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Title: SULFATION OF FUCOIDIN IN FUCUS ZYGOTES: INTERMEDIATES OF SULFATE TRANSFER

Abstract approved by: Redacted for privacy

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From previous work with algae and various animals, adenosine 5'-phosphosulfate (APS) and adenosine 3'-phosphate-5'-phosphosulfate (PAPS) were thought to be the intermediates in the transfer of inorganic sulfate to the sulfated polysaccharide fucoidin in embryos of the brown algae. In this study, high voltage paper electrophoresis (HVPE) was used as a technique to screen aqueous extracts of Fucus distichus L. for the presence for APS and PAPS. Since these compounds would be found in low concentrations, zygotes were pulsed for 60 min with carrier-free $^{35}SO_4$ in order to detect their presence.

A major finding was that 10 mM adenosine 5'-monophosphate (AMP) must be included in the extraction buffer to observe AP^{35}S and PAP^{35}S. Presumably, AMP protects the sulfate containing nucleotides from the action of hydrolytic enzymes during cell breakage. Evidence for the presence of APS and PAPS in F. distichus zygotes include: (1) comigration of ^{35}S radioactivity with authentic APS and PAPS during HVPE in three buffer systems; a) 0.1 M sodium citrate pH 5.5, b) 0.2 M Tris-HCl

pH 8.1, and c) 0.1 M sodium borate pH 10.0, (2) the presence of ^{35}S as a mixed acid anhydride linkage in the putative APS and PAPS regions from both thin layer chromatography and HVPE, (3) co-migration of ^{35}S activity with APS on polyamide and cellulose thin layer chromatographys, and (4) using high pressure liquid chromatography, the detection of adenine in the material co-migrating with PAPS during HVPE.

When zygotes were pulsed with $^{35}SO_4$, $AP^{35}S$ was detected throughout the first 24 hrs after fertilization. However, at the time when zygotes were actively sulfating fucoidin (from 8 hrs after fertilization), not only did the amount of $AP^{35}S$ increase, but $PAP^{35}S$ could be identified. Only at times of fucoidin sulfation was $PAP^{35}S$ detectable. These results suggested that APS and PAPS are the active intermediates in fucoidin sulfation and that the enzymatic conversion of APS to PAPS may be the limiting step to fucoidin sulfation prior to 8 hrs. The relevance of this data in determining the biochemical regulatory steps involved with fucoidin localization is discussed.

Sulfation of Fucoidin in *Fucus* Zygotes: Intermediates of Sulfate Transfer

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SULFATION OF FUCOIDIN IN *FUCUS* ZYGOTES: INTERMEDIATES OF SULFATE TRANSFER

I. Introduction

The processes involved in the differentiation of a complex embryo from a homogenous apolar, one celled zygote require an integration of genetic and spatial controls. The formation of an apolar zygote requires that specific cellular components become localized within the cell. Cytoplasmic localization is initiated in response to the extracellular microenvironment, usually in the form of a gradient. The partitioning of genetically identical nuclei into these areas of differing chemical environments can serve as a basis for cellular differentiation resulting from unique nucleo-cytoplasmic interactions. Therefore, differentiation during embryogenesis is a consequence of the unequal distribution of cytoplasmic components during oogenesis or shortly after fertilization (Davidson, 1976; Quatrano, 1978).

Differentiation is characterized by the appearance of cell specific products. The formation of a localized protuberance or rhizoid shortly after fertilization in *Fucus* embryos represents the first sign of polarity. This rhizoid eventually forms a unique cell-type of the two-celled embryo - the rhizoid cell. The rhizoid cell contains unique sulfated polysaccharides, organelles, and RNA (McCully, 1969; Quatrano, 1972; Nakazawa and Takamura, 1966) in its cytoplasm and cell wall that are not found in the thallus cell. The localization of these components in the rhizoid cell is the basis for cellular differentiation in the two-celled embryo, and may be used as a marker for differentiation.

The localization of the sulfated polysaccharide in the cell wall of the rhizoid cell of Fucus, is a prime example of cytoplasmic localization. The sulfated polysaccharide (fucoidin) localized in that part of the zygote wall which forms the rhizoid is an α-1, 2 linked fucan, characterized by an ester sulfate bond to the C-4 of the fucose residues (Percival and McDowell, 1967). Sulfate is incorporated into fucoidin between 8-10 hrs after fertilization and it is at this time that fucoidin is detectable (localized) by cytochemical and autoradiographic techniques (McCully, 1970; Hogsett and Quatrano, 1978; Brawley and Quatrano, 1979). Fucoidin is detectable only in the rhizoid cell as a mucilagenous layer around the rhizoid wall. The lack of sulfation prior to 10 hrs has been shown not to be a result of permeability barriers (Quatrano and Crayton, 1973).

The mucilagenous fucoidin layer around the rhizoid tip may function in the adhesion of the developing Fucus embryo to the substratum (Quatrano and Crayton, 1973). This function would be exceedingly important in the turbulent intertidal region where Fucus completes its life cycle. Histochemical evidence indicates that fucoidin acts as a bioadhesive in the brown alga Hormosira (Forbes and Hallam, 1979).

The appearance of fucoidin at this time raises questions concerning its mechanism of synthesis and accumulation. The accumulation of a localized, metachromatic Toluidin Blue O (TBO) stain (pH 2.0) in the rhizoid half of the cell at 8-10 hrs indicates the presence of fucoidin sulfate (McCully, 1970). Since TBO recognizes the SO_4 group, this accumulation could be the result of the unmasking of sulfate groups, the attachment of sulfate groups on a previously existing fucan, or the de

novo synthesis of the entire molecule. Any of these possibilities could occur throughout the cell at random with the resulting product transported to the site of rhizoid formation. Alternatively, they could occur at a predetermined site within the cell. The data discussed below indicates that the unsulfated fucan is present in the zygote before 8 hrs, and, that its sulfation occurs at random in the zygote followed by the transport of fucoidin sulfate to the rhizoid pole.

Only trace quantities of sulfate are found in fucoidin prior to 8 hrs and $^{35}SO_4$ incorporation into fucoidin cannot be detected at this time. It is not until 8-10 hrs after fertilization (several hours before rhizoid formation) that $^{35}SO_4$ incorporation into fucoidin is observed. From 14-18 hrs the amount of sulfation is sufficient to achieve a level of sulfation in fucoidin comparable to mature fronds (Quatrano and Crayton, 1973). In addition, Quatrano and Crayton (1973) demonstrated that the absence of sulfate in the growth medium prevented sulfation of fucoidin. Their data demonstrated that the localization of a sulfated fucoidin is directly dependent on the exogenous level of sulfate and not the removal of any component bound to a previously sulfated fucan.

Quatrano and Crayton (1973) also pulsed embryos with ^{14}C-labeled CO_2, fucose, and mannose and observed that there was a very low turnover rate of the carbon moieties of the fucoidin molecule at the time of sulfation. Also, the amount of fucoidin per zygote remained constant up to 24 hrs, strongly suggesting the lack of fucoidin turnover. All of this data is consistent with the proposal that at 10 hrs after fertilization there is not a *de novo* synthesis of an entire molecule, but

rather the sulfation of a previously existing fucan.

Callow et al. (1978) have demonstrated that the Golgi complex is the site of fucoidin sulfation in the Fucus embryo. The fucan becomes sulfated within Golgi-derived vesicles located randomly throughout the cytoplasm. The vesicles are then transported to the rhizoid pole and their contents deposited into the rhizoid wall (Quatrano et al., 1979; Brawley et al., 1976).

Sulfation has been shown to be necessary for the localization of fucoidin in the rhizoid cell. Hogsett and Quatrano (1978) found that by using the lectin ricin (which binds to the terminal galactose unit of both sulfated and unsulfated fucoidin) conjugated to the fluorescent dye FITC, they could observe the location of the sulfated fucan. Only embryos grown in the presence of sulfate were able to bind ricin in their rhizoid wall in sufficient quantities to be detected. Zygotes grown in the absence of sulfate contained the same amount of ricin-binding polymers (i.e. fucoidin) as those grown in the presence of sulfate, but they were not localized. Crayton et al. (1974) have shown that embryos grown in the absence of sulfate, but supplied with methionine (met-embryos) in order to facilitate protein synthesis, were prevented from sulfating fucoidin, but were able to become polar and form a 2-celled embryo with a rhizoid and a thallus cell, In addition, zygotes not supplied with sulfate do not adhere to the substratum further suggesting the role of fucoidin in adhesion. Brawley and Quatrano (1979) pulsed 2-celled met-embryos with $^{35}SO_4$ for 5 min to determine (autoradiographically) the localization of the sulfating sites. They observed equivalent sulfating activity in both cells, and even after a 2 hr chase, the ^{35}S-fucoidin

was not concentrated in the rhizoid cell. These results indicated that in the absence of sulfate, not only was the intracellular sulfation of fucoidin turned off, but the transport and secretion of the vesicles containing the unsulfated fucoidin into the rhizoid wall was prevented.

At this point, the role of sulfation in the localization of fucoidin can only be speculation. Perhaps sulfation gives the molecule a sufficient negative charge to be mobilized to the positive pole of an electrical field transversing the cytoplasm. Jaffe and Nuccitelli (1977) have shown that the Pelvetia zygote drives electrical current through its cytoplasm. The rhizoid pole is a site of entering cations which serve to generate a positive pole of an electrical field. Jaffe (1966) has shown that a field of approximately 100 mV/cm could be generated in the zygote. Since fucoidin must be charged (sulfated) to be localized in the rhizoid wall, it has been hypothesized that the electrical field may serve in localizing these particles at the rhizoid tip (Quatrano, 1978).

In order to study the biochemical mechanism operative in the localization of the sulfated polysaccharides, and to understand the regulation of the sulfation process, isolation and characterization of the primary acceptors was required. Sulfate must be activated before it can be used in biochemical reactions (Hilz and Lipmann, 1955; Robbins and Lipmann, 1957, 1958). The steps in sulfate activation in many systems are as follows:

$$(1)\quad ATP + SO_4^{=} + H^{+} \xrightarrow[EC\ 2.7.7.4]{ATP\ Sulfurylase} APS + PPi$$

$$(2)\quad APS + ATP \xrightarrow[EC\ 2.7.1.25]{APS\ kinase} PAPS + ADP$$

$$(3)\quad PPi + H_2O \xrightarrow[EC\ 3.6.1.1]{\text{inorganic pyrophosphatase}} O\text{-}PO_4 + 2\ H^+$$

Sulfate first reacts with ATP to form Adenosine 5'-phosphosulfate (APS) catalyzed by ATP sulfurylase (1). This reaction is "driven" to the right by the action of inorganic pyrophosphatase (3) and APS kinase (2) on the products. APS kinase catalyzes the reaction yielding adenosine 3'-phosphate-5'-phosphosulfate (PAPS) or "active sulfate" (2). PAPS has been shown to be the sulfate donor for sulfate ester formation in many systems (Roy and Truninger, 1970; Suzuki and Strominger, 1960). Since sulfation and protein synthesis are required for localization, it seems probable that the synthesis of one of these enzymes may be the limiting step for the localization of fucoidin to the rhizoid wall.

The purpose of this research was to: (i) establish the presence of APS and PAPS in Fucus embryos as the probable intermediates in the transfer of sulfate to fucoidin, and (ii) determine if the levels of APS and PAPS varied during development, and if a correlation could be made with the increased flow of sulfate through these intermediates at the time of fucoidin sulfation.

II. MATERIALS AND METHODS

A. Culturing Embryos

Receptacles of Fucus distichus L. were collected as soon as possible after exposure by the outgoing tide at Yaquina Head in Newport, Oregon. The receptacles were stored in the dark at 4-10°C in well aerated plastic bags for use during the subsequent 1-2 weeks.

Techniques used for handling and culturing embryos of F. distichus in artificial sea water (ASW) have been previously described (Quatrano, 1974). The embryos were cultured in large glass dishes containing 125 ml ASW at 15°C in the light (500 ft.c.). The embryos were labeled at the desired age by replacing the medium with fresh ASW (125 ml) containing 500 μCi carrier-free $^{35}SO_4$ ($H_2{}^{35}SO_4$) to the culture dishes. The zygotes were allowed to accumulate $^{35}SO_4$ for 1 hr, harvested, centrifuged, and resuspended in a homogenization buffer (Shawney and Nicholas, 1976 b) containing 0.1 M Tris-HCl (pH 7.5) and 10 mM adenosine 5'monophosphate (AMP). The zygotes were stored at -15°C in this buffer until needed.

B. Extraction, Separation and Characterization of Low Molecular Weight Intermediates

Zygotes were suspended in the homogenization buffer and ground in a scintered glass tissue grinder at 4°C. The homogenate was centrifuged at 3000 xg for 10 min and the supernatant (crude extract) was centrifuged again at 20,000 xg for 20 min. The resulting supernatant fraction (S_{20}) was heated in a water bath at 50°C for 30 min and stored at -15°C. After 12 hrs at -15°C the heated S_{20} was thawed, centrifuged at 20,000 xg for

30 min and the supernatant ($S_{20}H$) concentrated under reduced pressure and temperature. $S_{20}H$ was used directly for the detection of ^{35}S intermediates by HVPE.

$S_{20}H$ was spotted (50-75 µl/cm) on sheets of 3 MM paper (20 x 56 cm) and air dried. The spotted paper was wetted with appropriate running buffer and blotted dry. Extracts were electrophoresed at 5°C, 1750 V (80 mA) for 57 min in 3 buffer systems, each at a different pH: (i) 0.1 M sodium citrate pH 5.5; (ii) 0.2 M Tris-HCl pH 8.1; and (iii) 0.1 M sodium borate pH 10.0. Following electrophoresis, the paper was air dried and cut into 1 cm strips for the detection of ^{35}S activity. The strips were counted in a Packard Tri-Carb liquid scintillation counter (Model 2425) which operated at 86% efficiency for ^{35}S. Peaks of ^{35}S activity were then compared to known standards.

Autoradiography was used as an alternative to scintillation counting for detecting radioactivity. The air dried paper following electrophoresis was placed under a sheet of Kodak No-Screen X-ray film (NS-2T) and exposed for 3-7 days depending on the level of radioactivity. To develop the film, the exposed sheet was placed in Kodak liquid X-ray developer (4 min) followed by a 10 min wash in Kodak Rapid Fix. The film was then washed in tap water (20 min) and air dried.

^{35}S activity or a U.V. absorbing area from the paper electrophoretogram was eluted from the paper for determination of ester linkages or for electrophoresis in a different buffer system. For elution, pieces of paper were placed in a solution of 50% ethanol containing 2% (v/v) ammonium hydroxide at 4°C for 1 hr. The resulting solution was filtered (Whatman GF/C), concentrated as before and subjected either to electro-

phoresis in a different buffer system or hydrolysis.

To determine if the ^{35}S was present as an ester, a solution was hydrolyzed in 1N HCl for 1 hr at 37°C (Roy and Truninger, 1970). The hydrolysate was concentrated under reduced temperature and pressure and either frozen or subjected immediately to HVPE.

C. High Pressure Liquid Chromatography

Following HVPE, peaks of high ^{35}S activity corresponding to genuine PAPS, were eluted from the paper in 50% ethanol containing 2% NH_4OH. The eluate was made 6N with HCl and hydrolyzed in sealed tubes for 12 hrs at 105°C prior to subjecting it to high pressure liquid chromatography (De Lestang and Quillet, 1974). A μC18/reverse phase Porasil column (2.1 mm x 25 cm) was used, with a 2 min isocratic hold. The sample buffer was 0.02 M NH_4OAc. The flow rate was 2.0 ml/min with a running gradient of 0.0-30% ethanol.

D. Thin Layer Chromatography

Extracts were spotted on cellulose or polyamide thin layer chromatography plates (Brinkman Instruments Inc., Westbury, N.Y.) and allowed to run until the solvent front reached a point approximately 3 cm from the top of the plate. The solvent system was n-propanol: ammonia: water, 8:2:3 (Reuveny and Filner, 1976). Areas corresponding to known standards (APS, PAPS) were removed from the plates and assayed for ^{35}S activity as previously described.

III. RESULTS

The first objective of this research was to determine if APS and PAPS are present in Fucus embryos and actively transferring sulfate. The approach used to determine the primary acceptors of SO_4 was to analyze the low molecular weight ^{35}S components from synchronous cultures of zygotes. Numerous unsuccessful attempts were made to detect such molecules, and it was not until AMP was added to the homogenization buffer, presumably reducing the action of the nucleotidases on APS and PAPS (Shawney and Nicholas, 1976 b), that any reproducible results were obtained.

Embryos labeled with $^{35}SO_4$ at the time of rhizoid formation were immediately homogenized and the extract ($S_{20}H$) subjected to HVPE. Several peaks of ^{35}S activity were obtained when the extract was electrophoresed in a pH 5.5 citrate buffer (Fig. 1). Peaks II and III co-migrate with the expected sulfate intermediates APS and PAPS respectively. Peak IV is free SO_4 while the identity of the peak between I and II is not known.

To determine if these peaks were mixed acid anhydrides of sulfuric and phosphoric acid, the extract was subjected to an acid treatment sufficient to hydrolyze these linkages (Roy and Truninger, 1970) and electrophoresed in citrate buffer (Fig. 2). The activity in the peaks co-migrating with authentic APS and PAPS decreased upon hydrolysis, while the amount of free sulfate increased. Radioactivity in two peaks migrating slower than APS were unaffected, and therefore did not contain sulfate.

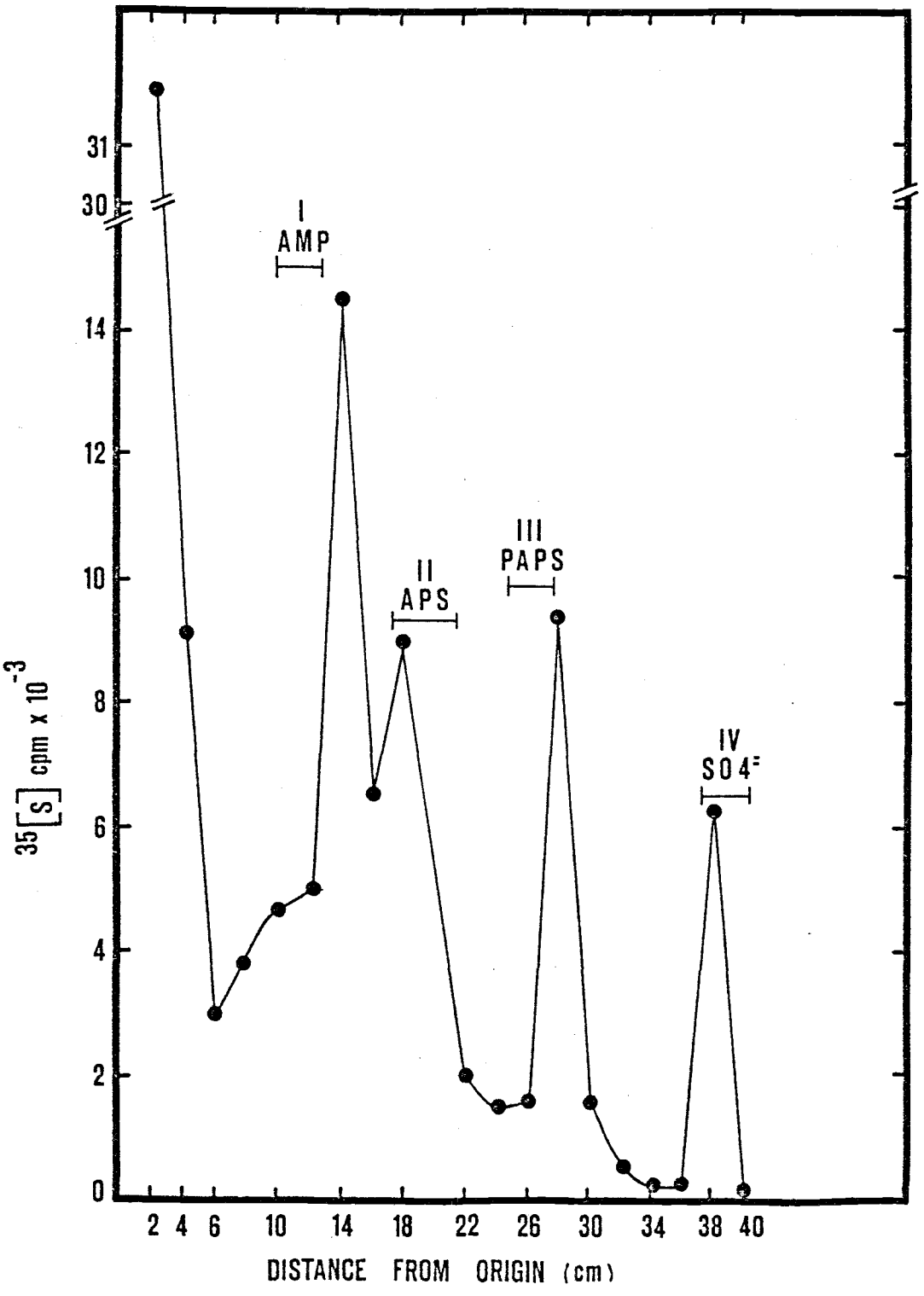

Figure 1. HVPE of a 20 hr embryo extract in citrate buffer (pH 5.5)

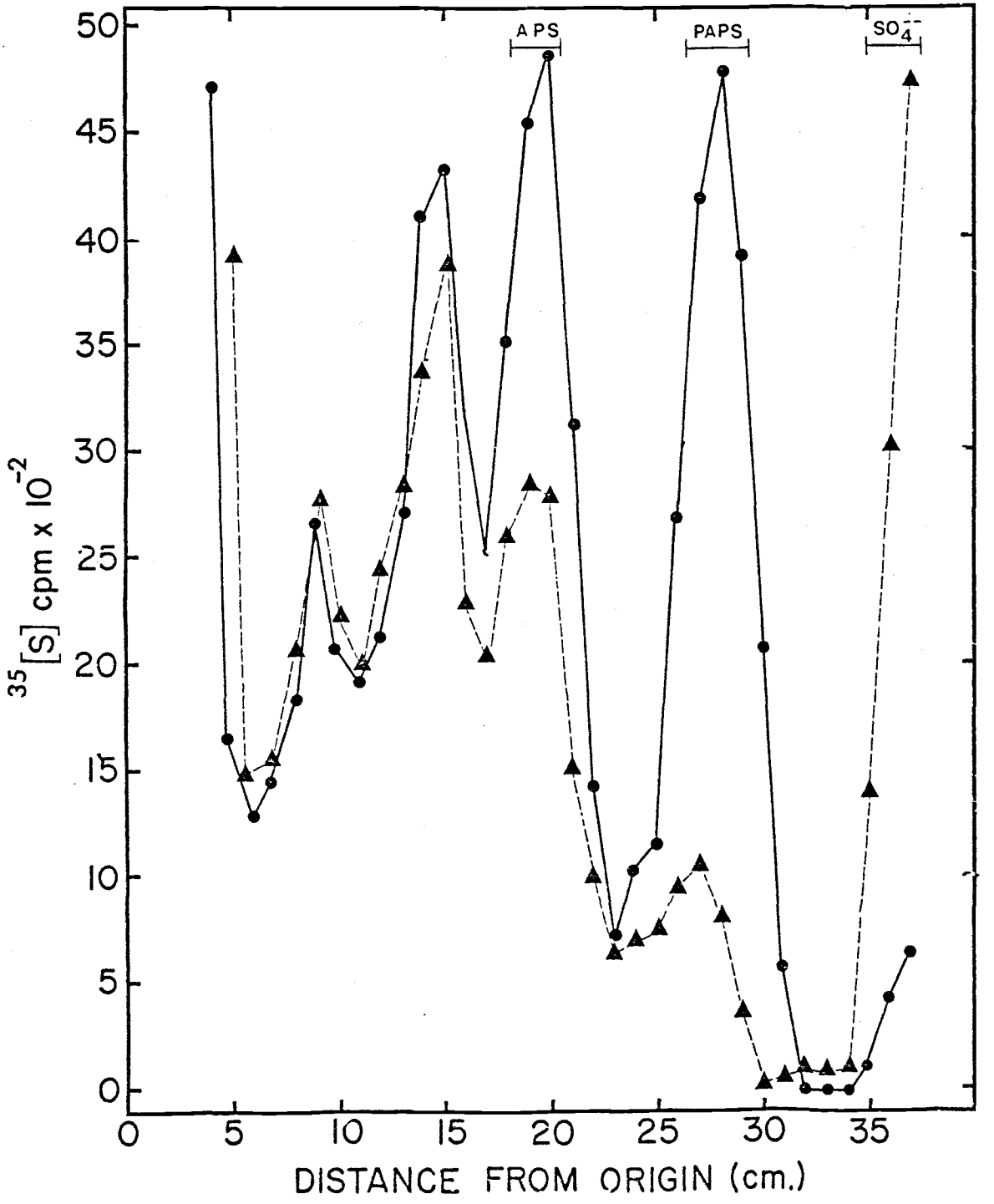

Figure 2. HVPE in citrate buffer (pH 5.5) of a 20 hr embryo extract (●-●). The same extract after acid treatment (1N HCl, 37°C, 1 hr) to hydrolyze sulfate esters (▲-▲).

To further purify the putative APS and PAPS molecules, the sulfated polysaccharides and other potentially interfering materials were removed from the extracts by ethanol precipitation (Reuveny and Filner, 1976). The ethanol soluble and insoluble fractions were then electrophoresed in citrate buffer (Fig. 3). The peaks of ^{35}S activity corresponding to APS and PAPS were now dominant in the ethanol soluble (ES) fraction.

Since migration during high voltage electrophoresis is dependent upon the charge on the molecule at a certain pH of the running buffer, the extract was electrophoresed and chromatographed in different buffer systems to substantiate the identity of the peaks. The ES fraction was electrophoresed in a borate buffer (0.1 M pH 10.0) and the dried paper subjected to autoradiography. The spots which developed corresponded exactly with authentic APS and PAPS (Fig. 4). Since each of these spots were well defined and highly radioactive, they were eluted from the paper and re-electrophoresed in the citrate buffer (Fig. 5). The spots again migrated with APS and PAPS. The extract was then subjected to electrophoresis in the Tris-HCl buffer (pH 8.1), and the activity again corresponded to authentic APS and PAPS. The ES fraction was also subjected to thin layer chromatography on cellulose and polyamide plates. In both cases, using autoradiography, evidence of APS was seen, but a spot corresponding to PAPS was never clearly delineated.

Since the PAPS peak was occasionally observed to migrate slightly faster than authentic PAPS during electrophoresis (see Figs. 1 and 3), it was thought that the intermediate might actually be cytosine 3'-phosphate-5'-phosphosulfate (PAPS) which has been found in Pelvetia (De Lestang and Quillet, 1974). To distinguish between PAPS and PCPS, material

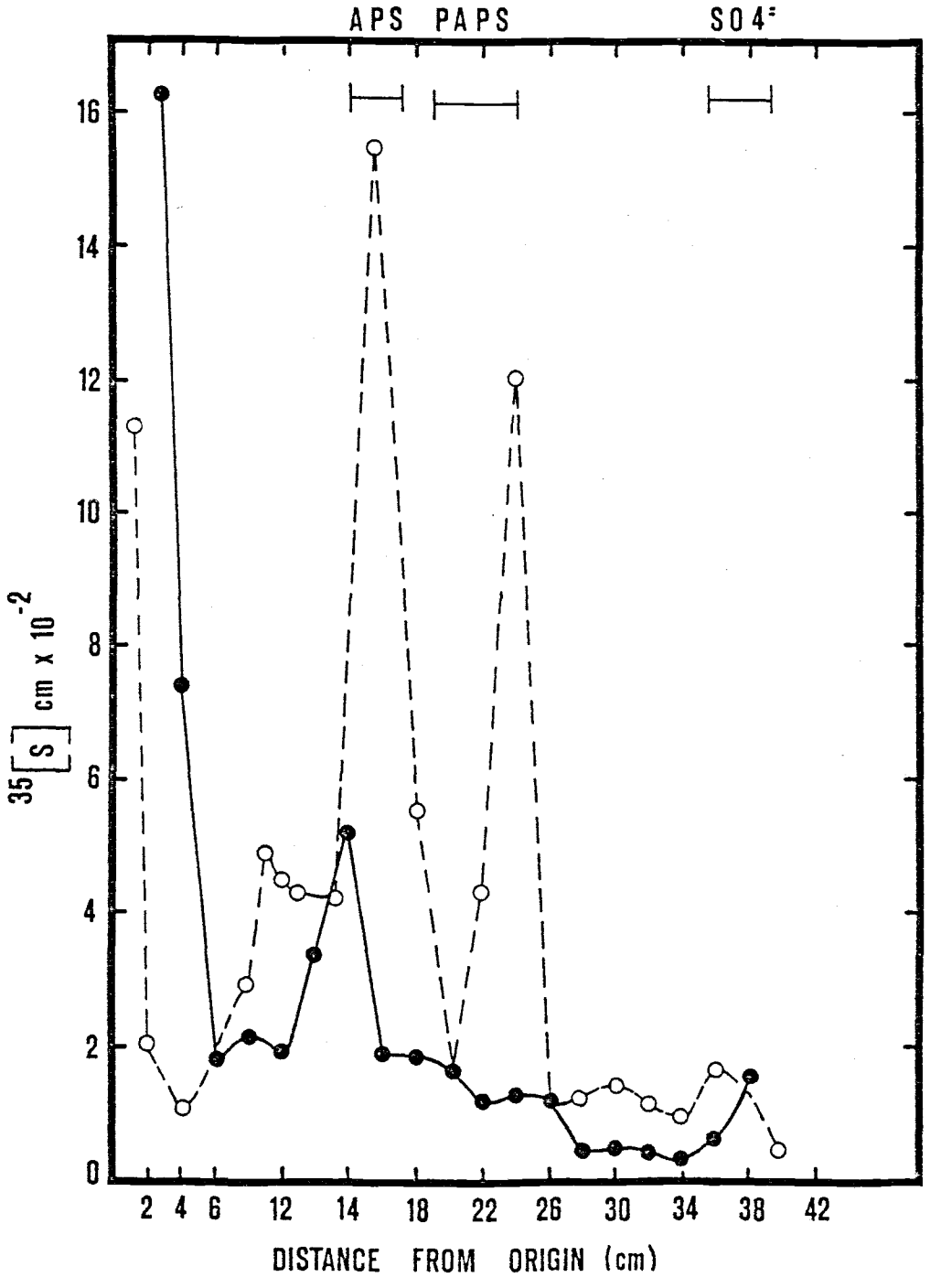

Figure 3. HVPE in citrate buffer (pH 5.5) of ethanol soluble (○-○) and insoluble (●-●) fractions from a 20 hr extract.

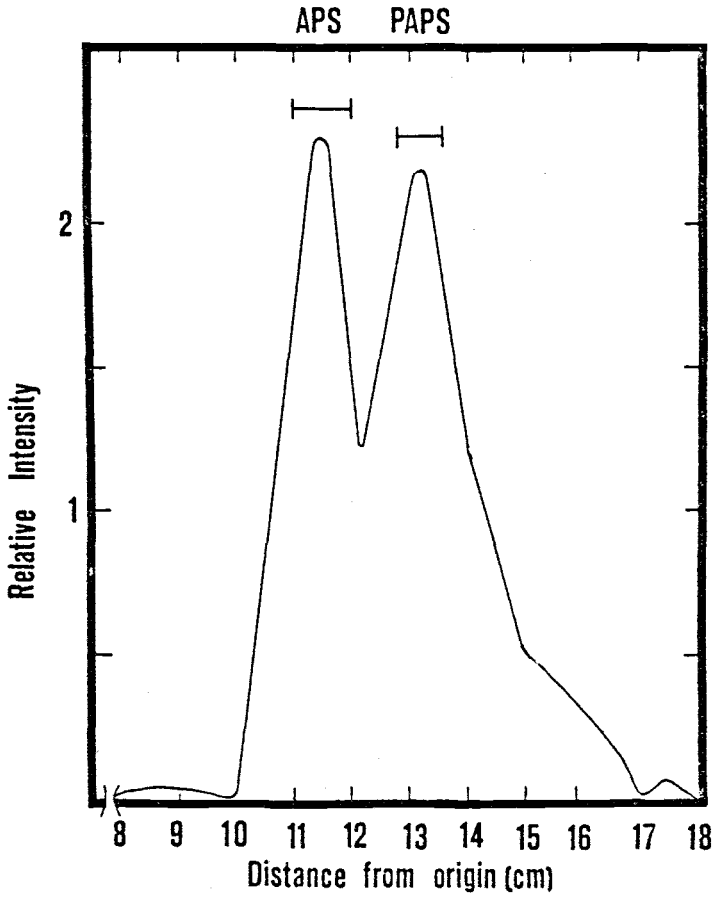

Figure 4. Densitometer scan of X-ray film exposed to dried electrophoresis paper from HVPE in borate buffer (pH 10.0) of a 20 hr embryo extract.

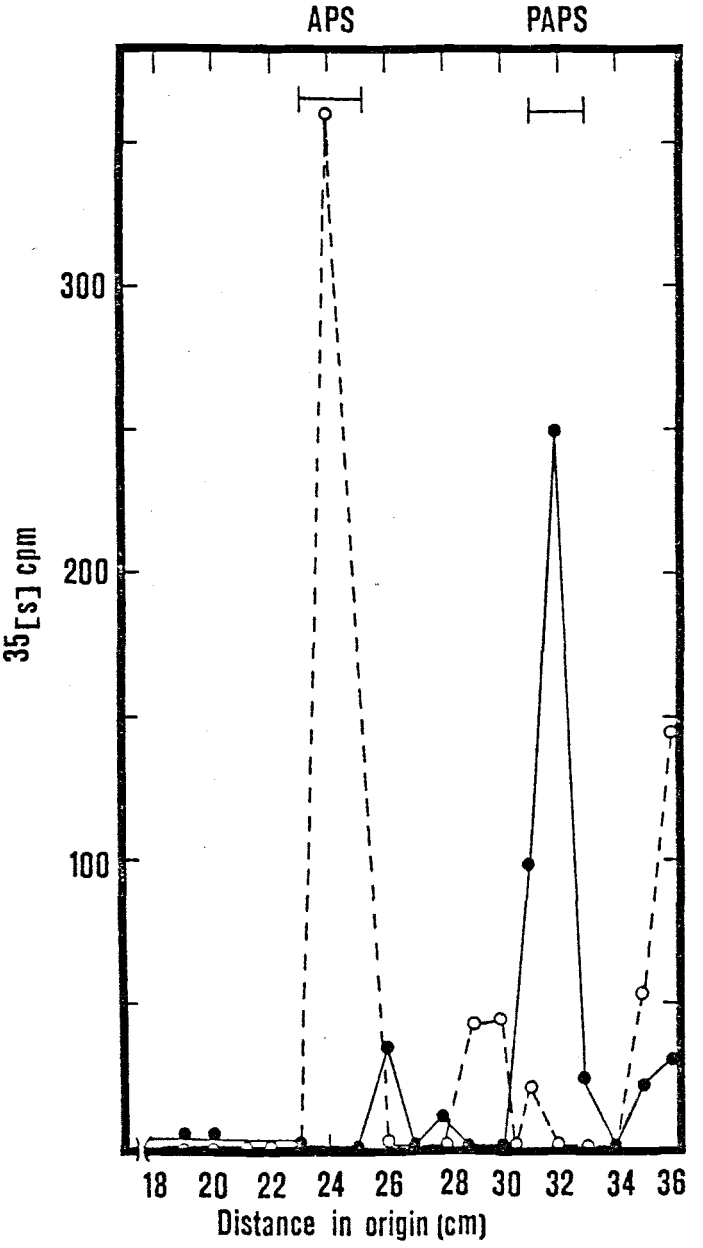

Figure 5. HVPE in citrate buffer (pH 5.5) of ^{35}S eluates from the APS (○-○) and PAPS (●-●) regions of a paper electrophoresed in borate buffer (pH 10.0).

eluted from the paper in the region of PAPS was hydrolyzed and subjected to high pressure liquid chromatography. As illustrated in Figure 6, the molecule was found to contain adenine, confirming the presence of PAPS.

The second objective was to determine if the levels of APS and PAPS varied during development and if a correlation could be made with the increase flow of SO_4 through these intermediates at the time of fucoidin sulfation. In this way the facotr which limits $^{35}SO_4$ incorporation into fucoidin prior to 8 hrs might be ascertained.

Approximately equal numbers of ^{35}S-labeled zygotes, 4 and 20 hrs old, were extracted and electrophoresed in citrate buffer. No PAPS was seen in the 4 hr embryos (Fig. 7) but small amounts of APS were present in the young embryos. This is probably due to the fact that APS functions to donate sulfate to the sulfur reductive cycle to form amino acids (Schiff and Hodson, 1973) needed for protein synthesis in the zygote (Quatrano, 1968). At 8 and 20 hrs, not only was the amount of $AP^{35}S$ enhanced, but a dramatic increase in the amount of $PAP^{35}S$ was clearly evident (Figs. 7 and 8). Ethanol soluble fractions of these extracts were acid hydrolyzed and again the ester nature of the sulfate linkages was demonstrated. Eight and 16 hr embryos had considerable acid labile activity in peaks corresponding to both APS and PAPS (Figs. 8 and 9). This data indicates that sulfate flow through these acceptors is first detected at the time of fucoidin sulfation.

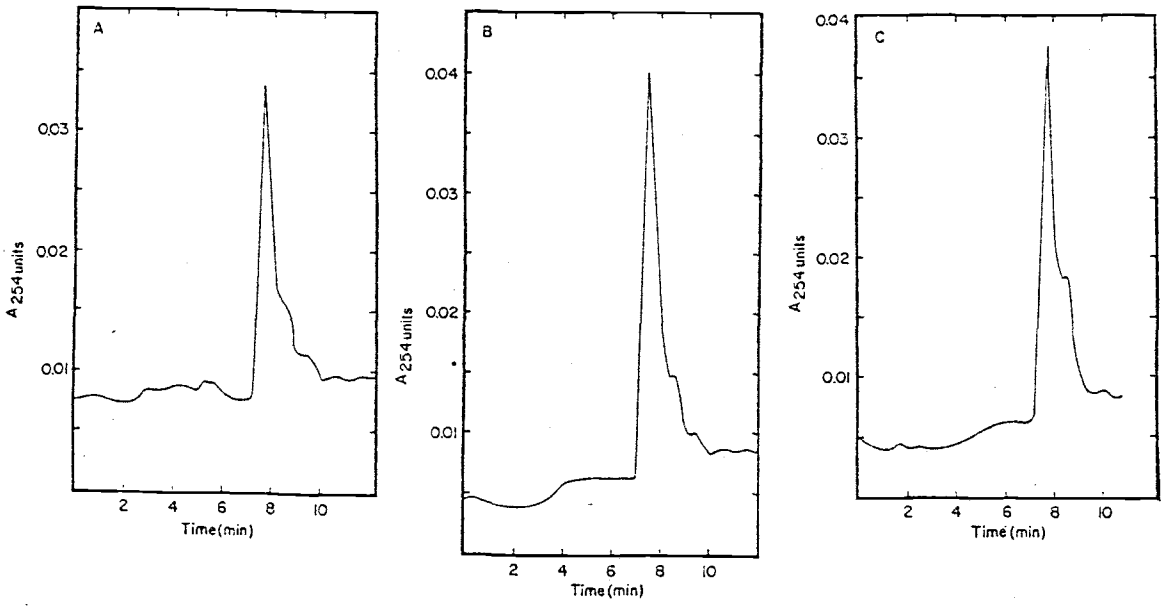

Figure 6. A_{254} scans of the material eluted from a high pressure liquid chromatography column: (A) 2 µg authentic adenine, (B) 2 µg authentic adenine plus 2 µl eluate from the PAPS region of a HVPE run in citrate buffer (pH 5.5), and (C) 2 µl eluate from the PAPS region of a HVPE run in citrate buffer (pH 5.5).

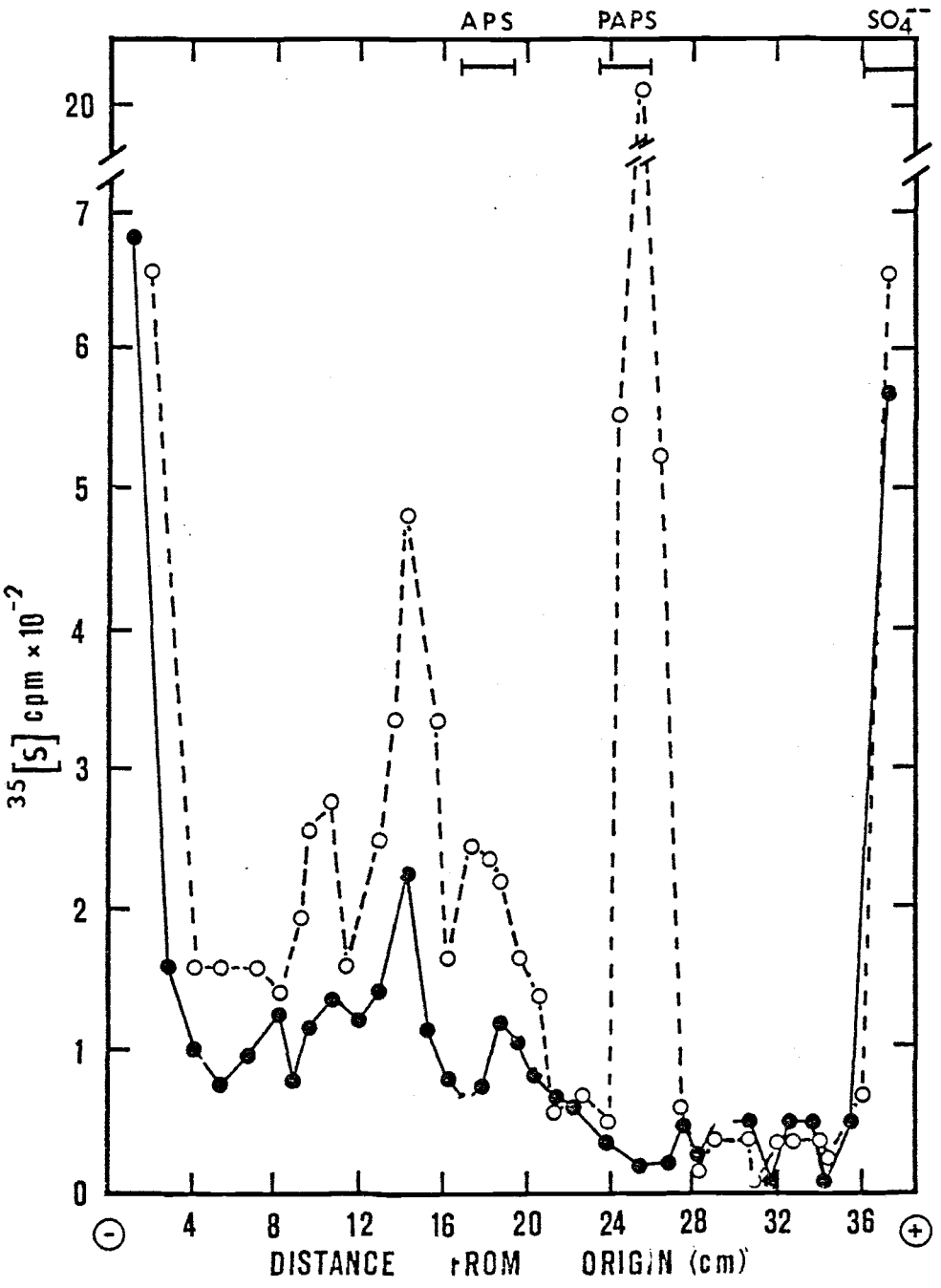

Figure 7. HVPE in citrate buffer (pH 5.5) of 4 (●-●) and 20 (○-○) hr embryo extracts.

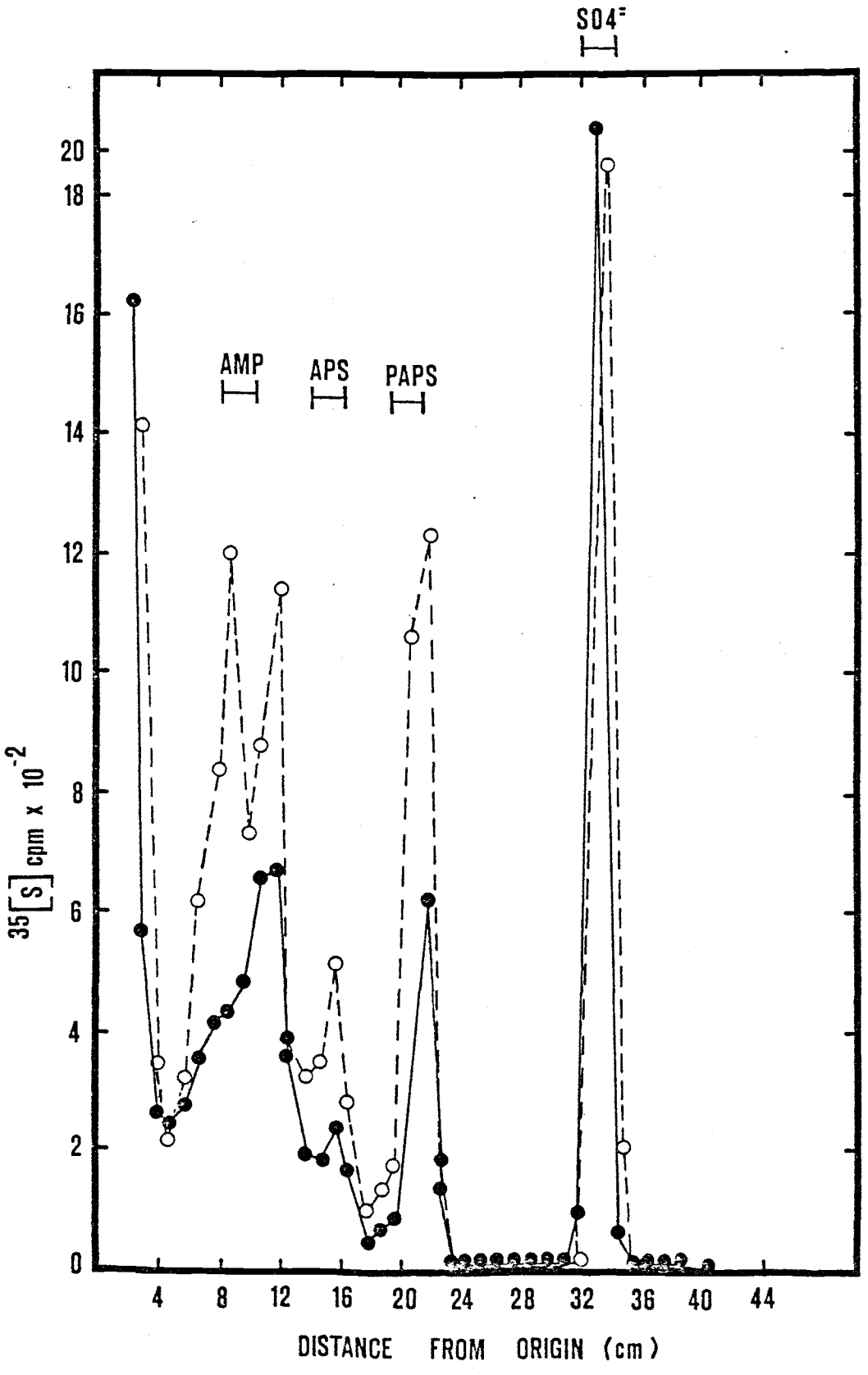

Figure 8. HVPE in citrate buffer (pH 5.5) of 8 (●-●) and 16 (○-○) hr embryo extracts.

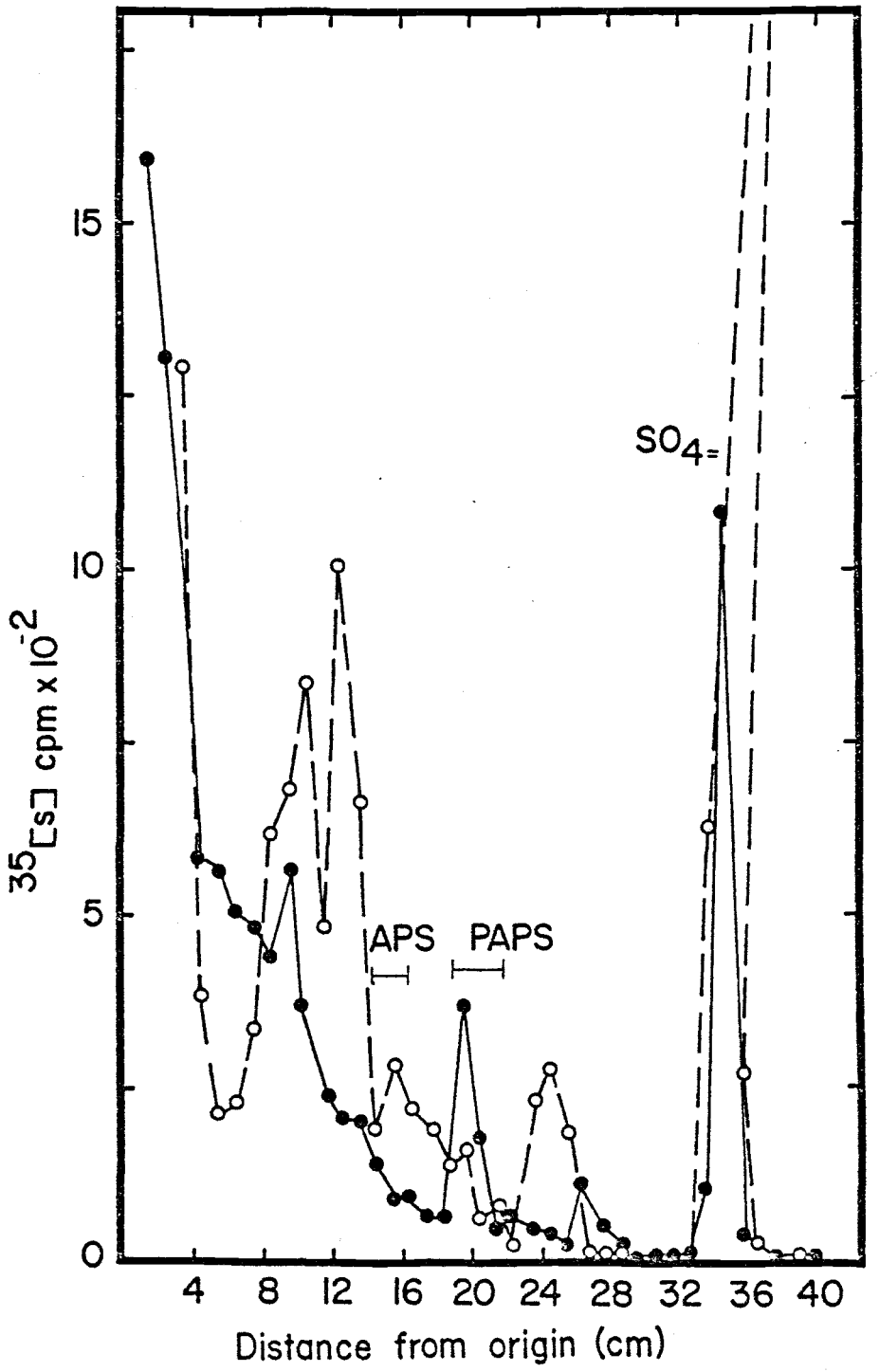

Figure 9. HVPE in citrate buffer (pH 5.5) of weak acid hydrolysates of 8 (●-●) and 16 (○-○) hr embryos.

IV. DISCUSSION

Differentiation is characterized by the appearance of cell specific products. In Fucus, the rhizoid cell of the two cell embryo is characterized by the accumulation of the sulfated polysaccharide fucoidin. Localization of this large molecular weight polymer in the rhizoid half of the zygote requires sulfation of an existing fucan as well as continued protein synthesis. The specific mechanism by which localization of macromolecules or particles occurs is not known nor has there been proposed a general hypothesis to suggest the relationship between localization of a particular macromolecule and gene activity.

Sulfate must be in a metabolically active form prior to its incorporation into a polymer such as fucoidin. The sulfate activating synthetic pathway operative in the brown algae has not received much attention, whereas the reductive assimilation and activation of sulfate in bacteria and higher plants has been studied extensively (Schiff and Hodson, 1973). In these systems the intermediates APS and PAPS have been identified as the metabolically active form of sulfate.

The demonstrated requirement for protein synthesis in the sulfation of the fucan polymer in Fucus suggests the enzyme(s) involved with sulfate assimilation, activation, and/or transfer to a polymer, be newly synthesized and consequently under a genetic control system. For this reason, we have attempted to monitor the flow of sulfate through these intermediates at various times in order to determine the probable limiting step to sulfation prior to 10 hrs and a possible genetic link.

Aqueous extracts of embryos ranging in age from 4-20 hrs were made

to determine the presence if any, of molecules co-electrophoresing with authentic APS and PAPS. A major problem with this type of procedure is the action of hydrolytic enzymes on the hypothetical intermediates. The occurrence of enzymes which rapidly hydrolyze APS and PAPS have been reported in a variety of organisms. PAPS has been shown to be degraded by 3'nucleotidase in rabbit liver (Brunngraber, 1958), rye grass (Robbins and Lipmann, 1957), and Chlorella (Goldschmidt et al., 1975) and by PAPS sulphatase in hen oviduct (Suzuki and Storminger, 1960) and sheep brain (Balasubrahaniam and Bachawat, 1962). The degradation of APS has been attributed to the action of APS sulphatase in hen oviduct (Suzuki and Storminger, 1960) and 5'nucleotidase in bull semen (Robbins and Lipmann, 1957). Enzymatic degradation of APS and PAPS has also been reported in extracts of Anabaena cylindrica (Sawhney and Nicholas, 1976 b). Numerous extraction buffers were utilized without success in attempts to protect the sulfur-containing nucleotides. Although Coughlin (1977) reported success in extracting APS and PAPS from Fucus with 60% ethanol we were unable to duplicate his results with an ethanol-based buffer. Only after 10 mM AMP was added to a 0.1 M Tris-HCl buffer (pH 7.5) to competitively inhibit the action of hydrolytic enzymes on APS and PAPS during extraction, was I able to obtain reproducible results.

All extracts were screened using HVPE (see Materials and Methods) for molecules co-migrating with authentic APS and PAPS. Figure 1 shows the presence of such ^{35}S-containing molecules when electrophoresed in a citrate buffer (pH 5.5). However, since the electrophoretic mobility of a molecule is dependent upon its charge, and consequently upon the pH of the running buffer, it was necessary to run

the extract in two additional buffer systems (pH 8.1 and 10.0) to confirm the sulfation of APS and PAPS. The co-electrophoresis observed at these pH's demonstrated that these ^{35}S-containing molecules possessed a charge identical to APS and PAPS at three different pH's (Figs. 1, 4, 5, 7, and 8). In addition, ^{35}S activity was also shown to migrate with authentic APS using thin layer chromatography on cellulose and polyamide plates. Since Coughlin (1977) did not electrophorese his extracts in different buffers, this is the first study that fully characterizes these intermediates and further documentation was necessary.

Mixed-acid anhydrides such as APS and PAPS contain a very reactive sulfatophosphate group. The group is quite acid labile (Roy and Truninger, 1970) and therefore a weak acid hydrolysis at 37°C is a fairly specific test for its presence. In our HVPE system we would expect, after weak acid hydrolysis, to see a loss of counts in the peaks corresponding to APS and PAPS, and an increase in free sulfate. The results of such a treatment are shown in Figure 2. The activity in the peaks co-migrating with APS and PAPS did show a marked decrease, offering further evidence that these molecules were APS and PAPS.

A disturbing piece of data was the fact that the activity corresponding to PAPS was occasionally noted to migrate slightly ahead of the authentic molecule (Fig. 1). This could be an indication that the molecule in question may contain the pyrimidine cytosine rather than the purine adenine. This was found to be the case in Pelvetia canaliculata (De Lestang and Quillet, 1974), also a member of the Fucales. When the peak of ^{35}S activity corresponding to PAPS was eluted from the paper, hydrolyzed, and subjected to high pressure liquid chromatography, the

molecule was found to contain adenine (Fig. 6). This combined with the electrophoretic and thin layer chromatography data, and the mixed acid anhydride analysis, confirms that the intermediates in sulfate metabolism in F. distichus zygotes are APS and PAPS.

Our next objective was to monitor the flow of sulfate through APS and PAPS at various times during development in an attempt to determine the limiting step in the sulfation process prior to 8 hrs. Extracts were made using 4, 8, 16, and 20 hr embryos (Figs. 7, 8, and 9). The 8, 16, and 20 hr extracts appear similar with respect to the level of APS and PAPS. The small amount of APS present at 4 hrs is most probably due to the fact that APS can donate sulfate to the reductive cycle,(e.g. for amino acid synthesis). The absence of PAPS at 4 hrs and its presence at 8, 16, and 20 hrs suggests that this may be the point at which fucoidin sulfation is controlled prior to 8 hrs.

The fact that the amount of ^{35}S incorporated into the two unidentified peaks did not vary at 4, 8, 16, and 20 hrs, indicates that sulfate uptake did not change appreciably during this time. This conclusion is also supported by earlier studies (Quatrano and Crayton, 1973) and cannot be used as an explanation of these results. According to the proposed pathway of sulfate utilization in brown algae (see Introduction, p. 5), these results could be explained by a block at the conversion of APS and PAPS. The fact that protein synthesis is necessary for sulfation also suggests that the actual regulatory step may be at the level of APS kinase synthesis. Direct measurement of APS kinase activity (c.f. Sawhney and Nicholas, 1976 a) with and without RNA and protein synthesis inhibitors at different times during development could determine the

level of control operating to relieve the block of fucoidin sulfation prior to 8 hrs. This study will now allow one to focus on the limiting biochemical reaction in the localization of a cell specific macromolecule and determine the genetic regulatory mechanisms involved.

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