AN ABSTRACT OF THE THESIS OF

SANDRA HONDA   for the degree of MASTER OF SCIENCE

BOTANY AND PLANT PATHOLOGY
in   (Developmental Biology)   presented on ____ May 5, 1978 ____

Title: PRELIMINARY BIOCHEMICAL EVIDENCE FOR AN ASCOSPORE COAT
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PROTEIN IN SACCHAROMYCES CEREVISIAE: PARTIAL
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CHARACTERIZATION AND TIMING OF SYNTHESIS
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Abstract approved: _____
                                         Dallice Mills

Sporulation in the yeast Saccharomyces cerevisiae involves

the process of meiosis which is accompanied by ascospore formation.

Using intact cells or spores, preliminary biochemical evidence is

given to identify ascospore wall components not found in vegetative

cell walls. Cell surface components are solubilized by treatment

with 8 M urea and subsequently dialyzed to precipitate hydrophobic

components. These urea-soluble hydrophobic components were

analyzed by SDS-polyacrylamide gel electrophoresis. The spore

wall component not present in vegetative cell wall extracts

appears to be proteinaceous and possesses no detectable carbo-

hydrate moiety as determined by periodic acid-Schiff reagent (PAS)

staining procedures. Further experiments indicate that a

component of snail gut enzyme, which is used to liberate ascospores

from asci, co-migrates with the spore wall protein. Due to the

hydrophobic nature of the ascospore wall, there is evidence

suggestive of some cytoplasmic binding to cell wall extracts.
It is unclear as to whether or not the ascospore wall protein
is sporulation-specific. Pulse-labeling experiments indicate
that the spore coat component is synthesized prior to ascospore
formation as early as zero to four hours after inoculation into
sporulation medium.

Preliminary Biochemical Evidence for an Ascospore Coat Protein
in Saccharomyces cerevisiae:
Partial Characterization and Timing of Synthesis

by

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A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed May 5, 1978

Commencement June 1978

APPROVED:

Redacted for Privacy

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Date thesis is presented _____ May 5, 1978 _____

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PRELIMINARY BIOCHEMICAL EVIDENCE FOR AN ASCOSPORE COAT PROTEIN IN SACCHAROMYCES CEREVISIAE: PARTIAL CHARACTERIZATION AND TIMING OF SYNTHESIS

I. INTRODUCTION

A. Overview of the Problem

Scientific curiosity has historically entertained a broad spectrum of questions concerning the development of an organism from fertilization to maturity. Growth, morphogenesis, and differentiation occur in synchrony at the molecular, cellular, and tissue and organ level. These processes are controlled temporally and spatially within the developing organism. Regulation of these processes in eukaryotes occurs at both the transcriptional and translational levels, as well as post-transcriptionally and post-translationally. The process of meiosis and ascospore formation in yeast is perhaps one of the simplest examples of intracellular differentiation in a developing eukaryotic system. Growth by mitotic budding is halted, cells enter meiosis, and ascospores are formed.

Diploids of the yeast Saccharomyces cerevisiae reproduce asexually by budding. A nutritional trigger promotes the diploid cells of the a/α mating type to undergo meiosis which is followed by ascospore formation. As a result, four haploid ascospores are produced within the ascus or original spore mother cell. These spores may germinate and fuse with one of opposite mating type to form a diploid cell.

B. Ascospore Formation

1. Physiology and Genetics

The genetic control of sporulation has been studied exten-sively. It is known that diploids of any mating type (a/a, α/α, a/α) are capable of reproducing mitotically. Only a/α diploids have the ability to undergo meiosis and spore formation (Roman et al., 1955). Both a/a and α/α strains will not sporulate and are blocked prior to spindle plaque replication (Roth and Halvorson, 1969; Roth and Lusnak, 1970; Roth, 1972). Evidently the earliest detectable biochemical event blocked in a/a or α/α strains is premeiotic DNA synthesis (Roth and Lusnak, 1970). Bud formation is associated with DNA replication in the normal mitotic cell cycle (Hartwell, 1971), but does not occur in sporulating yeast. Roth (1973) has also suggested that premeiotic DNA synthesis is different and under a separate genetic control from vegetative DNA replication on the basis of two sporulation-deficient mutants which do not exhibit premeiotic DNA synthesis. Premeiotic DNA synthesis appears to utilize recycled DNA precursors, while vegetative DNA synthesis does not (Simchen et al., 1972).

Neither mitochondrial or nuclear DNA is synthesized in a/a or α/α strains during the period when premeiotic DNA synthesis occurs in a/α diploids (Pinon et al., 1974). Chloroamphenicol inhibits sporulation (Tingle et al., 1972) suggesting that mito-chondrial protein synthesis is essential for sporulation. A diploid dominant sporulation mutant capable of synthesizing mito-

chondrial DNA was only able to initiate chromosomal DNA replication

providing evidence that gene products required for mitochondrial

premeiotic DNA synthesis are not essential for mitochondrial DNA

replication during sporulation. Ethidium bromide-induced mito-

chondrialess mutants have been used to analyze the role of mito-

chondria in the sporulation of S. cerevisiae (Kuenzi et al., 1974).

Extensive derepression of the respiratory system is critical for

sporulation and a functional mitochondrial genome is required

(Kuenzi et al. 1974). Once full derepression occurs, neither

the presence of the mitochondrial genome or its expression is

necessary for meiosis and ascospore formation.

Carbohydrate metabolism in a/a or α/α strains appears to be

basically identical with a/α strains during incubation in sporu-

lation medium (Kane and Roth, 1974). Trehalose, mannan, and glucan

are synthesized in strains which are heterozygous or homozygous

for the mating type alleles. The exception lies in the degradation

of glycogen. Sporulating a/α diploids degrade glycogen while a/a

and α/α strains do not. Glycogen degradation in a/α strains

accompanies ascospore formation suggesting that perhaps turnover

products are used to synthesize the ascospore wall.

All cells of a sporulating culture do not form spores.

Daughter cells sporulate poorly as compared with mother cells

(Yanagita et al., 1970). It appears that the capacity of a cell

to sporulate is dependent upon a variety of physiological factors.

Haber and Halvorson (1972) have found that cells which sporulate

cease to grow vegetatively immediately upon introduction into

sporulation medium. Cells which do not sporulate exhibit limited

vegetative growth. Ability to sporulate is also dependent on the

stage in the cell cycle that a cell is in when transferred to

sporulation medium (Haber and Halvorson, 1972). Cells in the

final third of the cycle sporulate most efficiently. These

data suggest that essential components which fluctuate throughout

the cell cycle are limiting in cells unable to sporulate. Other

hypotheses have been advanced to explain the cell cycle dependency

for sporulation. It has been postulated that specific functions

present at specific times during the cycle are limiting in unsporu-

lated cells (Tingle et al., 1972) and also that RNA and protein

content at the time of introduction into sporulation medium

influence the capacity to sporulate (Croes, 1967a).

RNA and protein content in sporulating cells show an initial

increase in total content followed by a decrease in RNA and

protein (Croes, 1967a,b; Esposito et al., 1969). Protein and

RNA turnover occurs in sporulating cells, presumably, to supply

the cell with an endogenous source of nitrogen which is not avail-

able from the medium (Esposito et al., 1969). Two sporulation-

deficient mutants have no endogenous proteinase-A activity during

sporulation (Chen and Miller, 1968), when activity rises in

sporulating wild type strains. It was suggested that proteinase-A

is involved in the degradation of proteins thereby providing a

mechanism for replenishing amino acid pools, which, in turn, may

be critical for sporulation. Sporulation is completely inhibited

by the addition of cycloheximide up to the stage at which mature

ascospores can be observed indicating that translation is a

necessary process in sporulation (Esposito et al., 1969; Darland,

1969).

To achieve efficient sporulation, cells must be primed for

respiratory activity. Cells grown under fermentative conditions

are able to sporulate best when harvested from stationary phase.

At this point, cells have changed from fermentative to oxidative

metabolism (Croes, 1967a,b; Esposito et al., 1969). Cells grown

in an acetate presporulation medium are capable of sporulating

when taken from an exponentially growing culture (Roth and

Halvorson, 1969).

The most prominent nutrient trigger which induces sporulation

is the absence of nitrogen coupled with the presence of a non-

fermentable carbon source (Miller, 1963b). The presence of

ammonium ions inhibits sporulation, but many nuclei do enter the

first stage of meiosis (Miller, 1963a; Miller and Hoffmann-

Ostenhof, 1964). Ammonium ions appear to prevent continued

synthesis of glyoxylate cycle enzymes and the ability of the cells

to use acetate as a carbon source (Gosling and Duggan, 1971).

However, in the presence of dihydroxyacetone, an effective carbon

source, ammonium ions do not affect sporulation (Miller, 1957).

The glyoxylate cycle is necessary for the synthesis of amino acids

during acetate metabolism (Kornberg, 1965). Croes (1967b) has

proposed that transfer to sporulation medium causes an increase in

the rate of protein synthesis without the equivalent production of

amino acids.

Continous exposure to acetate is not required throughout
sporulation. Cells transferred to distilled water from acetate
sporulation medium just prior to spindle plaque duplication
maintain their capacity to sporulate maximally (Darland, 1969).
It is also worthwhile to note that the induction process may
occur rapidly. Cells exposed to acetate for five to ten minutes
have an eight-fold increase in sporulation over those exposed
only to distilled water (Darland, 1969). However, Simchen et al.
(1972) found this period to be somewhat longer, in the range of
approximately three hours.

Simchen et al. (1972) have divided sporulation into four
physiological/developmental stages. After a brief exposure to
sporulation medium, cells which are able to sporulate in distilled
water without further exposure to acetate, but are able to revert
to vegetative growth when returned to presporulation medium are
in a stage of readiness. Readiness is a prolonged state beginning
before the onset of premeiotic DNA synthesis and extending until
the first cells complete their synthetic period. Readiness is
followed by a short-lived stage occurring at approximately the
same time as meiotic prophase. During this stage, cells which
are transferred back to vegetative medium fail to either grow
vegetatively by mitotic cell division or sporulate. A stage of
full commitment is demonstrated by the ability of cells to undergo
normal sporulation after re-exposure to vegetative medium.
However, cells can be committed to meiotic recombination without
being committed to the meiotic disjunction of chromosomes (Esposito

and Esposito, 1974).

2. Cytology

The process of meiosis and ascospore formation has been divided into several stages as defined by electron microscope studies (Moens, 1971; Moens and Rapport, 1971). During stage I the spindle plaque is single and has few microtubules associated with it. The spindle plaque duplicates at the onset of stage II and in preparation for meiosis I, the spindle plaques move in opposite directions and face one another. Stage III comprises the events which occur in meiosis I and nuclear elongation is observed. Nuclear division does not occur at the conclusion of meiosis I and a forespore wall is not associated with the elongated nucleus. Spindle plaques replicate for meiosis II during stage IV and meiosis II proceeds. Guth et al. (1972) could find no evidence of this second spindle plaque replication in freeze-etching studies. Early ascospore development is first observed during stage IV. Four nuclei appear to bud from the parental nucleus after binary nuclear elongation and first indications of the formation of the prospore or forespore wall are observed. Maturation of the ascospore occurs in stage V. Meiosis in yeast differs from other fungi and eukaryotes in that there is no dissolution of the nuclear membrane (Guth et al., 1972: Hashimoto et al ., 1960; Mundkur, 1961).

Lynn and Magee (1970) have made an elaborate ultrastructural study of ascospore formation by electron microscopy. It is

suggested that the spore membrane is derived from endoplasmic

reticulum. Lipid granules align at the newly formed spore plasma-

lemma. Spores begin to round up and cytoplasmic volume increases.

The nucleus becomes surrounded by two unit membranes which are

slightly separated at intervals around the forming spore. The

outer unit of the pair of unit membranes becomes partially

darkened and the darkened areas in particular appear to be in

close proximity to lipid granules. The endoplasmic reticulum

lies adjacent and parallel to the plasma membrane. The inner coat

is deposited such that the greatest amount of synthesis occurs

opposite the endoplasmic reticulum. Consequently, it is proposed

that the endoplasmic reticulum plays an important role in this

process. The ascospores ultimately are delimited by a pair of

unit membranes between which the spore wall is deposited (Beckett

et al., 1973). The wall consists of an inner electron transparent

layer and an outer electron dense layer (Beckett et al., 1973).

3. The Ascospore Wall

The structure and biosynthesis of the vegetative cell

envelope of Saccharomyces cerevisiae has been studied extensively

and reviewed thoroughly by Phaff (1972). In contrast, only a

sparse number of publications exist describing the structure and

development of the outer layers of the yeast ascospore. A large

portion of these investigations deal with cytological studies of

the developing spore.

The yeast ascospore is hydrophobic by nature and stains

readily with Sudan black suggesting the possibility of an outer-
most layer composed of lipid (Emeiss, 1958; Langeron and Luteraan,
1947; Schumacher, 1926). The presence of an outermost electron
dense layer (Fowell, 1969; Kreger-van Rij, 1969) characterized
by marked ultraviolet-absorbing properties (Miller et al., 1963)
in electron micrographs provides further evidence that is incon-
sistent with the hypothesis of an outer lipid layer. The electro-
phoretic mobilities of intact ascospores also show a marked
change prior to and after treatment with specific proteases or
the denaturing agent, urea (Briley et al., 1970). Electron micro-
graphs of thin sections through ascospores treated with 8M urea
revealed that the electron dense layer was completely removed
by the urea but small amounts of electron dense material remained
following treatment with chymotrypsin and papain.

A serological comparison between ascospores and asci and
between ascospores and vegetative cells has been reported (Snider
and Miller, 1966). Cross-agglutination assays of vegetative
cells, asci, or ascospores with rabbit-anti-vegetative cells,
rabbit-anti-ascus, or rabbit-anti-ascospore antibody or anti-
serum revealed that the vegetative cell envelope and ascus wall
are antigenically similar. However, the vegetative envelope
and the outer layer of the ascospore do not cross-agglutinate,
indicating antigenic dissimilarities. When vegetative cells or
asci are treated with periodate or pronase prior to cross-
agglutination assays, it was found that agglutination was not
affected periodate, but was affected by pronase. These

results suggest that the polysaccharides of the vegetative cell wall are weakly antigenic or non-antigenic. The protein component of the cell wall appears to be the primary source of antigen. Conflicting results have been reported by Suzuki et al. (1968) proposing that the $\alpha 1,2$ and $\alpha-1,3$ linkage groups of the cell wall mannan are the immunodominant groups in vegetative cells.

Glucosamine auxotrophs of Saccharomyces cerevisiae which were isolated by Whelan and Ballou (1975) are capable of undergoing meiosis. However, homozygous diploids segregate abnormal ascospores. These spores lack refractility, they are susceptible to βglucanases unlike wild type spores, and they are less "sticky" than wild type spores. Electron micrographs of thin sections through these spores show that they lack the outer electron dense layer. Kane and Roth (1974) have provided tentative evidence that mannan and glucan are spore wall components. Skinner et al. (1951) and Hashimoto et al. (1958) suggest that the inner electron transparent layer is the cell wall of the new vegetative cell.

Several significant sporulation-defective mutants of yeast which form abnormal ascospores have been isolated (Moens et al., 1974). These mutants appear to undergo meiosis and form four haploid nuclei. However, these nuclei do not become enclosed within the ascospore. As a result, four nuclei remain free in the ascus and four anucleate closed prospores are formed. The anucleate prospores do not form mature ascospore walls, that is, there is no insertion of ascospore wall material between unit

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membranes. This strongly suggests that this developmental stage
is controlled by the haploid nucleus which must be in contact
with the ascospore cytoplasm to exert control over wall deposition.

B. The Vegetative Cell Wall

The composition and structure of the vegetative cell envelope
of yeast has been reviewed in great detail by Phaff (1971) and
Bartnicki-Garcia and McMurrough (1971). The cell wall components
of Saccharomyces cerevisiae include a diverse group of macro-
molecules. Approximately 80% of the dry weight of cell wall
constituents is polysaccharide (Northcote and Horne, 1952; Falcone
and Nickerson, 1956; Miller and Phaff, 1958). Glucan and mannan
are the principal cell wall components. There is controversy as
to the exact nature of the glucan which composes the fibrillar
skeleton for the cell (Northcote and Horne, 1952; Houwink and
Kreger, 1953). It appears to be β-1,3 polymer with β1,6 side
chains (Misaki et al., 1968; Manners and Patterson, 1966). The
mannan is also branched with α-1,6-linked backbone and α-1,2,
α-1,3-linked side chains(Peat et al., 1961; Lee and Ballou, 1965).
A phosphate group is linked to the mannose (Cawley and Letters,
1968) in a molar ratio of 120 moles of mannose per mole of
phosphate (Stewart and Ballou, 1968) and it is diesterified (Mill,
1966). Chitin is present in a less soluble form in the bud
scars which contain approximately 50% chitin and 30-35% glucan
(Bacon et al., 1966). The presence of chitin in bud scars has
been confirmed by the ability of fluorescently-labeled lectin

specific for N-acetylglucosamine polymers to bind to bud scars (Yanagita et al., 1970). Ballou et al. (1977) have also observed that N-acetylglucosamine auxotrophs of Saccharomyces cerevisiae form chains of cells due to incomplete separation of newly formed buds. This can probably be attributed to the inability of mutants to form chitinous cross-walls in the budding process. The bulk of chitin occurs in a more soluble form and has been found throughout the cell wall (Sentandreau and Northcote, 1968). This more soluble form appears to form one type of linkage between mannan and protein in mannan-protein complexes (Sentandreau and Northcote, 1968) as had been suggested earlier by Eddy (1958) and Korn and Northcote (1960). Mannan-protein cell wall constituents have been reviewed recently by Ballou (1974).

The integrity of the cell wall can be attributed in great part to the wall proteins as evidenced by the ability of proteases to lyse cells (Nickerson, 1963). These proteins make up approximately 5-7% of the cell wall constituents and have a high sulfur content (Brown and Hough, 1966), which may contribute to stabilization of the cell wall by disulfide bridging. It has been shown that protoplast formation is often greatly facilitated by pre-incubation in 2-mercaptoethanol or other thiol reagents (Davies and Elvin, 1964). Lipids compose 1-2% of the cell wall (Eddy, 1958).

The components of the vegetative cell wall are proposed to be organized in the following manner. The branched structural mannan-protein forms the outer boundary of the cell wall (Mundkur,

1960). Located directly below is the mannan-protein enzyme

invertase (Preiss, 1958). Beneath the two mannan-protein layers

is a layer of chitin and glucan with protein in close association

with chitin (Phaff, 1971).

The fibrillar component of normal cell walls is the only

component regenerated in liquid medium by protoplasts of yeast

isolated by digestion of the cell wall with snail gut enzyme or

by autolysis. In a gel medium, both the fibrillar component

and amorphous matrix are regenerated (Nečas, 1971). This provides

a method by which the biosyntheses of these two components can be

studied separately. It appears that the fibrillar matrix is

composed primarily β-1,3-glucan. Farkas(1970) has also demon-

strated that 2-deoxyglucose, which interferes with polysaccharide

synthesis, completely inhibits the synthesis of fibrils. Studies

using the inhibitor cycloheximide have shown that wall matrix

synthesis is inhibited by cycloheximide with no effect on fibril

formation (Farkas, 1970).

D. The Bacterial Endospore

The formation of bacterial endospores in Bacillus is

similar to ascospore formation in Saccharomyces cerevisiae with

the exception that meiosis does not occur in prokaryotes. The

cytology of endospore formation has been described by Walker

(1970). The axial chromatin filament forms during stage I.

Stage II involves formation of the forespore septum by the

migration of a portion of the chromatin to one pole of the cell

and invagination of the cell membrane which encloses the chromatin. Total engulfment of the chromatin by completion of the forespore membrane and the development of the exosporium, if present, occurs in stage III. Polybetahydroxybutyrate granules appear in the vegetative cell during stage IV and the cortex is deposited between the layers of the forespore membrane. The cortex subsequently increase in width and differentiates into an inner dense layer or cortex membrane in stage V. The spore coat is deposited during stage V and is followed by maturation of the spore at stage VI. The endospore is liberated by the mother cell at the conclusion of the process in stage VII.

The structure of the bacterial endospore of several species of Bacillus bears a striking resemblance to the ascospore of Saccharomyces cerevisiae. The endospore of Bacillus cereus T possesses a loosely fitting exosporium as observed in electron micrographs of thin sections (Warth et al., 1963a,b). The exosporium consists of a basal layer and an external layer bearing hair-like projections. Matz et al. (1970) have determined the chemical composition to be 52% protein, 20% polysaccharide (0.3% glucose, 6.4% mannose, 3.6% glucosamine, 0.7% ribose), 12.5% neutral lipid, 5.5% phospholipid, and 3.8% ash. The protein appears to contain little methionine or cysteine (Matz et al., 1970) as opposed to the higher sulfur content of the proteins found in the major component of the coat protein (Kondo and Foster, 1967). The spore coat lies just internal to the exosporium and consists of two distinct layers, the outer being less soluble

than the inner (Warth et al., 1963a,b). The spore coat makes up

30-60% of the dry weight of the spore and contains as much as

80% of the total spore protein (Aronson and Fitz-James, 1968).

Protein is the principal component of the spore coat (Warth et al.,

1963a,b). The minor constituents include lipid, ash, carbo-

hydrate, and phosphate. The protein component appears to contain

no carbohydrate (Aronson and Fitz-James, 1971) and consists of

one to two species of polypeptide as determined by SDS-polyacryl-

amide gel electrophoresis and amino-terminal analysis (Aronson

and Horn, 1969). Immunological studies have shown that coat

maturation is inhibited by rifampicin and, presumably, is

dependent on RNA synthesis (Horn et al., 1973). Biochemical

studies indicate that precursor polypeptides of this layer appear

to be synthesized very early in sporulation, that is, during

forespore development and are accumulated for future deposition

(Aronson and Fitz-James, 1968). Cystine incorporation into the

spore coat is detected when well-developed coat layers can be

observed and is due to exchange between half-cystine residues

and disulfides or sulfhydryl groups within the coat polypeptide.

This cystine exchange appears to be essential for coat maturation

(Aronson and Horn, 1969). The spore coat layers can be recon-

stituted in vitro. Spores deprived of their coats become

susceptible to lysozyme (Aronson and Fitz-James, 1971).

In contrast, the spore coat preparations of Bacillus

subtilis appear to be heterogeneous (Kondo and Foster, 1967;

Spudich and Kornberg, 1968). Various disruptive procedures have

been employed resulting in the release of components differing in amino acid composition (Spudich and Kornberg, 1968). Kondo and Foster (1967) have also found that sequential chemical fraction- ation of spore coats from <u>Bacillus</u> <u>megaterium</u> yielded a hetero- geneous preparation based on biochemical criteria including amino acid composition, solubility in various reagents, analytical centrifugation, and X-ray diffraction.

External to the germ cell wall and internal to the spore coat is the cortex, which consists of a peptidoglycan. The glycan portion of the molecule contains β-1,4-alternate-linked N-acetyl- glucosamine-N-acetyl muramic acid. While the tetrapeptide moiety consists of L-alanyl-γ-D-glutamyl- (L)-<u>meso</u>-diaminopimelyl-(L)- D-alanine (Tipper and Gauthier, 1972).

II. MATERIALS AND METHODS

A. Organisms and Culture Conditions

Three strains of the yeast Saccharomyces cerevisiae were used
in these studies. Strain AP-1, a heterothallic adenine auxotroph,
was obtained from A. Hopper. The homothallic prototroph, SK-1,
and asporogenous a/α diploid auxotroph, DM10-2B, were obtained
from the laboratory of Robert Roth. The genotypes of AP-1 and
DM10-2B are presented below. The genetic nomenclature has been
described by Mortimer and Hawthorne (1973).

$$\text{AP-1:} \quad \frac{\text{ade-1} \quad \text{gall} \quad \text{lysl} \quad \text{his7} \quad \text{a} \quad \text{trpl} \quad + \quad + \quad \text{ural} \quad \text{ade-2}}{+ \qquad + \qquad + \qquad + \quad\ \alpha\ \quad + \quad\ \text{canl} \quad \text{ura3} \quad + \quad \text{ade-2}}$$

$$\text{DM10-2B:} \quad \frac{+ \qquad + \qquad \text{leu2-27} \quad \text{a} \quad + \quad \text{ade2-1} \quad \text{met2} \quad \text{ura3} \quad \text{m}}{\text{his4} \quad \text{leu2-1} \qquad + \qquad \alpha \quad \text{thr4} \quad \text{ade2-1} \quad \text{met2} \quad \text{ura3} \quad \text{m}}$$

Cultures were maintained on 1.5% agar plates containing
2% glucose, 2% peptone, and 1% yeast extract (YEP). All percen-
tages have been expressed as weight per volume unless otherwise
specified. Cells were grown in an acetate presporulation medium
at 30 C in an incubator shaker (New Brunswick Scientific Instru-
ments Co., Inc., New Brunswick, NJ) at 250 rpm. Acetate prespor-
ulation medium for AP-1 and DM10-2B contained 1% potassium
acetate, 1% peptone, 0.5% yeast extract, 0.5% ammonium sulfate,
and 0.17% yeast nitrogen base without amino acids and without
ammonium sulfate in 0.05M potassium phthalate buffer, pH 5.2
(PsP2) (Roth and Fogel, 1971). The medium was supplemented with
10 μg/ml adenine and uracil. SK-1 was grown in a vegetative

medium (YPA) (Fast, 1973) containing 1% potassium acetate, 2%

peptone, 1% yeast extract and the following amino acids and

nitrogenous bases at a final concentration of 50 μg/ml: adenine,

uracil, arginine, histidine, isoleucine, leucine, lysine, methio-

nine, and tryptophan.

Cells of strain AP-1 were labeled during vegetative growth

by growing cells in an acetate complete medium (Roth and Halvorson,

1969) which was supplemented with 10 μg/ml adenine and uracil in

the presence of ^{3}H-leucine at a final concentration of 2 μCi/ml

and specific activity of 70,000 mCi/mmole.

Upon reaching a cell density of 2 to 3 x 10^7 cells per ml

as determined using a hemacytometer according to the method of

Esposito et al. (1969), the cells were harvested by centrifugation

at 2500 x g for 5 minutes in a Sorvall RC-5 centrifuge and washed

twice with sporulation medium. Sporulation medium consists of

1% potassium acetate adjusted to pH 6.0. Cells were then suspended

in sporulation medium at 2 x 10^7 cells per ml and incubated in an

incubator shaker at 30 C for 24 to 30 hours. The efficiency of

sporulation was also determined by hemacytometer counts as

indicated above.

Cells labeled during sporulation were pregrown in acetate

presporulation medium (PsP2) as described above, but were sporu-

lated in a buffered sporulation medium to which $H_2^{35}SO_4$ was

added at specific times during sporulation at a final concen-

tration of 20 μCi/ml with a specific activity of 43 Ci/mmole.

Buffered sporulation medium contained 1% potassium acetate and

0.2M 2 N-morpholino ethane sulfonic acid (MES) at pH 5.6

(McCusker and Haber, 1977).

B. Enzymatic Digestion of Asci

Sporulating cells were collected 24-30 hours after inocu-

lation into sporulation medium by centrifugation at 2500 x g for

5 minutes and washed two times with 0.05M potassium phthalate

buffer, pH 5.2. Asci were suspended in 1% glusulase (Endo

Laboratories, Inc., Garden City, NY) in 0.05M potassium phthalate

buffer, pH 5.2 (Snider and Miller, 1966) and incubated in an

incubator shaker for 24 hours at 30 C. Digested asci were

harvested after 24 hours at 2500 x g for 10 minutes and washed

five times with intense vortexing. The cell pellets were stored

at -20 C with no effect on the ensuing extraction procedures and

were washed two times with sterile water prior to proceeding to

the extraction of spore coat material.

C. Spore Coat Extraction

Washed spore pellets obtained after glusulase treatment were

suspended in 8M urea at 1 to 5 x 10^8 cells per ml and incubated

with slow shaking (50-100 rpm) at 35 C. After a six-hour incu-

bation period, urea-soluble material was obtained in the super-

natant fraction after centrifugation at 2500 x g for 10 minutes.

The supernatant was dialyzed with several changes against a total

of approximately 20 volumes of distilled water at 4 C. Insoluble

material was subsequently collected as a pellet upon centrifu-

gation at 12,000 x g for 10 minutes and designated urea-soluble

spore coat material. For purposes of comparison and control,

vegetative cells were harvested, and washed, and subjected

directly to the urea treatment identical to that described for

ascospores.

D. Methods of Gel Electrophoresis

Analyses of urea-soluble components from spores and vegetative

cells were performed on 7.5% SDS-polyacrylamide slab (Studier,

1973) or disc (Laemmli, 1970) gels with a 5% stacking gel in a

tris-glycine buffer system (Laemmli, 1970) at 1 mA per gel.

Electrophoresis was concluded when the tracking dye, bromphenol

blue, migrated within 1 cm of the bottom of the gel, usually

three hours later.

Gels were stained for protein overnight in 0.2% Coomassie

brilliant blue R-250 in destaining solution, consisting of

methanol:glacial acetic acid:water in a 9:2:9 ratio. The gels

were stained and destained at room temperature. The gels were

scanned at 550 nm using a Gilford 240 spectrophometer equipped

with a Gilford 6040 recorder.

Protein concentrations of cold 10% trichloroacetic acid

insoluble material were determined by the method of Lowry et al.

(1951) prior to gel electrophoresis.

To identify carbohydrates, gels were stained by the periodic

acid-Schiff reagent method (PAS) as previously reported (Clark,

1964).

Radioactivity was identified by fluorography of gels as described by Bonner and Laskey (1974).

The molecular weights were determined by the method of Weber et al. (1972). Bovine serum albumin, gamma-globulin, catalase, and ovalbumin were used as molecular weight standards.

E. Pulse-Chase Methods

Pulse-chase experiments carried out in sporulating cultures were performed using $H_2{}^{35}SO_4$ as the radioactive isotope in MES-buffered sporulation medium as described above (Section IIA). Sporulating cells were labeled with $H_2{}^{35}SO_4$ at 20 µCi per ml with a specific activity of 43 mCi/mmole at 0 hour or 4 hours into sporulation. At the end of each four-hour labeling period, the cells were centrifuged at 2500 x g for 5 minutes and washed twice with buffered sporulation medium containing 0.05mM sodium sulfate. Labeled cells were resuspended in unlabeled medium obtained from centrifuged companion cultures to ensure minimal disturbance of the physiological state of the cell. Sterile aqueous sodium sulfate was then added to a final concentration of 0.05 mM to prevent further uptake of any remaining label. The cells were allowed to continue the sporulation process for an additional 16 to 20 hours at 30 C in an incubator shaker.

As a measure of uptake and incorporation of $H_2{}^{35}SO_4$ duplicate 0.2 ml aliquots of cells were removed from cultures at one hour intervals and placed directly into an ice bath. Uptake was assayed by collecting the cells on GF/A filters and

washing the filters twice with 15 ml ice-cold 1 mM sodium sulfate.

The amount of radioactivity incorporated was determined by the

method of Rodenberg et al. (1969). Two volumes of 10% trichloro-

acetic acid were added to the ice-cold samples which were then

incubated for 15 minutes at 100 C. Trichloroacetic acid-insoluble

material was collected on GF/A filters which were subsequently

washed twice with 15 ml of 5% trichloroacetic acid containing

50 µg/ml sodium sulfate followed by 10 ml of 95% ethanol. Radio-

active filters were air dried and placed in 5 ml of toluene

containing 4.2 mg PPO and 0.05 mg POPOP per ml and counted in

a Packard Tri-Carb Liquid Scintillation spectrometer (Model 2425)

which operated at 90% efficiency for ^{35}S and 56% efficiency for ^3H.

F. Preparation of Vegetative Cell Lysates

To prepare vegetative cell lysates from ^3H-labeled cultures,

cells were grown overnight at 30 C in the presence of ^3H-leucine

as described above (Section IIA). Cells were harvested by

centrifugation at 2500 x g for 5 minutes and washed two times with

sterile distilled water. Cells were resuspended at 5×10^7 cells

per ml in 8M urea in a 17 ml screw cap Pyrex test tube. Glass

beads (0.45 to 0.50 mm) were added to the meniscus of the cell

slurry and cells were disrupted with two 20-second agitations on

a Vortex Genie mixer separated by 15 second incubations in an

ice bath. Cell homogenates were centrifuged at 12,000 x g for

10 minutes to remove cell wall debris and the supernatant was

dialyzed against 20 volumes of distilled water at 4 C. The

dialysate was stored at −4 C.

G. Method of Determining the Amount of Non-Specific Binding of Cytoplasmic Components to the Ascospore

The amount of binding of cytoplasmic components to ascospore wall and the subsequent co-isolation of such components with ascospore wall material was demonstrated by the following experiments. The ^{3}H-vegetative cell lysate prepared as described in the previous section was mixed with a urea-treated sporulation preparation (see Section IIC) prior to centrifugation and dialysis. The mixture was then subjected to the remainder of the procedure for the extraction of spore coats, followed by gel electrophoresis and fluorography. The 1% glusulase in 0.05M potassium phthalate buffer, pH 5.2, which had been used to digest asci from an unlabeled culture was reused to digest asci from an unlabeled culture and the spore coats were extracted and analyzed as described above.

H. Urea-Extraction of Glusulase

A volume of 1.0 ml of glusulase was incubated in 10 ml of 8M urea for 6 hours at 35 C in an incubator shaker at 50 rpm. The incubated solution was then centrifuged at 2500 x g and the supernatant was dialyzed against 20 volumes of distilled water at 4 C. The dialysate was centrifuged and the pellet was analyzed by gel electrophoresis to determine the extent to which urea-extractable proteins from glusulase co-purified in

8M urea and migrated in the region of the spore coat protein upon gel electrophoresis.

I. Chemicals

Yeast extract, bactopeptone, yeast nitrogen base without amino acids and without ammonium sulfate, and agar were purchased from Difco Laboratories (Detroit, Michigan). Sigma Chemical Co. (St. Louis, Missouri) supplied urea, bovine serum albumin, catalase, amino acids, adenine, uracil, 2-mercaptoethanol, 2 N-morpholino ethane sulfonic acid, Trizma base, and Coomassie brilliant blue R-250. Glusulase was purchased from Endo Laboratories, Inc. (Garden City, New York). Gamma-globulin was obtained from Nutritional Biochemicals Co. (Irvine, California). Ovalbumin and ^{3}H-leucine were supplied by Schwarz/Mann Biochemical Co. (Orangeburg, New York). $H_2^{35}SO_4$ was purchased from New England Nuclear (Boston, Massachusetts). Potassium phthalate and periodic acid were supplied by J.T. Baker (San Francisco, California. Matheson, Coleman and Bell Manufacturing Chemists (Norwood, Ohio) were the source of glycine and ethylenediaminotetraacetic acid, disodium salt. Glycerol was purchased from American Scientific and Chemical (Portland, Oregon). Acrylamide, bis-acrylamide, N,N,N',N'-tetra-methylethylene diamine (TEMED) and sodium dodecyl sulfate (SDS) were obtained from Bio-Rad Laboratories (Richmond, California). Toluene, POPOP, PPO, potassium acetate, glucose, and sodium sulfate were purchased from Mallinckrodt Chemical Works (St. Louis, Missouri). Bovine serum albumin and gamma-globulin were the

gifts of Dr. Anne J. Anderson. Dr. R.O. Hampton generously

provided catalase and ovalbumin. The Schiff reagent used in

carbohydrate staining studies was a gift of Dr. Fred Rickson.

All three donors are faculty members of the Department of

Botany and Plant Pathology at Oregon State University (Corvallis,

Oregon).

III. RESULTS

Each of the three strains of the baker's yeast <u>Saccharomyces</u>

<u>cerevisiae</u> used in these studies sporulate with different

kinetics and efficiency (Figure 1). Strain AP-1 sporulates with

approximately 70% efficiency in a 24 hour period beginning at 10

to 12 hours after inoculation into sporulation medium. In

contrast, SK-1 sporulates to 100% efficiency in 15 hours beginning

at 8 to 10 hours and probably represents a much more synchronous

population of cells. Although this strain would seem to be

ideal for studies regarding the timing of a sporulation-specific

event, SK-1 may give misleading results because of its ability to

sporulate to a limited extent (less than 1%) in a vegetative

growth medium (J. Runnels, personal communication). Strain AP-1

is most representative of a majority of sporulating strains. The

asporogenous a/α mutant, DM10-2B, is blocked in the process of

premeiotic DNA synthesis (see Section 1B.1.).

The approach used to isolate the spore coat takes advantage

of the apparent hydrophobicity of the ascospore and the solubility

of the outermost electron dense layer in 8M urea (Briley <u>et</u> <u>al</u>.,

1970). Intact exponentially growing vegetative cells or asco-

spores isolated by enzymatic digestion of asci were incubated in

8M urea to solubilize outer wall components. This procedure did

not appear to distort the shape of the cells when observed under

the light microscope. Dialysis of urea-soluble material yielded

a heavy precipitate from a spore preparation and a barely

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Figure 1.   Sporulation kinetics of yeast strains AP-1,
            SK-1, and SM10-2B. Cells were harvested
            from acetate presporulation medium at 1-3 x 10^7
            cells per ml and resuspended in sporulation
            medium (1% potassium acetate, pH 6.0) at a
            density of 2 x 10^7 cells per ml. Sporulating
            cells were incubated at 30 C in an incubator
            shaker. AP-1 was also sporulated in buffered
            sporulation medium containing 0.2M MES.
                 AP-1;   SK-1;   DM10-2B;
                 AP-1 in buffered sporulation medium.
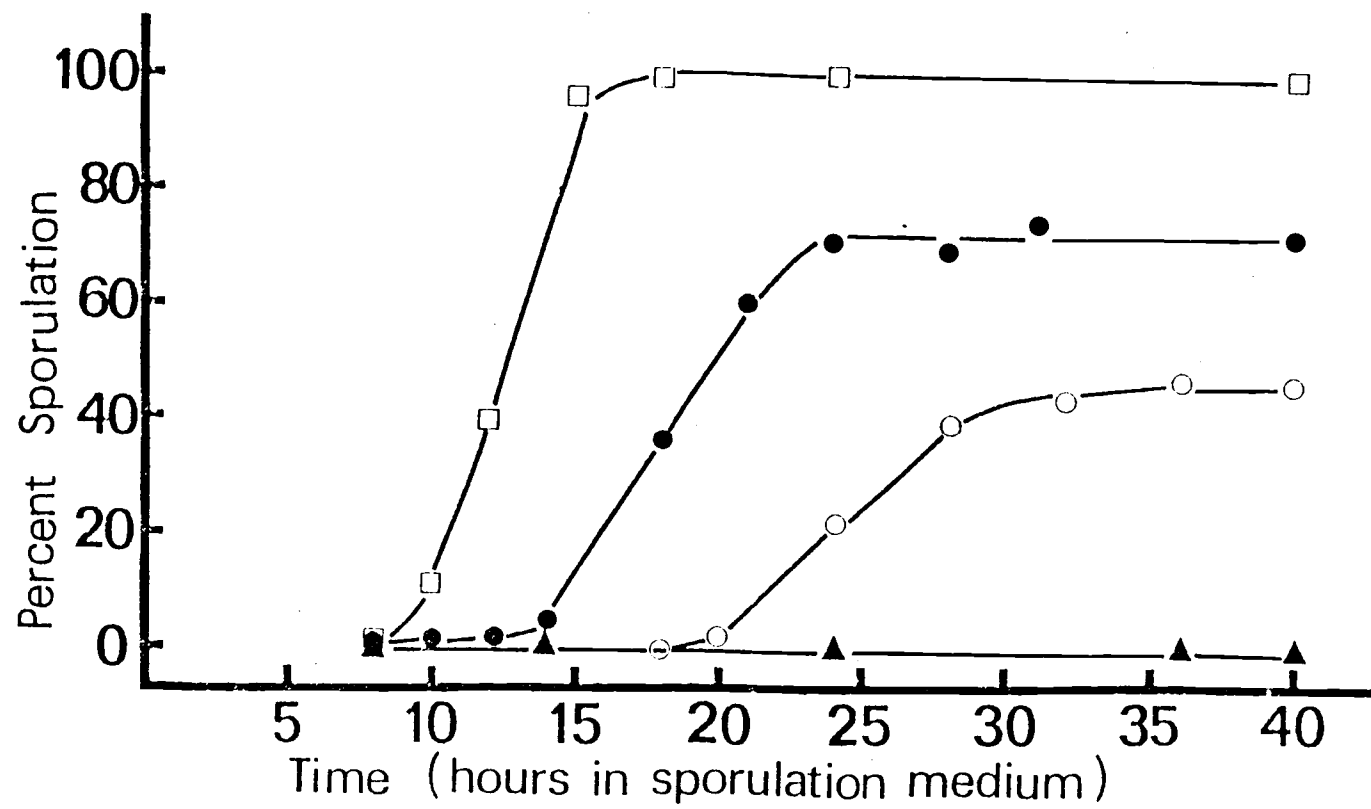
noticeable precipitate from vegetative cells. Approximately 10^8 sporulating cells were required to obtain 100 ug of protein as determined by the Lowry method (Lowry et al., 1951) from cold trichloroacetic acid insoluble material. On the other hand, in excess of 10^{11} vegetative cells were required to obtain 100 ug of protein with this procedure. To determine whether or not the cell wall constituents from vegetative cells and ascospores differed, the urea-soluble protein was precipitated during dialysis against water and solubilized in sample preparation buffer for Laemmli gels (Laemmli, 1970). Proteins from spores and vegetative cells of strain SK-1 show a distinct difference in banding patterns (Figure 2). A predominant protein band was extracted from ascospores which migrated with an R_f of approximately 0.79 (Figure 2B). No major proteins were isolated from vegetative cell extracts (Figure 2A).

A second strain, SK-1, was also treated in an identical manner and anlyzed by disc gel electrophoresis to ascertain whether the major protein was a component of ascospores of all strains or merely a protein which as unique to strain AP-1. Again, a marked difference was observed between the banding patterns of proteins from vegetative cells and spores (Figure 3). The relative mobility of the predominant band from the spore preparation of SK-1 appeared to be identical to that from AP-1 (Figure 3B) and no major proteins were isolated from vegetative cells (Figure 3A). A further indication that the urea-extractable protein was readily detectable only in sporulation-competent cells was indicated by

Figure 2.   Electrophoretic SDS-polyacrylamide gel patterns
            of 8M urea-soluble vegetative and ascospore
            wall components from intact cells of AP-1.
            Intact vegetative cells or ascospores were
            incubated for 6 hours in 8M urea at 35 C.
            Urea-soluble material was dialyzed extensively
            and the resulting precipitate was collected
            by centrifugation. The precipitate was
            analyzed on 7.5% SDS-polyacrylamide gels.
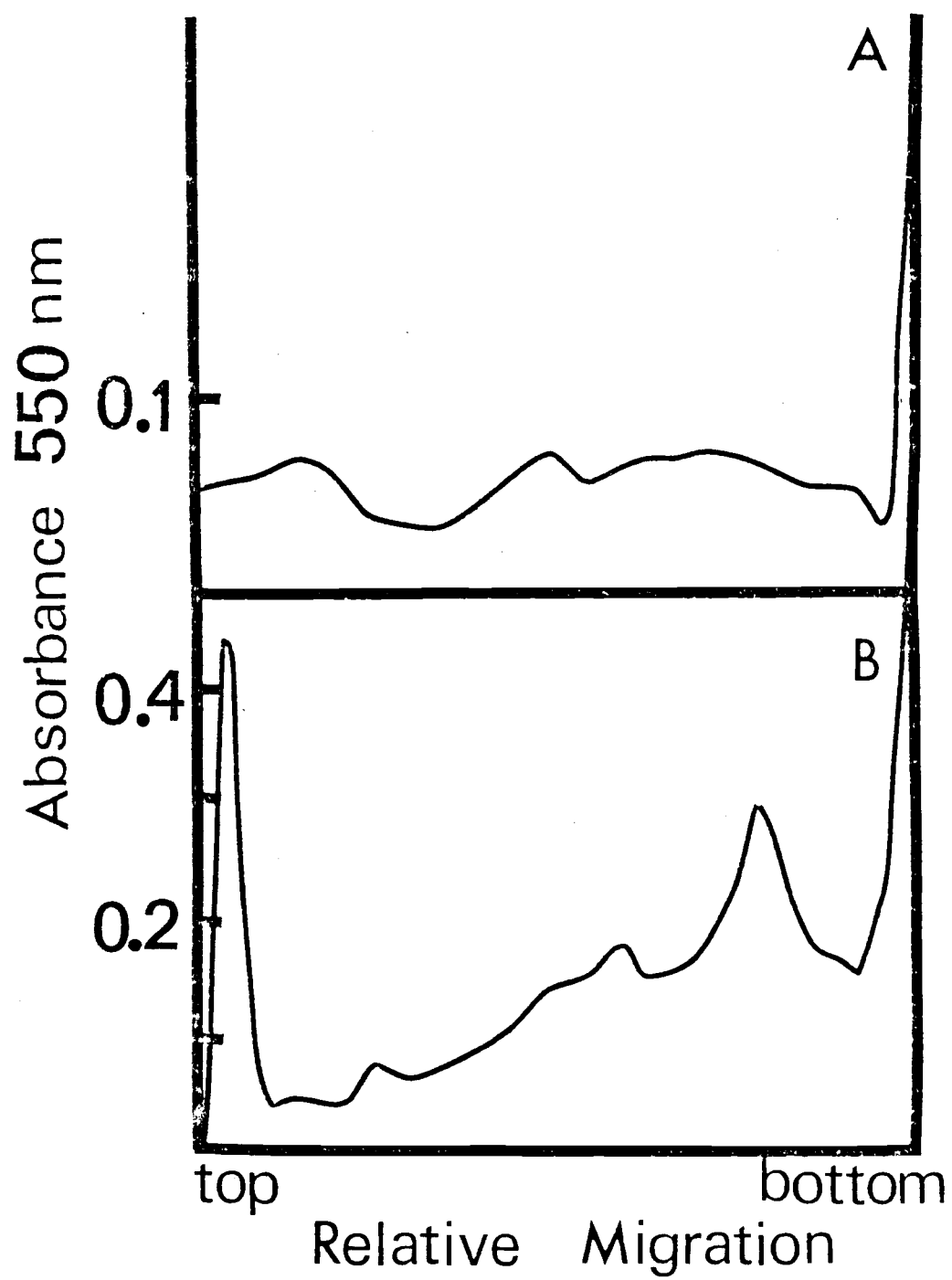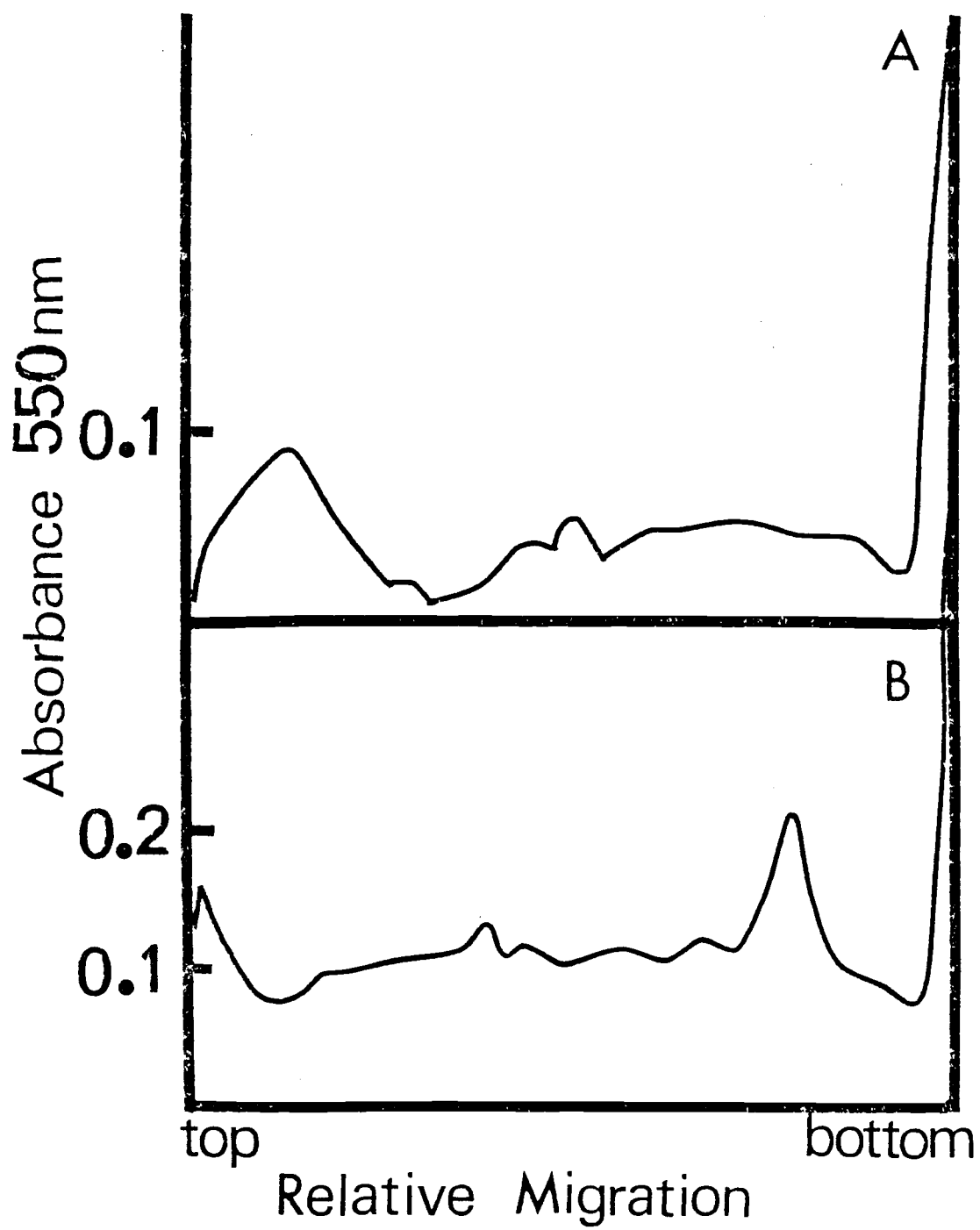   A, vegetative; B, spore.

Figure 3. Electrophoretic SDS-polyacrylamide gel patterns
of 8M urea-soluble vegetative and ascospore wall
components from intact cells of SK-1. Description
and legend same as for Figure 2.

the results obtained using the asporogenous mutant, DM10-2B.

Cells of DM10-2B were subjected to sporulating conditions and

subsequently incubated in urea, dialyzed and the precipitate

collected and analyzed by gel electrophoresis. The banding

pattern of the urea-soluble proteins do not correspond to the

major protein isolated from ascospores of strains AP-1 or SK-1

(Figure 4).

The predominant protein of ascospores does not appear to

contain a carbohydrate moiety that is detectable by periodic acid-

Schiff reagent (PAS) staining procedures (Clarke, 1964) (Figure 5).

The molecular weight of the component was determined to be

37,000 daltons from the relative migration in SDS-polyacrylamide

gel electrophoresis with standards of known molecular weights

(Figure 6).

Since the urea-soluble protein was present only in extracts

of sporulated cells, it was of interest to determine whether its

synthesis was limited to that period when cells were incubated in

sporulation medium or if reduced levels occur in vegetative cells.

Proteins were pulse-labeled in a buffered sporulation medium

(McCusker and Haber, 1977) to optimize uptake of label at all

intervals.

Two separate sporulating cultures were pulse-labeled with

20 μ Ci/ml of $H_2{}^{35}SO_4$ (sp. act. 43 Ci/mmole) during the interval

from 0-4 hours and 4-8 hours. Upon completion of the labeling

period, the cells were harvested and suspended in cell-free

unlabeled buffered sporulation medium that was obtained from

Figure 4.   Electrophoretic SDS-polyacrylamide gel patterns
            of 8M urea-soluble wall components from cells of
            DM10-2B after a 24 hour incubation in sporulation
            medium. Cells of DM10-2B were pregrown in an
            acetate presporulation medium to mid-log phase.
            Cells were then harvested and suspended in
            sporulation medium and allowed to incubate in
            an incubator shaker for 24 hours at 30 C, after
            which they were incubated for 6 hours at 35 C
            in 8M urea. Urea-soluble material was dialyzed
            extensively and the resulting precipitate was
            collected by centrifugation. The precipitate
            was analyzed on 7.5% SDS-polyacrylamide gels
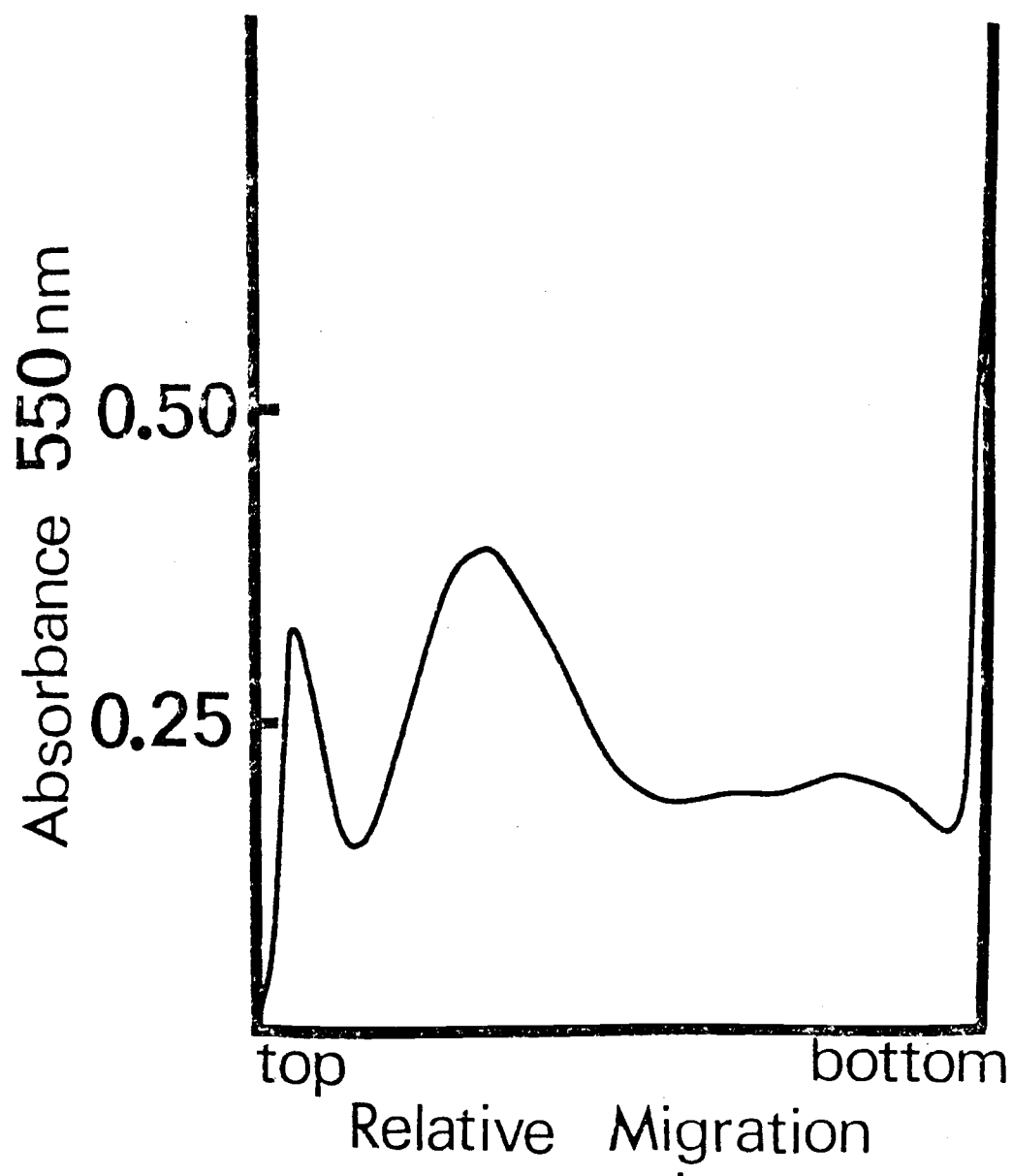   which were stained with Coomassie brilliant blue.

Figure 5.   Periodic acid–Schiff reagent (PAS) staining of
            urea-soluble ascospore wall components in 7.5%
            SDS-polyacrylamide disc gels from strain AP-1.
            A, gel stained with Coomassie brilliant blue;
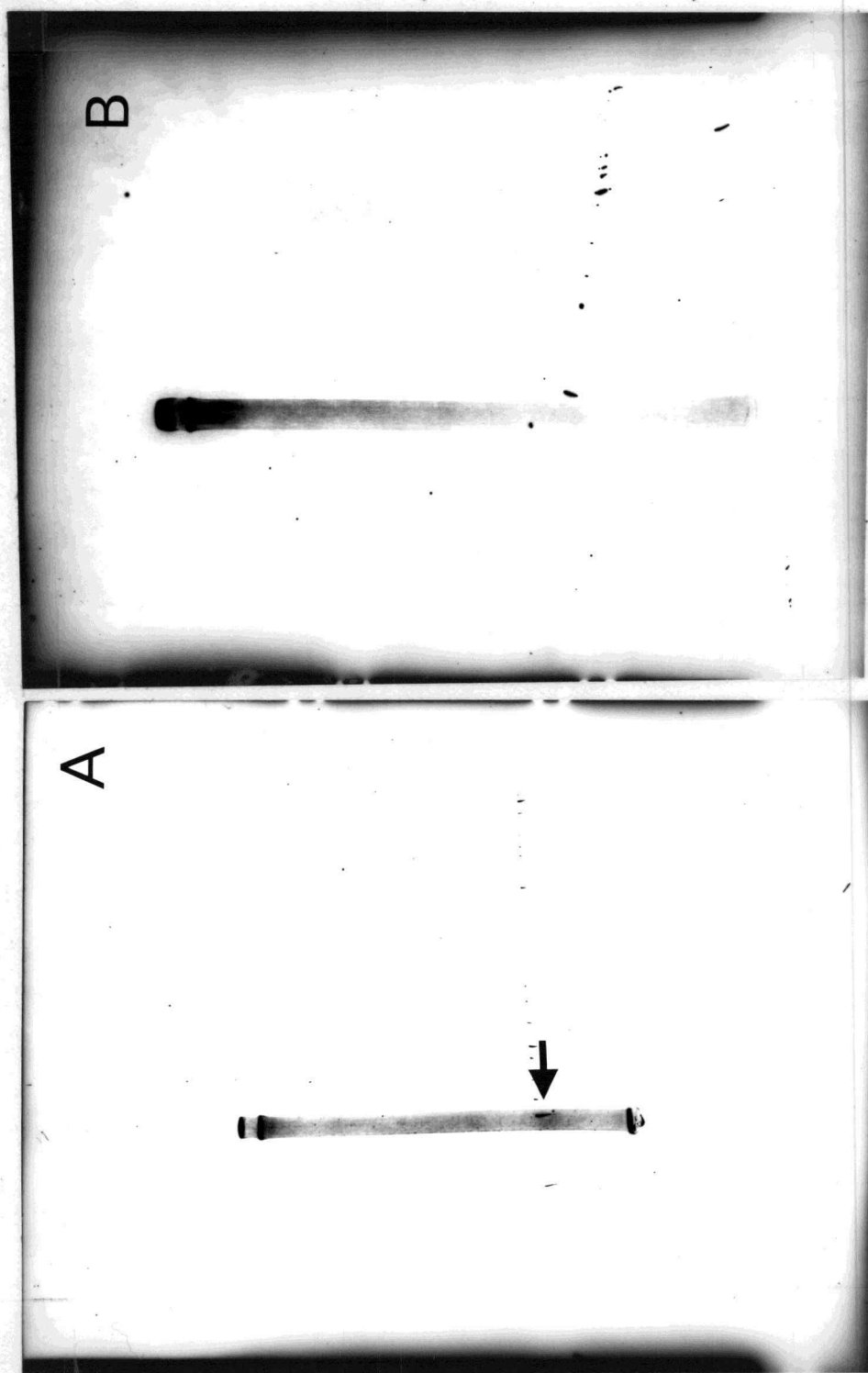   B, gel stained by the PAS method.

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Figure 6. Molecular weight determination of the predominant
ascospore wall component as determined by
SDS-polyacrylamide gel electrophoresis. Molecular
weight standards: bovine serum albumin (68,000),
catalase (58,000), γ-globulin, reduced (heavy,
50,000; light, 23,500), and ovalbumin (43,000),
lane 1; Ascospore wall extract from strain AP-1,
lane 2.
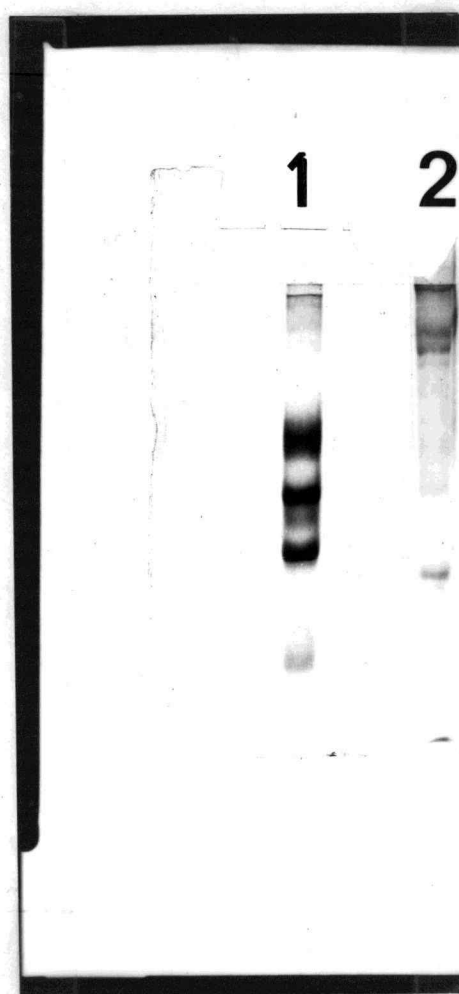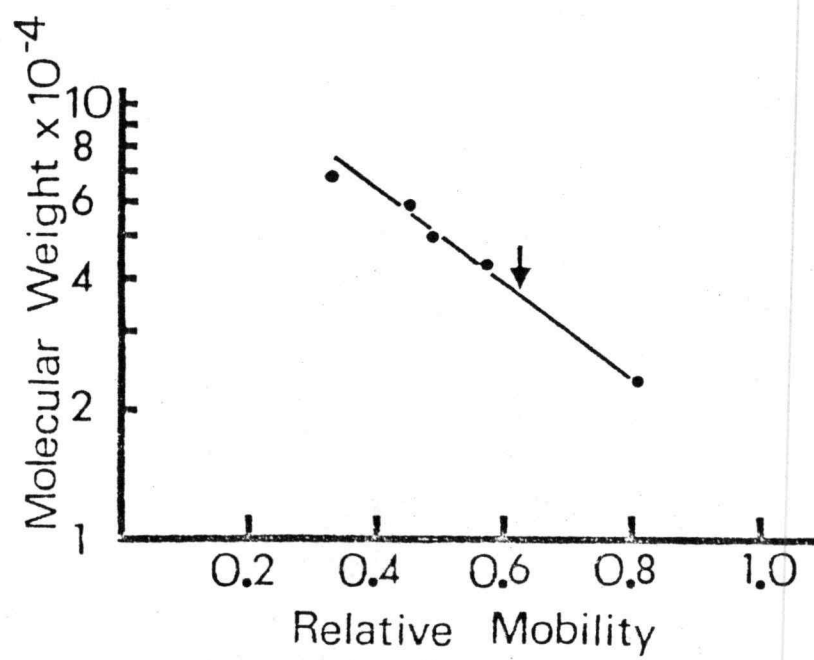
cultures which had been incubated under identical sporulating

conditions. Sterile aqueous sodium sulfate was added (final

concentration, 0.05 mM) to stop further uptake of $H_2{}^{35}SO_4$.

At this concentration no further uptake occurred as determined

by samples taken two and four hours after the end of the 0-4

hour pulse-labeling period (Figure 7A). Uptake and incorporation

were linear over the four-hour labeling periods (Figure 7). After

exposure to sporulating conditions for a total of 24 hours, spore

wall proteins were extracted and analyzed by SDS-polyacrylamide

slab gel electrophoresis and the radioactivity determined by

fluorography. The major urea-soluble protein was isolated from

each culture and the protein was radioactive (Figure 8) as

early as eight hours prior to ascospore formation.

The early synthesis of this protein during sporulation

(0-4 hours) suggested that perhaps it also may be synthesized

in the vegetative cell in a lower concentration which was not

readily detectable by Coomassie blue staining. This possibility

was examined by combining a labeled vegetative cell lysate with

ascospores and urea-extracting with subsequent analysis of extracts

as previously described. The vegetative cell lysate was obtained

from cells which were labeled overnight with ^{3}H-leucine at 2 µCi/ml

(sp. act. 70,000 mCi/mmole) and lysed in urea with glass beads by

vortexing (Section IIF). The lysate was centrifuged and the

supernatant dialyzed. The dialyzed vegetative lysate was added

to an unlabeled urea-soluble ascospore preparation and the mixed

preparation was further dialyzed and centrifuged at 12,000 x g

Figure 7. Uptake and incorporation of $H_2{}^{35}SO_4$ into sporulating cells of AP-1 during pulse-labeling. A chase was performed at the completion of each labeling period (arrow) and cells were assayed for inhibition of continued uptake two and four hours after the 0-4 hour labeling period. A, pulse-labeling period from 0-4 hours; B, pulse-labeling period from 4-8 hours.   uptake;   incorporation.
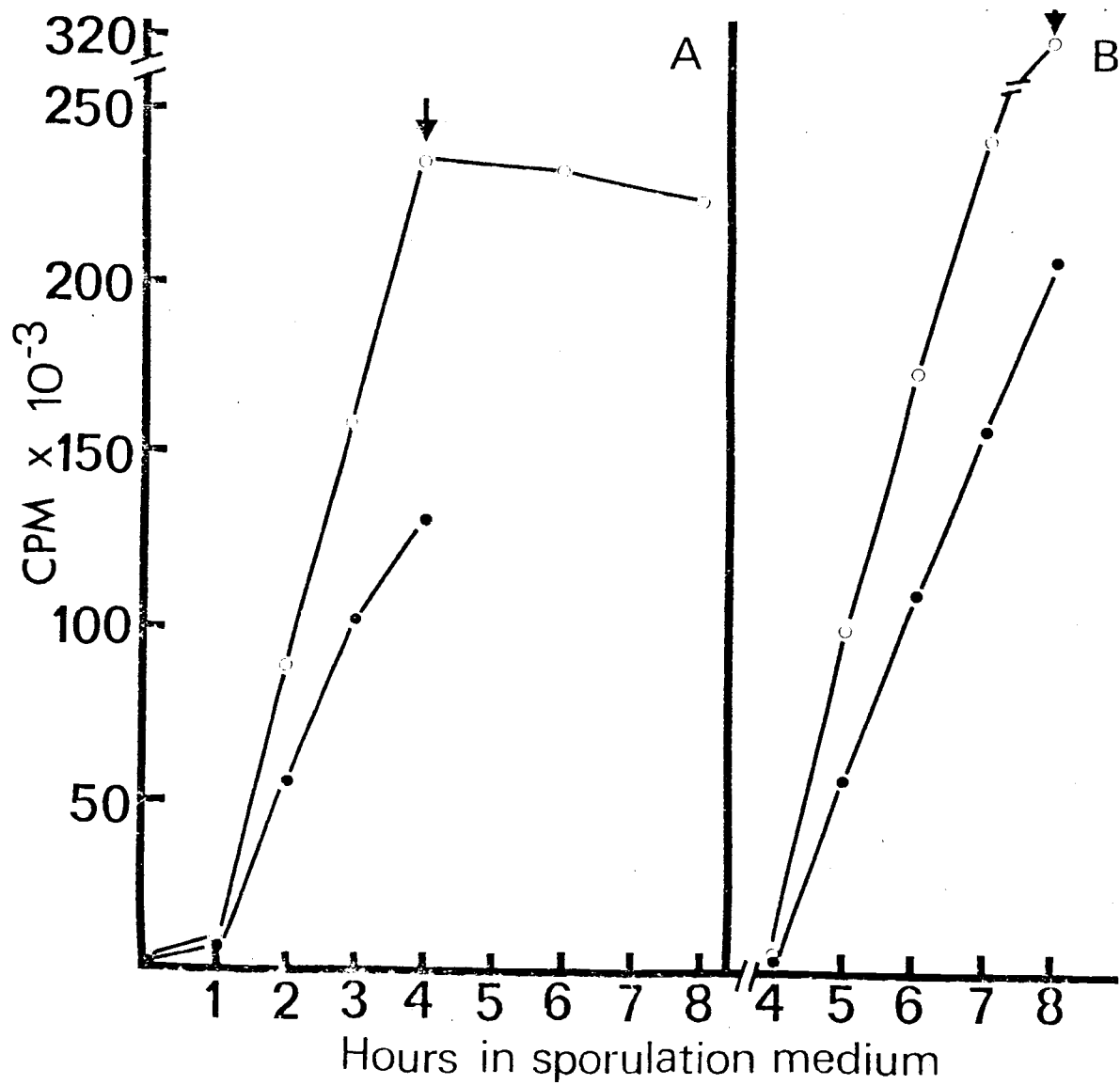
Figure 8.   Distribution of radioactivity of stained slab gels
from ^{35}S-pulse-labeled sporulating cultures of strain
AP-1.   Hydrophobic urea-soluble ascospore wall
components from cells labeled from 0-4 hours and
from 4-8 hours were electrophoresed on 7.5% SDS-
polyacrylamide gels, stained with Coomassie brilliant
blue, and exposed fluorographically.   A, stained gel;
B, fluorographic exposure of stained gel.   Lanes
1 and 3, urea-soluble ascospore wall components
pulse-labeled from 0-4 hours and added to the gel
in a lower concentration (lane 1) and higher concen-
tration (lane 3) to visualize the staining in the
gel.   Lanes 2 and 4, same as lanes 1 and 3, except
that pulse-labeling period is from 4-8 hours.

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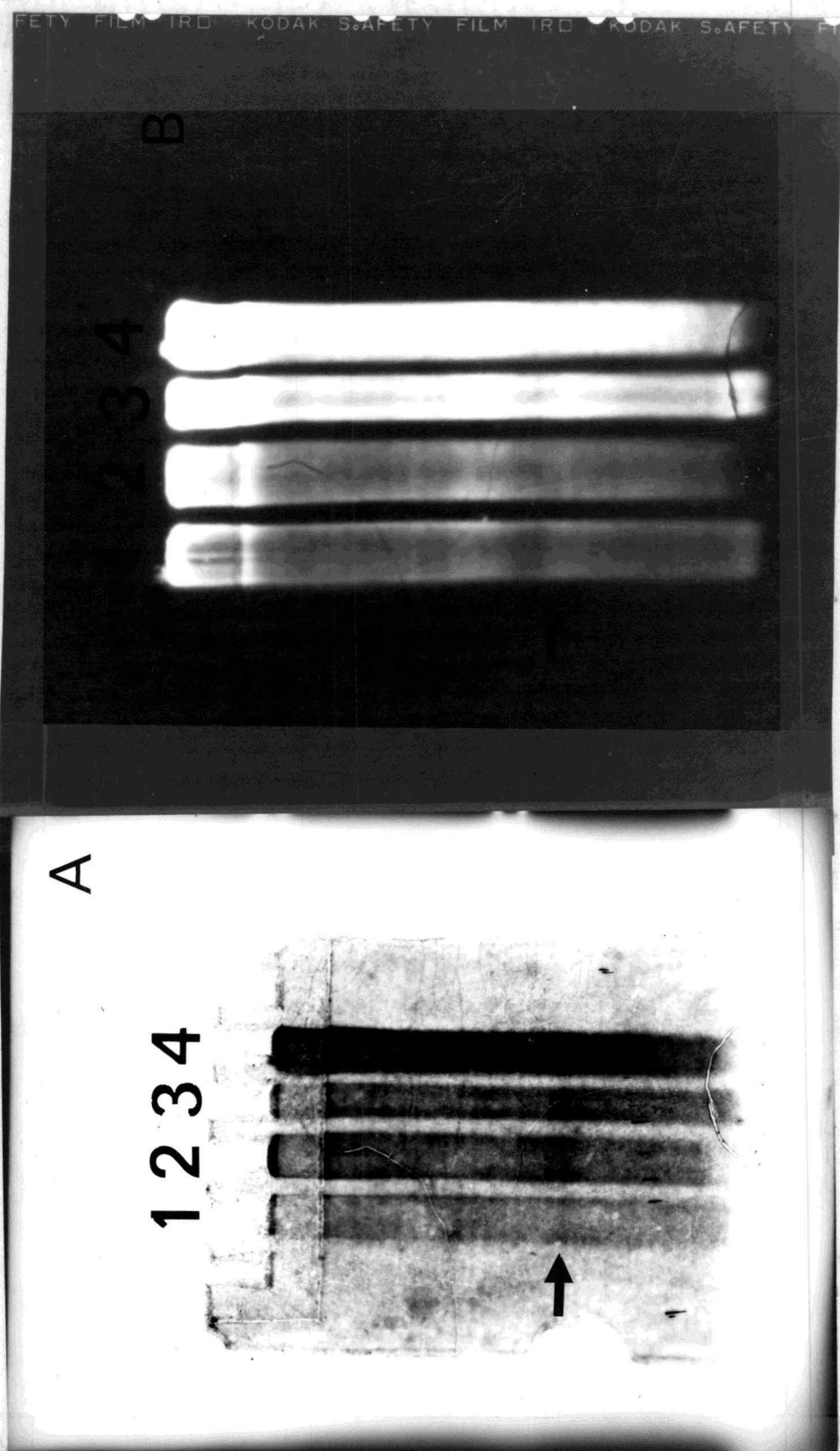
for 10 minutes. The resulting pellet was analyzed on 7.5% SDS-polyacrylamide gels and the radioactive banding pattern determined by fluorography. The vegetative lysate contained a protein which comigrated with the predominant urea-soluble protein (Figure 9).

It was also of additional interest to determine whether cytoplasm excluded from spores but included in the spore mother cell also bound to the ascospore wall and was subsequently co-isolated with it. Glusulase, which was previously used to digest a sporulating culture that had been pulse-labeled with $H_2{}^{35}SO_4$, was reused to digest asci obtained from an unlabeled sporulating culture. After digestion of asci, the ascospore walls were urea-extracted and the precipitate obtained upon dialysis was collected and analyzed by gel electrophoresis. Fluorographic exposures indicate that no labeled component in the spore-excluded cytoplasm was co-isolated with ascospore wall components (Figure 9).

Another consideration in analyzing the methods employed was to determine whether or not any glusulase components also contributed to some of the optical density observed in the region of the spore protein but would not be present in vegetative preparations which do not involve the use of glusulase. A 1.0 ml aliquot of glusulase was urea-extracted and the precipitate obtained after dialysis was electrophoresed on a 7.5% SDS-polyacrylamide slab gel (Figure 10) in conjunction with 100 µg of glusulase and an ascospore wall extract. It is important to note that no observable precipitate was detected upon urea-

Figure 9. Stained slab gel and distribution of radioactivity
on gel demonstrating cytoplasmic binding to ascospore
wall extracts of AP-1. A, stained gel; B, fluorographic
exposure of stained gel. Lane 1, ^{3}H-vegetative cell
lysate mixed with isolated ascospores prior to urea-
extraction as per Material and Methods; Lane 2,
unlabeled asci were incubated in the presence of
glusulase previously used to liberate asci from
a sporulating culture that had been pulse-labeled
with $H_2{}^{35}SO_4$. Lanes of stained gel correspond to
fluorographic exposure.

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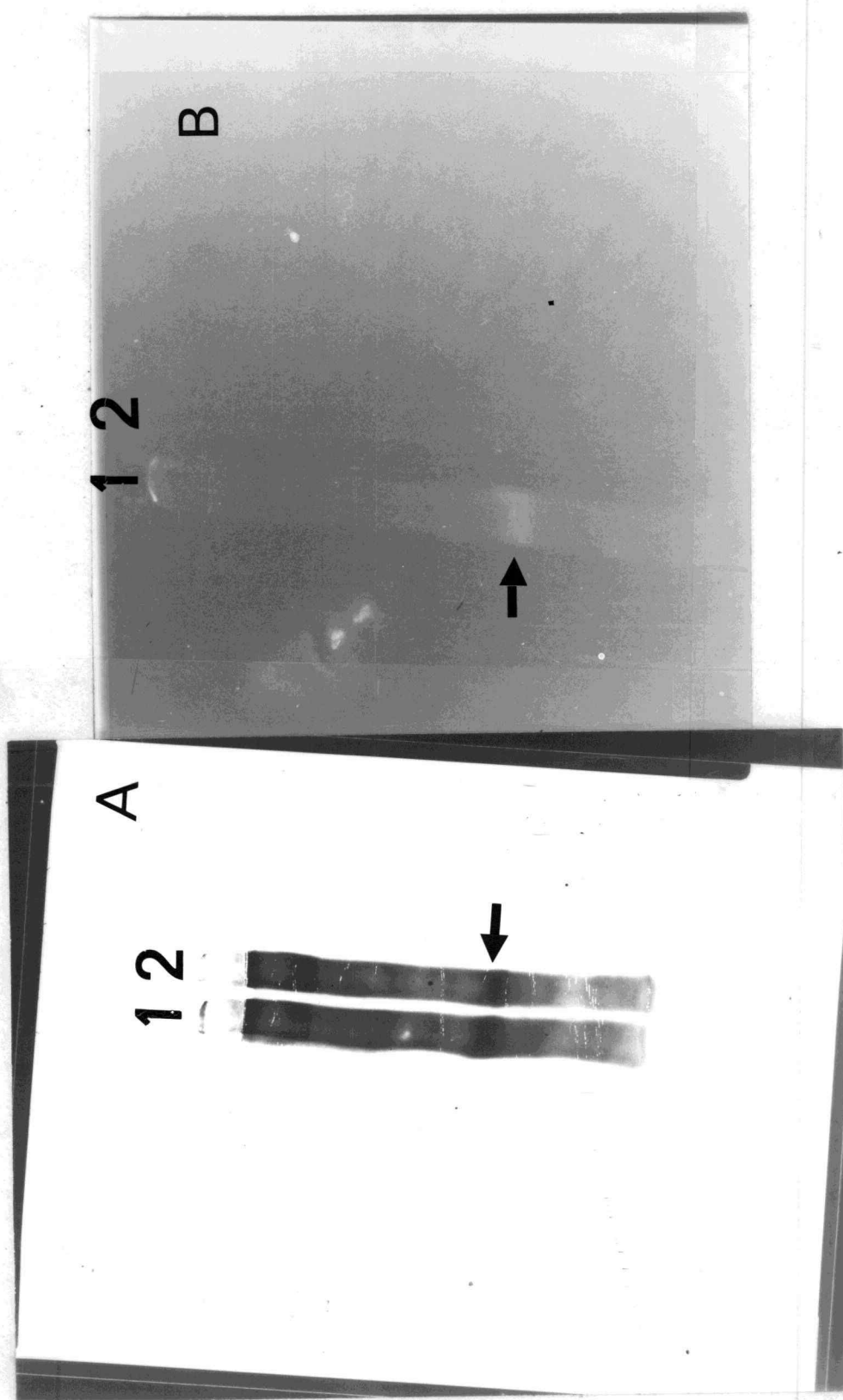
Figure 10. Comparison of electrophoretic mobilities of asco-
spore wall components of AP-1, 100 µg glusulase,
and 1.0 ml urea-extracted glusulase on 7.5% SDS-
polyacrylamide slab gels. Lane 1, ascospore wall
extract from AP-1; Lane 2, 1.0 ml urea-extracted
glusulase; Lane 3, 100 µg glusulase.

extraction and dialysis of concentrations of glusulase employed in ascospore wall preparations and subsequent gel electrophoresis. The original volume of urea-extracted glusulase which co-migrated with the ascospore wall component represents approximately 150 times of the volume used in the isolation of spore wall components.

IV. DISCUSSION

The differentiation of the ascospore wall is closely associated with the process of meiosis (Moens, 1971; Moens and Rapport, 1971) in Saccharomyces cerevisiae . Snail gut enzyme preparations used to digest asci contain a heterogeneous group of proteins, of which thirty or more have been studied (Holden and Tracy, 1950). Two-thirds of the enzymes actively degrade various carbohydrates. Although the component that has been identified as major ascospore wall component appears to be proteinaceous, other possibilities exist. These possibilities include:

i) a partial protein cleavage product stemming from harsh glusulase treatment,

ii) a carbohydrate-protein complex in which either the carbohydrate is not detectable by the staining procedures used or makes up only a miniscule fraction of the component, and/or

iii) the carbohydrate has been digested by the exposure to glusulase.

A previously described method for obtaining the outermost electron-dense layer of the spore from the yeast Saccharomyces cerevisiae (Briley et al., 1970)was used to identify a spore coat component. The tec components from intact cells has been employed in studies involving the bacterial endospore of Bacillus cereus (Horn et al., 1973) and the more fragile chick fibroblasts (Weston and Hendricks, 1972;

Yamada and Weston, 1974). Intact endospores of Bacillus cereus
were treated with 8M urea or 1% (w/v) sodium dodecyl sulfate
containing 50 mM dithioerythritol for three hours at 27 C to
obtain spore coats (Horn et al., 1973). A major cell surface
glycoprotein from normal chick fibroblasts also was isolated by
incubation of fibroblasts in Hank's balanced salt solution
containing 0.2M urea (Weston and Hendricks, 1972; Yamada and
Weston, 1974).

There is good evidence that N-acetylglucosamine is necessary
for the appearance of the outermost electron dense layer of the
spore coat. An N-acetylglucosamine auxotroph of Saccharomyces
cerevisiae has been isolated (Wheelan and Ballou, 1975; Ballou
et al., 1977) which undergoes meiosis, but forms aberrant spores.
These abnormal spores lack the outermost electron dense layer.
Other evidence from the electrophoretic mobility of intact
spores prior to and after protease treatment in addition to
electron micrographs of spores after identical treatment seem
to indicate that the outermost layer is proteinaceous (Briley
et al., 1970). These two studies suggest that the outer coat
is either: i) a glycoprotein or proteoglycan involving N-acetyl-
glucosamine as its carbohydrate moiety or ii) N-acetylglucosamine
serves as a cross link between the inner electron transparent
spore coat and the outermost electron dense layer. The second
possibility is particularly enticing as it is known that such a
complex does exist in normal vegetative cell envelopes (Sentandreau
and Northcote, 1968) and that the inner spore coat is thought to

be the new cell wall upon germination of the spore (Skinner et al.,

1951; Hashimoto et al., 1958).

The radioactive isotope of sulfur, ^{35}S, was chosen to

radioactively label sporulating cultures because of its high

specific activity. Adequate uptake and incorporation occur in

the absence of exogenous sulfate. The use of tritiated amino

acids to label proteins during sporulation may be less desirable

as carrier amino acid is often necessary for optimum uptake

and excess amino acid is necessary to inhibit further uptake

in pulse-chase studies. This exogenously-supplied amino acid may

act as a source of nitrogen to the sporulating cell if supplied

in sufficient amounts and could possibly inhibit the process of

sporulation to varying degrees depending on the interval of labeling.

During the course of each four-hour labeling period, uptake and

incorporation remain fairly linear (Figure 7). Breton and

Surdin-Kerjan (1977) have recently presented evidence that

sulfate uptake in Saccharomyces cerevisiae is mediated by a two-

enzyme system. Teh biphasic Lineweaver-Burk plot for sulfur

uptake is further substantiated by the identification of a strain

bearing two indipendent mutations each of which leads to altered

kinetics of slufate uptake. Sulfate permease I has a high affinity

for its substrate (K_m = 0.005 mM), while permease II has a lower

affinity (K_m = 0.35 mM). Exogenous methionine or S-adenosyl-

methionine regulate the synthesis of these permeases. Exogenously-

supplied sulfate at an initial concentration of 0.05 mM has been

shown to inhibit sulfur uptake. In the study described here,

0.05 mM sodium sulfate was used in pulse-chase experiments to inhibit further uptake of $H_2{}^{35}SO_4$. Cells analyzed two and four hours after a four-hour pulse labeling period revealed that the amount of radioactivity in the cell during the chase period was virtually constant (Figure 7).

The predominant spore wall protein appears to by synthesized early during sporulation during the periods of known high metabolic activity (Esposito et al., 1969; Hopper et al., 1974) and may also be present prior to sporulation in small quantities in the vegetative cell wall and cytoplasm. These speculations are drawn from the fact that a component from radioactively-labeled hydrophobic, urea-soluble, exponentially-growing vegetative cell lysates was co-purified with the predominant ascospore wall component (Figure 9). The vegetative cell may contain small amounts of the spore protein in the vegetative cell wall and/or cytoplasm, while the spore wall possesses substantially more.

It was of interest to determine whether or not glusulase enzymes were also isolated in the process of ascospore wall extraction. It was found that one component co-migrated with the ascospore wall component in SDS-polyacrylamide gels after urea-extraction and dialysis (Figure 10). The volume of glusulase which was urea-extracted is equivalent to 150 times the volume used to digest adequate numbers of asci to obtain 100 μg of protein as determined by the Lowry method (Lowry et al., 1951) of cold trichloroacetic acid-insoluble material. No observable precipitate was detected upon dialysis of urea-extracted glusulase

at concentrations of glusulase used in ascospore wall preparations and subsequent gel electrophoresis. These results seem to indicate that glusulase contributes a minor portion of the optical density observed in the region of the spore protein. This would not be observed in vegetative cell wall extracts as glusulase was not used in the vegetative cell wall extraction process.

Another consideration in the analysis of results is the observation that approximately 1000 times the number of vegetative cells are necessary to obtain an equivalent amount of protein as that obtained from ascospores. Spore wall preparations yieldeing 100 μg of protein as determined by the method of Lowry et al. (1951) from cold trichloroacetic acid-insoluble material were obtained from approximately 10^8 cells. However, greater than 10^{11} vegetative cells were required to obtain 100 μg of protein with this procedure. These results indicate that either the ascospore wall component is absent from vegetative cell walls or that it is present in quantities that cannot be detected by the method used.

In summarizing the results of this study, it has been demonstrated that the ascospore wall contains at least one component not detected in similar concentrations of the cell walls of vegetative cells. This wall component of ascospores can be detected in extracts from intact spores by solubilization in urea and subsequent dialysis of urea-soluble material yielding an insoluble fraction. The precipitate has been analyzed by SDS-polyacrylamide gel electrophoresis. A comparison between

vegetative cell wall and ascospore wall extracts indicates the existence of an ascospore wall component absent from or not detected in vegetative cell wall extracts having a molecular weight of approximately 37,000 daltons. This component appears to be proteinaceous. No carbohydrate moiety could be detected by the periodic acid-Schiff reagent method of staining. However, these results do not preclude the possibility that the component may be a carbohydrate-protein complex. This component appears to be synthesized as early as eight hours prior to ascospore formation. It is unclear as to whether the synthesis of the so-designated spore wall component is a sporulation-specific event.

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