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Influence of Ionizing Radiation on the Physiology of Spores of Cl. botulinum

Period: 29 December 1959 - 28 January 1963

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INTRODUCTION

The unusual resistance of bacterial spores to adverse conditions has been a source of fascination to many microbiologists since spores were first described by Koch (1876) and Cohn (1876). The spores of Clostridium botulinum have been of practical interest because of their importance in food spoilage. Much of the early work on botulinal spores was on elucidation of the factors affecting sporulation and germination (Knaysi, 1948; Wynn et al, 1948; 1951; 1954; 1955; 1956; and 1958; Treadwell et al, 1959; Hitzman et al, 1957; Rieman, 1957; and Perkins, 1962); and the general resistance of these spores to inimical agents (Curran, 1952).

Recently the chemical composition and fine structure of aerobic spores have received much study, directed especially toward elucidation of factors involved in resistance and induction of germination (Foster, 1957; Powell, 1953; Mayall et al, 1957; Halvorson, H., 1957; Young, 1959; Fitz-James, 1960; and Rode et al, 1962). However, similar studies with spores of Cl. botulinum are meager, due, perhaps, to the inherent problems of producing, harvesting and cleaning large masses of these spores.

During studies of aerobic spores, heat resistance has been correlated with a critical level of dipicolinic acid (DPA) (Foster, 1957; Keynan et al, 1961); radiation resistance has been correlated with a high sulfhydryl content (Vinter, 1957 and 1961); permeability has been associated with pore size, (Gerhardt, 1961, Black, 1962) a factor which in turn appears to be involved in resistance to chemicals. However, the mechanisms by which each compound or characteristic confers a specific resistance remains obscure.

The early part of this study was concerned with developing simple, rapid and efficient method for producing, harvesting and cleaning masses of spores of Cl. botulinum 62A and with reporting observations made during physiological and chemical studies on these spores. The latter part of the study was directed toward: (1) elucidation of the fine structure through studies of spores prepared for electron microscopy by fractionation, high level gamma irradiation and thin sectioning techniques; and (2) isolation and partial characterization of the morphological elements.

SELECTED LITERATURE SURVEY

Sporulation, the process by which vegetative cells produce spores, has been viewed rather differently at various periods during the past century. Behring (1889) considered sporulation to be an intermediate stage in the normal development of the bacterial cell, which could be partially or completely inhibited by some partial physiological damage short of total prevention of growth. Later, Knaysi (1948) advanced the hypothesis that endospores were formed by healthy cells facing starvation. In 1949, Foster and Heiligman enlarged upon Behring's hypothesis by stating that sporulation was a sequence of integrated biochemical reactions which were independent of vegetative growth and which could be interrupted at certain susceptible stages. Murrell (1962) has viewed sporulation as a special type of cell division in which the spore is a phenotypic expression of the vegetative cell nucleus.

The general nutritional and environmental factors affecting sporulation had been rather thoroughly investigated by 1957. The results of studies on environmental and nutritional factors affecting sporulation has been extensively reviewed by: Knaysi (1948); Curran (1957); Ordall (1957; and Murrell (1961). The range of environmental factors such as pH, temperature, and oxygen pressure are usually narrower for spore formation than for growth, although the optima are generally similar.

Temperature affects both the rate and percentage of sporulation. Sporulation occurs most rapidly at or near the optimum for growth and is decreased by temperatures above and below this optimum. Temperature

also affects the heat resistance of spores; those produced at higher temperatures having higher resistance than those produced at lower temperatures.

Those pH values which do not affect vegetative growth completely prevent sporulation. The range over which sporulation may occur varies widely with species, ranging from pH 5.4 in Cl. pasteurianum (Bowen et al, 1955) to pH 8.5, the highest value tried with B. mycoides (Hardwick et al, 1952). Williams (1929) observed that the pH of the medium falls rapidly from 7 to 5, and then rises above 8 during the final stages of spore formation and lysis. Halvorson (1957) observed that the initial fall in pH closely followed the first peak in the oxygen demand of the culture. During this period organic acids disappeared from the medium.

Oxygen has been found essential for sporulation in the genus Bacillus; the amount of oxygen required varies with the stage of sporulation. Halvorson (1957) observed that in broth culture of B. cereus var. terminalis the oxygen demand, which reached a peak of 600 µl./l/min. at 4 hours, coinciding with the maxiumum cell population and exhaustion of glucose, fell to 100 µl./l/min. by 9 hours when the first heat-stable spores had formed.

Oxygen has been found to inhibit sporulation in the genus Clostridium (Murrell, 1961). In five species sporulation was inhibited by 20 mm of oxygen pressure and in two species by 10 mm. In all cases sporulation was less at 10 mm oxygen pressure than at those pressures near 0 mm.

The inorganic requirements for sporulation have been rather extensively studied by Curran (1957), and Slepecky and Foster (1959). Ions which are required for sporulation include Ca ++, Mn ++, Fe ++(+), Mg ++, K +, Zn ++ and PO_4^{---} (Curran, 1957). Slepecky and Foster found that normal spore crops were formed over extremely wide ranges of concentrations of individual metal ions

in B. megaterium. For calcium the range was 1,805-fold, and for zinc, 2,270-fold. With the exception of copper which was very toxic, there was significant incorporation in the spores of each of the metals studied. The higher the concentration of an ion in the medium, the greater was its concentration in the spores. However, it was clear that a concentration mechanism was at work to account for the high accumulation of calcium, nickel, zinc, and manganese in the respective treatments. These data establish that the content of individual metals in bacterial spores can be varied over a wide range, and that under appropriate conditions metals other than calcium may be accumulated to a striking degree. In fact, it was obvious that maximal levels of other ions effectively competed with calcium for accumulation in spores. In the presence of these metals the absorption of calcium by the spores was markedly diminished as compared to calcium absorption in their absence. For example, maximal zinc, nickel, or manganese reduced the calcium content to a small fraction of what it was in the minimal treatments. Although the zinc, nickel, and manganese contents of the spores was substantially higher in these instances, the increases were not equivalent to the drop in calcium content, thus the substitution was not quantitative.

Numerous carbon compounds have been reported to affect sporulation rather than vegetative growth. Based on numerous reports from the literature Murrell (1961) has attempted a separation of the effects of various carbon sources on sporulation of some Bacillus and Clostridium species. In the studies upon which Murrell's separation was based, sporulation was determined by the ultimate appearance of heat stable spores. In view of recent fine structure studies on sporulation to be given later, it becomes obvious that sporulation is a lengthy process which might have been

interrupted rather than prevented. However, these studies have practical value in the development of protocols which give high yields of "normal" spores. Spores of B. megaterium grown in media with different carbon sources contained the same amounts of lipid, polysaccharide, nitrogen and ash (Tinelli, 1955).

The nitrogen source required for sporulation depends upon the synthetic capacities of the various organisms and varies from simple inorganic sources to complex amino acid mixtures and possibly peptides (Murrell, 1961). Clostridium botulinum 62A has been grown and sporulated on a chemically defined medium (Perkins, 1962), in which arginine was found to be important. The nature and concentration of the nitrogen compounds can affect the properties of the spores. A medium with more nitrogen and less minerals yielded spores of B. cereus with a higher nitrogen content, a slightly greater average weight, smaller volume and less ribonucleic acid. These spores germinated more rapidly during storage in wet suspensions (Fitz-James, 1957; Fitz-James et al, 1959). However, these same workers found that the amount of deoxyribonucleic acid was approximately constant for each of 24 species tested, regardless of the growth medium. The heat resistance of spores of B. cereus grown in a chemically defined medium was not affected by the removal of any one amino acid, and was independent of the amount of growth and sporulation (Williams et al, 1951).

The little that is known of the effect of various growth factors on sporulation suggests that the requirements are species dependent, as one would expect (Murrell, 1961).

Substances which specifically inhibit sporulation but not vegetative growth are called inhibitors. The saturated C_1-C_{14} fatty acids are commonly occurring inhibitors of sporulation which may be removed by treating the

medium with soluble starch or activated carbon (Foster et al, 1950). Rather surprisingly, asparagine (Tarr, 1932), beta alanine, L or DL-alanine and DL-aspartic acid (Foster et al, 1949) inhibit sporulation of some aerobes.

During sporulation rather dramatic biochemical changes are occurring within the cells. Some information is availabe on the synthesis and incorporation of spore components; however, an understanding of the metabolic sequences involved in sporulation is far from complete.

The concentration of amino acids, purines and pyrimidines in the intracellular pool falls during sporulation and some are incorporated into the spores. By antimetabolite inhibition studies it was shown that inhibition by any one analogue prevented the utilization of all the amino acids, purines and pyrimidines in the pool (Foster and Perry, 1954). This "all or nothing" phenomenon was interpreted as indication de novo synthesis of protein and nucleic acids from the low molecular weight substances during sporulation.

Synthesis of dipicolinic acid (DPA) commences as sporulation progresses. Its rate of formation is directly related to that of spore formation (Perry and Foster, 1955; Powell and Strange, 1956). In Clostridium roseum, heat resistant cells were formed 1-2 hours after the maximum amount of DPA appeared; however, some cells with staining properties and refractility of typical spores were produced before heat resistant cells appeared (Halvorson, H., 1957). Hashimoto et al (1960) observed that DPA synthesis started when the second coat began to form and before an increase in heat resistance had begun. Martin and Foster (1958) tested a number of common metabolites for their efficiencies as precursors of DPA and found that glutamic acid, aspartic acid, alanine, serine and proline were most efficient.

The uptake of calcium and iron followed the synthesis of DPA, the metal content of spores reaching up to 9 and 4 times respectively that of vegetative cells (Powell and Strange, 1956).

A method of obtaining rapid and nearly complete sporulation of Cl. roseum and B. cereus var. terminalis was developed by (Halvorson, H., 1957). The initial culture was obtained by inoculating the starting medium with about 1 x 10^6 spores/ml. Rapid and complete germination followed. Growth was allowed to proceed to the log phase then a 10 percent inoculum was transferred to a new batch of the same medium. In the third culture, growth and sporulation were allowed to proceed to completion. Under these conditions the cells were all close to the same physiological age; consequently, sporulation was complete in all cells within a narrow time interval.

Spores stored under a variety of conditions including refrigeration, desiccation and freezing remain viable and heat-stable for many years (Evans and Curran, 1960). However, within a relatively short time, usually minutes, of being placed in favorable conditions, these spores became permeable to dilute stains, lost phase refractility and became heat sensitive (Powell, 1957). These primary changes constitute "germination" (Campbell, 1957). Under favorable conditions the cell then swells, absorbs or ruptures the spore coat and elongates in preparation for division into two vegetative cells. These changes constitute "outgrowth" as defined by O'Brien and Campbell (1956).

Germination was measured quantitatively by the loss of heat resistance; permeability to dilute stains; loss of turbidity and loss of phase refractility. These methods gave good agreement in most instances (Powell, 1951; Campbell, 1957).

Heat activation (heat shock) has been an important part of the methods of most germination studies. It was found by Evans and Curran (1943) that, contrary to established belief, sub-lethal heat treatment greatly increased the rate and amount of germination. It was later observed that the germination requirements could also be changed by heat activation (Powell and Hunter, 1955). Heat shock activates the following: spore enzymes involved in glucose metabolism (Church and Halvorson, 1957); transaminases (Falcone and Caraco); pyrophosphatase (Levinson et al, 1958); and alanine deaminase (O'Conner and H. Halvorson, 1960). However, the mechanisms of heat activation remain obscure.

The classical concept of bacterial spore germination is contained in the following quotation from O'Conner and Halvorson (1960): "The germination of the bacterial endospore presents a unique opportunity in biology to examine the triggering of a poised system. As one can observe under the microscope, the conversion of a single refractile spore to a non-refractile one is almost instantaneous. This process is initiated by specific triggering agents which are themselves metabolized in the process (Halvorson and Church, 1957). From this and numerous other considerations it seems evident that the germination process has an enzymatic basis."

Based upon the concept of germination as being essentially enzymatic, the environmental factors of importance in the process have been temperature, osmotic pressure, pH, and oxygen. The effect of each of these factors is species specific and varies greatly from one species to another. Good reviews of the scattered information on germination are those of Murrell (1954, 1961).

The first study on elucidating chemical factors responsible for germination appears to be that of Hills (1949). Working with one strain of B.

anthracis, he showed that adenosine gave a significant effect at a concentration of 0.2 μg/ml. Hills in 1950 extended his observations to B. subtilis and B. cereus, both of which could be germinated by L-alanine. Subsequently, work on discovering metabolites capable of inducing germination proceeded at an exponential rate. Levinson and Sevag (1953) found one strain of B. megaterium which could be germinated by glucose alone; Woese (1958) found that several amino acids were capable of substituting for alanine; Powell (1957) observed that inosine could substitute for adenosine.

During germination studies with Cl. botulinum 62A, Treadwell et al (1958) found that complete germination was obtained in 2 hours in 5 percent yeast extract broth. Complete germination of heat activated spores was also obtained in vitamin-free casamino acids in 2 hours. However, only 20 percent germination was obtained when an amino acid mixture supposedly equivalent to 2.3 percent casamino acids.

During this period considerable work was being done on germination by non-metabolites and by physical means. Studies on germination by non-metabolic chemicals included those with: ethylene-diamine tetraacetic acid (Brown, 1957); ionic surfactants (Rode and Foster, 1960); hydrogen peroxide (Falcone et al, 1959); calcium dipicolinate (Reiman and Ordal, 1961); and pyridine carboxylic acids, salts of fatty acids, hydrazine, hydroxylamine and ammonium chloride (Rode and Foster, 1961). Rode and Foster (1961) achieved mechanical germination by electrodialysis and by abrasion with ground glass in a Mickle tissue disintegrator.

Because of the perplexing diversity of means of germinating bacterial spores, Rode and Foster sought some unifying mechanism to explain germination. Their results indicated that this unifying mechanism was based on ionic phenomena (Rode and Foster, 1962a, 1962b). These workers have shown

that B. megaterium QM B1551, a "glucose type" could not be germinated with deionized glucose, but could be germinated by a variety of inorganic salts alone. Glucose exerted an augmentative effect in combination with ions. Likewise, B. megaterium, Texas strain, an "L-alanine-inosine type" could not be germinated with L-alanine and inosine in the absence of ions; they were effective only in the presence of one of a large variety of salts. The need for inosine could be bypassed by various salts, e.g., ammonium propionate or salts of dipicolinic acid. L-alanine could be replaced by a variety of amino acids, provided suitable ions were present.

Powell and Strange (1953) observed that during the germination process spores of B. megaterium and B. subtilis excreted 30 percent of the cell dry weight. The materials excreted consisted mainly of amino acids, peptides, hexosamine attached to a non-dialysable peptide, and the calcium complex of dipicolinic acid (DPA). Approximately 50 percent of the total excreted solids was represented by DPA (Powell, 1953). In 1957, Powell further stated that the germination exudate was remarkably similar in all the Bacillus species studied. Surfactants caused the release of calcium dipicolinate and a nondialyzable mucopeptide, together with small amounts of dialyzable peptides and amino acids (Foster, 1959). He was also able to obtain the release of DPA by heating, by treatment with a variety of organic solvents, by mechanical abrasion, and by electrodialysis.

The effects of ionizing radiation on bacterial spores have been studied from several aspects. Among these, Woese (1959) studied the inactivation kinetics of several species of Bacillus; Powers et al (1960) studied free radical production in spores which were irradiated dry; and Anellis (1962) studied the relative radiation resistance of various isolates of Cl. botulinum. Foster (1959) observed that 30 percent of the DPA of B.megaterium

was released by prolonged ultraviolet irradiation. Levinson and Hyatt (1960) found that above 1.2 megarads of irradiation in an electron beam caused a sharp increase in spore stainability, with nearly all spores becoming stainable at 2.4 megarads. These workers also found that irradiation of Cl. sporogenes spores at 10 megarads caused loss of about 96 percent of the DPA.

Mechanical disruption of spores has been achieved by various procedures by a number of workers. As early as 1942 Curran and Evans used agitation with small glass beads to kill bacterial spores. A similar technique was used by King and Alexander in 1948 for the mechanical destruction of bacterial spores. In 1953 Fitz-James employed the Mickle disintegrator to break spores which he then treated with cytochemical reagents. Murrell (1955) and Powell and Hunter (1956) used shaking with glass beads to obtain spore extracts for enzyme studies. Murrell (1955) used the Hughes' press with glass powder to crush spores to obtain extracts for enzyme studies. Sonic disintegration with Superbrite glass beads was used to prepare spore extracts for enzyme studies (Halvorson and Chruch, 1957, Levinson et al, 1957). Later sonic oscillation in a Raytheon 10 kc was used, without glass beads, to remove the exosporia from spores of B. cereus 2005 and B. cereus var. terminalis, leaving viable, heat resistant spore bodies, which, during the long treatment necessary to disrupt a significant fraction of the spores the emptied spore coats are comminuted to submicroscopic particles (Berger and Marr, 1960).

Fractionation of one or another structural element from various spores has been accomplished. The most commonly isolated fraction has been the core extract (reviewed on page 12). Strange and Dark (1956) isolated spore coats from B. subtilis and B. cereus after breaking the spores in a Mickle tissue disintegrator. Hunnell and Ordal (1961) isolated spore

coats of B. coagulans by prolonged heating in 0.1 N HCl followed by treatment with pepsin, trypsin and lysozyme. Gerhardt and Ribi (1961) isolated exosporia from B. cereus var. terminalis by use of the Ribi Cell Fractionator. Kondo et al (1961) reported the isolation of exosporia and "stripped" spores; however, examination of their electron micrographs casts doubt on the conclusion that exosporia were isolated. Rather it appears that an impure fraction of spore coats was obtained.

Interest in the structure of bacterial spores has been studied by various techniques since the early days of bacteriology. These studies have, however, not produced agreement as to the structure of these cells. The results have been well summarized by Knaysi et al (1947) who stated: "A study of the literature reveals that there has been no agreement on the number and properties of the coats in the resting endospore, or on their behavior during germination. Brefeld (1881) believed in the existence of two coats; the outer one is shed and the inner one becomes the wall of the germ cell. Others (De Bary, 1884; Klein, 1889) wrote of a spore coat surrounded by weakly refractive slime layer. Meyer (1897, 1899) believed he had demonstrated two coats and a slime layer, all of which are shed. Preisz (1904, 1919) saw one coat and stated that the wall of the germ cell is formed during germination. Lewis (1934) considered a spore coat, possibly in two layers, and an outer, stainable layer, which is a residue of what he called the exosporium." The cytological processes involved in sporulation and germination was reviewed by Knaysi (1948).

The study by Knaysi et al (1947) of the endospore and life cycle of B. mycoides was among the early electron microscope studies of bacterial spores. In this study the investigators used only intact cells rather than thin sections. However, in 1953 Robinow published a study of spore structure by the use of thin sectioning techniques. From these studies he concluded

that in B. cereus the spore coat is a simple membrane, but in spores of B. megaterium two major layers could be distinguished. He found some evidence that these, in turn, were composed of layers of still finer membranes. An electron-transparent layer was observed immediately beneath the spore coat, in B. megaterium but not in B. cereus. The cores of both species consisted of basophilic matter which was remarkably dense and homogeneous.

In 1957 Mayall and Robinow published much superior electron micrographs and extended their observations on the integuments of B. megaterium. From this study they concluded: "the interior of spores of B. megaterium consists of two concentric regions. The inner one is readily stained and in electron micrographs is very dense. The outer one, or cortex, has no affinity for stains and is very transparent to electrons. It is in turn surrounded by two dense layers which are the spore coat. Cutting sections on to lanthanum nitrate increases the density of the cortex for electrons and provides a means of revealing its fine structure. In normal resting spores the cortex is smooth and featureless. Treatment of spores for half an hour with N/1 HNO_3 at room temperature loosens the fabric of the cortex and reveals a system of concentric strands (or lamellae) about 100 A thick and 60-80 A apart. In spores which have been protected against the action of acid by the addition to it of M/4 sodium fluoride the cortex looks entirely normal, but here and there it appears faintly laminated. It is postulated that the action of the acid leads to the removal from the cortex of a continuous electron scattering phase which normally permeates and obscures the spaces between the lamellae.

"The cortex occupies about 50% of the volume of the spore inside the spore coat. It is a special, intricately organized layer intervening between the dormant bacillus and the spore coat.

"On germination the inner table of the cortex becomes the cell wall of the new bacillus; the remaining layers suffer rapid and complete dissolution."

"The possibility is discussed that the cortex is the source of some of the components of the spore exudate of Powell and Strange (1953)."

From a study of 3 strains of B. megaterium, Fitz-James and Young (1959) concluded: "The alkali and acid insoluble residue phosphorus fraction of spores of Bacillus megaterium is composed largely of spore coats. Spores of strains that are rich in residue phosphorus possessed a single coat similar to the inner coat of the other strains. Carbon replicas emphasized the differences in surface structure of these spores. The presence or absence of a second coat altered the degree of differentiation of resting spores of B. megaterium to acid hydrolysis." However, an examination of electron micrographs of strains suggests that the differences in the surfaces result from differences in the sizes of exosporia. Furthermore, examination of electron micrographs of thin sections reveal more differences between sections of the same strains than between sections of different strains.

The importance of considering fixation effects in interpretation of observations made by thin sections was suggested by the work just described. Such effects were systematically investigated by Tokuyasu and Yamada (1959a) with B. subtilis. These workers compared osmium tetraoxide and potassium permanganate, individually, in sequence and in combination at a series of pH values and toxicities. These authors concluded: "In general, $KMnO_4$ fixation appeared to provide much better definition of the boundaries of various structures than did OsO_4." From thin sections fixed with 2 percent $KMnO_4$, these workers (1959b) concluded that: "The mature spore shows a very complex structure. The spore coat is composed of three layers, the middle of which consisted of 5 to 8 lamellae of thin membranes and interspaces,

both about 20 to 25 A thick. Between the inner layer of the spore coat and the spore cortex, a thin membrane with an affinity to the cortex can be observed. The spore coat is enclosed within two envelopes, one loosely surrounding the core, and the other adhering to it."

The effects of preparation on the appearance of thin sections was extended to cover sectioning procedures (Rode and Foster, 1962). These workers concluded that "abnormal spore structure" resulted from the solution of cellular constituents when fixed specimens were sectioned on water. This conclusion is questionable since spores which have been adequately fixed have become permeable to water, resulting in the removal of soluble substances during continued fixation the washing which is necessary to remove the fixative before dehydration. Thus, sectioning fixed specimens on water should cause no further loss. These authors concluded that dry sections of unfixed spores revealed spore structure in a more normal state.

Observation of fine structure in resting spores has been impossible since they are uniformly opaque and undifferentiated under the electron microscope; however, when the spores were induced to lose their DPA by biological germination, heat, abrasion or chemicals, they spores integument(s) became discernible (Rode and Foster, 1962).

The process of sporulation had been studied for many years by the use of cytological techniques and the light microscope. However, with the advent of thin sectioning techniques the submicroscopic aspects of sporulation have been investigated by a number of workers. Chapman (1956) described sporulation in B. megaterium and B. cereus as a process in which the spore proper is formed by differentiation in situ of the sporangial cytoplasm, following incorporation of nuclear elements. The spore coats appear to be formed by a condensation of sporangial and spore cytoplasm.

Hashimoto and Naylor (1958) described sporulation in Cl. sporogenes, the mature spores of which appeared to be composed of an exosporium, two outer membranes and an inner membrane. These investigators concluded that the two outer membranes appeared first and enclosed a portion of cytoplasm containing an area of low electron density. The exosporium was formed next and the inner coat appeared last. With the formation of the inner coat, a change in spore texture occurred, and it was at this point that the spore lost affinity for basic dyes.

In 1959a Young and Fitz-James published 3 electron micrographs showing a septum purported to be a "spore wall". Unfortunately, the series was not continued so that the claim was not supported. Also in 1959, Takuyasu and Yamada reported their studies of sporulation in B. subtilis, using potassium permanganate rather than the "conventional" osmium tetraoxide as fixative. These workers report: "At a very early stage, the pre-spore is seen to consist of what seems to be the nuclear material and granular substance, surrounded by a layer of dense material destined to become the innermost layer of the spore coat. At a subsequent stage, a light interspace is observed that is destined to become the spore cortex."

During 1960 several papers on the fine structure of sporulation were published. Among these was that of Hashimota et al (1960) on the development of fine structure, thermostability and synthesis of dipicolonate during sporogenesis in B. cereus var. terminalis. Also appearing in 1960 was the work of Takage et al on Cl. butyricum and Cl. botulinum type E. These workers concluded: "At the initial stage of sporulation, the nuclear site which was located corresponding to the expected place of spore formation, enlarged, became compact in contents, and this formed the spore primordium together with the surrounding cytoplasm. After this, at the

outside of the spore primordium, the dense and undulating outer spore coat began to form fragmentarily, gradually proceeding to envelop the whole spore. About at the same time, the differentiation of the core was initiated, and also the inner spore coat and cortex became distinct. The other nuclear sites except the one which was included in the sporulation process became contracted and finally disappeared during the process. The sporangium cells metamorphosed the ghost cells, the nature spores were liberated and thus the sporulation was completed." No attempt was made to obtain synchrony in the cultures on which these studies were made, thus there is no real basis for assuming that sequential steps of sporulation were demonstrated.

In 1960 the vividly interpreted work of Fitz-James on the participation of the cytoplasmic membrane in growth and spore formation established the participtation of this structure in sporulation of 4 species of Bacillus.

The participation of the cytoplasmic membrane in spore formation by B. coagulans was demonstrated by an elegant series of electron micrographs by Ohye and Murrell (1962). These workers concluded: "The developmental stages from the origin of the initial spore septum to the mature spore were investigated. The two forespore membranes developed from the double layer of cytoplasmic membrane. The cortex was progressively deposited between these two membranes. The inner membrane finally became the spore protoplasmic membrane, and the outer membrane part of the inner spore coat or the outer spore coat itself. In the mature spore the completed itegumenta around the spore protoplasm consisted of the cortex, a laminated inner coat, and a dense outer coat. No exosporium was observed."

The permeability of bacterial spores was investigated by Gerhardt and Black, (1961) and Black and Gerhardt (1961, 1962) by use of the space

technique of MacDonald and Gerhardt (1958). Gerhardt and Black tested more than 100 compounds for their uptake by dormant spores of B. cereus strain terminalis. The extent of penetration was found to be dependent on at least three molecular properties: (1) The dissociation of electrolytes usually resulted in high or low uptake predictable from their charge; (2) Lipid insolubility restricted permeation of small molecules; (3) The molecular weight of unsubstituted glycol and sugar polymers exponentially limited penetration to eventual exclusion at molecular weights above 160,000. From a generalized curve of these data, calculations indicated that the pore size varied from 10 to 200 A. Dormant spores did not take up specific germinants differently than they did other compounds, under conditions optimal for germination.

Glucose penetrated 40 percent of the weight of spores R^W40 of B. cereus strain terminalis in dormant spores. The glucose uptake was relatively independent of environmental variables, and thus was concluded to occur principally through a process of passive diffusion (Black and Gerhardt, 1961). After germination the permeability had increased to R^W51 (Black and Gerhardt), 1962). The authors contend that the germinated spores used in this study were prevented from growing out. However, germination was accomplished by placing the spores in a complex medium at 30° C for 30 minutes. Since fine structure studies of Mayall et al (1957) and Ohye and Murrell (1962) showed that under favorable conditions, extensive changes in spore integuments occurred very rapidly. Thus, the conclusion that no outgrowth had occurred is questionable.

MATERIALS AND METHODS

A. Production of Spores

The organism used was Clostridium botulinum 62A (ATCC 7948).

The TSP medium of Zoha and Sadoff (1958), contained 15 g trypticase, 10 g peptone, 2.5 g NaCl, 2.5 g K_2HPO_4 and 1000 ml distilled water. After adjustment to pH 8.0 with 5 N NaOH, the medium was dispensed and stored at 4° C to be sterilized immediately before use. Anaerobiosis was obtained by bubbling either N_2 with 5 percent CO_2 or natural gas through the sterilized medium for several minutes just prior to inoculation.

Synchronous growth was readily attained by starting with heat shocked spores and then serially transferring a 10 percent inoculum of cells which were in the exponential growth phase. The growth phase of the culture was easily followed by simple methylene blue stains. The logarithmic phase of synchronous cultures of Cl. botulinum 62A exhibits 3 distinct characteristics (note Fig. 30).

1. The cells occur in chains, usually 4 or more.
2. The cells are much larger than in older cultures.
3. The cells stain uniformly and intensely with methylene blue.

The time required for the culture to reach this phase depended upon the medium used, the size of inoculum, and the particular spore stock.

Initially 2 ml of an aqueous suspension of partially cleaned spores was heat shocked at 60° C for 10 minutes then added to 30 ml of medium. The resulting culture was incubated for approximately 2 hours then checked with a simple methylene blue stain. If the culture exhibited the characteristics typical of the exponential phase, the entire culture was used as inoculum for 300 ml of medium. This culture also reached the exponential phase in approximately 2 hours and was then transferred to 3000 ml of

medium. The resulting culture was incubated overnight on a magnetic stirrer at low speed. All cultures were incubated at 30° C. Cultures in which sporulation was above 90 percent were stored at 4° C for 3-7 days.

After the spores were free of sporangia, the cultures were put through a Sharples continuous centrifuge specially adapted to prevent aerosols. The spores were washed a minimum of 10 times at 4° C in distilled water.

B. Correlation of Spore Recovery, Dye Uptake and Dipicinolic Acid (DPA) Release During Prolonged Heating

Spore recovery. The number of spores surviving various intervals of heating at 65° C was determined by colony counts using 5 percent yeast extract (YE) agar at pH 8. Anaerobiosis was achieved by using oval tubes with 2 percent non-nutrient agar over-lays. After incubation at 30° C for 15-18 hours, colony counts were made with the aid of a Quebec Colony Counter (Wynn, 1951).

Dye uptake. To determine percentage of stainable and refractile spores a thin film was air dried and stained with methylene blue. To prevent spores from becoming stainable, heating of the films was avoided.

Dipicinolic acid release. After the desired interval of heating a 4 ml sample was removed to a Servall Centrifuge tube, cooled to 4° C and the spores sedimented by centrifugation in the cold. Concentrations of DPA in supernates was determined spectrophotometrically from the total absorption at 2600 to 2800 A (Young, 1959, Rode and Foster, 1960) in a Beckman DU Spectrophotometer.

C. Germination Studies

Five percent yeast extract (YE) broth at pH 8, which gave, in 20 minutes, 90 percent germination of heat shocked spores of Cl. botulinum,

was used as the standard of comparison in all germination studies. Based on the chemical composition of yeasts (Cook, 1958), the amino acid, growth factor, purine, pyrimidine, mineral, sugar and co-enzyme content of YE was simulated (Table 1). These compounds were autoclaved separately in distilled water for 10 minutes at 121° C, since all are reported to be stable to this heat (Meister, 1957; Clark, 1953; Hawk et al., 1947; Chargaff et al., 1955). These amino acid preparations which were not completely soluble were vigorously shaken prior to removing an aliquot for combining with other components of the medium.

One ml of each amino acid solution, 0.5 ml of glucose solution and 0.5 ml of inorganic salt solution were combined. After mixing all the vitamins into one solution and all the co-enzymes into another, 0.1 ml of each was added to the amino acid-glucose-mineral solution to give a total volume of 19.2 ml of medium. After adjusting to pH 8, heating at 100° C for 10 minutes and flushing with the N_2:CO_2 mixture the synthetic medium was inoculated with 1 ml of suspension containing 1 x 10^7 spores.

Germination studies using dialyzed YE were also performed. In these studies Dico yeast extract were placed in dialysis tubing, the tubing placed in 200 ml. of distilled water and stored at 4° C for later use. The material remaining in the dialysis tubing was dialyzed against flowing tap water for 24 hours.

The dialyzate was diluted 1:1 with distilled water and adjusted to pH 8 with 1 N NaOH.

The non-dialyzable material was diluted to 25 ml. and adjusted to pH 8 with 1 N NaOH.

Germination studies using 20 mmol $CaCl_2$:$2H_2O$ and 20 mmol DPA in tris buffer (Riemann et al,1960) were performed. The effect of chloramphenicol

TABLE 1. Concentration of components in synthetic germination media

Compound	gm per 100 ml	Compound	gm per 100 ml
Amino Acids			
L - alanine	2.7	DL - lysine	4.5
L - arginine	3.0	DL - methionine	0.6
DL - aspartic acid	6.8	L - phenylalanine	2.1
L - cystine	0.08	L - proline	2.1
DL - glutamic acid	6.4	DL - serine	1.3
glycine	2.6	DL - threonine	2.1
L - histidine	1.0	DL - tryptophane	0.6
DL - isoleucine	2.1	L - tyrosine	1.8
DL - leucine	3.7	DL - valine	2.9
Growth Factors			
folice acid	6.3	p-amino-benzoic acid	5.0
thiamine	20.0	pyridoxine	7.5
riboflavin	4.0	inositol	400.0
niacin	50.0	choline	480.0
Purines and Pyrimidines			
adenine	45.0×10^{-3}	uridine	13.0×10^{-3}
guanine	25.0×10^{-3}	thymine	20.0×10^{-3}
cytidine	25.0×10^{-3}		
Minerals			
K_2HPO_4	5.0×10^{-3}	Na_2CO_3	2.0×10^{-4}
$MgCl_2$	0.2×10^{-3}	Fe_2SO_4	2.0×10^{-4}
Coenzymes			
Co I (DPN)	10.0×10^{-3}	Co II (TPN)	10.0×10^{-3}
Miscellaneous			
glucose	4.01	ATP	10.0×10^{-3}

(CAP) on germination and outgrowth in 5 percent YE was determined by the routine procedure. In the first study, CAP was used at a concentration of 50 μg/ml. In a second study the effect of concentration of CAP over a longer period was determined.

D. Preparation of Germination Exudate

The spores of Cl. botulinum, in contrast to aerobic and some anaerobic (Hitzman et al, 1957) spores, could not be germinated by simple agents; consequently, preparation of germination exudate was rather involved. The germinating medium, prepared by placing 50 g YE in 100 ml distilled water and dialyzing against 900 ml distilled water, was the approximate equivalent of 5 percent YE. The YE dialyzate was adjusted to pH 7.8, transferred to an Erlenmeyer flask, boiled for 10 minutes and flushed with natural gas.

The inoculum consisted of 0.5 g lyophilized spores per 100 ml of YE dialyzate. The spores were sedimented by centrifugation when 70-80 percent became stainable. The supernate was decanted and checked for spores with a simple stain. The cell-free supernate was dialyzed against distilled water at 4° C, lyophilyzed, resuspended in a small volume of water and again dialyzed to give a non-dialyzable fraction which was used in subsequent analyses.

This method of preparation has limitations in that the exudate cannot be checked conveniently for free amino acids, minerals and other dialyzable substances in the presence of YE dialyzate. After removal of the germinating medium by dialysis, there remained only the non-dialyzable fraction of material released during germination.

E. Studies on Irradiated Spores

Preparation and radiation of spores. A heavy suspension of clean spores in distilled water was dispersed with a Waring blendor and dispensed into 7

ml polystyrene shoulder vials which were completely filled to minimize contact with oxygen. A temperature of 4° C was maintained during transit, irradiation and subsequent storage.

Microscopic observations. Irradiated and non-irradiated spores were observed in wet mount after treatment with simple methylene blue or carbol fuchsin with heat.

Determination of spore concentration. The concentration of spores in each lot was determined by direct counts using a Petroff-Hauser counting chamber and by optical density measurement in a Beckman DU spectrophotometer at 450 mμ. The number of viable spores was determined by colony counts using oval tubes with 5 percent YE agar at pH 8 with non-nutrient agar overlays.

Quantitation of material released. At 10 ml aliquot of each suspension was transferred to a heavy walled glass Servall tube and centrifuged. Five ml of supernate were removed, 5 ml of distilled water added, and the suspension again centrifuged. This procedure was performed 5 times to enhance the removal of compounds with low solubilities. The volume of 25 ml, equivalent to 9.40 ml of the original supernate was dried to constant weight at 110° C.

Analysis of control and irradiated spores. The spores which had just been washed 5 times were used for analyses by the methods given under "Analytical Procedures".

F. Preparation of Spore Coat Skeletons

Prolonged heating at 121° C. A suspension of clean spores or a mixture of spores and vegetative cells were autoclaved at 121° C and observed with the light microscope after treatment with methylene blue or carbol fuchsin. Heating was continued until most of the spores were reduced to empty shells.

Acid treatment. Cultures of Cl. botulinum containing mostly spores were harvested by centrifugation and dispersed in a small volume of water with a Waring blendor to give a heavy suspension. One volume of this suspension was mixed with two volumes of 0. 1 N HCl, autoclaved at 121° C for 90 minutes then centrifuged at 10,000 x g for 4 minutes. The resulting pellet was washed 5 times with distilled water. A second heating in 0.1 N HCl at 121° C for 15 minutes was followed by 5 washings. Another 15 minute heating period and another 5 washings were required to give a preparation free of stainable and ninhydrin positive materials.

G. Analytical Procedures

Solubility tests employing both inert and reactive solvents were performed on spores and spore coat skeletons. Intact spores were treated with methylene blue and examined with the light microscope for dye refractility and morphological changes while tests with spore coat skeletons were observed for decrease in turbidity and the "skeletons" were examined for a morphological integrity.

Elemental analyses were performed by the Schwarzkopf Microanalytical Laboratory, Woodside 77, N.Y.

Analytical procedures used in determining composition of spores and spore coat skeletons and free reactive groups in spore coat skeletons are shown in Table 2.

Spores and spore coat skeletons were exposed to a series of enzymes under conditions shown in Table 16.

H. Fine Structure Studies by Use of Spore Fractionation and High Level Gamma Irradiation

Three main morphological elements were identified in spores prepared for electron microscopy by fractionation and high level gamma irradiation.

TABLE 2. Analytical procedures

Compound or Group	Method	Reference
protein	ninhydrin phenol reagent	Hawk[1], p. 157 Methods[2] p. 448-450
glucosamine	indole reaction	Methods p. 98-99
calcium	wet ashing, then $KMnO_4$ titration	Methods, p. 1003 Powell[3]
reducing compounds	arsenomolybdate	Methods p. 85-86
phenols (p-unsubstituted)	Millon (5-nitroso-8-hydroxy-quinoline in conc. H_2SO_4)	Fiegl[4]
glucose fructose galactose pentose RNA DNA ascorbic acid trypthophane glutamic acid	anthrone	Methods, p. 94-95
carbohydrates furfural-yielding substance	Molisch (alpha-naphthol)	Hawk, p. 54
nitroso compounds	phenol-H_2SO_4	Fiegl, p. 165-166
$-CONH_2$* $-CSNH_2$* $-C(NH)NH_2$* $-CH_2NH_2$*	biuret	Hawk, p. 156
aldehydes* ketones*	2,4 dinitrophenyl-hydrazine	Cheronis[5] p. 122
active unsaturation*	$KMnO_4$ bromine	Cheronis, p. 86
alcohols,* phenols,* hydroxy acids,* hydroxy carbonyl*	$FeCl_3$	Cheronis, p. 117-118

Compound or Group	Method	Reference
aldehydes*	benzidine	Cheronis, p. 124
esters*	ferric hydroxamate	Cheronis, p. 121
aromatic amides*	hydroxamate test	Cheronis, p. 130
primary arylamines*	diazotization	Cheronis, p. 133

*Detection of these groups is dependent upon their occurrence in the uncombined state.

1. Hawk, Oser and Summerson, 1947.
2. Colowick and Kaplan, 1955.
3. Powell, 1953.
4. Fiegl, 1960.
5. Cheronis and Entrikin, 1947.

After numerous trial runs in which various techniques, including gradient centrifugation were tried, there was developed a relatively simple procedure whereby all 3 main morphological elements were isolated from the same spore suspension.

Electron microscopy of clean spores. Suspensions of spores which were produced as described under "Production of Spores" were placed on 150 mesh formvar coated grids and examined with the Phillips 100 electron microscope. Similar preparations were shadowed with heavy metal then micrographed.

Removal and isolation of exosporia. A suspension of 2.8 g of lyophyolized spores in 90 ml of 1 percent ethylene diamine tetraacetate (EDTA) was put through the Cell Fractionator at 45,000 psi. Electron microscopy of the effluent revealed nearly complete removal of the outer layer from the spores. The effluent was separated into an opalescent supernate and a firmly packed, 2-layered sediment by centrifuging for 45 minutes at 2000 x g. The upper layer of sediment was suspended in a small volume of distilled water by gentle washing with a rubber-bulbed pipette and by gentle rubbing with a round tipped glass rod. Electron microscopy revealed highly pure outer layer material which was subsequently washed 6 times with distilled water, lyophiolized and stored under refrigeration for future use.

Breakage of spore coats. The skinned spores resulting from the previous procedure were treated overnight with a saturated solution of EDTA after which they were washed 5 times with distilled water, suspended in 90 ml of distilled water and put through the Cell Fractionator 4 consecutive times under an atmosphere of nitrogen gas. The first two presses were at 45,000 psi and the last two at 50,000 psi. Great care was taken to prevent the contact of the material with oxygen during this and subsequent steps in fractionation. The Cell Fractionator was continuously flushed with

nitrogen and all subsequent manipulations were carried out in a hood under an atmosphere of nitrogen.

Isolation of spore coats and core material. Electron microscopy of the effluent revealed many broken and fragemented spore coats as well as many unbroken skinned spores and granular, extremely electron transparent material in the background. The effluent was transferred to a 200 ml glass bottle and centrifugred for 45 minutes at 1500 x g in an International PR-2 centrifuge. The supernate, which contained much amorphous, stainable material as well as broken second layers was decanted and centrifuged for 45 minutes at 1500 x g. The resulting supernate was visibly opalescent; light microscopy of stained samples revealed fine, uniformly granular material free of debris or spores. This material was shell frozen, lyophilized and stored at -30° C under a nitrogen atmospher

The pellet, composed largely of second layers, was further purified by differential centrifugation by 2 spins at 1000 x g, each for 30 minutes. Electron microscopy of the final pellet revealed better than 95 percent second layers.

The procedure for spore fractionation by use of the Ribi Cell Fractionator is shown diagramatically in Fig. 1.

Electron microscopy of gamma-irradiated spores. Clean suspensions of spores were prepared for irradiation as described under "Examination of Irradiated Spores" except that 50 ml polyethylene bottles were used. Samples which had received 3, 6, and 9 x 10^6 rads of gamma irradiation were examined with the electron microscope. The supernates were preserved for future examination while suspensions of the spores were put through the Cell Fractionator at 45,000 psi.

I. Fine Structure Studies by Thin Sections

In the early fine structure studies the fixative and conditions of fixation were varied to determine the optimum in each. The materials and methods are essentially standard for general fixation and methacrylate embedding (Servall, 1959).

Materials:

2 percent potassium permanganate in tap water

0.6 percent potassium permanganate in veronal-acetate buffer

1 percent osmium tetraoxide in veronal-acetate buffer

0.5 percent osmium tetraoxide in veronal buffer, pH 7.8

25, 50, 75, and 10^ percent ethanol

butyl methacrylate

methyl methacrylate

methacrylate mixture (1:9 ratio butyl:methyl methacrylates)

"Luperco CDB" (catalyst), 50 percent 2, 4-dichlorobenzoyl peroxide in dibutyl phthalate

Methods:

Spores. Spores which had been grown and cleaned as previously described and either lyophilized or stored in distilled water were used in preliminary studies. Later, skinned spores were also used.

Fixation. The fixatives used were: 2 percent potassium permanganate in tap water; 0.6 percent potassium permanganate in neutralized veronal acetate buffer; 1 percent osmium tetraoxid in neutralized veronal acetate buffer; 0.5 percent osmium tetraoxide in veronal buffer, pH 7.8; a mixture of 0.5 percent osmium tetraoxide and 0.6 percent potassium permanganate in neutralized veronal buffer; 0.6 percent potassium permanganate in buffer followed by 0.5 percent osmium tetraoxide in buffer;

in buffer and 2 percent potassium permanganate in water followed by 1 percent osmium tetraoxide in neutralized buffer. Fixation varied from 3 to 72 hours in the cold.

Initially, the penetration of fixatives into the spore was determined by the periodic removal of samples for making methylene blue stains and for inoculating into 5 percent yeast extract broth, after removal of the fixative by washing. The spores in these cultures were checked for germination and outgrowth by observing their refractility to dye and the general appearance and odor of the cultures as well as the morphological characteristics of any resulting vegetative cells. The results were correlated with satisfactory preservation of the spore core in thin sections. After satisfactory correlation was established, the time necessary for complete fixation was determined by periodically observing for loss of dye refractility by light microscopy of samples stained with methylene blue. After fixation, the spores were washed twice with tap water or buffer, whichever was used in the fixative.

Dehydration. The clean, fixed spores were dehydrated by serial transfer through 25, 50, 75, 95 and 100 percent ethanol. The spores were sedimented by centrifugation between ethanol changes.

Infiltration and embedding. Spores were impregnated at room temperature by successive exposure to the following embedding mixtures for 15 minutes: absolute ethanol: methacrylate mixture, 1:1; pure methacrylate mixture, methacrylate mixture plus 2 percent "Luperco CDG". After placing the embedded spores in the bottom of No. 00 gelatin capsules with capillary pipettes, the capsules were carefully filled with the methacrylate plus "Luperco CDB" and the tops placed on the capsules. The resin was polymerized at 45° C for 24 hours.

During all the steps of infiltration and embedding, care must be exercised to avoid incorporation of moisture into the resin as the presence of moisture produces soft blocks with poor cutting qualities.

Summary of Methacrylate Method

1. Suspend clean spores (wet pack or lyophilized) in fixative by use of applicator sticks.
2. Hold in the cold for desired time.
3. Check with a simple methylene blue stain; when fixative has penetrated spores they stain intensely.
4. When fixation is complete, sediment spores by centrifugation.
5. Wash spore pack twice with solvent used in the fixative (tap water or buffer).
6. Add 25 percent ethanol to the tube, suspend spores, hold in the cold for 15 minutes.
7. Sediment spores, decant supernate, add 50 percent ethanol, and resuspend spores. Hold in the cold for 15 minutes.
8. Sediment spores, decant supernate, add 75 percent ethanol, resuspend spores. Hold in cold for 15 minutes.
9. Sediment spores, decant supernate, add 95 percent ethanol. Hold at room temperature for 30 minutes.
10. Sediment spores, decant supernate, add 100 percent ethanol. Hold at room temperature for 30 minutes.
11. Sediment spores, decant supernate, add fresh 100 percent ethanol. Hold at room temperature for 30 minutes.
12. Sediment spores, decant supernate, add fresh 100 percent ethanol. Hold at room temperature for 30 minutes.
13. Sediment spores, decant supernate, add 1:1 ratio of ethanol;

methacrylate mixture. Hold at room temperature for 15 minutes.

14. Sediment spores, decant supernate, add pure methacrylate mixture. Hold at room temperature for 15 minutes.
15. Sediment spores, decant supernate, add methacrylate mixture plus 2 percent "Luperco CDB". Hold at room temperature for 15 minutes.
16. Sediment spores, decant supernate, add fresh methacrylate mixture plus catalyst. Hold for 15 minutes at room temperature.
17. Sediment spores, decant supernate, place spores in bottom of No. 00 gelatin capsules with capillary pipettes.
18. Gentle fill capsules with partially pre-polymerized methacrylate mixture plus catalyst.
19. Put tops on capsules.
20. Polymerize overnight at 45° C.

Breaking glass knives. An improved method of breaking glass knives, introduced by Dr. Kazue Fukushi*, greatly expedited this procedure. Strips of plate glass 4 x 16-24 x 1/4 inches were washed with warm water and detergent by gently rubbing with the fingers. After a distilled water rinse the strips were allowed to air dry. The strips were then scored across at 4 inch intervals with a diamond glass cutter, an applicator stick placed directly under the score and gentle pressure applied to effect a clean, straight break, giving 4 x 4 inch squares. Midway along one edge and extending about 3/4 inch into the square, scores were made. An applicator stick was placed under the score and gentle pressure applied to break the square into 2 rectangles, each 2 x 4 inches. A similar procedure was used to break the 2 x 4 inch rectangles into 2 x 2 inch squares. The 2 x 2 inch squares were marked with a diagonal score, slightly off center, and extending to within approximately 1/2 inch of the edges. To effect breakage,

* See acknowledgments.

this score was heated subterminally with a small hydrogen flame. This method, illustrated in Figure 2, is simple yet the percentage and quality of knives obtained are high.

Preparation for sectioning. Prior to sectioning, the gelatin capsules were removed by soaking the polymerized blocks in cold water for several hours then rinsing off the softened gelatin under warm flowing water. The thoroughly dry blocks were faced with a razor blade to give a very small cross section (Servall, 1959).

A trough for floating the sections was prepared by positioning electricians' tape perpendicular to the face of the knife, trimming the ends flush and sealing the lower edge of the tape to the knife with smoking hot beeswax.

Sectioning. Sections were cut with a Porter-Blum microtome, floated on distilled water, spread with xylene fumes and picked up on 150 mesh grids coated with formvar.

Micrographing. Thin sections were examined and micrographed with a Phillips 100 electron microscope at an accelerating voltage of 60 kv and with an objective aperture of either 25 or 40 microns.

Light microscopy. Simple methylene blue stains were prepared from each hourly specimen immediately upon its removal. Washing was avoided in order to preserve as much as possible the appearance and arrangement of the cells. Light micrographs of these specimens will be compared with corresponding electron micrographs of thin sections.

J. Fine Structure of Sporulation in a Synchronously Growing Culture of Cl. botulinum

The process of sporulation in Cl. botulinum was followed by light and electron microscopy of hourly samples removed from a synchronously growing culture, fixed with 2 percent potassium permanganate in tap water, dehydrated

with graded ethanol series and embedded in epoxy resin by use of reagents described by Luft (1961) and the protocol employed by Fukushi (1961).

Materials:

Synchronous culture of Cl. botulinum 62A in 5 percent YE

2 percent potassium permanganate in tap water

2 percent non-nutrient agar in tap water

50, 70, 90, and 100 percent ethanol

ethanol: propylene oxide, 1:1

ethanol: propylene oxide, 1:3

Mixture A

Epon 812 (Shell Chemical Co., San Francisco) ---62 ml
Dodecenyl succinic anhydride (DDSA) (hardener)
National Aniline Division of Allied
Chemical and Dye Corp., New York City ----100 ml

Mixture B.
Epon 812 ----100 ml
Methyl nadic anhydride (MNA) (hardener)
National Aniline Division of Allied
Chemical and Dye Corp., New York City ---- 89 ml

2,4,6-tri (dimethylaminomethyl) phenol (DMP-30)
(accelerator) Rohm and Haas Co., Philadelphia

propylene oxide (1-2-epoxy propane) Mathieson, Coleman and Bell, Norwood, Ohio; Eastman Organic Chemicals, Rochester, N.Y.

Methods:

Culture. Synchronous growth was obtained by inoculating 9 ml of 5 percent YE, pH 8, with 0.1 ml of heat shocked spores. After 2 hours at 30° C, the entire culture was transferred to 90 ml of YE, and the resulting culture incubated for 2 hours at 30° C. After checking with a methylene blue stain, the entire culture was transferred to 900 ml of YE. At hourly intervals, aliquots for thin sectioning were aseptically removed while anaerobiobis was maintained in the remaining culture.

Fixation. The cells in the hourly aliquots were sedimented

by centrifuging at 1500 x g for 20 minutes. After washing the pellet 2 times with tap water, the second time in a 12 ml conical tube, approximately 5 ml of 2 percent potassium permanganate in tap water was added and the cells gently suspended with applicator sticks. Hourly specimens, taken from the first through the twelfth hour, were held at 4° C for 4 hours while parallel suspensions of these hourly samples were held for 24 hours in the fixative. After fixation, the permanganate was removed by washing twice in tap water.

Dehydration. The clean, fixed cells were suspended in a few drops of 2 percent non-nutrient agar at 60° C. The suspension was dropped on a clean, glass slide by using a warmed capillary pipette. After the agar had solidified, it was cut into 1 mm cubes with a razor blade. Dehydration was accomplished by serial transfer through 90 and 100 percent ethanol.

Infiltration and embedding. The two mixtures, A and B, can be combined in any proportion to give a continuous and wide range of hardness. The ratios of A:B tried in this study were 8:2 and 9:1, with 9:1 proving more satisfactory. Mixtures A and B as well as DMP-30 are viscous liquids which must be carefully measured and thoroughly mixed (Luft, 1961).

The dehydrated cells were subjected to increasing concentrations of propylene oxide by using the series, ethanol-propylene oxide, 1:1; 1:3; and 0:1 for 30 minutes, each at room temperature. Impregnation with the resin was effected by the following procedure: proplyene oxide:Epon 812, 1:1, 60 minutes at room temperature; Epon 812, 2 hours at 40° C; fresh Mixtures A plus B, 60° C for 2 hours. Finally the cubes were placed in a No. 00 gelatin capsules and the capsules filled with Mixtures A plus B plus 1.5 percent DMP-30 (v/v). The blocks were settled slowly, the tops placed on the capsules and the resin polymerized at 60° C for about 24 hours.

Summary of Preparation of Specimens for Thin Sectioning

1. Remove 50 ml from a synchronously growing culture at hourly intervals.
2. Sediment by centrifugation.
3. Wash pellet with tap water, sediment by centrifugation.
4. Transfer pellet to 12 ml conical tube, wash with tap water, sediment by centrifugation.
5. Decant supernate, add 5 ml of 2 percent potassium permanganate in tap water,
6. Gently suspend cells by mixing with applicator stick.
7. Hold at 4° C for 4 hours.
8. Sediment by centrifugation.
9. Wash 2 times with tap water.
10. Make heavy suspension of cells in a few drops of 2 percent non-nutrient agar at 60° C.
11. Drop on glass slide with capillary pipette.
12. Cut solidified suspension into 1 mm cubes with razor blade.
13. Place cubes in 50 percent ethanol for 30 minutes at room temperature.
14. Decant, add 70 percent ethanol; hold for 30 minutes at room temperature.
15. Decant, add 90 percent ethanol; hold for 30 minutes at room temperature.
16. Decant, add 100 percent ethanol; hold for 30 minutes at room temperature.
17. Decant, add 100 percent ethanol; hold for 30 minutes at room temperature.
18. Decant, add 1:1 ratio of ethanol;propylene oxide; hold for 30 minutes at room temperature.
19. Decant, add 1:3 ratio of ethanol;propylene oxide; hold for 30 minutes at room temperature.
20. Decant, add 100 percent propylene oxide; hold for 30 minutes at room temperature.
21. Decant, add 1:1 ratio of Epon 812;propylene oxide; hold for 30 minutes at room temperature.

22. Draw off with capillary pipette, add Epon 812; hold for 2 hours at 40° C.
23. Draw off, add fresh Epon 812; hold overnight at 40° C.
24. Draw off, add Mixtures A plus B, 9:1 ratio; hold for 2 hours at 40° C.
25. Draw off, add fresh Mixtures A plus B; hold for 2 hours at 60° C.
26. Draw off, add Mixtures A plus B plus 1.5 percent DMP-30; hold for 2 hours at 60° C.
27. With Jewelers' forceps, place blocks in No. 00 gelatin capsules, one block per capsule.
28. Add Mixtures A plus B plus 1.5 percent DMP-30.
29. Settle blocks slowly.
30. Put tops on capsules.
31. Polymerize for 24 hours at 60° C.

K. Materials Released from Spore Coats

Nature of compounds released by irradiation, autoclaving and fixation. The ultraviolet spectra of compounds released by these treatments were determined spectrophotometrically on supernates from: (1) spores irradiated at 3, 6, and 9 megarads; (2) spores treated with 2 percent aqueous permanganate at 4° C for 3 hours; (3) spores autoclaved for 10 minutes at 110° C and 121° C for 15 minutes. Protein was determined by the ninhydrin method for amino acids. Calcium in the supernates was estimated by comparing their ultraviolet spectra with those of DPA and Ca:DPA in the 272-280 mu wave length region.

Materials released from skinned spores by EDTA treatment. Skinned spores were treated with saturated EDTA overnight in the cold, sedimented by centrifugation, and washed with distilled water. The supernatant EDTA solution and the wash water were combined for determination of the ultraviolet absorption spectrum.

L. Dehydrogenases and Proteases in Cytoplasmic Fraction

Methods for demonstrating the presence of active proteases and dehydrogenases in the core fraction are described below.

Materials:

A 1 percent aqueous solution of each of the following proteins was used for measurement of proteolytic activity:

casein, pure, Fisher

Albumin, crystallized bovine plasma, Armour

gelatin, purified, Difco

peptone, Difco

A 1/50 M aqueous solution of the following carbohydrates and organic substances were used to determine dehydrogenase activity:

glucose

1, 6-diphospho-fructose

sodium citrate

pyruvic acid

lactic acid

formic acid

sodium acetate

0.2 M phosphate buffer, pH's 5, 6, 7 and 8

quantitative ninhydrin reagents (Moore and Stein, 1948)

1/50,000 dilution of methylene blue

cytoplasmic fraction of spores

Methods:

The quantitative ninhydrin method (Moore and Stein, 1948) was used to follow proteolytic activity in anaerobic reaction tubes which contained:

0.2 M phosphate buffer	0.5 ml
dist. water	2.0 ml
protein solution	0.05 ml
cytoplasmic extract	0.5 ml

Methylene blue reduction, determined photometrically in a Klett-Summerson colorimeter with a No. 420 filter, was used to follow dehydrogenase activity. The anaerobic reaction tubes contained:

1/50 M substrate	0.5 ml
0.2 M phosphate buffer	0.5 ml
1/50,000 methylene blue	0.2 ml
dist. water	1.3 ml
cytoplasmic extract	0.5 ml

M. Miscellaneous

Phase refractility. Spores which had received 3, 6 and 9 megarads of gamma irradiation were examined in aqueous mounts under dark phase-contrast illumination both before and after exposure to 5 percent YE broth at pH 8.

Permanganate treatment of spores and spore coat skeletons. Normal clean spores and spore coat skeletons isolated by acid hydrolysis were treated with aqueous 2 percent potassium permanganate at 4° C. Normal and irradiated spores were exposed to 5 percent YE broth until they became phase dark, washed twice and treated with permanganate as indicated above. For comparison normal spores were germinated aerobically in YE broth containing 100 g/ml chloramphencol.

Treated specimens were examined with a dark phase contrast microscope. The same specimens were exposed to YE broth then again examined with dark phase contrast illumination.

The effect of permanganate treatment on dye resistance of normal and irradiated spores was determined on both suspensions and air dried films. The net weight increase of normal spores was determined by comparing dry weights before and after permanganate treatment. Treated spores were

prepared for spectrophotometric determination of DPA by heating for 5 minutes at 110° C and also by digestion with concentrated H_2SO_4 followed by adjustment with 5 N NaOH to pH 6.

Infrared spectra. Spore coat skeletons, 0.467 percent by weight, were pressed into a KBr pellet 1 mm thick. The conditions of spectral determination were: prism, NaCl; resolution, 1:1; gain, 5.2; speed, 4.2; supression, 0; and scale 5 cm/micron. A Perkin-Elmer instrument was used.

Effect of sonication on spores. Clean spores were weighed and suspended in either water or benzene containing an equal volume of Superbrite glass beads. The weight of spores used varied from 0.2-5.0 g and the volume of fluid from 50 to 150 ml. After treatment of suspensions for various intervals in a 10 kc Raytheon Sonic Oscillator at 10° C, samples were removed and refrigerated. Subsequently, air-dried films were treated with dilute methylene blue and examined for the general appearance of cells and background. Spores and large particles were sedimented by centrifugation at 3500 x g for 20 minutes. DPA in the resulting cell-free supernate was determined photospectrometrically and protein by the Millon and Folin-Ciocalteau tests.

RESULTS

A. Production of Spores

Large masses of clean spores (Fig. 3) were readily produced (as described on page 20) and lyophilized or frozen in distilled water.

B. Correlation of Spore Recovery, Refractility to Staining and Dipicolinic Acid Release During Prolonged Heating of Spores

The effect of prolonged heating on these three prominent characteristics commonly associated with "germination" (Campbell, 1957) are shown in Table 3 and Fig. 4. The loss of viability preceded and diverged from the loss of resistance to ordinary stains. The release of DPA was parallel to but lower than the loss of viability during the three hours of heating.

C. Germination Studies

The rates at which spores of Cl. botulinum became stainable in synthetic media were slower than that in YE broth. However, complete germination and heavy outgrowth of atypically large and granular cells had occurred after 18 hours incubation at 30° C. The percentages of germination after 60 minutes in various synthetic media are summarized in Table 4.

The components in YE which were responsible for initiating germination were found to be dialyzable (Table 5). Germination was not initiated by the non-dialyzable fraction of YE.

Dodine, (N-dodecyl-guanidine acetate), a compound known to change the permeability of certain fungi, caused no appreciable germination during two hours observation (Table 6); neither was germination initiated by an equimolar complex of Ca:DPA at a concentration of 20 m mol (Reiman et al, 1960).

TABLE 3. The effect of prolonged heating on viability, stainability and release of dipicolinic acid

Time of Heating	No. of Viable Spores	Percentage Staining	mg DPA Released
0 min	4.0×10^7	0	0
5 min	1.7×10^7	28	62.7
10 min	2.2×10^7	43	69.9
15 min	1.8×10^7	21	82.5
30 min	2.0×10^7	38	104.5
45 min	1.7×10^7	40	115.0
60 min	1.6×10^7	42	127.5
75 min	1.9×10^7	52	140.0
90 min	1.4×10^7	50	145.0
105 min	1.5×10^7	46	142.0
120 min	1.6×10^7	49	149.0
150 min	9×10^6	48	184.0
180 min	8×10^6	56	175.0
19 hrs	- - - -	85	260.0
boiled 15 min	- - - -	100	276.0

TABLE 4. Percentage germination in synthetic media after 60 minutes at 30°C

Medium	Per cent Germination
5% yeast extract	97
18 amino acids, 9 vitamins, 5 minerals 5 bases and glucose	50
18 amino acids, 9 vitamins, 5 minerals and glucose	41
18 amino acids, 5 bases, 5 minerals and glucose	42
18 amino acids, 5 minerals and glucose	44
18 amino acids and 5 minerals	31
18 amino acids and glucose	30
18 amino acids	30

TABLE 5. Percentage germination of Cl. botulinum 62 A in dialyzed and non-dialyzed yeast extract

Times Minutes	5% YE	Dialyzed YE	Non-dialyzable YE
0	2	2	2
15	70	76	33
30	76	94	12
45	86	96	7
60	81	99	2
75	91	98	1
90	91	100	6
105	100	100	1
120	100	100	9

TABLE 6. Percentage germination of Cl. botulinum 62 A in dodine

Time Minutes	5% YE	Dodine
0	2	2
15	70	26
30	76	36
45	86	25
60	81	28
75	91	20
90	91	23
105	100	19
120	100	21

D. Studies on Germination Exudate

Chloramphenicol in 5 percent YE broth had no effect on germination; however, 50, 75 and 100 micrograms of this antibiotic per ml of medium inhibited outgrowth for at least 5 hours (Table 8); thus, the exudate prepared by the procedure described on page 23 should be free of products of vegetative growth.

Analyses of germination exudates from untreated and irradiated spores revealed both qualitative and quantitative differences (Table 9).

When the arsenomolybdate test was performed on these exudates, particulate reducing material was detected. Further investigation revealed that all the reducing material could easily be removed from the non-irradiated exudate by centrifugation, leaving a clear supernate. However, when the exudate from irradiated spores was centrifuged, only part of the reducing material was removed. Although this material could not be removed by centrifuging in a Spinco preparative centrifuge, it was non-dialyzable, indicating a macromolecule. These data are summarized in Table 10.

E. Studies on Irradiated Spores

Determination of spore concentration. The results of duplicate colony counts are summarized in Table 7. The average colony count in the control was 69×10^7 and in the irradiated sample, 3×10^2, giving a kill of over 99.99 percent. Results of the direct count were: control, 6×10^8 and irradiated, 7×10^8. The absorbancies at 450 mu were: control, 1.10 and irradiated, 1.20 at 1×10^2 dilutions, indicating that the two spore suspensions contained essentially the same concentration of spores.

Analysis of control and irradiated spores. Morphologically, there was no apparent destruction of most of the irradiated spores; yet, at a dosage level of 3 megarads they had lost 14 times as much material on storage in distilled water at 4° C as did the control spores. The results of analysis

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TABLE 7. Effect on viability of 3 megarads of gamma irradiation

Dilution	Colony Count			
	Control		Irradiated	
10^2	----	----	2	4
10^3	----	----	10	0
10^5	TNTC	TNTC	--	--
10^6	TNTC	TNTC	--	--
10^7	73	63	--	--
10^8	8	7	--	--
10^9	1	0	--	--
10^{10}	0	0	--	--

TABLE 8. The effect of chloramphenicol (CAP) on germination and outgrowth of Cl. botulinum 62 A at 37°C

Time Minutes	Germination		Outgrowth	
	No CAP	50 µg CAP	No CAP	50 µg CAP
0	4	4	--	--
5	51	45	--	--
10	69	76	--	--
15	72	78	--	--
20	83	87	--	--
25	91	82	--	--
30	89	80	slight	--
60	98	99	slight	--
90	100	87	moderate	--
120	100	97	abundant	

for selected compounds in the supernates are presented in Table 11. The results of analyses of control and irradiated spores are given in Table 12. A considerable part of the material remains unidentified; however, there are no dramatic differences in the components in Table 12.

Light microscopy. Spores which had received 3 megarads of gamma irradiation remained refractile to staining. However, after exposure to 6 megarads, approximately 15 percent of the spores became stainable. Increasing the exposure to 9 megarads increased the percentage to 20 percent. For reasons which were not investigated, the percentage of spores which became stainable at 6 and 9 megarads varied somewhat from one lot of irradiated spores to another.

A hot carbol fuchsin stain revealed typical red, ellipsoidal bodies in the control and in a high percentage of the irradiated spores. The remaining bodies, as high as 15 percent, appeared as empty shells which did not stain. In wet mounts of irradiated spores stained with carbol fuchsin the same distinction could be made between typical spores and empty shells.

Quantitation of materials released by irradiation. The total amount of material released from the spores into the suspending water increased directly with the radiation dosage. The dry weights per ml of supernates were: control, 1.2 mg; 3 megarads, 3.8 mg; 6 megarads, 16.2 mg; and 9 megarads, 19.6 mg. The percentage losses in dry weight were: 3 megarads, 4.9; 6 megarads, 21.0; and 9 megarads, 25.5.

Calcium content of irradiated spores. The calcium content of spores varied inversely with the irradiation dosage. The percentages were: control, 0.32; 3 megarads, 0.14; 6 megarads, 0.12; 9 megarads, 0.11. The percentage losses were: 3 megarads, 56.2; 6 megarads, 62.5; and 9 megarads, 65.6.

TABLE 9. Analyses of germination exudates (percentage of dry weight)

Component	Control	Irradiated
protein (non-dialyzable)	18.0	42.0
hydrolyzable carbohydrate	0.0	1.4
glucosamine	3.5	1.4

TABLE 10. Distribution of reducing material in germination exudate

Time	X G	Control		Irradiated	
		Sed.	Sup.	Sed.	Sup.
5	4,000	blue	clear	blue	blue
15	17,000	- -	- -	blue	blue
60	68,000	- -	- -	blue	blue

TABLE 11. Partial analysis of supernatant liquids

Constituent	Control	Irradiated
DPA	28 µg/ml	300 µg/ml
amino acids	none	none
protein	none	420 µg/ml
calcium	none detectable	none detectable
glucose	15 µg	15 µg
glucosamine	none detectable	none detectable

F. Preparation of Spore Coat Skeletons

Prolonged heating at 121° C. Such treatment produced entities which appeared as typical refractile spores when treated with methylene blue and as empty shells when treated with hot carbol fuchsin. Very little disintegration of the shells was noted even after five hours of treatment (Table 13).

Acid treatment. The spore coat skeletons isolated by acid treatment appeared to be a combination of intact exosporia and spore coats from which cores and impregnating materials had been removed (Figs. 5 and 6). These skeletons, when exposed to methylene blue then examined with the light microscope, were indistinguishable from clean untreated spores. However, after treatment with hot carbol fuchsin the skeletons were faintly tinged empty shells while untreated controls were typical intense staining spores.

G. Analytical Procedure

Spore coat skeletons. The results of elemental analyses performed on spore coat skeletons isolated by acid treatment were: carbon, 56.69; nitrogen, 10.46; hydrogen, 8.25; oxygen, 17.29; sulfur 2.11; phosphorous 0.12. No amino nitrogen was found by the Van Slyke technique; however, a small insoluble residue remained in the nitrous acid at 230° C. The results of solubility and qualitative organic tests on spore coat skeletons are shown in Tables 14 and 15.

Enzyme treatment. Treatment of intact spores with the enzymes given in Table 16 produced no change observable by light microscopy; nor were detectable ninhydrin positive or reducing materials liberated into the supernates. Spore coat skeletons appeared to be unaffected by alpha and beta amylases, alpha glucosidase and cellulase.

H. Fine Structure Studies by Use of Spore Fractionation and High Level Gamma Irradiation

Spore fractionation by use of the Ribi Cell Fractionator. Electron microscopy of suspensions of clean spores of Cl. botulinum revealed electron

TABLE 12. Partial analysis of control and irradiated spores

Constituent	Percentage of Dry Weight	
	Control	Irradiated
DPA	14.0	14.0
protein	30.0	30.0
*reducing material as hexose	0.3	0.3
glucosamine	2.2	1.2
calcium	3.0	.6
lipid	20.0	not done

*This represents material which was hydrolyzable to reducing compounds by 1 N HCl at 95°C for 20 hrs.

TABLE 13. The effect of prolonged autoclaving on spores of Cl. botulinum

Time of Autoclaving	Microscopic Appearance
0 min	all refractile
15 "	all stainable
30 "	most stainable, few shells
60 "	" " " "
2 hrs	many with clear centers, dark rims
3 "	" " " " " "
5 "	most appear refractile

Cells treated with dilute methylene blue.

TABLE 14. Solubility of spore coat skeletons and intact spores

Solvent	Solubility	
	Spore Coat Skeletons	Intact Spores
water	-	-
0.1 N H_2SO_4	-	-
conc. HCl	-	-
1 N NaOH	-	-
5 N NaOH	-	-
absolute ethanol	-	-
xylene	-	-
benzene	-	-
carbon tetrachloride	-	-
diethyl ether	-	-
conc. H_2SO_4	+	+

TABLE 15. Characterization of reactive groups in spore coat skeletons

Compound or Group	Method	Reaction
protein	ninhydrin	-
	phenol reagent	-
	Van Slyke	-
glucosamine	indole reaction	-
reducing compounds	arsenomolybdate	+
phenols (p-unsubstituted)	Millon	+
glucose fructose galactose pentose RNA DNA ascorbic acid tryptophane glutamic acid	anthrone	-
indole nucleus	p-dimethylamino benzaldehyde	+
carbohydrates furfural-yielding substance	Molisch	-
nitroso	phenol H_2SO_4	-
$-CONH_2$* $-CSNH_2$* $-C(NH)NH_2$* $-CH_2NH_2$*	biuret	+
free indole	Kovac's reagent	-
glucose	cysteine $-H_2SO_4$	-
aldehydes*	2,4 dinitro-	+
ketones*	phenylhydrazine	+
active unsaturation	$KMnO_4$	+
	bromine	+
alcohols	$FeCl_3$	

Compound or Group	Method	Reaction
phenols* hydroxy acids* hydroxy carbonyl*	nitrous acid	+
aldehydes*	benzidine	-
esters*	ferric hydroxamate	-
aromatic amides	hydroxamate test	-
primary arylamines*	diazotization	-

*Detection of these groups is dependent upon their occurrence in the uncombined state.

TABLE 16. Treatment of spores with enzymes

Enzyme	Buffer	pH
alpha-amylase	tris	6.9
beta-amylase	citrate	5.2
alpha-glucosidase	citrate	5.2
cellulase	c'trate	5.2
lysozyme	citrate	6.2
trypsin	tris	7.2
pepsin	citrate	4.6
carboxypeptidase	tris	7.2

dense spore bodies surrounded by transparent membranes (exosporia, Fig. 7). Passage through the Ribi Cell Fractionator shattered all exosporia and an occasional spore coat (Fig. 8). In pure fractions the shattered exosporia were thin, electron transparent fragments with predominately angular breaks (Fig. 9). Purified skinned spores (Fig. 10) were capable of typical germination and outgrowth in YE broth at pH 8. After removal of impregnating material from the spore coat by treatment with EDTA or 9 megarads of gamma irradiation, many spore coats were broken by the cell fractionator, most frequently by mid-centric cleavage but with some ragged tearing into large fragments (Fig. 11). The spore coat so revealed was thicker and more electron dense but less brittle than exosporia. Close observation of the torn edges of fragments suggested a fibril structure. The purified fraction of second layer material (spore coats) is shown in Fig. 12).

Fine structure revealed by high level gamma irradiation. High level gamma irradiation was an unexpectedly useful tool in studying the fine structure of these spores. Three megarads of gamma irradiation produced little observable change in the spores (Fig. 13). However, 6 and 9 megarads produced a decrease in electron density at the periphery of the spore body, thus revealing the spore coat (Figs. 14 and 15). The electron absorbing characteristics and thickness of spore coats revealed by this method were the same as those revealed by spore fractionation.

When irradiated spores were put through the cell fractionator, all exosporia were shattered in all 3 treatments. In addition many spore coats were broken in the 6 and 9 megarad-treated samples (Figs. 16, 17 and 18). No naked cores were observed (Figs. 17 and 18). The typical morphological elements revealed here were also revealed in thin sections (Figs. 19-26).

I. Fine Structure Studies by Thin Sections

Thin sections of fixed spores. During the study of thin sections prepared by several techniques 3 important fixation effects were observed. These were: spine formation, vacuolization and layering.

Osmium tetraoxide alone caused the exosporium to shrink and adhere closely to the spore coat while the periphery of the spore coat was distorted, thus producing a polyhedron effect (Fig. 19). The simultaneous use of potassium permanganate and osmium tetraoxide reduced but did not eliminate spine formation (Fig. 20).

In specimens fixed for 4 hours there were many irregular vacuoles (Fig. 21) suggesting inadequate fixation and dehydration. Subsequent experiments revealed that spores which had been exposed to the fixative for varying lengths of time could be removed, washed and germinated. Germination after subjection to the fixative was slow but was nearly complete after 18 hours. Outgrowth appeared typical. Spores which were capable of germination and outgrowth were also resistant to staining with methylene blue. When the time of fixation was increased the spores became stainable with methylene blue and failed to grow out. Extensive vacuolization was not observed in these spores (Fig. 22). The time required for penetration of the core by potassium permanganate varied from experiment to experiment, being as short as 4 hours and as long as 8 hours. Osmium tetraoxide alone failed to penetrate these spores, even after 7 days in the refrigerator.

The appearance of spores fixed with potassium permanganate could be changed drastically by varying the time of fixation and the extent of washing after fixation. In "normal" spores which had been fixed for 4 hours then given 2 thorough washings before starting dehydration as many as 8 zones could be observed (Fig. 23). The outermost zone appeared as a somewhat

electron dense line (a); the second zone (b), was less electron dense and bounded inwardly by a narrow, sharp line of greater electron density (c). The fourth zone (d) was semi-transparent and variable in width. Inside was a wide, extremely transparent zone (e), bounded by a faintly differentiated line (f). Another semi-transparent zone (g) separated line (f) from line (h) which surrounded the core (i).

The cores of this and all other specimens were unusually electron dense and displayed small granules which were even more dense. Some cores contained semi-dense, S-shaped zones (Fig. 20), similar to those which have been ascribed to nuclear material in aerobic spores (Fitz-James, 1960; Tokyasu, 1960). As mentioned earlier, extensive vacuolization in some cores appeared to be artifacts of preparation. When the fixative was left on spores for 9 hours or more, only 4 zones were observed: the exosporium, outer dense zone, inner transparent zone and the spore core (Fig. 22). Fixation for 70 hours caused disintegration of parts of the spore coat (Fig. 24).

Skinned spores fixed with potassium permanganate appeared to be composed of a core and a double-layered coat (Figs. 21 and 24). The deposits of electron dense material, possibly manganous oxide, can be seen to penetrate irregularly into the spore coat.

Exhaustively washed free spores and twice washed endospores which had been fixed with potassium permanganate differed considerably in appearance. In the endospore the exosporium was well ballooned around the spore body, whereas it had been collapsed close to the spore coat except at the ends of exhaustively washed, free spores (Fig. 25 and 49). The space between the exosporium and the spore coat of endospores was filled with cytoplasmic-like material which was, in electron micrographs, indistinguishable from the cytoplasm of the mother cell (Fig. 49). The small amount of space remaining

between the exosporium and the spore coat in the exhaustively washed free spores appeared to be empty (Fig. 25).

The most informative specimens were thin sections of unfixed spores. In specimens which were simply dehydrated and sectioned on water (Fig. 26), the core had a very high density while the spore coat was extremely low in contrast. The density of the spore coat was slightly higher at the periphery. The exosporium cannot be seen. After exposure of such sections to osmium tetraoxide vapor the contrast of all structures was increased (Fig. 27); however, contrast of the cores and discontinuous zones at the periphery of the spore coat increased more markedly. The exosporium had become visible. Floating sections on one percent lanthanum nitrate increased the contrast of all structures rather uniformly (Fig. 28).

J. Fine Structure of Sporulation in a Synchronously Growing Culture

Light microscopy. Light micrographs taken during sequential stages of growth and sporulation showed changes in cell size, development of granulation, and gradual appearance of dye refractility. Figures 29 through 40 were prepared from methylene-blue stained specimens.

Changes in cell size and arrangement were obvious during the first 4 hours of growth (Fig. 29-32). At one hour the cells were relatively small and individually distinguishable within the chain. At 2 hours the cells had nearly doubled in diameter and individuals were not easily distinguished. Cells which were transferred at this stage continued vegetative growth, but by two and a half hours were committed to sporulation. By 3 hours the cells were still large but individuals were distinguishable. At 4 hours the diameter had decreased to that observed at one hour. The cell size and arrangement was nearly constant during the remaining stages of sporulation.

The gradual development of dye refractility is demonstrated in Figs. 33 through 40. At 5 hours granulation could be micrographed; at 6 hours it was pronounced. By 7 hours the irregular granulation had given way to a rather uniform, bipolar appearance. At 8 hours the bipolar appearance was more pronounced; at 9 hours this bipolar appearance was pronounced in every cell. At 10 hours many cells contained ovals which were approaching complete refractility. At 11 hours more than 90 percent of the cells contained fully refractile spores. At 12 hours sporulation was complete in essentially all cells.

Electron microscopy. Fine structure of the sporulation process is presented in Figs. 41 through 51. In sections through chains of cells taken from a 2-hour culture the cell walls appeared to be straight lines with which the cross walls formed at approximately 90 degree angles (Fig. 41). The cytoplasm was finely granular with no detectable differentiation. Chains of cells from a 3-hour culture contained segments in which the appearance had not changed and other segments in which the walls had assumed the curvature typically ascribed to these organisms (Fig. 42). The cytoplasm remained finely granular and without differentiation. The ends of cells from a 4-hour culture had become rounded so that individuals were easily distinguished (Fig. 43). The cytoplasm remained finely granular and without differentiation. The cytoplasmic membrane was a thin line just inside the cell wall. At 5 hours a subterminal, round structure (mesosome) had arisen, apparently by invagination of the cytoplasmic membrane (Fig. 44, cell A). In cell B of the same figure the round structure had migrated to the longitudinal axis of the cell and a septum was forming from the opposite side of the cell. At 6 hours the round structure had grown larger and the transverse septum extended completely across the cell (Fig. 45). The membranous character of the round structure may be seen faintly. At 7 hours (Fig. 46)

no dramatic change had occurred in cell A or B. However, in cell C the mesosome appeared to have migrated to the end of the cell, leaving behind a faintly visible primordial inner transparent zone. The cross septum had disappeared. Intermediate steps in this structural transformation remain to be elucidated.

There now ensued a phase of development during which the changes in appearance were rapid and straight forward. At 8 hours the round structure, now obviously a developing spore, is surrounded by an electron transparent zone (Fig. 47). The size, electron density and texture of the core did not change during the remainder of sporulation. The typical electron dense zone which was observed in sections of mature spores had not yet developed. At 9 hours the dense zone typically observed in fixed specimens was becoming evident (Fig. 48). At 10 hours the electron dense zone was complete and the developing exosporium was apparent (Fig. 49). At 11 hours the exosporium was nearly complete (Fig. 50). At 12 hours the exosporium was complete and the cytoplasm appeared to be disintegrating around the exosporium in some places (Fig. 51).

K. Release of Materials from Spore Coats

Nature of compounds released by irradiation, heating and fixation. The ultraviolet spectra of materials in the supernates are shown in Fig. 52. The spectra shown are those of mixtures; however, the characteristic peaks of DPA at 270 mu and Ca:DPA at 278 mu may be identified. Materials released by boiling and limited autoclaving contained little calcium; however, increased heating increased the amount of calcium which was released.

Release of DPA by treatment with EDTA. Clean, "normal" spores which had been treated overnight with a saturated solution of EDTA at 4° C contained 6 percent DPA while untreated spores from the same lot contained 10

percent DPA. Thus, the EDTA treatment caused a 40 percent decrease in the DPA content of these spores.

L. Enzymes in Cytoplasmic Fraction

Proteases. Only low level proteolytic activity was demonstrated with the first lot of cytoplasmic material, most likely due to the low concentration of protein present. In preparation of the second lot of cytoplasmic fraction, a much higher concentration of protein was obtained by using a higher initial concentration of spores. With this material, significant proteolytic activity was observed. The proteolytic activity observed on 4 substrates at 4 pH values after 30 minutes at 30° C are shown in Fig. 53.

Dehydrogenases. Only the second lot of cytoplasmic material was used in this experiment. Considerable dehydrogenase activity was observed even when no substrate was added, indicating the presence of reducible material in the spore core material. Of the seven carbohydrate substrates tried, significant dehydrogenase activity could be shown only with pyruvic and citric acids. The results are shown in Figs. 54-56.

M. Miscellaneous

Phase refractility. Spores which had received 3, 6 and 9 megarads of irradiation remained phase refractile, though the intensity was diminished. Although these spores were incapable of outgrowth they readily lost phase refractility in YE broth. Phase refractility could be restored to these spores by treatment with permanganate fixative for 3 hours. Furthermore, spore coat skeletons which had been isolated by acid treatment were phase dark, but could be made phase refractile by treatment with permanganate fixative. Phase refractility could be restored only partially to spores which were allowed to germinate in YE broth under optimal outgrowth con-

ditions before treatment with the permanganate fixative. Phase refractility could not be conferred upon vegetative cells or spores germinated aerobically in YE broth containing 100 μg/ml of chloramphenicol by fixation with permanganate for 3 hours. However, vegetative cells which had been exposed to the fixative for 24 hours were phase refractile. Control spores which had been rendered stainable by the permanganate fixative were somewhat tarnished but still phase refractile.

The phase refractility produced by treatment with permanganate was readily lost from all of these specimens when they were exposed to 5 percent YE at pH 8.

Effect of permanganate on spores. Control spores which were exposed to the fixative for three hours increased about 10 percent in weight even though they remained resistant to staining. Spores fixed 24 hours became stainable but showed no further net weight increase.

No DPA could be detected in spores which remained dye resistant but which had been exposed to permanganate for 3 or more hours at 4° C. However, DPA complexed with a small amount of calcium was detected in the supernatant fixative (Fig. 51).

X-ray diffraction. The measurements of reflections from x-ray diffraction plates are given in Table 17. Exosporia which were isolated by the cell fractionator exhibited 6 reflections while the spore coats exhibited only 4. However, these 4 were the same as the 4 of those in exosporia. After acid treatment of exosporia which had been isolated by the cell fractionator, exosporia exhibited 7 reflections. Acid treatment of exosporia had produced one new reflection, that at 23 mm. Spore coat skeletons exhibited 4 reflections not observed in exosporia or spore coats. However, three common reflections, (") those close to 19, 40, and 45, were observed

TABLE 17. Measurement of lines from x-ray diffraction plates

Exosporia Isolated by Cell Fractionator	Spore Coats Isolated by Cell Fractionator	Exosporia After Acid Treatment	Spore Coat Skeletons Isolated by Acid Treatment
*19-20 (S)"	*19 (S)"	19.5 (S)"	19 (S)"
			22 (M-W)
		23 (VW)	
			24 (M)
			33 (VW)
*35 (W)	*35 (W)	35 (M)	
			38 (W)
*40.5 (VW)"	*40 (VW)"	40.5 (M)"	40.5 (M-W)"
*44.5 (M)"	*44.5 (VW)"	44.5 (M)"	45 (M-W)"
47.5 (W)		48 (VW)	
51.5 (W)		52 (VW)	

Strength of Reflections:
S - Strong
M - Medium
W - Weak
VW - Very Weak

*Lines common to exosporia and spore coats isolated from spores which had been disrupted by the Cell Fractionator.

"Lines common to exosporia and spore coats both before and after acid treatment.

in the 4 specimens. These results strongly indicate that exosporia and spore coat skeletons are basically alike in chemical composition, since the probability of obtaining this degree of identity of reflections with very different compounds would be very low.

Infrared spectra of spore coat skeletons. Absorption peaks and groups to which they were attributed are shown in Fig. 57. Thus, spore coat skeletons contained the following groups; hydroxy or secondary amine; alkane; associated carbonyl; olifen; nitrate, amide or sulfon; asymetric -CH in aromatic or aliphatic compound; methyl; and oxygen group. Unfortunately, the spectrum reveals nothing of the organization of groups within the structure.

Dispersion of clumps of skinned spores. As previously mentioned, centrifugation of skinned spores resulted in a troublesome degree of tenacious clumping. Twenty-seven reagents (Table 18) were tried in an effort to disperse such clumps. Included were organic solvents, non-ionic solutions; solutions of various ionic concentrations; and solutions of various pHs. Of all the reagents tried, only EDTA dispersed clumps sufficiently to give a visibly homogeneous suspension. Because of the strong chelating properties of EDTA, this suggests competitive chelation as a possible mechanism for dispersing clumps.

Effect of sonication on spores. Microscopic examination of air dried films which had been treated with dilute methylene blue showed an early increase in stainable, finely particulate material in the background and an increase in the percentage of spores loosing dye refractility. As sonication was continued, the amount of background material increased; however, the percentage of stainable spores remained constant for a time then decreased. During the "steady state" about 50 percent of stainable cells

TABLE 18. Materials tried in an effort to disperse aggregates of skinned spores

Organic Solvents
- acetone
- benzene
- butanol
- carbon tetrachloride
- chloroform
- dioxane
- 95% ethanol
- ethylene chloride
- formamide
- mono-chloro-benzene
- 90% phenol
- polyethylene glycol, 400
- pyridine

Solutions of Electrolytes
- ammonium sulfate, saturated
- cesium chloride, saturated
- potassium chloride, 1 M, pH 7.2
- potassium chloride, saturated
- potassium chloride, saturated in formamide

Buffers
- acetate, 0.2 M, pH 3.0
- borate, 0.05 M, pH 8.8
- phosphate, 0.1 M, pH 6.5
- trihydroxyaminomethane, 0.01 M, pH 8.2

Miscellaneous
- EDTA (ethylenedinitrilo) tetraacetic acid tetrasodium salt
- glycerol, 10-40%
- sucrose, saturated

was frequently observed. Only a small percentage of grossly distorted but still recognizable spores were observed. The results of 3 experiments, 2 in water and one in benzene, are summarized in Table 19.

Cell-free aqueous sonicate gave an ultraviolet absorption spectrum which indicated but did not conclusively demonstrate DPA. However, heating the sonicate resulted in a typical DPA spectrum. Heating in a boiling water bath for 5 minutes was as effective in producing this shift in absorption spectra as was longer heating (Fig. 58). Increasing the time of sonication increased the relative amounts of calcium to DPA, as indicated by absorption maxima at 278 mu (Fig. 59).

TABLE 19. Effect of sonication on spores

Suspending Fluid	Min of Sonication	General Appearance	Percentage Stainable
water, I	0	no stainable background	5
	10	slight stainable background	30
	20	some stainable background material and distorted cells	36
	30	much background material lightly stained; few distorted cells	52
water, II	0	no stainable background	10
	30	not recorded	16
	60	not recorded	36
	90	much stainable material; no unbroken cells	--
	120	much stainable material; no unbroken cells	--
benzene	90	much stainable background material	50

DISCUSSION

Information obtained by the diverse techniques and innovations employed in this study contributed to an understanding of the basis of some of the peculiar spore properties and to the interrelations among them. Frequently information obtained by one technique was important to an understanding of more than one phenomenon. Thus, germination; phase refractility; location of DPA and calcium; resistance to adverse physical and chemical environments; and sporulation can be discussed as expressions of the basic chemistry and fine structure of this organism.

Germination

Bacterial spore germination has been widely believed to be an enzymatic process which is triggered by specific metabolites (O'Conner et al. 1960; Kenyan et al, 1962) and a vast amount of work has been done in finding such triggering agents for specific organisms (Hills, 1949; Levinson et al, 1953, 1962; Woese et al, 1958). Similar studies were conducted briefly on spores of Cl. botulinum with singularly uninspiring results. In view of our findings and those of others, especially Treadwell et al, (1958); Foster (1959); Rode and Foster (1960, 1962); and Black and Gerhardt (1961, 1962), the conviction grew that an understanding of germination depended upon a knowledge of chemistry and fine structure of the spore. Consequently, the emphasis of the study was shifted to chemistry and fine structure.

Chemistry

During chemical studies it was found that the DPA and calcium content of these spores was quite similar to that of aerobic spores and that a hexosamine containing peptide similar to that released during germination of B. megaterium was released from these spores. Thus, these studies support the

logical expectation that spores of Cl. botulinum are basically like aerobic spores in composition and behavior.

Fine Structure

Fine structures studies on spores of Cl. botulinum prepared for electron microscopy by fractionation, high level gamma irradiation, and thin sectioning techniques consistently revealed 3 main morphological elements. These elements were: an exosporium, a spore coat and a spore core. Furthermore, studies of spores prepared by each method yielded distinctive and detailed information which, when combined with other observations, enabled partial characterization of each structure.

Shattered exosporia exhibited a preponderance of angular breaks which suggested a crystallite structure which was confirmed by x-ray diffraction studies. The removal of the enclosed cytoplasmic-like material simply by washing and centrifugation suggested a porous structure. Both of these observations are in agreement with those of Gerhardt and Black (1961) and Black and Gerhardt, (1961, 1962) on exosporia of B. terminalis. The superficial function of the exosporium is clearly demonstrated by the typical resistance, germination and outgrowth of skinned spores.

The cytoplasmic-like material between the exosporium and spore coat appeared to be vegetative cytoplasm which was entrapped by the developing exosporium. The fortuitous presence of this material was clearly demonstrated by the typical resistance, germination and outgrowth of skinned spores and exhaustively washed spores from which this material had been removed. However, the completeness of removal of this material was not determined. So the presence of a small residuum of cytoplasmic-like material could account for several interesting observations; namely, the toxicity of botulinal spores (Anellis and Koch, 1962) and the observed presence of a

thin faint line of stain around the periphery of clean spores. The shifting of the spore body within the exosporium during washing was apparent from a comparison of clean whole spores and sections of endospores (Fig. 1 and 22). This was due, perhaps, to removal of the supporting matrix of cytoplasmic-like material.

From fractionation and irradiation studies, spore coats appeared to be composed of a somewhat electron dense skeleton impregnated with a mixture of substances which rendered it entirely electron dense. The spore coat was revealed only on release of the impregnating materials. These materials may be released by biological germination (Fig. 27), irradiation (Fig. 10), heating (Fig. 28) and partially by EDTA (page 50). The unusually tough spore coat was so weakened by removal of the impregnating compounds that it could be broken by 45,000 psi (Figs. 5 and 6). Not only the impregnating compounds but also the core material was removed during isolation of spore coat skeletons by acid treatment (Figs. 13 and 14). Thus it appears that the core is solubilized and removed through the pores. The porous spore coat could well result from a random arrangement of fibrils. Such an arrangement is suggested by the ragged tearing of the spore coats observed during fractionation (Fig. 5) and by the occasional observation of fibrils along torn edges (Fig. 6). The lack of lamellation in thin sections (Figs. 17 and 20) also suggests a random rather than an oriented arrangement of fibrils. This is in contrast to the oriented fibrils observed in B. megaterium (Robinow, 1957); B. subtilis (Tokuyasu, 1959) and B. terminalis (Hashimoto, 1960).

Compositional studies on spore coat skeletons left the nature of these structures a baffling problem. Negative results with the phenol reagent, ninhydrin, and the Van Slyke technique all indicated that these structures

were not protein. Further tests indicated the presence of an indole nucleus which could account for the 10 percent of nitrogen found in these structures. Negative anthrone, arsenomolybdate and cysteine-sulfuric acid reactions indicated that these structures were not polysaccharide. Both infrared spectrometry and spot tests indicated a high degree of aromaticity and the presence of various function groups. However, the organization of these structures and reactive groups into molecules and polymers constituted a problem beyond the interest and scope of this research.

The impregnating material contained calcium, DPA, and a hexosamine containing peptide.

The cytoplasmic fraction gave a typical protein reaction with ninhydrin and stained readily with dilute methylene blue. It also exhibited proteolytic and dehydrogenase activity.

Electron microscopic study of spore coats revealed by fractionation, gamma irradiation and heating (Figs.8,15 and 5) had consistently indicated a homogeneous structure with a smooth periphery. Therefore, the observation of two drastically different zones in thin sections of permanganate fixed skinned spores was surprising as was the spine formation observed in spores exposed to osmium tetraoxide. Obviously spine-formation was a fixation effect, the nature of which could be speculated upon but not proved. The difference in the zones of spore coats after fixation could only reflect differences in the outer and inner zones themselves. However, this difference in electron density did not distinguish between physical and chemical differences. The chemical composition of the coat layers could have been quite different yet lacked contrast in the electron microscope, but the fact that the spore coat skeletons could be isolated as a single structure by acid treatment which solubilized protein and the fact that

it broke as a homogeneous structure in fractionation strongly favored a continuous structure. Consequently, the possibility of a difference in structure being responsible for zoning during fixation appeared feasible.

Thus, sections of unfixed spores revealed a lack of contrast. Also, the spore coat of such sections reacted uniformly with osmium tetraoxide vapor indicating reactive homogeneity of the spore coat. Lanthanum nitrate, which has been reported to be specific for the cortex (Mayall et al, 1957) was deposited throughout the specimen, as indicated by increased contrast. However, it was accumulated excessively in the periphery of the coat rather than in the area which corresponds to the reported cortex of aerobic spores. Had lanthanum nitrate been applied to fixed spores, neither the accumulation in the periphery nor the increase in contrast of core could have been detected. Once the apparent conflicts have been resolved, the observations made during the study of thin sections are in accord with those made by other methods.

Thin sections of unfixed spores provided direct evidence on the physical state of the core, evidence subject only to the equation of dye refractility and impermeability of the spore core. Obviously the core must be rigid to permit sectioning. Since there is ample evidence that the small molecules do not penetrate into the core (nor presumably out) of spores which remain resistant to staining (Gerhardt, 1961; Black, 1962) the rigidity must be an innate property of the spore rather than a result of external treatment. The unusual electron density of the core in thin sections is suggestive of a high concentration of biological molecules, i.e., dehydration, the presence of heavy metals or a combination of both. The refractive indices of spores indicates a highly dehydrated state (Ross et al, 1957). Work currently in progress, while not yet conclusive, supports

the latter possibility, i.e., the calcium is distributed between the coat and the core. Rapid hydration with accompanying expansion of the gel and loss of calcium could account for the production of highly transparent granules of core material during fractionation. Thus, the sectioning qualities, the electron density, and the dispersing characteristics all clearly indicate that the core is composed of a rigid gel with a low water content.

The effect of the fixative on the spore core is reflected by an increase in rigidity and electron density. The distortion observed in cores of unfixed specimens but absent from cores of adequately fixed specimens (Figs. 24 and 20) demonstrate the increased rigidity resulting from fixation. The increase in electron density was best observed by comparing the cores in Fig. 24 with those in Fig. 25.

Phase refractility

Phase refractility of spores has been considered an absolute property. However, the appearance of irradiated spores demonstrated that phase refractility can decrease gradually and remain stable at the reduced level for a period of several months. It appears that gamma irradiation offers a controllable means of gradually removing the impregnating compounds from the members of a population, the extent of removal depending upon the dosage up to a certain level. However, the great difference between the amounts of material released by 3 and 6 megarads and the small difference between the amounts released by 6 and 9 megarads shows a decreasing response to dosage.

Phase refractility was alternately produced in and removed from spore coat skeletons and spores which had received massive doses of irradiation. The materials and methods used in these studies precluded the participation of enzymatic action and cores in the development of phase refractility.

Therefore, the development of phase refractility was, in this instance a physiocochemical phenomenon resulting from the deposition of refractile material in or on the spore coat. The materials deposited from the permanganate fixative were insoluble in water; thus phase refractility was retained in aqueous solution. However, the reduction products were soluble in 5 percent YE broth at pH 8, so phase refractility was rapidly lost. The pH and the quantity and quality of ions are doubtlessly involved in dissolving the permanganate reduction products from the spore coats. Unfortunately, these remain unidentified.

The mechanisms responsible for phase refractility and resistance to staining were shown to be different by two lines of approach. First, spores which had received 9 megarads of gamma irradiation became 10 to 30 percent stainable (this varied from one lot of irradiated spores to another) while all remained uniformly phase refractile. This difference could have either a qualitative or a quantitive basis. A higher level of compounds could be required for the maintanence of resistance to staining than for phase refractility. The present work provided no experimental evidence for favoring one possibility over the other. It is clear, however, that impermeability of the core is not necessary for natural phase refractility. Second, spores which have been treated with permanganate were phase refractile but stainable; the spores contained no detectable DPA while the DPA complexed with about half the normal amount of calcium was found in the supernatant fixative. Thus, the phase refractility observed here resulted from replacement of natural phase refractility with that artifically produced by a treatment which destroyed resistance to staining. This is direct evidence that conditions, either physical, chemical or both, necessary for phase refractility are less specific than those for resistance to staining. Again

there is no evidence to favor one possibility over the other, so the relation between the two remains obscure.

The location of a major portion if not all of the DPA in the spore coat was demonstrated in a straight forward way by two different methods. The first method is subject only to the qualification that so long as permanganate does not pass into the dye refractile, viable core, neither does DPA pass out. The first proof was furnished by the demonstration that spores which had been exposed to permanganate but which remained dye refractile and viable contained no detectable DPA while DPA was found in the supernatant fixative. Had permanganate penetrated the cores, then the spores would have become stainable and non-viable. The second proof was furnished by the demonstration that spores which had received 6 or 9 megarads of irradiation remained largely dye refractile yet contained practically no DPA while DPA was found in the supernate from such spores. Had irradiation destroyed the the impermeability of the spore coat, the cores would have taken up dye in both air dried smears and aqueous mounts rather than remaining refractile. Thus,no DPA was released from the core, but only from the integument(s) exterior to the permeability barrier.

The ultraviolet spectra of compounds released by irradiation and various heat treatments provided a firm indication of the location of calcium within spores. When DPA was released from spores by irradiation or by heat treatments which left most of the spores dye refractile, the spectra, particularly the absence of a peak at 278 mu, indicated that only a relatively small amount of calcium was released as compared to the amount released by heat treatments which rendered the spores stainable. This was true even when the dye refractile spores retained no detectable DPA. This observation strongly indicated that the calcium was distributed in integuments and cores and could be easily and sequentially released from each. Analyses of irradi-

ated spores confirmed the presence of calcium in 3, 6 and 9 megarad-treated spores while 6 and 9 megarad-treated ones contained no detectable DPA. The relatively small difference between the calcium contents of 6 and 9 megarad-treated spores could well result from the release of "internal" calcium from the greater percentage of spores which became permeable (stainable) at 9 megarads.

Resistance

The resistance of bacterial spores to inimical agents remains a problem of practical importance and theoretical interest. Thus, it appears that a consideration of resistance must form a part of any discussion of these peculiar cells. The present study contributed information which appears to have a direct bearing on resistance to: irradiation, staining, heating, and disruption, both chemical and physical.

Spores which had received 3 megarads of gamma irradiation, upon preliminary microscopic and chemical studies, revealed several facts. First, most of the dead, irradiated spores remained dye refractile, indicating that the lethal effect was internally located and was independent of the impermeability barrier. Second, in a small percentage of spores, the impermeability barrier had been breached, allowing spores to become stainable. Third, in still others the retaining membranes had been destroyed, and cores solubilized and released into the suspending medium, leaving intact but empty shells. Fourth, irradiation produced detectable changes in reducing material contained in germination exudate. In germination exudate released from non-irradiated spores, the reducing material was bound in particulate form which was easily removed from suspension by centrifugation. In contrast, the germination exudate released from irradiated spores appeared to be composed of units of two sizes, one particulate and one macromolecular.

Although several detectable changes occurred during irradiation, it is unlikely that these were directly related to irradiation death. It seems logical to suppose that death is due to DNA damage and enzyme inactivation, phenomenona which are well documented in other cells (Barron, 1954). A comparison of DPA and DNA reveals a striking similarity between their structures. Both contain a heterocyclic, resonant structure which is an efficient absorber of radiant energy (Sinsheimer, 1955). Thus, the presence of a high concentration of DPA (10-16 percent) located in a protective shell around the DNA-containing vital core would offer considerable radiation protection by its absorption of radiation. Other possibilities include: reduced production and mobility of free radicals in the core due to the anhydrous state of this structure (Powers et al, 1960); protection of the core from externally produced free radicals by the impermeability barrier; and presence of a high content of free-radical scavengers, such as sulfhydryl groups (Vinter, 1961).

Resistance to staining appeared to be due to failure of the stain to reach the core rather than to any pecularity of the core cytoplasm. This conclusion, by no means original, has been supported by: (1) the staining of spores by hot solutions or by prolonged exposure, presumably resulting in eventual penetration of the stain into the core; and (2) staining of biologically germinated spores, although the possibility of enzymatic changes in the core cytoplasm was not eliminated. This study has contributed further confirmatory evidence to this conclusion by demonstrating that some spores receiving large doses of irradiation at refrigeration temeratures become stainable and more significantly, that the core cytoplasm isolated by physical means and kept below 5° C, stained readily and typically with methylene blue. These observations are in agreement with

those of Gerhardt and Black (1961) who found that only 40 percent of the spore volume was penetrated by low moelcular weight solutes.

Heat resistance has long been thought to be due, in part at least, to an anhydrous core. The more recently demonstrated correlation of the DPA content and heat resistance of spores has been assumed, without proof, to establish a causal relationship. This has resulted from failure to appreciate the fact that spores grown under conditions leading to an atypically low DPA content may also be atypical in other respects not sought. This study provided no pertinent information on the role of DPA in heat resistance; however, the thin sectioning of unfixed, viable spores definitely and directly indicated that these structures were rigid. This fact, combined with the electron density of the unfixed thin sections, strongly indicated a high concentration of protein molecules, i.e., an anhydrous state of the core. Ross and Billings (1957) employed phase contrast and interference microscopy to demonstrate that the refractive indices of spores was similar to that of dehydrated protein.

The inertness, as judged by retention of dye refractility, of spore coats to various regents and enzymes (Tables 14 and 16) demonstrated why these spores were able to remain viable in adverse chemical environments. The inertness could, in turn, depend partially upon the high aromatic content of the spore coat skeletons which was demonstrated by spot tests and infrared spectra.

This resistance to physical disruption appears to be due to two factors, the unusually rigid state of the core and the tough, rigid spore coat. The behavior of the spore coat was reminiscent of bone structure in that it was composed of a pliant skeletal fraction unpregmated with a mixture of compounds which rendered it rigid. Only after partial removal, as indi-

cated by reduction in DPA content, yet retention of electron density, of the impregnating compounds, could the spore coat be broken.

Sporulation

The study of sporulation in this organism was gratifying because: (1) the time at which cells became irreversibly committed to sporulation was well established by repeated observations on synchronously developing cultures; (2) the gross changes in cell characteristics were recorded by light micrographs; and (3) fine structure changes were recorded in corresponding electron micrographs of thin sections.

As indicated by the method of producing spores and later confirmed by direct counts, the vegetative cells were committed to sporulation and thus stopped vegetative division well in advance of any structural evidence of sporulation. In fact, some 2 hours were necessary between commitment and visible evidence of sporulation. Once the cells were committed, even though there was no visible differentiation, transfer to fresh medium did not cause a reversion to vegetative growth. The factors responsible for this alteration in growth form are not known; however, it was obvious that the synthetic organization of the cell had undergone an irreversible change.

Light micrographs of methylene blue stained cells demonstrated the typical characteristics, such as changes in cell size and the uniformity and intensity of staining, with various growth phases. The cellular characteristics observed in light micrographs correlated well with fine structure which was observed in corresponding electron micrographs of thin section. Of particular interest was the filamentous appearance of cells from 2 hour cultures and the corresponding observation of the parallel lateral walls with which septa formed near 90 degree angles. No septa formation was observed after 3 hours, and only rarely at 3 hours.

A comparison of light and electron micrographs from 8 hour specimens was also of special interest. In light micrographs the cells had a pronounced dipolar appearance while electron micrographs revealed the presence of the inner transparent zone, which, from irradiation, heating and thin section studies seemed to constitute or to lie external to the impermeability barrier. Thus, the spore core was probably dye refractile at 8 hours, but enough vegetative cytoplasm still remained between it and the vegetative cell wall to partially obscure the picture.

Fine structure changes observed during the process of sporulation suggested that the cytoplasmic membrane, by invagination deposited an active site which mediated synthesis of spore materials in the mother cell. In this organism it seemed likely that the spore nucleic acids, protein and lipid were synthesised de novo as the spore developed rather than being separated from the mother cell by the spore septum. It was quite clear that the spore developed from the interior outwardly, i.e., the core was formed and then the integuments developed, the exosporium appearing last.

While this study was gratifying in elucidation of the events which occurred during sporulation, it was frustrating in that it brought clearly into focus the need for and lack of knowledge about the basic mechanisms underlying this and other forms of cell differentiation.

It has been postulated that sporulation occurs: in healthy cells facing starvation (Knaysi, 1945); and under various conditions which have in common the effect of reducing or stopping growth (Grelet, 1957). In aerobic spores which do not recycle (i.e., germinate, grow out and sporulate in the medium in which sporulation originally occurred) this postulate appears superficially to be acceptable. However, upon examination it becomes apparent that since the final appearance of mature spores was the

criterion for sporulation, the process might well have begun before the concentration of the nutrient became growth limiting. It is possible that sporulation could be initiated by metabolic changes which resulted in: a reduction in the concentration of a nutrient essential for vegetative poliferation; a shift in relative concentrations of nutrients; or the accumulation of a metabolic by-product.

In the anaerobes in which recycling is common this postulate as stated appears insufficient since both vegetative growth and sporulation subsequently occur in a medium in which sporulation has already occurred. This need not, however, exclude the possibility that during growth anaerobes produced changes in their environment, changes which in turn influence the mode of development of subsequent generations. Either one or a combination of the previously mentioned possibilities could account, not only for initial sporulation, but also for recycling, if one considers that each germinated cell develops into a vegetative cell which is influenced to produce a spore without so much as one vegetative cell division.

An extensive investigation involving correlated metabolic and fine structure studies would be required for elucidation of factors and interrelations responsible for initiation of sporulation. Such an approach to the study of sporulation appears to offer a model system for studying cell differentiation since it is a free-living, single-celled organism which can be induced to undergo a type of cell differentiation in a completely chemically defined medium. Furthermore, many of the cells synthetic capacities are already known, and as it is not part of an immensely complex system, it is conceivable that its synthetic capacities might be completely mapped. Thus, qualitative and quantitative changes in these systems could be followed and correlated with changes in the environment to give some understanding

of how differentiation (commitment to sporulation) is initiated and accomplished.

This discussion might well be concluded by briefly presenting the emergent concept of the structural and compositional characteristics of spores of Cl. botulinum 62A. The spore appears to be composed of three main morphological elements, an exosporium, a spore coat and a core. Of these, only the spore coat and core were essential for viability and typical spore properties. The spore coat was composed of two fractions, a flexible, skeletal fraction and an impregnating one which rendered the structure rigid and strong. The chemistry of both of these fractions was unique for biological material; no carbohydrate or protein could be demonstrated in the skeletal fraction while DPA, which constituted a great part of the impregnating material, has not been demonstrated in other biological structures. The impermeability barrier appeared to be located in or at the inner periphery of the spore coat. The core was an unusually rigid, gel, the rigidity due, apparently to its anhydrous state. It contained the essentials, such as nucleic acid and enzymes, for subsequent development into a new vegetative cell. Thus, the peculiar fine structure and chemistry account for the peculiar properties of these spores.

SUMMARY

1. A method for producing, harvesting and cleaning large numbers of spores of Cl. botulinum 62A was presented. After a preliminary correlation of morphological and staining characteristics with physiological age, the method enabled the rapid and easy microscopic determination of physiological age, thus, the proper time of transfer, greatly facilitating the synchronous culture of this organism by the activated culture technique.

2. Germination studies were conducted by the use of both chemical and physical agents. Chemical agents included metabolites and non-metabolites. Physical agents were high level gamma irradiation and sonication with superbrite glass bead. The criterion of germination was the loss of dye refractility as determined by the uptake of dilute methylene blue.

A. Chemical agents. Five percent YE, the active agents of which were dialyzable, was used as the standard of comparison in these studies. It was observed that the concentration of spores was important since germination was slow and incomplete in media which contained over 1×10^8 spores per ml. Chloramphenicol did not inhibit germination in YE broth.

The final percentage of germination of these spores in synthetic media was essentially complete, but the rate was much lower than that in YE broth.

DPA and calcium in equimolar concentrations produced no germination.

B. Physical agents. High level gamma irradiation rendered a variable percentage of the spores stainable; however, the loss of viability had preceeded this occurrence.

Sonication with superbrite glass beads, at the steady state, rendered approximately 50 percent of the spores stainable.

3. Spores which had received 3 megarads of gamma irradiation were studied culturally, microscopically and chemically.

Although 99.99 percent of the spores failed to produce visible colonies, they became stainable at an undiminished rate in 5 percent YE broth.

Most remained microscopically indistinguishable from control spores after treatment with dilute methylene blue. However, a hot carbol fuchsin stain revealed a variable percentage of empty but intact shells in irradiated spores.

Quantitative analyses of control and irradiated spores revealed no dramatic differences; however, qualitative analyses of germination exudate revealed marked differences in the state of aggregation of the reducing material. Exudate from control spores contained only particulate reducing material while that from irradiated spores contained both particulate and macromolecular reducing material.

4. Spore coat skeletons which were isolated by intermittent autoclaving of endospores in 0.1 N HCl for a total of 90 minutes exhibited a chemical composition unique for biological material because of the absence of carbohydrate and protein and the presence of a high degree of aromaticity and other chemical structures. The insolubility and unreactivity of this structure contribute to an understanding of some of the peculiar spore properties.

5. The fine structure of these spores was studied by electron microscopy of specimens prepared by 3 entirely different techniques. These techniques were fractionation, high level gamma irradiation and thin sectioning. Spores prepared by all 3 of these methods revealed 3 main morphological elements, exosporia, spore coats, and spore cores. By varying the techniques of preparation it was possible to identify certain effects of preparation and to confirm the identity of the genuine structural features just mentioned. Furthermore, each method of preparation yielded its own unique information which helped in further characterization of each structure.

Exosporia were thin, electron transparent membranes which were shattered by passage through the Ribi Cell Fractionator. The preponderance of angular breaks suggested a crystallite structure.

Spore coats of "normal" spores appeared to be composed of a tough somewhat electron dense skeleton impregnated with a mixture of compounds which rendered the structure rigid and very electron

dense. The impregnating compounds were removed for study by selective heating and irradiation; the skeletons were isolated by treatment of spores with 0.1 N HCl at 121° C.

Spore cores were unusual in 2 respects. First, the behavior of cores during the sectioning of unfixed specimens suggested that this structure normally occurs as a rigid gel. Second, thin sections of unfixed specimens were unusually electron dense for biological material. Taken together these observations suggest that the core is rather highly anhydrous. The presence of 0.11 percent calcium in the core doubtless contributed to the electron density and probably to the rigidity of this structure. This fraction exhibited proteolytic and dehydrogenase activity.

6. Fractionation of spores into their main morphological elements was accomplished physically by employing selective disruption and differential centrifugation.

7. The location of DPA in the spore coat was established, subject only to the equation of dye refractility and retention of impermeability by the study of spores which had received various levels of irradiation, heating and fixation.

8. The mechanisms responsible for death, dye refractility, phase refractility and DPA retention were segregated on the basis of observations made during studies employing various levels of irradiation. Death was equated with failure to produce visible colonies and occurred in spores which largely retained dye resistance, phase refractility, and DPA at 3 megarads. At 6 and 9 megarads of irradiation dye refractility was lost from a small but significant percent of spores which remained phase refractile. Apparently all DPA had been lost from both stainable and refractile members of the population since no DPA at all could be demonstrated.

9. Sporulation in a synchronously developing culture was studied on aliquotes which had been removed from the culture at hourly intervals and prepared for light and electron micrography. Smears for light micrography were air dried and stained with methylene blue. For electron micrography,

the cells were washed, fixed with potassium permanganate, dehydrated with ethanol and embedded in Epon.

From a study of these specimens it appeared that a structure arose from the cytoplasmic membrane, migrated to the longitudinal axis of the cell, and established a synthetic site for spore components. The core was synthesized first, then surrounded sequentially by the inner transparent zone, the outer dense zone, and the exosporium. The participation of the cytoplasmic membrane in establishing an active synthetic site for sporulation became evident; however, elucidation of its role in subsequent stages of the process will require investigation by techniques which give greater specificity in the electron image.

FIGURE 1

Spore fractionation by use of the Ribi Cell Fractionator. The protocol for separation of the main morphological elements is shown in this figure.

FIGURE 2

Breaking glass knives. Glass knives were broken from 4 inch strips of plate glass by scoring the glass as indicated by the broken lines then heating the score close to one end with a small hydrogen flame.

FIGURE 3

Production of large masses of spores. This was a typical specimen from a 7 liter culture showing complete sporulation after 15 hours at 30° C.

X 5,000.

FIGURE 4

Effect of prolonged heating on viability, dipicolinic acid release, and resistance to staining with dilute methylene blue. After 90 minutes, the loss of viability preceded and divered from the loss of resistance to ordinary stains. The release of DPA was parallel to, but lower than the loss of viability during the 3 hours of heating.

FIGURE 5

Spores after heating at 121° C with 0.1 N HCl for 90 minutes. Some spore coat skeletons (scs) were empty while others contained core remnants (cr). X 50,000.

FIGURE 6

Thin section of spore coat skeletons isolated by acid treatment then fixed with osmium tetraoxide. The density of the inner transparent zone (it) was slightly greater than that of the background. The inner dense zone (id) appeared to be the edge of the spore covering. Entrapped salt crystals (cry) were observed in some core spaces (cs). X 48,000.

FIGURE 7

Shadowed preparation of exhaustively washed free spores. The exosporium (exo) was an empty, collapsed, electron transparent membrane. The spore body (sb) was uniformly electron dense. X 58,000.

FIGURE 8

Effluent from the Ribi Cell Fractionator showing intact spore bodies (sb), shattered exosporia (exo), and an occassional spore coat (sc). X 63,000.

FIGURE 9

Shadowed preparation of purified exosporia. The preponderance of angular breaks suggested a crystallite structure for exosporia (exo). X 48,000.

FIGURE 10

Shadowed specimen of purified skinned spores. The spore bodies (sb) remained totally electron dense. X 40,000.

FIGURE 11

Effluent from passage of EDTA-treated, skinned spores through the Ribi Cell Fractionator at 45,000 psi. Although the spore body (sb) remained totally electron dense, many spore coats (sc) were broken.

FIGURE 12

Purified spore coats. The spore coats (sc) were broken, most frequently by mid-centric cleavage but with some ragged tearing into large fragments. These structures were thicker and more electron dense but less brittle than exosporia. Fibrils (f) were observed along the torn edges. X 43,000.

FIGURE 13

Clean, free spores after treatment with 3 megarads of gamma irradiation. The spore body (sb) remained uniformly electron dense with no change in appearance except for slight transition from smooth to angular contour. X 60,000.

FIGURE 14

Clean, free spores after treatment with 6 megarads of gamma irradiation. The electron density had decreased at the periphery arrow of the spore body (sb). X 60,000.

FIGURE 15

Clean, free spores after treatment with 9 megarads of gamma irradiation. Sufficient electron dense material has been removed to reveal the spore coat (sc) and the core (c). X 60,000.

FIGURE 16

Pressure cell effluent from passage of spores which had received 3 megarads of gamma irradiation. Passage of spores which had received 3 megarads of irradiation through the Ribi Cell Fractionator at 45,000 psi shattered all exosporia (exo), but left the spore bodies (sb) intact. X 40,000.

FIGURE 17

Pressure cell effluent from passage of spores which had received 6 megarads of gamma irradiation. Passage of spores which had received 6 megarads of irradiation through the Ribi Cell Fractionator at 45,000 psi shattered all exosporia (exo) and broke many spore coats (sc). X 40,000.

FIGURE 18

Pressure cell effluent from passage of skinned spores which had received 9 megarads of irradiation. Many spore coats (sc) were broken; however, no naked cores were observed. The spore coat (sc) and core (c) of unbroken spores were visible. Fibrils (f) were faintly visible along some broken spore coats. X 50,000.

FIGURE 19

Thin section of "normal" spores treated with osmium tetraoxide in veronal buffer, pH 7. The exosporium (exo) was shrunken close to the coat, thus becoming barely discernible. The spore coat (sc) was distorted, giving the appearance of spines (sp). An outer dense zone (od), an inner transparent zone (it), a core (c) and vacuoles (v) were also visible. X 45,000.

FIGURE 20

Thin section of spores fixed with a mixture of potassium permanganate and osmium tetraoxide. Spine-formation was reduced but still obvious. The exosporium was more evident and the spore coat not nearly so distorted. X 55,000.

FIGURE 21

Thin section of skinned spores after exposure to 2 percent aqueous permanganate for 4 hours. Many cores exhibited extensive vacuolization (v) An outer dense zone (od), an inner transparent one (it) and a core (c) appeared typical. The exosporium (exo) was missing. Nuclear material was indicated by the arrow. X 40,000.

FIGURE 22

Thin section of spores after exposure to 2 percent aqueous potassium permanganate for 9 hours. No vacuolization was observed in these spores. The unusually heavy deposit of electron dense material in the outer dense zone appeared to be due to the limited washing following fixation. X 40,000.

FIGURE 23

Thin section of "normal" spores after a four-hour permanganate treatment followed by thorough washing. The comparatively large number of gradations in electron density (a-h) indicated the importance of preparation effects on these spores. X 80,000.

FIGURE 24

Thin section of skinned spores fixed with permanganate for 70 hours and embedded in Epon, which caused no polymerization damage. The contrast of the inner transparent zone (it) had been increased and chunks of the integuments had been oxidized away (arrow). The width of the outer dense zone (od) was not increased by prolonged treatment. X 59,000.

FIGURE 25

Thin section of exhaustively washed "normal" spore after permanganate fixation. The space between the exosporium (exo) and spore coat (sc) appeared to be empty (es). X 46,000.

FIGURE 26

Thin section of unfixed spores. The core (c) was electron dense while the spore coat (sc) was almost witout contrast. Distortion of the core was pronounced (arrow). The exosporium was not visible. X 43,000.

FIGURE 27

Thin section from the same block as Fig. 26. After cutting, sections were exposed to osmium tetraoxide vapor for 30 minutes. The overall contrast was increased, but that of the core (c) and discontinuous dense zones (dd) were more markedly increased. X 47,000.

FIGURE 28

Thin sections from same block as Fig. 26. Sections were floated on one percent aqueous lanthanum nitrate. The contrast was increase uniformly in the core (c) and spore coat (sc). The exosporium (exo) had become visible. X 51,000.

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FIGURES 29-40.

Light micrographs of cells taken from a synchronous culture and stained with methylene blue. The cells consistently underwent changes in size, granulation and spatial relation to each other during the first 4 hours of growth.

FIGURES 29-32 (X 3,500)

Figure 29. At one hour the cells were relatively small and individuals were discernible within the chains.

Figure 30. At two hours the cells had doubled in diameter and individuals could be distinguished only with difficulty. The staining was uniform and intense.

Figure 31. At three hours the diameter of the cells had not changed, but individuals could be discerned within chains. Granulation was consistenly observed at this stage but could not be micrographed.

Figure 32. At four hours the diameter of the cells had returned to that observed at one hour. Individuals were easily discernible. Again granulation could not be micrographed.

FIGURES 33-36 (X 5,000)

Figure 33. At five hours granulation could be micrographed.

Figure 34. At six hours granulation was pronounced.

Figure 35. At seven hours granulation had given way to rather uniform, bipolar appearance.

Figure 36. At eight hours the bipolar appearance was more pronounced.

FIGURES 37-40 (X 5,000)

Figure 37. At nine hours definite oval light areas were apparent.

Figure 38. At ten hours many cells contained ovals which were approaching complete refractility.

Figure 39. At eleven hours more than 90 percent of the cells contained fully refractile spores.

Figure 40. At 12 hours sporulation was complete in essentially all cells.

FIGURES 41-51

Cells were removed from a synchronously growing culture at hourly intervals, washed, fixed, and embedded for thin sectioning.

Figure 41. Thin section of cells from a two-hour culture. In sections through chains of these cells the walls (cw) appeared to be straight lines with whic the cross walls (cws) formed at approximately 90 degree angles. The ctyoplasm (cyt) was finely granular with no detectable differentiation.
X 25,000.

Figure 42. Thin section of cells from a three-hour culture. Chains of these cells contained segments in which the walls (cw) were straight lines and with which the cross walls (cws) formed approximately 90 degree angles; in other segments the walls had assumed the curvature (arrow) typically ascribed to these organisms. The cytoplasm remained finely granular and without differentiation. X 25,000.

Figure 43. Thin section from a four-hour culture. The ends of most cells had become rounded so that individuals were easily distinguished. The cytoplasm remained finely granular and without differentiation. The cytoplasmic membrane (cm) was a thin line just inside the cell wall (cw). X 75,000.

Figure 44. Thin section of a five-hour culture. In cell A a subterminal, round structure, hereafter called a mesosome (m), had arisen, apparently by invagination of the cytoplasmic membrane (cm). In cell B the mesosome (m) had migrated to the longitudinal axis of the cell and a transverse septum (ts) had started forming from the opposite side of the cell. X 65,000.

Figure 45. Thin section of a six-hour culture. In cell A the mesosome (m) had enlarged and extended nearly to the opposite septum. In cell B the septum had formed completely across the cell, but the mesosome (m) remained attached. The membranous character of the mesosome was faintly visible. X 70,000.

Figure 46. Thin section of a seven-hour culture. The structure of cells A and B was similar to that observed in cells from six-hour cultures except that vacuolization had become extensive. However, the structure of cell C was startlingly different from that of the other cells. In cell C the transverse septum had disappeared and the mesosome (m) had migrated to the end of the cell, leaving a faintly visible primordium of the inner transparent zone (arrow). X 62,000.

Figure 47. Thin section of eight hour culture. The developing spore was clearly identifiable. The size, texture and electron density of the core did not change during the remainder of sporulation. The inner transparent zone was well developed, but the outer dense zone had not yet appeared. Vacuolization had become extensive. X 65,000.

Figure 48. Thin section of nine hour culture. The outer dense zone (od) typically observed in fixed specimens was becoming evident. Vacuolization remained extensive. X 55,000.

Figure 49. Thin section of ten-hour culture. The core and inner transparent zone were well developed. The outer dense zone was more pronounced than in the nine-hour culture; however, there was an area in which this zone was not in contact with the inner transparent zone, the only instance of such observation. The cytoplasmic-like material (clm) and exosporium (exo) were becoming evident. Vacuolization was still evident. X 100,000.

Figure 50. Thin section of eleven-hour culture. The core and inner transparent zone were complete; however, the outer dense zone was not so pronounced as in free spores. The exosporium was complete and enclosed considerable cytoplasmic-like material. Vacuolization had largely disappeared. X 50,000.

Figure 51. Thin section of twelve-hour culture. The main morphological elements appeared to be completely formed. The core (c), the inner transparent zone (it), the outer dense zone (od) and the exosporium (exo) were the same as those observed in free spores. The exosporium enclosed considerable cytoplasmic-like material. The mother cell appeared to be disintegrating around the exosporium in some places (arrow). X 55,000.

FIGURE 52

Ultraviolet absorption spectra of materials released from spores by heating, irradiation and fixation. The spectra shown were those of mixtures; however, the characteristic peaks of DPA at 270 mu and Ca:DPA at 278 mu were identifiable. Materials released by boiling and limited autoclaving contained little calcium; however, increased heating increased the amount of calcium which was released. The spectra of materials released by irradiation and fixation were typical of DPA complexed with but a small amount of calcium.

FIGURE 53

Proteolytic activity of cytoplasmic fraction on four substrates. After 30 minutes at 30° C, significant proteolytic activity was observed on peptone, casein hydrolysate and gelatin but not on purified casein.

FIGURE 54

Endogenous dehydrogenase activity of core cytoplasm. Sub-figure A shows the rate of dehydrogenase activity at 4 pH values when no substrate was added. Sub-figure B shows the effect of pH on dehydrogenase activity when no substrate was added.

FIGURE 55

Pyruvate dehydrogenase activity of core cytoplasm. Sub-figure A shows the rate of dehydrogenase activity at 4 pH values when pyruvic acid was added. Sub-figure B shows the effect of pH on the level of pyruvate dehydrogenase activity after 30 minutes at 30° C.

FIGURE 56

Citrate dehydrogenase activity of core cytoplasm. Sub-figure A shows the rate of dehydrogenase activity at 4 pH values when citric acid was added. Sub-figure B shows the effect of pH on the level of citrate dehydrogenase activity after 30 minutes at 30° C.

FIGURE 57

Infrared spectrum of spore coat skeletons. By use of a KBr pellet, the following groups were identified: OH/NH; -C/H; associated carbonyl; olifin; nitrite, amide or sulfon; CH bend, asymetric carbon in aliphatic compound; CH_3; and oxygen group. Not shown in the figure, but of great importance was a peak at 13.5 microns indicating aromaticity with o-substitution.

FIGURE 58

Ultraviolet spectrum showing the shift due to heating at 100° C. Without heating, the sonicate gave a spectrum which suggested, but did not conclusively demonstrate DPA. After heating for 5 minutes, the typical absorption peak at 270 was pronounced. The coincidence of the curves from 5 and 10 minute-heated samples indicated that 5 minutes was as effective in producing the shift as was 10 minutes.

FIGURE 59

Ultraviolet spectra showing the effect of time of sonication on the amounts of DPA released and the DPA:Ca ratio, as indicated by the peak at 276 mu.

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SUMMARY

Large masses of spores of Clostridium botulinum were produced rapidly and consistently by the activated culture technique. Synchronous growth was readily attained by following the growth phase of the culture with a simple methylene blue stain. When Cl. botulinum 62A was in the logarithmic phase the cells exhibited 3 distinct characteristics: (1) they occurred in chains, usually 4 or more; (2) they were much larger than in older cultures; and (3) they stained uniformly and intensely with methylene blue. The time required for the culture to reach this phase varied from 1.5 to 2.5 hours, and depended upon the medium, the size of inoculum and the particular spore stock. When freed of thin sporangia, the spores were harvested with a Sharple's continuous centrifuge especially adapted to prevent aerosols. The spores were washed a minimum of 10 times at 4° C in distilled water.

Investigation of the interrelation of viability, stainability and dipicolinic acid (DPA) release, three prominent characteristics commonly associated with "germination," during prolonged heating (heat shock) at 65° C revealed that the loss of viability was much greater than the loss of resistance to ordinary stains. The release of DPA correlated well with the loss of resistance to staining in the early stage. However, after an hour, the release of DPA was greater than the loss of resistance to stains.

Although germination in a chemically defined medium similar in composition to yeast extract was slow compared to that in 5 percent yeast extract broth at pH 8, incubation for 18 hours at 30° C produced complete germination. There was a heavy outgrowth of atypically large and granular cells but no sporulation. Rapid germination of Cl. botulinum was not attained with the germinants commonly employed with aerobic spores and spores of Cl. roseum.

Chemically spores of Cl. botulinum contained 10-16 percent DPA, 0.2-2 percent calcium, 20 percent lipid and 23 percent protein. The nondialyzable

fraction of germination exudate contained 15 percent protein,4 percent hexosamine, 4 percent reducing material, calculated as glucose, and 30 percent unhydrolyzable material.

Structures called spore coat skeletons were isolated by intermittent autoclaving in 0.1 N HCl and washing in distilled water. These structures, after subjection to a simple stain were morphologically indistinguishable from normal spores; however, electron microscopy revealed that they were empty bags free of electron dense materials. Moreover, these structures could not be stained with hot carbol fuchsin. Nitrogen, oxygen, hydrogen, sulfur and phosphorous, were detected in these structures, and the Van Slyke and ninhydrin methods of amino acid analysis gave negative results; however, various functional groups were identified by spot tests. These structures were not attacked by a variety of carbohydrases or proteases, and, of a number of solvents tried, they were soluble only in concentrated H_2SO_4. Spot tests and infrared spectra indicated a high degree of aromaticity.

X-ray diffraction studies indicated that exosporia, spore coat skeletons and spore coats contained the same basic chemical structure.

High level gamma irradiation and various levels of heating were employed in segregating the factors responsible for viability, resistance to staining, phase refractility and DPA release. Treatment of spores with 6 or 9 megarads of gamma irradiation or heating 5 minutes at 110° C resulted in non-viable spores which contained no detectable DPA, yet they remained largely dye and phase refractile. A small but significant percentage of spores had become stainable but remained phase refractile. The degree of phase refractility was reduced in irradiated spores.

Phase refractility could be produced in autoclaved spores and spore coat skeletons by treatment with 2% $KMnO_4$ or by impregnation with metal salt

solutions followed by precipitation of the metal ions as insoluble phosphates.

Three main morphological elements were identified by electron microscopy of spores prepared for examination by fractionation, high level gamma irradiation and thin sections.

These elements were: the exosporium, spore coat and spore core. Free spores which had been exhaustively washed appeared as electron dense bodies surrounded by electron transparent membranes which were empty and collapsed. Passage of heavy spore suspensions through the Ribi Cell Fractionator at 45,000 psi shattered exosporia but left spore bodies intact.

A pure fraction of exosporia obtained by centrifugation of the pressure cell effluent consisted of extremely electron transparent fragments with a preponderance of angular breaks. The spore bodies, hereafter called skinned spores, retained typical spore characteristics. Treatment of either control or skinned spores with 9 megarads of gamma irradiation decreased the electron density at the periphery of the spore body revealing the spore coat. Irradiation or treatment with a saturated solution of EDTA weakened the spore coats, apparently by removal of DPA, so that passage through the cell fractionator broke many coats. Differential centrifugation yielded pure fractions of spore coats. The core material remaining in suspension was ninhydrin positive and exhibited proteolytic and dehydrogenase activity.

During preparation of thin sections, osmium tetraoxide and potassium permanganate were used separately and in combination in water and in buffers. Two percent potassium permanganate in tap water gave the best results. Thin sections of fixed spores appeared to have 4 rather than 3 main morphological elements. The appearance of the exosporium and the core was what would be expected from spore fractionation and irradiation studies; however, the spore coat, which appeared as a single structure during fractionation and irradiation studies, appeared to be composed of 2 drastically different zones - an

outer dense one and an inner transparent one. This apparent conflict was resolved during the study of unfixed sections.

After the outer porous zone of the spore was dehydrated with ethanol, the still viable spores were embedded in methacrylate. Sections from the same block of such specimens were micrographed without treatment, after post-section staining with lanthanum nitrate and after post-section exposure to osmium tetraoxide vapor. In an unfixed specimen with no post-section treatment the density of the core was high while that of the covering was extremely low. The density of the covering was slightly greater at the periphery. The exosporium was not visible. The contrast of all structures was uniformly increased and exosporia became visible when specimens cut from the same block as the previous ones were floated on 1% lanthanum nitrate solution. Specimens cut from the same block and floated on water, were exposed to osmium tetraoxide vapor for 30 minutes and again the overall contrast was increased; however, contrast of the core and discontinuous zones at the periphery were more markedly increased. The uniform appearance of the spore coats in these 3 specimens indicated chemical homogeneity of the spore coat. Perhaps the dense zones observed after permanganate fixation were due to the deposition of reduction products in pores, the size of which decreased inwardly.

Once the main morphological elements were identified, it became of interest to follow their development. Obviously, for valid identification of stages in sporulation, a highly synchronous culture was necessary. Such a culture was obtained by the method which had been developed for routine spore production.

Aliquots were removed from this culture at hourly intervals and prepared for light and electron micrography. Smears for light micrography were air-dried and stained with methylene blue.

Light micrographs of the first 4 hours of growth demonstrated the changes in cell size and arrangement. At one hour methylene blue stained cells were

relatively small and individual cells in the chain were distinguishable. At 2 hours the cells had nearly doubled in diameter and individuals were not easily distinguished. Cells which were transferred at this stage continued vegetative growth, but by two and a half hours some were committed to sporulation. In 3 hour cultures the cells were large but individuals were distinguishable. At 4 hours the diameter had decreased to that observed at one hour. The cell size was nearly constant during the remainder of sporulation.

The gradual development of dye refractility was demonstrated in light micrographs of cells from 6-12-hour cultures. By 6 hours granulation was pronounced; at 7 hours granulation had given way to rather uniform, terminal light areas; at 9 hours definite oval light areas were apparent; at 10 hours many cells contained ovals which were approaching complete refractility; at 11 hours more than 90% of the cells contained fully refractile spores; at 12 hours sporulation was complete in essentially all cells.

For electron micrography, the cells were washed twice, fixed with 2% potassium permanganate in tap water at 4° C, dehydrated with ethanol and embedded in Epon. Sections were cut with a glass knife, mounted on formvar coated grids and examined in a Phillips 100 electron microscope. A series of electron micrographs demonstrated the fine structure of sporulation.

In electron micrographs of thin sections of cells, 1, 2, 3 and 4 hours old the cytoplasm was rather homogeneous with no differentiation. At 5 hours a subterminal, round structure arose, apparently by invagination of the cytoplasmic membrane, and migrated to the longitudinal axis of the cell. A septum, forming from the opposite side of the cell, was noted. At 6 hours the round structure grew larger, extending nearly to the opposite septum. In some cells the septum was formed completely across the cell, but the round structure remained attached. The membranous character of the round structure

was faintly visible. At 7 hours, the round structure appeared to be separated from the septum and was less membranous.

At 8 hours a great change had occurred. The round structure, now obviously a forespore, was surrounded by an electron transparent zone. The size, electron density and texture of the core did not change during the remainder of sporulation. Earlier work indicated that this structure functions as a permeability barrier. The electron dense zone which was characteristically observed in sections of clean, mature spores was absent.

At 9 hours the formation of a dense zone, typically observed in fixed specimens, was becoming evident, and at 10 hours it was complete. The developing exosporium was apparent. At 11 hours the exosporium was nearly complete; at 12 hours the exosporium was complete and the cytoplasm appeared to be disintegrating around the exosporium in some places.

The exosporium enclosed considerable cytoplasmic material which was indistinguishable from that of the mother cell. This material had been largely removed from exhaustively washed spores. However, the adherence of remnants of this material to the core could account for two earlier observations: first, the contraction of this material during alcohol dehydration could account for the increased density of the spore coat periphery in unfixed specimens; and second, the irregular dense zones noted in unfixed sections after post-section exposure to osmium tetraoxide vapor.

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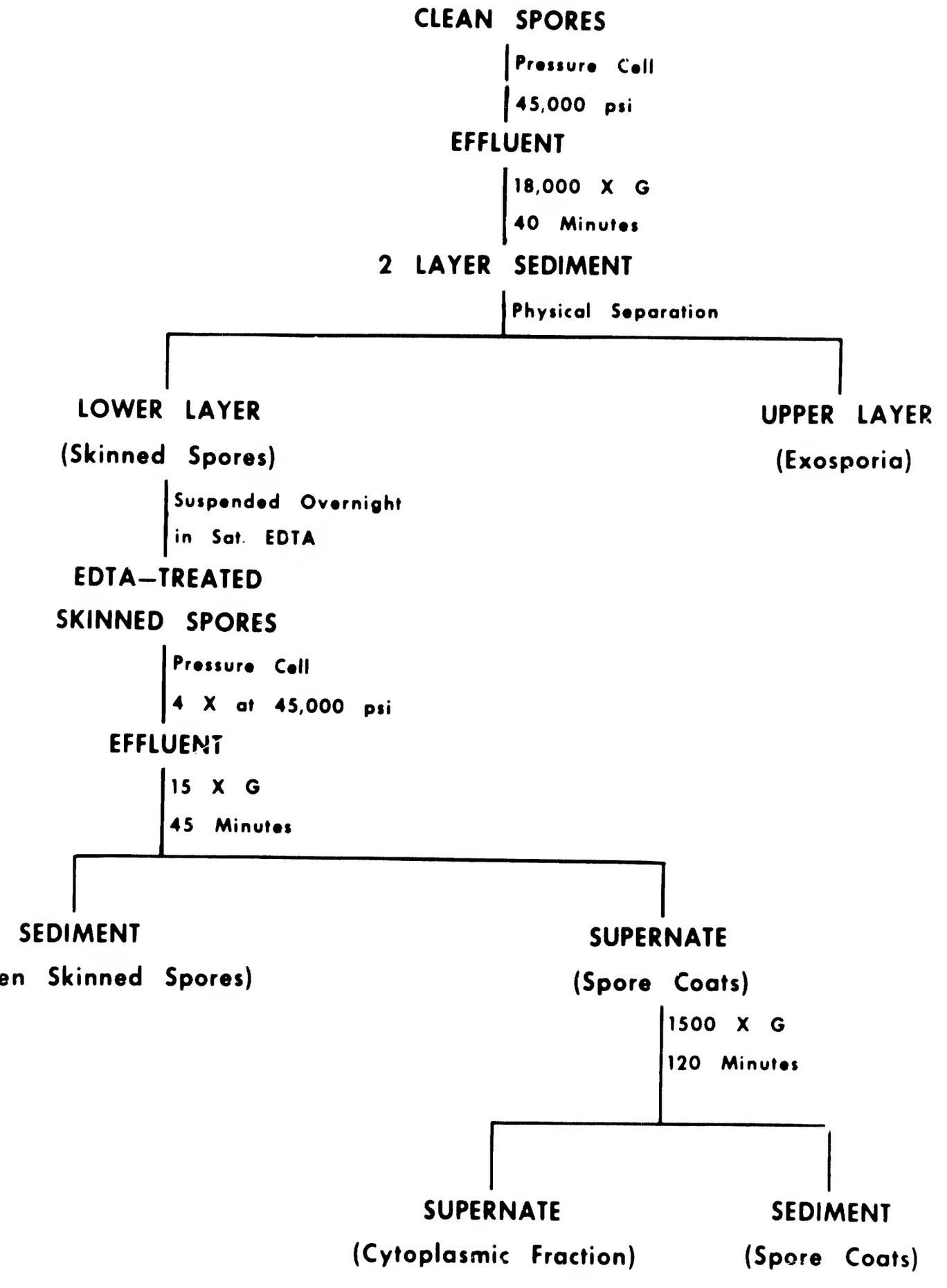
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Physical Separation
LOWER LAYER
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UPPER LAYER
(Exosporia)
Suspended Overnight
in Sat. EDTA
EDTA–TREATED
SKINNED SPORES
Pressure Cell
4 X at 45,000 psi
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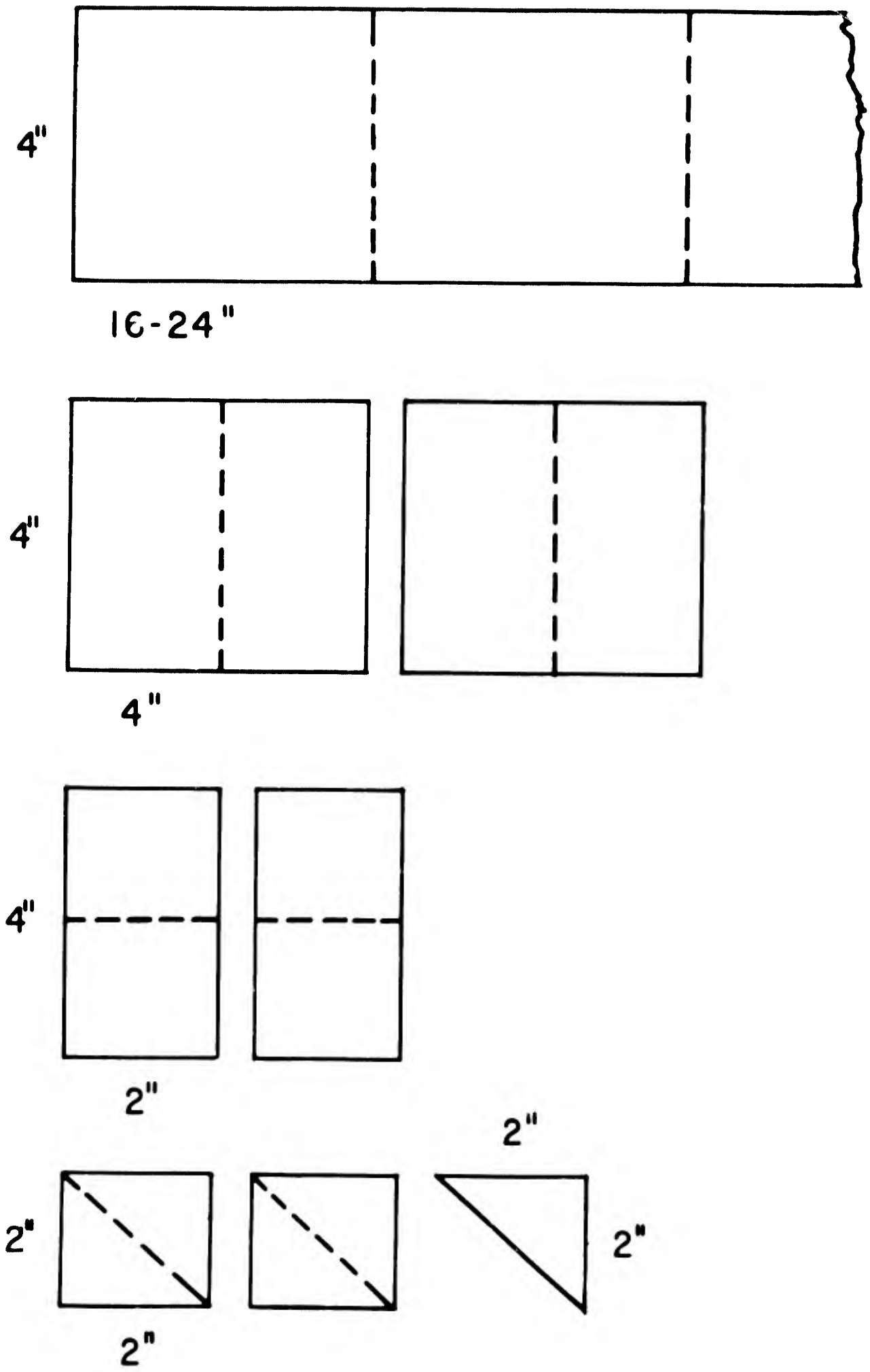

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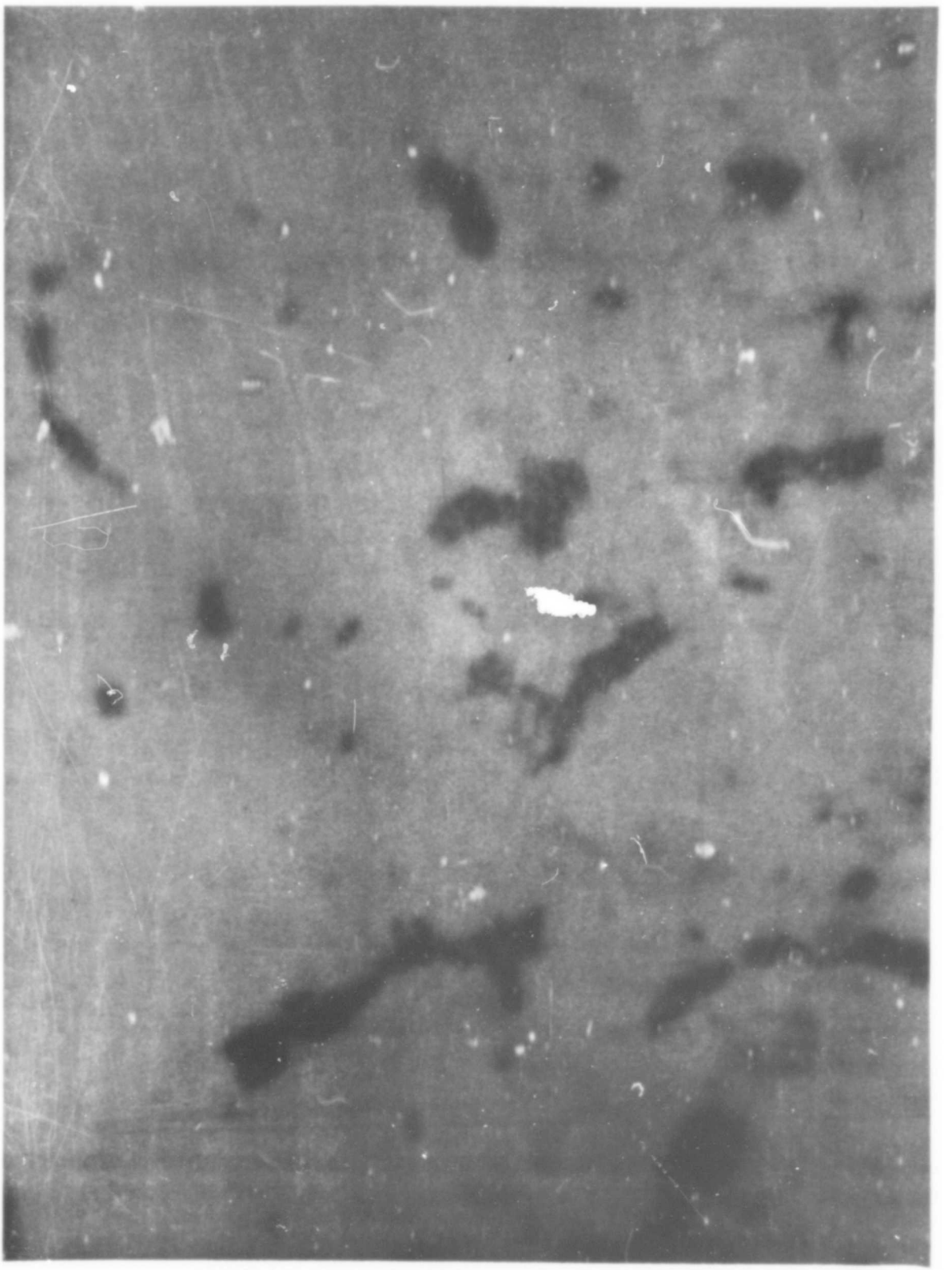

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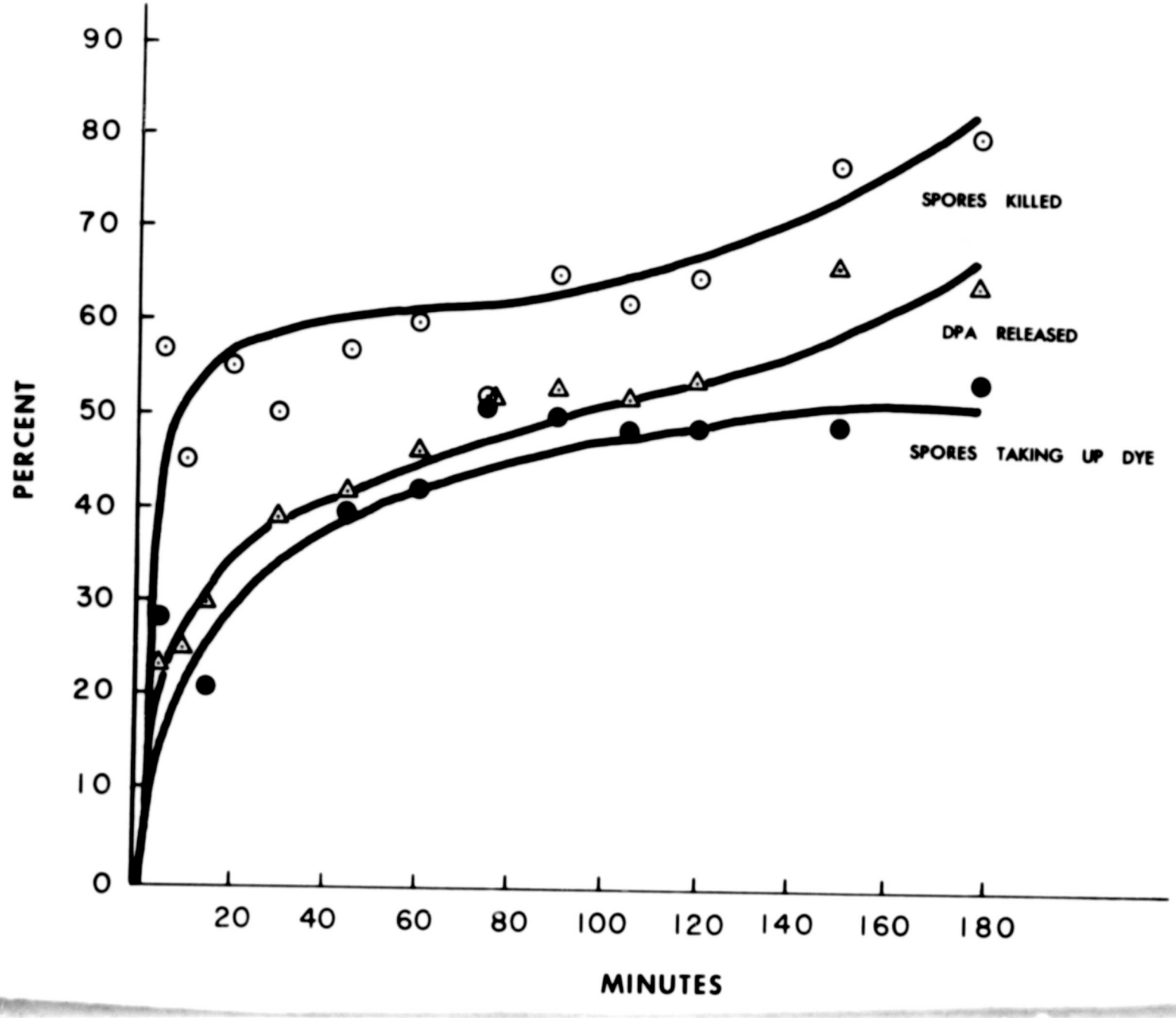
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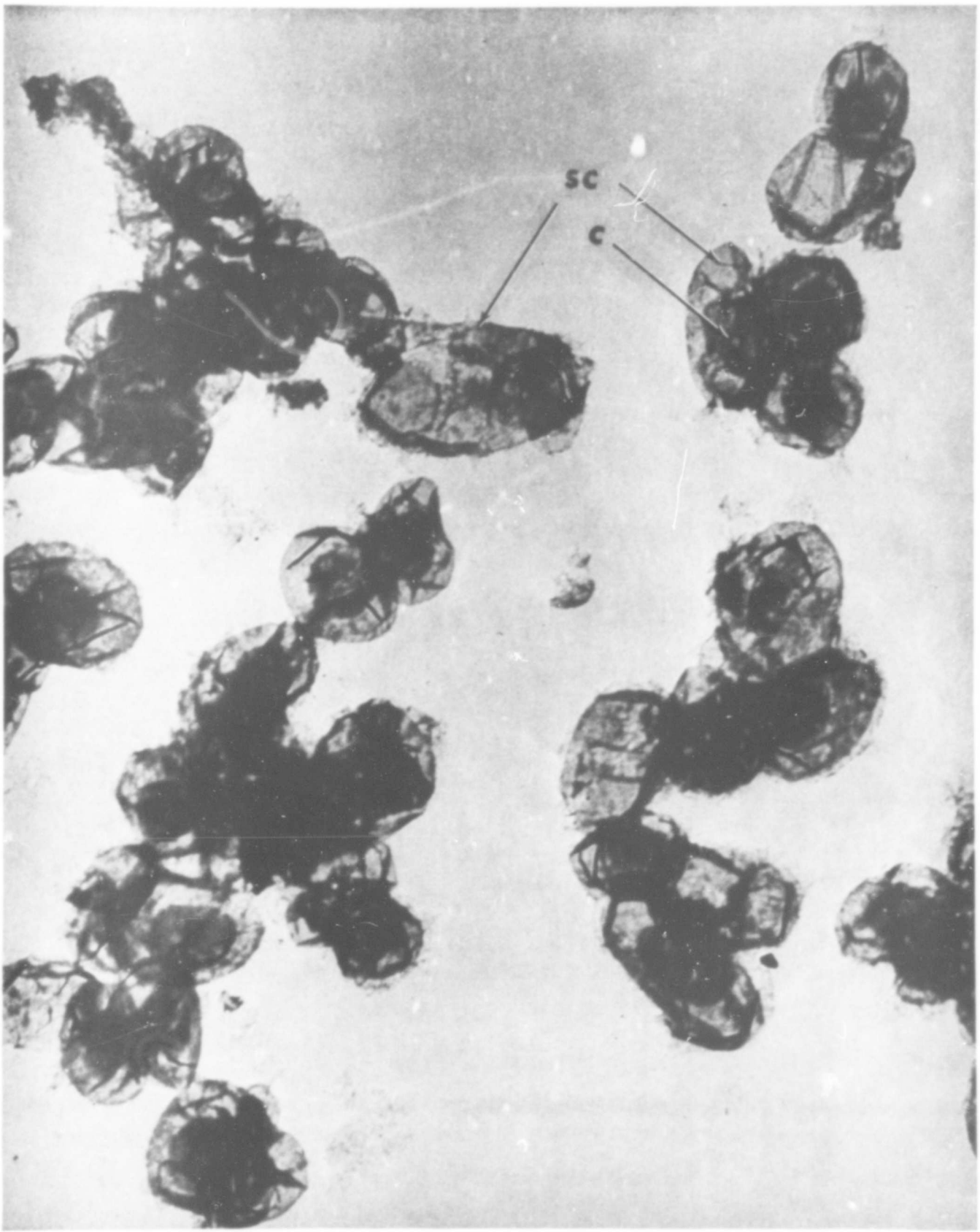

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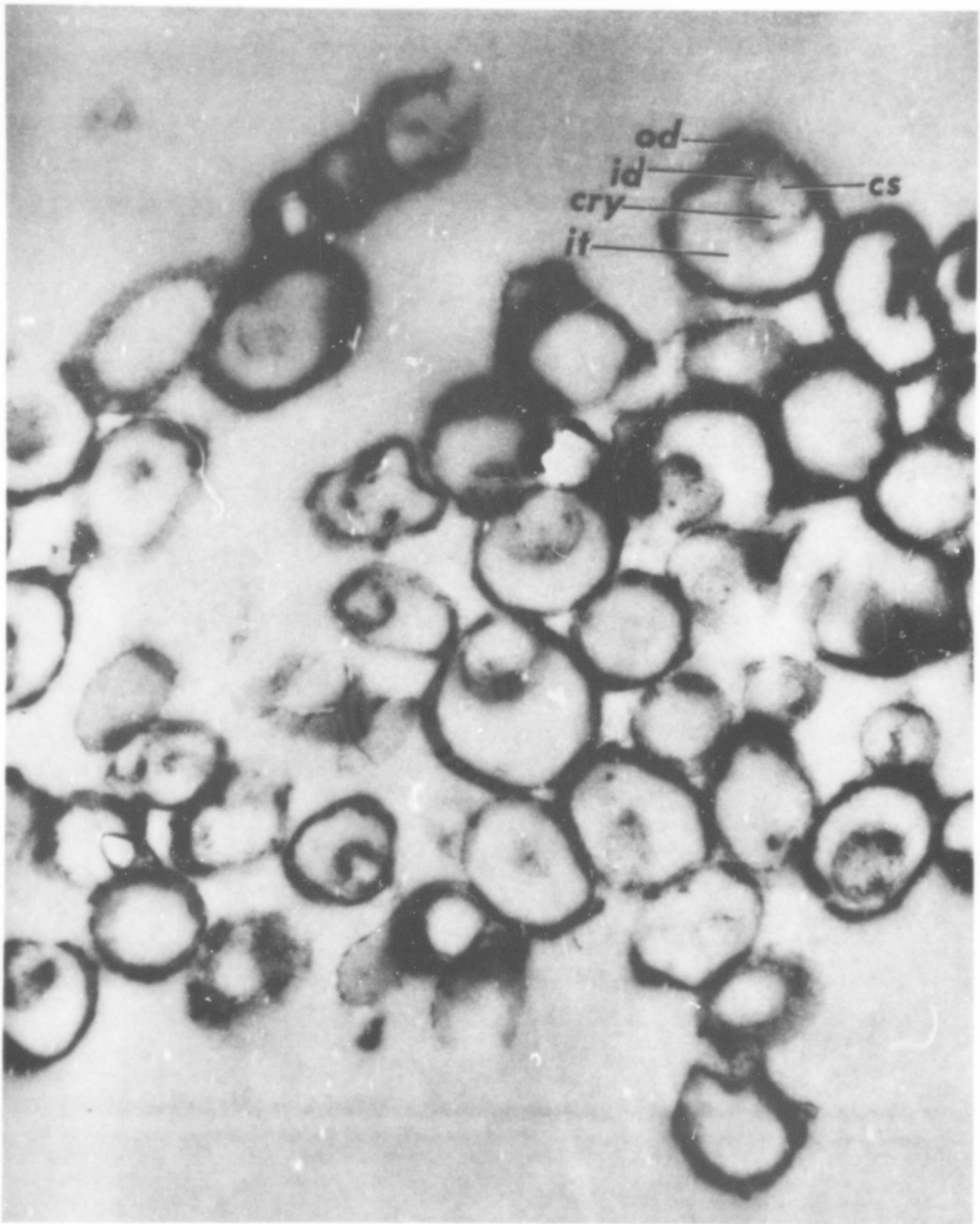

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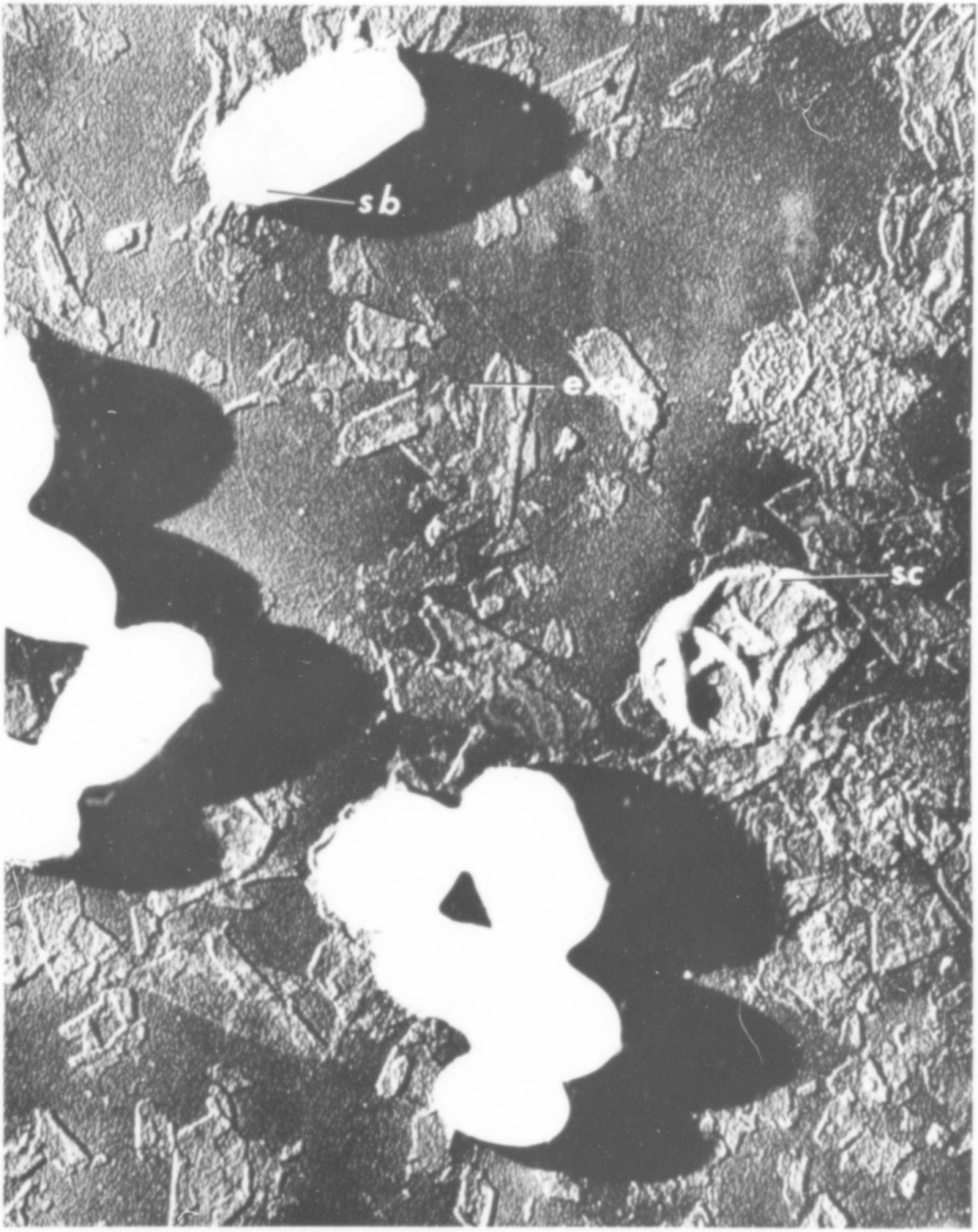

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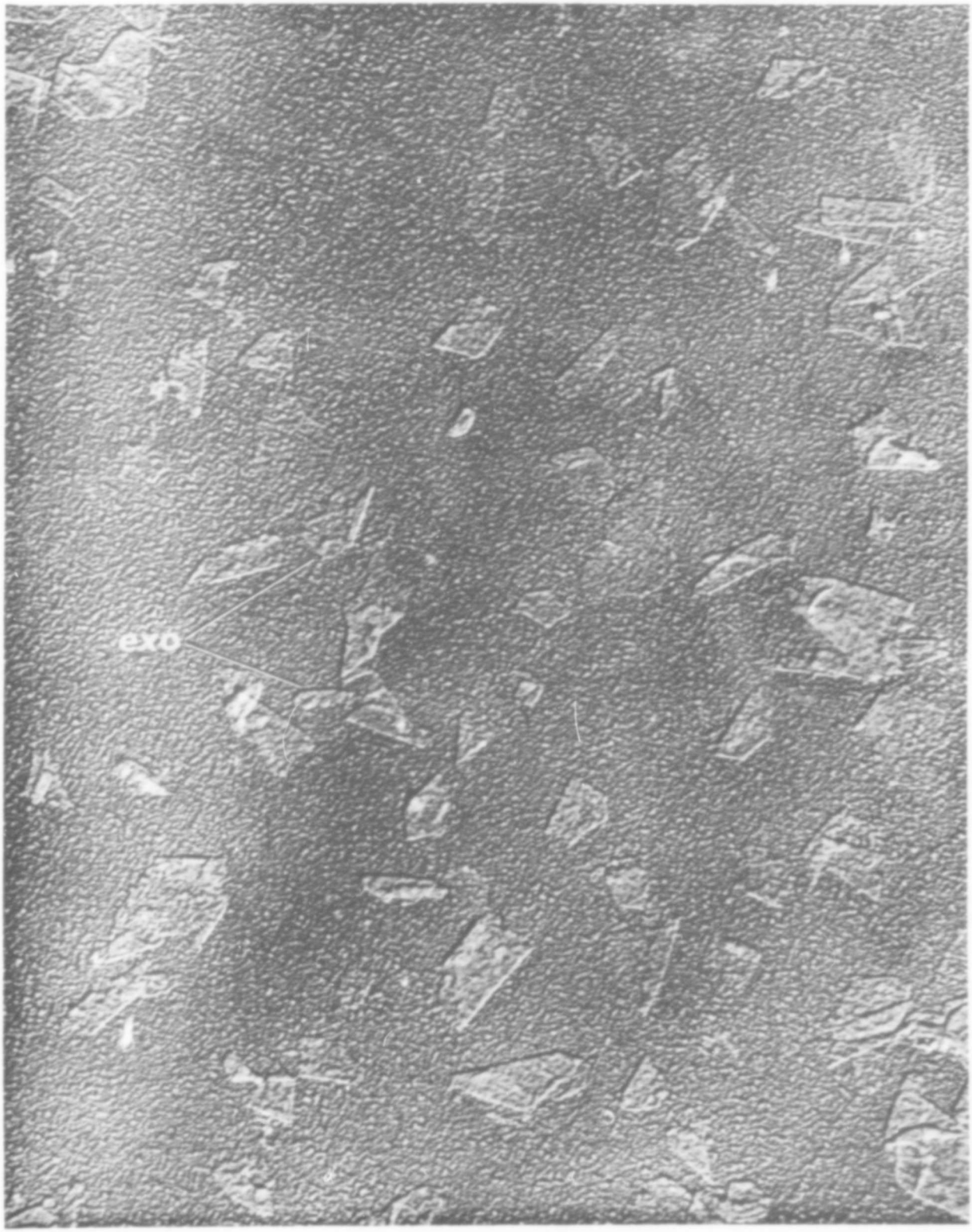

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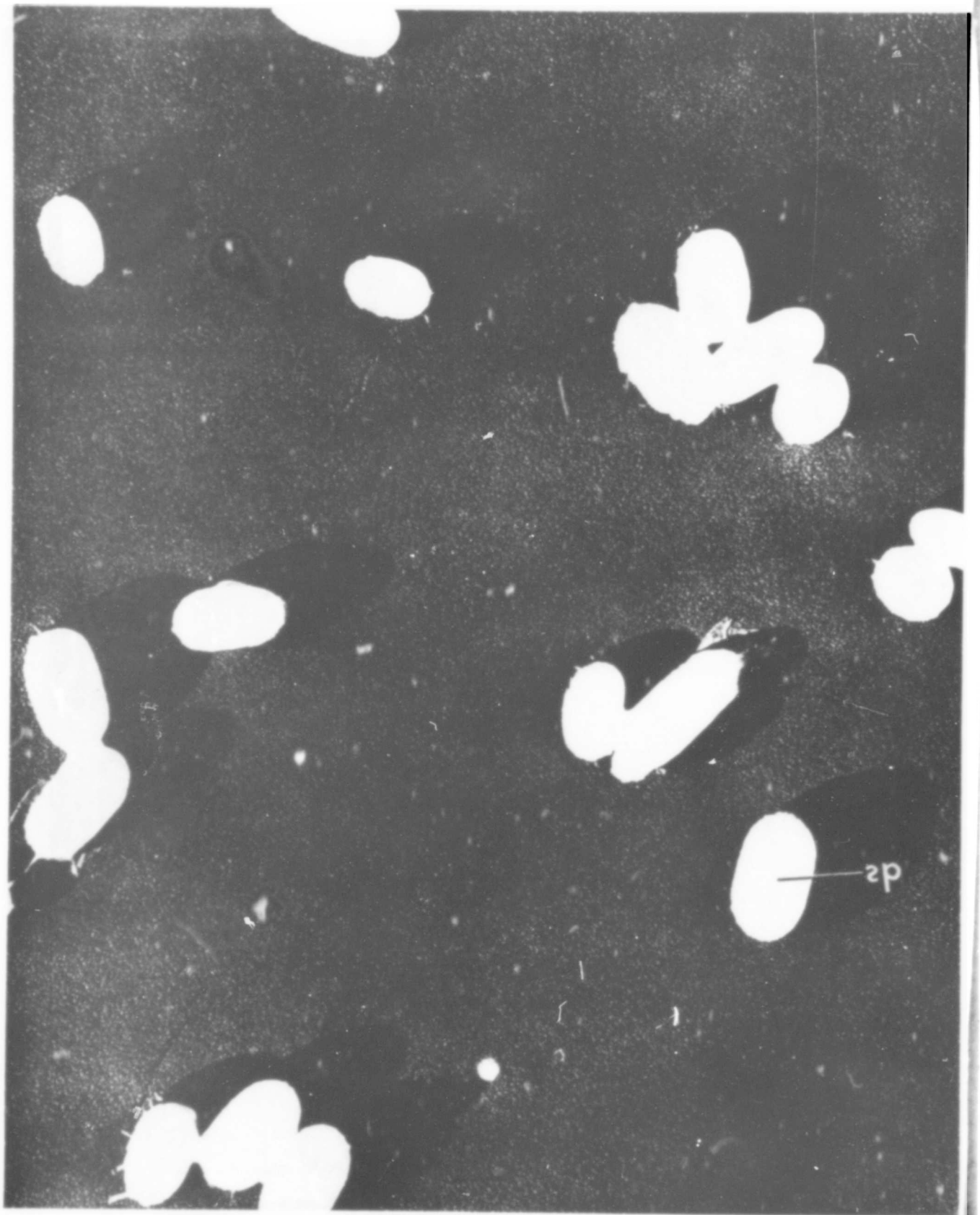

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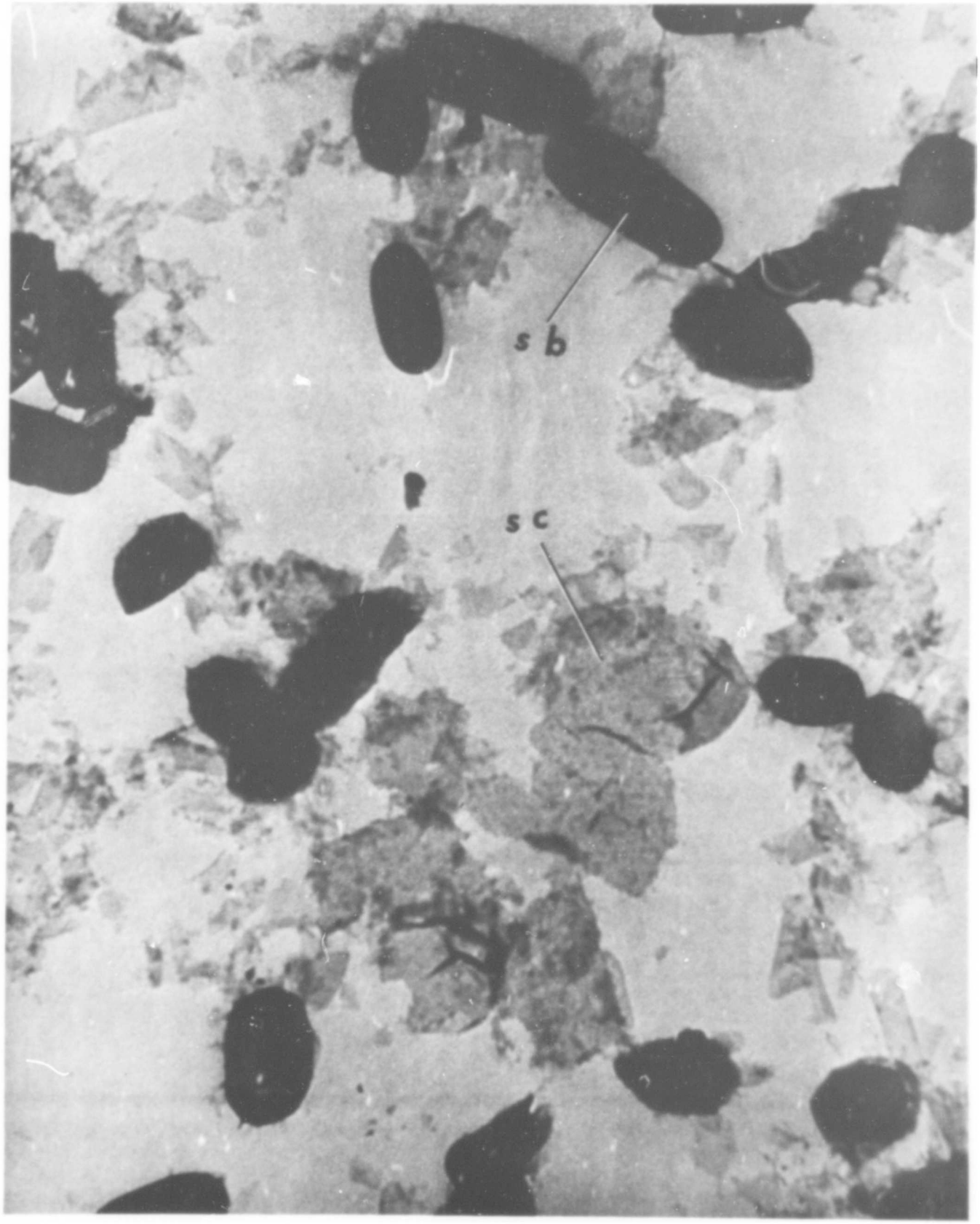

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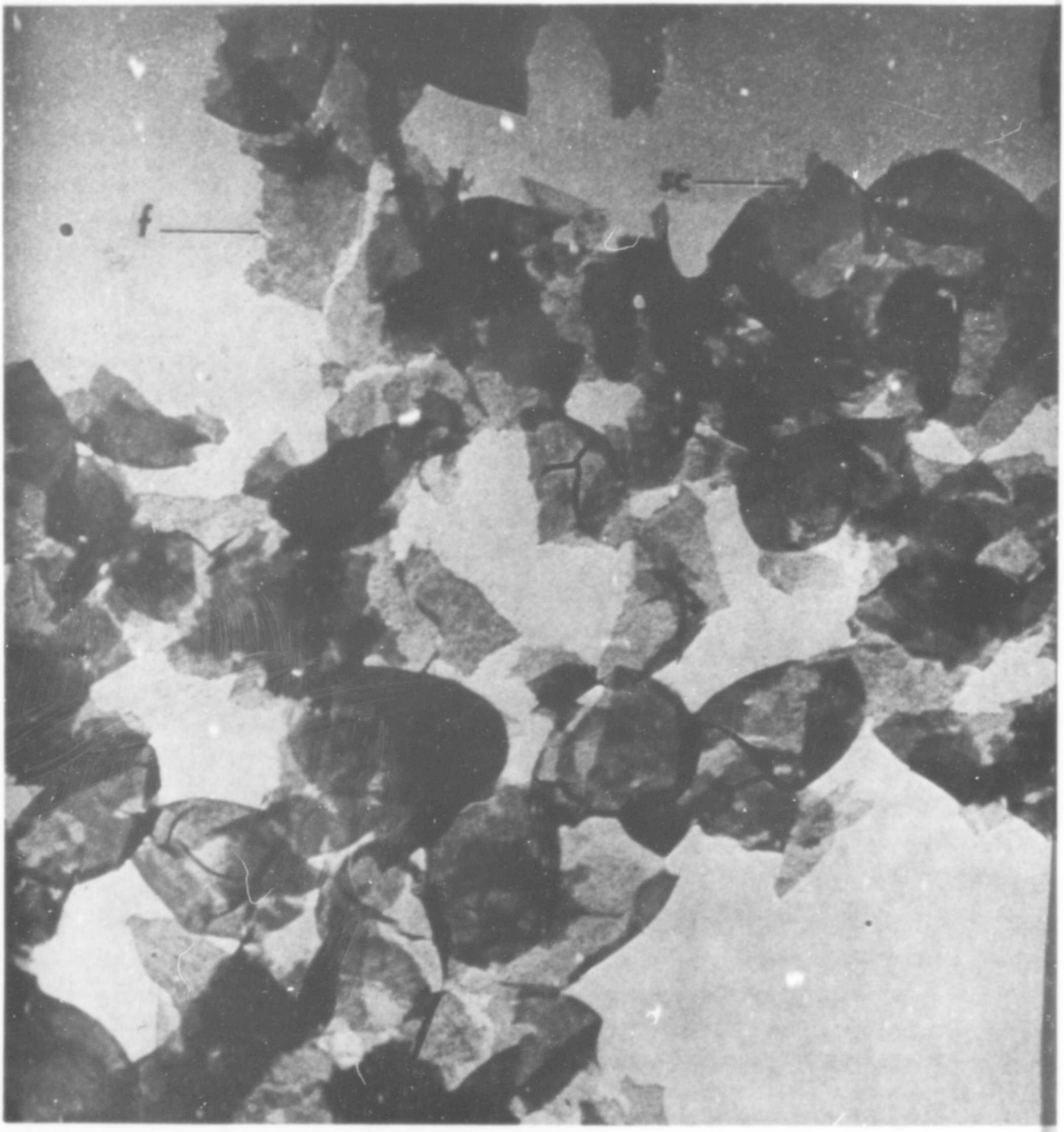

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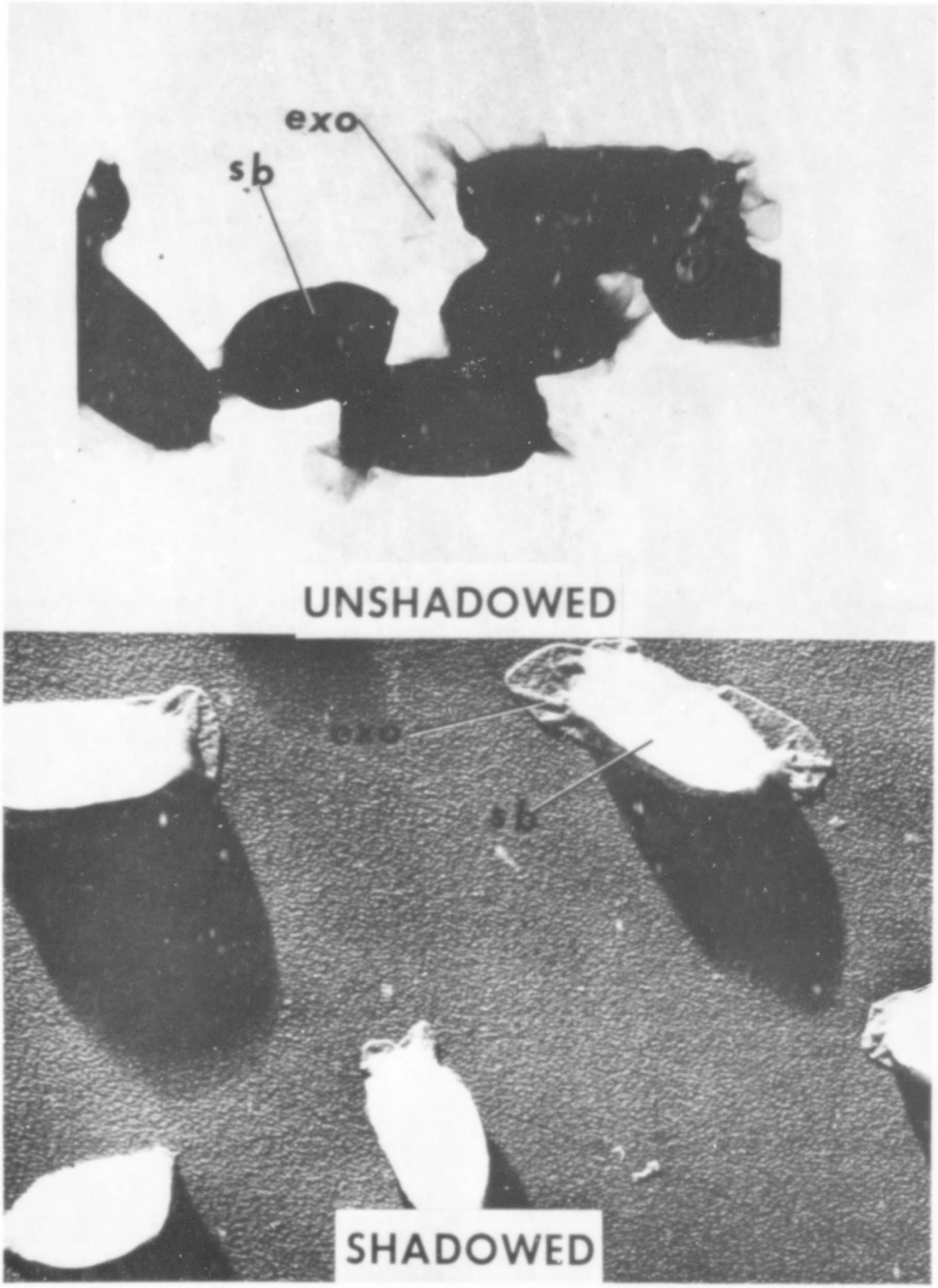

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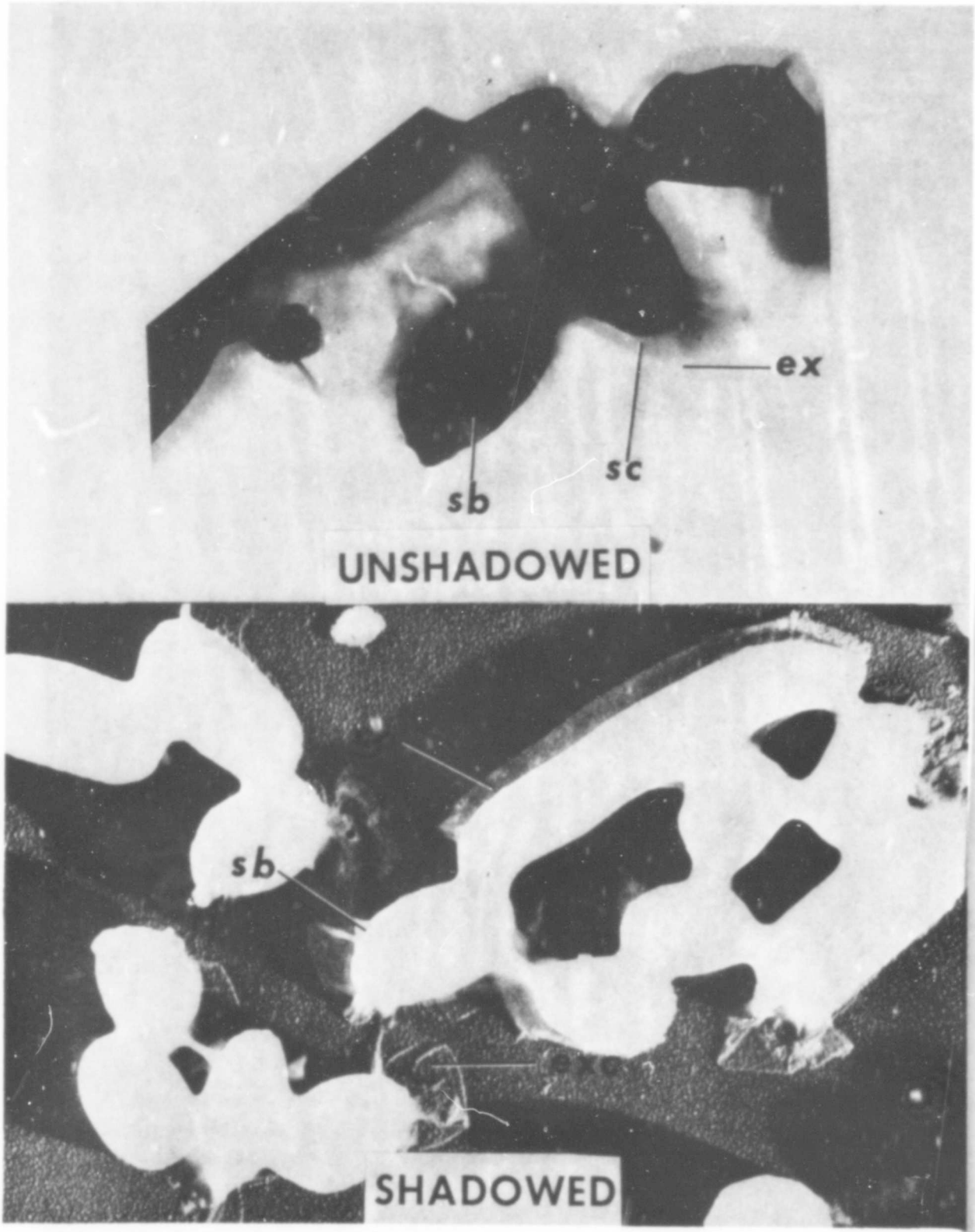

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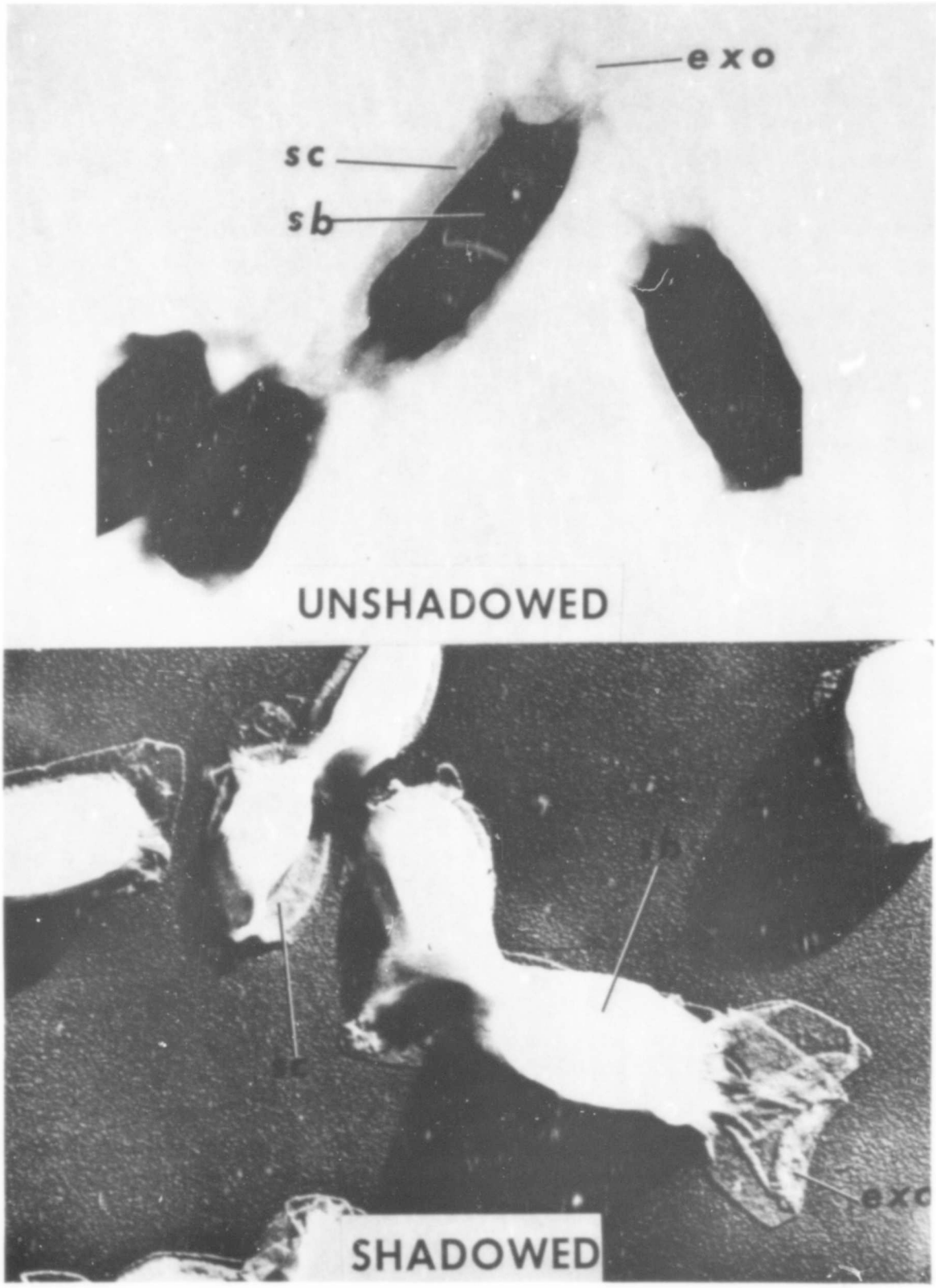

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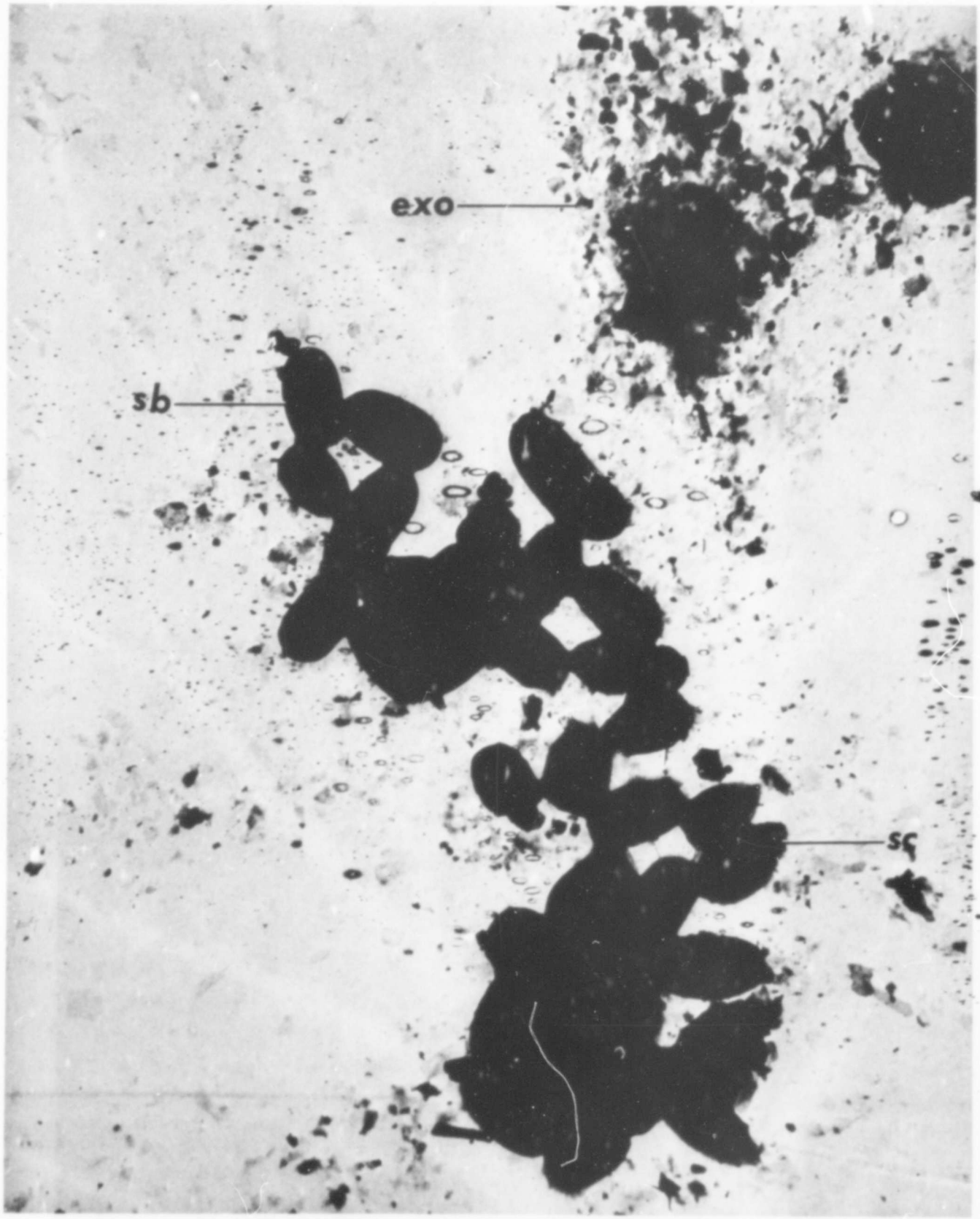

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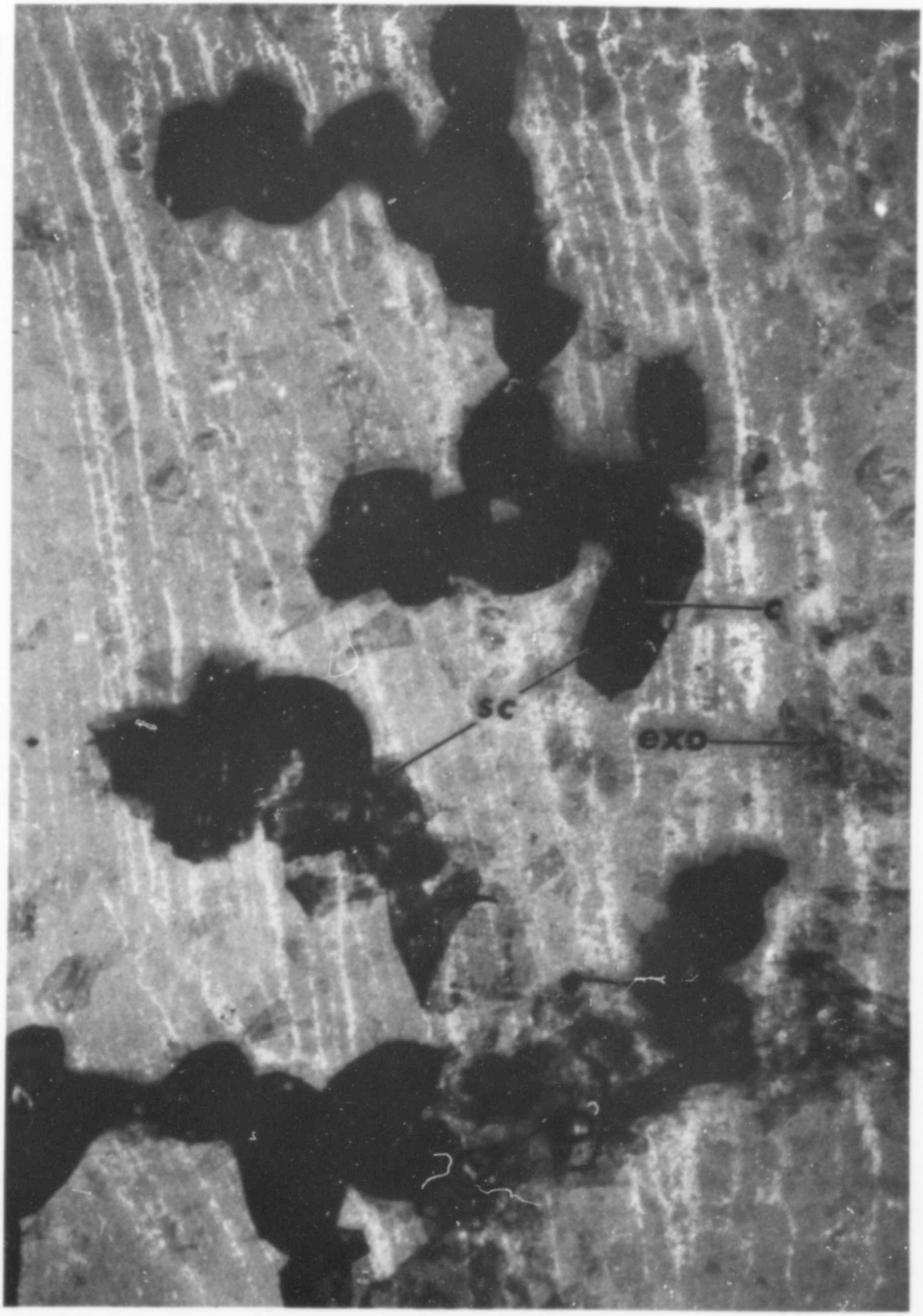

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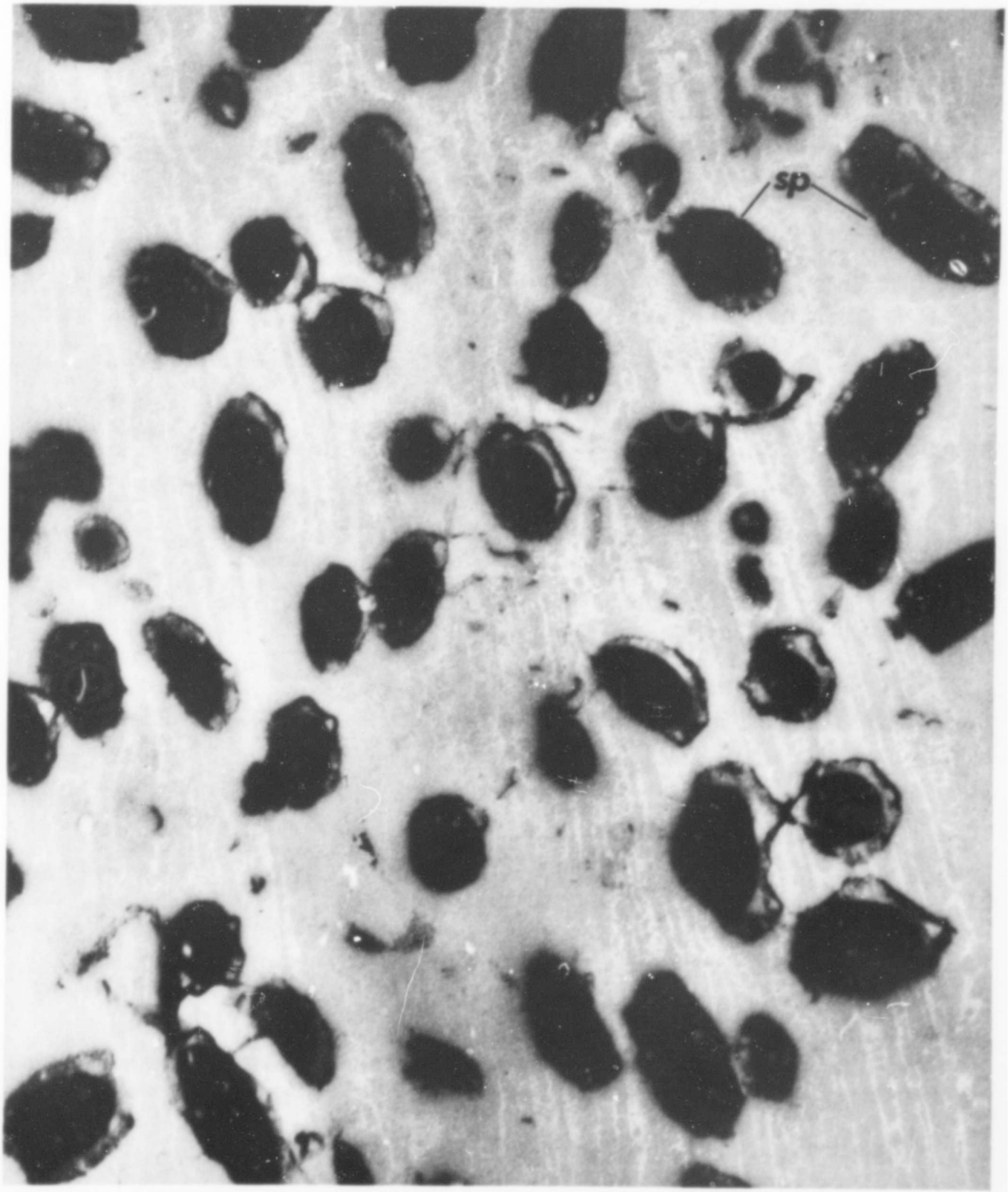

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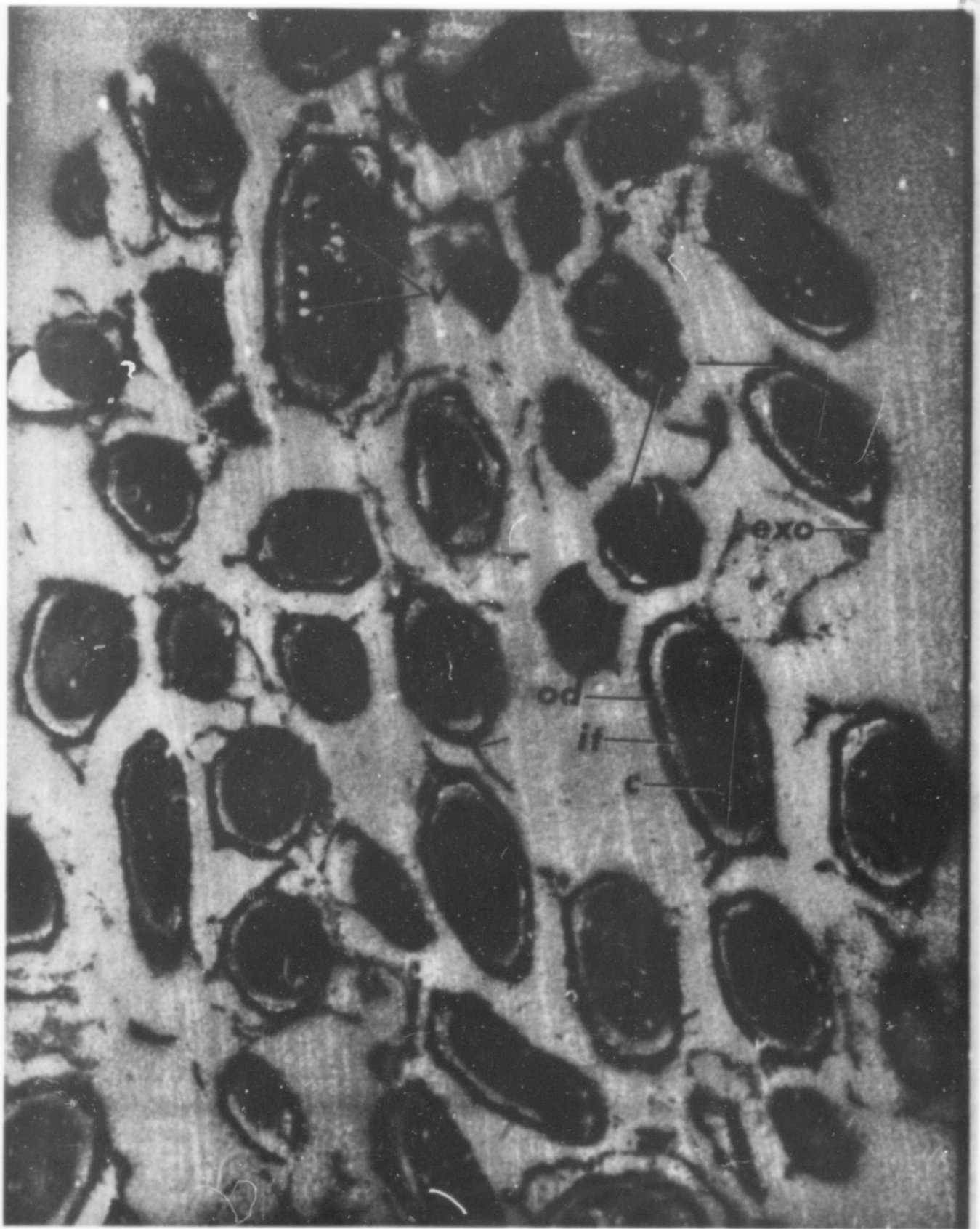

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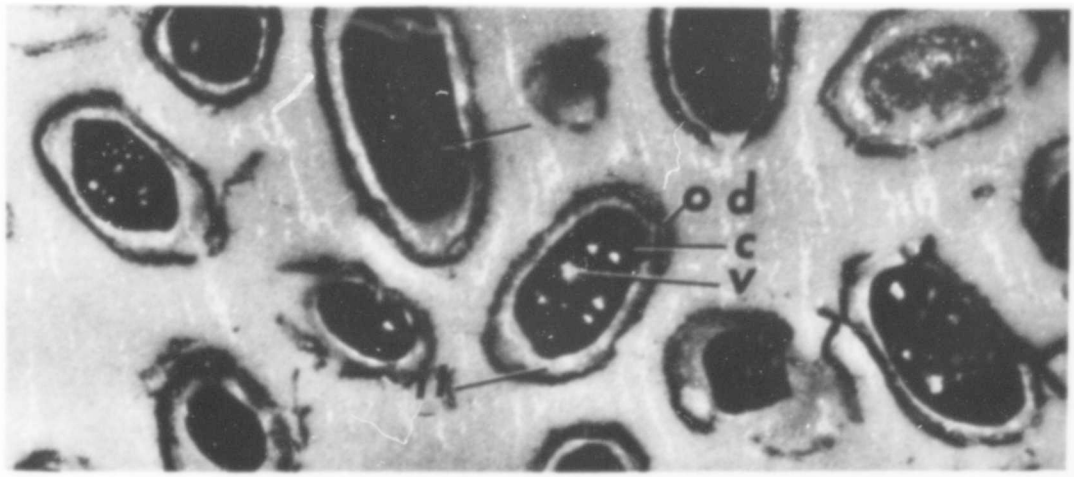

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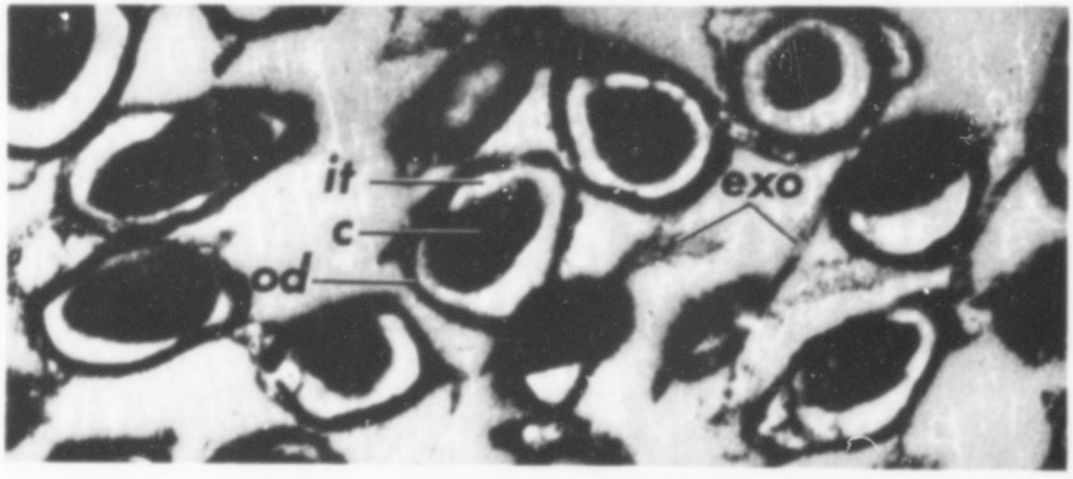

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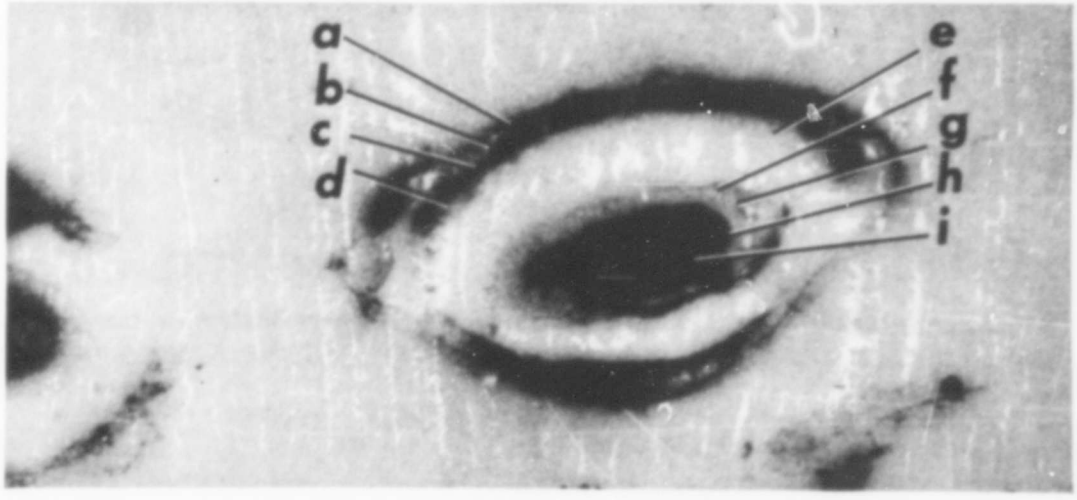

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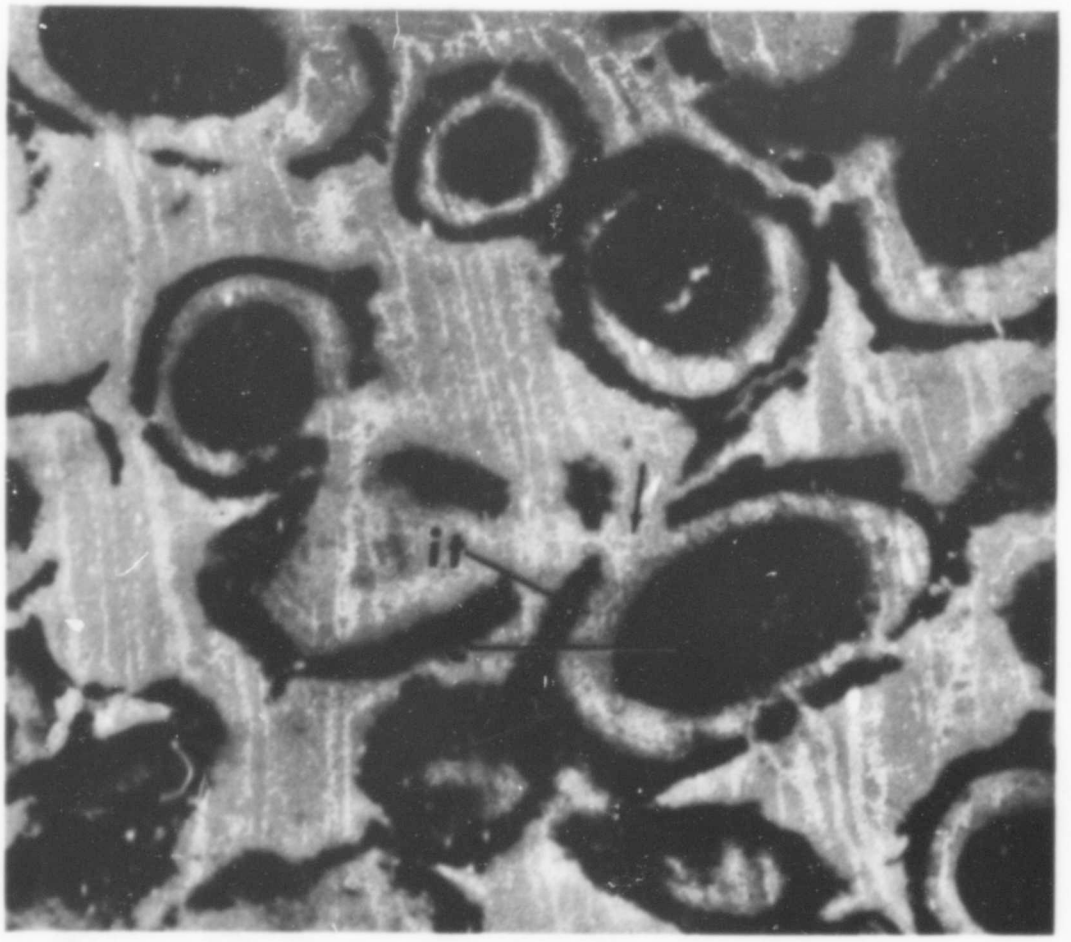

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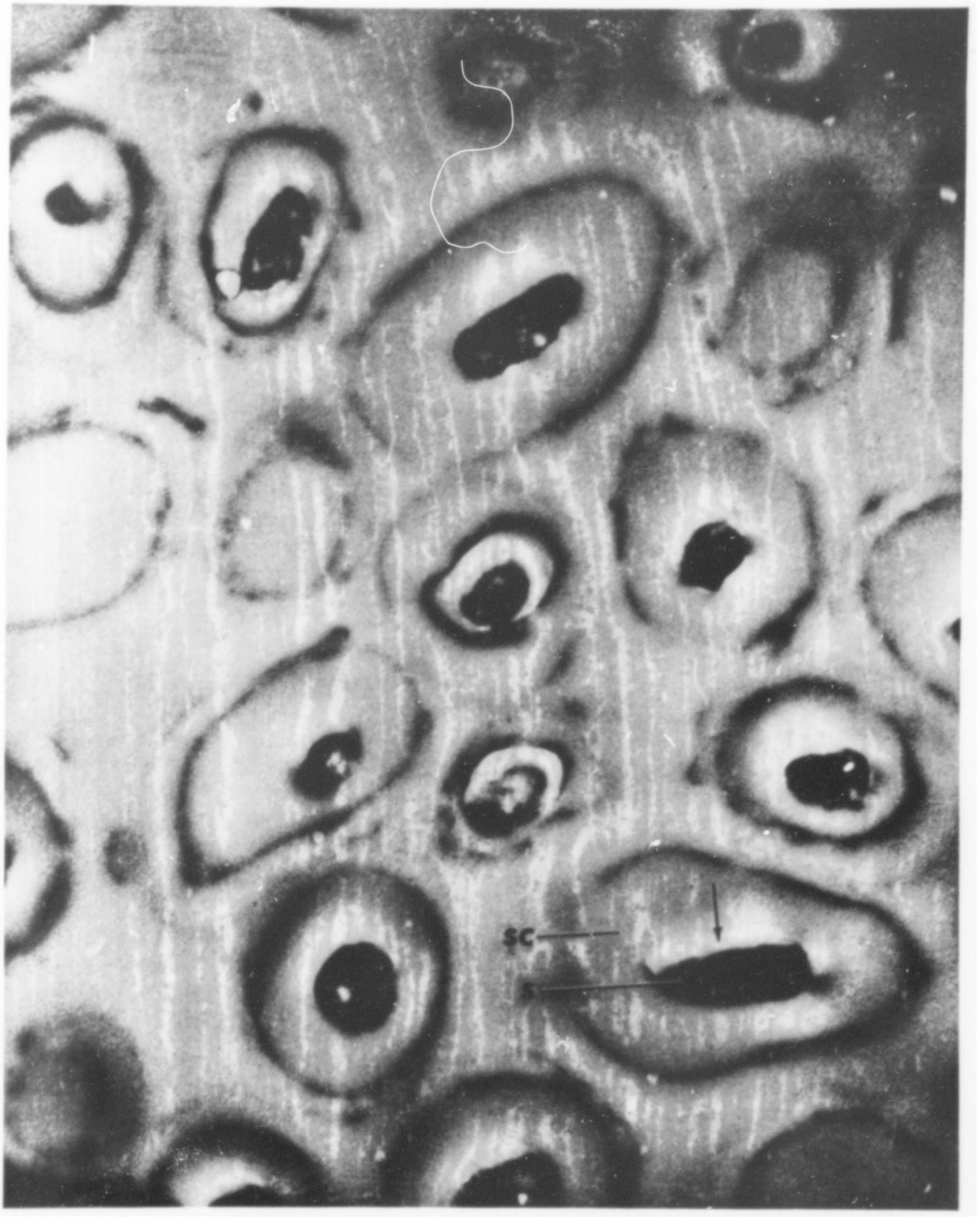

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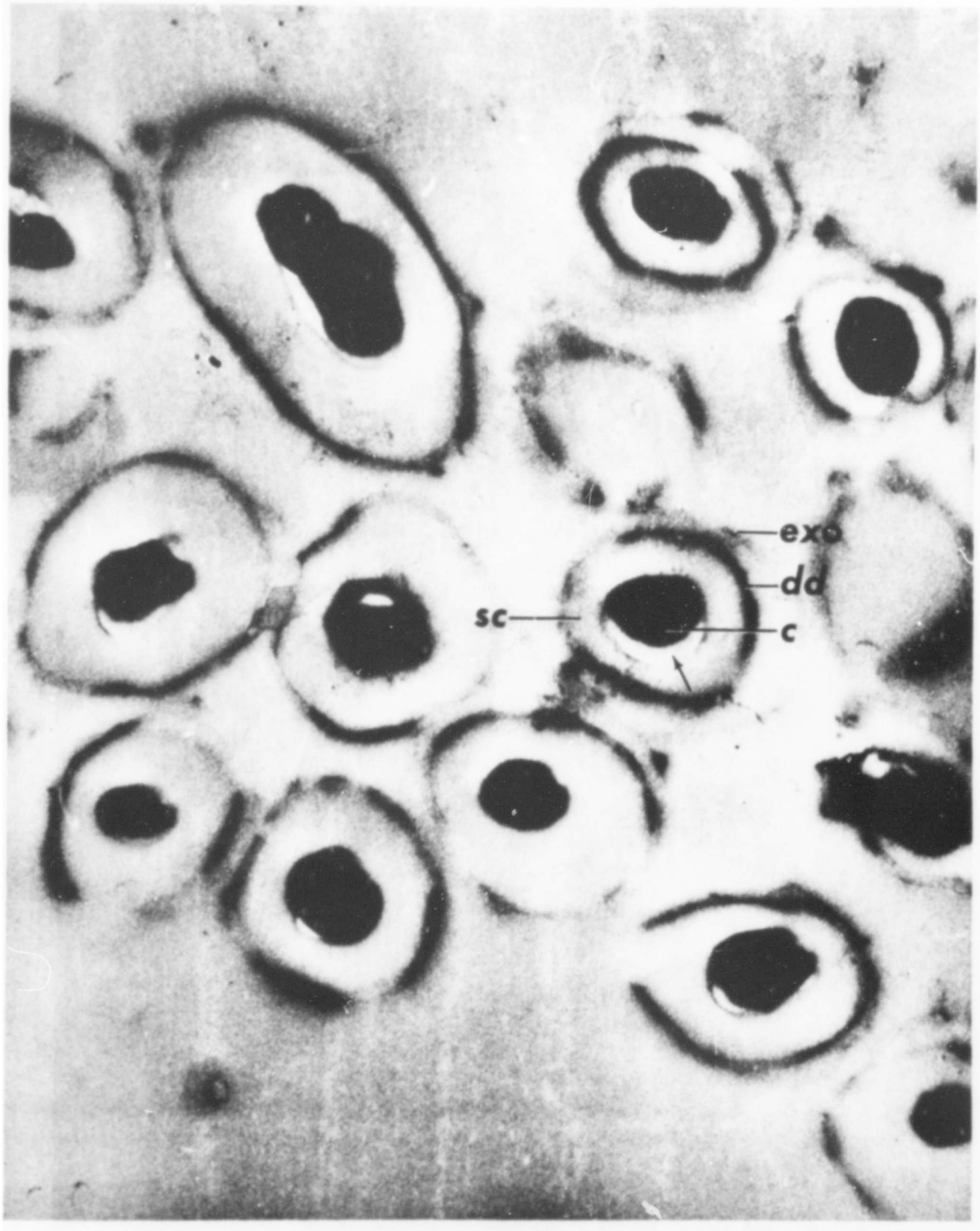

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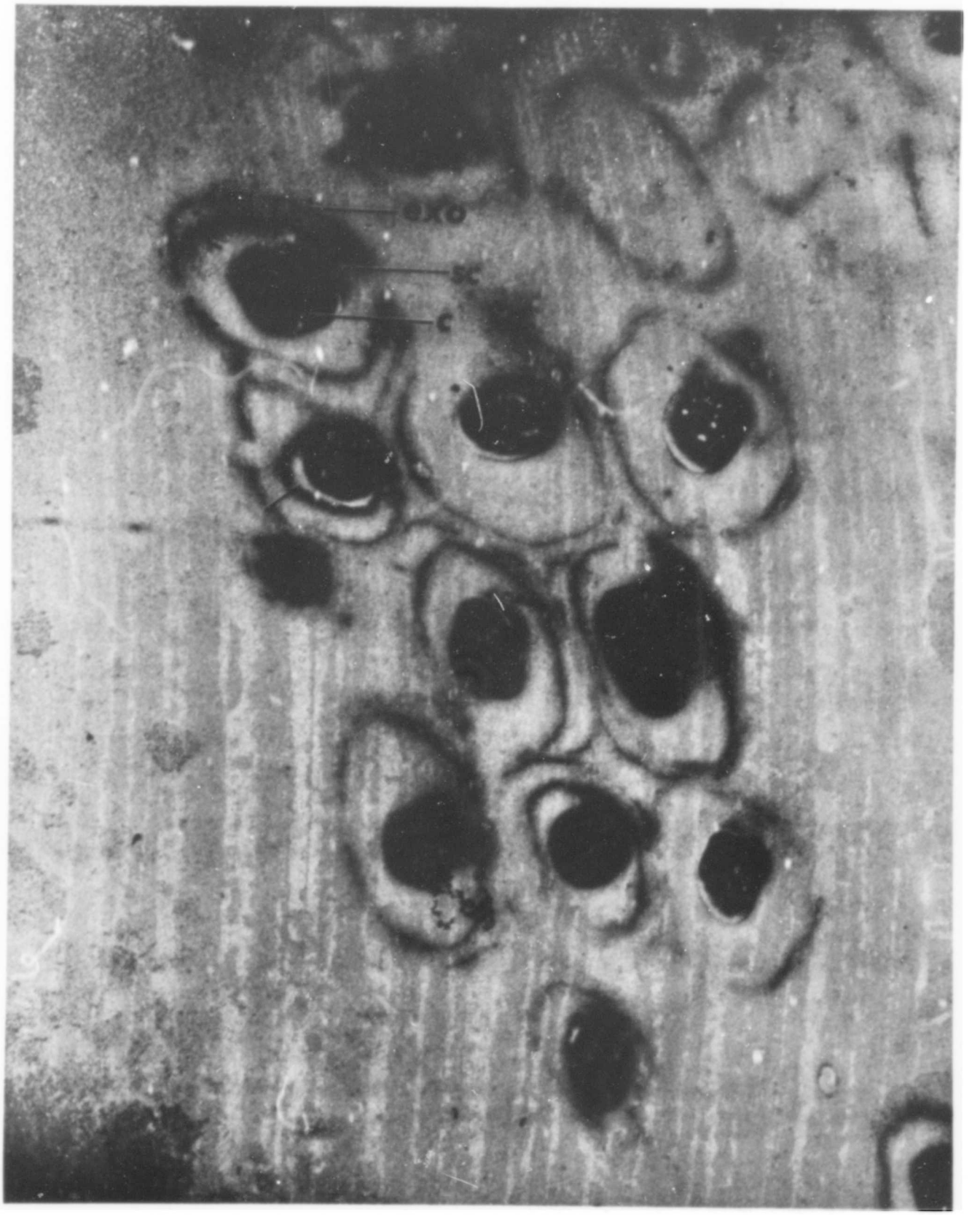

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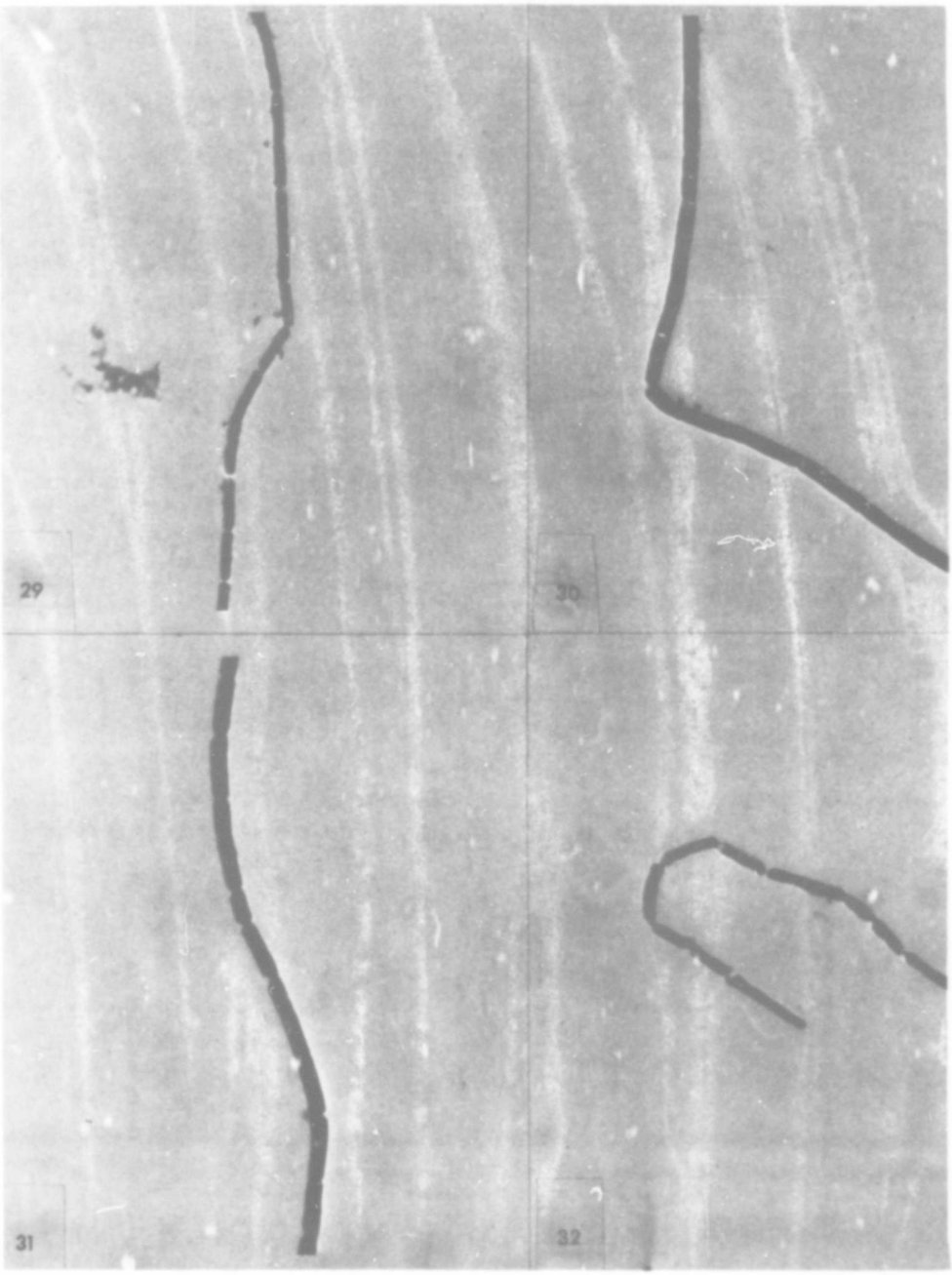
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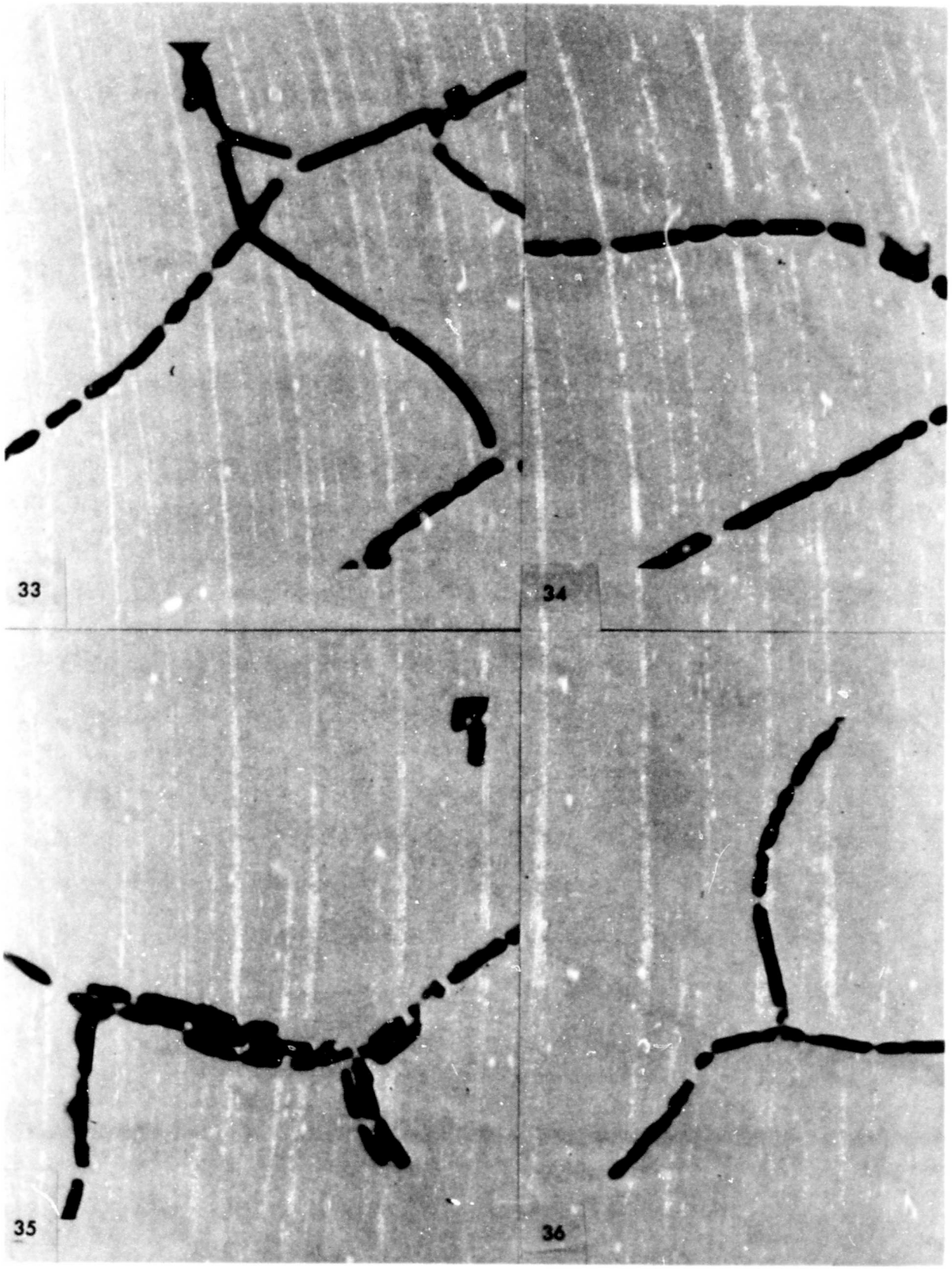
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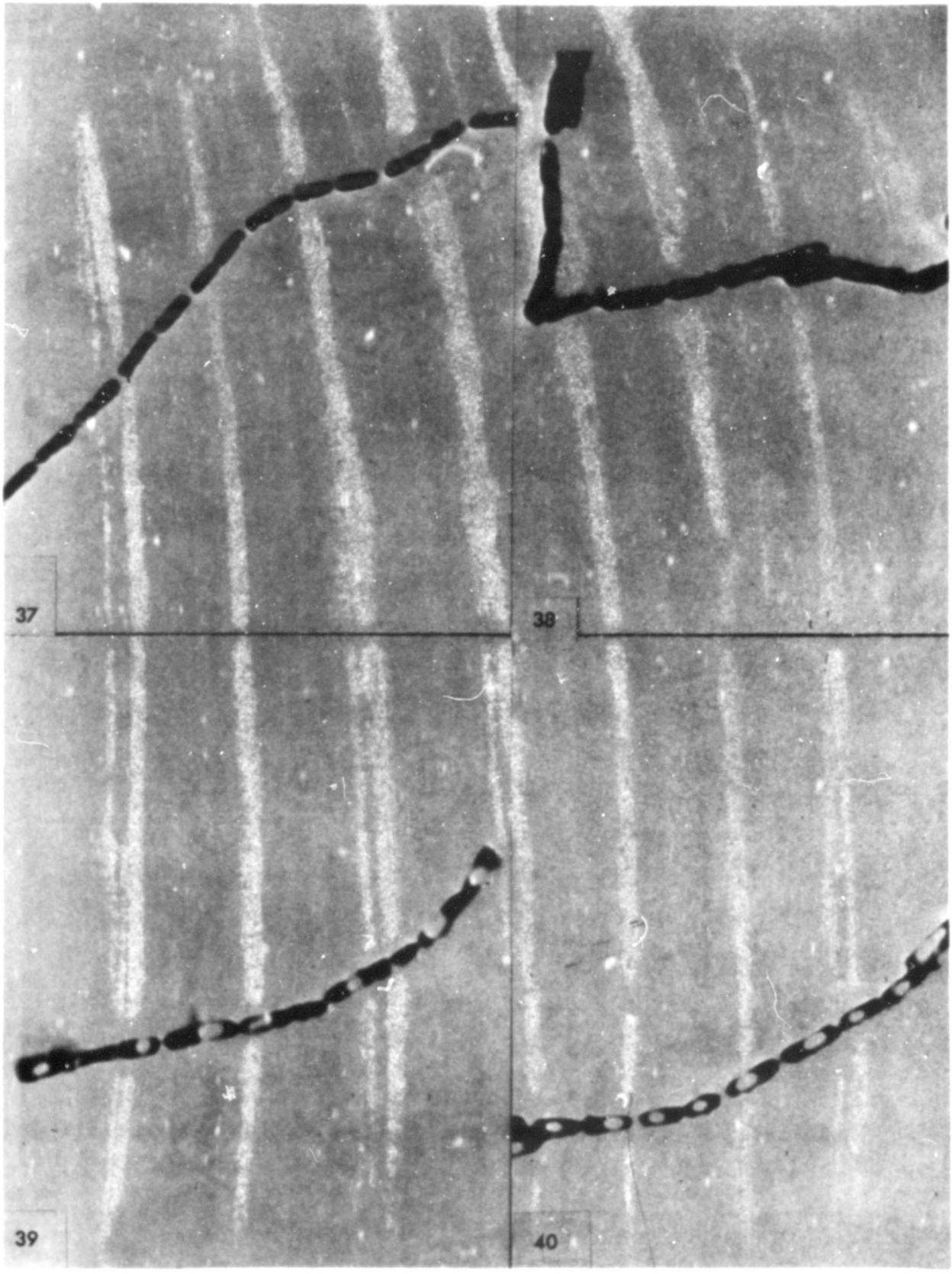
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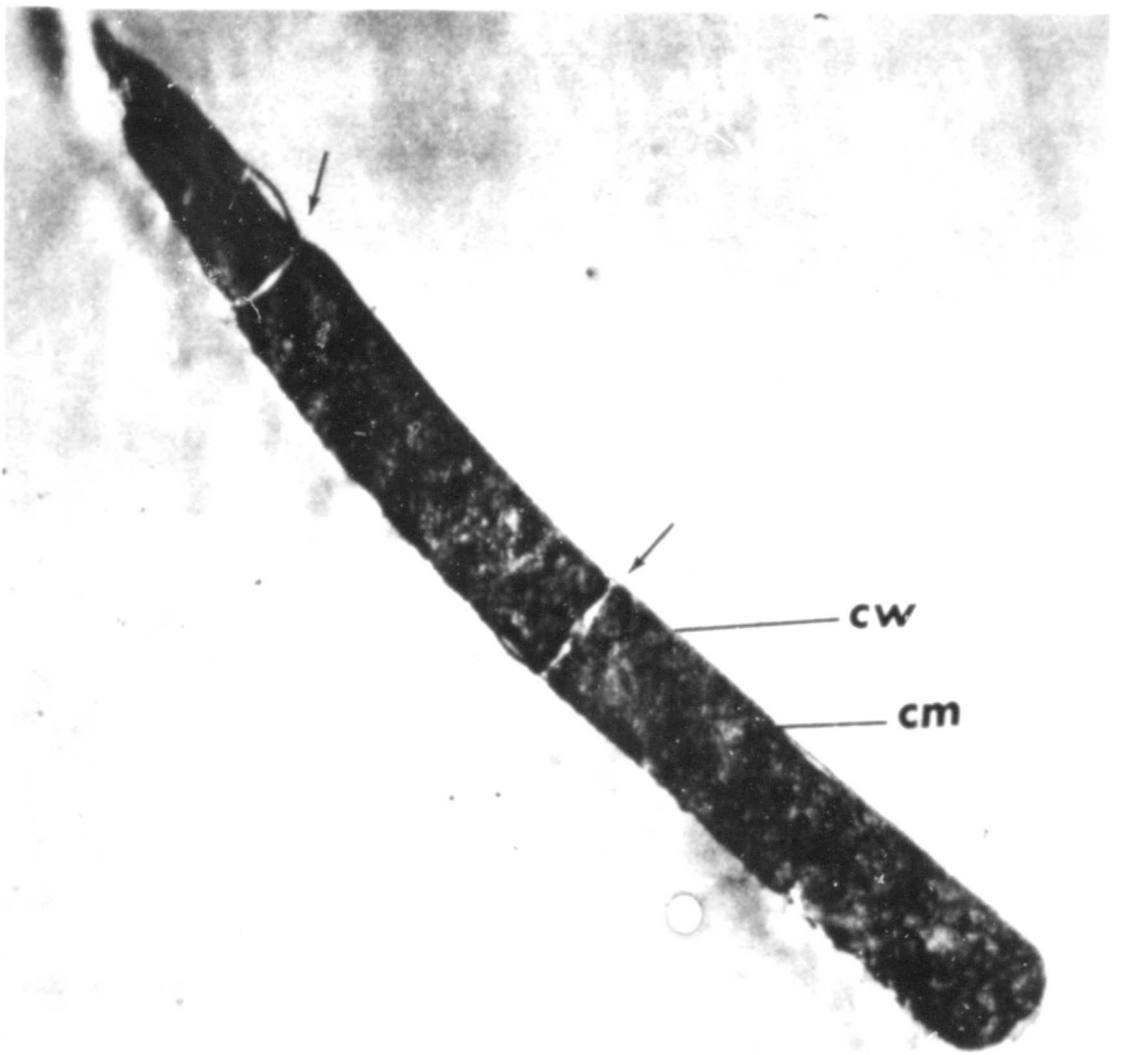

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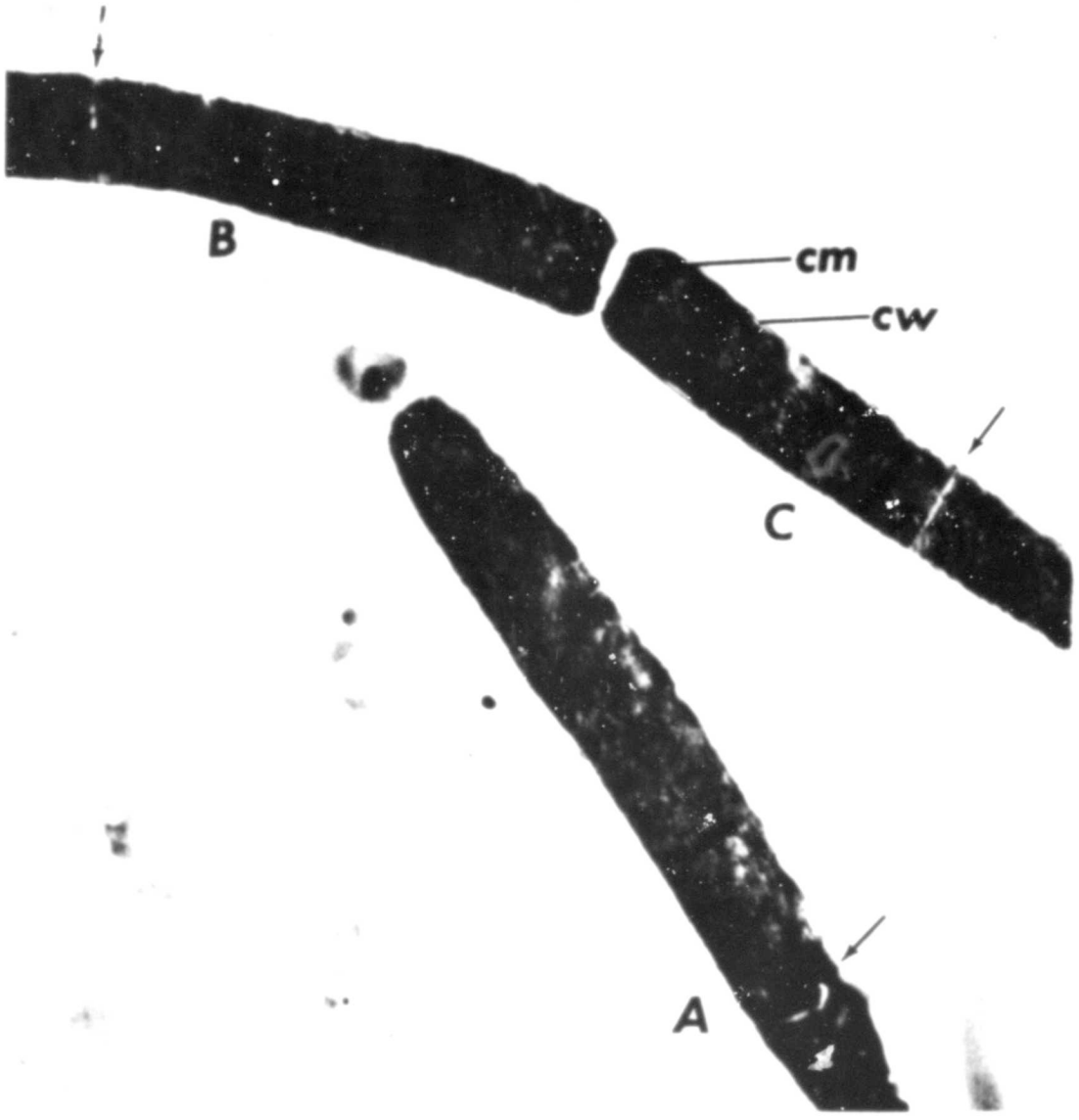

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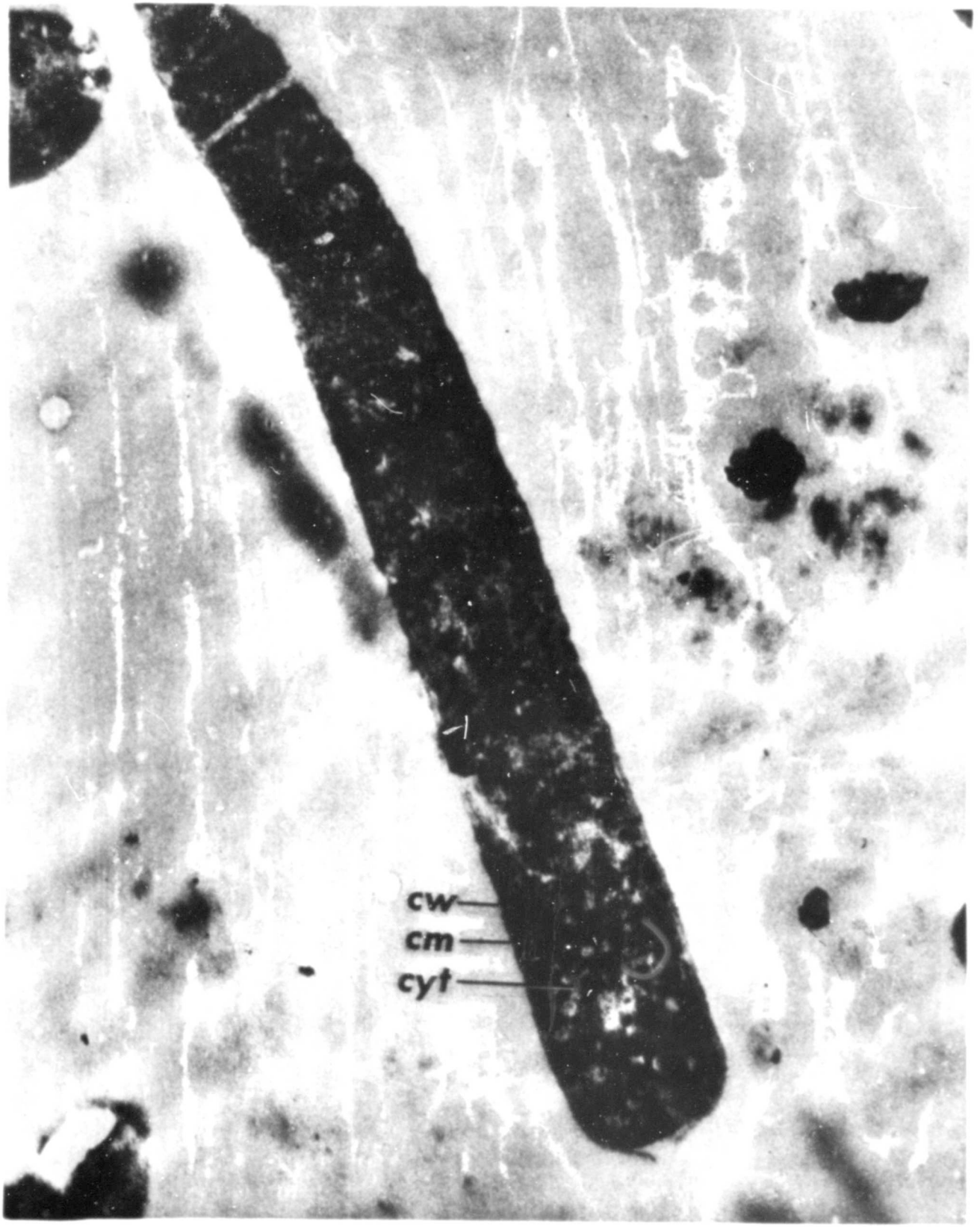

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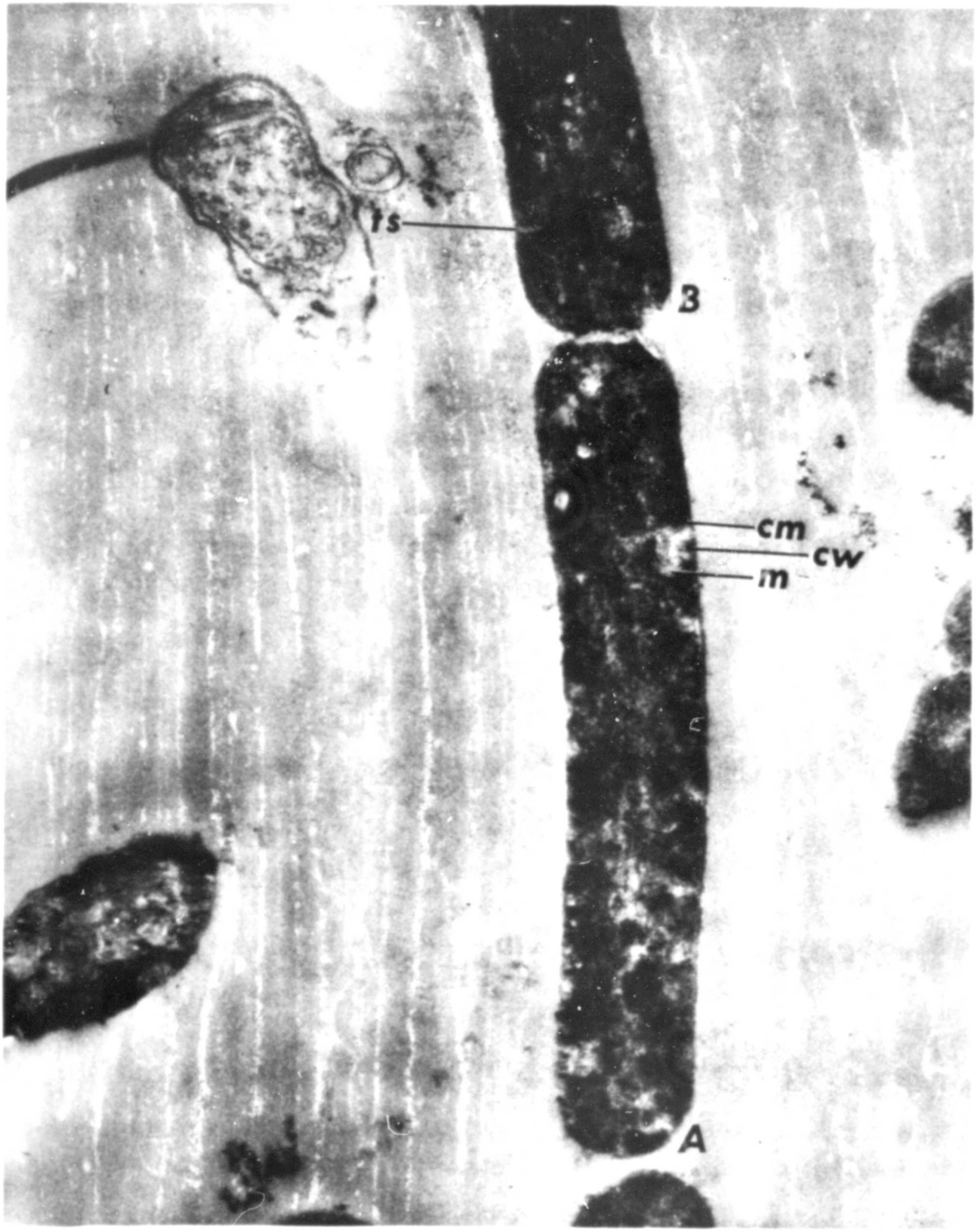

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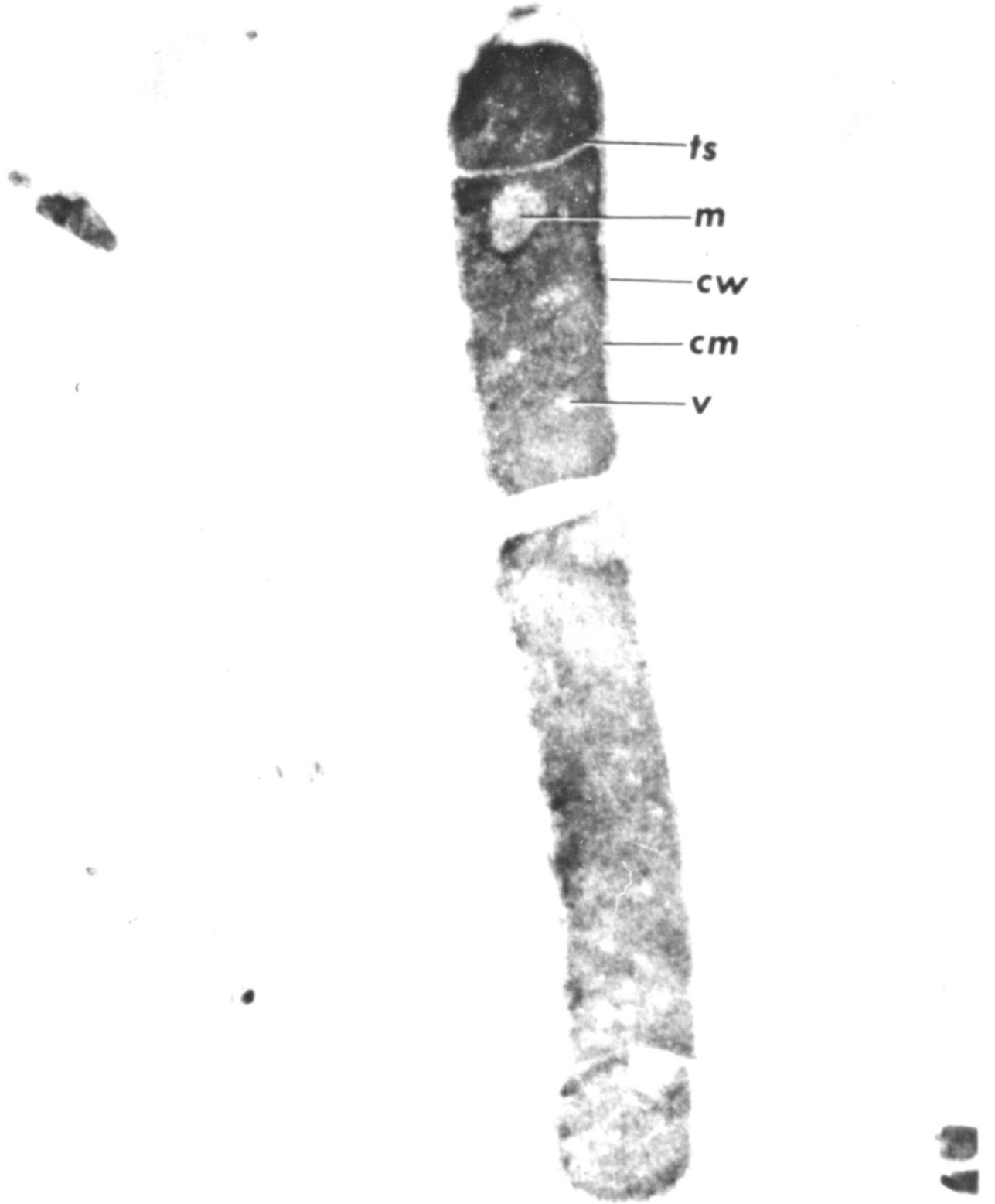

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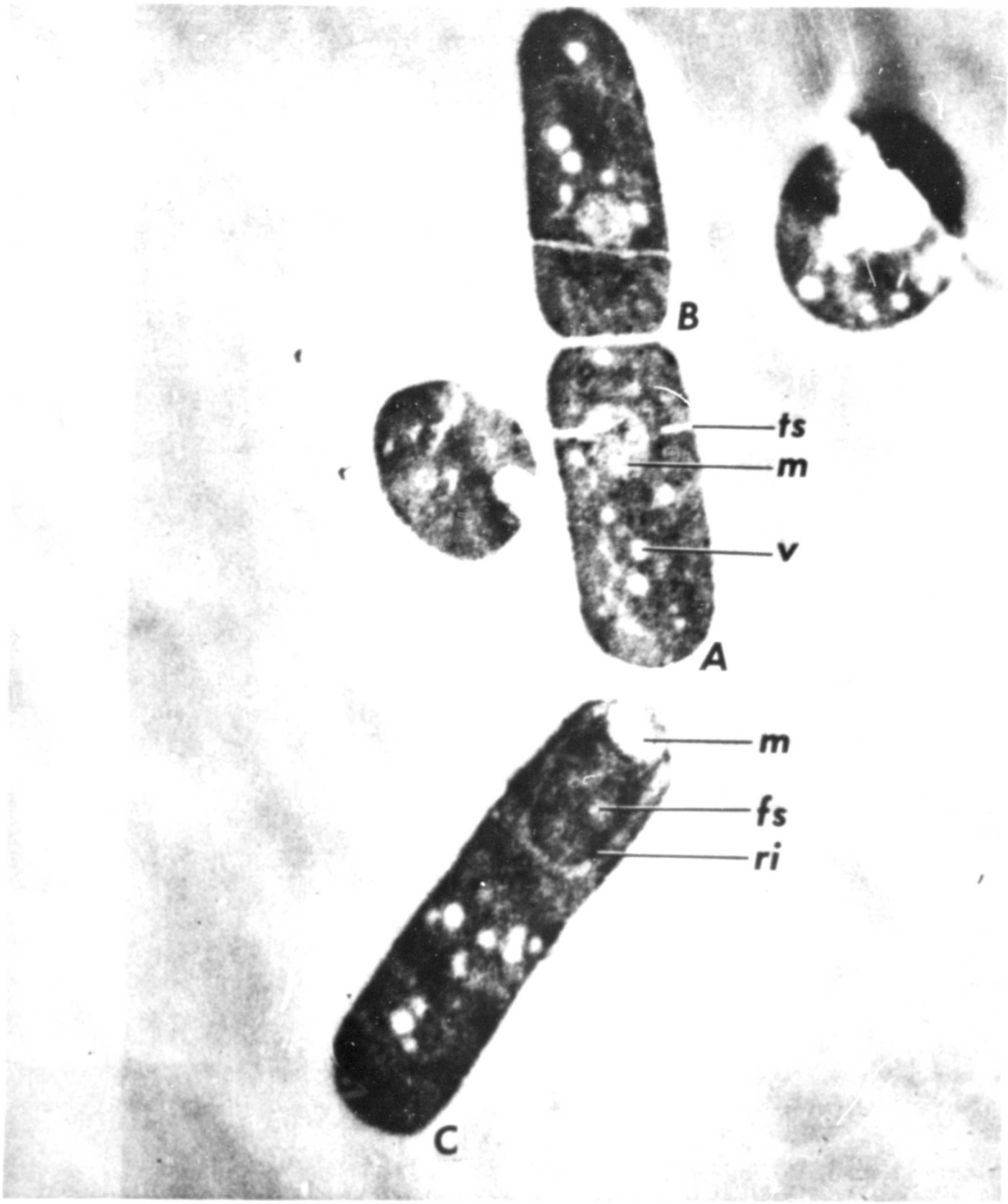

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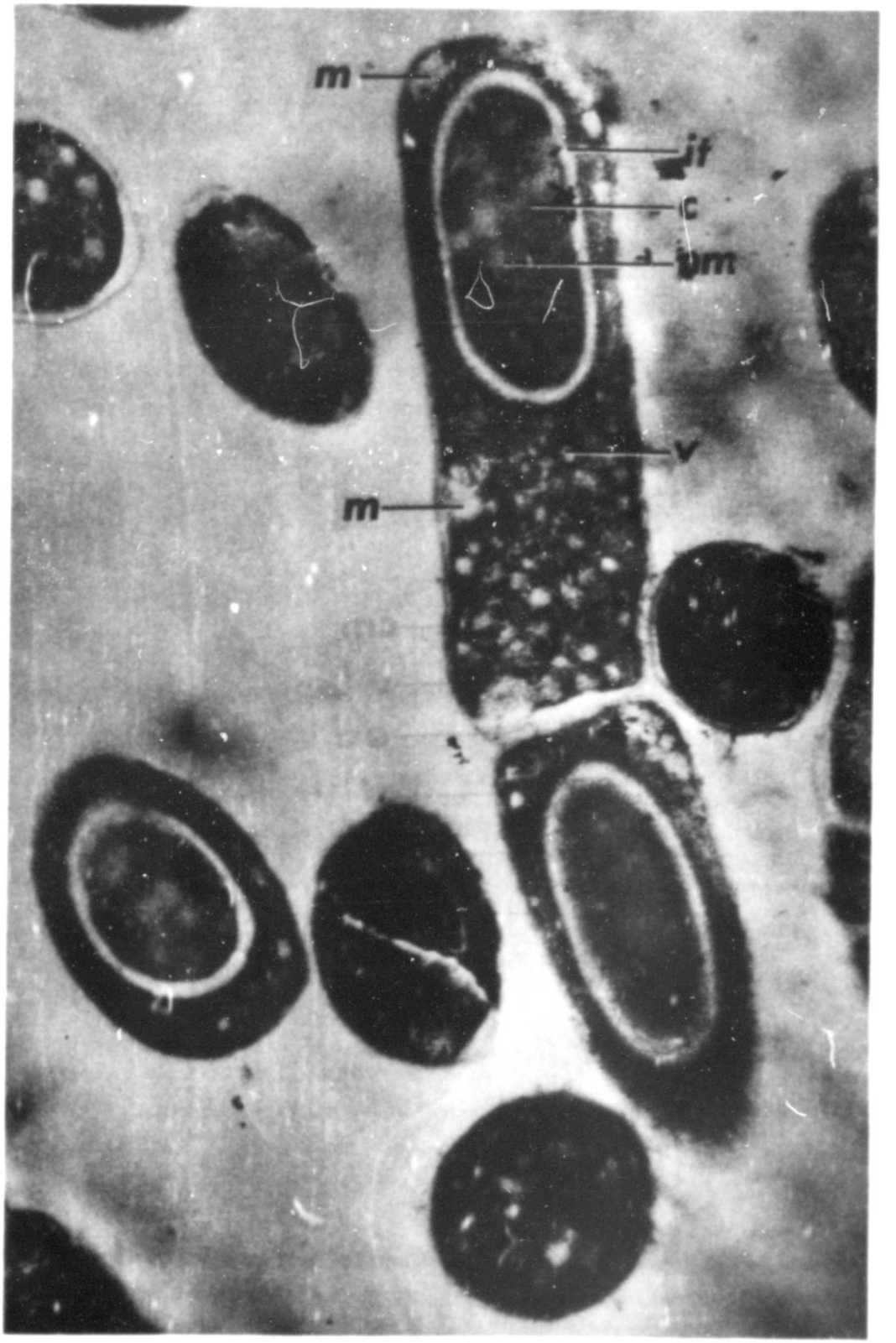

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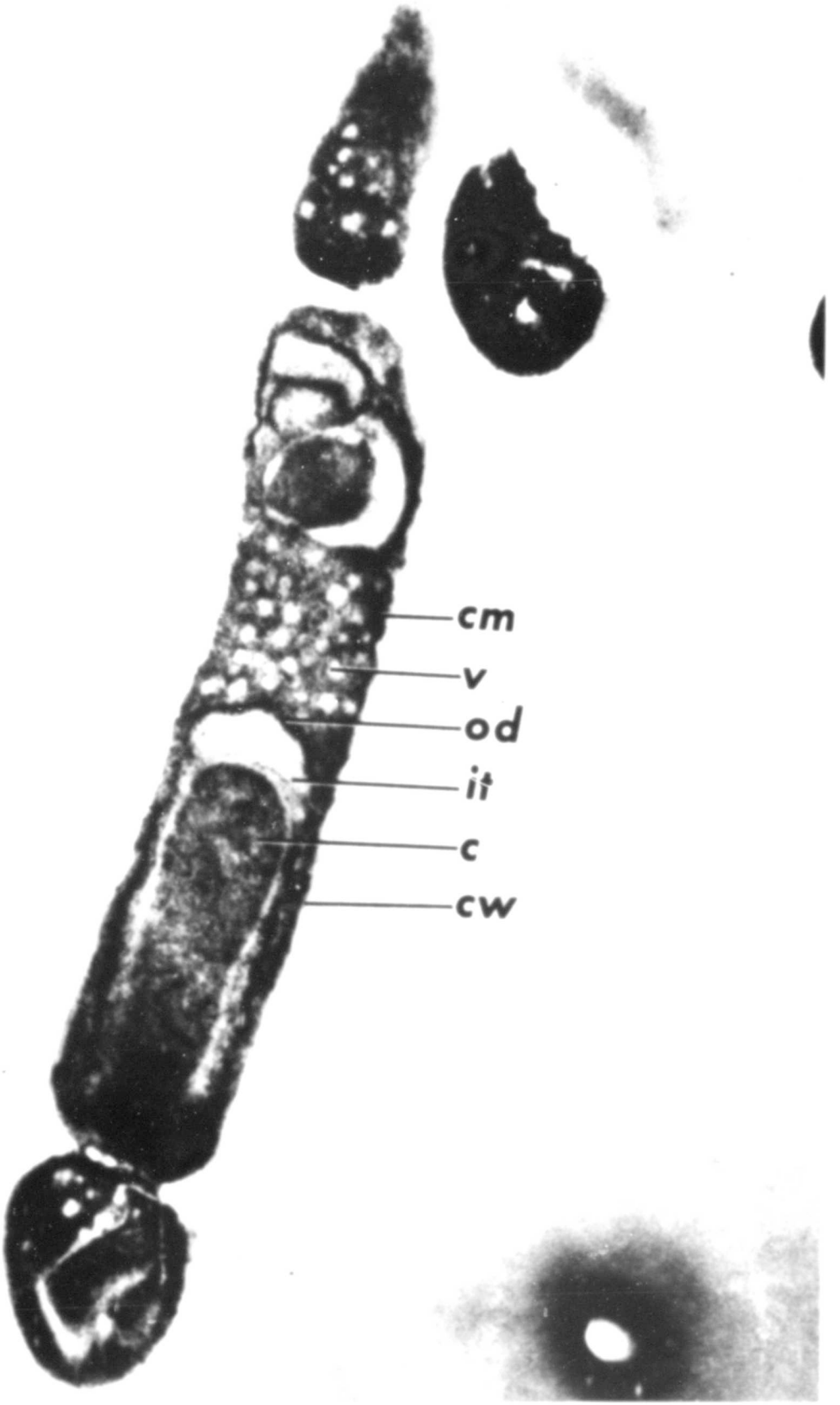

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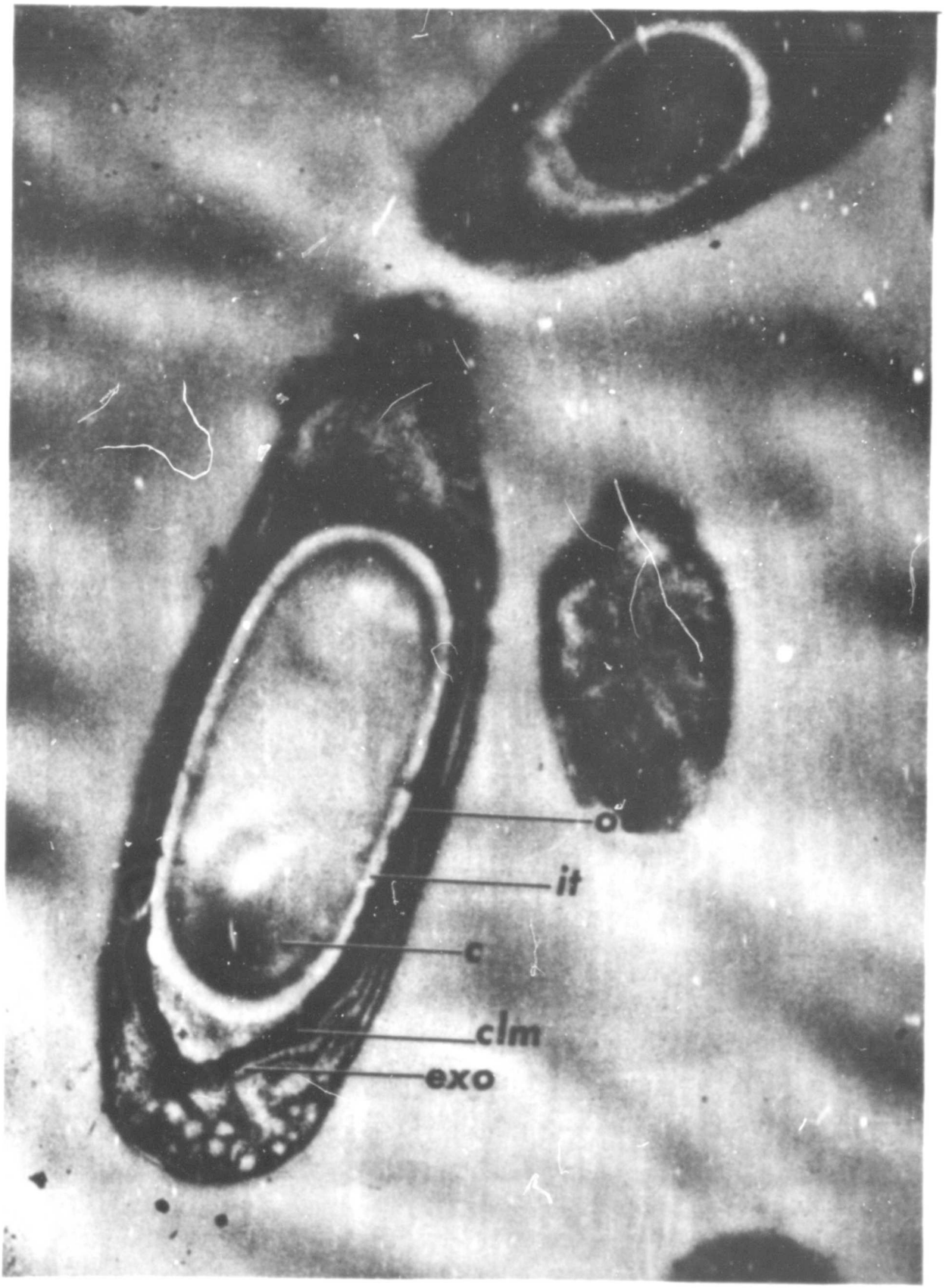

FIGURE 49

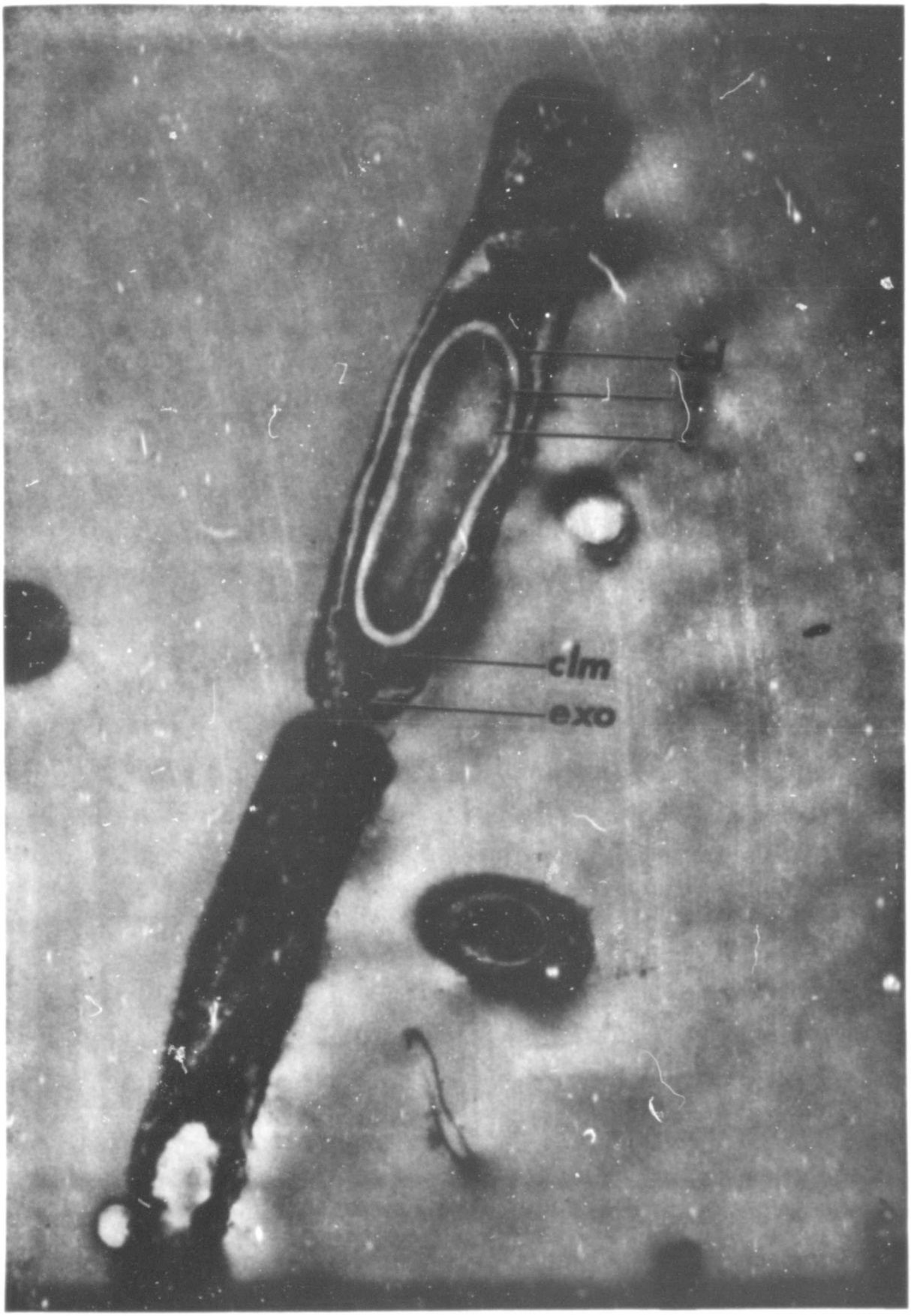

FIGURE 50

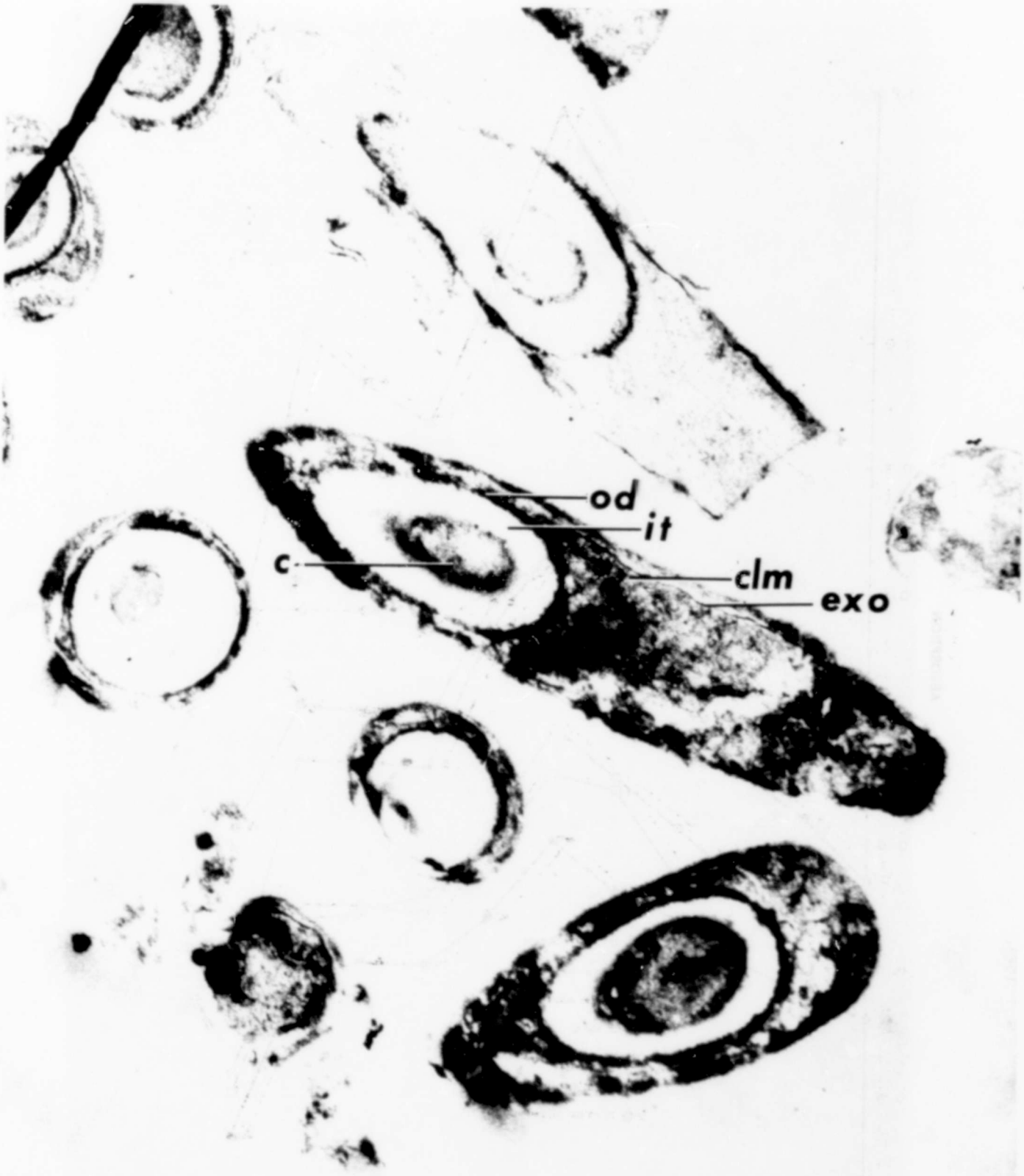

FIGURE 51

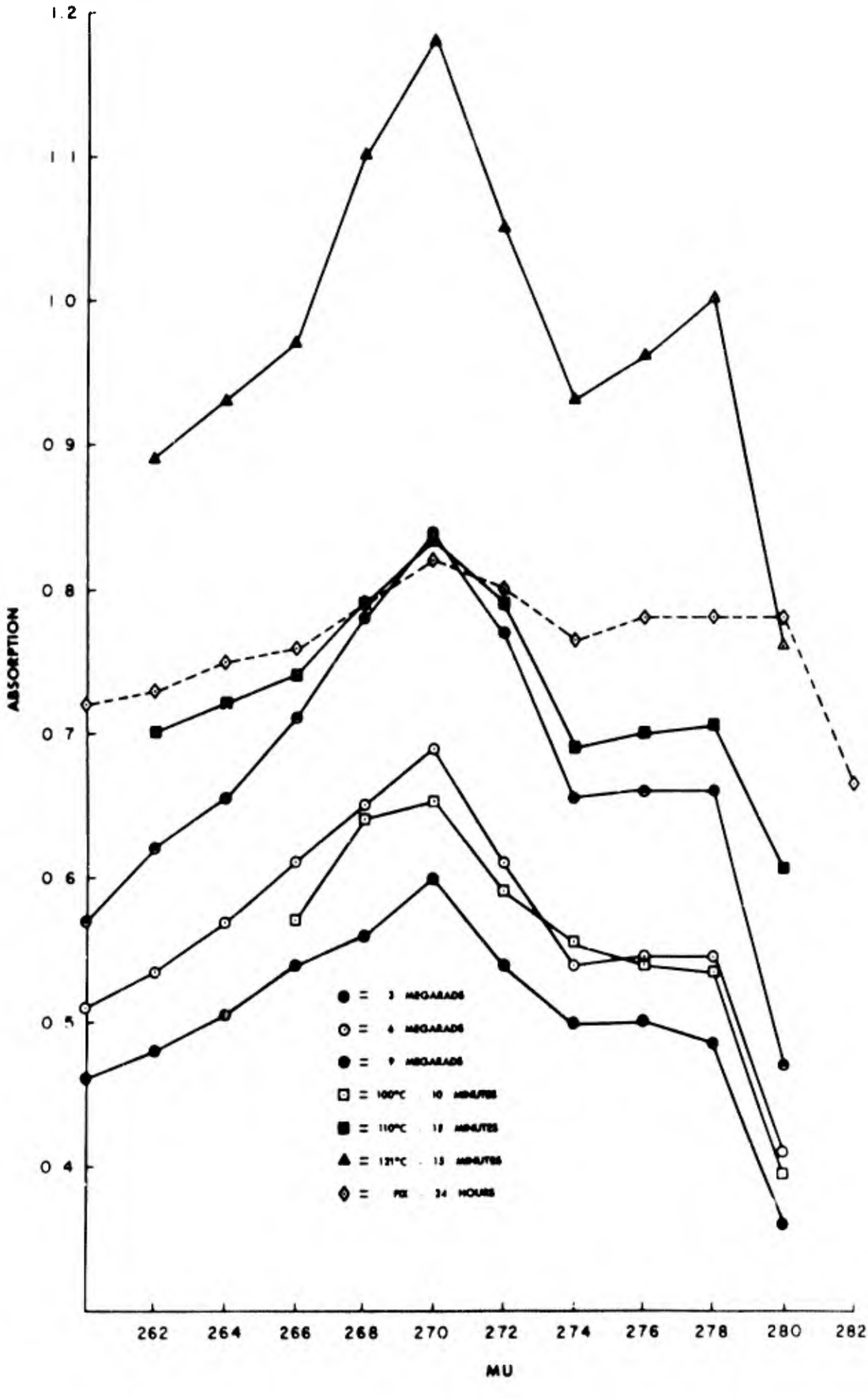
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
ABSORPTION
262
264
266
268
270
272
274
276
278
280
282
MU

FIGURE 52

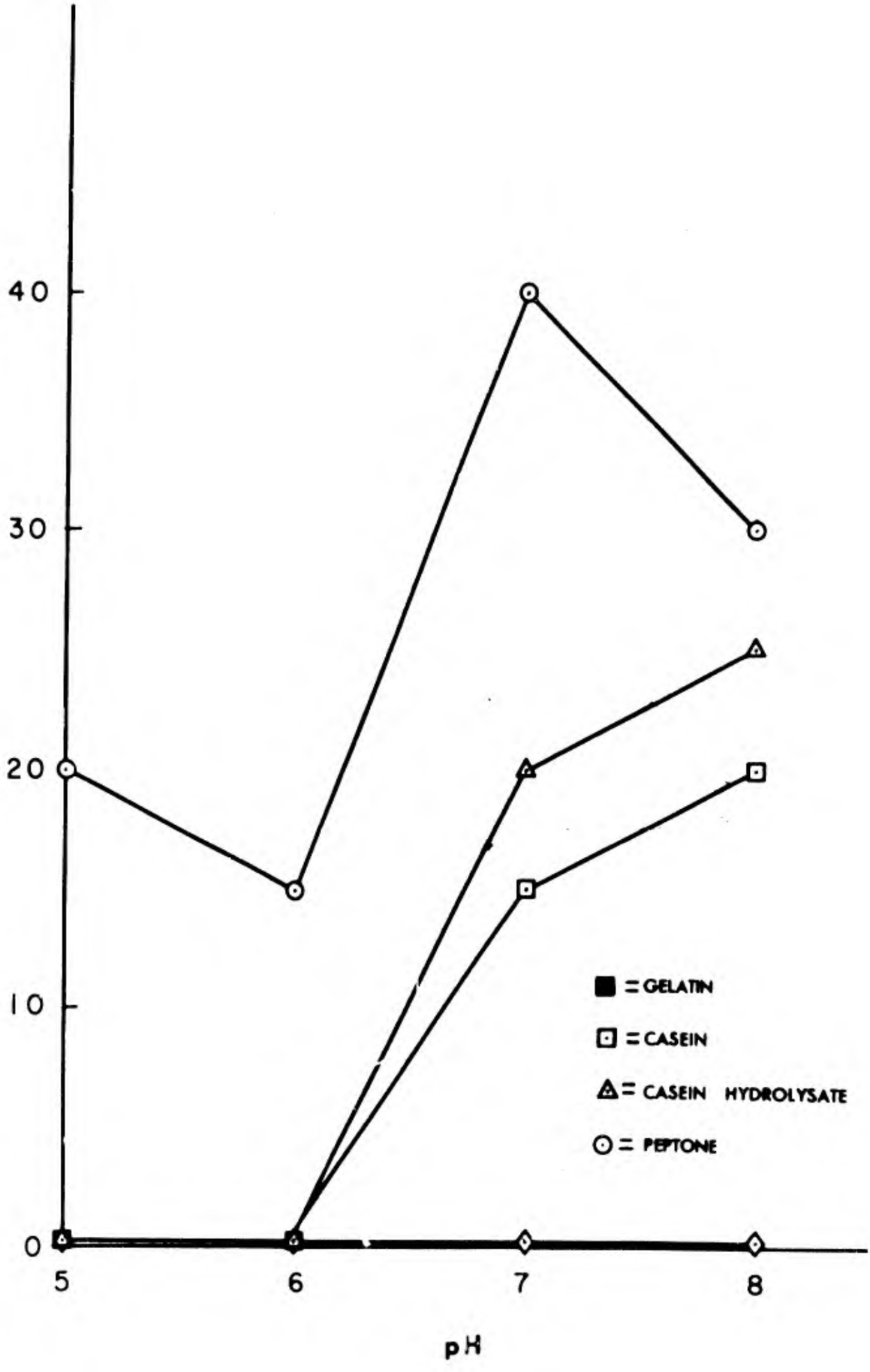
40
30
20
10
0
5
6
7
8
pH
= GELATIN
= CASEIN
= CASEIN HYDROLYSATE
= PEPTONE

FIGURE 53

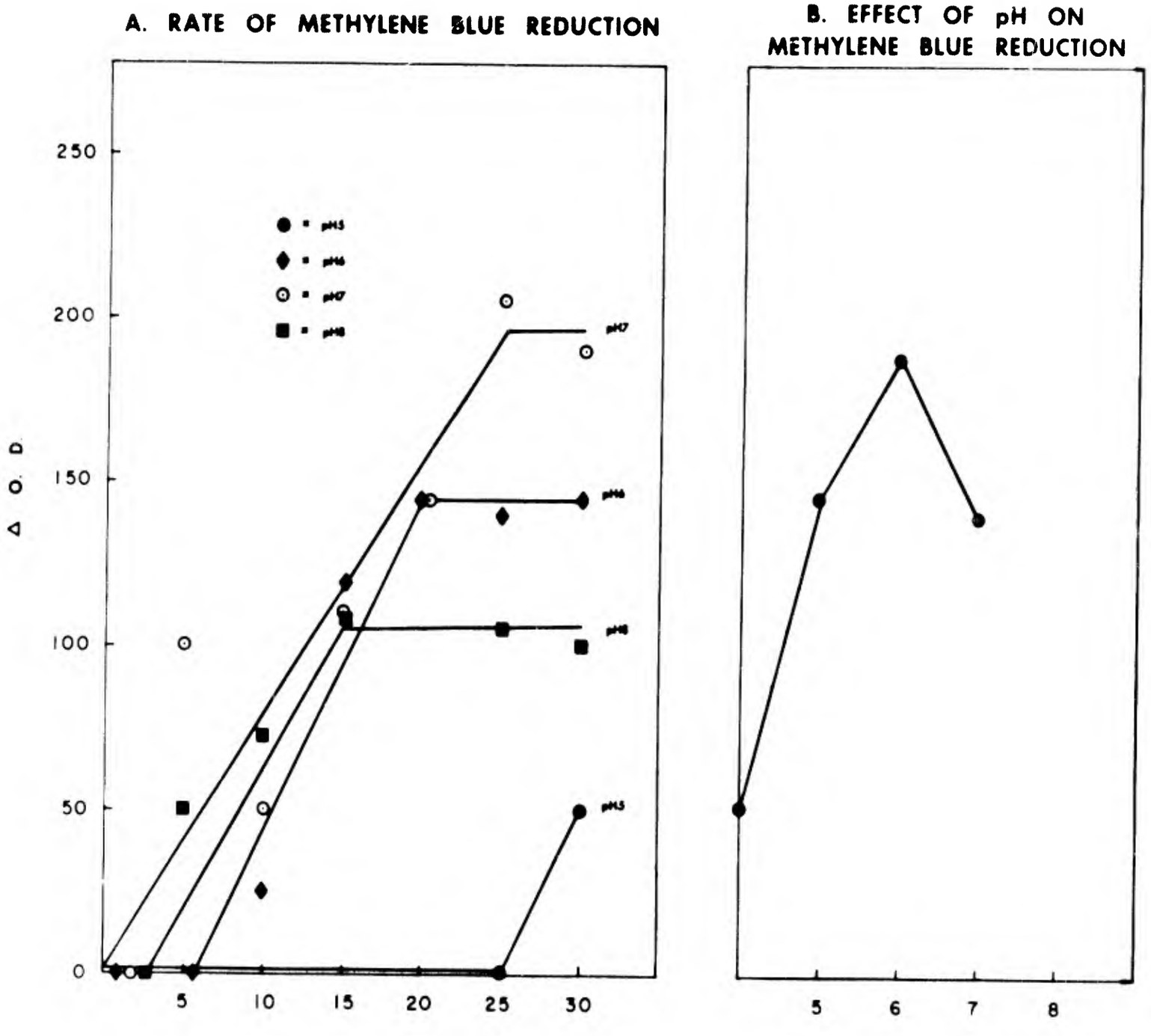
A. RATE OF METHYLENE BLUE REDUCTION
B. EFFECT OF pH ON METHYLENE BLUE REDUCTION
Δ O. D.
250
200
150
100
50
0
5
10
15
20
25
30
5
6
7
8

FIGURE 54

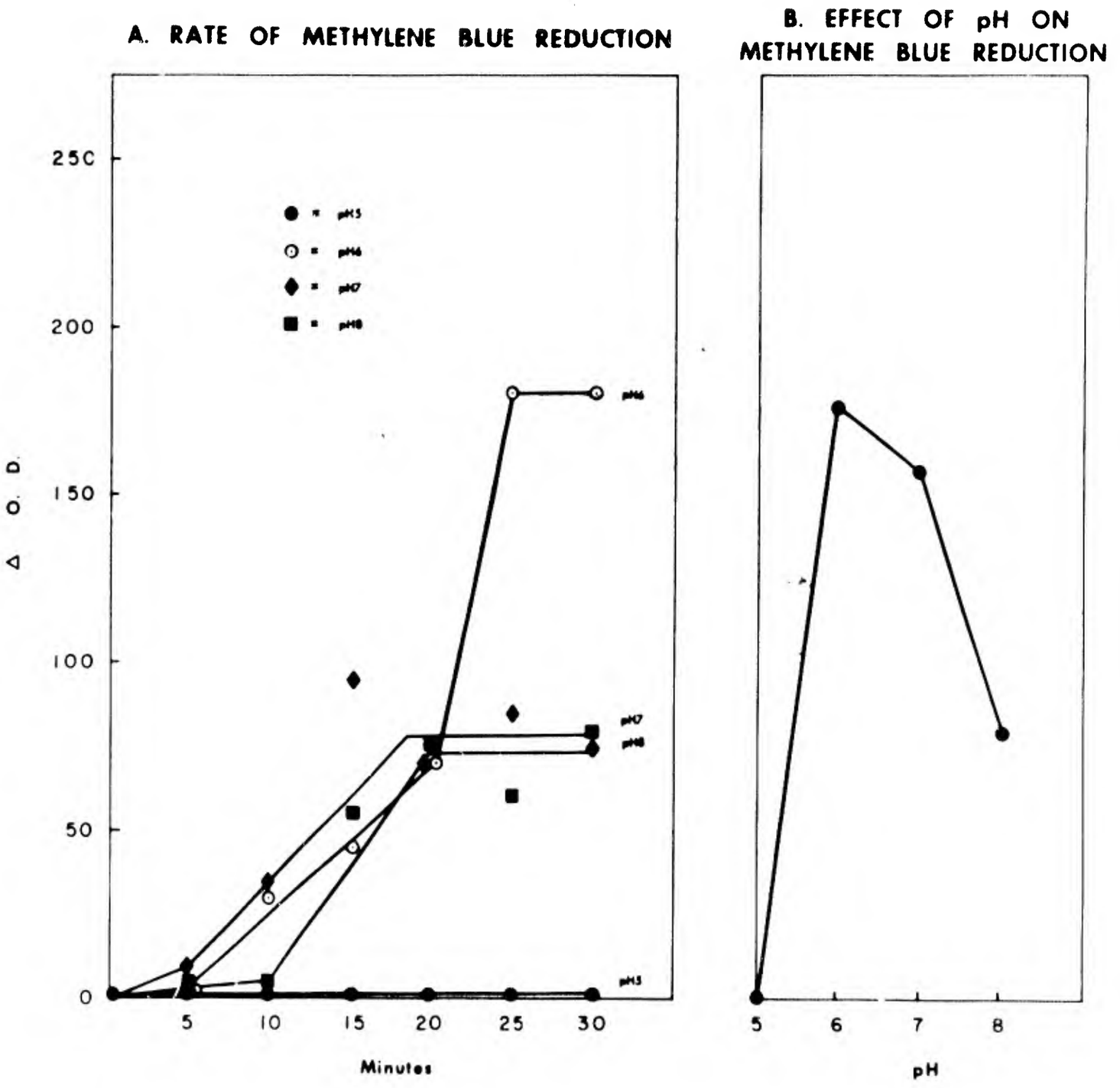
A. RATE OF METHYLENE BLUE REDUCTION
B. EFFECT OF pH ON METHYLENE BLUE REDUCTION
250
200
150
100
50
0
Δ O. D.
5
10
15
20
25
30
Minutes
5
6
7
8
pH

FIGURE 55

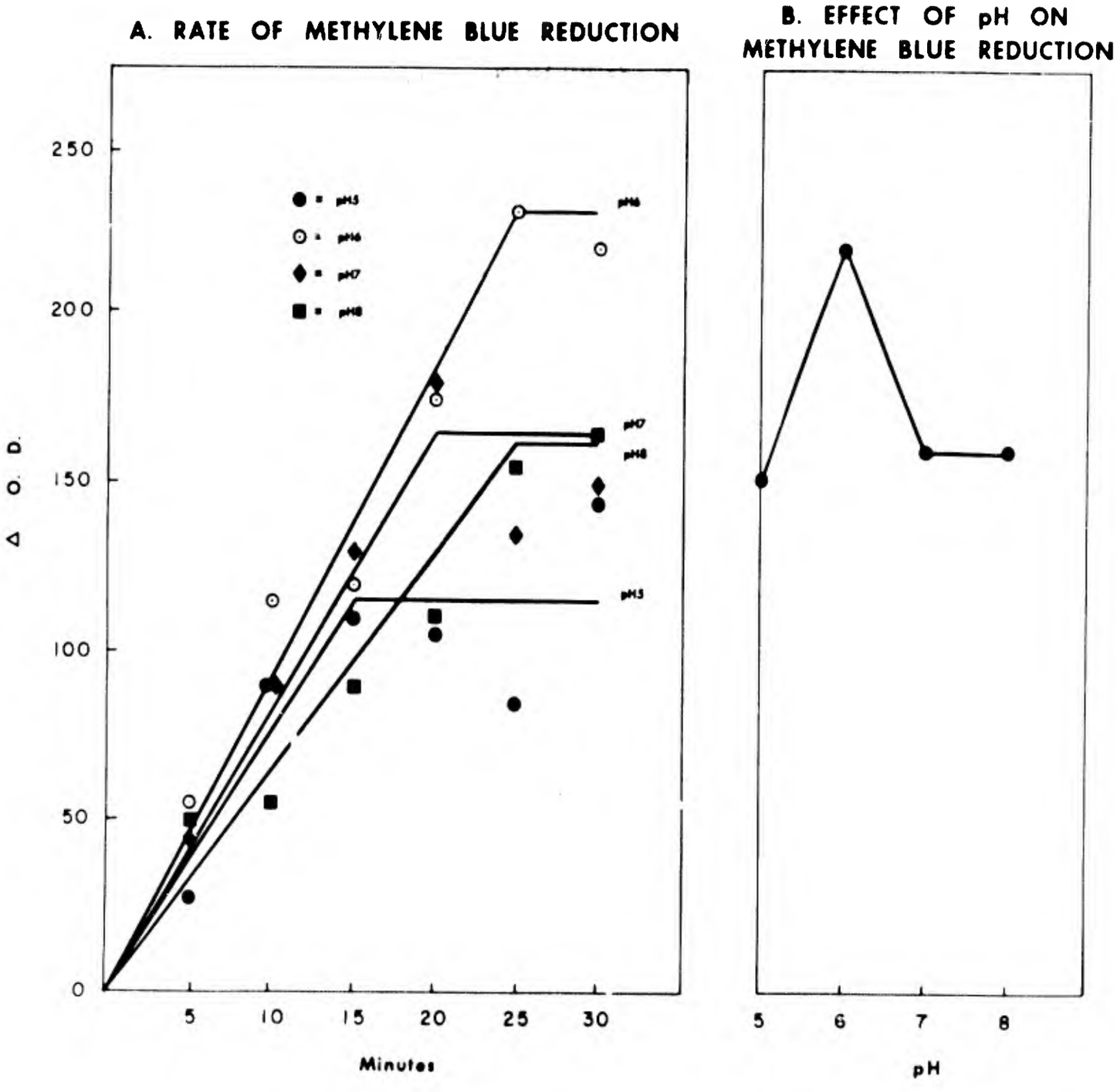
A. RATE OF METHYLENE BLUE REDUCTION
B. EFFECT OF pH ON METHYLENE BLUE REDUCTION
250
200
150
100
50
0
Δ O. D.
5
10
15
20
25
30
Minutes
pH
5
6
7
8

FIGURE 56

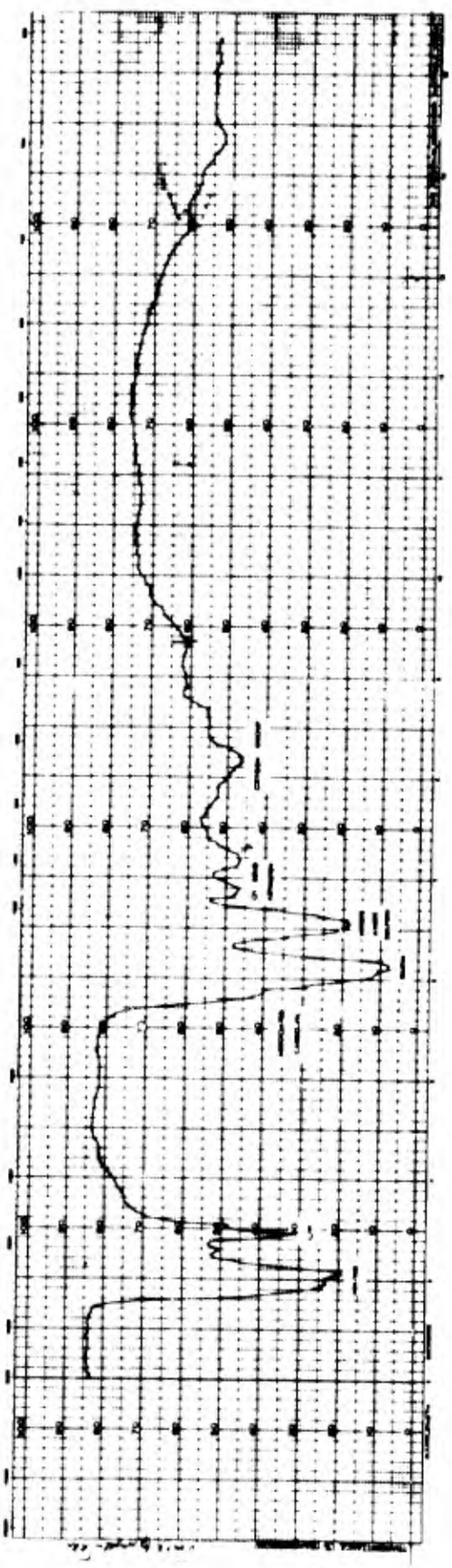

FIGURE 57

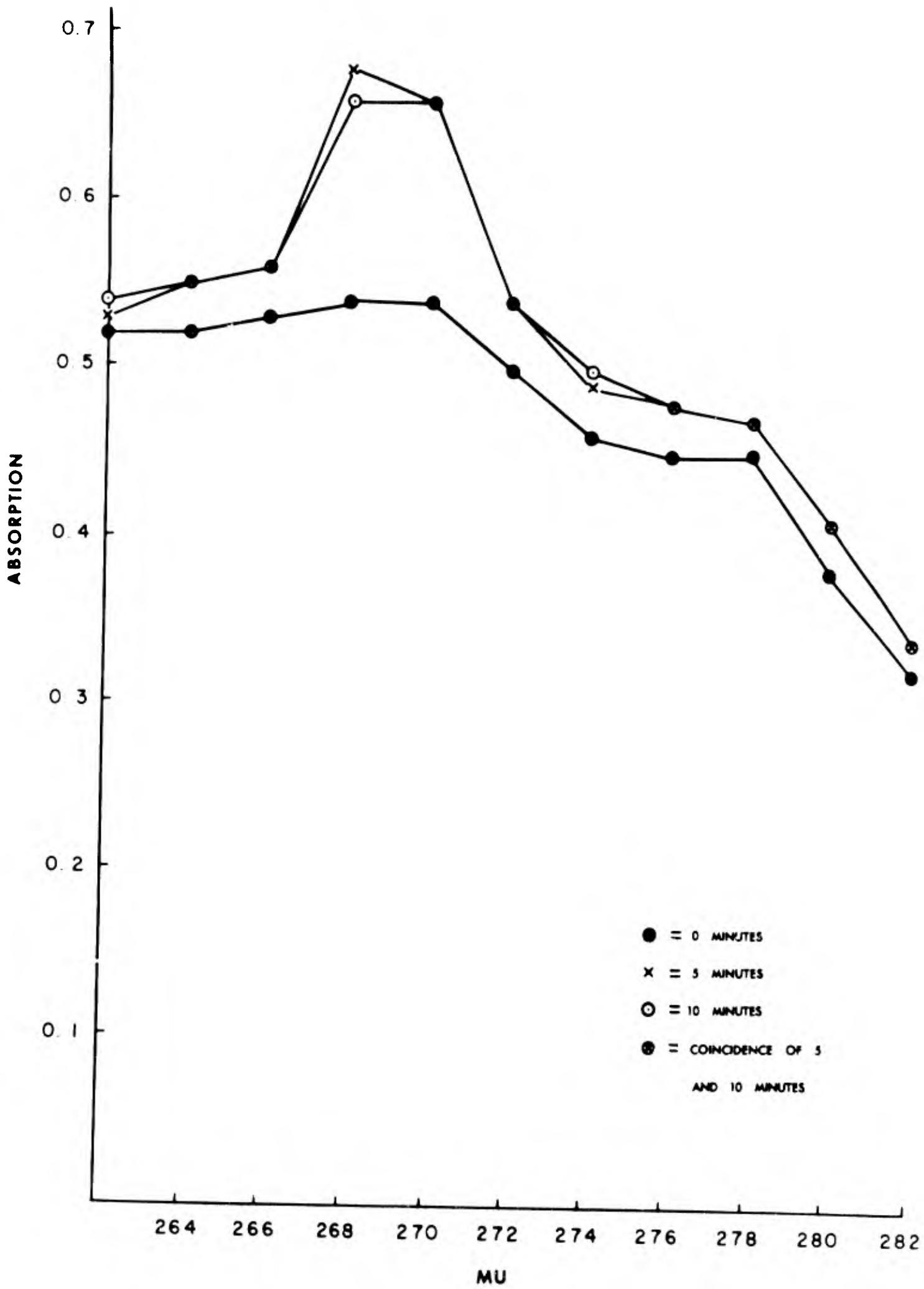
0.7
0.6
0.5
0.4
0.3
0.2
0.1
ABSORPTION
264
266
268
270
272
274
276
278
280
282
MU
● = 0 MINUTES
× = 5 MINUTES
⊙ = 10 MINUTES
⊛ = COINCIDENCE OF 5 AND 10 MINUTES

FIGURE 58

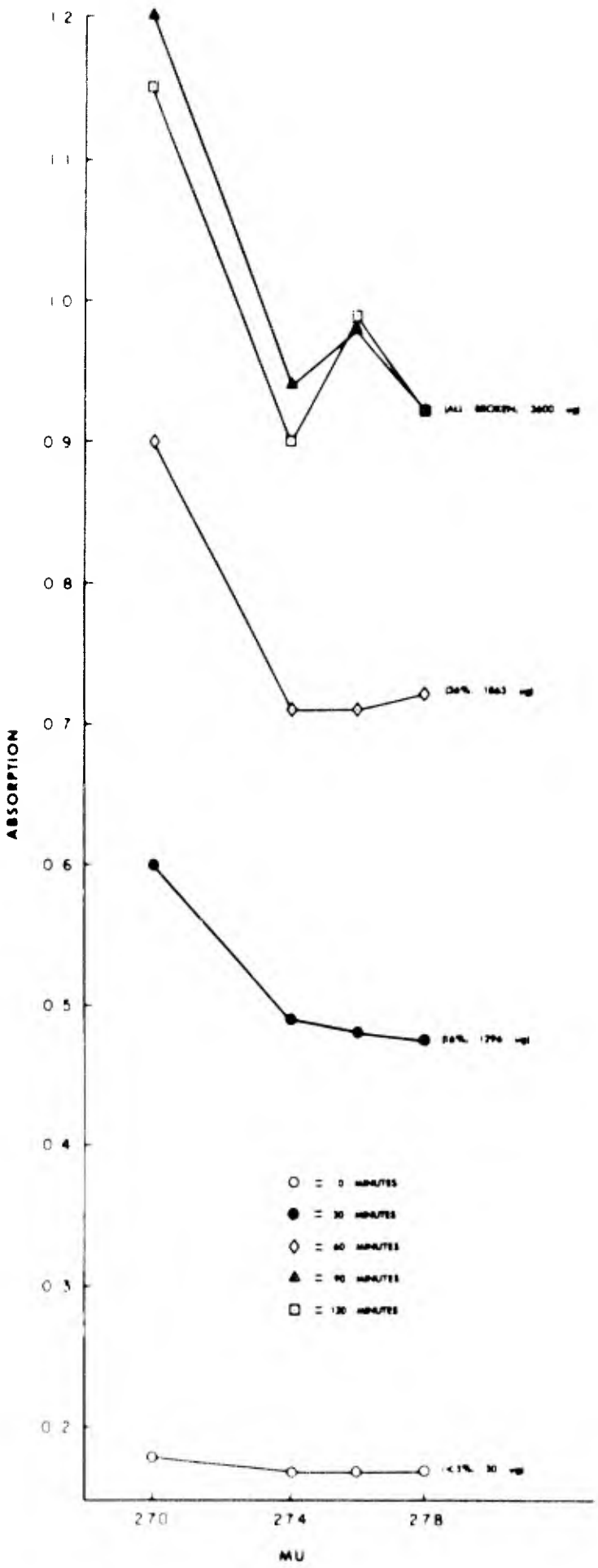

FIGURE 59