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ANIMAL PATHOGENIC CORYNEBACTERIA: ACID METABOLIC END PRODUCTS AND CYTOCHROMES

By

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ABSTRACT

ANIMAL PATHOGENIC CORYNEBACTERIA: ACID METABOLIC END PRODUCTS AND CYTOCHROMES

By

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The acid metabolic end products and cytochromes in selected animal pathogenic corynebacteria and C. diphtheriae have been studied.

The gas chromatographic profiles show that these corynebacteria are metabolically heterogeneous. C. pyogenes, similar to lactobacilli, produces large amounts of lactate and moderate amounts of acetate from glucose fermentation. There are no detectable acid end products produced by C. equi. C. renale appears similar to homofermentative lactic acid producing bacteria in producing lactic acid as the major acid end product. C. bovis produces major amounts of lactate and acetate from glucose. C. kutscheri produces major amounts of lactate and propionic acid and small amounts of succinic acid. C. pseudotuberculosis and C. diphtheriae, similar to propionibacteria, produce large amounts of propionic and acetic acids and in addition, both organisms produce major amounts of formic acid.

A b type cytochrome is detected in C. pyogenes, C. pseudotuberculosis, C. diphtheriae, C. bovis, C. kutscheri, C. renale,

while both cytochromes b and c are detected in C. equi grown under the same experimental conditions.

The results on acid end products from glucose of C. pyogenes and C. equi, in agreement with previous investigations, provided additional evidence that C. pyogenes and C. equi should be removed from the genus Corynebacterium and be reassigned to separate genera. The acid end products are consistent among the strains within a species and appear useful in differentiating animal pathogenic corynebacteria.

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I. INTRODUCTION

The genus Corynebacterium includes a wide variety of non-spore forming, Gram-positive, rod-shaped bacteria with irregular stained segments and sometimes granules (109). Cells frequently show club-shaped swellings and differing degrees of pleomorphism. Several species exhibit snapping division and cells are arranged in angles and palisades. Considerable differences exist among individual species in cell wall composition, motility, deoxyribonucleic acid base ratios and in a number of other physiological and biochemical characteristics (6, 25, 26, 29, 35, 36, 45, 48, 50, 92, 101, 105, 110). Based on these differences, the species of the genus Corynebacterium have been subdivided into three sections (92): human and animal parasites and pathogens, plant pathogenic corynebacteria, and non-pathogenic corynebacteria.

Plant pathogenic corynebacteria are morphologically similar to human and animal pathogens and parasites, but are considerably different otherwise (93). All plant pathogenic corynebacteria, with the exception of Corynebacterium facians, are distinct from human and animal pathogens in cell wall composition, serology, and in having distinctly higher deoxyribonucleic acid base ratios of 65-75% (28, 58, 92, 93, 105, 110). Furthermore, some plant pathogens, unlike human and animal pathogens, are motile (93).

Non-pathogenic corynebacteria are often poorly described mostly because heavy or in several cases sole reliance was placed on morphological criteria in their classification. In most cases there are insufficient data to permit differentiation of individual species of the group and therefore could be confused with members of at least 10 other genera (93).

There is considerable variation among the human and animal pathogenic corynebacteria in cell wall composition, hemolysis, catalase reaction, sugar metabolism, biochemical characteristics and in pathogenicity (23, 25, 26, 29, 36, 39, 50, 52, 93). It has been recognized for some time that certain Corynebacterium species differ markedly from the type species, C. diphtheriae, and as such belong in different genera (25, 26, 33, 47, 93). C. diphtheriae and related animal pathogens are characterized by the presence mainly of the sugars arabinose and galactose and the diamino acid meso-diaminopimelic acid (meso-DAP) in their cell walls (25, 26). However, C. pyogenes, which is one of the most frequently isolated bacterium from pyogenic infections in domestic animals (15), and C. haemolytium, which causes infections in humans and animals, contain the sugars rhamnose and glucose and the diamino acid, lysine (instead of meso-DAP), and share a cell wall polysaccharide antigen with Lancefield group G streptococci (93). While C. diphtheriae and the majority of the human and animal pathogens produce the enzyme catalase, C. pyogenes and C. haemolyticum are generally catalase negative (93). Several numerical taxonomic studies have

shown that C. pyogenes strains represent a well-defined group distinct from the genus Corynebacterium and should be placed in a different genus (40, 48). The metabolism of carbohydrates by C. equi is oxidative and no acids are produced from any carbohydrates (109). A number of studies have shown that C. equi is very closely related to a group of organisms referred to as Mycobacterium rhodochorous (28, 34, 40, 46, 48).

Anaerobic corynebacteria differed from the human and animal pathogens in lacking arabinose in the cell wall, in having L-DAP instead of meso-DAP and in lacking corynemycolic and corynemycolenic acid in cell lipids (93). An analysis of the acid end products from glucose catabolism showed that anaerobic to aerotolerant coryneforms, C. acnes, C. avidum, and C. granulosom, produce acetic and propionic acids as major metabolic end products and were therefore reclassified into the genus Propionibacterium (31, 69, 72, 73). However, at the present time, there is essentially no data on the metabolic end products produced by aerobic and facultative anaerobic human and animal pathogens with the exception of C. diphtheriae (103), which was shown to produce major amounts of acetic, propionic and formic acids and trace amounts of lactate and succinate.

Cytochromes of the a, b, and c-type were first reported in C. diphtheriae by Yaoi and Lamiya (112). The presence of cytochromes in the other human and animal pathogens has not been investigated, however.

The preceding data indicated a number of problems in the taxonomy of human and animal pathogens presently classified in the

genus Corynebacterium. One of the objectives of this investigation was to determine the metabolic end products produced by species of animal pathogenic and parasitic corynebacteria including C. equi, C. renale, C. pseudotuberculosis, C. pyogenes, C. kutscheri, and C. bovis and to: (1) determine the significance, if any, of the metabolic end products in their taxonomy; (2) compare the relatedness of the metabolic end products of these animal pathogens with those produced by anaerobic corynebacteria; and (3) assess the value of the metabolic end products in the rapid presumptive identification of important animal pathogens. A second objective of this investigation was to determine the distribution of cytochromes and the taxonomic significance of this characteristic in the taxonomy of animal pathogenic corynebacteria.

II. LITERATURE REVIEW

2.1. Taxonomy of Corynebacterium Species

Lehmann and Neumann (56) originally established the genus Corynebacterium for a group of mainly animal and human parasitic bacteria which are Gram-positive, club-shaped, predominantly aerobic, non-motile, and non-acid fast rods (56). Later on the word "coryneform" was used to describe any non-sporeforming, Gram-positive rods which have very little in common with C. diphtheriae, the type species of the genus. Examples of such organisms include a number of plant pathogens and others which are non-pathogenic and are widely distributed in nature, as saprophytes in soil, sea and lake water, and milk. Even worse, some of the organisms included in this genus at one time or another could be confused with organisms representing at least 10 other genera. As such there are perhaps few groups of bacteria in which the aberrant types are more numerous and more difficult to separate than in the genus Corynebacterium. Only a brief review of the taxonomic aspects of human and animal pathogenic or parasitic corynebacteria is presented here; taxonomy of plant pathogenic and non-pathogenic corynebacteria is not reviewed.

The doubtful taxonomic position of some of the species in genus Corynebacterium has been pointed out by many investigators. Cummins and Harris (25), and Cummins (26) observed that C. pyogenes

cell walls contain the sugars rhamnose and glucose and lysine whereas C. diphtheriae and related animal pathogens contain the sugars arabinose and galactose and meso-DAP in their cell walls. Furthermore, C. pyogenus shares a common cell wall polysaccharide antigen with Lancefield group G. streptococci (25). Robinson (91) examined the esterase, catalase and peroxidase activity of 24 Corynebacterium species by starch gel electrophoresis. He observed that C. pyogenes produced the enzyme esterase, whereas esterase was absent in other human and animal pathogen (91). Also, C. pyogenes is usually catalase negative while the other human and animal pathogens are catalase positive (92). A number of numerical taxonomic studies have also shown that C. pyogenes is quite distinct from the main group of animal pathogenic corynebacteria (8, 27, 28, 47, 90).

The classification of C. equi as a species of Corynebacterium has also come into question. Since some strains of C. equi are acid-fast in vivo, they were tentatively reassigned to the genus Mycobacterium (46). Gordon (34) pointed out that C. equi has the same morphological and physiological properties as M. rhodochorous. Cummins (26) detected the same major cell wall constituents, arabinose and galactose, meso-DAP and a common cell wall antigen in both C. equi and M. rhodochorous. To add to the confusion, M. rhodochorous is not recognized as a species in the eighth edition of the Bergey's Manual (93) and strains which formerly carried this epithet are treated as members of species within the genus Norcardia. Most recently Jones, based on her very extensive numerical taxonomic study (48), has reported that bacteria named "M. rhodochorous"

together with C. equi and a few other species form a distinct taxon separate from the genera Mycobacterium and Nocardia.

Taxonomy of the anaerobic coryneforms has also presented several problems similar to the aerobic corynebacteria. Douglas and Gunter (31) observed that C. acnes is similar to Propionibacterium in producing propionic acid as a major acid end product of glucose fermentation. Similar findings were reported by Moore and Cato (69), and they formally reassigned C. acnes to the genus Propionibacterium. There is widespread agreement at the present time that anaerobic coryneforms which produce major amounts of propionic and acetic acids from carbohydrate fermentation and have L-DAP, galactose and/or glucose and/or mannose in cell walls should be considered as Propionibacteria. C. acnes, C. avidum, and C. granulosum, which produce acetic and propionic acids as major metabolic end products, are now reclassified into the genus Propionibacterium in the eighth edition of the Bergey's manual. Cherry et al. (71, 72) found that the cellular fatty acid composition of C. acnes was essentially identical to that of Propionibacterium freudenreichii and P. shermanii. The common component was a saturated C_{15} branched-chain fatty acid. Johnson and Cummins (46) studied the DNA homology among the anaerobic coryneforms and classical propionibacteria. They showed a rather low level of homology (10-20%) between the anaerobic coryneforms and classical propionibacteria. A high degree of homology (85-100%) was observed among P. acnes strains in the P. avidum homology group and low levels of homology to strains in the P. granulosum homology group. The classical propionibacteria fell into four homology

groups. The G+C ratio of anaerobic coryneforms was 58 to 64% and that in classical propionibacteria is approximately 65 to 68%.

2.2 Morphological and Biochemical Properties of Selected Corynebacterium Species

C. pyogenes is a small, Gram-positive, pleomorphic, diphtheroid bacillus, frequently assuming an almost coccal form (93, 109). Small (1 mm) and larger (1-2 mm) colony variants are routinely seen on blood agar in 48 hours; on blood agar, the colonies (1-2 mm) are surrounded by a zone of β-hemolysis two to three times the diameter of the colony (93, 109). Soluble hemolysin produced is active against human, guinea pig, horse and rabbit erythrocytes (93) and against sheep and bovine erythrocytes (Reddy, unpublished data). It is aerobic to facultatively anaerobic and the type of metabolism is probably strictly fermentative (93). This organism ferments glucose, maltose and lactose (93) and a number of other sugars and sugar alcohols. Liquefaction of gelatin and serum, and acid, clot and peptonization reactions in litmus milk are characteristic of C. pyogenes. Nitrates are not reduced and indole is not produced. It is oxidase-negative and urease-negative. A large majority of the strains are catalase-negative but some strains are catalase-positive (93). There is little information available on the nutritional requirements of the organism. Growth of all strains improved by blood or serum. Hemin can for the most part replace the requirement for blood or serum (85). Hemin is apparently required for the synthesis of cytochrome b present in this organism (85).

There is considerable enhancement of growth on addition of bicarbonate to the growth medium (87).

C. equi grows well on all ordinary laboratory media, forming large mucoid, creamy white colonies of irregular shape; colonies may occasionally be pinkish in color (93, 109). It is a pleomorphic, Gram-positive rod variable in length between almost coccoid to medium-sized clubbed rods. It has been reported to be partly acid-fast on occasions (93). It is aerobic to facultatively anaerobic and catalase-positive. Carbohydrates are not fermented but oxidized by C. equi (109). It reduces nitrate, and is gelatin and urease-negative. It grows well at 37C as well as at 20C. This organism, unlike other animal pathogenic corynebacteria, is unusually resistant to 2.5% oxalic and 15% sulfuric acids. It is similar to C. diphtheriae and related animal pathogens in its cell wall composition (26).

C. renale is a relatively large, Gram-positive, catalase-positive organism with slightly pointed ends. It shows metachromatic granules and considerable pleomorphism (93, 109). This organism is distinct from other animal pathogens in having pili or fimbriae (15). The individual colonies are circular, punctiform, and approximately 1-5 mm in diameter (93) on nutrient or blood agar; colonies on milk agar are surrounded by a halo or a zone of clearing at the end of growing. Alkaline change in the litmus milk with peptonization is a characteristic feature of this organism (22, 93). It is strongly urease-positive. No hemolysis is seen on blood agar. C. renale is aerobic to facultatively anaerobic and ferments glucose with the

production of acid. Its cell wall composition is similar to that of C. diphtheriae and related animal pathogens.

C. pseudotuberculosis grows well on Loeffler's medium, and the colonies are circular, umbonate, opaque and non-hemolytic on blood agar. Growth is improved by the addition of blood or serum (93). It produces a toxic surface lipid analogous to cord factor of M. tuberculosis (93, 109). It ferments glucose, maltose and glycerol, and produces a soluble exotoxin (93, 109), which is similar to the toxin produced by C. diphtheriae (15). C. pseudotuberculosis is catalase-positive, aerobic to facultatively anaerobic and often shows club-shaped swellings and metachromatic granules.

C. bovis is aerobic to facultative, Gram-positive, catalase-positive rod-shaped bacterium, often showing club-shaped ends on nutrient agar plus 0.1% Tween 80; colonies are white to cream colored and circular with regular edges (93, 109). Serum, Tween 80 or egg yolk is required in medium for good growth of the organism (39). Almost all strains of C. bovis ferment glucose, maltose, glycerol and produce lipase and oxidase (93). Smith (98) and Skerman and Jayne-Williams (94) studied the nutrition of this organism and showed that it can grow with ammonium salts as the source of nitrogen, although casein hydrolysate accelerates growth. A number of strains require nicotinic acid for growth and most strains require unsaturated long-chain fatty acids.

C. kutscheri is an irregularly staining, Gram-positive slender rod-shaped organism often with club-shaped and sometimes

pointed ends (93); colonies on nutrient agar are yellowish or greyish white with serrated edges. It is aerobic to facultatively anaerobic and ferments glucose, maltose, fructose and sucrose (93) with the production of acid. It produces no change in litmus milk. The cell wall composition is similar to that in C. diphtheriae and related animal pathogens.

2.3 Disease Aspects of Selected Corynebacteria

2.3.1 C. pyogenes.--It is the most frequently encountered pus-forming organism in cattle, sheep and swine (18). It presumably occurs as a part of the normal flora on mucous surfaces of warm-blooded animals (15). It has been isolated from the tonsils of apparently healthy cattle and sheep. It is, therefore, believed that infections by C. pyogenes are primarily of endogenous origin, some accessory factor being needed for the initiation of the pathological process.

C. pyogenes produces a hemolytic exotoxin which is lethal when injected intravenously into rabbits and mice and dermonecrotic for guinea pigs and rabbits. This exotoxin does not appear to be related to that of C. pseudotuberculosis and C. diphtheriae (15). Strains of C. pyogenes tend to lose their ability to produce exotoxin rapidly and irreversibly on subculturing in the laboratory.

This organism is almost always found in necrotic and suppurative pneumonias in cattle, presumably as a secondary invader in most cases (15). It is frequently isolated from cases of purulent

metritis in cattle usually in association with staphylococci (15). In the U.S.A., the organism has been known to cause chronic abscessing mastitis mainly in lactating cows (7); secondary infection frequently results in suppurative arthritis which is usually fatal. In contrast, the same organism causes "summer mastitis" in dry cows during summer months in many parts of Europe (7). The organism causes umbilical infections and purulent polyarthritis in calves which usually lead to death (15). It is often isolated from cases of liver abscess in cattle, often in association with Fusobacterium necrophorum (89, 90).

C. pyogenes is one of the most common causes of mastitis in goats (15, 18). It is often isolated from cases of purulent pneumonia and joint infections in sheep, referred to abroad as "pyobacillosis" (15). Lesions in swine by this organism are very similar to those which occur in cattle. It is commonly encountered in suppurative pneumonia, joint infections and in most other suppurative conditions. C. pyogenes has been reported from cases of pyemia, gangrenous infections and abscesses in humans (20); metritis in horses (15); pyometra in dogs (15); traumatic reticulitis in buffalo (15); septicemias in birds (15); and pneumonias in cattle (15, 18).

2.3.2 Corynebacterium equi.--This organism was first described by Magnusson (61, 62) in Sweden from cases of purulent pneumonias in foals, often associated with pyemia. The pathogenicity of the organism is believed to be due, primarily, to its large

capsule which renders it resistant to phagocytosis and inhibits antibody formation by the infected host (15). The capsule is type-specific and two main antigenic types are recognized (14). It appears to be an important causal agent of severe and usually fatal suppurative pneumonias in foals normally between four weeks and two months of age (15). In these cases, thoracic lymph nodes, but not lymph nodes of the head, are greatly enlarged and suppurative. Foals could be infected by feeding or nasal instillation of virulent cultures. The primary source of infection is believed to be soil. Although in many cases the disease affects one or two foals in a herd, outbreaks of C. equi pneumonia in certain herds have been reported from Australia (15). The organism has been isolated from the uterine discharge of a number of mares (15).

In swine the infection occurs in cervical and submaxillary lymph nodes (15). In one study, the organism was isolated in as many normal as diseased lymph nodes of swine, suggesting that it is relatively low in its pathogenicity (15).

C. equi has been isolated from a case of chronic purulent pneumonia in a sheep in Australia (15), a caribou in India (15), and in a number of cases of pneumonia in humans (93).

2.3.3 Corynebacterium renale.--C. renale produces diphtheritic inflammation of bladder, ureters and in kidneys, principally in cattle (15, 18). It has been suggested that the predilection of the organism for tissues with high urea content may be associated with its potent urease activity. Accumulation of ammonia in the kidney

medulla has been noted during pyelonephritis (11). The natural habitat of C. renale appears to be the vaginal tract and penile urethra and prepuce of cattle and probably other ruminants (10) and most infections appear to be of endogenous origin. Evidence also indicates that in infected herds the bacterium is spread from animal to animal by contamination of the urogenital orifices with urine from diseased or carrier animals (15). Infection is generally thought to be of the ascending type originating in vagina or urethra although hematogenous origin of the infection can not be totally ruled out (15). The fact that the disease is much more common in cows than in bulls is in accordance with the concept that infection is of the ascending type because the shorter and wider urethra of the cow favors easier access of the infecting organism to bladder, ureters and kidneys (11). Pili in this organism are believed to play an important role in pathogenicity (111).

2.3.4 Corynebacterium pseudotuberculosis.--The organism is the primary cause of caseous lymphadenitis in sheep, of ulcerative lymphangitis in horses (associated with habronemiasis), of suppurative skin lesions in cattle, and of purulent arthritis in lambs (15). The modes in which infections with this organism are spread probably are by contact and skin shearing (15). In ulcerative lymphangitis in horses the nodules appear on the legs at first and then these break down to form ulcers, which exude a thick, greenish pus usually mixed with blood (15). In caseous lymphadenitis in sheep the affected lymph nodes are converted into large encapsulated

abscesses (15). The disease conditions caused by C. pseudotuberculosis rarely occur in cattle. However, it has been reported in a number of cases of tuberculosis like skin lesions in cattle in U.S.A. (27). In 1966 Lopez et al. (60) reported the first case of C. pseudotuberculosis in man. The clinical symptoms consisted of fatigue, muscular aches, tenderness and enlargement of the liver.

2.4 Metabolic End Products

Gas chromatography has been very useful in analyzing metabolic end products and in studying the fatty acid composition in bacteria (1, 2, 13, 41, 42, 64, 68, 70, 71, 75, 102). Holdeman and Moore (42) conclusively demonstrated that acid metabolic end products, as determined by gas chromatography, are very useful in the differentiation of different anaerobes. Suto et al. (102) employing gas chromatographic techniques showed that strains of C. acnes produce major quantities of propionate, acetate and succinate. Moore and Cato (69) showed that C. acnes is similar to propionibacteria in producing major amounts of propionic and acetic acids, as originally reported by Douglas and Gunter (31). Moss et al. (71, 72, 73) studied the fatty acid composition of C. acnes and several Propionibacterium species by gas chromatography. They observed that fatty acid profiles of C. acnes were similar to those of P. freudenreichii and P. shermanii (71, 72, 73). There is general agreement now that most anaerobic corynebacteria examined to date produce major amounts of propionic and acetic acids and should therefore be classified as propionibacteria (31, 69, 73).

Tasman and Branwijk (103) studied the glucose metabolism of C. diphtheriae. They suggested that glucose was metabolized to lactic acid, acetic acid, propionic acid, formic acid and carbon dioxide. It was different from obligate anaerobic P. acnes and other typical propionibacteria in this respect.

2.5 Bacterial Cytochromes

Cytochromes are heme proteins and they are composed of an iron-porphyrin prosthetic group attached by one or more covalent or noncovalent linkages to the protein part of the molecule. Cytochromes undergo reversible oxidation and reduction reactions and act sequentially in transferring electrons from flavo protein-quinone level to O_2, NO_3, SO_4 or some other electron acceptor. This passage of electrons through the electron transport chain permits a stepwise release of energy, part of which is converted into the phosphate band energy of ATP (51, 57). There have been several excellent reviews on bacterial cytochromes in the past several years (4, 74) and only a very brief review is given here.

In general, four major cytochrome classes are distinguished (4). In cytochrome c the heme group is covalently bound to the peptide chain (4). In the other cytochromes the heme group is more loosely bound. The b type cytochrome, for example, contains protoheme which is readily extractable with acid-acetone (104); cytochrome a contains heme a (4), and cytochrome d contains the iron-chlorin prosthetic group (4). The absorption maxima for each class of heme proteins is given in Table 1. There are also many subclasses of

Table 1.--Absorption spectra of cytochromes.

	cytochrome a	cytochrome b	cytochrome c	cytochrome d
absorption maxima in reduced form, nm				
α	600	563	550	620-630
β	-	532	521	-
γ	439	429	415	460

Source: Biochemistry, A. L. Lehninger, 1975. Worth Publishers, Inc. New York, N.Y.

each major group of cytochrome (4). Smith (96) pointed out that most of the obligate aerobic bacteria have relatively high concentration of cytochromes. Cytochromes have also been described from a number of anaerobic bacteria (38, 74, 83, 100). Electron-transport associated phosphorylation has been demonstrated in the obligate anaerobe Desulfovibrio gigas (82) as well as in Vibrio succinogenes (43, 44). A b-type cytochrome has also been observed in Bacteroides fragilis, an obligate anaerobe (86) and the cytochrome appears to mediate the transfer of electrons from NADH or H_2 to fumarate (86). Sperry and Wilkins (100) reported recently that the obligate anaerobe Eubacterium lentum contains cytochromes a, b and c and a carbon monoxide-binding pigment. The clostridia have long been regarded as not containing cytochromes; however, recently Gottwald and Andreesen (38) reported that there is a b-type cytochrome and a quinone present in C. formicoaceticum and C. thermoaceticum.

Pappenheimer et al. (80) indicated that C. diphtheriae has exceptionally high succinic dehydrogenase activity, which parallels its high cytochrome content. Both succinic dehydrogenase activity and cytochrome b content are directly proportional to the iron content of the cells. Ramlinson and Hale (84) reported that cells of C. diphtheriae grown in high iron medium did not excrete toxin or coproporphyrin, and showed a five to ten fold increase of heme components over the level associated with maximal excretion of toxin and porphyrin. Scholes and King (96) studied a slow growing, toxin-producing strain of C. diphtheriae, CN2000, which contains

a, b and c type cytochromes. The b type cytochrome, which was reduced by sodium dithionite, was not reduced by substrates. However, the b type cytochrome was reduced rapidly by substrates after the addition of menaquinone, suggesting that this strain is deficient in quinone. A slow growing mutant strain of PW 8 found by Pappenheimer et al. (81) was shown to lack a factor mediating the reduction of cytochrome C_{552} by b_{564}. Krogstad and Howland (54) suggested that the lack of a non-heme electron carrier between cytochrome b and c in the parental strain could have caused a block in menaquinone synthesis. In contrast to our knowledge about the cytochromes in C. diphtheriae, nothing is known about the presence of cytochromes in animal pathogenic corynebacteria except for one recent report which presented evidence for the presence of a b type cytochrome in C. pyogenes (85).

P. freudenreichii, P. rubrum, P. shermanii and P. pentosaceum were shown to contain cytochrome b, cytochrome a or a_1, cytochrome a_2 and a carbon-monoxide-binding pigment after anaerobic growth. Cytochromes function in the transfer of electrons from reduced pyridine nucleotides to fumarate to form succinate (30). The fermentation did not occur in the presence of air. Some other obligate coryneform bacteria such as P. acnes, P. avidum and P. granulosum showed a fermentation pattern similar to that mentioned above for the classical propionibacteria (71, 72, 73).

Goodfellow et al. (23) studied the distribution of isoprenoid quinone in Actinomyces and corynebacteria. They reported

that there was only one predominant menaquinine, which had eight isoprenoid units with one double bond hydrogenated, found in the majority of corynebacteria strains and representative of "rhodochorous" complex. However, C. bovis and C. glutamicum strains had menaquinine which contained nine isoprenoid units with one double bond hydrogenated, as the main menaquinine component found in Gordona, Mycobacterium and Nocardia.

III. METHODS AND MATERIALS

3.1 Bacterial Strains

The strains of corynebacteria selected for this study and their sources are given below.

C. bovis (BT 435-76), C. renale (CE 565-73), C. pseudotuberculosis (MM 120-71), C. kutscheri (MA 76-74), C. equi (EC 474-73, CE 222-70) and C. pyogenes (SS 7-74, BT 400-76, BT 343-76, BT 337-76, BT 331-76) were obtained from Dr. G. R. Carter, Diagnostic Microbiology Laboratory, Michigan State University.

C. pyogenes strain 5, C. diphtheriae strains 1, 2 and 3 were from the culture collection of the Department of Microbiology and Public Health, Michigan State University.

C. bovis (7715), C. renale (19412), C. pseudotuberculosis (19410), C. kutscheri (15677) and C. equi (10146, 7699, 6939, 7698) were obtained from the American Type Culture Collection, Rockville, Maryland.

Lactobacillus bulgaricus (3533) was obtained from the Institue for Fermentation Research, Osaka, Japan.

3.2 Culture and Preparation of Inocula

All cultures were routinely maintained on Bacto stock culture agar (SCA) which consisted of 500 g beef heart infusion, 10 g gelatin, 5 g isoelectric casein, 0.5 g dextrose, 4 g disodium

phosphate, 3 g sodium citrate and 7.5 g agar per liter. All media were sterilized by autoclaving at 115 C for 15 min. Slants of sterile SCA were inoculated with a loopful of lyophil material resuspended in 0.5 ml of Bacto Brain Heart Infusion (BHI) broth, incubated at 37 C for 24-48 h and stored at 4 C. Subcultures were made once every month to slants of SCA.

Inocula for various biochemical and physiological studies were prepared as follows. Stock cultures were transferred with a sterile loop to 3 ml of BHI broth contained in 12x75 mm foam-plugged tubes, incubated at 37 C for 24-48 h. A drop of this culture was then transferred to a fresh BHI broth and incubated as above. After two further transfers, these cultures then served as inocula for various studies. All solid and liquid biochemical media were inoculated with a loopful or a drop of these BHI broth cultures, respectively.

3.3 Biochemical Tests

A drop of actively growing broth cultures prepared as above was used to inoculate 3 ml of the differential biochemical test media. The sugars tested included glucose, maltose, lactose and sucrose, and these carbohydrate fermentation media were prepared by adding enough 10% filter sterilized sugar solution to sterile Trypticase Soy Broth (BBL) to give a final carbohydrate concentration of 1% in the medium. The carbohydrate media were aseptically dispensed in 3 ml amounts into 12x75 mm sterile foam-plugged test tubes containing a Durham tube, and phenol red (pH 6.8-8.4 red) was

used as the indicator. Inoculated tubes were incubated at 37 C for 48 h or longer as needed. Positive sugar fermentation was scored when the phenol red indicator turned yellow due to acid production. Positive gas production during fermentation of the sugars was scored when gas bubbles appeared in the Durham tube.

All biochemical tests were performed as per procedures described by Bailey and Scott (3). Unless otherwise mentioned, all media were dispensed in 5 ml amounts in 15x125 mm tubes. Unless otherwise mentioned, all inoculated test media were incubated at 37 C for 48 h. Esculin agar slants were prepared with 0.1% aesculin and 0.05% Ferric citrate in BHI agar (3). Formation of dark color is indicative of a positive test. For testing gelatin liquefaction, a medium containing 15% gelatin in Bacto BHI broth (3) was stabbed with a loopful of broth inoculum. After incubation, the gelatin cultures were refrigerated along with a control uninoculated gelatin tube. The uninoculated gelatin control solidified after refrigeration. Failure of the gelatin cultures to solidify was regarded as a positive test for hydrolysis of gelatin.

Litmus milk medium (3) contained 10 g skim milk powder and 0.5 gm litmus in 100 ml of distilled water. The results were read at the end of 4-8 days of incubation at 37 C for different cultures. The formation of a pink color indicated an acid reaction caused by the fermentation of lactose. A purple or blue (alkaline) color indicated no fermentation of lactose. Coagulation (clot) was indicated by the curdling of the milk. Peptonization was indicated

by digestion of the curd or milk protein by proteolytic enzymes giving a watery consistency to the medium.

Tryptone broth (3) dispensed in 3 ml amounts in 12x75 mm tubes was used to detect indole production. The presence of indole was detected by the appearance of a deep red color on adding 5 drops of Kovacs indole reagent (3) to the tube.

Nitrate test medium contained 0.5% tryptone, 0.5% neopeptone, 0.1% potassium nitrate and 0.1% glucose in 1000 ml distilled water (3), and was dispensed in 3 ml amounts in 12x75 mm tubes. Nitrate production was tested by adding the conventional nitrite reagents (3). Development of a red color was considered as a positive test. If no color, zinc dust was added and if a red color developed the test was considered negative and if no color developed after the addition of zinc dust, the test was read as positive.

Christensen urea agar (3) slant was used for the urease test. One loopful of culture was streaked on the slope of the slant medium. Urease-positive cultures turn the medium pink due to the production of ammonia.

The appearance of a dark purple color in 5 to 10 sec after adding a drop of oxidase reagent (53) to 24-48 h old Bacto BHI agar slant culture was read as positive oxidase test.

Rapid evolution of gas bubbles on addition of a few drops of 3% H_2O_2 to a 24-48 h BHI broth culture was read as positive catalase test.

BHI agar supplemented with 5% sheep blood was used for testing hemolysis by different corynebacteria.

3.4 Gas Chromatographic Analysis of Acid End Products

All strains of bacteria were grown in 10 ml of Bacto BHI broth supplemented with 0.3% dextrose contained in 18x150 mm foam-plugged tubes and incubated at 37 C for 72 h at which time all strains attained maximum growth in the medium used. The optical densities of the cultures were read at 600 nm using a Bausch & Lomb Spectronic 20 colorimeter. The purity of each strain was checked by examination of Gram-stained smears, examination of wet mounts under a phase contrast microscope and streaking a blood agar plate and looking for characteristic colony morphology (93, 109). The samples were then acidified to pH 2 or below by using about 0.1 ml of 50% aqueous H_2SO_4 (v/v) per 6 ml culture. The volatile fatty acid products were extracted from 2 ml of acidified culture with 1 ml ethyl ether as per procedures described by Holdeman and Moore (42). Non-volatile fatty acids were methylated by treating 1 ml of acidified culture with 2 ml methanol and 0.4 ml of 50% H_2SO_4 followed by heating at 55 C for 30 min and the methylated acids were extracted with 0.5 ml chloroform (42). The volatile and non-volatile fatty acid products were analyzed by a Dohrmann Gas Chromatograph employing a Resoflex column. The flow rate of Helium, the carrier gas, was 120 c.c./min. The column and thermal conductivity detector were maintained at approximately 118-120 C, while the injection port was maintained at 145 C.

3.5 Cytochrome Measurement

All corynebacteria, except C. pyogenes, were grown in 500 ml of Bacto BHI broth supplemented with 0.2% dextrose and contained in one-liter foam-plugged Erlenmeyer flasks at room temperature. C. pyogenes was grown at 37 C, in Bacto BHI broth supplemented with 0.2% dextrose and 0.0002% hemin. About 3 ml of 48 h old broth cultures grown in the respective media served as inoculum. Bacteria in the early stationary phase of growth were harvested by centrifugation in a Sorvall Centrifuge at 15,000x g for 15 min at 4 C and were washed three times with half-volumes of 0.02 M phosphate buffer (pH 7.0). The cells were then resuspended in the buffer solution to make a suspension of approximately 1 g cell mass/ml. The cells were broken in a French Pressure Cell at 15,000 P.S.I. The extracts were recentrifuged at 15,000x g for 10 min at 4 C to remove unbroken cells and large cell fragments. The supernatent is referred to as cell extract.

Difference spectra methods described by Chance (19) were used to examine the cell extract for cytochromes. All analyses were done at room temperature with a Cary 15 scanning split beam recording spectrophotometer using 1.5 ml Quarsil cuvettes with 10 mm light path. About 1.25 ml cell extract was filled into the cuvettes, a few crystals of sodium dithionite were used to reduce the extract in the sample cuvette and air was used to oxidize the extract in the reference cuvette.

3.6 Heme Extraction and Characterization

Procedures of Jacobs and Wolin (43) were used to extract the heme from the cytochromes. Cell extract containing approximately 76 mg of protein was lyophilized at -40 C. The lyophilized cell extract was mixed with 40 ml cold acetone and centrifuged at 15,000x g for 15 min at 4 C. This acetone wash was repeated one more time. For protoheme extraction, the acetone-washed pellet was extracted with acid-acetone (40 ml of cold acetone containing 1% of 2.4 N HCL). The extraction procedure was repeated twice, and the acid-acetone extract was dried under vacuum. The dried residue was then resuspended in 3.5 ml of pyridine and 3.5 ml of 0.2 N KOH to obtain pyridine hemochromogen. It was necessary to centrifuge some preparations at this point to remove the small amount of white precipitate which formed after addition of KOH. The difference spectrum of pyridine hemochromogen was obtained (at wavelengths from 650 nm to 400 nm) as per procedures described above. Sodium dithionite was added to reduce the protoheme.

The heme of cytochrome C, which remains in the residue after acid-acetone extraction, was resuspended in 3.5 ml pyridine and 3.5 ml of 0.2 N KOH and made into a uniform suspension by grinding in a tissue homogenizer. It was then scanned (at wavelengths from 650 nm to 400 nm) as per procedures described above. Sodium dithionite was used to reduce heme.

3.7 Protein Determination

Protein determination in the cell extracts was by the biuret method (24) in the presence of 0.06% sodium deoxycholate. Crystalline bovine serum albumin (Sigma) was used as the standard.

IV. RESULTS

4.1 Biochemical Characteristics

Certain key biochemical characteristics of Corynebacterium species included in this study were determined (Table 2) and were compared with the characteristics listed for these organisms in the eighth edition of the Bergey's Manual (93). These results, with a few exceptions, were similar to those previously reported for the respective species (18, 93).

C. pyogenes colonies were whitish, pin-point, convex and showed a clear zone of β-hemolysis on blood agar after 24-48 h incubation at 37 C. Cells were short, coccoid rods and were non-motile and non-sporing. This organism was catalase-negative, liquified gelatin, and produced acid, clot and peptonization in litmus milk. It was urease- and oxidase-negative. Glucose, maltose and lactose were readily fermented but sucrose fermentation was rather weak. This organism grew to about the same extent aerobically or anaerobically.

The strains of C. equi examined were coccoid rods, clearly capsulated and were catalase- and urease-positive and reduced nitrates. This organism grew well in BHI broth medium supplemented with dextrose but as previously reported (93, 109) no acids were produced from any of the carbohydrates tested, suggesting that this organism carried on oxidative metabolism of carbohydrate.

Table 2.--Selected biochemical characteristics of certain animal pathogenic corynebacteria and Corynebacterium diphtheriae.

Organism	CATALASE	HEMOLYSIS	GLUCOSE	MALTOSE	SUCROSE	LACTOSE	LITMUS MILK	NO_3 REDUCTION	GELATIN	INDOLE	OXIDASE	ESCULIN	UREASE	ANAEROBIC GROWTH
C. pyogenes	-	+	+	+	W	+	ACP	-	+	-	-	-	-	+++
C. equi	+	-	-	-	-	-	-	+	-	-	±	-	+	-
C. renale	+	±	+	-	W	K	-	-	-	-	-	+	+	++
C. bovis	+	-	+	+	+	-	-	-	-	-	-	-	-	++
C. kutscheri	+	-	+	+	+	-	-	+	-	-	-	+	+	++
C. pseudotuberculosis	+	+	+	W	-	-	-	W	-	-	±	-	+	++
C. diphtheriae	+	-	+	W	-	-	-	+	-	-	+	-	-	++

Key: + = positive
- = negative
± = variable

++ = good growth
+++ = excellent growth
K = alkaline
W = weak positive

A = acid
C = clot
P = peptonization

Strains of C. bovis were catalase-positive, fermented glucose, maltose, and sucrose, and did not hydrolyze urea.

The strains of C. renale examined were urease- and catalase-positive, fermented glucose and hydrolyzed esculin. All strains showed alkaline coagulation and peptonization in litmus milk after about eight days of incubation and this is a characteristic feature of this organism. Slight β-hemolysis was noted on sheep-blood agar plates after 24 h incubation at 37 C but this was a variable feature.

Nitrate was reduced and esculin was hydrolyzed by C. kutscheri. It was urease- and catalase-positive, and fermented glucose, maltose and sucrose, but not lactose.

C. pseudotuberculosis strain was catalase- and urease-positive, fermented glucose and produced β-hemolysis on sheep-blood agar plates after 24-48 h incubation.

C. diphtheriae strains typically produced acid from glucose and maltose but not from sucrose. They did not hydrolyze gelatin, hemolyze sheep blood or produce urease.

Animal pathogenic corynebacteria are described to be aerobic, facultatively anaerobic or microaerophilic. Yet no data were available on the ability, if any, of these organisms to grow under strictly anaerobic conditions. Our results showed that strains of all the species examined, with the exception of C. equi strains, were able to grow well in BHI agar stab both aerobically or under anaerobic conditions in Gas Pak anaerobic jar. There was no detectable growth of C. equi under anaerobic conditions.

The corynebacteria examined are readily distinguishable based on their biochemical reactions. All the Corynebacterium species examined in this investigation fermented glucose except C. equi. C. pseudotuberculosis is readily distinguishable from C. diphtheriae by its ability to show β-hemolysis on blood agar and by its ability to produce urease. C. pyogenes was the only organism studied that fermented lactose, liquified gelatin, produced acid, clot and digestion in milk and was catalase-negative. C. renale and C. kutscheri were the only two organisms that hydrolyzed esculin. C. renale is readily distinguishable from the others by the alkaline reaction and peptonization in litmus milk. Indole was not produced by any of the organisms.

4.2 Acid Metabolic End Products

The acid end products of C. diphtheriae and certain animal pathogenic corynebacteria are shown in Table 3 and the GLC profiles of each of the organisms are shown in Figures 1 to 6.

When grown in BHI broth, C. pyogenes appeared similar to lactobacilli (Figure 7) in producing large amounts of lactic acid and minor amounts of acetic acid (Figure 1). In contrast, Reddy and Cornell (85) showed that the same organism produced major amounts of acetic, formic and succinic acids and minor amounts (traces) of pyruvate and lactate in medium #10 of Caldwell and Bryant (16).

C. equi was not known to ferment any carbohydrates (93), and in agreement with this, it produced no detectable acid end products in BHI broth although it grew very well in this medium (see Table 3).

Table 3.--Acid metabolic end products of certain corynebacteria and L. bulgaricus.[a]

Organism	Volatile Acids[b]	Non-volatile Acids	Absorbance[c]
C. pyogenes	A to a	L s	0.8
C. equi	None	None	1.5
C. renale	(a p)	L (py s)	1.5
C. bovis	A	L py s oa	0.8
C. kutscheri	P	L s py oa	0.9
C. pseudotuberculosis	AFP	S py l oa	0.6
C. diphtheriae #1	AFP	l s (oa)	0.7
C. diphtheriae #3	AFP	fu py oa	0.92
C. bulgaricus[d]	A	L	0.83

[a]All organisms were grown in Bacto BHI broth supplemented with 0.3% dextrose and incubated at 37 C.

[b]A = acetate, F = formic acid, L = lactate, OA = oxalactate, P = propionate, PY = pyruvate, S = succinate, FU = fumarate. Upper case letters represent major products (1 m equivalent or greater per 100 ml); lower case letters represent minor products (less than 1 m equivalent per 100 ml). Figures in parentheses are produced by some strains, some times.

[c]Absorbance was measured in 18x150 mm tubes at 600 nm and refer to the maximum growth obtained by each organism in the medium used.

[d]Products of Lactobacillus bulgaricus have been determined and provided as a positive reference and for comparative purposes.

Figure 1.--Gas chromatographic profile of the volatile and non-volatile acid end products produced by C. pyogenes in BHI broth supplemented with 0.2% dextrose medium. Procedures used were those of Holdeman and Moore (42). Symbols used were the same as those in Table 3.

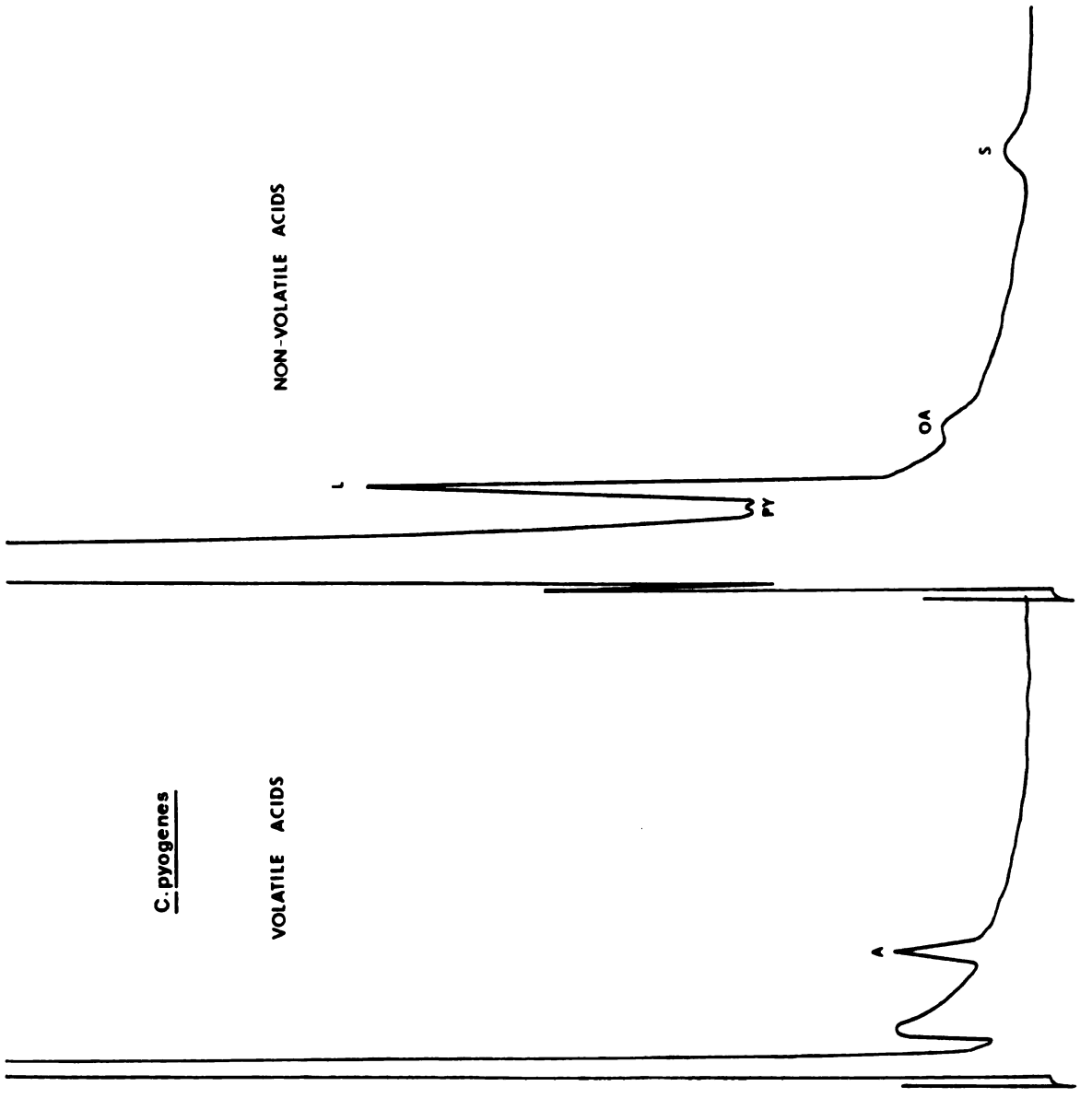

C. pyogenes
VOLATILE ACIDS
NON-VOLATILE ACIDS
A
L
PY
OA
S

Figure 2.--Acid end products produced by C. renale. Experimental procedures were as described in the legend for Figure 1 and the symbols used were the same as those in Table 3.

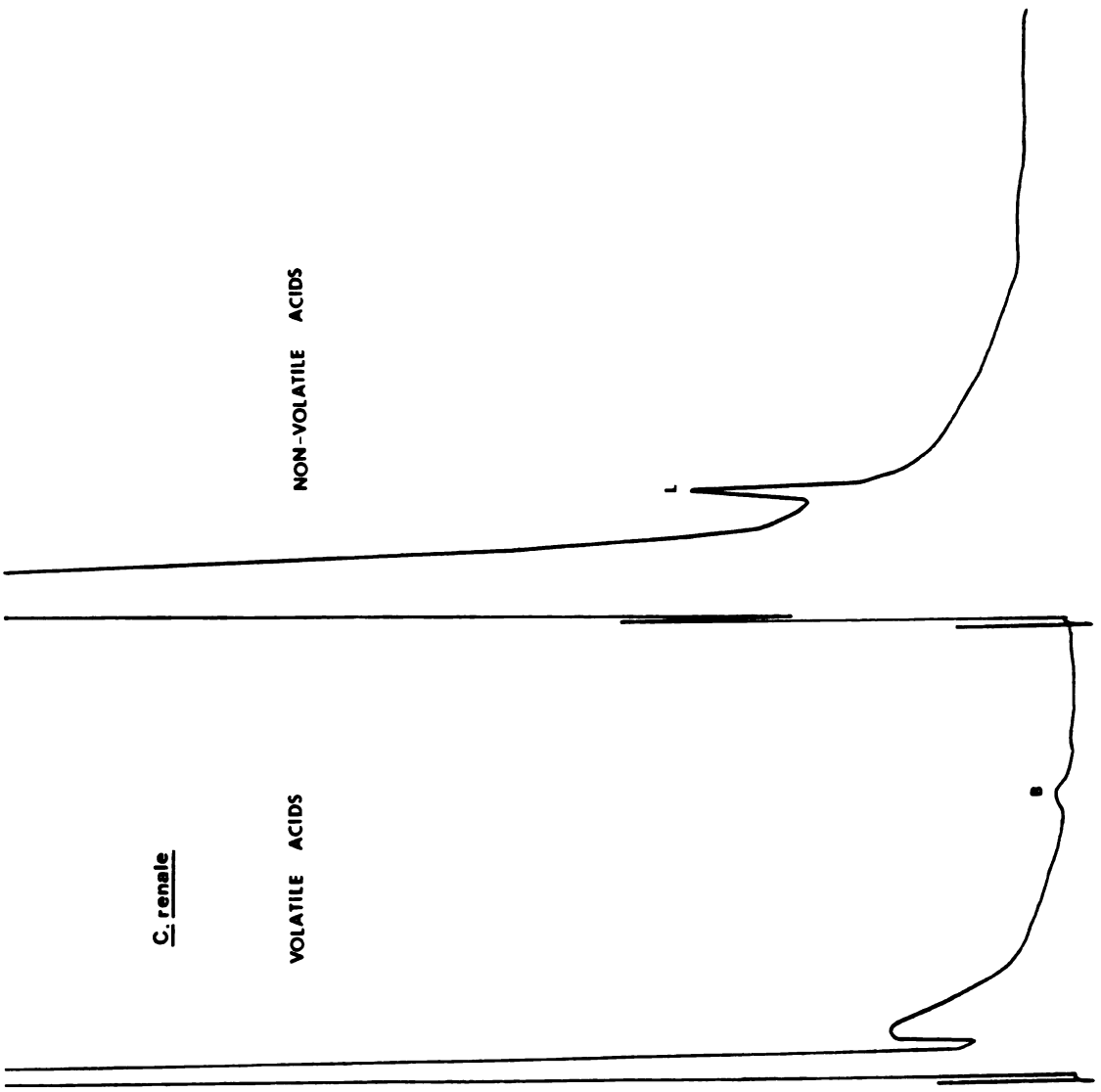

C. renale
VOLATILE ACIDS
NON-VOLATILE ACIDS

Figure 3.--Acid end products produced by C. bovis. Experimental procedures were as described in the legend for Figure 1 and the symbols used were the same as those in Table 3.

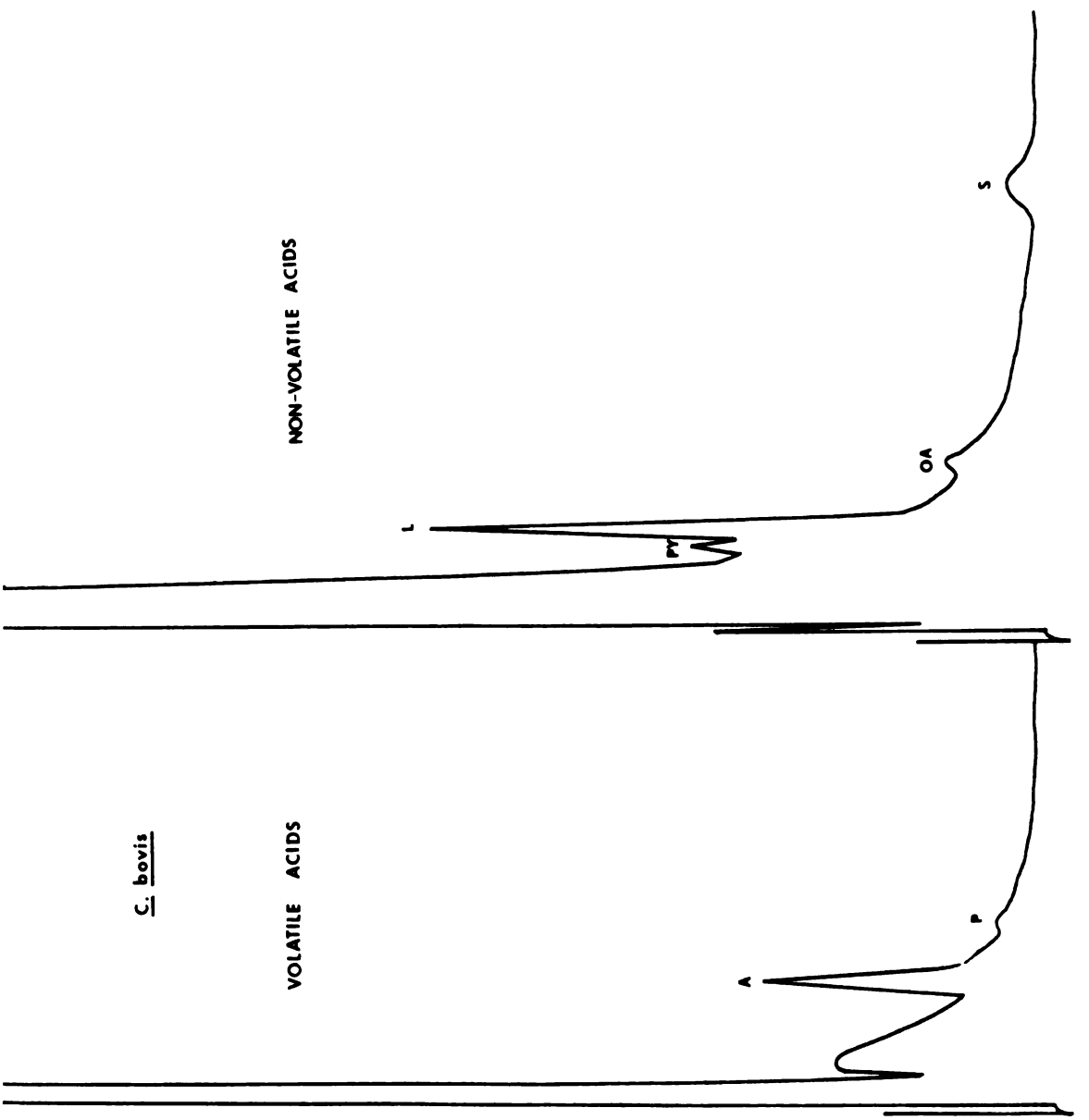

C. bovis
VOLATILE ACIDS
NON-VOLATILE ACIDS
A
P
L
PY
OA
S

Figure 4.--Acid end products produced by C. kutscheri. Experimental procedures were as described in the legend for Figure 1 and the symbols used were the same as those in Table 3.

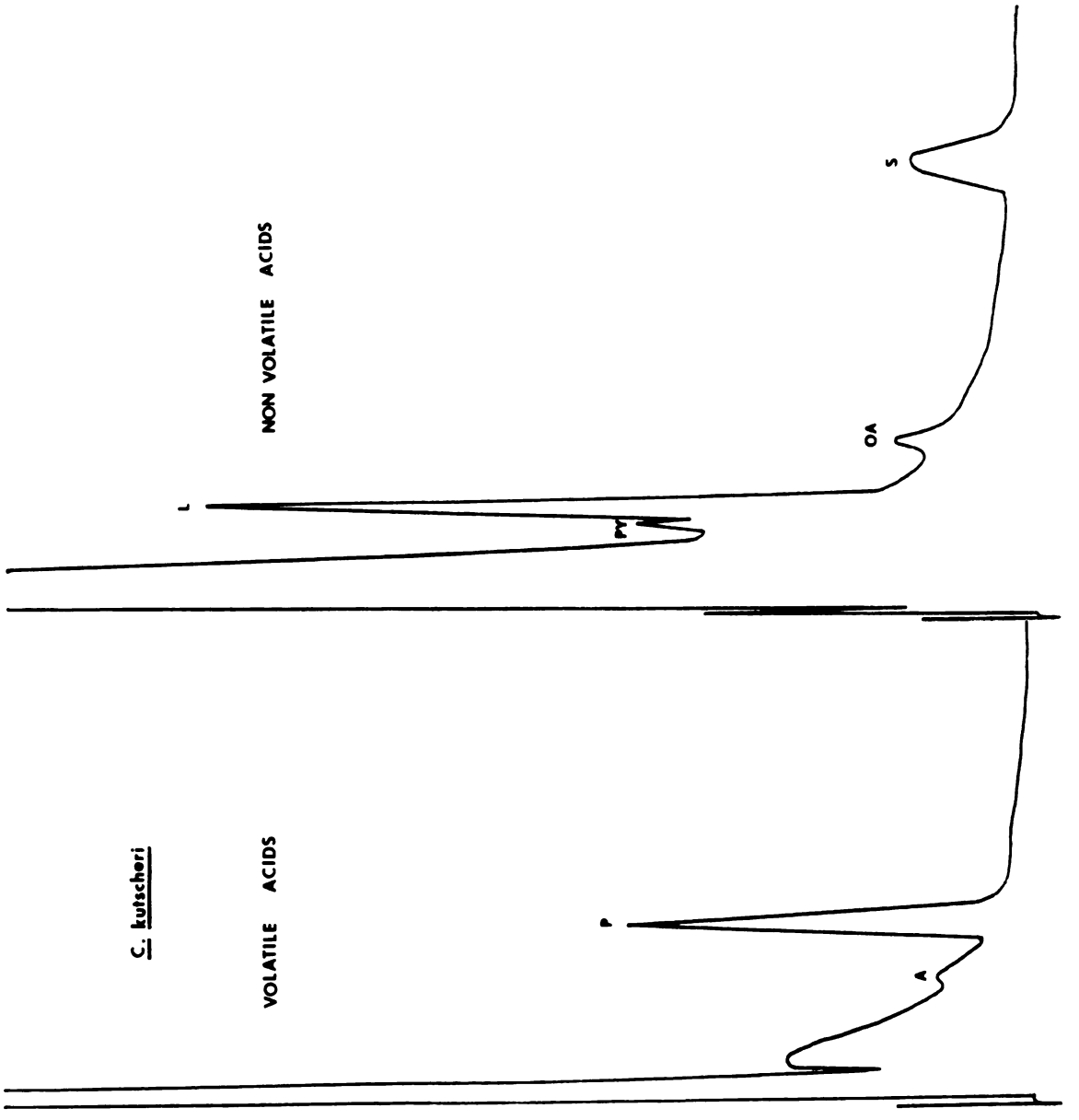

C. kutscheri
VOLATILE ACIDS
NON VOLATILE ACIDS
A
P
L
Py
OA
S

Figure 5.--Acid end products produced by C. pseudotuberculosis. Experimental procedures were as described in the legend for Figure 1 and the symbols used were the same as those in Table 3.

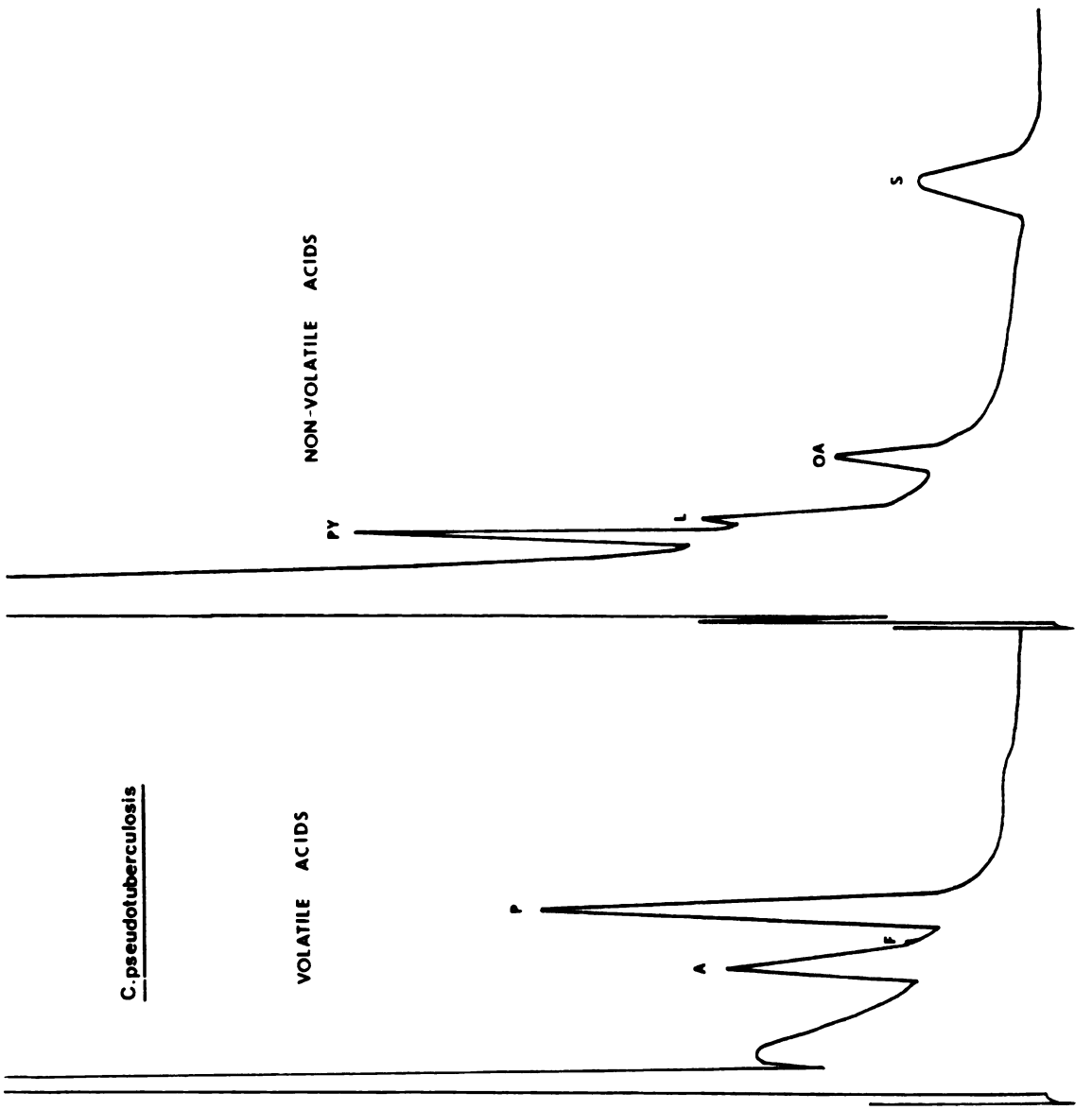

C. pseudotuberculosis
VOLATILE ACIDS
P
A
F
NON-VOLATILE ACIDS
PY
L
OA
S

Figure 6A.--Acid end products produced by C. diphtheriae 1. Experimental procedures were as described in the legend for Figure 1 and the symbols used were the same as those in Table 3.

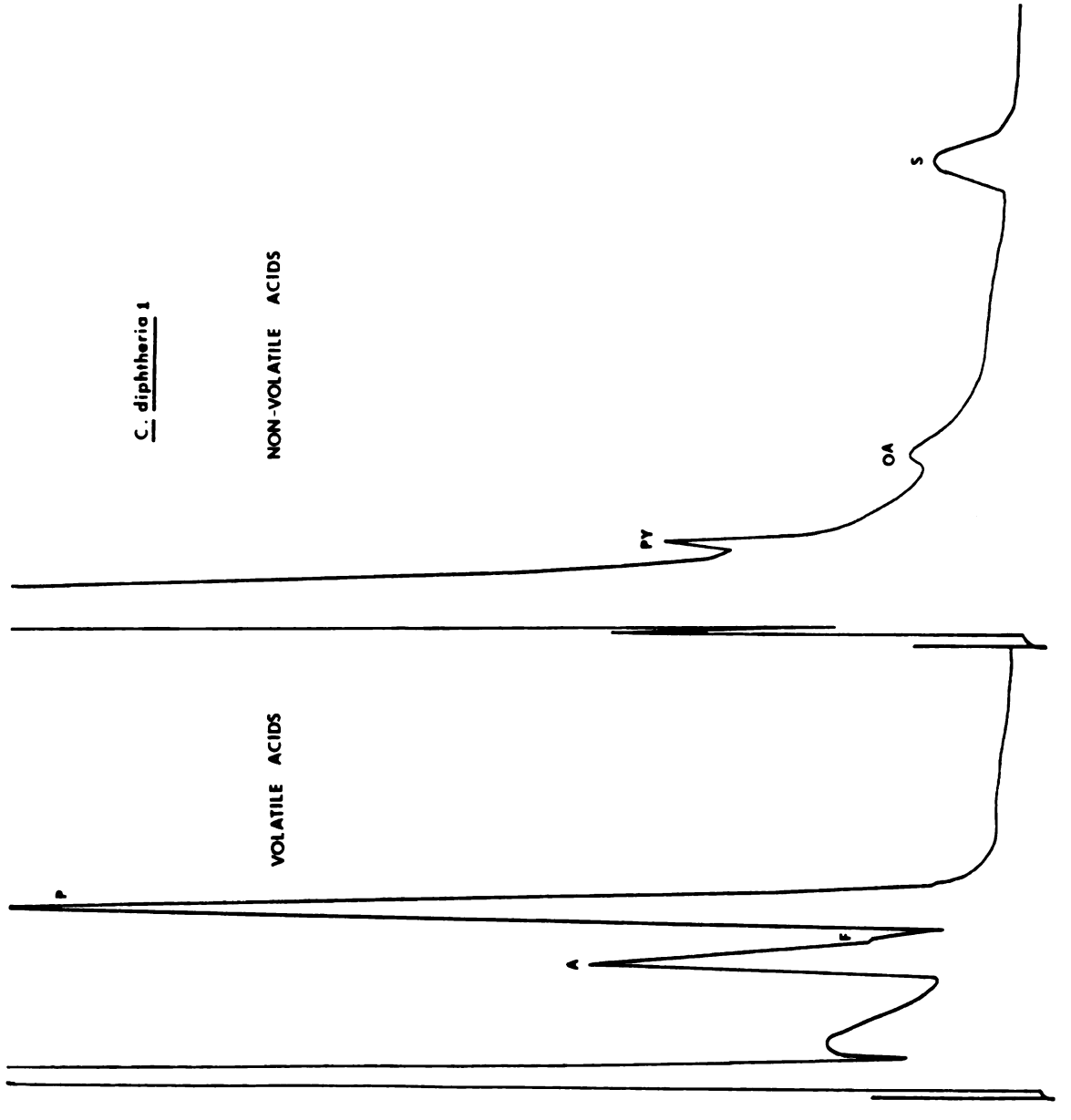

C. diphtheriae 1
NON-VOLATILE ACIDS
VOLATILE ACIDS
P
A
B
PY
OA
S

Figure 6B.--Acid end products produced by C. diphtheriae 3. Experimental procedures were as described in the legend for Figure 1 and the symbols used were the same as those in Table 3.

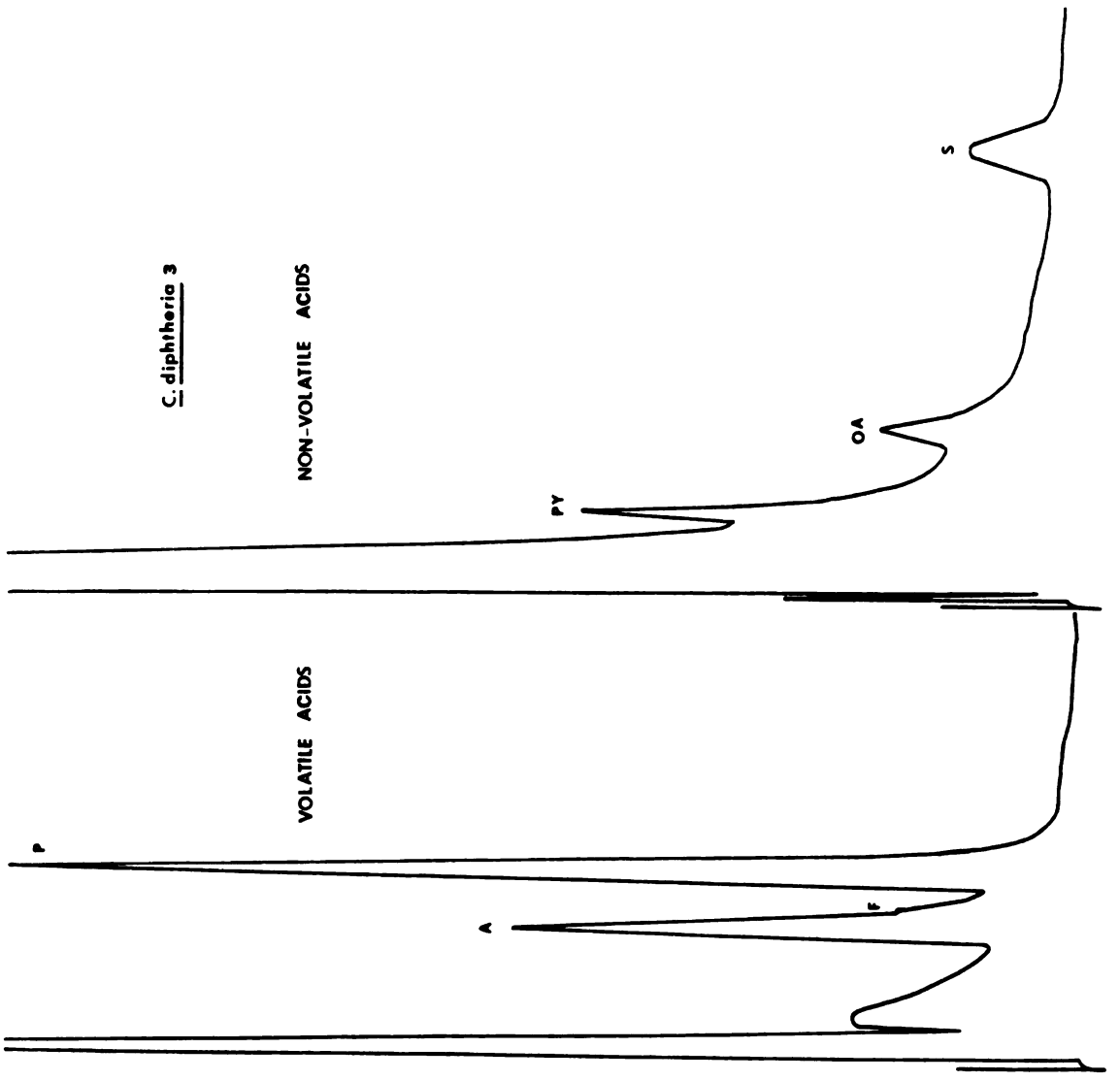
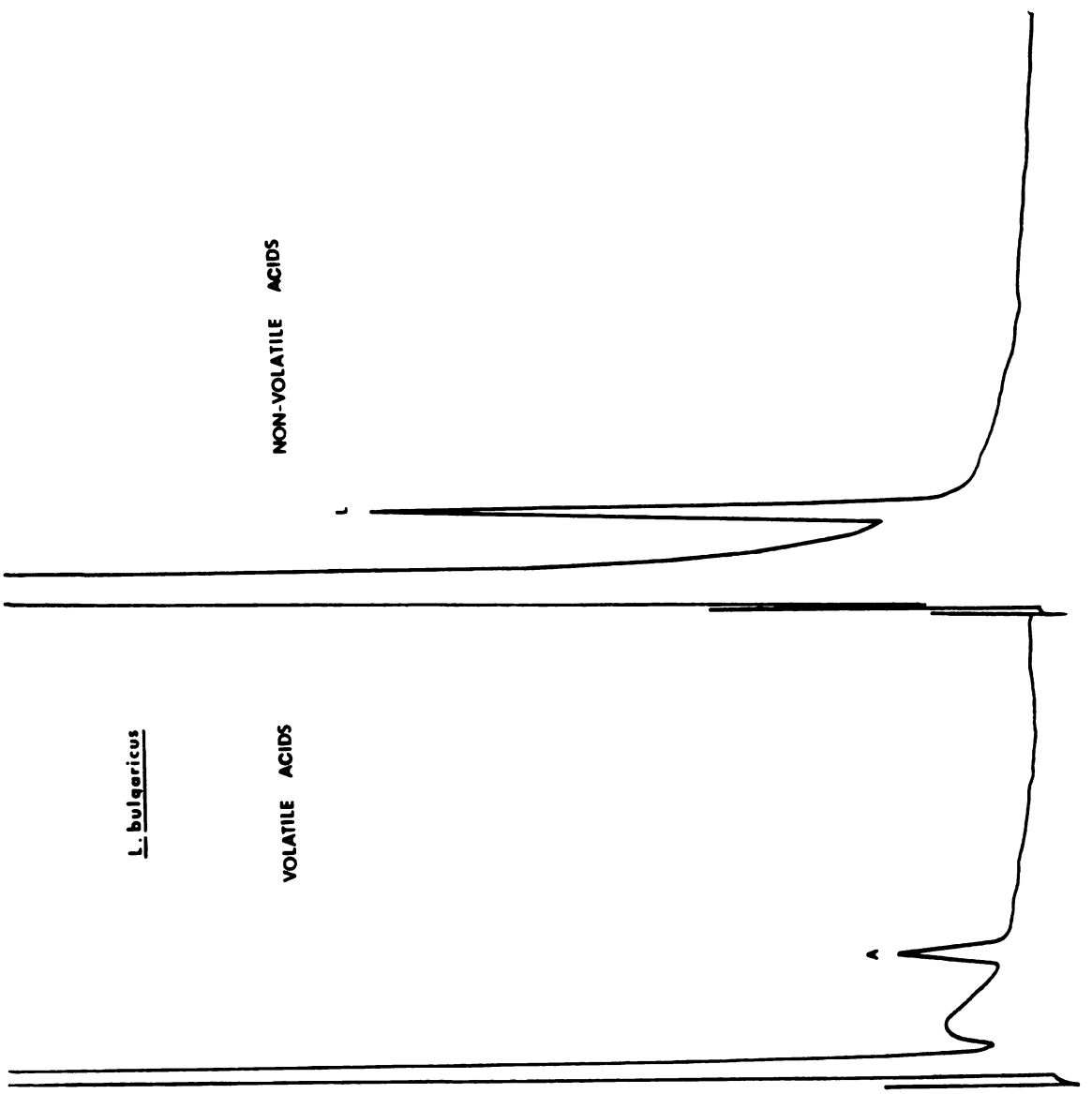

C. diphtheria 3
NON-VOLATILE ACIDS
VOLATILE ACIDS
P
A
F
PY
OA
S

Figure 7.--Acid end products produced by L. bulgaricus. Experimental procedures were as described in the legend for Figure 1 and the symbols used were the same as those in Table 3.


L. bulgaricus
VOLATILE ACIDS
NON-VOLATILE ACIDS
A
L

C. renale appeared similar to homofermentative lactic acid-producing bacteria in producing lactic acid as the major acid end product (Figure 2). A trace amount of propionate detectable was insignificant.

C. bovis was similar to C. pyogenes in producing major amounts of lactic and acetic acids (Figure 3). This organism also produced trace amounts of pyruvate, succinate and oxalactate.

C. kutscheri produced major amounts of lactic and propionic acids, considerable amounts of succinic acid (equivalent to 0.7 m eq of acid per 100 ml) and smaller amounts of pyruvate and oxalacetate (Figure 4).

C. pseudotuberculosis was similar to propionic acid bacteria in producing large amounts of propionic and acetic acids. However, it is different from propionibacteria in producing major amounts of formic acid. It also produced minor amounts of lactic and oxalacetic acids and considerable amounts of succinic and pyruvic acids (0.8 and 0.85 m eq per 100 ml, respectively).

Both strains of C. diphtheriae examined were similar to propionic acid bacteria in producing large amounts of propionic and acetic acids. In addition, one strain of C. diphtheriae produced major amounts of formic and moderate amounts of succinic acids and a small amount of pyruvic acid and a trace of oxalacetate (Figure 6A). A second strain of C. diphtheriae, in contrast, produced major amounts of formic and moderate amounts of pyruvic, oxalacetic and fumaric acids (Figure 6B), suggesting a metabolic lesion in succinic acid

production. The metabolic features of strains of C. diphtheriae appear very similar to those of C. pseudotuberculosis.

4.3 Cytochrome Study and Heme Characterization

The distribution of cytochromes and the characteristics of their heme derivatives in selected corynebacteria are shown in Table 4, and Figures 8 to 22. The difference spectrum of cell extracts of C. pyogenes showed absorption maxima for α, β, γ bands at 560, 530, and 428 nm (Figure 8) characteristic of a b-type cytochrome. The difference spectrum of the pyridine hemochromogen of the protoheme extracted from this cytochrome had absorption maxima at 558, 525, 423 nm (Figure 9) and this confirmed that it is a b-type cytochrome.

A b-type cytochrome detected in C. equi had absorption maxima at 562 and 425 nm for α and γ peaks, respectively (Figure 10). The absorption maxima of the pyridine hemochromogen for this cytochrome were at 556, 525 and 418 nm (Figure 11). C. equi in addition contained a c-type cytochrome which had α, β and γ absorption maxima at 555, 520 and 415 nm (Figure 10). The pyridine hemochromogen of the residue left after acid-acetone extraction of cell extract had absorption maxima at 550, 520 and 414 nm (Figure 12), indicative of the presence of a c-type cytochrome in C. equi.

C. renale contained a b-type cytochrome as revealed by the absorption maxima at 558, 520 and 423 nm (Figure 13) shown by a dithionite-reduced versus air-oxidized difference spectrum of the

Table 4.--Distribution of cytochromes in certain animal pathogenic corynebacteria and in *Corynebacterium diphtheriae*.[a]

Organism	Type of Cytochrome	Cytochrome Absorption Maxima (nm)			Type of Heme	Heme Absorption Maxima (nm)		
C. pyogenes	b	560	530	428	protoheme	558	525	423
C. equi	b	562	-	425	protoheme	556	525	418
	&							
	c	555	520	415	mesoheme	550	520	414
C. renale	b	558	520	423	protoheme	556	523	420
C. bovis	b	566	530	435	protoheme	556	526	424
C. kutscheri	b	560	528	432	protoheme	556	526	424
C. pseudotuberculosis	b	560	530	428	protoheme	556	523	418
C. diphtheriae, 1	b	558	525	425	protoheme	556	523	420
C. diphtheriae, 2	b	558	525	425	protoheme	556	523	418

[a]See materials and methods for experimental details.

cell extract. The absorption peaks of the pyridine hemochromogen of the extracted heme as expected were observed at 556, 523 and 420 nm (Figure 14), confirming the presence of a b-type cytochrome.

C. bovis and C. kutscheri contained a b-type cytochrome and the difference spectrum of the reduced protein in cell extracts had absorption maxima at 566, 530 and 435 nm and at 560, 528 and 432 nm, respectively (Figures 15 and 17). The pyridine hemochromogen derivative of the protoheme extracted from these two organisms had identical absorption maxima at 556, 526 and 424 nm (Figures 16 and 18).

The b-type cytochrome in cell extracts of C. pseudotuberculosis had α, β and γ absorption maxima at 560, 530 and 428 nm, respectively (Figure 19). The absorption maxima of the pyridine hemochromogen derivative of the protoheme were at 556, 523 and 418 nm (Figure 20).

The two C. diphtheriae strains used in this investigation were toxin producers. Dithionite-reduced versus air-oxidized difference spectrum had 525 and 425 nm (Figures 21A and 21B) and the corresponding pyridine hemochromogen had absorption maxima at 556, 523 and 420 (418) nm (Figures 22A and 22B). My results agreed with those of previous workers in showing that the toxin-producing strains of C. diphtheriae had only the b-type cytochrome (81).

Figure 8.--Difference spectra of a cell extract (10.4 mg of protein per ml) of C. pyogenes. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. 1.25 ml cell free extract was in each cuvette with 10 mm light path. All spectra were taken at room temperature in the wavelength range 650 to 400 nm.

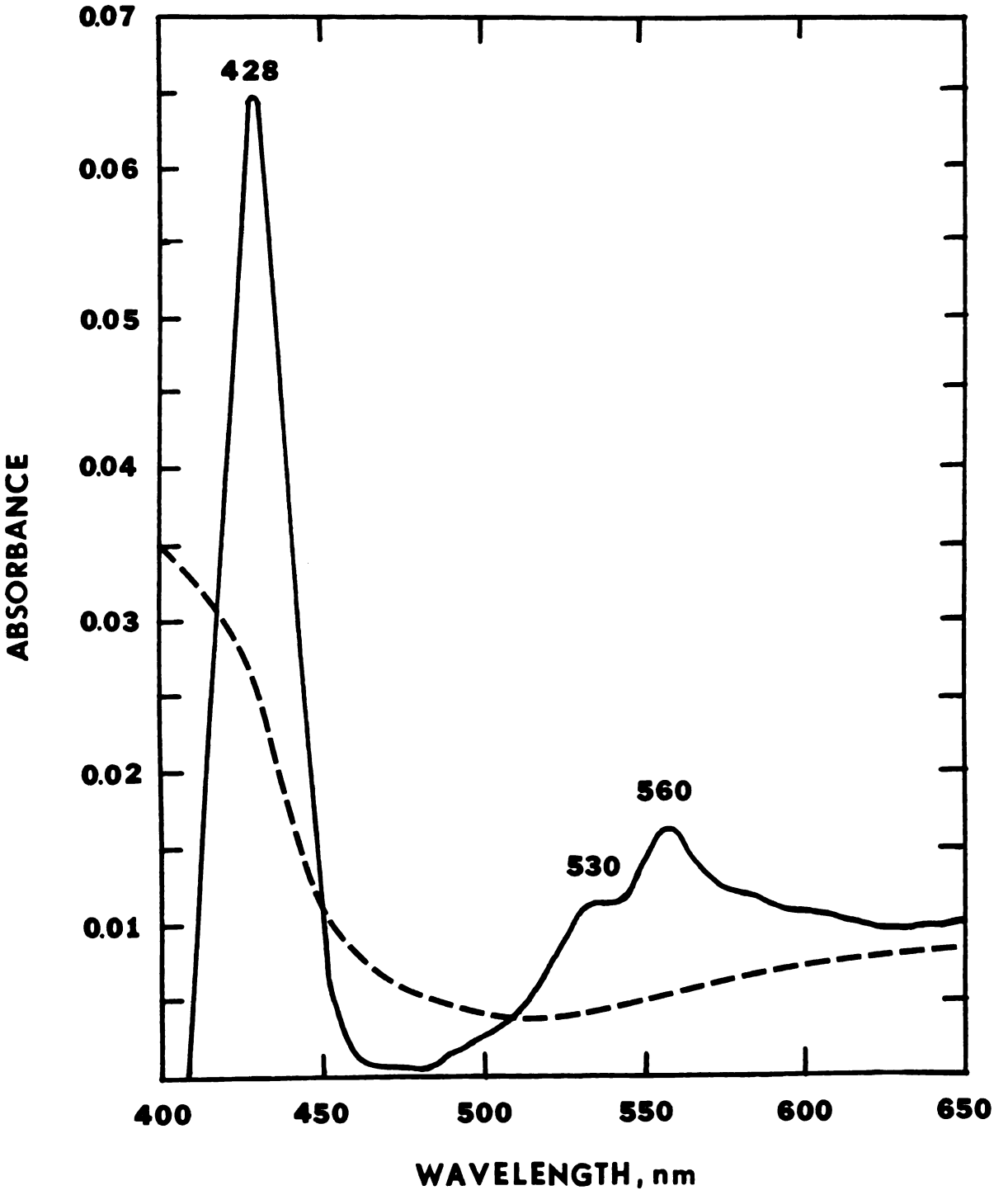

0.07
0.06
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
428
560
530
400
450
500
550
600
650
WAVELENGTH, nm

Figure 9.--Difference spectra of a protoheme extract (19 mg of protein per ml pyridine-KOH) of C. pyogenes. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

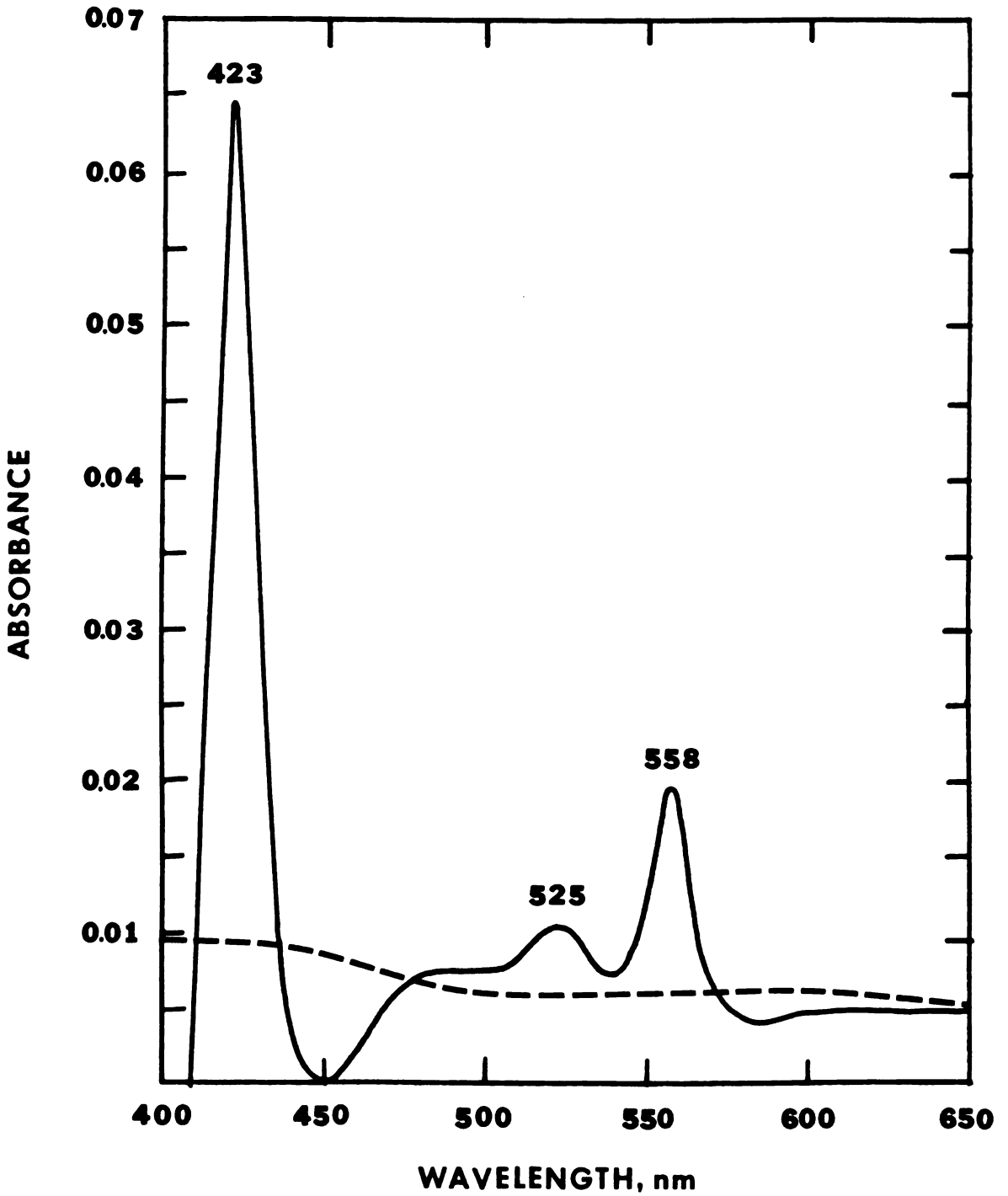

0.07
0.06
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
423
558
525
400
450
500
550
600
650
WAVELENGTH, nm

Figure 10.--Difference spectra of a cell extract (10.9 mg of protein per ml) of C. equi. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

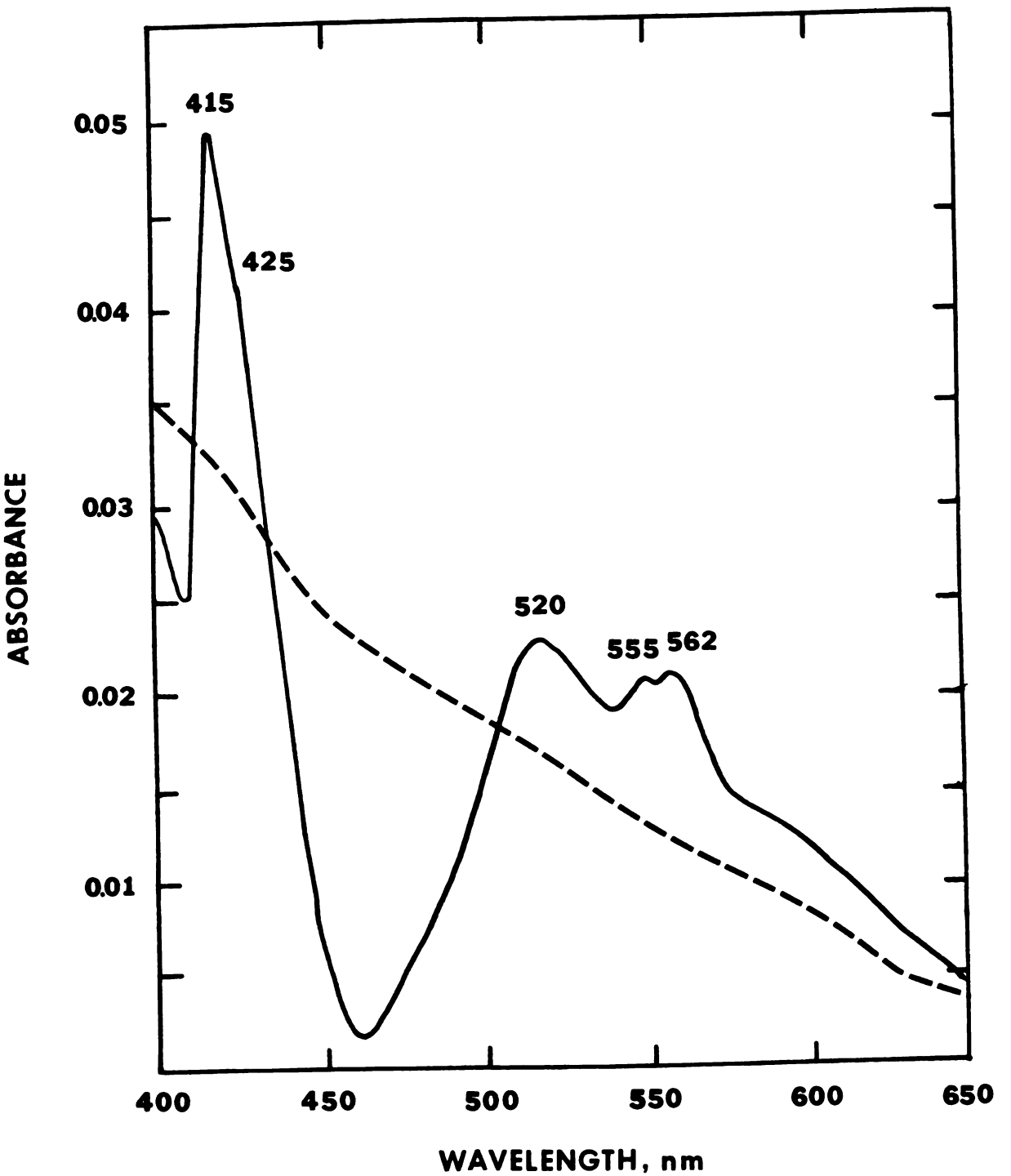

415
425
520
555
562
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
400
450
500
550
600
650
WAVELENGTH, nm

Figure 11.--Difference spectra of a protoheme extract (15.1 mg of protein per ml pyridine-KOH) of C. equi. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

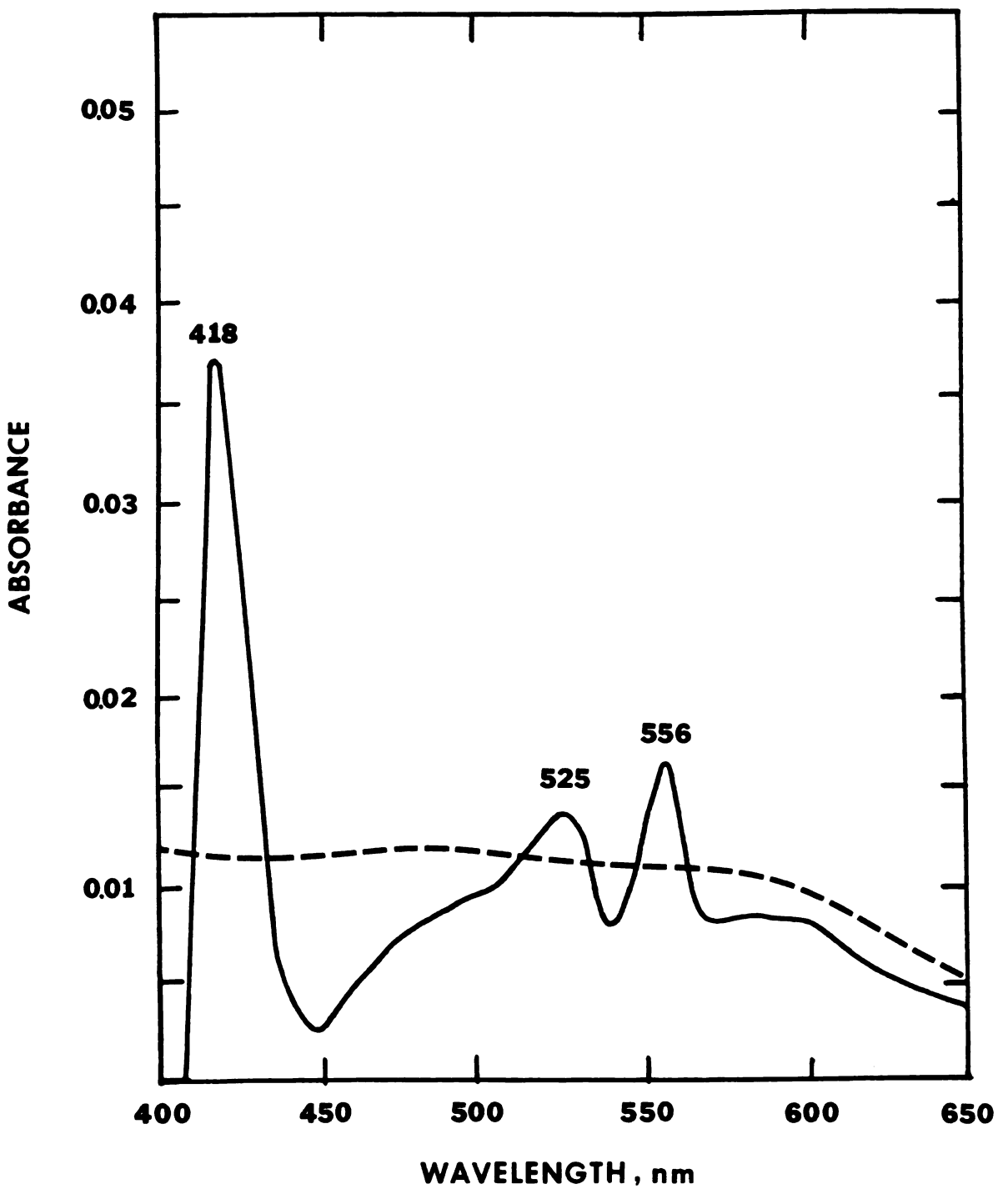

0.05
0.04
0.03
0.02
0.01
ABSORBANCE
418
525
556
400
450
500
550
600
650
WAVELENGTH , nm

Figure 12.--Difference spectra of a mesoheme extract (15.1 mg of protein per ml pyridine-KOH) of C. equi. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

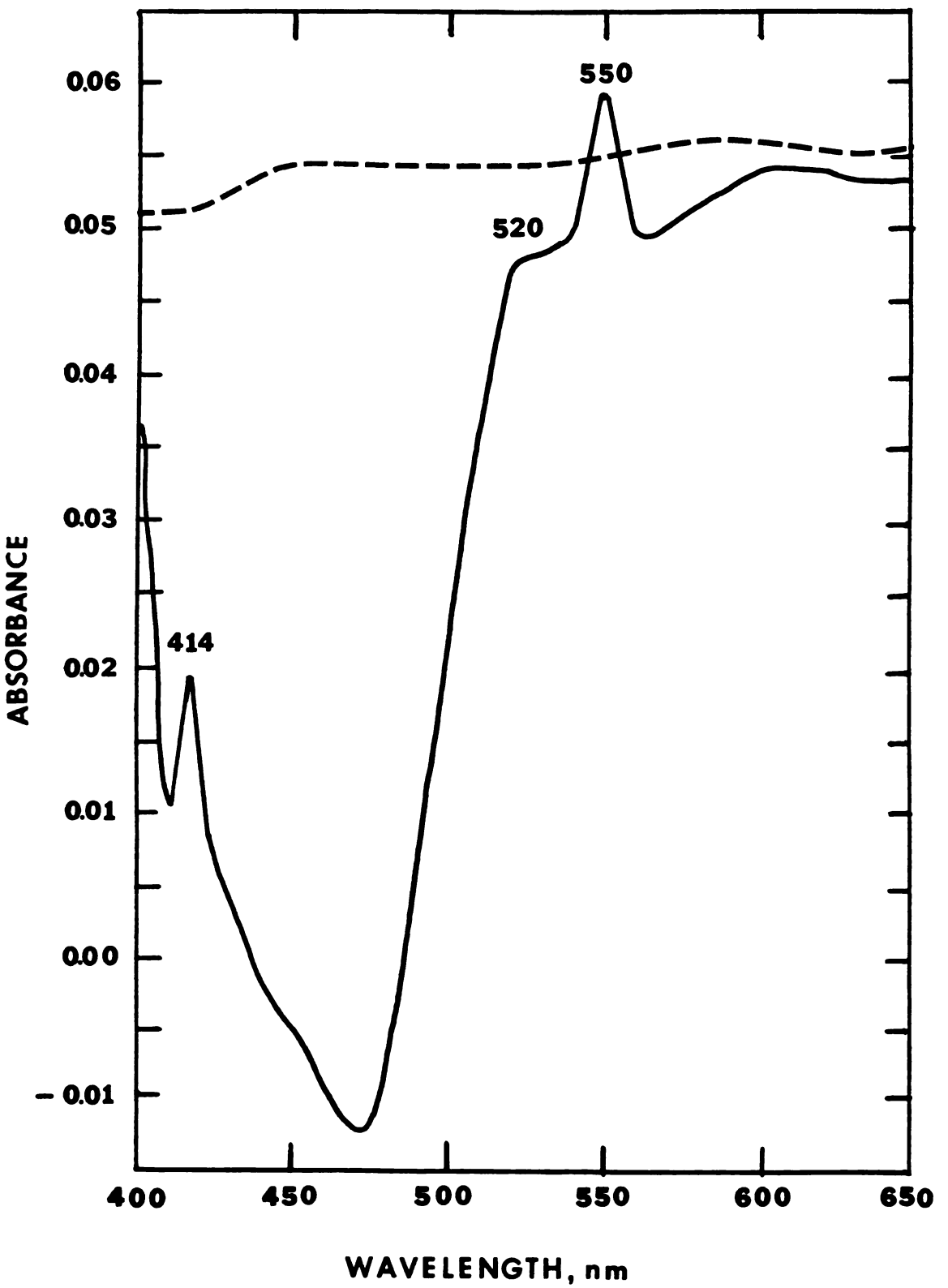

0.06
0.05
0.04
0.03
0.02
0.01
0.00
- 0.01
ABSORBANCE
550
520
414
400
450
500
550
600
650
WAVELENGTH, nm

Figure 13.--Difference spectra of a cell extract (12.3 mg of protein per ml) of C. renale. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

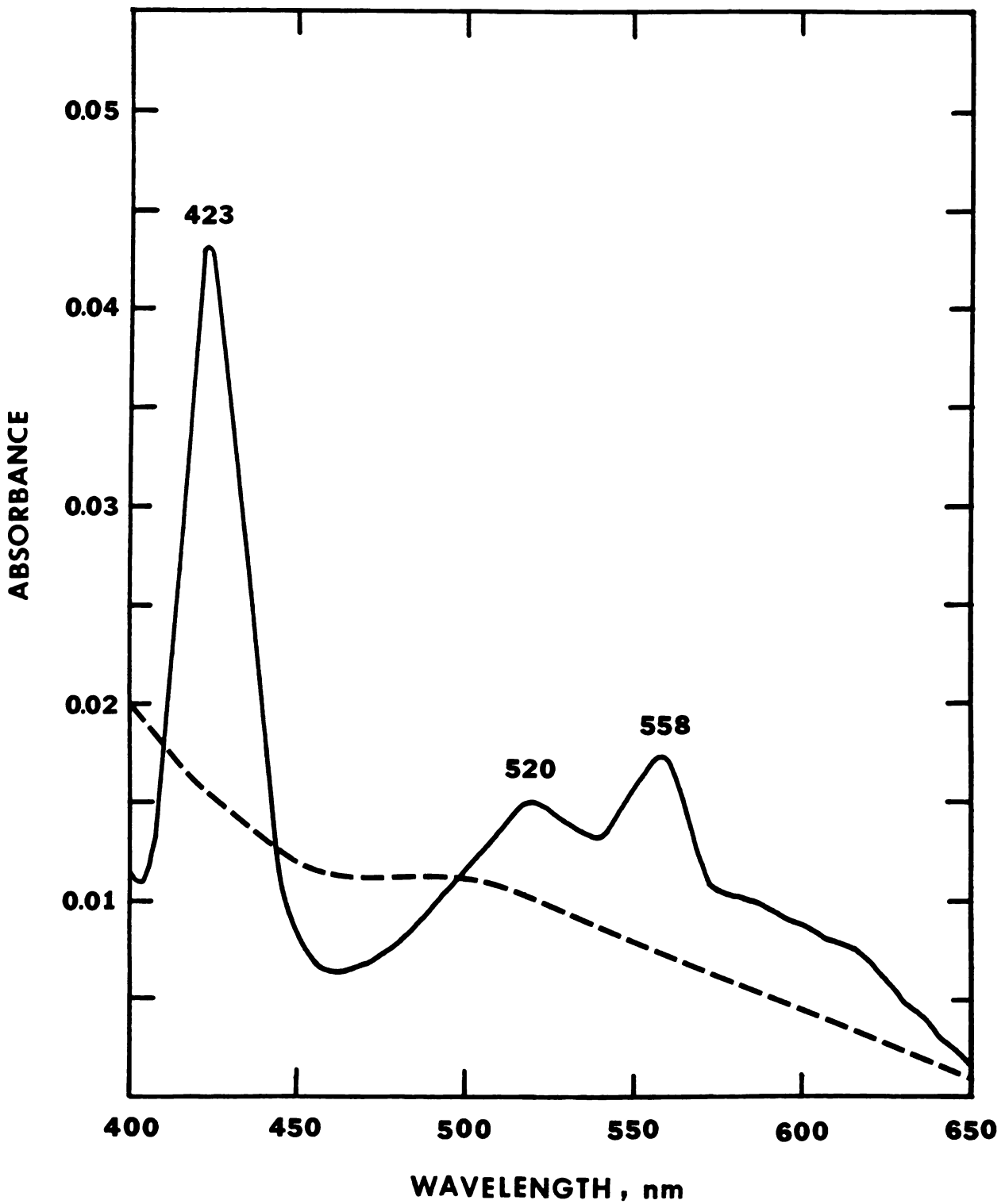

0.05
0.04
0.03
0.02
0.01
ABSORBANCE
423
520
558
400
450
500
550
600
650
WAVELENGTH , nm

Figure 14.--Difference spectra of a protoheme extract (5.2 mg of protein per ml pyridine-KOH) of C. renale. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

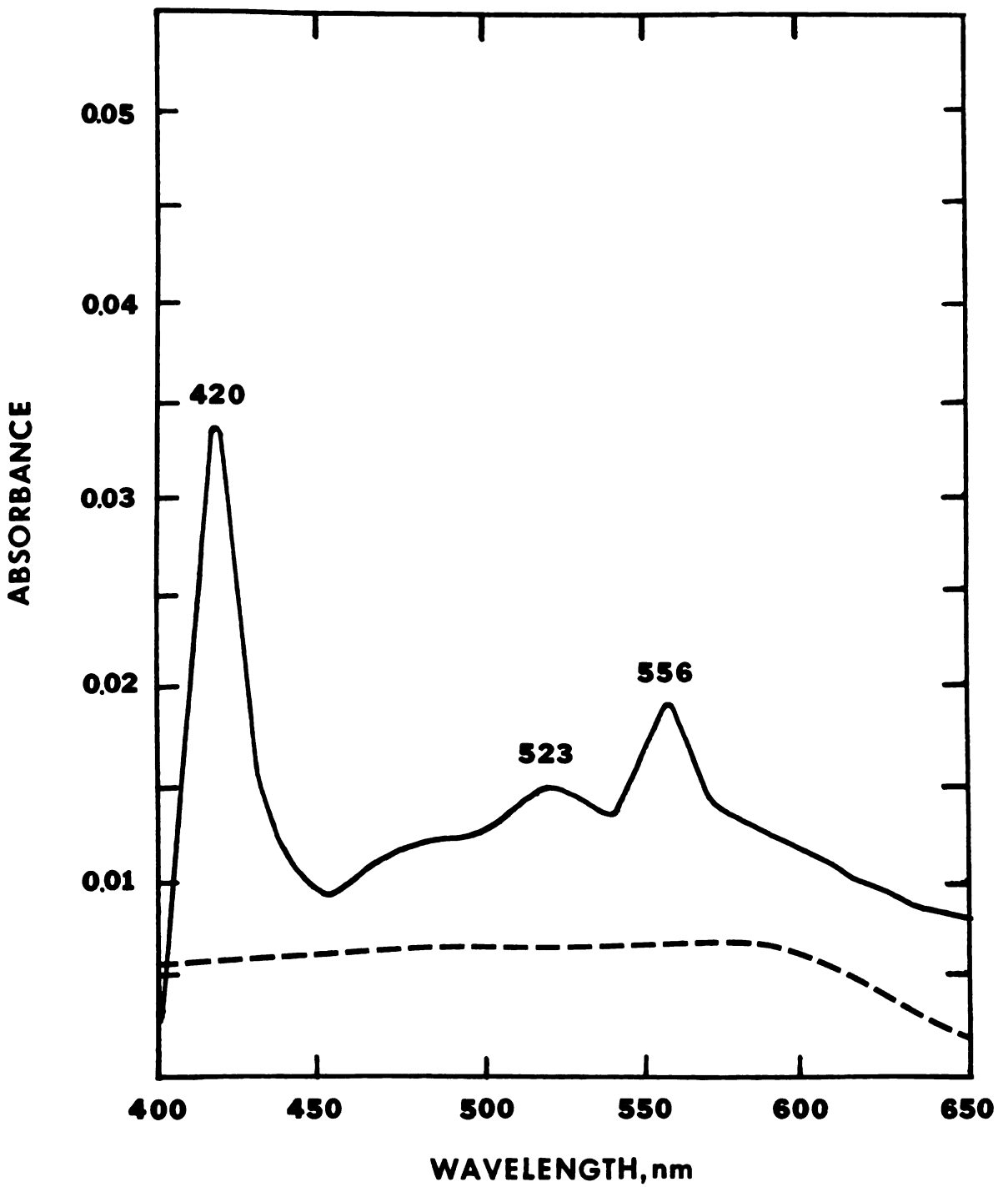

0.05
0.04
0.03
0.02
0.01
ABSORBANCE
420
523
556
400
450
500
550
600
650
WAVELENGTH, nm

Figure 15.--Difference spectra of a cell extract (6.0 mg of protein per ml) of C. bovis. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

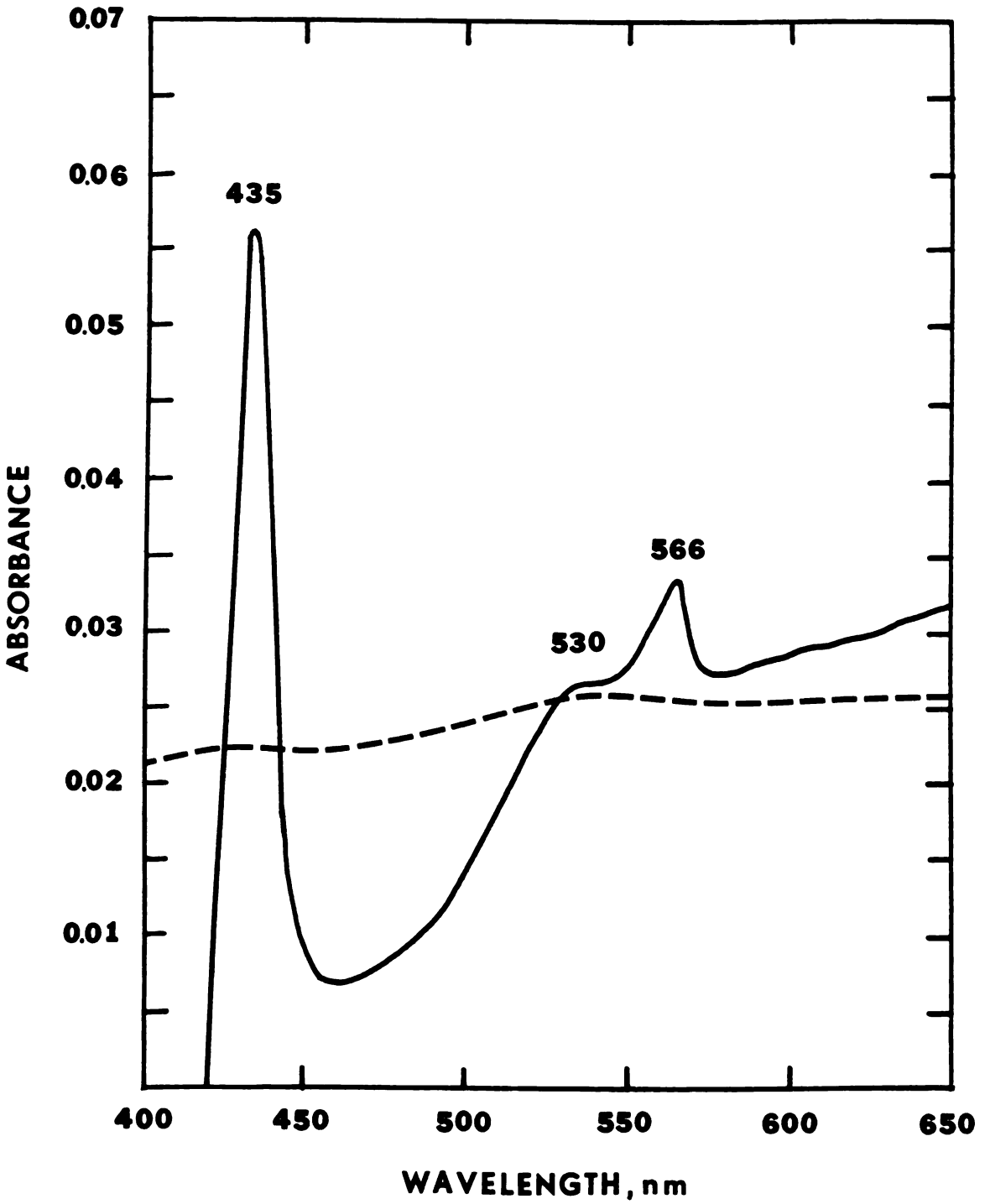

0.07
0.06
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
435
566
530
400
450
500
550
600
650
WAVELENGTH, nm

Figure 16.--Difference spectra of a protoheme extract (10.8 mg of protein per ml pyridine-KOH) of C. bovis. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

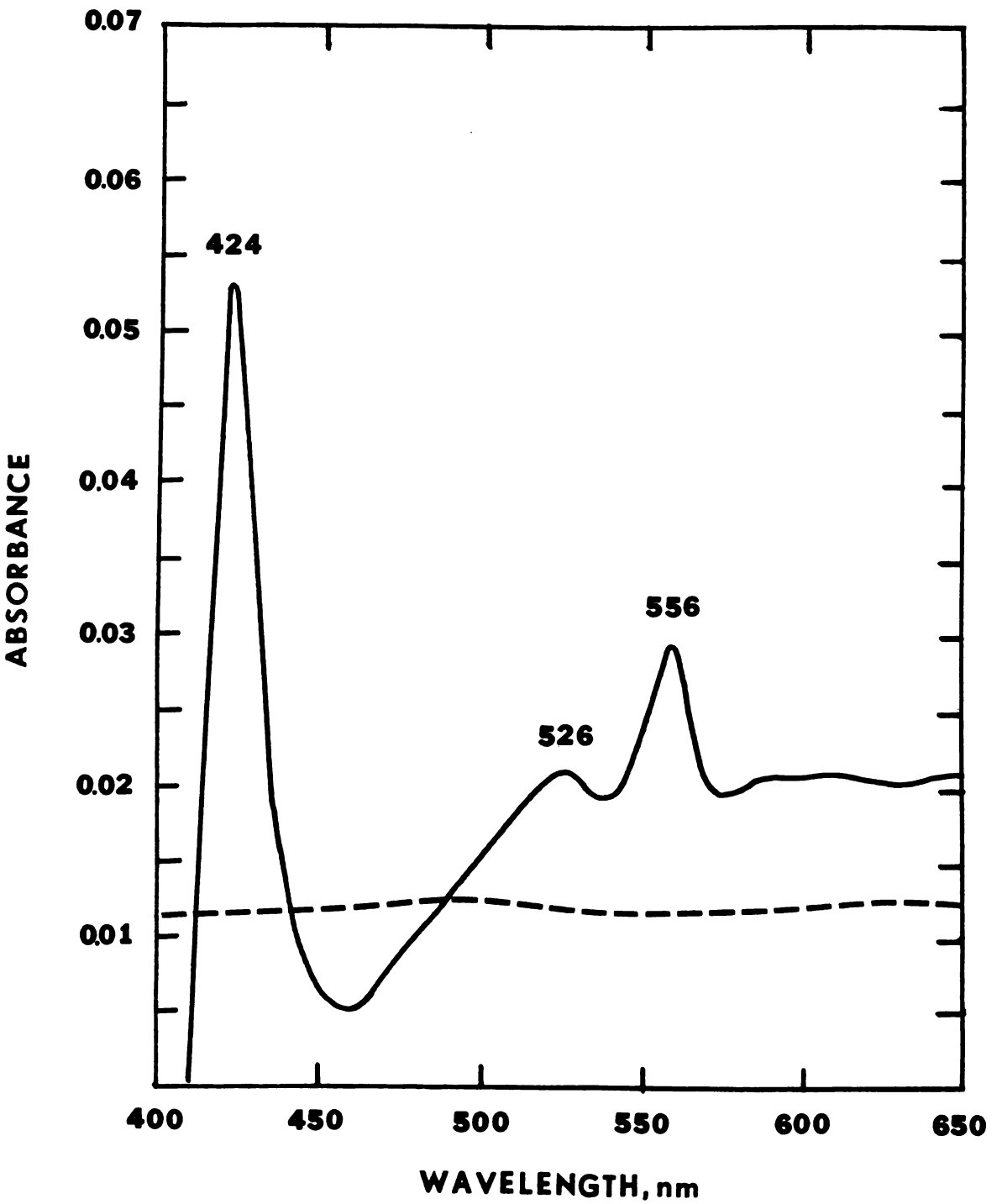

0.07
0.06
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
424
526
556
400
450
500
550
600
650
WAVELENGTH, nm

Figure 17.--Difference spectra of a cell extract (3.8 mg of protein per ml pyridine-KOH) of C. kutscheri. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

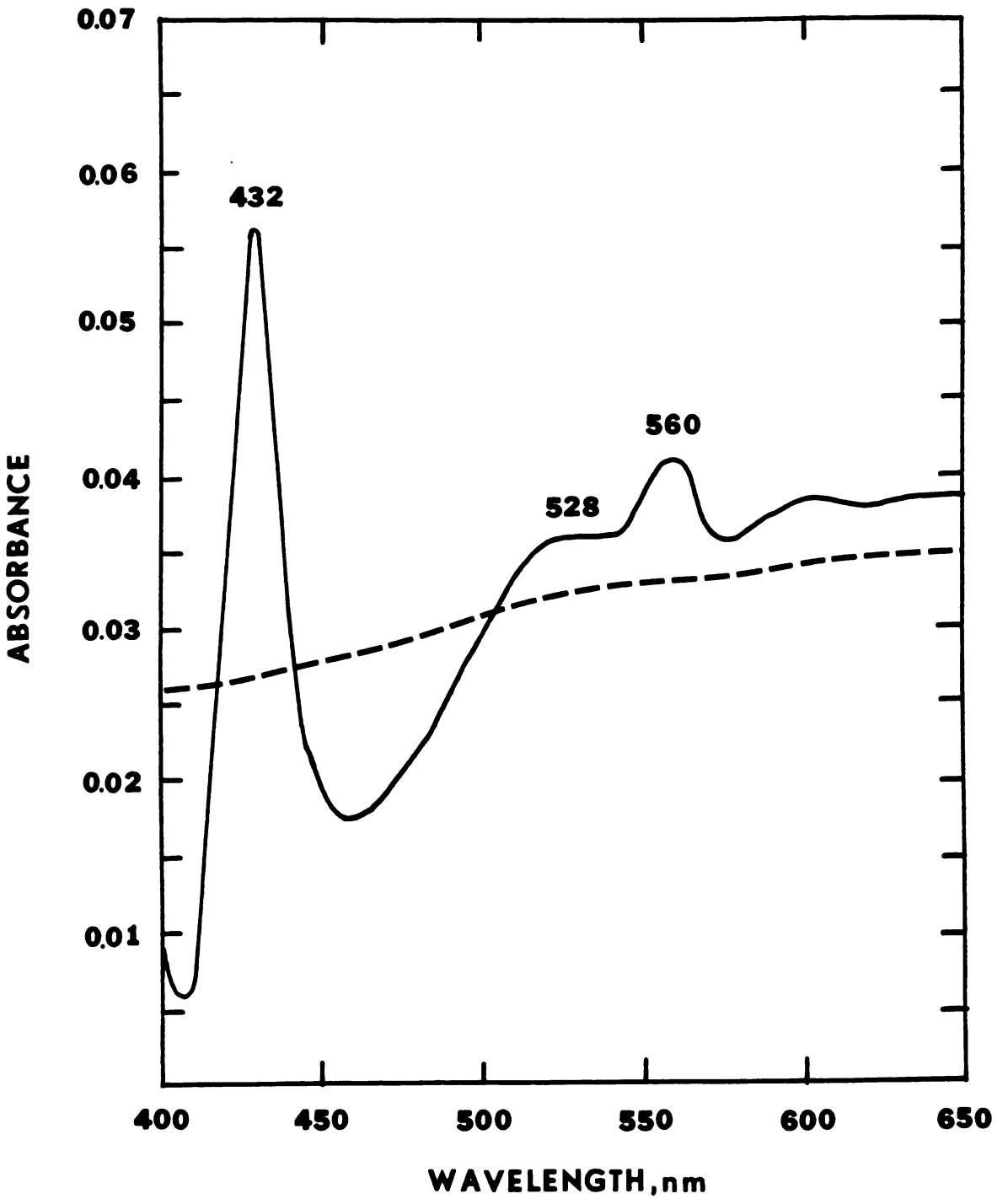

0.07
0.06
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
432
528
560
400
450
500
550
600
650
WAVELENGTH, nm

Figure 18.--Difference spectra of a protoheme extract (5.7 mg of protein per ml pyridine-KOH) of C. kutscheri. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

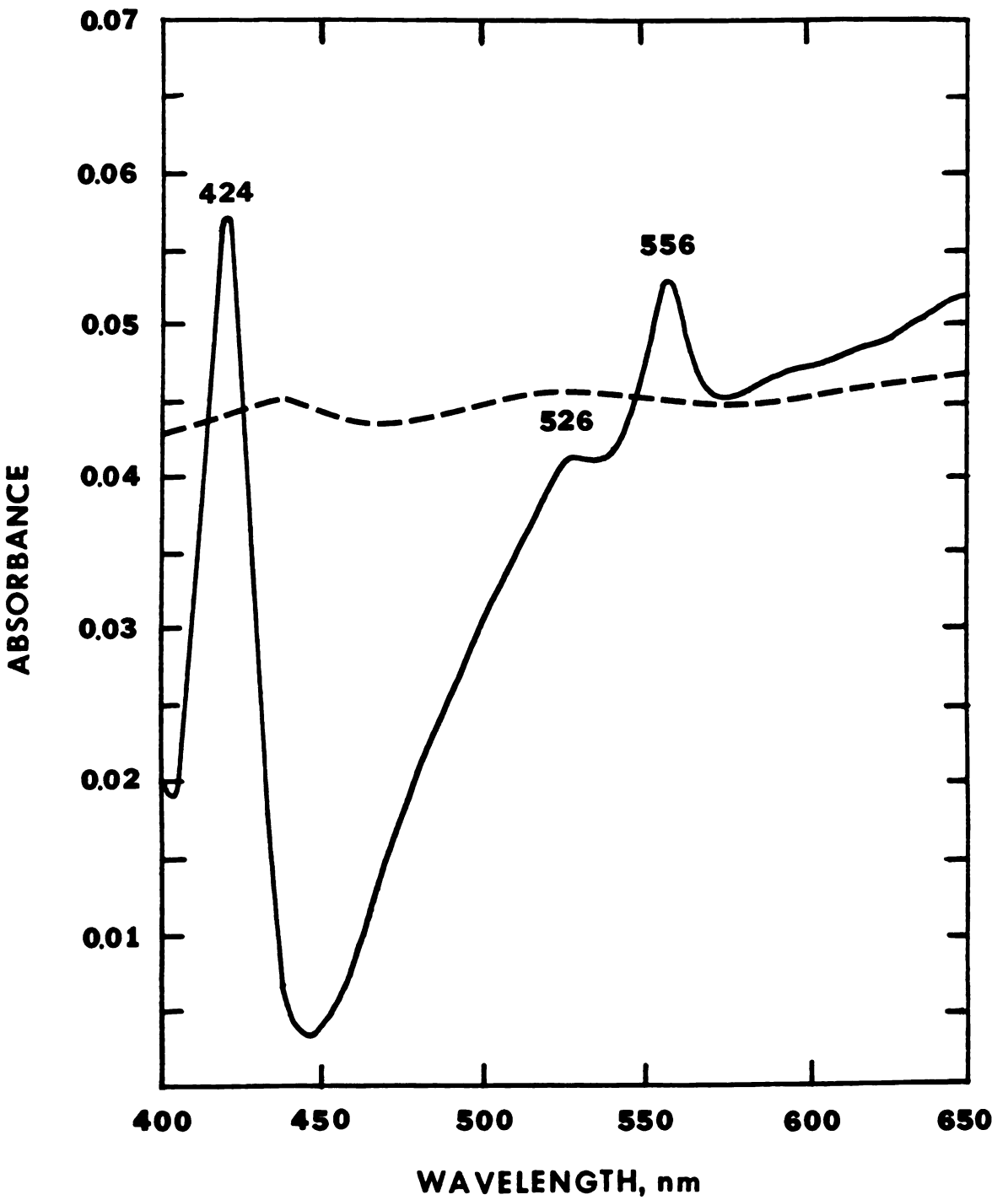

0.07
0.06
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
424
556
526
400
450
500
550
600
650
WAVELENGTH, nm

Figure 19.--Difference spectra of a cell extract (7.7 mg of protein per ml) of C. pseudotuberculosis. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

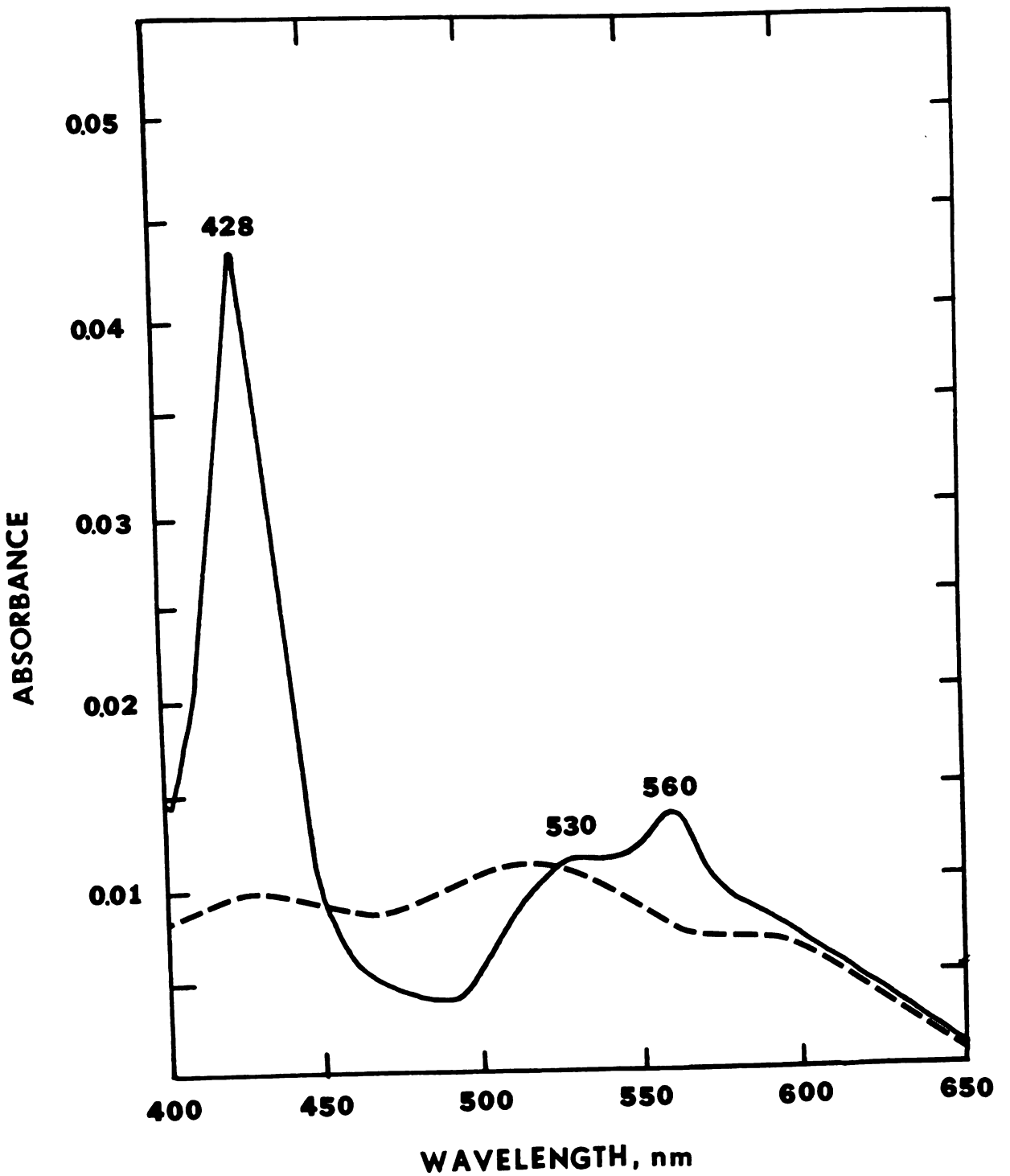

0.05
0.04
0.03
0.02
0.01
ABSORBANCE
428
530
560
400
450
500
550
600
650
WAVELENGTH, nm

Figure 20.--Difference spectra of a protoheme extract (15.2 mg of protein per ml pyridine-KOH) of C. pseudotuberculosis. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.

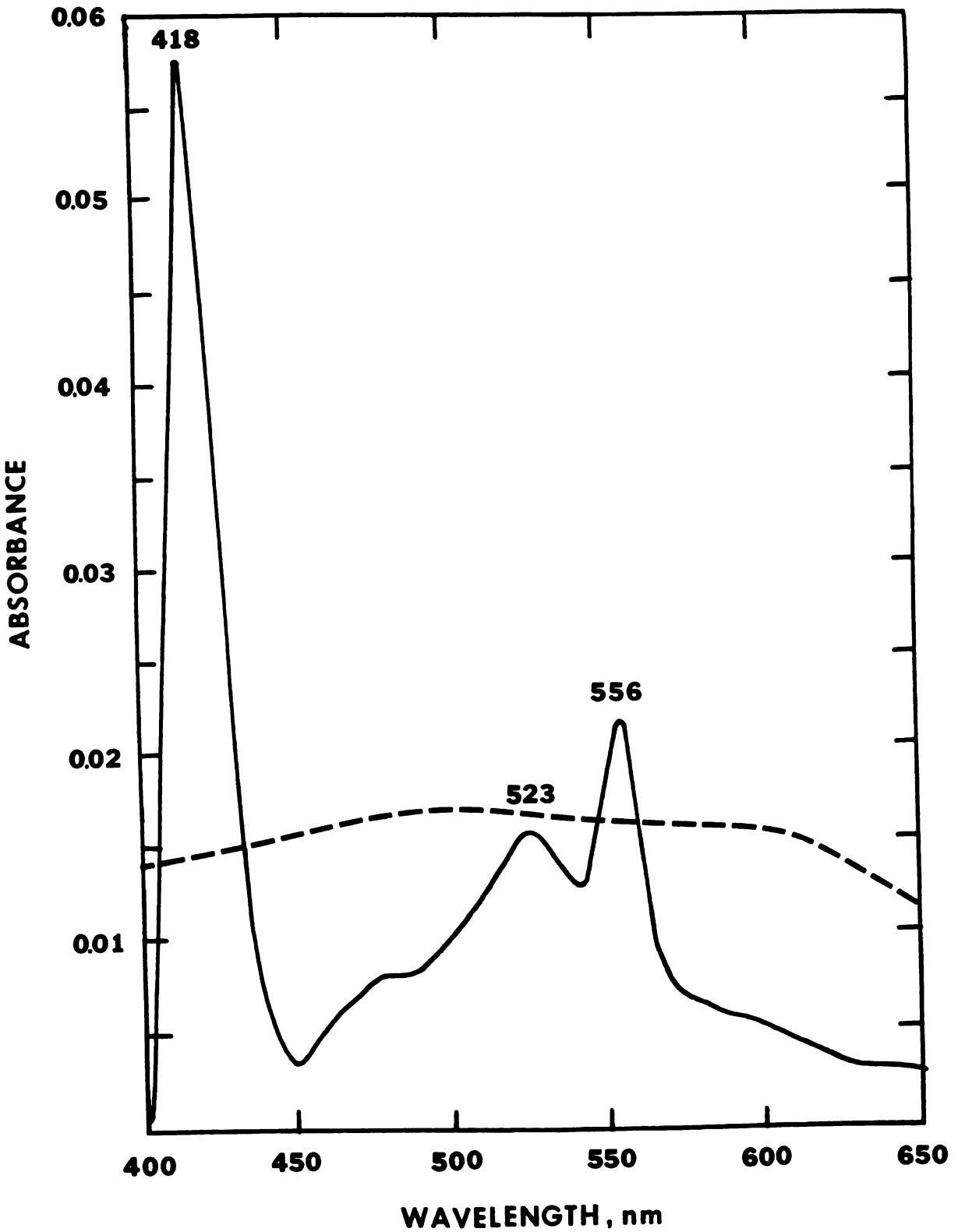
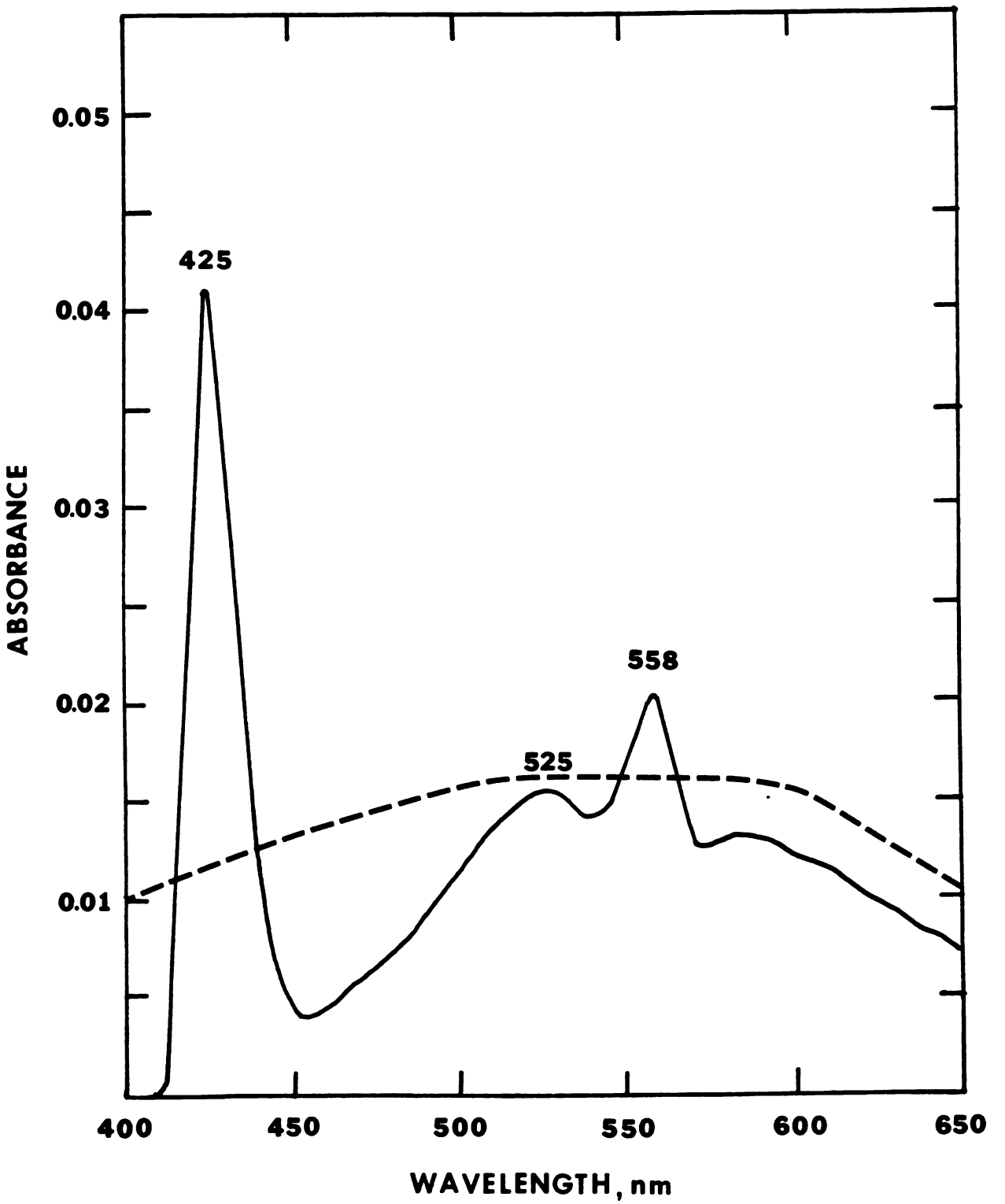

0.06
0.05
0.04
0.03
0.02
0.01
ABSORBANCE
418
556
523
400
450
500
550
600
650
WAVELENGTH, nm

Figure 21A.--Difference spectra of a cell extract (5.9 mg of protein per ml) of C. diphtheriae 1. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.


0.05
0.04
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0.02
0.01
ABSORBANCE
425
558
525
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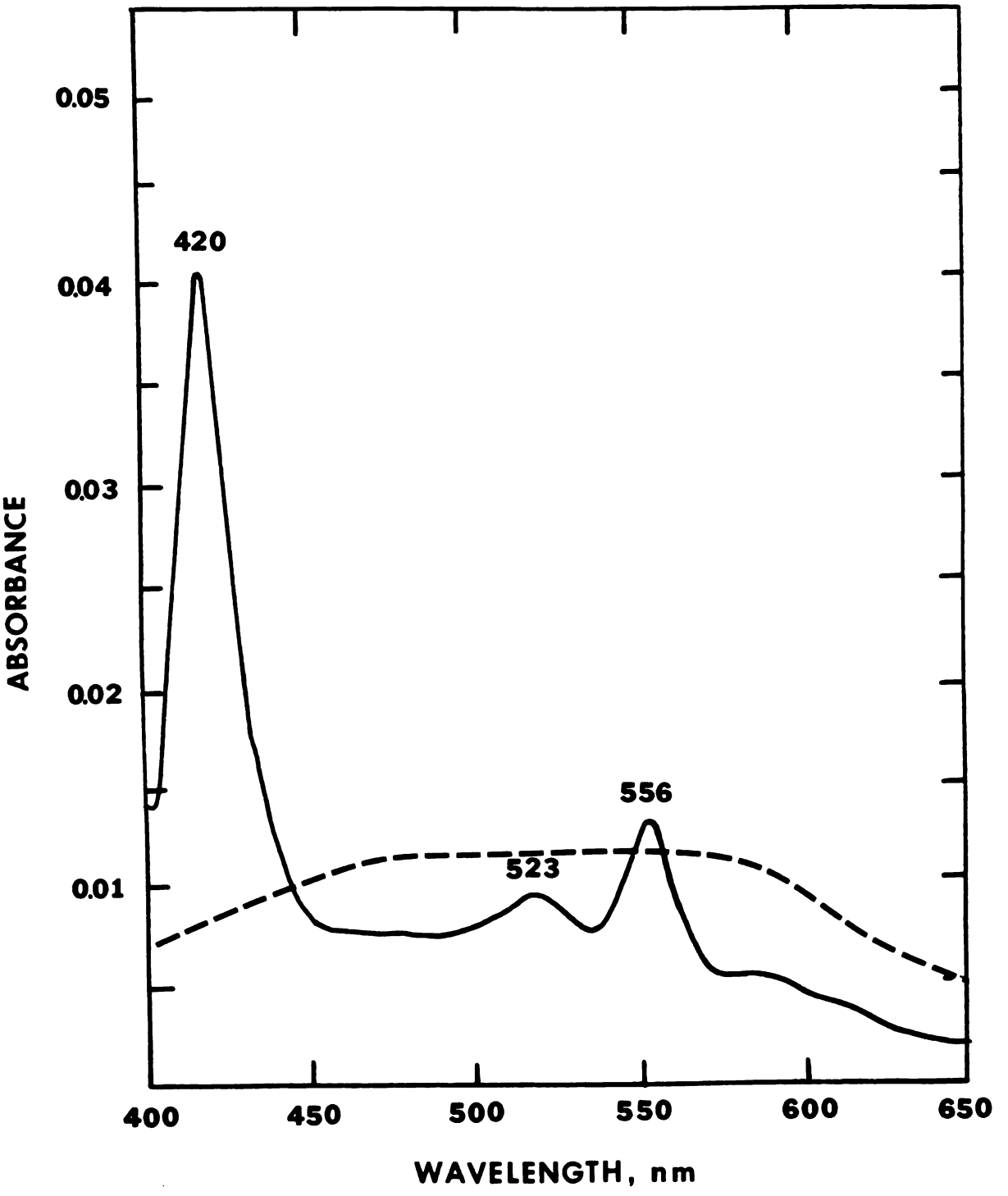
WAVELENGTH, nm

Figure 21B.--Difference spectra of a cell extract (6.9 mg of protein per ml) of C. diphtheriae 2. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.


0.05
0.04
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ABSORBANCE
420
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523
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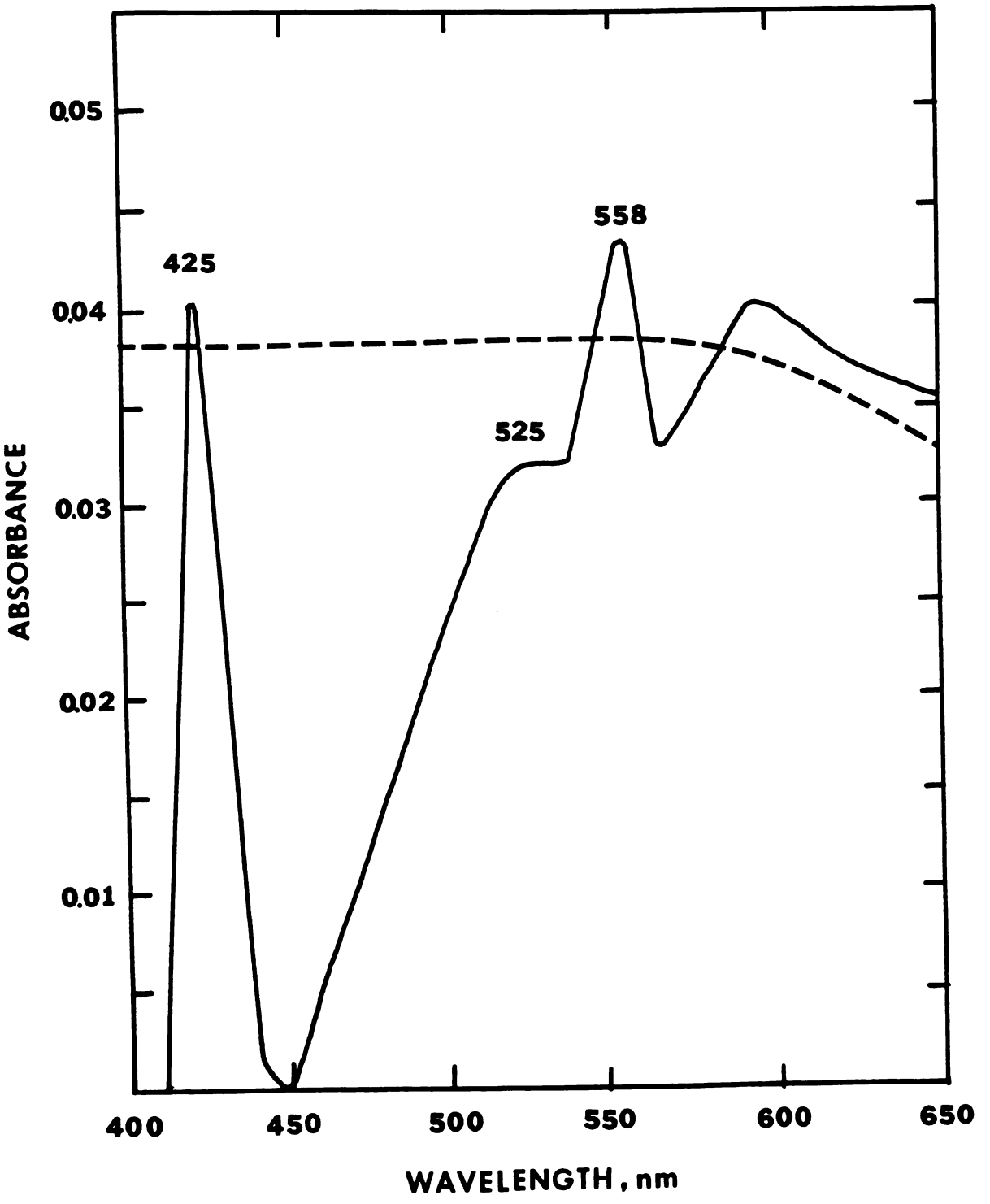
WAVELENGTH, nm

Figure 22A.--Difference spectra of a protoheme extract (10.9 mg of protein per ml pyridine-KOH) of C. diphtheriae 1. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.


0.05
0.04
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0.02
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ABSORBANCE
425
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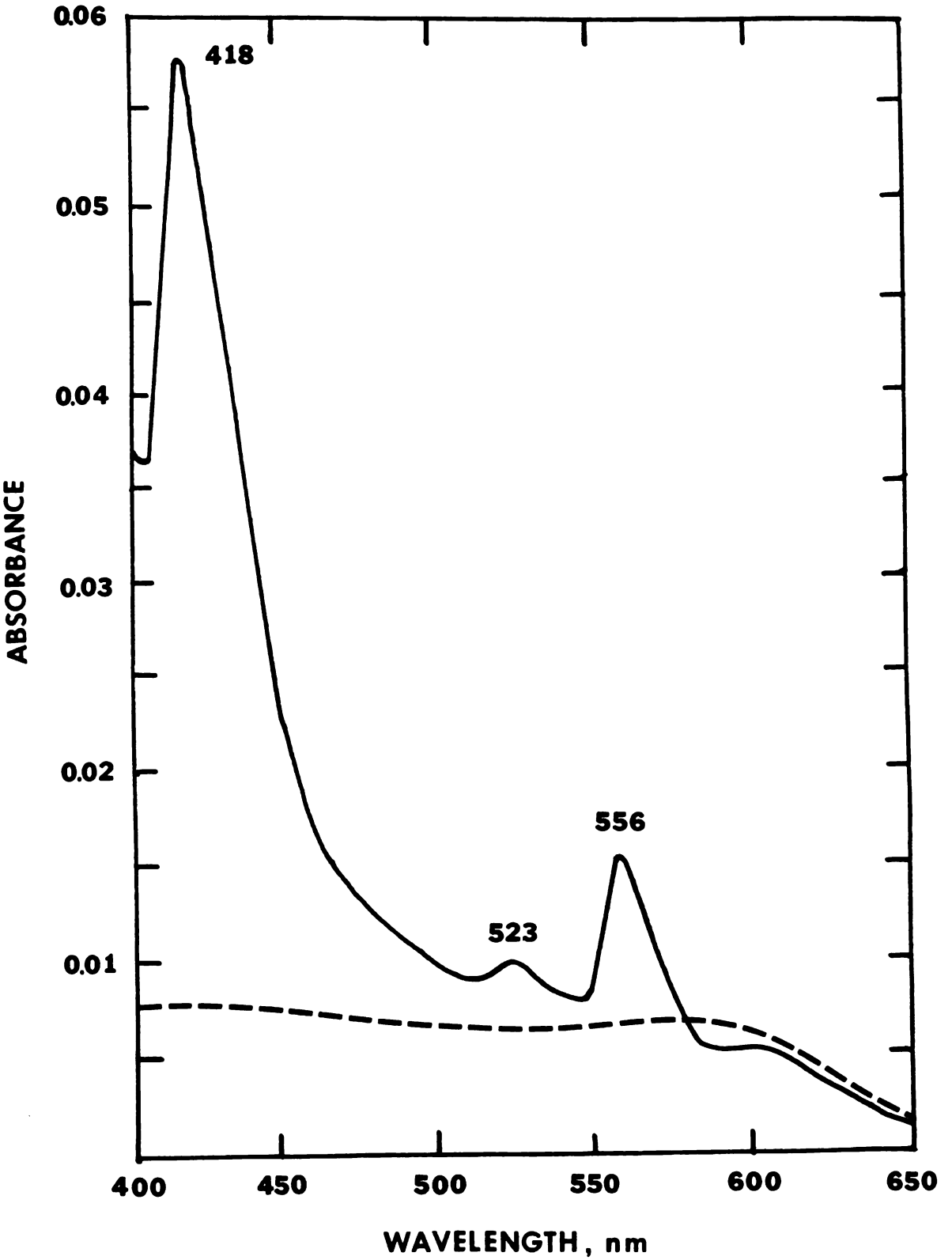
558
400
450
500
550
600
650
WAVELENGTH, nm

Figure 22B.--Difference spectra of a protoheme extract (7.2 mg of protein per ml pyridine-KOH) of C. diphtheriae 2. Air-oxidized versus air-oxidized (broken line) and dithionite-reduced versus air-oxidized (solid line) difference spectra. Other experimental conditions were as described for Figure 8.


0.06
0.05
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ABSORBANCE
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WAVELENGTH, nm

V. DISCUSSION

The strains representing various Corynebacterium species selected from the study appeared typical in their morphological and biochemical characteristics to the type strains described in Bergey's Manual (93) with a few exceptions. Although the manual indicates that C. renale does not produce hemolysin, the strain that was used in this study produced slight hemolysis on sheep blood agar after 24 h incubation at 37 C. According to Rogosa et al. (93) C. bovis strains are reported to be urease-positive and lack the ability to ferment sucrose; however, the C. bovis strain used in this study did ferment sucrose and did not hydrolyze urea. Carter (18) reported that C. bovis does not hydrolyze urea but did not mention about the organism's ability to ferment sucrose. C. kutscheri was reported to be negative for nitrate reduction but positive for urease (93); however, my results, in agreement with those of Carter (18), showed that this organism reduced nitrates and produced urease. Carter (18) indicated that strains of C. equi are variable in their ability to hydrolyze urea. However, the C. equi strains I used including four ATCC strains produced urease.

The biochemical tests showed that C. pyogenes was the only species tested that produced acid from lactose, produced acid coagulation and peptonization in milk, hydrolyzed gelatin, and was catalase-negative. According to the GLC profiles, C. pyogenes again

appeared different from others in producing large quantities of lactic acid and moderate amounts of acetic acid and in this respect it was similar to certain homofermentative lactobacilli and streptococci. Cummins (26) and Cummins and Harris (25) reported that the cell wall composition of C. pyogenes was similar to Streptococcus in containing rhamnose and lysine in its cell wall and in serology. Moreover, several numerical taxonomic studies also indicated that C. pyogenes bears little similarity to any of the other animal and human pathogenic corynebacteria (8, 29, 40, 49, 101). The results of the present study focusing on acid end products from glucose, in agreement with previous investigations, provide additional evidence that C. pyogenes should be removed from the genus Corynebacterium and be reassigned to a separate genus, probably to the genus Actinomyces as suggested by the data of Reddy and Cornell (85).

The results of this study showed that C. equi, unlike other human and animal pathogenic corynebacteria, did not produce detectable acid end products from glucose. These results agree with previous reports (33, 45, 46) which indicated that C. equi metabolized glucose oxidatively, rather than fermentatively. Cummins (26) reported that C. equi and the Rhodochorous group in Nocardia have the same major cell wall composition and a common cell wall antigen. A number of other studies have also shown that C. equi is distinctly different from C. diphtheriae and related animal pathogens and should be excluded from the genus Corynebacterium (34, 37, 46, 48). In view of these findings Goodfellow and Alderson (35) proposed that C. equi should be reclassified as Rhodococcus equi.

C. renale appears different from all other corynebacteria examined in producing lactic acid as the only significant acid end product. It is also different from the others in producing alkaline change and peptonization in litmus milk after six to eight days of incubation. C. renale is known to be similar to C. diphtheriae, C. pseudotuberculosis and C. kutscheri in cell wall composition (25, 26), mycolic acids (36), and antigenic relationships (93) but appears so different metabolically from these organisms that it is questionable whether it belongs in the genus Corynebacterium.

C. bovis is similar to C. pyogenes in producing major amounts of lactic acid and moderate amounts of acetic acid from glucose fermentation but differs from C. pyogenes in producing significant amounts of pyruvate. Further, the colony morphology, nutritional requirements and biochemical characteristics of C. bovis are distinctly different from C. pyogenes (93). Also, C. bovis is only infrequently encountered as an animal pathogen while C. pyogenes is a very common animal pathogen (15, 18).

C. kutscheri produced major amounts of lactic and propionic acids, moderate amounts of succinic acid and minor amounts of pyruvic and oxalacetic acids. These results showed that C. kutscheri is readily distinguishable from other animal pathogenic corynebacteria on the basis of acid metabolic end products alone and this should be of considerable help in its diagnosis. Besides, C. kutscheri is different from the other animal pathogenic cornyebacteria in biochemical characteristics also (18, 93).

C. pseudotuberculosis is similar to C. diphtheriae in morphology, in producing a toxin similar to that of diphtheria toxin, in cell wall composition and in being lysogenized by C. diphtheriae phages (17). The results of this study showed that both organisms are quite similar in producing major amounts of formate, acetate and propionate. However, C. pseudotuberculosis differed from C. diphtheriae in producing two fold greater amounts of pyruvic acid and relatively smaller amounts of acetic and propionic acids. Beta hemolysis on blood agar and urease activity should also be of help in distinguishing C. pseudotuberculosis from C. diphtheriae. It should be noted that C. pseudotuberculosis is easily distinguishable from other animal pathogenic corynebacteria studied on the basis of metabolic end products.

C. diphtheriae, the well-known causative agent of human diphtheria (6, 93), infrequently causes infections in animals also. C. diphtheriae strains examined were similar in producing major amounts of volatile products, acetate, formate and propionate, but showed considerable heterogeneity in the non-volatile acids produced. Strain #1 produced minor amounts of lactate and succinate and traces of oxalacetate; strain #3 produced major amounts of fumarate, pyruvate and oxalacetate but no succinate or lactate. Our results are similar to that of Tasman and Brandwijk (103) in showing significant differences among strains of C. diphtheriae in acid products produced. It should also be noted that C. diphtheriae and C. pseudotuberculosis are similar to propionibacteria in producing major

amounts of acetate and propionate and minor amounts of lactate and succinate. However, propionibacteria, apparently, either do not produce formate or produce only minor amounts of this acid, while C. diphtheriae and C. pseudotuberculosis produce major amounts of formate.

The use of gas chromatography as a chemotaxonomic tool has been well documented (1, 2, 41, 68, 70, 75, 102). The results of this study showed that gas chromatographic characterization of acid metabolic end products is quite useful in differentiating animal pathogenic Corynebacterium species. The gas chromatographic profiles were quite consistent among the strains within a species. Therefore, gas chromatographic analysis of acid end products of Corynebacterium may have a practical application in clinical laboratory diagnosis of various animal corynebacteria. The results presented here and those of Reddy and Cornell (84) are also consistent with the idea that C. equi and C. pyogenes are quite different from the other animal pathogens as previously discussed. With the exception of C. pseudotuberculosis, which is quite closely related to C. diphtheriae, the type species of this genus, the taxonomic status of others such as C. renale and C. bovis is an open question because the acid end products produced by these organisms are quite different from C. diphtheriae.

Previous studies showed that certain strains of C. diphtheriae contain cytochromes a, b and c while other strains of the same organism contain only cytochrome b (78, 81). With the methodology used

in this study only b type cytochrome was demonstrable in the strain of C. diphtheriae used. Although cytochromes other than b were not detectable in this study (with the exception of C. equi), it is possible that more sensitive procedures such as low temperature spectra in liquid nitrogen might reveal the presence of minute amount of other cytochromes present, if any.

The results show that all Corynebacterium species examined contained one major b type cytochrome; C. equi in addition contained a c type cytochrome. This is the first report of cytochromes in animal pathogenic corynebacteria as far as is known.

Several environmental factors affect the production of bacterial cytochromes and it is possible that under different growth conditions other cytochromes may be produced by these organisms. For example, oxygen tension is known to affect the composition of cytochromes (30, 59). The species of the genus Propionibacterium, which grew rapidly under anaerobic conditions and produced a, b and c type cytochromes, grew very slowly in an aerated medium, and the cytochrome synthesis was repressed by oxygen (30). Note that all the corynebacteria examined in this study were grown aerobically. The iron concentration in the medium is known to affect the cytochrome production. Pappenheimer (77, 79) suggested that cytochromes, which consist of a complex of apo-enzyme, iron and prophyrin, are normally formed by C. diphtheriae when there is enough iron in the medium, but that when iron is in short supply, apo-enzyme and porphyrin are not linked together, but are excreted into the medium.

The diphtheria toxin is the apo-enzyme that forms part of the cytochrome or succinooxidase respiratory system of C. diphtheriae. Therefore, if there is a high iron concentration in the medium, little toxin is produced and cytochrome is formed. Aerobacter indologenes, when grown on an iron sufficient medium, contained an a_1 type cytochrome similar to that found in E. coli, showed a moderate absorption band at 560 nm, and a weak band at 590 nm. However, growth in an iron deficient medium results in a loss of all the cytochrome bands (107). The age of the culture is also another factor that affects cytochrome production. Keilin (51) found that the cytochrome a_2 of Acetobacter pasteurianum varied with the culture medium as well as with the age of the organism.

Hemin was shown to be required for cytochrome synthesis in some anaerobic and facultatively anaerobic bacteria (74). Hemin was required for cytochrome b and furamate reductase production in Bacteriodes fragilis (63) and for cytochrome b production in Bacteriodes ruminicola (108). An obligate anaerobic bacterium, Eubacterium lentum, was shown to contain cytochrome types a, b and c (100). Only extracts of cells grown with hemin gave an alpha absorption maximum at 553 nm indicative of a c type cytochrome. In contrast, a and b type cytochromes could be shown in extracts (100) of E. lentum cells grown in the absence of hemin also. When cells were grown in the presence of 50 mM nitrate, cytochrome a with α absorption maximum at 605 nm was also detected (100). Reddy et al. (85) showed that hemin is stimulatory for the growth of C. pyogenes

and that cytochrome b synthesis in this organism was dependent on the presence of hemin in the medium.

Hemin, which was added to the medium of C. pyogenes, was not added to the medium used for growing other Corynebacteria species examined for the presence of cytochrome because hemin is not stimulatory to the growth of these organisms. Yet, all except C. equi had a b type cytochrome. However, both b and c were detected in C. equi grown under the same experimental conditions. The b and c type cytochromes detected in C. equi may function in electron transport phosphorylation (59, 106) during the oxidative metabolism of glucose as in the case of mycobactria and nocardiae to which it is closely related (21, 93).

In summary, the metabolic end products of these animal and human pathogenic corynebacteria are heterogenous. C. pyogenes, according to the acid end products from glucose as well as biochemical tests should be reclassified to Actinomyces. C. equi, oxidizing glucose instead of fermentation, differs from other animal pathogenic Corynebacterium species and its taxonomic position should be reclassified too. The gas chromatographic profiles were very consistent among the tested strains within a species. The gas chromatographic analysis of Corynebacterium species may have practical value for clinical diagnosis. With regard to the cytochrome study, the results don't yield any information of value for clinic diagnosis. The selected animal and human pathogenic Corynebacterium

species all contained b type cytochrome, and C. equi was found to possess c type cytochrome under the defined experimental conditions.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Abel, K., H. DeSchmertzing, and J. I. Peterson. 1963. Classification of microorganisms by analysis of chemical composition. I. Feasibility of utilizing gas chromatography. J. Bacteriol. 85: 1039-1044.

2. Azuma, R. and T. Suto. 1970. Validity of transfer of taxonomical position of Corynebacterium pseudopyogenes from genus Corynebacterium to genus Actinomyces. In H. Iizuka and T. Hasegawa (eds.), Culture Collection of Microorganisms, University Press, Baltimore, Md.

3. Bailey, W. R., and E. C. Scott. 1970. Diagnostic microbiology. pp. 331-354, 364-371. The C. V. Mosby Co., St. Louis.

4. Bartsch, R. G. 1968. Bacterial cytochromes. Ann. Rev. Microbiol. 22: 181-200.

5. Barksdale, L., K. Li, C. S. Cummins, and H. Harris. 1957. The mutation of Corynebacterium pyogenes to Corynebacterium Hamolyticum. J. Gen. Microbiol. 16: 749-758.

6. Barksdale, L. 1970. Corynebacterium diphtheriae and its relatives. Bacteriol. Rev. 34: 378-422.

7. Bean, C. W., W. T. Miller, and J. O. Heishman. 1943. A note on Corynebacterium pyogenes as the cause of bovine mastitis. J. Am. Vet. Med. Assoc. 103: 200-202.

8. Bousfield, I. J. 1972. A taxonomic study of some coryneform bacteria. J. Gen. Microbiol. 71: 441-455.

9. Bousfield, I. J., and M. Goodfellow. 1976. The 'rhodochorous' complex and its relationships with the allied taxa. In M. Goodfellow, G. H. Browwell and J. A. Serrans (eds.), The biology of the Nocardiae, Academic Press, London.

10. Boyd, W. L., and M. Bishop. 1937. Pyelonephritis of cattle and horses. J. Am. Vet. Med. Assoc. 90: 154-159.

11. Boyd, W. L. 1918. Pyelonephritis in cattle. Cornell Vet. 8: 120-121.

12. Bradley, S. G. 1971. Criteria for definition of Mycobacterium, Nocardia and the 'rhodochorous' complex. Adv. trantiess of Plant Sci. 28: 349-362.

13. Brooks, J. B., D. S. Kellogg, C. C. Alley, H. B. Short, H. H. Handsfield, and B. Huff. 1974. Gas chromatography as a potential means of diagnosing arthritis. I. Differentiation between staphylococcal, streptococcal, gonococcal and traumatic arthritis. J. Infect. Dis. 129: 660-668.

14. Bruner, D., W. W. Dimock, and P. R. Edwards. 1939. The serological classification of Corynebacterium equi. J. Inf. Dis. 65: 92-96.

15. Bruner, D. W., and J. H. Gillespie. 1973. Hagan's infectious diseases of domestic animals. pp. 151-166. Cornell University Press, Ithaca and London.

16. Caldwell, D. R., and M. P. Bryant. 1966. Medium without rumen fluid for nonselective enumeration and isolation of rumen bacteria. Appl. Microbiol. 14: 794-801.

17. Carne, R. H. 1962. Action of bacteriophages obtained from Corynebacterium diphtheriae on C. ulcerans and C. ovis. Nature (London) 217: 1066-1067.

18. Carter, G. R. 1976. Cornyebacterium, pp. 110-114. In Diagnostic procedures in microbiology, 2nd ed. Charles C. Thomas Publishers, Springfield.

19. Chance, B. 1954. Spectrophotometry of intracellular respiratory pigments. Science 120: 767-776.

20. Chlosta, E. M., G. K. Richards, E. Wagner, and J. F. Holland. 1970. An opportunistic infection with Corynebacterium pyogenes producing empyema. Am. J. Clin. Pathol. 53: 167-170.

21. Christensen, P. E. 1957. Studies on lysogenicity in C. diphtheriae. Acta Path. Microbiol. Scand. 41: 67-78.

22. Cobb, R. W. 1962. Cultural characteristics of some cornyebacteria of animal origin with special reference to C. bovis and C. pyogenes. J. Med. Lab. Technol. 20: 199-205.

23. Collins, M. D., T. Pirouz, and M. Goodfellow. 1977. Distribution of menaquinones in actinomycetes and corynebacteria. J. Gen. Microbiol. 100: 221-230.

24. Cornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. J. Biol. Chem. 177: 751-766.

25. Cummins, C. S., and H. Harris. 1956. The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. J. Gen. Microbiol. 14: 583-600.

26. Cummins, C. S. 1962. Chemical composition and antigenic structure of cell walls of Corynebacterium, Mycobacterium, Nocardia, Actinomycetes and Arthrobacter. J. Gen. Microbiol. 23: 35-42.

27. Daines, L. L., and H. Austin. 1932. A study of so-called skin-lesion and no-visible-lesion tuberculin-reacting cattle. J. Am. Vet. Med. Assoc. 80: 414-433.

28. Davis, G. H. G., and K. G. Newton. 1969. Numerical taxonomy of some named coryneform bacteria. J. Gen. Microbiol. 56: 195-214.

29. DaSilva, G. A. N., and J. G. Holt. 1965. Numerical taxonomy of certain coryneform bacteria. J. Bacteriol. 90: 921-927.

30. DeVries, W., W. M. G. van Wijek-Kapteyn, and A. H. Stouthamer. 1972. Influence of oxygen on growth, cytochrome synthesis and fermentation pattern in propionic acid bacteria. J. Gen. Microbiol. 71: 515-524.

31. Douglas, H. C., and S. E. Gunter. 1946. The taxonomic position of Corynebacterium acnes. J. Bacteriol. 52: 15-23.

32. Giddens, W. E. Jr., K. K. Keahey, G. R. Carter, and C. K. Whitehair. 1968. Pneumonia in rats due to infection with Corynebacterium kutscheri. Path. Vet. 5: 227-237.

33. Gordon, R. E., and J. M. Mihm. 1957. A comparative study of some strains received as Nocardiae. J. Bacteriol. 73: 15-27.

34. Gordon, R. E. 1966. Some strains in search of a genus--Corynebacterium, Mycobacterium, Nocardia or what? J. Gen. Microbiol. 43: 329-343.

35. Goodfellow, M., and G. Aldersan. 1977. The Actinomycete genus Rhodococcus: A home for the 'rhodochorous' complex. J. Gen. Microbiol. 100: 99-122.

36. Goodfellow, M., M. D. Collins, and D. E. Minnikin. 1976. Thin-layer chromatographic analysis of mycolic acids and other long chain components in whole-organism methanalysates of coryneform and related taxa. J. Gen. Microbiol. 96: 351-358.

37. Goodfellow, M., A. Lind, H. Mordarska, S. Pattyn, and M. Tsukamura. 1974. A. Cooperative numerical analyses of cultures considered to belong to the 'rhodochorous' taxon. J. Gen. Microbiol. 85: 291-302.

38. Gottwald, M., J. R. Andreesen, J. LeGall, and L. G. Ljundahl. 1975. Presence of cytochrome and menaquinone in Clostrium formicoaceticum and Clostridium thermoaceticum. J. Bacteriol. 122: 325-328.

39. Harrigan, W. F. 1966. The nutritional requirements and biochemical reactions of Corynebacterium bovis. J. Appl. Bacteriol. 29: 380-391.

40. Harrington, B. J. 1966. A numerical taxonomical study of some corynebacteria and related organisms. J. Gen. Microbiol. 45: 31-40.

41. Henis, Y., J. R. Gould, and M. Alexander. 1966. Detection and identification of bacteria by gas chromatography. Appl. Microbiol. 14: 513-524.

42. Holdeman, L. V., and W. E. C. Moore (eds.). 1975. Anaerobe laboratory manual. VPI. Anaerobe Laboratory, Blacksburg, Virginia.

43. Jacobs, N. J., and M. J. Wolin. 1963. Electron-transport system of Vibrio succinogenes. I. Enzymes and cytochromes of the electron-transport system. Biochim. Biophys. Acta 69: 18-28.

44. Jacobs, N. J., and M. J. Wolin. 1963. Electron-transport system of Vibrio succinogenes. II. Inhibition of electron transport by 2-Heptyl 4-hydroxyquinoline N-oxide. Biochim. Biophys. Acta 69: 29-39.

45. Jensen, H. L. 1952. The corynebacteria. Annu. Rev. Microbiol. 6: 77-79.

46. Jensen, H. L. 1966. Some introductory remarks on the coryneform bacteria. J. Appl. Microbiol. 29: 13-16.

47. Johnson, J. L., and C. S. Cummins. 1972. Cell wall composition and deoxyribonucleic acid similarities among the anaerobic coryneforms, classical propionibacteria, and a strain of Arachnia propionica. J. Bacteriol. 95: 893-900.

48. Jones, D. 1975. A numerical taxonomic study of coryneform and related bacteria. J. Gen. Microbiol. 87: 52-96.

49. Kamen, M. D., and L. P. Vernon. 1955. Comparative studies on bacterial cytochromes. Biochim. Biophys. Acta 17: 10-22.

50. Keddie, R. M., and B. L. Cure. 1977. The cell wall composition and distribution of free mycolic acids in named strains of corynebacteria and in isolates from various natural sources. J. Appl. Bacteriol. 42: 229-252.

51. Keilin, J. 1966. The history of cell respiration and cytochrome. pp. 140-164. The University Press, Cambridge.

52. Komura, I., K. Yamada, S. Otsuke, and K. Komagata. 1975. Taxonomic significance of phospholipids in coryneform and nocardioform bacteria. J. Gen. Appl. Bacteriol. 21: 251-265.

53. Kovacs, N. 1956. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature (London) 178: 703.

54. Krogstad, D. J., and J. L. Howland. 1966. Role of menaquinone in Corynebacterium diphtheriae electron transport. Biochim. Biophys. Acta 118: 189-191.

55. Kufe, W. D., and J. L. Howland. 1968. Oxidative phosphorylation in Corynebacterium diphtheriae. Biochim. Biophys. Acta 153: 291-293.

56. Lehmann, K. B., and R. O. Neumann. 1896. Bakteriologische Diagnostik. Lehman verlag, Munich.

57. Lehninger, L. A. 1975. Biochemistry. pp. 491-496. Worth Publishers, Inc., New York.

58. Lelliott, R. A. 1966. The plant pathogenic coryneform bacteria. 29: 114-118.

59. Lenohoff, H. M., D. J. D. Nicholas, and N. O. Kaplan. 1956. Effects of oxygen, iron and molybdenum on routes of electron transfer in Pseudomonas fluorescens. J. Biol. Chem. 220: 983-995.

60. Lopez, J. F., F. M. Wong, and J. Quesada. 1966. Corynebacterium pseudotuberculosis, first case of human infection. Am. J. Clin. Path. 46: 562-567.

61. Magnusson, H. 1923. Spezifische infektiose pneumonie bein fohlen. Ein neurer eitreneger beim pferde. Arch Wiss Prakt Tierheilk 50: 22-38.

62. Magnusson, H. 1938. Pyaemia in foals caused by Corynebacterium equi. Vet. Rec. 50: 1459-1468.

63. Macy, J., I. Probst, and G. Gottschalk. 1975. Evidence for cytochrome involvement in fumarate reduction and adenosine 5'-triphosphate synthesis by Bacterioides fragilis grown in the presence of hemin. J. Bacteriol. 123: 436-442.

64. Mandle, R. J., and T. J. Wade. 1976. The use of gas chromatography for the identification of microorganisms. pp. 163-180. In J. E. Prier, J. T. Bartola, and H. Friedman (eds.), Modern methods in medical microbiology, University Park Press, Baltimore, Md.

65. Mara, M., D. Klavodovka, F. Patocka, and M. Veselska. 1973. The effect of haemin on growth and cytochrome production in Corynebacterium haemolyticum. Acta Univ. Carolinae Med. 19: 197-206.

66. Mara, M., D. Klavodovka, and F. Patpcka. 1971. Correlation of growth and cytochrome b_1 production in Corynebacterium haemolyticum to the haemin concentration in the medium. Folia Microbiologica 16: 520-526.

67. Moitra, A. K. 1972. Incidence of Corynebacterium equi in bovine pneumonic lungs. Indian Vet. J. 49: 973-974.

68. Mitruka, B. M., and M. Alexander. 1968. Rapid and sensitive detection of bacteria by gas chromatography. Appl. Microbiol. 16: 636-640.

69. Moore, W. E. C., and E. P. Cato. 1963. Validity of Propionibacterium acnes (Gilchrist) Douglas and Gunter. comb. nov. J. Bacteriol. 85: 870-874.

70. Moore, W. E. C. 1970. Relationship of metabolic products to taxonomy of anaerobic bacteria. Int. J. Syst. Bacteriol. 20: 535-538.

71. Moss, C. W., and V. R. Cherry. Characterization of C_{15}-branched-chain fatty acids of Corynebacterium acnes by gas chromatography. J. Bacteriol. 95: 241-242.

72. Moss, C. W., V. R. Dowell, Jr., D. Farshtchi, L. J. Raines, and W. B. Cherry. 1969. Cultural characteristics and fatty acid composition of propionibacteria. J. Bacteriol. 97: 561-570.

73. Moss, C. W., V. R. Dowell, Jr., V. J. Lewis, and M. A. Schekter. 1967. Cultural characteristics and fatty acid composition of Corynebacterium acnes. J. Bacteriol. 94: 1300-1305.

74. Newton, J. W., and M. D. Kamen. 1961. Cytochrome systems in anaerobic electron transport. pp. 397-423. In I. C. Gunsalus and R. Y. Stainer (eds.). The Bacteria II. Academic Press, New York.

75. Oyama, V. I. 1963. Use of gas chromatography for the detection of life on Mars. Nature (London) 200: 1058-1059.

76. Pappenheimer, A. M., Jr. 1947. Bacterial toxins. Fed. Proc. 6: 479-484.

77. Pappenheimer, A. M., Jr. 1947. Diphtheria toxin. III. A reinvestigation of the effect of iron on toxin and porphyrin production. J. Biol. Chem. 169: 251-259.

78. Pappenheimer, A. M., Jr. 1959. Cytochromes of C. diphtheriae. Fed. Proc. 18: 590.

79. Pappenheimer, A. M., Jr., and E. D. Hendee. 1947. Diphtheria toxin. IV. The iron enzyme of Corynebacterium diphtheriae and their possible relation to diphtheria toxin. J. Biol. Chem. 171: 701-713.

80. Pappenheimer, A. M., Jr., and E. D. Hendee. 1949. Diphtheria toxin. V. A comparison between the diphtherial succinoxidase system and that of beef heart muscle. J. Biol. Chem. 180: 597-609.

81. Pappenheimer, A. M., Jr., J. J. L. Howland, and P. A. Miller. 1962. Electron transport systems in Corynebacterium diphtheriae. Biochim. Biophys. Acta 64: 229-242.

82. Peck, H. D. 1966. Phosphorylation coupled with electron transfer in extracts of the sulfate reducing bacterium Desulfovibrio gigas. Biochem. Biophys. Res. Commun. 22: 112-118.

83. Postgate, J. R. 1954. Presence of cytochrome in an obligate anaerobe. Biochem. J. 56: xi-xii.

84. Ramlinson, W. A., and J. H. Hale. 1949. Prosthetic groups of the cytochromes present in Corynebacterium diphtheriae with special reference to cytochrome a. Biochem. J. 45: 247-255.

85. Reddy, C. A., C. P. Cornell, and M. Kao. 1977. Hemin-dependent growth stimulation and cytochrome synthesis in Corynebacterium pyogenes. J. Bacteriol. 130: 965-967.

86. Reddy, C. A. 1967. Studies on DNA base composition and cytochromes in certain species of the genus Bacteroides. M. S. thesis, Univ. of Ill. Urbana.

87. Reddy, C. A., and C. P. Cornell. 1977. A study of certain nutritional and metabolic features of Corynebacterium pyogenes. Abstr. Annu. Mtg. Am. Soc. Microbiol. I 51.

88. Rizza, V., P. R. Sinclair, D. C. White, and P. R. Courant. 1968. Electron transport system of the protoheme-requiring anaerobe Bacteroides melaninogenicus. J. Bacteriol. 96: 665-671.

89. Roberts, D. S. 1967. The pathogenic synergy of Fusiformis necrophorus and Corynebacterium pyogenes. II. The response of F. necrophorus to a filterable product of C. pyogenes. Brit. J. Exptl. Pathol. 48: 674-679.

90. Roberts, D. S., N. P. H. Graham, J. R. Egertan, and I. M. Paronson. 1968. Infective bulbar necrosis (heel-abscess) of sheep, a mixed infection with Fusiformis necrophorus and Corynebacterium pyogenes. J. Comp. Pathol. 78: 1-8.

91. Roberts, R. J. 1968. A numerical taxonomic study of 100 isolates of Corynebacterium pyogenes. J. Gen. Microbiol. 53: 299-303.

92. Robinson, K. 1966. An examination of Corynebacterium species by gel electrophoresis. J. Appl. Bacteriol. 29: 179-184.

93. Rogosa, M., C. S. Cummins, R. A. Lettiott, and R. M. Keddie. 1974. Coryneform group of bacteria. pp. 599-632. In R. Buchanan and N. Gibbons (eds.), Bergey's manual of determinative bacteriology. The Williams and Wilkins Co., Baltimore, Maryland.

94. Skerman, T. M., and D. J. Jayne-Williams. 1966. Nutrition of coryneform bacteria from milk and dairy sources. J. Appl. Bacteriol. 29: 167-178.

95. Skerman, V. B. D. 1967. A guide to the identification of the genera of bacteria. pp. 174-177. The Williams and Wilkins Co., Baltimore, Maryland.

96. Scholes, P. B., and H. K. King. 1965. Electron transport in a Park-Williams strain of Corynebacterium diphtheriae. Biochem. J. 97: 754-765.

97. Smith, L. 1961. Cytochrome systems in aerobic electron transport. pp. 365-395. In I. C. Gunsalus and R. Y. Stanier (eds.), The bacteria II. Academic Press, New York.

98. Smith, R. F. 1969. Characterization of human cutaneous lipophilic diphtheroids. J. Gen Microbiol. 55: 433-443.

99. Sneath, P. H. A., and O. S. T. Cowan. 1958. An electro-taxonomic survey of bacteria. J. Gen. Microbiol. 19: 551-565.

100. Sperry, J. F., and D. T. Wilkins. 1975. Cytochrome spectrum of an obligate anaerobe, Eubacterium lentum. J. Bacteriol. 125: 905-909.

101. Stuart, M. R., and E. P. Pease. 1972. A numerical study of the relationships of Listeria and Erysipelothrix. J. Gen. Microbiol. 73: 551-565.

102. Suto, T., M. Minato, S. Ishibashi, R. Azuma, and K. Ogimoto. 1970. Gas chromatography as an aid for differentiation of strict anaerobes. pp. 387-395. In H. Iizuka and T. Hasegawa (eds.), Culture collection of microorganisms. University Park Press, Baltimore, Maryland and Manchester, England.

103. Tasman, A., and A. C. Branwijk. 1938. Experiments on metabolism with diphtheriae bacillus, II. J. Inf. Dis. 63: 10-20.

104. Teale, F. W. J. 1959. Cleavage of the haem-protein link by acid methyl ethyl ketone. Biochim. Biophys. Acta 35: 543-546.

105. Veldcamp, H. 1970. Saprophytic coryneform bacteria. Ann. Rev. Microbiol. 24: 209-240.

106. Vernon, L. P., and F. G. White. 1957. Terminal oxidases of Micrococcus denitrificans. Biochim. Biophys. Acta 25: 321-328.

107. Waring, W. S., and C. H. Werkman. 1944. Iron deficiency in bacterial metabolism. Arch. Biochem. 4: 75-87.

108. White, D. C., M. P. Bryant, and D. R. Caldwell. 1962. Cytochrome-linked fermentation in Bacteroides ruminicola. J. Bacteriol. 84: 822-828.

109. Wilson, G. S., and A. A. Miles. 1964. Topley and Wilson's principles of bacteriology and immunity. pp. 583-613. The Williams and Wilkins Co., Baltimore, Maryland.

110. Yamada, K., and K. Komagata. 1970. Taxonomic studies on corynebacteria. III. DNA base composition of corynebacteria. J. Gen. Appl. Microbiol. 16: 215-224.

111. Yanagawa, R., and K. Otsuki. 1970. Some properties of the pili of Corynebacterium renale. J. Bacteriol. 101: 1063-1069.

112. Yaoi, H., and H. Tamiya. 1928. On the respiratory pigment, cytochrome, in bacteria. Proc. Imp. Acad. 4: 436-439.


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