Effects of Sodium Deoxycholate on Selected Gram-Negative Bacteria

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EFFECTS OF SODIUM DEOXYCHOLATE ON SELECTED
GRAM-NEGATIVE BACTERIA

by

Anthony P. D'Mello

A Thesis Submitted to the Faculty of the Graduate School
of Loyola University of Chicago in Partial Fulfillment
of the Requirements for the Degree of
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LIFE

The author, Anthony Paul D'Mello, is the son of Mr. Ubaldin D'Mello and the late Mrs. Rosie D'Mello. He was born on April 18, 1948 in Karachi, Pakistan.

His elementary education was obtained at St. John Vianney and his secondary education at St. Patrick's High School, Karachi, where he graduated in 1964.

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Following graduation, he worked as a clinical microbiologist at the Seventh Day Adventist Hospital in Karachi, until he migrated to the United States of America in January 1975. From June 1975 to June 1976 he worked as a medical technologist for Rush-Presbyterian-St. Luke's Medical Center (Chicago, Illinois). In 1976 he became a registered microbiologist with the American Society of Clinical Pathologists.

In July 1976 he entered the Graduate School in the Department of Microbiology at Loyola University Medical Center.
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LIST OF ABBREVIATIONS

ATCC  American Type Culture Collection
°C     degrees centigrade
cfu    colony forming units
DNA    deoxyribonucleic acid
Fig.   figure
h      hour
kV     kilovolts
lb/in² pounds per square inch
M      molar
mCi    millicurie
mg     milligram
min    minute
ml     milliliter
mm     millimeter
mmol   millimoles
N      normal
OD     optical density
RNA    ribonucleic acid
TCA    trichloroacetic acid
TSA    trypticase soy agar
TSB    trypticase soy broth
μCi    microcurie
μm     micrometer
vol    volume
wt     weight
INTRODUCTION AND REVIEW OF LITERATURE

Deoxycholic acid is present as a conjugate of taurine and glycine in the bile of many animals including man and oxen (Haslewood, 1967), and plays a part in fat digestion. It is produced in the gut by the bacterial conversion of cholic acid.

The bacteriological media frequently used for the isolation of gram-negative organisms contain bile salts like sodium deoxycholate (Leifson, 1935; Anonymous, 1968, 1972) which inhibit the growth of gram-positive organisms.

The gram-negative bacteria have been shown to be relatively resistant to the action of detergents, a property attributed to the unique character of the cell wall structure (Nixdorff et al., 1977). The envelope of gram-negative bacteria is a complex structure which consists of an outer membrane, an intermediate layer composed of peptidoglycan and an inner cytoplasmic membrane (Costerton et al., 1974). The outer membrane acts as a permeability barrier for large oligo-peptides (Payne and Gilvary, 1968), neutral sugars with molecular weights more than 700 (Nakae, 1976) and hydrophobic dyes and antibiotics (Nikaido, 1976). It has been shown to exhibit marked stability to disruption by surface active agents. Detergent induced lysis is observed only when the outer membrane and peptidoglycan layers are disrupted with a lysozyme-ethylene diaminetetraacetic acid treatment (Godson and Sinsheimer, 1967; Birdsell and Cota-Robles, 1968) or the cells are metabolically unbalanced by pretreatment with
KCN (Woldringh and van Iterson, 1972) or UV irradiation (Swenson and Schenley, 1974). Ethylenediaminetetraacetic acid has been shown to affect the cell surface of *Escherichia coli* by causing the loss of a large fraction of the surface lipopolysaccharide (Leive et al., 1968) and thereby increasing its permeability to substances that normally cannot enter (Leive, 1965).

Surface active agents have been known for many years to possess bactericidal activity (Volko, 1946) and in some cases bacteriolytic activity as well (Godson and Sinsheimer, 1967). The bacteriolytic effect of ionic detergents are attributed to their ability to electrostatically bind to and denature cellular enzymes (Volko, 1946) or to disrupt the cellular membrane, causing the leakage of vital cellular components to the surrounding medium (Woldringh and van Iterson, 1972).

Several studies have indicated that the ionic detergent Triton X-100 has a specific solubilization effect on the cytoplasmic membrane of *E. coli*. Birdsell and Cota-Robles (1968) observed that this detergent lysed spheroplasts of *E. coli* which contained exposed regions of cytoplasmic membranes. DePamphilis and Adler (1971) isolated the outer membrane of *E. coli* spheroplasts with Triton X-100. Schnaitman (1971a, 1971b) purified cell walls of *E. coli* by using Triton X-100 to remove the contaminating cytoplasmic membrane fragments.

The murein layer of bacteria acts as a penetration barrier either directly or indirectly by virtue of its intimate association with the lipoprotein of the outer membrane (Braun et al., 1970). Nixdorff et al. (1977) studied the effect of sodium deoxycholate on
the solubilization of membrane vesicles composed of phospholipids alone. Disruption of the membranes was observed in the presence of deoxycholate. The incorporation of outer membrane protein protected these model membranes from the detergent action. This suggested that specific outer membrane proteins play an essential role in the stabilization of the outer membrane barrier during survival and growth of gram-negative bacteria in the presence of detergents.

The action of deoxycholate on bacterial cell surface is not understood well enough to permit specific interpretation, but the findings are in keeping with the conclusion that mutants tolerant to phage or antibiotics are altered in some surface component (Nagel de Zwais and Luria, 1967). In order to show that certain mutants of *E. coli* had altered membrane properties, investigators have determined that the mutants and the parents had different susceptibilities to deoxycholate (Nagel de Zwais and Luria, 1967; Lazdanski and Shapiro, 1972). Mutants of *E. coli* which develop tolerance to antibiotics (Miyosho and Yamagata, 1976) or T-even phages (Nishikawa and Maruo, 1976) have been found to have altered sensitivity to antibiotics, dyes and detergents. Since the effect of these various agents is determined by the integrity of the cell surface, it is suggested that mutation involving changes in the cell envelope is accountable for the increased sensitivity to sodium deoxycholate (Nishikawa and Maruo, 1976). Temperature sensitive mutants of *E. coli* (Hirota et al., 1970; Sturgeon and Ingram, 1978) and *Salmonella typhimurium* (Ciesla and Bagdashrian, 1972) have been shown to have increased sensitivity to deoxycholate, which suggests an altered cell envelope in these
mutants. Burman et al. (1972) found that when the penetration barrier of the murein layer was partially opened by growth of gram-negative bacteria in the presence of lysozyme and sublethal concentrations of ampicillin, increased penetration of sodium cholate was observed. Physiological data indicated that the increased sensitivity to cholate, induced by growth in the presence of ampicillin or lysozyme, was due to effects upon the murein layer.

**Proteus mirabilis** and **Proteus vulgaris** have been shown to exhibit swarming when grown on solid media (Buchanan and Gibbons, 1975). Hoeniger (1965) has shown that swarming of Proteus is accompanied by morphological changes in the cells. Nonswarming cells are about 2 to 4 µm in length, whereas swarm cells reach lengths of 80 µm and possess many more flagella per unit of surface area than the short forms. The swarming occurs with periodicity, that is, swarming proceeds for a period of time and then stops. The cessation of swarming is accompanied by division of the swarm cells to form short cells. These resulting short cells undergo a period of growth and then new swarm cells are formed at the periphery of the growth zone and swarming starts again. This zonation effect produces concentric rings of swarming.

Two theories have been proposed to explain the swarming phenomenon. According to the positive chemotaxis theory of Moltke (1927), the local over-population of bacteria exhausts the nutrients in the area and the resultant gradient of nutrients at the periphery encourages outward growth and movement. The other widely accepted theory is the negative chemotaxis hypothesis of Lominski and Lendrum (1947)
which proposes that swarming is a response to metabolic products which accumulate during growth of the non-swarming organisms. During growth of *Proteus* on a solid surface the cells excrete one or more toxic waste products, which inhibit cell division, thus leading to the formation of swarm cells. The toxic agent diffuses through the agar medium, out from the region of growth, establishing a gradient of the toxic substance. The swarm cells detect the gradient and move over the agar surface down the gradient towards a lower concentration of the toxic agent (negative chemotaxis). When the cells have moved to a subtoxic region, the inhibition of division is relieved and the swarm cells stop swarming and start dividing. The growth of short forms results once again in the production of toxic substances, swarm cells are again produced, and swarming starts again. Recent work of Williams et al. (1976) using nonchemotactic and nonswarming mutants has provided evidence against the involvement of chemotaxis in the swarming of *Proteus mirabilis*.

Several antibiotics and metabolic inhibitors that suppress swarming have been described, and a number of media containing specific inhibitors have been recommended for clinical use. Among these are media containing neomycin (Elston, 1965), sulfonamides (Holman, 1957), a combination of polymyxin, neomycin and fusidic acid (Lowbury et al., 1964) or sodium azide (Snyder and Lichstein, 1940). A variety of surface active agents have also been shown to inhibit the swarming of *Proteus* (Lominski and Lendrum, 1942).

The presence of 0.1% sodium deoxycholate in the medium has been shown to inhibit the swarming of *Proteus* and to render some gram-
negative flagellated bacteria non-flagellated and non-motile (Leifson, 1935). In *Vibrio alginolyticus* the inhibition of flagellation has been observed in the presence of bile salts (0.1%), sodium deoxycholate (0.1%), sodium taurocholate (2.5%) and sodium lauryl sulfate (0.05%) (DeBoer et al., 1975). All the detergents, except sodium taurocholate, were found to inhibit swarming of the vibrios.

The recommended methods for monitoring the environment for enteric bacilli call for the utilization of differential culture media some of which contain sodium deoxycholate as an inhibitory agent against gram-positive bacteria. Although deoxycholate is supposed to be innocuous to the enteric organisms, it influences the physiology of these bacteria (Leifson, 1935). Furthermore, growth-rate studies using this medium have not been reported and growth has only been scored as slight, fair and good.

The purpose of this research is to study the effects of sodium deoxycholate on selected gram-negative bacteria. The effect of deoxycholate on the growth parameters, oxygen consumption, and uptake of nutrients from the medium will be determined and an attempt will be made to investigate its effect on the synthesis of macromolecules, flagella, and the activity of certain enzymes and other cellular components.
MATERIALS AND METHODS

Cultures:

Escherichia coli ATCC 25922, Salmonella typhimurium ATCC 13311, Enterobacter cloacae ATCC 23355, Klebsiella pneumoniae ATCC 13883, and a strain of Proteus mirabilis isolated from clinical material were obtained from the stock collection of the clinical microbiology laboratory, Loyola University Medical Center. Stock cultures were maintained on trypticase soy agar (BBL) slants at 4°C and were transferred to new slants every four weeks. Cultures were tested periodically for gram reaction, indole production, citrate utilization, glucose, sucrose and lactose fermentation, reaction on triple sugar iron medium and growth on Eosin-Methylene Blue and MacConkey's agar. Antibiograms were determined by the Bauer-Kirby method (Bauer et al., 1966).

Chemicals:

Sodium deoxycholate was purchased from Difco Laboratories, Detroit, Michigan. Chemicals used for counting with the scintillation fluor; anisole, p-dioxane and 1,3-dimethoxyethane from Eastman Organic Chemicals, Rochester, New York. Crystalline 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(5-phenyloxazoyl)]-benzene (POPOP) from Packard Instrument Company, Downers Grove, Illinois. [U-14C] glucose, [1-14C] glucose and [U-14C] leucine from New England Nuclear, Boston, Massachusetts. All other reagents and chemicals were of analytical grade.
Measurement of Radioactivity:

Aliquots of 0.1 ml of the filtrates, the supernatants, the soluble fractions, or the dried membrane filters (Millipore Corporation, New Bedford, Massachusetts, 25 mm diameter) were added to 10 ml of scintillation fluor containing 18 g PPO, 60 mg POPOP, 900 ml p-dioxane, 150 ml anisole, and 150 ml 1,2-dimethoxyethane (Davidson and Feigelson, 1957). The $^{14}$C-activity was determined in a Packard Tri-Carb liquid scintillation spectrometer, model 3320. In this system $^{14}$C was counted with an eighty to eighty-five percent efficiency.

Determination of Dry Weight:

An 18 h TSB culture grown at 37°C was harvested by centrifugation, washed twice with saline and suspended in 0.1 M sodium phosphate buffer, pH 7.0, at densities of 100, 200 and 300 Klett units. To measure the dry weight, 10 ml of the cell suspension was added to tared aluminum foil planchets, which had been predried to a constant weight. The control planchet contained 10 ml of sodium phosphate buffer. The planchets were then dried to a constant weight in a 60°C drying oven.

Growth Studies:

Initial growth studies were performed by observing changes in optical density. A culture grown for 24 h in trypticase soy broth (BBL) was diluted in 0.85% sodium chloride and 0.1 ml of a $10^{-4}$ dilution was inoculated into capped Klett tubes containing 7.0 ml deoxycholate broth with different concentrations of deoxycholate. The tubes were incubated statically at 37°C and the change in cell density
was periodically determined with a Klett-Summerson photoelectric colorimeter using a no. 42 filter. The increase in optical density was taken as a measure of the growth of the organisms.

For growth studies using plate counts, an overnight culture grown in trypticase soy broth (TSB) at 37°C was harvested by centrifugation, washed in saline and a homogenous suspension made by manually shaking for 15 min in an Erlenmeyer flask containing glass beads (3 mm diameter). The density was adjusted to 10 Klett units by the use of a Klett-Summerson photoelectric colorimeter using a no. 42 filter. From this stock solution 1.0 ml aliquots of a $10^{-3}$ dilution were inoculated into deoxycholate medium. The flasks were incubated at 37°C and at intervals titrated by the pour plate method, using trypticase soy agar as the plating medium. Colonies developing after 48 h incubation at 37°C were counted. All samples were run in triplicate. Growth parameters were calculated according to the method of Monod (1949).

**Manometric Studies:**

**Calibration of Respiratometers.** Warburg Flask constant $K$, was calculated by the formula:

$$K = \frac{V_g \cdot 273 + V_f \cdot \alpha}{T} ,$$

where $p_0$.

$V_g$ was the volume of the gas in the closed system and equal to the volumes of flask and manometer.

$V_f$ was the volume of the fluid phase (3.2 ml).

$\alpha$ was the solubility of oxygen at a given temperature (0.024 at 37°C).
P₀ was the standard pressure, which is a constant equal to 760 mm mercury or 10,000 mm Brodie's manometer fluid at one atmosphere pressure as compared to mercury.

Calibration was performed as outlined by Lazarow (1949) using a Warburg Manometer Calibrator (Micrometric Instrument Co., 7929 Kinsman Ave., Cleveland, Ohio).

**Manometric Assay.** Cells for the Warburg studies were harvested from cultures grown at 37°C for 24 h in trypticase soy broth. The cells were washed twice with 0.1 M sodium phosphate buffer (pH 7.0) and a homogenous suspension was prepared by shaking in a 250 ml Erlenmeyer flask with glass beads. The suspension was adjusted to 100 Klett units on a Klett-Summerson photoelectric colorimeter using a no. 42 filter. This suspension contained 0.46 mg dry weight of cells per ml.

Oxygen uptake was studied according to the method of Umbreit et al. (1964) using glucose as a substrate. In a typical experiment 2.2 ml of the cell suspension was pipetted into the main flask of the Warburg vessel and 0.8 ml of 0.5% glucose with or without 0.1% (wt/vol) sodium deoxycholate in the side arms. The center well contained a folded strip of filter paper saturated with 0.2 ml of 40% KOH solution to absorb CO₂ present or produced by the bacteria during respiration. The manometers were equilibrated at 37°C for 30 min before taking the initial readings. Readings were taken every 15 min for 2-3 h and the rates of oxygen consumption were determined by the conventional manometric technique. The oxygen consumption values obtained were corrected for endogenous respiration (buffer in side arm) and
expressed as µl of oxygen consumed/mg dry wt of cells/h \( (Q_{O_2}) \).

**Effect of Deoxycholate on Unfractionated Cells:**

An 18 h aerated TSB culture of *E. coli* grown at 37°C was harvested by centrifugation. The cells were washed twice with saline and suspended in 0.1 M sodium phosphate buffer, pH 7.0, at a density of 200 Klett units. Sodium deoxycholate at a final concentration of 0.1% (wt/vol) was added to the experimental flask; the control flask did not receive any deoxycholate. Both flasks were incubated at 37°C on an oscillatory shaker incubator (Model R 26, New Brunswick Scientific Co., Inc., New Brunswick, N. J.) at a speed of 100 oscillations/min. At intervals, a 3.0 ml sample was removed and centrifuged at 10,000 x g for 10 min. The supernatant was saved for protein determination by the Lowry method (Lowry et al., 1951) using bovine serum albumin as standard and carbohydrate determination by the method of Scott and Melvin (1953), using glucose as standard.

In an alternate experiment, 1.0 ml of an overnight culture was inoculated into an Erlenmeyer flask containing 100 ml TSB and incubated at 37°C on an oscillatory shaker for 6 h, after which time the cells were harvested by centrifugation at 10,000 x g for 10 min. The cells were washed twice with saline and suspended in 0.1 M sodium phosphate buffer, pH 7.0, at a density of 200 Klett units. After 30 min incubation at 37°C, 5.5 µCi \([1^{14}C]\) glucose (specific activity 3 mCi/mmol) was added and incubation continued for an additional 30 min. Excess radioactivity not taken up by the cells was removed by centrifugation at 10,000 x g for 10 min and subsequent washing in cold
buffer. The cells were suspended in 0.1 M sodium phosphate buffer at a density of 100 Klett units and incubated at 37°C on a shaker water-bath, in the presence and absence of 0, 0.05, 0.1 and 0.2% (wt/vol) deoxycholate. At intervals of 15 min, 30 min, 1 h, 2 h, 4 h and 24 h, 1.0 ml samples of cell suspension were removed and centrifuged at 5,000 x g for 5 min. Aliquots of 0.25 ml of the supernatant were placed in scintillation fluid and assayed for radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320.

**Fractionation Studies:**

An 18 h TSB culture was harvested, washed twice with saline and suspended in 0.1 M sodium phosphate buffer, pH 7.0, at a density of 200 Klett units using a Klett-Summerson photoelectric colorimeter with a no. 42 filter. To 100 ml of bacterial suspension 1.35 μCi [U-14C] D-glucose (specific activity 237 mCi/mmol) was added. After labeling for 30 min, the cells were harvested, washed and suspended in 0.1 M phosphate buffer, pH 7.0, at a density of 200 Klett units. The cells were distributed in 4 tubes, deoxycholate added at final concentrations of 0, 0.05, 0.1 and 0.2% (wt/vol) and incubated at 37°C for 24 h. The bacteria were then fractionated by the method of Park and Hancock (1960). The washed cells were suspended in 5% trichloroacetic acid and maintained at 4°C for 10 min. The preparation was then centrifuged and the supernatant fluid tested for radioactivity (cold TCA soluble fraction). The pellet was suspended and extracted with 75% ethanol for 10 min at room temperature. The extract was centrifuged and the supernatant assayed for radioactivity (alcohol soluble
fraction). The alcohol insoluble pellet was treated with 5% TCA at 90°C for 6 min. The hot TCA soluble fraction obtained after centrifugation was assayed for radioactivity. The pellet was trypsinized for 75 min at 37°C in 2.0 ml solution of 0.05 M (NH₄)₂CO₃, 0.005 N NH₄OH (pH 8.2) and 0.1% (wt/vol) trypsin (E. H. Sargent Co., Chicago, Illinois). The preparation was passed through a membrane filter (Millipore Corporation, New Bedford, Mass.), pore size 0.45 μm, 25 mm diameter, to remove the remaining insoluble residue and the filtrate was tested for radioactivity (trypsin-insoluble protein fraction). Filters containing the residue were air-dried and placed directly in scintillation fluid (residue fraction).

Effect of Deoxycholate on the Uptake of [1-¹⁴C] Glucose:

A nephelometric flask containing 150 ml TSB was inoculated with 0.1 ml of a 18 h TSB culture and incubated on a rotary shaker at 37°C. The cells were harvested, washed twice with 0.1 M sodium phosphate buffer, pH 7.0, and suspended in the same buffer at a density of 200 Klett units. The cells were treated with deoxycholate at a final concentration of 0.1% (wt/vol). After 1 h incubation at 37°C, 0.2 μCi [1-¹⁴C] glucose (specific activity 15 μCi/mmol) was added. At intervals of 5, 15 and 30 min, 1.0 ml samples of cell suspensions were filtered through membrane filters (pore size 0.22 μm) and washed with three 1.0 ml volumes of cold 0.1 M phosphate buffer. The filters were dried, placed in vials containing scintillation fluor and assayed for radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320. The quantity of radioactivity retained on
the filter was taken to be a direct reflection of the total uptake of \([1-^{14}\text{C}]\) glucose by the bacteria.

In an alternative experiment after washing, the cells were suspended, at a density of 200 Klett units, in 'C' medium (Roberts et al., 1955) without glucose. The 'C' medium consisted of \(\text{NH}_4\text{Cl} 2\ \text{g}, \\text{Na}_2\text{HPO}_4 6\ \text{g}, \\text{KH}_2\text{PO}_4 3\ \text{g}, \\text{NaCl} 3\ \text{g}, \text{MgCl}_2 0.810\ \text{g}, \text{Na}_2\text{SO}_4 0.026\ \text{g},\) distilled water 1 liter. The cells were incubated on a shaker water-bath (Dubnoff Metabolic Shaking Incubator, Precision Scientific Co., Chicago, Ill.) for 30 min at a speed of 100 rev/min. Sodium deoxycholate and 1 \(\mu\text{Ci} [1-^{14}\text{C}]\) glucose (specific activity 3 mCi/mmol) were both added at the same time. At intervals of 15, 30, 60 and 120 sec, 0.2 ml samples were removed and filtered through 0.22 \(\mu\text{m}\) membrane filters. The filters were washed with three 1.0 ml volumes of ice cold 0.1 ml phosphate buffer, dried and assayed for radioactivity.

Chemical Analysis of Cells Grown in the Presence and Absence of Sodium Deoxycholate:

Media and Growth Conditions. Deoxycholate basal medium was prepared by dissolving the ingredients at double concentration and sterilized by filtration through 0.45 \(\mu\text{m}\) membrane filters. Three percent agar solution was sterilized separately by autoclaving at 15 lb/in\(^2\) pressure for 15 min. Equal volumes of broth and agar solution were mixed. Sodium deoxycholate at a final concentration of 0.1% (wt/vol) was added to the experimental flask and poured into petri plates. The plates were swabbed with a mid-logarithmic (6 h) TSB culture of \textit{Escherichia coli} ATCC 25922 and incubated at 37°C.
for 18 h. The growth was harvested in sterile saline and filtered through sterile gauze to remove any agar particles. When the bacteria were grown in broth containing deoxycholate, a white precipitate was formed which was insoluble in water and difficult to separate from the cells. This was probably due to the production of acidity from the fermentation of lactose, converting sodium deoxycholate to deoxycholic acid, which is insoluble in water. Furthermore, after washing, the cells gave a positive test for deoxycholic acid. To overcome this problem the cells were grown on agar plates and the growth washed thrice with saline and thrice with 0.05 M Tris-HCl buffer pH 7.2. The washed cells were lyophilized and stored at -20°C for further use. For chemical analysis the vials containing the lyophilized bacteria were warmed to room temperature and placed in a vacuum desiccator before weighing.

**Determination of Deoxycholate Present in the Cells.** Sodium deoxycholate present in the lyophilized bacterial cells was determined according to the method of Mosbach et al. (1954). A standard curve was made using cells grown in the absence of deoxycholate at a density of 1 mg dry wt/ml of 0.05 M Tris-HCl buffer, pH 7.2 and supplemented with different concentration of deoxycholate. The amount of deoxycholate present in deoxycholate-grown cells was determined at the same density. To 0.2 ml cell suspension 1.0 ml of 65% sulfuric acid was added and the suspension was incubated in a 60°C water-bath for 15 min. After the heating step the tubes were cooled to room temperature under tap water and absorption measurements made at 385 nm.
Extraction of Lipids. The procedure was as described by Bligh and Dyer (1959) for fish. This method was reported to be more convenient, less time consuming and to give a better yield of extracted lipid, than most other methods. It consisted of extraction of lipids in a monophasic system in which methanol, chloroform and water were in the proportion of 2:1:0.8 (vol/vol/vol). Lyophilized bacteria (100 mg) were suspended in 4.0 ml distilled water and allowed to rehydrate at room temperature for 1 h. Five ml of chloroform and 10 ml of methanol were added and the suspension vortexed for 3 min and left at 0°C for 10 min. The lipids were separated from the water-soluble material by diluting the extracting mixture with 5 ml chloroform followed by 5 ml water. The final ratio of methanol:chloroform:water, was 2:2:1.8 (vol/vol/vol). After centrifugation at 3000 x g for 30 min the chloroform layer was removed by gently inserting a pasteur-pipette through the water-methanol phase and through the pellet which formed at the interphase. The chloroform layer was placed in a tared scintillation vial and evaporated to dryness on a vortex mixer at 10°C. The scintillation vials were weighed and the difference in weight was taken to be the weight of the lipid extracted from the bacteria.

Effect of Deoxycholate on Macromolecular Synthesis. Cells that had been grown in the presence of deoxycholate, and subsequently washed, lyophilized and stored at -20°C, were fractionated by the method of Roberts et al. (1955), as modified by Kerridge (1959). This fractionation procedure is shown in the following diagram. DNA was determined by the diphenylamine method (Burton, 1956) using calf
Fractionation Procedure for Whole Cells (Roberts, Cowie, Abelson, Bolton and Britten (1951) as modified by Kerridge (1959).

\[20 \text{ mg Lyophilized Cells} + 10 \text{ ml (wt/vol) 5\% cold TCA at } 4^\circ\text{C for 1 h}\]

\[\text{centrifuge}\]

\text{Sediment I} \quad \text{Supernatant I (discard)}

\text{Wash with 10 ml (wt/vol) 5\% cold TCA} \quad \text{Extract with 10 ml (vol/vol) 75\% Ethanol at } 45^\circ\text{C for 30 min}

\[\text{centrifuge}\]

\text{Sediment II} \quad \text{Supernatant II}

\text{Extract with 5 ml Ethanol + 5 ml ether at } 45^\circ\text{C for 30 min}

\[\text{centrifuge}\]

\text{Sediment III} \quad \text{Supernatant III combine with Sup II, Add 10 ml dist. water}

\text{Extract X 3 with 0.5 N Perchloric Acid (5 ml) for 15 min at } 70^\circ\text{C}

\[\text{centrifuge}\]

\text{Sediment IV} \quad \text{Supernatant IV} \quad \text{Ether Fraction} \quad \text{Aqueous Layer}

\text{Dissolve in 10 ml N NaOH} \quad \text{1. Read OD at 260 nm} \quad \text{containing ethanol soluble proteins}

\text{Protein determined by Folin's Method} \quad \text{2. Determine RNA --Orcinol} \quad \text{Heat to dryness on}

\text{1. Read OD at 260 nm} \quad \text{2. Determine RNA --Orcinol} \quad \text{boiling water-bath}

\text{3. Determine DNA --Diphenylamine} \quad \text{Dissolve in 2 ml N NaOH}

\text{Estimate Protein with Folin's Reagent}
thymus DNA as standard, and RNA by the orcinol method (Hulbert, 1954) using Bakers' yeast RNA as standard.

**Effect of Deoxycholate on the Synthesis of Glucose-6-Phosphate Dehydrogenase.** Lyophilized cells were rehydrated by suspending in 0.05 M Tris-HCl buffer, pH 7.8, at a density of 10 mg dry weight per ml. The cells were allowed to rehydrate at 37°C for 1 h and were then broken by passage thrice through an Aminco French Pressure Cell operated at a motor driven pressure of 20,000 lb/in². The broken cells were centrifuged at 25,000 x g for 45 min and the supernatant separated and assayed for glucose-6-phosphate dehydrogenase activity by the method of Malamy and Horecker (1964). The 3 ml assay mixture contained 30 µmols of Tris-HCl buffer, pH 7.65, 30 µmols MgCl₂, 3 µmols glucose-6-phosphate, 1.5 µmols NADP (Sigma) and sample to make 3.0 ml. Change in absorbance was followed at 340 nm on a Gilford Spectrophotometer, Model 2000.

**Effect of Deoxycholate on Dehydrogenase Activity Measured by the Thunberg Technique:**

The effect of deoxycholate on the dehydrogenase activity of *E. coli* was measured by the Thunberg technique. To each of the Thunberg tubes was added 2 ml of 0.02 M substrate (glucose, sodium succinate or sodium fumarate), 2 ml of 0.1 M sodium phosphate buffer, pH 7.0 and 1 ml of 0.01% (wt/vol) methylene blue. An 18 h TSB culture of *E. coli* ATCC 25922 was harvested, washed twice with buffer and suspended in 0.1 M sodium phosphate buffer at a density of 300 Klett units. One ml of the bacterial suspension was placed in the
side cap. The joints of the caps were greased with a high vacuum grease and firmly placed on the tube. The tubes were evacuated using a vacuum pump, sealed firmly and placed in a water-bath at 37°C. After 15 min of temperature equilibration the contents were mixed and the reduction of the dye observed visually. Comparisons were made against a tube containing one-tenth the dye concentration and no substrate; this tube represented 90% reduction of the dye in the assay.

**Effect of Deoxycholate on Protein Synthesis:**

The effect of deoxycholate on the synthesis of protein was studied by the method of Hartwell and Magasanik (1963). A mid-logarithmic culture of *E. coli* was harvested, washed with saline and suspended in 'C' medium (Roberts et al., 1955) at a density of 200 Klett units. The cells were incubated at 37°C for 1 h. Sodium deoxycholate at a final concentration of 0.1% (wt/vol) and 0.7 μCi [U-14C] L-leucine (specific activity 287 mCi/mmol) were added and the flasks incubated at 37°C. At intervals, 1 ml suspensions were removed and added to an equal volume of 10% TCA containing 1% (wt/vol) casamino acids (Difco). The mixture was heated in a water-bath at 90°C for 30 min, cooled and filtered through membrane filters (Millipore Corp., New Bedford, Mass.), 0.22 μm pore size and 25 mm diameter. The filters were washed five times with 2 ml volumes of 5% (wt/vol) TCA containing 0.5% (wt/vol) casamino acids. The radioactivity retained on the filters was counted in a Packard Tri-Carb Liquid Scintillation Spectrometer, Model 3320.
Effect of Deoxycholate on Flagella Formation:

Bacteria were grown on deoxycholate agar plates with and without sodium deoxycholate. After 18 h incubation at 37°C, a suspension was made in distilled water and a small drop of the suspension was applied to 200 mesh copper grids coated with 0.25% formvar (Ernest F. Fullam, Inc., Schenectady, N. Y.). The cells were stained with 5% (wt/vol) uranyl acetate for 10 min and excess stain removed by rinsing in distilled water. Micrographs were taken with a RCA EM U 3 electron microscope at 50 kV.

Effect of Deoxycholate on the Swarming of Proteus mirabilis:

Plates of deoxycholate medium containing different concentrations of deoxycholate were inoculated in the center with a 24 h broth culture of P. mirabilis and incubated at 37°C. The inhibition of swarming of Proteus was measured by comparison with the control plate which was devoid of deoxycholate.

Effect of Deoxycholate on the Motility of Bacteria:

Bacteria were grown in deoxycholate broth containing different concentrations of deoxycholate. After 24 h incubation at 37°C, the motility was measured by the hanging drop method, using a phase contrast microscope.

Statistical Analysis:

Data were subjected to statistical analysis. A 't' test was performed as described by Remington and Schork (1970) for differences between sample means with unknown and unequal population variances.
The following formulae were used:

\[ \bar{x} = \frac{\sum x}{n} \]

where, \( \bar{x} \) = arithmetic mean of sample

\[ \Sigma = \text{summation of observations} \]

\[ n = \text{number of observations} \]

\[ s^2 = \frac{n\bar{x}^2 - (\Sigma x)^2}{n(n-1)} \]

where, \( s^2 \) = sample variance

\[ \bar{x}_1 = \text{mean of observations in the presence of deoxycholate} \]

\[ \bar{x}_2 = \text{mean of observations in the absence of deoxycholate} \]

\[ s_1 = \text{variance in the presence of deoxycholate} \]

\[ s_2 = \text{variance in the absence of deoxycholate} \]

\[ n_1 = \text{number of observations in the presence of deoxycholate} \]

\[ n_2 = \text{number of observations in the absence of deoxycholate} \]

Welch's approximation was used to calculate 'f', the degree of freedom.

\[ f = \frac{\left( \frac{s_1^2}{n_1} + \frac{s_2^2}{n_2} \right)^2}{\left( \frac{s_1^2}{n_1} \right)^2 + \left( \frac{s_2^2}{n_2} \right)^2} - 2 \]
RESULTS

Growth Studies:

The growth of gram-negative enteric organisms in deoxycholate broth causes the production of a white precipitate in the growth medium, which appears to be due to the lowering of pH from 7.3 to 4.4 on account of the fermentation of lactose. This situation interferes with optical density determinations, and thus the results of these experiments are inconclusive (Tables 1, 2). To overcome this problem the effect of deoxycholate on the growth of gram-negative bacteria was studied by the pour plate method.

Since sodium deoxycholate is used at a concentration of 0.1% (wt/vol) in bacteriological media, this concentration was included in all studies. The use of a small inoculum was advantageous (1.0 ml of $10^{-6}$ dilution of an overnight culture to 50 ml of broth which gave a final concentration of approximately $5 \times 10^2$ cfu/ml), because this medium is used for the isolation of gram-negative bacteria from water and food, where the bacteria may be present in minute quantities.

Suppression of growth was observed during the first 12 h in E. coli, S. typhimurium, Enterobacter cloacae and K. pneumoniae (Fig. 1-4). Examination of growth parameters (Tables 3-6) showed that the lag time constant, growth rate constant and generation time were all significantly affected at least at the 5% level in E. coli, S. typhimurium and K. pneumoniae. In E. coli and S. typhimurium, the lag time constant and generation time increased, while the growth rate
**Table 1.**

Effect of deoxycholate on the growth of *E. coli* ATCC 25922

<table>
<thead>
<tr>
<th>Concentration of deoxycholate (wt/vol)</th>
<th>Klett Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>0.00 %</td>
<td>34</td>
</tr>
<tr>
<td>0.05 %</td>
<td>0</td>
</tr>
<tr>
<td>0.10 %</td>
<td>0</td>
</tr>
<tr>
<td>0.20 %</td>
<td>0</td>
</tr>
</tbody>
</table>

A 10^-4 saline dilution of an overnight culture was inoculated into deoxycholate medium and growth measured turbidimetrically using a Klett-Summerson colorimeter with a No. 42 filter.
### Table 2.

Effect of deoxycholate on the growth of *S. typhimurium* ATCC 13311<sup>a</sup>

<table>
<thead>
<tr>
<th>Concentration of deoxycholate (wt/vol)</th>
<th>Klett Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6 h</td>
</tr>
<tr>
<td>0.00 %</td>
<td>40</td>
</tr>
<tr>
<td>0.05 %</td>
<td>0</td>
</tr>
<tr>
<td>0.10 %</td>
<td>10</td>
</tr>
<tr>
<td>0.20 %</td>
<td>0</td>
</tr>
</tbody>
</table>

<sup>a</sup> A 10⁻⁴ saline dilution of an overnight culture was inoculated into the deoxycholate medium and growth was measured turbidimetrically using a Klett-Summerson colorimeter with a No. 42 filter.
constant and total yield decreased (Tables 3, 4). In Enterobacter cloacae only the lag time constant increased while the effect on the other parameters was not statistically significant (Table 5). In Klebsiella pneumoniae the lag time constant and the generation time increased, while the growth rate constant decreased, the total yield was not significantly affected (Table 6).

Although the optical density after 24 h incubation at 37°C in the presence of deoxycholate was found to be higher than the control (Tables 1, 2), the viability counts gave lower cfu/ml, thus showing that the precipitate interfered with turbidimetric determinations.

Manometric Studies:

Since the growth parameters of E. coli were affected when grown in the presence of deoxycholate, an attempt was made to study its effect on oxygen consumption. The \( Q_{O_2} \) values for the uptake of oxygen by E. coli ATCC 25922 suspended in phosphate buffer, pH 7.0, containing glucose and deoxycholate in the final concentration of 0.013% (wt/vol) and 0.027% (wt/vol) respectively was reduced from 179 to 149 during the first hour and from 149 to 116 during the second hour (Table 7).

Effect of Deoxycholate on Efflux of \(^{14}C\) from Labeled Cells of E. coli:

Since surface active agents are used for the solubilization of membrane proteins (Birdsell and Cota-Robles, 1968; DePamphilis and Adler, 1971; Schnaitman, 1971a and 1971b; Rosenbusch, 1974) the effect of deoxycholate on the leakage and solubilization of cellular constituents from intact cells of E. coli was studied. Labeled cells of E. coli
Fig. 1. Growth curve of *Escherichia coli* ATCC 25922 in the presence and absence of 0.1% sodium deoxycholate.

A $10^6$ dilution of an overnight culture was made in sterile saline and 1.0 ml was inoculated into deoxycholate medium with and without sodium deoxycholate. The flasks were incubated at 37°C and at intervals titrated by the pour plate method. Colony counts were made after 48 h at 37°C. All samples were plated in triplicate. The points represent the mean value of the three separate experiments.

Symbols: o—o control flask; ••• flask containing 0.1% (wt/vol) deoxycholate.
Fig. 2. Growth curve of *Salmonella typhimurium* ATCC 13311 in the presence and absence of 0.1% (wt/vol) sodium deoxycholate.

The points represent the mean value of three separate experiments, each with triplicate plates.

Experimental conditions were identical to those of Fig. 1.
Fig. 3. Growth curve of Enterobacter cloacae ATCC 23355 in the presence and absence of 0.1% (wt/vol) sodium deoxycholate.

The points represent the mean value of three separate experiments, each with triplicate plates. Experimental conditions were identical to those of Fig. 1.
Fig. 4. Growth curve of *Klebsiella pneumonia* ATCC 13883 in the presence and absence of 0.1% (wt/vol) deoxycholate.

The points represent the mean value of three separate experiments, each with triplicate plates. Experimental conditions were identical to those of Fig. 1.
Table 3

Effect of Sodium Deoxycholate on the Growth Parameters
of E. coli ATCC 25922

<table>
<thead>
<tr>
<th>Growth Parameter</th>
<th>Concentration of Sodium Deoxycholate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 1000 µg/ml P Value</td>
</tr>
<tr>
<td>Lag Time Constant</td>
<td>119 (± 6.2) 154 (± 16.4) &lt;.05 b</td>
</tr>
<tr>
<td>Growth Rate Constant</td>
<td>0.99 (± .02) 0.73 (± 0.04) &lt;.001 b</td>
</tr>
<tr>
<td>Generation Time (min)</td>
<td>17.5 (± .93) 24.7 (± 1.14) &lt;.001 b</td>
</tr>
<tr>
<td>Total Yield (x 10⁹ cfu/ml)</td>
<td>3.90 (± .87) 2.23 (± 0.23) &lt;.05 b</td>
</tr>
</tbody>
</table>

a Dilutions of an overnight TSB culture were made in sterile saline and 1.0 ml of 5 x 10⁻⁶ dilution was inoculated into 50 ml deoxycholate broth. The data represents the average value for three separate experiments. Figures in parenthesis represent ± one standard deviation.

b Statistically significant.
Table 4

Effect of Sodium Deoxycholate of the Growth Parameters
of *S. typhimurium* ATCC 13311<sup>a</sup>

<table>
<thead>
<tr>
<th>Growth Parameter</th>
<th>Concentration of Sodium Deoxycholate</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 1000 µg/ml</td>
<td></td>
</tr>
<tr>
<td>Lag Time Constant</td>
<td>90 (+ 5.1)</td>
<td>&lt;.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Growth Rate Constant</td>
<td>0.86 (+ 0.01)</td>
<td>&lt;.025&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Generation Time (min)</td>
<td>21.6 (+ 0.62)</td>
<td>&lt;.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total Yield (x 10⁹ cfu/ml)</td>
<td>2.1 (+ 0.51)</td>
<td>&lt;.02&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>108 (+ 9.3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.71 (+ 0.03)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>25.6 (+ 0.87)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.59 (+ 0.21)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Dilutions of an overnight TSB culture were made in sterile saline and 1.0 ml of 1 x 10⁻⁶ dilution was inoculated into 50 ml deoxycholate broth. The data represents the average value of three separate experiments. Figures in parenthesis represent ± one standard deviation.

<sup>b</sup> Statistically significant.
Table 5

Effect of Sodium Deoxycholate on the Growth Parameters
of Enterobacter cloacae ATCC 23355

<table>
<thead>
<tr>
<th>Growth Parameter</th>
<th>Concentration of Sodium Deoxycholate</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control 1000 μg/ml</td>
<td></td>
</tr>
<tr>
<td>Lag Time Constant</td>
<td>43 (+ 10.2)</td>
<td>144 (+ 5.6)</td>
</tr>
<tr>
<td>Growth Rate Constant</td>
<td>0.83 (+ .04)</td>
<td>0.77 (+ .05)</td>
</tr>
<tr>
<td>Generation Time (min)</td>
<td>21.8 (+ 1.0)</td>
<td>23.6 (+ .95)</td>
</tr>
<tr>
<td>Total Yield (x 10⁹ cfu/ml)</td>
<td>1.40 (+ .56)</td>
<td>0.68 (+ .43)</td>
</tr>
</tbody>
</table>

a Dilutions of an overnight TSB culture were made in sterile saline and 1.0 ml of 5 x 10⁻⁶ dilution was inoculated into 50 ml deoxycholate broth.

The data represents the average value of three separate experiments. Figures in parenthesis represent ± one standard deviation.

b Statistically significant.
Table 6

Effect of Sodium Deoxycholate of the Growth Parameters of Klebsiella pneumoniae ATCC 13883<sup>a</sup>

<table>
<thead>
<tr>
<th>Growth Parameter</th>
<th>Concentration of Sodium Deoxycholate</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>1000 μg/ml</td>
</tr>
<tr>
<td>Lag Time Constant</td>
<td>45.6 (± 5.8)</td>
<td>99.6 (± 10.3)</td>
</tr>
<tr>
<td>Growth Rate Constant</td>
<td>0.91 (± .02)</td>
<td>0.80 (± .05)</td>
</tr>
<tr>
<td>Generation Time (min)</td>
<td>19.9 (± .67)</td>
<td>22.5 (± .92)</td>
</tr>
<tr>
<td>Total Yield (x 10&lt;sup&gt;9&lt;/sup&gt; cfu/ml)</td>
<td>1.6 (± .63)</td>
<td>1.3 (± .45)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Dilutions of an overnight TSB culture were made in sterile saline and 1.0 ml of 1 x 10<sup>-6</sup> dilution was inoculated into 50 ml deoxycholate medium.

The data represents the average value of three separate experiments.

Figures in parenthesis represent ± one standard deviation.

<sup>b</sup> Statistically significant.
Table 7

Effect of deoxycholate on oxygen consumption
in *E. coli*

<table>
<thead>
<tr>
<th>Time</th>
<th>Control</th>
<th>Deoxycholate Treated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st h</td>
<td>179 (± 9)</td>
<td>149 (± 10)</td>
<td>&lt; 0.0005 c</td>
</tr>
<tr>
<td>2nd h</td>
<td>149 (± 18)</td>
<td>116 (± 12)</td>
<td>&lt; 0.005 c</td>
</tr>
</tbody>
</table>

*E. coli* ATCC 25922 was grown in trypticase soy broth for 24 h at 37 °C, harvested, washed and suspended in 0.1 M phosphate buffer (pH 7.0) at a density of 0.45 mg dry wt/ml. Glucose was used as a substrate at a final concentration of 0.013% (w/v). The uptake of oxygen was studied in the presence of sodium deoxycholate at a final concentration of 0.027% (w/v). \(Q_{O_2}\) values have been corrected for endogenous respiration. The endogenous values were 12 (± 3) for control and 11 (± 3) for deoxycholate treated.

Results are the mean of five experiments. Figures in parenthesis represent ± one standard deviation.

Statistically significant.
were exposed aerobically to 0, 0.05, 0.1 and 0.2% deoxycholate and the $^{14}$C activity released was measured. The rate of release of radioactivity was high during the first 30 min (Fig. 5). At concentrations of 0.1 and 0.2% the activity found in the supernatants after 24 h was 10,550 cpm/ml and 18,700 cpm/ml, respectively, as compared with 6,800 cpm/ml in the control. Statistical analysis showed that the release of radioactivity was significantly higher with 0.1 or 0.2% deoxycholate but with 0.05% deoxycholate there was not a significant change.

Studies on supernatants of unlabelled cells incubated in the presence of deoxycholate showed a significant high amount of protein and carbohydrate when determined by the Lowry et al. (1951) and anthrone (Scott et al., 1953) methods, respectively. After 24 h the amount of protein in the supernatant of deoxycholate treated cells was 165 µg/ml as compared to 85 µg/ml in the control (Fig. 6). The amount of carbohydrate released in the supernatant of deoxycholate treated cells after 24 h incubation was 30 µg/ml as compared with 21 µg/ml in the control (Fig. 7).

**Fractionation Studies of Labeled Cells:**

Gram-negative bacteria, like *E. coli*, are enclosed by an envelope consisting of two separate membranes. The inner or cytoplasmic membrane, is the primary permeability barrier of the cell, containing specific permeases and transport proteins (Davis et al., 1973). The outer membrane provides the cell with a passive barrier to substances with molecular weights greater than several hundred daltons.
Fig. 5. Effect of deoxycholate on efflux of $^{14}$C from E. coli ATCC 25922.

A mid-logarithmic culture of E. coli ATCC 25922 was harvested, washed and suspended in 0.1 M sodium phosphate buffer, pH 7.0. The cells were labeled with [1-$^{14}$C] glucose, washed suspended in buffer and incubated at 37°C on a shaker water-bath. Various concentrations of deoxycholate were added. At intervals 1.0 ml suspensions were centrifuged and aliquots of the supernatant were counted. Values given are the mean of two flasks from the same experiment.

Symbols: 0, no deoxycholate added; Δ, 0.05% (wt/vol) deoxycholate; ●, 0.1% (wt/vol) deoxycholate; ▲, 0.2% (wt/vol) deoxycholate.
Fig. 6. Solubilization and leakage of protein from cells of *E. coli* ATCC 25922, incubated ○, in the absence of deoxycholate; ₀, in the presence of 0.1% (wt/vol) deoxycholate.

Results are the mean of 4 experiments. Bars represent ± one standard deviation.
Fig. 7. Solubilization and leakage of carbohydrate from cells of *E. coli* ATCC 25922 incubated 0, in the absence of deoxycholate; 0, in the presence of 0.1% (wt/vol) deoxycholate.

Results are the mean of 6 experiments. Bars represent ± one standard deviation.
The relative resistance of gram-negative bacteria to surface active agents is a property reflecting the unique character of the outer membrane complex of the cell wall of these organisms. This resistance has been most thoroughly documented through the extensive use of media containing surface-active agents for the specific isolation of enteric bacteria.

To study the effect of deoxycholate on various components of gram-negative bacteria, an 18 h culture grown in trypticase soy broth at 37°C was harvested, washed in saline, suspended in 0.1 M sodium phosphate buffer and labeled with \([U-^{14}C]\) D-glucose for 30 min. The excess label was removed by washing the cells with buffer and the cells were treated with deoxycholate for 24 h at 37°C. Fractionation was then carried out by the method of Park and Hancock (1960). The cold TCA fraction contained low molecular weight compounds. The ethanol fraction contained ethanol soluble proteins and lipids. Extraction with hot TCA removed nucleic acids from the cells. Treatment of the hot-TCA-insoluble fraction with trypsin converted 95% of the cell protein to soluble peptides. The residue consisted of mucopeptide.

Table 8 shows that deoxycholate caused leakage of low molecular weight compounds, ethanol soluble proteins, lipids and nucleic acids. The ethanol insoluble protein was slightly reduced but the peptidoglycan was unaffected. The residue which consisted of mucopeptide was not affected. In the presence of 0.1% deoxycholate the activity of cold-TCA-soluble fraction was reduced from 22,500 cpm to 8,350 cpm. The activity of the ethanol soluble fraction was reduced from 16,850
Table 8

Fractionation of labeled cells of *Escherichia coli* a

<table>
<thead>
<tr>
<th>Concentration of deoxycholate (wt/vol)</th>
<th>Counts/Minute per total volume of Fraction</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cold TCA Soluble</td>
<td>Ethanol Soluble</td>
<td>Hot TCA Soluble</td>
<td>Trypsin Degraded</td>
<td>Residue</td>
<td>Unfractionated Cells</td>
</tr>
<tr>
<td>--------------------------------------</td>
<td>------------------</td>
<td>-----------------</td>
<td>------------------</td>
<td>------------------</td>
<td>---------</td>
<td>---------------------</td>
</tr>
<tr>
<td>0.00%</td>
<td>22,500</td>
<td>16,850</td>
<td>33,000</td>
<td>17,900</td>
<td>36,050</td>
<td>139,650</td>
</tr>
<tr>
<td>0.05%</td>
<td>10,950</td>
<td>11,300</td>
<td>26,500</td>
<td>14,720</td>
<td>36,150</td>
<td>116,250</td>
</tr>
<tr>
<td>0.10%</td>
<td>8,350</td>
<td>9,750</td>
<td>19,750</td>
<td>13,240</td>
<td>36,240</td>
<td>114,000</td>
</tr>
<tr>
<td>0.20%</td>
<td>5,350</td>
<td>5,600</td>
<td>14,000</td>
<td>12,280</td>
<td>32,400</td>
<td>75,000</td>
</tr>
</tbody>
</table>

a After labeling resting cells of *E. coli* ATCC 25922 with [1-14C] glucose for 30 min, they were washed and suspended at a density of 760 μg/ml in 0.1M phosphate buffer. The cells were distributed in aliquots of 15 ml each into 4 tubes, deoxycholate was added at a final concentration of 0, 0.05, 0.10 and 0.20% and incubated at 37 C for 24 h. The cells were fractionated by the method of Park and Hancock (1960). Aliquots of each fraction were added to scintillation fluid and 14C activity counted. Whole cells were filtered and the counts/min of the filters obtained.
to 9,750. Loss of the activity was also observed in the hot-TCA and trypsin-soluble fractions, which were reduced from 33,000 to 19,750 and from 17,900 to 13,240, respectively.

**Viability Count:**

The effect of deoxycholate on the growth parameters, oxygen consumption and leakage or solubilization of cellular constituents from gram-negative cells led to the investigation of its effect on the viability of the cells. The possibility of leakage of proteins and carbohydrates due to cell death and lysis was determined by plate counts of cells incubated in phosphate buffer, pH 7.0, with and without deoxycholate.

Table 9 shows that in cells incubated in the absence of deoxycholate, there was a decrease in viability count, from $169 \times 10^7$ cfu/ml to $17.1 \times 10^7$ cfu/ml after 24 h incubation. However, in the presence of deoxycholate the death rate was still higher; cells incubated with 0.1% deoxycholate showed a decrease from $165.9 \times 10^7$ cfu/ml to $5.1 \times 10^7$ cfu/ml. The decrease in cell count was proportional to the concentration of deoxycholate present, although the difference between 0.1% and 0.2% deoxycholate was not significant.

**Effect of Deoxycholate on the Uptake of $^{14}$C Glucose:**

The effect of deoxycholate on the growth parameters of *E. coli* could be the result of alteration in the entrance of specific substances into the cells, thus reducing the availability of the required nutrients. To test this hypothesis experiments involving the exposure of *E. coli* suspensions to [1-$^{14}$C] glucose in the presence and absence
### Table 9

Determination of viable count of *Escherichia coli* cells incubated with deoxycholate \(^{a}\)

<table>
<thead>
<tr>
<th>Concentration of deoxycholate</th>
<th>Cfu/ml (x 10(^7)) (^{b})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>0</td>
<td>170</td>
</tr>
<tr>
<td>1/2</td>
<td>169</td>
</tr>
<tr>
<td>1</td>
<td>170</td>
</tr>
<tr>
<td>2</td>
<td>168</td>
</tr>
<tr>
<td>4</td>
<td>171</td>
</tr>
<tr>
<td>24</td>
<td>17</td>
</tr>
<tr>
<td>48</td>
<td>8</td>
</tr>
</tbody>
</table>

\(^a\) Bacterial cells were incubated in phosphate buffer, pH 7.0, at 37°C for 24 h. The viable count was determined by the pour plate method.

\(^b\) Data represent the mean value of two separate experiments, each plated in triplicate.
of 0.1% (wt/vol) deoxycholate were performed. After exposure to 0.1% deoxycholate for 30 min, 40% inhibition of the uptake of [l-14C] glucose was observed (Fig. 8).

Further experiments were carried out to determine the effect of different concentrations of deoxycholate on the uptake of glucose when both deoxycholate and glucose were added simultaneously. High specific activity glucose was used and readings were taken at shorter intervals. Uptake was very rapid during the first 15 sec and came to a plateau after 60 sec (Fig. 9). At 120 sec there was a 27.1% and 64.2% decrease in uptake of [l-14C] glucose when cells were exposed to 0.1 and 0.2% deoxycholate respectively. Beyond 120 sec the 14C contents in the bacterial cell decreased, probably due to the catabolism of glucose and the loss of radioactivity as 14CO2.

Determination of Amount of Deoxycholate Present in Lyophilized Cells:

In order to make a comparative study of cells grown in the presence and absence of deoxycholate, initial experiments, to determine the amount of deoxycholate present in the deoxycholate grown cells, were undertaken. Cells grown on deoxycholate agar at 37°C for 24 h were harvested, washed thrice with saline and thrice with 0.05 M Tris-HCl buffer, pH 7.2 and the deoxycholate contents of the cells determined by the method of Mosbach et al. (1954). Cells grown both in the presence and absence of deoxycholate were found to be free of deoxycholate. Thus equal weights of the cells could be used for analysis of lipids, proteins, DNA and RNA contents. However, to establish the binding of deoxycholate to the cells, further experi-
Fig. 8. Uptake of $^{14}$C glucose by E. coli ATCC 25922.

The results are the mean of 4 experiments. Bars represent $\pm$ one standard deviation. Results expressed after subtracting background count.

Symbols: $\bullet$- , cells treated with 0.1% (wt/vol) deoxycholate; $o$- , untreated cells.
Fig. 9. Effect of deoxycholate on the uptake of [1-$^{14}$C] glucose.

The results are the mean of three experiments. Bars represent ± one standard deviation.

Symbols: o--o, cells incubated in the absence of deoxycholate; •--•, cells incubated in the presence of 0.05% deoxycholate; Δ--Δ, 0.1% deoxycholate; ▲--▲, 0.2% deoxycholate; □--□, medium without cells.
ments using radiolabeled deoxycholate are necessary.

**Analysis of Lipid Content:**

Phospholipids constitute about 3 to 9% of the dry weight of gram-negative bacteria (Ames, 1968; Damaglou and Dawes, 1968). The variability in the estimates of *E. coli* lipid content (Ames, 1968; Damaglou and Dawes, 1968), probably reflects differences in extraction techniques and strain differences. The lipid content of a strain does not vary appreciably if the same growth conditions are maintained (Damaglou and Dawes, 1968). The lipid contents of *E. coli* ATCC 25922 grown in the presence and absence of deoxycholate was extracted by the method of Bligh and Dyer (1959). The results shown in Table 10 show that no significant difference was seen in the total lipid present in the cells grown under the two conditions.

**Effect of Deoxycholate on Macromolecular Synthesis in E. coli:**

Since cells incubated in the presence of deoxycholate lost some of their cellular constituents (Table 8), the synthesis of macromolecules in the presence of 0.1% deoxycholate was studied by chemical analysis of cells grown in the presence and absence of deoxycholate. Fractionation was carried out according to the procedure of Roberts et al. (1955) as modified by Kerridge (1959).

The cold TCA fraction is considered to represent the cellular pool, the ethanol-ether-soluble fraction comprises cellular lipids and a very small amount of alcohol soluble proteins, the perchloric acid extract contains nucleic acids and carbohydrates, the residue consists predominantly of ethanol-insoluble proteins.
Table 10

Determination of lipid contents of control and deoxycholate-grown cells

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Control Cells</th>
<th>Deoxycholate-Grown Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td>2</td>
<td>4.8</td>
<td>4.5</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>4.4</td>
</tr>
<tr>
<td>Mean (± SD)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>4.5 (± 0.3)</td>
<td>4.4 (± 0.1)&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

The lipid content of *E. coli* cells grown in the presence and absence of deoxycholate was extracted by the method of Bligh and Dyer (1959)

<sup>a</sup> SD = Standard Deviation.

<sup>c</sup> Not significant.
Table 11

Effect of deoxycholate on macromolecular synthesis in

E. coli ATCC 25922

<table>
<thead>
<tr>
<th>Trial No</th>
<th>Concentration of Deoxycholate µg/ml</th>
<th>RNA</th>
<th>DNA</th>
<th>EtOH Soluble Proteins</th>
<th>EtOH Insoluble Proteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>1.89</td>
<td>0.270</td>
<td>0.920</td>
<td>5.56</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1.79</td>
<td>0.293</td>
<td>0.628</td>
<td>6.64</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>1.86</td>
<td>0.270</td>
<td>0.872</td>
<td>6.60</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1.74</td>
<td>0.300</td>
<td>0.784</td>
<td>5.84</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
<td>1.89</td>
<td>0.278</td>
<td>0.864</td>
<td>6.64</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1.56</td>
<td>0.337</td>
<td>0.976</td>
<td>5.98</td>
</tr>
<tr>
<td>Mean (+ SD)</td>
<td></td>
<td>1.88 (± .02)</td>
<td>0.273 (+ .01)</td>
<td>0.855 (+ .03)</td>
<td>6.27 (+ .61)</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>1.70 (± .12)</td>
<td>0.310 (+ .02)</td>
<td>0.796 (+ .17)</td>
<td>6.15 (+ .43)</td>
</tr>
</tbody>
</table>

a E. coli was grown on deoxycholate agar and deoxycholate agar without deoxycholate. The growth was washed thrice with saline and thrice with 0.05 M Tris-HCl buffer, pH 7.2 and lyophilized. The lyophilized cells (20 mg) were fractionated according to the method of Roberts et al. (1955) as modified by Kerridge (1959)
The results of fractionation are shown in Table 11. No difference in nucleic acid and protein content was observed in the cells grown in the presence and absence of deoxycholate. The total nucleic acid content determined spectrophotometrically by absorbance at 260 nm gave results comparable to that of the combined orcinol and diphenylamine methods.

Effect of Deoxycholate on the Synthesis of Glucose-6-Phosphate Dehydrogenase:

The cytoplasmic enzyme glucose-6-phosphate dehydrogenase has been considered to be specific for NADP as its coenzyme. Thus the NADPH formed provides the bulk of the hydrogens used for reductive biosynthesis, particularly of fatty acids. The inhibition of oxygen uptake by cells incubated in the presence of deoxycholate led to the study of the effect of deoxycholate on the synthesis of glucose-6-phosphate dehydrogenase.

Cells grown in the presence and absence of deoxycholate were broken by passage through an Aminco French Pressure Cell. The suspension was centrifuged and the supernatant assayed for glucose-6-phosphate dehydrogenase activity by the method of Malamy and Horecker (1964). The results shown in Table 12 indicate that the synthesis of glucose-6-phosphate dehydrogenase, a cytoplasmic enzyme, was unaffected when the cells were grown in the presence of deoxycholate.

Effect of Deoxycholate on the Dehydrogenase Activity:

The oxidation of organic substrate catalyzed by dehydrogenases in the absence of oxygen was visualized using methylene blue as a
Table 12

Effect of deoxycholate on the synthesis of glucose-6-phosphate dehydrogenase\(^a\)

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Control Cells</th>
<th>Deoxycholate Grown Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>120.0</td>
<td>119.6</td>
</tr>
<tr>
<td>2</td>
<td>103.2</td>
<td>112.6</td>
</tr>
<tr>
<td>3</td>
<td>102.2</td>
<td>106.0</td>
</tr>
<tr>
<td>Mean (± SD)(^b)</td>
<td>108.5 (± 10.0)</td>
<td>112.7 (± 6.8)(^c)</td>
</tr>
</tbody>
</table>

\(^a\) Lyophilized cells stored at -20°C were rehydrated and broken by passage through an Aminco French Pressure Cell. The broken cells were centrifuged at 25,000 x g for 45 min and the supernatant separated and assayed for glucose-6-phosphate dehydrogenase activity by the method of Malamy and Horecker (1964).

\(^b\) SD = Standard Deviation.

\(^c\) Not significant.
hydrogen acceptor (Thunberg Technique). Under these conditions the methylene blue was reduced to a colorless leuco-compound. The enzymatic activity resulted in disappearance of the blue color, and the time required for 90% decolorization was chosen as the end point.

The dehydrogenase activity of cells of *E. coli* ATCC 25922, measured by the Thunberg technique was inhibited by the presence of sodium deoxycholate (Table 13). When glucose was used as a substrate the time required for decolorization of methylene blue was increased from 10.3 min in the control to 13.5, 16.0 and 26.0 min in the presence of 0.05, 0.1 and 0.2% deoxycholate respectively. In the case of succinate and fumarate, the control tubes were decolorized in 19.4 min and 37.5 min respectively, whereas the tubes containing deoxycholate failed to show any activity even after 120 min.

**Effect of Deoxycholate on Protein Synthesis:**

To investigate the cause of the effect of deoxycholate on the growth parameters, oxygen consumption and uptake of nutrients from the medium, protein synthesis in the presence and absence of deoxycholate was studied. Many antibacterial agents have turned out to be inhibitors of protein synthesis (Davis and Davis, 1968; Weisblum and Davis, 1968; Dienstag and Neu, 1972; Klainer and Perkins, 1972; Olenich and Hahn, 1972). The possible role of deoxycholate on translation was measured by studying the incorporation of labeled amino acid into bacterial protein. The incorporation of [U-\textsuperscript{14}C] leucine into newly synthesized protein molecules was studied by the method of Hartwell and Magasanik (1963). The results shown in Table 14
Table 13
Effect of deoxycholate on dehydrogenase activity,
measured by the Thunberg technique

<table>
<thead>
<tr>
<th>Trial No.</th>
<th>Substrate</th>
<th>Control</th>
<th>0.05 %</th>
<th>0.10 %</th>
<th>0.20 %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glucose</td>
<td>11.0</td>
<td>14.5</td>
<td>17.0</td>
<td>26.5</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>10.0</td>
<td>12.0</td>
<td>15.0</td>
<td>25.5</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>10.0</td>
<td>14.0</td>
<td>14.5</td>
<td>26.0</td>
</tr>
<tr>
<td>Mean (+ SD)</td>
<td>&quot;</td>
<td>10.3 (+ .58)</td>
<td>13.5 (+ 1.32)</td>
<td>16.0 (+ 1.41)</td>
<td>26.0(+ .50)</td>
</tr>
<tr>
<td>1</td>
<td>Na Succinate</td>
<td>19.0</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>19.0</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
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<tr>
<td>3</td>
<td>&quot;</td>
<td>19.5</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>4</td>
<td>&quot;</td>
<td>20.5</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>Mean (+ SD)</td>
<td>&quot;</td>
<td>19.4 (+ .48)</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>1</td>
<td>Na Fumarate</td>
<td>37.0</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>2</td>
<td>&quot;</td>
<td>37.5</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>3</td>
<td>&quot;</td>
<td>38.0</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
<tr>
<td>Mean (+ SD)</td>
<td>&quot;</td>
<td>37.5 (+ .50)</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
<td>&gt; 120</td>
</tr>
</tbody>
</table>

Notes:
- Tubes were compared against the control tube containing one-tenth the dye concentration and no substrate. Tubes devoid of substrates or cells did not decolorize after 2 h incubation.
Table 14

Effect of deoxycholate on protein synthesis a

<table>
<thead>
<tr>
<th>Time (Min)</th>
<th>Broth + $^{14}$C-leucine</th>
<th>Broth + Deoxycholate + $^{14}$C-leucine</th>
<th>Broth + Cells + Deoxycholate + $^{14}$C-leucine</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>52</td>
<td>97</td>
<td>19,200</td>
</tr>
<tr>
<td>15</td>
<td>61</td>
<td>85</td>
<td>33,000</td>
</tr>
<tr>
<td>30</td>
<td>73</td>
<td>99</td>
<td>34,000</td>
</tr>
<tr>
<td>45</td>
<td>78</td>
<td>94</td>
<td>30,000</td>
</tr>
<tr>
<td>60</td>
<td>81</td>
<td>98</td>
<td>32,000</td>
</tr>
<tr>
<td>90</td>
<td>93</td>
<td>103</td>
<td>36,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>21,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>31,600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35,500</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>30,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>35,000</td>
</tr>
</tbody>
</table>

a A mid-logarithmic culture of E. coli was harvested, washed and suspended in "C" medium at a density of 200 Klett units. Sodium deoxycholate and [U-$^{14}$C]-leucine was added and the incorporation of radiolabeled leucine into protein was determined by the method of Hartwell and Magasanik (21); see Materials and Methods.

b Results are of a single experiment using E. coli ATCC 25922. The values are expressed after subtracting the background count.
are inconclusive and show no evidence of protein inhibition.

**Effect of Deoxycholate on Flagella Formation:**

Leifson (1935) noted that motile bacteria taken from a medium containing 0.1% deoxycholate were devoid of flagella and were non-motile. To investigate the effect of deoxycholate on flagella formation under the electron microscope, negative staining using 5% uranyl acetate was performed on cell suspensions of *P. mirabilis*, *E. coli* and *S. typhimurium* grown on sodium deoxycholate agar. Reduction in the number of flagella per bacterial cell was observed when grown in the presence of 0.05% (wt/vol) deoxycholate (Fig. 10, 11) and complete inhibition in the presence of 0.1% deoxycholate (Fig. 10, 12, 14, 15, 17, 18).

No effect was observed on flagella or motility after treating the flagellated cells with 0.1% (wt/vol) deoxycholate for 6 h (Fig. 13, 16).

**Effect of Deoxycholate on Swarming of Proteus mirabilis:**

One of the advantages of using deoxycholate agar is that it inhibits the spreading of *Proteus* species, thus enabling plate counts to be performed. Lominski and Lendrum (1947) have observed that swarming of *Proteus* is motility stimulated and orientated by the negative chemotactic action of its metabolites. When *P. mirabilis* was inoculated on agar medium containing 0.05% and 0.1% deoxycholate complete inhibition of swarming was observed even after 32 h incubation at 37°C. The control plate containing no deoxycholate showed swarming after 6 to 8 h, whereas the plate containing 0.025% deoxy-
Fig. 10. Electron micrograph of *Proteus mirabilis* grown on deoxycholate basal medium. Bar in this and subsequent micrographs represents 1.0 µm.

Fig. 11. Electron micrograph of *Proteus mirabilis* grown on deoxycholate agar containing 0.05% sodium deoxycholate.
Fig. 12. Electron micrograph of *Proteus mirabilis* grown on deoxycholate agar containing 0.1% sodium deoxycholate.

Fig. 13. Electron microscope of *Proteus mirabilis* grown on deoxycholate basal medium and treated with 0.1% sodium deoxycholate for 6 h.
Fig. 14. Electron micrograph of *Escherichia coli* ATCC 25922 grown on deoxycholate basal medium.

Fig. 15. Electron micrograph of *Escherichia coli* ATCC 25922 grown on deoxycholate medium containing 0.1% sodium deoxycholate.
Fig. 16. Electron micrograph of *Escherichia coli* ATCC 25922 grown on deoxycholate basal medium and treated with 0.1% sodium deoxycholate for 6 h.
Fig. 17. Electron micrograph of *Salmonella typhimurium* ATCC 13311 grown on deoxycholate basal medium.

Fig. 18. Electron micrograph of *Salmonella typhimurium* ATCC 13311 grown on deoxycholate medium containing 0.1% sodium deoxycholate.
Fig. 19. Effect of deoxycholate on the swarming of *Proteus mirabilis* after 6 h incubation on deoxycholate medium containing
a) 0%; b) 0.025%; c) 0.05%; and d) 0.1% sodium deoxycholate.
Fig. 20. Effect of deoxycholate on the swarming of *Proteus mirabilis* after 24 h incubation on deoxycholate agar containing a) 0%; b) 0.025%; c) 0.05% and d) 0.1% sodium deoxycholate.
Effect of Deoxycholate on the Motility of Pathogens

A 30 to 40% decrease in motility was observed in typhimurium and P. mirabilis after 24 hours.
cholate, swarming was apparent after 14 h (Fig. 19, 20).

**Effect of Deoxycholate on the Motility of Bacteria:**

A 30 to 40% decrease in motility was exhibited by *E. coli*, *S. typhimurium* and *P. mirabilis* after 24 h growth at 37°C in broth containing 0.05% deoxycholate. In the presence of 0.1% deoxycholate about 80 to 90% motility was lost and the motile organisms exhibited sluggish movement (Table 15).
Table 15

Effect of deoxycholate on motility

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of Deoxycholate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00%</td>
</tr>
<tr>
<td><strong>E. coli ATCC 25922</strong></td>
<td>Highly motile</td>
</tr>
<tr>
<td><strong>S. typhimurium ATCC 13311</strong></td>
<td>Highly motile</td>
</tr>
<tr>
<td><strong>P. mirabilis</strong></td>
<td>Highly motile</td>
</tr>
</tbody>
</table>

a Motility was performed by the hanging drop method on a 24 h culture grown in deoxycholate broth at 37°C. Judgement of motility was based upon subjective estimates of population movement compared with the actively motile control.
DISCUSSION

The deoxycholate medium, which is used for the isolation of gram-negative bacteria from food and water, is inhibitory for the growth of gram-positive organisms. However, it has been reported by Leifson (1935) that the growth of the gram-negative bacillus, Salmonella gallinarum is completely inhibited. Leifson reported on the qualitative aspects of deoxycholate inhibition of growth in bacteria, but growth-rate studies were not done. The present study was therefore undertaken to investigate the effects of deoxycholate on the growth-parameters and other physiological characteristics, including oxygen consumption, uptake and efflux of nutrients and effect on flagella of some gram-negative bacteria. Escherichia coli was included in all studies because the isolation of this organism is considered as an indicator of fecal pollution in the analysis of water. Other organisms of clinical importance used in studying the effect of deoxycholate on the growth-parameters included Salmonella typhimurium, Enterobacter cloacae and Klebsiella pneumoniae.

Initially growth was measured by observing increases in optical density. This method gave inconsistent optical density readings (Tables 1 and 2) due to the precipitation of deoxycholic acid in the medium, and was therefore abandoned. The pour plate method, which has the advantage of measuring only viable organisms, was used in the growth-studies. The use of a small inoculum (about $1.5 \times 10^3$ cfu to 50 ml broth) was advantageous because the deoxycholate medium is
employed for the isolation of gram-negative bacteria from water and food, where the organisms may be present in very minute quantities.

When a fresh medium was inoculated with the organisms, multiplication of the bacteria did not start immediately, and a temporary lag, ranging from 45-120 min in the control and 100-155 min in the presence of deoxycholate, was observed. In the presence of deoxycholate a decrease in the number of viable colony forming units was also observed, which suggests a killing effect of the drug. An ideal medium for the isolation and detection of bacteria should be capable of detecting small quantities of organisms present in the sample. The use of deoxycholate medium may not be ideal for the detection of *E. coli* when these organisms are present in minute quantities and under stress of chlorination and freezing. The initial killing effect may be sufficient to eliminate these bacteria which will go undetected. In the presence of deoxycholate the lag was longer, probably because of slow synthesis of the necessary growth initiation factors (Lamanna et al., 1973).

The bacteria then recovered from the lag phase and entered the exponential phase of growth. The growth-rate constant of the organisms in the presence of deoxycholate was decreased from 0.99 to 0.73 (Table 3). This could be the result of inactivation of dehydrogenases and this possibility was examined by studying the uptake of oxygen in the presence and absence of deoxycholate. In the presence of deoxycholate the oxygen consumption decreased from 179 to 149 µl/mg dry wt of cells/h (Table 7).
The uptake of oxygen and the liberation of \( \text{CO}_2 \) are the end results of a series of metabolic reactions. In order to study the intermediate reactions, the dehydrogenase activity was estimated by the Thunberg technique, in which a hydrogen transferred from an enzymatically activated substrate to the methylene blue acceptor, reduced the dye to a leuco-compound. The results in Table 13 indicate that the inhibitory effect of deoxycholate was more pronounced with succinate and fumarate than with glucose as substrate. The delay in reduction of the dye suggests the inhibition of dehydrogenase activity, which may account for the lower rates of respiration in these organisms. These data suggest that succinic dehydrogenase, a membrane enzyme, was inhibited by deoxycholate.

The activity of the cytoplasmic enzyme glucose-6-phosphate dehydrogenase was not changed when prepared from cells grown in the presence of deoxycholate and then lyophilized. However, it is possible that the lyophilized cells used were not suitable for the study as the enzymatic activity may have been affected during the process of lyophilization. Since parallel studies on extracts of freshly grown cells were not compared, the results of the glucose-6-phosphate dehydrogenase assay are suggestive but not conclusive.

The oxidation of substrates entails entrance of these compounds into the bacterial cell. Since the addition of deoxycholate to the resting cells used in the manometric studies only showed decreases in exogenous respiration, it may be inferred that the effect of deoxycholate was on the uptake of the substrate into the bacterial cell. Furthermore, the presence of increasing amounts of cellular proteins
(Fig. 6) and carbohydrates (Fig. 7) released from cells incubated with deoxycholate provides evidence compatible with the suggestion that the drug caused alterations in permeability processes. The increased $^{14}$C activity released from the labeled cells in the presence of deoxycholate (Fig. 5) further supports this hypothesis. The possibility of solubilization of the outer layer of the cell wall cannot be ruled out as specific cell wall markers in the soluble material released from the cells after treatment with deoxycholate were not assayed. Further, since the cells were labeled in phosphate buffer, the radioactivity taken up may not have been distributed uniformly throughout the entire cell, and most of the label found in the supernatant may have come from the cytoplasm. The use of [1-$^{14}$C] glucose for labeling bacteria is not ideal. The results are difficult to interpret because the first carbon atom of glucose is preferentially lost in the hexosemonophosphate pathway and this $^{14}$CO$_2$ may not be representative of the total glucose metabolism. Use of [U-$^{14}$C] glucose would make the interpretation of the results much simpler.

The efflux of $^{14}$C in the presence of 0.1% deoxycholate was very rapid for the first hour, compared to the control, and then leveled off (Fig. 5). The rapid loss may be due to increased permeability during the first hour, although the cells later achieved stability. The suggestion of increased permeability is supported by the findings that in the presence of 0.2% deoxycholate the loss of $^{14}$C activity from the cells continued, which perhaps could be due to greater damage to the cell membrane. The bacterial cells suspended in buffer devoid of energy sources did not undergo cellular division, and, with the
possible death of some cells, proteolytic enzymes responsible for the breakdown of supernatant protein could have been released, and these enzymes could therefore account for the fall of the supernatant proteins after 24 h (Fig. 6). The death and lysis of bacteria was too low during the first 4 h (Table 9) to account for the increase in protein, carbohydrate and $^{14}\text{C}$ activity in the supernatant. However, the leakage and solubilization of cellular components could be responsible for the increased death rate of the cells incubated with deoxycholate.

The growth of a microorganism is the result of the synthesis of its cellular components. The entry of most nutrients into the cell is mediated by specific and non-specific permeases (Saier and Stiles, 1975). Biological membranes are selectively permeable to solutes such as sugars, amino acids, ions, etc., and transport processes are mediated by special systems localized in the membranes. The nature of these systems and the mechanisms by which they operate have been extensively studied in a wide variety of organisms; such examples include the amino acid permeases of _E. coli_ (Wargel et al., 1970) and _Pseudomonas aeruginosa_ (Kay and Gronlund, 1969), the tryptophan permeases of _Neurospora crassa_ (Wiley and Matchett, 1968), the carbohydrate permease of _Clostridium perfringens_ (Graves and Gronlund, 1969), and the histidine and aromatic acid permease of _Salmonella typhimurium_ (Ames, 1964). A phosphoenolpyruvate transferase system has been described in _E. coli_ (Kundig et al., 1964; Saier, 1977).

The inhibition of glucose uptake in the presence of deoxycholate may be due to its effect on the synthesis or the activity of an enzyme
involved in the transportation of the substrate into the cell. At concentrations of 0.1 and 0.2% (wt/vol) deoxycholate, the uptake of \(^{14}\text{C}\) glucose into the cells was inhibited up to 30 sec (Fig. 9), after which time an equilibrium was reached in the presence of 0.2% deoxycholate. It can be speculated that deoxycholate is affecting the activity as well as the synthesis of a protein involved in the transport of glucose. The inhibition was overcome in the presence of 0.1% deoxycholate, perhaps by the synthesis of a new enzyme resulting in the restoration of substrate uptake.

Radiolabeled glucose may be metabolized into different pathways and lost as \(^{14}\text{CO}_2\). This problem has been overcome by studying the transport for a very short period of time. However, the use of compounds which display very limited metabolism such as deoxyglucose or \(\alpha\) methyl glucoside in place of glucose or other metabolizable substrates would make interpretation of uptake studies much simpler.

Substances taken into cells and incorporated into cellular components must pass through metabolic pools before entering the various metabolic pathways (Britten and McClure, 1962; Britten, 1963). In this way, the pool size may directly influence the synthesis of macromolecules. Experiments involving the exposure of \(^{14}\text{C}\)-labeled cells to deoxycholate showed a loss of low molecular weight compounds (cold TCA fractions), ethanol soluble proteins, lipids, nucleic acids, and some ethanol insoluble proteins (Table 8). This loss of cell components may be partly due to the death of the cells incubated in buffer in the absence of an energy source. However, the decrease in viability during the first four hours was very slight (Table 9) as compared
to the loss of cellular carbohydrates and proteins in the supernatant (Fig. 6, 7). These results imply that the death of the organisms was the result and not the cause of cell impermeability.

The inhibition of flagella formation by 0.1% deoxycholate (Fig. 10-18) in *Proteus mirabilis*, *Escherichia coli* and *Salmonella typhi-murium* is responsible for the decreased motility in these bacteria (Table 15). It is possible that the flagella protein (flagellin), the hooks, and the basal structures were being synthesized, but could not be assembled in the presence of deoxycholate. Another possibility is that the apparatus for flagella synthesis is separate from that responsible for the synthesis of cellular proteins. This is in agreement with the findings of McClatchy and Rickenberg (1967), who have suggested that the mRNA for flagellin synthesis is stable and that the species of mRNA vary with respect to metabolic stability.

When bacteria are grown in media devoid of deoxycholate, and subsequently exposed to 0.1% sodium deoxycholate, no effect was observed on their organelles of locomotion. Although inhibition of swarming in *Proteus*, when deoxycholate was incorporated in the agar growth medium, was reported by Leifson (1935), no quantitative assay was performed. The present study shows that when *Proteus mirabilis* is grown on deoxycholate agar medium containing 0.05% sodium deoxycholate complete inhibition of swarming occurs. Since the presence of 0.05% deoxycholate had no appreciable effect on the physiology of gram-negative bacteria, its incorporation in the growth medium facilitates the isolation and enumeration of bacteria from specimens containing *Proteus* species.
The present work represents a basic study directed towards evaluation of the incorporation of deoxycholate for the selective isolation of gram-negative bacteria. Although suppression of the growth of gram-positive bacteria with deoxycholate was reported by Leifson (1935), the present study indicates that this medium influences the growth and physiology of gram-negative bacilli as well. Further improvement of this medium is suggested and it is recommended that a systematic search and evaluation of each medium component be carried out to identify the most desirable medium ingredients for a sensitive and reliable isolation of gram-negative, enteric bacilli present in environmental samples.

The classification of gram-negative bacilli of well known motile species is hindered by the production of non-flagellated organisms, when grown in the presence of deoxycholate. This should be curtailed in order to aid the laboratory in its tasks of accurately identifying gram-negative bacilli from environmental specimens.
SUMMARY

Sodium deoxycholate is used in a number of bacteriological media for the isolation of gram-negative bacteria. Initial experiments to study the effect of deoxycholate on the growth parameters showed an increase in the lag time constant and generation time, and a decrease in the growth rate constant and total yield of these bacteria. Further investigation showed reduction in oxygen uptake when the cells were incubated with deoxycholate and glucose used as substrates. Decreased uptake of glucose in the presence of deoxycholate suggests inhibition of a dehydrogenase in the cell. Using the Thunberg technique sodium deoxycholate was seen to inhibit the dehydrogenase activity when either glucose, succinate or fumarate were used as substrates.

Fractionation of labeled cells exposed to deoxycholate showed loss of cold-TCA-soluble, ethanol-soluble and trypsin-soluble fractions. However, the peptidoglycan was not significantly affected. Cells of E. coli incubated in phosphate buffer showed a higher death rate in the presence of deoxycholate. Moreover, the release of soluble cellular materials from the cell following treatment with deoxycholate suggests the solubilization of the cell envelope of E. coli.

When E. coli was grown in the presence of deoxycholate no effect was observed on the synthesis of lipids, DNA, RNA, protein and glucose-6-phosphate dehydrogenase activity. Although inhibition of protein synthesis was not observed, bacteria grown in the presence of sodium deoxycholate were observed to be non-flagellated and non-motile. This
implies that either the synthesis or the polymerization of flagellin may be inhibited by deoxycholate. The swarming of *Proteus mirabilis* was shown to be inhibited by the presence of 0.05% sodium deoxycholate.

The physiology of gram-negative bacilli has been shown to be influenced by the presence of sodium deoxycholate in the growth medium. Thus, the development of a more sensitive medium for the isolation of gram-negative enteric bacilli from environmental samples has been suggested.
LITERATURE CITED


Moltke, O. 1927. Contribution of the characterization and systematic classification of Bac. proteus vulgaris (Hauser). Levin and Munksgaard, Copenhagen.


APPORVAL SHEET

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Date: 9-6-79

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