responses to vagal stimulation; no effect was observed on the responses to direct injection of ACh, nor on the nictitating membrane.

The dialkyl-substituted amines displayed quite different properties: **38** and to a lower extent **40** appeared to be slightly hypotensive and to slow down the heart rate. *In vitro* **38** proved to be a competitive antagonist of norepinephrine. Lengthening of the alkyl chain causes the appearance of oxytocic activity: *in vitro* marked oxytocic activity, already evident for the lower homologs **44** and **45**, was exerted also by **46**, **48**, **49**, and **51**.

Some of quaternary derivatives, particularly **38** and **46**-methiodide, showed strong nicotinic properties. If given intravenously at 0.5–1 mg/kg, they caused a biphasic pressor response, characterized by a mild hypotension immediately followed by hypertension, which may be abolished by a pretreatment with hexamethonium (5 mg/kg sc). Previous adrenalectomy reduced this response considerably. The amides at 10 mg/kg ip in aqueous suspension, in the cat, appeared to have no appreciable effect on autonomic responses.

Of some interest is the spasmolytic activity of the disubstituted amides **16–21**, **23**, **24**, observed *in vitro* on the guinea pig ileum stimulated with histamine, ACh, and $BaCl_2$.

Antiexudative Property.—The dialkylamines **38** and **40** and the compounds **50–52**, given orally at the dose of 50 mg/kg, provided marked protection toward the foot edema produced by egg albumin in the rat.

Fungistatic Activity.—Unlike monoalkyl amino derivatives, quaternary compounds, and amido compounds, almost all tertiary amine derivatives have shown *in vitro* a mild activity toward *Candida albicans*, *Aspergillus niger*, and *Epidermophylon floccosum*. No appreciable effect toward gram-positive and gram-negative organisms was observed.

Discussion

The pharmacological study of the compounds examined has shown the following. (1) Few of the amides are effective on the central nervous system and exert a somehow depressant activity, synergistic with that of barbiturates; the lower homologs, such as the nonsubstituted amide and its N-monomethyl derivative exerted some protection toward pentylenetetrazole convulsant activity (see Table V). The N,N-disubstituted amides showed an interesting peripheral spasmolytic papaverine-like activity on smooth muscles. (2) In the series of amines lower monoalkyl-substituted members exert antidepressant and sympathomimetic effects qualitatively comparable to those of tranylcypromine and amphetamine, without modifying the brain and liver MAO activity (see Table V). In accordance with earlier observations by Zirkle, *et al.*,[3] we observed the importance of the *cis* and *trans* configuration for the appearance of the specific activity in the compounds studied: compound **29**, the *cis* analog of **28**, is devoid of any excitatory, anorexic, hyperthermic activities in the animal with a monoamine oxidase block, nor does it antagonize reserpine. Like the *cis*-phenethylamine derivatives,[12] our dialkylamino compounds appear endowed with antiepinephrine and oxytocic activity, the oxytocic properties being the more evident the longer the alkyl moiety. (3) The quaternary compounds are completely ineffective at the CNS level, but show significant nicotine-like properties.

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The Effects of Bile Acid Derivatives[1,2] on Bacterial Permeability and Enzyme Induction

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A series of twenty derivatives of cholanic acid has been tested for their abilities to accelerate cell swelling and to inhibit enzyme induction of a strain of *Pseudomonas aeruginosa*. The series included conjugated as well as unconjugated natural bile acids all of which bear a negative charge at physiological pH. These anionic substances may increase the rate of cell swelling but have no effect on enzyme induction. Evidence is presented that they increase bacterial permeability. Other anionic derivatives, not found naturally, behave similarly. Bile acids conjugated with N^1-trimethylethylenediamine, cholamine, are more potent in accelerating bacterial swelling. In addition, the cationic substances inhibit protein synthesis as evidenced by their inhibition of the induction of the enzymes which catabolize benzoic acid. Chenodeoxycholylcholamine, the more potent analog, approaches in effectiveness benzalkonium chloride (which is shown to have the same properties). The two effects on swelling and on enzyme induction are apparently not causally related. By altering the conditions of incubation, one can affect either cell swelling or enzyme induction.

When surface active agents are incubated with microorganisms, they apparently react with the cell membrane. Cell constituents such as potassium,[3] amino acids,[4] purines, and pyrimidines[5] diffuse into the medium, and protoplasts are rapidly lysed.[6] Anionic compounds are more active in acid solution probably because under these conditions the nitrogen groups in the proteins are more positively charged and thus facilitate ionic bonding.[7] In addition to ionic binding, other forces, possibly hydrophobic binding, must be involved in the interactions between anionic detergents and proteins. Thus, detergent may be associated

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(2) Parts of this paper were presented at the Fall Meetings (1966) of the American Society for Pharmacology and Experimental Therapeutics in Mexico City, and the National Academy of Sciences at Durham.

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with protein in excess of the stoichiometric amounts predicted by the number of cationic charges on the protein. In addition, the affinity between a given protein and detergent may be influenced by the size and structure of the hydrocarbon portion of the detergent.[8] Cationic compounds are more active in alkaline solutions and probably react with ionized acidic groups on both the protein and phospholipid of the membrane.[7] Added phospholipids counteract their effects.[9] MacGregor and Eliker[10] showed that ethylenediaminetetraacetate (EDTA) can make resistant strains of *Pseudomonas aeruginosa* sensitive to quaternary ammonium disinfectants which suggests that a divalent cation may have blocked the acidic groups by forming an un-ionized salt with them. Conversely, addition of calcium can, under certain conditions, antagonize the growth-inhibitory effect of benzalkonium chloride on *Escherichia coli*.[11]

The changes in the membrane which allow leakage of cell constituents could also allow the more rapid influx of added metabolites, and this might be the explanation for the increase in rate of respiration, glycolysis, and ammonia assimilation often seen when surfactants in low concentrations are added to microorganisms.[12]

In this paper the effects of various free and conjugated bile acids on the induction of enzymes which catabolize benzoic acid and on cell swelling of a strain of *Ps. aeruginosa* are described. This organism and most other gram-negative ones rapidly decrease in size when exposed to solutions of electrolytes or nonelectrolytes. This rapid, initial reaction is followed by a gradual swelling of the microorganism on continued incubation in a salt-containing medium.[13] When washed cells of *Ps. aeruginosa* are incubated in sucrose, essentially no secondary increase in cell size follows the initial shrinking. Addition of salt to the sucrose medium results in immediate swelling. Thus, the increase in cell size must be the result of the entry of electrolyte with its water solvent. The rate of swelling depends on three factors: the nature of the cation, the salt concentration, and the metabolic activity.[14] We have studied the effect of the bile acids on the swelling process and on enzyme induction of *Ps. aeruginosa*.

Experimental Section

A strain of *Ps. aeruginosa* maintained in this laboratory for 17 years was grown at 34° for 24 hr in 100 ml of Difco nutrient broth without shaking. The cells were centrifuged, washed twice with distilled H_2O, and suspended in distilled H_2O so that the absorbance (A) determined by the Coleman J. spectrophotometer at 500 mμ was between 0.160 and 0.180. This suspension (0.3 ml) was added to Na-K phosphate buffers of various molarities and pH 6.7 or 7.7 to a final volume of 2.0 ml.[14b] The compounds to be tested were added to the buffer before the addition of cells. After the initial reading, the tubes were incubated at 37° and the absorbance was determined after 20, 40, and 60 min. The results are expressed as change of absorbance. The inverse relationship between degree of swelling and absorbancy of a suspension was first described by Raaflaub[15] in studies of mitochondria. These changes in optical properties are attributed to change in translucency secondary to swelling or shrinkage. This has been utilized in bacterial studies.[13,14,16] Bernheim,[14] utilizing electron micrographs of the strain of bacteria used in these experiments, has documented the relationship. For the studies on enzyme induction, the oxidation of benzoic acid was used. Sleeper and Stanier[17] have shown that these enzymes are induced in *Pseudomonas*. The oxygen uptake was measured by the standard Warburg method. The conditions for the manometric experiments are described in the legend to Figure 2.

Unconjugated Bile Acids.—Cholic acid (Mann Research Laboratories, enzyme grade) was recrystallized twice from 70% EtOH. Chenodeoxycholic acid (Maybridge Research Chemicals) was recrystallized twice from benzene-EtOH as previously described.[18] Deoxycholic acid (Mann reagent grade) was recrystallized from acetone and then from 90% EtOH. Unconjugated bile acids used solely in the preparation of bile acid conjugates were obtained and purified by methods cited for each specific compound.

Preparation of Conjugated Bile Salts.—The following compounds were synthesized by the methods of Norman:[19] sodium taurodeoxycholate (**6**), sodium taurochenodeoxycholate (**7**), glycodehydrocholic acid (**14**), sodium glycodeoxycholate (**11**), sodium glycochenodeoxycholate (**12**), sodium taurocholate (**5**), and glycocholic acid (**10**). These compounds were chromatographically pure and had the reported melting characteristics. Sodium taurohyodeoxycholate (**8**) was prepared as described by Lee and Whitehouse.[20]

The synthesis of the following compounds by the mixed anhydride method of Norman[21] have been described:[18] glycohyodeoxycholic acid (**13**), 7,2-dihydroxycholanyltaurine (**9**), N-cholyl-*n*-5-aminovaleric acid (**15**), N-cholylaspartic acid (**16**), N-cholylaminoethylphosphonic acid (**17**), and N^1-cholyl-N^2-trimethylethylenediamine (cholylcholamine, **19**). The following compounds, synthesized by the mixed anhydride method, have not been previously reported: N-chenodeoxycholylaminoethylphosphonic acid (**18**) and N^1-chenodeoxycholyl-N^2-trimethylethylenediamine (**20**). These were prepared by procedures similar to those previously reported for the synthesis of their respective cholic acid analogs.

Other Compounds.—N^1-Trimethylethylenediamine chloride (cholamine, **21**) and pthalimidocholamine betaine (**22**) were synthesized by the method of Gabriel.[22]

Thin layer chromatography was used to ensure that the synthetic compounds and starting materials were chromatographically pure. The solvent systems and detection procedures employed for the bile salts and derivatives were the same as those cited previously.[18]

Results

The Effect of Bile Acids and Their Derivatives on Cell Swelling. (a) Anionic Compounds.—For purposes of comparison these negatively charged compounds have been grouped into four classifications: unconjugated bile acids, bile acids conjugated with taurine, bile acids conjugated with glycine, and bile acids conjugated with various amino acids. Their relative effectiveness in accelerating bacterial swelling is presented in Figure 1. Hyodeoxycholic acid (**4**) and its various conjugated compounds **8** and **12** had little

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or no effect on the rate of swelling. On the other hand, chenodeoxycholic acid (**3**), its taurine (**7**), its glycine (**12**), and its aminoethylphosphonic acid derivative

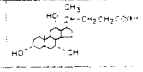
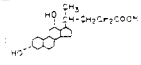
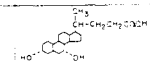
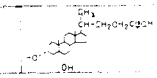
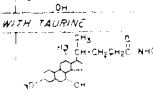
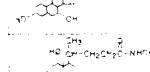
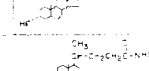
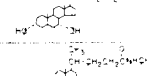
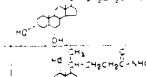
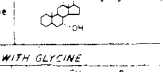
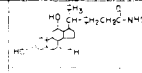
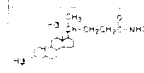
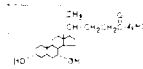
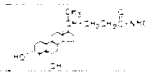
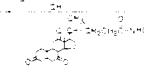
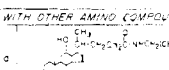
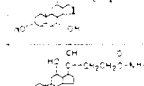
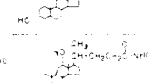
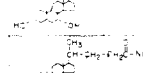
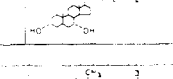
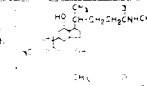
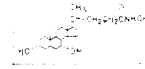
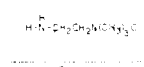
NO.	COMPOUND	FORMULA	CONC	RELATIVE ENHANCEMENT OF CELL SIZE[a] = ΔA OF BACT SUSPENSION + EXPTL CMPD / ΔA OF CONTROL	
			μg/ml	pH 6.7	pH 7.7
	UNCONJUGATED BILE ACIDS				
1	Cholic Acid		250	–	1.06
2	Deoxycholic Acid		250	–	1.48
3	Chenodeoxycholic Acid		250	–	2.30
4	Hyodeoxycholic Acid		250	–	1.20
	BILE ACIDS CONJUGATED WITH TAURINE				
5	Taurocholic Acid		250	0.98	0.86
6	Taurodeoxycholic Acid		250	1.44	1.22
7	Taurochenodeoxycholic Acid		250	1.33	1.54
8	Taurohyodeoxycholic Acid		250	1.09	0.98
9	7,12 Dihydroxycholonyltaurine		250	1.43	1.19
	BILE SALTS CONJUGATED WITH GLYCINE				
10	Glycocholic Acid		250	1.76	1.65
11	Glycodeoxycholic Acid		250	1.88	1.69
12	Glycochenodeoxycholic Acid		250	1.78	1.73
13	Glycohyodeoxycholic Acid		250	0.93	0.98
14	Glycodehydrocholic Acid		250	1.2	1.05
	BILE SALTS CONJUGATED WITH OTHER AMINO COMPOUNDS				
15	N-Cholyl-β-aminopropionic Acid		250	1.12	0.94
16	N-Cholylisoserine Acid		250	2	1.02
17	N-Glycylaminoethylphosphonic Acid		250	0.95	0.97
18	N-Chenodeoxycholylamino ethylphosphonic Acid		250	1.52	1.33
	CATIONIC COMPOUNDS				
19	N-Cholyl-N'-Trimethylethylenediamine (Cholylcholamine		50	1.26	1.52
20	N-Chenodeoxycholyl-N'-Trimethylethylenediamine (Chenodeoxycholylcholamine)		5	3	4.1
21	Cholamine		250	–	1.0
22	Phthalimidecholamine Betaine		250	–	1.09
23	Benzalkonium Chloride		5	30	40

Figure 1.—The effects of bile acids and their derivatives on cell swelling. The values represent the ratios of the decrease in absorbance at 500 mμ of the experimental to the control after 60 min of incubation.

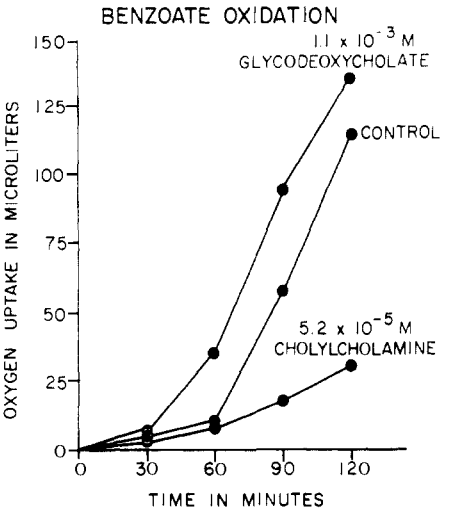


Figure 2.—The effects of glycodeoxycholate and cholylcholamine on the oxidation of benzoate, 1.7×10^{-3} *M*, 0.15 *M* Na–K buffer pH 7.7, 37°, final volume 2.0 ml, gas phase air. The concentration of cells was the same as described for the swelling experiments. When the experiments were prolonged an additional 60 min, no acceleration of oxygen uptake was observed in the flasks containing cholylcholamine.

(**18**) were invariably more potent than the corresponding analogs of cholic acid. In several cases (compare **1** with **3**, **5** with **7**, and **17** with **18**) the presence of a hydroxyl group at position 12 of the trihydroxycholanic acid abolished all activity. Deoxycholic acid and its derivatives, in general, were intermediate in activity between the trihydroxy acids and the chenodeoxycholic acid series. The active compounds showed a somewhat greater effect at pH 6.7 than at 7.7.

(**b**) **The Cationic Bile Salt Conjugates.**—Compounds **19** and **20** were more potent at the concentrations shown than their anionic congeners (Figure 1). In this group, bacterial swelling was greater at pH 7.7 than at pH 6.7.

The Effects of Inorganic Ions on the Acceleration of Cell Swelling by Bile Salt Derivatives.—Table I presents data which demonstrate that cholylcholamine and chenodeoxycholylcholamine are relatively more potent in accelerating bacterial swelling in 0.05 *M* than in 0.15 *M* phosphate buffer. In this regard, they mimic the behavior of benzalkonium chloride (**23**). On the contrary, glycodeoxycholic acid, which is a representative of the active anionic derivatives, has greater activity in the medium of higher ionic strength.

The swelling caused by the cationic bile salt derivative, cholylcholamine, is markedly inhibited by the presence of calcium (Table II). Calcium at this concentration also effectively blocked the action of chenodeoxycholylcholamine and benzalkonium chloride on cell swelling.

The Effects of Bile Acid Derivatives on Enzyme Induction.—The effects of cholylcholamine (**19**) and glycodeoxycholate (**11**) on the oxidation of benzoate are compared in Figure 2. Cholylcholamine prolonged the lag period which is characteristic of enzyme induction[17] and inhibited the oxidation of benzoate. The anionic compound shortened the lag period but had no effect on oxygen uptake.

Other anionic compounds (**3** and **12**) showed the same behavior as glycodeoxycholate. Chenodeoxycholylcholamine (**20**) and benzalkonium chloride (**23**)

TABLE I

BACTERIAL SWELLING IN SOLUTIONS OF DIFFERENT IONIC STRENGTH[a]

Compd	Concn, M	0.05 M buffer: $-\Delta A_{500}$, mμ 20 min	40 min	60 min	Relative swelling 60 min	0.15 M buffer: $-\Delta A_{500}$, mμ 20 min	40 min	60 min	Relative swelling 60 min
Control	...	35	43	54	1.00	84	106	117	1.00
Cholylcholamine (**19**)	4.8×10^{-5}	46	65	78	1.44	82	98	118	1.01
	9.6×10^{-5}	67	97	118	2.18	85	137	181	1.55
Chenodeoxycholylcholamine (**20**)	2.5×10^{-5}	62	95	123	2.28	89	137	174	1.49
	5.0×10^{-5}	110	135	150	2.78	141	209	233	1.99
Benzalkonium chloride (**23**)	1.4×10^{-5}	71	93	114	2.11	93	142	179	1.53
Glycodeoxycholic acid (**11**)	1.1×10^{-3}	38	52	63	1.17	170	182	190	1.63

[a] The effect of cholylcholamine, chenocholamine, and benzalkonium chloride on the rate of swelling (decrease in A_{500} with time) in 0.05 *M* and 0.15 *M* Na–K phosphate buffer pH 7.7, 37°. The term, "relative swelling" signifies ΔA_{500} of experimental incubation/ΔA_{500} of control. The molecular weight of benzalkonium chloride is set at 363.

TABLE II

EFFECT OF CALCIUM IONS ON BACTERIAL SWELLING[a]

Ca^{2+}, M	Cholylcholamine (**19**), M	$-\Delta A_{500}$, mμ 20 min	40 min	60 min	Relative swelling at 60 min
...	...	44	56	64	...
...	9×10^{-5}	76	118	149	2.32
2.3×10^{-4}	...	46	56	59	...
2.3×10^{-4}	9×10^{-5}	43	51	58	1.01

[a] The incubations were performed in Na–K buffer, 0.05 *M*, pH 7.7. The term "relative swelling" signifies ΔA_{500} of experimental incubation/ΔA_{500} of control.

acted qualitatively the same as cholylcholamine. However, as shown in Figure 3, chenodeoxycholylcholamine was twice as potent as cholylcholamine.

Experiments were performed with cells grown in the presence of 3 mg/100 ml of *o*-fluorobenzoate to preinduce the enzyme. The oxidation of benzoate by such cells was minimally inhibited by the quaternary compounds (Figure 4). The anionic salt, chenodeoxycholate, had no effect on oxygen uptake in contrast to its accelerating effect on uninduced cells. This indicates that the anionic compounds can increase the permeability of the cell to the inducer, benzoic acid.

Figure 3 shows that the cationic compounds inhibit enzyme induction more effectively in 0.15 *M* than in 0.05 *M* buffer. In 0.05 *M* buffer, low concentrations

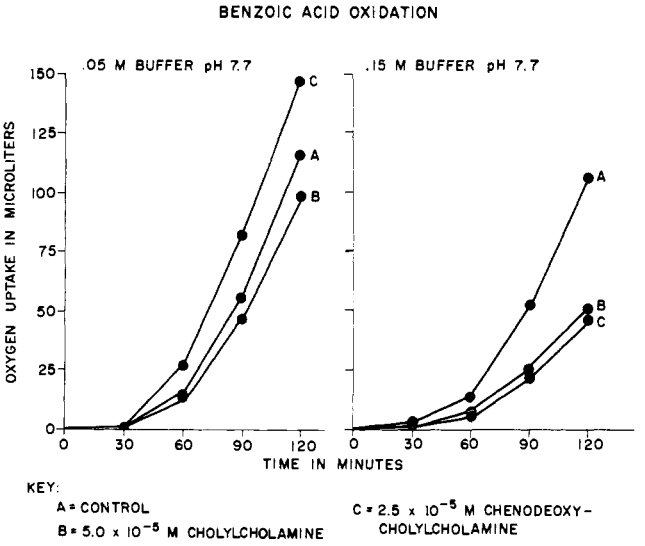


Figure 3.—The effect of cholylcholamine and chenodeoxycholylcholamine on the oxidation of benzoate in 0.05 *M* and 0.15 *M* Na–K buffer pH 7.7.

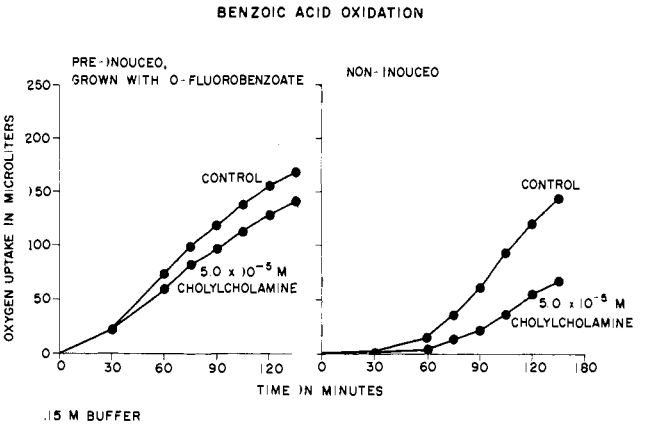


Figure 4.—The effect of cholylcholamine on the oxidation of benzoate by cells grown in media containing 3 mg/100 ml of *o*-fluorobenzoate compared with cells grown in regular media (noninduced). Conditions are the same as described for Figure 2.

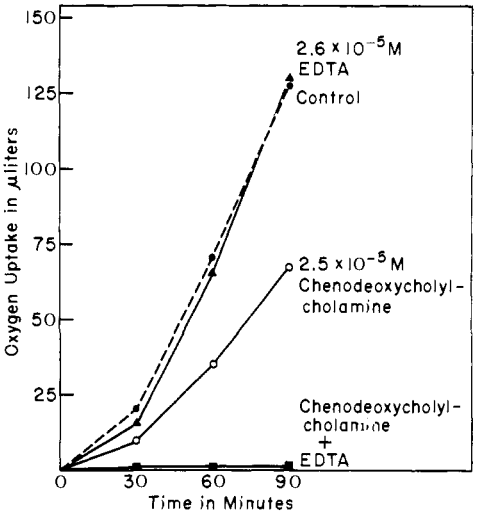


Figure 5.—The effect of ethylenediaminetetraacetate on the inhibition of benzoate oxidation by chenodeoxycholylcholamine.

may actually increase the rate of induction. Calcium ions abolish the inhibition of enzyme induction. Therefore EDTA should potentiate the inhibition. Figure 5 shows that it does.

Discussion

The effects of anionic bile salt detergents on augmenting bacterial cell swelling are in general accord with results previously reported by Bernheim[14a] for the

anionic alkyl- and alkenylsuccinic acids. In all cases greater relative activity was observed with increased buffer concentration.

The present survey of the bile salt compounds and their derivatives demonstrates that the chemical natures of both the steroidal and the ionic hydrophilic parts of the detergent molecule affect their abilities to accelerate swelling. Of the trihydroxycholanic acid group (the cholic acid series) only one compound (**10**) was active in this regard. The five others (**1**, **5**, **15**–**17**) did not have the ability to enhance bacterial swelling at the concentrations tested. Two series of dihydroxycholanic acid compounds are quite effective in enhancing cell swelling. The compounds of the chenodeoxycholic acid series are most active in this respect (**3**, **7**, **12**). Compounds of the deoxycholic acid series (**2**, **6**, **11**) were effective, although less so than the chenodeoxycholic series. The position of the α-hydroxyl group of the steroid nucleus as well as the number of hydroxyls is significant in determining activity. Thus the hyodeoxycholic acid series which has two α-hydroxyl groups at the 3 and 6 positions of the cholanic acid molecule has virtually no activity (see **4**, **8**, **13**).

When cholic acid (**1**) was conjugated with glycine, a compound was obtained which was active (the only active trihydroxy compound). Conjugation with glycine had no such effect on hyodeoxycholic acid or on the inactive triketo compound **14**.

The cationic cholanic acid compounds are noteworthy in that they are capable of accelerating swelling at much lower concentrations. In this regard, the most active one studied thus far approaches the potency of benzalkonium chloride compound (compare **20** with **23** in Figure 1). As with the anionic compounds, the cationic derivative **20** of chenodeoxycholic acid is more potent than the corresponding cholic acid analog **19**. Cholamine either as the free amine **21** or conjugated as an electrically neutral betaine **22** is inactive. This suggests that the positive charge of the cholamine moiety functions by orienting the detergent properly, most likely on the surface of the bacteria.

Since the cationic compounds do not inhibit the oxidation of benzoate once the enzyme is induced, the inhibition of oxidation in the noninduced cells may be attributed to an inhibition of enzyme synthesis. Calcium ions antagonize the inhibition which indicates that they are displacing the drug from a negatively charged site. Alternatively, the drug might displace endogenous calcium ions from some site within the cell where its presence is required for protein synthesis. However, EDTA by itself in the concentrations used had no effect on enzyme induction, but it markedly potentiated the potency of chenodeoxycholylcholamine. Therefore, the drug did not act by displacing endogenous calcium ions.

The following facts indicate that the effects of these cationic agents on enzyme induction and cell swelling are independent of one another. The anionic agents do not inhibit enzyme induction, although they cause cell swelling. The cationic agents do not inhibit enzyme induction in 0.05 *M* buffer, the condition in which they are most effective on cell swelling.

In work being completed, we have found that the growth of a number of gram-positive and gram-negative bacteria is inhibited by the bile acids conjugated with cholamine. These derivatives containing the positive charge in the side chain have been found to inhibit the growth of *Ps. aeruginosa*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptococcus hemolyticus* at concentrations between 3×10^{-4} and 2×10^{-5} *M*. Preliminary work suggests that the inhibition of growth and enzyme induction reflect the ability of these compounds to inhibit DNA synthesis.