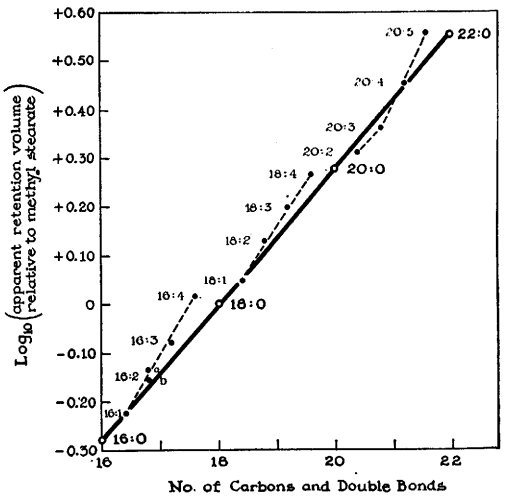


FIG. 1. Logarithms of retention volumes of long chain fatty acid methyl esters relative to methyl stearate (= 0); GLC at 197° on Reoplex 400 as stationary phase. Saturated normal homologs are shown by diagonal solid line. Groups of unsaturated acids are plotted in dashed lines. No. after colon indicates double bonds in acids of stated chain length (*i.e.* 16:4 = $C_{16}$-tetraenoic acid methyl ester). 16:2a = hexadeca-9,12-dienoic, 16:2b = hexadeca-6,9-dienoic acid.

400 serve to separate the mixtures in each. For example, in analysis of menhaden body oil fatty acids the fraction trapped between the trailing ends of the $C_{14}$- and $C_{16}$-saturate curves from an Apiezon M column can be collected and applied to a Reoplex 400 column. This mixture resolves cleanly into individual $C_{15}$ and $C_{16}$ acids, and in addition further separations in each group are based on numbers of double bonds. The success of this approach, an application of which has been described elsewhere by us(5), depends upon rigorous proof of stability of polyunsaturated fatty acid esters during GLC on Apiezon M.

*Summary.* Proof is presented that methyl esters of highly unsaturated long chain fatty acids are not significantly altered in chemical structure during gas-liquid chromatography with the stationary phase Apiezon M at 197°. Relative retention volumes of $C_{16}$, $C_{18}$, $C_{20}$ and $C_{22}$ polyenoic acids are listed for 2 stationary phases, non-polar Apiezon M and polar Reoplex 400. A system for rapid total analysis of complex fatty acid mixtures on a submilligram scale is described.

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## Inactivation of Amphotericin B, Chlorquinaldol, Gentian Violet and Nystatin by Bile Salts. (24308)

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Bile salts exert a variety of effects upon the action of different antibiotics(1). They enhance the antimicrobial activity of neomycin and penicillin against certain bacterial species, inactivate polymyxn, ristocetin and vancomycin and produce no effect whatsoever on other antibiotics. The present report is concerned with the effect of these salts upon the

TABLE I. Inactivation of Antimycotic Agents by 1% Bile Salts.

| Antimycotic agent | MIC in Basal broth (μg/ml) | MIC in Basal broth + 1% bile salts (μg/ml) | Increased MIC |
|---|---|---|---|
| Amphotericin B | .4 | 50 | 125 × |
| Chlorquinaldol | 6.0 | 60 | 10 × |
| Gentian violet | .2 | 25 | 125 × |
| Nystatin | 25.0 | 300 | 12 × |

activity of such diverse antimycotic agents as amphotericin B, chlorquinaldol, gentian violet and nystatin.

*Materials and methods.* The basal broth medium contained Trypticase (BBL) 7.5 g, Phytone (BBL) 1.3 g, sodium chloride 2.15 g, dipotassium phosphate 1.05 g, and glucose 10.0 g per liter of distilled water, adjusted to a pH of 7.2. Appropriate dilutions were prepared from the following sterile stock solutions: bile salts (Difco), 200 mg/ml in distilled water; amphotericin B (Fungizone®), 1 mg/ml in distilled water; chlorquinaldol (Steroson®), 5 mg/ml in 5% HCl; gentian violet, 1 mg/ml in distilled water; and nystatin (Mycostatin®), 5 mg/ml in propylene glycol. Bile salt and gentian violet stock solutions were sterilized by autoclaving at 15 lbs for 15 min., the others were prepared aseptically. All determinations of growth inhibition were performed in 5 ml amounts by the tube dilution method, except those involving gentian violet. These were performed in 10 ml amounts since readings of end points in the presence of the intense color imparted by higher concentrations of the dye was facilitated by the use of a greater volume. The standard inoculum employed throughout was 0.1 ml of an 18 hour culture of *Candida albicans* (MSH #005), grown at 37°C with an optical density of 0.18 at 650 mμ on the Lumetron colorimeter. Tubes were incubated at 37°C for 48 hours at which time they were read visually for the presence or absence of growth as indicated by formation of a distinct button of yeast growth at the bottom of each tube.

*Results.* Table I lists the minimal inhibitory concentrations (MIC) of each of the 4 antimycotic agents under study for the standard inoculum of *C. albicans* in basal broth as compared to the same medium to which bile salts had been added to yield a concentration of 1.0%. As is shown in the table, all were markedly but unequally inactivated by this concentration of bile salts. This is evidenced by the increased MIC of amphotericin B and gentian violet for *C. albicans* of 125 times, for nystatin of 12 times and for chlorquinaldol of 10 times.

To ascertain whether this finding is limited to the particular strain of *C. albicans* studied (MSH #005), 10 additional typical *C. albicans* strains were also investigated. One per cent bile salts completely neutralized the inhibitory effect of 2.0 μg/ml of gentian violet for all 10 strains, so that the described inactivation is not peculiar to the strain employed as the test organism.

Further studies were undertaken to elucidate the mechanism of inactivation of these antifungal agents by bile salts. Addition of the latter to unseeded medium in 0.5, 1 and 2% concentrations produces no change in the reaction of the medium either prior to or after 48 hours incubation at 37°C. Moreover, no difference was observed between the final reaction of the bile salts media and that of the basal medium after their inoculation with the test organism and growth at 37°C for 24 and 48 hours. Thus the effect of bile salts upon activity of antimycotic agents cannot be ascribed to any alteration of pH. Bile salts in the above concentrations also had no significant effect upon the growth curve of the test organism since growth after 24 hours and 48 hours at 37°C, measured photometrically, was essentially the same in the bile salts media as in the basal medium. Since the inactivating effect of bile salts on antifungal agents might conceivably be due to the surface tension depressing property of the former, it was decided to investigate the effect of other surface active agents. Two anionic compounds, di isopropyl napthalene sodium sulfonate (Naccosol A) and di hexyl sodium sulfosuccinate (Aerosol MA), and 2 non-ionic compounds, alkyl aryl polyether alcohol (Triton X-100) and polyoxyethylene sorbitan monooleate (Tween 80) were selected for these

studies. Cationic surface active compounds could not be tested because of their own marked growth-inhibiting activity against *C. albicans*. The minimal amount of bile salts and that of the detergents tested capable of neutralizing the inhibitory effect of a fixed amount of amphotericin B, 10 μg/ml or 25 times the MIC for C. *albicans*, was determined. It was found that whereas 0.4 mg/ml bile salts completely neutralized the growth inhibiting effect of the amphotericin B, 6.0 mg/ml of Naccosol A, 10 mg/ml of Aerosol MA, and 20% by volume of both Triton X-100 and Tween 80 all failed to do so. Higher concentrations of Naccosol A and Aerosol MA could not be tested since these concentrations were by themselves growth inhibitory for *C. albicans*. In the light of these findings it was concluded that inactivation of antimycotic agents by bile salts could not be attributed to the surface tension depressive action of the latter.

To ascertain whether bile salts exert their effect upon the microorganisms or upon the chemotherapeutic agent in the course of neutralizing the inhibitory action of antifungal compounds, the following experiments were performed: 10 ml quantities of the basal medium with and without bile salts in 0.5, 1 and 2% concentrations were seeded with a standard inoculum of *C. albicans* and incubated at 37°C for 48 hours. All tubes were then centrifuged at 2000 rpm for 15 minutes and the supernatant fluids poured off and replaced with an equivalent volume of sterile saline. The amount of each of the 4 antimycotic agents under investigation required to inhibit a similar sized inoculum of bile-exposed and unexposed *Candida* cells was then determined. No difference was observed between the concentrations of each drug required to inhibit the bile-treated and untreated cells thereby indicating that contact of *C. albicans* with bile salts for 48 hours at 37°C had not affected the susceptibility of the microorganisms. Minimal amounts of bile salts needed to neutralize the antimycotic effect of graded concentrations of gentian violet was then determined. Amounts of bile salts capable of annulling the *Candida* inhibiting activity of graded concentrations of gentian violet was directly proportional to concentration of dye employed (Fig. 1). This would indicate that the inactivating effect of bile salts is exerted upon the antimycotic drug rather than upon the organisms.

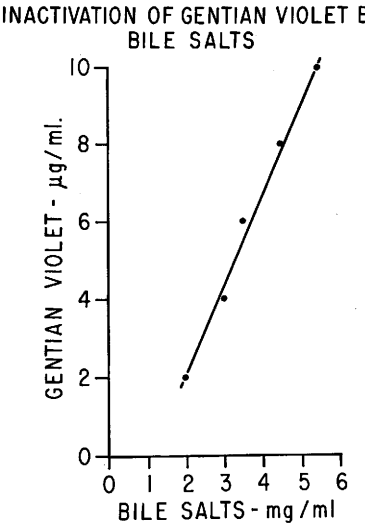


FIG. 1. Minimal amounts of bile salts capable of neutralizing *Candida* growth inhibitory effect of graded concentrations of gentian violet.

*Discussion.* The exact mechanism by which bile salts inactivate the different antifungal agents studied has not been established. Bile salts, in the concentrations employed, produce no pH change in the medium, exert no stimulatory or depressing effect upon growth of the test organism nor can their neutralizing effect upon antifungal agents be attributed to their property of surface tension depression. Our inability to demonstrate altered susceptibility of the microorganism plus existence of a straight line relationship between amount of bile salts needed to inactivate graded concentrations of gentian violet suggests that the former, in some undetermined manner, acts upon the chemotherapeutic agent itself rather than upon the fungus. Additional experiments to further resolve the mechanism of the reported observation as well as to determine the responsible component or components of the bile salts mixture are underway.

Each of the antimycotic drugs investigated ordinarily fails to produce significant blood levels in patients after oral administration.

This is also true of the 3 antibacterial agents previously found to be inactivated by bile salts, namely polymyxin, ristocetin and vancomycin(1). In the light of the findings herein reported, it may very well be that diminished or absent absorption of certain antifungal and antibacterial agents when administered orally is due to their inactivation by the high concentration of bile salts normally present in the intestinal tract rather than to their poor diffusion through the intestinal wall because of their large molecular size. Studies leading to reversal of bile inactivation of these antimicrobial agents could lead to their increased absorption and hence clinical usefulness.

*Summary.* One per cent bile salts inactivates amphotericin B, chlorquinaldol, gentian violet and nystatin to a considerable but variable degree depending upon the antimycotic agent. A number of factors possibly involved in the mechanism of this inactivation have been studied and are discussed.

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## Serum Cholesterol Determinations as Affected by Vitamin A. (24309)

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It was observed in this laboratory that Vit. A administration resulted in an increase in total serum cholesterol and that as the serum Vit. A level increased, serum total cholesterol increased proportionately. It was then necessary to determine if the elevation of serum Vit. A was associated with an apparent or real increase in total serum cholesterol.

The method used(1) for determining serum cholesterol was one widely used in clinical laboratories. Since the reagent ferric chloride is used both in this method and in measurement of Vit. A, it was felt that perhaps Vit. A was interfering with determination of cholesterol. It was therefore believed worthwhile to investigate the effect of Vit. A on the ferric chloride method of determining serum cholesterol.

*Methods and materials.* Serums were taken from a group of 5 patients, having the following Vit. A concentration as determined by the Carr-Price method(2); patient 1, 55; patient 2, 28; patient 3, 56, patient 4, 37, and patient 5, 40 $\mu$g% respectively. Of this group of serums, 3 had elevated cholesterol levels and 2 had normal cholesterol levels. Varying amounts of Vit. A alcohol equivalent to 15-150 $\mu$g per 100 ml serum were added to each serum (Tables I and II). In each case, a control tube was set up containing 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 ml respectively of Vit. A alcohol (3 $\mu$g per ml in Mallinckrodt chloroform) were added and the volume made up to 0.6 ml with chloroform.

A total cholesterol determination was then done on each of the 6 tubes from the 5 different serums by the method of Zak(3).

The same group of 5 serums was set up

TABLE I. Effect of Varying Concentration of Vit. A on Determination of Total Serum Cholesterol by the Zak Procedure(3).

| | mg % total cholesterol | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | 15 $\mu$ %* | 30 $\mu$ %* | 60 $\mu$ %* | 90 $\mu$ %* | 120 $\mu$ %* | 150 $\mu$ %* |
| Patient 1 | 403 | 417 | 425 | 455 | 480 | 510 | 545 |
| 2 | 363 | 363 | 380 | 415 | 445 | 470 | 500 |
| 3 | 321 | 326 | 330 | 360 | 390 | 420 | 445 |
| 4 | 249 | 253 | 260 | 295 | 325 | 360 | 390 |
| 5 | 205 | 208 | 210 | 230 | 255 | 280 | 300 |

* Vit. A added.