APPLIED MICROBIOLOGY, Oct. 1975, p. 530-535
Vol. 30, No. 4

Printed in U.S.A.

Convenient Non-Chromatographic Assays for the Microbial Deconjugation and 7α-OH Bioconversion of Taurocholate

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Received for publication 29 April 1975

We described two convenient assay methods to estimate bile acid deconjugation and bile acid bioconversion at the 7α-OH position by individual microorganisms grown in media containing taurocholic acid. The methods are based on (i) a selective chemical assay for taurine conjugates previously described and (ii) the use of a cell-free preparation of 7α-hydroxysteroid dehydrogenase from *Escherichia coli* to directly quantify 7α-OH groups. These non-chromatographic approaches have been applied to the study of three model strains of intestinal organisms, *E. coli*, *Bacteroides fragilis*, and *Clostridium perfringens*, grown in standard media in the presence of purified tritiated taurocholate. Assay results were confirmed by thin-layer chromatography solvent systems designed to separate conjugated from unconjugated bile acid and unmodified cholic acid nucleus from 7α-OH bioconversion product(s) (primarily 3α,12α dihydroxy, 7-keto-cholanoic acid). In addition, 7α-hydroxysteroid dehydrogenase activity was demonstrated in cell-free extracts of all three organisms. Of the three organisms, only *C. perfringens* was demonstrated to (i) deconjugate taurocholic acid, (ii) contain 3α-hydroxysteroid dehydrogenase activity, (iii) convert cholic acid into at least five labeled metabolites visible on thin-layer chromatography, and (iv) catalyze significant tritium exchange with water in the medium.

Secondary bile salts arise in the gastrointestinal tract as a result of bacterial action on primary bile salts. Metabolic actions of the intestinal flora on bile salts include: (i) deconjugation (removal of the amino acid taurine or glycine by enzyme-catalyzed amide hydrolysis); (ii) 7α-OH bioconversion {this term is applied to include oxidation of the 7α-OH group to a 7-keto group [by 7α-hydroxysteroid dehydrogenase (HSDH)], reduction to an alkene [by 7α-OH dehydroxylase], or possible stereoisomerization to the 7α-OH group [by isomerase]}; (iii) alteration of either the 3α- or 12α-OH group or degradation of the bile acid skeleton. It is generally accepted that reactions of categories (i) and (ii) are much more common than those described in (iii) (9).

A number of strains of *Clostridium* (1, 10) and a lesser number of strains of *Micrococcus*, *Bacteroides*, *Bifidobacterium*, *Eubacterium*, and *Veillonella* (5, 10, 14) have been demonstrated to deconjugate bile salts. Purified cell-free preparations of *Clostriduim perfringens* have been used to deconjugate bile salts in vitro (19, 20).

Oxidation of the 7α-OH group to the corresponding ketone has been observed in *Escherichia coli* (21). Cell-free preparations of 7α-HSDH have also been obtained from *E. coli* (2, 8, 15) and *Bacteroides fragilis* (17). The *E. coli* enzyme has been applied to bile acid analysis of duodenal aspirates (8, 16). Reduction of the 7α-OH group to an alkene has been demonstrated in anaerobes, such as anaerobic lactobacilli (18). The 7α-dehydroxylase enzyme is reported to be very unstable in cell-free preparations (2).

In studies performed on various strains of organisms grown on media containing bile acid, thin-layer chromatography (TLC) is generally used to detect bile acid alteration. It is the purpose of this communication to introduce non-chromatographic assays for estimation of percentage of bacterial deconjugation and percentage of 7α-OH bioconversion. These assays are verified in three model organisms by estimation of products of bioconversion from radiolabeled taurocholate (TC), after separation on TLC.

MATERIALS AND METHODS

Origin and growth of bacteria. *E. coli* (strain 23) and *B. fragilis* (strain 18) were obtained from clinical sources and have been previously described (16, 17). *C. perfringens* (Nair strain, ATCC 19574) was obtained from the American Type Culture Collection, Rockville, Md. 20852. These organisms were grown in 10 ml of brain heart infusion (BHI) broth (Difco) to which were added 0.1% (wt/vol) sodium thioglycolate and 10^{-3} M randomly tritiated TC (0.1

μCi) purified on TLC. After 4 days of incubation at 37 C the cultures were centrifuged at 6,000 $\times$ *g* to sediment the bacteria, and the supernatant fluids (spent media) were collected and subjected to chromatographic and non-chromatographic analysis for percentage of deconjugation and percentage of 7α-OH bioconversion.

Reagents. Unlabeled hydroxy-bile acids were products of Calbiochem, and 7-keto-deoxycholate (7k-DC) was from Steraloids. Nicotinamide adenine dinucleotide (NAD) and NAD phosphate were from Sigma Chemical Co. Azure A, glycine, NaOH, solvents, sulfuric acid, and sodium thioglycolate were from Fisher Chemicals. 3α-HSDH (EC 1.1.1.50) was obtained from Sigma. Cell-free extracts of 7α-HSDH from *E. coli* and from *B. fragilis* were prepared as previously described (16, 17).

Randomly tritiated taurocholic acid was obtained from New England Nuclear Corp. (250 μCi/0.137 mol of TC).

Assay for deconjugation. A volume of 0.6 ml of acetic anhydride was placed in separate 15-ml centrifuge tubes equipped with ground-glass joints and stoppers (Kontes Glass Co., Vineland, N.J.). To each tube was added a 30-μl aliquot of each of the spent culture media. Blank tubes (containing TC in sterile BHI broth) were run in parallel. The assay procedure for total taurine-conjugated bile acids was then performed as described by Christie et al. (4). A standard curve was also developed by the same procedure using 10 to 40 μl of sterile BHI broth containing 10^{-3} M TC (Fig. 1a). A blank curve from sterile BHI broth with TC was developed in parallel (Fig. 1a). The percentage of deconjugation was estimated from the absorbance at 645 nm of 30 μl of spent medium compared to the absorbance at 645 nm of 30 μl of the same medium in the absence of bacterial growth.

Assay for 7α-OH bioconversion. Quantification of 7α-OH groups in spent bacterial medium was based on previously described complete oxidation studies of bile acids with 7α-HSDH (15). Spent bacterial medium (200 μl) was introduced into a 3-ml cuvette containing 2.1 ml of water, 0.5 ml of 1.0 M sodium phosphate buffer (pH 9.5), and 0.1 ml of 53 mM NAD. The absorbance at 340 nm was read on a Beckman GB spectrophotometer and Beckman 10-inch (ca. 25-cm) recorder. A 100-μl aliquot of fresh *E. coli* 7α-HSDH solution (6 mg of lyophile/1.5 ml of water) was introduced. Absorbance readings were made at 15, 20, and 25 min. Blanks containing no TC were read before and after the addition of enzyme. A standard curve was developed on the absorbance changes at 340 nm by *E. coli* 7 α-HSDH at complete oxidation of 50 to 300 μl of 10^{-3} M TC in BHI broth.

Similar standard curves were constructed by the analogous use of NAD-dependent *B. fragilis* 7α-HSDH (17) and *Pseudomonas testosteroni* 3α-HSDH (12) (Fig. 1b). The percentage of 7α-OH bioconversion was calculated in spent bacterial medium on the basis of the disappearance of 7α-OH groups in spent bacterial medium compared to sterile medium (10^{-3} M) in TC.

Assay for total 3α-OH groups in spent bacterial medium. Spent bacterial medium (200 μl) was subjected to complete oxidation by purified 3α-HSDH (EC 1.1.1.50) from Sigma (100 μl of 1 mg of 3α-HSDH per ml). The reaction procedure was analogous to that described above.

TLC procedure for bile salt deconjugation. Unextracted spent bacterial medium (50 μl) was spotted directly onto a silica gel (20 by 20 cm) TLC plate (gel thickness, 0.5 mm). Five-microliter volumes of 3 $\times$ 10^{-2} M solutions of TC, C, and DC were spotted in parallel. The plate was chromatographed in a 1-h pre-equilibrated TLC chamber containing 40 ml of toluene-acetic acid-water, 10:10:1 (vol/vol/vol) (16), for 2.5 h. Selected lanes were sprayed with 3.5% molybdophosphoric acid reagent (E. Merck Laboratories) and developed at 140 C for 10 min. Corresponding lanes, not sprayed, were divided, scraped, and eluted with two 5-ml volumes of redistilled methanol. The eluates were reduced to dryness under a stream of nitrogen and subjected to the liquid scintillation counting protocol.

TLC procedure for 7α-OH bioconversion. Before TLC, bile salts in the spent bacterial media were chemically deconjugated and extracted. Two milliliters of spent medium was added to 2 ml of 2.5 N NaOH in a 25-ml glass test tube covered by a cotton-wool plug and aluminum foil. Samples were subjected to 120 C at 15 lb/in^2 in a Presto pressure cooker for 1.5 h. Samples were cooled to 0 C, transferred to centrifuge tubes equipped with ground joint glass stoppers, and acidified with 0.8 ml of concentrated HCl (final pH $\simeq$1.0). Acidified samples were twice extracted by two 5-ml additions of diethyl ether. After shaking and 5-min centrifugation at 500 $\times$ *g*, the top phases were removed. This was repeated once, and the combined top phases were reduced to dryness under a stream of nitrogen. The extracts were reconstituted in 0.5 ml of methanol-3% peroxide, 4:1 (vol/vol). A 50-μl aliquot of each extract was plated onto a silica gel TLC plate (40 by 20 cm; gel thickness, 0.5 mm). Five microliters of 3 $\times$ 10^{-2} M C and DC and 10 μl of 3 $\times$ 10^{-2} M 7k-DC were plated in separate lanes. The plate was chromatographed in a 2-h pre-equilibrated TLC chamber containing 70 ml of benzene-diozane-acetic acid, 75:20:2.0 (vol/vol/vol) (system S_1 of Eneroth [6]) for 24 h. Selected lanes were developed with 3.5% molybdophosphoric acid spray reagent as before.

Estimation of tritium as ^3H_2O. Spent bacterial medium was distilled on a small distillation apparatus (Kontes), and 25 μl of distillate was subjected to counting protocol.

Counting protocol. Spent bacterial media (25 μl) and deconjugated bile acid extracts were subjected to counting protocol. Eluates from scrapings after TLC of media extracts were also counted. All samples were added to glass vials containing 10 ml of toluene-Triton cocktail {5.87 g of 2,5-diphenyloxazole and 98 mg of 1,4-bis-[2]-(5-phenyloxazolyl)benzene in a solution consisting of 1 liter of toluene and 360 ml of Triton X-100}. Samples and quenched standards were counted for 20 min through a full tritium window for label recovery. From a standard quench curve (external standard ratio versus percentage of efficiency) the disintegra-

tions per minute were computed from the counts per minute for all quenched samples.

RESULTS

Assay for deconjugation. Although neither *E. coli* nor *B. fragilis* could deconjugate TC, *C. perfringens* (Nair strain) almost totally deconjugated TC (Fig. 2a) as established previously by Nair et al. (19, 20). Estimations of the percentage of deconjugation obtained non-chromatographically were within 2% of the chromatographic estimate (Table 1). The standard curve for the estimation of TC is essentially identical to that of Christie et al. (4) (Fig. 1a). As shown in this study, the assay method will quantify TC without measuring either free taurine or cholate. A nonspecific background due to the BHI broth itself was measurable, but contributed less than 10% of the color associated with the TC itself at a concentration of 1 mM. At the high end of this standard curve where we routinely worked, duplicates were reproducible within ±3%.

Assay for 7α-OH bioconversion. Both *E. coli* and *B. fragilis* were shown to oxidize the 7α-OH group yielding the 7-keto derivative (Fig. 2b). The percentage of 7α-OH bioconversion amounted to only 15% for both these organisms (Table 1). An absence of any DC was observed in the supernatant fluid after growth of both these organisms (Fig. 2b); thus, all the 7α-OH bioconversion appeared to be due to oxidation by these two bacteria. As previously demonstrated (16, 17), 7α-HSDH activity was present in cell-free sonicates of both organisms (Table 1).

As much as 28% 7α-OH bioconversion was measured non-chromatographically in *C. perfringens;* however, this figure could not be immediately confirmed by TLC, since no less than five labeled metabolites were revealed on TLC, one of which corresponded to 7k-DC (Fig. 2b). An NAD phosphate-dependent form of both 3α- and 7α-HSDH was demonstrable in this strain of *C. perfringens* (Table 1).

The standard curve for estimation of 7α-OH groups (Fig. 1b) represents quantitative oxidation of the 7α-OH groups present in supernatant fluid using *B. fragilis* 7α-HSDH, whereas oxidation with *E. coli* 7α-HSDH represents 96% oxidation of 7α-OH groups on the basis of the yield of reduced NAD, as estimated spectrophotometrically (11). Although some background absorption from the BHI medium was present, it did not interfere and, for each individual reaction mixture, was subtracted from the absorbance after complete oxidation. Oxidation of 3α-OH groups of TC in BHI medium by *Pseudomonas testosteroni* is quantitative, and the resulting standard curve cannot be distinguished from the *B. fragilis* 7α-HSDH oxidation curve. Although the estimations for 7α-OH bioconversion (Table 1) were made with *E. coli* 7α-HSDH, identical results were obtained with *B. fragilis* 7α-HSDH.

Estimation of 3α-OH containing bile salts in spent bacterial media. 3α-OH containing bile salts were recovered quantitatively in the supernatant fluids of *E. coli* and *B. fragilis*, but a 24% loss of 3α-OH containing bile acids was found in the supernatant fluid of *C. perfringens*.

Yields of tritiated bile salt. About 82 ± 5% of the labeled TC was recovered after subjecting 10^{-3} M TC in BHI broth to TLC (Table 2). Label losses on TLC were largely attributed to silica binding. Another source of label loss was due to tritium exchange with water, which amounted to 17% in the *C. perfringens* culture and 1.0% in the *B. fragilis* culture but was insignificant in the *E. coli* culture or sterile broth containing 10^{-3} M TC (Table 2). Label loss due to alkaline hydrolysis and ether extraction from acidified medium amounted to about 10% in all cases.

TABLE 1. *Bacterial deconjugation and 7α-OH bioconversion of TC estimated chromatographically and non-chromatographically*

Organism	% Deconjugation		% 7α-OH bioconversion		Demonstration of HSDH in cell-free preparations	
	TC estimation	TLC	7α-OH group estimation	TLC	7α-HSDH	3α-HSDH
E. coli 23	0.0	0.0	16	15	+(NAD)	–
B. fragilis 18	2.1	1.5	16	14	+(NAD)	–
C. perfringens (Nair)	95.0	97.0	28	—[a]	+(NADP)[b]	+(NADP)
None	0	0	0	0	–	–

[a] TLC estimation of 7α-OH bioconversion in *C. perfringens* was complicated by the formation of several metabolites from labeled TC.

[b] NADP, NAD phosphate.

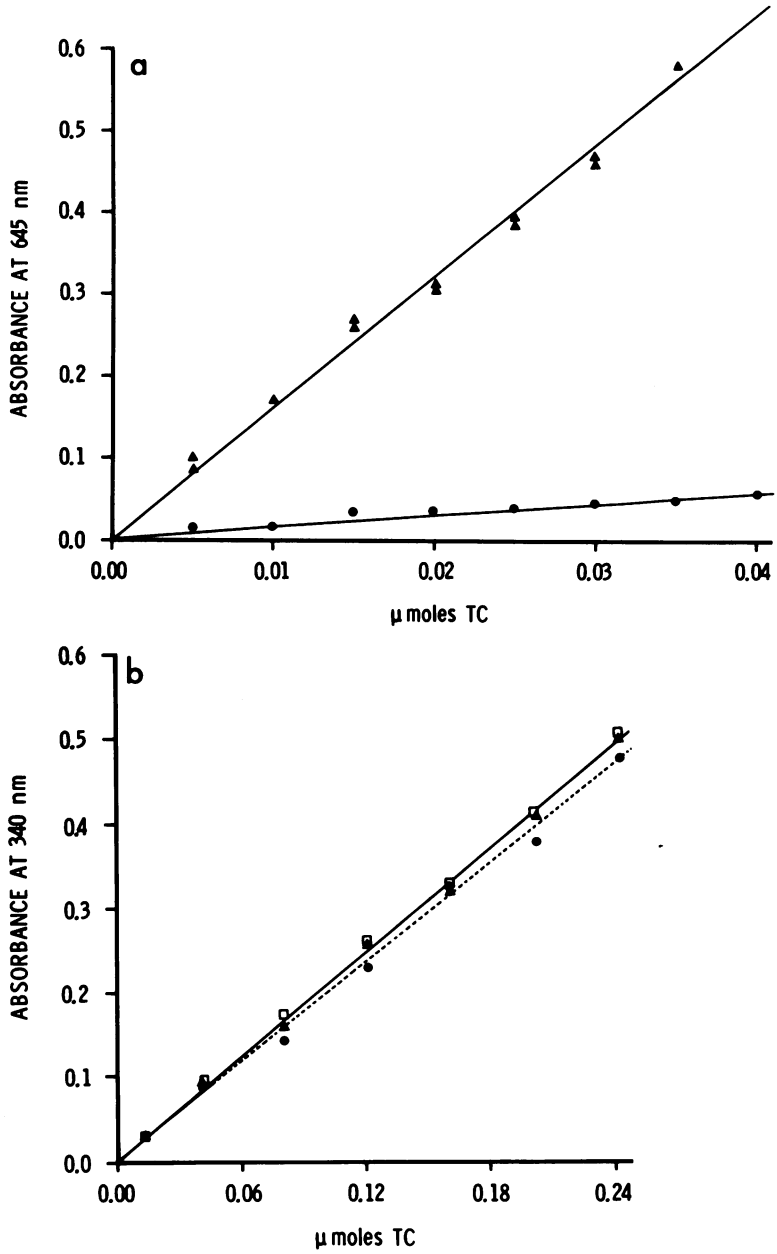

FIG. 1. *(a) Standard curve and blank curve for the estimation of taurine-conjugated bile acids (TC) in BHI broth. Absorbance values at 645 nm of the lower phase (see Materials and Methods) are plotted against the concentration of TC in BHI broth (▲) (duplicate analysis). Absorbance values of identical aliquots of BHI broth without TC (●) are represented in parallel (single analysis). (b) Standard curves for estimation of total 7α-OH groups and 3α-OH groups in TC and BHI broth. Increases in absorbance at 340 nm are plotted against the concentration of TC after complete oxidation by E. coli 7α-HSDH (●), B. fragilis 7α-HSDH (▲), and P. testosteroni 3α-HSDH (■).*

DISCUSSION

It must be emphasized that the non-chromatographic methods described above were designed to compliment rather than replace TLC procedures. TLC should be used when either of these rapid tests indicates significant metabolism of bile salts by the bacteria. Significant metabo-

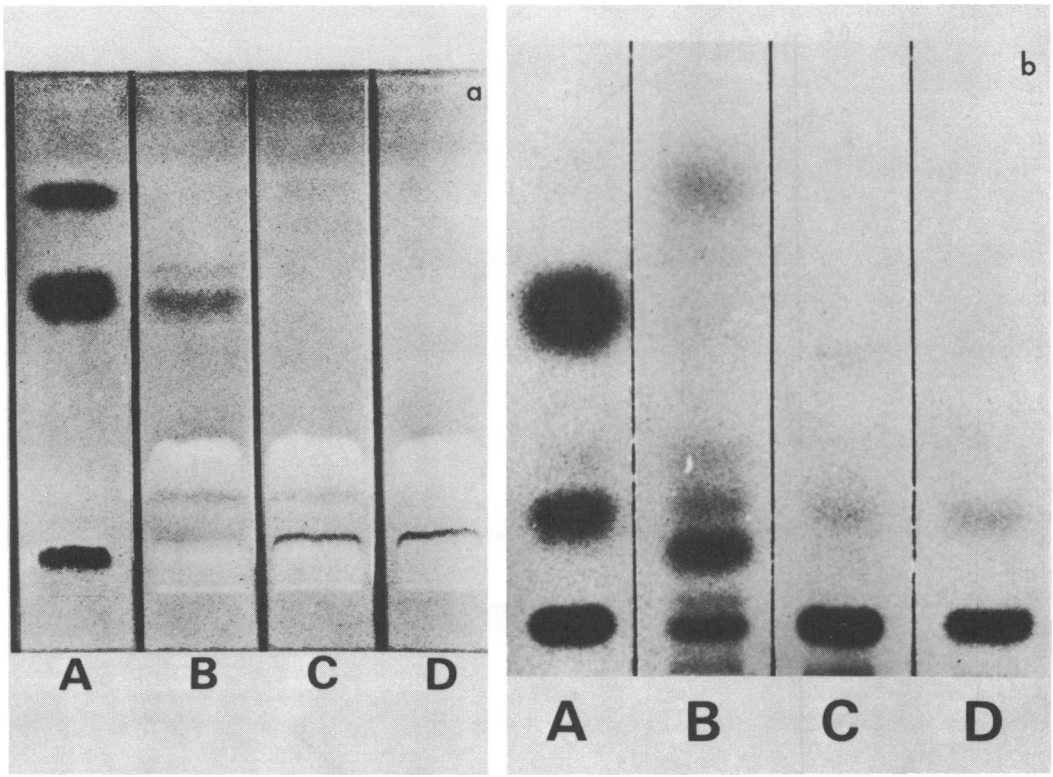

FIG. 2. (a) *TLC indicating deconjugation of TC (or lack of deconjugation). (A) From top to bottom, standard DC, C, and TC; (B) 50 μl of spent C. perfringens medium; (C) 50 μl of spent B. fragilis medium; and (D) 50 μl of spent E. coli medium, all originally containing 10^{-3} M TC. Solvent: toluene-acetic acid-water, 10:10:1 (vol/vol/vol). (b) TLC indicating 7α-OH transformation of the cholate nucleus of TC. (A) From top to bottom, standards DC, 7k-DC, and TC; (B) 20 μl of concentrated hydrolysate of spent C. perfringens medium; (C) 20 μl of concentrated hydrolysate of spent B. fragilis medium; and (D) 20 μl of concentrated hydrolysate of spent E. coli medium, all originally containing 10^{-3} M TC. Solvent: benzene-dioxane-acetic acid, 75:20:2.0 (vol/vol/vol).*

TABLE 2. *Overall label yields for TLC systems I and II*

Organism	Yield on TLC (% dpm) (system I)	% dpm in ^3H_2O (volatile fraction)	% dpm on TLC plate associated with TC and C bands (system I)	Yield on TLC (% dpm) (system II)	% dpm on TLC plate associated with C and 7k-DC bands (system II)
E. coli	86	0.03	98	78	95
B. fragilis	77	1.0	97	73	96
C. perfringens	60	17.0	99	52	38
None	84	0.03	98	76	91

lism was arbitrarily designated as >5% deconjugation or 7α-OH bioconversion. However, in view of the likelihood that many intestinal organisms do not metabolize bile salts, rapid tests for bile acid metabolism represent a considerable convenience in screening large numbers of organisms.

The application of non-chromatographic assays requires several routine precautions for meaningful results. The introduction of 0.2 ml of 50 mM TC into the BHI broth should be done with precision to ensure a final concentration of 1.0 mM in all tubes. A 1.0-ml amount of inoculum is introduced into 9.0 ml of BHI broth to give a final volume of 10 ml, the same as that for the control. Quantification of 3α-OH containing bile salts estimates losses of bile salts due to bacterial binding, uptake, or skeletal degradation or, alternatively, 3α-OH bioconversion. A loss of 3α-OH containing bile acids (>5%) in the

presence of bacteria also indicated the use of TLC.

These assays are specific for the groups they measure. The assay for taurine conjugates will not measure unconjugated bile acids or free taurine but will estimate taurine conjugates of transformed steroid nuclei (4). The assay for 7α-OH groups will not measure 3α-OH, 12α-OH, or 7β-OH groups or the corresponding ketones, the state of conjugation being inconsequential. It appears that the 7α-HSDH from *E. coli* and *B. fragilis* may be used interchangeably for the latter assay.

These assays have the advantage over TLC methods, since no errors due to label lost by silica binding, alkaline hydrolysis, and ether extraction or tritium exchange with water are encountered. But they have the disadvantage of not revealing bioconversions at the 12α-OH position or alterations in the nuclear skeleton. TC is an excellent model substrate, having four potentially measurable groups: the 3α-, 7α-, and 12α-hydroxyls and conjugated taurine. It appears to be one of the least inhibitory bile acids to bacterial growth (3).

The loss of label on incubation of TC with *C. perfringens* may be associated with some (or all) of the bioconversion reactions evidently present in whole cells. A much smaller percentage of labeled exchange (0.59%) was shown to occur by LaRusso et al. (13) on incubation of [2,2′;4,4′^{3}H]cholate with *C. paraputrificum* for 1 day only. The same group, however, showed as much as 35% label exchange in extract of feces where the population of *C. perfringens* is comparable to that of *C. paraputrificum* (7). Not surprisingly, as much as 55% loss of tritium has been observed when randomly labeled bile acids are introduced into the normal human enterohepatic circulation (22).

The loss in 3α-OH groups (24%) as well as 7α-OH groups (28%) in the presence of *C. perfringens* could be rationalized in part by binding of bile acids to the organisms. However, since both 3α- and 7α-HSDH have been demonstrated in cell-free preparations of this organism (Table 1), relatively minor amounts of 3-keto, 7-keto, and 3,7-diketo derivatives would be expected to form. A major metabolite (Fig. 2b, lane B) containing over 50% of the total label remains to be identified.

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