Exchange of tritium from randomly tritiated taurocholate by microbial bile salt oxidoreductases[1]

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Summary. Tritium distribution on randomly labelled taurocholate (TC) was estimated at 28%, 4%, 1% and <0.5% on the hydrogens opposite the 3α-, 7α- and 12α-OH groups and taurine moiety respectively. Anomalously, *C. perfringens* 3α-hydroxysteroid dehydrogenase (3α-HSDH) catalyzed tritium loss of 36% on formation of 7α-, 12α-dihydroxy-3-keto-5β-cholanoate, implying additional losses of tritium at other sites by this enzyme.

By enzymatic formation of ketones specifically at the 3[2-4], 7[5-7] or 12[4,8] positions of the bile salt molecule, tritium opposite the OH groups will be released into water. Limited information on the tritium distribution of commercially available randomly tritiated bile salts can be thus obtained. The use of tritiated bile salts in the estimation of human bile salt pools is controversial. On introduction of randomly tritiated bile salt into the enterohepatic cycle[9-12], label is partially removed by the enteric flora. Einarsson et al.[12] report a total tritium loss in the human enterohepatic cycle of 14% ± 2%, while Panveliwalla et al.[11] report a loss considerably greater and more variable and repudiate the use of randomly tritiated bile salts in such studies[10]. This communication describes the label distribution at the 3, 7 and 12 positions and taurine moiety of 1 preparation of randomly tritiated taurocholate (TC) and the anomalous loss of tritium by *C. perfringens* 3α-hydroxysteroid dehydrogenase (HSDH) and in whole cell cultures.

Materials and Methods. Randomly tritiated TC (3.3 Ci/mmole) and ^{14}C-24-cholate (10 mCi/mole) were from New England Nuclear and I.C.N., respectively. Radiolabelled TC and cholate were added to approximately 5 mg unlabelled TC and cholate and purified by TLC with toluene/acetic acid/water, (10:1:1, v/v/v) and chloroform/methanol/acetic acid (20:2:1, v/v/v) respectively. Duplicate columns of unlabelled bile salt were run in parallel and sprayed with p-hydroxybenzaldehyde reagent[13]. Unsprayed, labelled regions were scraped, twice eluted with methanol and eluates mixed (final volume 1 ml). Final specific activity was 10 mCi/mmole and 1.8 mCi/mmole, respectively. Approximately 1 μCi and 0.25 μCi of the respective isotopes were used per ml bacterial medium. The mixture was deconjugated by base hydrolysis[14], acidified to pH 3, extracted with ether and reconstituted to the original volume. Similarly, the deconjugated labelled cholate was used in dehydrogenase reaction mixtures. Oxidoreductase reaction mixtures consisted of 1.7×10^{-3} M NAD(P), 5×10^{-5} M radioactively labelled cholate in 0.17 M glycine/NaOH buffer pH 9.5 (total volume = 3 ml). Approximately 0.20 units of either 3α-HSDH *P. testosteroni*[2] (sigma), *C. perfringens*[3] or *E. lentum* preparations), 7α-HSDH (*E. coli*)[5,6] or 12α-HSDH (*Clostridium sp* group P)[8] were introduced. Reactions were allowed to go to completion. The reaction system was acidified to pH 3, extracted with ether and chromatographed (chloroform/methanol/acetic acid 20:2:1 v/v/v, sprayed[13], the products were twice eluted with methanol and eluates directly counted. Tritium losses were computed on the basis of the change in ^{14}C/^{3}H ratio. 10 ml volumes of selected strains of *C. perfringens* were grown in the presence of 10^{-3} M TC and isotope mixture; during the growth curve 25 μl aliquots were counted directly and with evaporation of the aliquot prior to counting[14]. Additionally, aliquots were assayed for the extent of 3α-OH and 7α-OH bioconversion and deconjugation[14]. Samples (3 ml) of spent bacterial medium were extracted, chromatographed and counted as described above.

Results and discussion. The distribution of tritium on randomly tritiated TC was 28%, 4%, 1% and <0.5% at the 3, 7 and 12 positions and on the taurine, respectively (table 1).

Table 1. Tritium exchange by cell-free preparations using radioactive cholate as substrate

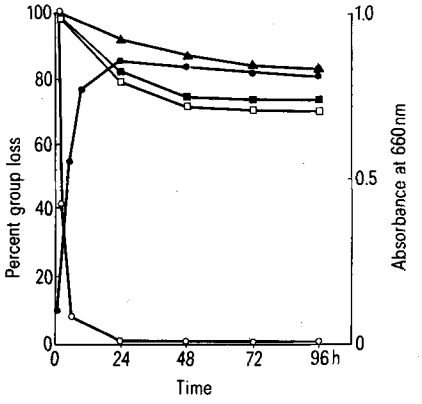
Reaction	Enzyme source	References	Cofactor	Major-5β-cholanoate(s) produced	Yield	Tritium exchanged %**
3α-HSDH	*P. testosteroni*	2	NAD	7α-,12α-diOH,3 keto-	100	27 ± 2
3α-HSDH	*C. perfringens*	3	NADP	7α,12α-diOH,3 keto- 3α-,7α-,12α-triOH-	75 25	35 ± 2 0 ± 1
3α-HSDH	*E. lentum**	4	NAD	7α-,12α-diOH,3 keto-	100	28 ± 2
7α-HSDH	*E. coli*	5, 6	NAD	3α-,12α-diOH,7 keto-	100	4 ± 1
12α-HSDH	*Clostridium* sp., group P	8	NADP	3α-,7α-diOH,12 keto-	100	1 ± 1
3α- and 12α-HSDH	*E. lentum*	4	NAD	7α-OH, 3,12 diketo-	100	29 ± 1

*Heat treated enzyme preparation, **average of duplicates and SD.

Table 2. Tritium loss on formation of 7α-,12α-dihydroxy-3 keto-5β-cholanoate by *C. perfringens* in whole cell cultures*

*C. perfringens*** strain	Tritium exchange in unpurified spent bacterial medium (%)	Tritium loss in remaining cholate (%)	Tritium loss on formation of 7α-,12α-dihydroxy-3 keto-5β-cholanoate (%)
1	22% ± 2%	6% ± 2%	37% ± 0%
4**	0% ± 0%	2% ± 2%	-
10	24% ± 1%	6% ± 1%	35% ± 1%
20	13% ± 0%	6% ± 2%	37% ± 2%
91	14% ± 2%	4% ± 2%	35% ± 2%

*Grown in the presence of ^{3}H-TC and ^{14}C-cholate for 96 h, **strain numbering system as previously described[3,15], ***a deconjugating but non-oxidizing strain of *C. perfringens*.



Growth of strain No. 21 *C. perfringens* and degradation of ^{3}H TC; ●——● absorbance of culture at 660 nm, ○——○ deconjugation of TC, ■——■ loss 3α-OH groups, □——□ apparent loss of 7α-OH groups and △——△ loss of tritium in crude spent bacterial medium.

However, about 36% of the tritium was lost on formation of 7α-, 12α-dihydroxy-3-keto-5β-cholanoate in vitro or in whole-cell *C. perfringens* cultures (table 2). The tritium loss (previously noted[14]) could be followed during the growth of *C. perfringens* cultures in TC-containing medium (figure). The appearance of this compound was verified by TLC. (Artifactual formation of methyl esters previously observed[14] was avoided by extraction at pH 3 instead of pH 1). Additionally, the loss of 3α-OH groups and apparent loss of 7α-OH groups (associated with the relative unreactivity of *E. coli* 7α-HSDH against this oxidation product[3]) closely paralleled the loss in label. Although the yield of 7α-, 12α-dihydroxy-3-keto-5β-cholanoate differed considerably from one strain to another, the percentage loss of tritium calculated from the ^{14}C/^{3}H ratio remained constant (table 2). Small losses of tritium were encountered in the remaining cholate in whole-cell cultures; none was measurable in vitro.

The discrepancy between the tritium lost from cholate by *C. perfringens* and that lost by *P. testosteroni* or *E. lentum* 3α-HSDH was rationalized by a stripping of protons from other sites on the steroid (possibly α- to C_3 position) on contact with the *C. perfringens* enzyme. These results support the conclusions of Panveliwalla et al.[11] in not recommending generally tritiated bile salt for human kinetic studies. Because of greatly differing *C. perfringens* populations in the human intestine[16], tritium loss, by this mechanism alone, could introduce a sizable and variable error in pool size estimation.

1 This work is supported by the National Cancer Institute.
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Absence of low molecular weight DNA polymerase activity from the nuclei of *Amoeba discoides*

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Summary. Amoeba discoides nuclear protein partially purified by passage through Sephadex G-200 showed 3 high-mol.-wt DNA polymerase activities which eluted in and just following the void volume. No low-mol.-wt (45,000 daltons) DNA polymerase β activity was detected. Nuclear protein layered on 5-20% sucrose gradients also showed an absence of low-mol.-wt DNA polymerase β. The void volume enzyme showed deoxyribonuclease activity, but no low-mol.-wt nuclease activity was detected.

DNA polymerase activity is found in many cellular structures[1]. A high-mol.-wt DNA polymerase found in the cytoplasm, DNA polymerase α[2] is the predominant activity found in growing cells[3-5], while DNA polymerase β is a well-characterized low-mol.-wt activity in the nuclei of many higher organisms. We wish to report the absence of a low-mol.-wt DNA polymerase activity from the nucleus of the large mononucleate Protozoan, *Amoeba discoides*.

Materials and methods. A. discoides ($_{T1}D_{13}$) were grown in mass cultures and nuclei were obtained as described previously[6]. Tritium-labelled DNA was obtained from *Tetrahymena pyriformis*[7] grown in proteose-peptone containing 2 µCi/ml ^{3}H-thymidine. Gel filtration with Sephadex G-200 and running buffer (20 mM Tris-HCl, pH 8.1, 1 mM EDTA, 1 mM β-mercaptoethanol, 0.02% sodium azide), and centrifugation on linear sucrose density gradients (5-20% sucrose in 50 mM Tris-HCl, pH 7.8, 1 mM EDTA, 1 mM β-mercaptoethanol, 50 mM KCl, 0.02% sodium azide) were carried out. The DNA polymerase assay composition was usually 50 mM Tris-HCl, pH 8.1, 10 mM $MgCl_2$, 30 mM KCl, 1 mM β-mercaptoethanol, 0.8 mM EDTA, 0.1 mM each of dATP, dGTP, dCTP, 0.1