PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 452-456 (1977)

A Comparison of Methods for Bile Salt Pool Size Measurements in the Prairie Dog Gallstone Model[1] (39955)

P. D. MEYER AND L. DENBESTEN[2]

Departments of Surgery, Veterans Administration Hospital, and The University of Iowa Hospitals and Clinics, Iowa City, Iowa 52242

Data defining bile salt pool sizes and composition during the development of cholesterol gallstones may clarify our understanding of the etiology of cholelithiasis. Bile salt pool size reduction is common among patients with gallstones (1, 2) and in populations predisposed to gallstones (3). Sequential measurements of bile salt pool size in an appropriate animal gallstone model should establish the sequence of events during the formation of gallstones, and the interrelationship of bile salt pool size to those events. Our finding that the prairie dog is an excellent model for the study of cholelithiasis (4, 5) has been confirmed by other investigators (6). Studies to clarify the relationship of the bile salt pool to gallstone formation in this model require a reliable technique for determining the effect of various perturbations on bile salt pool size and synthesis rates. The studies reported herein compare two methods for estimating pool sizes in small animals.

Acute experiments in the rat have relied on the "washing out" of the bile salt pool (7, 8), without confirmation that the bile salt pool measured accurately estimates the recirculating pool. Studies in humans using the isotope-dilution techniques (9) rely heavily on equilibration between the gallbladder subpool and recirculating pool. This assumption is especially important in the recently reported "one-sample" determinations of bile salt pool size (10). Since the prairie dog has a gallbladder, a "one-point" measurement of bile acid pool size might be unreliable if equilibration of the gallbladder and recirculating pool were incomplete. We, therefore, compared a modified isotope-dilution technique with the wash-out technique used on animals lacking gallbladders.

Methods. Two groups of adult (800- to 1000-g) prairie dogs were studied. One group was fed a control trace-cholesterol laboratory chow, the second was fed cholelithogenic chow (0.48 g of cholesterol/100 g) for 10 days. In previous experiments the cholelithogenic diet has induced microcrystalline cholesterol in bile after 8–14 days. Acute experiments to estimate the bile salt pools by isotope-dilution and wash-out techniques were carried out as follows: After a 6-hr fast, each animal was lightly sedated, and then received 0.30 μC of [^{14}C]cholic acid intravenously and a gavage feeding of a lipid-protein emulsion (1.6 g each of vegetable oil, milk solids, and peanut butter, suspended in water) to stimulate gallbladder contraction. All animals were ambulating within 30 min after administration of the isotope. Twelve hours later the animals were anesthetized with 50 mg of ketamine HCl and 0.5 mg of Acepromazine maleate. The abdomen was opened, the gallbladder was aspirated completely, the cystic duct was ligated, and the common duct was cannulated. The abdomen was closed, and hepatic bile was collected in 2-hr segments for 24 hr; animals were kept lightly sedated.

Bile volumes were recorded and bile acid concentrations were quantified by the method of Talalay (11). Bile aliquots were decolorized with 3% hydrogen peroxide, assayed for radioactivity, and corrected for quenching.

Calculations. Bile salt was calculated by the wash-out technique of Myant and Eder (12), adapted from Eriksson's original procedure (8). Pool size by isotope-dilution technique was determined by a modification for animal experimentation of the method of Duane *et al.* (10). Gallbladder bile salt

[1] This work was supported by a V.A. grant.

[2] Address reprint requests to L. DenBesten, M.D., Department of Surgery, University Hospitals, Iowa City, Iowa 52242.

ISSN 0037-9727

and bile salt from the first period hepatic collection were determined by mass, and radioactivity was also determined. The radioactivity administered (a_0) was corrected for losses by multiplying by e^{-kt}, where k is the fractional pool loss per unit time (daily synthesis rate divided by pool size). The technique of Duane *et al.* (10) (which mathematically assumes no losses, so that $k = 0$ and $e^{-kt} = 1$) was used to estimate pool size in computing k; the daily synthesis rate was assumed to be 24× the hourly synthesis rate as determined after wash out. The radioactivity from gallbladder bile and the first collection of hepatic bile was subtracted from this corrected radioactivity. This computation yields the radioactivity actually contained in the animal after the first hepatic bile collection. This radioactivity is divided by the specific activity of bile salt from the second-period hepatic bile collection; this constitutes the "pool remaining" in the animal after the first period hepatic bile collection. The sum of the bile salt in the gallbladder, first-period hepatic bile, and pool remaining constitutes the total bile salt pool. These calculations are summarized in the equation:

$$\text{Pool} = m_{gb} + m_{hc\ 1} + \frac{a_0 e^{-kt} - (a_{gb} + a_{hc\ 1})}{\text{sp act}_{hc\ 2}},$$

where m_{gb} = bile salt in gallbladder, $m_{hc\ 1}$ = bile salt from first hepatic collection, sp $\text{act}_{hc\ 2}$ = specific activity of bile salt from second 2-hr collection of hepatic bile, and a_{gb} and $a_{hc\ 1}$ = total radioactivity of respective samples. The ratio of specific activity (Rsa) of gallbladder bile salt to hepatic bile salt (second 2-hr collection) was calculated from quench-corrected activities and bile salt concentrations.

Results. All data refer to bile acid pools in the prairie dog. Data for control animals are summarized in Table I. The bile acid pool as calculated by the modified isotope-dilution technique was 333 ± 104 μmoles, and as calculated by wash out was 276 ± 84 μmoles. The average basal synthesis rate was 2.51 ± 1.16 μmoles/hr, determined as the "plateau" of the wash-out curve. The observed difference between the two methods varies in magnitude but is consistent and statistically significant ($P < 0.001$). Pool sizes computed from the specific activity of gallbladder bile (10) are included for

TABLE I. BILE SALT POOL SIZE MEASUREMENTS (micromoles)—CONTROLS.

I.D.	Pool SAcorr[a] (A)	Pool_{Σ}[b] (B)	Pool_{SAGB}[c]	e^{-kt}[d]	12·BR[e]	Rsa gb/2[f]
1	415	261	449	0.942	24.7	0.95
2	422	351	451	0.909	40.0	1.00
3	410	323	447	0.931	29.2	0.95
4	404	317	462	0.910	38.0	0.94
5	356	262	417	0.839	62.4	0.97
6	182	171	189	0.944	10.5	1.03
7	100	76	121	0.732*	31.2	1.13
8	312	299	319	0.942	18.8	1.04
9	352	284	383	0.895	39.0	1.02
10	461	407	563	0.951	23.0	0.83
11	368	336	373	0.971	11.0	0.98
12	259	242	293	0.904	26.0	0.98
13	290	254	324	0.876	38.4	1.03
$\bar{X}$ ± SD	333 ± 104	276 ± 84	368 ± 120	0.904 ± 0.062*	30.2 ± 13.9	0.99 ± 0.07

[a] Pool SAcorr = total bile acid pool size by modified isotope dilution.

[b] Pool_{Σ} = total bile acid pool size by wash-out technique.

[c] Pool_{SAGB} = total bile acid pool size calculated from administered radioactivity divided by specific activity of gallbladder bile.

[d] e^{-kt} = calculated fraction of pool retained from isotope administration until acute experimentation.

[e] 12·BR = basal secretion rate after wash out (in micromoles per hour) times 12 (hours between isotope administration and acute experimentation).

[f] Rsa gb/2 = ratio of specific activity of gallbladder bile to that of hepatic bile collected during second 2-hr collection period.

* Animal 7 < $\bar{X}$ − 2 SD; recomputed $\bar{X}$ = 0.918 ± 0.037.

comparison. The ratio of specific activities of gallbladder and stimulated hepatic bile was 0.99 ± 0.07.

Data for animals on the lithogenic chow are summarized in Table II. The pool size as measured by isotope dilution was 431 ± 137 μmoles, and as measured by wash out was 357 ± 120 μmoles ($P < 0.001$). Pool sizes computed from the specific activity of gallbladder bile (10) are included for comparison. Basal synthesis rate (i.e., the plateau of the "wash-out" curve) was 2.45 ± 1.06 μmoles/hr. The ratio of specific activities (Rsa) in this group was 0.84 ± 0.35.

Wash-out pools corrected for recovery of radioactivity agree very well with pools calculated by isotope dilution. In six animals, all radioactivity recovered during wash out was summated, a_Σ. The ratio a_0/a_Σ multiplied by the pool as calculated by wash out yields "corrected" wash-out pools that average 98% (range, 96–102%) of pools calculated by isotope dilution. In these same animals actual recovery of radioactivity ranged from 75 to 90% of that administered.

All animals demonstrated a classic wash-out pattern, reaching a wash-out plateau in 12–14 hr. None demonstrated increased bile acid synthesis due to released inhibition by the end of the 24-hr period.

Discussion. These studies indicate that the modified isotope-dilution technique yields values for bile salt pool sizes consistently higher than those of the wash-out technique. The observed differences in pool size measurement are apparently due not to overestimation by the isotope-dilution technique, but rather to underestimation by the wash-out technique. Pool sizes determined from the specific activity of gallbladder bile (aspirated at surgery) without correction for radioactivity losses are generally higher than those observed by our technique ($Pool_{SAGB}$, Tables I and II).

The pool calculated by the modified isotope-dilution equation as described is virtually identical with that calculated simply from the corrected activity and measured gallbladder specific activity, when mixing of the exogenous bile salt has clearly been adequate with all subpools (Rsa = 1.0). This would be expected when the gallbladder and hepatic bile equally well represent the specific activity of the mixed pool. Where recirculated bile salt has not yet equilibrated with gallblader contents (Rsa < 1.0), it has nevertheless been mixed thoroughly with itself, i.e., the specific activities of sequential hepatic bile samples are virtually identical. Hence, although the gallbladder contents constitute a localized subpool, the calculation of total pool size is computed accurately by the combination of mass measurement of the gallbladder pool and isotope measurement of the recirculating pool. The specific activity of the second hepatic collection has been used in these

TABLE II. BILE SALT POOL SIZE MEASUREMENTS (micromoles)—LITHOGENIC DIET.[a]

I.D.	Pool SAcorr	$Pool_\Sigma$	$Pool_{SAGB}$	e^{-kt}	12·BR	Rsa gb/2
1	468	398	468	0.903	48.0	1.24
2	518	459	534	0.920	43.2	1.08
3	618	N.A.[b]	708	N.A.[b]	N.A.[b]	0.92
4	547	459	31,000[c]	0.947	30.0	0.013[c]
5	395	263	2110	0.936	26.0	0.12
6	457	409	504	0.927	34.8	0.95
7	196	175	278	0.903	19.9	1.05
8	215	199	263	0.785	52.0	1.00
9	290	246	320	0.901	30.2	0.95
10	294	271	303	0.941	18.0	1.05
11	569	510	654	0.978	12.6	0.66
12	517	396	15,800[c]	0.936	34.3	0.02[c]
13	389	342	418	0.958	16.9	0.93
14	558	526	1750	0.971	16.6	0.17
$\bar{X}$ ± 1 SD	431 ± 137	358 ± 117	654 ± 626	0.924 ± 0.048	29.4 ± 12.7	0.84 ± 0.35[c]

[a] Abbreviations as in Table I.

[b] Not applicable, animal expired before wash-out completed.

[c] Animals 4 and 12 not included in calculation of $\bar{X}$, SD, on the supposition of *preexistent* gallbladder disease. When included, $\bar{X} = 0.73 \pm 0.44$.

calculations since we have found it to be slightly more stable than the first collection, probably due to transient effects of surgery during the first period's collection.

The observed difference in pool measurements cannot be attributed to the mathematical correction, e^{-kt}, which, in fact, lowers the computed pool size as measured by isotope dilution. This factor corrects for the activity presumably lost between isotope administration and the acute experiments. In actuality, this correction is very constant (0.92 ± 0.04) and may be ignored in comparative studies without altering relationships. Significantly, wash-out values corrected for isotope recovery (but multiplying by a_0/a_Σ) agree very well (96–102%) with values calculated by isotope dilution.We conclude, therefore, that the observed differences are due to an underestimation of the bile salt pool by the wash-out technique under the conditions of these experiments.

This underestimation is most consistent with a failure to recover a subpool of bile salt which had become equilibrated with the exogenous isotope. Completeness of aspiration of gallbladder contents is essential for accuracy in this method, but the fraction of the pool actually found in the gallbladder (10–40%) makes it unlikely that incomplete aspiration could account for the observed discrepancy. At most, an error of a few percent might result from incomplete gallbladder aspiration. Moreover, we have observed this same discrepancy in prairie dogs studied after cholecystectomy. A second source of error may be the secondary effects of surgery and anesthesia. The prairie dog is a wild animal, and must be anesthetized during the entire procedure. The combination of surgical trauma and anesthesia might sequester a portion of the recirculating pool. Evidence against this possibility, however, is the observation of a true "plateau" level of synthesis-secretion. Another possibility is that a portion of the pool, normally reabsorbed from the cecum and colon in this animal, is not adequately measured by the wash-out method under these conditions. This factor may be particularly important under these experimental conditions when we sought to maximize enterohepatic circulation by a high-lipid, high-protein "meal" at the time of isotope administration.

We are examining the effects of cholelithiasis on liver and gallbladder function, and these experiments will be the subject of future reports. Our data from these methodologic studies, however, suggest a tendency toward a slower rate of equilibration of recirculating bile salt with the gallbladder sub-pool, since Rsa is less than unity (0.84 ± 0.35). The reproducibility and extent of this effect will be further studied with this technique, since the modified isotope-dilution technique is appropriate for measuring pool size especially under these apparently nonequilibrated conditions. The apparent increase in bile salt pool size on the lithogenic diet (431 ± 137 vs 333 ± 104 μmoles for controls, $P < 0.05$) also requires further study, and is subject to at least two alternative interpretations. First, the two groups were from different purchase lots, and we have previously observed considerable variation between groups studied asynchronously. Second, the bile salt pools may in fact become enlarged, secondary to gallbladder stasis.

The ability to accurately measure bile salt pool size in an experimental model is an exceptionally valuable tool in determining the effects of certain perturbations on that model. The modified isotope-dilution technique yields a larger pool than does the wash-out technique in this model. The most consistent explanation for the observed differences in pool size measurement is that a combination of factors has made some portion of the pool, previously equilibrated with exogenous isotope, unavailable to "washing out" and measurement. The technique we describe will also avoid sources of error associated with "one-sample" determinations, e.g., based on the specific activity of gallbladder bile. Unless mixing has been proven to be adequate, such methods will tend to err due to nonequilibration of gallbladder bile salt with recirculating bile salt. The isotope-dilution technique we describe is accurate even in the presence of gallbladder stasis. Indeed, this technique not only measures pool size but should yield an index of stasis, since the Rsa is a reflection of the rate of equilibration of the gall-

bladder subpool with the recirculating pool.

With this technique we are determining the effects of several dietary and pharmacologic perturbations on bile salt pool size, synthesis rate, biliary stasis, and gallstone formation in a model with proven susceptibility to cholelithiasis and a bile composition similar to human bile.

1. Almond, H. R., Vlahcevic, Z. R., Bell, C. C., Jr., Gregory, D. H., and Swell, L., N. Engl. J. Med. **289,** 1213 (1973).
2. Grundy, S. M., Duane, W. C., Adler, R. D., Aron, J. M., and Metzer, A. L., Metabolism **23,** 67 (1974).
3. Vlahcevic, Z. R., Bell, C. C., Jr., Gregory, D. H., Buker, G., Juttijudata, P., and Swell, L., Gastroenterology **62,** 73 (1972).
4. Brenneman, D. E., Connor, W. E., Forker, E. L., and DenBesten, L., J. Clin. Invest. **51,** 1495 (1972).
5. DenBesten, L., Safaie-Shirazi, S., Connor, W. E., and Bell, S., Gastroenterology **66,** 1036 (1974).
6. Holzbach, R. T., Corbusier, C., Marsh, M., and Naito, H. K., J. Lab. Clin. Med. **87,** 987 (1976).
7. Mok, H. Y. I., Perry, P. M., and Dowling, R. H., Gut **15,** 247 (1974).
8. Eriksson, S., Proc. Soc. Exp. Biol. Med. **94,** 578 (1957).
9. Hofmann, A. F., and Hoffman, N. E., Gastroenterology **67,** 314 (1974).
10. Duane, W. C., Adler, R. D., Bennion, L. J., and Ginsberg, R. L., J. Lipid Res. **16,** 155 (1975).
11. Talalay, T., *in* "Methods of Biochemical Analysis" (D. Glick, ed.), Vol. 8, p. 119. Interscience, New York (1960).
12. Myant, N. B., and Eder, H. A., J. Lipid Res. **2,** 363 (1961).

Received April 27, 1977. P.S.E.B.M. 1977, Vol. 156.