Folates in Plasma and Bile of Man after Feeding Folic Acid–^{3}H and 5-Formyltetrahydrofolate (Folinic Acid)

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ABSTRACT During the 1st hr after feeding folic acid–^{3}H (^{3}H-PteGlu) to fasting human volunteers, plasma *S. faecalis* and ^{3}H activity were elevated to an equivalent degree, whereas after this, the ^{3}H activity exceeded *S. faecalis* activity, which suggests gradual conversion of folic acid–^{3}H to methyltetrahydrofolate-^{3}H (5-CH_3H_4 PteGlu). The increase of *L. casei* activity exceeded the increase of *S. faecalis* and ^{3}H activity, which is consistent with flushing of endogenous methyltetrahydrofolate from the tissues by the administered folic acid–^{3}H. Feeding of 5-formyltetrahydrofolate (±5$CHOH_4$PteGlu) produced a large increase of plasma *L. casei* activity and only a slight increase of *S. faecalis* and *P. cerevisiae* activity, which is consistent with very rapid conversion of folinic acid to methyltetrahydrofolate.

Bile folate concentration determined microbiologically was 2.3–9.8 times plasma folate. 40–80% of the bile folate was *S. faecalis*-active and 20–35% *P. cerevisiae*-active. Chromatography of bile folates on TEAE-cellulose showed several folates including four tentatively identified as 10-formyltetrahydrofolate (10-CHO-H_4PteGlu), 10-formylfolate (10-CHO-PteGlu), and/or 10-formyldihydrofolate (10-$CHOH_2$PteGlu), methyltetrahydrofolate, and possibly a triglutamate folate. After folate ingestion bile folate concentration increased rapidly. The distribution of bile folates measured by microbiological assay was similar after either folic or folinic acid feeding. Most of the ^{3}H label of folic acid–^{3}H appeared in the biological folates of bile rather than in the folic acid fraction, which shows that the administered folic acid was rapidly transformed to other folates.

Folate polyglutamate deconjugating enzyme activity was found to be much less than in serum. Polyglutamates of the type found in yeast were not found in bile.

It is suggested that biliary folate may reflect the hepatic intracellular oligoglutamate folate pool rather than the folate as it appears in the hepatic portal blood.

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INTRODUCTION

The concentration of folate in bile exceeds that in plasma and increases after ingestion of folic acid (1, 2). The form of the folate in bile is unknown although it recently has been suggested (3) that it is a monoglutamate form different from methyltetrahydrofolate (5-CH_3-H_4PteGlu), the predominant folate of plasma. Because of evidence suggesting that the form of folate appearing in the portal blood after feeding of folic acid (PteGlu) differs from that following ingestion of 5-formyltetrahydrofolic acid (folinic acid ±5-$CHOH_4$PteGlu) or other reduced folates[1] (4, 5), we undertook to determine if the pattern of bile folate reflects the form of folate absorbed from the intestine, or if biliary folate might reflect intracellular folate, which thereby provides an indication of the folate of liver cells. In contrast with a previous report (6), we have not observed significant folate deconjugase (conjugase, γ-glutamyl carboxypeptidase) activity in bile.

The pattern of folate activity in serum after feeding folic acid has been interpreted to indicate flushing of methyltetrahydrofolate from tissues followed by slower conversion of ingested folic acid to this coenzyme (4, 7, 8). We have studied the pattern of folate activity and of ^{3}H appearing in plasma after administration of folic acid–^{3}H and have found these to be consistent with this hypothesis.

METHODS

Assays of folate

Lactobacillus casei assay. The assay method was modified from that described by Baker et al. (9). Medium was prepared from a commercial mix (folic acid assay; Baltimore Biological Laboratories). Plasma was assayed at a final dilution in the medium of 1:100, whole blood at 1:1000, and bile at 1:100 and 1:1000 dilutions. Initial dilutions of plasma were made in 0.05 M phosphate buffer

[1] Whitehead, V. M., R. F. Pratt, and B. A. Cooper. 1970. Human intestinal conversion of folinic acid to 5-methyltetrahydrofolate. To be published.

with ascorbic acid, 150 mg/100 ml at pH 6.0, and 1 ml of these dilutions was added to 9 ml of prepared medium. The method for determining red cell folate has been reported previously (10). Plasma and whole blood dilutions, but not bile, were autoclaved for 4 min to precipitate proteins, centrifuged, and the supernatants assayed. Culture tubes were autoclaved for 20 min before inoculating with the assay organisms. After 18 hr at 37°C, growth of the organisms was compared with that in standard tubes containing 0.1–3.0 ng of folic acid per 10 ml of medium.

Streptococcus faecalis assay. This was based on the method of Teply and Elvehjem (11–13). The medium was purchased from Difco (bacto-folic acid assay medium). Plasma was assayed at final dilutions in the medium of 1 : 20 and 1 : 100, and bile at dilutions of 1 : 100 and 1 : 1000. Treatment of plasma and bile was the same as that described for the *L. casei* assay except that the plasma for the 1 : 20 dilution was added terminally after autoclaving the medium. The standard curves contained from 1.0 to 10.0 ng of folic acid per 10 ml of medium. As whole plasma was shown to alter the growth of *S. faecalis* in folic acid, an equivalent volume of fasting plasma was added terminally to a duplicate standard curve so that growth of the cultures containing the 1 : 20 plasma dilutions could be assessed more accurately.

Pediococcus cerevisiae assay. The method was based on that described in the Difco Manual (12) and by Sauberlich and Baumann (14). Medium was purchased from Difco (bacto-CF assay medium). The modifications of the basic method for assay of whole plasma were the same as described above for *S. faecalis.* Standard curves contained from 0.6 to 4.8 ng of DL-folinic acid per 10 ml of medium.

All assays were done in triplicate. After incubation of the inoculated assay media for the appropriate times, the optical densities of the cultures were measured with a colorimeter. The effect of the color of bile was corrected for by subtracting the optical density of a colorimetric blank made by adding an equivalent dilution of bile to uninoculated medium.

Column chromatography

Preparation of folic acid. ^{3}H-labeled folic acid (generally labeled) was purchased from Amersham/Searle (batch No. 20; specific activity 1.6 mCi/mg). Stepwise elution with phosphate buffers from a DEAE-cellulose column was used to purify the folic acid-^{3}H (15). The specific activity was verified by microbiological assay.

Fractionation of bile. Whole bile could not be chromatographed satisfactorily. Bile dialyzed for 24 hr against 2 volumes of water containing ascorbic acid (150 mg/100 ml) showed equilibration of the folate concentration inside and outside the dialysis bag, whereas 90% of the bilirubin stayed inside the bag. The entire dialysate was diluted with water so that the electrical conductivity was the same as that of the 0.02 M phosphate starting buffer (conductivity 1800 μmho). The diluted dialysate was applied to a 1 × 20 cm column containing TEAE-cellulose. Elution was started with enough 0.02 M phosphate buffer, pH 6.7, to make the total volume of bile solution and starting buffer 100 ml, and was followed by 200 ml of 0.1 M phosphate buffer, pH 6.7, and 200 ml of 0.25 M sodium chloride in 0.02 M phosphate buffer, pH 6.7. The flow rate was 1.25 ml/min, and 10-ml fractions were collected. All solutions contained ascorbic acid, 150 mg/100 ml, and collecting tubes contained 0.2 ml of 5% ascorbic acid. The column temperature was 3°C, and the collecting tubes were kept in a bath at 2°C. The fractions were kept refrigerated or frozen at −20°C until assayed. The folate activity of the chromatographed fractions was measured with the three assay organisms by adding 1–9 ml of prepared medium. 1 ml volumes of the eluated fractions were added to counting vials for liquid scintillation counting.

Fractionation of plasma. 5 ml of whole plasma was applied to a TEAE-cellulose column and eluted as for elution of bile. 10-ml fractions were collected. 1 ml aliquots from each fraction were assayed with *L. casei* and *S. faecalis* in duplicate, and 1 ml was added to vials for liquid scintillation counting.

Measurement of tritium activity in test solutions

Bile. 0.1 ml of each bile sample was added to 20-ml glass counting vials. One or two drops of 30% hydrogen peroxide were added to decolorize. The volume then was decreased to approximately 0.1 ml by evaporation in air. 0.3–0.5 ml of hydroxide of hyamine 10X was added, and the vials were allowed to stand 48 hr or more. 1.5 ml of 100% ethanol and 15 ml of toluene scintillator solution were added. Specimens were prepared in duplicate. The vials were counted in a Packard 3003 liquid scintillation spectrometer. Quenching correction utilized the automatic external standard compared with values obtained for a previously prepared tritiated toluene internal standard quenched with picric acid. The toluene scintillator solution contained 1.2 g of 2,5-diphenyloxazole (PPO), 40 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP), and 200 ml of toluene.

Plasma. The treatment of plasma was the same as that described for bile except that the decolorizing step was omitted. Efficiency of counting of tritium in plasma or bile was 21–30%.

Column eluate. 15 ml of dioxane scintillator solution was added to 1 ml of each fraction, and the radioactivity was measured as above. The scintillator solution contained 14 g of PPO, 0.6 g of dimethyl POPOP, 200 g of naphthalene, and 1900 ml of dioxane. Studies showed that this solution could hold 1 ml of water or buffer with full recovery of the radioactivity after correction for quenching. Quench correction was accomplished with automatic external standard referred to tritiated water standards quenched with picric acid.

Experimental procedure on patients

Eight volunteers, who had had cholecystectomy and common bile duct exploration for gallstones and who had a T tube in the common bile duct, were studied. The studies took place 5 or more days postoperatively when the patients were feeling relatively well and were able to eat. Each patient had been fasting for at least 8 hr on the morning of the study. Bile was drained continuously into the collecting tubes which were changed at 15- or 30-min intervals, covered, and kept on ice until the time of assay. In the last four experiments, 25 mg of ascorbic acid per 15 min of bile collection was added to the tubes prior to the bile collection. Blood for plasma and red cell folate was drawn from an antecubital vein into tubes containing 40 mg of sodium citrate and 10 mg of ascorbic acid for each 10 mg of blood.

After collection of control specimens, 100 ml of a solution containing 1.0 mg of folic acid with tritium content of 20 or 40 μCi was given orally to six patients. Com-

position of the test solution was verified by ^{3}H counting and folate assay on each occasion. Two other patients received 2.0 mg of DL-5-formyltetrahydrofolate (Leucovorin, Lederle), orally. Blood samples were taken at 30, 60, 90, 120, and 180 min after the oral folate. Bile collecting tubes were changed at 0, 15, 30, 45, 60, 75, 90, 105, 120, 150, and 180 min. Folate content was measured with the three assay organisms, and tritium content was measured as described, which converts the disintegrations per minute to nanograms of folate based on the specific activity of the administered folic acid-^{3}H. At the termination of the folic acid-^{3}H studies, 15 mg of folic acid was given intramuscularly to flush radioactivity into the urine. The folate assays were performed on the day the experiment was done.

In three patients, following the folate absorption study, 15 μCi of rose bengal-^{131}I was given intravenously, and bile was collected for 50–90 min to show the rate of appearance of ^{131}I. 5- or 10-min collections of bile were placed in counting tubes and counted in a well-type scintillation counter.

Studies on bile polyglutamates and conjugases

On seven occasions, bile was examined for the presence of polyglutamate folate and conjugase activity. Three studies were done at pH 6.0 (0.1 M phosphate buffer), and four at pH 4.6 (0.1 M citrate-phosphate buffer). 1 ml volumes of the reactant substances (bile, serum, or yeast extract solution) were added to buffer containing ascorbic acid, 150 mg/100 ml, to give final volume of 10 ml. Seven incubation solutions were prepared containing the reactants separately or in combinations. The solutions were incubated 90–180 min at 37°C. The incubation was ended by placing the tubes in a boiling water bath for 5 min to precipitate proteins. The supernatant solutions were separated by centrifugation and assayed at appropriate dilutions with *L. casei*. Control solutions were prepared by adding the same reactants to buffer in tubes in the boiling water bath so that enzymes were immediately inactivated. pH of the solutions did not change by more than 0.1 pH unit after adding the reactants to the buffer.

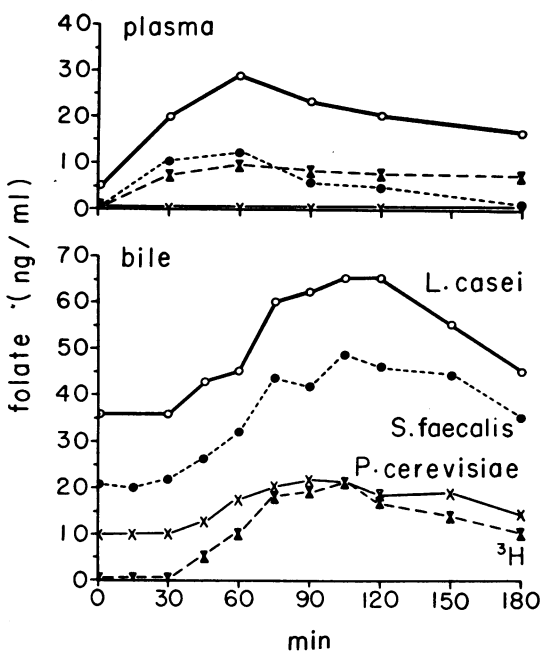

FIGURE 1 Folate levels in plasma and bile after feeding folic acid-^{3}H. Each point is the median value of biologically active folate or ^{3}H folate from five studies on patients given 1.0 mg of folic acid-^{3}H orally. Tritium activity has been converted to folate equivalent based on the specific activity of the administered folic acid. The symbols refer to the method of folate assay. Open circles joined by solid lines represent *L. casei*-active folate (○——○), closed circles joined by broken lines represent *S. faecalis*-active folate (●– –●), bows joined by broken lines (x– –x) represent ^{3}H folate, and ×'s joined by solid lines (×——×) represent *P. cerevisiae*-active folate.

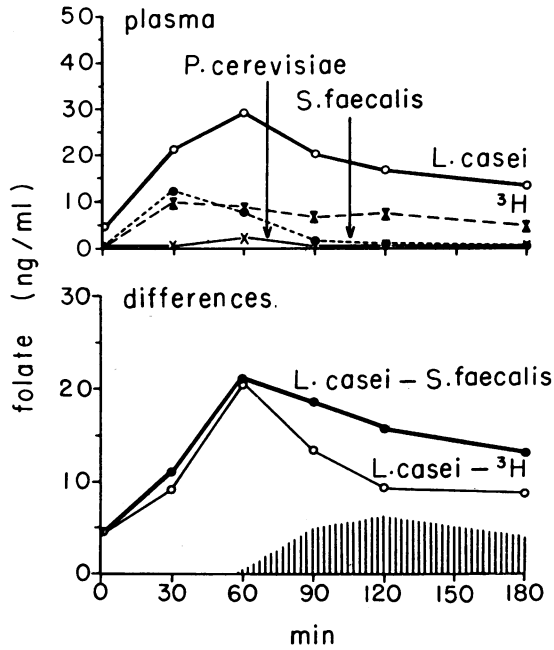

FIGURE 2 Plasma folates and ^{3}H after feeding folic acid-^{3}H (patient 4). 1.0 mg of folic acid labeled with 40 μCi ^{3}H was fed. Top, actual values are plotted. Bottom, differences between folate fractions are plotted. The area under the heavy line (●——●) represents methylfolate. The clear area under this line represents baseline plasma folate plus methylfolate flushed from the tissues. The area under the fine line (○——○) represents nonradioactive folate. The hatched area is the difference between the two lines and represents methylfolate synthesized from the administered folic acid.

RESULTS

Plasma and bile folates after folic acid-^{3}H ingestion (Figs. 1 and 2, Table I). Plasma and bile folates were followed in five patients after ingestion of 1 mg of folic acid-^{3}H (Table I). The patterns of plasma and bile folates in all patients were similar. Due to large differences between the bile folate levels in the different patients studied (Table I), it was determined that a plot of median

TABLE I

Summary of Data on Patients Given Folic Acid-^3H

Patient No.			1	2	3	4	5	6
Age (sex)			56 (F)	22 (F)	75 (F)	40 (M)	69 (F)	55 (M)
RBC folate*			930	292	258	169	256	253
Plasma	L.C.‡	Fasting	4.7	3.4	7.8	4.5	4.1	4.8
		Peak	30	25	44	29		22
		(Time)§	(60)	(90)	(90)	(60)		(60)
	S.F.	Peak‖	16.5	15.0	15.0	12.0		6.3
		(Time)	(30)	(60)	(60)	(30)		(30)
	P.C.	Peak	0		0	2		
		(Time)				(60)		
	^3H Peak		17	6.4	5.7	9.9		6.6
		(Time)	(60)	(60)	(90)	(30)		(60)
Bile	L.C.	Fasting	37	18	40	10	30	47
		Peak	60	46	172	45		140
		(Time)	(75)	(90)	(105)	(90)		(105)
	S.F.	Fasting	31	12	30	7		21
		Peak	49	40	115	35		100
		(Time)	(105)	(90)	(105)	(75)		(105)
	P.C.	Fasting	13	4.5	12	4		15
		Peak	18	8.5	34	6.4		42
		(Time)	(105)	(120)	(75)	(75)		(105)
	^3H Peak		15	23	17.2	21.5		27.5
		(Time)	(105)	(90)	(105)	(75)		(105)
Ratio bile/plasma			7.9	5.3	5.1	2.3	7.3	9.8

* Folate values are in ng/ml.

‡ L.C. = *L. casei*; S.F. = *S. faecalis*; P.C. = *P. cerevisiae.*

§ ( ) = Time in minutes of peak folate value.

‖ Fasting plasma values for S.F. and P.C. were 0 or below sensitivity of the assay (<2.0 and <0.3 ng/ml, respectively).

values for plasma and bile folates most closely represented the over-all pattern of folate levels after folic acid-^3H ingestion (Figs. 1 and 2).

The median values of these studies are plotted in Fig. 1 (top), and a single representative study of patient 4 is shown in Fig. 2. Plasma folate rose to a peak 60–90 min after feeding, and then gradually decreased. *S. faecalis* activity was negligible at time 0, but increased rapidly during the first 30 min, almost paralleling the increase of activity for *L. casei*. *S. faecalis* activity then decreased or increased at a slower rate while the *L. casei* activity continued to increase. No significant *P. cerevisiae* activity was found in the plasma except on one occasion after feeding folic acid to patient 4. Folate-^3H activity increased nearly parallel to *S. faecalis* activity initially, but by 60–150 min, the folate-^3H exceeded the *S. faecalis*-active folate and then decreased at a rate intermediate between that of *S. faecalis* and *L. casei*. These observations are summarized in Fig. 2 (lower) in which the differences between the parameters are plotted. *L. casei–S. faecalis* activity is assumed to indicate methylfolate concentration, and *L. casei–folate-^3H* to represent nonradioactive or endogenous folate. The shaded area, representing the differences between the two curves, should represent radioactive methylfolate.

Chromatography of plasma taken 30 min after ingestion of folic acid-^3H (patients 4 and 6) showed the presence of methyltetrahydrofolate and an *L. casei-S. faecalis*-active peak at the position of elution of folic acid (fraction 40). However, because of the dilution of folate during chromatography, radioactivity in the fractions was so low that counts were close to background.

Bile folate (Figs. 1 and 3, Table I). The total bile folate remained constant or increased slowly in the first 30–45 min after feeding folic acid-^3H in all the patients. A marked increase was then seen with elevation of folate to values from 45 to 172 ng/ml between 75 and 120 min after which bile folate concentration gradually decreased. *S. faecalis* activity in fasting bile was 45–80% of the *L. casei* activity. After administration of folic acid, the increase of *S. faecalis* activity was similar to that of *L. casei* activity in most of the patients. Fasting *P. cerevisiae*-active folate was 20–35% of the total bile folate. A small increase of *P. cerevisiae* activity was seen after oral folic acid in three patients, and a larger increase in patients 3 and 6. Folate-^3H rise tended to

parallel the total folate in three patients, but was less than the increase of folate in patients 3 and 6. The study performed in patient 4 is seen in Fig. 3.

Effect of feeding 5-formyltetrahydrofolate on plasma and bile folates (Fig. 4, Table II). Plasma and bile folates were measured in two patients after feeding 2 mg of DL-5-formyltetrahydrofolate a quantity equivalent in biological activity to the folic acid given to the other patients. The effect of this folate on plasma and bile folates was similar in the two patients; the mean values are plotted in Fig. 4. Most of the early rise of total plasma folate was due to folate active only for *L. casei* suggesting that the 5-formyltetrahydrofolate was more rapidly transformed to methyltetrahydrofolate than was folic acid. However, the appearance of some *S. faecalis*– and *P. cerevisiae*–active folate at 30 min suggests that some 5-formyltetrahydrofolate had been absorbed. Chromatography on TEAE-cellulose of serum taken after orally administered 5-formyltetrahydrofolate showed this folate to elute in the position of 5-formyltetrahydrofolate.[1] Changes in bile folate were similar to those found after administration of folic acid (Fig. 4). The absence of a large increase of *P. cerevisiae* activity suggests that little or no 5-formyltetrahydrofolate, a relatively stable folate, appeared in the bile.

Correction for delay in sampling of bile (Fig. 5). The rate of flow of bile out of the T tube was 0.25 to 0.4 ml/min. To calculate the delay between the presentation of the folate to the liver and its appearance in the bile, rose bengal–^{131}I was injected intravenously in three patients immediately after bile collection for folate. This material, which is rapidly excreted into the bile (16), appeared in the T-tube effluent 15–20 min after injection, and reached a maximum at 50–60 min. The study of patient 7 is seen in Fig. 5. It would appear that bile folate concentration increased about 10 min after the appearance of rose bengal–^{131}I, which suggests that the first folate increment was presented to the liver cell no more than 10 min after feeding. The peak concentration of folate was seen 55 min after the rose bengal–^{131}I peak.

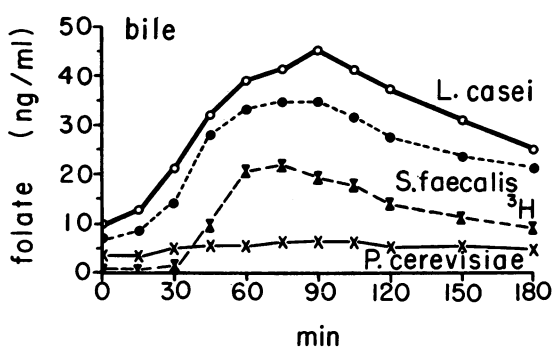

FIGURE 3 Bile folates and ^{3}H after feeding folic acid–^{3}H (patient 4). Same study as Fig. 2.

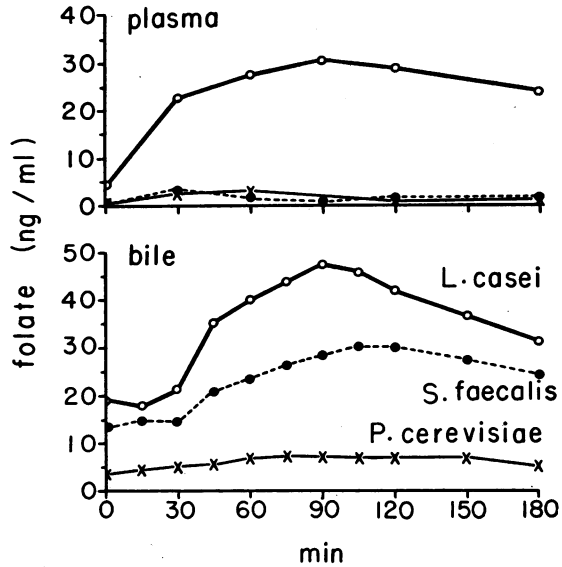

FIGURE 4 Plasma and bile folates after feeding 5-formyltetrahydrofolate. Each point is the mean value of folate from two studies on patients given 2.0 mg of DL-5-formyltetrahydrofolate orally. Open circles joined by solid lines represent *L. casei*-active folate (○——○), closed circles joined by broken lines represent *S. faecalis*-active folate (●--●), and ×'s joined by solid lines (×——×) represent *P. cerevisiae*-active folate.

Chromatography of dialysates of bile (Figs. 6 and 7). Dialysates of bile from patients 4, 5, 6, and 8 were chromatographed on TEAE-cellulose. Fig. 6 shows the

TABLE II

Summary of Data on Patients Given Folinic Acid

Patient No.			7	8
Age (sex)			90 (F)	27 (F)
RBC folate			312	194
Plasma	L.C.	Fasting	4.7	3.6
		Peak	30	30
		(Time)	(60)	(90)
	S.F.	Peak	3.8	2.1
		(Time)	(30)	(30)
	P.C.	Peak	3.3	
		(Time)	(30)	
Bile	L.C.	Fasting	21	16
		Peak	60	43
		(Time)	(105)	(120)
	S.F.	Fasting	15	12
		Peak	37	23
		(Time)	(105)	(90)
	P.C.	Fasting	5.5	3.0
		Peak	7.0	7.2
		(Time)	(105)	(90)
Ratio bile/plasma			4.5	4.4

See footnotes to Table I.

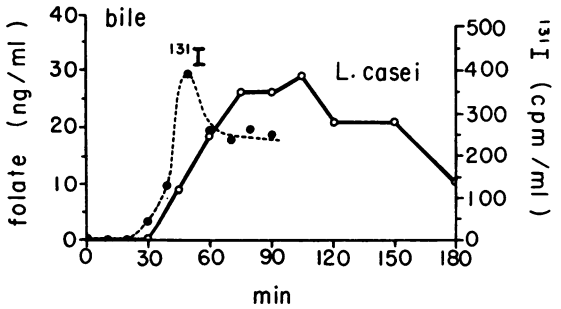

FIGURE 5 Comparison of excretion of folate and rose bengal-^{131}I in bile. The solid line (○——○) is the folate increment after 2.0 mg of DL-folinic acid given orally to patient 5. The broken line (●--●) is ^{131}I in bile after giving 15 μCi rose bengal-^{131}I intravenously immediately after completion of the folate study.

chromatogram of fasting bile from patient 6. The earliest folate fraction eluted in tube 12 and supported growth of all three assay organisms. Its position of elution and equal support of growth of the three assay organisms would be consistent with its tentative identification as 10-formyltetrahydrofolic acid (10-CHOH$_4$PteGlu). A second major peak eluted in fraction 16 and showed equal activity for *L. casei* and *S. faecalis*, but little for *P. cerevisiae*. A third eluted at fraction 18-19, the position of elution of 5-formyltetrahydrofolate and methyltetrahydrofolate. Most of this peak supported growth only of *L. casei* and probably represented methyltetrahydrofolate. A fourth major peak usually was seen at a later position, but before the position of elution of folic acid. This material supported growth of *L. casei* more than *S. faecalis* and has not been identified. Its mobility and biological activity would be consistent with a folate triglutamate.

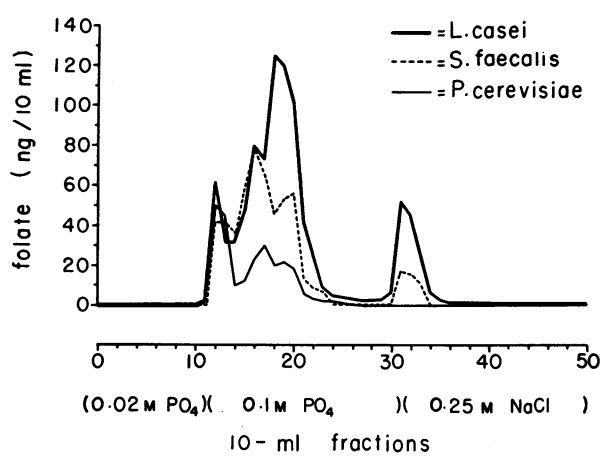

FIGURE 6 Fractionation of folates of fasting bile. The entire dialysate of 21 ml of fasting whole bile (patient 5) was applied to a TEAE-cellulose column and eluted with phosphate buffers and buffered sodium chloride.

A similar study is shown in Fig. 7, a chromatogram of bile collected from patient 4 during maximum bile folate concentration after feeding folic acid-^{3}H. Unlike the study illustrated in Fig. 6, in this study, ascorbic acid was not added to bile until 4 hr after collection, and biological assay was delayed 48 hr after the collection of the chromatographic fractions. These technical details probably were responsible for the greater folate-^{3}H than biological folate activity in fraction 12. The distribution of folate fractions is essentially similar in Fig. 7 to that of fasting bile in Fig. 6, except that a folate eluted at fraction 40 of Fig. 7 which supported growth of *L. casei* and *S. faecalis* but not *P. cerevisiae*. This is the position of elution of folic acid. It is apparent that ^{3}H activity appeared in most of the fractions, which indicates incorporation of the administered folic acid-^{3}H into several different folates.

Studies on bile polyglutamate folates and deconjugases (Table III). Table III shows one of seven studies done to determine if bile contains polyglutamates or deconjugase activity. Results of all seven studies were similar. Incubation of bile with yeast at pH 4.6 released from 0 to 10% (mean 4%) of the folate released following incubation of serum with yeast. Incubation of serum with bile caused no significant increase in total folate concentration, which suggests that polyglutamates larger than triglutamates (which are active for *L. casei* without deconjugase treatment) were not present. The folate activity of a mixture of serum, bile, and yeast incubated together was similar to that without bile present, which suggests that normal bile inhibited only slightly the action of serum deconjugase.

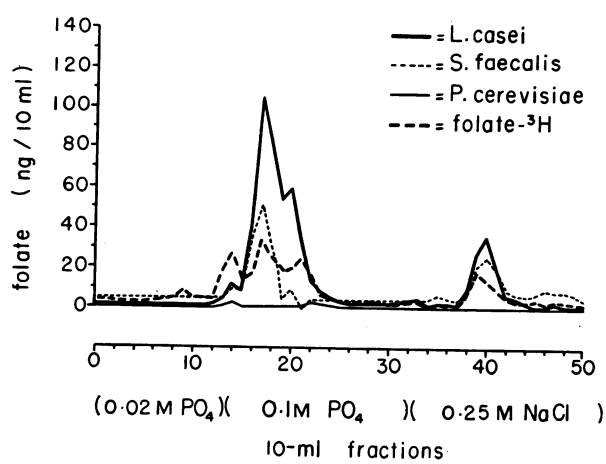

FIGURE 7 Fractionation of folates in bile after feeding folic acid-^{3}H. A dialysate of 35 cc of bile (patient 4) taken during the peak excretion of folate following administration of 1.0 mg of folic acid-^{3}H was chromatographed as in Fig. 6. ^{3}H activity has been converted to folate equivalent based on specific activity of administered folic acid-^{3}H.

DISCUSSION

Microbiological assay with three different assay organisms allows some indication of the forms of folate coenzymes present in the plasma and bile samples. Growth of *L. casei* is supported by all known pteroylmonoglutamates (folates) and by diglutamyl and triglutamyl forms as well. Growth of *S. faecalis* is supported by all pteroylmonoglutamates except N^5-methyltetrahydrofolate, by at least some pteroyldiglutamates, and by pteroic acid. Growth of *P. cerevisiae* is supported by all tetrahydropteroylmonoglutamates except N^5-methyltetrahydrofolate, and by no folates at a higher level of oxidation. Although it is probable that all folates may not support growth of a sensitive organism to the same degree, one may to a first approximation assume that *L. casei* measures total folate monoglutamate, *S. faecalis* measures the same group with the exception of N^5-methyltetrahydrofolate, and that *P. cerevisiae*-active folate represents tetrahydrofolates with the exception of N^5-methyltetrahydrofolate.

After feeding folic acid-^{3}H, most of the initial increment of plasma folate was due to a ^{3}H-labeled folate active for *L. casei* and *S. faecalis* but not *P. cerevisiae*. TEAE-cellulose chromatography of plasma showed that this folate had the mobility and microbiological activity of folic acid, but the radioactivity in the plasma was too low to confirm that the radioactivity was present in this folate fraction. This early appearing folate probably was folic acid. During the 1st hr after feeding the folic acid-^{3}H, an increase was observed in the *L. casei*-active, *S. faecalis*-negative fraction of total plasma folate which is presumed to be methyltetrahydrofolate. This was unassociated with an increase of tritium above the level accounted for by the folic acid fraction. After the 1st hr, the ^{3}H activity decreased more slowly than did the *S. faecalis* activity, which indicates that some of the ^{3}H-labeled material no longer supported growth of *S. faecalis* and was either converted to a form which would support only *L. casei* (i.e., methyltetrahydrofolate) or to a microbiologically inactive form. The simplest explanation for these observations is absorption of unaltered folic acid-^{3}H into the plasma together with flushing of nonradioactive methyltetrahydrofolate from the tissues, followed by conversion of some of the folic acid-^{3}H to methyltetrahydrofolate-^{3}H. It is probable that all of the ^{3}H remained associated with folate since folic acid-^{3}H has been found to be stable in serum for 3 hr in vitro (17). We have not excluded, however, the possibility that some of the ^{3}H activity in plasma was associated with a breakdown product such as *para*-aminobenzoyl glutamate.

Previous findings (1, 2) of high folate in bile have been confirmed. Unlike plasma folate, only 20–55% of bile folate was methylfolate. The evidence presented suggests that 10-formyltetrahydrofolic acid was the first major component eluted on TEAE-cellulose chromatography. It has been reported (18) that on DEAE-cellulose, 10-formyldihydrofolic acid and 10-formyl folic acid elute between the positions of 10-formyltetrahydrofolate and folinic acid. These compounds are growth factors for *L. casei* and *S. faecalis*, but not for *P. cerevisiae*. The second peak found on our chromatograms could contain one or both of these compounds. Immediate addition of ascorbic acid to bile after collection caused relatively more folate to appear in the first peak in studies on patients 5, 6, and 8 than in patient 4 in which ascorbic acid was not added to bile for about 4 hr. Of the 10-formyl folates, the tetrahydro one may be the predominant one, and the oxidized ones may have formed in the bile. Some of the administered folic acid was excreted unchanged into the bile, but most of it was transformed into biological folates. Considering the time taken for secreted bile to be sampled, the increase of bile folate concentration was rapid after folic acid administration.

TABLE III

Study on Polyglutamates and Deconjugase in Bile

Reactant added*	Control	After incubation
	ng folate per ml	
Bile (B)	16.0	16.0
Serum (S)	1.3	1.3
Yeast (Y)‡	15	14
B + S	18	18
B + Y	31	33
S + Y	13	125
B + S + Y	33	93

* 1.0 ml of each reactant was added to citrate-phosphate buffer to give a volume of 10.0 ml. Final pH was 4.6 ±0.1.
‡ Yeast was aqueous solution of yeast extraction with approximately 200 ng/ml total folate and 10% free folate.

5-Formyltetrahydrofolate was much more rapidly transformed to methyltetrahydrofolate than was folic acid after oral administration. This agrees with findings reported by Perry and Chanarin (5). A small proportion of the administered 5-formyltetrahydrofolate, however, did reach the peripheral circulation. The bile folates after 5-formyltetrahydrofolate feeding were similar to those after administration of folic acid.

Bile contained very little, or no, folate deconjugase activity by the assay method used here. This method is qualitative, but shows that bile contains less than 4% of the conjugase activity of an equivalent volume of serum. This observation disagrees with that of Bernstein and Gutstein (6) who reported extremely high levels of deconjugase in rat bile and human T tube bile. The reason for this disagreement is not apparent and may depend on

differences of assay conditions. However, the 4.6 pH used was in the range that they recommended. Expression of deconjugase activity as folate released per milligram of protein rather than per milliliter of plasma or bile would make the bile deconjugase activity appear relatively higher. Addition of serum deconjugase to bile caused little or no increase of folate activity, which suggests that significant quantities of folate polyglutamates of the type found in yeast were not present in bile.

The finding that there are several different folates in bile, that ^{3}H appears rapidly in these folates after folic acid-^{3}H administration, and that the folates in bile are similar after either folic or 5-formyltetrahydrofolate acid feeding is consistent with the supposition that bile folates reflect the intracellular oligoglutamate folates of liver rather than being the excretion of a proportion of the folate presented to the liver by the portal venous blood. Studies of the rate of appearance of label in bile folates may provide a useful tool for an in vivo investigation of the transport of folates into liver and their metabolic interconversion.

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