RAPID TRANSFER OF FOLIC ACID FROM BLOOD TO BILE IN MAN, AND ITS CONVERSION INTO FOLATE COENZYMES AND INTO A PTEROYLGLUTAMATE WITH LITTLE BIOLOGICAL ACTIVITY

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SUMMARY

1. [3H]Folic acid infused intravenously into patients with biliary fistulae appeared promptly in bile, coincident with 131I-labelled Rose Bengal injected simultaneously. The radioactivity was distributed among several fractions of biological folates present in bile and was associated with folic acid and with an unidentified folate which chromatographed on DEAE-Sephadex close to 10-formyltetrahydrofolyl mono-glutamate.

2. Based on affinity for DEAE-Sephadex and support of growth of Lactobacillus casei, Streptococcus faecalis and Pediococcus cerevisiae, we have tentatively identified some of the folates of human bile as 10-formyltetrahydrofolate, 10-formylfolate, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate and tetrahydrofolate or 5,10-methylene tetrahydrofolate. After infusion of folic acid, the formyltetrahydrofolates increased more rapidly than did 5-methyltetrahydrofolate.

3. The unidentified radioactive folate contained both the pteridine and p-aminobenzoate portions of folate. It appeared not to support growth of the test microorganisms and not to be bound to a protein or a chain of γ-glutamates. It was present in the bile of a dog injected with [3H]folic acid but was absent from extracts of liver. This material may be a transport form of folate or a special modification imposed on folic acid during transport across the liver.

Key words: bile, folate, folic acid, liver, filtration.

Bile folate concentration exceeds that of serum and ingested folic acid increases bile folate (Baker, Kumar & Swaminathan, 1965; Herbert, 1965). The variety of folates in human bile has prompted the hypothesis that human bile folate might reflect the distribution of oligoglutamyl folates in human hepatic cells (Pratt & Cooper, 1971). A portion of the folate in human portal

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venous blood appears rapidly in bile (Pratt & Cooper, 1971). We have studied the rate of appearance of folic acid in human bile after intravenous infusion, and compared this with that of Rose Bengal, a dye transported unchanged across hepatic cells within seconds of its presentation in hepatic blood (Turco, Ghemi, Molino & Segre, 1966). In addition, we have fractionated the folates in human bile sequentially after this infusion to determine if the infused label appears in folates other than folic acid, and other than those usually found in plasma, to determine if bile folate may reflect transport of folate into liver cells and its conversion there into oligo-glutamyl coenzyme forms of folate. A preliminary report of some of these observations has been published as an abstract (Lavoie & Cooper, 1972).

PATIENTS AND METHODS

Nine patients were studied. These had undergone cholecystectomy and surgical exploration of the common bile duct 3–12 days (median 4:5 days) before the study. The experimental nature of the study was explained to all subjects, and all volunteered. Ages ranged from 42 to 72 years; all except one were taking food by mouth at the time of the study. All were convalescing uneventfully, and in none was evidence of infection apparent. None received antibiotics after surgery. Serum and erythrocyte folates were normal in all but one of the patients. Erythrocyte folate was slightly low (165 ng/ml of erythrocytes) in one.

Folic acid labelled with $^3$H or $^{14}$C was purchased from The Radiochemical Centre, Amersham (batch 23, 78 mCi/mg, $^3$H label at 3', 5' and 9 positions; $^{14}$C batch 14, 125 mCi/mg, $^{14}$C on C-2), purified on DEAE-cellulose (Pratt & Cooper, 1971), mixed with folic acid purchased from Nutritional Biochemicals Co., Cleveland, Ohio, on the day preceding each experiment, to reduce its specific radioactivity to 40 pCi/mg, sterilized by filtration through Millipore 0.22 μm Swynnex filters, and stored in aliquots at $-70^\circ$C until used. Rechromatography of solutions so stored revealed a single major peak eluting in the position of folic acid.

Collection of samples

Bile was collected into containers containing mercaptoethanol to bring its final concentration in the bile sample to 10% (1 mol/l) and usually chromatographed within 2 h. In experiments in which many samples were taken, samples were stored at $-70^\circ$C for up to 8 weeks before processing. This storage appeared not to affect the folate content.

Bile and urine samples from 3 h to 27 h after infusion of radioactive folic acid were collected in dark amber bottles at room temperature, the volume was determined, 0.1 ml taken for radioactive counting, and 100 ml aliquots were stored at $-70^\circ$C for subsequent analysis. Mercaptoethanol was added to these samples to a concentration of 0.2 mol/l.

Folate infusion

After collection of baseline samples of blood, urine and bile, a bolus of 0.3 mg of folic acid (labelled with $^3$H and sometimes with $^{14}$C) was injected intravenously over 5–10 s. In experiments in which $^{131}$I-labelled Rose Bengal was injected, 15 μCi of this material was mixed with the folic acid and injected simultaneously. After the initial injection of folate, 0.7 mg of folic acid containing the remaining radioactive folate was infused over 10 min. Samples of bile, blood and urine were taken at intervals. At 3 h after the infusion, 15 mg of folic acid (Folvite, Lederle) was injected subcutaneously to flush as much as was possible of the radioactivity into
the urine. The radioactivity recovered from the urine varied from 8.5 to 61.7% of the injected dose (median 30%).

_Microbiological assay_

Microbiological assay of samples of blood, bile and urine utilized *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043) and *Pediococcus cerevisiae* (ATCC 8-81) and commercial mixes of assay medium (folic acid assay medium no. 11267, Baltimore Biological Laboratories, Difco folic acid assay medium no. 0318-15). Assay procedures were as described previously (Pratt & Cooper, 1971; Baker, Herbert, Pasher, Hutner, Wasserman & Sobotka, 1959; Teply & Elvehjem, 1945; Sauberlich & Baumann, 1948).

_Determination of radioactivity_

Samples of 0.1 ml of urine and plasma were transferred to counting vials (low-background vials, Packard Instrument Co.), mixed with 15 ml of Aquasol (New England Nuclear Corp.) and radioactivity was counted in a Packard Tri-Carb Liquid Scintillation spectrometer. Samples of bile were counted in the same way except that they were decolorized with 1–2 drops of 30% hydrogen peroxide for 24 h before addition of Aquasol. Counts of radioactivity were corrected for quenching and referred to 100% efficiency by using an automatic external standard referred to a standard curve prepared with $^3$H$_2$O. Radioactivity (d.p.m.) was referred to standards of known folate content and expressed as ng of infused folate. $^{131}$I radioactivity was determined in a well-type scintillation counter. Differentiation of $^3$H from $^{14}$C involved counting in two channels simultaneously and comparison of the results with counts obtained from known mixtures of the two isotopes.

_Fractionation of folates in bile_

As reported previously (Pratt & Cooper, 1971), human bile usually required pretreatment before folates could be fractionated on ion-exchange columns. Because the dialysis procedure used previously (Pratt & Cooper, 1971) was slow and provided incomplete recovery of folates, studies were undertaken with different ultrafiltration membranes. Samples diluted in phosphate buffer were ultrafiltered through a variety of commercial membranes under N$_2$ at 1035 kPa (150 lb/in$^2$). A Sartorius filter holder 47 cm in diameter was used to support the membranes. Ultrafiltration of 20 ml of ultrafiltrate required 3–5 h at room temperature with most of the membranes used.

With most of the membranes, the folate content of the first 1–4 ml of filtrate was lower than that of the bile sample. Folate in subsequent samples approached or exceeded that of unfiltered bile, suggesting that the capacity of the membrane to adsorb folates had been exceeded. As a result of these studies, Eastman filters UF-10 and HT-00 were selected for preparation of most bile samples.

The ultrafiltrate of bile was diluted to adjust its conductivity to about 6800 µmho, and was applied to a 0.9 cm x 30 cm column of DEAE-Sephadex (Nixon & Bertino, 1971). Elution utilized a gradient of phosphate buffer, pH 6.0, containing 0.2 mol/l mercaptoethanol: 250 ml of 0.8 mol/l potassium phosphate flowing into 250 ml of 0.1 mol/l potassium phosphate. Chromatography was conducted in the dark at 4°C; fifty samples of about 10 ml each were collected, and were assayed immediately after the completion of the chromatography. The column was standardized with folic acid, folinic acid and p-aminobenzylglutamate obtained.
Fractionation of bile and liver folates in a dog

A single study of bile and liver folates after folic acid infusion was carried out in a 30 kg mongrel dog to compare liver folates with bile folates. After induction of anaesthesia with pentobarbital, 500 μg of folic acid labelled with \(^{3}\text{H}\), corresponding to the same quantity per kg as infused in man, was infused intravenously exactly as for the studies in man. Bile, blood and urine samples were obtained from catheters placed previously in the common bile duct, femoral vein and bladder. At intervals after the infusion, biopsies of liver were taken. These were weighed and diluted 1:10 (w/v) in 0.1 mol/l potassium phosphate, pH 7.0, containing 0.2 mol/l mercaptoethanol. After homogenization in a glass homogenizer the samples were boiled for 4 min in a water bath and centrifuged at 1500 g for 15 min at room temperature (Whitehead, 1971); 0.1 ml of the supernatant was used for \(^{3}\text{H}\) counting, and the remainder was stored at \(-70^\circ\text{C}\).

Before chromatography, liver extract samples were thawed and incubated at 37°C for 1 h with an equal volume of normal human serum at pH 4.6. The pH then was adjusted to 6.0, and the mixture chromatographed. Folate in the human serum had been depleted previously with charcoal. Specimens of bile, urine and plasma were treated as were specimens from patients.

RESULTS

Rate of appearance of \(^{3}\text{H}\)folic acid in human bile after intravenous infusion

Because of the variable interval between the appearance of \(^{3}\text{H}\)folic acid in bile and its emergence from the T-tube, the rate of secretion of this material into bile was compared with that of \(^{131}\text{I}\)-labelled Rose Bengal injected simultaneously with the first bolus of folic acid. Results are illustrated in Fig. 1. The \(^{3}\text{H}\) radioactivity from the folic acid infused appeared in bile simultaneously with the \(^{131}\text{I}\) radioactivity of the Rose Bengal.

![Fig. 1. \(^{3}\text{H}\) and \(^{131}\text{I}\) in blood (---) and bile (— — —) after simultaneous injection of \(^{3}\text{H}\)folic acid (○) and \(^{131}\text{I}\)-labelled Rose Bengal (○). Radioactivity (d.p.m.) \((\times 10^{-2})\) of each isotope is plotted on the vertical axis against the time after injection of 12 μCi of \(^{3}\text{H}\)folic acid and 15 μCi of \(^{131}\text{I}\)-labelled Rose Bengal. Over the subsequent 10 min, indicated by the arrows, a further 28 μCi of \(^{3}\text{H}\)folic acid was infused intravenously. The total quantity of folic acid infused was 1 mg.](image-url)
The total \(^3\text{H}\) radioactivity which appeared in bile was about 0.3% of the injected dose, compared with median of 30% in the urine. Bile flowed freely from the T-tube orifice of all patients. No attempt was made to divert all of the bile secreted into the T-tube; in fact, the stools of all patients were brown at the time of the study.

In the experiment illustrated in Fig. 2, samples of bile, serum and biopsies of liver were obtained from a dog after intravenous infusion of \([^3\text{H}]\)folic acid. After ligation of the bile duct and complete diversion of bile into the collection tube, the rate of bile flow decreased rapidly. \(^3\text{H}\) radioactivity in serum and bile in Fig. 2 are plotted against the time of collection, but whereas the volume of serum samples was constant, the decreasing bile flow made standardization of the volume of bile samples impractical. The decreasing radioactivity in bile with time, illustrated in Fig. 2, thus reflects decreasing bile flow more than decreasing concentration of radioactivity in the bile. With this reservation, the pattern of serum and bile radioactivity with time in the dog was similar to that observed in man, whereas liver radioactivity reached a maximum at the end of the infusion and remained constant for 2 h before decreasing slowly.

**Fractionation of folate in bile and urine**

The folate content of human bile in these studies varied from 15 to 400 ng/ml, depending upon the interval after infusion of folic acid. These folates were fractionated on DEAE-Sephadex. The capacity of each fraction to support growth of the three test micro-organisms, and its \(^3\text{H}\) radioactivity, is plotted in Fig. 3, which is an average of nine chromatograms of bile from one patient 1–2 h after folic acid infusion. The same pattern was observed in chromatograms of bile from the other patients. Based on the sequence of elution of folates from the column (Nixon & Bertino, 1971), and their capacity to support growth of the test organisms
Fig. 3. $^3$H radioactivity and microbiological folate activity in human bile 1–2 h after infusion of $[^3$H]$\text{fol}$ic acid. Mean chromatogram of 2 ml of human bile, comparing $^3$H radioactivity (curve 4) and support of growth of $L$. $\text{casei}$ (curve 2), Strep. $\text{faecalis}$ (curve 1) and $P$. cerevisiae (curve 3) by each fraction. Results on the vertical axis are plotted as ng of folate activity per 10 ml of each fraction—the approximate volume of each fraction from the chromatographic procedure. As indicated in the text, increasing molarity of the eluting buffer partially inhibited growth of $L$. $\text{casei}$ in fractions eluted later from the column.

(Blakley, 1969), tentative identification of these folate fractions has been made as follows. Fractions supporting growth of $L$. $\text{casei}$, and peaking in tubes 17, 22, 25, 31 and 40, are consistent with monoglutamyl forms of 10-formyltetrahydrofolate (10-HCO $H_4$PteGlu), 10-formyl-folic acid (10-HCO PteGlu), 5-formyltetrahydrofolate (5-HCO $H_4$PteGlu), 5-methyltetrahydrofolate (5-Me-$H_4$MeGlu) and folic acid (PteGlu) respectively.

Folates in bile from a fasting patient were similar except that little or no activity was observed at tube 40. Folate measured with $L$. $\text{casei}$ became less than measured with Strep. $\text{faecalis}$ after the initial folates were eluted, owing to inhibition of growth of $L$. $\text{casei}$ by the increasing molarity of the eluting buffer.

$^3$H radioactivity appeared to coincide with several of the microbiologically active folate fractions, but most of the $^3$H radioactivity peaking at tubes 15–16 was observed consistently to precede the microbiological activity, suggesting that it might not be identical with the folates supporting growth of the micro-organisms. The $^3$H fraction peaking at tube 22 with the presumed 10-formylfolate coincided in position of elution with that of $p$-aminobenzylglutamate, a well-described breakdown product of folic acid (Johns, Sperti & Burgen, 1961). The $^3$H radioactivity in these tubes was inadequate to determine if it was accumulated by the micro-organisms during microbiological assay, and so the identity between the $^3$H radioactivity and the presumed 5-formylfolate remains uncertain.

**Sequential fractionation of $^3$H radioactivity in chromatograms of bile**

To study the sequence of labelling of the folate fractions in bile after folic acid infusion, bile
samples were collected from a single patient at intervals after infusion of \(^{3}\text{H}\)folic acid. The \(^{3}\text{H}\) radioactivity in each was fractionated on DEAE-Sephadex, and the radioactivity in each fraction was plotted against the time of collection (Fig. 4). Because of uncertainty about the identity of the microbiologically active and the radioactive fractions, \(^{3}\text{H}\) fractions were numbered sequentially in the order of their elution. Fractions I, II, III, IV and V in Fig. 4 correspond to the \(^{3}\text{H}\) fractions peaking at tubes 10, 15, 22, 31 and 40 in Fig. 3. Peaks II, III, IV and V correspond approximately in elution volume to the presumed folates 10-formyltetrahydrofolate, 10-formylyfolate, 5-methyltetrahydrofolate and folic acid respectively. Peak I corresponds in elution volume to one of the observed breakdown products of folic acid.

The \(^{3}\text{H}\) radioactivity in peak V (folic acid) increased rapidly during infusion and then decreased, indicating rapid appearance of unchanged folic acid in the bile and its early disappearance. The largest fraction of \(^{3}\text{H}\) radioactivity was in fraction II, which has been mentioned above.

\(^{3}\text{H}\) radioactivity in the other fractions increased more slowly than did that in fractions II and V, and decreased more slowly than did fraction V. Radioactivity associated with fraction I, almost certainly a breakdown product of folic acid, appeared and disappeared gradually.

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**Fig. 4.** \(^{3}\text{H}\) in human bile after infusion of \(^{3}\text{H}\)folic acid. Eleven bile samples were taken from a single subject after infusion of \(^{3}\text{H}\)folic acid. The \(^{3}\text{H}\) radioactivity eluting from chromatograms of these fractions on DEAE-Sephadex was divided into fractions, numbered in the order of their elution from the column. The distribution of \(^{3}\text{H}\) in these fractions is plotted against the time of completion of sample collection. The upper curve shows total \(^{3}\text{H}\). ●, Fraction I; ○, fraction II; ▽, fraction III; ▽, fraction IV; ×, fraction V.
Sequential changes in microbiological folate activity in bile

Sequential changes in the microbiological folate activity in the different folate fractions from the chromatogram were studied in eight of the samples taken from the subject studied in Fig. 4 (Fig. 5). Each point in Fig. 5 was calculated as follows: sequential samples (2 ml) of bile from

![Folate activity in bile](image)

Fig. 5. Folates in human bile after infusion of folic acid intravenously. Sequential changes in the amount of microbiologically active folate eluting from DEAE-Sephadex during fractionation of 2 ml samples of bile obtained at intervals from a single subject. The folate content in each chromatographic tube is plotted on the vertical axis. Identification of the folates is presumptive, based on their support of microbiological growth, and their affinity for DEAE-Sephadex. ×, Presumed 5-formyltetrahydrofolate; ▽, 10-formyltetrahydrofolate; ○, 5-methyltetrahydrofolate; ▽, 10-formylfolate; ●, folic acid.

In this experiment, there was a rapid increase of folic acid and of 5-formyltetrahydrofolate, with slower increments of other folates. The increase of 5-formyltetrahydrofolate was not associated with an increase of $^3$H radioactivity in this fraction (Fig. 4), and 10-formyltetrahydrofolate did not increase as did the $^3$H radioactivity in adjacent tubes (fraction II, Fig. 4). An identical pattern of change of microbiological activity was not observed in all patients. In some, folate activity associated with the presumed 10-formyltetrahydrofolate and 10-formylfolate fractions increased in addition to the 5-formyltetrahydrofolate. In none was a large increase in the 5-methyltetrahydrofolate fraction observed. The patient selected for the studies illustrated in Fig. 4 and Fig. 5 was folate-sufficient, with serum folate 6.7 and erythrocyte folate 451 ng/ml; fasting bile folate was 57 ng/ml, and she excreted 57% of the infused radioactivity
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in the urine during the 27 h after the infusion. This indicates that her store of folate in the liver probably was considerable.

Folates in bile and liver of a dog

Bile samples collected 15–65 min after the beginning of the infusion were pooled and chromatographed (Fig. 6). The pattern of radioactivity was similar to that in human bile, with a large radioactive fraction at tubes 15–16 which did not coincide with microbiologically active fractions. A sample of dog liver chromatographed after incubation at pH 4.6 with folate-depleted human serum did not contain a peak of radioactivity at tubes 15–16 corresponding to the one in bile. Several peaks of microbiologically active folates were observed in the extract of liver. Based on their support of growth of the three test organisms and their mobility on DEAE-Sephadex, these were tentatively identified as 10-formyltetrahydrofolate, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate and folic acid. Radioactivity was observed in several fractions of liver extract. This corresponded to several of the radioactive peaks observed in dog bile, but none corresponded to the fraction which eluted from the chromatogram of bile at tubes 15–16.

$^3$H radioactivity in human urine

Urine samples collected from the patients were fractionated on DEAE-Sephadex. The $^3$H fractions observed were similar to those reported by Nixon & Bertino (1972), but none corresponded exactly to the unidentified fraction in bile which eluted at tubes 15–16.

Characteristics of the $^3$H fraction in bile which eluted from chromatograms at tubes 15–16

The affinity of this material for DEAE-Sephadex appeared superficially to be identical with that of the microbiologically active folates presumed to be 10-formyltetrahydrofolate, but in all
cases it exceeded the microbiologically active folate in quantity and, in most cases, did not co-chromatograph with it.

Although the material was not identified, an attempt was made to determine some of its characteristics. The $^3\text{H}$ fraction re-chromatographed in the identical position when recovered after chromatography of bile on DEAE-Sephadex, and was adsorbed from bile by shaking with charcoal (Norit A). It appeared not to be bound to a peptide or protein because its mobility on DEAE-Sephadex was not affected by prior boiling in the presence of mercaptoethanol. It filtered through Sephadex G-25 more slowly than did $^3\text{H}_2\text{O}$, as does folic acid, whereas polyglutamylfolates filter before $^3\text{H}_2\text{O}$. It appeared not to be loosely associated with a binder of folic acid because incubation of bile with an excess of folic acid did not alter the mobility of the radioactive peak. It appeared not to be a polyglutamylfolate because of its early elution from the column, and because its mobility on DEAE-Sephadex was found unaltered by incubation with human serum at pH 4.6. It was not one of the well-described breakdown products of folic acid because it did not co-chromatograph with the peaks of extinction obtained when p-aminobenzoic acid was chromatographed, even when this material was incubated on the bench for several days to allow decomposition. No similar fraction was observed when $[^3\text{H}]$folic acid was mixed with bile, ultrafiltered and chromatographed, nor was a peak of extinction observed in this position when tetrahydrofolic acid and 5-methyltetrahydrofolate were allowed to decompose before chromatography.

To determine if the fraction contained both the pteridine and p-aminobenzoate portions of folate, a mixture of $[^3\text{H}]$folic acid labelled at 3', 5' and 9, and of $[^14\text{C}]$folic acid labelled in the pteridine ring, was infused into a patient; bile samples were collected, processed, and chromatographed. Both $^3\text{H}$ and $^{14\text{C}}$ radioactivity appeared in the fraction eluting in tubes 15–16 in approximately the same ratio as in the test solution given, indicating that the material contained both the pteridine and p-aminobenzoic acid portions of the molecule, and that if it were a breakdown product it could not be a fragment smaller than pteroic acid.

**DISCUSSION**

These studies describe the normal pattern of folate coenzymes in human bile and demonstrate that the concentration of some of them increased rapidly after intravenous infusion of folic acid. Although early reports suggested that most of the folate in human liver was free and not polyglutamyl, and that the largest fraction was 5-methyltetrahydrofolate, recent reports indicate that most folate in livers of mice (Sauberlich & Baumann, 1948), rats (Shin, Williams & Stokstad, 1972; Houlihan & Scott, 1972) and man (Whitehead, 1971) is in the polyglutamyl form. A considerable proportion of formylated folates has been reported to be present in human liver (Sauberlich & Baumann, 1948), and much more of oligoglutamates in rat liver have been reported to be formylated tetrahydrofolates and tetrahydrofolate itself than methyltetrahydrofolate (Shin et al., 1972). The distribution of biological folates which we have observed in bile is similar to these and supports the suggestion (Pratt & Cooper, 1971) that bile folate may reflect the oligoglutamylfolates of the hepatic cells. The studies illustrated in Fig. 5 demonstrate that intravenous infusion of folic acid results in rapid appearance of unchanged folic acid in bile and of a presumed formylated folate. The increase of concentration of other folates, including presumed methyltetrahydrofolates, was slower. This suggests that folic acid flushed presumed 5-formyltetrahydrofolate from the liver by displacing it from a pool or that
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the folic acid rapidly was converted into this material. The very small amount of \(^{3}\)H radioactivity appearing with this fraction in bile chromatograms (Fig. 3) would support the former suggestion.

\(^{3}\)H radioactivity appeared in bile rapidly after injection of \(^{3}\)Hfolic acid, as rapidly as did the \(^{131}\)I of Rose Bengal. This indicates rapid transfer of folic acid from the plasma to the liver cell. Of the \(^{3}\)H radioactivity infused, some appeared in bile as breakdown products of folic acid, some was associated with the microbiologically active folates, and much appeared as an unidentified fraction. The breakdown products indicate decomposition of folate in the liver or bile or selective accumulation into the bile of minimal quantities of impurities infused. In our studies, the delay between folate infusion and the appearance of \(^{3}\)H at the collection tube orifice was about 1 h, suggesting this period of incubation of the folate with bile in vivo (presuming almost immediate secretion of Rose Bengal). Folic acid stored at \(-70^\circ C\) after purification appeared not to decompose significantly, but re-chromatography after purification, freezing, storage and thawing sometimes demonstrated small amounts of \(p\)-aminobenzylglutamate and its products. Such a preparation of \(^{3}\)Hfolic acid was received by the patient who received simultaneous infusion of both \(^{3}\)Hfolic acid and \(^{14}\)Cfolic acid. His plasma after infusion contained small amounts of \(^{3}\)H-labelled breakdown products, but all of the \(^{14}\)C radioactivity chromatographed as folic acid.

Some of the \(^{3}\)Hfolate appeared in fractions of bile associated with biologically active folates, especially those tentatively identified as 5-formylfolate and 5-methyltetrahydrofolate. This may represent the rate of conversion of folic acid into these folates in human liver. Human bile appeared to contain the following folates: 10-formyltetrahydrofolate, 10-formylfolate, 5-formyltetrahydrofolate, 5-methyltetrahydrofolate and, after folic acid infusion, folic acid. Based on chromatographic mobility and support of growth for the test organisms, the first and fifth also could have been 5,10-methenyltetrahydrofolate, and 5,10-methylenetetrahydrofolate respectively, but this is less probable. The presence of apparent 5-formyltetrahydrofolate in large concentration was surprising, since this compound usually is considered to be outside of the usual folate metabolic pathways (Blakley, 1969). It is possible that other formylated folates were converted into this material during extraction and preparation. Samples were not heated nor exposed to extremes of pH, and so it is possible that a substantial proportion of human bile folate is in this form. We have not observed polyglutamylfolates in human bile (Pratt & Cooper, 1971).

The largest fraction of radioactive material appearing in bile after intravenous infusion of folic acid was not a recognized form of folate. It contained both the pteridine and \(p\)-amino-benzoate portion of the folate molecule and appeared not to be bound to a protein nor to a chain of \(\gamma\)-glutamates. It is possible that it was pteroic acid (Levy & Goldman, 1967), although it appeared not to support growth of \(Strep. faecalis\) significantly. Growth support by adjacent folates in tubes 14–16 in chromatograms indicates that growth inhibitors were not present in these tubes to explain the biological incompetence of the unknown material. Watanabe (1962) observed a similar radioactive fraction in the bile of rats after injection of \(^{3}\)Hfolic acid, and presumed it to be a ‘pteridine’.

Pratt & Cooper (1971) observed such a fraction in human bile, but presumed it to be identical with the first biological fraction eluting from the chromatogram. The absence of this fraction from dog liver homogenate, despite its presence in dog bile, and our failure to convert folic acid into it in bile incubated \(in vitro\), suggest that it is not one of the usual breakdown products
of folic acid and that bacteria in the bile probably were not responsible for its appearance (Levy & Goldman, 1967). These observations suggest that this modification of folic acid was specific to transport through the liver into the bile. It may be a transport form, or a fortuitous modification of folate (e.g. glucuronide), or it suggests that an enzyme system is present in liver which effects an unusual type of decomposition on folic acid secreted into the bile but not on that retained in the liver.

These studies indicate that when folic acid was presented to the liver after intravenous infusion in doses similar to the folate content of a high folate meal, some was rapidly cleared across the liver into the bile at a rate similar to the clearance of Rose Bengal. Some of this material appearing in the bile was unchanged folic acid, and some was a previously unreported metabolite. At the same time biologically active folate, some of which was probably 5-formyl-tetrahydrofolate monoglutamate and some 5-methyltetrahydrofolate monoglutamate, appeared to be flushed from the hepatic cells into the bile. The small recovery of radioactive folate coenzymes during the period of collection indicates that most of the folic acid presented to the liver entered the intracellular folate pools and was diluted by endogenous non-radioactive folate. The unidentified metabolite probably indicates the presence of a previously unreported enzyme system in liver capable of altering folic acid.

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