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ABSORPTION OF 5-METHYLTETRAHYDROFOLATE IN RAT JEJUNUM WITH INTACT BLOOD AND LYMPHATIC VESSELS

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Transport results from in vitro studies may not be applicable to in vivo situations. In this study, we extended our previous in vitro observations regarding the intestinal transport of 5-methyltetrahydrofolate to in vivo studies in the unanesthetized rat and examined the effect of the unstirred water layer on the absorption process. We used a well defined intestinal perfusion technique. Absorption of 0.5 and 5 μM 5-methyltetrahydrofolate proceeded in a linear manner for 40 min of perfusion at 0.31 and 1.74 nmol/100 cm per min, respectively. Absorption of 0.5 μM 5-methyltetrahydrofolate increased with increasing perfusate flow-rate from 0.5 to 2 to 4 ml/min, indicating an unstirred water layer influence on the absorption rate. Absorption of the substrate was saturable with an apparent K_t of 5.7 μM and V_{max} of 3.45 nmol/100 cm per min. Absorption was pH-dependent, and was inhibited by structural analogues. In contrast to the in vitro data, addition of glucose (20 mM) to the perfusate was unnecessary for in vivo absorption to proceed. Unconjugated cholic (5 mM) and deoxycholic (1 mM) acids and the organic anion rose bengal (0.1 mM) inhibited the absorption of 0.5 μM 5-methyltetrahydrofolate when added to the perfusate. Conclusions: the results of previous in vitro studies of 5-methyltetrahydrofolate intestinal transport are applicable to in vivo situations, except that luminal glucose was found to be unnecessary in the latter. The unstirred water layer modulated the absorption of 5-methyltetrahydrofolate, while unconjugated bile acids and rose bengal inhibited it.

Introduction

The natural folate derivative 5-methyltetrahydrofolate (5-CH_3H_4PteGlu) is the predominant circulating and storage form of folate in man [1–3]. We have previously shown that the mucosal-to-serosal transport of 5-CH_3H_4PteGlu in everted sacs of rat jejunum occurs by means of an active, carrier-mediated system which is pH-, Na^+-, energy- and glucose-dependent [4–6]. Although the everted sac technique is a useful method for examining transport processes for energy requirements, it has many physiological limitations. These include difficulty in providing sufficient oxygenation to the intestinal preparation in vitro, the existence of a relatively thick serosal muscle layer that acts as a diffusion barrier for the movement of the absorbed nutrients from the absorptive cells into the serosal compartment, and general viability problems. For these reasons and because no information is available on the effect of normal hormonal and nervous endogenous factors, normal blood and lymphatic circulations and the effect of the unstirred water layer on the transport of 5-CH_3H_4PteGlu, we examined the transport process of this compound in vivo in the unanesthetized rat

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using a well defined and established perfusion technique [7]. The results obtained confirmed the in vitro observations that a pH-dependent, carrier-mediated mechanism is involved in the transport of 5-CH_3H_4PteGlu. Our in vivo studies also show that the absorption process of 5-CH_3H_4PteGlu is limited by the thickness of the unstirred water layer and that the unconjugated cholic and deoxycholic acids and the organic anion rose bengal inhibit the absorption process.

Materials and Methods

Chemicals. The following materials were obtained commercially: unlabeled 5-CH_3H_4PteGlu, folic acid (PteGlu), methotrexate and 5-formyltetrahydrofolate (5-$CHOH_4$PteGlu) (Sigma Chemical Co.); 5-$^{14}CH_3H_4$PteGlu barium salt (58 mCi/mmol) (Amersham/ Searle Corp., Des Plaines, IL); scintillation cocktail, Ultrafluor (National Diagnostics, Somerville, NJ). The reduced folates used were the commercially available racemic mixture of the biologically active and inactive diastereoisomers. All other chemicals were of analytical quality. The radiochemical purity of the stock 5-$^{14}CH_3H_4$PteGlu was determined by cellulose precoated thin-layer chromatography using 0.1 M phosphate buffer (pH 7), containing 5% mercapto ethanol and found to be 97% radiochemically pure.

Methods. Unfasted male Sprague-Dawley rats weighing between 180 and 250 g were used. The animal was anesthetized with ether and the abdomen was opened by a midline incision. An inflow polyethylene catheter was inserted into the proximal end of the jejunum, 14 cm distal to the pylorus. An outflow L-shaped glass cannula was then introduced 50 cm distal to the inflow catheter. The catheters were secured by encircling ligatures which passed between the parallel end-vessels without obstructing blood vessels or lymphatics. The intestinal segment was flushed with phosphate buffer to remove any residual intestinal contents, and then flushed with air to minimize the amount of residual fluid left in the segment. The intestinal segment was then placed in the peritoneal cavity and the abdomen was closed. The animal was allowed to awaken and was placed in a restraining cage. The inflow cannula to the intestinal segment was then connected to a reservoir containing 30 ml of perfusate. The perfusate used was phosphate buffer exprising 110 mM NaH_2PO_4/35.2 mM NaCl/5.5 mM KCl/1.8 mM $MgSO_4$/20 mM glucose. 3 mg/ml sodium ascorbate was added to the incubation medium as an antioxidant. The pH of the perfusate, unless otherwise mentioned, was adjusted to 6 using 1 M NaOH. A totally occlusive roller pump (Buchler Instruments Inc., Fort Lee, NJ) was used to pump the perfusate from the reservoir through the inflow catheter and into the intestinal segment. The outflow cannula was allowed to drain by gravity back into the reservoir. The solution in the reservoir was stirred continuously with a magnetic stirrer. A forced air heating device and a thermostatic temperature controller (Thermistemp Model 74, Yellow Springs Instruments, Yellow Springs, OH) was used to monitor the animal's body temperature with a rectal probe and maintain the animal's temperature at 37 °C. The rate of 5-CH_3H_4PteGlu absorption was calculated by determining its rate of disappearance from the perfusate. Fluid shifts which might take place during the perfusion were assessed with the use of ^{3}H-labelled inulin as a nonabsorbable marker and the absorption rate of 5-CH_3H_4PteGlu was corrected for fluid shifts. ^{3}H- and ^{14}C-labeled radioactivity was determined by double isotope counting and calculating technique [8]. Perfusion was performed for 40 min and 100 μl aliquots in duplicate were removed from the reservoir at 10-min intervals and were added to scintillation vials containing 6 ml of scintillation fluid and analyzed subsequently for radioactivity. At the end of the experiment, the animal was killed by an overdose of ether and the perfused intestinal segment was removed and washed with 30 ml phosphate buffer. A 10 g weight was attached to the most dependent portion of the segment to insure a constant degree of bowel stretch during drying. After a 24-h drying period at 20 °C, the length of the segment was measured and recorded. The absorption results were expressed in terms of dry intestinal length since it correlates best with the intestinal surface area [9].

In preliminary experiments, we examined the possible adsorption of 5-CH_3H_4PteGlu to the tubing and reservoir and found that adsorption was less than 1% during 1 h of perfusion.

Statistical analysis. All the results presented in this paper are the mean ± S.E. of at least six observations in at least three animals. Data were analyzed using Student's *t*-test and regression analysis.

Results

Effect of time

The absorption of physiological (0.5 μM) and pharmacological (5 μM) concentrations of 5-CH_3H_4PteGlu as a function of time was examined in order to determine the linearity of the absorption process. Perfusion experiments were performed for 40 min and sampling was performed at 10-min intervals. The results showed that after an initial rapid disappearance of the substrate from the perfusate which is represented by the intersection of the lines with the *y*-axis and which is probably due to a rapid binding to the mucosal surface, the absorption of 0.5 and 5 μM 5-CH_3H_4PteGlu was constant with time for 40 min perfusion (Fig. 1). The rate of absorption of 0.5 and 5 μM 5-CH_3H_4PteGlu was calculated by linear regression analysis and was found to be 0.31 and 1.74 nmol/100 cm dry length per min, respectively. Subsequently, perfusion experiments were performed for 40 min and sampling were performed at 20, 30, and 40 min perfusion (there was an inconsistency in the amount of 5-CH_3H_4PteGlu absorbed at 10 min perfusion and for this reason we decided not to sample at this time).

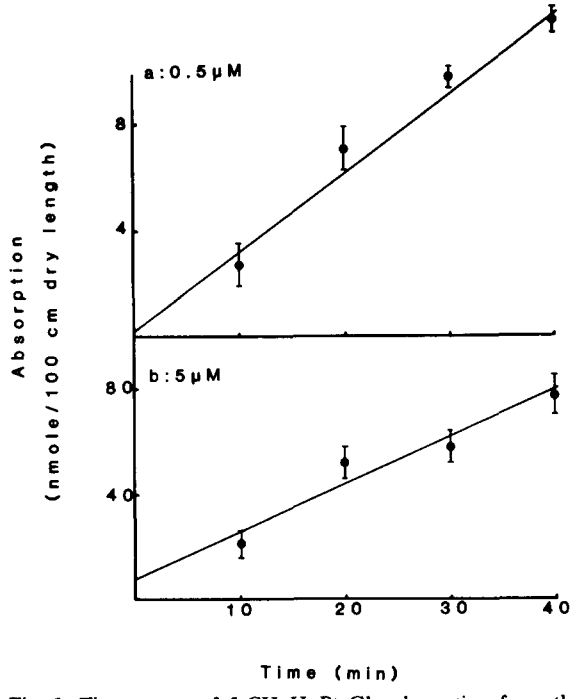

Fig. 1. Time-course of 5-CH_3H_4PteGlu absorption from the lumen of rat jejunum in vivo. Initial concentrations of 5-CH_3H_4PteGlu were (a) 0.5 μM and (b) 5 μM. Experiments were performed using phosphate buffer (pH 6) as perfusate. Flow-rate was 4 ml/min. Linearity of the absorption rate was calculated by means of regression analysis, $Y = mX + b$; r = 0.99 and 0.97, m = 0.31 and 1.74 and b = 0.25 and 8.5, for 0.5 and 5 μM 5-CH_3H_4PteGlu, respectively.

Effect of perfusate flow-rate

The absorption of 0.5 μM 5-CH_3H_4PteGlu was examined at perfusate flow-rates of 0.5, 2 and 4 ml/min [10] (Table I). An increase in the absorption of 5-CH_3H_4PteGlu was observed as the perfusion flow-rate was increased from 0.5 to 2 to 4 ml/min. The increase occurred mainly during the initial period of the perfusion, suggesting an effect of perfusate flow-rate on tissue uptake or binding to the substrate. Despite the increase in the perfusate flow-rate, water absorption remained constant (3–5%) throughout the 40 min of perfusion, indicating that the jejunal surface area did not change at higher perfusion rates [10]. 4 ml/min perfusate flow-rate was therefore chosen to be the standard rate for all subsequent experiments in order to minimize the influence of the unstirred water layer on 5-CH_3H_4PteGlu absorption.

Effect of pH

The absorption of 0.5 μM 5-CH_3H_4PteGlu was examined at different perfusate pH (Table II). The absorption of 5-CH_3H_4PteGlu was increased as the pH of the perfusate decreased from 7.5 to 5.5. The increase occurred mainly during the initial period of perfusion, suggesting an effect of pH on tissue uptake or binding to the substrate. Changes in perfusate pH were observed during the perfusion period when the pH was higher or lower than 6.0. We therefore decided to use pH 6 in all subsequent experiments because of its stability, its good approximation to the physiological pH of the proximal small intestine and because the absorption of 5-CH_3H_4PteGlu is near maximum at this pH.

TABLE I

EFFECT OF THE PERFUSATE FLOW-RATE ON THE ABSORPTION OF 5-CH_3H_4PteGlu

50 cm of the jejunum was perfused for 40 min with phosphate buffer (pH 6) containing 0.5 μM 5-CH_3H_4PteGlu. For *P* values, the amount of 5-CH_3H_4PteGlu absorbed after 40 min perfusion at flow-rates of 2 and 4 ml/min was compared to the amount absorbed at 0.5 ml/min using Student's *t*-test.

Perfusion rate (ml/min)	Absorption (nmol/100 cm dry length), after perfusion for			*P*
	20 min	30 min	40 min	
0.5	1.9±0.4	6.1±0.2	6.6±0.3	
2.0	2.8±0.1	7.4±0.8	9.2±0.6	< 0.01
4.0	7.1±0.8	9.8±0.4	12.0±0.5	< 0.01

Effect of concentration

The rate of absorption of 5-CH_3H_4PteGlu as a function of increasing the substrate concentration in the perfusate was examined at pH 6.0 over a range of concentrations from 0.1 to 12 μM (Fig. 2). The rate of absorption (nmol/100 cm dry length per min) at each specific concentration was calculated and plotted against the substrate concentration. The resultant curve conforms well to Michaelis-Menten kinetics with an apparent K_t of 5.7 μM and V_{max} of 3.45 nmol/100 cm dry length per min.

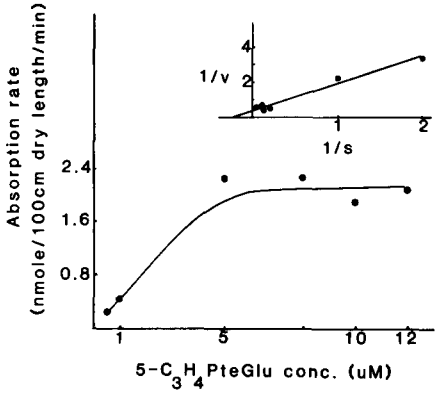

Fig. 2. Effect of concentration on the rate of absorption of 5-CH_3H_4PteGlu at perfusate pH 6.0 and flow-rate of 4 ml/min. The rate of absorption (nmol/100 cm dry length per min) at each concentration was calculated by linear regression analysis and plotted against the substrate concentration. Inset: plot of the reciprocal of the rate of absorption against the reciprocal of substrate concentration ($r = 0.98$, $m = 1.67$, $b = 0.29$).

Effect of structural analogues

The absorption of 5 μM 5-CH_3H_4PteGlu was examined at pH 6 in the presence of the structural analogues PteGlu, 5-$CHOH_4$PteGlu and methotrexate in the perfusate. The amount of 5-

TABLE II

EFFECT OF PERFUSATE pH ON THE ABSORPTION OF 5-CH_3H_4PteGlu

50 cm of jejunum was perfused for 40 min at a rate of 4 ml/min with phosphate buffer containing 0.5 μM 5-CH_3H_4PteGlu. Buffer pH was adjusted using 1 M NaOH and 1 M HCl. For *P* values, the amount of 5-CH_3H_4PteGlu absorbed after 40 min perfusion at different buffer pH values was compared to baseline value at pH 7.5 using Student's *t*-test.

Perfusate pH		Absorption (nmol/100 cm dry length), after perfusion for			*P*
Initial	Final	20 min	30 min	40 min	
7.5±0.01	6.9±0.10	1.6±0.4	3.4±0.5	5.6±0.5	
7.0±0.02	6.6±0.02	3.1±1.5	5.6±1.6	7.2±1.2	> 0.2
6.5±0.01	6.5±0.01	4.3±0.7	6.7±0.5	8.3±0.1	< 0.01
6.0±0.01	6.0±0.01	5.1±0.8	7.1±1.0	10.0±0.8	< 0.01
5.5±0.05	5.6±0.05	6.5±0.5	8.0±0.4	10.1±0.4	< 0.01
5.0±0.01	5.6±0.02	6.3±0.6	7.7±0.7	9.9±0.6	< 0.01

CH_3H_4PteGlu absorbed after 40 min perfusion was significantly less in the presence of an analogue than in its absence (control). Values of 77.0 ± 8.0, 13.0 ± 3.0 ($P < 0.01$), 17.1 ± 2.0 ($P < 0.01$) and 32.4 ± 2.0 ($P < 0.01$) nmol/100 cm dry length per 40 min were recorded for the untreated control and after the addition to the perfusate of 50 μM PteGlu, 5-$CHOH_4$PteGlu and methotrexate, respectively.

Effect of glucose

Glucose (20 mM) was found to be necessary for the transport of 5-CH_3H_4PteGlu in vitro [4]. Its removal from the incubation medium of rat jejunal everted sacs decreased the amount of 5-CH_3H_4PteGlu transported to 56% of the control value. However, our in vivo experiments showed no difference in the absorption rate of 5-CH_3H_4PteGlu (0.5 μM) in the presence (12.1 ± 1.4 nmol/100 cm dry length per 40 min) and absence (12.0 ± 0.5 nmol/100 cm dry length per 40 min) of glucose.

Effect of bile acids and rose bengal

Our previous in vitro studies using rat jejunal everted sacs have shown that unconjugated bile acid; cholic and deoxycholic acids inhibit the mucosal-to-serosal transport and tissue retention of 5-CH_3H_4PteGlu, while the conjugated bile acid taurocholate had no effect [11,12]. In this study, we reexamined the effect of cholic and deoxycholic acids and the effect of the organic anion rose bengal, which was shown to inhibit the transport of 5-CH_3H_4PteGlu in rat isolated hepatocytes [13], on the absorption of 0.5 μM 5-CH_3H_4PteGlu in vivo using the recycling perfusion technique described in Materials and Methods. Phosphate buffer (pH 6.1) was used as the basic perfusate. The results (Table III) showed that cholic acid has no effect and 1 mM concentrations, but decreased the absorption of 0.5 μM 5-CH_3H_4PteGlu to 44% of the control value when added at 5 mM concentration. Deoxycholic acid showed no effect at 0.1 mM concentration but depressed the absorption of 0.5 μM 5-CH_3H_4PteGlu to 43% of the control value when added at 1 mM concentration. Rose bengal (0.1 mM) also caused a decrease in the absorption of 0.5 μM 5-CH_3H_4PteGlu to 45% of the control value.

To examine the reversibility of the inhibitory effect of bile acids on the absorption of 5-CH_3H_4PteGlu, we pre-exposed the jejunum to 5 mM cholic acid and then examined the absorption of 0.5 μM 5-CH_3H_4PteGlu in the absence of cholic acid. A 50-cm segment of the rat jejunum was perfused for 40 min with phosphate buffer containing 5 mM cholic acid. The intestine was then flushed by a single pass of 60 ml phosphate buffer to wash out cholic acid solution and then flushed with air to minimize residual fluid. Perfusion of 0.5 μM 5-CH_3H_4PteGlu solution was then performed for 40 min. Control experiments were performed in a similar way except that cholic acid was not added. The results obtained showed that only 14% inhibition in the absorption of 0.5 μM 5-CH_3H_4PteGlu occurred when the intestine was pre-treated with 5 mM cholic acid compared to 56% inhibition observed when 5 mM cholic acid was added concomitantly with 0.5 μM 5-CH_3H_4PteGlu (Table III).

TABLE III

EFFECT OF THE UNCONJUGATED CHOLIC AND DEOXYCHOLIC ACIDS AND ROSE BENGAL ON THE ABSORPTION OF 5-CH_3H_4PteGlu

50 cm of the jejunum was perfused at a rate of 4 ml/min for 40 min with phosphate buffer (pH 6.1) containing 0.5 μM 5-CH_3H_4PteGlu and the compound under investigation. For *P* values, comparisons were made relative to control using Student's *t*-test.

Bile acid	Concn. (mM)	Absorption (nmol/100 cm dry length per 40 min)	*P*
Control		10.3 ± 1.3	
Cholic acid	0.1	10.4 ± 0.2	
	1.0	10.7 ± 0.4	
	5.0	4.5 ± 0.6	< 0.01
Deoxycholic acid	0.1	11.0 ± 1.1	
	1.0	4.4 ± 0.5	< 0.01
Rose bengal	0.1	4.6 ± 1.3	< 0.01

Discussion

The absorption of the naturally occurring folate derivative 5-CH_3H_4PteGlu was studied in the unanesthetized rat. The absorption rates of physio-

logical and pharmacological concentrations of 5-CH_3H_4PteGlu were constant with time up to 40 min of perfusion. The absorption of physiological concentrations of 5-CH_3H_4PteGlu (0.5 μM) was increased as the perfusate flow-rate increased from 0.5 to 2 to 4 ml/min. The increase in the absorption of 5-CH_3H_4PteGlu is not due to changes in the small-intestinal surface area, since net fluid absorption remained constant (3–5%) throughout the experiment despite the increase in the perfusate flow-rate (0.5–4 ml/min) (see also Ref. 10). It is clear from previous work [9,14–16] that the unstirred water layer at the luminal surface of the intestinal mucosa acts as a diffusional barrier for the movement of lipid- and water-soluble compounds from the bulk luminal phase to the brush-border membrane of the absorptive cell. The influence of the unstirred water layer is particularly significant at low concentrations of these compounds [15,16]. Decreasing the unstirred water layer resistance and increasing its surface area leads to an increase in the absorption of lipid- and water-soluble compounds [7,14–16]. In studies performed in this laboratory, it has been observed that increasing the perfusate flow-rate from 0.5 to 2 to 4 ml/min is associated with a gradual decrease in the unstirred water layer thickness and an increase in its surface area [17]. In the present experiments with 5-CH_3H_4PteGlu, more molecules of the substrate can reach the absorptive membrane of the microvilli at higher flow-rates, which could be due to a decrease in the unstirred water layer resistance and an increase in its surface area and due to an increase in the amount of 5-CH_3H_4PteGlu per unit surface area of the mucosa per unit time, and would therefore explain the increased absorption of the substrate observed (Table I).

The transport system of 5-CH_3H_4PteGlu in vitro is pH-dependent [4–6]. Our in vivo studies confirm this and suggest that an increase in luminal pH leads to folate malabsorption. Under normal physiological conditions, the intraluminal pH in the jejunum (the site of maximal folate absorption) is higher (pH 6.5–7.0) than the optimal pH for maximal absorption of the vitamin. However, the existence of the intestinal surface acid microclimate at the luminal surface of the enterocytes which has a pH of 5.5–5.8 as measured directly in the jejunum of the rat in vitro and in vivo and in man in vitro [5,18–21] provides the acidic environment for maximal absorption of folates.

The saturation kinetics observed in the absorption of 5-CH_3H_4PteGlu (Fig. 2) and the inhibitory effect of the oxidized, reduced and substituted structural analogues (Table III) suggest that a carrier-mediated system is involved in the absorption of 5-CH_3H_4PteGlu in vivo and confirm the previously reported in vitro observations [4]. Since the transport of 5-CH_3H_4PteGlu in vitro is energy dependent and occurs against a concentration gradient [4], it seems reasonable to suggest that the intestinal transport of 5-CH_3H_4PteGlu in vivo and in vitro occurs by means of an active, carrier-mediated process.

Compared to the in vitro situation [4], glucose in the perfusate was found to be unnecessary for the absorption of 5-CH_3H_4PteGlu in vivo. This finding indicates that sufficient glucose and/or other energy providing substrates are being delivered to the enterocytes from the intact circulation to supply energy for the absorptive process.

Conjugated and unconjugated bile acids are normal constituents of the intestinal lumen. In prior studies, we have shown that unconjugated but not conjugated bile acids are potent inhibitors of the transport of 5-CH_3H_4PteGlu in the rat jejunal everted sacs [11,12]. In this study, we reexamined the effect of the unconjugated cholic and deoxycholic acids and of the organic anion rose bengal on the intestinal absorption of physiological concentrations of 5-CH_3H_4PteGlu in the unanesthetized rat. We found that cholic and deoxycholic acids and rose bengal inhibit the absorption of 5-CH_3H_4PteGlu to different extents. The inhibition of 5-CH_3H_4PteGlu by these organic anions is not due to damage to the intestinal mucosa. This inhibitory effect is reversible following the removal of cholic acid from the perfusate. Moreover, bile acids at the concentrations used, cause no damage to the intestinal mucosa in vivo [22]. The reasons for the inhibition of 5-CH_3H_4PteGlu absorption by bile acids are not clear but may represent a competitive type of inhibition [11,12].

Under normal physiological conditions, bile acids in the proximal small intestine are in the conjugated forms [23]. Hence, folate absorption

proceeds unaffected [11,12]. However, in certain disease states associated with bacterial overgrowth of the small intestine, deconjugation and reduction of trihydroxy bile acids can occur leading to an increased concentration of unconjugated bile acids [24]. Increased concentration of unconjugated bile acids could lead to folate malabsorption and thus provide a partial explanation for folate malabsorption that is known to occur in diseases such as Crohn's disease, tropical sprue and amyloidosis [25]. Folate malabsorption and deficiency could theoretically occur following the long-term oral ingestion of unconjugated bile acids frequently prescribed for the dissolution of gallbladder stones [26].

In summary, the present in vivo studies confirm previous in vitro observations regarding the intestinal transport of 5-CH_3H_4PteGlu and show that the transport process is partly controlled by the unstirred water layer.

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