(containing factor VIII, which is probably the active principle concerned—Forbes and Prentice, 1973; Donati *et al.*, 1973) fails to aggregate the platelets of patients with the giant platelet syndrome, it aggregates von Willebrand's disease platelets normally in their own plasma—perhaps because it also contains the von Willebrand factor.

The exact nature of this platelet-plasma interaction and its place in the haemostatic mechanism—that is, the physiological stimulus for which ristocetin substitutes in vitro—have yet to be discovered. For the present, our findings merely serve to emphasize that it is as necessary for normal haemostasis as is ADP-mediated aggregation.

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# Formylation of Folate as Step in Physiological Folate Absorption

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## Summary

**Oral administration of folate analogues to rats is followed by a rise in plasma folate detectable only by micro-biological assay with Lactobacillus casei, suggesting methylation of folate during absorption. When using the everted rat gut technique with folate (PteGlu), dihydrofolate ($H_2$PteGlu), or tetrahydrofolate ($H_4$PteGlu) on the mucosal surface formyl folate (10-CHO-PteGlu, 10-CHO-$H_4$PteGlu, 5-CHO-$H_4$PteGlu) and methylfolate (5-$CH_3$-$H_4$PteGlu) are recovered from the serosal fluid. This indicates that absorption of PteGlu is followed by formylation and that the formyl group is further reduced to methyl, 5-$CH_3$-$H_4$PteGlu passing on to portal blood.**

## Introduction

Oral doses of reduced forms of folate monoglutamates such as dihydropteroylglutamic acid ($H_2$PteGlu), and tetrahydropteroylglutamic acid ($H_4$PteGlu) in man are followed by a rise in plasma methyltetrahydrofolate (5-$CH_3$-$H_4$PteGlu) (Baker *et al.*, 1965; Cohen, 1965; Chanarin and Perry, 1969; Perry and Chanarin, 1970; Pratt and Cooper, 1971; Whitehead *et al.*, 1972; Nixon and Bertino, 1972). In man, reduction and methylation of folate occurs in the small gut (Chanarin and Perry, 1969; Perry and Chanarin, 1970). Olinger *et al.* (1972), using isolated rat gut, reported that $^3$H-PteGlu was converted to methyltetrahydro folate and that this conversion was interfered with by methotrexate.

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In this paper we report evidence that folate is formylated as a first step and that methylation presumably arises from reduction of the formyl group.

## Materials and Methods

Pteroylglutamic acid labelled with tritium on the 3rd and 5th positions of the para-aminobenzoyl ring (Amersham) was used. $H_2$PteGlu and $H_4$PteGlu were prepared from $^3$H-folate by reduction with ascorbate and dithionite (Blakley, 1960; Silverman and Noronha, 1961).

5-Formyl-tetrahydrofolate (Lederle) was generally labelled with tritium by an exchange with tritiated water in the presence of a platinum catalyst (Amersham) and purified by chromatography on A25 DEAE-Sephadex followed by G-15 Sephadex (Nixon and Bertino, 1971). Tritium-labelled 10-formyl-$H_4$PteGlu and 5-$CH_3$-$H_4$PteGlu were prepared from the 5-formyl compound (Rabinowitz, 1963; Chanarin and Perry, 1967).

Folate analogues were identified by chromatography on A25 DEAE-Sephadex using a 0·9 by 27 cm column and elution by a gradient system consisting of 1·0 mol/l. phosphate buffer in the reservoir and 0·1 mol/l. phosphate buffer in the mixing chamber. Both buffers were pH 6·0 with 0·1 mol/l. 2-mercaptoethanol. Five-millilitre fractions were collected, radioactivity was counted, and microbiological activity determined using *Lactobacillus casei*, *Streptococcus faecalis*, and *Pediococcus cerevisiae*. Marker compounds as prepared above were used for further identification of folate analogues. Radioactivity was counted on a Wallac liquid scintillation counter.

Male Sprague-Dawley rats weighing 250 g were used to prepare everted sacs (Wilson and Wiseman, 1954). Three 15-cm segments, starting from the pyloric end of the gut, were prepared from each animal. One millilitre of Krebs Ringer bicarbonate solution pH 6·1 was introduced into each sac and the sacs were incubated in 25 ml Krebs Ringer bicarbonate containing glucose 28 mmol/l. The folate compounds under test were added to the mucosal fluid at a concentration of 10 nmols, and the sacs exposed to $O_2$-$CO_2$ (95/5%) for three minutes and incubated for one hour at 37°C. At the end of this period ascorbate (pH 6·0) was added to both serosal and mucosal fluids to give a 1% concentration. Folate analogues in these fluids were separated by chromatography and identified.

## Results

When 5 $\mu$g $H_2$PteGlu were given orally to three rats there was

an increase in serum *L. casei* activity from 15 ng/ml to 40 ng/ml after one hour. There was no detectable increase in the activity on *Str. faecalis* assay. This suggests that $H_2$PteGlu has been converted to 5-$CH_3$-$H_4$PteGlu by the intact animal during intestinal absorption.

The result of an experiment using everted sacs of rat gut wherein PteGlu was added to the mucosal fluid is shown in fig. 1. The serosal fluid contained three folate analogues—

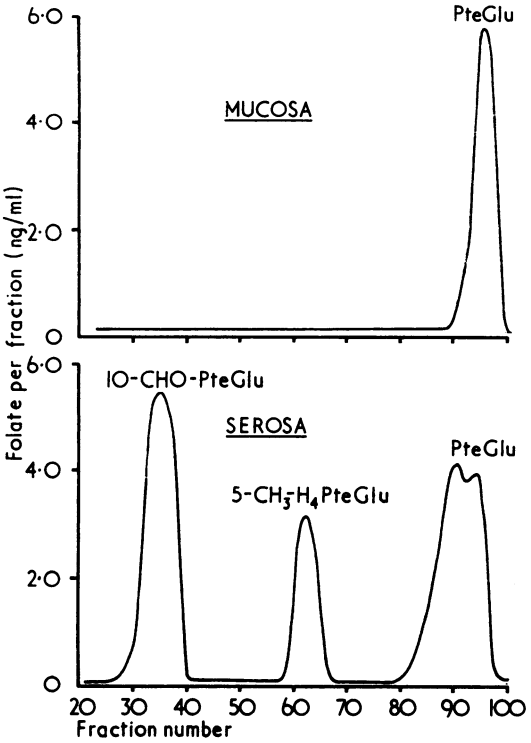


FIG. 1—5 μg PteGlu were placed on the mucosal side of an everted rat small gut preparation. After one hour mucosal and serosal fluids were collected and folate analogues identified (see text). Only PteGlu was present on the mucosal surface but after passage through the gut 10-CHO-PteGlu and 5-$CH^3$-$H_4$-PteGlu were found.

namely, PteGlu, 5-$CH_3$-$H_4$PteGlu, and 10-CHO-PteGlu. The latter was active with *L. casei* and *Str. faecalis* but not with *P. cerevisiae*. Folate peaks corresponded to peaks of tritium activity indicating derivation from the labelled mucosal folate.

A study with $H_2$PteGlu added to the mucosal fluid is shown in fig. 2. 5-$CH_3$-$H_4$PteGlu and 10-CHO-folates were present in the serosal fluid. In addition to 10-CHO-$H_4$PteGlu some 5-CHO-$H_4$PteGlu was present. Similar results were obtained when $H_4$PteGlu was added to the mucosal fluid.

## Discussion

Our results differ from those of Olinger *et al.* (1972) in that in addition to methyltetrahydrofolate we found various formyl analogues of folate. Tetrahydrofolate, in fact, does not accept a methyl group in toto but only single carbon units at the formaldehyde or formate state of reduction (Blakley, 1969). The accumulation of formyl derivatives as well as methyltetrahydrofolate in the serosal fluid of the everted sac as opposed to the intact animal is probably due to a negative feedback mechanism following a pile up of the methyl-form in the gut intestinal cells. Under normal circumstances the formyl group is further reduced to methyl. Butterworth (1973) recently reported the appearance of formylfolate in absorption studies in dogs.

With PteGlu as substrate 10-CHO-PteGlu appeared in the serosal fluid indicating that formylation can occur in the absence of reduction. Recently polyglutamate formation has been found

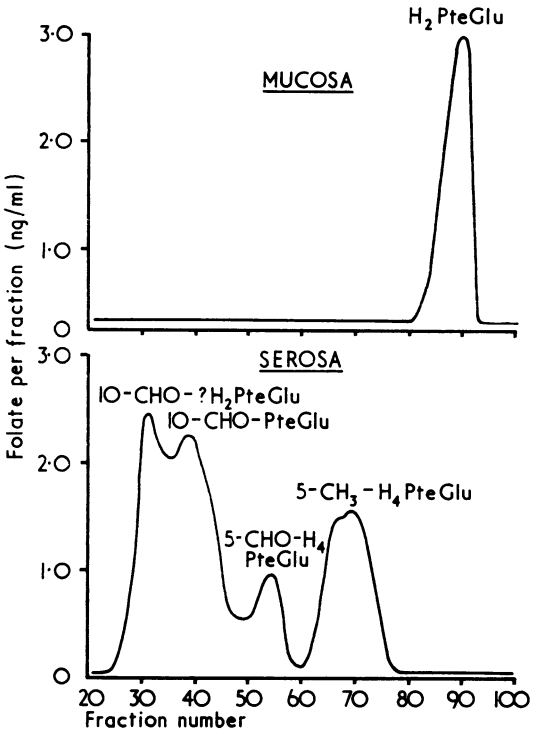


FIG. 2—5 μg $H_2$PteGlu were placed on the mucosal side of an everted rat small gut preparation. After one hour mucosal and serosal fluids were collected and folate analogues identified (see text). Only $H_2$PteGlu was present on the mucosal surface but after passage through the gut both 10 and 5-CHO-derivatives were present as well as 5-$CH_3$-$H_4$PteGlu.

in the liver of methotrexate-treated guinea pigs in the absence of reduction of the pteridine ring (Corrocher and Hoffbrand, 1972).

We are uncertain as to the significance of 5-formyl-tetrahydrofolate. This could arise as an artefact by conversion from the 10-formyl. Though oral 5-formyltetrahydrofolate gives rise to plasma 5-methyl-tetrahydrofolate (Chanarin and Perry, 1969; Perry and Chanarin, 1970; Pratt and Cooper, 1971; Nixon and Bertino, 1972), the latter reported that the 5-formyl group was removed and replaced by a formyl from another source. Should it be a physiological intermediary it would then be processed as described by Nixon and Bertino (1972).

We wish to thank Lederle Laboratories for the supply of folinic acid.

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