Factors influencing the release of assayable folate from erythrocytes

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SYNOPSIS The effects of pH, ascorbic acid, temperature, and time on folate release from whole blood haemolysates were investigated. Maximal yields of assayable folate were obtained within 10 minutes at room temperature in whole blood haemolysates prepared at pH 3.6 in aqueous ascorbic acid solution. When prepared over a period of two hours, haemolysates yielded maximal folate values within a much wider pH range (3.0 to 6.0). Released folate is shown to be extremely labile between pH 4.7 and 3.0 but the presence of ascorbic acid in a concentration of 25 mg% or more afforded complete protection. The possible nature of the plasma folate-releasing factor and the significance of these findings in relation to the laboratory estimation of whole blood folate are discussed.

Wolff, Drouet, and Karlin (1949) showed that human plasma contains a factor that is capable of releasing assayable folate from extracts of yeast incubated at 37°C and at pH 4.5. Toennies, Usdin, and Phillips (1956) demonstrated the importance of this factor for the release of the *L. casei* active material from erythrocytes. These workers found that the folate yield from erythrocytes is markedly enhanced by the incubation of whole blood at 37°C in phosphate buffer pH 6.0 to 6.1 before the precipitation of proteins by autoclaving. These conditions for 'maximal' folate release have since been adopted by most workers when estimating whole blood folate (Hansen and Nyström, 1961; Grossowicz, Mandelbaum-Shavit, Davidoff, and Aronovitch, 1962; Cooper and Lowenstein, 1964; Grzesiukowicz, Jennison, and Gowenlock, 1965). Recently, however, Hoffbrand, Newcombe, and Mollin (1966) found that incubation at 37°C does not increase the folate content of whole blood haemolysates when prepared in 1 g% ascorbic acid solution. Under these conditions the 'maximal' amount of folate is released at pH 3.6 to 3.8. This is at variance with the original observations of Toennies, Frank, and Gallant (1953) who found that the folate yield fell almost to zero at pH 4.0 and was maximal within a narrow pH range around 5.7, and so led Toennies *et al* in later work (1956) to consider pH 6.0 as optimal for plasma factor action.

In view of these contradictory data, experiments were carried out to investigate some of the factors affecting the release of assayable folate from erythrocytes. This paper reports on the influence of ascorbic acid, pH, and temperature on such release.

MATERIALS AND METHODS

The study was performed on blood samples obtained from healthy subjects with a normal peripheral blood picture.

Blood from each subject, 40 to 60 ml, was transferred into sterile heparinized Universal containers, and the plasma was separated by immediate centrifugation for half an hour at 1,000 *g*. Five ml of the plasma to be used for plasma folate estimation was stored at -20°C after the addition of 10 mg of dry ascorbic acid per ml. The remainder was recombined with enough of the red cells to give a packed cell volume (PCV) of 30% ± 2% in all experiments. Any subsequent dilutions were carried out with deionized water.

Haemolysis of whole blood in water or ascorbic acid solution for 10 minutes at room temperature, followed by freezing for one hour at -20°C and thawing for a further hour at room temperature, was adopted as a standard procedure for releasing assayable folate from erythrocytes in experiments 1, 2, and 3. Haemolysis at particular pH values was achieved by adding appropriate quantities of N/HCl or N/NaOH to the haemolysing fluids before adding blood.

The folate content of whole blood was extracted by combining 2 ml aliquots of haemolysates with 18 ml of 0.1 M phosphate buffer (pH 6.0 to 6.1) with ascorbic acid at a final concentration of 200 mg% and precipitating the proteins by autoclaving at 15 lb/sq in. for five minutes.

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Received for publication 2 July 1968.

The clear extracts, separated by centrifugation and filtration, were stored at -20°C until assayed at a final dilution of 1/600 to 1/800.

The *Lactobacillus casei* activity of whole blood and plasma extracts was estimated by the method of Waters and Mollin (1961), except that autoclaved ascorbate phosphate buffer was added to standard tubes.

CALCULATION OF RESULTS Whole blood folate values were calculated in $\mu\text{g}/\text{ml}$ of packed cells, using the formula:

$$\text{Whole blood folate} - \text{plasma folate} \times \left(1 - \frac{\text{PCV}}{100}\right) \frac{\text{PCV}}{100}$$

Comparisons of the amounts of folate released under differing conditions were sometimes carried out on the same blood specimen (experiments 1, 2, and 4). To facilitate analysis of the results, blood folate values in $\mu\text{g}/\text{ml}$ of packed cells were expressed in these experiments as percentages of the arbitrary optimum folate yield. This is defined as the amount of folate released, in $\mu\text{g}/\text{ml}$ of packed cells, when blood is haemolysed at a dilution of 1:10 in 1 g% aqueous ascorbic acid solution and the haemolysate is incubated for 90 minutes at 37°C . Such a haemolysate was therefore prepared from each blood specimen investigated in each experiment.

The pH determinations were carried out with a Beckmann model 72 pH meter.

RESULTS

EFFECT OF HAEMOLYSATE ASCORBIC ACID CONCENTRATION ON FOLATE RELEASE This was studied by preparing serial haemolysates from each of nine blood specimens at dilutions of 1:5 and 1:10 in aqueous ascorbic acid solutions containing 0 to 1 g%. The results are shown in Table I which relates the mean haemolysate pH value and the mean percentage folate yield to the corresponding ascorbic acid concentration. Table I shows that at both

dilutions there is an increasing folate yield with increasing ascorbic acid concentration. The increase in folate yield is related to the pH produced by added ascorbic acid and not to its concentration, i.e., at similar pH values, folate yield is similar irrespective of ascorbic acid concentration. The highest folate values are observed at pH 5.8 or less.

EFFECT OF pH ON FOLATE RELEASE Figure 1 relates yield to pH in a series of haemolysates prepared from five blood specimens in water containing no ascorbic acid. There is nearly 100% folate yield over the pH range of 4.8 to 6.0, almost no assayable folate at pH 3.0, and at pH 9.0 the folate yield ranges from 25% to 39%.

Figure 2 illustrates the relationship of folate yield to pH when haemolysis was carried out in 25 mg% aqueous ascorbic acid solutions using the same blood specimens as above. The pH for maximal folate release is now 3.0 to 6.0.

pH STABILITY OF RELEASED FOLATE Figure 3 shows the recovery of assayable folate at different pH values. In this experiment a large volume of whole blood haemolysate was prepared without ascorbic acid at pH 5.2 and subjected to the standard procedures for 'maximal' folate release. Aliquots of the haemolysate were then readjusted within pH range 3.0 to 11.0. In one aliquot 25 mg% ascorbic acid was added before reducing the pH to 3.5. Folate recovery was expressed as a percentage of the folate content of the original haemolysate. Figure 3 shows that folate recovery falls at pH values below 5.2 unless 25 mg% ascorbic acid is added to haemolysates before the pH reduction. This extreme lability of assayable folate and its complete protection by ascorbic acid explains the different pH ranges observed for maximal folate release in Figures 1 and 2.

TABLE I

EFFECT OF ASCORBIC ACID CONCENTRATION ON HAEMOLYSATE pH AND PERCENTAGE FOLATE YIELD¹

Blood Dilution 1:5				Blood Dilution 1:10			
Haemolysate Ascorbic Acid Concentration (mg %)	Mean Haemolysate pH	Mean Folate Yield (%)	SD $\pm$	Haemolysate Ascorbic Acid Concentration (mg %)	Mean Haemolysate pH	Mean Folate Yield (%)	SD $\pm$
0	7.47	47	8.8	0	7.29	40	12.0
20	7.35	47	9.2	22.5	6.87 ^a	61 ^a	10.0
40	7.25	53	8.7	45.0	6.63	75	6.0
80	6.9 ^a	63 ^a	5.6	90.0	5.83 ^a	95 ^a	3.6
200	5.84 ^a	95 ^a	4.3	225.0	4.48	95	5.8
400	4.73	98	3.4	450.0	4.13	97	4.0
600	4.41	97	2.9	695.0	3.9	99	5.2
800	4.27	98	1.9	900.0	3.67	98	4.2

¹The pH and folate values represent the means of results obtained for nine blood specimens at dilutions of 1/5 and 1/10.

² and ^a = similar folate values occurring at similar pH values irrespective of ascorbic acid concentration or blood dilution.

FIG. 1. Folate release related to pH in haemolysates prepared without ascorbic acid.

FIG. 2. Folate release related to pH in haemolysates prepared in 25 mg% aqueous ascorbic acid solution.

FIG. 3. The percentage recovery of assayable folate determined in the absence of ascorbic acid at various pH values and at pH 3.6 in the presence of 25 mg% ascorbic acid.

RATE OF FOLATE RELEASE AT DIFFERENT pH VALUES
Folate yield was determined in subsamples removed over a period of 30 minutes from haemolysates prepared at room temperature and at pH 3.6 and 5.8 respectively. The results are shown in Table II. These show that 'maximal' levels of assayable folate are obtained within 10 minutes from the initiation of haemolysis at pH 3.6. In contrast folate yield at pH 5.8 is still suboptimal at 30 minutes. The degree of haemolysis was tested for and found to be complete (100%) under both conditions within 10 minutes from the dilution of blood.

FOLATE RELEASE AFTER FREEZING AND THAWING COMPARED TO THAT AT 4°C Table III compares the

TABLE II
RATE OF FOLATE RELEASE AT ROOM TEMPERATURE IN ASCORBIC ACID CONTAINING HAEMOLYSATES PREPARED FROM TWO BLOOD SAMPLES

Blood Specimen				
No. 1		No. 2		
pH		pH		
3.6	5.8	3.6	5.8	
Incubation Time at Room Temperature (min)				
Percentage Folate Release				
5	52	34	62	25
10	99	47	102	31
15	97	66	95	38
30	100	78	96	47

mean folate values of three blood specimens prepared by the two methods at different pH values. At four pH values, haemolysates prepared at 4°C gave lower folate values but the differences are not significant for the number of specimens investigated ($P < 0.4 > 0.3$). For haemolysates prepared by freezing and thawing, the mean folate yield between pH 5.6 and 3.6 ranges from 92% to 100% which agrees with the findings in Figure 2. All folate values are expressed as percentages of the arbitrary optimal yield (folate release in haemolysates prepared in a g% ascorbic acid solution and incubated for 90 minutes at 37°C).

DISCUSSION

Toennies *et al* (1953, 1956) showed that the bulk of folate resides inside erythrocytes as microbiologically inactive precursors. They demonstrated the importance of a plasma 'enzyme' for the release of *L. casei*-

TABLE III

MEAN PERCENTAGE FOLATE YIELD IN THREE BLOOD SPECIMENS AFTER INCUBATION AT 4°C FOR TWO HOURS COMPARED AT DIFFERENT pH VALUES TO THAT OBTAINED AFTER FREEZING AND THAWING

	Haemolysate pH						
	7.0	6.6	6.2	5.6	4.7	4.1	3.6
Mean folate release after freezing and thawing (%)	22.0	49.0	66.0	92.0	94.0	95.0	100.0
SD ±	12.0	9.5	2.6	5.2	6.0	4.7	1.0
Mean folate release at 4°C (%)	22.0	49.0	60.0	88.0	88.0	91.0	97.0
SD ±	10.7	10.0	4.8	3.6	8.0	6.0	1.6
P				< 0.4 > 0.3	< 0.4 > 0.3		

active material from these precursors and found that such a release was best attained around pH 6.0 and a temperature of 37°C. The present findings, however, differ in many respects from those reported by Toennies and his colleagues, as regards conditions governing maximal folate yield in whole blood haemolysates.

Thus when whole blood haemolysates are prepared by the method described in aqueous solutions containing 25 mg% or more ascorbic acid, they yield their 'maximal' folate values within a wide pH range, namely, 3.0 to 6.0. It is demonstrated that ascorbic acid makes no significant difference in folate yield between pH 4.8 and 6.0. Within pH 4.7 to 3.0, however, released folate becomes progressively unassayable unless ascorbic acid is present in haemolysates to exert a protective effect. This extreme lability of the *L. casei*-active material at low pH and its complete protection by ascorbic acid has not been demonstrated previously, and partly explains why Toennies *et al* (1953), who did not include ascorbic acid in their experiments, found that the folate yield approached zero at pH 4.0 and was maximal around a much narrower pH range compared with that described in the present study.

Although the 'maximal' folate yield is between pH 3.0 and 6.0 in haemolysates prepared over two hours by the method described, folate, when timed at shorter intervals, was found to be released much more rapidly at the lower side of this pH range. Thus in two blood specimens investigated, the amounts released after 10 minutes at room temperature were 99%, and 102% of the arbitrary optimal yield at pH 3.6 compared with 47% and 31% respectively at pH 5.8 (Table II). A fall in pH alone, in the absence of plasma, was not associated with the release of assayable folate from erythrocytes. This means that the low pH enhances folate yield by potentiating the 'enzymatic' action of plasma on the erythrocyte folate precursor which in turn indicates that low pH values, *ie*, in the region of 3.6, are better for plasma factor action than those around 5.8. Folate is present inside the erythrocyte as conjugates of N⁵-methyltetrahydrofolic acid (Noronha and Aboobaker, 1963) which are bound to an intracellular protein (Iwai,

Luttner, and Toennies, 1964). It is possible that the fall in pH facilitates folate yield by breaking or denaturing the binding sites of the substrate inside the cell and so exposing it to enzymatic action. The enzyme involved has to be a plasma 'peptidase' capable of hydrolysing glutamyl peptide bonds and exhibiting optimal activity at a low pH. Such enzymes are usually present in biological material as precursors requiring activation before they can function. A notable example of this is the autocatalytic conversion of pepsinogen to pepsin below pH 5.0. Pepsinogen is known to occur in human plasma. Pepsin is generally recognized to have an optimum pH around 2.0. However, depending on the nature of peptide bonds of the substrate, its optimum pH may be higher (Bovey and Yanari, 1960). Investigations are being carried out with special reference as to whether plasma pepsinogen is involved in the release of assayable folate at a low pH.

Enzyme activity is recognized to extend over a limited pH range. In view of the fact that under the present experimental conditions the 'maximal' folate is yielded over a wide pH range (3.0 to 6.0), and also because of the demonstrated variable rate of folate release at two pH points within this range, the possibility of more than one plasma 'enzyme', each with different kinetics and pH requirements, cannot be excluded. The multiplicity of the plasma factor had already been suggested by Toennies and Phillips (1959) but their results were based on folate yield in incubation mixtures at pH 6.0. The elucidation of this problem depends upon purer forms of the substrate and enzymes to investigate the kinetics at different values for pH.

It seems that the enzymatic release of assayable folate from erythrocytes is predominantly pH dependent and that changes in temperature have no significant effect on folate yield over the period of two hours adopted. Thus in all experiments in the present work folate yield has been assessed without incubating haemolysates at 37°C but the results have been related as percentages to folate release from haemolysates prepared in 1 g% ascorbic acid solution incubated at 37°C for 90 minutes (the arbitrary

optimal yield). The occurrence, therefore, at a favourable pH of 'maximal' folate yields in these experiments indicates that a temperature of 37°C is not important for plasma factor action over the time adopted. Even when haemolysates are prepared at 4°C, the folate yield at any particular pH value is not significantly different from that obtained by freezing and thawing. There is no proof in the present work that the mechanical disruption of red cells by freezing and thawing is important for folate release. This procedure was adopted in preference to others, however, to ensure adequate haemolysis (an important requirement for the availability of substrate) under various conditions, *eg*, when using packed cells or preparing haemolysates at a high pH.

The finding in the present study of rapid 'maximal' folate yield (within 10 minutes) at room temperature in haemolysates prepared in ascorbic acid solutions at a final pH of 3.6 provides a convenient method for the laboratory estimation of whole blood folate in clinical material. Thus folate release preparations are simple to set up, and the method dispenses with the 90 minutes' incubation periods adopted in other methods. These recommended conditions are provided by using the method of Hoffbrand *et al* (1966) who prepare haemolysates in 1 g% aqueous ascorbic acid solution. When viewed in the light of the present results it will be seen that ascorbic acid at such a concentration, in addition to protecting released folate, drops the haemolysate pH to the desired value of 3.6 (see Table I). Further, these authors have shown that the folate content of such haemolysates was not affected by storage for up to five

months at -20°C. This method is, therefore, strongly recommended for the laboratory estimation of whole blood folate.

It is a pleasure to thank Professor R. H. Girdwood of the Department of Therapeutics, University of Edinburgh, and Dr S. H. Davies, consultant haematologist, South Eastern Regional Hospital Board (Scotland), for their indispensable help during the course of this work. I am very grateful also to Dr N. D. C. Finlayson and Dr D. J. C. Shearman of the Department of Therapeutics, and Dr N. C. Allan, consultant haematologist, Western General Hospital, Edinburgh, for their criticism and help in writing this paper.

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