Erythrocyte folate analysis: a cause for concern?

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Neural tube defects can be prevented by adequate intake of periconceptional folate, and inverse associations between folate status and cardiovascular disease and various cancers have been noted. Thus, there is renewed interest in the analysis of red cell folate (RCF) as an indicator of folate deficiency risk. Assessment of the assumptions that underpin RCF assays indicates that many are false. Published literature suggests that increased deoxy-hemoglobin (which can bind RCF electrostatically) yields more assayable folate, and increased oxy-hemoglobin (which cannot bind RCF) yields less assayable folate. It is argued that as deoxy-hemoglobin picks up oxygen and switches quaternary structure, any bound folate must, on purely theoretical grounds, become physically “trapped”. Venous blood taken for analysis is 65% to 75% saturated with oxygen, and pro-rata “trapping” will lead to serious underestimation of RCF. Hence, doubt is cast over the validity of all previous RCF values. Some strategies for accurately assessing RCF are suggested.

Spina bifida and related neural tube defects of anencephaly and encephalocele are major causes of perinatal, infant, and childhood mortality and morbidity. Although screening (maternal serum a-fetoprotein estimation or fetal ultrasound examination) has led to a fall in the number of children born with neural tube defects through termination of pregnancy (1, 2), it cannot prevent the development of these defects or the associated parental distress.

There is now firm evidence that the majority of neural tube defects can be prevented by an adequate intake of periconceptional folate, a B-group vitamin found especially in leafy green vegetables, some other vegetables and fruits, and whole-grain cereals. All studies (case-control and cohort studies; nonrandomized and randomized controlled trials) (3) showed a reduction in risk with increased intake of either dietary folates or supplemental folic acid. These studies have led to renewed interest in the analysis of red blood cell folate (RCF) as an indicator of folate deficiency risk in women of child-bearing age. Additional interest in the analysis of RCF as an indicator of folate deficiency risk in the general population is generated by the inverse associations between folate status and the risk of cardiovascular disease (4) and various cancers (5, 6).

Because of the increasing importance of folate nutrition to public health, a “round robin” interlaboratory comparison study was conducted to assess differences among methods (7). This exercise, as others before it (8, 9), demonstrated the large intraassay and interassay variation in whole-blood folate analysis. There are discussions in the literature about method comparisons (8, 10, 11) and possible sources of variability (9, 12–16). However, to date, the influence of the degree of hemoglobin (Hb) oxygenation on the estimation of RCF concentration has not been assessed critically. The degree of Hb oxygenation, however, could be of prime importance with respect to the accuracy and stability of both the microbiological (MA) and radio-folate-binding protein (RFBP) assays. This report questions currently held assumptions relating to RCF analysis and then evaluates critically the potential for gross inaccuracy in RCF analysis resulting from differences in the oxygenation state of Hb as a sample progresses through the analytical procedure.

Current method of RCF analysis

Venous whole-blood samples are transferred immediately to either EDTA or lithium-heparin coated tubes. A subsample is then hemolyzed by 10-fold dilution in a hypotonic aqueous solution [usually a freshly prepared solution of 1% ascorbic acid (AA) (7, 9, 10)]. The remaining blood is sent for general hematological analysis, which includes a hematocrit or packed cell volume. Subsequent to deconjugating the hemolysate folate (generally agreed to be polyglutamates of 5-methyltetrahydrofolic acid;...
5CH$_3$H$_4$-PteGlu$_n$) with plasma folate conjugase ($\gamma$-glu-x carboxypeptidase; EC 3.4.19.9) associated with the "whole-blood" sample, the folate monoglutamate (5CH$_3$-H$_4$-PteGlu) concentration is assessed by either MA or RFBP assays, which generally use folic acid (pteroyl monoglutamic acid; PteGlu), the parent and fully oxidized folate form, as the assay calibrant. The final answer is expressed (using the hematocrit or packed cell volume value of the original sample) as a folate concentration (µg/L or nmol/L) per liter of red blood cells.

ASSUMPTIONS
We make the following assumptions:
1. That 10-fold dilution of a whole-blood sample is sufficient to ensure complete lysis of all red blood cells;
2. That total lysis of all red blood cells will liberate erythrocyte folate completely to unfettered access by plasma folate conjugase and consequently ensure complete RCF deconjugation to the monoglutamate form before analysis by either MA or RFBP assay;
3. That allowing deconjugation to proceed for an excess of time, which would allow assayable folate concentration to plateau, is synonymous with ensuring complete deconjugation;
4. That AA is used in the hemolysate diluent as an "anti-oxidant", which will protect the assay from folate loss, because RCF exists in chemically reduced forms (5CH$_3$-H$_4$-PteGlu$_n$) that can potentially undergo oxidation;
5. That folate analysis is not affected by an acidic hemolysate pH causing perturbation in the pH of the terminal MA or RFBP assay, which would lead to an inappropriately increased/decreased response to deconjugated RCF (5CH$_3$-H$_4$-PteGlu) in comparison with the assay calibrant PteGlu;
6. That the MA or RFBP assay of folate is not affected by RCF binding to any erythrocyte or plasma component; and
7. That RCF assays are not affected by the degree of oxygenation of Hb in the hemolysates.

EVALUATION OF THESE ASSUMPTIONS
1. Red cell membranes enclose high concentrations of impermeant anions, principally Hb (5 mmol/L), 2,3-diphosphoglycerate (DPG; 5 mmol/L), and glutathione (2-3 mmol/L), and any osmotic imbalance creates an intolerable osmotic gradient, allowing the instant entry of water. Erythrocytes, when placed in a hypotonic medium, can swell by 60% to 70% before the critical hemolytic volume is reached, and the stretched membrane acquires transient holes that cause leakage of cellular contents (17). In our experience, visual inspection under a microscope confirms that all erythrocytes are hemolyzed when whole blood is diluted 10-fold with an aqueous hypotonic solution (e.g., 1% AA).
2. Knowing that 10-fold dilution of whole blood will produce total lysis is not synonymous with the assumption that there is complete liberation of RCF to unfettered access by plasma folate conjugase. Such an assumption would be false if there were any evidence for folate being bound to any erythocyte or plasma component (see evaluation of assumption 6).
3. The optimal deconjugation of folyl-polyglutamates with folate conjugase enzyme proceeds as a function of buffer, pH, amount of enzyme, temperature, and reaction time (18). Subsequent to erythrocyte hemolysis in 1% AA, RCF is deconjugated with naturally occurring plasma folate conjugase enzyme, with reaction time being the only variable utilized, at any given temperature, to optimize assayable folate. However, even if assayable folate is optimized, this does not infer that all RCF has been deconjugated. It only infers that all RCF made available to the plasma conjugase enzyme has been deconjugated.
4. The use of AA in hemolysates goes back to the 1950s. After it was observed that protein-free dialysates of whole blood had up to 100 times the microbiological folate activity of the same blood assayed after diluting with water and heating (19), it was further demonstrated that if bloods were diluted with a buffered solution containing AA and then autoclaved to precipitate proteins, folate activity comparable with that after dialysis was obtained in the clear protein-free supernatant solution (20). The addition of AA to the MA does not produce a higher growth response to the assay calibrant (PteGlu) (21), and hence the increase in RCF MA response is not an artifact of an underlying additional need for ascorbic acid to be incorporated into the assay growth medium. Because RCF exists in reduced forms that can potentially undergo oxidation, it has always been assumed that the only role of AA in RCF assays is as an antioxidant. The ubiquitous presentation of this theory has, inevitably, closed minds to the possibility of alternative theories by which the addition of AA could produce an increased MA (or RFBP) response. At this point, it may be pertinent to note that although AA technically has the ability to act as a prooxidant (especially in the presence of metal ions), the addition of AA to hemolysates has always resulted in an increase of assayable folate, thus ruling out the probability of it acting as a prooxidant in the context of erythrocyte folate analysis.

Data from a recent report (22) raise questions about the validity of the antioxidant role, at least over short-term hemolysate storage intervals, and hence compel examination of the merits of alternative theories. MA RCF values have been compared after various periods of hemolysate storage, in whole-blood diluted conventionally ($\times$10) with fresh 1% AA (pH 2.8), and the same blood diluted $\times$10 with 1% AA adjusted to pH 6.0 (AA-pH 6.0). After 1 day of storage, AA (pH 2.8) hemolysates had an assayable folate concentration 65% higher than AA-pH 6.0 hemolysates (22). For such a short period of storage, it can be argued that ascorbate ions would be present in both lysates at sufficient concentrations to exert an antioxidant influence, and therefore, the higher RCF values cannot be attributed to the antioxidant role of AA. Thus, any theory
that attributes an antioxidant role as the only effect of AA (pH 2.8) addition to hemolysates becomes untenable.

Dilution of whole blood with 1% AA (pH 2.8) lowers hemolysate pH to ~3.6. Human plasma folate conjugase (γ-glu-x carboxypeptidase; EC 3.4.19.9) exhibits maximum activity at pH 4.5 but functions across a broad spectrum from about pH 3.5–7.5 (23). Therefore, the addition of AA does not produce a hemolysate pH optimally appropriate for the action of plasma folate conjugase, any more than does dilution of neutral whole blood with water.

5. Both MA and RFBP assay response to deconjugated RCF (5CH$_3$H$_2$PteGlu) is pH dependent (12, 15). If the assay pH is not well chosen, these assays can display a disparate response toward 5CH$_3$H$_2$PteGlu, as opposed to their PteGlu calibrant. In our experience, the assay medium of the MA (at pH 6.2) is not perturbed by the appropriate addition of hemolysates in 1% AA. Commercial RFBP assays used currently are usually well buffered at their optimum pH, and it is unlikely that the addition of a small amount of an acidic hemolysate would compromise these assays. However, slight shifts in pH have been observed during sample processing using some RFBP assays (9), and therefore the onus is on users to verify the pH stability of their chosen kit before use.

6. A major clue as to why RCF values are higher when 1% AA (pH 2.8) is used as the diluent for whole blood is provided by the fact that assayable folate concentration in 1% AA-pH 6.0 lysates, although initially lower, increased during storage (22). This prompted the conclusion that the initial discrepancy was because of folate being bound rather than being destroyed. In reverse, the explanation for obtaining higher RCF values when using 1% AA (pH 2.8) as whole-blood diluent must, therefore, be because it releases at least some folate that would otherwise be bound. Therefore, the assumption that the availability of hemolysate folate for terminal MA or RFBP assay is not affected by folate binding to any erythrocyte or plasma component is false. Furthermore, it is clear from research affected by folate binding to any erythrocyte or plasma hemolysate folate for terminal MA or RFBP assay is not bound. Therefore, the assumption that the availability of

2.8) as whole-blood diluent must, therefore, be because it makes up of irregularly shaped polypeptide chains, do not fit each other precisely; through the center of the Hb molecule, along the axis of heterodimer symmetry (the molecular dyad axis) runs a central cavity that is populated by a variety of polar side chains. The cavity extends for a depth of 5 nm, with entry points at both the top ($\alpha_1\alpha_2$ entrance) and the bottom ($\beta_1\beta_2$ entrance) of the molecule. Its shape in horse oxy-Hb (which is very similar to human oxy-Hb) has been described (31) as most easily represented by two boxes, each ~2 nm long, 0.8 nm wide, and 2.5 nm deep (i.e., one-half the depth of the Hb molecule). One box separates the $\alpha$ chains from each other and the other the $\beta$ chains. The two boxes are set one above the other with their 2-nm-long axes at right angles to the dyad axis and to each other, each box being open at the top and bottom.

Hb is an allosteric protein that undergoes conformational change in quaternary structure from the T (tense) state (deoxy-Hb) to the R (relaxed) state (oxy-Hb) upon binding its first oxygen molecule such that the binding of additional oxygen molecules is enhanced. In other words, oxygen binds cooperatively to Hb. In contrast to myoglobin, which has a hyperbolic oxygen dissociation curve and 50% saturation at 1 torr oxygen pressure, Hb has a sigmoidal dissociation curve and 50% saturation at 26 torr. This lower affinity of Hb for oxygen gives it the latitude to be modified. Increasing concentrations of hydrogen ion and CO$_2$ promote the release of oxygen (the Bohr effect). An important ligand of human Hb is DPG (32). This highly anionic organic phosphate is present in human erythrocytes at approximately the same molar concentration as Hb and lowers profoundly the oxygen
affinity of Hb by cross-linking deoxy-Hb. Hb stripped of DPG loses its sigmoidal oxygen-binding relationship and exhibits an affinity for oxygen similar to myoglobin (29). DPG, with which folyl-polyglutamate can compete (27), binds to deoxy-Hb just inside the entrance to the β1β2 cavity (with a stoichiometry of 1-DPG/Hb tetramer) because it is stereochemically complementary to a constellation of six positively charged groups facing the central cavity of the Hb molecule. DPG cannot bind to oxy-Hb because the entrance to the cavity is reduced greatly as Hb switches from the deoxy- to the oxy-molecular state. In humans, it is DPG that promotes the sigmoidal oxygen-binding relationship with Hb, and its presence is essential for the release of oxygen in the tissues, allowing the discrepancy in oxygen saturation between arterial (about 96% saturated) and venous blood (about 64% saturated) (28).

In the transition from deoxy- to oxy-Hb, large structural changes take place at the α1β2 contact but only small ones at the α1β1 contact. The α1β1 heterodimer rotates, relative to the α1β2 heterodimer, by 15° and, at the same time, the two heterodimers move closer together. As a result, the oxy-Hb molecule has a more compact structure than deoxy-Hb, and the central cavity becomes smaller with the “box” separating the β chains, reducing from an open-ended slot about 0.8 nm wide to a box about 1.8 nm long and 0.5 nm wide (33). In combination with a slight alteration in tertiary structure, where the A and H helices of the two β chains move closer together, the β1β2 cavity in oxy-Hb becomes too small to accommodate DPG and, thus, expels it.

**The Binding of Folate to Hb**

It is clear from published research (24–27) that folate can be bound by Hb and that most, if not all, RCF in venous blood samples could be associated electrostatically with deoxy-Hb.

Folyl-polyglutamate binds within the β1β2 cavity, with an affinity similar to DPG (25), to deoxy-Hb tetramers with all three structural elements (pteridine moiety, p-aminobenzoyl portion, and glutamyl groups) contributing to the binding energy (27). The affinity increases with the number of glutamyl residues. Because the glutamate residues run through the central cavity, only one molecule can be bound per deoxy-Hb tetramer. Unlike DPG, which electrostatically binds at the entrance to the cavity, the pteroyl and p-aminobenzoyl groups of the folyl-polyglutamate are buried deep within the central cavity, nestled against an interior edge of the α1β1 interface, with the glutamate residues remaining at the entrance to the cavity where the first two or three glutamates interact with the DPG binding site. It is suggested that at least the fourth and higher glutamate residues exit the central cavity through the DPG binding site and either extend beyond the deoxy-Hb tetramer into the bulk solvent or, more likely, bind in a disordered way to the basic residues on the tetramer surface (27).

Folyl-polyglutamates do not bind to oxy-Hb tetramers, although removal of the pteridine moiety produces binding of the resulting p-aminobenzoylpolyglutamate residues (26). The (obvious) conclusion drawn is that the bulky pteridine residue is too large to penetrate the smaller β1β2 entrance to the central cavity, which arises as Hb switches from the T (deoxy-) to the R (oxy-) state, thus preventing the access of folate to the Hb-folate binding sites.

**Hb-folate binding and RCF analysis: a paradox?**

Removal of oxygen from oxy-Hb either directly [with N2 (30)] or indirectly [by increasing hemolysate acidity with AA (22)] produces more deoxy-Hb. However, in spite of the fact that it is deoxy-Hb that clearly binds folate (26), assayable RCF concentration increases. Conversely, direct oxygenation of Hb (30) produces a remarkable fall in assayable RCF, even though it is clear that the oxy-Hb tetramer does not bind folate (26).

It is easy to assume that an increase in the noncovalent electrostatic binding of RCF to deoxy-Hb should yield a decrease in assayable RCF and that, conversely, an increase in oxygenated Hb should, automatically, produce folate release and an increase in assayable RCF. However, although RCF binds to deoxy-Hb, this is not an impediment to analysis (30). The binding affinity of deoxy-Hb for polyglutamate folate is low, and it is only the high binding capacity of Hb (there are between 5 000 and 10 000 Hb molecules for every folate molecule) that leads to the conclusion that most, if not all, RCF is bound to deoxy-Hb (24–26) at the oxygen saturation (64% to 75%) (26, 28) associated with venous blood samples. Consequently, it is quite easy to envisage the scenario by which folate bound to deoxy-Hb is assayable. Once red blood cells have been lysed, plasma conjugase will compete for the pteroyl-polyglutamate (5CH3-H2-PteGlu, ) that is bound (with low affinity) to deoxy-Hb. Because most of the polyglutamate residues of the red cell folate reside outside of the entrance to the β1β2 central activity of deoxy-Hb, they are always accessible to plasma conjugase and the sequential removal of glutamyl residues, which will produce decreased folate binding affinity. Consequently, even if not initially successful in wrestling folate away from deoxy-Hb, the removal of glutamate residues from RCF by plasma conjugase will accelerate the dissociation of folate from deoxy-Hb and help to ensure complete deconjugation of RCF to the monoglutamate form (5CH3-H2-PteGlu). The monoglutamate form of the parent compound (PteGlu) has little affinity for binding to deoxy-Hb when compared with its polyglutamate counterparts (27), and because the addition of a methyl group has been shown to reduce binding affinity still further (27), it could be argued that both MA and KFBP should be able to assay fully deconjugated RCF in the presence of “folate-stripped” deoxy-Hb without the problem of substantial folate rebinding.

Why oxygenation of Hb should produce a substantial decrease in assayable RCF (30), even though it is clear that
the oxy-Hb tetramer does not bind folate (26), is puzzling. Although experimental evidence shows clearly that Hb, preexisting in the oxy-Hb state, does not bind folic acid polyglutamate (26), this does not mean that oxy-Hb cannot trap folate. Hb, although permanently in the presence of RCF, circulates in the body continuously, picking up oxygen in the lungs and depositing it in tissue. Returning, partially deoxygenated venous blood (conditions where RCF would be bound to deoxy-Hb) is pumped from the right ventricle of the heart via the pulmonary artery to the lungs. As soon as a deoxy-Hb molecule picks up its first molecule of oxygen, its quaternary structure abruptly snaps from the T-state to the R-state. If a deoxy-Hb molecule is carrying a molecule of bound folate when it converts to the R-state, then the folate must be trapped in the tetrameric cavity for the very same reason that folate cannot enter the cavity of a preexisting oxy-Hb molecule to be bound, i.e., the bulky pteridine moiety will prevent passage through the narrowed entrance of the β1β2 cavity present in the new quaternary structure. It is therefore irrelevant whether the pteridine, p-aminobenzoyl, and glutamate electrostatic bonding of the folate molecule with Hb is disrupted instantaneously, such that the folate molecule becomes dissociated from the Hb tetramer, because the folate molecule must remain trapped until the Hb molecule becomes fully deoxygenated once again. If true, previous conclusions that most, if not all, RCF in venous blood would be associated with deoxy-Hb, and not with oxy-Hb, should be re-assessed.

**Implications**

The implications for RCF assay are important, because even venous blood has substantial partial oxygen saturation (64% to 75%) (26, 28). Assuming, in the best-case scenario, that the percentage of oxy-Hb is no higher than the percentage of partial oxygen saturation, if RCF is “trapped” pro-rata to the proportion of oxy-Hb in venous blood, then RCF concentration could be underestimated by a factor of at least three- or four-fold. This casts serious doubt over all previously published RCF values. One report (30), in which whole blood was flushed with nitrogen, indicated that assayable RCF may only be increased up to twofold. However, deoxygenated red blood cells were subsequently lysed with an AA solution within which air (oxygen) was dissolved, and hence substantial amounts of RCF could have been retracted before being fully deconjugated.

Importantly, deoxy-Hb (which binds RCF) may keep folate in a state that can be successfully deconjugated and analyzed. The novel interpretation of folate-trapping by oxy-Hb, presented in this report, questions the effectiveness of the current method for RCF analysis.

**The Way Forward**

Although, superficially at least, it would seem easy to collect additional, practical evidence in support of our new theory, such thoughts would be naive. Even the simple step of producing a hemolysate creates a serious problem in that the concentration of naturally occurring allosteric effectors fall 10-fold, shifting the equilibrium of Hb to the R-state, which results in Hb tetramers having a much greater avidity for oxygen when in free solution than when originally contained within erythrocytes. This physical phenomenon must not only be neutralized, but the original affinity of Hb for oxygen must be reversed effectively; but how?

Strategies need to be developed that allow RCF to be deconjugated while all Hb is in the T-state quaternary structure; this will usually, but not exclusively (34, 35), mean that all Hb will need to be in the deoxy-Hb state.

Venous whole-blood samples could be equilibrated with a gas (e.g., 100% N₂, 100% CO₂) until the partial pressure of O₂ is reduced to a minimum. However, although it may be efficient at preventing Hb from carrying oxygen, CO should not be considered because it may mimic the allosteric effect of oxygen and cause Hb to snap to the R-state. This could itself provoke the trapping of RCF.

Because AA could protect hemolysates that are destined to be stored before analysis from oxidation, 10-fold dilution of deoxygenated blood (to engender complete lysis) should probably still be carried out with a solution of 1% AA, provided that the diluent is deoxygenated immediately before use. To avoid reoxygenation during the subsequent deconjugation step, hemolysates could be placed in containers, flushed with an appropriate gas, and sealed with a gas-tight closure.

The addition of chloride ions (a newly discovered allosteric effector) (36) or competitors for deoxy-Hb/folate binding such as DPG or inositol hexaphosphate [which has an even greater binding affinity and, thus, is even more potent than DPG in reducing the oxygen affinity of Hb (28, 32)] to the lysate diluent (1% AA) should be considered.

A search should be made for compounds that not only have a high binding affinity for Hb but can also cross-link the tetramer to permanently retain Hb in the T-state quaternary structure. In the interim, the usefulness of compounds that are already known to be synergistic with DPG (37, 38) should be examined.

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