CORRESPONDENCE

To The Editor:

In a recent issue of Blood (42:281, 1973), Waxman and Schreiber describe a competitive ligand-binding radioassay for serum folate using a commercial preparation of milk β lactoglobulin as a source of the binding ligand. This radioassay is quite similar to a procedure previously reported from their laboratory in which a dehydrated milk preparation was used as the source of folate-binding ligand. Since the major folate in serum, 5 methyl-tetrahydrofolate (methyl FH₄) is, for the most part, not bound to any protein, the authors assayed whole serum. There is, however, as noted by us previously, and later by Waxman and Schreiber, in the serum of some patients with either chronic myelogenous leukemia or folate deficiency a folate binder with determinants for PGA and not the natural methyl FH₄. Since this ligand will bind the ³H-PGA used in the radioassay, the concentration of tracer will be reduced in those assay reactions where the serum contains this binder. In their recent report, the authors discount the effect of this binder as “nonspecific.” We believe this is a serious disregard of one of the basic principles of competitive binding radioassays and raises a fundamental question about the validity of their method where the serum to be assayed contains a substantial amount of this folate binder.

It should be appreciated that, with all competitive binding radioassays, whether the binding ligand is an antibody or some other macromolecule, the contents of the reaction mixture for the standard curve and assay samples must be identical. This is particularly so with respect to the concentration of the tracer-labeled substrate and the binding ligand. Waxman and Schreiber stress the importance of the purity of the ³H-PGA, but it is a misconception that the tracer need be pure. It need not be pure, and it need not be the same as the substrate being assayed. If the impurity is a constant fraction of the total radioactivity, does not increase during the incubation period, and does not react selectively with any substance in the reaction mixture, it can be ignored. Of course, if the tracer is very impure, i.e., less than a few percent of the total radioactivity, then the error of separation of bound and free substrate and the determination of the radioactivity with statistical reliability can become major factors affecting precision.

Our criticism of the method of Waxman and Schreiber lies first with the fact that the standard curve reaction mixtures did not contain a volume of serum (folate free) equivalent to the assay reaction mixtures and, second, their failure to consider the binding of the ³H-PGA tracer when serum which contained the folate binder were assayed. This fault may be better appreciated by consideration of the following equations representing the reactants in the assay mixture:

\[
\begin{align*}
1. \quad & [³H-PGA] \quad [³H-PGA-BLG] \\
& + + \\
& [BLG] \quad [methyl FH₄-BLG] \\
& + + \\
& [methyl FH₄] \quad [³H-PGA] \\
& + + \\
& [methyl FH₄] \\
\end{align*}
\]

zero time  30 min incubation

In the above equation, which represents the reaction mixture for the standard curve, ³H-PGA is the labeled folic acid, BLG is the β lactoglobulin folate binder, and methyl FH₄ is the standard 5-methyltetrahydrofolate being assayed. The equation is shown as proceeding to the right with a single arrow because the reaction is stopped in 30 min with little likelihood of a state of equilibrium. At the end of 30 min, the free ³H-PGA is removed by coated charcoal, and the fraction of the total ³H-PGA which is bound is then determined. It is apparent from this equation that with the ³H-PGA and BLG held constant, and, with the latter sufficient to bind approximately 60% of the tracer (not, as the authors state, at the range of maximal binding of the ³H-PGA), the increase in the concentration of the methyl FH₄ will decrease the fraction of ³H-PGA bound to BLG.

Equation [2] below represents the reaction mixture for the assay of endogenous methyl FH₄ in a serum which also contains a binder with determinants for ³H-PGA (and not the reduced folate).

Blood, Vol. 43, No. 2 (February), 1974
CORRESPONDENCE

2. \[^{3}H\text{-PGA}\] \([^{3}H\text{-PGA-BLG}]\) + + [methyl FH\(_{4}\)] \([^{3}H\text{-PGA-FBP}]\) + + [FBP] \([^{3}H\text{-PGA-BLG}]\) + + [BLG] \([^{3}H\text{-PGA}]\) + + [methyl FH\(_{4}\)]

In this reaction, the methyl FH\(_{4}\) and folate binding protein, or FBP, both marked by an asterisk, are contained in the serum being assayed. This reaction is clearly different than the one for the standard curve, for it contains an additional binder of \(^{3}H\text{-PGA}\) not present in the standard reaction mixtures represented by equation [1].

This serum binder of \(^{3}H\text{-PGA}\) will have two effects: first, it will bind the \(^{3}H\text{-PGA}\) which would not have been bound to BLG as a consequence of the competing methyl FH\(_{4}\) in the serum and will result in a falsely high fraction of the \(^{3}H\text{-PGA}\) bound. The consequence of this will be a falsely lower serum folate concentration. A second effect of the serum folate binder will be to preferentially bind the \(^{3}H\text{-PGA}\) since it has a much higher affinity for folic acid than does the BLG. It is easy to demonstrate that more than 50\(^{\circ}\) of the \(^{3}H\text{-PGA}\) bound to BLG can be quickly dissociated by excess PGA, whereas PGA bound to the FBP in serum does not dissociate at all. Thus, with the \(^{3}H\text{-PGA}\) bound irreversibly to the serum binder, there is less \(^{3}H\text{-PGA}\) in the assay reaction mixture than in the standard reaction mixture. Such a system is untenable for a competitive reaction radioassay procedure, and even if the authors tried to correct for this serum binding of \(^{3}H\text{-PGA}\) (which they did not), the correction would have been inadequate because such mathematical manipulation would not have equated the reaction of equations [1] and [2].

We have encountered the same PGA binder in the serum of folate deficient patients (as we indicated previously\(^{2}\)) and occasionally in the serum of other subjects\(^{5}\) and have tried to overcome the problem in a number of ways. First, the sequential noncompetitive assay system, which is not complex but does require more time, is important because the methyl FH\(_{4}\) binds to the BLG before the addition of \(^{3}H\text{-PGA}\). By reducing the temperature to 4\(^{\circ}\)C, the dissociation of the methyl FH\(_{4}\) from the BLG is minimized, and the \(^{3}H\text{-PGA}\) titrates unoccupied binding sites on the BLG. A correction factor for binding of the \(^{3}H\text{-PGA}\) by the serum is then appropriately subtracted from the total \(^{3}H\text{-PGA}\) bound. The reaction mixtures for the standard curve also contain serum depleted of free folate and are, therefore, equivalent to serum assay reactions with respect to proteins.

The second way we try to circumvent the problem of the folate binder in serum is by using a very sensitive dose response curve which ranges from approximately 15 200 pg of methyl FH\(_{4}\). This permits us to assay 0.025 ml of serum. Studies in our laboratory with a partially purified folate binder, which is the same as the serum binder, indicate that there is a critically low concentration which causes dissociation of bound \(^{3}H\text{-PGA}\). This may account for our recent experience of a significant decrease in the number of serums which bind \(^{3}H\text{-PGA}\), as compared to the number containing the folate binder when we assayed 0.05 ml. With the 0.4 ml volume of serum assayed by Waxman and Schreiber, we would anticipate that a significant number of samples from folate deficient patients will have folate binder.

Some serums containing a high concentration of folate binder will bind \(^{3}H\text{-PGA}\) even when 0.025 ml is assayed. In such circumstances, the only way to assay the endogenous folate concentration is by extraction and deproteinization of the serum by boiling for a few minutes with ascorbate solution.\(^{2}\) We have encountered a number of such serums with a high concentration of folate binder which appeared to have a low concentration of folate when the whole serum was assayed, whereas significantly higher folate values were obtained when the extract was assayed. Similar findings were actually found by Waxman and Schreiber in Table 2 of their paper; six of the seven serums assayed after extraction had values 116\(^{\circ}\), 300\(^{\circ}\), greater than the folate concentration determined when the whole serum was assayed.

We believe the observation that commercial \(\alpha\) lactoglobulin can be used as a source of binding ligand for a folate assay will be very helpful. It is a far less pure preparation of folate binder than our own (i.e., folate bound/mg protein), but this may actually be a benefit for radioassay purposes because it appears to be more stable. We certainly find it far more convenient than preparing our own binder from milk and when used appropriately in a non-competitive system, we have obtained exquisite
sensitivity with a dose response curve extending from approximately 10-200 pg of methyl FH₄.

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MARIA DA COSTA, M.D.

REFERENCES


To the Editor:

Waxman and Schreiber recently described a modified radioassay for serum folate.¹ These authors, together with Herbert, previously² reported results on the first successful analysis of serum folate by radioassay, a very significant accomplishment, since microbiologic assay is both time-consuming and relatively imprecise. However, in the modified procedure of Waxman and Schreiber, a serious error is introduced by a faulty serum supernatant control which is supposed to correct for the binding of radioisotope to serum proteins. This error is due to adding nonradioactive PGA to the incubation mixture that serves as a serum supernatant control. Adding nonradioactive PGA to the serum supernatant control tube inhibits the binding of ³H PGA to the serum proteins and results in a much lower amount of radioactivity in the supernatant of this tube after charcoal separation. When this false low count in the serum supernatant control is subtracted from the count in the corresponding tube containing both serum and milk folate-binding protein, a falsely high net count is obtained. When the folate value of a serum sample is calculated by reference to a standard calibration curve by using this falsely high net count, a falsely low serum folate value is obtained. To obtain correct values, nonradioactive PGA should not be added to the serum supernatant control.

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ADDENDUM

I am sending this letter to the editors of both Blood and Clinical Chemistry since it is expected that both hematologists and clinical chemists would be interested in this folate radioassay.

REFERENCES

3. Tajuddin M, Gardyna H: Radioassay of
Correspondence


4. Rothenberg SP, da Costa M, Rosenberg

To the Editor:

We have reported the development of a sensitive radioassay for measurement of serum folate levels correlating well with microbiologic assay levels which utilized a stable, reliable, commercially available folate binder (beta lactoglobulin BLG), radioisotope (\(^{3}H\)PGA) and run in a rapid one-step simultaneous addition at room temperature. This radioassay obviated the need for a special radioisotope, preparation of purified milk folate binding protein and the more time consuming sequential additions at varying temperatures. These apparent advantages of the BLG-\(^{3}H\)PGA radioassay should allow it to be a useful, clinical method for replacing the more time-consuming, relatively imprecise and often artifactual microbiologic assay.

During the course of measuring serum folate levels we observed that most folate-deficient serums contained a specific binding protein for \(^{3}H\)PGA called FABP. Accordingly, an attempt was made to determine the effect of FABP on the folate levels and to derive a practical approach to deal with this problem. Our approach was to use a serum supernatant control containing stable PGA for the purpose of saturating the FABP to obtain radioactivity not absorbable to charcoal which was then subtracted from the \(^{3}H\) counts of each serum incubation mixture. It is this approach that is objectionable to Shaw, Rothenberg, and daCosta. We appreciate the rules of competitive binding radioassays and went to some length to establish that the contents of the tracer-labeled substrate and effective binding ligand capacity in the standard curve and assay samples containing FABP were not significantly different. This was based on the following observations: (1) The binding of \(^{3}H\)PGA to BLG was at near maximum at the concentration used for the radioassay and, when BLG concentration was held constant and the tracer reduced to one-half value, the binding and slopes of the curves were identical. (2) The per cent of \(^{3}H\)PGA (0.5 ng) bound by 0.1 mg BLG is not increased in the presence of 0.4 ml of dialyzed serum containing FABP. (3) The standard curve produced in the presence of dialyzed serum containing FABP is identical to that using buffer. (4) \(^{3}H\)PGA is not preferentially bound to serum FABP as compared to BLG; \(^{3}H\)PGA bound to BLG does not dissociate when dialyzed for 24 hr against 1000-fold excess PGA and equilibrium between binding of \(^{3}H\)PGA to BLG is reached within 30 min. These observations diminish the effects of FABP outlined in the letter by Rothenberg and daCosta. (5) The excellent recovery of added methyl-THFA to dialyzed serum containing FABP. (6) The finding of similar clinical diagnostic levels of folate in sera with FABP using whole serum as compared to serum extracts.

Other approaches to deal with serum FABP can be used, such as using lesser amounts of serum which tends to result in greater chance of error because of dilution or extraction of FABP which is tedious and subject to pH change, which may influence \(^{3}H\)PGA binding to BLG. This problem may be better dealt with using a sequential noncompetitive assay system which we previously described and used by Rothenberg, daCosta et al. However, this will merely diminish but not completely remove the problem of serum FABP.

We believe the use of stable PGA in the serum supernatant control allows this to be a practical approach in dealing with serum FABP in view of the above observations.

The end result is a reliable radioassay that correlates well with the microbiologic assay and with time and, perhaps, with further modifications as suggested by others working in this field, will become the clinical assay for measurement of serum and red cell folate.

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REFERENCES

5. Schreiber C, Waxman S: Measurement of folyl polyglutamates and red cell folate levels by \(^{3}H\)PGA radioassay. (submitted for publication)

NEWS AND VIEWS

XV CONGRESS OF THE INTERNATIONAL SOCIETY OF HEMATOLOGY TO BE HELD IN JERUSALEM: SEPTEMBER 1–6, 1974

The scientific program of the Congress will include the following sessions:

Section I: FRONTIERS OF HEMATOLOGY

<table>
<thead>
<tr>
<th>Subject</th>
<th>Chairman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Kinetics</td>
<td>F. Stohlman, Jr.</td>
</tr>
<tr>
<td>Physiological Ultrastructure</td>
<td>M. Bessis</td>
</tr>
<tr>
<td>Cell Enzyme Anomalies</td>
<td>E. R. Jaffe</td>
</tr>
<tr>
<td>Immunoproliferative Disorders</td>
<td>M. Seligmann</td>
</tr>
<tr>
<td>Autoimmune Hematological Disorders</td>
<td>R. S. Schwartz</td>
</tr>
<tr>
<td>Iron, Folate, Vitamin B(_1)2</td>
<td>C. A. Finch</td>
</tr>
<tr>
<td>Hemoglobin and Its Anomalies</td>
<td>D. J. Weatherall</td>
</tr>
<tr>
<td>Malignant Lymphoma</td>
<td>D. A. G. Galton</td>
</tr>
<tr>
<td>Myeloproliferative Disorders</td>
<td>F. A. Oski</td>
</tr>
<tr>
<td>Neonatal Hematology</td>
<td>J. F. Holland</td>
</tr>
<tr>
<td>Acute Leukemia</td>
<td>M. B. Zucker</td>
</tr>
<tr>
<td>Platelets</td>
<td>P. A. Marks</td>
</tr>
<tr>
<td>Hemopoietic Cell Differentiation and Maturation</td>
<td>M. Verstraete</td>
</tr>
<tr>
<td>Thromboembolic Diseases</td>
<td>D. Danon</td>
</tr>
</tbody>
</table>

These sessions are organized by their chairmen, who select their participants, also on the basis of the submitted abstracts.

Section II: TEACHING SESSIONS

<table>
<thead>
<tr>
<th>Subject</th>
<th>Faculty</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycythemia Vera</td>
<td>L. Wasserman, G. Astaldi</td>
</tr>
<tr>
<td>Nutritional Anemia</td>
<td>V. Herbert, C. E. Butterworth, V. Hoffbrand, J. Metz</td>
</tr>
<tr>
<td>Bone Marrow Failure</td>
<td>J. Bernard, W. Crosby, L. Sanchez-Medal</td>
</tr>
<tr>
<td>Bleeding Tendency</td>
<td>C. A. Owen</td>
</tr>
<tr>
<td>Iron Metabolism</td>
<td>Iron Panel, International Committee for the Standardization of Hematology</td>
</tr>
</tbody>
</table>