evidently represents an extreme, at least in its antibody titer, it constitutes an especially significant test case for B12 radioassays.

Some of the kits used to assay the specimen drawn in the third week of treatment were probably yielding spuriously increased results because of interference from antibodies or other proteins. However, because the three "boil" kits were in some disagreement with one another, it seems likely that mechanisms other than interference from antibodies may also have been operative: some kits may have been yielding spuriously low results on this specimen because of nonspecific binding problems or protein interference. Lower B12 values, as others have observed (6), are not necessarily "truer." Because no patient blanks were performed on the third-week sample for any of the kits involved in the study, however, this question cannot be settled. (Remember that the patient had already been started on treatment with B12 when the sample was drawn.)

The data presented by Higgins and Wu (6) in their recent Letter once again underline the importance of thorough testing by a variety of means to verify the clinical and numerical adequacy of any radioassay for vitamin B12.

References

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Disagreement of Anti-D Titers in Serum and Estimates of Bilirubin in Amniotic Fluid in a Case of Severe Rh Immunization

To the Editor:

We have recently seen an unusual problem in interpreting the results of a test for bilirubin in amniotic fluid, as determined by scanning the fluid through the spectral region between 340 and 550 nm (1) and noting the presence of interfering peaks. Peak height at 450 nm, from a baseline drawn tangentially to 550 and 380 nm, accurately reflects bilirubin concentration but also potential interferences by carotenoids. The test ("delta 450 test") usually shows a decreasing concentration of bilirubin as the fetus matures, but an abnormally high bilirubin concentration is possible in cases of excessive intra-uterine hemolysis, such as erythroblastosis fetalis. Polyhydran-
Total bilirubin in the cord serum was 14 mg/L, which correlated with the delta 450 bilirubin estimate of 15.2 mg/L. The free hemoglobin in the cord blood was 68 mg/L. The cord blood hematocrit was estimated to be less than 5%.

As shown by the data listed in Table 1, the mother had demonstrated an increasing anti-D titer throughout the pregnancy (highly suggestive of Rh immunization); however, the amniotic fluid concentrations of bilirubin were low and therefore inconsistent with the anti-D titers. The amniotic fluid did not show visible signs of hemolysis or mehemoglobin in any specimen. In fact, decreasing concentrations of bilirubin were observed at weeks 30, 31, and 32, and by week 35 the bilirubin estimation by the delta 450 test was quite low (estimated value of 0.4 mg/L).

The low result for bilirubin, and hence the failure of the delta 450 test to reflect accurately the apparent intrauterine hemolysis, was presumably due to the low hematocrit. We conclude that because of the low mass of erythrocytes in the fetus, there was insufficient hemolysis to show an increased bilirubin concentration in the amniotic fluid.

The reviewers of this Letter have suggested that another useful test in this situation would have been the determination of the IgG subclasses in the maternal blood.

References

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Microbiological and Radioimmunological Assays for Folic Acid In Whole Blood Compared: Effect of Zinc Nutriture

To the Editor:

Radioimmunoeaasys (RIAs) for folic acid in the blood, for which several kits are available, offer advantages of speed and technical ease over the microbiological assay. However, microbiological assay for folate in blood is still considered to be the most sensitive and reliable method. *Lactobacillus casei*, the organism long used for folate bioassay, responds similarly to all physiological derivatives of the pteridine moiety of the vitamin, a feature not true of most radioassays (1).

Several investigators have compared microbiological and RIA methods of analysis for folate in serum or whole blood (2–5). In most instances, the two methods were comparable with large numbers of samples, but unexplained discrepancies of individual samples have been noted (2, 3, 5). During a study of mild zinc deficiency in men we observed an apparent zinc effect on folate acid metabolism as reflected in the comparison of microbiological and radioimmunoassay of folic acid in whole blood.

Three men, ages 19 to 27, were fed diets on a three-day menu rotation, consisting of conventional foods chosen to minimize zinc content and variability of composition. These diets contained approximately 150 μg of folate and 3.5 mg of zinc per day (by analysis). One man's diet was supplemented with 400 μg of folic acid every other day. Zinc was supplemented according to the following protocol: 31 days of control, low zinc diet supplemented with 4 mg of zinc daily; 120 days of depletion, low zinc diet; 24 days of repletion, low zinc diet supplemented with 30 mg of zinc daily. The men's body weights were kept constant by adjusting energy intakes and expenditures. Details of these studies are published elsewhere (6).

Two folic acid assay methods were compared. We determined serum and erythrocyte folate activities by a microbiological procedure (7), using a chloramphenicol-resistant strain of *L. casei*, and also by a Becton Dickinson folate radioassay kit.1 Erythrocyte folate was then calculated by using values for whole blood, serum, and hematocrit.

As the tabulation shows, dietary zinc influenced the comparative results of the two procedures.

<table>
<thead>
<tr>
<th>Dietary Zn*, mg/day</th>
<th>Folate-unsupplemented</th>
<th>Folate-supplemented</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Microbiol.</td>
<td>RIA</td>
</tr>
<tr>
<td>8.0</td>
<td>312 ± 36 (2)</td>
<td>335 ± 30 (3)</td>
</tr>
<tr>
<td>3.5</td>
<td>353 ± 44 (17)</td>
<td>188 ± 44 (19)</td>
</tr>
<tr>
<td>33.5</td>
<td>367 ± 67 (4)</td>
<td>266 ± 79 (4)</td>
</tr>
</tbody>
</table>

*Mean ± SD (and no. of observations). b p < 0.001, Student's *t*-test. p < 0.004. *p* < 0.05.

The two methods appeared comparable during the initial control period. During zinc depletion, however, significant differences between the two folate methods were seen in both the folate-supplemented and un-supplemented groups, the apparent RIA folate values being significantly lower than those measured microbiologically. Microbiological values for erythrocyte folate concentrations remained essentially constant in the men who did not receive the folate supplement, regardless of the zinc content of the diet. On the other hand, apparent folate concentration as measured by the RIA procedure significantly declined when the men were receiving 3.5 mg of zinc per day. When dietary zinc was supplemented to 33.5 mg per day, the apparent erythrocyte folate increased slightly. When the diet was supplemented with folate acid, microbiologically measured erythrocyte folate concentration increased with time throughout the study, whereas erythrocyte folate measured by RIA did not change. No significant changes in plasma zinc were observed. Addition of exogenous zinc to pooled specimens of plasma or whole blood had no apparent effect on either folate assay.

Evidently, during zinc depletion there is a form of folate that is initially measured by RIA but then is converted to a metabolite that is poorly detected by RIA although still measurable microbiologically. Shane et al. (1) pointed out that most derivatives of folic acid that are metabolically important are equally measured microbiologically. In contrast, many forms of folate can give misleading results with RIA procedures because of different binding properties towards the binding protein used with the radioassay kits (1, 8).

Thus, in assessing the folate status of a patient by RIA, zinc nutriture must be considered as a nutrient that could produce falsely negative results. Further studies are in progress to establish the nature of these differences.

References
2. McGown EL, Lewis CM, Dong MH, Sauerbier HE. Results with commercial radioassay kits compared with microbiolog-