Use of Either Serum or Plasma for Folate and Vitamin B\textsubscript{12} Determinations

To the Editor:
Kubasaki et al. (1) reported that the literature regarding proper specimen-collec-
tion procedures for substances measured by radioassay or radioimmunoassay is poorly defined. Specifically, folate values for ethylenediaminetetraacetate (EDTA)-containing plasma were reported to be only one-third as large as those obtained by analyzing the corresponding serum or heparin-treated plasma by radioassay.

We have further investigated the effect of various anticoagulants on the values obtained for folate and vitamin B\textsubscript{12} with simultaneous radioassay (2-4).

Venous blood samples were collected from 30 healthy individuals, 25 to 45 years old. Five types of evacuated tubes were used to collect the blood samples: (a) Becton Dickinson no. 3206U, 5-ml capacity, no anticoagulant (red top); (b) Becton Dickinson no. 3206Q, 5-ml capacity, 6 mg of disodium EDTA (lavender top); (c) Monoject no. 0066, 4-ml capacity, 0.3 ml of 3.8% solution, equivalent to 10.8 mg of sodium citrate (blue top); (d) Monoject no. 00607, 5-ml capacity, 0.5 ml of 0.1 mol/liter solution, equivalent to 6.7 mg of sodium oxalate (black top); (e) Monoject no. 00647, 2.5-ml capacity, 286 USP units of sodium heparin (green top). We attempted to obtain a specimen for each tube from each individual, but this was not possible for all the subjects. The blood with no anticoagulant was allowed to clot for 15 min. All tubes were then centrifuged for 20 min at 1240 \( \times \) g. Plasma or serum was separated from packed erythrocytes and stored at \(-20^\circ\)C until assayed.

The SimulTRAC Radioassay Kit, Vitamin B\textsubscript{12} (57Co)-Folate 12SF (Schwarz/Mann, cat. no. 225517, lot CN4034A) was used to determine vitamin B\textsubscript{12} and folate. A Packard Model 5230 dual-channel gamma counter was used to determine the radioactivity with discriminator limits for 125I and 57Co set so that in either case the spillover into the other channel was <1%.

The reagents were brought to room temperature before the assay and the samples were analyzed in duplicate. No more than 50 tubes were run within a single assay. Two commercial controls were analyzed with each assay. Tubes containing clinical samples (100 \( \mu \)l) or standards were added to buffer and the tubes were heated at 100 \( ^\circ\)C (boiling water bath) for 45 min. The tubes were cooled to 20–25 \( ^\circ\)C, tracers and binders were added, and the tubes left at room temperature for 45 min. Dextran-coated charcoal suspension was added and the tubes were centrifuged for 15 min at 4 \( ^\circ\)C. The supernates were decanted and the radioactivity in the bound portion was counted. Standard curves were calculated (least squares linear regression) and the concentrations of vitamin B\textsubscript{12} and folate in the samples were determined by interpolation from the standard curve.

Regression analyses were performed to compare the folate values obtained in serum and those obtained in: (a) EDTA-treated plasma; (b) heparin-treated plasma; (c) sodium citrate-treated plasma, and (d) sodium oxalate-treated plasma. Analogue regres-
sions were calculated for the vitamin B\textsubscript{12} values. The results are shown in Table 1.

We conclude that the clinical signifi-
cance of a folate or vitamin B\textsubscript{12} value determined with this assay procedure is independent of the type of anticoag-
ulant/plasma or serum collected although statistically they may be different. Specifically, we find that samples of
EDTA-treated plasma may be used re-
liably to measure folate, in contrast to a previous report (1), in which a different assay procedure was used (5). Individual procedures should be evaluated simi-
larly before different biological matrices are assayed.

Table 1. Results of Regression Analysis

<table>
<thead>
<tr>
<th>Serum vs. plasma collected over</th>
<th>Folate y-intercept</th>
<th>Slope</th>
<th>Vitamin B\textsubscript{12} y-intercept</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDTA</td>
<td>0.98</td>
<td>-0.11</td>
<td>0.83</td>
<td>0.98</td>
</tr>
<tr>
<td>Heparin</td>
<td>0.97</td>
<td>-0.39</td>
<td>1.25</td>
<td>0.99</td>
</tr>
<tr>
<td>Citrate</td>
<td>0.90</td>
<td>0.65</td>
<td>1.23</td>
<td>0.98</td>
</tr>
<tr>
<td>Oxalate</td>
<td>0.95</td>
<td>-0.45</td>
<td>0.91</td>
<td>0.95</td>
</tr>
</tbody>
</table>

5. Kubasaki, N. P., Personal communication.

Artificial Alkaline Phosphatase Iosenzyme Band, Caused by Bilirubin, on Cellulose Acetate Electrophorograms

To the Editor:
Alkaline phosphatase isoenzyme separa-
tions by electrophoresis have been exten-
sively studied in the past 15 years (1). In our laboratory in the past two years, electrophorograms from 27 of about 1200 patients have shown a fast-migrating alkaline phosphatase (EC 3.1.3.1) band, which we do not find mentioned in the literature. Using cell-
lulose acetate medium, Rhone et al. (2) reported seeing five bands on electrophoresis. These included the two bands representing isoenzymes originating from the liver, and those originating from bone, placenta, and intestine (in order from anode to cathode). These facts led us to investigate this fast band that has apparent alkaline phosphatase activity.

The electrophoresis was done on cell-
lulose acetate strips, and with an Electrophoresis System (3), all supplied by Helena Laboratories, Beaumont, Texas 77704. Serum samples were applied to the strips three times in the same posi-
tion and electrophoresed for 19 min at 230 V. The buffer used was trisodium barbital (pH 8.8) and the staining re-

References
5. Kubasaki, N. P., Personal communication.

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Fig. 1. Electropherogram stained for alkaline phosphatase isoenzymes
A, albumin with added bilirubin; B, bilirubin solution in saline; C, patient's serum with artificial band in addition to placental and liver isoenzyme bands (poorly resolved); D, intestinal and placental isoenzyme control; E, patient's serum with liver isoenzyme band; F, intestinal and liver isoenzyme control; G, patient's serum with liver isoenzyme and artifactual band; and H, bone isoenzyme control

agent set consisted of α-naphthol
AS-MX (3-hydroxy-2-naphthol-2,4-xylidilide) and a diazo compound (Fast Blue RR), also from Helena Laboratories. A thin film of the stain was spread onto a second cellulose acetate strip and the electrophoresed strip was layered on top of this. This combination was incubated at 37 °C for 15 min, thus making the isoenzymes visible.

The fast band was identified by electrophoresing aliquots of the following solutions: (a) 200 mg of bilirubin per liter in a solution containing, per liter, 60 g of human albumin solution and 2 g of sodium carbonate; or (b) 200 mg of bilirubin per liter in a solution containing, per liter, 9 g of sodium chloride and 2 g of sodium carbonate; or (c) 60 g of albumin and 2 g of sodium carbonate per liter. The bilirubin used was Standard Reference Material No. 916 (National Bureau of Standards, Washington, D.C. 20234).

Alkaline phosphatase activities were measured by a kinetic modification of the method of Morgenstern et al. (4).

In reviewing about 1200 such alkaline phosphatase isoenzyme patterns run during the past two years, we found 27 patients whose sera showed the fast-migrating band. Twenty-six of the 27 also had abnormally high bilirubin concentrations (>10 mg/dL). To prove the association of this band with above-normal bilirubin, we electrophoresed and stained for alkaline phosphatase activity the following solutions: (a) bilirubin (200 mg/liter) plus human albumin (60 g/liter), (b) bilirubin (200 mg/liter), and (c) human albumin (60 g/liter). All three of these solutions, tested for alkaline phosphatase activity by a kinetic method, had insignificant (<3 U/liter) alkaline phosphatase activities but the albumin and bilirubin mixture had nonspecific staining in the albumin region (Figure 1). Figure 1 also shows the pattern from two patients whose serum showed the fast band in the same position as the nonspecific band for the albumin/bilirubin mixture.

We conclude that the alkaline phosphatase-like activity in the albumin region is merely an artifact due to a bilirubin/albumin complex that nonspecifically binds with this particular stain.

The separation by electrophoresis of albumin-bound bilirubin and free bilirubin (Figure 1, a and b) may be a useful method for measuring bound and free bilirubin in potential cases of bilirubin encephalopathy in the neonate. We are currently investigating this possibility.

References

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Accuracy of Immunological Methods for Measurement of Creatine Kinase Isoenzyme Activities

To the Editor:
In his recent evaluation of methods for creatine kinase fractionation, Morin (1) indicates that "the immunological method [2] has unacceptable reliability" and that "very broad variations and poor reproducibility" were observed.

The data presented in his Table 2 obtained by the immunological assay "essentially performed as described" are indeed unacceptable. Since the accuracy of an immunological procedure depends on the quality of the antisera used, one may ask how good was the antigen-binding capacity of Morin's antisera. For any quantitative approach a very careful evaluation of the inhibition and precipitation curves is fundamental. Surprisingly, Morin does not present these important data, thus rendering his study rather questionable.