plasma myoglobin, we were not able to single out any special features of their crises that could account for this finding.

In this study, we initially excluded patients who we thought were malingering. In addition, the question of infection without crisis was not studied because very few cases of infection without pain were seen, although this important problem deserves further study. We conclude that plasma αHBD may be useful in evaluating assay painful sickle cell crisis, especially if steady-state values are known. Increased plasma myoglobin concentrations appear to be a very specific but not very sensitive indicator of painful crisis. Plasma αHBD and myoglobin determination by radioimmunoassay may form the beginning of a battery of tests that will be useful to the clinician and researcher.

We thank Drs. Michael Harris and Michael Diaz for blood samples from their patients; Dr. Kurt Hirschhorn, Mount Sinai School of Medicine, and Dr. Ronald Nagel, Albert Einstein School of Medicine, for help with this project; and Mr. Robert Smith and Mrs. Helena Lipszyc for technical assistance. This work was supported in part from a grant given under the auspices of the March of Dimes Mount Sinai Sickle Cell Program no. 1-716; and NIH Grant #HL-21016.

References

Note added in proof: Plasma creatine kinase concentrations were also determined on the same samples, and the results revealed no increase in this enzyme during crises. In addition, fluctuations evidently produced by recent intra-muscular injections were observed. We conclude that creatine kinase is not useful in the evaluation of sickle cell crises.

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**High-Affinity Binding of Folate to a Serum Protein in Chronic Myelogenous Leukemia: Effect of Binder Concentration, pH, and Temperature**

Jan Holm, Steen Ingemann Hansen, and Jørgen Lyngbye

Serum from a patient with chronic myelogenous leukemia was subjected to anion-exchange chromatography on DEAE-Sepharose CL-6B. High-affinity binding of [3H]folate to front effluent, eluted at a low salt gradient, was studied in equilibrium-dialysis experiments (37 °C, pH 7.4). As suggested by the data, folate binding displayed positive cooperativity. Dilution of the binder solution resulted in a shift to a simple non-cooperative binding type and increased binding affinity. Furthermore, binding was inhibited at pH 5.0 and at low temperature (7 °C). This study demonstrates important similarities between high-affinity folate binding in milk and serum: positive cooperativity and dependence of binding affinity on concentration of binder. Identical mechanisms may underly these phenomena in milk and serum. The apparent relationship between binding type and concentration of binder shown herein seems to agree fairly well with recent observations on sera from groups of healthy persons.

The presence of a high-affinity folate-binding protein in serum has been established in several studies. A protein of this type was first identified in circulating granulocytes and serum from patients with chronic myelogenous leukemia (1). Subsequent studies demonstrated the presence of an immuno-

chemically similar folate binder in serum of pregnant women (2) and in such states as folate deficiency (3), liver disease (4), uremia (4), and even in normal individuals (4).

The lack of detailed information regarding the exact mechanism whereby high-affinity binding of folate in serum takes place prompted us to study this process under standardized conditions in serum from groups of healthy individuals (5, 6). In the present study this line of investigation has been extended to a patient with chronic myelogenous leukemia in whose serum a particularly high concentration of high-affinity binder had been found (7).

**Materials and Methods**

[3H]Folate with a specific activity of 40 kCi/mol was supplied by the Radiocohemical Centre, Amersham, U.K.

Venous blood was sampled from a 55-year-old man with chronic myelogenous leukemia (leukocyte count >250 × 10⁶/L and a peripheral blood smear completely predominated by promyelocytes, myeloblasts as well as stem cells) shortly after his admission to the hospital and before any medical treatment.

Pooled serum samples were subjected to anion-exchange chromatography on DEAE-Sepharose® CL-6B (Pharmacia, Uppsala, Sweden) as previously reported by us (5–7), and a 0.17 mol/L Tris buffer solution (pH 7.4) was prepared from front effluent fractions (eluted with 30 mmol/L NaCl) containing the high-affinity folate binder (5–7). Binding of [3H]folate to 600-μL aliquots of the latter solution was studied in equilibrium-dialysis experiments (external buffer volume 200 mL) at pH 7.4 (0.17 mol/L Tris buffer) or 5.0 (0.2 mol/L...
Table 1. Effect of Binder Concentration and Temperature on Parameters of High-Affinity Folate Binding in Serum

<table>
<thead>
<tr>
<th>Relative concentration of binder</th>
<th>Incubation temp., °C</th>
<th>H-I</th>
<th>Bq.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undiluted</td>
<td>37</td>
<td>1400</td>
<td>30</td>
</tr>
<tr>
<td>Fivefold diluted</td>
<td>37</td>
<td>2400</td>
<td>8</td>
</tr>
<tr>
<td>Fivefold diluted</td>
<td>7</td>
<td>2200</td>
<td>13</td>
</tr>
</tbody>
</table>

a Binding data from equilibrium-dialysis experiments (pH 7.4) at 37 and 7 °C with various folate binder concentrations were analyzed in Scatchard and Hill plots. Maximum folate bound (N) is given by the intersection on the abscissa of the Scatchard plot. Bq.D. indicates estimated folate concentration at half saturation of binding. Hill coefficients, n, were obtained from the regression lines:

\[ y = 1.34 (0.09) x - 2.0 (0.1), r = 0.95 (30 observations), n \text{ significantly } (p < 0.001) > 1.00. \]

\[ y = 1.18 (0.09) x - 1.1 (0.1), r = 0.94 (23 observations). \]

\[ y = 0.77 (0.08) x - 0.98 (0.08), r = 0.99 (six observations), n \text{ significantly } (p < 0.01) < 1.00. \]

acetate buffer), in accordance with a method we previously described (8).

Results

Equilibrium dialysis experiments with solutions containing high-affinity folate binder from chronic myelogenous leukemia serum were performed under different experimental conditions. Folate binding parameters were determined after analysis of the data in Scatchard and Hill plots (Figure 1 and Table 1).

A Scatchard plot (Figure 1) consisting of binding data obtained at 37 °C and pH 7.4 seemed to display a downward concavity. This finding, together with a Hill coefficient (n) of 1.34, is conventionally interpreted as positive cooperativity between at least two sites. The concentration of binding protein seemed to have a great impact on binding characteristics (Table 1). A decrease in the concentration of binder thus resulted in an increase of the binding affinity (the external folate concentration required for half saturation decreased), and a change to a simple non-cooperative binding type (n was no longer significantly higher than 1.00).

Experiments with low binder concentrations were repeated at 7 °C (Table 1). Maximum binding was virtually unchanged; but a decrease in binding affinity was observed, together with a change in binding type (n, the Hill coefficient, <1.00), apparently to negative cooperativity. Finally, folate binding was markedly depressed at pH 5.0 (Figure 1).

Discussion

Several important similarities between high-affinity folate binders in milk and serum have been observed, such as a relative molecular mass of 30,000, a weak affinity to anion-exchange columns at near-neutral pH (pI values ±7) and binding constants of 10^{-10} to 10^{-11} L/mol (6, 8, 10). By analogy with other systems (9) the two most important characteristics of high-affinity binding in milk—i.e., positive cooperativity and the dependence of binding affinity on the concentration of binder—may suggest the involvement of a polymerizing protein system in which the monomer prevailing at low protein concentrations has a greater affinity for the ligand than does the polymer (8-10). So far, this hypothesis has gained some support from ultracentrifugation studies demonstrating a remarkable aggregation tendency (a polymer consisting of more than 16 monomers) of highly purified folate binder from cow's milk (10). The aggregation tendency increased at higher binder concentrations, and the fact that addition of folate in particular enhanced aggregation (a polymer consisting of more than 32 monomers) gave further support to the idea of an intimate relationship between ligand binding and polymerization (10). As suggested by the present data, positive cooperativity and dependence of binding affinity on concentration of binder were apparently also typical characteristics of high-affinity folate binding in chronic myelogenous leukemia serum. In view of the many parallels that can be drawn between specific folate binding in milk and serum (cf. above) it seems justifiable to assume that identical mechanisms are underlying these phenomena in milk and serum. Furthermore, the effects of pH and incubation temperature on binding observed herein are also parallel to conditions in milk (8).

Another point of interest was the apparent relation between the concentration of binder in serum (expressed as maximum folate binding) and binding type, i.e., positive cooperativity disappeared after the binder solution was diluted (Table 1). This may explain the somewhat puzzling observation that high-affinity folate binding displayed negative cooperativity in a pooled specimen of serum from a group of healthy men, but positive cooperativity in a serum pool obtained from a group of pregnant women (5, 6). However, as was the case with the present sample of chronic myelogenous leukemia serum,

Fig. 1. Scatchard plot
High-affinity binding of ^{3}H\text{-}folate to front effluent obtained from serum after DEAE-Sephadex A-50 anion-exchange chromatography. Equilibrium-dialysis experiments at 37 °C in (O) 0.17 mol/L Tris buffer, pH 7.4, or (■) 0.2 mol/L acetate buffer, pH 5.0
a rather high concentration of high-affinity folate binder was found in the sera from pregnant women (6).

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References

Elimination of Inhibitors of Alkaline Phosphatase from 2-Amino-2-methyl-1-propanol
H. J. G. M. Derks, V. Borrias-van Tongeren, J. B. A. Terlingen, and J. C. Koedam

When 2-amino-2-methyl-1-propanol buffer solutions are extracted with chloroform, impurities inhibiting alkaline phosphatase are selectively removed and this source of variation is thereby greatly decreased. We also studied the effect of adding zinc sulfate to this buffer. A combination of chloroform extraction and zinc sulfate, added to give concentrations from 5 to 10 µmol/L, results in buffer of high and consistent quality. The alkaline phosphatase activities of 12 reference sera of different origin were determined with use of the purified buffers. Those sera, which closely resembled human serum in terms of alkaline phosphatase isoenzyme composition, behaved similarly to human serum. Use of such control sera is strongly recommended.

2-Amino-2-methyl-1-propanol (2A2M1P) and diethanolamine (DEA) are the two phosphorylating buffers most extensively used in determining the catalytic activity of alkaline phosphatase (EC 3.1.3.1) in serum. Several authors (1, 7) prefer 2A2M1P because of the presence of an inhibitor, probably monoethanolamine, in DEA. But 2A2M1P also often contains considerable amounts of other interfering substances. The inhibition caused by these impurities is negligible when the reaction is started by adding serum. But when the reaction is started with substrate, the inhibition increases with increasing interval of contact between serum and buffer before substrate is added (2). Williamson and Thompson (3) detected a substance in different lots of 2A2M1P, the concentration of which was proportional to the inhibitory effect. Mass-spectrometric work revealed the probable presence of substituted diamines that might inhibit the catalytic action by chelation of the zinc(II) ions necessary for the reaction.

In this paper, we describe experiments aimed at eliminating the effect of these inhibitors on alkaline phosphatase.

Materials and Methods
Seventeen lots of 2A2M1P were purchased from: Aldrich-Europe (Beerse, Belgium), J. T. Baker Chemicals BV (Deventer, The Netherlands), BDH Chemicals LTD (Poole, U.K.), Fluka AG (Buchs, Switzerland), Koch-Light Lab. Ltd. (Colnbrook, Bucks., U.K.), E. Merck (Darmstadt, F.R.G.), Serva (Heidelberg, F.R.G.), and Sigma (St. Louis, MO).
4-Nitrophenyl diosmidium orthophosphate was a product of BDH, Poole, U.K. (product no. 40030). All other reagents and solvents came from E. Merck, Darmstadt, F.R.G. In all experiments, doubly-distilled water was used in which no zinc was detectable by atomic absorption spectrometry (detection limit: 5 µg/L). The zinc content of the chloroform used in the experiments was stated by the manufacturer to be <50 µg/L. This was checked by extracting an aliquot of chloroform with 0.1 volume of a 1 mol/L solution of HNO3 in water. Analysis of the extract by atomic absorption spectrometry showed that the statement of the manufacturer was correct (found: 6 µg/L).
Control sera were purchased from Boehringer, Mannheim, F.R.G.; Merz & Dade, Dödingen, Switzerland; Hoffmann-La Roche, Basel, Switzerland; and Wellcome, Beckenham, U.K.
Frozen sera from patients and reconstituted control materials were left at room temperature for 20 h before the analyses. Electrophoresis was carried out on cellulose acetate with Helena (Beaumont, TX) equipment and reagents.

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