systematically to underestimate the proportion of Hb S, it should not be used for this purpose.

We thank Dr. James Eckman for providing several samples and Ms. Lorraine Bryan for assistance in obtaining these samples.

References

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Evidence That the Low-Affinity Folate-Binding Protein in Erythrocyte Hemolysate Is Identical To Hemoglobin
Steen Ingemann Hansen, Jan Holm, and Jørgen Lyngbye

Gel filtration studies on erythrocyte hemolysate demonstrated the presence of a folate binding protein, apparently of the low-affinity type, that co-elutes with hemoglobin. Further, the folate binder eluted with a low salt concentration after DEAE-Sephacore® CL-6B anion-exchange chromatography of erythrocyte hemolysate at pH 6.3. The chromatographic behavior of hemoglobin labeled with [3H]folate was so similar to that of the present binder as to suggest that the folate binder in erythrocytes is in fact hemoglobin.

Two types of folate binding proteins are present in serum: a group of low-affinity binders (K = 10³ L/mol), the quantitatively most important representative of this category being albumin (1-3), and a high-affinity binder (K = 10¹¹ L/mol) with characteristics similar to those of other specific folate binders in milk and leukocyte lysates (4-6). Because of its ionic properties, the latter trace protein can be separated from the low-affinity binders by anion-exchange chromatography of serum at near-neutral pH (6-7). However, we have recently reported the presence of a hitherto undescribed low-affinity binder which, in contrast with other binders of this type, co-elutes with the specific folate binder during anion-exchange chromatography of serum (12). The evidence presented suggested that this binder was identical to hemoglobin (12).

The present study further substantiates this hypothesis. By the combined use of gel chromatography and anion-exchange chromatography we demonstrate that a low-affinity folate binder, probably identical to hemoglobin, is present in erythrocytes.

Materials and Methods
Labeled folate was supplied by the Radiochemical Centre, White Lion Road, Amersham, Buckinghamshire HP7 9LL, U.K. The following three types of radiochemical preparations were used, [2,14C]folic acid, potassium salt (cat. no. CFA 333), with a specific activity of 55 Ci/mol and a radiochemical purity of 97-98%; [G-3H]folic acid, potassium salt (TRA. 34), with a spec. act. of 5 kCi/mol and a radiochemical purity of 95-97%; and [3',5'-7,9-3H]folic acid, potassium salt (TRK. 212), with a spec. act. of 29 kCi/mol and a radiochemical purity 95-97%. Hemoglobin was obtained from Sigma Chemical Co., St. Louis, MO 63178.

Specimens of venous blood (EDTA stabilized), drawn from 10 healthy volunteers, were pooled. After centrifugation at 1500 × g for 15 min, the plasma and buffy coat were discarded, and the erythrocytes were washed five times in isotonic NaCl solution (9 g/L) to remove contaminating plasma. In the last wash solution, no albumin was detectable by "rocket" immunoelectrophoresis (13) with monospecific antibody against albumin (Dakopatt, Copenhagen). The washed erythrocytes were diluted with an equal volume of distilled water and repeatedly frozen and thawed five to 10 times. The resulting hemolysate (hemolysis confirmed by microscopic examination) was dialyzed overnight at 4 °C vs 0.17 mol/L Tris buffer,

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Fig. 1. Gel filtration profile of erythrocyte hemolysate labeled with $[\text{H}]$folate, 100 nmol/L ($\triangle\ldots\triangle$) and 1250 nmol/L (■—■). Hemoglobin concentration, O—O. Abscissa, elution volume. Ordinate, cpm (left) and hemoglobin concentration (right).

Hemolysate and effluent obtained after this anion-exchange chromatography of hemolysate were incubated overnight at 25 °C in the Tris buffer with labeled folate before gel filtration, which was performed on a 5.3 cm$^2 \times$94 cm column of Ultrogel AcA 44 (LKB, Bromma, Sweden). The column was eluted (5 °C, flow rate 50 mL/h) with the Tris buffer (10). For molecular-mass calibration we used substances and proteins of known molecular mass as before (10).

Samples (400 μL) digested overnight in 1 mL of “Soluene-350” (Packard-Becker B.V., Chemical Operations, Groningen, The Netherlands) were decolorized when they were allowed to stand for 30 min at 37 °C after addition of 200 μL of 2-propanol and 200 μL of a 300 g/L solution of hydrogen peroxide. Liquid scintillation counting was done as previously described (10) after addition of 10 mL of “Dimilume” (Packard) scintillation fluid. Counting efficiencies were controlled by internal standardization with $[\text{H}]$toluene (spec. acty., 2.71 × $10^6$ dpm/g) and $[\text{C}]$toluene (spec. acty., 5.09 × $10^6$ dpm/g), both supplied by Packard.

Hemoglobin concentration was measured by measuring the absorbance of oxyhemoglobin at 540 nm.

**Results and Discussion**

Erythrocyte hemolysate labeled in the presence of increasing concentrations of $[\text{H}]$- or $[\text{C}]$folate was subjected to gel filtration. Two typical elution diagrams are shown in Figure 1. The peak eluted at 340 mL elution volume represents protein-bound $[\text{H}]$folate; the large peak at 500 mL elution volume represents unbound $[\text{H}]$folate. As can be seen, hemoglobin co-elutes with the first peak. Other chromatographic runs showed that the relative protein binding of folate, as assessed by integration of the peaks, was rather constant (2–3%) over a wide folate concentration range (10 nmol/L to 1 mmol/L).

Hemolysate was applied to an anion-exchange column and eluted with a low salt concentration (30 mmol/L NaCl) at pH 6.3. The effluent, which contained visible amounts of hemoglobin, was labeled with $[\text{H}]$folate before gel filtration. The elution profile (Figure 2) was similar to that of Figure 1.

Solutions of hemoglobin and of effluent obtained after anion-exchange chromatography of hemolysate were labeled with $[\text{H}]$folate before gel chromatography. The elution diagrams (not shown) were indistinguishable from those of Figures 1 and 2.

From these experiments we conclude that the folate binding protein in erythrocytes seems to be identical to hemoglobin. The binding seemed to be of the low-affinity type, inasmuch as relative binding was constant over a wide folate concentration range. In contrast with other low-affinity folate binders such as albumin (12), the erythrocyte binder possessed a weak affinity for anion-exchange columns at near-neutral pH. This is in fair agreement with the supposed identity of the binder, because P1 values within the pH range 7–8 were reported for hemoglobin (14). The physiological role of folate binding in erythrocytes is obscure, but hemoglobin may serve as a transport or storage protein from which folate is easily mobilized.

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**References**

8. Holm, J., Hansen, S. I., and Lyngbye, J., High affinity binding of
Radioimmunoassays for haloperidol are described, involving use of tritium- or $^{125}$I-labeled drug or tritium-labeled spiropelone, and a rabbit antiserum to a drug/bovine serum albumin conjugate. The $^{125}$I-labeled drug was prepared by the Chloramine T iodination technique. A radioimmunoassay for haloperidol is also described in which the antiserum is coupled to magnetizable solid-phase medium, and fluorescein-labeled haloperidol is used. The assays have acceptable accuracy, precision, and reproducibility, and are specific for haloperidol and similar butyrophenones, with no significant interference from known metabolites and other drugs. Only the radioimmunoassays have sufficient sensitivity to cover the whole range of haloperidol concentrations in serum. The radioimmunoassay can be used to monitor high concentrations of haloperidol in 150-μL samples or the complete concentration range of 1-mL serum samples that are extracted and concentrated before assay.

Additional Keyphrases: monitoring therapy • treatment of schizophrenia • drug assay • neuroleptic drugs

Haloperidol, a neuroleptic, is widely used as an intramuscular depot injection or orally, to suppress psychiatric disorders. Its use, however, frequently induces extrapyramidal dysfunction, including persistent and sometimes irreversible dyskinesias, which occur most frequently with high-dose regimens or prolonged treatment (1). Individual variations in steady-state concentrations of total drug (2-4) and “free” drug (4) in sera of patients on identical doses of haloperidol have been reported, but these concentrations, total and “free,” significantly correlate with daily dosage (3, 4). The optimal therapeutic effect in each patient therefore depends on individualized dosage and drug monitoring during the long therapy that is often required; such an approach would be expected to reduce the incidence of dyskinesias and other side effects. Specific, precise, and routine assays for neuroleptics should therefore be available.

Existing techniques for haloperidol assay include gas chromatography (5), “high-pressure” liquid chromatography (6), and gas chromatography–mass spectrometry (7), techniques not suited for use in routine analysis of many samples. A radioimmunoassay for haloperidol involving tritiated haloperidol (8) and a radioreceptor assay for antischizophrenic drugs, again involving a tritiated label (9), appear to be suitable for monitoring neuroleptic drugs, but tritiated tracers are relatively costly, and their use is time consuming and not readily applicable to rapid analysis and automation.

We report here the development and evaluation of a radioimmunoassay for haloperidol with use of $^{125}$I, which allows semi-automation of the method. In addition, we examined the potential of a fluoroimmunoassay for haloperidol in serum or plasma, in which antibody to haloperidol is coupled to the magnetizable solid phase and fluorescein-labeled haloperidol analog is the tracer. This technique enables rapid separation in immunoassays, obviates the need for centrifugation (10-13), and removes endogenous serum fluorophores and other interfering components in serum or plasma samples before the endpoint determination is made (14).

Materials and Methods

Materials

Haloperidol hydrochloride was from Searle Lab., Morpeth, Tyne and Wear, U.K.; trifluoperidol hydrochloride, spiroperidol hydrochloride, spirilen, moperone hydrochloride, and pipamperone hydrochloride were from Janssen Pharmaceutica N.V., Beerse, Belgium. The haloperidol metabolites were gifts from McNeil Laboratories, Inc., Fort Washington, PA 19034. The other drugs used in the specificity studies were gifts from their respective manufacturers.

The [3H]spiroperidol (spec. act. 22.1 kCl/mol) was from the Radiochemical Centre, Amersham, U.K., and the [3H]haloperidol (spec. act. 10.5 kCl/mol) was from I.R.E., Brussels, Belgium.