Identification and Purification of a New Non-Heme, Non-Ferritin Iron Protein (36367)

M. BOULARD, † M. DELIN,‡ AND Y. NAJEAN*
(Introduced by E. Beutler)

Laboratoire des Isotopes, Institut de Recherches sur Les Maladies du Sang, Dir. Pr Jean Bernard, Hôpital Saint Louis, Paris 10°

In a previous paper Najean *et al.* have proposed a "mamillary" model to account for iron metabolism in man and animals under normal conditions (1). The model is based on the concept that the biexponential fall-off curve of plasma radioactivity after *in vivo* labeling of transferrin with ^{59}Fe is due to a small non-heme, noncirculating iron pool (pool Q2 in the model, ref. 1) which can exchange iron with plasma transferrin. According to this model, the Q2 pool must be distributed throughout the whole body. If the model is to be validated, the characteristics of this small iron pool must be in good agreement with those calculated for Q2, from *in vivo* studies, using the mamillary model.

Materials and Methods. Adult Wistar rats fed with a standardized iron diet were injected intravenously with a tracer dose of ^{59}Fe (40 μCi/100 g, below U.I.B.C.) diluted in buffered saline. Blood samples were then withdrawn in order to calculate the variations in specific activity of pool Q2 according to the model (1). The animals were sacrificed at appropriate intervals following infusion of the tracer. Lung, liver, spleen, kidney and small intestine were then removed and washed or perfused in cold buffered saline. The tissues were homogenized in cold 0.006 *M* phosphate buffer, pH 7.2 and the homogenates were centrifuged at 80,000*g* at 4° for 1 hr. The clear supernatants were counted for ^{59}Fe radioactivity prior to a 5 hr dialysis in an ultrathimble[1] against the homogenizing buffer. The dialyzed and concentrated supernatants were counted once more for ^{59}Fe activity, in order to estimate the possible loss of iron due to dialysis. The dialysates were then subjected to ion-exchange chromatography on DEAE-cellulose, using the following stepwise elution process: (a) 0.006 *M* phosphate buffer pH 7.2; (b) 0.01 *M* sodium acetate in 2 *M* sodium chloride, pH 4; (c) 1.5 *M* phosphate buffer, pH 7.2; (d) 2 *M* phosphate buffer, pH 7.8. The elution was carried out at 4° and followed for absorbance at 278 and 410 nm on a LKB Uvicord II.[2] The radioactive fractions with maximal absorbance at 278 nm eluted with the acetate buffer were pooled and purified through an ascending SG-25 Sephadex[3] column poured in 0.1 *M* Tris–HCl buffer, pH 8.1, and run with the same buffer. The radioactive fractions collected from the Sephadex column were concentrated and precipitated by ammonium sulfate at a final concentration of 65%. The ^{59}Fe activity of the precipitated material was measured and it was submitted to acrylamide–agarose preparative electrophoresis according to the method of Uriel (2) with a Tris–glycine buffer, $I = 0.05$, pH 8.6. After location of the zones with nitroprussian blue for iron, and Coomassie's dye for protein, the radioactive ferroprotein was extracted from the gel and filtered through a Bio-gel P-30[4] column (2 × 45 cm) with a 0.1 *M* Tris–HCl buffer, pH 8.6. The radioactive iron material was desalted by a 6 hr dialysis in an ultrathimble against 0.006 *M* phosphate buffer, pH 7.2, and used for subsequent chemical

† Chargé de Recherches, Institut National de la Santé Publique et de la Recherche Medicale-Paris, France.

‡ Technicienne, Education Nationale.

* Pr Agrègé, Faculté de Médecine, Paris.

[1] Ultrathimble, Selectra-ultragaines. Schleicher & Schühl, Cassel, West Germany.

[2] LKB, AB. Stockholm, Sweden.

[3] Sephadex Pharmacia AB, Uppsala, Sweden.

[4] Bio-Rad Laboratories, Munich, West Germany.

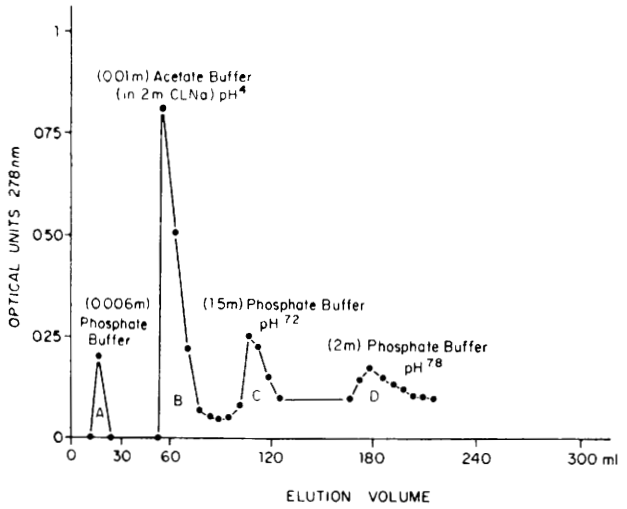

FIG. 1. DEAE-cellulose elution of a rat small intestine subjected to AEP isolation procedure. Peak "A" consists of minor contaminants. Peak "B" contains AEP. Peak "C" was not identified, but never contained ^{59}Fe radioactivity. Peak "D" consists of ferritin.

analysis. When the isolation procedure was applied to red blood cells, the hemoglobin was previously removed by washing the hemolysate on a cation exchange column at pH 6.

Protein assays were carried out according to the method of Lowry *et al.* (3) using crystalline serum albumin as a standard. Iron assays were performed as previously publisted (4). The mean value of the molecular weight of the acetate eluted ferroprotein (AEP) was estimated from the results of these two methods, assuming the presence of one atom of iron for each mole of protein. It was also measured by gel filtration according to the method of Whitaker (5) and finally by amino acid analysis of AEP since no impurities were seen after disc electrophoresis of AEP on 6% acrylamide gels. The electrophoresis was run for 1.5 hr at 4°, with a Tris–

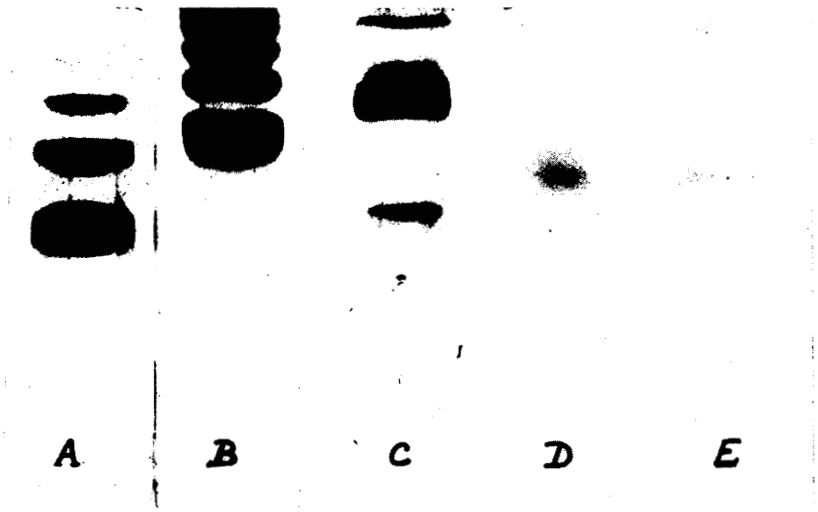

FIG. 2. Disc electrophoresis of AEP on 6% acrylamide gels. A = rat albumin. B = rat transferrin. C = rat ferritin. D = Crude AEP. E = Purified AEP. (See text for details).

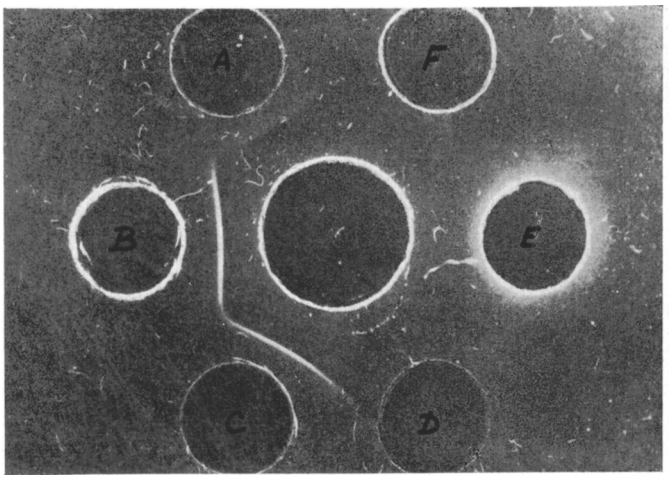

FIG. 3. Immunodiffusion of AEP against a rat ferritin antiserum (center well). A: 0.250 mg/ml ferritin. B: 2 mg/ml 6 × crystallized ferritin. C: 2 mg/ml rat ferritin obtained by AEP isolation procedure (See peak "D", Fig. 1). D: Crude AEP. E: AEP after ammonium sulfate precipitation. F: Purified AEP.

glycine buffer pH 8.6 at 13 V/cm. Alkaline hydrolysis of AEP was also carried out in order to detect tryptophan. Sugars and hexosamines were assayed according to Montreuil *et al.* (6) and to Elson-Morgan (7). These assays were carried out on paper chromatograms of hydrolysates of AEP. AEP was hydrolyzed for 8 hr, at 105°, with 0.5 HCl, under vacuum. Excess HCl was removed on a I R4B amberlite column (in OH-form) since evaporation of HCl destroys the monosaccharide residues. Warren's reagent was used for the detection of sialic acid in AEP (8). The heminic radioactivity precipitation test was carried out according to Labbe and Nishida (9). Immunological studies of AEP were done by immunodiffusion and immunoelectrophoresis of AEP against rat ferritin and rat plasma antisera.[5] The kinetics of iron in AEP were measured from experimental values of ^{59}Fe and protein amounts in purified AEP. The specific activity of AEP was expressed as cpm of ^{59}Fe per mg of protein. It was compared with the values obtained for Q2 when applying the mammillary model (1) to the animals used in the experiments. The exchange of iron between ^{59}Fe labeled AEP and transferrin was studied *in vitro* by a 1 hr incubation at 37° of 2 ml of normal rat plasma to which were added 500 cpm of ^{59}Fe labeled AEP. The transferrin was then precipitated by a specific antiserum and the precipitate was counted for ^{59}Fe activity. A control experiment with *in vivo* labeled transferrin was carried out in order to ensure that iron remained bound to the precipitate and was not released into the medium during the immunoprecipitation.

Results. The data presented were obtained on normal animals. The red cell count and plasma iron were measured in each animal used.

Elution and purification. Figure 1 shows the elution pattern on DEAE-cellulose of a rat small intestine submitted to AEP purification procedure. The acetate eluted ferroprotein isolated and purified as described in the experiment is present in normal rat liver, lung, kidney, spleen, small intestine (upper segment) and red blood cells. When rat transferrin and hemoglobin were submitted to the AEP isolation procedure, they were not eluted in the acetate phase—ferritin was always isolated in peak "D" (Fig. 1). Free $^{59}FeCl_3$, used as control, was not eluted from the column by any of the buffers used. Since higher amounts of AEP were found in the small intestine and since this tissue contains

[5] The antisera were prepared by Pentex, Inc., Kankake, IL, and Eurobio, Paris.

TABLE I. Amino Acid Composition of AEP.

Cys	1.02	Met	2.10
Asp	13.08	Ileu	3.08
Threo	1.09	Leu	8.04
Ser	1.03	Tyr	2.00
Glu	18.09	Phe	1.05
Pro	7.05	Lys	7.12
Gly	14.30	His	1.06
Ala	10.70	Arg	5.04
Val	6.07		

Absence of tryptophan was confirmed by alkaline hydrolysis.

less contaminating ferroproteins than liver or spleen, most of the chemical data were obtained on AEP prepared from small intestine.

Iron and protein content of AEP. After precipitation of AEP by ammonium sulfate there is no loss of iron from the ferroprotein, and the average amount of iron in AEP extracted from 2 g of small intestine is 8 μg. Using the method of Lowry *et al.* an average amount of 1,640 μg of AEP was found in 2 g of small intestine. However, when protein was estimated through nitrogen analysis the average result was 1,857 μg. The iron of AEP was always found in the Fe^{3+} state.

Molecular weight of AEP. According to the iron percentage in AEP and to protein estimations, the molecular weight of AEP is between 11,500 and 13,000, assuming the presence of one atom of iron for each mole of protein. Gel filtration of AEP indicated that the molecular weight was 14,000 $\pm$ 4,000. Since no impurities were found after disc electrophoresis (Fig. 2) AEP has been submitted to amino acid analysis. The results are shown in Table I. They are in good agreement with the preceding ones. They account for the discrepancy between the values obtained from Lowry's method and those given by nitrogen analysis, since they give evidence of only two residues of tyrosine in the molecule of AEP.

Chemical composition of AEP. No sugars and hexosamines were found in purified AEP. Rat transferrin and ferritin used as controls in these experiments gave positive results. Warren's reagent failed to demonstrate the presence of sialic acid in AEP. The presence of heme in AEP was analyzed by the precipitation test of Labbe and Nishida (9) which gave negative results, thus accounting for the absence of absorbance of AEP in the 410 nm region. The heme precipitation test proved to be efficient on ^{59}Fe labeled hemoglobin and on cytochrome *c* used as controls.

Immunological studies of AEP. When AEP was submitted to immunodiffusion against a rat ferritin antiserum, no precipitation line was observed between 2.5 mg/ml AEP and antiferritin, while a 0.250 mg/ml solution of ferritin still gave a sharp line. Immunoelectrophoresis of AEP against a rat plasma antiserum was also carried out and failed to demonstrate the presence of circulating iron binding proteins in AEP. The results of these studies are shown in Figs. 3 and 4.

Iron kinetics of AEP. The values of the specific activity of pool Q2 were determined

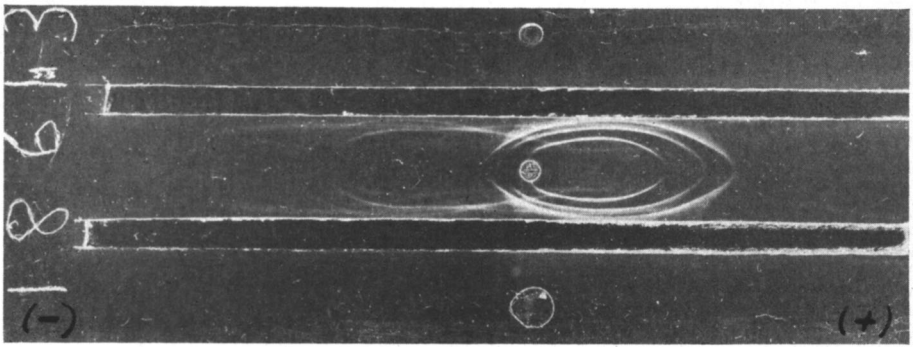

FIG. 4. Immunoelectrophoresis of AEP against a rat plasma antiserum. The upper and lower wells contained, respectively, 1 μl and 5 μl of 2.5 mg/ml AEP. There is no evidence of precipitation line between AEP and rat plasma antiserum.

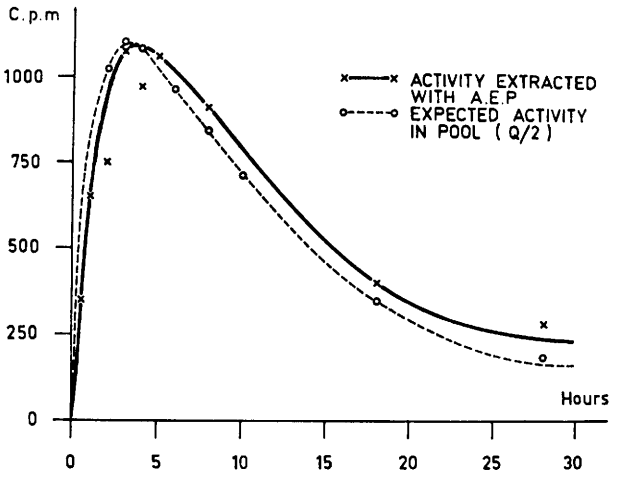

FIG. 5. Comparison of specific activity of AEP (x—x) with specific activity of Pool Q2 (O--O). The curves are parallel. These results are in favor of the identity of Pool Q2 and AEP.

according to previously published methods (1) in the animals used in the experiment. All the values were corrected for the decrease in ^{59}Fe activity, and whenever possible, all the samples of an elution procedure were counted the same day. The specific activity of AEP increases with time after injection of the isotope. The maximal value in AEP specific activity is reached between 2 and 3 hr after infusion of the tracer. The exponential decrease in specific activity of AEP occurs after the third hour after labeling. The mean slope in the normal rat is .055 hr^{-1} and T_{50} is 30 hr (1, 10). Figure 5 shows the comparison between the specific activities of pool Q2 and AEP. Both curves are resumably parallel.

Exchange of iron between ^{59}Fe labeled AEP and transferrin. After a 1 hr incubation with 500 cpm of ^{59}Fe labeled AEP, 2 ml of normal rat plasma were precipitated with a specific anti-transferrin serum. An average of 59% of the initial activity was always recovered with the precipitate after a 1 hr incubation. A control experiment using ^{59}Fe labeled transferrin showed that iron was not released into the medium during the precipitation process.

Discussion. The existence of metabolic pools of iron differing from the actually well-characterized iron binding or containing proteins is still a matter of controversy. On the one hand, different authors have presented evidence for iron proteins acting as heme precursors (11, 12) or as iron transfer proteins involved in iron metabolism in the membrane of the small intestine (13). On the other hand, the physiological and chemical heterogeneity of ferritin has been recently demonstrated (14, 15), and a new heminic component of the red cell has also been discovered (16). It should be pointed out also that mathematical analysis of iron kinetics are in favor of the existence of iron pools with short turnovers (17, 18).

Two hypothesis were at least plausible to account for the small pool in the mamillary model: (a) pool Q2 is kinetically homogeneous but chemically heterogeneous; (b) pool Q2 reflects the activity of a new ferroprotein. It is, in fact, unlikely that pool Q2 could have accounted for a "new" activity of an already well known iron protein, since these proteins are kinetically well defined. The chemical findings are in favor of the existence of a new ferroprotein. AEP is neither a glycoprotein, nor a hemoprotein, and could not be identified as an active ferritin subunit. Nor has it any of the characteristics of the ferroproteins described by other authors (11–13, 16–18).

The proposed hypothesis of the mamillary model supports the contention that pool Q2 contains little iron and is distributed

throughout the whole body. The model also implies that Q2 is noncirculating and is labeled prior to heme synthesis. All these conditions are fulfilled by AEP. Its Fe^{3+} state iron is readily exchangeable with transferrin iron, and AEP is no longer radioactive 72 hr after the tracer infusion.

Preliminary results have shown the presence of an acetate eluted ferroprotein in human spleen and small intestine. Human tissues were surgically removed from patients who were previously labelled with a tracer dose of ^{59}Fe. This human AEP behaves similarly to the ferroprotein extracted from rat tissue. Since variations of pool Q2 are known to occur in various hematological disorders, studies devoted to chemical analysis and metabolic significance of human (AEP) are underway.

The authors gratefully acknowledge Pr D. Hurez, Faculté de médecine d'Angers (France) and Dr. J. L. Preud'homme, Institut de Recherches sur les maladies du Sang (Paris) for helpful discussion and assistance in immunological studies of AEP; also Pr C. M. Lapiere, Université de Liège, Belgium, who has provided amino acid analysis of AEP.

1. Najean, Y., Dresch, C., and Ardaillou, N., Amer. J. Physiol. **213**, (2), 533, (1967).

2. Uriel, J., Bull. Soc. Chim. Biol. **48**, 969 (1966).

3. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

4. Boulard, M., Rev. Fr. Etud. Clin. Biol. **48**, 969 (1966).

5. Whitaker, J. R., Anal. Chem. **35**, 1963 (1956).

6. Montreuil, J., and Spik, G., "Microdosage des glucides," Vol. 1. Faculté des sciences, Lille (1967).

7. I. Smith, ed., "Chromatographic and Electrophoretic Techniques," Vol. I, p. 252. Interscience, New York (1960).

8. Warren, L., J. Biol. Chem. **234**, 1971 (1959).

9. Labbe, R. F., and Nishida, G., Biophys. Biochim. Acta **26**, 437 (1956).

10. Najean, Y., Thèse Sciences, Paris (1965).

11. Lothe, K., Falbe Hansen, I., Clin. Sci. **24**, 47 (1963).

12. Pollycove, M., and Maqsood, M., Nature (London) **194**, 152 (1962).

13. Manis, J., and Schachter, D., Proc. Soc. Exp. Biol. Med. **119**, 1185 (1965).

14. Horowitz, M. L., Ruettinger, M. J., and Munro, H. N., Biophys. Biochem. Acta **200**, 242 (1970).

15. Gabuzda, T. J., and Pearson, J., Biophys. Biochem. Acta **194**, 50 (1969).

16. Morrison, M., Reed, D. W., and Hultquist, D. E., Biophys. Biochem. Acta **214**, 389 (1970).

17. Pollycove, M., and Mortimer, R., J. Clin. Invest. **40**, 753 (1961).

18. Katz, J. H., J. Clin. Invest. **40**, 2143 (1961).

Received Oct. 18, 1971. P.S.E.B.M., 1972, Vol. 139.