Plasma Iron in Pyridoxine-Deficient Swine.* (32531)

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The method proposed by Ramsay(1) for measuring the total iron-binding capacity of plasma is based on the principle that iron bound to transferrin is not adsorbed to magnesium carbonate, whereas iron not bound to transferrin is completely removed by this adsorbant. In this method ionic iron is added to an aliquot of plasma in excess of the iron-binding capacity and the non-transferrin bound iron is removed by adsorption with magnesium carbonate. Iron-containing compounds such as colloidal iron, ferritin and hemoglobin are adsorbed completely or partially by magnesium carbonate. By measuring plasma iron before and after adsorption with magnesium carbonate, but without the addition of ionic iron, non-transferrin-bound iron compounds may be detected in plasma.

Recently, we have observed that the plasma iron concentration of pyridoxine-deficient swine consistently attained levels in excess of the total iron-binding capacity of the plasma. This suggested to us that the plasma of such animals contained iron which was not bound to transferrin.

The purpose of this study is to describe the alterations which occur in the transferrin-bound and non-transferrin-bound iron fractions in plasma during the course of pyridoxine-deficiency in swine and to present data pertinent to the characterization of the non-transferrin-bound iron fraction.

Methods. Swine of the Chester-White breed were housed in individual cages and started on the experimental diet at 3 weeks of age. Pyridoxine deficiency was produced in 24 pigs by feeding a purified diet supplemented with all of the vitamins except pyridoxine(2). Control animals received the same diet to which pyridoxine was added.

The methods used to determine plasma iron (PI) and total iron-binding capacity of the plasma (TIBC) have been described(1,3). Fractionation of total PI into transferrin-bound iron (TBI) and non-transferrin-bound iron (Non-TBI) was accomplished by measuring the iron content of plasma before and after adsorption with magnesium carbonate. The adsorbant was added to plasma in a concentration of 0.65 g/ml plasma and incubated with constant mixing for 45 minutes at 23°C. The TBI was defined as the amount of iron remaining in plasma after removal of the adsorbant. The Non-TBI fraction was obtained from the difference between the plasma iron concentration before and after adsorption with magnesium carbonate. With these conditions for adsorption, ionic iron added to porcine plasma *in vitro* was not adsorbed until the iron binding capacity of plasma was reached, beyond which adsorption was quantitative. Iron dextran, in concentrations less than 250 μg iron/100 ml of plasma, was quantitatively removed from plasma by magnesium carbonate. However, higher concentrations of iron dextran, as well as ferritin iron, iron dextrin, saccharated iron oxide and methemalbumin iron, were incompletely adsorbed from plasma. The *in vitro* addition of twice crystallized horse spleen ferritin to porcine plasma in concentrations ranging from 273 to 2560 μg iron/100 ml of plasma was associated with an increase in the measured plasma iron which equalled 31 to 58% of the ferritin iron added. Of the ferritin iron detected colorimetrically, 19 to 49% (mean 33%) was adsorbed by magnesium carbonate. Since all Non-TBI was not measured by the colorimetric method and since this fraction was incompletely adsorbed by magnesium carbonate, the data for TBI and Non-TBI are only semi-quantitative and the Non-TBI fraction is underestimated.

Gel filtration was accomplished at 4°C with a 2 × 43 cm column of cross-linked dextran gel Sephadex G-200 equilibrated with 0.9% sodium chloride, adjusted to pH 6.7 with sodium bicarbonate. Three ml plasma samples were eluted at a flow rate of 9.5 ml/hour with the solution used for equilibration. Five ml

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TABLE I. Plasma Iron Fractions in Control and in Pyridoxine-Deficient Swine.

Determination	Control	Pyridoxine-deficient
Total plasma iron (PI), μg/100 ml	157 ± 9	680 ± 49
Transferrin-bound iron (TBI), μg/100 ml	159 ± 8	459 ± 13
Total iron-binding capacity (TIBC), μg/100 ml	489 ±24	490 ± 19
% Saturation	32 ± 2	84 ± 3
Non-transferrin-bound iron (non-TBI), μg/100 ml	2 ± 1	233 ± 45

There were 10 animals in the control group and 24 animals in the experimental group. Values in the Table refer to mean and standard error of the mean. The values were selected between the 42nd and 70th days of the deficiency at which time plasma iron concentration in the experimental group was at the maximum.

aliquots collected on a fraction collector were analyzed for absorbancy at 280 mμ and for iron by the plasma iron method.

Results. All of the iron in the plasma of the control animals was bound to transferrin and the per cent saturation of transferrin ranged from 22 to 39 with a mean value of 32 (Table I). The values shown for the Non-TBI fraction are within the experimental error of the methods and are, therefore, insignificant. The total plasma iron was increased in all of the deficient animals, in confirmation of earlier studies (4,5). The increase in plasma iron was due to an increase in both TBI and Non-TBI fractions. In some animals the Non-TBI exceeded the TBI. The transferrin in the plasma of deficient animals was nearly completely saturated with iron.

The sequence with which these changes occurred during the development of the deficiency is shown in Fig. 1. The TBI increased early and was associated with a decrease in the TIBC with the result that the iron-binding protein became saturated. Simultaneously with the saturation of the transferrin, Non-TBI appeared and increased progressively until the 75th day of the deficiency. In the terminal phase of the deficiency, there was a decline in the Non-TBI fraction.

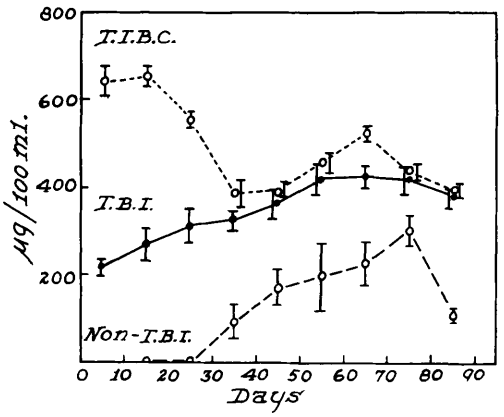

FIG. 1. Total iron binding capacity (T.I.B.C.), transferrin-bond iron (T.B.I.), and non-transferrin-bound iron (Non-T.B.I.) in 23 pyridoxine-deficient swine. Means ± one S.E.M. are illustrated.

The appearance of Non-TBI after the transferrin had become nearly completely saturated with iron, suggested the possibility that the iron in plasma in excess of the TIBC might be non-specifically and loosely bound to other plasma proteins. In this were the case, the Non-TBI would bind to apotransferrin if such were available. To test this hypothesis, plasma from an iron-deficient pig with a PI of 19 μg/100 ml and a TIBC of 372 μg/100 ml was added to plasma from pyridoxine-deficient animals. The unsaturated iron-binding capacity of the added plasma exceeded the Non-TBI present in the experimental plasma by 60 to 120 μg/100 ml. The Non-TBI was measured before and after a 2-hour incubation period at 23°C (Table II).

TABLE II. Non-Transferrin-Bound Iron Before and After Addition of Apotransferrin.

Experiment	μg/100 ml Before	After
1	75	74
2	81	76
3	142	160

No decrease in the Non-TBI was observed under these conditions.

Since transferrin is denatured and precipitated by heat and ferritin is heat stable (6), PI and Non-TBI were measured in the plasma of control and pyridoxine-deficient animals both before and after heat precipitation of the proteins (Table III). Only about

TABLE III. Heat Stability of the Iron Fractions in Plasma of Control and Pyridoxine-Deficient Pigs.

	Before		After*	
	Total PI	Non-TBI	PI	Non-TBI
Plasma	μg/100 ml			
Control	175	0	16	0
Deficient	580	222	200	185
"	633	105	120	95
"	650	156	180	142

* Plasma was heated at 65°C for 5 min with constant stirring and then cooled rapidly to 23°C. The coagulated proteins were washed once with water and the pooled supernatant solutions were dialyzed for 4 hr at 4°C against one % EDTA buffered with 0.01 M tris at pH 7.4 and then for 36 hr against 0.01 M tris-chloride, pH 7.4. Total PI, total plasma iron; Non-TBI, non-transferrin-bound iron.

10% of the iron in the control plasma remained after precipitation of the proteins and dialysis of the supernatant solution. Of this, none was found in the Non-TBI fraction. On the other hand, 83 to 91% of the iron in the Non-TBI fraction of the plasma from pyridoxine-deficient animals withstood this treatment and was recovered in the supernatant solution after heat precipitation of the proteins at 65°C.

Distinctive differences in the elution pattern of iron were demonstrated by fractionation of normal and pyridoxine-deficient plasma by gel filtration (Fig. 2). The filtration of normal plasma through Sephadex G-200 was associated with a single peak of chemically detected iron which was eluted with the final protein peak. In contrast, two clearly separated iron peaks were observed in the chromatographic profile of pyridoxine-deficient plasma. The elution volume of the second peak corresponded to the single peak observed in plasma from control swine. The first peak followed the void volume of the column. Iron appearing in the first peak was associated with a molecule which was excluded by the gel (mol. wt. greater than 200,000). Normal pig plasma to which horse spleen ferritin (Nutritional Biochemicals Corp.) had been added produced the same chromatographic profile with respect to iron as did pyridoxine-deficient plasma.

An attempt was made to label the Non-TBI fraction of plasma by the intravenous injection of 100 μc of transferrin-bound iron[59]

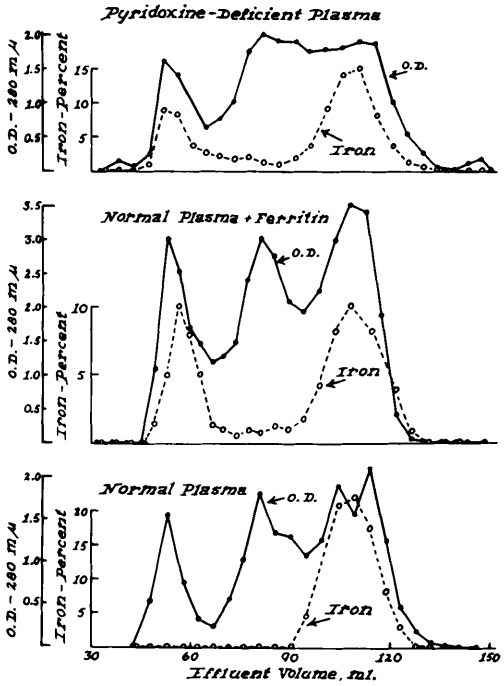

FIG. 2. Gel filtration (Sephadex G-200) of plasma from a control pig, control plasma to which horse-spleen ferritin had been added *in vitro*, and plasma from a pyridoxine-deficient pig.

to each of 3 pyridoxine-deficient pigs. The Non-TBI of these animals was 71 to 160 μg/100 ml at the time of injection. The radioactivity of plasma was not modified by adsorption with magnesium carbonate during the 31 day period following administration of the isotope and, therefore, detectable labeling of the Non-TBI fraction was not observed.

Discussion. The increased concentration of iron in plasma of pyridoxine-deficient swine is due in part to an increase in transferrin-bound iron and in part to the presence in plasma of an iron compound not bound to or by transferrin. The chemical nature of this Non-TBI has not been identified but it resembles ferritin in all respects. Ferritin, like the plasma Non-TBI, is adsorbed from plasma by magnesium carbonate; it does not relinquish its iron to transferrin at physiologic pH, and its heat stability and molecular size are similar to the iron compound in the Non-TBI fraction. Because we have been unable to prepare porcine ferritin suitable for immunization purposes, it has not been possible

to examine the immunologic properties of the Non-TBI. Anti-horse spleen ferritin failed to cross-react with fractions of pig liver or duodenal mucosa which presumably contained ferritin. However, ferritin is the only iron-containing compound of animal tissue origin known to have the properties which characterize the Non-TBI.

Ferritinemia is known to occur in human subjects with severe hepatocellular disease(7). However, clinical, biochemical and histologic evidence of liver disease in the pigs in this and in earlier studies(4,8) has been limited to centrilobular fatty infiltration. Therefore, it seems unlikely that in these animals the ferritinemia is secondary to liver disease.

A defect in heme synthesis is known to be present in pyridoxine deficiency. This is associated with a large non-heme iron pool in circulating erythrocytes(9). It is interesting to speculate that a red cell-to-plasma ferritin pathway may be present in such animals and account for the presence of the Non-TBI fraction in plasma. Since large iron pools are present in normoblasts, liver and other tissues, these may contribute to the plasma Non-TBI pool as well.

The demonstration of two functionally and perhaps kinetically distinct iron compartments (TBI and Non-TBI) in plasma will modify considerably the interpretation of ferrokinetic data in pyridoxine-deficient animals(10) since such calculations are based on the assumption of a single transferrin-bound plasma iron pool.

Summary. The hypersideremia which developed in swine deficient in pyridoxine was found to be due to the presence in plasma of non-transferrin-bound iron as well as to an increase in transferrin-bound iron. The non-transferrin-bound iron fraction was adsorbed on magnesium carbonate, not precipitated by exposure to 65°C for 5 minutes, not transferable to apotransferrin, and excluded by Sephadex gel G-200 (mol. wt. greater than 200,000). It is suggested that the non-transferrin-bound iron was present as ferritin.

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Ethanol Damage to Canine Oxyntic Glandular Mucosa.* (32532)

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The purpose of the work reported here was to determine the threshold concentration at which ethanol damages the oxyntic glandular area of the canine gastric mucosa, to discover whether ethanol is more damaging in acid than in neutral solution, to measure the rate of ethanol absorption over a wide range of concentrations, and to distinguish between effects of a high concentration applied to the mucosa for a short time and those of a lower concentration applied for a longer time. Changes in the barrier function of the mucosa (1,2) were used to assess the damaging effects of ethanol.

In the course of this work, acid secretion

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