Hemosiderin. Isolation from Horse Spleen and Characterization.*† (23272)

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Behrens and Asher(1) separated hemosiderin from horse spleens by utilizing fractional sedimentation with the aid of organic liquids of high specific gravities and analyzed this material for iron, phosphorus, calcium and nitrogen. Recently, Greenberg(2) described a procedure for separating hemosiderin by means of differential specific gravity centrifugation in a concentrated aqueous potassium iodide solution.

The present investigation is concerned with (1) fractional sedimentation of a water insoluble, iron rich complex (hemosiderin) from horse spleen by using salt solutions of relatively high specific gravities and (2) an analysis of some of the constituents of this material.

Methods. Isolation of Hemosiderin. Frozen horse spleens were thawed at 4°C. The tissues were cut into small pieces and 80 g portions were minced to fine suspension in 400 ml of cold 0.9% NaCl in a Waring blendor run at slow speed. The suspensions from 1000 g of spleen were pooled, filtered through a double layer of gauze, the filtrate was centrifuged and the supernatant solution discarded. The residue was extracted repeatedly with 1500 ml of 1 M NaCl to remove the nucleoprotein, detected as a precipitate after diluting the extract with water to yield a 1% saline solution. This step was essential for obtaining a final product free of gummy material. The extracted residue was stirred in 600 ml of saturated KBr solution (sp. gr. 1.38) and the mixture centrifuged. If a considerable amount of material floated on the surface of the supernatant solution, it was resuspended in KBr solution and recentrifuged. The residues were sedimented successively in a 4.42 M KI solution (sp. gr. 1.52) and in a saturated KI solution (sp. gr. 1.72). The hemosiderin was treated with distilled water until the washings were iodide free. The material was dried by lyophilization and stored over P_2O_5. *Analytical procedures. Iron.* The analysis was done by iodometric procedure(3). About 12 mg of hemosiderin were ashed in platinum boat with H_2SO_4 plus HNO_3(4). The ash was dissolved in 25 ml of 1 N HCl, the solution evaporated over open flame and finally brought to dryness on a steam bath. The residue was dissolved in 50 ml of 0.3 N HCl, one drop of bromine was added to convert the iron to the ferric form and the excess bromine was removed by boiling until the final volume was about 25 ml. Three grams of KI were dissolved in this solution, allowed to react with iron in the dark for 15 minutes, and the liberated iodine was titrated with 0.02 N sodium thiosulfate. *Phosphorus.* The ash obtained by above method was analyzed by the Fiske-Subbarow method(5). Nitrogen was determined by the micro-Dumas method.

Results. The weights of 9 spleens ranged from 550 to 1300 g and the yield of hemosiderin varied from a trace to 3 g/spleen. It was not possible to predict the yield of hemosiderin by gross inspection of spleens. Data for iron, nitrogen, phosphorus and ash concentrations of hemosiderins prepared from 9 individual horse spleens are shown in Table I. Variations in concentrations of these constituents indicate that hemosiderin does not have a constant composition. Iron concentrations vary from 24 to 36% which represent a range of 47 to 68% of the hemosiderins when expressed as $Fe(OH)_3$. An inverse relationship exists between iron and phosphorus and between iron and nitrogen; a direct relationship is observed between iron and ash concentrations.

In characterizing hemosiderin, Greenberg (2) calculated μg nitrogen/mg iron and obtained values of 320 and 290 for 2 preparations. In Table I the values for this ratio

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TABLE I. Iron, Ash, Nitrogen and Phosphorus Concentrations in Hemosiderin.

Sample	Iron (%)	Ash (%)	Nitrogen (%)	Phosphorus (%)	$\frac{\mu g\ N}{mg\ Fe}$
1	30.7, 31.7				
2	29.5, 29.6	47.5, 47.7			
3	24.5, 25.0			3.05, 3.28	
4	28.0, 28.1		5.40, 5.40	3.04, 3.05	192
5	35.7, 35.9		3.70, 3.90	1.58, 1.66	106
6	25.0, 26.4	49.2, 49.5	5.43	3.44, 3.74	211
7	31.0, 31.8	50.3, 50.8	4.51, 4.81	2.38, 2.61	148
8	34.5, 36.4		4.15, 4.30	1.62, 1.71	119
9	24.7, 25.8	44.0, 45.0			

range from 106 to 211 for 5 different preparations. Similar calculations applied to hemosiderin analyzed by Behrens and Asher gave values of 111, 179 and 196 for samples containing 35.9, 31.6 and 29.3% iron, respectively.

To test whether each of 3 samples of hemosiderin had a constant composition, they were fractionated in organic liquids(1) with specific gravities greater than that of saturated KI. According to the data in Table II, the fraction which sediments at sp. gr. 2.18 has an iron concentration of about 41% in contrast to the original iron value of 36%. It can be seen that the iron values for the succeeding fractions decrease at lower specific gravities. These results would indicate that hemosiderin is an inhomogeneous mixture with different iron contents.

After slowly agitating 50 mg of hemosiderin in 5 ml of water at room temperature in a sealed test tube for 24 hours, the supernatant solution had a faint yellow tinge and yielded no precipitate with 10% trichloroacetic acid. The test for iron with $K_4Fe(CN)_6$ was negative but on standing a blue precipitate developed, indicating that the yellow color was probably due to a small amount of colloidal iron hydroxide. In contrast, Behrens and Asher(1) and Behrens and Taubert(6) found that appreciable amounts of protein and iron were extracted from their hemosiderin samples, which indicates that hemosiderins prepared by different procedures yield different products.

TABLE II. Fractional Sedimentation of Hemosiderin by the Behrens-Asher Procedure(1). Hemosiderin was ground to fine powder in the Fisher Mortar Grinder.

	% iron in samples		
	2	5	9
Original	29.5, 29.6	35.7, 35.9	24.7, 25.8
Fraction with			
sp. gr. of: 2.18	No sed.	41.25, 41.60	No sed.
1.98	32.8, 33.1	36.1, 36.8	Traces
1.87	30.5, 30.9	32.6, 33.6	"
1.80	28.5, 28.5	No sed.	27.0, 27.4
<1.80	25.4, 25.8	" "	24.1, 25.0

Sp. gravity of 2.18: Ethylenebromide ($C_2H_4Br_2$)
1.98: Mixture of $C_2H_4Br_2$-CCl_4 (2:1)
1.87: " " " (2:2)
1.80: " " " (2:3)

Carbohydrates. After observing that hemosiderin gave a weak Molisch test, a hydrolysate of hemosiderin was routinely prepared by heating 100 mg of hemosiderin in 16 ml of 2 N H_2SO_4 in sealed tubes which were rotated slowly in boiling water for 4 hours. The hydrolysate was diluted to 64 ml with water and filtered. The filtrate was de-ionized by passage through a 13.2 x 2 cm column containing Dowex 50 (H^+ form), then through a 14.5 x 2 cm column containing Dowex-1-formate.‡ The de-ionized solution and column washings were dried by lyophilization.

The paper chromatogram for sugars present in the hemosiderin hydrolysate (Fig. 1) shows presence of galactose, mannose and fucose. According to the intensity of the color, concentrations of mannose and galactose are greater than that of fucose.

The (primary) cysteine-sulfuric acid reaction (PCyRI) for hexoses(8) was positive

‡ Dowex-1 anion exchange resin, 200 to 400 mesh, was washed with 1 N HCl and was converted to the formate by treatment with 0.3 M ammonium formate in 2 N formic acid until the chloride was completely displaced. Excess formate was removed by washing with water.

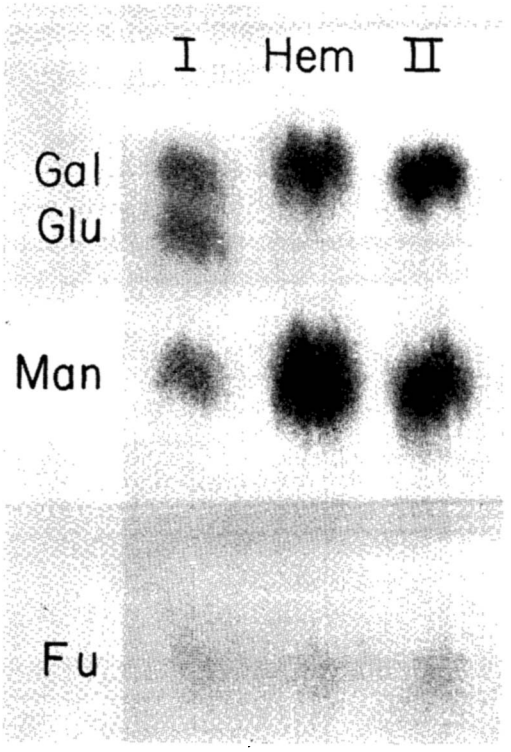

FIG. 1. Paper chromatograms of a hemosiderin hydrolysate (Hem) and of reference sugar mixtures (I and II). 100 mg hemosiderin were hydrolyzed and the deionized solution was lyophilized. Residue was dissolved in 50 μ of water and 20 μl aliquot was applied to Whatman No. 1 paper. The reference solutions contained (I) galactose (Gal) 60 μg, glucose (Glu) 60 μg, mannose (Man) 60 μg, and fucose (Fu) 30 μg; and (II) galactose 90 μg, mannose 120 μg, and fucose 30 μg in 20 μl of water. These sugars were chromatographed with a butanol-water-ethanol (10:2:1) mixture for 3 days and developed by spraying with aniline hydrogen phthalate(7).

and the results with different samples of hemosiderin indicated that the concentration of these sugars was approximately 1%. By determining the values for D_{396}-D_{427}, it was shown that fucose was present at a concentration of about 0.1%.

The Dische-Borenfreund indole reaction (9) and the Elson-Morgan test as described by Boas(10), performed on neutralized hydrolysates, indicated that hemosiderin contained about 1% hexose amine.

A positive Lieberman-Burchard test for cholesterol was obtained in a carbon tetrachloride extract of hemosiderin and several amino acids were demonstrable by paper chromatography in hemosiderin hydrolysates. Histochemical analyses of hemosiderin deposited in tissue cells have also demonstrated the presence of polysaccharides, lipids and proteins(11,12).

Discussion. The earlier attempts(13,14) to isolate hemosiderin and to characterize it chemically were unsuccessful. Satisfactory preparations of hemosiderin(1) were first obtained by fractional sedimentation of dried, powdered horse spleen in organic liquids. A comparison of the analytical data, obtained in the present investigation, with those reported for hemosiderin by Behrens and Asher(1) shows that the nitrogen, iron, ash and nitrogen/iron ratios are similar but phosphorus concentrations and amounts of soluble protein and colloidal iron compounds are different. Greenberg(2) found much higher nitrogen/iron values for hemosiderin prepared by his method and also reported the presence of significant amounts of ferritin. His observation that apoferritin-cadmium crystals could be prepared from hemosiderin was confirmed.

The combination of hexose amine, galactose, mannose and fucose observed in hemosiderin has also been found in plasma γ-globulin and in myeloma proteins(15,16). In addition, fucose has been found to be present in blood group substances(17) and in red cell stroma(18).

Schwietzer(19) used x-ray diffraction to characterize hemosiderin obtained from horse spleens(1) and concluded that this material may contain a crystallized form of iron oxide identical with that of the minerals limonite or lepidocrocite (rubin glimmer). Six samples of hemosiderin, prepared in this laboratory, did not show a crystalline structure by the x-ray diffraction method.§

The data in the present investigation corroborate the earlier work of Behrens and Asher(1) who showed that the insoluble iron-rich particles, obtained from horse spleen, represent a number of hemosiderins with varying iron concentrations. In view of these find-

§ The author is grateful to Dr. R. S. Mitchell of the Dept. of Geology, University of Virginia, for making these determinations.

ings, it would be appropriate to designate these materials as hemosidera.

Summary. 1. A method is outlined for isolating hemosiderin from horse spleen by sedimentation from salt solutions of different specific gravities. 2. The iron, nitrogen, phosphorus and ash concentration varied considerably in hemosiderin samples obtained from different spleens. Fractionation of these preparations by sedimentation in organic liquids showed that fractions could be obtained which varied in their iron concentrations from about 25 to 41% iron. 3. Hemosiderin has been found to contain hexoseamine, galactose, mannose and fucose.

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Fractionation of Radioactive B_{12}-Complex In Kidney Homogenates.* (23273)

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Rosenblum, *et al.*(1), demonstrated that among the various target organs of rats injected with radioactive B_{12} (referred to hereafter as B_{12}*) kidneys contained initially the greatest amount of radioactivity per gram of the wet organ. However, a major portion of its radioactivity disappeared within 21 days, Harte, *et al.*(2). In contrast, the amount of radioactivity initially retained by the liver and pancreas of these animals decreased only little even after as long as 90 days after injection. Furthermore, administration of nonradioactive B_{12} to rats previously injected with B_{12}* resulted in a decrease in radioactivity in liver and pancreas but an increase in kidneys. These unique properties of kidney prompted us to study the manner in which B_{12} is retained by this organ. To this end, the ability of the vitamin to combine with components of kidney tissue has been examined by various chemical fractionation procedures and differential centrifugation. The results of such studies are reported in this communication.

Methods. Preparation of Homogenates. Adult female rats from our stock colony were injected subcutaneously with 1.0 μg Co^{60}-

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