IRON METABOLISM IN EXPERIMENTAL ANEMIA

"Availability of Iron"

By P. F. HAHN, Ph.D., and G. H. WHIPPLE, M.D.

(From the Department of Pathology, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.)

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"Availability of iron" is a term much used in current writings, particularly in those articles dealing with various forms of anemia.

In 1930 Hill (9) introduced the dipyridyl method for the determination of iron. When dipyridyl is added to a buffered suspension of animal tissue or foodstuff which has first been reduced with hydrosulfite, a red color is produced which is due to the formation of an iron complex. Iron combined as hematin is said not to give this reaction.

Elvehjem and Hart and their associates (7, 17) claim that the dipyridyl reagent reacts only with the iron which is not bound in complex form (non-hematin iron), and that since hematin iron is not available for hemoglobin production, the amount of iron in the food which can be utilized by the body is measured by this method. We propose to offer evidence that such a premise is untenable.

Methods

The dipyridyl method for determination of "available iron" was as described by Shackleton and McCance (16) with the exception that in addition to filtration of the extracted tissue suspension, centrifugalization was used to produce a clearer solution for final comparison. The method as applied to tissues and foodstuffs is open to criticism. Many of the failings have been noted by Shackleton but it would seem that little has been done to correct them. Many unjustified assumptions must be made and certain weaknesses in the procedure shake one's confidence in the method accuracy. Inaccuracies may be introduced due to interference of natural pigments, use of a comparator in the final estimation, and superior buffering of some natural materials over the acetate-acetic acid. We include below a few values obtained by the method as used in different hands (16, 17, 18) to illustrate the validity of these criticisms (Table 1).

The method for determination of total iron was a modification of that originally reported elsewhere (6). The material was ashed by the wet procedure involving sulfuric and perchloric acids. After diluting to a known volume aliquots were transferred to 100 ml. centrifuge tubes and neutralized with 40 per cent NaOH using phenolphthalein as an indicator. The standards at this point were treated with concentrated sulfuric acid and all samples and standards received the same amount of NaOH, acid being added as found necessary in each case. In this way

TABLE I

	Material	Total Fe	"Available Fe"
		mg. per cent	*per cent*
Elvehjem, Hart, and Sherman	Pig's liver	—	60
Shackleton and McCance	" "	20	80
Hahn	" "	21	48
Elvehjem, Hart, and Sherman	Beef muscle	—	50
Shackleton and McCance	" "		10–25
Shackleton and McCance	Apricots (dry)	4.08	98
" " "	" (fresh)	0.37	95
Hahn	" (dry)	4.2	114
Smith and Otis	" (dry)	—	58
Sturgis and Farrar	Salmon bread	—	50
Hahn	Complete diet*	2.4	58
Shackleton and McCance	Salmon	0.89	94
Hahn	"	0.40	120
Elvehjem, Hart, and Sherman	Spinach	—	25
Shackleton and McCance	" (raw)	2.96	68
" " "	" (cooked)	4.15	57

* Salmon bread 200; Klim 20; salmon 75; salt mixture 1 gm.

the contamination of the standards and samples with iron by reagents was to a great extent constant in amount. After standing overnight they were centrifugalized for 20 minutes at about 2500 R.P.M., the supernatant liquid was poured off and the precipitate was dissolved in 2 ml. of 2/3 N HCl. The solution was transferred to a 25 ml. volumetric flask and the tube rinsed with 7 ml. of M/5 potassium acid phthalate solution and the rinsings added to the flask. Further rinsing of the tube was done with about 8 ml. of distilled water. 2 ml. of 0.2 per cent solution of 7-iodo-8-hydroxyquinoline-5-sulfonic acid were added and

after making to volume comparison was made in a colorimeter. If the readings of the unknowns varied by more than 10 per cent from the standards a correspondingly larger or smaller aliquot was taken and the determination repeated. This has been shown to be necessary due to the difference in color obtained at various iron concentrations as has been mentioned elsewhere (6).

Care of dogs, standard diets, and method procedures related to the anemic dogs have been described in detail (21).

EXPERIMENTAL OBSERVATIONS

A number of determinations were carried out with individual food materials as well as with a complete salmon bread diet. Comparison of the resulting values for "available iron" with those reported by others bears out the criticisms listed above. The procedure apparently does not give similar results in different hands.

TABLE 2

Dog No.	Tissue	Total Fe	"Available Fe"	"Available Fe"
		mg. per cent	*mg. per cent*	*per cent*
34-5	Liver	1.24	0.5	40
	Heart	2.91*	1.0	34
	Spleen	7.18†	1.6	22
30-115	Liver	3.16	1.4	44
	Heart	2.74*	1.4	51

* About 1/3 to 1/2 of this represents muscle hemoglobin iron (6).

† Some of the iron in this tissue is explained by blood not removable by perfusion.

Dogs with long continued anemia due to blood withdrawal fed a diet poor in iron will show complete exhaustion of all iron reserve stores (6). The iron remaining in the liver and other tissues appears to be essential to cell life, cannot be further reduced by any of the procedures tried, and cannot be drawn upon to produce new hemoglobin. This tissue or "parenchyma iron" may be in part cytochrome iron and it is probably not in inorganic form.

Table 2 gives the analysis of various organs of two dogs whose iron reserve had been completely exhausted by long continued anemia due to blood loss plus a diet very low in iron. The figures are self-

explanatory. Under ether anesthesia the viscera and other tissues were rendered blood-free by viviperfusion.

DISCUSSION

Iron salts frequently used (ferric and ferrous chloride), and presumably any ionizable iron salts, react quantitatively with the dipyridyl reagent and therefore are spoken of as 100 per cent available. Yet when fed to our standard anemic dogs in optimum amounts these salts are but 30 to 40 per cent available—using the same terms, and similar amounts of iron as given in meat or liver. Numerous experiments in dogs (20) given 40 mg. of iron *by mouth* per day for 14 days (total 560 mg. of iron) show a *net production* of 50 to 55 gm. new hemoglobin above the basal ration control output. If given *by vein* this same dose of iron will yield a 100 per cent return of hemoglobin, or 160 to 170 gm. hemoglobin net output. Therefore, this iron is 100 per cent "available" by the dipyridyl method and by intravenous test, but only about 35 per cent "available" when given by mouth. Furthermore, when larger doses of iron are given by mouth the "availability" falls off rapidly and with 400 mg. iron per day, or ten times the optimum dose, we record only double the production of new hemoglobin (95 to 100 gm. net output). The term "available iron" boils down to iron not in the form of hematin compounds, and what will happen to this "available iron" in the intestinal tract has no relation to the dipyridyl test but is conditioned by a great variety of factors, some known (4, 8) and others not recognized as yet.

From work in this laboratory it has been found in a long series of experiments that the feeding of 300 gm. of pig liver per day to a standardized anemic dog over a 2 week period will result in the production of an extra 95 gm. of hemoglobin over and above the control level. Assuming an average liver iron content of 20 mg. per cent, the extra iron intake would amount to 840 mg. during the feeding period. The iron corresponding to the surplus hemoglobin (95 gm.) is 318 mg. Therefore there is a return of 38 per cent of the metal as fed. This is somewhat less than the figure for the availability of iron in pig liver recorded above (48 per cent) as determined by one of us using the dipyridyl method, and considerably less than the values recorded by others, 60 per cent (17) and 80 per cent (16). The

physiological availability of iron again does not correspond to the "availability" as determined by dipyridyl.

Apricot feeding presents experimental data of interest in this connection. Feeding 100 gm. of dried apricots a day for 2 weeks to the standard anemic dog results in a surplus hemoglobin production of 42 gm. The iron contained is 100 per cent "available" (16) but amounts to only 4 mg. per 100 gm. of apricots—or 56 mg. per 14 days. If the iron was quantitatively changed to hemoglobin it would account for only 19 of the 42 gm. of hemoglobin actually produced.

It is not difficult to dissociate the iron from other potent factors (organic) in a variety of tissues. For example various fractions have been produced from liver, spleen, heart, and kidney which contained very small amounts of iron and yet showed much capacity to cause regeneration of new hemoglobin in anemic dogs ((12) see Table 5).

Liver ash (13) when fed to the anemic dog will produce about one-half as much hemoglobin as does the whole fresh liver. It would seem that the other half of the liver potency resided in the organic fraction.

Sturgis and Farrar (19) have shown that the salmon bread diet used in this laboratory (21) and the Cowgill diet (1) contained iron which was 50 per cent available by dipyridyl analysis. The Cowgill diet contains less iron but produces much more new hemoglobin in anemic dogs.

Even if iron were the only limiting factor involved in hemoglobin production in anemia our problem would not be simplified by the introduction of the term "available iron (dipyridyl)." The ultimate utilization of iron given orally is to a great degree governed by absorption. It is quite true that hematin iron is not absorbed to any great extent, if at all, but inorganic iron also is absorbed very poorly. Therefore, we have gained nothing by complicating the issue in stating that because 100 per cent of the metal in one of the inorganic salts reacts with dipyridyl it is all available *for absorption* when we know that actually very little will be absorbed.

In addition to this we know that iron is *not* the only limiting factor in the treatment of anemia. One or more organic factors are involved as can readily be appreciated from a survey of the work referred to above. Some would argue from results obtained in studying nutritional anemia in rats that the efficacy of liver in treatment of secon-

dary anemias in general was directly proportional to the available iron and copper content (7). This may be true as regards the milk nutritional anemia in rats. Here a deficiency in copper and iron has been produced and, as such, would be expected to respond to iron and copper therapy. It has been pointed out that nearly every article of food contains copper, in amounts varying from 0.1 mg. per kg. in celery to 44 mg. per kg. in fresh calves' liver (11).

There has yet to be found any condition in which a human being has been shown to be copper deficient or even notably low in copper as regards tissue content. If anything, quite the reverse has been found. Even the poorest of human rations contain significant quantities of copper. The tendency is for copper to be increased in many diseases, among which are anemias of various forms (3, 5, 14, 15). We do not deny that very small amounts of copper play a part in internal metabolism. Evidence has been forthcoming to show that it may influence the interchange of iron in the body (2, 10). But it has not been demonstrated to be lacking in any condition *except nutritional anemia in rats*, and so cannot be considered in any way as a limiting factor in the anemia of dogs due to blood loss or in secondary anemias of human beings generally.

SUMMARY

In experimental anemia in dogs due to blood loss the term "available iron" as determined by the dipyridyl test has no physiological significance. Iron salts (100 per cent available by dipyridyl) given in optimum dose (560 mg. per 2 weeks) will cause a net production of 50 to 55 gm. hemoglobin above the control base line in anemic dogs. This means that an iron salt which is rated as 100 per cent available by the dipyridyl test is only 35 per cent *physiologically available.*

The term "available iron (dipyridyl)" simmers down to iron not in the form of hematin compounds. The absorption of this "available iron" is conditioned by a great variety of factors, many unknown at this time.

Iron is indeed an elusive sprite whose "availability" or comings and goings cannot be determined in dogs by dipyridyl—perhaps only in part by studies of absorption and excretion.

Liver contains "available iron (dipyridyl)" but also *organic* factors

influencing hemoglobin regeneration in anemia as *liver ash* contains only about 50 per cent the potency of the whole liver.

One can readily dissociate the iron from other potent factors in various tissues. Fractions of heart, liver, spleen, and kidney may contain very little iron yet cause much hemoglobin regeneration in anemic dogs.

No investigator has reported any condition of copper deficiency in man or dog. In fact, in anemias copper is usually above normal concentration in the liver. It is unlikely, therefore, that in experimental anemia in dogs and in the various anemias of man, any significance attaches to the intake of copper.

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