Storage iron in 'muscle'

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SYNOPSIS In Bantu subjects with iron overload iron is visible in skeletal muscle cells and in the tissue histiocytes which lie between these cells. In the present study the concentrations of 'muscle' iron were measured chemically in subjects with varying hepatic storage iron concentrations. The results indicate that the concentrations of storage iron in 'muscle' are much lower than those in the liver. However, the muscle mass is so large that the total amount of iron present is at least equal to that in the liver in subjects with normal body stores. The concentrations of iron in 'muscle' are raised in subjects with iron overload but the degree to which they rise is far less than occurs in the liver; a thirtyfold increase in hepatic iron concentrations is associated with only a sixfold increase in 'muscle' iron. Experiments in rats revealed that storage iron in 'muscle' represents a relatively non-miscible pool which responds very little to acute changes in the iron environment.

Most of the body iron stores are situated in the liver, the spleen, and the bone marrow, but it has been shown that there is also an appreciable amount of storage iron in skeletal muscle. Roth, Jasinski, and von Bidder (1951) found that the mean concentration of storage iron in this tissue was 40 $\mu\text{g/g}$ wet weight in men and 21.6 $\mu\text{g/g}$ in women. However, Hallgren (1954) reported lower results, with concentrations between 5 $\mu\text{g/g}$ and 18 $\mu\text{g/g}$ in most adults. He found no correlation between the storage iron concentrations in muscle and liver. Sheldon (1935), on the other hand, observed high concentrations of iron in the skeletal muscles of patients with haemochromatosis, a finding which suggests that the amount of storage iron in this tissue increases in parallel with that in other organs such as the liver. Hallgren (1954) was not able to establish definitely whether the storage iron in muscles could be utilized for haemoglobin synthesis. He nevertheless believed that such iron was less available than that in the liver, spleen, and bone marrow. This contention was supported by the previous results of experiments carried out by Hahn and Whipple (1936) in dogs, since they found that there was no decrease in the muscle iron concentrations of animals venesected repeatedly over a period of two months. However, there is other evidence to suggest

that muscle iron stores are available for haemoglobin synthesis. For example, Roth *et al.* (1951) observed that the storage iron concentrations in muscles were lower in iron-deficient individuals than they were in normal human subjects.

The present investigation was carried out for two reasons. First, there is a lack of agreement concerning the amounts of storage iron normally present in muscles and, in addition, there is very little information on the degree to which these stores can increase in states of iron overload. It was felt that the problem might be answered by estimating the iron concentrations in the livers and muscles of a group, such as the Bantu, in which a large proportion of individuals have varying degrees of iron overload. These results could then be compared with those obtained in white subjects. A second objective of the current study was to try and find out experimentally what happens to muscle iron stores in animals when the total body iron content is altered by dietary means. The storage iron concentrations in liver and muscle were therefore measured serially in groups of rats maintained on diets with low, normal, and high iron contents.

MATERIALS AND METHODS

HUMAN STUDY It was established initially that the concentration of storage iron in muscles from the gluteal, pectoral, deltoid, thoracic, and lumbar regions did not differ by more than 10%. Thereafter specimens of pectoral muscle and of liver were collected from

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26 white and 25 Bantu subjects, and stored at minus 20°C until the analyses were performed. After thawing, the muscle fibres were separated from the fibrous connective tissue using glass microscope slides. Approximately 4 g muscle and 1 g liver were weighed accurately and homogenized in sufficient distilled water to give a final volume of 25 ml.

Various procedures were carried out on 5 ml aliquots of the homogenate. Total iron was determined by the thioglycolic acid method (McCance, Widdowson, and Shackleton, 1936) after digestion with sulphuric acid, nitric acid, and hydrogen peroxide. The concentrations of insoluble iron and of ferritin iron were estimated on a second aliquot. After centrifugation at $9,000 \times g$ for 20 minutes the supernatant solution was separated and the total iron content of the precipitate was determined by the method already described. The ferritin in the supernatant solution was precipitated with an antiserum prepared in rabbits. After adding the antiserum, the mixture was allowed to stand overnight at 4°C and was then centrifuged at $3,000 \times g$ for 20 minutes. The iron content of the deposit was determined after acid digestion as before. A third aliquot was used for determination of non-haem iron, using a modification (Gale, Torrance, and Bothwell, 1963) of the method of Brückmann and Zondek (1940). After adding 5 ml saturated sodium pyrophosphate and 5 ml 20% trichloroacetic acid, the mixture was stirred at 80°C for 10 minutes. It was cooled, filtered through acid-washed filter paper, and the iron content of an aliquot of the clear filtrate measured using the thioglycolic acid method. Finally, the method of Bothwell, Roos, and Lifschitz (1964) was used to determine the concentration of haemoglobin iron on a fourth aliquot. In this method the tissue is hydrolysed in normal sodium hydroxide and a pyridine haemochromogen determination is carried out on the supernatant solution. All results were expressed as $\mu\text{g/g}$ wet weight.

ANIMAL STUDY Weanling rats of the Wistar strain were divided into three groups. All were given a basic low iron diet similar to that described by Valberg, Taylor, Witts, and Richards (1961). Group 1 received no iron supplementation, while ferric citrate was added to the diets of groups 2 and 3 to give final iron concentrations of 0.025% and 0.4% respectively. At the start of the experiment six animals were killed. Specimens of muscles were obtained from the femoral and lumbar regions, and

the livers were perfused with saline through the portal veins in order to wash out most of the blood. Total iron concentrations were then determined in the same way as in the human study, except that the tissues were not stored before carrying out the analyses. The remaining animals were maintained on their respective diets, and at suitable intervals thereafter four animals from each group were killed and studied in the same way. The total duration of the experiment was 12 months.

RESULTS

HUMAN STUDY Before considering the significance of the observations it was important to establish the validity of the various measurements that were carried out. This was done by correlating the results obtained by the different methods. For example, it would be anticipated that the figure obtained by adding together the haem and non-haem fractions would be equal to the direct estimate of total iron. Similarly the figure for ferritin iron should approximate to the difference between haem iron and soluble iron (derived by subtracting insoluble iron from total iron). Furthermore, there should be reasonable agreement between the figure for non-haem iron and the sum of the insoluble and ferritin fractions.

The agreement of the methods was tested by deriving a linear regression equation of the form $y = Ax + B$ for each of the comparisons. The equations were derived using a least squares technique in which it was assumed that x was an independent variable and that y varied only with x . Regression equations for the various comparisons were derived separately for both the liver and muscle results.

The results of the comparisons that were made (Table I) indicate a reasonable degree of agreement, especially when it is realized that the estimations were carried out on separate aliquots from a number of tissues, many of which contained relatively low concentrations of iron. It was, however, apparent that the results obtained for ferritin iron using an immunochemical technique gave somewhat lower

TABLE I
REGRESSION EQUATIONS FOR COMPARISONS OF VARIOUS METHODS

	Tissue	Regression Equation	Coefficient of Correlation
Total iron versus haem iron + non-haem iron	Liver	Total iron = $0.93 (\text{haem iron} + \text{non-haem iron}) + 34.1$	0.99
	Muscle	Total iron = $0.92 (\text{haem iron} + \text{non-haem iron}) + 7.3$	0.99
Soluble iron ¹ - haem iron versus ferritin iron	Liver	(Soluble iron - haem iron) = $1.6 (\text{ferritin iron}) - 90.4$	0.94
	Muscle	(Soluble iron - haem iron) = $1.1 (\text{ferritin iron}) - 3.2$	0.88
Non-haem iron versus ferritin iron + insoluble iron	Liver	Non-haem iron = $1.1 (\text{ferritin iron} + \text{insoluble iron}) + 2.2$	0.99
	Muscle	Non-haem iron = $1.1 (\text{ferritin iron} + \text{insoluble iron}) - 0.9$	0.98

¹Soluble iron derived by subtracting insoluble iron from total iron.

values than were obtained by subtracting haem iron from soluble storage iron.

The concentrations of ferritin iron, haemosiderin iron, and total non-haem iron which were present in the livers and muscles of all the subjects studied were tabulated (Table II). The concentrations of storage iron in muscle were a good deal less than those in the liver, and this was especially so in siderotic subjects. If the 22 individuals with the lowest hepatic iron concentrations are considered, the relative mean figures in liver and muscle were 171 $\mu\text{g/g}$ and 26 $\mu\text{g/g}$ respectively. Comparable figures for the 17 individuals with the highest hepatic iron concentrations were 5,500 $\mu\text{g/g}$ for liver and 152 $\mu\text{g/g}$ for muscle. It was thus apparent that the concentrations of storage iron in muscle rose when the body iron stores were increased. However, an approximately thirtyfold increase in the hepatic iron concentration was accompanied by only a sixfold increase in muscle storage iron.

While these results indicate that the concentrations of hepatic storage iron are always a good deal greater than those in muscle, they give no idea of the total quantities stored in these two organs. If the livers are assumed to have weighed 1,700 g and the total body skeletal muscle mass to have been 30,000 g, the estimated quantities of iron in the liver and muscles at various storage iron levels can be calculated (Table II). It can be seen that the computed quantities in skeletal muscle were more than double those in the liver at the lower levels, but only approximately half those in the liver in subjects with markedly increased stores. It is, however, possible that the figures for muscle figures are falsely high since the actual concentrations present in subjects with reduced or normal stores were very low, and minor degrees of contamination could therefore influence the results significantly. Nevertheless, even if the results obtained with the sensitive and specific immunochemical technique for ferritin

are considered, the mean figure in subjects with hepatic iron concentrations less than 1,000 $\mu\text{g/g}$ was still 406 mg. These results therefore emphasize the fact that significant amounts of iron are normally stored in skeletal muscle, and that the quantities in individuals with iron overload may reach several grams.

In a further analysis, the proportions of storage iron present as ferritin and haemosiderin were compared. (In these calculations the insoluble iron fraction was assumed to represent haemosiderin.) The ratio of ferritin to haemosiderin iron for both liver and muscle was calculated in each individual. In this way it was possible to find out whether there was any change with increasing degrees of iron overload. The mean value for the ferritin/haemosiderin ratio was 0.84 in livers in which the iron concentration was less than 1,000 $\mu\text{g/g}$ as compared with a figure of 0.27 in those livers in which the concentration was more than 1,000 $\mu\text{g/g}$. Comparable figures for muscle were 0.85 at concentrations less than 100 $\mu\text{g/g}$ and 0.54 for those in which the concentration was more than 100 $\mu\text{g/g}$. These results indicate that the amounts of haemosiderin and ferritin present in liver and muscle are roughly the same at low and normal iron concentrations. When iron overload is present most of the hepatic iron stores exist as haemosiderin, but the trend is less evident in muscle. This is presumably due to the fact that the iron concentrations in muscle are still relatively low even in the presence of iron overload.

Finally, the location of the storage iron in muscle was studied histologically after staining with potassium ferrocyanide. Deposits of haemosiderin were visible only in those specimens with increased concentrations of storage iron on chemical analysis. In such circumstances iron was visible within muscle cells but significant deposits were also present in tissue histiocytes. It was, however, difficult to

TABLE II

TOTAL NON-HAEM IRON CONCENTRATIONS IN LIVER AND SKELETAL MUSCLE¹

No. of Subjects	Liver			Estimated Total Non-haem Iron Content (mg)	Muscle			Estimated Total Non-haem Iron Content (mg)
	Non-haem Iron Concentrations (μg/g)				Non-haem Iron Concentrations (μg/g)			
	Insoluble	Ferritin	Total		Insoluble	Ferritin	Total	
5	57	16	58	99	15	5	17	504
6	83	67	142	241	16	17	34	1,005
11	114	93	240	408	16	14	27	824
5	164	185	390	663	24	16	38	1,146
7	409	283	716	1,218	44	22	64	1,924
9	1,934	627	2,696	4,584	50	34	91	2,720
8	6,621	1,077	8,655	14,714	129	60	222	6,645

¹The subjects were arbitrarily divided as follows into seven groups, depending on the hepatic iron concentrations:—0-99 $\mu\text{g/g}$; 100-199 $\mu\text{g/g}$; 200-299 $\mu\text{g/g}$; 300-499 $\mu\text{g/g}$; 500-999 $\mu\text{g/g}$; 1,000-4,999 $\mu\text{g/g}$; > 5,000 $\mu\text{g/g}$.

FIG. 1. Comparison of total iron concentrations in the livers and muscles of rats maintained on diets with differing iron contents (closed stars = animals at beginning of study; open circles = low iron diet; open squares = normal iron diet; closed circles = high iron diet).

judge the relative proportions of iron in each site, since there was considerable variation between different muscles and even within the same muscle. It is therefore apparent that what has in the past been regarded as 'muscle iron' may, to a greater or lesser degree, represent 'reticuloendothelial iron'. At the same time it is entirely possible that the major portion of this iron is actually located within the muscle cells in individuals with normal amounts of storage iron. Because of these uncertainties it was felt that the term 'muscle' iron should be used to describe the chemical findings in the present study.

EXPERIMENTAL STUDY Although an attempt was made to modify the storage iron content of skeletal muscle in rats by manipulating the dietary iron content, the results were negative (Fig. 1). The mean total iron concentration was 21 $\mu\text{g/g}$ at the beginning of the experiment and by the end of the study there had been very little change in any of the three groups. Mean values were as follows: low iron diet 24.3 $\mu\text{g/g}$, normal iron diet 25.2 $\mu\text{g/g}$, and high iron diet 32.6 $\mu\text{g/g}$. In contrast, there was a marked rise in the hepatic iron concentrations of animals maintained on the diet, from a mean starting value of 286 $\mu\text{g/g}$ to 1,804 $\mu\text{g/g}$ at the end of the experiment. The group which was kept on a low iron diet had a mean hepatic iron concentration of only

50 $\mu\text{g/g}$ at the end of the study. These results suggest that 'muscle' iron stores in the rat are relatively non-miscible, and thus respond only slowly, if at all, to changes in the iron environment.

DISCUSSION

The present investigation was undertaken in order to define the quantities of iron normally stored in skeletal 'muscle' and to find out to what degree these stores expand in states of iron overload. In addition, animal experiments were carried out to ascertain how rapidly 'muscle' stores respond to changes in the iron environment. Before discussing the results in more detail it is important to define what is meant by 'muscle' stores. In subjects with normal amounts of storage iron in the body it was not possible to ascertain where the storage iron in muscle was located. However, in Bantu with iron overload, histological evidence was obtained which indicated that a significant proportion was situated in tissue histiocytes between the muscle fibres. The term 'muscle' iron is therefore a loose one, which describes a non-homogeneous storage depot in which the iron is probably distributed in varying proportions between muscle and reticuloendothelial tissues.

The present investigation has revealed that there

is a moderately good correlation between the concentration of storage iron in liver and 'muscle' in human subjects. For example, those individuals with heavy storage iron deposits in the liver have greater 'muscle' non-haem iron concentrations than do normal subjects. Results in subjects with low hepatic iron concentrations were, however, less predictable, and there was no corresponding marked fall in the 'muscle' iron values. This may be, in part at least, a function of the methodological difficulties involved in measuring very small amounts of iron in biological tissues. While the present results indicate that 'muscle' iron stores increase in parallel with those in the liver, the increase occurs at a much slower rate. With low stores the ratio of the liver non-haem iron concentration to the 'muscle' non-haem iron concentration was approximately 4 : 1. However, in the presence of greatly increased stores the ratio rose to approximately 40 : 1.

When these figures were related to the weights of the respective organs, it became apparent that the calculated figures for total storage iron in skeletal 'muscle' were significantly greater than those in the liver in subjects with low or normal stores. This was in contrast to the findings in individuals with iron overload, since under such circumstances the mean storage iron content of the liver was about double that in 'muscle'. The relatively large amount of iron normally present in 'muscle' was surprising and raised the possibility that some methodological error might account for them. In this context, it was noteworthy that the actual concentrations of iron that were measured were comparatively low, so that any extraneous contamination could influence results significantly. However, every effort was made to avoid such contamination, and the several different methods which were used were in reasonable agreement. Furthermore, if the results obtained for ferritin iron only are considered, then the mean amount of iron stored in 'muscle' at low and normal storage levels was still at least equal to that stored in liver (approximately 400 mg in 'muscle' versus 300 mg in liver).

These various results can be put further into perspective by relating them to figures obtained by other workers. There is evidence that the non-haem iron content of the liver in normal subjects is usually between 200 and 500 mg (Sheldon, 1935; Hallgren, 1954; Morgan and Walters, 1963; Mayet and Bothwell, 1964). The spleen normally contains only 15 to 20 mg non-haem iron (Roth *et al.*, 1951; Hallgren, 1954; Morgan and Walters, 1963). The third major site of iron storage is the bone marrow. Results obtained using an isotopic dilution technique in 24 normal living subjects suggest a mean figure of about 230 mg (Gale *et al.*, 1963), which is in close

agreement with results obtained by chemical methods (Hallgren, 1954). When these various figures are added together it is apparent that the liver, spleen, and bone marrow normally contain a total of between 500 and 1,000 mg storage iron. However, it has been estimated by the repeated phlebotomy technique that mobilizable iron stores are between 1,000 and 1,500 mg in normal male subjects (Haskins, Stevens, Finch, and Finch, 1952). It is therefore apparent that there must be another major site of iron storage, and the present results suggest that this is located in skeletal 'muscle'. While it is not possible to define the extent of this store with precision, it probably represents at least a third of normal body stores. In this context, it is of interest that data obtained on the ferritin content of skeletal muscles in dogs (Mazur and Shorr, 1950) suggest that about a third of the storage iron is contained in 'muscle' in this species also.

In an extension of the present investigation, rats were studied in an attempt to find out how responsive 'muscle' iron is to changes in iron nutrition. Three groups of animals were exposed for 12 months to diets with widely differing iron content. At the end of this time there were clear-cut differences between the hepatic iron concentrations in the animals maintained on low, normal, and high iron diets. In contrast, no such change was demonstrated in 'muscle'. It is, however, not clear whether the results in rats are applicable to human subjects, since the concentrations in the 'muscles' of animals with hepatic siderosis were much lower than would have been expected on the basis of the human studies. This suggests that there may be species differences, and that the 'muscle' storage iron concentration in human subjects more closely reflects their iron status than it does in rats. In an attempt to obtain more information on this point, muscle biopsies have been performed on two subjects with idiopathic haemochromatosis (Torrence, 1967). These patients had been venesectioned repeatedly over a number of years, and both manifested evidence of iron deficiency. In spite of this the total iron concentrations in the muscle specimens were 45 and 79 $\mu\text{g/g}$ respectively. These findings indicate that 'muscle' iron stores may also be relatively unavailable in human subjects.

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