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On the Distribution of Injected Radioactive Iron in Guinea Pigs and its Rate of Appearance in Some Hemoproteins and Ferritins

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I. INTRODUCTION

During the last ten years knowledge about the metabolism of iron in mammals has been rapidly growing [1-3]. One of the reasons for this development is the improved knowledge about the individual iron-containing substances that are present in the body. Another reason is the availability of radioactive isotopes of iron. The pathway of iron in the mammalian organism can now be outlined [1]. For convenience it will be considered in its parts.

1. Absorption. It seems to be a well established fact that the mammalian organism will take up iron from the food only to the extent that there is a need for it in the body. Thus an increase in iron uptake occurs after bleeding and during pregnancy or rapid growth [4-12]. Granick [13] has shown that ferritin can be demonstrated in the intestinal mucosa a few hours after iron is given *per os* to an irondepleted animal. This rapid formation of ferritin seems to be demonstrable wherever iron is resorbed [13-18]. The mechanism of the formation of ferritin or its protein component, apoferritin, has not yet been elucidated.

2. Transport. Numerous investigations on the transport form of iron in the plasma have been published. However, since the experiments to be reported in this paper do not enter this problem, the question of the transport form of iron will be left aside. References are found in some monographs and reviews [19].

3. Storage. From a functional point of view the body iron can be divided into two groups: the physiologically active tissue iron and the storage iron. The hemoglobin iron occupies an interposition. Normally it acts as a tissue iron with a welldefined function, but in cases of need the blood is deprived of a part of its iron for the maintenance of a necessary level of the life essential parenchymal tissue iron. The stores are supplied from the breakdown of hemoglobin and other heme-containing substances plus — when there is a need for it — intestinal resorption.

A close relationship has been demonstrated between the content of iron in tissues and the occurrence of reticuloendothelial cells [20,21]. Accordingly, liver, spleen, and bone marrow constitute the main stores of iron [22-24]. The endocellular storage iron in these organs, "hemosiderin", is very probably ferritin. Several investigators [25,26] have arrived at the conclusion that iron recently deposited in the stores is utilized earlier than iron which has been stored for longer time, independently of origin. This means that when tagged iron is given to an animal, it is not mixed with the total stored iron but rather with only "the surface" layer.

Ferritin is found in most other organs [27]. Intraperitoneally injected hemoglobin is, to a certain extent, broken down and stored as ferritin in the kidneys [15,16].

4. Utilization. Iron is taken from the stores for the building-up of heme-pigments. When a red cell is "worn out", the iron is liberated and pooled in the stores. From this state it is re-utilized for the synthesis of new hemoglobin [26] and probably also other hemoproteins.

5. Excretion. Normally the body seems to have little or no ability to excrete iron [8, 28–32]. Loss of iron under pathological conditions occurs — in addition to losses by bleeding — *e. g.* during myoglobinuria after electrical shock [33] or blast injuries [34]. Parenterally administered cytochrome c is partly excreted via the kidneys [17, 18, 35, 36,]. There is also a small excretion of iron into the urine and the intestinal lumen during the first days following a parenteral administration of inorganic iron [4, 8, 32].

From this pattern it is obvious that the body tends to keep itself in iron balance by avoiding losses and by taking up iron only when there is a need for it. Thus, it is clear that sooner or later all iron-containing substances in the organism must come to an equilibrium with each other as regards the iron, so that if radioactive iron is administered to a normal animal in iron balancy, all organs or iron compounds that can be isolated must finally arrive at the same specific activity.

Most animal experiments with radioactive iron have aimed at a study of the distribution of the supplied isotope or to follow a certain compound, *e. g.* the fate of the iron which is liberated upon the breakdown of hemoglobin or the catabolic processes to which parenterally administered cytochrome c iron is subjected. The animals have often been bled before the experiments, kept on an iron-restricted diet or pre-treated in some other way in order to facilitate and accelerate the changes to be studied. In this paper the aim is to study the distribution of administered iron in the body and the rate of its incorporation into the different pigments under *conditions as normal as possible.* For that reason the animals were kept on a normal diet and their iron nutritional state, as indicated by the hemoglobin level, determined previously to the injections. We also used as small doses of iron as possible to avoid deviations from the normal state. Since animals in iron-balance only absorb tiny amounts of iron from the gut, parenteral administration by intraperitoneal injections had to be used. For reasons of convenience intraperitoneal were preferred to intravenous injections.

In addition to a general, detailed study of the distribution and incorporation of iron, there are some specific questions which this investigation was intended to answer.

The incorporation of iron into erythrocytes in anemia has been studied in man [37–39], dog [6, 38, 40], rat [40, 41], rabbit [42], and guinea pig [40], and during normal conditions in man [37–39], rat [12, 41], and dog [38]. Therefore it was of interest to

follow the radioactivity in the red cells of normal guinea pigs. Moreover it was desirable for our purposes to know the specific activity of the blood iron since with our technic considerable amounts of blood were found to remain in the organs after the post mortal perfusion. As will be seen in the sequel, the specific activities of the myoglobin preparations could be obtained only after corrections for the contaminating hemoglobin. Finally we wanted to compare the rates of incorporation of radioiron into hemoglobin and blood catalase.

In the case of catalase there was another interesting problem. Bonnichsen [43] had found that the protein components of blood and liver catalases in horse very probably were identical. It may also be recalled that while blood catalase contains four protohematins per molecule [44,45], it has never, except in the case of guinea pig liver catalase, been possible to obtain a liver catalase preparation with more than 3.6 protohematins per molecule [46–48]. Less hematin is frequently found. The liver catalases, however, sometimes contain bile pigment, which brings up the total number of tetrapyrrolic compounds per molecule to four [46]. These observations raised the question of whether the blood and liver catalases are formed independently of each other or whether catalase is synthesized exclusively together with the red blood cells and secondarily deposited and broken down in the liver [48].

Very few direct determinations seem to have been made to find out the life length or rate of formation of any one of the parenchymal hemoproteins, either as regards the complete molecule or a part thereof, *e. g.* the iron atom or the porphyrin nitrogen atoms. There are, however, some observations, which can give an idea about the order of magnitude of these properties. Greenstein *et al.* [49] and others [50] extended earlier studies [51] on the behaviour of some respiratory pigments in cancer-bearing animals. The liver catalase activity was found to decrease considerably within a short time. However, the manometric technic is unsuitable for the assay of catalase activity [52]. Moreover, the very high hydrogen peroxide concentrations employed in the experiments would very likely destroy hemo-proteins. Again, *if* there is a cancer induced decrease in catalase activity in tumor-bearing animals, it can be explained in several ways: a hampered synthesis, or an accelerated disintegration of catalase, or both. The influence of some inhibitory agent, emanating from the tumor, could also be considered. In fact, the existence of a catalase inhibitor in tumor tissue has recently been claimed [53], but the results have not yet been confirmed. As no information whatsoever is available concerning the normal life time of the catalase molecule, we considered it necessary to investigate this problem in order to obtain a basis for a discussion of pathological conditions.

Not very much seems to be known about the rate of incorporation of iron into cytochrome c in normal animals [54]. By feeding radioiron to anemic,

growing animals cytochrome c with as much as 80 % specific activity of the isotope has been obtained [18]. This highly active material was one of the tools used to demonstrate definitely that parenterally administered cytochrome c is not utilized unchanged as a respiratory catalyst by the cells.

Still less has been done in the case of myoglobin. A few suggestions on the rate of incorporation of isotopic iron have been reported; however, no details were given [54]. It can be presupposed from the remarkable independency of the myoglobin content of muscles in anemia, muscular inactivity, and infectious diseases [55] that its turnover rate could be expected to be low.

These problems also determined what species should be used for our experiments. We considered it to be preferable to take several small rather than one big animal for each experiment in order to nullify individual variations. In our preliminary series of experiments it proved difficult to prepare pure catalase from rat livers. The preparation of catalase from rat blood, where the hemoglobin crystals adsorbed the catalase very firmly, was still more difficult. From guinea pig liver pure catalase can be prepared easily and rapidly, and the use of this species also involves the advantage that all the catalase iron occurs in protohematin [46]. These facts were considered to be favorable enough to counterbalance the difficulty raised by the low myoglobin content of guinea pig muscles and the fact that the available guinea pigs, weighing 500—1 000 g, were not adult and thus not strictly in iron balance. A certain increase in weight with formation of new tissue thus occurred during the experiment period. This fact had to be taken into consideration for the compounds with comparatively slow formation.

II. EXPERIMENTS

A. The animals

Male and non-pregnant female guinea pigs weighing 500—1 000 g were kept on a daily diet of oats, turnips and hay and fed with carrots once a week. During a preliminary period of 2—4 months the blood hemoglobin was checked in samples taken by heart puncture from every tenth animal, chosen at random.

Series no. 1 showed the average value of 14.5 ± 1.2 g * hemoglobin per 100 ml blood before the injection. The animals in series 2 were not examined for their hemoglobin level immediately before the injections; from table 12 it is obvious, however, that they were not iron-deficient. Series 3 was controlled with respect to hemoglobin and number of red and white cells. The results two days before the injection of radioactive iron were:

Hemoglobin	15.7 ± 0.4 g/100 ml blood
Red cells	8.69 ± 0.27 millions/mm^3
White cells	18.000 ± 4.000 per mm^3

* In this paper numerical values are given with their probable errors.

It was concluded that the animals were healthy and not deficient in iron. We are indepted to Dr S. Paléus for carrying out the blood controls.

The injection of the radioactive ferric ammonium citrate was made intraperitoneally in the mornings. The dosage was 0.05 mg Fe per 100 g body weight. It did not cause any trouble to the animals.

After the time given in the tables about 15 animals for each experiment were killed by a blow on the neck. The aorta was opened below the heart, the blood collected and the animal perfused with saline (about 1 liter in 15—20 min) through the distal end until the liquid flew almost colorless from the proximal opening. The perfusion fluid was collected and the hemoglobin determined.

The livers were homogenized; in series 1 and 2 a small aliquot was taken for iron and activity determinations, another for ferritin preparation, and the main part for preparing catalase. The spleens were taken separately for the preparation of spleen ferritin.

About one tenth of the musculature was dissected from the hind legs, which were more effectively freed of hemoglobin by the perfusion than the fore leg muscles, and used for myoglobin preparation ("myoglobin muscle"). The rest of the musculature ("cytochrome muscle") was liberated together with the skeleton from the skin and the other organs ("rest of animals") and used for the preparation of cytochrome c. Various methods were tried to grind or homogenize the rests of the animals, but without success. Since it was inconvenient for technical reasons to hydrolyze all the 14—16 rests in each experiment, three of them were chosen at random for analysis. The result was taken as representing the whole group. In the series 3 only liver ferritin and catalase, hemoglobin, blood catalase and cytochrome c were investigated.

B. Preparation methods

a. Myoglobin

The hind leg muscles of 14—16 guinea-pigs, forming one group, were cut off and weighed. The amount of pooled muscle tissue for myoglobin preparation weighed 370 ± 24 g. An equal amount of distilled water was added and the mixture homogenized immediately. The homogenate was weighed and about 2 % of it was taken for iron and activity determinations. The rest was put in the cold room and left for 2 days. After 2 days it was centrifuged, the insoluble residue discarded, the extract neutralised, and 0.25 volumes of basic lead acetate (20 %) was added. The precipitate was centrifuged off, and the excess lead removed from the supernatant by the addition of disodiumphosphate until no further precipitate was formed. The pH was maintained at neutrality during this operation [56]. The precipitate was centrifuged off and the supernatant was dialysed in the cold against 70 % neutralised ammonium sulfate. The precipitate formed was centrifuged off and discarded. The supernatant was dialysed against 80 % saturated, neutral ammonium sulfate solution in the cold. The precipitate was centrifuged off and dissolved in distilled water. This precipitate was labelled "70—80". The supernatant was dialysed against 90 % saturated neutral ammonium sulfate in the cold. The precipitate was centrifuged off and dissolved in distilled water (fraction "80—90 %"). The supernatant was dialysed against saturated, neutral ammonium sulfate in the cold and the precipitate treated as above (fraction "90—100 %"). A sample of each of the three fractions (70—80, 80—90, 90—100 %) was suitably diluted with *M*/50 phosphate buffer, pH 7 and CO from the gas-tap bubbled through them for 2—3 minutes. Solid sodium dithionite was then added and the bubbling continued for a further 5 minutes. The

solution was then transferred to a 1 cm Beckman cuvette, the cuvette was covered and readings taken immediately.

Acid acetone (1 ml concentrated hydrochloric acid per 100 ml acetone) was added to the dissolved precipitate in a quantity of about 4 times their volume. The precipitate was filtered or centrifuged off and the acetone, containing the dissolved hemin, evaporated on a water bath. After the acetone had evaporated, the precipitated hemin was filtered off on a glass filter. Both the original vessel and the filter were washed three times with *M* HCl to remove foreign iron. The precipitated hemin was then dissolved from the walls of the vessel and the glass filter with *M*/10 NaOH. This solution of hemin in *M*/10 NaOH was taken for Fe analysis and plating.

The specific activities of the different myoglobin fractions are given in Tables 11 and 14 under the heading "uncorrected" and of hemoglobin in Tables 9 and 12.

In some cases, when centrifugation was incomplete, some of the precipitate remained in the supernatant after 100 % ammonium sulfate saturation. It was found that even in this case the hemin could be isolated by treatment with HCl-acetone and subsequent evaporation as described. In these cases it was treated as a separate fraction and labelled "> 100 %".

As can be seen from Tables 11 and 14 under the heading "uncorrected" the specific activity decreased with increasing ammonium sulfate saturation. This would be expected if the precipitated myoglobin still contained some contaminating hemoglobin. Since in ammonium sulfate fractionation hemoglobin precipitates before myoglobin, the quantity of the former should decrease as the concentration of ammonium sulfate increases. On the other hand, if the quantity of hemoglobin in the precipitates 70—80 %, 80—90 %, 80—100 % were known and also the amount of radioactive iron it contained, and this value subtracted from the figures in Table 7 and under the heading "uncorrected", then all fractions should give similar values, *i. e.* the content of radioactive iron in pure myoglobin. In order to arrive at this figure, the relative hemoglobin and myoglobin contents of the different fractions had to be determined. The first aim was to get pure, hemoglobinfree myoglobin from guinea-pig muscle. This was obtained by re-dissolving and re-precipitating the fraction coming out between 90—100 % ammonium sulfate saturation. Because of the small amount of material, crystallisation was not attempted. Different absorption spectra of this re-precipitated fraction were taken and all data obtained agreed with those given in the literature for crystalline myoglobin. The absorption peaks of the CO-compound in the visible were at 579 and 542 mμ, the Soret band at 423 mμ. The absorption peak of the ferromyoglobin was found at 434 mμ and after re-oxygenation at 412 mμ. The numerical value of the light absorption coefficient at the absorption maximum after re-oxygenation was 105 $\pm$ 3 % of that of the reduced compound. The hematin content was determined as pyridine-hemochromogen. Table 1 gives the extinction coefficients, ε, of the CO-compounds of 6 such preparations, calculated for a 10^{-3} molar solution in 1-cm cuvettes. It can of course not be proven that these preparations were absolutely free from hemoglobin. As a matter of fact it has never been proved beyond doubt that even crystalline myoglobin preparations so far obtained from different species have been absolutely free from hemoglobin. However, as all data obtained with our preparations agree fairly well with those given for crystalline myoglobin, it seems reasonable to suppose that, as far as evidence at present suggests, hemoglobin can only be present, if at all, in very small quantities.

Assuming the absorption coefficients, recorded in Table 1, to be those of pure CO-myoglobin, the quantity of hemoglobin and myoglobin in a given sample with known CO-

Table 1. Extinction coefficients of guinea pig Co-myoglobin and Co-hemoglobin. Molarity of protohemin (determined as pyridine hemochrome) = 10^{-3}. d = 1 cm. The values of ε for Co-myoglobin are means of determinations on six different preparations, those for Co-hemoglobin means of three different samples.

Wave length mμ	CO-myoglobin ε	CO-hemoglobin ε
590	5.68 ± .65	2.99 ± .08
584	10.38 ± .16	5.80 ± .03
580	12.50 ± .15	8.75 ± .02
578	12.66 ± .23	—
576	12.36 ± .22	12.09 ± .03
574	12.15 ± .13	13.24 ± .08
572	11.84 ± .08	14.20 ± .04
570	11.70 ± .11	14.68 ± .01
568	—	14.76 ± .01
566	11.07 ± .06	14.52 ± .01
562	10.60 ± .03	13.19 ± .03
558	10.71 ± .13	12.17 ± .04
554	12.07 ± .30	11.77 ± .12
550	13.66 ± .30	12.55 ± .03
546	14.52 ± .05	13.79 ± .02
544	14.70 ± .09	14.44 ± .09
542	14.78 ± .15	14.84 ± .03
540	14.54 ± .20	15.13 ± .05
538	—	15.04 ± .05
536	—	14.86 ± .08

spectrum can be calculated. This procedure is essentially the same as the one employed by de Duve [57] for the simultaneous determination of myoglobin and hemoglobin. Because of the greater error inherent in the present determinations (owing to the material not being crystalline) the contents of myoglobin and hemoglobin were determined at several wavelengths and from these average values were taken.

Three isosbestic points were found in the spectra of the CO compounds of myoglobin and hemoglobin; at 575.7, 554.5 and 542.3 mμ. The hemin content of the fraction in question was calculated from the absorptions at the three points and an average of these values was used for further calculations. The hemin content by the pyridine hemochromogen method was determined in a few cases only, because of the scarcity of the material and the ammonium sulfate content of the fractions. In the few analyses performed, the values found agreed well with those calculated as above.

To make our procedure clear, the complete record of one experiment is included in extenso. The following account is for a group of 15 guinea-pigs killed 3 weeks after the injection of radioactive iron (expt. nr 27). The muscle extract was treated and fractionated as described. The spectra of the CO compounds of the redissolved fractions "70—80",

"80—90" and "90—100 %" were taken. The optical densities of the three fractions at the isosbestic points, as read in the Beckman spectrophotometer, were:

Table 2.

Isosbestic point mμ	Optical density of fractions "70—80"	"80—90"	"90—100"
575.7	0.365	0.404	0.568
554.5	0.3465	0.366	0.495
542.3	0.434	0.473	0.660

The optical densities of both CO-hemoglobin and CO-myoglobin at the isosbestic points in a 10^{-3} molar concentration are:

Table 3.

Isosbestic point mμ	ε
575.7	12.30
554.5	11.86
542.3	14.78

From these values the hemin content of the above mentioned three fractions was calculated.

Table 4. Hemin content calculated from the absorption of the CO compounds at the 3 isosbestic points.

Calculated from mμ	Hemin content of fractions "70—80"	"80—90"	"90—100"
575.7	19.3	21.4	30.1
554.5	19.0	20.1	27.2
542.3	19.1	20.8	29.1
Average μg hemin per ml	19.1	20.8	28.8

Since the spectra of the CO compounds of the above fractions were determined and their hemin content known from the above calculations, their absorption coefficients for a 10^{-3} molar solution could be calculated for different wave-lengths. Five wave-lengths, 580, 572, 570, 566 and 562 mμ were chosen, because the absorption coefficients of CO hemoglobin and CO myoglobin differ greatly at these wave-lengths. From the CO spectra and the hemin content, the absorption coefficients at the above wave lengths in this particular experiment were found to be:

Table 5.

Wave length mμ	Absorption coefficient for a 10^{-3} molar solution of fractions "70—80"	"80—90"	"90—100"
580	10.68	11.37	12.53
572	13.23	12.58	12.06
570	13.34	12.53	11.83
566	12.82	12.16	11.32
562	11.94	11.42	10.53

The absorption coefficients for CO-myoglobin and CO-hemoglobin are given in Table 1. From this table and the absorption coefficients of the three fractions given above, the myoglobin and hemoglobin content of the three fractions was calculated.

Table 6.

Calculated from extinction value at mμ	Percentage myoglobin content of fractions "70—80"	"80—90"	"90—100"
580	51.5	70.0	101.0
572	41.0	68.5	91.0
570	45.0	72.0	96.0
566	49.0	68.0	92.5
562	48.5	68.5	107.0
Average	47.0	69.5	97.5

This means that fraction "70—80" contained 53 %, fraction "80—90" 30.5 % and fraction "90—100" 2.5 % hemoglobin.

From Table 7 it can be seen that the specific activities of these three fractions were:

0.155 % for fraction "70—80"
0.1045 % for fraction "80—90"
0.0519 % for fraction "90—100"

The hemoglobin prepared from these animals showed a specific activity of 0.2085 %.

Thus if fraction "70—80" contained 53 % hemoglobin, the activity corresponding to this amount of hemoglobin has to be subtracted from the value actually found for this fraction. If we carry out these subtractions for all the three fractions, we come to the following results:

Table 7.

	Fractions		
	"70—80"	"80—90"	"90—100"
Spec. activity of preparation	0.155 %	0.1045 %	0.0519 %
Spec. activity due to hemoglobin	0.110	0.0635	0.0052
Spec. activity due to myoglobin	0.045	0.041	0.047

As can be seen, the three fractions give comparable activities, as they naturally should if they contained only myoglobin and no hemoglobin. The average value of the three fractions: 0.044 is taken to be the specific activity of the iron in myoglobin. As stated, the absolute purity of the fractions after this subtraction is still questionable. However, it seems reasonable to suppose that they contain very little, if any, hemoglobin. The values corrected in the way described here are found in Tables 11 and 14 in the column "corrected". It can be seen that the values for the three fractions lie much closer to each other than they did before the corrections. The agreement is in some cases very good. In some, however, the differences are still fairly pronounced.

b. Cytochrome c

In some preliminary experiments we tried to dissect the muscles from the skeletons. This procedure was, however, found to be tedious, and considerable losses could not be avoided in the experiments to be described in this paper. Therefore the dissection was omitted. The skeletons with the muscles *in situ* (except the hind leg muscles which were taken for the preparation of myoglobin) together with the hearts were thus used as sources for the cytochrome preparations. The material was homogenized and worked up according to the method of Keilin and Hartree [58], slightly modified. In guinea pigs extraction with sulfuric acid gave much higher yield than with trichloroacetic acid. The homogenates were extracted overnight with 2.5 volumes of 0.15 *N* sulfuric acid, neutralized, centrifuged, and the residues re-extracted for two hours with two volumes of 0.2 *N* sulfuric acid. To the combined extracts were added 500 g of ammonium sulfate per liter. Next day the inert material was filtered off and the cytochrome c precipitated from the filtrate with 1/40 of its volume of 20 % trichloroacetic acid. This crude cytochrome c was dialyzed against 0.1 % ammonia until the sulfate reaction became negative. At this stage the iron content was 0.25—0.30 %, which, however, included some non-cytochrome iron. The following procedure was therefore employed for the specific isolation of the cytochrome c iron.

The cytochrome c was precipitated from its aqueous solution by the addition of acid acetone (1 ml 5 *N* sulphuric acid per 100 ml acetone), centrifuged, and the supernatant examined spectrophotometrically for the presence of a Soret band. The precipitate was dissolved in water and re-precipitated, the procedure being repeated until the supernatant became free from any specific absorption in the region 380—420 mμ. Five washings were found to be necessary. The final precipitate was dissolved in 20—50 ml of a silver sulfate

Table 8.

Mixture	Pipetted of stock solns.			Mixture contained		Yield of Fe		Net c. p. m.	c. p. m. per
no.	Hemoglob. ml	Ferritin ml	Cytochrome ml	Fe μg	c. p. m.	μg	% of cytochrome Fe		μg isolated Fe
I	1.0	1.0	5.0	484.9	3 773	24.8	53.3	2.18	0.088
II	1.0	1.0	2.5	461.7	3 773	16.8	72.1	1.45	0.086
III	0.5	0.1	5.0	162.5	989	34.6	74.4	1.96	0.057

solution (800 mg silver sulfate per 100 ml solution). Next, 0.2 volumes of glacial acetic acid were added and the mixture digested for 90 minutes at 70° [59]. The protein residue was then precipitated with 5 volumes of ice cold acid acetone and filtered off. The acetone solution was cautiously evaporated in a current of air with slight warming. To the remaining water-acetic acid solution 10 ml of butanol were added. The hemin solution was washed in a separatory funnel twice with water, twice with 0.1 *M* HCl, and twice again with water, each time with twofold volume. A small amount of the hemin usually went into the hydrochloric acid, from which it could be re-extracted with ether. Sometimes a small precipitate appeared at the alcohol-water interphase. The washed butanol solution was therefore always filtered down into a combustion tube. The butanol was evaporated and the remaining hemin combusted with sulfuric acid in the ordinary way.

The specificity of this isolation procedure was tested on the following mixtures, from which the cytochrome iron was isolated as described above.

Stock solutions: Cytochrome c: Cow heart cytochrome c, prepared according to Keilin and Hartree [60] and with an iron content of 0.41 %, was made up to a solution containing 9.3 μg iron per ml. This cytochrome c iron had no radioactivity.

Hemoglobin: Guinea pig blood from animals, previously injected with radioactive ferric ammonium citrate, was hemolyzed with water. The solution contained 180.4 μg iron per ml with 8.48 c. p. m. per μg iron.

Ferritin: Radioactive ferritin from guinea pig liver was isolated as described in this paper. The material had an iron content of 258.0 μg per ml with 8.62 c. p. m. per μg. iron.

The artificial mixtures, to which the isolation procedure was applied, were made up and analyzed as follows (Table 8). The isolated iron was thus about 100 times less active than either of the radioactive compounds in the mixture. Since no Soret band was detectable in the acetone washings, the activity could hardly derive from the hemoglobin. The purification of the cytochrome c hemin is based on its distribution between the alcohol and water phases, and the degree of contamination therefore depends on the starting material. In the crude cytochrome c preparations the ratios between the cytochrome and the non-cytochrome iron could hardly be as unfavorable as in the artificial mixtures. It must also, however, be taken into consideration that the bone marrow may contain some compounds that follow the cytochrome c hemin closely during the isolation procedure. Nothing is known, for instance, about the mode of linkage between the prosthetic group and the protein part in verdoperoxidase, except that no splitting will occur with acid acetone. The data obtained in table 8 were considered to give satisfactory evidence in favour of the efficiency of our isolation procedure for cytochrome c iron.

c. Liver catalase

The livers (280—375 g) were ground in a Turmix blender with twice their weight of water and the insoluble residue centrifuged down. The extract was shaken with one tenth of its volume of an ethanol-chloroform mixture (5 : 1) and clarified by centrifugation. The catalase was precipitated from the supernatant by the addition of ethanol to 70 % by volume at room temperature, centrifuged down, immediately dissolved in distilled water and dialyzed against water in order to remove the rest of the ethanol. Dilute acetic acid was added to give a pH of 4.0 and a precipitate removed by centrifugation. The volume of the supernatant which contained all of the catalase was now 20—30 ml; one and a half volumes of saturated ammonium sulfate solution was added (60 % saturation) and the precipitated catalase centrifuged down, dissolved in a few ml of water and dialyzed a few hours to remove most of the ammonium sulfate. The solution was checked spectroscopically for the absence of hemoglobin.

A tenfold volume of acid acetone (1 ml of conc. HCl in 1 000 ml of acetone) was added to the catalase solution. After a few minutes stirring the precipitated catalase protein was centrifuged down. The acetone was now evaporated by keeping the solution in a water bath at about 40° and blowing air on the surface. The hemin then precipitated out from the acid water phase. It was collected in a small centrifuge tube, washed several times with n/l HCl and water, rinsed over to a small glass filter, dissolved in a little n/10 NaOH and sucked off. The hematin solution was combusted with 1 ml of concentrated sulfuric acid, and aliquots taken for iron determination and electroplating.

Isotopic control of the purification procedure for liver catalase

1. 10 mg of Fe^{59} (3.7 million c. p. m.) were mixed in the blender with 192 g of guinea pig liver, and catalase iron was isolated according to the above described procedure. It was completely inactive.

2. 25 mg of ferritin (= 1.8 million c. p. m.) were mixed in the blender with 192 g of liver. The isolated catalase iron was inactive.

3. A guinea pig liver catalase solution was dialyzed against a solution of Fe^{59} — ammonium citrate solution over night. The isolated catalase hemin iron was inactive.

4. 1.2 mg of protohemin and 1.87 mg of Fe^{59} (0.69 million- c. p. m.) were dissolved in acetone-HCl, the acetone removed and the hemin isolated according to the above procedure. The hemin iron was inactive.

These experiments indicate that the isolation procedure for liver catalase was very satisfactory.

d. Preparation of ferritin from liver and spleen

The livers or the spleens were ground and extracted with twice their weight of phosphate buffer m/50, pH 6.8, centrifuged and the residue washed once with the same amount of buffer. The extract was diluted with water to four times its volume and heated to 80° for one minute. A large precipitate was removed by centrifugation and the supernatant saturated to 70 % with ammonium sulfate. The precipitate, containing all of the ferritin, was dissolved in water and re-precipitated with ammonium sulfate to 0.4 saturation. The precipitate contained almost pure ferritin and was taken for analysis.

e. Preparation of blood catalase

The washed, red blood cells were hemolyzed by the addition of a two-fold volume of water and left over night at + 4°. The hemoglobin was denatured by the Tsuchihashi procedure: per 100 ml of hemolyzate 10 ml of ethanol-chloroform 3 : 1 were added and the mixture shaken. The denatured hemoglobin was removed by centrifugation and the clear supernatant evaporated in vacuo to a small volume (15—20 ml).

The absence of hemoglobin was checked spectroscopically. The catalase was precipitated by ammonium sulfate to 0.6 saturation and the precipitate dialyzed against water. The hemin was split off from the protein component by acetone-HCl and the former treated as described above for the liver catalase.

C. Technical procedure for activity determination

a. Combustion of samples

The standard procedure was as follows: Aliquots or the whole material were heated on a free flame in glass tubes or flasks with 1 to 50 ml concentrated sulfuric acid. A few glass beads prevented excessive bumping. When the volatile solvents had evaporated, the heating was continued with addition of known, small amounts of perhydrol at suitable intervals. The combustion was regarded as complete when no brownish color reappeared within 15 minutes after the last addition of hydrogen peroxide. Since ferric sulfate seems to be highly insoluble in concentrated sulfuric acid, one volume of water was added and the solution boiled for another few seconds. The sample was then rinsed over with distilled water to a graduate flask or cylinder, and aliquots taken for iron determination and electroplating. If possible, two or three determinations were made on each sample in addition to the blank. The blank values generally amounted to 1.0—1.4 μg in a final volume of 10 ml, the major part of the iron in the blank values deriving from the sulfosalisylic acid.

b. The iron determinations

were made essentially according to the sulfosalisylic acid method by Lorber [61]. The extinction coefficient, $\log I_0/I$ in 1 cm layer is = 0.100 at 424 mμ for 1 μg of Fe^{+++} per ml, if 0.1 ml of 20 % sulfosalisylic acid solution is used for every 5 ml of the final volume.

c. Preparation of the samples for plating

In the case of the samples from cytochrome c hemin, hydrogen sulfide was passed into the acid solution in order to precipitate the silver ions remaining from the splitting. The silver sulfide was centrifuged off and washed with water; the washings were pooled with the sample. If the washing of the butanol solution had been properly made, no silver sulfide could generally be detected.

The following procedure was the same in all cases: 0.500 mg carrier-Fe was added as ferric ammonium sulfate, concentrated ammonia was added in excess and hydrogen sulfide bubbled through. When very little material was available, as for instance sometimes blood catalase iron, we saved the samples used for the iron determination by adding carrier-Fe and precipitating with hydrogen sulfide. The iron sulfide was centrifuged down and washed three times with distilled water. It was then dissolved in two drops of 2 *N* hydrochloric acid and the tube kept on a boiling water bath under a pressure air current. During this time one drop of 2 *N* HCl was added four times in order to ensure the com-

plete removal of hydrogen sulfide. The dry residue was dissolved in 1 ml of saturated ammonium oxalate solution and one drop of methyl red solution added as indicator for pH-control during the electroplating. In some samples, especially those containing the bones, a precipitate of calcium oxalate appeared and had to be removed after three hours by centrifugation and washing of the precipitate with saturated ammonium oxalate. This procedure is essential, since the calcium oxalate seriously interferes with the plating.

The samples were transferred to the plating vessels by rinsing with saturated ammonium oxalate solution into the final volume of around 5 ml. The plating vessels were designed essentially according to Vosburgh *et al.* [62], but had considerably smaller dimensions, inner diameter 10 mm, height 50 mm.

d. Plating

50 m ampères during 2.5 hours was found to be sufficient for nearly complete plating on the central area (diameter = 10 mm) of the copper discs. The 0.5 mg of iron on this area does not give any measurable self-absorption [62] of the radiation of Fe^{59} or Fe^{55}.

The completeness of the plating was checked in all cases either by quantitative iron determinations in the solutions after plating and in the washings from the sulfide precipitate, or by determining the iron that was dissolved by hydrochloric acid from the copper discs after the counting. Correction for lost iron was made, if necessary. It never exceeded 50 μg Fe, and was generally only a few μg.

e. Counting

We used small bell-shaped Geiger-Müller counting chambers with mica windows 0.9—1.5 mg/cm^2, 27 mm diameter, as described by Hevesy [2]. They were filled with alcohol (10 mm pressure) + argon (90 mm).

Two recording circuits were used during the course of this investigation. One was built by AB. LKB-produkter, Stockholm, Sweden (Scale of 64); the other was constructed by Mr. K. O. Särnesjö in this institute (Scale of 16).

The background was 2.0—2.9 c. p. m.

In general at least 1 000 counts per sample were taken, for very weak samples only 500. The probable error was calculated according to the usual formula

$$K = \left[\left(\frac{2/3\sqrt{N}}{T}\right)^2 + \left(\frac{2/3\sqrt{n}}{t}\right)^2\right]^{\frac{1}{2}}$$

$$E\,\% = 100 \times \frac{K}{\text{net count}}$$

where N = total counts of sample in time T
n = background counts in time t

D. Radioactive material

The series 1 and 2 were injected respectively on May 7 and July 1, 1949, with ferric ammonium citrate prepared from a sample of Fe^{59} + Fe^{55} from A. E. C., Oakridge, Tenn. It was stated to have shown 0.0054 mc/mg of Fe^{59} (April 13, 1949) and 0.0086 mc/mg Fe^{55}. The series 3 was injected on February 20, 1950, with a mixture of 97 % of this

Table 9. Distribution of iron

Expt. no.	A = 19 B = 20				A = 22 B = 23			
Days	7				14			
	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe
Liver A	330	28.4	3.32	22.9	356	23.9	2.93	17.0
Liver B	280	20.5	3.57	17.7	419	26.6	2.38	15.3
Spleen A	10	3.8	0.23	0.2	8	3.3	0.25	0.2
Spleen B	8	2.8	0.23	0.2	12	3.9	0.27	0.3
Red blood cells A	250	127.6	0.16	4.8	201	102.6	0.33	8.2
Red blood cells B	208	106.1	0.13	3.4	188	95.7	0.38	8.8
Muscle for cytochrome A	2 900	259	0.09	5.7	2 550	88.5	0.17	3.6
Muscle for cytochrome B	2 700	270	0.09	5.9	2 750	85.6	0.24	5.0
Muscle for myoglobin A	305	4.8	0.19	0.2	265	4.5	0.21	0.2
Muscle for myoglobin B	297	4.1	0.17	0.2	344	5.0	0.19	0.2
Rest A	6 290	352	0.25	21.3	6 070	191	0.25	11.6
Rest B	5 100	160.5	0.37	14.4	5 440	224	0.13	7.0
Total A	10 085	775.6		55.1	9 550	413.8		40.8
Per animal	672	51.7			637	27.6		
Number of animals	A = 15 B = 15				A = 15 B = 15			
Total B	8 593	564.0		41.8	9 153	440.8		36.6
Per animal	573	37.6			610	29.4		

iron and 3 % of a highly potent Fe^{55} (0.4635 mc/mg, April 14, 1949). Our Geiger-Müller chamber could be determined from these data to count 15—25 % of the total radiation of Fe^{59} and 0.8 % of the radiation of Fe^{55}. In the series 1 and 2 about 98 % of the counts derived from Fe^{59} and the remainder from Fe^{55}; in the series 3, on the contrary, 10 % came from Fe^{59} and 90 % from Fe^{55} at the time the samples were counted.

For each series standard discs with suitable amounts of radioactive iron of the same mixture as was used for the injections were prepared. These discs were preserved until the series had been finished. Thus alterations in the counting equipment could be disclosed and corrected. Since our radioactive material consisted of a mixture of two isotopes, the decay of the radioactivity was corrected for by comparisons with the standard discs, and not by calculations from the half-lives of the isotopes.

III. DISCUSSION

A. Errors

Some of the data given in the Tables 4 and 15 may necessitate a comment on some possible source of errors.

and radioactivity (series 1).

A = 26 B = 27 21				A = 24 B = 25 28				A = 28 B = 29 35			
Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotope Fe
310	24.2	1.53	9.0	365	29.8	0.95	6.7	318	30.2	1.24	9.2
350	31.2	0.97	7.4	390	33.6	1.80	13.7	343	—	—	—
13	3.9	0.20	0.2	17	6.4	0.49	0.8	9	4.1	0.22	0.2
12	4.6	0.17	0.2	11	4.3	0.22	0.2	10	4.0	0.39	0.4
209	106.9	0.32	8.3	242	123.2	0.45	13.2	88	45.0	0.55	6.1
221	112.8	0.21	5.7	245	125.3	0.80	22.6	266	136	0.74	25.6
2 600	96.7	0.17	4.0	2 900	96.1	0.24	5.5	3 700	189	0.20	9.3
2 800	98.9	0.14	3.4	3 100	113.7	0.28	7.2	2 900	93.6	—	—
328	4.7	0.11	0.1	276	4.9	0.26	0.3	351	3.6	0.26	0.2
344	4.5	0.13	0.1	363	4.1	0.45	0.4	367	3.7	0.47	0.4
5 240	215	0.15	7.8	5 360	300	0.24	17.1	4 740	197	0.32	15.5
4 890	394	0.15	14.3	5 410	333	0.39	29.4	5 210	212	0.60	32.4
8 700	451.4		29.4	9 260	560.4		43.6	9 206	468.9		40.5
580	30.1			617	37.3			658	33.5		
A = 15 B = 15				A = 15 B = 15				A = 14 B = 14			
8 622	646.0		21.1	9 519	614.0		73.5	9 096			
575	43.0			635	40.9			649			

1. It can be seen in Tables 9 and 12 that the amount of hemoglobin iron obtained by the perfusion amounted in series 1 A—B and 2, as an average, to 7.7 mg per animal. Drabkin found the ratio between body mass and hemoglobin to be a consistent 12.7 g per kg body weight, for certain species examined by him (rat, dog. man, heifer, horse). On the assumption that this ratio is valid also in the case of guinea pig, the average amount of hemoglobin iron per animal in these series should be $12.7 \times 0.616 \times 0.34 \times 10^{-2} = 26.6$ mg. This demonstrates that only around 29 % of the blood had been washed out. It is to be observed that this calculation includes an error, since the total weights of the animals per experiment includes the calculated weight of the removed blood. This blood, however, has of course been replaced by saline, which remains in the bodies. The error is, however, not very large. If roughly one third of the blood had been removed from the bodies and replaced by saline, the error would increase the total weight of the animals by only three per cent. As an example of a very incomplete perfusion may be given experiment nr

28 (ser. 1 A, 35 days, Table 9), where 14 animals gave only 45 mg of hemoglobin iron. The major part of the blood obviously remained in the "musculature for cytochrome", which gave unusually high total iron values.

The remaining blood is thus to be taken into consideration when the values are to be interpreted.

2. In experiment nr 34 (ser. 2, 4 days, Table 12) the total iron is given as 464 mg for "musculature for cytochrome". This is 3—4 times more than was found in the corresponding fractions in the other experiments. The most likely explanation thereof is that some foreign iron has contaminated the material, *e. g.* a trace of metallic iron from a mill or a stand. In spite of all possible precautions an accident of this kind may of course happen during a series of experiments of this kind. This explanation is also supported by the low specific activity 0.13 %, only one third of what was found for the corresponding fractions in other experiments.

Contamination with even a few micrograms of foreign iron would of course influence the specific activities considerably in those cases where very small amounts of material were available, *e. g.* from blood catalase. Only 4.2—28.4 (mean 14.4) micrograms of iron from this substance could be obtained per experiment.

B. Rate of iron resorption via peritoneum

Granick and Hahn [63] observed that after intravenous injection into dogs of radioactive iron as ferric ammonium citrate, 41.3 and 60.0 per cent of the injected activity appeared in the liver ferritin after 1 and 2 hours respectively. It is known that the resorption of drugs and other substances given by peritoneal injection is very rapid. Therefore, an injected substance tends to reach a certain blood level at essentially the same rate, regardless of whether the substance is administered by intravenous or intraperitoneal injection. Our figures indicate, however, that the resorption of ferric iron, given as ferric ammonium citrate, must be regarded as comparatively slow. Maximum activity in the liver as well as in the liver ferritin were not reached until the second day after the injection, the values on the first day being considerably lower. It is remarkable that only part of the iron occuring in the liver after two days could be held back in the liver. The activity dropped markedly on the third day. After that time the values remain nearly constant for some days, whereupon they begin to decline to reach a level slightly above the equilibrium level.

Another evidence for the slow resorption via the peritoneum is given by the "rest" fraction (Table 12) which includes the intestine with the major part of peritoneum which on the second day had fallen to about 0.46 %, where it remained. On the first day the specific activity had its highest value, 0.81 %.

C. Recovery of injected iron

The recovery of the injected iron isotope in series 1 was 21.1—73.5 % and in series 2 was 74.5—91.3 %. No satisfactory account for the vanished iron can be given. The unavoidable losses of blood and other tissues at the sacrification were negligible. From series 1 and 2 it is evident that 35 days do not suffice to bring the various iron containing fractions in the body into isotopic equilibrium. From series 3 one can see that liver catalase and ferritin with their rapid rates, hemoglobin with its intermediate rate, and cytochrome c with its slow rate of incorporation tend to reach the same value after 59 days. That value is slightly above 0.50 %. The dilution factor for the injected isotopic iron is 0.84 % (= 0.05 × 100/5.97). If complete equilibration is assumed to be reached within two months, it would thus mean that only 60—65 % of the administered iron remained in the body. The possibility that a large part of the total iron had been lost somehow in series 1, which of course would lower the recovery, can be ruled out, since the total iron was the same in series 1 and 2 (Tables 9 and 12). In series 2 the recoveries were much higher. Feces and urine were not collected during the experiment, but we hesitate for several reasons to explain the losses by assuming excretion. There is no drift towards lower recoveries with increasing time. It seems unlikely that excessively high excretion took place during the first days after the injections, since small doses were given (0.8 % of the total body iron per animal) and no evidence for such high excretions can be found in the literature.

To determine the iron in the "rest" of the animals, 3 of the 14—16 bodies per experiment were randomly taken and hydrolysed (*cf.* p. 000). Since this iron fraction accounted for about half of the total iron, it is of course possible that the variations in per cent recovery can to a large extent be explained in this way.

In series 2, where a higher percentile recovery was found, the rate of incorporation of radioactive iron into all the isolated compounds was more rapid than in series 1. This finding is also difficult to explain. In fact, these discrepancies between series 1 and 2 led us to repeat the experiment with series 3.

Whatever may have been the course of these differences between series 1 and 2, this unknown effect did not influence the activity cycles of liver ferritin and liver catalase. They gave consistent values in all three series.

It has been reported for dogs [63] and rats [32] that the spleen plays a rather unimportant role in the iron metabolism. We found the same phenomenon in series 1, where the specific activity of the spleen iron remained at a rather constant and low level. Contrary to this, the spleen iron activity in series 2

Table 10. Per cent specific activity of

Expt. no.		A = 19 B = 20				A = 22 B = 23			
Days		7				14			
Spleen ferritin	A	0.22 ± .01	0.23 ± .01	0.31 ± .01	0.33 ± .01				
	B	0.22 ± .01	0.23 ± .005	0.25 ± .01	0.27 ± .01				
Liver ferritin	A	4.93 ± .09	4.80 ± .10	3.69 ± .08	3.70 ± .06				
	B	2.70 ± .05	2.66 ± .07	3.48 ± .08	3.36 ± .06				
Liver catalase	A	3.21 ± .07	3.13 ± .06	2.76 ± .06	2.89 ± .05				
	B	2.83 ± .06	2.62 ± .06	3.35 ± .07	3.22 ± .05				
Cytochrome c	A	0.077 ± .002	0.069 ± .002	0.067 ± .005					
	B	0.063 ± .004	0.071 ± .004	0.110 ± .003					

reached higher values and varied much more. Granick and Hahn [63] found that in a series of three dogs one obviously had a deranged mechanism for the conversion of intravenously administered iron (ferric ammonium citrate) to ferritin. They also supposed that the variations found in the ferritin contents of human livers could be explained by derangements of that probably enzymatic reaction. The amounts of ferritin, which we were able to prepare, varied from 0.4 to 9.2 mg in terms of iron. However, we are inclined to believe that these variations were due to preparative difficulties rather than to various contents in the organs.

D. Total iron and its distribution in guinea pig

Series 1 and 2 give information about the iron content and distribution in guinea pigs. The average values obtained from 226 animals in 15 groups were 5.97 mg of Fe per 100 g in series 1, 5.91 mg in series 2. The iron content of blood-free liver, equal to the difference between total iron and the hemoglobin-bound iron, was calculated in the following way: The iron in cytochrome, catalase and similar compounds was considered quantitatively negligible as compared to the iron in ferritin and hemoglobin. The latter was calculated according to the formula:

$$X = F \cdot \frac{b-c}{b-a}$$

iron in isolated compounds (series 1).

A = 26 B = 27				A = 24 B = 25				A = 28 B = 29			
21				28				35			
0.24 ± .01	0.22 ± .01	0.65 ± .01	0.58 ± .01	0.25 ± .01	0.24 ± .01
0.19 ± .01	0.17 ± .004	0.23 ± .01	0.21 ± .005	0.40 ± .01	0.38 ± .005
1.84 ± .04	1.68 ± .04	1.13 ± .03	1.14 ± .03	1.54 ± .03	—
1.35 ± .03	1.43 ± .03	2.15 ± .05	1.94 ± .04	1.68 ± .04	1.68 ± .04
2.74 ± .06	—	1.45 ± .03	1.37 ± .03	2.17 ± .06	1.86 ± .04
1.38 ± .02	—	2.29 ± .05	2.11 ± .05	1.70 ± .04	1.68 ± .04
0.099 ± .002	—	0.156 ± .003	—	0.207 ± .005	
0.064 ± .002		0.212 ± .005		0.285 ± .006	

where X = Hb—Fe in mg in the pooled livers from 14—16 animals
F = total iron » » » » » 14—16 »
a = specific activity of Hb—Fe
b = » » » liver ferritin iron
c = » » » total liver iron

An example may be given (exp. nr 35, ser. 2, 1 day, Tables 12 and 13):

$F = 33.2$ mg
$a = 0.09$ %
$b = (4.20 + 4.03) : 2 = 4.12$ %
$c = 3.72$ %

$$x = 33.2 \frac{4.12—3.72}{4.12—0.09} \text{ mg} = 3.3 \text{ mg}$$

Iron of the blood free organ = (33.2—3.3): 2.83 = 10.6 mg Fe per 100 g wet weight. In our incompletely perfused animals the hemoglobin iron amounted to 25.6 ± 2.0 % of the total iron in the bloodcontaining livers. The average value for iron on the blood free livers determined from series 1 and 2 was 6.4 ± 1.3 mg * per 100 g wet weight.

* In the calculation of the probable errors the values from the pooled organs from 14—16 animals were taken as individual values. The probable errors therefore refer to variations between the experiments, each of which includes 14—16 animals.

The "musculature for myoglobin" was probably more effectively perfused than the livers [32]. This was supported by the fact that the water extracts of muscle, submitted to fractionation with ammonium sulfate, turned out to contain very little hemoglobin which was of the same order of magnitude as myoglobin. In animals with high myoglobin content in their muscles, like seal, horse, beef, or man, the bulk of the tissue iron may be present in myoglobin. A calculation from some of the yields of myoglobin in the experiments disclosed that guinea pig muscles can not contain more than 0.03 % myoglobin of the fresh weight muscle, thus 10—20 times less than in man, even if due attention is given to incomplete extraction and losses during the preparation.

The total iron in the "musculature for myoglobin" was found to be 1.36 ± 0.05 mg per 100 g wet weight, calculated from series 1 and 2. To this quantity of iron myoglobin contributed around 0.1 mg, the hemoglobin not removed by perfusion about the same amount, and cytochrome c around 0.02 mg.

Roughly 1 mg of iron per 100 g of wet tissue is thus contained in components other than hemoglobin, myoglobin or cytochrome c. Since the specific activity of the iron in "musculature for myoglobin" was always higher than that of the iron in myoglobin and cytochrome c, and since the hemoglobin content was low, this 1 mg of iron must have had an activity slightly above the values presented here for "musculature for myoglobin". Thus there were considerable amounts of relatively highly active iron available for the synthesis of myoglobin. The fact that myoglobin, nevertheless, retained a very low specific activity for a long time (Table 11 and Fig. 1) is our main evidence for its slow formation and breakdown.

Table 11. Per cent specific activity of myoglobin fractions (series 1).

Fraction		7 days		14 days		21 days		28 days		35 days	
		Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.
»70— 80»	A	—	—	0.16	—	0.23	0.08	—	—	0.33	0.15
70— 80	B	—	—	0.10	0.00	0.16	0.05	0.17	0.00	0.48	0.19
80— 90	A	0.01	0.01	0.06	0.00	0.15	0.04	0.24	0.08	0.22	0.16
80— 90	B	0.05	0.04	0.09	0.02	0.10	0.04	—	—	0.34	0.26
90—100	A	—	—	0.07	0.01	0.07	0.06	0.14	0.10	0.17	0.17
90—100	B	—	—	0.08	0.03	0.05	0.05	0.17	0.11	0.29	0.28
> 100	A	—	—	—	—	—	—	0.11	0.07	—	—
> 100	B	—	0.04	—	—	—	—	0.15	0.09	—	—
Average	A		0.01		0.01		0.06		0.08		0.16
Average	B		0.04		0.02		0.05		0.07		0.24

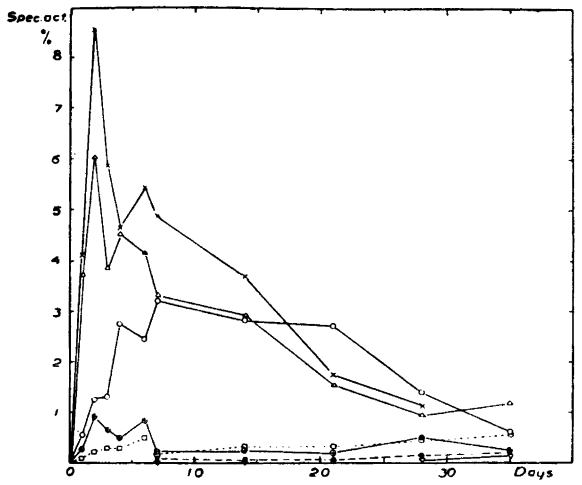

Fig. 1. Results of series 1 and 2.
——— × *Liver ferritin*
——— △ *Liver*
——— ○ *Liver catalase*
——— ⊕ *Spleen*
····· □ *Red blood cells*
– – – ● *Cytochrome c*
——— ⌓ *Myoglobin*

E. Rate of incorporation into various substances

a. Myoglobin. Specific activities significantly above zero are not achieved in myoglobin until after one month. At this time the above mentioned effect of the growth of the animals may already influence the results considerably. This means that the average life of the myoglobin molecule is probably still longer than would be estimated from our figures. On the other hand it must be born in mind that this does not necessarily apply to the myoglobin molecule as a whole. The protein part may be broken down and rebuilt again independently of the protohemin. Our results therefore only prove that the protohemin part of the molecule has a long lifetime. These observations correspond with the clinical observation mentioned in the introduction [55]. We would further like to draw attention to the fact that no rapid breakdown of protohemin in muscle tissue would be expected to occur since bile pigments do not normally appear in muscles in detectable amounts.

b. Cytochrome c. Cytochrome c takes up radioactive iron distinctly faster than the myoglobin does. In series 3 (Table 15) the activity increased steadily over the whole period of 59 days, but still at the end of the experiment it had not reached the equilibrium activity of around 0.5 %. In general the discussion on myoglobin is valid also for cytochrome c.

c. Hemoglobin and blood catalase. Conclusions regarding the average life time of the red blood cells can not be drawn from experiments with iron isotopes. We have not been able to find in the literature experiments on the life span of guinea pig erythrocytes. From our experiments, however, (*cf.* Figs. 1 and 2) it is evident that a comparatively rapid increase in specific activity

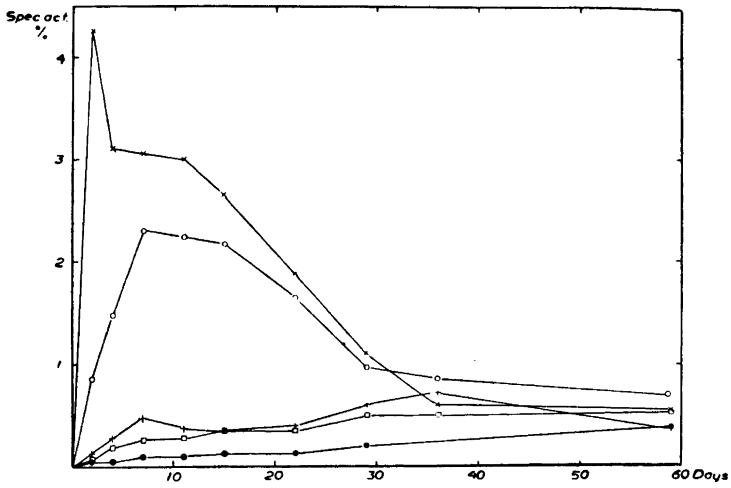

Fig. 2. Results of series 3.
× *Liver ferritin*
○ *Liver catalase*
\+ *Blood catalase*
□ *Red blood cells*
● *Cytochrome c*

occurs during the first week; the curve then flattens out more and more and approaches an equilibrium value asymptotically. The blood catalase activity gave rather scattered values owing to the technical difficulties encountered in preparing such minute enzyme quantities. In series 2 the activities of the blood catalase iron agreed reasonably well with the values for hemoglobin iron, with the exception of the three days value which came out lower for blood catalase. In series 3 the blood catalase activity was in most cases higher than the red blood cell activity. Because of the difficulties already mentioned in preparing sufficient amounts of blood catalase we can not, however, draw any other conclusions than that blood catalase is formed in the bone marrow simultaneously with the hemoglobin and probably takes its hemin iron from the same source as the hemoglobin.

d. Liver catalase, ferritin and blood catalase. As will be seen from Figs. 1 and 2 the specific activity of liver catalase iron follows the ferritin activity in a somewhat retarded cycle. After 4—5 days the catalase iron has reached half the activity of ferritin iron. The activity of the catalase later decreases, but as to be expected not quite as fast as the ferritin, so that the curves in series 3 intersect after 25 days. The average life time of catalase hematin can thus be

estimated to about 10 days, very much shorter than for myoglobin and cytochrome c. It is further obvious that the formation of liver catalase has nothing to do with that of blood catalase. They must be built up independently of each other in the liver and in the bone marrow. It is interesting to notice that these different organs are able to produce identical protein components, and it should be recalled that the liver acts as a hematopoietic organ during the fetal life.

e. Hemoproteins as possible precursors of stercobilin. It has been pointed out that a certain fraction ($\geq$ 11 %) of the stercobilin excreted by man cannot be derived from the hemoglobin metabolism. The possible role of catalase, cytochrome and myoglobin in the formation of that smaller fraction of stercobilin has been discussed.

Gray, Neuberger and Sneath [64] suggested that "the metabolism of porphyrins derived from myoglobin, catalase and possibly cytochrome c may account for the N^{15} content of the stercobilin excreted between the 30th and 80th day, but only for a small proportion of the high isotope content found at the initial period of the experiment". London, West, Shemin and Rittenberg [65] concluded that myoglobin could not be the sole source for the stercobilin not deriving from hemoglobin, but left the possibility open that myoglobin and the respiratory pigments might be the parent substance for that fraction of stercobilin.

From our experiments we are inclined to make the following comments.

Cytochrome c has a slow turnover number and can thus not participate in the "first" stercobilin peak. It may contribute to the "second" wave but owing to its low concentration only to a very restricted extent.

The blood catalase values of specific activity are quite close to those of hemoglobin. Any stercobilin deriving from blood catalase will therefore be included in the $\leq$ 89 % deriving from hemoglobin.

Liver catalase has a shorter life cycle than the two above mentioned substances. In man the N^{15} content of stercobilin has fallen from its initial high value to the minimum level on the 20th day. Thus liver catalase can contribute to the first "peak". However, from 500 g of guinea pig liver 700—800 mg catalase can be crystallized [46]. If the yield is assumed to be 1/3, and one guinea pig liver weighs 20 g, the total catalase content in one guinea pig liver is of the order of magnitude of 100 mg with the same hematin content as 25 mg of hemoglobin, corresponding to 0.06 ml blood. The guinea pig thus has roughly 300 times as much blood hematin as liver catalase hematin. The shorter life cycle of liver catalase as compared to hemoglobin is obviously still not short enough to give any appreciable contribution to the stercobilin production. Myoglobin is present in an amount of 1/5 of the hemoglobin in the human body [55]. The low turnover number, which from clinical observations can be supposed to be still lower in man than in guinea pig, diminishes the contri-

Table 12. Distribution of iron

Expt. no.	35				31			
Days	1				2			
	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe
Liver	283	33.2	3.72	28.4	326	27.2	6.01	34.4
Spleen	13	5.1	0.26	0.3	11	2.5	0.92	0.5
Red blood cells	248	126.5	0.09	2.6	220	112.1	0.23	5.4
Muscle for cytochrome	2 700	140.8	0.45	14.6	3 000	115.5	0.56	13.6
Muscle for myoglobin	355	5.7	0.27	0.4	378	3.8	0.61	0.5
Rest	4 790	241.0	0.81	45.0	6 220	327.0	0.46	31.7
Total	8 389	552.3		91.3	10 155	588.1		86.1
Number of animals	15				16			
Total/number of animals	559	36.8			635	36.8		

Table 13. Per cent specific activity of isolated compounds (series 2).

Exp. No.	35	31	30	34	32
Days	1	2	3	4	6
Liver	0.51 ± .01	1.26 ± .03	1.29 ± .03	2.60 ± .06	2.46 ± .05
Catalase	0.55 ± .01	1.35 ± .03	1.31 ± .03	2.91 ± .05	2.47 ± .05
Liver	4.20 ± .06	8.53 ± .18	5.52 ± .10	4.64 ± .09	5.48 ± .10
Ferritin	4.03 ± .07	8.62 ± .13	6.20 ± .08	4.66 ± .06	5.41 ± .07
Spleen	0.37 ± .01	1.00 ± .02	0.80 ± .01	0.54 ± .01	0.84 ± .01
Ferritin	0.39 ± .01	1.00 ± .02	0.77 ± .01	0.62 ± .01	0.86 ± .01
Blood	0.07 ± .01	0.18 ± .01	0.17 ± .02	0.28 ± .01	0.46 ± .01
Catalase	0.09 ± .01			0.25 ± .01	0.44 ± .01
Cytochrome *c*	0.069 ± .002	0.122 ± .003 0.118 ± .003	0.116 ± .003 0.121 ± .003	0.102 ± .002	0.199 ± .004

and radioactivity (series 2).

30 3				34 4				32 6			
Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe	Wet weight g	Total Fe mg	Spec. act. %	% recovered of isotopic Fe
311	25.3	3.85	20.5	299	33.7	4.52	32.1	356	26.4	4.16	24.7
13	3.38	0.65	0.5	13	5.0	0.49	0.5	12	2.3	0.85	0.4
272	138.7	0.29	8.5	251	128.0	0.27	7.3	292	149.0	0.50	16.8
3 000	150	0.36	11.4	3 000	464	0.13	12.7	3 000	124	0.53	14.8
435	4.9	0.52	0.5	408	7.2	0.27	0.4	405	4.5	0.49	0.5
6 120	308	0.51	33.1	4 830	278	0.49	28.7	5 940	210	0.52	24.5
10 151	630.3		74.5	8 801			81.7	10 005	516		81.7
16				16				15			
635	39.4			550				667	34.4		

bution of this important hematin fraction to the stercobilin production to a very low value. The initial peak in N^{15}-labelled stercobilin excretion can not derive from myoglobin metabolism.

The few experiments which we carried out with rats seemed to indicate that the liver catalase incorporated iron at about the same rate as in guinea pigs. If we compare this rate with the results of Greenstein *et al.* we find that the extremely rapid disappearance of catalase after implanting a liver tumor

Table 14. Per cent specific activity of myoglobin fractions (series 2).

Expt. no.	35		31		30		34		32	
Days	1		2		3		4		6	
	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.	Uncorr.	Corr.
70— 80 %	0.05	0.00	0.17	0.04	0.12	0.00	0.10	0.00	0.28	0.08
80— 90 %	0.03	0.02	0.08	0.05	0.07	0.04	0.08	0.04	0.20	0.11
90—100 %	0.01	0.01	—	—	0.06	0.02	0.05	0.03	0.14	0.08
Average		0.01		0.05		0.02		0.02		0.09

Table 15. Per cent specific activity of

Expt. no.	36	37	38	39
Days	2	4	7	11
Liver	4.27 ± 0.09	3.12 ± 0.08	3.07 ± 0.07	2.93 ± 0.08
Ferritin	4.25 ± 0.09	3.07 ± 0.07	3.07 ± 0.07	3.04 ± 0.07
		3.10 ± 0.06		
Liver	0.87 ± 0.02	1.48 ± 0.03	2.40 ± 0.06	2.33 ± 0.06
Catalase	0.81 ± 0.03	1.50 ± 0.03	2.26 ± 0.05	2.22 ± 0.05
			2.29 ± 0.04	2.17 ± 0.04
Cytochrome	0.038 ± 0.002	0.053 ± 0.005	0.105 ± 0.004	0.117 ± 0.004
C	0.037 ± 0.003	0.066 ± 0.003	0.107 ± 0.005	0.121 ± 0.004
Red blood	0.072 ± 0.003	0.164 ± 0.005	0.233 ± 0.006	0.274 ± 0.007
Cells	0.078 ± 0.002	0.176 ±0.006	0.227 ± 0.005	0.297 ± 0.007
Blood	0.12 ± 0.02	0.32 ± 0.01	0.47 ± 0.01	0.38 ± 0.02
Catalase	0.09 ± 0.02	0.25 ± 0.01		

can not be caused only by an inhibition of the normal synthesis of catalase but must be due at least to a substantial part by an increased destruction. The normal formation rate is not high enough to account for re-establishing a normal catalase level in one or two days after the removal of the tumor. It seems possible, however, that the rate of catalase formation could increase during catalase deficiency in analogy with the increased formation of hemoglobin in anemia. This interesting problem is worth while of reinvestigating with tracer technic and more reliable catalase activity determinations.

SUMMARY

1. The total iron content of normal guinea pigs weighing 500—1 000 g was determined and found to be 5.95 mg per 100 g body weight. The iron contents of blood-free liver and muscle tissue were estimated to be 6.4 ± 1.3 and 1.26 ± 0.05 mg respectively per 100 g tissue wet weight.

2. Radioactive iron in amounts of 0.05 mg per 100 g body weight was given intraperitoneally as ferric ammonium citrate. The distribution of radioactive

iron in isolated compounds (series 3).

40 15	41 22	42 29	43 36	44 59
2.79 ± 0.07	2.21 ± 0.05	1.25 ± 0.04	0.61 ± 0.03	0.55 ± 0.03
2.66 ± 0.06	1.86 ± 0.05	1.14 ± 0.03	0.59 ± 0.02	0.54 ± 0.02
2.54 ± 0.05	1.67 ± 0.03	0.94 ± 0.02		
2.21 ± 0.06	1.64 ± 0.04	0.94 ± 0.05	0.86 ± 0.05	0.64 ± 0.04
2.11 ± 0.04	1.65 ± 0.04	1.00 ± 0.03	0.86 ± 0.02	0.77 ± 0.04
0.135 ± 0.004	0.117 ± 0.005	0.215 ± 0.005	—	0.389 ± 0.009
0.135 ± 0.004	0.117 ± 0.005	0.220 ± 0.005	—	0.392 ± 0.009
0.343 ± 0.008	0.348 ± 0.009	0.381 ± 0.009	0.368 ± 0.010	0.527 ± 0.010
0.333 ± 0.007	0.344 ± 0.008	0.404 ± 0.009	0.388 ± 0.008	0.523 ± 0.012
0.35 ± 0.02	0.42 ± 0.02	0.59 ± 0.02	0.71 ± 0.02	0.36 ± 0.01

iron in different organs and the specific activities of the iron in some hemoproteins and ferritins were determined after time intervals up to 59 days.

3. Liver ferritin exhibited a very high specific activity with a maximum after two days, followed by a drop to a plateau, where the values remained for about a week. The activities then decreased asymptotically to a final value.

4. The liver catalase iron activity followed the ferritin in a retarded cycle, indicating that the half life of liver catalase hematin in normal guinea pigs is around 4—5 days. The importance of this finding is discussed.

5. The hemoglobin incorporated radioactive iron comparatively fast. The blood catalase followed the hemoglobin in this respect, as far as could be judged from the rather uncertain determinations on the small quantities of catalase iron isolated. It can thus be said with certainty that liver and blood catalase are formed independently of each other.

6. Myoglobin incorporated iron extremely slowly, muscle cytochrome c somewhat faster. The significance of these results are discussed with regard to previous clinical observations on myoglobin and experimental work on bile pigment production.

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