SOME ASPECTS OF RADIOIRON METABOLISM BY LEUCOCYTES*

by

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INTRODUCTION

A preliminary report by EVERETT[1] indicated that when radioiron is administered to rats, the leucocytes as well as erythrocytes incorporate enough of the radioactivity to produce single cell autographs. Although not mentioned in the report, the radioautographic evidence further indicated that within the first few days after administration of the radioiron the leucocytes incorporated more iron per cell than did the erythrocytes. The fact that leucocytes contain iron was noted in 1891 by MACALLUM[2,3] who demonstrated its presence by treating the cells with ammonium sulfide. Since he found iron in the cytoplasm of the leucocytes within the intestinal villi and only after oral administration of iron, he suggested that the leucocytes may be partially responsible for the transfer of the iron to the general circulation. BARKER[4] applying MACALLUM's technique to peripheral blood smears noted that the reaction of leucocytes was more pronounced than that of erythrocytes. It is doubtful, however, that under the conditions of this histochemical test that very much of the iron from hemoglobin would react. BARKER noted that the granules of eosinophils gave a very pronounced reaction to this test for iron. This was later substantiated by PETRY[5,6] who isolated the granules and found them to have a high iron content.

More recently, GILLMAN AND IVY[7] reported that the reticulo-endothelial system may function in the absorption and transport of iron since they found iron within the system following oral administration of ferrous ammonium sulfate. Subsequent studies by ENDICOTT AND GILLMAN[8] demonstrated that the iron within the lymph nodes and reticulo-endothelial cells was not derived from a test meal of radioiron.

It has been demonstrated that blood cells do not acquire iron following 15 minute *in vivo* or *in vitro* incubation with inorganic iron. Furthermore, studies of iron absorption and transport have indicated that plasma globulin is the primary vehicle of iron transport (LAURELL[9]). However, there have been no quantitative studies of the iron content of circulating leucocytes following iron administration. It is questionable, therefore, that there is any conclusive evidence as to whether leucocytes function in iron transport. There have been several iron containing enzymes identified in leucocytes which would contribute to the iron observed in these cells by the above investigators. AGNER[9] isolated an iron containing enzyme from the myeloid leucocytes which he identified as verdoperoxidase. Cytochrome C was identified in the cytoplasm of both the

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myeloid and lymphoid cells by HOFFMAN[10]. Catalase although previously thought to be present in leucocytes could not be identified by AGNER[11].

Reported here are studies designed to determine the magnitude of radioiron incorporation by leucocytes as compared to that of plasma and erythrocytes, and to ascertain whether the iron content of leucocytes is in excess of that present in their metabolic constituents. In addition it was hoped that this investigation would provide a basis for using radioiron as a label for leucocytes in subsequent studies of their origin and fate.

METHODS

The animals used in this investigation were male rats of the Spraque-Dawley strain weighing approximately 200 grams. Each rat was given a single do e of ^{59}Fe ferric chloride by either the intraperitoneal, intravenous, or oral route.

Two levels of iron were used, one of low and the other of high specific activity. In either case the amount of radioactivity given was from 15 to 30 microcuries. The activity varied in order to control the iron concentration. One to 9 milligrams of the low specific activity iron were used which was in excess of the animal's plasma iron binding capacity, as estimated from the human values reported by LAURELL[9]. Twenty nine to 60 micrograms of the high specific activity iron were administered, an amount considered to be in the physiological range. In brief our procedure was to obtain leucocytes for radioassay in a plasma suspension which could not be obtained entirely free of erythrocytes. The radioiron contributed by the erythrocytes was determined by assay of an equal number of erythrocytes in an essentially pure suspension. The plasma was removed by centrifugation and the remaining radioactivity was attributed to the number of leucocytes present. These assays were made at intervals from within a few hours to 21 days after iron administration.

In detail, whole blood was obtained from rats by heart puncture and immediately heparinized. All glassware used was silicone treated and care was exercised to avoid excessive agitation of the blood. A plasma suspension containing 10 to 30 times 10^6 leucocytes per cubic mm with no more than an equal number of red cells, was prepared by the differential sedimentation method, MINOR AND BURNETT[13], BUCKLEY *et al.*[14]. BUCKLY reported the variation of this differential sedimentation with time and concentration of fibrinogen (Bovine fraction I). It was found that one ml of a 3% fibrinogen solution per 5 ml of whole blood with incubation at 37° C for 30 minutes produced a satisfactory separation of rat blood. The erythrocyte suspensions were prepared by diluting whole blood with isotonic saline.

Cell counts were made in triplicate on both the leucocyte and erythrocyte suspensions using BUCKMAN'S AND HALLISEY'S[15] platelet diluting fluid to facilitate identification of the cells. The red cell suspension was diluted if necessary to approximate the concentration of red cells in the suspension of leucocytes. One ml aliquots of the leucocyte suspension were diluted to 5 ml with 0.9% NaCl in a 5 ml micro centrifuge tube. This was done to dilute the concentration of radioiron in the supernatant since resuspending the cells, once packed, was found to damage them severely. After dilution the cells were packed by centrifuging under refrigeration. The supernatant was transferred to a 10 ml volumetric flask. Five ml of 0.9% NaCl were added to the packed cells which were allowed to stand for 10 minutes and again centrifuged. This second supernatant was added to the first and the volume adjusted to 10 ml. One ml aliquots of this were plated in pyrex planchets for counting. The tubes containing the packed cells were inverted on filter paper to drain the residual fluid.

Aliquots of the red cell suspension were centrifuged without further dilution since the plasma content was negligible. The packed cells from both suspensions were then digested by the addition of 0.5 ml of concentrated nitric acid. This was facilitated by heating the samples in a boiling water bath. When clear the samples were transferred with capillary pipettes to pyrex planchets using three one half ml portions of water to rinse the tube and pipettes. The platings were evaporated slowly under an infrared lamp, and the activity was determined by conventional counting methods. The variation in counts per minute between duplicate samples was not more than one percent.

The activity of the erythrocyte was expressed as counts per minute per 10^7 cells. The activity of the leucocytes was determined by subtracting the activity contributed by the erythrocytes contaminating the leucocyte suspension and similarly expressed as counts per 10^7 cells. The value of 10^7 cells was chosen because this was the minimum number of leucocytes per ml of blood. The white cell counts of whole blood were found to vary between 1 and $3 \cdot 10^7$ per ml. The activity of the supernatant was recorded as counts/min ml $\cdot 5$ since this approximated the activity of the plasma per ml of blood. Investigation revealed that the platelets, which would also be contained in the leucocyte cell pack could be disregarded since they were non-radioactive. This was established by assay of pure platelet suspensions prepared by the method of DILLARD *et al.*[16].

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That the radioactivity was due entirely to radioiron was verified for three separate experiments by the procedure of VOSBURGH *et al.*[17] which is specific for iron. The platings of both the red and white residues were recovered after assay in 8 *N* HCl and extracted with *iso*propyl ether. The ether phase was extracted with 0.1 *N* HCl which was then plated for assay. The activity of these platings ranged between 85 and 90% of the original values.

Since there is a considerable amount of radioiron in the plasma protein for several days following iron administration, it seemed possible that the activity found in connection with the leucocytes was the result of surface adsorption or from the establishment of an equilibrium with the plasma iron by diffusion. If this were the case one would expect that by diluting the radioiron content of the plasma the distribution of activity between the cellular residue and the plasma supernatant would be altered. Dilutions of the plasma suspensions were made with 10 and 15 volumes of 0.9% NaCl and control plasma. These diluted preparations were compared with values obtained from an undiluted suspension. The ratio of activity between the cellular residue and the supernatant was not significantly changed by either diluent of volume.

Another approach to this problem was made by incubating a suspension of viable white cells, $25 \cdot 10^6$, from a nonradioactive rat, in labelled plasma obtained from a rat 24 hours after radioiron administration. The incubation was carried out at 37° C for 30 minutes with gentle agitation. The activity of the cell residue from this preparation was not significant. A similar approach was made by giving a rat an intravenous injection of iron labelled plasma. The white cells recovered from this animal 30 minutes after injection had no significant activity. Thus it seemed justifiable to assume that the radioiron content of the leucocytes was not the result of diffusion or adsorption.

RESULTS

Table I shows the results of the assays following the administration of excessive doses of radioiron. It is observed that although the leucocytes incorporated a considerable amount of iron, this was neither an immediate nor a prolonged phenomenon. The maximal levels of activity for the white cells were found at three days. At this time the activity of the leucocytes, although variable amongst the individual animals, was from 4 to 50 times that of an equal number of red cells. By the seventh day the activity of the leucocytes had declined and was of the same order of magnitude as that of the erythrocytes. During the following two weeks the activity of the leucocytes levelled off gradually to a more or less constant value.

TABLE I

EXCESSIVE DOSE OF RADIOIRON

Time Interval	*Rat No.*	*Iron milligrams*	*Counts per minute*		
			Erythrocytes 10^7 cells	*Leucocytes 10^7 cells*	*Supernatant ml·5*
		Intraperitoneal			
1 day	6	7.1	—*	7	50
1 day	13	7.1	—*	28	50
3 days	4	9.1	11	624	75
3 days	5	7.1	14	601	40
3 days	7	7.1	9	44	30
3 days	9	7.1	8	31	40
3 days	1	9.1	8	82	20
7 days	8	7.1	11	15	36
7 days	10	7.1	19	31	40
14 days	3	9.1	36	—*	11
14 days	11	7.1	17	26	30
14 days	12	7.1	16	3	15
21 days	14	7.1	25	4	6
		Intravenous			
3 days	15	1.01	6	10	15
8 days	18	1.01	4	3	15

* No measurable activity from 10^7 cells.

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There was an apparent correlation between the activity of the plasma and that of the leucocytes. It is noted that, with the exception of the period preceding the maximum uptake of iron by the white cells, the levels of activity in the plasma and leucocytes were of the same order of magnitude. Furthermore their activities declined at a similar rate.

The results following the administration of radioiron of high specific activity in physiological doses were similar to the above in regard to the uptake of iron by leucocytes, Table II. The only significant difference was that in this series the iron was utilized at a faster rate. The maximal values for leucocytes were obtained on the first day, and by the third day they had already declined. In this series the red cells attained higher values but as in the first series their activity never exceeded the maximal levels of the white cells.

TABLE II

PHYSIOLOGICAL DOSE OF RADIOIRON

Time Interval	*Rat No.*	*Iron micrograms*	*Counts per minute*		
			Erythrocytes 10^7 cells	*Leucocytes 10^7 cells*	*Supernatant ml·5*
		Intravenous			
4 hours	61	29	11	17	6,910
10 hours	61	29	11	40	1,200
1 day	23	29	118	665	795
1 day	24	29	132	351	590
1 day	27	29	75	590	625
3 days	21	29	300	80	290
8 days	22	29	254	21	
8 days	33	29	131	31	144
		Intraperitoneal			
4 hours	54	29	5	40	900
10 hours	54	29	21	50	425
1 day	30	29	29	154	220
1 day	60	29	31	1,216	984
1 day	28	29	21	2,211	180
1 day	35	29	17	102	330
1 day	50	60	34	597	
3 days	32	29	89	23	25
3 days	34	29	70	49	90
7 days	31	29	98	3	14
		Oral			
1 day	29	29*	81	225	
1 day	37	29*	10	81	
2 days	40	58*	27	11	

* Given with ascorbic acid.

Since the specific activity of the radioiron used was not constant throughout the series the ratios of activity amongst the blood components assayed were used to compare the results of individual experiments. Figs. 1 and 2 are graphic representations of the average ratio of activity of the plasma and leucocytes to that of the erythrocytes in the intraperitoneal series in which excessive and physiological doses of radioiron were

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used. These clearly indicate the early peak and the rapid fall of the leucocyte activity. In addition these graphs show the relationship between the activity of the leucocytes and plasma.

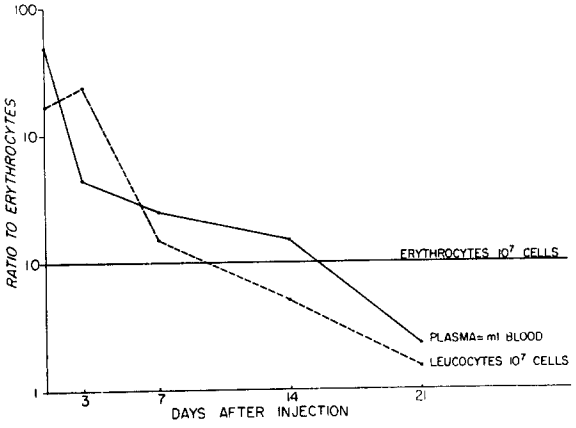

Fig. 1. Activity of leucocytes and plasma as compared to erythrocytes after excessive dose of radioiron (intraperitoneal).

Fig. 3 shows the results of assays made at intervals ranging from 2 hours to 7 days on one rat following a single intraperitoneal injection of radioiron. The radioactivity values of the leucocytes and plasma are recorded as activity of each per ml of blood. This demonstrates the actual magnitude of radioiron incorporation and eliminates the factor of variation between individual animals. It is observed that the amount of radioiron within leucocytes of the peripheral blood at 24 hours was 1.2% of the injected dose. This was calculated by assuming the total blood volume to be 5 ml per 100 g. At this same interval the plasma contained 1.4% of the injected dose.

It was observed that the ratio of reticulocytes to erythrocytes was higher in the red cell fraction contaminating the leucocyte suspension than it was in whole blood. At these early intervals the reticulocytes would contain proportionally more of the administered iron than the mature red cells, which would introduce an error in estimating the radioactivity of the red cells. Thus attempts were made to determine the magnitude

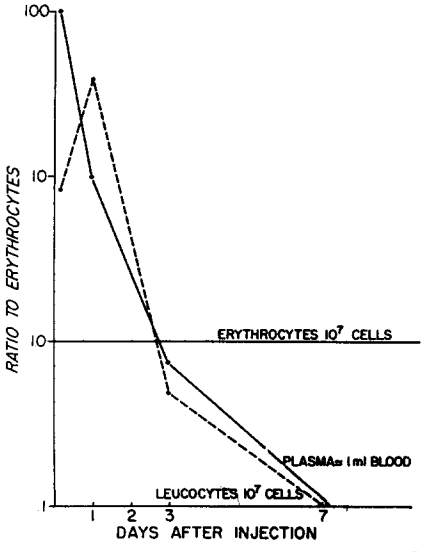

Fig. 2. Activity of leucocytes and plasma as compared to erythrocytes after physiological dose of radioiron (intraperitoneal)

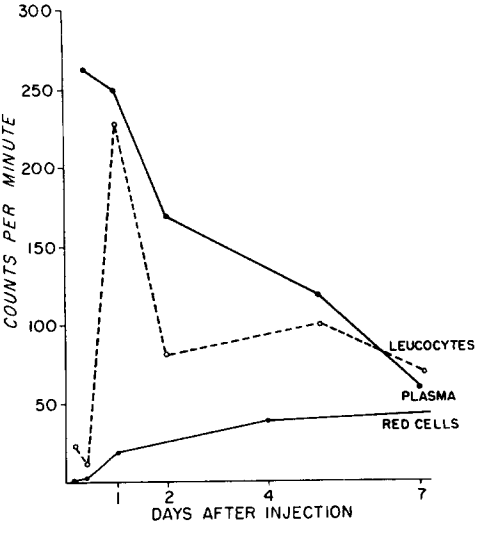

Fig. 3. Activity of leucocytes and plasma per ml of blood and of red cells in amounts equal to that of leucocytes. (Values obtained from a 335 g rat following intraperitoneal injection of 200 γ $^{59}FeCl_3$.)

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of this error in obtaining the values reported in Tables I and II. By varying the differential sedimentation of blood with a normal 2% reticulocyte count it was possible to obtain white cell suspensions contaminated with red cells that varied from 3 to 8% reticulocytes. From these data it was determined that the errors of leucocyte values reported did not exceed 5%. Furthermore it was found by preparing several white cell suspensions, from a single sample of blood, with ratios of white to red cells of 1:1; 2:1; and 5:1, that the white cell values for these suspensions varied not more than 5%.

The rather small quantity of leucocytes obtainable from rats has limited the attempts to identify the radioiron-containing fraction. However, the fractions containing the iron were shown to be readily soluble in water, in 0.1 *M* phosphate buffer of pH 6.8 or 7.4, and in dilute acid. In addition the extract lost no activity after dialysis in cellophane tubing against phosphate buffer of pH 6.8. On alkaline hydrolysis they yielded an insoluble residue containing 85 to 95% of the radioiron. These experiments suggested that the iron was bound to or was incorporated with one or more proteins. Attempts were made therefore to identify the radioactivity with known iron-containing proteins.

Following AGNER's[10] method for the isolation of verdoperoxidase it was found that essentially all of the radioiron was obtained in the discard fractions and that the crude VPO fraction had no significant radioactivity. These measurements were made using 15 ml samples of white cell supernatant from animals, each of which had received 50 μ of radioiron. Since this enzyme is restricted to the myeloid series and rat leucocytes are only 5% myeloid, these results are not surprising. A preparation of cytochrome C, isolated by THEORELL's[18] technique, was obtained from a similar sample of leucocytes and was found to contain 10% of the radioactivity. The remaining 90% was found in the ammonium sulfate precipitate (500 gm/liter) which was the residue from this cytochrome C extraction.

These data indicate that ferritin could be the radioiron containing protein. Attempts were made therefore to isolate ferritin by GRANICK's[19] method, but no crystalline ferritin was obtained from either the white cells or from the liver which normally is the main source of ferritin. Attempts were then made to isolate ferritin from leucocytes by the method of THEORELL[18]. This method is similar to GRANICK's procedure with the exception that the cadmium sulfate crystallization is replaced by precipitation in 0.4% saturated $(NH_4)_2SO_4$. THEORELL states that this procedure yields essentially a pure preparation of ferritin. By this method 12% of the radioiron content of the leucocytes was isolated as ferritin. When the heating step in the procedure, which had removed only 5% of the radioactivity by coagulation, was omitted, it was found that 90% of the radioiron was precipitated at 0.4% saturation, *i.e.* at the saturation precipitating ferritin. As yet no physical measurements have been made to determine if this fraction is in reality a heat labile form of ferritin.

DISCUSSION

It has been demonstrated that leucocytes incorporate considerable amounts of radioiron after intravenous or intraperitoneal administration. Although the data for oral administration are limited the same phenomenon was observed. It would appear that this incorporation is of sufficient magnitude to warrant consideration in studies of iron metabolism. Since the radioiron was found to be in a water soluble, non-dialyzable form which could be precipitated by $(NH_4)_2SO_4$, it was assumed to be associated with a

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soluble protein. Twelve per cent of the iron incorporated by leucocytes appeared to be ferritin. An additional 80% was found to have properties similar to ferritin but was evidently not stable on heating to 80° C since some of the iron could be removed by dialysis following heating. Since the heat stability of rat ferritin has not been studied, the instability of this fraction does not exclude its being ferritin.

Ferritin has not been identified as a normal constituent of blood, although AGNER[12] isolated a ferritin-like compound from erythrocytes. In addition MAZUR AND SHORR[20, 21] identified ferritin in the plasma of men and dogs under conditions of irreversible shock. These latter investigators were unable to identify ferritin by immunochemical tests in the blood of dogs under normal conditions. This is in line with GRANICK's finding[19], using immunochemical tests, that there was no ferritin in the red or white cells or plasma of horses. However, GRANICK[22] stated that the limit of sensitivity for the immunochemical test is 0.5 γ ferritin N per gram. If the unidentified iron in leucocytes is in the form of ferritin, the estimated quantity would be considerably less than the 0.5 γ gamma ferritin N per ml of white suspension and, therefore, would not be detectable by this immunochemical test. GRANICK[22] indicated that ferritin may well occur in almost all tissues but not in quantities sufficient for identification. Although ferritin is primarily an iron storage protein recent studies have indicated that it has other functions, see GRANICK[22] for review. Thus, to identify the protein bound iron of leucocytes as ferritin would not establish the significance of this fraction.

Approximately 10% of the radioiron content of leucocytes was found in the isolated cytochrome C. Verdoperoxidase, the only other heme protein reported to be present in leucocytes, was found to contain no significant amount of the radioiron 24 hours after iron administration. Although there may be other heme proteins in leucocytes it appears unlikely that they account for any of the unidentified fraction since the iron of this fraction is so readily liberated by hydrolysis or by heating to 80° C. Thus it appears that leucocytes incorporate iron in excess of their heme protein requirement.

Neither the mechanism by which iron enters the leucocytes nor the source from which it is obtained are known. It has been shown, however, that the radioiron of the plasma is not directly available to white cells which is in agreement with LAURELL's report[9]. Furthermore, it is observed that the leucocytes do not reach maximal levels of activity until a large per cent of the radioiron has left the plasma (Fig. 3). Numerous investigators, see ANDERSON[23] for a review, have shown that administered iron is taken up from plasma principally by the liver, spleen and bone marrow and to a lesser extent by the lymph nodes. Thus, it appears likely that leucocytes obtain iron within the reticulo-endothelium system. It is feasible that the iron could be incorporated during hematopoesis by the mononuclear or polymorphonuclear luecocytes or by both. Preliminary attempts have been made without success to correlate the cell types in the leucocyte series with the magnitude or radioiron incorporation. This was done by differential enumeration from smears made of the leucocyte suspensions at all intervals in hopes of ascertaining whether or not this phenomenon is restricted to a particular cell type. Work is in progress toward the development of a satisfactory method for assaying the various white cell types.

It has been shown in this study that leucocytes cease to incorporate any significant amount of the administered radioiron once the plasma content has been reduced to minimal values. This might be interpreted as indicating that the unidentified iron in leucocytes is associated with the transport mechanism since it is present only during

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the period of high plasma concentration. It may be of significance that the time intervals for the rapid increase and decline of radioiron content of leucocytes are similar to that of the liver ferritin cycle reported by THEORELL[18] following radioiron administration to guinea pigs. In contrast, THEORELL[18] demonstrated that the rate of incorporation of iron in hemoglobin, cytochrome and myoglobin was gradual and levelled off to values which remained constant for the duration of the 3 month period of study. However, since the life span of leucocytes is so brief, VAN DYKE *et al.*[24], it is perhaps unwarranted to compare directly the iron metabolism of leucocytes with that of other tissues.

In contrast to the proposal that the unidentified fraction is associated with transport mechanisms, it is of interest to note the changes in iron metabolism during conditions of chronic and acute infections. Numerous authors, principally CARTWRIGHT *et al.*[25], NEUKOMM[26], GILLMAN *et al.*[7], GREENBURG *et al.*[28] have demonstrated that under such conditions iron is diverted from hemoglobin production to the reticulo-endothelial system and that there is apparently a tissue affinity for iron primarily in inflamed or infected areas. It may be that this is an indication that leucocytes do require significant amounts of iron and that when the demand for leucocytes is increased they obtain iron at the expense of hemoglobin. Without further evidence there can be no definite conclusion drawn as to whether the unidentified iron incorporated by leucocytes is related to an inherent requirement of the cell or to a transport mechanism.

SUMMARY

1. A method for the quantitative radioassay of leucocytes is described.

2. It is shown that within the first few days after iron administration leucocytes incorporate considerably more radioiron per unit number of cells than do erythrocytes. This is true following intraperitoneal, intravenous or oral administration of physiological or excessive doses.

3. The radioiron content of leucocytes is shown to be approximately of the same magnitude as that of plasma at the time the leucocytes reach their maximal values. The radioiron all but disappears from leucocytes when the plasma values level off to a minimum.

4. Preliminary investigation reveals that the radioiron of leucocytes is apparently associated with a water soluble protein, 10% of which is extractable as cytochrome C. Of the remaining iron 12% is apparently ferritin and the rest has solubility properties similar to ferritin but is heat labile at 80° C.

5. The possible significance and methods of radioiron incorporation by leucocytes is discussed.

RÉSUMÉ

1. Une technique de dosage de la radioactivité dans les leucocytes est décrite.

2. Dans les premiers jours qui suivent l'administration de fer, les leucocytes incorporent beaucoup plus de fer radioactif par cellule que les erythrocytes. Et celà après administration par voie intrapéritonéale, intraveineuse ou bucale de doses physiologiques ou en excès.

3. La teneur en fer radioactif des leucocytes est à peu près du même ordre que celle du plasma, au moment où les leucocytes atteignent leur concentration maximum. Le fer radioactif disparaît presque complètement des leucocytes quand la teneur du plasma atteint son minimum.

4. Des expériences préliminaires montrent que le fer radioactif des leucocytes est apparemment associé à des protéines solubles dans l'eau, dont on peut extraire 10% sous forme de cytochrome C. 12% du fer restant est probablement sous forme de ferritine et le reste a des caractères de solubilité comparables à ceux de la ferritine mais est instable au-dessus de 80° C.

5. La signification possible et les voies de l'incorporation du fer radioactif par les leucocytes sont discutées.

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ZUSAMMENFASSUNG

1. Eine Methode zur quantitativen Bestimmung von radioaktiven Isotopen in Leucocyten wird beschrieben.

2. Es wurde gezeigt, dass innerhalb der ersten wenigen Tage nach der Verabreichung von Eisen Leucocyten beträchtlich mehr radioaktives Eisen per Einheit der Zellenzahl einbauen als Erythrocyten. Dies trifft zu bei intraperitonealer, intravenöser oder oraler Verabreichung von physiologischen Dosen und von Dosen im Überschuss.

3. Wie gezeigt wird ist der Gehalt an radioaktivem Eisen in den Leucocyten ungefähr von der gleichen Grössenordnung wie der des Plasmas, wenn die Leucocyten ihren Maximalwert erreichen. Das radioaktive Eisen verschwindet beinahe aus den Leucocyten, wenn die Plasmawerte auf ein Minimum zugehen.

4. Vorläufige Untersuchungen zeigen, dass das radioaktive Eisen der Leucocyten wahrscheinlich mit einem wasserlöslichen Protein verbunden ist, von dem 10% als Cytochrom C extrahierbar sind. 12% des zurückbleibenden Eisens sind wahrscheinlich Ferritin und der Rest hat dem Ferritin ähnliche Löslichkeitseigenschaften, ist aber labil bei der Einwirkung von Wärme bei 80°.

5. Die mögliche Bedeutung und Methoden zum Einbau von radioaktivem Eisen in Leucocyten werden besprochen.

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