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STUDIES ON HEPATIC FERRITIN IN TUMOR BEARING RATS

1. Chromatographic Isolation of Hepatic Ferritin

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Iron exists in the living body in the form of protein bound iron and alterations in the amount of the protein moiety depends on the change in the amount of iron. The role of transferrin in the metabolism of iron has been amply demonstrated by a number of investigators (1, 2, 3). It is a well known fact that the serum transferrin level is elevated in iron deficiency anemia. It was shown by Bothwell and Finch (4) that transferrin to iron ratio was of prime importance in iron transfer from serum to erythroblasts in the bone marrow.

It seems likely that a similar relationship as in the above exists between iron and iron bound protein in the tissues. Ferritin exists in the mammalian liver as one of the iron bound proteins and is concerned with the iron metabolism by regulating iron storage and iron supply depending on serum iron level. Finburg and Greenburg (5) demonstrated that hepatic ferritin synthesis was induced by the introduction of iron.

In a series of experiment on the metabolism of iron in cancer anemia, Urushizaki et al.(6) demonstrated a shortened life span of erythrocytes and decreased hepatic ferritin content in tumor bearing subjects, suggesting that the disturbance of iron metabolism in a tumor bearing state was

characterised by an impairment in the reutilization of hemoglobin iron in the reticulo-endothelial system. However, the hepatic ferritin iron was measured by Konno-Yoneyama's method(7) and ferritin protein was not studied in these experiments.

Consequently, an alternative method of isolating and measuring both ferritin iron and ferritin protein was required for the metabolic study of ferritin.

In 1965 Drysdale and Munro(8) devised a new method of isolating ferritin in a free state from other tissue proteins, based on the difference in chromatographic properties between ferritin and other tissue proteins. They used CM-cellulose and Sephadex G-200 column to achieve this purpose and obtained purified ferritin.

In the present work the authors investigated the method of Drysdale and Munro and confirmed the validity of this method for the quantitative measurment of hepatic ferritin content. By this method the hepatic ferritin content of tumor bearing rats was studied as a preliminary experiment on the hepatic ferritin biosynthesis in tumor bearing subjects.

Materials

Adult male albino rats (Gifu-strain, Japan) weighing approximately 150g were used. A group of rats were injected subcutaneously into one hind leg with approximately 10^7 freshly taken Yoshida sarcoma cells. Since tumor cell inoculation causes the death of the animals within 15 days, the experiments were carried out on the 7th day after the tumor cell inoculation.

Bled rats: Two ml of blood was drawn from the right jugular vein of normal rats and on the third day the rats were used for the experiments.

Rats leaded with iron: 500 g of iron in 1 ml of saline per 100g of body weight were injected intraperitoneally in the form of ferric ammonium citrate and on the second day the rats were used for the experiments.

Inflamed rats: The inflammatory stimulus used here consisted of injecting 1 ml of turpentine oil into a pouch on the back formed by a subcutaneous injection of 15 ml of air. This is the so-called granuloma pouch technique(9).

Herse spleen ferritin (cadmium free) was purchased from Pentex Co. Ltd, Illinoi, U. S. A..

Methods

All apparatuses were freed from iron by immersing overnight in a mixed solution of 3 volume of 18 % HCL and 1 volume of 30 % HNO_3, followed by repeated rinsings in deionised water. The reagents used for analysis were found to have a satisfactorily low iron content.

Determination of ferritin protein: Protein of the ferritin fraction was estimated by the method of Lowry, Rosebrough, Farr and Randall(10) using bovine serum albumin as a standard.

Determination of ferritin iron: Hill's bipyridyl method(11) modified by Drysdale and Munro(12) for the estimation of ferritin was chosen with Na_2SO_3 as the reducing agent. The ferritin containing solution was heated at 100°C in a water bath for 1 hour in the presence of Na_2SO_3, 2,2'-bipyridyl (0.05%) and acetic acid (6%,V/V). The color developed was read at 520 mμ after adjusting the volyme of the solution to 10 ml

with water.

Determination of hemoglobin and serum iron: Hemoglobin was determined by the cyanmethemoglobin method and serum iron was determined by Trinder's method with Nakao's modification(13).

Preparation of anti-sera for the heart supernatant of rat liver and for the horse spleen ferritin: These anti-sera were prepared by injecting rabbits with heat supernatant of rat liver and with commercial horse spleen ferritin respectively. Freund's complete adjuvant was added to the antigen solution to increase antibody production.

Immunoelectrophoresis was carried out in 1 % (W/V) agar gel in veronal buffer, pH 8.6, on a glass slide with LKB immunoelectrophoresis apparatus. The antigen-antibody precipitates were finally stained for protein with Ponceau S and for iron with Perl's stain.

Polyacrylamide gel electrophoresis: Gels 15 cm long containing 5 % of polyacrylamide in 50mM-tris-citrate buffer, pH 7.9, were run for 4 hours at 200 V. The antigen-antibody precipitates were stained as described for immunoelectrophoresis.

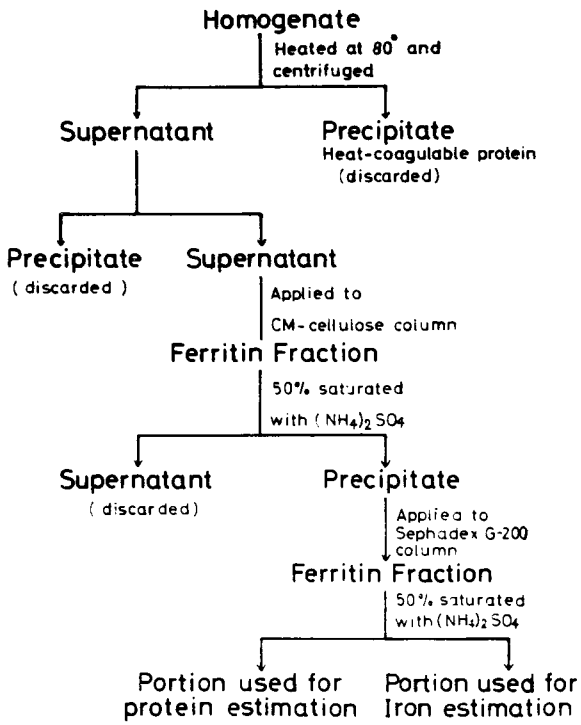
Experimental

PROCEDURE FOR THE ISOLATION OF HEPATIC FERRITIN

Isolation and determination of hepatic ferritin was carried out by the method of Drysdale and Munro with our minor modifications. The method consisted of four main steps (Scheme 1). The first step was the preparation of the heat supernatant fraction from rat liver homogenate. The second step was chromatography of the heat supernatant fraction on

CM-cellulose column. The ferritin containing fraction was subsequently subjected to ammonium sulphate fractionation. Finally the ammonium sulphate precipitate was passed through a Sephadex G-200 column for further purification. In the present experiment the final fraction was further subjected to ammonium sulphate precipitation in order to obtain a suitable concentration of ferritin for the determination of ferritin protein and it's iron.

Scheme 1 Modified method of Drysdale and Munro for the isolation of hepatic ferritin.



1) Extraction of heat supernatant fraction of rat liver:

The portal vein and the inferior vena cava above the diaphragm were severed to allow for blood drainage. Rats were killed in the bleeding process. Four grams of excised liver were homogenated in 4 volumes of water in a Potter-Elvehjem type homogenizer with a teflon pestle. The hemogenate was slowly heated in a water bath with continuous stirring at 75°C and the resulting precipitate was removed by centrifugation. This water soluble fraction is referred to as the heat supernatant fraction.

2) Chromatography of the heat supernatant fraction on CM-cellulose column:

The heat supernatant fraction was fractionated by the method of Drysdale and Munro(8). They demonstrated that if the pH was below 5.0 and the ionic stremgth was less than 0.015, ferritin was adsorped by C M-cellulose. The pH of the heat supernatant was adjusted to pH 4.8 with 0.2N-acetic acid and the resulting precipitate was removed by centrifugation.

The supernatant was passed through a column containing approximately 1 g of CM-cellulose equilibrated with a buffer, pH 4.8, containing 15m-equiv. of sodium acetate and 10m-equiv. of acetic acid per liter and the sample was allowed to flow with a flow rate of 20ml per hour. Under this condition ferritin was adsorped by CM-cellulose and thereafter the column was washed thoroughly with this buffer and the majority of the concomitant proteins in the heat supernatant fraction passed through the column. Subsequently the column was washed with a buffer containing 32m-equiv. of sodium acetate and 18m-equiv. of acetic acid. At this higher pH and ioni

strength some of the non-ferritin protein was removed and subsequent elution of ferritin occured more easily when the pH was subsequently increased to 5.5. A fraction containing ferritin was eluted by treatment with a buffer pH 5.5 containing 42.8m-equiv. of sodium acetate and 6,2m-equiv. of acetic acid per liter. The ferritin which is clearly seen as a yellow band (Fig. 1) can be collected in the next 20 ml of eluates. An ultra-violet spectrophotometric study of this fractionation showed three peaks, each of which were obtained by changing the buffer in a stepwise manner as described above (Fig. 2).

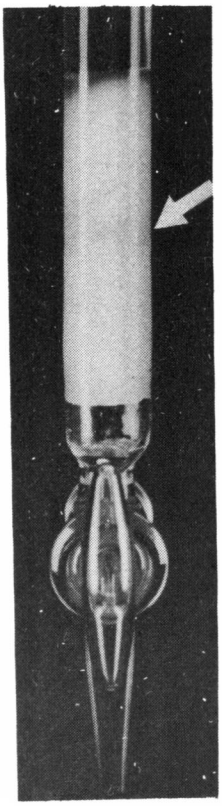


Fig. 1 A light brown bnad of ferritin fraction developing in the course of chromatographic procedure on CM-cellulose column.

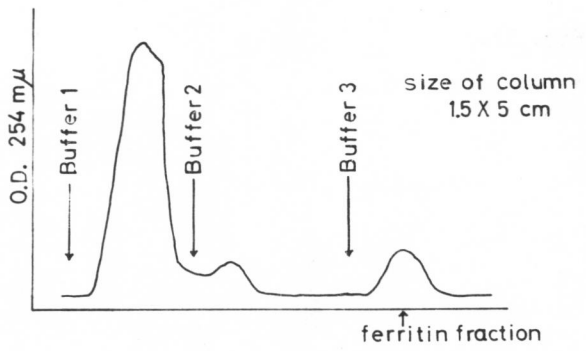


Fig. 2 Elution pattern of hepatic ferritin on CM-cellulose column.

3) The fractionation of the ferritin fraction by ammonium sulphate precipitation:

The ferritin was completely precipitated by half-saturation with ammonium sulphate overnight at 4°C and was collected by centrifugation at 100 g for 15 minutes, after which the supernatant fluid was discarded.

4) Sephadex G-200 gel filtration:

The ammonium sulphate precipitate of the ferritin fraction obtained from CM-cellulose column fractionation was dissolved in 0.5 ml of 10mM-phosphate buffer pH 7, for gel filtration on Sephadex G-200 column. Large molecules like ferritin with a molecular weight of more than 2×10^5 was excluded from this column and appeared in the first fraction after the elution of the void volume as shown by the ultra-violet spectrophotometry (Fig. 3).

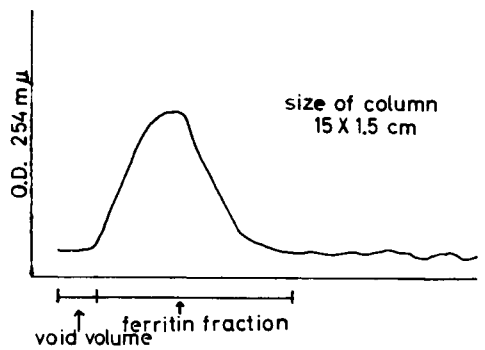


Fig. 3 Elution pattern of hepatic ferritin on Sephadex G-200 column.

Sephadex G-200 column was well swollen and equilibrated in 10mM-phosphate buffer, pH 7. It was packed 15 cm in height in a 1.5 cm diameter column and was washed with the same buffer under a pressure of 60 cm H_2O. An ultra-violet spectrophotometric study disclosed that the void volume was 7.5 ml and the first fraction containing ferritin was collected in another 10 ml of the eluate. This fraction was again precipitated with half-saturation with ammonium sulphate and the resulting precipitate was dissolved in 4 ml of distilled water. One portion of this was subjected to protein determination and another portion was used for iron determination.

IDENTIFICATION AND TESTS OF PURITY OF THE ISOLATED FERRITIN

1) Polyacrylamide gel electrophoresis:

The ferritin containing fraction in each step of ferritin isolation was subjected to polyacrylamide gel electrophoresis. The heat supernatant fraction showed several protein bands when stained with Ponceau S. The fraction obtained from CM-cellulose column chromatography showed two bands stained with Ponceau S, one band of which was the iron staining component as shown in Fig. 4. After Sephadex G-200 gel filtration, however, only one component which stained for iron and protein was obtained.

2) Immunoelectrophoresis:

Two sets of immunoelectrophoretic studies were performed. Anti-serum for the heat supernatant prepared from rabbits was used to confirm the process of purification of hepatic ferritin. Samples of the final purified ferritin preparation and of the heat supernatant fraction were tested by immunoelectrophoresis against this serum. The gel was stained both for

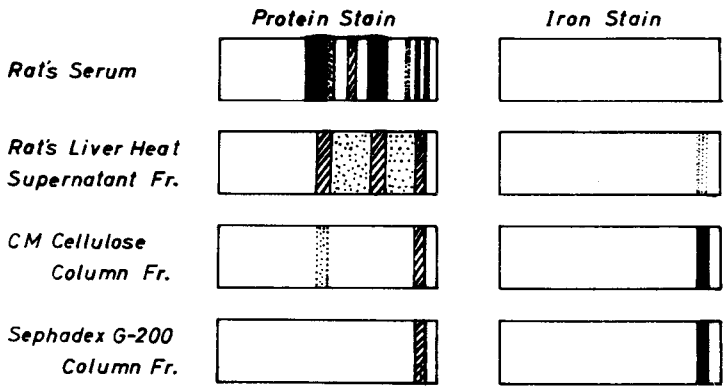


Fig. 4 Polyacrylamide gel electrophoresis of rat serum and of the fractions in each step of isolation of hepatic ferritin.

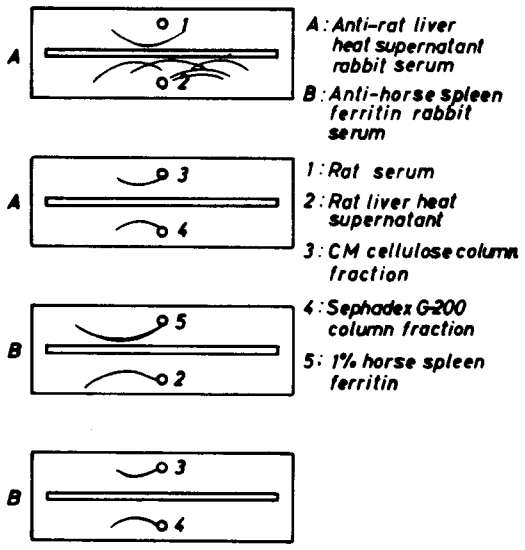


Fig. 5 Immunoelectrophoretic study of the fractions in each step of isolation of hepatic ferritin.

iron and protein after the completion of immune precipitation. At least five components were detected in the heat supernatant by immunoelectrophoresis. However, only one precipitin band was detected in the final purified ferritin fraction, which was stained both for iron and protein (Fig. 5).

For the identification of ferritin in the preparation obtained in the final process, anti-serum for horse spleen ferritin was used. Ibayashi et al. (14) and Ito (15) demonstrated that rat hepatic ferritin and horse spleen ferritin showed an identical antigenicity when tested by agar gel double diffusion. The final preparation showed a precipitin band for anti-serum of commercial pure horse spleen ferritin and thus the finally isolated fraction was identified as ferritin (Fig. 5).

Results

The data of serum iron, hemoglobin, hepatic ferritin content and iron to protein ratio of ferritin of normal control, rats bearing Yoshida sarcoma, bled rats, inflamed rats and rats loaded with iron are summarized in Table 1.

1) Normal control:

The body weight and the hematolo gical data of the normal control were standardized with care. The amount of hepatic ferritin iron and ferritin protein was expressed as γ/gram of dry tissue. Iron to protein ratio was expressed as iron to protein 100 (w/w) ratio, which gives the iron concentration in an unit of ferritin protein molecules.

The mean value of ferritin iron in normal control rats was 36.5γ ±

Table 1 Comparative data of serum iron, hemoglobin, hepatic ferritin iron, ferritin protein and iron to protein ratio.

	No. of rats	Serum iron (γ/dl)	Hb (g/dl)	Hepatic ferritin Iron*	Hepatic ferritin Protein**	Iron to protein ratio***
Normal controls	7	208.0 ± 14.53	14.1 ± 0.73	36.5 ± 14.46	310.3 ± 78.68	11.9 ± 1.40
Rats bearing Yoshida sarcoma	5	155.8 ± 42.40	9.96 ± 1.19	6.1 ± 3.73	62.4 ± 17.24	9.4 ± 5.06
Bled rats	5	92.8 ± 25.77	11.0 ± 1.69	10.0 ± 3.02	161.7 ± 37.80	6.2 ± 1.35
Inflammed rats	5	163.0 ± 22.57	12.4 ± 1.34	46.1 ± 4.82	410.2 ± 58.76	11.7 ± 1.54
Rats loaded with iron	5	223.2 ± 26.16	14.5 ± 1.31	61.9 ± 31.02	491.1 ± 100.69	12.1 ± 3.65

* γ/gram of dry tissue ** γ/gram of dry tissue

***Iron to protein 100(W/W) ratio

14.46, while the protein content of the ferritin was 310.3γ ± 78.68 and the iron to protein ratio was 11.9γ ± 1.04.

2) Rats bearing Yoshida sarcoma:

The mean survival time of rats after subcutaneous inoculation of Yoshida sarcoma cells was found to be 15 ± 2 days. Since metastasis to the abdominal cavity and to the liver became apparant 10 days after tumor cell inoculation, the experiment was carried out on the 7th day after tumor cell inoculation.

The mean ferritin iron content in rats inoculated with Yoshida sarcoma was 6.1γ ± 3.73, while the ferritin protein content was 62.4γ ± 17.24 and iron to protein ratio was 9.4 ± 5.06.

3) Bled rats:

Both the hepatic ferritin iron and the ferritin protein content of bled rats were significantly lower than those of normal control. The hepatic ferritin iron content of bled rats was 10.0γ ± 3.02 and the protein content of the ferritin was 161.7γ ± 37.80. Iron to protein ratio was 6.2 ± 1.35 .

Although iron to protein ratio was not significantly different between bled rats and tumor bearing rats, some of the tumor bearing rats showed a high iron to protein ratio, while all the bled rats showed low iron to protein ratio.

4) Inflamed rats:

Selye (9) produced an artificial abscess in rats by injecting turpentine oil into a pouch previously induced by a subcutaneous injection of air. He showed that the histological features were identical to inflammatory reaction. In the present experiment this turpentine abscess method was used to study the influence of inflammation on the hepatic ferritin content.

The hepatic ferritin iron content of inflamed rats was 46.1γ ± 4.82, while the ferritin protein content was 410.2γ ± 58.76 and iron to protein ratio was 11.7 ± 1.54. The fact that hepatic ferritin iron increased in the inflamed rats agrees with the report of Sugii (16) who demonstrated inoculation of staphylococcus aureus into rats increases hepatic ferritin iron.

5) Rats loaded with iron:

The hepatic ferritin content of rats loaded with iron was 61.9γ ± 31.02 and ferritin protein content was 491.1γ ± 100.69. Iron to protein ratio was 12.1 ± 3.65.

The fact that ferritin protein as well as ferritin iron increases in rats, loaded with iron suggests that iron acts as an inducer of ferritin protein synthesis in the liver as Finburg et al. (5) and Drysdale (8) demonstrated in their experiments.

Discussion

The procedure described above permits a quantitative determination of both ferritin iron and ferritin protein contents of the liver. The determination of tissue ferritin content has hitherto been exclusively dependent on antibody-precipitation techniques. With this technique tissue ferritin is precipitated with the antibody protein, which otherwise would interfere with the determination of ferritin protein content.

Until the above method of hepatic ferritin determination was used in our laboratory, hepatic ferritin was measured by Konno-Yoneyama's method (7). The PII fraction regarded as ferritin in Konno-Yoneyama's method contained other proteins and may have been contaminated with other form of iron. However, it was shown in the present experiment that the ferritii finally isolated was free from other proteins as shown by polyacrylamide gel electrophoresis and immunoelectrophoresis.

Drysdale and Munro raised a doubt on the validity of their method for the quantitative determination of tissue ferritin because of the incomplete recovery of the final isolated ferritin iron to that of heat supernatant. The recovery of the final isolated ferritin iron to that of heat supernatant was 78.4% $\pm$ 8.73 in our experiment. However, as Sugii (16) demonstratec approximately 10% of the PIII iron fraction existed in the heat supernatant

when iron was measured by Konno-Yoneyama's method. Thus if the iron of the PIII fraction can be subtracted from the iron in the heat supernatant, the recovery of the ferritin iron may become as high as about 90%.

The coefficient of variation of the amount of ferritin isolated from an aliquot part of pooled heat supernatant was 2.2% for ferritin iron and 8.3% for ferritin protein.

Inasmuch as the ratio of iron to protein of the purified ferritin is accurate regardless of recovery, the relationship between iron and protein in the study of ferritin can be readily clarified.

Although Granick (17) reported that the iron content of ferritin can vary between 0 and 23%, the ratio of iron to protein was fairly constant in normal controls, in rats loaded with iron and in inflamed rats. It is estimated from this observation that iron may act as an inducer of ferritin protein synthesis under conditions in which iron accumulates in the tissue.

A reduced hepatic non-hemin iron content in tumor bearing states has been reported by Hoshimima (18), Sawada (19) and Urushizaki (20). However, hepatic ferritin protein was not measured by any of these investigators. Rats bearing Yoshida sarcoma showed a higher iron to protein ratio than that of normal controls, whereas the ratio was significantly lower than that of normal control in all of the bled rats. This observation suggests that in tumor bearing rats a different mechanism from that of bled rats exists in the metabolic aspects of hepatic ferritin. Although Drysdale demonstrated in his turnover study of hepatic ferritin of normal rats that the synthesis and degradation of hepatic ferritin was controled by the uptake and release of iron in the tissue, it seems

likely that a depressed synthesis or accelerated degradation of hepatic ferritin protein may primarily occur in such a pathological condition as a tumor bearing state. Further investigations on the kinetic aspects of this process are in progress.

Conclusion

Chromatographic purification of hepatic ferritin originally introduced by Drysdale and Munro was tested by polyacrylamide gel electrophoresis and immunoelectrophoresis. As a result it was clarified that the finally isolated ferritin was free from contamination of other tissue proteins. The recovery of the final isolated ferritin to that of the heat supernatant was $78.4 \pm 8.73\%$.

This procedure was applied to the quantiative determination of hepatic ferritin in various conditions including tumor bearing state and the results were reported.

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