Small-Scale Isolation of Ferritin for the Assay of the Incorporation of ^{14}C -Labelled Amino Acids

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(Received 3 November 1964)

1. A new procedure is described for the isolation of pure ferritin from small amounts of tissue. After the removal of most of the tissue proteins by heat coagulation, the ferritin fraction was chromatographed successively on CM-cellulose and Sephadex G-200. 2. The isolated ferritin appeared to be free from other proteins, as judged by its sedimentation pattern in the ultracentrifuge, by electrophoresis on polyacrylamide gel and by immunoelectrophoresis. 3. After the injection of [^{14}C]leucine into a series of rats, the specific activity of liver ferritin isolated by the new procedure bore a constant relationship to that of liver ferritin separated by antigen-antibody precipitation. The procedure can thus be used to obtain ferritin of suitable purity for studies of amino acid incorporation. 4. The new procedure can be used to measure the total ferritin protein content of a tissue. It is not possible to use ultraviolet absorption for the measurement of ferritin protein because of considerable interference from the iron that it contains.

There are now a number of well-authenticated examples of mammalian tissue proteins whose concentrations are affected by specific stimuli. The alteration in tissue content of a protein is not always due to a change in the rate at which it is synthesized. It has been concluded that the increase in tryptophan-pyrrolase activity in the liver after the administration of tryptophan is due to stabilization of the enzyme by its substrate, with a consequent decrease in the rate of breakdown (Schimke, Sweeney & Berlin, 1964). Alterations in tissue protein content arising from changes in the rate of breakdown must therefore be distinguished from those due to changes in the rate of synthesis. To demonstrate the latter, it is necessary to isolate the specific protein and measure the incorporation of labelled amino acids from the free amino acid pool. Fineberg & Greenberg (1955b) have shown that there is a four- to five-fold acceleration in the rate of uptake of ^{14}C -labelled amino acids by guinea-pig-liver ferritin within 2 hr. of injecting iron salts. Their method of isolating ferritin was to prepare a specific antiserum that would precipitate this protein from liver preparations. This immunological method of isolation continues to be used in studies of ferritin biosynthesis (Loftfield & Eigner, 1958; Matioli & Eylar, 1964), but suffers from several disadvantages. The quantity of protein recovered in the antigen-antibody precipitate varies with the conditions of precipitation and demands careful standardization (Mazur & Shorr, 1950). In addition, the production of sufficient antiserum to the ferritin of small mam-

mals requires a prohibitively large number of animals (Mazur, Green & Carleton, 1960). An alternative procedure is to prepare an antiserum to the more readily available horse-spleen ferritin, which has been shown to cross-react with the ferritin of other species (Mazur & Shorr, 1948) and to give quantitative ferritin precipitation under suitable circumstances (Mazur *et al.* 1960), but is much less sensitive than homologous ferritin antiserum (Mazur & Shorr, 1950). Finally, some preliminary purification of tissue ferritin may be necessary to avoid adsorption of other proteins on to the antigen-antibody precipitate (Mazur & Carleton, 1963). The alternative method of isolating ferritin by crystallization with cadmium sulphate requires relatively large amounts of tissue and recoveries are incomplete (Fineberg & Greenberg, 1955a).

During a study of the mechanism of the induction of liver ferritin synthesis after the administration of iron, we have devised a new procedure for isolating small amounts of ferritin free from other tissue proteins, based on differences in chromatographic properties between ferritin and other proteins. Although DEAE-cellulose has been previously employed in the purification of rat-liver ferritin (Mazur *et al.* 1960), the final product was only 30% pure. Drysdale & Ramsay (1965) used CM-cellulose to separate ferritin from other iron-containing proteins present in tissues but their conditions do not exclude other proteins from the ferritin fraction. In our new procedure, several recognized general processes for the separation of ferritin have been

combined with chromatographic techniques on CM-cellulose and Sephadex G-200 to achieve a final purity suitable for measuring specific activities after the injection of labelled amino acids. With this method as many as 12 samples, each containing between 50 μ g. and 1 mg. of ferritin, can be conveniently processed simultaneously in 12 hr. of working time.

MATERIALS AND METHODS

All apparatus was freed from iron by immersion overnight in 6N-HCl followed by rinsing in deionized water. The analytical reagents were A.R. grade and proved to have a satisfactorily low iron content except for $(\text{NH}_4)_2\text{SO}_4$. This latter was freed from iron by boiling a saturated aqueous solution to which was added *o*-phenanthroline (0.01%), $\text{Na}_2\text{S}_2\text{O}_3$ (7.5 mm) and H_2SO_4 (25 mm). The iron-phenanthroline complex was extracted with propan-2-ol and the excess of alcohol was boiled off from the aqueous layer. The solution was then adjusted to approx. pH 7 by heating in the presence of a slight excess of aq. NH_3 (sp.gr. 0.88).

Preparation of crystalline ferritin and apoferritin. These were prepared from rat liver and horse spleen. Livers were obtained from adult male rats (150–200 g. body wt.), some of which received ferric ammonium citrate (20 μ g./ml.) in their drinking water to enhance the amount of ferritin recovered. Horse spleen was obtained from the slaughterhouse. Crystalline ferritin, prepared from each tissue by the method of Granick (1946), was recrystallized four times and chromatographed on CM-cellulose (capacity 0.7 m-equiv./g., Serva, Heidelberg, Germany) by the procedure of Drysdale & Ramsay (1965). Apoferritin was then prepared from the purified rat-liver ferritin by the dithionite method (Granick, 1946) followed by removal of traces of iron with 2,2'-bipyridyl (Richter, 1964a). Its identity was confirmed by crystallization.

Determination of protein. Protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with rat apoferritin used to obtain a calibration curve. The extinctions obtained were similar to those for crystalline ferritin, provided that the weight of ferritin used was corrected for content of iron as $(\text{FeOOH})_3\text{FeOPO}_3\text{H}_2$ (Granick, 1946).

Determination of ferritin iron. After the estimation of iron in ferritin, it was necessary to recover the protein. A procedure was therefore devised that would extract the iron from ferritin without hydrolysing the apoferritin to fragments that were no longer acid-precipitable. A modification of Hill's (1931) bipyridyl method for the estimation of iron in the ferrous state was chosen, with Na_2SO_3 as the reducing agent. The ferritin-containing solution was made up with water to 10 ml. in the presence of Na_2SO_3 (75 mm), 2,2'-bipyridyl (0.05%) and acetic acid (6%, v/v). After heating at 100° in a water bath for 1 hr., the colour developed was read at 520 m μ . Estimations of iron made by this method on a sample of purified ferritin were compared with the values obtained for total iron after digestion with a mixture of H_2SO_4 , HClO_4 and HNO_3 as described by Ramsay (1944). The values so obtained by our procedure did not differ significantly from the values given by the method for total iron ($96 \pm 3\%$), indicating that the new procedure measures all the iron in ferritin.

Determination of total ferritin iron in liver homogenates. An extract containing all the tissue ferritin was prepared from an aqueous rat-liver homogenate (200 mg./ml.) by heating at 80° (Laufferberger, 1937), followed by removal of the heat-coagulated tissue proteins by centrifugation. Ferritin was precipitated from this fraction by 50% saturation with $(\text{NH}_4)_2\text{SO}_4$ and left to stand for 2 hr. at 0° to allow complete precipitation; this was followed by iron determination on the precipitate, as described above.

Immunological isolation of ferritin from rat liver. (1) An antiserum to crystalline horse ferritin was prepared in rabbits by the method of Mazur & Shorr (1950). (2) This antiserum has been shown to be effective for the quantitative precipitation of rat ferritin under suitable conditions (Mazur *et al.* 1960). (3) With this antiserum, ferritin was precipitated from the supernatant fraction obtained by heating an aqueous liver homogenate to 80°, as described above. All precipitations were carried out in the presence of excess of antibody. Samples (2 ml.) of the heat supernatant fraction were incubated at 37° for 1 hr. with 3 ml. of the stock antiserum followed by standing for 18 hr. at 0° to complete precipitation (Mazur & Shorr, 1950; Mazur *et al.* 1960). The precipitates were collected by centrifugation and washed twice with 3 ml. of 0.9% NaCl. They were then taken up in 0.1 ml. of 0.1 N-NaOH, in which they were readily soluble, and the solutions were diluted to 10 ml. with water for subsequent estimations of protein content and radioactivity. Under these conditions, the antigen-antibody precipitate contained more iron than could be accounted for by the ferritin present in the heat supernatant fraction; this may represent transfer of iron from transferrin in the rabbit serum during incubation. Even when incubation was performed at 0° in the presence of 5 mm-EDTA, this transfer still takes place, as judged by iron recoveries. The iron content of the antigen-antibody precipitate is thus unsuitable as a measure of the amount of ferritin iron present.

Protein separation in polyacrylamide gels. Gels 13 cm. long containing 5% of polyacrylamide (with 5% cross-linkage) in 50 mm-tris-citrate buffer, pH 7.9, were run for 2 hr. at 200 v. They were stained simultaneously for protein and iron by immersion in a mixture of Ponceau S (0.05%) and potassium ferrocyanide (0.2%) in 3% (w/v) trichloroacetic acid and were washed with 5% (v/v) acetic acid.

Immunoelectrophoresis. Electrophoresis was carried out in 1% (w/v) agar gel on glass slides in veronal buffer, pH 8.6, with the LKB 6800A immunoelectrophoresis apparatus (LKB-Produkter A.B., Stockholm, Sweden). An antiserum was prepared by injecting rabbits with the heat supernatant fraction obtained after coagulating most of the liver proteins at 80°, as described above. Freund's complete adjuvant (Difco Laboratories, Detroit, Mich., U.S.A.) was added to the antigen to increase antibody production. This antiserum was then used to detect the proteins distributed in the agar gel after electrophoresis. The antigen-antibody precipitates were finally stained for protein with Ponceau S and for iron with ferrocyanide as described for the polyacrylamide gels.

Estimation of radioactivity. Protein precipitates were taken up in 0.1 N-NaOH and plated on disks of lens paper as described by Garrow & Piper (1955) to standardize self-absorption. Normally, samples containing 250–750 μ g. of protein were plated and were counted to a standard error of less than 3% with a Nuclear-Chicago Corp. gas-flow counter.

EXPERIMENTAL AND RESULTS

Procedure for the isolation of liver ferritin

The new method for the isolation of ferritin from small samples of rat liver is based on a combination of four procedures (Scheme 1). First, the well-known stability of ferritin to heating at 80° was used to separate it from the bulk of the tissue proteins, which are coagulated at this temperature (Laufberger, 1937). Secondly, this supernatant fraction

Scheme 1. Outline of the procedure for the isolation of ferritin, and the measurement of iron and protein content, and of its content of ^{14}C -labelled amino acids.

from the heat coagulum was chromatographed on CM-cellulose by modifying the procedure of Drysdale & Ramsay (1965), to eliminate some of the contaminating proteins. Thirdly, the ferritin fraction eluted from the CM-cellulose column was concentrated and further purified by precipitation at 50% saturation with ammonium sulphate (Mazur, Litt & Shorr, 1950). Finally, the ammonium sulphate precipitate was passed through Sephadex G-200 to remove the salt and traces of contaminating proteins. These steps are described in detail below.

Extraction of ferritin from liver homogenate. Each rat was killed by a blow on the head and the liver exposed. The portal vein and that part of the inferior vena cava above the diaphragm were severed to allow blood drainage. No further steps, however, were taken to remove residual blood. The excised liver was blotted, weighed and then homogenized in 4 vol. (w/v) of water in a Potter-Elvehjem-type homogenizer with a Teflon piston. The choice of 4 vol. is critical, being dictated by the need for an ionic strength sufficient to promote coagulation of most tissue protein on subsequent heating but low enough to allow adsorption of ferritin on the CM-cellulose (Drysdale & Ramsay, 1965). The homogenate was slowly heated, with continual stirring, in a water bath at 80° (Laufberger, 1937). About 80–90% of the liver protein is coagulated by this procedure. The supernatant fraction, which contains the tissue ferritin, was collected after removal of the coagulum by centrifugation and chilled to 2°. This fraction is referred to as the 'heat supernatant fraction'. A sample (usually 1 ml.) of this fraction was half-saturated with ammonium sulphate and ferritin iron was determined as described in the Materials and Methods section.

Chromatography of the heat supernatant fraction on CM-cellulose. The partial purification of the tissue ferritin on CM-cellulose was modified from the procedure of Drysdale & Ramsay (1965). They demonstrated that, if the pH is below 5.0 and the ionic strength less than 0.015, ferritin will be adsorbed by CM-cellulose. The chilled heat supernatant was therefore titrated to pH 4.8 with 0.2 N-acetic acid. A resulting precipitate, which appears to consist largely of haemoglobin, was removed by centrifugation. Usually 5 ml. of the pH 4.8 supernatant was added to a column of about 500 mg. of CM-cellulose that had been equilibrated with a buffer, pH 4.8, containing 15 m-equiv. of sodium acetate and 10 m-equiv. of acetic acid/l. and the sample was allowed to flow through under gravity. The columns were then washed thoroughly with this buffer, followed by washing with a buffer containing 32 m-equiv. of sodium acetate and 18 m-equiv. of acetic acid/l. At this higher pH and ionic strength some non-ferritin protein is removed, and subsequent

elution of ferritin occurs more easily when the pH is later increased to 5.5. These washings were discarded. A fraction containing ferritin was subsequently eluted by developing with a buffer, pH 5.5, containing 43.8m-equiv. of sodium acetate and 6.2m-equiv. of acetic acid/l. This ionic strength was found to give maximal ferritin recoveries with minimal contamination with other proteins. The ferritin, which may be clearly seen as a yellow band when in quantities greater than 25 $\mu\text{g.}$, separates from the top after a volume of about 5ml. of buffer has passed through, and can be collected in the next 15ml. of eluate. This chromatographic procedure gave optimum recoveries when carried out at low temperatures, as judged by recoveries of iron. The column procedure does not discriminate between different molecular species of ferritin. Samples (100–250 $\mu\text{g.}$) of apoferritin prepared from crystalline ferritin by the dithionite method were chromatographed and 70–80% was recovered in the ferritin fraction.

Ammonium sulphate fractionation. Ferritin can be fractionated according to its iron content with ammonium sulphate (Mazur *et al.* 1950). Those particles richest in iron are precipitated at 23% saturation whereas apoferritin-rich fractions require 50% saturation. Accordingly, the ferritin fraction obtained from chromatography on CM-cellulose was half-saturated with ammonium sulphate to ensure complete precipitation of all the ferritin species. The ferritin, which is completely precipitated after 2hr. at 0°, was collected by centrifugation at 1000g for 15min. and the supernatant fluid discarded.

Gel filtration. The preparation of crude ferritin, concentrated by the above precipitation with ammonium sulphate, was taken up in 0.5ml. of 10mM-phosphate buffer, pH 7, containing bromophenol blue (0.02%), for chromatography on Sephadex G-200. Ferritin, which has mol.wt. greater than 465 000 (Rothen, 1944), is completely excluded from this material and appears in the effluent immediately after the void volume. The dye was used to indicate the inner volume. The columns used were 12.5cm. long $\times$ 1.5cm. diam. It was usual to operate six to 12 columns fed from a common reservoir under a pressure of 60cm. H_2O . They were filled with a suspension of gel that was supported on a flat perforated bottom by a layer of lens paper. The bed volume was 12ml. and the void volume was 3.5ml. The samples were layered on to a protecting disk of glass paper and washed with 1ml. of buffer after they had drained through the surface. The final ferritin fraction was collected in the 2.5ml. effluent sample immediately following the void volume. For convenience, the first 6ml. eluted after the introduction of the sample was collected, thus including the void volume.

Tests of the purity of the isolated ferritin

It was first necessary to establish that the preparation obtained from the final Sephadex-gel filtration contained ferritin and then to test the preparation for the presence of contaminant proteins. For this latter purpose, gel electrophoresis, immunoelectrophoresis, ultracentrifugation and measurement of the specific activity of isotopically labelled ferritin were applied to the final preparation; the first two were also used to screen successive stages in purification.

Identification of ferritin in the eluate. The presence of ferritin in the final eluate from Sephadex-gel filtration was confirmed in three ways. First, the protein was shown to have a high iron content, preparations having up to 20% of iron. Secondly, crystals with the conformation characteristic of ferritin were obtained from the eluate by treatment with cadmium sulphate (Granick, 1943). Finally, by using a specific antiserum for ferritin, a precipitate was formed that contained up to 4–5% of iron. No other known protein contains these concentrations of iron, except haemosiderin, which unlike ferritin is removed in the coagulum formed by heating at 80° (Drysdale & Ramsay, 1965).

Gel electrophoresis. The fraction obtained at each of the four steps in the fractionation procedure was submitted to electrophoresis in polyacrylamide gels and stained for both protein and iron. The pictures obtained at successive stages of purification are shown in Fig. 1. The heat supernatant fraction of liver showed many discrete protein species, of which only a minor proportion stained for non-haem iron. This was normally present as one major band, accompanied by one or more minor iron-staining components. The presence of several ferritin bands agrees with the findings of Richter (1964b), who observed at least three electrophoretically distinct, but antigenically identical, components in crystalline rat ferritin, the major component comprising 75–85% of the total. After CM-cellulose treatment, only one major protein band other than iron-containing protein bands was found. This disappeared after ammonium sulphate precipitation, leaving only a small amount of protein migrating in front of ferritin. After gel filtration this disappeared and all of the protein components isolated from the final ferritin preparation took the Prussian blue stain for iron. This implies that no protein other than ferritin is present.

Ultracentrifugation studies. Samples of the final ferritin preparation were examined in the Spinco model E analytical ultracentrifuge to investigate its purity and also to examine the ferritin spectrum of particle densities, caused by the presence of particles with different iron contents and therefore of different densities (Rothen, 1944). One sample

Fig. 1. Polyacrylamide-gel electrophoresis of fractions obtained in successive steps during the isolation of ferritin, stained for protein (hatched bands and stippled areas) and for iron (solid bands). The origin is indicated by the arrow. (1) Heat supernatant fraction; (2) fraction obtained after chromatography on CM-cellulose; (3) fraction obtained on precipitation with $(\text{NH}_4)_2\text{SO}_4$; (4) fraction obtained after chromatography on Sephadex G-200.

Fig. 2. Absorption spectra of apoferritin (---) at a concentration of $30 \mu\text{g.}$ of protein/ml., ferritin (—) at a concentration of $30 \mu\text{g.}$ of protein/ml. and $7 \mu\text{g.}$ of iron/ml., and ferric ammonium citrate (.....) at a concentration of $7 \mu\text{g.}$ of iron/ml.

(approx. 5mg. of protein/ml.) was centrifuged at 44770 rev./min. (g_{av} 145650) in a 12mm. cell at 4° and photographs were taken at 4min. intervals with schlieren optics. A more dilute sample (0.5mg./ml.) was examined with u.v. optics. The sedimentation coefficients were calculated from both runs from the equation:

$$S = \frac{1}{\omega^2} \times \frac{d \log r}{dt}$$

where S is the sedimentation coefficient, ω is the angular velocity (radians/sec.), r is the distance of the boundary from the centre of rotation and t is the time (sec.). The results were expressed in Svedberg units (s). Under the above conditions, particles of 4s and greater can be resolved. To fractionate the ferritin species, another sample (approx. 10mg. of protein/ml.) was centrifuged at 25980 rev./min. (g_{av} 49040) and measurements were made at 8min. intervals with schlieren optics.

All the material detected with both optical systems had sedimentation coefficients in excess of 18s, that of apoferritin (Rothen, 1944). With u.v. optics, the absorption was all contained in a single diffuse boundary, most of the absorption being at about 60s. With schlieren optics the sedimentation coefficients also ranged from 20 to 60s, but the heavier fraction did not predominate.

The discrepancy in the relative amounts of ferritin densities observed by schlieren and by u.v. optics might be associated with the known high iron content of the heavier particles (Mazur *et al.* 1950). We therefore examined the contribution of the iron in ferritin to its absorption in u.v. light. Fig. 2 shows that ferritin absorbs strongly at $260 m\mu$, but an amount of apoferritin equivalent in protein content to the ferritin shown in Fig. 2 absorbed only 5% of the amount at this wavelength. The specific extinction coefficient ($E_{1\text{cm.}}^{1\text{mg./ml.}}$) was 0.95 at $280 m\mu$ for our rat apoferritin samples, which is comparable with the values 0.86–0.97 for horse apoferritin (Hoffmann & Harrison, 1963) and 1.01 for guinea-pig apoferritin (Friedberg, 1962). Ferric ammonium citrate at the same iron concentration as the ferritin sample also absorbed strongly in the u.v. range, though the curves were not identical (Fig. 2). The discrepancy between the relative amounts of ferritin densities by u.v. optics and by schlieren optics may therefore arise from the greater iron content of the denser particles, which would absorb extensively in the u.v. system but not in the schlieren system.

Immunoelectrophoresis. An antiserum was prepared, in rabbits, to the heat supernatant fraction, which might be expected to contain all possible contaminants of the ferritin preparation. Samples of the final purified ferritin preparation and of the

heat supernatant fraction were then tested by immunoelectrophoresis with this antiserum. The gels were stained for protein and iron. As would be expected from previous gel electrophoresis of this fraction (Fig. 1), at least six components were detected in the heat supernatant by immunoelectrophoresis (Fig. 3). However, only one antigenically active substance was detected in the final purified ferritin preparation (Fig. 3). This substance took the Prussian-blue stain for iron and was consequently identified as ferritin.

Specific activity of ^{14}C -labelled ferritin prepared by the new procedure and by immunological isolation

The new procedure for the isolation of ferritin was applied to studying the response of rat-liver

Fig. 3. Immunoelectrophoretic pattern obtained with the heat supernatant fraction (upper half of gel) and with the final purified ferritin fraction (lower half of gel), with an antiserum to the heat supernatant fraction in the central trough.

ferritin to the intraperitoneal injection of ferric ammonium citrate (300 μg . of iron/100g. body wt.) (J. W. Drysdale & H. N. Munro, unpublished work). Animals receiving iron and control animals not so injected were both given 5 μC of DL-[1- ^{14}C]-leucine/100g. body wt. and were killed 2hr. thereafter. Ferritin was isolated by the new chromatographic procedure and also by immunological precipitation directly from the heat supernatant fraction as described in the Materials and Methods section. To measure both the iron content and the specific activity/mg. of protein in the ferritin isolated by each procedure, the following steps were undertaken (Scheme 1). Samples of the ferritin preparations obtained by the chromatographic method and by antigen-antibody precipitation were estimated for protein content, and the remainder treated with the bipyridyl reagent for iron estimation. The protein precipitate formed during this reaction was spun down and the amount of iron was estimated colorimetrically on the clear supernatant fluid. This supernatant fluid was then recovered and combined with the precipitate. Perchloric acid was added to give a concentration of 0.35N. The precipitated protein was then spun down and washed once with 5ml. of 0.2N-perchloric acid. The precipitate was dissolved in 0.1N-sodium hydroxide and plated for counting as described in the Materials and Methods section. The specific activity was computed by relating the counts obtained after this treatment to the amount of protein measured before the addition of the bipyridyl reagent (see Scheme 1). To demonstrate that the

Table 1. Comparison of specific activities of ferritin samples isolated by the chromatographic procedure and as an antibody-ferritin precipitate from the same liver samples

Experimental details are given in the text.

Group of rats	Liver sample	Specific activity (counts/min./mg. of protein)		Ratio of specific activities
		Chromatographic ferritin	Antibody-ferritin precipitate	
Uninjected	1	120	28	4.3
	2	153	39	3.9
	3	180	51	3.5
	4	192	52	3.7
	5	200	50	4.0
	6	245	64	3.8
	7	262	76	3.5
	Mean	—	—	3.8
Injected with iron	8	381	99	3.8
	9	598	155	3.9
	10	751	180	4.2
	11	906	199	4.5
	12	1360	330	4.1
	13	1450	344	4.2
	Mean	—	—	4.1

use of the bipyridyl reagent does not lead to loss of protein by solubilization, a test was made of the recovery of ^{14}C activity from ferritin samples of known ^{14}C content. In a series of 15 such samples, the amount of activity recovered from the perchloric acid precipitate was $96 \pm 3\%$ of the activity of the original ferritin preparation.

Table 1 shows that the specific activity of the chromatographic ferritin was about four times that of the immunologically precipitated ferritin; this is due to the presence of antibody protein in the precipitate. This ratio was relatively constant in successive experiments, and, moreover, animals stimulated to synthesize ferritin protein by iron injection showed the same ratio as that found for the control animals. This implies that there is no significant contamination of the chromatographic ferritin with highly radioactive proteins, since these might be expected to cause wider variation in the ratio of specific activities, especially on iron stimulation. Consequently, the ferritin isolated by our method is suitable for specific activity measurements after the administration of labelled amino acids.

Estimation of total tissue ferritin

The procedure described above permits accurate measurement of the ferritin content of a tissue. The total ferritin iron can be measured on the supernatant fraction obtained after heat coagulation of the tissue proteins (Scheme 1). However, ferritin protein can only be accurately assayed on the preparation finally purified on Sephadex G-200. This does not provide a measure of the total amount of ferritin protein in the tissue, owing to incomplete recovery. However, from the ratio of protein to iron in the purified ferritin, combined with the estimate of total ferritin iron obtained from the heat supernatant fraction, it is possible to calculate the total amount of ferritin protein in the tissue.

DISCUSSION

Ferritin synthesis offers favourable circumstances under which to study adaptive protein formation in mammals, since the rate of ferritin formation increases rapidly after iron salts are injected into the animal (Fineberg & Greenberg, 1955b; Loftfield & Harris, 1956; Loftfield & Eigner, 1958). Since ferritin is not secreted from the cell, measurement of the total amount of ferritin in the tissue provides additional evidence about changes in its rate of synthesis and turnover. Procedures available for the measurement of tissue ferritin content and its uptake of labelled amino acids have hitherto been almost exclusively dependent on antibody-precipitation techniques, which are subject to the difficulties discussed above. Consequently, an

alternative procedure for isolating ferritin from small amounts of tissue would be advantageous.

The procedure proposed by us appears to fulfil the criteria for such a method. For several reasons, the ferritin finally isolated can be considered to be free from contaminating proteins. Ultracentrifugation has demonstrated the absence of significant amounts of molecules in the ferritin preparation with sedimentation coefficients less than 20s; most tissue proteins other than ferritin have lower sedimentation coefficients and are thus excluded. Examination of the final ferritin fraction by electrophoresis in a polyacrylamide gel and by immunoelectrophoresis showed no detectable proteins other than ferritin. Finally, after the injection of [^{14}C]leucine into a series of rats the specific activity of the liver ferritin isolated by the new procedure bore a constant relationship to that of liver ferritin separated by antigen-antibody precipitation, suggesting that both procedures isolate the same material.

The new procedure also permits the determination of small amounts of tissue ferritin, both by iron content and by protein content. Since the iron content of ferritin can vary between 0 and 23% (Granick, 1946), it is essential to include a measure of ferritin protein to obtain an accurate estimate of the amount of ferritin present. The precision of the ferritin measurement is determined by the accuracy of the final protein estimation. We have used the procedure of Lowry *et al.* (1951), which can detect 25 μg . of protein. At this level, nitrogen estimation can also be used, but demands an ultra-micro method (Fleck & Munro, 1965). It is not possible to use ultraviolet-absorption procedures to measure ferritin protein because its ultraviolet spectrum is masked by the intense ultraviolet absorption of the iron (Fig. 2), and, in consequence, the amount of ultraviolet light absorbed by a given amount of ferritin will depend mainly on its iron content.

This investigation was supported by U.S. Public Health Service Grant CA 07087 from the National Cancer Institute. We are grateful to Dr I. Aitken for advice on the immunological procedures used and to Miss Anne Morton for skilled technical assistance.

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