# FERRITIN SPECIES AND METABOLISM IN STRIATED MUSCLE

by

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Submitted to the Department of Nutrition and Food Science on November 5, 1975 in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

## ABSTRACT

The purpose of this thesis was to assess the functional advantage of having two ferritin proteins in rat cardiac muscle. This has been done by defining more clearly the structural and metabolic features of these two proteins under a variety of physiological conditions.

Molecular sizes and charges by quantitative polyacrylamide gel electrophoresis revealed that the slow and fast heart ferritins were significantly larger than other tissue ferritin monomers. In agreement with a higher electrophoretic mobility, the fast heart ferritin has a higher apparent valence than other tissue ferritin monomers. The slow and the fast heart ferritin had the same large and small subunits found in other rat tissue ferritins. Isoelectric focusing of the mixed slow and fast heart ferritins revealed only two bands.

Experimentally induced connective tissue cell hypertrophy in the hearts of rats produced an increase in the concentration of the slow ferritin suggesting its presence in the connective tissue cell coupled with a dramatic decrease in the concentration of the fast ferritin. Administration of excess iron to these hypertrophic animals produced an increase in the concentration of fast ferritin with no change in the slow indicating that the fast ferritin appears to be most related to the storage of iron. There was no change in the

concentrations of the slow and fast ferritin in muscle cell hypertrophy suggesting no direct relation between both ferritins and these cells.

Iron administration increased the rate of synthesis of both the slow and fast ferritins to the same degree in heart and diaphragms of male and female rats, while total heart and diaphragm protein synthesis was uneffected. However, in both sexes, the synthesis of the fast ferritin was greater than that of the slow with or without iron administration. Also, in both situations, preliminary studies indicated that the fast heart ferritin turns over more rapidly than the slow.

Red and white muscles of the rat and chicken were analysed for their ferritin content and suggested a positive relationship between ferritin and myoglobin for rat muscles but no correlation for chicken muscles. Thus this relationship is still unclear.

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## ABBREVIATIONS

| | |
|---|---|
| AFt | Apoferritin |
| Bis | N,N'-methylene bisacrylamide |
| % C | Bis/total gel conc (Bis + Acrylamide) x 100 |
| cpm | Counts per minute |
| Cu | Copper |
| dpm | Disintegrations per minute |
| DATDA | Diallyltartardiamide |
| Fe | Iron |
| g | Gram(s) |
| HFt | Holo-ferritin |
| $K_R$ | Retardation coefficient |
| $Y_o$ | Free electrophoretic mobility |
| mg | Milligram(s) |
| ml | Millilitre(s) |
| mm | Millimeter(s) |
| M | Molar |
| uC | Microcurie(s) |
| ug | Microgram(s) |
| nm | Nanometer(s) |
| OD | Optical density |
| PPO | 2,5-Diphenyloxazole |
| POPOP | P-Bis[2-(5-Phenyloxazolyl)-Benzene |
| i.m | Intramuscular |
| i.v | Intravenous |

| | |
|---|---|
| i.p | Intraperitoneal |
| s.c | Subcutaneous |
| SDS | Sodium dodecyl sulfate |
| % T | % Total gel concentration (Acrylamide + Bis) |
| UV | Ultraviolet |

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. Introduction

Ferritin, as the name suggests, is an iron-containing protein consisting of a number of protein subunits surrounding a core of ferric oxyhydroxide. It is present in a wide range of phyla from fungi, through the plant and animal kingdoms. Together with hemosiderin, ferritin accounts for about 20-30% of the total iron in the body of mammals(1,2). It is present in highest concentrations in the liver, spleen and bone-marrow, but has been identified in most tissues examine.

Differences in the electrophoretic mobility of ferritins from different species and different tissues within the same species have been reported. When Linder and Munro(3) first examined the electrophoretic migration of ferritins purified from different rat tissues on gel electrophoresis, they reported three distinctive ferritins: one characteristic of adult liver, also present in cardiac and skeletal muscle, one common to kidney, spleen and some hepatomas, and a third found only in cardiac and skeletal muscle. Other subsequent criteria have differentiated more ferritin species. Apart from differences in electrophoretic mobility, the two ferritin species present in striated muscle('slow ' and 'fast' ferritins) were found to have different antigenic properties and appear at different times during development.

The presence of two distinct ferritins in muscle suggests that there must be special features of iron metabolism peculiar to muscle. A related question is why there should be two

ferritin proteins in a single tissue. The presence of two such ferritins has also been identified in the human heart (4); (also, our own unpublished data) The heart in the human as well as in the rat is a very heterogeneous organ consisting of different cell types with a well-defined function assigned to each type. Cellular location is thus closely related to function and metabolism and the question arises whether the two ferritins are present in different cell populations. I have attempted to answer this question in the rat by investigating the structural and metabolic features of cardiac muscle ferritins.

The basic questions that I have attempted to answer in this thesis are as follows:

1. How do the slow and fast ferritin differ structurally and is there anything peculiar about them which would suggest a function different from that of other tissue ferritins?

To answer this question, we have analysed the heart ferritins in relation to other ferritins by quantitative polyacrylamide gel electrophoresis (225). Structural studies have also included subunit and amino acid analysis of the separated ferritins and iso-electric focusing to explore the possibility that the cardiac ferritins, which appear as two bands in electrophoresis on polyacrylamide gels, are actually composed of two families or groups of ferritins closely related in structure. These structural studies would not only be useful in considerations of their function but would provide additional information on general ferritin structure.

2. Are they present in the same cell type or in different cell types?

In a heterogeneous organ such as the heart, where estimates reveal that there are about three times as many non-myocardial cells as myocardial cells,(10) there is a strong possibility that slow and fast ferritins are present in different cell populations. Cellular location could thus be closely related to function, especially if there exists a relationship of one ferritin to supply of iron to myoglobin.

The tissue distribution of slow and fast ferritin poses the strong possibility of a relationship between one or both ferritins and myoglobin. Earlier studies where the concentrations of the two ferritins and myoglobin were compared in different muscles( 3 ) indicated that the concentration of all three proteins occurred in parallel, the highest being in the heart and the lowest in the thigh muscle. Furthermore, preliminary observations by Linder and co-workers( 3 ) indicate by $^{59}Fe$ uptake studies that the kinetics of uptake of label is compatible with entry first into fast ferritin, later slow ferritin and myoglobin. These studies thus suggest a positive relationship of fast ferritin iron to myoglobin, and also raise questions about the iron storage functions of slow and fast ferritin. Do both of them store iron, or does that depend on the cellular environment?

The questions of cellular location, function in relation to myoglobin and iron storage properties were explored together.

Ideally speaking, the question of cellular location and

function would be resolved by considering myocardial and connective tissue cells in culture. But this is very difficult to do in the adult rat heart. This problem has however been approached in the following way:

a) By determining the relative proportion of slow and fast ferritins in the myoglobin containing 'red' and the non-myoglobin containing 'white' muscles of the rat.

b) By analysing chicken red and white muscles for ferritin content

c) By measuring ferritin concentrations under conditions of cardiac hypertrophy experimentally induced in rats where one cell type preferentially enlarges.

d) By examining the response of the two ferritins to administration of excess (storage) iron.

3. Also related to cell type and function is whether the slow and fast ferritins are synthesized and degraded at different rates or whether they are independent of each other.

Early evidence by Linder and Munro( 3 ) indicated that the slow and fast ferritins occur at different times during development, suggesting they are independent of each other metabolically and perhaps in different cell types. Information about this aspect of muscle ferritin metabolism together with function and location would give us further insight into the advantage of having two such proteins within a single tissue. This was investigated by measuring muscle ferritin synthesis in the presence or absence of iron administration, the point being to see whether iron stimulates them equally or differently.

The iron metabolism of striated muscle has not been extensively studied. The identification of two ferritins in muscle suggest that there must be some specific features of iron metabolism peculiar to muscle. Further clarification of the role of the muscle ferritins would provide basic information on normal muscle function, as for instance the possible role of one or the other ferritin as an iron donor for myoglobin. It was thought that my studies of the behaviour of each cardiac ferritin in heart disease would also indicate whether significant changes in iron metabolism accompany cardiac hypertrophy and ischemia or suggest new means of assessing diagnosis of tissue damage specific to the heart.

# Literature Survey

## I. The Heart

The function of the heart is to maintain circulation so that an adequate supply of blood at sufficient head of pressure is delivered to all tissues. The efficiency of the heart as a pump depends on a sequential pattern of excitation and contraction proceeding in an orderly and co-ordinated manner from atria to ventricles. It is thus not surprising that the anatomy and histology of the heart is complicated, with a well-defined function assigned to each part.

The atrial and ventricular walls of the heart both consist of three main layers, the endocardium, myocardium, and epicardium.

### a) The Endocardium

This layer is in immediate contact with the blood and is lined with ordinary endothelium which consists of rounded or polygonal cells. Under the endothelium is a thin subendothelial layer. This contains collagenous and a few elastic fibres and fibroblasts. External to this layer is a thick layer of connective tissue containing mainly elastic elements and comprising the main mass of the endocardium. A subendocardial layer consisting of loose connective tissue binds the endocardium to the myocardium.

### b) The Myocardium

This is the contractile layer and consists of

numerous muscle cells. A good review on the fine structure of myocardial cells (6) has been dealt with elsewhere and does not concern this thesis. The organelles of chief interest to this thesis are the myofibrils, mitochondria, and the sarcoplasmic reticulum, accounting for the contractile elements, the energy supply and the contraction-relaxation system.

c) The Epicardium

The epicardium is covered on its free surface by a single layer of epithelial cells. Beneath the mesothelium ia a thin layer of connective tissue with flat networks of elastic fibres, blood vessels and many nervous elements.

This general anatomy and histology of the heart brings out the fact that the heart is a very heterogeneous organ consisting of different cell types in close association with each other. Due to the complexity of this relationship, adult rat heart cells have been difficult to isolate. Enriched fractions of cardiac cells have been obtained but these have been confined to embryonic hearts(7-9). However, very general estimates suggest that the heart contains three times as many interstitial cells as myocytes (10) though the volume occupied by non-myocytes is small (e.g. 30%) relative to muscle cells. This is an important point to remember when one looks at the biochemistry of two proteins such as the two ferritins in whole heart

homogenates as they may be present in different cell types.

Three physiological properties of heart muscle are autorhythmicity, conductivity and contractility. These properties are developed to different degrees in different regions of the heart. Autorhythmicity is most highly developed in the pacemaker region of the heart, a region which initiates impulses conducted to the rest of the heart. Other regions of heart muscle are specialized for the rapid conduction of the impulse i.e. the Purkinje fibres. These fibres have a large diameter, 50 to 70 μ relative to that of ordinary cardiac muscle fibres, and their sarcoplasm contains much glycogen and relatively few myofibrils (5). These large fibres are in between the endocardium and penetrate into the depths of the ventricular muscle, where they lose their specific structure and become more like ordinary cardiac muscle fibres(11).

## II. Cardiac Hypertrophy

The most universal manifestation of heart disease, whatever its cause, is cardiac enlargement. This enlargement may be either dilatation of blood vessels or hypertrophy of the tissue, but most often is a combination of both. While it is not always present in diseased hearts, its presence always indicates a diseased heart. Such a common feature of heart disease as hypertrophy must have considerable significance as a basic biological phenomena

as a clinical manifestation of disorders of this organ.

One of the most important functions of the interstitial cells is the formation of collagen while the myocytes are concerned with the contraction process. Just in terms of physiological importance to the heart under stress, it is reasonable to assume that the ability of the myocyte cells to cope with the increased work load is more important than that of the interstitial cells. However one fascinating aspect of experimental myocardial hypertrophy is that the interstitial cells are among the first cells to respond to an increased stress(10).

Thus one approach to the study of cardiac ferritin function and distribution is to investigate their metabolism under conditions of cardiac hypertrophy.

A. Methods of inducing cardiac hypertrophy

A number of experimental models have been used in the study of cardiac hypertrophy(12). The most important and often neglected aspect of experimental considerations of heart weight is its relationship to body weight. It has been shown (13) that if the logarithm of heart weight is plotted against that of body weight, a straight line results with growth. They find that the heart weight increases consistently at 0.75 times the rate of increase in body weight. This is important in comparisons of heart to body weight ratios and they are not valid unless the animals under consideration are near the same total body weight.

A difficulty often encountered with experimental animal models for cardiac hypertrophy is that they are sometimes not easily reproducible. A major cause of this is the variability amongst animals in their response to external stress. The following is a brief review of some of the methods used in the induction of cardiac hypertrophy.

a) Renal Hypertension

Cardiac hypertrophy in hypertension has usually been regarded as a secondary response to the increased blood pressure load. In some studies(14) the parallelism between the degree of hypertrophy and the level of hypertension has been confirmed. But it has been pointed out (15) that there are many exceptions to the above relationship, as on autopsy many patients with marked hypertension show little or no hypertrophy even though the duration and the severity of the disease do not differ substantially from those of patients who do show hypertrophy(16-19).

A simple and rather uncomplicated technique involves production of hypertension by unilateral nephrectomy with partial resection of the remaining kidney(20,21). Under such conditions there appears to be significant hypertrophy of the heart only 2 days after the kidney operation. However, in these experiments no measurement was made of the water content of the myocardium giving some doubt as to the origin of this early increase in heart weight.

More recently, the possibility that other factors in addition to arterial blood pressure are involved in

cardiac hypertrophy was examined in spontaneously hypertensive rats(22). These rats were bred at the Cleveland Clinic Foundation where the study was conducted and belonged to the F-26 generation derived from the spontaneously hypertensive Kyoto-Wistar strain developed by Okamoti and Aoki(23,24). They found that ventricular weight in spontaneously hypertensive rats (F26 generation, Okamoto-Aoki Strain) was significantly higher ($p < 0.001$) than that in the body weight matched American Wistar and Kyoto-Wistar normotensive rats. Plasma renin activity was significantly increased in younger spontaneously hypertensive rats before the development of established hypertension and with antihypertensive treatment such as $\alpha$ methyl dopa administration, plasma renin activity and ventricular weight was reduced ($p < 0.01$). These observations led to the conclusion that in the spontaneously hypertensive rat, blood pressure was not the sole factor contributing to the hypertrophy but that the renin-angiotensin system might play a permissive role enhancing myocardial hypertrophy.

b) Coarctation of Aorta

A more common method for the induction of cardiac hypertrophy in rats is aortic constriction. Proposed originally by Beznak (24-26) several groups have modified the actual experimental procedure. Beznak demonstrated the regression of heart weight upon body weight as a straight line and emphasized the need for consideration of terminal body weight in assessing experimental enlargement of the heart. She also

showed that loss of body weight, as in the case of starvation, does not alter the fundamental relationship of heart weight to body weight. She observed a 25% increase in heart weight 5 days after narrowing of the lumen of the abdominal aorta and a 32% increase in 21 days. The increase observed was due entirely to the hypertrophy of the left ventricle(24-26).

Many workers(27) have reported success by producing left ventricular hypertrophy by tieing off the ascending aorta with a circular clip made of silver wire 0.85 mm. thick to produce enlargement of the heart within 4 hours of the operation. The mortality rate was reported to be about 30%, the chief cause of death being acute pulmonary edema in the immediate postoperative period. The extent of hypertrophy varied from 10% to 60% above control values. This scatter according to the authors was probably due to the critical nature of the aortic stenosis. This operation is complicated and further requires the administration of positive pressure respiration produced by passing 95% $O_2$-5%$CO_2$ through an oral tube at the rate of 3 litres/minute with intermittent compression of the nose and mouth.

Constriction of the abdominal aorta close to the diaphragm (28) is somewhat less complicated than the above technique. Between a period of 11-22 days postoperatively, the increases in heart weight to body weight ratios are about 23% above control values. I will discuss this technique which I used in detail in the 'Methods' section.

Using the above techniques and as will be discussed in more detail in a subsequent section, cell proliferation in cardiac hypertrophy is predominantly if not exclusively limited to nonmuscular cells.

c) Treatment with Isoproterenol and other compounds

The sympathomimetic drugs such as isoproterenol are powerful inducers of myocardial hypertrophy (29). Amongst the various effects of these agents, are production of cardiac excitatory action. Isoproterenol is the most active of the sympathomimetic amines acting on the β receptors, with virtually no effect on the α receptors. More detailed actions of this drug on the smypathetic nervous system are reviewed by Goodman and Gilman(29).

Intravenous infusion of isoproterenol in man lowers peripheral vascular resistance, mainly in skeletal muscle but also in renal and mesenteric vascular beds, and diastolic pressure falls. Cardiac output is raised by the inotropic and chronotropic actions of the drug, combined with an increased venous return to the heart, thereby causing it to enlarge.

In several recent studies, isoproterenol has been shown to induce cardiac hypertrophy in rats at doses substantially below that which produces myocardial lesions. Doses of 5 mg/kg administered twice daily for 2 days produces both increased heart weight and microscopically detectable myocardial lesions; whereas smaller doses, 0.08 and 1.25mg/kgBW administered in the same way produced only hypertrophy

without myocardial lesions(30). Cellular metabolic effects of isoproterenol have been documented in dogs at a dose of 0.036mg/kg/day administered by continuous infusion for 6 days(31). Even lower doses of isoproterenol (0.02mg/kg/day) when administered subcutaneously, every day for a period of 13 and 31 days produced significant myocardial weight gain in rats, the increase in heart weight to body weight ratios being 23% above control values(32). At the concentration I used (as will be discussed later) I obtained a 20% increase of heart weight/body weight ratios above control animals given saline throughout the experimental period. Increased collagen content in the heart has been observed on the administration of this drug (70) suggesting that mainly the connective tissue elements are involved.

Recently talytoxin found in marine talythoa species (33) has been claimed as the most potent vasoconstrictor known. The material is derived from Palythoa colonial invertebrates and has a molecular weight of about 3300. It is a non peptide, polyhydroxy in nature and is lipophilic yet polar and highly water-soluble. As little as 25 ng. of polytoxin per kg. of body weight injected into dogs apparently induces powerful vasoconstriction and produces an elevated T-wave in their electrocardiogram, typical of patients suffering from variant angina. Though it has not yet been shown to produce hypertrophy future research may prove this compound to be very important because it is able to affect biologic systems at such low concentrations and is

likely that it operates at a fundamental biochemical level.

The old concept of myocardial damage in hyperthyroidism characterized by focal degeneration was observed in rats(34) following thyroxine treatment. Marked cardiac hypertrophy following dietary thyroid administration was produced(35) and mononuclear infiltration was found in older animals and not in younger animals. This age difference in the histologically demonstrable effect upon the myocardium may account for the difference in opinion as to whether there is myocardial damage in hyperthyroid-induced heart disease.

Rats and mice on administration of 3,3',3-triiodo-L-thyronine ($T_3$) developed cardiac hypertrophy(36). The extent of hypertrophy was dependent on the amount of $T_3$ in the diet: a 27% increment found in the heart weight of rats fed 20 µg $T_3$ for 35 days. This was not due to the water content of the myocardium as a marked hypermetabolism with accompanying increase in pulse rate was observed during the course of treatment with this compound. As has been discussed above, the isolated administration of either isoproterenol(isopropylnor-adrenaline) or desiccated thyroid is able to induce cardiac hypertrophy. The combined administration of both these drugs has an additive effect on heart weight(36). In these recent experiments on adult male rats with an average body weight of 200=220 grams isoproterenol was administered intraperitoneally at a dose of 0.5mg/kg body weight on the first day and 1.0mg per kg body weight throughout the subsequent days. Desiccated thyroid, containing 0.06

percent specifically bound iodine, was mixed with the diet. Among all the organs investigated in this study, only the weight of heart was significantly increased (18% above controls) and the maximum increase of the average absolute heart weight was observed on day 12. A 17.6 percent increase in blood pressure and a 62 percent increase in the minute blood volume was also observed.

d) Dietary Manipulation and Deficiencies

Physiologic and anatomic alterations in anemic heart disease have been extensively reviewed(38). In sickle cell anemia in humans some workers have found no cardiac hypertrophy(38). In the case of the nutritional anemias, enlargement of the heart has been reported(39). However this could be explained to be due to thiamine deficiency.

In dogs, cardiac hypertrophy in anemia induced by bleeding was observed(40). Though the authors did not describe dietary vitamin supplemantation, there was no alteration in body weight nor in serum proteins to suggest a nutritional deficiency. Documentation of their results by analysis of covariance showed conclusively increased heart weights ($p < .001$) in the experimental animals and there was no statistically significant increase in the water content of the myocardium of these animals.

After induction of chronic anemia by the administration of phenylhydrazine to rats for a period of approximately four weeks, cardiac hypertrophy was produced($p < 0.01$)(41).

Although heart weights were elevated even 28 days after cessation of treatment, a marked widening of the range of heart weight in these animals suggested the beginning of regression of the hypertrophy. In one study(42) anemia induced by phenylhydrazine treatment and hypertension induced by unilateral nephrectomy and ligation of the remaining kidney after capsular stripping were examined. Definite increases in heart weight resulted when both factors were present in the animals ($p=0.01$) as compared to animals with either hypertension or anemia alone ($p=0.05$). However, the overall effect was neither additive nor synergistic.

Beriberi heart disease is of much clinical interest though considerable debate still exists whether hypertrophy of the heart occurs in this thiamine deficiency state. A good review on the past and more recent investigations about this is available (43). For example, in recent experiments(43) there appeared to be hypertrophy when rats were put on a thiamine deficient diet, but these experiments were complicated by decreases in food intake, and close inspection of some of the data with consideration of terminal weights indicates that the increase in heart weights demonstrated was actually what would be expected in normal animals with such a weight loss.

Though hypertrophy was not clearly evident, the evidence for bradycardia and certain electrocardiographic changes that do sometimes accompanh it was strong. A histologic increase in fat, vacuolar degeneration and necrosis of the myocardium in deficient rats was also observed. It appears quite

obvious that the deficient animals probably had a marked decrease in intake of dietary protein and possibly other dietary components. Therefore it becomes difficult to accept the premise from these data that thiamine deficiency per se was responsible for the myocardial changes observed.

Experimental cardiac hypertrophy has also been observed in copper deficiency. In some(44), an increase in the heart weight of these animals has been reported which is out of proportion($p \leq 0.001$) to the degree of anemia present. In weanling rats fed a copper deficient diet for three weeks, cardiac hypertrophy also develops and regresses within 20 days of copper treatment(45). (I observed a 17-20% increase in heart weight/body weight ratios in the above case.) In a more intensive study on iron deficiency plus copper deficiency in rats(46) the hypertrophy of the heart was attributed to an increase in mitochondrial area in the myocytes. According to the authors marked enlargement of mitochondria displaces and distorts the myofibrils. These morphologic changes observed are distinct and in sharp contrast to those observed in experimental work hypertrophy and may represent lack of precursors required for cytochromes or other mitochondrial constituents.

A small but significant increase in heart weight (8% above controls) of chicks has been observed 24 hours after being placed on 6 percent dietary sodium chloride for 9 hours(47). Maximal hypertrophy was observed on the 9th day. Though the role of disturbed water metabolism in this

phenomenon is not very clear, the authors concluded that dilation of blood vessels and interstitial fluid along with the increase in cardiac protoplasmic mass was a quick response to Starling's law of the heart.

Other methods used in producing experimental cardiac hypertrophy include reaction to stress(48), occurrence in high altitude regions(49) and in athletes used to strenuous exercise(50). In these studies, conclusions were based on histological examination. Measurement of hypertrophy in terms of heart weight/body weight ratios were not considered.

B. Biochemical and Cellular Changes underlying Cardiac Hypertrophy

Enlargement of the heart may be due to enlargement of individual cells of a tissue or to an increase in cell numbers or both. Measurement of tissue DNA is one method of distinguishing the two processes since the total DNA in the organ should remain unchanged when cells increase in size, but should increase when the number of cells increases. In experimentally induced work overload of the left ventricle such as is produced in the rat by aortic banding, increase in the size of cells should result in a fall of the DNA concentration per mg of heart tissue since the increase in the heart weight would dilute the constant DNA content. An unchanged or increased DNA concentration would indicate DNA synthesis and the formation of new nuclei and probably new cells. An important point to note is that often DNA, RNA and other parameters are measured in the total heart. The heart

as seen earlier is a heterogeneous mixture of different cell types and it is difficult to assess changes in a particular cell type when one looks at the whole tissue.

1. DNA Content in hypertrophy

There are conflicting reports of the changes in the DNA content of the ventricular myocardium under conditions of cardiac hypertrophy. Some investigators report a decrease in DNA concentration (mg DNA per unit weight of tissue) with no change in the total DNA content of the heart(50-54). Others observe a moderate decline in DNA concentration with an increase in total DNA content(55,56). A third group suggests no change in DNA concentration and that the total increase in DNA content is proportional to the increase in cell mass(50, 52, 57-61). Still another group shows a definite increase in both DNA concentration and total DNA content of the heart under such conditions(62,63). Different laboratories employing different methods of induction such as DOCA induced hypertension(62), nutritional anemia (57-61) have also reported that cardiac hypertrophy is accompanied by a more or less proportional increase in DNA concentration. Histologically whether these agents act in a similar fashion is not documented. An important factor to be considered here in the interpretation of the conflicting data is the age and maturity of the cardiac tissues. The very large increase in DNA noted in some studies(63) may in part have resulted from their use of very young, rapidly growing animals.

## 2) Proliferation of Different Cell Types in Cardiac Hypertrophy

At this stage it is relevant to consider in brief the ability of muscle cells to multiply. Though authors disagree on the exact time at which cardiac muscle cells lose the ability to divide they agree that it is fairly early in life. There is reason to think that skeletal and cardiac muscle may not be exactly alike in this respect. In the developing skeletal muscle, DNA synthesis and mitotic cell divisions are incompatible with contractile activity(64). Once the dividing mono-nucleated myoblasts fuse to form multinucleated myotubes, DNA synthesis ceases in the myotubular nuclei. Results concerning the differentiating heart are less conclusive. Using electron microscopy, it has recently been shown that contractile cells of very young rats are able to divide under normal conditions, confirming earlier light microscopic studies (65,66).

During cardiac hypertrophy induced by aortic banding, abdominal aortic constriction or the administration of drugs the connective tissue nuclei appear to be quite active both in terms of mitotic activity and uptake of tritiated thymidine(67). Changes in collagen content after the onset of hypertrophy has been investigated. By the eleventh post-operative day following aortic banding in adult rats, the collagen content of the myocardium is reportedly increased by 67%(68,69), and enhanced DNA synthesis reaches a peak on the seventh day. Therefore new connective tissue nuclei

and collagen are both produced. Increased collagen content has been observed in cardiac enlargement induced by different methods: aortic constriction in rats(70), and pulmonary artery constriction in cats(71), administration of isoprenaline, long term adaptation to hypoxia and physical stress(70). Exceptions where collagen content did not change were cardiomegaly produced by anemia and the effects of thyroxine administration where the collagen content increased only moderately(70). In differentiated heart muscle cells in contrast to connective tissue cells, evidence available suggests that DNA synthesis is not resumed in cardiac hypertrophy(69,71), a situation similar to that of skeletal muscle hypertrophy(72,73).

3) RNA and Protein Synthesis in the Hypertrophic Heart

RNA and protein synthesis are activated in the myocardium, as the heart adapts to continuous stress such as by aortic banding(67) or abdominal aortic constriction (67,74,75 ). In such hearts, the relationship between RNA polymerase, adenyl cyclase and cyclic AMP has been explored using $\alpha$ -amanitin, a specific inhibitor of nucleoplasmic RNA polymerase II (76). An increase in RNA polymerase II activity and myocardial adenyl cyclase activity was found in rats with constricted aortas, and in inbred strains of the Syrian golden hamster that develop cardiomyopathy. The precise relationship between adenyl cyclase, cyclic AMP, protein kinase and the polymerases is yet to be explored, though it is known that in rabbit skeletal muscle and reticulocytes

protein kinase is activated initially be cyclic AMP and stimulates DNA-dependent RNA polymerase(77,78).

In line with the activation of RNA polymerase, recent studies(79) using [8-$^{14}$-C-] adenine precursor show that the rate of synthesis of ventricular RNA increases abruptly following abdominal aortic constriction and this is followed by an increase in DNA, suggesting according to the authors, the existence of two types of adaptation process: the initial response by cardiac muscle and interstitial cell components to form more RNA, and then the prolonged mesenchymal cell proliferation forming more DNA.

The exact mechanisms regulating protein synthesis, as the heart adapts to increasing stress are not known. Normally increased protein synthesis in a tissue is connected with an increase in polyribosomes. However, in cardiac hypertrophy, ribosomal yields from both cardiac muscle and liver were not elevated though the incorporation ability of cardiac muscle ribosomes in an in vitro system was increased(80).

It is important to mention here that almost all the work done in this area has considered total heart ribosomes and ribosomes not of individual cell types. The increase in polyribosomes observed in many cases in turn may be caused by the formation of messenger RNA (mRNA) and mobilization of translating ribosomes resulting in increased frequency of peptide chain initiation(81,82). Also, in higher animals an increase in protein synthesis can be regulated by an increase in ribosome content of these cells(83).

Sharp increases in ribosomal RNA and transfer RNA(tRNA) have been observed in hearts undergoing hypertrophy(84,85). In one case when the isolated heart was subjected to increased stress, an increase in the formation of mRNA was also found (86). Recent work shows that following two days of compensatory hyperfunction, ratios of translating polysomes to nontranslating ribosomal monomers and subunits increased from 1.0 to 1.5(87). These authors suggest that the increase in heart mass during compensatory hypertrophy depends both on an increased level of myocardial RNA and an increase in the proportion (of the total population of heart ribosomes) involved in translation of mRNA.

In other abnormal heart conditions in rats, such as surgical trauma, there is an increase in the incorporation ability of ribosomal preparations from both cardiac muscle and liver in an in vitro system(80). This increase is due to a temporary increase in the formation of polysomes. Investigations on the reaction of cardiac muscle and liver ribosomes to surgical trauma in adrenalectomized animals revealed a decrease in ribosomal yields from both liver and heart muscle(80). The authors suggest that the reasons for this could be the lower rate of formation of ribosomes and their subunits and/or faster degradation of these particles. Recently,(88) the RNA content in the heart muscle cells of the adrenalectomized rats and those with aortic constriction was measured by microspectrophotometry. In the adrenalectomized rats, the RNA content decreased about 32% below

that of the intact control rats by 7 days after operation. On the other hand, it decreased more abruptly in the adrenalectomized rats after aortic constriction. These results disagree with earlier findings (67,74,75) and it is possible that cardiac hypertrophy was never really induced by overloading the adrenalectomized rats. These experiments have been performed on heart cells and as methods for the separation of the different cell types become available, more insight can be gained into the response of a particular cell type to cardiac hypertrophy.

In relation to evidence for increased RNA synthesis in cardiac hypertrophy, there are also reports that purine synthesis is enhanced with the onset of cardiac hypertrophy (89). This, according to the authors was due to an increased "de novo" synthesis. With respect to adenine metabolism however, a recent study(90) could not differentiate between greater degradative activity with unchanged nucleotide synthesis and depression of the synthetic pathways 5 days after operative constriction of the aorta. Since adenosine is preferentially converted to inosine and hypoxanthine (91-93) for the synthesis of adenine nucleotides by the 'salvage pathway', the fractional adenosine levels in the fluid from hypertrophied hearts were always lowest in comparison to those of inosine and hypoxanthine.

To summarize these changes in cardiac hypertrophy, evidence available suggests there is an increase in total

DNA proportional to the increase in cell mass. Cell proliferation seems to be mainly in the connective tissue elements, as concluded from autoradiographic studies, collagen synthesis and uptake of tritiated thymidine by connective tissue nuclei. Overall protein synthesis is increased in the hypertrophic heart whether by an increase in RNA polymerase or an increase in the ratio of translating polysomes to nontranslating ribosomal monomers and subunits. The synthesis of purines under this condition was also increased.

C. Mechanisms Underlying the Regulation of Heart Weight

Cardiac muscle has a remarkable ability to adjust its mass to altered work loads. It is clear that a work overload stimulates RNA and protein synthesis (74,75) though the mechanism of regulation of these processes and adjustment to physiological demands is not yet known. Several suggestions have been offered for the stimulus that initiates cardiac cell enlargement and these have been extensively reviewed(94). Briefly they can be listed as follows:

1) Energy depletion

This hypothesis states that transient ATP depletion secondary to an increased work load triggers the synthetic processes. Though it is observed that cardiac ATP levels are transiently depressed immediately following constriction of the abdominal aorta(95)(presumably due to increased utilization) other investigators have noted no decrease in ATP content at later time periods. Though this hypothesis is supported by tissue culture experiments in the perfused rat heart(96)

where hypoxia stimulates cell growth and RNA synthesis, it has also been observed that anoxia and decreased energy stores inhibit protein synthesis in the isolated perfused heart and aorta(94). These facts therefore do suggest that if indeed energy depletion is observed, it must operate within a narrow range between stimulation and inhibition.

ATP depletion may preferentially stimulate mitochondrial biosynthesis to compensate for ATP utilization, as several laboratories have shown a selective increase in mitochondrial mass in the early stages of pressure-induced hypertrophy (95,97-99). A selective increase in mitochondrial mass is observed during copper deficiency together with hypertrophy of the heart(95). Furthermore, it appears from some of the above studies that during the initial period following constriction of the abdominal or ascendingaorta, the mitochondrial membranes appear to be synthesized preferentially. Studies involving measurements of respiratory enzyme activities(97-99), relative incorporation of isotope into different heart cell fractions(100) and incorporation of labelled precursors into mitochondrial proteins and RNA, do suggest that mitochondrial accumulation is an early event in cardiac hypertrophy.

However in chronic pressure-induced hypertrophy in contrast to the above situation, a decrease in relative mitochondrial volume and an increase in the ratio of myofibrillar to mitochondrial volume has been observed(101-105). Under such conditions, decreased contractility has been observed

in hypertrophied papillary muscles(106,107) while contractility is normal in volume-induced hypertrophy where proportional cardiac cell enlargement occurs(108). It remains to be established whether the decreased mitochondrial mass in the face of increased myofibrillar ATP utilization ia a factor in diminished cardiac function in pressure-induced hypertrophy. Independent control of mitochondrial and myofibrillar mass may account for the different patterns of subcellular change following different physiological stimuli.

2) Membrane Stretch is the Stimulus

The second general hypothesis is that membrane stretch itself is the stimulus. A possible mechanism by which increased stress may be translated into increased protein synthesis is by altering the properties of the plasma membrane, causing enhanced transport of precursors of proteins and RNA synthesis. In the isolated, perfused heart under conditions of increased pressure load, an accelerated uptake of $\alpha$ amino isobutyric acid has been noted(109), this uptake not being inhibited by cycloheximide.

3) Role of cyclic AMP

The role of cyclic AMP in cardiac muscle growth is far from clear. While elevated levels of cardiac adenyl cyclase have been obtained 15 minutes after increasing aortic pressure in the perfused rat heart(76), but in genetically inbred cardiomyopathic Syrian hamsters(77,78), a decrease has been observed following ascending aortic constriction.

In the latter case the synthesis of RNA and protein was stimulated in agreement with other studies (74, 75). This discrepancy in results may be explained by the location of the enzyme molecule. It is still not definitely established whether cardiac adenyl cyclase is localized exclusively at the cell surface membrane or whether other cell membranes contain independent adenyl cyclase.

It is not known whether energy depletion or muscle stretch is involved in the initial growth stimulus in cardiac hypertrophy. The two hypotheses are not mutually exclusive, though both may be operative. For example, ATP depletion may be primarily concerned with stimulating mitochondrial synthesis and stretch may be involved in accumulation of contractile elements. Mitochondria and myofibrillar elements may not increase in parallel.

III. Ferritin

Ferritin is an iron-storage compound and is therefore part of the total iron of the body. The approximate composition of the iron containing compounds in the adult, 70 kg. human have been summarized by Bothwell and Finch(110). The total body iron, according to them is 3-4 grams and approximately 65 percent of this is present as hemoglobin. Ferritin constitutes 30 percent of the total body iron. Myoglobin, the red pigment of muscle whose function is to store oxygen for utilization during muscle contraction comprises 3-5 percent of the total body iron. Other estimates show the

myoglobin concentration to be 10% of the body iron(111). The transport protein of iron, transferrin contains about 0.1 percent of the total body iron while the two iron-storage proteins ferritin and hemosiderin contain 30-35 percent of the total body iron.

A. Ferritin Distribution

The iron-storage protein ferritin is found in many organisms ranging from fungi(111) and plants(112) to annelid worms(113), insects(114), fish(115), amphibia(116), and mammals. Hemosiderin the other form of storage iron is less well defined. Histologically hemosiderin has long been defined as Prussian-Blue -staining granules visible under the light microscope. Biochemically, hemosiderin presents an insoluble non-heme iron complex with some protein, the ratio of iron to protein being higher than in ferritin (117). Studies on the intracellular distribution of ferritin and hemosiderin in rat liver by VanWyk et al (118) indicates that the reticuloendothelial cells of the liver i.e. the Kupfer cells store little if any as ferritin but retain iron as hemosiderin, whereas almost all of the ferritin of the liver occurs in the hepatocytes.

Also noteworthy from the same study is the fact that after repeated dosage (140 mg total iron) there appeared to be a limit to the capacity of the liver to convert iron into ferritin; only about one quarter of it appeared as ferritin compared with three quarters in control rats or rats treated with moderate doses (16-25 mg iron).

Ferritin and hemosiderin have two metabolic functions: to remove and store unneeded iron, and to provide a source of iron which can be drawn on when required. Under normal conditions, ferritin accounts for more than half of the iron in storage. As iron loading increases, the balance alters, and hemosiderin increases relative to ferritin. In extreme cases, hemosiderin forms massive deposits which may cause tissue damage(110,119-121). Severe siderosis (such overloading) is associated with scurvy in human subjects and in guinea pigs(122,123) and the shift in the balance of storage iron from ferritin to hemosiderin can be directly correlated with ascorbic acid depletion. A single dose of Vitamin C to scorbutic animals was found to decrease hemosiderin and increase ferritin with a net loss of storage iron from the spleen(123). This observation along with in vitro studies of ferritin reconstitution suggest that ascorbic acid may be required to provide $Fe^{2+}$ for ferritin formation while it would cause a net decrease in hemosiderin either if hemosiderin is produced by hydrolysis of $Fe^{3+}$ of if it is required for the release of hemosiderin iron.

Though iron can be mobilized from both ferritin and hemosiderin, estimates of the relative ease of iron release from the two storage components vary. Some authors feel that iron is more readily released from liver ferritin than hemosiderin after iron loss via bleeding in rats and mice (124), while others note no difference in the transfer of

iron from the two compounds from maternal liver in the pregnant rat to the foetus(125). The latter observation has been contradicted on the basis of $^{59}Fe$ uptake studies(121) which showed that iron stored as hemosiderin was less available for immediate utilization and so the balance is in favor of less release from hemosiderin.

There is evidence that iron can be transferred between ferritin and hemosiderin(120,122). The similarity in electron microscopic appearance (126,127), magnetic properties(120), X-ray diffraction patterns and Mossbauer spectra(127) of the two forms is consistent with the possibility that hemosiderin is a degradation product of ferritin in parenchymal cells produced by partial digestion of its protein with release of its micelles to form insoluble aggregates. However, these studies do not prove such a relationship since the polynuclear ferric hydrolysiate have similar structures(128) and these are the basis for the main criteria. Studies reported earlier in this survey(122,123) involving ascorbic acid and mobilization of iron from hemosiderin and subsequent incorporation into ferritin do not support the above idea. Furthermore in iron overload, the biosynthetic apparatus for ferritin and its ability to take up iron may become saturated and hemosiderin may be deposited as the major iron store independently of ferritin(118,129).

Early reports in the literature indicate that for the rat and some other species (mice, chickens, eels), the size of the liver iron stores differed with the sex of the animal

and in both sexes, these stores increased with age(130, 131). Linder and co-workers(132) have found in rats in the normal state the ferritin concentrations in the liver and kidney are greater in the female while there is no apparent sex difference in the heart ferritin levels. Iron administration to male and female rats for one week in the above study abolished the sex difference in the liver iron stores though in the kidney about twice as much ferritin was present in females. The heart ferritin though increased in both cases did not exhibit any difference in the concentrations in the two sexes. The larger amount of ferritin in the female rat can be attributed to more rapid synthesis as Linder et al (132) have shown as measured by the uptake of $^{14}$C-U-leucine. This is presumably due to the greater availability of iron as there is no change in the fractional degradation rate. Linder and Munro(1) further suggest that estrogen has an indirect effect on iron absorption and availability. There is no evidence however, that the livers of male and female rats have an inherently different capacity to make ferritin protein since synthesis and accumulation of ferritin in the liver becomes the same during iron overload.

In the past, ferritin has usually been considered to be an intracellular protein, identifiable in histologic sections or marrow smears (along with hemosiderin) by the blue color seen after staining by the Prussian Blue reaction.

It was detected in serum only in grossly pathologic states such as liver damage when release by necrotic cells might be expected. The development of an immunoradiometric assay for ferritin(133) has resulted in its demonstration as a normal constituent of serum and circulating blood cells(134).

In examining further the properties of human serum ferritin (135) it has been found to clear rapidly($t_{1/2}$ from 4-40 min) from the plasma and being taken up rapidly by liver parenchymal cells. Siimes and Dallman(135) have calculated on the basis of rat experiments that 18 mg iron would pass through the plasma as ferritin per day in a 70 kg. man. Although there are some problems in the characterization of specific tissue ferritins, the physical characteristics of serum ferritin are reportedly distinct on anion-exchange chromatography from human spleen, liver, heart or kidney ferritin(136). According to Worwood et al(136) the only cells found to have a component similar to serum ferritin are peripheral blood leukocytes, but even here, it rarely accounts for all the leukocyte ferritin. Therefore, whatever the origins of circulating ferritin, it is possible that its properties are modified either by a secretory process or by release from damaged cells. However, the failure to modify the characteristics of tissue ferritin by incubation with serum makes it unlikely that this type of interaction accounts for the difference. One of the difficulties of using ferritin levels as a measure of body iron stores is that concentrations in serum also vary with disease.

There is good evidence that serum ferritin concentration closely reflects body iron stores in iron deficiency and iron overload. In these situations, in contrast to other measures for determination of iron status (such as hemoglobin, transferrin saturation, and serum iron concentration) the serum ferritin concentration is better over-all at distinguishing differences in iron stores within the physiological range(134). From studies with patients having acute and chronic liver diseases, it was found that serum ferritin varied both with the serum transaminase level and with liver iron concentration(137). There was no correlation of serum ferritin with the serum iron or total iron binding capacity, though an extremely close correlation was found between serum ferritin and an empirical index derived from the product of the serum transaminase x liver iron concentration implying that the circulating level depended on both the degree of hepatocellular injury and liver iron store. It is likely that estimation of serum ferritin concentration will receive widespread use as a diagnostic tool.

B. Ferritin Structure

Ferritin consists of a protein shell (apoferritin) surrounding a core of ferric oxyhydroxide. The protein shell of horse spleen ferritin has been estimated to have a molecular weight between 430,000 and 480,000(138-141). The iron content can vary from none (apoferritin) to 5000 atoms of iron in the fully saturated holoferritin molecule(142) resulting in a two fold increase in molecular weight.

Ferritin structure is complicated by various types of heterogeneity and structural complexity, some of which may be methodological. Harrison and others(143) have reviewed this in detail and I will summarize the aspects relevant to this thesis.

a) On the basis of ultracentrifugation (density gradient centrifugation) two peaks, one sharp and colorless, one diffuse and colored are observed. This shows that apoferritin and ferritin have a broad distribution of iron atoms.

b) Gel filtration and analytical ultracentrifugation studies show three or more ferritin bands present in most ferritin preparations. These bands have been characterized as monomer, dimer, trimer, etc. The intermolecular bonds and biological significance is uncertain.

c) Gel electrophoresis studies demonstrate different migration rates for ferritins from different organs of the same animal. Confirmation of these observations by amino acid analysis and peptide maps suggest isoferritins with different primary structure implying more than one ferritin gene.

d) Iso-electric focusing studies show multiple peaks with different pI. It is suggested that isoferritins contain different proportions of subunits with different primary structure. The possibility still exists of some of the isoferritins being artifacts. I will expand on these aspects of ferritin heterogeneity further in this section.

1. Aggregation of Ferritin

Ferritins purified from various sources contain minor components which have been most extensively explored in the case of horse spleen ferritin. On the basis of ultracentrifugation studies where three components of horse spleen apoferritin(with sedimentation coefficients of 17s, 24s, and 32s) were recognized(144) it has been concluded that ferritin aggregates into dimers, trimers and oligomers confirming earlier studies. These minor components migrate slowly on electrophoresis and they have been analyzed by electron microscopy(145). After fractionation of horse spleen ferritin by starch gel electrophoresis and by gel filtration on Sephadex G-200, it was found by quantitative electron microscopy(145) that the monomer fractions contained up to 98% of monomer and oligomer fractions up to 76% oligomers. Furthermore amino acid analysis of monomer and oligomer enriched fractions showed no significant difference.

Consistent with this, Bjork and Fish(146) more recently estimated the molecular weight of the separated horse spleen ferritin major and minor components by sedimentation equilibrium to be 450,000 and 895,000. However, Bjork(147) who investigated the association behavior of a homogeneous preparation of horse spleen apoferritin concluded that both the monomer and dimer have a less regular shape than most typical globular proteins. Contrary to early studies by Richter and Walker(148), Williams and Harrison(145) found no evidence either for association of isolated monomers or

dissociation of oligomers under a variety of conditions. Oligomer stability has been recently investigated by Nitsu and Listowsky(149) who found that dissociation could be produced by the action of thiol reagents and conclude that the molecules may be linked by disulphide bridges. The multiple components of ferritin isolated from different tissues and species have not been subjected to this detailed technique. Part of my thesis has been the exploration of the size and apparent valence of ferritin monomers and oligomers using quantitative polyacrylamide gel electrophoresis.

2) Heterogeneity due to Electrophoresis and Isoelectric Focusing

Apart from such heterogeneity due to aggregation, there is considerable eveidence in the literature pointing to more basic differences between ferritin monomers in different species and different tissues within the same species.

It has been found by Alfrey and coworkers(150) using qualitative electrophoresis on cellulose acetate strips that normal human tissues contain at least five different ferritins including two in heart tissue. Contradictory to this observation, on the basis of quantitative polyacrylamide gel electrophoresis at different pH's and cellulose acetate strip electrophoresis Munro and workers(151) were unable to find significant differences between migration properties of human ferritins from different tissues. However in one heart preparation at pH 8.9, they detected two ferritin bands.

In the rat, Linder and others(152) have found at least three distinct ferritin species on discontinuous polyacrylamide gel electrophoresis; one characteristic of adult liver also present in heart and skeletal muscle, one common to kidney, spleen and some hepatomas, and a third found only in muscle and heart. More detailed studies on amino acid composition, sulfhydryl group content, proportions of subunits and distinctive peptide maps following trypsin digestion revealed however that ferritin extracted from adult kidney, neonatal liver, hepatic and renal tumors differed from adult liver ferritin and each other in some respect(153).

Rabbit marrow contains two different ferritin species migrating at different rates on starch blocks(154). On the basis of $^{59}$ Fe administration by way of the transferrin pathway and $^{55}$ Fe as heat-damaged labeled reticulocytes to rabbits, Gabuzda and Pearson (153a)concluded that the slower migrating species is present in the reticuloendothelial cells of the marrow where it accumulates the iron released from the degradation of old hemoglobin while the faster migrating species accumulates iron from plasma transferrin.

The question of what causes the different migration rates of isoferritins arises. Possible causes are differences in size or asymmetry (due to different numbers or arrangements of subunits or additional components).and/or differences in surface charge (due to different primary structures resulting from multiple genes or to post-translational changes

such as deamidation, acetylation, ion binding, etc). Part of this thesis is devoted to answering such questions.

In addition to the heterogeneity amongst tissue ferritins described above, Drysdale and workers(154,155) have reported a more extensive microheterogeneity amongst human tissue and horse spleen ferritins. On iso-electric focusing of human spleen,liver, kidney and heart ferritin in gels Drysdale(156) observes five or six bands when stained for protein. Some tissues (liver and spleen) have similar bands on iso-electric focusing though heart,kidney gave some bands with more acidic iso-electric points. Several bands have also been ovserved on the iso-electric focusing of tissues in idiopathic hemochromatosis(157), mammary and pancreatic carcinomas(158), early placenta and HeLa cells(159) and according to Drysdale this heterogeneity does not represent differences in iron content or aggregate size but differences in the structure of the apoferritin moeity(159).

On the basis of the subunit composition of human heart, liver, and horse spleen ferritins examined by sodium dodecyl sulphate and acetic acid urea gel electrophoresis, Drysdale (156) suggests that there are two subunits that mix together in a random fashion in different proportions to form the isomers. These subunit types are in the region of 15,000 to 20,000 molecular weight. Type B (15,000 molecular weight) appears to be most predominant in the more acidic bands of horse spleen ferritin on isoelectric focusing(156). Type A (20,000 mol.wt.) on the other hand predominates least in the

acidic iso-electric focused bands.

Some groups however feel that the multiple bands seen on iso-electric focusing result from the interaction of the protein with the constituents of the pH gradients used(159, 160). This idea is substantiated by earlier evidence(161, 162) where a single homogeneous macromolecule has been shown to interact with small constituents of buffer, ampholytes etc. Part of this discrepancy is probably due to the behaviour of ferritin on a isoelectric focusing gel. Being a large molecule it tends to sieve through the pores of the gel though many workers claim the gels they use to be minimally sieving.

3)Subunit Structure Studies

There has been some difference of opinion about the number and size of subunits in ferritin making up the protein shell. Originally, Harrison(138) concluded on the basis of x-ray diffraction and amino acid analysis that the number of ferritin subunits is 20, and the molecular weight of each subunit 22,000 to 27,000. On the other hand, Crichton (163,164) concluded for horse spleen ferritin on the basis of electrophoresis in sodium dodecyl sulphate and the methionine residue content stained after cyanogen bromide cleavage that the molecular weight of the subunit was 18,500 and hence the ferritin shell should contain 24 subunits. However, Linder and Munro(165) have shown by sodium docecyl sulphate electrophoresis that the ferritins in various tissues of the rat contain two-three major types of subunits of

molecular weights 19,500, 13,000, and 11,000. Double-labelling studies preformed by them show that synthesis and turnover of the two subunits occur at different rates and respond differently to iron treatment. This independence is consistent with the earlier evidence of Konijn, Baliga and Munro(166) that ferritin subunits of different molecular weights are made on different polysome populations, the smaller on the free and the larger on the bound polyribosomes. Niitsu and coworkers(167,168) have also recently reported multiple subunits using polyacrylamide gel electrophoresis in sodium dodecyl sulphate with horse, human, rat and rabbit ferritins. They conclude that ferritin consists of two polypeptide chains of 10-11,000 and 7-8,000 molecular weight, which aggregate to form the 19,000 subunit(168 ).

More detailed evidence reported by this group based on amino acid composition, $NH_2$-terminal analysis and investigation of detergent-induced breakdown products further confirms their hypothesis. On the other hand Drysdale(156) suggests on the basis of the subunit composition of human heart, liver and horse spleen ferritins examined by sodium dodecyl sulphate electrophoresis and acetic acid-urea gel electrophoresis there be two subunits that mix together in a random fashion in different proportions to form the isomers. He also feels that the structure of human ferritin subunits is such that they are not fragmented (as in the rat) by sodium dodecyl sulfate treatment. These subunit types both are in the region of 15,000 to 20,000 molecular weight.

C. Ferritin Protein Metabolism

1. Synthesis and Degradation

It is thought that the main function of ferritin is to store iron and release it on demand. Consequently, it can be expected that the ferritin concentration is regulated by changes in iron concentration in the environment. Iron administration increases the amount of ferritin iron and ferritin protein in the liver and other tissues. In many of the studies conducted so far, this has been observed after the administration of a single dose of iron. An increase in ferritin protein concentration can mean either that an increase in ferritin protein synthesis and/or a decrease in ferritin breakdown has occurred. Granick(169) postulated that iron combines with pre-existing apoferritin to form ferritin and that the iron moiety of ferritin protects the protein against breakdown. In this scheme, iron causes an increase in total ferritin concentration by retarding breakdown and not by increasing protein synthesis. However early work in guinea pigs(170) revealed that there was an increase in liver apoferritin synthesis following the administration of iron. In the case of the rat, Drysdale and Munro(171) have confirmed an increase in ferritin protein synthesis after the administration of iron intraperitoneally (ferric ammonium citrate:400 ug Fe/100 gm body weight). They showed a six-fold increase in the rate of labelling of ferritin protein within five hours after the iron injection, whereas the mixed liver proteins did not

show this response. This was followed by a two-fold increase in the ferritin concentration in the tissue. The synthesis of ferritin protein thus seems to be rapidly responsive to the availability of iron in the cell. There was also retarded breakdown.

The exact mechanism by which iron in fact stimulates ferritin synthesis is still unclear. Since administration of actinomycin D in adequate doses fails to block the response of ferritin synthesis to iron administration, it has been concluded (171) that iron does not act by stimulation of new messenger RNA synthesis. Drysdale and Munro(172) suggested that the regulation of ferritin synthesis by iron occurs via a cytoplasmic mechanism, and the iron could act by increasing the translation of ferritin messenger or at some step in the assembly of ferritin from its subunits.

More recently, it has been observed that addition of iron to a cell-free system stimulates the conversion of apoferritin subunits(173) to holoferritin indicating a post-translational mechanism. However very recently evidence for the effect of iron on the amount of ferritin mRNA has been obtained (174). Polysomes from normal and iron treated rats were incubated in a cell free system. The apoferritin products were compared using immunoprecipitation with ferritin antibody. The mRNA's from these polysomes were isolated and incubated in heterologous cell free systems prepared from Krebs II ascites tumour cells and from

wheat germ. The capacity of the two mRNA preparations to synthesize apoferritin was compared and it was found that iron increases the proportion of ferritin mRNA in the total polysomal mRNA population of the liver(174).

A special regulatory role for N-acetyl-seryl-tRNA in ferritin biosynthesis has been suggested(175). Based on the fact that N-acetyl-serine is the N-terminal amino acid of rat liver apoferritin the existence of N-acetyl-seryl-tRNA was sought by examining for its snzymatic formation in vitro. N-acetyl-seryl-tRNA was isolated directly from perfused iron-stimulated levers but absent from non iron stimulated livers. It still remains to be investigated how N-acetyl-seryl-tRNA donates the N-acetly-serine moiety during the biosynthesis of apoferritin,and if in fact it does it could be one of the regulating factors in apoferritin synthesis.

Comparisons of species variation of ferritin structure have been made using serological cross reactions(176,177), electrophoretic mobilities(178,179), amino acid composition (180-182) and finger printing(182,185). While human, horse and rat ferritins are serologically related(176), phytoferritin is not precipitated by antihorse serum(143). The specificity of each ferritin is that of its protein shell, apoferritin. It is interesting that protein subunits obtained from horse spleen ferritin by dissociation with sodium docecyl sulphate possess not only the antigenic specificity of horse spleen ferritin (and apoferritin) but also have additional antigenic specificity of their own distinct from associated apoferritin

(184). Using this fact, Richter and workers(184) raised rat liver ferritin subunit specific antibodies and by the technique of immunofluorescence studied the intracellular localization of ferritin positive and subunit positive sites. Ferritin positive immunofluorescence was over the Prussian-blue positive cytoplasmic granules in which high concentrations of ferritin are thought to be present based on electron microscopic studies.

Very recently, Richter and workers(185) have attempted to immunologically trace the biosynthesis of ferritin in cultured cells using the above techniques. Discrimination between ferritin subunits and ferritin/apoferritin was achieved based on the presence or absence of immunofluorescence when specific antisera was the primary reagent. Material possessing attributes of subunits was dispersed in the cytoplasm in agreement with the fact that ferritin is mainly synthesized on free polysomes that are present in the cytosol. Presence of iron in the media increased the ferritin directed immunofluorescence in the cytoplasm, again in agreement with the iron induction of ferritin synthesis(171). They also found that cytoplasmic ferritin directed immunofluorescence was at first rather diffuse and later became concentrated into clumps suggesting that after the molecules of ferritin (apoferritin) have been formed from subunits and iron, they collect as cytoplasmic domains in the vicinity of the nucleus. Richter and workers(184) proposed the following sequence in ferritin synthesis in cultured hepatome cells.

amino acids $\longrightarrow$ protein subunits $\xrightarrow{Fe^{++}}$ apoferritin

$Fe^{++} \rightarrow Fe^{+++}$ ferritin

Much of the work done on ferritin metabolism and all the work on the mechanism of iron regulation has been confined to the liver. But the effect of iron on deposition of ferritin in other tissues including the heart, kidney tumors and regenerating liver has been followed(153).

Studies on the rate of degradation of liver ferritin(171) suggest that the turnover of preformed ferritin can be varied by altering the supply of iron to the liver. Drysdale and Munro(154) have found that repeated iron dosage retards the rate of ferritin breakdown, whereas bleeding removes iron from the liver and accelerates the rate of loss of preformed ferritin. Studies on the sex difference in ferritin metabolism mentioned earlier by Linder and others(132) failed to observe the stabilizing effect of the higher iron pools on ferritin degradation in the livers of female rats as is seen with acute iron treatment(171). This may be explained by the fact that the excess iron in the liver of the female is already sequestered within ferritin and there may be little difference between sexes in the amount of free iron available to retard ferritin breakdown. Linder and others (132) also found a half life for ferritin protein of 58 hr. in male rats much less than early reports of Drysdale and Munro(171). This difference is probably due

to the label used in the determinations. Drysdale and Munro(171) used $^{14}$C-leucine which is much more subject to recycling than $^{14}$C-guanidino-arginine used by Linder and co-workers(132).

2. Developmental changes in ferritin metabolism

It is known for many animal species that during the last stages of gestation there is an increased rate of iron uptake by the foetus(186,187).

The maternal capacity for iron absorption from the diet increases during pregnancy, and in addition, maternal stores of iron are depleted. The carrier of maternal iron for this transfer is plasma transferrin which is made in increasing amounts during the last third of pregnancy(188). Transferrin binds to the fetal part of the placenta(189), itself not crossing the placental barrier. Its iron is rapidly transported to the placenta. In rats, it has been observed that 2,500 µg non heme iron from maternal liver is lost by the end of pregnancy, while at the same time, the total litter accumulated just this amount of iron(including heme iron) by the time of birth. In the absence of adequate maternal iron stores, the iron transferred to the fetus comes almost directly from the maternal gut after absorption from the diet(190). A more detailed elucidation of the role of ferritin in the placenta remains to be explored but it may relate to transfer of iron from mother to fetus .

Linder and co-workers(191 ) have shown that during the suckling period, ferritin deposited in the liver of the

fetus if rapidly depleted and by the end of three weeks, there is almost no liver ferritin left. As milk is a very poor source of iron (188-190 ) the iron stores that are laid down during the end of the gestational period provide iron for the growth of the infant during the first weeks of life. Linder and Munro (191) have studied the effect of iron administration at 3 weeks of age or earlier. Without iron administration small amounts of "neonatal" liver ferritin are present in liver at 21 days of age, but with massive iron dosage, new ferritin accumulates extensively. This migrates on gels like adult liver ferritin, thereby implying that iron injection has evoked the formation of the adult liver species. This normally appears only at a later age, probably because during the suckling period little iron is stored as most of it is used.

More detailed structural studies based on amino acid composition, content of tryptic peptides, available sulphydryl groups, subunit sizes and porportions showed differences between neonatal and adult liver(153). Tumor and neonatal ferritin were very similar in amino acid composition and showed many significant differences from adult rat liver ferritin. Similarly, the peptide patterns of neonatal liver ferritin and hepatome 3683F showed deletions from the adult liver ferritin pattern without emergence of new peptides. Thus it appears that neonatal liver ferritin is structurally quite different from that of adult liver ferritin.

Linder and Munro( 3 ) have also made some preliminary

observations on the cardiac ferritins during development. It appears that though both are present at birth, during weaning the slow ferritin disappears and appears a few weeks after the weaning period. This developmental pattern can be explained in light of the observations on neonatal ferritin. The slower migrating species is probably mobilized from the heart just as liver ferritin is for erythropoeisis.

To summarize the structural and metabolic features of the iron storage protein ferritin, that constitutes about 30% of the total body iron is very complicated (110). Contrary to the earlier view that ferritin is composed of a single molecular weight subunit species(138), evidence available suggests that ferritins purified from different rat tissues contain subunits of more than one(and in most cases three) size present in different proportions depending on the presence or absence of iron treatment. Additional evidence of structural heterogeneity is based on polyacrylamide gel electrophoresis where a distinct ferritin species unique to heart and skeletal muscle is observed and iso-electric focusing of different tissue ferritins(1,170a).

When iron is administered to the whole animal, there is a stimulation of ferritin protein synthesis. As actinomycin D fails to prevent this stimulation, it has been suggested that iron specifically increases the translation of apo-ferritin subunits and has been supported by evidence obtained in cell free systems immunofluorescence studies of subunit

response to iron treatment of HeLa cells. Recently a two fold increase in ferritin mRNA in liver cells has been found following pretreatment with iron suggesting increased transport of mRNA from nucleus to cytoplasm. A special role of a special N-acetyl-seryl-tRNA in ferritin biosynthesis has also been suggested.

IV. Iron Overload

Iron overload is caused by a number of factors, primary and secondary. Some of the categories of iron overload as suggested by Al Rashid(191) are as follows:

1) Primary

This category includes idiopathic hemochromatosis and congenital iron overload. Hemochromatosis is a condition of excess iron in all tissues in addition to excess iron in the normal storage organs (liver and spleen)(192,193). It can be idiopathic, meaning without an apparent explanation for increased body iron or cirrhosis and this suggests heredity.

2) Secondary

This category can be further divided into excessive iron intake and excessive iron absorption. Excessive iron intake has been well studied in the African Bantu tribes that eat their meals in iron pots and vessels(191) and this is referred to as 'African Bantu hemosiderosis'. Hemosiderosis is also a condition of iron storage but is distinguished from hemochromatosis in that the iron does not deposit in the parenchymal cells. Other conditions of excessive iron intake include Kashkin-Beck disease, alcoholic cirrhosis with hemosiderosis and medicinal therapy with hemosiderosis.

Excessive iron absorption can result from increased erythropoeitic activity, thalassemia, acquired hemolytic anemias and ineffective erythropoeisis as in pyridoxine-responsive anemia. Parenteral administration of iron and transfusion hemosiderosis also produce excess iron in tissues.

3) Other diseased conditions such as renal hemosiderosis and idiopathic pulmonary hemochromatosis also produce iron overload.

As defined earlier, hemochromatosis is a condition of excess iron in the normal storage organs(192,193). In this diseased condition, the iron is deposited in the parenchymal cells of the organs and the level of plasma ferritin increases (137). Furthermore, the disease is characterized by pigmentation of the skin and internal organs and is often associated with diabetes and cirrhosis of the liver(194). The disease may be idiopathic i.e. without an apparent explanation and it has been ascribed to heredity.

Hemosiderosis which has been classified as a secondary form of overload is secondary to excess iron intake and is also characterized by increased body iron. Though different authors have distinguished these two diseased in different ways(195), the following is a brief description of some of their distinguishing characteristics:

| Parameter | Criteria |
|---|---|
| Chelation Tests (using desferrioxamine that chelates iron excreted in the urine) | Amount of iron excreted in the urine increases |
| Differential desferrioxamine test. ($^{59}$Fe-gabelled ferrioxamine is given at same time as desferrioxamine so that ferrioxamine can be corrected) | Can distinguish between idiopathic hemochromotosis amd cirrhosis with secondary iron overload(198) |
| Tissue ascorbic acid levels | Low(198) |

Clearly,it is evident that both the histological and biochemical tests together would provide a correct diagnosis. The histological tests are necessary to establish whether one is dealing with primary hemochromatosis or secondary symptoms of excessive iron intake, absorption or therapy. No single test is conclusive in itself for diagnosing a condition such as hemochromatosis especially as so many variables are involved.

Hemochromatosis is an uncommon condition and the debate is raging whether it is an inherited disease or an acquired disease. The arguments in favour of hemochromatosis being an inherited disease(16) are as follows:

1. The disease may be idiopathic, without an apparent explanation for increased body iron or cirrhosis, and this suggests heredity.

2. Two or more cases have been reported in the same family.

3. Relatives of patients have had laboratory hematological abnormalities: in one study 9-20% of asymptomatic relatives had elevated plasma iron and increased percent saturation

of transferrin or both(199).

4. Patients with hemochromatosis withstand the withdrawal of blood containing the equivalent of several grams of iron before they develop anemia(200). This is unlike patients with alcoholic cirrhosis(a condition of liver damage), who have a slight increase in hepatic iron and develop anemia after relatively few phlebotomies(199).

5. It is not well substantiated that excess iron does produce histopathologic changes.(201-203). Therefore a genetic susceptibility to damage may be involved. Arguments that hemochromatosis is an acquired disease with multiple causes are the following:

1. If stainable iron is measured in the parenchymal cells of non-storage organs such as the heart, adrenals and pancreas, 10-16% of all autopsied persons in some hospital populations have it(204,205) indicating that it is not specific for hemochromatosis.

2. Experimental studies have shown that excess iron alone is not sufficient to cause the organ changes of hemochromatosis(199). If a lipotrope deficient diet which is a choline free or a folic acid free diet is fed to animals together with excess iron, iron absorption is increased. Cirrhosis develops as a result of the deficient diet independent of the iron. Pancreatic fibrosis develops, the reticuloendothelial system is impaired and excess iron is distributed in a manner similar to idiopathic hemochromatosis in storage organs for example the heart and the pancreas(206-208).

| | Hemochromatosis | Hemosiderosis |
|---|---|---|
| Body Iron | Increased(196) | Increased(196) |
| Transferrin | Completely Saturated (196) | |
| Serum Ferritin | Increased (137) | No data |
| Organs Involved | In addition to liver and spleen, organs that normally do not store iron---- pancreas, heart(192,193) | Localized to one or fewer organs in which there is tissue necrosis or red cell destruction or both as in pulmonary hemosiderosis or hemolytic anemia(195) |
| Tissue Damage | Cirrhosis of the liver pancreatic fibrosis (197) | Usually no tissue damage |
| Intracellular Location of Iron | Parenchymal cells such as liver hepatocytes (193) | Reticuloendothelial system such as Kupffer cells of the liver(193) |

A. Assessment and Measurement of Iron Overload

The following is a brief summary of the measures used to assess iron overload:

| Parameter | Criteria |
|---|---|
| Serum Iron Concentration | Above 200 ug/100 ml(196) |
| Transferrin Saturation | Complete(196) |
| Total Iron Binding Capacity | Reduced(137) |
| Serum Ferritin | Elevated(137) |
| Isoferritin Profile on isoelectric focusing | Abnormal(156) |
| Histochemical Assessment of tissue iron(grade0-4) | 3 or 4(198) |
| Hepatic iron measurement by biopsy | |

Two suggestions have been made to accomodate the above two arguments:

1. the majority of the cases are acquired secondary to other diseases but some are specific inherited diseases of iron absorption

2. the persons with hemochromatosis have an inherited metabolic defect which is made manifest by acquired or environmental conditions

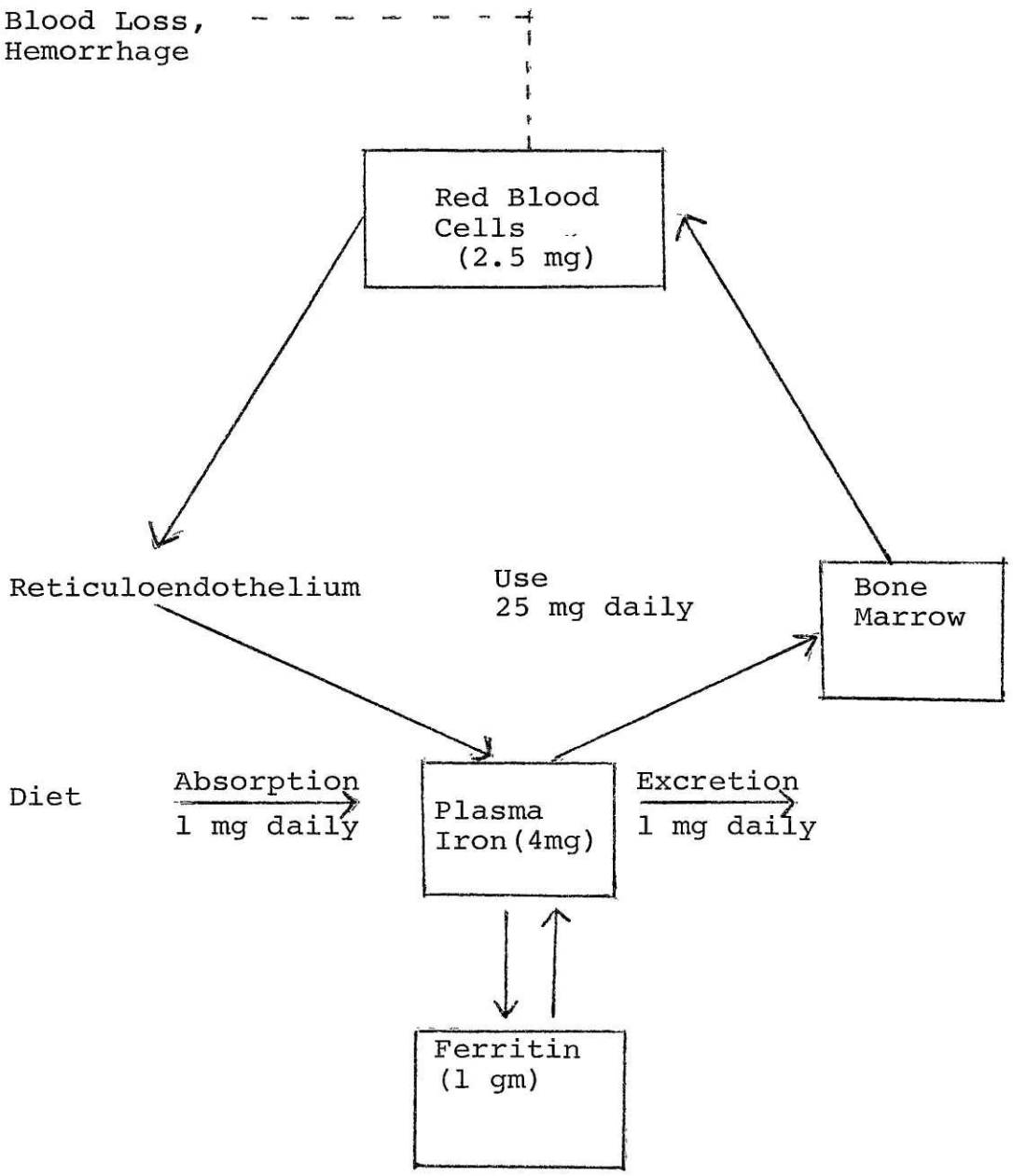
There is no completely satisfactory evidence to date for the existence of an inherited defect or for the contribution of an inherited defect to the acquired cases.

B. Biochemical features of hemochromatosis

1. Increased intestinal iron absorption

Because hemochromatosis is a disorder of iron metabolism, it is relevant to review the normal mechanisms for controlling the absorption,transport and excretion of iron. These aspects of iron metabolism have been extensively reviewed by Linder and Munro(209) and in hemochromatosis by (210).

The following diagram is a summary of the important concepts in iron metabolism that are relevant to the discussion of hemochromatosis:


Blood Loss,
Hemorrhage
Red Blood
Cells
(2.5 mg)
Reticuloendothelium
Use
25 mg daily
Bone
Marrow
Diet
Absorption
1 mg daily
Plasma
Iron(4mg)
Excretion
1 mg daily
Ferritin
(1 gm)

Via the plasma, iron is moved from place to place in the body, from one pool to another; from the small intestine where it is absorbed, to storage in the liver as ferritin and hemosiderin; from the spleen(reticuloendothelium) where red blood cells are destroyed, to the bone marrow where they are formed. Iron loss of about 1 mg/day is an obligatory loss for an adult human. The more constant excretory paths which account for this are the bile, sweat, urine as well as cells sluffed off in the intestine. Superimposed are variable losses such as (1) excessive sweating(depending on the individual or the environment, as in the tropics, illness which includes fever, etc); (2) bleeding, including menstrual bleeding which varies ten-fold among individuals; (3) the demands of pregnancy or lactation. Consequently the iron requirements of women are much higher. An important point here is that excretion of iron is not a major factor for controlling iron metabolism in the body.

Iron metabolism is carefully controlled at the level of the intestine which varies absorption according to need, and not at the level of excretion. If there is an appreciable loss of iron such as in hemorrhage, absorption of iron from the intestine may increase from 1 mg to 5 mg per day if available in the diet until the deficit is made up. When the iron deficit is made up, absorption by the intestine returns to the normal 1-1.5mg/day. Under such conditions, the bone marrow begins to repair the red cell mass immediately.

Hemochromatosis could thus be defined as a disorder of this complex system. Much more than the required amount of iron would be allowed to enter the body, and as there is no effective way of restoring balance by excess excretion, the excess iron is placed in storage in various organs. Over the years the iron stores build up until gradually the excess iron, it is thought, damages the organs that store it, the normal storage organs such as the liver and spleen as well as the non-storage organs such as the heart, pancreas, adrenals and anterior pituitary(206).

C. Iron Overload and the liver

There are a number of studies in the literature pointing to the effects of iron overload in the liver. Though it is not possible to cover the minor details in this brief review, I shall try and focus on some of the interesting conclusions drawn from these studies.

As discussed in an earlier section, it is felt that a number of other factors apart from iron overload cause hemochromatosis. Using rats fed a lipotrope deficient diet, which is choline and folic acid free, plus 6% ammonium citrate for two to twenty weeks, fatty liver, cirrhosis and excess body iron occurred(211). The iron was located not only in the normal storage organs of the liver, spleen and bone marrow but in parenchymal cells of nonstorage organs such as the pancreas and heart, similar to human hemochromatosis. On the basis fo these results the authors suggest that a block in the reticuloendothelial system, during rapid

accumulation of excess iron, could lead to the parenchymal accumulation of iron. In rhesus monkeys the effects of iron overload were studied with carbon tetrachloride, ethanol, or ethanol with dietary protein deficiency(212). Marked siderosis developed in several organs, with large deposits in the reticuloendothelial cells as compared with parenchymal cells. In the liver, parenchymal overload was least in animals given iron alone and was most marked in those given ethanol, whereas Kupfer cell iron was greatest in the former group and least in animals given iron and carbon tetrachloride. This can be explained on the basis that in some of the conditions employed for the above study, a fatty change may cause a blockade of reticuloendothelial cells leading to parenchymal distribution of iron in the absence of cirrhosis.

Electron microscopic studies of livers from adult female rabbits injected intravenously with ferrous sulphate (50 to 80 mg per kg body weight) revealed mitochondrial damage in parenchymal liver cells within two hours following injection(213). By eight hours, hepatic necrosis was evident by light microscopy and hepatic damage was at or near its maximum indicating that under these conditions iron is toxic for hepatocellular mitochondria. The doses used in this experiment are near the $LD_{50}$ for rats and in rabbits as well this is not a physiological situation. Under normal conditions ferritin accounts for more than half of the iron in storage. As already mentioned, when iron loading is increased the balance alters, hemosiderin increasing relative

to ferritin, and in extreme cases there are massive deposits of hemosiderin associated with tissue damage(110,119-121). As already mentioned, the bulk of the ferritin is present in the hepatocytes while hemosiderin is present in the Kupfer cells of the liver(118). Some evidence suggests that in idiopathic hemochromatosis, the deposition of hemosiderin is closely related to the intracellular conversion of ferritin (214). This conclusion was based on electron microscopic studies on hemosiderin granules isolated from the liver in two cases of idiopathic hemochromatosis where they found these granules contained 10 to 30 percent protein and up to 45 percent iron, almost insoluble in water and sparingly soluble in alkali. Much of the iron present was in the characteristic form of ferritin iron micelles.

## V. Iron and the Heart

### A. Distribution

Most of the iron present in the heart is heme iron. In the case of the rat, if one considers the total iron to be about 5.3 mg using Linder et al's(132) estimate, about 1% of the total iron in the body is present in the heart. Heart and skeletal muscle contain two ferritin species. Due to the high content of myoglobin in these muscles, the possibility of a relationship between it and the ferritins arises.

Linder and Munro(3) have compared the concentrations of slow and fast ferritin and myoglobin of rat heart, diaphragm

and thigh muscles. Their data show that cardiac muscle, diaphragm and mixed thigh muscles all contain two ferritins, and that on the basis of the iron content, these are present in about equal proportions in female rats. Though the concentration of fast ferritin iron in the three muscles seems to correlate closely with the myoglobin content, the myoglobin assay used in this experiment is in question. It is the method normally used but it gives a positive reaction with kidney (about 1 mg myoglobin per gm kidney). Though more detailed studies are needed to elucidate this relationship, the presence of an electrophoretically faster migrating ferritin species in all three muscles suggests that this ferritin may be a specific iron donor for myoglobin.

B. The heart and iron overload.

One of the characteristics of hemochromatosis is the deposition of iron in noniron storage organs such as the heart(191). It has been noted that iron deposits in the interstitial as well as the muscle cells(198) and iron deposition in the heart does not occur unless there has been deposition in the liver and other organs. This may be explained by the fact that deposition of iron occurs only when the other organs have been saturated and can accept no more free iron.

Grossly visible cardiac iron deposits, including those seen in cases of secondary iron overload, are always associated with cardiad dysfunction(196). Buja and Roberts (215) in a thorough, anatomical study of the problem claim that cardiac dysfunction depends upon the quantity of iron

deposited in individual muscle fibres and the number of fibres affected. Iron deposits are usually limited to the perinuclear areas of myocardial fibres and are present in few fibres in patients with little or no evidence of cardiac dysfunction, whereas iron deposits occupy large areas of individual myocardial fibres and are present in many fibres of patients with significant cardiac dysfunction.

Since in many cases, the pathologic changes do not appear to account adequately for the development of heart failure, it has been suggested that the latter is due to intracellular biochemical disturbances caused by hemosiderin deposits with resulting impairment in enzyme systems required for the glycolytic cycle in metabolism. Reports also exist for xanthine oxidase deficiency in patients with hemochromatosis (216). The interrelationship between the biochemical and pathologic conditions of this disease still remain to be explained.

## III. Materials and Methods

### 1. Animals and treatment

#### i) Rats and injections

Rats used throughout were males and females of the Fischer strain, purchased from Charles River Laboratories, Wilmington, Mass. and were maintained on regular chow and tap water unless otherwise stated. For purification of slow and fast heart ferritins, batches of forty to fifty animals were preinjected with Imferon (Lakeside Laboratories, Milwaukee) (25 mg iron/rat) twice at 2 day intervals over 4( days. In studies involving ferritin synthesis, 400 ug of iron/100g body weight (as ferric ammonium citrate neutralized) was injected intraperitoneally five hours before death since Drysdale and Munro(172) have shown that maximum ferritin synthesis in liver occurs at this time. A pulse dose of $^{3}$H-leucine [L-leucine, 4,5,-$^{3}$H(N)] (100 µcuries per 100 gm body weight) was administered intravenously via the tail vein 2 hours prior to death. In case of the double labelling experiment to measure relative turnover rates, $^{14}$C-U-leucine (20µCuries per 100 gram body weight) was also administered 3 days before $^{3}$H-leucine. Animals in all studies reported here were kelled either with ether or pentobarbitol (5mg per 100 gram body weight).

#### ii) Chickens and injections

Adult female hens (White, Leghorn) were obtained from Sparse Farms, Connecticut and housed in individual cages.

They were maintained on regular laboratory chow and tap water. Iron was injected intramuscularly into the breast as imferon (40 mg Fe per chicken) twice over five days.

iii) Induction of cardiac hypertrophy in rats

Cardiac hypertrophy was induced in rats by three methods. The first is that of Mallov and Alousi(28) involving constriction of the abdominal aorta as follows: Each animal was anesthetized either with pentobarbital (5 mg per 100 gram body weight) i.p. or ether, and a small section of the abdominal aorta was exposed. A stainless steel wire was placed alongside the section of the aorta, and the two are tied together by means of a ligature made with size 00 surgical silk so that circulation was completely cut off. The wire was then slipped out of the loop with the aid of a pair of curved forceps placed against the knot of the thread, permitting the return of circulation through a narrowed lumen. The authors recommend 0.89 mm diameter wire for 250-300 g. rats and 1.01 mm diameter for rats over 300 grams. I used 0.79 mm diameter wire for 150-200 g rats and 0.71 mm diameter wire for 110-120 g rats. Control animals were sham-operated by exposing the abdominal aorta. Mallov and Alousi reported heart weights were 13.4 and 21.7 % greater in experimental animals than in controls 11 and 22 days post-operatively. This method though did yield significant increases in heart weight to body weight ratio, was not easily reproducible (Table I) and I was unable to produce hypertrophy in young rats. [Cardiac hypertrophy can also be induced by constriction

TABLE I

METHODS OF INDUCTION OF CARDIAC HYPERTROPHY IN RATS

| Age, Sex, of animals at death and method of induction | Time allowed for onset of hypertrophy | Ratio of heart wt/body wt (HW/BW) Controls (mean±S.D.) | Experimental (mean ± S.D.) | % ↑ in HW/BW over controls |
|---|---|---|---|---|
| | | ($x10^{-3}$) | ($x10^{-3}$) | |
| Female Fischer Adult Rats (160 gm BW) Abdominal aortic constriction* | 14 days | 2.98,2.74 (2)** | 3.06,3.3 (2) | 12 |
| | 18 days | 2.84,3.2 (2) | 3.4 , 2.8 (2) | 2 |
| | 25 days | 2.81±0.12 (4) | 3.13±0.15 (5) | 9 |
| | 25 days | 2.93±0.11 (11) | 3.03±0.01 (11) | 3 |
| | 25 days | 3.2 , 2.9 (2) | 3.5 , 4.4 (2) | 29 |
| 100 gm BW | 21 days | 3.53±0.23 (5) | 3.35±0.35 (5) | - |
| | 21 days | 2.90±0.22 (10) | 2.92±0.11 (10) | - |
| Female Fischer Adult Rats (160 gm BW) Isoproterenol*** administration 2 ug/100g BW subcutaneously 1 x day | 14 days | 3.42±0.15 (3) | 3.5 ±0 (3) | 3 |
| | 21 days | 3.03±0.28 (3) | 3.5 ±0.14 (3) | 11 |

TABLE I (cont.)

| Age, Sex, of animals at death and method of induction | Time allowed for onset of hypertrophy | Ratio of heart wt/body wt (HW/BW) Controls (mean±S.D.) | Experimental (mean ± S.D.) | %↑in HW/BW over controls |
|---|---|---|---|---|
| Female Fischer Adult Rats (160 g BW) | 21 days | 3.17±0.17(3) | 3.97±0.46(5) | 25 |
| Isoproterenol administration 10 ug/100g BW 1 x day | 21 days | 3.01±0.22(6) | 3.58±0.12(9) | 16 |
| Weanling female Fischer rats (100 g BW) Copper deficient° diet fed for 21 days | 21 days | 3.81±0.29(18) | 4.45±0.36(21) | 17 |
| | | 3.96±0.37(20) | 4.41±0.29(20) | 11 |

° Dallman, P.R. (1967) J. Clin. Invest. 46, 1819-1827.

* Mallov, S., Alousi, A.A. (1966). Am. J. Physiol. 212, 1158-1164.

** The numbers in parentheses indicate the number of animals used

***Alderman, E.H., Harrison, D.C. (1971). Proc. Soc. Exp. Biol. Med. 136, 268-270.

of the ascending aorta near the fourth intercostal space(27). However, as the risk of death in rats due to pulmonary edema is rather high, I did not try this technique].

The second method, I used for inducing cardiac hypertrophy was copper deficiency. Weanling rats were fed a copper deficient diet (Nutritional Biochemicals) and distilled water for 21 days following which they were sacrificed while controls were fed regular chow and tap water. In all cases I obtained significant increases in heart weight to body weight ratios above controls. This proved to be a more reliable and less difficult technique than the abdominal aortic constriction.

The third method used to induce cardiac hypertrophy involved administration of 0.02 mg/kg isoproterenol once daily subcutaneously to rats for 13 to 31 days (32). According to these authors, this dose of isoproterenol produced a 14% heart dry weight gain over controls when the rats are treated for 13 days, and a 26% gain when the rats are treated for 31 days. At such a dose, other histological effects of isoproterenol are not visible.

2. Purification of tissue ferritins

Linder and Munro(216) have developed a generally applicable method of determining ferritin iron and protein concentrations in a wide range of tissues by combining partial chemical purification of ferritin with subsequent antibody precipitation. The tissue is homogenized in 4 vol. water plus 5 vol. 0.03N NaCl so that the final solution maintains a concentration of 0.03 N salt with the salts contributed by the tissue(216). The heat supernatant is titrated with

antiserum to precipitate ferritin iron and the amount of this iron estimated by Drysdale and Munro's(217) modification of Hill's(218) method per gram of liver is calculated (total ferritin iron per gram). Ferritin is then purified by three steps, namely pH5 treatment, ammonium sulphate precipitation, and filtration on Sephadex G-200. None of these steps discriminate between iron-containing holoferritin and iron-free apoferritin. The amount of iron in the Sephadex filterate is estimated(217) and for the estimation of ferritin protein, apoferritin is prepared.

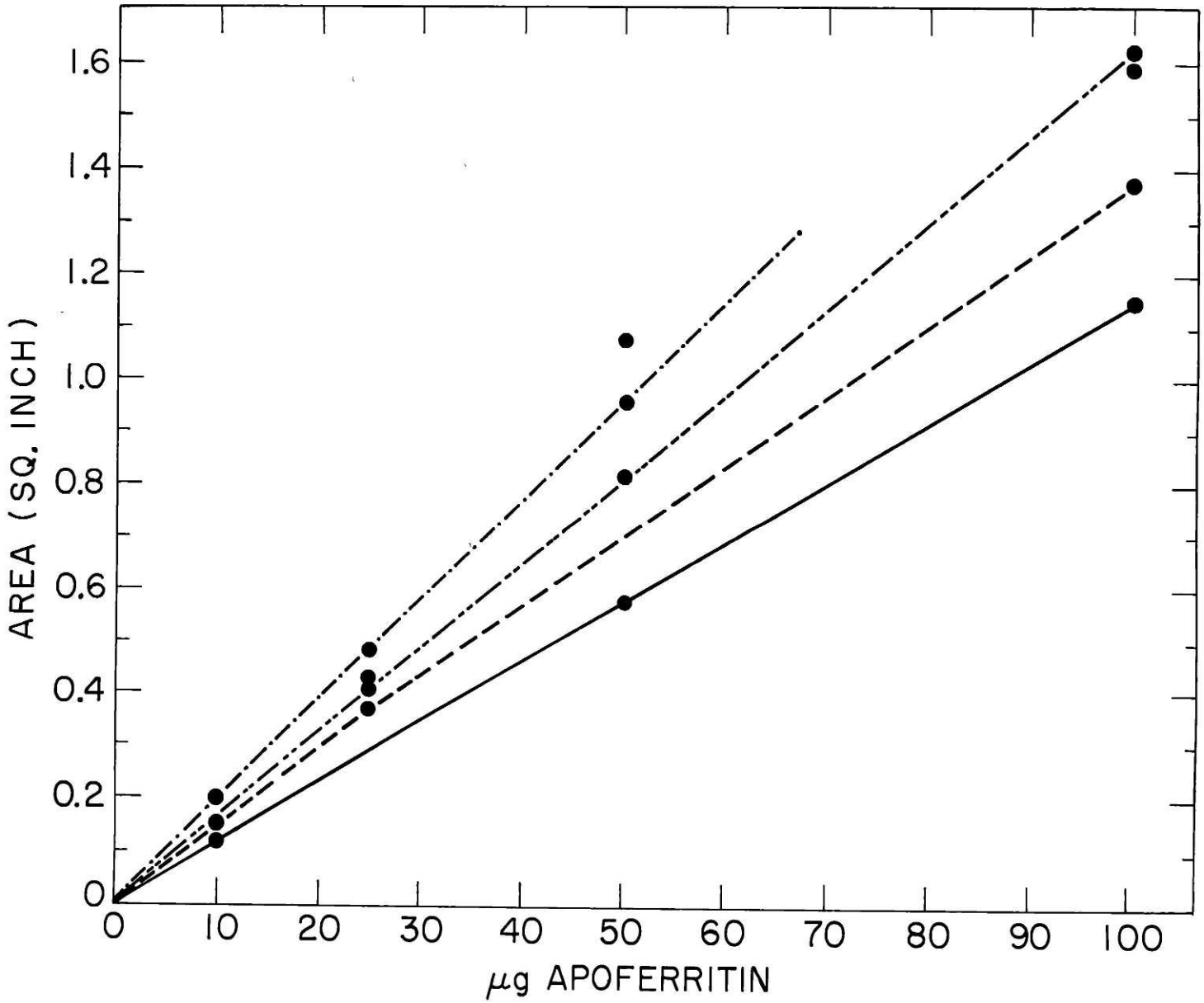
The iron is removed from the ferritin samples by dialysis for 6 hr against 1% thioglycollic acid in 0.1M acetate buffer pH5.4 [Crichton, R.R. unpublished]. This is followed by more extensive dialysis against 0.02M acetate buffer(pH5.4), and avoids denaturation and deposition of elemental sulphur which occurs when a combination of dithionate and bipyridyl is used (219).

The apoferritin mixture is then run on discontinuous polyacrylamide gels by the method of Ornstein-Davies(220). After staining with Amido Black and destaining in 7% acetic acid, the concentration of protein in each ferritin in the gels is determined by scanning at 280 mu. External rat liver apoferritin standards are run with each determination and there is a positive correlation between the concentration of protein and the area occupied under the peak it represents(Fig A).

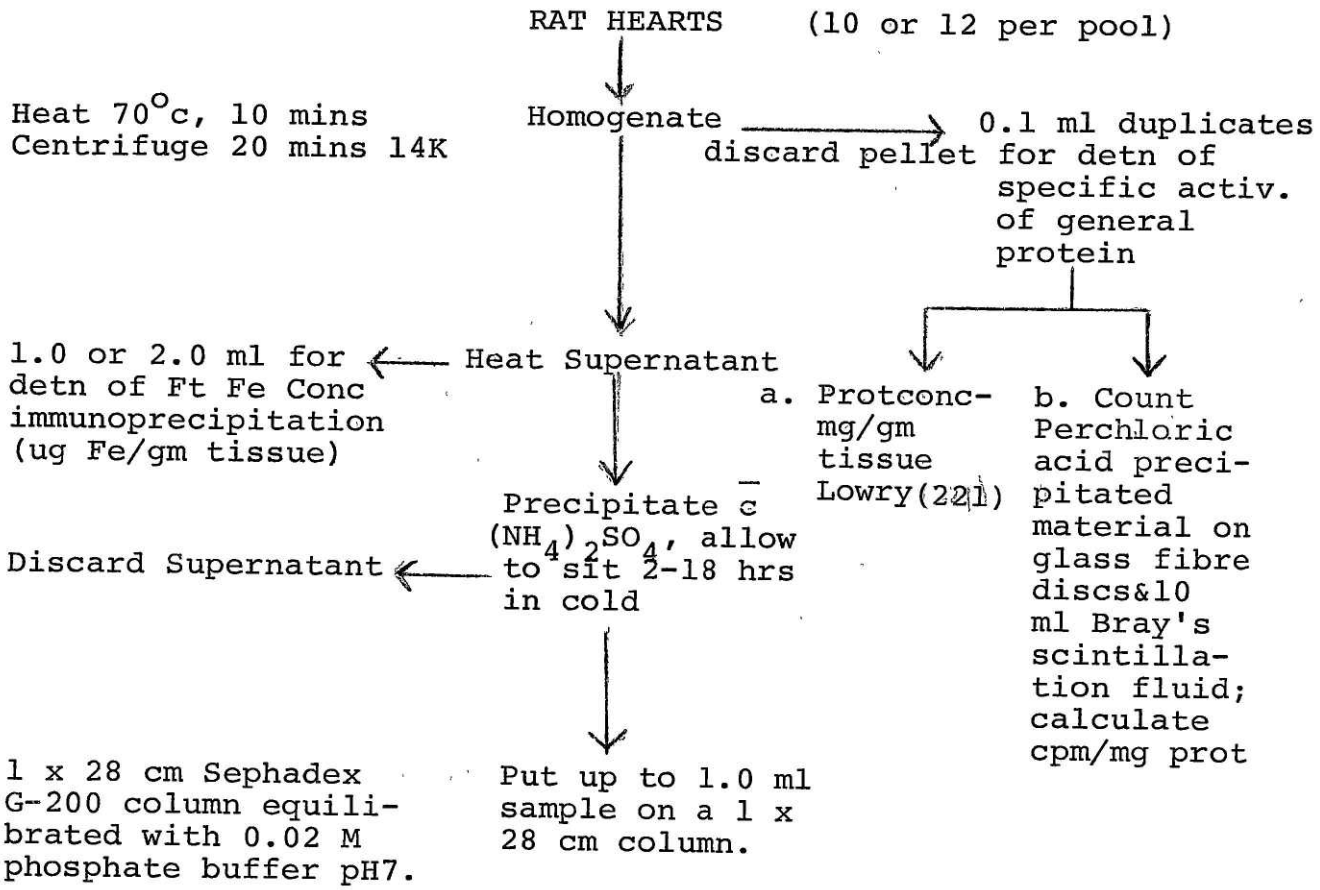
3. Measurement of Ferritin protein synthesis

In order to study the amount of labelled amino acid

Fig. A. Plot showing correlation between concentration of rat liver apoferritin and area it occupies under the peak it represents at OD 280 mu.


AREA (SQ. INCH)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
80
90
100
μg APOFERRITIN

incorporated over a 2 hour period, the procedure stated above was modified slightly as depicted




Continued on next page

6 ml Sephadex Filtrate

↓

Perform the following experiments with Seph Fraction in the order (1,2,3)

0.5 ml

1 Seph Fract.

Fe detn
µg Fe/ml SF

2 5.0 ml Seph Fract.

Thioglycollate treatment with dialysis

(Crichton, unpublished)

↓

Apoferritin

↓

0.5 ml Seph Frac

3 FE detn on individual bands

Concentrate by ultrafiltration apparatus using amicon so that 40-50 µg prot/gel

↓

(7% polyacrylamide gels with DATDA as cross linker)

( Anker)

Disc electrophoresis-Scan at 280 mµ. determination of ug protein. Stain with amido black and destain(7% acetic acid)

↓

Scan at 280 mµ or 540 mµ in comparison with standards (determine ug protein fast and slow ferritins)

← Gels Bands

↓

Dissolve in 2% periodic acid

↓

Scintillation Fluid-Triton X-114/xylene

(223)

cpm/µg Prot.

The 7% polyacrylamide gels were made using N,N[1] diallyltartadiamide (DATADA) as the cross linking agent instead of N,N[1] methylene bis acrylamide on a mole per mole basis as described (222). I ran several gels with different concentrations of acrylamide and DATDA and this concentration was chosen as the bands were most distinct and not at all diffuse. These gels were dissolved in 2% periodic acid (0.2 cm gel/0.5 ml periodic acid) at 37° C for an hour. The gels dissolved were then placed in a scintillation fluid consisting of Triton X-114 in xylene with PPO and POPOP as has been described (223). I had tried several different conditions of dissolving the gels with periodic acid and I found that at 37° for 1 hour, the dissolution was very good. Also, I determined the efficiencies of counting $^{3}H$ with the following scintillation fluids in the presence of the dissolved gel.

| Scintillation Fluid | Efficiency |
|---|---|
| Bray's Fluid | 6% |
| Triton X-114/xylene | 19% |
| Toluene/PPO | Two layers formed and thus discarded |

From the counts and the amount of ferritin present, the specific activity for each band was calculated. All results were corrected to efficiencies and were expressed as dpms.

4. Relative Turnover of Ferritin

Turnover of slow and fast ferritin was measured by the double isotope labelling technique of Schimke(224). Two groups of rats were either injected with 0.9% NaCl solution (controls) or iron dextran (25 mg iron per rat, as Imferon) daily for 4 days. Four hours after the first injection, they were given L-[U-$^{14}$C] leucine (20 µCi per 100 g body weight) by tail vein and, four hours after the last injection, L-[4,5-$^{3}$H] leucine (75 µCi per 100 g body weight) by the same route and killed 2 hours later. Heart ferritin was extracted by the methods described earlier from pooled hearts of each group. Slow and fast ferritin were separated on 7% polyacrylamide gels made with DATDA (as described earlier). Gels were stained with Amido Black and scanned to determine the relative proportions of slow and fast ferritin. The bands were cut out and dissolved in 2% periodic acid (as explained earlier). Radioactivity was counted with an Isocap-300 scintillation counter (Nuclear Chicago) by two programs each containing two channels. External standards were used(0.1 µCi $^{3}$H-leucine and 0.5µCi $^{14}$C-leucine) to determine the disintegrations per minute.

5. Preparation of 'slow' and 'fast' cardiac ferritin

A procedure for the separation of the two heart ferritins has been devised(Moor , J.R., Linder, M.C. unpublished). This technique involves first preparing pure total heart ferritin containing both species by the above isolation procedure. This is applied to disc electrophoretic gels containing 4.5%

acrylamide in the "separating gel." The bands separate well on electrophoresis and are excised and implanted separately in second gels encased in dialysis tubing. When reverse electrophoresis is applied, the ferritin band migrates into the liquid enclosed within the dialysis sac and can thus be recovered. Re-electrophoresis of the harvested ferritins shows only one species.

6. Quantitative Polyacrylamide Gel Electrophoresis

Holoferritin samples of different tissues obtained from adult Fischer female rats were isolated as described above. Samples of iron-containing holoferritins and the iron-free apoferritins were run in polyacrylamide gel electrophoresis at different gel concentrations. Standard proteins were run under identical conditions and included aldolase (MW=120,000), acetylcholinesterase (MW=240,000), horse spleen ferritin (MW=450,000) and thyroglobulin (MW=670,000) obtained from Worthington Biochemicals (USA), Nutritional Biochemicals (USA) and Sigma Biochemicals (USA).

These experiments were performed essentially by the Ornstein-Davis technique [Chrambach System 4192] (220) but using a range of acrylamide gel concentrations (%T) and 5% crosslinker a constant; %C is the ratio of bis acrylamide to the total gel concentration. The sample gel was omitted and the ferritin samples were applied on top of the stacking gel in sucrose. The stacking gel was 1.5 cm. long and separating gel 12.0 cm. The current applied was 2 mA per gel for 8 hours. Gels were polymerized taking into consideration some of the

experimental suggestions of Rodbard and Chrambach(225) namely (a) that the reagents were carefully purified; (b) that optimal polymerization conditions and catalyst concentrations were selected (c) that the pH of the operative resolving buffer was 9.5, and the ionic strength 0.015; (d) that the temperature was kept constant during polymerization and electrophoresis at 25° C. All $R_f$ measurements were made with respect to the tracking dye, Bromophenol Blue. All gels were stained for protein with Amido Black in 7% acetic acid.

7. Electrophoresis in minimally sieving gels

In order to minimize retardation of migration by sieving in gels of small pore size, the percentage of cross linking agent was increased (%C=20) while the concentration of acrylamide was maintained (%T=5) (225-227). This produces gels with large pores. The length of the separating gel was reduced to 6 cm while that of the stacking gel was kept at 1.5 cm. The experimental suggestions of Rodbard and Chrambach (225) as discussed in the earlier section were followed in this case as well. A current of 2mA per gel was applied for 3.6 to 4 hours. Duplicate gels were stained for iron with Prussian Blue and for protein with Amido Black in 7% acetic acid.

8. SDS electrophoresis in 10-15% gradient slab gels

Ferritin subunits were isolated and displayed by SDS polyacrylamide gel electrophoresis following immunoprecipitation or acetone precipitation of the protein and dissociation with

SDS(174). For immunoprecipitation, the ferritin samples were precipitated overnight with ferritin antiserum raised in rabbits against horse spleen ferritin. The immunoprecipitate was washed twice with a solution containing 1% Triton X-100, 1% deoxycholate, 10mM nonradioactive leucine, 10mM $Na_2HPO_4$, 150 mM NaCl, and then centrifuged through a discontinuous gradient (0.5 M:1.2M sucrose) containing the above detergent solution. The tubes were cut off, and the immunoprecipitates were dissolved in 25 ul of a solution containing 0.062M Tris-HCl(pH6.8), 2% SDS, 10% glycerol(v/v) and 5% mercaptoethanol. Alternatively, the acetone precipitates were prepared by treating the samples with equal volumes of acetone in the presence of detergent. The precipitates were allowed to sit overnight and washed with the detergent solution twice as described for immunoprecipitates and centrifuged in a similar fashion through a discontinuous gradient.

Acrylamide solutions used to make up the gel are as described by Maizel(228) for 10% and 15% gradient gel. A stacking gel was omitted as a 10-15% gradient was used. The dimensions of the slab used was 15 cm x 16 cm and the thickness was 0.1 cm. A constant voltage of 180 volts was applied for about $3\frac{1}{2}$ hours. Gels were stained with Coomassie Blue as described by Maizel(228) and destained in methanol/acetic acid in water.

9. Iso-electric focusing in polyacrylamide gels

Isofocusing gels were prepared using 4.15%T, 3.61%C

and 2% ampholine, either pH(4 — 6) or pH(3 — 10) with riboflavin as a polymerization catalyst at room temperature, 25°C. They were mixed in the following proportions(229).

| | |
|---|---|
| Acrylamide solution | 1 part |
| Ampholyte solution | 0.10 part |
| Riboflavin solution | 0.13 part |

Ferritin samples were mixed with ampholyte and 50% sucrose solutions in the following preportions

| | |
|---|---|
| Ampholyte solution | 1 part |
| Sample solution | 4 parts |
| Stock 50% Sucrose Solution | 1 part |

A total volume of 30 ul of sample was applied to the top of the column. Rat liver ferritin was dialyzed against water before the run to investigate if there were any ions present which could interfere with the isofocusing procedure.

The electrolytes used were 0.02M sodium hydroxide and 0.01M phosphoric acid. The apparatus used was an LKB. The gels were focused for about 18 hours at room temperature. The starting voltage was 100 volts which was maintained throughout the run. The steady drop in current throughout the run was an indication that the proteins were being focused. To avoid heating of the gels, water was run through the system during the entire period. Staining and destaining was by Vesterberg's(231) method. For each sample, at least four gels were run.

After staining and destaining, the bands were cut and the iso-electric pH's measured. The number of bands in each case was recorded.

# IV. Results

## A. Structural Studies

### 1. Size and charge of the rat heart and other tissue ferritins.

In earlier studies on polyacrylamide gels (3), differences in the electrophoretic migration of ferritin species were reported for adult rat liver, kidney and heart ferritins, the latter tissue showing two major ferritin bands.

Linder et al (3) classified the different rat tissue ferritins into three categories on the basis of their electrophoretic mobilities, one characteristic of adult liver but also in heart and muscle, one common to kidney, spleen and some hepatomas, and a third found only in muscle and heart. Although these studies provided evidence of the heterogeneity of the ferritins of rat tissues, they did not indicate whether the electrophoretic differences were based only on charge or whether size differences were also involved. In order to resolve the contributions of size and charge to the electrophoretic migration in polyacrylamide gels, samples of several purified rat tissue ferritins and horse spleen ferritin were analyzed by quantitative polyacrylamide gel electrophoresis (22). Standard proteins were run under identical conditions.

From the migration characteristics of these ferritins at several known gel concentrations, Ferguson plots (log relative mobility vs. gel concentration) (22) were constructed, and the molecular sizes and charges (apparent valences)

together with their statistical variability were obtained by applying the Rodbard computer programs (25) to the data. Ellipses were drawn describing the 95 per cent confidence limits of these data for size and charge and were used to identify those ferritins that differed in size and/or charge.

Before discussing the data and the results obtained, a few comments about the monomer-dimer relationship of ferritin are relevant. Ferritin, as has been discussed earlier, aggregates into dimers, trimers and oligomers. Experimental evidence supporting this comes from a number of studies on horse spleen ferritin (see Lit. Survey). I shall refer to the aggregate which directly follows the monomer ferritin band in electrophoresis (designated a dimer based on horse spleen ferritin) as the minor component, and monomer as the major component in this thesis. Higher oligomers were not seen in our gel runs.

Horse spleen ferritin has been studied extensively for its structural properties (see Literature Survey). A typical Ferguson plot for horse spleen ferritin is shown in Fig. 1.

The mobilities ($R_f$) relative to tracking dye (see Methods) were plotted against the gel concentration (%T) which, at constant cross-linkage (%C), determines the relative pore size of the gel and therefore the degree of molecular sieving (25) As may be seen, a very good linear correlation exists between log $R_f$ and %T for both the major (monomer) and minor components (dimer) of purified horse spleen ferritin ($r=0.998$). The slopes of the lines (or retardation coefficients, $K_R$) provide a measure of molecular size, while the y-intercepts

Fig. 1 A Ferguson Plot (log $R_f$ versus %T) for major and minor components of horse spleen holoferritin

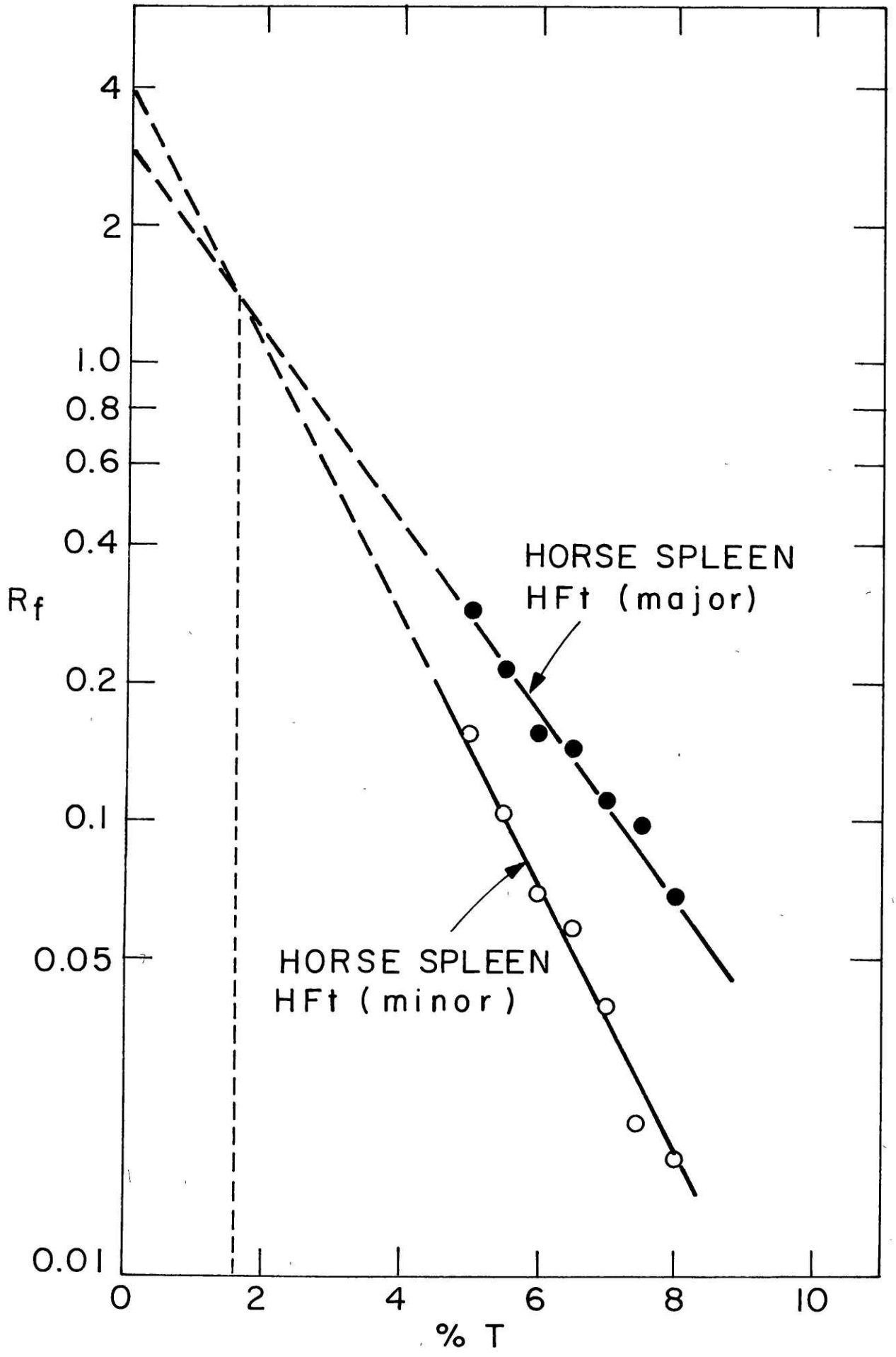
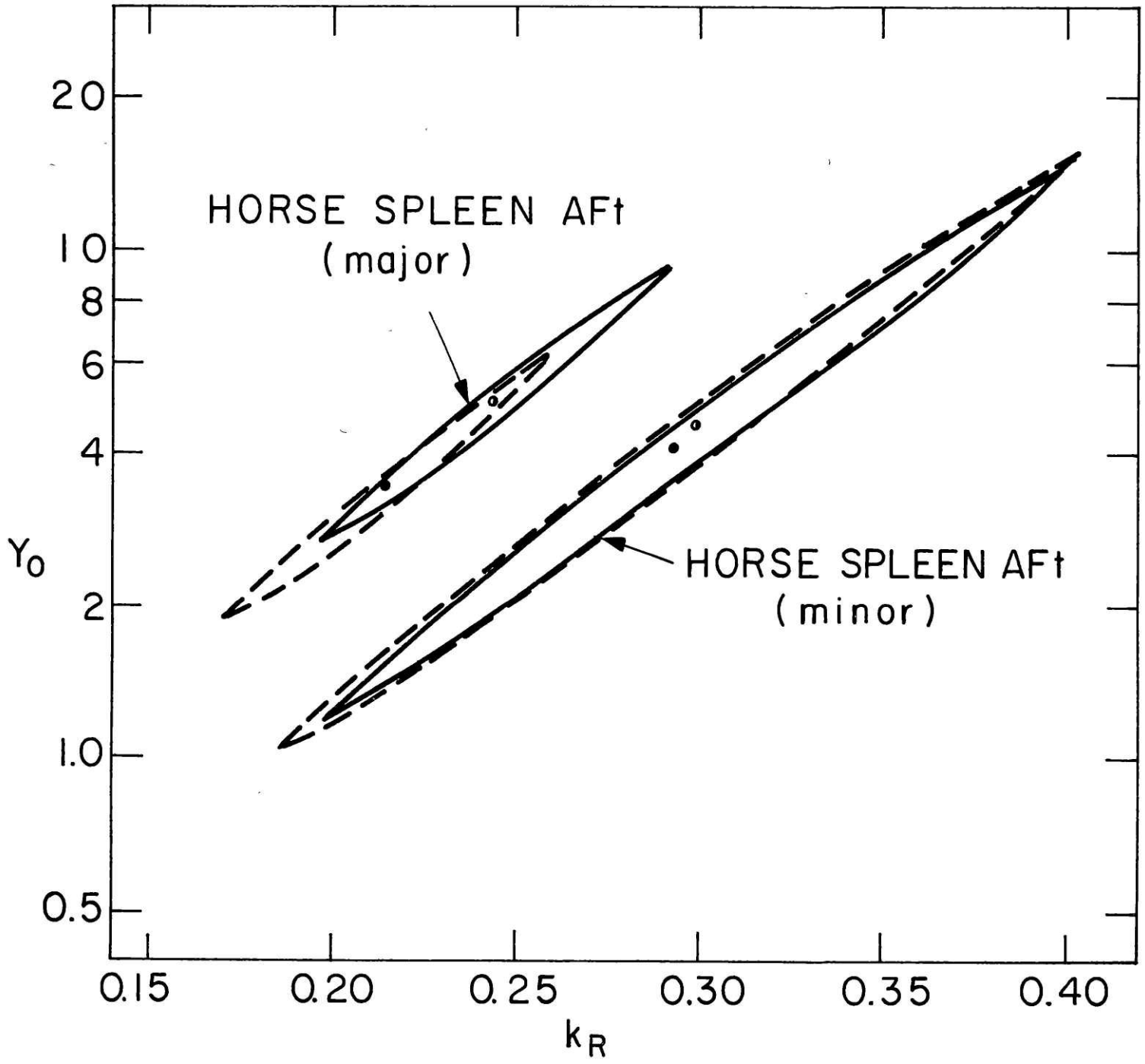

4
2
1.0
0.8
0.6
0.4
0.2
0.1
0.05
0.01
$R_f$
HORSE SPLEEN
HFt (major)
HORSE SPLEEN
HFt (minor)
0
2
4
6
8
10
% T

Fig. 2 Plot of 95% confidence limit ellipses for duplicate runs horse spleen apoferritin showing overlap of the two replications. The centers of the ellipses are indicated by symbols(● ◐ )


20
10
8
6
4
2
1.0
0.5
$Y_0$
HORSE SPLEEN AFt
(major)
HORSE SPLEEN AFt
(minor)
0.15
0.20
0.25
0.30
0.35
0.40
$k_R$

(relative free mobility $Y_0$) are related to the charge of the molecule. The data show that the major and minor components of horse spleen ferritin produce lines of different slopes ($K_R$) and thus the two components differ in size. However, the y-intercepts ($Y_0$) extrapolated from the experimental points show that the minor component has a similar relative free mobility to the major component, suggesting a similarity of charge. For 95% confidence ellipses (determined by the variability of $K_R$ and $Y_0$) the center of the ellipse is specified, and the extent of heterogeneity of size plus charge is indicated by overlap of the two ellipse outlines, and notably overlap of one outline with the center of the other ellipse. On the other hand, it is clear that the two components of horse spleen ferritin do not overlap (Fig. 2). To illustrate the reproducibility of the procedure, the data obtained in two runs of the same sample of horse spleen ferritin are shown (Fig. 2). The figure shows no significant difference of ellipses from the two runs either by the criteria of overlap of outlines, or overlap of center of one with the outline of the other for the two replications or by comparing the slope and intercept values in both cases statistically.

Earlier studies at a single gel concentration of polyacrylamide ( 152 ) had revealed no differences in migration patterns between iron-containing holo- and iron-free apoferritin, even though the molecular weights with and without iron differ by as much as 40 percent. This occurs because the iron is sequestered within the protein shell and does not

contribute to size or charge. In order to confirm that charge as well as size is unaffected by the iron core, it was considered desirable to determine by the more discriminating quantitative technique used above whether the migration characteristics would still be unaffected by the absence of iron in the ferritin core. As shown for ferritin from adult rat liver, there was either partial or complete overlap of the ellipses for apo- and holo-ferritins (Fig. 3). This was also true for the other ferritins applied to these analyses (not illustrated). Thus the behavior of ferritins in this system can be taken as representating that of the protein shell only. The procedure described was applied to rat holoferritins obtained from normal and malignant tissues. Fig. 4 demonstrates the ellipses obtained for the major components of ferritins of adult rat liver, kidney, and liver tumor (Morris hepatoma 3683F) compared with the ellipse for horse spleen holoferritin. None of the profiles overlap, thereby indicating non-identity of these ferritins in terms of the Ferguson plot (i.e., size and/or charge). This is in agreement with studies of amino acid and peptide composition (153)(see Literature Survey). The rat ferritins are grouped closely together in migration characteristics, and as a group they differ extensively from the ellipse for horse spleen ferritin. Within the rat group, the ferritin of liver tumor is nevertheless quite distinct from that of normal adult liver and also from that of kidney. This is displayed in more detail in Fig. 4A, in which the minor and major components of hepatoma ferritin are shown to differ from those of adult rat liver ferritin.

Fig. 3 Comparison of ellipses of apo(- -) and holo(-) ferritins prepared from rat liver (major component only). Centers of ellipses are shown by symbols( ● ○ ).

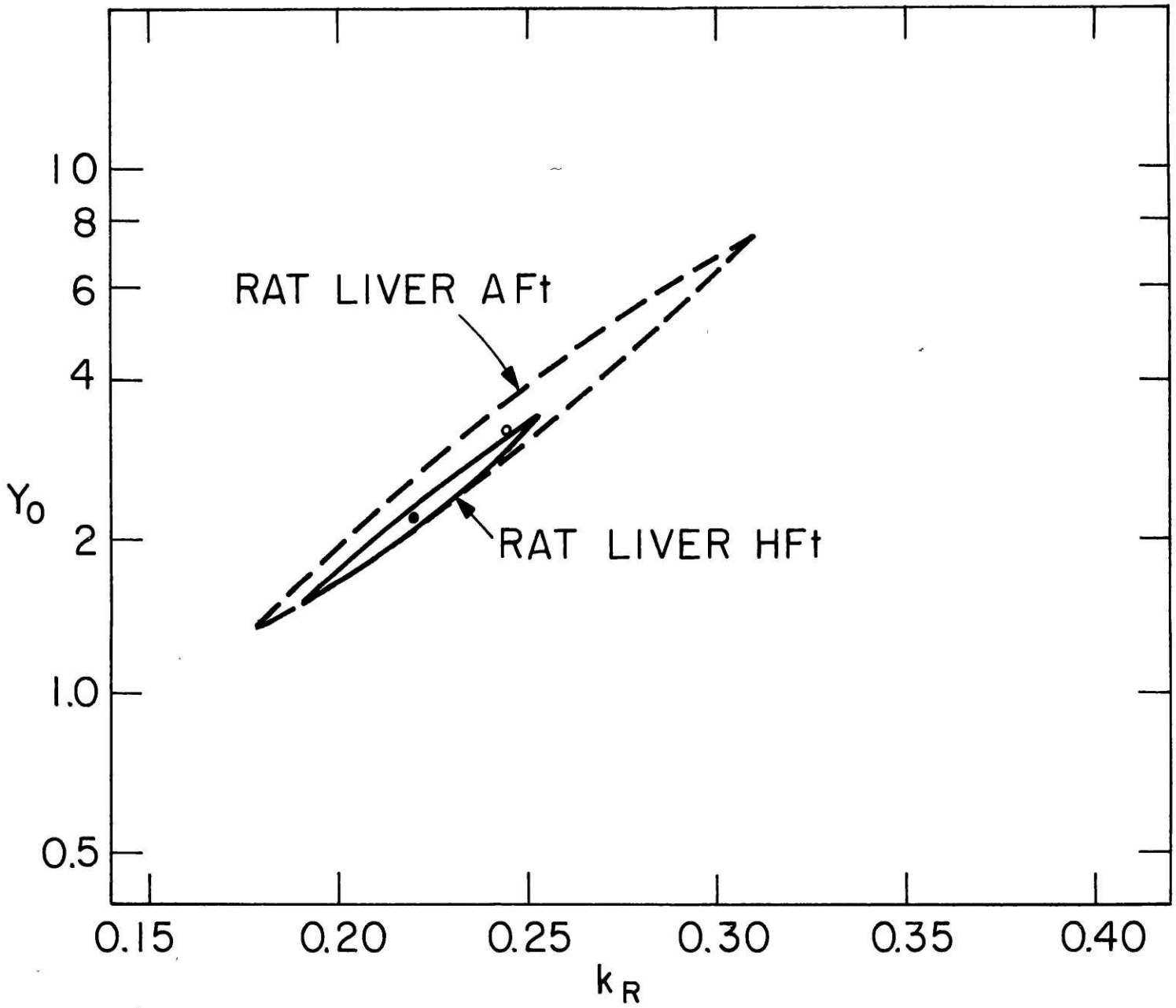

10
8
6
4
2
1.0
0.5
$Y_0$
RAT LIVER AFt
RAT LIVER HFt
0.15
0.20
0.25
0.30
0.35
0.40
$k_R$

Fig. 4 Plot of 95% confidence limit ellipses of the major conponents of holoferritins isolated from rat liver, hepatoma, kidney and from horse spleen. The centers of the ellipses are indicated by symbols(○ ◐ ● ▲).

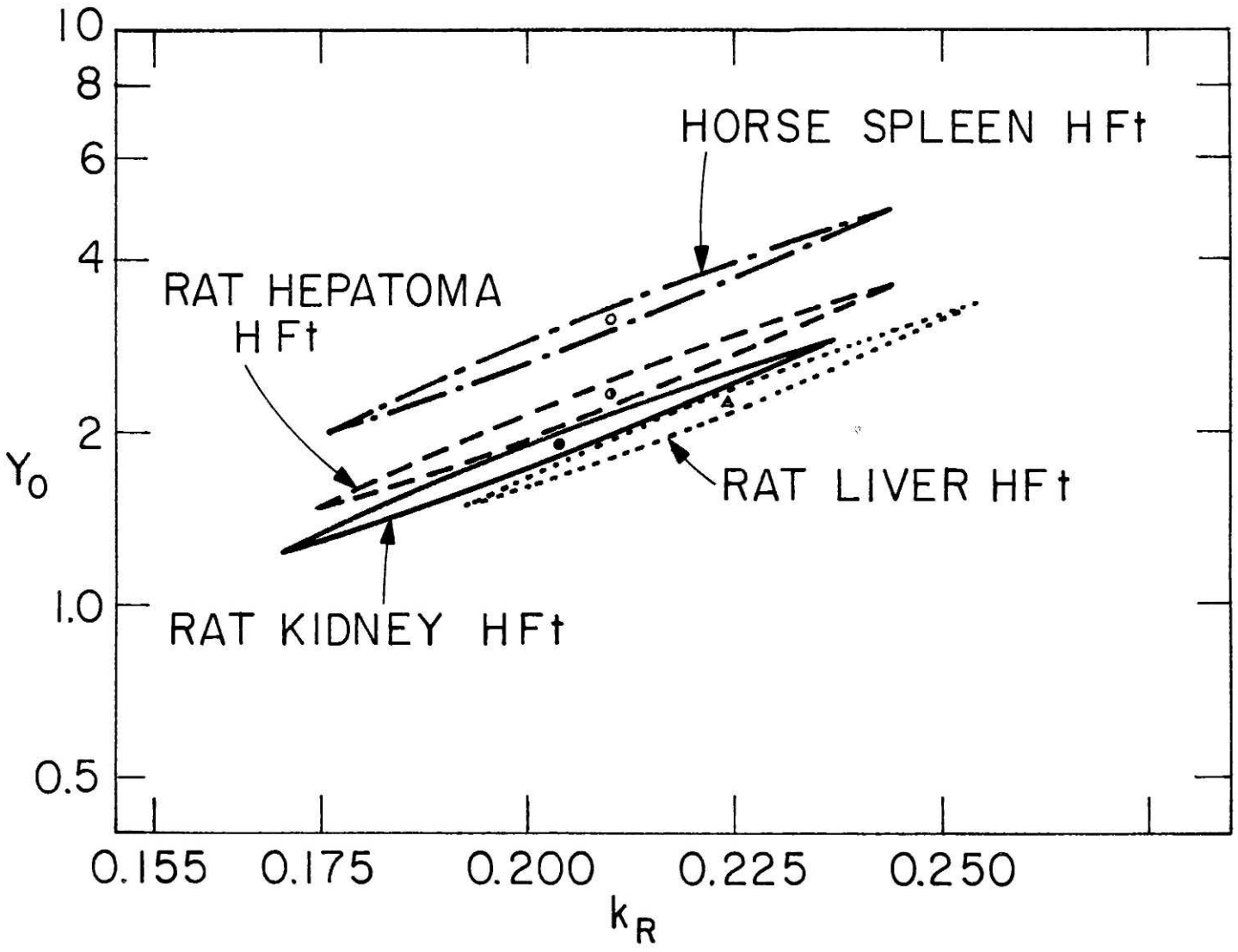

10
8
6
4
2
1.0
0.5
$Y_0$
HORSE SPLEEN HFt
RAT HEPATOMA HFt
RAT LIVER HFt
RAT KIDNEY HFt
0.155
0.175
0.200
0.225
0.250
$k_R$

Fig. 4a Plot of 95% confidence limit ellipses of holo-ferritins of rat liver and hepatoma 3683F showing major and minor components. The centers of the ellipses are indicated by the symbols(● ○).

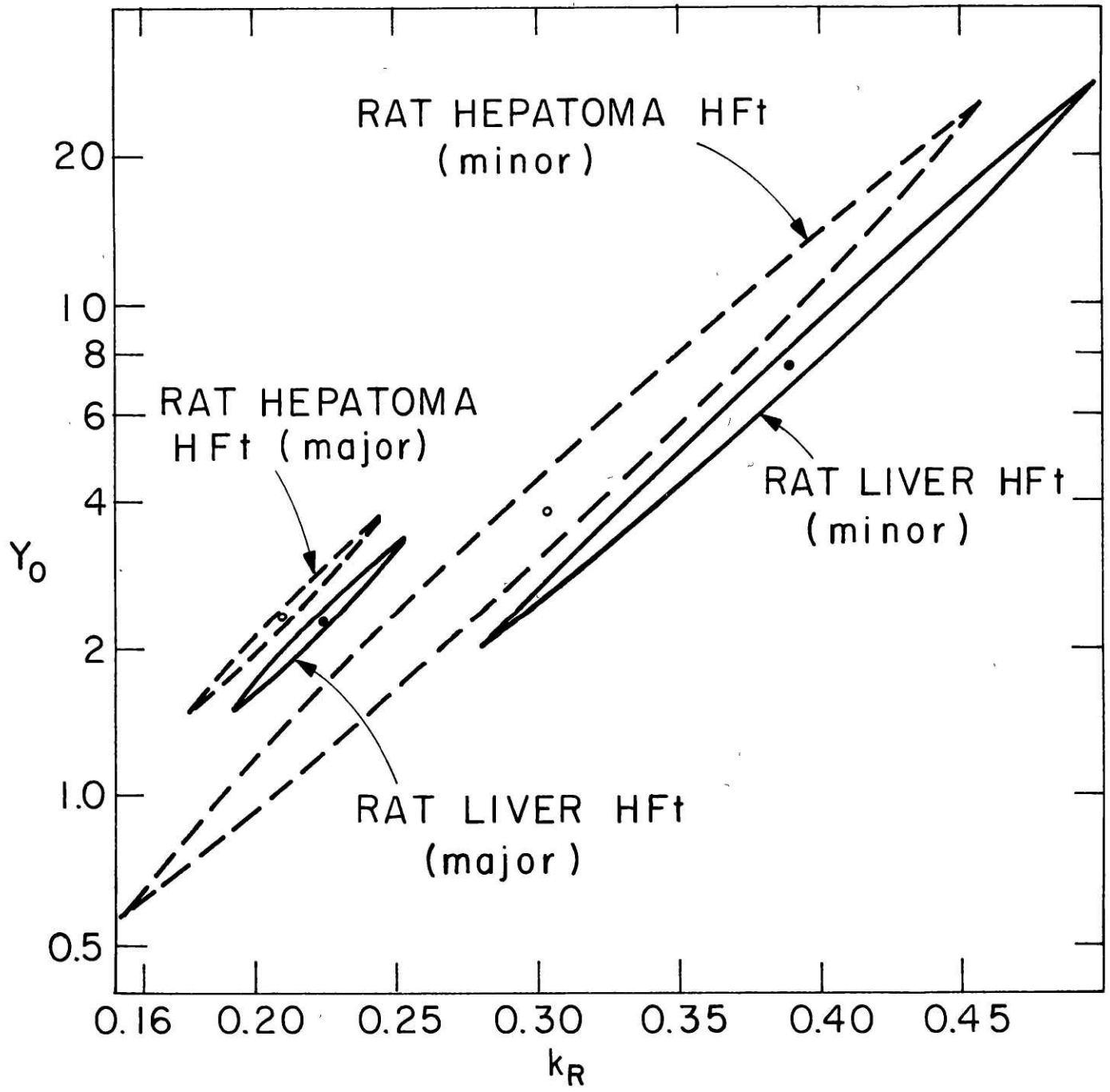

RAT HEPATOMA HFt
(minor)
RAT HEPATOMA
HFt (major)
RAT LIVER HFt
(minor)
RAT LIVER HFt
(major)
20
10
8
6
4
2
1.0
0.5
$Y_o$
0.16
0.20
0.25
0.30
0.35
0.40
0.45
$k_R$

Fig. 5 shows the results of analyses of migration of the two major (fast and slow) heart ferritin species, and as shown, the ellipses do not overlap. However, the slow rat heart ferritin ellipse partially overlaps with the major rat liver ferritin, indicating no discrimination when size and charge are lumped together by this technique in agreement with earlier studies of electrophoresis at one gel concentration ( 3).

Standards of known molecular weights (120,000 to 670,000) were used in this system in order to provide absolute values for molecular size and apparent valence. For each standard, the Ferguson plot relationship was checked and, as can be seen for acetylcholinesterase (MW 160,000) (Fig. 6), there is a linear relationship between log $R_f$ and %T. The data obtained for the standards were analyzed by the Rodbard computer programs to provide a standard curve for estimates of apparent valence, molecular radius and molecular size. As can be seen from Figs. 7 and 8, there were linear relationships between the molecular weight or molecular radius and the square root of the retardation coefficient, $K_R$. In order to compare the molecular weights obtained by PAGE with the theoretical values assigned to the standards by other techniques, I recalculated the molecular weights of the standards from the data obtained using the Rodbard computer programs. The calculated values for each standard using PAGE were very similar to the theoretical values based on their molecular weights and this supported the reliability of the technique. (No standards were available with sizes above 670,000).

Fig. 5 Plot of 95% confidence limit ellipses of holoferritins of rat liver and heart showing major and minor components and the presence of two heart ferritins. The centers of the ellipses are shown (Δ ● ○ ◐ ).

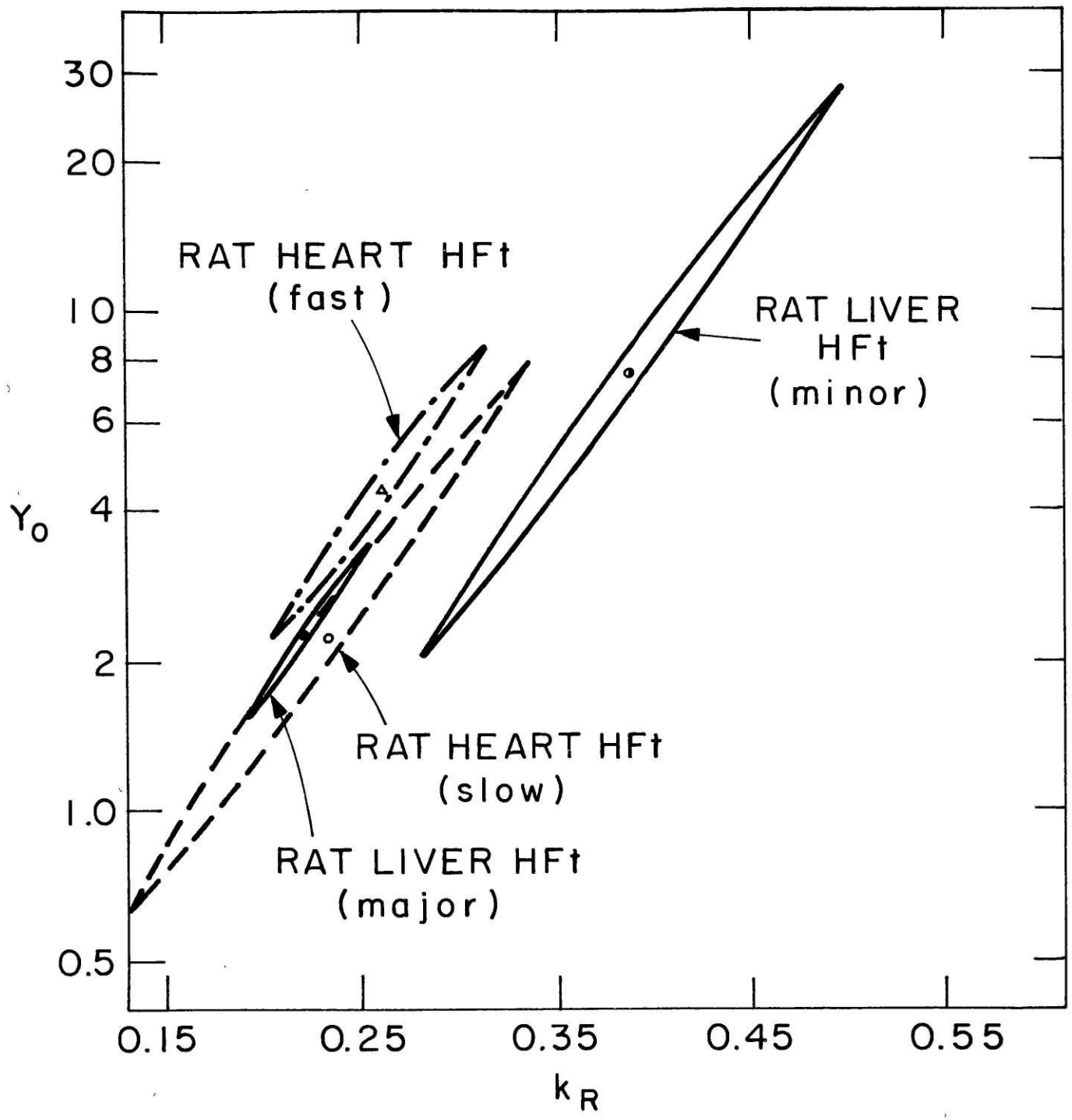

30
20
10
8
6
4
2
1.0
0.5
$Y_o$
RAT HEART HFt
(fast)
RAT LIVER
HFt
(minor)
RAT HEART HFt
(slow)
RAT LIVER HFt
(major)
0.15
0.25
0.35
0.45
0.55
$k_R$

Fig. 6 A Ferguson Plot (Log $R_f$ versus % T) for acetyl cholinesterase.

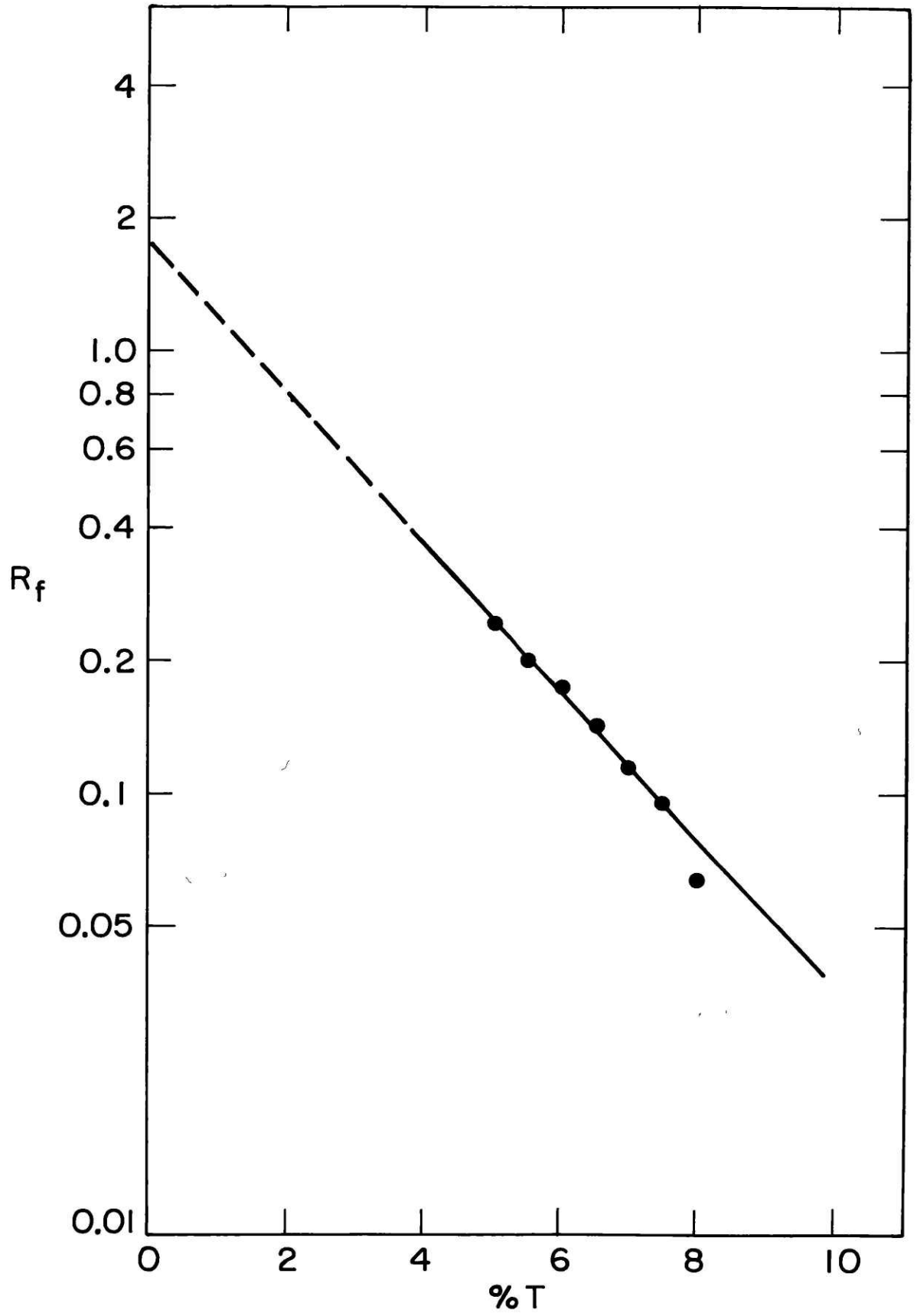

4
2
1.0
0.8
0.6
0.4
$R_f$
0.2
0.1
0.05
0.01
0
2
4
6
8
10
%T

Fig. 7 Linear relationship between molecular radius and $\sqrt{K_R}$ (retardation coefficient) for standard proteins

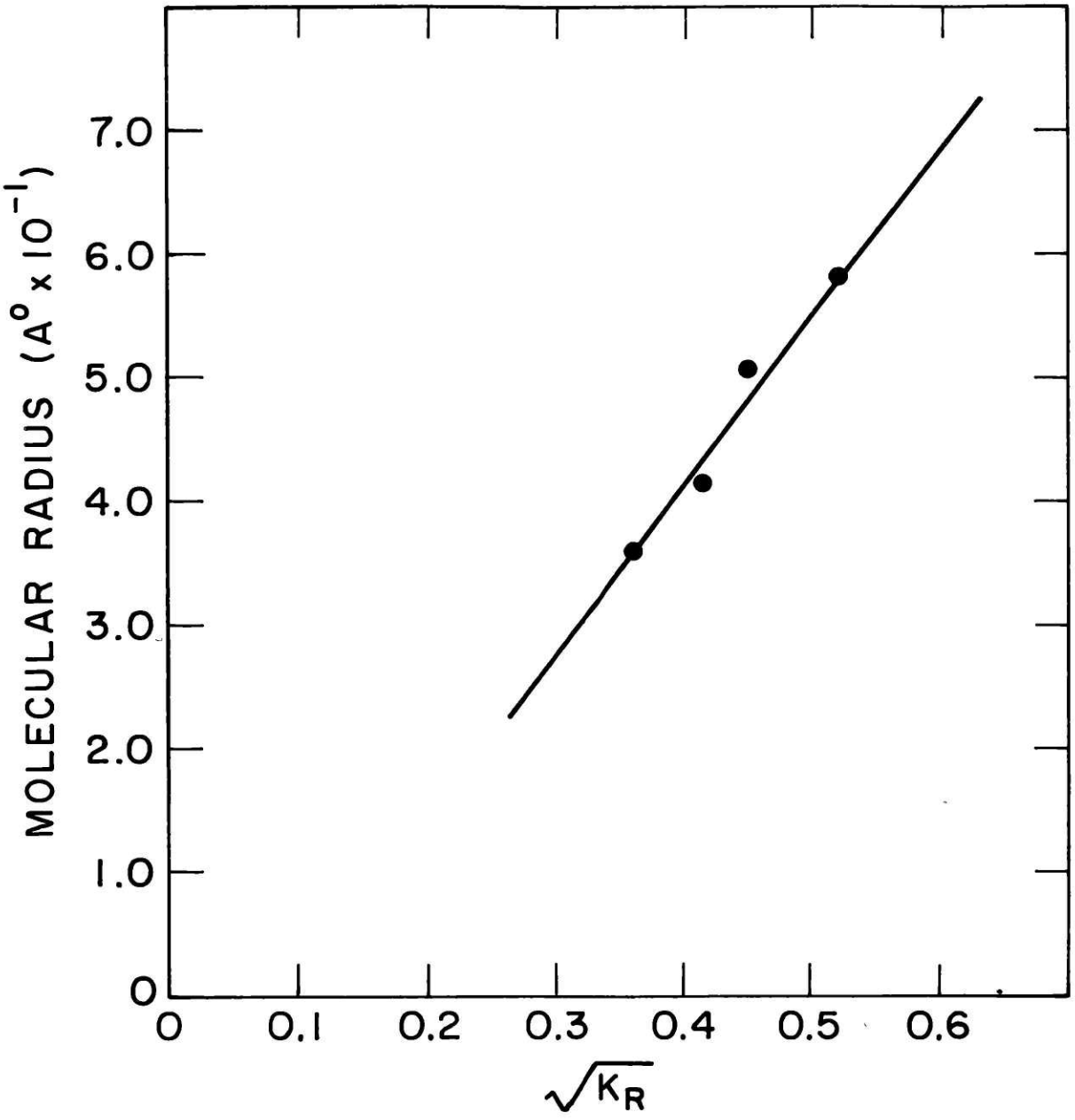
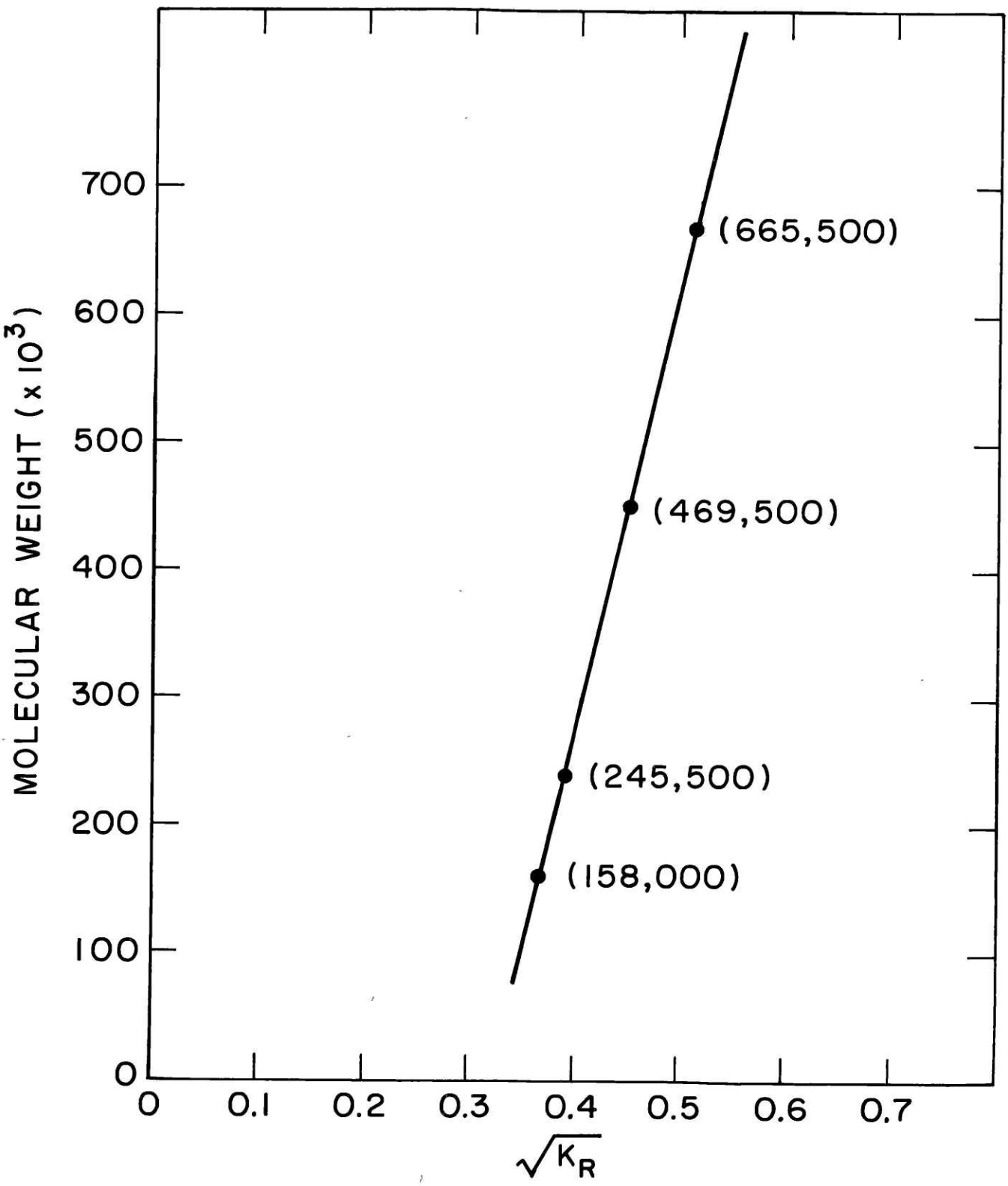

MOLECULAR RADIUS ($A^o \times 10^{-1}$)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
0
0.1
0.2
0.3
0.4
0.5
0.6
$\sqrt{K_R}$

Fig. 8 Linear relationship between molecular weight and $\sqrt{K_R}$ (retardation coefficient) for standard proteins. The values in parentheses are the calculated values for each standard using quantitative polyacrylamide gel electrophoresis


MOLECULAR WEIGHT (x 10³)
700
600
500
400
300
200
100
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
√K_R
(665,500)
(469,500)
(245,500)
(158,000)

The quantitative data obtained for all the ferritins was then analyzed by the Rodbard computer programs. Table II shows that the major components of all the ferritins analyzed had statistically indistinguishable molecular sizes falling within the range of 420,000 to 490,000 with the exception of the slow and fast heart species. These were significantly larger as judged from "t" tests of the values of $K_R$, with molecular weights of 530,000 and 626,000 respectively. This appears to be the first report of an apparent difference in the size of the protein shell of a tissue ferritin.

The apparent valences (charges) of these various ferritins are also reported in Table II. Using log $Y_o$ for statistical evaluation of differences, all rat ferritin monomers had valences that differed from that of horse spleen ferritin. Among rat tissues ferritins, the monomers of liver, hepatoma kidney and the slow heart ferritin had net charges that were statistically indistinguishable (30-40 net protons/mol.), whereas that of the fast heart ferritin was much greater (83 protons).

The migration properties of minor (dimeric) ferritin bands on the gels were compared with those of the monomer bands. The molecular sizes of the minor bands were larger than those of the major bands, and were not inconsistent with a doubling in size, in agreement with the results of others for horse spleen ferritin (143-145). However, charge differences varied, being either similar for major and minor forms (spleen ferritin), approximately twice for the minor form (rat hepatoma ferritin) or five times greater for the minor form (rat liver ferritin).

TABLE II

COMPUTED MOLECULAR WEIGHTS AND APPARENT VALENCES OF VARIOUS HOLO-FERRITINS

| | Retardation Coefficient ($K_R$) (Mean ± S.D.) | Apparent Molecular Weight ($\times 10^3$) | log Y (Mean° ± S.D.) | $Y_o$ | Apparent Valence (Net protons/ molecule) |
|---|---|---|---|---|---|
| Major Components | | | | | |
| Horse Spleen | 0.210±0.011° | 443 | 0.498±0.065°° | 3.15 | -51 |
| Rat Liver | 0.224±0.011° | 493 | 0.351±0.063° | 2.25 | -38 |
| Rat Hepatoma | 0.210±0.002° | 443 | 0.365±0.067° | 2.32 | -37 |
| Rat Kidney | 0.204±0.011° | 421 | 0.278±0.063° | 1.89 | -30 |
| Rat Heart (slow) | 0.234±0.016* | 531 | 0.351±0.090° | 2.24 | -40 |
| Rat Heart (fast) | 0.260±0.008** | 626 | 0.635±0.047** | 4.31 | -83 |
| Minor Components | | | | | |
| Horse Spleen | 0.276±0.013° | 685 | 0.519±0.074° | 3.30 | -67 |
| Rat Liver | 0.389±0.034°° | 1,100 | 0.875±0.179°° | 7.49 | -197 |
| Rat Hepatoma | 0.304±0.051° | 787 | 0.578±0.279° | 3.79 | -83 |

n = 14 except for the rat liver ferritin minor component (n=8) and rat hepatoma ferritin minor component (n=7)

Similar symbols for $K_R$ and for log $Y_o$ indicate that these values are not significantly different ($P > 0.1$ by "t" test). Different symbols indicates significant differences ($P < 0.01$) within major or minor groups.

TABLE III

COMPUTED MOLECULAR WEIGHTS, APPARENT VALENCES AND ELECTROPHORETIC BEHAVIOR OF MAJOR AND MINOR COMPONENTS OF HORSE SPLEEN AND RAT LIVER FERRITINS

| | Apparent Molecular Weight | Apparent Valence (Net Protons/molecule) | Electrophoresis in minimally sieving gels at pH 8.9 |
|---|---|---|---|
| HORSE SPLEEN (major) | 443,000 | -51 | migrate similarly |
| HORSE SPLEEN (minor) | 685,000 | -67 | |
| RAT LIVER (major) | 493,000 | -38 | minor band precedes the major band |
| RAT LIVER (minor) | 1,100,000 | -197 | |

In the case of rat liver and horse spleen ferritins, the relationship between major and minor components was explored further using a minimally sieving gel made with a high proportion of cross linking agent (20% C) which would greatly reduce differences due to size (.227). In these gels, the minor band now preceded the major band by a considerable distance (Table II). This agrees with the greater charge of the minor rat liver ferritin band shown by the Ferguson plot data (Table II). On the other hand, there was no separation of major and minor components of horse spleen ferritin in these large-pore gels, again in agreement with their similarity of charge calculated from the Ferguson treatment (Table II). The data thus suggest either the dimers have different shapes that affect their migration characteristics or the dimeric forms of ferritin may represent different kinds of associations of monomers in different tissues. It is also possible that the interface between the two monomeric units of the dimer obscures different proportions of acidic and basic groups in the various tissues, which affects the net charge of the molecule and electrophoretic migration.

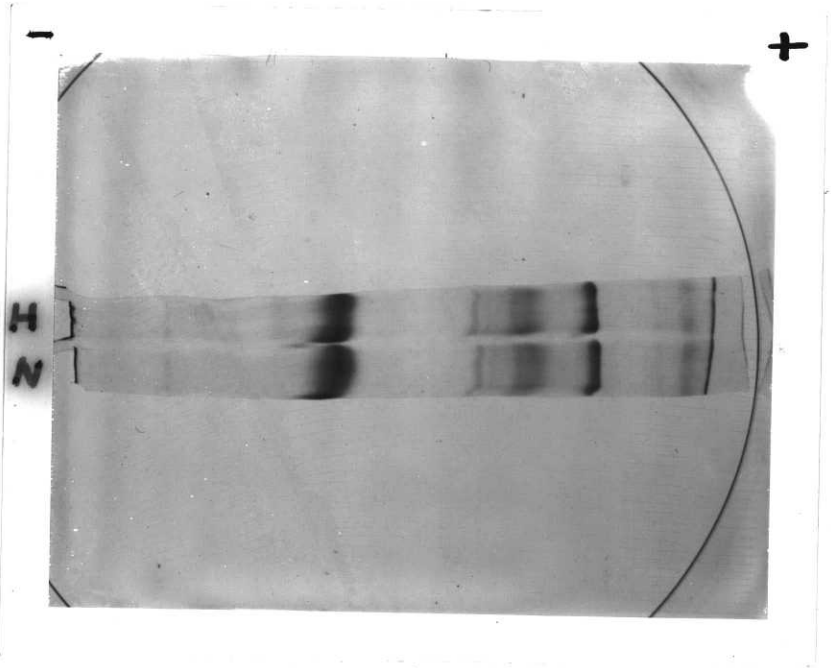
2. Subunit Composition of rat liver and heart ferritins

There has been some divergence of opinion about the size and number of subunits in ferritin. Originally, Harrison (138) concluded from X-ray diffraction data and amino acid analysis that horse spleen ferritin contains 20 identical subunits within the molecular weight range 22,000 to 27,000, but Crichton (163)concluded from a study of methionine residue

content after cyanogen bromide cleavage and sodium dodecyl sulphate electrophoresis that there were 24 identical subunits of molecular weight 18,500. However, Niitsu et al (167,168) and Linder et al (167) have reported multiple species of subunits in ferritins isolated from the tissues of various animals, in agreement with the finding of two species of polyribosomes in rat liver responsible for synthesis of ferritin subunits of 19,000 and 13,000 molecular weight respectively (166). Recently, Crichton has suggested that the initial heating of ferritin up to 70° in the purification procedure may activate proteolytic enzymes that break the major 19,000 subunit apart into two minor subunits, implying that the minor subunits observed on sodium dodecyl sulphate electrophoresis of holo- and apo-ferritin may be fragments of the major subunit, 19,000 (231).

I investigated this possibility by comparing dissociation of rat liver ferritin prepared with and without heat treatment. One preparation was a rat liver heat supernatant obtained after heating the homogenate to 70° for 10 minutes. The other was a liver supernatant kept cold throughout that was obtained after centrifugation of the homogenate for 20 minutes at 14K in the Sorvall. Both supernatants were fractionated on Sepharose 4B columns and the fractions were read at $OD_{280}$ and $OD_{320}$. Fractions containing the maximum absorbances of protein, ferritin were pooled together Ferritin in both samples was precipitated by antibody against horse spleen ferritin and the immunoprecipitates were run on a SDS-gradient gel (see Methods). As can be seen from Fig. 9,

Fig. 9 SDS electrophoresis of rat liver ferritin antibody precipitate in a 10-15% gradient gel. Ferritin samples were obtained with and without heating


−
+
H
N

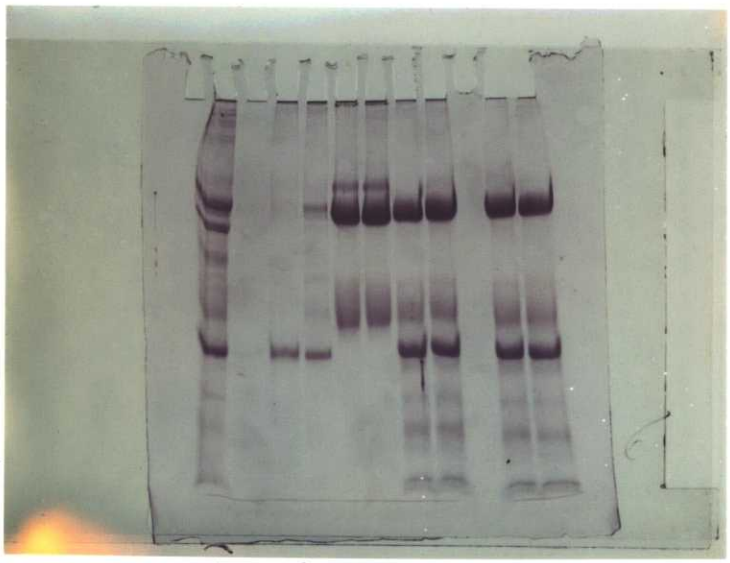
the minor subunits of rat liver ferritin were present even with no heat treatment. Both samples had identical subunit composition, the major 19,000 subunit and the minor 12,000 and 10,000 subunits. On the basis of these experiments, I conclude that heating the liver homogenate to 70° does not cause a breakdown of the 19,000 subunit to the minor subunits.

As we saw earlier by quantitative polyacrylamide gel electrophoresis, the slow and fast heart ferritin differed significantly from each other in terms of charge (apparent valence) and size. A difference in size between the two ferritins could thus mean either a larger subunit in one, or different proportions of the same subunits. It has not been possible to carry out extensive and elaborate subunit analyses on the individual heart ferritin species, primarily because of lack of enough material from the separate bands. Nevertheless, some preliminary experiments were performed on small quantities of each species, after separation.

It is known that the two ferritins differ immunologically (3), the faster migrating species requiring three times as much antibody for precipitation as the slower species. In our case there was not enough material to work out optimum conditions for precipitation of the individual ferritins with antibody, so the proteins were precipitated (separated and mixture) with acetone. The precipitates were dissociated with SDS and applied to SDS gradient gels (10-15% acrylamide). Included in the same electrophoretic run were rat liver ferritin immunoprecipitates, bovine serum albumin immunoprecipitate and

Fig. 10 SDS electrophoresis of acetone precipitates of heart ferritin mixture, slow and fast ferritin, immunoprecipitates of bovine serum albumin and rat liver ferritin in a 10-15% gradient gel.

10%



15%

H F S B RLFt

HEART MIXTURE

ALBU MIN

FAST AND SLOW Ft

RAT LIVER FERRITIN

an acetone precipitate of mixed heart ferritins. As can be seen in Fig. 10, the fast and the slow heart ferritins contained the same 19,000 major subunit. Probably because there was not enough material on the gel, the minor subunits are not visible, but they are present in the mixture of the two. (It was not possible by this technique to quantitate the relative proportions of the subunits in the mixture.)

3. Isoelectric focusing of rat heart and other tissue ferritins.

In addition to the known heterogeneity amongst tissue ferritins show by electrophoresis and other techniques, some work suggests that there may be a more extensive heterogeneity within ferritins from a single tissue using the technique of iso-electric focusing (170a). I investigated the iso-electric focusing of the rat tissue and horse spleen ferritins (see Methods for details). Ferritin being a large molecule tends to sieve on iso-electric focusing in polyacrylamide gels. Therefore, I performed some preliminary experiments with rat tissue ferritin, using minimally-sieving gels made with a high proportion of cross-linking agent, in this case, $N_1N^1$ - diallyltartardiamide. (see Methods). However, in spite of using different lengths of focusing time and different concentrations of acrylamide, ferritin did not appear to be resolved into distinct components but stayed as one diffuse band on the gel. Since I was unable to resolve the ferritins by this system, I used another system containing $N_1N^1$ - methylene bis acrylamide as the cross-linking agent and that

TABLE IV

ISOELECTRIC FOCUSING SPECIES OBTAINED BY ISOFOCUSING OF DIFFERENT TISSUE FERRITINS

| Ferritin* Species | pI** | | | | | |
|---|---|---|---|---|---|---|
| | 4.8 | 4.6 | 4.55 | 4.4 | 4.25 | 4.0 |
| Horse Spleen | - | - | - | + | + | + |
| Rat Kidney | - | + | + | + | - | + |
| Rat Hepatoma | - | - | + | + | + | + |
| Rat Heart | + | + | - | - | - | - |

* Rat liver ferritin was not completely resolved by this technique.

** The pI's were obtained by measuring the distance of the band and reading the pI from a calibration curve of pH vs. gel section of a blank gel included in the run.

The gels were made up using 4.15%T, 3.61%C, and 2% ampholine, pH 3-10.

was also reported to be minimally sieving (229). After focusing for 18 hours, the gels were stained with Coomassie Blue and after destaining, the number of bands and their pIs were recorded.

As can be seen from Table IV horse spleen, rat kidney and rat hepatoma ferritin contained multiple bands with pIs ranging from 4.0-4.6 . Rat heart ferritin mixture, on the other hand, contained only two bands with pIs 4.6 and 4.8 , in agreement with the electrophoretic studies showing the two distinct species. Also, this technique confirmed the purity of the rat heart ferritin mixture, since no other non-iron containing proteins were present. Another interesting feature is that none of the iso-electric focusing profiles of any of the ferritin species were identical, suggesting slight differences in the surface charge as revealed in the quantitative PAGE data.

Data for rat liver ferritin are not shown in Table IV because there still was an incomplete resolution by both techniques. The focusing time was varied from 2 hours to 24 hours with no success. The voltage was varied and this also did not provide any resolution. A thick, diffuse band at the beginning of the gel was observed. Furthermore, the rat liver ferritin sample was dialyzed against water to remove any ions that were inhibiting the iso-electric focusing of the protein. This did not change the picture and I did not pursue this any further.

## B. Tissue and Cellular location and function of Cardiac Ferritins

A major question this thesis has attempted to resolve is the functional advantage of having two ferritin species in the same tissue. A preliminary comparison made by Linder and Munro (31) shows that the muscle ferritin concentration parallels that of myoglobin in different muscles and the concentration of the two species is highest in the heart relative to other muscles. This suggests a possible relationship with one or both the ferritin species with myoglobin.

In line with the above suggested relationship is the possibility that the slow and the fast heart ferritins are present in different cell types. Estimates suggest that the heart contains three times as many non-myocardial cells as myocytes (10) and, ideally, separated heart muscle and connective tissue cells should be looked at (e.g. in culture) for the presence of a particular ferritin species. This separation is very difficult to achieve in the adult rat heart (see Literature Survey) and the problem of the ferritin myoglobin relationship and the cellular location of ferritin was, however, explored mainly in three ways. Ferritin concentrations were determined in the myoglobin-containing red and the non-myoglobin-containing white muscles of the rat and the chicken to enable us to associate a specific muscle ferritin with myoglobin. Furthermore, to give us insight into the cellular location of the two ferritin species which is probably related to their function, I have induced a specific

cell type hypertrophy experimentally where one cell type is metabolically more active than the other and enlarges, the concentrations and relative proportions of the two ferritin species were studied. These experiments are now described in greater detail.

1. Distribution of slow and fast ferritin in different muscle types of the rat.

It was suggested that I look at the soleus and the gastrocnemius muscles of the rat for the two ferritin species as these muscles were representative of non-myoglobin containing white and myoglobin containing red fibres. Looking at the data (Table V) with this assumption, the gastrocnemius has much more total iron than the soleus, since the soleus had a very low iron content.

The ferritin iron concentration as determined by the precipitation with horse spleen antibody (see Methods) showed that while the gastrocnemius had an appreciable amount of ferritin iron, none was detected in the soleus. The administration of iron produced a three and a half fold increase in the ferritin iron concentration of the gastrocnemius in line with the studies of iron administration on rat liver and heart. No ferritin was detected in the soleus even after iron administration, implying that it probably takes up much less iron. The bulk of the evidence in the literature, however, suggests that anatomically and histologically the soleus muscle is in fact a red muscle (232). However, the presence of little iron suggests little, if any, myoglobin could be present and, as

TABLE V

FERRITIN CONTENTS OF RED AND WHITE MUSCLES

| | RAT | | | | | | CHICKEN | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Gastrocnemius (red) | | Soleus (white?) | | Heart (mixed) | | Leg (red) | | Breast (white) | | Heart (mixed) | |
| | -Fe | +Fe[a] | -Fe | +Fe | -Fe | +Fe | -Fe | +Fe[b] | -Fe | +Fe | -Fe | +Fe |
| Total Fe (µg/g) | 400 | - | 10.0 | - | - | - | 12.9 | 33 | 6.5 | 20 | - | - |
| Total Ferritin Fe (µg/g) | 7.7 | 28.4 | ND* < 1 | ND* < 1 | 15 | 30 | 12.6 | 15.5 | 6.7 | 10.6 | 10.1 | 14.8 |
| Proportion Fast/Slow | ≤ 1 | > 1 | - | - | 3.6 | 4.0 | | | | | | |

[a] iron was given i.p. as Imferon, 25 mg Fe/rat 2 x 14 days

[b] iron was given i.m. (in the breast) as Imferon 40 mg/chicken 2 x/5 days

* immuno-precipitate from 1 ml heat supernatant gave 0.000 $OD_{520}$ - Fe, 0.002 OD + Fe (OD/µg Fe = 0.031)

[c] Adult Fischer female rats (160 g BW) and are adult chickens were used in this experiment

TABLE VI

RELATIVE ELECTROPHORETIC MOBILITY* OF FERRITINS FROM DIFFERENT MUSCLES OF THE RAT AND CHICKEN

| Species and Muscle | Age, Sex and Iron treatment | Relative Mobility ($R_f$) of slower migrating species (mean±S.D.) | Relative Mobility ($R_f$) of faster migrating species (mean±S.D.) |
|---|---|---|---|
| Chicken** | Adult Female | | |
| Leg | -Iron[a] | 0.173±0.008(4) | Not detected |
| Leg | +Iron[a] | 0.180,0.175 | Not detected |
| Breast | -Iron | 0.172±0.009(4) | Not detected |
| Breast | +Iron | 0.175,0.170 | Not detected |
| Heart | -Iron | 0.172±0.010(4) | Not detected |
| Heart | +Iron | 0.183,0.179 | Not detected |
| Rat | Adult Female | | |
| Gastrocnemius | -Iron[b] | 0.180±0.004(4) | 0.270±0.010(4) |
| Gastrocnemius | +Iron[b] | 0.160±0.010(4) | 0.230±0.060(4) |
| Soleus | -Iron | Not detected | Not detected |
| Soleus | +Iron | Not detected | Not detected |
| Heart | -Iron | 0.175±0.040(6) | 0.265±0.05(6) |
| Heart | +Iron | 0.183±0.06(6) | 0.257±0.03(6) |

* The Relative Mobility ($R_f$) is measured with respect to the tracking dye in the Ornstein-Davis system

** $R_f$ values for the only ferritin species detected in chicken muscle are placed in the column for the "slower" migrating species.

[a] In the case of the chicken, iron was given i.m. (in the breast) as Imferon, 40 mg Fe/chicken, 2 x 15 days

[b] For rats iron was given i.p. as Imferon, 25 mg Fe/rat, 2 x/4 days

I will discuss later, our observations show that the soleus is not a pure red muscle. Furthermore, the distribution of ferritin and myoglobin in the gastrocnemius and soleus appear to go in parallel, suggesting a link between the two.

The slow and the fast ferritin species in the gastrocnemius had a similar electrophoretic mobility on polyacrylamide gels measured relative to tracking dye ($R_{fs}$) as the ferritins of the rat heart and diaphragm. The proportion of the two bands in the gastrocnemius was approximately one-to-one, as determined by the ratio of the areas under the peaks when the gels were scanned at $OD_{280}$ . This ratio did not change with iron administration.

2. Distribution of slow and fast ferritin species in different muscle types of the chicken.

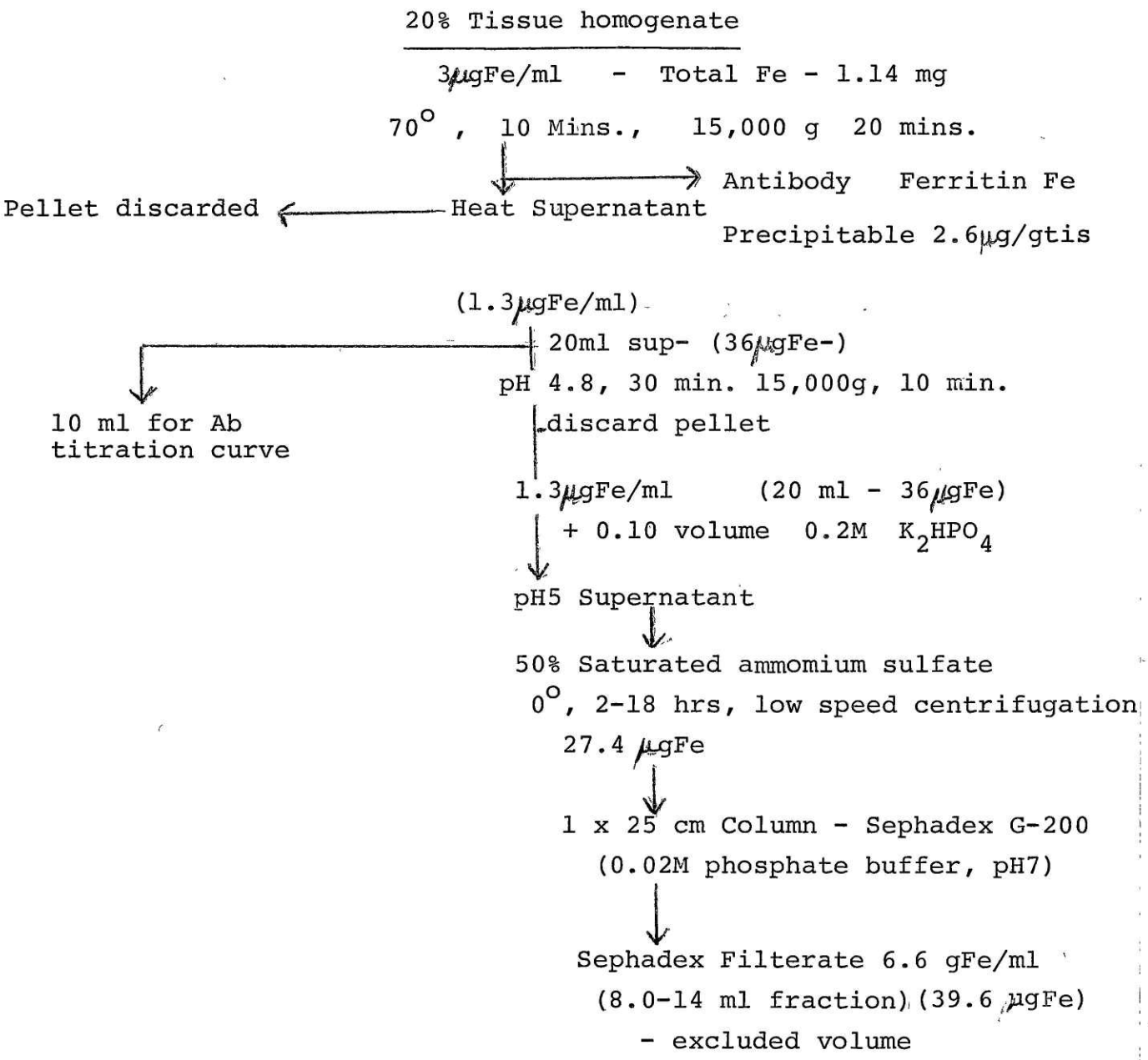
Since the findings on the rat soleus and gastrocnemius were inconclusive in demonstrating the distribution of ferritin in white and red muscle, I did some experiments on chicken leg (red) and breast (white) muscle. The advantage of this animal is that it has pure red and white muscle. Also, both tissues are present in adequate amounts to work with. I looked at the distribution of the two ferritins with and without prior administration of iron.

Though the total iron contents in the chicken leg and breast were lower than those of the gastrocnemius muscle of the rat, the ferritin iron levels in both muscle types were comparable (Table V). The "white" breast muscle has an appreciable amount of ferritin iron in it though slightly

less than the leg muscle and the heart. The administration of iron showed slight elevations in the ferritin iron content in all three chicken muscles though it was not as much as seen in the rat gastrocnemius. With these levels of total iron and ferritin iron in the muscles, a definite relationship between ferritin and myoglobin is indicated.

As can be seen from Tables V and VI, only a single ferritin species was detected in the leg, breast and heart of the chicken, both with and without the administration of iron. The electrophoretic mobility relative to tracking dye was the same in all three muscles and similar to that of rat liver ferritin (Table VI). In order to be sure that two ferritins migrating at the same rate but with different antigenicities were present, I did an antibody titration curve for the chicken muscles. An antibody titration curve using red muscle heat supernatant was constructed (Fig. 10). 1ml of heat supernatant was taken in each case and the amount of antibody increased from 0.3 ml to 2.0 ml. The immunoprecipitates were assayed for their iron content. As can be seen, the curve plateaued at 0.6 ml antibody added to 1ml of heat supernatant. This shows that since the curve is not biphasic, a single species is present. A similar titration curve was obtained for chicken white muscle. For chicken heart the amount of antibody necessary for maximum precipitation of 1ml of red muscle heat supernatant was used. The fact that chicken ferritin cross reacts with horse spleen ferritin antibody indicates that there must be antigenic determinants on the molecule similar to those for other ferritins.

Assay of Tissue Ferritin - Linder & Munro (1972)



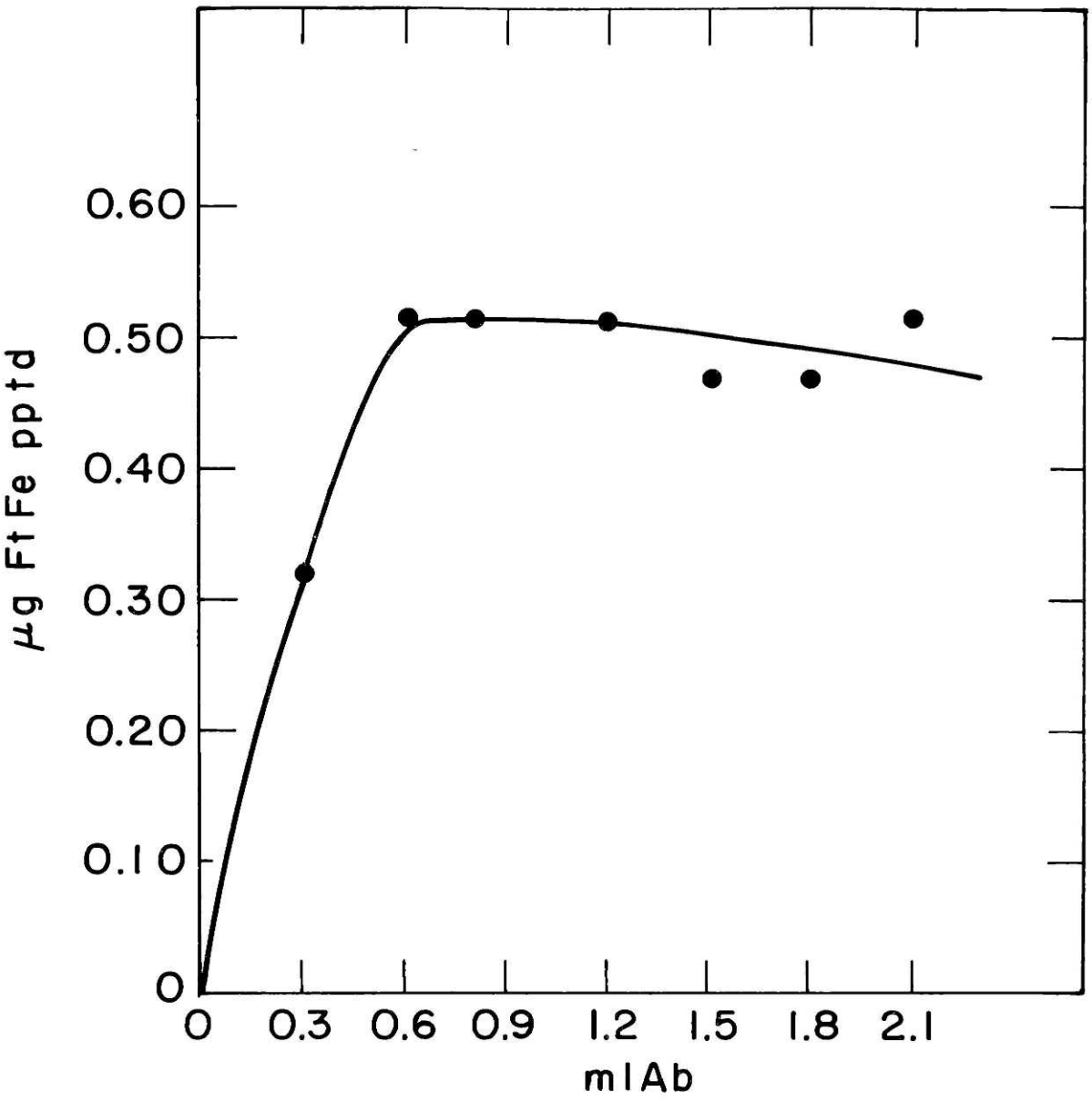
Furthermore, the recovery of iron was checked in the purification procedure because it was possible that there might be another ferritin which did not react with antibody but was lost during purification. Twenty mls of chicken red muscle heat supernatant fraction with a total of 36$\mu$g iron was purified and as can be seen from Fig. 11, there was virtually no loss of iron throughout the purification procedure implying that additional ferritins were not lost in this process.

### 3. Ferritin protein concentrations under conditions of cardiac hypertrophy.

Cardiac enlargement is a common feature of heart disease. It therefore must have considerable significance as a basic biologic phenomenon and as a clinical manifestation of disorders of this organ. Thus one approach to the study of cardiac ferritin function and distribution is to investigate the metabolism of the two ferritins under conditions of cardiac hypertrophy.

Different kinds of hypertrophy can be induced in experimental animals involving a preferential enlargement of a particular cell type. The relative distribution of ferritins under such conditions could provide indications of the cellular location of the two proteins. I tried to induce a connective tissue type of hypertrophy by the administration of isoproterenol and a muscle cell type of hypertrophy by feeding weanling rats a copper deficient diet for three weeks. The results obtained are discussed below:

Fig. 11 Antibody titration curve of chicken red muscle ferritin


0.60
0.50
0.40
0.30
0.20
0.10
0
μg Ft Fe ppt'd
0
0.3
0.6
0.9
1.2
1.5
1.8
2.1
ml Ab

(a) Isoproterenol induced hypertrophy.

Isoproterenol, one of the most active of the sympathomimetic amines acts on the $\beta$ receptors with virtually no effect on the $\alpha$ receptors. In my experiments administration of 10$\mu$g/100gm body weight subcutaneously everyday to rats for three weeks produced a 15% increase in heart weight/body weight ratios above controls that were given saline throughout the experimental period (Table VI).

In these hypertrophic animals there was a slight increase in the total ferritin iron concentration. More importantly however, there was a large change in the relative proportions of fast and slow ferritins in these hearts (lower line) suggesting a tremendous increase in the concentration of the slower migrating species and/or a tremendous decrease in the concentration of the faster migrating species. As can be seen, the concentration of the slow ferritin was indeed increased during hypertrophy and that of the fast ferritin was decreased, both contributing to the change in proportion.

If iron was administered to the hypertrophic animals there is a further increase in the heart weight to body weight ratios of the hypertrophied animals and thus in the degree of hypertrophy. This an an interesting observation and the enlargement observed could be the reaction to the additional stress imposed by the i.p administration of imferon.

Furthermore, there was a two fold increase in the concentration of ferritin iron in the tissue as is observed when normal animals are treated with iron (Table VII). As compared to the hypertrophic untreated controls, there was

TABLE VII

EFFECTS OF CARDIAC HYPERTROPHY ON FERRITIN CONTENT OF RAT

| | | Isoproterenol* | | |
|---|---|---|---|---|
| | | Control | Hypertrophy | |
| | | | Untreated | +Fe |
| Heart wt./(Body wt. x $10^{-3}$) (mean±S.D.) | I | 3.03±0.17(8) | 3.59±0.11(7) +18% | |
| | II | 3.30±0.18(12) | 3.54±0.09(5) +7% | 3.72±0.47(8) +13% |
| Total Ferritin Fe (µg/g) | | 13,10 | 13,16 | 26 |
| Ferritin Protein (µg/g) SLOW | | 9,11 | 25,39 | 20 |
| FAST | | 22,14 | 8, 1 | 45 |
| Ferritin Proportion FAST/SLOW | | 2.3,1.3 | 0.3,0.03 | 2.3 |

| | Copper-Deficient** | |
|---|---|---|
| | Control | Hypertrophy |
| Heart wt./(Body wt. x $10^{-3}$) (mean±S.D.) | 3.81±0.29(18) | 4.45±0.36(21) +17% |
| | 3.96±0.37(20) | 4.41±0.29(20) +11% |
| Total Ferritin Fe (µg/g) | 10,10 | 10 |
| Ferritin Protein (µg/g) SLOW | 18,14 | 3,18 |
| FAST | 7,10 | 2,8 |
| Ferritin Proportion FAST/SLOW | 0.4, 0.7 | 0.6,0.4 |

* Adult female Fischer rats (160-180 g BW) administered isoproterenol (10 µg/100g BW) S.C. daily for 21 days

** Weanling female Fischer rats fed a "low copper diet" for 21 days

The no. in parenthesis is the number of animals. The percentages below Heart wt/Body wt ratios are the percent increase in the ratio compared to controls

an increase in the relative proportion of the fast to slow ferritin, so that the proportion returned to the value for the saline injected (non-hypertrophied) controls. This was due to a tremendous increase in the concentration of the fast ferritin species. It thus appears that excess iron causes a preferential deposition of iron in fast ferritin in these animals suggesting that in this condition, this species is mainly concerned with the storage of iron. However, administration of iron to these hypertrophic animals appears to help restore the fine balance between the fast and the slow ferritin to control levels where the concentration of the fast ferritin is approximately twice that of the slow.

(b) Copper deficiency induced cardiac hypertrophy.

The administration of isoproterenol to rats in the isoproterenol study produced a connective tissue cell hypertrophy. In order to study the relative distribution of the fast and slow ferritin species in conditions of muscle cell hypertrophy, rats were put on a copper deficient diet for 3 weeks. I had several times tried to induce muscle cell hypertrophy by constriction of the abdominal aorta in rats, but I found the technique not very reproducible (see Methods).

Feeding weanling rats (females) a copper deficient diet for 3 weeks produced a 14% (average) increase in the ratio of heart weight/body weight (Table VII), a large number of animals were used in each batch. There was no apparent difference in the concentration of the ferritin iron in the hearts of the two sets of animals or in the relative proportion of fast ferritin to the slow ferritin (Table VII). However, because

the weight of the hearts was increased, though the relative proportion of the two species is the same. Thus on the basis of these observations, one cannot conclusively say that one of the ferritin species is present exclusively in the contractile cell. An interesting feature in the control weanling rats was that the concentration of the slower migrating species was greater than that of the faster migrating species, which is in contrast to the proportion of the two ferritins in adult female rats (Table VIII). This could be explained on the basis of a difference in the age of the animals.

C. Metabolism

1. Iron Induction of muscle ferritin snythesis in adult female and male rats.

Administration of a variety of iron compounds increases the ferritin content of different organs, whether measured as ferritin protein or ferritin iron. Linder and Munro ( 3 ) made some initial observations of the response of the rat cardiac ferritins to iron and found that the synthesis of both species was stimulated. I explored this in greater detail. The effect of iron administration on rat diaphragm was also studied.

Ferritin protein synthesis was determined by measuring uptake of labelled $^{3}$H-leucine into heart ferritins of control and iron injected adult female and male rats. Iron was administered as ferric ammonium citrate intraperitoneally five hours before sacrifice (see Methods).

Table VIII shows that in untreated rats, the concentration of the fast heart ferritin was greater than the slow heart

TABLE VIII

FERRITIN SYNTHESIS IN CARDIAC TISSUE OF ADULT RATS

FEMALES*

| | Controls | | Iron Injected** | |
|---|---|---|---|---|
| Total Protein (mg/g) | I | II | I | II |
| Ferritin Concn. (µg prot/gm) | 172 | 168 | 164 | 173 |
| SLOW | 11,9 | 14+ | 9,11 | 25+ |
| FAST | 40,36 | 14 | 39,37 | 10 |
| Ft Specific Act (cpm $^{3}H$/ µg prot***) | | | | |
| SLOW | 26.5,30.5 | 0.46 | 68.6,64.6 | 0.34 |
| FAST | 56.5,55 | 0.72 | 117,114 | 4.38 |
| Total Counts in ferritin (cpm/µg x µg ferritin/g) | | | | |
| SLOW | 294,292 | 12 | 650,654 | 14 |
| FAST | 2120,2110 | 21 | 4862,4858 | 70 |
| Total Protein Specific Act. (cpm $^{3}H$/mg) | 511,488 | 592,588 | 473,490 | 430,434 |
| % total counts in ferritin**** (slow plus fast) | 2.8 | 0.32 | 6.4 | 1.11 |

TABLE VIII(cont.)

MALES*

| | Controls | | Iron Injected** | |
|---|---|---|---|---|
| Total Protein (mg/g) | I | II | I | II |
| Ferritin Concn. (µg prot/gm) | 185 | 186 | 191 | 194 |
| SLOW | 26,23 | 35,32 | 39,37 | 50,54 |
| FAST | 24,25 | 23,20 | 24,25 | 20,20 |
| Ft Specific Act (cpm $^{3}H$/ µg prot***) | | | | |
| SLOW | 6.4,6.4 | 5.0,5.5 | 29,28 | 20,20 |
| FAST | 6.2,6.2 | 5.1,5.4 | 20,19 | 31,29 |
| Total Counts in ferritin (cpm/µg x µg ferritin/g) | | | | |
| SLOW | 158,158 | 178,176 | 1076,1075 | 1250,1240 |
| FAST | 147,145 | 96,99 | 498,499 | 2820,2825 |
| Total Protein Specific Act. (cpm $^{3}H$/mg) | 319,319 | 888,894++ | 137,308 | 694,688++ |
| % total counts in ferritin**** (slow plus fast) | 4.9 | 1.8 | 27.8 | 32.1 |

* I and II indicate two different sets of experiments:for females, 10 animals in each group, for males 14 animals in each group. Pooled samples from each group were analysed.

** Iron as ferric ammonium citrate (400 µg Fe/100 g BW) injected i.p. 5 hrs before death

*** $^{3}H$-leucine (100 µ Curies per 100 g BW injected i.v. 2 hrs before death. The two values in each case indicate duplicates of the same experiment.

+ $^{14}$C-U-leucine (10 µCi/100 g BW) were injected i.v. 2 hrs before death to 7 animals per group.

++ Using 0.05 ml homogenate for counts rather than 0.10 ml

TABLE IX

FERRITIN SYNTHESIS IN DIAPHRAGMS OF ADULT RATS

FEMALES*

| | Controls | | Iron Injected** | |
|---|---|---|---|---|
| | I | II | I | II |
| Total Protein (mg/g) | 106 | 93 | 125 | 103 |
| Ferritin Conc. (µg prot/g) | | | | |
| SLOW | 11.3 | 10.32 | 11.7 | 4.61 |
| FAST | 26.9 | 25.18 | 64.8 | 23.74 |
| Ft Specific*** Activity (cpm $^{3}H$/ leusine per µg) | | | | |
| SLOW | 13.97 | 0.67 | 44.57 | 3.65 |
| FAST | 9.69 | 0.57 | 12.48 | 5.74 |
| Total Counts in Ferritin (cpm/µg x ug ferritin/g) | | | | |
| SLOW | 120 | 7 | 236 | 27 |
| FAST | 199 | 15 | 363 | 225 |
| Total Protein Specific Activity (cpm $^{3}H$ leucine/ mg) | 867 | 832 | 858 | 696 |
| % Total Counts in slow plus fast ferritin | 0.35 | 0.30 | 0.56 | 0.35 |

TABLE IX (cont.)

MALES*

| | Controls | | Iron Injected | |
|---|---|---|---|---|
| | I | II | I | II |
| Total Protein (mg/g) | | 102 | 146 | 138 |
| Ferritin Conc. (µg prot/g) | | | | |
| SLOW | | 18.6 | 14.15 | 11.3 |
| FAST | | 5.5 | 22.10 | 2.6 |
| Ft Specific*** Activity (cpm $^{3}H$/ leucine per µg) | | | | |
| SLOW | | 1.04 | 23.18 | 2.82 |
| FAST | | 1.04 | 21.4 | 26.5 |
| Total Counts In Ferritin (cpm/µg x ug ferritin/g) | | | | |
| SLOW | | 13 | 214 | 10 |
| FAST | | 18 | 311 | 118 |
| Total Protein Specific Acitivty (cpm $^{3}H$ leucine/ mg) | | 563 | 589 | 568 |
| % Total Counts in slow plus fast ferritin | | 0.09 | 0.61 | 0.16 |

* I and II indicate two different sets of experiments performed under identical conditions; for females 10 animals in each group were analyzed.

** Iron as ferric ammonium citrate (400 µg Fe/100 g BW) injected i.p. 5 hrs before death

*** $^{3}H$-leucine (100 µCuries per 100 g BW) injected i.v. 2hrs before death.

ferritin in the female, but the two species appeared to be present in equal proportion in the male rats. Thus a sex difference appears to exist in the distribution of slow and fast heart ferritin in the normal condition in the rat. In the case of the male rat diaphragm (Table IX), the situation was reversed; the slower migrating species was more predominant. Thus the relative distribution of the two striated muscle ferritins depends on the sex of the animal and the type of muscle under consideration.

Iron administration stimulated slow and fast striated muscle ferritin synthesis in female and male rats. As measured by the uptake of $^{3}$H-leucine into ferritin protein (Tables VIII and IX). Total heart and diaphragm protein synthesis in males and female rats appeared to be unaffected by the administration of iron (Tables VII and IX). This situation is very much like rat liver where a substantial increase in ferritin synthesis is noted five hours after the administration of iron ( 171).

However, as can be seen in Tables VIII and IX, the degree of stimulation varied with the sex of the animal and the type of muscle under consideration. In female rat hearts, though the synthesis of both proteins was stimulated by the administration of iron, there was a greater incorporation of labelled amino acid into the fast ferritin species. This is because when one considers the total counts in ferritin, one takes into consideration the concentration of the protein in the tissue. In the case of the fast ferritin species, the concentration was much greater than the slower migrating species,

thereby explaining the higher total incorporation of labelled amino acid.

As mentioned earlier the relative proportions of the fast and the slow ferritin species are equal in normal male hearts. The administration of iron caused a change in this proportion as shown by the increase in the slow heart ferritin protein concentration. This was further supported by the finding of a six fold increase in total incorporation of $^{3}$H-leucine into the slow ferritin. Thus iron appeared to stimulate preferentially the synthesis of the slower migrating species in the male rat heart, and the reverse was true for the females.

In both female and male rats, there was no stimulation of total heart protein synthesis by iron, though there may have been a slight decrease in rate of synthesis in some cases (Expt. II, Males). It is also interesting that in the heart, there was a greater percentage of total counts in ferritin in males than in females, though in both cases there was an increased synthesis of ferritin following the administration of iron. In further contrast, if one looks at the female diaphragm the percentage of total counts incorporated into ferritin was greater than for the male diaphragm. It is hence important when one talks about slow and fast muscle ferritin synthesis to specify the sex of the animal and the muscle one is dealing with.

2. Relative turnover rates of slow and fast heart ferritin in female rats.

Since I have found that the slow and the fast ferritins were present in different amounts in the female rat heart,

and since the synthesis of both was stimulated by the administration of iron, I looked in a preliminary experiment at the relative turnover of the two species with and without iron administration. Double isotope labelling with $^{14}$C-U-Leucine and $^{3}$H-leucine over 3 days was used (see Methods for details) as a relative measure for the two species. The ratio $^{3}H/^{14}C$ reflecting how much $^{14}$C is left in a given species relative to the initial amount of radioactivity incorporated (the latter represented by the $^{3}$H activity).

Table IX shows that in untreated rats ratios of $^{3}H/^{14}C$ were different in the slow and fast heart ferritin. This implies that they are both degraded at different rates, the fast turning over more rapidly than the slow. This appears to be the case even after the administration of iron, though both proteins have higher relative turnover rates than in the untreated case. There is, however, no change in the relative turnover of total heart protein with the administration of iron in agreement with my earlier data where I found that total heart protein synthesis was not stimulated by the administration of iron.

TABLE X

TURNOVER RATE OF SLOW AND FAST HEART FERRITIN

To adult female Fischer rats 75 μ Curies per 100 gram body weight of $^{14}C$-u-leucine was administered i.v. by tail vein. Three days later 20 μ Curies per 100 gram body weight of $^{3}H$-leucine were administered by the same route and the animals were killed 2 hours later. During the entire period excess iron was present in one group. 25 mg Fe as imferon injected daily i.p. Five animals were in each group and the body weights were between 180-200 grams.

| | Untreated | | | Iron Injected | | |
|---|---|---|---|---|---|---|
| | $^{14}C$-dpm | $^{3}H$-dpm | $^{3}H/^{14}C$ | $^{14}C$-dpm | $^{3}H$-dpm | $^{3}H/^{14}C$ |
| Total heart protein (dpm/mg prot) | 130,70 | 310,300 | 2.37 | 124,800 | 282,200 | 2.26 |
| Slow heart ferritin (dpm/mg prot) | 120 | 3 | 0.030 | 140 | 150 | 1.1 |
| Fast heart ferritin (dpm/mg prot) | 260 | 220 | 0.86 | 260 | 950 | 3.6 |

TABLE XI

AMINO ACID COMPOSITION OF SLOW AND FAST HEART FERRITINS: RELATIVE RATIOS OF AMINO ACIDS

Ratio Relative to:

| Amino Acid | ASPARTATE | | ALANINE | | ISOLEUCINE | | PHENYLALANINE | | LYSINE | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Slow | Fast | Slow | Fast | Slow | Fast | Slow | Fast | Slow | Fast |
| asp | 1.00 | | 1.01 | 1.46*° | 3.58 | 4.38 | 3.95° | 3.71° | 1.33 | 1.93* |
| thr | 0.50 | 0.39 | 0.47 | 0.56 | 1.65 | 1.56 | 1.84 | 1.42° | 0.65 | 0.64 |
| ser | 1.08 | 0.67* | 1.09° | 0.99° | 3.90 | 2.98 | 4.70 | 2.52 | 1.46 | 1.29* |
| glu | 1.36 | 1.12 | 1.47 | 1.66 | 4.78 | 4.52 | 5.24 | 4.26 | 1.81 | 2.26* |
| gly | 1.52 | 0.72* | 1.54° | 1.05° | 5.36 | 3.63 | 6.84 | 2.67° | 2.08 | 1.36 |
| ala | 0.99 | 0.67* | 1.00 | | 3.54 | 2.98 | 3.90 | 2.53* | 1.24 | 1.28 |
| val | 0.56 | 0.38* | 0.57 | 0.63 | 2.01 | 1.80 | 2.17 | 1.40*° | 0.75 | 0.73 |
| ilen | 0.28 | 0.23 | 0.28° | 0.34° | 1.00 | | 1.09 | 0.85* | 0.37 | 0.45 |
| leu | 0.96 | 0.83 | 0.97?° | 1.21° | 3.37 | 3.58 | 3.17 | 3.05 | 1.22 | 1.64 |
| tyr | 0.32 | 0.16 | 0.32 | 0.26 | 1.18 | 0.78 | 1.60 | 0.65 | 0.45 | 0.34 |
| phe | 0.31 | 0.27 | 0.31 | 0.39 | 1.06 | 1.17 | 1.00 | | 0.38 | 0.53* |
| his | 0.28 | 0.33 | 0.28 | 0.48 | 0.97 | 1.42* | 0.93 | 1.27* | 0.35 | 0.68* |
| lys | 0.78 | 0.52* | 0.77° | 0.76° | 2.72 | 2.27 | 2.89° | 1.90 | 1.00 | |
| arg | 0.49 | 0.36 | 0.25° | 0.56 | 1.97 | 1.64 | 1.26 | 1.40 | 0.77 | 0.78 |
| pro (2) | 0.04 | 0.03 | 0.04 | 0.04 | 0.13 | 0.11 | 0.09 | 0.09 | 0.05 | 0.06 |
| cys (2) | 0.07 | 0.05 | 0.01 | 0.09 | 0.22 | 0.20 | 0.15 | 0.18 | 0.08 | 0.10 |
| meth (2) | 0.05 | 0.05 | 0.01 | 0.07 | 0.17 | 0.18 | 0.12 | 0.16 | 0.06 | 0.10 |

* differs from slow heart ferritin ($p < 0.05$)

° differ from rat liver ferritin ($p < 0.05$) (Linder, M.C., Moor, J.R., Munro, H.N., Morris, H.P. (1975) B.B.A. 386, 409-421.

No. of detns. in all cases were 4-5 except for proline, cysteine and methionine

TABLE XI A

AMINO ACID ANALYSES* OF SLOW HEART FERRITIN,

FAST HEART FERRITIN AND ADULT RAT LIVER FERRITIN

| Amino Acid | Slow heart Ferritin | Fast heart Ferritin | Adult Rat Liver Ferritin |
|---|---|---|---|
| asp | 474 | 726 | 489 |
| thr | 218 | 262 | 199 |
| ser | 517 | 501 | 204 |
| glu | 633 | 760 | 654 |
| gly | 710 | 610 | 295 |
| ala | 469 | 501 | 419 |
| val | 266 | 303 | 191 |
| ileu | 133 | 168 | 72 |
| leu | 447 | 602 | 628 |
| tyr | 156 | 131 | 117 |
| phe | 140 | 197 | 202 |
| his | 129 | 239 | 174 |
| lys | 360 | 382 | 248 |
| arg | 261 | 276 | 280 |
| meth | 23 | 30 | 20 |
| cys | 29 | 32 | - |
| pro | 19 | 17 | 207 |

Results expressed as residues/mole were calculated from amino acid ratios and ferritin molecular weights (Table II)

# V. Discussion

This thesis has attempted to define more clearly the structural and metabolic features of the striated muscle isoferritins (slow and fast) to provide an insight into the reason for the presence of two ferritins in a single tissue.

## 1. Ferritin Structure

Ferritin structure is complicated by various types of heterogeneity and structural complexity. Gel electrophoresis studies on the human (150), rabbit (153a) and rat (152) demonstrated different migration rates for ferritins from different organs of the same animal. In the rat, Linder and co-workers (3) reported three distinct ferritin species migrating at different rates: one characteristic of adult liver also present in cardiac and skeletal muscle, a second found in kidney, spleen and some hepatomes, and a third characteristic of cardiac and skeletal muscle. These differences in electrophoretic migration could have been differences in size and/or charge of the ferritins. Elaborate studies reported by Linder and workers (153) including amino acid composition analysis and peptide maps suggested that mainly charge differences were involved amongst the various rat tissue ferritins, thus accounting for their electrophoretic behavior. In order to investigate if apart from charge differences, size differences were also involved. I used the quantitative polyacrylamide gel electrophoresis technique (225).

Using this technique, I have found that all the rat tissue and horse spleen ferritins could be differentiated from each other based on their 95% confidence limit ellipses for retardation coefficient and relative free mobility (fig. 4-6) and in contrast to the lack of differentiation of some of these in electrophoresis at one acrylamide gel concentration. These observations are thus in general agreement with more laborius and elaborate studies of amino acid composition, sulphydryl group content, proportions of subunits and distinctive peptide maps following trypsin digestion which showed that ferritin extracted from adult kidney, neonatal liver, hepatic and renal tumors differed from adult liver ferritin and each other in some respect (153). The two heart ferritin ellipses differed from each other and the ellipse of the slow heart ferritin only partially overlapped with the ellipse of rat liver ferritin (Fig. 5) suggesting that there were slight differences in the size and/or charge of the slow heart and liver ferritins. This is in contrast to earlier electrophoretic studies (152) at one gel concentration in which slow heart and rat liver ferritin were found to have the same electrophoretic mobility. Part of the reason for the discrepancy may be that in this system where one is operating in a range of acrylamide gel concentrations, the small size and charge differences become apparent as the pore size of the gel decreases.

The molecular weight for all ferritin major components from different tissues except the heart ferritins

was in the range of 420-490,000. These estimates agree with available estimates in the literature (138-141) for the molecular weight of the major component of horse spleen apoferritin. The sedimentation-diffusion value, 480,000, measured by Rothen (140) and the molecular weight of 480,000 obtained from crystal unit cell and density measurements by Harrison (138) were both based on the measured partial specific volume ($\bar{V}$), 0.747. Using instead a $\bar{V}$ of 0.731-0.733 calculated from the amino acid composition, the above weights become approximately 440,000 as reported by Harrison (143) and 450,000 as reported by Crichton (233). Sedimentation equilibrium experiments on purified horse spleen apoferritin monomer by Bjork and Fish (146) give values in the range of 456,000. Light scattering experiments by Richter (148) for horse spleen apoferritin gave a value of 431,000. Within the error of our procedure our size estimations thus agree.

Our estimates of the molecular weight of the minor components shown to be dimers for horse spleen ferritin (143) are also consistent with dimer formation (Table III). The molecular weight for the horse spleen ferritin minor component (Table III) agrees with Bjork and Fish's (146) recent determination of 895,000. In the case of the rat liver ferritin minor component we obtained a molecular weight value greater than any previously reported for dimers (MW 1,100,000). However, on the basis of a statistical evaluation of the retardation coefficient ($K_R$), this difference was not statistically significant (Table II).

If there do exist differences in molecular sizes between the rat liver ferritin minor component and other dimers, this technique was not sensitive enough to detect them. Part of the reason for this insensitivity of PAGE is due to the fact that that at such high molecular weight ranges, there is little electrophoretic migration in the range of gel concentrations (4-8) used in this technique. Consequently, the errors in the estimate for size become very large.

It was of interest that in comparing the relative charges of major and minor components by PAGE, we found that relative charges varied considerably from one tissue to another. Charges of major and minor components were similar in the case of horse spleen ferritin, approximately twice as great for the minor component of rat hepatoma ferritin and five times greater for the minor as compared to major component of rat liver ferritin (Tables II, III). These observations in the case of horse spleen and rat liver ferritins were confirmed in minimally sieving gels (Table III).

Our results on the horse spleen ferritin monomers and dimers are compatible with Harrison's earlier work (144). In her experiments, horse spleen ferritin was fractionated by starch gel electrophoresis and by gel filtration on Sephadex G-200 to provide a separated monomer fraction which contained 98% monomer, and an oligomer fraction which contained 76% oligomers. The latter was determined by quantitative electron microscopy. Amino acid analysis of these monomer and oligomer enriched fractions showed no significant

differences between them in terms of the proportion of the various amino acids. This suggests that the horse spleen ferritin dimer is composed of two monomers clipped together which have the same composition as the unattached monomer. Our results on the charge relationships between hepatoma ferritin major and minor components would fit into the above scheme. In the case of rat liver ferritin, however, the minor component has a much greater charge than the major component, and there it is possible that the amino acid composition of the two components is very different. Thus the rat liver ferritin dimer could be the result of a different kind of dimerization process where more negatively charged groups are exposed on the surface of the minor component. Another possibility is that the dimerization process takes place in another part of the cell or even another cell type.

Part of the reason for the apparent charge of the rat liver ferritin dimer could be due to changes in the non-spherical shape of the molecule. The calculation of net charge and apparent valence by PAGE involves many assumptions, many of which relate to the shape of the molecule. Therefore, any peculiarity in the shape of the rat liver ferritin minor component could result in the very high apparent valence and net charge. However, this doesn't explain the result in non-sieving gels.

It is worth pointing out that Bjork (147) has recently studied hydrodynamic parameters of horse spleen ferritin such as

frictional ratio and intrinsic viscosity and has concluded that in solution both monomers and dimers of apoferritins have a slightly more extended or irregular shape than most typical globular proteins. This irregularity in shape of the horse spleen apoferritin molecule goes against x-ray diffraction studies by Harrison (144) on crystalline horse spleen ferritin where it appears that apoferritin is a molecule composed of a spherical shell of subunits with a hollow center where solvent molecules presumably take the place of the iron in ferritin. The rat tissue ferritins have not been subjected to either the x-ray diffraction techniques or the measurement of hydrodynamic properties and thus we have no information on peculiarities in the shape of these proteins.

To conclude, it appears that ferritin dimers may be formed in different tissues in different ways. The nature of the interactions between apoferritin molecules to form an oligomeric series is still controversial and uncertain. But in any event, it cannot be explained on the basis of a consistent mechanism of binding of just any two monomeric units from a general monomer pool in different tissue.

There has been some controversy about the size and total number of subunits of the ferritin protein shell. Originally, for horse spleen ferritin Harrison (138) concluded from x-ray diffraction data that there were 20 identical subunits, of about 22,000 to 27,000 molecular weight but Crichton (164) concluded on the basis of SDS electrophoresis and the methionine residue content after cyanogen bromide cleavage that the protein shell of horse spleen ferritin contains 24 identical subunits of molecular weight 18,500. Nowever, Niitsu et al (167, 168) and Linder et al (165) found multiple species of subunits in ferritins isolated from the tissues of various animals. Recently Crichton has suggested (231) that the smaller subunits observed by various groups (231) obtained following the dissociation of ferritin are in fact breakdown products of the major, 19,000 subunit due to proteolysis that occurs while heating the homogenate to $70^{o}$ in the purification procedure. I found (Fig. 9) that liver ferritin obtained with and without heating contained the same smaller that seems to be 12-13,000 and 10,000 subunits as well as the larger 19,000 subunit and in the same proportions. Thus, the smaller subunits of ferritin are not fragments of the larger (19,000) subunit. These observations support the independence of the large and small subunits but it isn't really a matter of compatibility shown by the double labelling studies of Linder et al (165) on rat liver apoferritin. These demonstrated that synthesis and turnover of the large 19,000 and small 13,000 subunits occur at different rates and that they respond differently to iron treatment. This independence is also

consistent with the evidence of Konijn, Baliga and Munro (166) that ferritin subunits of different molecular weights are made on different polysome populations, the smaller on the free and the large on the bound polyribosomes.

2. Structure of Heart Ferritins

One of the objectives of these structural studies was to determine how slow and fast ferritins of heart differed structurally from each other and other rat tissue ferritins. Fast ferritin had a higher electrophoretic mobility in polyacrylamide gels than other tissue ferritin monomers, required three times the amount of antibody for optimum precipitation, and both slow and fast heart ferritins appeared at different times during growth and development. On the basis of quantitative polyacrylamide gel electrophoresis, we found that the slow and fast heart ferritins differed on the basis of non-overlap of their 95% confidence limit ellipses (Fig. 5). As already mentioned, slow heart ferritin and rat liver ferritin (major component) also appeared to differ slightly. The heart ferritins have distinct amino acid compositions from themselves and adult rat liver ferritin. When the sizes of the heart ferritins is considered, this difference is even greater. Both the fast heart and adult liver ferritins contain the same relative concentrations of basic amino acids. The concentrations permole is different. Thus the striking differences in electrophoretic mobility observed in polyacrylamide gels is probably due to the effect of size more than charge. As has been discussed in "Results"

the slow and fast heart ferritins are significantly larger than other tissue ferritin monomers and the fast heart ferritin is larger than the slow heart ferritin as well.

There are many possibilities that may underline the apparent size difference between the heart ferritins and other tissue ferritin monomers. I explored a few of them. The first was that the iron content of the molecule might contribute to its size and shape. This was considered because there is a two-fold increase in actual molecular weight when there are 5,000 atoms of iron in ferritin, as when it is fully saturated (450,000 protein + M.W. x 5,000 for Fesalt) (163). As the apoferritins exhibited the same size behavior or gels as holoferritins (Fig. 3), this possibility was ruled out.

A second idea I explored was that there might be a larger subunit or subunits in fast heart ferritin. This did not appear to be the case as all three ferritins tested (slow and fast heart and liver ferritins) contained the same 19,000 major subunits and no larger subunits were detected in either of the heart ferritins (Fig. 10).

The third possibility was that the same sized subunits present in other ferritins are present in different proportions and total amounts in the slow and fast ferritins so that the total molecular weight of one exceeds the other and both exceed that of rat liver ferritin. I found that the mixture of the heart ferritins contained the same sizes of subunits as rat liver ferritin (Fig. 10) in agreement with the preliminary studies of Linder and co-workers (234) on separated heart ferritins. These studies also suggested that the proportion

of large to smaller subunits varied in the cases of slow and fast ferritins lending support to the concept of varying subunit proportions as the origin of the size differences observed.

The fourth possibility (which I have not pursued) is that the shape particularly of the slow and fast heart ferritin molecule may account for the apparent size difference calculated by quantitative polyacrylamide gel electrophoresis. In the earlier discussion on the monomer-dimer relationships of rat liver ferritin, I mentioned Bjork's (147) findings that horse spleen apoferritin monomer may in fact have an irregular shape than most typical globular proteins.
Such parameters have not been measured for the rat heart ferritins and may provide useful information.

There are a number of reports in the literature that individual ferritins may be fractionated into a series of different components by isoelectric focusing. Possible causes for differences in isoelectric point surface charge could be differences in primary structures of the subunits resulting from multiple genes or post-translational changes such as de-amidation, acetylation, iron binding, etc. Recently, Drysdale (156) has suggested on the basis of gel electrophoresis with sodium dodecyl sulphate or acidic urea using horse spleen ferritin that each of the bands seen on iso-electric focusing may contain different proportions of subunits of molecular weight 19,000 and 15,000, and hence that horse spleen ferritin consists of a family of stable structural variants that may be separated by iso-electric focusing.

However, no definitive evidence was presented to resolve this question.

We found that isoelectric focusing of tissue ferritins in polyacrylamide gels gave bands, none of the profiles being identical (Table IV). On the whole, the number of bands I obtained were less than Drysdale's (154) from studies of horse spleen ferritin and human ferritins. Unlike other rat tissue ferritins, the rat heart ferritin mixture showed two bands (Table VI) presumably corresponding to the two seen in electrophoresis. This observation also differed from those of Drysdale (156) on human heart ferritin which he found resolved into multiple bands on isoelectric focusing.

This disagreement between our studies on rat heart and Drysdale's could be attributed to a species difference or a technical difference. The gels used in our system are slightly different from Drysdale's and this may be an important factor.

There is considerable debate in the literature about the significance of multiple isoelectric focusing peaks. The widely debated issue is the limitations of the isoelectric focusing technique itself. It has been suggested by Richter (159) that the multiple isofocusing bands of ferritin result from interaction of the protein with the constituents of the pH gradients. He substantiates this by citing evidence (161, 162) that a homogenous macromolecule such as L-amino acid oxidase was shown to interact with small constituents of buffer, including the ampholytes used to obtain the pH gradients for gel isoelectric focusing.

Another interesting idea that has not been pursued is based on the fact that ferritin preparations are not homogeneous since they are a mixture of monomers and oligomers to begin with. On the basis of the information I obtained earlier on the relationship of sizes and charges of major and minor ferritin components, and Bjork's data (147) on the irregular shape of horse spleen apoferritin monomer and dimer, it is very possible that the dimers contribute to the multiple bands. Preparations of purified monomers and dimers ferritin have not been run individually in isoelectric focusing which would substantiate or refute the above suggestion.

Other ways of explaining the different surface charges of the isofocusing peaks could be different primary structures resulting from multiple genes, or post-translational changes such as de-amidation, acetylation, ion binding, etc. None of these have been studied.

To summarize my studies on the structural features of the rat heart ferritins indicate that they are different from each other and other tissue ferritin major components in terms of size and charge. Both heart ferritins and especially the fast ferritin appear to be of a unique and larger size than other tissue ferritins. Slow and fast ferritins, however, appear to have the same subunits as other ferritins at least as regards size and they are not composed of two families of hybrid molecules with slight charge differences and allow separation on isoelectric focusing. These structural features of the cardiac ferritins do suggest that the functions and capacities of the two heart ferritins may be quite different from that of other tissue ferritins.

### 3. Cellular Location and Iron Storage Functions of Slow and Fast Heart Ferritins

The heart is a heterogenous organ consisting of different cell types with specified functions. Thus in assessing the functional advantage of having two ferritins in the heart, it is important to determine the cellular location of the two ferritins and this may give further clues to their function. Since it is difficult to separate cardiac cells for analysis, I approached this problem by studying slow and fast ferritin concentrations in different muscles of the rat and chicken, and under conditions of experimental cardiac hypertrophy where one cell type preferentially enlarges. With these studies I also explored the possibility that one or both ferritins act as an iron donor for myoglobin synthesis.

Under conditions of connective tissue cell hypertrophy, there was a slight increase in the heart ferritin concentrations (Table VII). More importantly, there was a large change in the relative proportions of fast and slow ferritins in these hearts. This change in the relative proportion of the ferritins was due to an increased concentration of the slow ferritin and a decreased concentration of the fast ferritin (Table VIII), and if one considers the heart weight increase, the change was even greater. Thus, under conditions of experimental cardiac hypertrophy, where it is well documented that this only involves an increase in connective tissue cells (67) and connective tissue protein synthesis is enhanced (68, 69), there is a dramatic increase in the concentration of the slow ferritin which parallels the

increased synthesis. The fact that the concentration of slow heart ferritin is greatly enhanced in this situation of enhanced connective tissue cell proliferation, suggests that the slow heart ferritin may be present in the connective tissue cell. But this does not rule out the possibility that the slow and fast heart ferritin are both present in the connective tissue cell, especially since there is a decline in fast heart ferritin concentration as the concentration of the slow increases during connective tissue cell hypertrophy. This is compatible with the idea that both ferritins are in the same cellular location and fast ferritin gives up its iron to the metabolically more active slow ferritin form.

Another possibility is that there is a more fundamental precursor-product relationship between fast and slow heart ferritins. The rapid decline in the fast heart ferritin concentration and the tremendous increase in the slow heart ferritin concentration with no change in total ferritin concentration even suggests that the fast heart ferritin is being converted to the slow heart ferritin. On administration of excess iron, there is an increase in the concentration of the fast heart ferritin and a return of the proportion of the two heart ferritins to control values. The fast heart ferritin may thus be the main iron storage form being the first to give up its iron, thereby being converted to the slow form and the first to take up iron when it is administered in excess. Support for the fast ferritin being the initial product comes from the fact that the incorporation of radioactive leucine (Table VIII) is greater in fast heart ferritin

as compared to slow heart ferritin over the 2 hours time course examined. Further evidence for this concept is on the basis of $^{59}Fe$ uptake studies (Linder et al, unpublished) showing that the label first enters fast heart ferritin and over time appears in the slow heart ferritin and also myoglobin.

The fact that in contrast to connective cell hypertrophy in muscle cell hypertrophy there is no change in the relative proportions of slow and fast heart ferritins (Table VII) suggests one of two possibilities. The first is that both slow and fast heart ferritins are present in the connective tissue cell as stated above and thus one would not expect to find any change in their relative proportions under conditions of muscle cell hypertrophy. The other possibility is that the degree of hypertrophy in this case was not sufficient to see any marked change in the relative proportion of slow and fast heart ferritin. Another possibility of course is that there is no link between ferritin concentration and muscle cell hypertrophy.

The relationship between ferritin and myoglobin was explored by examining the concentrations of these proteins in white and red muscles of the rat and chicken (Tables V, VI). The question was whether the presence of slow or fast ferritins could be correlated with the presence of myoglobin. In support of the idea of a positive relationship, the gastrocnemius muscle of the rat contained appreciable amounts of total iron and ferritin and the soleus muscle of the rat contained very little iron and no ferritin (Table V). The data obtained for

total iron levels supports the idea that the soleus contains little myoglobin and is thus white. Even excess iron administration did not cause an increased deposition of iron in the soleus muscle and in this condition as well, no ferritin was detected. The possibility does exist that the soleus muscle doesn't take up iron.

Nevertheless, interpretation of the findings on the iron and ferritin content of the gastrocnemius and soleus muscles become more complicated when one becomes aware of the disagreement among anatomists about the histochemical classification of the soleus muscle. It is believed by many that it contains more than 80 percent of intermediate fibres (235). My data strongly support the latter. If the soleus were a pure red muscle, my data would indicate that there is a lot of heterogeneity among red muscles, since there is such a dramatic difference in their total iron and ferritin iron levels (Table V).

It becomes even more complicated when one considers the data on the distribution of ferritin in red, white and mixed muscles of the chicken. The presence of ferritin in white, non-myoglobin containing muscle (Table V) argues against the proposed relationship of a specific ferritin and myoglobin. It would be of interest in this respect to establish the cellular location of chicken muscle ferritin especially as techniques are available to isolate chicken muscle cells. It was also noted that the chicken muscles had less total iron than the rat muscles and only one ferritin species was present.

Chicken muscle ferritin also cross-reacted with horse spleen ferritin antibody (Fig. 11). It is thus serologically related to human, horse and rat ferritins (176) unlike phytoferritin which is not precipitated by antihorse ferritin serum. This indicates that there must be antigenic determinants on the molecule that are similar to those of other ferritins. Furthermore, chicken ferritins had the exact same electrophoretic mobility at one gel concentration as rat liver ferritin (Table VI) unlike human liver and horse spleen ferritins (143), which have a greater charge.

The absence of ferritin in soleus muscle of the rat also does not support a hypothesis that slow and fast muscle ferritins are present in the connective tissue cell. This is because white muscle contains more connective tissue cells than red muscle and the absence of the ferritins in white muscle implies that they are not present in that tissue. The other possibility is that the slow and fast heart ferritin are present in the connective tissue cells only in cardiac muscle and that their cellular location thus varies from muscle to muscle.

It also appears that the fast heart ferritin acts preferentially in iron storage and donation. Under conditions of cardiac hypertrophy, it preferentially gives up its iron (to the slow ferritin species) and on administration of excess iron, there is an increase only in the concentration of the fast heart ferritin. The fact that the fast heart ferritin acts mainly in iron storage goes against Linder and Munro's

(3) earlier suggestion that the slow heart ferritin is the primary iron storage protein and the fast heart ferritin an iron donor for myoglobin synthesis. Their conclusion was based on information on the developmental appearance of the two heart ferritins namely that, though they are both present at birth, the slow heart ferritin disappears sometime during suckling when storage iron is rapidly depleted and the slow form only reappears later in life, whereas the fast heart ferritin is present all along. It is possible that during growth and development the iron storage function fluctuates between the fast and the slow heart ferritins.

3. Metabolism of Low and Fast Heart Ferritins

Closely related to cellular location and function of the fast and slow ferritins is their metabolism, though the total concentration of heart ferritin in male and female rats is the same, in the female rat heart and diaphragm, the concentration of the fast ferritin is greater than that of the slow, the situation is reversed in the male diaphragm, and about equal in the case of the male heart (Tables VIII and IX). There is thus a sex difference existing in the proportion of slow and fast heart ferritins, similar to the sex difference observed in liver ferritin stores (132). The basis for the sex difference in liver ferritin stores probably is due to an effect of estradiol on intestinal iron absorption in the female rat, and that the extra iron absorbed was probably deposited as ferritin. Whether estradiol or any other hormone affects the relative contents of slow and fast heart ferritins is not known but should be investigated.

In both sexes, under normal conditions, there is much greater incorporation of labelled amino acid into fast ferritin (Tables VIII and IX) suggesting that there is probably more fast ferritin mRNA in the cell. In all cases, iron administration stimulates ferritin synthesis though the degree of stimulation of synthesis of the two ferritins with iron was not entirely predictable from one experiment to another and the stimulation of one was not always greater than for the others but synthesis of the fast ferritin species was greater than of the slow. The lack of predictability suggests some independence of regulation and perhaps also independent pools of mRNA for the two species. Further support for this concept comes from data for the relative turnover rates of the two ferritins (Table X) which again differed. Independent pools of mRNA for the heart ferritins would favor the idea that the two ferritins are located in different cells. At the same time there is the possibility that if slow and fast heart ferritin were present in the same cell, there are two different intracellular pools of ferritin mRNA.

To summarize, there are three possibilities regarding the location and function of slow and fast heart ferritins. The first is that they are present in different cell types. Our data suggest that the slow heart ferritin is probably present in the connective tissue cell. The metabolic studies pointing to different pools of mRNA and independent regulation of synthesis and degradation for slow and fast heart ferritin also suggest different cellular location. The

second possibility is that slow and fast heart ferritins are both present in the connective tissue cell. Support for this idea comes from the cardiac connective tissue cell hypertrophy experiments, where there were dramatic and opposing changes in the concentration and relative proportion of slow and fast heart ferritins, while no such change was apparent in muscle cell hypertrophy. However, data on the rat gastrocnemius and soleus muscles suggest instead that since the slow and fast ferritins were absent in the more predominantly connective tissue cell containing soleus muscle, they may in fact both be located in the muscle cell.

The third and possibly most interesting idea is that the fast heart ferritin may in fact be a precursor to the slow heart ferritin and this again suggests the presence of both species in the same cell. Strong support for this hypothesis is that the fast ferritin functions as an iron storage protein and gives up its iron as the iron content of the slow heart ferritin increases and takes up iron again when excess iron is administered. Furthermore, $^{59}Fe$ uptake studies (Linder and co-workers, unpublished) show the label to be taken up first by the fast ferritin which loses it to slow ferritin and myoglobin over time. The deciding question of whether there exists a precursor-product relationship would be to do a time course experiment and see the appearance/disappearance of label in each ferritin species. Which of these possibilities actually exists is difficult to say at this stage, but this thesis has defined more clearly some

structural and metabolic features of the ferritins and thus opens the doors to a lot of unexplored areas relating to cardiac ferritin function and metabolism.

## VI. Summary

Two distinct ferritins are found in cardiac and skeletal muscles of the rat. The purpose of this thesis was to assess the functional advantage of having two such ferritin proteins in a single tissue. This has been done by defining more clearly the structural and metabolic features of these two proteins under a variety of physiological conditions. Most of the work focused on rat cardiac ferritins as they are present in the highest concentration in striated muscle.

1. Molecular sizes and apparent valences of the heart ferritins in relation to other tissue ferritins were estimated by quantitative polyacrylamide gel electrophoresis. By this technique, all rat tissue and horse spleen ferritin monomers were distinguished from each other in terms of size and or/charge. The slow and the fast heart ferritins were significantly larger than other tissue ferritin monomers, and in agreement with a higher electrophoretic mobility, the fast heart ferritin had a higher apparent valence.

2. The molecular sizes of ferritin dimers were compatible with dimerization of monomer but dimer charges varied in relation to monomer charge from one tissue to another suggesting different mechanisms of dimer formation.

3. Examination of the subunits of rat liver ferritin indicated that heating had no effect on the presence of the

small ferritin subunits and that the slow and fast heart ferritins had the same large and small subunits found in other rat tissue ferritins. Isoelectric focusing of the mixed S & F heart ferritins revealed only two bands. Other tissue ferritins had multiple bands, no two of which were identical.

4. The metabolic studies were concerned with investigating the cellular location and function of the heart ferritins. The relationship between ferritin and myoglobin was examined by determining the ferritin content of red and white muscles. There was a positive correlation for rat muscles but no correlation for chicken muscles. Thus the relationship is still unclear. Chicken muscles had only one electrophoretically distinct ferritin in all muscles tested.

5. Experimentally induced connective tissue cell hypertrophy in the hearts of rats produced dramatic changes in the relative proportion of slow and fast ferritin. These changes were due to an increase in the concentration of the slow ferritin suggesting its presence in the connective tissue cell coupled with a dramatic decrease in the concentration of the fast ferritin. Administration of excess iron to the hypertrophic animals produced an increase in the concentration of fast ferritin, with no change in the slow, and the relative proportion of the slow and fast ferritins thereby returned to a normal value. Thus under these conditions

the fast ferritin appears to be the most related with the storage of iron. There was no change in the concentrations and relative proportions of slow and fast ferritin in muscle cell hypertrophy so no obvious conclusions may be drawn.

6. Iron administration increased synthesis of both the slow and fast ferritins in heart and diaphragm of male and female rats as measured by the uptake of $^{3}$H-leucine into ferritin protein. Total heart and diaphragm protein synthesis was unaffected by the administration of iron. There was a notable sex difference in the distribution of slow and fast cardiac ferritin in male and female rats. On the basis of average values, both ferritins were stimulated to the same degree however in both sexes synthesis of fast ferritin was greater than slow, with or without iron administration.

7. The relative turnover rates of slow and fast ferritins differed in the normal state, the fast turning over more rapidly than the slow. This was the case even after the administration of iron. Fast ferritin was more active in its synthesis, turnover and response to iron.

## VII. SUGGESTIONS FOR FUTURE RESEARCH

This thesis has tried to assess the functional advantage of having two ferritin proteins in a single tissue. It has broadly covered the areas of structure, cellular location and function, and metabolism. There are a number of questions that arise by studying the implications of the data presented Some of them are as follows:

1) Ferritin Structure.

The analysis of size and charges of rat tissue and horse spleen ferritin by quantitative polyacrylamide gel electrophoresis raises a number of questions about general ferritin structure which I was unable to pursue. The monomer-dimer relationship of rat liver ferritin and whether the two components are of the same or different peptide origins is worth investigating. On separated fractions of monomer and dimer, a number of structural analyses such as X ray diffraction, SDS subunit analysis, amino acid compositions, peptide maps and other studies will provide useful information. Isoelectric focusing studies would be useful on the separated monomer and dimer fractions to see if the multiple bands observed on isoelectric focusing samples were due to the dimers and oligomers present in the sample.

The cardiac ferritins differ in size but have the same large 19,000 molecular weight subunit. Hence the proportion and number of the larger and smaller subunits (that I detected

in the mixture but not in the separated slow and fast) which combine for the ultimate arrangement of their structure is of interest to determine. If the slow and fast ferritins can be separated on a large scale, by SDS electrophoresis in cylinderical gels followed by scanning in the Gilford Spectrophotometer at 280 mu, the relative proportions of the large and small subunits can be compared. Since we know the molecular weights of slow and fast heart ferritins as determined by PAGE the number of each size subunit could thus be calculated. The arrangement of the subunits in the protein shell can only be investigated with X ray diffraction techniques and may be difficult to determine in a protein containing multiple subunits.

Isoelectric focusing of the mixture of the heart ferritins shows only two bands. It would be possible to determine whichband belongs to which ferritin species by isoelectrifocusing of the individual slow and fast heart ferritins. Peptide maps of the heart ferritin mixture after trypsin digestion showed 7 additional spots as compared to adult rat liver ferritin. It would be useful to know which of these additional peptides belong to slow and fast heart ferritins and this can be determined with peptide maps of the individual species.

A relatively unexplored area in ferritin structure which could be important for our understanding of slow and fast heart ferritin structure is whether any of the subunits

have any carbohydrate moieties as suggested by Listowsky (personal communication). Ferritin samples would have to be assayed for the carbohydrate content and following that, the sugars involved would have to be characterized. Much work is needed in this area and it is possible that the larger sizes observed in slow and fast heart ferritins is due to there varying carbohydrate content.

2) Cellular Location and Function.

A basic question this thesis has begun to try to answer is the cellular location of the slow and fast cardiac ferritins. As has been stated many times in this thesis, the heart is a very heterogeneous organ consisting of different cell types. Techniques are now available for obtaining enriched cardiac muscle cell and interstitial cell fractions and the intracellular location of the ferritins could be studied with these methods. Optimum conditions will have to be worked out for obtaining these fractions. This aspect is a thesis in itself but is a very important point as all studies I have performed are on whole heart homogenates which automatically do not consider the individual cells synthesizing the proteins.

Apart from cell separation techniques, the intracellular location of the proteins may also be assessed using young rat hearts where the slower ferritin species is missing, by coupling autoradiography with immunofluoresence. Once the molecule is located, iron injected young rat hearts where

the slower migrating species is induced are subjected to the same procedure. These are difficult techniques but if performed would provide a great insight in the general metabolism of these proteins.

Closely related to the question of cellular location is that of cellular function, and in particular relationship with myoglobin. This relationship, may be explored in detail in the following manner.

(a) Precursor - Product relationship.

$^{59}Fe$ as ferric chloride in saline, may be administered intravenously via the tail vein to adult male and female rats. The incorporation of the label into fast ferritin, slow ferritin, myoglobin and blood can be measured after the administration of the tracer. The purpose would be to see which protein picks up the label first and whether the same protein donates iron to the other proteins. The disappearance of label in one protein followed by the appearance in another protein would establish a precursor-product relationship.

(b) Concentration relationship between ferritins and myoglobin

In the normal state, in order to establish relationships between the three proteins it may be useful to compare their concentrations in different muscles. I did not measure myoglobin concentration but rather total iron levels in my studies. It will also be useful to determine the concentrations of the ferritins and myoglobin in iron deficiency where it is known

that the myoglobin concentration is depressed ( 46 ). This would provide not only information about the ferritin-myoglobin relationship, but also on the iron storage functions of either or both ferritins.

3) Metabolism

An interesting possibility suggested by the metabolic studies is that the fast heart ferritin is a precursor to the slow heart ferritin. The deciding question of whether there exists such a precursor - product relationship at the level of protein synthesis would be to do a time course experiment. Either $^{3}H$- or $^{14}C$- leucine may be administered to the rats and the incorporation of the label in slow and fast ferritin protein over time maybe followed. If such a precursor-product relationship does exist, then the label would initially appear in the fast heart ferritin and gradually over time appear in the slow heart ferritin species.

I have not been able to do any work on the developmental aspects of slow and fast heart ferritin. It would be interesting to pursue the initial developmental observations of Linder and Munro (3) that though both proteins are present at birth, the slow heart ferritin disappears sometime during weaning and appears at a later stage in development. It would be important to see if any of the ferritins in the heart undergo the same changes as liver ferritin does throughout the developmental process ( 2 ). This would provide information about the iron storage properties of the proteins and more importantly

whether iron can be mobilized from the heart during development just as it is from the liver. The ferritin levels in the hearts of fetuses also would be useful in assessing the above problem.

Another relatively unexplored area is if their exists any relationship between serum ferritin and cardiac ferritin. The most important point to determine would be whether serum ferritin might be structurally similar to slow and fast heart ferritin thus suggesting a cardiac origin for serum ferritin. This can be done if adequate material is obtained using the quantitative polyacrylamide gel electrophoresis technique for estimations of sizes and charges, SDS subunit analyses and amino acid composition analyses.

A interesting but difficult question that maybe worth pursuing in the light of these studies is the role of cardiac ferritin in cardiac hypertrophy. In the light of the work on serum ferritin, one might look for the release of heart ferritins into the plasma in cardiac hypertrophy. Two ferritins have been identified also in the human heart. The problem with doing any studies on the human is that all work would have to be confined to hearts obtained on autopsy. Iron is known to be deposited in the heart under conditions of iron overload. It would be useful to see the concentrations of the two ferritins under these conditions.

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