CONTROL MECHANISMS IN PROTEIN SYNTHESIS AND TURNOVER

with Special Reference to Liver Ferritin

by

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ABBREVIATIONS

The following abbreviations have been used in the text.

ATP	adenosine 5 - triphosphate
CM-cellulose	carboxymethyl cellulose
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
s-RNA	soluble ribonucleic acid
PCA	perchloric acid
TCA	trichloroacetic acid

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INTRODUCTION

Proteins are essential constituents of all living organisms. The average mammalian cell may contain as many as 10,000 different proteins with a bewildering diversity of function. Considerable progress has recently been made in the elucidation of the chemical reactions involved in their biosynthesis and breakdown. Remarkably little, however, is yet known of the mechanisms controlling their rates of synthesis and turnover in cells. These mechanisms are central to an understanding of the processes of cell differentiation and of the neoplastic state. This work describes investigations into some of the factors which regulate the level of an iron storage protein called ferritin in rat liver. It is hoped that these studies might indicate similar control mechanisms operating for other mammalian proteins.

The protein content of mammalian organisms is maintained at a remarkably constant level. However, this obscures the wide differences in the rates of metabolism of different proteins within the various tissues and organs. The work of Schoenheimer (1942) and others clearly showed that all proteins are in a state of flux, being subject to continual degradation and resynthesis. Many factors are now known to influence the life span of a protein and its turnover rate i.e. the time required for a given amount of

protein to be replaced after degradation. The life span of a protein may be influenced by that of the cell or organelle in which it occurs; this may be relatively constant as in the case of the red blood cell (Shemin and Rittenberg, 1946) and the mitochondrion, (Fletcher and Sanadi, 1961). There are also large differences in the turnover time of proteins in the same subcellular fraction which result from a variety of factors such as diet and hormonal activities. The turnover time of many mammalian proteins may therefore represent the discontinuous turnover processes associated with the life span of cells or organelles together with other independent and apparently random reactions in the cell. Further details of this topic may be obtained in a comprehensive review by Neuberger and Richards (1964).

Total liver protein may have a half-life of between 40 and 80 hours (Thomson and Ballou, 1956; Buchanan, 1961a). This figure obscures the wide variations in the turnover of individual proteins, some of which like tryptophan pyrrolase may have a half-life of only 2.5 hours (Feigelson, Dashman and Margolis, 1959). In view of such differences it would clearly be more profitable for studies in protein turnover to examine a single protein. Liver ferritin has many advantages for this purpose since it may be readily isolated by crystallization with cadmium sulphate (Laufberger, 1937) or by immunological precipitation (Mazur and Shorr, 1948). Further, studies on its turnover rate should not be complicated by many of the factors discussed above since it is a soluble and non-secreted protein.

I. Control Mechanisms in Protein Synthesis and Turnover

Alterations in the tissue levels of proteins may result from changes in their rate of synthesis or breakdown. It is therefore of considerable importance to distinguish between these two processes in investigations of factors controlling the levels of proteins in tissues. The rate of synthesis of isolable proteins may be conveniently followed by measuring the incorporation of isotopically labelled amino acids at intervals which allow maximum uptake of label but which are sufficiently short to justify the neglect of turnover. Under steady-state conditions, the rate of turnover can be measured by determining the rate of incorporation of an amino acid of known constant specific activity or by determining the rate of loss of activity following a single administration of isotope. In order to demonstrate net increase or loss of a protein it is necessary to include measurements of the total amount of protein in the tissue or organ. Details of the theoretical considerations of the use of tracer kinetics for these purposes may be found in papers by Reiner (1955) and Buchanan (1961b).

Mechanism of Protein Synthesis

Considerable advances have been made in the last decade towards the elucidation of the steps involved in protein synthesis. This topic has recently been reviewed by Korner (1964) and will only be given in outline here.

A general summary of the chemical steps in protein biosynthesis is given in Fig.1. It is now generally accepted that the primary structure of a protein is determined by a template in the DNA molecules of the genes. The code for this template is contained in the sequence of deoxyribonucleotides. There is good evidence that three nucleotides code for one amino acid (Crick, Barnett, Brenner and Watts-Tobin, 1961; Khorana, cited by Ochoa, 1965), although there is also evidence of considerably degeneracy in the code (Crick et al., 1961; Weisblum, Benzer and Holley, 1962). By a process called transcription, this genetic information is transmitted from the DNA template to a cytoplasmic site of synthesis by a species of RNA called messenger RNA.

At some undefined stage, ribonucleoprotein particles called ribosomes become attached to the messenger RNA to form structures called polysomes which are believed to be the active units of protein synthesis. The number of ribosomes in a polysome often correlates well with the size of the polypeptide chain coded by the messenger RNA. The polysome responsible for the formation of the subunits of haemoglobin (M.W. 17,000) generally accommodates 5 ribosomes (Warner, Rich and Hall, 1962) whereas that responsible for the formation of myosin (M.W. 200,000) accommodates about 150 (Florini and Breuer, 1965). This is good

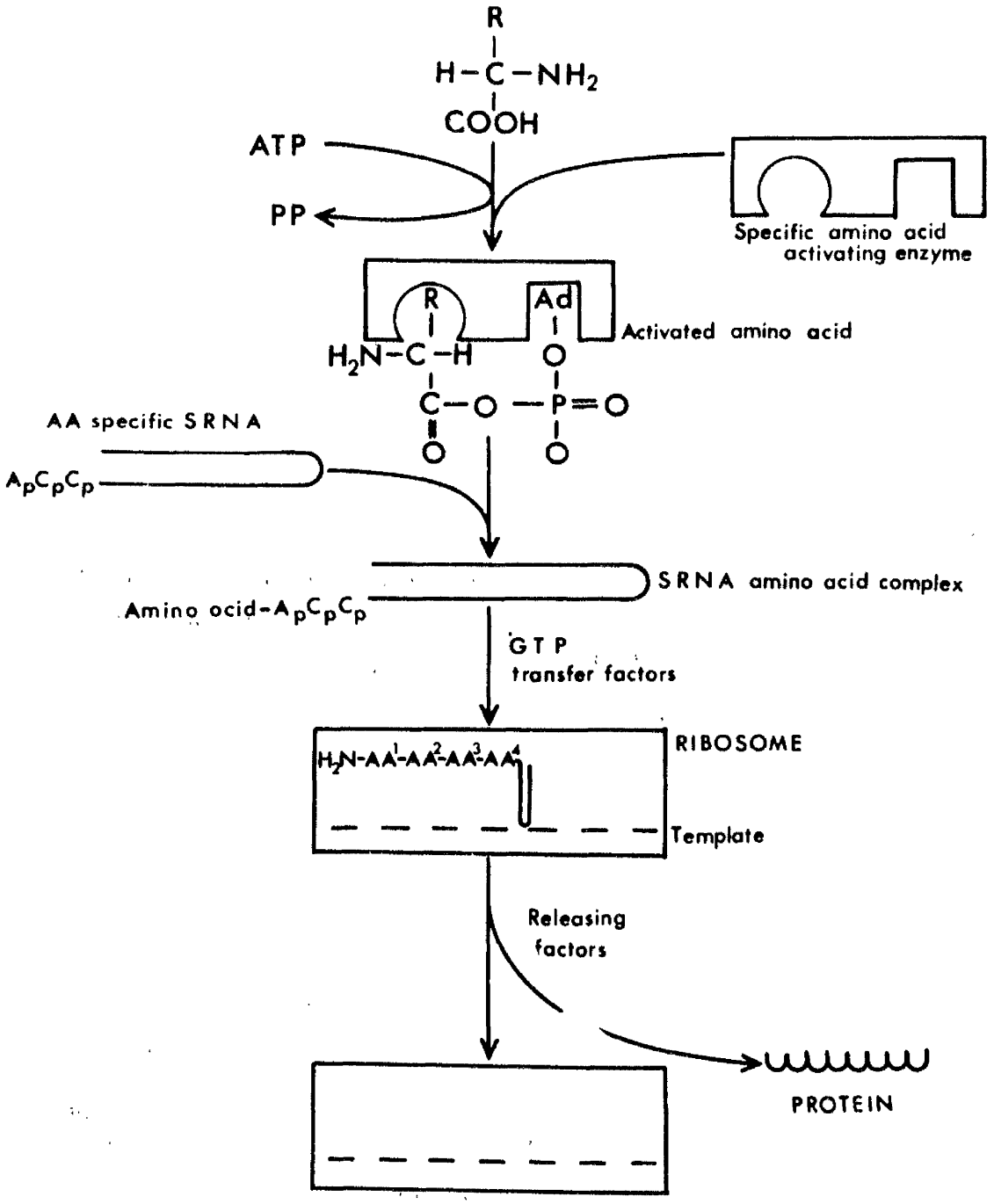


FIG. 1 Simplified scheme depicting the steps during the biosynthesis of protein.

from: Korner (1964)

"Mammalian Protein Metabolism," vol. 1, p. 188
Edited by Munro and Allison.

evidence for the existence of messenger RNA which until recently had only been a theoretical consideration to account for certain aspects of protein synthesis (Jacob and Monod, 1961). Amino acids are assembled into their correct order on the polypeptide chain, starting at the N-terminal end (Ditzmis, 1961; Bishop, Leahy and Schweet, 1960) by means of transfer RNA or sRNA molecules. These molecules, having bound a specific amino acid, are believed to form an active complex unit with the messenger RNA and a ribosome by means of an anticodon, complementary to that of the messenger RNA, in its own nucleotide sequence. The sRNA-bound amino acid then forms a peptide bond with the free carboxyl end of the polypeptide chain (Gilbert, 1963) which is attached to the ribosome by the sRNA molecule of the previous amino acid in the sequence. The latter sRNA molecule is discarded intact (Hoagland and Comly, 1960) and the incoming sRNA molecule renews the link between ribosome and polypeptide. This process is termed translation. The mechanism of release of the polypeptide after complete translation of the messenger RNA is not completely understood but appears to require magnesium ions and ATP (Simkin, 1958).

Intracellular Degradation of Proteins

Very little is yet known of the intracellular degradation of proteins. The presence in many cells of organelles called lysosomes (Applemans, Wattiaux and de Duve, 1955) which contain a variety of proteases suggests that

they may play a prominent role. Many factors, including hormones and vitamins, appear to affect the stability of these particles (Weissmann and Thomas, 1964). de Duve (1959) suggested that the enzymes normally sequestered within these organelles might be released in various physiological and pathological states. The precise function of lysosomes in the degradation of soluble proteins still remains to be demonstrated.

Control Mechanisms

Knowledge of the biochemical reactions in protein synthesis and breakdown may suggest certain stages at which these processes may be regulated. This knowledge does not, however, explain the mechanisms by which cells are able to regulate the rate of synthesis and turnover of their constituent proteins; mechanisms central to an understanding of the fundamental aspects of cell differentiation.

One of the most fruitful lines of research in this field has come from studies on enzyme induction and repression. Many of the earlier observations on these phenomena were made on bacterial systems in which the levels of certain enzymes, or groups of enzymes, could be altered by controlling the composition of the culture medium. An increase in enzyme activity was termed induction, a decrease - repression. However, there have recently been numerous demonstrations of increases in the activity of enzymes in mammalian tissues as a result of a variety of agents. These phenomena have

been loosely termed 'enzyme induction' in analogy with the bacterial systems, although it is now clear that different types of control mechanisms may be operative in many cases. Much of the experimental evidence from studies on enzyme induction has involved demonstrations of an increase in enzyme activity alone. However, the measured activity of an enzyme does not always reflect accurately the amount of enzyme protein present unless precautions are taken to ensure adequate supplies of necessary cofactors in the assay procedure. Further, increases in the tissue level of an enzyme may not depend on an increased de novo synthesis of the protein but arise from a decrease in the rate of its degradation. Many studies have failed to distinguish between these alternatives and consequently no unambiguous interpretation of results is possible. Nevertheless, much useful information has been obtained on control mechanisms in bacterial and mammalian systems resulting in useful working hypotheses which can now be tested experimentally. A brief review of current concepts of control mechanisms in the synthesis and turnover of protein in bacterial and mammalian tissues is given below.

Control Mechanisms in Bacteria

A common basis for the phenomena of enzyme induction and repression was proposed by Jacob and Monod (1961) to account for their observations on enzyme induction in wild and mutant strains of E.coli. Certain β-galactosides

were found to induce three enzymes, β-galactosidase, thiogalactoside transacetylase and galactose permease. Jacob and Monod suggested that the synthesis of these enzymes was controlled by a master gene which normally lay adjacent to the structural genes for these enzymes. The master gene was termed an operator gene and the whole complex, the operon. The rate of protein synthesis could be determined by the rate of synthesis of messenger RNA from the appropriate structural gene whose activity was normally inhibited in the absence of inducer by the operator gene. The inhibition in turn of the operator gene was considered to be effected by a repressor substance produced by another type of gene called a regulator gene. Enzyme induction resulted from an interaction of the inducer and the repressor substance, thus relieving the inhibition of the operator gene and subsequently the structural genes. Repression could occur by a direct action on the operator gene, or by the inactivation of an inducer substance. The scheme is depicted diagrammatically in Fig.2.

A considerable amount of evidence has been advanced in support of this type of control mechanism. An increase in synthesis of RNA has been shown to precede the increase in enzyme activity (Pardee and Prestidge, 1961). Some of this increase in RNA was concluded to be due to messenger RNA on the basis of hybridization experiments with appropriate segments of DNA (Attardi, Naono, Rouvière, Jacob and Gros, 1963;

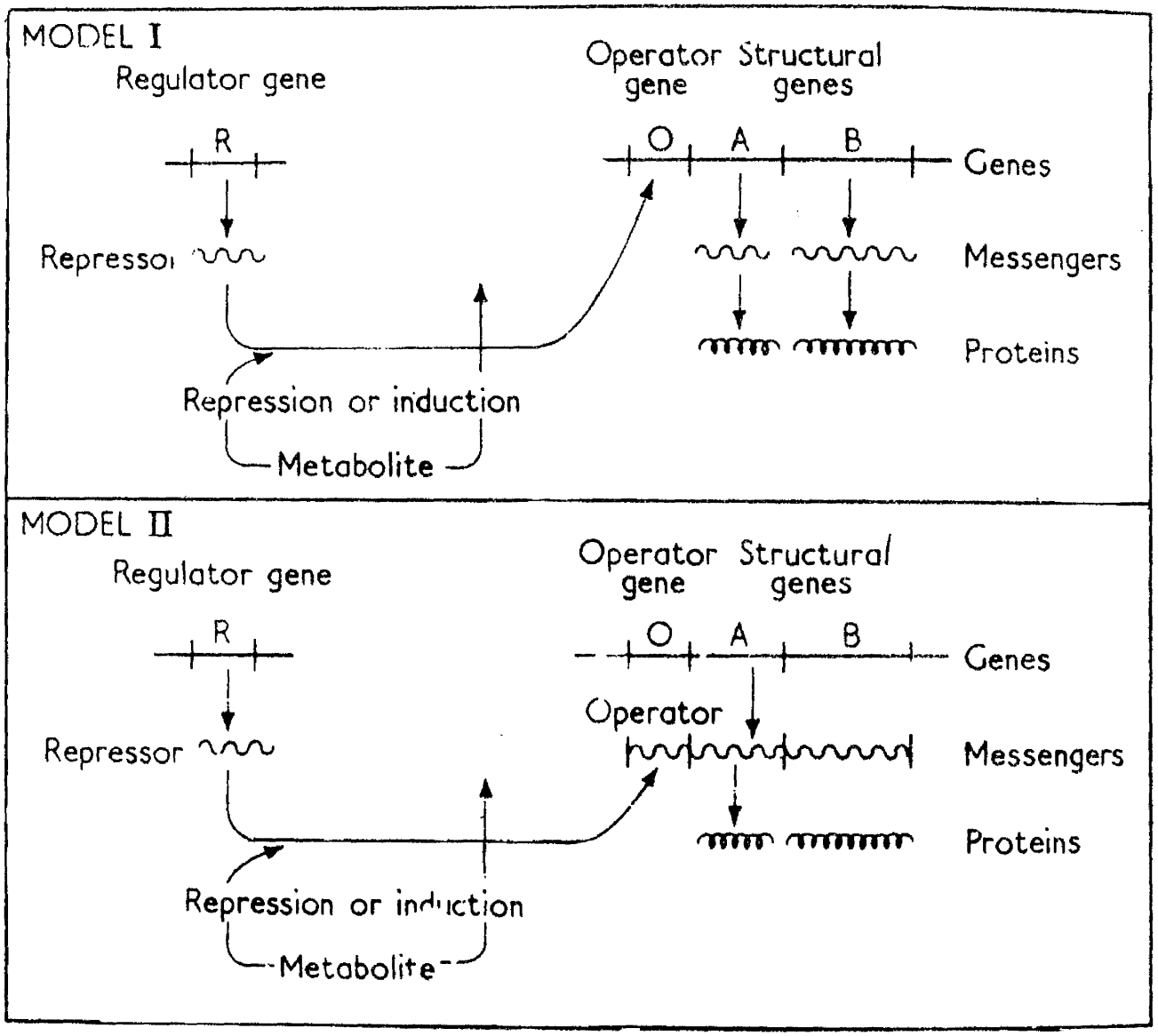


FIG. 2. Models of the regulation of protein synthesis.
from: Jacob and Monod (1961)
J. Mol. Biol., 3, p. 344

Spiegelman and Hayashi, 1963). Further, the molecular size of a fraction of RNA, believed to be the messenger RNA of the structural genes of the 'lac operon' was consistent with a polycistronic messenger (Guttman and Novick, 1963) as predicted by the theory.

Recent findings have necessitated modifications to this theory. It was originally supposed that the messenger RNA molecule would only code for one protein after which it would be degraded. However, the existence of polysomes in bacterial cells (Schaechter, 1963; Schlessinger, 1963) implies that more than one polypeptide chain can be formed at the same time from one messenger RNA molecule. This is supported by the kinetic studies of Levinthal, Kenyan and Higa (1962) indicating that the average messenger RNA molecule in B.subtilis may be used to produce as many as 15 copies of a protein. Further, since bacterial enzymes are generally considered to have little or no degradation (Hogness, Cohn and Monod, 1955; Koch and Levy, 1955), one would expect a polycistronic messenger, such as those from the histidine and lac operons (Ames and Hartman, 1963) to produce the same number of molecules of all proteins coded in the polycistronic message. However, Zabin (1963), has shown that the intracellular levels of β-galactosidase and thiogalactoside transacetylase differ by a factor of 35:1. To account for such anomalies, several authors have suggested a further control mechanism at the translational level which

determines the frequency of use of each part of the polycistronic message (Spiegelman and Hayashi, 1963; Ames and Hartman, 1963). In addition to these modifications, the theory of Jacob and Monod still leaves many interesting questions unanswered. For example, if the repressors are proteins as is generally believed, are they also subject to the same type of control mechanism? If so, the extension of this argument must lead to an exceedingly complicated situation. Nevertheless, despite certain unsatisfactory aspects of the experimental evidence, it seems likely that increases in protein synthesis in bacterial systems may be initiated at the transcription level as suggested by Jacob and Monod.

Control mechanisms in regenerating bacterial cells, however, only offer a useful guide to possible control mechanisms in non-dividing mammalian tissues. The extensive and rapid turnover of many mammalian proteins, as opposed to proteins in bacterial cells, introduces the possibility that other mechanisms such as changes in the rate of breakdown may operate to increase the tissue level of a protein. Further, the morphology and composition of the genetic apparatus in mammalian cells is vastly different from that in bacteria, especially in the amount of DNA-associated histone in the former. Histones once more are considered to be implicated in the regulation of gene activity in mammalian cells (Huang and Bonner, 1962; Allfrey, Faulkner and Mirsky, 1964) after a considerable period of neglect (Stedman and Stedman, 1950).

Control Mechanisms in Mammalian Tissues

It has been known for some time that certain agents such as hormones, dietary factors and specific substrates can affect the tissue levels of many mammalian enzymes and proteins (Knox, Auerbach and Lin, 1956). Such systems are now being extensively investigated since they offer excellent opportunities for studies of control mechanisms of protein synthesis and turnover. An increased rate of protein synthesis has been implicated in the hormonal induction of many enzymes since the observed increases are abolished by inhibitors of protein synthesis (Freedland and Harper, 1958; Segal, Beattie and Hopper, 1962; Goldstein, Stella and Knox, 1962; Schimke, 1964). Such a mechanism appears analogous to enzyme induction in bacteria where the increased differential rate of synthesis of the induced enzyme is considered due to an increased production of messenger RNA (Jacob and Monod, 1961). However, many increases in enzyme activity, notably those produced by injection of specific substrates do not appear to involve an increase in the rate of protein synthesis but involve either activation of preformed enzyme (Feigelson and Greengard, 1962) or stabilization of the enzyme by its substrate (Schimke, 1964). Some of the concepts of control mechanisms which have evolved from these studies and also some of the difficulties encountered in interpreting results are discussed below.

It is hardly surprising that hormones can alter enzyme activities in tissues in view of the obvious physiological and anatomical transformations produced by them e.g. the appearance of secondary sexual characteristics after androgen administration to castrated animals. Recent work has now indicated how some of the effects of hormones are initiated at the molecular level.

Glucocorticoid hormones, such as hydrocortisone, are known to increase the levels of certain liver enzymes involved in amino acid metabolism, e.g. tryptophan pyrrolase and tyrosine transaminase (Kenney, 1962; Schimke, Sweeney and Berlin, 1965). They also similarly effect certain gluconeogenic enzymes (Weber, Stamm and Fisher, 1965). On the other hand, insulin suppresses these gluconeogenic enzymes but induces certain glycolytic enzymes (Weber et al., 1965). These results need not be a direct consequence of the action of the hormones on the rate of protein synthesis or breakdown. For example, hydrocortisone is known to increase the level of amino acids in liver at the expense of muscle protein whereas insulin facilitates the entry of glucose into liver cells. Consequently, the observed effects of these hormones may be a secondary effect caused by alterations in the levels of substrates for these enzymes. Conversely, the injection of substrates may cause an unsuspected release of hormones such as those of the adrenal cortex, in response

to shock. The induction in liver of tyrosine transaminase by tyrosine may fall into this category, since normal adrenal function is necessary for this response (Knox, 1958). In addition, studies with hormones may be complicated by interactions of hormones *in vivo*. The stimulation of uterine metabolism by oestrogens has been concluded to result from an increase in activity of DNA-dependent RNA polymerase since the effect is abolished by pretreatment with actinomycin (Ui and Mueller, 1963). However, Lippe and Szego (1965) found that a significant proportion of the inhibiting influence of actinomycin pretreatment is due to an activation of adrenocortical secretion by the antibiotic, since endogenous adrenocorticoids are known to counteract the effect of exogenous oestrogens (Szego and Roberts, 1948). The co-ordination of hormonal activities and their close involvement with the levels of intracellular metabolites may therefore obscure differences in the action of hormones and substrates on enzyme induction *in vivo*.

Fortunately, a number of antibiotics have become available recently which interfere at different stages in protein synthesis. Two which have been extensively used for these purposes are actinomycin and puromycin, and their discerning use has proved a valuable tool in investigations of control mechanisms.

Actinomycins are a series of coloured peptide derivatives produced by strains of the streptomyces species.

The antibiotic has been shown to form complexes with DNA (Kirk, 1960; Kawamata and Imanishi, 1961) by binding guanine residues (Josse, Kaiser and Kornberg, 1961). It is thought to lie in the narrow groove of the DNA primer molecules (Hamilton, Fuller and Reich, 1963), thereby blocking the synthesis of messenger RNA by the DNA-dependent RNA nucleotidyl transferase.

Puromycin is produced by the actinomycetes, streptomyces alboniger, (Porter, Hewitt, Hesseltine, Krupka, Lowery, Wallace, Bohonos and Williams, 1952) and is composed of an aminonucleoside linked to p-methoxyphenylalanine. It is a structural analogue of the terminal group of phenylalanyl sRNA and is thought to operate at the level involving the sRNA-ribosomal complex, substituting for sRNA and being released with the incomplete polypeptide chain by the incoming sRNA (Allen and Zamecnik, 1962). The use of these antibiotics has indicated certain differences in the mechanism of enzyme induction by substrates and hormones which will be discussed below.

Hormone Induction

Agents such as puromycin which inhibit protein synthesis also inhibit the glucocorticoid induction in liver of the following enzymes:- ketoglutarate-tyrosine transaminase and tryptophan pyrrolase (Goldstein et al., 1962), glutamate-alanine transaminase (Segal et al., 1962), certain gluconeogenic enzymes (Weber et al., 1965). It has

therefore been concluded that these observed increases in enzyme activity are due to an increased rate of synthesis. In addition, enzyme induction by these and many other hormones (Williams-Ashman, 1964) is abolished by actinomycin which suggests that their primary effect is due to additional synthesis of RNA, particularly messenger RNA. This is supported by evidence of an increased synthesis of rapidly labelled RNA in liver nuclei following the administration of hydrocortisone (Jervell and Osnes, 1963) and certain apparently selective increases in similar RNA fractions in liver by a variety of hormones including androgens and oestrogens, insulin and thyroxine (Kidson and Kirby, 1964). As might be expected, these increases in RNA synthesis are abolished by actinomycin. On the basis of studies on the induction of dopa decarboxylase in the larval form of Chironomus by the insect metamorphosis hormone ecdysane, Karlson (1963) has proposed a unifying hypothesis of enzyme induction by hormones whereby the hormone modifies the rate of transcription of specific genes. Ecdysone was found to react with the nuclei of the epidermal cells (Karlson, Sekeris and Maurer, 1964) and to produce 'puff' regions of high RNA synthesis at certain loci on the giant chromosomes of the salivary gland. Further, RNA fractions isolated from the larva after hormone treatment were found to stimulate the synthesis of dopa decarboxylase in a cell free protein-synthesizing system prepared from rat liver

(Sekeris and Lang, 1964). This result was attributed to the presence in these fractions of the messenger RNA for dopa decarboxylase. In addition to the studies with actinomycin, other work in vertebrate and mammalian systems supports such a mechanism of hormone action. Aldosterone has been shown to react with the nuclei of cells of a target organ, toad bladder tissue, (Porter, cited by Davidson, 1965) and a similar reaction has been observed with TSH and guinea-pig thyroid cells (Greenspan and Hargardine, 1965). In addition, Begg and Munro (personal communication) have shown an increased activity of DNA-dependent RNA polymerase in fragments of thyroid nuclei after exposure to TSH.

In conclusion, the present evidence indicates that enzyme induction by many hormones involves an increased rate of synthesis of the enzymic protein as a result of an increased rate of messenger RNA production. The effects of many hormones are not very specific, since certain hormones, notably anabolic hormones, appear to affect many different organs. Any specificity of action must therefore reside in the target organ rather than in the hormone. Finally, in view of the complications arising from *in vivo* studies it would be most desirable to obtain more direct evidence of the primary site of action of hormones from studies *in vitro*, before accepting present concepts of their mechanisms of action.

Substrate induction

Many of the enzymes mentioned previously which are induced by hormones may also be apparently induced by their specific substrates or dietary factors e.g. tryptophan pyrrolase, arginase and ornithine transcarbymalase (Schimke, 1962). These inductions are generally much more specific than those of hormone induction and appear to involve a different mechanism since many are insensitive to actinomycin (Greengard, Smith and Acs, 1963) and therefore do not involve additional messenger RNA synthesis. The induction of tryptophan pyrrolase by tryptophan has been most extensively studied (Feigelson and Greengard, 1962; Greengard and Acs, 1962; Schimke, et al., 1963). The induction appears to involve at least two different mechanisms. Firstly, small amounts of substrate may activate preformed enzyme by increasing the availability of a haematin prosthetic group necessary for enzyme activity. Secondly, excess substrate may stabilize these enzyme molecules against degradation and thereby cause an accumulation of enzyme without affecting its rate of synthesis.

These studies stress the need to distinguish between alterations in enzyme levels as a result of changes in the rate of synthesis and changes in the rate of breakdown. In view of the widely different rates of turnover of liver enzymes, it seems possible that hormones may increase the rate of synthesis of many more enzymes than is generally

conceived. Thus the apparent specificities of a hormone for a given enzyme may only reflect a high rate of turnover of the enzyme.

II.

The Nature of the Present Problem

Despite the obvious advantages of ferritin for studies of control mechanisms in protein synthesis and turnover, remarkably little work has been done in this connection. Much of the present knowledge of its metabolism comes from its role in iron metabolism and its relationship to another iron storage substance called haemosiderin. Very little is yet known of the factors affecting the metabolism of the protein moiety despite the relative ease with which it may be isolated. The chemical nature of ferritin and the present knowledge of its metabolism are discussed below.

The Chemical Nature of Ferritin

Ferritin is now known to occur in a wide variety of cells including those of vertebrates (Granick, 1946), invertebrates (Roche, Bessis and Breton-Gorius, 1961) and plants (Hyde, Hodge and Birnstiel, 1962). It consists of

a homogeneous protein, apoferritin (M.W. 465,000, Rothen, 1944) in combination with variable amounts of an iron salt (Granick, 1946). Samples of the readily available ferritin from horse spleen may contain as much as 25% of their dry weight as iron (Laufberger, 1937). This is believed to be present in the form of a basic ferric phosphate to which Granick (1946) has given the empirical formula $(FeOOH)_8\ FeOPO_3H_2$. It may therefore be calculated that a single molecule of ferritin can contain as many as 2,500 atoms of iron. Under the electron microscope ferritin appears as a spherical shell of protein, 95-122Å in diameter (Farrant, 1954; Harrison, 1963), enclosing electron-dense iron micelles 27-30Å in diameter. These occupy a total diameter of 74Å (Harrison, 1963). The iron can be easily removed chemically from ferritin by dialysis against suitable reducing agents (Laufberger, 1937; Behrens and Taubert, 1952) to produce the iron-free protein moiety, apoferritin. Both ferritin and apoferritin can be crystallized from solution with cadmium sulphate (Laufberger, 1937) as isotropic cubic crystals, generally octahedra (Granick, 1946) or with caesium chloride as anisotropic, pseudocubic platy crystals (Richter, 1964). The early studies of Mazur and Shorr (1950) showed that apoferritin and ferritin had the same electrophoretic mobilities in the Tiselius apparatus and also the same immunological properties.

X-ray crystallography indicated the presence of subunits in apoferritin (Harrison, 1959). Later, chemical analyses suggested that there are 20 chemically identical subunits (Harrison, Hofmann and Mainwaring, 1962; Harrison and Hofmann, 1962). Since then, subunits (M.W. 25,000) have been obtained from dehydrated apoferritin by treatment with sodium dodecyl sulphate or by substituting the positive charges of lysine residues for negative charges by succinylation (Mainwaring, 1964; Harrison, 1964).

The quaternary structure of ferritin has been deduced from X-ray diffraction and electron microscopy. The protein shell is thought to consist of twenty subunits arranged in the form of a pentagonal dodecahedron (Harrison, 1963). The disposition of the iron micelles in this structure is still uncertain. Farrant (1954) thought that they consisted of four subunits at the vertices of a square. Kerr and Muir (1960), however, suggested that there are six subunits arranged at the vertices of a regular octahedron. Both structures would fit symmetrically into a regular dodecahedron since both are sub-groups of the dodecahedral group. Iron is thought to enter the molecule through channels about 8.5Å in diameter which may be seen by "negative staining" methods (Richter, 1959).

Polymorphism in Ferritin Molecules

Early studies using moving boundary electrophoresis suggested that ferritin and apoferritin prepared chemically from it had identical electrophoretic mobilities and were apparently homogeneous (Mazur and Shorr, 1950). Recently, however, samples of ferritin from many species have been separated into a series of multiple bands by electrophoresis in starch and acrylamide gels (Kopp, Vogt and Maass, 1963; Richter, 1964). This could be due either to differences in the genetically determined primary structure of the various ferritin fractions or to physico-chemical factors not involving differences in amino acid sequence. The various fractions cannot be distinguished by immunological procedures (Richter, 1964) but have constant mobilities. By contrast, Saadi (1964) maintains that each of the separated fractions, after elution and concentration, gives a similar electrophoretic pattern to that of the original mixture. In addition, in nearly all investigations, the relative abundance of each band increases with its electrophoretic mobility, the main band usually comprising 65-85% of the total (Richter, 1964). This, together with Saadi's observations, suggests an aggregation phenomenon, since similar gels segregate other substances, such as RNA (Tsanev, 1965), by virtue of their molecular size. However, Carnevali and Tecce (1964) have reported large differences in the relative abundance of the fractions obtained from

ferritin from different human livers. This observation is contradictory to a simple physical aggregation phenomenon unless the tendency for aggregation is an inherent property of some physico-chemical difference between the samples, such as a high iron content of the ferritin.

Some further observations are relevant to the problem of polymorphism in ferritin molecules. The observed heterogeneity is not due to differences in the iron content of the ferritin molecules since apoferritin prepared chemically from unfractionated ferritin exhibits a similar heterogeneity (Carnevali and Tecce, 1964). This is also supported by the fact that the bands on the gel are discrete, whereas there is a continuous spectrum of molecular saturation from 0 to 25% iron (Granick, 1946). By contrast, natural apoferritin (M.W. 465,000) separated from the iron containing fractions by ultracentrifugation is homogeneous on gel electrophoresis and has a similar mobility to the α main band in unfractionated samples of ferritin. Further, it is possible to separate the α band from the others by ammonium sulphate fractionation or by means of sucrose density gradients (Kopp, Vogt and Maass, 1963, 1964). These procedures are known to separate fractions of different iron content (Mazur, Litt and Shorr, 1950) but it seems possible that they might also separate fractions of different particle size.

In conclusion, the evidence suggests that the observed heterogeneity of ferritin molecules from one species may not be due to genetically determined differences in primary structure of the separated fractions but to some physico-chemical factor such as aggregation. The explanation for this aggregation is obscure. However, it is known that iron-rich ferritin molecules form crystalline aggregates in cells (Bessis and Breton-Gorius, 1957; Richter, 1958) which many regard as intermediates in the formation of haemosiderin, a rather ill-defined substance which has a higher iron content than ferritin. This suggests that the tendency for aggregation of ferritin molecules may increase with iron content and eventually give rise to haemosiderin in the cell.

In addition to the heterogeneity observed in the same samples of ferritin from one species, Richter (1965) has recently provided evidence of genetic differences in samples of ferritin from normal human liver and certain human carcinomas. He found that the ferritin from these carcinomas also separated into three fractions (α,- β and γ -) but their mobilities differed from those of the corresponding fractions from normal human liver and spleen. From the practical point of view, it is therefore valid to take all ferritin fractions from a single source to have the same amino acid structure and

presumably to be regulated by a common mechanism of protein biosynthesis.

Metabolism of Ferritin

The administration of many parenteral iron preparations leads to an early accumulation of ferritin in many tissues such as the liver, spleen and intestine (Granick, 1946; Fineberg and Greenberg, 1955; Heilmeyer, 1958; Shoden and Sturgeon, 1962). An increased rate of synthesis has been implicated in this response (Fineberg and Greenberg, 1955). Rat liver ferritin can be completely synthesised from the free amino acid pool in about 6 minutes (Loftfield and Eigner, 1958). Crude fractionation of ^{14}C-labelled ferritin molecules by ultracentrifugation indicated that amino acids were first incorporated into the fractions of the lowest iron content (Fineberg and Greenberg, 1955). This fraction was also claimed to have the highest level of ^{59}Fe incorporation (Mazur and Green, 1955), although their results also indicate a high level of incorporation into iron-rich fractions at early time intervals.

The turnover of the protein and iron moieties of ferritin need not be synchronous, since iron may be incorporated or removed from ferritin *in vivo* without an accompanying synthesis or degradation of the protein moiety (Mazur and Carleton, 1963). The incorporation of iron *in vivo* into ferritin is thought to require ATP and ascorbic acid (Loewus and Fineberg, 1957) and may involve xanthine oxidase (Green and Mazur, 1957). Both groups of workers

considered that only ferrous iron was involved in the incorporation or removal of iron from ferritin. Mazur, Baez and Shorr (1955) claim to have demonstrated the presence in ferritin preparations of a small amount of ferrous iron, about 0.2% of the total. They considered this fraction to be at or near the surface of the molecule and to be involved in the transfer of iron. Iron may also be incorporated *in vitro* into chemically prepared apoferritin (Bielig and Bayer, 1955; Loewus and Fineberg, 1957; Mazur and Green, 1959), but Harrison (1964) has found differences in the disposition of the iron in the micelles of the synthetic and natural ferritin.

Very little is known of the factors which affect the turnover of liver ferritin. Helwig and Greenberg, (1952) found that liver ferritin incorporated amino acids at a greater rate than mixed plasma proteins in guinea-pigs. Its specific activity decreased with a half life of about 2 weeks. By contrast, Fineberg and Greenberg (1955) computed the half life of rat liver ferritin to be about 40 hours. This ferritin had been obtained from animals which had been pretreated with iron before receiving the labelled amino acid. It is not certain whether the loss of ferritin occurs by direct degradation to free amino acids or whether some may be converted to haemosiderin (Shoden and Sturgeon, 1962).

It would be of considerable interest to determine how iron administration affects ferritin metabolism and also to discover how iron brings about the induction of ferritin. The early evidence of Fineberg and Greenberg (1955) suggests that an increased de novo synthesis of apoferritin is involved. In addition, since this present work was completed, it has been claimed (Yu and Fineberg, 1965) that the stimulant action of iron on amino acid incorporation into ferritin protein can be abolished by pretreatment with actinomycin. These authors concluded that the induction of ferritin protein by iron involves an increased rate of synthesis of its specific messenger RNA. Thus it would appear that the induction of ferritin by iron is analogous to the induction at the level of messenger RNA synthesis of many enzymes by hormones.

The proposed studies of the induction of ferritin by iron require a means of separating the protein in sufficiently pure form for accurate estimations of its specific activity following the injection of labelled amino acids. It would also be desirable to include a measure of the total amount of ferritin protein in liver. The methods available at present for ferritin isolation have several serious disadvantages which will be discussed later. In addition, although some methods are available for estimating the total amount of

ferritin iron in liver (Drysdale and Ramsay, 1965), they do not provide a reliable measurement of ferritin protein due to possible variation in the iron content of ferritin samples from 0-25%.

This work describes a completely new method for the isolation of samples of rat liver ferritin for the estimation of the incorporation of labelled amino acids. This has also allowed us to calculate the total amount of ferritin protein present in the liver. Samples of ferritin purified by our methods gave several discrete bands on electrophoresis in acrylamide gels. This apparent polymorphism has been investigated in view of the possibility that the bands may be derived from proteins with different amino acid sequences. This would suggest that each protein may have a separate control mechanism for its biosynthesis and so considerably complicate the proposed studies. The isolation procedures devised for the isolation of ferritin and the investigations into its apparent polymorphism are described in the first two sections of the experimental work. Later sections are concerned with kinetic studies of the induction of ferritin by iron followed by investigations of the effect of diet and actinomycin on the induction process. The remaining section deals with factors which affect the rate of turnover of ferritin. These studies have led us to propose a mechanism whereby iron affects the rate of synthesis and breakdown of rat liver ferritin.

GENERAL METHODS AND MATERIALS

This section is intended to cover general methods which were not devised or amended in the course of this research. The special techniques developed by us are described under the appropriate sections.

Animals

Adult male albino rats of 150-190 g. body weight were used in all studies. In no single experiment did the weights of the animals in the group differ by more than 5 g. The animals were housed under thermostatic conditions and were normally fed on stock diet. The use of a standard size of rat bred in our own animal house and maintained under controlled conditions was considered important in minimising variations in ferritin content of the liver due to age and diet (Kaufmann, 1962; Bhattacharya and Esh, 1964).

Diets

In investigations involving control of diet the isocaloric regimes of Munro and Naismith (1953) were followed. Stock animals were maintained on diets of adequate protein content or a protein-free diet for a period of four days after an initial 18 hour fast. Any injections were made immediately before the morning meal. The composition of the diets and amounts given to the animals are given in Tables 1, 2 and 3.

Table 1

Adequate protein mixture	Protein-free mixture
10% margarine	10% margarine
16.5% potato starch	45% potato starch
16.5% glucose	45% glucose
57% casein	

This mixture was fed as the second part of the diets shown in Table 3.

Table 2

Vitamin-Mineral-Roughage (V.M.R.) Mixture

6% sodium chloride

24% "446" salt mixture (Munro, 1949)

45% vitamin mixture

11% Agar

14% margarine

Table 3

Dietary Regime

Time	Diet	Weight given/150 g. body weight	
		Adequate protein group	Protein-free group
9.30 am	V.M.R.	1 g.	1 g.
	Glucose	5 g.	5 g.
5 pm	Adequate protein mixture	5.2 g.	---
	Protein-free mixture	---	5.2 g.

Apparatus

Glassware was freed from iron by immersion overnight in 6 N hydrochloric acid followed by thorough rinsing in deionized water.

Reagents

A.R. grade reagents were used when possible and most were found satisfactory for use except ammonium sulphate. This latter was freed from iron by boiling a saturated aqueous solution with O-phenanthrolene (0.01%), sodium sulphite (7.5 mM) and sulphuric acid (25 mM). The iron-phenanthrolene complex was extracted with propan-2-ol and the excess alcohol was boiled off from the aqueous layer. The solution was then adjusted to approximately pH 7 by heating in a slight excess of aqueous ammonia (sp.gr. 0.88). The iron content of the saturated solution was found to be less than 0.1 ug/ml.

Isotopes

DL-leucine-1-C14 (Code No.CFA 78) of specific activity 5-10 mc./mM and adenine-8-C14 sulphate (Code No. CFA 49) of specific activity 5-10 mc./mM were obtained from the Radiochemical Centre, Amersham. Both isotopes were dissolved in sterilized isotonic saline for injection.

Preparation of crystalline ferritin and apoferritin

These were prepared from rat liver and horse spleen. Livers were obtained from adult male rats (150-200 g. body weight), some of which received ferric ammonium citrate (20 ug/ml.) in their drinking water for three weeks to enhance the amount of ferritin recovered. Horse spleen was obtained fresh from the slaughterhouse. Crystalline ferritin prepared from each tissue by the method of Granick (1946), was recrystallised four times (Fig.3) and chromatographed on carboxymethyl (CM) cellulose (capacity 0.7 m-equiv./g., Serva, Heidelberg, Germany) by the procedure of Drysdale and Ramsay (1965). Apoferritin was then prepared from the purified rat liver ferritin by the dithionite method (Granick, 1946) followed by removal of traces of iron with 2,2' bipyridyl (Richter, 1964b). Its identity was confirmed by crystallization with cadmium sulphate (Fig.4). Samples were freeze-dried from 0.001 M ammonium acetate for use as a standard for protein estimation.

Estimation of Protein

Ferritin protein was estimated by the method of Lowry, Rosebrough, Farr and Randall (1951) using rat apoferritin to obtain a calibration curve (Fig.5a). The extinctions obtained were similar to those for crystalline ferritin, provided that the weight of

Fig. 3. **Horse spleen ferritin crystallized with cadmium sulphate.**

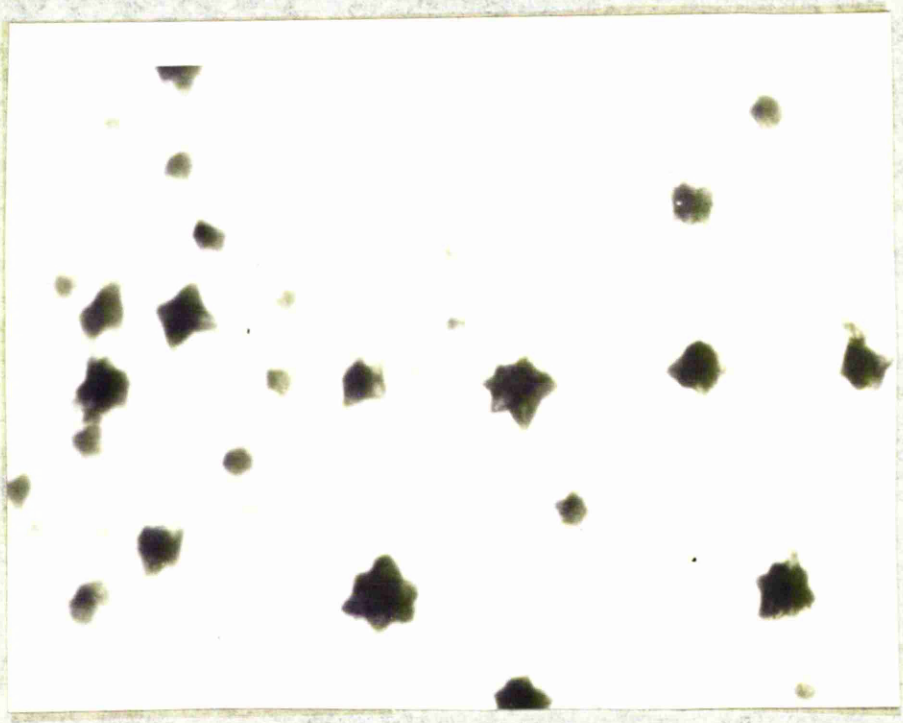
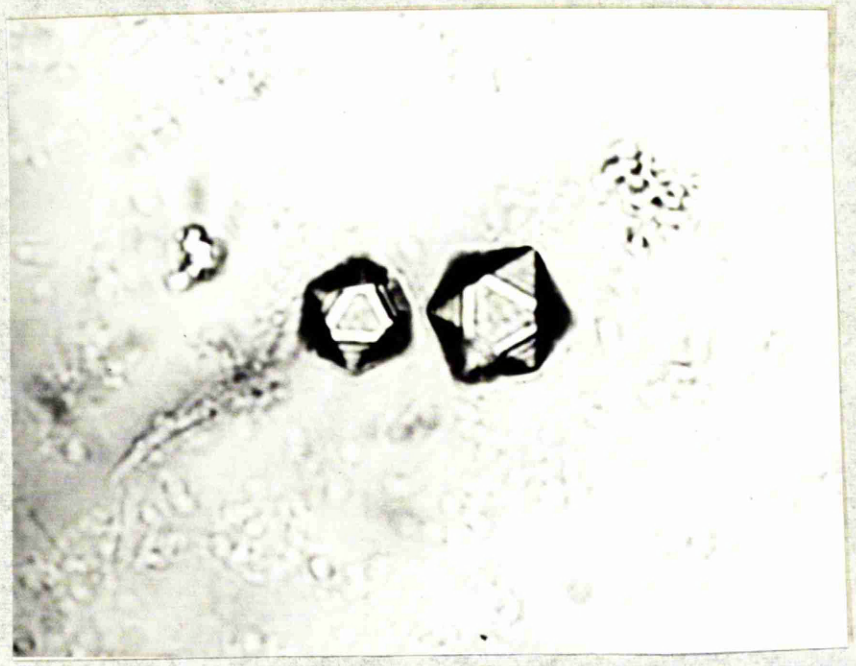


Fig. 4. **Crystalline rat apoferritin**



ferritin used was corrected for its content of iron as $(FeOOH)_8FeOPO_3H_2$ (Granick, 1946).

The protein content of samples of diluted liver homogenates was estimated by the same Lowry procedure. Serial dilutions of a homogenate of rat liver were used to construct a calibration curve (Fig.5b). As it did not seem practical to prepare a sample of mixed liver protein for a standard it was assumed that 100 ug mixed liver protein would have the same extinction as 100 ug bovine serum albumin. The protein concentrations shown in Fig.5b have been calibrated accordingly.

Estimation of Iron

After any necessary pretreatment, iron was reduced in solution with sodium sulphite and complexed with 2,2' bipyridyl (Hill, 1931) at pH 4 - 4.5. The optical density of the pink ferrous bipyridyl solution was measured at 520 mu and the iron content estimated from a calibration curve constructed from a ferric ammonium sulphate standard (Fig.6). This method is very robust yet allows accurate estimations of as little as 1 ug iron.

Total Iron

The total iron in samples (less than 0.2 ml. volume) was estimated as the ferrous bipyridyl complex after the solution had been digested with sulphuric, perchloric and nitric acid as described by Ramsay (1944).

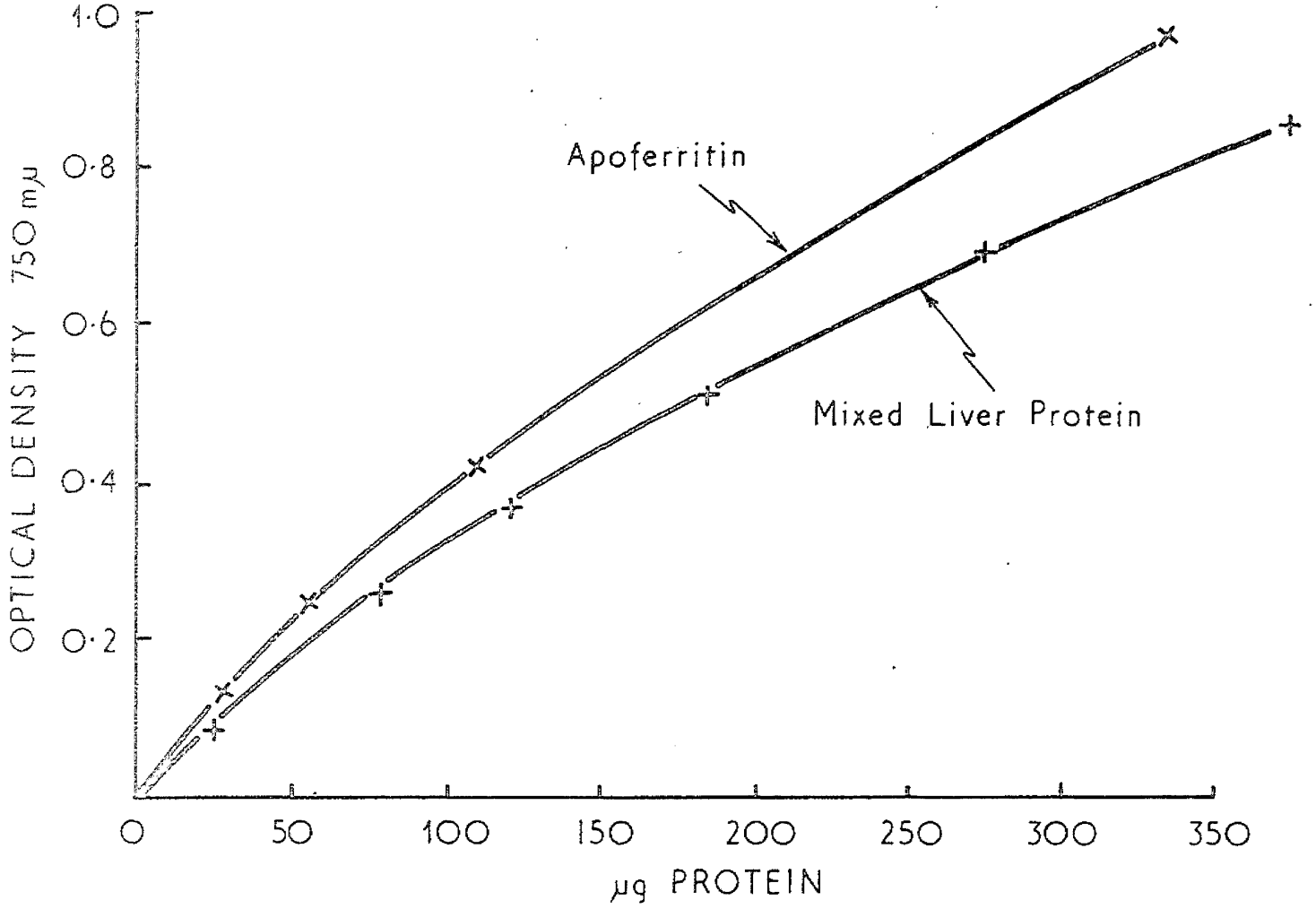


Fig. 5. Calibration curve for the estimation of ferritin protein and mixed liver protein by the method of Lowry et al. (1951). Final volume, 6.5 ml.

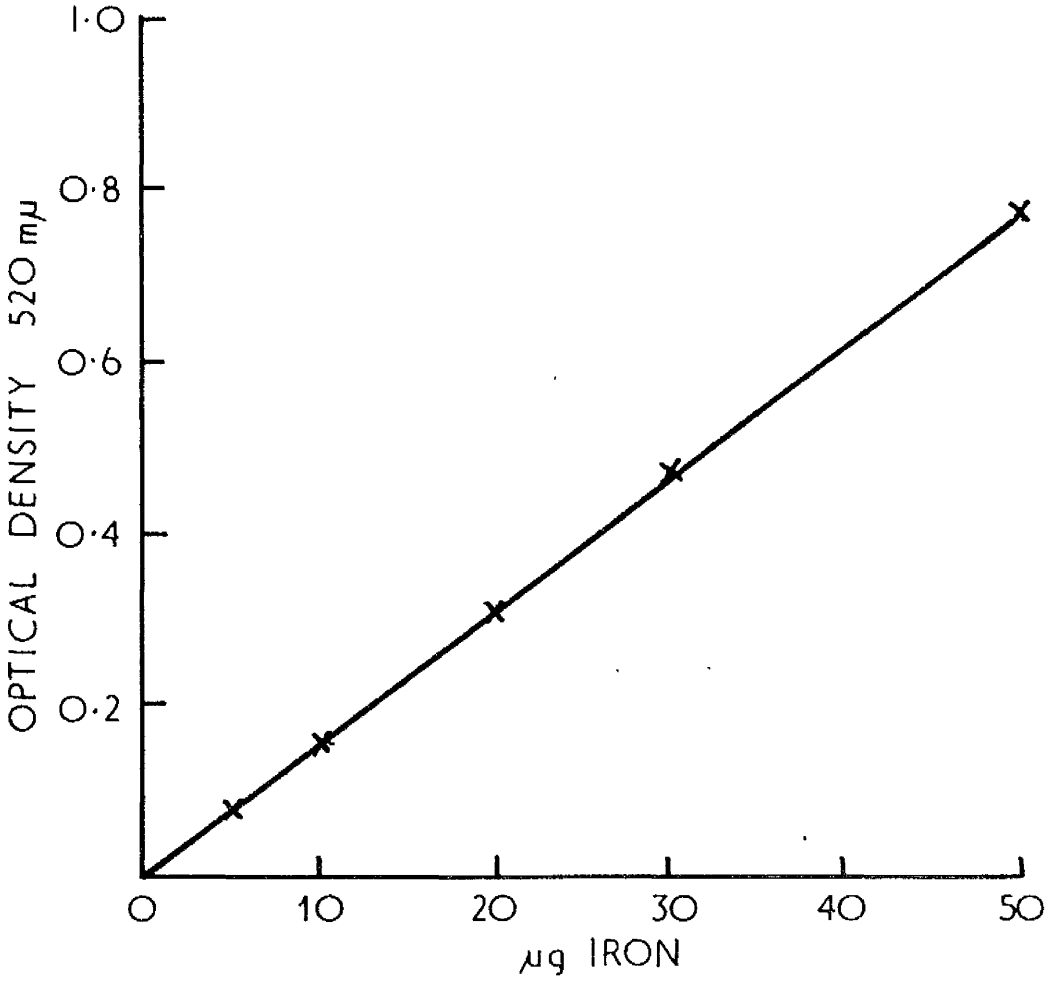


Fig. 6. Calibration curve for the estimation of iron as ferrous dipyridyl, final volume 10 ml.

EXPERIMENTAL AND RESULTS

Section 1: THE SMALL-SCALE ISOLATION OF FERRITIN FOR THE ASSAY OF THE INCORPORATION OF ^{14}C-LABELLED AMINO ACIDS

(i) Procedure for the isolation of rat-liver ferritin.

(ii) Tests of purity of the isolated ferritin.

(iii) Application of procedure to studies of the incorporation of ^{14}C-amino acids into ferritin

(iv) Comparison of specific activity of ^{14}C-labelled ferritin isolated by the new procedure and by immunological precipitation.

(v) Conclusion.

THE SMALL-SCALE ISOLATION OF FERRITIN FOR THE ASSAY OF THE INCORPORATION OF ^{14}C-LABELLED AMINO ACIDS

In order to follow the incorporation of ^{14}C-labelled amino acids into ferritin, methods were devised for its isolation from small samples of rat liver. In this section these procedures are described, together with tests of purity applied to the final preparation to determine its suitability for the above purpose.

Most methods of isolating ferritin for studies of the incorporation of ^{14}C amino acids involve immunological procedures (Fineberg and Greenberg, 1955; Loftfield and Eignor, 1958; Matioli and Rylar, 1964; Yu and Fineberg, 1965). These methods, however, have several serious disadvantages. The quantity of protein recovered in the antigen-antibody precipitate varies with the conditions of precipitation and requires careful standardization (Mazur and Shorr, 1950). In addition, the production of sufficient antiserum to the ferritin of small mammals requires a prohibitively large number of animals even when they have been suitably pretreated with iron preparations to enhance the amount of ferritin recovered. An alternative procedure is to prepare an antiserum to the more readily available horse-spleen ferritin, which has

been shown to cross-react with the ferritin of other species (Mazur and Shorr, 1948) and to give quantitative ferritin precipitation under suitable circumstances (Mazur, Green and Carleton, 1960). Such an antiserum, however, is much less sensitive than homologous ferritin antiserum (Mazur, and Shorr, 1950). Further, it is necessary to purify further crystalline horse-spleen ferritin as other non-ferritin antigens have been demonstrated in this preparation (Patterson, Suszko and Pruzanski, 1965). Finally, some preliminary purification of tissue ferritin may be necessary to avoid adsorption of other proteins on to the antigen-antibody precipitate (Mazur and Carleton, 1963). The alternative method of isolating ferritin after crystallization with cadmium sulphate and gel filtration (Patterson, Suszko and Pruzanski, 1965) requires relatively large amounts of tissue and recoveries are incomplete (Fineberg and Greenberg, 1955).

The procedures developed in the course of this work for isolating small amounts of ferritin involved differences in chromatographic properties between ferritin and other liver proteins. DEAE-cellulose has been previously employed in the purification of rat-liver ferritin (Mazur, Green and Carleton, 1960) but the final product was only 30% pure. Drysdale and Ramsay (1965) used CM-cellulose to separate ferritin from other iron-containing proteins but their conditions

do not exclude other proteins from the ferritin fraction.

In our new procedure, several recognized general processes for the separation of ferritin have been combined with chromatographic techniques on CM-cellulose and Sephadex G-200 to achieve a final purity suitable for measuring specific activities after the injection of labelled amino acids. With this method as many as 12 samples, each containing between 50 ug and 1 mg. of ferritin, can be conveniently processed simultaneously in 12 hr. of working time.

MATERIALS AND METHODS

Determination of Ferritin Iron

After the estimation of iron in ferritin, it was necessary to recover the protein for estimation of radioactivity. A procedure was therefore devised that would extract the iron from ferritin without hydrolysing the apoferritin to fragments that were no longer acid-precipitable. A modification of Hill's (1931) bipyridyl method for the estimation of iron in the ferrous state was chosen, with Na_2SO_3 as the reducing agent. The ferritin-containing solution was made up with water to 10 ml. in the presence of Na_2SO_3 (75 mM), 2,2'-bipyridyl (0.05%) and acetic acid 6% (v/v). After heating at 100° in a water bath for 1 hr., the colour

developed was read at 520 mμ. Estimations of iron made by this method on a sample of purified ferritin were compared with the values obtained for total iron after digestion with a mixture of H_2SO_4, $HClO_4$ and HNO_3 as described by Ramsay (1944). The values given by the two methods are given in Table 4 which shows that the values obtained by the ferritin iron procedure do not differ significantly from those given by the method for total iron. The recovery of iron was 96 ± 3% which indicates that the new procedure measures all the iron in ferritin.

Determination of total ferritin iron in liver

The method used was essentially that of Fulton and Ramsay (unpublished) as modified by Drysdale and Ramsay (1965). The procedure is based on the separation of the two major non-haem iron substances in liver, ferritin and haemosiderin, by heat coagulation. Ferritin, which is not in the coagulum, is thereafter separated from any haemoglobin which escaped coagulation by ammonium sulphate fractionation and ferritin iron estimated by procedures which avoid interference from haem iron as described above.

An extract containing all the tissue ferritin was prepared from an aqueous rat-liver homogenate (200 mg./ml.) by heating at 80° (Laufberger, 1937), followed by removal of the heat-coagulated tissue proteins by centrifugation. Ferritin was precipitated from this

Table 4

The Estimation of Iron in Ferritin

Optical Density at 520 mu of Ferrous Dipyridyl Solution.

New Method	Ramsay (1944)
0.219	0.249
0.226	0.224
0.235	0.233
0.264	0.235
0.255	0.238
0.287	0.252
0.275	0.233
0.244	0.242
0.247	0.232
0.249	0.242
0.219	0.220
0.229	0.226
Mean 0.236 ± 0.010	0.246 ± 0.021

The iron in 12 equal portions of a single preparation of horse ferritin was estimated by the method for ferritin iron described on page 34. The optical density values given by this method have been compared with those obtained from 12 similar portions using a published method for the estimation of total iron by wet ashing (Ramsay, 1944).

fraction by 50% saturation with $(NH_4)_2SO_4$ and left to stand for 2 hr. at 0° to allow complete precipitation. The iron content of the precipitate was estimated as described above.

Protein separation in polyacrylamide gels

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

Immunoelectrophoresis

Samples of antigen were applied to the wells of a 1% (w/v) agar gel on glass microscope slides in veronal buffer, pH 8.6 in the LKB 6800A immunoelectrophoresis apparatus (LKB-Produkter A.B., Stockholm, Sweden). Electrophoresis was usually carried out for 1 hour using a current of 2.5 ma/cm. after which the antiserum was applied to the central ditch and the gels stored for 20 hours at 20° to allow precipitation to occur. Non-precipitated protein and the excess antiserum were washed from the gel with physiological saline and the gels were dried by heating from an overhead lamp. The antigen-antibody precipitates were finally stained for protein

with Ponceau S and for iron with potassium ferrocyanide as described for the polyacrylamide gels.

Estimation of ^{14}C radioactivity in protein precipitates

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

Immunological isolation of ferritin from rat-liver

(a) Preparation of antiserum:

As it was considered that the production in rabbits of sufficient antiserum to rat ferritin would be too expensive in numbers of rats, an antiserum to the more readily available horse-spleen ferritin was prepared. Such an antiserum has been found effective for the quantitative isolation of ferritin from a suitable extract of rat liver by Mazur, Green and Carleton (1960). Horse spleen ferritin was prepared as described on page 30. Before use, the ferritin solution was chromatographed on columns of Sephadex G-200 (Pharmacia, Uppsala) in an attempt to remove toxic cadmium ions remaining after crystallization.

This proved a fortunate choice of precaution as it has since been shown (Patterson, Suszko and Pruzanski, 1965) that non-ferritin antigens are present in crystalline horse-spleen ferritin, but can be removed by chromatography on Sephadex G-200.

(b) Immunization

Twelve male Californian rabbits, each weighing about 1.5 Kg. were immunized with horse-spleen ferritin according to the method of Mazur and Shorr (1948). Before immunization, blood samples from each animal were tested for non-specific precipitations with rat ferritin and found to be negative. Each rabbit received a total of 30 mg. ferritin over a period of one month. The ferritin was given as a suspension (1 mg./ml.) of an alum precipitate (Kabat and Mayer, 1961) in saline on four consecutive days of each week. The dose was doubled each week throughout the month. During the first fortnight injections were made into the marginal ear vein. However, as four of the animals died in this period (apparently from anaphylactic shock,) and as the veins became occluded, all further injections were given intramuscularly into the hind legs.

About 50 ml. blood were withdrawn each week from the ear vein of each rabbit after the fifth week. The sera were stored separately in the refrigerator.

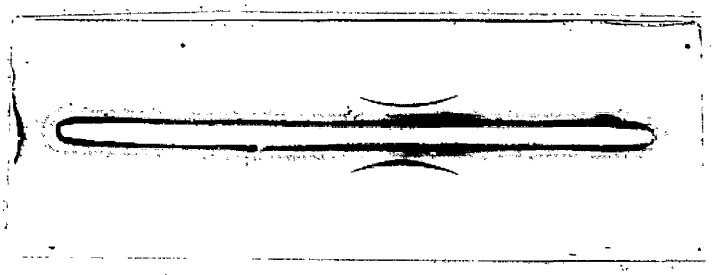
In order to maintain or boost the antibody level, each animal was given further doses of 10 mg. ferritin each month. Also, as a safeguard against possible iron deficiency, each was given an intramuscular injection of 200 mg. iron as "Imferon" (Benger).

(c) Tests of Specificity and antibody titre

On the fourth week after the first bleeding, the sera were tested for their specificity to rat ferritin in the proposed test system. This consisted of the supernatant fraction remaining after heating an aqueous (1:5 w/v) rat-liver homogenate to 80° followed by removal of the coagulated proteins by centrifugation. The uncoagulated fraction, which contains all the tissue ferritin, was concentrated about 10 fold by reverse dialysis against polyethylene glycol (M.W.20,000) and finally dialysed against veronal buffer pH 8.6, 0.07M before immunoelectrophoresis. The antiserum to the horse-spleen ferritin was used to detect any antigenically active proteins distributed in the gel (Fig.7).

Only one antigen-antibody precipitin band was detected. This stained with both Ponceau S and potassium ferrocyanide and was therefore considered to be ferritin. This demonstrates that only ferritin will be precipitated from the heat supernatant fraction with the antiserum to horse-spleen ferritin.

Fig. 7. Test of specificity of rabbit-antihorse ferritin serum for the precipitation of rat ferritin from a liver extract.



The antibody titre of this antiserum was estimated using the supernatant fraction, which remained after heating an aqueous (1:2 w/v) homogenate of normal rat liver to 80°, as a source of ferritin antigen. The concentration of ferritin iron in this preparation was estimated as before (page 35). Samples (1 ml.) were incubated with different amounts (0.01 - 6 ml.) of antiserum at 37° for one hour, followed by a further 18 hours at 0°. The resulting precipitates were collected by centrifugation, washed twice with 0.9% saline and taken up in 0.1 ml. of 0.1 N sodium hydroxide. The solutions were diluted to 10 ml. and their protein and iron content estimated. The precipitin curve thus obtained is shown in Fig.8.

The results showed that 1 ml. of antiserum would precipitate at least 100 µg. ferritin iron. Unfortunately, the iron content of the ferritin in this antigen preparation was not known and thus it was not possible to determine the amount of ferritin protein in it. However, later studies have shown that the iron content of liver ferritin from a normal rat is usually near 10%. On this assumption, 1 ml. of the antiserum should have precipitated at least 1 mg. of ferritin protein.

The complete precipitation of antigen(as judged by recoveries of iron) did not always result in a specific precipitate of constant composition. At low levels of antigen

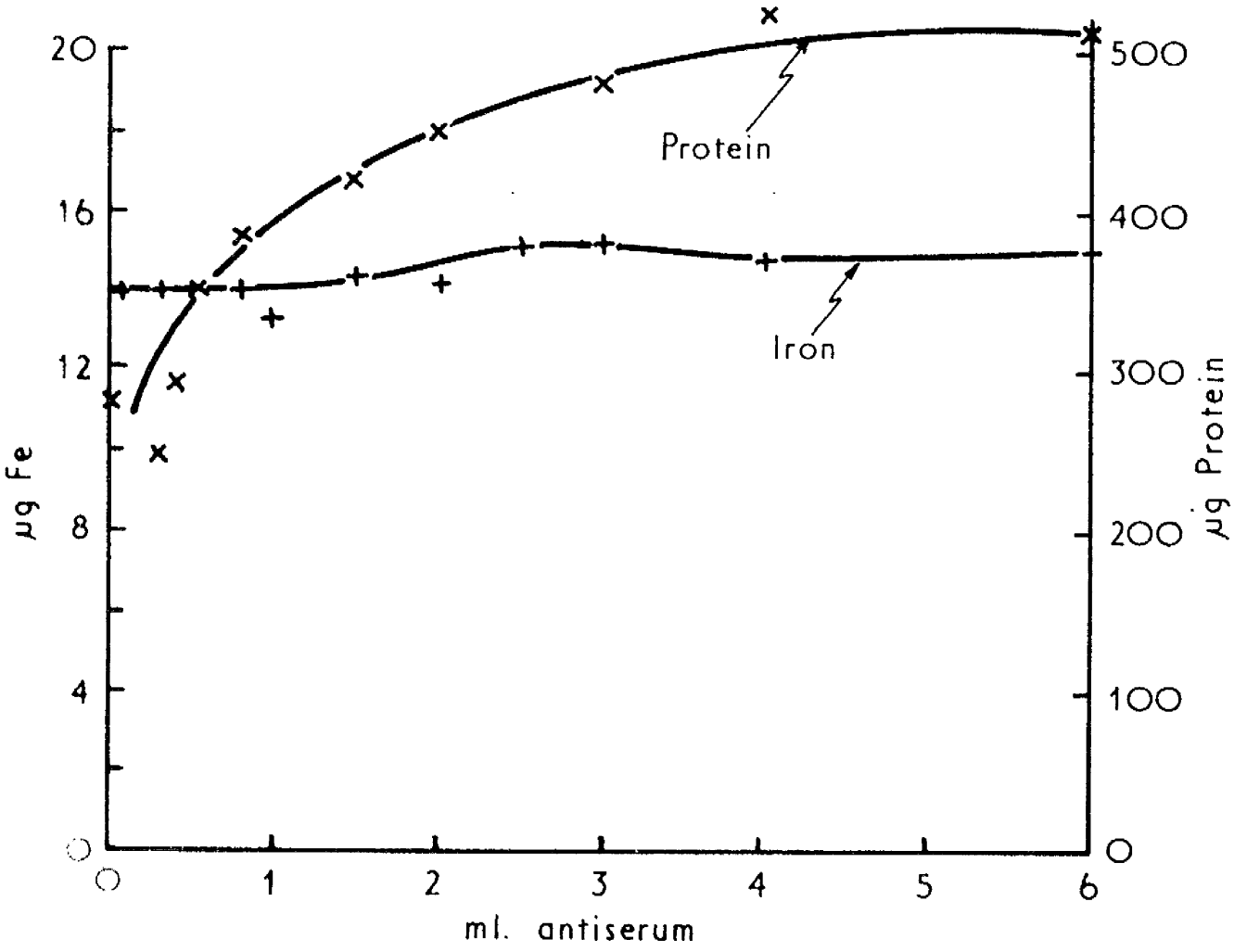


Fig. 8. Precipitation curve of rat ferritin by rabbit antihorse spleen ferritin. A liver extract (1 ml.) containing 13 µg. ferritin iron was used as a source of antigen. The amounts of iron and protein in the specific precipitate formed after incubation with increasing amounts of antiserum are plotted against the volume of antiserum used.

the amount of protein recovered in the specific precipitate was dependent on the initial amount of antibody. A constant composition of the specific precipitate was however obtained in regions of an excess of antibody.

(d) Immunological isolation of rat-liver ferritin

Ferritin was isolated by immunological precipitation from extracts of rat liver as described by Mazur and Shorr (1950). All precipitations were carried out in the region of excess of antibody. This was ensured by estimating the amount of ferritin iron in the liver extract before carrying out the immunological precipitation.

Samples (2 ml.) of the supernatant fraction remaining after heating a 1:5 (w/v) homogenate to 80° were incubated at 37° for one hour with 3 ml. of the stock antiserum. Under these conditions the composition of the antigen-antibody precipitate was relatively constant (Fig.8). The incubation was continued for a further 18 hours at 0° to ensure complete precipitation (Mazur and Shorr, 1950; Mazur, Green and Carleton, 1960). The resulting precipitates were collected by centrifugation and washed twice with 3 ml. of 0.9% saline. They were taken up in warm 0.1N sodium hydroxide, in which they were readily soluble, and the solutions were diluted to 10 ml. with water for subsequent estimations of protein content and radioactivity.

Under these conditions, the specific precipitate was found to contain more iron than could be accounted for by ferritin present in the heat supernatant fraction. In twenty-one different samples the specific precipitate contained between 0.3 and 8.6 (mean ± S.E; 3.9 ± 0.9) µg. more iron than that estimated to be present as ferritin by ammonium sulphate precipitation (page 35). The additional iron recovered in specific precipitate may represent transfer of iron from transferrin in the rabbit serum during incubation. This was quantitatively feasible since the volume of serum used could have contained as much as 12 µg. of transferrin iron. Such a transfer has been demonstrated in similar systems in vitro (Mazur, Green and Carleton, 1960). Even when incubations were performed at 0° in the presence of versene (0.005M) this transfer still occurred. The iron content of the specific precipitate was therefore considered unsuitable as a measure of the amount of ferritin iron present.

(i) PROCEDURE FOR THE ISOLATION OF RAT-LIVER FERRITIN

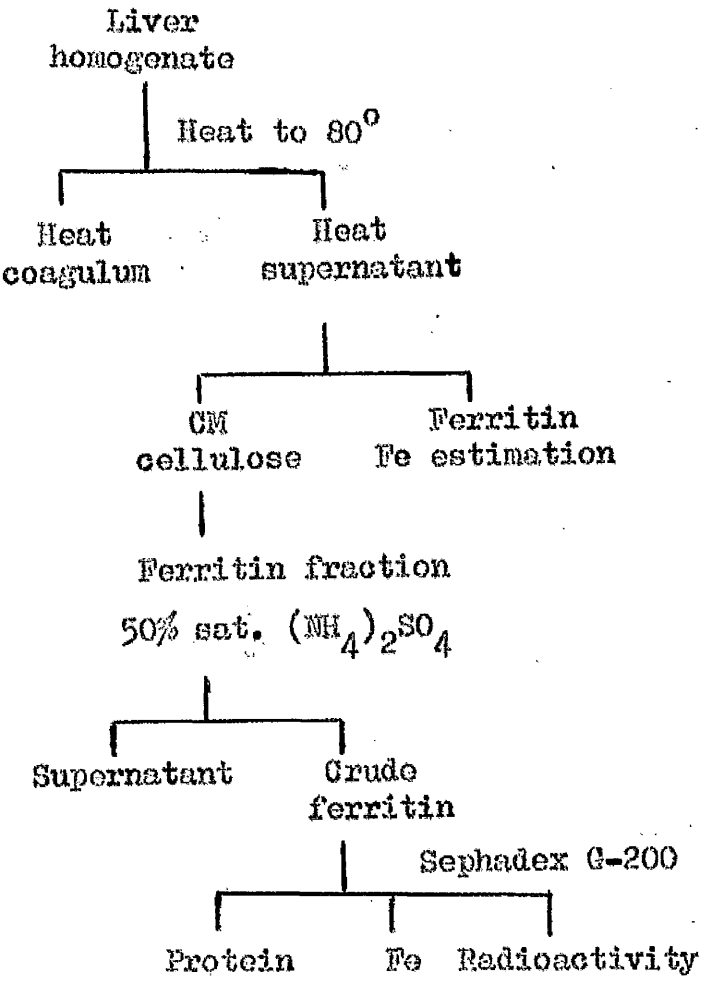
The procedure devised for the isolation of rat-liver ferritin can be considered in four parts (Fig.9). First, the well-known stability of ferritin at 80° in neutral solutions of low ionic strength was used to separate it from the bulk of the tissue proteins which are coagulated at this temperature (Laufberger, 1937). Secondly, the resulting supernatant fraction was chromatographed on CM-cellulose by a modification of the procedure described by Drysdale and Ramsay (1965). Thirdly, the chromatographic fraction containing ferritin was concentrated and further purified by precipitation with ammonium sulphate (Mazur, Litt and Shorr, 1950). Finally, the precipitate of crude ferritin was dissolved and fractionated on a column of Sephadex G-200 to remove the salt and traces of contaminating proteins. These procedures are described in detail below.

Extraction of ferritin from liver homogenate

Each rat was killed by a blow on the head and the liver exposed. The portal vein and that part of the inferior vena cava above the diaphragm were severed to allow blood drainage. No further steps were taken to remove residual blood. The liver was excised, blotted, weighed and finally homogenized in 4 volumes (w/v) of water in a Potter-Elvehjem-type homogenizer fitted with a Teflon (polytetrafluorethylene) piston. The choice of

Fig. 9

Scheme for the Isolation of Rat-Liver Ferritin



four volumes is critical, being dictated by the need for an ionic strength sufficient to allow coagulation of most of the tissue proteins yet low enough to permit the retention of ferritin by CM-cellulose (Drysdale and Ramsay, (1965). The homogenate was slowly heated, with continual stirring, in a water bath to 80°C. About 80-90% of the liver protein is thus coagulated and was subsequently removed by centrifugation. The supernatant fraction, hereafter referred to as the heat supernatant fraction, was chilled to 2° . Samples of this fraction were taken for the estimation of total ferritin iron in liver as described in the Materials and Methods section and other samples were used for the further purification of ferritin by chromatography.

Chromatography of the heat supernatant fraction on CM-cellulose

The further purification of ferritin from the heat supernatant fraction was accomplished by modifying the procedure of Drysdale and Ramsay (1965) to eliminate some of the non-ferritin proteins present in the chromatographic fraction obtained by their method. The above authors demonstrated that ferritin is retained by CM-cellulose from solutions of pH less than 5.0 and ionic strength less than 0.015. The chilled heat supernatant fraction was therefore titrated to pH 4.8 with 0.2N-acetic acid. A resulting precipitate, which appears to consist largely of

haemoglobin, was removed by centrifugation. Usually 5 or 10 ml. of the pH 4.8 supernatant was added to a column of about 500 mg. of CM-cellulose which had been equilibrated previously with a buffer, pH 4.8, containing 15 m-equiv. sodium acetate and 10 m-equiv. of acetic acid/l. The sample was allowed to flow through under gravity and the columns were then washed thoroughly with this buffer followed by a buffer, pH 4.9, containing 32 m-equiv. of sodium acetate and 18 m-equiv. of acetic acid/l. At this higher pH and ionic strength some non-ferritin protein is removed and the subsequent elution of ferritin occurs more easily. These washings were discarded.

Ferritin was subsequently eluted in a fraction obtained by developing the columns with a buffer, pH 5.5, containing 43.3 m-equiv. of sodium acetate and 6.2 m-equiv. of acetic acid/l. This treatment was found to give maximal ferritin recoveries and minimal contamination with other proteins. The ferritin, which may be clearly seen as a yellow band (Fig.10) when in quantities greater than 25 µg., separates from the top of the column after a volume of about 5 ml. of buffer has passed through. The fraction containing ferritin was collected in the following 15-20 ml. eluate.

Recoveries of ferritin, as judged by recoveries of ferritin iron, were optimal when all stages before the elution of ferritin were carried out in the cold room (4^{o}) with chilled buffers.

The Chromatography of Ferritin on CM-Cellulose and Sephadex G-200

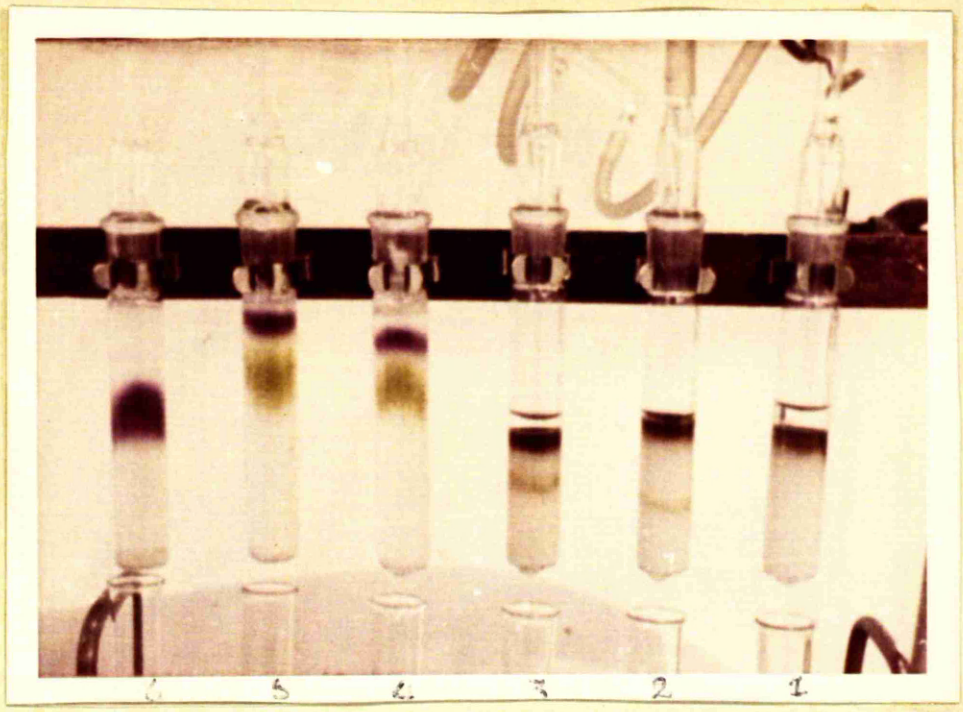


Fig. 10. Columns 1 - 3 show the elution of ferritin from columns of CM-cellulose after the addition of a liver extract (page 44). Columns 4 - 6 show the separation of ferritin from low molecular weight substances whose position is indicated by bromophenol blue. In column 6 the ferritin fraction has been completely eluted.

This chromatographic procedure did not discriminate between ferritin and apoferritin. Samples (100-250 µg) of apoferritin, prepared from crystalline rat ferritin as described on page 30 were chromatographed by this method and 70-80% was recovered under the conditions for the elution of ferritin.

Ammonium sulphate fractionation

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

Gel filtration

The precipitate, containing ferritin, resulting from the fractionation with ammonium sulphate was taken up in 0.4 ml. of 10 mM-phosphate buffer, pH 7.0, containing bromophenol blue (0.02%), for chromatography on Sephadex G-200 (Fig.10). Ferritin, which has a minimal molecular weight of 465,000 (Rothen, 1944) is completely excluded from the inner volume of this material and appears in the effluent

immediately after the void volume. The dye was used to indicate the inner volume of the gel. Although it appeared to interact slightly with the Sephadex, being eluted in a slightly larger volume than haemoglobin (which also penetrates the inner volume), it proved valuable for indicating columns with unsatisfactory flow properties.

The columns used were 12.5 cm. long and 1.5 cm. in diameter and were fitted with ground glass joints. It was usual to operate simultaneously 6 to 12 columns fed from a common reservoir under a pressure of 60 cm. water. They were filled with a suspension of gel which was supported on the flat perforated bottom by a layer of lens paper. The bed volume was 12 ml. and the void volume was 3.5 ml. The samples of crude ferritin were layered on to a protecting disc of glass paper and washed in with 1 ml. of buffer after they had drained through the glass paper. The final ferritin fraction was collected in the 2.5 ml. effluent sample immediately following the void volume. For convenience, the first 6 ml. eluted after the introduction of the sample were collected.

(ii) Tests of Purity of the Isolated Ferritin

Tests were carried out on the fraction eluted from the Sephadex gel to establish the presence of ferritin. This was demonstrated in three ways. First, the protein in this fraction was shown to have a high iron content, some preparations having up to 25% iron as estimated by the method for ferritin iron (page 34). Secondly, crystals with the conformation characteristic of ferritin (Granick, 1943) were obtained after concentrating this fraction and treating with cadmium sulphate. Finally, a brown precipitate containing up to 4-5% iron was obtained by immunological precipitation using an antiserum specific for ferritin (page 39). No other protein contains these concentrations of iron except haemosiderin which is removed in the coagulum formed by heating the homogenate to 80° and also by the chromatographic procedures on CM-cellulose (Drysdale and Ramsay, 1965).

Having established the presence of ferritin in this fraction, it was then examined for the presence of contaminant proteins. For this purpose, gel electrophoresis, immunoelectrophoresis, ultracentrifugation and measurements of the specific activity of isotopically labelled ferritin were applied to the final preparation; gel electrophoresis and immunoelectrophoresis were also used to screen successive stages in purification.

Gel electrophoresis

The fractions obtained at the successive stages in the fractionation procedure were submitted to electrophoresis in polyacrylamide gels and stained for both protein and iron (Fig.11) as described on page 36. The heat supernatant fraction of a rat-liver homogenate showed many discrete protein species, of which only a minor proportion stained for non-haem iron. This was normally present as one major band, accompanied by one or more minor iron-staining components. The presence of several ferritin bands agrees with the findings of Richter (1964b), who demonstrated at least three electrophoretically distinct, but antigenically identical components in crystalline rat-liver ferritin, the major component comprising 75-85% of the total. The supernatant fraction obtained after titrating the heat supernatant fraction to pH 4.8 showed a similar distribution of protein although there was less non-ferritin protein near the origin. After chromatography on CM-cellulose, only one major band which did not stain for iron was found. This band disappeared after ammonium sulphate precipitation, leaving only a small amount of protein which did not contain iron and migrated in front of ferritin. After gel filtration this disappeared and all of the protein components detected in the final ferritin preparation took the Prussian Blue stain for iron. This implies that no protein other than ferritin is present.

Screening of Ferritin Isolation Procedure by Electrophoresis in acrylamide gels

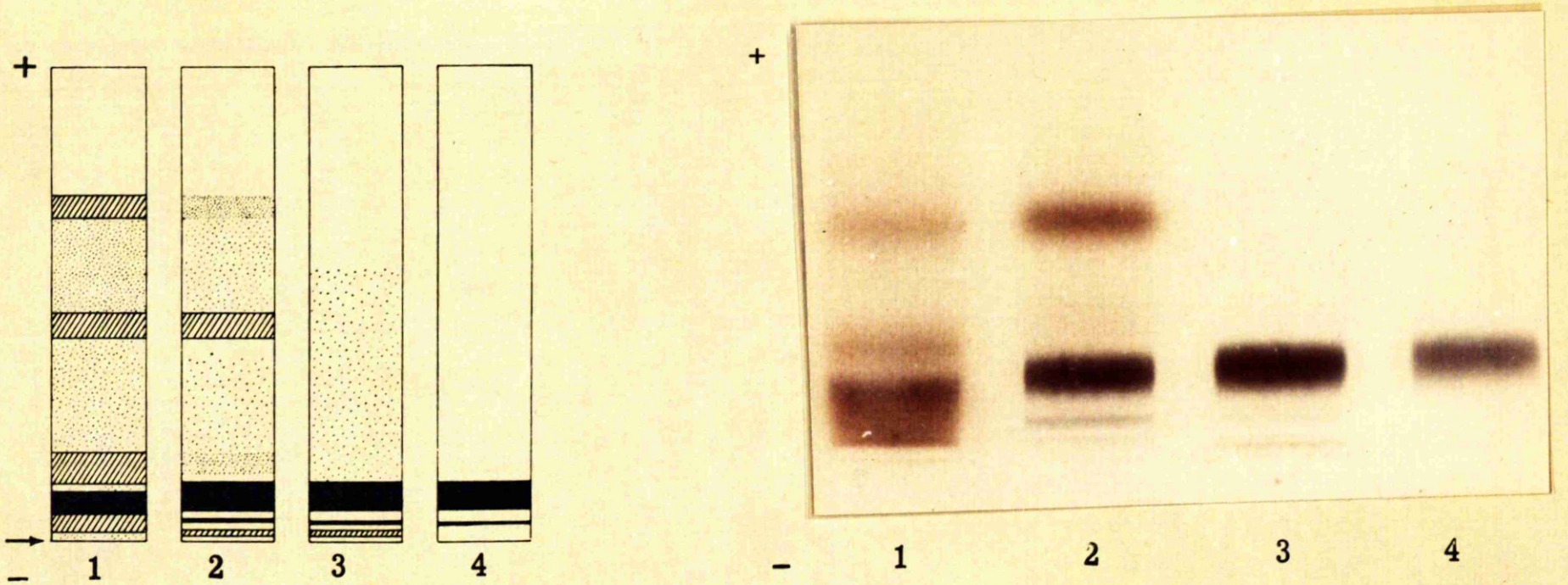
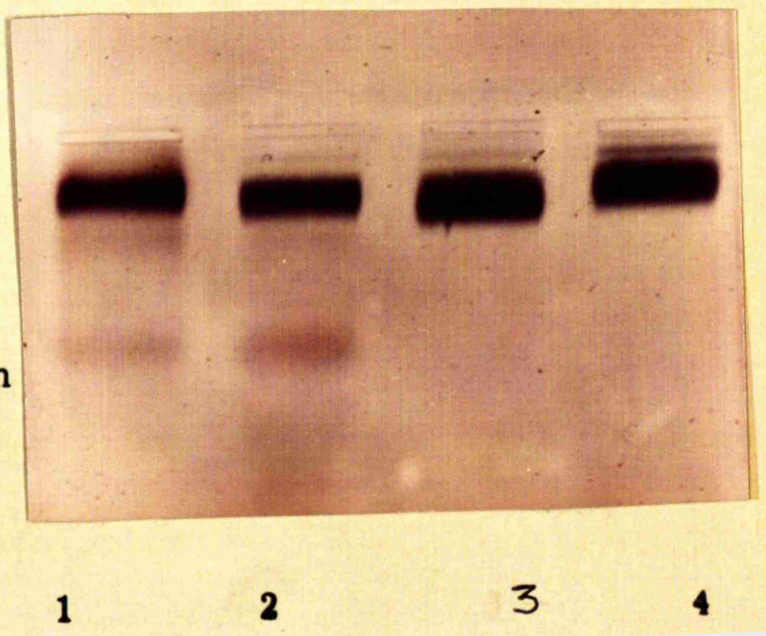


Fig. 11. Polyacrylamide-gel electrophoresis of fractions obtained at successive stages in the isolation of ferritin. The colour prints are very poor reproductions of the original gels. Prints from two different gels are shown. Since neither was satisfactory, a diagram is also given to show how the gels actually appeared. Protein is stained red with Ponceau S (hatched band and stippled areas in diagram) and iron is stained blue with potassium ferrocyanide (solid bands in diagram). The origin is indicated by the arrow. (1) Heat supernatant fraction. (2) Fraction obtained after chromatography on CM-cellulose. (3) Fraction obtained on precipitation with ammonium sulphate. (4) Fraction obtained after chromatography on G-200 Sephadex.



Ultracentrifugation studies

Samples of the final ferritin preparation obtained from livers of normal rats were examined in the Spinco model E analytical ultracentrifuge to investigate its purity and also to examine the ferritin spectrum of particle densities, caused by the presence of particles with different iron contents and therefore of different densities (Rothen, 1944). One sample (approx. 5 mg. of protein/ml.) was centrifuged at 44,770 rev./min (g_{av}. 145,650) in a 12 mm. cell at 4°. Photographs were taken at 4 min. intervals with schlieren optics. A more dilute sample (0.5 mg./ml.) was examined with u.v. optics. The sedimentation coefficients were calculated from both runs from the equation:

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

where S is the sedimentation coefficient, w is the angular velocity (radians/sec.), r is the distance of the boundary from the centre of rotation and t is the time (sec.). The results are expressed in Svedberg units (s). Under the above conditions, particles of 4s and greater can be resolved.

All the material detected with both optical systems had sedimentation coefficients greater than 18s, that of apoferritin (Rothen, 1944). With u.v. optics (Fig.12a), the absorption was all contained in a single diffuse

Tests of Purity of Ferritin by Ultracentrifugation

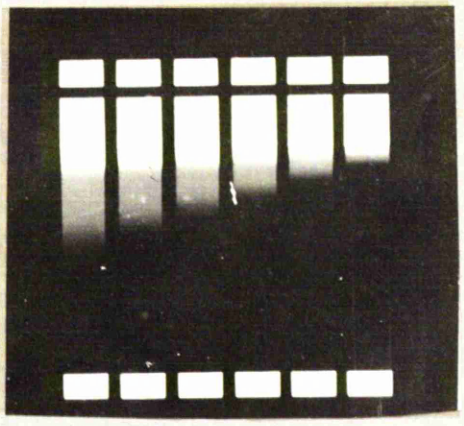


Fig. 12(a) U. V. pattern of ferritin from normal rats

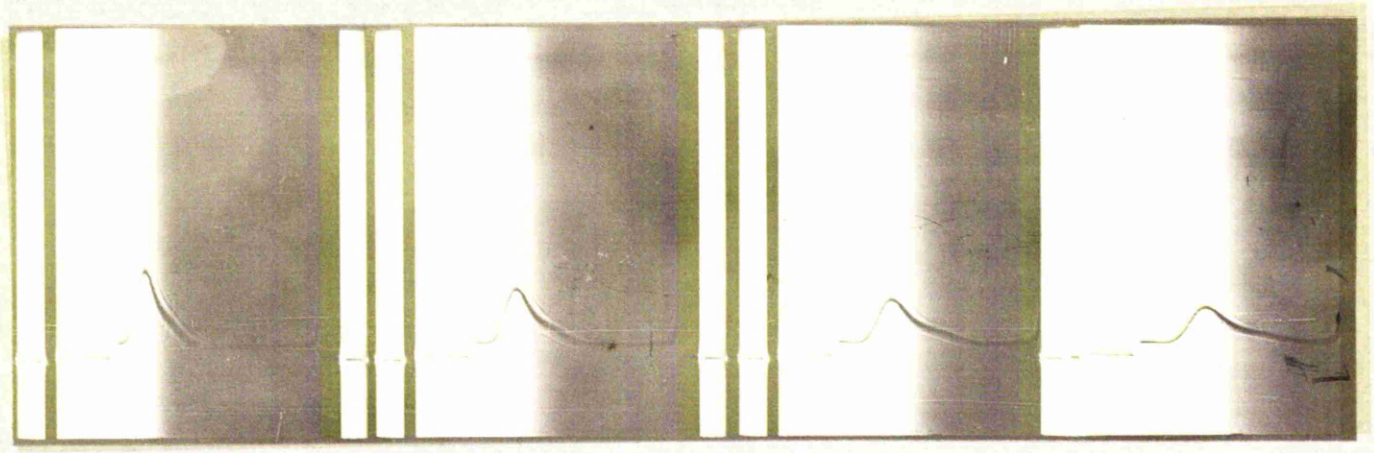
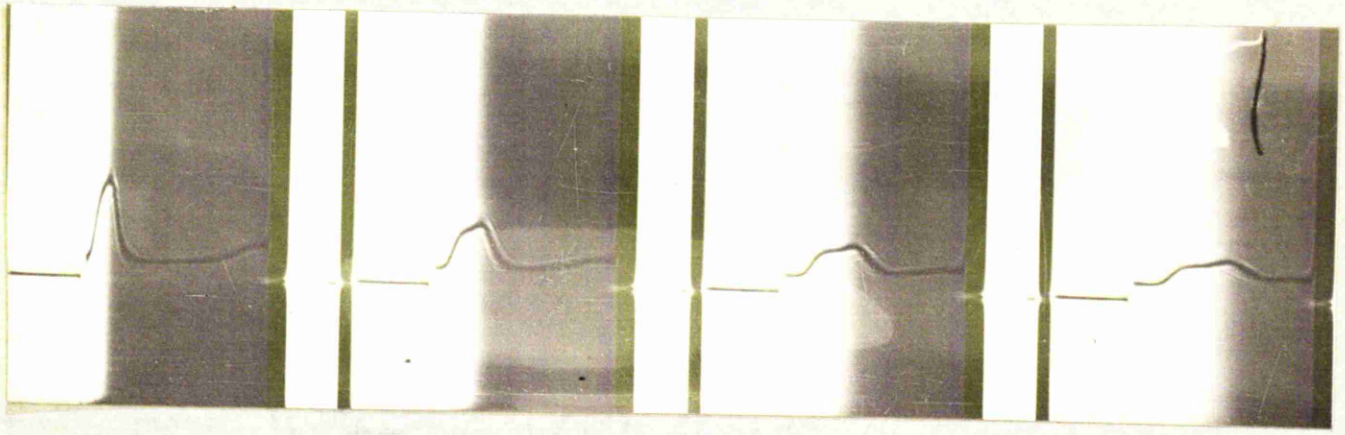


Fig. 12(b) (i) Schlieren pattern of ferritin from normal rats



(ii) Schlieren pattern of ferritin from iron-injected rats.

boundary, most of the absorption being near 60s. With schlieren optics, the sedimentation coefficients also ranged from 20 to 60s but the heavier fraction did not predominate. This was more apparent when samples (approx. 10 mg. of protein/ml.) of ferritin preparations were centrifuged at 25,980 rev./min. ($g_{av.}$ 49,040) in an effort to fractionate the ferritin species. Photographs were taken at 8 minute intervals (Fig.12b). Two samples of purified ferritin were examined. One sample was obtained from livers of normal animals while the other was obtained from animals which had received a single dose of 400 µg iron as ferric ammonium citrate four hours before death. Most of the ferritin particles from normal liver had sedimentation coefficients near 29s. However, in the case of iron-treated animals, the particles ranged in size from 21s to 60s, the highest concentrations being in the region of 60s and 43s. Similar changes in the distribution of ferritin molecules after iron treatment have been observed in later studies.

It seemed possible that the discrepancy in the apparent distribution of ferritin species observed by the u.v. and schlieren optics might be associated with the known high iron content of the denser particles (Mazur, Litt and Shorr, 1950). The possible contribution of the iron in ferritin to its absorption in u.v. light was therefore investigated.

The absorption spectra of solutions of apoferritin (30 µg of protein/ml.), ferritin (30 µg of protein/ml. and 7 µg of iron/ml.) and ferric ammonium citrate (7 µg of iron/ml.) were scanned between 250 mµ and 500 mµ in the Beckman DB Spectrophotometer (Fig.13). The results showed that the ferritin solution absorbed strongly at 260 mµ whereas a solution of apoferritin of equivalent protein content gave an absorption value of only 5% of that given by the ferritin solution at this wavelength. The specific extinction coefficient ($E\ \frac{1\ \text{mg./ml.}}{1\ \text{cm.}}$) was 0.95 at 280 mµ for the rat apoferritin sample, which is comparable with the values of 0.86-0.97 for horse apoferritin (Hoffmann and Harrison, 1963) and 1.01 for guinea-pig apoferritin (Friedberg, 1962). Ferric ammonium citrate solution of the same iron content as the ferritin sample also absorbed strongly in the u.v. range, and had a similar absorption between 250 and 300 mµ to the ferritin solution. These results therefore suggested that the discrepancy between the relative amounts of the different ferritin species shown by u.v. optics and by schlieren optics may arise from the greater iron content of the denser particles, which would absorb extensively in the u.v. system but not in the schlieren system.

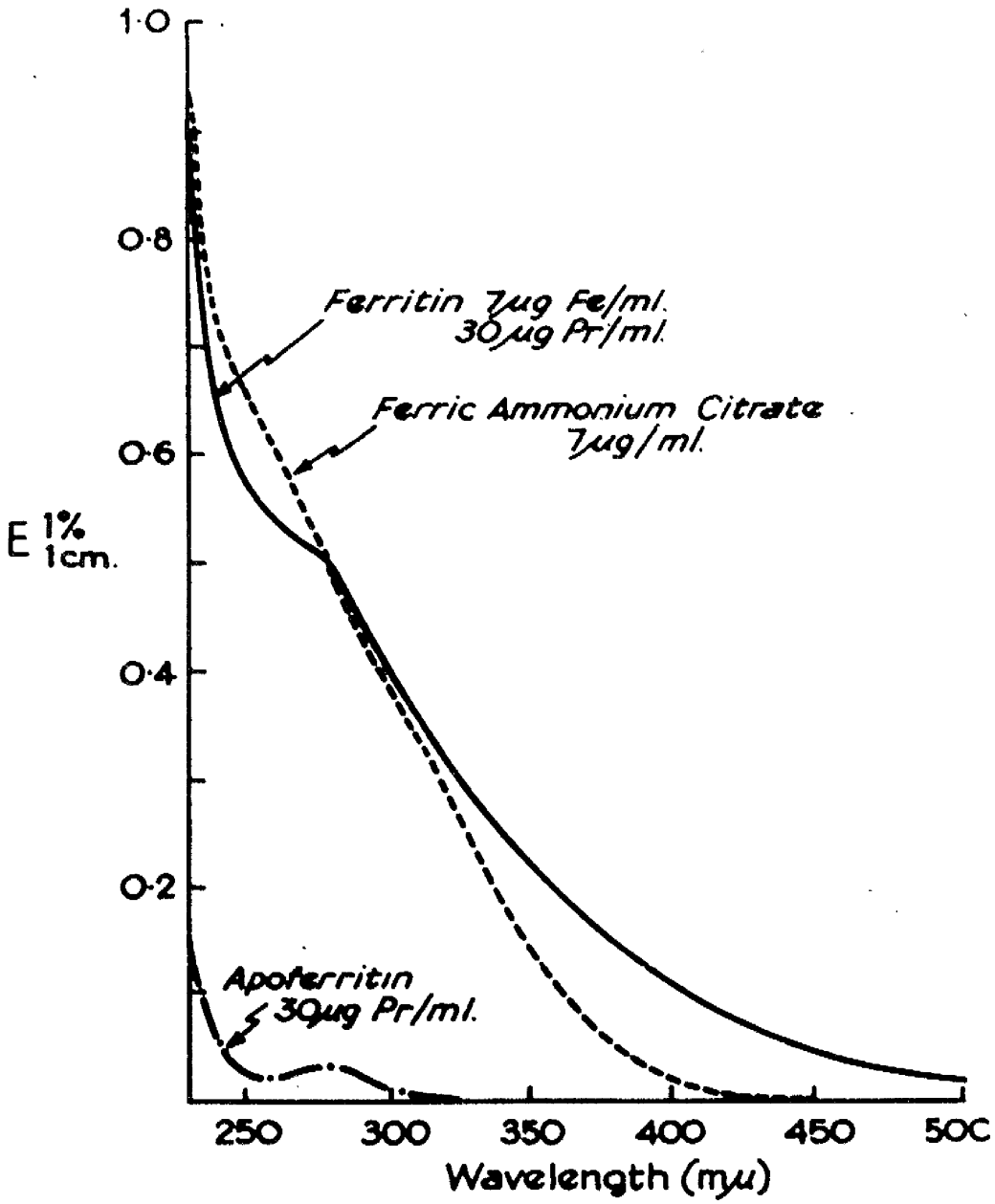


Fig. 13. Absorption spectra of apoferritin (–. –. –.) at a concentration of 30 µg. protein/ml., ferritin (_____) at a concentration of 30 µg. protein/ml. and 7 µg. iron/ml and ferric ammonium citrate at a concentration of 7 µg. iron/ml.

Immunoelectrophoretic studies

An antiserum was prepared in rabbits to the heat supernatant fraction (page 44) which might be expected to contain all the possible contaminants of the final ferritin preparation. Samples of the final purified ferritin preparation and of the heat supernatant fraction were examined by immunoelectrophoresis. The gels were stained for protein and iron as described on page 36 and were photographed (Fig.14).

As would be expected from previous electrophoretic studies with polyacrylamide gels (Fig.11), at least six components were detected by immunoelectrophoresis in the heat supernatant fraction. However, only one precipitin band was detected in the final ferritin preparation. This band stained with both the protein stain and the Prussian Blue stain for iron and was consequently identified as ferritin.

Test of Purity of Ferritin Preparation

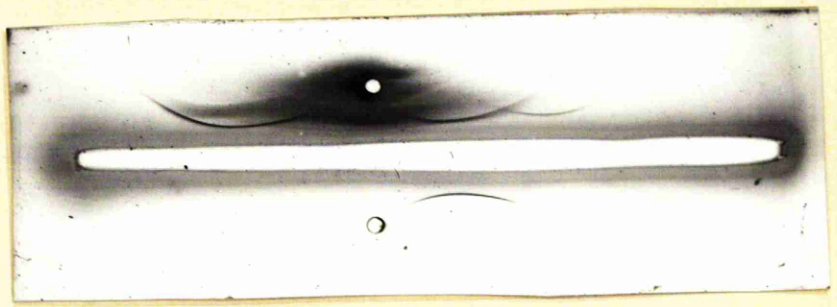


Fig. 14. Immunoelectrophoretic pattern obtained with the heat supernatant fraction (upper half of gel) and with the final purified ferritin fraction (lower half of gel), with an antiserum to the heat supernatant fraction in the central trough.

(iii) Application of Procedure to Studies of the Incorporation of ^{14}C-amino acids into Ferritin

The purified preparations of ferritin were used for estimations of iron content and specific activity (c.p.m./mg. protein) after labelling in vivo with ^{14}C-amino acids. Owing to the small amounts of ferritin normally available, procedures had consequently to be devised which allowed the complete recovery of protein after the iron estimations for subsequent estimation of radioactivity. The protein content of suitably diluted samples was estimated by the Lowry procedure (page 30) and also a known volume was taken for iron estimation as described on page 34. This method was chosen to obtain complete extraction of iron from ferritin and at the same time to prevent the production of fragments of protein which were no longer acid precipitable. The protein remaining after iron estimation was precipitated with acid and subsequently plated. The specific activity of the ferritin protein was then computed by relating the radioactivity observed in this precipitate to its protein content estimated separately. The details of these procedures and the various precautions found necessary are described below.

Recovery of protein after iron estimation

During the extraction of iron from ferritin, a precipitate of denatured or degraded apoferritin appeared which was subsequently deposited by centrifugation before the colorimetric estimation of iron. As it was desirable to be able to recover all of the apoferritin for the estimation of its radioactivity, the amount of the original ferritin protein recovered in this precipitate fraction was investigated. Nine different samples of purified ferritin solutions of known protein content were processed for the estimation of ferritin iron. The resulting precipitate formed from each solution was spun down and washed once with water. It was then taken up in 0.1N-sodium hydroxide and its protein content estimated by the Lowry method. The results are given in Table 5 which shows that 83 $\pm$ 4% of the protein estimated to be present in the original solution was recovered in the precipitate fraction after iron extraction.

This apparent loss of protein into the supernatant fraction might have been attributed to the loss of Lowry chromogenic groups on heating the protein at 100° in weak acid solution. This possibility was examined by investigating the recovery of ^{14}C-labelled ferritin protein from this precipitate fraction. Five different samples of purified ferritin were obtained from animals which had

Table 5

Recovery of protein after extraction of iron from ferritin

Protein (µg.) in original solution	Protein (µg.) recovered	% Recovery
76	66	87
81	62	77
88	81	92
137	103	75
145	117	81
146	109	75
184	170	92
198	210	106
283	190	67
	Mean ± S.E.	83 ± 4%

The amount of protein remaining in the precipitate fraction after the estimation of iron in different samples of purified ferritin was estimated by the Lowry method. The amount of protein recovered in this fraction has been compared with that estimated to have been present in the original solution.

received by intraperitoneal injection 5 μc ^{14}C-DL-leucine/ 100 g. body weight two hours before death. The ferritin in one portion of each sample was precipitated at 0.2N perchloric acid, washed twice with 5 ml. of 0.2N perchloric acid and plated for counting. Another equal portion of each solution was treated with the reagents for ferritin iron estimation and the resulting precipitate was washed once with 5 ml. of 0.2N perchloric acid and plated for counting. The results of this experiment are given in Table 6 which shows that, of the radioactivity precipitated from the original solution with perchloric acid, 80 ± 1% was recovered in the precipitate fraction formed during the extraction of iron from ferritin. These results therefore indicate that some protein material indeed remained in the supernatant fraction after iron estimation, as suggested by the previous findings.

In order to recover all of the protein after iron extraction, the supernatant fraction was recombined with the precipitate fraction after the estimation of iron. Bovine serum albumin (250 μg.) was added as carrier, followed by perchloric acid to a final concentration of 0.35N. After standing for at least one hour at 0°, the resulting precipitate was centrifuged down, washed once with 5 ml. of 0.2N perchloric acid and plated. Fifteen

Table 6

Recovery of radioactivity in precipitate fraction after extraction of iron from samples of ^{14}C-labelled ferritin

c.p.m. in PCA precipitate	c.p.m. in precipitate	% recovered in precipitate
45	34	76
64	53	83
73	58	79
77	63	82
155	126	81
	Mean ± S.E.	80 ± 1%

The amount of radioactivity recovered in the precipitate fraction after the estimation of iron in different samples of ^{14}C-labelled ferritin has been compared with that estimated to be present initially in the original sample. All samples were counted for 1000 counts.

different samples of purified ferritin, which had been previously labelled *in vivo* with ^{14}C-leucine were used to test the recovery of protein in the precipitate fraction obtained in this manner. The amount of radioactivity found in this fraction after the estimation of iron and the addition of carrier albumin and perchloric acid was compared with that given by direct precipitation with perchloric acid without iron estimation (Table 7). The results show that 96 $\pm$ 3% of the initial radioactivity was recovered in the precipitate fraction obtained by the above method. This procedure was therefore used for the estimation of the iron content and specific activity of ferritin in future experiments.

The pink complex formed between ferrous iron and 2 2'bipyridyl is known to be unstable below pH 3 (Hill, 1931). The acidification to 0.35N perchloric acid of the supernatant fraction after the extraction of iron was found to cause a considerable loss of optical density produced by this complex. Table 8 shows the effect of this concentration of perchloric acid on the optical density of ferrous bipyridyl solutions at various intervals after the addition of the perchloric acid. In view of this loss it was therefore necessary to complete the estimation of iron in the ferritin samples before precipitating the protein with acid. The above steps

Table 7

Recovery of ^{14}C-labelled ferritin after iron estimation

Radioactivity (c.p.m.) in original solution	Radioactivity (c.p.m.) recovered in precipitate	% Recovery
12	11	92
20	18	90
21	20	95
27	31	115
36	31	86
41	36	88
42	38	91
56	52	93
56	73	130
59	56	95
69	64	93
87	79	91
99	81	82
95	100	105
	Mean ± S.E.	96 ± 3

The amount of radioactivity in the precipitate fraction recovered from 14 different samples of ^{14}C-labelled ferritin after iron estimation and subsequent acidification to 0.35 N perchloric acid in the presence of carrier albumin has been compared with that precipitated from the original sample at 0.2N perchloric acid. All samples were counted for 1000 counts.

Table 8

Loss of optical density of ferrous bipyridyl in 0.35 N perchloric acid

Sample	Initial	Time after addition of perchloric acid (min.)		
		5	15	75
1	0.191	0.167	0.154	0.147
2	0.397	0.329	0.304	0.277
3	0.478	0.412	0.386	0.378
4	0.992	0.740	0.682	0.618

Samples of ferric ammonium citrate containing between about 10 and 70 µg. iron were treated with the reagents used for the estimation of ferritin iron as described on page 34. The optical density of each tube was read at 520 mu after the reaction was complete and then perchloric acid was added to give a final concentration of 0.35 N. The optical densities of the untreated samples may be compared with those given at various intervals after the addition of perchloric acid. The values of O.D. for the samples treated with perchloric acid have been adjusted to correct for the change in volume.

involved in the isolation of ferritin samples and the estimation of iron content and specific activity are given in Fig. 15 .

(iv) Comparison of specific activity of ^{14}C-labelled ferritin isolated by the new procedure and by immunological precipitation

The evidence from studies of gel electrophoresis, immunoelectrophoresis and ultracentrifugation studies suggested that only ferritin was present in the final fraction obtained after gel filtration. However, these tests do not exclude the possibility of some undetected contaminant which might have a rapid rate of amino acid incorporation and so cause serious errors in estimations of the specific activity of ^{14}C-labelled ferritin. This possibility was investigated by comparing the specific activities of samples of ^{14}C-labelled ferritin prepared by the new procedure with those given by ferritin isolated by immunological precipitation. The specific activity of the protein obtained by both methods was determined as described on page 56 .

Fifteen different samples of ^{14}C-labelled ferritin were obtained in the course of other investigations into the effect of iron on the incorporation of amino acids into ferritin. One group of animals received an intraperitoneal injection of ferric ammonium citrate (300 µg. Fe/100 g. body weight) while controls received saline. Both groups received 5 µc ^{14}C-DL-leucine/100 g. body weight at various

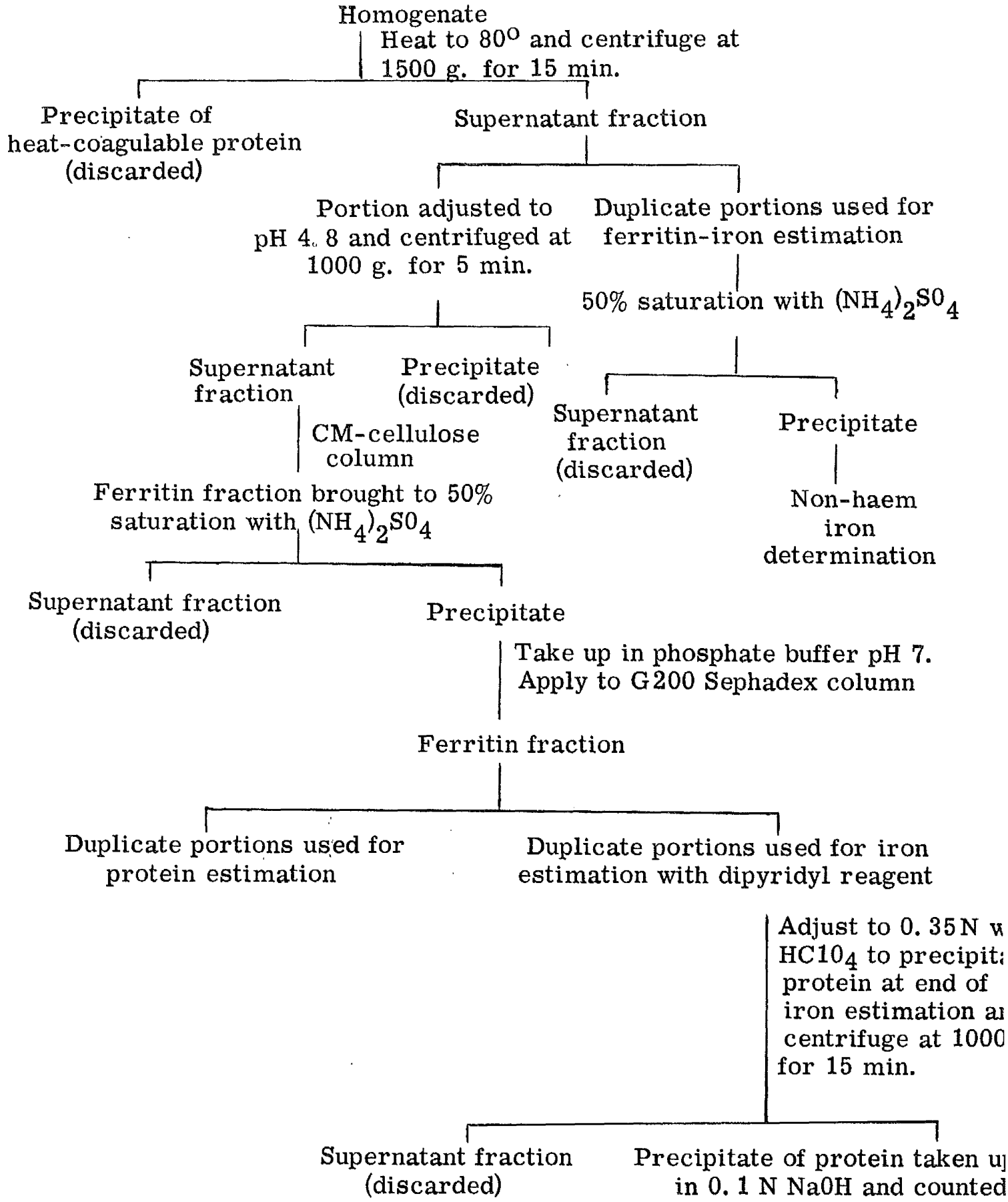


Fig. 15. Procedure for the isolation of ferritin and the estimation of its specific activity after labelling with ^{14}C-amino acids.

intervals after the iron and were killed two hours thereafter. The specific activity of the ferritin samples isolated by the chromatographic procedures and by immunological precipitation are given in Table 9.

The results show that the specific activity of the ferritin samples obtained by the chromatographic procedures was about four times that of those isolated by immunological precipitation; the lower result by the latter method is due to the presence of antibody protein in the precipitate. This ratio was essentially constant for samples obtained from controls as well as samples containing a higher level of radioactivity as the result of iron injection. This implies that there is no significant contamination of the purified ferritin preparations with highly radioactive proteins, since these might be expected to cause wider variation in the ratio of specific activities, especially on iron stimulation. Consequently, the ferritin isolated by the chromatographic procedures was judged suitable for the estimation of specific activity after the administration of labelled amino acids.

Table 9

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

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

Quantitative recovery of purified ferritin from liver homogenate

None of the steps in the purification of ferritin should cause any loss of ferritin. However, some loss may result from the chromatographic purification on CM-cellulose if chilled buffers are not used. Tests of recoveries of ferritin (as judged by the recovery of ferritin iron) were made on samples obtained from control and iron-treated animals. The results are given in Table 10 which shows that 81 $\pm$ 3% of the ferritin iron present in the original homogenate was recovered after our purification procedure.

The procedures described above also permit accurate measurements of the ferritin content of liver. The total ferritin iron can be measured on the supernatant fraction obtained after heat coagulation of the tissue proteins (Fig.9). Ferritin protein can only be accurately assayed on the preparation finally purified on Sephadex G-200. This does not provide a measure of the total amount of ferritin protein in the tissue, due to incomplete recovery. However, from the ratio of protein to iron in the purified ferritin, combined with the estimate of total ferritin iron in the heat supernatant fraction, it is possible to calculate the total amount of ferritin protein in the tissue.

Table 10

Recovery of purified ferritin from liver homogenate

Ferritin iron (µg.) in homogenate	Ferritin iron (µg.) recovered after purification	% Recovery
30	25	83
55	42	75
64	45	71
74	74	100
80	51	64
85	78	92
108	81	75
109	98	90
159	137	86
169	134	79
289	231	80
329	231	71
	Mean ± S.E.	81 ± 3%

The amount of ferritin iron in 12 samples of different liver homogenates used for the isolation of ferritin by chromatographic procedures, was estimated as described on page 35. The values have been compared with the total amount of ferritin iron recovered in the final preparation after the purification of the ferritin.

(v) CONCLUSION

The new procedure appears suitable for studying the incorporation of amino acids into rat-liver ferritin. The various criteria of purity applied to the purified ferritin preparation indicate that it is free from contaminating proteins. Ultracentrifugation has demonstrated the absence of significant amounts of molecules in the ferritin preparation with sedimentation coefficients less than 20s; most tissue proteins other than ferritin have lower sedimentation values and are thus excluded. Examination of the final ferritin fraction by electrophoresis in an acrylamide gel and by immunoelectrophoresis showed no detectable proteins other than ferritin. Finally, after injection of ^{14}C-leucine into a series of rats the specific activity of the liver ferritin isolated by the new procedure bore a constant relationship to that of liver ferritin separated by antigen-antibody precipitation, suggesting that both procedures isolate the same material.

The new procedure also permits estimation of small amounts of tissue ferritin, both by iron content and by protein content. Since the iron content of ferritin can vary between none and 23% (Granick, 1946), it is essential to include a measure of ferritin protein in order

to obtain an accurate estimate of the amount of ferritin present. The precision of the ferritin measurement is determined by the accuracy of the final protein estimation. The procedure of Lowry et al. (1951) was chosen, since the micro method can detect 5 µg. of protein. It is not possible to use ultraviolet absorption procedures to measure ferritin protein because its ultraviolet spectrum is masked by the intense ultraviolet absorption of the iron (Fig.13), and in consequence the amount of ultraviolet light absorbed by a given amount of ferritin depends mainly on its iron content.

EXPERIMENTAL AND RESULTS

Section 2: POLYMORPHISM IN FERRITIN

POLYMORPHISM IN FERRITIN

Ferritin and chemically prepared apoferritin each separate into a series of discrete bands on electrophoresis in starch or acrylamide gels (page 21). Richter (1964) attributed this separation to differences in the primary structure of the proteins in the separate bands. Rat-liver ferritin, obtained by our new procedure (page 43), separated into at least three discrete bands in acrylamide gels (Fig.11). If these bands represent genetically determined variations in the amino acid sequence of ferritin, then studies on control mechanisms of ferritin synthesis and turnover would be considerably complicated. Despite statements to the contrary by some authors, much of the experimental evidence suggests that the separated fractions represent different physico-chemical forms of a single protein. The arguments in favour of this viewpoint have already been presented (page 21).

This section describes investigations into the cause of the heterogeneity of ferritin after electrophoresis in acrylamide gels. The electrophoretic properties of ferritin and apoferritin on acrylamide gels have been examined under different conditions of electrophoresis. The relative electrophoretic mobilities and abundance of the various bands under different conditions suggested that a separation was being effected on the basis of particle size.

It was therefore desirable to obtain independent evidence by some other criterion to confirm these findings. One of the more obvious tools for this purpose was the analytical ultracentrifuge. However, earlier studies (page 50) showed that ferritin normally contains molecules with a wide spectrum of sedimentation coefficients between 20 and 60s due to variations in iron content. Thus it would be pointless to use ferritin for these studies since these iron-dependent differences in density of individual molecules would mask the presence of small amounts of material with different particle sizes. Accordingly, chemically prepared apoferritin was used since its individual molecules have the same density and since it also exhibits electrophoretic patterns in acrylamide gels similar to that of its parent ferritin (Kopp et al., 1964).

MATERIALS AND METHODS

Horse-spleen ferritin and apoferritin were prepared as described on page 30.

Electrophoresis

Acrylamide gels, 13 cm. long, were prepared at concentrations of 4, 5, 6.25 and 7.5% acrylamide with 5% cross-linkage in 0.05 M Tris-citrate buffer, pH 6.6. Solutions of approximately 2 mg. protein/ml. were used for all studies. The period of electrophoresis was adjusted to allow equal distance of migration of the main (α) band of ferritin. This was easily done by visual inspection of

the yellow ferritin band. Gels were run in the cold room (4°) to avoid overheating which occurred at room temperature during the prolonged electrophoresis needed with the more concentrated gels. After electrophoresis, the surface layer was sliced off with a wire in order to eliminate surface smears. Protein was stained red with Ponceau S and iron was stained blue with potassium ferrocyanide as described on page 36.

Densitometry

The relative intensities of the stained bands in the gels were estimated by densitometry on the Chromoscan instrument (Beckman) which was fitted with a device to allow the integration of the optical density profiles given by the bands.

Ultracentrifugation

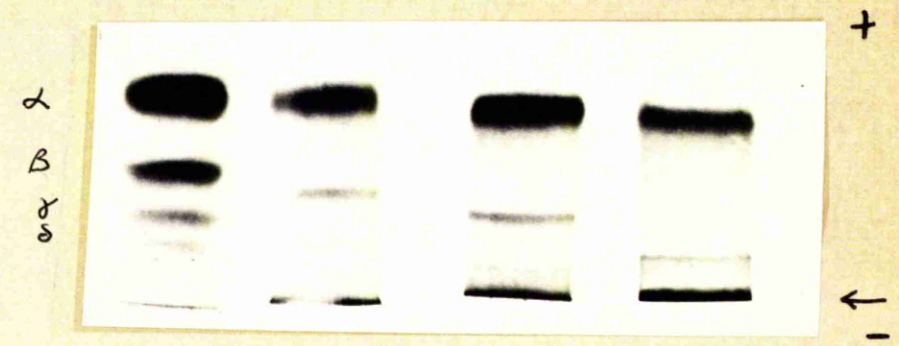
All studies were carried out in the Spinco Model E Analytical Ultracentrifuge.

EXPERIMENTAL AND RESULTS

(a) Electrophoresis in acrylamide gels

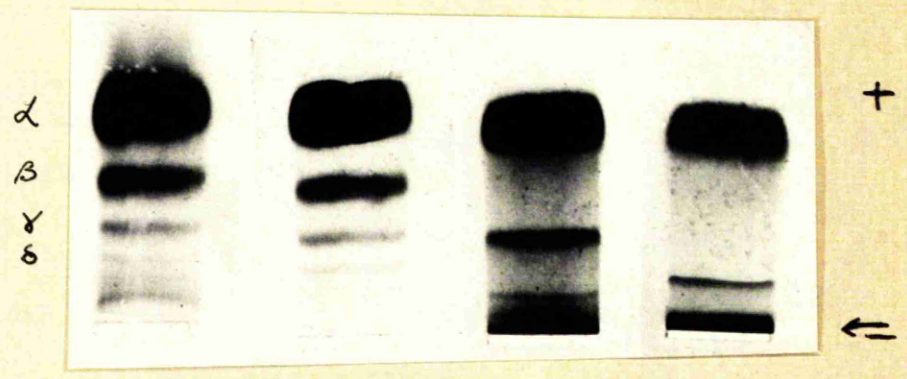
The electrophoretic patterns of ferritin and apoferritin in acrylamide gels ranging in concentration from 4 to 7.5% are shown in Fig.16. Similar electrophoretic patterns were given by both ferritin and chemically prepared apoferritin at any one gel concentration. The electrophoretic mobility of the main (α) band decreased considerably at the higher

Fig. 16. Electrophoresis of Ferritin and Apoferritin in Acrylamide Gels



Ferritin

% Acrylamide 4 5 6.25 7.5



Apoferritin

% Acrylamide 4 4 6.25 7.5

gel concentrations. Thus it took only 2 hours for this band to travel 3 mm. in a 4% gel but 20 hours to travel the same distance in a 7.5% gel.

One of the most striking findings was the wide variation in the number of bands observed at different gel concentrations. Only 2 bands entered the 7.5% gel but there was a progressive increase in the number entering the weaker gels; at least 6 bands were detected in the 4% gel. This progressive increase in the number of bands entering the gels was paralleled by a decrease in the amount of protein remaining at the origin. No material remained at the origin in the 4% gels. Further, the mobility of each minor band in relation to the α band decreased as the gel concentration increased. The β band migrated less than a quarter of the distance of the α band in the 7.5% gel but more than half the distance in the 4% gel. The mobilities of the other minor bands relative to the α band were also affected by gel concentration.

The above results suggested that these alterations in the relative electrophoretic mobilities of the minor bands with gel concentration are due to differences in the particle size of the protein in these bands rather than to differences in their net electrical charge. If the particles differed only in electrical charge one would expect them to have the same relative electrophoretic mobilities at all gel concentrations. Evidence from other electrophoretic

studies of proteins in starch gels supports this conclusion. Smithies (1962) showed that the electrophoretic mobilities of proteins in starch gels were considerably affected by gel concentration and that larger protein molecules were retarded to a greater extent than smaller molecules. He found that the relative retardations of different proteins in different gel concentrations were related to their particle size. Thus it is possible to compare the particle sizes of proteins which have the same net electrical charge (Smithies, 1962). This method was used to examine the particle size of the various ferritin fractions separated on acrylamide gels. The migration (m) of each of the minor bands relative to the α band was calculated from measurements taken from the photographs (Fig.16). These values were plotted against the reciprocal of the gel concentration ($\frac{1}{S}$) (Fig.17). The retardation coefficient (r) was calculated for each band from the equation $r = \frac{b}{m_r}$ where b is the slope of the plot of m versus $\frac{1}{S}$ and m_r is the migration at a fixed arbitrary reference gel concentration. The retardation coefficients of the β, γ and δ bands relative to the α band were 0.64, 1.03 and 1.29 respectively. These values approximate to a ratio of 2:3:4. The protein moiety of the α band has the same electrophoretic mobility as 18s apoferritin (Kopp et al., 1964). This must be the monomeric form since the twenty subunits of apoferritin have molecular weights of about 25,000 (Mainwaring, 1964).

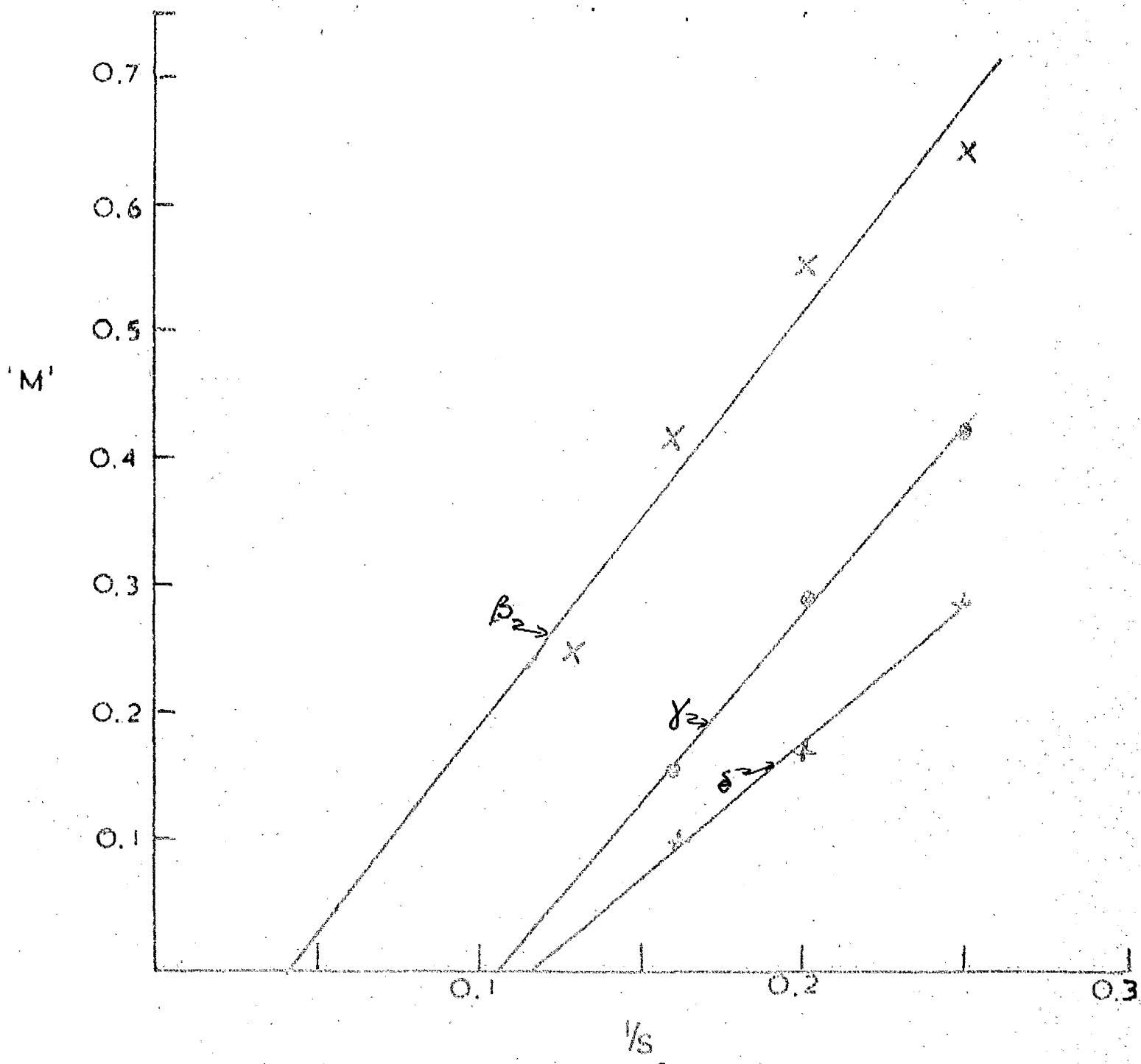


Fig. 17. Relative migrations of β, γ and δ bands of horse ferritin at four concentrations of acrylamide. The migration of the α band is taken as unity for each gel.

It has therefore been assumed by us that our α band is monomeric ferritin and that the β, γ and δ bands represent dimers, trimers and tetramers respectively, since they have relative retardation coefficients of 2:3:4.

Estimations were also made of the relative amounts of the α and β fractions at different gel concentrations by means of densitometry. In the case of the apoferritin preparation, the relative amount of the β band to the α band increased as the gel concentration was decreased. In the 7.5% gel, the amount of protein in the β band was only 12% of that in the α band but this rose to a value of 28% in the 4% gels. Similar trends occurred in the ferritin gels stained for iron. An exception to this picture was the amount of iron in the β band in the 7.5% gel which was 26% of that in the α band. This suggests that the β band has a higher iron content than the α band in this one instance. Unfortunately, not much reliance could be placed on this single observation in view of the considerable difficulties encountered in measuring the intensity of these coloured bands and it would be desirable to confirm these findings by some other method.

(b) Ultracentrifugation studies

Preliminary investigations with apoferritin solutions in salt concentrations adequate for accurate measurements of sedimentation coefficients showed the presence of one or two minor components sedimenting ahead of one major component.

This major component was assumed to be monomeric apoferritin M.W. 465,000, but was found to have a sedimentation coefficient of 14s, a value about 22% lower than that obtained by Rothen (1944). Subsequent studies showed that the sedimentation coefficient of this and the other components depended on the total protein concentration of the sample. Thus, accurate estimations of sedimentation coefficients would probably require extensive investigations over a wide range of protein concentrations and especially of dilute solutions. However, since the minor components comprised only a very small percentage of the total protein, it seemed probable that they would escape detection in dilute solutions. Since the main purpose of the study was to test for the presence of aggregates in apoferritin, no accurate measurements of sedimentation coefficients were attempted and conditions were selected which would favour the detection of minor components. Concentrated solutions of apoferritin (approximately 10 mg./ml.) were prepared in the same buffer as that used for gel electrophoresis i.e. 0.05 M tris-citrate pH 6.6, in order to compare the results given by both methods.

Samples of this preparation were centrifuged at 31,400 r.p.m. for 80 minutes. Photographs of the sedimenting peaks were taken with schlieren optics after 16 minutes at 8 minute intervals (Fig.18). At least 4

Schlieren Pattern of Ultracentrifugation of Apoferritin

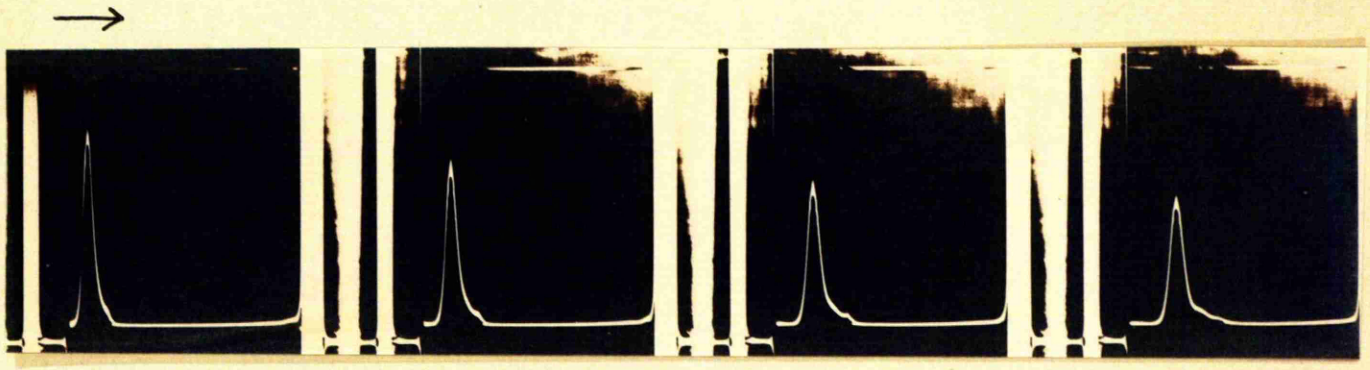


Fig. 18. A solution of apoferritin (approximately 10 mg. protein/ml. was centrifuged at 31, 400 rpm for 8 minutes. Photographs were taken with schlieren optics after 16 minutes at 8 minute intervals.

components were detected, the major component comprising about 80% of the total. The relative amounts of the minor components decreased with their increasing sedimentation velocity. These results are good evidence in favour of the presence of small amounts of aggregates in chemically prepared apoferritin.

Several interesting points emerged from these studies which might merit further investigation. First, since apoferritin and ferritin show identical electrophoretic patterns in acrylamide gels, it is possible that the distribution of the polymers observed in the gels represents that which was present in the tissue. However, since the relative proportions of protein in the α and β bands may be altered under different conditions of electrophoresis, it seems likely that some disaggregation and reaggregation can occur spontaneously. Secondly, comparison of the relative amounts of iron and protein in the α and β bands in the 7.5% gel may indicate a higher iron content in the β band than in the α band. The tendency for aggregation may therefore increase with increasing iron content of ferritin molecules. This would be in accord with the generally accepted conversion of ferritin to aggregates of haemosiderin. No firm conclusions can be drawn about factors affecting the state of aggregation of ferritin in tissues from this evidence of its behaviour in a concentrated solution. Many proteins such as albumin (Sogami and Foster, 1962), are known to aggregate

spontaneously in solution. The tendency for aggregation of such proteins may be altered by both pH and ionic strength.

Since this work was completed, Suran and Tarver (August, 1965) have published evidence which confirms many of these findings. They failed to detect any differences in the primary structure of the protein in the various bands separated by gel electrophoresis. They also found by ultracentrifugation studies that the α band, after reduction to apoferritin, consisted largely of monomeric ferritin. However, the β band contained about equal amounts of monomers and dimers. They considered that this heterogeneity was present in the β band in the gel. This is surprising in view of their findings that the α band consisted almost entirely of monomers and it seems likely that some disaggregation had occurred during the preparation of apoferritin from the band before ultracentrifugation studies.

The evidence from the present work and that of Sunar and Tarver (1965) suggests that it is valid for the purposes of future studies on ferritin induction to regard all ferritin molecules from one tissue as having the same genetically determined primary structure, and that the various bands represent different degrees of molecular aggregation.

EXPERIMENTAL AND RESULTS

Section 3: QUANTITATIVE ASPECTS OF THE INDUCTION OF LIVER FERRITIN BY IRON

(i) Time course of leucine incorporation into liver ferritin

(ii) Ferritin induction after iron injection

(iii) The effect of dose level of iron on the induction of ferritin

QUANTITATIVE ASPECTS OF THE INDUCTION OF LIVER FERRITIN BY IRON

The new procedures for the preparation of ferritin in sufficiently pure form for estimating its specific activity following injection of ^{14}C-labelled amino acids, combined with those for the estimation of the total amount of ferritin iron and protein in the whole liver, offered favourable circumstances for investigating the factors affecting the rate of ferritin synthesis and breakdown. This section deals with three quantitative aspects of ferritin synthesis:

(i) the time course of incorporation of ^{14}C-labelled amino acids into liver ferritin and mixed liver proteins

(ii) the time course of the induction of ferritin following a single injection of iron

(iii) the magnitude of this response to different dose levels of iron.

MATERIALS AND METHODS

Animals

Rats were fed stock diet and water ad libitum. They were fasted 18 hours before injection of labelled precursors to ensure uniformity of metabolic state.

Iron

Iron was given intraperitoneally in the form of a solution of ferric ammonium citrate. It was found to be

very important to adjust the pH of this solution to pH 7.4 before use to avoid obvious discomfort to the rats after injection.

Isolation of ferritin

Ferritin was isolated from liver samples as described on page 43. Measurements were made of the specific activity (c.p.m./mg. protein) and the iron/protein ratio of each sample. The total amounts of ferritin iron and ferritin protein in the liver of each animal were also estimated.

Measurement of ^{14}C-leucine uptake by mixed liver proteins

Samples (approx. 0.03 ml.) of the liver homogenate were heated at 80° in 0.05N NaOH for 5 minutes to solubilize protein and to dissociate leucine bound to S-RNA (Stephenson and Zamecnik, 1962). The samples were diluted to 10 ml. Portions (1 ml.) were taken for protein estimation by the Lowry method and the protein of other portions (5 ml.) was precipitated at 0.2N PCA. The precipitates were washed twice with 0.2N PCA and taken up in warm 0.1N NaOH and transferred quantitatively to planchets for counting as before (page 37).

EXPERIMENTAL AND RESULTS

I. Time course of leucine incorporation into liver ferritin

Fasted rats were injected with 5 μc (1-^{14}C)-DL-leucine/100 g. body weight and were killed at intervals up to 3 hours thereafter. The specific activities of samples of ferritin and of mixed liver proteins obtained from the livers

at the different time intervals were measured in counts/min./mg. protein and are presented diagrammatically in Fig.19. Statistical analysis of the data are given in Table 11.

The results show that uptake of ^{14}C-leucine into mixed liver proteins was already maximal at the 30 minute time interval after injection, but that labelling of ferritin rose more slowly and attained a plateau at 2 hours after injection. In view of this finding, a 2-hour time interval between injection of ^{14}C-leucine and removal of liver samples was used to allow maximum incorporation into both fractions.

II. Ferritin induction after iron injection

Fasted rats were given a single injection of 300 µg iron/100 g. body weight, control animals receiving saline. At intervals of 1, 3, 7, 10 and 22 hours thereafter, animals were injected with 5 µc ^{14}C-leucine/100 g. body weight and were killed 2 hours later. In this way, a "pulse-dose" effect was obtained so that the "instantaneous" (2-hour) incorporation was examined. The specific activities of samples of ferritin and mixed liver protein, together with the total amounts of ferritin iron and protein in the livers at these intervals, were estimated and are shown diagrammatically in Fig.20. Each point represents the mean value from 2 animals.

The results show that injection of iron caused a considerable, but transient, increase in the incorporation of

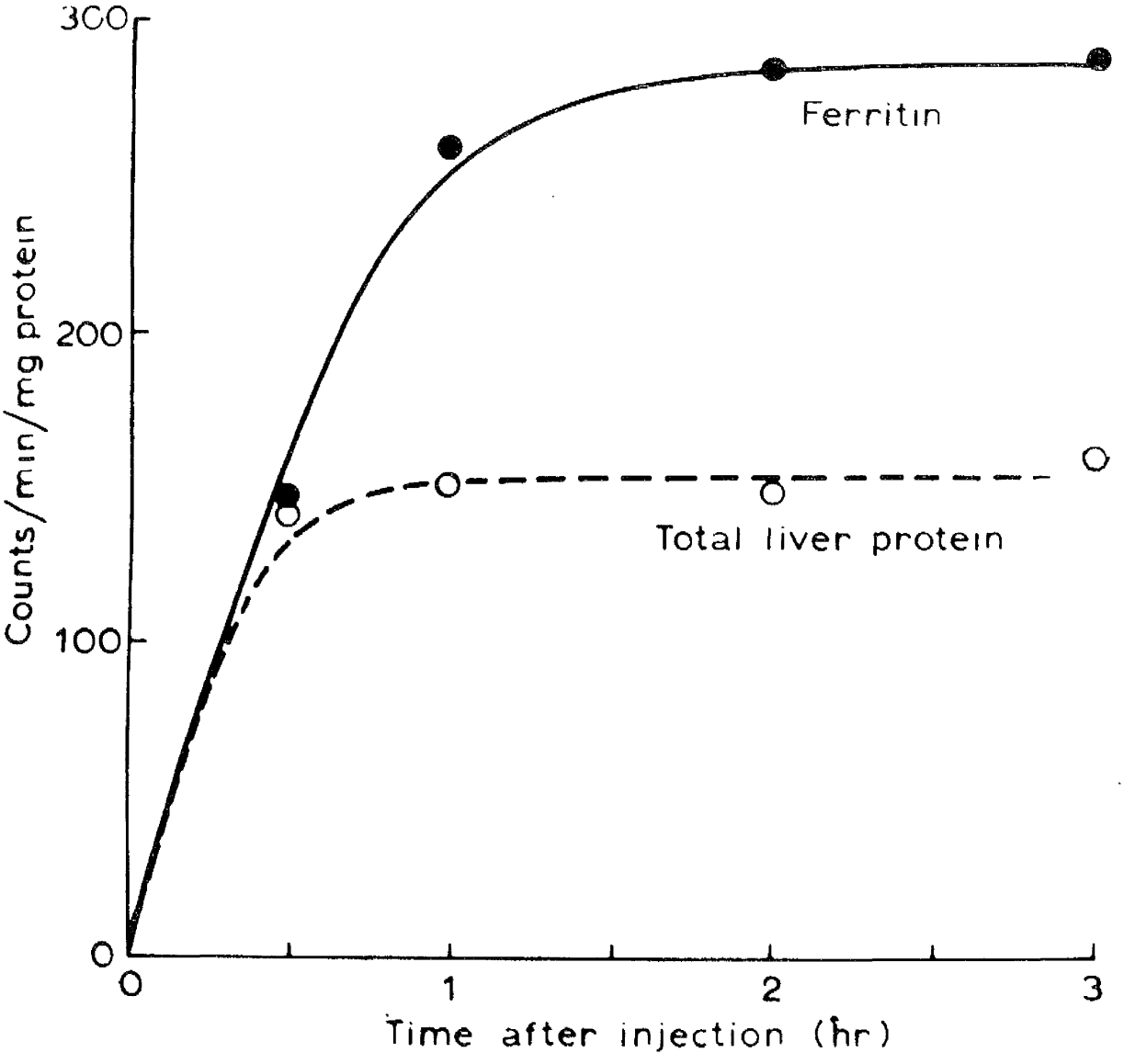


Fig. 19. Incorporation of ^{14}C-leucine into total liver protein and into liver ferritin at different times after injection of the isotope into rats. Each entry is the mean value obtained from 4 animals. Analysis of variance shows that the specific activity of the ferritin increases significantly with time, whereas that of total liver protein does not alter.

Table 11

Time course of incorporation of ^{14}C-leucine into ferritin and mixed liver protein

Ferritin

0.5	1 hr.	2 hr.	3 hr.	
260	326	395	300	
118	196	322	407	
84	243	222	206	
132	269	196	239	
594	1034	1135	1152	3915

CF = $(3915)^2/16$ = 957,951

Total sq. = 1084721 -CF = 126,770

Expts. = 991532 -CF = 33,581

Columns = 1009330 -CF = 51,379

Residual Error = 41810/11 = 3,800

Source of variation	Degrees of freedom	Sum of squares	Mean square	Variance ratio (F)
Total	15	126,770	-.	-.
Experiments	1	33,581	33,581	8.84
Columns	3	51,379	17,126	4.51 (sig. 5% level
Residual error	11	41,810	3,800	

^{14}C-leucine into ferritin. The maximal increase occurred when ^{14}C-leucine was injected between 2 and 3 hours after the iron and had subsided by 12 hours. The iron had no apparent effect on the incorporation of leucine into mixed liver protein. The single dose of iron also caused a significant increase in the total amount of ferritin iron and ferritin protein in the liver (Fig.20). Both values were maximal about 12 hours after iron injection but fell during the following 12 hours.

In order to support these quantitative findings, the data from these and later studies have been combined in order to show the total liver content of ferritin iron and protein and also the iron/protein ratio of ferritin in the livers of larger groups of control animals and of iron-injected animals. The data for the latter group were obtained from analysis of the livers of animals which had received a single intraperitoneal injection of 300 µg iron/100 g. body weight four hours before death. The full results and statistical analyses are given in Tables 12, 13 and 14. The average amount of ferritin iron in the livers of 28 uninjected rats was 0.166 ± 0.09 (S.E.) mg. iron/100 g. body weight and rose by about 60% to a value of 0.267 ± 0.016 four hours after iron injection. The average amount of ferritin protein in a group of 13 control animals was 1.59 ± 0.14 mg./100 g. body weight, whereas that in a group

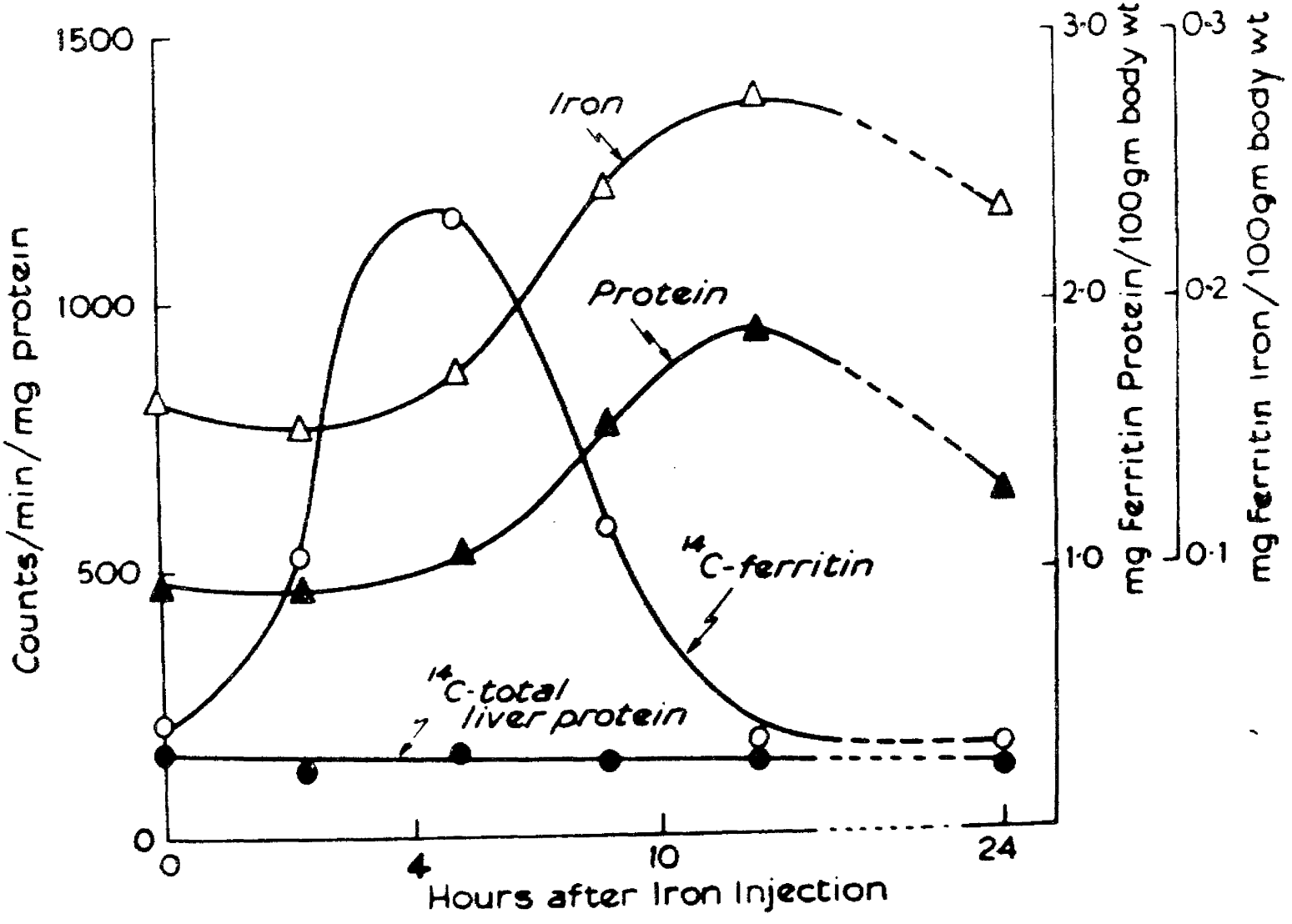


Fig. 20. Uptake of ^{14}C-leucine into liver ferritin and total liver protein after giving a 2-hour pulse dose of ^{14}C-leucine to rats at various time intervals after injection of 400 µg. iron/100 gm. body weight. The diagram also shows the amounts of ferritin protein and ferritin iron at these times. Each point is the mean result from 2 animals.

Table 12

Ferritin iron (mg./liver/100 g. body wt.) in livers of normal and iron-injected rats

Control			Iron-injected	
0.151	0.110	0.169	0.267	0.227
0.203	0.169	0.080	0.182	0.170
0.148	0.152	0.218	0.248	0.389
0.146	0.177	0.162	0.251	0.341
0.190	0.194	0.221	0.218	0.363
0.195	0.091	0.276	0.281	0.169
0.215	0.155	0.152	0.235	0.276
0.164	0.066	0.142	0.373	0.230
0.164	0.232		0.200	0.389
0.164	0.144		0.317	0.228

Summary of Statistics

Treatment	Number of rats	Degrees of freedom	Mean liver ferritin iron (mg./liver/ 100 g. b.wt.)	Sum of squares
None	28	27	0.166	0.057002
Iron	20	19	0.267	0.097732

Pooled variance $= s^2 = 0.154734/46 = 0.003364$

$s_{\bar{x}} = s^2(n_1+n_2)/n_1n_2$

$= 0.003364 \times 48/560 = 0.017$

$t = \bar{x}_2-\bar{x}_1/s_{\bar{x}} = 0.101/0.017 = 5.94$

$P < 0.01$

Table 13

Ferritin protein (mg./liver/100 g. body wt.) in livers of normal and iron-injected rats

Normal	Iron-injected
1.74	2.08
2.92	1.91
1.55	2.26
1.67	2.03
1.53	2.18
1.94	1.29
1.00	2.24
1.80	2.64
1.36	2.07
0.74	1.31
1.89	2.48
1.24	1.90
1.32	

Summary of Statistics

Treatment	Number of rats	Degrees of freedom	Mean liver ferritin protein (mg./liver/100 g. b.wt.)	Sum of squares
None	13	12	1.59	3.0931
Iron	12	11	2.03	1.7955

Pooled variance = s^2 = 4.888/23 = 0.2125

$s_{\bar{x}}$ = $s^2(n_1+n_2)/n_1n_2$ = 0.2125 x 25/156 = 0.185

t = $\bar{x}_1-\bar{x}_2/s_{\bar{x}}$ = 0.44/0.185 = 2.378

$P<0.05$

Table 14

The iron/protein ratio of liver ferritin from normal and iron-injected rats

Control		Iron-injected	
8.4	8.0	13.5	17.4
6.5	12.1	12.3	12.9
12.6	11.9	16.5	17.6
9.8	14.9	15.6	12.9
11.1	14.6	12.0	15.7
10.7	12.3	10.3	14.3
8.4	10.8	13.2	12.0

Summary of Statistics

Treatment	Number of rats	Degrees of freedom	Mean iron content of liver protein (%)	Sum of squares
None	14	13	10.86	79.44
Iron	14	13	14.01	64.20

Pooled variance = s^2 = 143.64/26 = 5.523

$s_{\bar{x}}$ = $2s^2/n$ = 11.046/14 = 0.885

$t = \bar{x}_1 - \bar{x}_2 / s_{\bar{x}}$ = 3.15/0.885

$P < 0.01$

The iron/protein ratio in the liver ferritin of a group of 14 rats which had received a single intraperitoneal injection of 300 µg. Fe/100 g. body weight at 4 hours before death was 14.0 ± 0.6%. This value is significantly greater than that of 10.9 ± 0.7% found in a similar group of control animals.

of 12 iron-injected animals was 2.03 ± 0.12 mg./100 g. body weight, an increase of about 30%. The greater increase in the amount of ferritin iron relative to the amount of ferritin protein can be attributed to an increased saturation of the pre-existing ferritin molecules with iron. The average iron control of ferritin molecules, expressed as the amount (mg.) of iron/100 mg. ferritin protein, was 10.9 ± 0.7% in a group of 14 normal rats and 14.0 ± 0.6% in another group of 14 rats that had received 300 µg iron/ 100 g. body weight at four hours before death. These differences are significant.

An important point in the assessment of the above results is the method of expression of the data. Following the 2-hour pulse with ^{14}C-leucine, estimation of rate of synthesis by means of specific activities is only valid when the liver levels of ferritin in the control and iron-injected animals are similar. Fig.20 apparently indicates that, 10 hours after iron injection, the rate of synthesis of ferritin in iron-injected rats had subsided to that in the controls, as judged by specific activity. However, owing to the increased level of ferritin protein in the livers of the iron-injected rats at this period, it can be calculated that the number of ferritin molecules being synthesised in these rats was in fact greater than that in the controls. By 24 hours after iron injection, there was little difference between the groups.

III. The effect of dose level of iron in the induction of ferritin

Fasting rats were injected with increasing doses of iron up to 600 µg./100 g. body weight. Two hours later, 5 µc ^{14}C-leucine/100 g. body weight were injected, and the rats were killed 2 hours thereafter. These conditions were chosen to allow a maximal response (Fig.20). Samples of liver ferritin were isolated and the specific activities of these samples and, also, of mixed liver protein were measured; the total ferritin iron and ferritin protein content of each liver was also estimated. The specific activities of the ferritin samples were related to those of the appropriate mixed liver protein samples. This avoids apparent differences caused by accidental differences in the levels of ^{14}C-leucine in the free amino acid pool in liver as a result of differences in uptake from the site of injection. The results of these experiments are given diagrammatically in Fig.21; each point is the mean result obtained from 4 rats.

Fig.21 shows that the magnitude of the response in ferritin biosynthesis increases with the dose of iron administered, the maximum being 10 times greater than that for animals not injected with iron. This is also accompanied by an increase in the ferritin iron and ferritin protein content of the liver, as in previous studies (page 75).

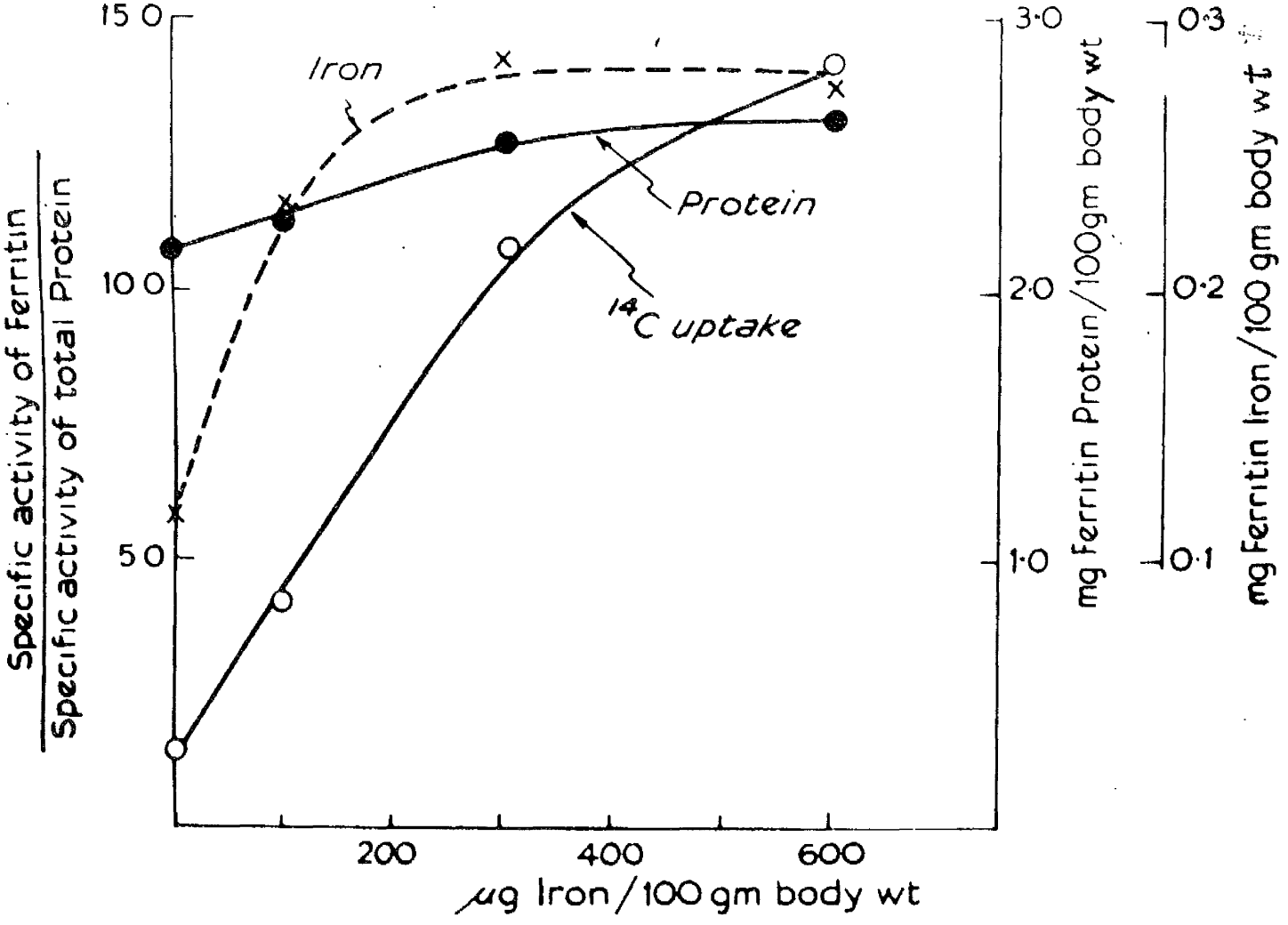


Fig. 21 The effect of increasing doses of iron on uptake of ^{14}C-leucine into liver ferritin and on the total amount of ferritin protein and iron in the liver. The animals were injected with ^{14}C-leucine 2 hours after giving iron, and were killed at 4 hours after iron injection. The uptake of ^{14}C-leucine is expressed as the specific activity of ferritin protein relative to that of total liver protein. Each point is the mean result obtained from 4 rats.

EXPERIMENTAL AND RESULTS

Section 4: INVESTIGATIONS INTO THE MECHANISM OF THE INDUCTION OF FERRITIN BY IRON

I Studies with Actinomycin D

(i) The inhibition of RNA synthesis by actinomycin D

(ii) The effect of actinomycin D on the induction of ferritin by iron

(iii) Addendum

II The Effect of Diet on the Induction of Ferritin by Iron

INVESTIGATIONS INTO THE MECHANISM OF THE INDUCTION OF FERRITIN BY IRON

There are many possible stages in ferritin synthesis at which iron could produce its stimulant effect. The induction of many mammalian and bacterial proteins appears to result from an increased synthesis of messenger RNA and a subsequent increase in the number of polysomes concerned with the synthesis of that protein. The possibility of a similar mechanism operating in the induction of ferritin by iron was therefore investigated. Two experimental approaches to this problem were made. First, the effect of actinomycin D on the induction was investigated to determine whether additional messenger RNA synthesis was involved. Secondly, the intake of dietary protein was varied to determine whether marked reductions in the level of liver RNA content resulting from protein deficiency would affect ferritin synthesis or its induction. The rationale for the use of these experimental approaches and the results obtained with them are described separately below.

I. The Effect of Actinomycin D on the Induction of Ferritin by Iron

Actino mycin D has been extensively used in studies of enzyme induction since suitable doses effectively inhibit RNA synthesis without immediately affecting protein synthesis (Reich, 1963; Revel, Hiatt and Revel, 1965). The induction of tryptophan

pyrrolase in rat liver following hydrocortisone administration is abolished by a single dose of 70 µg actinomycin D/100 g. body weight given intraperitoneally one hour before the inducer (Greengard and Acs, 1963). Similar dose levels also inhibit the methylcholanthrene stimulation of benzpyrene-hydroxylase activity in young rats (Gelboin and Blackburn, 1963). Levels of 150 µg. actinomycin D/100 g. body weight do not inhibit ^{14}C-amino acid uptake into liver protein up to 12 hours after injection (Revel, Hiatt and Revel, 1965).

The degree of inhibition of RNA synthesis in rat liver produced by dose levels of 70 and 150 µg. actinomycin D/100 g. body weight was first investigated. Since these dose levels effectively inhibited RNA synthesis they were used to study the effect of actinomycin D on the induction of ferritin by iron.

MATERIALS AND METHODS

Actinomycin D

Actinomycin D was a generous gift from Merck, Sharp and Dohme Research Laboratories (Pennsylvania). It was dissolved in 0.9% saline for injection.

Isolation and fractionation of whole cell RNA

The following procedures were used in studies of the uptake of ^{14}C-adenine into liver RNA. Whole cell RNA was extracted from a 1:20 liver homogenate in 0.25M sucrose by

the phenol method of Eason, Cline and Smellie (1963). Samples (0.4 ml.) containing 10-25 optical density units at 260 mμ were layered on to a linear gradient (3-30%) of sucrose in a volume of 4.6 ml. The gradients were spun at 41,500 g for 12 hours at 0° using the SW 39 rotor in the Spinco Model L ultracentrifuge. After centrifugation, the bottoms of the tubes were punctured with a No.14 hypodermic syringe needle (Record type) and fractions of 15 drops were collected. The samples were diluted to 3 ml. with water and the optical density measured at 260 mμ. Aliquots, 1 ml., were mixed with 8 ml. dioxane scintillation fluid (Nuclear Enterprises, Edinburgh) and the radioactivity was measured in a Nuclear Chicago scintillation spectrometer. The results were calculated as disintegrations/minute/fraction.

EXPERIMENTAL AND RESULTS

(i) The inhibition of RNA synthesis by actinomycin D

Fasting male rats (150 g.) were injected intraperitoneally with actinomycin D (70 μg/100 g. body weight) 1 hour before injection of [8-^{14}C] adenine sulphate (20 μc/100 g. body weight). Control animals received saline in place of the actinomycin D. After a further 4 hours, the animals were killed and samples of whole cell RNA were isolated from their livers. The purified RNA samples were resolved into components on a sucrose density gradient. The profiles of absorbancy and radioactivity so given are shown in Fig.22. This experiment was repeated

Effect of Actinomycin D on uptake of ^{14}C-adenine into Rat Liver RNA

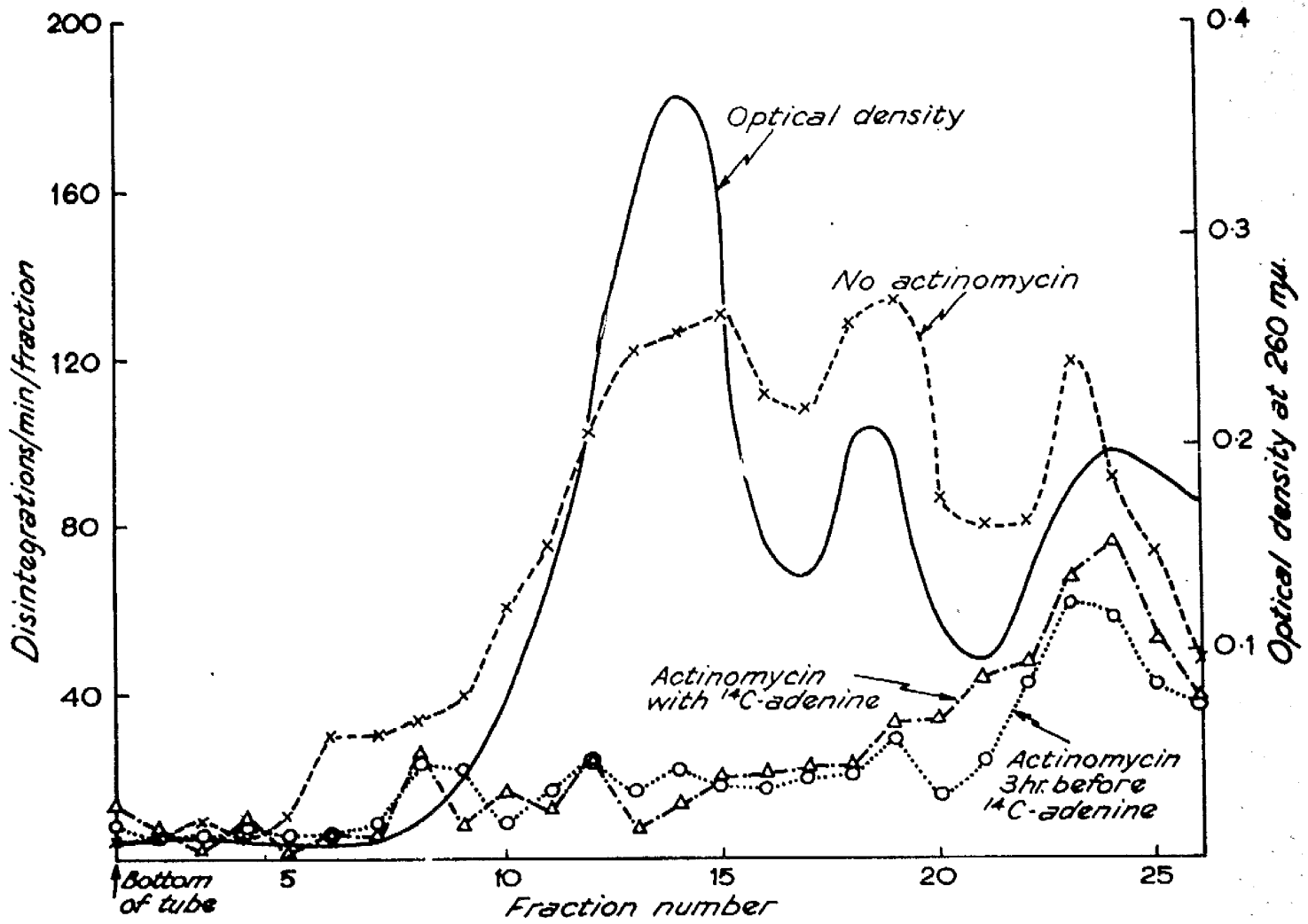


Fig. 22. Fasted male rats (180 g.) were injected intraperitoneally with 70 μg. actinomycin D per 100 g. body wt. and 1 hr. later with 20 μC. 8-^{14}C adenine sulphate per 100 g. body wt. Controls received injections of saline in place of the actinomycin. 4 hr. later, the livers were removed and RNA purified after phenol extraction. The RNA was centrifuged through a sucrose gradient (3-30%) for 12 hr. at 41 500 x g. Fractions of 15 drops were collected, the absorbancy was measured at 260 mμ and the total ^{14}C-activity per fraction was determined by scintillation counting. The continuous line represents the absorbancy profile for the control sample which was similar in pattern and position to that of the actinomycin-injected animal. The dotted lines show ^{14}C-activity per fraction for each group.

with dose levels of 150 µg actinomycin D/100 g. body weight injected at the same time or at an interval of 3 hours before the ^{14}C-adenine. The results of these experiments are shown in Fig.23.

These show that all treatments with actinomycin D inhibited the synthesis of whole cell RNA by about 80%; most of the remaining activity was present in RNA of low molecular weight. These findings are in agreement with evidence of suppression of RNA synthesis under similar conditions by Greengard, Smith and Acs (1963).

(ii) <u>The effect of actinomycin D on the induction of ferritin by iron</u>

Dose levels of 70 µg actinomycin D/100 g. body weight were first used in studies of its effect on the induction of ferritin by iron. Fasting male rats (150 g.) received this dose of actinomycin D 1 hour before a dose of 300 µg. Fe/100 g. body weight. Control animals not receiving iron or actinomycin were injected with physiological saline. At 2 hours after iron injection, all animals received 5 µc ^{14}C-DL-leucine/100 g. body weight and were killed 2 hours later. The specific activities of ferritin and mixed liver protein were measured as before (page 73). The results of these experiments are given in Table 15. This experiment was later repeated using dose levels of actinomycin D of 150 µg./100 g. body weight given at intervals up to 3 hours before iron injection. The results of this set of experiments are given in Table 16.

Effect of actinomycin D on the uptake of ^{14}C-adenine into Rat Liver RNA

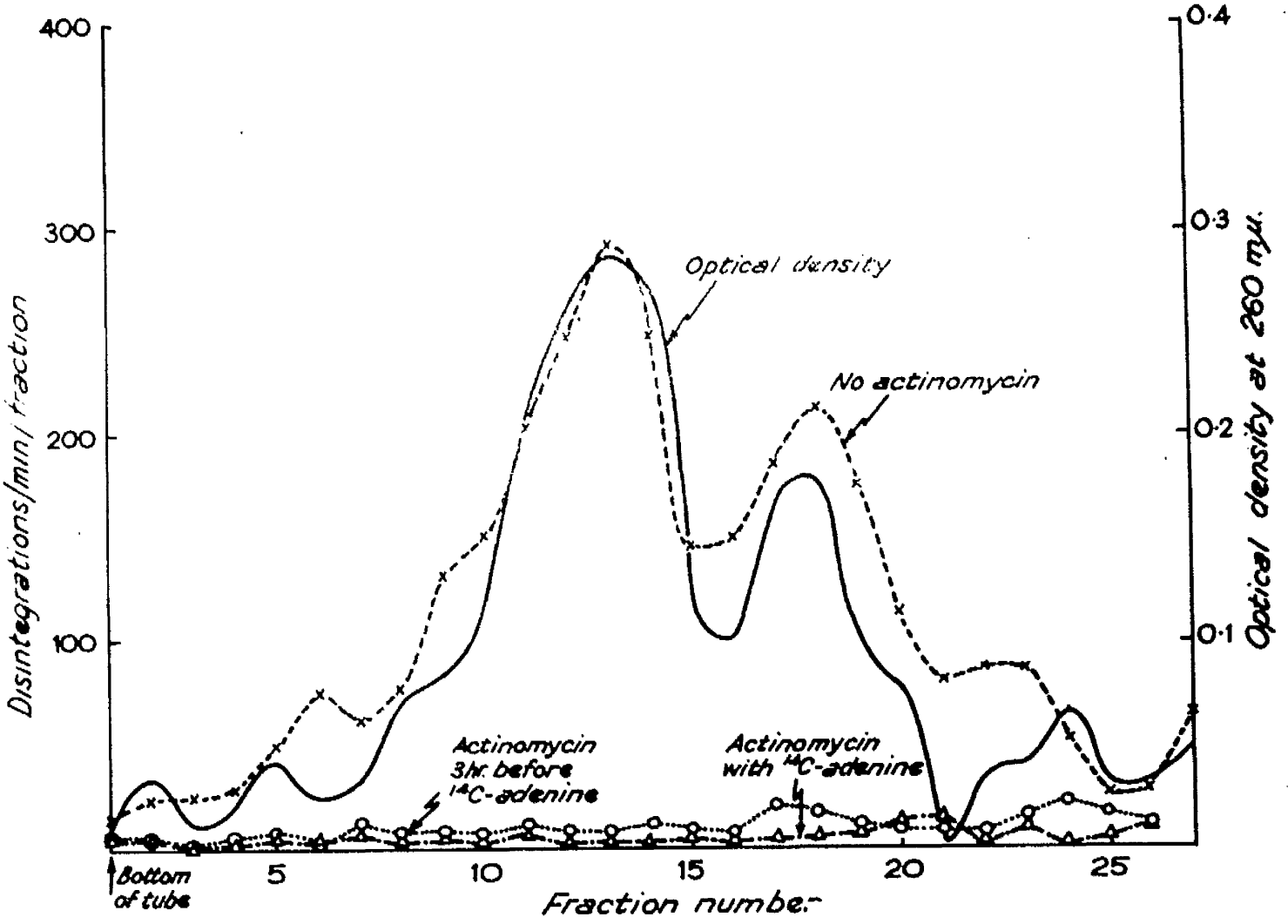


Fig. 23. Actinomycin D (150 μg./100 g. body weight) was injected at intervals of 0 and 3 hours before 20 μC 8-^{14}C adenine sulphate/100 g. body weight. The livers were removed 4 hours later and whole cell RNA was purified after phenol extraction. The RNA was fractionated on a sucrose density gradient and the absorbancy of the fractions was measured at 260 mμ. Total ^{14}C-activity per fraction was determined by scintillation counting. The continuous line represents the absorbancy profile for the control sample which was similar in pattern and position to that of the actinomycin-treated animal. The dotted lines show ^{14}C-activity per fraction for each group.

Table 15

Effect of Actinomycin D on Ferritin Protein Synthesis After Injection of Iron

Dosage/100 g. body weight		^{14}C Incorporation counts/min/mg protein		
Actinomycin	Iron	Into Ferritin protein	Into Mixed liver proteins	Ratio Ferritin / Mixed protein
-	-	305	190	1.6
-	300 µg.	1050	165	6.5
70 µg.	-	400	155	2.6
70 µg.	300 µg.	1100	160	7.2

Fasting male rats (180 g.) received 70 µg. actinomycin D/100 g. body weight by intraperitoneal injection, at 1 hour before 300 µg. Fe/100 g. body weight. Controls received saline at these times. All animals then received 5 µc ^{14}C-DL-leucine/100 g. body weight at 2 hours after the iron and were killed 2 hours thereafter. The specific activities of liver ferritin (page 57) and mixed liver proteins (page 73) were estimated. The entries in the table are the means of two experiments. Statistical analysis shows that the action of iron on ferritin biosynthesis is significant, and unaffected by actinomycin administration.

Table 16

Effect of Actinomycin D on Ferritin Protein Synthesis After Iron Injection

Interval between actinomycin and iron	Dosage/100 gm. body weight		^{14}C incorporation (counts/min/mg protein)		
	Act.	Fe	Mixed liver protein	Ferritin protein	Ratio: Ferritin / mixed protein
None	-	-	148	225	1.5
	-	400 µg.	171	725	4.3
0 hr.	150 µg.	-	130	410	3.1
	150 µg.	400 µg.	145	705	4.9
3 hr.	150 µg.	-	148	350	2.3
	150 µg.	400 µg.	129	890	6.9

Fasting male rats (180 g.) received 150 µg. actinomycin D/100 g. body weight at intervals of 3 hours and 0 hour before an injection of 400 µg. Fe/100 g. body weight. Controls received saline at these times. All animals then received 5 µc ^{14}C-DL-leucine/100 g. body weight at 2 hours after the iron and were killed 2 hours thereafter. The specific activities of ferritin and total liver protein were estimated as before. There were 4 rats in each group. Statistical analysis showed that iron significantly stimulated ^{14}C-uptake into ferritin and that this stimulus was unaffected by actinomycin administration.

In all experiments it was found that the increased uptake of ^{14}C-leucine into liver ferritin after iron administration was not suppressed by any of the pretreatments with actinomycin D. As before, there was no effect on total liver protein as a result of iron injection. Since actinomycin also did not affect incorporation into mixed liver protein, the ratio of ferritin uptake to mixed liver protein uptake increased 4-fold at 4 hours after iron administration in agreement with our earlier studies (page 75).

Since actinomycin treatment suppresses 80% of RNA synthesis during the period in which iron is acting (Figs.22 and 23), these results indicate that the increased ferritin synthesis as a result of iron injection is independent of additional messenger RNA formation.

In both series of experiments it was noticed that animals receiving only actinomycin showed a stimulation of ^{14}C-leucine uptake into ferritin, without any corresponding change in the uptake into mixed liver proteins. Similar increases in tissue enzyme activities following actinomycin treatment have been reported in other mammalian systems (Garren, Howell, Tomkins and Crocco, 1964; Rosen, Raina, Milholland and Nichol, 1964; Eagle and Robinson, 1964). This increase in ferritin synthesis may be due to an influx of iron to the liver from other organs as a result of actinomycin injection.

(iii) Addendum:

Since this work was completed, a conflicting report has appeared about the effect of actinomycin D on the induction of liver ferritin by iron (Yu and Fineberg, 1965). For their studies, these authors used liver slices which were obtained from rats after injections of actinomycin and iron. They used three groups of animals: (1) uninjected controls; (2) animals receiving 400 µg. Fe/100 g. body weight; (3) animals which received 1.8 mg. actinomycin D/100 g. body weight at 1 hour before iron injection. Tissue slices were prepared 2 hours after iron injection from the livers of these animals and were incubated with ^{14}C-leucine for 2.5 hours in Tyrode's solution. Liver ferritin was isolated from these slices by immunological precipitation. They found that the total radioactivity recovered, as ferritin, from the liver slices of the iron-injected rats was much higher than that obtained from uninjected control animals. When the donor rat was injected with 1.8 mg. actinomycin D/100 g. body weight at 1 hour before iron, the uptake of ^{14}C-leucine into ferritin fell to the level of the uninjected controls. No control animals receiving actinomycin alone were examined. In addition, no checks were made on the rates of synthesis of other proteins to determine whether the treatments with iron and with actinomycin D affected only the synthesis of ferritin or whether it had a general action on protein synthesis.

Nevertheless, the authors concluded that actinomycin D prevented the induction of ferritin by iron by blocking the synthesis of messenger RNA.

It is well known, however, that ribosomal preparations from livers of rats receiving only 0.5 mg. actinomycin D/100 g. body weight show considerable impairment of their ability to incorporate amino acids. These preparations also show a reduction in the number of polysomes with an increase in monomeric ribosomes (Stachelin, Wettstein and Noll, 1963; Revel, Hiatt and Revel, 1964). These effects are not a direct consequence of the inhibition of RNA synthesis but are due to some toxic effect of high concentrations of the antibiotic on the polysomes (Revel, Hiatt and Revel, 1964). In view of these findings, it might not seem surprising if the incorporation of amino acids into ferritin, as well as into other proteins, was substantially reduced by dose levels of 1.8 mg. actinomycin D/100 g. body weight, as used by Yu and Fineberg (1965).

The limited supplies of actinomycin D available to us did not permit extensive investigations into the effect of such massive doses on protein synthesis and ferritin induction. As a result we were able only once to repeat the experiment of Yü and Fineberg (1965). We were careful, however, to include the necessary controls which these authors had neglected. This experiment is described below. As far as was practicable, the experimental procedures of Li and Fineberg were followed.

Four pairs of animals were used for these studies. They were injected thus:

(1) physiological saline (3) iron only
(2) actinomycin D (4) actinomycin D + iron

Fasting male rats (100 g.) received 1.8 mg. actinomycin D/ 100 g. body weight, given in 4 separate subcutaneous sites to avoid direct contact with the liver. At 1 hour later, 400 µg. Fe/100 g. body weight were given intraperitoneally. Controls received saline in place of either actinomycin or iron. Two hours after the iron injection, the animals were killed and their livers removed. Tissue slices, 35 mm. in section, were prepared with a McIlwain and Buddle (1952) tissue chopper. Samples (1 g.) were incubated at 37° for 2.5 hours with 4 volumes of Tyrode's solution containing 1 µc ^{14}C-DL-leucine/ml. After incubation, the slices were homogenized and samples of the homogenate were taken for the estimation of the specific activity of mixed liver protein (page 73). The remainder of the homogenate was heated to 70° for 5 minutes and centrifuged to separate ferritin from heat coagulable proteins. Ferritin was precipitated from samples of this supernatant fraction by a specific antiserum (page 41) and the amounts of protein, iron and radioactivity in this specific precipitate were measured (page 56). The results of this experiment are given in Table 17.

Table 17

The Effect of Actinomycin D and Iron Given in vivo on the Uptake of ^{14}C-leucine into Protein in Liver Slices

Actinomycin D (1.8 mg./100 g. b.wt.)	Iron (400 µg. Fe/100 g. b.wt.)	Mixed protein (c.p.m./mg. protein)
-	-	938
-	+	466
+	-	179
+	+	224

Fasting rats (100 g.) were injected with 1.8 mg. actinomycin D/100 g. body weight at 1 hour before 400 µg. Fe/100 g. body weight. Controls, not injected with actinomycin D or iron received saline at these times. At 2 hours after iron injection, the animals were killed. Liver slices were incubated in Tyrode's solution containing 1 µc. ^{14}C-leucine/ml. After incubation the slices were homogenized and the specific activity of mixed liver protein was measured as before (page 73). The entries in the table are the means of two experiments.

The values for the incorporation into ferritin have not been reported because of uncertainty about the isolation procedure reported by Mu and Fineberg (1965) and used by us in this experiment. It gave extremely low and erratic recoveries of ferritin in our hands. In most cases, less than 4 µg. of ferritin iron were recovered from the liver samples, which is only about 10% of the amount one would expect from such samples (Kaufmann, 1962; see also page 75). These poor recoveries were not due to defects in the immunological precipitation technique, since the antiserum was of known high potency and the conditions of precipitation were carefully standardized (page 41) and had been successfully used previously (page 58). The most likely explanation is that ferritin was lost into the precipitate fraction during the heat coagulation procedure. It is this author's experience that a satisfactory separation of ferritin from other proteins by heat coagulation only occurs over certain small ranges of pH and ionic strength. In addition, the stability of ferritin at 70-80^{0} is considerably affected by the presence of certain anions, such as phosphate, which are abundant in Tyrode's incubation medium.

Despite this, much interesting information was obtained about the effect of these high doses of actinomycin on the level of protein synthesis in liver slices. These results offer an explanation for the conflicting evidence presented by the present author and Mu and Fineberg (1965).

Table 17 shows that the incorporation of amino acids into the mixed proteins of slices of liver from actinomycin-treated animals was less than 20% of that in control animals. Moreover, the incorporation into general liver protein was considerably decreased as a result of iron injection in animals which did not receive actinomycin.

No evidence is available from the paper by Yü and Fineberg (1965) that they were aware of these depressing effects of iron and actinomycin on total liver protein synthesis in their experimental procedures. They found a 4-5 fold increase in the incorporation of amino acids into ferritin as a result of iron injection in the absence of actinomycin. However, in view of our findings above, it seems probable that the absence of a stimulant effect of iron after actinomycin injection was due to the general decrease in the rate of synthesis of all proteins and was not due to a direct interference of actinomycin in the induction of ferritin by iron. This might have been apparent to the authors if they had run control animals receiving actinomycin but no iron.

In consequence, we are confident that actinomycin does not affect ferritin induction by iron when the antibiotic is given at a level sufficient to prevent messenger formation but not to intoxicate the general mechanism of protein synthesis.

II. The Effect of Diet on the Induction of Ferritin by Iron

Decreases in the intake of dietary protein cause marked reductions in the amount of protein, ribonucleic acid and phospholipid in the liver (Kosterlitz, 1947; Munro, 1964). The decrease in the total protein content of liver does not reflect a uniform decrease in the individual levels of all the constituent proteins. Some proteins, such as the enzyme xanthine oxidase, are extremely sensitive to protein depletion whereas others, such as catalase and alkaline phosphatase, remain relatively immune (Miller, 1950; Fisher, 1954). Fractionation of whole cell RNA from liver on sucrose density gradients indicates that ribosomal RNA is the predominating class of RNA. It therefore seems justified to assume that the large loss of whole cell RNA observed on protein depletion will result in a marked decrease in the number of ribosomes and consequently polysomes in the liver cell. The losses of protein and ribonucleic acid are also accompanied by structural changes in the nucleus (Lagerstedt, 1949) and endoplasmic reticulum (Munro, 1964). All of these changes are promptly reversed when the intake of dietary protein is restored to normal.

Recent work in this laboratory has shown that diets inadequate in protein or containing an incomplete amino acid mixture lower the protein-synthesising capacity of the microsome fraction in liver. This may be due to an accompanying

breakdown of liver polysomes into monosomes and disomes (Fleck, Shepherd and Munro, 1965). Such changes in polysome patterns have been attributed to differences in their content of messenger RNA (Wettstein, Staehelin and Noll, 1963; Staehelin, Wettstein and Noll, 1963), although Fleck et al. (1965) consider that some other mechanism may be responsible for the early changes in polysome pattern shortly after feeding an incomplete amino acid mixture. It was therefore of considerable interest to investigate the effect of changes in dietary protein on the induction of ferritin by iron in view of its profound effects on both the amount of RNA and the structure of polysomes. Described below are investigations into the effect of dietary protein level on the response in liver ferritin synthesis to iron administration.

MATERIALS AND METHODS

Animals and Diets

Adult male albino rats weighing 150-190 gm. were housed in individual cages under thermostatic conditions at 25°. The dietary arrangements were modelled on those used in previous experiments in which the diet was supplied in two portions, all of the dietary protein being in the evening meal (Munro and Naismith, 1953). The compositions of the diets are given in Tables 1, 2 and 3 in the general Methods and Materials section (page 28). Two groups of animals were maintained on these diets for 4 days. On the morning of the 5th day, half of the animals on the adequate protein diet were

given 2 g. casein dispersed in water containing 0.15 g. sodium bicarbonate. They were allowed to eat this for one hour before injection of iron and isotopes. The remaining animals on this diet and on the protein-free diet were kept fasting. In this way, rats were prepared for injection of iron in three dietary conditions: (a) those absorbing amino acids; (b) those fasting after an adequate intake of protein; (c) animals that had been depleted by 4 days on a protein-free diet.

EXPERIMENTAL AND RESULTS

At each replication of the experiment, one rat from each dietary group was kept as a control. The remainder received a single dose of 300 µg. Fe/100 g. body weight. At intervals of 1, 3, 7 and 10 hours thereafter, individual animals received 5 µc ^{14}C-DL-leucine/100 g. body weight by intraperitoneal injection and were killed two hours later. In this way, the rate of liver protein synthesis was measured by a 2 hour pulse dose which had been shown previously to allow maximum incorporation into ferritin and mixed liver proteins (page 74). The specific activities (c.p.m./mg. protein) of ferritin and mixed liver protein were estimated as before (page 73) and their ratio was calculated. The total amounts of ferritin iron and ferritin protein in the liver were also measured. The results are given in Table 18, and some have been presented diagrammatically in Fig.24 and Fig.25.

The rate of incorporation of ^{14}C-leucine into liver ferritin increased considerably in all groups after iron

Table 18

Effect of Protein Intake on the Total Amount of Ferritin Iron and Ferritin Protein in Rat Liver after Iron Administration

Time after iron injection (hours)	Amount of ferritin iron/ liver/100 g. body weight			Amount of ferritin protein liver/100 g. body weight			Fe/protein (mg. Fe/100 mg. protein		
	H.P. fed	H.P. fasting	L.P.	H.P. fed	H.P. fasting	L.P.	H.P. fed	H.P. fasting	L.P.
0	1.58	1.47	1.49	0.21	0.16	0.22	13.4	10.9	14.3
3	2.09	1.82	2.25	0.22	0.27	0.29	14.8	15.0	13.6
5	2.42	1.56	2.24	0.27	0.21	0.38	11.4	13.5	17.7
9	2.69	2.26	3.18	0.28	0.25	0.52	10.8	11.0	16.3

Mean results of 4 rats per time interval for the adequate protein fed (H.P. fed) and for the protein-free diet (L.P.) and of 3 rats per interval for the adequate protein fasting (H.P. fasting).

The Effect of Diet on the Induction of Ferritin by Iron

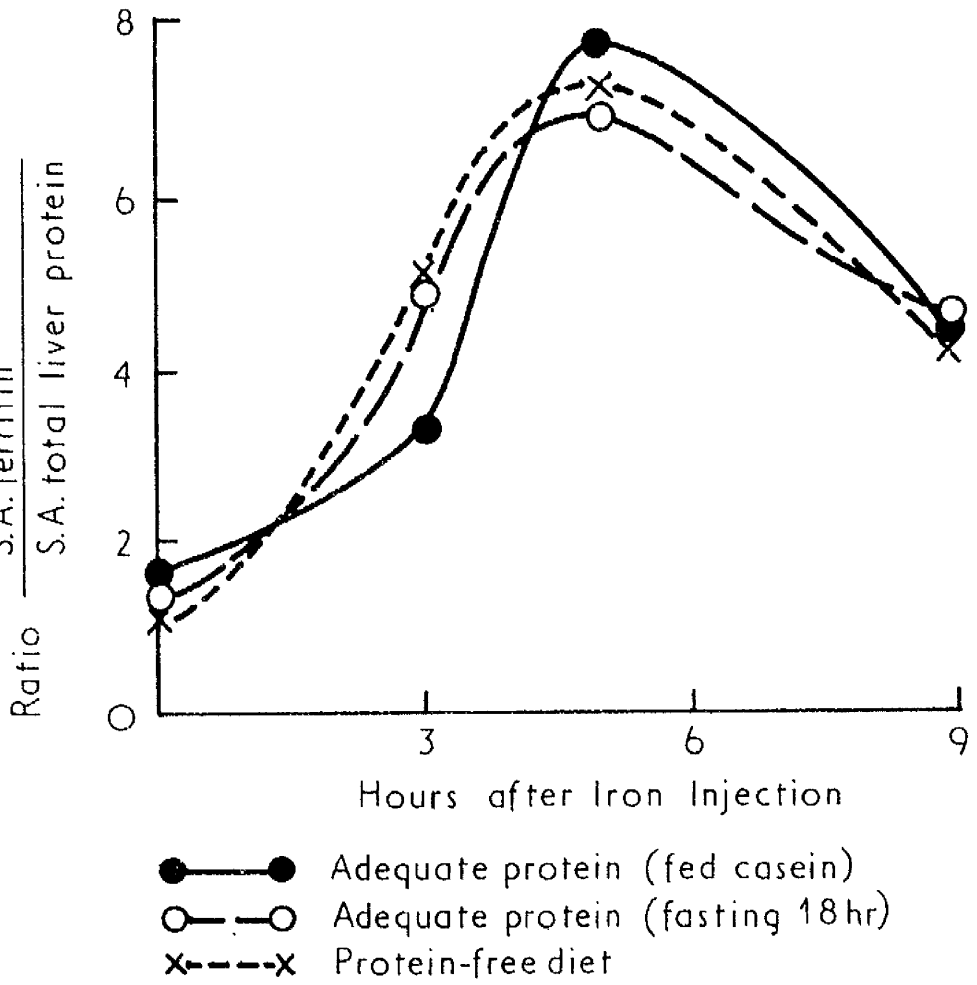


Fig. 24. Uptake of ^{14}C-leucine into liver ferritin and total liver protein after a 2-hour pulse dose of ^{14}C-leucine given at various time intervals after injection of 300 μg. Fe/100 g. body weight.

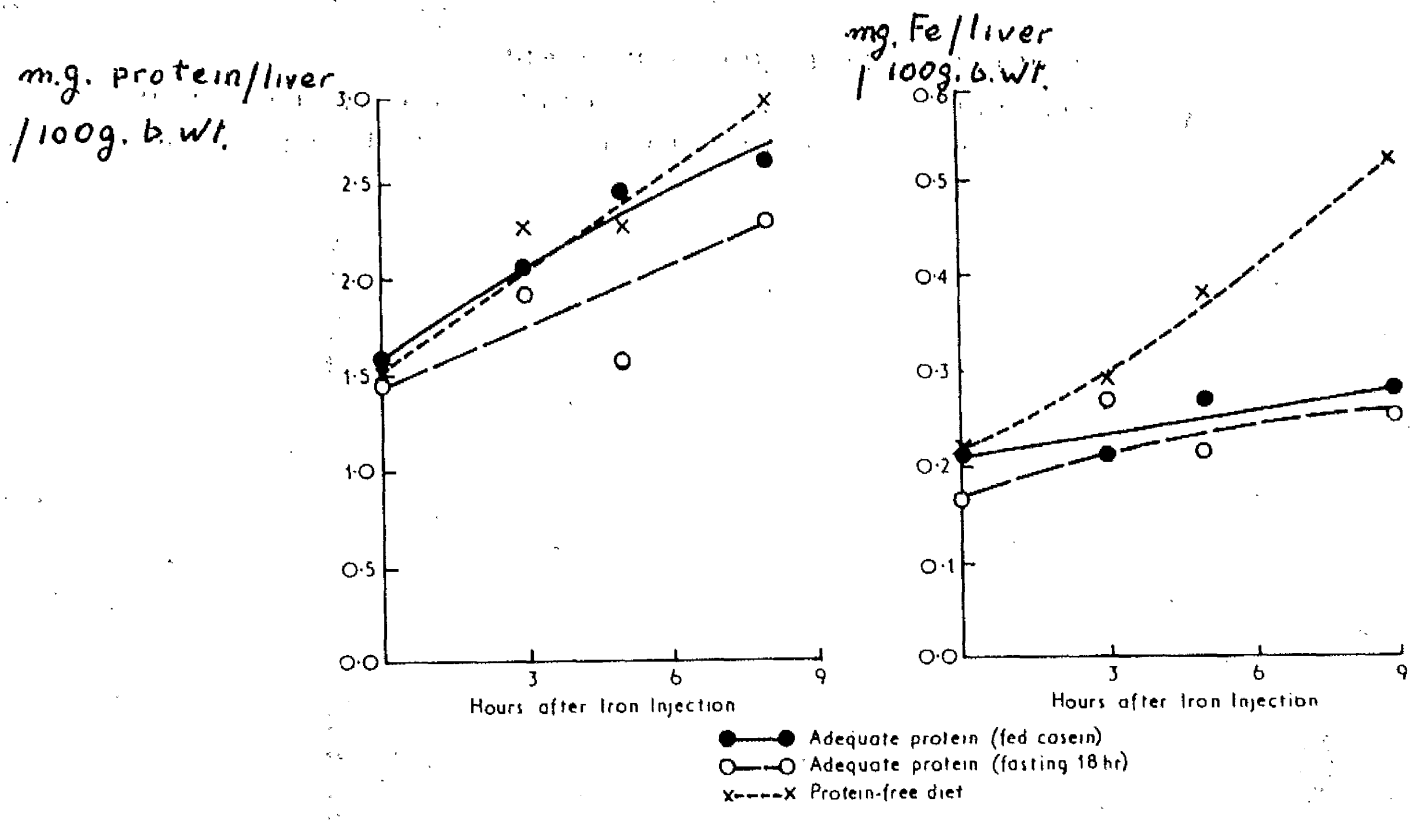


Fig. 25. The Effect of Diet on the Amounts of Liver Ferritin Iron and Ferritin Protein after Iron Injection.

injection (Fig.24). As in previous studies (page 75), the maximum uptake was obtained about 5 hours after iron injection. There was no apparent difference among the groups in either the magnitude of the response to iron or in the period over which it was active.

Fig.25 shows that, during the 9-hour period after iron injection, there was an increase in the total amount of ferritin in the liver; the increment was not affected by previous diet. However, the ferritin iron content of the liver was only slightly increased after injecting iron into the two groups of animals receiving protein in the diet, but was markedly increased in the case of rats on the protein-free diet. This may be due to differences in the distribution of parenteral iron between the liver and other tissues as a result of diet. The results also show that the levels of ferritin in liver are not greatly affected by diet. This may be due to the central role of ferritin in iron metabolism and the continuous recycling of iron through the liver from the haemopoietic tissues.

In conclusion, it would appear that the mechanism by which iron stimulates the incorporation of amino acids into ferritin is unaffected by the nutritional status of the animal. Since protein depletion probably results in a decrease in the number of polysomes in liver, these findings confirm those obtained with actinomycin, namely that the induction of ferritin by iron does not require additional RNA synthesis, particularly messenger RNA.

EXPERIMENTAL AND RESULTS

Section 5: THE EFFECT OF PARENTERAL IRON ON LIVER FERRITIN METABOLISM

(i) Turnover studies of unfractionated ferritin in normal and iron-induced rats.

(ii) Procedure for the fractionation of ferritin molecules according to particle size.

(iii) Distribution of radioactivity in ferritin subfractions after ^{14}C-leucine injection.

(iv) Search for a labile precursor of ferritin.

THE EFFECT OF PARENTERAL IRON ON LIVER FERRITIN METABOLISM

The inability of actinomycin D to suppress the induction of ferritin by iron thereby ruled out a mechanism involving additional messenger RNA synthesis and led us to explore other possible mechanisms by which iron administration could affect ferritin metabolism. The turnover of liver ferritin was studied in normal and iron-treated animals to investigate the effect of parenteral iron on the rate of breakdown of ferritin. The results of these experiments suggested that iron might affect the stability of the ferritin protein by converting labile fractions of low iron content into stable fractions of higher iron content. In order to test this hypothesis, attempts were made to fractionate ferritin molecules according to iron content. Sucrose density gradient techniques were devised for the separation of an artificial mixture of horse ferritin and chemically prepared apoferritin. This allowed us to select conditions for the separation of rat-liver ferritin into a wide spectrum of molecules of differing iron content. These methods were then used in investigations into the distribution of radioactivity in ferritin fractions from normal and iron-treated rats at different intervals after ^{14}C-leucine injection. The results of these investigations offered an explanation for the regulation of the turnover of ferritin in relation to iron administration and led us to suggest a mechanism for the induction of ferritin by iron. This section contains 4 parts:-

(i) Turnover studies of unfractionated ferritin in normal and iron-induced rats.

(ii) Procedure for the fractionation of ferritin molecules according to particle size.

(iii) Distribution of radioactivity in ferritin subfractions after ^{14}C-leucine injection.

(iv) Search for a labile precursor of ferritin.

(i) TURNOVER STUDIES OF UNFRACTIONATED FERRITIN IN NORMAL AND IRON-INDUCED RATS

The rate of turnover of ferritin in normal and iron-induced rats was followed by measuring the rate of loss of ^{14}C-labelled ferritin molecules over a period of 72 hours after a single injection of ^{14}C-leucine.

EXPERIMENTAL AND RESULTS

Liver ferritin synthesis was induced in a group of 18 rats by a single injection of 400 µg. Fe/100 g. body weight. At 2 hours after iron injection the animals were given 5 uc. ^{14}C-DL-leucine by intraperitoneal injection. Six of these animals were given 4 further injections of iron at 12 hourly intervals between the period of 24 and 72 hours. A third group of 12 rats served as controls and were injected only with ^{14}C-leucine. Three animals from each group were sacrificed at intervals of 12, 24, 48 and 72 hours after isotope injection. In all three groups i.e. controls, single iron injection, multiple iron injections, measurements were made of the total amount of

ferritin iron, and ferritin protein, per liver. The specific activities of the ferritin and of the mixed liver proteins were estimated. From these data, it was possible to compute the total amount of radioactivity present in liver ferritin protein in relation to body size (Fig.26).

The rate of loss of labelled ferritin molecules from the livers of the control group indicated a half-life of about 72 hours, which was similar to that observed for mixed liver proteins. Following a single injection of iron, the level of activity in ferritin at 12 hours after injection was about six times greater than that at the same interval in the control group. During the period between 12 and 24 hours after isotope injection, there was a rapid loss of label from the liver ferritin of this group; the rate of loss of radio-activity in this interval indicated a half-life of about 15 hours. Thereafter the rate of loss of radioactivity decreased to that of the controls. When iron was repeatedly administered to a similar group between the period of 24 and 72 hours, the rate of loss of radioactivity was considerably retarded.

A larger series of studies were made on similar groups of rats to confirm these findings. The experimental design used above was replicated, except that rats were killed at only two intervals, viz. 12 and 72 hours after isotope injection. The total amounts of ferritin protein and ferritin iron in the liver were estimated as were the specific activities of the ferritin and of the mixed liver proteins. The data for the

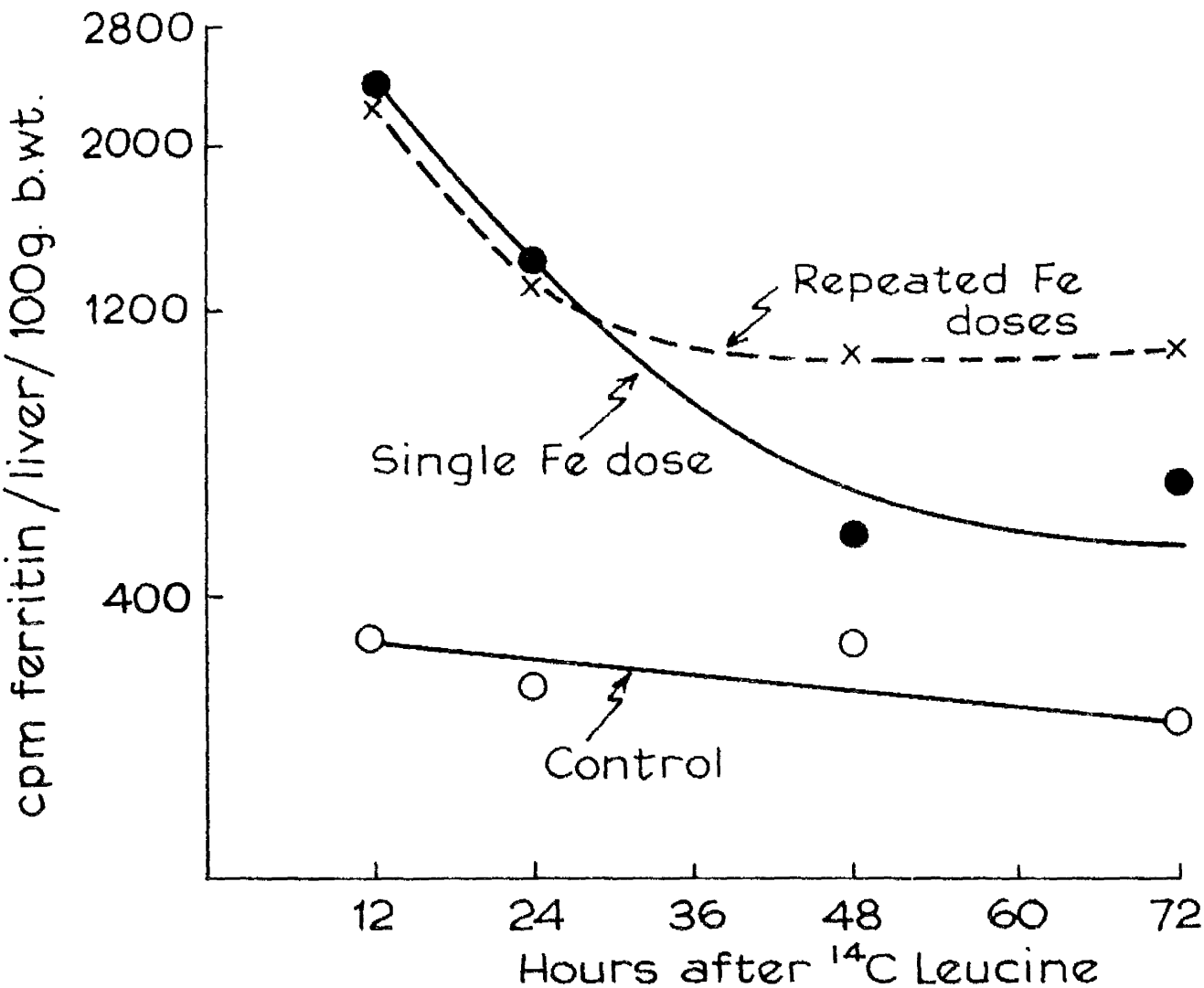


Fig. 26 Turnover of rat liver ferritin after injection of ^{14}C-leucine. Two groups of rats received 400 μg. iron/100 gm. body weight and one uninjected group acted as controls. ^{14}C-leucine was injected 2 hrs. later and the rats were killed at 12, 24, 48 and 72 hours thereafter. One of the iron-injected group received an additional 400 μg. Fe/100 gm. body weight at 12 hr., 24 hr., 36 hr. and 48 hr. Each point is the mean result from 3 animals.

uptake of ^{14}C-leucine by ferritin were standardized by relating the specific activity of ferritin to that of mixed liver proteins. This corrected for accidental differences in the amount of isotope reaching the liver and also for possible differences in the level of free leucine in the liver. The total amount of radioactivity in the liver ferritin in relation to body size was computed and the results are given in Table 19.

The results show that iron administration produced an increase after 12 hours in the amounts of ferritin protein and ferritin iron in the liver. The total amounts of ferritin protein and of ferritin iron/liver/100 g. body weight were 1.5 mg. and 0.22 mg. respectively in the control group and 2.3 mg. and 0.27 mg. respectively in the iron-injected group. At 72 hours, the amount of ferritin protein in the group that had received the single injection of iron was still 2.5 mg. whereas the amount of ferritin iron was now 0.40 mg. Consequently, there was an increase in the iron content (parts Fe per 100 parts protein) of ferritin in this group from 11.5% at the 12 hour interval to 15.5% at the 72 hour interval. However, the group receiving repeated administration of iron showed a much larger increase in the amounts of ferritin protein and ferritin iron, the values being 3.6 mg. and 0.84 mg./liver/100 g. body weight. Further, the iron content of the ferritin of this group had increased to a value of 23%.

Table 19

Effect of Parenteral Iron on the Turnover of Liver Ferritin

Ferritin content and radioactivity in liver in control animals, animals given a single dose of iron, and animals given repeated iron doses

Group	Time after dose	Ferritin Fe/ liver/100 g. b.wt. (µg.)	Ferritin Pr./ liver/100 g. b.wt. (mg.)	Fe/Pr. Ratio	$\frac{\text{S.A. Ferritin}}{\text{S.A. - T.L.P.}} \times 100$	Total cpm ferritin / S.A. - T.L.P.
Control	12h	219	1.51	13.0	225	280
	72h	186	1.47	12.2	153	230
Single dose	12h	261	2.31	11.5	824	1890
	72h	395	2.46	15.5	345	860
Multiple doses	12h	271	2.25	12.7	880	1860
	72h	855	3.56	23.0	415	1460

Results are mean of 4 rats at 12 hrs. and 5 rats at 72 hrs.

In agreement with the previous experiment, the total amount of radioactivity in the liver ferritin at the 12-hour interval was 6-7 times higher in the livers of the iron-induced group than in the corresponding controls. About 55% of the total amount of the labelled ferritin molecules had, however, disappeared by 72 hours from the livers of the group that received a single injection of iron whereas only 20% was lost from the group which had received repeated administrations of iron.

It was therefore concluded that the administration of excess iron can stabilize preformed ferritin by converting it into fractions of higher iron content. In order to test this hypothesis, attempts were made to fractionate ferritin molecules according to their iron content. These investigations are described below.

(ii) PROCEDURE FOR THE FRACTIONATION OF FERRITIN MOLECULES ACCORDING TO IRON CONTENT

Ferritin fractions of different iron contents may be separated by ammonium sulphate fractionation or by differential centrifugation of an aqueous solution. Kopp et al. (1964) succeeded in separating two electrophoretically distinct fractions, which probably represent ferritin in different degrees of aggregation, by centrifugation through sucrose density gradients. This method seemed suitable for our purposes but, unfortunately, no details of the procedure were published.

Accordingly, we first experimented with sucrose density gradients to establish conditions for the separation of an artificial mixture of horse ferritin and chemically prepared apoferritin. As a result of these studies we were able to select conditions for the separation of normal rat ferritin into a wide spectrum of molecules of different iron content.

EXPERIMENTAL AND RESULTS

Sucrose density gradients, covering a wide range of sucrose concentrations, were prepared in cellulose acetate tubes suitable for the SW 39 Spinco rotor to give a final volume of 4.6 ml. Samples (0.4 ml.) of a mixture of horse ferritin and chemically prepared apoferritin in water, containing approximately 100 ug. protein and 20 ug. iron, were layered on top of the sucrose gradient and centrifuged for various intervals and speeds at 0° in the SW 39 head of the Spinco Model L ultracentrifuge. After centrifugation, the bottom of the tubes were punctured with a No.14 Record fit hypodermic syringe needle and 10 fractions of 30 drops were collected. The volume in each tube was adjusted to 1.0 ml. by the addition of 0.5 ml. water. Samples (0.1 ml.) were taken for the estimation of protein using a micro Lowry method (final volume 1.2 ml.). The optical densities of these solutions were measured using the micro cells, 1 cm. light path, in the Beckman D.B. Spectrophotometer. The remaining solution was used for iron estimation in a volume of 5 ml. By a process of trial and error, conditions were

eventually obtained which appeared to give a satisfactory separation of iron-rich ferritin from apoferritin. Fig.27 shows the distribution of iron and protein obtained from the separation of a mixture of ferritin and apoferritin on three different sucrose density gradients after centrifugation at 79,500 g. for 90 minutes at 0°. The gradient of 0-0.25 M sucrose was considered most favourable for our purposes since it allowed a good separation of iron-rich ferritin and apoferritin and also a considerable fractionation of the iron-rich ferritin molecules.

These conditions were then used to study the distribution of iron and protein in a sample of normal rat-liver ferritin. This was obtained by the usual chromatographic procedures (page 43). A sample, containing approximately 0.5 mg. ferritin protein/ml., was precipitated at 50% saturation with ammonium sulphate (page 46). The precipitated ferritin was collected by centrifugation and the supernatant discarded. After thorough draining, the ferritin precipitate was taken up in 0.4 ml. water and applied to the top of the sucrose density gradient. The gradient was centrifuged at 79,500 g. for 90 minutes at 0° and the fractions collected as before. The distribution of iron and protein in the separated fractions are shown in Fig.28.

Fig.28 shows that most of the iron was found towards the bottom of the tube whereas the protein was widely distributed throughout the gradient. That this fractionation

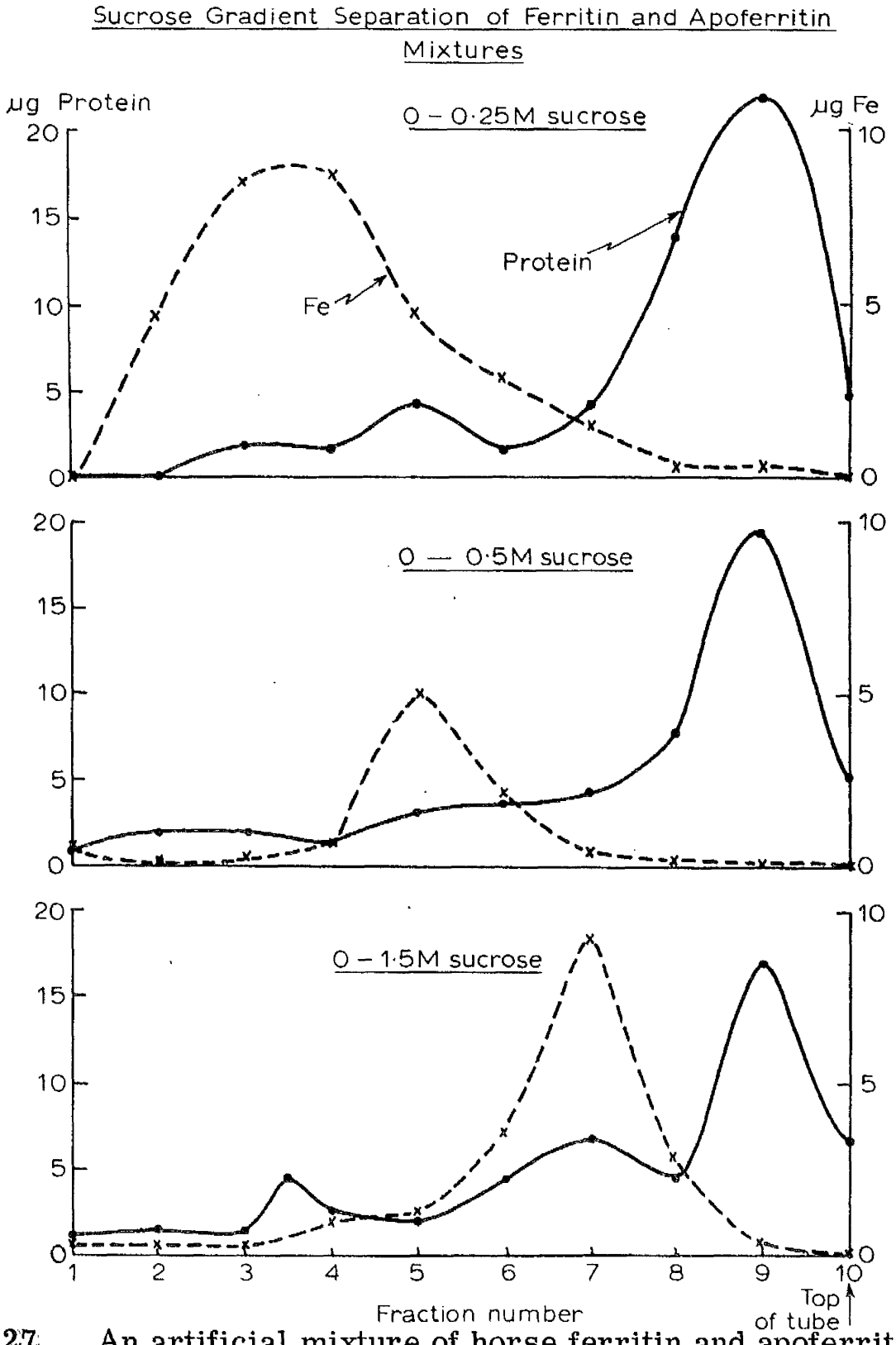


Fig. 27. An artificial mixture of horse ferritin and apoferritin, containing approximately 20 µg. iron and 100 µg. protein, was centrifuged through the above sucrose density gradients at 79, 500 g. for 90 minutes at 0°. The distribution of iron (----) and protein (____) in the separated fractions was determined as described on page 97.

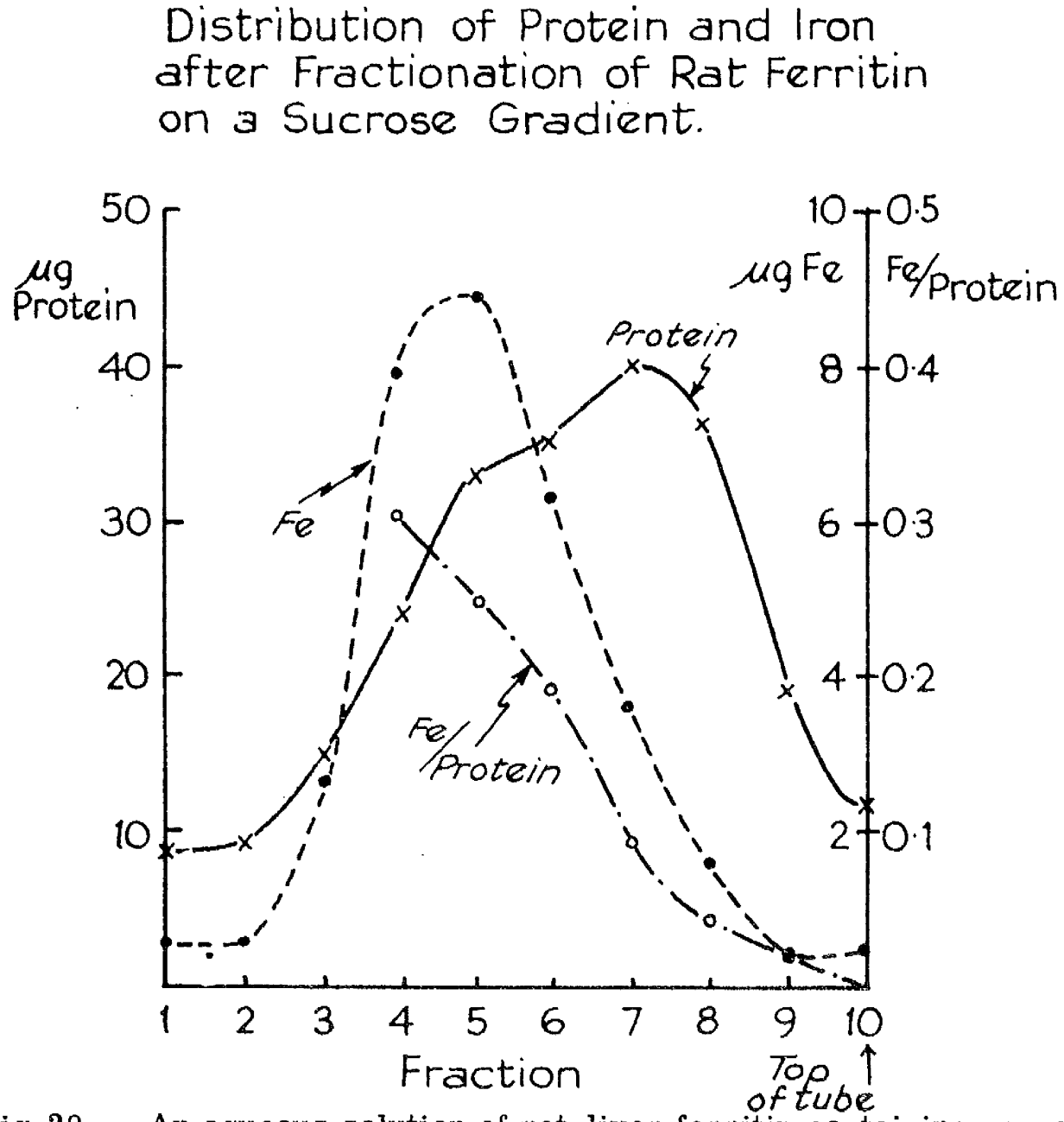


Fig. 28. An aqueous solution of rat-liver ferritin containing approximately 250 µg. protein and 30 µg. iron was centrifuged through a sucrose density gradient of 0-0.25 M at 79, 500 g. for 90 mins. at 0°. The amounts of iron and protein in the separated fractions were determined as described on page 97.

was dependent on differing iron content of ferritin molecules may be seen from the decreasing iron/protein ratio as one progressed to the top of the gradient. It is not known whether this fractionation was due solely to differences in the sedimentation velocity of the different fractions or whether sedimentation equilibrium was attained. From the practical point of view, the gradient allowed us to obtain a reproducible separation of molecules according to iron content. The top of the gradient provided a protein fraction very low in iron content which may be equated with apoferritin. The question of whether a completely iron-free form of ferritin exists in the cell will be deferred until the general discussion of the results is presented. At the moment, we may note that on most occasions, traces of iron were found in the first two fractions at the top of the gradient.

(iii) DISTRIBUTION OF RADIOACTIVITY IN FERRITIN SUBFRACTIONS AFTER ^{14}C-LEUCINE INJECTION

The fractionation procedure devised as described above was used to study the distribution of radioactivity in liver ferritin obtained from normal control rats and iron-injected rats at intervals up to 72 hours after ^{14}C-leucine administration.

EXPERIMENTAL AND RESULTS

Three groups of rats were used for these studies: (1) controls; (2) induced, single iron injection; (3) induced, multiple iron injections. A control group was injected with 5 μc. ^{14}C-DL-leucine/100 g. body weight. Rats were killed at 0.5, 1, 2, 4, 12 and 72 hours after isotope injection. Ferritin synthesis was induced in a second group of rats by a single injection of 400 μg. Fe/100 g. body weight given 2 hours before isotope injection. Animals in this group were killed at 0.5, 2, 12, 24 and 72 hours after isotope injection. A third group was treated similarly to the second group except that the rats received 4 further injections of 400 μg. Fe/100 g. body weight at 12-hourly intervals commencing 24 hours after isotope injection. The specific activities of ferritin and mixed liver protein obtained at these various intervals were estimated; the total amounts of ferritin protein and ferritin iron were also measured. Samples (0.5 mg.) of ferritin were fractionated on a sucrose density gradient according to iron content and the distribution of iron, protein and radioactivity in the separated fractions was determined. The results have been expressed as counts per minute per ferritin subfraction in the total amount of liver ferritin/100 g. body weight and are shown diagrammatically in Fig.29 (controls) and Fig.30 (iron-treated rats). In this way, the distribution shown in the figures is that which would have

Turnover of Ferritin Subfractions in Normal Rats

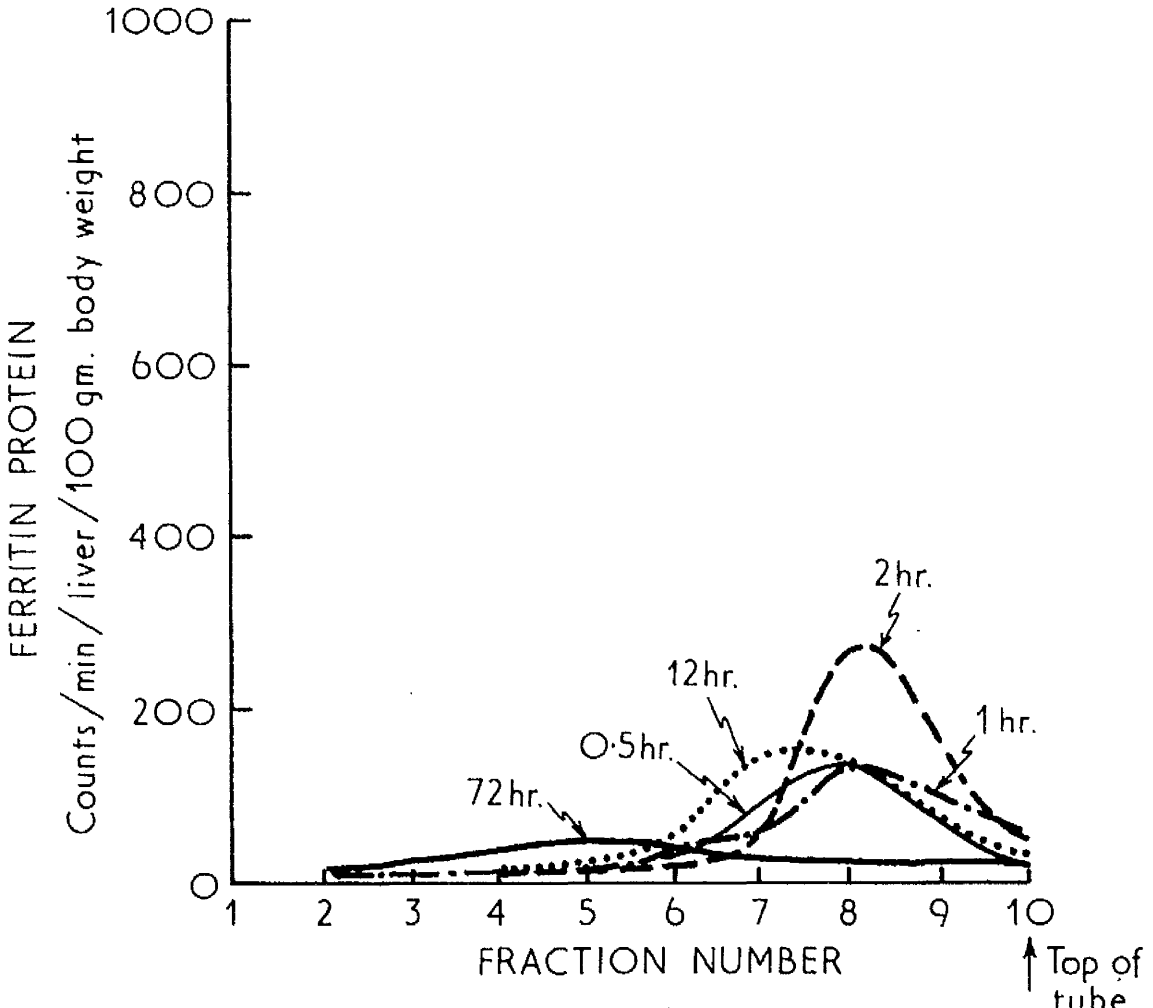


Fig. 29. Fasting rats were injected intraperitoneally with 5 μc. ^{14}C-DL-leucine/100 g. body weight and were killed at 0.5, 1, 2, 12 and 72 hours thereafter. Liver ferritin was fractionated according to iron content on sucrose density gradients. The total radioactivity in each fraction in relation to body weight was estimated. Each result is the mean from at least 3 experiments.

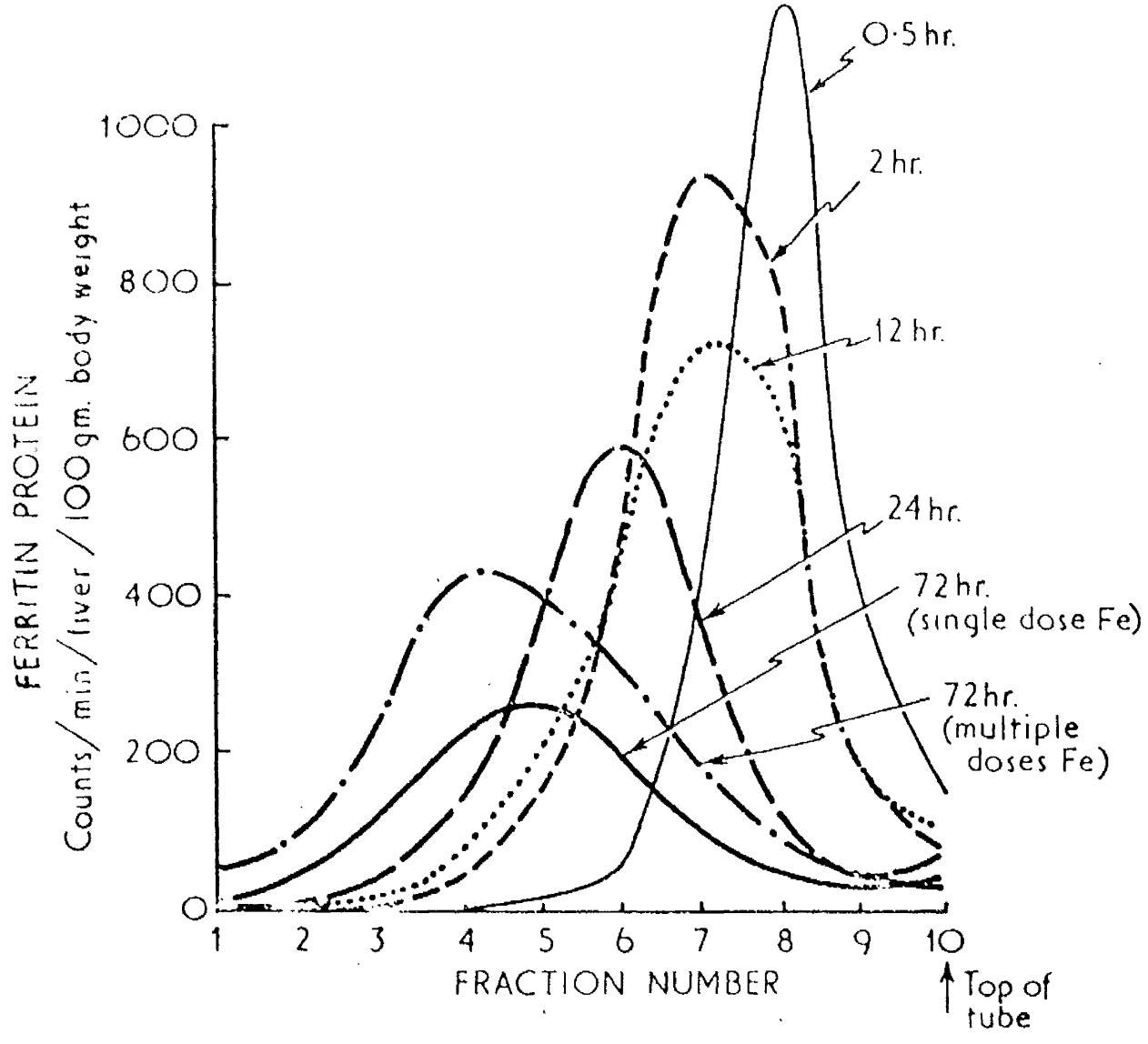


Fig. 30. Fasting rats were injected intraperitoneally with 400 μg. Fe/100 g. body weight followed at 2 hours later with 5 μc. ^{14}C-DL-leucine/100 g. body weight. Some of the rats later received additional injections of 400 μg. Fe/100 g. body weight at 24, 36, 48 and 60 hours after isotope injection. Animals were killed at 0.5, 2, 12, 24 and 72 hours after isotope injection. Liver ferritin was fractionated according to iron content on sucrose density gradients. The total radioactivity in each fraction in relation to body weight was estimated. Each result is the mean from at least 3 experiments.

been obtained if the entire ferritin content of the liver had been fractionated. Both Fig.29 and Fig.30 show that the labelling of ferritin molecules at the earliest intervals after isotope injection was restricted, in all groups, to the fractions of lowest iron content. The level of radioactivity in the iron-injected group was, however, considerably greater than in the controls. As time progressed, there was a general reduction in the level of labelling, accompanied by a progressive movement of the radioactivity down the gradient into the iron-rich forms. Fig.30 also shows the effect of continued iron administration on the distribution of ^{14}C-leucine inferritin molecules at 72 hours. In agreement with earlier findings (page 94), the amount of radioactivity retained in the ferritin of this group was considerably greater than that retained by the group receiving only a single injection of iron. Further, most of the radioactivity inthe ferritin isolated at 72 hours from the group receiving multiple iron injections was found in molecules of higher iron content than that from the group receiving only one injection of iron. This finding coincided, as before (page 95) with a greater saturation with iron of ferritin from the group that received multiple injections of iron. Thus, the increased retention of preformed ferritin inthis group appears to be associated

with increased iron content of ferritin molecules. These findings are considered good evidence in support of the earlier conclusion that iron regulates ferritin turnover by transforming unstable fractions of low iron content into stable fractions of higher ironcontent.

(iv) The Search for a Labile Precursor of Ferritin

The above studies suggested that excess iron stabilizes preformed ferritin molecules by converting them into more stable fractions with a higher iron content. This concept might be extended to provide a mechanism for the induction of ferritin by iron whereby iron acted by stabilizing some labile precursor of ferritin. Such a precursor might be synthesized at a rapid rate but also be rapidly degraded unless trapped as stable ferritin by the insertion of iron. This would imply that, even in the uninduced state, there is also a rapid synthesis of this precursor which however, never appears as stable ferritin because of the low iron supply.

Apoferritin immediately came to mind as the most likely candidate for this role. The work of Fineberg and Greenberg (1955) had led them to conclude that apoferritin was the initial product of ferritin synthesis since at the

earliest interval their apoferritin fraction was more highly labelled. Our fractionation studies also showed that newly synthesised ferritin, in both normal and iron-induced rats, first appears in the fractions with the least iron. However, our earlier studies on the uptake of ^{14}C-leucine into unfractionated ferritin in control animals (page 73) provided no evidence of a rapidly synthesised ferritin fraction in normal rats, since it took 2 hours for the ferritin to attain its maximum level of incorporation (Fig. 19). Our subsequent fractionation studies have shown that the level of ^{14}C-leucine incorporation in normal rats at short time intervals never approached the levels necessary for it to be considered the precursor of ferritin (Figs. 29 and). Our results, therefore, indicated that iron-free apoferritin is not the precursor of ferritin. This might be resolved if iron had the effect of stabilizing the turnover of the subunits of ferritin so that the first stable product is a ferritin species containing a small amount of iron, but not apoferritin. It will be noted above that we do not claim that our gradient isolates apoferritin, but only an iron-poor fraction.

It was decided to reinvestigate the rate of incorporation of ^{14}C-leucine into ferritin in normal rats to exclude the possibility that the earlier studies using a chromatographic separation of ferritin (page 32) had failed to detect a fraction with a very short half-life,

perhaps in the form of ferritin subunits. It seemed reasonable to assume that an antiserum for ferritin might also be immunologically active against ferritin subunits if they were present in the liver extract. Consequently, this experiment contains two separate investigations. The first was concerned with measuring the rate of incorporation of ^{14}C-leucine into chromatographically isolated ferritin in normal animals to test for the presence of a fraction of ferritin with a very short half-life. Secondly, by immunological precipitation, it was hoped to measure the rate of incorporation into both soluble subunits of ferritin, which might be in the liver extract, as well as the entire ferritin complement. This latter investigation might help to elucidate the nature of the ferritin precursor.

EXPERIMENTAL AND RESULTS

Normal rats were each injected with 5 μc ^{14}C-DL-leucine/100 g. body weight and were killed at intervals of 15, 30, 45, 60 and 120 minutes thereafter. Liver ferritin was isolated by the usual chromatographic procedures, and its specific activity measured. In addition, samples (1 ml.) of the heat supernatant fraction (page 44) used for the chromatographic isolation of ferritin were incubated with 3 ml. rabbit antihorse ferritin (page 37) and the specific activities of the resulting precipitates were measured as before (page 73). The results are given in Table 20.

Table 20

Uptake of ^{14}C-leucine into Ferritin in Normal Animals

Time after injection (mins.)	Ferritin (isolated by column chromatography) (c.p.m./mg. protein)	Ferritin + Subunits (isolated by immunological precipitation) (cp.m./mg. protein)	Ratio Chromatographic ferritin / antibody ferritin	Mixed liver protein (c.p.m./mg. protein)
15	95	22	4.3	156
30	107	31	3.5	151
45	210	50	4.2	194
60	261	64	4.1	218

Fasting male rats were injected with 5 µc ^{14}C-leucine and then killed at 15, 30, 45 and 60 minutes after isotope injection. Liver ferritin was isolated by the usual chromatographic procedures (page 43) and also by immunological precipitation (page 41). The specific activities of these fractions and of mixed liver protein were estimated as before (page 58 and page 73). Each entry in the table is the mean from at least two experiments.

There was no evidence of a high level of incorporation of ^{14}C-leucine into any of the samples of chromatographically purified ferritin at very early intervals after isotope injection. The incorporation of ^{14}C-leucine into the ferritin of these normal rats was slow and took 2 hours to reach its maximum level. These findings are in agreement with the previous experiment (page 74) and rule out the possibility of a high rate of turnover of a significant part of the ferritin.

The results obtained with ferritin fractions obtained by immunological precipitation were also in agreement with these findings. There was no evidence of a high level of incorporation into immunologically precipitated material at any interval up to 2 hours after isotope injection when maximum incorporation was attained. The ratio of the specific activity of ferritin isolated by chromatographic procedures to that of the material in the specific precipitate was about 4:1. This ratio is similar to that found in previous experiments (page 73) where conditions excluded the possibility of highly labelled subunits.

In conclusion, these experiments have excluded the presence of a labile fraction in ferritin in normal rat liver which would act as a precursor for ferritin. The data do not allow us to decide whether the induction of

ferritin by iron occurs through an effect of iron on the rate of turnover of ferritin subunits or not. If such subunits exist in any quantity, they are either unstable at 80° (the temperature used in isolating ferritin) or they are held on some structure which is precipitated at this temperature, such as the polysomes. These conclusions, however, must be regarded with considerable reserve until it is established whether rabbit antihorse ferritin serum will precipitate ferritin subunits.

DISCUSSION.

DISCUSSION

The induction of liver ferritin by iron offers an unusually favourable opportunity for studying control mechanisms in protein synthesis and turnover. This study was greatly facilitated by our new procedures for measuring the total liver content of ferritin iron and ferritin protein and the incorporation of labelled amino acids into ferritin. With these procedures, we were able to investigate independently some of the factors regulating the rate of synthesis and turnover of this protein. Unlike other workers (Fineberg and Greenberg, 1955; Yu and Fineberg, 1965) we were able to obtain quantitatively reproducible responses which allowed us to select suitable conditions for studying the induction of ferritin by iron, because of careful breeding and selection of animals to ensure uniformity.

In agreement with Fineberg and Greenberg (1955), we found that iron accelerated the de novo synthesis of ferritin by a demonstrable increase in amino acid incorporation. The increased rate of synthesis produced by iron was maximal within four hours of the iron injection. At this interval there was a significant increase in the levels of ferritin iron and ferritin protein in the liver. As the increase in ferritin iron was greater than the increase in ferritin protein, it was concluded that the incorporation of iron into ferritin may occur independently of a proportional change in protein synthesis. Similar conclusions were reached by Mazur, Green and Carleton (1963).

The recent work of Yu and Fineberg (1965) led them to conclude that the effect of iron on ferritin induction could be abolished by actinomycin D. They proposed that the induction process depended on an increase in messenger RNA synthesis. Our results, however, directly contradict this conclusion. We found that dose levels of 70 and 150 µg. actinomycin D/100 g. body weight given at intervals up to 3 hours before iron, did not prevent the induction of ferritin by iron. These dose levels were shown to inhibit RNA synthesis by more than 80% without markedly depressing general protein synthesis. In view of this conflict, we were prompted to attempt to repeat the experiments of Yu and Fineberg (1965). The results of these experiments show that their massive doses of actinomycin D (1.8 mg./100 g. body weight) reduced the rate of general protein synthesis in the liver to a level of only 20% of the control values. These authors were apparently unaware of the toxic effects of their dose levels of the antibiotic, owing to the omission of certain necessary controls in their experiments. It seems apparent that their observed failure of iron to stimulate ferritin synthesis could be entirely attributed to the depressing effect of actinomycin D on general protein synthesis and not to a direct effect on the induction of ferritin. We therefore feel confident in concluding that dose levels of actinomycin D which effectively block RNA synthesis without immediately affecting

general protein synthesis do not inhibit the induction of ferritin by iron. Consequently, the increase in ferritin synthesis as a result of iron administration does not depend on additional messenger RNA synthesis. This conclusion was also supported by evidence that the time course of induction and the magnitude of the response of liver ferritin synthesis to iron were unaffected by the intake of dietary protein, which is known to alter the RNA content of the liver cell.

In order to explore other mechanisms by which iron might operate, we investigated the effect of parenteral iron on the turnover of ferritin. These studies suggested that the turnover of ferritin was greatly influenced by the amount of iron in the liver; repeated iron administration apparently stabilized preformed ferritin. By fractionating ferritin according to iron content, it was found, in agreement with Fineberg and Greenberg (1955), that newly formed ferritin molecules had a very low iron content. With time, the number of labelled molecules remaining in the liver decreased and those remaining were found predominantly in the iron-rich fractions. Repeated iron administration considerably reduced the rate of loss of prelabelled molecules. The labelled molecules which survived had a considerably higher iron content than those from animals which had received only a single injection of iron. This implies that the extra iron stabilized some of the preformed ferritin by converting it into fractions with a higher iron content. This apparent

increase in stability of ferritin molecules as a result of repeated iron administration is compatible with the fact that iron-rich ferritin is less susceptible to proteolytic degradation in vitro than apoferritin (Mazur and Shorr, 1950).

The above findings, that iron administration decreased the rate of breakdown of liver ferritin, led us to consider that a similar mechanism might be responsible for the observed increase in ferritin synthesis as a result of iron administration. This mechanism is depicted diagrammatically in Fig.31. It is suggested that some labile fraction of the ferritin spectrum or a precursor, is synthesised at a rapid and constant rate but is normally degraded unless trapped as stable ferritin by iron. This mechanism is compatible with many of our findings. It would account for the observed increase in amino acid incorporation into ferritin after iron administration in the absence of additional messenger RNA synthesis, as well as for the stabilization of preformed ferritin by extra iron. The absence of an effect on the induction process when cell RNA is depleted by diet is not incompatible with this thesis nor is the increased response with dose level of iron, since it could be argued that the rate of production of the precursor was not rate-limiting in either case under the experimental conditions.

This mechanism is analogous in some respects to the induction of certain mammalian enzymes by their substrates. The increase in arginase activity in suspensions of liver cells

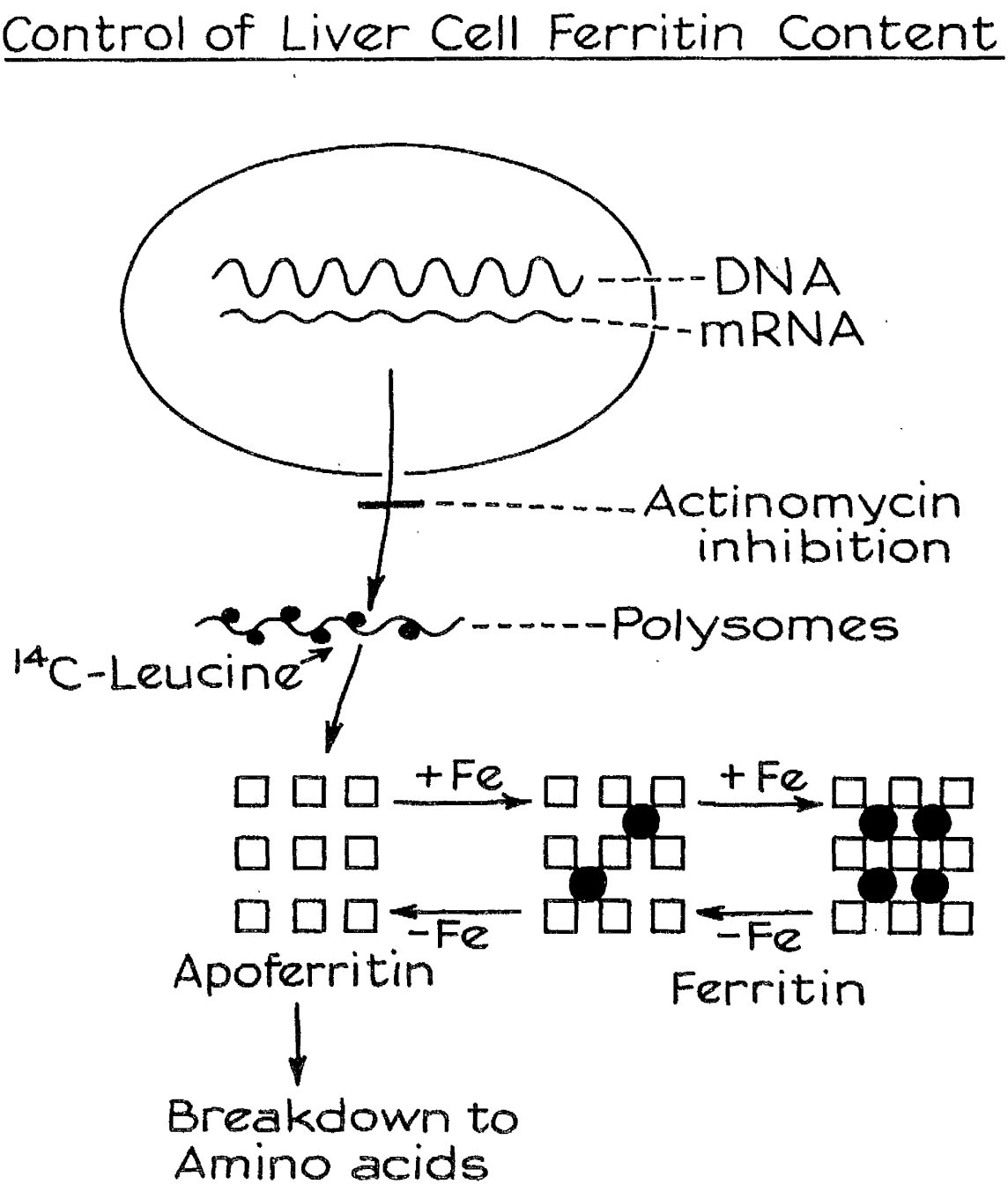


Fig. 31 Suggested mechanism for the regulation of ferritin synthesis and turnover by iron.

after the addition of glutamine does not require an increase in messenger RNA synthesis (Eliasson, 1965). This increase is apparently due to some effect of glutamine on the turnover of arginase and is not due to an activation of preformed enzyme. The induction of tryptophan pyrrolase by tryptophan also does not involve an increase in messenger RNA synthesis. The increased enzyme activity following tryptophan administration involves two processes: (a) activation of the apoenzyme by combination with an iron-porphyrin cofactor (Greengard and Feigelson, 1964); (b) accumulation of enzyme as a result of substrate stabilization in the presence of continued enzyme synthesis (Schimke, Sweeney and Berlin, 1965). The stabilization of ferritin by iron appears to parallel this second process. There is no evidence, however, that tryptophan can stimulate enzyme synthesis as iron can stimulate ferritin synthesis. In support of their findings, Schimke et al. (1965) have shown that tryptophan protects tryptophan pyrrolase against denaturation and proteolytic attack in vitro, possibly by maintaining the enzyme in a conformational state which resists proteolytic degradation. It seems appropriate to mention once more that similar observations of stability to proteolysis have been made with ferritin and apoferritin (Mazur and Shorr, 1950).

It follows from our proposed mechanism for ferritin induction by iron that one would expect to find the same rapid turnover of the ferritin precursor in the normal animal as in

the iron-induced animal. Owing to the low turnover of iron in normal animals, only a little is deposited as ferritin. Our fractionation studies showed that the newly formed ferritin molecules had a very low iron content. These results pointed to the possibility that the ferritin precursor was apoferritin, as suggested by Fineberg and Greenberg (1955). However, no evidence of such a rapidly-labelled precursor was found on fractionation of the ferritin of control animals. These results would therefore indicate that the subunits, and not apoferritin, must be the immediate precursor of ferritin. We have as yet, no direct experimental evidence for this conclusion. The failure to demonstrate a rapid turnover of immunologically precipitated material does not preclude the presence of subunits in the liver extract since there are many technical problems which must first be resolved. One of the most obvious tasks is to establish a method for isolating subunits from the liver cell, since an antiserum to ferritin might not precipitate its subunits. The problem of identifying the immediate precursor of ferritin will be deferred till later in this discussion.

The relatively slow response of ferritin synthesis to iron administration appears at first sight incompatible with our proposed mechanism. The incorporation of ^{14}C-leucine into ferritin only attained its maximum when isotope was given at two hours after iron injection. It is known, however, that the uptake of iron by liver from a dose of 500 µg. iron as

ferric chloride is already considerable after only 30 minutes (Nagarajan, Sivaramakrishnan and Brahmanandam, 1964). We have recently confirmed these results using ferric ammonium citrate labelled with ^{59}Fe (these data are not given in the thesis). In this case, uptake was almost maximal at 30 minutes. The question therefore arises why there is a lag period before the iron shows its effect on ferritin synthesis. The only rational explanation is that ferritin is not the primary depository in the liver of the parenteral iron. Certain observations of others lend support to this explanation. Thus, it is known that the distribution of a parenteral dose of iron is determined by the form in which the iron is administered (Nagarajan et al., 1964). Further, the distribution of iron within an organ is also determined by the form of the iron administered. Saccharated iron oxide is rapidly removed from the blood and appears in the Kupffer cells of the liver as a granular deposit (Shoden and Sturgeon, 1962). Later, some of this iron is transferred to ferritin in the parenchymal cells. By contrast, the iron in iron dextran preparations such as Imferon (Benger Ltd.) is only slowly removed from the blood and gives rise directly to ferritin in the parenchymal cells. It therefore seems likely that the iron in ferric ammonium citrate is treated as that in saccharated iron oxide and gives rise to some primary iron deposit which acts as a reservoir for ferritin synthesis. This explanation is

consistent with the observations of Richter (1964) that HeLa cells grown in tissue culture first incorporate ferrous sulphate into granular bodies before the appearance of ferritin.

There are many outstanding problems which have yet to be resolved before our proposed mechanism of ferritin induction by iron can be confirmed. Firstly, it would be desirable to demonstrate that iron depletion results in an increased breakdown of ferritin. Some earlier work indicates that this is so. Granick (1951) found a large decrease in the amount of crystallizable ferritin in tissues of iron-deficient animals. We have attempted to confirm this evidence by demonstrating a loss of prelabelled ferritin molecules after the administration of an iron-chelating agent called Desferal (Ciba Laboratories Ltd., Sussex). In an exploratory experiment (not reported here) we were able to demonstrate a dramatic loss of ^{14}C-labelled ferritin at 48 hours after an injection of 10 mg. Desferal/100 g. body weight. If these results can be confirmed, they would substantiate our belief that the stability of ferritin *in vivo* is regulated by its iron content.

Secondly, the precise nature of the precursor of ferritin and the mechanism by which iron affects its turnover still awaits elucidation. One likely mechanism would be a direct participation of iron in the synthesis of the polypeptide chain of the subunits. If this were so, it might be possible to identify the polysomes responsible for their synthesis by

labelling the polysomes with ^{59}Fe. Alternatively, the iron might operate in the aggregation of the subunits into ferritin. It is not yet known whether this aggregation occurs on the polysomes or in the cell sap. This would be best tackled by devising methods for the isolation of the subunits. However, it might be possible to study this process by pre-labelling the subunits and then investigating the effect of iron on their aggregation under conditions which inhibit the further incorporation of amino acids. A somewhat similar approach has recently been used to study the aggregation of the subunits of thyroglobulin (Seed and Goldberg, 1965; Sellin and Goldberg, 1965).

Our evidence suggests that there may be two mechanisms operating in the regulation of ferritin synthesis and turnover by iron. The first mechanism involves an increased synthesis of ferritin protein, possibly by some catalytic effect of iron on the synthesis of ferritin subunits or on their subsequent aggregation. The second mechanism involves an apparent stabilization of the completed ferritin molecule by iron. It is tempting to speculate that similar mechanisms might operate in other proteins which contain subunits or a non-protein moiety such as a metal or a prosthetic group.

Certain recent evidence suggests that this may indeed be the case. Thus, iron may regulate the rate of translation of the subunits of haemoglobin by a direct action on the polysome (Rabinovitz and Waxman, 1965). Alternatively,

or in addition, the level of the haem prosthetic group may regulate the rate of synthesis of globin (Lovere and Granick, 1965). These latter authors suggested that haem acts at the ribosomal level to stimulate globin synthesis. However, it seems possible that haem might produce its effect by facilitating the aggregation of the subunits. Similarly, the level of iodine may regulate the aggregation of the subunits of thyroglobulin (Seed and Goldberg, 1965). Thus the rate of synthesis of the subunits of proteins need not determine the rate of formation of the completed molecule. Further, Tatibana and Cohen (1965) have suggested that the conversion of the precursor of carbamyl phosphate synthetase into active enzyme may occur by an aggregation of its subunits by a process independent of protein synthesis. Little is known of how these transformations occur, but it seems likely that they are enzymically controlled similarly to the formation of disulphide bonds linking the subunits of insulin and ribonuclease (Goldberger, Epstein and Anfinsen, 1963; Goldberger, Goldwyn and Anfinsen, 1964).

In addition to mechanisms affecting the rate of synthesis or aggregation of subunits, there may be others which act by converting an inactive, unstable precursor into an active stable protein. An example of this might be the mechanism involved in the formation of glycoproteins. It is known that the protein moiety and carbohydrate prosthetic group are formed in different compartments of the cell. The completed

molecule first appears in the membranes of the endoplasmic reticulum where the insertion of the carbohydrate prosthetic group occurs (Sarcione, 1964; Cook, Laico and Eylar, 1965; Hallinan, 1965).

As discussed earlier, factors which stabilize completed protein molecules may cause an accumulation of protein by decreasing its rate of breakdown. This type of mechanism has been concluded to account for the substrate induction of tryptophan pyrrolase (Schimke et al., 1965) and also for the increased retention of prelabelled ferritin molecules as a result of repeated iron injection (page 110). One might expect other enzymes to be controlled by a similar process in view of the general property of stabilization of enzymes by substrates in vitro. It also seems likely that coenzymes might operate in a similar manner by stabilizing their apoenzymes. Before accepting the stabilization of proteins by associated smaller molecules, it would be desirable to demonstrate an increased retention of prelabelled protein molecules in the absence of protein synthesis. The stabilization phenomenon is not the only possible explanation for an increased retention of protein under conditions allowing the synthesis of new protein. Assuming an unaltered rate of degradation, an influx of unlabelled protein as a result of substrate administration would reduce proportionately the degradation of the labelled protein.

There is obviously a vast new area now open for exploration. Many of the mechanisms suggested above for specific proteins may overlap or prove to be wrong in the light of new evidence. However, one intriguing aspect which emerges is that stabilization of the polypeptide chain may occur at all levels from the polysome to the completed protein.

Two points remain abundantly clear. First, the rate of messenger RNA production alone does not determine the rate of synthesis of mammalian proteins, and secondly the rate of synthesis of mammalian proteins does not necessarily determine their levels in tissues.

SUMMARY

SUMMARY

I. The Small-scale Isolation of Ferritin for the Assay of the Incorporation of ^{14}C-labelled Amino Acids

1. A new procedure is described for the isolation of ferritin from small amounts of rat liver. After the removal of most of the tissue proteins by heat coagulation, the ferritin fraction was chromatographed successively on CM-cellulose and Sephadex G-200.

2. The isolated ferritin appeared pure as judged by its sedimentation pattern in the ultracentrifuge, by electrophoresis in polyacrylamide gel and by immunoelectrophoresis.

3. After labelling with ^{14}C-leucine, the specific activity of ferritin isolated by the new procedure bore a constant relationship to that of liver ferritin separated by antigen-antibody precipitation. The ferritin isolated by the new procedure is therefore of suitable purity for studies of amino acid incorporation.

4. The new procedure can be used to measure the total ferritin protein content of a tissue. This is valuable since it is not possible to measure ferritin protein by ultraviolet absorption because of considerable interference from the iron it contains.

II. Polymorphism in Ferritin

1. The isolated ferritin was found to separate into several discrete bands on electrophoresis in acrylamide gels. The patterns obtained from ferritin and chemically prepared ferritin were identical. The number of bands entering the gel decreased with increasing gel concentration. The migration of each band relative to the main (α) band also decreased with increasing gel concentration. It was concluded therefore that the bands separated by electrophoresis in acrylamide gels represents ferritin in different degrees of aggregation.

2. Ultracentrifugation of apoferritin confirmed the presence of small amounts of aggregated material.

III. Quantitative Aspects of the Induction of Liver Ferritin by Iron

1. The uptake of ^{14}C-leucine into mixed liver proteins was maximal at 30 minutes after isotope injection. The uptake into ferritin in normal rats was much slower and attained a plateau at 2 hours after isotope injection.

2. A single dose of iron caused a considerable, but transient, increase in the incorporation of ^{14}C-leucine into ferritin but had no effect on the incorporation into mixed liver proteins. The maximal increase occurred when isotope was injected 2-3 hours after the iron.

3. There was a significant increase in the total amount of ferritin iron and ferritin protein in the liver as a result of the iron injection. The administration of iron led to an increased iron content in liver ferritin at 4 hours after iron injection.

4. The magnitude of the response in ferritin biosynthesis increases with the amount of iron administered.

IV. Investigations into the Mechanism of the Induction of Ferritin by Iron

1. Dose levels of actinomycin D which effectively blocked RNA synthesis did not inhibit the induction of ferritin by iron.

2. The induction of ferritin by iron was unaffected by protein depletion.

3. It was concluded that the induction of ferritin by iron did not require additional messenger RNA synthesis and that the amount of available messenger RNA was not rate-limiting in these studies.

V. The Effect of Parenteral Iron on Liver Ferritin Metabolism

1. Liver ferritin in normal rats has a half-life similar to that of mixed liver proteins.

2. During the period 12-24 hours after isotope injection, the half-life of ferritin in iron induced animals was only 15 hours.

3. Repeated iron administration considerably increased the retention of prelabelled ferritin. It was concluded that excess iron stabilizes preformed ferritin.

4. A procedure was devised for the fractionation of ferritin according to iron content by sucrose density gradient centrifugation. Labelling of ferritin molecules from livers of normal and iron-injected rats first occurs in fractions of lowest iron content. With time, there was a general reduction in the level of radioactivity, accompanied by a progressive movement of radioactivity into iron-rich fractions.

5. These studies confirmed that repeated iron administration considerably reduces the rate of loss of prelabelled ferritin. The labelled molecules which survived had a higher iron content than those from animals which had received only a single injection of iron. It was therefore concluded that the administration of excess iron stabilized preformed ferritin molecules by transforming them into fractions of higher iron content.

6. There was no evidence of a fraction of ferritin in normal rats with a rapid rate of turnover in samples of ferritin isolated by chromatographic procedures, nor could such a fraction be demonstrated in material precipitated

from the heat supernatant fraction with a ferritin antiserum .

These results indicated that the increased synthesis of ferritin as a result of iron administration is due to a catalytic effect of iron on the aggregation of subunits bound to polysomes.

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Publications arising from this work:

1. Small-scale isolation of ferritin for the assay of the incorporation of ^{14}C-labelled amino acids.
J.W. Drysdale & H.N. Munro (1965) Biochem.J. 95, 851.

2. Failure of actinomycin D to prevent induction of liver apoferritin after iron administration.
J.W. Drysdale & H.N. Munro (1965) Biochim.biophys. Acta, 103, 185.

3. Induction of rat liver ferritin after iron administration.
J.W. Drysdale & H.N. Munro (1965) in Abstracts of the 2nd Meeting of the Federation of the European Biochemical Societies, A113, Vienna.

4. Control mechanisms in liver ferritin synthesis and turnover.
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SUMMARY

CONTROL MECHANISMS IN PROTEIN SYNTHESIS AND TURNOVER WITH SPECIAL REFERENCE TO LIVER FERRITIN

by

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The aim of this thesis was to investigate factors which regulate the synthesis and turnover of mammalian proteins. Liver ferritin was chosen for these studies since it is known that parenteral iron leads to an increased synthesis of this protein. It was hoped that these investigations might indicate common control mechanisms for the regulation of synthesis and turnover of other mammalian proteins.

In order to carry out these studies, procedures were devised for the isolation of ferritin in sufficiently pure form to allow measurements of the uptake of ^{14}C-labelled amino acids to be made. This was achieved by chromatographic techniques on carboxymethyl cellulose and Sephadex G-200. The final product was judged to be pure by its sedimentation pattern in the analytical ultracentrifuge, by electrophoresis in acrylamide gels and by immunoelectrophoresis. After labelling with ^{14}C-leucine, the specific activity of ferritin isolated by the new procedure bore a constant relationship to that

of liver ferritin isolated by immunological precipitation. The ferritin isolated by the new procedure is therefore of suitable purity for measurements of amino acid incorporation. In addition, procedures were devised for the estimation of the total liver content of ferritin. Owing to the considerable interference from iron in ferritin it was not possible to measure ferritin protein by ultraviolet absorption and another established procedure was used.

The molecular complexity of the isolated ferritin was examined. It separated into several discrete bands on electrophoresis in acrylamide gels. These differences were concluded to be due to the presence of several aggregates of one type of protein, and not to several genetically different protein species.

The new isolation method was used to study ferritin induction in the liver after intraperitoneal injection of iron. This was done by injecting ^{14}C-leucine and observing the level of radioactivity in the isolated liver ferritin. In control animals, the uptake of ^{14}C-leucine took 2 hours after isotope injection to attain a plateau. The time course of the induction of ferritin by iron was followed by using this 2-hour pulse dose for measuring the rate of ferritin synthesis. A single injection of iron produced a considerable

but transient, increase in the incorporation of ^{14}C-leucine into ferritin but had no effect on the uptake into mixed liver proteins. The magnitude of this response to iron was dose-dependent.

Amounts of actinomycin D which were shown to be effective in blocking RNA synthesis did not inhibit the induction of ferritin by iron. Further, the induction was unaffected by protein depletion. It was concluded that the induction of ferritin by iron did not require additional messenger RNA synthesis and that the amount of available messenger RNA is not rate-limiting in this induction.

The turnover of liver ferritin was also examined at time intervals up to 72 hours after ^{14}C-leucine injection. The half-life in normal rats was found to be about 72 hours. However, the half life of newly synthesised ferritin in iron-injected rats was reduced to about 15 hours at 12-24 hours after a single injection of iron. Repeated iron administration considerably retarded the loss of label, suggesting that excess iron stabilizes preformed ferritin. By fractionating ferritin according to iron content on sucrose density gradients, it was shown that newly synthesised ferritin first appears in the fractions of lowest iron content.

With time, there was a progressive movement of radioactivi into iron-rich fractions. The labelled ferritin molecule which survived in animals receiving multiple iron injections had a higher iron content than those from animals which had only received a single injection of iron. It was therefore concluded that the repeated administration of iron stabilized preformed ferritin molecules by transforming them into fractions with a higher iron content and that these are more resistant to degradation.

There was no evidence of a rapidly turning over subfraction in ferritin which would act as a precursor for ferritin, nor was there any evidence of such a fraction in material precipitated from a liver extract with a ferritin antiserum. These results indicate that the increased synthesis of ferritin resulting from iron administration may be due to a catalytic effect of iron on the aggregation of subunits bound to polysomes. Thereafter, iron stabilizes completed ferritin molecules in a manner analogous to stabilization of enzymes by their substrates.

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