STUDIES ON COLLAGEN BIOSYNTHESIS

by

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INTRODUCTION

The work to be presented in this thesis is concerned with the biosynthesis of collagen which is the major protein constituent of connective tissues. All these tissues contain this protein and its nature and concentration contribute largely to the variations of structure and function of connective tissues which occur in different organs. For example, very fine fibrils of collagen are found in the vitreous humor and cornea of the eye, these fibrils which are few in number are of necessity far smaller than those of organs such as tendon. In tendon the fibrous bundles are arrayed in a parallel manner, conferring high tensile strength whilst in dermis a "woven" pattern contributes to the elasticity of skin. In the very specialized tissue, bone, the collagen fibers, are invested with mineral salts which confer hardness and rigidity to the structure. With the exception of the Protozoa, collagen has been found in every class of animals studied (126) but it is far more abundant in vertebrates than in invertebrates, indeed in the former it may constitute up to a quarter of total body protein (55).

Collagen is frequently regarded as a relatively metabolically inert protein and this may be true when compared to the proteins found in such organs as liver and muscle. However, in times of growth or repair, synthesis

takes place quite rapidly, and the removal of collagen is also quite dramatic as is seen in the involuting uterus which loses 72% of its collagen within eight days post partum (87). Klemperer et al (71) also focused attention upon collagen metabolism and the consequences of its alteration in the now famous concept of "diffuse collagen disease."

Before the investigations into the biosynthesis of collagen are described, it is necessary to consider the chemical and physical properties of the protein insofar as they bear upon its biological function.

Collagen Structure

a) Amino acid composition

"Colla-genic" material was known to antiquity as a material which gave rise to "Colla" or glue upon wet heating and it was upon gelatin, the main substance used for glues, that the earliest amino acid analysis of collagen was carried out by Dakin in 1920 (21). The necessity of using gelatin which is now recognized as denatured collagen, was due to the then current belief that native collagen was insoluble in water and in dilute salt solutions.

Dakin showed that about one third of the amino acid residues of gelatin were glycine and that a further one quarter to one third were composed of proline plus hydroxy-

proline. This latter amino acid has still not been definitely identified in any other animal protein, although it is known to occur in the cell walls of several higher plants (140) (74).

Subsequent amino acid analysis carried out with increasingly elegant techniques, served to verify the early work of Dakin and culminated in the complete analysis of Ox-Hide gelatin by Bowes and Kenten (12) who showed that gelatin contains unusually large concentrations of glycine (26.3%) and proline (15.1%), together with striking amounts of unusual amino acids, i.e. hydroxyproline (14.0%) and hydroxylysine (1.3%).

b) Amino Acid Sequence

The complete amino acid sequence of collagen is not yet known and a discussion of the development of the present state of knowledge is outside the scope of this dissertation; however, a summary of the current concepts will be presented insofar as they bear upon the secondary and tertiary structure of this protein.

In 1954, Schroeder, Kay et al (132) were able to isolate forty-five peptides from a partial acid hydrolysate of gelatin. By applying the classical dinitrophenylation technique to these peptides they obtained results which led them to suggest that the sequences gly-pro-hypro-gly or

gly-pro-hypro-gly-pro-hypro-gly may occur frequently in gelatin.

Later work led them to postulate a sequence P-X-G-P, where P is either proline or hydroxyproline, G is glycine, and X is any amino acid other than proline, usually hydroxyproline or alanine. This general structure was in good agreement with all peptides isolated. This work was supported in the following year by Kroner, Tabroff and McGarr (73) who reported the isolation of peptides from steer hide collagen with the sequence gly-pro-hypro-gly.

c) Polypeptide Configuration

The orientation of the polypeptide chains came under consideration long before the concept of collagen as a molecular species emerged. Astbury in the late 1930's studied many fibrous proteins by x-ray diffraction techniques and found that the apparent distance between amino acid residues of collagen was smaller than that observed for other fibrous proteins such as silk fibroin, and Astbury (4) felt that this could be accounted for on the basis of the very large content of imino acids and glycine. He postulated that the presence of the large pyrrolidone side chains of proline and hydroxyproline together with the insignificant hydrogen side chain of glycine would distort the backbone. of the peptide chain into an alternating cis-trans configuration, which would thus shorten the distance between two

adjacent residues.

Another notable prediction of the configuration was made in 1942 by Huggins (63), who suggested that a spiral arrangement of a polypeptide chain fitted the steric requirements of collagen with its unique amino acid composition far better than the cis-trans arrangement put forward by Astbury.

It is now felt by many workers that the x-ray diffraction pattern of collagen can best be interpreted in terms of a macromolecule containing three chains coiled in a helical fashion about each other to form a coiled coil, (17) (113) (114) (118), rather than the single Helix proposed by Huggins. This view was first put forward by Pauling and Cory in 1951 (100).

The various models proposed, whilst all conforming to the same basic pattern, differ in the way in which the three chains are phased in relation to one another and particularly in which hydrogen bonds are used to hold the molecule together. Of late, the models proposed by Rich and Crick (118) have come into favor and are now generally accepted by most workers in the field.

In these models which are simply called structures I and II, the three chains are thought to be about 5Å apart and to wind around each other forming a left handed super helix around an axis lying between them. The pitch of this

super helix is 28.6Å. Structures I and II differ in the orientation of proline and hydroxyproline residues. In Structure I, hydroxyproline hydroxyl groups are inwardly directed and can take part in interchain hydrogen bonding along with the CO and NH groups associated with peptide bonds of glycine residues. Structure II, which Rich and Crick believe to be sterically more satisfactory, has the hydroxyl groups of hydroxyproline arranged radially and thus available for hydrogen bonding with another tropocollagen molecule. This complex subject was reviewed very thoroughly in 1961 by Rich and Crick (119).

d) The Molecular concept of collagen structure

Investigations in 1942 with the electron microscope by Hall Jakus and Schmitt (54) showed collagen fibrils to have a repeating band pattern with a periodicity of 620 to 650 Å, and in the same year, Bear (5) was able to demonstrate an axial periodicity of 640 Å by use of x-ray diffraction techniques.

With improvements in electron microscopic techniques many finer periods were recognized within the major 640 Å periods (130) and Bear (6) suggested that the bands, or dark lines seen on electron micrographs represent regions rich in polar amino acids (dicarboxylic and diamino) whilst the interbands represent the areas rich in glycine, proline, hydroxyproline and alanine. This apparent

concentration of non polar residues is supported by the sequence studies previously described.

It would seem logical to equate the major 640 Å bands with the length of single collagen macromolecules arranged in sequence along a fiber axis, but, that this is not so, will be seen later. It is first necessary, however, to introduce intervening work on the solubility of collagen in various salt solutions which contributed much to the molecular concept of collagen.

The earliest demonstration of the solubility of collagen was made by Zachariades in 1900 (158) followed by Nageotte in 1927 (92) and Leplat in 1933 (75). All these workers extracted rat tail tendon with dilute acetic or other organic acids and were able to show solubilisation of protein. Nageotte (92) was also able to reprecipitate a fibrous material from his extract which was microscopically similar to teased collagen fibers. Much later, Tustanovskii (142) used citric acid buffers to obtain solutions of collagen, whilst Harkness et al (56) used slightly alkaline phosphate buffers. More recently, Gross et al (47) and Jackson and Fessler (65), used neutral salt solutions to the same end. The metabolic significance of these various extractable fractions will be discussed in another section of this thesis, their importance to the elucidation of structure must now be considered.

If the ionic strength of a solution of collagen is raised, a fibrous precipitate is obtained which appears identical under the electron microscope, to native collagen, and this method is frequently used as a purification technique.

In 1950 however, Gross, Highberger and Schmitt, (46) (58), by varying this procedure, were able to produce precipitates which did not resemble native collagen. Two types of material were obtained; Segment long Spacing (SLS) by the addition of ATP to collagen solutions, and Fibrous long Spacing (FLS) upon addition of α1 acid glycoprotein. Both these materials demonstrated an axial periodicity of 2600 - 3000 Å, about four to five times that of native collagen. As the names suggest FLS appears fibrous with a longer spacing than normal, whilst SLS occurs only as short segments with a total length of 2600 - 3000 Å, Schmitt (128) interpreted their occurrence as follows:

If the collagen macromolecule is assumed to have a length of 2600 - 3000 Å, and to be assymmetrical as far as the amino acid composition of the end regions is concerned, i.e. A.....B, then these molecules can aggregate together in various ways.

SLS can be visualised as a parallel array of molecules,

A.....B
A.....B
A.....B

FLS is an antiparallel array with end to end association,

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A..........BA..........B
B..........AB..........A
A..........BA..........B
```

whilst native collagen is a parallel array but with an overlap of about one quarter of a molecular length.

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....BA..........BA..........BA....
  ....BA..........BA..........BA....
    ....BA..........BA..........BA....
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If a large enough model such as this is constructed the regions represented by AB give the appearance of banding across the whole model which coincides with that seen for native collagen.

In 1956 Boedtker and Doty (10) were able to show by light scattering, osmometry, viscosity and flow birefringence that the molecular weight of collagen was about 345,000 and that its dimensions were about 2900 Å by 14 Å. This was subsequently confirmed by Doty and Nishihara (23) and by Hall (53) who was able to photograph collagen molecules under the electron microscope. In order to differentiate this collagen monomer from the much broader term "collagen", Schmitt, Gross and Highberger (129) coined the term "tropocollagen", and this term will be used in this thesis to refer to the collagen macromolecule.

e)Subunits of the Collagen Molecule

The collagen denaturation process has been studied extensively, (15) (23) (33) (96), and it is found that fairly mild heating, i.e. 30 °C to 35 °C, of an aqueous solution at acid pH, causes marked loss of viscosity and of specific optical rotation (23). The rate at which the viscosity falls being identical to the rate at which the specific rotation falls (23). Since these two parameters are dependent upon two different physical properties of the protein, Doty and Nishihara (23), consider that this reflects a very rapid, all or none denaturation. A solution which is "half denatured" as measured by either of the above methods, is considered to consist of a mixture containing completely denatured molecules and completely native molecules, rather than of molecules each of which is "half denatured". The material thus obtained is termed "parent gelatin", it contains no intact tropocollagen molecules when examined electron microscopically (117), yet no peptide bond hydrolysis has taken place, and Boedtker and Doty (10) suggested that this conversion broke the bonds holding the three polypeptide chains together and that they then separated and became randomly coiled.

Ultracentrifuge studies however demonstrated the presence of two components in parent gelatin obtained from rat skins (97), calf skin and ichthyocol (23), which were

termed α and β gelatins and were subsequently separated by ammonium sulfate fractionation (99). It was estimated that the β component had a molecular weight, twice that of the α component (23) (97). This of course does not agree with the above proposition of Boedtker and Doty, since on the basis of their suggestion, only one component should occur, if the three chains are assumed to be identical.

The relationship of these fractions to the tropocollagen molecule was not easily visualised until 1961 when Piez, Lewis et al (106), demonstrated four components of parent gelatin derived from rat skin tropocollagen. These components were separated by chromatography on carboxy methyl cellulose, and it was found that two of them had sedimentation constants which were almost identical to that previously described for the α component and were consequently termed α_1 and α_2. Two others, termed β_1 and β_2 were very similar to β gelatin.

Piez et al (107) have recently reported detailed amino acid analyses of α and β components isolated from rat, carp and dogfish collagen, and although some species differences are evident the same general pattern is seen in all. On the basis of these results and by studying the ratios in which the four components occurred in collagen extracted from animals of different ages, they proposed that the tropocollagen molecule consists of subunits (α_1 & α_2) of approximately

equal size but of differing amino acid composition. There are two α_1 chains and one α_2 chain per molecule and these can become cross linked to form the two β components which are found upon denaturation, i.e.

$$1\alpha_1 + 1\alpha_2 = 1\beta_1$$

and

$$1\alpha_1 + 1\alpha_1 = 1\beta_2$$

In vivo, the degree of this cross linking may well be directly related to the age of the particular tropocollagen molecule, thus a newly formed molecule may dissociate completely into three α chains upon denaturation (i.e. no cross linking) whilst a somewhat older molecule will dissociate into one α and one β . A still older molecule may have become completely cross linked across all three of its chains and will not dissociate at all. Evidence for this is presented in several reports of the existence of a so-called γ gelatin or γ tropocollagen which can be renatured into a native state (3) (117) (144) (145) and it is suggested (3) that this component has arisen from a completely cross linked tropocollagen in which only the interchain hydrogen bonds have been broken during the transition to parent gelatin.

No evidence is available concerning the nature of these interchain cross links other than that they are probably covalent, since they withstand the denaturing action

of 5M Guanidine and 2M Potassium Thiocyanate, which disrupt hydrogen bonds only (107). Since collagen contains no cystine or cysteine, disulfide bridges are not possible. Bonds other than α peptide links which have been reported, are α and β aspartyl esters (9) γ glutamyl (30) and ϵ-amino of lysine (84). It is not clear whether these contribute to intermolecular or intramolecular stability, but some evidence has been put forward to suggest that they may be involved in both.

Veis et al (145) have described a gelatin which has a higher molecular weight than tropocollagen and they consider this to consist of aggregates of tropocollagen molecules held together by heat stable bonds which may be related to those just described.

Gross[1] has shown that when a solution of tropocollagen is warmed at neutral pH for long periods, an opaque gel is formed which contains many collagen fibrils. If this is subsequently converted to parent gelatin the ratio of β component to α component is greater than the ratio in the original tropocollagen. This suggests that the bonds which are thought to form as a molecule ages in a tissue can also form in vitro.

By incubating the gel with trypsin or chymotrypsin, Gross could then reduce the amount of β component and produce a material which upon denaturation gave solely

[1] Gross, J. Personal Communication. November 1962.

α component. There was no evidence of peptide bond splitting. The well known esterase activity of trypsin and chymotrypsin is thus thought to be implicated in splitting of intramolecular bonds. The experiment does not however give any information about the nature of these bonds since the two enzymes used have a notoriously catholic choice of substrates (36). Gross was also able to reduce the amount of β component which appeared upon denaturation of a solution of tropocollagen which had not been subjected to gelling at 37 °C, by use of the same enzymes. It thus appears that the intramolecular bonds found in solutions of native collagen are of the same general type as those formed during the artificial "ageing" brought about by gelling.

It has been suggested that a defect in formation of these intramolecular bonds is responsible for the connective tissue pathology known as osteo lathyrism (81) (152).

Summary of Collagen Structure

From the foregoing, the current concept of a collagen molecule emerges as a rigid, rod like molecule with dimensions of 14 Å x 2900 Å. Its molecular weight is usually accepted as being 345,000 although Piez (107) has recently suggested that 300,000 is more correct. It consists of three polypeptide chains mutually coiled in a helix and held together with hydrogen bonds which become supplemented with stronger covalent forces as the molecule ages.

This molecular species can be isolated by extraction of a tissue and it can be reaggregated in vitro in a variety of ways to give, amongst others, a fibril which appears identical with native collagen.

The questions next to be answered are concerned with the origin of these native collagen fibrils in vivo, and the relevance to the in vivo situation of fibril formation as found in vitro.

COLLAGEN BIOSYNTHESIS

Collagen biosynthesis can be conceptually divided into two parts, Protein synthesis and Fibrogenesis.

Protein synthesis is concerned with the formation of the tropocollagen molecule and is thought to be an intracellular mechanism. Fibrogenesis is concerned with the aggregation of tropocollagen into fibers, and may be largely independent of cells, as shown in a previous section. This conceptual division will be used in the description of biosynthesis which follows.

Role of the Fibroblast in Collagen formation

Since 1847 when Schwann (133) first described the connective tissue cell, there has been speculation about the mechanism by which these cells (now termed fibroblasts) synthesised the mass of connective tissue which surrounds them. Virchow (146) considered that the cells secreted a soluble material which formed into fibrils outside the cell, a view which is surprisingly close to current concepts as will be seen later. Schwann (133) believed that collagen fibers developed directly from cell cytoplasm, whereas the extreme opposite view was held by Nageotte (91) who gave the cell no role whatsoever in fibrogenesis.

In more recent times, however, it has become quite apparent that the fibroblast is intimately concerned with collagen biosynthesis and many workers have devised methods of achieving concentration of these cells within a tissue. This has been approached in many ways, which are described below, but in general they are all based upon the introduction of a foreign body or chemical irritant into an animal. Inflammation ensues, followed by rapid infiltration of fibroblasts and rapid local collagen formation.

Induced models of Collagen Biosynthesis

a) Carageenan Granuloma

Carageenan is a sulphated mucopolysaccharide of high molecular weight which is extracted from the seaweed Chondrus crispus (7). Robertson and Schwarz (123) found that a subcutaneous injection of this material administered to a guinea pig, produced the growth of a large mass of collagen rich tissue within a few days, and the model has subsequently been used by many other workers for the study of the metabolism of collagen and other connective tissue components (64) (76) (79) (135). The histology of the granuloma has been described in detail by Williams (151).

The use of the carageenan granuloma is however limited by the fact that resorption is taking place concurrently with synthesis (64), indeed after a period of about

fourteen days, resorption seems to exceed synthesis and the entire mass disappears in about four to six weeks (64). The presence of the injected carageenan in the tissue may also complicate the interpretation of results obtained from this model.

b) Polyvinyl sponges

The subcutaneous implantation of small polyvinyl alcohol sponges as first described by Grindley and Waugh (39) has provided much useful information (11) (28) (31) (51) since the sponges become infiltrated with cells and rapid collagen formation proceeds. The sponges and their contained connective tissue are very conveniently removed and the sacrifice of the animal is not necessary. Indeed, even serial studies on the same animal are possible, as for example, with a primate or other expensive animal.

Other induced sources of rapid collagen formation which have been used relatively less frequently than carageenan granulomas or sponges are subcutaneously implanted wire mesh cylinders (127) and granulomas produced by introduction of irritants such as Silicic acid (16). A somewhat more physiologically normal tissue has been frequently used and will be described in detail in a later section. This is the tissue which forms in an excised wound, the so called granulation tissue.

In addition to the artificially induced models just described, much information has been obtained from the study of naturally occurring tissues such as skin (41) (42) (47) (68), chick embryos (110) (112), tendon (101) and from tissue cultures of the above (69) (157).

COLLAGEN PROTEIN SYNTHESIS

Intracellular Localisation

It was not until 1940 that fairly conclusive evidence implicating the Fibroblast in collagen biosynthesis was presented by Stearns (137) (138). She observed living cells in a transparent chamber in a rabbit's ear and noticed that a material was separated from the cell cytoplasm, which, to quote her,"was apparently utilized in the production of fibrils".

More recent investigations with the electron microscope have shed more light upon this subject and it now appears that collagen is organized intracellularly into very fine beaded filaments which are concentrated at the periphery of the cell (109) (149). In places the cell membrane is very poorly defined and it is proposed (35) (110) (157) that rupture or dissolution at this point allows the escape of the "collagen precursor" material contained in the cytoplasm. This hypothesis would seem to provide a bridge between the original theories of Schwann and Virchow, but, as is shown in the short review by Chapman in 1962 (13) it must still be considered a hypothesis and the subject is by no means closed.

The localisation within the fibroblast, of the site of collagen synthesis was first investigated by

Lowther et al (79) who incubated slices of carageenan granuloma with C^{14} proline.

Collagen was extracted and purified from subcellular fractions of this tissue, and the fraction isolated from the microsomes showed the highest specific activity. This indicated that synthesis is intimately associated with this fraction or that newly synthesised collagen accumulates there in vivo.

This work was confirmed and extended by Prockop et al (112) who followed the in vitro formation of hydroxyproline containing protein by some subcellular fractions isolated from chick embryos.

The localization of collagen synthesis in the cell microsome fraction is of course in accord with the current theory of protein synthesis which will be very briefly presented here for the purpose of orientation.

Protein Synthesis - (for review see (150))

Knowledge of the mechanisms concerned in the synthesis of proteins has increased very greatly during the past few years and the subject is today developing more rapidly than probably any other in biochemistry. The current theories are concerned with two main subjects, a) the preparation of free amino acids for incorporation into protein, and, b) their incorporation in a predetermined sequence rather than in a random manner.

The first part, the activation step, takes place in the cell cytoplasm under the influence of enzymes which are jointly referred to as "pH 5 enzyme system". It is felt that there is a specific enzyme for each amino acid, and that through their mediation an amino acid is combined with ATP to form an enzyme-amino acyl-adenylate complex. This complex is transferred to a relatively low molecular weight Ribonucleic acid which exists in the cytoplasm and is referred to as soluble or transfer Ribonucleic acid (SRNA or TRNA). The SRNA then "transports" the amino acid to the site of protein synthesis, the so called ribosome. As with the activating enzymes there are thought to be specific SRNA's for each amino acid.

The second part of the mechanism concerned with the correct alignment of amino acids now comes into play. The information concerning the order of amino acids in a protein is contained in the nuclear Deoxyribonucleic acid (DNA) and is dependent upon the order of nucleotide bases in a particular DNA molecule. This information or code can be transferred to yet another ribonucleic acid molecule, which is usually referred to as messenger RNA, which then becomes attached to the surface of the ribosomal particle. Due to the length of messenger RNA molecules it is probable that aggregates of several ribosomes are necessary for each messenger RNA (148).

The SRNA moiety of the SRNA amino acid complex can "recognize" the code which is contained in the messenger RNA template on the ribosomal surface and this ensures that amino acids are lined up in the correct order upon this template. The ribosomal electron transport system (136) then supplies the requisite energy for peptide bond formation and the completed protein is released into the cell cytoplasm.

Metabolism of characteristic Amino Acids

Since Stetten (139) showed that the hydroxyproline of collagen is almost exclusively derived from proline, a controversy has existed regarding the location of the hydroxylation step in the collagen biosynthetic pathway. It was earlier suggested (52) (123) that a proline rich collagen-like precursor existed which was subsequently hydroxylated, but attempts to demonstrate this have not been successful (124).

An alternative is that proline is hydroxylated after it has been activated in some way but before it has been incorporated into a protein molecule (124). On the assumption that proline is incorporated into protein by the currently accepted mechanism of protein synthesis, it has been suggested that this step could occur at the SRNA-proline complex stage, or alternatively following the

activation step to prolyladenylate (22). Meister et al (85) point out however that the former would lead to quite random distribution of prolyl-SRNA and Hydroxyprolyl-SRNA upon the ribosomal template which is thought to "recognize" only the SRNA moiety, which would be identical for proline and hydroxyproline if hydroxylation occurred following complex formation.

They propose therefore, that a portion of the proline pool is activated and then proceeds normally to unite with its specific SRNA, whilst a further portion, destined to become collagen hydroxyproline is activated, hydroxylated and complexed with its own specific SRNA. Meister et al do not make any suggestion as to the nature of this "activation" step but it may be at the pH5 enzyme-prolyl adenylate.stage. It is possible that the absence of a specific pH5 enzyme which activates hydroxyproline accounts for its inability to enter collagen directly.

Evidence which suggests that hydroxylation takes place prior to incorporation into peptide linkage has been presented by Gerber et al (34) who found that the specific activity of urinary free hydroxyproline was greater than that of skin collagen hydroxyproline or of urinary peptide bound hydroxyproline in rats which had received labeled proline. Doughaday and Mariz (22) have presented similar results from an in vitro study of rat cartilage.

The finding of Linstedt and Prockop (78) that urinary free hydroxyproline and urinary bound hydroxyproline have the same specific activities need not detract from the theory of "hydroxylation after activation", it does, however, suggest that the only path which "hydroxylated-activated-proline" can follow is into a collagen molecule and thence into the urine by subsequent protein breakdown.

As is the case with hydroxyproline, little is known about the hydroxylation of lysine. It has been shown, however, that the ratio of specific activities of collagen lysine to collagen hydroxylysine is always 1:1 (108), after introduction of labeled lysine.

The Extracellular Phase - Fibrogenesis

The mechanism of collagen fiber growth outside the cell has been largely elucidated through the study of the so called soluble collagens, which have been referred to in an earlier section. Nageotte (93) was the first to ascribe a biological precursor role to the collagen which he was able to solubilise from rat tail tendon. When this solution, in dilute acetic acid, was left standing in contact with the original fibers, or was treated with salt, a precipitate occurred which appeared similar to native collagen fibrils. Nageotte postulated that this precipitation from solution

may be the method by which collagen fibers were formed in vivo, and he coined the term "précollagène" for the collagen fraction which was soluble. Much later, Orekhovitch and co-workers (96), were able to obtain solutions of collagen by extracting various tissues with citrate buffers at acid pH. The name "procollagen" was given to this material since it had a faster uptake of C^{14} glycine than the insoluble skin residue (98).

Harkness et al (56) reported a faster uptake of labeled glycine into alkaline pH, phosphate buffer soluble collagen than into procollagen or insoluble collagen. In 1957, Jackson (64), in a study of the uptake of C^{14} glycine into collagen formed in a carageenan granuloma, compared the metabolic activities of insoluble collagen, procollagen and neutral salt soluble collagen, which latter had previously been described by Jackson and Fessler (65). He found the collagen extracted with neutral salt solution to be metabolically more active than either of the other two fractions and hence deemed it more likely to be the true precursor than procollagen.

Once extracted and purified, however, all these fractions are interconvertible (40). They all have the same physical and chemical properties and are identical with tropocollagen, the basic collagen molecular species. As previously mentioned, however, different degrees of

intramolecular cross linking may be present.

The differences of metabolic activity previously described are thus probably reflections of biological differences rather than chemical differences, such as would be expected of a precursor-product relationship.

Gross showed (43), that when a neutral solution of tropocollagen is warmed to 37°C a rigid gel is formed containing fibrils identical to native collagen and that upon cooling, the gel reverts to a clear solution. The gel becomes increasingly insoluble upon cooling, as the length of time of incubation at 37°C is extended, and this led Gross (44) to postulate that the process of this artificial fibril formation is one of aggregation of tropocollagen molecules into a polymer, the strength of whose intermolecular linkages increased with time.

The complementary in vivo experiment of Jackson and Bentley (68) served to confirm this theory. They used increasing concentrations of sodium chloride solutions sequentially, to extract collagen from the tissues of guinea pigs which had been injected with C^{14} glycine. A short time after injection, the most highly radioactive material was extracted with the lowest concentration of salt. This peak of radioactivity was shifted into collagen extracted with progressively higher ionic strength as the time after isotope injection was

increased.

It was thus proposed that newly formed collagen molecules become bound to the surface of a preformed fibril, perhaps by hydrogen bonding, and that the strength of these bonds increases with time. Progressively higher ionic strength solutions are therefore required to break these bonds as the individual molecule and the fibril as a whole, age.

Indications of such a mechanism had been previously noted by Jackson and Williams (66) who were able to alter the reticulin staining properties of tissue by a prior extraction with sodium chloride solution.

It can thus be seen that previously used terms such as "Neutral Salt Soluble Collagen", "Acid Soluble Collagen" etc., are but operational definitions and are not related to absolute precursor compounds. A further refinement has been provided by Wood (156) who has produced evidence for a nucleation or seeding phenomenon connected with collagen fibril growth from solution. Wood suggests that the collagen fraction which initiates fibril formation may be material containing a high proportion of the β fraction of Orekhovitch, which has been described in another section.

ASCORBIC ACID DEFICIENCY AND COLLAGEN FORMATION

Many of the symptoms of scurvy as described in the eighteenth century, (see Lind (77)), were concerned with the abnormalities of connective tissues such as the nonhealing of wounds and fractures. It was not until 1926, however, that experimental evidence of a mechanism was put forward by Wolbach and Howe (155), who were able to demonstrate histologically, a failure of collagen fiber formation in wounds of scorbutic guinea pigs. Subsequent work has confirmed this defect in collagen production in such models as Healing wounds (25) (2), carageenan granulomas (123), tendons healing after cutting (72), and in sponge implants (53).

The earliest effect of scurvy which can be seen, is a drastic lowering of the content of extractable collagen in the tissues of guinea pigs deprived of ascorbic acid (41) and, as has been described in another section, this is taken to mean a slowing of synthesis. This can be reversed in a matter of twenty-four hours after administration of ascorbic acid, either locally (49), or systemically (52).

The mechanism of the interaction between ascorbic acid and collagen biosynthesis is quite unclear and it is hoped that some of the data to be presented in this thesis will cast light upon the problem, by differentiating between some of the previously proposed mechanisms.

One mechanism suggested in 1953 by Robertson and Schwartz (123) is that a collagen like protein is formed in scurvy, in which the place of hydroxyproline in a peptide chain is taken by proline, these residues only becoming hydroxylated upon administration of ascorbic acid. The existence of such a proline rich protein was supported by Gould and Woessner (52) for some years, but upon closer investigation by Gross (42) and by Robertson et al (124) the hypothesis could not be substantiated.

Alternative sites for the effect have been enumerated by Mitoma and Smith (86) to be as follows:

Since as has been previously described, collagen hydroxyproline is derived from proline, then a defect in the hydroxylation of proline prior to the formation of a peptide chain could be responsible for the lack of collagen synthesis. Secondly, a block in the incorporation of "active" hydroxyproline into the complete collagen molecule or thirdly, the failure to form fibers from a soluble precursor collagen may be involved. A fourth possibility put forward by Gross (45) and supported by Gould (50) is that previously formed collagen, in particular newly synthesised material, is much more labile in scurvy than normal.

Mitoma and Smith (86) were unable to find any effect of scurvy upon the hydroxylation of proline in guinea pigs and believe that the second possibility is the most

likely, Other workers, however, notably Meister et al (85) and van B. Robertson (122) feel that the most probable site of interaction is at the hydroxylation step. Neither of these workers offered conclusive proof of this, but they were able to demonstrate far greater incorporation of labeled hydroxyproline into a peptide bound form, after addition of ascorbic acid to an incubation mixture of scorbutic guinea pig tissue and labeled proline.

An experiment designed to distinguish between some of these possibilities was thus carried out and is described in the following section.

EXPERIMENTAL

The material to be presented here is divided into eight sections. Each describes a separate experiment together with a discussion of the results of that experiment.

The conclusions which can be made on the basis of all eight experiments are discussed more fully in a general discussion which follows the Experimental section..

EXPERIMENT I

Introduction

The investigations into the effect of scurvy which were carried out by other workers prior to the present experiment (1961) had been mainly concerned with its effect upon total collagen synthesis (2) (25) (52) (72) (123), and other than the work of Gross (41) little information was available about the extractable collagen content of scorbutic animals. This is of particular importance since it is now accepted that newly formed tropocollagen is "extractable" in contrast to older "non extractable" material. Similarly, nothing was known about the incorporation of labeled amino acids into this fraction during scurvy, and it was felt that investigations of this point would help to differentiate between some of the proposals which had been made regarding the site of interaction between scurvy and collagen biosynthesis.

The following experiment in which the incorporation of C^{14} glycine into collagen extractable with two different salt concentrations was carried out to determine whether, a) collagen was initially synthesised as rapidly in scorbutic as in normal guinea pigs, and b) whether once synthesised, this material became aggregated normally.

Methods

Sixty-eight young albino guinea pigs of either sex were weighed thrice weekly until a uniform rate of growth had been attained. The animals were fed McDonald scorbutigenic diet #5 (14) (See appendix 1) supplemented with 50 mg ascorbic acid orally every other day during this period. Forty-four of the animals were deprived of the supplement for four days and then all animals were injected subcutaneously in the abdomen with 5 mls of a 1% solution of carageenan in physiological saline[1].

The granulomas thus produced were allowed to develop for seven days at which time all animals were injected intraperitoneally with 50 μC of 2-C^{14} glycine in 1 ml of physiological saline. Animals were killed in groups of three or five at the following time periods after the isotope: 30 minutes, 1, 1.5, 3, 6, 8, 12 and 18 hours. The granulomas were excised and frozen immediately, care was taken that skin or underlying muscle did not contaminate the sample. Adrenal glands were also removed and pooled for assay of ascorbic acid (121). The normal animals showed a value of 75 micrograms ascorbic acid per gram of tissue, whilst the scorbutics contained 6.5 micrograms per gram.

[1] The carageenan was a gift of Marine Colloids, Inc., Rockland, Me.

Granulomas from each group were pooled, frozen in liquid nitrogen and crushed in a stainless steel mortar (147). After weighing, the tissue was extracted three times with a 0.14 M NaCl solution buffered to pH 7.6 with 0.02 M phosphate buffer, and the three extracts were pooled. Three further extractions were made with a 1.0 M NaCl solution buffered as above, and these extracts were pooled. Ethanol was added to the extracts until a fibrous precipitate appeared, a concentration of 14% being required for the 0.14 M NaCl extract and 28% for the 1.0 M NaCl extract.

The precipitates were removed by centrifugation and dissolved in 0.01 M acetic acid followed by dialysis against a large volume of acetic acid of the same concentration.

All previous manipulations were carried out at 4°C to prevent collagen denaturation.

The partially purified solution was heated to 60°C for two hours, a procedure which converts collagen to parent gelatin. These gelatin solutions were next dialysed against large quantities of sodium maleate/maleic acid buffer pH 7.0 and incubated at 37°C with collagenase [1], 1 unit per 10 mls. solution. At the end of twenty-four hours the color developed with ninhydrin had reached a maximum and undigested protein was precipitated by adding perchloric acid to a concentration of 6%. Since collagenase has a very high specificity for collagen, or rather for the X-gly-pro-y

[1] Highly purified C.welchii collagenase was the gift of Dr. P. M. Gallop.

sequence which is virtually unique to collagen (134) (143), the small peptide fractions remaining in solution were considered to be entirely derived from collagen. Excess perchloric acid was precipitated by neutralisation with potassium hydroxide and aliquots of this solution were then removed for analysis of total nitrogen by the Kjeldahl method. Further aliquots were removed, evaporated to dryness, dissolved in hydroxide of hyamine (115) and assayed for radioactivity by liquid scintillation spectrometry (see appendix 2).

Results and Discussion

The results are presented in figs. 1a, 1b, 1c, 1d, and 1e, and in Table I.

By a comparison of figs. 1a and 1b, it can be seen that the specific activity of the collagen extracted from scorbutic granulomas with both salt concentrations, is considerably lower than similar material obtained from normal animals. This at first glance, may be interpreted as a lowered rate of synthesis which would of course present such a picture.

If, however, the aggregation of newly formed collagen is interfered with by scurvy, then material formed prior to administration of the label would still be extractable with 0.14 M NaCl in the scorbutic animal, whereas

Fig. 1a Specific activities of collagen extractable from carageenan granulomas of normal guinea pigs.

Solid line = 0.14 M NaCl extractable collagen.

Broken line = 1.0 M NaCl extractable collagen.

Fig. 1b Specific activities of collagen extractable from carageenan granulomas of scorbutic guinea pigs.

Solid line = 0.14 M NaCl extractable collagen.

Broken line = 1.0 M NaCl extractable collagen.

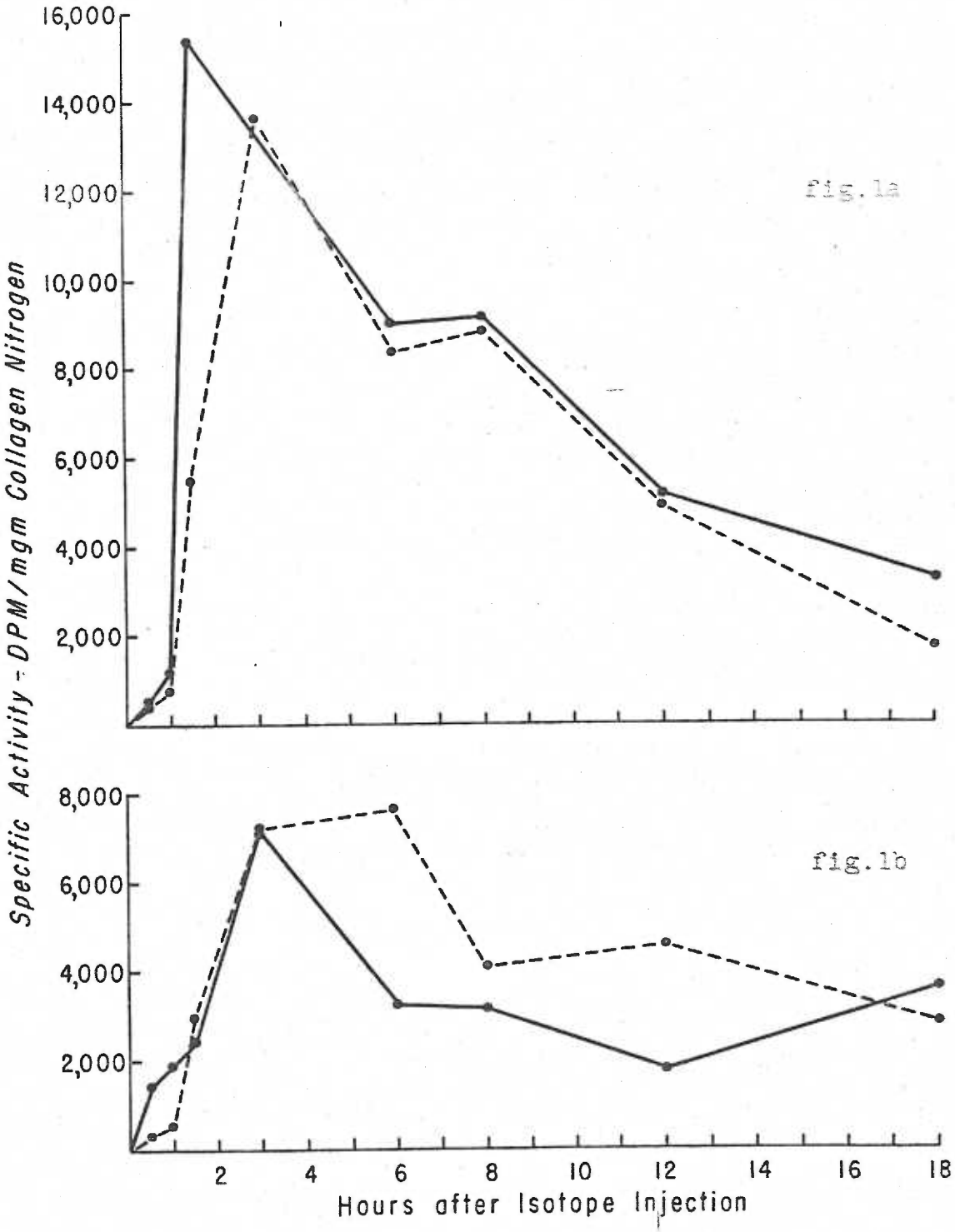

16,000
14,000
12,000
10,000
8,000
6,000
4,000
2,000
Specific Activity - DPM/mgm Collagen Nitrogen
8,000
6,000
4,000
2,000
2
4
6
8
10
12
14
16
18
Hours after Isotope Injection

fig. 1a

fig. 1b

Fig. 1c — Total radioactivities of extractable collagen purified from 1 gm. of carageenan granulomas from normal guinea pigs.

Solid line = 0.14 M extractable collagen
Broken line = 1.0 M extractable collagen

Fig. 1d — Total radioactivity of extractable collagen purified from 1 gm. of carageenan granulomas from scorbutic guinea pigs.

Solid line = 0.14 M extractable collagen
Broken line = 1.0 M extractable collagen

Fig. 1e — Percentage of the total extractable collagen radioactivity found in the 1.0 M NaCl extractable fraction.

i.e. $\frac{(\text{DPM/Gm. Tissue in 1M extract}) \times 100}{(\text{DPM/Gm. Tissue in 1M} + \text{0.14 M extracts})}$

Solid line = Normal animals
Broken line = Scorbutic animals

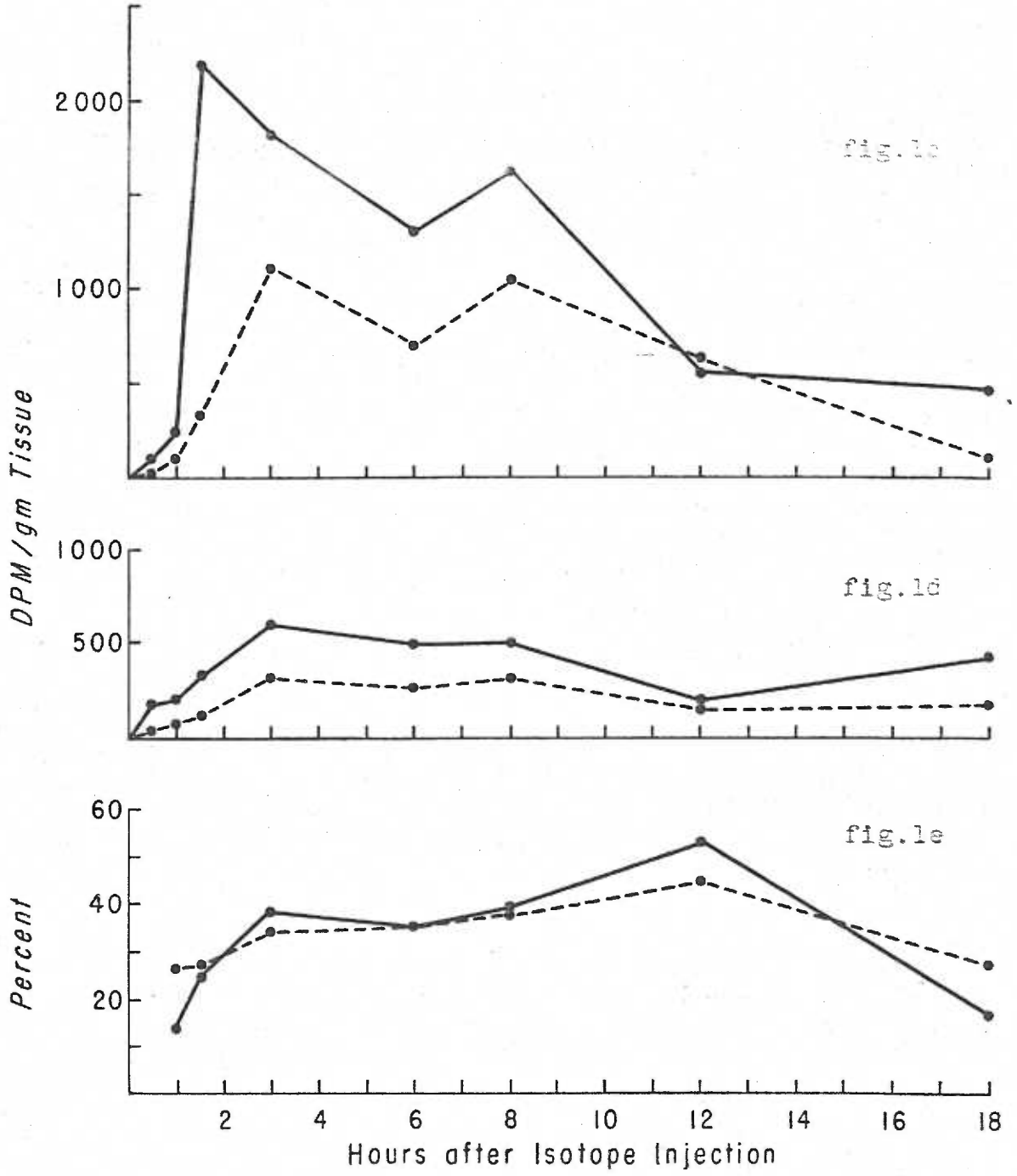

2000
1000
DPM/gm Tissue
fig.1a
1000
500
fig.1d
60
40
20
Percent
fig.1e
2
4
6
8
10
12
14
16
18
Hours after Isotope Injection

TABLE I

Time After Isotope (Hours)	Total Extractable Collagen Purified (Mgm.N/Gm. Granuloma)				Specific Activity (DPM/Mgm.N)				Total Activity (Collagen DPM/Gm. Granuloma)			
	0.14M		1.0M		0.14M		1.0M		0.14M		1.0M	
	N	S	N	S	N	S	N	S	N	S	N	S
½	.163	.117	.081	.105	508	1492	385	374	83	174	30	39
1	.228	.106	.062	.055	1072	1946	609	1339	244	206	38	74
1½	.140	.147	.130	.045	15644	2564	5421	3097	2190	377	705	139
3	.137	.082	.081	.044	13174	7271	13724	7198	1805	596	1112	317
6	.145	.153	.086	.036	8968	3290	8307	7584	1300	503	714	273
8	.176	.154	.119	.078	9193	3219	8812	4115	1618	496	1048	321
12	.110	.108	.126	.035	5052	1854	4949	4676	556	200	624	164
18	.134	.123	.057	.060	3363	3600	1647	2926	451	442	94	176
MEANS	.154	.124	.093	.057								

KEY

0.14 M = Collagen extractable with 0.14 M NaCl at pH 7.6.
1.0 M = Collagen extractable with 1.0 M NaCl at pH 7.6
N = Normal guinea pigs
S = Scorbutic guinea pigs

collagen formed at an equivalent time in the normals would be cross linked to an extent which made it unextractable with 0.14 M NaCl. This would effectively dilute the radioactivity appearing in this fraction from the scorbutic animals. The same of course holds true for the 1 M NaCl extractable material. If, on the other hand, insoluble, preformed fibers are broken down by disaggregation as suggested by Jackson (64) and this breakdown is enhanced in scurvy (50) then a similar dilution effect would be noted.

As an attempt to overcome these dilution effects, total extractable collagen radioactivity per gram of tissue is plotted in Figs. 1c and 1d, and it can be seen by comparison of these figures that the total amount of labeled amino acid incorporated into extractable collagen by one gram of scorbutic granuloma is considerably less than normal.

Aggregation

It was shown by Jackson and Bentley (68) that the degree of cross linking between collagen molecules in a tissue is reflected by the ionic strength of the medium required to extract those molecules into solution. If aggregation were not taking place in scurvy then it would be expected that the ratio of collagen extractable with a high ionic strength medium to that extractable with a lower ionic

strength; would be lower than normal. The ratio of incorporated radioactivities should also be lower than normal. Fig. 1e, shows such a comparison and it suggests that once a molecule has been synthesised in a scorbutic granuloma, it becomes cross linked into larger aggregates at the same rate as in the normal animal, at least to the extent which is reflected by 1.0M NaCl extractable material.

In the above experiment, material which had been precipitated by ethanol and digested with collagenase was considered to represent total extractable collagen and this may not be valid. Although differences between "scorbutic collagen" and "normal collagen" as far as their precipitability with ethanol etc. are concerned, have not been previously described, the possibility of their existence cannot be neglected. This is particularly important since the differences between the total purified collagen levels per gram of tissue, in the two groups are not significant, even at the 10% level of probability (Table I), whereas in other tissues studied the level of extractable collagen has been found to be much reduced (41).

It is possible that this anomalous behaviour can be ascribed to the use of the carageenan granuloma as a model. The collagen formed in this situation has been described as inherently unstable, (64), and it was thus felt that less ambiguous results would be obtained if another

tissue such as skin were used for future experiments.

In summary, the experiment shows that even though the total amount of collagen being synthesised in a scorbutic granuloma is lower than normal, the rate of its aggregation does not appear to be impaired.

EXPERIMENT II

Introduction

Experiment I offered no evidence about the occurrence of collagen breakdown, and also, even though the results indicated no alteration of the aggregation mechanism in its early stages, subsequent failure to aggregate can not be ruled out.

The present experiment, by making use of amino acids labeled with different radioactive atoms, was designed to determine whether increased breakdown occurred and at the same time investigate the early stages of synthesis in a tissue which is physiologically more normal than the carageenan granuloma, i.e. skin.

Collagen was prelabeled by giving an injection of C^{14} glycine, forty-eight hours prior to an injection of tritiated glycine. It was felt that any aggregation failure or any breakdown would be reflected in increased C^{14} activity in the extractable collagen and that the tritium activity would indicate any alteration of biosynthesis.

Methods

Forty-two young guinea pigs were placed on a vitamin C free diet supplemented as in Experiment I (see appendix 1) and frequently weighed until a uniform

growth rate was achieved. Half of the animals were deprived of the supplement for eleven days whereupon all animals received 40 μC of 2-C^{14} glycine. The glycine was injected intraperitoneally in three aliquots at eight hour intervals. Forty-eight hours following the last C^{14} glycine injection, all animals were injected intraperitoneally with 50 μC 2-H^3 glycine and decapitated at the following time intervals after injection, 0.5, 1.5, 2, 3, 4, 6 and 12 hours. Adrenals were removed as before for assay of ascorbic acid and the experimental group were found to be truly depleted. (Normal 70.1 micrograms per gram tissue. Scorbutic 5.3 micrograms per gram tissue.)

Dorsal skin was removed, crushed after freezing in liquid nitrogen, weighed and extracted three times with 0.14 M NaCl buffered as before at pH 7.6. The tissue was then extracted three times with 1.0 M NaCl as before. All extractions and purifications were carried out at 4°C.

The total collagen extracted with each medium was determined by assaying hydroxyproline on an aliquot following hydrolysis with 6 n HCl at 140°C for three hours (94). The hydroxyproline values were multiplied by a factor of 7 (67) to convert to collagen. Collagen, in the remainder of the extract, was precipitated with ethanol as in Experiment I and redissolved by dialysis against 0.01 M acetic acid.

Collagen was purified by precipitating with sodium chloride and redissolving in 0.01 M acetic acid.

This procedure was repeated twice, aliquots of the final acetic acid solution being used for determination of hydroxyproline, and radioactivity. Radioactivity was determined on a Tri-Carb liquid scintillation spectrometer, and by the use of appropriate pulse height analysis techniques it was possible to distinguish between C^{14} and Tritium activities (see appendix 2).

Results and Discussion

The results are presented in figs.2a, 2b, 2c, Table I and in Table 2.

Incorporation of C^{14} Glycine

The C^{14} specific activities in both extractable collagen fractions of the scorbutic animals (fig.2b) are quite constant and are at the same low level as those of the normal (fig.2a). This fact suggests that increased breakdown of preformed collagen via the "extractable collagen pool" is not a factor in scurvy. If it were, one would expect the C^{14} activities in the scorbutics to be much higher due to the disaggregation of high specific activity material formed at a time when the C^{14} glycine pool was high. This disaggregation would make prelabeled collagen available for extraction along with newly synthesised material and thus add C^{14} activity to the

extractable fractions.

If such disaggregation into macromolecules does occur, even though the C^{14} activity is low, then it must be assumed that they are immediately split into dialysable fragments so that no increase in the extractable collagen pool is discernible. The only proteolytic enzyme which is known, with any certainty, to be capable of this digestion is collagenase, but this enzyme has only been isolated from species of Clostridia (80). Reports have however appeared of the presence of an uncharacterized enzyme with weak collagenolytic activity in uterus (154) pancreas (62) and metamorphosing tadpole tails (48).

The similarity of the C^{14} activity also adds weight to the suggestion that aggregation is not impaired, since if it were, a "piling up" of C^{14} labeled extractable collagen would be expected to occur. The above reservations on the immediate hydrolysis by collagenase must also be made in this situation.

The low level of activity seen in both groups of animals thus probably reflects synthesis still occurring in the presence of a much reduced C^{14} glycine pool (57), and is in accord with that level of activity demonstrated after 48-60 hours by Jackson (64).

Fig. 2a Specific activities of extractable collagen from the skin of normal guinea pigs. H^3 glycine was injected at time 0 and C^{14} glycine 48 hours previous to time 0.

Solid line = 0.14 M NaCl extractable collagen.
Broken line = 1.0 M NaCl extractable collagen.

Fig. 2b Specific activities of extractable collagen from the skin of scorbutic guinea pigs. H^3 glycine was injected at time 0 and C^{14} glycine 48 hours previous to time 0.

Solid line = 0.14 M NaCl extractable collagen
Broken line = 1.0 M NaCl extractable collagen

N.B. Ordinates in both figures should be multiplied by a factor of 10.

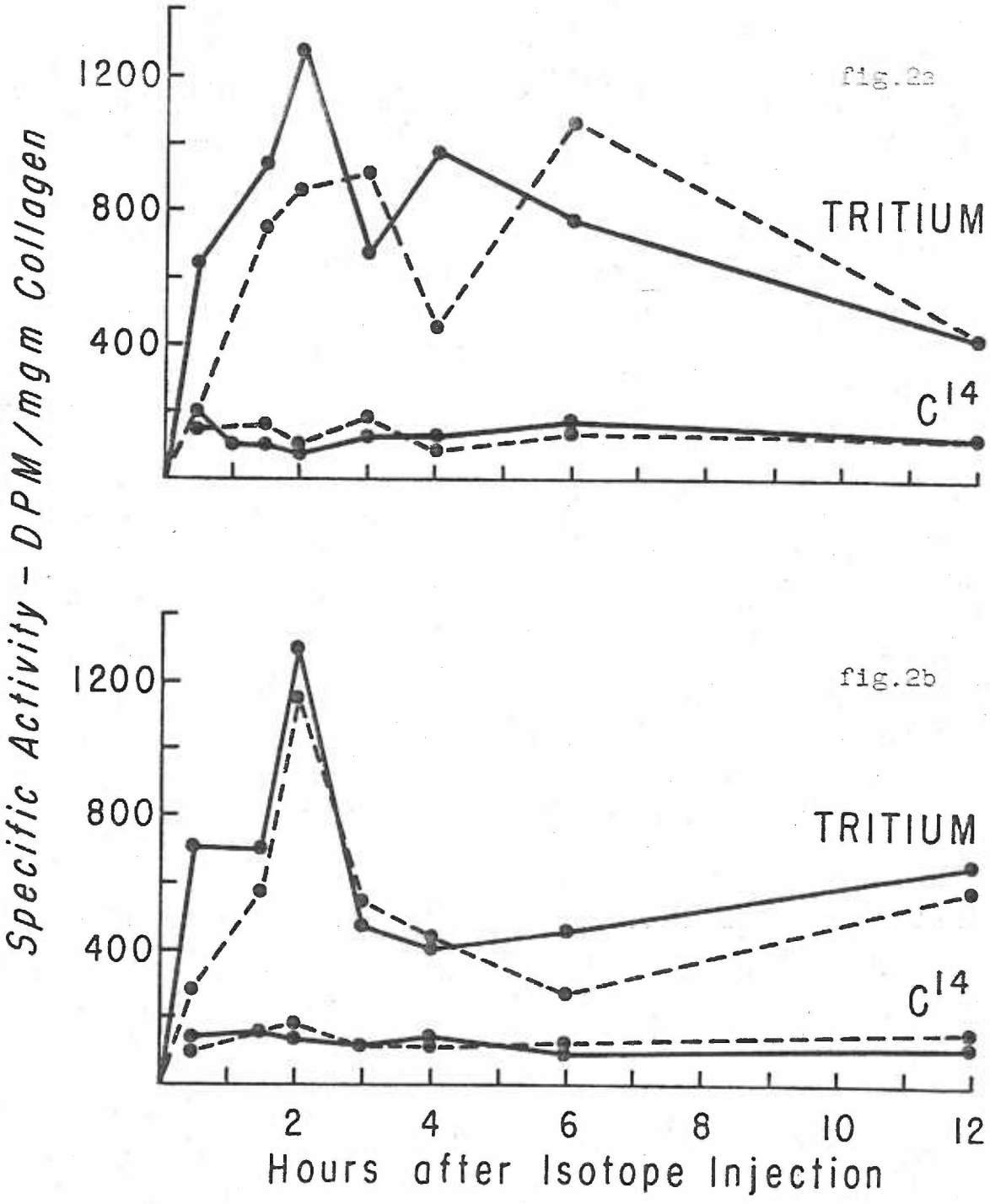


fig.2a
1200
800
400
TRITIUM
C14
fig.2b
1200
800
400
TRITIUM
C14
2
4
6
8
10
12
Hours after Isotope Injection
Specific Activity - DPM/mgm Collagen

TABLE 2

Time After Isotope (Hours)	Total Extractable Collagen (mgms. per gm. skin)				Tritium Specific Activity (DPM/mgms. Collagen)				Total Collagen Tritium (DPM/Gm. skin)			
	0.14M		1.0M		0.14M		1.0M		0.14M		1.0M	
	N	S	N	S	N	S	N	S	N	S	N	S
½	1.13	.21	2.4	.6	6437	7116	1934	2987	7274	1494	4642	1792
1½	.84	.45	1.6	1.1	9380	6994	7529	5739	7900	3147	12046	6313
2.	.64	.41	1.5	.6	12822	12899	8673	11538	8206	5289	13009	6923
3	1.12	.54	3.2	1.8	6725	4745	9113	5213	7532	2562	29161	6383
4	.68	.33	2.1	.8	9707	4021	4537	4103	6600	1327	9528	3282
6	.78	.27	1.6	.6	7743	4513	10544	2750	6039	1218	16870	1650
12	1.39	.23	4.3	.8	4139	6444	4144	5726	5753	1482	17819	4581

KEY

0.14 M = Collagen extractable with 0.14 M NaCl at pH 7.6
1.0 M = Collagen extractable with 1.0 M NaCl at pH 7.6
N = Normal guinea pigs
S = Scorbutic guinea pigs

Tritiated Glycine Uptake

The difference between the normal and experimental animals as seen in the tritium specific activities (Figs. 2a and 2b) is most marked. The initial peak at two hours in the normal is closely paralleled in the scorbutic animals. Two hours later, however, a second peak of activity is seen in the normal, which is completely absent in the scorbutic animals.

The fact that collagen extracted with 0.14 M NaCl at the two hour time point has identical specific activities in both cases does not mean however that collagen synthesis is unimpaired. Table 2 shows that the total amount of extractable collagen per gram of skin is very much reduced in scurvy, as of course is the total amount of radioactivity incorporated into collagen.

From this, we infer that the skin is not synthesising as much collagen per unit time, but that the collagen which is synthesised is normal as far as its content of labeled glycine is concerned. This can be explained in two ways; either scorbutic skin contains a smaller number of fibroblasts synthesising collagen normally, or conversely, it contains a normal complement of fibroblasts whose synthetic pathway is inhibited or slowed down in some way.

In the light of some recent work by Peach et al (101)

it seems possible that both of these possibilities may occur. Peach presents electron micrographs of healing tendon from scorbutic guinea pigs, which show that there are far fewer fibroblasts in this tissue than in similar material from normal animals. The endoplasmic reticulum of these cells is altered in form and much reduced in amount. Ross and Benditt (125) also showed changes in the morphology of Endoplasmic reticulum of wound tissue from scorbutic guinea pigs

Caution must be used however in making interpretations of these results, since the guinea pigs used in both of the above experiments were losing weight and were probably suffering from general malnutrition rather than simple vitamin C deficiency.

The occurrence of the second peak in the normal animals indicates that the early phase of collagen synthesis is far more complicated than was hitherto suspected. Any interpretations of the scorbutic curves, which lack this peak, must therefore remain purely speculative until such time as the normal mechanism is better understood.

Multiple Peak Phenomenon

Previous studies (64) (68) (112) of the uptake of labeled amino acids into extractable collagen have shown a

rise to a single peak as the protein became labeled, followed by a fall which was interpreted as being due to the transfer of collagen into a non extractable fraction (68) and also to the decreasing radioactivity of the free amino acid pool (57). These studies however did not include such frequent time points as those used in the present study, or else were concerned exclusively with later time points such as eight to twelve hours. The time points used in Experiment I in this thesis, were also such that if a peak had occurred between three and six hours after isotope injection, it would have been missed. (An interesting shoulder does occur, however, in Fig. 1a at 6 to 8 hours.) It is thus apparent that in order to investigate this phenomenon further, very frequent time points must be used.

Many possible explanations exist which could account for the appearance of a phasic uptake curve and an enumeration of these possibilities is given below.

1. The result may be an artefact.

2. Tritium on the number two carbon atom of glycine may be exchangeable with hydrogen to such an extent that relabelling of cold glycine becomes possible.

This is not a likely explanation as was shown by Rittenberg et al (120) in experiments with deuterium. Upon boiling amino acids deuterated in positions other than the

amino and carboxyl groups, with 20% HCl solutions, no loss of deuterium was noted even after four days of this harsh treatment.

A small amount of exchange (8.5%) was, however, observed when glycine was used, but this would no doubt be lower under less extreme conditions.

Since the carbon-tritium bond is somewhat stronger than the carbon-deuterium and the carbon-hydrogen bond (70), any possible exchange would be expected to be less when this heavier isotope is used as in Experiment II, than in the Rittenberg deuterium work.

3. The first peak may be caused by incorporation of glycine into a collagen precursor molecule, perhaps a peptide of relatively low molecular weight.

This is unlikely since the radioactivity measurements were made upon relatively highly purified collagen prepared by a method which has been previously shown to isolate material which is homogenous in the ultracentrifuge, and whose concentration of hydroxyproline and tyrosine compares with material purified by other methods (65).

4. The existence of a very small amount of a glycine containing impurity with an extremely high specific activity compared to collagen could provide an explanation of one of the peaks seen. It would be necessary to suppose

that such an impurity, if it exists, has a different rate of turnover than collagen.

5. Newly formed collagen may be extractable with 0.14 M NaCl solution for a short time, i.e. up to three hours after synthesis, at which time it is rendered temporarily unextractable by some mechanism and the specific activity of the extracted collagen falls. Later this collagen may become available for extraction once more and the second rise occurs.

A mechanism which renders collagen temporarily unextractable is of course purely speculative. The site at which this may occur is also completely unknown. It is nevertheless conceivable that at some time during protein synthesis, collagen molecules become detached from the ribosomal templates and attached to another site within the fibroblast, perhaps the endoplasmic reticulum, perhaps the cell membrane, and collagen in the process of transfer from one site to another, may have different solubility properties than collagen which is attached to a subcellular particle. It is of interest in this connection that Porter and Pappas (110) remarked that during the preparation of tissue for electron microscopy, fibrils seen at the cell surface could not be washed off.

6. Collagen may be synthesised in pulses. This of course depends upon many factors such as penetration of

the cell by free amino acids, "activation" of these, formation of a peptide chain, and release of this from the cell. Any or all of these processes may be visualised as taking place in a discontinuous manner which then accounts for the apparent discontinuity of collagen synthesis.

A telling argument against this hypothesis is that it would require the synthetic activities of all, or at least a very large majority of the fibroblasts in a tissue, to be "in phase". For a tissue which has been in existence for many months, i.e. skin, this possibility seems quite remote.

7. It could be argued that some of the collagen which is represented by the first peak is labile and breaks down to its constituent amino acids. This would make the labeled amino acid available for reincorporation into another protein molecule, but if this amino acid were to mix freely with the total body pool of labeled amino acid the supplementation of the specific activity of this pool would be negligible.

If however the products of breakdown were not freely miscible with the total pool, but were to remain in some intracellular pool, then the supplementation of the label in this pool may be appreciable.

Evidence for this sort of phenomenon is not directly available in mammalian cells but is for some micro

organisms (18) (20).

Two operationally distinct pools of amino acids are recognized, one is termed the expandable pool and is concerned with concentrating amino acids from the medium, whilst the so called internal pool is concerned with supplying amino acids for protein synthesis and other metabolic pathways. Once radioactive amino acids are incorporated in this internal pool, their specific activities and those of the proteins into which they are incorporated cannot be influenced by the addition of unlabeled amino acids to the medium. The authors interpret this to mean that once an amino acid is incorporated into the internal pool then it is not in competition with extracellular amino acids. This concept will be dealt with more fully in the general discussion section.

EXPERIMENT III

Introduction

In order to confirm that the double peak phenomenon was not an artefact, Experiment II was repeated, using supplemented guinea pigs only.

Method

Thirty-three albino guinea pigs were fed a standard diet of triangle rabbit pellets (see appendix 1) supplemented orally with ascorbic acid as before and weighed until a uniform growth rate was achieved. All animals were injected intraperitoneally with 60 μC of 2-H3 glycine in 1 ml of physiological saline, and decapitated at the following time intervals after injection; 1, 1.5, 2, 2.5, 3, 3.5, 4, 5, 6, 8, 10 hours.

Collagen was extracted and purified as described in Experiment II.

Total extractable collagen and the specific activity of the purified collagen was also determined as described in Experiment II.

Results and Discussion

The specific activities of the 0.14 M NaCl extractable collagen are presented in Fig. 3. All other data is

presented in Table 3.

The occurrence of a third peak in the specific activity curves (fig. 3) appears to complicate their interpretation even further and does not appear to be in good agreement with the results of Experiment II. However, no measurements were made in Experiment II at intervals between six and twelve hours after administration of isotope and any peak occurring in this position, i.e. ten hours as in the present experiment, would be missed.

The fact that the Experiment is repeatable and that multiple peaks occur as before, suggests that the results of Experiment II were not "accidental" and that further investigations into the problem are in order.

Fig. 3 Specific activity of collagen extracted with 0.14 M NaCl from skin of normal guinea pigs following injection of H^3 glycine.

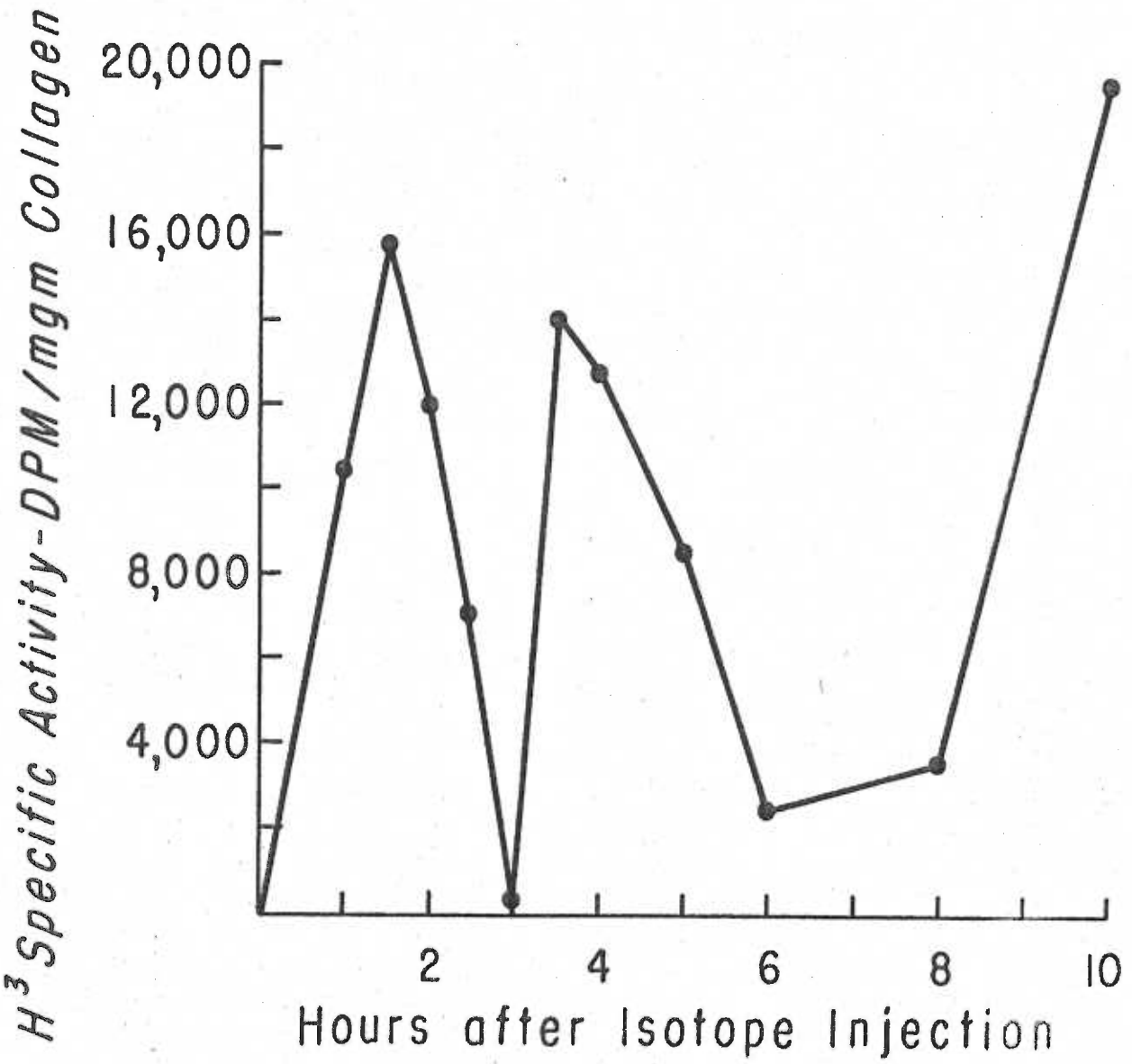

H³ Specific Activity-DPM/mgm Collagen
20,000
16,000
12,000
8,000
4,000
2
4
6
8
10
Hours after Isotope Injection

TABLE 3

Time After H^3 glycine	0.14 M NaCl Extractable Collagen			1.0 M NaCl Extractable Collagen		
	Mgms. per gm. skin	Specific Activity DPM per mgm Collagen	Total Collagen DPM per gm. skin	Mgm. per gm. skin	Specific Activity DPM per mgm Collagen	Total Collagen DPM per gm. skin
1	.593	10402	6168	--	631	--
1.5	.398	15753	6270	2.130	435	927
2	.394	12020	4735	1.914	471	901
2.5	.455	7041	3204	1.744	977	1704
3	.465	330	153	1.624	1013	1645
3.5	.463	14063	6511	1.914	272	521
4	.509	12770	6500	2.398	297	712
5	.396	8623	3415	2.939	0	0
6	.428	2514	1076	1.341	0	0
8	.417	3623	1511	2.470	0	0
10	.291	20067	5839	2.361	1459	3445

EXPERIMENT IV

Introduction

The question of the lability of the tritium on the number 2 carbon atom of glycine still remains to be answered, and in addition, the existence of the third peak at ten hours after isotope, also requires confirmation.

2-C^{14} glycine was therefore used in Experiment IV and further time points were included.

Methods

The experiment was carried out in an identical manner to Experiment III, with the exception that the animals were injected with 35 μC of 2-C^{14} glycine instead of with H^3 glycine, and were decapitated at the following times after this injection; 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 10, 18 hours.

Results and Discussion

The results are presented in Fig. 4 and Table 4. From these results, it can be concluded that the multiple peak is not brought about by any tritium lability which may exist, since the phenomenon is repeatable when C^{14} glycine is used.

The existence of a third peak (Fig.4) at about ten hours after isotope is also confirmed, although it does not attain the height relative to the other peaks which is seen in Fig.3.

This of course may be due to the fact that samples were taken relatively infrequently at this time, that is, in Experiment IV the actual peak may be on either side of ten hours and may in fact rise to a much higher level than seen. The accurate placing of these peaks would necessitate sampling at such frequent time points as to be economically prohibitive, it does not seem necessary, however, to accurately pin point the times, but rather to find a rational explanation for the occurrence of multiple peaks.

Fig. 4 Specific activity of collagen extractable with 0.14 M NaCl from skin of normal guinea pigs following injection of C^{14} glycine.

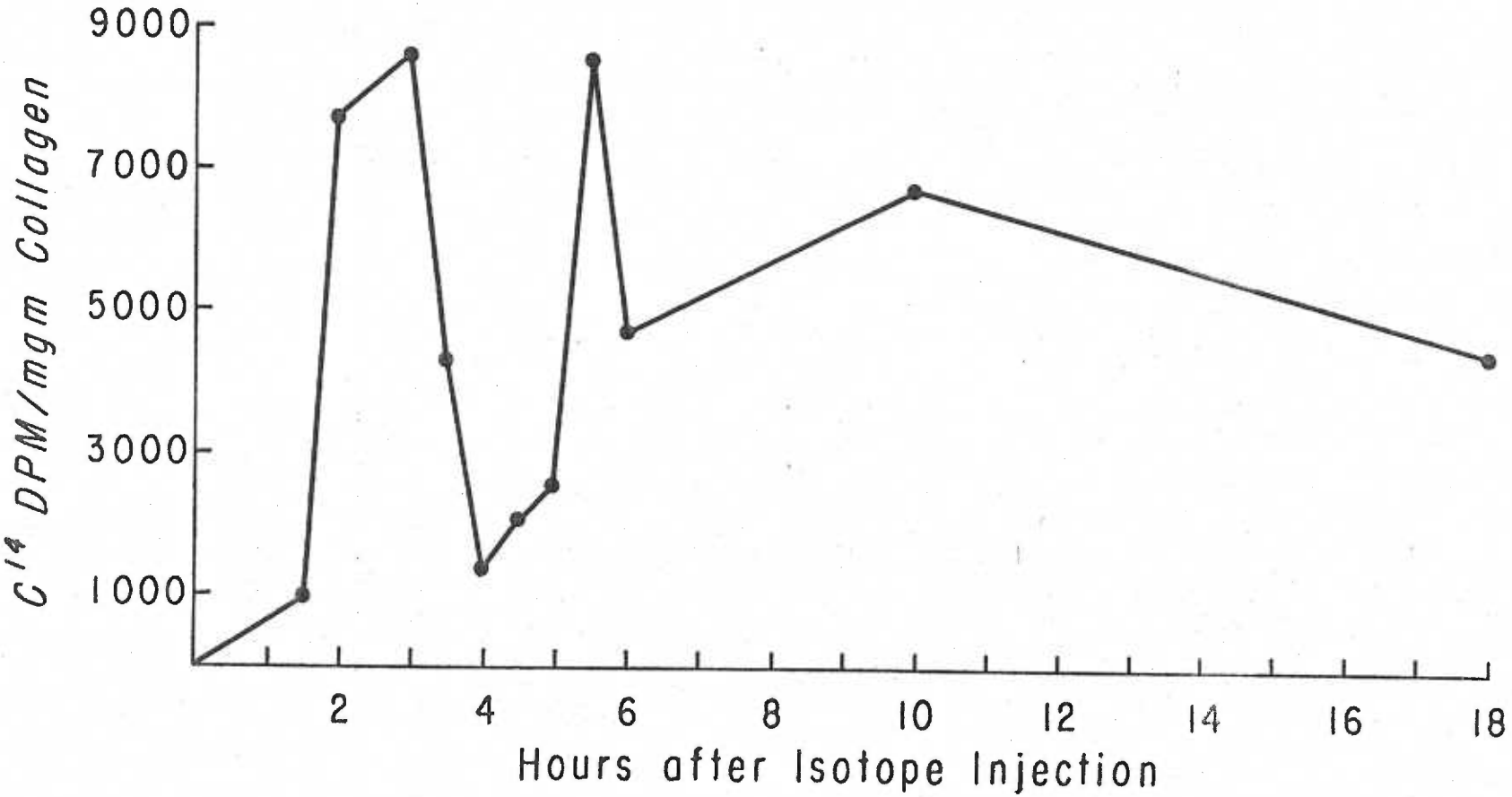

9000
7000
5000
3000
1000
C14 DPM/mgm Collagen
2
4
6
8
10
12
14
16
18
Hours after Isotope Injection

TABLE 4

Time After C^{14} glycine (in hours)	0.14 M NaCl Extractable Collagen			1.0 M NaCl Extractable Collagen		
	Mgms. per gm. skin	Specific Activity DPM per mgm. Collagen	Total Collagen DPM per gm. skin	Mgm. per gm. skin	Specific Activity DPM per mgm. Collagen	Total Collagen DPM per gm. skin
1.5	0.558	945	527	5.32	59	314
2	0.330	7695	2539	5.81	676	3927
2.5	0.637	Lost	Lost	7.02	706	4956
3	0.453	8678	3931	476	797	3794
3.5	0.556	4287	2383	5.56	545	3030
4	0.758	1426	1080	4.86	187	909
4.5	0.651	2113	1375	5.99	111	665
5	1.073	2614	2805	5.24	337	1766
5.5	0.858	8504	7296	7.34	1287	9447
6	0.769	4768	3667	6.08	576	3502
10	0.723	6681	4830	6.07	1654	10040
18	.0.971	4427	4298	7.47	1969	14708

EXPERIMENT V

Introduction

It has already been suggested that retention of newly synthesised collagen upon particulate components of the fibroblast may occur during synthesis and that reduced extractibility may be the resultant of this (8).

This reasoning can be extended to include the third peak (Fig.4) (Fig.3) which may be associated with the extracellular phase of synthesis, i.e. aggregation.

We would need to assume that the low specific activities seen between 6 and 8 hours (Fig.3) are associated with reduced extractability just prior to release of collagen from the cell.

On the basis of this reasoning it was felt necessary to investigate the uptake of labeled amino acid into collagen extractable from tissue which had been fractionated centrifugally into the classical cell fractions, microsomes, mitochondria and debris.

Previously, Lowther et al (79) had demonstrated that after incubation of carageenan granuloma slices with labeled proline, collagen which had incorporated label, was extractable from each of these three particulate fractions. The highest specific activity was found in the microsomal collagen which led the authors to suggest that this was the

primary site of collagen synthesis.

The preparation of subcellular components from skin has not however been previously reported and an experiment in which this is attempted is here described.

Methods

Preparation of Homogenate

Guinea pig skin was shaved with electric clippers and sliced into thin strips about 2 mm wide. The strips were placed in ice cold 0.25 M sucrose and homogenized for one minute in a Virtis 45 homogenizer at maximum speed (about 45,000 rpm). The brei was filtered through several thicknesses of cheese cloth to remove large pieces of connective tissue and homogenized for two minutes in a ground glass Potter-Elvehjem homogenizer, packed in ice.

Differential Centrifugation

A procedure described by Schneider and Hogeboom (131) for use with liver was employed. The brei was centrifuged for ten minutes at 700 x g. The sediment, assumed to consist of a mixture of nuclei, unbroken cells, cell debris, and extracellular debris was washed twice with 0.25 M sucrose at 700 x g for ten minutes.

The supernate and washings from this procedure were centrifuged at 5000 x g for ten minutes and the sediment washed twice at 24000x g for ten minutes. This sediment was labeled, mitochondrial fraction.

The supernatent fluid and washings were centrifuged for one hour at 105,000 x g and the sediment washed twice at 105,000 x g for one hour. This sediment was labeled, microsomal fraction.

The above procedures were carried out in a spinco model L ultracentrifuge, using a #30 rotor at 2°C.

Extraction of Collagen

Since 0.25 M sucrose will not solubilise collagen from these subcellular fractions [1], they were considered to contain collagen which had been associated with them in vivo, and an attempt was made to extract this material.

The sediments were suspended overnight at 2°C in 0.14 M NaCl solution buffered as previously described. The supernatent fluid, following clarification on the ultracentrifuge at 105,000 x g was brought to 20% NaCl concentration and the collagen precipitate removed by centrifugation. The precipitate was hydrolysed in 6 N HCl at 140°C for three hours and collagen was estimated by determining the hydroxyproline content using a micro

[1] Green, N.M. Personal Communication. March 1962.

method (153).

Results and Discussion

From a total weight of 29.5 gms of skin it was possible to obtain only extremely small quantities of collagen from the three subcellular fractions, as is seen in Table 5.

TABLE 5

Fraction	Total Hypro. extractable (Micro gms)	Total Collagen extractable (Micro gms)	Hypro. per gm skin (Micro gms)	Collagen extract-able per gm.skin
Nuclear	2.92	20.44	0.098	.686
Mitochondrial	3.34	23.38	0.113	.791
Microsomal	2.04	14.28	0.069	.483

The amounts of collagen which can thus be obtained from subcellular fractions of skin are far too low to permit purification or even hydroxyproline isolation to be carried out, and it seems clear that the amounts of tissue which would have to be processed to make an experiment on skin feasible, would be so large as to be economically prohibitive.

Hypro. = Hydroxyproline.

The paucity of cells in skin compared with liver and with such tissues as a carageenan granuloma is no doubt the reason for this low yield. By comparison, Lowther et al (79) were able to extract much more collagen from subcellular fractions of carageenan granuloma, e.g.

Nuclear fraction	160 Mgms.collagen/gm.Tissue
Mitochondrial fraction	38 Mgms.collagen/gm.Tissue
Microsomal fraction	3.8 Mgms.collagen/gm.Tissue

A more cellular material would therefore be preferable for the experiment in which the labeling of collagen on subcellular fractions was to be followed [1].

In view of the objections to the use of the carageenan granuloma which have previously been described, wound granulation tissue was chosen as a model. It was necessary however to demonstrate the phasic nature of labeled amino acid uptake into collagen extractable from this tissue as a whole, before the cell fractionation studies could be undertaken. This will be described in the following experiment.

1 Following the completion of the work here presented, a report was published by Urivetzky, et al (143) in which microsomes were successfully isolated from skin of 1 day old rabbits. Up to 160 gms. of skin were used in the preparation but no figures are presented describing the yield of microsomal material.

EXPERIMENT VI

Introduction

The rate of collagen formation in healing skin wounds was measured by Dunphy and Udupa in 1955 (24) who showed that after an initial lag period of four to five days, collagen was rapidly produced for a further ten days until its concentration leveled off at about 50% of that normally found in skin. This work was largely confirmed by Hosoda in 1960 (61) who did not however study wounds older than twelve days and consequently observed no plateau in collagen concentration. No plateau was observed in a study by Abercrombie et al (1) but this is probably due to loss in water after the twelfth day, which gives an apparent continued rise of collagen concentration if determinations are made on a wet weight basis. The work Hosoda and of Abercrombie et al was based upon wet weight whilst Dunphy et al used dry weight of tissue as a reference. A similar study carried out by Grillo et al (37) was largely in agreement with the work of Dunphy and Udupa except that the collagen content of the wounds studied by Grillo et al rose very rapidly for the first twelve days and then continued to rise more slowly for about thirty days, at which time it approximated that of normal skin. Much other work carried out with healing wounds has been

directed towards the study of factors which influence the rate of wound healing per se. Attempts have been made to accelerate the process by the use of such agents as Adenosine monophosphate (116) Bacterial endotoxins (32) and dietary proline and hydroxyproline (102).

Other investigations have been directed towards factors which are known to have a deleterious effect upon the rate of wound healing, such as cortisone (104) Rheumatoid arthritis (60) and protein depletion (103).

All these studies are in agreement however that collagen concentration in wound granulation tissue is increasing rapidly six to twelve days after wounding, and it was thus felt that at this time, the granulation tissue of an open wound would provide a useful model for investigation of collagen biosynthesis in subcellular "organs". Before this fractionation can be attempted it is first necessary to confirm the presence of a multiple peak phenomenon in the tissue as a whole.

Experimental

Fifty young guinea pigs of about 300 gms. weight, which were growing at a uniform rate, were used. The animals' backs were shaved and cleaned with alcohol. Light ether anesthesia was used whilst a circular piece of full thickness skin about 5 cm. in diameter was removed with

curved scissors. No special attempt was made to prevent infection which rarely occurred, but the very few animals whose wounds did become infected were not used in the experiment.

Ten days after wounding, all animals were injected intraperitoneally with 45 μC of 3, 4,H^3 proline in 1ml. physiological saline and killed at the following times after injection: 2, 2.5, 3, 4, 5, 5.5, 6, 7, 8, 10 and 12 hours.

The scab was removed from the wounds and discarded. At this time the wounds had contracted to a little more than half their original area but the original skin edges were quite clearly distinguishable from the newly formed tissue. The granulation tissue was removed, care being taken that skin from the wound margin did not contaminate it. The tissue was immediately frozen in liquid nitrogen and crushed as previously described. Collagen was extracted as before with 0.14 M NaCl solutions but no purification procedures were carried out.

The total extract was dialyzed salt free, evaporated to dryness on the steam bath and was hydrolyzed in 6 N HCl for three hours at 140°C. Hydroxyproline was determined on an aliquot of this hydrolysate.

Hydroxyproline was isolated from the hydrolysate by thin layer chromatography as described by Myhill (88) (89) (see appendix 3), eluted from the chromatogram

and its concentration determined (153) followed by radioassay as before.

Results and Discussion

The results are presented in Fig.5 and Table 6, a multiple peak of specific activities is again seen, confirming that collagen synthesis in granulation tissue follows an apparently similar pathway to that in skin. The third peak is less pronounced than in skin and occurs at a somewhat earlier time, but as previously pointed out the disparity may possibly be due to sampling frequency.

It is felt therefore that this occurrence of a multiple peak in the uptake curve permits the use of this tissue in the proposed investigations of isotope incorporation into the collagen found in association with subcellular fractions.

The possibility that one of the peaks is due to the synthesis of something other than collagen can be rejected on the basis of this experiment. Due to the fact that the specific activities presented here are on the basis of isolated hydroxyproline rather than upon collagen, and since the hydroxyproline found in the extract was non dialysable it was thought probable that it was associated with some large molecular species, perhaps as a peptide or as a complex with soluble RNA.

Fig. 5 Specific activity of hydroxyproline isolated from 0.14 M NaCl extractable collagen of guinea pig wound granulation tissue following injection of H^3 proline.

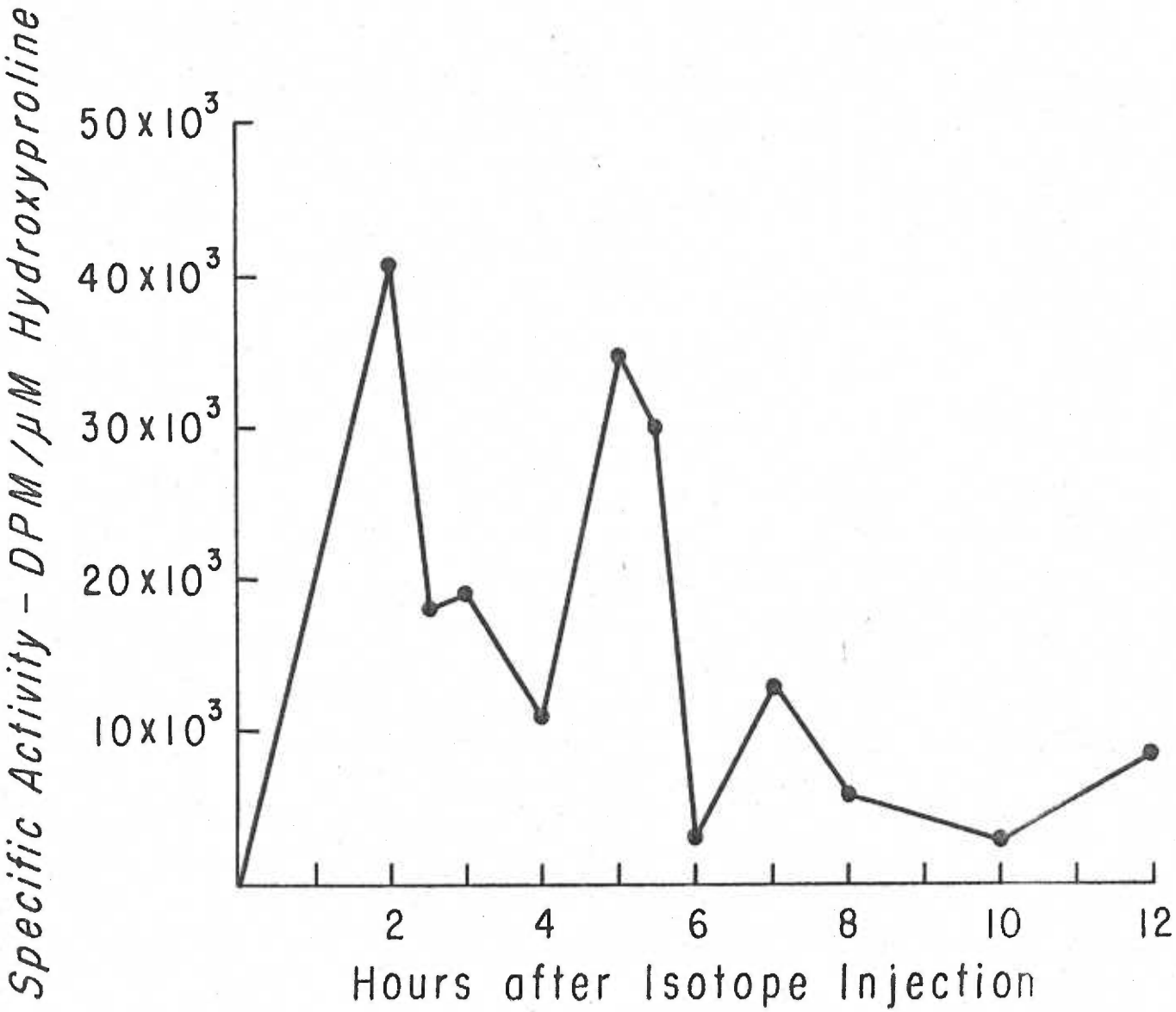

Specific Activity - DPM/μM Hydroxyproline
50x10³
40x10³
30x10³
20x10³
10x10³
2
4
6
8
10
12
Hours after Isotope Injection

TABLE 6

Time After Isotope (hours)	Total Extractable Collagen micro gms. Hydroxyproline per gm. tissue	Specific Activity DPM/micro gms. Hydroxyproline	Total Extractable Collagen Hydroxyproline DPM.gm.tissue
2	33.723	40741	10480
2½	27.016	18340	2380
3	55.398	19257	8144
4	40.132	11004	3371
5	60.627	34584	15840
5½	83.48	29999	19007
6	11.233	3013	253
7	20.451	13100	2000
8	44.07	5764	1936
10	22.71	3013	506
12	29.857	8515	1950

It is felt that the latter possibility is remote but cannot be wholly discounted. Such a complex may well be extracted with the salt solutions used to extract collagen but is is unlikely that it would behave as collagen in the purification method used in Experiments II, III and IV, which gave essentially the same results as the present experiment. Indeed the disappearance of the absorption maximum at 256 mμ which is associated with RNA, is one of the criteria frequently used in this laboratory as a check on the purification procedure used. This method was first suggested in this context by Grillo and Gross (38).

The unique occurrence of hydroxyproline in collagen means that any peptide bound hydroxyproline is either bound into a complete collagen molecule or into a smaller peptide precursor of collagen. If such a precursor exists, (it has never been described) and its presence indeed accounts for one of the peaks of specific activity in this experiment, then that peak should be much reduced in Experiments II - IV, where collagen, was highly purified. This is not the case and it is therefore concluded that the hydroxyproline specific activities shown in Fig. 5 represent hydroxyproline incorporated into a complete collagen molecule.

EXPERIMENT VII

Introduction

Since the previous experiment had shown that open wound granulation tissue was a suitable one in which to study"the multiple peak phenomenon", the following experiment was carried out. It is an attempt to investigate whether the transfer of newly formed collagen from one subcellular "organ" to another is responsible for the fluctuations of specific activity of collagen extracted from tissue as a whole.

Methods

Wounds were made as previously described on forty-eight young guinea pigs and allowed to heal for ten days. At which time all animals were injected intraperitoneally with 50μC 3,4, H^3 proline. The guinea pigs were decapitated at the following times after isotope injection; 2, 2.5, 3, 4, 5, 5.5, 6, 8. Wounds from six animals were combined for each time point.

Wound granulation tissue removed as described in experiment VI, was homogenized and subjected to differential centrifugation exactly as employed for skin in Experiment V.

The fractions were named Sed. 700 g, Sed. 5000 g,

and Sed. 105,000 g according to the g value employed to bring about their sedimentation in the ultracentrifuge. These terms are purely operational corresponding to the more frequently used terms, debris and nuclei, mitochondria, and microsomes, which are also operational terms and do not necessarily correspond to "organs" existing within the cell before its disruption. Samples of the sedimented pellets were taken for electron microscopic examination.

Collagen extraction

The various sediments were twice extracted overnight at 2°C with 0.14 M NaCl solution prepared as before.

After clarification on the centrifuge the extracts were dialysed exhaustively, evaporated to dryness and hydrolysed in 6 N HCl for three hours at 140°C. Total hydroxyproline in the extract was determined (153) and a known amount of unlabeled carrier hydroxyproline added to the material isolated from the Sed. 105,000 g fraction. This was considered necessary due to the relatively small amounts of collagen extractable from this fraction.

Hydroxyproline was isolated by thin layer chromatography (see appendix 3) and radioactivity assayed as before.

A correction was made when specific activities were calculated to allow for the carrier hydroxyproline added, if any.

Results and Discussion

The very large differences between the amounts of the three sediments and hence of the amount of collagen extractable from them would make comparison of total activity difficult and probably valueless. For this reason, specific activities only are presented in this experiment.

These specific activities are presented in Table 7 and in Figs. 6a, 6b and 6c.

Electron micrographs of the three fractions isolated are presented in plates 1, 2 and 3.

Very obvious differences can be seen between the specific activity curves of the three subcellular fractions, but no clear cut separation of the peaks is seen, i.e. it is not possible to ascribe each peak to a separate fraction. The results are anomalous in that the highest initial peak is seen in the Sed. 5000 g fraction, thus implicating mitochondria in the first phase of collagen synthesis.

Fig. 6a Specific activity of collagen hydroxyproline extracted from Sed. 700 g fraction of wound granulation tissue following injection of H^3 proline.

Fig. 6b Specific activity of collagen hydroxyproline extracted from the Sed. 5000 g fraction of wound granulation tissue following injection of H^3 proline.

Fig. 6c Specific activity of collagen hydroxyproline extracted from the Sed. 105,000 g fraction of wound granulation tissue following injection of H^3 proline.

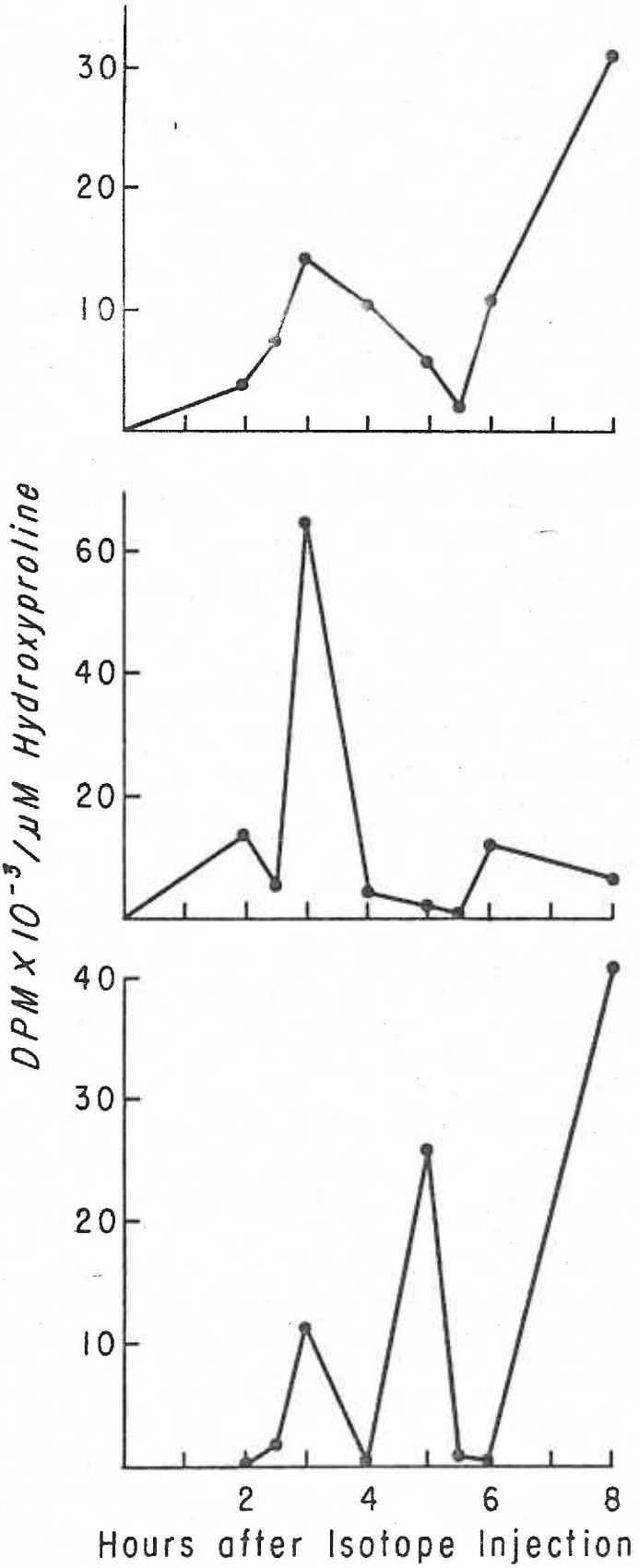


fig.6a

fig.6b

fig.6c

TABLE 7

Specific Activities DPM/Micro. M Hydroxyproline

Time After Isotope (Hours)	Sed. 700 g fraction	Sed. 5000 g fraction	Sed. 105000 g fraction
2	3891	13739	0
2.5	7571	4943	1843
3	14451	65156	11504
4	10464	4621	0
5	5809	1310	26290
5.5	2099	293	974
6	10672	11790	0
8	31100	5994	41267

Plate 1 Electron micrograph of the Sed. 700 g fraction of wound granulation tissue from guinea pigs. Magnification x 26,800.

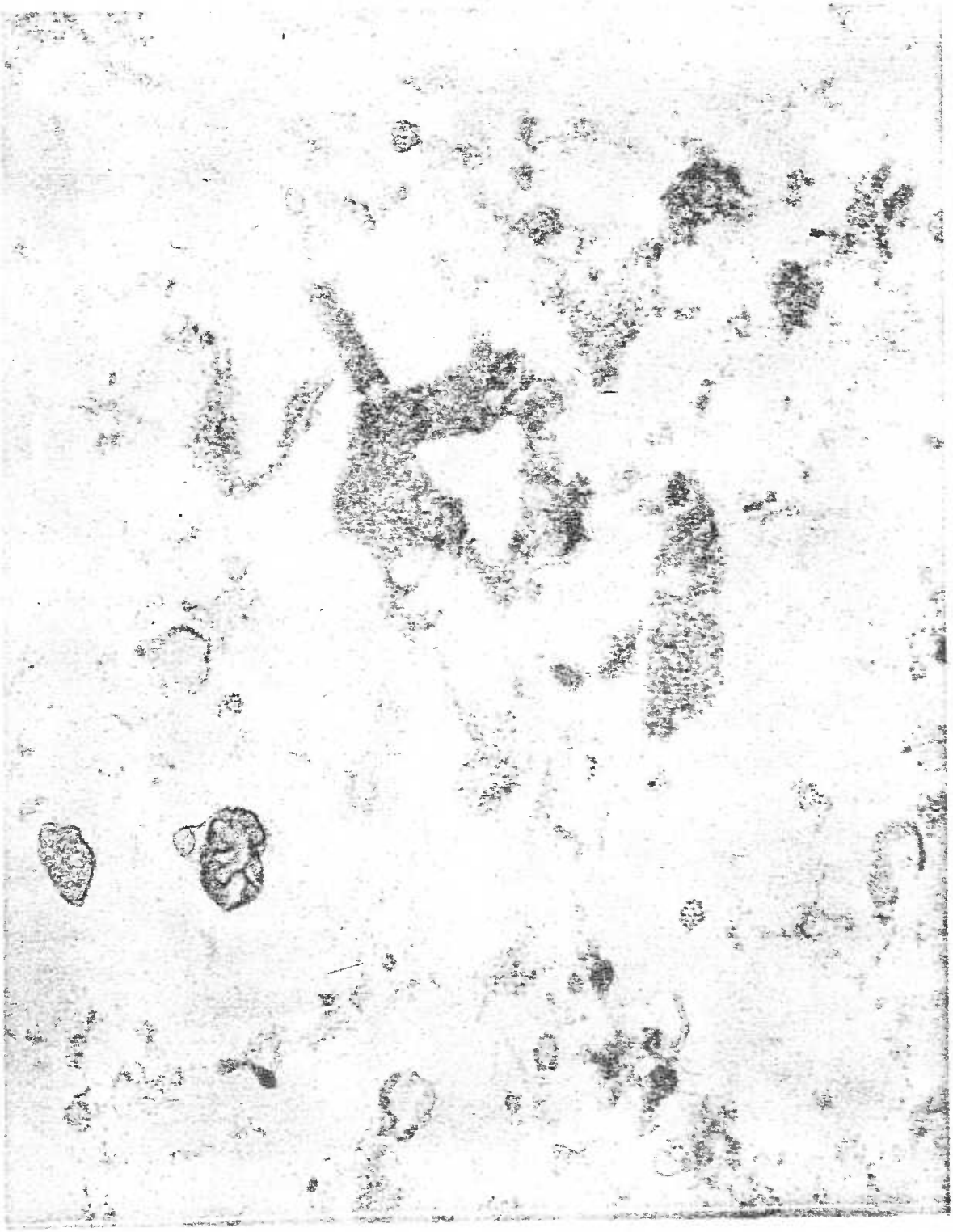


Plate 2 Electron micrograph of the Sed. 5000 g fraction of wound granulation tissue from guinea pigs. Magnification x 26,800.

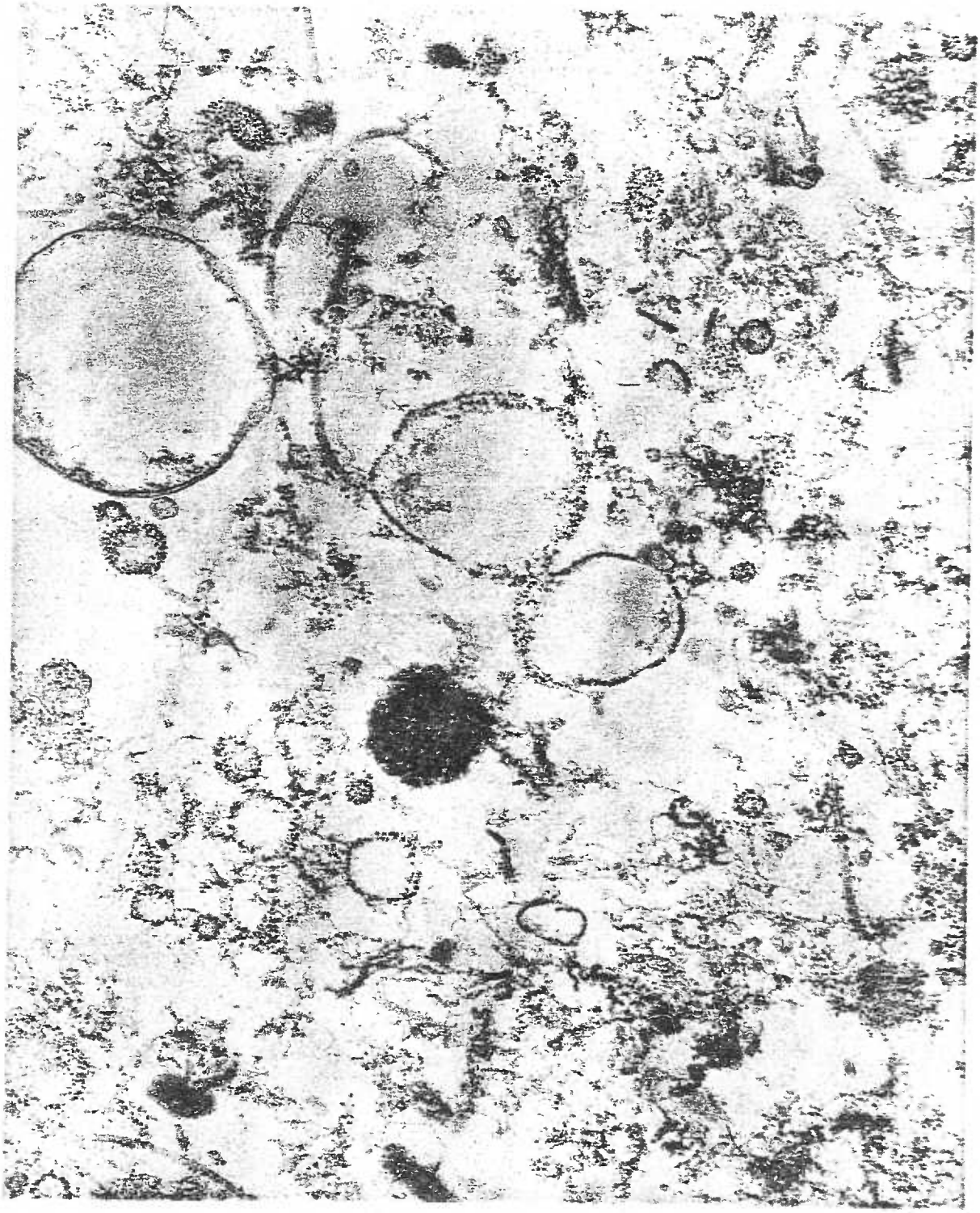
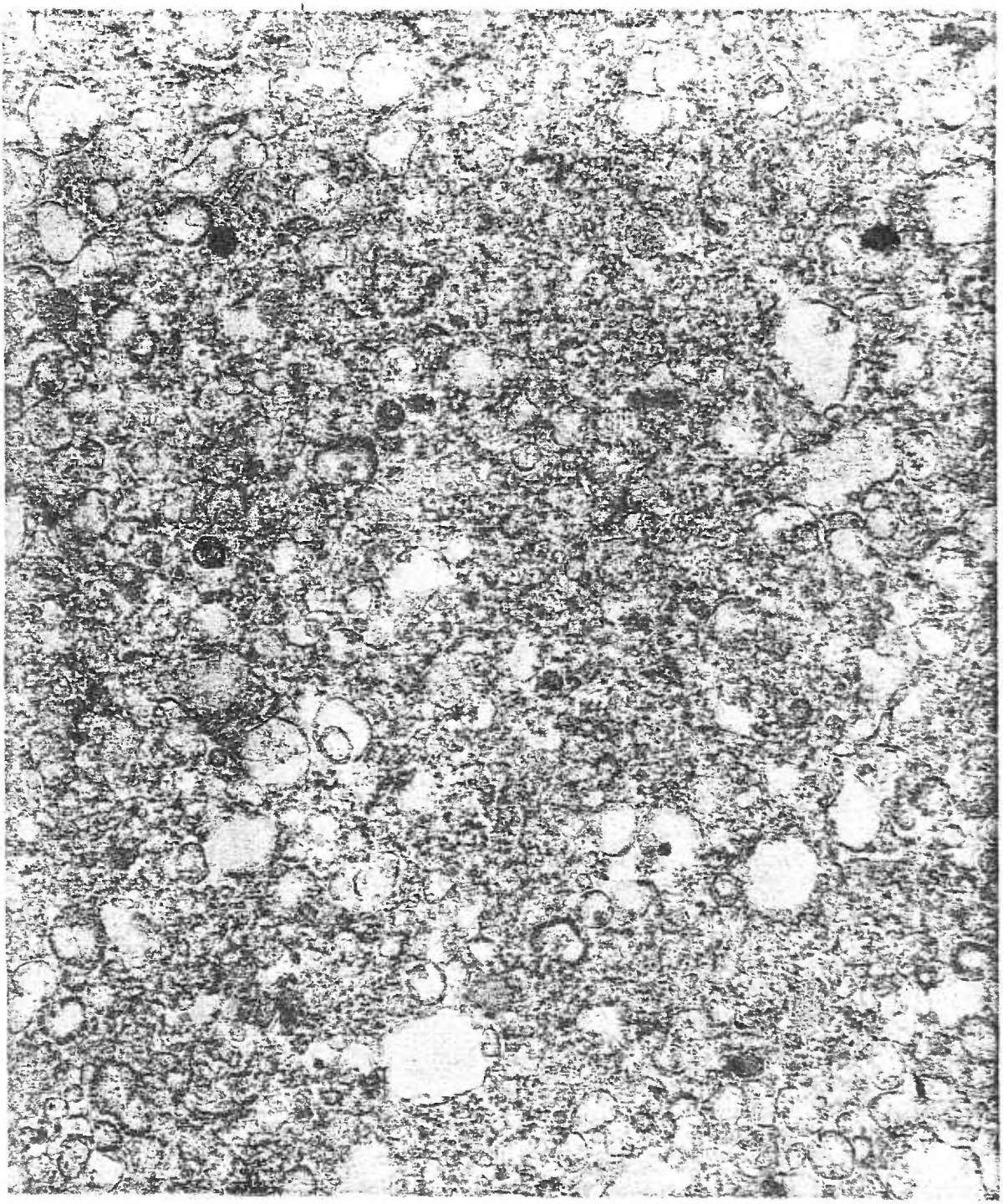


Plate 3 Electron micrograph of the Sed. 105,000 g fraction of wound granulation tissue from guinea pigs. Magnification x 26,800.



Lowther et al (79) on the other hand always found the highest activity in collagen isolated from the microsomal fraction of carageenan granulomata. Examination of Plate 2 to a certain extent resolves this anomaly since the Sed. 5000 g fraction is seen to contain numerous beaded vesicles which are thought to represent endoplasmic reticulum. The Sed. 105000 g fraction (Plate 3) contains large numbers of much smaller vesicles, most of which are not beaded, together with many free particles which may be free ribsosomes.

The Sed. 5000 g fraction also contains several striated collagen fibrils, together with some much finer fibrillar material, perhaps intracellular collagen filaments. No identifiable mitochondria are seen.

Peach et al (101) demonstrated in 1961 that the endoplasmic reticulum of fibroblasts in regenerating tendon was composed of beaded vesicles with very irregular sizes, and it can be calculated from Peach's plates that the range of diameters of these vesicles is about 1.7 to 13 microns, there being a continuous distribution between these sizes.

The largest vesicles seen in Plate 2 have a diameter of about 13 microns whilst the smallest in Plate 3 have diameters of about 1.9 microns and these figures agree very well with those calculated from Peach's plates. It is

thus proposed that the differential centrifugation concentrates the smaller of these vesicles in the Sed.105000 g fraction and the larger in the Sed.5000 g fraction.

The Sed. 700 g fraction (Plate 1) appears quite amorphous and no interpretation can be made of this plate.

Transfer Postulate

The results do appear to subscribe to the postulate that collagen becomes soluble or insoluble according to its locus within the cell. No peak is seen at five hours in Figs. 6a and 6b whilst a definite peak occurs at this time in the Sed. 105000 g fraction (Fig. 6c). The Sed.5000 g fraction (Fig.6b) demonstrates no peak at eight hours whilst both other fractions do (Figs. 6a and 6c).

Due to the apparent inhomogeneity of the Sed.700 g fraction and Sed. 5000 g fraction, as shown in plates 1 & 2, it would be difficult to correlate the observed peaks with any single subcellular component found in these fractions. The Sed. 105000 g fraction on the other hand appears quite homogeneous (Plate 3) and still demonstrates three peaks of activity which thus suggests that the above "transfer postulate" is incorrect.

It thus becomes obvious that the technique of isolating subcellular components from liver cannot be directly applied to connective tissue. The extreme.

difficulty of homogenisation is a large factor, but more important is the apparent disparity between the rates at which components from the two tissues sediment in the centrifuge. The development of a new technique for the homogenisation and differential centrifugation of connective tissue which would produce "clean" homogeneous fractions is no doubt desirable, but is a major undertaking which it was decided not to attempt at this time.

Due to the appearance of multiple peaks in the Sed. 105,000 g fraction, it is probable that either breakdown and resynthesis, or pulsatile synthesis as described in items 7 and 6 respectively, (pages 56 & 57) is responsible for the multiple peak. For reasons previously stated it is felt that pulsatile synthesis is not likely to occur.

EXPERIMENT VIII

Introduction

This experiment was designed to confirm the occurrence of multiple peaks of specific activity in the Sed. 105,000 g fraction, which as was shown in the previous experiment is the only fraction which can be obtained in a relatively homogeneous state from connective tissue homogenates. This fraction corresponds to the microsome fractions of Lowther et al (79) and of Prockop et al (112) which is considered by both of these workers to be the site of collagen synthesis.

Methods

The experimental procedure was the same as that described for Experiment VII with the following exceptions.

Each animal was injected with 40 μC 3,4,H^3 proline.

Animals were killed and wounds removed at the following time intervals after injection, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 7, 8, 9, 10, 12 hours.

No attempt was made to fractionate the homogenate into Sed. 700 g and Sed. 5000 g fractions by centrifugation due to the inhomogeneity of fractions thus prepared. A single fraction was instead obtained by centrifuging for ten minutes at 10000 x g. This fraction was washed twice

at 17,000 x g and discarded.

The supernatent and washings from this were centrifuged for one hour at 105,000 x g the sediment washed twice as before, and labeled Sed. 105,000 g fraction. The above procedure is essentially that described by Prockop et al (112) for the isolation of chick embryo microsomes.

Collagen was extracted from the Sed. 105,000 g fraction, dialysed and hydrolysed, as previously described.

Isolation of Hydroxyproline

The method of Prockop et al (111) was employed for the separation of hydroxyproline. (The method is described in detail in appendix 4, together with trial runs performed on standard samples of gelatin and labeled amino acids.)

In brief, the method consists of treating the hydrolysate with an oxidising agent, the oxidation products of all amino acids other than hydroxyproline are preferentially soluble in organic solvents, and these are removed with chloroform and toluene. Upon heating, the Pyrrole-carboxylic acid which is the initial oxidation product of hydroxyproline is converted to pyrrole which is extracted into toluene.

The pyrrole content and the radioactivity can

both be conveniently determined on this solution.

Results and Discussion

Fig. 7 and Table 8 show the specific activities of collagen extracted from the Sed. 105,000 g fraction, a multiple peak of specific activity is again seen which is in agreement with the results of Experiment VII.

The confirmed presence of a multiple peak of specific activities in the subcellular fraction which is considered to be the site of protein synthesis shows that at certain times, more label is being incorporated into newly synthesised collagen than at others.

It can be seen that collagen synthesis does not cease at times when the specific activity is low since collagen is always extractable at these times, and this mediates against the pulsatile synthesis idea. Incorporation of labeled amino acids is nevertheless higher at certain times and it is felt that this is due to a greater availability of labeled amino acid at this time, a point which will be developed in detail in the General Discussion section.

Fig. 7 Specific activity of collagen hydroxyproline extracted from the Sed. 105,000 g fraction of wound granulation tissue following injection of H^3 proline.

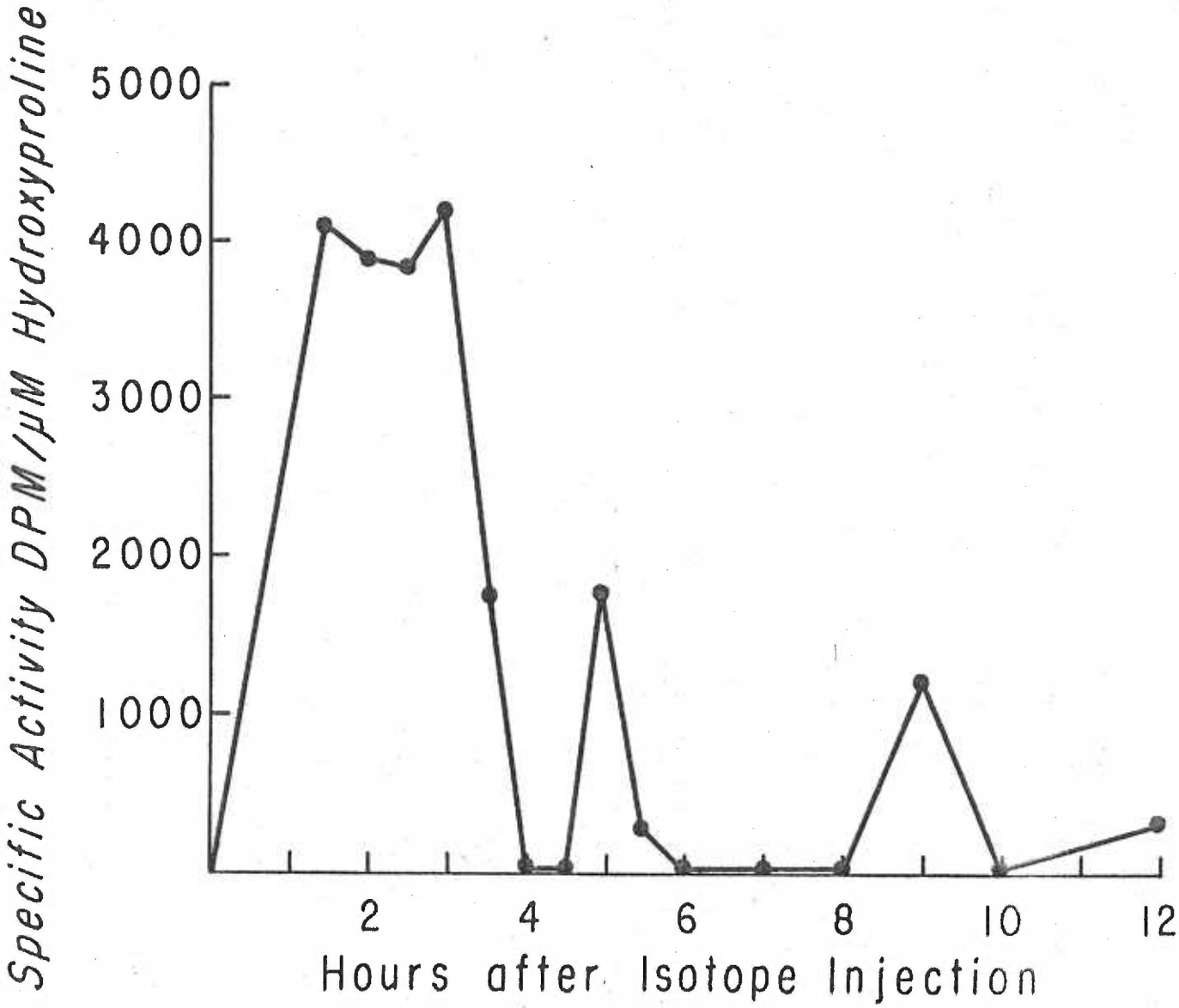

Specific Activity DPM/μM Hydroxyproline
5000
4000
3000
2000
1000
2
4
6
8
10
12
Hours after Isotope Injection

TABLE 8

Time	Sed. 105000 g fraction Specific Activity DPM/Micro. M Hydroxyproline
1.5	4100
2	3936
2.5	3854
3	4255
3.5	1895
4	0
4.5	0
5	1786
5.5	309
6	0
7	0
8	0
9	1238
10	0
12	354

GENERAL DISCUSSION

Some speculations upon the origin of the multiple specific activity peaks were presented in a previous section, and subsequent experiments carried out which were intended to discriminate between these speculations and perhaps eliminate some of them.

At this point it is proposed to summarise the evidence for or against the various possibilities noted, in an attempt to arrive at a reasoned hypothesis explaining the multiple peak phenomenon.

Explanation number 1, page 54, that the result is an artefact is considered quite unlikely since the existence of multiple peaks has been subsequently demonstrated many times. It can be shown to occur in skin, and wound granulation tissue when the radioactivity is measured either on collagen as such, or upon hydroxyproline isolated from collagen. Claycomb [1] has also been able to demonstrate a multiple peak of radioactivity in collagen isolated from the gingiva of the same animals used in some of the author's experiments.

The possibility of relabelling taking place due to the lability of tritium in glycine, followed by a subsequent uptake of this relabeled amino acid can be discounted for many reasons. As previously described

[1] C. K. Claycomb. Personal communication, January 1963.

(page 54) this is unlikely in theory. In addition, no change is seen in the uptake pattern when C^{14} glycine is used instead of H^3 glycine (Experiment IV).

Perhaps the most telling argument however can be made from results of Experiments where 3,4,H^3 proline was used. In this case one of the tritium atoms on the #4 carbon or 25% of the tritium label of proline is known to be lost upon hydroxylation (27) and yet no change is seen in the uptake pattern.

The possibility that contaminating proteins are responsible for one or more of the peaks whilst collagen is only represented by a single peak is not thought to be tenable for the following reason.

The use of labeled proline and the isolation of its product, labeled hydroxyproline gives the same results as the use of labeled glycine and isolation of collagen. As previously stated, no other animal proteins contain hydroxyproline, which suggests that the results do in fact represent only collagen biosynthesis.

The suggestion that newly formed collagen is transferred from one intracellular site to another and that its extractability is affected as a result of this transfer was previously made by Bentley and Jackson (8) in a preliminary report of this work. The fact that a multiple peak phenomenon can still be demonstrated in a relatively

homogeneous microsomal fraction of wound tissue makes this suggestion unlikely however.

The experiments offer little evidence for or against possibility number 6 page 56. However, as previously stated, in order for pulsatile synthesis to produce a discontinuous uptake curve, a large majority of cells in the tissue must be supposed to be in phase with one another. They must all be assumed to start making collagen together and stop together. Newly formed collagen is nevertheless always extractable from tissue, and even from microsomal fractions and we can thus assume that its synthesis is continuous rather than discontinuous. In the experiments presented here it is the incorporation of label which is discontinuous and this is felt to reflect variation in the availability of labeled precursor i.e. variation of the specific activity of free amino acid or of some more immediate precursor.

The existence in the extracts of a small molecular weight collagen precursor containing both glycine and hydroxyproline is a possibility. It is unlikely however that such a macromolecule would behave in a similar manner to collagen in the purification technique used. It is to be expected that any such "impurity" would be removed differentially in the experiments where collagen was purified and so cause a marked diminution of the first

peak relative to the others. Such a diminution is not seen when the results from purified collagen are compared with those where hydroxyproline was isolated from hydrolysates of crude extracts. As previously stated, it is unlikely that the soluble RNA amino acid complex which is known to be a precursor macromolecule, would occur in the rigorously purified collagen extracts.

Breakdown and Resynthesis Hypothesis

It is felt that of all the possible explanations of the "multiple peak phenomenon", breakdown of newly synthesised material followed by re-utilisation of the freed amino acids, is the most likely and thiswill be expounded in some detail.

For reasons previously stated, it is felt that the radioactivities presented, particularly in Experiments II to IV, represent the incorporation of label into complete tropocollagen molecules. This being so, it follows that a molecule synthesised at one time, i.e. about 4.5 hours after isotope injection, contains a smaller amount of labeled amino acid than similar molecules synthesised at previous and subsequent times, i.e. 2.5 hours and 5.5 hours.

Since all these tropocollagen molecules are assumed by definition to contain the same total amount of any one amino acid then the ratio of labeled to unlabeled

must differ at the three time points given as examples. This ratio is of course the definition of specific activity.

One way in which the specific activity of a newly synthesised molecule can be changed is that the synthetic mechanism distinguishes between labeled and unlabeled precursor molecules, a so called isotope effect. It is highly improbable that positive and negative isotope effects will operate during alternate hours in one system.

A second influence upon the specific activity of a newly synthesised protein molecule is specific activity of the free amino acid pool, i.e. the ratio of labeled to unlabeled.

It is known that following the injection of C^{14} glycine the specific activity of the free glycine in plasma falls exponentially and that after a short period of equilibration the tissue free glycine behaves similarly (58).

This, together with the fact that collagen is progressively aggregated into material which is unextractable with any particular salt concentration (68) gives rise to the classical uptake curve of labeled glycine into extractable collagen (57) (64) which will be examined in Fig.8.

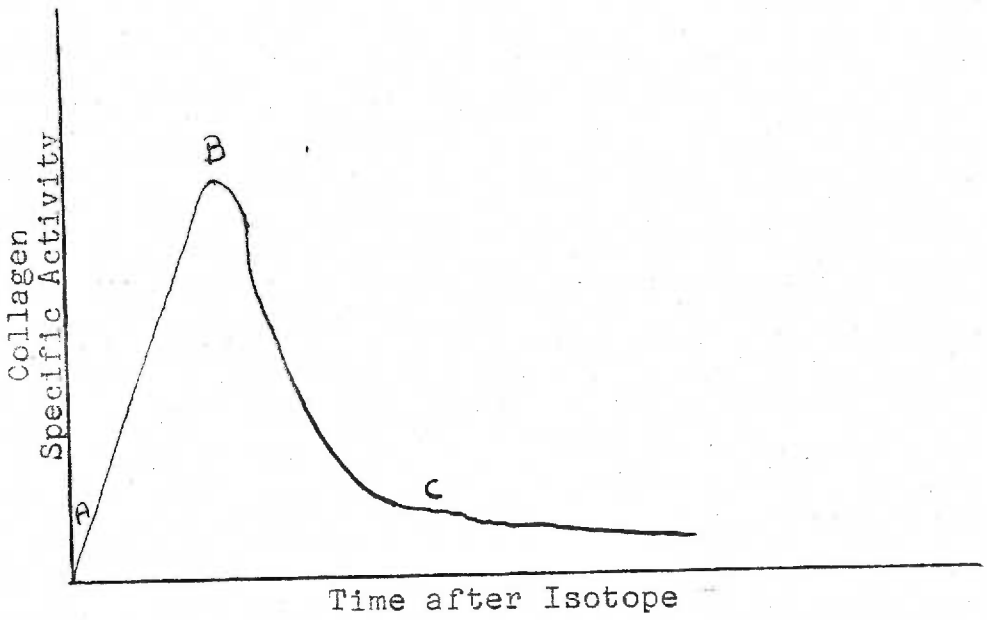


Fig. 8

Explanation Fig. 8

Point A - Collagen is being synthesised from a free pool of high specific activity and for a period of time this collagen is extractable with e.g. 0.14 M NaCl solution. An amount of collagen formed prior to the administration of isotope is also extractable and the specific activity of the collagen thus extractable at A is low.

A to B The specific activity rises for two reasons, a) more labeled collagen is added to the extractable "pool" by synthesis and, b) preformed unlabeled collagen is removed from the "pool" by aggregation.

B to C - The specific activity falls for two reasons, a) the specific activity of the free amino acid pool is falling and hence newly synthesised collagen has incorporated less of this radioactive precursor than material synthesised between A and B. b) The relatively highly labeled material synthesised between A and B is being removed from the extractable "pool" by aggregation.

The curves resulting from the present experiment do not follow this pattern however but rise to second and third peaks as time progresses and it is felt that this can only be due to a rise in the specific activity of the collagen precursor amino acid pool. Since it has been shown that the plasma and tissue free pools do not show any subsequent rises after the first one (57) then it is postulated that these pools do not represent the immediate precursor of collagen.

It was first shown by Cowie and Walton (18) (19) in 1956 that two functionally different pools of nucleic acid bases are present in the Candida utilis cell.

Later (20) they described analagous pools of amino acids in the same organism and these they named "concentrating pool" and "conversion pool". The former accumulates amino acids within the cell to a higher concentration than that of the medium. The amino acids in this pool are exchangeable with those of the medium and

indeed the pool does not exist in the absence of amino acids in the medium.

The conversion pool on the other hand is of constant size even when no exogenous amino acids are supplied, in this case the amino acids of this pool are derived from new synthesis.

If labeled amino acids are made available to the organism they are taken up by the "concentrating pool" and transferred to the "conversion pool", and thence into protein. If the labeled medium is now replaced by unlabeled medium, the amino acids of the "concentrating pool" freely exchange with the medium and the specific activity of this pool falls. The specific activity of the conversion pool is however virtually unaffected, as is that of the protein which is being synthesised. Cowie suggests that the contents of this conversion pool are held in association with some macromolecule, probably protein, and that this explains their nonexchangeability. A similar experiment, in which bone chips were incubated with labeled proline followed by washing and transfer to an unlabeled medium has recently been described by Flanagan and Nichols (29). They found that collagen was progressively labeled for as long as six hours after removal of the labeled medium, the experiment being terminated at this time.

This is taken to mean that a certsin amount of the labeled proline within the cell is not exchangeable with the medium proline and is used preferentially for collagen synthesis.

Intracellular Recycling

It is suggested that a similar "conversion pool" exists in vivo in fibroblasts and that this accounts for the multiple peak phenomenon. This can best be explained by means of a diagram based upon the uptake of labeled proline into microsomal collagen (experiment 8).

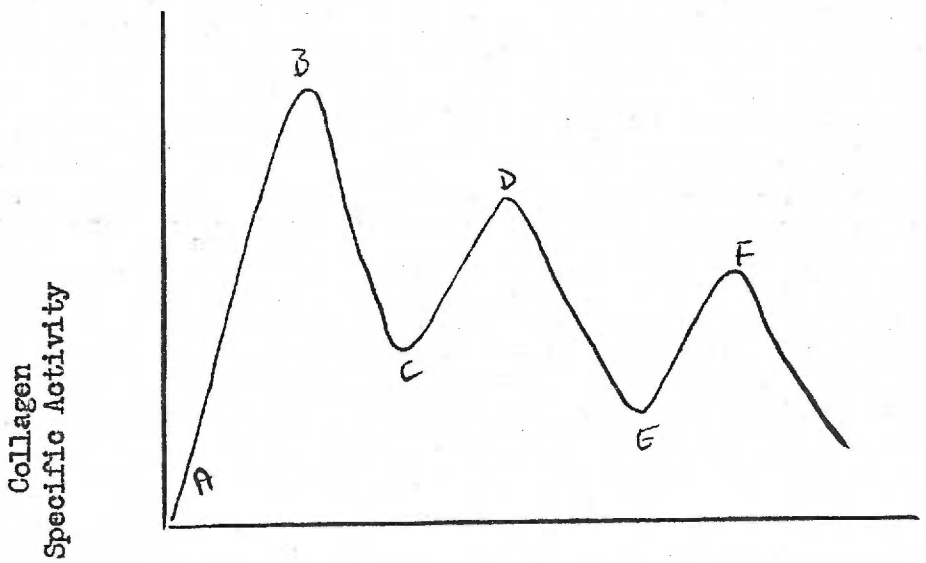


Fig. 9
(for explanation see text)

A to B - The collagen specific activity rises as newly formed collagen is added to unlabeled collagen which is still associated with the endoplasmic reticulum. At the same time the specific activity of the conversion pool is increasing as it gains labeled proline from the concentration pool, this also contributes to the rise of collagen specific activity.

B to C - The specific activity falls because labeled collagen is being removed from the endoplasmic reticulum and replaced by less highly labeled material, newly formed from the conversion pool whose specific activity is lowered at this time.

It is suggested that some of the highly labeled collagen formed between A and B is now broken down by some mechanism which releases its constituent amino acids into the conversion pool only. Since this pool is conceived to be non-exchangeable with extracellular amino acids, the net result is that more labeled proline is available for conversion to hydroxyproline and incorporation into collagen. The resultant increase of specific activity in the conversion pool then leads to the second rise of collagen specific activity seen between C and D.

Subsequent breakdown and resynthesis account for the third rise seen from E to F.

Over and above this of course much collagen escapes

or is secreted from the cell and gives rise to the progressive labeling of total collagen which is well known.

Support for this hypothesis is found in the results of an experiment carried out by Myhill [1] upon the same animals used in Experiment VIII. Myhill isolated and purified collagen from the skin of these animals and measured the specific activities of the isolated proline and hydroxyproline. He found that a multiple peak was present in the specific activities of both but that the proline peaks always preceded the hydroxyproline peaks. Thus at any one time the specific activities of the two imino acids were different even after corrections had been made for the loss of tritium from the number 4 carbon atom of proline, which takes place upon hydroxylation. This discrepancy may be attributed to the time required for hydroxylation and is in agreement with the work of Peterkofsky et al (105) who demonstrated a twenty minute lag between the appearance of labeled proline in protein, and labeled hydroxyproline in protein when chick embryo microsomes were incubated with C^{14} proline.

If the conversion pool proline specific activity rises due to collagen breakdown then a lag will occur before a similar rise is seen in the hydroxyproline. Collagen formed at this point will thus contain "relatively hot" proline and "relatively cold" hydroxyproline, since

[1] Myhill, D. - Personal Communication. January 1963.

this hydroxyproline was formed before the postulated breakdown which supplemented the proline specific activity. At the end of the hydroxylation lag, the conversion pool hydroxyproline specific activity will be higher than that of the proline since this has fallen due to its removal into protein. Collagen formed now will contain "relatively hot" hydroxyproline and "relatively cold" proline.

The above hypothesis makes certain suggestions which are not proved in the data presented here and indeed are not susceptible of proof.

For example, it is suggested that some newly formed collagen is broken down to its constituents whilst some is not. This breakdown cannot occur immediately upon synthesis or the enrichment of the activity in the conversion pool would be continuous. A lag between synthesis and breakdown must therefore be postulated to account for the discontinuity of the specific activity curves.

It is also basic to the hypothesis, that breakdown products, i.e. amino acids, must remain in the conversion pool so that dilution of radioactivity will not occur.

Even though no direct evidence is available to support either of these contentions, it is felt that at the present time the breakdown and resynthesis hypothesis is the best explanation of the "multiple peak phenomenon".

Vitamin C deficient animals

On the basis of the above hypothesis it is possible to offer tentative interpretations of the curves obtained from the collagen of scorbutic animals. No peaks of specific activity subsequent to the first are seen in collagen extracted from the skin of these animals (Fig.2b). It can thus be suggested that none of the newly formed collagen is broken down in these animals after its initial synthesis. It must be stressed however that the total amount of collagen synthesised per unit weight of tissue is always smaller in scorbutic animals (Table 2).

An alternative suggestion is that breakdown does occur but that the amino acids thus released are not held in the conversion pool available for new synthesis.

It is not possible to distinguish between these two possibilities from the data presented. Indeed, until more information is available about the proposed breakdown and resynthesis mechanism, interpretations of possible alterations in this mechanism can be nothing but speculative.

A somewhat different disturbance of the normal pattern of labeled amino acid incorporation into protein has recently been noted by Gross [1] in the lathyritic chick embryo. Multiple peaks of collagen specific activity were seen in bones from this animal following injection of labeled proline onto the chorioallantoic membrane, and these peaks

[1] Gross, J. Personal Communication. August 1962.

were greatly exaggerated when lathyrism was induced by the injection of amino-propionitrile.

Gross feels that the lathyritic defect is brought about by the breakdown of collagen and his results could therefore be used as evidence in favor of the breakdown and resynthesis hypothesis presented here. It must be borne in mind however that the chick embryo is a completely closed system and may not be at all analagous to mammalian tissues due to the obvious possibilities of metabolite recycling.

SUMMARY

The uptake of radioactively labeled amino acids into collagen extractable from skin and wound granulation tissue has been studied at very frequent time intervals following administration of the tracer. A characteristic rising and falling of collagen specific activities was seen in all experiments even when the collagen was extracted from a centrifugate which is considered to represent endoplasmic reticulum.

Several possible explanations of this phenomenon are considered and attempts made to eliminate some of them experimentally. On the basis of this, a hypothesis is put forward which is felt to be the most logical interpretation of the results.

It is suggested that soon after collagen is formed, some is broken down and that its constituent amino acids are utilised preferentially for the synthesis of new collagen.

Other experiments indicate that this mechanism is affected in vitamin C deficiency, no breakdown and resynthesis being evident in this condition.

APPENDIX I

Guinea Pig Diets

Triangle Rabbit Pellets with Greens 16%

Ingredients	
Alfalfa meal	40.0%
Wheat Millrun	15.0%
Ground Barley	18.05%
Ground Oats	10.0%
Soybean meal	7.5%
Cottonseed meal	5.0%
Cane Molasses	2.5%
Salt	0.5%
Minerals	1.45%

(Defluorinated phosphate, Ground Limestone, Potassium Iodide, Copper Sulfate, Manganese Sulfate, Magnesium Sulfate, Cobalt Carbonate, Fenous Sulfate, Zinc Sulfate.)

APPENDIX I

Vitamin C Free Diet

MacDonald Guinea Pig Basal Diet #5 (14)
(Compounded for us by Ralston Purina Co., St. Louis, Mo.)

Ground Oats	15%
Ground Wheat	13% (See note 1)
Ground Dried Beet Pulp	25%
Linseed Oil meal	12.5% (See note 2)
Skim milk powder	15%
Fish meal	5%
Brewers dried yeast	10%
Bone Char	4% (See note 3)
Salt (iodized)	0.5%

NOTES:

1. 5 lbs. of this may be replaced if desired by an equal weight of molasses for greater ease in pelleting.

2. Purina substitute solvent extracted soy bean meal on an equivalent protein basis.

3. Purina substitute diCalcium phosphate to give equivalent calcium and phosphorous levels.

4. If animals are to be maintained on this diet for prolonged periods they should be given supplemental vitamins A, D and E. It is of course completely lacking in vitamin C.

APPENDIX 2

Radioactivity determination by liquid scintillation spectrometry

Apparatus and Materials

The Tri-Carb liquid scintillation spectrometers 314 X and 314 EX-2 (Packard Instrument Co., La Grange, ILL.) were used throughout.

20 ml. low potassium vials #3001-1A with tin foil lined toluene resistant caps were obtained from Wheaton Glass Co., Millville, N.J.

Fluor Solution

0.4% 2, 5 diphenyloxazole (PPO) and 0.01% (POPOP) p-bis(2-(5-phenyloxazolyl) benzene, in toluene was used as a Fluor.

1 M Hydroxide of Hyamine 10-X in methanol was used as supplied from Packard Instrument Co.

Techniques - Sample preparation

When intact protein or hydroxyproline isolated by chromatography were counted, a measured aliquot of their solutions in water, containing a predetermined amount of sample, was placed in a spectrometer vial. Where possible, between 1 and 5 mgms. of protein was counted. The solution was evaporated to dryness in a desiccator and 1 ml. of Hydroxide of Hyamine solution (115) was added.

The tightly capped vials were heated at 56°C for a few hours until a clear solution was obtained. After cooling, 10 mls. of Fluor solution were added and the vials placed in the spectrometer to dark adapt. This was necessary since the fluor is excited by ultraviolet light from room lighting. Two hours of dark adaption was routinely employed.

In Experiment VIII where hydroxyproline was converted to pyrrole prior to isolation, no hyamine was needed to solubilise the samples in toluene.

Counting Techniques

When C^{14} or H^3 compounds are counted alone in the spectrometer, instrument settings are chosen which present maximum efficiency coupled with minimum background on one of the scalers only, the data on the other scaler being ignored.

Each sample was counted several times until a sufficient number of counts had been accumulated. The minimum counting time which would give statistically significant reproducibility was determined by the method presented on page 126.

After the initial count rate of each sample had been determined 1 ml. of internal standard in toluene containing a known amount of radioactivity; expressed as

as, disintegration per minute (DPM) was added to each. The internal standards consisted of C^{14} Benzoic acid or H^3 Toluene. The samples were then recounted as before. The increase in counts per minute caused by addition of standard permitted the counting efficiency for each sample to be determined,

$$\text{i.e.} \quad \frac{\text{Increase in CPM}}{\text{DPM added}} = \text{efficiency}$$

The initial count rate of each sample could then be converted to DPM,

$$\text{i.e.} \quad \frac{\text{Initial CPM}}{\text{efficiency}} = \text{DPM}$$

and,

$$\frac{\text{DPM}}{\text{amount of sample counted}} = \text{Specific activity}$$

Double Label Counting - (Okita et al (95))

Instrument settings were chosen which cause most of the counts due to tritium to fall on one scaler and most of the C^{14} counts on the other.

After initial counts are completed, tritium internal standard is added and the ratio of counts falling on the two scalers is determined. C^{14} is then added and a similar ratio determined. Okita et al (95) derived equations into which the above ratios are substituted, the equations are presented below together with specimen calculations.

Calculation of Specific activities

N_1 = Net cpm on scaler 1

N_2 = Net cpm on scaler 2

H_1 = net tritium cpm on scaler 1

C_2 = Net C^{14} cpm on scaler 2

a = Ratio of $\frac{\text{Increase due to } H^3 \text{ standard on Scaler 2}}{\text{Increase due to } H^3 \text{ standard on Scaler 1}}$

b = Ratio of $\frac{\text{Increase due to } C^{14} \text{ standard on Scaler 2}}{\text{Increase due to } C^{14} \text{ standard on Scaler 1}}$

$$H_1 = \frac{b\,N_1 - N_2}{b-a}$$

$$C_2 = \frac{b(N_2 - aN_1)}{b-a}$$

$$H^3DPM = \frac{H_1}{\text{Scaler 1 } H^3 \text{ efficiency}}$$

$$C^{14}DPM = \frac{C^2}{\text{Scaler 2 } C^{14} \text{ effieiency}}$$

SAMPLE CALCULATIONS

Single Label Counting

Mean initial count rate	=	1263 cpm
Mean background	=	16 cpm
Mean net initial count rate	=	1247 cpm
Mean count rate after addition of 584 DPM of same isotope	=	1650 cpm
Increase	=	403 cpm
Efficiency	=	$\frac{403}{584}$ = .69
Absolute count rate	=	$\frac{1247}{.69}$ = 1807 DPM
Weight sample counted	=	1.7 mgm
Specific activity	=	$\frac{1807}{1.7}$ = 1063 DPM/mgm

Double Label Counting

	Original Counts Per Minute		Counts Per Minute After Addition of 11475 DPM H 3		Counts Per Minute After Addition of 1633 DPM C 14	
	T_1	T2	T1	T2	T1	T2
$\bar{X}$ C.P.M.	257	497	958	1481	1173	2364
Increase = I =			701	984	215	883

Scaler 1 background = 50

Scaler 2 background = 65

$N_1 = T_1$ - background = 217

N_2 = T2 - background = 432

Scaler 1 H^3 efficiency = 0.061

Scaler 2 C^{14} efficiency = 0.51

$H^3 \frac{(I_2)}{(I_1)} = a = \frac{984}{701} = 1.40$

$C^{14} \frac{(I_2)}{(I_1)} = b = \frac{883}{215} = 4.10$

$aN_1 = 303$

$bN_1 = 890$

$b-a = 2.70$

$H_1 = \frac{bN_1 - N_2}{b-a} = 169$

H^3 DPM $= \frac{169}{0.061} = 2770$

$C^2 = \frac{b(N_2 - aN_1)}{b-a} = 195$

C^{14} DPM $= \frac{195}{0.51} = 382$

Mgms. sample counted = 5.35

H^3 specific activity in DPM per mgm. = 518

C^{14} specific activity in DPM per mgm. = 71

Determination of Minimum Counting Time

This is determined by substitution into the following equation [1]

$$t_s = \frac{K^2}{(\sigma N_s - N_b)^2} \quad (N_s + \sqrt{N_s \times N_b})$$

where t_s = minimum time that a sample must be counted so that the standard error will be within $\pm \sigma$ % of the true count rate.

σ = level of probability

N_s = Total count rate

N_b = Background count rate

K = Normal curve function i.e. 1.96 for 5% level of probability

example - if expected count rate is 50 cpm above background which is 16. Then at the 5% level of probability.

$$t_s = \frac{(1.96)^2}{\left(\frac{5}{100}(66-16)\right)^2} \quad (66 + \sqrt{66 \times 16}) = 60 \text{ mins.}$$

[1] Dr. D. A. Rigas - Personal Communication. February 1961.

APPENDIX 3

Imino Acid separation by Thin Layer Chromatography

Apparatus

Desaga-Brinkmann apparatus [1] was used.

The applicator was non adjustable giving a standard layer of 250μ thickness. The glass plates used were 8" x 8".

Reagents

Nitrous acid reagent was prepared by slowly adding 3 mls. of 40% aqueous sodium nitrite to 10 mls. of concentrated HCl in an ice bath.

Proline and hydroxyproline standards were purified by chromatography on Dowex 50 x 8 (H) resin.

Procedure

Protein was hydrolysed in 6N HCl for three and one half hours at 140°C in sealed tubes. Hydrolysates were decolorised with activated charcoal, filtered and evaporated to dryness under a stream of nitrogen. Excess nitrous acid reagent (1 ml/mgm of amino acids) was added to the hydrolysate residue, allowed to stand at room temperature for ten minutes then heated to 140°C until the color was faintly yellow. The acid mixture was evaporated in a steam bath and the residue extracted once with 5 mls. of ether to

[1] Supplied by Brinkmann Instruments, Inc.
115 Cutter Mill Rd., Great Neck, N.Y.

remove deamination products. Imino acids are insoluble and loss of nitroso derivatives was slight.

After ether extraction, the imino acids were taken up in 0.1 ml of 70% ethanol. This left much of the salt behind. This solution was used for chromatography.

Chromatography

Cellulose powder (MN300, Macherey, Nagel & Co. Duren) [1] was suspended in distilled water and plates were spread using the applicator.

40 gm. of cellulose in 200 mls of water was found suitable. Plates were air dired and stored in a desiccator.

The unknowns and marker spots of nitroso imino acids were applied to the plates in 10-20 microliter volumes containing 100-200 microgms. for 5 hours in butanol, acetic acid, water solvent (63;27;10 v/v), plates were dried at 110°C for 10 - 15 minutes. Marker spots were sprayed with 0.2% ninhydrin in acetone or 0.4% isatin in N-Butanol containing 4% acetic acid and heated for a few minutes at 110°C. Positions of the unknowns were marked by reference to the sprayed spots. A slightly larger area approximately 1 cm. beyond the apparent spot was scraped off the plate with a razor blade.

The cellulose was extracted twice with 10 mls of boiling distilled water.

[1] Supplied by Brinkmann Instruments, Inc.
115 Cutter Mill Rd., Great Neck, N.Y.

Determinations of proline, hydroxyproline and radioactivity are made on this eluate.

The determination of hydroxyproline by the method of Woessner (153) and the Proline determination of Troll and Lindsley (141) work equally well for the nitroso derivatives as for the imino acids themselves.

The method can be used for mixtures containing up to 0.5 mgm. of each imino acid per spot.

The overall recovery is between 58 and 63%.

APPENDIX 4

Hydroxyproline isolation by the toluene extraction method of Prockop et al (111)

Materials

The following solutions were used: 0.2 M chloramine-T (Eastman Organic Chemicals) in distilled water, prepared daily; 0.2 M sodium pyrophosphate, pH 8.0, in distilled water; 1.0 M sodium thiosulfate in distilled water; toluene (American Chemical Society specifications); 1.0 M Tris buffer, pH 8.0, in distilled water.

p-Dimethylaminobenzaldehyde or Ehrlich's reagent (analytical grade; Matheson, Coleman and Bell) was prepared in the following manner. Concentrated sulfuric acid (27.4 ml) was slowly added to 200 ml of absolute alcohol in a beaker and the mixture was cooled. In another beaker, 120 g of p-dimethylaminobenzaldehyde were added to 200 ml of absolute alcohol, and then the acid-ethanol mixture was slowly stirred into the second beaker. The solution can be kept in the refrigerator for several weeks. The crystals which precipitate upon cooling can easily be redissolved by warming the solution briefly [1].

A phosphor solution was made by dissolving 15 g of 2,5-diphenyloxazole (Pilot Chemicals, Inc.) and 50 mg of

[1] Occasionally it is necessary to recrystallize the p-dimethylaminobenzaldehyde from ethanol to minimize absorbancy of the reagent blank.

p-bis(2-(5-phenyloxazolyl) benzene (Pilot Chemicals, Inc.) in 1.0 liter of toluene. If the solution is protected from light, it can be stored in a refrigerator for several weeks.

The culture tubes used were screw capped with Teflon liners, 200 mm in length and 25 mm in outside diameter (Kimax).

Procedure

Samples were hydrolysed in 6 N HCl at 140°C for three hours. The hydrolyzates were evaporated to dryness and redissolved in about 5 mls of distilled water.

Oxidation and Conversion to Pyrrole

The pH was adjusted to 8.0 0.5 with dilute NaOH, and the final volume was made up to 8 to 10 ml. Pyrophosphate buffer (2ml) was added, and the sample was oxidized at room temperature with 0.5 to 2.0 ml of chloramine-T solution. After twenty minutes, oxidation was stopped with 20 ml of 1.0 M sodium thiosulfate.

Since the immediate oxidation products of hydroxyproline present at this stage are water-soluble, labeled organic-soluble materials, which would later interfere with the isotope counting, were removed by several extractions with chloroform and toluene. The pH was readjusted to 8.0 if necessary, and 6 ml of Tris buffer and 10 ml of chloroform were added. The top was secured and the tube shaken vigorously 100 times. The sample was

centrifuged briefly at low speed to insure separation of phases, and the chloroform layer was drawn off from the bottom. The chloroform must be removed completely to avoid quenching during counting. The solution was saturated with a liberal excess of sodium chloride and the aqueous phase was extracted four times with 10 to 15 ml of toluene, and a fifth time with 11.5 ml of toluene. The tube was shaken vigorously 100 times for each toluene extraction and centrifuged at low speed for complete separation of phases. The pH should be checked after each extraction, and occasionally it is necessary to add dilute alkali to maintain the pH at 8. A total of 10 ml of toluene from the final extraction was placed in a counting vial to serve as a blank for scintillation counting.

Conversion of Pyrrole

The residual toluene layer was discarded, and the tube was capped and placed in a boiling water bath for 25 minutes. This is sufficient to convert the intermediate oxidation products to pyrrole. The sample was then cooled under running tap water, 11.5 ml of toluene were added, and the tube was again shaken vigorously 100 times. After centrifugation, 10 ml of the clear toluene phase were transferred to a counting vial for radioactive measurement.

Color Reaction

A 0.5-ml aliquot of the same toluene solution was diluted with 4.0 ml of toluene, and 2 ml of the Ehrlich's reagent were rapidly mixed into the toluene. The color was allowed to develop at room temperature for 15 minutes, and the absorbancy at 560 mμ was determined. With a Beckman model B spectrophotometer, 0.10 μmole of redistilled pyrrole gives an absorbancy of 1.48 ± 0.01 units. The toluene aliquot must be free from any of the aqueous phase, since small amounts of sodium thiosulfate will cause the color to fade rapidly.

Effectiveness of Method

In order to check upon the usefulness of the method, trial runs were carried out to determine,

1) the amount of contamination of the isolated hydroxyproline derivative with radioactivity due to proline,

2) the recovery of hydroxyproline.

1) To three tubes containing varying amounts of unlabeled gelatin hydrolysate a sample of C^{14} proline was added and the separation procedure carried out.

Results

Sample No.	Micro gms. Hypro. added	DPM C^{14} Proline added	DPM found in pyrrole fractions	Percent added DPM
1	1160	41,388	138	.33%
2	290	41,388	119	.287%
3	116	41,388	230	.55%

2) 9.65 mgms. of gelatin were hydrolysed and the hydroxyproline content determined, i.e.
.108 mgm. hydroxyproline/ml.

A 1 ml. sample was carried through the separation procedure and the pyrrole determined.

Results

0.80 μM pyrrole recovered and this represents 0.80 μM of hydroxyproline.

Since 0.108 mgm. hydroxyproline was added = .82 μM

therefore recovery = $\frac{.80 \times 100}{.82}$ = 97.5% recovery

These results showed that the method of separation was suitable for use in Experiment VIII.

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