METABOLISM OF COLLAGEN IN MAMMALIAN TISSUES

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ABSTRACT The amino acid composition of collagen is described and the status of knowledge about the synthesis of its unique amino acids, hydroxyproline and hydroxylysine, presented. This is followed by a schematic overview of collagen metabolism. Scurvy and lathyrism, the only two abnormalities of collagen metabolism which can now be reasonably elucidated at a molecular level, are then discussed in some detail. The paper concludes by stressing the importance of recognizing the role of histoarchitecture and of interactions of collagen with other compounds when studying collagen or its metabolism in the whole animal.

A consideration of the metabolism of collagen in the mammalian organism and of modifying factors, must take into account not only the molecular structure of collagen but also its relation to other constituents of the tissue of which it is a part. The behavior of collagen, a polyelectrolyte, is modified by other polyelectrolytes commonly associated with it, *e.g.* mucopolysaccharides and (muco)-proteins, by smaller ions, and even by such non-electrolytes as urea. Although collagen shares some of the molecular and histologic characteristics of other proteins and is probably affected to some extent by most of the factors which affect protein metabolism in general such as caloric and nitrogen intake, hormones, etc. (1, 2), this paper will concern itself with problems of metabolism peculiar to collagen. Some of the more complex aspects of collagen structure which must be accounted for in metabolism have been discussed in preceding papers, but even its primary structure is unusual and may permit specific metabolic abnormalities.

AMINO ACID COMPOSITION

When the amino acid compositions of collagens from a variety of mammalian tissues and species are examined and compared with each other and with the amino acid composition of other proteins, it is apparent that the amino acid compositions of the several collagens are quantitatively as well as qualitatively very similar. This is not the case for hemoglobins, serum albumins, or many enzymes all of which may show great variation in amino acid composition among species. In terms of the cur-

rent view that the peptide chains of all proteins are synthesized on a ribonucleic acid template whose structure is maintained genetically, this uniformity of composition suggests that collagen appeared relatively early in mammalian evolution and that appreciable variation in composition is not compatible with successful function. Although it is in general implicitly assumed that synthesis of collagen polypeptides takes place on the ribosome (3, 4), the possibility that synthesis of the collagen polypeptide involves a different mechanism in which the structure is not immediately specified by a nucleic acid template has been suggested (5).

This impressive uniformity of mammalian collagen structure should not obscure the small differences between collagens from various tissues in the same species (4). These appear to be real and may have functional significance.

The composition of collagen as shown in Table I shows the unusual distribution

TABLE I

AMINO ACID COMPOSITION OF MAMMALIAN COLLAGEN

	Residues, *per cent*		
Glycine	33.5	Valine	2.4
Proline	12.0	Threonine	1.9
Hydroxyproline	9.5	Isoleucine	1.1
Alanine	10.5	Phenylalanine	1.2
Glutamic acid	7.2	Methionine	0.6
Arginine	4.6	Hydroxylysine	0.8
Aspartic acid	4.8	Histidine	0.5
Serine	3.6	Tyrosine	0.3
Lysine	3.0	Cystine	0.0
Leucine	2.4	Tryptophan	0.0

Typical data compiled from Eastoe (6) and Piez (7).

and nature of the amino acids. Collagen lacks two amino acids, tryptophan and cysteine, found in most other animal proteins, and has an exceptionally low content of aromatic amino acids. One amino acid, glycine, makes up a third and the imino acids, proline and hydroxyproline, a fifth of the amino acid residues. Hydroxyproline and hydroxylysine are found in collagen but in very few other proteins.

Hydroxyproline accounts for 9 to 10 per cent of the amino acid residues of collagen, and has been found in no other mammalian protein except elastin where it is present to the extent of about 1 per cent. The difficulties of purification of elastin and its constant association with collagen in tissues suggest that hydroxyproline in elastin may be a contaminant. The singular and massive occurrence of hydroxyproline in collagen has made it a useful measure for collagen and collagen metabolism. The hydroxyproline of collagen is predominantly 4-hydroxyproline, but, as has recently been recognized, as much as 4 per cent of the total may be 3-hydroxyproline (7).

The concentration of hydroxylysine in collagen is smaller and more variable within

a species than that of hydroxyproline, ranging from 0.8 per cent of the total residues in skin collagen to 2.8 per cent in dentin collagen (8). Despite the spate of amino acid analyses of protein since the advent of automatic analysis, hydroxylysine has turned up in only two other mammalian proteins, trypsin (9) and serum gamma globulin (10).

HYDROXYPROLINE AND HYDROXYLYSINE SYNTHESIS

The synthesis and incorporation of these hydroxyamino acids into collagen pose special problems for collagen synthesis and offer sites where the metabolism of collagen but probably no other protein may be affected.

Some of the earliest studies using *N*-15–labeled amino acids in animals showed that hydroxyproline could be derived from proline. This was not unexpected, but Stetten's (11) later observation that proline was a far more effective precursor of collagen hydroxyproline than hydroxyproline itself was indeed surprising and unique. The finding was confirmed and shown not to be due to an inability of hydroxyproline to get into the cell (12). The relation of lysine as a precursor of hydroxylysine in collagen is similar (13). These observations are understandable, if the hydroxyamino acids are not readily activated for protein synthesis and if proline and lysine are already committed to collagen synthesis when hydroxylation occurs. The suggestion of Stetten (11), still favored by some workers, is that proline is built into a peptide linkage and that some of the proline is then hydroxylated to yield the hydroxyproline-containing collagen peptide. This scheme is illustrated in Fig. 1.

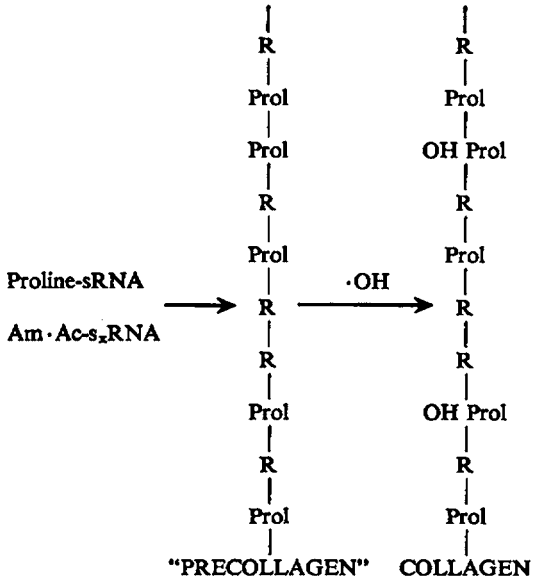

FIGURE 1 Suggested hydroxylation of proline in protein in collagen synthesis.

The search for a proline-rich, hydroxyproline-poor polypeptide precursor of collagen has been extensive but fruitless. The first compounds recognized as collagen during collagen synthesis have the same percentage composition of proline and hydroxyproline as mature collagen and the only hydroxyproline-containing proteins isolated appeared to contain the usual ratio of proline to hydroxyproline of collagen (14). Because of the possible very transitory existence of such an intermediate, negative evidence is not too decisive. Isotope distribution in collagen proline and hydroxyproline after administration of labeled proline, and in lysine and hydroxylysine after lysine administration, has been measured in order to obtain at least indirect evidence for an intermediate of this type. The results have been conflicting and discussion has centered about the purity of the isolated collagen and the statistical validity of the assays (15). However, the data of Green and Lowther (12) are, in general, accepted as valid and they obtained ratios of hydroxyproline to proline specific activity higher than 1.6 in collagen synthesized in the presence of proline-C-14, whereas the highest ratio to be expected if the proline were hydroxylated after incorporation into the protein would be 1.

These and similar data (16) suggest that proline and hydroxyproline in collagen may be derived from separate pools of proline which arise after it is bound but before formation of the peptide chains of collagen. Some of the possible stages at which hydroxylation might occur are illustrated in Fig. 2. This suggestion received con-

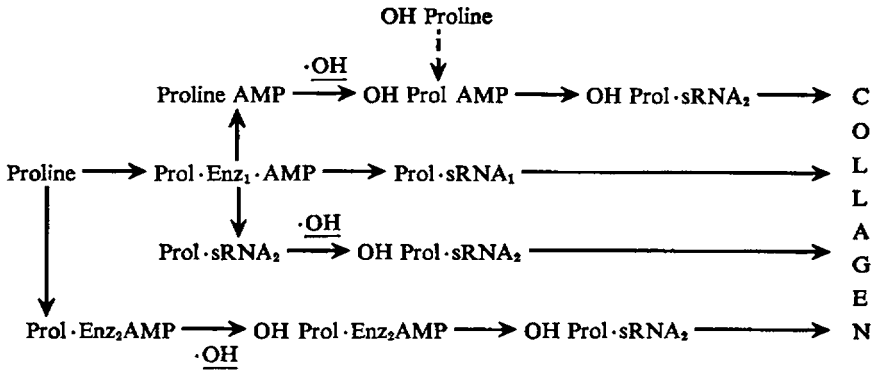

FIGURE 2 Possible sites for hydroxylation of proline to hydroxyproline in collagen synthesis.

siderable support when Manner and Gould (17) were able to isolate a s-RNA-hydroxyproline from a collagen-synthesizing system and show that the hydroxyproline was derived from proline. Meister (18) has been able to dissociate proline hydroxylation from collagen synthesis with puromycin. Highly labeled free hydroxyproline found when cartilage slices were incubated with proline-C-14 could have arisen from the hydrolysis of a labile intermediate (19). The observation by Mitoma (20) and by Stetten (11) that some free hydroxyproline is incorporated directly

into collagen can also be fitted into this scheme if some activation of hydroxyproline occurs.

Although the nature of the proline intermediate which is hydroxylated has not yet been identified, the nature of the hydroxylation has been somewhat clarified within the past year. Ebert (21), using tritium-labeled proline derived from dehydroyproline, showed that only one tritium atom was lost during hydroxylation. This would exclude consideration of mechanisms involving a hydration step either preceding or following a dehydrogenation, for such mechanisms predict loss of two tritium atoms. Complementary studies by Prockop (22) and by Fujimoto (23) using O^{18} showed that the oxygen of the hydroxyl group was derived from atmospheric oxygen and could not have come from water. Thus, this hydroxylation (Fig. 3) is similar to that proposed for hydroxylation of steroids and some aromatic com-

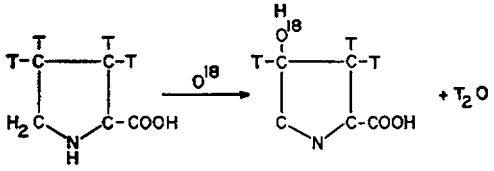

FIGURE 3 Mechanism of hydroxylation of proline.

pounds. The possibility that an oxygenase catalyzes this reaction has been suggested but no evidence for such an enzyme is available.

The hydroxylations yielding hydroxylysine or the minor hydroxyprolines have not been investigated in detail but we assume similar mechanisms are operative.

AN OVERVIEW OF COLLAGEN METABOLISM

Fig. 4 is a scheme of over-all collagen metabolism. Many details are omitted and in so far as it is based on a personal evaluation of data presently available it may be in error. The scheme is intended to serve only as a basis for the following discussion of abnormalities of collagen metabolism.

The early stages of collagen synthesis take place within the fibroblast; amino acids are activated, proline is hydroxylated to hydroxyprolines and lysine to hydroxylysine. Peptide subunits of about 250 amino acids and molecular weight 30,000 are assembled on the ribosome. Carbohydrate of unknown composition is added to aspartic acid residues at one end of the peptide and serves to link it with another peptide (24), thus alpha chains ($2\alpha_1$, and $1\alpha_2$) each containing four of these peptides and having molecular weight 120,000 are formed. The three alpha chains spontaneously associate to form the typical coiled coil of tropocollagen with molecular weight 360,000. Tropocollagen is secreted by the cell or possibly the free alpha chains are secreted and associate extracellularly.

At this stage the tropocollagen is extracellular and readily soluble in cold neutral salt solutions. Stronger bonds are gradually developed between the α subunits; they

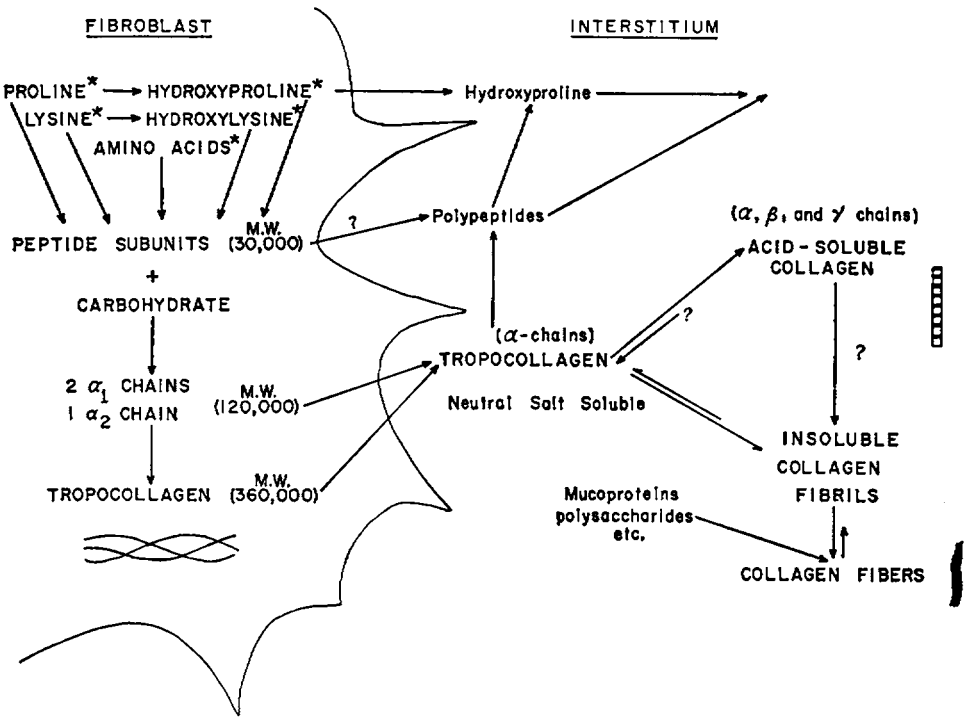

FIGURE 4 Collagen metabolism.

form β and γ components and eventually the insoluble collagen fibril. The nature of these bonds has not been clarified but it has been suggested that they include covalent bonds involving the carbohydrate moiety of collagen. The evidence that the series of collagen fractions obtained by extracting with increasing concentrations of neutral salt are precursors of the insoluble collagen fibril is rather conclusive (25). The collagen soluble in dilute acid was likewise considered as one in this series of precursors. Newer data (26) make this unlikely and we include it in the scheme as a metabolic by-product. The conversion of insoluble fibrils to the typical collagen fibers observed in the light microscope probably involves the formation of interfibrillar bonds. In tissues this takes place in the presence of mucoproteins, polysaccharides, lipids, etc. which either by occlusion or chemical interaction become part of the fiber and affect its morphology and behavior.

The metabolic inertia of the bulk of insoluble collagen has been amply demonstrated (27). While there seems to be essentially no turnover in the usual sense a rapid breakdown occurs under some circumstances; *e.g.*, during remodeling of bone and tissue and in the involuting postpartum uterus. This catabolism of insoluble collagen appears to involve a reversion to the neutral salt-soluble tropocollagen (28, 29) which may then be hydrolyzed to peptides and amino acids.

During the course of collagen synthesis, free hydroxyproline or hydroxyproline-containing peptides may appear as by-products at various stages up to and including tropocollagen. A fraction of these, not further metabolized, may appear in the urine. It has been suggested that this excretion of hydroxyproline may be used as a measure of collagen synthesis (30). The data, in general, seem to justify this interpretation but it cannot be accepted without reservation since tropocollagen and its breakdown products can also derive from catabolism of collagen. The elevated excetion of hydroxyproline as a result of burns probably arises from collagen breakdown (31).

ASCORBIC ACID DEFICIENCY

Of the several conditions in which collagen structure or metabolism is presumed affected only two have so far found, at least partial, explanations at the molecular level. These are scurvy and lathyrism. Scurvy, or more specifically ascorbic acid deficiency, is accompanied by a number of defects of which the inability to form normal collagen appears primary. This is seen most strikingly at sites of potential rapid collagen synthesis during tissue repair. Wolbach and other pathologists described in detail the morphologic phenomena associated with vitamin C deficiency a number of years ago (32), but only within the past decade have there been adequate biochemical demonstrations of the lesion and an investigation of its molecular basis (16). Typical of these studies are our own using the granuloma induced by carrageenan, a polygalactosan sulfate derived from a seaweed, Irish moss. This substance when injected subcutaneously into guinea pigs stimulates the rapid formation of a massive granuloma. When the animal is adequately supplied with ascorbic acid the granuloma obtained at 14 days contains about 14 to 16 per cent collagen. The granuloma growing in a guinea pig deprived of ascorbic acid during the 14 days contains about 2 to 3 per cent collagen. At this time the animal is not suffering from inanition, a complicating factor in many studies of scurvy. We have a scorbutic tissue in an essentially normal animal. If these guinea pigs are given ascorbic acid for 3 days, the collagen concentration increases to that of the normal granuloma.

In considering the various ways in which ascorbic acid might be involved in this rapid synthesis of collagen in new tissue it was necessary not only to examine the role of ascorbic acid in the steps of collagen synthesis and breakdown but also to determine first whether it acted directly on the collagen-synthesizing tissue or whether the primary action was somewhat removed, for example, on blood vessels or endocrine system. A direct relationship between collagen concentration and asorbic acid concentration in granulomas, as well as other indirect evidence suggested a local action of ascorbic acid (33). The enhanced conversion of proline to collagen hydroxyproline when ascorbic acid was added *in vitro* to suspensions of scorbutic granuloma cells finally demonstrated unequivocally the local action of ascorbic acid (34). These data and other *in vivo* measurements of collagen synthesis (16) showed

that a major consequence of an ascorbic acid deficiency was decreased collagen synthesis, not increased catabolism.

Gross's observation that the amount of tropocollagen was markedly decreased in skin of scorbutic guinea pigs when compared with pair-fed controls, suggested that the basic defect resided before the level of synthesis of the collagen molecule and not in the formation of intramolecular bonds leading to stabilization of the molecule (35). He also found that ascorbic acid had no specific effect on spontaneous *in vitro* fibril formation from tropocollagen. Confirming this *in vivo,* Jackson and Bentley (personal communication) found no impairment of conversion of soluble collagen to collagen fibers during an ascorbic acid deficiency. Incorporation of proline into proteins other than collagen was found to be unimpaired during ascorbic acid deficiency as was synthesis of an induced enzyme (16, 36). No generalized defect of peptide bond synthesis is found concomitant with the inability to synthesize collagen. The defect apparently lies somewhere between proline activation and synthesis of the protein. Because of decreased labeling of hydroxyproline in the several collagen fractions from the scorbutic granuloma, we suggested that during an ascorbic acid deficiency the conversion of proline to hydroxyproline was impaired. On the other hand, Mitoma (36) interpreted his studies of urinary excretion of hydroxyproline during scurvy as indicating that hydroxylation of proline was not decreased but that some defect existed so that hydroxyproline could not be built into a collagen peptide and was rapidly catabolized.

Earlier this year, Stone and Meister (37, 18) presented data which seem to resolve this question. The transfer of tritium from proline tritiated on positions 3 and 4 to water was measured (see Fig. 3) and found to be much lower in minces of scorbutic granuloma than in normal minces. The technique used was a measure of proline utilization rather than hydroxyproline accumulation and in conjunction with the C-14 incorporation data, provides fairly direct evidence for a marked decrease in the formation of hydroxyproline from proline in scurvy.

It now appears that the basic defect in collagen metabolism resulting from an ascorbic acid deficiency is impaired synthesis of at least one of the amino acids peculiar to collagen, hydroxyproline. Possibly hydroxylysine synthesis is also impaired.

What is the role of ascorbic acid in this hydroxylation? It might be as the coenzyme of the postulated, but elusive, oxygenase catalyzing hydroxylation, but about 2 years ago we speculated on the basis of a variety of suggestive evidence, which I will not now review, that the activity of ascorbic acid depended on its ability to form the monodehydro-ascorbyl radical and to catalyze the formation of hydroxyl radicals (16). Since then, although no decisive evidence has appeared, additional suggestive observations have been made. The mechanism of hydroxylation of proline, which was then unknown, has been elucidated (see above) and is consistent with an attack by a hydroxyl radical and not with dehydrogenation and hydration. The finding of

a second hydroxyproline isomer in collagen may reflect the relative non-specificity of free radical mechanisms. One more hydroxylation, that of γ-butyrobetaine to carnitine, has been shown to be ascorbic acid–dependent (38). We have recently shown that in the carrageenan granuloma D-ascorbic acid can replace the natural L-ascorbic acid in collagen synthesis, a lack of sterospecificity more consistent with the suggested free radical function of ascorbic acid than with a function as a coenzyme (39)

LATHYRISM

This condition originally observed in animals eating a diet of sweet pea, *Lathyrus odoratus,* meal, results in bone deformities, scoliosis, exostoses, and in aortic aneurysms (40). It can be reproduced by administration of the toxic agent of the sweet pea, β-aminopropionitrile glutamate, or other nitriles. Although abnormal metabolism of collagen as well as other connective tissue elements had been suspected, the microscopic appearance of the collagen and its concentration in a variety of tissues from lathyritic animals appeared essentially normal. A few years ago, several workers, almost simultaneously (41), found that a much larger percentage of the collagen of lathyritic animals than of comparable normal animals could be extracted with neutral salt solutions. The lathyritic factor was either solubilizing collagen fibers or blocking the process of fibril formation at the stage of tropocollagen or perhaps doing both. The difficulty in demonstrating a lathyritic effect in older animals and the striking effects demonstrable in young, rapidly growing animals and fetuses suggested that impaired fibril formation was the more likely. Several groups using labeled amino acids have been able to show that isotope piles up in the soluble collagen, very little being built into the insoluble fibril (42, 43).

As discussed earlier, fibril formation from tropocollagen appears to be a spontaneous phenomenon not requiring enzymes. Gross has studied this phenomenon following the precipitation and resolution of salt-soluble collagen when its temperature is first raised to 37° and then returned to 0°. The longer tropocollagen from normal animals is held at 37°, the less soluble it becomes at 0°. Purified lathyritic tropocollagen, on the other hand, retains the ability to redissolve upon cooling even after prolonged standing at 37°. Although a tropocollagen molecule is synthesized it appears to be defective in its ability to form fibrils (41). Studies of subunit structure of collagen confirmed this. Acid-soluble lathyritic collagen has been shown to have a ratio of 3 α chains to 1 β chain whereas in acid-soluble normal collagen the ratio is 1; and the data of Martin, Piez, and Lewis (44) show a normal or even supranormal incorporation of glycine-C-14 into α chains but very little transfer of isotope to β chains.

These results suggest that lathyritic agents lead to synthesis of defective α chains which no longer have the ability to cross-link adequately. The lathyritic and normal

collagens have the same amino acid composition but it appears that the lathyritic collagen also contains rests of the toxic aminonitriles (45). Gallop (24) has suggested that these might be combined with carbonyl groups of the carbohydrate moiety of collagen preventing the latter's function as a cross-linking agent. The exact nature of the defect remains to be elucidated.

Whereas in scurvy, a defect in the synthesis of an amino acid essential for collagen results in decreased collagen formation, the defect in lathyrism leads to synthesis of an abnormal collagen. In scurvy new connective tissue lacking collagen has essentially no strength, breaking down under the slightest stress; and hemorrhage results from even the hydrostatic pressure in capillaries. In lathyrism, on the other hand, the new connective tissue, while somewhat weaker than normal (46), becomes basically more plastic and tends to deform rather than rupture. In neither disorder is the molecular defect complete; *i.e.*, some synthesis of normal collagen persists and the pathology one sees is a resultant of the stresses and the need for new collagen. In both conditions evidence for an effect on already synthesized collagen has on occasion been presented (47, 48). This cannot be accounted for by the above explanations, if mature collagen is metabolically inert. While turnover studies are in agreement that the half-life of most insoluble collagen is very long, this is not true of collagen at all sites (49), and, as Gross pointed out (50), extensive remodeling of collagenous structures may take place in response to a variety of stimuli. Such breakdown combined with defective synthesis probably explains the loss of insoluble collagen which has been observed in both ascorbic acid deficiency and lathyrism.

HISTOLOGIC ASPECTS OF COLLAGEN METABOLISM

In addition to the two conditions discussed, there is evidence that a number of other factors acting in the living animal will preferentially affect collagen (51). Thus, growth hormone, estrogen, deoxycorticosterone, chronic anaerobiosis, and dilantin appear to enhance collagen deposition whereas parathyroid hormone, cortisol, and related steroids decrease collagen concentration. There are also a number of diseases in which it has been suggested that collagen metabolism is more or less specifically involved. These would include the so called collagen diseases or collagenoses and of course the congenital abnormalities of connective tissue so beautifully collated by McKusick (52).

Our present knowledge of collagen structure and metabolism in these various conditions is extremely limited and it would be rash to make interpretations at the molecular level similar to those for lathyrism and ascorbic acid deficiency. Those of us who have worked with either of these problems know the large number of false starts before the present stories emerged which despite their reasonableness and beauty may also be wrong.

The difficulties of elucidating the molecular basis of a presumed change in col-

lagen or its metabolism are compounded by the many factors at the histologic level which affect collagen. A brief consideration of these aspects of collagen and how they may affect its metabolism concludes this presentation.

Essentially all of the collagen in the body, and this is a sizable amount, making up about 30 per cent of the total proteins, is located outside of cells. Although it is possible using rather mild extraction procedures to dissolve some of this collagen, and this is referred to as soluble collagen, most of the collagen is not soluble in body fluids and there is no evidence for transport of collagen. Any soluble collagen *in vivo* probably exists as a microphase contiguous to the collagen fibrils. Thus, unlike most other extracellular proteins, collagen is synthesized and broken down where collagen is found. Anything which affects the fibroblast, however indirectly, might be expected to affect collagen.

Although collagen is ubiquitous in the organs of the body, its density and distribution in tissues are highly variable. It may appear as very fine fibrils in the vitreous humor or as coarse compact bundles in tendon and skin. The availability of the collagen molecule to substances which interact be it the nitriles of lathyrism or collagenolytic enzymes must differ in these tissues by several orders of magnitude. Some of the collagenous tissues are very vascular permitting a rapid exchange of soluble substances with blood, and rapid mobilization of cellular elements from blood, while others such as cartilage or heart valves are essentially avascular and exchange is relatively slow. In some tissues such as tendon, there are so few cells that they have very little impact on the collagen; in other tissues, for example, basement membrane, collagen exists in close proximity to many actively metabolizing cells and is exposed to their secretions or upon cytolysis to their contents.

Not only the cellular environment of collagen varies from one tissue to another but also the chemical environment of collagens may be strikingly different. Collagen is found closely associated with minerals in bone and teeth, with large amounts of mucopolysaccharides, which in each case may differ, in cartilage, skin, vitreous humor, etc., and with unsaturated lipids in reticulin. The association with different substances may not only alter the appearance and reactivity of the collagen, but primary abnormalities in the metabolism of these substances may easily be misinterpreted as abnormalities of collagen.

The metabolism of many individual proteins in the body may be considered a random process from the molecular point of view—one molecule has the same chance of catabolism as any other. Collagen molecules on the other hand are each part of a connective tissue continuum so that in spite of the similarity of basic molecular structure the metabolism of collagen in each tissue requires special consideration in which the role of histoarchitecture is commensurate with molecular mechanisms.

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