RECENT TRENDS IN THE BIOCHEMISTRY OF THE GROUND SUBSTANCE OF THE CONNECTIVE TISSUES

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THE UBIQUITY of members of the anatomical group loosely defined as *connective tissues* is a matter of long-standing record. The same comment applies to the convention that, despite the differing biological roles which they are required to play and their characteristically different mechanical character at macroscopic level, the individual members of the connective tissue group exhibit certain basic similarities which first become apparent at microscopic level. A common biochemical approach is to develop these features of similarity to a point where an idealised composite of all connective tissues can be devised. Use of such a model allows initial broad generalisations to be drawn. On the other hand, the same model must subsequently be employed to establish not only morphological but fundamental biochemical differences which exist between the tissues.

A model connective tissue is usually conceived as containing relatively few cells per unit volume compared to a highly cellular organization such as liver and additionally differentiated from the latter by the presence of a significant quantity of extracellular material. This material is characterised by the presence of organised fibrillar elements, composed variously of the biochemically distinct proteins collagen, elastin and reticulin, these being embedded in an amorphous medium termed the ground substance.

In this context, it is fitting that laboratories in the Cardiff area should be connected in studies on all aspects of pulmonary dust disease and in particular of coal miner's pneumoconiosis, progressive massive fibrosis and silicosis. The need for such work is presented with considerable impact in the observations of Gough (1947). Much has already been established about the statistical incidence and pathological characteristics of such dust diseases, but their true aetiology is still a matter for conjecture (Policard, 1962). However, from the simplified histological standpoint the human lung lesion in such conditions shows characteristically an increased 'fibrosis' when compared

to the surrounding tissue, the fibres usually being described as collagenous. As yet there is no clear indication whether such fibroses arise according to the partially established pattern of inflammatory response in which the earliest recognisable biochemical changes include alterations in the ground substance of connective tissues followed only subsequently by *de novo* formation of additional fibrous material. It is for this reason that the research group housed in the Department of Biochemistry is designed to assist in the clarification of fundamental biochemical changes associated with general lung dust diseases from the aspect of disturbed connective tissue metabolism. Such an approach must obviously take into account the many recent developments concerning the nature and properties of the structural elements of the connective tissues and in particular the intrinsic complexity of the ground substance.

The Ground Substance

There is now no doubt that histological examination of connective tissues using conventional techniques provides a wholly incomplete and frequently artificial picture of the complexity of the ground substance. It has even been established that long-used methods of tissue fixation may even facilitate the extraction of ground substance constituents (see Szirmai, 1963). Thus, even for relatively gross histological observations such methods must be replaced by the use of modified tissue fixatives (Quintarelli, Scott and Dellovo, 1964a) or alternatively frozen section techniques in which 'fixation' is virtually carried out in the dye bath (Szirmai, 1963). Methods have become available for differentiating some of the ground substance components by selective physico-chemical *blocking* and *unblocking* in the presence of quaternary ammonium compounds (Kelly, Bloom and Scott, 1963), by selective staining in the presence of solutions of varying pH or electrolyte concentration (Szirmai, 1963; Saunders, 1964; Quintarelli,

Scott and Dellovo, 1964b) and by selective enzymic digestion (Zugibe, 1962 a and b). However, such techniques continue to be the province of the specialist in the field, rather than methods of routine applicability. As such, much reliance must still be placed on approaches based on the extraction of connective tissue components followed by their characterisation using physical, chemical and biochemical methods. Even in this regard studies initiated during the middle of the last century and subsequently prosecuted with increasing vigour are still the subject of continuous contemporary reappraisal.

Polysaccharide-Protein Complexes of the Ground Substance

The Polysaccharides

Early studies on these materials invariably involved treatment of connective tissues under relatively vigorous conditions such as prolonged digestion with proteolytic enzymes followed by extraction with strong salt solutions or even dilute alkali. The resultant extracts yielded, by several methods of purification, varying amounts of one or more of a group of carbohydrate high polymers. These have been variously and synonymously termed *acid mucopolysaccharides*, *acid aminopolysaccharides* or more recently *acid glycosaminoglycans* (Jeanloz, 1960). In defining the purity of such materials early workers attached much significance to the absence of amino acid and polypeptide residues from the polysaccharide preparations and when demonstrated such 'contamination' was frequently attributed to inefficiency of purification procedures. Structural studies (reviewed by Jeanloz, 1963) on these compounds reveal a pattern of similar molecular organization. Thus, in the main they are relatively high molecular weight polysaccharides having linear chains containing hexosamine residues. The latter may be either D-glucosamine or D-galactosamine each occurring in a state in which the amine grouping is blocked. The hexosamines alternate regularly in the polymer chains with other monosaccharide residues which may be hexuronic acids, such as D-glucuronic acid and L-iduronic acid or a hexose such as D-galactose.

All of the polysaccharides are also poly-anions, the negative charge arising either from the carboxyl group of the above uronic acids or from the presence of ester bound sulphate, or both. Accepted structures show the potentially charged groups arranged regularly along

the polymer chains. As both carboxyl and sulphate groups are fully ionized at physiological pH the resultant highly negative character is likely to have considerable bearing on the functions attributed to the polymers in the native state.

The acidic glycosaminoglycans recognised to date fall into four classes, differentiated by features of chemical fine structure:

A. Polysaccharides having anionic character conferred solely by hexuronic acid constituents e.g., *hyaluronic acid* and *chondroitin*.

B. Polysaccharides having anionic character resulting from the dual presence of hexuronic acid and O-sulphate groups e.g., *chondroitin 4-sulphate* (syn. *chondroitin sulphate-A*), *chondroitin 6-sulphate* (syn. *chondroitin sulphate-C*) and *dermatan sulphate* (syn. *chondroitin sulphate-B*, β -*heparin*).

C. Polysaccharides having O-sulphate groups as sole functional anionic entities e.g., *keratan sulphate* (syn. *keratosulphate*).

D. Polysaccharides characterised by the presence of hexuronic acid, O-sulphate and N-sulphate residues e.g., *heparan sulphates* (syn. *heparitin sulphates*). Heparin and ω -heparin may also be included in this class for the sake of completeness.

The compositions of each of these materials is shown in Table 1 and the major repeating periods of the polysaccharides from Classes A, B and C are summarised in Figs. 1, 2 and 3 respectively. These should be considered to be structures derived on the principle of best analytical evidence. While there is little doubt that they account for the format of the major portion of the polysaccharide chains there are indications that some regions of the polymers are not constructed in this way. Such evidence will be dealt with later. It should also be noted that variability of sulphate analysis is not an uncommon occurrence and that acid glycosaminoglycan preparations with sulphur to nitrogen ratios both greater and less than unity have been isolated. Conclusive data allowing the assignment of wholly acceptable repeating period structures for heparan sulphates (and for heparin and ω -heparin) has yet to be obtained (see Muir, 1964).

Aside from the significant contributions which they have made in structural work on the acid glycosaminoglycans, elegant studies made by Professor Karl Meyer and his colleagues lay a foundation for the concept that connective tissues fulfilling distinctive biological functions are also provided with differing

TABLE 1
MAJOR CONSTITUENTS OF THE ACIDIC GLYCOSAMINOGLYCANS

Polysaccharides	Hexosamine	Other Monosaccharide	N-Acetyl Group	O-Sulphate Group (s)	N-Sulphate Group
CLASS A					
Hyaluronic Acid	D-Glucosamine	D-Glucuronic Acid	+	—	—
Chondroitin	D-Galactosamine	D-Glucuronic Acid	+	—	—
CLASS B					
Chondroitin 4-Sulphate	D-Galactosamine	D-Glucuronic Acid	+	+	—
Chondroitin 6-Sulphate	D-Galactosamine	D-Glucuronic Acid	+	+	—
Dermatan Sulphate	D-Galactosamine	L-Iduronic Acid	+	+	—
CLASS C					
Keratan Sulphate	D-Glucosamine	D-Galactose	+	+	—
CLASS D					
Heparan Sulphates	D-Glucosamine	D-Glucuronic Acid	+	+	+
Heparin	D-Glucosamine	D-Glucuronic Acid	—	+	+

FIG. 1.—Structures of the repeating periods in hyaluronic acid (a) and chondroitin (b).

complements of the acidic polysaccharides (Meyer, Davidson, Linker and Hoffman, 1956). Table 2 is a summary of part of the original work plus some additional information, giving analyses for tissues of average chronological age. On the other hand, it should also be stressed that the content of acid glycosaminoglycans must not be considered to be static throughout life. Thus, apart from variations due to metabolic turnover, changes both in overall content and in the concentration ratios of individual polysaccharides as a function of age have also been observed. For example, embryonic pig skin polysaccharides are comprised of 9.5% dermatan sulphate, 71% hyaluronic acid and about 20% of a mixture of chondroitin 4-sulphate and chondroitin 6-sulphate. Analyses of adult pigskin reveals a sharp change in ratio corresponding to 64%

FIG. 2.—Structures of the repeating periods in chondroitin 4-sulphate (a), chondroitin 6-sulphate (b) and dermatan sulphate (c).

FIG. 3.—Structure of the repeating period in keratan sulphate.

TABLE 2

DISTRIBUTION OF ACIDIC GLYCOSAMINOGLYCANS IN MAMMALIAN CONNECTIVE TISSUE
POLYSACCHARIDES

Tissues	Hyaluronic Acid	Chondroitin	Chondroitin 4-Sulphate	Chondroitin 6-Sulphate	Dermatan Sulphate	Keratan Sulphate	Heparan Sulphate	Heparin
Synovial Fluid	+	—	—	—	—	—	—	—
Cartilage	—	—	+	+	—	+	—	—
Bone	—	—	+	+	—	+	—	—
Tendon	+	—	—	+	+	—	—	—
Ligamentum Nuchae	+	—	+	—	+	+	—	—
Skin	+	—	±	±	+	—	+	+
Aorta	+	—	+	—	+	—	+	+
Vitreous Humor	+	—	—	—	—	—	—	—
Cornea	—	+	+	—	—	+	—	—
Sclera	—	—	+	+	+	—	—	—
Umbilical Cord	+	—	—	—	—	—	—	—

dermatan sulphate, 30% hyaluronic acid and some 0.1% of the chondroitin 4-sulphate-chondroitin 6-sulphate mixture (Loewi and Meyer, 1958). Similar effects have been observed in rat and in man (Loewi, 1961; Prodi, 1964). In addition Kaplan and Meyer (1959) recorded that in human costal cartilage there is a decrease in the overall content of acidic glycosaminoglycans with age. The same authors have shown that in phase with this decrease the constituent polysaccharides change from exclusively chondroitin 4-sulphate in foetal costal cartilage to a mixture of chondroitin 6-sulphate and keratan sulphate in cartilage from the same location in adults. These selected examples can be considered to be representative of phenomena which are likely to become the rule for all connective tissues rather than exceptional observations.

The distributions summarized in Table 2 are based on analyses of 'bulk' samples of material where the morphological integrity of the tissue is destroyed at an early stage of the extraction procedure and therefore correspond to average results for fairly large volumes of tissue. As such they give no indication whether the polysaccharides are arranged homogeneously throughout the whole ground substance or whether in fact they are the subject of concentration and localisation effects in relation to the fine structure of the tissue. Such information is clearly of prime importance to assessments of the functional significance of individual acid glycosaminoglycans. Antonopoulos, Gardell, Szirmai and de Tyssensk (1964) have recognised the need for means to carry out precise surveys. These authors are responsible for the development of methods which allow alternate tissue sections (10-40 μ thick) to be examined qualitatively by histological means and quantitatively by micro-scale modifications of established chemical methods. Data collected

in this way allows the construction of concentration (and tentative identity) profiles through a given tissue. The methods have been applied to studies on bovine nasal septum and rabbit intervertebral disc (Antonopoulos and others, 1964) and to epiphyseal plates from normal and rachitic dogs (Hjertquist, 1964a, b). The resulting observations establish that the acid glycosaminoglycans are subject not only to gross variation from tissue to tissue and to age fluctuations in the same tissue but also to three dimensional variations in a given tissue of defined age.

The Complexes

The 'pure' polysaccharides required for structural studies do not exist as such at tissue level and are, in a sense, chemical artifacts produced by the relatively vigorous means used for extraction. Application of milder methods, such as physical disintegration of the tissue by high speed homogenisation, yields the acid glycosaminoglycans linked firmly to protein moieties by covalent bonds (see for example Shatton and Schubert, 1954; Gerber, Franklin and Schubert, 1960). Erroneous implications should not be drawn from the persistent use of the generic nomenclature of polysaccharide-protein *complexes* for these materials when obviously the term *compound* would be far more appropriate.

In recent years the most extensively studied complex is that derived from various kinds of cartilage. Preliminary work conceived the cartilage *chondromucoprotein complex* as consisting of numerous polysaccharide chains (largely chondroitin 4-sulphate or 6-sulphate plus some keratan sulphate) firmly attached to a median, non-collagenous protein core (Partridge, Davis and Adair, 1961). It is a characteristic of the cartilage complexes that

the polysaccharide moieties are cleaved irreversibly from the protein chain by treatment with dilute alkali (Muir, 1958; Partridge and Davis, 1958; Malawista and Schubert, 1958; Partridge and Elsdon, 1961). However, the work of Muir (1958) established that when the isolated complex from cartilage was exhaustively degraded with proteolytic enzymes *in vitro* the polysaccharide preparations subsequently isolated were still firmly associated with serine-rich polypeptide stubs. Such observations focussed considerable attention on the possibility that serine residues in the original core material were intimately concerned in the linkage of polysaccharide and protein. The value of this initial work is illustrated by the recent isolation from a chondroitin sulphate-protein complex of carbohydrate residues linked directly to serine (Rodén, Gregory and Laurent, 1964). Evidence has been presented (Dr. L. Rodén, personal communication) that a novel carbohydrate linkage sequence, comprising xylose and galactose moieties, is involved as a bridge between the serine hydroxyl and the characteristic repeating periods of the glycosaminoglycan chain. These observations are by no means unique to the chondroitin sulphate-protein complexes as similar linkage arrangements are also found in heparin-polypeptide complexes (Lindahl and Rodén, 1964). Heparan sulphate-protein complexes are likely to be bonded in an analogous fashion (Jacobs and Muir, 1964).

Treatment of glycosaminoglycan-protein complexes with alkali and analysis of the products of reaction provides a relatively rapid means for assessing the participation of serine and threonine in glycoprotein linkage (Anderson, Seno, Sampson, Riley, Hoffman and Meyer, 1964; Anderson, Hoffman and Meyer, 1965). Studies using these alkaline degradation methods reveal the involvement of hydroxylated alkyl amino acid residues in linkages between protein and chondroitin 4-sulphate, chondroitin 6-sulphate and skeletal keratan sulphate. However, bonds in the corresponding complexes of chondroitin, dermatan sulphate and corneal keratan sulphate are suggested to be of the asparaginyl-glycosyl type as found in α_1 -glycoprotein (Professor K. Meyer, personal communication).

There is no clear indication whether a single type of acid glycosaminoglycan may be attached to protein cores of different amino acid constitution. However, it has been shown that bovine nasal septum cartilage yields two varieties of chondroitin sulphate-protein complex, distinguished by their differential sedimen-

tation in the ultracentrifuge (Gerber, Franklin and Schubert, 1960). The large particle fraction (designated PP-H) has a substantially higher protein content than the lighter (so-called PP-L) fraction. Both PP-H and PP-L also contain small amounts of materials recognisable as keratan sulphate and sialic acid. Differences have been noted in the PP-H: PP-L ratio in cartilages from varying anatomical locations. Thus, human costal cartilage, in contrast to bovine nasal septum, contains a large proportion of PP-H and a correspondingly smaller amount of PP-L, both fractions having a higher content of keratan sulphate and proportionately less chondroitin sulphate. Furthermore, human costal cartilage PP-L shows two distinct components on electrophoresis (Schubert, 1964). No information is yet available regarding a similar pattern of behaviour for the protein complexes of other acid glycosaminoglycans.

Lastly, it is necessary to record that hyaluronic acid exists naturally in firm combination with protein (Rogers, 1961). Even in this instance complicating features have arisen since it has been suggested that up to 10% of the hyaluronic acid molecule may not be constructed according to the accepted repeating period plan (Montgomery and Nag, 1963).

The examples given are designed to show the new approaches which are being made in studies on the chemical constitution of the ground substance. However, they also serve to illustrate the complexity of the situation which exists in a highly vascular organ such as lung where biochemical events take place simultaneously at the interfaces of several distinct types of connective tissue.

The Biological Significance of the Complexes

It is now known that the biosynthesis of the complexes takes place inside the appropriate connective tissue cells. For example, studies with inorganic [35 S]-sulphate and [14 C]-labelled amino acids reveal the concentration of the radioisotopes predominantly in the chondrocytes of bovine costal cartilage slices *in vitro* (Campo and Dziewiatkowski, 1962). The same studies point to the simultaneous formation of the polysaccharide and the protein moieties of the chondroitin sulphate-protein complex. However, it is still not clear at what stage in the synthesis union between polysaccharide and protein is achieved. Godman and Lane (1964) have shown the localisation of [35 S]-sulphate, visualised autoradiographically, in vesicles of the juxtannuclear Golgi apparatus of chondro-

cytes, suggesting that the sulphation of the carbohydrate moieties at least occurs in this region of the cell. Subsequently, it is believed that the molecules of the intact complex are conveyed through the cytoplasm to the periphery of the cell in vesicle-like structures and then discharged into the extracellular matrix via stomata in the cell membrane (Godman and Porter, 1960; Sheldon and Robinson, 1960; Godman and Lane, 1964).

Once in the extracellular environment there is no doubt, for the chondromucoprotein complex of cartilage at least, that the biological stability and consequent functional significance of the polysaccharide chains is directly dependent on the integrity of the protein core. The remarkable finding that intravenous injection in young animals of the proteolytic enzyme papain produces the now renowned 'flop-ear' rabbits (see Thomas, 1956) may be explained in terms of *in situ* degradation of the protein core of the ear cartilage complex. In addition to the effect on ear cartilage the simultaneous and more widespread action of the enzyme throughout the body is illustrated by the disappearance of the matrix in all forms of cartilage as evidence by basophilic and metachromatic staining reactions. Similar experiments show the disappearance of extractable chondroitin sulphate from these tissues (Tsaltas, 1958) and its appearance in blood and urine (Bryant, Leder and Stetten, 1958). Papain introduced in this way shows greatest activity in young animals and towards cartilage, older animals becoming progressively more resistant presumably in phase with changes in the constitution of the ground substance. It is now known that such findings are not restricted to the injection of papain but also accompany the administration of ficin and bromelain (McCluskey and Thomas, 1958), while chondroitin sulphate is also liberated from cartilage by the action of plasmin (Lack and Rogers, 1958; Anderson, 1962). An endogenous protease, liberated *in vivo* or *in vitro* from mammalian lysosomes by the action of vitamin A etc. and its action on chondroitin sulphate-protein complexes, has been the subject of extensive examination (see Dingle, 1964, for the most recent review). To date attention has been devoted almost exclusively to studies on the degradation of cartilage protein-polysaccharide complexes and in consequence there is no appropriate information regarding the biological stabilities of the other materials.

In terms of percentage dry weight the protein complexes of the acidic polysaccharides appear

to represent in many instances only an insignificant part of the ground substance of the connective tissues. However, considered as centres of intense anionic character there can be little doubt that in the tissues they are likely to exist as a focus for the concentration of inorganic cations. They must also participate in electrostatic interactions with proteins exhibiting cationic properties at physiological pH. Obviously, highly ionic arrangements of this sort can only exist at tissue level in a state solvated with water molecules. The concept of 'dry weight' thus has little bearing on the physical existence of the complexes in the biological environment. It is conceived (see Schubert, 1964) that the complexes occur naturally as diffuse, extended macromolecular structures, each occupying an aqueous 'domain' that is large in comparison to the absolute weight of the complex. The interaction of each domain is envisaged as producing a three dimensional network, the pore size of which must obviously be governed by the degree of solvation, the intensity of charge and the chemical and physical fine structure of each complex. Experimental evidence suggests that apart from a differential ability to bind cations according to the nature of the constituent glycosaminoglycans (Dunstone, 1960, 1962; Mathews, 1964) such three-dimensional molecular organizations should also be able to entangle particles (Laurent, 1964) and exclude large solutes (Gerber and Schubert, 1964). Viewed in this way it is tempting to suggest that the apparently precise biological allocation of structurally different anionic polysaccharide-protein complexes is a direct function of their variable properties in controlling ion-binding, water content and even the porosity of the tissue as a whole.

Opinions vary as to the extent to which the acidic glycosaminoglycans and their protein complexes participate in collagen fibrogenesis, both in the normal tissue and during the course of an inflammatory response. In some instances it has been suggested that because of their highly regular repeating character the polysaccharides may function intimately as templates or organizers in determining the size and geometrical arrangement of collagen fibres in the connective tissues (Meyer, 1960). Studies designed to explore the effect of chemically-pure acidic polysaccharides on the formation of collagen fibres *in vitro* tend to contradict this view. Gross (1959) found that a number of acidic glycosaminoglycans were

without measurable effect on the rate of collagen fibril formation. The observations of Wood (1960) were partially at variance with the earlier findings in so far as chondroitin 4-sulphate, chondroitin 6-sulphate and a preparation rich in keratan sulphate accelerated fibril formation markedly. Dermatan sulphate and hyaluronic acid had relatively little effect, while heparin actually inhibited precipitation. It is now obviously essential to repeat such *in vitro* studies on collagen fibre formation as a function of the presence of the intact polysaccharide-protein complexes and not merely the polysaccharides themselves. In the meanwhile it must be considered tentatively that the complexes are concerned with fibre binding and the limitation of collagen mobility in the three dimensional net of the ground substance.

Acid Glycosaminoglycans in the Inflammatory Response.

The last two decades have seen an expanding interest in the biochemical events involved in the processes of inflammation and wound repair. Perhaps the most astonishing feature of the ensuing work is the diversity of techniques and materials used in the induction of the inflammatory focus. Broadly speaking these fall under four major headings.

a. *Wounding.* Included in this section are observations on various cutaneous incisions and burns induced in mouse, rat, guinea pig, rabbit and man, together with studies on regenerating tendon in rat and guinea pig and bone fractures in various experimental animals.

b. *Injection or implantation of 'resorbable' substances.* The most widely examined responses here are granulomata induced subcutaneously, intraperitoneally and even in the cornea of several species of experimental animal by the introduction of agar, carrageenin, alginate acid, croton oil, oil of turpentine etc.

c. *Injection or implantation of 'non-resorbable substances'.* Materials used in such experiments have included sections of polyvinyl sponge, cotton pellets, perforated stainless steel cylinders, mineral particles including talc, silica, coal and asbestos these being located in subcutaneous, intraperitoneal and pleural regions of various animals.

d. *Lesions induced topically by irritation or other means.* Examples here include skin irritation produced by various hydrocarbon oils and exposure to high frequency current or ionizing radiations.

It is surprising to note that the biochemical changes associated with connective tissue com-

ponents apparent in each of these instances follows an almost identical broad sequence irrespective of the species of animal employed, differences in anatomical location and variations in the causative agent. Observed deviations from the sequence are almost invariably of degree rather kind.

Where measured it has been found that the earliest detectable biochemical event involves a sharp, time-based increase in the nucleic acid (both DNA and RNA) content of the tissue in the vicinity of the inflammatory focus evidencing considerably elevated cellular activity. Such observations have been attributed both to the invasion of the area by cells and to increased cell division.

Within a very short period after injury histochemical observations also show pronounced changes associated with the acid glycosaminoglycans of the ground substance. Both metachromatic and colloidal iron staining reactions are considerably intensified, a maximum usually being reached in 72-96 hours. Thereafter, there is a gradual decline in the intensity of stain in healing wounds and in areas of resorbing granulomata but a persistence in both surrounding non-resorbable material.

The results of microscopic examination are substantiated by biochemical studies. Invariably the latter have involved analyses made for total hexosamine as a measure of overall glycosaminoglycan content, or for the individual hexosamines D-glucosamine and D-galactosamine as an index of the presence of the corresponding parent polysaccharides. Alternatively, the magnitude of the uptake of inorganic [³⁵S] sulphate has been used as an indication of altered synthesis of sulphated glycosaminoglycans. The amounts of total hexosamine and [³⁵S] sulphate in inflamed areas have been shown to increase abruptly, in some instances in parallel with the content of DNA and usually within 24 hours after injury. Maxima are reached in both series of determinations in a matter of days, and may persist at these levels even after the nucleic acid content has declined to 'normal' values. The rate of decline of hexosamine and [³⁵S] sulphate values, like the staining reactions, is a function of the material or technique used to produce the lesion and a persistence at levels above 'normal' is noted in areas surrounding non-resorbable material. Usually, in healing wounds and resorbable granulomata a return of hexosamine level to average values for the tissue is recorded by the time active formation

of collagen fibres is already under way. In some instances the actual nature of the acidic glycosaminoglycans responsible for these analytical changes has been examined but the polymers produced and their ratios one to another are not unexpectedly varied according to the site of injury. However, as a general rule non-sulphated polymers such as hyaluronic acid tend to appear before the sulphated polysaccharides. No observations have yet been made concerning the protein moieties associated with the polysaccharides. In this regard it has been shown that the acid glycosaminoglycans of inflamed areas tend to become progressively more resistant with time to extraction with neutral salt solutions.

The significance of such changes in the ground substance in relation to fibrogenesis remains unclear, but may be considered tentatively as preparing the extracellular environment for the appearance of newly formed collagen. (Because of the large number of publications in this field individual references have been excluded to ensure continuity).

Studies on Normal and Diseased Lung

There are few recorded attempts to apply such methods to the study of normal lung and to pulmonary dust diseases in experimental animals and man. The interpretation of the results of such studies is also likely to be a complex matter. The connective tissues of the average normal lung are considered to contain dermatan sulphate, heparan sulphates and heparin (see Muir, 1964), each presumably stabilised as its protein complex. The presence of the chondroitin sulphates and keratan sulphate has also been detected in the vicinity of major airways, the content of the polymers increasing in progressive massive fibrosis in man (A. G. Lloyd, unpublished results). Constitutional studies will also have to take into account the various mucous secretions (Jakowska, 1963).

Studies on age-related changes in the connective tissues of lung have been directed largely to observations on the fibrous elements. Thus, it is now accepted generally that the proportion of lung 'collagen', measured in terms of hydroxyproline analyses per unit weight, increases in age in mice, rats, guinea pigs and humans (Elster and Lowry, 1950; Slutskii and Sheleketina, 1959; Briscoe, Loring Chvapil, 1957, 1960; Chvapil and Roth, 1964; and McClement, 1959; Boucek, Noble and Marks, 1961). However, there is also tentative evidence for the doubling of the elastin content

of human lungs between the ages of 25 and 75 with no alteration in collagen content (Pierce, 1962).

Variation in the polysaccharide constitution of lung in experimental animals and man has been studied only through the medium of hexosamine analysis. Thus, Saltzman, Schauble and Siecker (1961) record little change with age of overall hexosamine content and in the ratio of galactosamine and glucosamine in lung parenchyma in rabbit and man. The same authors indicate statistically significant alteration of these values in lung parenchyma in certain cases of chronic disease. In contrast to such regions Saltzman, Siecker and Green (1963) indicate that there are significant age related decreases in bronchial cartilage hexosamine content and the galactosamine: glucosamine ratio in the absence of lung disease. It is interesting to compare these results with analogous age-related effects in rib cartilage and nucleus pulposus (Shetlar and Masters, 1955; Stidworthy, Masters and Shetlar, 1958; Kaplan and Meyer, 1959; Davidson and Woodhall, 1959). On the other hand, bronchial cartilage from emphysematous lung maintains a persistently higher ratio of galactosamine to glucosamine with advancing age in contrast to the progressive decrease observed with senescence in specimens derived from normal lung.

In relation to pulmonary dust disease Harington, Marasas, Melamed, Sutton and Dreosti (1960) were unable to show appreciable increase in total hexosamine content of guinea-pig lung after dusting for periods up to 70 days. However, these observations should be considered simultaneously with the report of Bailly, Kilroe-Smith and Harington (1964) which shows a substantial increase in lung weight in dusted guinea pigs, the increase being due largely to materials low in hydroxyproline content. Lastly note should also be taken of observations of excessive production of hyaluronic acid in pleural mesotheliomas (Wagner, Munday and Harington, 1962).

Conclusions

It must be clear from the foregoing that much remains to be elucidated regarding the nature and properties of connective tissues, both in the normal lung and during pulmonary dust diseases. Furthermore, such work must continue to proceed hand in hand with advances in general biochemical knowledge in relating to the connective tissue field as a whole, due regard always being given to studies on the mucous secretions of the respiratory tract.

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