Invertebrate Collagens

Marked differences from vertebrate collagens appear in only a few invertebrate groups.

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Summary. The collagens of all major invertebrate phyla have been studied, but characterization has been thorough in only a few classes and in no case in the detail (such as sequence analysis) known for vertebrate collagen. Biochemical data on insect collagen are particularly sparse. Invertebrate and vertebrate collagens are strikingly similar, with some notably unique features in annelids and nematodes. Present data do not support the suggestion that invertebrate collagens resemble vertebrate basement membrane collagen. In invertebrates, as in vertebrates, collagens of specific tissues show differences that probably reflect individual tissue requirements.

Collagen, the principal protein of metazoan connective tissue, is the subject of a large literature, encompassing diverse interests in chemistry, biology, medicine, and industrial technology. As a family of proteins with unique structural features, collagen has been a focus of structure-function correlation studies as well as studies interrelating successive levels of structural organization from amino acid sequence to the anatomically defined fibril. As a central component of connective tissue, collagen is also of interest for its role in tissue development and differentiation, as well as in such medically relevant processes as wound healing and pathological fibrosis.

Invertebrates are estimated as constituting 95 percent of all animal species (*1*), yet most collagen studies have centered on the vertebrates. Advances in vertebrate collagen biochemistry (*2*) in the past 20 years have revealed the amino acid sequence for subunit polypeptide chains (*3*), the biochemical polymorphism of intraspecies collagens (*4*), details of the posttranslational modifications of collagen precursors (for example, hydroxylation of proline and lysine residues) (*5*), and the molecular definition of several genetic disorders of collagen maturation (*6–8*). In part, progress has been speeded by the similarity of all vertebrate collagens: except in refined details, new insights based on a given vertebrate species apply to other vertebrates.

Comparative studies, particularly involving nonvertebrate collagens, however, can expand our picture of structural modifications compatible with a basic definition of collagen, and can reveal new functions over a wide range of organismic needs. An early review of comparative collagen biochemistry (*9*) summarized much data published before 1962, including that on the microscopic and molecular structure. Because that review predated the era of amino acid sequence analysis and—to a large extent—of collagen-related enzymology, x-ray diffraction data and amino acid composition dominated the comparison of collagens from various invertebrate phyla and among vertebrate classes. More recent reviews of invertebrate collagens are of specialized interest (*10*).

A crucial question in comparative studies concerns the definition of collagen; the exploration of collagens differing from the vertebrate "norm" implies the need to define the limits that include proteins under this common category. As was noted in 1963 (*9*), a still-valid general criterion common to proteins that differ in many other properties is a characteristic wide-angle x-ray diffraction pattern. From the compositional viewpoint, this structural feature correlates well with the occurrence of glycine as approximately one-third of the amino acid residues, and, in turn, with the collagen triple-helix, whose native structure requires hydrogen bonds linking three polypeptide chains in approximate register. This model holds for all proteins so far studied that conform to the x-ray pattern noted, and that include certain other (although not necessarily all) of the common compositional criteria of collagens, such as a relatively high content of the pyrrolidine amino acids, proline or hydroxyproline (or both). The triple-helix itself is believed to require that each individual chain contain a glycine residue in each third sequence position, as $(Gly\text{-}X\text{-}Y)_n$ (*11*). While this central set of related properties can be used to define all proteins classed as collagens, other features considered characteristic of collagen show considerable variation. Thus, by the criteria above, there is at least one collagen with no measurable hydroxyproline (*12*) or with little (*13*) or no (*14*) hydroxylysine, and there are collagens with rather different banding periods in the electron microscope (*9, 15*), or with no banding at all (*9, 16*).

Collagen Distribution Among Phyla

The presence of *trans*-4-hydroxy-L-proline as a component of cell wall proteins has been documented in both higher plants and algae (*17*); both 4-hydroxyproline and 3,4-dihydroxyproline are present in siliceous cell walls of diatoms (*18*). There is, however, no clear evidence that the plant proteins containing hydroxyproline are collagens, although proline residues in the precursor proteins of plants are substrates for the prolyl hydroxylase of animal origin (*17*), an enzyme required for collagen processing. Proteins associated with the shells of a number of molluscs and brachiopods also contain hydroxyproline, although, as in the plant proteins noted, these proteins have not been unequivocally identified as collagens (*19*). Neither collagen nor hydroxyproline has been reliably identified in bacteria.

The distribution of hydroxyproline, therefore, is considerably wider than that of typical collagen-class proteins, which are now believed to be limited to metazoan forms. Examples of every metazoan phylum studied, however, have been found to contain collagen as defined by molecular or microscopic structural features. Table 1 summarizes the distribution of collagen among invertebrate phyla. Because most of the

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0036-8075/78/1110-0585$02.00/0

Table 1. Invertebrate phyla in which collagen has been studied morphologically or chemically.

Phylum	Subphylum, class, or genus
Porifera	*Ephydatia* (*98*), *Euplectella* (*99*), *Halichondria* (*48*), *Haliclona* (*95, 100*), *Hypospongia* (*66*), Ircinia (*101*), *Scypha* (*99*), *Suberites* (*94*)
Coelenterata	*Actinia* (*24, 25, 27, 67, 94, 102*), Aiptasia (*103*), *Aurelia* (*104*), *Briareum* (*105*), *Calliactus* (*94*), *Hydra* (*34, 68, 97*), Lobophyllia (*106*), *Metridium* (*28, 48, 66, 74, 75, 94, 107*), Muricea (*108*), *Virgularia* (*109*)
Platyhelminthes	*Bipalium* (*90*), *Diphyllobothrium* (*48*), *Fasciola* (*24, 67, 92*)
Nematoda	*Ascaris* (*14, 31–33, 35–37, 39, 45, 47, 49, 51, 52, 55, 80–82, 84, 94, 110–112*), Nematospiroides (*113*), *Nippostrongylus* (*113*), *Panagrellus* (*38, 85, 114*)
Annelida	Hirudinea (*115*), *Lumbricus* (*15, 39, 45, 48, 54, 61, 62, 76, 79, 94, 107, 111–113, 116, 117*), other Oligochaeta (*15, 40, 47, 115, 117–120*), Polychaeta (*41, 42, 72, 115, 120*)
Acanthocephala	*Macracanthorhynchus* (*26, 47*)
Mollusca	Cephalopoda (*29, 48, 57, 72, 73, 107, 121*), *Haliotis* (*29, 72*), *Helix* (*60, 67, 115, 122*), *Mytilus* (*48, 99, 107, 115, 123, 124*)
Arthropoda	Arachnida (*125*), Crustacea (*20, 21, 29, 48, 72, 89*), Insecta (*12, 22, 88, 91, 99, 126*), Onychophora (*127*)
Echinodermata	*Asterias* (*128*), Holothuridea (*48, 58, 69, 70, 71, 95, 129*), *Strongylocentrotus* (*69, 95, 99*), *Paracentrotus* (*30, 130*)
Chordata	Ascidiacea (*48, 72*)

studies on invertebrate collagen through 1961 have been reviewed already (*9*), Table 1 includes, for the most part, studies since 1962 and is an effort to present the most significant contributions over a wide range of collagen-focused interests, both morphological and chemical. In some listings in Table 1 [for example (*20*, *21*)], crude extracts containing hydroxyproline are described but not well characterized as typical collagens. Table 1 is not intended to be exhaustive, but to contain most of the major recent references.

The most thoroughly studied invertebrate collagens are those of nematodes and annelids, particularly *Ascaris* in the former phylum and *Lumbricus* and related oligochaetes in the latter (Table 1). The composition and other features of coelenterate collagen, especially that of sea anemones, have also been reported in some detail. Studies of other phyla are scattered and often somewhat superficial. Surprisingly few intensive studies of insect collagen are reported, perhaps because of the relative inaccessibility of collagen-rich tissues, in contrast to the ease of obtaining, for example, worm cuticles. Studies of insect collagens have been chiefly morphological, concentrating on fibrillar collagen (*22*).

Molecular Weight and Subunit Composition

All of the well-characterized vertebrate collagens are molecules with a molecular weight of approximately 300,000 and assemblies of three peptide chains of approximately equal length; fundamental features of collagen structure are dependent on this model (*23*). Except for type I collagen, which has the subunit formula $[(\alpha 1)_2\alpha 2]$, vertebrate collagens appear to consist of three identical chains (*4*). By comparison, there is only scattered information on the molecular weight and subunit composition of invertebrate collagens. In a few instances, the collagen of specific invertebrates appears to resemble the vertebrate collagen norm. *Actinia* collagen was estimated to consist of three α chains totaling 300,000 molecular weight (*24, 25*), as was the collagen of *Macracanthorhyncus* (*26*). The stoichiometry of cyanogen bromide peptides from *Actinia* collagen further indicated that the α chains were identical (*27*); a similar conclusion came from studies of the collagen of *Metridium* (*28*), another sea anemone. On the basis of sedimentation velocity, intrinsic viscosity, subunit gel electrophoresis and ion-exchange chromatography, Kimura and Matsumura (*29*) concluded that the collagen of abalone foot muscle consisted of three identical α chains each having a molecular weight of about 100,000, but that collagen derived from octopus, squid, crab, and lobster resembled vertebrate type I collagen in the subunit formula $[(\alpha 1)_2\alpha 2]$. Insoluble collagen from an echinoderm (*30*) also appears to conform to the $[(\alpha 1)_2\alpha 2]$ subunit formula.

Studies of the cuticle collagens of *Ascaris* and earthworm have indicated molecular weights and probable subunit structure very different from those of vertebrate collagens or from those of the invertebrate collagens noted above. Detailed data have been reported for *Ascaris*. An unusual feature of *Ascaris* cuticle collagen is its extreme aqueous insolubility in the native state, but its considerable solubility under reducing conditions, such as treatment with mercaptoethanol (*31, 32*). From these and related findings, a model has been inferred involving individual polypeptide chains held together by disulfide bonds; the unreduced collagen particles were estimated as having a molecular weight of about 900,000, and they are converted after disulfide reduction to particles having a molecular weight of 62,000 (*32*). When the latter subunits were denatured by heat or guanidine hydrochloride, they lost the native structure (in terms of high viscosity and optical rotation) but retained their molecular weight (62,000), suggesting that the native subunit formed a triple helix by self-folding of the individual chains (*32, 33*). The studies of Evans *et al.* (*14*) have required a modification of this model by a seemingly more reliable molecular weight of 52,000 for the subunit and evidence for three chromatographically separable and compositionally different chains of this molecular weight. There is no adequate model depicting how the small subunits, approximately half the molecular weight of vertebrate α chains, are arranged to form the postulated 900,000-molecular-weight, cross-linked molecule, and whether the 900,000-molecular-weight units are composed of three different kinds of subunit, or if there are several kinds of large molecule, each consisting of identical subunits with a molecular weight of 52,000.

Evidence for other disulfide-linked invertebrate collagens—that of *Hydra* nematocysts (*34*) and of the muscle layer of *Ascaris* (*35*), and possibly of the intestinal basement membrane of *Ascaris* (*36, 37*)—suggests that this kind of cross-link may not be a rarity. Disulfide cross-linking has also been demonstrated in both procollagens and certain mature collagens of vertebrate origin (*5*). Although there is little chemical information on the collagen of nematodes other than *Ascaris*, recent data describe several chain sizes, ranging from a molecular weight of 89,000 to 105,000 in *Panagrellus* cuticle (*38*). In the same study (*38*), extensively incorporated azetidine-2-carboxylate in the cuticle collagen failed to alter collagen synthesis, suggesting more plastic conformation than in the vertebrate collagens.

The annelid cuticle collagens represent yet a different set of molecules with

respect to molecular weight. An earlier estimate (*39*) of *Lumbricus* cuticle collagen indicated a molecular weight of 1.9 million for the native molecule and subunits of approximately 600,000, after denaturation by heat or chemical treatment. In approximate agreement, the molecular weight of cuticle collagen of the oligochaete *Pheretima* was estimated as about 1.7 million (*40*) as was that of the cuticle collagen of *Nereis*, a polychaete annelid (*41*). A later study of denatured *Nereis* cuticle collagen (*42*) revealed the existence of two kinds of subunit comparable to the $[(\alpha 1)_2\alpha 2]$ formulation, but with subunit molecular weights of 470,000. The annelid cuticle collagens may exist as assemblies of unprecedentedly long chains, five to six times the length of the polypeptide subunits of vertebrate collagen. If each is a unique sequence, as appears to be the case for the various vertebrate α chains, they represent the largest polypeptide chains, up to approximately 6000 residues, known in nature.

Amino Acid Composition

As was noted in Gross's review (*9*) and more fully documented with data of the past 10 years (*3*), the composition of all vertebrate collagens—especially of the interstitial, that is, nonbasement membrane types—is closely similar. Sequence information also shows a high degree of homology in the distinct polypeptides of vertebrate interstitial origin, homology both with respect to the subunits of types I to III, but also of each type in various vertebrate species. In contrast, invertebrate collagens display very different amino acid compositions. No simple generalizations concerning composition have emerged from the considerable number of species studied in different phyla. Glycine, as expected, is the least variable residue, approximating 30 to 33 percent (on a molar basis) in most species (*43*). The early values for glycine in *Ascaris* cuticle collagen were significantly lower than 300 residues per 1000 residues (*9, 39*), and recent analyses of the separated purified chains (*14*) agree in yielding about 270 residues per 1000. It is now known that procollagen chains contain less than one-third glycine residues (*3*) as do the C1q complement component (*3*) and various fractions of basement membrane collagen (*44*), because of the presence of closely associated or peptide-bond linked nonhelical regions. *Ascaris* collagen chains may also contain sequence regions atypical of helical collagen. It is notable that a single analysis of *Ascaris* cuticle collagen based on material extractable with trichloroacetic acid (*45*) gave a glycine value close to 33 percent residues.

Among invertebrate collagens, differences in the content of hydroxylated amino acids, especially differences in the ratios of proline to hydroxyproline and lysine to hydroxylysine, are most clear. Earthworm cuticle collagen and *Ascaris* cuticle collagen provide a striking example, since the ratio of proline to hydroxyproline is close to 20 for *Ascaris* cuticle and close to 0.05 for earthworm cuticle (*39*). Nematodes other than *Ascaris* appear to have been little studied with respect to cuticle collagen composition, but a recent report (*38*) indicated that the cuticle of *Panagrellus*, a small free-living nematode, also has a high ratio of proline to hydroxyproline. The extreme example, however, is the silk of the sawfly *Nematis ribesii*, which qualifies as a collagen by virtue of its wide-angle x-ray diffraction diagram (*46*) and contains about 33 percent glycine residues and about 4 percent hydroxylysine residues, but is devoid of hydroxyproline (*12*). 3-Hydroxyproline is also quite variably distributed in invertebrate collagens, from little [earthworm cuticle (*13*)] or none [*Ascaris* cuticle (*14*)] to values up to two residues per hundred in the sea anemone *Actinia* (*27*) or close to three per hundred for *Allolobophora* body wall (*47*). Reported values for 3-hydroxyproline in sea anemone collagen differ (*24, 28, 48*) and may reflect analytical errors or the unavailability of pure reference samples of this difficultly obtained compound. Analyses of the collagen of *Actinia* in our own laboratory, with the use of a pure reference standard, agree with the highest values reported (*27*).

An effort to codify collagen composition and to search for evolutional correlates (*43*) was based on clusters of three amino acid groups (hydrophobic, hydroxylic, and polar), and the two-dimensional mapping of points for separate species on the coordinates of a triangular grid, one coordinate for each of the three groups. By these criteria, a rather small area (S range) included all striated collagens, both from vertebrate and many invertebrate species, and from collagens both of ectodermal and mesodermal origin. Vertebrate basement membrane collagens, with relatively high values for the polar and the hydrophobic groups, fall in an area distinct from the major S area. Earthworm cuticle collagen, with its high content of hydroxy amino acids and low content of polar amino acids, and *Ascaris* cuticle collagen, with its low hydroxy amino acid and high polar amino acid content, are also out of the S range, and differ more from each other than do any other two collagens plotted. No species or phylum correlations were disclosed by this somewhat arbitrary classification, but, rather, differences in tissue origin: The two worm cuticles, vertebrate basement membrane collagens, and the remaining 90-odd collagens of various tissue and species origin formed four distinct classes.

Other attempts to classify collagen composition across species and phylum lines have focused on the hypothesis that the stability of a given collagen, as determined by the midpoint of denaturation temperature, T_D, or midpoint of stretching temperature, T_s, correlates with its content of hydroxyproline, of proline, or of total pyrrolidine amino acids. Josse and Harrington (*39*) demonstrated that a plot of T_D as a function of hydroxyproline or proline frequency showed a linear relation for approximately ten collagens of vertebrate origin. In each such plot, however, both *Ascaris* and earthworm cuticle collagen fell far from the vertebrate line, as was expected from the extremely different values of the ratio of proline to hydroxyproline in each cuticle collagen. When the frequency of proline plus hydroxyproline was plotted, however, the now-curvilinear line relating these values to T_D included, in a reasonable way, both cuticle collagens.

When data were used from additional invertebrates, including body wall collagens of other earthworm and nematode species, a simple correlation between frequency (proline plus hydroxyproline) and T_t (melting point of tropocollagen) could no longer be demonstrated (*47*). It would seem likely that not only the number of pyrrolidine residues, but their sequence position [whether in the X or Y position of -Gly-X-Y-sequences (*49*)] and the frequency of dipyrrolidine residues (*49*) influence collagen stability. In addition, recent evidence (*5*) has revived the older hypothesis (*50*) that hydroxyproline contributes more to collagen stability than can be accounted for simply by its pyrrolidine status.

Intraspecies Collagen Differences

One aspect of amino acid composition is the question of collagen polymorphism within a given species. The existence of chemically distinct collagens in individual species is well established for vertebrates (*4*); the full implications of tissue-specific and genetically different collagens are not yet understood, but seem relevant to tissue differentiation and

functional specificity. The first clear example of marked differences in the composition of collagens from different tissues of the same animal appeared in analyzing the cuticle and body wall collagens of *Ascaris* and of *Lumbricus* (*45*), as is shown in the later data of Table 2. This finding was confirmed both for *Ascaris* (*47, 51*), *Lumbricus* (*47*), and additional worm species (*47*). An additional collagen species, the basement membrane collagen from *Ascaris* intestine, markedly different in composition from the cuticle collagen, has also been described (*36*). It seems likely (Table 2) that at least three collagen species can be obtained from *Ascaris*—that from the cuticle (*14, 39*), that from the muscle layer (*51*), and that from intestinal basement membrane (*36, 37, 52*). The last-named collagen has not been purified, so that the analytic values for the amino acids do not represent collagen alone; however, ratios of those amino acid residues (hydroxylysine, 4-hydroxyproline, 3-hydroxyproline) that are probably unique to the collagen component suggest that the intestinal basement membrane contains a collagen (or collagen-like sequences) distinct from that of *Ascaris* cuticle or muscle.

Amino Acid Sequence

In contrast to the extensive sequence information for vertebrate collagens (*3*), there are only fragmentary and somewhat indirect data for any invertebrate collagen. Despite marked composition differences, however, vertebrate collagens show a pattern of segment-long spacing (*53*) remarkably like that of two invertebrate collagens, from *Fasciola* and *Actinia* (*24*) which are themselves quite distinct phyla. Since the staining patterns are believed to represent gross sequence information in terms of the alternation of clusters of charged and of uncharged residues (*3*), it is apparent that, at this level of resolution, these striated invertebrate collagens are closely similar to typical vertebrate interstitial collagens. Even identity of a banding pattern could permit considerable variation of composition and amino acid sequence, as long as residue substitutions were allowed only within the broad categories of stained (that is, polar) or unstained (nonpolar) sequences.

Direct sequence information is limited to only a few studies. Goldstein and Adams (*54*) reported that most, if not all, of the 4-hydroxyproline residues of earthworm cuticle collagen are confined to the X position of the Gly-X-Y triplets, and they isolated and identified two tripeptides from a collagenase digest of this collagen; one, H-Gly-Hyp-Ala-OH (*54*), the other H-Gly-Hyp-Ser-OH (*13*), accounting together for 10 to 15 percent of total hydroxyproline. This is in contrast to all present information about vertebrate sequences (*3*), in which 4-hydroxyproline is confined to the Y position.

It is of interest that X-position hydroxyproline was first predicted for earthworm cuticle collagen to account for observed stability (*49*). On a related basis, it may be inferred that a substantial fraction of prolyl residues of *Ascaris* cuticle collagen occurs in the Y position (*49*). This is consistent with the substrate activity of *Ascaris* cuticle collagen for a vertebrate prolyl hydroxylase (*55*) since the specificity of the vertebrate hydroxylases is limited to the Y-position prolyl residue (*56*).

The unusual feature of hydroxyproline in the X position of earthworm cuticle collagen triplets has not been established for other invertebrate collagens, although only little data bear on this question. Isemura *et al.* (*57*) found that, in a number of short glycopeptides isolated from cuttlefish skin collagen, 4-hydroxyproline as well as hydroxylysine was present only in the Y position; the same finding was made for sea cucumber collagen (*58*). No systematic sequencing of an invertebrate collagen chain seems to have been reported, although a subunit chain (approximately 1000 residues) of *Actinia* collagen has been separated into 11 cyanogen bromide peptides ranging in size from 13 to 231 residues (*27*).

Collagen-Linked Carbohydrates

The interstitial collagens of vertebrate origin are glycoproteins containing a small number of hydroxylysyl residues substituted with galactose or glucosylgalactose (*4*). Vertebrate basement membrane collagens contain a relatively large number of hydroxylysyl residues, up to four residues per hundred, most of which are glycosylated with glucosylgalactose units (*44, 59*); small quantities of other sugars, including fucose, mannose, hexosamine, and sialic acid, are also associated with preparations of basement membrane collagen (*59*); their binding to collagen-like peptide sequences is not well characterized.

In contrast to the low carbohydrate content of vertebrate bulk collagens (that is, nonbasement membrane), many invertebrate collagen preparations were early noted to be rich in carbohydrate (*9, 60*). Since Gross's review (*9*) and a more recent summary of collagen-associated carbohydrates (*19*), carbohydrates linked to several invertebrate collagens have been more precisely identified and characterized with respect to the linkage region. Thus, the high D-galactose content of earthworm cuticle collagen, 12 to 14 percent by weight, could be largely accounted for as 2-*O*-α-D-galactopyranosyl-D-galactose and *O*-α-D-galactopyranosyl(1 → 2)-*O*-α-D-galactopyranosyl-(1 → 2)-D-galactose (*61*), the disaccharide unit predominating over the trisaccharide in a ratio of about 4:1 (*62*). Both serine and threonine residues are the amino acids bound glycosidically to these saccharides (*62*), in contrast to the glycosidic linkage of galactose to hydroxylysine in vertebrate collagens (*63, 64*). The fact that purified earthworm cuticle collagen contains only traces of hydroxylysine has been noted above.

The reported large quantity of galactose in the cuticle collagens of two polychaetes of the *Nereis* group (*42*) suggests that galactose oligosaccharides and their possible linkage to serine and threonine may be general among annelids. In contrast to earthworm cuticle collagen, *Ascaris* cuticle collagen is reported to contain only small quantities of hexoses (*39, 65*). Collagen from a variety of other invertebrates follows the vertebrate pattern in containing glucosylgalactosylhydroxylysine or galactosylhydroxylysine. These include the sponge *Hippospongia* (*66*), the coelenterates *Metridium* (*66*), *Actinia* (*67*), and *Hydra* (*68*), the echinoderms *Thyone* (*69, 70*), *Stichopus* (*58*), and *Holothuria* (*71*), the molluscs *Octopus*, *Todarodes*, and *Haliotis* (*72*), the arthropods *Panulirus* and *Portunus* (*72*), the prochordate *Halocynthia* (*72*), and the intestinal collagen—probably of basement membrane origin—of the annelid *Nereis* (*72*) and the nematode *Ascaris* (*36, 51*). Where examined quantitatively, these saccharide units are present in invertebrate collagens in greater frequency than in vertebrate interstitial collagen and, in this respect, resemble vertebrate basement membrane collagens. As in vertebrate collagens, substituted hydroxylysine residues occur in the Y position of Gly-X-Y sequences (*57, 58*). In cuttlefish skin, the glycosylated hydroxylysines (Hyl*) occur in the sequence Gly-A-Hyl*-Gly-B-Arg, where A and B are any of a variety of amino acids (*57, 73*). Y-position Hyl* has also been reported in the echinoderms *Stichopus* (*58*) and *Holothuria* (*71*).

Another similarity with vertebrate basement membrane collagen is the presence in some invertebrate collagens of a

different type of saccharide unit, containing fucose, xylose, mannose, and hexosamines. Such a heteropolysaccharide unit linked to *Metridium* collagen chain has been described (*74*). Partial sequencing indicated that the saccharide unit was linked to asparagine in a peptide sequence containing 4-hydroxyproline; the saccharide portion contained fucose, mannose, and *N*-acetylglucosamine among other sugars (*75*). Isolation of purified α and β units containing both this heteropolysaccharide as well as the more familiar glucosyl-galactosyl-Hyl units (*28*), indicated that both kinds of saccharide units are bound to the same collagen chain. The hydroxyproline-free collagen-like silk of the sawfly, referred to above, contains only nonglycosylated hydroxylysine residues; these were glycosylated on incubation with uridine diphosphate galactose and the appropriate galactosyltransferase from rat kidney (*12*), suggesting that the cells synthesizing the silk-collagen lacked the appropriate enzymes for glycosylation.

Table 2. Amino acid composition of the collagen of several tissues of earthworm and *Ascaris*. All values are residues per 1000 total residues. Reference source of analytic values is shown in parentheses. All samples were purified collagens except for *Ascaris* intestine: this was whole basement membrane.

Amino acid residue	Earthworm		*Ascaris*		
	Cuticle (*13*)	Body wall (*47*)	Cuticle (*14*)	Muscle (*51*)	Intestine (*36*)
3-Hyp	2	28	*	†	*
4-Hyp	160	67	20	122	24
Asp	58	65	55	57	82
Thr	49	36	16	13	47
Ser	88	52	19	16	50
Glu	81	97	54	80	124
Pro	9	53	357	103	87
Gly	348	352	265	326	148
Ala	100	59	53	63	61
Val	16	21	12	13	53
Met	*	3	8	6	11
Ile	15	14	10	24	36
Leu	27	35	14	54	61
Tyr	1	6	2	6	26
Phe	6	12	6	14	20
1/2 Cys	*	4	30	3	37
Trp	†	†	2	†	12
Lys	16	22	30	19	22
Hyl	*	12	*	40	11
His	1	9	7	3	22
Arg	23	51	28	38	66

*Not detected. †Not reported.

Collagen-Associated Enzymes

Vertebrate studies dominate the data on enzymes responsible for the post-translational modification of collagen. Of these enzymatic steps, only proline hydroxylation has been investigated in invertebrates, but even these few studies suggest that a wealth of diversity will be found in enzymatic adaptations to form functionally differing collagens.

One example of such diversity concerns the enzyme responsible for hydroxylating proline residues in earthworm cuticle collagen. As already noted, earthworm cuticle collagen contains much or all of its hydroxyproline in the X position of Gly-X-Y sequences, a position apparently forbidden in all vertebrate collagens studied. This anomalous sequence distribution of 4-hydroxyproline residues could have resulted from a prolyl hydroxylase of conventional specificity acting on X-position prolyl residues in an unusual sequence that promoted their hydroxylation. Alternatively, the relevant earthworm hydroxylase might have inherent specificity differences for residue-position, as compared to vertebrate hydroxylases, in the ability to act on X-position prolyl residues. That the second explanation holds was reported by Adams and Lamon (*76*), who found that the synthetic polytripeptide $(Gly\text{-}Pro\text{-}Ala)_n$, not a substrate for vertebrate enzymes (*76, 77*), was a good substrate for a partly purified hydroxylase obtained from the subcuticular epithelium of earthworms. The enzyme has been more extensively purified (*78*) and shows many similarities to vertebrate prolyl hydroxylases, particularly in its cofactor requirements. Its substrate specificity pattern is complex since, when allowed to act either on natural collagens or on synthetic polytripeptides, each containing the internal sequence -Gly-Pro-Pro-, it hydroxylates only the Y-position proline (*76*), exactly as the vertebrate hydroxylases do.

In an earlier study, Nordwig and Pfab (*79*) had reported that, with synthetic polytripeptides containing the internal sequence -Gly-Pro-Pro-, in which either prolyl residue was radioactive, a homogenate of whole-earthworm body wall acted to hydroxylate prolyl residues in either position, and furthermore formed a substantial proportion of the position isomer 3-hydroxyproline as well as 4-hydroxyproline. Adams and Lamon (*76*), using similar radioactive substrates, enzyme preparations, and incubation conditions, were unable either to repeat or explain these findings, but noted that their partly purified hydroxylase did not catalyze appreciable formation of 3-hydroxyproline.

The prolyl hydroxylase from the muscle of *Ascaris* also resembles vertebrate hydroxylases in cofactor requirements, but differs in its dependence on O_2 concentration (*80*). Unlike the vertebrate enzymes, which show a typical hyperbolic saturation curve in plotting reaction velocity against O_2 concentration, the *Ascaris* muscle enzyme is increasingly inhibited by O_2 at concentrations exceeding 1 percent; the apparent values of K_m (the Michaelis constant) for O_2 are, however, similar for both the *Ascaris* muscle and chick embryo enzyme (*80*). Inhibition of the *Ascaris* muscle enzyme by O_2 was confirmed by Chvapil *et al.* (*81*), who showed also that a prolyl hydroxylase was present in the cuticular fluid obtained after freezing whole worms. This enzyme differed from the muscle enzyme in lack of inhibition by O_2, and in a higher K_m for O_2. In vivo findings consistent with these enzymatic differences indicated that, after incubation of living worms in atmospheres of elevated O_2 concentration, the cuticle collagen, but not that of muscle, showed increased prolyl hydroxylation (*82*). Prolyl hydroxylase has also been reported in sea urchin larvae (*83*) and in *Ascaris* eggs (*84*). The *Ascaris* egg enzyme (*84*), unlike the muscle enzyme, is not inhibited by high O_2 concentration, an observation consistent with the development of *Ascaris* eggs in an aerobic environment. A prolyl hydroxylase with features similar to that of the vertebrate enzymes has been purified from the free-living nematode *Panagrellus* (*85*). In analogy with the *Ascaris* enzymes, it is probable that more than one tissue-specific enzyme may exist in the small nematodes as well, although the difficulty of isolating individual tissues in such small animals precludes an easy test of this analogy.

The two major classes of collagenolyt-

ic enzymes are those of *Clostridia* (*86*) and those of vertebrate origin (*87*). The bacterial enzymes, which have not been completely defined in terms of isoenzyme multiplicity and detailed substrate specificity, have the general characteristic of cleaving collagen or collagen-like polypeptides in such a way as to release tripeptides whose NH_2-terminal residue is glycine (*86*). The vertebrate enzymes cleave each of the three helical chains of native vertebrate collagens at a single bond between Gly-Leu and Gly-Ile (*87*), producing two fragments of quite different length.

Collagenases of invertebrate origin, only few of which have been carefully studied, differ in some respects from both of the above collagenase types. A collagenase from crab hepatopancreas (*88, 89*) has been purified to homogeneity and resembles vertebrate collagenases in cleaving native helical collagen into two fragments of unequal size, but differs from vertebrate collagenases in its capacity to further degrade one of the initial scission products. This enzyme shows both trypsin-like and chymotrypsin-like substrate activity and inhibition features. *Bipalium kewense*, a land planarian that feeds on earthworms, contains a pharyngeal collagenase (*90*) which, when purified, degrades native vertebrate collagen to small fragments. A recently reported collagenase from *Hypoderma* larvae (*91*) is of special physiological interest, in that this insect larval form migrates in the connective tissue of a vertebrate host. The enzyme was purified to homogeneity, resembles the vertebrate collagenases in cleaving calf skin collagen into a large and small fragment, and has the surprisingly low molecular weight of 16,000. Other reports of collagenase activity, as in *Fasciola* (*92*), have been short reports without intensive enzyme characterization.

Collagen Cross-Links

This subject has become an important specialty in studies of vertebrate collagens, and concerns tensile strength and other functional aspects of mature collagens. Various cross-links have been defined chemically in vertebrate collagens, including the disulfide bonds of basement membrane and type III collagens (*5*). As was noted above, such collagen linkages were first found in the invertebrate collagens.

Cross-links more distinctive of collagen are aldimine and aldol links arising from the condensation of aldehydic groups, or of an aldehyde and amino group, each condensation partner on a separate peptide chain. Such aldehyde groups are formed by oxidation of the terminal carbon of lysine or hydroxylysine, and appear to be specific for collagen and elastin (*93*). Examples of this kind of cross-link are sparse in invertebrates, although there is no reason to doubt their wide distribution in many invertebrate collagens. Bailey (*94*) reported dihydroxylysinonorleucine as the major radioactive peak after borohydride reduction and acid hydrolysis of earthworm cuticle collagen or of the body collagen of several sea anemones; the body wall of sponge (*Suberites*) yielded a small peak of this reduced cross-link and a prominent peak of the reduced aldehyde, hydroxynorleucine. It is of interest that *Ascaris* cuticle collagen yielded no lysine- or hydroxylysine-derived cross-links, consistent with the alternative mode of disulfide linkage in this collagen. It is surprising that earthworm cuticle, which contains little or no hydroxylysine (see above), revealed an apparent hydroxylysine-derived cross-link. This finding may be a function of how carefully separated the cuticle is from underlying cells and their attached basement membrane.

Similar findings by Eyre and Glimcher (*95*) were in agreement for the sea urchin *Strongylocentrotus* and the sea anemone *Thyone*, but noted a more complex pattern from the body wall of the sponge, in which lysinonorleucine was the most prominent peak. Subsequent studies of *Thyone* collagen suggested that the cross-link, which had been reduced to dihydroxylysinonorleucine, existed as a glycosylated unit in the original collagen (*70*).

General Conclusions

The above survey of invertebrate collagen reveals the blend of biochemical similarities and differences—both among the invertebrates and in comparison with vertebrates—that characterizes most comparative biochemical studies. It is perhaps tautologic to note that basic features of collagen structure (triple helical structure, distribution of glycine residues, and x-ray diffraction pattern) are similar in all animals studied, but it may be significant that possible quasi collagen-like molecules that seem only distantly related to the vertebrate collagens have not been found. Whether molecules containing partial collagen-like sequences like that of the C1q complement component also occur in invertebrates is not yet known, but seems likely from recent studies of *Ascaris* intestinal basement membrane (*36, 37*). Broad generalizations that might separate invertebrate from vertebrate collagens may yet emerge, but present data do not suggest these. Of the striking similarities in collagens across all phylum lines so far studied, perhaps most impressive is the finding that segment long spacing banding (*24*) indicates a virtually identical alternation of polar and nonpolar regions in the sea anemone, the liver fluke, and the mammal. Among both invertebrates and vertebrates, there are collagens made up of three identical peptide chains and of peptide chains of differing sequence; both vertebrates and invertebrates show intraspecies collagen polymorphism at the level of composition; intraspecies sequence differences are established for vertebrate collagens and can be inferred for invertebrates from the marked composition differences. Both groups of animals possess collagens that contain disulfide cross-links or lysine- or hydroxylysine-derived cross-links (or both). The enzymatic features of the hydroxylation of prolyl and lysyl residues appear similar for both large groups of animals, as do the presence and formation of glycose units. Under the latter heading, many invertebrate collagens contain both the glucosylgalactosyl-linked hydroxylysyl residues first demonstrated in vertebrate collagens as well as the heteropolysaccharide type of unit found also in vertebrate basement membranes.

One generalization which has been repeatedly suggested is the resemblance of invertebrate collagens to vertebrate basement membrane collagen. In discussing the high ratio of hydroxyproline to proline in earthworm cuticle collagen, an early comparison of this kind (*45*) noted the relatively high hydroxyproline content of kidney cortex collagen, first reported by Windrum *et al.* (*96*). Other investigators have more explicitly pointed out the resemblance of a *Metridium*-derived collagen (*75*) to glomerular basement membrane collagen with respect to the high 3-hydroxyproline content, the presence of cysteine, the somewhat low glycine content, the high glycosylated hydroxylysine, and the presence of a heteropolysaccharide. A similar composition resemblance of *Hydra* mesogleal collagen and vertebrate basement membrane collagen was noted by Barzansky and Lenhoff (*97*). While this comparison seems valid for selected invertebrate collagens, it does not fit other invertebrate collagens in the annelid and nematode cuticles, which differ markedly from each other and differ from vertebrate

basement membrane collagens both in possessing little or no hydroxylysine and in a different mode of glycosylation. In contrast, collagens of certain invertebrate tissues resemble vertebrate interstitial collagens more than they do vertebrate basement membrane collagens, while some invertebrate collagens of basement membrane origin, such as that of *Ascaris* intestine, show clear differences in composition from those typical of vertebrate basement membrane collagen (*36*).

No inclusive generalization relating all or even many invertebrate collagens to vertebrate basement membrane collagen appears valid. In addition, there appear to be no strong compositional similarities (across phylum lines) between collagens secreted by epithelium or endothelium. Thus, the collagens of annelid cuticle, of nematode cuticle, and of renal tubular or glomerular basement membrane, all secreted by sheets of epithelial or endothelial cells, differ markedly.

While, in general, similarities between vertebrate and invertebrate collagens appear more impressive than differences, it should be noted that unique features of collagen structure and synthesis have been described in specific groups of invertebrates. The sequence distribution of 4-hydroxyproline residues in earthworm cuticle collagen, along with the specificity of the associated prolyl hydroxylase of earthworms, is one example; the presence of di- and tri-galactosyl units linked to serine and threonine residues in annelid cuticle collagens is another. Other such singularities are to be expected and would seem to reflect the much greater range of structure-function adaptations among the invertebrates as a whole, compared with the narrow and rather homogeneous subphylum of vertebrates. It would seem that, in many biochemical features, collagen is a remarkably stable molecule, both in structure, synthesis, and degradation, throughout all metazoan phyla. In particular groups of animals, however, the needs of a given tissue can require highly individual modifications of collagen form and function.

References and Notes

1. R. D. Barnes, *Invertebrate Zoology* (Saunders, Philadelphia, ed. 3, 1974).
2. G. N. Ramachandran and A. H. Reddi, Eds., *Biochemistry of Collagen* (Plenum, New York, 1976).
3. K. A. Piez, in (*2*), pp. 1–44.
4. E. J. Miller, *Mol. Cell. Biochem.* **13**, 165 (1976).
5. D. J. Prockop, R. A. Berg, K. I. Kivirikko, J. Uitto, in (*2*), pp. 163–273.
6. C. M. Lapiere and B. Nusgens, in (*2*), pp. 377–447.
7. J. Uitto and J. R. Lichtenstein, *J. Invest. Dermatol.* **66**, 59 (1976).
8. K. I. Kivirikko and L. Risteli, *Med. Biol.* **54**, 159 (1976).
9. J. Gross, in *Comparative Biochemistry*, M. Florkin and H. S. Mason, Eds. (Academic Press, New York, 1963), vol. 5, pp. 307–346.
10. A review by A. Bairati [*Boll. Zool.* **39**, 205 (1972)] emphasizes banding patterns in electron micrographs among different phyla. A review in the Japanese language [S. Kimura, *Leather Chem.* **21**, 62 (1975)] deals with some chemical features of invertebrate collagens.
11. The usual three-letter code for amino acid residues is used here: Ala, alanine; Asp, aspartic acid; Arg, arginine; Cys, cysteine (or 1/2 cystine); Glu, glutamic acid; Gly, glycine; His, histidine; Hyl, 5-hydroxylysine; 3-Hyp, 3-hydroxyproline; 4-Hyp, 4-hydroxyproline; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; X and Y signify any amino acid residue.
12. R. G. Spiro, F. Lucas, K. M. Rudall, *Nature (London)* **231**, 54 (1971).
13. A. Goldstein and E. Adams. *J. Biol. Chem.* **245**, 5478 (1970).
14. H. J. Evans, C. E. Sullivan, K. A. Piez, *Biochemistry* **15**, 1435 (1976).
15. S. Humphreys and K. R. Porter, *J. Morphol.* **149**, 33 (1976).
16. G. B. Pierce, in *Chemistry and Molecular Biology of the Intercellular Matrix*, E. A. Balasz, Ed. (Academic Press, New York, 1970), vol. 1, pp. 471–506.
17. R. Kuttan and A. N. Radhakrishnan, *Adv. Enzymol.* **37**, 273 (1973).
18. D. Sadava and B. E. Volcani, *Planta* **135**, 7 (1977).
19. S. Hunt, *Polysaccharide-Protein Complexes in Invertebrates* (Academic Press, New York, 1970).
20. H. C. Thompson, Jr., and M. H. Thompson, *Comp. Biochem. Physiol.* **27**, 127 (1968).
21. ———, *ibid.* **36**, 189 (1970).
22. D. S. Smith and J. E. Treherne, *Adv. Insect Physiol.* **1**, 401 (1963); D. E. Ashhurst, *Q. J. Microscop. Sci.* **105**, 391 (1964); *Annu. Rev. Entomol.* **13**, 45 (1968).
23. G. N. Ramachandran and C. Ramakrishnan, in (*2*), pp. 45–84.
24. A. Nordwig and U. Hayduk, *J. Mol. Biol.* **44**, 161 (1969).
25. A. Nordwig, H. Nowack, E. Hieber-Rogall, *J. Mol. Evol.* **2**, 175 (1973).
26. G. D. Cain, *Arch. Biochem. Biophys.* **141**, 264 (1970).
27. H. Nowack and A. Nordwig, *Eur. J. Biochem.* **45**, 333 (1974).
28. R. L. Katzman and A. H. Kang, *J. Biol. Chem.* **247**, 5486 (1972).
29. S. Kimura and F. Matsuura, *J. Biochem. (Tokyo)* **75**, 1231 (1974).
30. I. Pucci-Minafra, R. Galante, S. Minafra, *J. Submicrosc. Cytol.* **10**, 53 (1978).
31. O. W. McBride and W. F. Harrington, *J. Biol. Chem.* **240**, 4545 (1965).
32. ———, *Biochemistry* **6**, 1484 (1967).
33. ———, *ibid.*, p. 1499.
34. R. Blanquet and H. M. Lenhoff, *Science* **154**, 152 (1966).
35. D. Fujimoto, T. Ikeuchi, S. Nozawa, *Biochim. Biophys. Acta* **188**, 295 (1969).
36. B. D. Peczon, J. H. Venable, C. G. Beams, Jr., B. G. Hudson, *Biochemistry* **14**, 4069 (1975).
37. C. H. Hung, M. Ohno, J. W. Freytag, B. G. Hudson, *J. Biol. Chem.* **252**, 3995 (1977).
38. J. Leushner and J. Pasternak, *J. Exp. Zool.* **204**, 155 (1978).
39. J. Josse and W. F. Harrington, *J. Mol. Biol.* **9**, 269 (1964).
40. H. Utiyama, K. Sakato, K. Ikehara, T. Setsuiye, M. Kurata, *Biopolymers* **12**, 53 (1973).
41. S. Kimura, *Bull. Jap. Soc. Sci. Fish.* **37**, 419 (1971).
42. ——— and M. Tanzer, *Biochemistry* **16**, 2554 (1977).
43. T. Matsumura, *Int. J. Biochem.* **3**, 265 (1972).
44. N. A. Kefalides, *Int. Rev. Conn. Tissue Res.* **6**, 63 (1973).
45. D. Fujimoto and E. Adams, *Biochem. Biophys. Res. Commun.* **17**, 437 (1964).
46. K. M. Rudall, in *Treatise on Collagen*, B. S. Gould, Ed. (Academic Press, New York, 1968), vol. 2A, pp. 83–137.
47. B. J. Rigby, in *Symposium on Fibrous Proteins*, W. G. Crewther, Ed. (Plenum, New York, 1968), pp. 217–225.
48. J. Pikkarainen, J. Rantanen, M. Vastamäki, K. Lampiaho, A. Kari, E. Kulonen, *Eur. J. Biochem.* **4**, 555 (1968).
49. N. V. Rao and W. F. Harrington, *J. Mol. Biol.* **21**, 5771 (1966).
50. K. H. Gustavson, *Nature (London)* **175**, 70 (1955).
51. D. Fujimoto, *Biochim. Biophys. Acta* **168**, 537 (1968).
52. B. D. Peczon, L. J. Wegener, C. H. Hung, B. G. Hudson, *J. Biol. Chem.* **252**, 4002 (1977).
53. R. R. Bruns and J. Gross, *Biochemistry* **12**, 808 (1973).
54. A. Goldstein and E. Adams, *J. Biol. Chem.* **243**, 3550 (1968).
55. D. Fujimoto and D. J. Prockop, *ibid.*, p. 4138.
56. G. Cardinale and S. Udenfriend, *Adv. Enzymol.* **41**, 245 (1974).
57. M. Isemura, T. Ikenaka, Y. Matsushima, *J. Biochem. (Tokyo)* **74**, 11 (1973).
58. M. Isemura and T. Ikenaka, *Experientia* **33**, 871 (1977).
59. N. A. Kefalides, *J. Invest. Dermatol.* **65**, 85 (1975).
60. A. P. Williams, *Biochem. J.* **74**, 304 (1960).
61. L. Muir and Y. C. Lee, *J. Biol. Chem.* **244**, 2343 (1969).
62. ———, *ibid.* **245**, 502 (1970).
63. W. T. Butler and L. W. Cunningham, *ibid.* **241**, 3882 (1966).
64. R. G. Spiro, *ibid.* **242**, 4813 (1967).
65. M. R. Watson and N. R. Sylvester, *Biochem. J.* **71**, 578 (1958).
66. R. L. Katzman, M. H. Halford, V. N. Reinhold, R. W. Jeanloz, *Biochemistry* **11**, 1161 (1972).
67. A. Stemberger and A. Nordwig, *Z. Physiol. Chem.* **355**, 721 (1974).
68. B. Barzansky, H. M. Lenhoff, H. Bode, *Comp. Biochem. Physiol. B* **50**, 419 (1975).
69. R. L. Katzman, A. K. Bhattacharyya, R. W. Jeanloz, *Biochim. Biophys. Acta* **184**, 523 (1969).
70. D. R. Eyre and M. J. Glimcher, *Proc. Soc. Exp. Biol. Med.* **144**, 400 (1973).
71. M. Isemura, R. K. Zahn, K. Schmid, *Biochem. J.* **131**, 509 (1973).
72. S. Kimura, *J. Biochem. (Tokyo)* **71**, 367 (1972).
73. N. Isemura, T. Ikenaka, Y. Matsushima, *Biochem. Biophys. Res. Commun.* **46**, 457 (1972).
74. R. L. Katzman and R. W. Jeanloz, *ibid.* **40**, 628 (1970).
75. R. L. Katzman and A. L. Oronsky, *J. Biol. Chem.* **246**, 5107 (1971).
76. E. Adams and M. Lamon, *ibid.* **252**, 7591 (1977).
77. K. I. Kivirikko, D. J. Prockop, G. P. Lorenzi, E. R. Blout, *ibid.* **244**, 2755 (1969).
78. N. V. Rao, *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **36**, 753 (1977).
79. A. Nordwig and F. K. Pfab, *Biochim. Biophys. Acta* **181**, 52 (1969).
80. D. Fujimoto and D. J. Prockop, *J. Biol. Chem.* **244**, 205 (1969).
81. M. Chvapil, M. Boucek, E. Ehrlich, *Arch. Biochem, Biophys.* **140**, 11 (1970).
82. M. Chvapil and E. Ehrlich, *Biochim. Biophys. Acta* **208**, 467 (1970).
83. C. H. Ellis and G. D. Cain, *Am. Zool.* **10**, 318 (1970).
84. G. D. Cain and D. Fairbairn, *Comp. Biochem. Physiol. B* **40**, 165 (1971).
85. J. R. A. Leushner and J. Pasternak, *Can. J. Zool.* **56**, 159 (1978).
86. A. Nordwig, *Adv. Enzymol.* **34**, 155 (1971).
87. J. Gross, in (*2*), pp. 275–317.
88. A. Z. Eisen and J. J. Jeffrey, *Biochim. Biophys. Acta* **191**, 517 (1969).
89. A. Z. Eisen, K. O. Henderson, J. J. Jeffrey, R. A. Bradshaw, *Biochemistry* **12**, 1814 (1973).
90. J. Phillips and M. H. Dresden, *Biochem. J.* **133**, 329 (1973).
91. C. Boulard and R. Garrone, *Comp. Biochem. Physiol. B* **59**, 251 (1978).
92. R. M. Howell, *Nature (London)* **209**, 713 (1966).
93. This subject has been reviewed by M. L. Tanzer [in (*2*), pp. 137–162].
94. A. J. Bailey, *FEBS Lett.* **18**, 154 (1971).
95. D. R. Eyre and M. J. Glimcher, *Biochim. Biophys. Acta* **243**, 525 (1971).
96. G. M. Windrum, P. W. Kent, J. E. Eastoe, *Br. J. Exp. Pathol.* **36**, 49 (1955).
97. B. Barzansky and H. M. Lenhoff, *Am. Zool.* **14**, 575 (1974).
98. L. DeVos and F. Rozenfeld, *J. Microsc. (Paris)* **20**, 15 (1974).
99. D. F. Travis, C. J. Francois, L. C. Bonar, M. J. Glimcher, *J. Ultrastruct. Res.* **18**, 519 (1967).
100. R. Garrone and J. Pottu, *J. Submicrosc. Cytol.* **5**, 199 (1973).
101. R. Garrone, J. Vacelet, M. Pavans de Ceccatty, S. Junqua, L. Robert, A. Huc, *J. Microsc. (Paris)* **17**, 241 (1973).
102. A. Nordwig and U. Hayduk, *J. Mol. Biol.* **26**, 351 (1967); H. Nowack, R. Timpl, A. Nordwig, *Eur. J. Immunol.* **4**, 698 (1974).
103. J. M. Gosline and H. M. Lenhoff, *Comp. Biochem. Physiol.* **26**, 1031 (1968).

104. B. J. Rigby and M. Hafey, *Aust. J. Biol. Sci.* **25**, 1361 (1972).
105. M. S. Silberberg, L. S. Ciereszko, R. A. Jacobson, E. C. Smith, *Comp. Biochem. Physiol. B* **43**, 323 (1972).
106. S. D. Young, *Int. J. Biochem.* **4**, 393 (1974).
107. J. Pikkarainen and E. Kulonen, *Comp. Biochem. Physiol. B* **41**, 705 (1972).
108. W. M. Goldberg, *ibid.* **49**, 525 (1974).
109. M. Kalyani, *Biochim. Biophys. Acta* **221**, 135 (1970).
110. D. Fujimoto, *ibid.* **140**, 148 (1967); W. F. Harrington and G. M. Karr, *Biochemistry* **9**, 3725 (1970); P. V. Hauschka and W. F. Harrington, *ibid.*, p. 3734; *ibid.*, p. 3745; *ibid.*, p. 3754; S. Fuchs and W. F. Harrington, *Biochim. Biophys. Acta* **221**, 119 (1970); A. Maoz, S. Fuchs, D. Michaeli, *ibid.* **243**, 106 (1971); D. Michaeli, G. Senyk, A. Maoz, S. Fuchs, *J. Immunol.* **109**, 103 (1972); D. Fujimoto, *J. Biochem. (Tokyo)* **78**, 905 (1975).
111. W. F. Harrington and N. V. Rao, *Biochemistry* **9**, 3714 (1970).
112. A. Maoz, S. Fuchs, M. Sela, *ibid.* **12**, 4246 (1973).
113. T. P. Bonner and P. P. Weinstein, *J. Ultrastruct. Res.* **40**, 261 (1972); B. J. Rigby and M. S. Robinson, *Nature (London)* **253**, 277 (1975).
114. J. Leushner and J. Pasternak, *Dev. Bol.* **47**, 68 (1975); J. Pasternak and J. R. A. Leushner, *J. Exp. Zool.* **194**, 519 (1975).
115. B. Baccetti, *Monit. Zool. Ital.* **1**, 23 (1967).
116. M. D. Maser and R. V. Rice, *Biochim. Biophys. Acta* **74**, 283 (1963); *J. Cell Biol.* **18**, 569 (1963); D. Fujimoto and E. Adams, *Biochim. Biophys. Acta* **107**, 232 (1963); R. E. Coggeshall, *J. Cell Biol.* **28**, 95 (1966); Y. C. Lee and D. Lang, *J. Biol. Chem.* **243**, 677 (1968).
117. M. D. Maser and R. V. Rice, *Biochim. Biophys. Acta* **63**, 255 (1962).
118. J. M. Burke and R. Ross, *Tissue Cell* **7**, 631 (1975); S. Humphreys and K. R. Porter, *J. Morphol.* **149**, 53 (1976).
119. B. Baccetti, *J. Cell Biol.* **34**, 885 (1967).
120. C. O. Hermans, *J. Ultrastruct. Res.* **30**, 255 (1970); S. Kimura, *Bull. Jpn. Soc. Sci. Fish.* **37**, 432 (1971); P. Valembois, *J. Microsc. (Paris)* **10**, 347 (1971); S. Kimura and M. Tanzer, *J. Biol. Chem.* **252**, 8018 (1977).
121. S. Hunt, M. E. Grant, S. J. Liebovich, *Experientia* **26**, 1204 (1970).
122. A. Abolins-Krogis, *Cell Tissue Res.* **156**, 217 (1975).
123. A. F. Krivis and C. O. Chiu, *Microchem. J.* **20**, 315 (1975).
124. J. P. Pujol. M. Rolland, S. Lasry, S. Vinet, *Comp. Biochem. Physiol.* **34**, 193 (1970).
125. B. Baccetti and G. Lazzeroni, *Tissue Cell* **1**, 417 (1969).
126. E. Harper, S. Seifter, B. Scharrer, *J. Cell Biol.* **33**, 385 (1967); D. E. Ashhurst and N. M. Costin, *Tissue Cell* **6**, 279 (1974); *J. Cell Sci.* **20**, 377 (1976).
127. H. R. Hepburn and J. J. A. Heffron, *Cytobiology* **12**, 481 (1976).
128. T. Matsumura, *Comp. Biochem. Physiol. B* **44**, 1197 (1973).
129. T. Matsumura, M. Shinmei, Y. Nagai, *J. Biochem. (Tokyo)* **73**, 155 (1973); T. Matsumura, *Conn. Tissue Res.* **2**, 117 (1974).
130. I. Pucci-Minafra, S. Minafra, F. Gianguzza, C. Casano, *Boll. Zool.* **42**, 201 (1975); S. Minafra, I. Pucci-Minafra, C. Casano, F. Gianguzza, *ibid.* **42**, 205 (1975).

NEWS AND COMMENT

EPA and Toxic Substances Law: Dealing with Uncertainty

The associate administrators of the Environmental Protection Agency (EPA) often gather at breakfast Monday mornings for an informal exchange on the ongoing battle to protect the environment. So frequently have the Sunday papers carried a story indicting yet another chemical as a threat to health and the environment that the offending substance has come to be ruefully referred to as the "chemical of the week."

The point underscored is that chemicals are ubiquitous in the environment and that some of them are dangerous. Chemicals also contribute significantly to American living standards, and regulating them involves substantial economic consequences. In 1976 Congress put EPA in charge of resolving this dilemma when it enacted the Toxic Substances Control Act (TSCA), which provided for the first comprehensive regulation of the chemical industry.

EPA's most obvious problem is the estimated 63,000 chemicals already in commerce and others coming into use at a rate of perhaps 1000 a year. The real difficulties for EPA, however, lie in the fact that the means of establishing long-term effects of these chemicals are imperfect. In regulating toxic substances, therefore, EPA must contend with a considerable measure of scientific uncertainty. At the same time, if the agency is not to be overwhelmed by sheer numbers of chemicals, it must set workable priorities for determining which chemicals to test and how to test them. It is not surprising, therefore, that now, at the end of the second year since the passage of TSCA, questions are being raised as to whether the law is enforceable or, to put it another way, whether EPA is capable of enforcing it.

TSCA is complicated and controversial legislation. The complexity is in part a product of the controversy. Six years of negotiations and debate were necessary before Congress passed it. Adversaries throughout the process were representatives of chemical industry and environmental groups. The tension persists, with the former group typically arguing that EPA is being too tough and the latter that the agency is not tough enough.

When it passed, TSCA was regarded as a substantial improvement on previous environmental laws: second-generation environmental legislation that built on experience. The major departure from earlier law were the provisions aimed at preventing cancer-causing substances from ever reaching the environment. As EPA administrator Douglas M. Costle is fond of saying, TSCA makes EPA a preventive health agency as well as an environmental agency. To protect EPA from being swamped by the sheer number of chemicals, the new law was designed to give the agency greater leeway in setting priorities for choosing which chemicals to regulate—enabling it, so to speak, to deal with worst things first.

TSCA is second-generation legislation also in the sense that it reflects the reaction of recent years against no-holds-barred federal regulatory activity and requires EPA to perform a balancing act between economic and environmental imperatives.

For a federal agency, the heart of any law is the section which sets forth its authority. TSCA states that the act's authority over chemicals "should be exercised in such a manner as not to impede unduly or create unnecessary economic barriers to technological innovation while fulfilling the primary purpose of this Act to assure that such innovation and commerce in such chemical substances and mixtures do not present an unreasonable risk of injury to health or the environment."

The law does not define "unreasonable risk" and, in the nearly 2 years since TSCA was signed into law, EPA has been mainly engaged in adding flesh to the spirit of the statute through the tortuous and time-consuming process of writing administrative regulations aimed at giving "unreasonable risk" a definition which is defensible.

To be sure, other circumstances have contributed to the slow pace of TSCA implementation. TSCA was enacted at the end of the Ford regency. It was not until nearly a year after the bill became law that EPA filled the post of director of its Office of Toxic Substances (OTS) with the appointment of Steven D. Jellinek, who came to EPA from the post of staff director of the Council on Environmental Quality. The last year has been spent hiring staff, including most of the upper and middle management, while at the same time formulating policy and get-

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