BASEMENT MEMBRANE AND INTERSTITIAL COLLAGEN CONTENT OF WHOLE ANIMALS AND TISSUES

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SUMMARY: A method for estimating total basement membrane collagen in whole animals or tissues was based on measuring 3-hydroxyproline and 4-hydroxyproline of samples. Basement membrane collagen is 1% or less of total mouse collagen, but is a substantial fraction of kidney cortex collagen. In agreement with earlier analysis by a different method, total mouse collagen is 2-3% of wet weight.

The collagen of mammals is now believed to comprise several genetically and structurally distinct types (1), all of which contain trans-4-hydroxy-L-proline as one-eighth to one-twelfth of total residues; collagen is the only significant source of 4-hydroxyproline in vertebrates (2). Basement membrane collagen differs from the other collagen types ("interstitial" collagens) both in amino acid and carbohydrate composition (3). A prominent difference is the content of trans-3-hydroxy-L-proline. This amino acid, unique to collagen, was discovered only recently (4,5) and is present at low levels in interstitial collagens: only one residue occurs in the 1052 residues of the α-1 chain of ratskin or calfskin collagen, the complete sequence of which is given in (6). In contrast, collagens purified from glomeruli (7), the anterior lens capsule (8), or whole kidney cortex (9) contain between 18 and 25 3-hydroxyproline residues per thousand residues. The marked difference in 3-hydroxyproline content of the known basement membrane collagens and of interstitial collagens prompted an attempt to

estimate total basement membrane collagen in whole animals or tissues by analyses of total 3-hydroxyproline and 4-hydroxyproline.

EXPERIMENTAL

Unlabeled trans-3-hydroxy-L-proline was obtained as noted in (9); samples of this compound were tritiated by an exchange method (New England Nuclear Corp., Boston, Mass.) and then purified by chromatography on an amino acid analyzer column. [2-^{14}C]-4-hydroxy-DL-proline was purchased from Amersham-Searle Corp., Arlington Heights, Ill. and purified as for 3-hydroxyproline. For analyses, whole mice or tissues (human kidney cortex or choroid plexus) were cut into small pieces and hydrolyzed in refluxing 6 N HCl (30 ml per g of tissue, 24 hours). 3-Hydroxyproline and 4-hydroxyproline were measured in hydrolyzates by an isotope dilution method. After hydrolysis, [^{3}H(G)]-trans-3-hydroxy-L-proline (1 x 10^{6} dpm, 200 nmoles) and [2-^{14}C]-4-hydroxy-DL-proline (1 x 10^{6} dpm, 50 nmoles) were added to the hydrolyzates, insoluble material was removed by filtration, and the clear brown-to-yellow filtrate was evaporated to dryness several times, then redissolved in water. An appropriate sample was placed on a Dowex 50-H^{+} column (X8, 200 mesh, 3.5 x 30 cm); the column was washed thoroughly with water and eluted with 0.5 N HCl. The radioactive eluates were pooled and usually purified further by passage through Dowex 1-acetate (X8, 100-200 mesh, 1 x 10 cm). On occasion a subsequent Dowex 50 step was required. Samples of the radioactive eluates were then pure enough to permit direct measurement of 3-hydroxyproline and 4-hydroxyproline on the amino acid analyzer (9); fractions corresponding to these peaks were obtained by a split-stream technique (9) and their radioactivity was measured. From such data the original content of each amino acid was calculated.

Calculation of the mg of basement membrane and interstitial col-

lagen in a sample was based on the relationships

$$\text{4-Hydroxyproline (}\mu\text{moles)} = aB + bI$$

$$\text{3-Hydroxyproline (}\mu\text{moles)} = cB + dI$$

where B and I are respectively mg of basement membrane and interstitial collagen, a and b are respective coefficients (μmoles per mg) for the concentration of 4-hydroxyproline in basement membrane and in interstitial collagen, and c and d are respective coefficients for the concentration of 3-hydroxyproline in basement membrane and in interstitial collagen. From the above, B and I can be readily expressed in terms of the analytic data for total μmoles of 3-hydroxyproline and 4-hydroxyproline in a sample, as

$$B = \frac{d(\text{4-Hydroxyproline}) - b(\text{3-Hydroxyproline})}{ad - bc}$$

$$I = \frac{a(\text{3-Hydroxyproline}) - c(\text{4-Hydroxyproline})}{ad - bc}$$

Estimates of basement membrane and interstitial collagen by the procedure described clearly depend both on analytical values for the two hydroxyprolines and on the concentration coefficients chosen. Values for interstitial collagen are based on the known sequence of the α-1 chain of rat (calf) skin collagen (6), for which b is taken as 1.18 (111 μmoles per 94 mg) and d as 0.0106 (1 μmole per 94 mg); coefficients for basement membrane collagen are less certain because purified collagen from only a few specific sources have been characterized; based on available analyses (7-9), an average value for a is taken as 1.37 (125 residues per 1000 residues, or 125 μmoles per 91 mg) and for c as 0.23 (21 μmoles per 91 mg). For a given set of coefficients, the ratio B/I (or %B/(B+I)) in a tissue sample is determined by the 4-hydroxyproline/3-hydroxyproline ratio. The curves of Fig. 1 show this relationship for the 3-hydroxyproline content selected for basement membrane collagen (Curve A), as well as for an arbitrary lower value of 10 residues per 1000 (c= 0.11, curve B).

RESULTS AND DISCUSSION

Table 1 presents several sets of data on whole mice as well as on two tissues of human origin. In considering the validity of these data, certain potential sources of error were considered. Foremost among these was possible destruction of either amino acid under acid hydrolysis conditions in the presence of a complex mixture of tissue components. An addition experiment (Experiment 3, Table 1) showed that free 3-hydroxyproline and 4-hydroxyproline,

TABLE 1. 3-HYDROXYPROLINE AND 4-HYDROXYPROLINE CONTENT OF WHOLE MICE AND OF TWO HUMAN TISSUES. Mice were 2-year old males (ICR strain); experiment 1 utilized 3 mice, experiments 2 and 3, one mouse each. Human kidney cortex was histologically normal tissue from a 43 year old female (Experiment 1) and a 25 year old male (Experiment 2). Human choroid plexus was pooled material from two males (71 and 83 years old). In Experiment 3 (whole mice), 10 μmoles of 3-hydroxyproline and 500 μmoles of 4-hydroxyproline were added to the mouse tissues before hydrolysis; the values shown are the totals recovered less the quantity of each added compound.

Tissue (Experiment)	3-Hydroxyproline (μmoles/gram)*	4-Hydroxyproline (μmoles/gram)*	Ratio $\frac{\text{4-Hyp}}{\text{3-Hyp}}$	%† $\frac{\text{B}}{\text{B+I}}$
Whole Mice				
(1)	0.31	29.8	96	0.8
(2)	0.32	30.3	95	0.8
(3)	0.32	29.8	93	0.9
Kidney Cortex				
(1)	0.88	12.1	15	36
(2)	0.81	13.6	17	29
Choroid Plexus	9*	340*	38	10

*For all samples except choroid plexus, this ratio is based on wet weight of whole animals or tissues. The pooled choroid plexuses were thoroughly rinsed in water to remove blood, then lyophilized and weighed; values shown are μmoles/gram dry weight.

†See Fig. 1 and text.

added to a mouse carcass in HCl solution before hydrolysis, were recovered almost quantitatively from a whole-mouse hydrolysate.

Another source of error is epimerization of either amino acid to the cis-form; this has been reported at only a low level for 4-hydroxyproline as a free amino acid or in collagen (10,11), but might be accelerated in a complex hydrolysis mixture. Because we added the radioactive tracers as the trans-forms only after hydrolysis, our method would underestimate each amino acid by the quantity of the cis-epimer formed during hydrolysis. Parenthetically, our reason for adding the tracers after rather than before hydrolysis was based on our concern that ^{3}H might be lost by exchange from the 3-hydroxyproline tracer, and indeed separate experiments showed that 25% of ^{3}H was lost from our tritiated sample of 3-hydroxyproline when subjected to acid hydrolysis either as the pure compound or when hydrolyzed with mouse tissues. For this reason, explicit estimates were made of the extent of epimerization of 3-hydroxyproline and 4-hydroxyproline obtained from whole-mouse hydrolysates; this reaction appeared not to exceed 6% for 3-hydroxyproline and 10% for 4-hydroxyproline.

Using the coefficients corresponding to curve A (Fig. 1), the data of Table 1 may be interpreted as follows: Perhaps surprisingly, considering its ubiquity, basement membrane collagen represents only a small fraction of total mouse collagen, probably less than 1%; if the lower value for 3-hydroxyproline content of basement membrane collagen is chosen (Curve B, Fig. 1), basement membrane collagen still represents only about 2% of total collagen.

In contrast, kidney cortex and choroid plexus give 4-hydroxyproline/3-hydroxyproline ratios indicating a substantial fraction of basement membrane collagen: between 30% and 40% for kidney cortex and approximately 10% for choroid plexus (Table 1). The evalu-

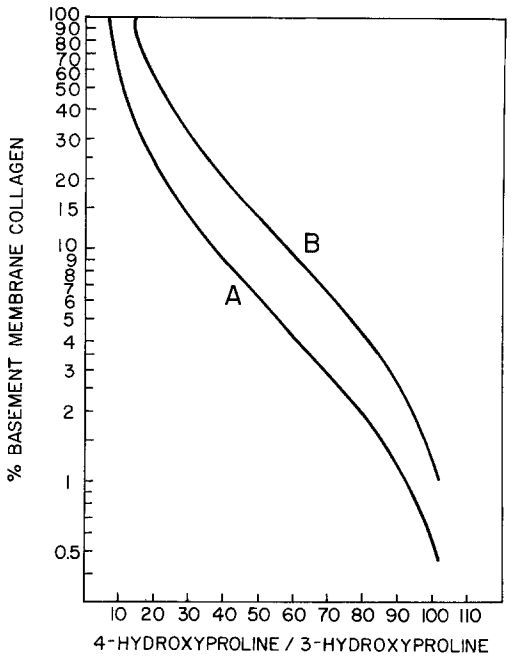

FIG. 1. SEMILOGARITHMIC PLOT OF PERCENT BASEMENT MEMBRANE COLLAGEN AS A FUNCTION OF THE 4-HYDROXYPROLINE/3-HYDROXYPROLINE RATIO. The ratio B/(B+I) is obtained from the expressions for B and I (see text) and is equal to $(dR-b)/(a-b+(d-c)R)$, where a,b,c,d are the coefficients defined in the text and R is the 4-hydroxyproline/3-hydroxyproline ratio. Concentration coefficients are as noted in the text for curve A; curve B is based on a lower assumed value of 3-hydroxyproline in basement membrane collagen as 10 residues per 1000 residues (c = 0.11).

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ation of percent basement membrane collagen is sensitive to the choice of coefficients in this range of the 4-hydroxyproline/3-hydroxyproline ratio (Fig. 1): thus if the lower coefficient for 3-hydroxyproline is chosen (Curve B, Fig. 1), kidney cortex will appear to contain over 70% basement membrane collagen.

The data of Table 1 imply that kidney contains a significant fraction of total body basement membrane collagen. If we apply data from human kidney cortex to mouse kidney and use a value for the mouse of 1.5 g of kidney per 100 g of body weight (12), we calculate that mouse kidney makes up approximately one-fifth of total

body basement membrane collagen. Analyses of swine and rabbit kidney cortex, to be reported separately, give 4-hydroxyproline/3-hydroxyproline ratios similar to those for human kidney cortex.

Our data for 4-hydroxyproline of whole mouse hydrolysates agree closely with those of Harkness et al (13), based on extraction of mouse tissues with hot trichloroacetic acid and colorimetric determinations on hydrolyzed extracts. Although the value for total mammalian body collagen is often erroneously cited as considerably higher, both our data and those of Harkness et al agree on a value of about 25 mg of collagen per g fresh weight of mice.

The procedure outlined would seem applicable to the analysis of any normal or pathological tissue, as well as of whole animals. It could, for example, determine if an extensive fibrotic lesion, such as that seen in experimental hydronephrosis (14), represents proliferation of basement membrane collagen, interstitial collagen, or of both types. It might also detect age- or disease-related changes in total collagen as well as in basement membrane collagen. While some uncertainties exist in the choice of concentration coefficients, our method appears suitable for the estimation of total basement membrane collagen in tissues, a determination that would seem difficult or impossible by morphologic means.

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