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Changes in the Mucopolysaccharide Composition of Bovine Heart Valves with Age

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The morphology of human heart valves changes throughout the average lifetime. Some of the changes, recognizable in all four types of heart valve in the absence of disease, are associated with the aging process (Lev & McMillan, 1961). Preparatory to studies on human valves, we have investigated some of the age-dependent biochemical properties of bovine heart valves. The biosynthetic activity of bovine heart valves declines continuously with increasing age of the animal (Boström, Moretti & Whitehouse, 1963). This also happens in another connective tissue, cartilage, the mucopolysaccharide composition of which changes with age (Whitehouse & Boström, 1961, 1962). We have

therefore examined the composition of each of the heart valves from a large number of new-born calves and old cattle and found distinct differences in their mucopolysaccharide content.

Three acidic mucopolysaccharides have been identified as constituents of pooled bovine and porcine heart valves (Deiss & Leon, 1955; Meyer, Davidson, Linker & Hoffman, 1956), namely hyaluronate, chondroitin sulphate C and derman sulphate (also known as chondroitin sulphate B or 'β-heparin'). Some new fractionation procedures involving the use of Rivanol (6,9-diamino-2-ethoxyacridine lactate) were developed for the quantitative isolation of small amounts of these polysaccharides from heart valves.

A brief report of this investigation has been published (Whitehouse & Moretti, 1963).

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MATERIALS AND METHODS

Chilled fresh hearts were obtained from calves 2-6 days old and from cattle 8-10 years old within 2 hr. of slaughter and immediately dissected. The excised valves were washed free of blood, immersed in acetone and then dried at room temperature. All preparations of dried valves were stored at -15° .

Twice-crystallized papain was obtained either from British Drug Houses, Poole, Dorset, or from Sigma Chemical Co., St Louis, Mo., U.S.A. Ovine-testicular hyaluronidase was the commercial product marketed by Bengers Laboratories, Holmes Chapel, Cheshire.

Rivanol (Ethodin, Acrinol) was a gift from Bayer Products, Surbiton, Surrey.

The following polysaccharides were used as reference compounds: streptococcal hyaluronate, having $[\alpha]_D^{20} -58^{\circ}$ (from Dr P. W. Kent, Oxford); chondroitin sulphate A isolated from calf cartilage, having $[\alpha]_D^{20} -20^{\circ}$ and containing 5% of galactose (from Dr S. Gardell, Lund, Sweden); commercial heparin (116 i.u./mg.; Boots Pure Drug Co., Nottingham); and a preparation of derman sulphate (β -heparin) originally isolated from ox lung (from Dr L. Rodén, Chicago, U.S.A.). This last-named material had been purified by precipitation with Benedict's reagent (Cifonelli, Ludowieg & Dorfman, 1958) and was further purified by exhaustive digestion with hyaluronidase. The final product had $[\alpha]_D^{20} -53^{\circ}$ and contained no glucosamine. Chondroitin [35 S]sulphate was obtained by papain digestion of calf-tracheal-cartilage slices that had been incubated *in vitro* with sodium [35 S]sulphate (Whitehouse & Boström, 1962).

Extraction of mucopolysaccharides from heart valves. Acetone-dried valves were chopped finely, extracted with two changes of chloroform-methanol (1:1, v/v) with intermittent shaking at room temperature over 2 days and finally dried at 100° for 3 hr.

Weighed portions of these defatted dried tissues were digested at 65° with crystalline papain (2 mg./100 mg. dry wt. of tissue) in 0.1M-acetate buffer, pH 5.8, containing cysteine (5 mM), EDTA (disodium salt) (5 mM) and NaCl (1.0M). After incubation for 18 hr., when most of the tissue was in solution, more papain (1 mg./100 mg. dry wt. of tissue) and cysteine were added and the digestion was continued for a further 24 hr. at 65° .

The digests were clarified by centrifuging and the residues were discarded. (These residues were very small, accounting for only 1-3% of the undigested tissue.) Trichloroacetic acid was then added at 3° to give a final concentration of 5% (w/v); the precipitated nucleates and protein were removed by centrifuging at 3° . Polysaccharides were precipitated at 3° from the supernatants by the addition of 3 vol. of ethanol saturated with sodium acetate. The precipitated mucopolysaccharides were washed with ethanol and redissolved in water. These solutions were stored at -15° .

Fractionation of mucopolysaccharides. Sulphated mucopolysaccharides were isolated from solutions containing the mixed mucopolysaccharides by the method of Whitehouse & Boström (1961), which utilizes the formation of a complex with Rivanol at acid pH.

The pH of portions of the solutions was adjusted to 1.5 with 0.5N-HCl, and excess of Rivanol was added (3 mg./mg. of total mucopolysaccharide, calculated from the amino

sugar content). The samples were kept at room temperature for 3 hr. and then centrifuged. The precipitates (P-1), which contained the sulphated mucopolysaccharides, were washed twice with 0.05N-HCl. The supernatants and washings were combined and the pH was adjusted to 3.2 (with thymol blue as an external indicator) by adding 1M-glycine with shaking. The non-sulphated acidic mucopolysaccharides were obtained as precipitates (P-2) which were washed once with diluted Rivanol. The supernatant was designated the 'neutral fraction'.

Other samples of the solutions containing the total mucopolysaccharides were digested with hyaluronidase (400 i.u./mg. of mixed mucopolysaccharides) at 50° in 0.1M-acetate buffer, pH 4.6, containing NaCl (0.15M). After incubation for 2.5 hr., more hyaluronidase was added (200 i.u./mg. of mucopolysaccharides) and the digestion was continued for a further 2.5 hr. Sufficient ammonium formate was then added to the hyaluronidase digests to give a final concentration of 0.5M. The addition of excess of Rivanol (1 mg./mg. of mucopolysaccharide initially present) precipitated the hyaluronidase-resistant mucopolysaccharide fraction (P-3) which was washed once with 0.5M-ammonium formate and once with diluted Rivanol. The products of hyaluronidase action on chondroitin sulphate and hyaluronate were not precipitated under these conditions.

Monosaccharide components of the mucopolysaccharide fractions. The mixed polysaccharides and individual fractions (P-1, P-2, P-3 and the residual neutral fraction) were assayed for amino sugars after acid hydrolysis as described below. Other samples of P-1, P-2 and P-3 were dissolved in the minimum volumes of 2M-potassium acetate by warming in a water bath at 60° . Colorimetric analyses for uronic acid were made on samples of these solutions, by both the carbazole and orcinol methods, as described below.

Analytical methods. Polysaccharide sulphates were determined turbidimetrically with Rivanol (1 mg./ml.) in 0.8M-ammonium formate (Whitehouse & Boström, 1961), with derman sulphate and chondroitin sulphate A as standards. Under these conditions hyaluronate, papain and hyaluronidase give no turbidity. Amino sugars were determined by a modification of the Elson-Morgan reaction (Bolognani, Coppi & Zambotti, 1958) after hydrolysis with 4N-HCl for 8 hr. at 100° and adsorption on columns of Dowex 50 (X8) (Boas, 1953). Diaminoethoxy-acridinium ions liberated after hydrolysis of the Rivanol-mucopolysaccharide complexes were retained on the Dowex 50. The uronic acid content was determined by using glucuronolactone as a standard (a) with carbazole in the presence of borate ions (Gregory, 1960), and (b) by the following procedure with orcinol: 1 ml. of the polysaccharide solutions (containing 5-50 μ g. of uronic acid) was heated with 2 ml. of the reagent (0.2% orcinol in 11N-HCl containing 6 mg. of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ /100 ml. of acid) in a boiling-water bath for 40 min. and the extinction was determined at 660 $m\mu$. Diaminoethoxyacridinium ions were removed, before determination of the uronic acid content with carbazole, by adsorption on alkali-washed fuller's earth (50 mg./ml. of solution). Nucleic acids were determined by measuring the ratio of the extinctions at 260 and 280 $m\mu$, with a purified preparation of yeast RNA as a standard.

Glucosamine and galactosamine were separated by paper chromatography (Fischer & Nebel, 1955) with a running time of 16 hr. at 20° . The amino sugars were stained on chromatograms with alkaline silver nitrate at room tem-

perature (Trevelyan, Procter & Harrison, 1950) or with ninhydrin, applied as a 0.25% solution in acetone containing 2% (v/v) of pyridine, the papers being dried at 65° in a humid-air oven for 15 min. For semiquantitative determinations of the glucosamine:galactosamine ratio, dried ninhydrin-treated chromatograms were sprayed with ethanolic cupric nitrate (Fischer & Dorfel, 1953) and dried at room temperature. The extinctions of the red ninhydrin-copper complexes formed by the amino sugars were measured at 510 m μ after the appropriate zones had been cut out and each eluted with 3 ml. of methanol. Areas of equivalent size from the background were similarly eluted. By this method 2 μ g. of amino sugar could be determined.

Amino sugars were also separated by ionophoresis in sodium molybdate, pH 5.0 after they had been caused to react with α -naphthyl isocyanate by the procedure described by Scott (1962). The amino sugar derivatives so formed were detected by their fluorescence in ultraviolet light.

Other procedures. Optical rotations were measured with an E.T.L.-N.P.L. recording polarimeter. The ³⁵S-labelled materials were assayed at infinite thinness with an end-window Geiger-Müller tube, counting with 2% efficiency. Mucopolysaccharide preparations were submitted to ionophoresis on paper (Lash & Whitehouse, 1960a): Rivanol-mucopolysaccharide complexes were dissolved in piperidine, these solutions were applied to the paper and ionophoresis was conducted in 0.1 M-sodium carbonate buffer, pH 10.4.

RESULTS

Qualitative aspects of the fractionation and analyses. The mixed polysaccharides precipitated with ethanol and sodium acetate from papain-digested heart valves contained no more than 10% by weight of nucleic acids. By the use of Rivanol, these mixtures were resolved into four fractions. Fractions P-1 contained at least two sulphated mucopolysaccharides, one of which was resistant to hyaluronidase digestion and was subsequently isolated as fractions P-3. Each P-3 fraction was characterized as derman sulphate by the negative optical rotation, constituent amino sugar (galactosamine) and a low uronic acid content (calculated as

glucurone) determined by the carbazole method; the uronic acid content, determined by the orcinol method, was in every case equivalent to that of the amino sugar. The ratio of the carbazole and orcinol values for the uronic acid content of P-3 fractions, from all four types of valves of both young and old animals, fell within the range 0.35-0.39 (Table 1). The ratios of the carbazole and orcinol values for authentic hyaluronate, chondroitin sulphate A, derman sulphate and heparin were 1.1, 1.1, 0.32 and 2.6 respectively. Only traces of glucosamine were contained in fractions P-3 and did not exceed 6% of the galactosamine content; glucosamine-containing sulphated mucopolysaccharides such as keratosulphate and heparin or heparitin sulphates, which are resistant to hyaluronidase digestion, could therefore be excluded as constituents of this fraction. No material absorbing at 260 m μ was present, so that the high orcinol values could not be ascribed to residual RNA.

Fractions P-1 likewise contained galactosamine and only negligible amounts of glucosamine. So the second polysaccharide sulphate that is present together with derman sulphate in this fraction, and that is susceptible to hyaluronidase, must be a chondroitin sulphate (A or C). That this predominated in the mixed fraction from each of the calf valves was shown by the comparatively high ratios of the carbazole and orcinol values, approaching unity, which are characteristic of polysaccharides containing glucuronic acid (Table 1). Fractions P-1 from the valves of old cattle gave a rather lower ratio of the carbazole and orcinol values, indicating a higher proportion of derman sulphate.

The third acidic fraction isolated from all valves (P-2) was hyaluronate; it had a single amino sugar constituent (glucosamine), a uronic acid content equivalent to that of the amino sugar, and a ratio of the carbazole and orcinol values of 1.1. The absence of sulphate in these fractions was indi-

Table 1. *Determination of uronic acids in polysaccharide fractions from heart valves*

The fractions were prepared as described in the text. The uronic acid contents were determined by the carbazole or orcinol methods, as described in the text, with glucuronolactone as the standard. The ratio of the two values is given for each fraction.

		Fraction P-1 (derman sulphate and chondroitin sulphate)		Fraction P-2 (hyaluronate)	Fraction P-3 (derman sulphate)
Animals	Valves				
Young calves	Aortic	0.71		1.00	0.36
	Pulmonary	0.81		1.10	0.39
	Mitral	0.90		0.94	0.35
	Tricuspidal	0.87		1.07	0.38
Old cattle	Aortic	0.50		1.10	0.37
	Pulmonary	0.51		1.15	0.39
	Mitral	0.66		0.97	0.38
	Tricuspidal	0.67		1.03	0.38

cated by their mode of isolation and also by their complete solubility in 0.5M-ammonium formate. Even such poorly sulphated polysaccharides as corneal chondroitin with an ester sulphate content of 4% give a turbidity with Rivanol in 0.5M-ammonium formate (M. W. Whitehouse, unpublished work). Further evidence that these fractions lacked any ester sulphate was given by their immobility when subjected to paper ionophoresis in 0.05M-trichloroacetic acid; for this purpose the fractions were redissolved in ammonium formate and the Rivanol was removed with fuller's earth.

These findings confirm previous observations on the mucopolysaccharide composition of (pooled) heart valves (Deiss & Leon, 1955; Meyer *et al.* 1956), and establish that these three acidic mucopolysaccharides are present in all four valves.

A fourth amino sugar-containing fraction, the 'neutral fraction', was present, together with excess of Rivanol, in the supernatants after the precipitation of hyaluronate at pH 3.2. An increase of the pH of this fraction by the addition of dilute sodium hydroxide did not precipitate any further polysaccharide, thus excluding the presence of an unsulphated derman. (The *pK* of iduronic acid is rather higher than the *pK* of glucuronic acid; Mathews, 1959). This Rivanol-soluble fraction had a negligible uronic acid content (Table 2), and was therefore composed of non-acidic aminopolysaccharides.

Quantitative aspects of the analysis. Significant differences were found between the quantities of the total and individual acidic mucopolysaccharides in calf valves and in the corresponding valves from old cattle. The combined acidic mucopolysaccharides constituted about 2.5–3.5% of the dry weight of the calf valves but only 1.4–1.8% of the dry weight of the valves from old cattle. These values are calculated from the amino sugar (or uronic acid) content of fractions P-1 and P-2 listed in Table 2.

The proportion of derman sulphate in the polysaccharide sulphate fraction (P-1) was higher in all the valves from old cattle (Table 3). This was indicated by higher yields of the fraction P-3 recovered after digestion with hyaluronidase, measured as amino sugar or uronic acid (orcinol method), by higher turbidity values with Rivanol in 0.8M-ammonium formate after hyaluronidase action, and by lower ratios of the carbazole and orcinol values for the uronic acid content of the mixed polysaccharide sulphate fractions (P-1). The turbidity values obtained with Rivanol after digestion with hyaluronidase, which give a measure of the residual derman sulphate, are expressed in Table 3 as percentages of the turbidity values given by the mixed polysaccharide sulphate

Table 2. *Quantitative analyses for total aminopolysaccharides and individual mucopolysaccharide fractions from heart valves*

Animals	Valves	Total aminopolysaccharides										The fractions were prepared, and the amino sugar and uronic acid (determined with orcinol) contents were measured, as described in the text. N.D., Not determined.									
		'Neutral fraction'					Fraction P-1 (derman sulphate and chondroitin sulphate)					Fraction P-2 (hyaluronate)					Fraction P-3 (derman sulphate)				
		Amino sugar (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Uronic acid (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)	Amino sugar (100 mg. dry wt. of tissue)
Young calves	Aortic	1350	216	0	0	960	900	1.06	1.06	215	195	1.10	1.10	135	141	0.96	0.96	—	—	103	(Lost)
	Pulmonary	1420	316	1.0	1.0	637	585	1.08	1.08	230	208	1.08	1.08	145	122	1.18	1.18	122	122	145	122
	Mitral	1400	N.D.	2.6	2.6	890	790	1.12	1.12	166	166	1.00	1.00	96	121	0.79	0.79	121	121	96	121
	Tricuspidal	1380	N.D.	1.2	1.2	820	830	0.98	0.98	228	210	1.08	1.08	166	156	1.06	1.06	222	222	200	200
Old cattle	Aortic	863	142	0	0	552	494	1.11	1.11	176	160	1.10	1.10	205	240	0.86	0.86	240	240	205	205
	Pulmonary	758	105	0	0	457	450	1.01	1.01	111	111	1.00	1.00	120	119	1.00	1.00	119	119	120	120
	Mitral	960	N.D.	N.D.	N.D.	428	472	0.91	0.91	156	156	1.00	1.00	156	156	1.00	1.00	156	156	156	156
	Tricuspidal	1125	N.D.	N.D.	N.D.	469	312	1.11	1.11	156	156	1.00	1.00	156	156	1.00	1.00	156	156	156	156

fraction before digestion of the chondroitin sulphate with hyaluronidase. The sensitivity of this turbidimetric assay is largely dependent on the charge density of the mucopolysaccharide molecules. It is unlikely that this would be the same for both the chondroitin sulphate(s) and derman sulphate(s) in heart valves of different ages, especially if the mucopolysaccharide fractions differ in molecular homogeneity and degree of sulphation. Therefore differences in the proportion of derman sulphate in fractions P-1 from the heart valves from young and old cattle deduced from the turbidity values given in Table 3 are of qualitative significance only. They are not strictly comparable with other values given in Table 3 which are proportions deduced from analyses for the monosaccharide components of fractions P-1 and P-3. These colorimetric analyses are independent of the heterogeneity, or charge density, of the derman sulphate(s) and chondroitin sulphate(s).

These analyses indicate that the amount of derman sulphate (fraction P-3) in each of the old valves was higher than in the corresponding calf valves (Table 2). Another notable change with age is the loss of the polysaccharide sulphate that is digestible with hyaluronidase and presumed to be

chondroitin sulphate A. Thus calf valves contained approx. 1.8–2.5 mg. of chondroitin sulphate/100 mg. of dry defatted tissue, whereas the valves from old cattle had only 0.7–1.2 mg./100 mg. of tissue. The hyaluronate content of all four calf valves (0.5–0.6 mg./100 mg. of tissue) was significantly higher than in the corresponding valves from old cattle (0.3–0.4 mg./100 mg. of tissue). These changes are summarized in Table 4.

The only notable difference between the mucopolysaccharide content of individual valves was between the two semilunar valves. Pulmonary valves contained at least 20 % less of the polysaccharide sulphates, i.e. chondroitin sulphate and derman sulphate together, than the aortic valves. This difference was also found by turbidimetric assays with Rivanol (in 0.8 M-ammonium formate) when the following values (expressed as arbitrary turbidity units) were obtained with preparations of polysaccharides different from those previously analysed (Table 2) but from the same age group of animals: young aortic, 270; young pulmonary, 214; old aortic, 158; old pulmonary, 128. This difference was also noted in animals of intermediate age (1–5 years) and is of especial interest because aortic valves have a higher rate of metabolism and

Table 3. *Derman sulphate as a proportion of the total polysaccharide sulphate in different valves*

The derman sulphate content was measured as the amino sugar and uronic acid (determined with orcinol) contents of fractions P-3 and expressed as a percentage of the corresponding measurements on fractions P-1, and turbidimetrically after hyaluronidase digestion. Experimental details are given in the text.

Animals	Valves	Calc. from analyses for		
		Amino sugar	Uronic acid	Rivanol turbidity
		Derman sulphate (% of total polysaccharide sulphate)		
Young calves	Aortic	14.1	15.7	7.4
	Pulmonary	16.1	(Lost)	14.5
	Mitral	16.3	15.4	11.1
	Tricuspidal	11.7	14.6	12.1
Old cattle	Aortic	30.0	31.5	20.0
	Pulmonary	43.7	44.4	25.9
	Mitral	47.8	50.7	36.6
	Tricuspidal	25.6	28.8	32.0

Table 4. *Comparison of the acidic mucopolysaccharide content of the valves of old cattle and those of the corresponding valves of young calves*

The fractions were prepared, and the analyses carried out, as described in the text.

Valves	Acid mucopolysaccharide content of valves of old cattle Acid mucopolysaccharide content of valves of young calves × 100 (%)			
	Total	Hyaluronate	Chondroitin sulphate	Derman sulphate
Aortic	62	73	47	123
Pulmonary	76	76	50	194
Mitral	51	67	30	141
Tricuspidal	60	68	48	125

incorporate inorganic [^{35}S]sulphate into polysaccharide sulphates more readily than pulmonary valves from the same animal heart (Boström *et al.* 1963). These differences in composition and metabolism may reflect the higher pressure to which the aortic valves are exposed.

Tests of the reliability of the procedures employed. The validity of the fractionation procedures and hence of the analytical results reported in the Tables was checked as follows. Chondroitin [^{35}S]sulphate (68 μC) added to papain digests of heart valves was quantitatively recovered in the polysaccharide fraction precipitated with ethanol and sodium acetate. Artificial mixtures of polysaccharide sulphates (1 mg. of chondroitin sulphate or derman sulphate or both) and 1 mg. of hyaluronate, alone and added to extracts of heart valves, were recovered in yields of 95 % or greater by precipitation with Rivanol at pH 1.5 and 3.2 respectively, as determined by amino sugar, uronic acid, turbidity and radioactivity assays. Derman sulphate (1 mg.) added to hyaluronidase digests was recovered in 98 % yield by precipitation with Rivanol in 0.5M-ammonium formate. Under these conditions keratosulphate (200 $\mu\text{g.}$) formed a precipitate but no material containing uronic acid was precipitated from hyaluronidase digests of chondroitin sulphate or hyaluronate. Diaminoethoxyacridinium ions present in solutions of Rivanol-precipitated polysaccharide, redissolved in 2M-potassium acetate or 2M-ammonium formate, were completely removed (measured by the loss in extinction at 270 $m\mu$) by shaking with alkali-washed fuller's earth and subsequent centrifuging. The recovery of polysaccharides in the supernatant was 98 %.

The orcinol method for determining uronides gave equal colour yields from glucuronic acid and galacturonic acid. Iduronic acid, the constituent uronic acid of derman sulphate, apparently gives the same colour yield as glucuronic acid since the ratio of the amino sugar content to uronic acid content, determined by this procedure, was unity for authentic purified derman sulphate and for the polyglucuronides, hyaluronate and chondroitin sulphate (after correction for the keratosulphate content of the latter). Heparin gave a lower colour yield than its theoretical uronic acid content. Ribose (as yeast RNA) and galactose gave colour yields, measured at 660 $m\mu$, equal to 116 % and 22 % respectively that of glucuronolactone, and Rivanol did not interfere.

This orcinol method for determining uronic acids in a polysaccharide differs principally from those previously described (e.g. Rodén & Dorfman, 1958) in substituting Cu^{2+} ions for Fe^{3+} ions in the reagent and extending the time of heating. The hydrochloric acid concentration employed reproduces the conditions of the classical Tollens

method for determining uronic acids by decarboxylation with 21 % acid.

Analyses of the hyaluronidase preparation showed that 600 units, used to digest 1 mg. of the unfractionated mucopolysaccharides, contained not more than 2.4 $\mu\text{g.}$ of amino sugar and 7.5 $\mu\text{g.}$ of uronic acid (orcinol method). This represents the maximum contamination of the heart-valve mucopolysaccharides from this source. Even in 0.2M-ammonium formate, i.e. under much less stringent conditions than were used for precipitating derman sulphate in these experiments, 7500 units of this enzyme gave no precipitate with Rivanol.

DISCUSSION

Use of Rivanol as a reagent. Rivanol proved to be a versatile reagent for the measurement and fractionation of very small amounts (5 mg. or less) of polysaccharides obtained from calf valves. It offered some distinct advantages over the cationic detergents commonly used to precipitate acidic mucopolysaccharides (Scott, 1960) in forming immediate precipitates that could be separated by centrifuging without recourse to additives (such as described by Schiller, Slover & Dorfman, 1961). Rivanol was about five times as sensitive a reagent as cetylpyridinium salts for the turbidimetric determination of acidic mucopolysaccharides. A fairly accurate measure of the individual mucopolysaccharide fractions was also obtained simply by measuring the absorption due to the diaminoethoxyacridinium ion (at 410 $m\mu$ ϵ^{20} is about 6950; at 362 $m\mu$ ϵ^{20} is about 14 900; at 270 $m\mu$ ϵ^{20} is about 15 500) in the solutions obtained by redissolving the washed precipitates in aqueous ammonium formate or potassium acetate (0.5M salt solutions for hyaluronate and 1.8M for chondroitin sulphate or derman sulphate). Two precautions must be observed with this reagent: (a) solutions containing Rivanol should not be exposed to excessive light, especially direct sunlight, and (b) diaminoethoxyacridinium ions must be removed by adsorption or extraction (Whitehouse & Boström, 1961) before determining the uronic acid content of mucopolysaccharides with carbazole in concentrated sulphuric acid.

Effect of age on the polysaccharide composition of bovine heart valves. The mucopolysaccharide composition of both the semilunar and atrioventricular heart valves evidently changes with time. There are several reports that the mucopolysaccharide content alters with age in certain other connective tissues. These include human, bovine and invertebrate cartilage (Kaplan & Meyer, 1959; Lash & Whitehouse, 1960b; Anderson & Odell, 1960; Whitehouse & Boström, 1962), human nucleus pulposus (Hallén, 1958, 1959; Davidson & Wood-

hall, 1959), human, pig and rat skin (Loewi, 1961; Loewi & Meyer, 1958; Schiller & Dorfman, 1960), and human aorta (Bertelson & Marcker, 1961; Kaplan & Meyer, 1960; Zugibe, 1962). The decrease in hyaluronate and increase in derman sulphate in the heart valves with age follows the pattern in skin and aorta. The smaller change in the hyaluronate content of the valves on aging compared with other tissues, e.g. skin, may indicate less degeneration and loss of flexibility in valvular tissue. The loss of chondroitin sulphate from heart valves with age resembles that described in skin, aorta, nucleus pulposus and cartilage. Although heparitin sulphate(s) occurs in bovine and human aortae (Meyer *et al.* 1956; Kaplan & Meyer, 1960; Muir, 1961), we obtained no evidence for its presence in either aortic valves or other bovine heart valves.

SUMMARY

1. Aortic, pulmonary, mitral and tricuspidal valves from new-born calves and from cattle 8–10 years old have been analysed for their content of acidic mucopolysaccharides. Hyaluronate, a chondroitin sulphate and derman sulphate were found in each valve.

2. The derman sulphate content of valves from old cattle is higher than that of valves from calves.

3. Each of the valves from old cattle contained less hyaluronate and much less chondroitin sulphate than the corresponding calf valve.

4. Procedures involving the use of Rivanol are given for the quantitative fractionation of hyaluronate and polysaccharide sulphates in less than 10 mg. of a mucopolysaccharide mixture.

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