Mucopolysaccharides of Whole Human Spleens in Generalized Amyloidosis *

THOMAS BITTER † AND HELEN MUIR ‡

(From the Medical Unit, St. Mary's Hospital, London, England)

Amyloidosis is a morphological concept (2) the diagnosis of which has been based on histological critera (3). "Primary" and "secondary" amyloidosis and the forms of amyloidosis seen in conjunction with familial Mediterranean fever (FMF) and myeloma are all grouped under one name because of morphological similarities, although it is not certain that they represent a single disease. Electron microscopy has, however, revealed characteristic fibrils in all forms of amyloidosis (4–6). Some of the stains that are taken up by amyloid material suggest that it may contain mucopolysaccharides (MPS) (7). Nevertheless, chemical investigations have not previously shown any marked increase in extractable metachromatic material (8) nor in uronic acid (9) or sulfate-containing compounds (10), although heparan sulfate has been isolated from a few amyloid-bearing organs (11, 12). The purpose of the present study was a systematic chemical investigation of the MPS in an extended series of cases and controls that has not hitherto been carried out.

Methods

Twenty patients with primary and secondary amyloidosis were collected, including one after myeloma and one after FMF. Table I gives some clinical findings and the distribution of amyloid in the spleens. The degree of infiltration was assessed arbitrarily as follows: + = partial, ++ = subtotal, and +++ = total replacement by amyloid. The kidneys were also infiltrated with amyloid

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in all patients, and the adrenals in all those examined (Patients 4, 17, and 18 were omitted). The liver was involved significantly in Patients 6 and 7 and to a lesser degree in Patients 1 to 3, 8, 10, 12, 14, and 17 to 19. The gastrointestinal tract, however, was involved throughout its length only in Patients 11 and 15, and the thyroid showed little involvement except in the capillaries and periacinar interstitial tissue of Patients 1, 4, 11, and 20. Amyloid was found in the pancreas of Patients 1, 3, 10, 12, 19, and 20, and in the small blood vessels of the myocardium of Patients 5, 8, 11, and 18. None had been jaundiced, none except the patient with juvenile rheumatoid arthritis (Patient 1) had noticeable lymph gland enlargement, and none had signs of neurological disorder. The diagnosis of rheumatoid arthritis in Patients 2, 3, 4, 5, and 7 met the requirements established by the American Rheumatism Association (15). Urinary infection of variable duration was observed in Patients 3, 9, 13, and 15 and had been treated with sulfonamides, antibiotics, or both.

Samples of fresh or acetone-fixed spleen tissue were stained with methyl violet, the periodic acid-Schiff reagent, Congo red, and iodine, respectively. Iodine staining was often found unsatisfactory when applied to sections prepared after formalin fixation and paraffin embedding. Amyloid deposits were recognized by typically localized hyaline lesions showing affinities for at least three of the dyes mentioned above.

Eighteen spleens were obtained from individuals who had died suddenly as road casualties, from cerebrovascular accidents, coronary arterial occlusions, or suicide, and who were found at postmortem examination to be free from inflammatory or neoplastic disease. The ages of these controls were chosen to match those of the patients and were grouped as follows: under 31 years, 34 to 46 years, and over 49 years. Spleens from some controls within each age group were pooled before extraction.

All reagents used were of analytical grade except for enzymes, amino sugars, 9-aminoacridine hydrochloride, ethylenediaminetetraacetic acid, and acetone for fixation and storage of tissues.

Analytical methods. Uronic acid was determined by the modified carbazole method of Bitter and Muir (16), hexose by the anthrone method (17), and hexosamine by a modification of the procedure of Cessi and Piliego (18) after hydrolysis of the MPS in 4 N HCl at 100° C for 8 hours. Sulfate was determined by a modification of the method of Jones and Letham (19). All these analyses were done in duplicate and repeated if agreement was less than ± 2%. Average values are given in Table II.

TABLE I

Diagnosis of patients with amyloidosis and distribution of lesions in the spleen

Patient	Sex	Age	Salient clinical features*	Spleen weight	Peni-cillar arteries	Sinusoids red pulp	Folli-cles	Trabe-culae	Larger vessels
		years		*g*					
1	M	19	Juvenile rheumatoid arthritis, onset at age 3½.	508	+	+	−	+	−
2	F	40	Rheumatic fever at age 8, chorea until age 15, rheumatic heart disease for 32 years, cardiac failure for 7 years (valvotomy, steroids).	272	−	−	+		−
3	F	45	Rheumatoid arthritis for 14 years (gold, chloroquine, steroids). Chronic bronchitis, bronchiectasis for 15 years. Hyperthyroidism for few months at age 33 (carbimazole).	225	+	+	−		−
4	F	51	Rheumatoid arthritis for 10 years (gold, salicylates, phenylbutazone, prednisone). Chronic bronchitis.		−	−	++		−
5	M	62	Rheumatoid arthritis for 20 years. Bronchiectasis (gold—see Reference 13).	577	+	+++	−	+	−
6	M	50	Ankylosing spondylitis with polyarthritis of small joints for 14 years (gold, steroids). Pulmonary tuberculosis for 12 years, pulmonary mycetoma.	400	+	+	−		−
7	F	45	Tuberculosis of right knee at age 15 (arthodesis). Disseminated miliary tuberculosis at age 34 (INH, PAS, streptomycin). Rheumatoid arthritis for 27 years (salicylates, prednisolone).†	153	+	−	−	+	+
8	M	34	Tuberculosis of both upper lobes at age 7 (untreated for 10 years, then streptomycin). Bronchiectasis; tuberculosis of left hip for 23 years. Addison's disease (prednisone).	220	+	+	−		−
9	M	44	Pulmonary tuberculosis (thoracoplasty). Chronic discharging empyema, gastric ulcer (gastrectomy), and malignant hypertension (pentolinium).	175	+	−	−		−
10	M	51	Tuberculosis? Admitted in uremic coma, died during dialysis. No charts available. Necropsy showed healed tuberculosis of left hip; hyperparathyroidism.	154	+	+	−		−
11	M	35	Osteomyelitis of left femur for 17 years after acute septicemia (unsuccessful removal of sequestra).	270	+	+	−		−
12	M	40	Chronic osteomyelitis of sacrum, one rib, and mandible for 24 years (tetracycline, unsuccessful removal of sequestra).	270	+	+	−		−
13	M	39	Subphrenic abscess for 4 months, pyonephrosis (drainage, antibiotics).	319	+	+	−		−
14	F	75	Recurrent "chest infections" for 60 years (antibiotics). Osteoporosis with compression fracture of spine.	129	+	+	−		+
15	M	45	Familial Mediterranean fever for 12 years (tetracycline, phenylbutazone). Malaria at age 25.	640	+	+	−		−
16	M	75	Carcinoma of right renal pelvis (see Reference 14).	100	−	−	++		−
17	M	83	Hypernephroma of right kidney; aortic stenosis without cardiac failure, gastric ulcer.	145	−	−	+		−
18	F	51	Myeloma, intermittent idiopathic erythema nodosum for 25 years; chronic pyelonephritis; and episodes of jaundice.	150	+	−	−		+
19	F	55	Primary nonfamilial amyloidosis.	120	+	+	−	−	−
20	F	60	Primary nonfamilial amyloidosis.	160	+	+	−	−	−

* Treatment, if any, stated in parentheses.
† PAS = para-aminosalicylic acid.

The ratio of glucosamine to galactosamine in the hydrolysates was determined after evaporating to dryness and redissolving in 0.33 N HCl by ion exchange chromatography (20) scaled down fivefold by using a small column (6 × 180 mm) of Zeokarb 225 (H⁺, 0.8%; water regain, 1.55). Satisfactory resolution was achieved only when 10- to 40-μ particles of resin were used. Mixtures of 2 to 25 μg of each amino sugar were separated and recovered quantitatively, whereas 0.5 μg in a fraction could be detected by the modified hexosamine method. The elution volumes of glucosamine and galactosamine were 20 and 24 ml, respectively. Fractions of 0.8 ml were collected at a rate of 4 ml per hour.

Extraction procedure. The spleens were sliced and placed in acetone at room temperature. The acetone was changed twice during the following 3 days. The tissue was stored in acetone at room temperature for up to 12 months to await extraction. Lipoids were removed (21), and the fat-free dry weight was obtained after the solvent had been removed in a current of air at 40° C for 6 hours and then overnight *in vacuo* over phosphorus pentoxide at 60° C. The dry tissues from Patients 4, 11, 13, and 15 were each suspended in 400 ml of 0.2 M sodium acetate, pH 5.7, and digested by activated papain [1] (22) at 60° C for 48 hours. The other spleens were similarly treated,

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[1] Supplied by Lights & Co. Ltd., Colnbrook, Bucks, Great Britain.

TABLE II

Analysis and calculated composition of MPS mixture extracted from spleens of patients with amyloidosis and of controls*
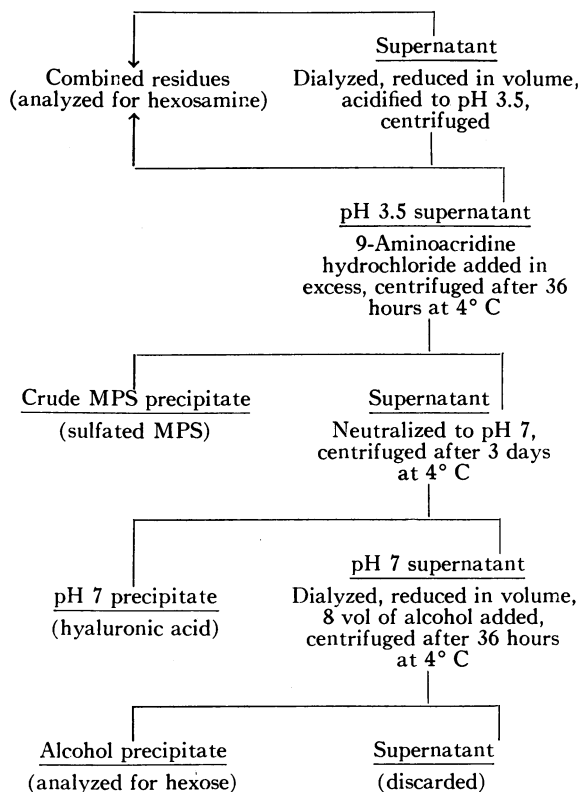
Predisposing disease	Patient	Sex	Age	MPS content	Molar ratio of glucosamine:galactosamine	Proportion of total MPS resistant to hyaluronidase	Molar ratio of uronic acid:hexosamine	MPS composition calculated from analyses			
								Heparan sulfate content	Heparan sulfate	Dermatan sulfate	Chondroitin sulfate A/C
			years	mg/100 g dry wt		% of total MPS		mg/100 g dry wt	% of total MPS		
Juvenile rheumatoid arthritis	1	M	19	151	0.994	65	1.0	75	50	15	35
Tuberculosis	8	M	34	120	1.24	65	1.05	67	55	10	35
Osteomyelitis	11	M	35	20	0.90	70	1.22	9.5	47		
Subphrenic abscess	13	M	39	111	1.64	69	1.37	69	62	8	30
Osteomyelitis	12	M	40	170	1.55	45	1.11	103	61	8	31
Rheumatic fever	2	F	44	321	0.53	77	1.11	111	35	10	55
Tuberculosis	9	M	44	77	1.16	71	1.09	41	54	23	23
Tuberculosis and rheumatoid arthritis	7	F	45 {34–45}	116	1.82	71	1.14	75	65	6	29
Rheumatoid arthritis and bronchitis	3	F	45	124	1.14	72	1.12	66	53	19	28
Familial Mediterranean fever	15	M	45	129	1.78	68	1.3	83	64	4	32
Ankylosing spondylitis and tuberculosis	6	M	50	41	1.87	69	1.56	27	65	4	31
Rheumatoid arthritis and chronic bronchitis	4	F	51	183	1.35	64	1.09	105	58	6	36
Tuberculosis?	10	M	51	33	1.07	65	1.24	17	52	13	35
Myeloma	18	F	51	43	1.37	67	1.54	25	58	9	33
Primary amyloidosis	19	F	55	47	1.30	76	1.41	27	57	19	24
Primary amyloidosis	20	F	60 {50–83}	81	0.64	69	1.12	32	39	30	31
Rheumatoid arthritis and bronchiectasis	5	M	62	490	0.416	55		144	29	26	45
Recurrent chest infections	14	F	75	103	2.10	70	1.30	70	68	2	30
Carcinoma of renal pelvis	16	M	75	147	1.40		1.49	86	58		
Hypernephroma	17	M	83	70	2.07		2.00	47	67		
Control spleens Single Pooled 1		M	14 {14–30}	127	0.33	25	1.11	32	25	0	75
Two		F	23;30	137	0.30	40		32	23	17	60
1		M	35 {35–46}	82	0.36	33	1.06	22	27	6	67
1		F and M	40 {35–46}	70 / 60	0.36 / 0.32	41 / 24	1.15	19 / 15	27 / 24	14 / 0	59 / 76
Nine		F and M	50–89 {50–89}	34	0.36	30	1.03	9	27	3	70
1		F	69 {50–89}	17	0.33	37	1.15	4	25	12	63

* Details given in Methods.   MPS = mucopolysaccharides.

DIAGRAM I

Fresh spleen

Sliced, stored in acetone, defatted, dried, weighed.
Rehydrated, digested with papain/pronase, centrifuged.

Combined residues	Supernatant
(analyzed for hexosamine)	Dialyzed, reduced in volume,
	acidified to pH 3.5,
	centrifuged

pH 3.5 supernatant

9-Aminoacridine
hydrochloride added in
excess, centrifuged after 36
hours at 4° C

Crude MPS precipitate	Supernatant
(sulfated MPS)	Neutralized to pH 7,
	centrifuged after 3 days
	at 4° C

pH 7 supernatant

pH 7 precipitate	Dialyzed, reduced in volume,
(hyaluronic acid)	8 vol of alcohol added,
	centrifuged after 36 hours
	at 4° C

Alcohol precipitate	Supernatant
(analyzed for hexose)	(discarded)

but the small undigested residue was subsequently suspended in 0.66 M borate, pH 8.4, containing 0.02 M $CaCl_2$ and digested for 48 hours with pronase [2] (23). The amount of each crude enzyme used was 1% of the dry tissue. Similar amounts of enzyme were added at 4-hour intervals in the daytime and the pH adjusted when necessary. The small amount of insoluble residue that remained was removed by centrifugation. The supernatant was dialyzed against 80 L of distilled water for 72 hours in the presence of toluene and then reduced to 400 ml by rotary evaporation. The solution was brought to pH 3.5 with 1 N HCl and the resulting precipitate collected by centrifugation after standing for 6 hours at 4° C. It was combined with the residue from proteolysis ("Combined residues," in Diagram 1, which illustrates the procedure used to extract MPS from whole spleens) and the hexosamine content determined after hydrolysis. The MPS in the supernatant were precipitated by adding 100 ml of 9-aminoacridine hydrochloride [1] solution saturated at 60° C (24). This precipitate, referred to as "crude MPS fraction" in Diagram 1, was collected after a minimum of 36 hours at 4° C by centrifugation and was then washed with 0.3% 9-aminoacridine solution. The MPS

[2] Supplied by Kaken Co., Tokyo, Japan.

were redissolved by converting them to sodium salts by exchange with Zeokarb 225 (Na, 8% 200-mesh), which took up the aminoacridine. The resin was washed four times by resuspending with an equal volume of water and centrifuging. The solution and washings were combined and reduced to approximately 10 ml. Four vol of ethanol half saturated with sodium acetate and cooled to −15° C was added. The resulting precipitate was collected after 36 hours at −15° C by centrifuging at 3,000 rpm at −10° C for 30 minutes. It was washed with 80% ethanol and with ethanol and acetone, dried at 60° C *in vacuo* for 16 hours, and weighed. The amount of MPS in the crude product was estimated from the uronic acid content (16).

Hyaluronic acid was obtained in small amounts from most of the samples of spleen MPS when the supernatants from the precipitation of sulfated MPS with 9-aminoacridine at pH 3.5 were adjusted to pH 7 (Diagram 1, "pH 7 precipitate"). The resulting scanty precipitates were collected after 4 days at 4°, converted to the sodium salts as described above, and the uronic acid contents determined.

The pH 7 supernatants (Diagram 1) were dialyzed to remove excess aminoacridine and reduced to approximately 150 ml, and 6 vol of ethanol was added. The hexose contents of the resulting precipitates were determined (17).

Examination of the MPS composition of specimens and fractions. In addition to determining the ratio of glucosamine to galactosamine in the MPS, we determined the proportion resistant to testicular hyaluronidase (hyaluronate lyase E.C. 4.2.99.1) as follows. Samples whose uronic acid content had been previously determined were incubated with 400 TRU per mg MPS of highly purified enzyme [3] at 37° C for 24 hours under toluene, fresh enzyme (200 TRU per mg MPS) being added every 2 hours during the day. After dialyzing for 72 hours at 4° C in previously heated dialysis tubing (25), the MPS that had not been digested by hyaluronidase were isolated via 9-aminoacridine and the recovered uronic acid was determined. 9-Aminoacridine did not precipitate oligosaccharides separated by Sephadex G-50 from hyaluronidase digests of chondroitin sulfate.

Some of the MPS samples were fractionated as their cetyl pyridinium (CPC) salts on cellulose columns according to the procedure of Buddecke (26) except that after eluting with 0.5 N $MgCl_2$ the salt was changed to 0.65 N KCl followed by 0.8 to 2 N KCl in stepwise concentration increments of 0.1 N, 30 ml of solution being used at each step. This improved the resolution of the MPS mixtures.

The MPS from spleens 4 and 5 were separated into two fractions by preparative electrophoresis in glass fiber (27) using 0.1 M phosphate buffer, pH 7.2. The total uronic acid in each fraction was determined and also the proportion of MPS resistant to hyaluronidase, as well as the ratio of glucosamine to galactosamine after hydrolysis of each fraction.

[3] 20,000 turbidity-reducing units (TRU) per mg, a gift of A. B. Leo, Hälsinborg, Sweden.

Electrophoresis of 5 to 10 μg of the MPS specimens was carried out on cellulose acetate strips at pH 7 in 0.1 M phosphate buffer, applying 8 v per cm for 2 to 3 hours. The strips were stained with 0.1% aqueous toluidine blue. The MPS mixture from human aorta was also applied to each strip to compare the resolving capacity of the strips.

The presence of heparin in the MPS specimens was sought by the CPC method of Schiller, Slover, and Dorfman (28). The antithrombin activity of several samples and fractions of MPS was determined by the procedure of Owren (29) and also by that of Douglas (30).

Results

In nineteen spleens with amyloid infiltration confirmed by histology, there was a significant though variable increase in total MPS, as compared with control spleens of corresponding ages (Figure 1 and Table II), although the total amounts were not large, in agreement with previous findings (8–10). There was, however, a more notable increase in a component containing glucosamine, which was resistant to hyaluronidase. This is assumed to be heparan sulfate (Figure 2). First, no heparin was detected in any of the samples on cellulose acetate electrophoresis (Figure 3) or by CPC fractionation (28) of 12 specimens; second, there was no evidence that keratan sulfate was present because only traces of hexose were
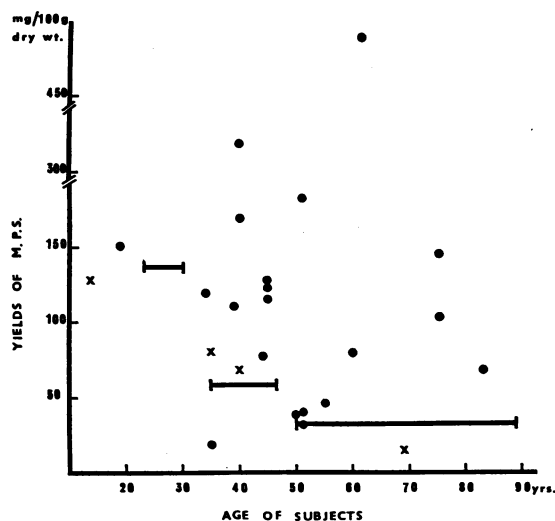
FIG. 1. TOTAL MUCOPOLYSACCHARIDE (MPS) CONTENT OF SPLEENS WITH AMYLOID INFILTRATION AND OF CONTROL SPLEENS FROM SUBJECTS OF DIFFERENT AGES. ● = amyloid spleens, × = single control spleens, and |——| = pooled control spleens.
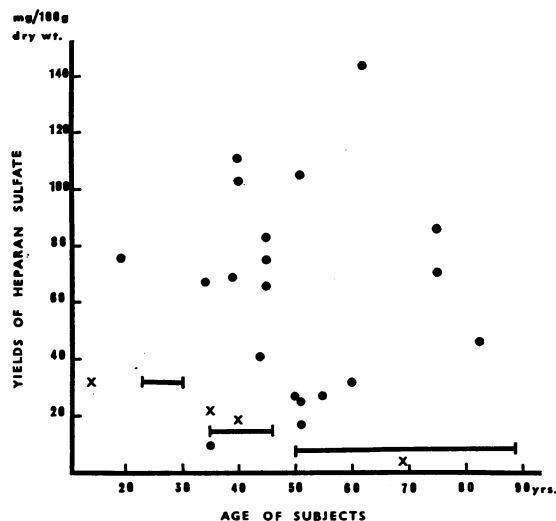

FIG. 2. HEPARAN SULFATE CONTENT OF SPLEENS WITH AMYLOID INFILTRATION AND OF CONTROL SPLEENS FROM SUBJECTS OF DIFFERENT AGES. ● = amyloid spleens, × = single control spleens, and |——| = pooled control spleens.

found by the anthrone reaction (17) in the MPS specimens, and they all had molar ratios of uronic acid to hexosamine greater than unity (Table II). Heparan sulfate thus accounted for much of the increase in total MPS that resulted in a marked rise in the relative proportion of heparan sulfate in the MPS specimens from amyloid spleens (Figure 4). The exceptions were spleens 2 and 5 where the total increase in MPS was most pronounced. Here there was a comparable increase in MPS containing galactosamine (Table II) so that little change in the ratio of glucosamine to galactosamine resulted.

The MPS from both control and diseased spleens had low antithrombin activities that were comparable and similar to that of the MPS from the urine of a patient with Hurler's syndrome.

As all the MPS specimens except that from spleen 5 had been precipitated at pH 3.5, the hyaluronic acid present in the spleens was not precipitated until the supernatants containing 9-aminoacridine were adjusted to pH 7 (Diagram 1, "pH 7 precipitate"). The amounts were approximately 2 to 5% of the total MPS estimated by uronic acid content.

The MPS only from spleens 4 and 5 were separated into two fractions by preparative electrophoresis in glass fiber (27) because there was not enough material from the other spleens. Com-
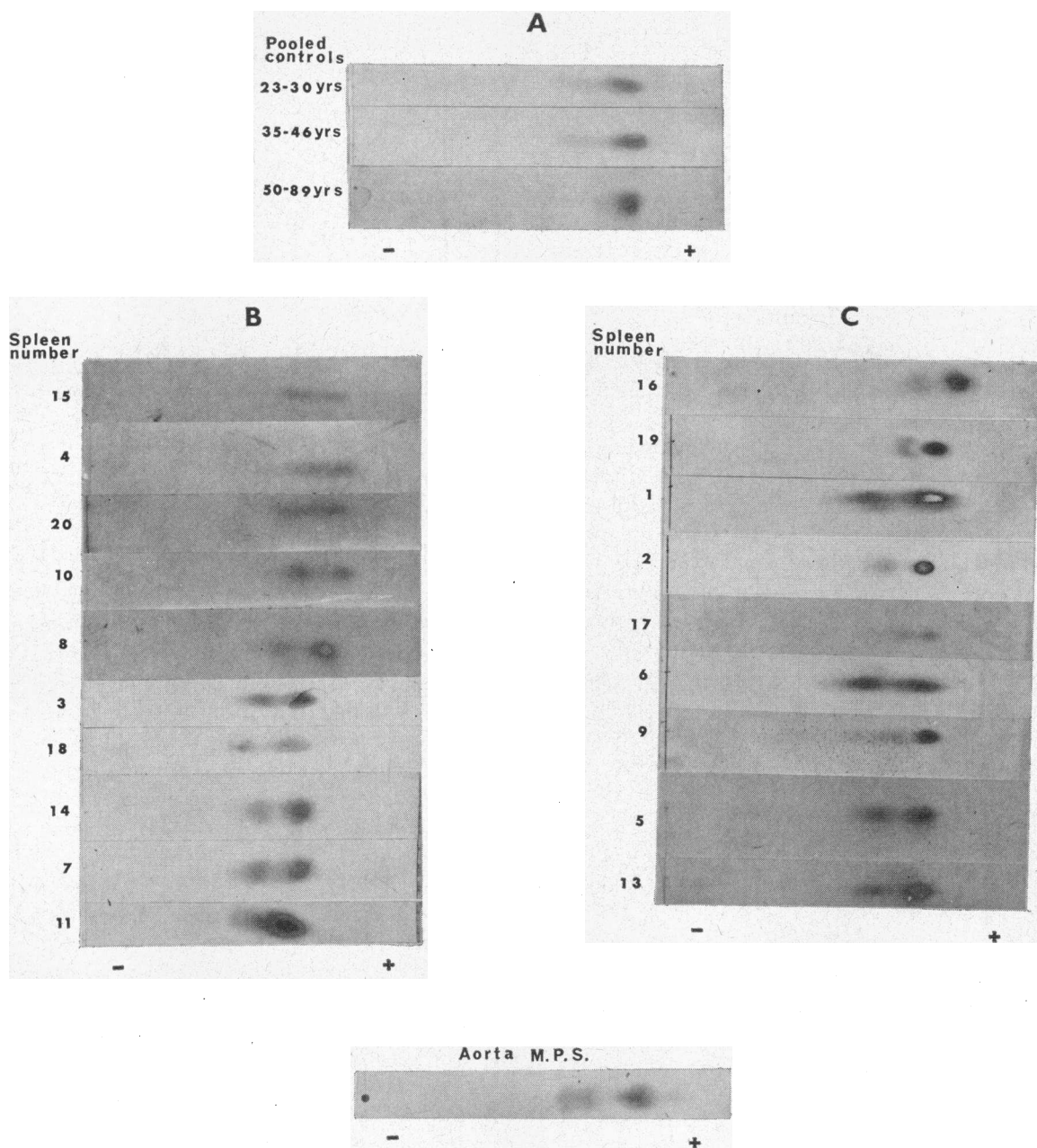
FIG. 3. CELLULOSE ACETATE ELECTROPHORESIS OF SPLEEN MPS AT pH 7.2. The strips were photographed after staining with 0.1% aqueous toluidine blue. *A*) MPS from pooled control spleens. Note that there is only one component. This had the same mobility as chondroitin sulfate, the main component of human aorta MPS, a typical pattern of which is shown at the bottom. Single control spleens (not shown) likewise had one component of similar mobility. *B*) and *C*) MPS from spleens with amyloid infiltration. Note that all have varying amounts of a second minor component. The major components had the same mobility as chondroitin sulfate from aorta. Each specimen was compared on the same strip with a standard MPS mixture from aorta to account for variation between runs not performed simultaneously, since the relative distances travelled on different strips were not directly comparable.

plete spleens were not always available, portions being removed for other purposes.

The approximate proportions of each compound in the two electrophoretic fractions of the MPS from spleen 5, shown in Table III, were deduced as follows. The faster fraction whose mobility was similar to that of chondroitin sulfate contained some glucosamine and more hyaluronidase resistant material. It would therefore appear to consist of a mixture of heparan sulfate and some dermatan sulfate, together with chondroitin sulfate A or C. The fraction of lower mobility contained some hyaluronic acid, because only in this instance were the MPS precipitated by 9-amino-acridine at pH 6.0. This fraction, after it had been submitted to electrophoresis three times to remove any contaminating material of faster mobility, contained only glucosamine. CPC fractionation yielded two components. One corresponded to hyaluronic acid, since it was eluted by 0.25 N MgCl$_2$ (31), whereas the other was eluted by 0.6 to 0.9 N KCl. This salt concentration was similar to that which eluted a heparan hemisulfate of aorta (32, 33). After removing hyaluronic acid with hyaluronidase, the remaining resistant MPS of the slow fraction had a ratio of sulfate to hexosamine of 0.79 : 1. Thus, the slow electrophoretic
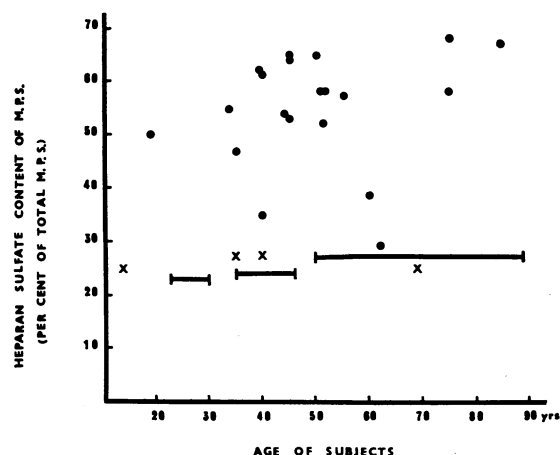

FIG. 4. THE PROPORTION OF HEPARAN SULFATE (EXPRESSED AS PER CENT) IN THE TOTAL MPS OF SPLEENS WITH AMYLOID INFILTRATION AND OF CONTROL SPLEENS OF DIFFERENT AGES. ● = amyloid spleens, × = single control spleens, and |———| = pooled control spleens.

fraction of spleen 5 would appear to contain a small amount of hyaluronic acid and a heparan of relatively low sulfate content. This had no significant antithrombin activity, since 347 μg had activity equivalent to 0.1 IU of heparin in the thrombin clotting time test (30).

CPC fractionation of both electrophoretic frac-

TABLE III

Composition of fast and slow electrophoretic fractions of MPS of spleen of Patient 5

Electro-phoretic fraction	Analyses of MPS		Composition of MPS mixture deduced from analyses		Contribution to total MPS of spleen
		% of fraction		% of fraction	% of total MPS
Fast	Hyaluronidase resistant	49	Chondroitin sulfate	51*	42
	MPS containing glucosamine	13	Heparan sulfate	13	11
	MPS containing galactosamine	87	Dermatan sulfate	36	29
				100	
Slow	CPC† precipitate eluted by 0.25 N MgCl$_2$	18	Hyaluronic acid	18	3
	CPC precipitate eluted by 0.6–0.9 N KCl	82	Heparan sulfate of lower charge density‡	82	15
	MPS containing glucosamine	100		100	100
	MPS containing galactosamine	0			

* Approximation.
† CPC = cetyl pyridinium.
‡ When purified, the molar ratio of sulfate to hexosamine was 0.79.

tions of MPS of spleen 4 showed them to be similar to the two fractions from spleen 5. Because the MPS had been precipitated at pH 3.5 there was no hyaluronic acid in the slow electrophoretic fraction.

The MPS from the other diseased spleens also showed two electrophoretic fractions on cellulose acetate, although the relative proportion of the slower component seemed to be variable (Figure 3). (The MPS from Patient 12 alone was not tested.) In contrast, the MPS from normal spleens migrated as single spots with the mobility of chondroitin sulfate.

Part of spleen 13 was extracted with 2 and 4 M CaCl$_2$ and with 6 and 8 M urea successively for 7 days each at 4° C. An insoluble metachromatic residue remained, from which the MPS were isolated after proteolysis and found to contain glucosamine almost exclusively.

The supernatants remaining after hyaluronic acid had been precipitated (Diagram 1, "pH 7 supernatant") were examined in 14 patients and in all the controls. Alcohol precipitated large amounts of material from the pH 7 supernatant that accounted for 0.7 to 1% of the dry weight of tissue in both groups. Hexose (17) accounted for 21 to 54% of this material in the specimens from diseased spleens and 43 to 45% in those from controls.

There was no significant variation with age in the ratio of glucosamine to galactosamine of the MPS from control spleens (Table II, Figure 4), although there was a progressive decline in total MPS content (Figure 1).

Discussion

The diagnosis of generalized amyloidosis had been established by biopsy in the majority of living patients and was confirmed independently at necropsy in all instances. In Patients 3, 4, 6, and 7, two concurrent predisposing conditions were present.

Spleens were selected in preference to other organs because of the high incidence of amyloid lesions in the spleen in generalized amyloidosis. Moreover, at the start of this investigation, control spleens were found to contain very small quantities of MPS, whereas in two of the diseased spleens first examined there were considerable

amounts of glucosamine-containing MPS. The liver normally contains heparan sulfates (11), which would obscure the pathological significance of changes in glucosamine-containing MPS. Kidneys are frequently affected by many of the diseases predisposing to amyloidosis, and any changes found might not be due to amyloid infiltration. Although the spleens from all the patients contained amyloid material, histological sections showed that the degree of infiltration was not very extensive, which perhaps accounted for their relatively small increase in total MPS.

Losses of MPS were reduced as far as possible. Aqueous fixation was avoided (34–37), and only molecules smaller than commercial heparin would have escaped on dialysis (38) in heated tubing (25). The hexosamine in the combined residues (Diagram 1) amounted to 4 to 8% of the total MPS hexosamine and may not be due to MPS in any case (39) but to glycoprotein (8, 40). It was impossible to estimate losses of MPS in the supernatants from which they had been precipitated because these contained hexose-rich material (Diagram 1, "pH 7 supernatants") that interfered with the determination of uronic acid. This material resisted proteolysis and therefore did not dialyze. It probably consisted of the carbohydrate-rich moieties of glycoproteins derived from plasma and from those which accumulate in amyloid lesions (7, 8, 40). Neither could the total MPS content of whole spleens be determined by hexosamine or uronic acid analysis because of interference by glycoproteins, nor by sulfate determinations because of interference by nucleic acids. The recoveries were therefore assessed on recoveries of standard mixtures submitted to each stage of the isolation procedure. With the same procedure, high yields of even urinary MPS had been obtained (35). When cartilage chondroitin sulfate was precipitated with 9-aminoacridine, less than 1 μg per ml of uronic acid was detected in the supernatant (after excess aminoacridine had been removed), and 79% of keratan sulfate was recovered from a solution containing 45 μg per ml. To reduce losses even further the dialyzed solutions of spleen MPS were concentrated tenfold before the MPS were precipitated.

Possible ways of isolating amyloid material from the rest of the spleen by mechanical (41, 42), chemical (43, 44), and physicochemical methods

(9, 45, 46) have been discussed by Calkins, Cohen, and Larsen (10). None of these were considered suitable for the present investigation because they were not quantitative. Although amyloid fibrils have also been separated by differential centrifugation (47), they may not constitute the whole of amyloid material. Whole tissue was therefore used so that over-all quantitative changes might be discerned before using more refined methods for separating amyloid material that would unavoidably be less quantitative, even though it could be argued that there would be no direct evidence that the MPS isolated were located in the amyloid lesions. Recent studies with radioactive sulfate demonstrate increased synthesis of MPS at sites where amyloid is subsequently deposited (48, 49) and at these sites during the course of the disease (50). Since amyloidosis was apparently the only disease common to all the spleens of the present series, it is not unreasonable to suppose that any consistent changes found would be the consequence of amyloid infiltration.

Earlier investigators (8–10 and 51–53) have found no significant differences between the MPS contents of amyloid and normal organs. The small increase found here in most of the diseased spleens (Figure 1) may be due to more complete isolation of MPS and also to more sensitive methods for determining uronic acid (16) and hexosamine (18), which enabled 0.5 μg of either sugar to be detected. The composition of crude MPS mixtures could thus be deduced without purification. Unless infiltration were uniform, however, good correlation is unlikely between histological assessment of amyloid infiltration and changes in minor constituents such as MPS. Most of the available spleen was required for chemical analysis, curtailing histological examination. Heparan sulfate, contrary to a previous report (12), was found in control spleens. In amyloid spleens, however, it may consist of two forms separable by electrophoresis. The MPS from all the diseased spleens had a minor component of somewhat lower mobility than the rest (Figure 3). In Patient 5 where it was isolated, it proved to be a heparan of relatively low sulfate content. This spleen also contained another heparan sulfate that migrated with chondroitin sulfate (Table III). Both varieties would have been estimated together by glucosamine analysis in the other patients. If the

minor component of lower mobility, apparently absent in control spleens (Figure 3), is exclusive to amyloid material, it would be particularly difficult to correlate over-all MPS changes with histological findings.

The molar ratio of sulfate to hexosamine of the heparan of lower mobility from spleen 5 was somewhat greater than that of a low-sulfated heparan from aorta (33) but was similar to that of one fraction separated by Cifonelli and Dorfman (54) from heparin side fractions. There appear to be a number of heparan sulfates, however, which have yet to be fully characterized (55), and both varieties in amyloid spleens may differ from that in healthy organs.

The absence of heparin is not unexpected because it is probably formed only by mast cells (56), which are not conspicuous in the development of amyloidosis (57–60). The low antithrombin and antithromboplastin activities of the MPS from both diseased and control spleens are probably due to the activities of dermatan (61) and heparan (62) sulfates, which were present in all specimens. The absence of keratan sulfate is also explicable because it has been found as a normal constituent only of cornea and parts of the skeleton (63–65).

The presence of characteristic fibrils in all forms of amyloidosis (4–6) would suggest that amyloid is a specific lesion despite differences in staining properties and the variety or absence of predisposing conditions and concomitant plasma protein changes (45, 66–68). Amyloid material is largely protein (9, 51), but it is not yet known whether it is the same protein in diseases as different as myeloma, familial Mediterranean fever, chronic inflammatory disease, and infectious complications of agammaglobulinemia (69). Most forms of generalized amyloidosis where there is great variation in morphology (70) and clinical features (71, 72) were represented by the present 20 patients. The consistent increase in heparan sulfate, particularly in relation to the other MPS (Figure 4) would suggest that heparan sulfate may be involved in the deposition of amyloid protein. If it were bound by some nonspecific process, the other MPS might be involved also, but they showed no consistent changes (Table II). A report (73) that chondroitin sulfate increased in the liver of a patient with amyloidosis conflicts with

the results of Meyer, Davidson, Linker, and Hoffman (11). Furthermore, no heparan sulfate was identified in the diseased (73) or normal (74) liver, an organ from which heparan sulfate was first isolated (75).

The fibrous nature of amyloid protein may account for its insolubility (46, 76, 77), although why it should be deposited as fibrils (4–6) remains to be explained. The process may be analogous to the nucleation of collagen fibrils, which is affected differently by different MPS (78), only very small amounts of MPS being incorporated into the fibrils. The amount of heparan sulfate in amyloid material thus need not be large but enough to make the fibrils insoluble and resistant to peptic digestion (2, 7, 40, 79). The resistance of "fibrinoid" deposits to proteolysis appears to be associated with the presence of metachromatic material (80–81). The finding that a glucosamine-containing MPS remained in the insoluble residue after part of spleen 13 had been extracted with calcium chloride and urea provides some support for this. Deposits that stain like amyloid appear around visceral blood vessels after intravenous injection of sulfated cellulose (82). Heparan sulfate may perhaps precipitate amyloid protein in a similar way. Christensen, Hjort, and Bertelsen (58) consider, however, that MPS appear late in the development of amyloid lesions, but in Case 13 the short duration of the predisposing condition in a relatively young patient where the proportion of heparan sulfate was one of the highest does not support their views. Furthermore, in experimental amyloidosis sulfate turnover is increased in the initial stages of the development of lesions (48–50).

Hyaluronic acid was found only in trace amounts. It would not, therefore, appear to be involved in the pathological change in the spleen of the present series of patients. It has been reported, however, to be involved in cardiac amyloid (83), which Benditt and Eriksen (76) found lacked the singularly insoluble proteins of amyloid lesions elsewhere.

The MPS changes in amyloid degeneration do not appear to be associated with aging (Figures 1 and 2). Normal spleens, like other tissues, showed a progressive decline with age in MPS content. Unlike cartilage (84), intervertebral disc (85), or skin (86), however, the relatively

constant ratio of glucosamine to galactosamine (Table II) suggests that there is no gross change with age in MPS composition in normal spleens.

Summary

Acid mucopolysaccharides (MPS) were extracted quantitatively from the spleens of 20 patients with generalized "primary" and "secondary" amyloidosis and from the spleens of 18 healthy subjects of comparable ages who had died suddenly from accidents or suicide.

The MPS content of control spleens decreased with age, but the MPS consistently contained 25 to 27% of heparan sulfate.

The total MPS per gram dry weight increased in all but one of the spleens with amyloid infiltration compared with controls of comparable ages. This was largely accounted for by a rise in the relative proportion of heparan sulfate in the MPS from all amyloid-bearing spleens, even in the spleen that had no increase in total MPS. This change occurred irrespective of the type of amyloidosis or predisposing disease and showed no correlation with age.

All the MPS specimens from amyloid-bearing spleens had a minor component of lower electrophoretic mobility that was absent in control specimens. This component was isolated in one case and proved to be a heparan sulfate of relatively low sulfate content.

Part of the spleen from the patient with the most acute amyloidosis was extracted with $CaCl_2$ followed by urea. The MPS extracted from the metachromatic residue contained glucosamine.

We suggest that heparan sulfate may be involved in the deposition of protein in amyloid lesions in the characteristic insoluble fibrillar form seen in the electron microscope.

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