and sand rat nuclear inclusion agent formed plaques reaching maximum plaque sizes of 10 mm, 7–8 mm, 7 mm, 4 mm, and 2–2.5 mm, respectively within a period of 14 days. This cell culture remained viable up to 24 days with herpes T plaques. The rest of the viruses destroyed the rabbit kidney primary cells within 2–3 weeks.

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Glycosaminoglycans in Experimental Amyloidosis* (32836)

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Amyloidosis in animals is easily induced experimentally and serves as a useful model to study pathogenesis of amyloid disease as it occurs in man. The chemical nature of material recognized as amyloid and the mechanisms that are involved in amyloid formation are not well understood. It is still unknown whether amyloidosis is a single disease, appearing with varied clinical and anatomic expressions, or exists as several diseases. To explore such problems further chemical studies of amyloid material seem warranted.

The major components of amyloid consist of fibrillar protein (1), but complex carbohydrates are found in much smaller amounts (2–7). Carbohydrate materials occur, at least in part, as glycosaminoglycans [acid mucopolysaccharides (MPS)], and are suggested to contribute to the characteristic tinctorial quality of amyloid. The MPS are of particular interest, since they might play a basic role in formation of amyloid, and their characterization could aid in differentiation of specific forms of the disease.

As a further study of MPS in amyloidosis, these polysaccharides were determined in multiple organs from mice with amyloidosis induced experimentally. The results were compared to observations in human forms of the disease (6,7).

Experimental. Amyloidosis was produced in male C57 BL10J mice, 6–7 weeks old, obtained from Jackson Laboratory. The mice were divided into two groups, 50 animals each, consisting of mice with amyloidosis and controls. To induce the amylidosis the mice were given subcutaneous injection daily for a period of 120 days of 25–30 mg of casein, according to the method of Janigan (8). Those mice dying during the course of injections after 90 days were frozen and later pooled with those surviving the injection period. In order to obtain a maximum amount of tissue, no effort was made to study the sequence of amyloid production, or to quantitate the amount occurring in each mouse. At the end of the

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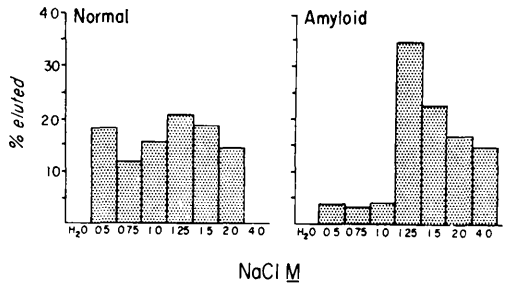

FIG. 1. Fractionation of acid mucopolysaccharides (MPS) isolated from splenic tissue from mice with induced amyloidosis and controls. The increasing molarity of NaCl fractionates on the resin groups of MPS compounds (please refer to text for further information) which allows quantitation of individual MPS by multiple analyses. Dowex - I × 2, Cl^-, 200–400 mesh.

period, the mice were killed, and selected organs (liver, spleen, kidney, and heart) were collected. A complete duplicate set was studied. The formation of amyloid deposits in tissues was verified by histologic examination of several animals, using the following stains: Congo red, crystal violet, periodic acid–Schiff, and hematoxylin–eosin. The format of inducing the amyloid in mice is well outlined in the experiments of Janigan, and similar anatomic changes have been reported in many earlier investigations of experimental amyloidosis.

Preparation of crude glycosaminoglycans (MPS). Glycosaminoglycans were extracted from dry, defatted tissue by a method described earlier (9). Briefly, this consisted of extraction of the tissue with alkali, digestion of the extract with proteolytic enzymes, removal of peptide material with trichloroacetic acid, dialysis of the resulting polysaccharide solution against distilled water, and concentration to a small volume. The total content of MPS isolated from the tissues was estimated by uronic acid analysis (10).

Fractionation of MPS by column chromatography. The isolated mixture of MPS was resolved by fractionation on Dowex 1 X2 (200-400 mesh) Cl^- column, 1.0 × 50 cm, by eluting with a stepwise increasing concentration of NaCl (0.5–4.0 *M*) (11). The effluent fractions were dialyzed, concentrated, and analyzed for uronic acid content. The MPS were then quantitated by additional analyses; the procedures for characterization and quantitation of the individual MPS in tissues with amyloid disease have been reported earlier (7). The resin technique fractionates MPS, due to their polydispersity and varying degree of sulfation, essentially into groups of compounds, and further chemical analyses are needed for quantitation of individual MPS. As an example, fractionation of polysaccharides from control and amyloid splenic tissues on Dowex columns is shown in Fig. 1. In general, the fractionation separates hyaluronic acid at 0.5 *M* NaCl; chondroitin and heparan sulfate, the latter with increasing sulfate content, from 0.75 to 1.5; chondroitin sulfates A, B, and C, 1.25–2.0; heparin and keratosulfates at 2.0 and 4.0 *M* concentrations of NaCl. On the basis of previous studies on a number of tissues and by many analytic procedures, including uronic acid, hexosamine, acetyl, sulfates, nitrogen, hyaluronidase digestions, optical rotations, chromatographic and electrophoretic techniques, individual MPS have been identified. However, fairly large amounts of materials are needed for all of these analyses, and extensive characterization of individual MPS was not possible with the amount of tissue available in the current studies. Recently, gas–liquid chromatographic techniques (12,13) for the analysis of uronic acids and hexosamines were developed and the study of much smaller quantities of MPS is now possible. In these experiments certain samples considered of similar composition were necessarily pooled for analysis. By this approach a certain "average" content of six individual MPS was obtained.

Analysis of fractions by gas–liquid chromatography (GLC). Fractions obtained from resin columns were analyzed for uronic acids and hexosamines by GLC using methods previously described (12–14). The MPS were hydrolyzed in 4 *N* HCl for 16 hours in sealed tubes at 100–105°C for hexosamine analysis. Trimethylsilyl (TMS) derivatives were prepared using hexamethyldisilazane and methylformamide (HMDS) (12). Samples to be analyzed for uronic acid were hydrolyzed in concentrated formic acid at 100–105°C for 20 hours in sealed tubes under nitrogen. The TMS derivatives of uronic acids were prepared by the method of Sweeley (14), and

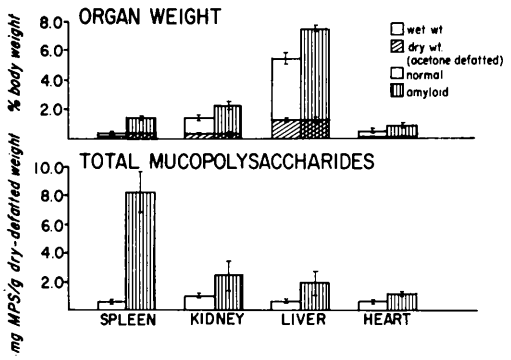

FIG. 2. Comparison of organ weights, wet and dry, defatted, from mice with amyloidosis and controls. The bar graphs at the bottom illustrate total amounts of MPS isolated from various organs in experimental amyloidosis and normal mice. The values are averages of two complete experimental sets.

the conditions of GLC have been described in detail elsewhere (13).

Results. Histologic examination of organs revealed the morphologic appearance and localization of amyloid similar to descriptions referred to earlier. The degree of involvement of the spleen, as expected, seemed to be most severe, but the liver and kidneys were also generously infiltrated with amyloid.

When the weight of organs was compared to body weight, all organs showed increases relative to body weight. Splenic changes showed as much as a fivefold increase over normals, whereas kidney and heart weights were almost double, and liver increased approximately 50% (Fig. 2). On a dry, defatted weight basis, however, the differences were less marked. The spleen still demonstrated a weight increase of three times that of controls. The differences in wet and dry, defatted weight changes suggest a marked increase in water concentration and/or lipid-extractable material in the tissues, induced by the disease. The presence of lipid in amyloid material has been shown by Kim, *et al.* (15), although it is unknown if the lipids form an integral part of amyloid.

Certain changes in content of MPS were observed in all tissues, illustrated at the bottom of Fig. 2. The total MPS values are shown based on uronic acid content, which represents approximately one third of the parent compounds. A marked increase in total MPS content occurs in tissues infiltrated with amyloid; in these experiments the spleen showed a striking 14-fold increase. Control animals showed very little variations in content of MPS within the group, whereas greater variability was found in mice with amyloidosis, which likely reflected differences in total response to casein injection.

The MPS were then studied further to assay for a specific composition of each tissue. The results of analyses of the mixture of compounds from amyloid spleen as fractionated on the resin columns is shown in Table I. Fraction I, representing a small amount of the total MPS, contained hyaluronic acid but, in addition, glycopeptide materials which interfered with quantitating the hexosamines and uronic acid; hence, no values for the sugars are reported in the table. Fraction V (1.5 *M*) as shown in Table I was indicated to be heparan sulfate and showed the presence of iduronic acid, which is now believed to be one of the constituents of this polysaccharide (16). Since the quantity of polysaccharides available were so small (note the total amount of uronic acid material available to study), no attempt was made to differentiate chondroitin sulfates A and C. The results of the analyses on the different organs for both groups of mice are shown in Fig. 3. For best comparison the estimation of the amount of MPS is expressed according to the dry, defatted weight of tissue for four organs from mice with amyloidosis and the control mice. The most characteristic change was the increase of heparan sulfate in all organs studied, including the heart. In the spleen the heparan sulfate increase was of a magnitude of 48-fold. Interestingly, CS-B showed a moderate increase in all of the organs, with somewhat smaller increases of the other MPS. These observations are quite similar to the results found for study of secondary amyloidosis in humans.

Discussion. Sorenson and others (17, 18) have suggested, after very careful and intricate histochemical and electron microscopic studies, that the experimental model of casein-induced amyloid produced in mice is similar to secondary amyloidosis as observed in humans. On a chemical basis, observations by Meyer

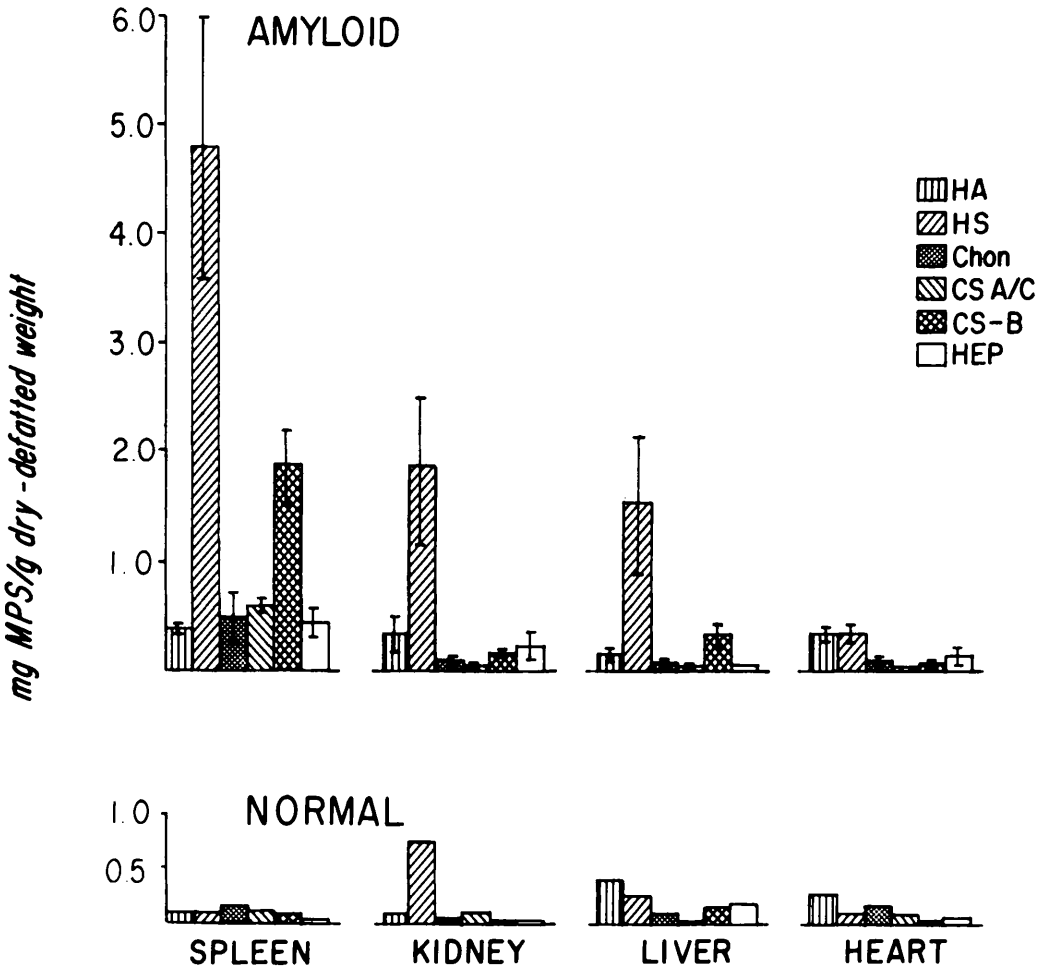

FIG. 3. Acid mucopolysaccharides from four organs from mice with amyloidosis and controls. The remarkable and consistent change is noted in increased content of heparan sulfate. An increase of chondroitin sulfate B was noted in the spleen. (HA, hyaluronic acid; HS, heparan sulfate. Chon, chondroitin. CS, chondroitin sulfates; HEP, heparin).

and associates (3), Bitter and Muir (6), and others (5), including our own laboratory (7), have all indicated that there is an accumulation of heparan sulfate, principally in organs involved in secondary amyloidosis. The findings in mice are similar and suggest that this experimental model closely mimics secondary forms of the disease as it occurs in humans. In a form of cardiac amyloidosis, large amounts of hyaluronic acid also accumulate. Unfortunately, the present state of knowledge concerning the MPS and their precise role in biologic functions does not allow further interpretation of their contribution to the pathogenesis of amyloidosis.

The results, however, suggest certain alterations in the metabolism of MPS. It is now clearly established that MPS exist in native form in tissue as MPS–protein complexes. The detection of a defect either in the protein, polysaccharide, or the linkage of the two would reflect changes in the metabolism of MPS. An alteration in the formation of the complex could occur, for example, by inhibition of an enzyme that brings about the linkage of MPS to protein, or in the synthesis of the protein moiety of the complex.

It is interesting to conjecture at this time, in view of the rather current attention to induction and repression mechanisms in protein

TABLE I. Analyses of MPS from Murine Amyloid Spleen after Dowex-1 Fractionation.

Fraction (NaCl *M*)	Total uronic acid (mg)	Gas–Liquid Chromatography: Hexosamine Glucosamine (%)	Hexosamine Galactosamine (%)	Uronic acid Glucuronic (%)	Uronic acid Iduronic (%)	MPS	%
0.50[a]	0.15	present	present	present	—	HA[b]	3.6
0.75	1.75	83.4	16.6	83.4	16.6	HS	35.0
1.00						Chon	7.0
1.25							
1.50	0.95	100.0	trace	71.6	28.4	HS	22.8
2.00	1.32	16.0	84.0	32.3	67.7	CS-A/C	5.3
4.00						CS-B	21.2
						Hep & KS (?)	5.1

[a] Neutral sugars, peptides present.

[b] For abbreviations, refer to Fig. 3 legend; KS, keratosulfate.

synthesis (19), that altered protein synthesis provoked by the continual casein injection without incriminating an immunologic basis is a factor in the production of the amyloid fibrils. Such a mechanism was suggested by studies of Teilum in 1954 (21) by attempts to inhibit the metabolism of proteins with cortisone and nitrogen mustard in proliferating mesenchymal cells. The relationship of the specific MPS involved in amyloidosis to the fibrillar protein being deposited remains to be defined.

These studies were able to demonstrate quantitative changes only and a consistent increase of one MPS, heparan sulfate. This observation establishes amyloidosis as a somewhat unique problem in MPS chemistry which is apt to excite further interest in the disease.

Summary. Acid mucopolysaccharides were studied in casein-induced amyloidosis in mice. The MPS were isolated and quantitated from four organs, spleen, liver, kidney, and heart. Heparan sulfate largely accounted for the increased content of MPS in all the tissues involved in amyloidosis, with changes quite similar to secondary amyloidosis in man. Experimental amyloidosis in mice is a reproducible model that is very useful in studying metabolic and pathogenetic aspects of a form of human amyloidosis.

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