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THE FATE OF INJECTED MUCOPOLYSACCHARIDES *

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The general outline of the biosynthesis of the mucopolysaccharides (MPSs) has been fairly well established by isotope studies. On the other hand, the mechanism and extent of removal from the tissues of these substances is still quite obscure. The half-life of hyaluronic acid and of chondroitin sulfate has been calculated as 2 to 4 and 7 to 16 days, respectively, from isotopic studies of rabbit skin (1) and rat cartilage (2), although there was considerable variation with age (3).

The disposal of locally injected polysaccharides has been studied by histochemical methods. On subcutaneous injection of chondroitin sulfate B (ChS-B) and heparitin sulfate (Hep.S.), metachromatic granules appear within 2 hours in mononuclear cells. These granules dissolve over the next 24 to 48 hours to give diffuse metachromasia in the extracellular spaces. ChS-A, on the other hand, requires 5 to 6 hours for maximal intracellular granule formation (4).

The urinary excretion of intravenously injected heparin has been reported to vary between 0 and 40 per cent by various investigators (5, 6). Recently, Loomis has shown that about 20 per cent of $S^{35}O_4$-labeled heparin appears in the urine 2 hours after injection (7). Piper found that only 2 to 4 per cent of a heparin preparation was dialyzable and assumed from this that it could not be filtered at the glomerulus but must be secreted by the renal tubule (8). From clinical experience in the use of heparin as an anticoagulant, it is known that the activity disappears in 4 to 6 hours after intravenous injection in doses of 0.5 to 1.0 mg per kg of body weight.

The urinary excretion of mucopolysaccharides has been studied in normal individuals and in some pathological conditions. Ordinarily the daily excretion in normal men and women is about 8 to 15 mg (9), expressed as chondroitin sulfate. Hyperexcretion of polysaccharides has been well documented in only one disease, Hurler's syndrome, in which quantities ten or more times that excreted by normal individuals have been found in the urine. Furthermore, in contrast to the finding that normal persons excrete mainly ChS-A or C, or both, the MPSs in Hurler's syndrome have been identified as ChS-B and Hep.S. (10). The present studies were undertaken in order to obtain information on the disposal of polysaccharides injected in a dog and in man by studying their disappearance from the circulation and their urinary excretion. One previous study had shown that only 5 to 10 per cent of a crude chondroitin sulfate preparation from cartilage, when injected intravenously into rabbits, was excreted in 2 days (11).

METHODS

The mucopolysaccharides were isolated and characterized by methods previously described (12). The analyses and sources of the substances used in the present experiments are summarized in Table I. Eight volunteers, aged 30 to 66 years, were studied. In none was there evidence of impaired renal function; 2 were normals, 1 had a chronic infection, and 5 had malignancies of varying severity. Each of the MPSs was dissolved in water to a concentration of 10 mg per ml. Twenty ml of blood was withdrawn, and through the same needle the MPS solution was injected intravenously at a dose of approximately 1 mg per kg. Complete mixing was assumed to have occurred after 5 to 10 minutes, when another blood sample was withdrawn. During the next 3 to 4 hours, three to five further blood samples were collected.

In the experiments with human volunteers, urine was collected as follows. After hydration by mouth, the patient was instructed to void when ready. At this time, the injection of MPS was given. Urine during the following 4 hours was collected, and blood was drawn simultaneously.

In the dog, after collection of a 24-hour control sample of urine, MPS was injected intravenously. The urine

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TABLE I

Composition of the MPS used in the experiments *

	Uronic acid						Hexosamine (17)	
	Carbazole	Orcinol	Hexosamine	$[\alpha]_D$	Sulfate	Nitrogen	Galactosamine	Glucosamine
	%	%	%		%	%		
ChS-A (fibroid tumor)	38.0	20.0	27.1	−24	11.5	2.88	4+	±
ChS-B (gargoyle liver)	15.2	35.4		−53	11.8		4+	0
ChS-C (shark)	29.4	22.3	23.0	−16	15.2		4+	0
ChS-C (chondrosarcoma)	30.0	20.0	28.3	−20	19.3	2.64	4+	0
Hep.S. (Upjohn heparin side fraction)†	45.6	22.6	28.7	+69	13.2		±	4+

* Abbreviations: MPS = mucopolysaccharide; ChS = chondroitin sulfate; Hep.S. = heparitin sulfate.

† We thank Dr. Coleman of the Upjohn Company for a generous supply of a crude fraction from which the purified material was prepared.

for the following 24 hours was again collected, except in one case in which an indwelling catheter was used to collect samples at frequent intervals over a 2-hour period after the injection of ChS-B. Blood was collected during the 4-hour interval as in the humans.

The method of Bollet, Seraydarian and Simpson (13) was used for the isolation of MPS from the serum. The blood was allowed to clot; the serum was removed and dialyzed for 3 days against 0.0063 M phosphate buffer of pH 5.8. A precipitate of the MPS together with the euglobulins was thereby obtained. This was redissolved in alkaline NaCl solution, and the protein precipitated with perchloric acid and removed by filtration. The filtrate was dialyzed against tap water for 24 hours. Protamine sulfate solution was then added to the dialyzed solution. After standing in the cold for 0.5 hour, the precipitated materials were recovered by centrifugation at 32,000 G for 1 hour. The precipitate was dissolved in 2 M sodium acetate, adjusted to pH 5 with glacial acetic acid, and analyzed for uronic acid by the carbazole reaction. The results are expressed as micrograms of mucopolysaccharide uronic acid (MPS-UA) per 100 ml of serum.

The urine was analyzed by a modification of the method of DiFerrante and Rich (9). Two aliquots of each sample were taken. To each was added 1/30 vol of 5 per cent aqueous cetylpyridinium chloride (CPC)—to one immediately after collection and to the other after dialysis against running tap water for 3 days. Dialysis was resorted to in order to rule out the possibility that the presence of salts in the urine may have prevented the precipitation of a desulfated product. The precipitate was dissolved in 2.5 ml of a 5 per cent solution of sodium acetate. To this solution 5 ml of 95 per cent alcohol was added. The resultant precipitate which formed overnight at 4° C was dissolved in water and analyzed for uronic acid by the carbazole reaction.

Control values for Subject D.K. on 3 different days were 150, 173, and 181 μg per 100 ml. Control values for a dog showed a wider variability, being 227, 267, 290, 340, and 410 on 5 different days.

The recovery of MPS was studied by adding 3.9 mg of chondroitin sulfate A to 80 ml of citrated blood, equivalent to the maximum found in the *in vivo* experiments. The mixture was incubated at 37° C. Six aliquots of 10 ml each were withdrawn at intervals over a 4-hour period, and values of 1,290, 1,380, 1,300, 1,320, 1,345, and 1,311 μg per 100 ml were obtained. These figures indicate the reproducibility of the method at this concentration. It further shows that MPS is not metabolized in the blood under these conditions. Similar data were obtained for ChS-B.

Reliability of the method for measuring varying amounts of urinary MPS was determined by adding known amounts of ChS-C to 10-ml aliquots of urine. The recovery of 4, 20, 50, and 80 μg per ml was 80, 88, 90, and 99 per cent, respectively.

In addition to the acute experiments in which only one dose of MPS was given, the results of repeated injections were studied in a female dog weighing 20 kg. Solutions of 100 mg of MPS in 5 ml of water were administered intraperitoneally each day over an 18-week period, 6 days per week. Urine was collected over chloroform in a metabolic cage, from which it was removed twice daily, filtered, and stored at 4° C over chloroform. The urine for an entire week was pooled and studied separately after separation from the chloroform.

RESULTS

Four patients were given ChS-C intravenously. The results of a typical experiment in Patient W.P., with carcinoma of the breast, are sum-

marized in Table II. She weighed 51 kg and received 46 mg of ChS-C from shark cartilage. It is apparent that urinary excretion cannot account for the disappearance of the MPS from the circulation. The observed concentration of 1,070 μg per 100 ml of MPS-UA agrees well with the calculated value of 1,010 μg per 100 ml, assuming a blood volume of 8 per cent of body weight. The control figure for urinary excretion of MPS per minute and per milliliter is within the expected range, as calculated from the known daily excretion of MPSs.

The data obtained from four patients given ChS-C intravenously are summarized in Figure 1, illustrating the rate of disappearance of the ChS-C from the blood. For purposes of graphic representation, the absolute values have been translated into percentages, with the peak concentration minus the control concentration being equal to 100 per cent. (These peak values in the three patients other than W.P. were 798, 839, and 1,122 μg per 100 ml.) It can be seen that in three of the four cases the results fall on a smooth curve, and in all cases, within 4 hours the serum concentration of MPS-UA has returned to the control level. Urinary excretion was measured after injection in one patient other than W.P., and again no increase was found.

In Figure 2, similar data are plotted for two separate experiments done in a 25 kg dog which received 25 and 30 mg of ChS-A intravenously, obtaining peak concentrations of 1,038 and 1,227 μg of serum MPS-UA per 100 ml serum, respectively. There was no detectable difference in the disappearance curve of ChS-A and of ChS-C. Furthermore, the excretion of MPS in the urine of the dog during the 24 hours prior to the injection was equal to the excretion during the 24 hours immediately following the injection, so that one may conclude that, as is the case with ChS-C, the disappearance of injected ChS-A from the serum is not due to urinary excretion.

Figure 3 is a composite of the data obtained from four experiments in which ChS-B was given intravenously in a dose of approximately 1 mg per kg, twice in the dog and in two different patients. It is apparent that the disappearance of this substance from the blood occurs at a considerably

TABLE II

Concentration of MPS in serum and urine of Patient W.P. after intravenous injection of 46 mg of ChS-C of shark cartilage *

Sample no.	Time after injection	Serum MPS-UA	Urine MPS-UA	Urine MPS-UA
	min	*μg/100 ml*		*μg/min*
1	−1	140	6.9	5.0
Injection	0			
2	6	1,070		
3	30	657	7.2	5.6
4	75	411		
5	210	169	9.7	5.7

* For abbreviations, see Table I. Also: MPS-UA = mucopolysaccharide uronic acid.

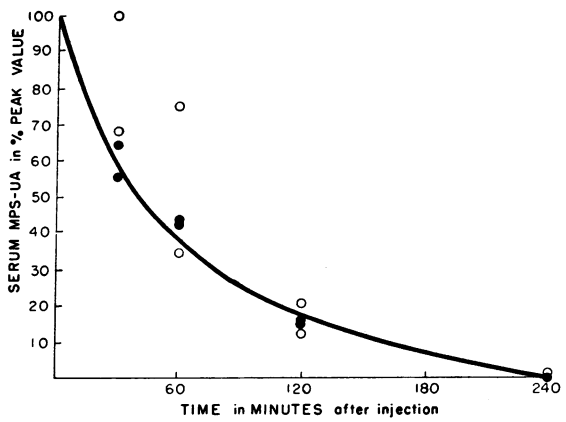

FIG. 1. RATE OF DISAPPEARANCE OF CHONDROITIN SULFATE C FROM THE SERUM OF FOUR SUBJECTS AFTER INTRAVENOUS INJECTION IN A DOSAGE OF APPROXIMATELY 1 MG PER KG. ● = ChS-C from shark cartilage (2 subjects); ○ = ChS-C from chondrosarcoma (2 subjects).

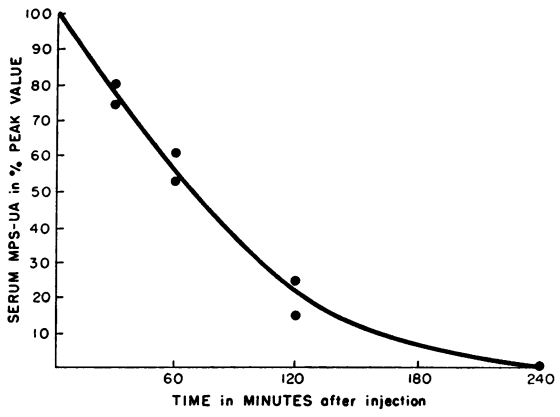

FIG. 2. RATE OF DISAPPEARANCE OF CHONDROITIN SULFATE A FROM THE SERUM OF A DOG AFTER INTRAVENOUS INJECTION IN A DOSAGE OF APPROXIMATELY 1 MG PER KG.

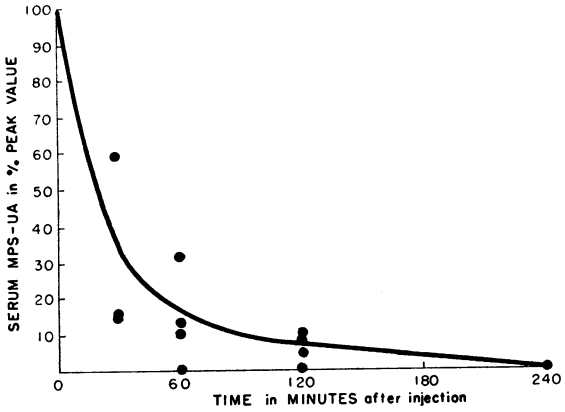

FIG. 3. RATE OF DISAPPEARANCE OF CHONDROITIN SULFATE B FROM THE SERUM OF A DOG AND OF THREE HUMAN SUBJECTS AFTER INTRAVENOUS INJECTION OF APPROXIMATELY 1 MG PER KG.

faster rate than that for the previous two mucopolysaccharides used. Evidence that this is at least in some part due to urinary excretion is presented in Tables III and IV.

Table III shows the rapid fall-off of serum concentration coincident with a marked increase in the rate of excretion of MPS-UA in the urine, which undoubtedly represents the injected ChS-B. One can calculate that the increased MPS-UA in the urine is equivalent to 17 mg of ChS-B, which represents 43 per cent of the injected dose.

Table IV shows similar data on the urinary excretion in the dog. In this case the serum levels were not measured. The total excretion of MPS-UA which presumably represents ChS-B is 1.07 mg, equivalent to 7.1 mg of ChS-B. This is 35 per cent of the injected dose, which is in reasonable agreement with the figure found in Patient H.P.

TABLE III

Concentration of MPS-UA in serum and urine of Subject H.P. after intravenous injection of 40 mg ChS-B *

Sample no.	Time after injection	Serum MPS-UA	Urine MPS-UA	
	min	*μg/100 ml*	*μg/ml*	*μg/min*
1	−1	154	5.4	6.1
Injection	0			
2	5	820		
3	38	246	29.2	34.5
4	77	212		
5	136		5.8	15.3

* For abbreviations, see Tables I and II.

TABLE IV

Rate of excretion and total excretion of MPS-UA after injection of 20 mg of ChS-B in a dog *

Time after injection	Urine flow	Urine MPS-UA	Total excretion of MPS-UA of ChS-B†
min	*ml/min*	*μg/ml*	
−20		0.5	
22	5.4	7.6	844
52	4.9	1.8	191
60	4.5	1.0	18
72	4.5	1.0	18
78	3.9	0.5	0
90	2.8	0.4	0

* For abbreviations, see Tables I and II.

† Calculated by multiplying urine output during designated time period by concentration of urine MPS-UA less the control concentration.

TABLE V

Disposition of MPS 4 hours after injection into a dog at dose of 5 mg per kg *

	Excreted in urine	Remaining in blood	Unaccounted for (presumably in tissues)
	%	%	%
ChS-A	6	28	66
ChS-B	30	0	70

* For abbreviations, see Tables I and II.

In an attempt to determine whether the increased urinary excretion accounts for the more rapid disappearance of ChS-B from the circulation or whether it is more rapidly taken up by the tissues, five times the doses (in milligrams per kilogram) of ChS-A and B were given to the dog on different days, so that 4 hours after the injection of ChS-B the serum MPS level had returned

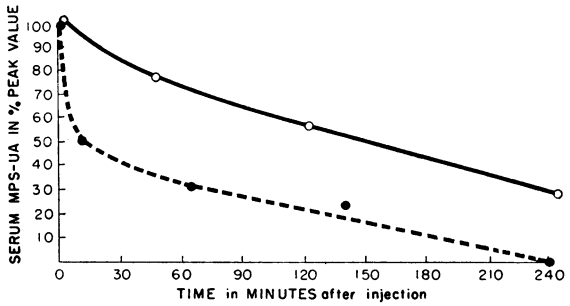

FIG. 4. RATE OF DISAPPEARANCE OF CHONDROITIN SULFATE A (——) AND B (- - - -) FROM THE SERUM OF THE DOG AFTER INTRAVENOUS INJECTION OF 5 MG PER KG OF EACH.

TABLE VI

Data on urinary excretion of MPS in the dog following long-term intravenous administration

Week no.	Urine vol	Type of MPS inj.*	Total MPS excreted	Recovery of inj. MPS†	Analysis of 25% alc. fraction							Analysis of 50% alc. fraction							Increase in reducing sugar	
					Wt	Uronic acid: Carbazole	Uronic acid: Orcinol	$[\alpha]_D$	SO_4	Hexosamine: Galact.	Hexosamine: Gluco.	Wt	Uronic acid: Carbazole	Uronic acid: Orcinol	$[\alpha]_D$	SO_4	Hexosamine: Galact.	Hexosamine: Gluco.	A-adapted enzyme	Heparitin-adapted enzyme
	L/wk		*mg*	%	*mg*	%	%		%			*mg*	%	%		%			%	%
1	3.4	B	173		138	11	38	−59	14	4+	0	15	14	22	−26		3+	1+		
2	2.7	B	225		175	12	39	−61	16	4+	±	50	21	29	−34		4+	±	21	12
3	3.1	B	215		199	12	49	−66	14	4+	±	16	23	26			4+	1+	22	8
4	4.0	B	184	30	155	13	40	−63	18	4+	0	29	25	32	−32		3+	1+	22	7
5	3.5	B	227		181	15	39			4+	±	46	25	30			3+	2+	9	23
6	3.4	B	163		137	14	41			4+	±	26	22	29			3+	2+	11	13
7	5.4		28									25	26	23			3+	2+	6	21
8	5.8		15									13	34	26			4+	1+		
9																				
10	5.3	C	79	11								79	32	22	−17	14	3+	1+		
11																				
12	4.9	HS	240		94	38	27			1+	4+	146	32	29			±	4+		
13	4.6	HS	290		90	42	24	+53		1+	4+	200	45	21	+54		1+	4+		
14	4.2	HS	219		40	37	25			1+	3+	179	46	25			1+	4+		
15	5.3	HS	231	39	43	40	27			1+	3+	188	45	22			1+	3+		
16	4.4	HS	239		104	43	23			±	4+	135	46	24			1+	4+		
17	5.3	HS	272		150	46	24	+55		±	4+	122	41	22	+46		1+	4+		
18	4.9		15																	

* B = chondroitin SO_4-B; C = chondroitin SO_4-C; HS = heparitin SO_4.
† Control value of 15 mg/week is subtracted.

to the baseline, while still remaining elevated 4 hours after the injection of A (Table V). At this dosage, 6 per cent of the ChS-A was recovered in the urine, and 30 per cent of ChS-B was excreted. After 4 hours, approximately equal amounts of ChS-A and B are unaccounted for in blood or urine, suggesting that the rate of tissue uptake from the circulation is similar for the two substances, despite the fact that a portion of the ChS-B is rapidly excreted in the urine. The rates at which ChS-A and ChS-B disappear from serum are shown in Figure 4. It can be seen that ChS-B leaves the blood stream more rapidly than A, probably because the former is more rapidly excreted in the urine than the latter, as is also seen in Tables III and IV.

In Table VI are summarized data on the urinary excretion of repeated injections of ChS-B, C, and Hep.S. The table summarizes the total quantity of the excreted MPSs and their analyses. Thirty, 11, and 39 per cent (corrected for control excretion), respectively, of ChS-B, C, and Hep.S. were isolated from the urine.

Analysis of the major fractions during the first 6 weeks indicates that the ChS-B injected is apparently excreted unchanged in the urine (compare with Table I).

The analyses of the minor fractions for this 6-week period showed that after 4 weeks of ChS-B injection, the dog was beginning to excrete small amounts of a glucosamine-containing fraction in her urine. Fractions from weeks 5 to 7 were combined, and unsuccessful attempts were made to isolate a glucosamine-containing fraction from the mixture. Enzymatic digestion with extracts obtained from ChS-A- and from Hep.S.-adapted organisms, respectively, suggests the presence of Hep.S. in this fraction (10). It should be noted that week 7 is the week immediately following the last injection of ChS-B, during which time the excretion of ChS-B ceased, while the excretion of the presumed Hep.S. continued, along with the normal excretion of ChS-A or C.

As can be seen from a comparison of Tables I and VI, the fraction of ChS-C isolated from the urine did not differ significantly from that of the injected polysaccharide.

DISCUSSION

It has been demonstrated in these experiments that sulfated mucopolysaccharides injected in a single dose disappear from the circulation of man and dog at a fairly rapid rate. The urinary excretion, however, of the four types of polysaccharides injected in a single dose or, in the dog, in repeated doses, varied considerably with the type of polysaccharide injected. After injection of a moderately large amount of ChS-A or C, no significant amount was demonstrable in the urine. In contrast, after injection of ChS-B, 40 per cent was recovered unchanged in the urine. On injection of a heavier dose into the dog, only 6 per cent of ChS-A was excreted versus 30 per cent of ChS-B, while 28 per cent of A versus 0 per cent of B remained in the circulation after 4 hours. On daily injection of ChS-C, the recovery in the urine was 11 per cent, versus 30 per cent of B and 39 per cent of Hep.S. The fate of the unrecovered polysaccharide is unknown.

These experimental data appear at first sight to be in disagreement with the data of Dziewiatkowski (14) and of Dohlman (15) on the fate of injected S^{35}-labeled ChS (presumably mainly A) in the rat. The quantities injected, especially by Dohlman, were far greater than those used in these experiments. In their experiments, 50 to over 80 per cent of the injected label was excreted within 24 hours in the urine, some as inorganic sulfate, despite the fact that in tissue homogenates no sulfatase activity could be demonstrated with ChS as substrate. In our own laboratory, the search for such a sulfatase also has been unsuccessful. How can the claim of extensive *in vivo* degradation and desulfation be reconciled with the excretion of chemically unaltered polymers in our experiments? The most probable explanation appears to be a twofold mechanism for handling these polysaccharides: one, the intracellular uptake presumably by mononuclear and reticuloendothelial cells leading to extensive degradation; the other, the minor path, the excretion of the unchanged polymers by the kidney. The first pathway probably is nonspecific and identical with the postulated mechanism of the degradation of antigens. From the known molecular weights of the polysaccharides, it would appear that the route of disposal depends on the quantity and molecular size of the polymers.

The mucopolysaccharides of lower molecular weight, ChS-B and Hep.S., are excreted to a larger extent than ChS-A and C, which have higher molecular weights (16). The fact that the normal excreted urinary polysaccharides consist almost exclusively of ChS-A cannot be explained at this time.

The next most noteworthy finding in our experiments seems to be the appearance, on long-continued injection of ChS-B, of a polysaccharide with properties indicating Hep.S. It is hoped that a more prolonged experiment will lead to the unequivocal isolation of such a fraction. Such an experiment would greatly simplify our understanding of the genetic defect of Hurler's syndrome, the original object of these experiments, as it would indicate that the basic defect involved just one mucopolysaccharide.

SUMMARY

The blood levels and urinary excretion of chondroitin sulfate (ChS) A, B, and C and of heparitin sulfate (Hep.S.) have been determined after injection in man and dog. ChS-C of shark cartilage or of human chondrosarcoma disappeared from the blood after a single injection in four persons in less than 4 hours, while no significant increases of polysaccharide were found in the urine. A similar disappearance rate from the blood was found in a dog on injection of ChS-A. In one human and one dog, ChS-B disappeared from the blood faster than A or C, with over 40 per cent of the injected dose appearing in the urine. On injection of higher doses of ChS-A and B in the dog, the blood level with A remained elevated after 4 hours, with a urinary excretion of 6 per cent, while with B it had returned to the baseline with an excretion of 30 per cent. Repeated injections of ChS-C, B, and Hep.S. in the dog led to the excretion of 11, 30, and 39 per cent, respectively. The chemical composition of the isolated polymers remained unchanged. In view of extensive degradation of chondroitin $S^{35}O_4$ reported by other authors, it has been concluded that two different paths of disposal of the circulating polysaccharide exist—one, by direct excretion by the kidney, the other, by intracellular degradation. On prolonged injection of ChS-B, the urinary polysaccharide contained a glucosamine-containing polymer, which may be Hep.S., indicating its induced excretion by ChS.B.

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