Chemical and Pharmacological Studies on N-Resulfated Heparin. (27372)

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During a recent study on the biological effects of heparin and heparinoids it was desirable to compare the biological activities of synthetic heparinoids with those of heparin using labeled compounds. It was apparent that the amount of heparin, as well as the specific radioactivity of the biosynthetic heparin prepared by other workers(1,2,3) was low. Thus a semi-synthetic S^{35} labeled heparin was prepared by the N-resulfation of N-desulfated commercial heparin. A similar method was recently reported(4) using non-radioactive materials. The synthetic technics used, and the biological comparison of the N-resulfated heparin to commercial heparin are the subjects of this communication.

Methods. I. *Chemical.* A. *N-desulfation of heparin.* Na heparin (USP) was converted to heparinic acid by passing a 4% solution through a column containing 10× excess of IR-120 resin (H^+). The resulting solution of heparinic acid (final concentration 3%) was kept at 55°C to cause desulfation (Table I). The loss of N- and O-sulfate was followed by titration of aliquots to pH 7.5 before and after passage through a column of IR-400 resin (OH^-). Changes in optical rotation and viscosity were also measured. The N-desulfated heparin was recovered by passing the solution through IR-400 resin (OH^-) to remove $SO_4^=$ followed by neutralization to pH 7.5 with NaOH and lyophilization.

B. *N-resulfation.* Radioactive pyridine · $S^{35}O_3$ and trimethylamine · $S^{35}O_3$ complexes were prepared by known methods from $ClS^{35}O_3H$ and the amine(5). Equal amounts, by weight, of N-desulfated heparin, Na_2CO_3 and trimethylamine · $S^{35}O_3$ were added simultaneously to water such that the final concentration of heparin was 50 mg/ml. The resulting solution was kept at 55°C while being agitated for 24 hours. The clear solution was then passed consecutively through IR-400(OH^-) and IR-120(H^+) resins to remove $SO_4^=$, $CO_3^=$, and Na^+ and $(CH_3)_3N$. The effluent was neutralized to pH 7.5 with NaOH and dialyzed against tap water overnight followed by lyophilization. N-resulfation with pyridine · $S^{35}O_3$ was carried out in the same manner except that the reaction was run for only 2 hours at room temperature.

TABLE I. N-Desulfation of Heparin.

Time, hr	% N-desulfation	% O-desulfation
1.5	16	< 1
3	28	
4.5	33*	1
6	47	
24	74	4
48	78	
72	79	12

* This figure was confirmed independently by the S^{35} analysis following N-resulfation. At room temperature 5 days are required to achieve the same amount of N-desulfation.

II. *Biological.* Mongrel dogs and Carworth Farm Wistar rats were used throughout. Radioactivity measurements were performed as previously described(6). Lipemia clearing was measured by a modified Grossman technic(7) and anticoagulant activity was measured by the Lee-White whole blood clotting time.

Results. I. *Chemical.* The results of the N-desulfation and N-resulfation procedures are shown below in Tables I and II. No changes were observed during the 72-hour run from the initial readings of $[\alpha]_D^{24} \pm 45.5°$ $\pm 2°$ (C = 2.64% as Na heparin) and the intrinsic viscosity $[\eta] = 0.125$ (in N/4 NaCl). Recovery of the N-desulfated and N-resulfated heparin was quantitative.

II. *Biological.* A. *Urine, blood and bile levels in acute catheterized dogs.* Pentobarbitalized dogs were injected i.v. with 200 U.S.P. units/kg of N-S^{35} resulfated heparin and periodic blood, urine and bile samples were obtained. The radioactivity and biological lipemia clearing activity in the blood dropped rapidly following the initial peak, while urine and bile radioactivity rose steadily (Fig. 1). The secretion in the bile was less than 1%

TABLE II. N-Resulfation of N-Desulfated Heparin.

Desulfation conditions	u/mg of N-desulfated product	Resulfating reagent	u/mg of resulfated product	c/m/mg
4 hr, 54°	20	$Py \cdot SO_3$	76*	2.8×10^5
1 ", 54°	85	"	131	7.1×10^4
4 ", 54°	27	$(CH_3)_3N \cdot SO_3$	128	5.6×10^5

* Incompletely N-resulfated.

TABLE III. Clearing and Clotting Activity of Heparin* in Dogs.

	Time after inj. (hr)				
	0	½	1	2	3
			N-resulfated heparin		
Clearing activity (CU)	.04	3.65	3.00	.88	.31
Clotting time (min.)	10.5	>30	>30	15	8.5
			Commercial heparin		
Clearing activity (CU)	.20	3.40	2.40	1.30	.30
Clotting time (min.)	11	>30	>30	—	25

* 200 u/kg i.v.

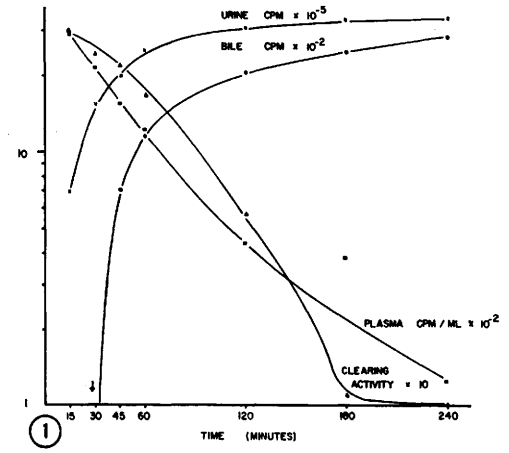
of that in the urine over the entire time course. Similar results were obtained on each of the 4 dogs studied. B. *Urinary excretion in dogs treated semi-acutely.* Two hundred U.S.P. units/kg/dog was administered to 16 dogs; however, they received this dose by 2 different routes and for different periods of time. A single i.v. injection was given daily for 3 days to 2 dogs; 11 dogs received an s.c. injection daily for 2 days and 3 dogs received only a single s.c. injection. The mean 24-hour urinary excretion for 10 dogs after an s.c. injection was 26% with a range of 9.3-38.2% of the injected radioactivity. One week after the last s.c. injection the mean urinary excretion for 7 dogs had reached 61.4% with range of 47.6-76.7% of the injected radioactivity. Seven days after 3 i.v. injections to 2 dogs 81 and 92% of the injected radioactivity had been excreted in the urine.

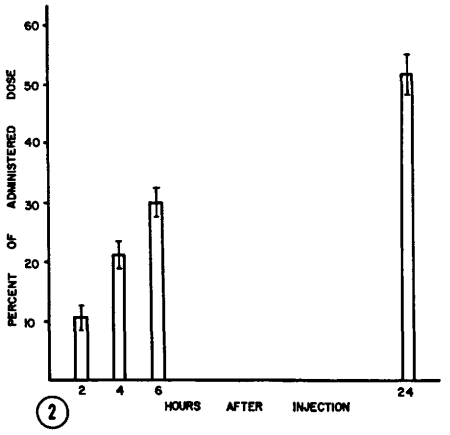
C. *Urinary excretion in rats.* Twenty-four hours after a single subcutaneous dose (500 or 1000 u/kg) of N-S^{35}-resulfated heparin, 51.5 ± 3.6%* of the injected dose of S^{35} had been excreted (Fig. 2). In order that measurable levels of the isotope would be obtained the rats received higher doses than the dogs.

D. *Tissue retention studies.* (Rats and dogs). In Fig. 3, the percentages of administered S^{35} found in rat liver are illustrated. The peak concentration 4 to 6 hours after injection is significantly higher ($p<0.05$) than that found after 24 hours. Although an identical experiment was not performed on dogs, it was observed at the end of 4-5 hours in some acute experiments (Results II. A) that approximately 35% of the injected dose of S^{35} was present in the dogs' liver. When liver tissue was obtained 24 hours after the last dosage from 10 dogs on the semi-acute experiments, 11.6 ± 0.92% of the S^{35} still remained in the liver. Another group of 10 dogs analyzed 7 days after dosing, had 4.59 ± 0.45% of the injected S^{35} in the liver. Other tissues in the dog were examined for radioactivity, but only the lungs and the kidneys had reliably measurable concentrations of S^{35}. The amount present in the lung and kidney 24 hours after dosage was less than 1% of the administered S^{35} and there was no measurable S^{35} in these tisues 7 days after dosage.

E. *Biological activity.* Table III and Fig. 1 illustrate the time course of lipemia clearing activity and clotting time in dogs given an intravenous injection of N-S^{35}-resulfated or commercial heparin. It is apparent from these data that the biological activity rises and falls rapidly for both the lipemia clearing

* s.e. $= 6/\sqrt{n}$.





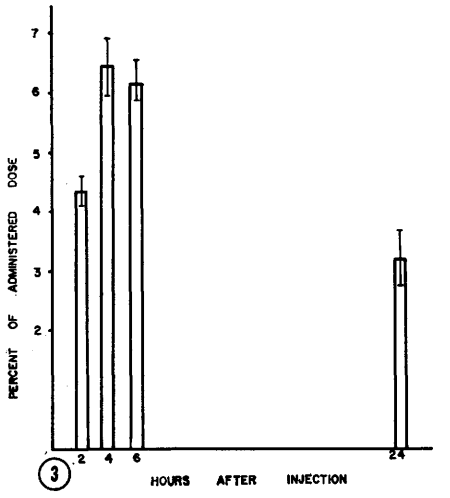


FIG. 1. Plasma, urine, bile radioactivity and clearing activity in one dog given 200 u/kg N-S^{35} resulfated heparin i.v. containing 1.03 × 10^7 cpm. Urine and bile radioactivity is cumulative while plasma measurements were per ml. Clearing activity expressed as clearing units/ml. All values adjusted by the factors expressed in the graph for comparison purposes.

FIG. 2. Cumulative rat urinary excretion of radioactivity after a single s.c. inj. (1000 u/kg) of N-S^{35} resulfated heparin expressed as the avg % of administered dose of radioactivity ± s.e.m. (n = 4).

FIG. 3. Rat liver concentrations of radioactivity after a single s.c. inj. (1000 u/kg) of N-S^{35} resulfated heparin expressed as the avg % of administered dose of radioactivity ± s.e.m. (n = 8).

and the anticoagulant activity. At approximately 3 hours, all values are at the control level. This is identical to observations using a commercial heparin preparation.†

Discussion. Comparisons of the biological activity and disposition of N-S^{35}-resulfated heparin with commercial heparin and literature reports on biosynthetic radioactive heparin indicate there has been no major change in the heparin molecule due to N-desulfation and resulfation. In Table III commercial heparin and N-S^{35}-resulfated heparin are compared. No major differences either in quantitative response or temporal characteristics of these responses were noticed. Experiments using the subcutaneous route of administration were also performed with identical results for commercial heparin and N-S^{35}-resulfated heparin when anticoagulant or clearing activity was measured.

Eiber, Danishefsky and Borelli(8), using S^{35} heparin obtained biosynthetically from dogs injected with $Na_2S^{35}O_4$, studied the disposition of heparin S^{35} after i.v. administration of 2 mg/kg (approximately 200 μ/kg) to dogs. They found 28% of the injected radioactivity in the liver 24 hours after a single i.v. injection which is greater than the findings of 16% (2 dogs) of the injected radioactivity in the liver 24 hours after a single subcutaneous injection reported here. When a daily s.c. injection was given for 2 days and the liver radioactivity measured 24 hours after the last dose, only 10% (5 dogs) of the injected radioactivity was found in the liver. No data are available which are directly comparable to Eiber, Danishefsky and Borelli since a different route of administration was used but the indications of a high liver con-

† Lipo-Hepin®; Riker Laboratories.

centration of S^{35} while the blood was essentially without radioactivity, indicates hepatic retention of heparin or heparin metabolites. This liver retention appears to have a biological half-life of slightly less than 1 week. Seven days after injection, 2 dogs which received a single s.c. injection of N-S^{35} heparin, had an average of 4.7% of the injected radioactivity still in the liver while 5 dogs which received 2 consecutive daily s.c. doses had an average of 3.8%. This is ½ to ⅓ of the amount found after 24 hours and indicates the liver is capable of turning over the S^{35} tag which is derived from the heparin molecule.

Loomis(3) followed plasma radioactivity, clotting time and urinary excretion of biosynthetic S^{35} heparin in a single dog and the data presented in Fig. 1 are comparable to his curves. Loomis' cumulative urine concentration curve has a rapid slope for the first hour and then slows down, while his plasma S^{35} shows a similar rapid decrease in activity which is parallel to the clotting time curve. The urinary cumulative curve for N-S^{35} heparin also increases rapidly for the first hour and then starts to plateau while the plasma radioactivity falls rapidly. In Table III clotting time values of >30 minutes until approximately 2 hours after injection reflect the same rapid return to control levels as does Loomis' curve.

The bile data indicate a route of elimination of heparin which has not been previously reported. Since heparin is not appreciably absorbed through the intestine the secretion of heparin into the bile indicates that only approximately 1% of the exogenous heparin is excreted with the feces. The interesting phenomenon presented here which confirms other investigators is that the urinary excretion, biliary excretion or tissue accumulations examined do not reflect the rapid loss of heparin itself or heparin activity in the blood. The heparin appears to be diffusely spread throughout the body after it rapidly leaves the blood. Radioactivity was present in all 16 tissues examined but aside from kidney, lung and liver the concentrations were less than 1% 24 hours after dosage. Even when the dogs were given i.v. heparin S^{35}, where approximately 90% was in the urine, a material balance could not be achieved.

Since this is the first report on chemically obtained S^{35} heparin, it is necessary to comment on the comparison of N-resulfated heparin with commercial heparin. Optical rotation, viscosity, sulfur content and infrared data were not different from those of the original heparin. The *in vitro* and *in vivo* anticoagulant potency was also equal to that of U.S.P. heparin (>120 μ/mg).

Heparin which is N-desulfated beyond 33%, suffers certain irreversible changes which were reflected mainly in that the N-resulfation did not restore full anticoagulant activity even though the physical constants mentioned were similar to those of the starting heparin. The most probable irreversible processes responsible for the lowered activity would be either depolymerization or O-desulfation or both. Extensive depolymerization which would manifest itself by changes in rotation, viscosity and dialyzability, was not observed confirming earlier observations(9). It is possible that an undetectable amount of depolymerization could have occurred which would only be reflected by a lowered anticoagulant activity after N-resulfation. The magnitude of the more likely irreversible process, O-desulfation, could not be checked independently. The usual sulfur analyses are not of a sufficient accuracy to follow the small amount of O-sulfate which direct titration of aliquots indicates to be lost after a 24-hour hydrolysis at 54°C. Therefore, it is possible that approximately 1% of the O-sulfate is irreversibly removed during the preparation of 33% N-desulfated heparin. The 33% N-desulfated heparin represents the best choice for preparing S^{35}-heparin. The trimethylamine · SO_3 reagent is preferred over the pyridine · SO_3 since it is much more stable. An investigation of the position of the label in biosynthetic S^{35} heparin using similar hydrolytic technics is under way.

On the basis of the data described it would appear that the S^{35} resulfated heparin described here is essentially identical with natural heparin and the chemical manipulations did not alter the molecule. Anticoagulant activity of the N-S^{35} resulfated heparin can be

maintained at greater than 120 U.S.P. units/mg and specific radioactivity of 10^5 or more cpm/mg can be obtained. The semi-synthetic process is simple enough to permit its use to further knowledge about the metabolism and mode of action of heparin.

Summary. A method for synthesis of radioactive heparin of high unit activity and radioactivity has been described using a process of N-desulfation and N-resulfation. Comparison of the biological identity of the radioactive material with commercial heparin has been made. N-resulfated S^{35} heparin appears to be similar to biosynthetic S^{35} heparin in both its clearing and anticoagulant activity as well as its ultimate fate and distribution in the body.

$ClS^{35}O_3H$ was prepared by the reaction of $H_2S_{35}O_4$ and PCl_5, followed by distillation under nitrogen. We wish to thank Marshall Draper for working out this method and supplying us with the $ClS_{35}O_3H$. The authors also wish to acknowledge the technical assistance of C. deFusco, T. Hooper and M. Horne.

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Iodoacetamide Induced Gastric Ulcers in Rats.* (27373)

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Chemicals such as acetylsalicylic acid(1), cortisone(2), histamine(3), cinchophen(4,5), and reserpine(6) when administered in proper concentration are capable of producing ulcers in stomachs of animals. Many of the experimental ulcers, however, have a tendency to perforate, heal quickly or cause profound emaciation of the animal(7). Production of gastric ulcers in a majority of animals without perforation or emaciation would therefore be advantageous in experimental studies of pathogenesis. Iodoacetamide feeding will frequently produce chronic deep ulcers in the secretory part of the rat stomach. Because of the apparent good health and sustained weight gain in these rats, it is believed that the production of ulcers by iodoacetamide feeding may provide a useful method in future investigations of pathogenesis.

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Method. Four to 6 Sprague-Dawley rats of different age and sex were housed in one open-bottom mesh cage. Control and test rats were fed Purina Chow and water *ad libitum*. One gram of iodoacetamide was dissolved in 20-30 ml of water buffered with 2% NaOH to pH 6.5-7.5 and then diluted to 100 ml. Each day either 5 or 10 ml of 1% solution was diluted further to 100 ml. Final concentration of iodoacetamide in drinking water was either 50 or 100 mg per 100 ml. Water consumption usually increased from 4 to 10 ml per day as the rats gained weight. Depending upon the variables indicated, the consumption of iodoacetamide by individual rats varied from 2.0 to 10 mg per day. A 1% solution of iodoacetic acid was prepared in a similar manner and administered in drinking water in another assay. Rats in the control and test groups were fed for periods of 20 to 93 days (Table I). When the experiments were terminated autopsies were per-