Incorporation of Sulfate-S\textsuperscript{35} and Glucosamine-1-C\textsuperscript{14} into Heparin During Perfusion of Isolated Rat Liver

By Nicola Di Ferrante, M.D., Ph.D., Ann L. Meyers, M.S., and Leon L. Miller, M.D., Ph.D.

The addition of sulfate-S\textsuperscript{35} to rat blood perfusing an isolated, surviving rat liver causes association of some of the isotope with several plasma protein fractions in a pattern similar to that obtained when the isotope is injected in a living rat. Preparative starch block electrophoresis (pH 8.6) of aliquots of the perfusing plasma indicates that approximately 80% of the bound radioactivity is associated with the \(\alpha_1\) globulin and albumin fractions while 20% is present in a pre-albumin peak of low protein but considerable hexuronic acid content. Ninety per cent of the radioactivity associated with the pre-albumin peak is dialyzable through cellophane tubing but none of it is precipitated upon addition of \(\text{BaCl}_2\) and carrier \(\text{Na}_2\text{SO}_4\). These findings are consistent with the possibility that the pre-albumin peak represents a small molecular weight, protein-free, sulfated acid mucopolysaccharide capable of acting as sulfate acceptor in presence of the enzymatic systems of the liver involved in sulfate activation and transfer. The data to be presented indicate that the heparin routinely added to the perfusing fluid is responsible for at least a significant part of the pre-albumin peak. In fact, during perfusion of an isolated surviving rat liver, heparin becomes labeled with sulfate-S\textsuperscript{35} and glucosamine-1-C\textsuperscript{14}.

**General Methods**

The heparin used was a commercial preparation purified by chromatography on ectorola\textsuperscript{*} anion exchanger modified cellulose according to the method of Anseth and Laurent. The material was eluted from the exchanger by 2 M NaCl and by 1 N NaOH. These eluates were combined, desalted, dried, and the heparin recovered was utilized for these experiments. On analysis, it was found to have nitrogen 1.60, sulfur-sulfate 10.97, hexuronic acid 31.2%. Carrier free sodium sulfate-S\textsuperscript{35} and D-glucosamine-1-C\textsuperscript{14} HCl (100 \(\mu\)g/32.5 \(\mu\) mole) were used.

The rat liver perfusion was performed with the apparatus and technique described by Miller et al. All rats used were males of the Sprague-Dawley strain, weighing 300 to 400 g. The liver donors were fasted for 18 hours before the liver was removed. The blood donors were not fasted. The liver was perfused for five hours, at a rate of 1.8 ml/min, with 100 ml of diluted rat blood (75 ml of freshly withdrawn blood, 25 ml of saline) containing 75 mg of purified heparin. Two aliquots of the perfusing fluid, 10 ml each, were exhaustively dialyzed against distilled water for three days. The dialysates were reduced in volume, and the amount of free glucosamine present was measured using the chromatographic technique described by Gardell. Approximately 100 \(\mu\)g of glucosamine were found to be present in 100 ml of perfusing fluid. In a typical experiment 1 mc of sulfate-S\textsuperscript{35} and 100 \(\mu\)c of glucosamine-1-C\textsuperscript{14} HCl were added to the perfusion at zero time. Aliquots of blood (5 ml) were withdrawn from the apparatus at one-half, one, two, three, four, and five hours after the beginning of

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the perfusion. Bile was collected throughout the experiment. At the end of the perfusion, the residual blood was collected and replaced with two 50-ml aliquots of saline, in order to rinse the liver and the apparatus.

**Results (and special methods)**

**ISOLATION OF HEPARIN FROM BLOOD SAMPLES**

After centrifugation of the six blood samples, each plasma supernatant (3.75 ml) was placed on a 150 X 7 mm column of ECTEOLA. Stepwise elution was performed with 10 ml of 0.05 N HCl, followed by 10 ml of 0.1 M, 0.25 M, 0.5 M, 1.0 M, 2.0 M, and 4.0 M NaCl in 0.05 N HCl, the final elution being performed with 10 ml of 1 N NaOH. The bile collected was treated similarly. The first seven eluates were neutralized with NaOH; the last one with HCl. All were scanned for hexuronic acid content with the modified Dische reaction described by Bitter and Muir. For each plasma sample, more than 90% of the hexuronic acid-containing material was eluted by 2 M NaCl in 0.05 N HCl and by 1 N NaOH. These two eluates were neutralized, pooled, and dialyzed against distilled water. The nondialyzable material was reduced in volume and precipitated with cetyltrimethylammonium bromide without addition of carrier heparin. The precipitate obtained was washed with 95% ethyl alcohol saturated with NaCl, and dried with ether. The material recovered weighed 42 mg. Moreover 10.8 mg of heparin were calculated to be present in the dialysate on the basis of its hexuronic acid content. Duplicate 1-mg aliquots of the recovered heparin (found to contain 33% hexuronic acid) were digested for C14 and S35 counting (table 1).

With distilled water, twice with 95% ethyl alcohol saturated with NaCl, dried with ether and eventually dissolved with 10 ml of distilled water. Duplicate 2 ml aliquots from each solution were processed for C14 and S35 counting in a liquid scintillation spectrometer according to the methods described by Jeffay and co-workers. Figure 1 shows the results obtained.

The heparin present in the 75 ml of residual blood and in the saline used for rinsing the liver and the apparatus was isolated in similar manner, using a 300 X 30 mm ECTEOLA column and 200 ml volume for each solvent. The hexuronic acid-containing eluates (2 N NaCl in 0.05 N HCl and 1 N NaOH) were neutralized, pooled, and dialyzed against distilled water. The nondialyzable material was reduced in volume and precipitated with cetyltrimethylammonium bromide without addition of carrier heparin. The precipitate obtained was washed with 95% ethyl alcohol saturated with NaCl, and dried with ether. The material recovered weighed 42 mg. Moreover 10.8 mg of heparin were calculated to be present in the dialysate on the basis of its hexuronic acid content. Duplicate 1-mg aliquots of the recovered heparin (found to contain 33% hexuronic acid) were digested for C14 and S35 counting (table 1).

**Figure 1**

Incorporation of $^{34}S$O$_4$ and glucosamine-1-C$^{14}$ into heparin calculated to be present in blood at the time of each sampling.
HEPARIN LABELING DURING LIVER PERFUSION

TABLE 1
Specific Activity of Heparin Recovered at End of Perfusion, Expressed as Counts per Minute per Mg of Heparin and as Per Cent of Dose Employed per Mg of Heparin

<table>
<thead>
<tr>
<th>Isotope</th>
<th>Counts per min/mg Before dialysis</th>
<th>After dialysis</th>
<th>Per cent dose employed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>C14</td>
<td>1384</td>
<td>1080</td>
<td>0.51 x 10⁻³ (0.17 x 10⁻³ μmole glucosamine HCl)</td>
</tr>
<tr>
<td>S2⁵</td>
<td>4000</td>
<td>2800</td>
<td>0.57 x 10⁻³</td>
</tr>
</tbody>
</table>

* Calculated from specific activity values obtained after dialysis.

DIALYSIS OF HEPARIN-CETYL PYRIDINIUM COMPLEX AGAINST WATER

Ten mg of recovered heparin were precipitated with 10% cetyl pyridinium chloride. The precipitate was washed several times in the centrifuge with aliquots of a solution containing 0.025 mole of Na₂SO₄ and 0.005 mole of glucosamine HCl per liter. The precipitate was then suspended in 10 ml of the same solution and transferred to a dialysis bag. After exhaustive dialysis at 4°C against frequently changed distilled water, the heparin-cetyl pyridinium complex was transferred to a centrifuge tube, washed with ethyl alcohol saturated with NaCl and dried with ether. The specific activity of the heparin recovered (31% hexuronic acid) was measured and compared with the predialysis values. It was found to have decreased 30% for S²⁵ and 22% for C¹⁴ (table 1).

SELECTIVE HYDROLYSIS OF SULFAMIDO GROUPS

Duplicate 2-mg aliquots of recovered heparin were dissolved in 2 ml of 0.04 N HCl and heated at 100°C for 90 minutes. A third aliquot, dissolved in water and similarly heated, was used as a control. After neutralization, each solution was quantitatively transferred to a centrifuge tube and precipitated with 0.5 ml of a 2% solution of cetyltrimethylammonium bromide. After 24 hours at 4°C the precipitates were collected by centrifugation and washed twice with 3 ml of distilled water. Supernatants and washes from each precipitate were digested for S²⁵ counting. Each precipitate was washed with 95% ethyl alcohol saturated with NaCl, dissolved in water, and digested for S²⁵ counting. 58.0% and 60.5% of the total S²⁵ radioactivity of the sample were found in the supernatants, only 1.8% of the total S²⁵ radioactivity being present in the supernatant of the control sample.

MEASUREMENT OF THE REDUCING POWER OF ORIGINAL AND RECOVERED HEPARIN

The reducing power of the original heparin and of the heparin recovered from the residual blood was measured with the method of Park and Johnson⁶ using glucose as a standard. Values of 0.018 and 0.025 μmole glucose per mg of heparin were found, indicating that the recovered heparin had undergone some depolymerization.

ASSAY OF ANTICOAGULANT ACTIVITY OF ORIGINAL AND RECOVERED HEPARIN

The anticoagulant activities of the original heparin and of the heparin recovered from the residual blood were measured using the prothrombin, thrombin, and recalcification time as criteria. They were found to be similar, both preparations being slightly more active than a commercial preparation used as a standard (fig. 2).

CHROMATOGRAPHY OF BILE ON ECTEOLA COLUMN

Between 2 and 2.5 ml of bile were collected during each perfusion. Most of the hexuronic acid-containing material present in a bile sample was eluted from the ECTEOLA column by 0.05 N HCl, only a small amount being eluted by 1 N NaOH (fig. 3). While no hexosamine was found in a hydrolysate (4 N HCl, 100°C for 8 hours) of the 0.05 N HCl eluate, trace amounts were found in the hydrolysate of the 1 N NaOH eluate. One-ml aliquots of each eluate from the ECTEOLA column were processed for S²⁵ counting and the results are presented in figure 3.

Another bile sample, obtained from a similar experiment, was diluted to 10 ml with distilled water and precipitated with 2 ml
of 2% cetyltrimethylammonium bromide. The precipitate obtained was washed three times with 10 ml of 0.025 M Na$_2$SO$_4$, then with 95% ethyl alcohol saturated with NaCl and eventually dried with ether. Dissolved in 5 ml of distilled water (to contain 15 μg of hexuronic acid per ml) and assayed for anticoagulant activity, this precipitate prolonged slightly the recalcification time but not the prothrombin or the thrombin time (Fig. 2).

**CONTROL EXPERIMENT**

A control experiment was performed by circulating for five hours, in the perfusion apparatus, 75 mg of heparin, 500 μg of sulfate-S$^{35}$, and 50 μg of glucosamine-1-C$^{14}$ HCl, in absence of the liver. The heparin recovered at the end of the perfusion was found to be without any S$^{35}$ and C$^{14}$ activity. However, measurement of its reducing power demonstrated a twofold increase from the preperfusion value. This indicates that the moderate degree of depolymerization undergone by heparin during the experiments may be a result of the isolation procedures and it is not related to possible metabolic activities of the liver.

**Discussion**

Heparin isolated from the perfusing fluid at different intervals shows an increasing uptake of S$^{35}$ and C$^{14}$. 58 to 60% of the bound S$^{35}$ was removed by 0.05 N HCl at 100°C and represents sulfamido-S$^{35}$. Korn, working with heparin synthesized by slices of mouse mast cell tumor, found 48% loss of S$^{35}$ activity under similar conditions. The uptake of sulfate-S$^{35}$ by heparin does not necessarily indicate synthesis of the polymer. In fact, it may represent only sulfation of hydroxyl or amino groups which were desulfated during the process of original extraction or as result of postulated, but hitherto not demonstrated, metabolic activities of the liver.

In a recent study, Capps and Shetlar have demonstrated that after injection of glucosamine-1-C$^{14}$ into rabbits, the acid mucopolysaccharides extracted from the liver contain the isotope only in the glucosamine moiety. Therefore, it is possible to assume that in our experiments, the uptake of C$^{14}$ by heparin indicates that glucosamine-1-C$^{14}$ has become part of the polymer. This uptake would be evidence of synthesis if the labeled aminosugar could be demonstrated to be within the polymer and not only at the terminal end of it or, if the amount of labeled aminosugar incorporated were larger than the amount present at the reducing end of the polymer. Neither of these two possibilities has been proved for the labeled heparin recovered at the end of the perfusion. However, the
amount of reducing groups per mg of recovered heparin \((0.25 \times 10^{-3} \text{ mmole glucose})\) exceeds 100 times the amount of C\(^{+}\) glucosamine incorporated \((0.17 \times 10^{-3} \text{ mmole})\) and this finding suggests that the latter may have been added to the terminal end of some polymer chains.

Since the biological activity of the heparin recovered remains unchanged, the liver perfusion recommends itself as a useful technique for biological labeling of heparin.

After injecting S\(^{35}\)-labeled heparin (sulfamido-S\(^{34}\)) intravenously into dogs Levy and Petracek \(^{19}\) noticed that approximately 1% of the isotope was excreted in the bile and concluded that the bile represents a minor route of elimination for circulating heparin. In our experiments, approximately 2% of the S\(^{35}\) used in the perfusion was excreted in the bile. After chromatography of a bile sample on ECTEOLA, most of the isotope was found in the first two eluates, along with considerable amounts of material containing hexuronic acid but not hexosamine (fig. 3). This pattern of elution suggests that the isotope was excreted as inorganic sulfate or sulfate esters of small molecular weight and that the hexuronic acid-containing material represents glucuronide esters normally present in bile.

A small amount of the S\(^{35}\) present in the bile was, however, eluted from the ECTEOLA column by 1 N NaOH, along with material containing hexuronic acid and hexosamine. Moreover, a small amount of hexuronic acid-containing material was precipitated from the bile with cetyltrimethylammonium bromide. While the amount recovered did not allow adequate analyses, measurement of its anticoagulant activity demonstrated absence of antiprothrombin and antithrombin activity and a moderate effect on the recalcification time. It is possible that this material represents products of heparin breakdown which are eliminated through the bile.

**Summary**

Sulfate-S\(^{34}\) and glucosamine-1-C\(^{14}\) are incorporated into heparin which has been added to the blood perfusing an isolated, surviving rat liver. Although both labels are firmly bound to heparin, such uptake does not necessarily indicate synthesis of the polymer.

Despite the fact that the perfused heparin has undergone some moderate depolymerization, its biological activity remains unchanged.

The presence in the bile of material containing hexuronic acid and hexosamine but deprived of anticoagulant activity suggests...
that products derived from heparin breakdown may be eliminated in the bile.

Acknowledgment
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References

AUTHORS' CORRECTION
The name of the second author of reference number 1 on page 144 of the August issue, volume 15, 1964, following "Principle Factor Waveforms of the Thoracic QRS Complex" was inadvertently omitted. The names of the authors should read—Abildskov, J. A., and Wilkinson, R. S., Jr.
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