# PREPARATION AND CHARACTERIZATION OF THE HIGH MOLECULAR WEIGHT [$^3$H]HYALURONIC ACID

Peter CHABREČEK[a], Ladislav ŠOLTÉS[b], Hynek HRADEC[c], Jiří FILIP[c] and Eduard ORVISKÝ[d]

[a] *Institute of Biotechnology, Slovak Technical University, 812 37 Bratislava*
[b] *Institute of Experimental Pharmacology, Slovak Academy of Sciences, 842 16 Bratislava*
[c] *Institute for Research, Production and Application of Radioisotopes, 102 27 Prague 10*
[d] *Research Institute of Rheumatic Diseases, 921 01 Piešťany*

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Two methods for the preparation of high molecular weight [$^3$H]hyaluronic acid were investigated. In the first one, hydrogen atoms in the molecule were replaced by tritium. This isotopic substitution was performed in aqueous solution using $Pd/CaCO_3$ as the catalyst. In the second method, the high molecular weight hyaluronic acid was alkylated with [$^3$H]methyl bromide in liquid ammonia at a temperature of -33.5 °C. High-performance gel permeation chromatographic separation method was used for the isolation and characterization of the high molecular weight [$^3$H]hyaluronic acid. Molecular weight parameters for the labelled biopolymers were: $\overline{M}_w$ = 128 kDa, $\overline{M}_w/\overline{M}_n$ = 1.88 (first method) and $\overline{M}_w$ = 268 kDa, $\overline{M}_w/\overline{M}_n$ = 1.55 (second method). The high molecular weight [$^3$H]hyaluronic acid having $\overline{M}_w$ = 268 kDa was degraded further by specific hyaluronidase. Products of the enzymatic depolymerization were observed to be identical for both, labelled and cold biopolymer. This finding indicates that the described labelling procedure using [$^3$H]methyl bromide does not induce any major structural rearrangements in the molecule.

Hyaluronic acid (HA, hyaluronan, hyaluronate) is an unbranched high molecular weight polysaccharide which consists of repeating disaccharide units of glucuronate β-1,3-N-acetylglucosamine linked together by β-1,4-bonds. This biopolymer operates in the extracellular matrix of connective tissues and plays an important role in affecting the cellular behaviour[1,2].

Many biochemical and biophysical studies on HA have been facilitated considerably by the use of radiolabelled polysaccharides. Examples of such fields of research include the degradation of HA after its injection into the body, as well as the interaction of HA with other macromolecules and with the cells[3,4,5].

Although, several methods for the labelling of the hyaluronic acid have been published the preparation of high molecular weight hyaluronic acid with a high specific activity still presents an unresolved problem. Biosynthetic methods in which the hyaluronic acid is synthesized from a radioactive precursor (glucose or N-acetylglucosamine)

added to the cell or tissue cultures are usually laborious, costly, and yield a limited amount of HA, often of unknown specific activity[6,7,8].

Chemical radiolabelling methods are applied rarely. Orlando et al.[9] prepared a radiolabelled hyaluronic acid derivative using controlled periodate oxidation of HA, followed by the product reduction with [$^3$H]$NaBH_4$. Molecular weight parameters of the resulting labelled HA derivatives were characterized, however, insufficiently. Hook et al.[10] prepared [$^3$H]hyaluronic acid by acetylation of N-unsubstituted hexosamine residues with [$^3$H]acetic anhydride. Although the resulting [$^3$H]hyaluronic acid was characterized by its molecular characteristics, radiochemical purity, etc., the described labelling process is relatively tedious. Raja et al.[11] prepared [$^{125}$I]radiolabelled hyaluronic acid derivative, yet with low molecular weight.

In this paper we report two simple and rapid methods for radiolabelling of the native hyaluronic acid. We also characterize these labelled hyaluronic acid derivatives, with regard to the molecular weight parameters and their behaviour under specific enzymatic degradation treatment.

## EXPERIMENTAL

### Materials

A sample of hyaluronic acid isolated from rooster combs was obtained from Movis (Holíč, Czechoslovakia). It was characterized in our previous papers[12,13].

[$^3$H]methyl bromide with high specific activity (1.5 TBq mmol) was from the Institute for Research, Production and Application of Radioisotopes (Prague, Czechoslovakia).

Bovine testicular hyaluronidase (hyaluronate 4-glycanohydrolase, E.C. 3.2.1.35, 4 567 nkat/mg) was purchased from SEVAC (Institute of Sera and Vaccines, Prague, Czechoslovakia).

Hydroxyethyl starch (HES) standards with the weight-average molecular weight from $\overline{M}_w$ = 53.8 to 937 kDa were used for calibration of the chromatographic system. They were kindly provided by Dr Kirsty Granath (Pharmacia Fine Chemicals, Uppsala, Sweden).

### Sample Preparation

*Method 1*): The solution of hyaluronic acid (10 mg) in water (0.5 ml) was added to 10 mg of the catalyst (10% Pd/$CaCO_3$) in the reaction flask (volume 1 ml). The reaction flask, equipped with a magnetic stirrer, was joined to the tritiation aparature. The flask was then cooled with liquid nitrogen and the tritiation apparatus was evacuated. Carrier free tritium gas (200 GBq) was transferred to the reaction flask by the Toepler pump and the reaction mixture was stirred at room temperature for 100 min. The content of the flask was frozen and the residual tritium was returned into the reservoir. Water and the labile radioactivity were removed by lyophilization in a closed system. Redistilled water (2 ml) was added to the product and the catalyst was centrifuged. Water and the residue of the unstable bonded tritium were removed by repeated lyophilization in a closed system. The sample was then fractioned by gel-permeation chromatography (GPC) and analysed by GPC and liquid scintillation couting (LSC).

*Method 2*): Ethanol (0.6 ml) and diethyl ether (9 ml) were added to the aqueous solution of hyaluronic acid (5 mg of HA in 0.5 ml of redistilled water). The white suspension was evaporated under vacuum to a constant weight. The reaction apparatus, flushed with anhydrous ammonia, was then connected to the reaction flask. Anhydrous ammonia (3 ml) was added to the flask by distillation and the mixture was

stirred under reflux (-33.5 °C). Natrium (2 mg) was then added to the mixture. After decolourizing of the blue mixture, 5 GBq of [$^3$H]methyl bromide (dissolved in 0.5 ml of toluene) was added. The reaction mixture was refluxed for 1 h. Ammonia was evaporated and the residue was dissolved in redistilled water. The solution was evaporated under vacuum to a constant mass. The residue was dissolved again in redistilled water and after fractionation by GPC, analyzed by means of the gel-permeation chromatography and liquid scintillation counting.

Analytical Procedures

The high-performance gel permeation chromatographic (HPGPC) system employed comprised a high pressure pump HPP 5001 (Laboratorní přístroje, Czechoslovakia), eight-port switching valve Model PK 1 (Vývojové dílny, Czechoslovak Academy of Sciences), two stainless-steel columns (250 × 8 mm i.d.) connected in series, packed with Separon HEMA-BIO 1000 (TESSEK Ltd., Czechoslovakia; mean particle size 10 μm) and a differential refractometric detector RIDK 102 (Laboratorní přístroje, Czechoslovakia).

Chromatographic experiments were carried out at room temperature. The mobile phase purged by helium was composed of 0.1M aqueous $NaNO_3$ solution. Flow rate of the eluent was 0.4 ml/min. [$^3$H]hyaluronic acid derivatives dissolved in the mobile phase used were injected as 0.05% solutions. The injected sample volumes were 100 μl. $^3$H activity was determined in collected fractions (0.33 ml). Aliquots of 50 μl were mixed with 10 ml Insta-Gel (Packard, U.S.A.) and measured by means of liquid scintillation spectrometer Packard 300 CD.

Enzymatic degradation of hyaluronate samples was carried out in 0.067M phosphate buffer (pH 7.4) at 37 °C. Hyaluronidase solution (1 ml, 1 600 nkat) was added to 1 ml of the hyaluronate solution (1 mg) and the mixture was incubated for 24 h. The digestion was terminated by heating the mixture in a boiling water bath for 5 min. After cooling to room temperature, each sample was centrifuged at 10 000 *g* for 10 min. The supernatants were analysed by gel-permeation chromatography and liquid scintillation counting. The molecular weight decrease was used as an indicator of sample depolymerization.

## RESULTS AND DISCUSSION

Both the methods presented for radiolabelling of original sodium hyaluronate samples are new. Each method affords different results.

The Method *1*) in which hydrogen atoms were replaced by tritium, yielded 100 MBq of a tritiated sample, with a specific activity of 20 MBq/mg. Three fractions of radiolabelled hyaluronate were obtained from this sample by chromatographic separation (Fig. 1). Approximate values of the molecular weight parameters for [$^3$H]hyaluronic acid derivative fractions are given in Table I. On the basis of these results it is clear that degradation process is initiated by using this radiolabelling method. This process resulted not only in fractions with small molecular weight, but also in broadening of fraction polymolecularities. Only a trace of the activity was found to be bound to the polysaccharide fraction with the highest $\overline{M}_w$. The major amount of activity was bound to low molecular weight fractions of the labelled hyaluronic acid derivative.

Method *2*) in which the original high molecular weight hyaluronic acid was alkylated with [$^3$H]methyl bromide at -33.5 °C, yielded a radiolabelled hyaluronate sample with a specific activity ≈ 80 MGq/mg. Since [$^3$H]methyl bromide had a high specific activity (1.5 TBq/mmol), the specific activity of the resulting hyaluronate sample was also high.

Only two fractions of radiolabelled hyaluronate were obtained by chromatographic separation of this sample (see Fig. 2). The molecular weight parameters of these fractions are given in Table II. Marked differences were observed in the distribution of the [$^3$H] activity in hyaluronate samples prepared by different methods. While the high molecular weight fraction of the hyaluronate sample prepared by Method *1*) exhibited a very low amount of activity, the high molecular weight fraction of the hyaluronate sample prepared by Method *2*) carried the major amount of activity. Figure 2 shows the distribution of radioactivity in the sample prepared by Method *2*).

The high molecular weight fraction of the [$^3$H]hyaluronate sample prepared by the Method *2*) and separated from the low molecular weight co-products can be useful for providing the labelled [$^3$H]hyaluronic acid for metabolic and enzymatic experiments, as well as for studies on the interaction between hyaluronic acid and other biological macromolecules. This fraction has been characterized further by specific degradation

TABLE I
Molecular weight parameters of fractions of the [$^3$H]hyaluronic acid derivative prepared by the Method *1*) (of Experimental)

| Fraction | $\overline{M}_w$, kDa [a] | $\overline{M}_w/\overline{M}_n$ [b] |
|---|---|---|
| 1 | 128.00 | 1.88 |
| 2 | 13.50 | 1.37 |
| 3 | 1.26 | 1.31 |

[a] $\overline{M}_w$ represents the weight-average molecular weight; [b] $\overline{M}_w/\overline{M}_n$ is polymolecularity.

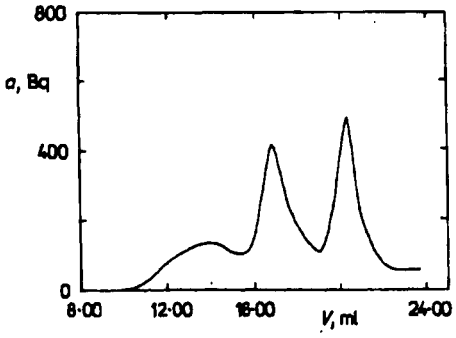


FIG. 1
Chromatogram of the [$^3$H]hyaluronic acid derivative sample prepared by the Method *1*) (radioactivity profile). *V* is elution volume, *a* is $^3$H activity; for details see Experimental

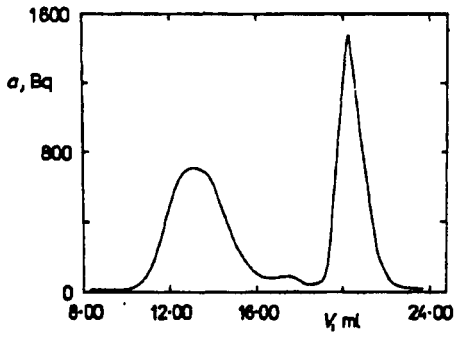


FIG. 2
Chromatogram of the [$^3$H]hyaluronic acid derivative sample prepared by the Method 2) (radioactivity profile). *V* is elution volume, *a* is $^3$H activity; for details see Experimental

with hyaluronidase E.C. 3.2.1.35. Degradation by the enzymatic attack resulted in the same low molecular weight product as in the case of the unlabelled hyaluronic acid (Fig. 3). This finding indicates that the labelling procedure did not induce any major structural changes in the hyaluronate molecule. Shift of the chromatographic peak of the radiolabelled HA in comparison to unlabelled HA is caused by degradation of HA during the labelling process.

Two methods for the radiolabelling of HA were evaluated combining GPC, LSC and enzymatic analysis. The results obtained clearly proved that the newly developed Method *2*) of hyaluronate tritiation with [$^3$H]methyl bromide is the procedure of choice. The high molecular weight [$^3$H]hyaluronic acid fraction with $\overline{M}_w$ = 268 kDa and with the polymolecularity $\overline{M}_w/\overline{M}_n$ = 1.55 is fully suitable as a radiolabelled tool for studying the fate, biochemistry, and behaviour of high molecular weight hyaluronic acid.

TABLE II
Molecular weight parameters of fractions of the [$^3$H]hyaluronic acid derivative prepared by the method *2*) (cf. Experimental)

| Fraction | $\overline{M}_w$, kDa [a] | $\overline{M}_w/\overline{M}_n$ [b] |
|---|---|---|
| 1 | 268.00 | 1.55 |
| 2 | 1.26 | 1.32 |

[a] $\overline{M}_w$ represents the weight-average molecular weight; [b] $\overline{M}_w/\overline{M}_n$ is polymolecularity.

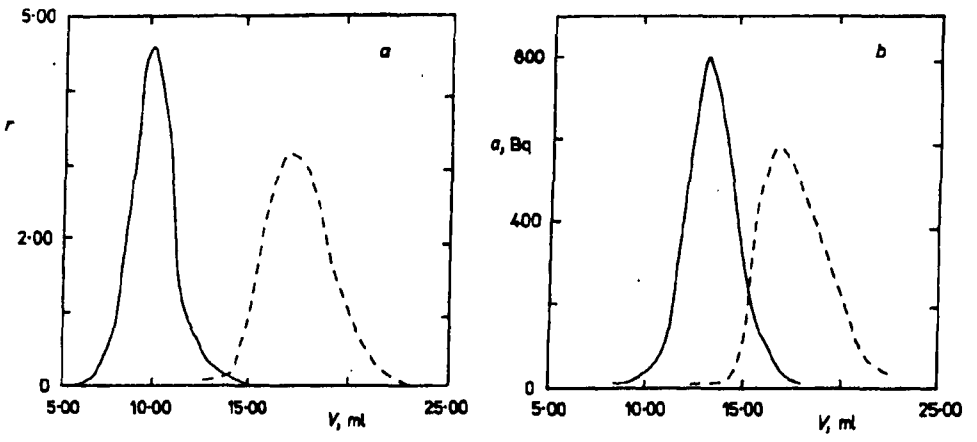


FIG. 3
Chromatograms of the hyaluronic acid derivative before (full line) and after (dashed line) the enzymatic treatment. *a* Refractivity profile, *b* radioactivity profile. *V* is elution volume, *a* is $^3$H activity, *r* is refractometric detector response; for details see Experimental

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