

## Cross-linked Gels of Hyaluronic Acid

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Recent work on the interaction between dextran and other macromolecules has shown that the polysaccharide increases the activity of other solutes, probably due to a sterical exclusion of solvent.<sup>1,2</sup> The effect of dextran seems to be the same when the polysaccharide is in solution as when it exists in the form of a cross-linked gel.<sup>3</sup> Hyaluronic acid, a polysaccharide isolated from connective tissue, shows exclusion properties in solution, which exceed those of dextran<sup>4-6</sup> and which may be of great biological importance. These properties have mainly been determined by equilibrium dialysis<sup>4,6</sup> but the great potentiality of gel filtration<sup>3</sup> has induced us to investigate the possibility of making cross-linked hyaluronic acid gels similar to cross-linked dextran gels.<sup>7</sup>

Hyaluronic acid with a weight-average molecular weight of approximately  $1.5 \times 10^6$  prepared from umbilical cord was used.<sup>5</sup> The polysaccharide was precipitated with ethanol and dried with ethanol and ether and then ground in a mortar to a fine powder. 0.2 M sodium hydroxide was added to the powder and the mixture was placed overnight in a refrigerator so that the polysaccharide could swell to a thick suspension. 1,2,3,4-Di-epoxybutane was added to a concentration of 7% (v/v) and sodium borohydride to a concentration of 0.1% (w/v). After stirring, the mixture was kept at 50°C for 2 h. Acetic acid was added to approximately neutral pH and the gel formed was washed repeatedly in distilled water.

The following final concentrations of hyaluronic acid were used in the reaction mixtures: 0.9; 5.0; 7.1; 12.0; and 17.5 g/100 ml. A gel was formed at all concentrations except the lowest. The gel obtained from 5% hyaluronic acid was precipitated with alcohol (80–90%) and dried with ether and then used for chemical analysis. The following values were found: nitrogen<sup>8</sup> 2.33%; ash 7%; ash after

addition of sulfuric acid, 10.6%; hexosamine<sup>9</sup> after 7 and 11 h of hydrolysis at 100°C in 6 N HCl, 24.2%; uronic acid<sup>10</sup> 16.9%; acid titration of carboxyl groups,  $1.57 \times 10^{-3}$  equiv./g. The analytical values are calculated as percent of dry weight. The equivalencies nitrogen:hexosamine:uronic acid:sodium (average from ash determinations):carboxyl groups are accordingly 1.00:0.81:0.52:0.97:0.95.

The low value of uronic acid, must in view of the higher sodium and carboxyl values, be assumed to be due to an interference of the crosslinking groups in the carbazole reaction. The hexosamine value may be too low for the same reason. The equivalency between nitrogen, sodium and carboxyl groups indicates that no appreciable deamination has occurred during the crosslinking reaction.

The gels had high swelling capacities in distilled water. An exception was the gel, which had been dried; it could not swell to its original volume again. The swelling was strongly dependent on the ionic strength. The approximate water regains of gels crosslinked at concentrations of 12 and 17.5 g/100 ml are shown in Table 1. The volumes of the gels were

Table 1. Water regain at various sodium chloride concentrations of two hyaluronic acid gels cross-linked in concentrations of 12.0 (A) and 17.5 (B) g/100 ml.

	Distilled water	0.001 M	0.01 M	0.1 M	1 M
A	920	790	400	200	50
B	380	280	160	80	50

determined by centrifuging them in graded centrifuge tubes and their hyaluronic acid concentrations were estimated from nitrogen determinations.

Preliminary experiments indicate that it is possible to use hyaluronic acid gels for gelfiltration.

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## Preparation of 2-<sup>13</sup>C, 3-<sup>13</sup>C and <sup>15</sup>N Enriched Pyrroles. Conversion of Furan to Pyrrole

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In order to get further information on the molecular structure of pyrrole<sup>1</sup> by microwave investigation of isotopically substituted species we have prepared the necessary small quantities of 2-<sup>13</sup>C, 3-<sup>13</sup>C and <sup>15</sup>N enriched pyrroles. The microwave results will be published elsewhere. In a similar study on furan,<sup>2</sup> 2-<sup>13</sup>C and 3-<sup>13</sup>C enriched furans were synthesized, each in ca. 230 mg quantity, enrichment 22%. What remained of these samples (170 and 130 mg, respectively) was converted to the corresponding pyrroles by the action of gaseous NH<sub>3</sub> and a catalyst, but prior to these final experiments it was necessary to investigate the reaction between "ordinary" furan and NH<sub>3</sub> to ensure a reasonable yield of known purity. A 40–50% yield of this reaction has been reported recently<sup>3,4</sup> but this applies to the conversion of larger quantities. <sup>15</sup>N enriched pyrrole was prepared by pyrolysis of the ammonium salt of mucic acid, (CHOH)<sub>4</sub>(COO\*NH<sub>4</sub>)<sub>2</sub>, enriched to 33% in \*N.

*Conversion of furan to pyrrole.* Initially, a dozen experiments each involving 500 mg furan were performed. Under comparable conditions (*vide infra*) the aluminium silicate cracking catalyst used by Yur'ev

(kindly given to us by A/G Deutsche Kalichemie) was found superior to  $\gamma$ -Al<sub>2</sub>O<sub>3</sub> formerly used. The optimal temperature was fixed to 390°–410°C, a 50° rise or decrease in temperature causing a considerable smaller yield. Variation of the furan/NH<sub>3</sub> mole ratio from 1:5 to 1:8 indicated the advantage of working close to the latter limit which should not be exceeded because of the subsequent separation of reaction products. In literature, the magnitude of the optimal space velocity,  $v_s$ , defined as ml normalized gas volume per hour per ml catalyst, has never been given, although it is of great practical interest, it being widely independent of the scale of the experiment. We found that 100 <  $v_s$  < 200 gave satisfactory yields of 40–50% (*vide infra*).

In these 500 mg experiments the correct mixture of gaseous furan and NH<sub>3</sub> was fed at room temperature and atmosphere pressure to a U-shaped tube (i.d. 15 mm) containing 30 ml catalyst, the catalyst containing part of the U-tube being immersed in a heated metal-bath. The reaction products were condensed in a trap, cooled by dry ice-acetone. Removal of most of the excess of NH<sub>3</sub> took place *in vacuo* by pumping off controlled volumes of vapor fractions occurring at  $p = 300$ –100 mm Hg and  $t = -45^\circ$ –0°C. At 0°C, 5.00 ml of benzene was added to the remainder. The resulting solution of furan, pyrrole, H<sub>2</sub>O, NH<sub>3</sub> etc. in benzene was analyzed quantitatively on a gas chromatograph, (Perkin Elmer 116 E, column "R" [polypropyleneglycol], length 2 m). In this way any ambiguity in stating the true yield of the reaction, dating from a more or less wasteful separation of reaction products, was avoided.

In these 500 mg experiments the desired mixture of furan and NH<sub>3</sub> was obtained by mixing a needle-valve regulated stream of pure NH<sub>3</sub> (at room temperature and a pressure slightly higher than one atmosphere) of velocity 50 ml/min, with a stream of a mixture of furan and NH<sub>3</sub> (2:1) of the same temperature and pressure. This latter stream was fed to the NH<sub>3</sub>-stream by a mechanical pump which introduced Hg at a constant rate of 10 ml/min, displacing the furan/NH<sub>3</sub> mixture from a flask of 300 ml volume. In the final experiments involving 170 and 130 mg furan, respectively, all of the correct 1:7 furan/NH<sub>3</sub> mixture was contained in a 500 ml flask and introduced at a rate of 50–60 ml/min by the action of the pump.