INDUCTION OF CELL DIFFERENTIATION

II. THE ISOLATION OF A CHONDROGENIC FACTOR FROM EMBRYONIC CHICK SPINAL CORDS AND NOTOCHORDS

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SUMMARY

A chondrogenic compound has been isolated in chromatographically homogeneous form from spinal cords and notochords of 4.5-day old chick embryos. This compound stimulates the formation of vertebral cartilage in explanted chick somites.

INTRODUCTION

In the preceding paper it has been shown that a nucleotide containing component extracted from the spinal cord and notochord simulates the chondrogenic action of the intact tissues by promoting cartilage formation in explanted somites[1]. Addition of very minute quantities of this component to isolated and explanted chick somites induces cartilage formation. The cartilage which forms is demonstrable four days after addition of this component. The phenomenon of vertebral cartilage induction by the intact spinal cord or notochord has been examined extensively[2].

This paper is concerned with the isolation of the chondrogenic factor and gives some criteria for its homogeneity.

MATERIALS AND METHODS

Preparation of spinal cords and notochords

Spinal cords and notochords were removed from 4.5 day old chick embryos by crude dissection. The head, tail, limb buds and embryonic digestive tract were removed, and the spinal cord and notochord were dissected free of adhering tissues[1] (somites, kidney, epidermis). The spinal cord and notochord from 1000 embryos were stored at —20° in 0.9 % NaCl until extraction procedures were started.

Preparation of adsorbent

The charcoal (grade S X II, Dutch Norit Company) was refluxed for 1 h under vigorous stirring with 10% aqueous acetic acid, filtered over a Büchner funnel and washed with distilled water until all acetic acid had been removed. The washed charcoal was mixed with an equal portion (by weight) of Celite 535 (N.V. Profiltra,

Amsterdam) by adding the celite in small portions to a vigorously stirred aqueous suspension of charcoal. The charcoal–celite mixture was then poured into the column and subsequently washed with distilled water, 8% octan-2-ol (B.D.H. Laboratory reagent) in ethanol, 95% ethanol and distilled water. After this washing the column is ready for use.

Preparation of crude extract

Spinal cords and notochords of 1000 chick embryos were homogenized, without addition of any liquid, in a Waring Blendor, for 5 min at 0°. The mince was transferred to a beaker with 15 ml of 0.25 *N* perchloric acid and stirred for 5 min in the cold. The mixture was neutralized with 0.5 *N* potassium hydroxide solution, using a drop of phenol red as an indicator and centrifuged at 0° for 20 min at 12000 × *g*. The clear supernatant fluid was withdrawn and adsorbed onto a charcoal–celite column (2.5 × 15 cm). The column was extensively washed with water (approx. 300 ml) and subsequently eluted with 10% aqueous pyridine. The eluate (approx. 250 ml) was collected, frozen and thawed 3 times to precipitate some collodial material and finally centrifuged. The clear supernatant fluid was lyophilized, yielding approximately 40 mg of crude extract per 1000 spinal cords and notochords.

Dowex-1 chromatography

This was performed essentially as described by HURLBERT, SCHMITZ, BRUNN AND POTTER[3]. Dowex-1 X8 (200–400 mesh) in the chloride form, was washed with 3 *M* sodium formate until the wash water was completely free of chloride ions. The resin (450 g) was washed with 6 l of 6 *N* formic acid. After placing in the column (0.9 × 12 cm) it was washed with 88% formic acid and with water until neutrality. The lyophilized charcoal eluate was redissolved in a small amount of water and adsorbed onto the column. Elution of the nucleotides was achieved with a formic acid gradient by pumping 4 *N* formic acid into a continuously stirred mixing vessel of 250 ml content employing an LKB Miniflow Precision Micropump. The mixing chamber was initially filled with distilled water. The flow rate was 25 ml/h. The extinction of the eluate ($\lambda = 260$ mμ) was continuously measured with an LKB Uvicord equipped with an automatic recorder. In as much as some of the eluted compounds seemed to be acid labile, the eluate was continuously neutralized with 4 *N* ammonium

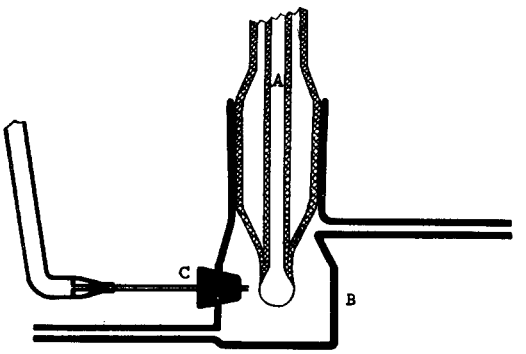


Fig. 1. Vessel for continuous neutralisation of column eluates. A, Combined glass electrode and calomel electrode, Radiometer GK 264, type B; B, glass vessel; C, rubber stop cock, equipped with injection needle for base supply.

hydroxide. For this purpose the outlet of the Uvicord was connected to a small mixing vessel (see Fig. 1), equipped with an electrode (Radiometer GK 264 combined electrode, type B), a small magnetic stirrer and an ammonium hydroxide supply. The electrode was connected to a Radiometer Titrator TTT, which regulates the base supply, so that a final pH of about 5 is constantly ensured. In the beginning of the elution a large proportional band was used, which was reduced in the course of the elution. Finally the eluate was collected automatically at 12 min intervals.

Elution with an ammonium formate gradient was performed in the same manner, the neutralization step being omitted.

Desalting of the eluate

The combined fractions from one peak of the chromatogram were desalted by a slight modification of the method of OKASAKI[4]. A small amount of charcoal–celite mixture (1:1) was successively washed, by suspending and centrifuging the mixture, with 50% ethanol containing 0.7% of concentrated ammonia, 0.1 *N* hydrochloric acid and then distilled water until the washings were neutral.

The eluate was adjusted to pH 5 and added to the washed charcoal–celite mixture under vigorously stirring for 0.5 h.

The adsorbent was centrifuged off and washed four times with distilled water. The nucleotides were eluted with 50% ethanol (v/v) containing 0.7% of concentrated ammonia (v/v). The eluate was concentrated *in vacuo* at room temperature in a flash evaporator. Using adenosinemonophosphate as a test substance, a recovery of 80% was obtained.

Paper electrophoresis

The lyophilized charcoal eluate was applied to Whatman paper No. 3MM. Electrophoresis was performed at pH 6.5 in pyridine–acetic acid–water (10:1:89, v/v) with the pherograph of WIELAND AND PFLEIDERER[5] at 0°, for 2 h at 40 V/cm.

Paper chromatography

Descending paper chromatography on Whatman paper No. 1, was carried out according to PALADINI AND LELOIR[6] at pH 7.5. Phosphate containing spots were made visible with the ferric chloride–sulfosalicylic acid procedure of RUNECKLES AND KROTKOV[7] or by the molybdenic acid spray of HANES AND ISHERWOOD[8]. Elution of spots was performed according to the method of SANGER AND TUPPY[9].

Chondrogenic activity of nucleotide fractions

Somites were dissected from chick embryos and cultured in the presence of the nucleotide fractions according to previously published methods[1]. A chondrogenically active fraction caused large nodules of cartilage to form in clusters of explanted somites.

RESULTS

As demonstrated earlier, the chondrogenic activity was present in the 10% aqueous pyridine (v/v) eluate from the charcoal–celite column[1]. This nucleotide fraction has been analyzed further by means of ion exchange chromatography on Dowex-1. The

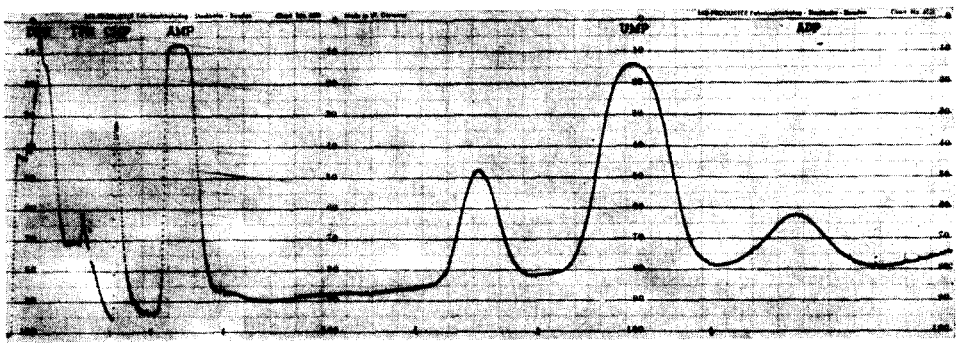


Fig. 2. Gradient elution chromatography of charcoal–celite eluate (10% aqueous pyridine) on Dowex-1 X8 (200–400 mesh). Column dimensions: 0.9 × 12 cm. Mixing chamber: 250 ml of water. Gradient: 4 *N* formic acid, none linear. Collected in 5-ml fraction at a constant flow rate of 25 ml/h. The abscisse of the graph is divided in centimeters, 2 cm correspond to 1 h.

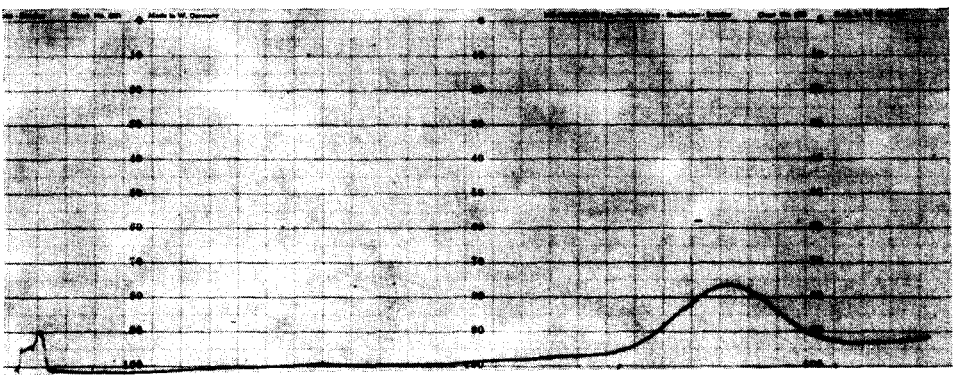


Fig. 3. Rechromatography of fraction No. V, on Dowex-1 X8 (200–400 mesh) in the formate form. Gradient with 1 *M* ammonium formate. Experimental conditions as in Fig. 1.

most relevant part of the elution diagram is shown in Fig. 2. The resolution of the component nucleotides was checked with a synthetic mixture of nucleotides. So far spinal cords and notochords from 70000 chick embryos have been processed in batches of 1000. With the above described techniques the chromatogram chart in Fig. 2 is exactly reproducible. After elution of ADP, small amounts of ATP and uridine activated carbohydrates may be eluted with 4 *N* formic acid plus ammonium formate[3]. Each fraction eluted from the Dowex-1 column was tested for its chondrogenic activity. In order of emergence from the column only fraction No. 5 showed definite chondrogenic activity. Out of 75 somite cultures 55 formed cartilage after the addition of a solution containing this fraction. Somite cultures receiving the other fractions did not show any activity beyond that of the control cultures. Control cultures received extracts from tissues other than the spinal cord or notochord, or received no extracts[1].

The chondrogenic fraction appeared to be homogeneous when rechromatographed on Dowex-1 employing an ammonium formate gradient (Fig. 3). Paper electrophoresis of this fraction at pH 6.5 yielded one sharp band which exhibited blue quenching when observed under ultraviolet-light and positive reactions with the ammonium molybdate reagent for phosphate, with ninhydrin for amino acids and with an acidic

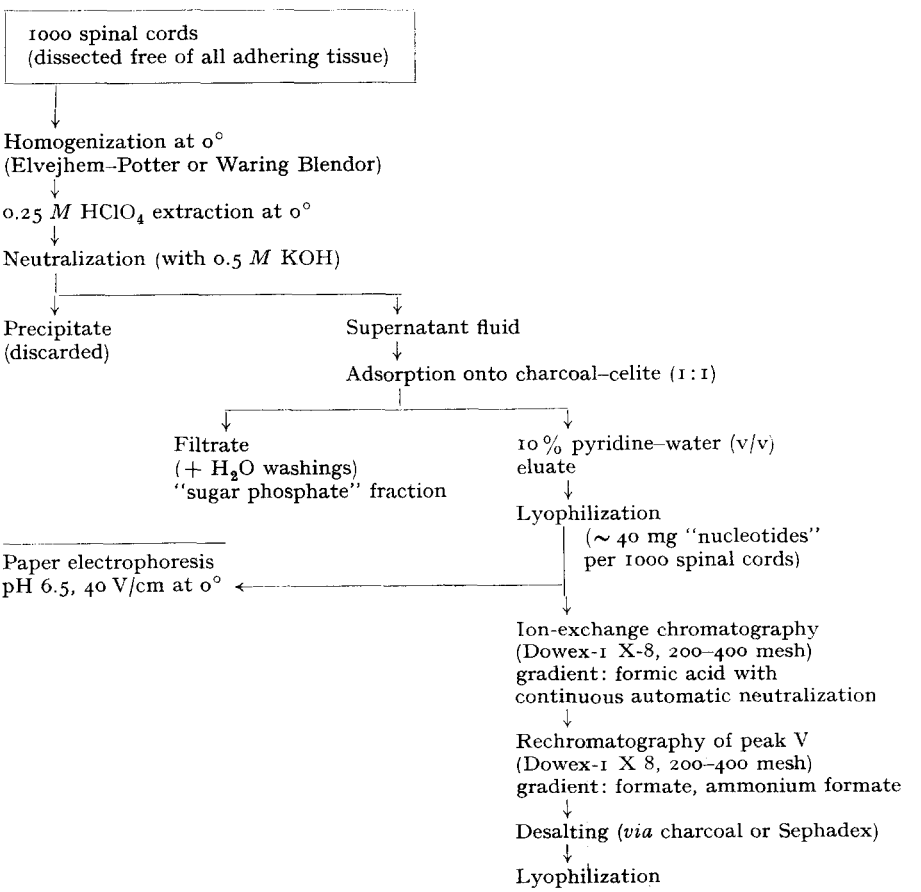
solution of aniline phthalate for reducing carbohydrates. The electrophoretic mobility of this zone was approx. $+ 7.2 \cdot 10^{-6}$ cm²/sec/V. Paper chromatographic analysis of the same fraction in ethanol ammonium acetate at pH 7.5 yielded a single spot with similar positive reactions possessing an R_{AMP} value of 1.42.

The same chondrogenic fraction was also obtained by means of zone electrophoresis at pH 6.5 of the crude charcoal eluate thus omitting ion exchange chromatography on Dowex-1. In this case besides 7 or 8 phosphate containing fractions, a zone with the same electrophoretic mobility ($+ 7.2 \cdot 10^{-6}$ cm²/sec/V) was also observed, giving positive reactions with ninhydrin, with ammonium molybdate and with aniline phthalate. This zone was well separated from the other phosphate containing compounds. In addition, two other zones were observed, which gave positive reactions with ammonium molybdate and ninhydrin reagents and possessed electrophoretic mobilities of $34.5 \cdot 10^{-6}$ cm²/sec/V and $38.5 \cdot 10^{-6}$ cm²/sec/V respectively.

When the anodically migrating zone ($+ 7.2 \cdot 10^{-6}$ cm²/sec/V) was eluted and chromatographed in the Paladini–Leloir system only one spot was observed with an R_{AMP} value of 1.42 as mentioned above.

SCHEME I

METHOD OF ISOLATION OF CHONDROGENIC FACTOR



When larger amounts of this material were prepared by zone electrophoresis at pH 6.5, eluted from the paper, lyophilized and rechromatographed on Dowex-1 employing the above described technique only a single peak was obtained which was found to be identical in allocation and properties with the above mentioned peak 5 obtained after Dowex chromatography of the entire charcoal eluate.

DISCUSSION

A factor which induces somite cells to differentiate into cartilage cells has been isolated from chicken embryonic spinal cords and notochords by two independent methods. It has been shown that both procedures yield the same biologically active products, which are homogeneous as judged by paperchromatography, column chromatography and paper electrophoresis. As far as the authors are aware, this is the first isolation of an embryonal inducer in a pure state.

The column chromatographic method is suitable for preparative isolation procedures, leading to the exact chemical composition of the factor, work which is now in progress. Preliminary experiments have definitely identified cytidine monophosphate and the amino acids aspartic acid, threonine, serine, glutamic acid, glycine, alanine and valine in different ratios, and in addition a reducing hexosamine. Besides a cytidine nucleotide, spectral evidence shows the presence of a guanosine nucleotide.

The paper electrophoretic method lends itself as a suitable tool for working with labelled compounds, leading to the proper allocation of the factor in different cell compartments. Preliminary experiments toward this end have shown that at the age of 4.5 days the chondrogenic factor is only present in the 100000 $\times$ g supernatant, obtained after the removal of the cell debris, nuclei, mitochondria, microsomes and pH 5 fraction by fractional centrifugation.

Further experiments in this direction may shed some more light on the problem of the enzyme forming system in as far as cartilage is composed of a matrix of chondroitin sulphate and protein.

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