INDUCTION OF CELL DIFFERENTIATION

I. THE *IN VITRO* INDUCTION OF VERTEBRAL CARTILAGE WITH A LOW-MOLECULAR-WEIGHT TISSUE COMPONENT

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SUMMARY

A nucleotide-containing fraction has been obtained from a cold perchloric acid extract of the chick embryonic spinal cord and notochord that simulates the chondrogenic action of the intact inducing tissues. This fraction induces the formation of cartilage in explanted somites. Upon partial purification of this fraction the ability to induce cartilage formation has been restricted to one nucleotide-containing component.

INTRODUCTION

Of the many induction systems that have been described, one of the most rewarding in which to study the biochemistry of embryonic induction is that of cartilage formation. Since the major molecular components of cartilage are readily identifiable, and since some information is available regarding their biosynthesis[1], it is feasible to analyze events attending their induction and subsequent synthesis[2].

This paper is concerned with the processes involved during a comparatively late stage of embryonic development, *viz.* vertebral chondrogenesis. Past work has suggested that the ventral half of the embryonic spinal cord produces a transmissible substance that passes to the somite cells, there inducing the formation of vertebral cartilage, whereas the notochord induces cartilage formation in somite cells only when the cells are in direct apposition to the notochordal sheath[3]. Moreover, these specific chondrogenic influences occur within the first few hours of association between the tissues, even though cartilage cannot be detected histologically or biochemically until the fourth day of culture[2].

The work reported below has led to the isolation and tentative characterization of a chondrogenic factor of relatively small molecular weight that can be extracted from the embryonic spinal cord and notochord. This factor elicits the formation of cartilage in somites, thereby simulating the biological action of the intact inducing tissues.

MATERIALS AND METHODS

In order to perform biochemical analyses pertinent to the problem under investigation, it was necessary to use small pieces of tissues. Most of the cultures to be extracted varied in size between 0.5–1.0 mm^3. Since the intent of the experiments was to isolate nucleotides, the phosphorus compounds were labelled by growing the tissues in the presence of radioactive inorganic phosphate, $Na_3{}^{32}PO_4$. The tissues readily incorporated the radioactive isotope into the phosphate-containing compounds, although the specific activity was not determined. Although radioactive phosphorus has a half-life of only 14.3 days, it was used satisfactorily to label, extract and partially characterize the chondrogenic factor in the inducing tissues.

When it was established that an active chondrogenic factor was present in the inducing tissues, chemical extractions were performed on large numbers of unlabelled spinal cords and notochords[4].

Preparation of tissues

Embryonic chick somites, spinal cords and notochords were isolated by methods previously published[5]. All tissues were taken from 2.5–3 day incubates (stages 16–18, HAMBURGER AND HAMILTON[6]), with the exception of the large numbers of 4.5 day unlabelled spinal cords used for chemical extractions. The latter tissues were obtained by cutting them free of surrounding tissues.

Labelling of tissues

Inducing tissues (notochord and spinal cord), reacting tissues (somites) and chondrogenic cultures (inducing tissues plus somites), were grown in the presence of radioactive inorganic phosphate (20 μC $Na_3{}^{32}PO_4$/0.1 ml SIMMS'[17] balanced salt solution per dish of 5 explants). These tissues were placed upon lens paper floating on a liquid nutrient medium[8], and incubated at 38° for 24 h before extraction. Chondrogenic cultures (inducing tissues plus somites) were incubated for longer intervals, until cartilage appeared.

Extraction of tissues

Varying amounts of tissues, most frequently 20 cultures, were extracted with 1.0 ml of 0.25 *M* perchloric acid at 0° for 15 min. After this the solution was neutralized with potassium hydroxide. Following centrifugation the supernatant fluid was adsorbed onto a charcoal–celite (1:1) column (6 × 0.4 cm). The charcoal–celite column was prepared according to THRELFALL[9].

The charcoal–celite filtrate (*i.e.* substances not adsorbed onto the column) was found to contain sugar-phosphates, and will hence be spoken of as the "sugar-phosphate" fraction. This fraction was analyzed separately by means of paper electrophoresis and paper chromatography.

The acid-soluble nucleotides which were adsorbed onto the charcoal–celite column were eluted with 10% aqueous pyridine[9]. This eluate will be called the "nucleotide fraction", since its electrophoretic and chromatographic patterns, its positive reaction with the WADE-MORGAN colorimetric method[10], and its ultraviolet spectrum all indicate the presence of nucleotides.

Electrophoresis and chromatography

Both radioactive fractions (nucleotide and sugar-phosphate) were analyzed by means of paper (Whatman 3 MM) electrophoresis in pyridine–acetic acid–water (100:10:890, v/v) at pH 6.5, 30 V/cm at 0° for 2.5 h. The air dried electropherograms were then scanned for radioactive components with a chromatogram scanner (Nuclear Chicago). One of the components of the sugar-phosphate fraction that appeared to be unique to the inducing tissues was eluted from the electropherogram and chromatographed in the solvent of Paladini-Leloir[11] (Whatman 1 paper, 95% ethanol–NH_4Ac (pH 3.8), (7.5:3, v/v)). The dried chromatogram was scanned for radioactive components, yielding R_F values corresponding to hexose phosphates, and giving a positive test with ammoniacal silver nitrate solution[12]. The other components have not yet been analyzed.

Preparation on larger scale

For further characterization of the nucleotide fraction, spinal cords and notochords from 1000 4.5-day chick embryos were prepared and analyzed as described in the following paper[4].

Testing extracts for chondrogenic activity

All somites were grown on nutrient agar[13]. Each extract or fraction to be tested was dissolved in 1.0 ml of balanced salt solution, sterilized through a Swinny Hypodermic Adaptor (Millipore Filter Corp., Bedford, Mass.) and made up into a liquid nutrient medium (1.0 ml balanced salt solution–1.0 ml horse serum–0.5 ml embryo extract). Approximately 0.25 ml of this nutrient medium was placed in each dish containing 10–20 clusters of 6–8 somites. Control cultures received either plain nutrient medium or nutrient medium containing extracts of tissues other than spinal cord or notochord. The amount of extract or fraction added to the cultures was too small to quantitate, but according to the dilutions of the extract yielded by 1000 spinal cords and notochords, approximately 0.5–1.0 μg of the nucleotide fraction was added to each dish of somite cultures.

Assay for induction of cartilage

Cartilage in living cultures was determined visually through the dissecting microscope. Tissues were also fixed in acetic acid–ethanol (1:3, v/v), sectioned and stained with aqueous thionin (1:1000 w/v) for metachromic material.

RESULTS

Characterization of extracts

Nucleotide fractions from radioactive cultures were analyzed by means of paper electrophoresis and scanning of the air dried electropherograms for radioactive components. The nucleotides moved as a broad band, without resolution of separate component nucleotides (Fig. 1). The peaks on the scanning charts indicate the amount of activity present (scale in Figs. 1–3 is 10000 counts/min; Fig. 4, 3000 counts/min), but no attempt has been made yet to quantitate the data. Nucleotide fractions similar to the one in Fig. 1 were eluted from the electropherogram, made up into a nutrient medium solution, and added to somite clusters. The characterization of these fractions

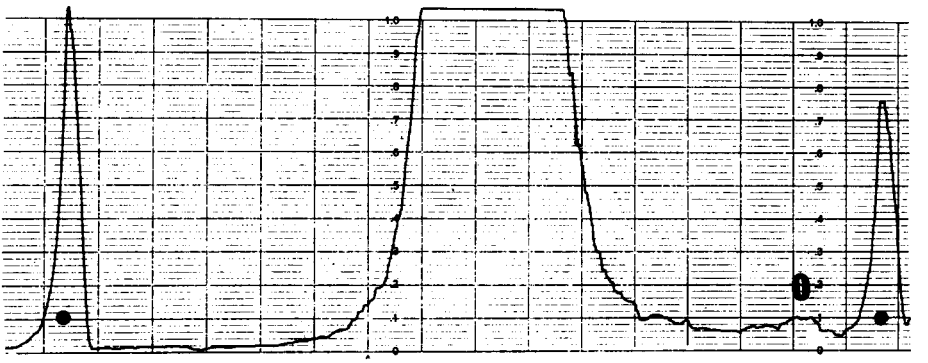

Fig. 1. Paper electrophoresis of partially purified cold perchloric acid extract (nucleotide components) from inducing tissues. Radioactive phosphate components move as a broad band. In Figs. 1–3 all conditions are the same. Electrophoresis at pH 6.5, 30 V/cm, at 0° for 2.5 h. The corresponding chart for the electropherogram was matched for radioactive components with the aid of radioactive markers (black dots on charts). Full scale on the chart represents 10000 counts/min.

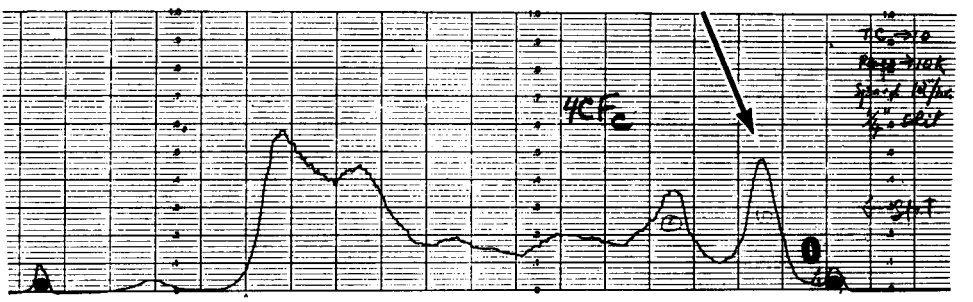

Fig. 2. Paper electrophoresis of components of perchloric acid extract not absorbed on charcoal–celite column (filtrate). The radioactive sugar-phosphate component found only in inducing tissue shown by arrow. Other components have not been analyzed. All other conditions similar as in Fig. 1.

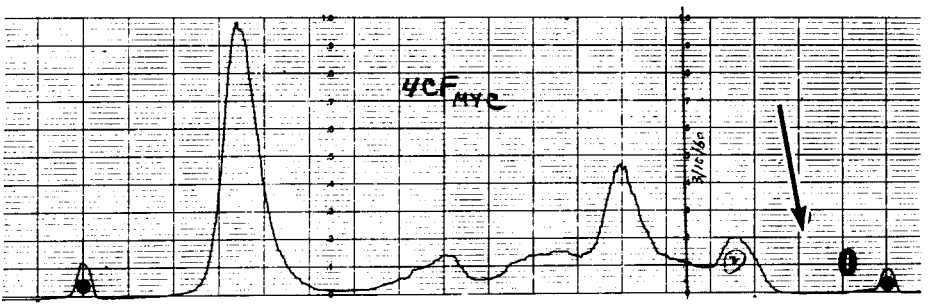

Fig. 3. Paper electrophoresis of charcoal–celite filtrate from non-induced somites. This shows the absence of the unique component (arrow). All other conditions same as in Fig. 1. Similar electropherograms are obtained from non-inducing tissues (*e.g.* muscle, limb buds).

as "nucleotides" is based upon analytical tests performed on combined fractions of 80–100 3-day spinal cords. These fractions showed electrophoretic and chromatographic mobilities comparable to known nucleotide standards and gave positive tests typical of nucleotides. Further characterization of this fraction is given in the following paper[4].

The sugar-phosphate fractions upon electrophoretic separation contained five major peaks (Fig. 2), one of which was present only in extracts of the inducing tissues and somites which had been exposed to the inducing tissues. Somites which had not been induced possessed only four of the major peaks in the sugar-phosphate fraction (Fig. 3). The extra peak has not been completely identified yet, but upon elution and re-chromatography it yielded two phosphorylated compounds with R_F values indicating the presence of a hexose phosphate and a hexosamine phosphate (Fig. 4). The hexosamine compound gave a positive Morgan-Elson reaction as modified by REISSIG *et al.*[14], indicating the presence of N-acetylhexosamine phosphate.

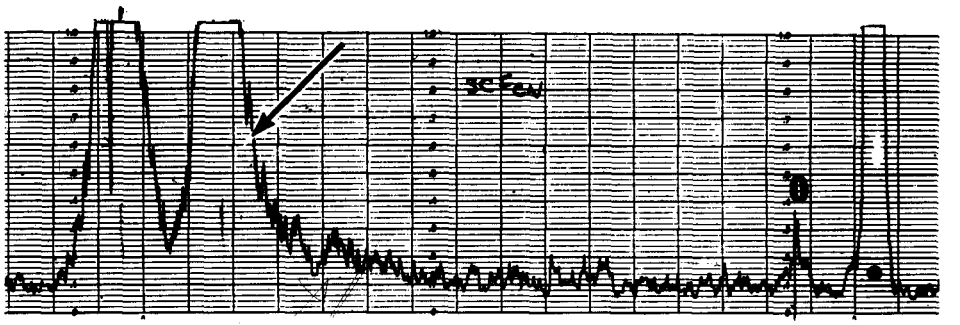

Fig. 4. Paper chromatography of radioactive sugar-phosphate component shown by arrow in Fig. 2. Descending chromatography 14 h, 95% ethanol–1.0 *M* NH_4Ac, pH 3.8 (7.5:3, v/v). The phosphate-compound indicated by the arrow gave a positive Morgan-Elson reaction. Full scale on the chart represents 3000 counts/min.

Effect of extracts upon somite cultures

The extract thus yielded two fractions; a fraction containing sugar phosphates, and one containing nucleotides. Sugar-phosphate and "nucleotide fractions" from notochords and spinal cords were added separately to somite cultures to determine whether the extracts were capable of simulating the action of the intact inducing tissues. The extracts were taken from 3-day old tissues approximately 1.0 mm^3 in size, which had been grown in the presence of radioactive inorganic phosphate.

The sugar-phosphate fraction had no observable effect, but the "nucleotide fraction" and the non-fractionated perchloric acid extract from as few as eight 3-day spinal cords caused cartilage to appear regularly in embryonic somites. Subsequent experiments using non-radioactive extracts or "nucleotide fractions" from large numbers of 4.5-day spinal cords and notochords gave the same results.

Extracts of the notochord or the ventral half of the spinal cord induced cartilage formation, whereas extracts of the dorsal half of the spinal cord had no effect upon the somites. Moreover nucleotide extracts from other embryonic tissues (ectoderm, endoderm, muscle, limb buds) did not induce cartilage in somites.

Out of 228 cultures of explanted somites, 186 have been induced to form cartilage by adding the "nucleotide fractions" (Table I). After the addition of the "nucleotide fraction", the induced somites formed cartilage on the fourth day of culture. Control cultures of somites without the addition of an active "nucleotide fraction", or with the addition of a total yeast perchloric acid extract prepared in a similar fashion, yielded 38 small nodules of cartilage in 304 cultures.

Somites of varying ages were used to test the chondrogenic activity of "nucleotide fractions". When young somites were used (stages 16–17) fewer control cultures formed cartilage. Control cultures of stage 16 were completely negative with respect

TABLE I

CHONDROGENIC ACTIVITY OF NUCLEOTIDE FRACTIONS

Age of somites	*Number of cultures*	*Number of cultures forming cartilage*	*Incidence of cartilage (%)*
Stage 16			
Controls	20	0	0
Plus extract	10	9	90
Stage 17			
Controls	80	9	11
Plus extract	53	44	83
Stage 18			
Controls	194	29	15
Plus extract	165	134	81

to cartilage formation whereas 9 out of 10 cultures receiving "nucleotide fraction" produced cartilage (Table I). Not only was the incidence of chondrogenesis less in the controls than that in the treated cultures, but the amount of cartilage per cluster of somites was also considerable less and formed later in the life of the culture. The low incidence and small quantity of cartilage formation in the control cultures of older somites is in agreement with the idea that there is a time at which the somites become induced *in vivo*, and if explantation occurs during this interval there will occur some cases of seemingly spontaneous cartilage formation[5].

Resolution of the nucleotide fraction

To identify the components of the "nucleotide fraction", and to determine which component was the chondrogenic factor, combined "nucleotide fractions" from 4.5-day spinal cords and notochords were passed over the Dowex column. The resolution of the nucleotides eluted in the formic acid gradient is shown in Fig. 5. Each component

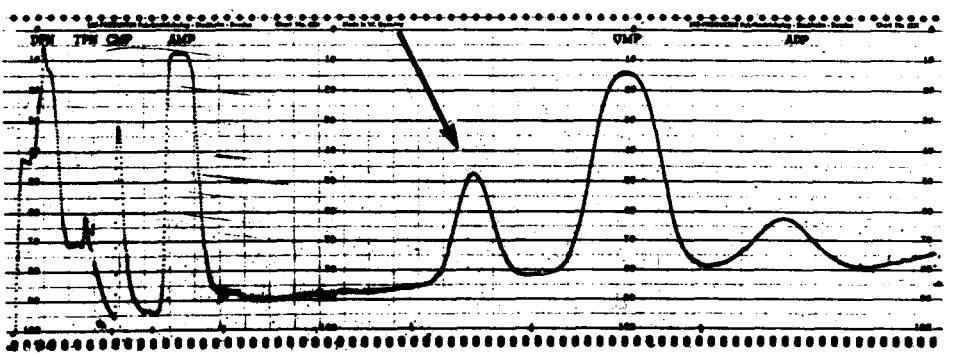

Fig. 5. Dowex 1 X 8 (200–400 mesh) chromatography of nucleotide fraction obtained from 4.5 day old spinal cords and notochords. Column dimensions: 12 × 0.9 cm. Elution and characterization as specified in Fig. 2 of the following paper[4]. Nucleotide-containing component capable of chondrogenic induction indicated by arrow.

was then lyophilized, redissolved in balanced salt solution, and tested for chondrogenic activity. The only component possessing such activity corresponded to the peak indicated by the arrow in Fig. 5. Out of 75 cultures, 55 formed cartilage after the addition of a nutrient medium containing this component. The other nucleotide components had no inducing effect, each fraction being tested on 20 cultures. Details on the methods of isolation and identification of this component an a larger scale is published in the following paper[4].

DISCUSSION

Previous work has shown that explanted somites form significant quantities of cartilage only when exposed to the embryonic spinal cord or notochord. This reaction is specific in the sense that no other tissues or agents have been found to induce the same chondrogenic response in somites[3].

There is little doubt that in the induction of vertebral cartilage, a nucleotide containing component extracted from the spinal cord and notochord simulates the chondrogenic action of the intact inducing tissues. According to the methods of isolation, this component must be of relative small molecular size. The "nucleotide component" is fractionated from a perchloric acid extract, the other fraction (sugar-phosphate) possessing no chondrogenic activity.

At present we can not say whether this component represents the only one capable of simulating chondrogenic induction, or whether the spinal cord and notochord are the only tissues containing such a component. An explanation of the biological action of this cartilage promoting factor and its significance to biosynthetic processes will have to wait until more work has been done.

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