Quantitative analysis of type X-collagen biosynthesis by embryonic-chick sternal cartilage

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We have performed a quantitative analysis of the various collagens biosynthesized by organ cultures of whole embryonic-chick sternum and its separate anatomical regions corresponding to the zones of permanent hyaline and presumptive-calcification cartilages. Our studies demonstrated that embryonic-chick sternum devotes a large portion of its biosynthetic commitment towards production of Type X collagen, which represented approx. 18% of the total newly synthesized collagen. Comparison of the collagens biosynthesized by the permanent hyaline cartilage and by the cartilage from the presumptive-calcification zone demonstrated that Type X-collagen production was strictly confined to the presumptive-calcification region. Sequential extraction of the newly synthesized Type X collagen demonstrated the existence of two separate populations. One population (approx. 20%) was composed of easily extractable molecules that were solubilized with 1.0 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4. The second population was composed of molecules that were not extractable even after repeated pepsin digestion, but became completely solubilized after treatment with 20 mM-dithiothreitol/0.15 M-NaCl buffer at neutral pH. These results suggest that most of the Type X collagen normally exists in the tissue as part of a pepsin-resistant molecular aggregate that may be stabilized by disulphide bonds. Quantitative analysis of the proportion of Type X collagen relative to the other collagens synthesized in the cultures indicated that this collagen was a major biosynthetic product of the presumptive-calcification cartilage, since it represented about 35% of the total collagen synthesized by this tissue. In contrast, the permanent hyaline cartilage did not display any detectable synthesis of Type X collagen. When compared on a per-cell basis, the chondrocytes from the presumptive-calcification zone synthesized approx. 33% more Type X collagen than the amount of Type II collagen synthesized by the chondrocytes from the permanent-hyaline-cartilage zone. Subsequently, it was demonstrated that Type X collagen is a structural component of chick sternum matrix, since quantitative amounts could be extracted from the region of presumptive calcification of 17-day-old chick-embryo sterna and from the calcified portion of adult-chick sterna. The strict topographic distribution in the expression of Type X collagen biosynthesis to the zone of presumptive calcification suggests that this collagen may play an important role in initiation or progression of tissue calcification.

INTRODUCTION

The molecular heterogeneity of hyaline-cartilage collagens has been well documented (Bornstein & Traub, 1979). Although initially it had been thought that this tissue contained exclusively Type II collagen (Miller, 1971), Burgeson, Hollister and co-workers (Burgeson & Hollister, 1979; Burgeson *et al.*, 1982) were first to demonstrate the existence of three additional collagen chains, designated 1α , 2α and 3α , in human and bovine articular cartilage. The presence of small amounts of these collagen chains has been reported in various other cartilagenous tissues (Shimokomaki *et al.*, 1980; Ayad *et al.*, 1981; Reese & Mayne, 1981). In addition, these and other studies (Reese *et al.*, 1982; von der Mark *et al.*, 1982; Ricard-Blum *et al.*, 1982; Gibson *et al.*, 1982; Bruckner *et al.*, 1983; Duance *et al.*, 1984; Yasui *et al.*, 1984) have identified a distinct collagen molecule, characterized by the presence of interrupted helical

domains held together by intermolecular disulphide bonds, which yields several smaller molecular species after limited proteolytic digestion and disulphide-bond reduction. This collagen, which has recently been termed 'Type IX collagen' has been extensively studied and characterized (van der Rest *et al.*, 1985) and a cDNA coding for a region of one of the polypeptide chains of this molecule has been identified (Ninomiya & Olsen, 1984).

More recently, several laboratories have reported the biosynthesis of a short-chain collagen by embryonic-chick chondrocytes (Gibson *et al.*, 1982, 1983; Schmid & Conrad, 1982a, b; Schmid & Linsenmayer, 1983; Capasso *et al.*, 1984), and mammalian growth-plate cartilage (Remington *et al.*, 1983, 1984; Sussman *et al.*, 1984). Characterization of this collagen from long-term cultures of chondrocytes from the hypertrophic zone of embryonic-chick tibiotarsus showed that it is composed of 59000- M_r non-disulphide-bonded polypeptides con-

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; NEM, *N*-ethylmaleimide; SDS, sodium dodecyl sulphate; DTT, dithiothreitol; MEM, Minimal Essential Medium.

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taining a 14000- M_r non-collagenous domain and a 45000- M_r triple-helical pepsin-resistant collagenous region (Schmid & Linsenmayer, 1983). This collagen, which was previously termed 'G collagen' (Gibson *et al.*, 1982) and 'SC collagen' (Schmid & Linsenmayer 1983), has been recently classified as 'Type X collagen' (Schmid & Linsenmayer, 1985). Although several studies failed to show the presence or biosynthesis of Type X collagen in embryonic-chick sternum (Reese *et al.*, 1982; Kielty *et al.*, 1984, 1985), a recent report showed that this tissue is capable of its biosynthesis in an organ-culture system (Gibson *et al.*, 1984). In the present study we have performed a detailed quantitative study of the relative proportions of the various collagens synthesized by whole chick sternum and by the two distinct anatomical regions composed of permanent hyaline cartilage and cartilage that will undergo calcification. We found that Type X collagen is exclusively synthesized by cartilage from the presumptive-calcification region and that it represents a major biosynthetic product of this tissue. When calculated on a per-cell basis, the amount of Type X collagen synthesized by the chondrocytes from the presumptive-calcification region was approx. 33% greater than the amount of Type II collagen synthesized by chondrocytes from the permanent hyaline cartilage during the same period of culture. Sequential extraction and electrophoretic characterization of the newly synthesized collagens indicated the existence of two distinct populations of Type X collagen molecules. A minor population (approx. 20%) was composed of easily solubilized molecules, whereas the remainder was remarkably resistant to extraction even after repeated proteolytic digestion, but became completely solubilized after treatment of the tissues at neutral pH with DTT. These results suggest that most of embryonic-chick sternum Type X collagen exists in the tissue as part of a pepsin-resistant molecular aggregate that may be stabilized by disulphide bonds.

EXPERIMENTAL

Biosynthetic studies

Sterna from 17-day-old White Leghorn chick embryos were removed and carefully dissected from surrounding perichondrium. The caudal region corresponding to the permanent hyaline and the cephalic region corresponding to presumptive-calcification cartilages respectively were separated with a scalpel. Whole sterna and cartilage from these two distinct zones were minced and incubated in separate culture dishes for 24 h (37 °C; CO₂/air, 1 : 19) in tissue-culture medium (NCTC 135/Minimum Eagle's Medium/Earle's Basic Salt solution) containing 10% (v/v) foetal-calf serum, ascorbic acid (100 µg/ml) and 10 µCi of [U-¹⁴C]proline/ml (275 mCi/mmol; Amersham International). β-Aminopropionitrile was not included in order to resemble more closely the conditions of cartilage-matrix biosynthesis *in vivo*.

Collagen solubilization

After incubation, the media were removed and the minced tissues were separately homogenized with a glass/glass tissue grinder in 1.0 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, at 4 °C, and the following mixture of proteinase inhibitors: 5.0 mM-disodium EDTA, 0.2 mM-PMSF, 5.0 mM-NEM and 1.0 mM-*p*-aminobenzamidine

hydrochloride. The homogenized tissues were sequentially extracted under non-denaturing conditions by using the method previously described for the solubilization of heart-valve collagen (Jimenez & Bashey, 1978). Briefly, the homogenates were extracted with the 1.0 M-NaCl/Tris buffer for 72 h at 4 °C and subsequently twice each with 0.5 M-acetic acid, followed by 0.5 M-acetic acid/pepsin (1 mg/ml), and then 0.15 M-NaCl/20 mM-DTT/50 mM-Tris/HCl, pH 7.4. The remaining pellet was extracted again with the acetic acid/pepsin solution for 48 h at 4 °C. After removal of this final supernatant by centrifugation at 16000 *g* for 30 min, the residue was suspended in 0.5 mM-acetic acid and after mild shaking became completely solubilized. Each of the extracts was extensively dialysed against 0.15 M-NaCl/50 mM-Tris/HCl, pH 7.4, containing proteinase inhibitors at 4 °C and total ¹⁴C radioactivity was determined in a liquid-scintillation counter. Other portions were hydrolysed in 6 M-HCl for 18 h and, after evaporation, their [¹⁴C]hydroxyproline contents were assayed by the method of Juva & Prockop (1966).

Fractional NaCl precipitation of the extracted collagens

Fractional NaCl precipitation of the extracted collagens from acidic solutions was performed essentially as described by Shimokomaki *et al.* (1980). For this purpose, portions of the extracts were dialysed against 0.5 M-acetic acid containing progressively increasing concentrations of NaCl. Fractions precipitating at 0.8 M, 1.2 M and 2.2 M-NaCl were obtained and were removed by centrifugation at 16000 *g* for 30 min and dialysed against 0.15 M-NaCl/50 mM-Tris/HCl, pH 7.4, at 4 °C for further studies.

Proteolytic digestion

Samples used for collagenase digestion were dialysed against a buffer containing 0.15 M-NaCl/5.0 mM-CaCl₂/50 mM-Tris/HCl/10 mM-NEM/0.2 mM-PMSF, pH 7.4. Purified bacterial collagenase (Form III, Advanced Biofactures) was added (150 units/ml) and the samples were digested for 6 h at 37 °C. The reaction was terminated by the addition of EDTA to a final concentration of 10 mM and cooling to 4 °C. For digestion with pepsin, samples were dialysed against 0.5 M-acetic acid, and after dialysis they were digested in 0.5 M-acetic acid containing pepsin (300 µg/ml) for 6 h at 15 °C. The reaction was terminated by addition of 0.1 ml of 6 M-NaOH. The digested samples were then dialysed at 4 °C against 0.1 M-ammonium bicarbonate and freeze-dried.

Gel electrophoresis and fluorography

Labelled proteins were examined by SDS/polyacrylamide-slab-gel electrophoresis in SDS/8%-(w/v)-polyacrylamide gels under reducing and non-reducing conditions as described previously (Yoshimura *et al.*, 1981). Samples were electrophoresed for 16 h at a constant voltage of 40 V. In certain experiments, samples were electrophoresed in SDS/4–10%-polyacrylamide gels in a Tris/borate buffer (Benya, 1981; Remington *et al.*, 1983). To determine the identity of the Type X collagen obtained before and after proteolytic digestion, two-dimensional peptide mapping after CNBr cleavage was performed exactly as described previously (Remington *et al.*, 1983). After electrophoresis the gels were processed for fluorography with EN³HANCE (New England

Nuclear) and exposed to X-Omat AR film (Eastman Kodak).

Quantitative analysis of the relative proportions of various collagen types synthesized in culture

To determine the relative proportions of the various collagen types synthesized during the organ cultures, representative fluorographs of the extracts were scanned in a linear-drive densitometer at 540 nm and the areas under each collagen peak were quantified employing a planimeter. In the case of the 1.0 M-NaCl and acetic acid extracts, the samples were first submitted to limited proteolytic digestion with pepsin to remove non-collagenous proteins and the non-collagenous regions of the various collagens as well as to cleave the large molecular species of Type IX collagen. All the fluorographs examined were from samples electrophoresed under reducing conditions to allow dissociation of the disulphide-bonded Type IX-collagen polypeptides.

To compare the biosynthetic commitment towards production of Types II and X collagens between the chondrocytes from the presumptive-calcification region and those from the permanent hyaline regions on a per-cell basis, parallel experiments were performed to determine the number of chondrocytes present in each of the separate regions. For this purpose, sterna obtained exactly as described for the biosynthetic experiments were divided into the cephalic and caudal regions, and the separate regions were digested in 10 ml of MEM containing 2.5% trypsin (Sigma) and clostridial collagenase (1 mg/ml; Worthington, CLSPA; sp. activity 402 units/mg) for 90 min at 37 °C. This treatment resulted in complete dissociation of the cells from their matrix. The isolated chondrocytes were collected by centrifugation at 1500 *g* for 15 min, resuspended in MEM and stained with Trypan Blue and the cell yields determined by using a haemocytometer. In two separate experiments it was found that the number of cells per sternum obtained from the permanent-hyaline-cartilage region was substantially greater than that obtained from the zone of presumptive calcification (2614.4×10^3 and 2174.25×10^2 as against 990.3×10^3 and 872.0×10^3 respectively).

Extraction of Type X collagen from intact embryonic- and adult-chick sterna

To further demonstrate that Type X collagen is a structural component of chick sternum matrix, 50 sterna from 17-day-old chick embryos and 25 sterna from adult chicks were removed and cleaned, and the anatomical regions corresponding to the permanent-hyaline-cartilage and presumptive-calcification regions were separated exactly as described above. After washing, the tissues were homogenized separately and the collagens were extracted and fractionally precipitated by procedures identical with those outlined above. The extracted collagens and the various fractions were examined by SDS/polyacrylamide-slab-gel electrophoresis and the gels were stained with Coomassie Blue dye.

Amino acid analysis of purified type X collagen from adult-chick sterna

Type X collagen extracted from the calcification region of adult-chick sterna as described above was purified by repeated fractional NaCl precipitation under acidic conditions. The highly purified samples were hydrolysed under N₂ and their amino acid composition was determined in a Beckman amino acid analyser.

RESULTS

Extraction of newly synthesized collagen

Table 1 shows the relative effectiveness of the extraction procedure. Initial homogenization and extraction of whole sterna with 1.0 M-NaCl solubilized less than 15% of the newly synthesized collagen. Subsequent extractions with 0.5 M-acetic acid and repeated limited pepsin digestion were less effective, and more than 75% of the newly synthesized collagen remained insoluble. The subsequent treatment of the pepsin-resistant collagens with a neutral NaCl buffer containing DTT under non-denaturing conditions (Jimenez & Bashey, 1978) resulted in its complete solubilization. The patterns of collagen solubilization from the two separated zones were very similar to the extraction pattern from whole sterna.

Table 1. Extractability of newly synthesized collagen from whole sternum and from the separated permanent hyaline and presumptive-calcification regions

Whole sterna and cartilage from the separated permanent hyaline (caudal) and presumptive-calcification (cephalic) sternal regions from twenty 17-day-old chick embryos were separately minced and labelled with [¹⁴C]proline as described in the Experimental section. After the tissues had been labelled, they were homogenized in 1.0 M-NaCl/15 mM-Tris/HCl buffer, pH 7.4 containing a mixture of proteinase inhibitors and sequentially extracted as previously described (Jimenez & Bashey, 1978). Aliquots of the extracts were hydrolysed after extensive dialysis and their [¹⁴C]hydroxyproline contents assayed by the method of Juva & Prockop (1966).

Extractant	Whole sternum		Permanent hyaline cartilage		Presumptive-calcification cartilage	
	$10^{-3} \times [^{14}\text{C}]\text{Hydroxyproline (d.p.m.)}$	% of total	$10^{-3} \times [^{14}\text{C}]\text{Hydroxyproline (d.p.m.)}$	% of total	$10^{-3} \times [^{14}\text{C}]\text{Hydroxyproline (d.p.m.)}$	% of total
1.0 M-NaCl	827.0	13.6	357.7	10.7	442.3	11.8
0.5 M-Acetic acid	177.3	2.9	134.9	4.1	29.2	0.8
Pepsin (1 mg/ml)	313.5	5.2	315.0	9.5	258.2	6.9
First DTT	3871.5	63.8	2040.8	61.2	2332.4	62.4
Second DTT	753.0	12.4	306.9	9.2	535.2	14.3
Pepsin after DTT	122.2	2.0	179.6	5.4	142.5	3.8
Total extracted	6004.5		3334.9		3684.5	

However, a slightly greater proportion of the newly synthesized collagen was solubilized from the zone of presumptive calcification with the use of reducing agent (82% as against 76%).

Gel electrophoresis

The labelled proteins present in each of the extracts from whole sternum and from the separate anatomical zones were examined by SDS/polyacrylamide-gel electrophoresis before and after limited pepsin digestion. The collagenous bands migrating into the gels were identified from comparison of their relative migration to those of standard collagen chains and from their susceptibility to digestion with purified bacterial collagenase. Furthermore, in certain experiments, [^{14}C]hydroxyproline assays were performed on the separated bands after they were cut out from the gels and hydrolysed as described previously (Remington *et al.*, 1983).

Electrophoretic and autoradiographic examination of the 1.0 M-NaCl extracts from whole sternum and from the two separate regions under reducing conditions is shown in Fig. 1. The pattern of labelled proteins present in the samples from whole sternum (lane 1) showed a prominent band migrating in the position corresponding to $\alpha 1(\text{II})$ chains and several additional bands migrating above it. From previous studies, including two-dimensional CNBr-peptide analysis, it was concluded that three of these bands correspond to the pre-pepsin forms of 1α , 2α and 3α chains (Remington, M. C., Bashey, R. I., Brighton, C. T. & Jimenez, S. A. unpublished work). In addition, one major (arrow) and several minor bands were found migrating below the $\alpha 1(\text{II})$ chains. The major band migrated in a position corresponding to approx. 60000 M_r , as estimated from comparison with the mobility of Type II collagen CNBr-peptide standards. Comparison of the electrophoretic patterns of labelled molecules present in the 1.0 M-NaCl extracts from the presumptive-calcification and permanent hyaline regions (compare lanes 2 and 3) showed one remarkable difference between these populations. In the extracts from the presumptive-calcification zone the 60000- M_r band was very prominent and represented approx. 18% of the total radioactivity migrating into the gel and about one-third of the radioactivity migrating in the $\alpha 1(\text{II})$ position. By contrast, this band was entirely absent in the extracts from the permanent hyaline cartilage, even when overloaded samples were examined. To further clarify the nature of the molecules migrating in the 60000- M_r position, 1.0 M-NaCl extracts from both regions were electrophoresed in 4–10%-(w/v)-polyacrylamide gradient gels under reducing and non-reducing conditions and before and after enzymic digestion with pepsin and collagenase (Figs. 2a and 2b). The 60000- M_r band present in the extracts from the presumptive-calcification region (arrow in Fig. 2a) did not change its electrophoretic mobility when examined under reducing and non-reducing conditions (compare lanes 1 and 2 in Fig. 2a), indicating that it contained non-disulphide-bonded molecules. After limited pepsin digestion a more rapid migration of the 60000- M_r band into a 45000- M_r position (arrowhead) was observed (compare lanes 1 and 3 in Fig. 2a), indicating the proteolytic removal of non-collagenous domains. As expected, the resulting band did not change its electrophoretic mobility when the analysis was carried out under reducing or non-reducing conditions (compare lanes 3 and 4 in Fig. 2a). These findings are consistent with

Fig. 1. SDS/polyacrylamide-gel electrophoresis fluorograph of 1.0 M-NaCl extracts from whole sternum and from the regions of presumptive-calcification and permanent hyaline cartilage

Samples of 1.0 M-NaCl extracts were denatured in the presence or in the absence of 1% (v/v) 2-mercaptoethanol and 1% SDS as described in the Experimental section and were electrophoresed in 8%-polyacrylamide gels as described previously (Yoshimura *et al.*, 1981). 1, Whole sternum extract; 2, presumptive-calcification-cartilage extract; 3, permanent-hyaline-cartilage extract.

the previously described electrophoretic behaviour of chick Type X-collagen chains before and after pepsin treatment (Schmid & Conrad, 1982a; Gibson *et al.*, 1982; Kielty *et al.*, 1984). The collagenous nature of the labelled molecules migrating in this position was confirmed by their disappearance after collagenase digestion (lane 5 in Fig. 2a). Similar analysis of the 1.0 M-NaCl extracts from the permanent-hyaline region (Fig. 2b) failed to show any radioactive bands migrating in the 60000- M_r region before pepsin digestion (lanes 1 and 2) or in the 45000- M_r region after pepsin digestion under reducing or non-reducing conditions (lanes 3 and 4). Additional bands were observed migrating below $\alpha 1(\text{II})$ -chains in the pepsin-digested 1.0 M-NaCl extracts from both regions, but were more prominent in the samples from the presumptive-calcification region. These bands displayed electrophoretic mobilities similar to those of Type IX collagen after pepsin digestion and disulphide-bond reduction as described previously (Reese *et al.*, 1982; Gibson *et al.*, 1983; Yasui *et al.*, 1984).

Fig. 2. SDS/polyacrylamide-gel-electrophoresis fluorograph of pepsin-digested 1.0 M-NaCl extracts from presumptive-calcification cartilage (a) and permanent hyaline cartilage (b)

Samples of intact or pepsin-digested 1.0 M-NaCl extracts were denatured and electrophoresed under reducing and non-reducing conditions as described in Fig. 1 and were electrophoresed in 4–10% polyacrylamide exponential-gradient gels as described previously (Benya, 1981; Benya & Shaffer, 1982; Remington *et al.*, 1983). 1, Electrophoresis without reduction of intact extract before pepsin digestion; 2, as lane 1, after reduction; 3, electrophoresis of pepsin-digested extract without reduction; 4, as lane 3, after reduction; 5, electrophoresis of collagenase-digested extract after reduction.

The electrophoretic analysis of the 0.5 M-acetic acid-extracted samples displayed a similar pattern to that of the 1.0 M-NaCl extracts, except that only negligible amounts of radioactivity migrated below the $\alpha_1(II)$ chains (results not shown). No significant differences were noted between the samples from whole sternum, presumptive-calcification region or permanent hyaline cartilage, although in some experiments small amounts of Type X collagen chains ($60\,000\ M_r$) were seen in the extracts from whole sternum and from the presumptive-calcification cartilage (results not shown).

The electrophoretic patterns of the labelled proteins present in the pepsin extracts from whole sternum and from the separated zones are shown in Fig. 3. These extracts contained mainly $\alpha_1(II)_p$ chains, with a small proportion of pepsin-cleaved 1α and 2α chains ($1\alpha_p$ - and $2\alpha_p$ -), migrating above the $\alpha_1(II)_p$ chains. The pepsin-cleaved 3α chain ($3\alpha_p$) presumably co-migrated with the $\alpha_1(II)_p$ chains. Several radioactive bands migrating below the $\alpha_1(II)_p$ chains were observed when the pepsin extracts were examined under reducing conditions. Although in some experiments three or four bands were seen quite distinctly (M_r 84000, 69000, 54000 and 35000), in most cases only one prominent 69000- M_r band was demonstrated (arrow). These bands are similar to those observed after pepsin digestion of the 1.0 M-NaCl extracts described above and they correspond to Type IX-collagen

polypeptides electrophoresed after pepsin digestion and subsequent reduction (Reese *et al.*, 1982; Gibson *et al.*, 1983; Yasui *et al.*, 1984). No significant differences were detected between extracts from the two sternal regions (compare lanes 2 and 3). No radioactivity migrating in the 45000- M_r position of pepsin-treated Type X-collagen chains was detected in any of these extracts.

Electrophoretic analysis of the samples obtained after extraction of the pepsin-digested tissues with DTT is shown in Fig. 4. In contrast with the pepsin extracts described above, the DTT extracts from whole sterna contained a large amount of radioactivity migrating in the position of pepsin-cleaved Type X collagen (lane 1, arrow). A comparison between the extracts from the presumptive-calcification and permanent-hyaline-cartilage zones revealed striking differences (compare lanes 2 and 3), since only the samples from the presumptive-calcification zone displayed a very intense band migrating in the 45000- M_r position expected for pepsin-digested Type X-collagen chains. These chains were not detected in the extracts from the permanent-hyaline-cartilage zone, even when overloaded gels were examined. The collagenous nature of these molecules was demonstrated by their susceptibility to collagenase digestion (results not shown) and by determination of their [^{14}C]hydroxyproline content after excision of the band from the gel and subsequent hydrolysis. The degree

Fig. 3. SDS/polyacrylamide-gel-electrophoresis fluorograph of pepsin extracts from whole sternum and from the regions of presumptive-calcification and permanent hyaline cartilage

Samples of pepsin extracts were concentrated by freeze-drying and electrophoresed as described in Fig. 1. 1, Whole sternum extract; 2, presumptive-calcification-cartilage extract; 3, permanent-hyaline-cartilage extract. The subscript p denotes the chains resulting after limited proteolytic digestion with pepsin. The M_r markers were obtained from comparison with the electrophoretic migration of Types I and II collagen CNBr-peptide standards.

Fig. 4. SDS/polyacrylamide-gel-electrophoresis fluorograph of DTT extracts from whole sternum and from the regions of presumptive calcification and permanent hyaline cartilage

Samples of DTT extracts were electrophoresed as described in Fig. 1. 1, Whole sternum extract; 2, presumptive-calcification-cartilage extract; 3, permanent-hyaline-cartilage extract.

Table 2. Fractional NaCl precipitation from 0.5 M-acetic acid solutions of DTT extracts from whole sternum, permanent hyaline and presumptive-calcification regions

DTT extracts from whole sterna, permanent hyaline and presumptive-calcification regions obtained as described in the Experimental section were centrifuged at 3000 *g* for 30 min to remove insoluble collagen and then sequentially precipitated by dialysis against solutions of 0.5 M-acetic acid containing increasing concentrations of NaCl as described by Shimokomaki *et al.* (1980). Fractions precipitating at 0.8, 1.2 and 2.2 M-NaCl were obtained. The precipitates were collected by centrifugation and dialysed against 0.15 M-NaCl/50 mM-Tris/HCl, pH 7.4, and their radioactivity determined.

Source of DTT extract	10 ⁻³ × Initial extract radioactivity (d.p.m.)	[NaCl] (M) . . .		1.2		2.2	
		0.8		10 ⁻³ × Precipitate radioactivity (d.p.m.)	% of total	10 ⁻³ × Precipitate radioactivity (d.p.m.)	% of total
Whole sternum	1890.7	1268.9	67.1	21.4	1.1	600.4	31.8
Permanent hyaline cartilage	708.6	401.2	56.6	97.0	13.7	210.4	29.7
Presumptive-calcification cartilage	955.4	398.4	41.7	140.2	14.7	417.0	43.7

Fig. 5. SDS/polyacrylamide-gel-electrophoresis fluorograph of fractionally precipitated DTT extracts from presumptive calcification cartilage

Samples of DTT extracts from the presumptive-calcification region were fractionally precipitated from a solution of 0.5 M-acetic acid by dialysis against buffers containing increasing concentrations of NaCl as described by Shimokomaki *et al.* (1980). The resulting fractions were resuspended and dialysed against 0.5 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4 at 4 °C and electrophoresed as described in Fig. 1. 1, Initial extract; 2, 0.8 M-NaCl fraction; 3, 1.2 M-NaCl fraction; 4, 2.2 M-NaCl fraction.

of hydroxylation of proline residues in the molecules migrating in the 45000- M_r position was 52.6%. As expected for collagen molecules extracted after proteinase and DTT treatment of the tissues, there was no change in electrophoretic mobility when the same extracts were electrophoresed after pepsin digestion or with and without reduction (results not shown).

Fractional NaCl precipitation

To characterize the collagens present in the DTT extracts further, aliquots were fractionally precipitated with NaCl under acidic conditions as described by Shimokomaki *et al.* (1980). The results shown in Table 2 demonstrate that substantial amounts of collagen were found in the 2.2 M-NaCl precipitates from the presumptive-calcification region. This fraction contained as much collagen as the 0.8 mM-NaCl precipitates. Electrophoretic analysis and fluorography of the fractions obtained from the presumptive-calcification zone extract are shown in Fig. 5. As expected, the 0.8 M-NaCl precipitates contained mainly $\alpha_1(\text{II})_p$ chains and the 1.2 M-precipitates contained $1\alpha_p$, $2\alpha_p$ and $3\alpha_p$ chains as well as Type IX-collagen chains. The 2.2 M-NaCl precipitates from

Fig. 6. Two-dimensional CNBr-peptide mapping of the 60000- and 45000- M_r forms of Type X collagen

Samples of the 1.0 M-NaCl containing the 60000- M_r species and DTT extracts containing the 45000- M_r species from the presumptive-calcification cartilage shown in Figs 1 and 4 were examined by two-dimensional peptide mapping after CNBr cleavage exactly as described previously (Remington *et al.*, 1983). The direction of electrophoresis is shown by long arrows. The arrowheads show the peptides from the 60000- M_r (60 K) and the small arrows the peptides from the 45000- M_r (45 K) pepsin-digested species. The peptides from Type II collagen are shown with their respective M_r values.

these extracts contained essentially pure pepsin-cleaved Type X-collagen chains.

CNBr-peptide pattern analysis

To demonstrate further that the 60000- and 45000- M_r molecules present in the 1.0 M-NaCl and DTT extracts from the region of presumptive calcification respectively represent the intact and pepsin-cleaved forms of Type X collagen, these samples were analysed by two-dimensional CNBr-peptide mapping involving SDS/polyacrylamide-gradient-gel electrophoresis as described previously (Remington *et al.*, 1983). The CNBr-peptide maps shown in Fig. 6 demonstrate that the 60000- M_r species yielded four main peptides (arrowheads) with apparent M_r values of 24000, 13000, 10000 and 6000–8000 in decreasing order, whereas the 45000- M_r species yielded three main peptides (arrows) all migrating below the 10500- M_r Type II-collagen CNBr-peptide marker. Both forms of collagen yielded two peptides with identical electrophoretic mobility (lower arrowheads and upper and lower arrows). The 24000- and 13000- M_r peptides (upper arrowheads) are unique for the 60000- M_r molecules, whereas a low- M_r peptide (middle arrow) was exclusively found after CNBr cleavage of the 45000- M_r molecules. These results conclusively demonstrate that the 60000- and 45000- M_r molecules represent the intact and the pepsin-cleaved forms of Type X collagen and suggest that the 24000- and 13000- M_r peptides derived from the 60000- M_r molecules contain the non-collagenous extensions of the intact Type X collagen.

Table 3. Relative proportions of ^{14}C radioactivity in each of various collagen chains in extracts from permanent hyaline and presumptive-calcification regions

Aliquots of extracts from permanent hyaline and presumptive-calcification cartilage were electrophoresed in SDS/8% polyacrylamide slab gels after pepsin digestion and disulphide-bond reduction as described in the Experimental section. After fluorography the gels were scanned at 540 nm in a linear-drive densitometer and the areas under the peaks corresponding to each of the collagen chains were integrated with a planimeter. The values shown are averages of values obtained with two separate preparations of extracts and were calculated by multiplying the relative integrated peak area of each collagen chain by the total ^{14}C in each extract and are expressed in c.p.m. of [^{14}C]proline. Values in parentheses are the percentages of ^{14}C radioactivity present in each collagen from the total ^{14}C proline in each extract shown. The sum values in certain extracts are less than 100% because minor peaks were not included in the Table.

Extractant . . .	[^{14}C]Proline (c.p.m.)									
	Permanent-hyaline-cartilage extracts					Presumptive-calcification-cartilage extracts				
	NaCl	Acetic acid	Pepsin	DTT/pepsin	All extracts	NaCl	Acetic acid	Pepsin	DTT/pepsin	All extracts
Total ^{14}C in extract . . .	201.4	72.2	172.9	569.2	1015.6	151.9	15.3	110.4	490.5	768.2
^{14}C in:										
$1\alpha_p + 2\alpha_p$	65.5 (32.5)	8.7 (12.3)	13.2 (7.7)	32.1 (5.7)	119.4 (11.8)	37.5 (24.7)	1.5 (10.2)	13.7 (12.4)	15.7 (3.2)	68.4 (8.9)
$3\alpha_p + \alpha_1(\text{II})_p$	131.1 (65.1)	60.6 (83.6)	110.7 (64.7)	415.9 (73.9)	718.5 (70.4)	71.6 (47.1)	13.1 (84.8)	80.0 (72.5)	181.0 (36.9)	345.6 (44.9)
Type IX	4.8 (2.4)	2.9 (4.1)	48.9 (28.6)	92.2 (16.2)	148.8 (14.6)	16.1 (10.6)	0.4 (2.5)	16.7 (15.1)	51.0 (10.4)	84.2 (10.9)
Type X	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	26.7 (17.6)	0.4 (2.5)	0 (0)	234.1 (47.7)	262.1 (34.2)

Quantitative analysis of the relative proportion of type X-collagen synthesis

In order to compare the relative proportion of the various collagens synthesized during the organ culture, the fluorographs from each of the extracts were examined by densitometric analysis and the areas under each collagen type were quantified. The 1.0 M-NaCl and 0.5 M-acetic acid extracts were first submitted to limited proteolytic digestion with pepsin to remove non-collagenous proteins and to cleave the non-helical regions of the various collagens. All samples analysed were from electrophoretograms obtained under reducing conditions to allow dissociation of disulphide-bonded chains. The results indicated that approx. 18% of the newly synthesized collagen from whole sternum was composed of type X-collagen chains (results not shown). This proportion increased to about 35% in the zone of presumptive calcification. In contrast, the permanent-hyaline-cartilage region did not display any detectable biosynthesis of Type X collagen (Table 3). The results also show that 1α , 2α and Type IX chains together represented a small proportion of the total collagen synthesized (19.8%) of the biosynthesized collagen. This proportion was slightly greater in the extracts from the permanent hyaline cartilage (26.2%).

Subsequently, the rates of protein and collagen biosynthesis based on the absolute number of cells obtained from the separate regions after their dissociation from the matrix were compared. These experiments demonstrated that the chondrocytes from the presumptive-calcification zone were substantially more active than those from the permanent-hyaline-cartilage region, since they incorporated approx. 2.5-fold more [^{14}C]proline and synthesized almost three times as much collagen (Table 4). The synthetic rates of all collagens were higher in

cartilage from the presumptive-calcification region. Furthermore, when the rates of synthesis of Type II and Type X collagens were calculated on a per-cell basis, it was found that the chondrocytes from the presumptive-calcification zone synthesized about 33% more Type X collagen than the amount of Type II collagen synthesized by the chondrocytes from the permanent-hyaline-cartilage zone (248.7 as against 187.4 d.p.m./24 h per 10^3 cells).

Extraction of Type X collagen from embryonic- and adult-chick sterna

To demonstrate that Type X collagen is a structural component of embryonic- and adult-chick embryo sternum matrix, the separated regions from embryonic- and adult-chick sterna corresponding to zones identical with those used for the biosynthetic experiments were sequentially extracted and the extracted collagens were fractionally precipitated and identified as described in the Experimental section. The results shown in Fig. 7 demonstrate that quantitative amounts of Type X collagen could be extracted from both embryonic- and adult-chick sterna. Furthermore, the strict topographic distribution of Type X collagen, which was exclusively confined to the region of presumptive calcification (in embryonic sternum) or calcification (in adult sternum), was further documented. The extractability of Type X collagen from the tissues and its fractional-NaCl-precipitation behaviour were similar to those found in the biosynthetic studies, since only a minor amount was solubilized with pepsin, but its complete extraction was accomplished with the use of the neutral-salt/DTT buffer (results not shown). The extracted Type X collagen precipitated at 2.2 M-NaCl from a solution of 0.5 M-acetic acid and had an electrophoretic mobility identical with

Table 4. Comparison of the rates of [¹⁴C]proline incorporation, hydroxy[¹⁴C]proline synthesis and production of various collagens on a per-cell basis between permanent hyaline and presumptive-calcification cartilage

The total amounts of [¹⁴C]proline incorporated and [¹⁴C]hydroxyproline synthesized by organ cultures from the separate regions during a 24 h labelling period were corrected for the number of cells obtained by enzymic dissociation of identical tissue specimens in parallel experiments as described in the Experimental section. The amount of hydroxy[¹⁴C]proline in each collagen was calculated from densitometric analysis of fluorographs from each extract as described in Table 3, and in selected cases was corroborated by direct determination of [¹⁴C]hydroxyproline after excision of the corresponding bands from the gels and subsequent hydrolysis.

	Incorporation, synthesis or production * in:	
	Permanent hyaline cartilage	Presumptive-calcification cartilage
Total [¹⁴ C]	784.4	1728.5
Total [¹⁴ C]hydroxyproline	265.1	727.1
[¹⁴ C]Hydroxyproline in:		
1 α , 2 α collagen	31.3	64.7
Type II collagen†	187.4	326.5
Type IX collagen	38.7	79.3
Type X collagen	0	248.7
Degree of hydroxylation (%)† . . .	33.8	42.1

* Each value is an average for two separate experiments and represents d.p.m./24 h per 10³ cells.

† Degree of hydroxylation = $\frac{[^{14}\text{C}]\text{hydroxyproline}}{\text{total } ^{14}\text{C}} \times 100$

‡ Includes collagen 3 α -chains.

Fig. 7. SDS/polyacrylamide-gel electrophoresis of 2.2 M-NaCl fractions from DTT extracts from the presumptive-calcification and permanent hyaline cartilage from 17-day-old chick-embryo sterna and from the calcified and cartilagenous regions of adult-chick sterna

Embryonic and adult chick sterna were extracted as described in the Experimental section and the DTT extracts were fractionally precipitated from a 0.5 M-acetic acid solution. The 2.2 M-NaCl fractions were electrophoresed after dialysis against 0.15 M-NaCl/50 mM-Tris/HCl buffer, pH 7.4, at 4 °C under reducing conditions. After electrophoresis the gel was stained with Coomassie Blue dye. 1, Chick-embryo sternum collagen standards; 2, fraction from 17-day-old chick-embryo presumptive-calcification cartilage; 3, fraction from 17-day-old chick-embryo permanent hyaline cartilage; 4, fraction from adult-chick calcified sterna; 5, fraction from adult-chick non-calcified sternal cartilage.

that of pepsin-cleaved newly synthesized Type X-collagen molecules.

Amino acid analysis of purified Type X collagen from adult-chick sterna

A highly purified preparation from a DTT extract of the calcification cartilage from adult chick sterna was utilized for amino acid analysis. The results shown in Table 5 indicate that the amino acid composition of adult-chick sternum Type X collagen is very similar to that of Type X collagen from cultured chick chondrocytes and from embryonic-chick tibiotarsus reported previously (Schmid & Linsenmayer, 1983; Kielty *et al.*, 1984). Our results confirmed the absence of cysteine and the high proportion of methionine residues characteristic of Type X collagen.

DISCUSSION

Although previous studies have examined the biosynthesis of minor collagens by embryonic-chick sternum and the synthesis of 1 α , 2 α , 3 α and Type IX collagens has been conclusively demonstrated, the results regarding the production of Type X collagen by this tissue have been controversial. Except for recent reports describing the biosynthesis of Type X collagen in embryonic-chick sternum organ cultures (Gibson *et al.*, 1984) and its detection employing immunohistological methods (Schmid & Linsenmayer, 1985), most studies have failed to demonstrate its presence or biosynthesis in this tissue (Gibson *et al.*, 1982; Reese *et al.*, 1982; von der Mark *et al.*, 1984; Kielty *et al.*, 1984; Yasui *et al.*, 1984; Kielty *et al.*, 1985). The results presented here clearly demonstrate that the embryonic chick sternum devotes a large proportion of its biosynthetic commitment towards the synthesis and production of Type X collagen. The Type X collagen synthesized by the embryonic-chick sternum

Table 5. Amino acid composition of purified Type X collagen from adult chick sterna

Amino acid analysis of a highly purified preparation of Type X collagen from a DTT extract of the calcification region of adult chick sterna was performed after hydrolysis as described in the Experimental section. Further abbreviations: 4-Hyp, 4-hydroxyproline; Hyl, hydroxylysine.

Amino acid	Composition (residues/1000 residues)*		
	Chick sterna	Chick tibiotarsus†	Cultured chondrocytes‡
4-Hyp	112	101	112
Asp	33	41	35
Thr	22	18	11
Ser	23	30	16
Glu	89	93	85
Pro	107	113	136
Gly	310	312	316
Ala	56	65	53
$\frac{1}{2}$ -Cys	0	0	0
Val	21	20	25
Met	17	14	23
Ile	27	22	28
Leu	57	56	56
Tyr	5	2	3
Phe	11	13	11
Hyl	35	31	35
Lys	21	26	20
His	16	7	7
Arg	38	37	28

* Average for two separate analyses.

† From data published by Kielty *et al.* (1984).

‡ From data published by Schmid & Linsenmayer (1983).

organ cultures was shown to be similar to Type X collagen from chick-embryo tibiotarsus (Schmid & Conrad, 1982*a,b*; Kielty *et al.*, 1985) and from cultures of chick chondrocytes grown in three-dimensional gels (Gibson *et al.*, 1982). In agreement with the latter authors, we found that tissue extracts obtained without proteinase digestion contain intact Type X-collagen chains that migrate electrophoretically with an apparent M_r of 59000–60000. These chains do not contain disulphide-linked subunits, since their electrophoretic mobilities do not change after reduction. Limited proteolytic digestion of the 59000–60000- M_r species resulted in cleavage of non-helical regions, yielding 45000- M_r proteinase-resistant domains.

The evidence obtained from the sequential-extraction studies indicates the existence of two separate Type X collagen populations. The minor one, which is composed of highly soluble molecules, represents approx. 20% of the total Type X collagen biosynthesized during the culture period. The major population is composed of molecules that are normally present in the matrix in the form of a pepsin-resistant molecular aggregate. Confirmation that the molecules in these two pools represent the soluble and the pepsin-resistant forms of Type X collagen was obtained from two-dimensional CNBr-peptide mapping, which showed at least two peptides with identical electrophoretic mobilities in both populations. The existence of these two distinct populations of Type X collagen molecules suggests that the soluble pool may contain precursor molecules that eventually become

deposited and stabilized in the matrix through the formation of pepsin-resistant bonds or as part of a molecular aggregate that renders these molecules inaccessible to pepsin. Elucidation of these possibilities, however, will require pulse-chase studies to demonstrate the precursor-product relationship between these two pools.

The resistance of Type X collagen to pepsin extraction and the complete solubilization of the pepsin-resistant molecules with a buffer containing a reducing agent suggest that these molecules are stabilized in the tissue by disulphide bonds. These findings, however, do not accord with the absence of cysteine residues in Type X collagen reported previously and confirmed by our own results. To explain this discrepancy, it can be postulated that the Type X collagen molecules in the matrix are surrounded by a disulphide bonded non-collagenous protein that prevents the escape of the Type X-collagen molecules into the extractant. Reduction of the disulphide bonds or treatment with a high-ionic-strength buffer will result in the dissociation of the non-collagenous protein and the consequent release of the previously cleaved Type X collagen.

When the biosynthetic products of the presumptive-calcification and the permanent hyaline cartilage were compared, a striking difference between these populations was found, in that only the presumptive-calcification cartilage displayed synthesis of Type X collagen. This remarkable topographic distribution in the expression of Type X collagen synthesis raises some questions regarding the interpretation of the previous findings of Type X collagen synthesis by cultures of chick sternal chondrocytes in three-dimensional gels (Gibson *et al.*, 1982). These studies suggested that the particular culture environment was responsible for a change in the phenotypic expression of chondrocyte collagen genes, resulting in a shift from the biosynthesis of Type II to that of Type X collagen. Our results indicate that embryonic-chick sternum cartilage contains a population of chondrocytic cells whose biosynthetic commitment is primarily directed towards the synthesis of Type X collagen. It is therefore possible that a preferential expansion of this chondrocyte subpopulation during long-term cultures in the studies of Gibson *et al.* (1982) rather than a change in chondrocyte phenotypic expression may have been responsible for the observed increase in Type X-collagen synthesis.

The quantitative analysis of the relative proportions of the various collagens synthesized by organ cultures from the two separate sternum zones demonstrated that, on a per-cell basis, the chondrocytes from the presumptive-calcification region displayed a remarkably greater rate of biosynthetic activity than did the cells from the permanent-hyaline-cartilage region. Chondrocytes from the presumptive-calcification region incorporated greater than 2-fold more [14 C]proline and synthesized about 3-fold more collagen than did the chondrocytes from the permanent-hyaline-cartilage region. Furthermore, the amount of Type X collagen produced by these cells on a per-cell basis was 33% greater than the amount of Type II collagen produced by the chondrocytes from the permanent-hyaline-cartilage region.

The demonstration of quantitative amounts of Type X collagen in extracts from embryonic- or adult-chick sternum clearly indicate that this collagen is an integral component of the tissue matrix. These studies also

confirm its strict anatomical distribution to the zone of calcification. Although Kielty *et al.* (1984, 1985) performed similar studies, they were not able to demonstrate the presence of Type X collagen in 17-day-old-chick sterna. The remarkable resistance of Type X collagen to solubilization even after repeated proteolytic digestion might explain this discrepancy, since these authors employed only pepsin digestion to extract the tissues. The strict topographical distribution of Type X collagen synthesis to the region of presumptive calcification, coupled to the remarkable metabolic activity of the cells from this region, suggests that the chondrocytes from the presumptive-calcification region may be intricately involved in the process of endochondral bone formation. Further understanding of the role of Type X collagen, a major biosynthetic product of these cells, may help with the understanding of the intimate mechanisms responsible for this important physiological process.

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REFERENCES

- Ayad, S., Abedin, M. A., Grundy, S. M. & Weiss, J. (1981) *FEBS Lett.* **123**, 195–199
- Benya, P. D. (1981) *Collagen Relat. Res.* **1**, 17–30
- Benya, P. D. & Shaffer, J. D. (1982) *Cell* **30**, 215–224
- Bornstein, P. & Traub, W. (1979) in *The Proteins* (Neurath, H. & Hill, R. L., eds.), vol. 4, pp. 411–632, Academic Press, New York
- Bruckner, P., Mayne, R. & Tuberman, L. (1983) *Eur. J. Biochem.* **136**, 333–339
- Burgeson, R. E. & Hollister, D. W. (1979) *Biochem. Biophys. Res. Commun.* **87**, 1124–1131
- Burgeson, R. E., Heboa, P. A., Morris, N. P. & Hollister, D. W. (1982) *J. Biol. Chem.* **257**, 7852–7856
- Capasso, O., Tajana, G. & Cancedda, R. (1984) *Mol. Cell Biol.* **4**, 1163–1168
- Duance, V. C., Wotton, S. F., Voyle, C. A. & Bailey, A. J. (1984) *Biochem. J.* **221**, 885–889
- Gibson, G. J., Schor, S. L. & Grant, M. E. (1982) *J. Cell. Biol.* **93**, 767–774
- Gibson, G. J., Kielty, C. M., Garner, C., Schor, S. L. & Grant, M. E. (1983) *Biochem. J.* **211**, 417–426
- Gibson, G. J., Schor, S. L. & Grant, M. E. (1984) *J. Cell. Biol.* **99**, 208–216
- Jimenez, S. A. & Bashey, R. I. (1978) *Biochem. J.* **173**, 337–340
- Juva, K. & Prockop, D. J. (1966) *Anal. Biochem.* **15**, 77–83
- Kielty, C. M., Hulmes, D. J. S., Schor, S. L. & Grant, M. E. (1984) *FEBS Lett.* **169**, 179–184
- Kielty, C. M., Kwan, A. P., Hulmes, D. F., Schor, S. L. & Grant, M. E. (1985) *Biochem. J.* **227**, 545–554
- Miller, E. J. (1971) *Biochemistry* **10**, 3905–3911
- Ninomiya, Y. & Olsen, B. R. (1984) *Proc. Natl. Acad. Sci. U.S.A.* **81**, 3014–3018
- Reese, C. A. & Mayne, R. (1981) *Biochemistry* **20**, 3905–3911
- Reese, C. A., Wiedemann, H., Kuhn, K. & Mayne, R. (1982) *Biochemistry* **21**, 826–830
- Remington, M. C., Bashey, R. I., Brighton, C. T. & Jimenez, S. A. (1983) *Collagen Relat. Res.* **3**, 271–278
- Remington, M. C., Bashey, R. I., Brighton, C. T. & Jimenez, S. A. (1984) *Biochem. J.* **224**, 227–233
- Ricard-Blum, S., Hartmann, D. J., Herbage, D., Payen-Weran, C. & Ville, G. (1982) *FEBS Lett.* **146**, 343–347
- Schmid, T. M. & Conrad, H. E. (1982a) *J. Biol. Chem.* **257**, 12444–12450
- Schmid, T. M. & Conrad, H. E. (1982b) *J. Biol. Chem.* **257**, 12451–12457
- Schmid, T. M. & Linsenmayer, T. F. (1983) *J. Biol. Chem.* **258**, 9504–9509
- Schmid, T. M. & Linsenmayer, T. F. (1985) *Dev. Biol.* **107**, 373–381
- Shimokomaki, M., Duance, V. C. & Bailey, A. J. (1980) *FEBS Lett.* **121**, 51–54
- Sussman, M. D., Ogle, R. C. & Balian, G. (1984) *J. Orthop. Res.* **21**, 134–142
- van der Rest, M., Mayne, R., Ninomiya, Y., Seidah, N. E., Chretien, M. & Olsen, B. R. (1985) *J. Biol. Chemistry* **260**, 220–225
- von der Mark, K., van Menxel, M. & Wiedemann, H. (1982) *Eur. J. Biochem.* **138**, 629–633
- Yasui, N., Benya, P. D. & Nimni, M. E. (1984) *J. Biol. Chem.* **259**, 14175–14179
- Yoshimura, M., Jimenez, S. A. & Kaji, A. (1981) *J. Biol. Chem.* **256**, 9111–9117

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