

Calcification inhibitors in human ligamentum flavum

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Abstract

To examine the presence of substances which inhibit calcification in human ligamentum flavum, the calcification inhibitory effect of human ligamentum flavum extract obtained by extraction of the tissue with 3% Na_2HPO_4 was determined by the in vitro calcium uptake of the ligamentum flavum matrix. In addition, grafts of extracted and non-extracted dry ligamentum flavum matrices were transplanted into the dorsal muscles of rats, and calcification in the grafts was examined radiologically and histochemically. In order to determine whether or not component cells of human ligamentum flavum produce calcification inhibitors, ligamentum flavum cells were cultured, and the crystal inhibitor activity of the culture medium was measured by the seed test using hydroxyapatite as the nucleus of precipitation. The calcification reaction system demonstrated that the ligamentum flavum extract contains an inhibitory factor for calcium uptake by the ligamentum flavum matrix. The seed test revealed that human ligamentum flavum cells produce calcification inhibitor activity. The inhibitor activity was fractionated into two peaks by gel filtration of the culture medium.

[Key words: Human ligamentum flavum, Calcification, Calcification inhibitors]

Introduction

Numerous substances are known to inhibit the formation and growth of calcium phosphate crystals in vitro (1,3). Among these substances, pyrophosphate, magnesium, citrate and albumin are believed to be involved in the regulation of various processes of mineralization in vivo (3). In addition to these substances, it has been demonstrated that substances having in vitro crystal inhibitor activity are present in plasma ultrafiltrate (8,10,17), urine (2,8), human saliva (7), bone (14,16), cartilage (4), fetal mice skin (9), and connective tissues such as bovine and human Achilles tendons (15).

In 1967, Thomas and Tomita (19) found that extraction of bovine tendon with 3% Na_2HPO_4 converts that tissue to a matrix capable of inducing calcification. Quittner and Wadkins (15) reported in 1978 that the inhibitory substance in the extract has a molecular weight of 85,000-100,000. Various factors regulating calcification are present in hard tissues. These factors include calcification inhibitors such as osteonectin (16), osteocalcin (14), proteoglycans (4), and phosphoprotein (18). In 1988, Ohya et al (13) obtained two fractions, a high molecular weight fraction and a low molecular weight fraction, which have in vitro calcification inhibitor activity from the medium of rat calvaria cell culture.

In the present study, calcification inhibitors in the human ligamentum flavum were extracted and their inhibitor activity was

assessed, on the basis of the fact that the clinical manifestation of spinal stenosis can be caused by calcification and ossification of the ligamentum flavum. The production of calcification inhibitors by component cells of the ligamentum flavum was also examined.

Materials and Methods

Materials

Lumbar ligamentum flavum, obtained during surgery from 63 patients ranging in age from 18 to 80 years (mean 54 ± 18 years), were used in the present study. The patient group was comprised of 25 individuals with lumbar disc herniation, 36 with degenerative lumbar spinal stenosis and 2 with cauda equina tumor. The ligamentum flavum obtained during surgery was stripped of adventitious tissue as thoroughly as possible, rinsed with cold distilled water, and immediately frozen at -80°C . The total weight of the ligamentum flavum obtained was 121 g.

Extraction of calcification inhibitors

According to the methods of Thomas and Tomita (19) and Quittner and Wadkins (15), the ligamentum pieces, prepared after thawing the frozen specimen, were suspended in 20 ml of 3% Na_2HPO_4 per gram of ligament (containing 1.0 mM NaN_3) at pH 9.2, and stirred slowly and continuously for 6 days at 4°C . The tissue was then removed, and the phosphate extract was

lyophilized and dissolved in enough distilled water to obtain approximately 25% of its original volume. This solution was dialyzed against distilled water at 3°C for 3 days, using cellulose dialysis tubing (Nacalai Tesque Inc, Kyoto) with an exclusion limit of 8,000 daltons. After dialysis, the solution was lyophilized to dryness, and the dry residue was then suspended in distilled water and dialyzed repeatedly until inorganic phosphate reached a concentration of less than 0.1 mM (p-methylaminophenol reduction method). The solution thus obtained was centrifuged at 100,000 G for 2 h, and the supernatant was lyophilized to dryness to obtain the extract containing the inhibitor.

Tissue extracted with 3% Na₂HPO₄ and non-extracted tissue were rinsed with distilled water for 2 h at 4°C and lyophilized for use as matrices in the reaction system for calcification.

Calcium uptake by the matrix in the reaction system for calcification

The in vitro crystal inhibitor activity of the extract was assayed in the following reaction system. A 100-mg of extracted or non-extracted ligamentum flavum matrix was added to 25 ml of a mixture of 17.5 mM barbital buffer (pH 7.4), 125 mM NaCl, 2.0 mM ⁴⁵CaCl₂ (350,000-400,000 cpm) (370 GBq-2.77 TBq/g calcium, New England Nuclear, Boston, MA) and 1.2 mM potassium phosphate (pH 7.4). The mixture was stirred while incubating at 37°C. 2000 µg Streptomycin and 2000 units of penicillin were added to all

incubations. At 2, 4, 6, 8, 12, 18, 24, 36 and 48 h of incubation, a 1 ml aliquot of the soluble phase was obtained, and the radioactivity was measured with a liquid scintillation counter (LS 3801, Beckman, U.S.A.) to determine the uptake of calcium by the matrix. In the reaction system using extracted matrices, changes in radioactivity in the presence of 16 mg of the ligamentum flavum extract, 1 mM NaF or phosvitin (250 μ g/ml) were also examined.

Histological observation of calcification after transplantation of ligamentum flavum matrices

Grafts of ligamentum flavum lyophilized before and after extraction were transplanted into the bilateral dorsal muscles of 10 SD rats (Japan SLC, Shizuoka). Each graft was trimmed as precisely as possible to the size of 5 x 5 x 3 mm, and sterilized with ethylene oxide gas. The bilateral dorsal muscles were incised symmetrically, and a graft prepared from non-extracted tissue was transplanted on the left side, and extracted graft on the right side. The fascia was not sutured. Two rats each were sacrificed 1, 2, 4, 8 and 12 weeks after transplantation, and the grafts on both sides were extirpated with the dorsal muscle. The specimens were immediately subjected to roentgenography using a Softex CSM-2 X-ray apparatus (Softex Co., Tokyo) with Fuji Softex N100 films at 50 KV, 12 mA, for 4 s. For histological examination, the specimens were fixed in 10% neutral formalin,

embedded in paraffin, and cut into serial sections, 6 μ m in thickness. The sections were then stained using hematoxylin-eosin stain and von Kossa stain.

Crystal inhibitor activity in ligamentum flavum cell culture system

a. Cell culture

The ligamentum flavum obtained during surgery from 23 patients ranging in age from 20 to 78 years (mean: 52 \pm 17) were used. Each specimen was washed with saline immediately after the resection, followed by careful removal of the surface layer of the tissue under magnification. The tissue was digested in 0.1% trypsin (Wako Pure Chemicals, Tokyo) for 20 min, and treated with 0.05% collagenase (Wako Pure Chemicals, Tokyo) for 1 h at 37° C while stirring. After filtration through a #250 nylon mesh (Abe Science, Chiba), the filtrate was centrifuged at 1,000 rpm for 10 min to separate cells. After washing three times with Ham's F12 medium (Flow Laboratories, Inc., McClean, VA), cells were seeded at a cell density of 40,000 cells/25-cm² flask in 5 ml of Ham's F12 medium supplemented with 10% fetal calf serum (Bocknek, Toronto) and incubated at 37° C under a 5% CO₂-95% air atmosphere for 14-21 days until they became confluent. The cell layer was then washed three times with 0.9% (w/v) NaCl solution, and 5 ml of Dulbecco's modified essential medium (DMEM) (Flow Laboratories, Inc.,) supplemented with 0.1% albumin (fatty acid-free). After 24 h of incubation under the same conditions, the

culture medium was collected and subjected to measurement of crystal inhibitor activity. DMEM supplemented with 0.1% albumin was kept for 24 h under the same conditions as those in the above cell culture, and used as the control. Streptomycin 100 μ g/ml and penicillin 100 U/ml were added to each medium.

b. Measurement of crystal inhibitor activity (seed test)

The crystal inhibitor activity was determined according to the seed test described by Rufenacht and Fleisch (17). First, the solubility of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ was determined by the following procedure. To DMEM, 50 mM HEPES (Dojin pharmacochemical Lab., Kumamoto) and 0.1% NaN_3 (Wako Pure Chemicals, Osaka) were added, and the pH was adjusted to 7.4. A 0.5-ml sample of this medium was mixed with 2.5 mg of $\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$ in a 15-ml polystyrene tube (Corning 25311) (Iwaki Glass, Tokyo) and incubated at 37°C for 60 min, using a shaker with a frequency of 300 rpm. The sample was then centrifuged at 7,500 G for 4 min, and the concentrations of Ca^{2+} and inorganic phosphate (Pi) in the supernatant were determined by the ionic electrode method using an ionized calcium measuring apparatus (NOVA 2, Nova Biomedical, U.S.A.) and by p-methylaminophenol reduction, respectively. Solutions of 0.33 M CaCl_2 and 0.25 M Na_2HPO_4 were added to the remaining portions of the above medium to adjust to the Ca^{2+} and Pi concentrations determined. Hydroxyapatite (HAP) (Nippon Chemical, Tokyo) was added to 0.5 ml aliquots of the adjusted medium to prepare a

series of hydroxyapatite suspensions at 11 different concentrations ranging from 0.01 to 3.16 mg/ml. All samples were incubated at 37° C for 60 min while stirring and centrifuged at 7,500 G for 4 min.

The Ca^{2+} concentration of the supernatants was then measured by the ionic electrode method. The obtained values were plotted on the ordinate of a scale that linearizes the Gauss integral (probability paper), whereas the logarithm of the amount of apatite added was plotted on the abscissa. From this plot, the amount of apatite necessary to induce a 50% decrease in the initial Ca^{2+} concentration was obtained, and this value was regarded as the index of the crystal inhibitor activity of the sample.

c. Crystal inhibitor activity of each medium fraction after gel filtration

Ligamentum flavum obtained from a 21-year-old woman with lumbar disc herniation was used. The specimen was subjected to cell culture in Ham's F12 medium supplemented with 10% fetal calf serum, followed by DMEM medium not supplemented with serum or albumin. The DMEM medium cultured with ligamentum flavum cells was fractionated by column chromatography (column diameter: 1.5 cm, height: 47 cm) using Sephadex G25 (particle size: 50-150 μm , Pharmacia, Uppsala), and the inhibitor activity of 20 fractions was determined by the seed test. Another DMEM not supplemented with serum or albumin was kept under the same conditions for 24 h

and used as the control.

Results

Calcium uptake by the ligamentum flavum matrix in the reaction system for calcification

A total of 145 mg (dry weight) of human ligamentum flavum extract was obtained, corresponding to about 1.2 mg per gram of wet weight of ligamentum flavum. In the reaction system, calcium uptake by the extracted matrix increased with time in a quadratic curve; the values were significantly higher than those in the non-extracted matrix. In the reaction system using an extracted matrix, calcium uptake was suppressed in the reaction solution (25 ml) containing 16 mg of the extract (Fig. 1). Changes in calcium uptake in the presence of 1 mM NaF or 250 mg/ml phosvitin are shown in Fig. 2. NaF caused a marked increase in calcium uptake (open squares in Fig. 2). A combination of NaF and 16 mg of the extract induced a slight decrease in uptake (closed squares in Fig. 2). Phosvitin caused a far greater inhibition of uptake than did the extract (open triangles in Fig. 2).

Radiologic findings of the matrix transplantation in paraspinal muscles

Grafts of extracted ligamentum flavum matrices showed an uneven opacity one week after the transplantation (Fig. 3-b), and this finding was more conspicuous at 4 weeks (Fig. 3-d). The

shadows increased in opacity with time, showing a radial pattern of extension suggestive of crystalline growth (Figs. 3-f, h). The extent of shadows measured using an eye piece scale was clearly smaller than that in grafts of extracted tissue (Figs. 3-a, c, e), however, there were evidently calcified shadows in grafts of non-extracted ligamentum flavum matrices 12 weeks after transplantation (Fig. 3-g).

Histological findings

Vascular connective tissue proliferation accompanied by foreign body giant cell and round cell infiltration was found in the area surrounding the grafts of both extracted and non-extracted matrices (Fig. 4). These changes reached a peak at 2 weeks, followed by mature connective tissue (Figs. 4, 5, 6 and 7).

The transplanted matrices had a fibrous structure with apertures of varying size. A few small round cells entered these apertures by 2 weeks after transplantation (Fig. 5), but no extension of the cell infiltration was noted with time. Although there was a considerable difference among individuals, calcification observed by von Kossa stain was generally manifested as small grains in the graft from 1 week after transplantation, and the calcareous deposits became enlarged over time (Figs. 6 and 7). There was no ossification throughout the observation period.

Crystal inhibitor activity in human ligamentum flavum cell culture

In the seed test of media cultured with ligamentum flavum cells, the mean crystal inhibitor activity determined by the amount of hydroxyapatite (HAP) required to obtain 50% inhibition of calcification was $617.8 \pm 182.1 \mu\text{g HAP}/500 \mu\text{l}$ ($n=23$), whereas it was $372.8 \pm 89.6 \mu\text{g HAP}/500 \mu\text{l}$ with the control medium ($n=7$) ($p < 0.01$) (Table 1). When the inhibitor activity in relation to age was examined in three different age groups (20-40, 41-60, and 61-78 years), the inhibitor activity tended to increase with an increase in age, but the difference was not significant (Table 2). The seed test of fractions of the culture medium obtained by Sephadex G25 chromatography showed three peaks of inhibitor activity. The first peak agreed almost completely with the void volume, and the second peak was of lower molecular weight. The third peak almost corresponded to the only peak obtained with the control medium, suggesting a pre-existing Mg^{2+} (Fig. 8).

Discussion

The concentration of calcium and phosphate in extracellular fluids are supersaturated with respect to hydroxyapatite dissolution (11, 12), but no calcification of connective tissue occurs under normal conditions, suggesting the existence of an inhibitory mechanism in the tissue.

The calcification inhibitors in the present study were

extracted according to the methods of Thomas and Tomita (19) and Quittner and Wadkins (15) for the extraction of inhibitor in the Achilles tendon. In the present study however, since the amount of ligamentum flavum obtained during surgery from each patient was very small, specimens from patients of various ages were pooled for extraction. In order to prevent contamination from blood, the ligamentum flavum specimens were carefully rinsed with distilled water immediately after removal. It is clear from the results that the human ligamentum flavum matrix after extraction with 3% Na_2HPO_4 exhibits calcification activity. As pointed out by Quittner and Wadkins (15), this change in the ligamentum flavum can be explained by the following two hypotheses; 1) phosphate specifically promotes dissociation from the tissue of substances calcification-inhibitory in situ, and 2) phosphate alone, or in combination with endogenous Ca^{2+} , binds at appropriate sites of the matrix and thereby provides centers for nucleation and growth of calcium phosphate crystals. The results of the present study suggest that at least the substance which inhibits calcification was removed through the process of extraction. It is reported that the calcification inhibitor in bovine Achilles tendon has a peptidoglycan structure with a molecular weight of 85,000-100,000 (15). In the present study, although identification and measurement of the molecular weight was not possible because of the limited specimens, the molecular weight is presumed to be greater than 8,000 from the exclusion

limit of the cellulose dialysis tubing used. The addition of 1 mM NaF to the calcification reaction system increased the calcium uptake by the matrix. These results are consistent with the previously reported finding that calcium uptake by the collagenous matrix from bovine Achilles tendon is stimulated by NaF in the range of 2×10^{-5} to 2×10^{-3} M (21). Wadkins and Luben (21) reported that the calcification process mediated by the collagenous matrix is less sensitive to methylenediphosphonate and phosphonoacetate in the presence of NaF. This coincides with the results of the ligamentum flavum extract examined in the present study. It is therefore possible that the ligamentum flavum extract inhibits calcification through a mechanism similar to that of methylenediphosphonate or phosphonoacetate.

Phosvitin, a phosphoprotein in egg yolk, has a molecular weight of 20,000-30,000. According to Quittner and Wadkins (15), phosvitin inhibits calcification mediated by matrix from bovine Achilles tendon, showing 67% inhibition at 100 μ g/ml, and 78% (at 2 hat 200 μ g/ml. The promotion of calcification by NaF and the potent inhibitor activity of phosvitin found in the present study are consistent with the findings obtained in bovine Achilles tendons by Quittner and Wadkins (15) and Wadkins and Luben (21).

The above-mentioned in vitro results are consistent with the histological findings obtained in the human ligamentum flavum matrix transplanted into the dorsal muscles of rats. However,

the fact that calcification also occurred in the grafts of non-extracted ligamentum flavum tissues at a later stage suggests that the substance extracted with 3% Na_2HPO_4 does not interfere with every step of the calcification process.

The crystal inhibitor activity of the culture medium from human ligamentum flavum cell culture, as determined by the seed test indicated that the cultured cells produce inhibitors for the formation of calcium phosphate crystals. In this experimental system, there was no significant relationship between age and the inhibitor activity of the ligamentum flavum; the values were nearly constant. Thus, it seems that the inhibitors in the culture medium are not related to age (Table 2). The inhibitor activity was separated into three peaks after gel filtration; the first and second peaks (indicated by arrows in Fig. 8) represent inhibitors produced by the culture cells. The third peak seems to indicate the inhibitor activity of pre-existing Mg^{2+} in the medium. Ohya et al (13) demonstrated three peaks of the inhibitor activity, corresponding to a high molecular weight of over 4,000, a low molecular weight and magnesium, by Bio-Gel P4 column chromatography of the culture medium from rat carvarium cell culture. They speculated that the high molecular weight inhibitor activity might originate either from the substances that are absorbed onto bone mineral from body fluids, or from soluble components of the matrix. They also pointed out the possibility that the culture cells produce non-collagenous bone

proteins such as osteocalcin, osteonectin, proteoglycans or phosphoprotein. With regard to the low molecular weight fraction, Ohya et al stated that pyrophosphate and citrate are present at low concentrations, and show negligible activities, and that most of the activity originates from other unknown inhibitors (13). In plasma ultrafiltrate, magnesium, citrate and pyrophosphate are reported to represent only part of the inhibitor activity, indicating the presence of unknown inhibitors (10). Although the inhibitors in the present study which separated into two fractions are also unknown, it is possible that proteoglycans and phosphoprotein are included.

It is considered that, unlike simple precipitation of calcium phosphate crystals from solution, the biological mechanism of calcification consists of mutual relationships among cells forming calcified tissues, organic matrices, minerals and regulatory factors (1,3,6,11,12,20). Various hypotheses have been proposed for the in vivo calcification mechanism. In one hypothesis, it is speculated that biological tissues have an inhibitory mechanism for the precipitation of minerals (1,3,5,11,12), and that the inhibition ceases when hard tissues are formed. Although this hypothesis cannot explain the entire process of calcification, a distinct inhibitory mechanism for calcification in human ligamentum flavum has been shown. Thus, it appears that changes in this mechanism are involved in the clinical cases of calcification or ossification of the ligamentum

flavum.

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legends for illustrations

Fig. 1. Crystal inhibitor activity of the ligamentum flavum extract: ○—○ Time-course changes in calcium uptake by 100 mg of extracted ligamentum flavum matrix (n=3), ●—● changes in the presence of 16 mg of ligamentum flavum extract (n=3), △—△ changes by non-extracted ligamentum flavum matrix (100 mg) (n=3).

Fig. 2. Effects of 1 mM NaF and 6.25 mg phosvitin on the reaction system for calcification: ○—○ Time-course changes in calcium uptake by 100 mg of extracted ligamentum matrix, ●---● changes in the presence of 16 mg of ligamentum flavum extract, □—□ changes in the presence of 1 mM NaF, ■—■ changes in the presence of 16 mg of ligamentum flavum extract and 1 mM NaF, △--△ changes in the presence of 6.25 mg of phosvitin.

Fig. 3. Softex images of extracted and non-extracted ligamentum flavum matrices after transplantation into the dorsal muscles in rats. a) non-extracted matrix at 1 week, b) extracted matrix at 1 week, c) non-extracted matrix at 4 weeks, d) extracted matrix at 4 weeks, e) non-extracted matrix at 8 weeks, f) extracted matrix at 8 weeks, g) non-extracted matrix at 12 weeks, h) extracted matrix at 12 weeks.

Fig. 4. Histological appearance of the peripheral area of non-extracted ligamentum flavum matrix 4 weeks after transplantation.

Non-specific fibrous reaction and foreign body giant cells can be seen. (H.E., x 100)

Fig. 5. Histological pictures of extracted (A) and non-extracted (B) ligamentum flavum matrices 2 weeks after transplantation. **A)** Calcareous deposits can be seen in the outer layer of the graft. Marked round cell infiltration and proliferation of vascular connective tissue are evident in the peripheral area of the graft (H.E., x 40). **B)** No calcareous deposits are detectable in the graft. (H.E., x 40).

Fig. 6. von Kossa stained pictures of extracted (A) and non-extracted (B) ligamentum flavum matrices 2 weeks after transplantation. **A)** Calcareous deposits are more numerous in the outer layer of the matrix (x 40). **B)** Calcification deposits are present in a part of the graft, but are clearly less conspicuous than those in (A) (x 40).

Fig. 7. Histological pictures of extracted (A) and non-extracted (B) ligamentum flavum matrices 8 weeks after transplantation. **A)** Numerous calcareous deposits are distributed throughout the entire graft. Cellular infiltration in the peripheral area of the graft has disappeared (von Kossa, x 40). **B)** Calcareous deposits are located in only a part of the graft. There is almost no cellular infiltration in the peripheral area of the graft (von Kossa, x 40).

Fig. 8. Crystal inhibitor activity of each fraction obtained by column chromatography of medium cultured with *ligamentum flavum* cells (seed test). Culture medium (3 ml) was separated on a Sephadex G 25 column (1.5 x 47 cm), and the crystal inhibitor activity of each fraction (3 ml) is expressed in the amount (μg) of hydroxyapatite. ●—● Culture medium from *ligamentum flavum* cell culture. Arrows indicate the fractions having inhibitor activity specific to this medium. ○-----○ Control medium. The peak centering on the fraction number 21 seems to represent the inhibitor activity of Mg^{2+} .

Table 1. Crystal inhibitor activity in medium cultured with ligamentum flavum cells and in control medium

	n	Crystal Inhibitor Activity (μ HAP)
		mean \pm SD
Medium cultured ligamentum flavum cells	23	617.8 \pm 182.1 *
Control medium (0.1% albumin)	7	372.8 \pm 89.6 *

HAP, hydroxyapatite

* $p < 0.01$

Table 2. Crystal inhibitor activity in relation to age

Age (yr)	n	Crystal Inhibitor Activity (μgHAP) mean \pm SD
20-40	6	565.9 \pm 86.0
41-60	8	580.7 \pm 197.5
61-78	9	685.5 \pm 209.4

HAP, hydroxyapatite

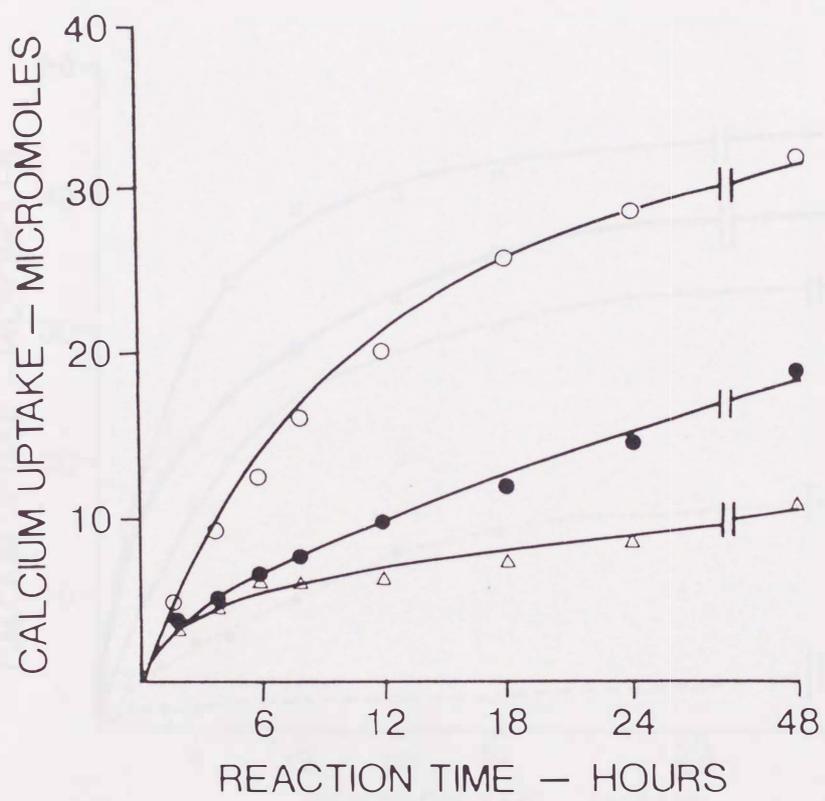


Fig 1

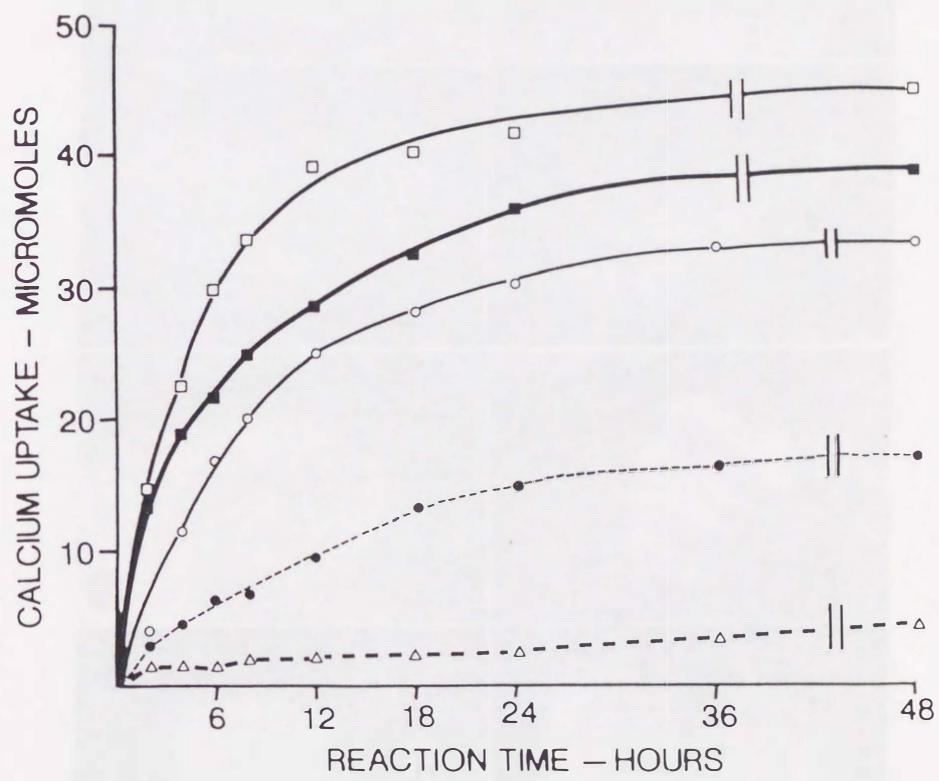


Fig 2

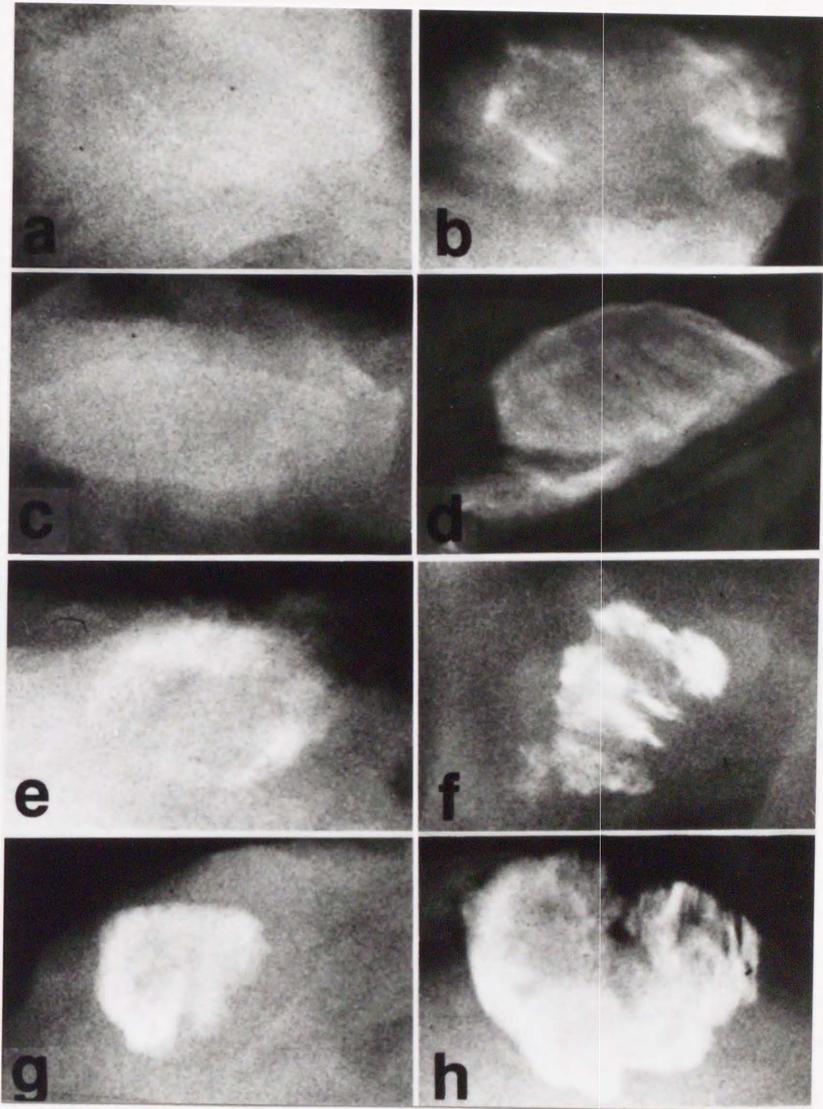


Fig 3

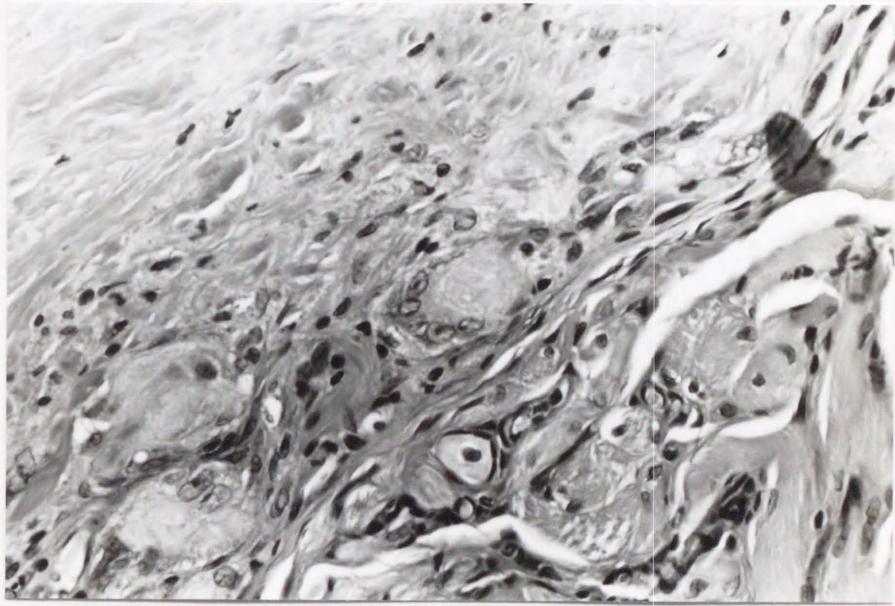


Fig 4

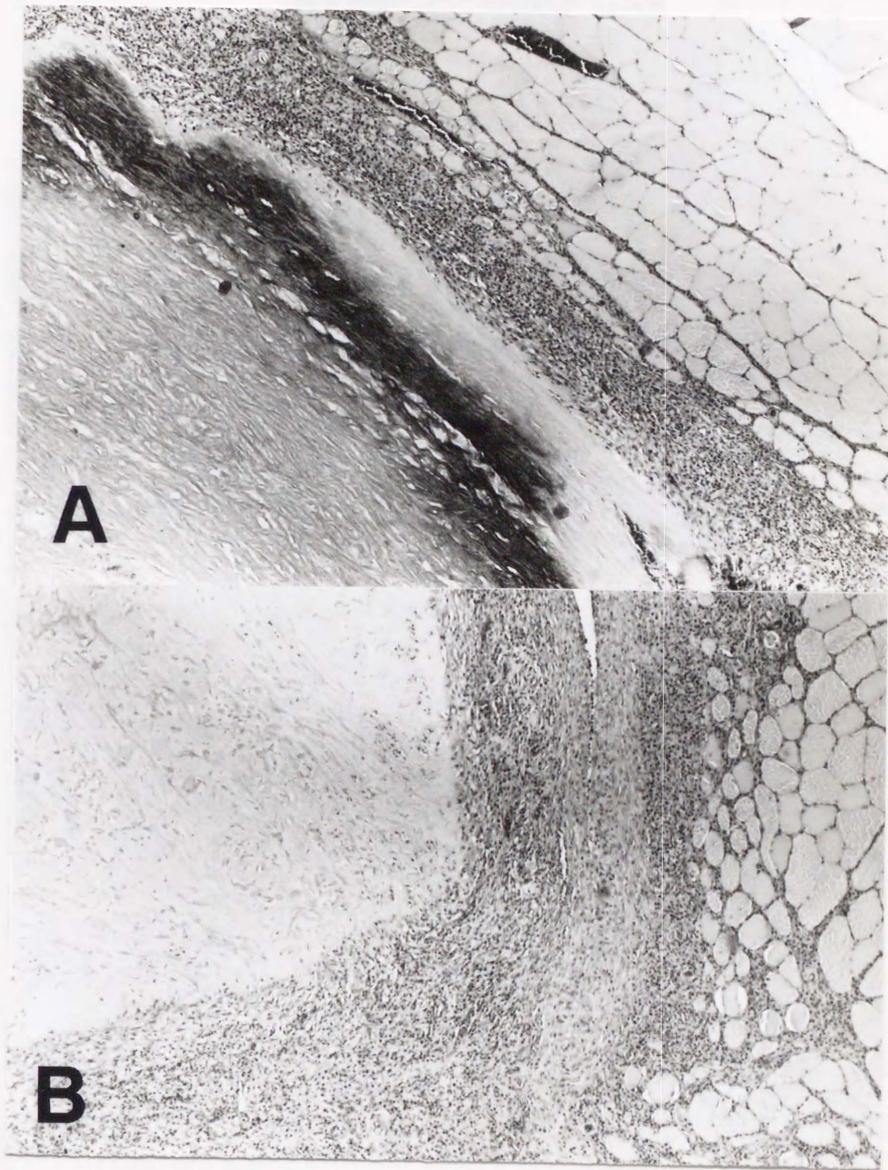


Fig 5

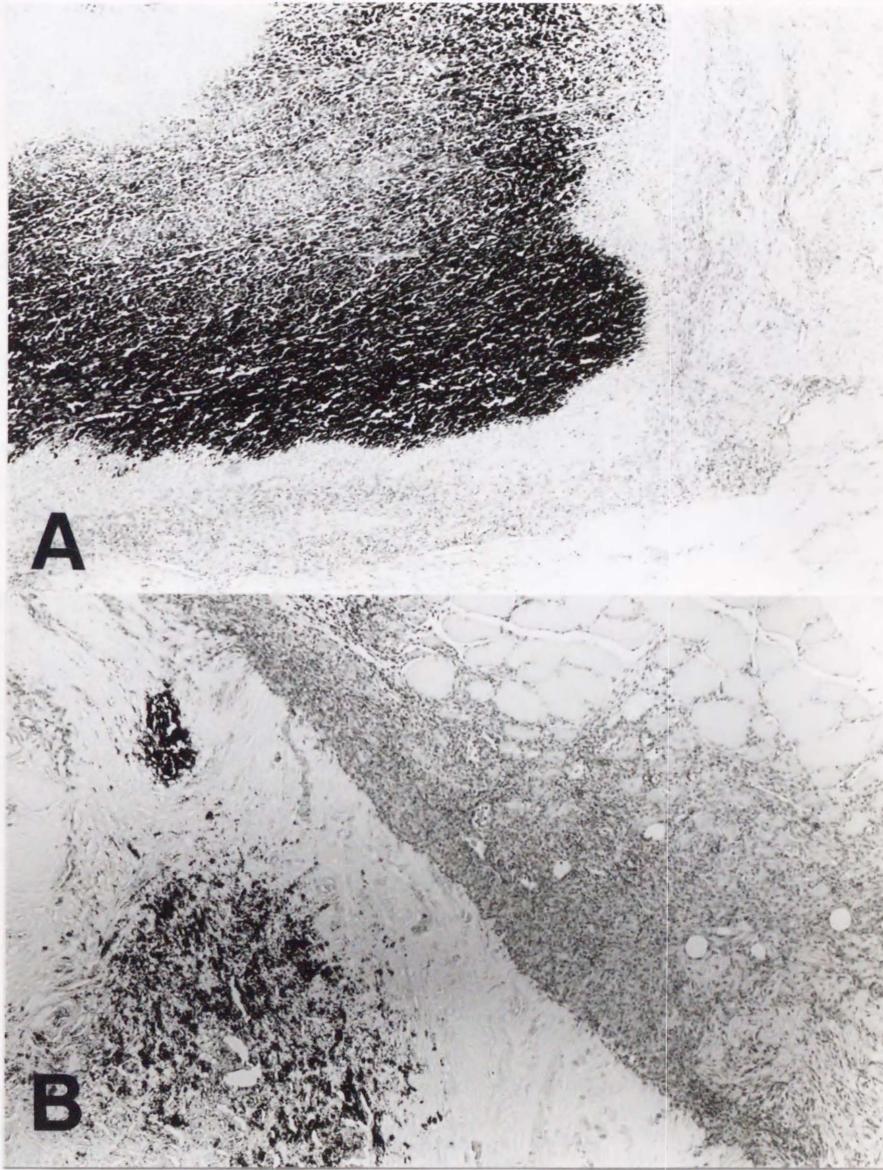


Fig 6

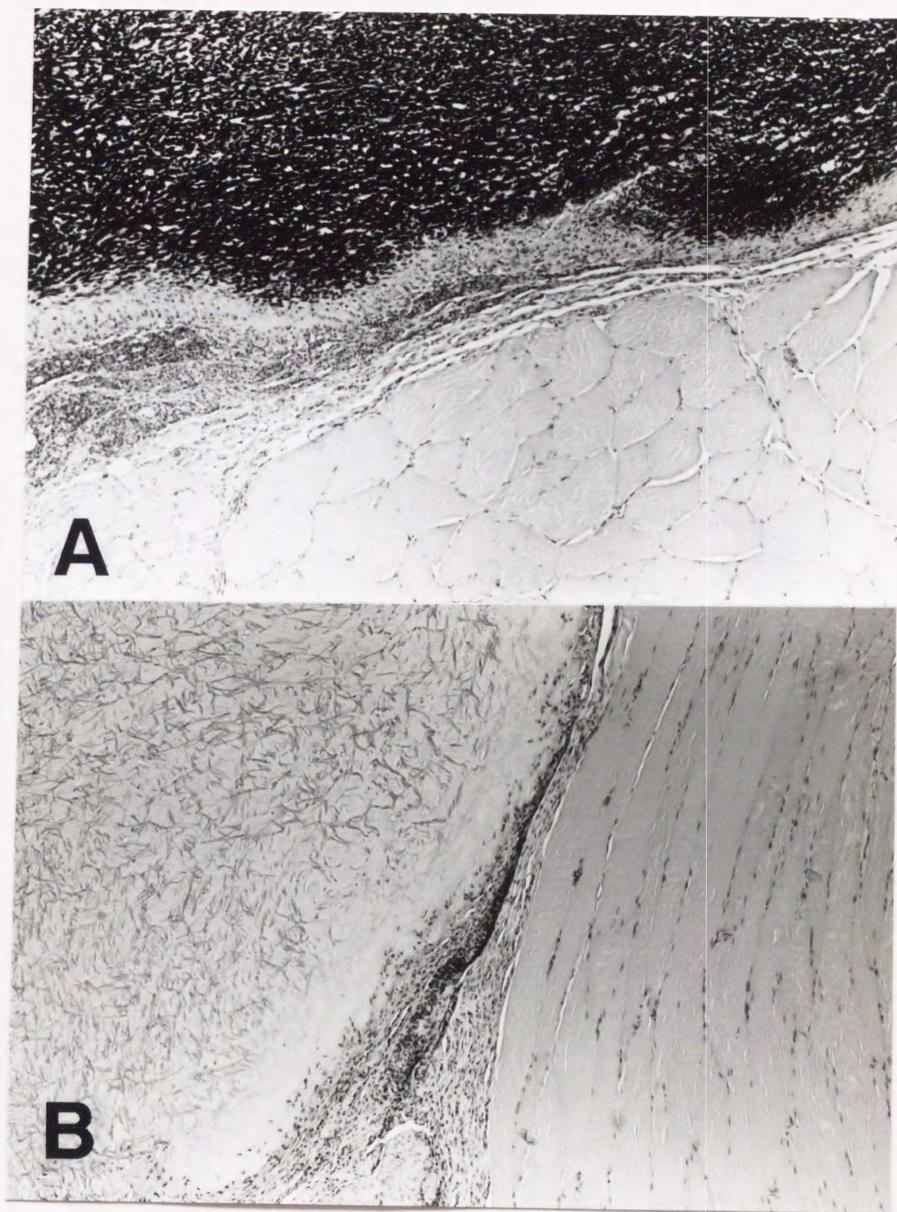


Fig 7

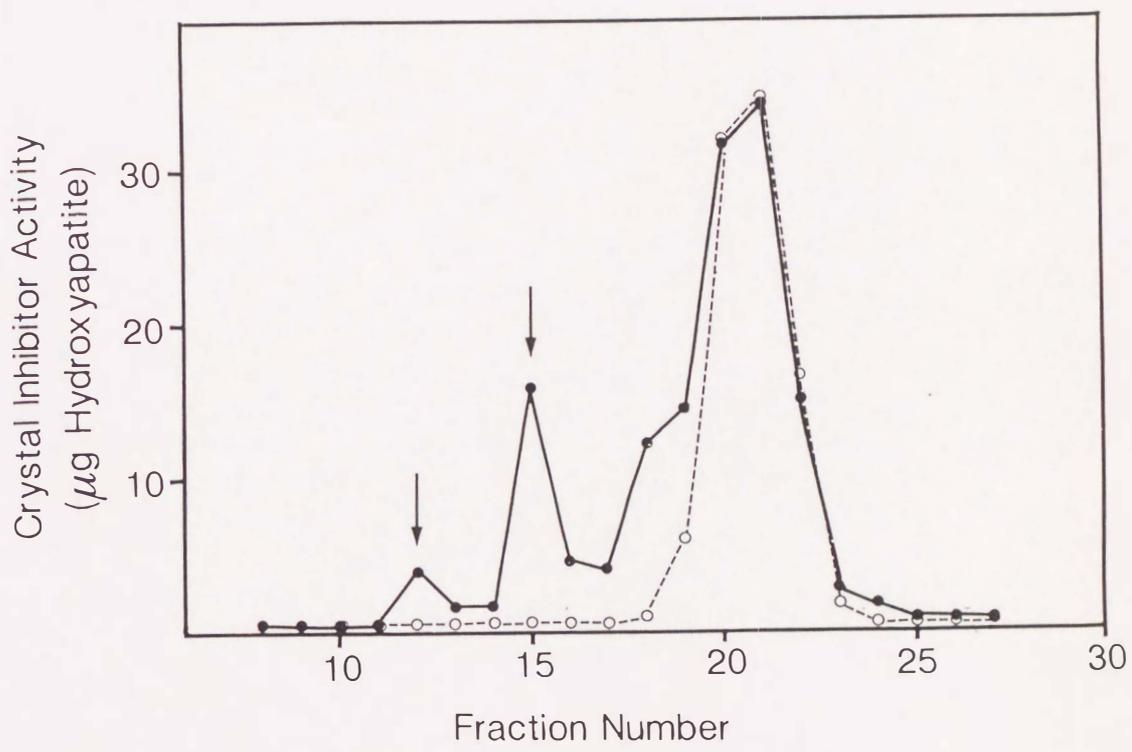
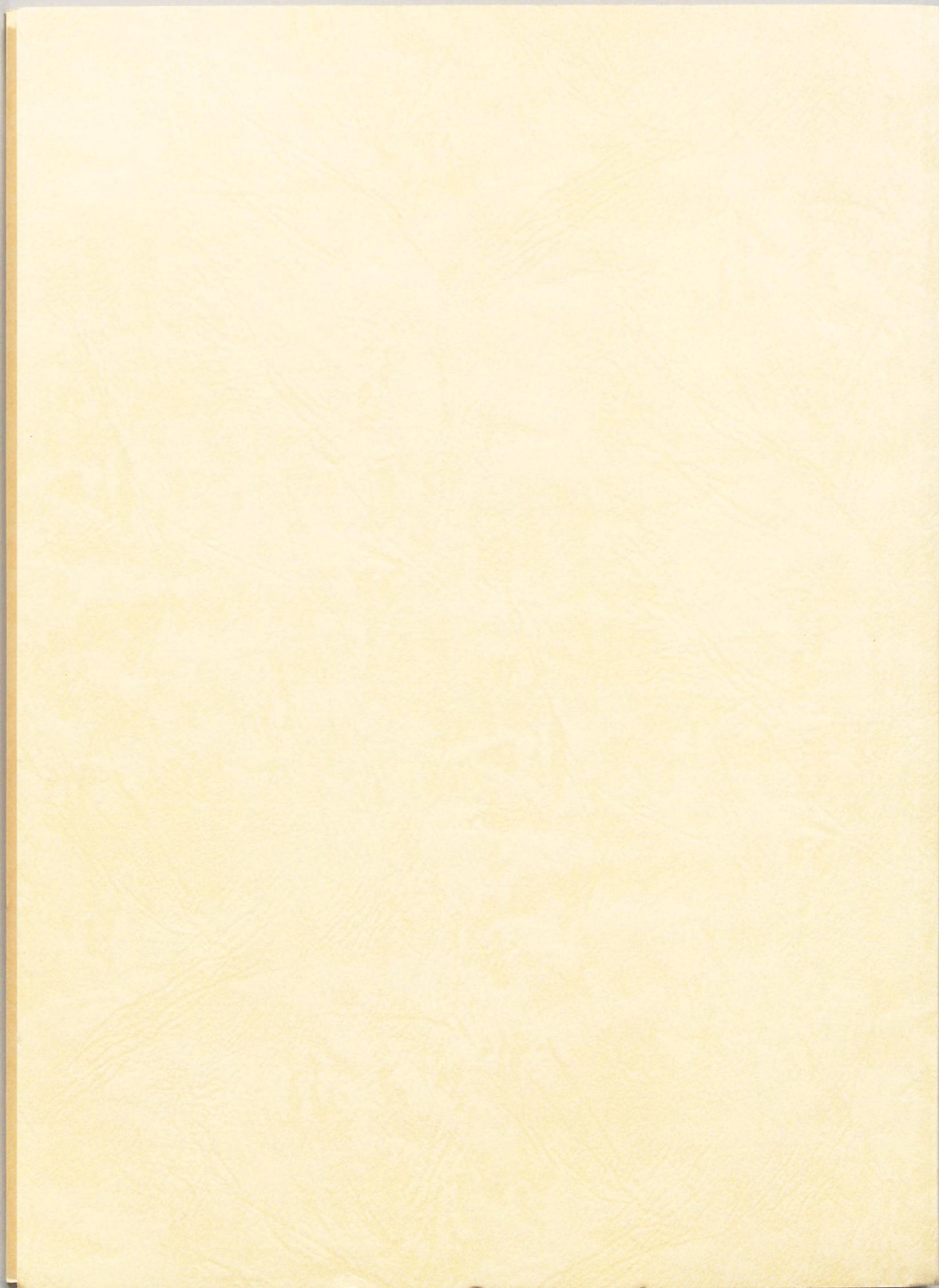


Fig 8



Inches 1 2 3 4 5 6 7 8
cm 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19

Kodak Color Control Patches

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Blue	Cyan	Green	Yellow	Red	Magenta	White	3/Color	Black
Light Blue	Light Cyan	Light Green	Light Yellow	Light Red	Light Magenta	White	Light Skin	Black
Dark Blue	Dark Cyan	Dark Green	Dark Yellow	Dark Red	Dark Magenta	White	Dark Skin	Black

Kodak Gray Scale



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A 1 2 3 4 5 6 **M** 8 9 10 11 12 13 14 15 **B** 17 18 19

