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The Antigenicity of Chondromucoprotein

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Summary. Antibody was obtained in rabbits following immunization with porcine chondromucoprotein and complete adjuvant. Several constituents of the antigen were only revealed by prior hyaluronidase digestion, while an antigenic constituent revealed without hyaluronidase treatment was found to be shared by chondromucoproteins from several other species. Cross-reactivity was confirmed by tanned cell haemagglutination-inhibition and by delayed hypersensitivity reactions in guinea-pigs. Electrophoresis of chondromucoprotein gave some separation of the immunologically distinct constituents. The results suggest that chondromucoprotein may be made up of several species-specific proteins as well as a polysaccharide-peptide of common occurrence in several species. No evidence for antibody directed against chondroitin sulphate was found.

The antibody has been used for the localization by immunofluorescence of chondromucoprotein in sections of tissue.

INTRODUCTION

Only very few examples are known of protein-free polysaccharides acting as antigens. It is therefore not surprising that chondroitin sulphate, which occurs widely-distributed in connective tissue has not been found to be antigenic (Boake and Muir, 1955; Quinn and Cerroni, 1957). In its biological state, however, chondroitin sulphate, and probably also other acid polysaccharides, is co-valently bound to protein, and by suitable means can be extracted from cartilage as such a complex (Muir, 1958; Malawista and Schubert, 1958; Partridge and Davis, 1958; Partridge, Davis and Adair, 1961). This has been found to be antigenic in experimental animals by several workers (White, Sandson, Rosenberg and Schubert, 1963; Di Ferrante, 1964; Loewi, 1964). We report here a study of the antigenic constituents of porcine chondromucoprotein, including an antigenic similarity of a constituent found in various species. We have also used the antibody for histological localization of chondromucoprotein by immunofluorescence.

MATERIALS AND METHODS

The material used throughout this work was a preparation of chondromucoprotein (CMP) extracted from porcine thyroid and cricoid cartilage. Details of extraction and analysis were described by Muir (1958), and are summarized in Table 1. For tests of antibody cross-reactions, bovine CMP was prepared from nasal septal cartilage by the method of Malawista and Schubert (1958). Analysis showed 20·5 per cent hexuronic acid (Dische), 19·3 per cent hexosamine (Elson and Morgan) and 34·0 per cent protein (Lowry). A two-dimensional chromatogram of an acid hydrolysate showed no evidence of hydroxyproline. Other samples of CMP were similarly extracted from rabbit and guinea-pig nasal septa. Another batch of bovine chondromucoprotein was provided by

courtesy of Dr Matthews of Chicago, and human chondromucoprotein was a gift from Dr A. J. Anderson. Horse cartilage chondroitin sulphate was provided by Dr J. E. Scott, as was a sample of hyaluronic acid prepared from pleural fluid. Another sample of hyaluronic acid had been prepared from embryo pig skin (Loewi and Meyer, 1958). Keratosulphate was provided by Dr K. Meyer of New York. Chondromucoproteins subjected to hyaluronidase digestion were dissolved in 0·9 per cent saline at 10 mg/ml to which was added ovine, salt-free testicular hyaluronidase (Seravac Laboratories) at 150 μg/ml. This was incubated for 48 hours at 37° with a small amount of toluene.

TABLE 1

ANALYSIS OF CHONDROMUCOPROTEIN FRACTIONS FROM PIG THYROID AND CRICOID CARTILAGE

	Unfractionated chondromucoprotein*	Faster electrophoretic fraction†	Slower electrophoretic fraction†	Papain treated chondromucoprotein‡
Nitrogen	3·92	3·06	4·82	2·84
Hexosamine	27·5	32·0	27·5	31·0
Sulphate	14·5	15·04	13·0	16·6

* Prepared according to Muir (1958) and purified by two precipitations with 5-aminoacridine.
† Obtained by preparative electrophoresis on compressed glass fibre at pH 7·2 (Muir and Jacobs, 1965).
‡ Obtained by papain digestion of unfractionated chondromucoprotein (Muir, 1958).

Immunization and testing

Rabbits were immunized by foot-pad injection of 2 mg CMP dissolved in saline, emulsified with Freund's complete adjuvant. A week later a similar injection was given subcutaneously and a third injection a month after the second. One week later, the animals were bled. Subsequent blood samples were taken following further similar immunization. Guinea-pigs were immunized with 1 mg CMP emulsified with complete adjuvant given in divided doses in the foot-pads, followed 1 week later by 0·5 mg intracutaneously. Approximately 10 days later, guinea-pigs were tested for circulating antibody as well as delayed hypersensitivity by intracutaneous injection of 0·1 ml of saline containing an appropriate amount of antigen. At the same time, 0·3 ml of a 2 per cent solution of Coomassie Blue (Feinberg and Dewdney, 1963) was given intravenously, producing an immediate blue reaction at the antigen injection site in immunized guinea-pigs, but blanching in time to reveal a later delayed reaction at the same site. Results were read immediately and at 4, 24, and 48 hours.

Serology

Sera were tested for antibodies by capillary precipitation and by Ouchterlony gel diffusion. Tanned cell haemagglutination was carried out according to Stavitsky (1954). The final antigen concentration used for coating was 2·5 mg/ml, whether CMP had had prior treatment with hyaluronidase or not. The cell suspension was incubated for 30 minutes at 37° prior to washing. Uncoated tanned cell controls were used with every experiment. Agglutination inhibition tests employed a solution of the test material in 1 per cent normal rabbit serum in buffer; and this was used for serial dilutions; incubation of the antibody-inhibitor mixture at 37° for 30 minutes preceded the addition of sensitized cells. The amount of potential inhibitor per Perspex tray well varied from 0·1 to 100 μg.

Complement fixation was performed according to the method of Donnelley (1951). Passive cutaneous anaphylaxis was carried out in guinea-pigs by the method of Ovary and Bier (1953), but allowing 18 hours for fixation of antibody in the skin. Serum globulins were fractionated on DEAE-cellulose columns by the method of Lospalluto, Chegoriansky, Lewis and Ziff (1960). Ultracentrifugation with a sucrose density gradient was performed as described by Kunkel (1960). Immunoelectrophoresis in agar was done in barbiturate–HCl buffer at pH 8·2. The fluorescent antibody technique was applied to sections of cold-alcohol fixed tissues as well as to cryostat-cut sections of fresh-frozen material. The sandwich method was used, in which incubation of the section with rabbit antibody was followed by fluorescent goat-anti-rabbit conjugate.

RESULTS

Precipitating antibody to pig CMP was found in the sera of immunized rabbits. This could be shown by capillary precipitation when the antigen had been treated with hyaluronidase. Double diffusion in agar showed multiple lines (Fig. 1) with hyaluronidase-treated CMP, but only one line, near the antibody well, with untreated CMP. No line

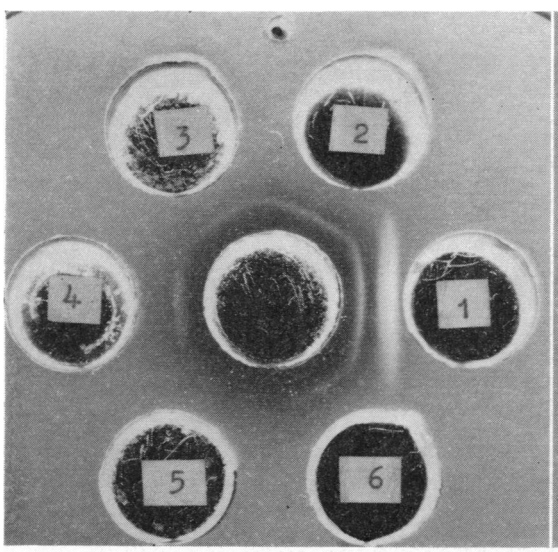

FIG. 1

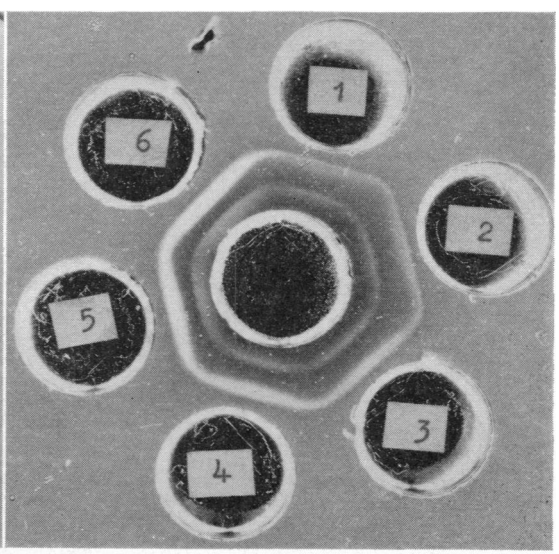

FIG. 2

FIG. 1. Reaction of rabbit anti-chondromucoprotein (centre well) with porcine and bovine CMPs. 1 = Hyaluronidase-digested porcine CMP, 10 mg/ml; 2 = same without hyaluronidase digestion; 3 = bovine CMP, 1 mg/ml; 4 = same after hyaluronidase digestion; 5 = bovine CMP, 1 mg/ml; 6 = pig CMP, 1 mg/ml.

FIG. 2. Effect of increasing duration of hyaluronidase digestion of CMP. 1 = 15 minutes, 2 = 2 hours, 3 = 5 hours, 4 = 8 hours, 5 = 24 hours, 6 = 48 hours. Antibody in centre well.

was seen when hyaluronidase was diffused against the immune serum and no lines were seen with sera from rabbits not immunized with CMP. The strength of the immune precipitation lines increased with the duration of hyaluronidase treatment of the antigen (Fig. 2). When antibody to CMP was diffused against pig serum, only a very fine precipitation line was produced, and this appeared to be continuous with a faint line running between the two main CMP lines. Absorption of anti-CMP serum with an equal volume

of pig serum failed to remove this fine line. That only an insignificant portion of antibody was directed against pig serum was further shown by tanned cell haemagglutination as described below. Absorption of antibody with an equal volume of pig serum did not interfere with the main lines against CMP. The minor line was lost, presumably owing to dilution of antibody. Treatment of CMP by papain left only a very weak line near the antibody well.

It could further be shown by gel diffusion that the antibody cross-reacted with CMP from species other than the immunizing one. Cross-reaction with bovine CMP is shown in Figs. 1 and 3. The main cross-reacting component was regularly found to correspond to the minor, non-hyaluronidase dependent component of pig CMP, but in some cases another finer line was seen in addition. Weaker cross-reactions were obtained with human and with guinea-pig CMP, but no line was found with rabbit CMP. These lines of cross-reaction could be shown with or without hyaluronidase treatment of the antigens. No lines were obtained when antibody was diffused against bovine, human or guinea-pig serum. The antibody was also diffused against acid-mucopolysaccharides which had been freed from protein by proteolysis. No reactions were noted with chondroitin sulphate or hyaluronic acid with or without hyaluronidase treatment. Keratosulphate similarly failed to react.

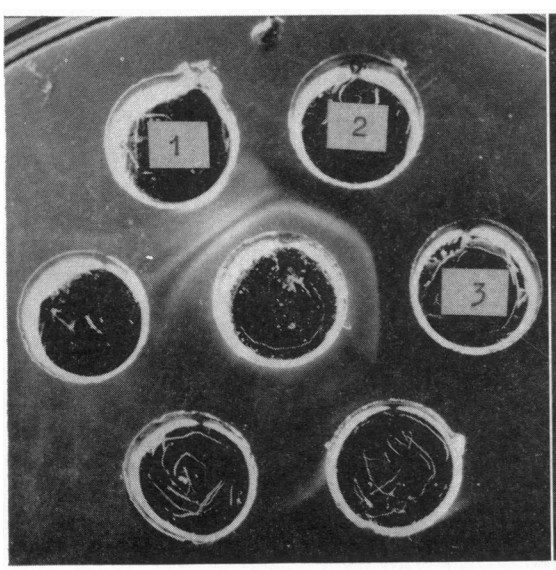

FIG. 3

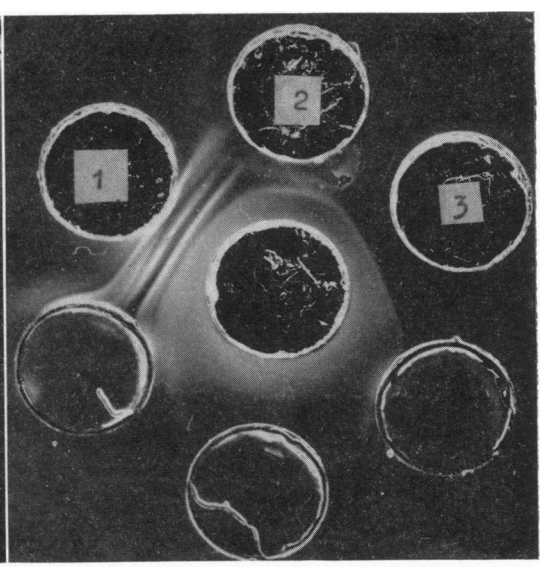

FIG. 4

FIG. 3. Cross-reaction of antibody to porcine CMP with bovine CMP. 1 = Hyaluronidase-digested porcine CMP; 2 = hyaluronidase-digested bovine CMP; 3 = bovine CMP. Antibody in centre well.

FIG. 4. Examination of CMP fractionated by electrophoresis. 1=Hyaluronidase-digested CMP; 2 = faster-moving electrophoretic fraction (see text); 3 = CMP. Antibody in centre well.

In view of the evidence suggesting several antigens in pig CMP, one non-hyaluronidase dependent and cross-reacting, and several (at least four lines were seen with sera obtained after prolonged immunization) species-specific ones shown after hyaluronidase treatment, attempts were made to obtain the several constituents separately. Preliminary electrophoresis in agar showed two components. Two fractions were obtained by preparative

electrophoresis on compressed glass fibre (Muir and Jacobs, 1965). Although there was no distinct separation, the faster moving fraction showed a line of identity (Fig. 4) with the component of unfractionated CMP that appeared without hyaluronidase digestion. Hyaluronidase treatment of this component produced no additional precipitation lines. The slower moving electrophoretic fraction showed after hyaluronidase treatment lines corresponding to those of hyaluronidase treated unfractionated CMP. A weak non-hyaluronidase dependent line, however, was still present near the antibody well.

TABLE 2

TANNED CELL HAEMAGGLUTINATION WITH VARIOUS ANTIGENS

Coating antigen	Reciprocal of titre
Pig CMP, hyaluronidase treated	640,000
Pig CMP	12,000
Bovine CMP, hyaluronidase treated	128,000
Hyaluronidase	<100
Pig serum	<100
Pig CMP, papain treated	<100

The CMP antigen–antibody system was further explored by tanned cell haemagglutination and inhibition. Table 2 shows titres obtained with a rabbit serum in a typical experiment. No agglutination was seen in controls employing normal rabbit serum or unsensitized tanned cells. Cells coated with pig serum showed a titre of $<1:100$, and absorption of sera with pig serum only caused insignificant reduction of titre against CMP. The considerable increase in titre shown with treatment of pig CMP with hyaluronidase reflects the effect of this treatment on the appearance of precipitin lines already described.

Results of inhibition studies, which were designed to explore cross-reaction of antigens, are shown in Table 3. When two tubes or more at the end of a row of doubling dilutions showed no agglutination, when read against the control row containing no inhibitor,

TABLE 3

INHIBITION OF AGGLUTINATION OF CELLS COATED WITH DIFFERENT CMP PREPARATIONS BY CMPs

Coating antigen	Inhibiting Antigen					
	A	B	C	D	E	F
Pig CMP	4	N.D.	3	3	0	0
Pig CMP, hyaluronidase treated	3	5	0	0	0	0
Bovine CMP, hyaluronidase treated	5	5	4	5	N.D.	0

Numerals refer to numbers of tubes showing inhibition (see text).
A: Pig CMP, 10 μg; B: pig hyaluronidase-treated CMP, 10 μg; C: bovine CMP, 10 μg; D: bovine hyaluronidase-treated CMP, 10 μg; E: Chon.SO_4 ± hyaluronidase, 100 μg; F: papain-treated pig CMP ± hyaluronidase, 100 μg.
N.D. = not done.

significant inhibition was considered to have occurred. In Table 3, the numbers of such tubes showing inhibition are given. Non-specific inhibition was tested for by adding the same inhibitors to an ovalbumin rabbit-anti-ovalbumin system: no inhibition was produced. A few experiments with rabbit anti-pig CMP anti-serum and rabbit CMP failed to show cross-reaction by agglutination or inhibition tests.

The cross-reactivity of CMPs could further be demonstrated in sensitized guinea-pigs. Immunization with pig CMP produced antibody in this species, but titres were lower

than in rabbits. Only faint precipitin bands were seen, but tanned cell haemagglutination titres up to 1 : 51,200 were produced, with complement fixation up to 1 : 64 and passive cutaneous anaphylaxis. In sensitized guinea-pigs, active cutaneous anaphylaxis was regularly obtained with pig CMP, but evidence of cross-reaction with other CMPs was only rarely obtained in this way. When reactions were read at 24 hours, however, extensive cross-reactivity was found. Table 4 presents a summary of these findings.* Reactions were seen in all animals tested with the immunizing antigen, and in all those tested with human or bovine CMP, although only pig CMP had been used for immunization. Not all the guinea-pigs tested responded to rabbit CMP and none to guinea-pig CMP. No reactions were observed with porcine, bovine, human, rabbit or guinea-pig sera, and skin tests with chondroitin sulphate, hyaluronic acid and keratosulphate also proved to be negative. None of the antigens produced reactions in unsensitized animals. Guinea-pigs immunized with guinea-pig CMP failed to react with any of the antigens employed. Some of the animals were tested with CMPs that had undergone hyaluronidase digestion, hyaluronidase activity being subsequently inhibited by 3×10^{-4} M $FeCl_3$. Reactions were similar to those seen with undigested CMP.

TABLE 4

TWENTY-FOUR-HOUR SKIN REACTIONS IN GUINEA-PIGS IMMUNIZED WITH PIG CMP

Pig CMP 10–100 μg	Bovine CMP 10–100 μg	Human CMP 100 μg	Rabbit CMP 50–100 μg	Guinea-pig CMP 100 μ–1 mg	Sera 1 : 100
20/20	14/14	6/6	8/11	0/16	0/9

Number of animals responding are given as a fraction of the total number tested with each antigen.

With the aid of the fluorescent antibody technique, both cartilage and soft tissues could be stained by rabbit antibody to pig CMP (Figs. 5–8). A dense narrow zone of fluorescence was seen outlining the lacunae of cartilage, with some staining of chondrocytes. Cartilage matrix showed zones of finely stippled fluorescence. The latter was enhanced by hyaluronidase treatment of the sections. Control sections stained with unrelated rabbit antibody or absorbed anti-CMP serum showed occasional stained chondrocytes, but no other fluorescent staining. Sections of kidney and other soft tissues showed prominent selective staining of basement membranes. Absorption of anti-pig CMP serum with pig serum or collagen did not interfere with the ability to stain. Cross-reactivity of the antibody was shown by its ability to stain bovine, human and guinea-pig cartilage. Fluorescent staining of CMP will be further considered elsewhere.

The nature of rabbit antibody to pig CMP was examined in a variety of ways. Immunoelectrophoresis in agar showed a line in the γ_2-globulin region (Fig. 9). This was stronger when the antigen had been treated with hyaluronidase. On chromatography on DEAE-cellulose, the major portion of the antibody activity was eluted with 0·01 M buffer at pH 7·0. On centrifugation in a sucrose density gradient, the highest antibody titre was found in the midzone, thus confirming the association of antibody activity with 7S

* Reactions were considered positive when a raised, erythematous zone of at least 6 mm diameter, often with central necrosis, appeared.

(*Facing p.* 124)

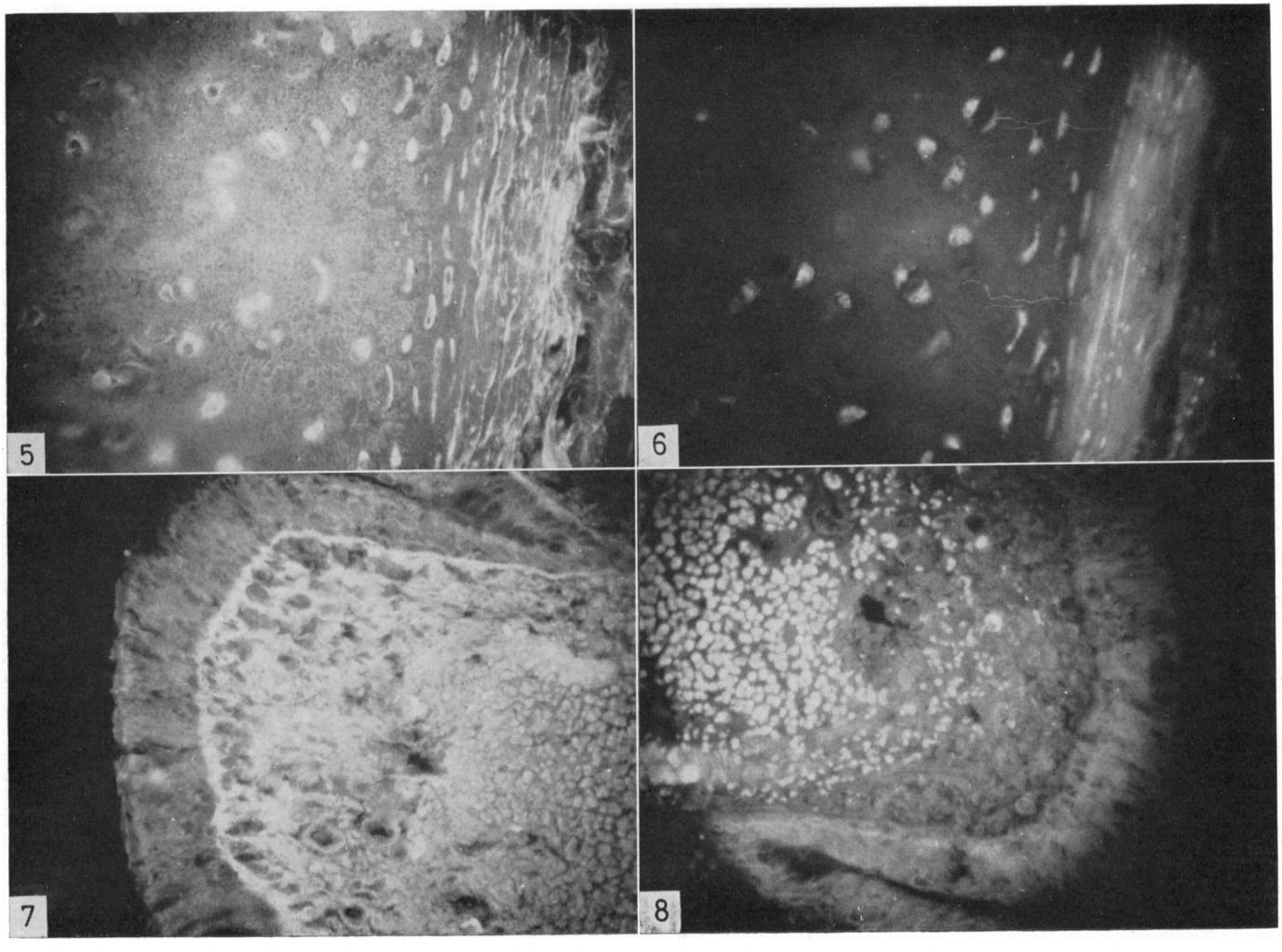

FIG. 5. Pig tracheal cartilage treated with rabbit antibody to CMP, followed by goat-anti-rabbit fluorescent conjugate.
FIG. 6. As Fig. 5, but rabbit anti-dextran serum substituted for anti-CMP.
FIG. 7. Pig tracheal mucosa treated with rabbit anti-CMP, followed by goat-anti-rabbit fluorescent conjugate. Note staining of basement membrane. Fibres cut at right angles show the auto-fluorescence of elastin.
FIG. 8. As Fig. 7, but rabbit anti-dextran serum substituted for anti-CMP.

globulin. Complement fixation was also associated with γ_2-globulin, but there appeared to be another and separate complement-fixing peak eluted from a DEAE-cellulose column with 0·30 M buffer at pH 5·0, suggesting a macroglobulin with antibody activity.

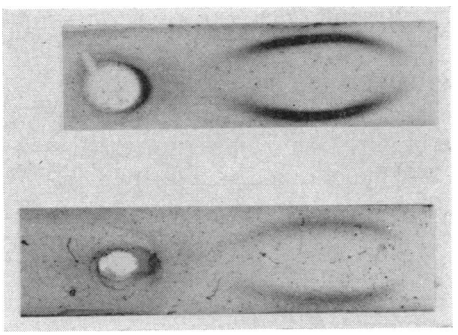

FIG. 9. Electrophoresis of anti-CMP sera, rabbit above, guinea-pig below. Anode to left. Lines are developed with hyaluronidase-treated porcine CMP. Slow (γ_2) globulin line only has appeared.

Data on guinea-pig antibody to pig CMP are summarized in Table 5. Activity in the γ_1 globulin is shown by haemagglutination and P.C.A. activity, although no γ_1 precipitin line is seen on immunoelectrophoresis.

TABLE 5

CHARACTERISTICS OF PEAKS OF GUINEA-PIG ANTIBODY AGAINST PIG CMP OBTAINED BY CHROMATOGRAPHY ON DEAE-CELLULOSE

	Globulin fraction		
	I	II	III
Eluting buffer	0·01 M, pH 7·0	0·1 M, pH 6·0	0·3 M, pH 5·0
Protein concentration (μg/ml)	730	860	530
Haemagglutination titre (reciprocal)	256	128	256
Complement fixation	+	0	0
P.C.A.	0	+	0

DISCUSSION

The results indicate that chondromucoprotein (CMP) can act as an antigen in both rabbits and guinea-pigs. This contrasts with the reported failure of antibody production against chondroitin sulphate (Blake and Muir, 1955; Quinn and Cerroni, 1957) and against hyaluronic acid (Humphrey, 1943). Our data further show several antibodies, presumably directed against different parts of the CMP complex. Like Sandson (personal communication) we find that most of the antigens are only revealed after digestion with hyaluronidase, although the immunizing antigen had not been so digested before injection. Di Ferrante (1964) has, however, reported that no precipitation bands were detected when antiserum was diffused against hyaluronidase-treated light fraction of bovine CMP. We cannot, at the moment, offer an explanation for this discrepancy. The results of hyaluronidase digestion, the failure of the antibody to react with or to be inhibited by chondroitin sulphate or other acid mucopolysaccharides, and the lack of cutaneous reaction to chondroitin sulphate in guinea-pigs immunized with CMP, suggest that we are not

dealing with an antibody directed against chondroitin sulphate, as Di Ferrante and Pauling (1964) infer from their work. Nevertheless, the immunologically minor, non-hyaluronidase requiring constituent that we obtained from CMP, consists predominantly of polysaccharide (Table 1) and it is this constituent which mainly figures in the cross-reaction of antibody with CMP from different species. This antigenic site may be associated with the region of linkage between carbohydrate and a series of amino-acids, including serine. Such a configuration is likely to be shared by CMP from different species, whereas the major part of the protein constituents appears to have species-specificity. The protein nature of the major hyaluronidase-requiring constituents was also shown by the lack of reactivity of material that had been digested with papain.

Cross-reaction with CMP from several species was also shown by skin-testing CMP-immunized guinea-pigs. Amongst CMPs tested the only one producing no reaction was guinea-pig CMP. Tests with hyaluronidase-treated antigens produced no bigger or earlier responses, and the delayed nature of the reactions could not, therefore, be attributed to degradation of the CMP at the skin site by hyaluronidase prior to reaction. The reactions, appearing 24 hours after challenge and preceded by only negligible 4-hour reactions, therefore have to be regarded as mainly delayed hypersensitivity reactions. No reaction was seen with papain-treated CMP. It is most likely that the target of the delayed skin reaction was the protein moiety of CMP. Delayed cross-reaction between related protein carriers similarly modified by attachment of chemically similar haptens has been shown repeatedly. Purified polysaccharides, on the other hand, have consistently been found to be incapable of eliciting a delayed hypersensitivity reaction (Freund and Bonanto, 1944; Maurer and Manssmann, 1958), and the work of Holborow and Loewi (1962) showed delayed hypersensitivity to blood group substance to be directed against the protein rather than the carbohydrate constituent.

The fluorescent antibody reaction with CMP is a welcome addition to the armamentarium for the study of ground substance mucopolysaccharides. Apart from the expected sites in cartilage, the method demonstrated basement membranes in various organs, indicating the presence of CMP or at least a closely related protein in this situation. Cartilage from several species could be stained in this way, again showing the cross-reactivity already revealed by other methods. It was further found that rabbit cartilage could be stained by rabbit antibody against pig CMP. This is another illustration of the capacity of the rabbit to form antibodies reactive with homologous tissue constituents upon immunization with related heterologous materials (Asherson and Dumonde, 1962).

An interesting feature of the guinea-pig antibody was the demonstration by agar electrophoresis of only a γ_2 component, in the absence of the usual γ_1 line, which occurs in sera after immunization of guinea-pigs with protein antigens. This is reminiscent of the finding by Bloch, Kourilsky, Ovary and Benacerraf (1963) of γ_2 antibody activity only in guinea-pigs immunized with *E. coli* the antibody being apparently mainly directed against the specific polysaccharide of this organism. Our finding, however, with CMP antibody differed from that with *E. coli* polysaccharide; although we obtained no precipitin line in the γ_1 region, chromatography produced a γ_1 fraction which gave a P.C.A. reaction, as well as tanned cell haemagglutination. Its separation from γ_2 antibody was shown by inability to fix complement, while the γ_2 antibody did not give a P.C.A. reaction. We may infer that the polysaccharide constituent has modified the CMP protein, so that it does not act quite as other protein antigens when given with complete adjuvant to guinea-pigs.

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