Immunology, 1975, **28**, 781.

Immunological Properties of Procollagens Obtained from the Culture Medium of Dermatosparactic Cells

EUNDUCK PARK,* R. L. CHURCH† AND M. L. TANZER*

* *Department of Biochemistry and* † *Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut, U.S.A.*

(*Received* 12*th August* 1974; *accepted for publication* 1*st October* 1974)

Summary. Rabbit antisera were produced against purified calf dermatosparactic procollagen and against the purified procollagens obtained from the culture medium of calf dermatosparactic cells. These antisera and their derived γ-globulins were characterized by immunoprecipitation, double immunodiffusion and immunoelectrophoresis.

Antiserum directed against dermatosparactic procollagen cross-reacted with the two different forms of procollagen obtained from the culture medium of dermatosparactic calf cells. Antiserum directed against one of these procollagens, namely (pro $\alpha 1)_2$ pro α_2, cross-reacted with dermatosparactic procollagen and also cross-reacted with the other procollagen, (pro $\alpha 1')_3$.‡ Antiserum directed against procollagen (pro $\alpha 1')_3$ cross-reacted with dermatosparactic procollagen and with the procollagen (pro $\alpha 1)_2$ pro $\alpha 2$.

None of the antisera reacted with authentic calf skin collagen, or with the collagen extracted from the cell layer of the dermatosparactic calf cells in culture. Reduction and alkylation of the procollagens abolished the antigen–antibody reactions, while prior digestion of the antigens with bacterial collagenase did not eliminate the immunological reaction. Antigenic determinants in the cell culture procollagens were found at the COOH-terminal non-collagen peptide as well as at the NH_2-terminal non-collagen peptide.

INTRODUCTION

The heritable connective tissue disease in cattle, called dermatosparaxis, is probably caused by the lack of an active processing enzyme, procollagen peptidase (Lenaers, Ansay, Nusgens and Lapiere, 1971; Lapiere, Lenaers and Kohn, 1971). Fibroblasts from the skin of a dermatosparactic calf have been isolated and established as clonal strains in culture (Church, Lapiere and Tanzer, 1973). These cells secrete procollagen into the medium in the form of two distinct molecules, which are also present in extracellular fibres (Church, Yaeger and Tanzer, 1974). One of the molecules is composed of pro $\alpha 1$ and pro $\alpha 2$ chains, as well as a larger polypeptide, θ chain, while the other molecule is composed only of pro $\alpha 1$ chains and θ chain (Church *et al.*, 1974; Tanzer, Church,

‡ The designation pro $\alpha 1'$ indicates that the primary structure of this polypeptide differs from the pro $\alpha 1$ chain (Church, Lapiere and Tanzer, 1973).

Correspondence: Dr R. L. Church, Department of Anatomy, University of Connecticut Health Center, Farmington, Connecticut 06032, U.S.A.

Yaeger, Wampler and Park, 1974). The cell culture procollagen molecules contain non-collagen peptide extensions at both the NH_2 and COOH termini, while the dermatosparactic procollagen extracted from the skin of the mutant contains only an NH_2-terminal non-collagen extension.

The dermatosparactic procollagen extracted from the mutant skin has been shown readily to elicit antibodies, which are primarily directed against the NH_2-terminal non-collagen appendage (Timpl, Wick, Furthmayr, Lapiere and Kühn, 1973; Kohn, Iserky, Zupnik, Lenaers, Lee and Lapiere, 1974; Park, unpublished results). Preliminary results indicated that these antibodies react with one of the procollagens which are secreted by the dermatosparactic calf cells in culture.

MATERIALS AND METHODS

Preparation of procollagen obtained from the culture medium

Clonal cell strains of calf dermatosparactic (CD) cells were grown and maintained as previously described (Church *et al.*, 1973). [^{3}H]Proline-labelled procollagens were isolated from the culture medium by a three-step procedure (Church *et al.*, 1974). Briefly, this method consists of an initial precipitation of total procollagen by 20 per cent ammonium sulphate, followed by dissolving the precipitate in potassium phosphate, pH 7·6, ionic strength 0·4. Insoluble proteins were removed by centrifugation and the procollagens were precipitated by the slow addition of ethanol to 18 per cent at 4°. This precipitate was harvested and then mixed with DEAE–cellulose which had been equilibrated with 0·01 M Tris–HCl, pH 7·4, containing 2 M urea. The mixture was applied to the top of a DEAE–cellulose column (2·5 × 20 cm), equilibrated with the same buffer, and eluted as previously described (Church *et al.*, 1973). Two procollagens eluted from the column.

This procedure consistently yielded procollagen type (pro $\alpha 1)_2$ pro $\alpha 2$ at least 96 per cent pure and procollagen type (pro $\alpha 1')_3$ at least 93 per cent pure, as determined by SDS–polyacrylamide electrophoresis.

Preparation of antiserum

Antiserum to purified dermatosparactic procollagen was produced in six adult rabbits. Antisera to the two different forms of cell culture procollagen were produced in individual adult rabbits.

All rabbits received a subcutaneous injection of 0·25 mg of antigen which had been previously dissolved in 0·05 per cent acetic acid and emulsified with an equal volume of Freund's complete adjuvant. Two weeks after the initial innoculation, a subcutaneous injection of 0·25 mg of antigen in Freund's incomplete adjuvant was given. Booster injections of 0·1 mg of antigen dissolved in PBS were given to those rabbits whose primary response was weak. Blood was collected by ear-bleeding 7 days after the last injection. Animals were killed by cardiac puncture 4 weeks after the last injection. Purification of immunoglobulin was carried out by standard methods (Williams and Chase, 1967).

Quantitative determination of precipitating antibody

Quantitative precipitin tests were carried out as described by Campbell, Garvey, Cremer and Sussdorf (1970). A hundred microlitres of undiluted antisera or 100 μl of

preimmunization rabbit sera were added to 100 μl of procollagen solution, in borate–saline buffer, pH 8·4. The reaction mixtures were incubated initially for 1 hour at room temperature and then at 4° for 2 days. The immunoprecipitates were centrifuged for 1 hour at 3000 rev/min, following which the supernatant fluids were assayed by the interfacial ring test, and the precipitates were washed twice by centrifugation in cold borate–saline buffer. The amount of the protein in the redissolved (0·1 N NaOH) precipitates was determined by the Lowry method (Lowry, Rosebrough, Farr and Randall, 1951).

Double immunodiffusion

After placing antigen in the wells, the diffusion plate was incubated for 30 minutes at room temperature and then overnight at 4°. Antiserum was added, the plate was first incubated for 30 minutes at room temperature and then at 4° for 2 days. The concentration of antigen (1 mg/ml or 3 mg/ml) was consistent throughout the various experiments, and the amount of antiserum was constant at 20 μl per well.

Immunoelectrophoresis

Immunoelectrophoresis of native antigens and enzyme-treated procollagens was performed as described by Williams (1971). A voltage gradient of 6 V/cm was used during electrophoresis in 1·0 per cent agar, without special provisions for cooling; electrophoresis was performed for 2 hours. After applying the antiserum, the plates were incubated at 4° for 2 days.

Immunodiffusion plates and immunoelectrophoresis plates were stained with amido black (Crowle, 1961).

Enzyme digestions

Rabbit tumour collagenase digestion. The reaction mixture contained 1 ml of the substrate solution (3 mg of native procollagen) in 0·025 M Tris, pH 8·0–0·2 M NaCl–0·0025 M $CaCl_2$ buffer, and 50 μl of rabbit tumour collagenase. The reaction mixtures were incubated for 16 hours at room temperature. The reaction was stopped by the addition of excess EDTA (McCroskery, Wood and Harris, 1973).

Bacterial collagenase digestion. The procollagens were dissolved in 0·1 M NaOH at a concentration of 2·0 mg/ml; 0·2 ml of this substrate solution was chilled to 0°. Sixty micromoles of Hepes buffer (pH 7·2) were added, then 60 μl of 0·15 N HCl were added and the final pH was 9·0. The other components of the reaction mixture were 1·25 μM NEM, 0·25 μM $CaCl_2$, and protease-free bacterial collagenase (20 μg). The tubes were incubated at 37° for 90 minutes with shaking. The reaction was stopped by the addition of excess EDTA (Peterkofsky and Diegelmann, 1971).

Procollagen peptidase digestion. The standard enzyme assay included 0·1 ml of the substrate solution (0·6 mg of native procollagen per millilitre), 0·05 ml of 0·01 M Hepes buffer, pH 7·2 and 0·1 ml of enzyme, in a final volume of 0·25 ml (Kohn *et al.*, 1974). Tubes were incubated at 26° for 6 hours. The reaction was stopped by addition of excess EDTA.

All digests were assayed by SDS–polyacrylamide electrophoresis.

Reduction and alkylation

Cell culture procollagen and dermatosparactic procollagen were reduced and alkylated in 8 M urea by standard methods (Hirs, 1967).

Preparation of subunits of procollagen from the culture medium

[^{3}H]Proline-labelled procollagens were subjected to 4 per cent polyacrylamide gel electrophoresis in the presence of SDS. All gels were fractionated by means of a Gilson gel fractionator. The peaks of θ, pro α1, and pro α2 were separately pooled and each was centrifuged to sediment acrylamide and the supernatant fluids were extensively dialysed against water and lyophilized.

RESULTS

CHARACTERIZATION OF ANTISERA DIRECTED AGAINST DERMATOSPARACTIC PROCOLLAGEN

The antisera gave a typical immunoprecipitation curve which showed an equivalence point (Fig. 1). Twenty microlitres of antiserum were able to precipitate 10 μg of dermatosparactic procollagen at equivalence and this antiserum–antigen ratio was used in subsequent studies. At the equivalence point, the antibody–antigen binding ratio was about

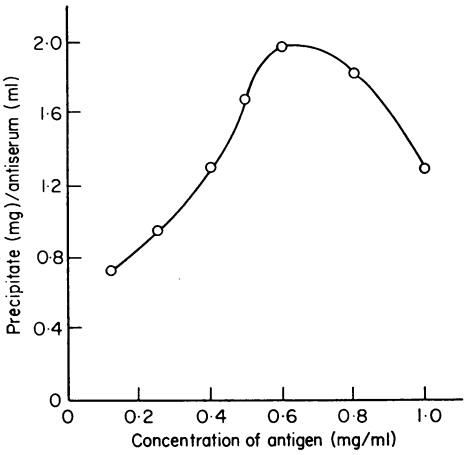

FIG. 1. Quantitative precipitation of dermatosparactic procollagen with anti-dermatosparactic procollagen serum.

TABLE 1

ANTIBODY CONCENTRATION IN THE ANTISERUM DIRECTED AGAINST EITHER DERMATOSPARACTIC PROCOLLAGEN OR THE PROCOLLAGENS ISOLATED FROM THE CELL CULTURE MEDIUM

Group*	Animal	Immunogen	Amount of antibody (mg/ml of serum)
I	Rabbit (3)*	Native dermatosparactic procollagen	2·7
II	Rabbit (3)*	Native dermatosparactic procollagen	5·1
III	Rabbit (1)*	Native procollagen, $(\text{pro } \alpha 1)_2$ pro $\alpha 2$	5·8
IV	Rabbit (1)*	Native procollagen, $(\text{pro } \alpha 1')_3$	8·6

* For groups I and II, and III and IV, respectively, the same immunization schedules were applied. Numbers in parentheses correspond to the number of animals in each group.

2·3. Separation of the immune γ-globulin from the antiserum revealed that the original antibody concentration in the serum ranged from 2·7 to 5·1 mg/ml (Table 1).

CHARACTERIZATION OF ANTISERA DIRECTED AGAINST CELL CULTURE PROCOLLAGENS

The gel precipitation reaction was investigated employing concentrated antibody solutions (Fig. 2). A marked dependence on antibody concentration was found and no

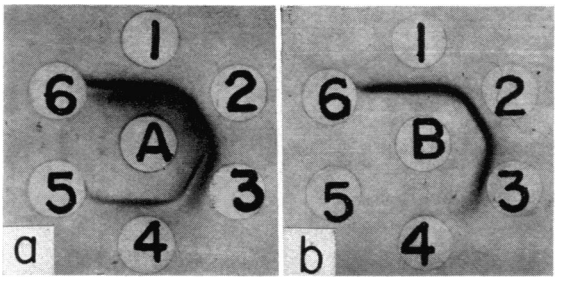

FIG. 2. Double immunodiffusion of antiserum against cell culture procollagens. (a) (A) Cell culture procollagen, (pro $\alpha 1)_2$ pro $\alpha 2$, 1·0 mg/ml; (1) antiserum against cell culture procollagen, (pro $\alpha 1)_2$ pro $\alpha 2$, 38·6 mg/ml; (2) 19·3 mg/ml; (3) 9·7 mg/ml; (4) 4·8 mg/ml; (5) 2·4 mg/ml; (6) 1·2 mg/ml. (b) (B) Cell culture procollagen, (pro $\alpha 1')_3$, 1·0 mg/ml; (1) antiserum against cell culture procollagen, (pro $\alpha 1')_3$, 48·2 mg/ml; (2) 24·1 mg/ml; (3) 12·1 mg/ml; (4) 6·1 mg/ml; (5) 3·1 mg/ml; (6) 1·6 mg/ml.

reaction could be observed below 4·8 mg/ml (Fig. 2). Much higher concentrations of antibody were present in the serum directed against cell culture procollagen than in the corresponding dermatosparactic procollagen antiserum (Table 1). About 5·8 mg/ml of antibody was obtained in the serum against (pro $\alpha 1)_2$ pro $\alpha 2$ procollagen, 8·6 mg/ml in the serum against (pro $\alpha 1')_3$ procollagen, in contrast to an average of 3·9 mg/ml in the serum against dermatosparactic procollagen.

THE IMMUNOLOGICAL RELATIONSHIP BETWEEN DERMATOSPARACTIC PROCOLLAGEN AND PROCOLLAGEN FROM THE CULTURE MEDIUM

In initial studies using the antisera against dermatosparactic procollagen to test the cell culture procollagen, the results indicated that dermatosparactic procollagen gave a line of identity with procollagen (pro $\alpha 1)_2$ pro $\alpha 2$, and its isolated polypeptide subunits (Fig. 3a). Thus, this cell culture procollagen is immunologically equivalent to dermatosparactic procollagen and has similar antigenic determinants in the NH_2-terminal non-collagen extension.

Although the antibody to the procollagen (pro $\alpha 1)_2$ pro $\alpha 2$ gave a line of identity with procollagen (pro $\alpha 1')_3$ (Fig. 3b) the antibody to (pro $\alpha 1')_3$ showed only partial cross-reactivity with (pro $\alpha 1)_2$ pro $\alpha 2$ (Fig. 3c). These results indicate that the antiserum to (pro $\alpha 1')_3$ contains some antibodies which are not present in the antiserum to the (pro $\alpha 1)_2$ pro $\alpha 2$ molecule.

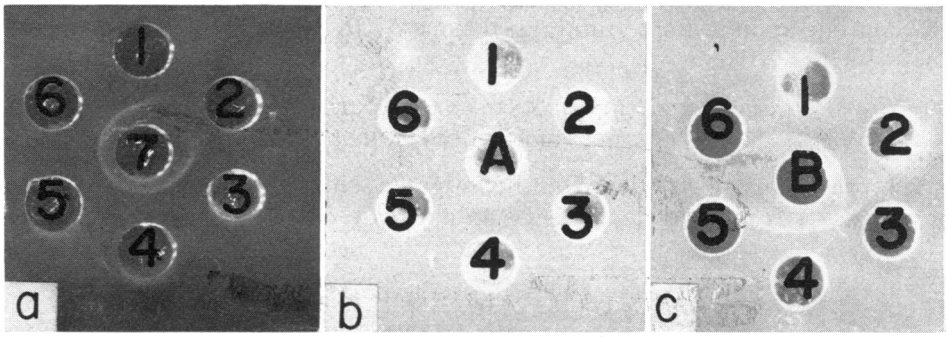

FIG. 3. Double immunodiffusion analysis of procollagens. (a) (7) Antiserum against dermatosparactic procollagen; (1) pro α1 of cell culture procollagen, (pro α1)$_2$ pro α2; (2) pro α2 of cell culture procollagen, (pro α1)$_2$ pro α2; (3) θ chain of cell culture procollagen, (pro α1)$_2$ pro α2; (4) and (6) procollagen (pro α1)$_2$ pro α2; (5) the antigen, dermatosparactic procollagen. (b) (A) Antiserum against cell culture procollagen, (pro α1)$_2$ pro α2; (1) cell culture procollagen (pro α1)$_2$ pro α2, 1 mg/ml; (2) cell culture procollagen (pro α1)$_2$ pro α2, 3 mg/ml; (3) cell culture procollagen (pro α1′)$_3$, 1 mg/ml; (4) cell culture procollagen (pro α1′)$_3$, 3 mg/ml. (c) (B) Antiserum against cell culture procollagen (pro α1′)$_3$; the antigen contents of wells numbers 1–4 are the same as in 3b.

PROCOLLAGEN-SPECIFIC ANTIGENIC DETERMINANTS IN CELL CULTURE PROCOLLAGEN (PRO α1)$_2$ PRO α2

Immunoelectrophoresis of cell culture procollagen (pro α1)$_2$ pro α2, using the antiserum directed against it, showed two major precipitin bands (Fig. 4a). The immunoprecipitation line at the origin well was markedly decreased when less antigen was used (Fig. 4b). This line is probably due to aggregated procollagen which does not undergo electrophoresis. Preincubation of the antigen with a highly purified animal collagenase yields two segments of unequal length; the small segment is from the COOH-terminus of the procollagen and the large segment is from the NH_2-terminus (Tanzer *et al.*, 1974). Immunoelectrophoresis of these digestion products demonstrated the presence of four major precipitin bands (Fig. 4c). The band at the origin well was again attributed to aggregated procollagen, which did not undergo electrophoresis. In order to identify the other bands, the enzyme digest was tested against an antiserum which contained antibodies directed only against the NH_2-terminal procollagen extension. The results showed two immunoprecipitation lines in addition to the line near the origin well (Fig. 4d). The most anodal of the bands is thought to contain the NH_2-terminal fragment, while the one closer to the origin may either be uncleaved substrate or NH_2-terminal fragment still bound to enzyme. Comparison of Fig. 4c and 4d shows that 4c contains an extra band which is furthest from origin; this band probably is the COOH-terminal fragment.

Similar results were obtained when identical studies were done with cell culture procollagen (pro α1′)$_3$ and its antiserum.

Procollagen peptidase, which excises the intact NH_2-terminal peptide extension of procollagen (Kohn *et al.*, 1974), was used to obtain two segments; NH_2-terminal noncollagen peptide and the intact collagen portion containing the COOH-terminal noncollagen extension. Cell culture procollagen (pro α1)$_2$ pro α2 and dermatosparactic procollagen were each treated with procollagen peptidase. The results (Fig. 4e) show that two precipitin bands were observed in wells 1 and 4 which contain enzyme-treated culture procollagen, whereas one band was present in wells 2 and 5, which contain enzyme-treated

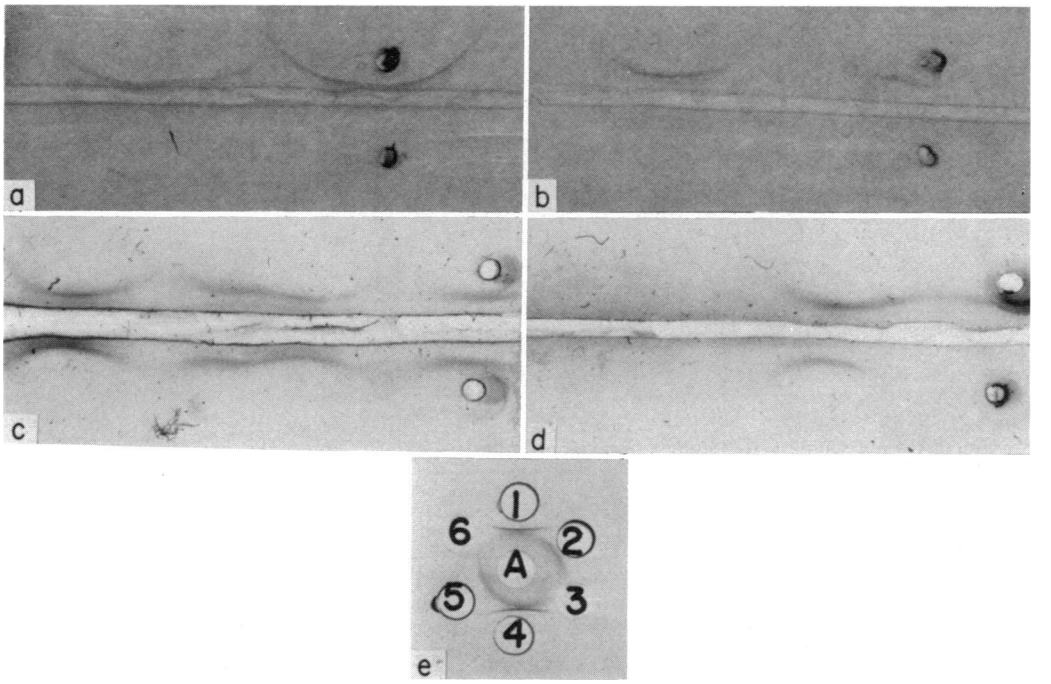

FIG. 4. Immunoelectrophoresis of cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$ against antiserum to the antigen (a) 3 mg/ml, (b) 1 mg/ml. Cell culture procollagen previously digested with rabbit tumour collagenase (c) against antiserum to cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$ and (d) against antiserum to dermatosparactic procollagen. (e) Immunodiffusion analysis of procollagens digested with (1) and (4) procollagen peptidase, cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$; (2) and (5) dermatosparactic procollagen. (A) Antiserum against cell culture procollagen, (pro $\alpha 1)_2$ pro $\alpha 2$. The cathode is to the left in (a)–(d).

dermatosparactic procollagen. These results suggest that the inner precipitin line, which shows a complete line of identity, is probably the NH_2-terminal non-collagen extension peptide. The outer line is probably the collagen portion which contains the COOH-terminal extension.

The above antisera produced against culture medium procollagen were assayed against calf serum in order to determine if the serum used in the culture medium contributed antigens to the purified procollagen samples used to inject into the rabbits. Both immunodiffusion and immunoelectrophoresis assays failed to demonstrate any reactive antigens in the calf serum when tested against the purified medium procollagen. However, when foetal calf serum was assayed by immunodiffusion against medium procollagen type (pro $\alpha 1)_2$ pro $\alpha 2$, a strong immunoprecipitation band was observed. Further studies in which this reactive material was further purified from the foetal calf serum showed that the immunoreactive material was in fact procollagen of a type indistinguishable from the culture medium procollagen type (pro $\alpha 1)_2$ pro $\alpha 2$. Therefore, foetal calf serum was never used in studies involving the purification of cell culture procollagen.

STRUCTURAL REQUIREMENTS OF THE ANTIGENIC DETERMINANTS FOR PROCOLLAGEN

The antisera directed against the cell culture procollagens did not react with calf skin collagen or with the collagen extracted from the cell layer of the cells in culture.

Reduction and alkylation of native procollagen destroyed its antigenic activity as measured by the gel diffusion test (Fig. 5a). No loss of activity was observed after complete digestion of the procollagen with bacterial collagenase (Fig. 5b). However, more rapid

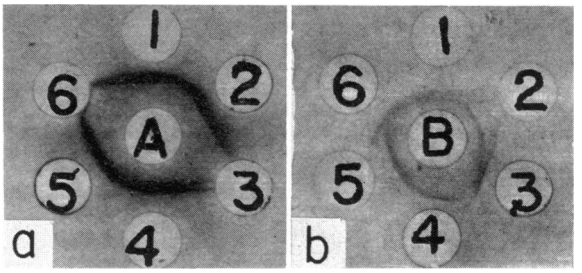

FIG. 5. Double immunodiffusion pattern of procollagens. (a) (A) Antiserum against cell culture procollagen, (pro $\alpha 1)_2$ pro $\alpha 2$; (1) and (4) native cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$, 1 mg/ml; (2) and (5) native cell culture procollagen (pro $\alpha 1')_3$, 1 mg/ml; (3) and (6) reduced and alkylated cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$. (b) (B) Antiserum against cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$; (1) cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$ treated with bacterial collagenase; (2) and (5) dermatosparactic procollagen treated with bacterial collagenase; (3) and (6) native cell culture procollagen (pro $\alpha 1)_2$ pro $\alpha 2$; (4) native dermatosparactic procollagen (4).

migration of the enzyme-digested antigen was observed (well number 1), compared to intact antigen in (wells numbers 3 and 6). Similar results are seen when enzyme-treated dermatosparactic procollagen (wells numbers 2 and 5) is compared with intact dermatosparactic procollagen (well number 4).

The intact and enzyme-digested procollagen (pro $\alpha 1)_2$ pro $\alpha 2$ showed double immunodiffusion lines (wells numbers 1, 3 and 6) in this pattern. The innermost line of identity is thought to be the true result while the outermost line is probably an artifact due to aggregation of the antigen.

DISCUSSION

Two groups of workers have previously immunized rabbits with calf dermatosparactic procollagen (Timpl *et al.*, 1973; Kohn *et al.*, 1974). The major difference in their results was that, in the case of Timpl *et al.* (1973), a small proportion of the antibodies were directed against the collagen portion of the procollagen molecule. This seemed to be due to immunization with large amounts of procollagen (15 mg) because immunization with 0·5 mg or less produced antibodies which only reacted with the NH_2-terminal procollagen extension (Isersky, personal communication). In the present report, we also obtained antisera specific for the procollagen appendage. These antisera showed full immunological identity with the two cell culture procollagens and with two of the isolated polypeptide chains of procollagen (pro $\alpha 1)_2$ pro $\alpha 2$. Conversely, immunization of rabbits with the isolated cell culture procollagens produced antisera which showed full immunological identity with calf dermatosparactic procollagen. The data also showed that reduction and alkylation of the antigens abolished serological reactivity, as previously noted (Timpl *et al.*, 1973).

The detection of COOH-terminal procollagen extensions in the cell culture procollagens, in conjunction with the amino acid composition of COOH-terminal peptides released by an animal collagenase (Tanzer *et al.*, 1974) suggested that antisera to these extensions

might also be produced. The data support this possibility and reinforce the point that the procollagen extensions at both NH_2 and COOH termini are more potent immunogens than the main portion of the collagen triple-helix. Indeed, earlier studies have shown that one of the major antigenic determinants in collagen is a *short* COOH-terminal procollagen extension (Becker, Timpl and Kühn, 1972). We interpret the present results to indicate that the long extension at the COOH-terminus in procollagen provides additional antigenic determinants, similar to the situation at the NH_2-terminal end. Fig. 6 illustrates

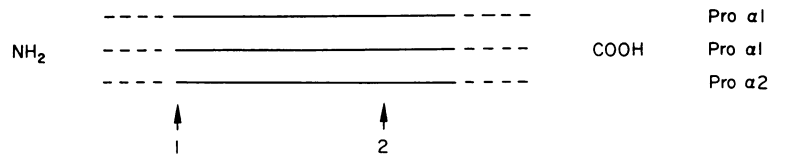

FIG. 6. Schematic diagram of procollagen (pro $\alpha 1)_2$ pro $\alpha 2$ or (pro $\alpha 1')_3$ and the points of attack of: (1) procollagen peptidase; (2) rabbit tumour collagenase. The collagen helical region is depicted by the solid lines while the non-collagen peptide extensions are shown by the dashed lines.

the procollagen extensions at each end of the procollagen molecule and shows the approximate point of attack of the enzymes used to help localize the antigenic determinants.

The immunological cross-reactivity of calf dermatosparactic and cell culture procollagens is consistent with their origin from the same species and with their related molecular structures. The presence of additional antigenic determinants in one cell culture procollagen compared to the other may reflect the proposed unique genetic origins for each of these proteins (Church *et al.*, 1973). The molecular basis for this immunological difference remains to be elucidated.

ACKNOWLEDGMENTS

We thank the following workers for the materials they provided: Dr L. D. Kohn, National Institute of Health, Bethesda, Maryland; Dr E. D. Harris, Jr and Mr P. A. McCroskery, Dartmouth Medical School, Hanover, New Hampshire.

Valuable discussions with Dr E. L. Becker (Department of Pathology, University of Connecticut Health Center, Farmington, Connecticut) and the helpful assistance of Mr S. W. Kegeles are greatly acknowledged.

This research work was supported by the USPHS (grants numbers AM-12683 and AM-17220) and the NSF (grant number GB-31077).

REFERENCES

BECKER, U., TIMPL, R. and KÜHN, K. (1972). 'Carboxyterminal antigenic determinants of collagen from calf skin.' *Europ. J. Biochem.*, **28,** 221.

CAMPBELL, D. H., GARVEY, J. S., CREMER, N. E. and SUSSDORF, D. H. (1970). 'Quantitative determination of precipitating antibody.' *Methods in Immunology*, 2nd edn, p. 246. W. A. Benjamin, New York.

CHURCH, R. L., LAPIERE, C. M. and TANZER, M. L. (1973). 'Identification of two distinct species of procollagen synthesized by a clonal line of calf dermatosparactic cells.' *Nature: New Biology*, **244,** 188.

CHURCH, R. L., YAEGER, J. A. and TANZER, M. L. (1974). 'Isolation and partial characterization of procollagen fractions produced by a clonal strain of calf dermatosparactic cells.' *J. molec. Biol.*, **86,** 785.

CROWLE, A. L. (1961). *Immunodiffusion*, 1st edn, p. 265. Academic Press, New York.

FAHEY, J. L. (1967). 'Chromatographic separation of immunoglobulins.' *Methods in Immunology and Immunochemistry*, Volume I (ed. by C. A. Williams and M. W. Chase), p. 321. Academic Press, New York.

HIRS, C. M. W. (1967). 'Reduction and *S*-carboxymethylation of proteins.' *Methods in Enzymology*, 1st edn, volume II, p. 199. Academic Press, New York.

KOHN, L. D., ISERSKY, C., ZUPNIK, J., LENAERS, A., LEE, G. and LAPIERE, C. M. (1974). 'Calf tendon procollagen peptidase: its purification and endo-

peptidase mode of action.' *Proc. nat. Acad. Sci. (Wash.)*, **71,** 40.

LAPIERE, C. M., LENAERS, A. and KOHN, L. D. (1971). 'Procollagen peptidase: an enzyme excising the coordination peptides of procollagen.' *Proc. nat. Acad. Sci. (Wash.)*, **68,** 3054.

LENAERS, A., ANSAY, M., NUSGENS, B. V. and LAPIERE, C. M. (1971). 'Collagen made of extended α-chains procollagen, in genetically-defective dermatosparactic calves.' *Europ. J. Biochem.*, **23,** 533.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. and RANDALL, N. J. (1951). 'Protein measurement with the Folin phenol reagent.' *J. biol. Chem.*, **193,** 265.

MCCROSKEY, R. A., WOOD, S., JR and HARRIS, E. D., JR (1973). 'Gelatin: a poor substrate for a mammalian collagenase.' *Science*, **182,** 70.

PETERKOFSKY, B. and DIEGELMANN, R. (1971). 'Use of mixture of proteinase-free collagenase for the specific assay of radioactive collagen in the presence of other proteins.' *Biochemistry*, **10,** 988.

TANZER M. L. CHURCH R. L. YAEGER, J. A., WAMPLER, D. E. and PARK, E. (1974). 'Procollagen: intermediate forms containing several types of peptide chains and non-collagen peptide extensions at NH_2 and COOH ends.' *Proc. nat. Acad. Sci. (Wash.)*, **71,** 3009.

TIMPL, R., WICK, G., FURTHMAYR, H., LAPIERE, C. M. and KÜHN, K. (1973). 'Immunochemical properties of procollagen from dermatosparactic calves.' *Europ. J. Biochem.*, **32,** 584.

WILLIAMS, C. A. and CHASE, M. W. (1967). *Methods in Immunology and Immunochemistry*, 1st edn, volume 1, p. 321. Academic Press, New York.

WILLIAMS, C. A. (1971). *Methods in Immunology and Immunochemistry* 'Immunoelectrophoretic analysis (IEA)' (ed. by C. A. Williams and M. W. Chase), 1st edn, volume 3, p. 234. Academic Press, New York.