

# The Antigenic Properties of Some Synthetic Poly-Iminoacids

## II. THE ANTIGENICITY OF POLYPEPTIDES RELATED TO COLLAGEN; PEPTIDES CONTAINING HYDROXYPROLINE AND ACETYL-HYDROXYPROLINE

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**Summary.** The antigenic properties of some synthetic polymers containing hydroxyproline and acetyl-hydroxyproline have been tested in guinea-pigs and rabbits by active cutaneous anaphylaxis, delayed skin hypersensitivity reactions, PCA, tanned cell agglutination and fluorescent antibody microscopy.

The antigenic relationships between these polymers, collagen and acetylated collagen have been investigated. The results obtained suggest that acetyl-hydroxyproline is a common antigenic determinant in both acetylated copolymers and acetylated collagen.

Poly-hydroxyproline and poly-acetyl-hydroxyproline were found not to be antigenic in rabbits or guinea-pigs. Rabbit antiserum against acetylated collagen has been used to stain acetylated tissue sections by immunofluorescence. Absorption studies indicate that acetyl-hydroxyproline groups are important antigenic determinants as shown by the considerable decrease in specific fluorescence when the rabbit anti-acetylated collagen is absorbed with a synthetic polymer containing acetyl-hydroxyproline.

The overall results are discussed in terms of the structural similarity existing between collagen and some of the polymers used in this work.

### INTRODUCTION

Since proline and hydroxyproline constitute 25 per cent of the residues of collagen, the antigenic properties of synthetic polypeptides containing these iminoacids have some bearing on the problem of the antigenicity of collagen. The similarity of the helical structure of this fibrous protein with some of these polymers is also of interest when their possible antigenic relationships are considered.

We have already reported on synthetic polypeptides containing proline (Jasin and Glynn, 1965); the present work describes the antigenic properties of some polymers of hydroxyproline and acetyl-hydroxyproline and their antigenic relationship to collagen and acetylated collagen.

### MATERIALS AND METHODS

Table 1 lists the polymers used in this work and some of their characteristics. The abbreviations shown in the Table are those employed throughout this paper. A more

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detailed description of their properties as well as the methods of immunization and antibody detection will be found in our previous paper (Jasin and Glynn, 1965).

### Materials

Extracted collagen was kindly supplied by Dr. G. Bowes of the British Leather Manufacturers Research Association. Two forms were used: one, a 'standard hide powder', had been obtained by the treatment of bovine hide with saturated lime water, the other

TABLE I  
LIST OF POLYPEPTIDES TESTED

<i>Polymer</i>	<i>Symbol abbreviation</i>	<i>Helix</i>	<i>Molecular weight</i>	<i>Solubility in water</i>	<i>Observations</i>
Poly hydroxy-L-proline Form II	p.H.	Left	11000	Soluble	—
Poly acetyl hydroxy-L-proline Form I	FIp.acH	Right	16000	Insoluble	—
1 : 1 : 1 Poly-L-proline-glycine acetyl hydroxy-L-proline Form II	p.PGacH	Left	>4000	Partially soluble	Inhomogeneous
1 : 1 : 1 Poly-L-proline-glycine hydroxy-L-proline	p.PGH	Left	>4000	Partially soluble	Inhomogeneous
1 : 1 Poly-L-proline-glycine Form II	p.PG	Left	>4000	Partially soluble	Inhomogeneous
Poly-L-proline Form II	p.P170	Left	17000	Soluble	—

by extraction of the middle layer of ox hide with 0.1 M disodium hydrogen phosphate. Rat neutral salt soluble collagen was extracted with a solution containing 0.14 M NaCl, 0.01 M sodium phosphate (pH 7.0) at 4° for 24 hours from finely minced chilled skin of weanling rats. The extract was spun at 1750 g for 30 minutes in the cold and the supernate after dilution used for skin testing.

Ox gelatin was obtained from the Soci t  des Produits Chimiques Coignet, Brussels, Belgium.

Crystalline bovine serum albumin (BSA) and bovine fraction V were supplied by Armour Co.

### Methods

Gelatin fragments of different molecular weights were obtained by controlled acid hydrolysis according to Courts (1954). A method using non-aqueous media (Green, Ang and Lam, 1953) was used to acetylate collagen and BSA. Collagen and its acetylated form as well as acetylated BSA were insoluble in water; before using them for immunization and skin testing 1 per cent suspensions in saline were subjected to sonication at 4° in an MSE Ultrasonic Disintegrator No. 3000 at 1.6 amp. Collagen and acetylated collagen were sonicated for 3 minutes, acetylated BSA for 30 seconds.

Each batch of the finely suspended sonicate was analysed for hydroxyproline after acid hydrolysis (Prockop and Udenfriend, 1960) as modified by Jasin, Fink, Wise and Ziff

(1962). Using this mild sonication procedure only 4-7 per cent of the insoluble material was found in the sonicate.

Rabbits were immunized with 0.5 mg. of hide powder or the acetylated sonicates. Details of immunization and tests for immediate and delayed reactivity are given in our previous paper (Jasin and Glynn, 1965).

*Immunofluorescent ultraviolet microscopy* was done following Coons and Kaplan (1950). In order to minimize non-specific staining both test sera and fluorescein conjugates were diluted with an equal volume of 20 per cent bovine fraction V in phosphate buffer saline pH 7.0 (Nakhla and Glynn, personal communication). Tissues were either snap frozen in a dry ice-acetone bath or fixed in 95 per cent ethanol at 4° (Sainte-Marie, 1962).

Acetylation was carried out on the fixed tissue sections by immersing them in a 1:1 v./v. solution of acetic acid-acetic anhydride for 18 hours at 20°; they were then washed in 95 per cent ethanol. Control sections were left in 95 per cent ethanol. Both were rehydrated through graded ethanol and finally kept in buffered saline, pH 7.0, prior to incubation with the test antisera.

For enzyme treatment, the sections were rehydrated through graded ethanol prior to acetylation, then incubated with a 2.5 per cent solution of ovine testicular hyaluronidase (Seravac Laboratories, Colnbrook, Bucks.) at 37° for 1 hour, washed in buffered saline and dehydrated. Control sections were treated in the same way but incubated with buffered saline only.

## RESULTS

Table 2 shows the results obtained in guinea-pigs immunized with the synthetic polypeptides, collagen and acetylated collagen. It is interesting to note that the homopolymers tested were not antigenic. Hide powder was a very weak antigen, but in its acetylated form sensitized all animals injected. In the case of p.PGacH the proportion of responding animals increased with the use of incomplete Freund's adjuvant and still further with complete adjuvant; delayed skin reactions were obtained only when the copolymer was given with complete adjuvant.

These results were confirmed by tanned cell agglutination tests (Table 3). Sera from guinea-pigs and rabbits repeatedly injected with homopolymers and from rabbits injected with the copolymers failed to show any agglutination while sera from either species immunized with acetylated collagen agglutinated tanned red cells coated with p.PGacH.

PCA tests were performed with all the negative sera and confirmed the results obtained with the tanned cell agglutination procedure.

Precipitating antibodies were not detected in any of the sera and complement fixation tests using guinea-pig anti-p.PGacH were negative with a wide range of antigen concentrations.

Table 4 shows the pattern of cross-reactions between the different antigens, tested in guinea-pigs by active cutaneous anaphylaxis. Animals immunized with p.PGH cross-reacted with polymers containing proline or proline and glycine but no reaction was obtained with either p.H or p.acH. With the acetylated polymer similar results were seen and in addition the animals reacted to FIp.acH. It is interesting to note that guinea-pigs sensitized with acetylated collagen cross-reacted not only with FIp.acH but also with polymers containing a poly-hydroxyproline sequence. These animals reacted also to hide powder and neutral salt soluble acetylated collagen. No reactions were seen using acetylated BSA. In addition, guinea-pigs immunized with p.PGH and p.PGacH were

TABLE 2

SKIN TESTS OF GUINEA-PIGS IMMUNIZED AND TESTED WITH COLLAGEN, ACETYLATED COLLAGEN AND SYNTHETIC POLY-PEPTIDES CONTAINING HYDROXYPROLINE AND ACETYL HYDROXYPROLINE

Figures show numbers positive/numbers tested.

Antigen	Immunizing dose (µg.)	Complete Freund's adjuvant		Incomplete Freund's adjuvant		No adjuvant	
		Immediate reaction	Delayed reaction	Immediate reaction	Delayed reaction	Immediate reaction	Delayed reaction
p.PGH	150*	4/5	4/5	—	—	—	—
	↓ 500	4/5	4/5	—	—	—	—
p.PGacH	150	9/9	9/9	—	—	—	—
	500	4/4	4/4	5/8	0/8	1/5	0/5
	↓ 500	—	—	5/8	0/8	1/4	0/4
p.H.	150	0/5	0/5	—	—	—	—
	↓ 500	0/5	0/5	—	—	—	—
	↓ 1000†	0/5	0/5	—	—	—	—
Flp.acH	150	0/5	0/5	—	—	—	—
	↓ 500	—	—	—	—	—	—
	↓ 1000†	0/5	0/5	—	—	—	—
Calf collagen	150	0/5	2/5	—	—	—	—
	↓ 150	2/5	2/5	—	—	—	—
Acetylated calf collagen	100	10/10	10/10‡	—	—	—	—

\* Downward arrow indicates reinjection.

† Intraperitoneal route.

‡ 7/10 were Arthus reactions with necrosis.

TABLE 3

AGGLUTINATION TITRES OF SERA FROM GUINEA-PIGS AND RABBITS IMMUNIZED WITH PEPTIDE OR COLLAGEN DERIVATIVES AND TESTED AGAINST TANNED RED CELLS CORRESPONDINGLY COATED

Immunizing and coating antigen	Reciprocal of titre						
	Guinea-pig sera					Rabbit sera	
p.PGH	20	20	80	160		< 10	< 10
p.PGacH	640	640	2560	320		< 10	< 10
p.H	< 10	< 10	< 10	< 10		< 10	< 10
Flp.acH*	< 10	< 10	< 10	< 10		< 10	< 10
Calf collagen†	< 10	< 10	< 10	< 10		< 10	< 10
Acetylated calf collagen*	1600	800	800	400	1600	2560	2560

\* Tanned cells coated with p.PGacH.

† Tanned cells coated with p.PGH.

tested with ox gelatin fragments of different molecular weights, rat neutral salt soluble collagen, egg albumin, and human  $\gamma$ -globulin and gave negative immediate and delayed skin reactions.

The availability of antisera reacting with an acetyl-hydroxyproline group prompted

TABLE 4

CROSS-REACTIVITY TO DIFFERENT SYNTHETIC POLYMERS AND COLLAGEN DERIVATIVES SHOWN BY IMMEDIATE SKIN REACTIONS IN IMMUNIZED GUINEA-PIGS

<i>Immunizing antigen</i>	<i>Test antigen</i>							
	<i>p.PGH</i>	<i>p.PGacH</i>	<i>FIIp.P170</i>	<i>p.PG</i>	<i>p.H</i>	<i>FIp.acH</i>	<i>Acetylated collagen</i>	<i>Collagen</i>
p.PGH	4/5	4/5	4/5	4/5	0/5	0/5	0/5	0/5
p.PGacH	5/5	5/5	5/5	5/5	0/5	5/5	1/5	0/5
Acetylated calf collagen	3/5	5/5	0/5	N.D.*	4/5	5/5	5/5	3/5
Calf collagen	0/5	0/5	0/5	0/5	0/5	0/5	0/5	2/5

\* N.D. = not done.

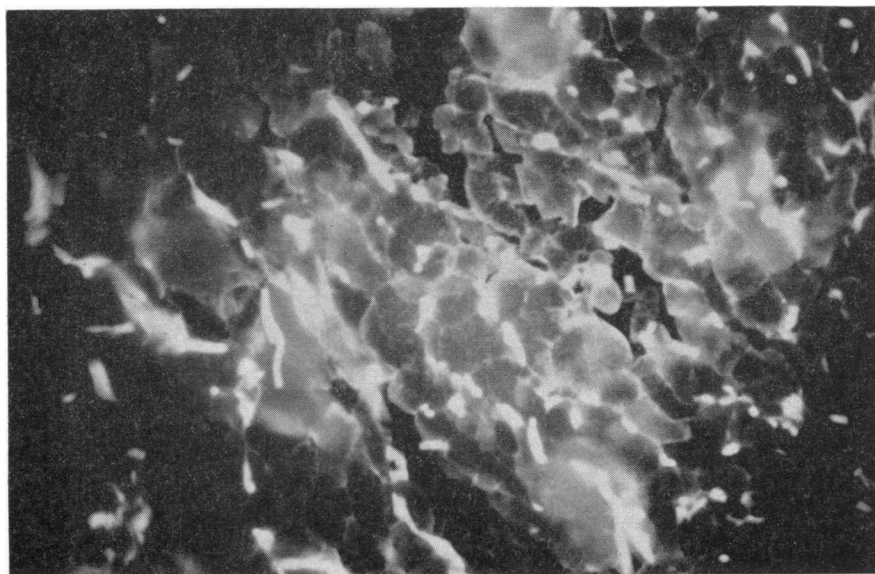
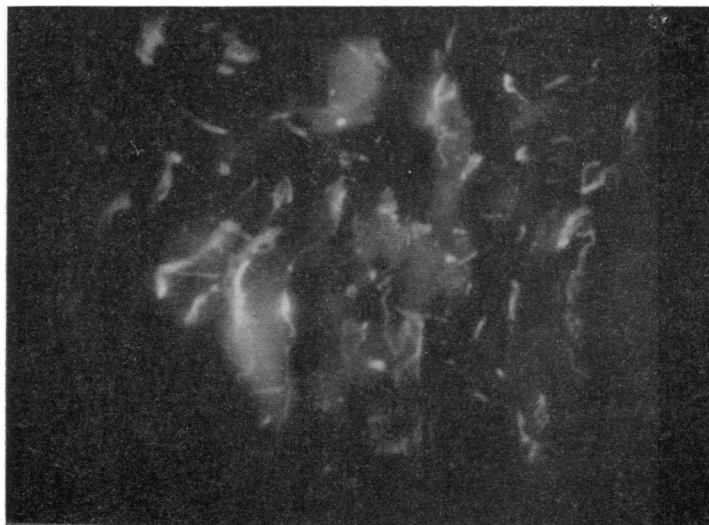
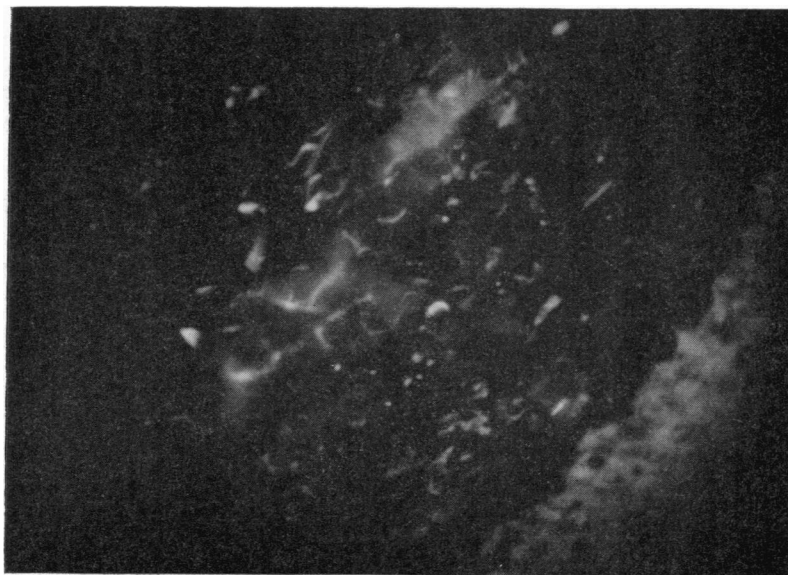


FIG. 1. Acetylated human skin incubated with rabbit anti-acetylated collagen serum and stained with fluorescent goat anti-rabbit  $\gamma$ -globulin.  $\times 250$ .

us to investigate its fixation by acetylated tissues rich in collagen. Fig. 1 shows a section of acetylated human skin treated with rabbit anti-acetylated calf collagen and stained with fluorescein-conjugated goat anti-rabbit  $\gamma$ -globulin. The thick fluorescent lines represent blueish collagen autofluorescence while the specific apple green fluorescence is seen as a fine contour around the thick collagen bundles of the deep dermis. Fig. 2 shows a control section using an unrelated hyperimmune rabbit serum and Fig. 3 represents non-acetylated human skin treated as in Fig. 1.



**FIG. 2.** Acetylated human skin incubated with an unrelated hyperimmune rabbit serum and stained as in Fig. 1.  $\times 250$ .



**FIG. 3.** Normal human skin treated as in Fig. 1.  $\times 250$ .

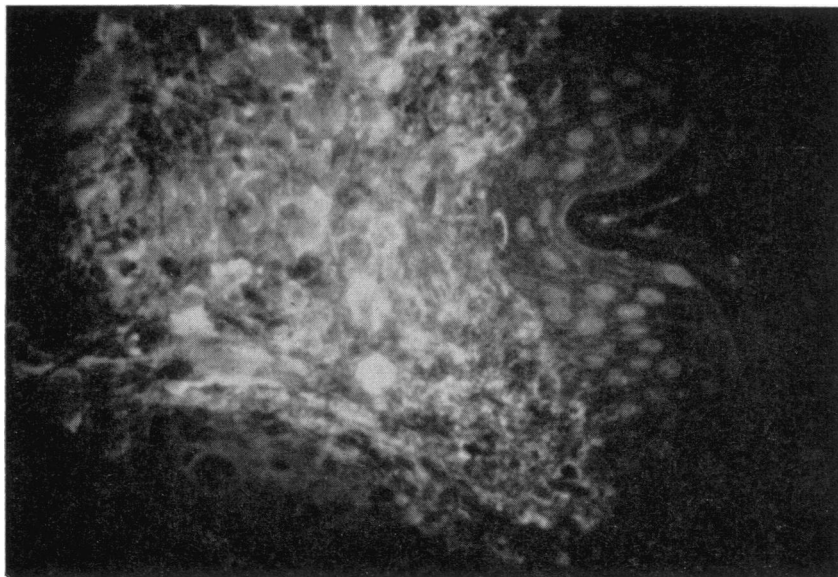


FIG. 4. Acetylated human skin incubated with rabbit anti-acetylated collagen serum absorbed with p.PGH and stained as in Fig. 1.  $\times 250$ .

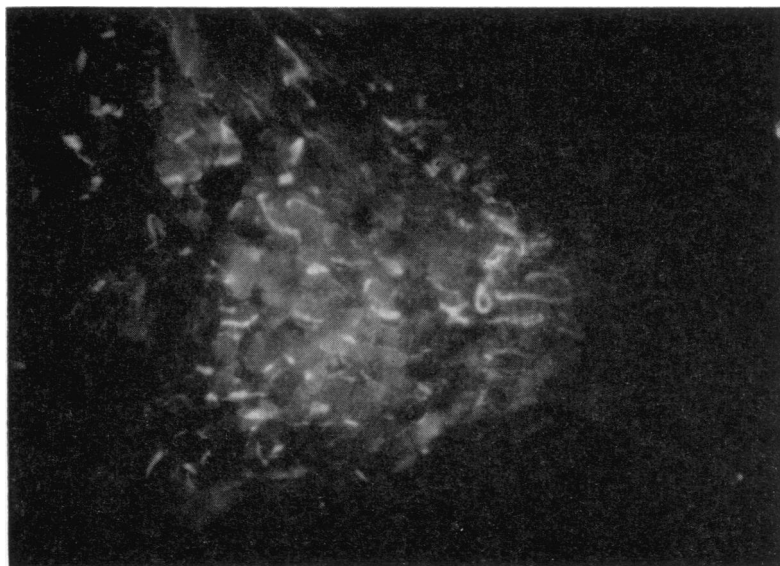


FIG. 5. Acetylated human skin incubated with rabbit anti-acetylated collagen absorbed with p.PGacH and stained as in Fig. 1.  $\times 250$ .

In order to show what was the contribution of the antibodies directed against the acetyl-hydroxyproline groups, the rabbit antiserum was absorbed with 0.200 mg./ml. of either p.PGH (Fig. 4) or p.PGacH (Fig. 5). It is readily apparent that p.PGH is not able to decrease collagen staining while a great deal of the specific fluorescence has disappeared from the section treated with the antiserum absorbed with the acetylated terpolymer. Absorption with hide powder and acetylated BSA failed to decrease the intensity of the specific fluorescence or to alter the staining pattern.

Fig. 4 also shows the pattern obtained in the superficial dermal layers. The thin collagen fibres adjacent to the epidermis are well stained while the basement membrane is not. Treatment of the sections with hyaluronidase prior to acetylation failed to render stainable the collagen presumably present in the basement membranes.

Guinea-pig anti-p.PGacH sera were also used to treat acetylated skin sections but non-specific fluorescence made it impossible to ascertain the presence of *bona fide* staining.

Rabbit antiserum against acetylated collagen was also used to treat human skin from senile elastosis in order to find out if this substance contained hydroxyproline available for acetylation. Neither the control nor the acetylated elastotic material showed any specific uptake of fluorescence.

## DISCUSSION

The above results show that both p.PGH and p.PGacH are antigenic in guinea-pigs. In a previous paper (Jasin and Glynn, 1965) we showed that a homopolymer, poly-L-proline, and a proline-glycine copolymer were antigenic in guinea-pigs and not in rabbits. The addition of hydroxyproline or acetyl-hydroxyproline has not altered this property. Furthermore cross-reactivity studies (Table 4) suggest that in the case of p.PGH the antibody is mainly directed against the poly-proline sequences; no reaction was obtained when the animals were tested with p.H. Owing to the different polymerization rates of the three amino acid anhydrides these polymers are very likely to contain long poly-proline or hydroxyproline sequences (C. H. Bamford, personal communication), and furthermore the water soluble fraction of p.PGH and p.PG was rich in imino-acid (Jasin and Glynn, 1965), so it is not surprising that animals immunized with these polymers cross-reacted with poly-proline.

The cross-reactivity results in Table 4 also show, in animals injected with p.PGacH, the presence of antibody directed against one or more acetyl-hydroxyproline groups. The following considerations support the possibility that this particular antigenic determinant consists of only one acetyl-hydroxyproline group: the immunizing antigen was in its Form II and the guinea-pigs reacted to Form I of p.acH (for a discussion of the secondary structure of the polymers tested see Jasin and Glynn, 1965); one animal cross-reacted with acetylated collagen which is unlikely to have a hydroxyprolyl-hydroxyproline sequence; and lastly, acetyl-hydroxyproline seems to behave as a hapten in this system. This last conclusion arises from our observation that guinea-pigs immunized with p.PGacH developed delayed skin hypersensitivity reactions to p.PG and p.P170 but were negative with FIp.acH suggesting that the poly-proline sequence acted as a 'carrier' and the acetyl-hydroxyproline groups as haptens. Furthermore p.acH with a molecular weight of 16,000 was not antigenic.

It is well known (Ram and Maurer, 1957, 1958) that antisera against acetylated antigens will cross-react extensively with the native molecules. In the present work we have



found that the guinea-pigs immunized with acetylated collagen reacted also with the native hide powder. We should point out that the possibility of an acetylated impurity giving rise to this cross-reactivity cannot be ruled out. The fact that some animals immunized with acetylated collagen reacted also to p.H would indicate that the cross-reactions involved collagen and not an impurity.

Our polymers and especially p.PGH are structurally very similar to collagen: both presumably have sequences of gly-pro-hypro; their secondary structure is almost identical in that they share the poly-proline Form II type helix (Cowan and McGavin, 1955; Harrington and von Hippel, 1961). In addition, the three chain association is present in both, the only difference being the presence of a coiled coil structure in the case of collagen which only slightly alters the configuration of each individual chain (Crick and Rich, 1955). In spite of this similarity we have failed to demonstrate any cross-reactivity to collagen in animals immunized with the copolymers. The reactions obtained with p.H in guinea-pigs immunized with acetylated collagen are likely to indicate the presence of some antibody directed against only one hydroxyproline ring. From our results it is clear that the antibodies to our synthetic polymers were mainly directed either against one acetyl-hydroxyproline group or against a poly-proline sequence, and we must conclude that the differences in structure between poly-proline and collagen are great enough to make these two immunologically non-cross-reacting. This conclusion is supported by the work of Sela and Arnon (1960) showing that anti-gelatin antibodies do not cross-react with peptides similar to ours. It should also be noted that Schmitt, Levine, Drake, Rubin Pfahl and Davison (1964) have shown that the main antigenic determinants in collagen are the non-collagenous polar telopeptides which do not contain iminoacids.

The fluorescent staining of acetylated skin sections clearly demonstrates the presence of antibodies against acetyl-hydroxyproline. This is shown by the absence of staining in non-acetylated tissue sections, the failure to absorb the antibody activity with collagen, acetylated BSA, or p.PGH, and the considerable reduction of fluorescence when the antiserum was absorbed with p.PGAcH.

Although the collagen bundles and smaller fibrils were well stained it was not possible to detect any fluorescence in the basement membranes even after hyaluronidase treatment. The absence of staining of this structure suggests that either there is no collagen present, or the hydroxyproline is not available for acetylation or if acetylated the antibody cannot reach the antigenic groups. The presence of mucoproteins covering this structure could well explain our findings. In this context, we may note that Loewi (1965) in our laboratory has been able to stain basement membranes using an antiserum against the non-poly-saccharide fraction of chondro-mucoprotein.

The application of the fluorescent antibody technique to the study of elastotic degeneration of the skin was prompted by the suggestion made some years ago by Loewi, Glynn and Dorling (1960) that this form of degeneration might result from acetylation of collagen. Many of the features of the elastotic material as shown by histological staining and enzyme digestibility can be imitated by collagen after acetylation and the availability of an antibody with specificity for acetylated hydroxyproline provided an excellent opportunity to test this hypothesis. The entirely negative result obtained makes the hypothesis untenable. Moreover the failure to induce staining by previous acetylation suggests that the elastotic material is neither collagen nor a collagen derivative, unless its hydroxyproline residues are in some way protected from the acetylation process as they are apparently in the basement membrane between epidermis and dermis.

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## REFERENCES

- COONS, A. H. and KAPLAN, M. H. (1950). 'Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of a fluorescent antibody.' *J. exp. Med.*, **91**, 1.
- COURTS, A. (1954). 'The N-terminal amino acid residues of gelatin. 2. Thermal degradation.' *Biochem. J.*, **58**, 74.
- COWAN, P. M. and MCGAVIN, S. (1955). 'Structure of poly-L-proline.' *Nature (Lond.)*, **176**, 501.
- CRICK, F. H. C. and RICH, A. (1955). 'Structure of polyglycine. II.' *Nature (Lond.)*, **176**, 780.
- GREEN, R. W., ANG, K. P. and LAM, L. C. (1953). 'Acetylation of collagen.' *Biochem. J.*, **54**, 181.
- HARRINGTON, W. F. and VON HIPPEL, P. H. (1961). 'The structure of collagen and gelatin.' *Advanc. Protein Chemistry*, **16**, 19.
- JASIN, H. E., FINK, C. W., WISE, W. and ZIFF, M. (1962). 'Relationship between urinary hydroxyproline and growth.' *J. clin. Invest.*, **41**, 1928.
- JASIN, H. E. and GLYNN, L. E. (1965). 'The antigenicity of some synthetic poly-iminoacids. I. Antigenic properties of poly-L-proline.' *Immunology*, **8**, 95.
- LOEWI, G., GLYNN, L. E. and DORLING, J. (1960). 'Studies on the nature of collagen degeneration.' *J. Path. Bact.*, **80**, 1.
- LOEWI, G. and MUIR, H. (1965). 'The antigenicity of chondromucoprotein.' *Immunology* (In press).
- PROCKOP, D. J. and UDENFRIEND, S. (1960). 'A specific method for the analysis of hydroxyproline in tissues and urine.' *Analyt. Biochem.*, **1**, 228.
- RAM, J. S. and MAURER, P. H. (1957). 'Immunochemical studies on some acetylated proteins; acetylated rabbit serum albumin an antigen in rabbits.' *Arch. Biochem. Biophys.*, **72**, 119.
- RAM, J. S. and MAURER, P. H. (1958). 'Modified bovine serum albumin. IV. Characterization and immunochemical studies of the O-acetylated derivative.' *Arch. Biochem. Biophys.*, **74**, 119.
- SAINTE-MARIE, G. (1962). 'A paraffin embedding technique for studies employing immunofluorescence.' *J. Histochem. Cytochem.*, **10**, 250.
- SCHMITT, F. O., LEVINE, L., DRAKE, M. P., RUBIN, A. L., PFAHL, D. and DAVISON, P. F. (1964). 'The antigenicity of tropocollagen.' *Proc. nat. Acad. Sci. (Wash.)*, **51**, 493.
- SELA, M. and ARNON, R. (1960). 'Studies on the chemical basis of the antigenicity of proteins. 3. The role of rigidity in the antigenicity of polypeptidyl gelatins.' *Biochem. J.*, **77**, 394.