Chemical Analysis of the Dermis in Scleroderma.* (32415)

R. FLEISCHMAJER AND S. KROL (Introduced by John H. Moyer)
Section of Dermatology, Department of Medicine, Hahnemann Medical College and Hospital, Philadelphia, Pa.

Scleroderma is a striking example of a mesenchymal disorder. Histochemical studies at different stages of the disease showed no abnormalities in the ground substance and collagen fibers(1,2). Electronmicroscopic studies of the collagen fibers revealed a normal periodicity(1,3). Rasmussen *et al*(4) noted that the collagen in scleroderma is normal in its physical property of hydrothermal shrinking. Hydroxyproline content, on a weight basis, amino acid composition and X-ray diffraction studies of collagen fibers failed to disclose any abnormalities(5). Harris *et al*(6) reported a decrease in acid soluble collagen with normal distribution of alpha and beta components. Seville(3) noted by electron microscopy, an increase in amorphous materials in the ground substance proportional to the degree of swelling and homogenization of the dermis. Musso(7) also suggested an increase of the ground substance in scleroderma which was very susceptible to trypsin digestion. Denko and Stoughton (8) found an increased uptake of S35 ($Na_2S^{35}O_4$) by skin of patients with scleroderma, although these authors did not suggest that this finding was indicative of an increase in acid mucopolysaccharides. Boas and Foley (9) referred to an increase in hexosamines in the corium and subcutaneous tissue from scleroderma although this data was not published. The main purpose of this investigation is to report the results of some chemical analyses performed on the total dermis, ground substance, and collagen obtained from 9 cases of scleroderma.

Materials and methods. Skin specimens were obtained from 9 cases of scleroderma with the following clinical diagnosis: diffuse scleroderma (Cases 1 and 2), acrosclerosis (Cases 3, 5, 6, 7, 8, 9) and localized scleroderma (Case 4). The specimens of Case 1 and 2 were obtained during autopsy while the others following regular skin biopsies.

Total dermis. The subcutaneous fat was carefully trimmed, the epidermis removed by stretching and scraping with a scalpel, and the samples weighed before and after lyophilization to determine dry weight and water content. The dry specimens were processed through a Wiley micro-model mill in order to convert them into a fine powder. Aliquots were hydrolyzed with 6 N HCl at 110°C for 20 hours in vacuum sealed tubes and used to determine the total hydroxyproline content by the method of Stegemann (10). Hexosamines were determined by the method of Boas(11). Eight normal control specimens from fresh autopsy material were obtained from the medial incision and subjected to similar analysis.

Ground substance. In order to perform chemical analysis of the ground substance, 2 scleroderma (Cases 1 and 2) and 8 normal dermal specimens of 400 mg, dry weight each, were extracted in a homogenizer at high speed with a 0.15 M sodium chloride solution for 20 minutes at 4°C. This operation was repeated 4 times. The extracts were dialyzed against distilled water for 72 hours and brought to dryness by lyophilization. The following chemical analyses were performed: total proteins(12), tyrosine(13), hydroxyproline, hexoses (anthrone) and hexosamines. The proteins of the NaCl extracts were studied by acrylamide gel electrophoresis (Cases 1, 2, 3) using a modification of the Raymond's technic(14) which consisted of an adaptation of the Ornstein method(15) to the vertical slab gel electrophoresis. The spacer gel consisted of 3.5% acrylamide, 1 N HCl, Tris (tris (hydroxymethyl) aminomethane) and TEMED (N, N, N', N'-Tetramethylethylenediamine), pH 6.7, while the running gel consisted of 7% acrylamide gel in Tris glycine buffer, pH 8.9. Samples were run for 1 hour at 300 volts (110 milliamperes) and stained for proteins with amido black.

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Collagen-bound hexoses, hexosamines and free aldehydes. The subcutaneous fat was carefully trimmed, the epidermis removed by stretching and scraping with a scalpel and the dermis cut into small pieces and lyophilized. The dry specimens were passed through a micro-mill and converted into a fine powder. Aliquots were extracted with 0.15 M NaCl solution in a Virtis homogenizer for 20 minutes at 4°C (20 ml/g dry weight). This operation was also performed with 0.1 N Na_2HPO_4 in a similar fashion to that described above. The same operation was repeated with 0.1 M citrate buffer, pH 3.8 to remove the acid soluble collagen. The residue was dialyzed against distilled water at 4°C for 72 hours and autoclaved at 20 lb pressure for 8 hours to precipitate the elastic tissue and convert the insoluble collagen into gelatin. After centrifugation, the supernatant was decanted, filtered, lyophilized and labelled as gelatinized insoluble collagen. To determine the purity of this gelatin, 2 samples of normal and 2 of scleroderma were subjected to amino acid analysis by the method of Piez and Morris(16). There were no significant differences in the amino acid composition of the insoluble collagen between normal and scleroderma specimens, confirming a previous report(5). The gelatins were subjected to hexoses and hexosamines determinations. Free aldehyde groups were estimated in 3 scleroderma and 16 control specimens by the method of Sawicki *et al*(17) using formaldehyde as a standard. Gelatins processed as described above, were hydrolyzed for 15 hours in 4 N HCl, at 110°C in vacuum sealed tubes. Free-aldehydes were also determined in non-hydrolyzed gelatins.

Results. Chemical analysis performed on the total dermis is reported in Table I. Hydroxyproline content of scleroderma dermis, estimated on a weight basis, did not show significant differences from the controls. Hexosamine determinations revealed a significant increase in 5 cases. In Case 1 the increase in total hexosamines was noted only in the specimen obtained from the dorsal aspect of the hands, which was the most severely affected area.

The chemical analysis of the dermal sodium chloride extracts is reported in Table II. This fraction represents soluble components of the ground substance. No significant differences were disclosed when compared with the normal controls. The amount of neutral salt-soluble collagen was reduced, while the concentration of total proteins, hexoses and hexosamines, was within normal range.

Acrylamide gel electrophoresis of the sodium chloride extracts of normal human dermis reveals a series of proteins some of which represent serum components while others seem to be local products of the ground substance. In a previous study, human and rabbit dermal sodium chloride extracts were compared with their autologous serum proteins(18). This investigation revealed that there are two major protein fractions in the dermis. One, the so-called "skin albumin" which shows a faster electrophoretic

TABLE I. Hydroxyproline and Hexosamines of Total Dermis.

		Hydroxyproline	Hexosamines
		mg/g dry wt	
Scleroderma*			
Case 1	Chest	100	2.16
	Abdomen	101	2.17
	Arms	98	2.26
	Forearms	94	2.12
	Hands	97	3.20
Case 2		94	2.68
" 3		97	3.30
" 4†		97	2.41
" 5		96	3.10
" 6		98	3.40
" 7		97	2.40
Controls‡		101 ± 3	2.01 ± .29

* Performed in duplicates.
† This case was previously reported (R. Fleischmajer *et al*, Arch. Dermat., 1966, v94, 531).
‡ Reported as mean and standard deviation of 8 normal adult specimens.

TABLE II. Chemical Analysis of the Crude Dermal Extract with 0.15 N NaCl.

	Case 1*	Case 2*	Controls†
	mg/g dry wt		
Proteins	18.43	20.90	24.00 ±7
Hydroxyproline	.25	.10	.65 ± .18
Tyrosine	1.13	1.81	1.61 ± .2
Hexoses	.90	1.00	.83 ± .08
Hexosamines	1.05	.93	.89 ± .13

* Average of 2 determinations.
† Reported as mean and standard deviation of 8 samples.

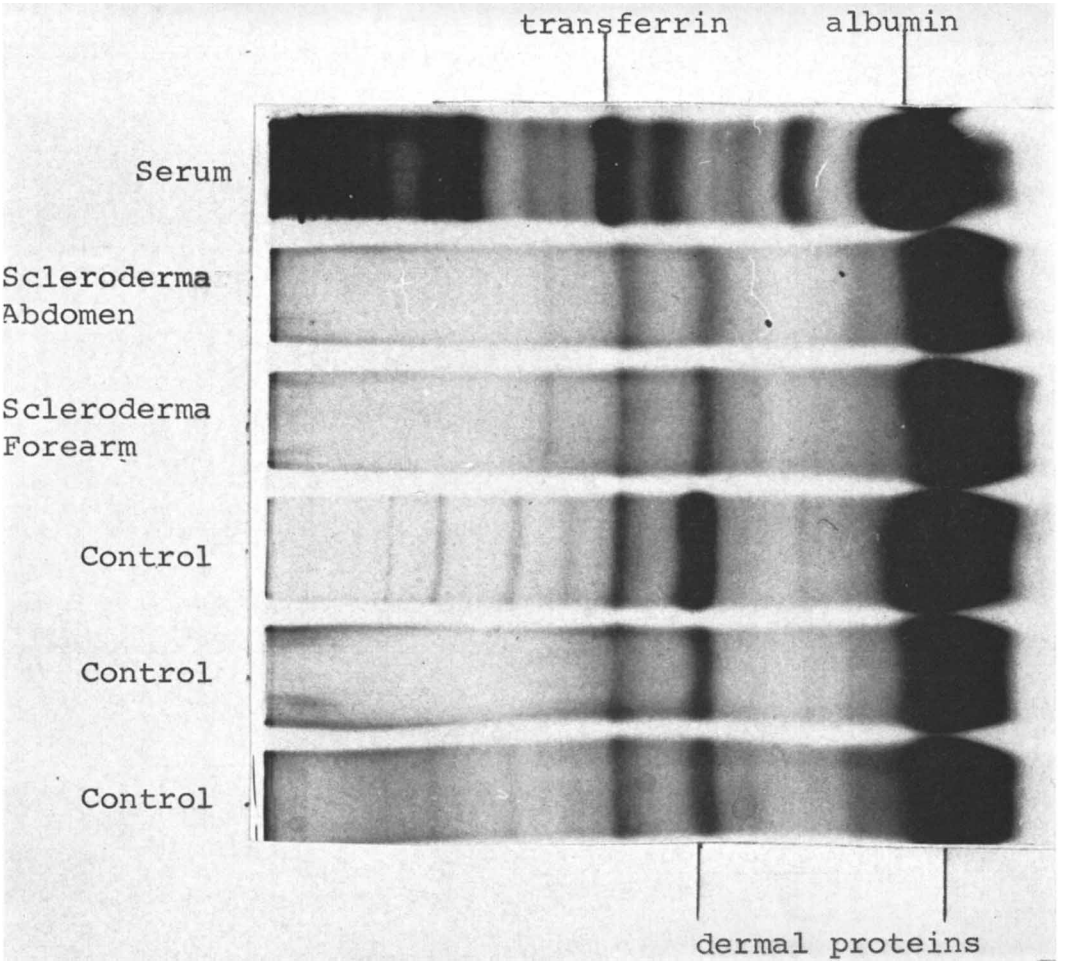

FIG. 2. Acrylamide gel electrophoresis of 0.15 M NaCl dermal extracts. The scleroderma specimens (Case 1) show no significant qualitative changes from the controls.

motility than the autologous serum albumin. The other fraction is present between the transferrin and albumin regions, and is not seen in human or rabbit plasma. The protein patterns of the NaCl extracts from scleroderma dermis were essentially similar to those observed from several normal controls (Fig. 1).

The results of the estimations of collagen-bound hexoses and hexosamines are reported in Table III. The concentration of collagen-bound hexoses was normal in all scleroderma specimens with the exception of Case 4, who had the localized form of this disease. On the other hand, a significant increase in collagen-bound hexosamines was noted in all specimens. In Case 9, two biopsy specimens were obtained, one from the forearm which was markedly indurated, and one from the back which was clinically normal. Determination of collagen-bound hexosamines revealed a 3-fold increase in the affected area. The estimations of free aldehydes performed on hydrolized and non-hydrolyzed gelatins, revealed no significant differences from the controls.

Discussion. The homogenous appearance of the collagen in scleroderma has been frequently associated with fibrosis, namely, an increase of collagen fibers. Our findings and a previous study(5) do not support this concept. This is further substantiated by the

TABLE III. Bound Hexoses and Hexosamines of Gelatins.

	Hexoses	Hexosamines
	mg/g gelatin	
Scleroderma*		
Case 1	6.10	1.06
" 2	5.70	.85
" 3	—	2.40
" 4	11.90	2.40
" 8	6.40	1.11
" 9	6.20	1.89
Controls†	6.12 ± .28	.50 ± .08

* Reported as average of 2 to 4 determinations.
† Reported as mean and standard deviation of duplicate analysis of 9 normal specimens.

following observation. Case 1 showed marked skin induration of the hands, to the degree that movement of joints was practically absent, while the forearms, upper arms, chest and abdomen revealed skin induration ranging from moderate to mild, respectively. Yet, the yield of total dermal hydroxyproline content was about the same for all areas. This study failed to reveal significant changes in the concentration of soluble dermal proteins in systemic scleroderma. Moreover, analysis of these proteins by acrylamide gel electrophoresis did not disclose the presence of abnormal components. Thus, the hypothesis suggesting qualitative or quantitative changes in the proteins of the ground substance was not substantiated. The normal concentrations of hexoses and hexosamines in the NaCl extracts seems to rule out an increase in neutral or acid mucopolysaccharides. The concentration of collagen-bound hexoses and free aldehydes in scleroderma was essentially normal. On the other hand, a significant increase in collagen-bound hexosamines was noted in all scleroderma specimens. In this regard, it is interesting to note that increase in serum hexosamines levels is frequently present in scleroderma(19).

TABLE IV. Free-Aldehydes in Gelatins.*

	Hydrolyzed	Non-hydrolyzed
	mg/g gelatin	
Case 1†	1.40	1.10
" 2	1.24	1.14
" 3	1.30	—
Controls‡	1.31 ± .26	.92 ± .11

* Free-aldehydes were measured as formaldehyde.
† Reported as average of 3 determinations.
‡ Mean and standard deviation of 16 normal specimens performed in duplicates.

The increase in collagen-bound hexosamines in scleroderma is difficult to interpret, although one may hypothesize that this alteration may be in some way related to the homogenous appearance of the collagen, either by participation of these sugars in inter and or intramolecular cross-linkages or as components of a more complex molecule, namely an acid mucopolysaccharide or a glycoprotein.

Summary. Analysis of the total dermis from scleroderma revealed a normal concentration of hydroxyproline while hexosamines were increased in a significant number of cases. A 0.15 M NaCl dermal extract revealed normal concentration of hexoses and hexosamines, thus, suggesting no increase in neutral or acid mucopolysaccharides. The concentration of soluble dermal proteins was normal and separation by acrylamide gel electrophoresis revealed no significant qualitative changes. Gelatins obtained from scleroderma insoluble collagens revealed a normal concentration of hexoses and free aldehydes although the hexosamines were significantly increased. The possible implications of this last finding are discussed.

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Influence of Unsaturation on Fibrinolytic Activity of Salts of Fatty Acids. (32416)

M. J. SURGALA, E. D. BEESLEY, AND R. R. BRUBAKER*
(Introduced by G. G. Wright)
Biological Sciences Laboratory, Fort Detrick, Frederick, Md.

Sodium salts of long chain saturated fatty acids have been reported to accelerate blood clotting and artificial thrombus formation *in vitro*(1,2) and to cause massive thrombosis and death in dogs(3,4). Unsaturated fatty acids are relatively inactive. We have found recently that sodium salts of long chain fatty acids can induce lysis of bovine fibrin films, and in this case it appeared that the unsaturated acids were more active than the saturated. No lysis occurred on heated plates. These preliminary findings are confirmed and extended in the present report.

Materials and methods. Fibrin films were prepared by dissolving Armour bovine fibrinogen (2.5 mg/ml) and Parke, Davis bovine thrombin, topical (50 NIH units/ml) in sodium borate buffer pH 7.7(5). Fibrinogen solutions were sterilized by filtration and 10 ml volumes were added to 8.5 cm Petri dishes. With the plates on a level surface, 0.5 ml of thrombin solution was added dropwise to the fibrinogen while the mixture was gently swirled to assure thorough distribution. Highly purified fatty acids were purchased from Applied Science Laboratories, State College, Pa. They were converted to their sodium or potassium salts by gentle warming with sodium (test no. 1) or potassium (tests 2-5) hydroxide and were adjusted to pH 8.1 with HCl. Two-fold dilutions of the fatty acids in sodium borate buffer were placed on fibrin films in 0.02 ml amounts. The lowest concentration which caused liquefaction sufficient for complete perforation of the fibrin film after incubation at 37°C for at least 4 hours was taken as the end point. (Identical results were obtained with oleate when the system was adjusted to pH 7.4).

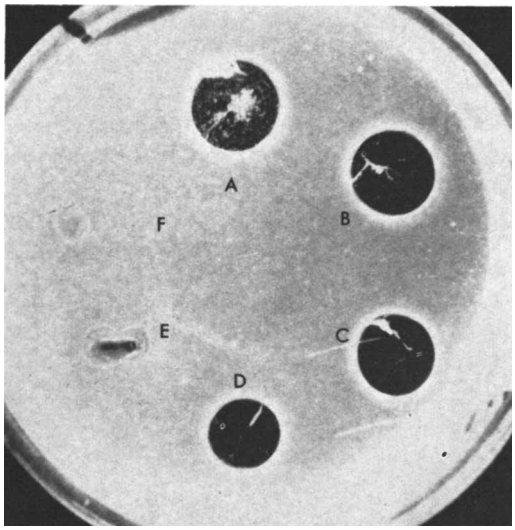

FIG. 1. Lysis of unheated bovine fibrin by sodium myristate. (A) 20 mM, (B) 10 mM, (C) 5mM, (D) 2.5 mM, (E) 1.25 mM, (F) 0.63 mM.

Results and discussion. Fig. 1 shows the activity of 2-fold dilutions of sodium myristate. At concentrations of 20, 10, 5, 2.5 and 1.25 mM, 0.02 ml amounts placed on the fibrin film caused complete perforation of the film. Liquefaction caused by 0.63 mM concentration was insufficient to perforate the film.

Saturated fatty acids having 16, 18, 20, 22 and 24 carbons were compared with acids with one unsaturated bond (Table I). Palmitic and palmitoleic acids appear to be

*Present address: Dept. of Microbiology, Michigan State Univ., East Lansing, Michigan.