METABOLISM OF ACID MUCOPOLYSACCHARIDES IN THE SHWARTZMAN PHENOMENON*

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The two-stage inflammatory state observed by Sanarelli (1) and by Shwartzman (2) is considered an interesting, although not entirely understood, reaction of tissue. The reaction localized to skin, referred to as the Shwartzman phenomenon, represents a special type of inflammatory change, one requiring "preparation" of tissue by intracutaneous injection, usually of an endotoxin from Gram-negative bacteria, and "initiation" of the lesions after a specified time interval by an intravenous, challenging, or provoking injection of the same or a variety of unrelated substances. Evidence has been offered to indicate that polymorphonuclear white blood cells (3), leucocytic-platelet thromboses (4), altered local enzymatic reactions (3, 5), and possibly vascular reactive chemical substances, *i. e.* adrenaline and serotonin (6, 7), play a part in production of the hemorrhagic reaction. Upon histologic study, the lesions assume a hemorrhagic necrosis and "fibrinoid" change (8) observed in many inflammatory patterns which involve connective tissues (9). And as an experimental model, the Shwartzman phenomenon has often been studied because of a similarity to certain clinical states.

Observations by histologic means (9) and experiments with the use of sulfated polymers (10) have implicated the acid mucopolysaccharides (MPS) in the pathogenesis of the lesion produced by the Shwartzman phenomenon. Up to the present time, little information has been available on the changes of the MPS in Shwartzman lesions except as observed by histochemical methods. The development of technics for isotopically labeling and for isolating MPS offers an opportunity to study the MPS quantitatively. The following experiments represent an attempt to evaluate metabolic alterations of the MPS within tissues involved in the Shwartzman phenomenon.

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Materials and Methods

Animals.—Male rabbits, 4 to 6 pounds in weight and obtained from a single commercial breeder, were used in the experiments. They were maintained on a diet of Purina rabbit pellets and water, given freely.

Bacterial Endotoxin.—E. coli endotoxin, a trichloracetic acid soluble fraction (11), was used throughout the experiments and was supplied through the kindness of Dr. A. Braude of the Department of Medicine, University of Pittsburgh Medical School, Pittsburgh.

Radioactive Compounds.—Uniformly labeled [1]glucose-C^{14} and C^{14} carboxy-labeled sodium acetate[2] prepared from $BaC^{14}O_3$ were used as precursors of the MPS. The administration of radioactive compounds will be described in more detail later.

Production of the Localized Shwartzman Phenomenon.—Since the concentration of MPS in skin is low, it was desirable to produce lesions involving large segments of tissue to permit isolation of these compounds. Through a series of preliminary experiments, it was found that approximately 1.5 mg. of endotoxin administered intracutaneously, followed by 0.3 to 0.5 mg. given intravenously, could be tolerated by most of the rabbits studied. The hair on the back and sides of the animals was clipped, and the midline was marked. Endotoxin, dissolved in isotonic saline (1.0 mg./3 ml.), was distributed unilaterally over a large area of skin by the injection of 12 to 15 discrete intracutaneous wheals, and a challenging injection of 0.5 mg. was given intravenously 20 to 24 hours later. Inflammatory and hemorrhagic areas developed at the sites of intracutaneous preparation and reached a maximum hemorrhagic intensity during the next 24 hours.

By this procedure of skin preparation and by the pooling of tissue from several animals, adequate amounts of skin could be obtained, and the adjacent heterolateral skin, which was not directly involved with lesions, could serve as a control tissue. An example of the lesions is shown in Fig. 1.

Additional animals were handled simultaneously but received no endotoxin. Thus, three types of skin samples were obtained: normal skin from control rabbits and Shwartzman lesions and unprepared skin from the animals receiving endotoxin. The amount of tissue obtained as lesions from an individual rabbit varied according to the number and extent of lesions which formed. The variations compared with control sites are indicated in Table I. The lesions, which were edematous and hemorrhagic, particularly at 24 hours, lost considerable weight (approximately 16 per cent) in preparation for extraction of MPS.

Isolation of the Mucopolysaccharides.—The MPS were isolated by the method described by Schiller and associates (12). Tissues were ground, hemorrhagic fluid was decanted, defatted with acetone, and air-dried. Pooled samples of dry-defatted tissues were extracted with alkali and digested with trypsin. Protein precipitation was performed with trichloroacetic acid, and a crude preparation of MPS was obtained by precipitation with alcohol. The MPS, a mixture, was then separated by zone electrophoresis on celite with a 0.1 M PO_4 buffer, pH 6.9, into two fractions, hyaluronic acid (HA) and chondroitin sulfate (CSA). The sulfated fraction consists of a mixture of CSA-A and CSA-B, but primarily B, and in these experiments no further attempts were made to fractionate this group. The amount of MPS was determined by recovery from the electrophoretic procedure by precipitation and collection of a dried sample or by a carbazole determination of hexuronic acid (13). For samples of CSA (which gives low

[1] Obtained from Isotopes Specialties Co., Inc., Burbank, California.

[2] Prepared with the kind assistance of Dr. J. Meyer, Veterans Administration Hospital, New Orleans, Louisiana.

values by the carbazole method due to L-iduronic acid in CSA-B), a factor of 1.6 was found to give similar values to samples which could be recovered.

Assay of Radioactivity.—The amount of radioactivity of the MPS was determined by combustion to $BaCO_3$ and counting with a continuous gas-flow windowless counter. Quadruplicate determinations were made. Results were corrected for background, converted to "infinite thickness," and expressed as counts per minute. Except for certain samples of CSA, discussed below, a counting error within 5 per cent was maintained.

TABLE I

Amount of Skin Samples Obtained from Rabbits with Shwartzman Lesions and from Control Animals

Study interval	Weight of tissues, *gm./rabbit**		Dry-defatted tissue, *per cent of wet weight*‡	
	Mean	Range	Mean	Range
12 hrs.				
Controls	43.5	(36.1–50.9)	30.5	(28.3–32.7)
Shwartzman experimental				
control skin	53.6	(48.8–58.5)	30.7	(28.0–33.4)
lesions	26.7	(23.7–29.6)	24.0	(20.4–27.5)
24 hrs.				
Controls	42.0	(30.3–87.0)	30.4	(28.0–33.6)
Shwartzman experimental				
control skin	38.8	(17.4–89.0)	34.0	(30.5–41.3)
lesions	26.7	(16.0–35.7)	22.3	(20.9–27.1)
5 days				
Controls	36.8	(27.5–54.4)	29.7	(24.8–39.4)
Shwartzman experimental				
control skin	44.6	(17.9–67.8)	29.9	(27.2–32.2)
lesions	21.6	(10.2–42.7)	24.2	(21.5–28.3)

* Half of actual amounts obtained from control animals are shown. Values for the Shwartzman lesions represent amount of tissue after dissection from uninvolved skin and, if expressed on basis of surface area, would be greater than control sites due to inflammatory edema.

‡ Acetone-extracted, ground tissue expressed as per cent of skin samples after grinding. Approximately 3 per cent of control tissues was lost in grinding procedure; the percentages of tissue, edema, and hemorrhagic fluid lost for the lesions were 5, 16, and 7 for 12 hour, 24 hour, and 5 day studies, respectively.

EXPERIMENTAL

Two approaches, with different isotope-labeling methods, were used to study the MPS. In one, radioactive glucose was given at the time of the second or challenging dose of endotoxin and the *incorporation* of the radioactive precursor into the MPS was observed (14, 15). Other experiments used the technic of *in vitro dilution* of previously prepared, radioactive MPS fractions which were added to extracts of inactive tissue samples.

Incorporation of Glucose-C^{14} into the Mucopolysaccharides.—The method of production of the Shwartzman lesions has already been described. Twenty to 24 hours after the initial preparation of skin sites with endotoxin, the challenging intravenous injection of endotoxin was given and, in addition, 20 μc./kg. of body weight of radioactive glucose in isotonic saline was administered intravenously to both prepared and control animals. The rabbits were usually handled in groups of six, two controls and four with lesions. Two or three rabbits which survived the initial preparation with endotoxin, matched by weight with one control, were used for isotope injection. The animals were sacrificed, and dorsal skin was harvested at three intervals, 12 hours, 24 hours, and 5 days, after injection of glucose and challenging endotoxin. This procedure was repeated with several groups until adequate quantities of tissue could be obtained for study. Four rabbits (excluding discarded animals) with lesions and two control animals supplied adequate tissue for the 12 hour study, six with lesions and two controls were used for the 5 day study; samples of tissue for the 24 hour period were obtained from a total of twelve animals with lesions and four controls in order to perform three complete procedures. The replicate experiments enabled an estimate of the variation for over-all experimental conditions (approximately $\pm$ 12 per cent). Since conditions are similar, a comparison can be made of more detailed incorporation studies in normal rabbits performed by Schiller and associates (15). Those studies present decay curves of the time course of isotope incorporation into MPS adequate to allow calculations of turnover rates.

Results of the incorporation experiments are summarized in Table II. Most apparent is the increased radioactivity in samples obtained from the animals with lesions, which demonstrated comparable radioactivity for tissue directly involved in the lesions and for adjacent control skin. An exception to the symmetrical change was observed for CSA from skin lesions of 24 hour duration. Differences from normal animals in the amount of incorporation into CSA were observed only at the later time intervals.

Dilution of Previously Labeled Radioactive Mucopolysaccharides.—Highly labeled MPS compounds were prepared by injecting 1.5 mc. of sodium acetate–C^{14} into normal rabbits and sacrificing the animals 24 hours later. The MPS were isolated from skin by the technic described. CSA was also obtained from cartilage (predominantly CSA-A) (16) because of the isolation of an inadequate quantity of the skin CSA. Although the CSA tracer material is derived from another tissue source and is of a different composition, previous studies indicate that the compounds CSA-A and B are frequently isolated from tissues as a mixture, are electrophoretically similar, and require additional technics for their separation (17).

The carrier MPS, estimated to be approximately one-tenth the content of MPS contained in samples to be extracted, were added to extracts of equal

quantities of dry-defatted tissue from the three experimental conditions, skin from control rabbits, control skin from rabbits receiving endotoxin, and the Shwartzman lesions. For these experiments the skin to be studied was pooled from rabbits not receiving radioactive precursor. A total of fifty-six rabbits was used in these studies. The MPS were isolated as described for the incorporation experiments, and their radioactivity was determined. From the degree of dilution of radioactivity of exogenous MPS an estimate was made of the content of MPS contained in the original samples of tissue.

TABLE II

Incorporation of Glucose-C^{14} into Mucopolysaccharides of Shwartzman Lesions and Control Tissues

Study interval	Hyaluronic acid, *counts/min.**			Chondroitin sulfate, *counts/min.**		
	Controls	Shwartzman experimental		Controls	Shwartzman experimental	
		Control	Lesion		Control	Lesion
12 hrs.	174	282	246	104	105	89
24 hrs.‡	235	377	433	108	120	229
	(192–271)	(310–411)	(397–469)	(86–128)	(108–125)	(208–249)
5 days	110	243	162	68	125	131

* Corrected for background and infinite thickness.

‡ Average values and ranges of radioactivity of mucopolysaccharides from triplicate experiments.

The results of three series of experiments performed with samples of tissue obtained at 24 hours and 5 days are presented in Table III. (Two additional experiments were performed for the 24 hour period with the use of labeled CSA from skin, but, because of inadequate data for CSA fraction, are not presented. Results observed for the HA fraction are similar to those presented in Table III.) In general, the results indicate a considerably greater degree of dilution by tissue containing lesions for HA and CSA at both 24 hours and 5 days.

The data shown in Table IV represent an analysis of the amount of MPS isolated in all the experiments. These results are presented for comparison with those obtained from the dilution studies. Since the isolation procedures are not quantitative, the studies with a dilution technic help corroborate the observation that the lesions contained a greater concentration of MPS. Approximately 50 per cent more HA was isolated from skin involved in Shwartzman lesions and, although less consistent, studies of CSA demonstrated almost a twofold increase in this fraction. A slightly greater than normal concentration was observed in the control skin of animals with lesions.

Comment.—Incorporation of radioactivity by administration of a precursor

of the MPS from a technical standpoint is somewhat simpler and is a more reproducible procedure than the studies of dilution of added or carrier MPS. Degree of incorporation alone, however, is not a valid estimate of absolute change of the compounds, and without determination of amounts, the MPS

TABLE III

Dilution of Added C^{14}-labeled Mucopolysaccharides by Isolates from Shwartzman Lesions and Control Tissues*

Study interval	Hyaluronic acid			Chondroitin sulfate‡		
	Controls	Shwartzman experimental		Controls	Shwartzman experimental	
		Control	Lesion		Control	Lesion
24 hrs.§						
Radioactivity, *counts/min.*	129	108	78	25	11.9	9.8
Dilution	14.8	17.6	24.5	4.5	9.4	11.4
Calculated MPS content, *mg./100 gm. tissue*	296	352	490	64	134	163
5 days‖						
Radioactivity, *counts/min.*	122 (122–123)	112 (101–123)	93 (85–101)	20.6 (9.6–31.6)	8.4 (7.2–9.6)	7.9 (7.3–8.5)
Dilution	15.3	17	20.7	5.4	13.3	14.2
Calculated MPS content, *mg./100 gm. tissue*	306	340	414	77	190	203

* Seven mg. of hyaluronic acid of 1910 counts/minute and 5 mg. of chondroitin sulfate-A of 112 counts/minute were added to each set of alkali-extracted tissue (solubilized 35 gm. of dry-defatted skin).

‡ The low values are not within 5 per cent counting accuracy; however, they are suggestive of direction of change.

§ Results of one experiment.

‖ Results of duplicate experiments. Values obtained for control animals from 24 hour and 5 day studies could be considered as triplicate determinations, which indicate reproducibility of dilution procedure.

pools, involved interpretation of actual differences is difficult. This becomes apparent from observations of similarity of incorporated radioactivity of fractions from skin with and without lesions from the same animals. Since recovery of MPS from tissue is not complete, the use of a method such as the dilution technic becomes necessary.

The problem may arise that the dilution studies are not true tracer experiments. In the isolation of the MPS fractions considerable degradation occurs. After addition of isolated (partially degraded or lower molecular weight) compounds, are equivalent amounts of materials being lost throughout the stages of isolation? Information to answer this question is not available, but it is obvious that the recoveries of the MPS vary widely. Calculated from content of MPS estimated by the dilution method, the average recovery of HA was approximately 46 (39 to 49) per cent, as compared to 36 (13 to 60) per cent

TABLE IV

Amount of Mucopolysaccharide Isolated from Skin Containing Shwartzman Lesions and Control Sites

Results are given in mg./100 gm. dry-defatted tissue.*

Study interval	Hyaluronic acid			Chondroitin sulfate		
	Controls	Shwartzman experimental		Controls	Shwartzman experimental	
		Control	Lesion		Control	Lesion
12 hrs.‡	145	183	307	64	33	81
24 hrs.	145	172	219	31	33	61
	(74–206)	(72–235)	(85–320)	(24–44)	(27–37)	(53–71)
5 days	148	134	182	46	23	72
	(123–168)	(83–176)	(131–244)	(41–51)	(17–27)	(53–82)

* Calculated from weight of compounds isolated or by carbazole determinations. Refer to text for further details.

‡ Results of one experiment.

for CSA. Results for normal animals observed in these experiments are in a similar range as reported for rats (18).

The studies of HA, technically, are more satisfactory than for CSA. Several reasons may account for the difference. Hyaluronic acid is a larger molecular weight material, the major MPS of skin, and is more easily freed from protein in tissue. Moreover, the tracer HA material in these experiments came from the same tissue source and the results are more consistent. For observations of CSA by the dilution method, it would have been desirable to use material with a higher level of specific activity. The data of low radioactivity could not be considered strictly quantitative in adhering to an accepted 5 per cent variation in counting technic. But, despite the limitations a direction of change—one of increased MPS fractions, HA and CSA, within the lesions—is indicated by both sets of observations, isolation and MPS dilution.

In relating the changes of the MPS within the Shwartzman lesions, one must consider the differences of tissue to be extracted. The skin involved in lesions

was dissected free of uninvolved tissue, and upon gross examination was found to be extremely edematous and hemorrhagic. Upon grinding, in preparation for extraction of MPS, a considerable amount of hemorrhagic fluid was expressed and discarded. The question may be posed whether the necrosis of the tissue with destruction of cells allows a relatively greater concentration of connective tissue (MPS-yielding material) during the process of dehydration and defatting with acetone. Necrosis could conceivably result in an apparently greater concentration of MPS per dry weight of tissue. Also, another interpretation may be made if calculations are expressed on original wet weight materials which are given in Table I. If applied, there is no significant change in concentration of MPS within lesions, but on the basis of area the total amounts may be increased. (Such corrections do not apply to *incorporation* of isotope. Those data are compared for specific activity and are relative, irrespective of the condition of tissue extracted or amount of MPS isolated.) However, analyses based on dry weight seem to be the best means for comparing the different tissues, since the reduced percentage of residual dried tissue may be an artifact due to inflammatory edema. Further evidence that an increased concentration of MPS actually occurs is reflected by comparison of both controls which are similar as dried tissue but different in the calculated content of MPS. In addition, the data shown in Table III suggest a continued increase of CSA after 5 days; this observation agrees with experiments that demonstrate that CSA is synthesized more slowly than is hyaluronic acid (14, 19).

Another explanation for the increase of MPS is deposition from blood into the lesions. That deposition may be a factor is suggested by a higher specific activity at 24 hours of CSA samples obtained from lesions than from heterolateral skin (Table II). CSA has previously been shown to be present in serum (20), white blood cells (21), and platelets (22). Incorporation into these sources may be more rapid and could contribute to the isolation of a more highly labeled compound from hemorrhagic skin. Unfortunately, comparative rates of incorporation for CSA from different sources, including blood, were not determined and are unavailable in other investigations. The continued increase of CSA after 5 days by isolation from tissue with less hemorrhage, nevertheless, favors primarily an increased rate of synthesis of this compound.

DISCUSSION

The Shwartzman phenomenon has been studied extensively by histologic methods. As part of the necrotic process a "fibrinoid degeneration" is observed, which is similar to that seen in many inflammatory reactions involving connective tissue. Impressions from histologic observations indicate that MPS take part in the reaction; however, beyond information concerning these compounds obtained by non-specific histochemical technics, little is known of their role in the Shwartzman reaction. Methods to isolate the MPS and to study

these compounds with isotopes enable a more direct and quantitative approach to determine alterations of the different substances within tissue. Earlier studies (23) of experimental serum sickness with the use of radioactive acetate reflected only slight increases in incorporation of a precursor without changes in the amount of MPS isolated. A limitation of that study was the selection of skin as the source of MPS. Serum sickness produces small (microscopic) vascular lesions, and the skin from these animals represented an admixture of diseased and normal tissue. Contrary to this, the localized cutaneous lesion of the Shwartzman phenomenon offers good experimental conditions; gross lesions can be produced with adjacent tissue as a control from the same animals.

The present experiments suggest metabolic changes, both quantitative and qualitative, of two MPS fractions. Of particular interest is the greater than normal incorporation of glucose (higher specific activity) into MPS similarly from both lesions and control tissue of animals receiving endotoxin. Although the implication has been made that the rate of incorporation is not a direct reflection of pool size of precursors (24), the results obtained with animals with Shwartzman lesions suggest the degree of incorporation to be secondary to glucose metabolism altered by illness. It is possible that one unit such as glucose may be rate-limiting in synthesis of complex MPS. Consequently, caution is needed in interpreting incorporation studies and a direct comparison with normal animals in a steady state may not be valid. In these experiments within the limits already discussed, a primary change of MPS occurs in the lesions as compared with a control tissue of heterolateral skin. The data may be interpreted as indicating an increased biosynthesis of MPS since the calculations of fractional turnover rates were greater both for HA and CSA isolated from the lesions (Table V).

It is interesting to speculate on the role of the MPS in the tissue reactivity of the Shwartzman phenomenon. Proper evaluation of these compounds is difficult with currently available information. Although mucopolysaccharides are often discussed as a group, uniformity of function cannot be attributed to individual MPS, even in a single tissue, such as skin. Striking individual differences in chemical and physicochemical properties are known to exist. For example, a considerable antithrombin activity has been demonstrated for CSA-B (β-heparin) (25), and this physiologic property could be a factor in the extensive hemorrhage of the lesions. In addition, these compounds form part of the matrix of blood vessel walls and could be of importance in "intactness" of blood vessels. Vascular permeability, although not observed to be abnormal in the early phase of development of the Shwartzman reaction (26), could still play a role in production of a hemorrhagic lesion. Adhesiveness associated with viscosity of MPS may be a factor in formation of platelet and white cell thrombi. A number of MPS are also known to compose a part of granulation tissue (27) and are metabolically active in inflammation provoked by a variety of methods.

Thus, the role of these substances may not be *special* to Shwartzman lesions but may be part of the inflammatory reaction of tissue to injury. The questions which are posed may be clarified by future investigations of connective tissue with improved methods of study.

TABLE V

Comparison of Pool Sizes and Turnover Rates of Mucopolysaccharides in Rabbits with Shwartzman Lesions and Control Tissues

	Hyaluronic acid			Chondroitin sulfate		
	Controls	Shwartzman experimental		Controls	Shwartzman experimental	
		Control	Lesion		Control	Lesion
Pool size,* *mg./100 gm. tissue*	301	346	452	70	162	183
Fractional turnover rate,‡ *mg./100 gm./day*	77	96	126	10	19	26

* Average values obtained from 24 hour and 5 day studies, which were determined by a dilution method as described in text and presented in Table III.

‡ $\frac{\text{pool size}}{t^{1/2} \times 1.44}$—The values for turnover rates are rough approximations based on curves obtained for three time intervals covering formation and healing of Shwartzman lesions. The curves were constructed by comparison with studies in normal rabbits and estimates of turnover rates of mucopolysaccharides in rats. A summary of those experiments is presented by Dorfman and Schiller (24). The actual decay curves which are derived in normal animals as well as in diseased animals are not single exponential, but the difficulty of reproducing detailed experiments and the little additional information to be gained justify the simplified calculations.

SUMMARY

The effect of the Shwartzman phenomenon on the metabolism of the acid mucopolysaccharides (MPS), hyaluronic acid and chondroitin sulfate, from rabbits with localized lesions was studied. Quantitative changes of MPS were observed by two techniques—the *in vivo* incorporation of glucose C^{14} into the MPS and the *in vitro* dilution of C^{14}-labeled MPS. The results indicate an increased concentration of both MPS fractions in the skin lesions and suggest an increased biosynthesis of the compounds at the site of the lesions. Deposition of chondroitin sulfate from blood may have contributed in part to the concentration of this substance in the lesions. The MPS contribute to the inflammatory state of the Shwartzman phenomenon but the specific roles of these compounds need to be defined by further study.

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EXPLANATION OF PLATE 53

FIG. 1. Hemorrhagic lesions of the Shwartzman phenomenon of 24 hour duration in the dorsal skin of a rabbit. Multiple lesions were produced unilaterally and are shown on the subcutaneous surface. The adjacent heterolateral skin was used in the experiments as a control.

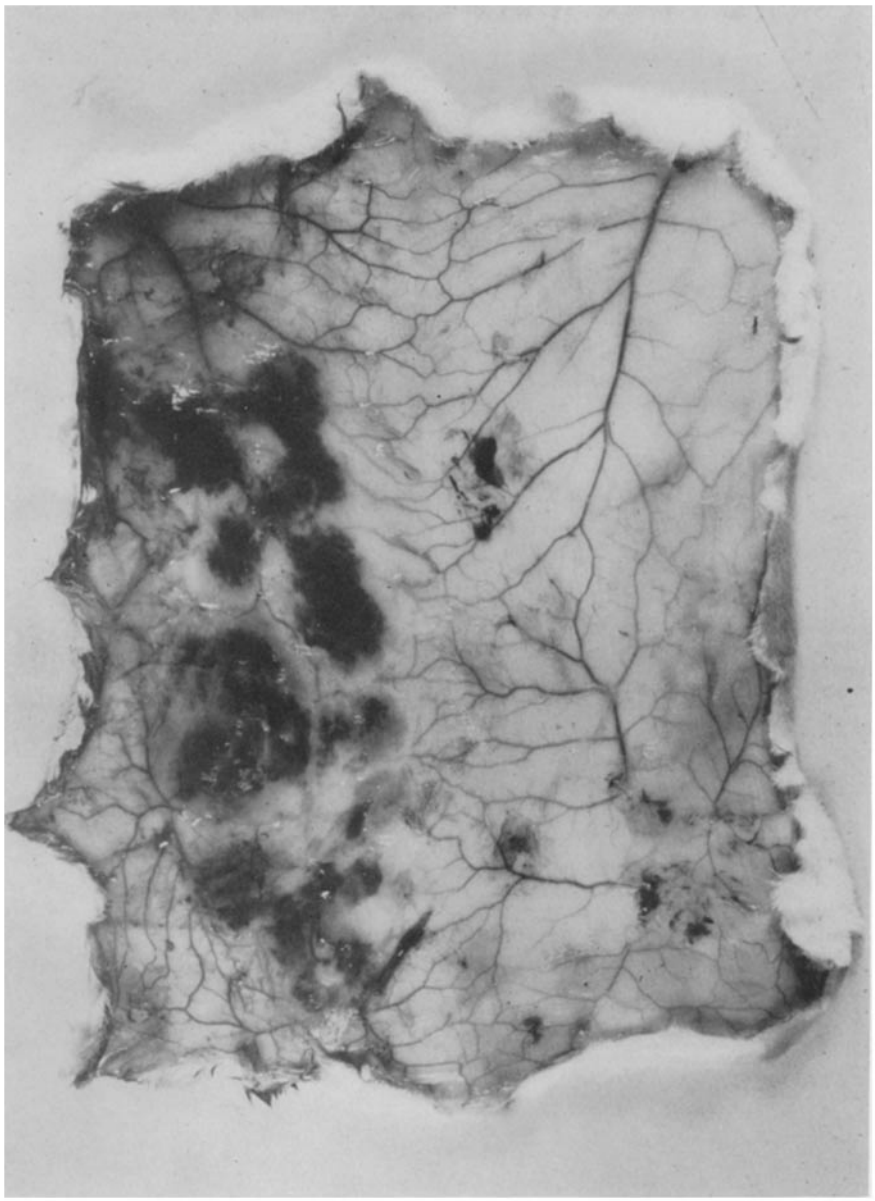

FIG. 1

(Berenson and Dalferes: Mucopolysaccharides in Shwartzman phenomenon