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DEMONSTRATION OF A MACROMOLECULAR COMPLEX SOLUBILIZED FROM GUINEA PIG EPIDERMIS BY ULTRAVIOLET LIGHT

A DISSERTATION

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in partial fulfillment of the requirements for the

degree of

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BY

OWEN BLACK, JR

Oklahoma City, Oklahoma

1969

DEMONSTRATION OF A MACROMOLECULAR COMPLEX SOLUBILIZED FROM GUINEA PIG EPIDERMIS BY ULTRAVIOLET LIGHT

APPROVED BY

DISSERTATION COMMITTEE

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DEMONSTRATION OF A MACROMOLECULAR COMPLEX SOLUBILIZED FROM GUINEA PIG EPIDERMIS BY ULTRAVIOLET LIGHT

CHAPTER I

INTRODUCTION

Ultraviolet light irradiation of skin produces many diverse alterations in its normal morphology and metabolism. The literature is replete with documentation of the effects of ultraviolet light (UV) on skin, including the formation of tumors (1), enzyme activity changes (2, 3), glycogen storage (4), lysosomal alterations, and lipid changes. Emphasis in this literature survey will be placed on lipid and sterol alterations because of the diversity of the UV effects already documented, and because of the nature of the present study.

Efforts to find the primary agent or agents responsible for the series of alterations characterizing epidermal changes have met with less success. Epstein (5) has shown that UV induces the formation of kinins in human skin. Majno (6) showed morphological evidence that histamine, which is released from mast cells by UV, causes abnormal vascular permeability and acute inflammation. Fukuryama (7) presented evidence that UV decreased the epidermal cell ribonucleic acid and protein synthesis. Ottolenghi (8), in work on mitochondrial enzyme activities, showed UV produced oxidation of lipids in proportion to the period of

irradiation. The oxidation products, in turn, inhibited certain enzyme reactions.

Many workers have furnished evidence for the involvement of lysosomes in the UV response. Weissman (9) showed that lysosomal protease was released by UV and that its release could be inhibited by hydrocortisone. Wills (10) stated that the release of lysosomal hydrolases by UV was mediated by the formation of lipid peroxides. Vitamin E was reported to decrease this effect. Johnson (11) assayed the lysosomal acid phosphatase content of irradiated and non-irradiated mouse ears and suggested that the decrease in activity found was due to the enzyme being released and either binding to substrate or being removed by serum.

There is evidence to show lipid and sterol involvement in the UV response. In the early 1930's, several workers reported that the formation of UV-induced tumors was primarily due to the effect of UV on cholesterol. One of the investigators in this field, Roffo (12), maintained that UV acted on cholesterol, in vivo, to form a carcinogen having a phenanthrene nucleus. His work, and the work of others (1, 13) was based on evidence of increased cholesterol in tissues of animals treated with daily exposure to UV irradiation for up to several weeks. Rix (14) found that rubbing cholesterol into the skin of pigs resulted in increased erythema response upon exposure to UV. This work has been supported by the more recent work of Wells (15) and Simko (16) who showed that long exposure of rat skin to UV produced a significant increase in skin cholesterol. Horlick (17) exposed rats to daily doses of UV, injected them with ^{14}C-acetate, and measured the changes in radioactivity in various

lipid fractions after six to eight days. He presents evidence that the increase in cholesterol in irradiated skin is due to increased synthesis of cholesterol. The concentration of fatty acids was found to be decreased but they showed an increased turnover. He stated that the treatment appeared to have an overall stimulating effect on lipid metabolism of skin. The work of Baumann (18) on mouse and on guinea pig skin, and the work of Finagin (19, 20) and Simko (21) on guinea pig skin, however, showed no change in skin cholesterol. Kawaguchi (22) stated that guinea pig skin increases its cholesterol content significantly when irradiated with sunlight; but only slightly when irradiated with UV light. Logan (23) compared the erythematous response in guinea pig skin from an injection of a lecithinase (from microorganisms) to the UV response. The evidence indicated that since the lecithinase activity could be blocked without affecting the UV response, the permeability was not due to lecithinase activity.

Rauschkolb, et al. (24), showed, by irradiating in vitro in an oxygen environment, that cholesterol content of human skin was decreased by a significant amount. They also showed that about half the total cholesterol appears to be bound, or is not otherwise easily extracted with chloroform. They suggested that the 3-hydroxy position of cholesterol might be bound to protein. Roffo reported earlier (25), however, that the cholesterol content in human skin increased from 7% to 150% with UV radiation.

Johnson (26) suggested that phospholipids play some role in the response by showing an increase in the incorporation of ^{32}P in phospholipid fractions of irradiated mouse ears, while acid soluble and nucleic

acid phosphorus were not altered. On the other hand, Tichner (27) presented evidence that mouse skin, irradiated at 0°C in vitro, contained less phospholipid phosphorus. He indicated this loss was due to a significant destruction of both ethanolamine plasmalogen and phosphatidyl ethanolamine. His results showed a compensatory increase in ribonucleic acid phosphorus. Pora (28) studied the effects of long periods of UV irradiation on the fixation of ^{32}P in the skins of rats and frogs. He showed that a 16 day exposure to UV, followed by injection of ^{32}P, resulted in increased incorporation into acid soluble phosphorous and lipid phosphorous and decreased incorporation in nucleic acids in both animals.

There is evidence both for and against any serum cholesterol changes in humans irradiated with ultraviolet light. Malczynski (29) showed that normal humans exhibited a 20-40% increase in serum cholesterol within a few minutes after irradiation. Ornstein (30) reported, however, that there was no change in serum cholesterol levels with irradiation.

Photochemical reactions of fatty acids, cholesterol esters, and cholesterol have been studied. Hais (31) showed that unless precautions were taken, aqueous emulsions of cholesterol, exposed to daylight for several days, would undergo photolysis. This work was supported by Claude (32) who showed that the in vitro irradiation of dry films of cholesterol and cholesterol esters resulted in the cleavage of the sterol ring. The ester bond was not hydrolyzed. He found that the esters containing unsaturated fatty acids appeared to be more liable to photolysis that those with saturated fatty acids. Ottolenghi (8) and

Baker (33) showed that UV irradiation of fatty acids resulted in their oxidation. Waravdekar (34) reported that aqueous extracts of irradiated linolenic acid contain materials toxic to livers of mice.

Ultraviolet light irradiation of guinea pigs was found by the author to result in the appearance of opalescence in the supernatant fraction of 0.3 M sucrose epidermal homogenates. This observation implies that UV alters some epidermal cellular component which results in the liberation of material having a different index of refraction in solution, seen as opalescence. A study of the chemical, physical, and biological properties of this material will aid in the understanding of the interaction of UV and skin. In particular, knowledge of the constituents will lend insight into the nature and locus of UV injury in skin. Insight into some of the physical and biological properties will lend a better understanding of the structure and physiological function of cellular components. This study is thus concerned with the isolation and partial characterization of the opalescent material. Techniques used in characterizing this material include chemical assays, liquid chromatographic, gas chromatographic, thin layer chromatographic, electrophoretic, immunologic, and electron microscopic techniques.

CHAPTER II

EXPERIMENTAL METHODS AND RESULTS

In the course of preparing epidermal homogenates in 0.3 M sucrose for other experimental procedures, it was observed that the supernatant of irradiated preparations was consistently more opalescent than that of non-irradiated samples. Preliminary experiments verified this observation and disclosed that this material was of a large size and contained protein, and that it was sensitive to osmotic pressure changes. This opalescence was studied in the following manner. Because of the large size gel filtration was selected for isolation of the opalescent material. Several experimental conditions were varied to study the factors affecting the extent of appearance of the opalescent material. Since several workers (35-39) have shown a relationship of tissue proteins to serum proteins, immunological and electrophoretic techniques were utilized to show the association of serum proteins with these tissue proteins. The morphology of the material was investigated by electron microscopy because the initial experiments showed this material had a large structure, and that it was sensitive to osmotic pressure changes. Chemical assays, thin layer and gas liquid chromotography, and an enzyme assay were utilized to further characterize the opalescent material. Because of certain similarities of this opalescence with opalescence produced by the action of snake venom phospholipase A on tissue (40), experiments were designed

to compare the products of this enzyme to the UV opalescent material.

Only white or albino male guinea pigs, weighing from 350 to 600 gm, were used for this study. Hair was removed from the dorsal surface of the animals just prior to irradiation by clipping and shaving with an electric razor.

Selection of Standard Irradiation Procedure

Two methods were used in the irradiation of the animals. Initially the animals were irradiated with a bank of four Westinghouse Sun Lamps (500 microwatts/cm^2) as described previously (2). The irradiation period with this procedure was three hours (12 MED[1]). This procedure was used in the initial experiments in isolating the opalescent material, most of which are not reported here.

The second method utilized light from a Burdick mercury-vapor lamp[2] arranged 23 inches lamp-to-back. The irradiation period with this source was 12 minutes (12 MED). The methods appeared to produce equivalent amounts of opalescence. Since the shorter period of irradiation for this procedure was more satisfactory, most of the data reported in this study utilized this method of irradiation.

Isolation of the Opalescent Material

Preparation of Homogenate

After a period of time post irradiation, which varied from zero

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[1] MED, minimal erythemal dose, is defined as that dose of UV required to produce a barely perceptible reddening of the skin. For this light source one MED has been determined to be 15 minutes.

[2] QA 450N, 410 Watt (15,000 microwatts/cm^2), Burdick Corporation, Milton, Wisconsin.

to four hours according to the experiment, the animals were sacrificed by cervical luxation. The pelts were removed and scraped with a razor blade (41). The pelts were scraped carefully to minimize dermal tissue contamination (42). The tissue was weighed and homogenized in glass tissue grinders with aliquots (10 ml per gm wet tissue weight) of cold 0.3 M sucrose. All sucrose utilized in these experiments contained 0.02% merthiolate to retard microbial growth. Merthiolate was not found to have any effect on the experiments. The homogenates were transferred to centrifuge tubes and centrifuged for 10 minutes at 700 x g. The supernatant was centrifuged at 25,000 x g for 15 minutes. This second supernatant was used for the column chromatography.

Gel Filtration

Sephadex G-200 was prepared by equilibrating the gel in 0.3 M sucrose at 2°C, according to manufacturer's instructions.[3] Chromatographic columns were prepared with the Sephadex, either as a small column (2.4 x 36 cm) for single animal experiments, or a larger column (5 x 73 cm) for large scale isolation. All column chromatography was performed in a cold room at 2°C. Following application of the sample the columns were eluted with 0.3 M sucrose. The eluate was collected in a fraction collector and the fractions were assayed for protein by the method of Lowry, et al. (43). An elution pattern of protein of irradiated and non-irradiated tissue supernatant fractions is seen in Figure 1. This graph, and all subsequent graphs of protein profiles, are shown with ordinates designated as relative absorbance, i.e., the absorbance derived

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[3]Pharmacia Fine Chemicals, Piscataway, New Jersey.

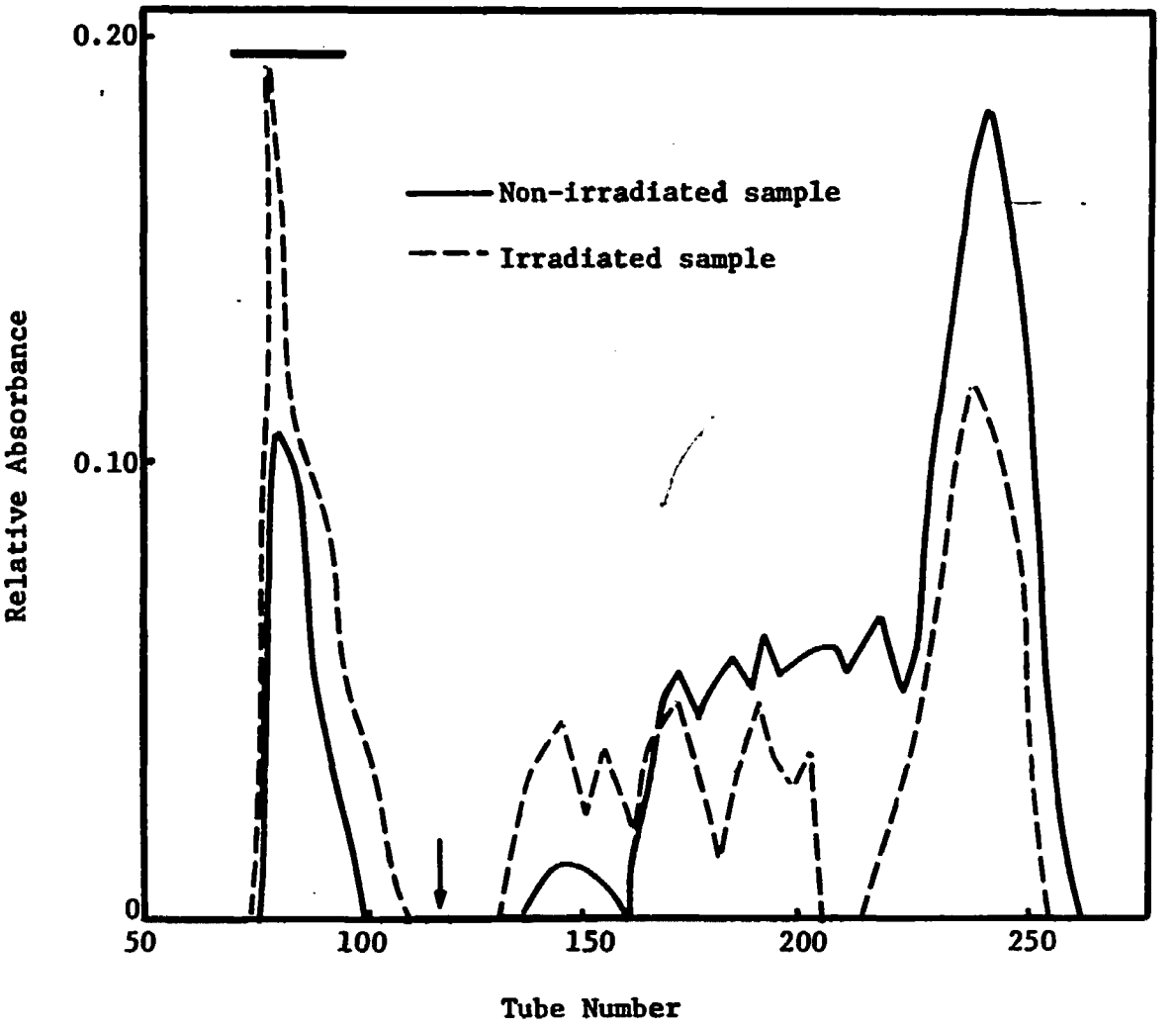

Figure 1--Graph of protein in fractions from Sephadex columns comparing an irradiated preparation (8.11 gm wet tissue weight) with a non-irradiated preparation (9.37 gm wet tissue weight). The material eluted prior to the arrow (which denotes void volume) was excluded by the gel. The bar above the initial peak denotes opalescent fractions.

from the Lowry assay procedure and is the spectrophotometric reading at 500 nm. The abscissa is designated as tube number which is the number of the fraction collected in sequence in the fraction collector. The figure shows the opalescent material (in both irradiated and non-irradiated samples) is excluded by the gel and eluted in the void volume (shown by the small arrow). This implies a molecular weight greater than 200,000. Equilibration and elution of the columns with sucrose was necessary since any procedure which lowered the osmotic pressure resulted in an irreversible aggregation or precipitation of the material. The opalescent fractions were pooled and reduced in volume by one of two methods. In one method, the fractions were pooled in a graduated cylinder and dialysis tubing filled with 20M (20,000 molecular weight) polyethylene glycol flakes[4] (PEG) was placed in the cylinder. These tubes were replaced as needed to reduce the volume to the desired quantity. In the other method, the fractions were pooled and placed in dialysis tubing, laid in a pan, and covered with PEG flakes. The flakes were changed until the volume was reduced as desired.

Conditions Affecting Extent of Appearance

Irradiation In Vitro

An experiment was performed to test whether serum protein might be contributing to the opalescence. A guinea pig was sacrificed, clipped, and shaved. The pelt was removed and placed in a petri dish with 10 ml Eagle's Minimum Essential Medium.[5] The pelt was irradiated with

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[4]Union Carbide Corporation, New York, New York.

[5]Grand Island Biochemicals Co., Grand Island, New York.

the Burdick lamp as described above and incubated four hours at 37°C. The pelt was processed in the usual manner. The supernatant was applied to a small Sephadex column and eluted with the sucrose solution. The protein profile is shown in Figure 2A. This profile is similar to that in Figure 2B, which is of an animal irradiated *in vivo* and processed identically. The amount of opalescent material does not appear to depend on the degree of vascular circulation, hence serum proteins probably do not materially contribute to the opalescence.

Time Following Irradiation

To determine what effect time following irradiation had on the appearance of the opalescent material, the following experiment was performed. An animal was sacrificed, clipped, shaved and the pelt removed and irradiated *in vitro*. The pelt was processed immediately and the supernatant applied to the Sephadex column. Its protein pattern is seen in Figure 2C. There appears to be a greater response when the tissue is examined immediately than after a four hour delay.

Thermal Treatment

To test whether this opalescence was a specific response to ultraviolet light or a general injury response, a series of thermal injury experiments were performed. In these experiments an excised pelt was placed in contact with a metal beaker containing water at 59°C for 10 minutes (44). The pelt was processed immediately and the protein profile recorded. A non-treated pelt was processed identically and its protein profile was noted. The profiles are seen in Figure 3. It can be seen that there is essentially no difference between non-treated and heat-treated epidermis.

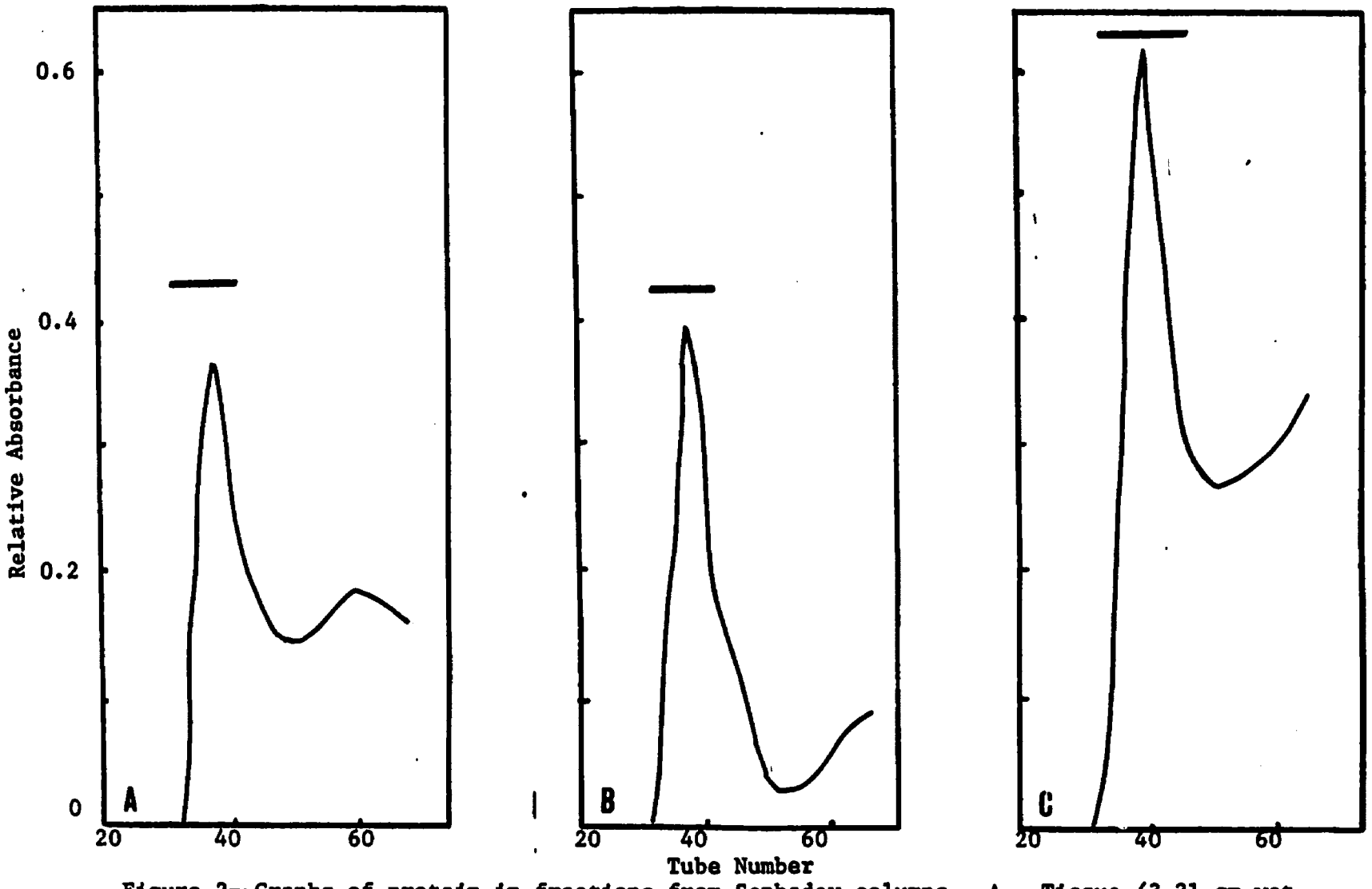

Figure 2--Graphs of protein in fractions from Sephadex columns. A - Tissue (3.21 gm wet weight) from pelt irradiated in vitro and processed after four hour delay. B - Tissue (2.82 gm wet weight) from pelt irradiated in vivo and processed after four hour delay. C - Tissue (2.98 gm wet weight) from pelt irradiated in vitro and processed immediately. Bar denotes opalescence.

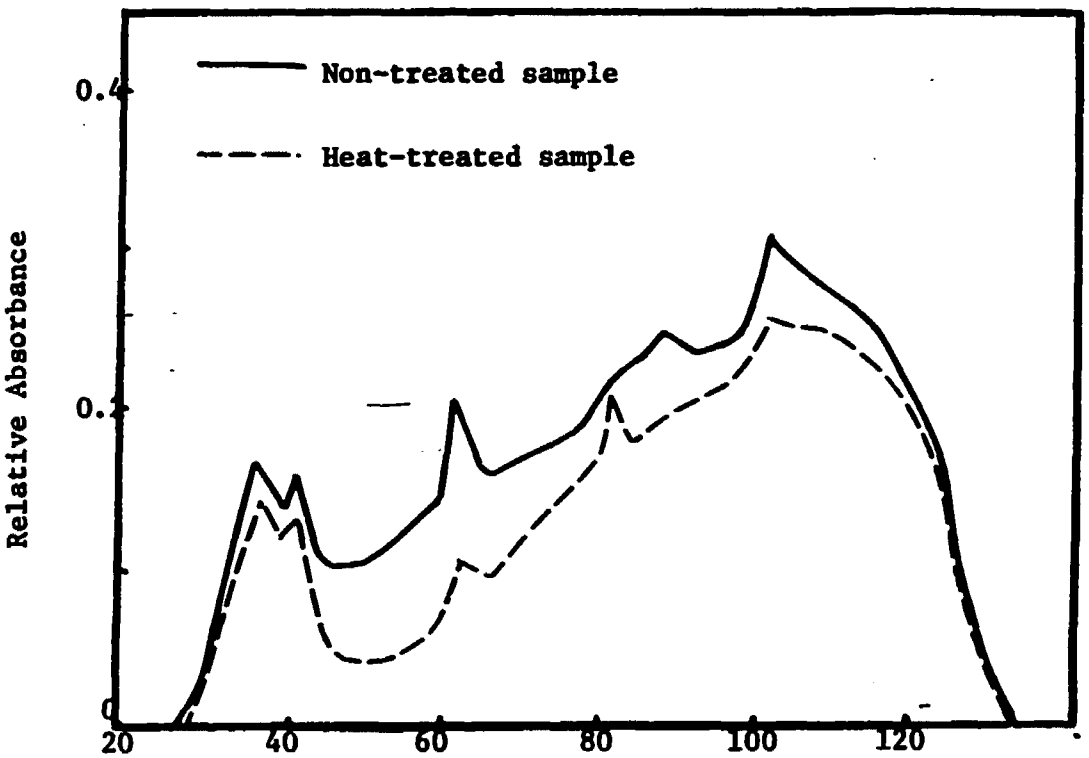

Figure 3--Graphs of protein in fractions from Sephadex columns of heat-treated (3.80 gm wet weight) and non-treated samples (2.32 gm wet weight).

Homogenization in Saline

Epidermis was homogenized in 0.9% NaCl to test what effects homogenization in solutions of lower osmotic pressure would have on isolation of the opalescent material. Two animal pelts were prepared, one irradiated and one non-irradiated, and their epidermal scrapings were homogenized in cold 0.9% saline. The homogenates were centrifuged in the usual manner and the supernatants applied to small Sephadex G-200 columns and eluted with 0.3M sucrose. The protein profile is seen in Figure 4. Both irradiated and non-irradiated profile peaks (opalescent fractions) are larger than the non-irradiated sucrose-homogenized profile peak, seen in Figure 3. This would suggest that homogenization of tissue in saline fragments or solubilizes the tissue which results in formation of the opalescent material. The irradiated opalescent peak is only slightly higher and could possibly be due to animal variation. In Figure 5, the saline control from Figure 4, the non-treated control from Figure 3, and the irradiated profile from Figure 2C, have been incorporated into one graph to show the relative effects these treatments have on the tissue. Treatment of epidermis with UV or homogenization of the tissue in saline appears to produce quantitatively similar results in opalescent fractions. Homogenization of non-treated tissue will produce some opalescent material, much less, however, than treated tissue.

Dose-dependence Experiments

To show whether the response observed was dependent on the dose or irradiation, a series of experiments was performed changing the period of irradiation. With these experiments, single animals were prepared as described and the pelts irradiated for periods of 0, 0.75, 1.5, 3.0,

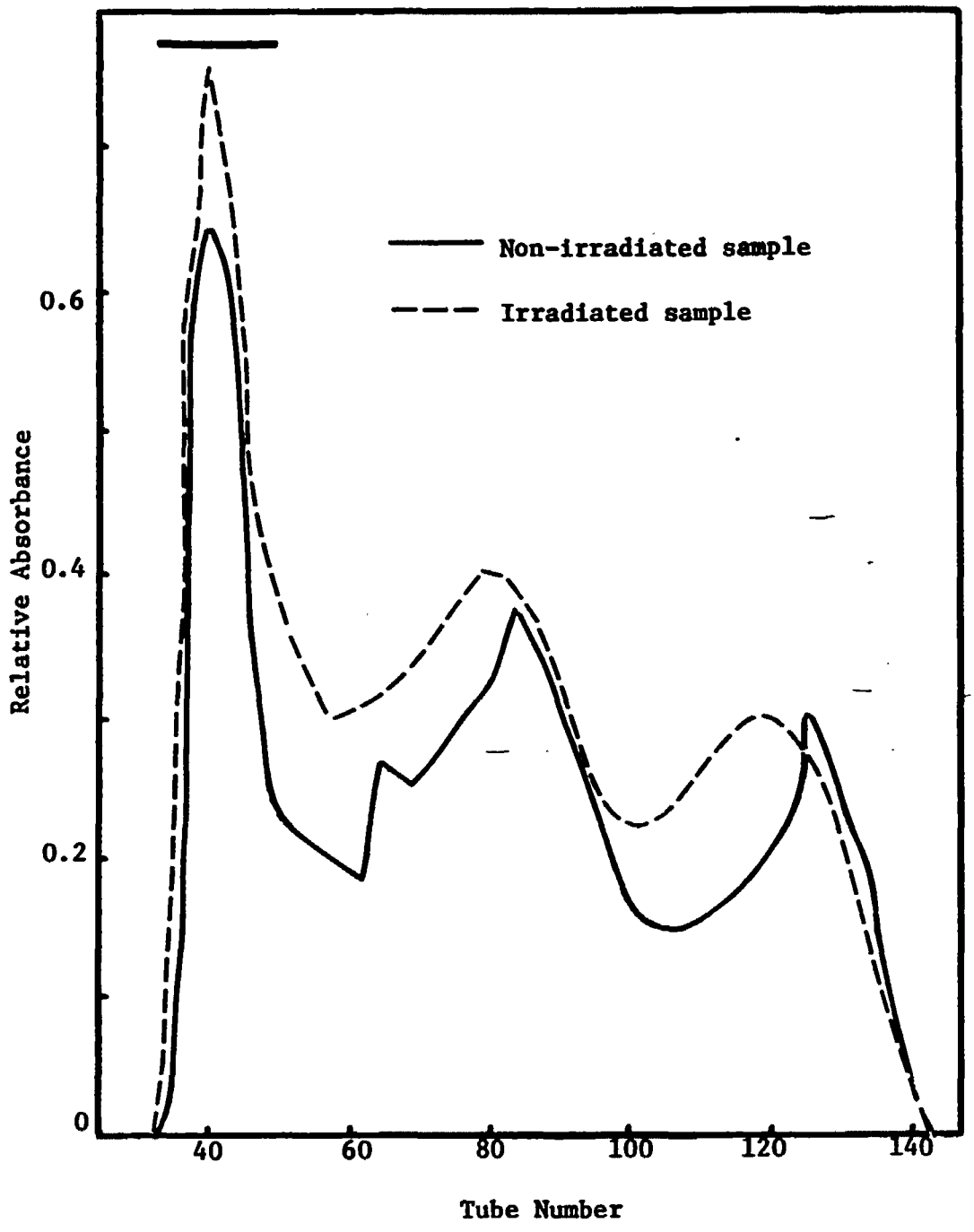

Figure 4--Graph of protein in fractions from Sephadex columns of irradiated saline-homogenized (2.90 gm wet weight) and non-irradiated saline-homogenized (3.12 gm wet weight) samples.

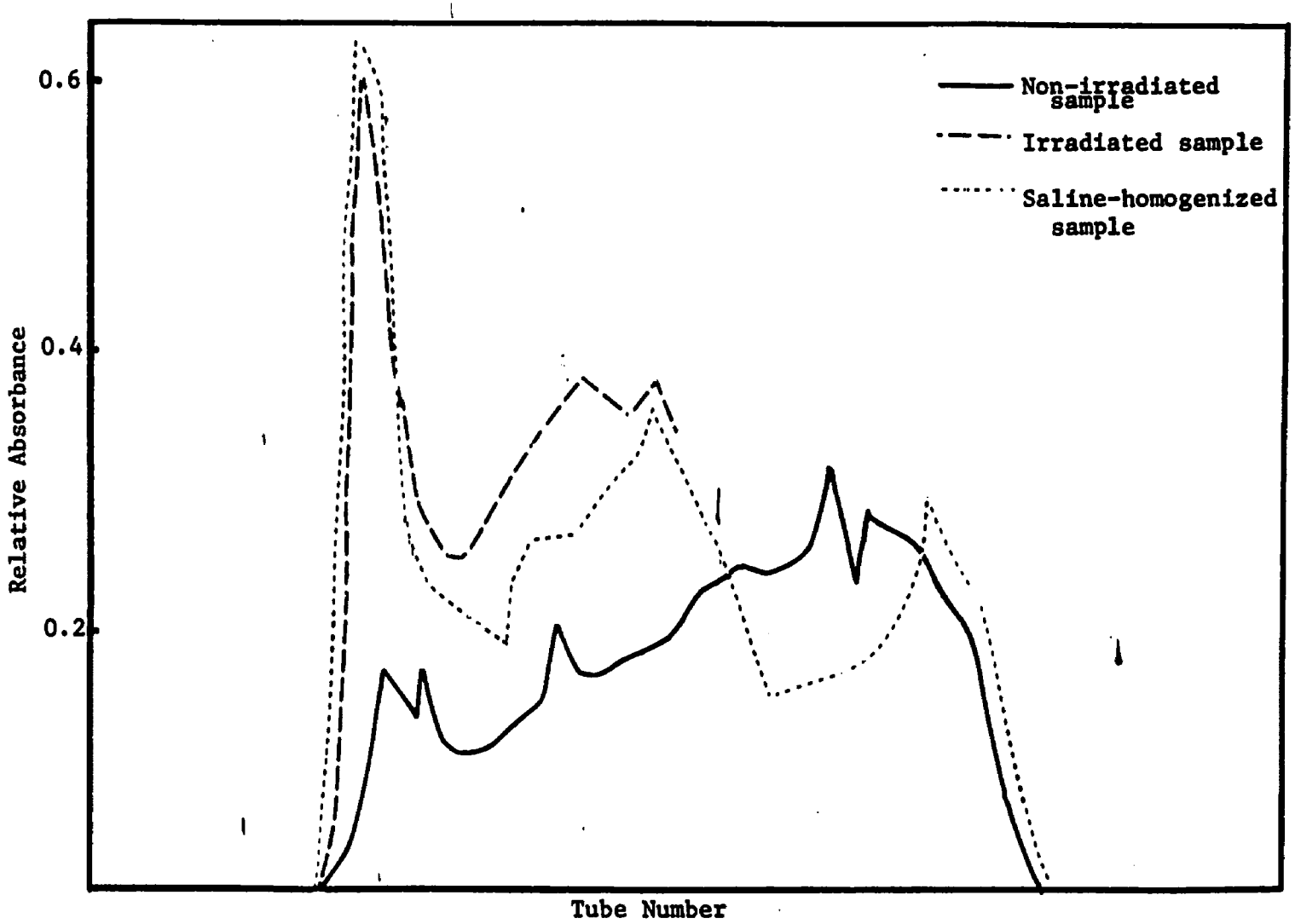

Figure 5--Graph of non-irradiated sucrose-homogenized protein profile from Figure 3 (2.32 gm wet weight), non-irradiated saline-homogenized profile from Figure 12 (3.12 gm wet weight), and irradiated sucrose-homogenized profile from Figure 2C (2.98 gm) for comparison of treatments.

and 6.0 minutes. The pelts were scraped, homogenized, centrifuged, and applied to the column as soon as possible. The opalescent fractions were pooled and extracted with chloroform-methanol as will be described later. The chloroform-methanol extract was taken to dryness, resuspended, and aliquots were assayed for total cholesterol as will be described below. The results, shown in Table 1, indicate that irradiation periods of 1.5, 3.0, and 6.0 minutes appear to produce equivalent amounts of opalescent material, and the 0 and 0.75 minute periods produce equivalent amounts. The total cholesterol values for 0 and 0.75 minute irradiation periods and the values for the 1.5 and 6.0 minute periods are within one standard deviation of the mean of the non-irradiated and irradiated values, respectively, from Table 4. The value for the 3.0 minute period is two standard deviations of the mean from the irradiated means, but the deviation is probably not biologically significant in view of the values of the periods on either side of the 3.0 minute period. These data would suggest that an exposure between 0.75 minutes (0.75 MED) and 1.5 minutes (1.5 MED) is sufficient to cause the solubilization of the opalescent material.

Characterization of the Opalescent Material

Electrophoresis

Aliquots of irradiated and non-irradiated samples were applied to Sepraphore III[6] cellulose acetate strips (2.5 x 17 cm), presoaked according to the manufacturer's instructions. The strips were placed in an electrophoresis chamber[6] with cold 0.05 ionic strength Tris-barbital-

[6]Gelman Instrument Co., Ann Arbor, Michigan.

TABLE 1

INDEPENDENCE OF RESPONSE TO DOSE ADMINISTERED

Period of Irradiation* (min)	Total Cholesterol / Wet Tissue Weight (mg/gm)
0	0.082 (1)
0.75	0.098 (2)
1.50	0.121 (2)
3.00	0.151 (1)
6.00	0.139 (1)

*Equivalent to MED.

All assays performed in triplicate. Number of animals assayed in parenthesis.

Average of three non-irradiated total cholesterol assays from (Table 4) $\pm$ standard deviation of the mean = 0.091 $\pm$ 0.018 mg total cholesterol per gm wet tissue weight. The average of three irradiated values = 0.130 $\pm$ 0.010 mg total cholesterol per gm wet tissue weight.

sodium barbital buffer, pH 8.8 (HR Buffer[7]) and electrophoresed at one milliampere per strip for one hour. The strips were removed and stained with Ponceau S stain for five minutes. Excess stain was removed with four, two minute rinses in 0.5% acetic acid. The strips were blotted and photographed immediately, as resolution decreased with drying of the strips. The electrophoresis of samples of irradiated and non-irradiated opalescent material and guinea pig serum are seen in Figure 6. The epidermal opalescent material samples appear to migrate with α-globulins.

Immunology

Immunological techniques were employed to investigate the relationship of these tissue proteins to serum proteins, and to assist in determining the number of proteins associated with the opalescent material. To prepare an antigen to be used for the production of antiserum, twelve guinea pigs were processed in the usual manner. The opalescent material was isolated from the Sephadex column, concentrated with PEG, and mixed with an equal volume of Freund's complete adjuvant (45)[8]. The mixture was injected subcutaneously on all four flanks of a white rabbit (46). The rabbit was bled at intervals by cardiac puncture. The blood was allowed to coagulate at room temperature for one hour then refrigerated overnight. The serum was withdrawn, centrifuged, and the precipitate discarded. This serum was used as the antiserum without any further treatment. Within two weeks after injection, double diffusion experiments showed the rabbit's serum contained enough antibody to form an

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[7]Gelman Instrument Co., Ann Arbor, Michigan.

[8]Difco Laboratories, Detroit, Michigan.

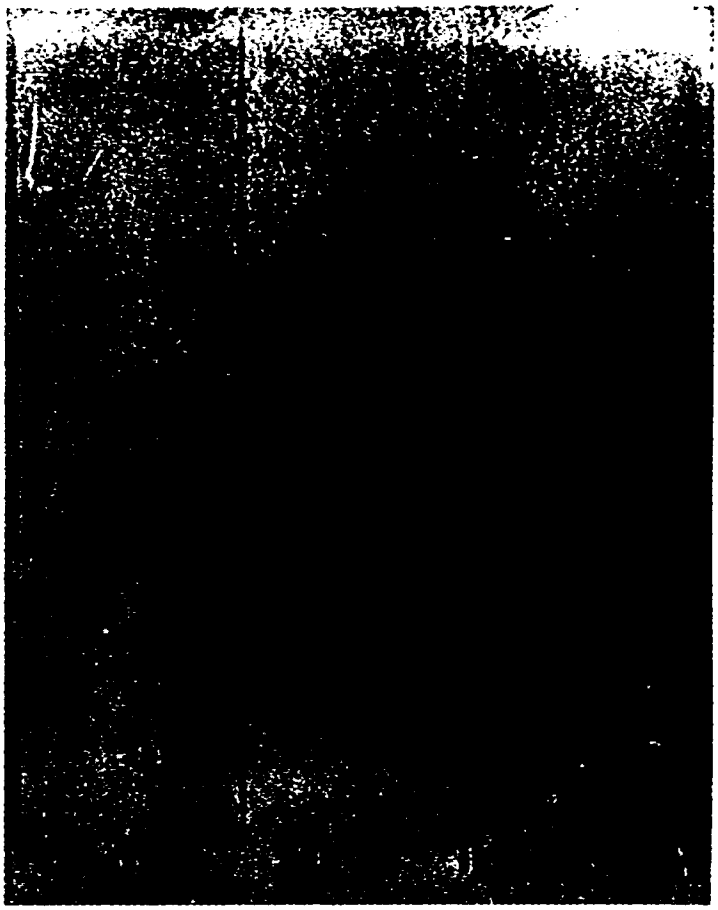

Figure 6--Cellulose acetate strips comparing the electrophoretic mobility of irradiated and non-irradiated (control) opalescent material to guinea pig serum.

antigen-antibody reaction to the opalescent material. Commercially available rabbit antiguinea pig serum antiserum[9] was also utilized.

Double diffusion experiments were performed by the method of Ouchterlony (47) to show the immunological relationship between irradiated and non-irradiated (control) samples of the opalescent material. Double diffusion was performed in 1% agarose plates (Ago-Immunodiffusion Plates[9]). The results, shown in Figure 7, indicate a single band of immunological identity between these two samples. A single band of identity was also seen between these two bands and a serum protein (see Figure 8). Commercial antiguinea pig serum also showed a single band of identity to the opalescent material.

Immunoelectrophoresis experiments were performed by the method of Kahn (48) to show which serum protein was reacting to the antiserum. Cellulose acetate strips were presoaked in buffer, loaded with the sample, and electrophoresed for one hour at one milliampere per strip. The strips were cut in half longitudinally. One section was stained with Ponceau S stain, the other was placed on agar plates. The strips were laid in parallel a short distance apart so that troughs could be cut equidistant between the strips for the placement of antisera. The strips on the agar were overlayed with 0.9% saline, allowing the saline to be absorbed through the strips into the agar. Agar plates were made by pouring melted 1.0% Special Noble Agar[10] in 0.05 ionic strength Tris-barbital buffer, pH 8.8, onto glass plates 8 x 10 cm. The plates were placed on water soaked pads in petri dishes and incubated at room

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[9]Mann Research, Inc., New York, New York.

[10]Difco Laboratories, Detroit, Michigan.

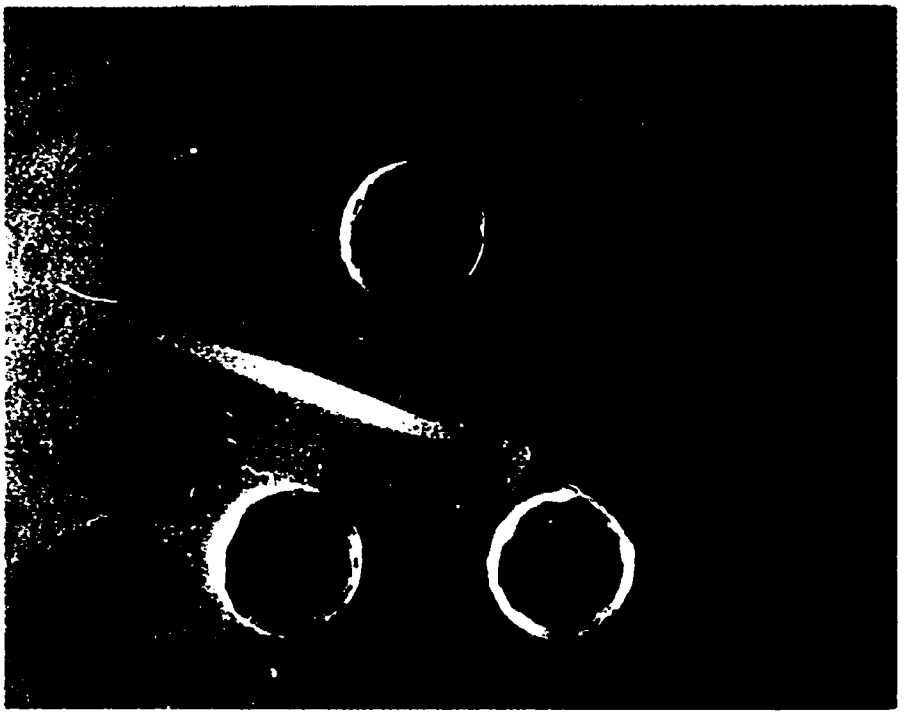

Figure 7--Double diffusion agar plate showing a single band of identity between irradiated (I), non-irradiated opalescent material (C), and opalescent material antiserum (As).

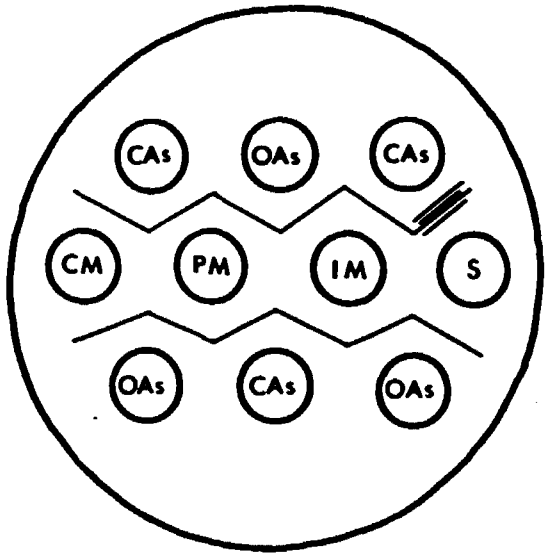

Figure 8--Double diffusion agar plate illustrating single band identity reactions of irradiated material (IM), non-irradiated material (CM), phospholipase A-treated material (PM), and serum (S) to commercial antiguinea pig serum antiserum (CAs) and opalescent material antiserum (OAs).

temperature. In other experiments, serum was electrophoresed and a portion stained as above. The fractions so outlined were cut out and placed separately on the agar next to the antisera troughs. Double diffusion experiments were performed on guinea pig serum fractions on whole cellulose acetate strips or on fractions isolated as described above. Antiserum to the opalescent material was placed in the troughs, shown in Figure 9A. The only bands detected were those in the α_1- and α_2-globulin areas. The single band found with the whole strip would indicate a partition between these two serum fractions. Similar experiments with irradiated opalescent material show a single band (see Figure 9B) migrating with the α-globulins.

Electron Microscopy

The gel filtration experiments indicated this material was of a large size. The sensitivity of this material to osmotic pressure changes, suggesting that a structural element might be involved, prompted the use of electron microscopy. An aliquot of irradiated opalescent material was air dried on formovar-coated copper grids and stained with a solution of 1% phosphotungstic acid, pH 7.2. The grids were dried and examined by electron microscopy. Photographs were made, one of which is seen in Figure 10. Note the spheroidal, lipid-like globular shape and the lack of structure. The average diameter of the spheroid is 0.1 microns.

Chemical Analysis

To study the chemical nature of both irradiated and non-irradiated opalescent samples, portions were subjected to several

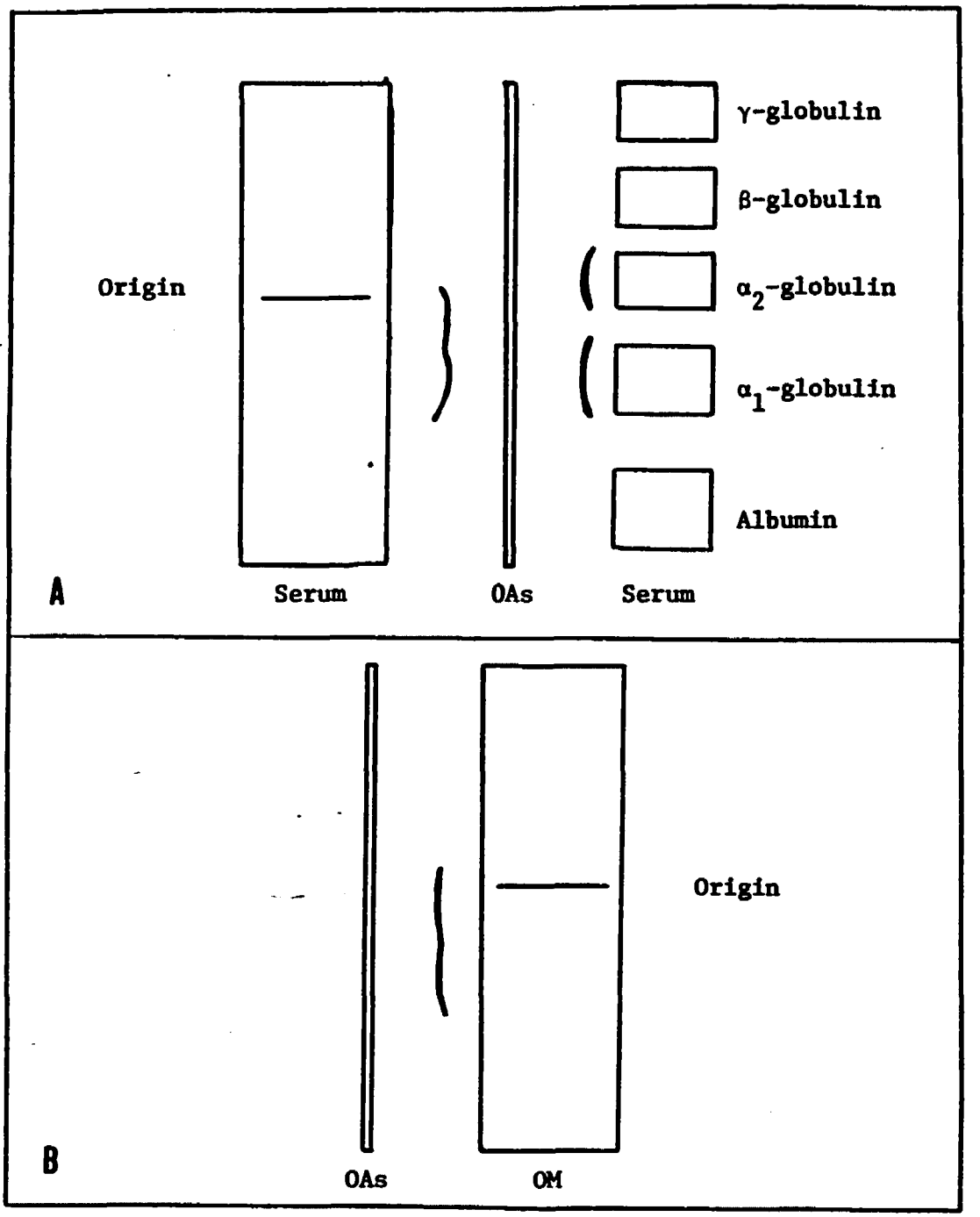

Figure 9--Immunoelectrophoresis patterns. A - Pattern illustrating the single band reaction between irradiated opalescent material antiserum (OAs) and serum proteins. B - Pattern illustrating the single band reaction between the irradiated opalescent material (OM) and its antiserum (OAs).

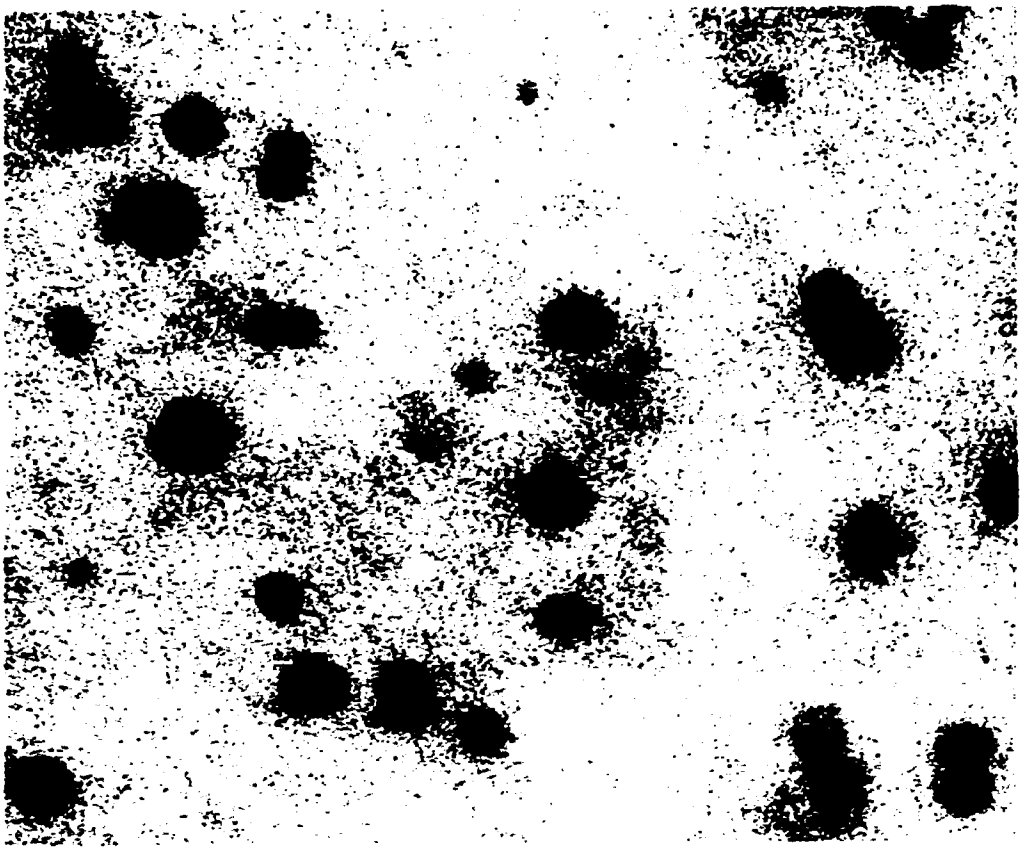

Figure 10--Electron micrograph of the irradiated opalescent material negatively stained with phosphotungstic acid. Enlarged 61,250 times.

chemical assays. Aliquots of irradiated samples were subjected to acid hydrolysis and the hydrolysate assayed for sialic acid, total hexose, and amino sugars. Other irradiated and non-irradiated samples were assayed for total cholesterol, lipid phosphorus, free fatty acids, free cholesterol, and for protein.

Acid hydrolysis. Opalescent material, derived from epidermal scrapings (weighing 15.25 gm) of six irradiated guinea pigs, was concentrated with PEG to 15 ml. A 12 ml aliquot was placed in dialysis tubing and dialyzed against one liter of 0.01 M acetate buffer, pH 5.5. The buffer was changed every two hours until carbohydrate assays on the lyophilized dialysates, for two successive changes, were negative. The sample was then concentrated to nine ml and sufficient concentrated HCl was added to make the solution 8N. The sample was placed in a hydrolysis tube and frozen in a dry ice-acetone bath. While frozen the tube was flushed with CO_2 and sealed. The tube was then placed in a preheated beaker of sand at 110°C and heated for five hours. The hydrolysate was removed and placed in vacuo over NaOH and P_2O_5 until dry. The dried hydrolysate was resuspended with five ml distilled water. This solution was assayed for amino sugars, total hexoses, and sialic acid.

Amino sugar assay. The method of assay was, with one change, that of Gardell (49). Half the quantity of ethanol prescribed was used. This increased the sensitivity of the assay. Triplicate assays were performed utilizing 0.3 ml of the hydrolysate. Glucosamine was used as standard and assayed simultaneously. The results, adjusted to the total amount present in the sample derived from the 15.25 gm wet weight epidermal tissue, are presented in Table 2 as mg/gm wet tissue weight.

TABLE 2

ANALYSIS OF PRODUCTS OF HYDROLYSIS
OF OPALESCENT MATERIAL

Assay	mg/gm Wet Tissue Weight
Protein	0.336 ± 0.026
Amino Sugar	0.017 ± 0.001
Total Hexose	0.039 ± 0.001
Sialic Acid	0.007 ± 0.001

Values are averages of triplicate assays ± the standard deviation of the mean.

Total hexose assay. Total hexose was assayed by the anthrone method (50). Aliquots of 0.2 ml of the hydrolysate were assayed in triplicate along with aliquots of glucose as standard. These results, corrected to the total sample, are presented in Table 2 as mg/gm wet tissue weight.

Sialic acid assay. The sialic acid content was measured by means of the resorcinol assay of Svennerholm (51). A 0.5 ml aliquot of the hydrolysate was diluted to 3.1 ml with distilled water and one ml aliquots were assayed in triplicate. These values were compared to N-acetylneuraminic acid as standard, treated in an identical manner. These values are seen in Table 2, corrected to the total sample, as mg/gm wet tissue weight.

Lipid extraction. Aliquots of the opalescent material from both irradiated and non-irradiated samples were extracted with 20 volumes of chloroform-methanol (2/1; vol./vol.) by the method of Folch, et al. (52). To this mixture was added one volume 0.9% saline. The mixture was shaken vigorously and allowed to stand in the cold until the chloroform layer (lower layer) was clear. The chloroform layer was withdrawn and taken to dryness in a flash evaporator. The contents were resuspended with aliquots of chloroform-methanol and transferred quantitatively to a screw-capped tube. The volume was reduced further under nitrogen. The extracted material was tested to determine whether one extraction was sufficient to remove the lipids from the sample. The pellet centrifuged from the aqueous phase was subjected to 4.5 hours refluxing with 20 ml chloroform-methanol in a Soxhlet apparatus.[11]

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[11] Kontes Glass Co., Vineland, New Jersey.

The material so extracted was assayed for cholesterol as described below. No cholesterol could be detected.

Cholesterol and lipid phosphorous assays. The non-irradiated (control) chloroform-methanol extracts were compared to the irradiated extracts to determine if these samples differed in their cholesterol or lipid phosphorous content. Assays for cholesterol were performed by both the Lieberman-Burchard method (53) and by the method of Zak (54). The phosphate assay was that of Dryer (55) and was performed on the same chloroform-methanol extract used for the Lieberman-Burchard assays, allowing a comparison between cholesterol and lipid phosphorus. With these experiments, 0.2 ml aliquots of the extracts were assayed in triplicate for cholesterol and 0.2 ml was assayed for phosphorus. Disodium glycerol phosphate pentahydrate was used as phosphorous standard. Protein assays, performed on the material prior to extraction with chloroform-methanol, were used to compare with the cholesterol and lipid phosphorus assays. These data are presented in Table 3, comparing the cholesterol and lipid phosphorus both to protein and to wet weight of epidermal scrapings. These data shows an increase of cholesterol with irradiation treatment by either comparison. Lipid phosphorus values appear to be decreased after irradiation.

The Lieberman-Burchard method of assay, without correction, is not a true measure of cholesterol, since esters give a higher chromagen value than free cholesterol. Since these samples contained cholesterol esters, it was decided to utilize the Zak method of assay, measuring the total cholesterol directly and measuring the free cholesterol as the digitonide. As seen in Table 4, the increase in total cholesterol values

TABLE 3

CHOLESTEROL AND LIPID PHOSPHOROUS ASSAYS ON CHLOROFORM-METHANOL EXTRACTS OF OPALESCENT MATERIAL

		Wet Wt. (gm)	Cholesterol* (mg)	Lipid P. (mg)	Protein (mg)	Cholesterol / Protein (mg/mg)	Lipid P. / Protein (mg/mg)	Cholesterol / Lipid P. (mg/mg)	Cholesterol / Wet Wt.≠ (mg/gm)	Lipid P. / Wet Wt.≠ (mg/gm)	Protein / Wet Wt.≠ (mg/gm)
Control	1	9.37	0.419	1.240	1.352	0.31	0.92	0.32	0.045	0.132	0.144
	2	5.55	0.180	0.388	0.582	0.31	0.98	0.46	0.032	0.070	0.105
	Avg.					0.31	0.95	0.40	0.036	0.101	0.124
Irradiated	1	8.11	1.500	0.680	1.623	0.92	0.42	2.21	0.185	0.084	0.200
	2	6.42	0.560	0.266	0.930	0.60	0.29	2.11	0.087	0.042	0.145
	Avg.					0.76	0.36	2.16	0.136	0.063	0.176
Difference						+0.45	-0.59		+0.100	-0.038	+0.052

*Lieberman-Burchard Assay
≠Wet Tissue Weight
Each experiment used three animals. All assays in triplicate.
Standard deviation of the mean not greater than ± 0.005 for any assay.

TABLE 4

FREE AND TOTAL CHOLESTEROL ASSAYS* ON CHLOROFORM-METHANOL EXTRACTS OF OPALESCENT MATERIAL

		Wet Weight (gm)	Total Cholesterol (mg)	Free Cholesterol (mg)	Total Cholesterol** / Wet Weight ≠ (mg/gm)	Free Cholesterol / Wet Weight ≠ (mg/gm)
Control	1	6.32	0.864	0.384	0.137	0.061
	2	3.82	0.198	0.088	0.052	0.023
	3	4.84	0.444	0.198	0.092	0.041
	Avg.				0.094	0.042
Irradiated	1	8.19	1.076	0.636	0.131	0.078
	2	6.32	0.924	0.540	0.146	0.085
	3	6.86	0.764	0.488	0.112	0.071
	Avg.				0.130	0.078
Difference					0.036	0.036

*Zak Method (54). Triplicate assays.
**Standard deviation of the mean for control mean = 0.018; for irradiated mean = 0.010.
≠ Wet tissue weight.

with irradiation is the same as the increase in free cholesterol values. This implies that the change in cholesterol in the irradiated tissue is due to the increase of free cholesterol.

Thin Layer Chromatography

Thin layer chromatography (TLC) was used to compare the samples qualitatively for neutral lipid and phospholipid changes. Thin layer plates were prepared by spreading an aqueous slurry of Silica Gel G onto glass plates (20 x 20 cm) with an adjustable spreader[12] to a thickness of 500 microns. The plates were dried at 110°C for 30 minutes. The plates were developed in either chloroform/methanol/water (65/25/4) for phospholipids (56), or petroleum ether/diethyl ether/acetic acid (90/10/1) for neutral lipids (57). The phospholipids were detected by spraying with Zinzadze reagent (58). The neutral lipids were either sprayed with an aqueous 50% sulfuric acid solution containing 1% dichromate and heated in an oven at 100°C, or placed in a closed container with iodine vapor. The lipids were characterized by chromatographing the unknown extract solutions with known standard lipids.

Phospholipids. The chloroform-methanol extracts were examined qualitatively for differences between irradiated and non-irradiated samples. No qualitative differences could be observed. As is seen in Figure 11, the samples did not appear to contain cardiolipin, a constituent of mitochondria and lysosomes.

Neutral lipids. Examination of the extracts for neutral lipids revealed that the two fractions appeared to be similar except for fatty

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[12]Research Specialties Co., Richmond, California.

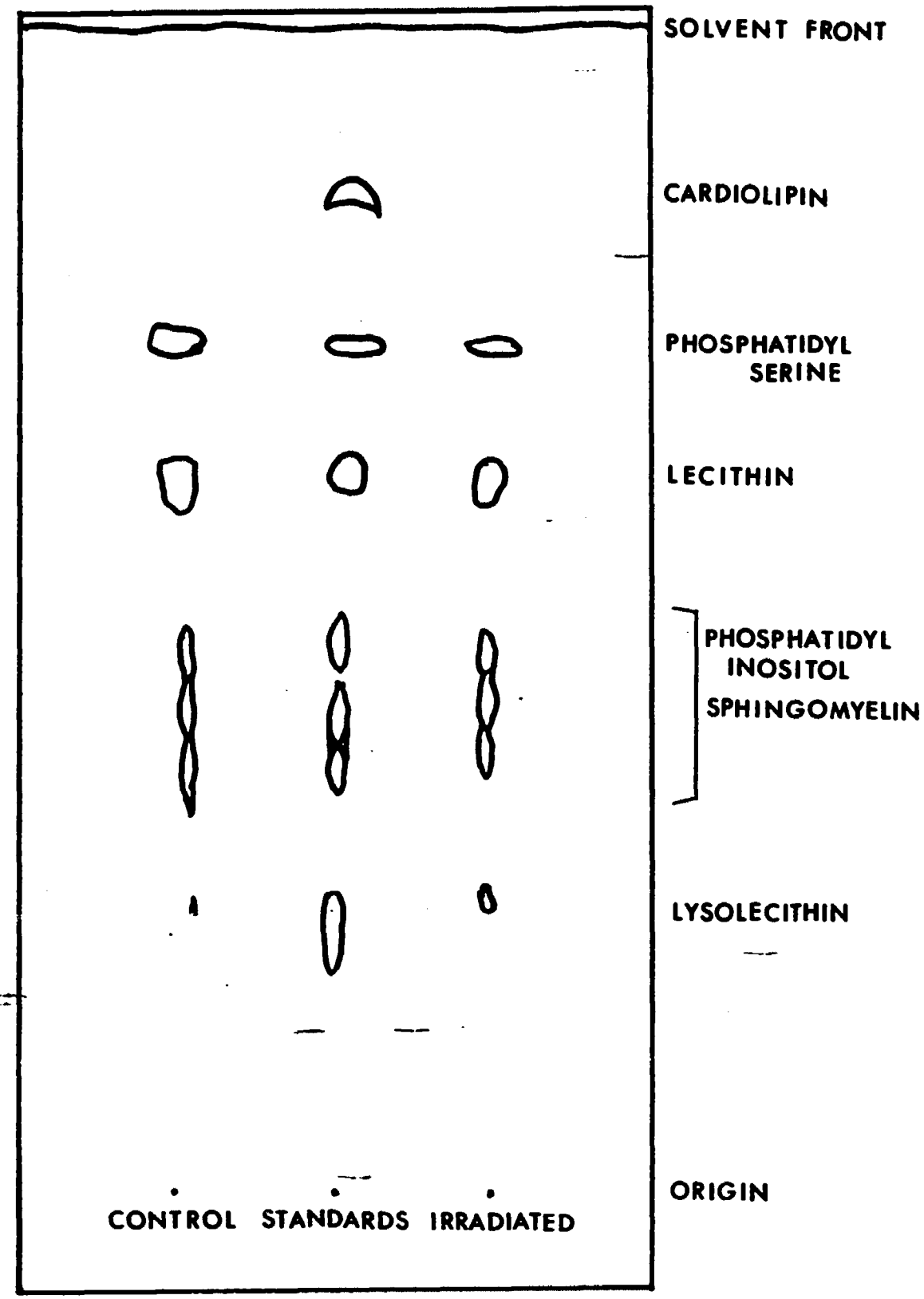

Figure 11--Tracing of thin layer plate illustrating phospholipids of non-irradiated (control) and irradiated samples.

acids of the irradiated sample. As is seen in Figure 12, the two samples seem equivalent in other lipids except fatty acids, where the irradiated fatty acid appears to be in greater quantity. Based on this evidence gas-liquid chromatography was used to examine the fatty acid composition of the samples.

Gas Liquid Chromatography

The evidence from the TLC of neutral lipids of irradiated samples, showing the apparent increase in fatty acids, prompted an investigation into the fatty acid composition of both samples. Fatty acids were isolated from thin layer plates. The materials migrating with fatty acid standards were scraped from the plates and eluted with the chloroform-methanol mixture. One three ml aliquot of solvent was shaken with the silica gel, allowed to stand 10 minutes, then centrifuged. The silica gel was washed with an additional two ml of solvent. The supernatant washings were combined and reduced under nitrogen to 0.5 ml. To this mixture was added one ml BF_3-Methanol (BF_3 methyl ester kit, 14% w/v[13]) following the procedure of Morrison (59). The mixture, in a screw cap tube, was placed in a boiling water bath for 30 minutes. The tubes were allowed to cool and one ml water was added with shaking. Two ml of N-hexane were used to extract the methyl esters from the mixture. An additional two ml hexane were used to repeat the extraction. The hexane fractions were combined and the volume reduced under nitrogen. These samples were then submitted to gas-liquid chromatography. The column used was a diethylene glycol succinate column (185 x 0.5 cm) utilizing Chromasorb W (AW), 80-100 mesh, as stationary phase. Argon carrier flow

[13]Applied Science Laboratory, Inc., State College, Pennsylvania.

Figure 12--Thin layer plate showing neutral lipids. Cholesterol (C), non-irradiated opalescent material (CP), phospholipase-treated sample (PLP), irradiated opalescent material (IP), and palmitic acid (P).

rate was 59 ml/minute. A flame detector was used with hydrogen flow rate 53 mm/min and air flow rate 306 mm/min. Temperature of the injector was 260°C, column 188°C, and the detector 221°C. The results are seen in Table 5. The data suggest that there is no difference in the fatty acid composition between the two samples.

Microsomal Assay

Because the centrifugation procedure for the homogenates would not remove all the microsomes, a series of experiments was performed to test: (a) whether there was indeed any microsomal contamination in the opalescent material, and (b) whether the microsomal content in the supernatants was altered with UV treatment.

The assay procedure was that of Bottomley (60), utilizing the reaction of alkaline phosphatase (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) as a marker enzyme. Stock solutions were made up as: 48.9 mg of p-nitrophenylphosphate[14] per 10 ml water; 0.5M tris-HCl buffer, pH 10.3; and 0.1M $MgCl_2$ as cofactor. Test tubes were prepared with 0.4 ml of a solution containing 10 parts substrate, 10 parts buffer, and 0.2 parts cofactor. These tubes were frozen until required for assay. A 0.2 ml aliquot of the sample to be assayed was added to the thawed tubes. The mixture was incubated at 37°C, until the color developed, then the reaction was stopped with the addition of one ml 0.25 N NaOH. The optical density was determined in a spectrophotometer at 410 nm. With this assay procedure 1.0 optical density unit equals 0.1 micromole substrate hydrolyzed per ml. In one experiment the assays were

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[14]Sigma, St. Louis, Missouri.

TABLE 5

FATTY ACID PERCENT COMPOSITION

FA	Percent Composition					
	Control			Irradiated		
	1	2	Avg.	1	2	Avg.
16:0	21.1	21.4	21.3	19.4	22.6	21.0
16:1	8.5	8.2	8.4	10.8	9.2	10.0
Unknown	7.0	9.9	8.5	None*	None*	--
18:0	14.5	14.6	14.6	22.8	24.4	23.6
18:1	16.7	20.5	18.6	15.5	17.7	16.6
18:2	10.5	14.1	12.3	11.8	11.0	11.4
18:3	8.9	6.5	7.7	10.0	9.4	9.7
Unknown	12.9	5.1	9.0	9.6	5.9	7.8

*Found to be masked by the 18:0 peak. Percent composition of control unknown plus 18:0 sums to the irradiated 18:0 value.

performed on an aliquot of the supernatant before application to the Sephadex column and on an aliquot of the pooled opalescent material after passage through the column. The results indicated that essentially all the activity applied could be recovered in the opalescent fractions. Therefore a series of experiments was undertaken to compare the alkaline phosphatase activity in equivalent samples of supernatants from homogenates of irradiated and non-irradiated halves of a single animal pelt. The results, seen in Table 6, show that there was essentially no difference between the two samples.

Solubilization with Phospholipase A

Because of certain similarities of the response of UV irradiated epidermis to that of phospholipase A[15] (phosphatide acylhydrolase, EC 3.1.1.4) in snake venom (40), an experiment was performed to test the effect of that enzyme on epidermal homogenates. A guinea pig was sacrificed and the epidermis removed as described for the non-irradiated preparations. The scrapings were homogenized in cold 0.15 ionic strength tris buffer, pH 8.8, that was made 0.3M in sucrose. The homogenate was allowed to equilibrate to room temperature before adding one mg phospholipase A. The mixture was incubated at room temperature with stirring for one hour. The usual centrifugation pattern was followed. The supernatant was applied to a small Sephadex G-200 column and eluted in the usual manner. The protein profile may be seen in Figure 13 (as compared to a control protein profile in Figure 3). The figure shows that a larger quantity of opalescent material is derived from the tissue with this enzyme treatment than with UV irradiation. The experiment was

[15]Sigma, St. Louis, Missouri.

TABLE 6

ALKALINE PHOSPHATASE ACTIVITY IN SUPERNATANTS

Animal	Alkaline Phosphatase Activity (μM/min/gm Wet Tissue Weight)	
	Non-Irradiated	Irradiated
1	0.041	0.046
2	0.028	0.032
3	0.033	0.037
4	0.031	0.031
Avg.	0.033	0.036

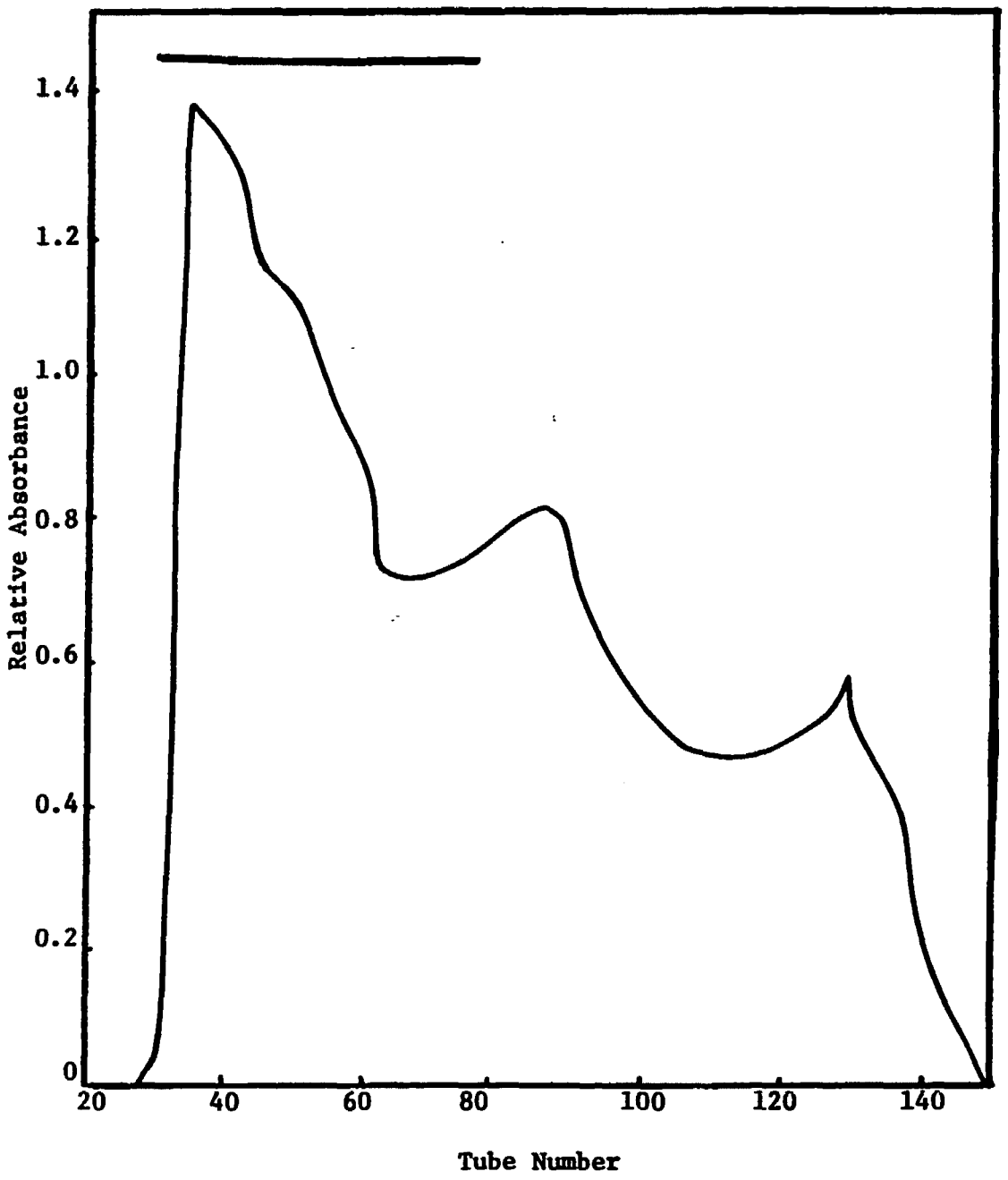

Figure 13--Graph of protein in fractions from Sephadex column of non-irradiated homogenate (2.93 gm wet weight) treated with phospholipase A. Bar above initial peak denotes opalescent fractions.

repeated with equivalent results. As is noted in Figure 8, the material from the phospholipase A-treated epidermis is immunologically identical to both irradiated and control, with either the opalescent material antisera or the serum antisera. The electrophoresis pattern was identical to that of the irradiated opalescent material. The neutral lipid pattern, seen in Figure 12 as PLP, is similar to the irradiated pattern in having the fatty acid spot. There was no apparent difference from the irradiated material in the phospholipid pattern, including the lack of cardiolipin.

CHAPTER III

DISCUSSION

The evidence presented is consistent with the hypothesis that ultraviolet light irradiation of guinea pig epidermis results, in part, in the specific solubilization of a particular intracellular component, probably an organelle.

Solubilization or fragmentation of membranes can be accomplished *in vitro* by phospholipase A (40, 61) or by sonication (62). In either case, a large molecular weight, opalescent, globular material is released, not unlike the material formed by the action of UV light. The product of the reaction of phospholipase A on epidermal homogenates resembles the UV photoproduct when compared by immunological, column and thin layer chromatographic, and electrophoretic techniques. The protein released or solubilized by the enzyme was shown to be immunologically identical and electrophoretically similar to the UV photoproduct.

Rosenberg (62) showed, in his work on the solubilization of the erythrocyte membrane by sonication, that the opalescent product formed was excluded from a Sephadex G-200 column, in a manner similar to the UV photoproduct. He showed by electron microscopy that the opalescent material consisted of spherical globules varying in diameter from 0.02 to 0.04 microns. The globular material in this study averaged 0.1 micron in diameter. Gurd (63), in reference to low density

lipoprotein, states that the agents which disrupt the stable lipid complex lead to aggregations of larger particles which, in turn, increase the turbidity of the solution.

Homogenization of epidermal tissue in 0.9% saline also appears to cause solubilization of components of that tissue. This is indicated by the difference in magnitude between opalescent peaks of saline-homogenized and sucrose-homogenized samples.

There are several considerations in support of the specificity of the solubilization by UV. First, thermal injury, known to produce vascular changes and a biphasic edema response in guinea pig skin (44), fails to produce the opalescent material in quantities greater than the untreated control. Second, no qualitative changes in fatty acids, neutral lipids, or phospholipids in the UV-treated samples were apparent. One might anticipate that if fatty acid oxidation were involved there would be a change in the composition of the fatty acids or a change in the phospholipid or neutral lipid fatty acids. Third, of the chemical assays performed the only quantitative changes detected were; (a) the increase in total cholesterol, free cholesterol, and protein, and (b) the decrease in lipid phosphorous. A loss of lipid phosphorous following UV treatment was also found by Tichner (27). The destruction of phosphatidyl plasmalogen and phosphatidyl ethanolamine, noted by Tichner, were not detected in this study since no quantitative estimates were made of phospholipid changes. The increase in total cholesterol appears to be due to an increase in the free cholesterol. There is a possible relationship between protein and cholesterol in the irradiated samples. This relationship is supported by the following evidence: (a) both are

increased in the irradiated samples; (b) evidence presented in this study shows the serum protein, immunologically identical to the opalescent material, migrates with α_1- and α_2-globulins; (c) Ling states (64) that the cholesterol-binding globulin, migrating electrophoretically with the α-globulins, may be a series of several serum proteins; (d) Bailey (65), in studying the dynamic exchange of cholesterol in cell cultures, showed that the exchange of cholesterol through the cellular membrane was mediated by certain serum α-globulins. This was true for endogenously synthesized cholesterol as well. Fourth, because the response can be provoked in vitro, the direct participation of serum proteins seems unlikely. Fifth, the short time period between insult and assay, as seen in the dose-dependence experiments, precludes an enzymatic mechanism. Thus the appearance of the opalescent material seems to be a specific in vivo response to UV irradiation.

The equivalent alkaline phosphatase activity in the irradiated and non-irradiated samples would indicate the microsomal contents are also equivalent, therefore the microsomes appear to be unaltered by UV treatment. Since the neutral lipids (except free cholesterol) and phospholipids in the opalescent material do not appear to be qualitatively different from microsomal components (66), perhaps the neutral lipids and phospholipids present in the opalescent material are derived from microsomes. This would imply the free cholesterol increase is not due to an increase in microsomal content. This would also suggest that the decrease in lipid phosphorus was not a decrease in microsomal lipid phosphorus, or that its loss is not related to the presence of microsomal alkaline phosphatase enzyme activity. It would appear, therefore,

that the solubilization, perhaps resulting (as in Tichner's work (27)) from the loss or breakdown of the lipid phosphorus bearing moiety, results in the liberation of the cholesterol-protein complex. Microsomal contamination might well explain the glycoprotein nature of the opalescent material since glycoproteins have been associated with microsomes (66). The size and osmotic pressure sensitivity of microsomes could also explain the initial observations in the isolation of the opalescent material by gel filtration.

It would appear, from the failure of the response of opalescent material to show dose dependency with exposures greater than one MED, that: (a) there is, perhaps, a threshold of energy from UV irradiation required to produce the solubilization, i.e. about one MED; (b) a depletion of the precursor material (i.e. production of opalescent material) with one MED exposure is perhaps, required for erythema to occur. The formation of the opalescent material might involve the release of a finite amount of an erythrogenic agent, such as the kinins suggested by Epstein (5).

Nix, in electron microscopic work on human epidermis (67) and guinea pig epidermis (68), has shown that large membrane-bound vacuoles appear within an hour following exposure to UV and remain for at least 12 hours. These vacuoles were observed to contain material which resembled intercellular space material. A similar observation was made by Lane (69) in UV irradiated cell cultures of KB cells. Nix also observed that besides vacuole-containing cells there were cells without vacuoles which contain diffused cytoplasm of decreased density. Other cells, he noted, contained vacuoles without membranes, suggesting a

transitory stage between the diffuse-appearing cells and the membrane-bound vacuoles. Since the quantity of opalescent material in this study is only slightly decreased after a four hour delay post irradiation, perhaps the vacuoles observed by Nix and Lane contain this opalescent material.

Thus, the evidence from this study and from the literature is consistent with the hypotheses that approximately one MED of ultraviolet light solubilizes or fragments an intracellular organelle causing a decrease in structural organization and a local increase in soluble protein-bound cholesterol. Homogenization of this tissue in a slightly hypertonic medium would likely result in an aggregation of these lipid-containing fragmented components, hence, the observed opalescence. The absence of cardiolipin suggests that at least mitochondria and lysosomes are not involved since these tissues contain cardiolipin (70, 71). The microsomes, with high phospholipid content (66), probably offers a better substrate for the phospholipase A enzyme than does the plasma membrane which has a lower phospholipid content. Since the material resulting from the enzyme incubation appeared to be similar to the UV product, perhaps microsomal tissue is a locus of UV damage. However, the alkaline phosphatase enzyme assay (showing no change with treatment) and the failure of the response to show dose dependency (giving maximum response after one MED) indicates that perhaps not all microsomal tissue is involved equally and that some portions are more labile to UV than others. The increase in the amount of free cholesterol is, perhaps, part of the repair mechanism, since the work of DeGier (72) shows that the cholesterol appears to decrease membrane permeability. The reorganization of

structural elements could possibly result from the accumulation of the solubilized material into vacuoles, first by aggregation into membrane-less vacuoles, then by incorporation into membrane-bound vacuoles, perhaps to be acted on by lysosomes.

The opalescent material could thus be the result of UV damage to some cellular component, probably elements of the endoplasmic reticulum or Golgi apparatus. This damage is manifest <u>in vitro</u> in the aggregation of lipid-containing cellular components when the tissue is homogenized, and is observed as opalescent material. The damage is observed by electron microscopy as vacuoles.

CHAPTER IV

SUMMARY

Ultraviolet light irradiation of guinea pigs results in the appearance of opalescent material in the supernatant fraction of 0.3 M sucrose epidermal homogenates. To lend further insight into the interaction of UV and skin, some of the physical and biochemical properties of this material were studied. The opalescent material, isolated by gel filtration was shown to have a large size. The amount of material was not affected by vascular circulation. It occurred in greater quantity when isolated immediately following irradiation than when isolated after a four hour delay. The material appeared to be a specific response to UV injury, since thermal injury failed to provoke the response. The response was not dose dependent and appeared to require one MED exposure to UV.

The protein associated with the material had an electrophoretic mobility similar to the α-globulins and was immunologically identical to a serum α-globulin. Electron microscopic evidence indicated the material had a spheroidal, lipid-like globular appearance, averaged 0.1 microns in diameter, and lacked definite structure. Chemical evidence indicated the opalescent material contained a glycoprotein moiety since it contained amino sugars, hexoses, and sialic acid. The material was shown to contain increased amounts of cholesterol (as free cholesterol)

and protein, and decreased amounts of lipid phosphorus when compared to non-irradiated preparations. Gas liquid and thin layer chromatography showed that fatty acids, neutral lipids, and phospholipids were not qualitatively altered. Alkaline phosphatase enzyme assays indicated that although microsomes were present their content was not altered with UV light treatment. The material solubilized by UV was found to be immunologically, electrophoretically, and chromatographically similar to material solubilized by phospholipase A.

The evidence is consistent with the hypothesis that ultraviolet light irradiation of guinea pig epidermis results in the specific solubilization of an intracellular organelle. This solubilization, perhaps, leads to aggregations of larger particles which results in increased turbidity of the solution. Concomittant with, or as a result of the solubilization, there is an increase in a free cholesterol-protein complex and a decrease in lipid phosphorous. Electron microscopic evidence from other workers shows large vacuoles appearing in UV irradiated epidermis, which perhaps, may contain this opalescent material.

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