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Changes in the Phospholipids of Mouse Skin after Ultraviolet Irradiation *in vitro*

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If a mouse is exposed to ultraviolet radiation, changes occur in the skin that ultimately lead to malignancy (see Blum, 1959, for a complete discussion). In this phenomenon a primary photochemical change may be postulated to occur from which other observed changes derive. Such a photochemical change would be observed with irradiation of the skin *in vitro*, when complications such as inflammation and repair are eliminated. Further, if such irradiation *in vitro* is carried out at low temperature, accompanying or succeeding thermal reactions are correspondingly decreased.

The literature on photochemical changes in nucleic acids and their derivatives (e.g. Hollaender, 1941; Rapport & Canzanelli, 1950) and the known efficiency of ultraviolet light in producing mutations (Hollaender, 1955) suggest that the nucleic acids are the most likely component of the skin to show photochemical change. Such changes, however, were not observed in our experiments, but there were striking changes in the phospholipids. Significant changes were found to be limited to phospholipids containing ethanolamine and were most marked in the ethanolamine plasmalogen fraction.

Preliminary reports of some of the present work have been given (Tickner, 1959, 1960, 1961).

MATERIALS AND METHODS

Chemicals used throughout were of A.R. or, where available, M.A.R. grade.

Adult mice of strain A of either sex were used. The animal was killed by a blow on the head, the hair was shaved off with a scalpel and the mouse was flayed. The skin was freed of subcutaneous tissue by blunt dissection and divided into quarters. Alternate quarters were pooled and weighed. One of the weighed halves was spread in a quartz Petri dish covered with a quartz cover; the other half was similarly spread out in a glass Petri dish that was then enclosed in a closely fitting metal container. The two dishes were placed side by side in a deep-freeze cabinet (Frigidaire JCR 35) at -20° and irradiated with an un-screened medium-pressure mercury lamp (Hanovia, model 10) for 16 hr. at a distance of 50 cm. Under these conditions the temperature of the specimen did not exceed 0° , as judged by the use of a thermometer in apposition to the Petri dishes and also by the persistence of ice in the dish itself. For some experiments, more skin was required than could be obtained from one mouse. In these experiments individual skins were quartered as before and alternate quarters assigned to experimental or control groups.

Separation into and measurement of the main phosphorus-containing groups (acid-soluble phosphorus, phospholipids, RNA phosphorus, protein phosphorus and DNA phosphorus) was carried out by modifications of methods of Schmidt & Thannhauser (1945) and Logan, Mannell & Rossiter (1952). Skin was dispersed in ice-cold water with

a micro wet-grinder (Townson and Mercer Ltd.) to give a dispersion containing 200 mg. fresh wt. of skin in 1 ml. Quadruplicate samples of the dispersion were treated as follows. To 1 vol. was added 2.5 vol. of ice-cold 10% (w/v) trichloroacetic acid. The supernatant (acid-soluble phosphorus) was removed and the precipitate suspended in a further 2.5 vol. of ice-cold 10% trichloroacetic acid, spun and the supernatant added to the acid-soluble phosphorus fraction. These procedures were carried out in a room at -10° . Subsequent manipulations were carried out at room temperature. The precipitate was suspended in 1 vol. of water and 4 vol. of ethanol. The supernatant was recovered after centrifuging, the precipitate washed successively with 5 vol. of ethanol and three times with 5 vol. of ethanol-diethyl ether (3:1, v/v). The precipitate was spun down between each wash and the supernatants were added to the phospholipid fraction.

Two samples of the extracted precipitates were incubated at 37° in 2 vol. of N-KOH with shaking for 16–20 hr. They were cooled nearly to 0° , and neutralized with ice-cold 6N-HCl. Ice-cold 10% trichloroacetic acid (1 vol.) was added and the precipitate spun down. The supernatant (RNA) was saved and the precipitate washed with 1 vol. of 5% trichloroacetic acid. The precipitate was spun down and the washings were added to the RNA fraction. The precipitate was suspended in 5 vol. of 5% trichloroacetic acid and heated for 15 min. at 90° . The supernatant (DNA) was saved after centrifuging and the procedure was repeated with a further 2.5 vol. of 5% trichloroacetic acid, the washings being added to the DNA fraction.

The other two samples remaining after the extraction of phospholipids were treated as follows. Each precipitate was suspended in 5 vol. of 5% trichloroacetic acid and heated for 15 min. at 90° . The supernatant (total nucleic acids) was saved after centrifuging and the procedure was repeated with a further 2.5 vol. of 5% trichloroacetic acid. The supernatants were combined.

The phosphorus content of the solutions was determined as follows. The organic solvents of the phospholipid fraction were evaporated over an infrared lamp. To the residue, or directly to the aqueous samples with the other fractions, 0.5 ml. of 10N- H_2SO_4 was added and heating continued by means of the infrared lamp (which avoids bumping) until charring began. To the hot mixture was added 0.2 ml. of conc. HNO_3 . This digestion was carried out in tubes 200 mm. $\times$ 24 mm. with ground-glass necks. Heating was carried on until acid fumes appeared, when the tube was almost closed by inserting into it a filter funnel with the upper end of its stem closed by a glass bead. Heating was then continued for a further $\frac{1}{2}$ hr. The digest was allowed partially to cool, when 10 ml. of water was added and the whole brought briefly to the boil. After cooling, the phosphorus content of the digest was estimated by the method of Berenblum & Chain (1938). The phosphorus derived from phosphoprotein was calculated by directly determining the orthophosphate present in the RNA fraction before digestion. Total nucleic acids and DNA were determined by ultraviolet spectrophotometry on samples of the appropriate fractions. Extinctions were measured at 268.5 m μ against heated trichloroacetic acid as blank (Logan *et al.* 1952). Samples of the total nucleic acid fraction were assayed for deoxyribose by the diphenylamine reaction and for ribose by the orcinol reaction as described by Schneider (1945).

In other experiments, the phospholipids were extracted by the method of Daoust & Hooper (1957) or by the method of Folch, Lees & Sloane-Stanley (1957). The choline content of lipid extracts was estimated by the method of Wheeldon & Collins (1958*a*). The ethanolamine and serine were measured by the following modifications of the method of Wheeldon & Collins (1958*b*): considerable difficulty was encountered in the removal of unchanged fluorodinitrobenzene by distillation, and it was therefore separated from the kephalin derivatives by chromatography on silicic acid-Hyflo Super-Cel (2:1, v/v). A suitable sample of lipid extract was treated with fluorodinitrobenzene as described and the solution containing dinitrophenylated kephalin was evaporated to dryness under reduced pressure. The residue was dissolved in 1 ml. of benzene and applied to the prepared column. The column was washed successively with 20 ml. of benzene and 25 ml. of chloroform to remove the unchanged reagent and the phospholipid derivatives were eluted with 10 ml. of chloroform-methanol (2:1, v/v). Estimation of the phosphorus content of the washings showed that 1% of that applied was eluted in the benzene fraction, whereas none could be detected in the chloroform wash. The yellow dinitrobenzene derivatives of the kephalin fraction were seen to stick firmly at the top of the column during washing and to move as a compact band during elution with chloroform-methanol. As these derivatives are light-sensitive (Wheeldon & Collins, 1958*b*) all glassware used was painted black.

The phospholipids were further analysed by the method of controlled hydrolysis and paper chromatography as described by Dawson (1958) with the following minor modifications: paper chromatography (Whatman paper no. 541) without preliminary washing was used; each run was made in 6 hr. with paper 10 in. square in the 'universal' chromatography apparatus described by Smith (1958). A preliminary dip of the paper in ninhydrin, to show ethanolamine and serine, was followed by use of a dip reagent (Burrows, Grylls & Harrison, 1952) for the detection of phosphate. Because of the difficulty of quantitative transfer to the paper, the phosphorus content of each solution was determined by direct estimation and the relative quantities of each component were determined by the chromatographic separation.

RESULTS

The distribution of phosphorus in irradiated and non-irradiated skin and the change in each fraction due to irradiation are shown in Table 1. There was a significant fall in the content of lipid phosphorus after irradiation, about one-half of which appeared in the acid-soluble fraction; the remaining portion became more resistant to extraction by lipid solvents and appeared in the RNA fraction after alkaline hydrolysis.

No significant changes in ultraviolet absorption and sugar content after irradiation occurred in this fraction, showing that the increased 'RNA phosphorus' observed was derived from phospholipid and did not represent any change in the RNA itself. The decrease in lipid phosphorus was con-

firmed in a second series of experiments in which the phospholipids were extracted in different ways.

Changes in the ethanolamine, serine and choline content of the phospholipids are shown in Table 3. Changes occurred both in the choline and ethanolamine fractions although only the latter was statistically significant.

Table 4 shows results obtained with controlled hydrolysis and paper chromatography. Only the lipids containing ethanolamine were affected. The content of ethanolamine plasmalogen was lowered markedly, to 28 % of the control value; the content of phosphatidylethanolamine was decreased significantly, but to a much less degree. The phosphatidylethanolamine estimate, however, included a small quantity of cyclic glycerophosphoric acid. In one experiment where clear-cut separation was achieved this substance was present to the extent of 0.7 μ mole/g. in the control skin and 0.57 μ mole/g. in irradiated skin.

DISCUSSION

A discrepancy is shown by considering the results in Tables 1 and 4. In the experiments of Table 1 about 30 % of the phospholipid phosphorus disappeared, about 10 % appearing in the acid-soluble fraction. In the experiments of Table 4 only about 8 % of the phospholipids disappeared. In Table 1 experiments the tissue was dispersed in water, precipitated with trichloroacetic acid and the precipitate extracted with ethanol-ether. In Table 4 experiments the tissue was dispersed in chloroform-methanol, followed by washing of the lipid extract with dilute potassium chloride. In Table 1 experiments the remainder of the missing phospholipid phosphorus appeared after alkaline hydrolysis.

These facts suggest that the missing phospholipid shown in Table 4 corresponded to that part of the phospholipid which appeared as increased acid-soluble phosphorus in Table 1, and that the remainder of the lost phospholipid of Table 1 indicates that it is more difficult to extract phospholipids of irradiated skin with ethanol-ether from a trichloroacetic acid precipitate of an aqueous dispersion than it is if the tissue is dispersed directly into chloroform-methanol. The restriction of changes in the phospholipids after irradiation to ethanolamine plasmalogen, and to a much lesser extent to phosphatidylethanolamine, is not easy to explain. The presence of the vinyl ether group may render some bond or bonds on the distal side of the P-O-C link or the P-O-C link itself susceptible to disruption by ultraviolet irradiation, but this appeared to be inoperative in serine or choline plasmalogen.

The constitution of the fatty acid and fatty

Table 1. *Distribution of phosphorus in mouse skin and the effect of irradiation*

Six experiments, each with analyses in duplicate, were done. Mean values $\pm$ s.e. are given. The differences are significant (Student's *t* test) for acid-soluble phosphorus and RNA phosphorus ($P < 0.01$) and highly significant for phospholipid phosphorus ($P < 0.001$).

Fraction	Control (μ moles of P/g. wet wt. of skin)	Irradiated as percentage of control
Acid-soluble	6.92 $\pm$ 0.98	111 $\pm$ 2.5
Phospholipid	8.23 $\pm$ 1.32	71 $\pm$ 5.5
Protein	0.52 $\pm$ 0.067	100 $\pm$ 2
RNA	6.00 $\pm$ 0.80	115 $\pm$ 3.5
DNA	7.10 $\pm$ 0.84	104 $\pm$ 3

Table 2. *Changes in phospholipid phosphorus due to irradiation*

Extraction was by two different methods. All experiments were carried out in duplicate. Mean values $\pm$ s.e. are given. Both differences are significant ($P < 0.01$ by Student's *t* test).

Method	No. of experiments	Control (μ moles of P/g. wet wt. of skin)	Irradiated as percentage of control
Schmidt & Thannhauser (1945)	7	7.84 $\pm$ 1.25	73 $\pm$ 5
Daoust & Hooper (1957)	8	11.05 $\pm$ 0.39	85 $\pm$ 4.5

Table 3. *Choline, ethanolamine and serine content of lipids of mouse skin, and changes due to irradiation*

Each experiment was carried out in duplicate. Mean values $\pm$ s.e. are given. The difference is significant for ethanolamine ($P < 0.05$ by Student's *t* test).

Substance	No. of experiments	Control (μ moles/g. wet wt. of skin)	Irradiated as percentage of control
Choline	10	5.4 $\pm$ 0.45	83 $\pm$ 9.5
Ethanolamine	4	3.5 $\pm$ 0.45	71 $\pm$ 5.5
Serine	4	3.4 $\pm$ 0.95	98 $\pm$ 6

Table 4. *Phospholipids of mouse skin*

Analysis was by controlled hydrolysis and paper chromatography (Dawson, 1958). All values (μ moles of P/g. wet wt. of skin) are given as means of seven experiments, each with analyses in duplicate, $\pm$ s.e. Both 'X' and 'Y' showed wide variations.

Fraction	Control	Irradiated
Alkali-labile		
Phosphatidylcholine	5.4 $\pm$ 0.08	5.4 $\pm$ 0.24
Phosphatidylethanolamine	2.3 $\pm$ 0.11	1.8 $\pm$ 0.14
Phosphatidylserine	1.7 $\pm$ 0.08	1.5 $\pm$ 0.12
Phosphoinositides	0.8 $\pm$ 0.15	0.8 $\pm$ 0.14
Acid-labile		
Choline plasmalogen	0.43 $\pm$ 0.07	0.59 $\pm$ 0.09
Ethanolamine plasmalogen	1.06 $\pm$ 0.08	0.30 $\pm$ 0.07
Serine plasmalogen	0.24 $\pm$ 0.06	0.28 $\pm$ 0.07
Resistant		
Sphingomyelin	1.08 $\pm$ 0.15	1.12 $\pm$ 0.15
'X' (approx. R_F 0.4)	0.48	0.62
'Y' (approx. R_F 0.2)	0.31	0.55

aldehyde moieties of phosphatidylethanolamine and of ethanolamine plasmalogen may differ from other phospholipids in such a way that they are more photo-labile than the latter. Although no direct evidence is available for mouse skin, Gray & Macfarlane (1961) showed that in pig spleen, lung and kidney, kephalin and kephalin plasmalogens contain fatty acids and aldehydes of longer mean chain length than lecithin and choline plasmalogens. Further, the average number of unsaturated bonds/molecule of fatty acid is greater in kephalin plasmalogen (3.2 in pig spleen) than in kephalin, lecithin or choline plasmalogen (1.6, 1.1 and 1.9 respectively).

Published work on the effect of ultraviolet irradiation on phospholipids has been scanty. Kollath (1927) suggested on the basis of experiments on the growth of *Haemophilus influenzae* that lecithin was the material through which ultraviolet rays exert their effects on tissues. Kögel (1937) showed that irradiated lecithin partially lost its power to reduce a silver-Metol-acid reagent, and Roffo & Correa (1938) stated that irradiation of lecithin gave rise to phosphoric acid and fatty acids, glycerol and choline.

According to Spiegel-Adolf (1935) the protective power of a kephalin solution for colloidal gold was destroyed by intensive ultraviolet irradiation.

The splitting of simple phosphate esters by ultraviolet light is known (cf. Bandurski & Axelrod, 1951; Bamann, Gubitz & Trapmann, 1961) but no explanation has been given for the restriction of the effect of ultraviolet light to phospholipids containing ethanolamine and in particular the ethanolamine plasmalogen.

To follow the possible physiological significance of these findings Johnson & Mier (1962) have studied the phospholipid turnover of mouse skin after ultraviolet irradiation *in vivo*. They found that after irradiation with ultraviolet light the phospholipids of skin showed an increased incorporation of 32 P relative to control skin, whereas values for acid-soluble phosphorus and values for nucleic acids were not changed from the control values. Results obtained *in vitro* may thus reflect changes occurring *in vivo*.

SUMMARY

1. When the skin of mice of strain A was irradiated *in vitro* with ultraviolet light the phospholipid fraction was found to diminish and the phosphorus that disappeared from this fraction appeared as acid-soluble phosphorus or as 'ribonucleic acid phosphorus'.

2. The fractions of phospholipid affected were ethanolamine plasmalogen (72 % destruction) and, to less extent, phosphatidylethanolamine (21 % destruction).

3. No significant changes were seen in deoxy-ribonucleic acid or ribonucleic acid with the methods used.

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The Reaction of Protein Thiol Groups with some Disulphides

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Although the occurrence in Nature of mixed disulphides between a protein and non-protein moiety has often been proposed, only a few examples are known. The fractional protein SH content of the serum albumins is probably due partly to mixed disulphide formation with cystine (King, 1961), and there is evidence for mixed disulphide formation between glutathione residues and the protein sulphide residues of human haemoglobin (Huisman & Dozy, 1962) and of glutathione reductase (Mize, Thompson & Langdon, 1962). The protective effect of certain thiols and disulphides (e.g. cystamine) against radiation damage in animals may be due to their combination with sensitive enzyme SH groups to form mixed disulphides, thus protecting them from oxidation. Cystamine and its derivatives react readily with a number of proteins to give mixed disulphides by a thiol-disulphide exchange reaction (Eldjarn & Pihl, 1956):

If such mixed disulphides are normally present in biological systems, it is likely that they are formed analogously by reaction between protein SH groups and the physiologically occurring non-protein disulphides, cystine or oxidized glutathione.

The results of a study of the reaction of these two substances with protein SH groups are presented below.

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MATERIALS AND METHODS

Materials. Sodium *p*-chloromercuribenzoate (L. Light and Co. Ltd., Colnbrook, Bucks.) was purified and standardized by iodine titration (Boyer, 1954). The extinction at 247 mμ in 2% (w/v) metaphosphoric acid in 33 mm-phosphate buffer is linear with concentration. Bovine serum albumin (fraction V) and bovine γ-globulins (fraction II) were obtained from Armour Ltd., Eastbourne, and human serum albumin (fraction V) was from the Blood Transfusion Service, Royal Infirmary, Edinburgh. Bovine haemoglobin solutions were prepared from fresh bovine erythrocytes by haemolysis with water, prolonged dialysis against water at 4° and removal of stroma by centrifuging at 1000g for 30 min. Solutions were stored at -10°, thawed when required and standardized as oxyhaemoglobin in ammonia solution by measurement of the extinction at 576 mμ and comparison with standards (C. D. Keeler Ltd., London). Cystine was determined by the naphthoquinonesulphonate method (Sullivan & Hess, 1937).

Determination of protein thiol groups. The protein was dissolved in water and to the solution (1.0 ml. containing up to 0.1 μmole of protein) was added 0.2M-phosphate buffer, pH 7.4 (0.5 ml.), and either sodium *p*-chloromercuribenzoate (0.2 ml.; 0.16 μmole) or water (0.2 ml.). The volumes were adjusted to 2 ml., mixed and, after 10 min., 6% (w/v) metaphosphoric acid (1 ml.) was added. The precipitated proteins were sedimented by centrifuging and the extinction of the protein-buffer-*p*-chloromercuribenzoate supernatant was measured in a spectrophotometer (Unicam SP. 500) at 247 mμ. in a silica cell (1 cm. light-path) against that of the protein-buffer-water supernatant. The extinction found was subtracted from the extinction of a solution of the *p*-chloromercuribenzoate (0.2 ml.; 0.16 μmole) in 2% (w/v) metaphosphoric acid in 33 mm-phosphate buffer, pH 7.4, read against a buffer-metaphosphoric acid blank. From the decrease in ex-