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FURTHER STUDIES ON THE PEROXIDATION OF THE SURFACE LIPIDS OF THE SKIN OF RODENTS

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In a previous study (5) we reported that surface lipids of the skin of rats contain normally substantial amounts of peroxides. This amount increased with a vitamin E-deficient diet and decreased with a diet deficient in essential fatty acids. The formation of lipoperoxides in the skin-surfaces increased when the rats were exposed to sunlight.

These studies have been elaborated in investigations into the relationship between the composition of the diet and the peroxide content of the skin-surface lipids. An attempt was made to study in detail the effects of the peroxidation process on the chemical composition of the lipids. Furthermore, the peroxide content of the skin-surface lipids of other rodents were determined and compared with that of the rat. The results of these investigations are reported in this paper.

Material and Methods

Animals and diets. The first experiment was carried out as described previously (5). Eight groups of 6 male Wistar rats of an average weight of 50 g were fed a basal fat-free diet [see Table 2 (5)], supplemented as shown in Table 1 of the present paper. The amount of tocopherol acetate given to the vitamin E-supplemented groups was 10 times higher than that given to the corresponding groups in our previous experiment.

A number of rodents, *viz.* rats, mice, hamsters, rabbits and guinea-pigs, of comparable young age were used in the second experiment. They were housed in similar conditions in the same room and given a commercial diet[1] consisting of a mixture of vegetable and animal products supplemented with salts and vitamins.

Extraction of skin-surface lipids. The skin of the rats were extracted as described previously (5). The other rodents were treated with suitable amounts of acetone in the same way. The extracts were filtered and evaporated *in vacuo* at temperatures not exceeding 35°C to complete the removal of acetone. The lipids were dissolved in peroxide-free petrol ether in 25 ml measuring flasks. Aliquots were taken for determinations of peroxide and dry matter. The remaining solutions were stored at −20°C for further chemical examination.

Determination of lipoperoxides. The determinations were made by the colorimetric thiocyanate method (7) reported previously (5), with the following modification: since lipoperoxides appear to react slowly with ferrous ions when large amounts of acetone are present, the determination was carried out on the evaporated extract dissolved in petrol ether.

Determination of tocopherol. An estimate of the tocopherol content of the skin lipid extracts was made based on the reaction with the stable free radical diphenylpicrylhydrazyl (4). Two ml of the skin extracts in petrol ether was pipetted into a

[1] Pelleted rabbit diet, Karensmølle Ltd., Viby J., Denmark.

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Table 1. *Supplements given to a basal fat-free diet of rats*

Group number	1	2	3	4	5	6	7	8
Vitamin E[1]		+		+		+		+
Cholesterol[2]			+	+				
Ethyl linoleate[3]					+	+		
Ethyl linolenate[3]							+	+

[1] D,l-*α*-tocopherol acetate (Ephynal®, F. Hoffmann-La Roche & Co., Basel), 500 mg added to 5 g of the vitamin mixture.

[2] Commercial product, recrystallized from ethanol, mixed in the diet at a level of 1 %.

[3] The Hormel Institute, Austin, Minnesota, U.S.A. Each animal in the respective group was given 0.1 ml of the ester orally daily with a syringe-pipette. Care was taken to prevent contamination of the fur with the oil.

test tube. One ml of a solution of the free radical in methanol and 0.5 ml of chloroform was added. The optical density at 517 mμ was read after 10 minutes, and the decrease in optical density calculated. Tocopherol contents were calculated on the basis of the extinction coefficient for diphenylpicrylhydrazyl $\varepsilon = 11.9 \times 10^3\ M^{-1}\ cm^{-1}$.

Chromatographic methods. The methods of Nikkari (10) were adopted and similar adsorbents and solvents for chromatography were chosen.

Visualization of tocopherol was made by the method of Glavind and Hølmer (8).

Visualization of peroxides was made by an adaption of Glavind *et al.*'s method (6) for histochemical demonstration of peroxides. The following spray-reagent was prepared immediately before use: four mg hemin is dissolved in 3 ml pyridine-acetic acid (1 : 2), and 0.75 ml of the solvent added to 25 mg leuco-dichloro-phenolindophenol in 3.5 ml ethanol. The lipoperoxides appear bluish-red on the chromatographic plate.

Quantitative thin-layer chromatography was carried out in the following way: about 100 mg lipid from each extract dissolved in petrol ether was applied as an 18 cm long streak on two 20 × 20 cm plates with a 0.25 mm layer of silicagel G and developed twice in benzene-chloroform (1 : 1). After drying, two zones heavily loaded with lipid could be identified with the naked eye: one broad zone in the front (zone 1) and one narrow band which had migrated only a few centimeters (zone 3). The two zones, the area between them (zone 2) and the area from the application band to zone 3 (zone 4) were scraped off separately. The powder was eluted four times with chloroform or chloroform : methanol (2 : 1) (zones 3 and 4). The eluates were evaporated to small volumes, transferred to tared tubes, evaporated to dryness and weighed. Zone 3 (free sterols) could not be observed with the naked eye in the extracts from rats on the stock diet but was identified by the cherrish-red color produced by spraying with 50 % sulfuric acid and heating.

Results

The rats were fed the experimental diets for a period of 34 days. Ny symptom of vitamin E deficiency was observed during the period.

Slight scaliness of feet and tail was seen in all the groups. After 34 days the feet appeared normal in the linoleic ester supplemented group 5. These findings are in agreement with the observation reported previously (5) concerning transient deficiency symptoms in rats supplemented with essential fatty acids.

Redness and loss of hair around the mouth was observed in the groups supplemented with ethyl fatty esters. The symptom occurred very early and was most

Table 2. *Dietary influence on the content of lipoperoxides and tocopherol in the rat skin-surface lipids*

Group no.	Animal weight, grams[1]	Skin-surface lipids extracted after: 4 weeks		two days later		
		Mg extracted per rat	Peroxide μequiv./g	Mg extracted per rat	Peroxide μequiv./g	Tocopherol[2] mg/g
1	154 ± 10	76.5	297	59.3	121	0.00
2	158 ± 14	85.0	105	50.5	19	0.16
3	150 ± 7	66.0	411	55.8	111	0.04
4	136 ± 4	72.7	100	55.2	16	0.22
5	151 ± 3	66.1	402	47.5	303	0.01
6	158 ± 5	68.7	164	45.7	59	0.30
7	168 ± 10	68.5	353	45.0	150	0.03
8	153 ± 4	60.5	91	48.7	31	0.13

[1] Average weights and standard errors.
[2] Tentative determinations. See text for details.

pronounced in the vitamin E-deficient groups, especially in the linoleate-supplemented group. It was not seen in groups 1–4 and had not been observed in our previous experiments when the supplement of fatty acids was given in the form of triglycerides. The symptom is probably the same as was observed by Thomasson and Gottenbos (13) as the result of feeding methyl and ethyl esters of fatty acids. However, these authors found this effect after feeding non-essential fatty acid esters rather than essential fatty esters.

The skin of the rats was extracted after four weeks on the experimental diets and again after two days. The total lipids and peroxides found in the individual groups are presented in Table 2.

Results of the determination of tocopherol in the second extracts are presented in the last column of Table 2. The reagent diphenylpicrylhydrazyl reacts not only with tocopherols but also, though more slowly, with lipoperoxides. Therefore, the decrease in optical density after a short lapse of time (10 minutes) was used as a tentative method. The determination of small amounts of tocopherol in the presence of high concentrations of peroxides presents a difficult problem, and the figures can only be considered as rough estimates.

An estimate of the tocopherol content by thin layer chromatography was also made (Fig. 1). A yellow zone was observed when a chromatogram of the skin lipids of the vitamin E-supplemented group, Group 6, was sprayed with diphenylpicrylhydrazyl. No such zone was observed for the corresponding vitamin E-deficient group, Group 5, but when lipid of Group 5 was applied in combination with α-tocopherol, a yellow zone was also observed. The yellow zones were of equal position and size, though the migration was slower than that of the α-tocopherol standard. However, the chromatograms were overloaded with neutral lipids which formed a large transparent zone in front of the chromatograms and have probably distorted the R_f-values of the tocopherols. The size of the zone indicates an order of magnitude for the tocopherol content of the Group 6 skin lipids of 0.2 mg per gram lipid.

Two of the groups (3 and 4) were maintained on the diets for a total of 14 weeks after which skin-surface lipids were extracted. The animals were kept in the dark for 3 more days during which time the skin was extracted on the first and again on the third day. Analytical results from these samples are presented in Table 3.

A comparative chemical study on the first extract from Group 3 and the last from Group 4 (peroxide content 285 and

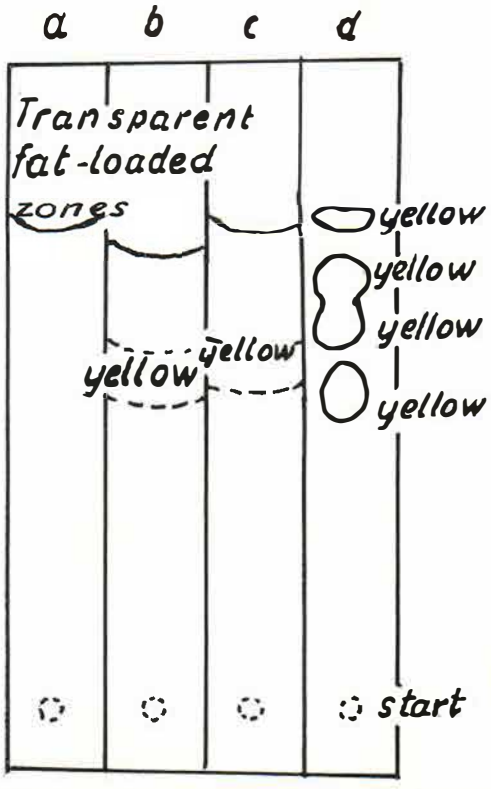


Fig. 1. Identification of tocopherol in rat skin-surface lipids by thin-layer chromatography.

a Skin lipids Group 5 (vitamin E-deficient diet).
b Skin lipids Group 6 (vitamin E-supplemented diet).
c Skin lipids Group 5 + 2.4 μg α-tocopherol.
d 2.4 μg α-tocopherol (fastest migrating spot) + 4.5 μg β- + 4 μg γ- + 3 μg δ-tocopherol (slowest migration).

Silicagel G. About 11 mg skin lipids dissolved in 100–150 μl chloroform was applied in chromatograms a–c. Development: Cyclohexane-ether (80 : 20) to 14.5 cm. Spray reagent: Diphenylpicrylhydrazyl in methanol. Tocopherols appear as yellow spots on a violet background.

Table 3. *Influence of repeated extraction on the lipoperoxide content of the skin-surface lipids of rats, maintained in the dark*

Extraction no.	Days in darkness	Skin – surface lipids from			
		Group 3		Group 4	
		Mg extracted per rat	Peroxide μequiv./g	Mg extracted per rat	Peroxide μequiv./g
1	0	171	285	150	44
2	1	71	107	75	21
3	3	122	53	135	9

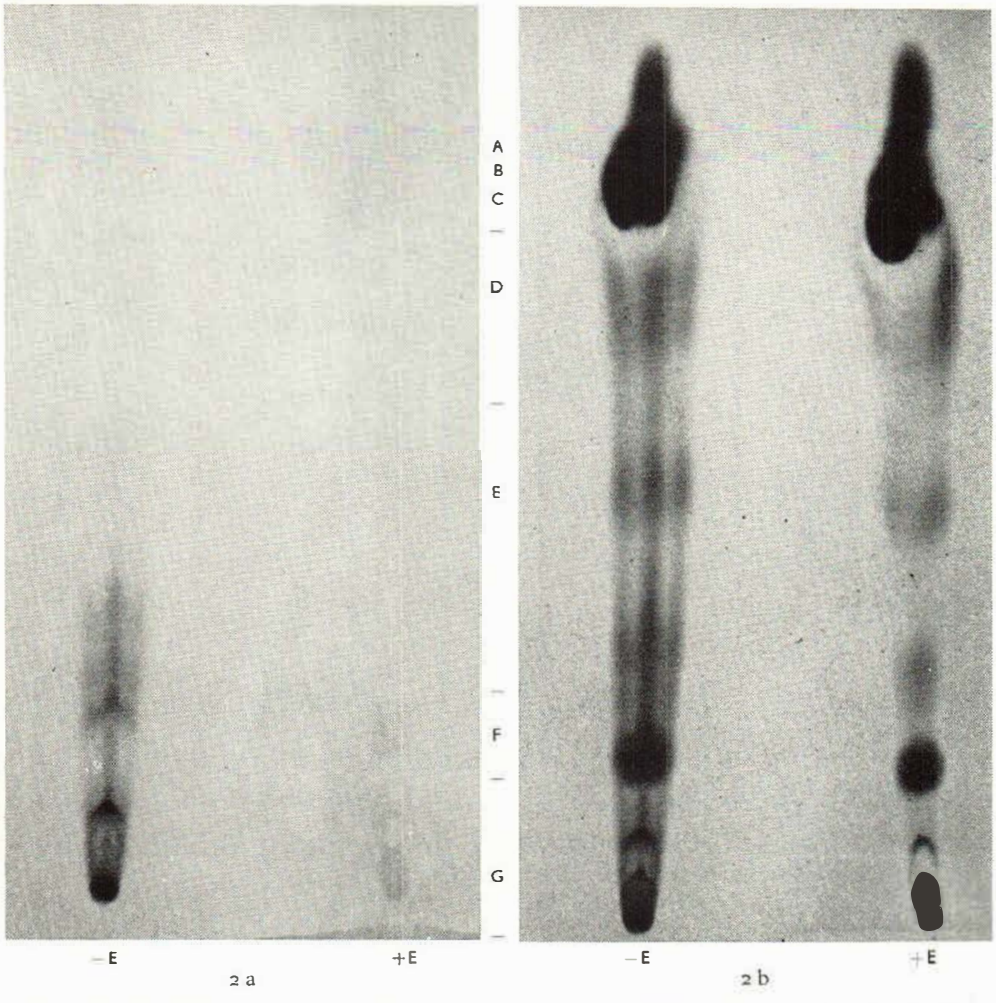


Fig. 2. Identification of lipoperoxides in thin-layer chromatograms of rat skin-surface lipids. +E: Vitamin E-supplemented diet. Rats maintained for 3 days in the dark. Surface lipids collection period 2 days following two successive skin extractions. Peroxide content 9 μequiv./g lipid. −E: Vitamin E-deficient diet. Rats housed in ordinary laboratory conditions without the exclusion of light. Last previous skin extraction 10 weeks earlier. Peroxide content 215 μequiv./g lipid. Silicagel G. Development: Twice in benzene : chloroform (1 : 1).—Fig. 2 a: Spraying: Hemin-leuco-dichlorophenolindophenol reagent for visualization of lipoperoxides—Fig. 2 b: Same plate after spraying with 50 % sulfuric acid and charring. Letters A–G: Designation of fractions used by Nikkari (10).

9 μequiv./g, respectively) was attempted.

Thin layer chromatograms are presented in Fig. 2. The same plate was sprayed, first with the leuco-dichlorophenolindophenol—hemin reagent to visualize the lipoperoxides (Fig. 2 a), and next with 50 % sulfuric acid, followed by heating until charring had taken place (Fig. 2 b).

Densitometric tracings of chromatograms of the two extracts after spraying with sulfuric acid only followed by charring are shown in Fig. 3.

The results of a comparison of the two extracts by quantitative TLC are presented in Table 4.

The results of the determination of total lipids and peroxides in the skin of some common rodents are presented in Table 5.

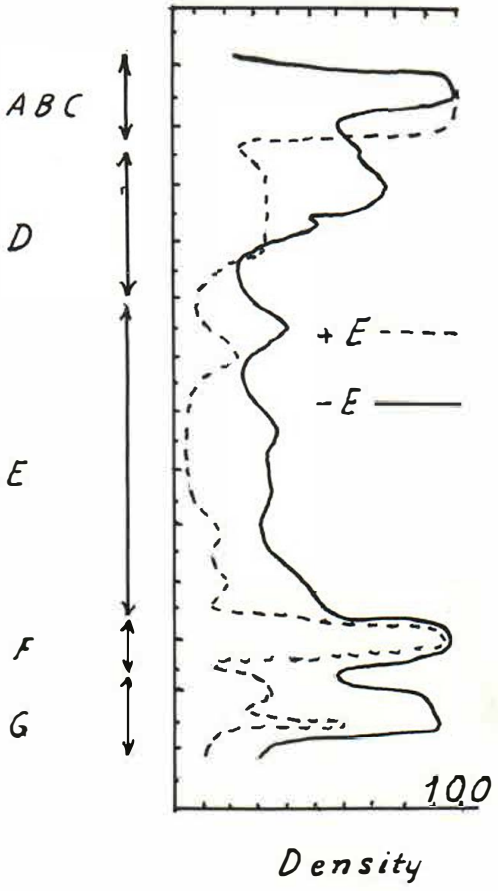


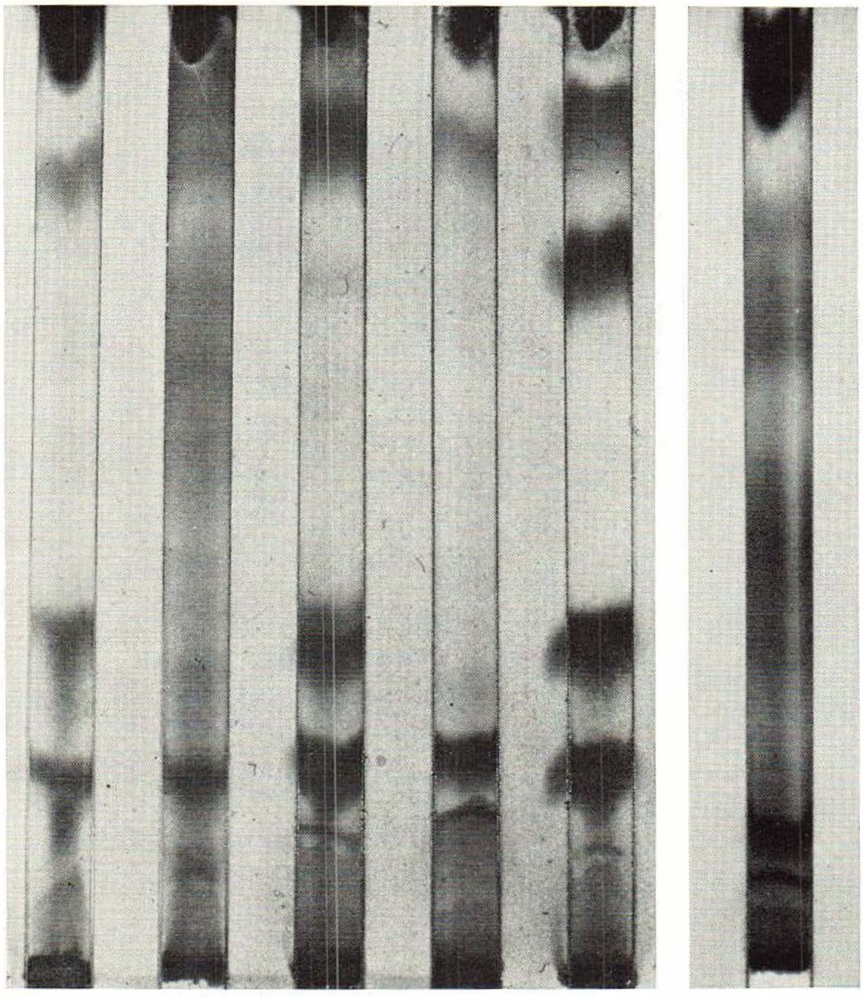
Fig. 3. Densitometric tracing of thin-layer chromatograms of rat skin-surface lipids. +E and −E: Same extracts as in Fig. 2.

Silicagel G. 0.6 mg substance administered to the plate as a streak of 1 cm. Development: Twice in ether : cyclohexane (20 : 80) to 15 cm height. Spraying with 50 % sulfuric acid and charred. Ordinates: Migration of spot. Abscissae: Densitometric reading (Densicord Recording Electrophoresis Densitometer, Photovolt Corp., New York). Letters A–G: Designation of fractions used by Nikkari (10).

Table 4. *Quantitative thin-layer chromatography of rat skin-surface lipids*

Zone	Designation of Nikkari[1]	Percentage composition according to Nikkari[1]	Percentage composition of skin-surface lipids from rats on diet: fat-free + vitamin E[2]	fat-free − vitamin E[2]	stock, first extraction[3]	stock, second extraction[3]
1	ABC	64.6	85.2	73.0	52.5	68.4
2	DE	13.5	7.9	13.0	19.5	16.0
3	F	9.1	3.6	6.9	8.8	5.8
4	G	12.9	3.2	7.1	19.2	9.8
Peroxide content, μequiv./g			9	285	800	300

[1] Nikkari (10), p. 77.
[2] Same extracts as in figs. 2–3.
[3] Extracts from rats on stock diet. Second extraction carried out after an interval of four days.



4 a 4 b

Fig. 4 a: Thin-layer chromatography of skin-surface lipids of some common rodents. From left to right: Mice, rats, hamsters, guinea pigs, rabbits. — Fig. 4 b: Thin-layer chromatography of lipid extract from rat ceruminous glands. Silicagel G. Application: About 0.5 mg lipid in a 1 cm streak. Development: Twice in ether : cyclohexane (20 : 80). Visualization: Spraying with 50 % sulfuric acid and charring.

Table 5. *Lipoperoxide content of skin-surface lipids of some rodents*

Species	Number of animals	Average weight, grams	First extraction		Extraction 2 days later	
			Mg per animal	Peroxide μequiv./g	Mg per animal	Peroxide μequiv./g
Mouse	4	24	23	41	14	26
Rat	6	195	113	400	54	140
Hamster	5	87	20	62	9	41
Guinea pig	2	277	57	40	33	42
Rabbit	2	1820	173	11	109	19

Extraction of skin lipids was carried out twice with an interval of two days between the extractions.

Thin-layer chromatograms of the first extracts are shown in Fig. 4 a.

The rats were killed after the second extraction, and the ceruminous glands extirpated. Adherring non-glandular tissue was removed and the glands, 76 mg from 6 animals, were extracted with chloroform : methanol (2 : 1) in a tissue homogenizer; 17 mg lipid was obtained after evaporation. A thin-layer chromatogram of this lipid together with chromatograms of skin-surface lipids of the same animals are shown in Fig. 4 a.

Discussion

The results presented in Table 2 agree with those of our previous studies (5). The first extracts of the vitamin E-deficient groups had peroxide contents of 300–400 μequiv./g both in the essential fatty acid-supplemented and in the non-supplemented diet groups. The extracts collected two days later showed a peroxide content of about 300 in the linoleic acid-supplemented Group 5, and lower values in the other groups. Nikkare [see p. 93 (10)] found an octadecadienoic acid content of 3.1 % of the unsubstituted fatty acids of sebum from rats fed a diet containing 17 % linoleic acid, and only 0.1 % when a fat-free diet was fed. More highly unsaturated fatty acids were not found among the main constituents of sebum. Linoleic acid esters probably autoxidize most easily, but the amount is not sufficient to account for the high lipoperoxide concentrations observed. Once the peroxide-forming reaction is started other substances will also form peroxides under the conditions at the rat skin-surface. Free sterols form very easily peroxides, ester bound sterols and monoenoic esters less easily. The greater part of the waxes of the skin-surface lipids must be considered quite resistant to autoxidation.

Consistently smaller amounts of peroxide were found in the groups supplemented with viamin E than in the corresponding non-supplemented groups. Nevertheless, peroxide values of about 100 μequiv./g were observed. Even a dietary supplement as high as 0.5 mg tocopherol acetate per g diet did not completely protect the lipids on the rat skin-surface against autoxidation.

The lower peroxide contents in the vitamin E-supplemented group indicate that this vitamin is secreted in the sebum. As mentioned above, the results (Table 2, last column) can only be considered as rough estimates. They do indicate, however, that the skin surface lipids of the groups supplemented with 0.5 mg α-tocopherol acetate per g diet contain about 0.2 mg α-tocopherol per g lipid. Comparative thin-layer chromatograms of the lipids from the vitamin E-supplemented and the corresponding non-supplemented groups indicate a tocopherol content of the same order of magnitude (Fig. 1).

In our previous studies only one tenth the amount of tocopherol acetate (0.05 mg per g diet) was given. The peroxide content was higher than in the present study, but definitely lower than in the vitamin E-deficient groups. When rats are fed a supposedly sufficient stock diet a very high peroxide content is found (Table 5). The conclusion appears justified that vitamin E is secreted in rat sebum, but that it is normally destroyed at a fast rate by the powerful autoxidation processes occurring on the skin-surface. Only in special circumstances (high intake of vitamin E, repeated removal of skin lipids) do the lower peroxide contents observed indicate the persistence of substantial amounts of vitamin E.

To our knowledge the presence of vitamin E in the rat skin surface lipids has not been demonstrated previously. Attempts to show its occurrence in human sebum have been made by Mackenna *et al.* (9) and by Festenstein and Morton (3). They concluded that the amounts were small and could not exceed 0.2–0.3 mg per g lipid.

It is seen in Table 2 that the amount of lipid secreted is consistently lower in the groups supplemented with essential fatty acids than in the other groups. Similar findings were reported in our previous paper (5) and by Nikkari (10). The results indicate that the increased production of sebum

probably forms part of the essential fatty acids deficiency syndrome in rats. It is rather an early symptom which in this study was observed after four weeks.

A profuse secretion of cerumen which covers the tympanic membrane of hamsters fed a fat-deficient diet has been observed in this laboratory (1). This was also observed in rats (2) and can be prevented by feeding of linoleic acid. It seems possible that the profuse secretion of cerumen and the increased secretion of lipids from the sebaceous glands are caused by related mechanisms. From a functional view-point the ceruminous glands can be considered as special sebaceous glands. The size of the glands permits the easy obtaining of lipid extracts for examination. A thin-layer chromatogram of such an extract, which has not undergone autoxidation, is shown in Fig. 4 b. The major bands resemble those of a chromatogram of rat skin-surface lipids (Fig. 4 a), but the broad grey zones representing autoxidation products are absent. The glandular extracts are supposedly representing predominantly non-secreted cerumen. The similarities between these chromatograms suggest a biochemical relationship between sebaceous and ceruminous glands in the rat.

Two extracts were chosen for a more detailed study of the influence of the autoxidation process on the composition of the lipids. One of the extracts was made from the animals of Group 3 (vitamin E-deficient) after a total of 14 weeks on the diet. It had a content of 285 μequiv. of peroxide per gram lipid. The other extract was obtained from the corresponding vitamin E-supplemented group, 3 days longer on the diet. During this time the animals were kept in the dark and the skin was extracted twice to remove as efficiently as possible the peroxide-containing lipids formed during the preceeding period. By these precautions it was possible to obtain an extract containing only 9 μequiv. of peroxide per gram lipid.

The two extracts are considered comparable. Both represent sebum synthesized in the rat on essentially the same diet deficient in essential fatty acids. However, the lipids of the first extract had been allowed to autoxidize freely, while autoxidation was suppressed as much as possible in the second extract by feeding of antioxidant, exclusion of light and repeated skin extractions.

The most extensive studies on the composition of skin sebum of the rat have been made by Nikkari (10) who examined sebum collected for four days following a previous extraction. He removed the free fatty acids which accounted for only 1 % of the total lipids, and examined the remainder by quantitative thin-layer chromatography. He designated and identified the fastest moving fraction *A*, as paraffins and squalene (10, p. 65). The next two fractions, *B* and C, the bulk of the material, represented various aliphatic and sterol monoesters. Fraction C was studied in more detail by Nikkari and Haahti (11) and consisted mainly of diester waxes. Fraction *F* consisted of free sterols. The nature of fraction *D* and *E* running between the esters and the free sterols, and *G*, the most polar lipids, has not been definitely established. The unidentified fractions account for about one fourth of the lipids.

The chromatographic location of the lipoperoxides (Fig. 2 a) can be compared with the chromatogram after charring (Fig. 2 b). Nikkari's fraction F is easily identified by the color reaction of the free sterol with sulfuric acid. The large part of the lipoperoxides are located in two zones of fraction G; one zone on the spot of application, and the other half way up zone F. The latter corresponds to the migration of hydroperoxides while the non-migrating spot may contain more than one polar group. Peroxides are also present in fraction E; they might be peroxides with both oxygen atoms bound to carbon.

When the chromatograms are compared, however, it is seen that the charred chromatogram of the autoxidized extract contains, at least in fraction D, substances other than the lipoperoxides in larger amounts than in the extract in which autoxidation had been suppressed. The difference between the two extracts is still better seen after scanning (Fig. 3). The

difference is probably due to secondary products formed during the autoxidation but they do not contain peroxide-groups. On the other hand, even the extract with the very low peroxide content of only 9 μequiv./g gave three definite bands in the zones D–E and also contained materials in zone G.

It can be seen from the results of the quantitative thin-layer chromatography (Table 4) that the extract of the group "fat-free + vitamin E" in which very little autoxidation had taken place, about 3 % of polar lipids (Nikkari's fraction G) and 8 % of the fractions D + E was found. These figures probably represent the amounts of unidentified compounds of the surface lipids secreted by the skin. In the group "fat-free – vitamin E", the values increased to 7 % of fraction G and to 13 % of fractions D + E. The increases probably represent autoxidation products; very likely the increase of fraction F is also due to products of autoxidation migrating with the sterols. The total amount of autoxidation products account for about 10 % of the extracts from the "fat-free minus vitamin E" group. They may be still higher in the extracts of the rats fed the stock diet. The impact of autoxidation on the composition of the lipids is illustrated by the fact that while zone 3 (free sterols) from rats on a fat-free diet could be observed as a thin fatty streak in the chromatograms, no definite zone could be observed with the naked eye on those from rats on stock diet.

It is seen in Table 5 that the high content of peroxides in the skin-surface lipids is a characteristic of the rat. Much less peroxide was found in the lipid extracts of other rodents. A simple explanation of this difference may be the different compositions of the sebum of these animals. Wheatley and James (14) found 3.1 % of diunsaturated and 1.7 % of highly unsaturated C_{18} acids in the saponifiable fraction of rat sebum, but no polyunsaturated acids at all in guinea pig, rabbit or mouse. Next to the polyunsaturated fatty acids free sterols probably form peroxides most rapidly. The very low peroxide content of rabbit sebum can be explained by the finding of Wheatley and James that rabbit sebum had the lowest content of sterols, especially "fast acting" sterols.

A content of lipoperoxides of 10–60 μequiv./g corresponds to less than 1 % of the total surface skin lipids in rodents other than rats. In rats this concentration will be about 5–10 %. Concerning possible biological functions of skin-surface lipids on which certain speculations were brought out in our previous paper (5), species difference must now be taken in consideration.

Carcinogenic hydrocarbons and azo dyes are rapidly destroyed by autoxidizing linoleic acid (12). The powerful autoxidative processes in the skin-surface of the rat may be active when carcinogens are tested. In this way the composition of the skin-surface lipids could contribute to explain the different susceptibilities of rodents species to these carcinogenic compounds when applied by skin painting.

The difference between the rat and other rodents is clearly seen in the thin layer chromatogram in Fig. 4. Nikkari's zones D–E from the rat appear as a continuous grey zone, but for the other animals as a few definite bands. Thus a quantitative elucidation of the chemical composition of sebum would probably be an easier task to carry out in rodents other than the rats.

If studies on the composition of rat sebum—such as it is secreted by the glands—are attempted, our data suggest that care should be taken to suppress autoxidation. Feeding a high content of tocopherol, maintaining the animal in a dark environment and, eventually, skin extractions with acetone repeated even more frequently than in our present studies should enable one to accomplish this aim.

SUMMARY

The influence of essential fatty acids and vitamin E supplements to a fat-free synthetic diet was investigated in relation to the peroxide content of the rat-skin surface lipids. Ethyl linoleate produced the highest peroxide content. A high dietary supplement of vitamin E resulted in only a partial

decrease of the peroxide content. It is concluded that vitamin E is secreted in the skin but is normally destroyed during the autoxidation process. Tentative estimations of tocopherol in the skin-surface lipids of the vitamin E-supplemented rats were made. The composition of slightly and moderately autoxidized rat skin-surface lipids was compared by chromatographic methods; lipoperoxides and secondary autoxidation products accounted for about 10 % of the lipids in these samples. Only small amounts of peroxides were found in the skin-surface lipids of mice, hamsters, guinea-pigs and rabbits. The exceedingly high lipoperoxide content of the rat is explained by the fact that the rat is the only common rodent which excretes large amounts of linoleic acid in the skin-surface.

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