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PRELIMINARY THIN-LAYER CHROMATOGRAPHIC CHARACTERIZATION OF THE RAT HARDERIAN GLAND LIPID*

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SUMMARY

The lipid of the Harderian gland, which incorporates a considerable proportion of 1-^{14}C-labelled acetate which had been injected into rats (and mice) could not be resolved from the main lipid fraction of the gland by TLC, either before or after saponification.

The main lipid and acetate-incorporating fraction agrees in its chromatographic behaviour with the sterol ester band. About 83 % of the radioactivity of the lipid extract from the Harderian gland (24 h after the application of 1-^{14}C-acetate) was found in this band.

Small amounts of free cholesterol were found in the Harderian gland extract. The cholesterol band was not appreciably labelled.

The main lipid of the unsaponifiable matter of the Harderian gland (about 99 % of the radioactivity of this fraction 24 h after ^{14}C-acetate injection) travels in hexane–ethyl acetate (80:20) and chloroform–diethyl ether–acetic acid at a rate slightly below that of dihydrolanosterol. On a $AgNO_3$-impregnated layer it is retarded, with respect to dihydrolanosterol, and more so than lanosterol.

The unsaponifiable material gives a dull yellow colour and whitish yellow fluorescence with the $SbCl_3$–acetic anhydride reagent.

Acetate labelled with carbon-14, both in positions 1 and 2, was administered to rats and mice and macroautoradiographs were obtained[1,2] by methods previously published[3] (Fig. 1). Even in the first moments, high radioactivity was noted in the orbit of the animals; in less than one hour after the administration, this site contained the highest radioactivity per unit weight and 24 h after the administration it was practically the only one still containing appreciable radioactivity.

Slices of the areas concerned showed the bright red fluorescence characteristic of the protoporphyrin-rich Harderian gland (glandula palpebrae tertiae profunda),

* Dedicated to Professor E. LEDERER on the occasion of his 60th birthday.

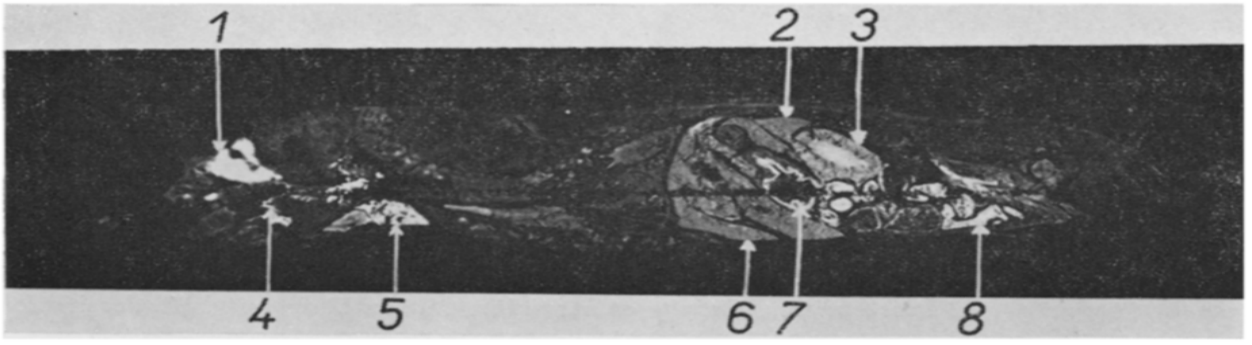


Fig. 1. Autoradiogram of a section through a rat 5 h after the administration of 100 μC of sodium 2-^{14}C acetate to a 100 g animal. 1 = Harderian gland; 2 = spleen; 3 = kidney; 4 = oral mucosa; 5 = submandibular gland; 6 = liver; 7 = gastric mucosa; 8 = intestine.

which is a lipid-producing retrobulbar gland present in animals possessing a third eyelid.

The function of this gland and the reason for its high content of porphyrin in some rodents[4], has not yet been explained, though chromodacryorrhoea, *i.e.* secretion of coloured "tears" by this gland, has often been studied by pharmacologists as a quantitative test for cholinergic stimulation[5]. According to FIGGE AND DAVIDHEISER[6] Harderian glands resemble in some respects the sebaceous glands of human subjects, though according to the electron optical investigations of WOODHOUSE AND RHODIN[7], Harderian gland exhibits a merocrine type of secretion. A lubricating function is often ascribed to the secretion of Harderian gland[8]. Incidentally, protoporphyrin is also present in the sebum of human adults[9].

In a preliminary communication[2] we described the time-course of radioactivity in the submandibular gland (as representative of salivary glands), Harderian glands and spleen (Fig. 2). Hydrochloric acid–nitric acid digests were separated into an aqueous and toluene-soluble (lipid) fractions. While the aqueous fraction was more radioactive in the case of the submandibular gland, the opposite was the case for the Harderian gland.

When lipid extracts from the Harderian gland were subjected to TLC, the bulk of the phosphomolybdic acid stainable lipids as well as the bulk of the radioactivity travelled at a rate typical of sterol esters, with an R_F below that of squalene and above that of triglycerides[10,11].

Comparison with lipids from other organs showed that the predominance of this fraction is a rather characteristic feature of the Harderian gland.

MATERIAL AND METHODS

Reference compounds

Cholest-7-en-3β-ol (lathosterol) was obtained as a gift from Messrs. Ikapharm, Ramat-Gan, Israel, lanosterol and dihydrolanosterol from Dr. J. W. COPIUS-PEEREBOOM (Rijkszuivelstation, Leiden), cholesterol esters from Dr. Č. MICHALEC (Faculty of General Medicine, Prague), 7-dehydrocholesterol from Dr. V. JANATA (Research Institute of Pharmacy and Biochemistry, Prague) and cholestan-3β-ol from Dr. V. CERNÝ (Czechoslovak Academy of Sciences, Prague). The remaining steroid

samples were from the collection of Dr. O. SIBLÍKOVÁ (Research Institute of Pharmacy and Biochemistry, Prague).

Biological material

Organs from white rats (local breed of Wistar type) were obtained and submerged in ethanol within 20 min after death. In the early stages of the work, the red fluorescence was used as the guide in dissecting the contents of the orbit. The organs were ground with 0.5 g sand and approximately 1 ml ethanol per 0.3 g of tissue. On average, one pair of Harderian glands from a 200 g rat weighed about 0.3 g.

Administration of radio-acetate

Sodium 1-^{14}C-acetate (24 mC/mmole, Isocommerz, GDR), was dissolved in 0.5 ml saline and injected in the saphena vein. Unless otherwise indicated, 1 μC (0.42 μmole) per 1 g body weight was administered.

Solvent partition and saponification procedure

Solvent partition. To one volume of the ethanolic suspension were added 3 volumes of water and 3 volumes of light petroleum (b.p. 60–80°). This mixture was then shaken and the layers allowed to separate. The upper layer was used for further study.

Saponification and fractionation of the alkaline digest. The lipid extract (either the light petroleum layer from the basic partition or an eluate from chromatograms) was evaporated to dryness under argon in subdued light and the residue dissolved in a solution of 2 *M* KOH in 95 % ethanol (about 1 ml ethanolic KOH corresponded to about 0.1 g of the tissue from which the extract had been prepared). The solution was refluxed for 2 h under a stream of argon and in darkness*.

After saponification 2.5 volumes of light petroleum (b.p. 60–80°) and 1.5 volumes of water were added to 1 volume of the alkaline digest and the whole gently shaken. The upper layer was then removed, and the lower layer was repeatedly shaken with further volumes of petroleum. The pooled petroleum extract was then washed twice with half its volume of water. It was used for application on the chromatogram as the "unsaponifiable" fraction.

The aqueous layer was acidified and repeated extractions were carried out, first with light petroleum, then with ether.

Acid digestion of tissues

Tissue samples were digested for 24 h at 50° with a mixture of nitric and hydrochloric acid (1:3)[13]. One millilitre of the acid mixture was taken for 0.2 g of the tissue. The digest was shaken with an equal volume of toluene and 0.1 ml samples from both layers were added to the scintillation fluid and counted (Fig. 2).

Thin-layer chromatography

Layers

Kieselgel HF_{254} or GF_{254} was used. The layer was spread as a 25 % (w/v) aqueous suspension on a glass support (20 cm × 20 cm) with the help of a glass rod

* Failure to observe these precautions (*cf.* ref. 12) or the use of peroxide-containing ether resulted in the formation of a number of both more and less polar compounds, often to such a degree that the main unsaponifiable material completely disappeared.

(provided with adhesive tape collars 0.7 mm high) and activated by heating to 105° for 1 h. Average thickness of the dry layer was found to correspond to 33.3 mg per cm^2.

Chromatographic development

Most of the chromatograms were run in an S-type chamber (with the support and lid 2.1 mm apart). A tank of 6 l volume per 400 cm^2 of thin layer was also used in some cases. No attempt was made to ensure reproducibility of the separation by adjustment of temperature, atmospheric humidity etc. Room temperature varied from day to day between 18 and 21°.

Application of samples

The samples were applied with pipettes. Transverse origin lines were formed as a series of contiguous spots. Light petroleum and ethanol were used as solvents for the application of samples.

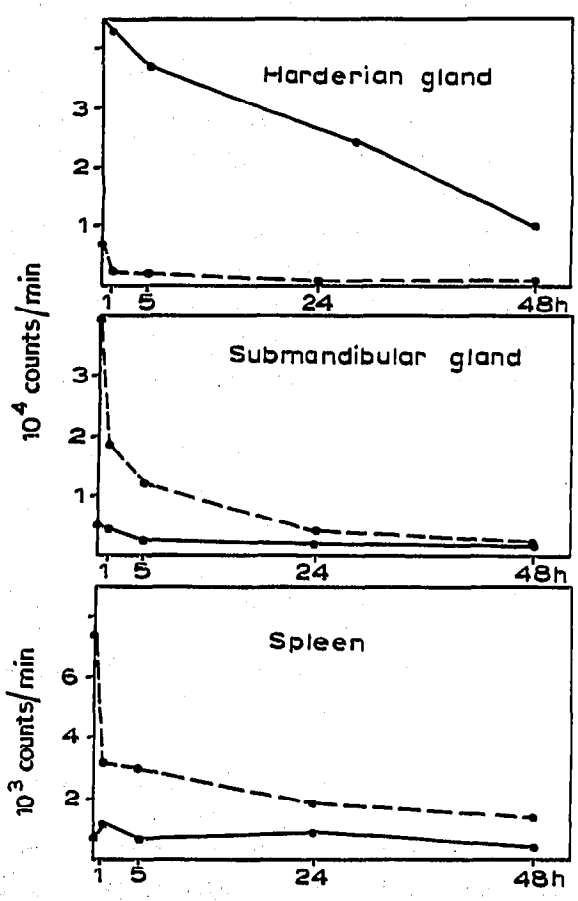


Fig. 2. Acid digest (see Methods section) of the respective tissue was divided into the aqueous and the toluene layer and aliquots of both layers representing 20 mg of the original tissue were counted. Each point represents the average from only two animals. Solid lines refer to the lipid (toluene) layers, dashed lines to the aqueous layers. Note different scale of the ordinate for the spleen.

Solvent systems

(All proportions of solvents are given in volumes at room temperature).

S 1. Light petroleum (b.p. 60–80°)–diethyl ether–acetic acid (90:10:1)[10].

S 2. Hexane–ethyl acetate (80:20)[14].

S 3. Chloroform–diethyl ether–acetic acid (97:2.3:0.5)[15].

S 4. As S 3, but silver nitrate (in the proportion of 1.6 g per 4 g silica gel) was added to the suspension from which the layer was prepared[15].

S 5. The layer was first impregnated by chromatographic ascent with a 5 % (v/v) solution of liquid paraffin (Czechoslovak Pharmacopoeia *2* grade) in light petroleum (b.p. 60–80°), dried first at room temperature and then at 100° for 20 min. Propan-1-ol–methanol-water (15:82:3) was used as the mobile phase[16]. Wedge-shaped lanes were mostly used[14].

Detection reactions

D 1. After spraying with distilled water, major lipid-containing zones are less transparent than the background.

D 2. Viewing in short-wave ultraviolet light (max. 254 nm) from a low-pressure mercury discharge lamp provided with a filter UG5 (ref. 17). Fluorescence quenching of Kieselgel HF_{254} is observed.

D 3. Fluorescence in near ultraviolet light (max. 367 nm) from an analytical mercury discharge lamp with Wood's glass envelope.

D 4. A 10 % (w/v) solution of phosphomolybdic acid (Spolana) in methanol to which 4 ml conc. HCl per 100 ml water had been added. After spraying, the plate was heated to 100° until blue spots appeared on yellow background[18].

D 5. Antimony trichloride was left in contact with chloroform overnight and 25 ml of the saturated solution was mixed with 2.5 ml acetic anhydride immediately before spraying the layer[18]. The layer was then placed in an oven (105°) and heated until coloured spots of steroids reached full intensity (about 20 min). The colour in both visible light and fluorescence in ultraviolet light (367 nm) were noted.

D 6. The Liebermann reaction[19]. The chromatogram is sprayed with a sulfuric acid–acetic anhydride (1:3) mixture.

D 7. Saturated solution of $K_2Cr_2O_7$ in concentrated sulfuric acid[18].

Records of the plates

A sketch was made or the layers were photographed. Visible colours (D 4 and D 5) were photographed in daylight using ordinary panchromatic film without a filter. Fluorescence excited by the 367 nm light was photographed with panchromatic film (Ilford ASA 400/DIN 27) and a "gelbmittel" filter Panchromar (Lehmann und Balzer). Exposure was about 3 sec, aperture 2 when the source (125 W) was 65 cm from the plate. Colour transparencies were also taken using an inversion film (15 DIN).

Autoradiography of TLC chromatograms

The layer was placed in contact with an ORWO macro-autoradiography film AF 4 for 4–6 days unless otherwise indicated.

Removal of plate material for scintillation counting

After a graphic or photographic record of the visualized chromatogram or autoradiogram had been made, rectangular sections of the layer were scraped off with

a spatula and added to a scintillation liquid SLT-31 (Spolana) consisting of 2-phenyl-5-(4′-biphenylyl)-oxadiazole-1,3,4 (PBD) and 1,4-di-[2-(5-phenyloxazolyl)]-benzene (POPOP) in 7.5 ml toluene and 2.5 ml ethanol. Liquid scintillation counting was performed using the automatic equipment (Mark I) of Nuclear Chicago. It has been shown by internal standardization that the correction on the basis of the Ba/tritium channel ratio of the external standard counts is satisfactory to account for quenching caused by silica gel, the detection reagent and lipid; the plot of efficiency *vs.* channel ratio did not differ markedly from that constructed by the use of other (dissolved) quenchers.

Removal of plate material for further chromatography

Only part of the chromatogram was sprayed with a reagent and the orientation on the remaining part was guided by fluorescence, quenching, colour, and changed transparency of some of the zones. Alternatively, detection was carried out with water (D 1) which was then evaporated. The bands were then scraped off and eluted either with ethanol followed by light petroleum or with acetone–ethanol (1:1).

RESULTS

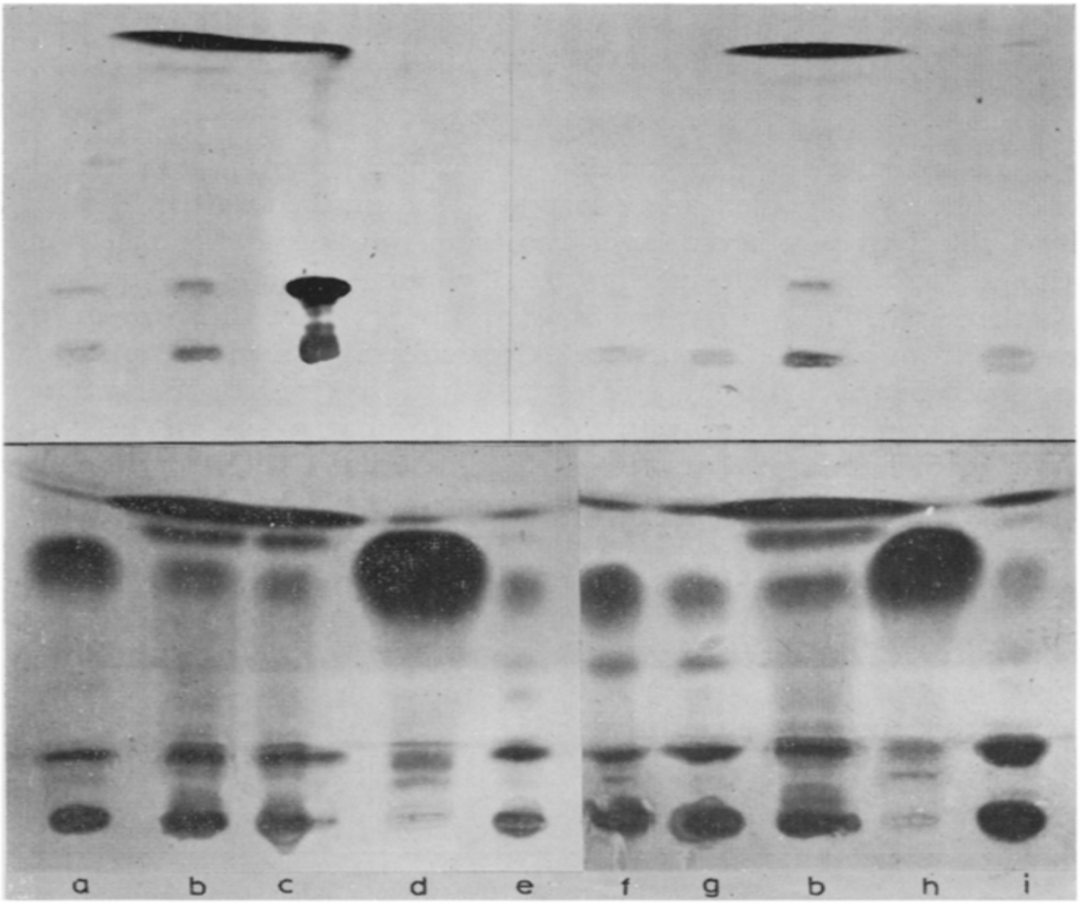
Comparison of the Harderian gland lipids with those of other tissues or secretions

The light petroleum layers of the ethanolic extracts (see Methods section) were separated by TLC. Fig. 3 shows such a chromatogram. Detection was carried out by autoradiography (upper half) and by phosphomolybdic acid D 4 (lower half of the figure). Strongest band of the Harderian gland lipid corresponds to the sterol ester fraction (verified by reference samples of cholesterol esters). This band was also the most strongly labelled by ^{14}C-acetate *in vivo*. Additional bands stained with phosphomolybdic acid appeared at the origin and close to it (probably mainly phospholipids); at the position of free cholesterol and/or diglycerides (cholesterol detected with the $SbCl_3$ reagent D 5); at the position of triglycerides and at a position which would correspond to methyl oleate according to MALINS AND MANGOLD[10] in S 1. The origin spot and that of cholesterol are also seen in the autoradiogram. Fig. 4 gives quantitative information on the distribution of radioactivity in the Harderian gland extract.

The extracts of the submandibular gland, depot fat, spleen, brain, and liver exhibited too low a radioactivity to show up clearly in the autoradiogram. Stained chromatograms showed minor bands at the sterol ester position in the case of the brain and liver; well marked origin and cholesterol bands in the case of the submandibular gland, liver, kidney and brain; cholesterol band in the case of the spleen and interscapular fat. Neutral lipids were fairly strong in the liver and submandibular gland, highly predominant in the case of fatty tissue and just above detection limit in the brain and spleen. The lipid of the Harderian gland is thus rather unique, not only in view of its high incorporation of exogenous acetate, but also with respect to the pattern of its composition.

In other chromatograms Harderian gland lipid was compared with the extracts of various secretions; in some cases the unsaponifiable fraction was also prepared and investigated.

In several solvent systems the extract of cut *rat hair* (which contains sebum constituents) mainly showed a spot in the position of free cholesterol (or lathosterol)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In *butter* from cow's milk the triglyceride fraction was predominant. Weak spots were observed in the position of free cholesterol and sterol esters.

Radioactivity distribution before and after saponification

Twenty-four hours after the administration of ^{14}C-acetate to seven animals, Harderian glands were obtained, pooled and processed as described in the Methods section. Aliquots from the light petroleum layer and from the "unsaponifiable" and "fatty acids" fraction as well as the original light petroleum extract were analyzed by TLC. Fig. 4 shows the result. The ordinate of the graph refers to 10^3 disintegrations per minute in a sample from about 0.1 g tissue. After subtraction of the silica gel background, radioactivity in the main ("sterol ester") band of the original extract amounted to 83 % of the overall activity applied on the chromatogram. In the unsaponifiable fraction the main band accounts for 99 % of the radioactivity applied on the chromatogram. The ratio of the radioactivity summed over the chromatogram of the "unsaponifiable" fraction and of that summed over the chromatogram of the "fatty acid" fraction was 61 : 39.

Chromatographic comparison of the main Harderian gland lipid fraction with reference compounds

Table I gives the positions and detection reactions of some reference compounds

TABLE I

SOME TLC $R_{cholesterol}$ VALUES AND DETECTION REACTIONS

	S2 HF_{254}	*S3* GF_{254}	*S4* GF_{254}	*D5 Daylight colour*	*D5 Fluorescence (367 nm)*	*D7 Immediate daylight colour (cold)*
Dihydrolanosterol	1.42	1.25	1.23	greyish blue (yellow border)	whitish pink (yellow border)	greenish white
Lanosterol	1.40	1.25	1.20	violet or grey	orange (centre red, yellow border)	greenish white
Harderian gland lipid (after saponification)	1.30	1.20	1.05	dull yellow	whitish (grey, yellow or pink hue)	greenish white
3β-6β-Diacetoxy-cholestan-5α-ol	1.12	0.90	1.05	brown	orange-yellow	greenish white
Stigmasterol	1.01	0.98	1.03	violet or grey	orange	greenish white
Cholesterol	(R_F 0.38)	(R_F 0.45)	(R_F 0.5)	violet (bluish)	orange (centre red)	violet
Cholestan-3β-ol	0.96	0.95		purplish brown	pink	greenish (then brown)
Lathosterol	0.93	0.96	1.03	greyish brown	whitish (centre brown)	greenish white
7-Dehydrocholesterol*	0.93	0.97	0.50	red, then blue	orange (centre brown)	blue
Ergosterol*	0.85	0.98	0.45	red, then blue	orange (dark)	blue-grey

* Data refer to the main spot of the inhomogeneous sample.

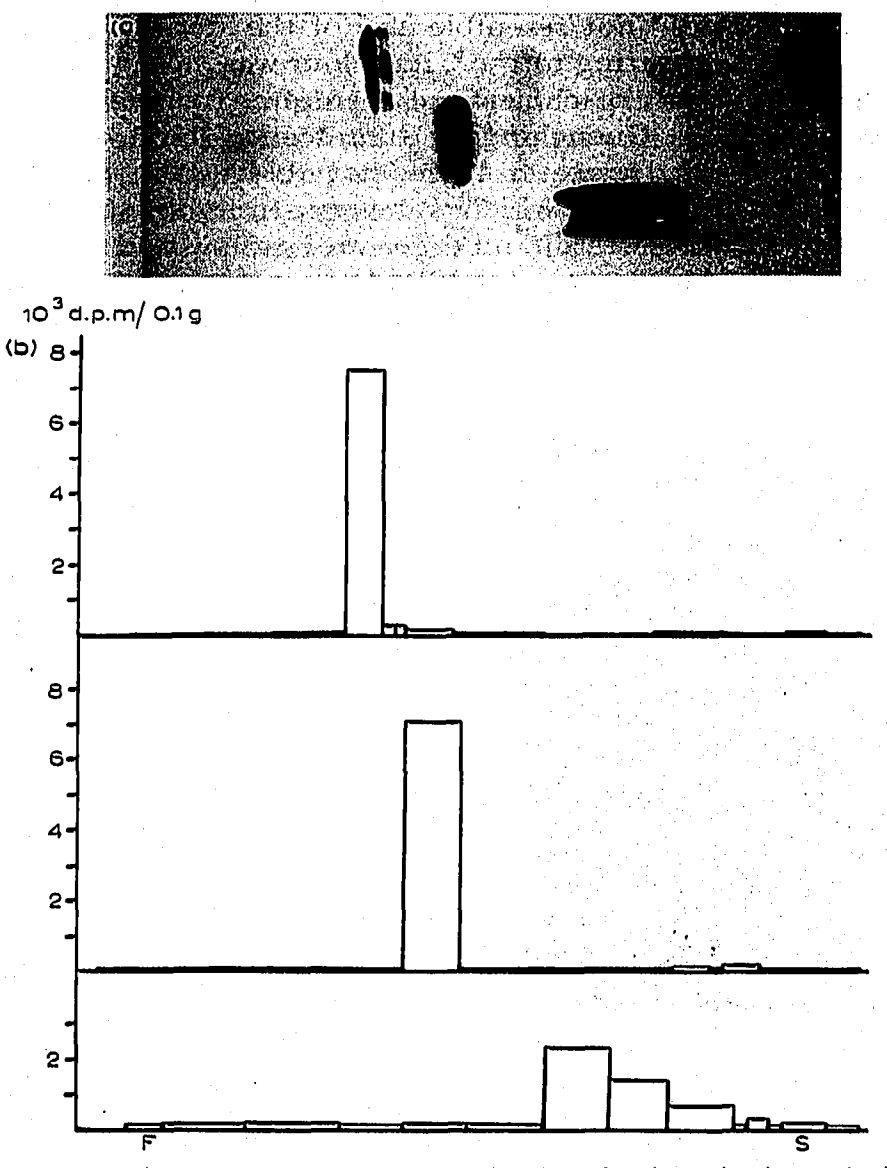

Fig. 4. Harderian glands were obtained 24 h after the i.v. administration of 100 μC 1-^{14}C-acetate to 200 g rats. 1 = An aliquot (corresponding to 100 mg of the gland) of the light petroleum layer obtained from the ethanolic extract. 2 = An aliquot from the unsaponifiable fraction prepared from the lipidic extract. 3 = An aliquot from the diethyl ether extract of the acidified aqueous layer resulting after alkaline digestion and light petroleum extraction. (a) Detection with phosphomolybdic acid (D 4). (b) Radioactivity (in disintegrations per minute per 0.1 g of tissue) of the silica gel in scraped off rectangles. The heights of the blocks correspond to the overall activity of the respective section, without reference to its width. Correction for quenching was made using the channel ratio method with a Ba external standard. The quenching curve was established by the addition of known radioactivities to some of the samples.

together with those of the main Harderian gland lipid fraction, both before and after chromatography. The $R_{\text{cholesterol}}$ values should not be taken as characteristic constants of the substances. In most cases, they were calculated from two duplicate estimations which sometimes differed widely. The sequence of the spots, however, remained constant for a given solvent system. It may be significant to note, on silver-nitrate impregnated layers (S 4), the decrease of the R_F of the Harderian gland lipid relative to the positions of lanosterol and cholesterol.

Its yellow (ochre) colour with D 5 did not resemble any of the sterols tested. After spraying with 50 % sulfuric acid and heating to 150°, its colour was more yellowish and fluorescence more reddish than that of cholesterol (before charring set in). Liebermann's reaction (D 6) was negative. Quenching of fluorescence (D 2), which was seen in case of 7-dehydrocholesterol and ergosterol, was not observed in case of the Harderian gland lipid. Fluorescence (367 nm), which was very strong for some of the components of the impure 7-dehydrocholesterol and ergosterol sample and of the wool fat, was not observed in case of the Harderian gland lipid.

Two of the chromatograms from which the data of Table I were compiled are shown in Figs. 5 and 6.

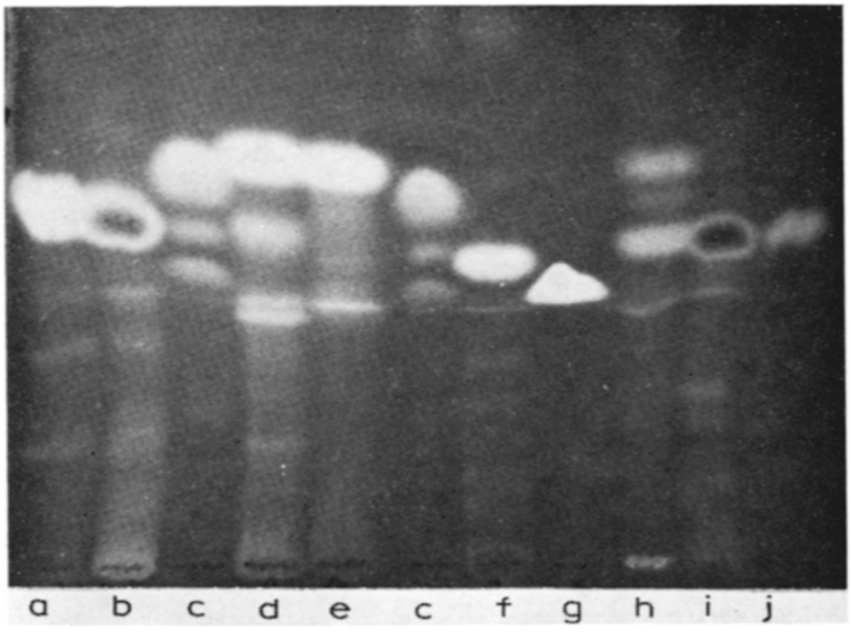

Fig. 5. Photograph of fluorescence with $SbCl_3$ (D 5). System S 3. a = Cholesterol; b = dehydrocholesterol; c = unsaponifiable fraction of the Harderian gland lipid; d = lanosterol; e = dihydrolanosterol; f = lathosterol; g = 3β,6β-diacetoxycholestan-5α-ol; h = unsaponifiable fraction of wool fat; i = ergosterol; j = stigmasterol.

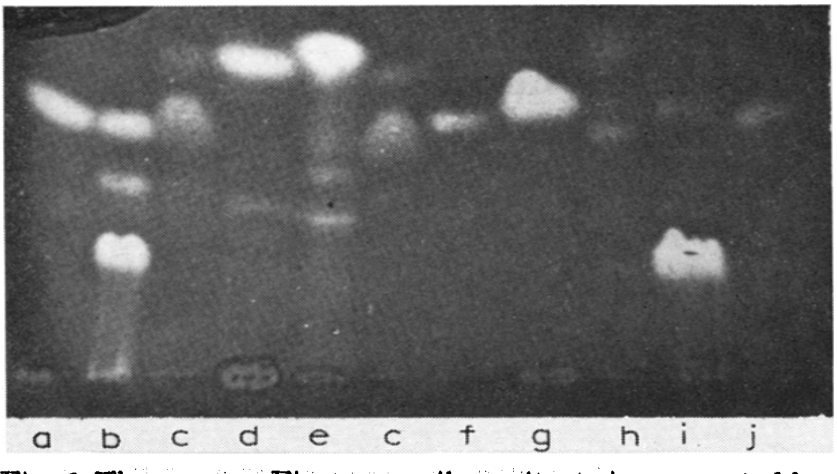

Fig. 6. The same as Fig. 5 on a silver nitrate impregnated layer (S 4).

DISCUSSION

The very high proportion of radioactivity due to the labelled acetate that is incorporated in the lipid of the mouse and rat Harderian gland shows that considerable amounts of lipid are formed in this gland, to which no attention has been devoted so far from the point of view of lipid metabolism. This lipid chromatographs on thin layers with the sterol ester fraction. As this fraction happens to be carried by the second front in the S 1 and S 2 system, its compactness is no indication of homogeneity. It is more polar than the hydrocarbon fraction and less polar than triglycerides, judging from its TLC behaviour. After saponification of the lipid extracted from the gland of rats injected with ^{14}C-labelled acetate 24 h before death (or of the "sterol ester" band therefrom), part of the radioactivity passes into the fatty acid fraction, yet the bulk appears with the unsaponifiables. The fatty acid fraction has not been investigated in any detail in the experiments reported in this paper.

As far as the unsaponifiable fraction is concerned, some very tempting speculations come to mind. (1). If the lipid were cholesterol or one of its precursors (such as lathosterol[20,21]) and if it were resorbed (directly into the blood-stream, or after passage through the lacrimal duct and subsequent ingestion), considerable amounts of cholesterol of blood and tissues could be derived from this source. Any experiments on cholesterologenesis in whole animals would be incomplete if such a rich source were neglected. (2) If the lipid were a provitamin D, the gland could become an important source of the vitamin, since eyes are more exposed to sunlight than skin which is covered with fur; both rats and mice mostly hide themselves in dark places during the day. Protoporphyrin could act by modifying the course of photochemical reactions.

Both these speculations as well as others have no foundation as long as the chemical nature of the Harderian gland lipid remains obscure.

Cholesterol was ruled out very early in this work since neither its chromatographic position nor detection reactions (*e.g.* the Liebermann reaction, $SbCl_3$ colour and fluorescence) agreed with that of the main constituent of the Harderian gland unsaponifiable matter. Free cholesterol was shown by TLC as a minor constituent of the extract, which carried no detectable radioactivity derived from ^{14}C-acetate. In some experiments, cholesterol was also observed as a minor constituent in the unsaponifiable fraction obtained from the "sterol ester" band, yet it remains to be shown whether or not this was a contaminant due to incorrect sectioning of the undetected chromatogram.

Lathosterol was the next guess[2] since it is the main sterol of the epidermis of rodents[22] and is also present in the epidermis of other animals[23]. But even this was ruled out after a reference sample was obtained from Ikapharm, since lathosterol had an R_F value close to cholesterol and thus definitely lower than that of the main Harderian gland unsaponifiable lipid in the TLC solvent systems investigated. This is in agreement with the literature according to which lathosterol travels as a compound of the same or even a higher[24] polarity than cholesterol. Similar considerations also apply to the provitamin, 7-dehydrocholesterol, which was also found to migrate with cholesterol in our system.

Cholestan-3β-ol, whose esters have been found in human smegma[25] and in the preputial gland of rats, was also considered, but the relative decrease of the R_F of the

Harderian gland lipid on $AgNO_3$ impregnated layers made a saturated compound unlikely.

Other sterols which could have a polarity similar to that of the main Harderian gland lipid are 14-norlanosterol, agnosterol and lophenol (4α-methyl-5α-cholest-7-en-3β-ol). Agnosterol is unlikely, since the Harderian gland lipid showed no quenching of the 254 nm induced fluorescence whereas agnosterol contains a conjugated double bond*. In addition, none of the fifteen or so $SbCl_3$-fluorescent bands of the unsaponifiable fraction of wool fat resembled the Harderian gland lipid in the colour of their fluorescence. An isomer of agnosterol, without conjugation of double bonds, is not ruled out. Lophenol has only one double bond, whereas the decrease in the R_F of the Harderian gland lipid on $AgNO_3$-impregnated layers suggests several double bonds. No sample of norlanosterol was available for comparison.

There is no argument at the moment which would allow us to attribute definitely a steroid structure to the lipid we are trying to characterize. A hydrocarbon structure is unlikely since the material (a) occurs originally in bound form from which it is released by alkaline hydrolysis; (b) its R_F is much lower than that of hydrocarbons. An unsaturated fatty or terpenoid alcohol, on the other hand, remains a possibility to be considered.

Further work is in progress towards the isolation of the lipid in pure form in order to check its homogeneity and to study some of its properties more closely.

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* The same argument rules out provitamins D since these substances contain a $\Delta^{5,7}$-conjugation.

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