

LIPID COMPOSITION OF THE EGG SHELL OF RHIPICEPHALUS SANGUINEUS

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Abstract: Observations of thin layer chromatograms revealed the presence of wax esters, cholesteryl esters, unsaturated hydrocarbons, and lecithin in the egg shells of R. sanguineus. Observation of gas chromatograms revealed the presence of palmitate and oleate with lesser amounts of stearate and linoleate. Free fatty acids, triglycerides, paraffin, and free cholesterol were not found. Amino acids could not be detected in the lipid fraction.

Although biochemical analysis has been carried out on several varieties of insects, the investigation of Jaskoski and Butler (1971) and the present study are the first to describe the chemical components of a tick egg shell. The egg shell lipids of the tick Rhodnius prolixus are not extracted by lipid solvents and the term used to describe them by Beament (1945) is lipidized protein. Thin layer chromatography was used by Jaskoski and Butler (1971) to determine the amino acid composition of the protein component of the egg shell of R. sanguineus. The amino acids of this tick were found to be similar to those of the silkworm Bombyx mori, the cricket Acheta domesticus, and the fruit fly Drosophila melanogaster as determined by Tomita (1921), McFarlane (1962), and Wilson (1960), respectively.

Arthur (1948) reported on the layer of the egg shell of Ixodes ricinus secreted by Gene's organ. These eggs were found to rapidly shrivel and dry if they were not allowed to be covered with the secretion of Gene's organ. This layer was found to attract moisture and is presumably of a waxy nature. When the egg clusters were treated with ether, the integrity of the cluster would soon disappear.

Lees and Beament (1948) reported that a wax secretion from Gene's organ, an organ unique to the ticks, serves to waterproof the eggs. Without this secretion, the eggs of Ornithodoros moubata become completely shrivelled and hard. In other tick species such as I. ricinus, a portion of the external lipid layer was found to be secreted by the common oviduct and the vagina. By interfering with the normal process of Gene's organ in this species, partial waterproofing was still observed. The egg shell lipid layer of O. moubata when extracted with hot chloroform was found to have two fractions in a 6 to 1 ratio, a white waxy solid and a soft yellow grease. The lipid layers of O. moubata and I. ricinus measured 0.47 μ and 1.76 μ respectively.

The outer layer of the egg shell of Metatetranychus ulmi Koch is described by Beament (1951) as a hard, transparent, waxy coating of an inert lipid material that is unsaturated in nature. This lipid layer has a high melting point, no proteinaceous material, and its function, unlike in ticks, is not to waterproof the eggs.

The egg shells of the mite, Petrobia latens Muller were studied by Lees (1961). He described the outer wax layer as having a melting point of 165°C., insoluble in a cold benzene or chloroform, or ethanol, but soluble in hot benzene or chloroform. The carbon chain lengths probably range from 16 to 36 as in other hard insect waxes as suggested by Chibnall et al (1934) and Beament (1945).

The investigation reported here was undertaken to determine the nature of the lipids in the egg shell of Rhipicephalus sanguineus. Possible application of this kind of a study relates to tick control at a most vulnerable stage of the life cycle, the egg.

MATERIALS AND METHODS

Oviposition was induced in a fully engorged tick by the method of Kohls (from Lutz, 1937). The eggs produced were examined under the dissecting microscope and were found to measure 0.5 mm by 0.3 mm in the form of translucent red ovals.

The vacated shells of Rhipicephalus sanguineus are translucent white ovals that are split along a thickened area, the "hatching line" as described by Jaskoski and Butler (1971).

A batch of egg shells* was first cleaned of macroscopic debris by hand using two sharp dissecting probes under the dissecting microscope. They were weighed on an analytical balance at 6 mg. The egg shells were fragmented in 100 ml triple distilled water using a Sonifier Cell Disruptor** for five minutes at which time almost all of the egg shells were broken and had settled to the bottom. The egg shells were removed from the water by centrifugation, and then washed three times with triple distilled water. The small pellet of egg shell fragments produced was drained by inverting the tube and allowed to dry at room temperature for one hour. This procedure for cleaning the egg shells is a modification of the method used by Clegg and Morgan (1966) for Fasciola hepatica eggs.

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**Heat Systems Company, Melville, L.I., New York.

The clean dry egg shells were resuspended in 25 ml anhydrous ether and transferred to a 125 ml Erlenmeyer flask. This suspension was shaken on a Burrell Wrist-Action Shaker for ten minutes and the ether fraction was decanted and stored in the refrigerator at 3°C. A fresh 25 ml portion of ether was added to the egg shells and again shaken for 10 minutes. This ether extraction was repeated three times a day for three days, the other fractions being centrifuged, decanted and collected in a single vessel.

Further extraction of the lipid layer was made using the method of Schmit and Wynne. The egg shells were soaked in 10 ml chloroform-methanol (70/30 v/v). This was then centrifuged at 2000 rpm for 10 minutes, the liquid phase being added to the ether soluble lipid fraction. This was repeated three times.

The other half of the lipid-containing solvent was prepared for gas chromatography by reduction to dryness in a 25 ml test tube using a stream of dry nitrogen. A fresh mixture of 9 ml absolute ethanol and 1 ml 33% KOH was added to the lipids to saponify them. This was then heated on a water bath for 30 minutes at 55°C. Fifty percent HCl was added to this drop by drop until the solution became acidic. The fatty acids were then extracted using 10 ml hexane three times. The hexane fraction was then washed twice with 10 ml portions of triple distilled water, and dried for 24 hours over sodium sulfate. The hexane fraction was then dried under nitrogen in a 25 ml volumetric flask. Ten ml of 5 percent perchloric acid in methanol was added and heated on a water bath for 20 minutes at 55°C. The methyl esters thus synthesized were extracted three times with 10 ml hexane-ether (50/50 v/v). The esters were washed three times with distilled water and dried again over sodium sulfate. One ml chloroform containing 50 mg C₁₇ was added to the residue as an internal standard.

The gas chromatograph (Barber-Coleman flame ionization, thermal conductivity, He carrier gas at 200°C.) was programmed from 100°C. to 215°C. at 3°C./min and had a 6' column of 6 percent DEGS on 80/100 mesh Diatroport S. Two µl of the above solution was injected into the chromatograph.

Thin layer chromatography was employed to determine the lipid classes using glass plates 20x20 cm and 5x20 cm coated with a layer of Silica Gel 250 µ thick. These plates were prepared by the method of Stahl (1965).

The solvent systems used to develop the chromatograms were ethylene dichloride, acetic acid, 99:1; n-hexane, diethyl ether, acetic acid, 99:10:1; and petroleum ether, diethyl ether, acetic acid, 80:20:1. In all cases the chromatography chambers were allowed to become saturated with the solvents for 24 hours before the development of the plates. The distance of the solvent front migration was between 12 cm and 15 cm for all chromatograms.

Visualization was accomplished in most cases using the iodine vapor technique. Other methods used include charring with chromic-sulfuric acid, or spraying with 2,7-dichlorofluorescein, phosphotungstic acid, or ninhydrin.

RESULTS

Use of thin layer chromatography techniques revealed the presence of highly mobile non-polar lipids with high R_f values for the types of solvent systems used. The uppermost area in the chromatogram of the tick lipids corresponds with standard solutions of cholesteryl esters, particularly cholesteryl

oleate and cholesteryl palmitate. The lower weight cholesteryl esters have a slightly decreased mobility and do not correspond with the unknown. This region sprayed with 10 percent alcoholic phosphotungstic acid revealed a red color that indicated the presence of cholesteryl esters. Since only a fraction of this area reacted positively to phosphotungstic acid, it is certain that other lipids are present in this area.

Squalene, an unsaturated branched hydrocarbon (C₃₀), showed a high mobility corresponding with this area of the tick lipids, as well as similar tailing effects. The dark I₂ color is similar to the unknown, lending evidence that there is a high degree of unsaturation in the tick lipids. When the charring technique is used to visualize the chromatograms, both the unknown and squalene demonstrated rapid breakdown in this area.

An effort was made to test for saturated hydrocarbons, that is, long chain alkanes with strong C-H and C-C alpha bonds that are not readily attacked by hydrolysis or oxidation. These include mineral oil, lubricants, and paraffin. These saturated hydrocarbons tend to repel the I₂ visualization due to their lack of weak pi bonds and thus have a characteristic faded area that is not present in the egg shell fraction.

Natural waxes are not saturated and appear in the unknown. Pure beeswax is a mixture of waxes, the main component being myricyl palmitate. This is the fatty acid ester of myricyl alcohol and palmitic acid. Other bases for these esters can be oleic and linoleic acids which have been determined as bases for the lipids in the unknown. It is thus apparent that the uppermost area of the tick egg shell lipids corresponded with natural wax esters, cholesteryl esters, and unsaturated hydrocarbons.

Under all types of solvent systems used, lecithin was found to correspond to an area of the unknown that is somewhat less mobile than that mentioned above. Under long wave ultraviolet light, lecithin and its tailing effects exhibit a light violet color that corresponds with the tick egg shell lipids. Several sources of lecithin were used including synthetic and bovine. It is likely that the fatty acids involved are palmitic and oleic as they would be cleaved from the lecithin molecule during the preparation of the unknown for gas chromatography.

Cholesterol and other sterols were repeatedly sought in the fraction and are present in only trace amounts, if at all. The area of the chromatograms where cholesterol would appear, if present, was sprayed with phosphotungstic acid and the results were negative.

The use of TLC revealed that only trace amounts of triglycerides are in the egg shell lipids. Free fatty acids have been compared to the unknown and no indication of their presence was indicated. The unknown lipids were compared to several lipids from both classes and negative results were constant.

Spraying the unknown on the chromatogram with 0.5 percent ninhydrin revealed that no amino acids were present.

The methyl esters of the total lipid fraction of the tick egg shells as obtained by extraction and synthesis as mentioned above indicate the presence of palmitate and oleate, with smaller amounts of stearate and linoleate.

SUMMARY

- 1) The lipid fraction of the egg shells of Rhipicephalus sanguineus was analyzed using the techniques of thin layer and gas chromatography. The outer wax layer was separated from the inner protein or shell layers by extraction using anhydrous ether, chloroform-methol (70/30 v/v), and hexane-ether (50/50 v/v).
- 2) Using thin layer chromatography, it was shown that the egg shells contain wax esters, cholesteryl esters, unsaturated hydrocarbons, and lecithin.
- 3) By gas chromatography, palmitate and oleate with lesser amounts of stearate and linoleate were present.
- 4) Free fatty acids, triglycerides, paraffin, and free cholesterol were not found.

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