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Contribution to the Histochemistry of the Cuticle and Cuticular Structures of Trematodes

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Abstract. The cuticle of the liver fluke *Fasciola hepatica* Linné, 1758 and of *Echinostoma revolutum* (Fröhlich, 1802) Dietz, 1909 consists of two layers—the surface layer of the cuticle and the cuticle proper. The basement layer of connective tissue is not considered part of the cuticle. With histochemical reactions acid mucopolysaccharides can be confirmed in the external surface layer of the cuticle; in the proper cuticular layer the upper limiting zone is formed by a PAS-positive mucopolysaccharide. The granules of the lower part of the proper cuticle are formed by an acid mucopolysaccharide. In addition to the mentioned polysaccharides also cysteine, arginine, tyrosine and an abundance of lipoid substances were found especially in the trematode *E. revolutum*. The substance of integumentary scales and collar spines may be characterized as a scleroproteid with a high refraction index, a high cysteine content and a lower content of arginine and tyrosine. No polysaccharides were confirmed in the scales, but lipoid substances were plentiful. In the collar spines of *E. revolutum* it is possible to distinguish a surface layer and a medullary layer, the latter possessing almost the same staining properties as the basic substance of the scales. Contrary to the surface layer of the collar spine, the medullary layer contains more arginine and more lipoid substances but the accumulation of cysteine is substantially higher on the surface of these collar spines.

Generally, in the cuticle of platyhelminthes two or three layers have been distinguished, while, at the same time, the cells producing the cuticle have mostly been taken for a submerged epithelium. KOWALEWSKI (1895) first drew attention to the mutual connection of these cells with the cuticle. Recent electron microscopic works confirmed quite clearly the fused cytoplasm of subcuticular cells, which may possibly be held as an argument in the solution of the ancient dispute on the origin of the cuticle. Already HYMAN (1951) has pointed out that the submergence of groups of epithelial cells under the muscle layer can be traced in the land planarian *Bipalium*. Should that be also the case with trematodes this may concern the ectodermal body surface enclosing the surface muscle system. This muscle system is enclosed in the respective connective tissue component, which some earlier writers considered correctly a layer of mesodermal origin and which shows clearly that it does not belong to the cuticle.

Electron microscopic research of THREADGOLD (1963) has shown that the cuticle is formed by the direct outer syncytial layer of subcuticular cells. Its extensions passing through the fibrous basal layer connect the syncytial layer with the principal part of the cells proper containing a nucleus, a multitude of mitochondria and the Golgi apparatus. At the cuticular surface there are minute canals and bladders, lower down numerous mitochondria, the endoplasmatic reticulum and vacuoles. The syncytial layer is marked off by a basement membrane, distinguishable by electronmicroscopy, belonging to the basal fibrous layer and not to the cuticle.

Despite of the elucidation of the submicroscopic structure of the cuticle, the general histological picture has not yet been confronted with the electron microscopic picture. A histochemical study of the cuticle has made this correlation possible. Although some electron-optical works have also been concerned with the structure of the cuticular formations, only the scales of some trematodes, namely of the species *Fasciola hepatica* and *Haematoloechus medioplexus* have been studied so far. No research work either by electron microscopy or histochemically has been done on the collar spines, a characteristic sign of the species *Echinostoma revolutum*. For these reasons we have tried to identify the substance structure of these formations by histochemical methods.

MATERIAL AND METHODS

Echinostoma revolutum (Fröhlich, 1802) Dietz, 1909 were dissected from the small intestine of domestic ducks and *Fasciola hepatica* Linné, 1758 from the liver of fallow deer. The cuticle and cuticular structures were studied in both species.

The adult trematodes *Echinostoma revolutum* were washed in saline, placed between two slides and fixed with neutral formol after Baker. The trematodes *Fasciola hepatica* were fixed in 10 % formol together with the surrounding liver tissue. After embedding in paraffin the serial sections were stained with histological methods: Böhmer's hematoxylin-cosin, Weigert's hematoxylin-cosin, Mallory's phosphowolfram-hematoxylin, trichrome after Masson and Goldner, the regressive method after Giemsa, the illustration of reticular fibers after Gomori, the illustration of acid resistent structures after Ziehl-Neelsen. Metachromasy was ascertained with toluidine blue and thionine. For the study of polysaccharides we used Best's carmine with the saliva test and mucicarmine, also the PAS method with acetylation, desacetylation and with the saliva test, Hale's method modified after MOWRY (1958), also in combination with the PAS method and with control staining with a solution of only colloidal iron. Of the amino acids arginine was proved with Sakaguchi's method modified after BAKER (1947), tryptophan after ADAMS (1957) with dimethylamino-benzaldehyde (DMAB), tyrosine with Millon's reagent, cystine with performic acid in combination with alcian blue (PFA-AB) after PEARSE (1960) and also in a method with 2.2′-dihydroxy-6.6′-dinaphthyldisulphide (DDD) after BARNETT and SELIGMAN (1952) in combination with thioglycolic acid. For the cysteine proof a method with DDD and a method with p-nitrobromacetophenon (pNBAF) in the modification after GERSTEYN (1958) was used.

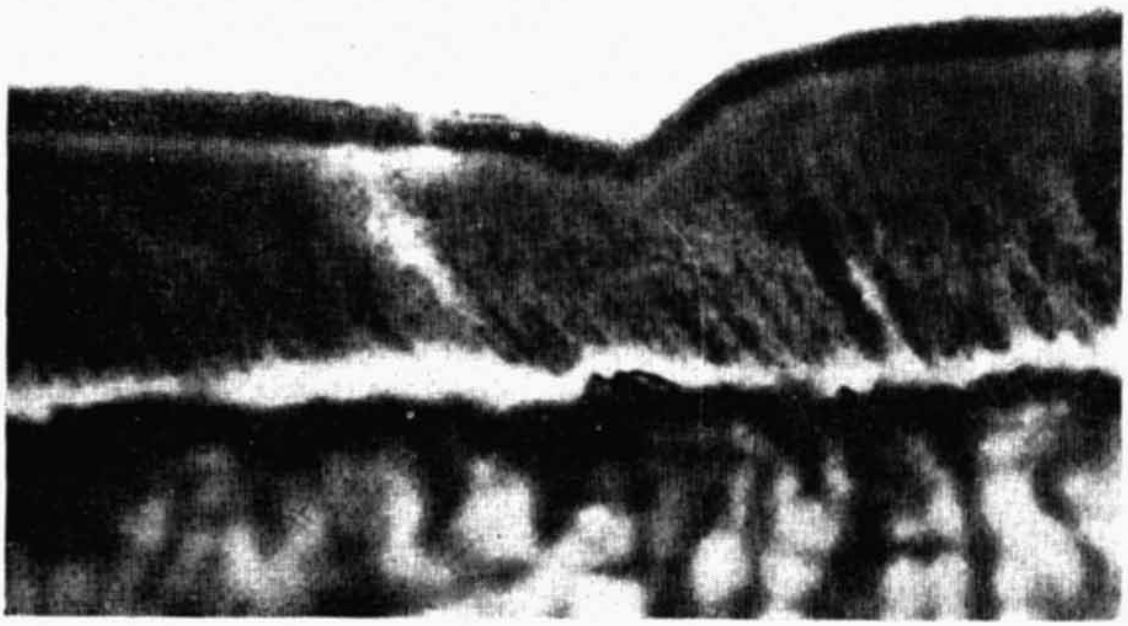

Fig. 1

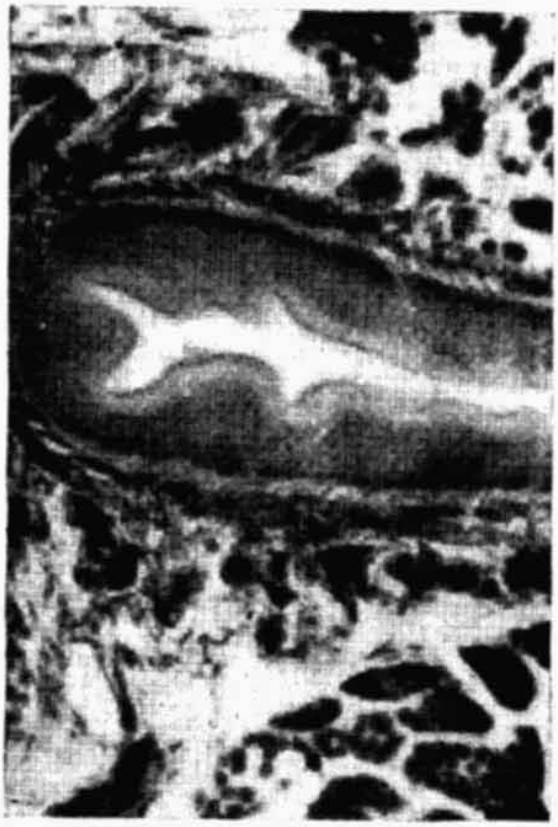

Fig. 2

Fig. 3

Fig. 1

Fig. 2

Fig. 3

RESULTS

1. Morphology of the cuticle

The cuticle consists of a surface layer and of the proper cuticular layer. In the latter, two zones can be distinguished, one passing gradually into the other. Its lower part contains various granules and fibrous formations, which become less numerous towards the top and finally disappear. Under the surface layer, the proper cuticular layer forms a boundary brim, sometimes exhibiting a distinct staining character. The proper layer of the cuticle of both species lies on a layer with connective tissue properties, which stains green with Goldner, red with van Gieson and becomes distinctly silver impregnated with the method after Gomori. Small openings, through which the connecting extensions of the subcuticular cells pass, can be observed in the connective tissue layer.

The cuticle of both trematodes is not of equal thickness. It is thinnest in the cephalic part and thickest at the sites where the largest scales are situated. Contrary to the distinct differentiation of the connective tissue layer, numerous methods have failed to differentiate the individual cuticular layers. Hematoxylin staining makes the whole cuticle appear like a basophilic complex; after van Gieson, only the surface layer staining reddish-voilet and the proper cuticle (yellow) are distinguishable. The trichrome staining method after Masson and Goldner reveals a good differentiation of the proper cuticular layer (red granules). In sections stained after Giemsa and with Mallory's phosphowolfram-hematoxylin, the surface layer appears pink, the granules of the proper cuticular layer stain violet. Only Gomori's method makes a detailed differentiation of all cuticular layers possible, which is similar to some histochemical methods (see Tab. 1). The surface layer becomes so distinct, because silver is precipitated in the fine tubulose invaginations of the surface of this layer and, contrary to that, the upper limiting zone of the proper cuticle does not stain at all with the method after Gomori. The other parts are brownish and the characteristical granules in the lower half black (Plate I).

In our studies of the polysaccharides we found that contrary to *F. hepatica* the surface layer in *E. revolutum* stains intensively with mucicarmine and Best's carmine and that this colouring remains even after the saliva test. Also the PAS positivity is very high, but disappears after acetylation. This finding confirms the high accumulation of a polysaccharide which is not glycogene. In *F. hepatica* the PAS positivity in this layer was found to be considerably lower and it could not be clearly distinguished after desacetylation. Also when demonstrating acid mucopolysaccharides we observed a more intensive colouring of the surface layer in *E. revolutum* than in *F. hepatica*. Especially marked differences occurred in the grade of metachromasy in both species, because staining with toluidine blue and thionine caused a red colouring in *E. revolutum* and a violet colouring in *F. hepatica*. The upper limiting zone of the proper cuticular layer, which communicates with the surface layer, reacts quite clearly as a neutral mucopolysaccharide. In the lower part of the proper

Table 1. Staining properties of the individual cuticular layers

Methods	External layer		Proper layer of cuticle		
	Echinostoma revolutum	*Fasciola hepatica*	Upper limiting zone	Matrix	Granular structure on basement part
Best's carmine	intensive red	–	–	–	–
Saliva test + Best's carmine	intensive red	–	–	–	–
Mucicarmine	intensive red	–	–	–	–
PAS	++++	++	+++	++	+
Acetylation + PAS	–	–	–	–	–
Desacetylation + PAS	++++	–	+++	++	+
Saliva test + PAS	++++	++	+++	++	+
Hale-PAS	intensive blue	blue	red	light blue	blue
Colloidal Fe after MÜLLER	intensive blue	blue	–	light blue	blue
Hale	intensive blue	blue	–	greenish-yellow	blue
Toluidine blue	red	violet	–	light blue	violet
Thionine	red	violet	–	–	rose
Alcian blue pH 0.2	+++	+	–	–	+++
PFA-AB	+++	+	–	–	+++
Thioglyc. acid + DDD	red		red	red or pink	–
DDD	red		red	red or pink	–
DMAB	–		–	–	–
Millon	+		+	+	–
Sakaguchi	+		+	+	–

cuticular layer granules are distinguished, which react distinctly as acid mucopolysaccharides with a contrasting manifestation of metachromasy, when toluidine blue or thionine is used.

Protein reactions revealed the presence of SH-groups in the whole cuticle and also a moderate positivity of tyrosine and arginine. In accordance with it, the proper cuticular layer stains greenish-yellow in the complete staining method after Hale. The whole cuticle of *E. revolutum*, when stained with Sudan black B took on an intensive black colour, which incapacitated the differentiation of its individual parts. The staining of the cuticle of *F. hepatica* was less intensive and in a more prolonged differentiation a more intensive colouring of the upper limiting zone of the proper cuticular layer occurred in correlation with an increased PAS positivity.

2. Morphology of the integumentary scales and collar spines

The cuticular formations are attached to the outer side of the connective tissue layer, i.e. to the connective tissue membrane; they either only touch this membrane (as the scales of *E. revolutum*) or their bases are slightly immerged into it (scales of *F. hepatica*), or most parts of their length are immerged into it as this is the case with the collar spines of *E. revolutum*, which almost appear as if enveloped in a

connective tissue sheath. To what extent these spines are immerged into the connective tissue sheath depends on the stage of contraction or dilatation of the muscles, which push the spines either in or out.

The external cuticular layer covers continuously the entire body surface of both trematodes and is perforated only by the points of the collar spines of the species *E. revolutum*. The scales breaking only through the proper cuticular layer have their protruding points completely covered with the external layer of the cuticle. The scales of *F. hepatica* are spaced in chess-board fashion throughout the body. They are of various size, their dimensions ranging from 49 by 28 to 84 by 63 μ. In the substance of the scales canaliculi resembling a fibrous structure are present; they vary in length, the longest extending up to the point of the scale and are clearly visible on longitudinal and transverse sections. The scales of the trematode *E. revolutum* are placed on the anterior extremity; from the cephalic collar down to the level of the first testis; they are smaller than those of *F. hepatica* (dimensions 33 by 15 μ). Contrary to *F. hepatica* no fibrous structures could be confirmed in their ground substance. The scales are arranged like a chess-board, decreasing gradually in size and density as their distance from the collar increases.

The cephalic collar of *E. revolutum* consists of 37 spines—5 on the ventral side, of which the two biggest are in the aboral row (90 μ—100 μ by 21 μ) and the smallest (65 μ—70 μ by 10 μ) is closest to the centre. The lateral spines are aligned, the dorsal spines in two rows—the oral and aboral row. The dimensions of the lateral and dorsal spines are approximately the same — 80—95 μ by 21 μ. The collar

Table 2. Results of different staining methods of collar spines and integumentary scales

Staining method	Collar spines		Scales		
	E. revolutum		*E. revolutum*	*Fasciola hepatica*	
	external layer	medullar layer	ground substance	ground substance	canaliculi
Böhmer's hem.-eosin	red	pink	pink	pink	red
Van Gieson	yellow	yellow	yellow	yellow	red
Mallory phospho-wolfr. hem.	blue only at base	—	blue	blue	—
Goldner	dark red	red	red	red	red
Masson	red	pink	red	pink	red
Gomori	yellowish to brown	black	yellowish or colourless	yellowish	black
Giemsa	red	blue	red and blue	blue	pink
Zichl-Neelsen	red	red	red	pink to red	—
Millon	−/+	−/+	−/+	−/+	—
Sakaguchi	+/++	+++	+/−	++/−	—
DNFB	+	−	+	+	—
pNBAF	+++	−	++	++/+++	—
DDD	++++	++	+++	++++	—
Thiogl. DDD	++++	++	+++	++++	—
Sudan black B	+/++	++++	++++	++++	—

spines consist of an external and an internal layer. The fibers observed in the scales of *F. hepatica* were not found in them.

Our results show that the substance constituting the scales has a very distinct character. It is highly refractive; in histological methods it stains picric yellow after van Gieson, red with trichrome after Masson and Goldner, violet in Mallory's phosphowolfram-hematoxylin, intensive purple after Ziehl-Neelsen. In the method after Gomori and Rogers it does not reduce silver nitrate. Its staining properties are not clean-cut in the method after Giemsa, some scales stain red, others blue, according to the grade of differentiation with acetic acid. Histochemical reactions to polysaccharides are negative, but highly positive to the SH-groups. Negative are also the reactions to the SS-groups, moderately positive in the reaction after Sakaguchi and Millon. Highly positive are these reactions in staining with Sudan black B, which confirms a high content of lipoid substances. All these reactions prove that the substance concerned is a scleroproteid with a high refractive index and a high cysteine content. In addition it contains also a little arginine and tyrosine. A characteristic feature is also the high content of lipoid substances (Plate II).

The collar spines exhibit a differentiation in a higher accumulation of arginine and lipoid substances in the medullar layer than in the external layer, while the proof of SH-groups is evident principally in their external layer. It is therefore possible to speak of a morphological differentiation of the cortex layer of these spines, manifested also by an increased refractivity index.

DISCUSSION

The structure of the cuticle and also the staining of its individual components can be best distinguished in trematodes with an extremely developed cuticle, such as *Fasciola hepatica*. In the proper cuticular layer two distinct components can be distinguished; with trichrome after Masson the matrix stains blue and the rows of minute granules red. The external cuticular layer can also be differentiated with some staining methods; especially in Gomori's method the precipitated silver fills the fine tubules of this layer. The tubules of this species have been illustrated in electron microscopy by THREADGOLD (1963) and BJÖRKMAN and THORSELL (1964) and by BURTON (1966) in *Gorgoderina* sp. All the writers called them tubulose invagination of the surface membranes, THREADGOLD considering the deeper situated contoured cavities under these invaginations to be pinocytotic vesicles. In our opinion, these are the basement parts of the tubules, nipped off during sectioning from their main portion, opening on the surface. This is in agreement with the findings by BJÖRKMAN and THORSELL (1964) about the experimental resorption of ferritin, which, as they remark, is better classified as "transmembranosis" then as pinocytosis. The system of tubules in the external layer extends its surface, but seems also responsible for its rather high lability, which lead LOGACHEV (1955) to designating it as "pars decidua". This external layer is also formed in *E. revolutum*, in which the

granules of the proper cuticular layer are rather dispersed than aligned. The external cuticular layer of *E. revolutum*, which is absent only in the cephalic part, consists in most parts of the body of an acid mucopolysaccharide. In *F. hepatica* it covers the whole body. The upper limiting zone of the proper layer is PAS-positive in both species. The granules of the proper cuticular layer are histochemically in agreement with the substances of the external cuticular layer. In *F. hepatica* the reaction of the external layer and the granules of the proper cuticular layer to the acid mucopolysaccharide is not so distinct as in *E. revolutum* (Tab. 1). Similar observations were made by MONNÉ (1959). The fine granules in the basement part of the proper cuticle can be identified as mitochondria in correlation with the electron microscope. Especially their characteristic arrangement in strings induced LOGACHEV to call this part of the cuticle "pars baculosa". The protein component of the external layer is formed by arginine, cysteine and tyrosine. Also the proper cuticular layer consists, in addition to the granules of the acid mucopolysaccharide, of a protein component, in which also a small amount of arginine, cysteine and tyrosine could be confirmed. Our observations are substantially in agreement with the results of BJÖRKMAN, THORSELL and LIENERT, finding the cuticle of *F. hepatica* of a proteinic nature with a border of mucopolysaccharides or mucoproteins. Contrary to their observations we could not detect glycogene in the cuticle. Also SHAPIRO (1961) found mucoproteins in the proper cuticular layer of *Zygocotyle* and *Haematoloechus*. She also mentions the finding of lipoproteins, which is in agreement with our findings of a heavy accumulation of lipoid substances.

In the past, little attention has been given to the histochemistry of cuticular structures and only fragmentary references on their substance composition are found in the literature. Only MONNÉ (1959) published a more detailed study on the scales of *F. hepatica*, *E. revolutum* and *Alaria alata* without mentioning the reactions of the collar spines of *E. revolutum*. BURTON (1964) described in the species *Haematoloechus medioplexus* the detailed structure of integumentary scales consisting of a crystallic protein, its structure suggesting a lattice. Similar to our observations and to those of SHAPIRO (1961), Monné could confirm neither polysaccharide in the scales. According to his statement the scales do not contain a "polyphenol-quinone-tanned protein", because they do not reduce the ammoniacal solution of silver nitrate. Neither could the basic scale substance of both trematodes studied reduce this solution (Gomori, Roger), but it became reduced on the internal layer of the collar spines of *E. revolutum* and on the fibrous canaliculi inside the scales of *F. hepatica*. MONNÉ (1959) concluded that the scales of the three trematode species mentioned by him are formed by a "hard" protein, which is highly Gram-Weigert-positive and stains in the method after Azan and in Altman's picrate fuchsin intensively red. In connection with these findings he also mentions that some forms of vertebrate keratine are also Gram-Weigert-positive, but he did not carry out the proof of cysteine and cystine in the scales. Our findings enable us to conclude that the scales and spines of trematodes are formed by a scleroproteid, which partly resembles keratine, but is characterized only by the presence of the SH-groups.

CONCLUSIONS

1. The histochemical analysis has proved that the cuticle of the trematodes *Fasciola hepatica* and *Echinostoma revolutum* is formed by a lipoprotein complex. The characteristic granules in the proper cuticular layer consist of an acid mucopolysaccharide. Another important observation is the density of acid mucopolysaccharides in the external layer of the cuticle and of neutral mucopolysaccharides in the upper limiting zone of the cuticle proper.

2. The scales and spines of these trematodes are formed by a scleroproteid with a characteristically high content of only cysteine and also with a multitude of lipoid substances.

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EXPLANATIONS TO THE PLATES

Plate I

Fig. 1. Surface of the body of *Fasciola hepatica*. The external cuticular layer is clearly visible as a dark strip, interrupted by darker vertical lines corresponding to the canalicular invaginations of the surface membrane and are filled with precipitated silver. The light external zone of the proper cuticle differentiates distinctly the external cuticular layer. In the proper cuticle distinct granular formations partly arranged in strings can be viewed. The basis of the proper cuticle is not stained and communicates closely with the basement connective tissue layer, which is not distinct. Gomori (× 1600).

Fig. 2. Fold in the surface of the body of *Echinostoma revolutum*. Granular formations are visible in the proper cuticular layer, however, its surface zone merges with the surface cuticular layer in a homologous surface band, which is little stained. Mallory's phosphowolfram-hematoxylin (× 700).

Fig. 3. Two portions of the surface of the body of *Echinostoma revolutum* in a fold. The basement border of the proper cuticle is clearly visible; on the surface of the body the external cuticular layer is distinctly tinged. In comparing its thickness with the situation on Fig. 2 it becomes evident that in this figure the external layer is formed by an undifferentiated surface layer of the cuticle and the surface zone of the proper cuticle. Best's carmine after the saliva test (× 700).

Plate II

Fig. 1. Part of the section of *Fasciola hepatica* in the gall ducts, filled with cell exudate. On the surface of the trematode the distinct cuticular scales are more intensively tinged than the proper cuticle. The scales reach into the muscular connective tissue layer under the cuticle. Giemsa (× 145).

Fig. 2. Tangential section through the connective tissue layer under the cuticle of *Echinostoma revolutum*. Deformations of this layer, caused by pressure of the base of the cuticular spines, are visible. Gomori (× 1450).

Fig. 3. Longitudinal section through the body surface of *Fasciola hepatica*. More intensively stained are the scales, the bases of which are pressed into the connective tissue layer under the cuticle. Also more intensively stained is the external layer of the cuticle, while the external border zone of the proper cuticle stained less. Sudan black B (× 700).