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Comparative Topo-optical Investigation of Cardiac Glycoside Localization

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Summary. The aldehyde-bisulfite-toluidine blue (ABT) reaction is a selective topo-optical test of vicinal-OH and amino-OH groups. The localization of cardiac glycoside was investigated morphologically. After digoxin the sarcolemma membranes, capillaries and sinus node showed strong basophilia and negative birefringence. The topo-optical reaction are useful for the histopathological examination. The new method gives the possibility to the digoxin intoxication with a high specificity.

Key words: Poisoning, digoxin – Digoxin poisoning – Glycoside localization, topo-optical reaction

Zusammenfassung. Die Aldehyd-bisulfit-Toluidinblau (ABT) Reaktion wird in Geweben als selektive topo-optische Testmethode der vicinalen-OH-Gruppen und der Amino-OH-Gruppen angewandt. Die Lokalisation der herzwirksamen Glycoside wurde morphologisch untersucht. Nach experimenteller Digoxinintoxikation wurde das Reaktionsprodukt in den Sarcolemma-Membranen, den Sinus-Knoten und an den Kapillaren lokalisiert. Für die topo-optische Reaktion ist die durch die orientierte Farbstoffbindung herbeigeführte starke Doppelbrechung kennzeichnend. Die Unterschiede in der Doppelbrechung bieten eine Möglichkeit zur neuartigen Analyse in der Histopathologie bei Glycosidvergiftung.

Schlüsselwörter: Vergiftung, Digoxin – Digoxinvergiftung – Digoxinlokalisation, topo-optische Reaktion

Introduction

Carbohydrate moities are common building blocks of great variety of biological structures such as polysaccharide, glycoproteins and glycolipids. The PAS reaction developed for the selective demonstration of vicinal-OH groups. However, it does not produce anisotropic effects in the reactive structure and therefore it can not be used to study the molecular order of vicinal OH groups by polarization microscopy. This aim could only be achieved by selective and oriented topo-optical staining reaction (Romhányi et al. 1969, 1974, 1975).

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Even 200 years after medical introduction of digitalis compounds for the treatment of heart failure, there remains some controversy about the basic mechanism of its action. It is generally accepted that digitalis compounds are specific in their inhibition of Na-K activated membrane ATP-ase, the enzyme controls active Na-K transport.

Digitalis is still one of the most frequently used drugs. The risk accompanying treatment justifies the revision of indication contra-indication according to stricter criteria. The radioimmunological determination of the serum cardiac glycosida level and the purified preparations have provided new pharmacokinetic informations. Intoxication with cardiac glycosides are frequent (Carruthers et al. 1974; Lown et al. 1972; Ogilvie and Ruedy 1967; Sótonyi et al. 1981).

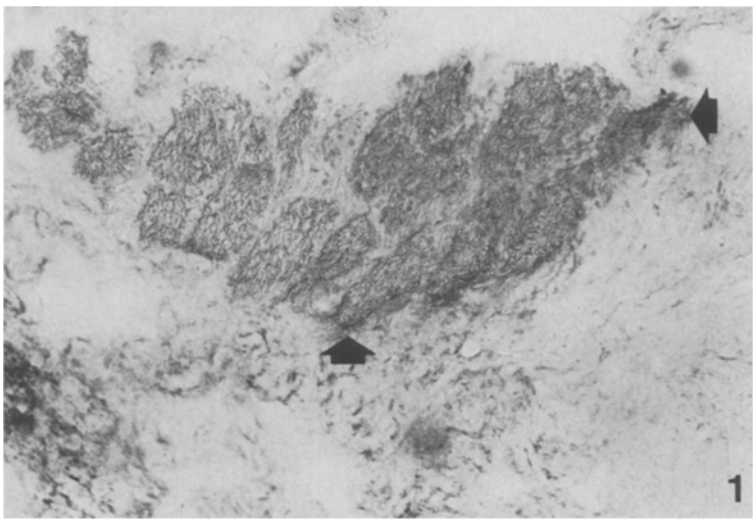
In spite of these findings cardiac glycoside poisoning has a great clinical as well as forensic medical aspect. Morphological findings, autopsy and histological examinations revealed non-specific alterations after cardiac glycoside poisoning (Asplund et al. 1971; Brandes and Suchowsky 1954; Dearing et al. 1943; Kisser 1969; Selesky et al. 1978; Taubert and Shapiro 1975). In this field it is necessary to know the correct morphological localization of cardiac glycoside. The aldehyde-bisulfite-toluidine blue (ABT) reaction is a new method for the study of vicinal-OH and amino-OH groups of polysaccharides. The new possibilities of the demonstration of carbohydrate components are suitable for the study of their ultrastructural organization (Fischer 1976; Romhányi et al. 1974, 1975). Since there are no satisfactory morphological methods for investigation of digitalis or other cardiac glycosides binding, in this paper the adaptation of ABT reaction, which is useful for demonstration or digoxin in toxic doses is discussed.

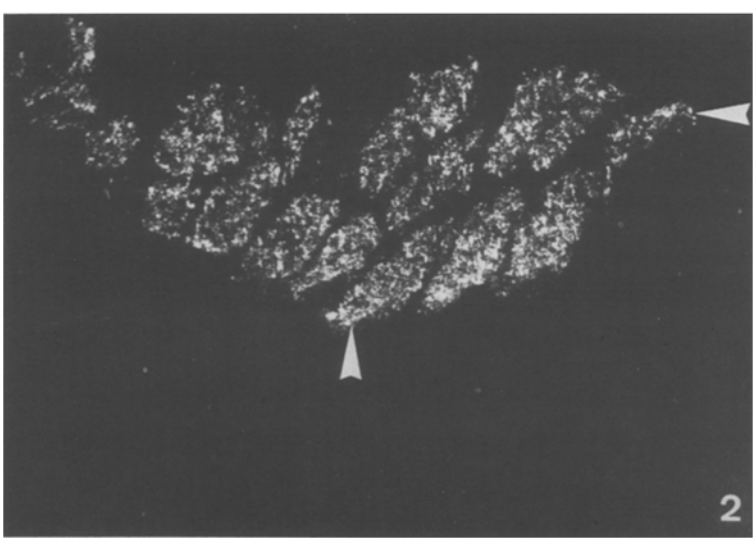
Material and Methods

The experiments were carried out on mongreal dogs (12–14 kg). Cardiac glycoside, Digoxin (Richter, Hungary) was administered after slowly i.v. application of 0.15 mg/kg body weight per day. Glycoside level of serum were continuously checked by radioimmunassay. For measurement of serum digoxin concentration the commercially available ^{125}J-radio-immunoassay Kit were used (New England Nuclear, USA). When cardiac glycoside concentration in the blood plasma was between 40–60 ng/ml the morphological examination was carried out. Under pentobarbital anaesthesia (30 mg/kg) the artificial ventilation was maintained with room air, the heart was taken out. Randomly selected atrial and ventricular tissue samples were used. The tissues for the topo-optical staining were unfixed and investigated in 5–10 μ thick cryostat sections (Cryo-Cat Microtome, American Opt. Co). The sections on glass slides were treated with 0.1% periodic acid for 30 min then after rinsing in destilled water, saturated Na-bisulphite for 30 min. After short rinsing with water the slices were stained for 5 min with toluidine blue at pH 1.0 (0.1% toluidine blue in 0.1 N HCl). The dye solution was dropped into the slides. After staining, the dye solution was blotted off with good quality filter paper and then 1% potassium ferricyanide solution was dropped into the slides, for 10 min. Subsequently the sections were covered with 20% gum arabic containing 1% potassium ferricyanide. The dried slices were sealed with Canada balsam.

Polarization optical examinations were carried out with a Leitz Ortholux polarization microscope equipped with compensators. Quantitative measurements were made with rotating compenzators (1/4 λ plate).

The enzyme treatment was performed with 1% Diastase (Serva, Heidelberg) and 1% Amylase (Sigma, Heidelberg) in destilled water at 37°C for 1–2 h. For more details of type and theory of the topo-optical reactions see Romhányi and Deák (1969) and Módis (1974).



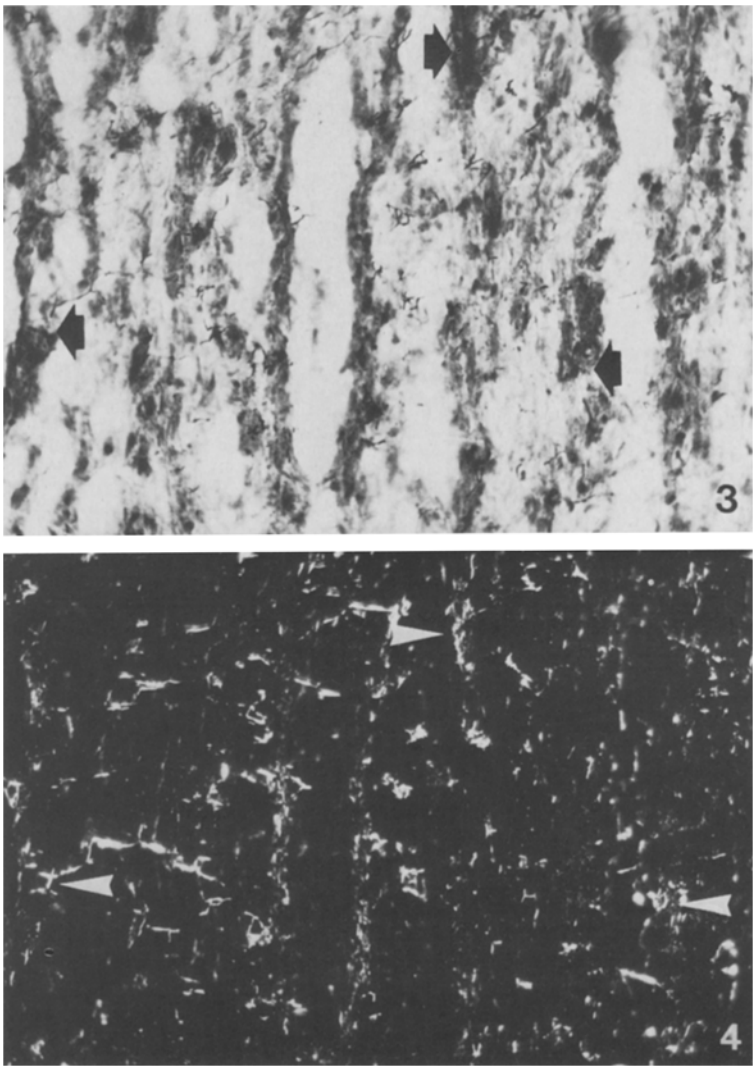


Figs. 1 and 2. ABT reaktion shows the basophilia *(arrows)* and between crossed polar negative birefringence *(arrows)* of the sinus node. × 650, × 650

The topo-optical examinations were also used in 10 human cases where glycoside was determined with radioimmunoassay method. In these cases the concentration of cardiac glycoside was 120–260 ng/ml. As controls untreated dogs were used.

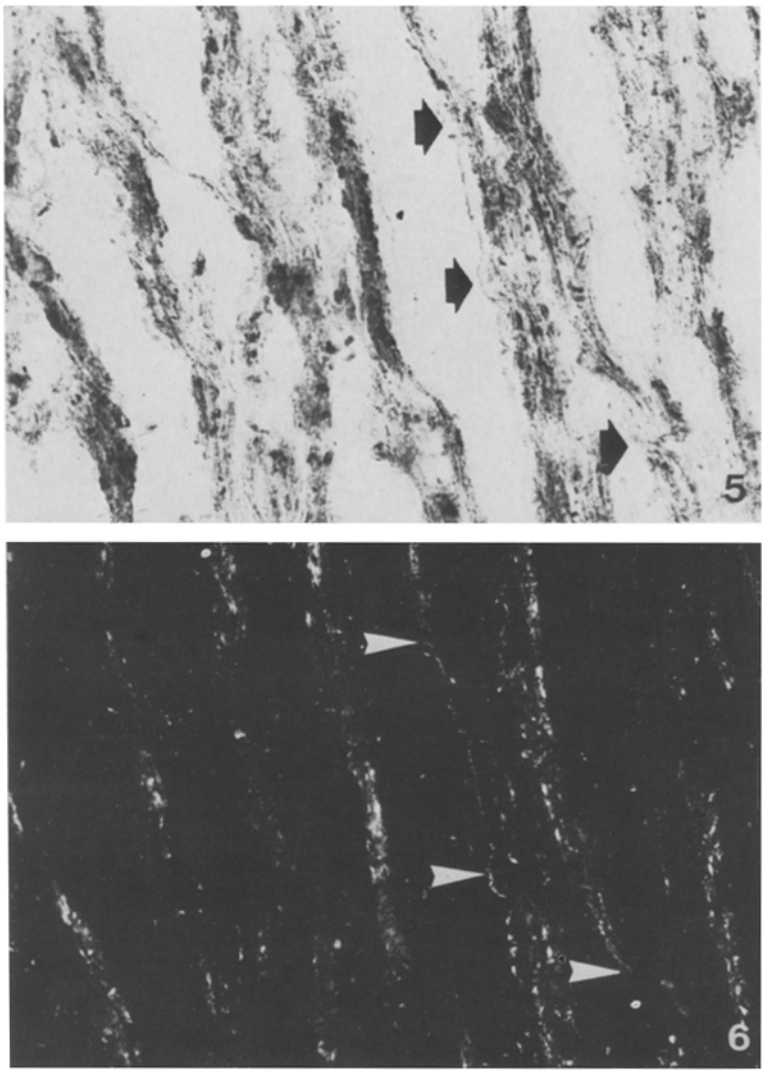
Results

After the ABT reaction by acute digitalis intoxication the sinus node (Figs. 1 and 2) the capillaris (Figs. 3 and 4) and the sacrolemma membranes (Figs. 5 and 6) showed intensive metachromatic basophilia with very strong birefringence with 30–40 nm retardation, negative to the surface.



Figs. 3 and 4. ABT reaktion. Under light microscope *(arrows)* and between crossed polar *(arrows)*. Intensive basophilia and birefringence of the capillaries. × 280, × 280

The strong binding of the drug was indicated by the appearance of dichrosim and the characteristic green color of polarization. The localization appeared to be in extracellular connection with the outer side of sarcolemma membrane. In unstained preparations no measurbalc birefringencc was observed. The birefringence was negative and this indicated thc presencc of sugar components in tangentially oriented pattern in the membrane. The enzymatic treatment altcred the intensity of ABT reaction. Glycogen granules were rendered strongly basophilic and slightly metachromatic due to the ABT reaction. Localization was intracellular. The glycogen had a granulated birefringence, while the polari-

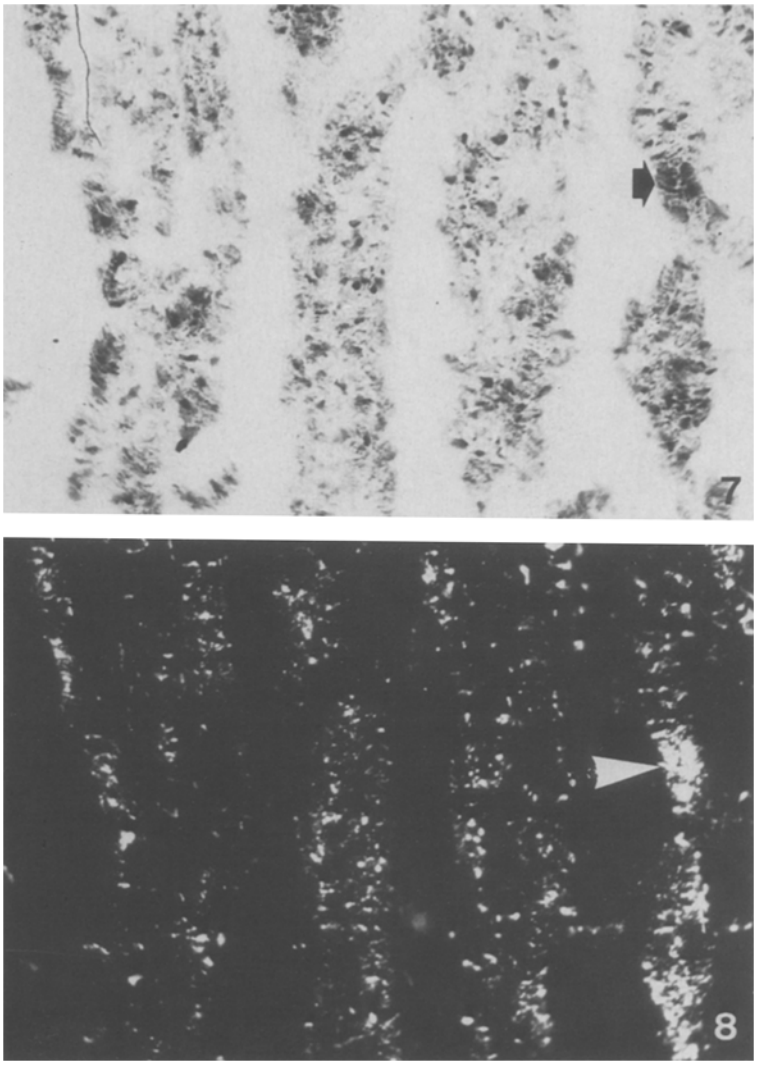


Figs. 5 and 6. ABT reaktion with high basophilia and birefringence *(arrows)* of the outer side of sarcolemma membrane. × 380, × 380

zation color was red and enzymatic treatment had a strong effect. In human cases the ABT reaction was strong in sinus node and on the sarcolemma membranes.

In the untreated dogs we did not find a strong positive reaction at the sarcolemma membrane. The sarcolemma membrane showed very weak effect of basophilia and birefringence with 5–10 nm retardation. Only the glycogen granules were rendered strongly basophilic and slightly metachromatic by the ABT method and strongly birefringent revealing red polarization colours (Figs. 7 and 8).

A topo-optical reaction was strong positive by the human sections and it compared favourably with the radioimmunoassay result.



Figs. 7 and 8. ABT reaktion in the untreated material with very weak effect of basophilia and birefringence. × 380, × 380

Discussion

The ABT method was introduced into histochemistry by Malinin (1970) who found that the metachromatic basophilic staining with this method was characteristic of glycogen. The molecular mechanism of the ABT reaction and ultrastructural basis of the birefringence induced by oriented binding of toluidine blue has been outlined (Fischer 1976; Romhányi et al. 1975). The molecular mechanism of topo-optical reaction is well known. By periodic acid the vicinal-OH and amino-OH groups of the carbohydrate components are changed into dialdehydes and on the addition of bisulfite they aquire a strong negative charge.

The acid radicals formed in this way bind the basic toluidine blue dye molecules even in strong acid (pH 1.0) solutions. With lincarly oriented polysaccharide chains, the ABT reaction, owing to oriented dye bindings, induces a birefringence of negative character to the length of the polysaccharide chains. The heart glycosides among other characteristics are cardioactive steroid a compounds that contain unsaturated lacton rings at C17 and sugar chains at C3 which through their respective radicals have been fitting for ABT reaction. The method utilizes the birefringence produced by the oriented binding of toluidine blue to the vicinal-OH groups in the sugar component of cardiac glycosides. The ABT reaction induced an intensive basophilia and strong negative birefringence on the capillary, sarcolemma membrane and sinus node.

According to our observations the ABT reaction is indeed suitable for the selective demonstration of carbohydrate components of cardiac glycosides. In our new results the lacton ring after chemical treatment is also available for the ABT reaction. The difference between the polarization optical findings are useful for the demonstration of glycogen and cardiac glycosides. Carbohydrate moities such as polysaccharides, glycoproteins and glycolioides which are in the sarcolemma membrane showed different measurable topo-optical effects. In untreated conditions the basophilia and birefringence shows weak effect. After binding the cardiac glycoside, retardations were strong with green polarization colour suggesting high degree of dye molecula binding. Periodic acide is known to transform the vicinal OH groups into dialdehyde groups (Barka and Anderson 1963; Romhányi et al. 1974, 1975), which after bisulfite addition react with toluidine blue at pH 1.0. The splited terminal glucose residue had remaind under topo-optical reaction too.

The double lipid layer of glycolipids and the sialic acide chains of glycoproteins have partially inhibited the periodic acid oxidation of the vicinal OH groups and oriented toluidine blue binding. After enzymatic treatment the topo-optical ABT-reaction is stronger positive for it cancells the inhibition (Makovitzky 1978). The membrane lipids play a role in orienting the dye molecules bound by the negative side group of the membrane protein structure. Digitalis glycosides and their related compounds have long been among the most important drugs used in the treatment of various forms of cardiac insufficiency. Consequently, the rats of intoxication are high. In spite of such intoxications the problem remains in the clinical and forensic medical practice (Dökert 1979; Greef 1981; Haustein et al. 1980; Shillingford 1980; Smith and Haber 1973; Sótonyi et al. 1981, 1982). Forensic medical aspects of cardiac glycoside intoxication plays a serious role in toxicology.

The exact laboratory determination is possible of cardiac glycoside by radioimmunoassay, but outside of ABT reaction there is no satisfactory morphological method for investigation of glycosides binding. The findings suggest that ABT reaction is suitable to both, molecular analysis digitalis molecular as well as for the histopathological examinations. The ABT reaction is a usefulle morphological method for the investigation of cardiac glycoside intoxication.

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