DETECTION OF INTERMEDIATE PRODUCTS OF OXIDATION OF LIPIDS IN TISSUE SECTIONS

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It is nowadays possible to detect certain intermediate products of lipid metabolism in tissue sections histochemically. This applies, in particular, to products of oxidation of lipids such as peroxides and aldehydes. The reactions of Schiff and of Glavind [5] are well suited to this purpose.

Schiff's reaction is widely used in histochemistry for the detection of substances capable of forming aldehyde groups as a result of certain forms of treatment. This group includes, in particular, substances such as deoxyribonucleic acid, acetalphosphatides and polysaccharides.

The property of restoring the color of the dye fuchsin-sulfuric acid (leukofuchsin) is possessed by bromine and by certain ketones [2, 3, 7, 12] in addition to aldehydes. The possibility of the participation of these substances in the reaction is, however, doubtful. The solution of this problem is important to the histochemistry of the ketosteroids. Oster [9, 11] is inclined to regard Schiff's reaction with ketones as a "pseudoreaction," because the product formed is rose pink in color and not the typical purple. At the same time Oster and Mulinos [10] have shown that the product of the reaction between tissue aldehydes and fuchsin-sulfuric acid can be decolorized with alkalis and the initial color restored with acids. This type of result is unattainable with the pink-staining product of combination of fuchsin-sulfuric acid with ketone. They suggest the procedure of successive alkalization and acidification as a test for the more precise identification of a substance detected by means of Schiff's reagent. Lison [8] pointed out some time ago that a reaction with fuchsin-sulfuric acid may also be given by unsaturated fatty acids and that this reaction involves ethylene groups. Subsequent investigations, however, have shown these claims to be unfounded. It has been shown that oleic and other unsaturated fatty acids are able to react with fuchsin-sulfuric acid only after preliminary oxidation and the formation of aldehyde groups [2, 11].

There are claims in the literature that the oxidation of unsaturated fatty acids, especially in the presence of light and heat, takes place relatively rapidly [6]. This arouses misgivings that the formation of aldehydes from unsaturated lipids may take place in the process of histological treatment of the material. In order to test this hypothesis we carried out the following experiments.

Pure oleic acid was injected subcutaneously into rats; three hours later the animals were sacrificed and the skin at the site of injection was excised, together with the subcutaneous cellular tissue and muscle, and immersed in 10% formalin. After fixation for 24 hours the material was rinsed in tap water and embedded in gelatin by the usual method. Sections were cut with the freezing microtome and Schiff's reaction carried out with them, after which they were stained with Nile blue sulfate.

In all cases the results of the Schiff reaction were negative. After staining with Nile blue, cavities containing oleic acid, staining a dense blue, were clearly seen in the subcutaneous fatty tissue. The experiments

thus showed that oleic acid does not give a reaction with fuchsin and sulfuric acid, and that in the process of the usual histological treatment and embedding in gelatin decomposition of oleic acid and formation of aldehydes do not take place.

Experiments in which Schiff's reaction was performed on sections of normal skin and subcutaneous cellular tissue also gave negative results. Consequently, the lipids of normal skin do not contain aldehydes, and in the process of histological treatment of the material aldehyde formation does not occur. Prolonged storage of the material in the air or in formalin leads to the formation of both carboxyl (giving a blue stain with Nile blue sulfate) and aldehyde groups (positive reaction with Schiff's reagent). The histochemical study of intermediate products of oxidation of lipids thus requires that the material used should be fresh or fixed in formalin for not longer than 24-48 hours.

Schiff's reaction has been used as an indicator of the decomposition of lipids in experiments in vitro by Fellenberg [4], Inikhov [1] and others.

Glavind's [5] method has been suggested comparatively recently, and it consists of the oxidation of leuko-2-6-dichlorophenolindophenol with tissue peroxides with the formation of indophenol, which is readily soluble in lipids and is shown by its red color. To accelerate the reaction a solution of hemin in a mixture of pyridine and glacial acetic acid is used as a catalyst. Leuko-2-6-dichlorophenolindophenol is prepared from sodium 2-6-dichlorophenolindophenolate by reduction with ascorbic acid.

This method has been tested by us in vitro with hydrogen peroxide, with oleic acid, with a solution of oleic acid in ethyl alcohol, with formaldehyde, and also in tissue sections from rats receiving preliminary subcutaneous injections of oleic acid before sacrifice. The problem of whether unsaturated fatty acids and aldehydes may give a positive reaction was of great interest, for it indicated the specificity of the method. The results obtained are given in the table.

Comparison of the Results of the Reaction of Fatty Acids, Aldehydes and Peroxides with Glavind's Reagent and with Sodium 2-6-Dichlorophenolindophenolate

Material	Results of reaction	
	with Glavind's reagent	with sodium 2-6-dichlorophenolindophenolate
Hydrogen peroxide	Red	Red
Oleic acid	Not stained	Red
Solution of oleic acid in ethyl alcohol	Not stained	Red
Formalin	Not stained	Blue
Oleic acid injected subcutaneously	Not stained	Red

Of all the substances which we investigated, only hydrogen peroxide thus gave a stain. Neither oleic acid, as representative of the unsaturated fatty acids, nor formalin, as representative of the aldehydes, gave staining with Glavind's reagent. The findings that Glavind's method reveals peroxides but not aldehydes and acids are also confirmed by model experiments in which we compared the results of staining the lipids of the skin and subcutaneous cellular tissue after preliminary oxidation by means of three methods: Nile blue sulfate, fuchsin-sulfuric acid and Glavind's method.

During microscopic investigation of this material it may be observed that the distribution of the areas giving a positive reaction with each of these methods does not always coincide.

We know that 2-6-dichlorophenolindophenol is an indicator giving a red color in an acid medium and a blue color in an alkaline. Analysis of our findings (see table), however, shows that the acid-alkaline color changes

characteristic of 2-6-dichlorophenolindophenol are by no means so of its leuko-form (Galvind's reagent). Consequently, the color changes observed cannot be attributed to changes in the pH of the medium.

When Galvind's reaction is carried out with normal human skin, the fat cells of the dermis and the subcutaneous cellular tissue acquire a weak pale pink color. This weak staining is evidently the result of the presence of a small quantity of peroxides, formed in the lipids during the time elapsing since the moment of death. The fat cells of the skin and of the subcutaneous cellular tissue of the rat, investigated directly after decapitation of the animal, do not show this coloration.

Our observations showed that it is unnecessary to obtain the pure leuko compound in a dry form. In the reaction it is quite suitable to use solutions of this compound obtained ex tempore by the reduction of an alcoholic (50° alcohol) solution of sodium 2-6-dichlorophenolindophenolate by means of addition of ascorbic acid (also in the form of a solution in 50° alcohol). To preserve the relative proportions of the individual ingredients in the reagent mixture as recommended by the authors, it is better to use a 0.3% solution of sodium 2-6-dichlorophenolindophenolate. The concentration of ascorbic acid is not of fundamental importance, but it must be high enough to produce minimal dilution of the mixture as a result of its addition.

According to the results of the authors of the method [5], excess of ascorbic acid may retard the reaction, so that addition should be made carefully, a drop at a time, precisely to the moment when the blue-green color changes to brown. To 4 ml of the resulting mixture is added 0.35 ml of a previously prepared solution of hemin in pyridine and glacial acetic acid (hemin 40 mg, pyridine 10 ml, glacial acetic acid 20 ml; this solution is stable and may be kept for a long time).

Sections are laid out on slides and dried with filter paper to remove excess of water, in order to prevent loss of hemin. The mixture of reagents is poured onto the sections for 3-5 minutes. The sections are then thoroughly washed in distilled water and examined immediately (they may be mounted in glycerol), for the reaction is unstable and the color disappears in about one hour. Material containing peroxides gives a red color.

SUMMARY

Schiff's reaction (for aldehydes) and Glavind's reaction (for peroxides) may be used for histochemical detection of intermediate products of fat oxidation. The question of the specificity of these methods is discussed and the technique of conducting these reactions on tissue sections is presented on the basis of literature data and the author's investigations.

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