that swelling of mitochondria is connected with the mechanism of coupling the electron transfer to the synthesis of ATP.

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SC 2319

A modified benzidine method for the chromatographic detection of sphingolipids and acid polysaccharides

Many sphingolipid and acid polysaccharide molecules of current biological interest can be detected by virtue of the reaction of their secondary amide group. When the Cl-substituted derivative of this amide is prepared and then combined with benzidine, a blue reaction product is formed[1] (Fig. 1). The modified method to be described differs chiefly from the foregoing method in two respects:

(1) The color reaction is more sensitive in practice (down to 5–10 μg).

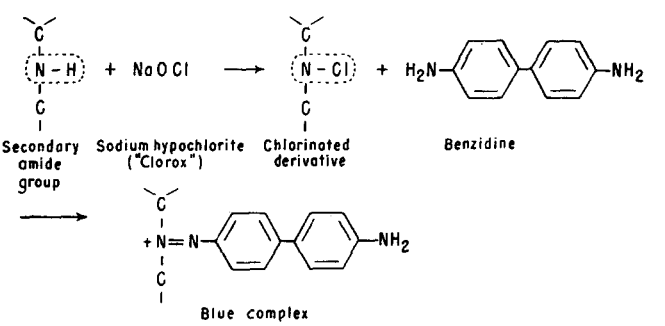

Fig. 1. One schematic simplification of the "Clorox–benzidine" method for detection of secondary amide groups. (This is but one of several theoretical possibilities and is thus neither the only possible nor the only correct reaction.)

(2) The color reaction is more stable. Using the present technique, sphingolipids and acid polysaccharides have been stained and differentiated both by thin-layer chromatography and on paper chromatograms.

Thin-layer chromatograms were run on silica gel G, and paper chromatograms were run on Whatman No. 1 using a variety of standard solvent systems. The chromatograms are first dried in a ventilated hood and then sprayed with the "Clorox" reagent. This reagent was found empirically to give the best results. It is prepared by adding 5 ml of commercial "Clorox" brand bleach* to 50 ml benzene, analytical grade, and then by adding 5 ml glacial acetic acid. The "Clorox" reagent must be used immediately. After spraying, air drying in the hood suffices to remove all unbound Cl_2 from thin-layer chromatograms. However, paper chromatograms are placed in tap water for two rinses of 1–2 min duration, and are then allowed to air dry until damp.

Chromatograms are then sprayed with the benzidine reagent. To prepare this, 0.5 g benzidine (Matheson, Coleman and Bell), and one small crystal of KI are dissolved in 50 ml of 50% ethanol and then filtered. This solution is kept out of direct light both during filtration and storage and is used within 2 h after preparation. Alternatively, paper chromatograms may be dipped in the benzidine reagent.

The technique described above is used to identify sphingolipids. For acid polysaccharides, there are only two minor modifications. Water is substituted for benzene in the "Clorox" reagent. Paper chromatograms are rinsed in 95% ethanol instead of water. For best results, it is important to avoid exposure of the test materials either to NH_3 or to other alkali vapors both before and after staining. It may be noted that "Clorox" brand commercial bleach was the only product of a number tested which served as a satisfactory chlorine donor. This bleach contains 5¼% NaClO, is essentially free of caustic, contains no stabilizer, has a lower pH, and a higher oxidation potential than have other commercial sources of NaOCl (ref. 4).

Reacting molecules yield a distinct medium-dark blue color against a white background with thin-layer chromatography. On paper chromatograms the blue is seen against a pale yellow background. Relevant purified control molecules which did not contain a secondary amide group gave no reaction. The sensitivity for sphingolipids is in the order of 5–10 μg in a single application spot 0.7 cm in diameter and 5–10 μg for a chromatographed spot on thin-layer chromatograms. Sensitivity for a chromatographed spot on paper is 15–20 μg. The following sphingolipids were tested and all gave a blue color: cerebronylceramide, *N*-palmitoyldihydrosphingosine, phrenosine (cerebron), kerasin, cerebronhydrate, cerebron sulfuric acid ester (sulfatide), and sphingomyelins. Gangliosides stained on thin-layer chromatograms only.

When acid polysaccharides are stained on paper chromatograms, the color intensity varies with the nature of the compound tested. Thus, hyaluronic acid, sulfated hyaluronic acid, and chondroitin sulfate B form one group of acid polysaccharides staining only a very pale blue, whereas chondroitin sulfates A and C stain a more intense light blue, and heparin and heparitin sulfate stain the most intense dark blue color. When cellulose powder is used for thin-layer chromatography, the staining differences are even greater. For example, the chondroitin sulfates and hyaluronic acids give no reaction, whereas heparin and heparitin sulfate spots stain an intense blue. In this regard, it may be of interest that heparin and heparitin sulfate

* The Clorox Co., 850 42nd Avenue, Oakland 1, Calif. (U.S.A.).

contain *N*-sulfated functional groups. By contrast the hyaluronic acids and chondroitin sulfates have only *N*-acetylated functional groups[2,3]. The observation that different acid mucopolysaccharides have different staining intensities may be of some value in distinguishing among acid mucopolysaccharides which have similar R_F values, particularly after thin-layer chromatography.

The blue color reaction has remained stable on paper strips for at least 1 year during which the strips were protected from direct light. The blue color may also be preserved on thin-layer chromatograms if the thin layer is first treated with a plastic coating (Brinkmann Instruments, New York), then removed with transparent plastic tape, and finally stored out of direct light. Studies are now in progress to determine whether the color intensity of various sphingolipids can be related to molar quantities.

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Comparative chromatographic and dissociative behaviour of avian and amphibian lipovitellin*

The sedimenting granules in hen's egg yolk and the platelets of frog's eggs both contain a phosphoprotein (phosvitin) and a lipoprotein (lipovitellin). The corresponding proteins from both species are similar in several respects but they may not be identical[1]. Lipovitellin from avian egg yolk has been separated into two components, α- and β-lipovitellin, by column chromatography on hydroxyapatite, and both have been shown to dissociate into two subunits in alkaline solvents[2,3]. Apart from their differential affinity for hydroxyapatite (β- eluted at a phosphate buffer concentration of 0.6 M, α- at 1.1 M), these two avian lipovitellins can be distinguished by their protein phosphorus content (α- has 0.6%, β- 0.3%) and the pH required for equivalent dissociation (α- requires pH 10.5 and β- pH 7.8 to produce 50% dis-

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