A SPECTROPHOTOMETRIC METHOD OF SELECTING ORCEIN DYES FOR HISTOLOGICAL DEMONSTRATION OF HEPATITIS B SURFACE ANTIGEN¹

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Spectrophotometric measurements were made on to quantitate batch variations of orcein dyes. Three types of spectra were observed among eleven batches tested. Four showed bimodal curves with a peak absorption at 520 nm and smaller peak at 595 nm (Type A). Five showed a single peak at 520 nm (Type B) and two synthetic orceins gave a single broad peak at 510 nm (Type C). Modified Shikata's orcein stain for hepatitis B surface antigen was tested with these reagents and large variations in staining capacities were noted. An immunoenzyme method was performed for confirmation and comparison of orcein reaction. Type A orcein gave the best results and staining specificity was found to increase in proportion to the amount of absorption at 595 nm. Results of staining with good batches of orcein were equally satisfactory with immuno-peroxidase staining. The latter method being expensive and time-consuming, orcein staining was found to be method of choice and a spectrophotometric analysis may facilitate choice of the batches.

For histological studies of hepatitis B surface antigen (HBs) Shikata (7, 8) developed an orcein staining which was easy to perform and applicable to routine paraffin embedded materials. Since the procedure had such a technical simplicity, it was rapidly utilized in many institutes on a routine basis (5). The method was found, however, to be less specific and less sensitive than immunofluorescent (8) or immuno-peroxidase staining (1) when tested in some laboratories. Lipofuscin, ceroid pigments and bile plugs were frequently stained. Heavy background coloration obscured weak positive cytoplasmic staining.

To resolve the nonspecificity of the staining we examined batch variations of the dyes and tried to correlate the staining qualities with the results of spectrophotometric data in an effort to develop a rapid method of selecting the most suitable orcein. For confirmation of the orcein-positive reaction an immunoperoxidase technique was adapted.

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MATERIALS AND METHODS

Orcein Eleven batches of orcein purchased from five different manufacturers were studied. The batch data are listed in Table 1. #2 dye solution was prepared from #1 dye in the list and has been used in our laboratory for the past two years. #11 and 12 were synthetic orcein.

Specimens Five paraffin blocks of cirrhotic liver obtained at autopsy or biopsy were employed. Four of them were seropositive and one negative for HBs. All specimens were fixed in phosphate-buffered 10% formalin, embedded and sectioned in the usual way.

Modified Shikata's orcein staining

Solutions

Acid orcein solution: 0.5 g of each orcein was dissolved in 100 ml of 70% ethyl alcohol. To acidify the solution 1.3 ml of concentrated hydrochloric acid was added.

Oxidizing solution: 0.3 ml of concentrated sulfuric acid was mixed with 100 ml of stock 0.15% potassium permanganate solution.

4% Sodium bisulfite solution.

Procedure

- 1) Deparaffinize and bring sections to water.
- 2) Oxidize in oxidizing solution until sections turn brown for two and half min.
- 3) Reduce the sections in 4% sodium bisulfite solution for twenty sec. Sections should be pale white at the end of treatment.
- 4) Rinse well in distilled water and transfer slides to the staining solution and allow to stand for two hr.
- 5) Remove excess staining solution in water and then briefly in 70% alcohol and further dehydrate in absolute alcohol.
- 6) Clear in xylene and mount.

Our procedure is a slight modification of Shikata's orcein stain (7, 8). Counter-stain was usually avoided for the sake of staining cleanliness. When desired, short dips in Mayer's hematoxylin (10–20 sec) or Giemsa stain after Pinkus and Hunter (6) were performed.

Immunoperoxidase technique An unlabeled immunoenzyme stain after Afroudakins (1) was performed. Rabbit antiserum to HBs was obtained commercially from the Hoechst Company, W. Germany. Goat anti-rabbit Ig G was prepared by the MBL Company, Nagoya, Japan. Cross reactions to human, swine and sheep serum proteins were abolished by the appropriate absorption. Anti horseradish peroxidase serum from rabbits was purchased from the MBL Company. Other reagents and procedure are described in the literature cited above.

Spectrophotometric measurement A part of each orcein dye solution was further diluted with 1.3% hydrochloric acid in 70% ethyl alcohol to obtain an optimal absorption pattern. Absorption curves were recorded with a Hitachi digital spectrophotometer type 624 and type 056 recorder. (Hitachi Company, Tokyo, Japan)

RESULTS

Using our modified orcein staining HBs antigen was demonstrated in the cytoplasm of the liver cells as perinuclear inclusion bodies or as peri-sinusoidal diffuse staining. The quality of stain, however, differed significantly from batch to batch. Staining specificity and intensity were compared with the results of an immunoperoxidase staining (Fig. 1). Degree of nonspecific background coloration of the cellular cytoplasm, lipofuscin, ceroid pigments, hyaline bodies and erythrocytes was examined.

Finally, the quality of the orcein was classified as either A) excellent, B) fair, or C) poor. The results are shown in Table 1. The best dye among those tested was #1 dye, which stained the virus antigen brilliantly with almost no background staining. #2 orcein, a two-year-old solution which had been used for staining hundreds of biopsy slides, proved to be equally satisfactory. #7, 8 and 10 stains could achieve optimal staining intensity with minimal background coloration, but they stained lipofuscin, ceroid and hyaline bodies. Synthetic orceins also gave minimal non-specific staining but the virus inclusions were stained weakly. The rest of the dyes stained HBs inclusions intensely but caused intense background and nonspecific staining. A nonspecific reaction produced with the poorer grade orcein made the recognition of the weak peri-sinusoidal virus staining difficult since the peri-sinusoidal inclusions were devoid of sharp outlines (Figs. 2 and 3).

Three types of absorption spectra were observed by the spectrophotometric analysis (Fig. 4). Type A had a spectrum with a maximum absorption at 520 nm and a smaller peak at 595 nm. It was obtained with #1, 2, 7, 8 and 10 orceins. The amount of relative absorption at 595 nm wavelength varied from batch to batch and was found to closely parallel the lack of non-specific staining. Type B was monophasic curve with a peak at 520 nm. It was recorded with #3, 4, 5, 6 and 9 dye solutions. Type C spectra with a broad peak absorption at 510 nm characterized

TABLE 1. *Batch data, summary of absorption spectrum analysis and classification of the staining quality of orcein dyes.*

#	Source and batch data	type of absorption spectrum	staining quality
1.	E. Merck, Batch 7396154	type A	A
2.	Two year old #1 dye solution	type A	A
3.	E. Merck, Batch 4088933	type B	C
4.	E. Merck, Batch 5226406	type B	C
5.	E. Merck, Batch 4143850	type B	C
6.	E. Merck, Batch 6312091	type B	C
7.	Wako Junyaku, LG 8372	type A	B
8.	Nakarai Kagaku, M6G 4718	type A	B
9.	Wako Junyaku, DMK 5455	type B	C
10.	BDH Chemicals, Batch 2466870	type A	B
11.	Chroma Gesellschaft, 5A 238	type C	C
12.	Tokyo Kasei, AG 01	type C	B

Staining quality: A-excellent, B-fair, C-poor. See the text for classification of the absorption spectra.

FIG. 1. HBs inclusion bodies in hepatocytes stained by an unlabeled immunoperoxidase method. The dark material represents peroxidase reaction products deposited at the sites of HBs antigen. $\times 160$

FIG. 2. Perinuclear cytoplasmic inclusions of HBs antigen stained with #1 (left) and #5 (right) orcein. Photomicrographs are taken from similar areas in adjacent sections. $\times 320$

FIG. 3. Peri-sinusoidal diffuse inclusion bodies of HBs antigen stained with #2 (left) and #6 (right) orcein. Photomicrographs are from similar areas in adjacent sections. Absence of background staining with #2 dye makes a clear recognition of antigenic distribution possible. $\times 320$

FIG. 4. Spectrophotometric absorption curves for the three types of orcein. Types A, B, and C are represented by the spectra for #1, 5 and 11 respectively.

#11 and 12 synthetic orceins.

DISCUSSION

Our modification of Shikata's orcein stain was to ensure the adequate oxidation

of sections and to reduce nonspecific staining. It has been proved useful for the demonstration of the HBs antigen in liver cells when select batches of the dyes are employed. Although an immunoperoxidase technique gives slightly more intense staining, the performance of a good orcein almost equals that of the immunological method. Even the smaller amounts of antigen located at the peripheral portions of the hepatocyte cytoplasm could be detected when no background coloration is present.

Natural orcein is derived from an extract of lichens occurring in semitropical countries and synthetic orcein is manufactured from orcinol (2, 4). It has been used as an elastic tissue stain or chromosome stain in acid alcohol or in acetic acid mixture. The exact formula for both types of orcein, however, is not known (4). Considerable variability in composition and staining quality has been reported (3) and the standardization of the stain is considered difficult (2). Chromatographic analysis by Engle and Dempsey (4) showed both natural and synthetic orcein could be separated into four fractions with different light absorption patterns. They also ascertained the differences in the staining ability of the fractions. Spectrophotometric studies of orcein have been made by some investigators (3) to reveal any significant difference between individual dye batches. But no difference that can account for the variations in the staining properties was found. We used this assay method and compared the results with the capabilities of dyes to react with HBs.

We found that a bimodal absorption curve with peaks at 520 and 595 nm is a characteristic of a fine quality of the dye and the amounts of absorption at 595 nm wave length parallel its good performance. These findings will facilitate the choice of better batches of orcein and may provide a clue for the constant production and standardization of a good orcein for the histological demonstration of HBs.

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