LOCALIZATION OF CYTOCHROME b_5 IN RAT ORGANS AND TISSUES BY IMMUNOHISTOCHEMISTRY*

M. Tavassoli, J. Ozols, G. Sugimoto, K.H. Cox, and U. Muller-Eberhard
Departments of Biochemistry and Hematology,
Scripps Clinic and Research Foundation
La Jolla, California 92037
and
Department of Biochemistry
University of Connecticut
Farmington, Connecticut 06032

Received July 12,1976

We employed an immunohistochemical technique for a study of the localization of cytochrome b_5 in paraffin-embedded tissues of the rat. The presence of cytochrome b_5 was limited to the centrilobular areas of the liver, the distal tubules and the collecting ducts of the kidney and the absorptive cells of the villi in the small intestine.

Cytochrome b_5 has been reported to be present in many tissues (1), but the evidence is invariably based on the spectral properties of this protein. Recently, however, the occurrence of proteins in mammalian tissues with spectral characteristics indistinguishable from those of cytochrome b_5 has been recognized. One such protein has been isolated from porcine kidney (2). This preparation has a molecular weight of 120,000 and cannot be reduced by NADH-cytochrome b_5 reductase purified from bovine or human liver (Ozols and Gerard, unpublished results). Another example of a cytochrome b_5-like protein is the bovine liver arylsulfatase. Cohen and Fridovich observed that sulfite oxidase, partially purified from acetone powder of bovine liver, exhibits an absorption spectrum which is almost identical with microsomal cytochrome b_5 (3). This preparation, however, is not reduced by microsomal NADH-cytochrome b_5 reduc-

*This work was supported by USPHS Grants # AM-16501 (MT), NP-134 (JO), CA-17735, HD-04445 (UME) and RCDA #AM-70551 (MT).

tase (Ozols, unpublished results). Baker's yeast lactate dehydrogenase also shares the spectral properties of cytochrome b_5 as well as similarities in amino acid sequence of the heme binding peptide, yet they do not cross-react immunologically or possess microsomal cytochrome b_5 functions (4). These observations indicate that spectral evidence per se is insufficient for documenting the presence of hemoprotein with cytochrome b_5 function in a given tissue. Therefore, we initiated immunological studies in an attempt to verify the presence of and to study the tissue localization of cytochrome b_5.

In a previous communication, we described an adaptation of the immunoperoxidase method for the demonstration of cytochrome b_5 and hemopexin in formalin-fixed paraffin embedded sections of rat liver (5). With a modification of this method, we have now examined the localization of cytochrome b_5 in various tissues in the rat.

MATERIALS AND METHODS

Preparation of cytochrome b_5 and its antibody:

Cytochrome b_5 was purified from rat liver. The preparation contained *ca.* 150 amino acid residues and a blocked NH_2-terminus (6). Two antisera were developed in goats by injecting 2 separate preparations of cytochrome b_5. The antisera were absorbed with lyophilized rat serum to remove possible contaminating antibodies (7), and the antibody containing IgG fraction was isolated by column chromatography using Whatman DE52. Both antisera showed a single precipitation line over a wide range dilution of antigen and antiserum by double diffusion in 1.0% agarose in PBS* containing 0.2% cholate (pH 7.4). The sharpest antigen-antibody precipitate line was observed with undiluted antiserum and an antigen concentration of *ca.* 0.3 mg/ml. Furthermore, when the antiserum was reacted with cholate -solubilized rat liver microsomes, a single immunoprecipitate formed merging in complete identity with the purified cytochrome b_5-anti-cytochrome b_5.

Tissue preparation:

Tissues were obtained from Sprague-Dawley rats (200-300 g) and cut into small pieces and slices no thicker than 4 mm. They were fixed in 10% phosphate buffered formalin. After dehydration, they were embedded in paraffin and 5 μ sections were obtained and mounted on albumin-coated glass slides (8). The microsomes of liver, lung, spleen, and kidney were isolated in 0.25 M sucrose, solubilized in cholate (personal communication, E.F. Johnson) and tested for the presence of cytochrome b_5 by double diffusion in agarose.

*Phosphate buffered saline, PBS; horseradish peroxidase, HRP; horseradish peroxidase-antiperoxidase immune complex, PAP; gamma globulin, IgG.

Immunoperoxidase methods:

Two immunohistochemical methods were used for demonstration of cytochrome b_5 in tissue (9). In the "sandwich" method, anti-HRP antiserum was developed in a goat and the horseradish peroxidase-antiperoxidase immune complex was prepared by adding equivalent amounts of HRP and anti-HRP. The immune precipitate was then solubilized by additon of excess peroxidase according to the method described by Sternberger (10). This soluble PAP complex is homogenous with a 3:2 ratio of HRP to anti-HRP. Donkey anti-goat IgG, purchased from Miles Laboratories and rabbit anti-goat IgG prepared in our laboratory was used to "sandwich" with the PAP*complex and the cytochrome b_5 antiserum. Deparaffinized sections were sequentially treated with goat anti-rat cytochrome b_5, rabbit anti-goat IgG and PAP. Each step consisted of a 60 minute incubation at room temperature in a moist box with 1 drop of antiserum per tissue section, followed by a 30 minute wash in PBS at 37° and a 5 minute drying period.

For the conjugated peroxidase method, HRP-conjugated rabbit anti-goat IgG was purchased from Miles Laboratory. Treatment with the conjugate then replaced the last two steps in the "sandwich" method.

Control experiments:

Cytochrome b_5 antiserum was absorbed with increasing amounts of antigen and the absorbed antisera were used in parallel experiments. In addition, experiments were carried out in which normal goat serum or PBS replaced the anti-cytochrome b_5 antibody.

Peroxidase staining and blocking of endogenous peroxidase:

Benzidine base or 3'-3' diaminobenzidine were used as substrate for staining HRP, followed respectively by counterstaining with Mayer's Haemalum or methyl green. Endogenous peroxidase activity was blocked with either cold methanol (containing 0.01% H_2O_2) for 20 minutes or by a substrate different from the one used for staining HRP, as previously described (11, 12).

RESULTS AND DISCUSSION

The cholate-solubilized microsomes of the liver but not those of kidney, lung, or spleen showed a precipitation line when developed with the anti-cytochrome b_5. With the immunoperoxidase technique, the presence of cytochrome b_5 could be identified in sections of liver, kidney and small intestine. No staining was observed in sections of pancreas, colon, testes, ovary, adrenal gland, striated and cardiac muscles, lung, spleen, or bone marrow. Nonspecific background staining was found particularly in lung, spleen and other collagen containing organs. The staining pattern was, however, light and diffuse and could easily be differentiated from the dark,

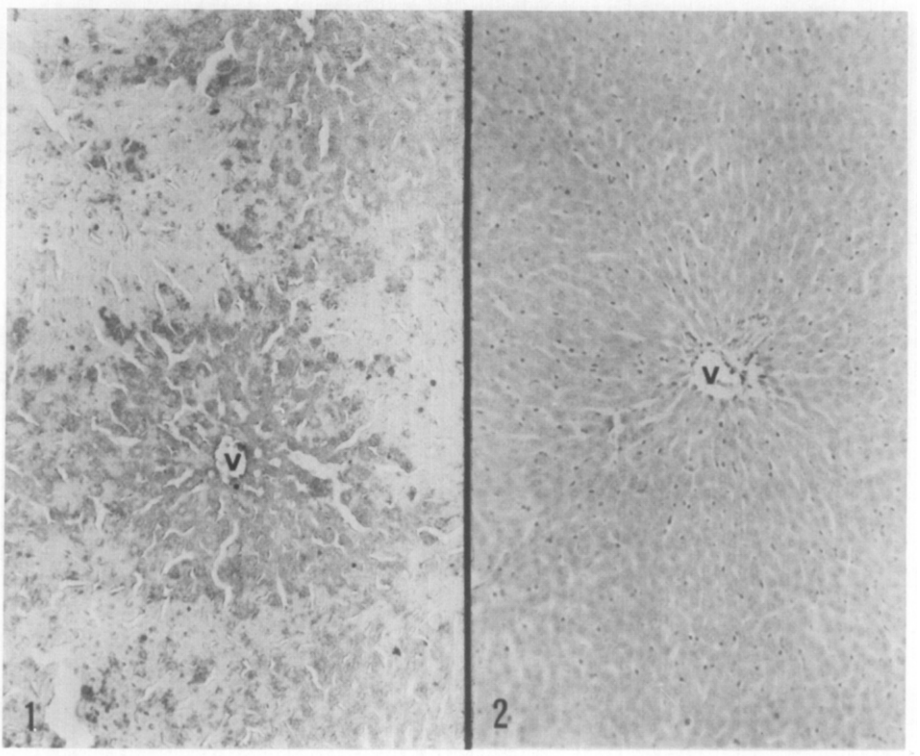

Figure 1: Immunoperoxidase distribution of cytochrome b_5 in rat liver. Note the centrilobular location of protein antigen. Portions of 2 hepatic lobules are shown and one of the central veins is identified (V). DAB substrate with methyl-green counterstain. The photograph was taken with a red Kodak Wratten filter #25 (A) from a color photograph.

Figure 2: Control experiment in which goat anti-rat cytochrome b_5 antibody is replaced by normal goat serum. Specifications as in Figure 1.

well-delineated specific staining observed in liver, kidney and small intestine. The non-specific staining of collagen has been reported by others and is believed to be the result of non-specific linkage of antibody to the charged collagenous elements of connective tissue (12).

In the liver, the distribution of cytochrome b_5 was limited to centrilobular areas (Figs. 1,2). The distal tubules of the kidney and the collecting ducts stained specifically, the distal tubules giving a particularly strong reaction (Fg. 3). Glomeruli and proximal tubules did not stain. The

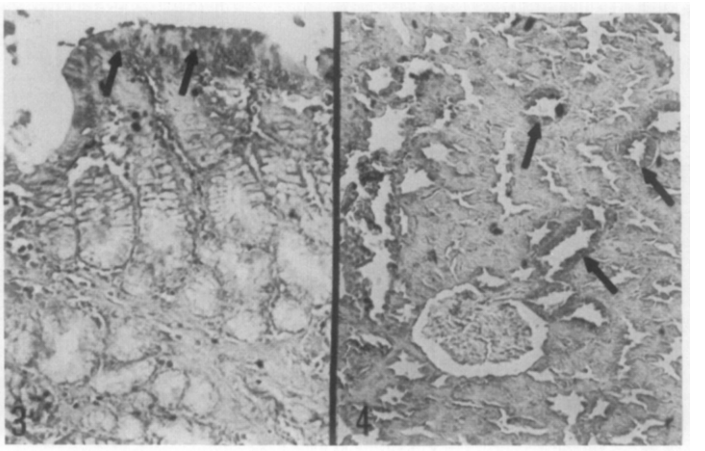

Figure 3: Immunoperoxidase distribution of cytochrome b_5 in rat jejunal mucosa. Note the positive reaction (arrows) in the absorptive cells. The Brunner glands do not react with the stain. Benzidine substrate with neutral red counterstain. A green Kodak Wratten filter #11 (X1).

Figure 4: Distribution of cytochrome b_5 in renal cortex. Note the positive reaction in the distal tubules (arrows). A glomerulus and the proximal tubules are unreactive to the stain. Specifications as in Figure 3.

distribution of protein antigen in the small intestine was limited to the absorptive cells of the villi with an increasing gradient from the base toward the tip (Fig. 4). Brunner glands, goblet cells, argentaffin cells and Paneth cells did not react with the immunoperoxidase stain.

Both the "sandwich technique" and HRP-conjugated antibody technique gave similar results. The conjugated antibody technique was considerably less time-consuming and equally sensitive.

Control sections in which specific anti-b_5 antiserum was replaced by normal goat serum, showed background staining in collagen containing organs but no specific reaction was seen. This background staining was not seen with PBS and was increasingly reduced by gradual dilutions of normal goat serum. Absorption of the specific antiserum with increasing amounts of cytochrome b_5 antigen eliminated the specific reaction.

Cytochrome b_5 is an essential component of the hepatic microsomal electron transport system which catalizes the desaturation of fatty acids and under certain conditions may participate in the hydroxylation and N-demethylation of a wide variety of endogenous and exgenous lipid-soluble compounds. Previous attempts to study its distribution in tissues have utilized its spectral properties which can no longer be considered the unique property of this protein (2,3). Immunohistochemistry has recently been found promising for the identification and localization of tissue protein antigens. The application of immunohistochemistry for localization of cytochrome b_5 has been shown by electron microscopic observations of Remacle et al (13). They used hybrid antibodies labeled with ferritin to demonstrate the association of cytochrome b_5 exclusively with the smooth endoplasmic reticulum of rat hepatocytes.

We have previously reported on the feasibility of using the immunoperoxidase technique to demonstrate the localization of this protein in paraffin embedded sections (5). We now have extended these studies to other tissues of the rat. Our findings indicate that the presence of cytochrome b_5 is limited to the liver, kidney and intestinal tissues.

Our failure to demonstrate whether this protein can be demonstrated in the tissues may depend on these 3 factors: 1) the amount of cytochrome b_5 in some tissues may be below the level of the sensitivity of the method applied, 2) the structure of cytochrome b_5 from various tissues may differ sufficiently so that no immunological cross-reactivity could be observed with this technique, 3) the proteins with spectral characteristics of cytochrome b_5, reported in other tissues may not indeed be the liver microsomal cytochrome b_5. The recognition that several other proteins may have spectral characteristics similar to those of cytochrome b_5, has strengthened this last possibility.

REFERENCES

1. Strittmatter, P. (1967) Methods Enzymol. 10, 553-556.
2. Mangum, J.H., Klingler, M.D., and North, J.A. (1970) Biochem. Biophys. Res. Commun. 40, 1520-1525.
3. Cohen, H.J., and Fridovich, I. (1971) J. Biol. Chem. 246, 367-373.
4. Guiard, B., Lederer, F., and Jacq, C. (1975) Nature 255, 422-423.
5. Muller-Eberhard, U., Yam, L., Tavassoli, M., Cox, K., and Ozols, J. (1974) Biochem. Biophys. Res. Commun. 61, 983-988.
6. Ozols, J. (1975) In: Solid-Phase Methods In Protein Sequence Analysis, R.A. Laursen, ed., Pierce Chemical Company, 211-228.
7. Vladutiu, G.D., Bigazzi, P.E., and Rose, N.R. (1973) J. Histochem. Cytochem. 21, 559-567.
8. Lillie, R.D. (1965) Histopathological Technic and Practical Histochemistry, p. 371, 3rd ed., McGraw-Hill, London.
9. Avrameas, S. (1970) Intern. Rev. Cytol. 27, 349-385.
10. Sternberger, L.A., Hardy, P.H., Jr., Cuculis, J.J., and Meyer, H.G. (1970) J. Histochem. Cytochem. 18, 315-333.
11. Burns, J., Hambridge, M., and Taylor, C.R. (1974) J. Clin. Path. 27, 548-557.
12. Taylor, C.R., and Burns, J. (1974) J. Clin. Path. 27, 14-20.
13. Remacle, J., Fowler, S., Beaufay, H., and Berthet, J. (1974) J. Cell Biol. 61, 237-240.