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Studies on the Distribution and Immunohistochemical Localization of Xanthine Oxidase in Bovine Tissues

(xanthine oxidase/enzyme distribution/fluorescent antibody)

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Distribution of xanthine oxidase activity in bovine tissues was investigated by biochemical assay and cellular localization of the enzyme was studied using a fluorescent antibody technique. XOD activity was detected in the homogenate of liver, spleen, duodenum, lung and kidney. Liver and spleen had at least five times higher activity. Fractionation of liver homogenate revealed the presence of enzyme activity only in the high speed supernatant fraction. Immunochemical properties of XODs in the soluble fractions from each organ homogenate were studied using antibody to milk XOD. The enzyme in these organs and that from milk revealed complete immunological identity. Immunohistochemically, in cryostat sections fixed with a strong fixative such as Carnoy's solution etc. the enzyme was localized as follows : Liver ; in the cytoplasm of hepatic cells. Duodenum ; in the apical portion of Brunner's gland epithelium. Kidney ; in some of the epithelium of the proximal tubuli. Mammary gland ; in the apex of alveolar epithelium and in the margin of alveolar spaces. Although the choice of the fixatives for immunohistochemical studies was empirical, possible reasons for lack of effect with fixatives employed in the present study are discussed.

We have studied the demonstration of oxidoreductases in mammalian tissues using immunohistochemical methods (1, 2, 3, 4). Utilizing fluorescent antibody technique and immunochemical methods not only the localization of the enzymes in tissues but also their organ specificty (3) or the differential distribution of the isozymes within a tissue (4) was clearly revealed as there is a high degree of specificity of antigen-antibody reaction.

Xanthine oxidase (XOD*), a flavin enzyme containing flavin adenine dinucleotide, iron and molybdenum, catalyzes the oxidation of hypoxanthine to xanthine and the latter to uric acid (see 5 for review). It also functions in the release of iron from liver ferritin to the serum iron-binding protein, transferrin (6) and may play a role in other types of iron transfer. It is of interest to examine the tissue localization of this enzyme in healthy and disease states correlated with its function. Methods for histochemical demonstration were reported by Bourne (7) and Sackler (8) using various tetrazolium com-

* Abbrebiations used : XOD ; xanthine oxidase, PBS ; phosphate buffered saline, AHP ; 2-amino-4-hydroxypteridine, FITC ; fluorescein isothiocyanate, AD ; antigenic determinant.

pounds. The histochemical reaction, however, varied with the different tissues examined as did the fixatives employed (7, 8, 9). Moreover, we did not confirm the specificity of the reaction because of the coloration on the tissue incubated in control medium, i. e. medium without substrate.

In the present paper distribution of the enzyme activity in bovine tissues and in subcellular fractions from the liver was studied using biochemical assay and immunological identification of xanthine oxidases found in each organ homogenates was determined. For the immunohistochemical demonstration of the enzyme in bovine tissues, effect of various fixatives was examined in frozen sections.

MATERIALS AND METHODS

All chemicals were of analytical grade and were not purified further. All were from Nakarai Chemical Co. Kyoto, unless otherwise stated.

Biochemical Assay of Enzyme Activity in Organ Homogenates and in Subcellular Fractions

Approximately 15 g of fresh tissues from bovine liver, spleen, duodenum, lung and kidney were minced with a pair of scissors and homogenized in a Waring blender in 2 volume (w/v) of ice-cold medium of the following composition : 0.25M sucrose, 0.01M $MgCl_2$ and 0.2M sodium phosphate buffer at pH 7.4. Each homogenate was passed through two sheets of gauze to remove tissue fragments. For the assay of XOD activity 0.1ml of homogenate or supernatant fraction (see below), which had been diluted ten times with phosphate buffer, was added to 1.9 ml of 0.2 M phosphate buffer, pH 7.1 and 1.0 ml of 5×10^{-6} M 2-amino-4-hydroxy pteridine (AHP). The increase in fluorescence (λ_{em} = 430 nm, λ_{ex} = 350 nm) which occurs when AHP is oxidized in phosphate buffer to isoxanthopterin was followed at 25°C for 30 min (10), for which the rate of oxidation was linear. One unit of enzyme activity was defined as the amount of enzyme that forms 10 μmoles of isoxanthopterin from AHP per hr at 25°C. The specific activity were expressed in terms of units per kg protein. Protein was determined in an aliquot of each sample by the micro-Kjeldahl method (11). All determinations were carried out in duplicate.

To study subcellular distribution of enzyme activity, the nuclei, mitochondoria, microsomes and supernatant fractions were prepared from liver homogenates as described by Sackler (8) and each fraction was tested for XOD activity.

Preparation of Antisera and Fluorescein Isothiocyanate-Labeled Conjugates

Antibody to the enzyme was obtained by injecting male albino rabbits with either XOD preparation from milk which was purchased from Boehringer Mannheim GmbH or its purified form obtained by preparative disc electrophoresis. The locations of the enzyme in an polyacrylamide gel were visualized by immersing the gel in a histochemical reaction mixture (see below for composition) (Fig. 1). A disc containing XOD was cut and homogenized with Freund s complete adjuvant (Difco Laboratories, Detroit, Mich.) to immunize

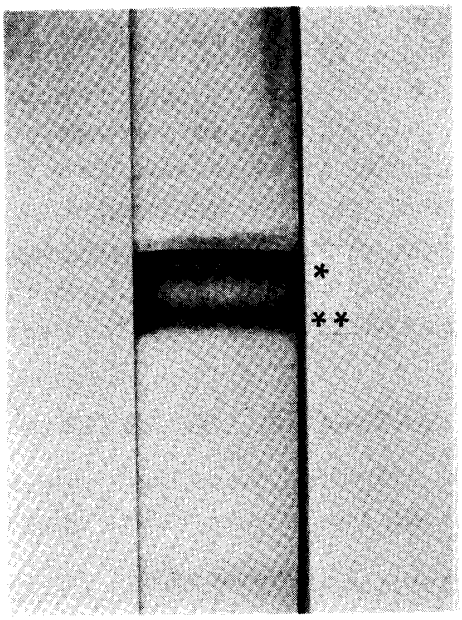

Fig. 1. Preparative disc electrophoresis of xanthine oxidase from milk on polyacrylamide gel. A gel was cast in a 5 ml glass syringe and electrophoresed for 3.5 hr at 6 mA/gel. The gel was incubated in a histochemical reaction mixture for enzyme activity. Two discs possessing enzyme activity were found (*, **). A fast moving band (**) was cut out and homogenized with FCA to immunize rabbits.

rabbits. Globulin fractions of the antisera were prepared by precipitating three times with one-third saturated ammonium sulfate. The globulins were then conjugated with fluorescein isothiocyanate (FITC) (Baltimore Biological Laboratory, Inc., Baltimore, Md.) (12). The conjugates were then purified by fractionation on DEAE-cellulose (0.94 mEq/g, Brown Company, Berlin, N. H.) column.

Assay for Specificity of Antisera

Specificity of antibody was examined by Ouchterlony's double gel diffusion and by immunoelectrophoresis. The precipitin lines formed in agarose plates were stained for XOD activity by incubating for 5—10 min in 0.2 M phosphate buffer at pH 7.4 containing 2.5×10^{-3} M hypoxanthine and 1 mg/ml nitro-blue tetrazolium. The plates were also stained with amino black 10B for protein.

Preparation of Sections, Staining with FITC-Labeled Conjugates and Fluorescent Microscopy

Frozen sections were prepared as described previously (13). They were fixed with one of the following fixatives at 0°C for 5—30 min : Carnoy's solution, 100% ethanol, 95% ethanol, 1% acetic acid in 95% ethanol (acidified ethanol), ether-ethanol (1 : 1), acetone, 10% buffered formalin, 2.5% glutaraldehyde in cacodylate buffer. After a thorough washing with phosphate buffered saline (PBS), they were stained with FITC-labeled anti-XOD conjugate for 12—16 hr at room temperature. After washing out the unreacted conjugate, the slides

were mounted in glycerol-PBS (9 : 1) and were then examined under a Carl Zeiss transmitted-light fluorescence microscope with a filter combination for UV excitation and No. 41 Schott s suppression filter for the observation of green fluorescence and tissue structure. The specificity of the staining was examined by either blocking test, i. e. sections were incubated with non-labeled antibody prior to staining with FITC-labeled conjugate, or staining with control conjugates which had no relation to the enzyme under study.

Test for the Preservation of Antigenic Determinants after Fixation

Gammaglobulin fractions of anti-XOD were insolubilized by cross-linking according to the method of Avrameas and Ternynck (14). Insoluble protein was washed with PBS several times and packed by centrifugation. The packed material was frozen and sectioned at 5—6 μ thickness in a cryostat. XOD was immobilized by antigen-antibody reaction on the thin sheets of anti-XOD. These XOD preparations were treated with one of the fixatives and processed in the same manner as the immunohistological tissue specimen to observe the intensity of fluorescence specific for XOD.

RESULTS

Biochemical Studies

XOD activity was detected in all tissues examined. As shown in the left half of Table I, liver and spleen homogenates had at least five times higher enzyme activities than did the duodenum, lung and kidney. Cell fractionation study (Table II) revealed that almost all enzyme activity in liver was recovered in the high speed supernatant fraction, i. e. supernanant obtained after centrifugation at 100, 600 g for 60 min. To see if such was the case in the other organ homogenates, enzyme activity in the supernatant fraction from each tissue was assayed and compared taking the liver XOD activity as 100% (the right

TABLE I. *Comparison of Xanthine Oxidase Activity in Homogenate and Soluble Fraction among Various Tissues*

Tissue	Homogenate		Soluble practice	
	Total activity (units*/kg tissue)	%	Specific activity (units/kg protein)	%
Liver	19	100**	660	100**
Spleen	22	116	550	83.3
Duodenus	1.3	6.8	91	13.8
Lung	2.8	14.7	180	27.3
Kidney	1.0	5.3	45	6.8

Enzyme activity was measured by fluorometry as described in Materials and Methods.

* One unit of enzyme activity was defined as the amount of enzyme that forms 10 μmoles of isoxanthopterin from 2-amino-4-hydroxy pteridine per hr at 25°C.

** Enzyme activity in homogenate or in soluble fraction of liver was taken as 100%.

TABLE II. *Intracellular Distribution of Xanthine Oxidase Activity in Bovine Liver*

Fraction*	Total activity (units/kg tissue)	Recovery (%)	Specific activity (units/kg protein)
Homogenate	15.6	100	416
Nuclei	0.32	2.1	61
Mitochondria	0.25	1.6	68
Microsome	0.33	2.1	53
Supernatant	14.7	94.2	660

* Subcellular fractions were prepared from bovine liver homogenate as described by Sackler (8).

half of Table I). As was observed in the homogenates, the spleen had as high activity (83%) as did the liver, whereas the other organs showed much lower activity (6.8—27.3%).

Immunodiffusion and Immunoelectrophoretic Studies

Antiserum (Ab I) procured by immunizing rabbits with XOD preparation (Boehringer) without further purification developed a contaminating immune precipitin line when tested on an agarose plate against either enzyme solution or normal bovine serum. The antiserum was incubated with insolubilized bovine serum to remove contaminating antibody. Fig. 2 shows that only one precipitin line was formed between the absorbed antiserum (Ab I′) and enzyme solution. No lines were formed between Ab I′ and bovine serum. When the

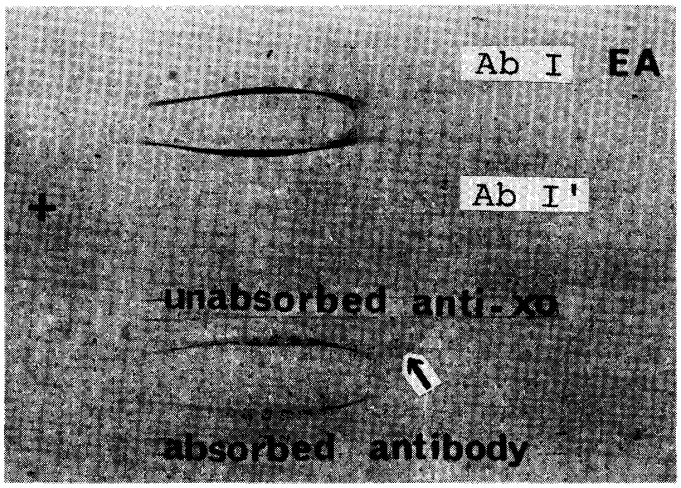

Fig. 2. Test for the specificity of Ab I and Ab I′. Xanthine oxidase (Boehringer) was electrophoresed simultaneously on two agarose plates. Unabsorbed antiserum (Ab I) and absorbed antiserum (Ab I′) was put into the upper and lower trenches, respectively. After forming precipitin lines and then washing with PBS, the upper slide (EA) was stained for enzyme activity and the lower for protein. Protein stain revealed a contaminating precipitin line (↑) overlapping the specific one between unabsorbed antiserum (Ab I) and XOD. Absorbed antiserum (Ab I′) formed only one specfic precipitin line as revealed by both protein and enzyme-activity stain.

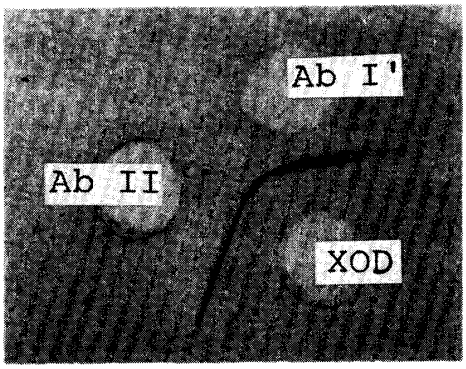

Fig. 3. Crude gammaglobulin preparations from absorbed anti-XOD (Ab I') and antiserum prepared by immunizing rabbits with XOD purified by polyacrylamide gel electrophoresis (Ab II) showed complete immunological identity. The precipitin lines formed between two antisera and XOD solution fused on an agarose gel plate.

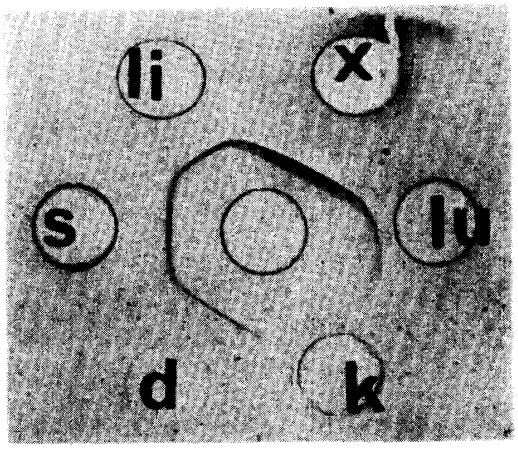

Fig. 4. Xanthine oxidases in supernatant fractions from various organ homogenates formed a single fusing precipitin line against anti- milk XOD (center well). This shows immunochemical identity of XODs of various sources with XOD from milk and with each other. XOD from milk (well X), liver (li), spleen (s), duodenum (d), kidney (k) and lung (lu). A faint precipitin line formed between kidney XOD and anti-XOD was not reproduced photographically.

plate was immersed in a histochemical reaction mixture, the precipitin line revealed enzyme activity (Fig. 2)

Anti-XOD antiserum (Ab II) produced in rabbits immunized with polyacrylamide gel-purified XOD gave one precipitin line against XOD. Two kinds of antiserum (Ab I' and Ab II) showed complete immunological identity by double gel diffusion test (Fig. 3) and immunoelectrophoretic assay. Since these antisera also showed comparable immunohistochemical staining after conjugation with FITC, they will hereafter be termed anti-XOD, without additional designation.

XOD in the supernatant fraction of each tissue was concentrated by precipitating in 60% saturated ammonium sulfate and resolving in one-fifth volume of PBS. XOD was tested for immunochemical identity by Ouchterlony's double

gel diffusion technique. As shown in Fig. 4, all precipitin lines formed between anti-XOD and each of enzyme preparations from various sources fused completely.

Histologic Studies

Among the fixatives examined only a few were effective for the demonstration XOD in some tissues examined.

Liver : Carnoy's solution proved best for staining frozen sections of the liver. In the cytoplasm of hepatic cells specific fluorescence was observed diffusely (Fig. 5). Intensity of the fluorescence varied from cell to cell. Sections fixed with 2.5% glutaraldehyde gave a faint specific fluorescence, but the autofluorescence caused by the fixative interfered with the observation of specific fluorescence. Kupffer cells or any nucleus were not fluorescent regardless of

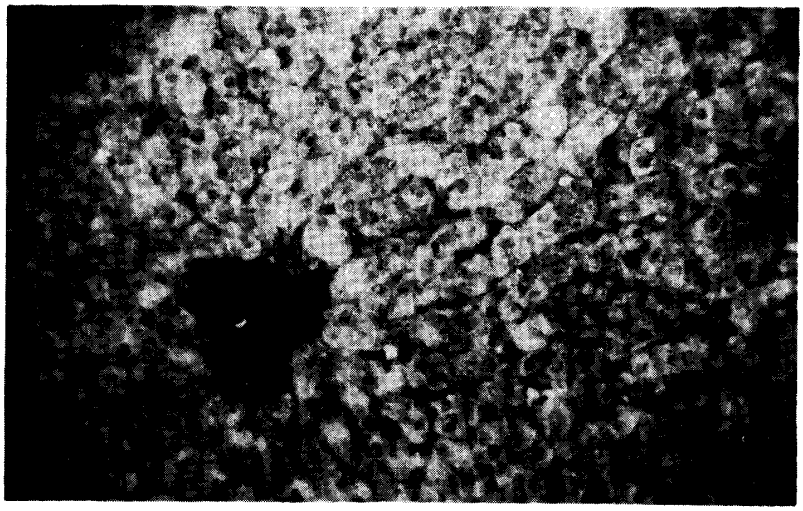

Fig. 5. Liver, frozen section, fixed with Carnoy's solution for 5 min at 0°C, stained with FITC anti-XOD. Cytoplasm of hepatic cells showed diffuse bright fluorescence. Intensity of the fluorescence varied from cell to cell. × 260.

Fig. 6. Duodenum, frozen section, fixed with acidified ethanol for 30 min at 0°C. Apical portions of epithelium of Brunner's gland were fluorescent. × 260.

the staining procedure.

Spleen : Specific fluorescence could not be discerned from the non-specific after fixation with Carnoy's solution. No other fixatives stained the materials.

Duodenum : Apical portion of epithelium of Brunner's gland was weakly fluorescent after acidified ethanol fixation (Fig. 6). No specific staining was observed in the epithelium of the intestinal glands or villus or goblet cells.

Kidney : Only some of epithelium of proximal tubuli showed a faint fluorescence using Carnoy's fixation. No fluorescence was observed in glomeruli or Bowman's capsel.

Lung : No specific staining was observed.

Mammary gland : The apex of the alveolar epithelium and the margin of the alveolar space stained after fixation with Carnoy's solution, 95% ethanol or acidified ethanol.

Effect of Fixatives on the Preservation of Antigenic Determinants of XOD

To investigate the cause(s) of failure for many fixatives to demonstrate specific fluorescence in such tissues as the liver which has high enzyme activity, effect of fixation on the preservation of antigenic determinats (ADs) of XOD was examined. XOD was immobilized onto insoluble anti-XOD by antigen-antibody reaction on slide glasses. After treatment with one of the fixatives in the same manner as was used for the histological sections, anti-XOD conjugate was applied to examine the intensity of the fluorescence specific for XOD. Compared with non-fixed XOD, all fixed specimens showed variable but specific fluorescence. This suggests that fixed XOD retained sufficient ADs to react with the conjugate.

DISCUSSION

XOD activity was detected herein in the homogenates of bovine liver, spleen, duodenum, lung and kidney (Table I) as reported in rat tissues (8, 15). Total activity recovered varied depending upon the organ ; liver and spleen having much higher activity than the other organs. This distribution pattern of the enzyme among tissues was different from that of rat (8, 15) indicating the variation among species.

To examine organ specificity and cellular localization of XOD by immunological methods, antibody to XOD from bovine milk was procured using rabbits. Two kinds of antisera were obtained ; one (Ab I) from rabbits immunized with XOD (Boehringer) and the other (Ab II) from those immunized with purified XOD by polyacrylamide gel electrophoresis. A contaminating immune system found in Ab I was removed using insolubilized bovine serum as an immunoadsorbent. This absorbed antiserum (Ab I') and Ab II being specific and identical as anti-XOD were used for further studies without discrimination. As shown in Fig. 4, XODs in the supernatant fraction from each organ had immunochemical identity with XOD from milk. The presence of two discs possessing the enzyme activity seen on an acrylamide gel after electrophoresis of milk XOD (Fig. 1) and slight difference observed in electro-

phoretic mobilities on agarose plates among XODs from various sources (data not shown) suggest that there might be isozymes, irrespective to the immunochemical identity of XOD molecules. Difference in electrophoretic mobilities between XODs from rat liver and from duodenum on starch gel and on acrylamide gel were reported by Sackler (8), but there are apparently no reports regarding immunological identity of XODs from various organs.

Enzyme histochemical demonstration of XOD activity on tissue sections has been reported by Bourne (7), Wohlrab (9) and Sackler (8) using tetrazolium compounds for an artificial electron acceptor, but the staining as a result of histochemical reactions varied among the tissues examined or fixatives employed. Furthermore specificity of the reactions could not be confirmed because of the presence of the reaction products on control sections. This lack of specificity is probably best explained by a low specificity of the enzyme towards both substrate and electron acceptor and by the nature of tetrazolium compounds as they are reduced by sulfhydryl groups in tissues (16).

Immunohistochemical methods have an advantage over enzyme histochemical techniques in that they are based on highly specific antigen-antibody reaction. Any substances possessing ADs can be localized immunohistochemically, provided enough are preserved in the area where they function. As reported in the present study, XOD was demonstrated by fluorescent antibody technique in the cytoplasm of hepatic cells after fixation with Carnoy's solution (Fig. 5), in epithelium of Brunner's gland of duodenum by acidified ethanol fixation (Fig. 6) and in alveolar cells of mammary gland fixed with one of three kinds of fixatives. Many other fixatives examined in this study failed to locate the enzyme in such tissue sections as liver which showed the presence of high enzyme activity by biochemical assay. Difficulties encountered in immunohistochemical demonstration of XOD seemed to be partly the same as those experienced in enzyme histochemical demonstrations (7, 8). As shown by cell fractionation studies by us and others (8) XOD was a soluble enzyme located in the high speed supernatant fraction of organ homogenates. XOD might be lost from cryostat sections during fixation. The experimental result that many fixatives employed in this study preserved ADs of XOD at least to the extent that FITC-labeled conjugates could react would support this explanation. It should be noted, however, that the intensity of immunofluorescence in sections is a function of local concentration of ADs, provided factors from fluorescent antibody are constant. Such is an expression of the sum of such factors as primary antigen concentration, loss of antigen itself during fixation and loss of ADs caused fixatives.

Although Carnoy's solution gave the best result in some tissues, it also resulted in a non-specific staining in spleen sections. In addition, such strong fixatives may destroy cellular fine structures and tissues could not be precisely observed under high resolution microscopy. These difficulties may be overcome by designing better processes for preparation of tissue sections for immunohistochemistry.

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