

FINAL PROGRESS REPORT

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Robert E. Smith, Ph.D.  
Instructor of Pathology  
School of Medicine  
Stanford University  
Stanford, California 94305

Present Address: Senior Electron Microscopist  
Biological Research Division  
Eli Lilly and Company  
Indianapolis, Indiana 46206



The principle objectives for which funds were allocated in this grant were to support original research in: (1) the development of a technique for identifying subcellular sites of enzymes hydrolyzing  $\beta$ -naphthylamine substrates, and (2) to study the sites of reaction product localization in cells of various tissues where specific enzymes had been shown biochemically to be high in physiologic or pathologic states. Research support for these endeavors was from September 1, 1966 through April 30, 1970, a period of 3-1/2 years. All studies were conducted in close collaboration with Dr. Stanley Ellis and his laboratories at the National Aeronautics and Space Administration Laboratories, Ames Research, Moffitt Fields, California.

INTRODUCTION

In order to define the subcellular sites of enzymes hydrolyzing  $\beta$ -naphthylamine substrates, it was first necessary to develop a reliable technique to be used in electron microscopy. Prior to September 1966 there had been no investigations as to what substrates and azo dye could be used to identify proteases at the level of the electron microscope. Previous to this date all work concerning the subcellular localization of hydrolytic enzymes had been formulated through investigations using the classic lead salt procedure for acid phosphatase by George Gomori. Such observations could be taken only as general, and the necessity for the development of a specific technique to identify individual enzymes became even more desirable. For a period of two years various avenues of technical procedures were conceived and tried. Extensive studies were laid out using numerous azo dyes in hopes of increasing the stability in deposition of reaction product. These trial and error procedures are not recorded in this report

since it is believed that such comment would not serve a useful purpose in better understanding of the working procedure outlined in the materials and method section.

By the time of our progress report covering the period from April 1, 1968 to February 28, 1969, we had established that as far as  $\beta$ -naphthylamine substrates were concerned the addition of the methoxy group in position four was necessary. At that time we were using the azo dye salt Fast Blue B as the capturing agent of the released 4-methoxy- $\beta$ -naphthylamine upon hydrolysis of the various substrates. Continued electron microscopic studies clearly established that the coupling rate of this dye was slow, permitting diffusion of reaction product, principally to lipoprotein components of cellular structure. In our searches for an azo dye that would aid in stabilization of reaction product we ultimately discovered that hexazotized pararosaniline in proper concentration and slightly buffered before addition to substrate gave the best results, i.e. speed of coupling, stability of reaction product, and lipophobic properties. It could be used in media preparations from a pH 4.5 to 7.4 for the localization of enzymes hydrolyzing specific  $\beta$ -naphthylamine substrates in the acid or alkaline ranges. The enzyme identified as dipeptidyl arylamidase III by Dr. McDonald, using the substrate Lys-Ala-4-methoxy- $\beta$ -naphthylamine has served as the model substrate-enzyme for the principle developments of the azo dye metal chelation procedures for localizing peptidases at the electron microscopic level.

In the last year of our research program it became clear that certain peptidases did not lend themselves well, either by the nature of their synthetic substrate or by the activators required in the media, for cytochemical localization. Consequently, studies were begun in June of 1969 to take advantage of the peroxidase labeling antibody techniques for the subcellular localization of enzymes. Pure preparations of dipeptidyl arylamidase I (cathepsin C) were obtained by Dr. McDonald. Antibodies were made to this enzyme and peroxidase coupled. This avenue of investigation predominated until termination of our financial support. The results obtained for the short period of time proved to be exceedingly fruitful, and have since been applied by ourselves and other investigators in a wide variety of applications.

## MATERIALS AND METHODS

### Procedure for Localizing Proteases Hydrolyzing Derivatives of 4-Methoxy- $\beta$ -Naphthylamine

#### 1. Fixation of Tissue

The choice of fixative and method of prefixing tissue before cytochemical reactions is extremely important. Conditions are somewhat variable depending on the particular tissue to be studied and the substrate-media for the enzyme to be localized.

- a. All enzymes under study thus far except DAP-II in some tissues withstand acetone fixation (1-4 hr fixation 2-4°C, i.e. trypsin-like proteinase in acrosome); however subcellular structure is less satisfactory than when aldehyde fixatives are used.
- b. Some enzymes that poorly withstand glutaraldehyde fixation (i.e. Cathepsin C) are well localized in formal-phosphate buffer pH 7.4 fixative, (formalin prepared from paraformaldehyde).
- c. Some enzymes withstand glutaraldehyde-phosphate buffer fixation (i.e. Gly-Pro-4MeO $\beta$ NA and Leu-4MeO $\beta$ NA) better than glutaraldehyde-cacodylate buffer fixation.
- d. Fixation with distilled quality glutaraldehyde at 1.5% is strongly recommended over commercial grade glutaraldehyde, and higher per cents.
- e. The addition of 1% formalin prepared from paraformaldehyde to 1.5% glutaraldehyde-phosphate appears to have no additional effects on enzyme survival and is recommended where tissues are not perfused with fixative.

Routine fixation procedure: Fix tissue slices not exceeding 1.0 mm in diameter for 3-4 hour at 4°C in 1.5% glutaraldehyde containing 1% sucrose and buffered at pH 7.4 with phosphate as used by Milloning, 290-305 mOsm.

#### 2. Thickness and Quality of Sections

Substrates have a limited tissue penetration capacity. It is advisable to use 20-25 $\mu$  sections. Depth of tissue penetration appears to be decreased in proportion to the length and complexity of the peptide chain. Substrate penetration

is less of a problem with sections cut from fixed organelle pellets. At the electron microscopic level, diffusion of enzyme can clearly be identified with frozen sections as apposed to nonfrozen sections.

### 3. Substrates and Media

The structural features of substrates for the various proteases has been derived from the biochemical literature. The following have been studied:

- a. Pro-Arg-4MeO $\beta$ NA and Ser-Tyr-4MeO $\beta$ NA for Cathepsin C.
- b. CBZ-di Gly-Arg-4MeO $\beta$ NA for Cathepsin B<sub>1</sub> and trypsin.
- c. Bz-Arg-4MeO $\beta$ NA for trypsin.
- d. Lys-Ala-4MeO $\beta$ NA for DAP-II.
- e. Gly-Pro-4MeO $\beta$ NA for DAP-IV.
- f. Arg-Arg-4MeO $\beta$ NA for DAP-III.
- g. Leu-4MeO $\beta$ NA for aminopeptidase.

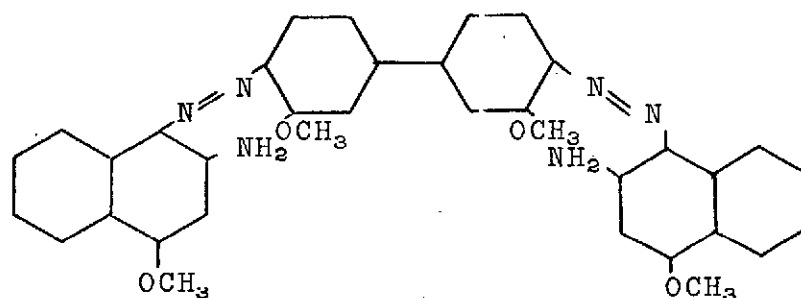
The choice of buffer, molarity, pH, and presence or absence of activators and inhibitors are important controlling factors. Example: Equal volumes of 2mM CBZ-di Gly-Arg-4MeO $\beta$ NA and 2mM sodium cacodylate buffer pH 4.8 + 2 Mercapto ethanol, and coupling agent for lysosomal localization of cathepsin B<sub>1</sub>, or a change to pH 6.7 for secretion granule localization.

### 4. Importance of Methoxy Group in 4 Position

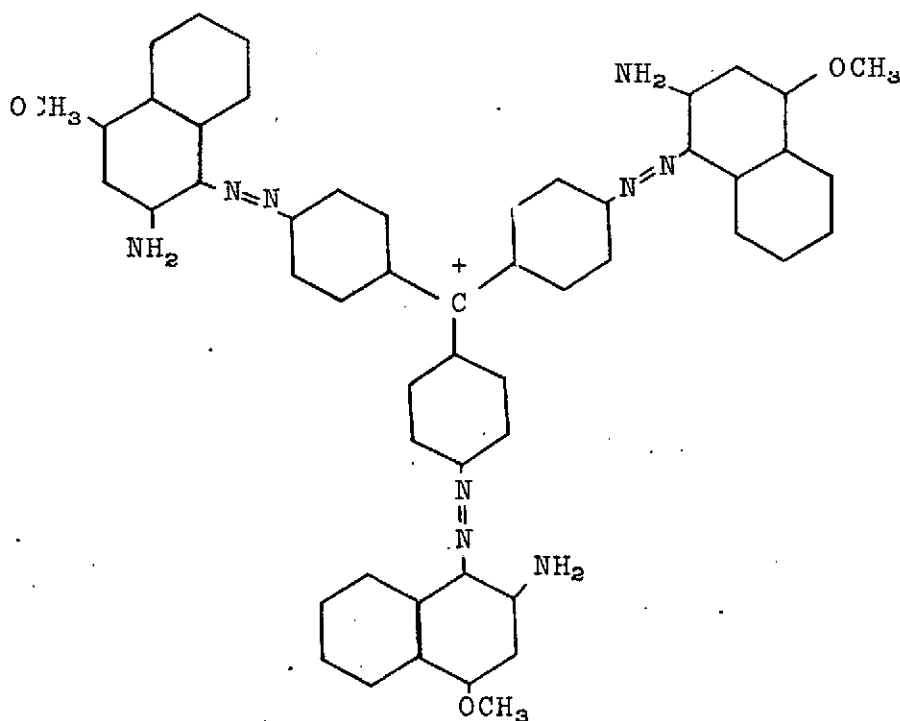
- a. Increase in rate of substrate hydrolysis (Nachlas, M. M., et al., J. Biophys. Biochem. Cytol. 7, 261-264, 1960).
- b. Increase in stability of reaction product, less lipophilic.
- c. Increase in visual color.
- d. No change in fluorescent properties from  $\beta$ NA, consequently biochemical studies can be carried out with the less expensive  $\beta$ NA derivatives without the methoxy group.

5. Coupling Agents

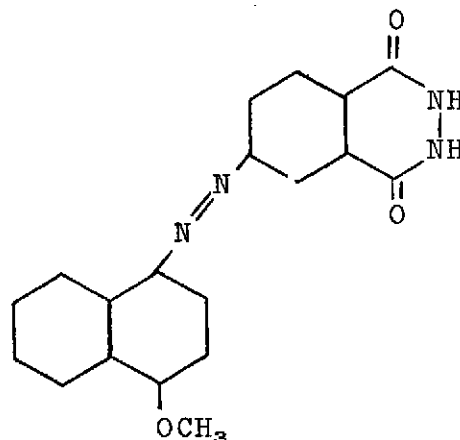
- a. The speed of azo-coupling is extremely important for electron microscopic localization of enzyme. Example: Fast Blue B gives best color reaction on 20 $\mu$  sections but at EM level it can be determined that color intensity is a result of permitting free 4 MeO $\beta$ NA diffusing slightly before capture, (slow coupling). Hexazotized pararosanilin is a faster coupling agent, permitting no diffusion of 4 MeO $\beta$ NA but less color distinction.
- b. Not all azo dyes will permit the later chelation of osmium or some lesser metal between the nitrogens of the azo group and the amine. Although Fast Garnet GBC is a very good coupling agent its chelation properties are poor, limiting its use to color reactions and light microscopy.



4MeO $\beta$ NA/Fast Blue B1



4 MeO $\beta$ NA/Hexazotized Pararosanilin



4 MeO $\beta$ NA/4-aminophthalhydrazide (Seligman, A. M., et al.  
J. Histochem. and Cytochem. 18:542, 1970)

- c. Osmium from O<sub>5</sub> O<sub>4</sub> is not chelated with Fast Blue B, however, copper from CuSO<sub>4</sub> and mercury from Hg(C<sub>2</sub>H<sub>3</sub>O<sub>2</sub>)<sub>2</sub> are.
- d. Osmium is chelated with hexazotized pararosaniline, and in our hands to a lesser extent with 4-aminophthalhydrazide.

## 6. Chemicals and Their Preparation

### a. Preparation of Substrates

1. Lys-Ala 4MeO $\beta$ NA 2mM 43.5 mg in a 50 ml volumetric flask.
2. Leu-4MeO $\beta$ NA 2mM 32.3 mg in a 50 ml volumetric flask.

### b. Preparation of Buffers

1. 2mM Sodium cacodylate pH 5.8.
2. 2mM Tris-HCl pH 7.2.

### c. Preparation of Hexazotized Pararosaniline Dye

1. 3 gram pararosaniline in a 50 ml glass stopper volumetric flask - adding 25 ml of 2N HCl (not all PRA will go into solution).
2. 1 gram sodium nitrate NaNO<sub>3</sub> in 25 ml distilled H<sub>2</sub>O in a 25 ml volumetric flask.

Diazotization by adding 25 ml of  $\text{NaNO}_2$  into the 50 ml volumetric flask containing pararosanilin - stopper volumetric with glass stopper, shake back and forth in hand, stopping intermittently and loosen glass stopper to permit release of some gas.

Azo dye for coupling: To 10 ml of diazotized PRA (now a clear solution ruby-brown in color) add 10 ml Na acetate (14 grams  $\text{Ca}_3(\text{COO})_2 \cdot 3\text{H}_2\text{O}$  to 100 ml distilled  $\text{H}_2\text{O}$ ). Then add 1 ml 1N NaOH pH should be 4.6.

Diazotization and preparation of coupling dye should be prepared fresh. The azo dye should be used in media within 15 minutes, with incubations at  $37^\circ\text{C}$  not exceeding 40 minutes for effective use of coupler.

d. Preparation of osmium see Smith and Fishman, J. Histochem. and Cytochem. 17:4, 1969.

e. Preparation of Medias:

Media I - 20 ml of 2mM Leu-<sup>1</sup>MeO $\beta$ NA and 20 ml 2mM Tris-HCl pH 7.2 buffer in an Erlenmeyer flask. Three to five minutes before use add 6 mg Fast Blue B and adjust pH to 7.1 if necessary.

Media II - 20 ml of 2mM Leu-<sup>1</sup>MeO $\beta$ NA and 20 ml 2mM Tris-HCl pH 7.2 buffer in an Erlenmeyer flask. 3 to 5 minutes before use add 2.5 ml of PRA azo dye for coupling. Adjust pH to 7.1 with 1N NaOH.

Media III and IV - 20 ml distilled  $\text{H}_2\text{O}$  in place of Leu substrate.

Media V - 20 ml of 2mM Lys-Ala-<sup>4</sup>MeO $\beta$ NA and 20 ml 2mM sodium cacodylate pH 5.8 buffer in an Erlenmeyer flask. 3 to 5 minutes before use add 6 mg Fast Blue B and adjust pH to 5.8 if necessary.

Media VI - 20 ml of 2mM Lys-Ala-<sup>4</sup>MeO $\beta$ NA and 20 ml 2mM sodium cacodylate pH 5.8 buffer in an Erlenmeyer flask. 3 to 5 minutes before use add 2.5 ml of PRA azo dye for coupling and adjust pH to 5.8 if necessary.

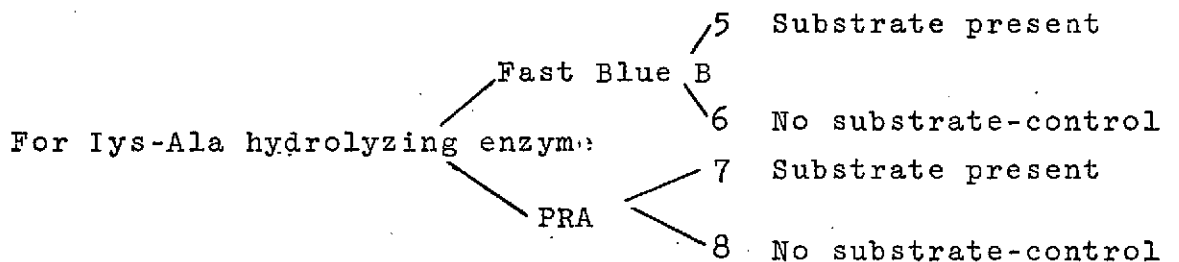
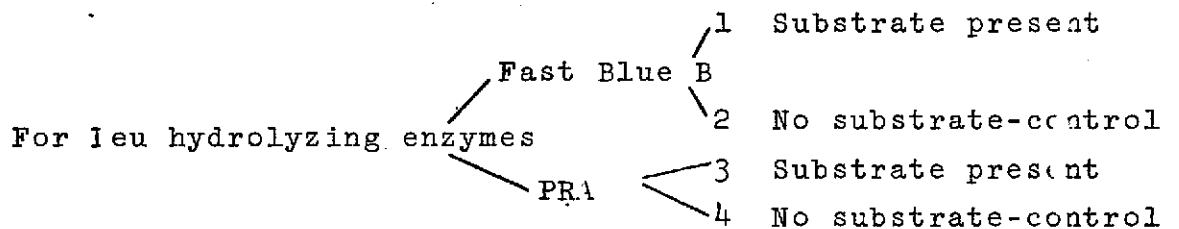
Media VII and VIII - 20 ml distilled H<sub>2</sub>O in place of Lys-Ala-substrate.

f. Other Chemicals:

1. Fast Blue B salt - 6 mg for 40 ml media.
2. Cupric Sulfate CuSO<sub>4</sub> - 500 mg/50 ml volumetric with 50 ml distilled H<sub>2</sub>O.
3. Thiocarbohydrazide TCH - 500 mg/50 ml volumetric with 50 ml warm distilled H<sub>2</sub>O.

7. Technique for Localizing Enzymes Hydrolyzing Substrate Leu-4 MeOBNA and Lys-Ala-4MeOBNA

1. Fix tissue slices for 3 to 4 hours in 1.5% glutaraldehyde-phosphate buffered pH 7.4, 4°C.
2. Wash tissue slices for at least 24 hours in veronal-acetate buffer pH 7.4 (containing 7% sucrose) 4°C.
3. Cut 20-25 μ nonfrozen sections and store at 4°C in veronal-acetate buffer as above for 13-24 hours.
4. Place sections in 8 20 ml beakers containing Veronal-acetate buffer.





5. Sections in beakers 1 to 4 pipet off V-A wash buffer, add Leu-substrate, incubate at 37°C. Beakers 1 and 2 for 5 minutes and beakers 3 and 4 for 10 to 15 minutes.  
  
Sections in beakers 5 to 8 pipet off V-A wash buffer, add Lys-Ala-substrate, incubate at 37°C. Beakers 5 and 6 for 4 minutes, and beakers 7 and 8 for 8 to 12 minutes.
6. Quickly pipet off media from beakers 1 to 8 after incubation times and wash 3 times with ice cold veronal-acetate wash buffer pH 7.4.
7. Pipet off last buffer wash of beakers 3, 4, 7, and 8 - add 1.5% aqueous OsO<sub>4</sub> pH 7.5 + at room temperature for 1 hour at 35°C.
8. Pipet off last buffer wash of beakers 1, 2, 5, and 6 - add 1% Cupric Sulfate for 5 minutes at room temperature (red sections with copper chelation will turn a darker red).
  - a. Pipet off Cupric sulfate and wash 3 times with V-A wash buffer.
  - b. Pipet off wash buffer and add 1% thiocarbohydrazide for 5 minutes at room temperature. (Solution of TCH should be slightly warm.)
  - c. Pipet off TCH and wash 3 times with V-A wash buffer.
  - d. Pipet off last wash buffer and may then choose to post-fix with 1% OsO<sub>4</sub> veronal-acetate buffered pH 7.2 for 20 minutes 4°C.
9. All beakers 1 to 8, containing sections after osmium treatment are carried through dehydration and embedding according to steps 10, 11, and 12 page 5. Smith and Fishman, J. Histochem. and Cytochem. 17, 1969.

## 8. Value of Technique

Easy adaptation to enzyme with appropriate substrate-media. Broad application to all tissues, whereas enzyme-antibody labeled with peroxidase is dependent on purity of enzyme before attaining antibody, with limitation to tissue and animal specificity.

Procedure for Localizing Enzymes (Antigens) by Peroxidase Labeling of Antibody

1. Fixation of Tissue

Enzyme-antigens, in general, appear not to withstand fixation as well as hormone-antigens; therefore choice of fixative, method of fixative, and period of fixation are more critical than with substrate hydrolyzing procedures.

2. Technique for Localizing Enzymes

- a. Peroxidase labeling of antibody for Cathepsin C was prepared by Dr. K. McDonald, NASA - Ames, as follows:

Preparation of Peroxidase-Labeled (Second) Antibody

The labeling procedure is basically that of Nakane and Pierce (1967). A goat antiserum to rabbit  $\gamma$ -globulin was obtained from Antibodies Incorporated (Davis, California). One ml of this antiserum precipitated 5 mg of rabbit  $\gamma$ -globulin. The  $\gamma$ -globulin fraction was precipitated from 20 ml of goat antiserum by slowly adding, with mixing, an equal volume of a 28% (W/V) aqueous solution of ammonium sulfate. All solutions were maintained at 0-5°C throughout the procedure. The  $\gamma$ -globulin was sedimented by centrifugation at 10,000 x g for 10 min. The supernatant was discarded, and the  $\gamma$ -globulin washed by resuspending the precipitate in a solution of 18% (W/V) ammonium sulfate. The protein was recovered by centrifugation, washed once, and dissolved in 2 ml of phosphate-buffered saline (8% NaCl-0.01 M sodium phosphate, pH 7.2). The solution was transferred to a dialysis bag using an additional 2 ml of buffered saline as rinse, and dialyzed for 24 hr against 3 l of cold buffered saline to remove contaminating ammonium sulfate. The dialysis was repeated for an additional 24 hr. The  $\gamma$ -globulin solution was recovered using additional buffered saline as wash. The final solution (5 ml) was held on ice over night. Some protein sedimented and was eliminated. The concentration of soluble  $\gamma$ -globulin was estimated to be 35 mg per ml by absorbance at 280 m $\mu$ , assuming 1 mg per ml for unit A<sub>280</sub>.

A 4.3 ml quantity (150 mg protein) of the prepared goat anti-rabbit  $\gamma$ -globulin solution was taken for conjugation with horseradish peroxidase. The addition of 1.7 ml of 0.5 M sodium carbonate buffer, pH 10, gave a final pH of 9.5. The pH was adjusted up to 10.1 by adding, with vigorous stirring, about 0.1 ml of 7 M NaOH. To this

solution, 150 mg of horseradish peroxidase (Sigma type II) was added, followed by 0.75 ml of a 0.5% solution, in acetone, of the bifunctional conjugating reagent 4, 4'-difluoro-3,3'-dinitrodiphenyl sulfone. The latter was added dropwise with stirring. The solution, now turbid, was maintained on a shaker in a cold room for 5 hr. The reaction mixture was then dialyzed 24 hr against 3 l of phosphate-buffered saline. The suspension was recovered and centrifuged at 10,000 x g for 10 min. About 7.6 ml of clear supernatant was recovered that was held on ice and stirred magnetically while an equal volume of a saturated solution of ammonium sulfate was slowly added to precipitate the conjugated and unconjugated  $\gamma$ -globulin away from free horseradish peroxidase. The precipitate was sedimented at 12,000 x g for 10 min. The precipitate was washed twice in 10 ml of cold 50% saturated ammonium sulfate. The washed precipitate was dissolved in 4 ml of phosphate-buffered saline and dialyzed against 3 l of the same. The dialysis medium was changed four times at about 24 hr intervals. The peroxidase-antibody conjugate was recovered after dialysis as a clear, amber-colored solution. Rinse solutions were used to bring the final volume to 6 ml. The peroxidase activity of the conjugate was tested as follows, and found to be highly reactive. A little of the conjugate was transferred on a glass rod to a peroxide solution, prepared according to Graham and Karnovsky (1966), that contained 0.025% 3,3'-diaminobenzidine tetrahydrochloride-0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6. The solution was darkened instantly by the conjugate, and the resulting stain could be precipitated by adding a little  $\text{OsO}_4$  as a 2% aqueous solution.

The peroxidase-labeled antibody was then adsorbed with acetone-dried rat liver powder obtained from Mann Research Laboratories (New York City). No charcoal was added. About 600 mg of the liver powder was washed repeatedly (5 times) with 30 ml of cold 0.8% NaCl-0.01 M phosphate buffer, pH 7.2. After each washing the liver powder was recovered by centrifugation at 20,000 x g for 20 min. The washed rat liver powder was then resuspended in the 6 ml solution of peroxidase-labeled antibody, and the suspension was agitated in the cold for 1 hr. The mixture was centrifuged at 20,000 x g for 20 min, and the peroxidase-labeled antibody was recovered in the supernatant. Finally, the supernatant was centrifuged at 100,000 x g for 30 min to remove aggregates of the conjugate that would otherwise interfere with immunohistochemical localization by electron microscopy. The peroxidase-labeled antibody, which was stored frozen, was usually diluted 200 fold with buffered saline just prior to the treatment of sections.

b. Subcellular Localization

1. Fat liver tissue was fixed with 1.5% glutaraldehyde-phosphate buffered pH 7.4, 300 mOsm; first with fixative at room temperature and perfusion of the liver in situ through the hepatic portal vein for about 2-5 minutes. Perfused liver was removed from the animal and slices cut with one dimension not exceeding 1 mm were fixed additionally for 1 hour at 4°C. An alternate fixing procedure was the removal of the liver from the animal, the cutting of thin slices of tissue, and fixing in cold formal-phosphate buffer pH 7.4 fixative for 8 hours.
2. After period of fixation in either glutaraldehyde or formal, tissue slices were washed in phosphate buffer containing 4.5% sucrose (PBS) 4°C for at least 24 hours.
3. Nonfrozen sections 20-25µ were cut, using an Oxford Vibratome, and returned to fresh PBS for 18-24 hours.
4. Sections were placed in 16 5 ml glass stoppered weighing bottles. All PBS was pipet off and 3 ml of Rabbit Anti-Cathepsin C was added as follows:

Bottles 1, 2, 3, 4 - Rabbit Anti-Cathepsin C Stock  
Bottles 5, 6, 7, 8 - Rabbit Anti Cathespin C 1:10 Dilution  
Bottles 9, 10, 11, 12 - Rabbit Anti Cathespin C 1:50 Dilution  
Bottles 13, 14, 15, 16 - Rabbit Anti Cathespin C 1:100 Dilution

Dilutions were made in pH 7.2 0.05 M phosphate buffer. Sections were reacted for 24 hours at 4°C, and an additional 6 hours at room temperature with constant agitation.

5. Antibody solution was removed and sections washed in PBS for 12 hours or overnight at 4°C.
6. PBS was removed and sections were reacted with peroxidase-labeled goat antiserum to rabbit gamma globulin for 12 hours at 4°C as follows:

Bottles 1, 5, 9, 13 - PGAR 1:5 Dilution  
Bottles 2, 6, 10, 14 - PGAR 1:10 Dilution  
Bottles 3, 7, 11, 15 - PGAR 1:50 Dilution  
Bottles 4, 8, 12, 16 - PGAR 1:100 Dilution

7. Second antibody solution removed and sections washed in PBS overnight 4°C.

8. Remove PBS and react with 3,3'-diaminobenzidine without peroxide for 1 hour 4°C. (25 mg/100 ml of 3,3'DAB in 0.05 M Tris buffer pH 7.6.)
9. Can test for approximate time required for staining reactions by taking a wood applicator stick and dipping it first into peroxidase labeled antibody and then into 3,3'DAB with peroxide. A reaction is indicated by brown color of stick. React sections with 3,3'DAB with peroxide for 10 minutes at room temperature. (One part 30% peroxide to 2 parts distilled H<sub>2</sub>O to make 10%.) (0.05 ml of 10% peroxide to 10 ml 3,3'DAB, filter with Whatman filter paper #2.)
10. Remove second 3,3'DAB and wash 3 times over a period of 18 to 24 hours with PBS 4°C.
11. Remove last PBS wash and add 1.5% O<sub>3</sub>O<sub>4</sub> prepared according to Smith and Fishman, J. Histochem. and Cytochem. 17, 1969, at room temperature with constant agitation for 2 hours.
12. Dehydrate through ETOH and araldite to araldite, avoiding the use of propylene oxide.

The best immunochemical results with the Rabbit Anti Cathepsin C preparation used in this procedure were obtained with a 1:10 dilution of Rabbit Anti Cathepsin C and a 1:100 dilution of Anti Rabbit Goat gamma globulin. Less dilute preparations showed increasing nonspecific staining with 3,3'diaminobenzidine. Electron opacity due to osmium chelated by oxidized 3,3'DAB was limited to dense-body-type lysosomes.

#### RESULTS AND DISCUSSIONS

Investigations using the substrate Leu 4-methoxy-8-naphthylamine, a capture with hexonium pararosaniline, and the final chelation of osmium has identified the hydrolyzing enzyme with the membrane system of cells as was shown biochemically in 1968 by Emmelot and others et al. Our studies of rat liver cells showed this enzyme to be localized on cell membranes with intense deposition in the areas of the parcanaliculi. The study of cells in the anterior pituitary of the rat showed the deposition of reaction product on cell membranes and on the membranes of secretion granules contained within the cell. The deposition of reaction product on the cell membrane however showed no increase or decrease with changes in the physiological state of the gland and release of secretion granules from specific cells.

Our studies for the localization of reaction product for DAP-II have been carried out in rat thyroid, anterior pituitary, hyperplastic alveolar nodular tissue from mouse mammary gland, and rat endometrium. The observations clearly show the substrate to be hydrolyzed by an enzyme contained within the dense-body-type lysosomes of these tissues, and levels of hydrolysis are related to the physiologic condition of these structures. This is clearly demonstrated by the group of six pictures of two microns sections showing the localization of reaction product in lysosomes of rat anterior pituitary cells with reference to the state of lactation and post lactation. Such observations in the pituitary have established the identification of this enzyme with the process of hormone degradation as first proposed cytochemically by Smith and Farquhar, and subsequently biochemically by McDonald and others.

Peptidases hydrolyzing other substrates have also been investigated at the light and electron microscopic level. The substrate Pro-Arg-4-methoxy- $\beta$ -naphthylamine has been used for the subcellular localization of Cathepsin C. This enzyme is identified with the lysosomes of liver, and an intense deposition of reaction product is clearly demonstrated when animals are pretreated with glucagon; thus confirming that this enzyme is the specific peptidase involved in the autolytic events described by Dieter, *et al.* Two substrates have been studied in relationship to kidney function. The enzyme hydrolyzing the substrate Gly-Pro-4MeO $\beta$ NA at pH 7 has been identified with lysosomes of the proximal kidney tubular cells. A trypsin like hydrolyzing enzyme using the substrate CBZ-Di-Gly-Arg-4MeO $\beta$ NA is present in certain cells, and the enzyme within secretory-like granules. Furthermore, preliminary observations suggested that the substrate CBZ-Di-Gly-Arg-4MeO $\beta$ NA in a range of pH 4.5 to 5.6 may be identified with the enzyme Cathepsin B. Subsequent work along these lines are presently being conducted.

The results obtained from the peroxidase labeling of antibody limiting to the localization of Cathepsin C parallel those with the cytochemistry using the substrate Pro-Arg-4-methoxy- $\beta$ -naphthylamine. The deposition of osmium in peroxidase procedure and osmium by the cytochemical procedure is in lysosomes and autophagic structures in the liver cells of the glucagon treated animals; consequently a cross-reference for the subcellular localization of this specific peptidase is achieved.

In conclusion the major goals of this research project were reached under the period of time that the grant was in effect. With the establishment of a cytochemical procedure for the localization of peptidases at both the light and electron microscopic level we are presently, in our new position, pursuing their broad application

in medical research by not only identifying these enzymes in normal and pathological tissues but also in the development of bioassay procedures to permit us to determine where within tissue cell fraction the organelle localization of a specific enzyme is active. Certainly this general cytochemical technique will be used for the identification of numerous enzymes by varying simply the peptide leakages, and therefore serve for identification of numerous peptidases. These applications in the field of protein chemistry are anticipated to be of value in identifying the sequential degradation of proteins whether into functional complexes such as proinsulin to insulin or to break-down of non-released secretion granules into amino acid pools.