BIOCHEMICAL AND HISTOCHEMICAL STUDIES OF ESTERASES IN LIVER, KIDNEY, AND SPLEEN OF YOUNG AND ADULT MICE, STRAIN C 57 BL/6

Thesis for the Degree of Ph. D.
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BIOCHEMICAL AND HISTOCHEMICAL STUDIES OF ESTERASES IN LIVER, KIDNEY, AND SPLEEN OF YOUNG AND ADULT MICE, STRAIN C 57 BL

presented by

S. Arthur Reed

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Richard G. Tennent
Major professor

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BIOCHEMICAL AND HISTOCHEMICAL STUDIES OF ESTERASES IN LIVER, KIDNEY, AND SPLEEN OF YOUNG AND ADULT MICE, STRAIN C 57 BL/6

By

S. Arthur Reed

AN ABSTRACT OF A THESIS

Submitted to
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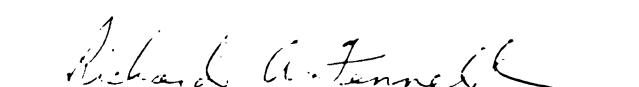
1962

Approved 

ABSTRACT

BIOCHEMICAL AND HISTOCHEMICAL STUDIES OF ESTERASES IN LIVER, KIDNEY, AND SPLEEN OF YOUNG AND ADULT MICE, STRAIN C 57 BL/6

by S. Arthur Reed

The esterases of homogenates of adult liver, kidney, and spleen of C 57 Bl/6 mice were separated by starch gel electrophoresis and then exposed to 5 inhibitors in an effort to classify these enzymes more completely. The inhibitors, sodium taurocholate, eserine sulfate, benzaldehyde, sodium fluoride, sodium arsanilate, were selected because of their frequent use as a standard method of classification of the esterases. The effect of these same inhibitors on the enzyme activity of tissue homogenates was also quantitatively measured by a colorimetric technique.

Adult liver showed the highest level of esterase activity, while kidney was much less reactive, and spleen had the least activity. Inhibitor studies indicated that the activity was due largely to esterase in liver and kidney, and that lipase may not exist in the spleen.

Zymograms of adult liver contained 10 bands of esterase activity, whereas 5 bands appeared in kidney, and 3 bands in the spleen. When exposed to the inhibitors, no uniform pattern of sensitivity of the bands was evident. Results indicate that classification of esterases on the

basis of selective sensitivity to inhibitors is not entirely adequate.

The development of esterase activity in liver, kidney, and spleen of newborn and maturing mice was studied quantitatively by starch gel electrophoresis, and with histochemical localization techniques. In all 3 organs there was a gradual increase in activity, reaching adult levels in liver, kidney, and spleen at about 10 days, 25 days, and 5 days, respectively, remaining relatively constant thereafter. In the zymograms of these organs, new bands of esterase activity appeared at various age intervals postpartum. In the liver the number doubled during the first 30 days of life of the animal. Possible changes in cellular mechanisms that might cause this addition of enzymes are discussed.

Esterase activity was localized in the parenchymal cells of immature liver among large numbers of enzymatically inactive hemopoietic cells and fat globules. The increase in esterase activity in maturing liver seems to be caused partly by the addition of new esterases within each cell, as demonstrated by the zymograms, and partly to the gradual decrease in the number of hemopoietic cells, thereby removing their diluting influence.

Esterase active cells in adult spleen were arranged in a circular pattern within the white pulp, but this pattern was not apparent in tissues from young animals up to 15 days of age.

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Distribution of esterase activity in adult kidney agreed closely with previous studies and no major variations could be observed. In newborn animals only a few nephrons located in the juxtamedullary region of the kidney showed esterase activity. The nephrogenic region of the outer cortex was negative. Increase in esterase activity with age is probably a result of a gradual addition of new nephrons as they attach to the collecting duct, mature, and become active. During the latter stages of development of this organ further increase in enzyme activity may be due to elongation of active nephrons.

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CURRICULUM VITAE

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candidate for the degree of

Doctor of Philosophy

Final examination: June 15, 1962.

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INTRODUCTION

Many schemes for the classification of esterases have been proposed based upon the activity of this group of enzymes toward a number of substrates and their sensitivity toward a variety of inhibitors.

Gomori (1952a) divided the esterases into two subgroups, the aliesterases and the cholinesterases. The former group was subdivided into lipases which have a predilection for splitting fats and oils, and esterases which cleave simpler esters of monohydric alcohols. Differences between the two groups are not absolute and some enzymes occupy an intermediate position, having characteristics in common with both lipase and esterase.

Gomori (1952b) found that lipase activity was inhibited by benzaldehyde and accelerated by sodium taurocholate. Esterase activity was inhibited by sodium taurocholate, sodium fluoride, and sodium arsanilate.

Richter and Croft (1942) have shown that the aliesterases are resistant to concentrations of eserine sulfate as high as 10^{-3}M, while the cholinesterases are completely inhibited by 10^{-6}M eserine.

The cholinesterases were further subdivided into true or specific cholinesterases with acetylcholine as the

substrate preference (Mendel and Rudney, 1943) and a resistance to diisopropyfluorophosphate (DFP) (Koelle and Friedenwald, 1949), and the nonspecific or pseudocholinesterases, which show a preference for other choline esters (Nachmansohn and Rothenberg, 1944) and a sensitivity to DFP.

Chessick (1954) maintained that the only reliable differential feature of esterases was their degree of sensitivity to eserine. This author found that esterases are divisible into eserine resistant (aliestereases) and eserine sensitive esterases (cholinesterases).

Gomori (1955) divided the esterases into hepatic, pancreatic, and general types on the basis of histochemical substrate preference. The hepatic type cleaved alpha naphthyl acetate most readily and was inhibited by sodium fluoride, DFP, and E-600 (diethyl-p-nitrophenyl phosphate). The pancreatic type, present in pancreas and anterior pituitary, was insensitive to fluoride but sensitive to DFP and E-600. The general type, present in the stomach, kidney, and most organs, was insensitive to DFP and E-600.

Pearse (1961) maintained that the classifications of esterases cited in the preceding paragraphs are inadequate. He used the established biochemical practice of dividing the group into A, B, and C esterases. This classification can be done on the basis of inhibitor and activator studies of Pepler and Pearse (1957), Aldridge (1953), and Hess and Pearse (1958). The A esterases are not inhibited by E-600

up to 10^{-3}M and hydrolyze acetate esters faster than butyrates. The B esterases are inhibited by concentrations of E-600 as low as 10^{-8}M and hydrolyze butyrates equal to or faster than acetates. The C esterases are activated by 10^{-4}PCMB (p-chloromercuribenzoate).

Procedures for the demonstration of esterases. The natural substrates for lipases are true fats and oils, but have the disadvantage of being insoluble in water and are, therefore, unsuitable for histochemical localization procedures because they do not readily penetrate the cell surface. Because of this difficulty, early work on esterases was entirely limited to quantitative studies using tissue homogenates and extracts. For a thorough review of this early work the reader is referred to Ammon and Jaarma (1950).

Localization of enzyme activity within tissue sections became possible with Gomori's (1945) introduction of long chain (C_{12}-C_{18}) fatty acid esters of sorbitan and mannitan (Tweens) producing a compound soluble in water. Enzymatic hydrolysis of Tweens yielded fatty acids which were combined with calcium salts of the incubation medium to form insoluble calcium soaps. The latter were transformed into lead soaps and ultimately into a brownish black lead sulfide precipitate by immersion of the tissues in ammonium sulfide.

The naphthol procedure for localization of esterase was introduced by Nachlas and Seligman (1949). Following hydrolysis of beta naphthyl acetate the naphthol liberated

was combined with a diazonium salt in the culture medium to form an insoluble dye at the site of enzyme activity. Other substituted naphthols have been developed including alpha naphthol and naphthol AS (the anilide of 2-hydroxy-3-naphthoic acid) in combination with a variety of fatty acids (Gomori, 1952b).

On the basis of localization patterns, Malaty and Bourne (1955) maintained that beta naphthyl acetate and alpha naphthyl acetate are hydrolyzed by the same enzyme, but that incubation of the tissue sections in naphthol AS acetate produced a different distribution pattern of enzyme active areas. Gomori (1952b) attributes this difference to a separate naphthol AS esterase. Pearse (1954) suggests that the difference may be due merely to the lack of ability of the complex naphthol AS molecule to penetrate some cell membranes. The pH optimum for the two substrates lies in the range of pH 7.3 - 8.4 (Pearson and Defendi, 1957).

Barrnett and Seligman (1951) and Holt (1952) introduced a new principle for the histochemical localization of esterases based upon the hydrolysis of indoxyl acetate to release indoxyl. The latter is oxidized by atmospheric oxygen to form an insoluble, highly colored dark blue indigo dye at the sites of enzyme activity. Holt (1954) modified the procedure by the use of 5-bromoindoxyl acetate as substrate and added an equimolar solution of potassium ferrocyanide and potassium ferricyanide for the oxidation of

5-bromoindoxyl to a highly insoluble, intensely chromogenic 5,5' dibromoindigo at the sites of enzyme activity. The pH optimum of this reaction is in the range of pH 4.8 - 5.8 (Pearson and Defendi, 1957).

Inhibitors. Wilstätter and Memmen (1924) found that sodium taurocholate at concentrations of $2x10^{-2}M$ accelerated the hydrolysis of methyl butyrate and triacetin by pancreas homogenates. Gomori (1948), who used the Tween procedure, noted an increased staining of pancreatic tissue in the presence of sodium taurocholate and less intense staining of liver and kidney sections. Nachlas and Seligman (1949), using beta naphthyl acetate and beta naphthyl laurate as substrates, demonstrated a slight inhibition of liver and kidney enzyme activity, whereas pancreatic activity was markedly accelerated. This effect was most pronounced with the lauric acid ester which is preferentially acted upon by lipases. Taurocholate accelerates lipase activity (present in large quantities in the pancreas) and slightly inhibits the action of esterases.

Eserine sulfate is used as a specific inhibitor of cholinesterase. Richter and Croft (1942) demonstrated complete inhibition of acetylcholine hydrolysis with eserine at a concentration of $10^{-6}M$ whereas esterase activity in either methyl butyrate or tributyrin substrate solutions was unaffected with concentrations of eserine as high as $10^{-3}M$.

Benzaldehyde has been cited as an inhibitor of lipase (West and Todd, 1956). In quantitative studies using pancreatic lipase Weinstein and Wynne (1936) observed a strong inhibition of triproprionin hydrolysis with benzaldehyde ($3x10^{-3}M$).

Loevenhart and Peirce (1907) found that sodium fluoride inhibited esterase activity of hepatic and pancreatic extracts when incubated in either ethyl butyrate or olive oil. The degree of inhibition was 100 to 1000 times greater with the former than with olive oil, indicating a preferential but not specific inhibitory effect upon esterase. Rona and Haas (1923) demonstrated little influence of sodium fluoride upon pancreatic extract but a marked inhibition of liver and kidney enzyme. When beta naphthyl acetate was used as substrate, Nachlas and Seligman (1949) obtained essentially the same results with sodium fluoride. These authors also suggest that the inhibitory effect of sodium fluoride is primarily upon esterase and not lipase. Schnitka and Seligman (1961) demonstrated a sensitivity of enzymes in the non-droplet regions of the cytoplasm of rat kidney cells to sodium fluoride, whereas perinuclear droplets in the epithelial cells lining the proximal convoluted tubule were unaffected.

Inhibition of esterases with sodium arsanilate (atoxyl) has been demonstrated by a number of investigators. Rona and Pavlovic (1922) noted that arsanilate has a much greater

inhibitory effect upon liver and kidney esterase activity toward tributyrin than that of pancreas. Gomori (1948), using the Tweens, and Nachlas and Seligman (1949) using naphthol substrates, reported similar findings.

Koelle and Friedenwald (1949) demonstrated that 10^{-6}M diisopropylfluorophosphate (DFP) completely inhibited the nonspecific or pseudocholinesterases, whereas true cholinesterases (acetylcholinesterase) were only partially inhibited. Thus, this inhibitor has been employed to subdivide the cholinesterases into these two groups.

Aldridge (1953) has shown that certain of the esterases are resistant to E-600 (diethyl-p-nitrophenyl phosphate) at concentrations as high as 10^{-3}M, while others are sensitive to low concentrations (10^{-7}M).

Starch gel electrophoresis. The introduction of zone electrophoresis in starch gel by Smithies (1955) made it possible to obtain a finer resolution of proteins in biological materials than had previously been possible. Hunter and Markert (1957) adapted this technique to the study of enzymatic activity of the separated protein bands.

Markert and Hunter (1959) resolved as many as 10 separable esterases from mouse liver tissues with this technique and characterized the specificities of many bands with the use of a variety of substrates. Only a single inhibitor, eserine, was used in this study. Barron et al. (1961) have demonstrated 9 electrophoretically separable

esterase bands in brain tissues using alpha naphthyl acetate as substrate.

One of these bands has been identified as acetylcholinesterase by the use of eserine sulfate as a selective inhibitor. Two other potential inhibitors, cysteine, and p-chloromercuribenzoate (PCMB) were without effect on the activity of the enzymes.

Localization of enzyme activity in immature organs. Few studies have been made on esterase activity of organs in embryos and young organisms. Hunter (1951) found esterase to be present in pancreas, liver, lungs, and thyroid of 15-20 mm mouse embryos, but the kidney was unreactive in embryos of any age.

Using Tweens as substrates, Buno and Marino (1952) demonstrated esterase activity in the hepatic rudiment of 3 day old chick embryos. Moderate reaction was present in the tubules of the mesonephros, and after 18 days of incubation the convoluted tubules of the metanephros were positive. Verne et al. (1952) reported true lipase activity in the pancreas of rat embryos in the middle of gestation. Esterase activity did not appear in the kidney until the end of gestation, and activity in the liver was not detected until 3 days postpartum.

Objectives of this study. In this study the homogenates of adult liver, kidney, and spleen were subjected to

electrophoresis in starch gels and then exposed to 5 different inhibitors in an effort to more completely classify the individual esterases which make up the spectrum of activity within the tissues. The inhibitors were selected because of their extensive use in other investigations and because their effect upon esterases is well known.

For comparative purposes, both with previous studies and with the results of the above mentioned starch gel studies, the effect of these same inhibitors on the enzyme activity of tissue homogenates was quantitatively measured by a colorimetric technique.

There is a large body of information concerning the activity and localization of esterases in adult mammalian tissues, but less is known of these enzymes in developing tissues of embryo, newborn, and maturing organisms, and their ontogeny in relation to the corresponding ontogeny of the organs has been investigated to a limited degree.

In an attempt to study the relationship between these two phenomena, organs from mice of varying ages were subjected to 3 different techniques. (1) Quantitative colorimetric experiments were performed on tissue homogenates to measure possible increases or decreases in esterase activity that might accompany maturation. (2) The tissue homogenates were also subjected to starch gel electrophoresis to follow the variations in activity of individual enzymes. (3) Histochemical localization studies were performed on

tissue sections to detect any changes in sites of enzyme activity as a result of the developmental processes.

MATERIALS AND METHODS

Experimental animals. The experimental animals used throughout this study were inbred mice of strain C 57 Bl/6. The original breeding colony was obtained from Dr. W. F. Dunning of the Cancer Research Laboratory, University of Miami, Coral Gables, Florida. The inbred character of this strain was maintained by brother x sister matings. The animals were fed a constant diet of Wayne Mouse Breeder Blox. Just prior to the removal of the tissues the adults were sacrificed by cervical dislocation and the immature animals by decapitation.

Colorimetric quantitative procedure. The esterase and lipase enzyme activity of the liver, kidney, and spleen of various ages of mice from birth to maturity were measured following the colorimetric procedure of Nachlas and Seligman (1949). The sensitivities of these enzymes in adult tissues to various inhibitors were also measured. Inhibitors employed were; sodium taurocholate (1.65×10^{-2}MO) eserine sulfate (10^{-5}M), benzaldehyde (2.8×10^{-2}M), sodium fluoride (7.14×10^{-2}M), and sodium arsanilate (10^{-1}M).

The tissues were quickly excised from the animal, trimmed of any excess fatty material, rinsed briefly in distilled water and blotted to remove clinging hair and

blood, and then weighed. Tissues were homogenized in a Kontes glass homogenizer following addition of distilled water (1 to 1 dilution). Final concentrations of tissues in the test solutions are listed below:

Tissue	Substrate	
	beta naphthyl acetate	beta naphthyl laurate
Liver	0.005 mg/ml	0.01 mg/ml
Kidney	0.01 mg/ml	0.02 mg/ml
Spleen	0.05 mg/ml	0.1 mg/ml

Five-tenths ml of tissue homogenate was added to each of a series of duplicate test tubes. In the experiments using inhibitors 1 ml of each inhibitor was added to each 0.5 ml sample of homogenate and allowed to stand for half an hour to permit inhibition of the enzyme to take place.

The substrate solution was prepared by adding 5 ml of a 4 mg/ml solution of beta naphthyl acetate or laurate in acetone through a submerged pipette to a mixture of 20 ml of 0.2 M phosphate buffer, pH 7.4, and 7.5 ml of distilled water. In the case of beta naphthyl laurate a cloudy solution results. The final concentration of substrate in this solution of 0.2 mg/ml.

Five ml of this substrate solution were then added to each test tube containing homogenate. The incubation was allowed to proceed at room temperature for 30 minutes when

beta naphthyl acetate was the substrate of choice. With beta naphthyl laurate the length of the incubation was increased to 240 minutes at 37.5°C.

The remainder of the procedure was carried out in the dark as much as possible. This precaution was found to be necessary since the colored reaction product proved to be light sensitive (Page, personal communication).

At the end of the incubation period the enzyme activity was halted by placing the test tubes in an ice water bath. One ml of a 4 mg/ml solution of diazo blue B in cold (4°C) distilled water was then added to each test tube and thoroughly mixed. This coupler solution was prepared immediately before use and kept cold. After 2 minutes, during which the coupling reaction takes place, 1 ml of a 40 per cent trichloroacetic acid solution was added to stop the reaction.

The purple pigment was then extracted by vigorously shaking the contents of each test tube with 8 ml of ethyl acetate. The tubes were centrifuged for 5 minutes and the supernatant ethyl acetate containing the colored reaction product was transferred to a colorimetric tube. Color density was determined in a Bausch and Lomb Spectronic 20 colorimeter at 540 mu. Enzyme activity was expressed as mg of beta maphthol liberated per mg of tissue per hour.

Zone electrophoresis in starch gel. The modification of Smithies (1955) starch gel electrophoresis technique developed by Hunter and Markert (1957) was used to separate the enzymes.

The hydrolyzed starch and stock borate buffer solution used in preparing the starch gel strips were obtained from the Connaught Medical Laboratories, Toronto, Canada. Instructions for mixing the reagents are supplied with each batch of starch.

Starch and buffer were added to a stoppered vacuum flask and heated over a Bunsen burner with a continuous agitation until the solution boiled vigorously. The flask was placed under vacuum and quickly returned to normal pressure to remove air bubbles. The hot liquid was poured into plastic trays with inside dimensions of 22.5 cm x 2.3 cm x 0.8 cm, covered with a flat plastic strip and allowed to cool overnight in the refrigerator.

The tissues to be subjected to electrophoresis were first homogenized and diluted with distilled water to the following concentrations; liver--10 mg/ml, kidney and spleen--200 mg/ml. Slots were cut in the starch gel strips for insertion of homogenates about 8 cm from one end using two single edged razor blades soldered together. The volume of the slit produced was 0.2 ml.

Tissue homogenates were suspended in crude potato starch paste (about 0.5 g/ml of homogenate). Small pieces of filter paper soaked in the homogenate were used in other experiments.

The mixture of homogenate and starch was inserted by means of a small hypodermic syringe fitted with a 16 gauge

needle. After insertion, the exposed surface of the homogenate paste was covered with a layer of vaseline, heated so that it was just liquid (about 50° C). Three filter paper electrodes were placed at each end of the starch gel strip and the entire tray was wrapped in saran wrap to prevent desiccation. The ends of the filter paper electrodes were then placed into the electrophoretic borate bridge buffer (0.23M, pH 8.9).

To carry out the electrophoretic separation of the enzymes a current of 4 to 6 milliamperes, with a voltage drop of 6 volts per cm was maintained on each gel strip for a 4 hour period. The power supply was a Heathkit variable voltage regulated power unit (Heath Company, Benton Harbor, Michigan). The voltage was measured with a Heathkit vacuum tube voltmeter and kept at about 144 volts for the 24 cm distance across the starch gel tray.

At the end of the 4 hour electrophoretic period the gel strip was removed from the tray and sectioned longitudinally into 3 thin strips. The upper and lower of the 3 strips were placed in a 0.2M phosphate buffer solution (pH 7.4), while the middle strip was placed in buffer containing an inhibitor. Inhibitors used were sodium taurocholate (10^{-2}M), eserine sulfate (10^{-3}MO) benzaldhyde (2.8×10^{-2}M), sodium fluoride (10^{-2}M), and sodium arsanilate (10^{-3}M).

At the end of one-half an hour the alpha naphthyl acetate substrate dissolved in acetone (4 mg/ml) was added

to the buffer solution to produce a final concentration of 20 mg of substrate per 100 ml of buffer, and the strips were permitted to incubate for another half hour in the presence of the inhibitors with periodic agitation. All strips were then transferred to a fresh buffer solution containing fast garnet GBC (4 amino-3,1' dimethyl azo benzene) and allowed to couple for another half hour. Final concentration of the coupler in the buffer was 8 mg/ml.

The strips were then rinsed in tap water and photographed on Kodak panatomic X film which was developed in D-11 developer.

The term, zymogram, which has been suggested to refer to these starch gel strips containing the visualized enzyme patterns (Hunter and Markert, 1957) will be used in this study.

Histochemical localization procedure. The enzymes under investigation were localized in liver, kidney, and spleen tissues using the 5-bromoindoxyl acetate procedure of Holt and Withers (1958), and alpha naphthyl acetate substrate in combination with either diazo blue B, fast red TR, or fast garnet GBC as couplers.

All reactions were carried out on fresh, frozen tissues sectioned on a Harris-International microtomecryostat. After removal from the animal the tissues were placed on a brass chuck and lowered into a Dewer flask containing a dry ice--acetone mixture (-78°C) so that the chuck, but not the

tissues, was submerged. Freezing of the entire mass was accomplished within 15-30 seconds. If care was taken to orient the tissues, all 3 organs could be frozen in a single block and simultaneously sectioned for and subsequent staining.

The chuck holding the frozen tissue block was then transferred to the cryostat where the optimum temperature for sectioning was controlled between -14° and -18°C. Sections were cut at 8 microns. The cut section was picked up from the knife blade on a warm glass coverslip held with a rubber bulb suction pickup. The tissue thaws immediately upon contact with the coverslip and must be rapidly transferred to the incubating solution to prevent desiccation.

If the tissue was to be stained with hematoxylin and eosin, the hematoxylin was placed directly on the sections immediately upon their removal from the cryostat. The usual procedure of dehydrating the tissue sections in a series of alcohols, staining with eosin, and clearing in xylol was then followed. The coverslip was affixed to the microscope slide with Canada balsam.

When the Holt and Withers technique was employed, 5 to 10 drops of a solution containing 1.3 mg of 5-bromoindoxyl acetate dissolved in 0.1 ml absolute ethanol, 2.0 ml of 0.1 M tris (hydroxymethyl) aminomethane--HCl buffer (pH 7.2), 1.0 ml of 0.05 M potassium ferricyanide, 1.0 ml of 0.05 M potassium ferrocyanide, 1.0 ml of 0.1 M Ca Cl_2 was placed on

the tissue section affixed to the coverslip. The tissues were allowed to incubate in this solution for 30 minutes, after which they were washed briefly in distilled water and mounted on a slide with glycerine jelly.

Other sections were incubated for 30 seconds to 2 minutes in 20 ml of a cold Krebs Ringer solution, to which was added 1 mg of alpha naphthyl acetate, dissolved in 0.5 ml acetone, and 5 mg of diazo blue B just before using. Fast garnet GBC, and fast red TR (5-chloro-o-toluidine) were substituted as coupling agents in other experiments.

A Sudan IV stain in 50 per cent ethanol (Pearse, 1961) was employed in some sections to visualize more clearly the location and size of fat globules in the cells. The stain was placed on the tissues immediately following their incubation in the substrate solutions. After 2 minutes the excess stain was removed with a brief rinse in 50 per cent ethanol and the tissue was then transferred to distilled water.

Nuclei were counterstained with Mayer's carmalum or Harris' hematoxylin to aid in intracellular localization of enzyme reactions.

All photomicrographs were taken through an American Optical Microstar microscope with attached camera using achromat lenses on Kodak panatomic X film and developed in D-11 developer.

RESULTS

Colorimetric determinations. A sensitivity curve (Fig. 1) was established for the Bausch and Lomb Spectronic 20 colorimeter using the reagents employed for ascertaining esterase activity with known amounts of beta naphthol. Optimum sensitivity for the extracted azo dye was found to be at a setting of 540 mu. This agrees with the data of Seligman and Nachlas (1950).

A calibration curve was established by addition of fixed increments of beta naphthol (0.004 mg - 0.020 mg) to reagents used in the test solutions. Variations in per cent transmittance of light through the solutions, when presented graphically, formed an essentially linear curve (Fig. 2). These observations agree with those of Seligman and Nachlas (1950) and Fennell and Pastor (1958). The presence of the inhibitors in the incubating medium did not alter the readings of either curve.

Enzyme activity of the adult tissues, expressed as mg of beta naphthol liberated per mg of tissue per hour, in the presence of either beta naphthyl acetate or beta naphthyl laurate alone and in combination with inhibitors is shown in Table I. Liver was enzymatically the most reactive of the three glands tested, with kidney showing a much lower level of activity, and spleen with the least

Figure 1. Transmittance spectrum of azo dye obtained by coupling beta naphthol (0.001 mg per ml) with diazo blue B. Ordinates, per cent transmittance of light; abscissae, wavelength in millimicrons. All readings were made with a Bausch and Lomb Spectronic 20 colorimeter.

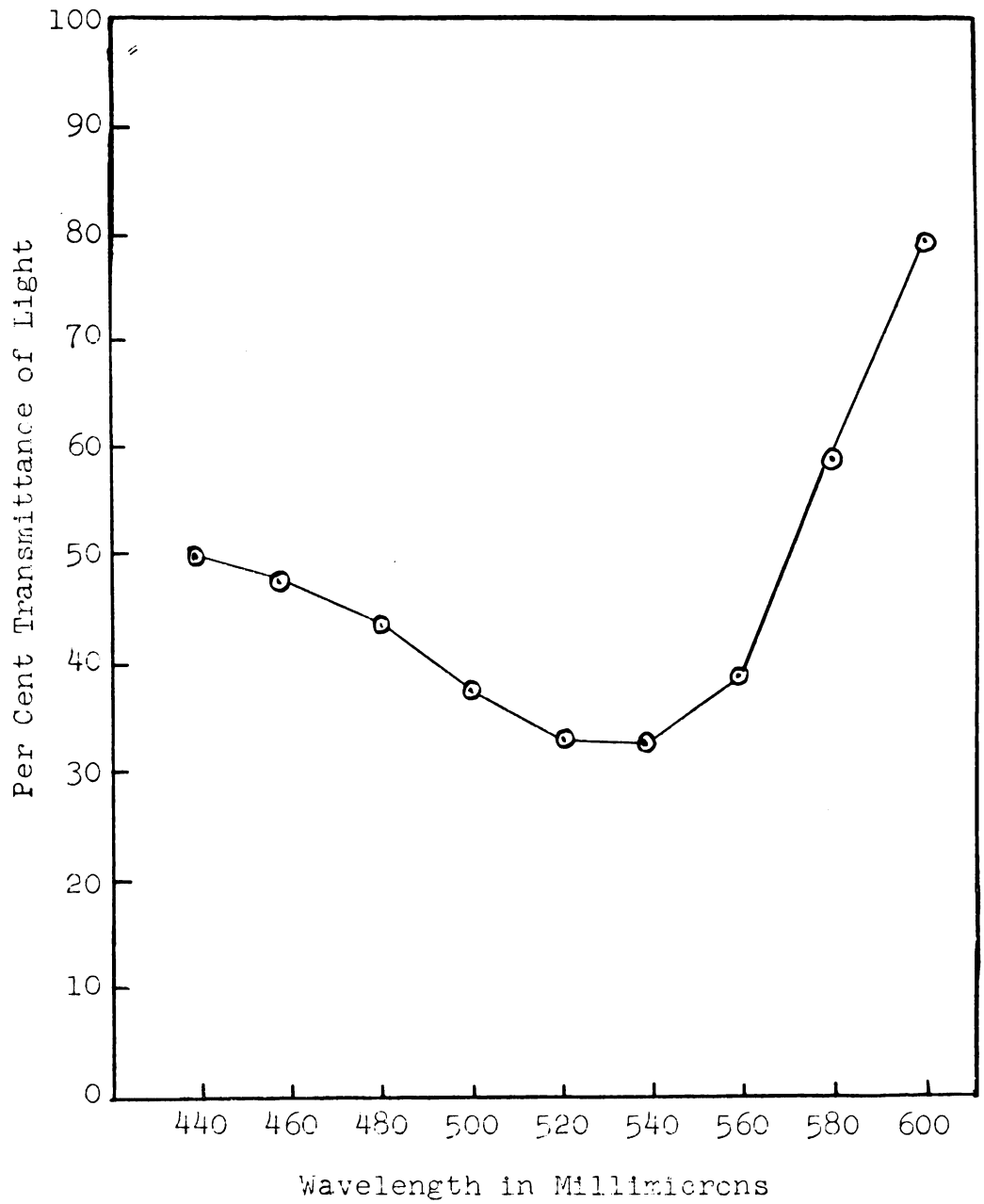

100
90
80
70
60
50
40
30
20
10
0
Per Cent Transmittance of Light
440
460
480
500
520
540
560
580
600
Wavelength in Millimicrons

Figure 2. Calibration curve for beta naphthol. Fixed increments (0.002 mg - 0.020 mg) of beta naphthol were coupled to diazo blue B. Ordinates, per cent transmittance of light at 450 mu, plotted on a logarithmic scale; abscissae, milligrams of beta naphthol. Each point on the curve is the arithmetic mean of 4 experiments.

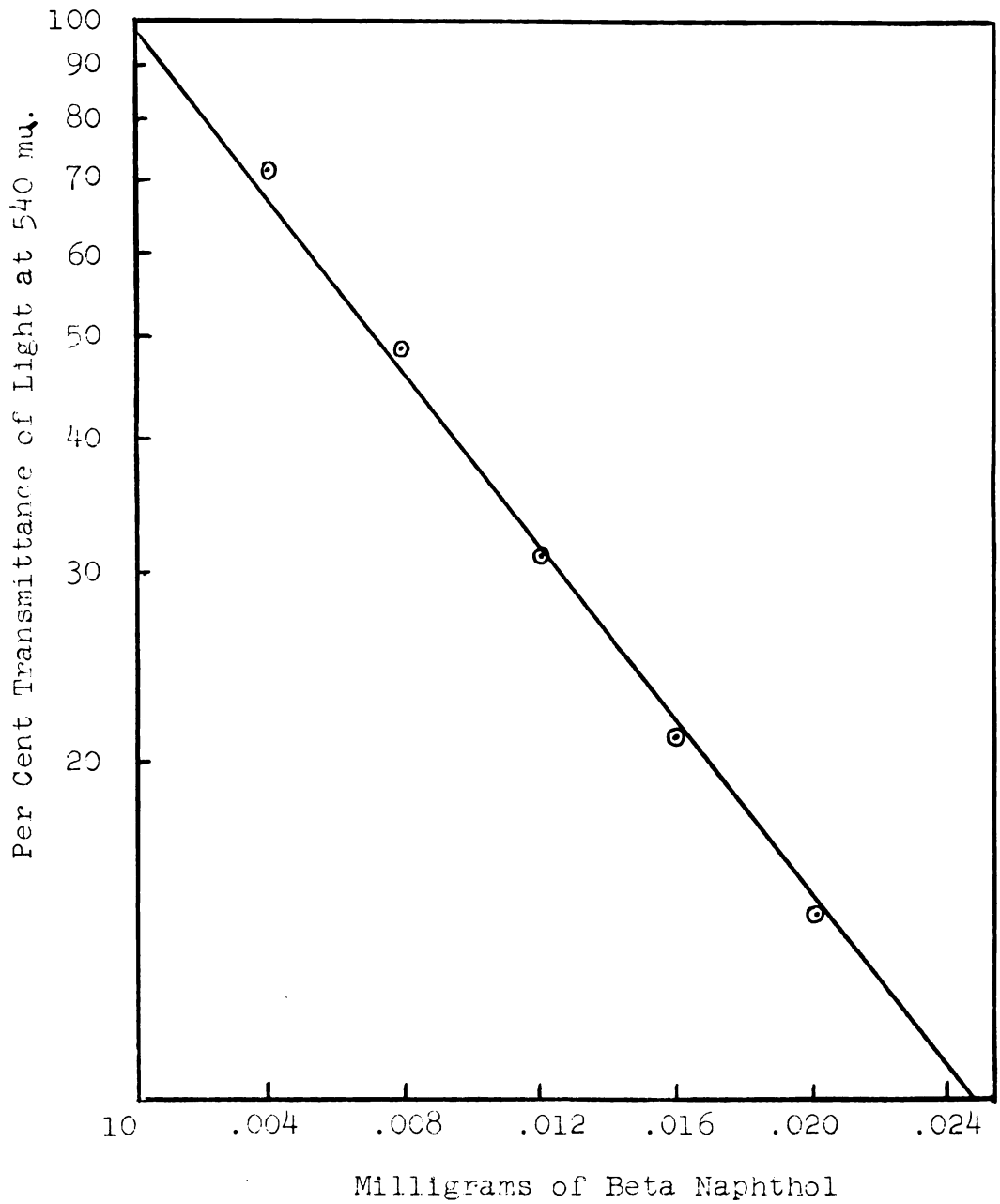

100
90
80
70
60
50
40
30
20
Per Cent Transmittance of Light at 540 mμ.
10
.004
.008
.012
.016
.020
.024
Milligrams of Beta Naphthol

TABLE I.--Quantitative measurements of esterase and lipase activity of liver, kidney, and spleen homogenates, and the effects of various inhibitors on esterase activity. Substrates, beta naphthyl acetate and beta naphthyl laurate. Values expressed as mg beta naphthol released per mg tissue per hour. Each value is the arithmetic mean of three experiments run in duplicate.

Organ	Inhibitors					
	Control	Sodium taurocholate	Eserine sulfate	Benz-aldehyde	Sodium fluoride	Sodium arsanilate
	Substrate--beta naphthyl acetate					
Liver	3.40	1.38	1.68	1.00	1.16	0.32
Kidney	0.92	0.52	0.78	0.42	0.22	0.22
Spleen	0.108	0.032	0.092	0.034	0.042	0.096
	Substrate--beta naphthyl laurate					
Liver	0.036	0.060	0.081	0.021	0.014	0.014
Kidney	0.035	0.052	0.031	0.015	0.012	0.013
Spleen	0 027	0.028	0.024	0.007	0.008	0.009

activity. The reaction with beta naphthyl laurate was much lower in all three organs than with beta naphthyl acetate.

Effect of inhibitors. When beta naphthyl acetate was used as a substrate, sodium taurocholate inhibited esterase activity of tissues in all glands. In the presence of eserine sulfate, naphthol released by liver and kidney esterase descreased from 3.40 to 1.68 mg/hr, and 0.92 to 0.78 mg/hr, respectively, whereas spleen esterase was not appreciably affected. Benzaldehyde and sodium fluoride inhibited enzyme activity of all glands. Sodium arsanilate strongly inhibited liver esterase, and moderately affected kidney, whereas spleen esterase was only slightly inhibited.

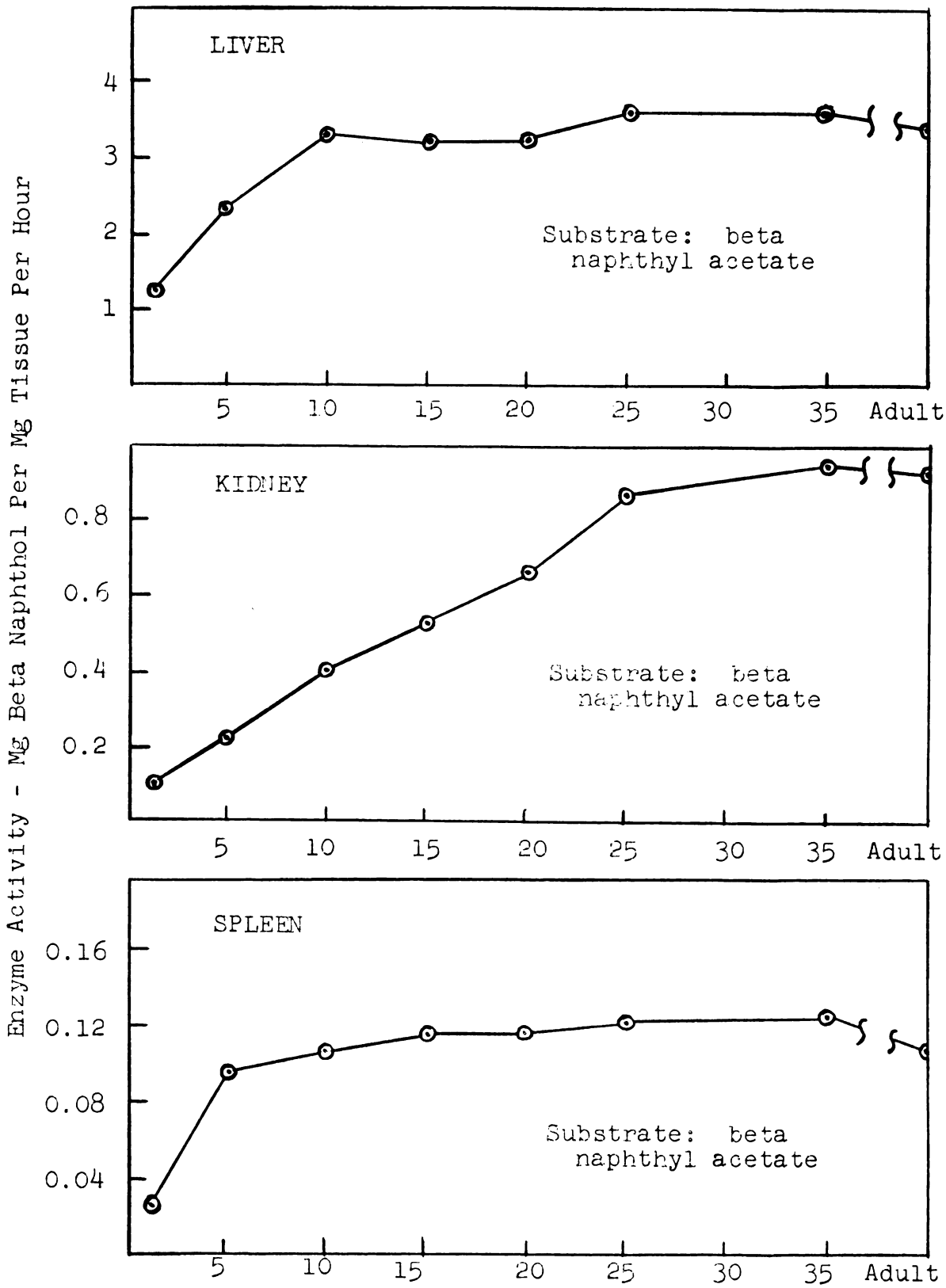
When beta naphthyl laurate was used as a substrate, sodium taurocholate accelerated enzyme activity of the liver and kidney, but had little effect on spleen esterase (Table I). Eserine sulfate stimulated esterases of spleen and kidney. Benzaldehyde, sodium fluoride, and sodium arsanilate inhibited the esterase spectrum of enzymes of tissues from all glands used for experimentation.

Quantitative measurements of enzyme activity in young animals. Table II and Figure 3 show that esterase activity of liver when incubated in beta naphthyl acetate increased from a minimum in 1 day old mice to a maximum in 35 day mice. In the younger age group, 1.2 mg of naphthol was released from the substrate per mg of tissue per hour, whereas in 35

TABLE II.--Quantitative measurements of enzyme activity of liver, kidney, and spleen homogenates of mice of various ages. Substrates, beta naphthyl acetate and beta naphthyl laurate. Values expressed as mg of beta naphthol released per mg tissue per hour. Each value is the arithmetic mean of three experiments run in duplicate.

Organ	Postpartum Age of Organism							
	1 Day	5 Days	10 Days	15 Days	20 Days	25 Days	35 Days	Adult
	Substrate--beta naphthyl acetate							
Liver	1.20	2.32	3.28	3.20	3.25	3.32	3.52	3.40
Kidney	0.10	0.22	0.40	0.52	0.65	0.86	0.93	0.92
Spleen	0.028	0.096	0.108	0.116	0.116	0.122	0.126	0.108
	Substrate--beta naphthyl laurate							
Liver	0.0125	0.0375	0.040	0.036	0.043	0.033		0.036
Kidney	0.0011	0.0038	0.009	0.012	0.018	0.033		0.035
Spleen	0.0045	0.013	0.017	0.025	0.024	0.025		0.027

Figure 3. The relation between age of mice and esterase activity of liver, kidney, and spleen when incubated in beta naphthyl acetate. Ordinates, enzyme activity expressed as mg beta naphthol liberated per mg tissue per hour; abscissae, age in days postpartum of animal. Each point on the curve represents the arithmetic mean of three experiments run in duplicate. All tissue homogenates incubated for 30 minutes at room temperature.


LIVER
4
3
2
1
Substrate: beta naphthyl acetate
5
10
15
20
25
30
35
Adult
KIDNEY
0.8
0.6
0.4
0.2
Substrate: beta naphthyl acetate
5
10
15
20
25
30
35
Adult
SPLEEN
0.16
0.12
0.08
0.04
Substrate: beta naphthyl acetate
5
10
15
20
25
30
35
Adult
Enzyme Activity - Mg Beta Naphthol Per Mg Tissue Per Hour
Age in Days

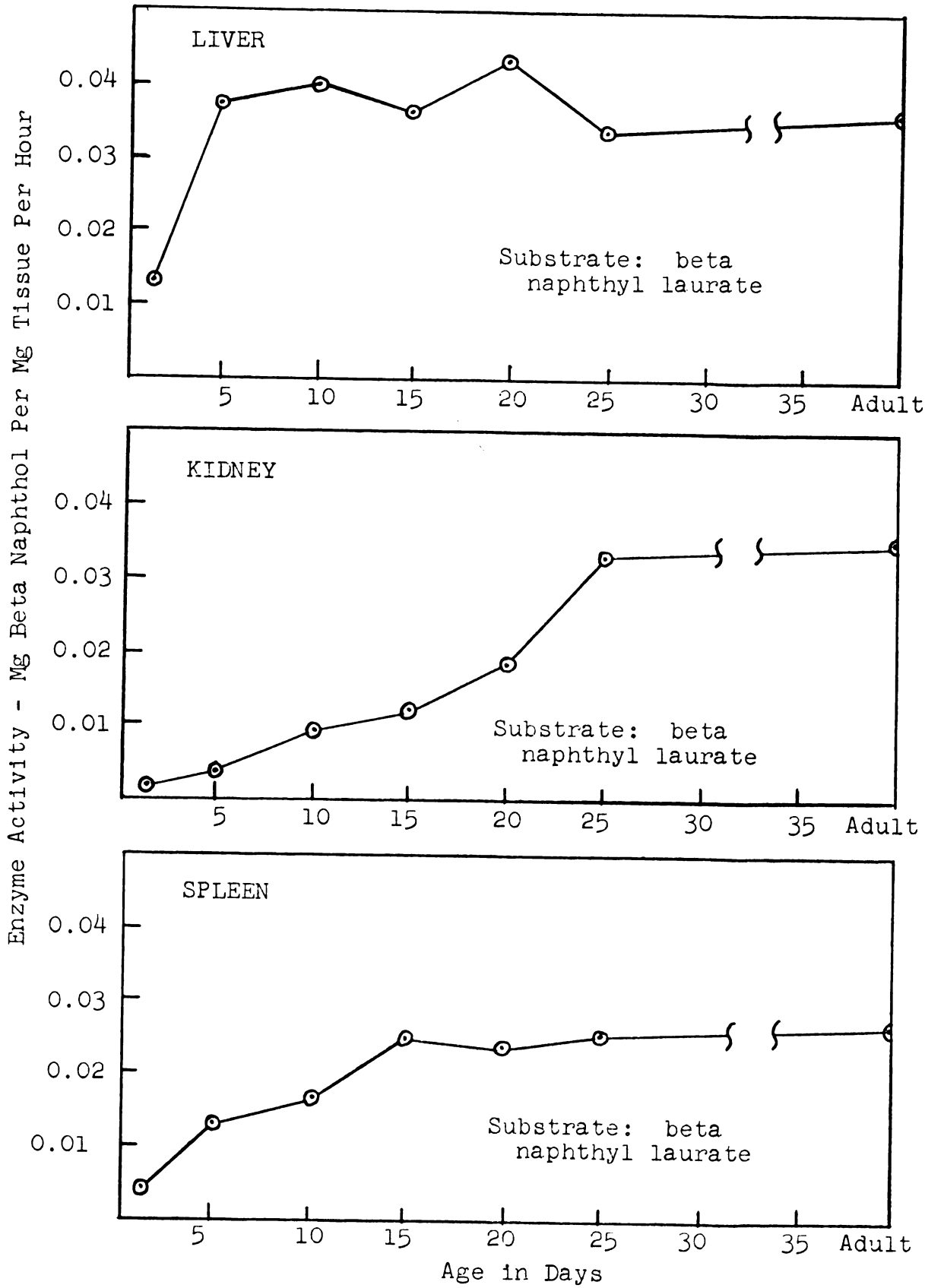
day mice 3.52 mg was released under essentially the same conditions. Adult levels of esterase activity for liver, kidney, and spleen were reached at approximately 10, 25, and 5 days, respectively.

Figure 4 shows that the adult level of esterase activity of liver tissues, when incubated in beta naphthyl laurate, was reached in 5 day old mice, whereas esterase activity of the spleen and kidney reached adult levels in mice at about 15 and 25 days of age, respectively.

When adult levels of enzyme activity were reached in these glands it varied only slightly, even in mice as old as 1 year. The sex of the animals likewise showed little influence on levels of activity. Therefore, adult levels expressed here are considered representative for tissues of any adult animal.

Electrophoretic studies of adult glands--Effect of inhibitors. After subjecting liver homogenate to electrophoretic separation, 10 distinct bands were identified when alpha naphthyl acetate was used as a substrate and fast garnet GBC as a coupler (Fig.5). The bands were numbered L1 to L10 inclusive, beginning with the band farthest from the origin. When filter paper was used to suspend the homogenate the same number of components was obtained, but the results were much less uniform than when the homogenate was suspended in crude potato starch. In all electrophoretic studies there was a strong esterase reaction at the site of

Figure 4. The relation between age of mice and esterase activity of liver, kidney, and spleen when incubated in beta naphthyl laurate. Ordinates, enzyme activity expressed as mg beta naphthol liberated per mg tissue per hour; abscissae, age in days postpartum of animal. Each point on the curve represents the arithmetic mean of three experiments run in duplicate. All tissues incubated for 240 minutes at 37.5°C.


LIVER
0.04
0.03
0.02
0.01
Substrate: beta naphthyl laurate
5
10
15
20
25
30
35
Adult
KIDNEY
0.04
0.03
0.02
0.01
Substrate: beta naphthyl laurate
5
10
15
20
25
30
35
Adult
SPLEEN
0.04
0.03
0.02
0.01
Substrate: beta naphthyl laurate
5
10
15
20
25
30
35
Adult
Enzyme Activity - Mg Beta Naphthol Per Mg Tissue Per Hour
Age in Days

insertion, indicating that one or more esterases did not migrate in the electrical field.

The intensity of enzymatic activity of the separate bands varied considerably in a single experiment, but each band was essentially similar in degree of mobility and intensity in repetitive experiments.

The individual bands of the liver zymograms showed great variation in sensitivity to the inhibitors. In low concentrations sodium taurocholate was without visible effect on the activity of the components, but in higher concentrations all bands were equally inhibited. Eserine sulfate moderately inhibited bands L1 and L2 and completely inhibited bands L3 and L4. The remaining bands were unaffected. Bands L4 and L5 were completely inhibited by benzaldehyde and the activities of the remaining bands were affected to a moderate degree. Sodium fluoride completely inhibited bands L3, L4, L9, and L10, and affected the other bands slightly. Finally, sodium arsanilate completely inhibited bands L1 and L4 and slightly inhibited band L2.

Five esterase active components in adult kidney homogenate were separated electrophoretically and proved to be resistant to most inhibitors (Fig. 6). Sodium taurocholate, eserine sulfate, and benzaldehyde were without noticeable effect on the bands. Sodium fluoride inhibited band K5 and sodium arsanilate slightly inhibited band K1.

Adult spleen homogenate, following electrophoretic separation, showed only 3 distinct components (Fig. 7).

Sodium taurocholate was without effect, whereas eserine sulfate slightly inhibited bands S1 and S3. Benzaldehyde markedly inhibited band S1 and sodium fluoride moderately inhibited all 3 bands. Sodium arsanilate completely inhibited band S1.

Esterase patterns in zymograms of young animals. Tissues of liver, kidney, and spleen, of young animals from birth to 30 days of age and embryonic liver were subjected to electrophoresis at 5 day age intervals. In all glands the number of esterases increased with age.

Liver tissues removed from an embryo with a crown-rump length of 20.5 mm produced 5 bands which corresponded roughly in position to bands of adult liver following electrophoretic separation (Fig. 8). A 6th band appeared at 1 day of age, the 7th at 5 days, the 8th at 10 days, and the 9th and 10th at 30 days.

Kidney homogenate of 1 day old mice produced 4 electrophoretically mobile esterases (Fig. 9). This same number of esterases persisted until the mice reached 30 days of age, when the number increased to 5 bands, and then remained constant throughout the life of the individual.

Two bands appeared in zymograms of spleen homogenate of 1 day old mice (Fig. 10). The most mobile band increased in activity through the 25th day. A 3rd band appeared in the 30 day age interval.

Histochemical localization of esterases. The combination of alpha naphthyl acetate and diazo blue B, when used for incubation of tissue sections, produced a strong azo dye precipitate in esterase active areas of individual cells. The azo dye was violet in color and not well localized in the tissues (Fig. 13). The reaction proceeded at such a rapid rate that considerable diffusion of the dye was apparent. Clouds of precipitate were released into the incubation medium and agitation of the medium was necessary to facilitate uniformity of staining in the highly reactive areas of the cells.

The reaction in the presence of alpha naphthyl acetate and fast red TR coupler proceeded at a much slower rate and better localization of enzyme activity was observed (Fig. 12). The precipitate was yellowish brown in color and limited to discrete particles within the cytoplasm of the cells.

The most precise localization was obtained with 5-bromoindoxyl acetate as substrate. Sites of enzyme activity were localized as small, deep blue granules evenly distributed throughout the cytoplasm (compare Figs. 12, 13, with 14). With all 3 localization techniques the enzyme activity was limited to the cytoplasm and in no instance were esterase positive areas localized in the nuclei of cells.

The reaction was strong in all parts of the liver except in arteries, veins, and bile ducts, which did not show visible reactive areas (Fig. 14). The intensity of the reaction was slightly stronger in the liver cords surrounding the central vein than in tissue adjacent to the portal veins (Fig. 15). Large crystals of precipitate were always observed on the surface of fat globules within the cells when 5-bromoindoxyl acetate was used in substrate. These crystals appeared to grow from a single locus to cover a large part of the globule (Fig. 17).

In liver sections of embryonic and young animals many hemopoietic loci were identified. Megakaryocytes were seen interspersed between the cords of liver parenchymal cells. Clusters of cells with deep staining nuclei were also abundant. These cells exhibited morphological features of normoblasts (Figs. 18 and 19) and, along with the megakaryocytes, consistently showed no esterase activity.

In embryonic liver the esterase active cells were randomly distributed among the more numerous immature blood cells (Fig. 22). Subsequent to birth the hemopoietic cells decreased in number and could not be identified in tissue sections of 15 day old mice (Fig. 16).

Fat globules were also large and abundant within liver cells of newborn animals, but both size and number of globules had a tendency to decrease in older animals (Fig. 20).

In the adult spleen esterase activity was limited to a small number of isolated cells located mainly in the white pulp. Some of these enzymatically active cells were arranged to form a circular pattern within the white pulp, whereas other of these cells were distributed at random throughout the organ (Fig. 24). The substrate and coupler of choice was alpha naphthyl acetate and diazo blue B. The enzyme activity of pulp spleen cells was low and this combination produced a visible reaction, even though there was considerable diffusion of the reaction product (Fig. 25).

In the spleen of newborn and young mice the circular arrangement of esterase active cells was never observed. On the other hand, esterase active cells were randomly distributed throughout the tissues (Fig. 27, 28). Megakaryocytes were also commonly seen in these sections but showed no esterase activity (Fig. 28).

Esterase activity of the adult kidney was limited mainly to the convoluted tubules and the loop of Henle of nephrons. The glomerulus was free of enzymes as were all branches of the renal arteries and vens (Fig. 30, 32), but Bowman's capsule showed intense activity (Fig. 36). The proximal and distal convoluted tubules were highly active as was the straight segment of the thick descending limb of Henle's loop (Fig. 33). The thin ascending segment, where identifiable, was only slightly reactive. The collecting tubules comprising a portion of the medullary

rays and those of the medulla were either negative or only slightly positive (Fig. 32).

In the kidney of the newborn animals only a small number of nephron elements reacted positively and these were located mostly in the juxtamedullary region (Fig. 39). Many tubules, located between the active nephrons, did not display any enzyme activity. Furthermore the entire area of the outer cortex was negative (Fig. 34, 38).

DISCUSSION

Quantitative measurements of adult organs. Various substrates have been employed to quantitatively measure the activity of esterases and lipases in organ homogenates. The esterases preferentially act upon compounds possessing fatty acids of short chain length, whereas lipases have a predilection for longer fatty acid esters.

Svanborg (1953) utilized Tween 20 and 40 to measure esterase activity of mouse liver and kidney. Both glands showed appreciable activity. Liver tissue was 1 to 2 times as active as kidney in the presence of the first substrate and 3 times more active when Tween 40 was used. In both organs Tween 20 was hydrolyzed more rapidly than Tween 40, indicating that the predominant enzyme was esterase(s).

Nachlas and Seligman (1949) studied the esterolytic powers of liver, kidney, and pancreas of 6 species of animals with 3 substrates of beta naphthol of varying fatty acids (acetate, laurate, and palmitate--stearate). Kidney and liver (esterase) hydrolyzed the acetate most readily and showed a reduced activity toward the laurate. Pancreas (esterase and lipase) hydrolyzed all 3 substrates.

Huggins and Moulton (1948) measured the esterase activity of liver, kidney, and spleen on p-nitrophenyl

proprionate (C_3). This substrate was hydrolyzed very readily by liver enzymes, less by kidney, and least by spleen.

Similar results were produced in the present study with beta naphthyl acetate as substrate Liver was most reactive of the 3 organs with kidney and spleen showing much lower levels of activity. The inhibition of activity by sodium taurocholate, sodium fluoride, and sodium arsanilate, all esterase inhibitors, indicates that the activity was due largely to esterase and not lipase. Furthermore, the inhibition by eserine sulfate indicates that some cholinesterase(s) exists in these organs.

Enzymes of adult organs hydrolyzed beta naphthyl laurate much less readily, indicating low amounts of lipase. In the case of liver and kidney, sodium taurocholate accelerated activity, suggesting that enzyme activity was due, in part, to lipase. It is interesting to note that taurocholate was without effect on spleen esterase, indicating that lipase may not exist in this gland. Gomori (1946), using Tweens, also failed to demonstrate lipase activity in histological sections of spleen.

Electrophoretic separation of enzymes in adult tissues. Markert and Hunter (1959), using starch gel electrophoresis, produced zymograms of a number of mouse tissues and demonstrated 10 distinct bands of esterase activity in liver,

5 bands in kidney, and 7 bands in spleen. They also showed a sensitivity of certain of these bands to eserine sulfate. Allen et al. (1958) have shown 11 bands to exist in zymograms of rat liver. In the present study zymograms of liver, kidney, and spleen exhibited 10, 5, and 3 bands, respectively.

The significant feature of all of these studies was the fact that the total esterase activity of an organ was not due to a single esterase or even a small group, but rather to a complex array of enzymes, each of which contributes partially to the total activity. When the results of these various studies are compared it is evident that the zymograms from similar tissue of different organisms, even of closely related species, are remarkably similar in number, intensity of activity, and electrophoretic mobility of the individual components. However, different organs from a single individual produce distinctly different patterns of position and activity.

Further evidence that esterases are not molecularly identical was shown by their activity toward the alpha naphthyl acetate substrate and their sensitivity to inhibitors. Thus, bands 1, 2, and 3 of the zymograms of the various organs studied showed similar electrophoretic mobility, but reacted in a distinctly different manner to the substrate as indicated by the variations in staining intensities of the bands. Furthermore, these components did not show similar sensitivities to the inhibitors. To cite

a specific example, band L3 of the liver was completely inhibited by eserine sulfate, while in the spleen S3 was only slightly affected, and in the kidney band K3 was not visibly affected.

Pronounced sensitivity of band L3 to eserine suggests that this enzyme is a cholinesterase. Hunter and Markert (1957) also demonstrated a band from the liver in this same position (labeled band G in their study) to be eserine sensitive. Allen et al. (1958) reported similar results by use of eserine on rat liver, but could show no corresponding sensitivity in the zymograms of adrenal medulla. They concluded that this lack of correlation may indicate the presence of distinctly different esterases in these 2 organs.

It appears, therefore, that each organ has within its cells a group of enzymes which are unique to that organ and do not exactly correspond to enzymes of other organs.

The enzymes which migrate in the starch gel column are not a complete representation of all the esterases within these tissues, since an appreciable amount of enzyme activity could be observed at the site of sample insertion at the end of the electrophoretic period. Markert and Hunter (1959) demonstrated that this electrophoretically immobile component was made up of approximately 50 per cent of the total esterase activity of the homogenate. It is possible that much of this enzyme activity at the origin

represents some of the same enzymes which migrate, but remain behind as a result of strong adsorption by the starch used for suspension of the sample (Hunter and Burstone, 1960).

On the other hand, the mobile component could represent the more soluble enzymes in the homogenate, whereas the immobile fraction could be composed of insoluble proteins, or enzymes that are bound tightly to particulate structures within the cells, and as a consequence too large to migrate through the starch gel.

The terms lyoenzymes and desmoenzymes, first used by Willstätter and Rhodewald (1932), have been applied to these two fractions by Nachlas et al. (1956). Using a quantitative technique in tissue sections, these authors have shown that, in the absence of fixation, about 51 per cent of the lyoenzymes diffused out of the tissue slice within 20 minutes. The desmoenzymes which remained behind were largely responsible for the reaction which takes place in histochemical studies of enzyme distribution within tissue sections. This percentage agrees closely with the measurements cited above in the starch gels. It was also shown in this study that various fixatives markedly reduced the quantity of diffusible enzymes.

Further work is needed to ascertain the nature of this immobile fraction in the electrophoretic studies. Tissues could be subjected to fixation prior to homogenization and electrophoresis in an attempt to alter the amount of mobile

components. The new technique of disc electrophoresis, where the "effective pore size" of the polyacrylamide gel (Canal Industrial Corporation) can be varied, might be used to measure the size of the components presently immobile in starch gel.

The nature of the mobile esterases can, in part, be interpreted from the studies employing inhibitors. In low concentrations sodium taurocholate (10^{-3}M) was without effect upon the enzyme activity. In higher concentrations, however, all mobile bands appeared to be equally inhibited. Since this compound has been shown to be an inhibitor of esterase and an accelerator of lipase, the results would indicate that these mobile bands are all of the esterase classification.

However, when the effects of the various inhibitors on the activity of the bands are compared a confusing picture emerges. Sodium taurocholate, sodium fluoride, and sodium arsanilate, which are frequently employed as inhibitors of esterase, show completely different patterns of inhibition. Some bands that were markedly affected by one compound were untouched by another (compare bands L8, L9, and L10 of liver in the presence of fluoride and arsanilate, Fig. 5). Benzaldehyde, known as an inhibitor of lipase, affects many of the same enzyme bands as the esterase inhibitors. These results indicate that classification of the esterases based upon use of inhibitors is by no means

adequate and should be viewed with caution.

Electrophoretic studies of developing organs. Results of the electrophoretic experiments on tissues from various ages of young mice clearly demonstrated that there was a progressive addition of distinctly new bands of enzyme activity as well as an increased activity of those already present. This was most apparent in liver tissue, where the number of bands doubled, and less so in kidney and spleen, where only slight changes in zymogram patterns were noted.

The addition of new esterases seems to be a result of developmental changes occurring within the organs, since a fixed zymogram pattern was reached and maintained constant as the animal approached maturity, and remained so even in mice as old as 1 year.

New bands of activity seemed to appear in two different ways. The position and intensity of bands L8 and L9 in the liver zymograms indicate that they might have been produced as a result of a splitting of a single band of the next younger age interval. This was also true of bands K4 and K5 in the kidney zymograms. On the other hand, band L5 in the liver began as a faint region of activity where none existed in zymograms made with tissues of younger mice. It is interesting to note that band L8 was only partially inhibited by sodium fluoride, whereas band L9 was completely inhibited.

In order to produce these results the enzyme located at the site of the original band would have to be modified in its net molecular charge, which would alter the electrophoretic mobility. Alteration of the active sites of the enzymes would also be necessary to cause the differing sensitivities to the inhibitors.

Markert and Møller (1959) have also shown changes in zymograms of embryonic and adult pig tissues in a study of lactic dehydrogenases. They suggest a number of possible hypotheses to explain their findings. Enzyme-forming sites may produce a variety of different proteins at progressive periods of ontogeny depending upon the raw materials available to it, or the protein-forming mechanism itself may change structurally, causing the variation in enzyme production. After the enzyme molecule has been produced its activity could be altered by its structural rearrangement within the maturing cell. Various methods of attachment might change the net molecular charge and the electrophoretic mobility of the molecule.

Just what the developmental changes are that occur within the cells to produce these new bands of activity is unclear and existing knowledge of genetic regulation of enzyme synthesis does not account for these phenomena.

Comparison of quantitative studies and localization of enzymes in developing organs. Liver. In the developing liver of most mammalian species the important function of

hemopoiesis during the embryonic growth of the organism gradually subsides, so that at the time of birth little or no evidence of this process is found normally (Bloom, 1938). However, in the mouse, blood forming elements persist in this organ for some time after parturition. Deane (1944) reported mouse liver to be composed about equally of liver parenchyma and hemopoietic cells at birth.

During certain periods of fetal life, blood formation in the liver may reach such an intense degree that the parenchyma becomes distorted and often is quite inconspicuous among the masses of developing blood cells (Bloom, 1938). These hemopoietic islands of cells gradually decrease as the animal ages and have been reported to disappear between the 9th and 13th day of life (Deane, 1944).

In the present study the disappearance of these blood forming cells followed a similar pattern. They were present in large numbers in embryonic tissues and at birth. At 10 days of age the number had decreased considerably, until at 15 days they could not be demonstrated.

Another prominent feature of fetal and immature liver tissue is the large amount of fat present, reaching a maximum level at time of parturition (Stieve and Kaps, 1937). At this time large fat globules can be observed filling many cells. In the rat (Rice and Jackson, 1934) they appear as medium sized droplets in both parenchyma and Kupffer cells. The size and number of these globules gradually

decrease in this animal, and by 4-1/2 weeks of age there is little fat visible. In the mouse (Doljanski, 1960) this decrease continues for 1 to 2 weeks, at which time the adult liver lipid concentration is reached.

By applying Sudan black stain to the liver sections in the present study, large globules were made visible in tissues from animals of 1 day of age. Their presence was still quite evident at 10 days. Beyond 15 days of age the fat was limited to very small droplets evenly distributed throughout the cells.

The data from the quantitative colorimetric studies show a gradual increase of esterase activity up to the 10th day of age, when adult levels are reached. However, if a comparison is made of histological sections in the various ages of tissue studied (Figs. 14, 16, 17, 21, and 22) it appears that there is no great increase in enzyme activity within the individual cells. Electrophoretic studies of young tissues revealed that part of the increase in activity might be due to an addition of new esterases, but probably does not account for the threefold increase indicated by the quantitative studies. The increase in esterase activity, therefore, does not seem to be merely a result of addition of enzymes within the active cells.

Observations cited previously in this study demonstrated that the fat globules and hemopoietic cells were esterase inactive. Moloney et al. (1960) have also shown

that megakaryocytes and red blood cell precursors in human bone marrow smears are inactive. The presence of these cells in large quantities within embryonic and newborn liver tissue would therefore serve to dilute the enzyme activity within the parenchyma cells, producing low readings in quantitative studies. This diluting influence would lessen with the gradual disappearance of this inactive material, thereby increasing total activity.

Spleen.--In histological sections of the spleen from newborn mice only isolated areas of esterase activity could be demonstrated and they appeared as discrete particles within widely scattered cells. Megakaryocytes and hemopoietic cells were also present. These cells are commonly seen since the spleen is an important accessory blood forming organ in embryos and young individuals (Jordan, 1938).

The small amount of esterase activity in the sections was reflected in the low readings obtained in the quantitative studies. The gradual increase in activity during the first 15 days of life corresponded to the appearance of increased number of esterase active cells. The latter were not identified as to cell type, nor was the significance of their peculiar circular arrangement in the white pulp apparent.

Wachstein et al. (1961) have reported staining of mononuclear cells in the red and white pulp in rabbit and rat spleen. In addition there was striking staining of the

sinusoids in the rabbit. Pearson and Defendi (1957) suggest that esterase active cells in the spleen may be macrophages, based upon their size and random distribution, but carefully point out that this has yet to be demonstrated.

Kidney.--Localization of esterase activity within the adult kidney in many animals has been intensively studied and details of the distribution of these enzymes are well known.

Gomori (1946) using the Tween technique, Nachlas and Seligman (1949) with beta naphthyl acetate, and Barrnett and Seligman (1951) with indoxyl acetate all reported similar localization patterns within kidney. All parts of the nephron reacted positively. The proximal and distal convoluted tubules showed marked activity, while the other tubule elements were moderately active. Glomeruli and all medullary collecting tubules were only weakly positive, or negative.

Recently a number of workers have produced detailed studies of precise intracellular localizations of esterases within various cells of the kidney. The reader is referred to papers by Holt and Withers (1958), Wachstein (1955), Wachstein et al. (1961), and Schnitka and Seligman (1961).

The localization of esterase activity in the various regions of the nephron of the adult kidney already described in this study agrees closely with reports cited above. No major variation in localization patterns could be noted.

Before an attempt is made at correlating the quantitative increase in emzyme activity with the histochemical

localization in the various ages of kidney tissue, the details of maturation of this organ should be discussed. In the embryo clusters of metanephrogenic cells form in small vesicular masses around the terminal branches of the growing collecting duct. Each of these cell masses develops into the secretory portion of the uriniferous tubule and finally connects to the collecting duct (Patten, 1958).

During this development the tubule takes on a characteristic S-shape and cell nuclei are large and stain deeply with hematoxylin. New nephrons continue to arise from the embryonic tissue around the branching upper ends of the tree-like straight collecting tubules. Thus, the youngest and least well developed nephrons are found in a zone in the outer cortex of the kidney, i.e., the nephrogenic zone (Valdes-Dapena, 1957).

In the rat, nephrogenesis continues at a rapid rate for 2 weeks after birth. Kittleson (1917) has shown that the number of nephrons doubles during this postnatal period.

Baxter and Yoffey (1948) have studied uptake and storage of trypan blue in the nephrogenic zone, demonstrating that the cells of this region do not stain with this vital dye as do the proximal convoluted tubules in the adult organ. They concluded that the ability to store trypan blue must be related to the functional differentiation of the nephron and that the developing tubules were not yet active. With older animals the amount of dye storage in the nephrogenic zone

increased, and at the end of the 4th week it was maximal in all layers of the cortex.

In the present study developing nephrons were common in the outer region of the cortex and could be easily identified in hematoxylin and eosin stained sections by their characteristic shape and deep staining nuclei. The amount of nephrogenous tissue gradually declined with age so that by the 15th day it could not be demonstrated.

If adjacent sections were stained, one with hematoxylin and eosin, and the other for esterase, it could be shown that the developing tubules were not active enzymatically (Fig. 34 and 38). Verne et al. (1952), using Tweens, reported a lack of esterase activity in the nephrogenous zone, termed the cortex corticus by them. It is interesting to note that the appearance of esterase activity coincided with the appearance of cells with phosphatase activity, both enzymes showing feeble activity at first and gradually increasing. It is possible that the neprhon remains inactive until it connects with the collecting tubule, although no evidence can be presented in support of this view.

Quantitative data in this study show a gradual, almost linear increase in esterase activity as the kidney matures. It seems likely that this increase is due to the continued addition of an increasing number of active nephrons at a uniform rate as they are formed and mature in the cortical regions of the kidney.

However, this production of nephrogenous tissue cannot be demonstrated in histological sections beyond the 15th day, whereas the quantitative increase in enzyme activity continues until 25 days of age.

Allen (1951) maintains that growth in size of the human kidney results from elongation of the tubules of the nephron following the period of nephrogenesis. It is probable that the increase in esterase activity in the later stages of maturation of the kidney is caused by this increase in length of tubules, implying a parallel increase in number of esterase active cells.

SUMMARY

1. Quantitative measurements of esterase and lipase activity were made on homogenates of liver, kidney, and spleen of adult C 57 Bl/6 mice. Levels of activity compared closely with previous reports in a variety of animals. Liver was most reactive, with kidney showing a much lower level of activity, and spleen with the least activity.

2. Application of inhibitors to the homogenates indicated that enzyme activity was due largely to esterase in the liver and kidney, and that lipase may not exist in the spleen.

3. Electrophoretic separation of enzymes in adult tissues produced zymograms consisting of 10 bands in the liver, 5 bands in kidney, and 3 bands in spleen. Sensitivity of band L3 of the liver to eserine sulfate indicated that it was a cholinesterase.

4. The electrophoretically separated enzymes were exposed to sodium taurocholate, sodium fluoride, sodium arsanilate (esterase inhibitors), eserine sulfate (cholinesterase inhibitor), and benzaldehyde (lipase inhibitor). No uniform pattern of sensitivity of bands in the zymograms was apparent, indicating that use of inhibitors for classification of esterases is not entirely adequate.

5. The enzyme activity of organs from young mice was measured quantitatively at 5 day age intervals from birth to maturity. In all 3 organs there was a gradual increase in esterase activity, reaching adult levels at about 10 days in liver, 25 days in kidney, and 5 days in spleen, remaining relatively constant thereafter.

6. Electrophoresis of homogenates of tissues of young mice demonstrated an increase in number of esterase bands at definite age intervals. This increase was most pronounced in liver, where the number of bands doubled, and less so in kidney and spleen. Two different means of formation of new bands are indicated, one by an apparent molecular change in a single band, producing two different enzymes, and also by the formation of an esterase where none existed previously. Possible changes in cellular mechanisms that might produce these results are discussed.

7. Esterase enzyme activity was localized in the parenchyma cells of embryonic and young liver sections. Large numbers of esterase inactive hemopoietic cells and fat globules were also identified. As the organism matures, the number of hemopoietic cells gradually decreases, until at 15 days of age they could not be demonstrated. Quantitative increase in enzyme activity of the liver is probably due partly to the addition of new esterases within each cell, as demonstrated by the electrophoretic studies, and partly to the decrease in enzymatically inactive cells, thereby removing their diluting influence.

8. Esterase activity in histological sections of adult spleen was localized in cells arranged in a circular pattern within the white pulp. This pattern was not apparent in tissues from young animals up to 15 days of age.

9. Esterase activity in the kidney was limited mainly to the proximal and distal convoluted tubule and Bowman's capsule. The glomerulus was negative, and low activity was observed in the loop of Henle and all parts of the collecting tubule. This pattern of localization agrees closely with many previous studies of this gland in a large number of species.

10. In the kidney of newborn animals, only a few nephrons located in the juxtamedullary region showed esterase activity. The nephrogenic tissue in the outer regions of the cortex was negative. Quantitative increase in esterase enzyme activity during the early developmental period is probably a result of a gradual addition of new neprhons as they attach to the collecting ducts, mature, and become active. During the latter stages of development of this organ the continued increase of enzyme activity may be due to elongation of active nephrons.

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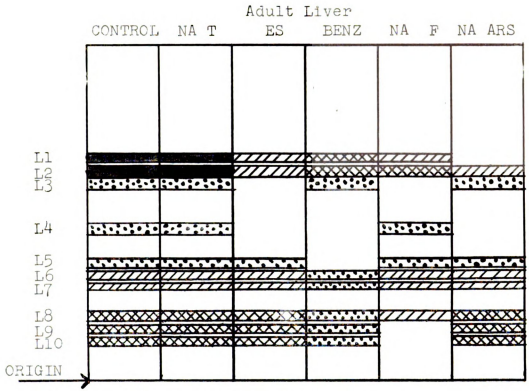
Figure 5a. Adult liver. Diagrammatic representation of zymograms showing the sensitivity of esterases to various inhibitors. Location, size, and intensity of the bands are indicated by degrees of stippling and crosshatching. Inhibitors employed were; sodium taurocholate (NA T), final concentration, 10^{-2}M; eserine sulfate (ES), 10^{-3}M; benzaldehyde (BENZ), 2.8×10^{-2}M; sodium fluoride (NA F), 10^{-2}M; sodium arsanilate (NA ARS), 10^{-3}M.

Figure 5b. Photograph of a sample of zymograms from which the above diagram was interpreted.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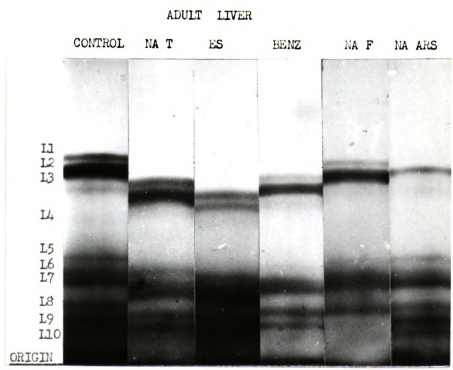
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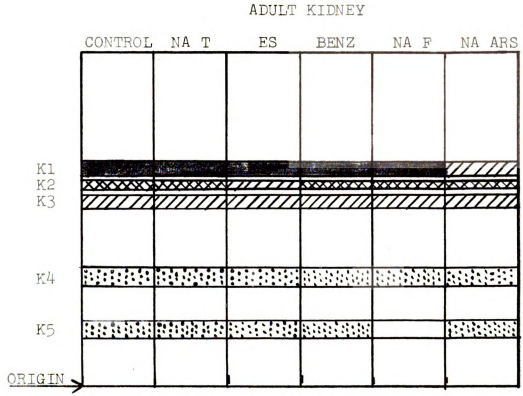
Figure 6a. Adult kidney. Diagrammatic representation of zymograms showing the sensitivity of esterases to various inhibitors. Location, size, and intensity of the bands are indicated by degrees of stippling and crosshatching. Inhibitors employed were: sodium taurocholate (NA T), final concentration, 10^{-2}M; eserine sulfate (ES), 10^{-3}M; benzaldehyde (BENZ), 2.8×10^{-2}M; sodium fluoride (NA F), 10^{-2}M; sodium arsanilate (NA ARS), 10^{-3}M.

Figure 6b. Photograph of a sample of zymograms from which the above diagram was interpreted.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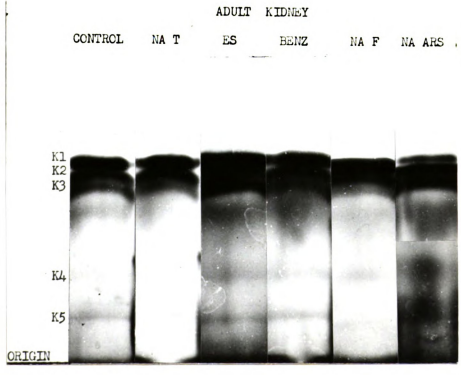
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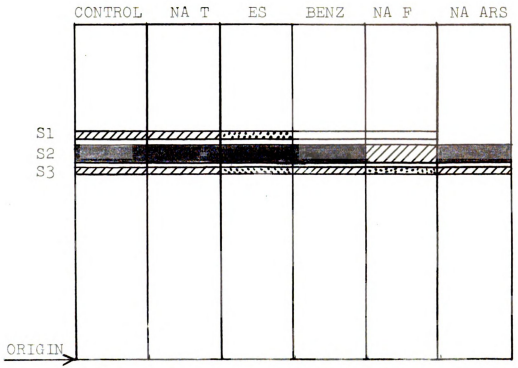
Figure 7a. Adult spleen. Diagrammatic representation of zymograms showing the sensitivity of esterases to various inhibitors. Location, size, and intensity of bands are indicated by degrees of stippling and crosshatching. Inhibitors employed were: sodium taurocholate (NA T), final concentration, 10^{-2}M; eserine sulfate (ES), 10^{-3}M; benzaldehyde (BENZ), 2.8×10^{-2}M; sodium fluoride (NA F), 10^{-2}M; sodium arsanilate (NA ARS), 10^{-3}M.

Figure 7b. Photograph of a sample of zymograms from which the above diagram was interpreted.

ADULT SPLEEN



ADULT SPLEEN

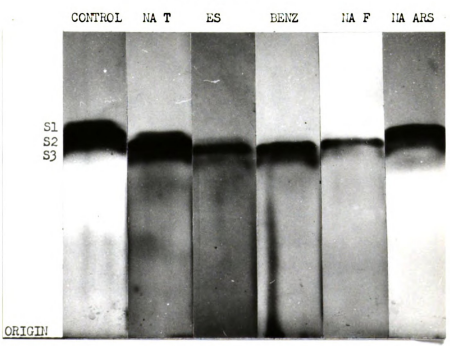
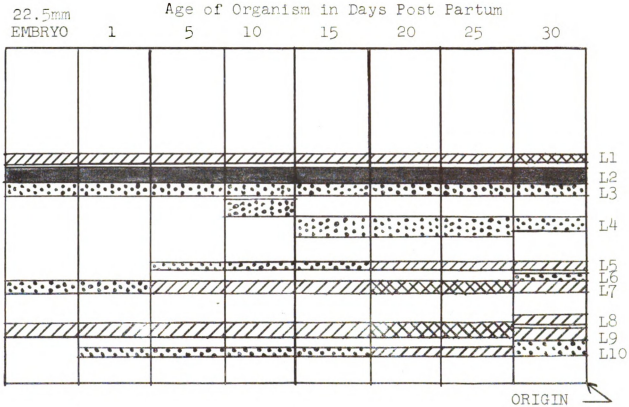


Figure 8a. Liver. Diagrammatic representation of zymograms from tissues of various ages of mice showing the change in size, position, and intensity of existing bands and appearance of new bands as the organism matures. Numbering of the adult bands is shown at the right.

Figure 8b. Photograph of a sample of zymograms from which the above diagram was interpreted.

LIVER



LIVER

AGE OF ORGANISM IN DAYS POSTPARTUM

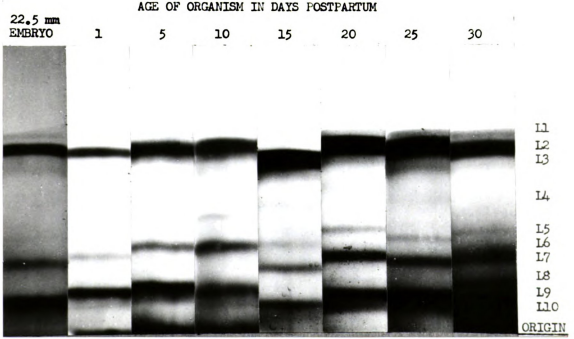
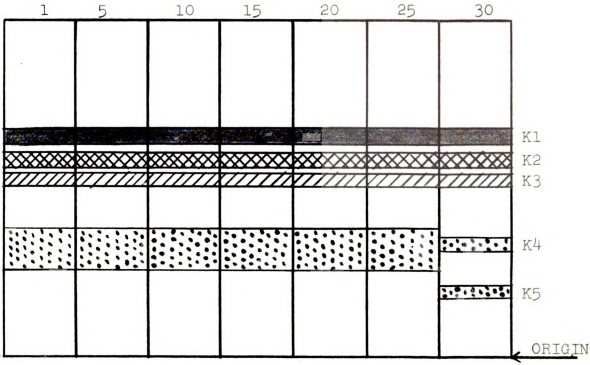


Figure 9a. Kidney. Diagrammatic representation of zymograms from tissues of various ages of mice showing change in size, position, and intensity of existing bands and appearance of new bands as the organism matures. Number of the adult bands is shown at the right.

Figure 9b. Photograph of a sample of zymograms from which the above diagram was interpreted.

KIDNEY

Age of Organism in Days Post Partum



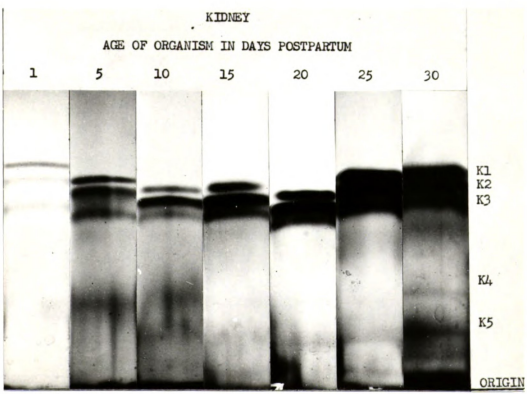
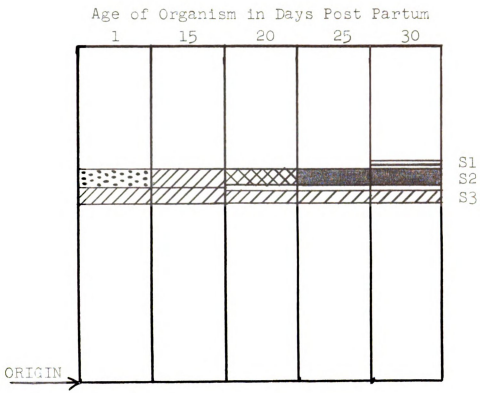


Figure 10a. Spleen. Diagrammatic representation of zymograms from tissues of various ages of mice showing change in size, position, and intensity of existing bands and appearance of new bands as the organism matures. Numbering of the adult bands is shown at the right.

Figure 10b. Photograph of a sample of zymograms from which the above diagram was interpreted.

SPLEEN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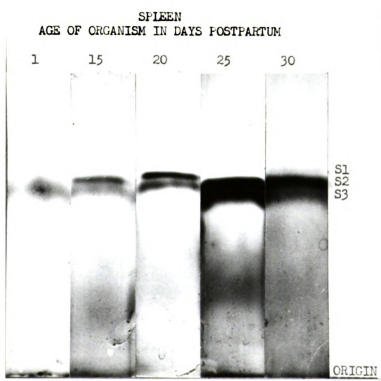
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Figure 11. Adult liver. Hematoxylin and eosin stain. X430.

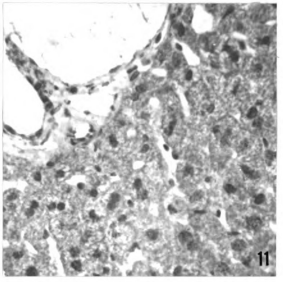
Figure 12. Section adjacent to the previous figure. Alpha naphthyl acetate substrate, fast red TR coupler. Activity is localized as discrete granules within the parenchymal cells, blood vessels are negative. X430.

Figure 13. Section adjacent to the previous figure. Alpha naphthyl acetate substrate, diazo blue B coupler Gross staining of cells showing diffusion which occurs with this coupler, substrate combination. Blood vessels show false positive localization. X430.

Figure 14. Liver, 15 days of age. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum. Esterase activity localized as discrete dustlike particles within the parenchymal cells. Arteriole, bile duct, and vein are all negative. Note the lack of hemopoietic cells and fat globules X970.

Figure 15. Liver, 20 days of age. 5-bromoindoxyl acetete. Enzyme activity surrounding the central vein is slightly more intense than at the periphery of the lobule.

Figure 16. Liver, 15 days of age. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum. Section through a central vein of a lobule. Note of lack of enzyme activity in the nuclei of cells. X430.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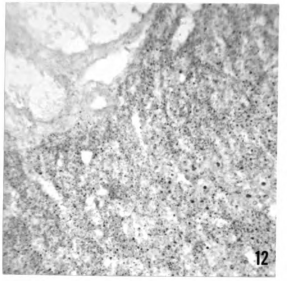
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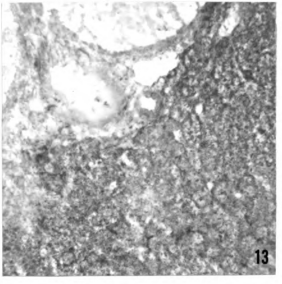
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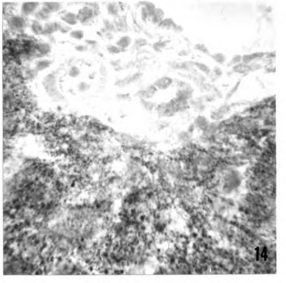
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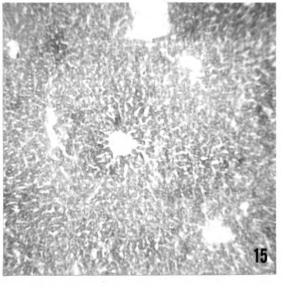
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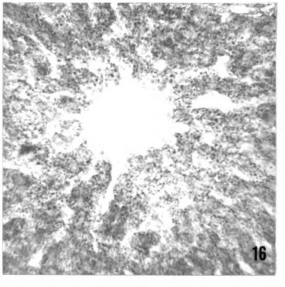
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Figure 17. Liver, 10 days of age. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum, fat globules stained with Sudan IV. Large clusters of precipitated materials appear on the surface of each fat globule. A megakaryocyte, showing no enzyme activity, appears in the center. X970.

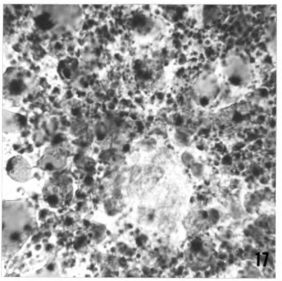
Figure 18. Liver, 1 day of age. Feulgen stain. Large, clear areas are fat globules. Megakaryocyte and numerous hemopoietic cells are present. X970.

Figure 19. Liver, 1 day of age. Hematoxylin and eosin stain. Islands of hemopoietic cells and megakaryocytes are prominent features in sections of liver from newborn mice. X430.

Figure 20. Liver, 1 day of age. Fat globules stained with Sudan black are large and numerous. X430.

Figure 21. Liver, 1 day of age. 5-bromoindoxyl acetate. Nuclei are counterstained with Mayer's carmalum. Esterase active cells are interspersed between the enzymatically inactive hemopoietic cells. X430.

Figure 22. Liver, 11 cm embryo. 5-bromoindoxyl acetate. Enzyme activity sparsely distributed among masses of hemopoietic cells. X970.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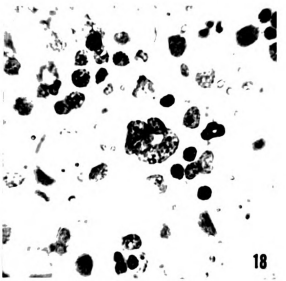
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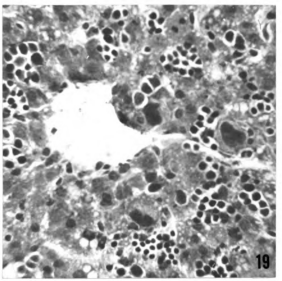
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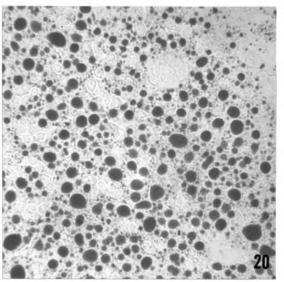
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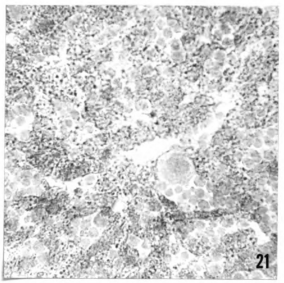
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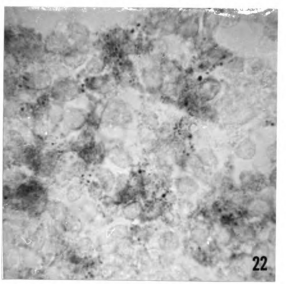
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Figure 23. Adult spleen. Hematoxylin and eosin. X100.

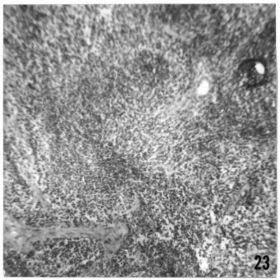
Figure 24. Section adjacent to previous figure. Alpha naphthyl acetate substrate, diazo blue B coupler. Esterase active cells appear individually throughout the tissue with some arranged in a circular pattern. X100.

Figure 25. Adult spleen. Alpha napthyhl acetate substrate, diazo blue B coupler. Esterase activity appears to be localized within individual cells. Some diffusion into the surrounding tissue is evident. X430.

Figure 26. Spleen, 15 days of age. Alpha naphthyl acetate substrate, diazo blue B coupler. Adult pattern of esterase active cells becomes apparent at this age. X100.

Figure 27. Spleen, 5 days of age. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum. Esterase active cells localized as discrete particles within widely scattered cells. X970.

Figure 28. Spleen, 1 day of age. Alpha naphthyl acetate substrate, diazo blue B coupler. Enzyme activity is very sparse. A megakaryocyte is present. X430.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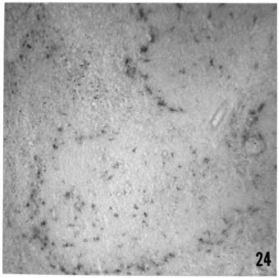
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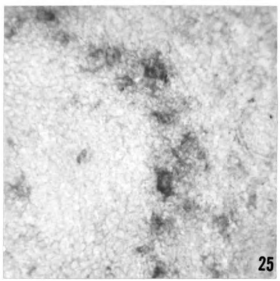
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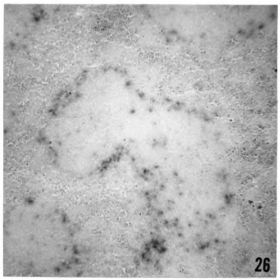
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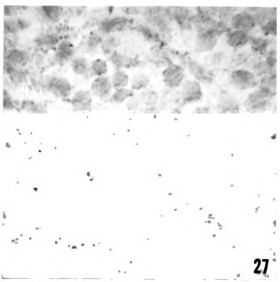
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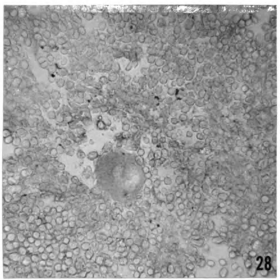
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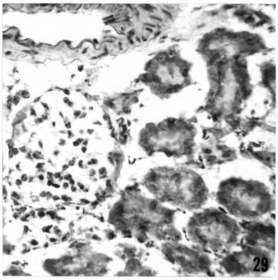
Figure 29. Adult kidney. Hematoxylin and eosin stain. Section in cortex showing an artery, glomerulus, and convoluted tubules. X430.

Figure 30. Section adjacent to previous figure. Alpha naphthyl acetate substrate, fast red TR coupler. Glomerulus and artery are enzymatically negative. Bowman's capsule and sections through the convoluted tubules are strongly positive. X430.

Figure 31. Adult kidney. Hematoxylin and eosin stain. Section through a vein and artery surrounded by glomeruli, convoluted tubules, and collecting ducts. X100.

Figure 32. Section adjacent to previous figure. Alpha naphthyl acetate substrate, fast red TR coupler. Convoluted tubules positive. Artery, vein, glomerulus, collecting tubules negative or only slightly positive. X100.

Figure 33. Kideny, 15 days of age. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum. The thick segment of Henle's loop shows a positive reaction. Collecting tubules are only weakly positive. X430.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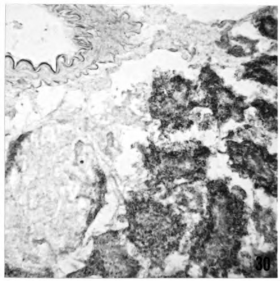
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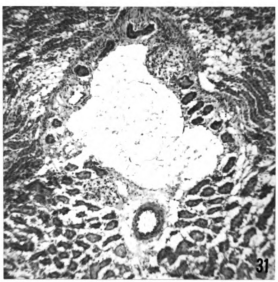
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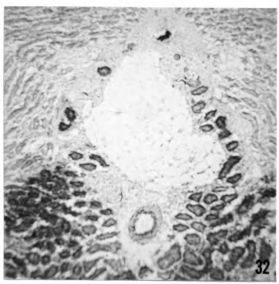
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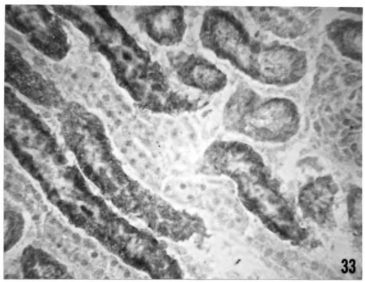
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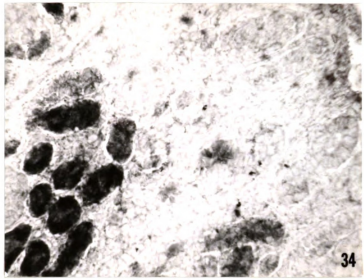
Figure 34. Kidney, 15 days of age. Alpha naphthyl acetate substrate, diazo blue B coupler. The nephrogenic tissue fails to show esterase activity. X430.

Figure 35. Kidney, 10 days of age. Hematoxylin and eosin stain. Nephrogenic tubules with characteristic S -shape and large, dark staining nuclei are present. X430.

Figure 36. Kidney, 15 days of age. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum. Bowman's capsule and convoluted tubules show a strong positive reaction. X430.

Figure 37. Kidney, 1 day of age. Hematoxylin and eosin stain. Nephrogenic tissue in upper right corner of photograph. X430.

Figure 38. Section adjacent to previous figure. Alpha naphthyl acetate substrate, diazo blue B coupler. Developing tubules are enzymatically inactive. X430.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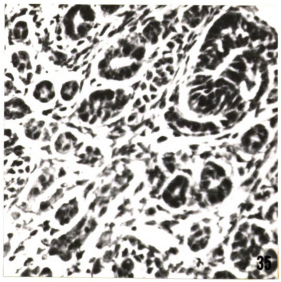
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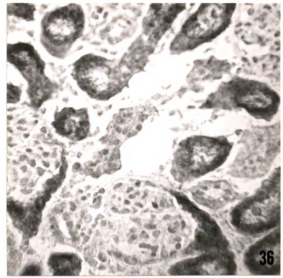
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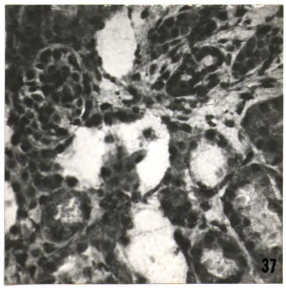
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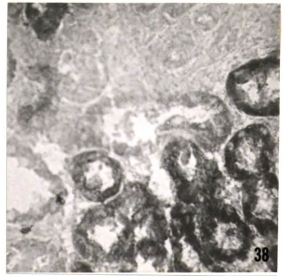
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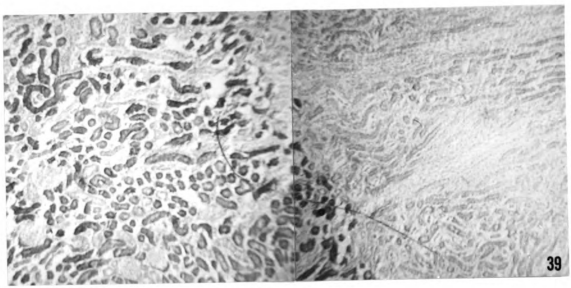
The figures on the opposite page are composites of two contiguous photographs which were matched and glued together.

Figure 39. Adult kidney. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum. Nephrons show maximal activity in all regions of the cortex.

Figure 40. Kidney, 15 days of age. 5-bromoindoxyl acetate. Nuclei stained with Mayer's carmalum. Nephrogenic tissue cannot be demonstrated at this age. The entire cortex contains esterase active tubules. X100.

Figure 41. Kidney, 1 day of age. 5-bromoindoxyl acetate. Nuclei counterstained with Mayer's carmalum. A few nephrons in the juxtamedullary region show enzyme activity. The nephrogenic zone in the outer cortex is negative. X100.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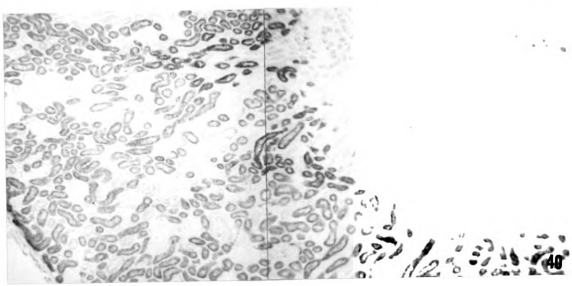
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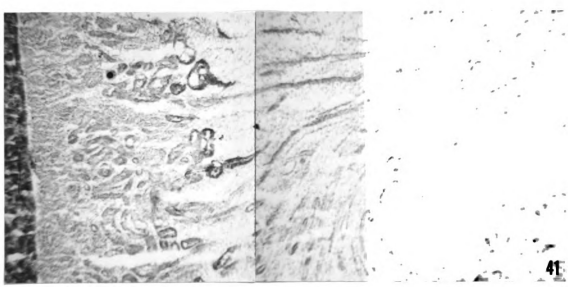
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