Munkres, K. D. (1968), *Ann. N. Y. Acad. Sci. 151*, 294.
Murachi, T., Inagami, T., and Yasui, M. (1965), *Biochemistry 4*, 2815.
Naughton, N. A., Sanger, F., Hartley, B. S., and Shaw, D. C. (1960), *Biochem. J. 77*, 149.
Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.
Pallini, V., Ricci, C., and Martelli, P. (1965), *Ital. J. Biochem. 14*, 407.
Penhoet, E., Kochman, M., Valentine, R., and Rutter, W. J. (1967), *Biochemistry 6*, 2640.
Petrack, B., Greengard, P., Craston, A., and Kalinsky, H. J. (1963), *Biochem. Biophys. Res. Commun. 13*, 472.
Petrack, B., Greengard, P., Craston, A., and Sheppy, F. (1965), *J. Biol. Chem. 240*, 1725.
Preiss, J., and Handler, P. (1958a), *J. Biol. Chem. 233*, 488.
Preiss, J., and Handler, P. (1958b), *J. Biol. Chem. 233*, 493.
Rosenberg, R. M., and Klotz, I. M. (1960), *in* A Laboratory Manual of Analytical Methods of Protein Chemistry, Vol. 2, Alexander, P., and Block, R. J., Ed., New York, N. Y., Pergamon Press, p 144.
Schaffer, N. K., May, S. C., Jr., and Summerson, W. H. (1953), *J. Biol. Chem. 202*, 67.
Schoellmann, G., and Shaw, E. (1963), *Biochemistry 2*, 252.
Smillie, L. B., and Hartley, B. S. (1964), *J. Mol. Biol. 10*, 183.
Steers, E., Craven, G. R., and Anfinsen, C. B., Jr. (1965), *J. Biol. Chem. 240*, 2478.
Su, S., Albizati, L., and Chaykin, S. (1969), *J. Biol. Chem. 244*, 2956.
Su, S., and Chaykin, S. (1971), *Methods Enzymol. 18B*, 185.
Teipel, J. W., and Hill, R. L. (1968), *J. Biol. Chem. 243*, 5679.

Multiple Pancreatic Lipases. Tissue Distribution and Pattern of Accumulation during Embryological Development†

William S. Bradshaw‡ and William J. Rutter*

ABSTRACT: Two lipases, designated A and B, have been detected in extracts of rat pancreas and in pancreatic juice. These enzymes can be distinguished from each other and from other esterolytic enzymes on the basis of their electrophoretic mobility and chromatographic properties as well as their substrate specificity and the differential effects of sodium taurocholate on their activity. Significant levels of lipases A and B are apparently found only in the pancreas and pancreatic juice; these enzymes were not detected in a number of other embryonic and adult tissues. Total lipase activity was measured in rudiments obtained from developing rat and mouse embryos and from postnatal and adult animals. A low but significant level of lipase A activity was found in the earlier stages of development of the rat pancreas (12 to 14 days gestation). This level was significantly higher than that present in other tissues of the embryo and, hence, is considered to be developmentally significant. The lipase specific activity of isolated rudiments increases dramatically (over 1000-fold) in embryos from 15 to 18 days gestation reaching a second plateau of activity characteristic of the late embryonic and newborn pancreases. The quantitative and temporal aspects of this pattern of development were duplicated in 13-day embryonic rat pancreases cultured *in vitro*. Lipase B was first detectable in the 18-day embryonic rat pancreas; the level slowly increases until birth at which time lipase B represents approximately 10% of the total lipase activity. Subsequently, however, the relative level increases substantially, for in the adult animal, lipase B contributes more than 50% of the total activity found in pancreatic extracts. The biphasic developmental pattern of lipase specific activity is similar to that found for a number of other specific exocrine proteins during pancreatic development and supports the concept of a biphasic process of differentiation in the exocrine cells.

Pancreatic lipase (EC 3.1.1.3, glycerol-ester hydrolyase) is one of the specific exocrine enzymes secreted by the pancreas in the pancreatic juice. Studies on purified pancreatic lipase from hog (Marchis-Mouren *et al.*, 1959; Entressangles *et al.*, 1966) indicate that the enzyme specifically hydrolyzes the primary ester bonds of emulsified triglycerides of long-chain fatty acids. The purified hog enzyme has been shown to be electrophoretically and chromatographically homogeneous (Marchis-Mouren *et al.*, 1959) and only a single lipase was detectable in rat pancreas by means of immunoelectrophoresis (Pascale *et al.*, 1966). However, a partially purified hog lipase has been resolved by Sephadex chromatography into two active components of significantly different molecular weight (Sarda *et al.*, 1964). The conversion of the "rapid" lipase to the "slow" form by methanol–ether extraction suggested that the former was a lipid-bound derivative of the latter.

We report here the results of studies aimed at determining (1) the number of distinct proteins contributing to pancreatic lipase activity; (2) the distribution of these lipases in other adult or embryonic tissues; (3) the patterns of accumulation of lipase activity in the developing pancreas *in vivo* and *in vitro*.

The developmental profiles of lipase activity emphasize

† From the Departments of Biochemistry and Genetics, University of Washington, Seattle, Washington 98105. *Received March 20, 1970.* The experimental work described in this paper was supported in part by the U. S. Public Health Service Grant No. HD-02126 and National Science Foundation Grant No. GB 4273.

‡ Present address: Department of Zoology, Brigham Young University, Provo, Utah 84801.

* Present address: Department of Biochemistry and Biophysics, University of California, San Francisco Medical Center, San Francisco, Calif. 94122; to whom correspondence should be addressed.

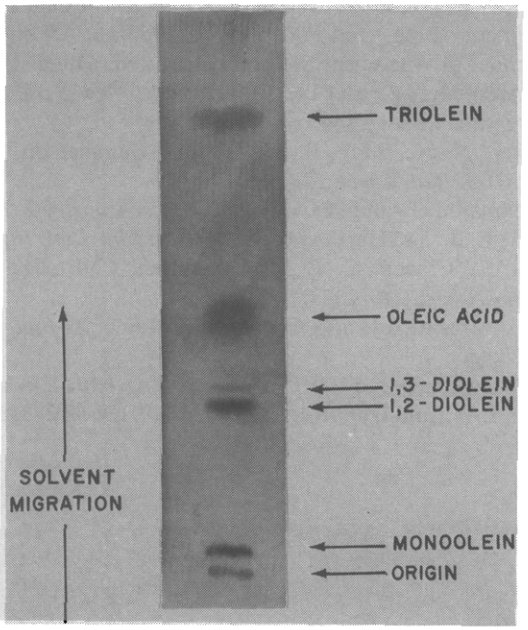


FIGURE 1: Hydrolysis of triolein by pancreatic lipase. Extracts of a reaction mixture to which a crude sonicate of adult rat pancreas had been added were resolved by thin-layer chromatography as described in *Methods*. Lipid spots were visualized in iodine vapor and their identities confirmed by comparison with standard samples.

the stringent and multiphasic nature of the regulation of specific protein synthesis in pancreatic differentiation.

Methods

Radioassay of Pancreatic Lipase Activity. PREPARATION OF EMULSIFIED SUBSTRATE. Triolein (5 μl; 4.6 mg; unlabeled, >99% purity from Applied Science Laboratories) and benzene (10–20 μl) containing approximately 5×10^{-3} μmole and 2×10^{6} cpm of [^{14}C]triolein (specific radioactivity 35.4 mCi/mmole, radiochemical purity >98%, Nuclear-Chicago Corp.) were added to 1.0 ml of an aqueous medium consisting of 3.6% gum arabic (Sigma Chemical Co.), 0.1 M NaCl, 5×10^{-4} M $CaCl_2$, and 7.4 mM sodium taurocholate (Sigma Chemical Co.) in 0.05 M sodium borate buffer (pH 8.5). Mixing was carried out for 10 minutes using a Sorvall Omni-Mixer (approximately 40,000 rpm); the mixing canister was partially submerged in ice water during the last 8 min. Aliquots of the labeled triolein (10–20 μl) in benzene were stored before use at room temperature in sealed-glass ampoules under nitrogen to prevent oxidation. Assays performed in the presence of an amount of benzene corresponding to the initial concentration revealed that there was no effect of the organic solvent on lipase activity.

Some of the earlier results were obtained with a different assay procedure employing the incubation technique described by Borgström (1964) and using [^{14}C]tripalmitin as substrate. Labeled and unlabeled substrate in benzene were added to the reaction tubes. Following evaporation of the solvent and addition of the aqueous medium the stoppered tubes were mixed thoroughly on a Vortex mixer and the contents incubated with enzome. Significantly higher rates and more reproducible emulsions were obtained by using the liquid substrate (triolein) and mechanical emulsifying techniques described above.

INCUBATION OF ASSAY. The emulsion (50 μl; containing a total substrate concentration of 10^{-2} M in terms of oleic acid equivalents) was incubated at 37° for 5 min with enzyme and sodium borate buffer in a final volume of 70 μl in stoppered (6 × 10 mm) glass culture tubes, previously flushed with nitrogen. Shaking during the incubation period had no effect on activity. The reactions were terminated by the addition of 10 μl of 1 N HCl. Carrier oleic acid (10 μg; >99% purity, Applied Science Laboratories) was added to each tube.

RESOLUTION OF HYDROLYSIS MIXTURE. The entire reaction mixture was extracted three times with 50 μl of chloroform. Following separation of the chloroform and aqueous layers by centrifugation at 2000*g* for 1 min, the components of the hydrolysis mixture were resolved by thin-layer chromatography (tlc) on silica gel G (according to Stahl, Brinkmann Instruments, Inc.). Chloroform extracts (three 50-μl portions) were withdrawn from the incubation tubes and applied by means of a Hamilton micro-syringe to tlc plates (5 × 20 cm) previously activated by heating at 100° for at least 30 min. The plates were developed using a solvent system consisting of petroleum ether (bp 30–60°)–ether–acetic acid (60:40:1, v/v) in a tank lined with absorbent paper saturated with the solvent. About 45-min development time was required for the solvent front to reach the top of a plate (20 cm). The plate was then removed from the tank and let stand 15 min while the solvent evaporated. Lipid spots on each plate were visualized by incubating the plate in a tank containing solid iodine crystals. The pattern of lipid spots from a typical plate processed in this manner is shown in Figure 1. After marking the lipid-containing areas, the plates were heated a few minutes on a hot plate until the yellow color in each spot disappeared.

DETERMINATION OF REACTION RATES. The zone containing the oleic acid and a small area on either side of it were sectioned into fractions each of which was removed with a razor blade and placed into a separate scintillation vial and counted in Kinard (1957) solution in a Packard Tri-Carb liquid scintillation spectrometer. The remainder of the plate (excluding the origin), containing all glyceride species, was counted in a separate vial. The counting efficiency was 80–85% as determined with [^{14}C]toluene as standard. At least ninety per cent of the initial counts in each incubation was recovered by this procedure. Counts in oleic acid were determined by subtracting the counts at the base of each peak from the total counts under that peak of radioactivity. The calculation of per cent hydrolysis was then based on the ratio of counts in oleic acid peak to total counts on each plate. In each group of assays, reaction mixtures lacking enzyme were processed in an identical fashion in order to determine the background level of counts in oleic acid (presumably contributed by nonenzymatic hydrolysis). This value never exceeded 0.6% hydrolysis. After subtraction of the per cent hydrolysis in the blank, the net microequivalents of free acid liberated during the incubation period was readily calculated (Bradshaw, 1968).

One unit of lipase activity was defined as the quantity of enzyme liberating 1 μequiv of free acid (either oleate or palmitate) per min under the conditions of the assay. The specific activity was defined as units of lipase activity per milligram of protein. The protein concentration was determined using a modification (Rutter, 1967) of the method of Lowry *et al.* (1951). Crystalline bovine serum albumin was employed as a standard.

ASSAY CHARACTERISTICS. Rates obtained from assays utilizing different emulsion preparations were normalized by comparison with the rates obtained for a standard lipase preparation. The standard used was a 0.024% solution of pancreatic lipase (PL III, 15–25 units/mg, Worthington Biochemical Corp.). Rates for this standard varied by a factor

no greater than 1.5 from emulsion to emulsion. Using our assay system, the average specific activity of this standard preparation was 35 μmoles of oleic acid/min per mg of protein. The value obtained for measurements performed using a potentiometric titration (Marchis-Mouren *et al.*, 1959) was 23. Increasing the substrate concentration in the same volume failed to give increased rates. It is thus presumed that lipase is functioning at or near maximum velocity under these assay conditions.

The results of several lipase assays performed during increasingly longer incubation periods showed that linear hydrolysis was dependent on the total accumulation of free fatty acid produced and was not directly a function of incubation time; the decreases in rate seen routinely at 15–20% hydrolysis may reflect an inhibition of the reaction by hydrolytic products. This same limitation of enzymatic rate was observed in experiments measuring activity against enzyme concentration. The lipase activity of rat pancreas was proportional to enzyme concentration over the same range (15–20%) of hydrolysis.

Assays containing either bovine serum albumin or boiled pancreatic protein at concentrations equivalent to those of active pancreas preparations exhibited the same control values. On the other hand, high levels of foreign tissue (for example, up to 400 μg of protein of a sonicate of rat liver) added to an incubation mixture, reduced the activity by as much as 60% though linearity persisted. The liver proteins in concentrations up to 400 μg assayed alone exhibited no detectable activity. The inhibitory effect of high concentrations of other foreign tissue may be due to substrate binding by extraneous protein (bovine serum albumin likewise inhibited and is known to bind fatty acids and lipids), or by addition of a significant quantity of endogenous unlabeled triglyceride to the system by some other direct interference with catalysis.

Colorimetric Lipase Determination. Seligman *et al.* (1949) and later Kramer *et al.* (1963) developed a colorimetric assay for pancreatic lipase based on the hydrolysis of chromogenic naphthyl alkanoates in the presence of various bile salt activators. We have modified these procedures as follows. A solution containing 0.2 ml of enzyme, 0.5 ml of 0.4 M Tris-chloride buffer (pH 7.4), and (when present) 0.1 ml of sodium taurocholate (17.8 mg/ml) in a volume of 0.8 ml was preincubated at 37° for 5 min. The reaction was then initiated with the addition of 0.2 ml of β-naphthyl stearate (5 mg/ml in methoxyethanol, Mann Research Laboratories), β-naphthyl nonanoate (Nutritional Biochemicals), α-naphthyl acetate (K & K Laboratories), or other naphthyl ester (β-naphthyl laurate, Mann Research Laboratories). Following an incubation period (37°) of routinely 3 min, the reaction was stopped with 0.2 ml of Fast Blue RR dye (4 mg/ml, Dajac Laboratories). (However, a linear response to enzyme was obtained during at least a 15-min period.) After 3 min, 0.6 ml of glacial acetic acid was added and the tube was agitated. After an additional 5 min, the insoluble precipitate was extracted with 2.0 ml of ethyl acetate, and the tubes centrifuged for 10 min at 2000*g*. The absorbance at 540 mμ was determined using a reagent blank without enzyme. β-Naphthol was employed as a standard, and activities were reported as micromoles of β-naphthol produced per min per mg of protein. Under those conditions a linear colorimetric response was obtained up to an absorbance of 1.6 (20 μmoles of β-naphthol).

Cellulose Acetate Zone Electrophoresis. Electrophoresis was performed on cellulose polyacetate strips (Sepaphore III, Gelman Instrument Co.). Applying the samples 2.5 in. from the end of the 3.75-in. strip resting in the cathodic section of the buffer chamber enhanced the mobility of several protein bands of interest. Electrophoresis was carried out at pH 8.6 (0.05 M Veronal) and 300 V (approximately 1 mA/strip) for 2.5 hr at 4°. Protein bands were detected with Coomassie Brilliant Blue R-250 (Colab Laboratories, Inc.).

Esterolytic Activity Staining. Following electrophoresis, the cellulose acetate strips were placed, application side down, on an assay mixture solidified in agar in 100 × 15 mm petri dishes (Falcon Plastics). Two per cent Special Noble Agar (Difco) in 0.05 M Veronal buffer (pH 8.6) was boiled for several minutes until homogeneous and maintained at 60°. To 10 ml of the agar solution was added 10 ml of Veronal buffer containing 10 mg of Fast Blue RR salt and 0.2 ml of a stock substrate solution (2% naphthyl alkanoate in dimethylformamide) at the same temperature. The agar–reaction mixture was solidified (5 ml/Petri dish) and stored for as long as 4 hr at 4° before use. On incubating electrophoretic strips at 37°, proteins with esterase activity appeared as brownish or red bands depending upon the A or B configuration of the substrate, respectively. In those assays testing taurocholate activation, electrophoretic strips were removed from the buffer chamber and placed on a glass plate covered with Parafilm. Each strip was then cut longitudinally down the center with a razor blade and one-half was assayed on an agar plate containing 2.3×10^{-3} M sodium taurocholate, the other half on a plate lacking this bile salt. In some instances esterase inhibitors were added to the incubation mixture. These included: diethyl-*p*-nitrophenyl phosphate (E-600),[1] eserine sulfate, *p*-chloromercuribenzoate (K & K Laboratories), and diisopropyl fluorophosphate (DFP, Sigma Chemical Co.).

Quantitative Assay for Electrophoretically Resolved Enzymes. In some experiments, the proteins separated by electrophoresis were eluted from cellulose acetate strips and assayed for activity against triolein. Strips were placed on Parafilm and cut transversely into sections of equal width parallel to the origin. These sections were then cut into very small pieces which were introduced into tubes containing the [^{14}C]triolein-containing reaction mixture. The specific activities obtained in this manner have been reported in terms of microequivalents oleic acid hydrolyzed per minute per milligram of protein applied to the electrophoretic strip. The amount of original activity that was recovered after electrophoresis by this technique was a function of the amount of protein applied to a strip: at relatively low levels of protein (5–10 μg) 90% of the initial activity was recovered, while with increased amounts, 100–200 μg of protein, as little as 2–3% activity was recovered.

Animals and Determination of Gestational Age. Sprague-Dawley rats (Northwest Rodent Co., Pullman, Washington), were employed in this study both as a source of tissues, and in the production of enzymes. Mouse embryos were obtained from crossing DB A/2J males with BALB/CJ females, supplied by Roscow B. Jackson Memorial Laboratories, Bar Harbor, Maine.

Pregnant females were detected by daily examination for the presence of vaginal plugs. Fertilization (day zero of embryonic life) was assumed to have taken place at noon on the day when a plug was discovered. (Mating usually takes place at midnight ±3 hr, and it is known that fertilization occurs

[1] Abbreviations used are: G-2, α-naphthyl acetate; C-9, β-naphthyl nonanoate; C-12, β-naphthyl laurate; C-18, β-naphthyl stearate; DEAE, diethylaminoethyl; E-600, diethyl-*p*-nitrophenyl phosphate, Paraoxon.

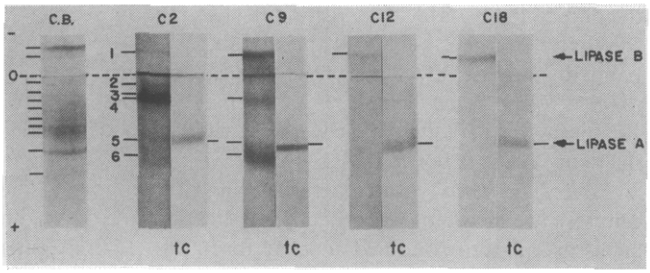


FIGURE 2: Resolution of pancreatic lipases A and B by zone electrophoresis on cellulose acetate. A crude sonicate of adult rat pancreas was prepared from 800 mg of fresh tissue in 200 μl of water containing 10^{-3} M phenylmethyl sulfonyl fluoride and 10^{-4} M ethyl *p*-guanidinobenzoate. Three-microliter aliquots containing 150 μg of protein were applied to cellulose acetate strips and electrophoresis carried out under the conditions described in Methods. One of the strips was fixed for 2 min in 20% sulfoselicylic acid and then immersed for 5 min in Coomassie Blue (CB). Other strips were stained for esterase activity against α-naphthyl acetate (C-2), β-naphthyl nonanoate (C-9), β-naphthyl laurate (C-12), and β-naphthyl stearate (C-18). The right half of each strip, labeled (taurocholate), was assayed for enzyme activity in the presence of sodium taurocholate, the left half in its absence. The incubation periods at 37° for each half-strip were 5 min (+tc) and 2 hr (−tc). Short horizontal dashes indicate bands detected visually. + and − indicate anode and cathode of the buffer chamber, and) denotes the application origin.

approximately 12 hr later (Asdell, 1946).) The gestation period for rats is 22 days, for mice, 19 days. The average litter size for rats was 10–12, for mice, about 8.

Rhesus monkey pancreas was obtained through the Regional Primate Center, and human pancreas from University Hospital, University of Washington, Seattle. The human pancreatic juice was the kind gift of Dr. Patricia J. Keller, Department of Oral Biology, University of Washington, Seattle. All other animals tissues were obtained freshly frozen through Lab Associates, Inc., Kirkland, Washington.

Tissue Preparation. Adult male rats (250–300 g) were sacrificed by decapitation, adult mice by cervical dislocation. Tissues were removed immediately, minced with scissors in iced beakers, and introduced into microfuge (Beckman Instrument Co.) tubes. Some organs including pancreas, parotid gland, and duodemun, were freed from fat and extraneous material under a dissecting microscope prior to mincing.

Excised embryonic pancreas rudiments were also collected in microfuge tubes, care being taken to transfer as little as possible of the dissecting medium. Pancreas rudiments were transferred using pasteur pipets drawn out to a very fine bore. For early embryonic states (11–15 days) when a large amount of tissue was required, pancreases from several litters were pooled and stored at −20° prior to sonication and assay. No difference in lipase activity could be detected between pancreases assayed immediately after dissection and those stored at −20° for as long as 4 weeks.

An appropriate quantity of tissue suspended in a known volume of distilled water in the microfuge tubes was disruped when submitted to sonication for 2 min (Branson Sonifier Model S-125, Heat Systems Co., Melville, N. Y.). Maximum power was applied (power supply setting of 8) and an output of 8–11 A was maintained over the sonication period. The tubes were taped on the bottom of a plastic beaker and covered with ice and water. During the sonication period the Sonifier probe was allowed to touch the surface of the tube (about 0.5 cm below the surface of the liquid in the beaker) and was moved along its length thus keeping the contents in constant agitation. Appropriate experiments on activity showed that release of soluble protein and enzyme activity reached a maximum after 1 min. When sonicates were prepared in this manner and examined under a phase-contrast microscope, only cell debris could be detected; no intact cells could be detected.

Following sonication, the microfuge tubes were centrifuged at 4° for 2 min (Spinco microfuge) at a constant speed of 15,000 rpm, and the supernatant assayed for enzyme activity and protein content.

Collection of Pancreatic Juice. Pure pancreatic juice was obtained from adult male rats by cannulation of the pancreatic duct at a point immediately before its junction with the duodenum. A second cannula inserted in the bile duct between the liver and pancreas was used to divert the flow of bile, hence the juice obtained from the first tube was bile free. Following the operation, animals were placed in a restraining cage and fed *ad libitum* on their regular diet. Pancreatic juice was collected in small tubes in ice for immediate use or frozen in tubes packed in Dry Ice. In successful experiments, 5–10 ml of juice was collected per day for 3–4 days beginning 24 hr after the operation. The protein content of the juice was 18 mg/ml.

Organ Culture Methodology. Organotypic cultivation of embryonic pancreas was carried out according to Clark and Rutter (1968). Intact pancreatic rudiments were placed on Millipore Filters (0.80 μ, ultrathin HA filters, Millipore Filter Corp.) in sterile Petri dishes containing a medium consisting of Eagles Basal Medium, Earle's salts (Grand Island Biochemical Co.), 2 mM glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin, and 10% freshly prepared chick embryo extract. Cultures were maintained at 37° in an atmosphere of 95% air–5% CO_2. The medium was changed every 2 days.

Results

Resolution of Lipases A and B by Zone Electrophoresis on Cellulose Acetate. Extracts of adult rat pancreatic tissue (fresh or frozen immediately after sacrifice of an animal) were subjected to zone electrophoresis on cellulose acetate. Typical results are shown in Figure 2. The first strip (left) was stained for protein with the dye Coomassie Blue; eleven distinct bands were visualized by this procedure. The next four strips were tested for esterolytic activity toward homologous naphthyl esters of increasing fatty acid chain length. Six bands of hydrolytic activity against the C-2 and C-9 substrates were detected. Only two of these, bands 1 and 5, were also capable of cleaving the C-12 and C-18 homologs under these conditions. Band 5 was designated lipase A and band I lipase B. Band 5 (lipase A) was further characterized by a striking increase in activity against all substrates in the presence of taurocholate. Lipase B (band I), on the other hand, was significantly inhibited by this bile salt. From the time required for development of an equivalent color intensity, the reaction rates for these substrates decrease in the order C-9 > C-2 > C-12 > C-18 for both lipases A and B. By this same criterion, the activity of lipase A toward all naphthyl alkanoates in the presence of taurocholate was greater than that of lipase B in its absence.

In these experiments approximately 0.15 mg of pancreatic protein was applied to the strips. This quantity was necessary for visualization of the weakly active esterases, bands 2 and 6. Under these conditions, in the presence of taurocholate the development of color in lipase A using α-naphthyl acetate

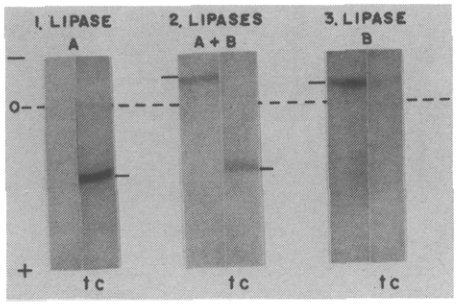


FIGURE 3: Separation of lipase A and B activities. Crude lipase preparations from rat pancreas were submitted to electrophoresis on cellulose acetate, and to lipase activity staining with and without taurocholate by procedures described in Methods. β-Naphthyl stearate was the substrate. (1) An aliquot (11.5 μg of protein) from a sonicate of 18-day embryonic pancreas containing lipase A; (2) an aliquot (9.7 μg of protein) of stage IV (acetone precipitate) from the procedure of Melius and Simmons (1965) containing lipases A and B; (3) an aliquot (0.5 μg of protein) of the effluent from a DEAE-cellulose column according to Vandermeers and Christophe (1968) beginning with fresh adult pancreas.

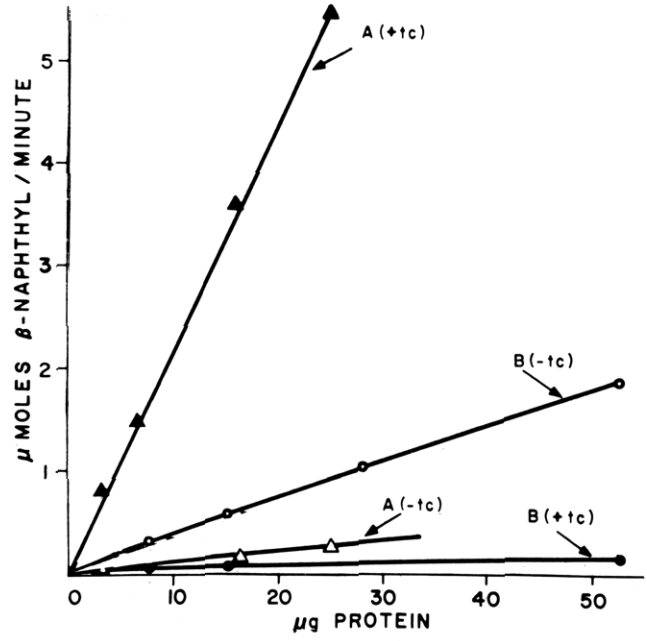


FIGURE 4: Differential effects of taurocholate on lipases A and B. Aliquots of increasing protein content from the rat pancreas preparation described in Figure 3 were assayed colorimetrically for lipase activity using β-naphthyl stearate as substrate. Triangles, 18-day embryonic rat pancreas. Circles, effluent from DEAE-cellulose column of adult pancreas purified according to Vandermeers and Christophe (1968). Open symbols, without sodium taurocholate. Closed symbols, with sodium taurocholate.

or β-naphthyl nonanoate required less than 5 min. Continued incubation of such plates resulted in gradual diffusion of the color so that detection of the other bands of activity was not possible in this system. On the other hand, 2 hr was required for maximum color development of bands in the corresponding half of the strip incubated without the bile salt. Thus, the apparent differences seen in Figure 2 in the activity of bands 2, 3, 4, and 6 in the presence and absence of taurocholate are due to the difference in incubation time. Actually, the minor bands of activity are neither inhibited nor activated by inclusion of taurocholate in this test system.

The effect of inhibitors was tested in this system 10^{-5} M diethyl-*p*-nitrophenyl phosphate (E-600) inhibited nearly all the activity of both lipases A and B, either with or without taurocholate. Diisopropyl fluorophosphate (10^{-2} M) at (10^{-2} M) resulted in marked but not complete inhibition of both lipases, but had little or no effect on either enzyme at a concentration of 10^{-5} M. These observations agree with these reported for the purified lipase from hog pancreas (Desnuelle *et al.*, 1960; Sarda *et al.*, 1964). Neither eserine sulfate nor *p*-hydroxymercuribenzoate at a concentration of 10^{-3} M had any detectable effect on either lipase activity.

Only lipases A and B were detected in pancreatic juice from rat (Figure 7). The respective electrophoretic mobilities, substrate specificity, and response to taurocholate of the lipases in pancreatic juice were identical with those from whole tissue preparations. Two analogous lipases were also detected in human pancreatic juice (Figure 6). These results support the conclusion of two lipase activities and suggest that both activities are found in the zymogen granules.

Separation of Lipase A and Lipase B Activities. Further evidence suggestive that lipase A and B are distinct enzymes comes from the detection in both crude and purified lipase preparations of one activity in the absence of the other. The two activities are not, therefore, procedural artifacts. As shown in Figure 3, only lipase A was detected in extracts of the pancreas from 18-day embryos. The purification procedure of Vandermeers and Christophe (1968) carried out on adult rat pancreas yielded lipase B free of lipase A. On the other hand, when a butanol extraction procedure used by Melius and Simmons (1965) for the purification of lipase from hog pancreas was carried out on frozen, adult rat pancreas, a preparation containing both lipases A and B resulted. When a homogenate of fresh, adult rat pancreas containing both lipase activities was chromatographed on DEAE-cellulose at pH 8.2, the cationic lipase B appeared in the first void volume, while the anionic lipase A remained on the column. These observations then indicate lipases A and B are discrete entities, and suggest a means for resolution and partial purification of each enzyme.

Differential Effects of Taurocholate on Lipase A and B Activities against Naphthyl Esters. A quantitative estimation of the specific effects of sodium taurocholate on the lipase A and B catalyzed hydrolysis of β-naphthyl stearate is presented in Figure 4. Values for the activity ratio +taurocholate/−taurocholate were 18, 2.3, and 0.08 for the preparation of lipases A, A + B, and B respectively.

Activity toward Triolein. Lipases A and B both catalyze the hydrolysis of emulsified triolein (Figure 5). Following zone electrophoresis of a crude homogenate of adult rat pancreas, one-half of a cellulase acetate strip was assayed by activity staining using naphthyl ester substrates, and the other half was cut transversely into fractions which were assayed for hydrolytic activity against radioactive triolein. Two peaks of activity were detected which corresponded with the electrophoretic mobilities of lipases A and B as determined by colorimetric activity staining of the complementary strip. In contrast to their relative activity against naphthyl esters, however, lipase B possessed greater activity toward triolein than did lipase A. Thus, the enzymes have a different substrate specificity. The ratio of lipase A to lipase B activity in this experiment was 0.5, a value which is maximal because the lipase B fractions were assayed under nonoptimal conditions.

Attempts to Interconvert Lipase A and Lipase B. The possibility that one of the lipases was a derivative of the other was tested by attempting to interconvert the forms, either

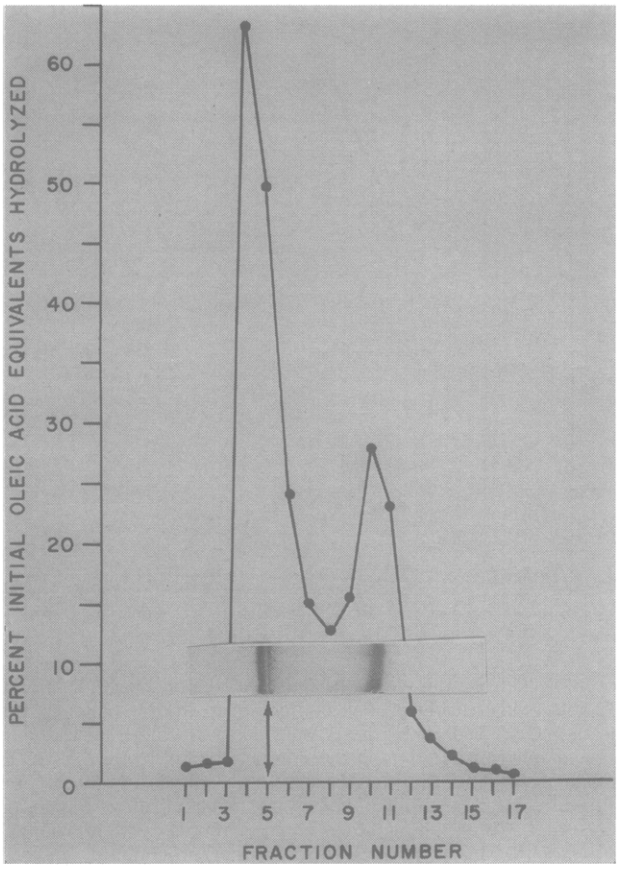


FIGURE 5: Lipase A and B activities toward triolein. The photograph shows one-half of a cellulose acetate strip upon which electrophoresis of adult rat pancreatic proteins had been performed and subsequently assayed for esterolytic enzyme activity using β-naphthyl stearate plus sodium taurocholate as substrate. The other half of the strip was cut transversely into fraction 5 mm in thickness. Each fraction was cut into small pieces and assayed for lipase activity after an 8-min incubation at 37° in a reaction mixture containing emulsified triolein. The distance between fractions along the abscissa is drawn to a scale corresponding exactly to that of the photograph. The double-headed arrow inidicates the application origin.

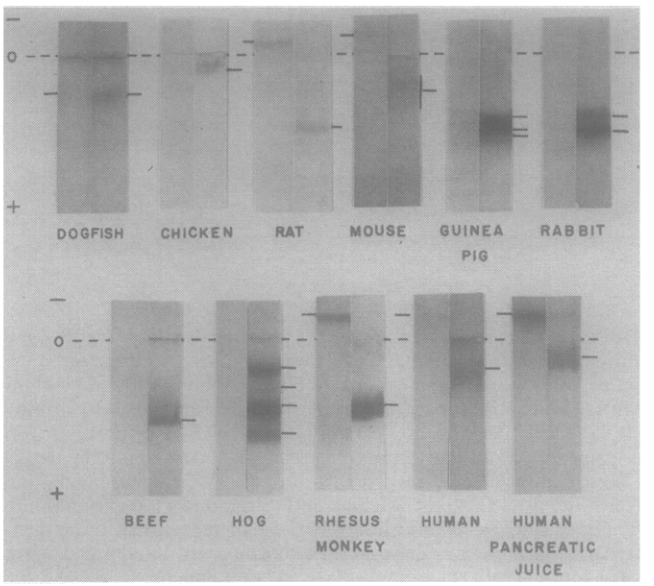


FIGURE 6: Pancreatic lipase multiplicity in various species. All tissues surveyed here were taken from adult animals. Rat, mouse, and rhesus monkey pancreases were taken from freshly sacrificed animals. Human pancreas, obtained through University Hospital, Seattle, was frozen 24 hr after death of the individual. All other tissues and human pancreatic juice were obtained freshly frozen. Quantities of these tissues from 0.5 to 0.8 g wet weight were sonicated in 200 μl of distilled water containing 10^{-3} M phenylmethyl sulfonyl fluoride and either 10^{-3} M ethyl *p*-guanidinobenzoate or 0.1% soybean trypsin inhibitor. Zone electrophoresis and lipase activity staining using β-naphthyl stearate were as described in Figure 2.

by removing or adding lipid materials, or by treatment with proteinases. In view of the report of Sarda *et al.* (1964) that a high molecular weight lipase activity in hog pancreas could be converted to a form of lower molecular weight by methanol–ether extraction, preparations, containing only lipase A or lipase B were subjected to extraction by lipid solvents. Aliquots of the enzymes were extracted for 90 min at 4° with equal volumes of butanol, chloroform–methanol (3:1, v/v), and methanol–ether (1:1, v/v. After centrifugation, cellulose acetate electrophoresis was performed on the precipitates and the aqueous and organic solvent phases which resulted. No indication of conversion of lipase A to lipase B was found. When the solvent-treated enzymes were assayed for lipase activity colorimetrically, losses in total activity were incurred, but the ratios of these activities in the presence and absence of taurocholate was not altered for either lipase A or B. Similarly, no "transformation" of the one lipase form to the other was observed when triolein and oleic acid were added to these preparations.

The alternative possibility, that a second lipase was produced by proteolytic modification of the first was tested by observing the effects of trypsin on crude pancreatic extracts. Sonicates prepared from pancreases of 18-day embryonic rats (containing only lipase A) and from 28-day postnatal rats (containing both lipases A and B) were incubated in the presence of a quantity of trypsin sufficient to convert all of the chymotrypsinogen in the system to chymotrypsin after a 10- to 20-min incubation at 37°. In control experiments, aliquots of each enzyme were incubated with no additions, and with the protease inhibitors *p*-nitrophenyl *p*-guanidinobenzoate, and phenylmethyl sulfonyl fluoride 10^{-3} M. Samples were then removed and subjected to zone electrophoresis, and assayed for lipase activity by staining, using naphthyl esters as substrates. In the control treatments, the qualitative lipase patterns of the two pancreas preparations were unchanged after incubation for as long as 24 hr. In the presence of trypsin, the lipase A activity in embryonic pancreas decreased somewhat over the incubation period but at no time was lipase B activity detectable. Similarly the relative distribution of the two lipases did not change during trypsin incubation with the postnatal rat pancreas preparation. We conclude that no significant interconversion of the two enzymes occurred under the conditions employed.

Multiple Pancreatic Lipases in Various Species. Multiple pancreatic lipases have been detected in several species by the zone electrophoresis of crude extracts on cellulose acetate followed by esterolytic activity staining (Figure 6). Single lipases of distinct electrophoretic mobilities, activated by taurocholate, were observed in dogfish and chicken. One, two, three, and four lipase bands all activated by taurocholate (analogous to rat lipase A) were observed in beef, rabbit, guinea pig, and hog, respectively. No activity inhibited by taurocholate (like lipase B) was observed in any of these species. However, one band stimulated by taurocholate and another inhibited by taurocholate were detected in mouse, rhesus monkey and human. In the mouse, the taurocholate-

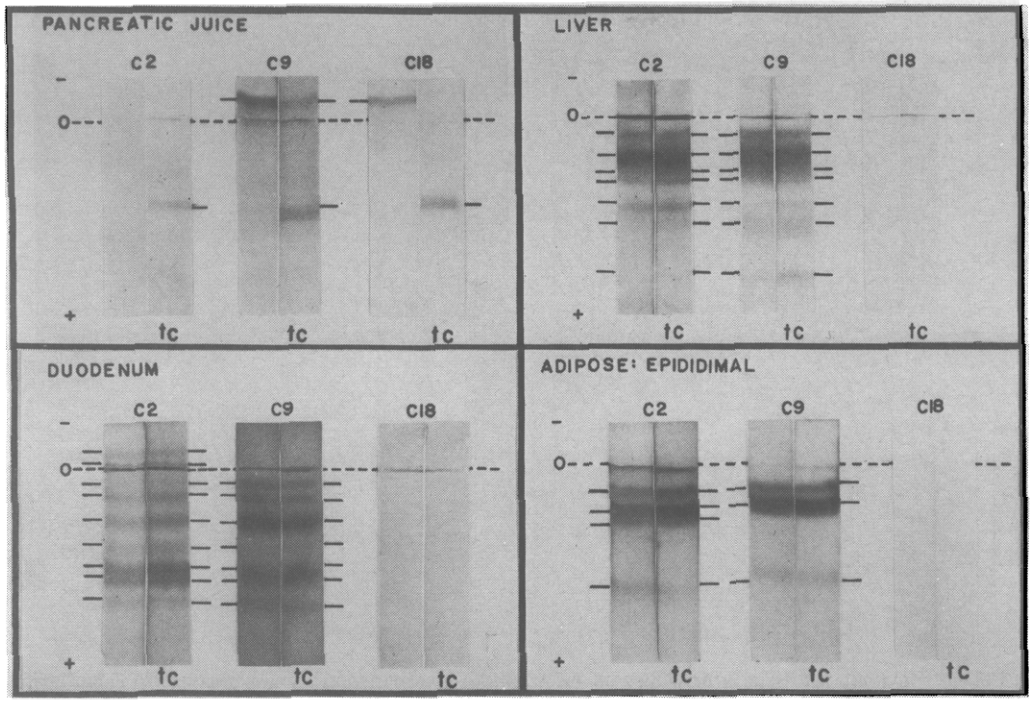


FIGURE 7: Tissue distribution of lipases and esterases in the rat. Crude sonicates of tissues from adult male rats were prepared, subjected to electrophoresis on cellulose acetate, and assayed by activity staining as detailed in Figure 2. The quantity of protein applied to a strip varied from 30 to 130 μg. Letter assignments for the various bands were made on the basis of electrophoretic mobilities of preparations processed under identical conditions in the same buffer chamber, and also mixing experiments in which joint preparations containing two or more tissues were applied to a single electrophoretic strip. (+) Activation by taurocholate. (−) Inhibition by taurocholate

stimulated activity was always diffuse and required a much longer time for color development than did the rat activity. This pattern was not altered when either fresh or frozen tissues from a number of mouse strains were tested. The taurocholate-activated band from human pancreatic tissue and human pancreatic juice was likewise diffuse and exhibited a mobility different from the rat enzyme.

Distribution of Esterases and Lipases A and B in Rat Tissues. A number of tissues of the adult rat have been screened for the presence of esterases and of lipases A and B, using the criteria employed above. Representative zymograms from preparation of tissues from a single male adult are shown in Figure 7. Pancreatic juice is included as a reference for the two lipases. This experiment reveals multiple esterase activities in rat tissues. For example, at least nine esterases were resolved in a sonicate of the duodenum. However, only the pancreas exhibited measurable hydrolytic activity toward β-naphthyl stearate. Moreover, none of the activity bands was significantly affected by taurocholate. The most mobile esterase in liver showed slight activation by the bile salt, but its R_F (0.8) was clearly greater than that of pancreatic lipase A (0.43).

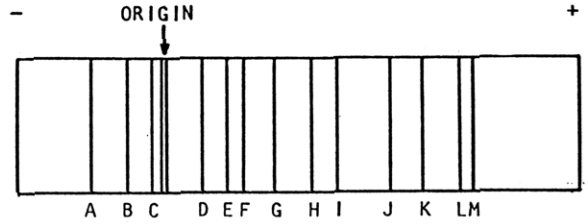
Table I summarizes a more extensive series of electrophoretic analyses of esterase activities in adult rat tissues. Each tissue studied exhibited a unique complement of esterases. Some activities (bands D, E, and K) were common to most of the tissues examined; others (bands B, C, and M) were restricted to a few tissues. None of the tissues contained bands H and A, that is enzyme activities with the properties of lipases A and B.

In addition to the qualtitative studies described above, radioactive lipase assays using emulsified triglycerides were performed on a number of adult and embryonic tissues. Lipase activity was not detectable in most nonpancreatic tissues. In certain tissues (*e.g.*, liver) the activity was above control values but there was no proportionality between apparent activity and protein concentration over a 100-fold range. Thus, we believe this apparent activity is probably due to nonspecific hydrolysis, rather than low endogenous levels of lipase. At any rate, the specific lipase activities in the pancreas were at least 10,000-fold and most often 100,000-fold higher than in any nonpancreatic tissue.

Attempts to measure lipase activity in extracts of nonpancreatic tissue after zone electrophoresis (Figure 8) also failed to reveal significant activity. The negative results obtained with this technique for preparations of adult liver, duodenum, and epididimal fat pad, and heads from embryos in the 11th and 12th day of gestation contrasted with our ability to detect significant lipase activity in pancreatic rudiment of a 14-day rat embryo. Thus the levels of activity if they exist in other tissues must be lower than those present in the earliest phase of pancreatic development (Rutter *et al.*, 1968a,b).

Ontogeny of Pancreatic Lipase Activity. The profile of lipase activity measured in pancreatic rudiments excised from embryonic and postnatal rats is shown in Figure 9. A low but significant level of lipase activity was present in the pan-

CHART I



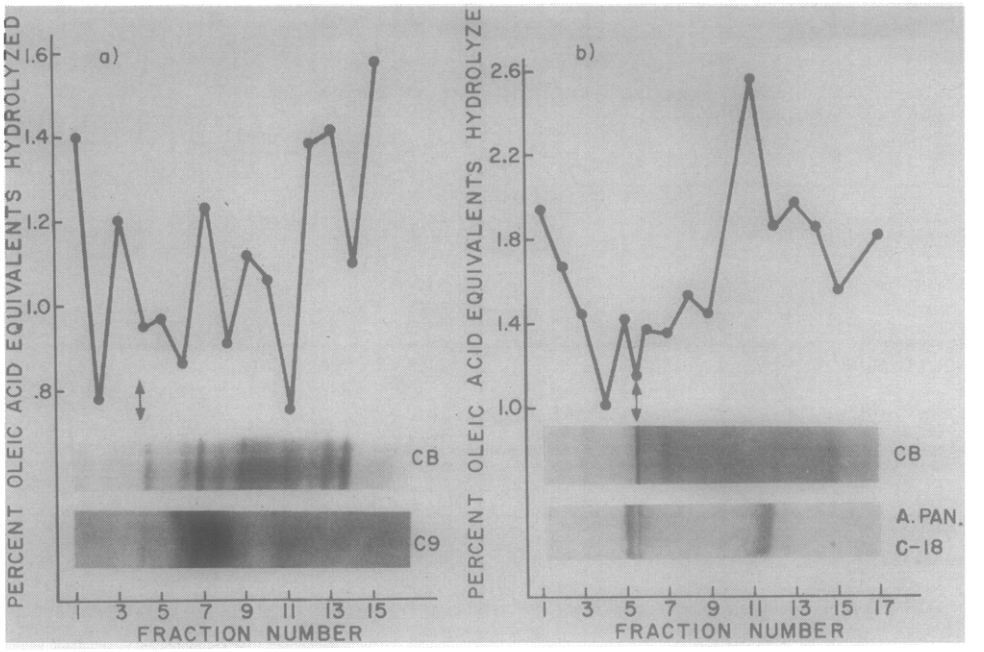


FIGURE 8: Levels of lipase activity in rat tissues following zone electrophoresis. Lipase activity was assayed using both trioloin and naphthyl esters as described in Figures 2 and 5. (a) Adult rat liver. Protein (0.41 mg) was applied to the strip. The background level of activity in a control reaction mixture with no additions was 1.1% hydrolysis. (b) 14-day embryonic rat pancreas. 70 μg of protein was applied to the strip. Background activity in the no additions control was 1.67% hydrolysis. A. Pan., adult pancreas.

TABLE I: Tissue Distribution of Lipases and Esterases in the Rat.[a,b]

Tissue	Total No.	Bands of Estereolytic Act. α-Naphthyl Acetate	β-Naphthyl Nonanoate	β-Naphthyl Stearate	Effect of Taurocholate
Pancreas	6	A DEF HI	A F HI	A H	A (+)[a] H (−)[d]
Pancreatic juice	2	A H	A H	A H	A (+)[a] H (−)[d]
Duodenum	9	BCDE G IJKL	DE G IJKL		
Liver	7	DEFG IJ M	DEFG IJ M		M (+)[c]
Kidney	8	DEFG IJ M	EFG K M		M (+)[c]
Spleen	5	DE G JK	DE G K		
Adipose					
Epididimal	4	DEF K	DE K		
Brown	4	DEF K	DE K		
Subserosal	4	DEF K	DE K		
Parotid	5	B DE JK	DE K		
Submaxillary	4	DE JK	DE K		
Brain	3	DE K	DE K		
Heart	5	DEF JK	DE K		
Testis	4	DEF I	DEF I		

[a] See Chart I for key. [b] Crude sonicates of tissues from adult male rats were prepared, subjected to electrophoresis on cellulose acetate, and assayed by activity staining for esterase activities as described in Methods. The quantity of protein applied to a strip varied from 30 to 130 μg. Letter assignments of the various bands were made on the basis of electrophoretic mobilities of preparations processed under identical conditions in the same buffer chamber, and also mixing experiments in which joint preparations containing two or more tissues were applied to a single electrophoretic strip. [c] (+), activation by taurocholate. [d] (−), inhibition by taurocholate.

creas at day 12 and persisted through day 15. Then a dramatic rise in lipase specific activity began on the 16th day and continued until day 19 when a second plateau was reached at a specific activity three orders of magnitude higher than that at day 12. The inflection point of this curve occurred at approximately 17 days. Considerable (fourfold) variation in the specific lipase activity existed in the pancreas of adult males and females. However, no correlation of activity with age or nutritional state was detected.

Lipase specific activity measurements were also carried out

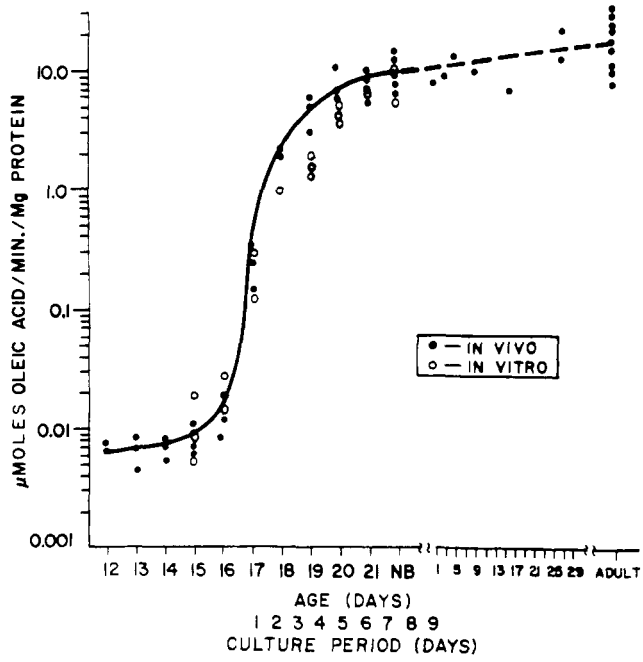


FIGURE 9: Lipase activity in the developing rat pancreas. Whole rudiments of embryonic rat pancreas, and pancreases cultured *in vitro* were collected, and prepared for assay of both protein content and lipase activity using [^{14}C]triolein as substrate by procedures described in Methods. NB = new born.

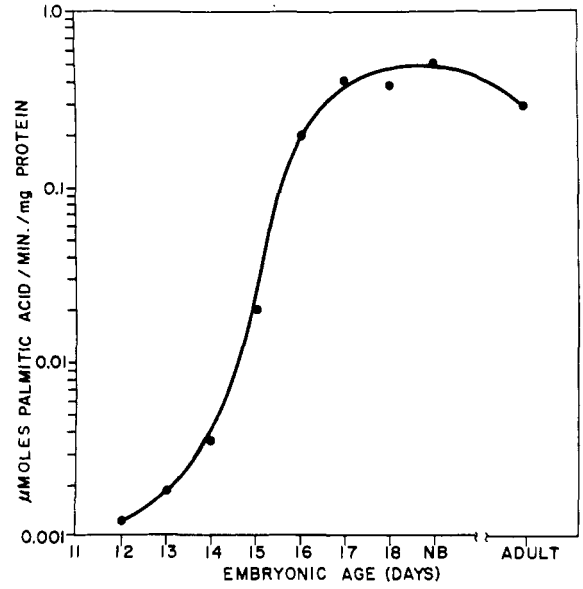


FIGURE 10: Lipase activity in the developing mouse pancreas *in vivo*. Whole rudiments of embryonic mouse pancreas were collected and prepared for assay of both protein content and lipase activity using [^{14}C]tripalmitin as substrate by procedures described in Methods.

on embryonic rat pancreases maintained in organ culture. Essentially normal development of whole 13-day pancreatic rudiments (including both epithelial and mesenchymal elements) can be achieved *in vitro*. Differentiation in these pancreases at the morphological level closely parallels that *in vivo* (Pictet *et al.*, 1972). As shown in Figure 9, the specific lipase activity was also indistinguishable in the *in vivo* and *in vitro* systems.

A similar profile for lipase activity in the pancreatic rudiments of mouse embryos is shown in Figure 10. The sigmoidal character of the curve and magnitude of the developmental increment resembled those for the rat. However, the developmental profile in the mouse is shifted 36–48 hr to later embryonic ages.

The observations of constant specific lipase activity during the early phase of pancreatic development and the lack of detectable activity in other tissues raise the question whether lipase activity is present in the presumptive exocrine cells from the initiation of pancreatic development. The pancreatic diverticulum in the rat begins to emerge from the gut at approximately the 20–25 somite state (11 days). Dissection of early diverticula from 25 somites (11-day) or 35 somites (12-day) embryos was too tedious to be practical, so lipase assays were carried out on regions of the gut isolated during these periods and compared to those of other somite as well as the head regions. The results are presented in Table II. Lipase activity was readily detected in 12-day embryonic gut sections. Specific activity was about tenfold lower in the 11-day gut sections, but was still severalfold higher than the background level of activity seen with the head and somite fractions of the same embryo. The total amount of lipase activity found in an 11-day gut section corresponds roughly to that found in 100 cells of the isolated 13-day pancreatic rudiment (this value correlates well with the approximate size of the pancreatic diverticulum at 25 somites).

These observations, semiquantitative as they are, support strongly the contention that lipase A is present at a low steady-state concentration within the pancreas but not present in other tissues during the entire early phase of morphogenesis from the formation of the initial pancreatic diverticulum until the period of cytodifferentiation begins at approximately 15 days.

Lipases A and B in Development. Embryonic and postnatal rat pancreases were tested for lipases A and B by the cellulose acetate electrophoresis technique with the activity stain. As shown in Figure 11, lipase A accounted for nearly all of the activity in the embryo and, indeed, for most of the activity through 3 weeks after birth. At days 15 and 16, lipase A activity could only be detected using β-naphthyl nonanoate but

TABLE II: Lipase A Activity in Sections of Early Rat Embryos.[a]

Tissue	Lipase Act.		
	% Hydrolysis	Sp Act.[b]	Act.:11-Day Gut
12-day Gut	0.16	8×10^{-3}	16
12-day Somites	0[b]	$<1 \times 10^{-4}$	$<2 \times 10^{-1}$
12-day Head	0[b]	$<2 \times 10^{-4}$	$<4 \times 10^{-1}$
11-day Gut	0.03	5×10^{-4}	1
11-day Somites	0[b]	$<1 \times 10^{-4}$	$<2 \times 10^{-1}$
11-day Head	0[b]	$<8 \times 10^{-5}$	$<1 \times 10^{-1}$

[a] Rat embryos were divided into heads, guts (including all internal organs except heart), and somites (limbs and early vertebrae). Sonicates were prepared from these tissues, submitted to zome electrophoresis, and subsequently assayed for lipase A activity by the procedures outlined in Methods. Quantities of protein from 30 to 80 μg were applied to the cellulose acetate strips. [b] Micromoles of oleic acid per minute per microgram of protein applied to strip.

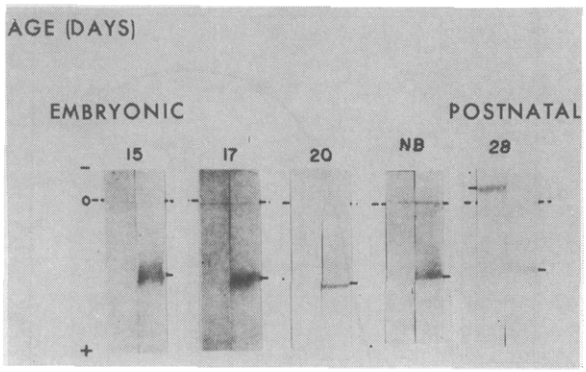


FIGURE 11: Ontogeny of pancreatic lipases A and B in the rat. Details of the procedure used are described in Figure 2.

at subsequent embryonic days, the less reactive β-naphthyl stearate could be used with successively decreasing concentrations of protein. Lipase B was first detectable at about 19-days gestation age. A lipase distribution (A:B = <0.5) characteristic of the adult organ was not observed until approximately the time of weaning (28-days postpartum).

The relative activities of lipases A and B during pancreatic development were estimated by cellulose acetate electrophoresis of the appropriate pancreatic extract and subsequent assay of sections of the strips for hydrolytic capacity against labeled triolein. This method is inherently highly sensitive and specific (if cumbersome), but its utility was limited by a troublesome and as yet uncontrolled variation in the hydrolytic rates observed with "control" segments of the cellulose acetate in practice strips, remote from the area containing lipases A or B. This fact thus limited the sensitivity of the assay. Using this procedure, however, a significant level of lipase B activity was readily demonstrated in the pancreases of 19-day embryos. Before this time, lipase B activity was not detectable; the limits of sensitivity of the assay indicate the ratio of lipase A: lipase B was at least 150. From 19 days *in utero* until 28 days after birth, the lipase A to B ratio still remains rather high (28 to 5) but the lipase A to B ratio is less than 1 in older animals.

Discussion

Multiplicity of Pancreatic Lipases. The present description of lipases A and B from rat pancreas is, to our knowledge, the first demonstration of multiple independent pancreatic lipase activities in a single species. Resolution of these two activities was affected by electrophoresis on cellulose acetate as well as by column chromatogrpphy on DEAE-cellulose. At pH 8.2–8.6, lipase A behaves as an anion and lipase B as a cation. Lipases A and B also exhibit different substrate specificities. Lipase A cleaves naphthyl alkanoates at a much higher rate than does lipase B, while the reverse is true for the hydrolysis of emulsified triolein. The most striking difference between the two enzymes, however, is in the effect of sodium taurocholate upon their activities toward naphthyl alkanoates. Lipase A activity is strongly enhanced by taurocholate while lipase B activity is inhibited by this bile salt.

A number of previous studies (Marchis-Mouren, 1965; Vandermeers and Christophe, 1968; Pascale *et al.*, 1966) have detected only a single lipase activity in hog and rat pancreas by chromatographic, immunological, and electrophoretic procedures. Matson and Volpenhein (1966) have however reported a single lipase activity in lyophilized rat pancreatic juice, whose activity to triolein is inhibited by bile salts at pH 8. In other systems, for example, hog pancreatic lipase, the stimulatory effect of taurocholate is well known (Borgstom, 1964). Thus, there is precedent for both effects of bile salts. We have shown that the purification procedure developed by Vandermeers and Christophe (1968) for rat pancreatic lipase yields only lipase B while that of Melius and Simmons (1965) developed for the hog pancreatic lipase but applied to the rat pancreas yields a mixture of lipases A and B. The basis for the resolution of lipases A and B by DEAE-cellulose chromatography is presented here. The resolution of taurocholate-activated and taurocholate-inhibited lipase activity from the pancreas and/or pancreatic juice of the mouse, rhesus monkey, and the human suggests the presence of both activities in these species, as well. On the other hand, taurocholate-inhibited lipases are not found in a number of other species (although two, three, and four bands of taurocholate-activated lipase were found in rabbit, guinea pig, and hog pancreatic extracts, respectively). These results then suggest a widespread, but not ubiquitous distribution, of lipase A and lipase B activities, and furthermore indicate the likelihood of multiple forms of lipase A in several species.

The resolution of two activities with different catalytic properties is not conclusively indicative of two discrete enzymes. This is especially true for an enzyme like lipase which operates at the interphase between aqueous and nonaqueous phases. Sarda *et al.* (1964), for example, report a marked change in the mobility of lipase activity of G-200 Sephadex by treatment with methanol–ether in the presence of deoxycholate. The authors suggest a lipid-bound aggregate as the high molecular weight species. In addition, there is always the possibility of proteolytic conversion in pancreatic secretions. The transformation of zymogens to active enzyme is well known. Recently a zymogen form of phospholipase A has been reported (Arnesjö *et al.*, 1967; De Haas *et al.*, 1968). In the present instance, however, all attempts to demonstrate conversion of one lipase activity to the other have been unsuccessful. The present report of multiple lipases is not the only instance of redundancy in specific exocrine proteins. Three distinct amylases were recently purified from rabbit pancreas (Malacinski and Rutter, 1969). Moreover, two trypsinogens (Marchis-Mouren, 1965; Pascale *et al.*, 1966) and two insulins (Smith, 1966) have been reported in the rat.

Distribution of Pancreatic Lipases. Both lipases A and B appear restricted to the pancreas. Considerable experimental effort was made to detect these enzymes in other tissues but no significant activity toward long-chained naphthyl alkanoates or toward triolein was evident. At least, the specific activity in adult rat pancreases was 10^4–10^5 times greater than any other tissues tested. Lipase activity, if present, in these other tissues was within the limits of sensitivity of the assay procedures employed. These results, then, suggest that the pancreatic lipases are cell specific proteins, and there is strongly selective expression of these genes in the mammalian systems studied. The case for unique distribution of several other pancreatic exocrine proteins has been presented elsewhere (Rutter *et al.*, 1967, 1968a).

Developmental Profile of Lipase Activities. The sensitive, highly specific assay employed here has allowed the detection of lipase activity throughout the course of pancreatic development. Parsa *et al.* (1966) observed lipase activity in the embryonic pancreas. They noted the significant decrease between days 19 and 18 and by linear extrapolation of their assay values suggested the appearance of this enzyme on day 15. The present work emphasizes that the enzyme is present

even in the earlier pancreatic rudiments and probably from the initial formation of the pancreatic diverticulum. The accumulation of lipase activity is a sigmoidal function of the developmental stage. Its specific activity is low and relatively constant during the early stages of development (12–15 days in the rat) and then is increased by three orders of magnitude to another steady-state level—that present in the fully differentiated tissues. A similar developmental profile is seen for the mouse pancreas, though over a slightly different period of gestation age. Two facts of considerable developmental significance arise from these data. The first is that the lipase level is apparently a characteristic of this period of differentiation. This level of activity is considerably higher than that present in nonpancreatic embryonic or adult tissues tested. (The specific activity of the youngest pancreatic rudiment isolated (a 12-day embryo) is at least 100-fold higher than that present in surrounding embryonic tissues including gut and somites or in adult tissues.) Furthermore, although the level of lipase within the cells of the early pancreatic rudiment is low compared to those of the adult pancreatic exocrine cell, it nevertheless represents significant synthetic activity. If the turnover numbers of mouse and hog pancreatic lipase are assumed to be identical (1.2×10^4 mmoles/min per mg of lipase) and the molecular weight of the pancreatic lipase is assumed to be 38,000 (Sarda *et al.*, 1964), then employing the relationship 13,000 cells/mg per protein for 12-day embryonic rat pancreas and 1600 cells/mg per protein for the fully differentiated rat pancreas (W. R. Clark and W. J. Rutter, unpublished data, 1968) it can be calculated that on the average, 700 molecules of lipase are present in each pancreatic cell during this early phase of development. Finally, other members of the exocrine pancreatic proteins have been detected during this early period. Relative activities are similar those in the differentiated state when each is present at much higher levels (Rutter *et al.*, 1967, 1968a).

The early accumulation of lipase may coincide with the beginning of pancreatic organogenesis (the primary developmental transition). The constant specific activity maintained over the early developmental period is suggestive of a single differentiative state which we have termed the "protodifferentiated" state (Rutter *et al.*, 1967, 1968a). The rapid increase in specific activity observed in pancreatic rudiments between 15 and 18 days of embryonic age occurs during the period when cytodifferentiation (development of an extensive endoplasmic reticulum and golgi apparatus together with zymogen granules) occurs within the exocrine cells (Kallman and Grobstein, 1964; Rutter *et al.*, 1964a,b). The fully differentiated cell contains somewhere between five and ten million lipase molecules, an increase of four orders of magnitude on a cellular basis (or three orders of magnitude on a protein basis since the protein content per cell increases about eightfold during this period). This period of striking accumulation of specific exocrine proteins in cytodifferentiation is considered evidence of a specific regulatory transition. More extensive morphological and enzymatic evidence in support of this multiphasic concept of differentiation is presented and discussed elsewhere (Rutter *et al.*, 1967, 1968a,b).

It is emphasized that lipase activity in the embryonic pancreas is primarily lipase A. The other exocrine specific proteins studied have similar but not identical developmental profiles. The pattern of development of lipase B, on the other hand, is singularly different. It is not detected until the later stages of embryonic life, but becomes the dominant lipolytic activity in the adult. This suggests that the level of lipase B is regulated by different effectors than is lipase A and perhaps the rest of the exocrine proteins. Independent regulation of the synthesis of certain exocrine proteins has been realized for some time, at least changes in the diet effect the enzymatic constituents independently (Reboud *et al.*, 1964, 1966). Lipase B activity might be influenced by the diet or by the levels of specific hormones since these change during the period of dramatic changes in the relative levels of lipase B activity. Another possibility should also be considered. Our studies on very early embryonic pancreases have been carried out primarily on the dorsal pancreas. The ventral pancreas develops independently and fuses with the dorsal pancreas at about 18–19 days—approximately the time that lipase B is first detected. It seems possible therefore that lipase A is produced by the dorsal and lipase B by the ventral pancreas. This can be readily tested using a recently developed culture procedure (Spooner *et al.*, 1971).

Finally, the closely similar developmental profiles of pancreatic rudiments developing *in vitro* or *in vivo* suggest that the mechanisms of regulation of lipases A and B and other exocrine proteins during development may be effectively investigated using the *in vitro* system.

References

Arnesjö, B., Barrowman, J., and Borgström, B. (1967), *Acta Chem. Scand. 21*, 2897.

Asdell, S. A. (1946), Patterns of Mammalian Reproduction, Ithaca, N. Y., Comstock Publishing Co.

Borgström, B. (1964), *J. Lipid Res. 5*, 522.

Bradshaw, W. S. (1968), Ph.D. Thesis, Univ. of Illinois.

Clark, W. R., and Rutter, W. J. (1968), Ph.D. Thesis, Univ. of Washington.

De Haas, G. H., Postema, N. M., Nieuwenhuizen, W., and Van Deenen, L. L. M. (1968), *Biochim. Biophys. Acta 159*, 118.

Desneuelle, O., Sarda, L., and Ailhaud, G. (1960), *Biochim. Biophys. Acta 37*, 571.

Entressangles, B., Sari, H., and Desnuelle, P. (1966), *Biochim. Biophys. Acta 125*, 597.

Kallman, F., and Grobstein, C. (1964), *J. Cell Biol. 20*, 399.

Kinard, F. E. (1957), *Rev. Sci. Instr. 28*, 293.

Kramer, S. P., Aronson, L. D., Rosenfeld, M. G., Sulkin, M. D., Chang, A., and Seligman, A. M. (1963), *Arch. Biochem. Biophys. 102*, 1.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Malacinski, G. M., and Rutter, W. J. (1969), *Biochemistry 8*, 4382.

Marchis-Mouren, G. (1965), *Bull. Soc. Chim. Biol. 47*, 2207.

Marchis-Mouren, G., Sarda, L., and Desnuelle, P. (1959), *Arch. Biochem. Biophys. 83*, 309.

Mattson, F. H., and Volpenhein, R. A. (1966), *J. Lip. Res. 7*, 536.

Melius, P., and Simmons, W. S. (1965), *Biochim. Biophys. Acta 105*, 600.

Parsa, I., Marsh, W. H., and Fitzgerald, P. J. (1966), *2nd Int. Symp. Cell. Chem., Ohtsu, Japan.*

Pascale, J., Aurameas, S., and Uriel, J. (1966), *J. Biol. Chem. 241*, 3023.

Pictet, R., Clark, W. R., Renold, A. E., and Rutter, W. J. (1972), *Develop. Biol.* (in press).

Reboud, J. P., Marchis-Mouren, G., Pasero, L., Cozzone, A., and Desnuelle, P. (1966), *Biochim. Biophys. Acta 117*, 35.

Reboud, J. P., Pasero, L., and Desnuelle, P. (1964), *Biochem. Biophys. Res. Commun. 17*, 347.

Rutter, W. J. (1967), *in* Methods in Developmental Biology,

Wilts, F. A., and Wessells, N. K., Ed., New York, N. Y., Thomas Y. Crowell Co., p 671.
Rutter, W. J., Ball, W. D., Bradshaw, W. S., Clark, W. R., and Sanders, T. G. (1964a), *Exp. Biol. Med. 1*, 110.
Rutter, W. J., Clark, W. R., Kemp, J. D., Bradshaw, W. S., Sanders, T. G., and Ball, W. D. (1968a), *in* Epithelial-Mesenchymal Interactions, Fleischmajer, R., Ed., Baltimore, Md., Williams & Wilkins, p 114.
Rutter, W. J., Kemp, J. D., Bradshaw, W. S., Clark, W. R., Ronzio, R. A., and Sanders, T. G. (1968b), *J. Cell. Physiol. 72*, Suppl. 1.1.
Rutter, W. J., Wessells, N. K., and Grobstein, C. (1964b), *J. Nat. Cancer Inst. 13*, 57.
Sarda, L., Maylie, M. F., Roger, J., and Desnuelle, P. (1964), *Biochim. Biophys. Acta 89*, 183.
Seligman, A. N., Nachlas, M. M., and Mollono, M. L. (1949), *Amer. J. Physiol. 159*, 337.
Smith, L. (1966), *Amer. J. Med. 40*, 662.
Spooner, B., Walther, B., and Rutter, W. J. (1970), *J. Cell Biol. 47*, 235.
Vandermeers, A., and Christophe, J. (1968), *Biochim. Biophys. Acta 154*, 110.

Purification and Properties of Dihydrofolate Reductase from an Amethopterin-Resistant Strain of *Streptococcus faecium*†

L. D'Souza, P. E. Warwick,‡ and J. H. Freisheim*

ABSTRACT: Dihydrofolate reductase from an amethopterin-resistant strain of *Streptococcus faecium* has been isolated and purified (*ca.* 360-fold) employing a procedure that involves sonication of intact cells, filtration through Bio-Gel P-150, and chromatography on hydroxylapatite and DEAE-Sephadex. Two forms of the enzyme (I and II) were separated following hydroxylapatite chromatography. Rechromatography of form II enzyme on hydroxylapatite resulted in the conversion of II to I as judged by electrophoretic analysis. Form I enzyme was isolated in *ca.* a 40% overall yield following DEAE-Sephadex chromatography. At pH 6.5, the turnover number was 6000 moles of dihydrofolate reduced min^{-1} $mole^{-1}$ of enzyme at 25°. The enzyme showed a single protein band following polyacrylamide gel electrophoresis and was monodisperse in the ultracentrifuge ($s_{20,w}$ = 2.10 S). The reductase had a molecular weight of *ca.* 20,000 as judged by sedimentation equilibrium analysis, gel filtration, and amethopterin titration. Amino acid analyses indicated the presence of one cysteine residue and nine tryptophan residues per mole of protein. The circular dichroic absorption spectrum of the enzyme revealed the presence of an aromatic side-chain Cotton effect consisting of three distinct ellipticity bands in the 250- to 310-nm region.

Dihydrofolate reductase (EC 1.5.1.3), which catalyzes the TPNH-dependent reduction of dihydrofolate to tetrahydrofolate, has been obtained in essentially pure form from chicken liver (Kaufman and Gardiner, 1966; Kaufman and Pierce, 1971; Huennekens *et al.*, 1970), L-1210 lymphoma (Perkins *et al*,. 1967), *Streptococcus faecium* (Nixon and Blakley, 1968), and *Lactobacillus casei* (Gundersen *et al.*, 1972) among others. This enzyme appears to be the main, if not the only, target site for the 4-amino-4-deoxy analogs of folic acid.

Elevated levels of dihydrofolate reductase have been reported for certain cell types exhibiting amethopterin resistance as compared to that observed in the corresponding sensitive parent strain. This phenomenon has been observed in amethopterin-resistant strains of various bacteria (Freisheim *et al.*, 1972; Crusberg *et al.*, 1970; Dunlap *et al.*, 1971; Nixon and Blakley, 1968; Sirotnak and Salser, 1971) as well as in certain mammalian (Hakala, 1965) and tumor cells (Sartorelli *et al.*, 1964; Friedkin *et al.*, 1962).

The results of this study describe the purification and certain molecular properties of dihydrofolate reductase from an amethopterin-resistant strain of *S. faecium*. A comparison of certain properties of the enzyme isolated in the present study to those of the dihydrofolate reductases isolated by Nixon and Blakley (1968) suggest that different strains of amethopterin-resistant *S. faecium* were employed.

Materials and Methods

Materials. The amethopterin-resistant strain of *S. faecium* (ATCC 8043) was a gift from Dr. Carl Smith, University of Cincinnati. The characterization of this strain in terms of specific growth requirements is being done by Dr. Smith and coworkers. Dihydrofolate was prepared from folic acid (Calbiochem) by the dithionite method of Futterman (1957) as modified by Blakley (1960). TPNH was obtained from P-L Biochemicals. DEAE-Sephadex A-50 was obtained from Pharmacia Fine Chemicals. Hydroxylapatite (Bio-Gel HT) and Bio-Gel P-150 were purchased from Bio-Rad Laboratories. The hydroxylapatite was mixed with an equal weight of Celite (Baker) as a support to obtain better chromatographic flow rates. Dialysis tubing (Union Carbide) was treated as described by Kaufman and Gardiner (1966). All other chemicals were of reagent or analytical grades.

† From the Department of Biological Chemistry, College of Medicine, University of Cincinnati, Cincinnati, Ohio 45219. *Received November 18, 1971.* This work was supported by a grant (CA-11666) from the National Cancer Institute, National Institutes of Health. Paper II in a series on folate-dependent enzymes.

‡ National Science Foundation Predoctoral Trainee.