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Determination of Carboxypeptidase in Human Pancreatic Juice.* (24626)

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While properties and specificities of proteolytic enzymes of human pancreatic juice are well characterized, few quantitative measurements are available. Lack of data stems from 2 difficulties: obtaining samples of juice and presence of enzymes as zymogens. Human pancreatic juice is now available in adequate amount by collecting it through plastic catheters left in pancreatic duct for drainage after sphincterotomy(1). Total proteolytic activity of pancreatic juice in healthy and diseased pancreas has been determined(2). However, few data are available concerning relative amounts of trypsinogen, chymotrypsinogen, and procarboxypeptidase enzymes activated by trypsin. Procarboxypeptidase was recently isolated and studied by Neurath, *et al.*(3), although its existence as proenzyme requiring activation by trypsin had been established by Anson(4) as early as 1937. Measuremnt of carboxypeptidase in pancreatic juice is facilitated by availability of the synthetic substrate, carbobenzoxyglycyl-l-phenylalanine, first synthesized by Bergmann (5). This substrate is quite specific for carboxypeptidase and is not affected by trypsin or chymotrypsin(6). The enzyme splits off the phenylalanine moiety, liberating alpha-amino group which can be conveniently followed by ninhydrin color. The method used here is a modification of assay described by Neurath *et al.*(6). The ninhydrin reagent of Stein and Moore(7) is used for color development. The method previously described (2) for activation of total pancreatic juice proteolytic activity (utilizing calcium and minute amounts of trypsin) required 18 hours at 4°C and was somewhat variable. We devised a method utilizing commercial duodenal extract for production of enterokinase preparation which is simple to prepare, stable, and which completely activates trypsinogen of pancreatic juice in 60 to 75 minutes at room temperature. The presence of calcium ion in this extract retards autolysis of the trypsin.

Methods. These procedures were applied to measure active carboxypeptidase and total carboxypeptidase after activation of procarboxypeptidase with duodenal extract in human pancreatic juice. Pancreatic juice samples were collected from 12 patients with surgical fistulas at various intervals during their postoperative course. 1. *Reagents*—A. .1M veronal buffer (PH 7.8). B. .06M carbobenzoxyglycyl - l - phenylalanine (Mann Fine Chem.). C. 5% LiCl. D. 8% ninhydrin (Dougherty Chem. Co.) in methyl cellosolve mixed with equal volume of freshly prepared stannous chloride (16 mg dissolved in 10 ml of pH 5. citrate buffer). E. *Duodenal extract containing enterokinase*—400 mg Viodenum (whole duodenum defatted and dehydrated at low temperature, VioBin Corp., Monticello, Ill.) is suspended in 10 cc distilled water containing 200 mg $CaCl_2$ and shaken gently 18 hours at 4°C. After centrifuging, the supernatant is used as activator. This preparation is stable for at least one month if kept frozen. F. 50% N-Propanol. 2. A *standard curve* was prepared by subjecting known amounts of carboxypeptidase, varying 1.5-39 mg/per ml to procedure described below. Values were plotted against optical density. Carboxypeptidase suspension (Worthington Co.) was dissolved in 10% LiCl at 0°C. The exact amount of carboxypeptidase in this solution

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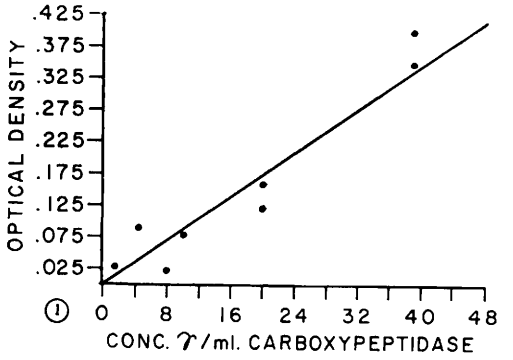
TABLE I. Active and Total Carboxypeptidase in Pancreatic Juice following Recovery from Operation.

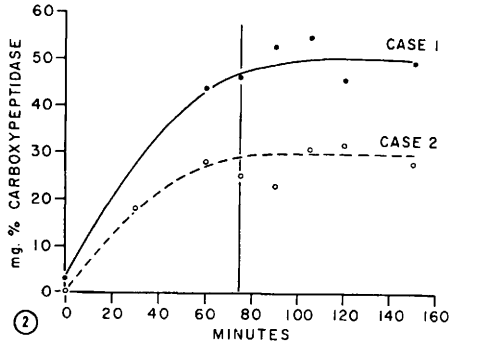
Patient	Days post-op.	mg % carboxypeptidase Active	Total
M.L.	3	31.	57.
	10	3.4	43.
J.F.	1	113.	119.
	8	6.	
C.M.	1	14.	97.
	5	4.8	17.5
T.F.	1	38.	130.
	4	2.5	40.
E.J.	1	52.	
	3	2.5	5.
Avg, 12 patients	10+	3.9 (2.2–6.2)	52.8 (28.3–134)

extract as described above. This mixture was then diluted 1:25 and a 0.25 ml aliquot was used as the enzyme mixture.

Results. Forty determinations of active and total carboxypeptidase were made on pancreatic juice from 12 patients. Samples were collected at various intervals following operation beginning on first postoperative day. Samples collected during first 3 postoperative days showed high concentration of enzyme (Table I). In 3 instances active carboxypeptidase represented a significant part of total carboxypeptidase. In one patient a sample was analyzed on 18th postoperative day following pancreatographic study, which produces a transient inflammatory reaction(9). Active trypsin in this sample was 3.91 mg %. Normal human pancreatic juice contains no more than 0.2 mg % of active trypsin(2). Active carboxypeptidase was 119 mg % and total was 127 mg %. Evidence from pancreatographic studies and, in a few instances, direct examination at operation indicates that with proper treatment the pancreas resumes normal function 10 to 14 days after operation (9). Results of analyses of pancreatic juice collected beyond this period were considered normal, in that the pancreas had recovered from operative procedure. Analysis of 12 samples showed uniformly that there was a significant amount of active enzyme present in pancreatic juice (Table I). Average value for active carboxypeptidase was 3.9 mg %, range 2.2-6.2 mg %. Total enzyme concentration, which represents the active plus procarboxypeptidase, had average value of 52.8 mg %, range 28.3-134.0 mg %.

Discussion. Patients from whom pancreatic juice was collected were kept on naso-gastric suction for the first 3 postoperative days. In the absence of acid in the duodenum the pancreas secreted a very small amount of juice containing high concentration of enzymes. When feeding was started, the secretin produced by contact of food and acid with the duodenal mucosa, caused an increased secretion of water and bicarbonate by the pancreas, diluting the enzymes (Table I). Concentration of active carboxypeptidase was also high during this first postoperative period but diminished after a few days. Similar findings were previously noted when proteolytic activity was measured(2). High active trypsin content was correlated with presence of acute inflammation since subsidence of the acute process was accompanied by almost complete disappearance of active trypsin. High ratio of active to total carboxypeptidase found during the first few postoperative days may have been due to activation of procarboxypeptidase by active trypsin present in such cases. Thus in one patient in whose juice active carboxypeptidase was 94% of total carboxypeptidase, active trypsin was found to be markedly elevated. Values reported here for active carboxypeptidase in normal pancreatic juice were significantly higher than those reported for active trypsin which rarely rose above 0.2 mg %(2). This amount of trypsin should be adequate to activate the larger amount of carboxypeptidase found. Indications that carboxypeptidase is a rather stable proteolytic enzyme first became evident during activation studies with enterokinase. Procarboxypeptidase was almost completely activated in 60-75 minutes but little of the activity was lost during prolonged incubation at 37°C even after 3 hours. On the other hand, although trypsinogen also attained maximal activation at 75 minutes, decline thereafter was rapid due presumably to autodigestion (Fig. 3). It was also found that pancreatic juice could be kept at least 24 hours at 5°C without loss of either active or procarboxypeptidase. The stability of this enzyme and its high specificity for this





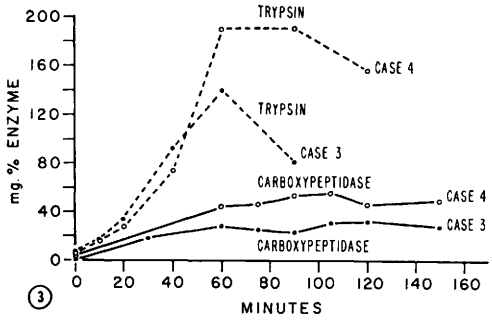


FIG. 1. Carboxypeptidase—standard curve.
FIG. 2. Activation curve 37°C. 1 ml pancreatic juice + 0.1 ml duod. extract.
FIG. 3. Comparative stability after activation.

was estimated on the basis of its nitrogen content using the value of 15.4% nitrogen cited by Neurath(3). Kinetic studies were undertaken to compare activity of standard carboxypeptidase with the highly purified preparations of Neurath *et al.*(3). A proteolytic activity coefficient, C = 13, agreed reasonably with values found by Neurath under similar conditions(8). The curve obtained was linear and first order as expected. Subsequently, all pancreatic juice values were read from the curve (Fig. 1). 3. *Procedure.* A. Activation of Pancreatic Juice. Procarboxypeptidase in pancreatic juice with 0.1 ml of duodenal enterokinase preparation at 37°C. Experiments were carried out to find optimal time of activation. Samples were taken from the activation mixture at convenient intervals and assayed for carboxypeptidase activity. The results of 2 such studies are shown in Fig. 2. B. *Incubation mixture* which contained 0.5 ml veronal buffer (pH 7.8), 0.5 ml 0.06M carbobenzoxyglycyl-l-phenylalanine, and 0.25 ml 5% lithium chloride, was brought to 37°C in water bath. At zero time 0.25 ml of enzyme solution was added. In exactly 15 minutes the reaction was stopped by rapidly pipetting *0.2 ml* of incubation mixture into a tube containing 1.8 ml water immersed in boiling water bath. After at least 5 minutes this tube was cooled and 0.2 ml aliquot pipetted into a tube graduated at 10 ml. One ml of the ninhydrin reagent was added and the tube kept in boiling water for 20 minutes. Then the tube was cooled and diluted to 10 ml with 50% n-propanol in water. This was read against a blank in a colorimeter at 550 mm. The blank was prepared by pipetting the enzyme solution into tube containing incubation mixture immersed in boiling water. After 5 minutes this was cooled and a 0.2 ml aliquot was added to 1.8 ml distilled water. 0.2 ml aliquot of this solution was treated with ninhydrin as above. 4. *Determination of carboxypeptidase in pancreatic juice.* A. Active carboxypeptidase—Since a small amount of proteolytic activity as measured by casein digestion (without activation) was present in human pancreatic juice(2), studies were undertaken to determine if active carboxypeptidase was also present. Pancreatic juice was always collected in ice bath. Freshly collected pancreatic juice was diluted 1:10 in water and assayed for carboxypeptidase A significant amount of active carboxypeptidase was found in all samples of human pancreatic juice flowing directly from pancreatic ducts. B. Total carboxypeptidase was measured by first activating pancreatic juice with duodenal

type of synthetic substrate should permit its estimation in blood and urine. Accurate measurement of the blood levels of proteolytic enzymes of pancreatic origin has not been achieved. The presence of inhibitors, enzymes of similar properties and specificities, and complicated activation systems have raised doubts as to the reliability of these methods. Attempts to measure carboxypeptidase in serum and urine are in progress and preliminary studies suggest that this is possible.

Summary. 1. A method is described for determination of carboxypeptidase in human pancreatic juice. Eighteen samples of pancreatic juice gave an average value for active carboxypeptidase of 3.9 mg % with a range of 2.2-6.2 mg %. Total enzyme concentration representing active enzyme plus procarboxypeptidase gave an average value of 52.8 mg % with a range of 28.3-134.0 mg %. 2. A significant amount of active carboxypeptidase was found in fresh pancreatic juice. High values of active and total carboxypeptidase were found initially postoperatively but these declined on about the third postoperative day. 3. Carboxypeptidase was found to be more stable to autolysis than trypsin.

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Metachromatic Sulfatides in Cerebral White Matter and Kidney.* (24627)

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Of considerable biological interest is the striking metachromasia of normal myelin with toluidine blue(1). Color change of this blue dye toward red is consistent with the presence of compounds rich in negatively-charged acid groups(6). While acid mucopolysaccharides are commonly associated with metachromatic staining, it is less fully appreciated that acidic lipids may also cause intense metachromasia. These strongly charged anionic groups might be expected to bind cations. Such a process might be relevant to bioelectric potentials involved in the nerve impulse and to rapid movements of K+, Na+, Ca++, etc. across membrane systems in other body tissues.

In previous staining experiments, made *in vitro* on naturally-occurring sulfatides, it was shown that at least part of the normal lipid metachromasia of white matter may be related to sulfuric acid ester groups(2,7).

It was thus of interest to extend these staining observations to a measurement of lipid sulfuric ester groups in 2 tissues where cation transfer is biologically prominent. In the present study normal, metachromatic white matter was contrasted with normal kidney, where lipid metachromasia is negligible. Pertinent diseases which afford a range of sharply contrasting values were also included. Results bear ultimately on the intriguing function of myelin and other sulfatides in health and disease.

Materials and methods. Applicability of partitioning, separation, and identification(3, 4,8,9,11) methods used in this study were previously verified(2,7), (Witmer, F., and Austin, J. to be published). Histologically nor-

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