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Multiple Molecular Forms of α-Amylase from the Rabbit*

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ABSTRACT: Electrophoretic variants of α-amylase were observed in various vertebrate species (lungfish, chicken, beef, guinea pig, rat, rabbit, and human). Three variants of the enzyme were isolated from the rabbit pancreas and another single amylase from the parotid gland. Some of their catalytic and molecular properties have been defined. The amino acid compositions, molecular weights (approximately 54,000), and catalytic properties of the enzymes were very similar. The number of peptides observed in tryptic maps closely corresponds to the predicted number of trypsin-sensitive bonds. This finding and the results of ultracentrifugal studies in 6 M guanidine hydrochloride suggest the molecule is composed of a single subunit.

Reproducible differences in the tryptic maps of each of the enzyme preparations were also detected. These results are compatible with the postulate that the molecules have unique sequences, and are thus the products of separate genes. However, phenotypic modifications of a single gene product are not ruled out, especially since the enzymes appeared somewhat heterodisperse on ultracentrifugation in 6 M guanidine hydrochloride (possible degradation?). The difficulties in resolving differences in primary structure from modifications of a single structure in multiple closely related (homologous) molecules are emphasized.

The enzyme α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) is a prominent constituent of the mammalian pancreas and, in some species, the parotid glands. Preliminary evidence has suggested that a number of molecular forms may contribute to the amylase activity measured in pancreatic and salivary extracts. For example, Marchis-Mouren and Pasero (1967) demonstrated two forms of amylase in the hog pancreas, and Sick and Nielsen (1964) detected two amylase isozymes in the mouse pancreas, while Lamberts *et al.* (1965) reported the existence of four electrophoretically distinct amylases in human saliva. In no instance, however, has the molecular basis of these variants been defined. These variants might possess a unique primary structure and hence be products of different genes. On the other hand, if the enzyme were composed of multiple subunits, then the biosynthesis of more than one subunit could result in both heteromeric and homomeric combinations yielding hybrid molecules which possess, as in the case for lactic dehydrogenase (Appella and Markert, 1961) and aldolase (Penhoet *et al.*, 1967), different electrophoretic mobilities. Alternatively, phenotypic alterations such as binding of carbohydrate residues or limited proteolysis could generate variants with differing electrophoretic mobilities from a single molecular species.

The pancreas and parotid glands share a similar physiological function and are largely composed of similar cell types. It is not known, however, whether the pancreatic and parotid amylase molecules are identical and hence the products of the same gene(s) in these glands. A comparative study of crystalline human salivary and pancreatic amylase made by Bernfeld *et al.* (1950) suggested that the proteins were closely related or identical. More recently, McGeachin *et al.* (1966) found the rabbit pancreatic and salivary amylases to be immunologically identical. These experimental results, though they certainly indicate a close similarity in the molecules, do not prove identity.

The present investigation was undertaken with the purpose of isolating the amylases from the pancreas and parotid glands of the rabbit and determining the basis of the molecular variation in amylases from these tissues. A characterization of the amylases, including molecular weight determinations and amino acid and peptide compositions, were carried out.

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Experimental Procedure

Tissues. Rabbit pancreatic tissue was obtained from Lab Associates, Kirkland, Wash. Parotid gland tissue was purchased from Pel-Freeze Biologicals, Rogers, Ark. After the animals were sacrificed, the tissues were removed immediately and quick frozen on Dry Ice. They were stored at −20° until used.

Reagents. A solution of high molecular weight glycogen was prepared by adding 95% ethanol to a 5% aqueous solution of oyster glycogen (Nutritional Biochemicals Corp., Cleveland) with stirring. When an ethanol concentration of 40% was attained, the precipitate was collected by centrifugation at 10,000*g* for 10 min and air dried. This material was redissolved in distilled water to a final glycogen concentration of 2%.

The charcoal–Celite mixture was prepared with activated charcoal (British Drug Houses, Ltd., Poole, England) according to the method of Schramm and Loyter (1966). After the mixture was packed into a chromatography column it was washed with 0.02 M Tris-HCl (pH 8.0) for 48 hr.

Assays. Amylase was assayed by the method of Bernfeld (1955). The Fisher Scientific Co. (Chicago) brand of soluble starch was employed as substrate and a unit of enzymatic activity is defined as 1 mg of maltose hydrate equivalent produced in 3 min at 30°. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The specific activity of the enzymes is expressed as units of activity per milligram of protein.

Zone Electrophoresis. Electrophoresis was performed in 0.05 M acetate buffer (pH 5.5) at a constant current of 1 mA/strip for 5 hr on 6.75-in. cellulose acetate strips (Gelman Instrument Co., Ann Arbor). The zones of amylase activity were located by placing the strip on the surface of a layer of starch–agar containing 0.05 M histidine-HCl (pH 6.5), 0.8% Noble agar (Difco, Detroit), and 0.5% soluble starch. The Petri dish containing the starch–agar and the strip was incubated at 37° for 10–40 min and the zones of starch hydrolysis were detected by removing the strip and irrigating the plate with 0.25 M potassium iodide–0.0035 M iodine solution.

Analytical Disc Gel Electrophoresis. Electrophoresis was performed at pH 4.5 in a 7.5% polyacrylamide gel system devised by Reisfeld *et al.* (1962). A constant current of 3 mA/gel column (0.5 × 5.0 cm) was applied for the time required for the tracking dye (methyl green) to migrate the length of the column (approximately 1.5 hr). The protein in the gel column was stained with 1% Amido Black 10 B in 7% acetic acid. The gels were destained by electrophoresis.

Amino Acid Composition. Protein samples were extensively dialyzed against 0.01 M potassium phosphate (pH 6.9) and aliquots containing 2.75 mg of enzyme were lyophilized. Hydrolysis of the protein was carried out with constant-boiling hydrochloric acid in evacuated, sealed tubes at 110° for 24, 48, and 120 hr. A small crystal of phenol was included in each tube. After hydrolysis the HCl was removed at 50° with a rotary evaporator. The amino acid analysis was performed with a Beckman Model 120C automatic amino acid analyzer according to the procedure of Spackman *et al.* (1958). The tryptophan content was estimated by a spectrophotometric method (Edelhoch, 1967). The values for serine and threonine were obtained by extrapolation to zero times of hydrolysis.

Peptide Maps. Amylase at a concentration of 5 mg/ml was extensively dialyzed against 0.1 M ammonium bicarbonate (pH 8.2). EDTA, β-mercaptoethanol, and urea were added to final concentrations of 0.006, 0.1, and 8.0 M, respectively. The resulting protein solution was maintained at 45° for 4 hr with occasional stirring. Recrystallized iodoacetamide was added slowly to a final concentration of 0.5 M and 6.0 N sodium hydroxide was added dropwise to maintain the pH at 8.2 (Craven *et al.*, 1965). The mixture was allowed to stand in the dark for 20 min and then a tenfold molar excess of β-mercaptoethanol over iodoacetamide was added. Stirring was maintained for 45 min and then the resulting amidocarboxymethylated protein was extensively dialyzed against 0.1 M ammonium bicarbonate. The protein solution was lyophilized, weighed, and suspended in 0.2 M ammonium bicarbonate (pH 8.6) to a final concentration of 5 mg/ml. Bovine trypsin (TPCK[1]-treated, Gallard-Schlesinger Chemical Manufacturing Corp., Carle Place, N. Y.) was added in an amylase:trypsin ratio of 100:1. After 8-hr stirring at room temperature a second 100:1 aliquot of trypsin was added and stirring continued for 4 hr longer. The reaction mixture was centrifuged and the supernatant was lyophilized. The peptides (3 mg) dissolved in 20 μl of redistilled water were applied to 40 × 56 cm sheets of Whatman No. 3MM filter paper. Chromatography was carried out in butanol–acetic acid–water (4:1:5, v/v) for 16 hr and then the sheets were air dried for 24 hr. The sheets were rotated 90° and electrophoresis was performed for 1 hr at 2600 V in pyridine–acetic acid–water (1:10:293, v/v) at pH 3.6 (Katz *et al.*, 1959). The peptides were localized with a 0.25% ninhydrin–acetone solution.

Enzyme Purification and Experimental Results

Electrophoretic Variants of Amylase. A survey of the pancreas and salivary glands of several species (lungfish, chicken, beef, guinea pig, rat, and human) revealed the presence of electrophoretic variants in a variety of vertebrates. These data are summarized in Figure 1. Zone electrophoresis of crude extracts of the pancreas and parotid gland revealed the presence of multiple forms of amylase in the rabbit also (Figure 2). A single zone of amylolytic activity was observed in the parotid extract, whereas three electrophoretically distinct amylase activities were detected in crude extracts of the pancreas. These variants are referred to as P_1, P_2, and P_3, in order of increasing electrophoretic mobility toward the cathode.

It is known that amylase is present (McGeachin and Potter, 1960) and synthesized (McGeachin *et al.*, 1960, 1964; Rutter *et al.*, 1961; Arnold and Rutter, 1963) in mammalian liver. The electrophoretic mobility of the liver amylase was examined by applying a crude extract of liver to the cellulose acetate electrophoresis system described here. A single zone of amylase activity with a unique electrophoretic mobility somewhat greater than that of the pancreatic and parotid amylases was observed. The concentration of the liver amylase was, however, too low to permit isolation of the amylase by the methods employed here.

[1] Abbreviation used is: TPCK, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone.

TABLE I: Purification Procedure for Rabbit Pancreatic Amylases.[a]

Step	Units/ml	Total Units	Sp Act.	Recov (%)	-Fold
I, Crude extract	640	948,000	53	100	
II, Ethanol fraction	145	579,000	58	61	1
III, Glycogen precipitate	7,800	234,000	1,170	25	20
IV, Autodigestion	36,000	234,000	740	25	14
V, Charcoal column	3,530	131,000	1,220	14	23
VI, DEAE-cellulose chromatography					
Isolation of P_3	1,210	18,700	1,210	2	23
VII, Preparative disc gel electrophoresis					
Isolation of P_1	500	(10,500)[b]	1,000	1[b]	18
Isolation of P_2	945	(30,500)[b]	975	3[b]	18

[a] The experimental details are given in the Experimental Procedure. [b] From two DEAE-cellulose column chromatography runs; total units were pooled and applied to the preparative disc gel apparatus; the resultant figures were therefore divided by two for presentation in this table.

Enzyme Purification. Rabbit pancreatic and parotid amylases were purified by a modification of a procedure devised by Schramm and Loyter (1966). The enzymes were precipitated out of a crude extract with high molecular weight glycogen. Then glycogen dextrins were removed from the enzyme by either a modified charcoal column procedure or by digestion with amyloglucosidase. All of the operations, unless stated otherwise, were carried out at 4°. The purification procedure for the pancreatic and parotid amylase is summarized in Tables I and II. The methods employed were similar except different procedures were employed for the removal of the glycogen dextrins.

STEP I: CRUDE EXTRACT. Frozen tissue was diced into small pieces, thawed, and suspended in three volumes of 0.012 M potassium phosphte buffer (pH 6.9) containing 3.0×10^{-3} M calcium chloride, 4.0×10^{-3} M sodium chloride, 1.0×10^{-3} M phenylmethylsulfonyl fluoride (Calbiochem, Los Angeles), and 0.002% Triton X-100 (Rohm and Haas Co., Philadelphia). The mixture was homogenized in a Sorvall Omni-Mixer at top speed for 3 min and then centrifuged at 12,000*g* for 45 min. The pellet was reextracted by rehomogenizing in more of the same buffer and recentrifuging. The supernatants were combined and centrifuged at 35,000*g* for 1 hr. The pellet was discarded.

STEP II: ETHANOL PRECIPITATION. Reagent grade 95% ethanol was passed through a Dry Ice–methanol cooling bath into the crude extract with stirring. After addition of ethanol to 40% (v/v), the mixture was stirred for an additional hour and then centrifuged at 10,000*g* for 20 min. The pellet, which, on occasion, contained more than 60% of the amylase activity, was resuspended in the buffer of step I and reprocessed in the same fashion.

STEP III: GLYCOGEN PRECIPITATION. The 2% glycogen solution, prepared as described in Experimental Procedure, was added dropwise, with stirring, to the ethanol-fractionated supernatant. Glycogen (1 mg) was added per 400 units of

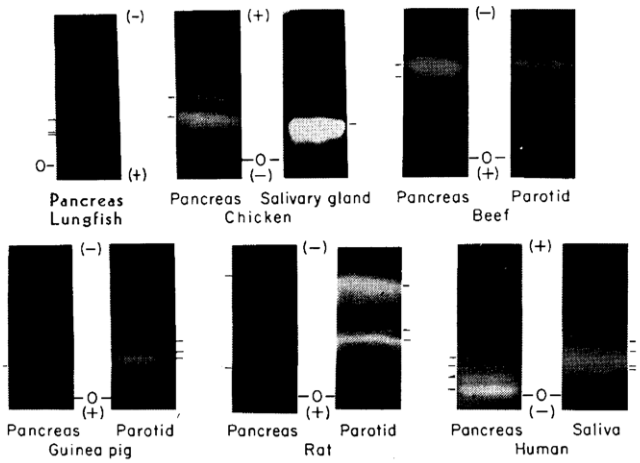

FIGURE 1: Cellulose-acetate zone electrophoresis of crude extracts of the pancreas and salivary glands of various vertebrates.

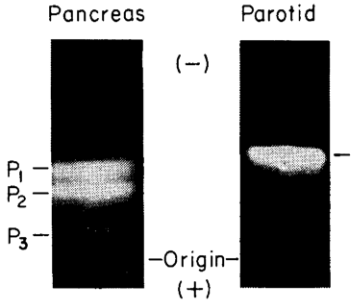

FIGURE 2: Cellulose-acetate zone electrophoresis of crude extracts of rabbit pancreas and parotid gland. Electrophoresis was performed in 0.05 M acetate buffer (pH 5.5) for 5 hr. The amylolytic activity was resolved by placing the cellulose acetate strip on a layer of starch–agar, removing the strip after 20 min, and irrigating with a potassium iodide–iodine solution (see Experimental Procedure section). Identical patterns were obtained when the glycogen-purified enzymes (step V) were run in this electrophoresis system.

TABLE II: Summary of Procedure for Purification of Rabbit Parotid Amylase.[a]

Step	Units/ml	Total Units	Sp Act.	Recov (%)	-Fold of Purificn
I, Crude extract	1,900	1,415,000	211	100	1
II, Ethanol fractionation	320	390,000	533	28	2.5
III, Glycogen precipitate	10,400	336,000	900	24	4.3
IV, Autodigestion	19,300	231,200	920	16	4.5
V, CM-Sephadex chromatography	6,400	205,000	994	14	4.7

[a] The experimental details are given in the Experimental Procedure.

amylase. The mixture was stirred for 30 min, then centrifuged at 12,000*g* for 1 hr. The supernatant was discarded and the pellet was washed once with 40% ethanol.

STEP IV: GLYCOGEN DIGESTION. The glycogen precipitate was suspended in 0.012 M potassium phosphate buffer (pH 6.9) containing 3.0×10^{-3} M calcium chloride, 4.0×10^{-3} M sodium chloride, and 5.0×10^{-4} M phenylmethylsulfonyl fluoride to a final protein concentration of 20 mg/ml. The mixture was kept at room temperature for 2 hr with occasional stirring. The pH was then adjusted to 8.5 with 1.0 M ammonium hydroxide and the mixture centrifuged at 6000*g* for 5 min. The supernatant fluid was readjusted to pH 6.0 with 1.0 M acetic acid and then placed on ice. The pellet was resuspended in more of the same buffer and reprocessed in the same way. The precipitates which formed overnight on ice were collected by centrifugation at 30,000*g* for 30 min.

STEP V: REMOVAL OF GLYCOGEN DEXTRINS. Glycogen dextrins were still present in the enzyme preparation in the ratio of approximately 0.1 mg/ml of protein. The dextrins were removed from the pancreatic amylases by passage over a charcoal–Celite column. For this purpose the pancreatic amylase–glycogen precipitate was suspended in 0.02 M Tris-HCl (pH 8.0) at a final protein concentration of 20 mg/ml. The glycogen content was determined colorimetrically (Dubois *et al.*, 1956), then a saturated glucose solution (in the same buffer) was added with stirring until the precipitate was dissolved and would remain in solution when centrifuged at 25,000*g* for 30 min at 25°. This enzyme solution was passed over a charcoal–Celite column (length to width ratio of 10:1) at room temperature. The size of the column was important for full recovery of the enzyme and was determined by the glycogen content of the enzyme preparation; 1 ml of charcoal–Celite was used for each 4.0 mg of glycogen. The enzyme was eluted from the column with the Tris-HCl buffer (pH 8.0) at a rate of 0.2 ml/min. The flow rate was obtained by applying positive pressure.

When the parotid–amylase glycogen complex was treated with charcoal under the above conditions which disassociated the pancreatic–amylase glycogen complex, the enzyme was irreversibly adsorbed to the charcoal column. The following method was therefore devised for the removal of the limit dextrins. The parotid–amylase glycogen precipitate was suspended in 0.05 M acetate buffer (pH 5.5) to a final protein concentration of 10 mg/ml. Crystalline amyloglucosidase (Pazur and Kleppe, 1962), was then added to a final concentration of 0.10 mg/ml and the mixture was incubated with occasional stirring at 25° for 4 hr.

After treatment with amyloglucosidase the parotid amylase preparation was dialyzed against 0.005 M potassium phosphate (pH 6.5) and applied to a CM-Sephadex (Pharmacia, Uppsala) column (2.0 × 30.0 cm) equilibrated with the same buffer. The column was first washed with two volumes of the initial buffer, then with 0.05 M potassium phosphate (pH 6.5). Fractions (10 ml) were collected.

The data in Figure 3 reveal that a single symmetrical peak was eluted from the column. The pooled fractions of the peak showed a single band of protein in the analytical disc gel system and a single band of amylase activity on cellulose acetate strip electrophoresis (Figure 3).

The preceding procedures produced a single amylase from the parotid and a mixture of three amylases from the pancreas as detected by zone electrophoresis (Figure 2), just as detected in crude extracts of these tissues. The following operations were designed for the quantitative separation of each of the pancreatic amylase enzymes.

STEP VI: DEAE-CELLULOSE COLUMN CHROMATOGRAPHY OF PANCREATIC AMYLASES. The dextrin-free pancreatic amylase

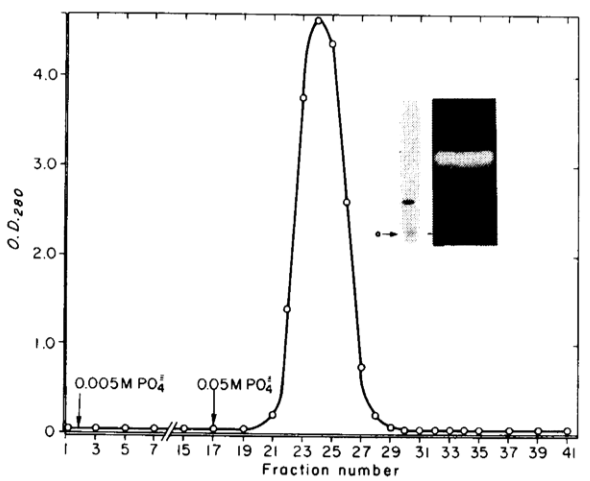

FIGURE 3: CM-Sephadex column chromatography of parotid amylase. The amylase from step IV, treated with α-glucosidase as described in the text, was applied to a CM-Sephadex column equilibrated with 0.005 M potassium phosphate (pH 6.5). After washing with two volumes of the initial buffer the enzyme was eluted off the column with 0.05 M potassium phosphate (pH 6.5). Acrylamide gel electrophoresis revealed that the protein preparation was homogeneous and cellulose-acetate zone electrophoresis demonstrated that a single zone of amylase activity was present.

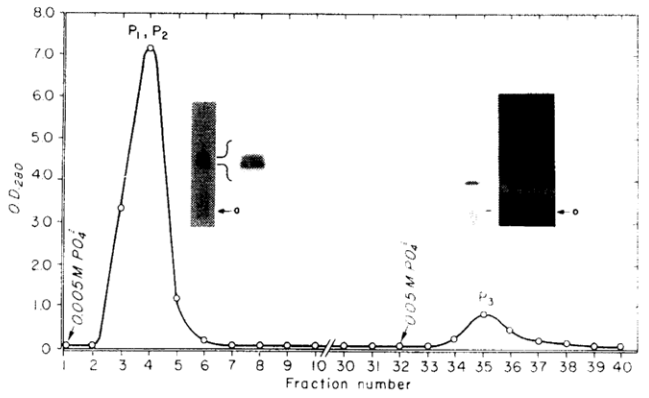

FIGURE 4: DEAE-cellulose column chromatography of pancreatic amylases. The material from step V was applied to a DEAE-cellulose column equilibrated with 0.005 M potassium phosphate (pH 6.9). After the first protein peak was eluted the column was washed with 0.05 M potassium phosphate (pH 6.9). Acrylamide gel electrophoresis revealed that the first peak contained two proteins, P_1 and P_2, whereas the second peak contained a single protein, P_3, and a single electrophoretic variant of amylase activity.

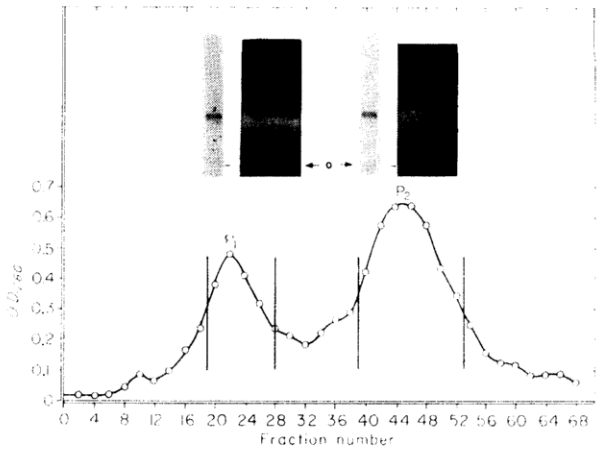

FIGURE 5: Preparative disc gel electrophoresis of a P_1, P_2 mixture. The first peak from a DEAE-cellulose column was electrophoresed at pH 4.5 in a 200-ml 7.5% acrylamide gel. The anode was placed in the upper reservoir and electrophoresis was carried out at 40 mA for approximately 18 hr. Vertical lines in the graph indicate the fractions which were pooled. The pH of the pooled samples was adjusted to 8.0 with 0.1 M NH_4OH and they were then concentrated and analyzed.

preparations were dialyzed against 0.005 M potassium phosphate buffer (pH 6.9) and applied to a DEAE-cellulose (DE 32; Whatman, England) column (1.0 × 20.0 cm) equilibrated with the same buffer. Three void volumes were collected in 4-ml fractions, and then the elution of the column was continued with 0.05 M potassium phosphate (pH 6.9). As can be seen in Figure 4, two peaks of protein were obtained. The fractions in each peak were pooled and aliquots were subjected to electrophoresis in the analytical disc gel system and on cellulose acetate strips. As can be seen in Figure 4, the first peak from the DEAE-cellulose column was shown to contain two protein bands on disc gel electrophoresis (corresponding to P_1 and P_2) and the second peak a single protein band and zone of amylase activity corresponding to P_3.

The pooled fractions from the first peak were dialyzed against 0.007 M β-alanine–acetic acid (pH 4.5) and concentrated to 10 mg/ml in an ultrafiltration apparatus (Amicon) in preparation for large-scale disc gel electrophoresis.

STEP VII: PREPARATIVE DISC GEL ELECTROPHORESIS. A Büchler Model Poly-Prep apparatus (Jovin *et al.*, 1964) was employed. Each of the following buffer solutions was adjusted to pH 4.5: the upper reservoir buffer containing 0.035 M β-alanine–0.014 M acetic acid; the insert buffer containing 3.75 M acetic acid–0.60 M potassium hydroxide; and the lower reservoir and elution buffer containing 0.25 M acetic acid–0.12 M potassium hydroxide. A 200-ml gel was cast, which contained 7.5% acrylamide, 0.1% *N,N'*-methylenebisacrylamide, 0.14% ammonium persulfate, 0.37% *N,N,N',N'*-tetramethylethylenediamine, and 0.035 M β-alanine–0.014 M acetic acid. The instrument was then prerun by applying a current of 15 mA for 16 hr and eluting at the rate of 0.1 ml/min. Then a 30–100-mg sample of a mixture of P_1 and P_2 in 15 ml of 0.007 M β-alanine–0.0028 M acetic acid containing 0.5 M glucose was layered onto the surface of the gel. A current of 15 mA was applied for 45 min, then the current was raised to 40 mA. Elution was carried out at a rate of 1.0 ml/min and the temperature of the apparatus was maintained at 15°. Fractions (6 ml) were collected and, as Figure 3 indicates, two protein peaks were obtained.

Electrophoresis on the analytical gel electrophoresis system revealed that the peaks were homogeneous with respect to protein and zone electrophoresis on cellulose acetate revealed that each peak corresponds to a single amylase activity, P_1 or P_2 (Figure 5).

The specific activities of the enzymes purified by this method are approximately equal to the specific activities of other amylases purified in this manner (Loyter and Schramm, 1962) and by other methods (Shainkin and Birk, 1966). A carbohydrate determination of the material treated for the removal of glycogen dextrins revealed that both the charcoal column and the amyloglucosidase methods removed all detectable (at least 90%) carbohydrate. A sample of P_1 was kindly analyzed by Professor R. Rinzler for the neutral carbohydrates by the alditol–acetate method (Lehnhardt and Winzler, 1968). Insignificant quantities were found.

Catalytic Properties of the Enzymes. The effects of pH (4.0–9.0), chloride ion concentrations (1.0×10^{-5}–1.0×10^{-2} M), and calcium ion concentrations (1.0×10^{-5}–1.0×10^{-2} M) on the rates of starch degradation were observed. A maximal rate at pH 6.5 (0.05 M histidine-HCl) was observed. The chloride ion concentration in the reaction mixture which produced a 50% maximal stimulation of starch hydrolysis was 40×10^{-4} M for each of the enzymes; calcium ion 4.0×10^{-4} M in the reaction mixture produced a 50% maximal stimulation in catalytic rate of each of the four enzymes. Superficially, then, the properties of the pancreatic and parotid enzymes appear very similar.

Molecular Weights. The molecular weights were determined in collaboration with Dr. David Teller using the high-speed equilibrium method of Yphantis (1964). A Beckman Model E ultracentrifuge with a Model AN-D rotor was employed. The initial protein concentration was 0.75 mg/ml in 0.05 M Tris-HCl (pH 8.0) and the rotor speed was 26,000 rpm. The centrifuge was operated at 5° and the data from a Rayleigh interference optical system were treated according to Teller *et al.* (1969). A partial specific volume of 0.721 ml/g was

TABLE III: Molecular Weight Determinations of Rabbit Pancreatic and Parotid Amylases.[a]

	M_w	M_n	M_z
Pancreatic Amylases			
P_1	48,900	53,600	58,800
P_2	46,800	55,300	55,700
P_3	44,400	53,000	57,500
Parotid Amylase	50,700	54,700	54,300

[a] The high-speed equilibrium method of Yphantis (1964) was employed. The data from a Rayleigh interference optical system were treated according to Teller *et al.* (1969).

calculated from the amino acid compositions, and this value was employed in all of the computations. The molecular weights of the four enzymes are given in Table III.

The discrepancy between M_n, M_w, and M_z values indicates heterogeneity in the preparations, especially in the pancreatic enzymes. The M_w values of both pancreatic and salivary enzymes vary between 53,000 and 55,000 and are very similar to molecular weight values (55,000 ± 2,000) recently determined for rat parotid and pancreatic amylases (T. G. Sanders and W. J. Rutter, unpublished). In addition, the value 54,800 for the rat parotid amylase can be calculated from the $s_{20,w}$ of 4.6 S and $D_{20,w}$ of 7.3×10^{-7} cm²/sec determined by Loyter and Schramm (1962) and employing a partial specific volume of 0.72 calculated from the amino acid composition. The value of 20,000 found by Vandermeers and Christophe (1968) by gel exclusion chromatography is, no doubt, artifically low, probably because of selective binding of the amylase to the gels employed. Kranz *et al.* (1965) have determined the molecular weight of human salivary amylase to be 55,200.

An attempt to define the subunit composition in the molecule was made by determination of the molecular weight of P_1 amylase in guanidine hydrochloride (Kawahara *et al.*, 1965). After dialyzing the enzyme at two concentrations (0.52 and 0.23 mg per ml) for 5 days against 6 M guanidine-HCl, 0.1 M β-mercaptoethanol, and 0.05 M Tris-HCl (pH 8.0), aliquots were subjected to high-speed equilibrium centrifugal analysis (Teller *et al.*, 1969). The calculated molecular weights were 21,200 (M_n), 40,400 (M_w), and 50,100 (M_z). These results indicate the heterogeneity of the sample; nevertheless, the method employed selectively emphasizes the lower molecular weight components. The higher values of M_w and M_z indicate substantial quantities of material of higher molecular weight (approaching that of native amylase) not dissociated by 6 M guanidine-HCl under reducing conditions. A further analysis of the molecular weight distribution of molecules over the ultracentrifuge cell suggests that the population does not reflect a monomer–dimer equilibrium, but a heterodisperse mixture of larger and smaller molecules. That the larger molecules are likely to be intact amylase chains is suggested by more comprehensive studies performed on the closely related but more homogeneous rat pancreatic amylase (T. G. Sanders and W. J. Rutter, unpublished). This enzyme is apparently a single polypeptide chain since this enzyme in 6 M guanidine-HCl appears homogeneous and the molecular weight is identical with that of the native enzyme. The data obtained with the rabbit pancreatic enzymes are thus interpreted in terms of monomeric molecules.

TABLE IV: Amino Acid Compositions of Pancreatic and Parotid Amylases Residues per 54,000 g of Protein.[a]

Amino Acid	P_1	P_2	P_3	Parotid	Rat Pancreatic Amylase[b]
Aspartic acid and asparagine	69.5	72.2	74.2	71.7	82.7
Threonine	22.5	23.5	25.7	25.5	20.7
Serine	37.4	32.7	34.8	36.5	28.0
Glutamic acid and glutamine	34.7	35.2	36.7	33.8	29.7
Proline	19.4	20.0	20.6	19.8	18.7
Glycine	56.0	56.8	54.6	56.0	49.1
Alanine	26.7	26.7	26.3	26.4	33.4
Half-cystine	9.6	10.5	10.6	11.8	10.8
Valine	37.7	39.6	40.4	41.2	35.5
Methionine	6.7	6.2	7.6	7.4	10.2
Isoleucine	26.6	27.7	24.8	26.9	25.7
Leucine	25.8	29.2	26.1	25.0	23.8
Tyrosine	20.0	21.1	19.9	21.0	16.0
Phenylalanine	23.1	23.5	25.0	23.7	23.8
Lysine	22.3	20.3	19.1	20.8	23.1
Histidine	10.7	9.9	9.2	9.3	12.5
Arginine	29.1	27.2	26.1	26.1	26.2
Tryptophan	10.5	8.0	8.2	8.4	14.9

[a] Values represent averages of triplicate determinations performed after 24-, 48-, 72-hr hydrolysis in 6 N HCl as described in Experimental Procedure. [b] Taken from Vandermeers and Christophe (1968).

Amino Acid Compositions. The amino acid analyses of the four enzymes are given in Table IV. A high degree of similarity of the amino acid compositions is readily apparent; the rabbit amylases comprise a group of very closely related proteins. The largest variance in amino acid content is about 20% and usually the determinations are within 10%, close to the uncertainty of the methods employed. Certain of the differences detected may, however, be related to the molecular properties. For example, the acidic and amide residues 105, 107, 111, 106 and the total basic residues (arginine, lysine, and histidine) 51, 47, 45, and 47 for P_1, P_2, P_3, and parotid amylase, respectively. Thus, the difference is 43, 50, 57, and 50. Thus, if the amide residues (not determined) were relatively constant in the pancreatic enzymes, the relationship of acidic and basic residues in the molecules would serve to predict the relative electrophoretic mobilities of the molecules.

These amino acid compositions of the rabbit enzymes are also very similar to those of the rat pancreatic amylase (Vandermeers and Christophe, 1968; T. G. Sanders and

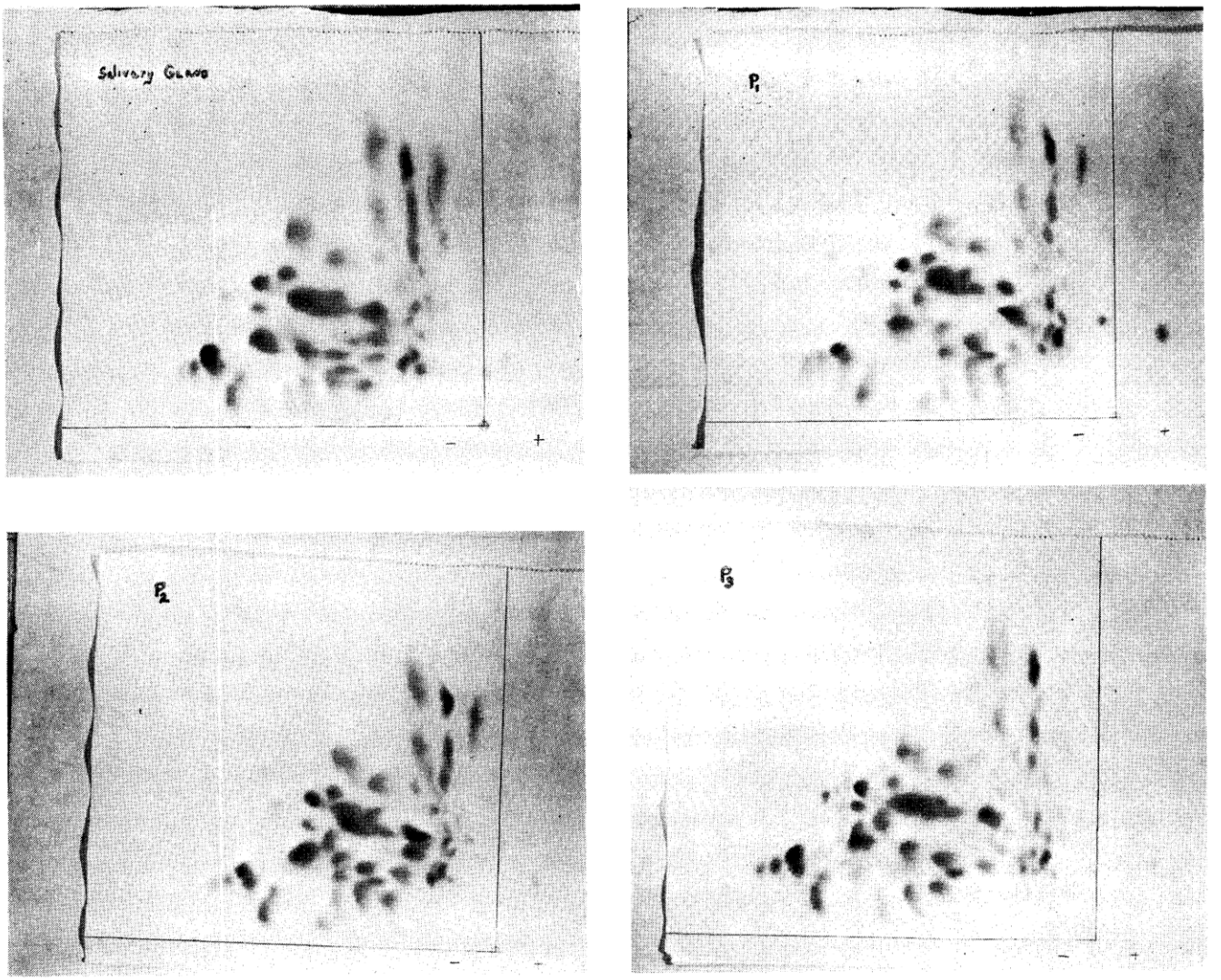

FIGURE 6: Peptide maps obtained from chromatography and electrophoresis of tryptic digestion products of the amide-carboxymethylated derivatives of the three pancreatic amylases, P_1, P_2, P_3, and the parotid amylase. The experimental details are described in the text.

W. J. Rutter, in preparation) and human salivary amylase (Muus, 1954).

Peptide Maps. A total of approximately 50 lysine and arginine residues are present in each of the enzymes and therefore a complete digestion with trypsin would be expected to yield approximately 50 peptides for a single subunit enzyme. Peptide maps of the four enzymes are presented in Figure 6. Approximately 50 peptides were resolved from each protein. An over-all similarity in the patterns is obvious, but a spot-by-spot comparison reveals only about 50% are common to all of the proteins. Similar experiments were carried out with several different enzyme preparations; the peptide maps obtained were essentially identical with those produced here. The general results, therefore, were highly reproducible; thus, the molecules appear to be different at the amino acid sequence level.

Discussion

The purification procedure presented in this report provides a convenient method for obtaining 100-mg to 1-g quantities of the individual amylase variants from rabbit pancreas and parotid glands. Similar procedures can no doubt be employed to obtain single amylase species from the organs of other species. The removal of limit dextrins from amylases purified by glycogen precipitation has frequently been particularly difficult to achieve (M. Schramm, 1967, personal communication; Kulka and Duksin, 1964). The modified charcoal column procedure is useful, but perhaps less generally satisfactory than the alternative method involving treatment with amyloglucosidase. This should provide a general method for removal of residual glycogen dextrins from proteins.

The procedures employed, however, for the purification and resolution of the dextrin–amylase complex and subsequently for the separation of the enzyme variants apparently jeopardize the molecular integrity of the resulting material. Precautions were taken to avoid proteolytic degradation; quick frozen tissues were used; phenylmethylsulfonyl fluoride (an inhibitor of serine proteases) (Fahrney and Gold, 1963) was included in the extracts, and all operations (except removal of dextrins by degradation) were carried out at low temperature. Nevertheless, significant heterodispersity existed

in the final product as indicated by the ultracentrifugal experiments. Of course, this molecular heterogeneity could be a result of adventititous contamination by other proteins or peptides. We believe because of the discriminative procedures used in the isolation procedures that it is more likely that the isolated amylases have been subjected to limited proteolytic degradation. The specific activity of the preparations, for example, remains high in comparison with other preparations.

In spite of the possibility of limited proteolysis of the molecular species isolated, a number of observations suggest that the three pancreatic enzymes and the parotid amylase are unique molecules. The activities can be readily resolved by electrophoresis in both crude and in purified preparations; furthermore, the relative proportions of the molecules and their mobilities are not altered significantly by incubation in the presence of pancreatic extracts. Even though the molecules are very similar in their superficial catalytic properties, molecular size, and general amino acid composition, there appear to be significant, if minor, differences in the amino acid compositions. The peptide maps, furthermore, provide presumptive evidence that the enzymes as isolated are different at the level of amino acid sequence. The heterogeneity in the preparations, however, demands a cautious interpretation of these experimental results and allows only an equivocal interpretation of the differences in peptide maps. Whereas tryptic "nicks" would not influence the peptide patterns, proteolytic attack by other enzymes would produce new peptides. If the degradation were indiscriminant throughout the molecules, then the new peptides would be largely blurred out in the resulting maps. On the other hand, selective hydrolysis of certain peptide linkages would produce alterations in the peptide maps. Since the number of peptides corresponds closely to the number of tryptic-sensitive amide bonds, extensive nontryptic hydrolysis cannot have occurred. Furthermore, the electrophoretic mobility differences of the pancreatic components remain unchanged in the crude and highly purified preparations. Thus, we believe the peptide maps reflect the true differences in the primary structure of the enzymes. There is apparently no difference in the aldehyde carbohydrate content for the various amylases like that detected in RNase variants of the beef (Plummer and Hirs, 1963). On the other hand, the possibility that other phenotypic modifiers are present is not eliminated by the present work. The possibility that the pancreatic proteins could be members of a hybrid set is ruled out on several counts: the pancreatic set of isozymes do not make up an electrophoretically ordered series of proteins as do the hybrid series of aldolase (Penhoet *et al.*, 1966), lactic dehydrogenase (Markert and Moller, 1959), and triosephosphate dehydrogenase (Lebherz and Rutter, 1967). The electrophoretic mobility of P_1 is not intermediate between P_2 and P_3. Peptide maps also give no indication of additivity of spots as would be expected in a hybrid molecule (Penhoet *et al.*, 1967); rather, the molecules appear to be composed of a single subunit. This conclusion is supported by the ultracentrifugal studies in guanidine hydrochloride which indicated a prominent proportion of high molecular weight material approaching that of the native enzyme remaining under dissociating conditions and by analogy with the more convincing studies of the rat parotid amylase. Furthermore, the number of peptides seen on peptide maps corresponds to the number of tryptic-sensitive amide bonds as would be expected for a single subunit molecule. None of the pancreatic enzymes appears to be identical with the parotid amylase. Thus, the results are most reasonably interpreted in terms of four unique amylases (three pancreatic and one salivary amylase), each products of different genes. This conclusion should be substantiated by further structural studies of the intact molecules. This may perhaps be achieved by resolving the intact molecules from the degraded molecules by gel filtration of the molecules in 6 M guanidine hydrochloride.

The conclusion that the salivary and pancreatic amylases are distinct sets of molecules is of some relevance to the mechanisms of cytodifferentiation in these two homologous organs; thus, differentiation in these tissues must proceed largely through the expression of different sets of genes. That this may generally be true in other vertebrates is suggested by the different electrophoretic patterns of amylases obtained from pancreatic and parotid glands of a number of species (Figure 1). This contention is supported by the recent studies on the parotid and pancreatic amylases of the rat (T. G. Sanders and W. J. Rutter, in preparation). In some instances, however, for example in beef, the electrophoretic mobilities of the amylases are similar and the production of a single enzyme species by both tissues is not ruled out.

It seems likely that still another amylase is synthesized in rabbit tissues. Amylase is known to be synthesized in the liver, for example (Arnold and Rutter, 1963; McGeachin *et al.*, 1964). The finding of the present studies that the liver amylase has unique electrophoretic mobility suggests that it is different from the pancreatic and parotid amylases. This general conclusion is also supported by immunochemical studies performed in other species (McGeachin *et al.*, 1966).

The possibility that each of the variants has modified catalytic properties should be examined in detail. A more definitive study of the catalytic properties of the enzyme, for example, with well-defined substrates (Roberts and Wheland, 1960) as well as a definition of the products of the starch hydrolysis reaction should be carried out. An investigation into the physiological function of the enzymes should perhaps also be extended to include a study of the levels of the individual variants and the regulation of their biosynthetic rates, both during the embryological stages of development when these enzymes are first being synthesized (Rutter *et al.*, 1964) and during postnatal stages of growth. Techniques for investigation of such parameters are presently available (Palla *et al.*, 1967). Rebout *et al.* (1966) have recently shown that the compositions of the diet of adult rats and insulin have a marked effect on the levels of the various pancreatic exocrine enzymes. Neither of these parameters affects the level of the parotid enzymes (Palla *et al.*, 1967). An examination of the biosynthetic rates of each of the pancreatic amylases might provide meaningful information about the relationships of these proteins to pancreatic and salivary functions.

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