Molecular Properties of Rat Pancreatic and Parotid α-Amylase*

Thomas G. Sanders† and William J. Rutter‡

ABSTRACT: The α-amylases purified from the pancreas and parotid glands of the rat have been shown to be readily distinguished by both disc gel electrophoresis and the technique of isoelectric fractionation. In addition, the latter procedure resolves two major species of the pancreatic enzyme. Pancreatic and parotid amylase have also been shown to be nonidentical by double diffusion analysis *vs.* rabbit antibodies to the pancreatic enzyme. Amylase preparations from both sources have a molecular weight of 56,000 g/mole in either the presence or absence of 6 M guanidine hydrochloride–0.1 M 2-mercaptoethanol indicating an apparent single polypeptide chain subunit structure. The amino acid compositions of the amylase isolated from the two organs show a general similarity but significant differences in the amount of several residues are evident. These results are consistent with the hypothesis that pancreatic and parotid amylase are each products of different genes and eliminate the possibility of subunit hybridization as a cause of the observed multiple molecular forms of the enzyme. A further comparison of the amino acid compositions of amylases from a wide variety of sources suggests that sequence homology exists among the mammalian, fungal, and bacterial α-amylases but reveals no such evolutionary relationship between these enzymes and the β-amylases of plants.

The mammalian pancreas and parotid glands possess a similarity of physiological function and morphological structure which extends to a prominent constituent of both organs, the enzyme α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1). The existence of multiple molecular forms of the enzyme from both sources has been documented in a number of species. Two forms of the pancreatic enzyme have been observed in the hog (Marchis-Mouren and Pasero, 1967; Rowe *et al.*, 1968) and also in the mouse (Sick and Nielsen, 1964) while Lamberts *et al.* (1965) and Muus and Vnenchack (1964) both reported four variants in human saliva. In none of these cases, however, has the molecular basis of this variability been defined, and the question of whether the pancreas and parotid enzymes are identical and hence the product of the same genes has remained unanswered. Comparative studies of the crystalline human pancreatic and parotid enzymes by Bernfeld *et al.* (1950) and an immunological examination of the two rabbit proteins (McGeachin *et al.*, 1966) suggested that the enzymes were identical, while a genetic study of the mouse by Sick and Nielsen (1964) suggested the opposite conclusion.

The ambiguity of these results has prompted a reexamination in this laboratory of the molecular basis of amylase variants in the vertebrate pancreas and parotid gland. In a recent investigation, Malacinski and Rutter (1969) observed electrophoretic variants in a number of vertebrate species (lungfish, chicken, beef, guinea pig, rat, rabbit, and human). Three forms of the pancreatic enzyme and the single amylase component present in the parotid gland were isolated from the rabbit and a comparison with regard to amino acid composition, molecular weight, and peptide mapping pattern suggested that the parotid enzyme was not identical with any of the pancreatic species. The present study represents a further inquiry into the relationship of pancreatic and parotid amylases and examines similar parameters of the rat enzymes with additional focus on the immunological nonidentity of the proteins. The results substantiate the conclusion that pancreatic and parotid amylases are unique molecules and thus the product of distinct genes.

Materials and Methods

Amylase was assayed by reductometry with dinitrosalicylate, using the method of Bernfeld (1955) at 30° with all volumes reduced by one-half. One unit of activity is defined as that quantity yielding 1 mg equiv of maltose hydrate in 3 min at 30°. Protein was determined either according to Lowry *et al.* (1951) with bovine serum albumin as standard or spectrophotometrically using a value of $E_{cm}^{1\%} = 16.4$ determined for the chromatographically purified pancreatic enzyme. Specific activity is expressed as units per milligram of protein. Carbohydrate was measured using the phenol-sulfuric acid method of Dubois *et al.* (1956) with glucose as standard.

Enzyme preparation used rat pancreatic and parotid tissues collected from freshly killed Sprague–Dawley rats, frozen on Dry Ice, and stored at −20°.

Rat pancreatic amylase was prepared by both the methods of Loyter and Schramm (1962) and Vandermeers and Christophe (1968) with several modifications as detailed below. In the case of the former procedure both 10^{-3} M benzamidine·HCl and 10^{-4} M *p*-nitrophenyl-*p'*-guanidinobenzoate (Chase and Shaw, 1969) were included in the initial homogenization buffer (0.012 M potassium phosphate–0.004 M NaCl–0.003 M $CaCl_2$ (pH 6.9), filtered before use) in an effort to inhibit proteolysis. Preparations were terminated following the glycogen digestion step due to difficulties encountered both in the 0° reprecipitation of the enzyme at pH 7.0 and also in subsequent charcoal–Celite chromatography. After dialysis of the resultant preparation, a significant quantity of carbohydrate remained (approximately 0.3 mg/mg of protein).

* From the Departments of Biochemistry and Genetics, University of Washington, Seattle, Washington 98105. *Received March 20, 1970.* This investigation was supported by both U. S. Public Health Service Grant No. HD-02126 and National Science Foundation Grant No. GB-4273. This work constitutes a portion of the thesis of T. G. S., submitted in partial fulfillment of the requirements for the Ph.D. degree, University of Illinois, 1970.

† Present address: Department of Zoology, University of British Columbia, Vancouver, British Columbia, Can.

‡ Present address: Department of Biochemistry, University of California, San Francisco, California 94122; to whom correspondence should be sent.

In contrast, the procedure of Vandermeers and Christophe (1958) afforded a preparation apparently free of carbohydrate (<0.01 mg/mg of protein). Modifications employed in this procedure included addition of proteolytic inhibitors (*vide supra*) to the homogenization medium (0.2 M Tris-Cl, pH 8.5) and inclusion of 10^{-3} M benzamidine·HCl alone in all chromatography buffers. The initial chromatography in 0.013 M Tris-Cl (pH 8.2) utilized DEAE-Sephadex A-50 (Pharmacia) instead of DEAE-cellulose and was followed by gel filtration in Sephadex G-100 with an elution buffer of 0.005 M Tris-Cl (pH 7.0) containing 10^{-3} M benzamidine·HCl. The use of DEAE-Sephadex *vs.* DEAE-cellulose for chromatography of the pancreatic enzyme afforded a substantially greater purification in this step (5.0× *vs.* 3.6×) and resulted in the absence of lipase in the recovered "breakthrough" material.

Rat parotid amylase was prepared according to Loyter and Schramm (1962) with the appropriate modifications indicated above. The resultant enzyme contained somewhat less carbohydrate (0.2 mg/mg of protein) than the pancreatic preparation. All preparations were stored at −20°.

Preparative isoelectric focusing (Svensson, 1962; Vesterberg and Svensson, 1966) utilized an 110-ml column and pH 7–10 or 8–10 carrier ampholyte (LKB Instruments, Stockholm) in the manner suggested by the manufacturer. The density gradient consisted of 0–50% (w/v) sucrose with a final ampholyte concentration of approximately 1% (w/v). Enzyme (5–20 mg) was dialyzed overnight against 1% glycine and applied to the column in place of one of the "less dense" solutions. Fractionation was performed at 4° for 40 hr with a final voltage of 900 V after which 150–200 fractions were collected, analyzed, and pooled for further study.

Analytical isoelectric focusing in acrylamide gels were performed essentially according to Wrigley (1968) using a pH 3–10 gradient. The sample was included in the gel and polymerization was effected with ammonium persulfate. Fractionation was allowed to proceed for 3 hr with a final voltage of 250 V. After fractionation, gels were fixed and washed in 10% trichloroacetic acid to remove the ampholytes, stained in a 0.0125% solution of Coomassie brilliant blue R-250 (Colab) in 10% trichloroacetic acid, and destained by further washing in 10% trichloroacetic acid.

Disc gel electrophoresis followed the procedure of Reisfeld *et al.* (1962) using a 7.5% polyacrylamide system. Gels were fixed in a 5% trichloroacetic acid, 5% sulfosalicyclic acid solution, stained in a 0.0125% solution of coomassie blue in this solvent, and destained by several changes of the same solvent.

Molecular weights were determined by the high-speed equilibrium method of Yphantis (1964) in the An D rotor of a Spinco Model E ultracentrifuge. Native enzyme samples were dialyzed overnight *vs.* 0.01 M Tris-Cl (pH 7.5) and centrifuged at 5° and 26,000 rpm in 3-mm columns. Initial protein concentrations were 0.25, 0.50, and 0.75 mg per ml. Denatured enzyme samples were prepared by dialysis against 6 M guanidine hydrochloride (Mann, Ultra Pure grade), 0.1 M 2-mercaptoethanol–0.01 M Tris-Cl (pH 7.5) for 4 days at room temperature. In the case of the parotid enzyme, this was followed by chromatography on Sephadex G-100 in the same buffer to remove carbohydrate and then a second dialysis. Centrifugation was carried out at 20° in 2.5-mm columns at 28,000 and 32,000 rpm for parotid and pancreatic amylases, respectively. In all cases, the external medium was employed as diluent and reference solvent. A modified Nikon microcomparator (Teller, 1967) was used to read the Rayleigh interference plates and the data were analyzed using the computer techniques of Teller *et al.* (1969). A partial specific volume of 0.721 ml/g was calculated from the amino acid compositions of the proteins (McMeekin *et al.*, 1949) and solvent densities were determined pycnometrically.

Amino acid analysis samples were extensively dialyzed *vs.* 0.01 M Tris-Cl (pH 7.5) and 1-mg aliquots were lyophilized and then hydrolyzed at 110° for 24, 48, 72, and 96 hr in evacuated, sealed tubes with 0.5 ml of constant-boiling hydrochloric acid. After removal of HCl, and standing at pH 6.5 for 8 hr (Moore and Stein, 1963), the analyses were performed using a Beckman Model 120B amino acid analyzer either according to Spackman *et al.* (1958) with 3-(2′thienyl)-2-aminopropanoic acid and 2-amino-3-guanidopropanoic acid added as internal standards (Walsh and Brown, 1962) or, alternatively, utilizing the modified one-column procedure of Dévényi (1968). Values for threonine and serine were extrapolated to zero times of hydrolysis and valine and isoleucine values were extrapolated to infinite time. Methionine, cysteine, and cystine were determined on separate samples as methoionine sulfone and cysteic acid after oxidation with performic acid according to Hirs (1967).

For the preparation of rabbit antibodies to chromatographically purified rat pancreatic amylase an emulsion of 1 ml of enzyme (approximately 10 mg/ml) and 1 ml of complete Freund's adjuvant (Difco) was prepared by forcing this mixture repeatedly through an 18 gauge needle. Approximately 0.2 ml was then injected into each footpad and the remainder administered by several 0.1-ml intradermal injections into the skin of the neck and back. Occasional subsequent injections consisted of 1 ml of emulsion administered *via* the intradermal route only. Rabbits were bled weekly from the marginal ear vein, beginning 2 weeks following the initial injections. After the clot was allowed to form at room temperature and retract overnight in the cold, the serum was decanted and stored at −20°. γG-Immunoglobulin was purified by ammonium sulfate fractionation and chromatography on DEAE-cellulose essentially as described by Deutsch (1967) and Fahey (1967). Double diffusion analysis (Ouchterlony, 1948) in 1.5% Noble agar (Difco) in saline utilized microscope slides prepared with an LKB gel cutter.

Results

The enzymes employed in the present study were prepared by the methods of Vandermeers and Christophe (1968) and Loyter and Schramm (1962) (see Materials and Methods) and were judged to be pure on the basis of several criteria including specific activities comparable to those reported in the literature, disc gel electrophoresis, and ultracentrifugal analysis. The parotid enzyme had a specific activity of 2780 u/mg (Loyter and Schramm (1962) reported 2700 u/mg). In comparison to a published value of 620 u/mg (Vandermeers and Christophe, 1968), the chromatographically purified pancreatic enzyme had a specific activity of 780 u/mg while that of the glycogen precipitation isolate was 620 u/mg.

The result of disc gel electrophoresis of each of the preparations is shown in Figure 1. Irrespective of the method of purification, pancreatic amylase moves as a single broad band which in some cases was suggestive of a doublet. The parotid enzyme consists of one major component together with a minor, faster moving form. Most of the gels show some trailing due to protein precipitation in the region of the origin; this is especially true in those preparations containing carbohydrate but could be partially prevented by smaller sample loads (as in gel 6). Electrophoresis of mixtures of the parotid

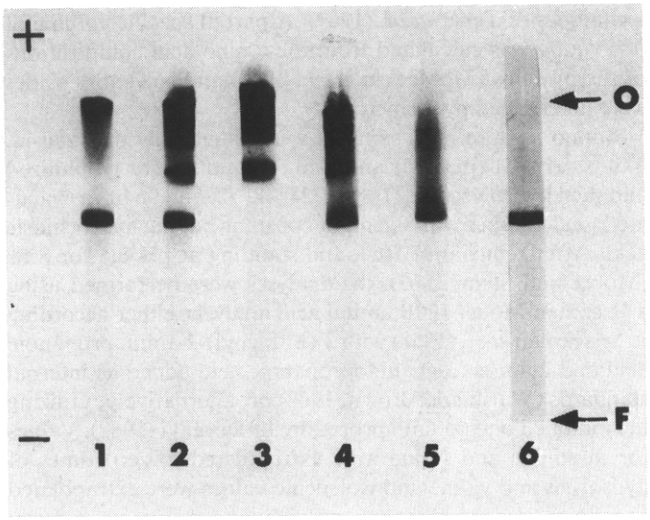

FIGURE 1: Disc gel electrophoresis of purified rat pancreatic and parotid amylases. 20-μg (1–5) or 10-μg (6) samples of each preparation were used and treated according to Materials and Methods. (1 and 6) Pancreatic amylase (chromatographically purified); (3) parotid amylase; (5) pancreatic amylase (purified by glycogen precipitation); (2) mixture of 1 and 3; (4) mixture of 3 and 5. 0 indicates gel interface, F is the position of the dye band.

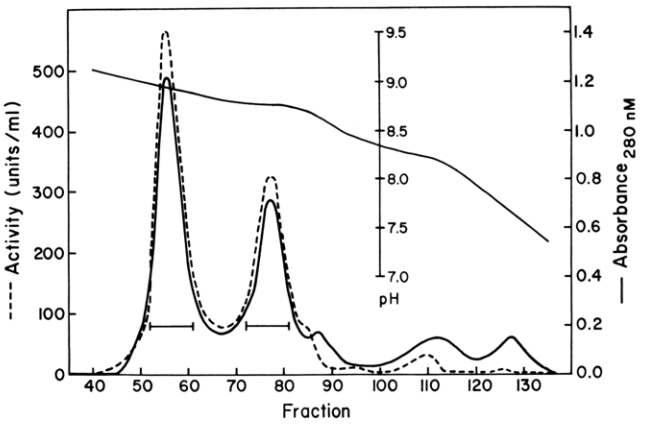

FIGURE 3: Preparative isoelectric fractionation of purified rat pancreatic amylase. Chromatographically purified enzyme (approximately 7 mg) was treated as described in Materials and Methods and the indicated portions of the two major species were retained for amino acid and molecular weight analysis.

enzyme and either pancreatic preparation provided the initial suggestion that each was a unique molecule.

Analytical isoelectric fractionation, as shown in Figure 2, confirmed the marked difference between the pancreatic and parotid amylases. In addition, this technique clearly resolved two major forms and several minor components of the chromatographically purified pancreatic enzyme and also confirmed the second minor species of parotid amylase. Furthermore, as illustrated in Figure 3, preparative isoelectric fractionation (see Materials and Methods) of the chromatographically purified pancreatic preparation resolved two major species with isoelectric points of 8.77 and 8.95 and specific activities of 745 and 755 units per mg, respectively. In addition, several minor components of lower isoelectric point and activity were detected. The pattern thus duplicated that shown analytically, and subsequently the amino acid composition of both major forms and the molecular weight of the most cathodic species were determined (*vide infra*). The absence of two major species in the glycogen precipitated pancreatic preparation (but rather the occurrence of one diffuse zone in the same region, Figure 2) is presumably due to the presence of carbohydrate. An alternative explanation, the occurrence of proteolytic degradation in the other preparation resulting in the two forms, although not rigorously eliminated, is not indicated by the results of the ultracentrifuge studies discussed below.

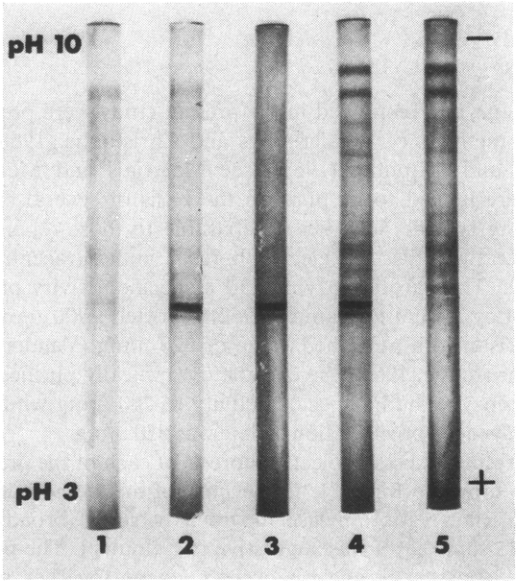

FIGURE 2: Analytic isoelectric fractionation of purified rat pancreatic and parotid amylases. Samples (20 μg) of each preparation were employed and treated as described in Materials and Methods. (1) Pancreatic amylase (purified by glycogen precipitation); (3) parotid amylase; (5) pancreatic amylase (chromatographically purified); (2) mixture of 1 and 3; (4) mixture of 3 and 5.

The demonstration of multiple forms of amylase required evidence that the heterogeneity observed was not the result of proteolysis. In order to examine this possibility, and to test the hypothesis that heterogeneity was the result of hybrid enzyme molecules formed from homomeric and heteromeric contributions of polypeptide chain subunits, an extensive study of the molecular weights of the amylases from both sources was undertaken. As shown in Table I, the pancreatic enzyme (G-100 effluent of the chromatographically purified preparation) has a native molecular weight (M_w) of approximately 56,000 g/mole; in guanidine hydrochloride–mercaptoethanol solutions it is evident that some heterogeneity exists in this preparation as evidenced by small differences in M_N, M_w, and M_z. However, the quantity of "light" material required to inject this degree of dispersity in the sample is of the order of 15%,[1] insufficient to account *via* extensive proteolytic degradation for the two major species observed isoelectrically although it may indicate the source of the several minor species. The molecular weight data for the most cathodic form of the enzyme (isoelectric point = 8.95) isolated by

[1] Calculated using the point-by-point values of the weight-average molecular weight (M_w) and integrating by trapezoidal approximation the value of C_1 obtained from the equation $C_1 = C_t(M_2 - M_w)/M_1$ (Seery, 1968), where C_1 and C_t refer to the concentration of "light" component and total concentration, respectively, at the point under consideration, and M_2 and M_1 are the weight-average molecular weights of the intact molecule (55,000 g/mole) and that arbitrarily assigned the light component (27,500 g/mole).

TABLE I: Molecular Weights of Rat Pancreatic and Parotid Amylases.

Sample	Molecular Weight × 10^{-3} (g/mole)[a]							
	Native				Guanidine·HCl			
	M_N	M_w	M_z	M_{z+1}	M_N	M_w	M_z	M_{z+1}
Pancreatic (G-100 effluent)	53.4	56.3	57.0	54.7	46.7	50.6	53.7	62.8
Pancreatic (isoelectric fraction)					53.0	54.0	54.5	55.1
Parotid					53.3	57.6	58.9	61.6

[a] Whole cell averages.

preparative isoelectric fractionation is shown and is monodisperse. Similarly, the parotid enzyme (examined only in guanidine hydrochloride–mercaptoethanol due to the carbohydrate contaminant) appears to be essentially intact with a molecular weight of 56,000 g/mole.

The amino acid compositions of rat parotid amylase and the two major isoelectrically resolved species of the pancreatic preparation are given in Table II, together with data for the later enzyme recalculated from that of Vandermeers and Christophe (1968). The agreement between the results of these authors (obtained with an unresolved mixture) and the present determinations is satisfactory, generally being within 10%; furthermore, although small variations between the two pancreatic forms are evident, these differences are not considered significant. Although the amylases derived from the two tissues show pronounced differences in electrophoretic mobility and apparent isoelectric point, the amino acid compositions are also markedly similar. However, in this case certain significant differences are apparent, principally in tyrosine, but reproducible, albeit smaller, differences were also noted in threonine, alanine, and phenylalanine.

In order to more precisely compare the amino acid composition of the enzymes shown here and a number of other α- and β-amylases from a wide variety of sources, the deviation function: $D = [\Sigma(X_{1,i} - X_{2,i})^2]^{1/2}$ (*cf.* Harris *et al.*, 1969), was employed, where $X_{1,i}$ represents the mole fraction of amino acid i in a protein and $X_{2,i}$ is the mole fraction of the same amino acid in the protein to which it is being compared. The sum of the squares of the deviations over all amino acids yields a value of D which is thus a measure of the divergence of amino acid compositions between the proteins. The results of the application of this analysis to a number of amylases are shown in Table III together with an estimate of per cent of total residues predicted to be identical in sequence on the basis of an examination of a large number of protein classes for which the sequence is known (C. E. Harris and D. C. Teller, in preparation). It has previously been domonstrated (C. E. Harris and D. C. Teller, in preparation) that values of D such as calculated here *within* each of the mammalian, plant, and microorganism amylase groups ($D = \geq 0.07$) are generally observed with proteins for which substantial evidence for evolutionary homology has been documented. Similarly, values of $D = >0.11$ are generally associated with protein comparisons for which there is no *a priori* reason to believe an evolutionary relationship exists, as in the case of the cytochrome *c* (human) and bovine α-chymotrypsin comparsions illustrated. The interpretation of composition divergence values in the range 0.07–0.11 with respect to evolutionary homology is equivocal and other criteria must also be utilized; it is thus suggested that the fungal and bacterial α-amylases are related to those of mammalian origin since, in addition to the relatively low D value observed ($D_{av} = 0.0786$), both groups have monomer molecular weights around 50,000 g/mole and possess a requirement of one Ca^{2+} ion per mole of enzyme for the maintenance of structural integrity. In contrast, the plant β-amylases do not have a metal requirement, and furthermore are sensitive to sulfhydryl reagents (French, 1960) and thus the possibility of homology based on D is small.

The question of the relationship or identity of the amylase species present in the pancreas and parotid glands was further examined by double diffusion analysis (Ouchterlony, 1948), the results of which are presented in Figure 4. Antiserum prepared to the chromatographically purified pancreatic enzyme

TABLE II: Amino Acid Compositions of Rat Pancreatic and Parotid Amylases.

Amino Acid	Moles/56,000 g of Protein			
	Pancreatic Amylase (IEP = 8.95)[a]	Pancreatic Amylase (IEP = 8.77)[a]	Parotid Amylase[a]	Pancreatic Amylase[b]
Asx	86.1	81.9	82.0	85.7
Thr	24.0	23.8	18.5	21.5
Ser	32.5	35.3	35.9	29.0
Glx	32.4	34.1	32.5	30.8
Pro	21.2	20.0	20.3	19.4
Gly	50.7	52.0	54.5	50.9
Ala	35.0	35.0	29.0	34.6
½-Cys	10.0	9.9	10.1	11.1
Val	34.7	36.0	37.4	36.9
Met	9.7	10.1	10.5	10.6
Ile	24.9	24.1	23.2	26.6
Leu	23.6	23.1	23.6	24.6
Tyr	16.1	16.0	21.0	16.5
Phe	26.0	24.6	28.4	24.7
Lys	26.9	25.7	25.1	23.9
His	13.1	13.3	12.3	13.0
Arg	28.1	26.3	25.3	27.2
Total	495.0	491.6	489.6	487.0

[a] This work. [b] Taken from Vandermeers and Christophe (1968).

TABLE III: Divergence of Amino Acid Compositions of α- and β-Amylases.

Comparison	Composition Divergence, D		Estimated Amino Acid Sequence Homology	
	Range	Average	Lower Limit (%)[f]	Upper Limit (%)[f]
Mammalian α-/mammalian α-amylases[a]	0.0119–0.0771	0.0426	47–88 (69)	82–97 (89)
Plant β-/plant β-amylases[b]	0.0512–0.0911	0.0640	36–64 (55)	77–87 (84)
Bacterial and fungal α-/bacterial and fungal α-amylases[c]	0.0463–0.0725	0.0595	49–68 (58)	82–88 (85)
Mammalian α-/plant β-amylases	0.0670–0.1274	0.0963	10–53 (32)	67–83 (76)
Mammalian α-/bacterial and fungal α-amylases	0.0710–0.0918	0.0786	36–50 (43)	77–83 (80)
Plant β-/bacterial and fungal α-amylases	0.0664–0.1137	0.0904	20–53 (35)	71–83 (77)
Cytochrome c[d]/amylases	0.1490–0.1834	0.1724	0–0 (0)	53–62 (55)
Chymotrypsin[e]/amylases	0.0972–0.1430	0.1231	0–31 (12)	63–75 (68)
Cytochrome[c]/chymotrypsin	0.1884	0.1884	0 (0)	51 (51)

[a] Compositions taken from Malacinski and Rutter (1969); Muus (1954); Caldwell *et al.* (1954). [b] Compositions taken from Gertler and Birk (1965); Thoma *et al.* (1965); Waldschmidt-Leitz *et al.* (1964). [c] Compositions taken from Pfueller and Elliott (1969); Akabori *et al.* (1956); Narita *et al.* (1966). [d] Compositions taken from Dayhoff and Eck (1967). [e] Composition taken from Walsh and Neurath (1964). [f] Data in parentheses taken from D_{av}; other values refer to the range of D values.

cross-reacts only weakly with a homogenate of parotid gland but shows identity between the pure enzyme used as antigen and that present in the pancreatic homogenate. Furthermore, experiments using the pure enzymes and the same serum or the γG-immunoglobulin fraction purified from it indicate that the two different pancreatic preparations are immunologically identical in spite of the presence in one of a substantial carbohydrate contaminant; however, both possess antigenic determinants distinct from the parotid enzyme as shown by pronounced spur formation.

Discussion

Although Vandermeers and Christophe (1968) noted two disc gel electrophoretic forms of rat pancreatic amylases at pH 5.8, this was not observed in the present case under somewhat different conditions (pH 4.3). However, at pH 5.4 using a disc gel system consisting of citric acid and ϵ-aminocaproic acid as trailing ion (M. M. Sanders, unpublished data), two active species of rat pancreatic amylase were resolved (J. Kemp, unpublished data). Furthermore, utilizing the high degree of resolution of isoelectric fractionation, two species of the enzyme were observed. In contrast, Pascale *et al.* (1966) detected a single form of the enzyme in extracts of rat pancreas subjected to agarose gel immunoelectrophoresis. The two forms may represent different allelic forms (as has been observed, for example, with bovine carboxypeptidase A (Walsh *et al.*, 1966; Petra and Neurath, 1969)), the products of gene duplication, or alternatively, posttranslational modification (*e.g.*, acetylation, phosphorylation, glycosylation) of a single polypeptide chain may be implicated as in the case of bovine ribonuclease (Plummer and Hirs, 1964). However, since multiple forms have been observed in both the pancreas and parotid glands of a number of species and single individuals, the former possibility seems least likely (*vide infra*).

The molecular weight value of 56,000 g/mole found for both rat pancreatic and parotid proteins is in good agreement with the results for the several rabbit enzymes (53,000–55,000 g/mole) (Malacinski and Rutter, 1969) and human salivary amylase (55,200 g/mole) (Kranz *et al.*, 1965). In addition, a result of 54,800 g/mole for the rat parotid enzyme may be calculated from the $s_{20,w}$ of 4.6 S and $D_{20,w}$ of 7.3×10^{-7} cm^2/sec determined by Loyter and Schramm (1962) using a partial specific volume of 0.72 ml/g calculated from the amino acid composition. As discussed by Vandermeers and Christophe (1968), the low value of 20,000 g/mole determined for the rat pancreatic enzyme by gel filtration is presumably an artifact of amylase adsorption to the gel matrix. The molecular weight of the hog pancreatic enzyme (45,000 g/mole) determined by Danielsson (1947) from $s_{20,w} = 4.5$ S, $D_{20,w} = 8.05$ cm^2/sec, and $\bar{v} = 0.70$ ml/g is also at variance with these data but probably in error due to the low value for the partial specific volume as has been pointed out by Fischer and Stein (1960). In addition, a more recent examination of the hog enzyme by McGeachin and Brown (1965) yielded an $s_{20,w}$ of 4.67 S while Rowe *et al.* (1968) report s values in various buffers of 4.3–4.65 S which when corrected for solvent viscosity and density give $s_{20,w}$ of the order of 4.5–4.8 S. It is thus likely that a reevaluation of the molecular weight of hog pancreatic amylase by more precise methods (*e.g.*, sedimentation equilibrium) would result in a value of the order of 53,000–55,000 g/mole comparable to those obtained here and in better agreement with the value of 51,000 g/mole calculated from the amino acid composition (Caldwell *et al.*, 1954).

The use of guanidine hydrochloride–mercaptoethanol solutions as a dissociating agent for the purpose of examining the molecular weight and number of polypeptide chains in proteins is well documented (Lapanje and Tanford, 1967). In the present case, it is clear from the congruity of the results in the presence and absence of dissociating agents that the amylases from both rat pancreas and parotid glands are composed of single polypeptide chains with molecular weights of 56,000 g/mole. This result is in agreement with the finding by Marchis-Mouren *et al.* (1963) of a single C-terminal lysine for rat pancreatic amylase but in contrast to the claim of McGeachin and Brown (1965) of three N-terminal residues (phenylalanine, alanine, and glycine) and hence three chains in the hog pancreatic enzyme. Thus, the multiple molecular forms observed in this study cannot be due to hybridization of sev-

eral different constituent subunits as has been demonstrated for lactate dehydrogenase (Appella and Markert, 1961) and aldolase (Penhoet *et al.*, 1967). The conclusion that the α-amylases of the pancreas and parotid gland of the rat and rabbit (Malacinski and Rutter, 1969) are single polypeptide chains of molecular weight *ca.* 55,000 g/mole may presumably be extended to this class of proteins in the vertebrates in general. Thus, in cases where greater than two enzyme species are present in a single organism (*e.g.*, human saliva (Muus and Vnenchack, 1964; Lamberts *et al.*, 1965)) either gene duplication or posttranslational modification must have occurred to account for the observed multiplicity.

It should be noted that the use of molecular weight analysis in guanidine hydrochloride–mercaptoethanol solutions as a criterion of molecular integrity is valid only for internal peptide-bond breakage; the loss of a small peptide from either the C- or N-terminal end of the polypeptide chain would not be revealed by the techniques employed. In this sense, the conclusion that the two forms of the pancreatic enzyme do not represent the products of proteolytic digestion can only be tentative; however, the congruence of amino acid compositions argues that neither is a degradative form of the other.

As noted above, the differences in amino acid composition between the rat pancreatic and parotid amylases suggest that the enzymes differ in primary sequence; however, it is not possible, at present, to distinguish between the possibilities of gene duplication of posttranslational derivatization as a source of the observed heterogeneity in the pancreatic enzyme. If gene duplication has arisen, subsequent mutation to yield altered sequence has not been extensive; in addition, preliminary *in vitro* experiments suggest the incorporation of *N*-acetylglucosamine into both variants.

The further phylogenetic comparative analysis of amino acid composition indicates sequence homology exists within each of the mammalian, plant, and fungal and bacterial amylase classes. In addition, it is probable on the same basis that the α-amylases of microorganisms and mammals are evolutionarily related, but less likely that the β-amylases of plants are similarly derived.

The contention that the rat pancreatic and parotid amylases do contain significant differences in primary sequence is strongly supported by the immunological results, although previous immunological studies (McGeachin *et al.*, 1966) indicated that the pancreatic and salivary amylases of a number of species were identical or, at least, very similar. The results of the present study demonstrate pronounced immunological differences between the pancreatic and parotid enzymes of the rat (as implied by previous data (McGeachin *et al.*, 1961)) and thus confirm the conclusion of Malacinski and Rutter (1969) and Sick and Nielsen (1964) that the amylases of the pancreas and parotid glands are, in fact, nonidentical proteins and hence the product of different genes.

As discussed previously (Malacinski and Rutter, 1969), this conclusion is of importance to a consideration of the mechanisms of control of biosynthesis of the amylases in these two organs and also in the liver, in which a third distinct amylase (McGeachin *et al.*, 1966) is synthesized (Arnold and Rutter, 1963). The temporal differences in the embryonic accumulation of the pancreatic and parotid enzymes (Rutter *et al.*, 1964) and the different responses in the adult animal to changes in either the composition of the diet (Reboud *et al.*, 1966) or serum insulin levels (Palla *et al.*, 1967) must, therefore, reflect the activity of different gene systems rather than selective control of the same gene in different organs of the same animal.

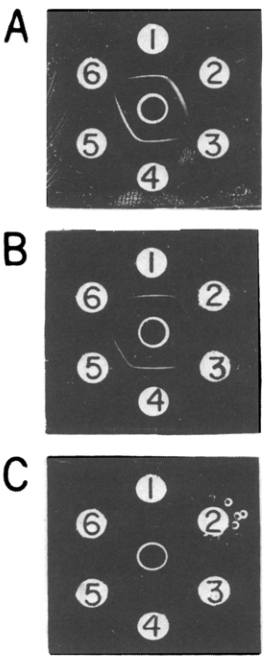

FIGURE 4: Double diffusion immunological analysis of rat pancreatic and parotid amylases. The center well of A and B contained 20 μl of antiserum to the chromatographically purified pancreatic enzyme; the center well of C contained 20 μl of a 10-mg/ml solution of γG-immunoglobulin purified from the serum used in A and B (see Materials and Methods). The outer wells contained: (A) (1 and 4) chromatographically purified pancreatic amylase (0.5 mg/ml); (2 and 5) crude homogenate of rat pancreas (1000 amylase units/ml); (3 and 6) crude homogenate of rat parotid gland (2500 amylase units/ml); (B) (1 and 4) chromatographically purified pancreatic amylase (0.5 mg/ml); (2 and 5) pancreatic amylase purified by glycogen precipitation (0.5 mg/ml); (3 and 6) parotid amylase (1.0 mg/ml); (C) as in part B.

Acknowledgment

The authors wish to thank Dr. David Teller, Dr. Thomas Horbett, and Mr. Curtis Harris for assistance in the molecular weight determinations and comparison of amino acid compositions, and Dr. Elliott Shaw for the kind gift of *p*-nitrophenyl-*p'*-guanidinobenzoate.

References

Akabori, S., Okada, Y., Fujiwara, S., and Sugae, K. (1956), *J. Biochem.* (*Tokyo*) *43*, 741.

Appella, E., and Markert, C. L. (1961), *Biochem. Biophys. Res. Commun. 6*, 171.

Arnold, M., and Rutter, W. J. (1963), *J. Biol. Chem. 238*, 2760.

Bernfeld, P. (1955), *Methods Enzymol. 1*, 149.

Bernfeld, P., Duckert, F., and Fischer, E. H. (1950), *Helv. Chim. Acta 33*, 1064.

Caldwell, M. L., Dickey, E. S., Hanrahan, V. M., Kung, H. C., Kung, J. T., and Miscko, M. (1954), *J. Amer. Chem. Soc. 76*, 143.

Chase, T., and Shaw, E. (1969), *Biochemistry 8*, 2212.

Danielsson, C. (1947), *Nature* (*London*) *160*, 899.

Dayhoff, M. O., and Eck, R. V. (1967), Atlas of Protein Structure and Sequence, New York, N. Y., National Biomedical Research Foundation, p 86.

Deutsch, H. F. (1967), *in* Methods in Immunology and Immunochemistry, Vol. 1, Williams, C. A., and Chase, M. W., Ed., New York, N. Y., Academic Press, p 319.
Dévényi, T. (1968), *Acta Biochem. Biophys. Acad. Sci. Hung. 3*, 429.
Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.
Fahey, J. L. (1967), *in* Methods in Immunology and Immunochemistry, Vol. 1, Williams, C. A., and Chase, M. W., Ed., New York, N. Y., Academic Press, p 321.
Fischer, E. H., and Stein, E. A. (1960), *Enzymes 4*, 313.
French, D. (1960), *Enzymes 4*, 345.
Gertler, A., and Birk, Y. (1965), *Biochem. J. 95*, 621.
Harris, C. E., Kobes, R. D., Teller, D. C., and Rutter, W. J. (1969), *Biochemistry 8*, 2442.
Hirs, C. H. W. (1967), *Methods Enzymol. 11*, 59.
Kranz, D., Mutzbauer, H., and Schulz, G. V. (1965), *Biochim. Biophys. Acta 102*, 514.
Lamberts, B. L., Meyer, T. S., and Losee, F. L. (1965), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 24*, 441.
Lapanje, S., and Tanford, C. (1967), *J. Amer. Chem. Soc. 89*, 5030.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Loyter, A., and Schramm, M. (1962), *Biochim. Biophys. Acta 65*, 200.
Malacinski, G. M., and Rutter, W. J. (1969), *Biochemistry 8*, 4382.
Marchis-Mouren, G., and Pasero, L. (1967), *Biochim. Biophys. Acta 140*, 366.
Marchis-Mouren, G., Pasero, L., and Desnuelle, P. (1963), *Biochem. Biophys. Res. Commun. 13*, 262.
McGeachin, R. L., and Brown, J. H. (1965), *Arch. Biochem. Biophys. 110*, 303.
McGeachin, R. L., Pavord, W. M., Widmer, D. N., and Prell, P. A. (1966), *Comp. Biochem. Physiol. 18*, 767.
McGeachin, R. L., Reynolds, J. M., and Huddleston, J. I. (1961), *Arch. Biochem. Biophys. 93*, 387.
McMeekin, T. L., Groves, M. L., and Hipp, N. J. (1949), *J. Amer. Chem. Soc. 71*, 3298.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 7*, 819.
Muus, J. (1954), *J. Amer. Chem. Soc. 76*, 5163.
Muus, J., and Vnenchack, J. M. (1964), *Nature (London) 204*, 283.
Narita, K., Murakami, H., and Ikenaka, T. (1966), *J. Biochem. (Tokyo) 59*, 170.
Ouchterlony, O. (1948), *Acta Pathol. Microbiol. Scond. 25*, 186.
Palla, J. C., Ben Abdeljlil, A., and Desnuelle, P. (1967), *Biochim. Biophys. Acta 136*, 563.
Pascale, J., Avrameas, S., and Uriel, J. (1966), *J. Biol. Chem. 241*, 3023.
Penhoet, E., Kochman, M., Valentine, R., and Rutter, W. J. (1967), *Biochemistry 6*, 2940.
Petra, P. H., and Neurath, H. (1969), *Biochemistry 8*, 2466.
Pfueller, S. L., and Elliott, W. H. (1969), *J. Biol. Chem. 244*, 48.
Plummer, T. H., and Hirs, C. H. W. (1964), *J. Biol. Chem. 239*, 2350.
Reboud, J. P., Marchis-Mouren, G., Pasero, L., Cozzone, A., and Desnuelle, P. (1966), *Biochim. Biophys. Acta 117*, 351.
Reisfeld, R. A., Lewis, V. J., and Williams, D. E. (1962), *Nature (London) 195*, 281.
Rowe, J. J. M., Wakim, J., and Thoma, J. A. (1968), *Anal. Biochem. 25*, 206.
Rutter, W. J., Wessells, N. K., and Grobstein, C. (1964), *Nat. Cancer Inst. Monograph 13*, 51.
Serry, V. L. (1968), Ph.D. Thesis, University of Washington, Seattle, Wash.
Sick, K., and Nielsen, J. T. (1964), *Hereditas 51*, 291.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30*, 1190.
Svensson, H. (1962), *Arch. Biochem. Biophys., Suppl. 1*, 132.
Teller, D. C. (1967), *Anal. Biochem. 19*, 256.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 164*, 66.
Thoma, J. A., Koshland, D. E., Jr., Shimke, R., and Ruscica, J. (1965), *Biochemistry 4*, 714.
Vandermeers, A., and Christophe, J. (1968), *Biochim. Biophys. Acta 154*, 110.
Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20*, 820.
Waldschmidt-Leitz, E., Grafinger, L., and Westphal, M. (1964), *Hoppe-Seyler's Z. Physiol. Chem. 339*, 36.
Walsh, K. A., and Brown, J. R. (1962), *Biochim. Biophys. Acta 58*, 596.
Walsh, K. A., Ericsson, L. H., and Neurath, H. (1966), *Proc. Nat. Acad. Sci. U. S. 56*, 1339.
Walsh, K. A., and Neurath, H. (1964), *Proc. Nat. Acad. Sci. U. S. 52*, 884.
Wrigley, C. (1968), *J. Chromatogr. Sci. 36*, 362.
Yphantis, D. A. (1964), *Biochemistry 3*, 297.