Amelogenins

PURIFICATION AND PARTIAL CHARACTERIZATION OF PROTEINS FROM DEVELOPING BOVINE DENTAL ENAMEL

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1. Procedures are described for the purification of amelogenin electrophoretic components and their analysis for homogeneity by polyacrylamide-gel electrophoresis at both acidic and alkaline pH values. 2. Most of these components belonged to two main groups, termed the J group and the C group after their major electrophoretic components. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis indicated that, within each group, proteins were of similar size, but the C-group proteins were larger than those of the J group. 3. By sedimentation-equilibrium ultracentrifugation and amino acid analysis, the four J-group components were found to be very small proteins (mol. wt. 5500-3000) and, except for one, similar in amino acid composition. The components of the C group were found to be proteins of moderate size (mol. wt. 16800-16100) with very similar amino acid compositions. A third minor amelogenin group of intermediate size was also found, but not further analysed. Details of the results of the ultracentrifuge studies are given in a supplementary paper that has been deposited as Supplementary Publication SUP 50014 at the National Lending Library for Science and Technology, Boston Spa, Yorks. LS23 7BQ, U.K., from whom copies can be obtained on the terms indicated in *Biochem. J.* (1973) 131, 5. 4. Two of the J-group components were similar to amelogenins isolated by other workers. 5. All amelogenins analysed were rich in proline, glutamic acid, histidine and methionine, and contained no half-cystine. Their amino acid compositions, combined with their molecular weights, serve to distinguish the amelogenins from both collagens and keratins.

Developing dental enamel contains a large amount of protein (Eastoe, 1963; Burgess & Maclaren, 1965; Kaneko, 1967) which is almost completely removed from the enamel during its mineralization (Burgess & Maclaren, 1965; Stack, 1967). Enamel proteins from human, porcine, and bovine enamel have been found to have similar distinctive amino acid compositions, i.e. high proline, glutamic acid, leucine and histidine contents, and very low cysteine contents (Eastoe, 1960, 1963; Piez, 1961; Glimcher *et al.*, 1961, 1964; Burgess & Maclaren, 1965). The enamel proteins from at least five other species exhibit this same sort of composition (Levine *et al.*, 1967). Eastoe (1965) suggested calling these proteins amelogenins because the amino acid composition of the enamel-protein mixture was unlike that of either collagens or keratins.

Studies by Burgess & Maclaren (1965), Nikiforuk & Simmons (1965), Fincham (1968, 1971), Katz *et al.* (1969) and Nikiforuk & Gruca (1969) showed that the amelogenins are more electrophoretically heterogeneous at alkaline pH in the presence of urea (20 electrophoretic components) than at acid pH (nine electrophoretic components). Nikiforuk &

Gruca (1969) showed that six of the amelogenin electrophoretic components demonstrated at basic pH in the presence of urea were immunologically identical.

Burgess & Maclaren (1965) employed fractional precipitation to separate bovine amelogenin electrophoretic components into two groups with differing amino acid compositions. Burgess *et al.* (1965) used gel filtration at acid pH to attempt further purification of the major components of each of these two groups. They isolated one fraction from each group; one fraction (termed J) contained very low-molecular-weight aggregating proteins, whereas the other (termed C) contained non-aggregating higher-molecular-weight proteins. Subsequent polyacrylamide-gel electrophoresis at acid pH by J. Huang & R. C. Burgess (unpublished work) showed that the gel-chromatographic preparations J and C were electrophoretically heterogeneous. Fincham (1968) confirmed that homogeneous electrophoretic components could not be obtained by gel filtration at acid pH.

Glimcher *et al.* (1964) exploited differential solubilities to separate the amelogenins into a neutral and an acid soluble fraction. Mechanic *et al.* (1967) and Katz *et al.* (1969) by means of ultracentrifugation

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and gel filtration, characterized the neutral soluble amelogenins as a group composed of several components capable of aggregating to produce very high-molecular-weight complexes; sedimentation equilibrium at neutral pH (Mechanic *et al.*, 1967) indicated a molecular weight of between 1.9×10^6 and 2.9×10^6 for these proteins.

Mechanic *et al.* (1967), by using gel filtration at neutral pH, were not able to separate the neutral soluble amelogenins into fractions with constant elution volumes or a constant amino acid composition. Mechanic (1971) and Nikiforuk & Gruca (1971) by using gel filtration at neutral or basic pH in the presence of dissociating agents, failed to produce a separation of the amelogenins.

Nikiforuk & Simmons (1965) attempted a separation by ion-exchange chromatography without success. Fincham (1971) was able to separate a single component by a combination of ion-exchange chromatography and gel filtration. Seyer & Glimcher (1971) used free-flow preparative electrophoresis, combined with preparative polyacrylamide-gel electrophoresis at alkaline pH in the presence of urea to isolate four homogeneous amelogenin components. Their analytical polyacrylamide-gel electrophoresis system was that described by Katz *et al.* (1969).

The aims of the present investigation were: (i) to obtain information about amelogenin monomer molecular-weight heterogeneity; (ii) to find a combination of techniques that would separate most of the amelogenin electrophoretic components; (iii) to identify the separated components in acidic and alkaline analytical gel-electrophoretic systems used by other workers, to allow a correlation of present results with previous work; (iv) to characterize the separated components with respect to amino acid composition and molecular weight.

Experimental

Materials

All chemicals employed were of analytical grade and were obtained from BDH Chemicals, Toronto, Ont., Canada; Eastman-Kodak Co., Rochester, N.Y., U.S.A.; Fisher Scientific Co. Ltd., Toronto, Ont., Canada; and Canadian Laboratory Supplies Ltd., Toronto, Ont., Canada. Tooth germs were obtained from Canada Packers Ltd., Toronto, Ont., Canada, at the time of slaughter and packed in ice during transport. Gel filtration was carried out by using Sephadex G-25 (superfine grade), and Sephadex G-75 (superfine grade) obtained from Pharmacia (Canada) Ltd., Montreal, P.Q., Canada. Ion-exchange chromatography was carried out by using sulphopropyl Sephadex C-25 (SP-Sephadex C-25) and quarternary aminoethyl Sephadex A-25 (QAE-Sephadex A-25) also obtained from Pharmacia

(Canada) Ltd. Dialysis tubing was obtained from Fisher Scientific Co. Ltd. Ultrafiltration was carried out with apparatus and membranes obtained from Amicon Corp., Lexington, Mass., U.S.A. All aqueous solutions were prepared with deionized water. Aq. 6M-urea solutions were freed of u.v.-absorbing material by passage through a column (7.5 cm $\times$ 8 cm) of Norit-A charcoal (BDH Chemicals).

Amelogenin preparations

Enamel powder was obtained from first-molar tooth germs of six-month-old calves (Burgess & Maclaren, 1965). Pooled samples of enamel were used after analytical polyacrylamide-gel electrophoresis had shown identical electrophoretic components in enamel obtained from six individual animals (F. M. Eggert, unpublished work). A preparation termed 'Mixed' and prepared in the same way as the acid-soluble, chloride-insoluble material (Burgess & Maclaren, 1965), served for comparison during electrophoretic analysis. The C-group (EDTA, pH 11-insoluble fraction) and the J-group (EDTA, pH 11-soluble pH 7-insoluble fraction) preparations served as the starting materials for further purification. These preparations were obtained as described by Burgess & Maclaren (1965), with a change in the concentration of EDTA to 0.25 M from 0.1 M.

Analytical polyacrylamide-gel electrophoresis

Acid system (pH 3.2). This system [10% (w/v) final total polyacrylamide concentration], was prepared as follows. The solutions used included Stock buffer (0.75 M-formic acid, 0.25 M-NaOH); solution A: 3.2 ml of Stock buffer, 0.32 ml of β -dimethylamino-propionitrile, diluted to 10.0 ml with water; solution B: 40.0 g of acrylamide, 1.1 g of *NN'*-methylenebisacrylamide, diluted to 100.0 ml with water; solution C: 0.48 g of ammonium persulphate diluted to 5.0 ml with water (stored at 3°C and made fresh weekly).

To 9.0 ml of water was added 0.75 ml of solution A, 2.25 ml of solution B and 0.075 ml of solution C, in that order. Gels, 3 cm long, were cast in glass tubes (internal diam., 5 mm), and carefully overlaid with 3 mm of water. After gel formation (25 min at room temperature), the gels were overlaid with electrode buffer (pH 3.2, a 1:100 dilution of Stock buffer), and pre-run (anode at top) in a Buchler Polyanalyst apparatus at room temperature for 60 min at a constant voltage of 300 V, with a constant voltage/milliamperage electrophoresis power supply. The electrode buffer in both electrode compartments was then replaced with fresh buffer.

Protein samples were prepared as 0.5% (w/v) solutions in water, and layered on to the gels under the electrode buffer as 20 μ l of a 1:1 dilution of the protein solution with 6M-urea. Electrophoresis was done

at room temperature at a constant 300 V for 20 min. The gels were then removed from the glass tubes, and stained with Coomassie Brilliant Blue R (Machrome no. 1137) according to the methods of Chrambach *et al.* (1967). After being left in 10% (w/v) trichloroacetic acid for several days, the gels were photographed by using a fluorescent light box, a red filter and Kodak Panatomic X film.

Alkaline system (pH 8.8, 6M-urea). By using the gel system of Katz *et al.* (1969), which contains a 5% (w/v) total polyacrylamide concentration, gels 3 cm long were cast in glass tubes (internal diam., 5 mm) and carefully overlaid with 3 mm of water. After gel formation (20 min at room temperature), the gels were overlaid with 0.05 M-Tris-glycine electrode buffer (pH 8.8), and pre-run (cathode on top) at 8°C and a constant 2.5 mA/gel until the voltage reached 150 V. Then the upper gel surface was flushed by means of a Pasteur pipette, and the protein sample layered on to the gel. Protein samples were prepared as for the acidic system. Electrophoresis was carried out at a constant voltage of 160 V at 8°C for 40 min. The gels were stained and photographed as for the acidic system.

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis. The 'Mixed', J-group, and C-group preparations were analysed, by using as molecular-weight standards: bovine serum albumin and lysozyme (Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.); trypsin and chymotrypsin (Mann Research Laboratories Inc., New York, N.Y., U.S.A.); horse heart cytochrome *c* (Dr. G. R. Williams, Department of Biochemistry, University of Toronto, Toronto, Ont., Canada); and crystalline zinc insulin (Connaught Laboratories, Toronto, Ont., Canada).

Sample preparation consisted of mixing together: 10 µl of aq. protein solution [0.5% (w/v)], 20 µl of an aq. solution containing 1.0% (w/v) sodium dodecyl sulphate and 1.0% (v/v) β-mercaptoethanol, and 10 µl of 6M-urea, followed by incubation at 37°C for 30 min. Before electrophoresis 3 µl of Bromophenol Blue [0.05% (w/v)], and 20 µl of 6M-urea were added. Gels (5 cm long) with 10% (w/v) total polyacrylamide concentration were prepared by using solutions made according to Weber & Osborn (1969). The electrode buffer consisted of a 1:4 dilution of the gel buffer with water.

The split-gel technique of Dunker & Rueckert (1969) was used. The reference protein (reduced chymotrypsin) and the test protein were layered under the upper electrode buffer. The gels were run at room temperature and a constant 7 mA/gel until the Bromophenol Blue band had run 3/4 of the length of the gel. Thereafter, the gels were removed and stained as for regular electrophoretic analyses.

A photographic enlargement (2×) of the gels was used for measurements to the nearest 0.1 mm. Relative migration distances were calculated by dividing

each protein migration distance from the cathode end of the gel by that of the B chain of chymotrypsin. These results were then plotted to produce a molecular-weight standard-curve according to the method of Shapiro *et al.* (1967).

Purification of the J-group electrophoretic components

Gel filtration. A 0.5% (w/v) solution of J-group preparation (10 ml) was chromatographed on a column (2.5 cm × 45 cm) of Sephadex G-25 (superfine grade) with 0.05 M-formic acid as eluent. A flow rate of 0.5 ml/min was maintained by means of a peristaltic pump. The column effluent was monitored at 280 nm by means of a dual-beam spectrophotometer. Fractions (3 ml) were collected, and fractions corresponding to each peak of the elution profile were pooled, freeze-dried, weighed and then subjected to electrophoresis and amino acid analysis.

Ion-exchange chromatography. Protein (50 mg) from the largest peak in the gel filtration elution profile was dissolved in 10 ml of 0.1 M-sodium citrate-6M-urea buffer, pH 3.6. A column (2.5 cm × 45 cm) of SP-Sephadex C-25, maintained at 8°C with a water jacket, was equilibrated with 0.1 M-sodium citrate-6M-urea buffer, pH 3.5. The sample was applied to the column, allowed to flow into the ion-exchanger, and then 400 ml of the pH 3.6 buffer was applied. The eluent was monitored at 280 nm, the flow rate adjusted to 0.5 ml/min by means of a Mariotte bottle, and 12-min fractions were collected. A pH-gradient derived from a series of 0.1 M-sodium citrate-6M-urea buffers was then applied to the column by means of a nine chambered Technicon Autograd. The following buffers (100 ml of each) were used in successive chambers: pH 3.7, 4.5, 4.6, 4.8, 4.8, 5.4, 5.9 and 6.5. The flow rate was adjusted to 1 ml/min, and 12 min fractions were collected. The column effluent was monitored as above. For selected fractions the pH was determined to the nearest 0.05 pH unit. All pH measurements were carried out by using a Sargent model S-300700-10 combination glass electrode, and a Fisher Accumet model 310 pH-meter.

Having applied the above procedure to four 50-mg batches of starting material, the pooled solutions corresponding to each peak were titrated to pH 3.6 with 0.1 M-citric acid-6M-urea buffer, reappplied to a column of SP-Sephadex C-25 and rechromatographed as above.

Sections of dialysis tubing were heat-treated at 90°C for 7 days to render them less permeable to low-molecular-weight proteins (Kupke, 1961). Individual peaks obtained from ion-exchange chromatography were dialysed separately against six changes of 3 litres of water, over a period of 3 days, at a temperature of 3°C. A crystal of chlorobutanol was added to the dialysis reservoir to prevent contaminating growths.

After dialysis the precipitated proteins were re-dissolved by dialysis against a minimum volume of 0.005 M-formic acid. Both the contents of the dialysis bag and the formic acid of the dialysis reservoir were pooled for each sample. By using a Diaflo UM-05 membrane, these pooled solutions (approx. 150 ml) were concentrated to a volume of 10 ml and then dialysed in the ultrafiltration chamber with 150 ml of 0.05 M-formic acid. The material from each peak was then freeze-dried, weighed and subjected to electrophoresis, amino acid analysis and ultracentrifugation.

Purification of the C-group electrophoretic components

Gel filtration. A sample (250 mg) of the C-group preparation was dissolved in 50 ml of 0.05 M-formic acid and chromatographed on a column (5 cm × 100 cm) of Sephadex G-75 (superfine grade), by using 0.05 M-formic acid as eluent. A flow rate of 0.5 ml/min was maintained by means of a peristaltic pump. The column was kept at 8°C by means of a water jacket, the column effluent monitored at 280 nm, and 12 min fractions were collected. Pooled fractions from each peak of the elution profile were freeze-dried, weighed and subjected to electrophoresis and amino acid analysis.

Ion-exchange chromatography. Ion-exchange chromatography, by using QAE-Sephadex A-25, was performed on the material of the main gel-filtration peak. A portion (150 mg) of the sample was dissolved in 15 ml of 0.1 M-Tris-HCl-6 M-urea buffer, pH 9.5. A column (5.0 cm × 60 cm) of the ion-exchanger was equilibrated with the above buffer. The column was kept at 4°C. The sample was applied to the column by upward flow; by using a peristaltic pump a flow rate of 7 ml/h was maintained. Then 600 ml of the pH 9.5 starting buffer was applied at the same flow rate and 32-min fractions were collected. The column effluent was monitored at 280 nm. A pH-gradient was then applied to the column by means of a nine-chambered Technicon Autograd. The following 0.1 M-Tris-HCl-6 M-urea buffers (300 ml of each) were used in successive chambers; three at pH 9.5, three at pH 9.0 and three at pH 8.5. The flow rate and fraction size was the same as above. The gradient was followed by 500 ml of 0.1 M-Tris-HCl-6 M-urea buffer, pH 8.5. The pH of selected fractions was measured to the nearest 0.05 pH-unit.

The major peaks of the ion-exchange elution profile were dialysed separately in untreated dialysis tubing at 3°C for 48 h against three changes of 3 litres of water. The non-diffusible material (approx. 150 ml) was concentrated to 10 ml by means of ultrafiltration with a Diaflo UM-10 membrane. The concentrated solutions corresponding to each peak were chromatographed on a column (2.5 cm × 90 cm) of Sephadex G-25 (superfine grade) with 0.05 M-formic

acid as eluent. The flow rate was maintained at 0.8 ml/min, the column effluent was monitored at 280 nm and 12-min fractions were collected. The pooled fractions corresponding to the major peaks of the elution profiles were freeze-dried, weighed and subjected to electrophoresis, amino acid analysis and ultracentrifugation.

Amino acid analysis

Freeze-dried sample (0.5 mg) was dissolved in 3.0 ml of glass-distilled, constant-boiling HCl. The vial containing this solution was sealed under vacuum, and hydrolysed at 110°C for 24 h. The hydrolysates were dried at 60°C in an evaporator and analysed on a commercial Beckman model 120 C amino acid analyser by a modification of the method of Spackman *et al.* (1958). Corrections of 5 and 10% were made for destruction of threonine and serine respectively. Samples for tryptophan analysis were hydrolysed and analysed according to the method of Matsubara & Sasaki (1969).

Ultracentrifugation

The u.v.-absorption maximum for each of the purified amelogenin fractions dissolved in 0.05 M-formic acid, in the range of 260–290 nm, was 275 ± 2 nm. At 275 nm the relationship between the absorbance and the protein concentration was linear for each preparation up to the maximum concentration studied (2 mg of protein/ml).

The protein partial specific volumes were calculated from the protein amino acid compositions as described by Cohn & Edsall (1943). Protein (0.36 mg/ml and 0.72 mg/ml) dissolved in 0.05 M-formic acid was used for ultracentrifugation. Sedimentation was carried out at a temperature of 20°C in a Beckman model E analytical ultracentrifuge equipped with a photoelectric scanner and an u.v.-light source. Standard low-speed sedimentation-equilibrium experiments (Schachman & Edelstein, 1966) were carried out by using an AN-F titanium rotor, and 12-mm double-sector filled-Epon centrepieces with sapphire windows. All samples were scanned at 275 nm, and runs were continued for 8 h after there was no measurable change in absorbance at the inner end and outer end of the cell. The results of absorbance relative to radial distance were recorded on a strip chart.

All measurements were made on the strip chart to the nearest 0.1 mm. Inter-hole distance, recorder response to internal absorbance standards, and at least 80 pairs of absorbance versus radial-distance measurements were carried out. The straight-line relationship of absorbance versus concentration for the proteins allowed direct use of the strip-chart absorbance measurements in the calculations. The

information was placed on data cards, and analysed by using an IBM 360 computer and a program devised by D. Kells, Biochemistry Department, University of Toronto. This program converted absorbance values in terms of chart divisions into actual absorbance values and then calculated and plotted $\ln c$ versus $r^2/2$, where c = protein concentration in terms of absorbance at 275 nm and r = radial distance. Weight-average molecular weights were calculated from the slope of this line. The molecular weight as a function of concentration was determined and plotted as described by Schachman & Edelstein (1966).

Results

Terminology of electrophoretic components

Scheme 1 provides a summary of the steps employed in the separation of the amelogenin electrophoretic components. For each step in the separation, the separation medium, the buffers employed, the starting protein preparation and the fractions obtained are identified. The electrophoretic components in the final fractions are also indicated. Once the two major amelogenin groups had been separated by

selective precipitation, the amelogenin monomers were separated by a combination of methods that exploited their molecular-weight and charge differences.

The electrophoretic component terminology used is based on that of Burgess & Maclaren (1965), who described the electrophoresis of amelogenin preparations on starch gels at pH 3.6 in the presence of urea. Plate 1 represents the results of electrophoresis of 'Mixed', J-group and C-group preparations in both the acidic and the basic polyacrylamide-gel systems. In the acidic system, the 'Mixed' preparation contains all the electrophoretic components present in the other two preparations, and all but one of the components reported by Burgess & Maclaren (1965). In the acidic system, the J-group preparation contains the major component j and the minor components h, i and k. Since these components were isolated as a group, it was decided to name the group, and the preparation, after its major component. In the same system, the C-group preparation contains the major component c and the minor components a, b and e, as well as other components which apparently represent components h, i and j. It was decided to term the components a, b, c and e, the C group of components, and the whole preparation the C-group preparation, after its major component.

Step

Scheme 1. *Method of purification of the amelogenin electrophoretic components*

Scheme 1 identifies the separation medium and the buffers employed at each step in the purification. The starting protein preparation for each step and the fractions obtained are indicated. Electrophoretic components in the final fractions are identified. Fraction and electrophoretic component designations are described in the text.

Table 1. *Results of sodium dodecyl sulphate–polyacrylamide-gel electrophoresis*

Split-gel sodium dodecyl sulphate–polyacrylamide-gel electrophoresis of molecular-weight-standard proteins and the 'Mixed', J-group and C-group amelogenin preparations was performed as described in the Experimental section. Relative mobilities are given relative to the B chain of chymotrypsin.

Protein	Relative mobility	Molecular weight	Reference
Bovine serum albumin	0.578	66 500	Schachman (1963)
Trypsin	0.863	23 800	Schachman (1963)
Lysozyme	1.009	14 400	Schachman (1963)
Chymotrypsin (B chain)	1.000	13 927	Moroux & Ravery (1966)
Horse heart cytochrome <i>c</i>	1.036	12 500	Margoliash <i>et al.</i> (1962)
Chymotrypsin (C chain)	1.095	10 157	Moroux & Ravery (1966)
Insulin	1.153	5 800	Schachman (1963)
C group	0.851	25 000	
Intermediate component	0.938	18 000	
J group	1.147	<10 000	

In the basic system, the 'Mixed' preparation presents an electrophoretic pattern very much like that described by Katz *et al.* (1969) and Seyer & Glimcher (1969, 1971) for the neutral soluble enamel proteins in the same analytical system. A similar pattern was also reported by Nikiforuk & Gruca (1969) and Fincham (1971), by using different polyacrylamide-gel systems at basic pH in the presence of urea.

In the basic system the J-group preparation contains one major and at least eight minor electrophoretic components. In the same system, the C-group preparation is represented by a series of seven slowly moving bands. These bands were numbered from 1–7, starting with the one closest to the cathode. A number of these C- and J-group components were subsequently isolated. It was then possible to determine the relative mobility of these bands in both the acidic and the alkaline electrophoretic systems.

Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis

Relative mobilities for the standard proteins were obtained (Table 1). These results, when plotted by the methods of Shapiro *et al.* (1967), produced a straight line except for the insulin value, as was expected from the results of Dunker & Rueckert (1969).

The 'Mixed' preparation contained one major fast-moving component, one major slow-moving component, and also a faint minor component with an intermediate mobility. The C- and the J-group preparations each contained only a single component having mobilities corresponding to the slow- and fast-moving components respectively in the 'Mixed' preparation.

Purification of the J-group electrophoretic components

Gel filtration. The result of chromatography of a 50 mg freeze-dried sample on Sephadex G-25 (super-

fine grade) is shown in Fig. 1. The eluate from 1000 mg of J-group preparation was pooled into four fractions (J1, J2, J3 and J4); after freeze-drying it yielded 280 mg of J1, 160 mg of J2, 240 mg of J3 and 200 mg of J4. Plate 2(a) and Table 2 respectively represent the results of analytical electrophoresis and amino acid analysis on samples from each fraction. Fraction J3 contained the highest proportion of electrophoretic component j along with substantial amounts of components h, i and k in the acidic system, as well as a minimal number (5) of components in the alkaline system. Although fraction J4 contained component j as its major component and had the least number of electrophoretic components in both systems, the faint staining of the protein bands and the low absorbance at 280 nm suggested that its total protein content was low. The amino acid analyses indicated that fraction J3 contained a high amount of tyrosine and glycine and a low amount of alanine. No cysteine was demonstrated in any of the fractions after acid hydrolysis and analysis for tryptophan was not carried out.

Ion-exchange chromatography. The material of the J3 fraction was subjected to ion-exchange chromatography on SP-Sephadex C-25 utilizing 0.1 M-sodium citrate–6 M-urea buffers to provide a pH-gradient. The results of chromatography of a 50 mg sample are shown in Fig. 2. The fractions, termed J3s1, J3s2, J3s3, J3s4, J3s5 and 6 and J3s7, were obtained from four 50 mg samples of J3 starting material, and were combined and rechromatographed under the same conditions as described above. Fractions J3s1, J3s2, J3s3 and J3s4 produced single peaks, each eluting at approximately the same position as the parent peak. Fractions J3s5 and 6 eluted as a broad peak with several minor shoulders in the same position as the parent peaks. Fraction J3s7 eluted at the position of the parent peak, but as two peaks which overlapped considerably. Recovery of freeze-dried material from

Table 2. Amino acid composition of amelogenin 'Mixed', J-group and C-group preparations and gel-filtration fractions

Values are expressed as mol/100mol. Fraction designations are the same as those in Fig. 1 and Fig. 3.

Preparation	... 'Mixed'	C group	C1	C2	C3	C4	J group	J1	J2	J3	J4
Amino acid											
Lys	1.2	1.1	1.0	0.5	1.7	1.5	2.0	1.1	2.1	2.2	3.0
His	5.8	6.9	7.1	5.8	7.1	4.1	6.8	15.7	8.0	6.4	6.0
Arg	1.4	1.4	1.0	0.4	2.6	2.9	1.6	0.3	2.4	1.7	2.6
Asp	6.1	4.2	3.3	2.1	3.8	4.7	4.2	3.5	4.4	3.3	2.2
Thr	3.1	2.6	2.2	2.3	3.4	3.3	2.9	3.2	2.5	2.6	2.6
Ser	5.5	4.9	3.9	3.7	7.6	8.7	5.9	4.5	5.0	6.3	6.4
Glu	15.0	17.3	19.8	22.3	18.5	14.2	10.8	16.1	8.7	8.1	7.5
Pro	25.8	26.7	27.6	30.8	17.0	18.2	22.0	20.9	25.3	22.0	22.3
Gly	6.2	4.0	4.0	3.8	6.8	9.5	8.8	4.1	5.7	10.5	11.1
Ala	2.0	2.3	2.4	2.3	2.0	1.7	1.2	2.2	1.2	0.2	0.1
Cys	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Val	3.8	3.8	3.5	3.1	5.3	3.3	3.9	5.4	3.0	2.4	2.3
Met	4.3	6.3	5.8	5.5	4.4	3.5	4.9	4.2	6.0	6.6	9.1
Ile	3.1	3.2	4.9	3.3	2.5	3.3	4.1	3.5	4.5	4.3	2.9
Leu	8.9	9.0	9.1	10.0	9.4	9.9	8.4	10.2	13.0	7.9	7.3
Tyr	5.8	3.6	2.6	2.7	4.2	7.0	10.2	4.6	5.5	13.2	13.4
Phe	2.5	2.3	2.0	1.5	3.7	4.2	2.3	0.5	1.6	2.2	1.3

200mg of the J3 preparation was: 14mg of J3s2, 8mg of J3s3, 22mg of J3s4 and 6mg of J3s7, with J3s1, J3s5 and J3s6 present in such small amounts that none was recovered. Analytical polyacrylamide-gel electrophoresis (Plate 2b) and amino acid analysis (Table 3) were performed on the freeze-dried fractions. All electrophoretic components isolated by ion-exchange chromatography corresponded to components present in the J3 starting material. In the acidic system fractions J3s2, J3s3, J3s4 and J3s7 represent the single electrophoretic components h, i, j and k respectively. In the alkaline system fractions J3s3 and J3s4 were preparations of single components, whereas J3s2 (h) contained one major electrophoretic component, and very minor amounts of three other components. Fraction J3s7 is represented by two electrophoretic components designated as k1 and k2. Both of these components have a slower mobility than component j; the component k1 has the slower mobility and both components are present in the J3 fraction during electrophoresis at alkaline pH. It has therefore been possible to identify the electrophoretic components h, i, j and k of the acidic system as individual electrophoretic components in the alkaline electrophoretic system. It can also be seen that the relative mobility of components in the acidic system: h, i, j and k (slowest to fastest), is reversed in the alkaline system.

All of these components contained high amounts of glutamic acid and proline, with no detectable cysteine. Components i, j and k contained higher

amounts of serine, glycine and tyrosine and lower amounts of leucine and alanine than component h. Tryptophan was found in components h and j.

These components were also subjected to molecular-weight determination by means of sedimentation-equilibrium studies (Table 4). Plots of $\ln c$ versus $r^2/2$ (Supplementary Publication SUP 50014) were almost linear for the analyses of components i and j, indicating molecular-weight homogeneity and minimal aggregation over most of the concentration range available for study. Plots of molecular weight versus protein concentration obtained at different experimental speeds (Supplementary Publication SUP 50014) indicated the presence of high-molecular-weight material in each preparation. The preparation of component j contained the least amount of high-molecular-weight material. It was not determined for any of these preparations whether the high-molecular-weight material was an aggregate of the electrophoretic component or a contaminant. At low protein concentrations the plots became linear for components h, i and j, and the apparent molecular weight changed minimally with decreasing protein concentration. Least-squares straight lines were fitted to the results representing the linear portions of these plots and were extrapolated to zero protein concentration to determine molecular-weight values. The plot for component k was non-linear at low protein concentrations, indicating the presence of a low-molecular-weight contaminant. Only an approximate molecular weight was determined for this

Fig. 1. Gel filtration of the J-group preparation

Fractionation of J-group preparation (50mg of protein) on a column (2.5 cm $\times$ 45 cm) of Sephadex G-25 (superfine grade) in 0.05 M-formic acid. The flow rate was 0.5 ml/min; 3 ml of effluent were collected per tube; —, E_{280} . Tubes were pooled into fractions J1, J2, J3 and J4 as indicated in the Figure. V_0 is the void volume.

component from the slope of the plot of $\ln c$ versus $r^2/2$, by fitting a least-squares straight line to the results representing the approximately linear portion of the plot near the top of the cell.

Purification of the C-group electrophoretic components

Gel filtration. The results of chromatography of 250 mg of C-group preparation on Sephadex G-75 (superfine grade), with 0.05 M-formic acid as eluent, are shown in Fig. 3. When the effluent from two such separations was pooled into four fractions (C1, C2, C3, and C4), as indicated, and freeze-dried, it yielded 15 mg of C1, 170 mg of C2, 125 mg of C3 and 25 mg of C4. These fractions were then subjected to electrophoretic analysis (Plate 3a) and amino acid analysis (Table 2).

Analysis in the acidic system showed that fraction C2 represented a purification of electrophoretic component c, with component a as a contaminant. Analysis in the alkaline system indicated that fraction C2 contained components which appeared to be the same as those of the C-group preparation.

Amino acid analysis showed that none of the

Fig. 2. Ion-exchange chromatography of the J3 fraction

Fractionation of J3 fraction (50mg of protein) on a column (2.5 cm $\times$ 45 cm) of SP-Sephadex C-25. The starting buffer was 0.1 M-sodium citrate-6 M-urea, pH 3.5. The sample was eluted with a pH-gradient from pH 3.7-6.5 consisting of 0.1 M-sodium citrate-6 M-urea buffers. The flow rate was 1 ml/min; 12 ml of effluent were collected per tube; —, E_{280} ; •, pH. Tubes were pooled into fractions J3s1, J3s2, J3s3, J3s4, J3s5 and 6 and J3s7 as indicated in the Figure.

EXPLANATION OF PLATE I

Analytical polyacrylamide-gel electrophoresis of amelogenin 'Mixed', J-group and C-group preparations

Electrophoretic components are identified at the sides of (b) which is the key for (a). The direction of migration was from the top to the bottom in each case. Acidic system: gel dimensions were 3 cm $\times$ 5 mm; the total polyacrylamide concentration was 10% (w/v). A continuous buffer system consisting of 0.02M-sodium formate, pH 3.2, was employed. Electrophoresis was carried out at 300 V for 20 min. The stain was Coomassie Brilliant Blue. Alkaline system: gel dimensions were as above; the total polyacrylamide concentration was 5% (w/v). The gel buffer consisted of 0.05M-Tris-glycine-6M-urea, pH 8.8. Electrophoresis was carried out at 160 V for 40 min. The same stain as above was used.

EXPLANATION OF PLATE 2

Analytical polyacrylamide-gel electrophoresis of the fractions obtained during the purification of the J-group electrophoretic components

(a) J-group preparation gel-filtration fractions. (i) Results of electrophoretic analysis under the same conditions as those described for the acidic system in Plate 1. (ii) Results of electrophoresis under the same conditions as those described for the alkaline system in Plate 1. J1, J2, J3 and J4 refer to the fractions in Fig. 1. J group refers to the J-group preparation applied to the Sephadex G-25 (superfine grade) column. 'Mixed' refers to the 'Mixed' amelogenin preparation. The direction of migration is from the top to the bottom in both the acidic and the alkaline systems. (b) Analysis of the fractions obtained after ion-exchange chromatography on SP-Sephadex C-25 of the J3 gel-filtration fraction shown in Fig. 1. (i) and (ii) refer to the acidic and alkaline systems respectively, as in Plate 2 (a). J3s2, J3s3, J3s4 and J3s7 refer to the fractions in Fig. 2. 'Mixed' refers to the 'Mixed' amelogenin preparation. The direction of migration is from the top to the bottom in both systems.

EXPLANATION OF PLATE 3

Analytical polyacrylamide-gel electrophoresis of the fractions obtained during the purification of the C-group electrophoretic components

(a) C-group preparation gel-filtration fractions. (i) and (ii) refer to the acidic and alkaline systems, respectively, as in Plate 2(a). C1, C2, C3, and C4 refer to the fractions in Fig. 3. C group refers to the C-group preparation applied to the Sephadex G-75 (superfine grade) column. 'Mixed' refers to the 'Mixed' amelogenin preparation. The direction of migration is from the top to the bottom in both the acidic and the alkaline systems. (b) Analysis of the fractions obtained after ion-exchange chromatography on QAE-Sephadex A-25 of the C2 gel-filtration fraction shown in Fig. 3. (i) and (ii) refer to the acidic and alkaline systems respectively, as in Plate 2(a). C2q4, C2q5 and C2q6 refer to the fractions in Fig. 4. 'Mixed' refers to the 'Mixed' amelogenin preparation. The direction of migration is from the top to the bottom in both systems.

Table 3. *Amino acid composition of amelogenin ion-exchange chromatography fractions (electrophoretic components)*

Values are expressed as mol/100mol. Fraction designations are the same as those in Fig. 2 and Fig. 4. N.D., Not determined; Tr., trace.

Electrophoretic component	Fraction ...	C2q4 c'4	C2q5 c'5	C2q6 c'6	J3s2 h	J3s3 i	J3s4 j	J3s7 k (k1, k2)
Amino acid	...							
Lys		0.5	0.5	0.6	2.4	2.5	2.4	1.8
His		7.1	7.7	8.1	5.9	6.6	6.6	7.0
Arg		0.5	0.4	0.6	2.2	2.3	2.0	1.6
Asp		2.4	2.6	2.9	4.9	3.1	2.6	3.0
Thr		2.0	2.1	2.2	2.8	3.0	2.6	3.0
Ser		2.9	2.9	2.9	4.4	6.7	6.6	6.5
Glu		18.1	19.9	19.3	8.2	8.6	7.7	9.2
Pro		33.2	31.0	30.9	29.8	24.5	22.6	24.2
Gly		3.2	3.2	3.1	5.1	11.9	11.6	12.2
Ala		1.7	2.0	2.1	2.3	0.2	0.1	0.4
Cys		0.0	0.0	0.0	0.0	0.0	0.0	0.0
Val		3.0	3.0	3.1	2.6	1.9	2.6	3.0
Met		5.9	5.3	5.0	4.9	2.9	4.9	4.1
Ile		3.3	3.2	3.2	3.6	3.6	4.1	4.0
Leu		10.2	9.7	9.9	11.0	7.2	6.7	7.2
Tyr		3.1	3.2	3.2	5.8	11.4	11.9	10.5
Phe		1.9	1.7	1.7	2.4	2.5	2.5	2.3
Trp		0.8	1.6	1.2	1.8	Tr.	2.4	N.D.

Table 4. *Low-speed sedimentation-equilibrium ultracentrifugation of the amelogenin ion-exchange fractions*

The solvent was 0.05M-formic acid. The protein partial specific volume ($\bar{v}$) was calculated from the protein amino acid composition by the method of Cohn & Edsall (1943).

Amelogenin fraction	Electrophoretic component	Protein partial specific volume ($\bar{v}$)	Molecular weight
J3s2	h	0.744	5500
J3s3	i	0.731	4300
J3s4	j	0.733	4500
J3s7	k(k1, k2)	0.731	(3000)*
C2q4	c'4	0.740	16500
C2q5	c'5	0.736	16800
C2q6	c'6	0.736	16100

* Preliminary estimate only.

fractions contain detectable amounts of cysteine after acid hydrolysis. The composition of fraction C2 is characterized by higher contents of glutamic acid, proline, and leucine, and by lower contents of lysine and arginine as compared with the other C fractions. Fraction C3 is distinguished by being the only amelogenin fraction found in this study to contain more glutamic acid than proline. It was decided to use fraction C2 as the starting material for the purification of the C-group electrophoretic components demonstrated in the alkaline system.

Ion-exchange chromatography. The C2 gel-filtration fraction was subjected to ion-exchange chromatography on QAE-Sephadex A-25, utilizing a series of 0.1M-Tris-HCl-6M-urea buffers to provide a pH-gradient for the elution of the sample. The results of chromatographing 150mg of C2 fraction in this system are shown in Fig. 4. The fractions collected were dialysed, and then chromatographed on Sephadex G-25 (superfine grade), with 0.05M-formic acid as the eluent. Each fraction eluted from the Sephadex column as a very sharply defined peak at the

Fig. 3. Gel filtration of the C-group preparation

Fractionation of C-group preparation (250mg of protein) on a column (5cm×100cm) of Sephadex G-75 (superfine grade) in 0.05M-formic acid. The flow rate was 0.5ml/min; 6ml of effluent were collected per tube; —, E_{280} . Tubes were pooled into fractions C1, C2, C3 and C4 as indicated in the Figure. V_0 is the void volume.

void volume of the column. The material of each fraction was freeze-dried and subjected to electrophoretic and amino acid analysis. These fractions represent partial or complete purifications of many of the C-group components demonstrated in the alkaline electrophoretic system (G. A. Allen, R. C. Burgess & F. M. Eggert, unpublished work). The present study concentrated on the analysis of fractions C2q4, C2q5 and C2q6, since these represented the major peaks of the elution profile.

The results of electrophoretic analysis of the C2q4, C2q5 and C2q6 fractions for both the acidic and the alkaline systems are shown in Plate 3(b). In the acidic system all three purified preparations have component c as their major component. In addition, these fractions contain similar amounts of components a and e as contaminants. In the alkaline electrophoretic system these purified preparations each contain as their main component one major electrophoretic component of the C group. Since each of these components is represented in the acidic system primarily as component c, it was decided to designate the C-group components in the alkaline system with the prefix c'. Thus for the C2q4 preparation the major electrophoretic component is c'4; for the C2q5 fraction it is c'5; for the C2q6 fraction it is c'6. Each

preparation contains the neighbouring components as contaminants.

The results of amino acid analysis of the C2q4, C2q5 and C2q6 fractions are given in Table 3. Tryptophan was found in all three fractions, but cysteine was not detectable after acid hydrolysis. There are no striking differences in the compositions of these three fractions; minor differences in histidine, glutamic acid, proline and methionine content are evident. All three preparations are characterized by a high proline, glutamic acid, leucine and histidine content.

The three fractions were subjected to molecular-weight analysis by means of sedimentation-equilibrium centrifugation (Table 4). The plot of $\ln c$ versus $r^2/2$ for fraction C2q5 was linear (Supplementary Publication SUP 50014), indicating molecular-weight homogeneity. The plots for fractions C2q4 and C2q6 (Supplementary Publication 50014) were linear except for the very bottom 5% of the solution in the cell. It was not determined whether the material at the bottom of the cell represented a high-molecular-weight contaminant or an amelogenin aggregate. Plots of molecular weight versus protein concentration (Supplementary Publication 50014) indicated the presence of this high-molecular-weight component. However, except at high concentrations these plots were linear and the apparent molecular weight changed minimally with decreasing protein concentration. As is described above for the J-group components, the plots for all three C-group components were extrapolated to give molecular-weight values at zero protein concentration.

Discussion

Electrophoretic analysis at both acidic and alkaline pH values has shown that the 'Mixed' amelogenin preparation (present study) and the neutral soluble enamel protein preparation (Katz *et al.*, 1969) contain the same components. Comparison of the amino acid compositions of these amelogenin preparations (Burgess & Maclaren, 1965; Glimcher *et al.*, 1964) supports this conclusion.

Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis provided information about amelogenin molecular-weight heterogeneity. Three amelogenin groups of different size were demonstrated, all with apparent molecular weights of less than 25100. The group with the largest size represented the C group of electrophoretic components, that with the smallest size the J group of components. The minor group with intermediate size was not identified. These results were supported by gel filtration of these preparations on Sephadex G-75 by using 6 molal guanidine hydrochloride as eluent (F. M. Eggert, unpublished work), which demonstrated the same three amelogenin size groups. Under favourable

Fig. 4. Ion-exchange chromatography of the C2 fraction

Fractionation of C2 fraction (150 mg of protein) on a column (5 cm $\times$ 60 cm) of QAE-Sephadex A-25. The starting buffer was 0.1 M-Tris-HCl-6 M-urea, pH 9.5. The sample was eluted with a pH-gradient from pH 9.5-8.8 consisting of 0.1 M-Tris-HCl-6 M-urea buffers. The flow rate was 7 ml/h; 32 min fractions were collected into tubes; — E_{280} ; ●, pH. Tubes were pooled into fractions C2q4, C2q5 and C2q6 as indicated in the Figure.

conditions amelogenin aggregation can be prevented, and then their moderate to small size and limited molecular-weight heterogeneity becomes apparent.

The results from ultracentrifugation (Supplementary Publication SUP 50014) permitted an accurate extrapolation to zero protein concentration of the plot of apparent molecular weight versus protein concentration for components j, c'4, c'5 and c'6, thereby yielding accurate molecular weights for the major J-group and C-group components. Amelogenin monomer molecular weights determined by sodium dodecyl sulphate-polyacrylamide-gel electrophoresis were higher than the molecular weights determined by ultracentrifugation. It is unlikely that this behaviour of the amelogenins is due to incomplete reduction of disulphide bridges, since no cysteine was detected in these proteins after acid hydrolysis. Identical sample preparation produced the expected linear plot (Shapiro *et al.*, 1967) for the standard proteins. The amelogenins seem to behave anomalously during sodium dodecyl sulphate-polyacrylamide-gel electrophoresis, somewhat like ribonuclease (Shapiro *et al.*, 1967; Dunker & Rueckert, 1969).

The order of electrophoretic migration of the J-group components was reversed by a change from acidic to alkaline pH; their elution from the cation-exchanger is in the order of increasing electrophoretic mobility at acid pH. Relative rates of component migration within the J group appear to be related primarily to net charge. Their fast migration rates, which are faster than those of the C-group components in both electrophoretic systems, are apparently due to their low molecular weights.

Amino acid analysis indicates a close relationship between components i, j and k. These components are distinguished by high contents of aromatic residues (approximately 13-17% of total amino acid residues); their very low alanine contents (0.1-0.4%) indicate the presence of only trace amounts of contaminants. Absence of cysteine and alanine and a high content of glycine, glutamic acid and proline, give these amelogenins an unusual composition. The higher content of alanine and leucine, lower content of serine and glycine, as well as its higher molecular weight, distinguish component h from the other J-group components.

All of the J-group amelogenins have molecular

weights in the 3000–5500 range. Since the extreme values of this range represent molecular-weight estimates for aggregating components, future studies may find an even narrower molecular-weight range for the J-group monomers.

Seyer & Glimcher (1971) reported isolation of electrophoretic components from the neutral soluble protein mixture, which they termed E1, E2, E3 and E4. Components E3 and E4 correspond to components h and j, respectively, an identification which can be made with some confidence since the same analytical electrophoresis system was used in both studies. None of the components isolated in the present study corresponded to components E1 and E2. The amino acid compositions reported for components E3 and E4 agree with those of components h and j respectively, although their analysis of component E4 (j) indicates the presence of more contaminating alanine. Results of their gel-filtration studies of components E3 (h) and E4 (j) established a molecular-weight range in agreement with results from ultracentrifugation in the present study.

Electrophoretic analysis of the C1 gel-filtration fraction at acid pH indicates a purification of electrophoretic components a and b. However, this preparation shows all the C-group components at alkaline pH. Amino acid analysis of the C1 and C2 fractions indicates a similarity in their composition. Since the C1 fraction elutes from Sephadex G-75 before the C2 fraction, components a and b seem to represent semi-stable aggregates of the major C-group components at acid pH, which are only disaggregated in the urea-containing alkaline electrophoretic system.

Glimcher *et al.* (1964) and Mechanic *et al.* (1967)

reported the isolation of a minor amelogenin group which they termed the acid-soluble enamel proteins. This group was characterized by a high content of proline, glutamic acid and leucine, but with the proline content being lower than that found in any other amelogenin preparation. Fractions C3 and C4 have this same sort of amino acid composition; fraction C3 has the lowest ratio of proline to glutamic acid content of any of the preparations of this amelogenin group.

The C-group components c'4, c'5 and c'6 have identical electrophoretic mobility at acid pH. These components also have almost identical molecular weights (Table 4) and amino acid compositions (Table 3). Elution from the anion-exchanger is in the order of increasing electrophoretic mobility at alkaline pH; fractions of low electrophoretic mobility being eluted first. The differing electrophoretic mobilities of these components at alkaline pH appear to be due primarily to charge differences. These components are also distinguished as a group by their high contents of proline, glutamic acid, leucine and histidine. It is not surprising that Nikiforuk & Gruca (1969) obtained reactions of immunological identity for the C-group amelogenins. However, their conclusion that the C-group electrophoretic components demonstrated in the alkaline electrophoretic system are primarily higher-molecular-weight aggregates is not supported by the results of chromatography, electrophoresis or ultracentrifugation of the amelogenins in the present study.

The slow electrophoretic migration of the C-group components at alkaline pH is not explained by their amino acid compositions determined after acid

Table 5. *Summary of amelogenin characterization*

Sources of results are identified by Table, Scheme or Plate. The results from the present study suggest a division of the amelogenin monomers into three groups on the basis of size. These groups are here identified by Roman numerals.

Amelogenin monomer size group	Molecular weight (Table 4)	Fraction or electrophoretic components (Scheme 1)	Electrophoretic component group (Plate 1)	Amino acid compositions
I	16800–16100	c'4, c'5, c'6	C group	Similar within this group; unlike those of other groups (Table 3)
II	Between 16100 and 5500 (gel-filtration)	C3, J1	Uncertain	Dissimilar within this group; also unlike those of other groups (Table 2)
III	5500 (strongly aggregating)	h	J group	Composition unlike other group III members or those of members of other groups (Table 3)
III	4500–4300	i, j	J group	Similar within this group; unlike those of other groups (Table 3)
III	3000 (strongly aggregating)	k (k1, k2)	J group	Similar within this group; unlike those of other groups (Table 3)

Table 6. *Amino acid compositions of purified amelogenin electrophoretic components*

Values are expressed as mol of amino acid/mol of protein. N.D., Not determined.

Electrophoretic component ... Amino acid	c'4	c'5	c'6	h	i	j	k (k1, k2)
Lys	1	1	1	1	1	1	0-1
His	10	11	11	3	3	3	2
Arg	1	1	1	1	1	1	0
Asp	4	4	4	2	1	1	1
Thr	3	3	3	1	1	1	1
Ser	4	4	4	2	3	3	2
Glu	26	29	27	4	3	3	2-3
Pro	48	46	44	14-15	9-10	9	7
Gly	5	5	4	2-3	5	5	3
Ala	2	3	3	1	0	0	0
Cys	0	0	0	0	0	0	0
Val	4	4	4	1	1	1	1
Met	9	8	7	2	1	2	1
Ile	5	5	5	2	1	2	1
Leu	15	14	14	5	3	3	2
Tyr	5	5	5	3	4	5	3
Phe	3	3	2	1	1	1	1
Trp	1	2	2	1	0	1	N.D.
Total	146	148	141	46-48	38-39	42	27-29

hydrolysis since each appears to contain twice the number of acidic versus basic residues. Ion-exchange chromatography in the present study indicates that the major C-group components have an alkaline isoelectric point. This evidence is supported by isoelectric-focusing experiments (F. M. Eggert & R. C. Burgess, unpublished work), which also indicate an alkaline isoelectric point for the C-group components. Burgess & Maclaren (1965) found a high amide N content in the C-group preparation. These findings suggest the presence of asparagine and glutamine residues in the C-group amelogenins with a corresponding decrease of the total number of free carboxyl groups and an elevation of the isoelectric point. These amide groups would not be apparent after acid hydrolysis for routine amino acid analysis as was carried out in the present investigation.

Amino acid compositions of whole enamel matrix (Levine *et al.*, 1967) and of the bovine amelogenins isolated in the present study explain histochemical results obtained by stains specific for tyrosine and tryptophan, but do not explain intense staining of the matrix with stains for disulphide groups or free thiol groups (Suga & Gustafson, 1963).

The pH- and concentration-dependent amelogenin aggregation, and the considerable intragroup similarities explain difficulties in separating the amelogenins by any single method. It is now apparent that

the major groups of amelogenins present a simpler system than was previously supposed, merely on the basis of electrophoretic analysis of the 'Mixed' (neutral-soluble protein) preparation (Burgess & Maclaren, 1965; Katz *et al.*, 1969) or gel filtration of this preparation at neutral or alkaline pH values (Mechanic *et al.*, 1967; Mechanic, 1971; Nikiforuk & Gruca, 1969). The results of the present investigation allow a description of the amelogenin monomers in specific terms that are summarized in Table 5.

When the results of amino acid analysis are expressed in terms of mol of amino acid/mol of protein (bearing in mind the uncertainty of the molecular-weight value for component k) in Table 6, it is evident that it could be possible for the components of the J group to be derived from the components of the C group, or as a second possibility, both the C- and the J-group amelogenins might be derived from a larger precursor protein.

In considering the relationship of amelogenins to mammalian collagens and keratins, it is apparent that amino acid composition, and/or size (Eastoe, 1963; Lewis & Piez, 1964; Fincham *et al.*, 1965; Jeffrey, 1970) distinguish the amelogenins, as isolated in this study, from members of the above two groups of proteins. It seems reasonable to consider the amelogenins as unique secretory products of specialized epithelial cells.

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