Biochem. J. (1977) **167**, 23–29
Printed in Great Britain

Enzymically Iodinated Human Salivary Proteins

FRACTIONATION AND CHARACTERIZATION BY COLUMN CHROMATOGRAPHY AND ELECTROFOCUSING

By SEPPO S. SARIMO* and JORMA TENOVUO†
**Department of Biochemistry and †Institute of Dentistry, University of Turku, SF-20500 Turku 50, Finland*

(*Received* 17 *January* 1977)

Human salivary proteins were enzymically iodinated by the ^{125}I–lactoperoxidase system. The proteins were than subjected to DEAE-cellulose column chromatography, preparative column electrofocusing and thin-layer polyacrylamide-gel electrofocusing. The radioactivity in the resolved protein pools and bands was determined. Results show that salivary proteins differ in their susceptibility to iodination carried out by this enzymic method. Two major iodine-binding protein fractions were discovered: one behaved like serum albumin on electrofocusing and was most susceptible to iodination by lactoperoxidase, and the other had pI characteristics similar to those of salivary amylase. The physiological significance of the iodination of salivary proteins, which can also take place *in vivo*, is discussed.

It has been known for years that various peroxidases in the presence of H_2O_2 or peroxide-generating systems are capable of iodinating tyrosine and different proteins (see references in Marchalonis, 1969). It is also well documented that human whole-mouth saliva contains all the necessary components for the enzymic iodination of its proteins *in vivo*, although the oral conditions may not always be the most favourable for this process to take place (Tenovuo, 1976). In our previous studies we showed that when endogenous salivary peroxidase or commercial milk lactoperoxidase were used *in vitro*, only a few salivary-protein fractions contained a considerable amount of covalently bound ^{125}I, as measured by one-dimensional polyacrylamide-gel electrophoresis (Tenovuo & Sarimo, 1977).

The present paper describes the analysis of the enzymically [^{125}I]iodinated human salivary proteins by DEAE-cellulose column chromatography, column electrofocusing and thin-layer polyacrylamide-gel (flat-bed) electrofocusing. The last method allows an excellent resolution of proteins on the basis of the difference in their isoelectric points. Protein staining combined with the determination of bound radioactivity provides us with the detailed information of the species of human salivary proteins susceptible to enzymic iodination.

Materials and Methods

Chemicals

Lactoperoxidase (EC 1.11.1.7) was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Carrier-free $Na^{125}I$ in 0.1 M-NaOH was obtained from NEN Chemicals, Dreieichenhain, W. Germany, at a concentration of 10 mCi/ml. Aquacide III, which was used for the concentration of protein solutions, was purchased from Calbiochem, San Diego, CA, U.S.A. Bovine serum albumin (Sigma) was used as a reference protein in flat-bed electrofocusing and in enzymic protein iodination.

Collection and treatment of saliva samples

Paraffin-stimulated whole-mouth saliva (200–250 ml per experiment) was collected from several persons and centrifuged immediately in a Sorvall Superspeed RC-2 centrifuge for 15 min at 4°C and 23500*g*. The supernatant was at first concentrated 8–10-fold by using Amicon ultrafiltration apparatus TCF 10 (membrane UM20E) and was subsequently dialysed against 0.01 M-$\beta\beta$-dimethylglutarate buffer, pH 7.2, overnight. The final saliva sample, containing 3–6 mg of protein/ml, was subjected as such (procedure A) or after enzymic iodination (procedure B) to chromatographic and electrophoretic fractionations.

Iodination of salivary proteins

The reaction mixture for enzymic iodination of unfractionated salivary proteins contained, per ml of concentrated and dialysed saliva: 50 μCi of carrier-free $Na^{125}I$, 60 μmol of H_2O_2 and 50 μg of milk lactoperoxidase. Incubation was for 30 min at 30°C, and the reaction was terminated by the addition of 2-mercaptoethanol to a final concentration of 20 mM. Unchanged [^{125}I]iodide was removed by

extended dialysis against 0.01 M-sodium phosphate buffer, pH 7.0, at 4°C.

When protein pools from preparative column electrofocusing were subjected to iodination, the reaction mixture contained (per ml): 0.4–4 mg of salivary proteins, 40 μg of milk lactoperoxidase, 40 μCi of $Na^{125}I$, 400 nmol of KI and 400 nmol of H_2O_2. The reaction was for 30 min at 30°C and was stopped by adding 2-mercaptoethanol to a final concentration of 7.5 mM. Before analysis by flat-bed electrofocusing all the reaction mixtures were dialysed to remove unbound [^{125}I]iodide as described above. In the kinetic experiments, portions of reaction mixtures were spotted directly on Millipore filters and treated as described below under 'Measurement of radioactivity.'

DEAE-cellulose chromatography

Both uniodinated and [^{125}I]iodinated saliva samples were first fractionated on a DEAE-cellulose (230–270 mesh; Schleicher und Schull, Dassel, Germany) column (1.7 cm × 32 cm) equilibrated with 0.01 M-$\beta\beta$-dimethylglutarate buffer, pH 7.2. During elution a linear NaCl gradient from 0.0 to 0.5 M was used. The protein content and the radioactivity of column eluates were determined.

Column electrofocusing

Preparative isoelectric focusing of salivary-protein pools I and II (see Fig. 1) after DEAE-cellulose chromatography was performed on an LKB column (volume 100 ml, inner dimensions 33 cm × 2.5 cm) at 2°C. Sucrose-density-gradient and electrolyte solutions were prepared according to the manufacturer's directions. The column was loaded with 2.5 ml of carrier ampholyte, pH 3.5–10 (Ampholine; LKB-Produkter, Bromma, Sweden), and with 50 ml of DEAE-cellulose desalted previously on a Sephadex G-25 column (5.7 cm × 29 cm). The focusing was allowed to continue for approx. 30 h at the maximum voltage (300 V). The column was emptied through a LKB Uvikord u.v.-analyser, which monitored proteins at 280 nm. Each 1 ml fraction was analysed for pH and radioactivity.

Combined fractions which represented the protein pools Ia, Ib and Ic and IIa, IIb, IIc and IId from preparative electrofocusing (see Fig. 2) were dialysed against 0.01 M-phosphate buffer, pH 7.0, to remove Ampholine, and were subsequently concentrated approx. 5-fold by using the polysaccharide concentration material Aquacide III.

Flat-bed electrofocusing

The LKB 2117-101 Ampholine electrofocusing kit for polyacrylamide gel and the LKB 2117-301 Multiphor basic unit were used in analytical thin-layer polyacrylamide-gel electrofocusing. The LKB Ampholine PAGplates (pH 3.5–10) contained 2.4% (w/v) Ampholine carrier ampholytes and had a gel concentration of 5% acrylamide and a degree of cross-linkage of 3%. Protein samples (15–30 μl, corresponding to 5–20 μg of protein) were applied and electrophoresis was performed at 4–6°C according to the manufacturer's instructions.

After the formation of the pH gradient and when the electrophoretic focusing of proteins was complete in about 90 min, the PAGplates were placed in fixing solution (17.25 g of sulphosalicylic acid and 57.5 g of trichloroacetic acid in 150 ml of methanol and 350 ml of water) for 0.5–1 h. Staining was for 10 min at 60°C in staining solution (0.115 g of Coomassie Brilliant Blue R250 in 100 ml of destaining solution) and destaining was in 500 ml of ethanol and 160 ml of acetic acid diluted to 2 litres with water (destaining solution). Plates were photographed for documentation and then subjected to radioautography or cut into thin slices (9 mm × 2 mm) for the localization of protein-bound ^{125}I radioactivity.

Measurement of radioactivity

The ^{125}I incorporated into proteins was measured by the method described by Marchalonis (1969). In the kinetic studies or when the final protein-bound radioactivity was determined, portions (5–15 μl) of reaction mixture were quickly applied to cellulose acetate membranes (HAWP 02 500, HA 0.45 μm, Millipore Corp., Bedford, MA, U.S.A.). Immediate fixing of proteins and removal of unbound [^{125}I]-iodide were accomplished with the fixative solution (methanol/acetic acid/water; 5:1:5, by vol.) on a Millipore filtering apparatus. Washed discs were placed in capped plastic vials and counted for radioactivity in a LKB–Wallac 1230 Ultrogamma counter.

For the measurement of radioactivity in the different fractions from DEAE-cellulose column chromatography and column electrofocusing, samples (25 μl) were spotted on Whatman no. 2 filter-paper discs which were counted for radioactivity as described above.

Association of ^{125}I label with specific proteins was demonstrated on electrofocused PAGplates either by direct counting of the radioactivity in individual gel slices in the gamma counter as described above or by radioautography against Kodalith Ortho Film, type 3.

Protein assay

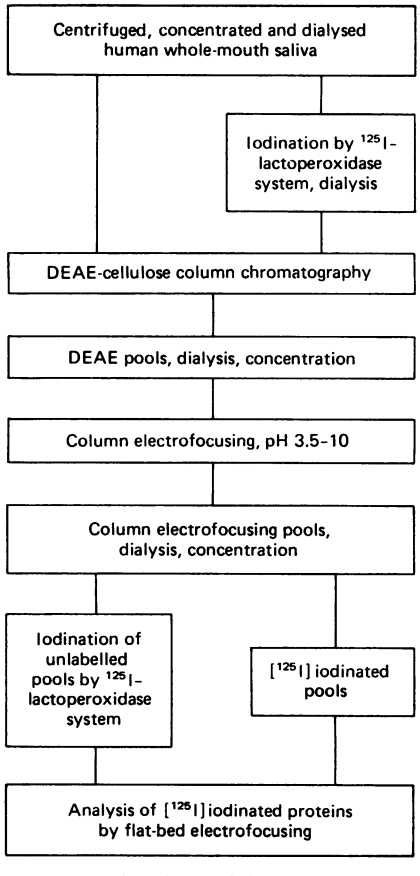
Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Special attention was given to the removal (by dialysis) of Ampholine from the pools of column electrofocusing before chemical protein assay.

Results

General outlines of experiments

Two approaches were used in the analysis of the possible differences in the susceptibility of human salivary proteins to enzymic iodination by the ^{125}I–lactoperoxidase system (Scheme 1). First, proteins in the dialysis residue of concentrated saliva were labelled before fractionation by DEAE-cellulose column chromatography and column electrofocusing; secondly, the unlabelled protein pools from preparative fractionations were labelled individually to determine their iodination susceptibility. In both cases the proteins of the different pools were finally studied by flat-bed electrofocusing.

Protein and radioactivity contents of all preparations were determined throughout the fractionations, and results are presented as percentages of the total. Ratios of radioactivity content to protein content were used when relative susceptibilities of different protein species to enzymic iodination *in vitro* were compared.



Scheme 1. *General outlines of the experiments to test the susceptibility of human salivary proteins to enzymic iodination in a lactoperoxidase-catalysed reaction in vitro*

Enzymic incorporation of [^{125}I]iodide into human salivary proteins

Confirming our previous observations (Tenovuo & Sarimo, 1977) and in agreement with the results of Marchalonis (1969), the enzymic iodination of salivary proteins occurred only when both H_2O_2 and peroxidase were present in reaction mixtures. The incorporation of [^{125}I]iodide into enzyme protein alone was negligible under the conditions used. The amounts of [^{125}I]iodide bound to the proteins of two different salivary-protein samples is shown in Table 1. These preparations are two major salivary-protein pools separated by DEAE-cellulose column chromatography and preparative electrofocusing. Serum albumin was used as a reference protein. It is also shown in Table 1 that, with the amount of lactoperoxidase used, the reaction was completed within 10min at 30°C.

Preparative fractionation of salivary proteins

Both labelled and unlabelled salivary-protein preparations were subjected to two preparative fractionations before a more detailed analysis by analytical electrofocusing. Since practically identical protein-fractionation patterns were obtained, only the results obtained with [^{125}I]iodinated proteins are presented here.

Fig. 1 illustrates protein and ^{125}I content of the material eluted from a DEAE-cellulose column. Proteins were separated into two major peaks. The distribution of radioactivity, featuring three distinctive peaks, followed mainly that of proteins. Collected fractions (2ml each) were combined on the basis of protein distribution. Pool I from the DEAE-cellulose column contained the two radioactivity peaks eluted first and pool II the third one. The percentages of protein and radioactivity in pools I and II are presented in Table 2.

Preparative column electrofocusing of desalted pools I and II was subsequently performed. The results show that pool I (Fig. 2*a*) contained one major and two minor protein peaks with pI values 3.2, 6.1 and 7.5 respectively. The ^{125}I-labelling pattern of the eluted material followed roughly the distribution of protein. In pool II (Fig. 2*b*) nearly all the proteins containing radioactivity were located in the pH region of 3.5–6.5.

The fractions (2 ml each) of the major protein and/or radioactivity peaks were combined and pools Ia, Ib, Ic and IIa, IIb, IIc, IId were formed. They were further dialysed to remove Ampholine and then analysed for protein and radioactivity (Table 2).

Table 1. *Effect of protein concentration on the incorporation of [^{125}I]iodide into salivary proteins and serum albumin at 30°C*
Protein samples were two major protein pools from column electrofocusing. The amount of lactoperoxidase in each reaction tube was 2.5 μg. Of a total volume of 40 μl, 10 μl was removed for counting of protein-bound radioactivity. Approx. 15–20s lag between the initiation of reaction and the removal of the first sample (zero time) should be considered. Background radioactivity was 55c.p.m.

	Reaction time (min)	Salivary protein (μg/reaction mixture) ...	$10^{-3} \times {}^{125}I$ incorporation (c.p.m.) None	12.5	1.25	0.125
Salivary-protein pool Ib	0		—	13.3	4.2	2.4
	5		—	106.1	18.0	4.9
	10		—	99.5	15.3	6.2
Salivary-protein pool IIb	0		—	25.6	10.0	1.8
	5		—	117.6	20.0	4.4
	10		—	109.2	20.0	5.6
Bovine serum albumin	0		—	25.6	5.8	1.3
	5		—	169.7	32.5	5.4
	10		—	184.1	34.2	4.3
Lactoperoxidase (alone)	0		0.24	—	—	—
	10		0.35	—	—	—
	25		0.44	—	—	—

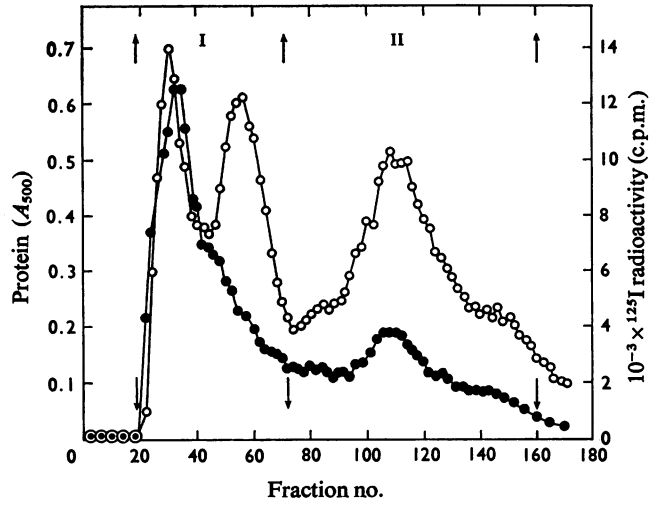


Fig. 1. *Fractionation of [^{125}I]iodinated human salivary proteins by DEAE-cellulose column chromatography*
●, Protein content of eluted material; ○, ^{125}I radioactivity in the eluate. The arrows between the symbols I and II indicate the regions from which fractions were combined to form salivary-protein pools I and II.

Flat-bed electrofocusing

Flat-bed electrofocusing on PAGplates over a pH gradient was used for the final analysis of [^{125}I]-iodinated-protein pools from preparative fractionation. Fig. 3 illustrates the protein and radioactivity profiles of three major iodine-carrying protein pools of human saliva [^{125}I]iodinated before preparative fractionation. Proteins were detected by Coomassie Brilliant Blue staining, and protein-bound ^{125}I was located by measuring the radioactivity of thin gel slices. The pH of specific areas of the gel was obtained from a pH-gradient curve obtained by using a surface pH-electrode.

The protein patterns indicate that these salivary-protein pools are still far from homogeneous. The

Table 2. *Comparison of the protein and radioactivity contents of various salivary-protein pools iodinated by the lactoperoxidase method*

Salivary proteins were fractionated by DEAE-cellulose column chromatography and column electrofocusing. Values for protein and radioactivity contents are percentages of the total radioactivity or protein eluted in that pool. The first values are protein and radioactivity determinations from final protein pools (combined fractions). Values in parentheses were obtained by adding up the values of protein and radioactivity determinations of individual fractions eluted from the electrofocusing column.

	Percentage content		
Preparation	Protein	Radioactivity	Radioactivity/protein
Whole-mouth saliva	100	100	1
DEAE pools:			
I	63	46	0.73
II	37	54	1.46
Column electrofocusing pools:			
Ia	20 (19)	4 (16)	0.20 (0.84
Ib	31 (26)	38 (26)	1.23 (1.00)
Ic	17 (16)	16 (12)	0.94 (0.75)
IIa	7 (10)	10 (12)	1.42 (1.20)
IIb	9 (12)	21 (23)	2.33 (1.92)
IIc	10 (8)	9 (8)	0.90 (1.00)
IId	7 (10)	2 (3)	0.28 (0.30)

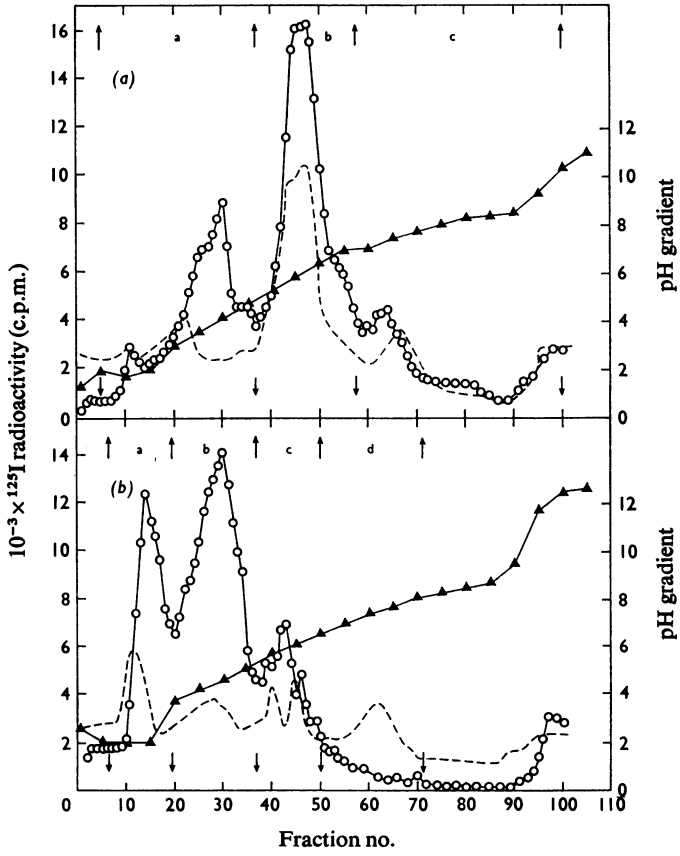


Fig. 2. *^{125}I-labelling patterns of salivary-protein pools in pH gradients of preparative electrofocusing columns*
(*a*) Pool I and (*b*) pool II from DEAE-cellulose column: ----, relative protein content monitored at 280nm; ○, ^{125}I radioactivity across the fractions; ▲, pH (Ampholines to form a pH gradient from 3.5 to 10 were used). Arrows with letters a to d between indicate the regions where fractions were combined to prepare the samples for further analysis.

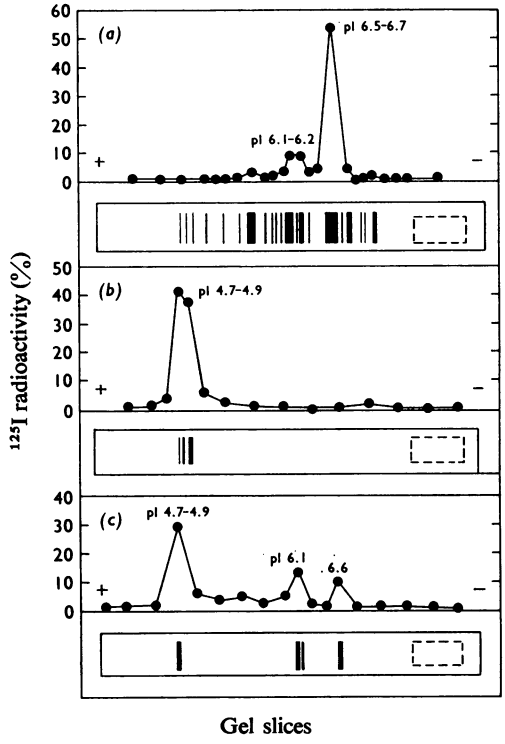


Fig. 3. *Protein and ^{125}I-labelling patterns of iodinated salivary proteins in thin-layer polyacrylamide-gel electrofocusing in a pH3.5–10 gradient*

Three samples were [^{125}I]iodinated salivary-protein pools from previous fractionation on DEAE-cellulose column chromatography and column electrofocusing: (*a*) pool Ib, (*b*) pool IIb and (*c*) pool IIc. The sample application point is indicated by broken lines. Abscissa scale: gel slices (gels were sliced between the symbols, ●). The ^{125}I radioactivity is expressed as percentages of total radioactivity measured at the appropriate gel section.

radioactivity, however, is concentrated mainly in the three pH areas corresponding to proteins with pI values 4.7–4.9, 6.1–6.2 and 6.5–6.7. One zone, with the lowest pI and with the most effective iodine-binding capacity, was focused at the same pH as the reference protein serum albumin, which gave a similar multiband focusing profile. Iodinated and uniodinated serum albumin had identical pI values. The proteins with pI between 6.1 and 6.7 most probably represent the salivary amylases, which are known to be focused at this pH (Pronk, 1976).

Discussion

All peroxidases do not possess an equal capacity to catalyse the iodination of proteins (Marchalonis, 1969). Other properties, such as their ability to inhibit the growth of certain streptococci, also differ (Jago & Morrison, 1962). Milk lactoperoxidase and endogenous salivary lactoperoxidase have, however, very similar properties with respect to their ability to iodinate tyrosine and proteins and to inhibit the growth of cariogenic *Streptococcus mutans* (Tenovuo, 1976). The fractionation patterns of [^{125}I]iodinated salivary proteins produced by these two peroxidases showed no significant differences when analysed by polyacrylamide-gel electrophoresis (Tenovuo & Sarimo, 1977). Therefore it should be acceptable that our iodination reaction mixtures *in vitro* were enriched with commercial milk peroxidase to increase the endogenous iodination capacity of the system.

It is also known that proteins vary in their susceptibility to enzymic iodination, which probably reflects the accessibility of tyrosine groups in the molecule (Marchalonis, 1969). Thus it was not surprising to discover a considerable variation in the ability of different salivary-protein species to bind [^{125}I]iodide in a lactoperoxidase-catalysed reaction. Of course, the discovered differences may also reveal the proteins that might function as effective iodine carriers.

The results of both preparative column electrofocusing and analytical flat-bed electrofocusing indicate that most of the ^{125}I radioactivity covalently bound to salivary proteins is focused at two separate pH regions, namely between pH4–5 and 6–7. Since iodination often does not change the electrophoretic properties of proteins [Marchalonis (1969) and the present results with serum albumin], the observed isoelectric points can be considered to characterize native proteins and can be used to identify individual protein bands.

The multiple protein bands that focused at pH4.7–4.9 most probably represent the serum albumin. This was confirmed by using commercial bovine serum albumin and human serum, as reference, which both gave a similar multiband focusing pattern at pH4.7–4.9. It was also discovered that bovine serum albumin and our pI 4.7–4.9 salivary-protein fraction (pool IIb) had an equal capacity to bind radioactive iodide to their tyrosine residues.

The proteins that focused at pH6–7 are obviously isoenzymic species of salivary amylase. Most of the proteins of human parotid saliva with pI 6–7 seem to possess saccharolytic activity, as shown by Pronk (1976). That they can bind a considerable amount of iodine is in agreement with our previous results obtained by polyacrylamide-gel electrophoresis (Tenovuo & Sarimo, 1977).

Could the iodination of certain human salivary proteins have any physiological significance? Our preliminary experiments have shown that the incorporation of [^{125}I]iodide into salivary proteins can be achieved in newly collected saliva by endogenous peroxidase and H_2O_2 (S. S. Sarimo & J. Tenovuo, unpublished work). However, the organic binding of

iodide to proteins in the thyroid gland is a more common mechanism. In blood, iodine in the form of the hormone thyroxine (protein-bound iodine) is bound to a specific plasma globulin, thyroxine-binding globulin, or to another protein called pre-albumin (White *et al.*, 1964). In human saliva only inorganic iodine has been reported to be present (Logethetopoulos & Myant, 1956; Weiss *et al.*, 1962). Our earlier observations indicate, however, that human salivary peroxidase is capable of iodinating salivary proteins as effectively as is commercial milk lactoperoxidase. The salivary glands are known to secrete iodine in the saliva at a 20–100 fold higher concentration than that found in the plasma (Schiff *et al.*, 1947). The present results suggest the possible occurrence of iodoproteins in human saliva. The significance of these iodoproteins may be related to the non-thyroidal metabolism of iodine. Iodination *in vitro* of tyrosine-containing peptides and proteins, e.g. serum albumin, produces compounds which, when administered, exert a thyroxine-like effect on the metabolic rate. Indeed, thyroxine has been isolated from hydrolysates of these iodinated proteins (White *et al.*, 1964).

We are indebted to Professor Pekka Halonen, head of the Department of Virology, University of Turku, and to Mr. Olli Suominen, Wallac Oy, Turku, for permission to use their gamma counter and Multiphor basic unit respectively. The skilled technical assistance of Mrs. Aila Lähteenmäki and Mrs. Leena Tuomarila is gratefully acknowledged. Financial support for this work was provided by the Finnish Dental Society.

References

Jago, G. R. & Morrison, M. (1962) *Proc. Soc. Exp. Biol. Med.* **111**, 585–588

Logethetopoulos, J. H. & Myant, N. B. (1956) *J. Physiol. (London)* **134**, 189–194

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275

Marchalonis, J. J. (1969) *Biochem. J.* **113**, 299–305

Pronk, J. C. (1976) *Abstr. Int. Symp. Electrofocusing Isotachophoresis, Hamburg*

Schiff, L., Stevens, C. D., Molle, W. E., Steinberg, H., Kumpe, C. W. & Stewart, P. (1947) *J. Natl. Cancer Inst.* **7**, 349–354

Tenovuo, J. (1976) *Acta Odont. Scand.* **34**, *Suppl.* **71**

Tenovuo, J. & Sarimo, S. S. (1977) *Scand. J. Dent. Res.* in the press

Weiss, M., Weiss, G., Terroux, K. G. & Burgen, A. S. (1962) *Nature (London)* **194**, 186–187

White, A., Handler, P. & Smith, E.-L. (1964) *Principles of Biochemistry*, p. 832, New York