The distribution of proteolytic and alkaline phosphatase activities in human saliva treated with sodium deoxycholate

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Sodium deoxycholate was used to release peptidase and alkaline phosphatase activities from saliva sediment. The degree of the release was determined at a variety of detergent concentrations and incubation periods. By use of 0.4% of this detergent 80% of the alkaline phosphatase found in the sediment was released. A twofold increase in peptidase in the soluble fraction (10,000 g supernatant) and a 10% increase in protein resulted in the same concentration of sodium deoxycholate. The distribution of the enzymes in saliva supernatant and the enzymes released from sediment were determined on the substrates gelatin, poly-L-lysine HBr, haemoglobin, α-N-benzoyl-L-arginine ethyl ester-HCl, p-tosyl-L-arginine methyl ester-HCl, L-lysine-*p*-nitroanilide HBr and *p*-nitrophenyl phosphate. The study indicates that the peptidase(s) soluble in human saliva without detergent are derived from saliva sediment.

Key-words: Human saliva; proteolytic enzymes; sodium deoxycholate

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The presence of alkaline phosphatase activity (*Chauncey et al.*, 1954) and an enzyme or a mixture of enzymes with proteolytic activity in human saliva has been reported by several authors (*Chauncey et al.*, 1954; *Chauncey*, 1961; *Chauncey & Shannon*, 1966; *Mäkinen*, 1966; *Söder*, 1972).

If whole saliva is centrifuged at 20,000 g around 40% of the proteolytic activity is found in the supernatant and 60% remains in the sediment (*Chauncey*, 1961; *Söder*, 1972). It is believed that the activity found in whole saliva originates from epithelial cells, leucocytes or microorganisms normally present in the oral cavity.

The release of proteolytic and alkaline phosphatase activities from saliva sediment has not been studied so far.

To release and isolate different biological activities from cells or parts of cells detergents are often used. Triton X-100 has been used to extract proteolytic activity from erythrocyte plasma membranes (*Bernacki & Bosman*, 1972). Sodium deoxycholate (DOC) has been used for disruption of influenza B virus particles (LEE strain) (*Laver*, 1963). This detergent

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has also been used for isolation of ribosomes from cell and tissue homogenates (*Golub & Clegg*, 1969) and for extraction of proteins from pig lymphocyte plasma membranes (*Allan & Crumpton*, 1971; *Allan et al.*, 1972).

The aim of the present investigation was to study the distribution of the proteolytic activity and alkaline phosphatase activities in human whole saliva by solubilizing and extracting these enzymes with DOC.

MATERIAL AND METHODS

Saliva. Paraffin stimulated mixed whole saliva, 25 ml, was collected at three different times from each of 4 men and 3 women after their mouths were rinsed with distilled water, in the morning about three hours after individual oral hygiene. The saliva samples were collected in cups placed in an ice bath and treated immediately in three different experiments.

Experiment I

The effect of sodium deoxycholate on the solubilization of proteolytic and alkaline phosphatase activity in human saliva. To samples of pooled saliva from seven subjects, solid DOC was added in concentrations up to 0.60% (w/v). The mixtures were incubated at 25°C for 1 hour. At the end of the incubation period, aliquots were withdrawn and the rest of the samples centrifuged at 10,000 g for 15 min at 4°C. As a control one part of saliva was first centrifuged at 10,000 g and then the supernatant was treated in the same manner.

Experiment II

The influence of incubation time with DOC on the release of proteolytic and alkaline phosphatase activity in saliva. To pooled saliva 0.40% DOC (final concentration) was added and incubated at 25°C. Aliquots were withdrawn after 15 min, 1.0, 2.0, 4,0, and 8.0 h., and centrifuged at 10,000 g for 15 min at 4°C. The sediments were resuspended in distilled water. As a control saliva was incubated without DOC.

Experiment III

The distribution of the proteolytic and alkaline phosphatase activities in three subfractions of saliva. From each of seven saliva samples an aliquot was withdrawn (E) and the rest of the samples were centrifuged at 10,000 g for 15 min at 4°C. The sediments were washed twice with 0.05 M tris-HCl buffer, pH 8.1, recentrifuged and the supernatant fluids and the washes were pooled (supernatant-e_1). The sediments were resuspended and incubated for 3 hours at 25°C with 0.40% DOC in 0.05 M tris-HCl buffer, pH 8.1, centrifuged, washed twice and centrifuged again. The pool of these supernatant fluids and the washes were called supernatant-e_2. The final sediments were resuspended in 0.05 M tris-HCl buffer, pH 8.1 (e_3). The volume of the fractions (e_1 and e_2) were twice the volume of saliva (E).

Fractions e_1 and e_2 were dialyzed against 0.05 M tris-HCl buffer, pH 8.1. Fraction E and e_3 were tested on gelatin and *p*-nitrophenyl phosphate. Fraction e_1 and e_2 were tested on gelatin, haemoglobin, poly-L-lysine HBr, L-lysine-*p*-nitroanilide HBr, p-tosyl-L-arginine methyl ester-HCl, *α*-N-benzoyl-L-arginine ethyl ester-HCl and *p*-nitrophenyl phosphate.

Enzyme assays. Proteolytic activity was determined according to the following methods:

1. *Gelatin as substrate* (USP gran Fisher S-C Co., N. J., USA). Viscosimetric determinations were performed at 37°C

on a reaction mixture consisting of 3.0 ml of 4.0% (w/v) gelatin in 0.2 M tris-HCl buffer, pH 8.8, and of 1.0 ml of enzyme solution. The activity was expressed in Hultin units (H.U.). (*Hultin*, 1946, 1948; *Lundblad*, 1949; *Söder*, 1967).

2. *Poly-L-lysine hydrobromide (PLL) as substrate* (Pilot Chemical Inc., Watertown, Miss., USA) (molecular weight 150.000). Viscosimetric determinations were performed at 37°C on a reaction mixture consisting of 3.0 ml of 0.75% (w/v) poly-L-lysine hydrobromide in 0.2-M tris-HCl buffer, pH 8.8, (containing 0.02 M $CaCl_2$) and of 1.0 ml of enzyme solution. The activity was expressed in Hultin units (H.U.). (*Fasman et al.*, 1961; *Söder*, 1967; *Lundblad & Johansson*, 1968).

3. *Haemoglobin as substrate* (Sigma Chemical Company, St. Louis, Mass., USA). The enzyme solution (1.0 ml) was incubated 24—48 hours at 37°C with urea-denatured bovine haemoglobin (1.0 % w/v) in 0.1 M tris-HCl buffer, pH 8.1. The absorbance of released material not precipitated by trichloracetic-acid was measured spectrophotometrically at 280 nm. The enzyme activity was expressed as μmoles of tyrosine released per minute. (*Anson*, 1938; *Ruyssel & Lauwers*, 1963).

4. *α-N-Benzoyl-L-arginine ethylester-HCl (BAEE) as substrate* (Sigma Chemical Company). The absorbance at 255 nm of the reaction mixture, consisting of 3.0 ml of 1 mM BAEE in 0.1 M tris-HCl buffer, pH 8.1, (containing 0.02 M $CaCl_2$) and 0.20 ml enzyme solution, was measured during the linear period of substrate hydrolysis at 25°C. The activity was expressed in μmoles of substrate converted per minute. (*Bergmeyer*, 1965; *Kétzdy et al.*, 1965).

5. *p-Tosyl-L-arginine methylester-HCl (TAME) as substrate* (Nutritional Biochemical Corporation, Cleveland, Ohio, USA). The absorpance was determined at 247 nm in an assay system like the BAEE system. (*Hummel*, 1959).

6. *L-lysine-p-nitroanilide dihydrobromide (LPA) as substrate* (Nutritional Biochemical Corporation). The reaction mixture consisted of 2.5 ml of 1.2 mM LPA in 0.1 M tris-HCl buffer, pH 8.1, (containing 24 mM $CaCl_2$) and of 0.50 ml of enzyme solution. The absorbance was measured at 410 nm at zero time and after 20 hours of incubation at 37°C. The activity was expressed in μmoles of liberated *p*-nitroanilide per minute. (*Erlanger et al.*, 1961).

The alkaline phosphatase activity. Alkaline phosphatase activity was determined according to a modification of the method described by *Linhardt & Walter* (1965). The assay system consisted of 1.0 ml of 5.5 mM *p*-nitrophenyl phosphate (PNPP), of 0.5 mM $MgCl_2$ in 0.05 M glycine-NaOH buffer, pH 10.5, and of 0.2 ml of the test solution. After 2 to 5 hours of incubation at 37°C, 2.0 ml of 0.1 M NaOH was added. In the control the test solution was added after the NaOH addition. The optical density was measured at 410 nm and the amount of released *p*-nitrophenol was calculated (*Linhardt & Walter*, 1965). The phosphatase activity was expressed in μmoles *p*-nitrophenol liberated per minute.

The protein content. The protein content was calculated by the method of *Warburg & Christian* (1942) from the absorbances at 260 and 280 nm. The amount of sediment was determined as the optical density (turbidity) of the resuspended sediment at 525 nm. The sediments were diluted to give an optical density between 0.25 and 0.75.

All spectrophotometric measurements were carried out with a Zeiss spectrophotometer PMQ II.

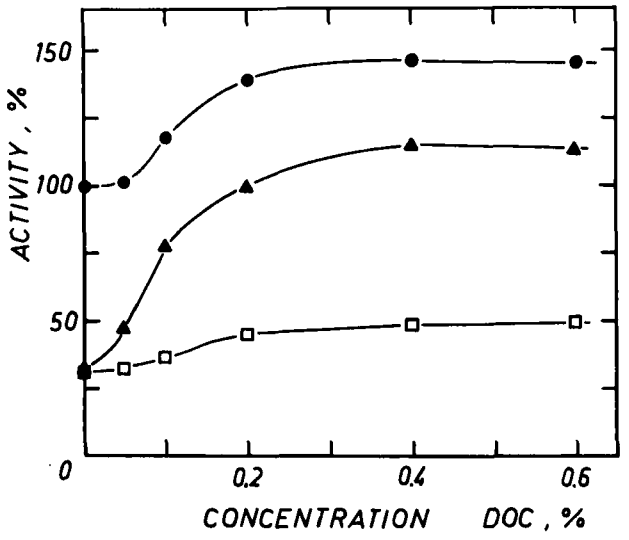

Fig. 1. The effect of increasing concentrations of DOC on the gelatinolytic activity in human saliva. The activity at pH 8.8 was determined in uncentrifuged saliva (●), in supernatant (▲), and in control (□).

RESULTS

Experiment I

With increasing concentrations of DOC up to 0.40% the gelatinolytic activity increased to 145% in the uncentrifuged saliva and in the supernatant from 31% to 114% of the activity in saliva. In the control the activity increased from 31% to 49% (Fig. 1).

With increasing amount of DOC up to 0.40%, the alkaline phosphatase activity in the supernatant increased from 27% to 98% of the activity in uncentrifuged saliva. DOC at the concentrations tested did not increase the activity in the

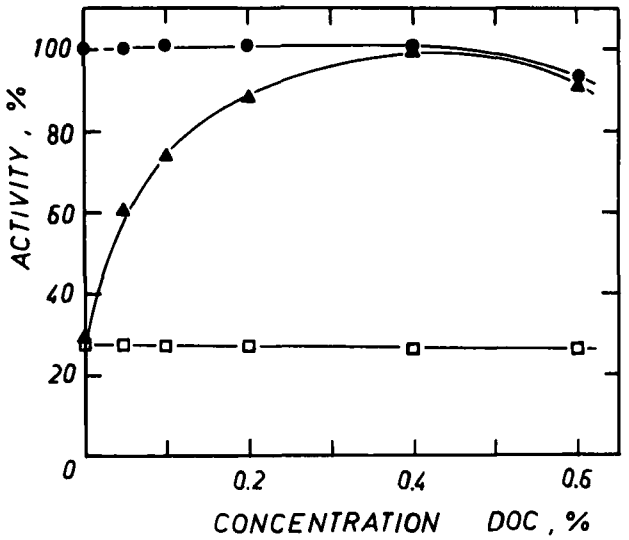

Fig. 2. The effect of increasing concentrations of DOC on the alkaline phosphatase activity at pH 10.5 in human saliva. The activity was determined in uncentrifuged saliva (●), in supernatant (▲), and in control (□).

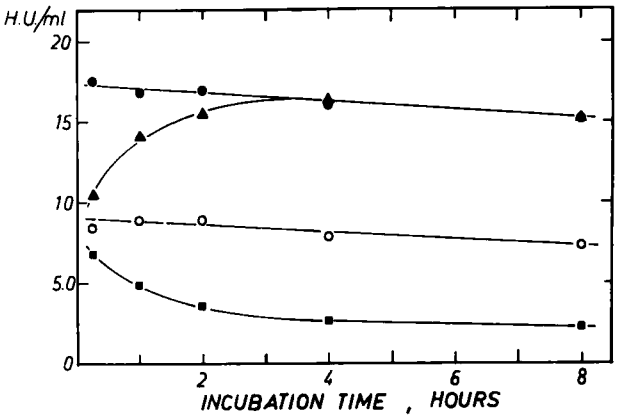

Fig. 3. The effect of incubation time with 0,4% DOC on the gelatinolytic activity in human saliva. The activity at pH 8.8 was determined in uncentrifuged saliva (●), in supernatant (▲), in resuspended sediment (■) and in control (○).

uncentrifuged saliva and the control (Fig 2).

Experiment II

The gelatinolytic activity in saliva increased to 195% after 15 min incubation compared to saliva without DOC. Between 15 min and 4 hours the gelatinolytic activity in the supernatant increased from 10.5 to 16.4 H.U./ml. During the same period of time the activity in the resuspended sediment decreased from 6.8 to 2.6 H.U./ml (Fig. 3).

DOC at a concentration of 0.40% did not increase the alkaline phosphatase activity in the uncentrifuged saliva (Table

Table I. *The effect of incubation time with DOC on the alkaline phosphatase activity at pH 10.5 in human saliva. As a control the activity was determined without DOC treatment*

Incubation time	DOC	Alkaline phosphatase activity		
Hours	%	μmoles/(min × ml × 10^3)		
		Uncentrifuged saliva	Supernatant	Resuspended sediment
0.25	0	41	16	24
	0.4	43	37	9
1.0	0	41	—	—
	0.4	42	42	9
2.0	0	41	—	—
	0.4	42	43	8
4.0	0	41	—	—
	0.4	41	43	8
8.0	0	42	—	—
	0.4	42	44	9

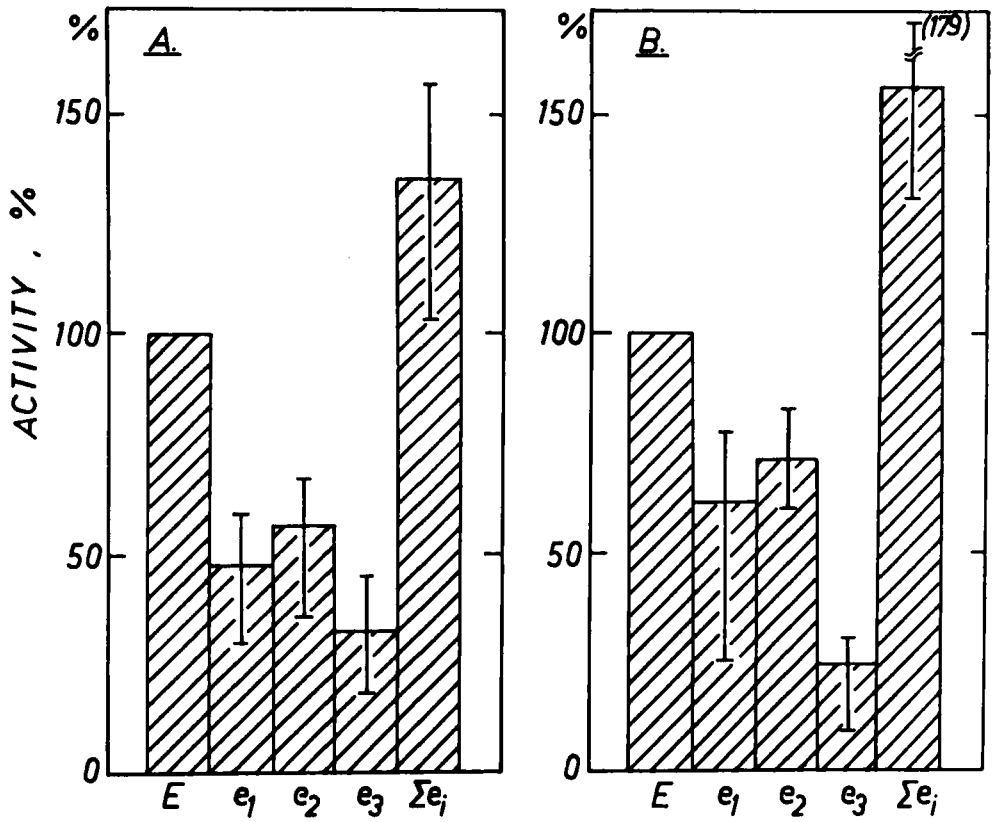

Fig. 4. The distribution of gelatinolytic (A) and alkaline phosphatase activity (B) in subfractions of human saliva: supernatant-e_1, supernatant-e_2 and resuspended sediment (e_3). The activity at pH 8.8 is expressed as per cent of activity in the saliva (E). The individual variations are indicated.

I). However, the activity in the supernatant increased to 230% after 15 min incubation compared to the supernatant without DOC. The activity showed no significant increase even after 8 hours of incubation. In the resuspended sediment the activity was decreased in proportion to the increase of the activity in the supernatant (Table I).

Experiment III

The distribution of the gelatinolytic activity and the alkaline phosphatase activity of the saliva is presented in Fig 4. Almost 50% of gelatinolytic activity in the saliva (E) was found in the supernatant-e_1. The activity released from the sediment by 0.40% DOC (in supernatant-e_2) was only slightly higher than in the supernatant-e_1. In the final sediment (e_3) some activity still remained. The total activity of e_1, e_2 and e_3 was 36% higher than the gelatinolytic activity originally found in saliva (E).

The distribution of the alkaline phosphatase activity (Fig 4B) showed a pattern similar to the gelatinolytic activity. The total alkaline phosphatase activity of e_1, e_2 and e_3 corresponded to more than 150% of the activity in the saliva (E).

The protein content and the activities of supernatants-e_1 and e_2 on different

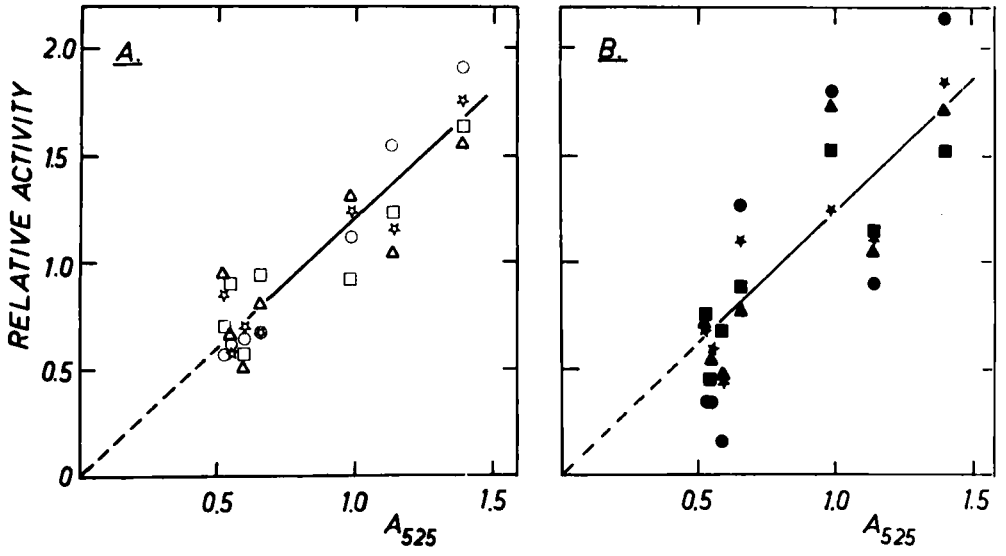

Fig. 5. The relative proteolytic activity in supernatant-e_1 (A, open symbols) and supernatant-e_2 (B, closed symbols) of saliva samples from seven subjects as assayed on gelatin (□ ■), haemoglobin (○ ●), poly-L-lysine (☆ ★) and L-lysine-*p*-nitroanilide (△ ▲) plotted against the turbidity at 525 nm of the resuspended sediment (e_3).

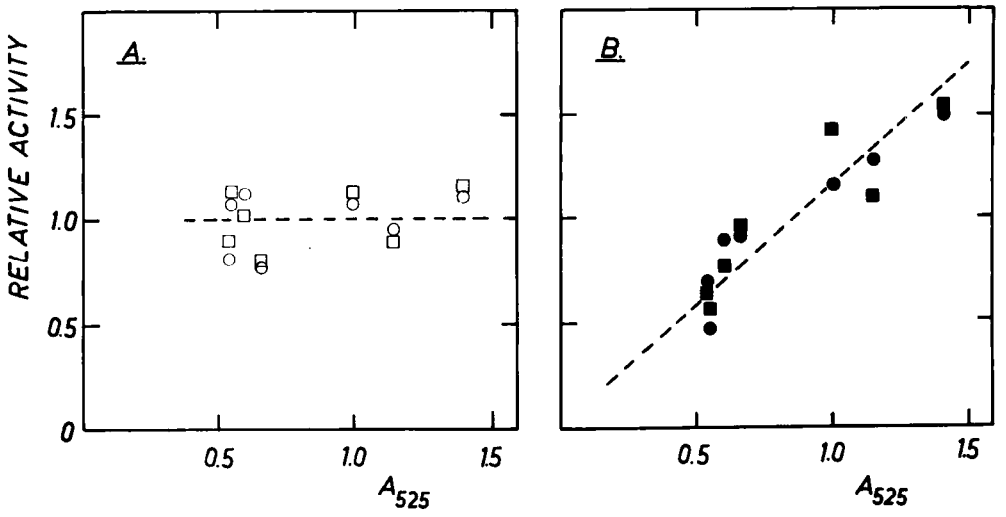

Fig. 6. The relative esterase activity in seven saliva specimens.
A. The relative esterase activity in supernatant-e_1 assayed on BAEE (○) and TAME (□) at pH 8.1 plotted against the turbidity at 525 nm of the resuspended sediment.
B. The relative esterase activity in supernatant-e_2 assayed on BAEE (●) and TAME (■) at pH 8.1 plotted against the turbidity at 525 nm of the resuspended sediment.

Table II. *The protein content and the activity on different substrates of seven specinens of supernatants e_1 and e_2. The mean value and the range was given in units per volume corresponing to 1.0 ml saliva*

		Activity							
		Gelatin	PLL	Haemo-globin	LPA	BAEE	TAME	PNPP	Protein
		H.U.		μmoles/(min $\times$ 10^4)					mg
Superna-	mean	6.7	0.67	6.6	1.33	107	86	147	3.24
tant-e_1	range	3.9–10.9	0.42–1.18	3.3–10.3	0.86–2.05	83–122	69–98	24–219	2.28–4.32
Superna-	mean	8.2	0.73	15.4	2.05	44	59	159	0.34
tant-e_2	range	3.7–12.6	0.32–1.34	2.2–33.7	0.93–3.84	23–66	31–90	73–244	0.12–0.64

substrates are summarized in Table II. The mean value of the activity released by DOC (e_2) was higher than that originally found activity (e_1) for all substrates except BAEE and TAME. However, there were large individual variations for all the tested substrates.

The relative proteolytic activities (individual activity/mean activity) in the supernatants e_1 and e_2, assayed on gelatin, haemoglobin, PLL and LPA were plotted against the turbidity at 525 nm of the resuspended sediment (Fig. 5). There appeared to be a relationship between peptidase activity and the amount of sediment.

The relative esterase activities of fractions e_1 and e_2 in different specimens as assayed on BAEE and TAME, were plotted against the turbidity at 525 nm (Fig. 6). The figure shows that whereas the relative esterolytic activity in supernatants-e_1 was not dependent of the amount of sediment, that of supernatants-e_2 was.

When the esterolytic activity of the specimens on BAEE were plotted against the corresponding activity on TAME, there was a linear correlation between these activities, but these were different for e_1 and e_2 (Fig. 7).

DISCUSSON

One problem in studying the proteolytic activity of human saliva is that slightly more than half of the activity is bound to the 20,000 g sediment (*Söder*, 1972).

Extraction of proteins from cell membranes with detergents has been widely used and the results have varied from complete solubilization to removal of one or more components (*Estrugo et al.*, 1977; *Baccino et al.*, 1971, *Philippot*, 1971; *Allan & Crupton*, 1971; *Dunnick et al.*, 1972; *Bachorik & Dietrich*, 1972). *Crumpton* (1971) reported that sodium deoxycholate used to solubilize the membranes had little effect on specific molecular interactions.

The present investigation has shown that DOC released proteins to a small extent from saliva sediment. Some of the released proteins were proteases and alkaline phosphatase. The degree of solubilization of the enzymes investigated was dependent on the concentration of

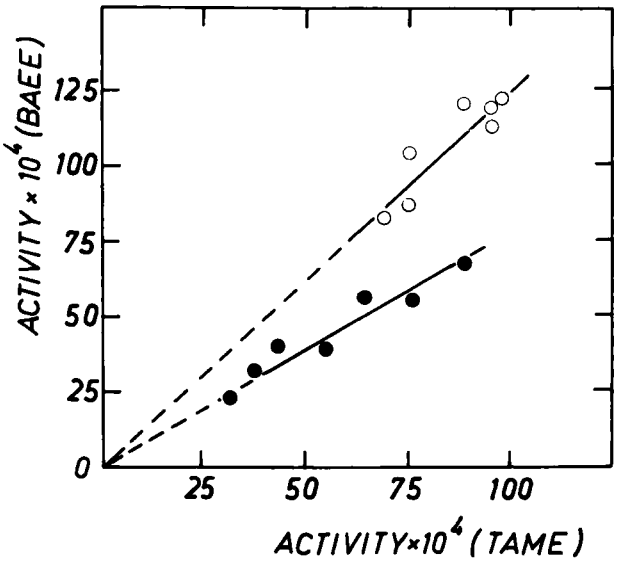

Fig 7. The esterase activity in supernatant-e_1 (○) and supernatant-e_2 (●) from seven different saliva specimens assayed on BAEE and TAME. The activity at pH 8.1 is expressed as μmoles of ester hydrolyzed per minute and per volume (corresponding to 1.0 ml saliva).

the detergent and the incubation time (Fig 1, 2 and 3; Table I).

Allan & Crumpton (1971) found that the degree of solubilization of pig lymphocyte plasma membranes was also dependent on the concentration of the membranes, as well as the absence or presence of salts, and temperature. The same factors seem to be relevant for saliva sediment. The range of sediments and salt in saliva from different persons would not appear to influence the degree of solubilization of the enzymes under the incubation conditions used since the release of enzymes was found to be proportional to the amount of sediment (Fig. 5 and 6).

The increase in the gelatinolytic activity of the supernatant reflects a release of enzyme from sediment (Fig. 1). The apparent activation of the activity in uncentrifuged saliva could be described as a transformation of the enzyme from a »bound» inactive to a »soluble» active form (Fig 1 and 3). The activation of the supernatant treated with DOC may be related to the same mechanism, because »bound» enzyme could be present in the 10,000 g supernatant.

Alkaline phosphatase, which was not activated by DOC, may have a lesser degree of binding to the saliva sediment than proteolytic enzymes, as it was released faster (Table I). *Allan & Crumpton* (1971) found that the different degree of solubilization of 5′-nucleotidase and leucine naphthylamidase from plasma membranes could probably reflect different degrees of binding to the membranes.

The solubilization of the sediment by DOC was determined by the change in absorbance (turbidity) at 525 nm. There was a low degree of solubilization of saliva sediment by DOC. *Dunnick et al.* (1972) has used this method to determine the solubilization of rat liver plasma membranes.

In this paper a good correlation between the amount of sediment and the proteolytic activity assayed on the substrates gelatin, haemoglobin, PLL and LPA was found both in fraction e_1 and e_2 (Fig.

5 and 6). This relationship suggests that most of the proteolytic activity detected in saliva was derived from epithelial cells, leucocytes or microorganisms. *Taylor* (1959) demonstrated proteolytic activity in the superntant of saliva and the sediment and suggested that the activity was from lysed buccal mucosa cells rather than from salivary glands. *Chauncey* (1961) showed that most of the proteolytic activity in whole saliva was contained in the sediment, which could be fractionated in two components, microorganisms and mammalian cellular elements such as epithelial cells and leucocytes. The mammalian cellular elements contributed from 60—80% of the total activity.

In fractions e_1 and e_2 there must be more than one peptidase, which is indicated by the different slopes of the correlation lines for e_1 and e_2, when TAME activity is plotted against BAEE activity (Fig. 7). The esterolytic activity on TAME and BAEE was probably due both to proteases and esterases, because of the different distribution of activities on the more specific proteolytic substrates (Table II). Purification of the enzymes in e_1 and e_2 and inhibitor studies are in progress.

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