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STUDIES ON ORAL ENZYMES

VII. PYRIDOXAL-5-PHOSPHATE DEPENDENT CLEAVAGE OF L-CYSTINE AND RELATED COMPOUNDS BY ENZYME PREPARATIONS FROM HUMAN ORAL CAVITY AND CERTAIN MICRO-ORGANISMS

by

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INTRODUCTION

The action of enzymes on disulphide bonds is often coupled with the simultaneous oxidation of $NADH_2$ or $NADPH_2$[1]). The enzymes involved act on reduced NAD or NADP using different compounds (e.g., disulphide compounds) as acceptors. Cystine reductase ($NADH_2$:L-cystine oxidoreductase, EC 1. 6. 4. 1) has been demonstrated in plants and yeast (*Romano & Nickerson*, 1954); glutathione reductase ($NAD(P)H_2$:glutathione oxidoreductase, EC 1. 6. 4. 2) has been shown to occur in animal tissues, plants, yeast and bacteria (*Mapson & Goddard*, 1951; *Racker*, 1955; *Rall & Lehninger*, 1952; *Suzuki & Werkman*, 1960; *van Heyningen & Pirie*, 1953), as well as lipoamide dehydrogenase ($NADH_2$:lipoamide oxidoreductase, EC 1. 6. 4. 3) (*Basu & Burma*, 1960; *Hager & Gunsalus*, 1953; *Massey*, 1960; *Sawage*, 1957; *Straub*, 1939). These three enzymes are all flavoproteins. In addition, there is also protein disulphide reductase (reduced-NAD(P):proteindisulphide oxidoreductase, EC 1. 6. 4. 4) which breaks disulphide bonds in protein molecules. There are also several other oxidoreductases affecting the enzymic reactions of sulphur containing compounds, e.g., those acting on sulphur groups of donors (sub group 1. 8.) or acting on H_2O_2 as acceptors (sub group 1. 11.) (*Florkin & Stötz*, 1965).

Recently, cystathionases (L-homoserine hydro-lyase, deaminating, EC 4. 2. 1. 15) from *Neurospora* and liver have been shown to catalyze the

[1]) Abbreviations: NAD = nicotinamide-adenine dinucleotide; NADP = nicotinamide-adenine dinucleotide phosphate.

degradation of cysteine to pyruvic acid, ammonia, and thiocysteine (*Flavin*, 1962; *Cavallini, DeMarco, Mondovi & Mori*, 1960; *Flavin & Slaughter*, 1964). The enzyme preparations from *Neurospora* can utilize L-cystine as a substrate. More recently, the cleavage of L-cystine to pyruvic acid and thiocysteine, by soluble enzyme preparations from *Brassica* species, has been demonstrated (*Tishel & Mazelis*, 1966; *Mazelis, Beimer & Creveling*, 1967). This enzyme requires pyridoxal-5-phosphate for activity.

The enzymic cleavage of disulphide bonds in human oral tissues, or in oral cavity in general, may be important since it is known that human dentine and enamel contain cystine, although at low concentration (*Eastoe*, 1963; *Weidmann*, 1965). In addition, the keratins, major components of skin and other epidermal structures, contain variable amounts of the sulphur containing amino acids. The oral cavity also contains a variety of sulphur-containing compounds derived from food, micro-organisms, etc. The enzymic transformations of such compounds constitute a portion of oral biochemical processes. This investigation, then, concerns the existence and the characteristics of those enzymes acting on L-cystine and related compounds in the human mouth.

MATERIALS AND METHODS

1. Chemicals

[^{35}S]L-Cystine and [3-^{14}C]DL-cystine hydrochloride (specific activity 39.5 mC/mmole and 43 mC/mmole, respectively) were purchased from The Radiochemical Centre (Amersham, England). The following compounds were obtained from K & K Laboratories (Plainview, New York, N. Y., USA): L-djencolic acid, L-cystine dimethyl ester dihydrochloride, L-cystine diethyl ester dihydrochloride, DL-cystathionine, DL-lanthionine, 1(+)*meso*-lanthionine, *meso*-cystine, L-cysteic acid, cysteamine hydrochloride (β-mercapto ethylamine), and L-homoserine. The following compounds were obtained from Mann Research Laboratories, Inc. (New York, N. Y., USA): S-methyl-L-cysteine, O-phospho-DL-serine, O-methyl-DL-serine, O-phospho-DL-threonine, and the following from Fluka AG (Buchs SG, Switzerland): L-cysteine, L-cystine, DL-homocysteine, DL,DL-allocystathionine, and taurine. S-Methylglutathione was purchased from Calbiochem (Luzern, Switzerland), and L-cystine di-2-naphthylamide (bis(2-naphthylamide) L-cystine) from Koch-Light Laboratories, Ltd. (Colnbrook, England). Of the coenzymes used in this study, pyridoxine hydrochloride, thiamine, and riboflavine were obtained from Hoffman-La Roche & Co. (Basel, Switzerland), $NADPH_2$ from C. F. Boehringer & Söhne GmbH (Mannheim, Germany), and pyridoxal-

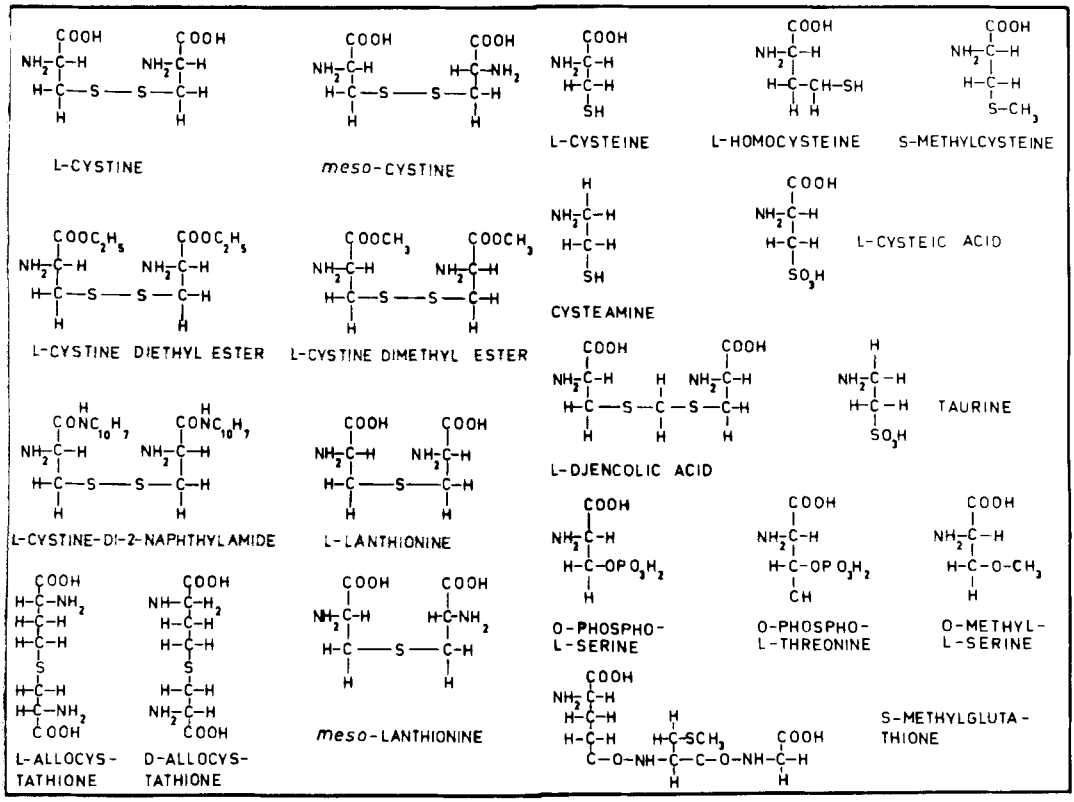
5-phosphate from Fluka AG. Unless otherwise stated, all of the other reagents were obtained from E. Merck AG (Darmstadt, Germany). In Table I the structure of the substrates used is given. All of the commercial preparations were used with no further purification.

2. Estimation of L-cystine cleaving activity

At the beginning, three separate methods for assaying cystine (and cysteine) cleaving activity were to be considered: to measure the H_2S, ammonia and pyruvic acid produced. Obviously these products could be produced from

Table I

Structure of the compounds used as substrates for various enzyme preparations in the study. For some substrates only one of the stereoisomers is shown, although a mixture of them was used in the experiments (see list in Materials section).



cystine (and from cysteine) in more than one way and could undergo secondary reactions. With crude preparations the methods may thus measure the activity of more than one enzyme. Because preliminary experiments had shown that ammonia and H_2S rapidly undergo secondary reactions, the measurement of pyruvic acid was selected to estimate cystine cleaving activity. Also the latter compound did participate in secondary reactions, but it also accumulated in the reaction mixtures at higher concentrations than H_2S and ammonia. However, due to the secondary reactions, the results obtained by measuring pyruvic acid do not give strictly comparable results with different enzyme preparations.

a) *Keto acid method.* The reaction mixture was composed of the following ingredients: 0.5 ml of buffer-substrate solution, 0.1 ml of enzyme solution, and 50 μl of water or proper enzyme activator or other solution containing the compound to be tested. Throughout the work, 0.01 M tris-HCl buffer, pH 7.2 was used. The reaction mixtures were incubated for different periods, depending on the activity of the enzyme preparation involved; the exact incubation time is given with each experiment in RESULTS. The substrate-buffer solutions or suspensions were made as described by *Mazelis, Beimer,* and *Creveling* (1967), to a concentration of 0.01 M. In cases of insoluble substrates, homogenous suspensions were prepared from them, and these suspensions were then used as such in the reaction mixtures where the compounds also remained undissolved, forming saturated solutions. Enzyme reactions were stopped by adding 0.5 ml of 10 % trichloroacetic acid. The resultant mixtures were centrifuged where necessary. In these centrifugations, the Laboratory Centrifuge Universal Junior III was used at 4500 rpm (3100 g) for 10 min., at room temperature. The supernatant fluids were assayed for pyruvate colorimetrically by the total keto acid method of *Friedemann* and *Haugen* (1943). Pyruvic acid (Fluka AG) was used as a standard.

b) *Use of labelled compounds.* The enzymic cleavage of [^{35}S]L-cystine and 3-^{14}C]DL-cystine was estimated in the following way. 5 mg of unlabelled L-cystine was first weighed into the bottom of small test tubes. Most of this cystine remained undissolved in the reaction mixture. Thus it was ascertained that the reaction mixture had been saturated with cystine during the experiment. 50 μl of 0.01 M tris-HCl buffer, pH 7.2, was then added to the tubes, followed by 50 μl of enzyme solution. Finally, 5 μl of labelled cystine solution was put into the tubes, and the contents were well mixed and incubated for various time periods. The 5 μl of labelled compounds had been obtained from a solution made by suspending the commercial cystine preparations (altogether 0.5 mc of both ^{14}C- and ^{35}S-labelled) in 1 ml of water at 25°C. Due to the low solubility of cystine in water, about 5 mg/100 ml

water for DL-cystine and about 11 mg/100 ml water for L-cystine (*Dawson et al.*, 1962), it was calculated that the 5 μl amount of labelled cystine solutions corresponded to an increase of only 0.5—1.0 μg in the total cystine content of the reaction mixtures. It is to be noted that, when these labelled cystine solutions were added to the reaction mixtures, the latter already were saturated by cystine. Hence it was approximated that 0.5—1.0 μg of the labelled compounds had been precipitated in the reaction mixtures. Labelled cystine from the undissolved part was then randomly dissolved in the reaction mixture in the course of the reaction and picked up by the enzyme molecules.

After these processes the slightly turbid mixtures were rapidly centrifuged in a Universal Junior III centrifuge. The clear supernatant solutions were then kept in an ice bath, after which these reaction mixtures were analyzed on thin layer plates (20 × 20 cm) coated with a Silica Gel G layer (thickness 0.35 mm), adhering closely to the instructions of *Randerath* (1964). 25 μl of the clear reaction mixtures were applied in small quantities to the plates, at 3 cm distances from each other, with the aid of a heating fan. The plates were then developed with 96 % ethanol so that the front of the solvent came within 15 cm of the applied spots. The amino acids were made visible with the modified ninhydrin reagent of *Moffat* (1959). The radioactivity on the plates was finally estimated with a thin layer scanner and a methane gas flow counter (Laboratorium Prof. Berthold, Wilbad, W.-Germany). Special blank tubes were prepared by replacing the enzyme solution with water or by omitting the 5 mg amounts of substrate. These mixtures were then handled in the above way.

A general recommendation can be made for oxidizing an amino acid mixture with performic acid before chromatography (*Hirs*, 1956), in order to convert cysteine and cystine into the more stable cysteic acid. By omitting such an oxidation, several autoxidation products are formed from cysteine and cystine. A portion of the experiments in this study were performed without this procedure, while in another portion the oxidation with performic acid was done in the following manner. The enzyme reactions involved were stopped by adding 4 ml of performic acid. The mixtures were then allowed to stand for 15 min. at room temperature, and the performic acid was then removed by repeated freezedrying (thrice). The residues were finally dissolved in 0.1 ml of water, and aliquots of 25 μl were applied to the thin layer plates as previously related. The performic acid had been prepared by treating 90 ml of formic acid with 10 ml of 30% hydrogen peroxide for 10 min. at room temperature. The resultant product was then used immediately. By this procedure all of the uncleft cystine, and the cysteine possibly formed during the reaction, was oxidized. Hence any lowering of the cysteic acid

peak on the thin layer plates could be used as an indication of enzyme activity causing cystine to cleave to other compounds than cysteine, e.g., to pyruvic acid. The formation of keto acids was also estimated by studying the thin layer plates after chromatography in UV-light.

c) Estimation by amino acid analysis. The reaction mixtures with L-cystine as substrate were also analyzed on a Beckman Unichrom Amino Acid Analyzer in order to determine the consumption of L-cystine during the reaction. No attempt to identify the other ninhydrin positive compounds in the amino acid analyses was made due to the considerable alterations in the amount and nature of the reaction products resulting from the crudeness of the enzyme preparations used. These experiments were set up in the following way. 5 mg of L-cystine was weighed into the bottom of small test tubes. 1.0 ml of 0.01 M tris-HCl buffer, pH 7.2, was then added, followed by 0.5 ml of enzyme solution. In the blank tubes the enzyme solutions were replaced with water. These reaction mixtures were incubated for 3 hr. at 37°C, and the reactions were stopped by adding 2 ml of 1 % picric acid. The quantitative precipitation of the proteins in the mixtures was ascertained by adding more picric acid, allowing the precipitates to form for one hour. The precipitates were then spun down in the Universal Junior III centrifuge, and the yellow and clear deproteinized solutions were passed through a Dowex® 2 × 8 or 2 × 10 column (Fluka AG) according to standard methods used in amino acid analysis (Beckman Unichrom Amino Acid Analyzer, Instruction Manual, 1966). The obtained colourless solutions were finally freeze-dried, and the residues were dissolved in 2 ml of 0.01 N HCl and then analyzed for their cystine content with the Analyzer.

3. Enzyme preparations from the oral cavity

In this investigation the keto acid forming activity of the following samples obtained from the human oral cavity was studied: whole saliva, parotid saliva, bacterial plaque, carious dentine, and normal dentine. The bacterial plaque means, in this case, that whitish material which was easily removed from the surface of the teeth when collecting whole saliva by paraffin stimulation. The whitish material remained as a pellet at the bottom of the centrifuge tube when a whole saliva sample was centrifuged. Bacterial plaque was also collected from the surface of teeth with an excavator. This material may have had a composition differing from that obtained from whole saliva, because the latter, centrifuged material originated from other oral surfaces as a result of the chewing of paraffin. Carious dentine means here that dentine obtained from extracted teeth with advanced caries. All of the aqueous enzyme preparations obtained from these materials were stored

at —20°C, between experiments. The enzyme preparations were diluted in proper ratios with cold water just before use.

A detailed description of making the enzyme preparations is presented in the following.

Whole saliva was collected from four persons (2 male, 2 female) by stimulating the secretion with paraffin (m.p. 52 °C). No attention was paid to the clinical condition of the dentition of the persons. The material was allowed to run into test tubes placed in an ice bath and centrifuged immediately thereafter at 19500 rpm (45900 g) for 10 min. at 4 °C in rotor SS-34 of the Sorvall Superspeed RC-2B centrifuge. Throughout this work the centrifugal forces quoted were average figures. The pellet was preserved for further handling. The supernatant fluids were stored at —20 °C. When a sufficient amount of centrifuged whole saliva had been obtained in daily collections, the samples were thawed and pooled. The evident turbidity was removed by centrifugation at 19500 rpm (45900 g) for 10 min. at 4 °C, and the supernatant fluid was then designated as the whole saliva supernatant.

The pellets obtained after centrifuging the pooled whole saliva samples were suspended in cold (about 4 °C) 0.01 M tris-HCl buffer, pH 7.2 (about 1 ml per the original 10 ml volume of collected saliva), so that several 10 ml suspensions were obtained. These 10 ml suspensions containing oral micro-organisms (and some epithelial cells) detached from the surface of the teeth, the tongue and the oral mucosa through the rubbing action of chewing paraffin, were treated for 10 min. with the MSE ultrasonic disintegrator (Measuring & Scientific Equipment Ltd, London, England), 100 Watt Model, using a titanium probe, end diameter 19 mm. During the treatment the sample was kept in ice to prevent over-heating. The resultant solution was centrifuged at 19500 rpm (45900 g) for 20 min. at 4 °C, and the supernatant fluid was designated the enzyme preparation representing whole saliva sediment and is later called Bacterial Plaque I. This kind of enzyme preparation may be claimed to represent only such soft bacterial plaque which is easily removed from oral surfaces. Thus in addition, as mentioned earlier, bacterial plaque was collected directly from the surface of the teeth with an excavator. This material was obtained from four persons, altogether about 0.2 g at a time, and it was immediately suspended in 1 ml of cold (about 4 °C) 0.9 % sodium chloride solution. The suspension was centrifuged in glass tubes at 14500 rpm (23500 g) for 15 min. at 4 °C, and the clear supernatant fluid was designated as an enzyme preparation representing an extract which contained products of bacterial metabolism, e.g. extracellular proteolytic enzymes, and is later called Bacterial Plaque II.

The parotid saliva was collected from four persons, using the Carlson-Crittenden device (*Shannon, Prigmore & Chauncey*, 1962). The parotid saliva collected in this way was always completely clear and did not require any centrifugation. The enzyme preparations from the carious and healthy dentine were prepared from extracted teeth. After extraction, the teeth were stored for about 10 days at 4 °C before further treatment. The carious areas of the teeth were detached with a cyrette, and the fragments obtained were ground for 10 min. at room temperature in a porcelain mortar into as fine particles as possible and treated for 3 hr. with 0.01 M tris-HCl buffer, pH 7.2, stirring the mixture with a magnetic mixer at about 4 °C. 10 ml of buffer was used for each 2 g of dry material. The extracts were centrifuged at 19500 rpm (45900 g) for 10 min. at 4 °C, and the supernatant solutions were designated as enzyme preparations obtained from carious dentine. The hard normal dentine was crushed in a percussion mortar of steel. The resultant fine powder was then treated as above, and the final supernatant fluids were designated enzyme preparations representing the activity in normal dentine.

4. Enzyme preparations from cultivated micro-organisms

For this investigation 10 different micro-organism species or strains were chosen. A number of them were related to those constantly occurring in the human mouth. The test organisms are listed in Table II together with certain informations on their source and cultivation. The final cultivations were performed under identical conditions in order to allow a reliable comparison of the results. All cultivations were carried out at 37 °C. The lactobacilli were maintained as stab cultures in P-agar*, then transferred from this to 7 ml of Bacto-Micro Inoculum Broth** and cultivated for 24 hr. without aeration. The contents of the 7 ml tubes were finally transferred, aseptically, into 500 ml of pre-warmed TSHGA-medium***. Other test organisms were maintained as slope cultures on P-agar and transferred from these into 5 ml tubes of the TSHGA-medium, where the cells were cultivated for 24 hr. as lactobacilli. The contents of the 5 ml tubes were then transferred aseptically into the 500 ml TSHGA-medium, as above.

All of the test organisms were grown in the 500 ml TSHGA-medium for 4—24 hr. as indicated in Table II. Growth was followed turbidimetrically with a Klett-Summerson colorimeter employing filter No. 62. The incubations were stopped when the growth had clearly reached the middle of the exponential growth phase. This was ascertained in preliminary experiments. The growth mediums were aerated (except in the case of Str. salivarius, which did not grow well under aeration) by agitating the medium with a mixer (Heidolph Laborrührer, Typ RZR, Heidolph Elektro KG, Kelheim/Donau, Germany) using a steel propeller at 150 rpm.

The cells were harvested from the growth medium by centrifugation at 19500 rpm (49500 g) for 10 min. at 4 °C and washed twice with 5 ml of cold (about 4 °C) 0.9 % NaCl solution in the centrifuge tubes. After pouring off the last washing solution, 2—7 ml of cold 0.01 M tris-HCl buffer, pH 7.2, (1 ml per the original turbidity reading of 20 obtained with the colorimeter) was added to the pellet, and the cells were suspended in it. The resultant suspension was then treated with the MSE ultrasonic disintegrator, as earlier

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* The solid P-agar was composed of the following ingredients per 1000 ml distilled water: 10 g Bacto-peptone, 3 g yeast extract, 1.5 g meat extract, 20 g agar. The pH of the medium was adjusted to 6.5—6.6 and the medium was autoclavized for 20 min. at 121 °C.

** The Bacto-Micro Inoculum Broth was made by rehydrating 37 g of the commercial medium in 1000 ml distilled water. The medium was autoclavized for 15 min. at 121 °C. The frequency of transfers using these solid mediums varied from 4 to 5 weeks. The cultures were maintained at 4 °C between transfers.

*** The TSHGA-medium contained the following ingredients per 1000 ml distilled water: 5 g sucrose, 5 g Bacto-tryptone, 10 g glucose, 5 g lactose, 5 g yeast extract, 2.5 g gelatine, and 0.7 g ascorbic acid. The pH of the medium was adjusted to 6.7—6.8.

Table II

List of micro-organisms used in the study and some details about their cultivation. The first column gives the names of the test organisms and the second column the length of the incubation time in the final cultivation in 500 ml of TSHGA medium. In the other columns further details on the cultivations are given.

Organism	Length of the second incubation time (hr.)	Mg cells (dry weight) in 1 ml of growth medium at the end of cultivation	Turbidity reading reached	Final pH of the growth medium[c]
Streptococcus salivarius[a]	24	0.25	110	4.5
Streptococcus pyogenes ATCC 6636[b]	4.1	0.20	78	4.5
Streptococcus pyogenes (haemolyticus) ATCC 9342[b]	6.5	0.19	80	4.3
Streptococcus sp. ATCC 9854[b]	4.5	0.22	103	4.8
Lactobacillus fermenti ATCC 9338[b]	9.0	0.18	82	5.3
Lactobacillus lactis ATCC 8000[b]	4.8	0.20	90	4.5
Lactobacillus casei ssp. rhamnosus ATCC 7469[b]	8.0	0.15	50	5.3
Escherichia coli 113[b]	3.0	0.32	115	5.4
Escherichia coli 154[b]	5.0	0.21	88	5.2

[a]Isolated and cultivated from human whole saliva at the Institute of Dentistry, University of Turku, Turku, Finland.

[b]Supplied by Apteekkitavaraintarkastuslaboratorio, Helsinki, Finland. The numbers refer to the American Type Culture Collection (ATCC) numbers, and in the case of the Eschetichias to numbers given by the supplier.

[c]Measured at 37 °C with glass electrode just before harvesting the cells.

described. The supernatant solutions finally obtained were used as enzyme preparations and stored at —20 °C between experiments. The clear growth mediums were not saved for investigation because it was found that their cystine cleaving activity was rather low.

5 Dry weight of micro-organisms

The dry weight of the micro-organisms studied was determined from one 10 ml sample from the same growth phase from which the cells had been harvested for enzyme studies. The drying was conducted with centrifuged pellets [at 23500 g (1400 rpm) at 4 °C for 10 min., washed thrice with cold

0.9 % NaCl] at 65 °C until a constant weight was reached. For most test organisms a colorimeter reading of 100 (with the Klett-Summerson colorimeter) corresponded to 0.2—0.3 mg dried cells per 1 ml growth medium.

6. Column chromatography

The gel filtrations were conducted on Sephadex® columns. In the preparation of gels and in conducting the fractionations, the instructions of the manufacturer (Pharmacia, Uppsala, Sweden) were followed. DEAE-cellulose chromatography was carried out on columns packed with a product of Schleicher & Schüll (Dassel/Kr. Einbeck, Germany). A fraction of 200—230 mesh was sieved from the commercial p·eparation. In preparing the material, in packing the columns, and in conducting the fractionations, the instructions presented by *Peterson* and *Sober* (1962) have been followed.

7. Determination of protein concentration

In this investigation the protein concentrations were determined using the Folin-Ciocalteu method (*Layne*, 1963). Bovine serum albumin (crystallized and lyophilized, Sigma Chemical Company) was used as a standard.

RESULTS

When preliminary experiments had shown that centrifuged human whole saliva possessed enzymes capable of forming keto acids from L-cystine, a number of other oral enzyme preparations were also tested. The results are given in Table III, which shows that the enzyme preparation made by ultrasonic treatment from whole saliva sediment (Bacterial Plaque I) displayed the highest enzyme activity. This was evidently due to the occurrence of micro-organisms capable of synthesizing enzymes which transformed L-cystine into a variety of degradation products, among others to keto acids. This formation of keto acids (presumably pyruvic acid) from L-cystine may be said to have taken place with a simultaneous, or subsequent, formation of inorganic sulphate, ammonia, and some S-containing compounds not yet positively identified. The activity of various oral enzyme preparations cleaving L-cystine seemed to be rather low in most cases. Hence any end product of this cleavage reaction, except of keto acids, was present in the reaction mixture in rather low quantities, or else was rapidly transformed to other compounds. In addition, attempts to purify the enzyme(s) involved in these transformations failed, as is discussed later in more detail, probably

Table III

Ability of various oral enzyme preparations to form keto acids from L-cystine. The reactions were carried out for 3 hr. in 0.01 M tris-HCl buffer, pH 7.2, with 0.01 M L-cystine and without the presence of added pyridoxal-5-phosphate. The enzyme activity is expressed as μmoles of pyruvic acid formed per min. and per mg protein ($\times 10^6$).

Source of enzyme preparation	Activity
Supernatant fluid of whole saliva	611
Parotid saliva	280
Whole saliva sediment (Bacterial Plaque I)	5800
Plaque extract (Bacterial Plaque II)	605
Extract of carious dentine powder	390
Extract of normal dentine powder	10

because of their insignificant rate of appearance in the oral cavity and especially because of their low stability. These are the reasons why, when using crude and complex enzyme preparations, identification of the exact end products was not possible. Hence, only the terms »pyruvic acid forming activity», or »L-cystine cleaving activity» are used.

Because the observed enzymic formation of keto acids would be expected to require the participation of a coenzyme, some possible compounds were studied, *i.e.*, $NADPH_2$, pyridoxal-5-phosphate, and pyridoxine (all tested at 0.77×10^{-3} M). Only pyridoxal-5-phosphate increased the rate of the formation of pyruvic acid. In addition, several other compounds not expected to activate the cleavage of L-cystine were also investigated in order to determine their possible effect on the reaction. These compounds and their concentrations in the reaction mixtures were as follows: thiamine HCl (0.77×10^{-3} M), riboflavine (4.0×10^{-5} M), nicotinic acid (0.77×10^{-3} M), p-aminobenzoic acid (0.77×10^{-3}) M), calcium panthotenate (0.77×10^{-3} M), and NADP (0.77×10^{-3} M). These compounds had no effect on the formation of pyruvic acid. The efficiency of pyridoxal-5-phosphate as a coenzyme in the keto acid formation may, however, be dependent on the structure and nature of the substrate when a crude enzyme preparation was used. For example, the formation of keto acids from DL-lanthionine, *meso*-cystine and L-djencolic acid took place at different rates, depending on the presence or absence of the added coenzyme in the reaction mixture. The formation of keto acids (measured as pyruvic acid) from these compounds was increased, using pyridoxal-5-phosphate, by 10, 30, and 75 %, respectively.

When it had been found that pyridoxal-5-phosphate increased the rate of the formation of keto acids from L-cystine and L-djencolic acid, the effect of EDTA (at several concentrations ranging from 0.166×10^{-2} M to 0.83×10^{-3} M in the reaction mixture) and certain metal cations (*i.e.* Fe^{+++}, Cr^{+++}, Al^{+++}, and Cu^{+} ions, at 0.166×10^{-5} M, in the form of metal chlorides) was tested with a dialyzed enzyme preparation. A portion (2 ml) of an enzyme preparation obtained from Bacterial Plaque I by ultrasonic treatment was dialyzed for 24 hr. against 10 l of water at +1 °C. The faint turbidity formed during the dialysis was removed by centrifugation at +1 °C. An equal amount of undialyzed enzyme preparation from the same original lot was studied as well. Subsequent experiments with these preparations showed that EDTA did not inhibit the reaction at any of the concentration used. An inhibitory effect of about 5 or 10 % was occasionally obtained, this being explained as an unspecific effect. Further, the metal salts tested had no clear effect either. The most clearly observed result was a lowering, by 30 to 40 percents, of the activity in the enzyme preparation, this evidently being due to protein denaturation. The results seem to indicate that the formation of keto acids do not involve in this case the initial formation of a metal chelate of a Schiff's base between catalyst and substrate. The formation of such a base may not be, however, an absolute requirement, as will be shown in the discussion. It seems certain, on the basis of the results, that the observed formation of keto acids is also due to an enzyme, and not only, for

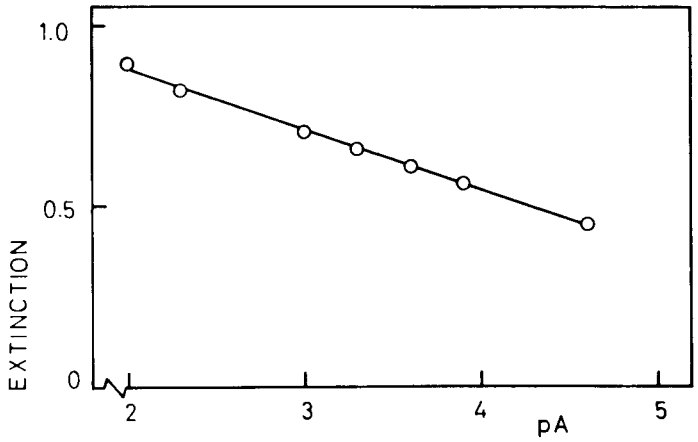


Fig. 1. Effects of various concentrations of pyridoxal-5-phosphate on the enzymic cleavage of L-cystine by enzyme preparations obtained by ultrasonic treatment of whole saliva sediment (Bacterial Plaque I). Incubation time: 3 hr. The concentrations of pyridoxal-5-phosphate have been given as pA (pActivator). The enzyme activity is given in extinction (this concerns Figs. 2—4 as well).

example, to a non-enzymic catalysis by the coenzyme itself. Such a non-enzymic cleavage of L-cystine and L-djencolic acid did occur, caused by pyridoxal-5-phosphate, but these effects were taken into account in appropriate blank tubes.

It was also found necessary to test the effect of various concentrations of pyridoxal-5-phosphate. The results from such experiments are given in Fig. 1, which shows that the enzyme preparations made by ultrasonic treatment from oral micro-organisms (from Bacterial Plaque I) evidently already contained considerable amounts of activating compounds, e.g., pyridoxal-5-phosphate. However, the added coenzyme clearly induced even higher enzyme activity.

When the effect of the enzyme concentration was studied (Fig. 2), it appeared that the rate of the cleavage of L-cystine was found to be directly proportional to the amount of the enzyme present in the reaction mixture. The cleavage of L-cystine was also found to be linear with time. This is shown in Fig. 3. Dependence of the rate of the cleavage on various concentrations of L-cystine is given in Fig. 4. It is seen that, under the conditions given, a saturation of the enzyme was obtained when the initial amount of

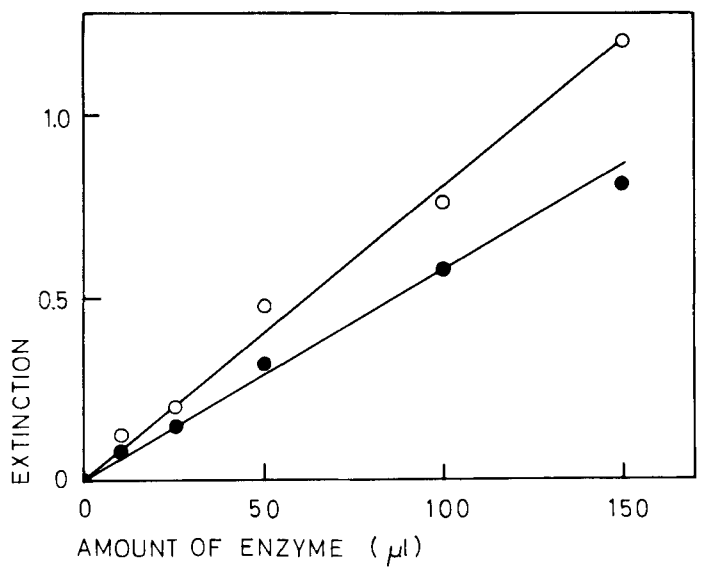


Fig. 2. Dependence of the cleavage of L-cystine on the quantity of enzyme preparation (the same enzyme preparation as given in the legend to Fig. 1). Incubation time 3 hr.
o in the presence of 2×10^{-5} M pyridoxal-5-phosphate. • in the absence of added coenzyme.

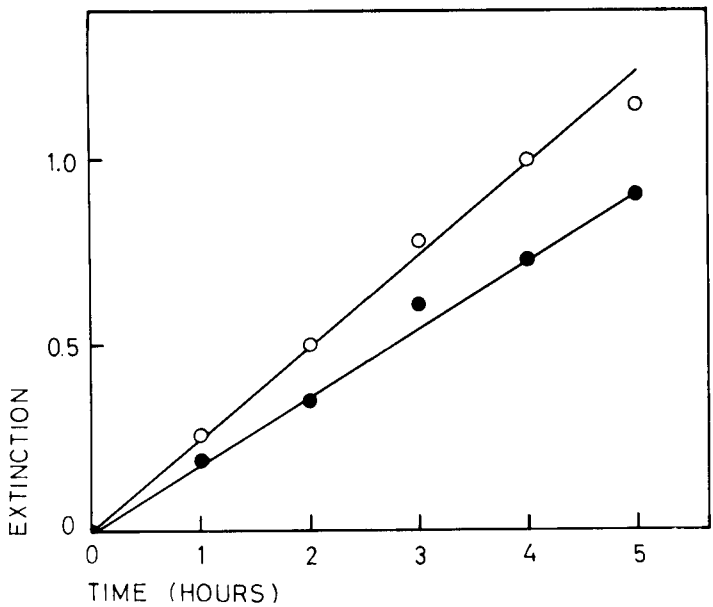


Fig. 3. Rate of cleavage of L-cystine by the same enzyme preparation mentioned in the legend to Fig. 1. o in the presence of 2×10^{-5} M pyridoxal-5-phosphate. • in the absence of added coenzyme.

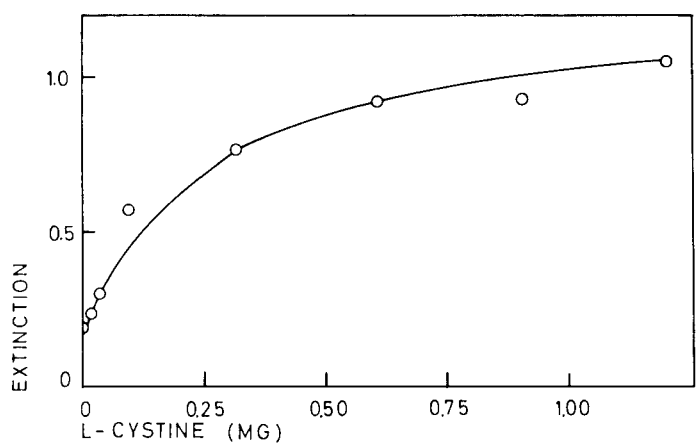


Fig. 4. Dependence of the rate of cleavage of L-cystine on substrate concentration. The same enzyme preparation was used as given in the legend to Fig. 1. Incubation time 3 hr.

L-cystine in the reaction mixture was 0.15 mg and that substrate inhibition occurred at higher substrate concentrations. The above experiments (Figs. 1—4) were carried out with an enzyme preparation obtained by disintegrating the whole saliva sediment (Bacterial Plaque I) with ultrasonic treatment. Similar results were obtained also with enzyme preparations obtained by extracting excavated plaque by buffer (Bacterial Plaque II).

The formation of keto acids from L-cystine and several related compounds by two enzyme preparations obtained from whole saliva is given in Table IV. The cleavage of L-djencolic acid and L-cystine and some closely related compounds (*i.e.*, *meso*-cystine, DL-lanthionine, 1(+)*meso*-lanthionine, and

Table IV

Formation of keto acids from various L-cystine related compounds using two oral enzyme preparations. The reactions were carried out for 6 hr. in 0.01 M tris-HCl buffer, pH 7.2 using 0.01 M substrate concentrations (without added pyridoxal-5-phosphate). The results are given as µmoles of pyruvic acid formed per min. and per mg protein ($\times 10^6$).

Substrate	Source of the enzyme preparation	
	Whole saliva supernatant	Bacterial Plaque I
L-Cystine	525	4,350
meso-Cystine	568	5,230
L-Cystine diethyl ester	568	450
L-Cystine dimethyl ester	21	19
L-Cystine di-2-naphthylamide	139	563
L-Cysteine	165	434
DL-Homocysteine	128	564
L-Cysteic acid	201	564
Cysteamine	165	216
Taurine	201	547
O-Phospho-DL-serine	0	43
O-Methyl-DL-serine	460	1,040
O-Phospho-DL-threonine	367	800
DL-Lanthionine	201	5,500
1(+)*meso*-Lanthionine	220	5,200
L-Djencolic acid	881	10,000
DL-Cystathionine	498	3,777
DL, DL-Allocystathionine	553	3,480
S-Methyl-L-cysteine	155	1,521
S-Methylglutathione	460	690

DL-cystathionine) was most rapid. When using L-djencolic acid it was found that the amount of pyruvic acid, estimated in μmoles per min. and per mg protein (about 0.01 μmoles), was always the highest of all the compounds tested. When calculating that the reaction was carried out for 360 min. and with an initial amount of 0.5 μmoles of L-djencolic acid in the reaction mixture, it was found that the amount of enzyme in the reaction had produced about one μmole of pyruvic acid from one μmole of L-djencolic acid. It was

Table V

Formation of keto acids from various compounds related by their structure to L-cystine using enzyme preparations from various micro-organisms. The reactions were carried out in the absence of added pyridoxal-5-phosphate for 6 hr. in 0.01 M tris-HCl buffer, pH 7.2 using 0.01 M substrate concentrations. The results are given as μmoles of pyruvic acid formed per min. and per mg protein ($\times 10^6$).

Substrate	Source of the enzyme preparation								
	Str. salivarius[a]	Str. pyogenes[b]	Str. pyogenes[b]	E. Coli[b]	E. coli[b]	E. Coli[b]	L. fermenti[b]	L. lactis[b]	Streptococcus sp.[b]	L. casei ssp. rhamnosus[b]
L-Cystine	608	200	950	90	130	306	893	430	358	
meso-Cystine	638	244	1,300	58	239	234	1,345	565	460	
L-Cystine diethyl ester	38	16	68	19	35	134	8	233	120	
L-Cystine dimethyl ester	105	48	65	10	46	225	218	295	169	
L-Cystine di-2-naphthylamide	181	34	46	76	93	190	167	286	447	
L-Cysteine	0	0	0	0	0	0	0	78	0	
DL-Homocysteine	58	35	33	82	60	0	116	157	101	
L-Cysteic acid	43	30	37	18	44	190	77	308	161	
Cysteamine	0	35	50	0	15	162	69	192	228	
Taurine	354	538	103	45	54	252	141	365	447	
O-Phospho-DL-serine	0	0	0	0	0	114	38	250	92	
O-Methyl-DL-serine	322	46	138	121	44	408	638	319	406	
O-Phospho-DL-threonine	64	31	92	31	55	255	0	265	484	
DL-Lanthionine	1,190	362	1,710	105	119	360	2,060	740	448	
l(+)*meso*-Lanthionine	1,100	350	1,620	95	170	370	2,010	660	390	
L-Djencolic acid	2,708	1,050	5,820	130	98	127	5,860	3,390	566	
DL-Cystathionine	611	105	582	77	37	676	514	466	369	
DL, DL-Allocystathionine	434	123	384	24	53	210	532	467	368	
S-Methyl-L-cysteine	537	119	710	67	35	475	710	607	484	
S-Methylglutathione	128	15	0	50	63	229	140	314	850	

a, b. These markings refer to Table I.

interesting to find that the formation of keto acids from other closely related compounds was only approximately half of that formed from L-djencolic acid.

L-Djencolic acid and L-cystine were also the most rapidly cleft substrates when the keto acid-forming activity of several crude bacterial enzyme preparations was studied (Table V). As stated earlier, some of these microorganisms are related to those commonly occurring in the human mouth. Thus the test organisms selected served merely as examples of the general ability of various bacteria to form enzymes capable of cleaving L-cystine and related compounds. Due to the evident differences of the bacterial enzyme preparations involved, the results may not be strictly compared with each others. However, it seemed that *Str. pyogenes* 6636 and *L. lactis* possessed considerable enzyme activity under the conditions set up. It is also to be noted that although the solubility of *meso*-lanthionine is 0.022 g in 100 ml of water at 25 °C, while that of the DL-form is 0.15 g under the same conditions (*Greenstein* & *Winitz*, 1961), the cleavage of both was almost the same (this is revealed in Table IV as well). Hence, more substrate was dissolved in the reaction mixture from the undissolved portion of the *meso* form as it was transformed by the enzymes, permitting equal rates of cleavage with the two substrates, despite of differing solubilities.

The formation of keto acids from L-cystine was inhibited by L-cysteine, although the concentrations for the appearance of that phenomenon had to be rather high, *i.e.* about equimolar concentrations of the substrate (at almost 10^{-2} M). The same inhibition was found also with dithiothreitol. No inhibition by L-cysteine and dithiothreitol was found in the concentration range 0.8×10^{-5} M to 1.6×10^{-3} M.

It was thought that free cysteine or other compounds with a thiol group would arise in measurable amounts in the cleavage of L-cystine. Hence many of the reaction mixtures were also titrated spectrophotometrically with p-chloromercuribenzoate according to *Benesch* & *Benesch* (1954). However, no increase in the amount of SH-groups was found. This may have its explanation in other reactions, where the evidently formed cysteine, for example, was rapidly utilized to form other sulphur compounds. Experiments carried out for the detection of inorganic sulphate, with slight modification of *Antonopoulos* (1962), revealed that the use of L-cystine as substrate for a disintegrated plaque enzyme preparation (Bacterial Plaque I) caused the formation of inorganic sulphate in the reaction mixture. This formation of sulphate was not shown to be stoichiometric and varied to a certain extent, depending on the enzyme preparation used.

Several attempts to form pyruvic acid from L-cystine were made in order to show it to be stoichiometric with the disappearance of the substrate.

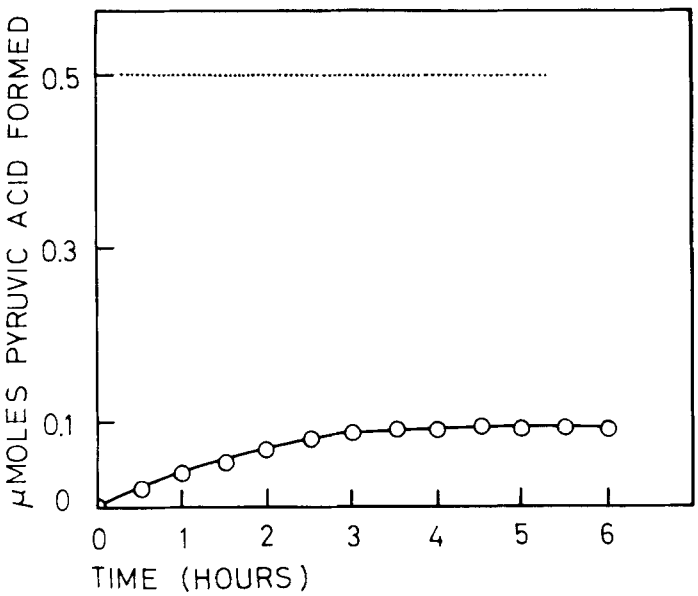


Fig. 5. Production of keto acids, measured as pyruvic acid, from 0.5 μmoles of L-cystine (*i.e.* a tenth of that used in the experiments of Fig. 3). The dotted line in the upper part of the Figure represents the initial amount of L-cystine in the reaction mixture. The lower line describes the formation of pyruvic acid. See text for details.

These resulted in failure. The failure is thought to be due, in part, to the crudeness of the enzyme preparations used. The formed keto acid, e.g., pyruvic acid, might have been rapidly utilized by other enzymes to form other products. This is also shown in Fig. 5 which indicates that the pyruvic acid formed in these experiments never reached the double (1.0 μmoles) value of μmoles of L-cystine initially present (0.5 μmoles) in the reaction mixture, if it is assumed that two pyruvic acid molecules are formed from one molecule of L-cystine. Nor had it reached even the half (0.5 μmoles), if it is assumed that only one molecule of pyruvic acid is formed from one L-cystine molecule. Inasmuch as all the undissolved substrate disappeared during most of the reactions studied, it may be stated that products other than pyruvic acid were also formed, or that the pyruvic acid was itself consumed as it was liberated at the active site of the enzyme molecules. Attempts to detect the formation of ammonia during the enzymic reactions, by the method of *Searsy et al.* (1961), failed too, apparently for the same reasons as above.

An attempt was made to fractionate the keto acid forming enzyme(s) (in Bacterial Plaque I) by DEAE-cellulose chromatography and Sephadex gel filtration with G-100 and G-200 gels. These experiments did not produce any positive results, only low activity was obtained in some of the fractions after DEAE-cellulose chromatography, with L-cystine as a substrate and pyridoxal-5-phosphate as a coenzyme. The activity of these fractions proved to be too low to permit closer study. The same can be said of the Sephadex gel filtrations.

The results obtained by analyzing reaction mixtures with L-cystine as substrate, using an amino acid analyzer, are given in Fig. 6, which shows that the substrate was indeed consumed during the reaction by enzymic catalysis. Several other unidentified ninhydrin-positive peaks (not shown) were also obtained. The relative amount of compounds correlated to these peaks was modified in terms of the nature of the plaque enzyme preparation and the conditions used.

The experiments with labelled cystines are documented in Figs. 7—9. In Fig. 7 it is shown that [3-^{14}C]DL-cystine and [^{35}S]L-cystine had been broken down by an enzyme preparation obtained by disintegrating plaque micro-organisms with ultrasonic treatment. Two points are to be noted in Fig. 7. First, a portion of the substrate had already spontaneously broken down, probably to cysteine or cysteic acid; this is seen from the curves describing the substrate blank. Second, the enzyme blank (actually not a

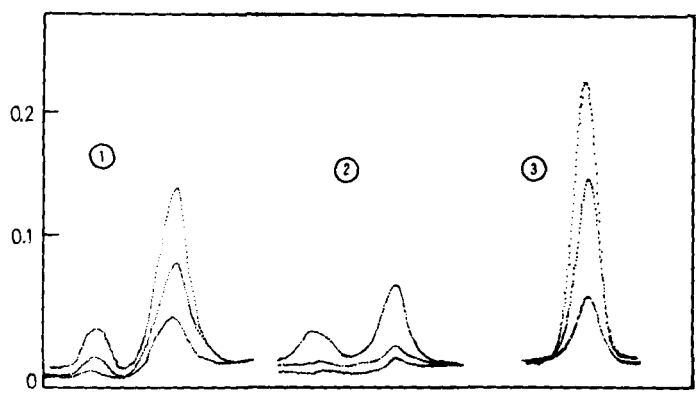


Fig. 6. Patterns showing the consumption of L-cystine by enzymes from a Bacterial Plaque I preparation (the same as given in the legend to Fig. 1). 1: pattern given by the reaction mixture; 2: pattern given by enzyme; 3: pattern given by substrate. The ordinate is ninhydrin color as recorded continuously with the amino acid analyzer. The highest peaks represent cystine.

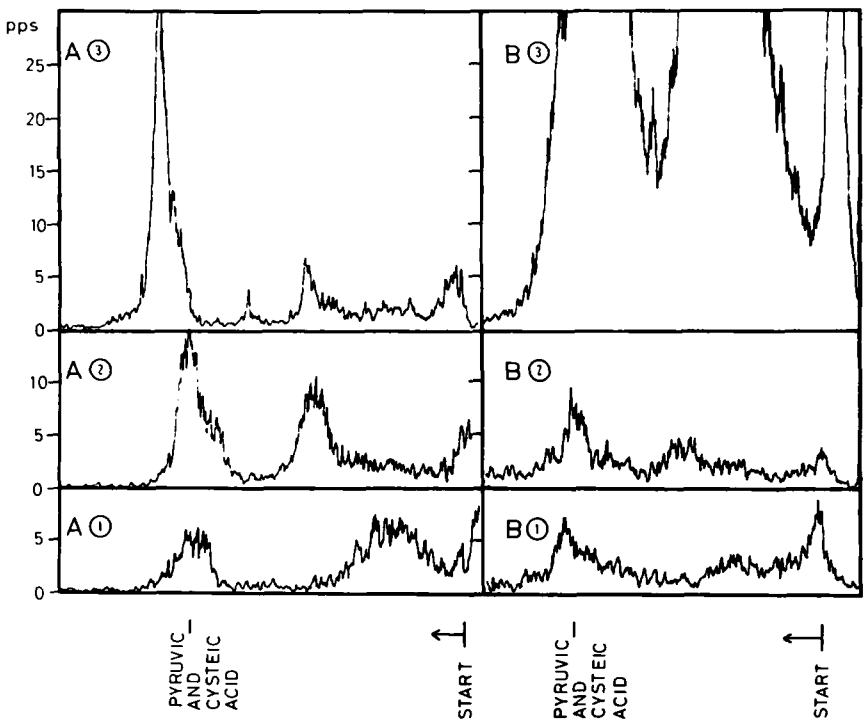


Fig. 7. Radioscans of the reaction mixture containing L-cystine as substrate, pyridoxal-5-phosphate (25 µl of 0.25 mM solution), and enzyme solution (Bacterial Plaque I) analyzed by thin layer chromatography. Incubation time: 1 hr.; development distance (ethanol: water): 15 cm. The instrument settings were: operation voltage 3040 V; time constant 3 sec.; sensitivity 400 mV: measuring range 30 pps; scanning speed 600 mm/hr.; slit width 2 mm, without window. A: experiment with [^{14}C]DL-cystine; 1A: substrate blank (omitting the enzyme but containing both labelled and unlabelled aubstrate). 2A: reaction mixture; 3A: »enzyme blank» (omitting the 5 mg amount of unlabelled L-cystine, but still containing the 5 µl amount of the labelled substrate). B: experiment with [^{35}S] L-cystine, 1, 2, and 3, as above. In B3, the entire labelled substrate was considered to have been broken down, hence the high peaks.

blank mixture, since still containing labelled cystine) gave the clearest result; evidently all of the labelled cystine had been cleft during the reaction. The essential reaction mixtures containing the unlabelled substrate (5 mg) had seemingly low reaction rates because the labelled cystine molecules were randomly picked up by the enzyme. Still, as a matter of fact, even here the cleavage of L-cystine was certainly more rapid. Most of the reacting cystine molecules were unlabelled and were therefore not detectable by the thin layer scanner. It was also found that the distribution of various labelled

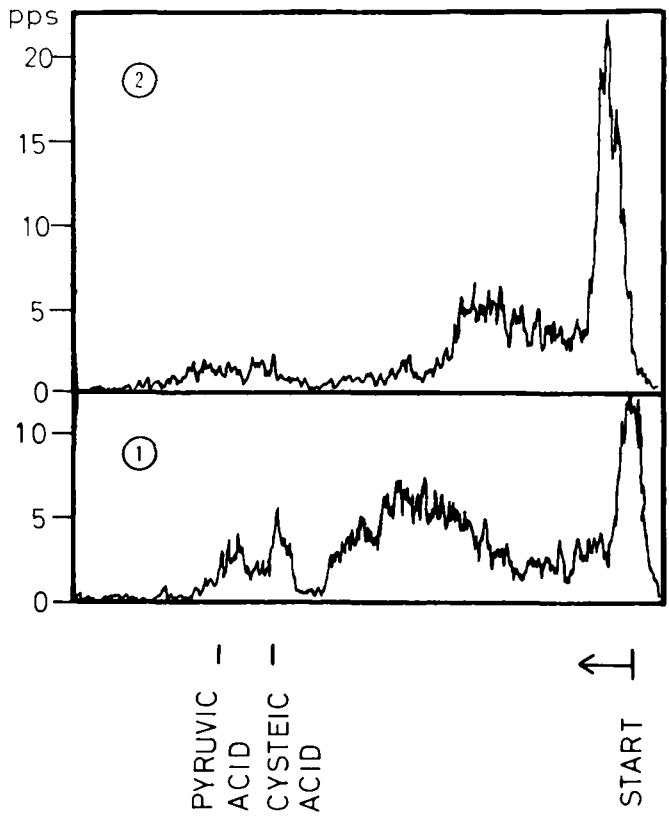


Fig. 8. Effect of $NADPH_2$. Radioscans of the reaction mixtures containing L-cystine as substrate, $NADPH_2$ (25 μl of 0.012 M solution), or water, and enzyme solution (Bacterial Plaque I), analyzed by thin layer chromatography. Only [^{14}C]DL-cystine was used. 1: reaction mixture containing $NADPH_2$; 2: corresponding substrate blank (containing $NADPH_2$). Other details as for Fig. 6.

cleavage products of L-cystine differed, depending on whether [3-^{14}C]DL-cystine or [^{35}S]L-cystine was used. In Fig. 8 the effect of $NADPH_2$ on the cleavage of L-cystine is shown. The presence of the reduced coenzyme in the reaction mixture caused the cleavage of L-cystine in a way different from that in its absence. Finally, Fig. 9 presents the effect of enzyme concentration on the cleavage of L-cystine. In these experiments the reactions were stopped by adding performic acid as described in the *Methods* section. An increase in the amount of the enzyme resulted in an increase in the dissolution of substrate from the undissolved portion and hence also an increase in the

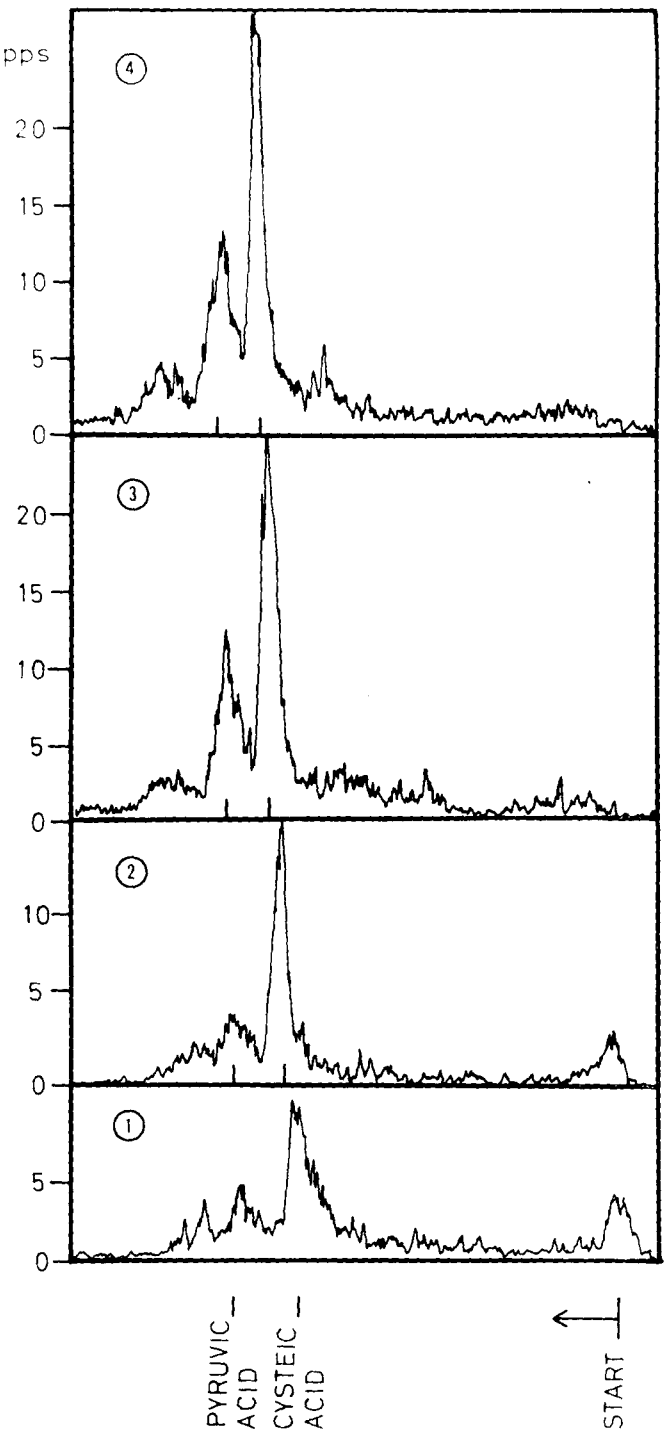


Fig. 9. Effect of enzyme concentration. Radioscans of reaction mixtures containing L-cystine as substrate and varying amounts of enzyme preparation (Bacterial Plaque I) analyzed by thin layer chromatography. No coenzyme was added. The labelled substrate was [^{14}C] DL-cystine, and the reaction were stopped by performic acid as described in the *Methods* section. Amounts of enzyme added: 1. blank (water instead of enzyme); 2: 5 μl; 3: 20 μl; 4: 50 μl. Other details as for Fig. 6.

transformation of L-cystine to various cleavage products. When the reaction mixtures were treated with performic acid before chromatography, the cystine cleaving activity in all cases was seen to lower the radioactivity of the cysteic acid spots on the thin layer plates. On the other hand, this lowering was accompanied by the appearance of radioactivity in areas where pyruvic acid had been assumed to be present (based on comparison of Rf values).

Fig. 7 shows that the substrate already contained at least three ^{14}C-labelled compounds which were also stainable by ninhydrin. In these experiments 95 % ethanol was used as solvent and performic acid oxidation was not carried out before the chromatography. Simultaneous experiments with commercial preparations of cysteine revealed the same spots: the lowest at the application spot; the middle one as an extended spot above the former, corresponding cystine; and the highest one as a sharp-edged area, possessing an Rf value of about 0.7, evidently corresponding to cysteic acid. It is clear that the commercial cysteine preparations contained cystine and cysteic acid, or else they were formed during the incubation (without enzyme or coenzyme) or during the chromatography. However, in all of the experiments with labelled cystines, the presence of enzymes in the reaction mixture caused an increase in the radioactivity of that compound possessing the highest Rf value (about 0.7). In the solvent systems used in the study (96 % ethanol: water, 63:37, w/w, and *n*-butanol:acetic acid:water, 60:20:20:, w/w/w; results with the latter are not shown) both cysteic acid and pyruvic acid possessed rather similar Rf values. These were then used as an indication of the formation of either or both of these compounds in the enzyme reactions.

DISCUSSION

Cysteine and cystine may be important in the oral cavity because of the ease with which a pair of sulphydryl groups of cysteine can be oxidized to give -S-S- bonds of cystine, and vice versa. In the constantly varying conditions of human oral cavity, possibilities always exist for this reversible reaction. For example, all cells in general, including micro-organisms, contain glutathione which reacts rapidly in its oxidized form, as well as nonenzymically, to cysteine, forming cystine and reduced glutathione. Oxidized glutathione is formed, for example, in reaction to ascorbic acid. It could be thus claimed that in the oral cavity also reduction of cystine to cysteine is accomplished by the reversible reaction just mentioned. The cleavage of the disulphide bond of cystine through the action of other reducing agents (cyanides, heavy metals, etc.) is also to be considered.

It has been thought that no enzyme is required to catalyze an enzymic interconversion of cystine to cysteine. Still, so-called disulphide reductases (EC 1.6.4.1) may create this reaction in the oral cavity just as they do in plants and yeast (*Romano & Nickerson*, 1954). The existence in the oral cavity of such an enzyme, however, was not significantly proven by the present results. It is known further that this enzyme requires the cooperation of a reduced coenzyme, e.g. reduced NADP. The experiments with reduced NADP, presented in Fig. 8, may in part support the action also of a disulphide reductase, whereas those given when presenting the results with various coenzymes do not. This ambiquity cannot, of course, be explained easily; still it may very well be a consequence of the use of different enzyme pereparations.

The main sources of the keto acid-forming activity seemed to be the soft bacterial plaque on the teeth and the masses of micro-organisms on other oral surfaces. These enzyme reactions occur in a variety of microorganisms, as also shown by the examples in this paper (Table V). In the deeper layers of this bacterial plaque, cysteine (and hence also cystine) can be converted anaerobically by several routes, among which: the desulphydrase (EC 4.4.1.1) reaction and via transamination (EC 2.6.1.3) with subsequent desulphuration of the keto acid. As for the cysteine desulphydrase reaction (EC 4.4.1.1), it has been shown that the actual substrate is cystine, which is always present in solutions of cysteine, where even traces of cystine catalyze the desulphuration of cysteine (*Flavin*, 1962; *Cavallini, Mondovi, DeMarco & Scioscia-Santoro*, 1962; *Cavallini, DeMarco, Mondovi & Mori*, 1960; *Thibert, Diederich & Kosicki*, 1967).

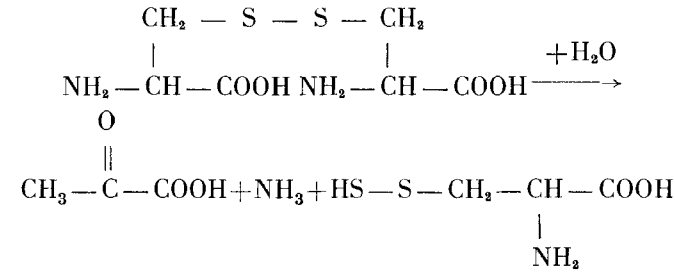
The aerobic formation of pyruvic acid from cysteine via the cysteine sulphinic acid pathway (EC 4.1.1.29) has been demonstrated in animal tissues (*Bergeret, Chatagner & Fromageot*, 1956; *Sörbo & Heyman*, 1957), but it may operate also in micro-organisms, e.g. on the surface layers of plaque.

It is worth observing that the metal cations tested had no effect on, nor did EDTA inhibit, the formation of keto acids from cystine when pyridoxal-5-phosphate was added to the reaction mixture. The formation of a Schiff's metal chelate may not, as well, be an absolute requirement. It has been shown, for example, that pyridoxal phosphate also forms the prosthetic group of α-glucan phosphorylase (EC 2.4.1.1). Pyridoxal phosphate is attached to this enzyme through its aldehyde group, but without forming a Schiff's base. The phosphate group is important for the action of the enzyme, but it is not exchanged during the reaction (*Illingworth, Jansz, Brown & Cori*, 1958; *Kent, Krebs & Fisher*, 1958). *Braunstein* (1960) also concludes

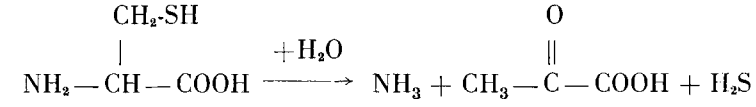
that the experimental evidence of the involvement of metals in activating pyridoxal-5-phosphate enzymes is highly contradictory. Further, *Mahler* and *Cordes* (1966) state that most pyridoxal dependent enzymatic reactions do not require metal ions for activity. Finally, *Thibert, Diederich* and *Kosicki* (1967) have shown that in a model system L-cysteine reacts even in the absence of added metal. An explanation of this sort remains a strong possibility for the results obtained in this study.

It should be remembered that cysteine and cystine certainly form only a small portion of pyruvic acid found in saliva and plaque, more being formed from other amino acids occurring in higher amounts, *i.e.* from glycine, alanine, arginine, valine, glutamic acid, etc. Thus the role of the present enzyme reactions in saliva and plaque must not be over-estimated, their relative signifigance depending on the amount of proper substrates available. However, as a producer of H_2S, the cleavage of cystine and cysteine in the oral cavity is constantly noticeable. Another possible role of these enzymes, the action as protein disulphide reductases in the degradation of oral tissues, remains to be investigated.

In conclusion, the cleavage of cystine, suggested on the basis of the results in this study, may be pictured as:



The corresponding reaction with cysteine would be



When cystathione is the substrate, it is concerted by cystathionase to cysteine, ammonia and a-ketoglutaric acid. All of these enzymic reactions require pyridoxal-5-phosphate as coenzyme. In addition, the evident involvement of reduced nicotinamide-adenine dinucleotide phosphate must then be considered.

Acknowledgement, The skillful technical assistence of Mrs. Irma Rintanen, Aila Karelius and Raili Turta is gratefully acknowledged. The author wishes also to thank the Yrjö Jansson Foundation, Helsinki, Finland, for financial support in carrying out this research project.

SUMMARY

The enzymic cleavage of L-cystine and 18 other related compounds was studied with enzyme preparations obtained from the human oral cavity and from certain cultivated micro-organisms related to those constantly occurring in the human mouth. The cleavage of the substrates was measured 1) by a keto acid method; 2) by using labelled cystines, determining the reaction on thin layer plates with a thin layer scanner and methane gas flow counter; and 3) by determining the consumption of L-cystine by the enzymes using an amino acid analyzer. The keto acid-forming activity was highest in the bacterial plaque. Lower activity was found in centrifuged whole saliva, parotid saliva, and carious dentine. Practically no activity was found in normal dentine. Of the microorganisms, a strain of *Str. pyogenes* and *L. lactis* possessed considerable L-cystine cleaving activity. Pyridoxal-5-phosphate was found to act as a coenzyme in the reactions, but no evidence for the initial formation of a metal chelate of a Schiff's base between the catalyst and substrate was observed. The most rapidly cleft substrates by all of the enzyme preparations studied were L-cystine, L-djencolic acid *meso*-cystine, DL-lanthionine, l(+)*meso*-lanthionine, and DL-cystathionine

RÉSUMÉ

Etudes sur les enzymes buccaux. VII. Action du phosphate du 5-pyridoxal sur la dissociation de la L-cystine et de composés apparentés, par des préparations enzymatiques provenant de la cavité buccale humaine et de certains micro-organismes.

La dissociation enzymatique de la L-cystine et de 18 autres composés apparentés a été étudiée au moyen de préparations enzymatiques provenant de la cavité buccale humaine et de la culture de certains micro-organismes apparentés à ceux qui sont constamment présents dans la cavité buccale humaine. La dissociation des substrats a été mesurée 1) par méthode céto-acide; 2) en utilisant des cystines marquées, la réaction étant déterminée sur plaques de couches minces avec un appareil à balayage pour couche mince et un compteur à débit de gaz méthane; 3) en déterminant la destruction de la L-cystine par les enzymes en utilisant un analyseur d'amino-acides. C'est la plaque microbienne qui présentait l' activité de formation d'acide cétonique la plus élevée. Une activité plus basse a été trouvée dans la salive totale centrifugée, dans la salive parotidienne et dans la dentine carieuse. Dans la dentine normale, on ne trouvait pratiquement pas d'activité. Parmi les micro-organismes, une souche de *Str. pyogenes* et *L. lactis* possédaient

une activité de dissociation de la L-cystine considérable. Le phosphate du 5-pyridoxal agissait comme coenzyme dans les réactions, mais on n'a pas observé de preuve de la formation initiale d'un complexe métallique chélaté d'une base de Schiff entre le catalyseur et le substrat. Les substrats qui étaient dissociés le plus rapidement dans toutes les préparations enzymatiques étudiées étaient la L-cystine, l'acide L-djencolique, la *méso*-cystine, la DL-lanthionine, la l (+)*méso*-lanthionine et la DL-cystathionine.

ZUSAMMENFASSUNG

Untersuchungen über Enzyme in der Mundhöhle VII. Pyridoxal-5-Phosphat abhängige Abspaltung von L-Cystin und nahe verwandten Verbindungen durch Enzympreparaten der Mundhöhle und gewissen Mikro-organismen.

Die enzymatische Abspaltung von L-Cystin und 18 nahe verwandten Verbindungen wurde mit Enzympräparaten untersucht, die aus Mundbakterien und gewissen den Mundbakterien ähnlichen Mikroorganismen entnommen wurden.

Die Untersuchung erfolgte 1) durch Ketosäureverfahren, 2) durch Anwendung von markierten Cystinen mit einer nachfolgenden Reaktionsmessung durch Dünnschichtplatten mit Hilfe von Dünnschicht-Scanner und 3) durch Messung des Abnehmens der Cystin wegen der Enzymtätigkeit mit einem Aminosäureanalyzator. Die stärkste Aktivität in der Ketosäurenbildung wurde in der Bakterienplaque festgestellt. Die Aktivität war schwächer im zentrifugierten Speichel, im Parotisspeichel und im kariösen Dentin. Im gesunden Dentin wurde praktisch keine Aktivität festgestellt. Von den untersuchten Mikroorganismen besass ein Bakterienstand von *Str. pyogenes* und *L. lactis* eine erheblich grosse Menge L-Cystin abspaltender Aktivität. Für die enzymatische Bildung von Ketosäuren war die Gegenwart von Pyridoxal-5-Phosphat in der Reaktionsmasse erforderlich. Dafür aber, dass zwischen dem Enzym und dem Substrat eine der Schiff'schen Base ähnliche Metallchelatbildung entstanden sei, wurden keine Beweise erzielt. In allen untersuchten Substraten spalteten sich L-Cystin, L-Djenkolsäure, *meso*-Cystin, DL-Lanthionin, l(+)*meso*-Lanthionin, und DL-Cystathionin am schnellsten ab.

ADDENDUM

While this paper was in press, studies on the disulphide reductase of *Achromobacter starkei* by *Ruiz-Herrera, Amezcua-Ortega* and *Trujillo* was published (J. Biol. Chem. 243: 4083—4088 (1968)). The enzyme was purified appr. 90-fold. Their success suggests that it might be possible to purify an oral disulphide reductase after such an enzyme has been reliably identified in the human oral cavity.

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