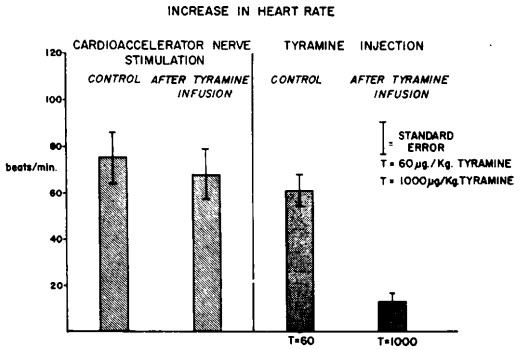

FIG. 1. Average increase in heart rate produced by stimulation of right cardioaccelerator nerve before and after infusion of 200 µg/kg/min of tyramine for 1 hr is shown on the left side of this figure. On the right half, average increases in heart rate produced by 60 µg/kg tyramine in control period and 1000 µg/kg tyramine after tyramine infusion are shown.

tions of this amine almost completely (2). In the present study it was also shown that the physiologic response to tyramine was essentially abolished after a similar infusion. However, there was no significant reduction in chronotropic and inotropic responses to cardioaccelerator nerve stimulation. In a recent study of the mechanism of development of tachyphylaxis to phenylalkylamines, it was suggested that the response of the nictitating membrane to nerve stimulation was unaffected after the development of amphetamine tachyphylaxis(7). Since, in the studies reported herein, nerve stimulation was evidently capable of releasing NE, when the store of NE available to release by tyramine was depleted, it is clear that some of the "available" NE can be released by nerve stimulation but not by tyramine. Thus, in addition to the concept that the NE in sympathetic nerve endings may exist in "bound" and "available" compartments, it must be considered that the NE available for release is further subdivided functionally. This subdivision may result either from heterogeneity of the readily releasable NE, or conceivably from the saturation of an intermediate step in the release of NE by tyramine.

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Desulfuration of Thiourea by Saliva.* (27944)

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The enzymatic desulfuration of thiourea by a cytoplasmic particulate system prepared from sheep thyroid has been described by Maloof and Spector(1). A known association exists between phenylthiocarbamide (PTC) non-tasting, and pathologic thyroid states(2-5). It has also been shown that persons who are relative non-tasters for PTC are relative non-tasters of thiourea and other thioamides such as D,L-goitrin and propylthiouracil(4,6). Because of these findings it was hypothesized that a thioamide desulfurating enzyme might be the common bio-

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chemical link between thyroid function and PTC tasting.

This report describes the enzymatic desulfuration of thiourea by human saliva. Preliminary comparative studies have been made of salivary desulfuration by P.T.C. tasters and non-tasters as well as by various animals.

Methods and materials. Collection of human saliva from volunteers was done without stimulation by repeated spitting into a clean vessel. Animal saliva was obtained by passive collection during general anesthesia; while rattlesnake venom (*Crotalus adamanteus*) was obtained by milking the poison gland into a small beaker. Parotid saliva was obtained from a device attached by suction around the opening of Stensen's duct. The saliva was centrifuged to remove cells and debris and then the human saliva was passed through a Seitz filter. After dialysis of this filtrate against distilled water for 24 hours, it was lyophilized.

S^{35} thiourea was obtained from New England Nuclear Corp. The specific activity was 96 mc per mmole.

The incubation mixture containing saliva, S^{35} thiourea, thiocyanate, ascorbic acid, and 0.15 M Tris buffer‡ pH 7.8 was immersed in a waterbath at 38-39°C for 1 hour. Table I details the concentrations used in a typical determination. The reaction was terminated by immersing the tube in boiling water for 1 minute or, in experiments involving different temperatures, by adding 40% trichloroacetic acid.

Analytical methods. At termination of the reaction 0.1 ml of the mixture was applied in 0.01 ml amounts along a 1.5 cm line on Whatman No. 1 paper. The ascending chromatography system and identification of the spots was as described by Maloof and Spector(1) except that the thiosulfate was located by ultraviolet quenching. In ethanol-ammonium acetate both thiosulfate and sulfate have the same Rf (.24) whereas thiourea has an Rf of .74. The areas on the paper were cut out and placed directly into a toluene scintillation counting solution§ and counted in an Automatic Tri-Carb Liquid Scintillation Spectrometer (Packard Instrument Corp., Model 314A). The over-all counting efficiency of the S^{35} thiourea on the paper was 35%. The amount of desulfuration was represented by the following formula:

$$\frac{\text{cpm on } SO_4 \text{ spot} - C}{\text{total cpm applied to paper}} \times \text{m}\mu\text{moles thiourea} = \text{m}\mu\text{moles thiourea desulfurated}$$

where C is the counts per minute on the sulfate spot from the boiled control reaction. A small amount of non-enzymatic desulfuration occurs with storage or heating.

Protein determinations were by the method of Layne(7).

Taste sensitivity for phenylthiocarbamide was determined as previously described(4).

Electrophoretic separation of sulfite and thiosulfate from sulfate was done according to the method described by Maloof and Spector(1). Thiocyanate has an Rf of .81 in the ethanol-ammonium acetate system mentioned above.

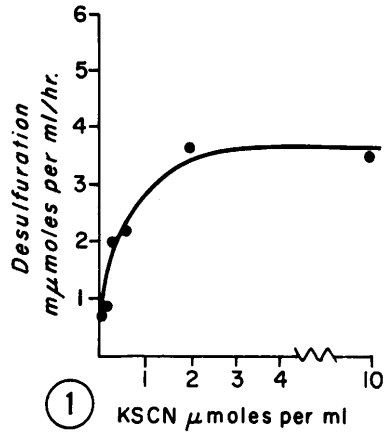
TABLE I. Requirements for Desulfuration of Thiourea by Dialyzed and Lyophilized Saliva. Complete system contained dialyzed and lyophilized saliva (0.41 mg protein), 10^{-2} M KSCN, 5×10^{-4} M ascorbic acid, 3.95×10^{-5} M S^{35} thiourea, 0.15 M Tris buffer, pH 7.8 in a final volume of 1.0 ml incubation, 1 hr at 39°C.

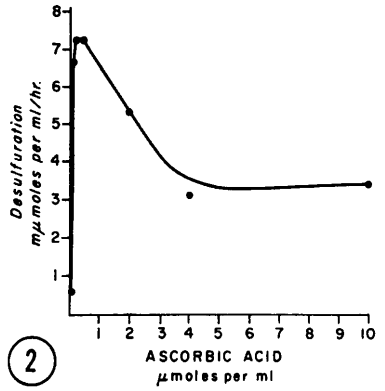
System	Thiourea desulfurated, mμmoles
Complete system	7.3
Idem minus ascorbic acid	.8
" minus thiocyanate	1.7
" (with boiled saliva)	.0
" (without saliva)	.0

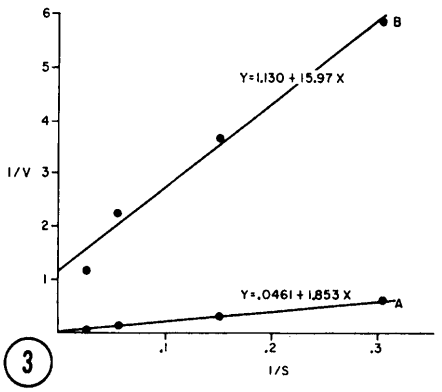
Results. Table I represents a typical desulfuration experiment and demonstrates the strict requirement for thiocyanate and ascorbic acid. Dialysis with subsequent controlled addition of these cofactors is requisite for uniformity of results. Variable small amounts of these activators are present in fresh undialyzed saliva. Thiocyanate saturates the system at 2×10^{-3}M (Fig. 1). Substitution of thiocyanate by perchlorate, cysteine, or reduced glutathione resulted in complete

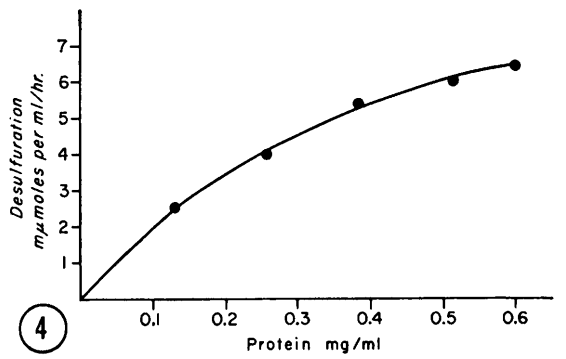
‡ Tris, (tris hydroxymethyl) aminomethane.

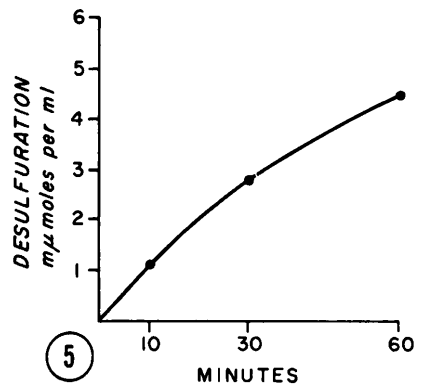
§ 5.0 g P.P.O. (2,5-diphenyloxazole), 200 mg P.O.P.O.P. (1, 4-bis-2-(5 phenyloxazolyl)-benzene) dissolved in 1 liter of toluene.











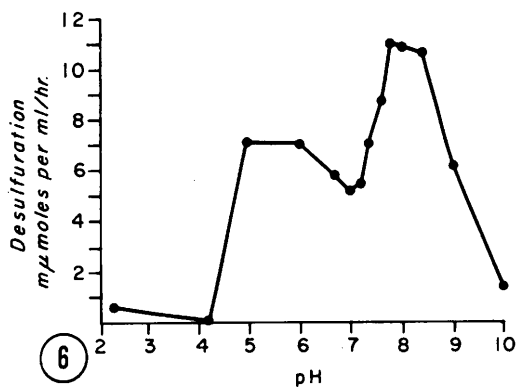


FIG. 1. Amount of thiourea desulfurated as a function of thiocyanate. Reaction conditions were as in Table I except concentration of ascorbic acid was 10^{-2}M. Protein concentration, 0.4 mg/ml.

FIG. 2. Amount of thiourea desulfurated as a function of concentration of ascorbic acid. Incubation conditions as in Table I except for changes in ascorbic acid concentration as shown and concentration of thiocyanate was maintained at 10^{-2}M. Protein concentration, 0.4 mg/ml.

FIG. 3. Lineweaver-Burke plots of uninhibited reaction (A) and reaction inhibited by 3×10^{-4}M propylthiouracil (B). Reaction conditions were as detailed in Table I except for variation of substrate. 0.5 mg protein/ml.

FIG. 4. Amount of thiourea desulfurated as a function of protein concentration. Incubation conditions as in Table I.

FIG. 5. Amount of thiourea desulfurated as a function of time. Incubation conditions as in Table I except reaction mixture contained 0.5 mg protein/ml.

FIG. 6. Effect of pH on desulfuration of thiourea. Reaction conditions were as in Table I except for composition of buffers. At pH's of 6.0, 6.7, 7.0, 7.2, 7.4, 7.6, 7.8, 8.0, 8.2, 8.4, and 9.0 the buffer was Tris .15M. At pH 2.3 a potassium chloride buffer 0.08M was employed and at pH's of 4.2 and 5.0 a 0.025M potassium acid phthalate buffer was used. At pH 10 the buffer consisted of 0.025M boric acid and 0.025M KCl.

inactivity but potassium iodide in a concentration of 10^{-3}M gave some measurable desulfuration. The requirements for ascorbic acid are between 2.5-5.0 $\times$ 10^{-4}M (Fig. 2). DPNH can be substituted for ascorbic acid, but is less effective.

The identified reaction products include sulfate (about 55% of the products), thiosulfate or sulfite (9%) and a protein bound fraction (36%). No thiocyanate was found. We were unable to separate thiosulfate from sulfite.

Passage through a bacterial filter did not reduce enzymatic activity. After 1 hour's centrifugation at 105,000 $\times$ *g* (Spinco model L) over 90% of the enzyme action remained in the supernatant fraction. Storage of filtered *non-dialyzed* saliva at room temperature for 24 hours reduced desulfurase activity by 60% but storage at 7°C or freezing for up to 3 months did not alter activity. No activity was lost by heating to 70°C for 15 minutes. Boiling for 1 minute completely inactivated the enzymatic reaction as did the addition of 40% trichloroacetic acid in a volume equal to one-tenth of the reaction solution.

Reconstituted dialyzed and lyophilized material was slightly less active than the freshly collected saliva.

The reaction system is saturated with thiourea at a concentration of 40 mμmoles per ml. The Lineweaver-Burke Plot (Fig. 3) suggests a first order reaction. Ks was calculated to be 40.162 and Vmax 21.69. S^{35} cystine was not desulfurated in this system.

The desulfuration of thiourea is a function of protein concentration and of time (Fig. 4, 5).

The pH optimum is between 7.8 and 8.4 (Fig. 6). The optimum temperature for the reaction is between 38 and 40°C.

The results of inhibition studies are presented in Table II. In Fig. 3 the Lineweaver-Burke plot for the propylthiouracil-inhibited reaction is given and this result does not suggest a competitive inhibition. Desulfuration of thiourea is inhibited 26% by anaerobiosis.

TABLE II. Inhibition of Salivary Desulfuration of Thiourea. Incubation conditions as in Table I.

	Concentration, M	Inhibition, %
Sulfate	1×10^{-2}	0
Thiosulfate	1×10^{-3}	100
"	1×10^{-4}	27
"	1×10^{-5}	0
Sulfide	1×10^{-4}	37
Urea	1×10^{-2}	0
Perchlorate	1×10^{-2}	0
Iodide	1×10^{-2}	0
Cyanate	1×10^{-3}	100
Iodoacetate	1×10^{-2}	100
"	1×10^{-3}	34
Dinitrophenol	1×10^{-2}	0
Glutathione (reduced)	1×10^{-3}	100
" "	1×10^{-4}	42
L-goitrin	1×10^{-3}	100
"	1×10^{-4}	87
Propylthiouracil	1×10^{-3}	100
"	1×10^{-4}	85
Cysteine	1×10^{-3}	73

Saliva from 4 PTC-tasters was compared with that of 4 non-tasters. No significant differences in enzymatic activity were observed in freshly collected filtered saliva, in dialyzed, or in reconstituted dialyzed and lyophilized samples.

The salivas from a lamb and a lion cub were shown to contain desulfurases. Saliva from a heifer calf did not. Fresh rattlesnake venom did not desulfurate thiourea. The desulfurase activity in samples of human parotid saliva was equal to that of whole saliva.

Discussion. The desulfuration of thiourea by human saliva fits the common criteria for a first order enzyme reaction. There is no assurance that only a single enzyme is present; in fact, pH studies (Fig. 6) and preliminary analysis of starch electrophoresis data suggest there is more than one enzyme.‖

The parotid appears to be one source of the enzyme; other salivary glands have not been investigated. Particulate fractions of the posterior superficial portion of sheep

‖ Shepard, T. H., unpublished data.

tongue were found to have desulfurase activity but similar preparations from the anterior portion of the tongue and from the liver were inactive. The posterior portion of sheep tongue differs from the anterior portion by containing the circumvallate papillae and taste buds in addition to the glands of Von Ebner. The source of the activity in this area is not known.

The sheep thyroid particulate enzyme described by Maloof and Spector(1) is similar to the salivary enzyme in requiring the 2 cofactors thiocyanate and ascorbic acid, but the salivary enzyme has stricter requirements for ascorbic acid. Both ascorbic acid and thiocyanate have been detected in normal human saliva. The salivary enzyme is for the most part water soluble whereas the sheep thyroid enzyme is particulate(1). The pH optima differ. Sheep thyroid desulfurase is inhibited by iodide, sulfate and dinitrophenol whereas the salivary enzyme is not. Both enzymes are inhibited by cysteine, reduced glutathione, and thiosulfate(1). Neither enzyme was inhibited by urea or perchlorate (1).

The hypothesis that thioamide desulfuration represents a biochemical link between genetically inherited PTC non-tasting and abnormal thyroid metabolism has not been supported by these studies.

Summary. A soluble enzyme system from human saliva which desulfurates thiourea has been shown to be heat-labile, non-dialyzable and temperature and pH dependent. Thiocyanate and ascorbic acid are necessary cofactors. Propylthiouracil appears to behave like a non-competitive inhibitor. The products of this reaction are protein bound sulfur, thiosulfate or sulfite, and sulfate. Lamb and lion cub saliva desulfurate thiourea, but rattlesnake venom and calf saliva do not. Preliminary studies of saliva from phenylthiocarbamide tasters and non-tasters have shown no differences in desulfuration activity.

The authors appreciate advice generously given by Dr. Melvin Fried.

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Relation Between Myelination and Binding of Sodium and Potassium in Rat Brain.* (27945)

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Using a dialysis technic to measure extent of binding, Hayden *et al.*(1) suggested that such binding of sodium and potassium in guinea pig brain homogenate may be related to its myelin content. In view of conflicting reports by other investigators(2-5), it was felt that the study should be verified in another species and in animals of various ages. Differences in technic and different uses of the term "binding" undoubtedly account for most of the discrepancies reported. Stone and Shapiro(2), for example, filtered brain and muscle homogenates through a cellophane membrane under negative pressure at 3-4°C and found that 20% of potassium would not diffuse through the cellophane

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