Journal of Clinical Investigation
Vol. 43, No. 10, 1964

The Induction of Proteolysis in Purulent Sputum by Iodides *

JACK LIEBERMAN AND NATHANIEL B. KURNICK

(From the Veterans Administration Hospital, Long Beach, and the Department of Medicine, the University of California Center for Medical Sciences at Los Angeles, Calif.)

Iodide, in the form of potassium or sodium iodide, is widely used as an expectorant for patients with viscid sputum. The iodides are thought to act by increasing the volume of aqueous secretions from bronchial glands (1). This mechanism for the effect of iodides probably plays an important role, but an additional mechanism whereby iodides may act to thin viscid respiratory secretions was demonstrated during a study of the proteolytic enzyme systems of purulent sputum.

Purulent sputum contains a number of proteolytic enzymes, probably derived from leukocytes (2–4). These proteases, however, are ineffective in causing hydrolysis of the native protein in purulent sputum and do not appear to contribute significantly to the spontaneous liquefaction of these purulent secretions. On the other hand, these intrinsic proteases are capable of causing proteolysis after the exudative protein is separated from deoxyribonucleic acid (DNA) by the action of deoxyribonuclease (DNase) or high concentrations of sodium chloride (5).

During these studies an attempt was made to evaluate the effectiveness of various salts for inducing proteolysis in purulent sputum, ostensibly by liberating protein from nucleoprotein complexes. Sodium iodide was found to induce more rapid proteolysis than any other salt tested, but we were able to demonstrate that the mechanism did not primarily involve the dissolution of nucleoprotein complexes. In addition, iodinated tyrosine and thyronine compounds were found to have even greater proteolysis-inducing activities than inorganic iodide.

Methods

Source of sputum specimens. Purulent sputa were obtained from 17 patients with cystic fibrosis and from 22 with other types of pulmonary problems (Table VIII). Two specimens of pus were obtained and studied in a similar fashion. The sputa were collected in glass jars kept in the patients' home freezers at approximately $-10°$ C over a 2- to 5-day period and then were kept frozen at $-20°$ C up to 3 weeks. Sputa collected from hospitalized patients were frozen after a 4-hour collection period.

Preparation of sputum homogenates. Individual sputum specimens were homogenized in distilled water with a Potter-Elvehjem homogenizer to form 10% (wet weight to volume) homogenates. The 10% homogenates were diluted further in some experiments. In certain experiments fresh sediments were prepared by centrifugation [1] of the 10% aqueous sputum homogenates at 30,000 $g \times 15$ minutes at 0° C. The supernatant fluids were decanted, and the sediments were washed where indicated by resuspension to the initial volume followed by recentrifugation.

Assay of proteolysis. Proteolysis was determined routinely by measuring the liberation of trichloroacetic acid (TCA)-soluble protein fragments by the Folin-Ciocalteu (F-C) test. The 10% homogenate of sputum and the reagent whose proteolysis-stimulating effect was being tested were both adjusted to the appropriate pH (see below) with 0.1 N NaOH or HCl, before being mixed in equal volumes. The mixture was then incubated in duplicate in a 37° C water bath. Two-ml samples were removed at suitable intervals, added to 4.0 ml 16% TCA, and centrifuged at 30,000 g for 15 minutes. The supernatant fractions (2.0 ml) were mixed with 4.0 ml of 1 N NaOH in colorimetric cuvettes, and 1.0 ml of F-C reagent (diluted 1:3 with distilled water) was added to each cuvette and mixed immediately. Exactly 5 minutes later the absorbance was measured on a Coleman Junior spectrophotometer at 675 mμ against a reagent blank. Proteolysis is expressed as the optical density developed in a given period of incubation. A similar proteolytic assay utilizing F-C reagent was used with

* Submitted for publication October 9, 1963; accepted June 15, 1964.

Presented in part before the Western Section of the American Federation for Clinical Research, Carmel, Calif., January 30, 1963.

Aided by a grant from the California Research and Medical Education Fund of the Tuberculosis & Health Association of California and grants HE1880 and CA-0382-07 from the National Heart and National Cancer Institutes, U. S. Public Health Service.

[1] Servall refrigerated automatic centrifuge, SM-24 rotor.

extraneous protein substrates such as casein, hemoglobin, gelatin, and albumin.

Three other methods for detecting proteolysis were utilized to confirm the observation of iodide-induced proteolysis. The first of these methods assayed the tyrosine content of the TCA-insoluble sediment, instead of the supernatant fluid, with the F-C reagent as follows: After centrifugation of the TCA mixture, the sediment was washed once with 10% TCA, then dissolved in a volume of 1 N NaOH equal to that of the initial TCA mixture. Two ml of the dissolved sediment was then mixed with 4.0 ml 1 N NaOH and finally with 1.0 ml of the F-C reagent; the absorbance was measured on the spectrophotometer at 675 mμ against a reagent blank.

In a second supplementary method of assaying proteolysis, the absorbance of the TCA-supernatant fluid was measured directly with a Beckman DU spectrophotometer at 280 mμ against a water blank.

The third supplementary method for assaying proteolysis involved the assay of alpha-amino nitrogen with Ninhydrin according to the method of Moore and Stein (6). A 0.2-ml sample of the TCA-supernatant fluid was mixed with 1.3 ml of 0.1 N NaOH (to adjust to pH 5.0) and with 2.0 ml of the Ninhydrin solution. The mixture was heated in a boiling water bath for 20 minutes, followed by the addition of 15 ml of diluent (a 50: 50 mixture of *n*-propanol with water) and vigorous shaking for 1 minute. The mixture was poured into colorimeter tubes and the absorbance determined against a reagent blank at 570 mμ on a Coleman Junior spectrophotometer.

Preparation of iodinated tyrosine and thyronine solutions. The iodinated organic compounds used in this study were difficult to dissolve in neutral buffer solutions. The following procedure was used routinely for preparing 25.0 ml of such solutions in 0.05 M, pH 7.5 sodium phosphate buffer. The iodine compound was weighed and then dissolved in 2.0 ml of 0.1 N NaOH. Fifteen ml of distilled water and 2.5 ml 0.5 M, pH 7.5 sodium phosphate buffer were then added, and the mixture was brought to pH 7.5 with 0.1 N HCl while being stirred constantly. The volume was adjusted to 25.0 ml with distilled water.

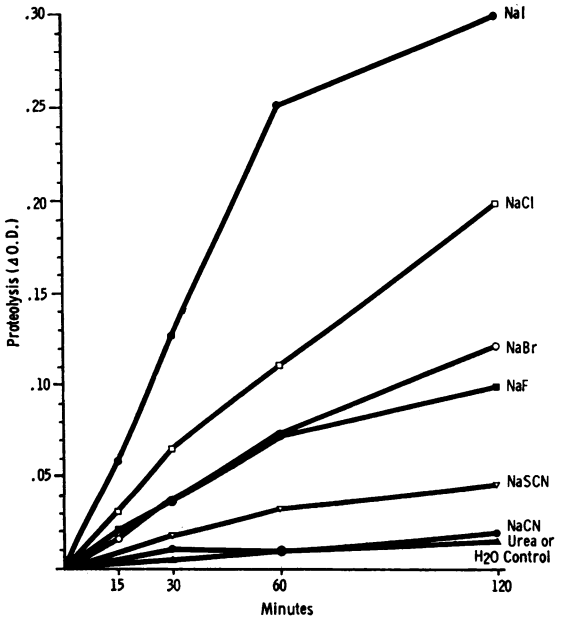

FIG. 1. INFLUENCE OF VARIOUS IONS AND UREA ON PROTEOLYSIS IN PURULENT SPUTUM AT pH 7.5 (CONCENTRATION OF EACH ADDITIVE =1 M).

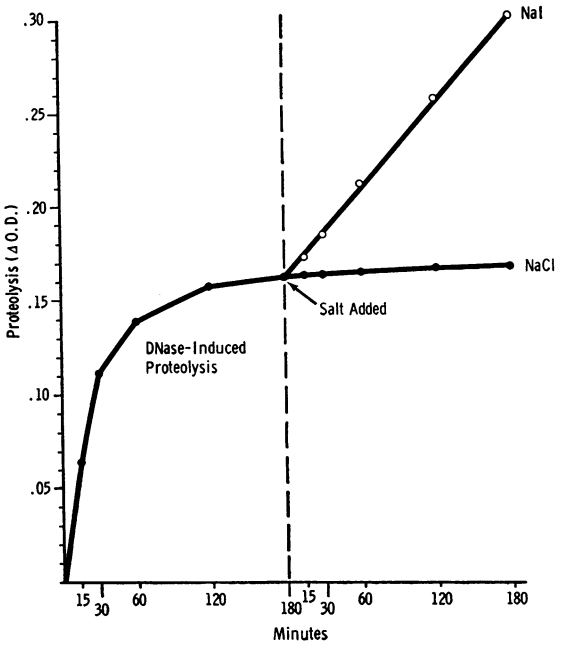

FIG. 2. ADDITIVE EFFECTS OF 1 M NaI OR NaCl ON DNASE-INDUCED PROTEOLYSIS IN PURULENT SPUTUM.

Reagents. Reagent grade chemicals were used. Soybean-trypsin inhibitor (5× crystalline);[2] L-3,5-diiodothyronine and its analogues, L-3,5-diiodotyrosine, D-3,5-diiodotyrosine, L-3,5-dibromotyrosine, and tyrosine;[3] L-3,3′,5-triiodothyronine, L-thyroxine, D-thyroxine, L-3-monoiodotyrosine, and DL-3,5-diiodothyronine;[4] deoxyribonuclease (1× crystallized);[5] and trypsin (2× crystalline)[2] were obtained commercially.

Results

The experimental data to be described were obtained from experiments with individual sputum specimens, but each experiment was repeated with sputa obtained from at least four other individuals. The absolute proteolytic activity varied from pa-

[2] Nutritional Biochemicals Corp., Cleveland, Ohio.

[3] Cyclo Chemical Corp., Los Angeles, Calif.

[4] California Corporation for Biochemical Research, Los Angeles, Calif.

[5] Worthington Biochemical Corp., Freehold, N. J.

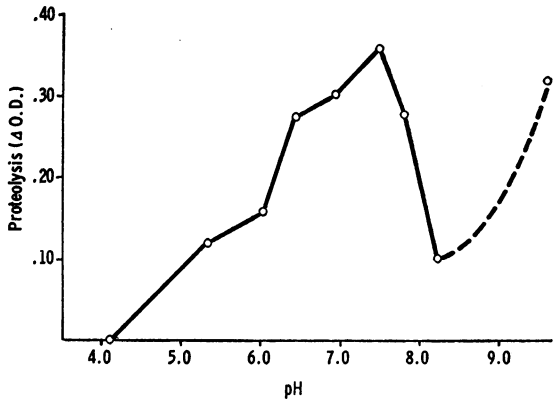

FIG. 3. OPTIMAL pH CURVE FOR PROTEOLYSIS INDUCED BY 1 M NaI IN A HOMOGENATE OF PURULENT SPUTUM.

tient to patient (4), but the qualitative results described below were characteristic of all specimens examined.

Effect of various electrolytes

Figure 1 shows the rates of proteolysis at pH 7.5 in 5% sputum homogenates in the presence of various 1 M salt solutions or urea or with distilled water as a control. Sodium iodide (or potassium iodide) caused a higher rate of proteolysis than that resulting from any of the other electrolytes tested, although the other halogens, namely, chloride, bromide, and fluoride, also caused some proteolysis. Sodium thiocyanate induced only minimal proteolysis. The effect of sodium cyanide or urea was insignificant and did not differ appreciably from the control.

A difference between iodide- and chloride-induced proteolysis was demonstrated by the experiment shown in Figure 2. The proteolysis resulting from the action of DNase (1.0 mg DNase + 5.0 ml sputum homogenate + 5.0 ml 0.05 M sodium phosphate buffer, pH 7.5) appeared to be complete by the end of a 3-hour incubation period (Figure 2). At this time an equal volume of 2 M NaCl was aded to one portion of the reactant mixture, and 2 M NaI was added to another portion of the mixture. The addition of NaCl did not result in further proteolysis, but the addition of NaI renewed the proteolytic action.

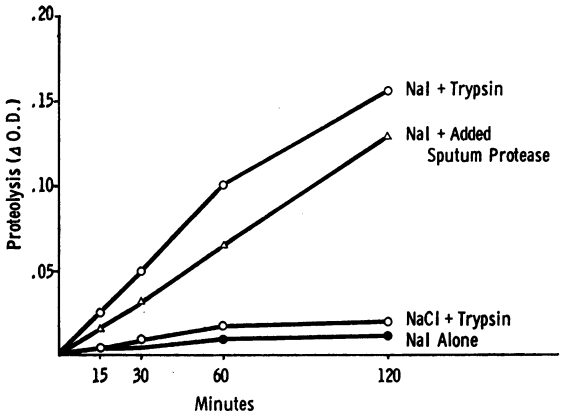

FIG. 5. INFLUENCE OF 1 M NaI OR NaCl ON PROTEOLYSIS BY TRYPSIN (0.2 MG PER ML) OR SPUTUM PROTEASE (0.5 ML OF UNHEATED SPUTUM HOMOGENATE PER 5.0 ML) WITH 80° C-HEATED SPUTUM AS SUBSTRATE (0.025 M SODIUM PHOSPHATE BUFFER, pH 7.5).

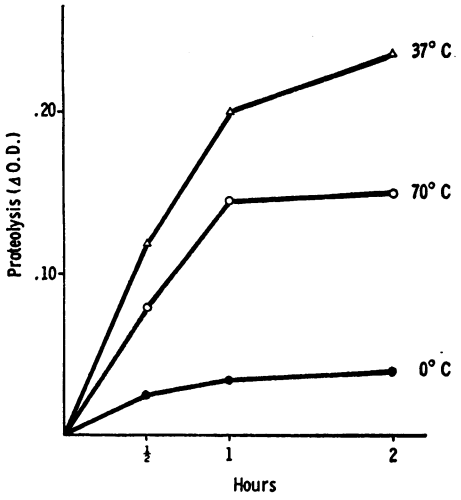

FIG. 4. EFFECT OF TEMPERATURE UPON PROTEOLYSIS INDUCED BY 1 M NaI IN A HOMOGENATE OF PURULENT SPUTUM (0.025 M SODIUM PHOSPHATE BUFFER, pH 7.5).

Enzymatic nature of iodide-induced proteolysis

Optimal pH. The proteolytic effect of sodium iodide was studied at the various pH levels shown in Figure 3. An optimum between pH 7.0 and 7.5 was demonstrated. At pH values above 9.0 the high electrolyte concentrations caused hydrolysis that is nonenzymatic, as was previously observed with NaCl (5).

Effect of temperature. Proteolysis was more active at 37° C than at 70° or 0° C (Figure 4). The proteolytic reaction could be prevented by boiling the sputum preparation or by heating at 80° C for 1 hour before mixing with sodium iodide (Figure 5, bottom curve). The native proteases within purulent sputum were found to be

completely destroyed by heating at 80° C (3). The addition of trypsin or of a small amount of unheated sputum to the heated sputum preparation resulted in active proteolysis in the presence of 1 M sodium iodide, but not with 1 M sodium chloride.

Effect of soybean-trypsin inhibitor. Soybean-trypsin inhibitor (SBTI) has been observed to inhibit a "heat-labile" protease (inactivated at 65° C) in purulent sputum when tested upon a casein substrate, but not to inhibit a "heat-stable" protease (3). A similar result was obtained when sputum protein, instead of casein, was used as substrate. SBTI (2.3 mg per ml) caused 70% inhibition of iodide-induced proteolysis (1-hour incubation) in an unheated sputum homogenate, but did not significantly inhibit iodide-induced proteolysis of a 65° C-heated sputum preparation (Table I).

Optimal concentration of sodium iodide

Various concentrations of sodium iodide were tested with a sputum homogenate to determine the effect of iodide concentration upon the induction of proteolysis. Two types of sputum preparations were used for this experiment: (A) a routine sputum preparation that had not been heated before the experiment and (B) another portion of the same sputum homogenate that had been heated at 65° C for 1 hour to destroy the "heat-labile" protease. As can be seen in Figure 6, iodide caused more proteolysis in the unheated sputum during a 1-hour incubation period than in the 65° C-heated preparation. Both systems demonstrated optimal enhancement of proteolysis at 1 M NaI concentration.

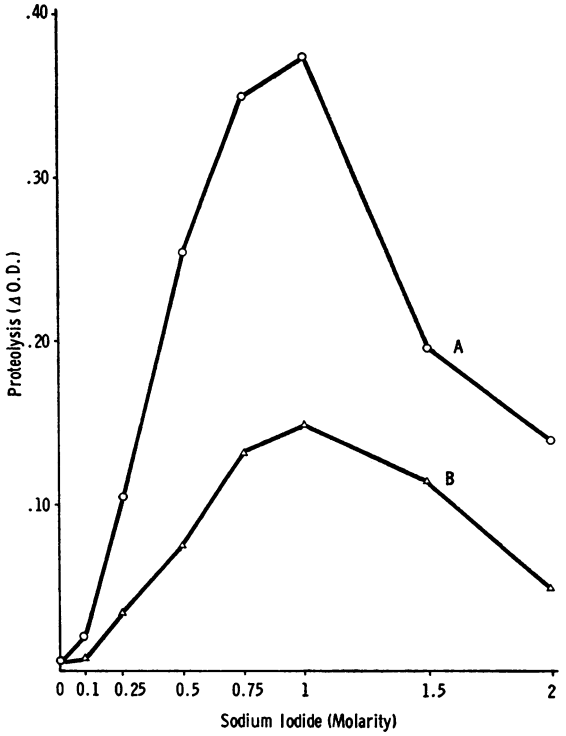

FIG. 6. OPTIMAL NaI CONCENTRATION FOR THE INDUCTION OF PROTEOLYSIS IN (A) AN UNHEATED SPUTUM HOMOGENATE AND (B) A SPUTUM HOMOGENATE PREVIOUSLY HEATED AT 65° C FOR 1 HOUR. The unheated sputum preparation contains a "heat-labile" protease that is destroyed by heating at 65° C for 1 hour.

TABLE I

*Effect of soybean-trypsin inhibitor (SBTI) upon the induction of proteolysis by 1 M sodium iodide in purulent sputum**

Sputum preparation	Spontaneous proteolysis (OD)†			NaI-induced proteolysis (OD)†						% Inhibition
				Without SBTI			With SBTI			
	0 hr	1 hr	ΔOD	0 hr	1 hr	ΔOD	0 hr	1 hr	ΔOD	
Unheated sputum homogenate	0.201 0.205	0.200 0.203	0	0.237 0.240	0.483 0.487	0.248	0.210 0.205	0.280 0.271	0.068	70
65° C-heated sputum homogenate	0.191 0.190	0.183 0.191	0	0.220 0.220	0.291 0.292	0.072	0.205 0.215	0.274 0.276	0.065	10

* SBTI powder (7.0 mg) was mixed with samples (1.5 ml) of unheated and of 65° C-heated sputum homogenates and allowed to stand at room temperature for 20 minutes. NaI (1.5 ml of 2 M NaI in 0.05 M sodium phosphate buffer, pH 7.5) was added to these samples and to samples of the same sputum homogenate lacking SBTI. The mixtures were incubated at 37° C and assayed for proteolysis with Folin-Ciocalteu (F-C) reagent as described under methods. Spontaneous proteolysis was measured in a mixture of 1.5 ml sputum homogenate with 1.5 ml, 0.05 M, pH 7.5 sodium phosphate buffer.

† OD = optical density of simultaneous duplicate experiments.

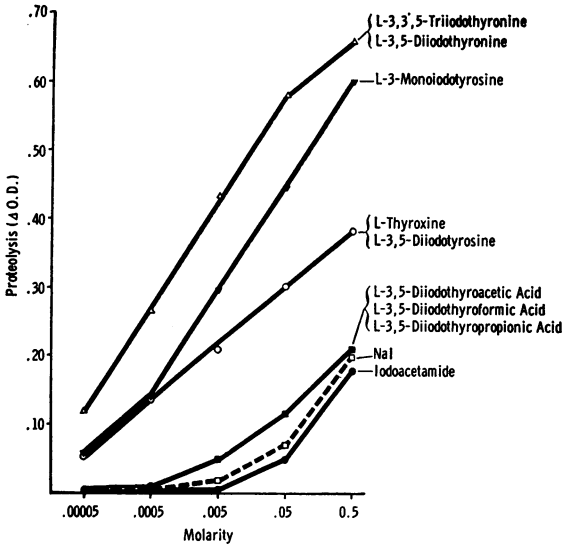

FIG. 7. INFLUENCE OF ORGANIC IODIDES ON PROTEOLYSIS IN PURULENT SPUTUM (1-HOUR INCUBATION) COMPARED TO NaI.

Concentrations of NaI as low as 5 mM appeared capable of inducing measurable proteolysis during a 1-hour incubation period. However, when the incubation period was extended to 24 to 48 hours, enhanced proteolysis could be detected with concentrations of NaI as low as 0.05 mM (Table II). Spontaneous proteolysis was also measurable during this prolonged incubation but did not equal that occurring in the presence of NaI.

Effect of iodinated tyrosine and thyronine compounds

Various iodinated tyrosine and thyronine compounds were tested for their ability to induce proteolysis in purulent sputum. Figure 7 shows the level of proteolysis induced by these compounds at different molar concentrations during a 1-hour incubation period. At 0.5 M concentration, the most active compounds, di- and triiodothyronine and monoiodotyrosine (MIT), produced approximately 3 times as much proteolysis as sodium iodide. Tetraiodothyronine (thyroxine) and diiodotyrosine (DIT) were also more active than sodium iodide, but less active than monoiodotyrosine or di- and triiodothyronine. Analogues of diiodothyronine, in which the alanine chain is replaced by propionic, formic, or acetic acids, were much less effective than the thyronine compound and showed no greater proteolysis-inducing action than did sodium iodide. Iodoacetamide, an inhibitor of many enzymes, promoted proteolysis in sputum similar to sodium iodide.

Tyrosine itself had no proteolysis-inducing action, and substitution of bromide ion for iodide on the tyrosine did not allow proteolysis to proceed (Figure 8).

Optimal concentration. The graphs in Figure 7 demonstrate that 0.005 to 0.0005 M concentrations of the iodinated tyrosine and thyronine compounds induced proteolysis equal to that resulting from 0.5 M sodium iodide. Further increases in concentration of these organic compounds promoted even higher rates of proteolysis (Figure 7), but this was shown to be dependent upon the concentration of sputum being used as substrate (Figure 9).

A series of dilutions of heat-inactivated (80° C for 1 hour) sputum homogenate was mixed with equal volumes of a series of DIT concentrations

TABLE II

*Effect of low concentrations of NaI and prolonged incubation upon proteolysis of purulent sputum**

Time of incubation	Proteolysis							
	5 mM NaI		0.5 mM NaI		0.05 mM NaI		Spontaneous proteolysis control	
	OD	ΔOD	OD	ΔOD	OD	ΔOD	OD	ΔOD
0 hr	0.168, 0.170 → 0.169		0.163, 0.165 → 0.164		0.160, 0.168 → 0.164		0.160, 0.165 → 0.163	
24 hr	0.365, 0.363 → 0.364	0.195	0.305, 0.307 → 0.306	0.142	0.221, 0.225 → 0.223	0.059	0.200, 0.206 → 0.203	0.040
48 hr	0.470, 0.473 → 0.472	0.303	0.413, 0.417 → 0.415	0.251	0.310, 0.321 → 0.316	0.152	0.251, 0.255 → 0.253	0.090

* 5% sputum homogenate in 0.025 M sodium phosphate buffer, pH 7.5.

in 0.05 M sodium phosphate buffer, pH 7.5. Trypsin (0.5 ml of 2.0 mg per ml in 0.001 N HCl) was added to each mixture and to DIT blanks (no sputum) and incubated at 37° C for 1 hour. The rate of proteolysis was determined in the usual manner, and the control values for trypsin autolysis in DIT were subtracted. Figure 9 shows that maximal proteolysis was induced in the more dilute sputum homogenates by lower concentrations of DIT than were needed to induce maximal proteolysis in the more concentrated sputum homogenates. This direct relationship between DIT and sputum concentration is suggestive of a stoichiometric reaction. When the concentration of sputum homogenate was in excess relative to DIT [i.e., homogenate concentration (wt/vol) : DIT (molarity) > 1], the rate of the reaction was a function of DIT concentration. Similarly, when the concentration of DIT was in excess relative to the sputum concentration, the rate of the reaction was a function of the sputum concentration. Concentrations of sputum greater than 5% resulted in the same curve as that for 5% (Figure 9), indicating that substrate was present in excess.

Importance of the stereochemical structure of iodinated tyrosine and thyronine. Figure 10 shows the comparative rates of proteolysis caused by dextro- and levorotatory compounds in equimolar (0.005 M) concentrations. The mixture of DL-

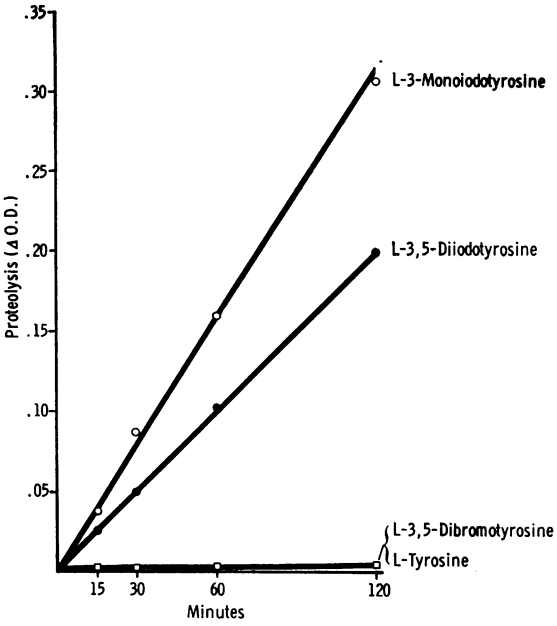

FIG. 8. EFFECT OF IODINATION OR BROMINATION UPON THE PROTEOLYSIS-INDUCING ACTION OF TYROSINE (5 mM) IN PURULENT SPUTUM.

diiodothyronine and the D isomers of thyroxine and diiodotyrosine are much less active than the pure L compounds. Thus, the stereochemical structure of the amino acid to which the iodide is attached appears to be important for causing maximal proteolysis within the purulent sputum.

This enhanced role for the L isomers was shown

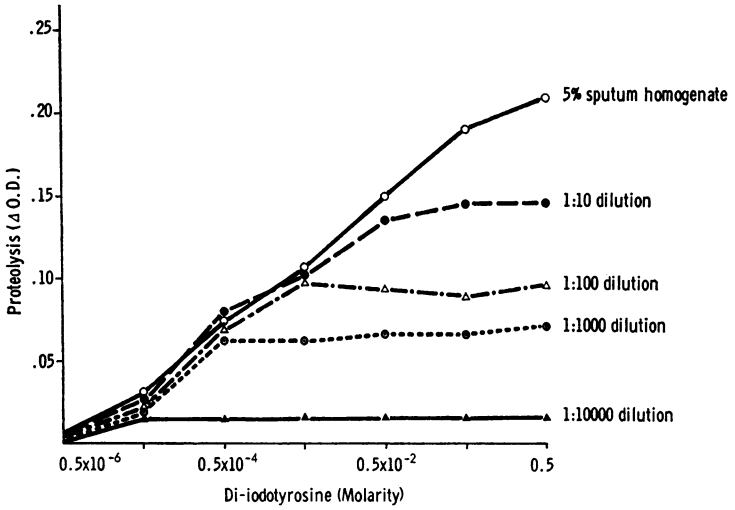

FIG. 9. EFFECT OF DIIODOTYROSINE (DIT) CONCENTRATION ON DIGESTION OF VARIOUS DILUTIONS OF AN 80° C-HEATED SPUTUM HOMOGENATE BY TRYPSIN.

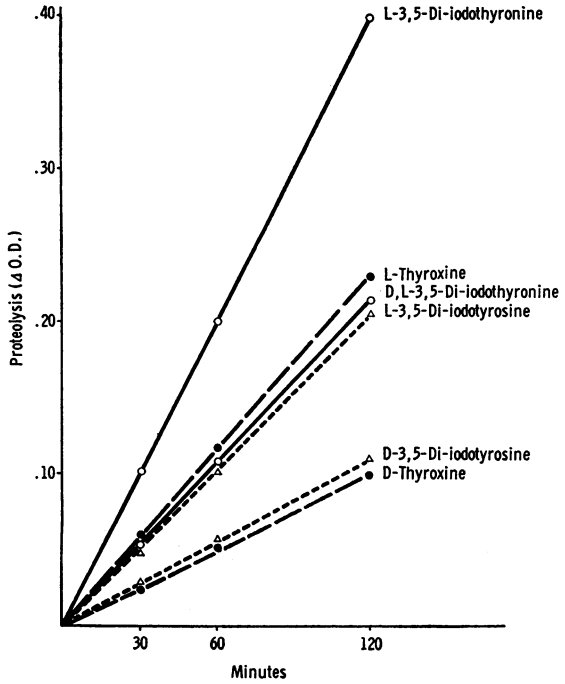

FIG. 10. COMPARISON OF DEXTRO- AND LEVOROTATORY ISOMERS FOR THE INDUCTION OF PROTEOLYTIC ACTIVITY IN PURULENT SPUTUM.

to depend upon the integrity of cells in the sputum preparation (Table III). When the cells contained in the sputum were disrupted by dissolution in 2 M NaCl, or by repeatedly washing the sediment with distilled water (which does not remove the enzyme from the sediment), or by the addition of Triton X-100, the D and L isomers produced equal effects in promoting proteolysis in the sputum preparation (Table III).

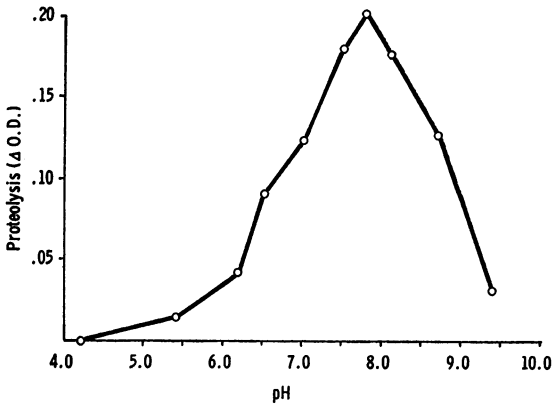

FIG. 11. OPTIMAL pH CURVE FOR PROTEOLYSIS INDUCED BY 5 mM DIT IN A HOMOGENATE OF PURULENT SPUTUM.

Optimal pH for the induction of proteolysis with DIT. Samples of 5% sputum homogenate containing 0.005 M DIT were assayed for proteolysis at various pH values during a 1-hour incubation period. Figure 11 demonstrates that maximal activity occurred at pH 7.5 to 8.0.

TABLE III

Comparison of proteolysis resulting from dextro- and levorotatory thyroxine in acellular and cell-containing sputum homogenates

Sputum preparation	Proteolysis (OD)						Ratio of proteolytic activity in L- to D-thyroxine
	With L-thyroxine			With D-thyroxine			
	0 hr	2 hr	Activity (ΔOD)	0 hr	2 hr	Activity (ΔOD)	
A. Cell-containing homogenates*							
Homogenate in physiological saline	0.405 0.410	0.685 0.680	0.275	0.400 0.405	0.530 0.525	0.125	2.20
B. Acellular homogenates†							
Sediment washed 5 to 6 times with distilled water (resuspended to original volume)	0.428 0.430	0.745 0.740	0.314	0.417 0.422	0.720 0.710	0.296	1.06
Homogenate in distilled water treated with Triton X-100	0.460 0.455	0.795 0.797	0.339	0.435 0.445	0.765 0.763	0.324	1.05
Homogenate in 2 M NaCl	0.419 0.422	0.829 0.825	0.407	0.421 0.428	0.794 0.788	0.367	1.11

* Microscopic examination reveals intact cells.
† Microscopic examination reveals no cells, only amorphous material.

TABLE IV

*Effect of 5 mM diiodotyrosine (DIT) upon proteolysis of various substrates at pH 7.5 by sputum protease, trypsin, and chymotrypsin**

Enzyme	Substrate: Casein† 1% — Proteolysis (ΔOD)** No DIT	With DIT	% Change	Denatured hemoglobin‡ 1% — No DIT	With DIT	% Change	Gelatin§ 1% — No DIT	With DIT	% Change	Albumin‖ 1% — No DIT	With DIT	% Change	Sputum protein¶ 5% — No DIT	With DIT	% Change
			%			%			%			%			%
Sputum protease, 10% wt/vol extract in 1 M NaCl	0.265	0.237	−10.5	0.312	0.293	− 6.5	0.210	0.183	−4.3	0.040	0.015		0.001	0.106	+
Trypsin, 0.2 mg/ml	0.649	0.640	− 1.4	0.316	0.387	+22.4	0.263	0.272	+3.4	0.466	0.488	+ 4.7	0.000	0.175	+
Chymotrypsin, 5,000 Armour U/ml††	0.404	0.497	+23.0	0.504	0.522	+ 3.6	0.281	0.205	−6.0	0.196	0.172	−12.5	0.000	0.101	+

* 2.5 ml of each substrate in 0.05 M phosphate buffer, pH 7.5 (2% wt/vol solutions, except sputum protein, 10% solution) was mixed with an equal volume of either the phosphate buffer or 0.01 M DIT in the buffer. 0.5 ml of each enzyme (type and concentration listed in the table) was added to each substrate mixture, and the tubes were incubated at 37° C for 1 hour. Samples (2.0 ml) were mixed with 4.0 ml of 16% trichloroacetic acid (TCA) at 0 hour and 1 hour. Proteolysis was assayed with the F-C reagent as described under Methods.
† Vitamin-free casein, Pfanstiehl Laboratories, Inc., Waukegan, Ill.
‡ Hemoglobin (denatured), Nutritional Biochemicals Corp., Cleveland, Ohio.
§ Knox unflavored gelatin, Knox Gelatine, Inc., Johnstown, N. Y.
‖ Albumin, human (crystallized), Mann Research Laboratories, Inc., New York, N. Y.
¶ Sputum heated at 80° C × 1 hour at pH 8.0 to destroy intrinsic protease activity.
** OD values = change in optical density, average of duplicate assays.
†† One-tenth the concentration of chymotrypsin was used with casein substrate.

Substrate specificity for enhanced proteolysis with DIT

DIT was tested for its effect upon the activities of sputum protease, trypsin, and chymotrypsin with substrates other than the sputum itself. Casein, denatured hemoglobin, gelatin, and human crystalline albumin (1% concentrations) were used as substrates (Table IV). The data show that 0.005 M DIT has no activating or inhibitory effect upon these proteases with these other substrates, but that DIT does allow proteolysis of sputum protein. We have also observed that the autolysis of trypsin and chymotrypsin is enhanced by the presence of DIT, necessitating the inclusion of blanks containing only the enzyme and DIT. Appropriate blanks were also needed when the sputum protease was studied with other substrates, since the 0.5 ml of sputum homogenate used as enzyme was also readily hydrolyzed in the presence of DIT.

Effect of DIT upon boiled sputum substrate

A 10% sputum homogenate in distilled water was placed in a boiling water bath for 30 minutes and was then tested for *a*) the presence of a trypsin inhibitor and *b*) the influence of DIT on tryptic digestion of the boiled sputum. The purpose of this experiment was to determine whether or not the enhancement of proteolysis in sputum by DIT could be due to the inactivation of a protease inhibitor by DIT.

One-half ml samples of trypsin (0.2 mg per ml) were mixed with 0.5-ml samples of the boiled sputum homogenate and incubated at room temperature for 30 minutes before the addition of 5.0 ml of 1% casein (in 0.05 M sodium phosphate buffer, pH 7.5). The mixture was incubated at

TABLE V

*A test of trypsin-inhibitory activity by a boiled sputum homogenate**

Time of incubation	Hydrolysis of casein by trypsin: In presence of boiled-sputum homogenate — OD	ΔOD	In presence of distilled water — OD	ΔOD
0 hr	0.175, 0.175 → 0.175		0.110, 0.105 → 0.107	
15 min	0.390, 0.400 → 0.395	0.220	0.320, 0.315 → 0.318	0.211
30 min	0.585, 0.580 → 0.583	0.409	0.535, 0.540 → 0.537	0.430

* See text for procedure.

TABLE VI

*Effect of 5 mM diiodotyrosine (DIT) upon the proteolysis of boiled sputum homogenate by trypsin**

Time of incubation	Proteolysis of boiled sputum				Trypsin autolysis				Net proteolysis of boiled sputum with DIT (B minus D)
	A Without DIT		B With DIT		C Without DIT		D With DIT		
	OD	ΔOD	OD	ΔOD	OD	ΔOD	OD	ΔOD	
0 hr	0.257 0.260		0.597 0.603		0.059 0.057		0.767 0.753		
30 min	0.265 0.269	0.008	0.775 0.786	0.181	0.063 0.068	0.008	0.829 0.837	0.073	0.108
60 min	0.281 0.277	0.020	0.995 1.000	0.398	0.075 0.078	0.019	0.910 0.925	0.158	0.240

* 2.5 ml boiled 10% sputum homogenate or H_2O for control + 2.5 ml 0.01 M DIT in 0.05 M, pH 7.5 sodium phosphate buffer or buffer alone + 0.5 trypsin (2.2 mg per ml).

TABLE VII

*Assay of iodide-induced proteolysis by various techniques**

	Method of assay							
	Folin-Ciocalteu reagent				Absorbance at 280 mμ		Ninhydrin reagent	
	TCA-supernate		TCA-sediment		TCA-supernate		TCA-supernate	
	OD	ΔOD	OD	ΔOD	OD	ΔOD	OD	ΔOD
A. Spontaneous proteolysis								
0 hr	0.155 0.153		0.550 0.551		0.252 0.250		0.500 0.505	
30 min	0.150 0.155	0	0.550 0.555	0	0.243 0.251	0	0.490 0.500	0
60 min	0.157 0.158	+0.004	0.547 0.565	−0.005	0.247 0.249	0	0.485 0.485	0
120 min	0.160 0.160	+0.006	0.543 0.540	−0.008	0.250 0.253	+0.001	0.510 0.500	+0.002
B. NaI (1 M)-induced proteolysis								
0 hr	0.210† 0.205		0.470 0.475		1.431‡ 1.426		0.515 0.515	
30 min	0.250 0.253	+0.044	0.421 0.424	−0.050	1.468 1.471	+0.041	0.530 0.530	+0.015
60 min	0.327 0.321	+0.116	0.385 0.387	−0.087	1.517 1.509	+0.084	0.575 0.580	+0.063
120 min	0.430 0.429	+0.222	0.295 0.291	−0.180	1.596 1.590	+0.164	0.640 0.675	+0.143
C. DIT (0.005 M)-induced proteolysis								
0 hr	0.573§ 0.610		0.361 0.365		1.461§ 1.498		0.520 0.560	
30 min	0.679 0.675	+0.085	0.305 0.300	−0.060	1.620 1.598	+0.120	0.580 0.580	+0.040
60 min	0.725 0.718	+0.129	0.240 0.243	−0.121	1.732 1.728	+0.250	0.610 0.620	+0.075
120 min	0.820 0.861	+0.249	0.157 0.150	−0.210	2.004 1.961	+0.053	0.680 0.700	+0.150

* Samples of a 10% purulent sputum homogenate were mixed with equal volumes of 0.05 M, pH 7.5 sodium phosphate buffer, or 2 M NaI in the same buffer, or 0.01 M DIT in the buffer, and incubated at 37° C. The mixtures were sampled at intervals and mixed with 2 parts 16% TCA. The TCA-supernatant fluids and sediments were then subjected to the various assays as described under methods.

† The slightly higher TCA-supernatant fluid OD and lower TCA-sediment OD, as compared to that seen with spontaneous proteolysis, is due to the immediate nonenzymatic release of a small amount of acid-soluble material by the NaI.

‡ Sodium iodide and TCA react to form a compound that absorbs at 280 mμ and is associated with a yellow discoloration of the solution. A blank containing only TCA and NaI gives an absorbance of slightly greater than 1.0 OD, but the absorbance increases with time. For this reason the measurement of absorbance at 280 mμ is a poor method for measuring proteolysis in the presence of NaI. The reaction of NaI with TCA did not affect the Folin-Ciocalteu (F-C) color development, however.

§ The initial high OD is due to the added DIT and to the immediate nonenzymatic release of a small amount of acid-soluble material by the DIT. This effect is apparently more marked on the absorbance at 280 mμ than on the intensity of the F-C reaction, because of the greater slope of the absorbance:concentration curve at 280 mμ.

37° C and sampled at 15- and 30-minute intervals. Comparison to a similar preparation utilizing distilled water instead of boiled sputum homogenate revealed that the sputum did not inhibit trypsin activity (Table V).

When the boiled sputum homogenate was used as substrate for trypsin (0.2 mg per ml final concentration), it resisted proteolysis completely in the absence of DIT but was hydrolyzed by trypsin in the presence of 0.005 M DIT (Table VI).

Since trypsin-inhibitory activity could not be demonstrated in the boiled sputum preparation, and since DIT can induce tryptic proteolysis of the boiled sputum, this action of DIT probably is not dependent upon the removal of a protease inhibitor.

Other methods of assay

Data obtained from four different methods for assaying proteolysis are shown in Table VII. All four methods showed minimal spontaneous proteolysis in a 10% homogenate of purulent sputum during a 2-hour incubation, but all the assay

TABLE VIII

A survey of DIT-induced proteolysis in purulent sputum and pus

Age	Sex	Diagnosis	Units ATEEase activity*	1-hr spontaneous proteolysis	1-hr DIT-induced proteolysis
37	M	Asthma	0.25	0.003	0.036
46	M	Pneumonia	0.5	0.002	0.041
69	M	Asthma	0.5	0.006	0.061
65	M	Emphysema	1.0	0.004	0.132
47	M	Chronic bronchitis	1.1	0.002	0.118
43	M	Chronic bronchitis	1.25	0.005	0.079
35	F	Bronchitis	1.6	0.008	0.050
71	M	Tuberculosis	2.0	0.003	0.156
46	M	Bronchogenic carcinoma	2.1	0.002	0.195
43	M	Bronchitis	2.6	0.000	0.221
43	M	Bronchiectasis	4.0	0.002	0.210
57	M	Lung abscess	5.6	0.006	0.115
74	M	Inactive tuberculosis	5.9	0.002	0.118
39	M	Bronchiectasis	6.0	0.004	0.257
46	M	Inactive tuberculosis	7.5	0.000	0.140
40	M	Asthma	9.8	0.000	0.138
4	M	Cystic fibrosis	12.5	0.000	0.132
35	M	Asthma	15.0	0.000	0.244
8	M	Cystic fibrosis	17.5	0.000	0.257
68	M	Emphysema	18.0	0.004	0.202
13	F	Cystic fibrosis	20.0	0.000	0.274
27	M	Pneumonia	20.6	0.002	0.258
76	M	Bronchogenic carcinoma	22.5	0.003	0.206
76	M	Asthma	22.5	0.006	0.078
13	M	Cystic fibrosis	23.0	0.006	0.172
6	M	Cystic fibrosis	26.3	0.003	0.245
11	M	Cystic fibrosis	26.3	0.000	0.276
7	M	Cystic fibrosis	28.1	0.001	0.294
73	M	Emphysema	29.0	0.000	0.079
54	F	Bronchiectasis	32.0	0.001	0.222
14	M	Cystic fibrosis	40.0	0.000	0.257
73	M	Bronchiectasis	42.5	0.002	0.235
10	M	Cystic fibrosis	48.0	0.004	0.179
	F	Pus from right kidney abscess†	50.0	0.001	0.070
16	F	Cystic fibrosis	51.3	0.006	0.228
	F	Pus (empyema)†	56.0	0.005	0.104
16	M	Bronchitis	50.0	0.000	0.142
12	M	Cystic fibrosis	71.0	0.003	0.255
9	F	Cystic fibrosis	75.0	0.000	0.289
7	F	Cystic fibrosis	79.0	0.000	0.249
13	M	Cystic fibrosis	80.6	0.000	0.244
9	F	Cystic fibrosis	82.0	0.000	0.258
10	F	Cystic fibrosis	85.0	0.000	0.285
15	F	Cystic fibrosis	100.0	0.004	0.294

* ATEE = *N*-acetyl-L-tyrosine ethyl ester.
† Prepared as 10% homogenates in distilled water.

techniques demonstrated that active proteolysis took place in the presence of 1 M NaI or 0.005 M DIT.

Reproducibility of the DIT effect on proteolysis in purulent sputa

Forty-two specimens of purulent sputum and two samples of pus were prepared as 10% homogenates in distilled water. Each specimen was assayed with 0.01 M *N*-acetyl-L-tyrosine ethyl ester (ATEE) substrate as an estimate of protease content (4). Samples (2.5 ml) of each homogenate were then mixed with equal volumes of 0.05 M sodium phosphate buffer, pH 7.5, to measure spontaneous proteolysis, or with 0.01 M DIT in the buffer at pH 7.5, and incubated at 37° C. At intervals of 0, 30, 60, and 120 minutes, 1.0-ml samples were removed and mixed with 2.0 ml 16% TCA. The TCA-supernatant fluid was then tested for the degree of proteolysis with the F-C reagent.

DIT-induced proteolysis was linear with time. Table VIII contains the data for spontaneous proteolysis as compared with DIT-induced proteolysis for the 1-hour incubation period. The specimens are listed in the order of increasing activity with ATEE substrate. None of the specimens showed any appreciable spontaneous proteolysis during the 1-hour incubation, but all of them showed enhanced proteolysis in the presence of 0.005 M DIT. The DIT-induced proteolysis was roughly proportional in rate to the hydrolysis of ATEE by the sputum specimens, up to an ATEE hydrolytic activity corresponding to 25 U. Sputa that contained higher levels of ATEE hydrolytic activity did not demonstrate further increases in DIT-induced proteolysis because of the limiting concentration of DIT (Figure 9).

Discussion

In the past it has been thought that iodides act to thin purulent respiratory secretions simply by increasing the volume of aqueous secretion from the bronchial glands. However, our experiments suggest that iodides may have a more specific role and that they liquefy purulent secretions by inducing the enzymatic hydrolysis of protein. This action of iodides requires the presence of a proteolytic enzyme: either inherent leukocytic proteases or an added protease such as trypsin. Since the reaction is enzymatic, inhibition of the enzyme prevents proteolysis.

Although the data demonstrate optimal proteolysis at a concentration of 1 M sodium iodide, considerable proteolysis was apparent at 0.005 M (Table II and Figure 7). Even lower concentrations may be clinically effective, since the amount of proteolysis that must take place to reduce the viscosity of sputum is very slight compared to the levels of proteolysis attained under optimal *in vitro* conditions. For example, we have demonstrated effective liquefaction of whole purulent sputum by the addition of trypsin (7), although the level of measurable proteolysis resulting from tryptic activity is minimal.

The mechanism whereby iodides induce proteolysis does not appear to depend upon the dissociation of nucleoprotein complexes and thus differs from the manner in which deoxyribonuclease and high NaCl concentrations act. The reaction is substrate specific in that proteolysis of some protein substrates other than sputum, such as casein, albumin, hemoglobin, and gelatin, was not affected by the iodides. In addition, this effect of iodides is not enzyme specific, since the action of trypsin and chymotrypsin upon a heated sputum preparation (lacking inherent protease activity) was also enhanced by the iodides. The iodides appear to act by altering the potential protein substrate in sputum so that it is more readily hydrolyzed by proteolytic enzymes. The nature of this protein substrate and the changes caused by the iodides is under study. The splitting of glycoproteins, analogous to the splitting of nucleoproteins by DNase, is suggested to be the mechanism whereby iodides induce proteolysis.

The naturally occurring iodinated thyroidal compounds were much more effective for initiating proteolysis in a homogenate of purulent sputum than were the inorganic iodides. Lower concentrations of these natural compounds were required, and faster rates of proteolysis resulted. It is possible that the reaction is specific for iodinated tyrosine or thyronine and that inorganic iodide must first form iodinated amino acids to have an effect. In the absence of iodination, on the other hand, the parent amino acid (tyrosine) lacked this activity, as did brominated tyrosine. The action of the organic iodine compounds appeared

to result from a stoichiometric reaction between the compound and sputum protein, with either the organic iodine compound or the sputum protein being the limiting factor in determining the rate of proteolysis when the other was present in excess.

Use of both dextro- and levoisomers of the iodinated thyronine and tyrosine compounds suggests that some of the substrate affected by the iodides is intracellular. The levorotatory compounds were consistently more effective than the dextrorotatory compounds, but these differences were eliminated by disrupting the cells contained in the sputum preparation.

These observations raise the question of whether thyroxine and its precursors directly affect proteolysis *in vivo* in cellular metabolism. Evidence that thyroid hormone influences enzymatic activity has long been sought, but very few such reactions have been found to be affected by thyroxine *in vitro* (8, 9). Barker has shown such an effect in carbohydrate oxidation (10), but the effect lacked specificity since it could be produced by metabolically inactive analogues of thyroxine. The test system employed by Barker, however, was essentially a cell-free system, and possibly *in vivo* specificity of thyroid hormone depends upon its ability to cross the cell membrane. In our experiments no difference between the D and L compounds was observed when a cell-free system was used, but a definite advantage of the metabolically active L compounds over the D isomers was apparent when cells were contained in the system. However, the reaction is not completely specific for metabolically active compounds, since iodinated tyrosines (MIT and DIT), thought to be metabolically inactive, similarly enhance proteolysis in sputum. Other metabolically inactive analogues of thyroxine (diiodothyropropionic, -acetic, and -formic acids) are much less active than iodinated thyronine and tyrosine, suggesting some selective advantage for the thyroidal hormones. The order of effectiveness of the organic iodides for inducing proteolysis (diiodothyronine = triiodothyronine > monoiodotyrosine > thyroxine = diiodotyrosine) is not correlated with the metabolic activity *in vivo*. The difference between the metabolic effect *in vivo* and the proteolytic-enhancing effect *in vitro* may depend upon differences in penetration of viable cells by the iodinated compounds.

The lowest concentration of DIT or L-thyroxine that enhanced proteolysis measurably in our system was 5 μM. This is approximately two orders higher than is likely to occur in tissues. However, factors of time and local concentrations (of hormone, proteolytic enzyme, and of specific sensitive substrates) may provide suitable conditions for physiologic effects.

As a result of these studies, certain historical observations concerning the use of iodides appear to have a scientific basis. The use of iodides for dissolving syphilitic gummas, and the fear of using iodides in tuberculosis because of the possibility of breaking down tubercles and spreading the infection, could both be related to the proteolytic action resulting from iodides in exudates. In 1915, Jobling and Petersen (11) investigated the action of iodides on necrotic material and concluded that iodides acted to soften tubercles and syphilitic gummas by causing a general reduction in antitryptic activity, thereby allowing the proteolytic breakdown of the caseous material. These early studies closely parallel our own, but we found no evidence for the implication of a protease inhibitor. Indeed, we have demonstrated that boiled sputum lacks an inhibitor of trypsin, but nevertheless its proteolysis by trypsin is enhanced in the presence of organic iodides. Our experiments suggest that the iodides interact with the substrate to produce enhanced susceptibility to proteolytic enzymes.

The highly effective action of iodinated-tyrosine compounds upon the induction of proteolysis within purulent sputum (or other purulent material) suggests that such compounds may become important therapeutic adjuncts in the treatment of chronic respiratory disease by aiding in the liquefaction of viscid respiratory secretions. We have observed that whole sputum from patients with cystic fibrosis is liquefied when mixed with DIT *in vitro,* indicating that a reduction in viscosity accompanies proteolysis. Compounds such as monoiodotyrosine and diiodotyrosine, in contrast to the iodinated thyronines, have no systemic calorigenic effect and may be found useful for thinning respiratory secretions without causing calorigenic side effects. The clinical effective-

ness of such compounds, when administered by aerosol, is now being investigated.

Summary

Iodides were found to induce proteolysis in purulent sputum from patients with cystic fibrosis and other forms of respiratory disease. Proteolysis was shown to be enzymatic in nature and to depend upon the presence of natural leukocytic proteases or an added protease such as trypsin. The reaction was inhibited by conditions that inhibited the proteolytic enzyme. Naturally occurring iodinated thyroidal compounds were much more effective for inducing proteolysis than were the inorganic iodides. The L-isomers of these thyroidal compounds promoted a higher rate of proteolysis than did the D-isomers, when intact cells were present in the sputum preparation.

The question is raised as to whether thyroxine and its precursors affect proteolysis *in vivo* in cellular metabolism. The possible clinical use of iodinated tyrosine compounds is suggested for thinning viscid purulent respiratory secretions.

Acknowledgment

We are indebted to Mrs. Betty M. Trimmer for her skilled technical assistance.

References

1. Goodman, L., and A. Gilman. The Pharmacological Basis of Therapeutics, 2nd ed. New York, Macmillan, 1955, p. 817.
2. Opie, E. L. The enzymes in phagocytic cells of inflammatory exudates. J. exp. Med. 1906, **8**, 410.
3. Lieberman, J., and N. B. Kurnick. Characterization of the proteolytic activities in purulent sputum. Submitted for publication.
4. Lieberman, J., and N. B. Kurnick. Proteolytic enzyme activity and the role of deoxyribonucleic acid in cystic fibrosis sputum. Pediatrics 1963, **31**, 1028.
5. Lieberman, J., and N. B. Kurnick. Influence of deoxyribonucleic acid content on the proteolysis of sputum and pus. Nature (Lond.) 1962, **196**, 988.
6. Moore, S., and W. H. Stein. Photometric Ninhydrin method for use in the chromatography of amino acids. J. biol. Chem. 1948, **176**, 367.
7. Lieberman, J. Enzymatic dissolution of pulmonary secretions. Amer. J. Dis. Child. 1962, **104**, 342.
8. Barker, S. B. Biochemistry *in* The Thyroid, 2nd ed., S. C. Werner, Ed. New York, Harper & Row, 1962, p. 102.
9. Barker, S. B. Mechanism of action of the thyroid hormone. Physiol. Rev. 1951, **31**, 205.
10. Barker, S. B. *In vitro* action of thyroxine analogs on succinate and malate oxidation. Endocrinology 1957, **61**, 534.
11. Jobling, J. W., and W. Petersen. The therapeutic action of iodin. Arch. intern. Med. 1915, **15**, 286.