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EFFECTS OF TRYPAN BLUE ON THYROID SECRETION

INHIBITION OF PURIFIED CATHEPSIN D FROM BOVINE THYROID

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Previous studies have demonstrated that trypan blue directly inhibits thyroid secretion when the dye is administered in vitro or in vivo. To further study the mechanisms of inhibition, cathepsin D (EC 3.4.23.5) (thyroidal acid proteinase) has been purified from bovine thyroid. Trypan blue inhibited the proteolysis of both ^{125}I-labeled thyroglobulin and ^{125}I-labeled hemoglobin in both crude lysosomal enzyme preparation and purified endopeptidase and the inhibition was competitive. Inhibition was also observed when the dye was allowed to prebind to either purified enzyme or purified substrate. Inhibition of cathepsin D is shown to account for part of the inhibition of thyroid secretion.

Introduction

Previous studies with trypan blue have demonstrated direct inhibition of thyrotropin-induced thyroid secretion from whole mouse thyroids in vitro [1]. Thyroid secretion, measured by butanol extractable-^{125}I products released to the media, was inhibited whether trypan was administered in vitro or in vivo. Two possible mechanisms were demonstrated: a decrease in colloid droplet formation indicating decreased pinocytosis and an inhibition of thyroglobulin proteolysis by a crude extract of thyroid lysosomal enzymes.

The purpose of the present studies is to examine the inhibition of thyroid secretion by employing purified cathepsin D (EC 3.4.23.5), an acid protease that cleaves proteins on the carboxyl side of aromatic amino acids [2] and the predominant endopeptidase found in thyroid lysosomes [2–6]. Determination of the effects of trypan blue on the activity of this enzyme and on other lysosomal cathepsins has been made. Inhibition of thyroglobulin proteolysis was observed when either purified enzyme or thyroglobulin was allowed to preincubate with the dye.

Materials and Methods

Purification of cathepsin D

Purified cathepsin D was prepared from frozen bovine thyroids by minor modifications of the procedures of Barrett [7] and Smith et al. [4]. Homogenization was accomplished with a Polytron (Brinkman) followed by overnight incubation at pH 3.6, 4°C (autolysis step of Barrett [7]). As recommended by Smith et al. [4], two acetone fractionations were employed: the first was performed at pH 3.6 recovering protein precipitating between 20 and 85% acetone, and the last at pH 7.0, recovering protein precipitated between 36 and 70% acetone. This material was chromatographed on DEAE-cellulose and the fractions containing protease activity were rechromatographed on Sephadex G-100 [4]. Active fractions were pooled, lyophilized, and stored at −10°C, pH 4.8 with no loss of activity for periods up to 1 year.

Crude lysosomal enzyme

Throughout the purification and subsequent studies, purified protease was compared to crude lysosomal enzyme preparation. This was prepared from fresh bovine thyroid 800–15 000 $\times g$ particulate fraction as detailed earlier [1].

Substrates and ^{125}I labeling

Hemoglobin. Hemoglobin was extracted from packed human blood cells by the method of Shapland [8]. Because trypan blue interfered with the A_{280} measurement of hemoglobin degradation, it was necessary to prepare ^{125}I-labeled hemoglobin for the studies employing this dye. Hemoglobin was further purified by column chromatography on Sephadex G-100 and iodinated by the method of Hunter and Greenwood [9] to a specific activity of 0.4 μCi/μg.

Thyroglobulin. ^{125}I-Thyroglobulin was prepared by in vivo labeling techniques employing mice [10]. ^{125}I-thyroglobulin was purified by column chromatography on Bio-gel 1.5 A (Bio-rad), equilibrated and eluted with 0.2 M KCl/0.02 M KH_2PO_4 (pH 7.4).

Enzyme assay methods

Hemoglobin assay for protease. Enzyme purification was monitored at each step using the modified hemoglobin assay of Anson [11]. After incubation the samples were allowed to stand in an ice bath prior to centrifugation. The absorbance at 280 λ of the supernatants was measured and compared to non-incubated blanks. Enzyme activity was expressed as hemoglobin units/mg protein, calculated using Barrett's definition of a hemoglobin unit: change of absorbance at 280 λ of 1.000/h [7]. In order to compare activity yields with those employing other methods, it was determined spectrophotometrically that one hemoglobin unit was equivalent to 2.76 μmol tyrosine [4,12,13].

To determine the effect of trypan blue, the procedure was identical, except that instead of unlabeled hemoglobin, 50–250 ng ^{125}I-labeled hemoglobin was incubated for 20 min and 0.2 ml of 5% bovine serum albumin (Sigma) was added prior to trichloroacetic acid precipitation for more complete precipitation of undigested substrate. Precipitates were washed with another 2 ml trichloroacetic acid, and each sample's supernatant and wash were pooled for gamma counting. Trichloroacetic acid-soluble products were expressed as a percent of total ^{125}I.

^{125}I-Thyroglobulin assay. Microgram quantities of in vivo-labeled thyroglobulin were combined with enzyme protein in 0.2 M acetate (pH 4.1) and incubated for 2–4 h. Products of thyroglobulin digestion were quantitated after butanol extraction as previously described [1,14].

Carboxypeptidase A (cathepsin A) (EC 3.4.16.1). *N*-Carbobenzoxy-L-glutamyl-L-tyrosine (Sigma) was dissolved in 100% ethanol and diluted with 0.16 M acetate buffer (pH 5) to a concentration of 30 μmol/ml (0.03 M). 0.5 ml was incubated for 2 h at 37°C with 0.5–1.5 units of enzyme in sucrose, in a final volume of 1.5 ml [13]. Final ethanol concentration was less than 10%. Nonincubated samples were used as controls. Tyrosine liberated was measured with a ninhydrin reaction [15].

Cathepsin B assay (EC 3.4.22.1). Cathepsin B activity was assayed using *N*-benzoyl-L-arginine amide-HCl (Sigma), 30 μmol in 0.1 M citrate buffer (pH 5.4) with 0.3–1.0 unit enzyme in a final volume of 1.5 ml. Incubation was for 1 h at 37°C, GSH (10 mM) was the thiol activator used, and nonincubated samples were controls. Cathepsin B activity was determined by measuring the ammonia in the trichloroacetic acid supernatant directly with phenol reagents [16] or after diffusion into 0.2 M H_2SO_4 employing Conway diffusion chambers [17].

Cathepsin C assay (EC 3.4.14.1). Glycyl phenylalanine-amide acetate, 30 μmol in 0.1 M phosphate buffer (pH 7.5), or 0.1 M acetate buffer (pH 4.1), was incubated with 0.3–0.9 unit enzyme from fresh homogenates for 1 h at 37°C in a volume of 1.5 ml and containing 10 mM GSH. Cathepsin C activity was measured as ammonia liberated utilizing the Conway diffusion method and phenol reagents above [16,17] and samples incubated without enzyme were used as controls.

Analytical electrofocusing. Electrofocusing was performed to analyze preparations of purified enzyme, using the LKB Multiphore with a polyacrylamide gel containing Ampholines 4–9 (LKB PAGplate). 50–100 μg protein were applied at the anode and focused for 1.5 h at 25 W constant power. Polyacrylamide gel plates were stained with Coomassie brillant blue G-250 and destained with methanol in acetic acid to delineate protein bands [18].

In some cases, instead of staining protein bands, focused polyacrylamide gel plate strips were cut into 0.5 cm sections and each section eluted with 0.25 M sucrose overnight. The resultant eluates were tested for protease activity using the hemoglobin and thyroglobulin assays.

Prebinding of trypan blue. Aliquots containing 0.025–1.0 mg thyroglobulin were incubated with 0.4 mM trypan blue in 0.15 M NaCl, acetate-buffered (pH 4.1), for 1 h at 37°C. Unbound trypan was removed by filtration on Sephadex G-25 (1 × 10 cm), eluted with the same buffered saline. Previous experiments had demonstrated that unbound trypan blue was adsorbed to Sephadex and that no detectable trypan could be eluted from this column ($<10^{-6}$ M is level of detectability). Bound and free trypan blue concentrations were calculated by A_{590} measurements. ^{125}I-labeled thyroglobulin with trypan attached in this way was employed in the assay described above and results compared with samples using thyroglobulin preincubated alone.

Purified enzyme preparations were preincubated with trypan in the same buffer. Activity of the enzyme bound with trypan was measured, using ^{125}I-labeled hemoglobin and ^{125}I-labeled thyroglobulin in assays above, and compared to the activity of enzyme preincubated and eluted without trypan present.

Results

Purification of cathepsin D

Purified cathepsin D was 320–1000-fold more active in hydrolyzing hemoglobin than thyroid homogenate. A representative purification is shown in Table I. The second acetone fractionation was found to be essential for decreasing the quantities of protein to be applied to DEAE-cellulose. The inactive protein (probably thyroglobulin) eluted in the barrier volume from DEAE immediately preceding the peak of enzyme activity. At least 6–9 g cellulose/g protein was a critical requirement in this chromatographic step so that enzyme activity was retarded and eluted as a separate peak rather than in the downslope of the barrier volume. The effectiveness of the DEAE step dropped to 5-fold purification rather than 12–20-fold if this sequence and cellulose to protein ratio was not maintained. Active enzyme consistently eluted from Sephadex G-100 with the same R_F as ovalbumin, in agreement with molecular weight determinations for cathepsin D of 45 000 [4,7].

Electrofocusing of the purified cathepsin D at pH 4–9 demonstrated protein bands from pH 4.9 to 6.6. Only three areas contained proteolytic activity when proteins were eluted and assayed using hemoglobin or thyroglobulin; protein with isoelectric points (p*I*) of 6.6, 5.9–6.0 and 5.6. The active protein with a p*I* value of 6.6 accounted for 62% of the original cathepsin D activity, and appeared to be the major isoenzyme. Enzyme protein focusing at 5.9–6.0 accounted for 31%, and that focusing at 5.6 represented less than 7% of the total. The remaining protein bands were without enzyme activity.

Testing of purified cathepsin D for other proteases

Incubation of up to 2 units of purified cathepsin D demonstrated no activity against the artificial sub-

TABLE I

POOL VI CATHEPSIN D PURIFICATION

	Total protein (g)	Total activity hemoglobin (units)	Activity hemoglobin (units/mg protein)	Activity recovery (%)	Purification (-fold)
Defatted thyroid	400				
Homogenate	100	10 000	0.10	100	–
15 000 × *g* supernatant	73	10 000	0.14	100	1.4
pH 3.6 autolysis, supernatant	7	8000	1.10	81	11.0
20–85% acid acetone	1.5	5000	3.00	50	30.0
36–70% neutral acetone	0.5	3000	4.00	30	40.0
DEAE-cellulose fractions	0.02	1500	50.00	15	500.0
G-100 fractions lyophilized	0.007	500	90.00	5	900.0

TABLE II

TESTING OF PURIFIED CATHEPSIN D FOR OTHER PROTEASES

Relative activity = activity of the enzyme assayed per hemoglobin unit.

	Carboxypeptidase A		Cathepsin B		Cathepsin C	
	Specific activity pkat/mg (±S.E.)	Relative activity	Specific activity pkat/mg (±S.E.)	Relative activity	Specific activity pkat/mg (±S.E.)	Relative activity
Homogenate	38 (±2) $n = 8$	0.55 (±0.02)	15 (±2) $n = 6$	0.57 (±0.09)	14 (±1) $n = 14$	0.51 (±0.03)
900-fold purified cathepsin	0 $n = 6$	0	245 (±47) $n = 10$	0.01 (±0.003)	0 $n = 7$	0
Lysosomal extract	153 (±7) $n = 2$	0.91 (±0.04)	58 (±2) $n = 9$	0.32 (±0.05)	0 $n = 5$	0

strates employed for carboxypeptidase A and cathepsin C. However, minor contamination of the purified enzyme with cathepsin B was observed (Table II). When the purified enzyme was compared to crude homogenate, a 15-fold purification of cathepsin B activity had been accomplished compared to a 900-fold purification of cathepsin D. Relative carboxypeptidase A, cathepsin B, and C activities for crude lysosomal enzyme preparation (P_{15}) are also shown in Table II. Crude enzyme preparations employed in these studies had always been freeze-thawed; and absence of cathepsin C was compatible with previous findings that freezing inactivates this enzyme [2].

Thyroglobulin proteolysis by crude or purified protease

In experiments comparing equivalent activity of purified cathepsin and crude (P_{15}) enzyme, more thyroglobulin was hydrolyzed/unit time by the purified cathepsin. This may indicate an increase in activity, but more likely is the result of contamination of the crude preparation with significant amounts of unlabeled thyroglobulin.

0.05 U (10^{-8} M) cathepsin was required to produce measurable products of hydrolysis during 4 h incubation. This was equivalent to the molarity of thyroglobulin substrate. 10-times that quantity released 25% butanol extractable-^{125}I (Fig. 1). Addition of GSH to purified cathepsin did not enhance this hydrolysis, confirming the nonthiol dependence of this protease (Fig. 1).

Prolonged incubations of ^{125}I-labeled thyroglobulin with the purified enzyme were compared to the crude enzyme preparation with and without GSH (Fig. 2). Slightly more purified enzyme was employed to better compare with the GSH-stimulated crude enzyme. Increased butanol extractable-^{125}I release was observed with the purified enzyme over the first 6–8 h, but it then appeared to level off with only a slight increase up to 24 h of incubation. Crude enzyme (P_{15}) without GSH gradually released butanol extractable-^{125}I over the 24 h. When crude enzyme was incubated with GSH, butanol extractable-^{125}I release continued to increase for the 24 h of incubation and reached 35% of the total ^{125}I-labeled thyroglobulin.

Effect of trypan blue on cathepsin proteolysis of ^{125}I-labeled thyroglobulin and ^{125}I-labeled hemoglobin

Employing very low concentrations of both labeled substrates it was possible to show that proteolysis by purified cathepsin D was inhibited by low

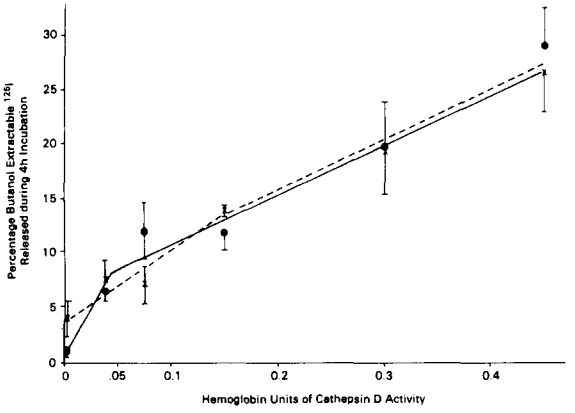

Fig. 1. Enzyme concentration curve showing various concentrations of purified cathepsin D incubated with 6.5 μg ^{125}I-labeled thyroglobulin alone (●———●) or in the presence of 10 mM reduced glutathione (X———X).

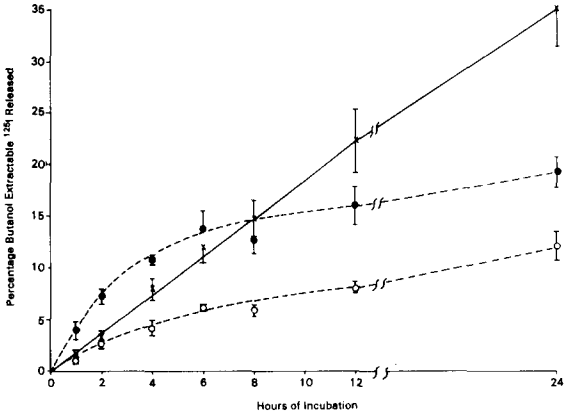

Fig. 2. Effects of crude and purified cathepsin on thyroglobulin proteolysis. Butanol-extractable iodine released from ^{125}I-labeled thyroglobulin incubated with three enzyme preparations: 0.4 units purified cathepsin D (●———●), 0.3 units crude lysosomal extract (○———○), and 0.3 units lysosomal extract with 10 mM reduced glutathione (X———X). Standard error of the mean indicated by vertical lines for each point on this and subsequent figures.

concentrations of trypan blue, and increasing the dye concentration increased the inhibition observed (Table III). When the three cathepsin D isoenzymes were eluted from electrofocusing gels, the proteolytic activity of all three against ^{125}I-labeled thyroglobulin was completely inhibited by $4 \cdot 10^{-4}$ M trypan blue (data not shown).

When $4 \cdot 10^{-4}$ M trypan blue was added in the assays for cathepsin B (P_{15}-enzyme preparation) and C (fresh thyroid homogenate) no inhibition was observed. Inhibition of carboxypeptidase A could not be studied because the dye interfered with measurement of the reaction products after ninhydrin reaction.

Effect of increasing substrate concentration on the inhibition of cathepsin D proteolysis by trypan blue

Using fixed concentrations of trypan blue, inhibition of proteolysis of ^{125}I-labeled thyroglobulin decreased as the substrate concentration increased indicating competitive inhibition. Lineweaver-Burk plots produced a value for K_m of $2.6 \cdot 10^{-8}$ M ^{125}I-labeled thyroglobulin with 6 μg ($8 \cdot 10^{-8}$ M) or 0.5 unit of purified cathepsin D (Fig. 3). The apparent K_m increased as trypan blue concentrations were

TABLE III

EFFECTS OF VARIOUS CONCENTRATIONS OF TRYPAN BLUE ON PROTEOLYSIS WITH 0.1 UNIT CATHPESIN D

Trypan blue	Percentage butanol extractable-^{125}I released during 4 h incubation with 6.5 μg ^{125}I-labeled thyroglobulin			Percentage trichloroacetic acid soluble-^{125}I released during 20 min incubation with 50 ng ^{125}I-labeled hemoglobin		
	N	Mean ± S.E.	*P* *	*N*	Mean ± S.E.	*P* *
None	3	13.8 ± 0.7		4	11.6 ± 1.6	
10^{-9} M	3	13.3 ± 1.9	>0.4	–		
10^{-8} M	3	10.3 ± 1.5	<0.025	–		
10^{-7} M	3	10.3 ± 2.4	<0.025	4	9.1 ± 1.6	>0.1
10^{-6} M	3	9.2 ± 1.1	<0.01	4	6.9 ± 0.1	<0.1
10^{-5} M	3	4.4 ± 0.1	<0.001	4	2.3 ± 0.03	<0.001

* *P*, probability, assessed by Student's *t*-test.

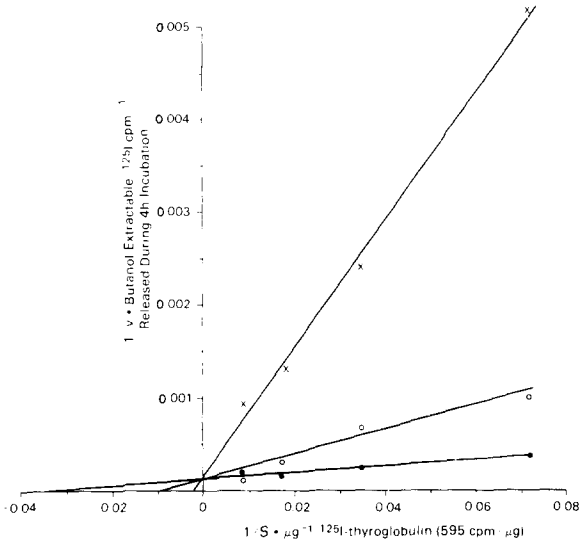

Fig. 3. Reciprocal plot showing comparison of butanol-extractable iodine released by 0.5 units cathepsin (pool I) incubated with increasing concentrations of ^{125}I-labeled thyroglobulin alone (●———●) and with two concentrations of trypan blue: $5 \cdot 10^{-6}$ M (o———o) and $5 \cdot 10^{-5}$ M (X———X).

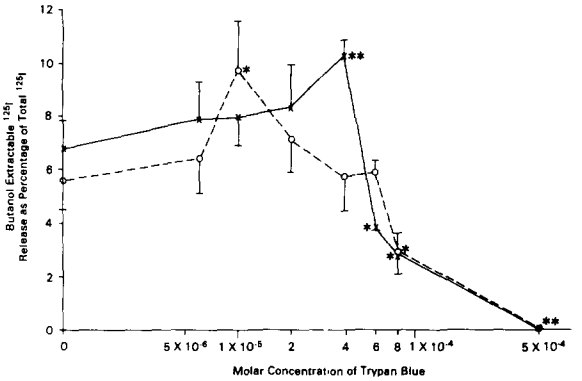

Fig. 4. Effects of trypan blue on thyroglobulin proteolysis by two concentrations of protease. Comparison of butanol-extractable iodine released from excess (800 μg) ^{125}I-labeled thyroglobulin incubated for 4 h with 0.8 units cathepsin (o———o) and 1.6 units cathepsin (X———X), when increasing concentrations of trypan blue were present. * Based on Student's t-test analysis, probability of significant difference from samples containing no trypan blue (P) < 0.05. ** $P < 0.01$.

increased, while V did not decrease below control. The increased substrate requirement necessary to reach half-maximum velocity is consistent with competitive inhibition.

In these experiments, stimulation of butanol extractable-^{125}I release and increased V were observed at very low inhibitor concentrations, as substrate increased. Since stimulation had never been observed with our previous in vitro and in vivo experiments, experiments were conducted with an unlimiting quantity of ^{125}I-labeled thyroglobulin, two concentrations of purified enzyme and various concentrations of inhibitor (Fig. 4). Slight stimulation of butanol extractable-^{125}I release was observed with both enzyme concentrations, but a 4-fold increase in molar concentration ($1 \cdot 10^{-5}$ M vs. $4 \cdot 10^{-5}$ M) of the dye was required to produce this stimulation at the higher enzyme concentration, possibly indicating increased importance of dye-enzyme interaction over that of dye-substrate.

Experiments using increasing concentrations of ^{125}I-labeled hemoglobin as substrate demonstrated that inhibition of cathepsin D activity was competitive (data not shown).

Preincubation of substrate and enzyme with trypan blue. To determine whether trypan blue inhibition was due to interaction with substrate or enzyme, ^{125}I-labeled thyroglobulin and cathpesin D were preincubated with dye and the excess dye was removed by Sephadex chromatography. Prebound thyroglobulin or enzyme was subsequently incubated with appropriate untreated enzyme or substrate. As shown in Table IV, prebinding the dye to either thyroglobulin or cathepsin D resulted in significant inhibition of butanol extractable-^{125}I release from thyroglobulin proteolysis employing various concentrations of both substrate and enzyme. Trichloroacetic acid soluble-^{125}I resulting from hydrolysis of ^{125}I-labeled hemoglobin was also inhibited when enzyme was pretreated with trypan blue.

Discussion

Cathepsin D was purified from bovine thyroid in order to study the effects of trypan blue on the proteolytic activity of this isolated enzyme. Purification (usually greater than 700-fold) was comparable to that achieved by others [4,5,7,12,19–21]. The purified enzyme demonstrated characteristics consistent with cathepsin D: hydrolysis at acid pH,

TABLE IV

INHIBITION OF CATHEPSIN D ACTIVITY AFTER PREBINDING TRYPAN BLUE TO SUBSTRATE OR ENZYME AND REMOVING EXCESS INHIBITOR

	n	% of total ^{125}I released *		*P*
		Without trypan blue	With trypan blue	
A. After preincubation of thyroglobulin				
Enzyme concentration				
0.4 U cathepsin D	6	14.1 ± 0.5	3.4 ± 0.7	0.001
0.2 U cathepsin D	6	5.7 ± 0.2	1.3 ± 0.2	0.001
0.1 U cathepsin D	6	2.0 ± 0.5	0.5 ± 0.2	0.005
B. After preincubation of enzyme				
Substrate concentration				
55 μg [^{125}I]Thyroglobulin	4	12.7 ± 1.1	5.9 ± 0.1	0.001
35 μg [^{125}I]Thyroglobulin	4	14.8 ± 1.5	5.7 ± 0.9	0.01
200 ng [^{125}I]Hemoglobin	4	16.7 ± 1.3	4.8 ± 0.5	0.001
100 ng [^{125}I]Hemoglobin	4	20.7 ± 1.8	7.2 ± 1.5	0.005

* % of total ^{125}I released represents: (1) butanol extractable-^{125}I as a percentage of total ^{125}I released in 4 h incubation with ^{125}I-labeled thyroglobulin as substrate; or (2) trichloroacetic acid soluble-^{125}I as a percentage of total ^{125}I released in 20 min incubation with ^{125}I-labeled hemoglobin as substrate.

molecular weight of approx. 40 000–45 000, nonthiol dependence. Three isoenzymes with p*I* values of 5.6, 6.0 and 6.6, corresponding to those found in human and chicken liver by Barrett [7,19], were identified.

The purified cathepsin contained no carboxypeptidase A or cathepsin C activity. Cathepsin B activity was one-sixieth of that present in thyroid homogenate and was not detectable at concentrations of cathepsin used in assays (≤0.5 units). Cathepsin E (M_r 100 000) activity was not specifically examined, however no large molecular weight endopeptidase activity was detected on the gel filtration step of purification [22].

Reduced glutathione has been shown to enhance thyroglobulin proteolysis by a crude lysosomal enzyme preparation [1,14] but it does not increase the activity of purified cathepsin D. As previously proposed [23,24] this may be through the increased dissociation of thyroglobulin by GSH-protein transhydrogenase and thus increased exposure of susceptible bonds. It may also be the result of removal of thiol-dependent exopeptidases which act only after cathepsin D to increase butanol-soluble products.

Trypan blue inhibited thyroglobulin proteolysis by the purified cathepsin D as was previously demonstrated with the crude enzyme, with and without GSH [1]. ^{125}I-Labeled hemoglobin proteolysis was also inhibited by the dye. Lloyd and Beck [25] have reported similar inhibition of acid protease activity by trypan blue. Trypan blue attached to thyroglobulin, as well as to both crude and purified enzyme (and probably to most other proteins), and inhibition was observed when the dye was allowed to prebind to either substrate or enzyme. The dye did not inhibit the action of cathepsin B and C against their substrates, and it was impossible to test inhibition of carboxypeptidase A because of interference with product determination (A_{280}). Therefore, it is concluded that the dye's major effect on in vitro thyroglobulin degradation is inhibition of endopeptidase activity.

The inhibition of cathepsin D activity was competitive in type and was overcome by increasing substrate concentration. The slight increase in velocity observed with low concentrations of trypan blue relative to thyroglobulin (less than 100 : 1 molar ratio) may indicate that the dye interferes with or

binds to the site of enzyme-substrate interaction, and at these concentrations enhanced binding is affected.

Early in these experiments it became evident that equimolar concentrations of thyroglobulin and protease were being employed. There are, however, many potential and actual sites for endopeptidase cleavage in both hemoglobin and thyroglobulin and usually two susceptible peptide bonds must be cleaved to yield trichloroacetic acid-soluble or butanol-soluble products. Comparison of the release of these soluble products from the two labeled substrates revealed that trichloroacetic acid- and butanol-soluble products from thyroglobulin were equal, but release of butanol-soluble products from hemoglobin did not occur. This indicates differences in primary structure of the two substrates and the occurrence of very proximate, susceptible bonds in thyroglobulin allowing the release of iodoamino acids or very small peptide fragments containing iodoamino acids.

Trypan blue represents another inhibitor of cathepsin D and in comparison to the pepsin inhibitor diazoacetylnorleucine methyl ester [3,4], Cu^{2+} was not required and inhibition was competitive. Trypan blue also inhibited proteolysis when it was prebound to the substrate. Pepstatin, another inhibitor of most acid proteinases, like trypan blue, has no effect on thiol-dependent proteases [26] and appears to inhibit competitively [27].

Trypan blue does inhibit the action of both crude and purified protease and this action could represent a major site of the inhibition of thyroid secretion observed in vitro and in vivo [1]. Inhibition of protease does not explain the marked decrease in colloid droplets or phagolysosomes that was observed in mouse thyroid [1]. Indeed, if inhibition of lysosomal enzymes was the only mechanism of action of the dye, one might expect an increase in colloid droplets containing undigested thyroglobulin. In preliminary observations the decrease in colloid droplets has been confirmed by electron microscopic studies and fusion of colloid droplets with lysosomes appears to occur normally. By fluorescence microscopy, rapid entry of trypan blue into the colloid space and attachment to protein have been observed and this dye-protein complex may represent the mode of entry of the dye into the thyroid follicular cell and/or may interfere with the process of pinocytosis. These and other studies of trypan blue action on pinocytosis are in progress.

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