INHIBITION OF PROTEOLYSIS OF CYTOSOL PROTEINS BY LYSOSOMAL PROTEASES AND OF MITOCHONDRIA OF RAT LIVER BY ANTIBIOTICS

S. Grisolia, J. Rivas, R. Wallace and J. Mendelson

Department of Biochemistry
University of Kansas Medical Center
Kansas City, Kansas 66103

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SUMMARY. Chloramphenicol and cycloheximide inhibit the proteolysis of cytosol proteins by lysosomal preparations and of mitochondria from rat liver; streptomycin has no effect. The % inhibition of proteolysis by chloramphenicol is the same at pH 5.0, at neutral pH or at pH 8.5. The findings presented show for the first time a marked inhibition of proteolysis by lysosomal enzymes and of mitochondrial preparations. The need for caution in interpreting inhibition experiments by antibiotics as solely due to inhibition of protein synthesis is pointed out.

The inhibitory effect of antibiotics on protein synthesis is well known; moreover, it has been proposed from time to time that these compounds would also inhibit protein degradation (1). It has been shown recently that the activity of liver tyrosine aminotransferase is indeed increased by injection of tetracycline in rats, probably due to inhibition of enzyme degradation "in vivo". That this is the likely explanation is strengthened by the fact that tetracycline protects the enzyme in homogenates or when incubated with rat liver lysosomes (2).

We have found that chloramphenicol and cycloheximide, albeit at high concentrations, inhibit the proteolysis of cytosol proteins by lysosomal preparations and of mitochondria from rat liver in a broad pH range. Thus, although doubts have been expressed that antibiotics would inhibit proteolysis in cell-free extracts (1), indeed the inhibition by tetracycline of tyrosine aminotransferase inactivation by rat liver lysosomes is small, i.e., ∿20% (2), the findings presented here show profound inhibition of proteolysis with lysosomal extracts and with mitochondrial preparations.

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Rubio and Grisolia have shown recently that the bulk of proteolysis exhibited by mitochondrial preparations at neutral pH is likely due to lysosomal contamination (3). Since the % inhibition of proteolysis by lysosomal and mitochondrial fractions of rat liver by chloramphenicol is the same at pH 5.0, at neutral pH and even at pH 8.5, it confirms that the proteolysis of mitochondria is indeed mostly due to the contaminant lysosomal content.

MATERIALS AND METHODS. [^{14}C]-leucine (50 μCi/ml, 312 mCi/mmol) was purchased from Schwarz-Mann, Orangeburg, New York. [^{3}H]-leucine (1000 μCi/ml, 60,000 mCi/mmol) was from Amersham/Searle, Arlington Heights, Illinois. Aquasol (scintillation solution) was obtained from New England Nuclear, Boston, Mass. Chloramphenicol, Cycloheximide and Streptomycin Sulfate were from Sigma Chemical Co., St. Louis, Mo. All other chemicals were of analytical grade.

Male Holtzman rats, weighing 250-350 g, were used. Rats were injected I.P. with either 25-100 μCi [^{14}C]-leucine or 125 μCi [^{3}H]-leucine (specific activity adjusted to 1000 mCi/mmol), and killed by decapitation 5-8 h later. Liver mitochondria were prepared by the method of Schneider (4), except that a third wash with 0.15 M KCl was done. The mitochondria were then resuspended in cold distilled water, broken by homogenization for 20 s with an Ultra-turrax and frozen once in a dry ice-acetone bath. These, and all other liver fractions, were kept frozen until use. The mitochondrial supernatant was centrifuged at 48,000 x g at 4°C for 30 min to obtain a cytosol fraction. To each ml of the resulting supernatant 0.56 g ammonium sulfate was added (85% saturation). After centrifugation (10,000 x g for 15 min), the precipitate was taken, dissolved in cold water and dialyzed against running tap water overnight.

Highly purified lysosomes and extracts thereof were prepared by a modification (5) of the method of Ragab, et al. (6). The method of Lowry, et al. (7) was used for determining protein in the lysosomal preparations. Protein in all other fractions was determined by a biuret method (8). Bovine serum albumin was used as a standard.

To assess radioactivity in the liver fractions, aliquots were precipitated with cold trichloroacetic acid to a final concentration of 5%. The precipitate was washed once with 5% trichloroacetic acid, dissolved in 0.2 M KOH, transferred to a scintillation vial, neutralized with 6 N HCl, and after addition of 10.0 ml Aquasol counted in a Nuclear Chicago Isocap/300 liquid scintillation system. All the samples were counted long enough to give results within 5% or less statistical error.

Proteolytic activity was determined as follows: reaction mixtures, containing 50 mM potassium phosphate buffer at specified pH, protein and (when used) antibiotics were incubated at 37°C. The amounts of antibiotics are given as is customary in w/v; they were neutralized and added at the specified pH's. In some cases when using lysosomes citrate buffer at pH 5.0 was used together with 2-mercaptoethanol. At 0 time and at the indicated times, aliquots were taken and mixed with trichloroacetic acid to a final concentration of 5% and then centrifuged. Aliquots of the supernatants were counted in scintillation vials as described above. Protein breakdown was determined as the amount of acid-soluble radioactivity relative to that initially found in protein (i.e., acid-precipitable form) or as ninhydrin-positive material by the method of Spies (9) with leucine as a standard. All measurements were corrected for endogenous values.

TABLE I. Effect of chloramphenicol, cycloheximide and streptomycin on the proteolysis of rat liver mitochondria.

EXPT.	ANTIBIOTIC ADDED	% INHIBITION OF PROTEIN HYDROLYSIS BASED ON	
	mg	RADIOACTIVITY	NINHYDRIN
1	None	--	0
	Chloramphenicol, 2.5	--	42
2	None	0	--
	Chloramphenicol, 0.5	16	--
	" 1.0	36	38
	" 2.5	48	--
	" 5.0	62	60
	" 8.0	67	--
3	None	0	--
	Chloramphenicol, 3	32	--
	" 6	43	--
	" 9	59	--
	Cycloheximide, 6	29	--
	" 12	52	--
	Streptomycin, 24	0	--

Reaction mixtures contained in 1.0 ml 50 μmoles potassium phosphate buffer, pH 7.4, the following amounts of mitochondrial protein: 5 mg (experiment 1), 12.5 mg containing 950 cpm ^{14}C (experiment 2), or 19.2 mg containing 22,850 cpm ^{3}H (experiment 3), and the stated amounts of antibiotics. The hydrolysis without antibiotics calculated on the basis of either radioactivity or ninhydrin-positive material liberated was as follows: experiment 1, 0.31 μmoles aminoacid formed/mg protein at 15 h; experiment 2, 11% protein breakdown in the absence of chloramphenicol; 0.29 μmoles aminoacid formed/mg protein at 3 h; experiment 3, 8.4% protein breakdown in the absence of antibiotics at 3 h.

RESULTS AND DISCUSSION. Although chloramphenicol is generally thought of as an inhibitor of protein synthesis by prokaryots, Ibrahim, et al. (10) have shown that aminoacid incorporation into protein is inhibited by chloramphenicol with rat liver mitochondria. As shown in Table I, by liberation of trichloroacetic

acid-soluble radioactive material (with both ^{14}C- and ^{3}H-labelled proteins) and by increase in ninhydrin-positive material, there is inhibition of mitochondrial proteolysis at neutral pH with increasing concentrations of chloramphenicol. As shown, cycloheximide, a typical inhibitor of protein synthesis by animal tissues, is also inhibitory, while streptomycin, which has no effect on protein synthesis by eukaryots, is not.

Table II, Expt. 1 illustrates the effect of pH on the proteolysis of mitochondria and the inhibitory effect of chloramphenicol thereon. It is interesting that the mitochondrial preparation still showed marked activity at neutral pH, more so than could be expected from the lysosomal activity on the cytosol proteins (see below). This may reflect both the presence of some non-lysosomal proteases and the fact that the mitochondrial proteins behave differently from the cytosol proteins which are known to be excellent substrates for lysosomal proteases (11). As shown in Table II, Expt. 2, using [^{14}C]-leucine labelled cytosol proteins and at different concentrations of lysosomal protein, chloramphenicol inhibits markedly lysosomal activity. As shown, proteolysis is roughly proportional to the amount of lysosomal protein added and the % inhibition is approximately the same at both levels of lysosomal protein. There was little proteolysis in the absence of lysosomal protein.

Table II, Expt. 3 illustrates with cytosol proteins labelled with [^{3}H]-leucine the remarkable decrease in activity of the lysosomal preparation when the pH was changed from 5.0 to 7.4. Since the activity with lysosomes drops so much with the increase in pH, the data we have included in the table for pH 7.4 are only from experiments done with a ratio of lysosomes to cytosol protein 10 times higher and with a longer incubation time than at pH 5.0. Under these circumstances, it is possible to assess the proteolytic activity of the lysosomal preparations. It can be calculated from the data that the activity of the purified lysosomal preparation drops about 100-fold with the change in pH from 5.0 to 7.4. Again, at all pH's, chloramphenicol inhibited the proteolysis by about the same %.

TABLE II. Effect of chloramphenicol on the hydrolysis of mitochondrial and of cytosol proteins at several pH's.

EXPT.	pH	INCUBATION TIME	CHLORAMPHENICOL ADDED	
			NO	YES
		(h)	(% protein hydrolyzed)	
1	5.0	2	40	23
	6.0	2	33	20
	7.4	3	9.5	5.5
	"	6	14	7
	"	24	20	11
	8.5	3	13.5	4.8
	"	24	16	9
2	5.0*	2	15.5	3.5
	5.0**	2	31	9.5
3	5.0	2	8	2.4
	7.4	4	0.9	0.3
	8.5	4	0.3	0.2

For Expt. 1, incubation mixtures contained in 1.0 ml 19.2 mg ^{3}H-labelled mitochondrial protein (17,550 cpm), 50 µmoles potassium phosphate buffer at indicated pH's, and 6 mg chloramphenicol when used. For Expt. 2, incubation mixtures contained in 1.3 ml 6.28 mg ^{14}C-labelled cytosol protein (2830 cpm); 150 µmoles citrate buffer, pH 5.0; 0.25 µmoles mercaptoethanol and 0.07(*) or 0.14(**) mg lysosomal protein. For Expt. 3, incubation mixtures contained in 1.3 ml 1) at pH 5.0, 3.6 mg ^{3}H-labelled cytosol protein (10,400 cpm); 65 µmoles citrate buffer, pH 5.0; 0.25 µmoles mercaptoethanol; and 0.07 mg lysosomal protein; 2) at pH 7.4 and 8.5, 1.45 mg ^{3}H-labelled cytosol protein (4160 cpm); 65 µmoles potassium phosphate buffer, pH 7.4 and 8.5, respectively; 0.25 µmoles mercaptoethanol; and 0.28 mg lysosomal protein. For Expts. 2 and 3, 7.8 mg chloramphenicol were added when used.

Fig. 1 illustrates increased inhibition of proteolysis by lysosomal proteases with the concentration of chloramphenicol. Also, as shown, cycloheximide but not streptomycin has an inhibitory effect. It should be noted that because of the high concentrations of antibiotics used, the effect of high salt concentra-

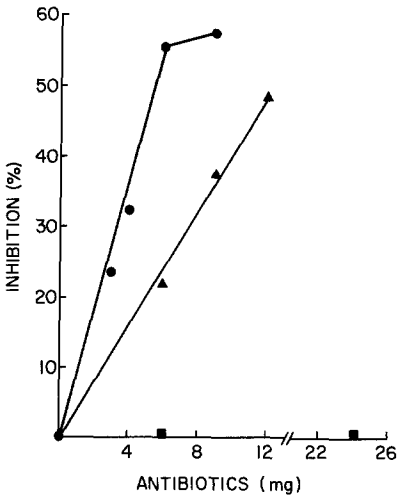

Fig. 1. Effect of antibiotics on the hydrolysis of cytosol proteins by lysosomal extracts. Incubation mixtures contained in 1.3 ml 3.6 mg ^{3}H-labelled cytosol protein (10,400 cpm); 65 µmoles citrate buffer, pH 5.0; 0.25 µmoles mercaptoethanol; and 0.07 mg lysosomal protein. 4 h incubation. Protein hydrolysis without antibiotics was 11%. ● - chloramphenicol; ▲- cycloheximide; ■- streptomycin.

tions was tested; no effect on proteolysis by either lysosomal or mitochondrial preparations was noted by the addition of up to 24 mg NaCl per ml incubation. Of course, it is not feasible to do experiments at higher levels of chloramphenicol due to solubility. As shown with mitochondria and cytosol, the inhibition progresses with the concentration of antibiotic and then levels off. This indicates that the antibiotic may inhibit one family of proteases and not others. It should be noted that the inhibition of phenylalanine synthesis by chloramphenicol and other antibiotics with yeast and rat liver polysomes also levels off at high concentrations of antibiotic (2).

As extensively illustrated by other workers, it is very difficult to differentiate between the proteases of lysosomes which indeed have at least 10 endopeptidases (11). Thus, it is a difficult task to assign a role and/or to be sure that one or more peptidases are involved in a certain effect. Perhaps the inhibition by antibiotics demonstrated here may serve to differentiate or separate some eukaryotic proteases into antibiotic-sensitive and insensitive enzymes.

Inasmuch as the extent of inhibition by chloramphenicol is similar with

extensively purified lysosomal preparations and with mitochondrial preparations, the results give added support to earlier findings suggesting that the bulk of mitochondrial protein degradation is due to lysosomes (3,12). Since the inhibition by chloramphenicol is moderate even at high concentrations of the antibiotic, while that exhibited by cycloheximide is much lower and that of streptomycin is nil, it is apparent that these antibiotics may be generally used in the bacteriostatic range without serious interference with degradation of proteins by lysosomal enzymes. On the other hand, cases are known of micro-organisms which grow even in the presence of 500 to 1000 μg chloramphenicol per ml, e.g. Clostridium perfringens, Histoplasma capsulatum, and even others, e.g. Endamoeba histolytica, which seems entirely resistant (13). Moreover, it seems that the concentration needed, in some cases e.g. 250 μg of chloramphenicol per ml (2), for extensive inhibition (80-90%) of polyphenylalanine synthesis by rat liver and yeast polysomes may inhibit some proteases.

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