In Vitro Catabolism of Heterologous and Homologous Serum Albumin by Lysosomal Enzymes[1] (37017)

GARY L. CURTIS AND WAYNE L. RYAN
University of Nebraska College of Medicine, Omaha, Nebraska 68105

In the sequence of events leading to the production of antibodies, it is generally believed that macrophages play an important but unknown role. The macrophage may in some manner "process" an antigen into antigenic material which can be further utilized by the T and/or the B lymphocytes (1, 2).

Foreign protein is believed to be catabolized prior to initiating antibody synthesis (3–6). Catabolism of heterologous protein is thought to occur in the lysosomes of cells, particularly in the macrophages of the reticuloendothelial cells (7, 8). Although the complete role of lysosomes in biological systems is not known, one proposed function of lysosomes has been that of regulating the amount of foreign protein which is made available to antibody-forming cells (9).

The fact that homologous and heterologous protein degradation results in two different phenomena, *i.e.*, homologous protein degradation resulting in the production of amino acids which are made available to the amino acid pool for reutilization in protein synthesis (10) and heterologous protein degradation resulting in the production of antibodies, lead to the investigation of the catabolism of heterologous and homologous protein by lysosomes. Cathepsin D is believed to be the proteolytic enzyme responsible for much of the proteolytic activity found in lysosomes (11). Ryan and Lee found a correlation between the *in vivo* antigenicity and *in vitro* catabolism of a protein. They demonstrated that the more antigenic the protein, the less its degradation by liver lysosomes (12). This paper compares the degradation of homologous and heterologous protein by lysosomal proteases under *in vitro* conditions optimal for cathepsin D activity (13).

Materials and Methods. Preparation of homogenates. Liver and spleen homogenates were prepared from female Wistar rats and New Zealand female rabbits. Animals were sacrificed by cervical dislocation after fasting for 24 to 48 hr. The livers and spleens were rapidly removed and washed free of blood with cold saline. During the working period, all the homogenates were kept near 5°. Following mincing, the livers and spleens were placed in iced saline and homogenized with the TRI-R STIR-R Model K43 using a teflon pestle and glass container. Homogenates were frozen and thawed six times, filtered through two layers of cheese cloth, and protein content and enzymatic activity determined. Protein determinations were performed by the method of Lowry *et al.* (14).

Assay of cathepsin D. Proteolytic activity (cathepsin D) was assayed by a modification of the method of Press *et al.* (11). Denatured bovine hemoglobin substrate (Mann Research Lab) was suspended in water and dialyzed at 2° for 3 days. The hemoglobin concentration was adjusted to 2% (w/v) with water. Two milliliters of the 2% hemoglobin solution were added to 2.9 ml of 0.4 *M* citrate buffer (pH 2.8). To this solution was added 0.1 ml of enzyme solution. The enzymic mixture was incubated at 37° for 1 hr and 4 ml of 10% trichloroacetic acid was added to stop the proteolytic reaction. The solution was heated at 60° for 10 min, centrifuged at 120*g* for 30 min, and filtered through Whatman No. 3 filter paper. The absorption of the filtrate at 280 nm was measured in the Beckman DB dual beam spectrophotometer.

Catabolism of serum albumin. Incubations

[1] This work was supported by a Research Grant from the John A. Hartford Foundation and by PHS Grant 3 T01-HD00018-10S1 HDPB.

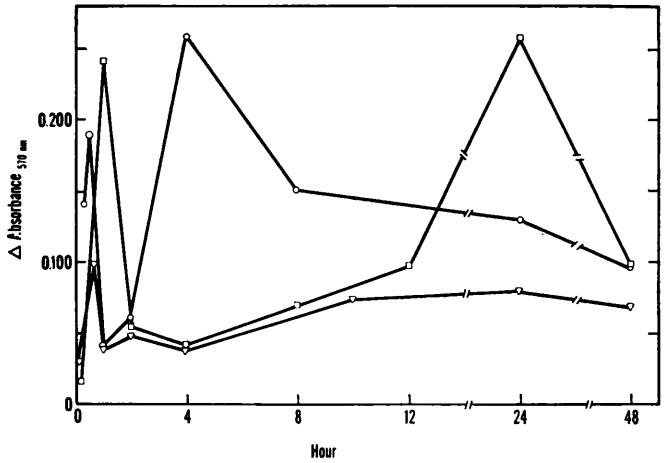

FIG. 1. Ninhydrin reaction of retained peptides (greater than 1,000 mol wt and less than 10,000 mol wt) resulting from rat serum albumin incubated with (○) rabbit spleen homogenate, (□) rat spleen homogenate, and (▽) rat serum albumin alone. Equal amounts of peptide sample were reacted with ninhydrin for 15 min at 60° and 4 ml *n*-propanol-H_2O (1:1 v/v) was added and read at 570 nm. Similar results were obtained from two experiments. The values used to calculate each point on the graph varied less than 6%.

of heterologous and homologous spleen homogenates with rat serum albumin substrate were performed in an Amicon Diaflow Ultrafilter apparatus. The apparatus was modified in such a manner that the magnetic stirrer would not touch the membrane, but was supported by a "collar" that was placed between the stirrer and the membrane. The stirrer was free to rotate, but would not damage the membrane during prolonged use.

One-hundred-milligram rat serum albumin with 0.0028 units of activity of either rabbit (0.70 units/mg) of rat spleen (0.43 units/mg) homogenate were placed within the modified ultrafilter which had a volume of 10 ml. The controls consisted of 100 mg rat serum albumin or the homogenates alone. The pressure was maintained at 55 psi on the ultrafilter cell by means of an Accuflo pump which pumped a 0.1 *M* formate buffer, pH 3.0. The temperature was maintained at 37°. A membrane (Amicon, UM-10), which retains particles with a molecular weight greater than 10,000, was placed on the bottom of the ultrafilter apparatus. The filtrate (solution which went through the UM-10 membrane) was continuously collected and separated at prescribed intervals. The filtrate from the UM-10 membrane was then passed through another ultrafiltration membrane (Amicon UM-2), which excludes anything greater than 1,000 molecular weight (15). The fraction which passed through the UM-10, but not the UM-2 membrane (retained fraction) was subjected to ninhydrin analysis. The fraction which passed through both the UM-10 and the UM-2 membranes (ultrafiltrate) was subjected to ninhydrin and amino acid analysis.

Amino acid and peptide estimation. For the ninhydrin analysis of peptides (retained fraction) and amino acids (ultrafiltrate), the samples were lyophilized and the retained fraction resuspended in 6.0 ml 0.1 *N* $NaHCO_3$ and the ultrafiltrate fraction was resuspended in 6.0 ml H_2O. One ml of solution was mixed with 1 ml ninhydrin and placed in the water bath at 60° for 15 min in sealed test tubes. Two milliliters *n*-propanol-water (1:1 v/v) was added, and the mixture was read in a DB spectrometer at 570 nm.

Amino acid analysis. Two milliliters of the ultrafiltrate fractions were analyzed for amino acids on the Beckman Model B Amino Acid Analyzer. To obtain amino acid analysis of the ultrafiltrate fraction following acid hydrolysis, 2 ml of ultrafiltrate fraction were added to 2 ml of 12 *N* HCl, sealed in a vacuum, and hydrolyzed and analyzed for amino acids on the Beckman Model 120 B Amino Acid Analyzer.

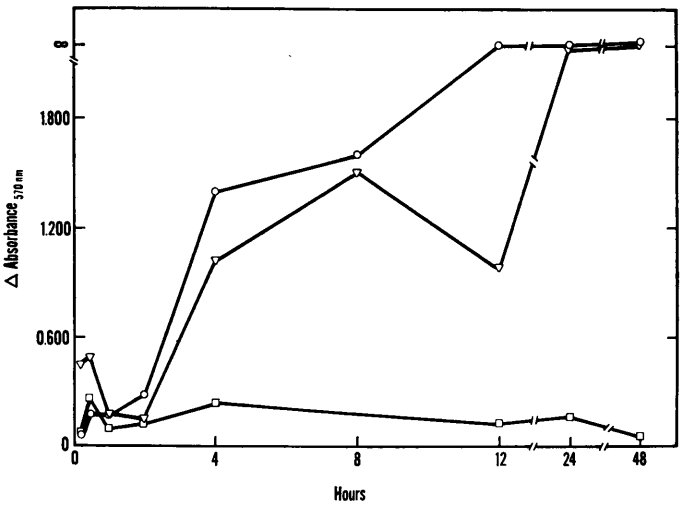

FIG. 2. Ninhydrin reaction of ultrafiltrate fraction (less than 1,000 mol wt) resulting from rat serum albumin incubated with (○) rabbit spleen homogenate, (▽) rat spleen homogenate, and (□) rat serum albumin alone. Equal amounts of ultrafiltration fraction was reacted with ninhydrin for 15 min at 60° and 4 ml *n*-propanol-H_2O (1:1 v/v) was added and read at 570 nm. Similar results were obtained from two experiments. The values used to calculate each point on the graph varied less than 6%.

Comparative extent of heterologous and homologous degradation of serum albumin. To determine the substrate concentration curve and kinetics of catabolism, rabbit or rat liver and spleen homogenates were adjusted to the same enzyme activity as judged by the hemoglobin assay. Increasing concentrations of rat and rabbit serum albumin (Fraction IV, Pentex Co.) was added to the test tubes. The homogenates in 0.5 ml saline were thoroughly mixed with the albumin substrates. The albumin was in 2.5 ml of a 0.25 *M* formate buffer, pH 3.0. Following incubation for one hour at 37°, 3 ml of a 10% trichloroacetic acid solution was added to each test tube. They were heated for 10 min at 60° and centrifuged at 1,000 rpm for 30 min, then the precipitate was removed by filtration through Whatman No. 3 filter paper. One milliliter of supernatant was mixed with 1 ml ninhydrin and placed in the water bath at 60° for 15 min. Two milliliter *n*-propanol-water (1:1 v/v) was added to the reactants and read at 570 nm.

Results. A comparison of the peptides from homologous and heterologous spleen degradation of serum albumin can be seen in Fig. 1. The heterologous degradation produces peptides in greater quantities more rapidly (4 hr), while homologous degradation does not produce detectable peptides until 24 hr.

For a quantitative comparison of the amino acids resulting from homologous and heterologous spleen degradation of serum albumin, the ultrafiltrate fraction was reacted with equal amounts of ninhydrin for 15 min at 60°, after which 4 ml *n*-propanol-water (1:1 v/v) was added and the samples read at 570 nm. The results are seen in Fig. 2. Both the heterologous and hon degradation gives similar levels of nin reactive substances during the reactic tested.

Amino acid analysis was performed on the ultrafiltrate resulting from heterologous and homologous degradation at 4, 8, and 48 hr. The results of the analysis are shown in Table I. It appears that homologous degradation produces more amino acids than does heterologous degradation. However, because the ultrafiltrate contains molecules less than 1,000 molecular weight, the possibility of small dipeptides and tripeptides coming through the UM-2 membrane along with the amino acids was considered. The ultrafiltrate fractions were hydrolyzed in 6 *N* HCl for 36 hr at 110°, lyophilized, and the hydrolyzates ana-

TABLE I. Amino Acid Analysis of the Ultrafiltrate Fraction (Less Than 1,000 mol wt) Resulting from Rat Serum Albumin Incubated with Rabbit Spleen Homogenate and Rat Spleen Homogenate for 4, 8, 48 hr.[a]

	4 hr		8 hr		48 hr	
	Rabbit spleen homogenate	Rat spleen homogenate	Rabbit spleen homogenate	Rat spleen homogenate	Rabbit spleen homogenate	Rat spleen homogenate
Aspartic acid	0.035	*	*	0.008	*	0.071
Threonine	0.017	*	*	0.008	*	0.035
Serine	0.196	0.187	0.026	0.107	0.142	0.428
Glutamic acid	*	*	*	*	*	*
Glycine	0.160	0.187	0.071	0.071	0.142	0.285
Alanine	0.071	0.127	0.062	0.133	0.142	0.499
Valine	*	*	*	0.062	*	0.285
Isoleucine	0.026	0.499	*	0.026	*	*
Leucine	0.151	1.535	0.303	0.857	0.583	0.196
Tyrosine	0.026	0.428	*	0.008	*	*
Phenylalanine	0.026	0.732	*	0.053	*	*

* $<$ 0.003 μmoles.

[a] Amino acid values are expressed as μmoles/unit Cathepsin D/min.

lyzed on the amino acid analyzer. The results are seen in Table II. There was a large increase in amino acids from the acid hydrolysis of the heterologous degradation (rat serum albumin incubated with rabbit spleen homogenate). This is a much greater increase than is seen with the homologous degradation. It appears, therefore, that heterologous degradation produces considerably more small peptides (dipeptides and tripeptides, less than 1,000 mol wt) than does homologous degradation.

For a comparison of the extent of homologous and heterologous degradation of albumin, liver and spleen homogenates from rat and rabbit were adjusted to the same enzymatic activity as determined by the hemoglobin assay and incubated with rabbit or rat serum albumin.

The results are seen in Figs. 3 and 4. The rabbit liver homogenate with rat serum albumin gives the greatest degradation, while the rat liver homogenate gives the greatest degradation with the rabbit serum albumin. In both cases the heterologous liver degrades protein to a greater extent than does homologous liver. This is not the case for spleen; heterologous spleen does not in both cases (rat and rabbit albumin substrates) give greater degradation than does homologous degradation. The rat spleen homogenate does not degrade rabbit serum albumin to a greater extent than does rabbit spleen homogenate.

Heterologous liver demonstrates greater activity towards serum albumin than does homologous liver. Heterologous liver also demonstrates greater activity toward serum albumin than does either heterologous or homologous spleen at pH 3.0.

Discussion. The initial amino acid analysis on the ultrafiltrate fraction (less than 1,000 mol wt) from the rabbit and rat spleen homogenate degradation of rat serum albumin demonstrated a larger production of amino acids for the homologous degradation (*i.e.*, the rat spleen homogenate degradation) than the heterologous degradation (*i.e.*, the rabbit spleen homogenate degradation). However, because there was the possibility of this less than 1,000 mol wt fraction containing small dipeptides and tripeptides, acid hydrolysis was carried out on each sample followed by amino acid analysis. This demonstrated that the heterologous (rabbit spleen) degradation produced far more small peptides (less than 1,000 mol wt) than did the homologous (rat spleen) degradation. The importance these small peptides (less than 1,000 mol wt) may have in initiating an immune response warrant further investigation. It is at this time

TABLE II. Amino Acid Analysis of the Ultrafiltrate Fraction (Less than 1,000 mol wt) Resulting from 4 hr Incubations of Rat Serum Albumin with Rabbit Spleen Homogenate and Rat Spleen Homogenate.[a]

	Rabbit spleen homogenate			Rat spleen homogenate		
	Initial values	Following acid hydrolysis	Factoral increase	Initial values	Following acid hydrolysis	Factoral increase
Aspartic acid	0.035	1.392	40	*	0.187	13
Threonine	0.017	0.910	50	*	0.157	11
Serine	0.196	0.857	4	0.187	0.404	2
Glutamic acid	*	2.249	250	*	0.461	32
Glycine	0.160	0.785	5	0.187	0.491	3
Alanine	0.071	1.749	25	0.127	0.562	4
Valine	*	0.874	100	*	0.041	3
Isoleucine	0.026	0.499	20	*	0.288	20
Leucine	0.151	1.535	10	0.461	0.723	1.6
Tyrosine	0.026	0.428	16	*	*	0
Phenylalanine	0.026	0.732	27	*	0.273	20

* $<$ 0.003 μmoles.

[a] Amino acid values are expressed as μmoles/unit cathepsin D/min. Values given are prior to and following acid hydrolysis. The % increase indicates increase in μmoles following acid hydrolysis over the level observed prior to hydrolysis.

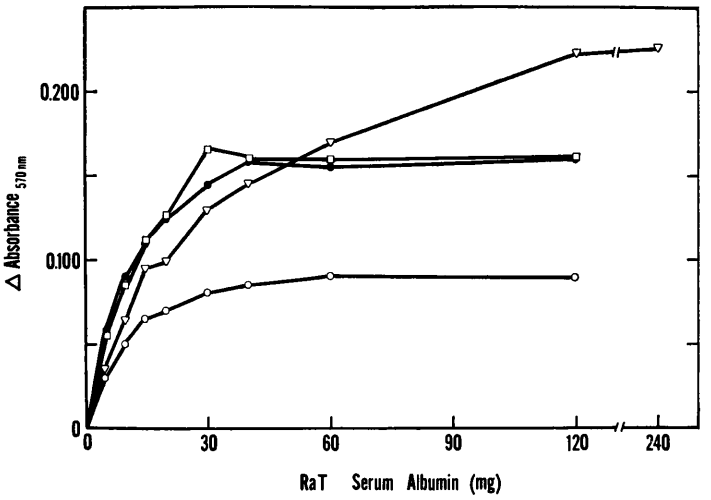

FIG. 3. Substrate concentration curve. Rat serum albumin incubated with rabbit liver homogenate (▽), rabbit spleen homogenate (□), rat liver homogenate (●), and rat spleen homogenate (○). Rat spleen and liver homogenates and also rabbit spleen and liver homogenates were adjusted to the same enzymatic activity as determined by the hemoglobin assay. Rat serum albumin in 2.5 ml of 0.25 *M* formate buffer, pH 3.0, was placed in test tubes in increasing concentrations. The homogenates (0.5 ml) were added and incubation was for 1 hr at 37°. At the end of the incubation, 3 ml of 10% TCA was added and the solution was heated at 60° for 10 min, centrifuged to remove precipitate, and filtered through Whatman No. 3 filter paper. One milliliter filtrate was reacted with 1 ml ninhydrin and heated at 60° for 15 min. Two milliliter *n*-propanol-water (1:1 v/v) was added and read at 570 nm. The controls and reagent blanks received TCA prior to incubation. Values are an average of four experiments. The values used to calculate each point on the graph varied less than 5%.

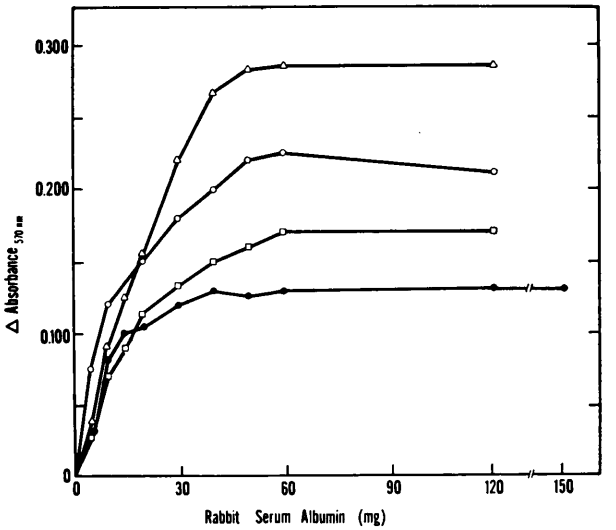

FIG. 4. Substrate concentration curve. Rabbit serum albumin incubated with rat liver homogenate (△), rabbit liver homogenate (○), rabbit spleen homogenate (□), and rat spleen homogenate (●). Rat spleen and liver homogenates and also rabbit spleen and liver homogenates were adjusted to the same enzymatic activity as determined by the hemoglobin assay. Rabbit serum albumin in 2.5 ml of 0.25 *M* formate buffer, pH 3.0, was placed in test tubes in increasing concentrations. The homogenates (0.5 ml) were added and incubation was for 1 hr at 37°. At the end of the incubation, 3 ml of 10% TCA was added and the solution was heated at 60° for 10 min, centrifuged to remove precipitate and filtered through Whatman No. 3 filter paper. One milliliter filtrate was reacted with 1 ml ninhydrin and heated at 60° for 15 min. Two ml *n*-propanol-H_2O (1:1 v/v) was added and read at 570 nm. The controls and reagent blanks received TCA prior to incubation. Values are an average of four experiments. The values used to calculate each point on the graph varied less than 5%.

not known how or if these peptides could stimulate T or B cells. However, it has been demonstrated that peptides of this size can pass through the lysosomal membrane (16).

To compare the extent of homologous and heterologous degradation, rat and rabbit liver and spleen homogenates were used to degrade rat and rabbit serum albumin. It was found that the rabbit liver homogenate gives the greatest degradation of rat serum albumin compared to the degradation of this substrate by rabbit spleen, rat liver, and rat spleen. Similarly, rat liver homogenate gives the greatest degradation of rabbit serum albumin compared to the degradation of this substrate by rat spleen, rabbit spleen, and rabbit liver. Rabbit spleen homogenate gave greater degradation of rabbit serum albumin than did rabbit spleen homogenate.

We report that liver lysosomes are more active toward heterologous protein than homologous protein. It has previously been demonstrated that liver is capable of differentially removing foreign erythrocytes from the circulation (17). The liver, in addition, can differentiate between aggregated and soluble bovine serum albumin (18).

The significance of this more extensive degradation of heterologous rather than homologous protein by liver lysosomes may be related to the suggestion that lysosomes may function as intracellular regulators controlling the amount of antigenic material which is made available to the cytoplasm (9, 12).

The lesser degradation that is seen with homologous protein might maintain a state of immunological paralysis to homologous protein in the body. In order for foreign protein to result in an immune response, intracellular lysosomes may have to degrade much of the protein to avoid overloading the immune system and causing immune paralysis.

Summary. The products of hydrolysis and reaction rates for the degradation of homologous and heterologous serum albumin by lysosomal enzymes from spleen and liver were compared. The products of the degradation of homologous and heterologous serum albumin are similar with the exception of the small peptides with a mol wt less than 1000. A greater quantity of these small peptides were found in the degradation of heterologous serum albumin. Liver lysosomal enzymes hydrolyze heterologous serum albumin more rapidly and to a greater extent than homologous serum albumin.

The ability of an animal to distinguish between native and foreign proteins may be dependent upon the proteolytic enzymes which catabolize such proteins. The greater degradation of foreign protein by liver suggests that mechanism for differentiation of homologous and heterologous protein may reside with lysosomal enzymes.

1. Shortman, K., and Palmer, J., Cell. Immunol. **2**, 399 (1971).

2. Feldmann, M., J. Exp. Med. **135**, (5), 1049 (1972).

3. Lapresle, C., and Webb, T., Ciba Foundation Symposium Cellular Aspects Immunity, p. 44 (1960).

4. Ehrenreich, B. A., and Cohn, Z. A., J. Reticuloendothel. Soc. **5**, 230 (1968).

5. Argyris, B. F., J. Immunol. **99** (4), 744 (1967).

6. Catawzaro, P. J., Graham, R. C., Jr., and Schwartz, H. J., J. Immunol. **103**, 618 (1969).

7. Fishman, M., and Adler, F. L., J. Exp. Med. **117**, 595 (1963).

8. Ehrenreich, B. A., and Cohn, Z. A., J. Cell. Biol. **38**, 244 (1968).

9. Gabathuler, M. P., and Ryser, J. J-P, Proc. Roy. Soc. London (Biol. Sci.) **173**, 95 (1969).

10. Schultze, H. E., and Heremans, J. F., "Molecular Biology of Human Proteins," Chap. 2, p. 450. Elsevier, New York (1966).

11. Press, E. M., Porter, R. R., and Cebra, C., Biochem. J. **74**, 501 (1960).

12. Ryan, W. L., and Lee, J. W., Immunochemistry **7**, 251 (1970).

13. Iodice, A. A., Leong, V., and Weinstock, I. M., Arch. Biochem. Biophys. **117**, 477 (1966).

14. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

15. Zipilivan, E. M., Hudson, B. G., and Blatt, W. F., Anal. Biochem. **30**, 91 (1969).

16. Lloyd, J. B., Biochem. J. **121**, 245 (1971).

17. Nelson, E. L., and Buras, N. S., J. Immunol. **90**, 412 (1962).

18. Jeunet, F. S., Cain, W., and Good, R. A., *in* "Cellular Recognition" (R. T. Smith and R. A. Good, eds.), p. 295. Appleton–Century–Crofts, New York (1969).

Received Sept. 29, 1972. P.S.E.B.M., 1973, Vol. 142.