Physicochemical Properties of Bovine Muscle Particulate Cathepsin

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Some of the fundamental properties of cathepsin from bovine muscle granules were studied. Chemical (Triton X-100, acetone, hydrogen peroxide, L-adrenaline, vitamin A palmitate, and lowering pH) and physical (hypotonic sucrose, blending, freezing and thawing, and heating at 37° C.) lysis treatments freed cathepsin from muscle particulate components sedimented at 10,000 G. pH optima for muscle lysosomal cathepsin occurred at pH 4.0 and 9.0, but greatest activity was found at pH 4.0. The enzyme had a temperature optimum at 45° C. and an energy of activation between 30° and 45° C. of 4.66 kcal. per mole. Its activity was not affected by sulfhydryl reagents but was enhanced by the addition of 0.04*M* ferrous ions. The enzyme was purified by salt and organic solvent fractionations and the purity determined electrophoretically. The purified enzyme was similar to cathepsin D from spleen.

Most of the early biochemical studies on hydrolytic enzymes originating from subcellular particulate, or granular components, were done on preparations of rat liver by De Duve (*9*, *10*). He has shown in a series of experiments that a special group of cytoplasmic subcellular particles distinct from mitochondria and microsomes, termed lysosomes, contain a number of different hydrolases.

Limited study shows the presence of distinct particles in muscle tissue that contain hydrolases (*31*), but these particles and their constituent enzymes have not been studied thoroughly. Very little is known about the properties of cathepsins and other enzymes associated with particulate matter from muscle tissue.

The objective of this research was to study particulate cathepsin from bovine skeletal muscle and to determine if the behavior of this enzyme is analogous to that of other hydrolases associated with lysosomes from different sources.

Materials and Methods

Particle Source. Skeletal muscle tissue was obtained from bovine diaphragm crus muscle immediately after exsanguination. The separable fat and connective tissue were removed and the muscle was cut into small strips and ground in a meat grinder. One kilogram of ground tissue was dispersed in 10 liters of cold 0.25*M* sucrose containing 0.01*M* EDTA and adjusted to pH 7.4. Homogenization was accomplished by blending in a cold Waring Blendor for 45 seconds. The homogenate was filtered through muslin and centrifuged at 500 G for 10 minutes to remove debris and nuclei, and then at 10,000 G for 30 minutes to sediment granular components. The sediment was suspended in a small volume of 0.25*M* sucrose and aliquots were removed for treatments which disrupt lipoprotein membranes.

Lysis Treatments of Particles Sedimented at 10,000 G. Chemical treatments included incubation at 4° C. for 1 hour with Triton X-100, acetone, and hydrogen peroxide or incubation at 37° C. for 45 minutes in the presence of 110μM L-adrenaline or 19.1m*M* vitamin A palmitate. Physical treatments consisted of incubation at 4° C. for 1 hour in the presence of hypo-osmotic concentrations of sucrose, repeated freezing by carbon dioxide and thawing, blending for various times in a Waring Blendor, heating at 37° C. for various time intervals, and lowering pH by the addition of 0.1*M* citrate buffer. Each treatment was accompanied by an appropriate control system.

Following each of the above treatments, the experimental and control samples were centrifuged at 20,000 G for 30 minutes and the supernatant was assayed for cathepsin activity.

Measurement of Activity. Protease activity was measured by a method similar to that of Anson (*2*) as modified by Sliwinski, Doty, and Landmann (*28*). The reaction was carried out at 37° C. for 2 hours in pH 4.0 acetate buffer. Specific activity was calculated as the change in absorbance at 274 mμ of trichloroacetic (TCA)–soluble substances between zero and 2 hours of reaction per milligram of protein times 1000. Protein was determined by a biuret method (*18*). The method of Udenfriend and Cooper (*33*) was used to measure tyrosine when L-adrenaline was used in the enzyme-substrate mixture.

Cathepsin A, B, and C activities were determined by using carbobenzoxy-L-glutamyl-L-tyrosine, benzoyl-L-arginine amide, and glycyl-L-phenylalanine amide as substrates. Rates of hydrolysis were measured by titration of carboxyl groups in acetone with alcoholic hydrochloric acid (*22*).

Purification. The zinc-ethanol method of Cohn *et al.* (*5*) as modified by Snoke and Neurath (*30*) for fractionating proteins was used to purify cathepsin from Triton X-100-lysed bovine muscle granules.

Polyacrylamide Gel Electrophoresis. Electrophoresis was carried out as described by Parrish and Bailey (*24*).

Electron Microscopy. Particles described above from bovine diaphragm muscle were washed with 0.25*M* sucrose and separated by centrifugation (20,000 G). They were then suspended in a small volume of

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0.06*M* sucrose and a sample was placed on a grid coated with Formvar and air-dried. The specimens were viewed with an Akashi Tronscope. Another sample was suspended in water, transferred to a grid coated with Formvar, and air-dried. Gomori staining medium (*16*) was applied, followed by drying in air for 24 hours before microscopic study. Similar suspensions were viewed under a Wild phase contrast microscope.

Results

Effect of Lysis Treatments on Particulate Cathepsin Activity. All chemical treatments recorded in Table I freed cathepsin from the particles studied. The most effective additive studied for releasing cathepsin was 19m*M* vitamin A palmitate and further study (Table II) revealed that this constituent stimulated activity at much lower concentrations. The rate of increase in activity was greatest at the lower concentrations of the vitamin (0.1 to 1.9m*M*), but all concentrations of the vitamin studied increased activity.

Table III contains results on release of cathepsin by incubating granules in buffers of varying pH for 45 minutes at 4° C. At pH 7.0, little cathepsin was released from the granules, but as pH was lowered activity gradually increased and at pH 3.0 was eight times as great as at pH 7.0.

Data obtained for cathepsin activities by exposing particles to hypotonic concentrations of sucrose for 60 minutes at 4° C. are graphically depicted in Figure 1. Decreasing sucrose concentration caused progressive increase in cathepsin activity.

Freezing and thawing of granules three times resulted in a 20-fold increase in cathepsin activity (Figure 2). However, after three freezings and thawings the activity decreased until at 16 freezings and thawings the activity was less than half that of the maximum.

Data obtained by blending granules in a Waring Blendor for various time intervals are plotted in Figure 3. Blending granules 1 minute resulted in 10-fold increase in activity, but subsequent blending resulted in only slight increase.

In Figure 4 are results obtained by heating granules at 37° C. for 30, 60, 90, and 120 minutes followed by centrifugation. Activity progressively increased during the first 90 minutes of heating at this temperature and, at the end of this period, was five times the initial value. Subsequent heating resulted in diminished activity.

pH Optima. Results obtained by assaying the supernatant from Triton X-100-lysed particles for cathepsin activity between pH 2 and 10 are illustrated in Figure 5. Activity maxima were obtained at pH 4.0 and 9.0, although the activity at pH 4.0 was four times as great as at pH 9.0. Minimal activity was found at pH 10.0.

Energies of Activation. An Arrhenius plot of activities obtained from bovine granular cathepsin at 1°, 10°, 20°, and 30° C. is shown in Figure 6. The energy of activation from these data was 3.70 kcal. per mole and the temperature coefficient was 1.29. A similar plot of enzyme activities at 30°, 35°, 40°, and 45° C. was used to compute activation energy at these higher temperatures. The activation energy was 4.66 kcal. per mole and the activity coefficient was 1.36.

Table I. Effect of Chemical Treatments on the Release of Cathepsin from Bovine Muscle Granules

Treatment	Treatment: Time, hr.	Treatment: Temp., ° C.	Specific Activity
Control	1–4		48.7[a]
Triton X-100 (0.2%)			78.0[a]
Control	1–4		32.8[a]
Acetone (5.0%)			94.8[a]
Control	1–4		20.2[a]
Hydrogen peroxide (1.0%)			82.8[a]
Control			20.8[b]
L-Adrenaline (110μM)	3/4	37	291.5[b]
Control	3/4	37	40.2[a]
Vitamin A palmitate (19.1m*M*)			339.1[a]

[a] Change in absorbance at 274 mμ per mg. of protein per ml. × 1000.
[b] Change in absorbance at 450 mμ per mg. of protein per ml. × 1000 (*33*).

Table II. Effect of Vitamin A Palmitate on the Release of Cathepsin from Bovine Muscle Granules

Vitamin A Palmitate Concn., m*M*	Specific Activity[a]
0	40.2
0.02	36.4
0.10	41.3
0.19	82.2
0.95	154.0
1.90	179.1
9.50	246.2
19.10	339.1

[a] Change in absorbance at 274 mμ per mg. of protein per ml. × 1000.

Table III. Effect of pH upon Release of Cathepsin from Bovine Muscle Granules

pH of Granule Suspension	Specific Activity[a]
3.0	131.0
3.5	100.9
4.0	97.5
4.5	49.2
5.0	40.7
5.5	39.8
6.0	35.7
6.5	30.0
7.0	16.1

[a] Change in absorbance at 274 mμ per mg. of protein per ml. × 1000.

Purification by Zinc-Ethanol Fractionation. Results obtained from zinc-ethanol fractionation of protein from granules sedimented from bovine crus of the diaphragm muscle are shown in Table IV. The specific activity of the initial enzyme preparation was

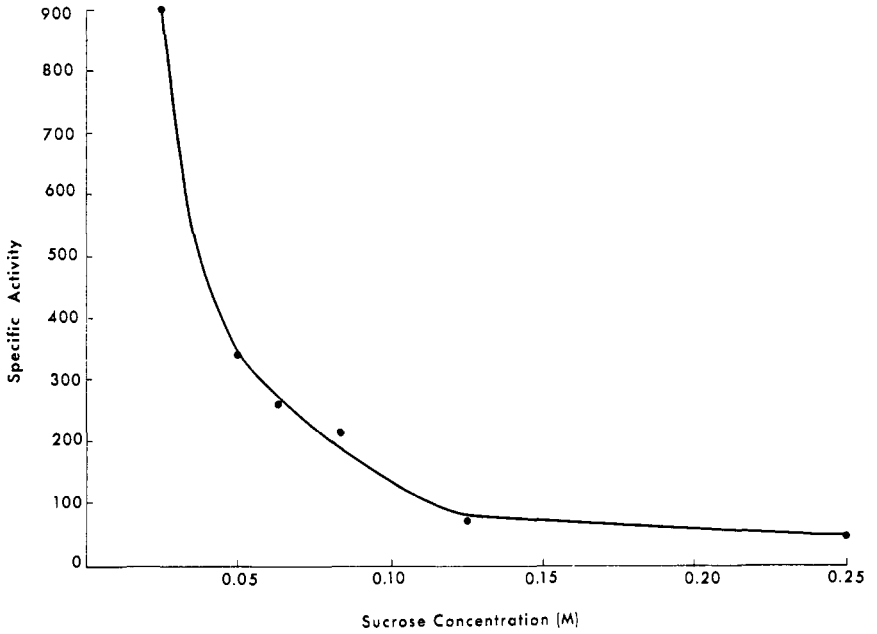

Figure 1. Liberation of bovine muscle particulate cathepsin in hypotonic sucrose

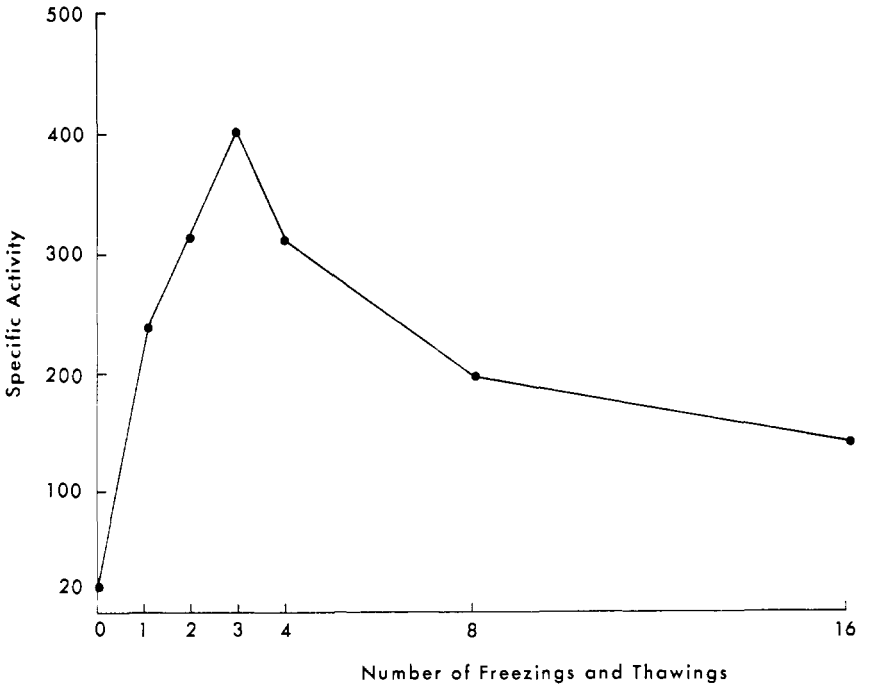

Figure 2. Liberation of bovine muscle particulate cathepsin by repeated freezing and thawing

100. Protein precipitated at pH 4.0 following addition of 1*M* hydrochloric acid had a specific activity of 101 and that of the supernatant was 110.7. The most active fraction was that obtained by precipitating the protein of the supernatant as the zinc salt in 19% ethanol (fraction A). Specific activity was 3450.0, resulting in a 34.5-fold increase in purity of the enzyme in the supernatant following lysis and a 170-fold increase in activity of the supernatant prior to lysis of particles sedimented at 10,000 G. Some enzyme remained in fraction B (pH 7.5 precipitate) and in the final supernatant.

Activation and Inhibition. Reaction mixtures containing 0.01*M* cysteine, 1m*M* iodoacetic acid, 0.1m*M* *p*-chloromercuribenzoate, and 1.5m*M* EDTA were without effect on the bovine muscle particulate cathepsin activity or the zinc-ethanol–purified preparation of bovine muscle granules. On the other hand, 0.04*M* ferrous ions in the reaction mixture increased the activity of the crude cathepsin from this source and the zinc-

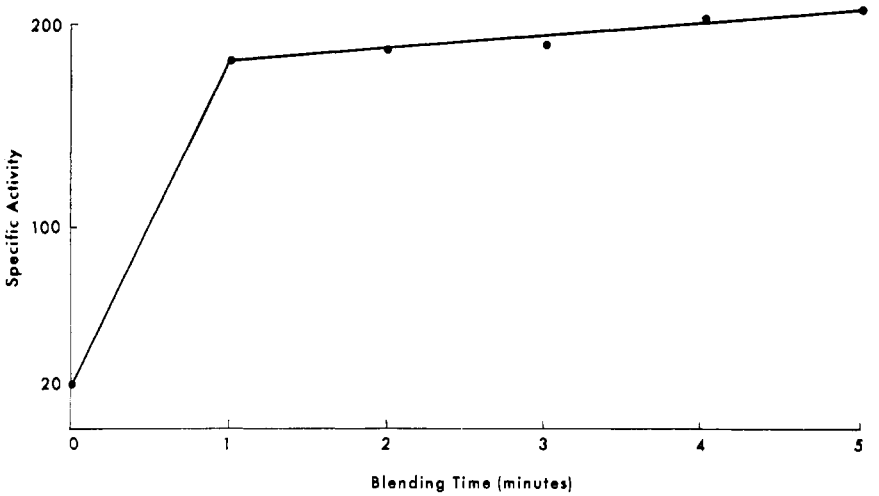

Figure 3. Liberation of bovine muscle particulate cathepsin by blending

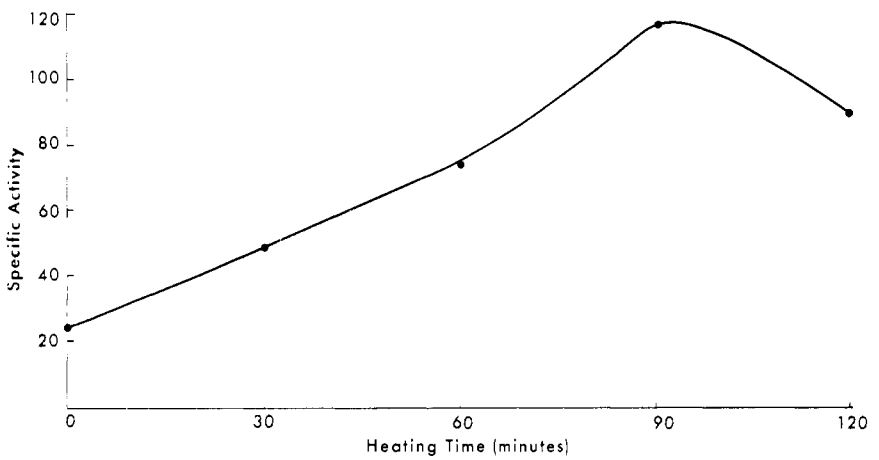

Figure 4. Thermal activation of bovine particulate cathepsin at 37° C.

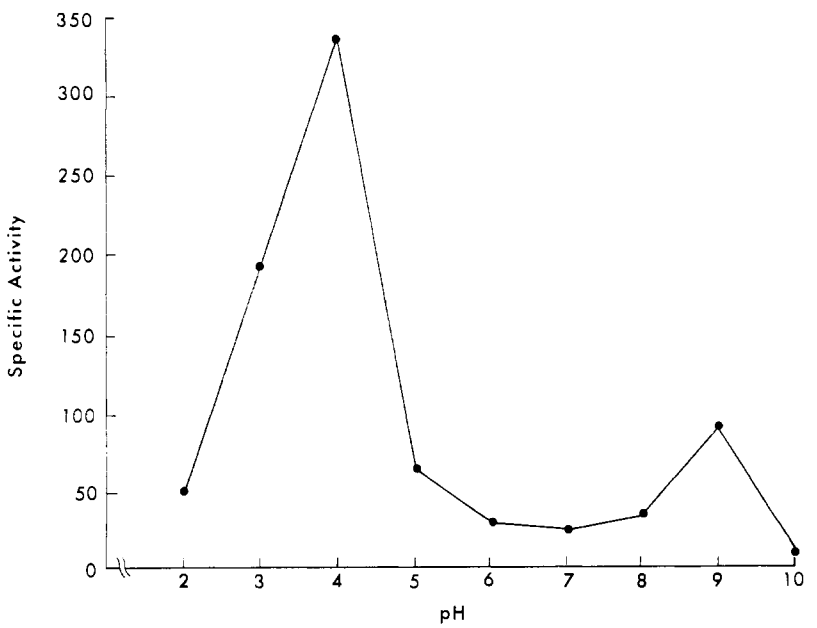

Figure 5. Effect of pH on activity of free cathepsin

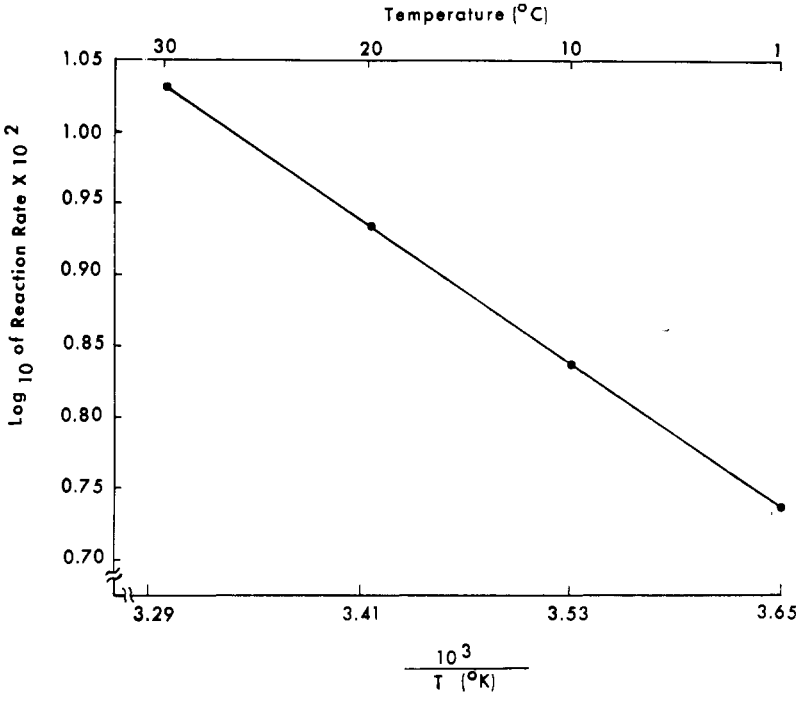

Figure 6. Arrhenius plot of bovine muscle particulate cathepsin activity between 1° and 30° C.

Table IV. Effect of Precipitation with Acid, Zinc-Ethanol, and Alkali on Bovine Granular Cathepsin Activity[a]

Treatment[b]	Specific Activity[c]	Purification
Acid precipitate at pH 4.0	101.4	1.01
Supernatant after acid precipitation	110.7	1.10
Zinc-ethanol precipitation at pH 5.8 (fraction A)	3450.0	34.50
Alkaline precipitation at pH 7.5 (fraction B)	180.0	1.80
Final supernatant	156.0	1.56

[a] Cathepsin released from granules by 0.1% Triton X-100; specific activity 100.1.
[b] Treatments similar to those described by Snoke and Neurath (*30*).
[c] Change in absorbance at 274 mμ per mg. of protein per ml. × 1000.

ethanol-purified cathepsin 10- and 30-fold, respectively.

Crude cathepsin from bovine muscle granules catalyzed hydrolysis of the substrates for cathepsins A, B, and C. The rate of hydrolysis of glycyl-L-phenylalanine amide was approximately twice that of the substrates for cathepsins A and B but under the conditions employed the activities of these enzymes were relatively low. No evidence of hydrolysis of the substrates for cathepsin A, B, or C could be detected with the zinc-ethanol-purified cathepsin from bovine muscle granules.

Polyacrylamide (Cyanogum) Gel Electrophoresis. Bovine muscle particulate preparations contained proteins that migrated as six electrophoretic bands (Figure 7). Following fractionation by pH adjustment and addition of ethanol (fraction A), only one protein band was detected by the electrophoretic method used. This protein had an electrophoretic migration rate of 4.82×10^{-5} sq. cm. volt^{-1} sec.$^{-1}$ in tris(hydroxymethyl)-aminomethane-borate buffer at 20° C., pH 8.8, and ionic strength of 0.08. The protein was present in all muscle preparations studied.

Microscopic Study of Particles. An electron micrograph of an unstained preparation of bovine muscle particles sedimented at 10,000 G is shown in Figure 8. Preparations stained with Gomori stain and viewed under the electron microscope were similar in appearance to electron micrographs of purified rat liver lysosomes (*9*) following lead staining. Under the phase-contrast microscope the particles appeared as dark oval disks but detail was lacking.

Data from all microscope studies of these particles revealed that they were extremely heterogeneous in size (0.3 to 1.0 micron) and appearance, and in no case was the degree of purity sufficient to allow unequivocal morphological identification.

Discussion

The successful use of lipid solvents, oxidants, and treatments that affect osmotic conditions to increase cathepsin activity of particulate matter from muscle sedimented at 10,000 G suggests that this enzyme is contained within a particle surrounded by a lipoprotein membrane. The response of these particles to osmotic

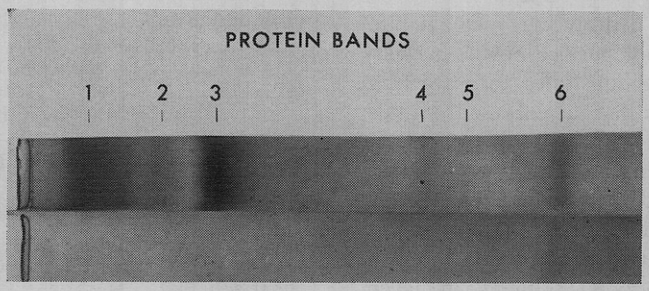

Figure 7. Polyacrylamide vertical gel electrophoresis patterns of bovine muscle particulate proteins

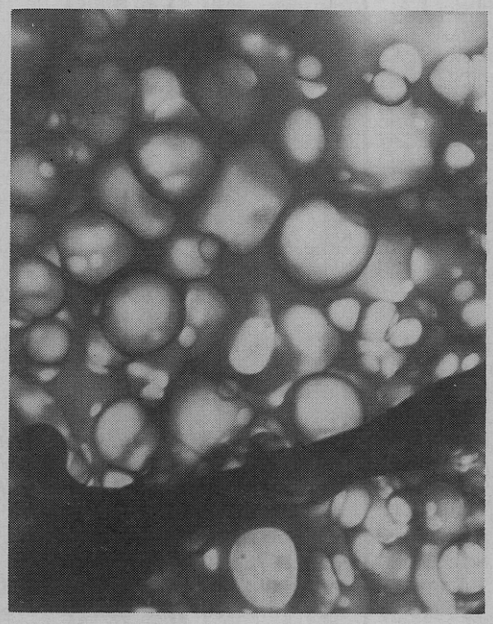

Figure 8. Electron micrograph of particulate matter sedimented at 10,000 G

× 5000, photographically enlarged × 2

shock and other lysis treatments associated these particles with lysosomes isolated from mammalian organ tissue (*9*, *34*). Evidence from microscopic study of these particles also related them morphologically to organ cellular lysosomes (*9*).

Data reported here indicate that L-adrenaline and vitamin A palmitate have a weakening effect on the lipoprotein membrane to release enzyme. Although Gordon and Zak (*17*) have shown that physiological doses of L-adrenaline activate rabbit muscle cathepsin, this hormone has little effect on the activity of purified porcine sarcoplasmic cathepsin (*24*).

Work of Dingle (*12*), Fell, Dingle, and Webb (*13*), and Weissmann and Thomas (*35*) indicates that vitamin A causes lysis of cellular lipoprotein membranes. Dingle demonstrated that vitamin A caused lysis of liver lysosomes to release cathepsin (*11*, *12*) and this was attributed to weakening of the lysosomal membrane. Other hormones such as cortisone, cortisol, etiocholanolone, and progesterone have been shown to release hydrolases from lysosomes (*35*).

Both acid and alkaline pH optima are common for cathepsins from different sources (*1*, *4*, *8*, *20*, *23*, *32*). The greatest activity for cathepsin from many species in hydrolysis of hemoglobin is near pH 4.0. The pH optimum for rat liver lysosomal cathepsin has been reported to be 3.6 (*15*). In respect to low pH optimum these enzymes are similar to cathepsin D.

The lack of effect of cysteine, iodoacetate, and *p*-chloromercuribenzoate on the activity of muscle particulate cathepsin when hemoglobin is used as substrate suggests that enzyme originating from this source does not require sulfhydryl groups for activity and, in this respect, is also similar to cathepsin D from spleen (*25*).

Ferrous ions have been reported to activate bovine sarcoplasmic cathepsin at acid pH (*29*), and similar results were found for porcine sarcoplasmic cathepsin (*24*). The similarity of the energies of activation and other activities of cathepsin from the sarcoplasmic fraction of bovine and porcine (*24*) muscle and bovine particulate cathepsin is evidence that the activities of these enzymes are essentially the same during the catalytic hydrolysis of hemoglobin. Heating of free enzymes apparently activates these enzymes very little compared to other hydrolases (*21*). The sarcoplasmic cathepsins previously studied by many investigators must be localized initially in subcellular or similar particles and released by osmotic shock during extraction with different media.

The low activity of protein from bovine muscle particles and that in the sarcoplasmic fraction in hydrolysis of synthetic substrates for cathepsins A, B, and C indicates that enzymes necessary for hydrolysis of these substrates are either low in concentration or are inhibited. Many previous data concerning activity of enzymes from lysosomes or similar particles in catalytic hydrolysis of substrates for cathepsins A, B, and C are conflicting.

Protein from mitochondrial fractions of rat liver has been found to hydrolyze the synthetic substrates of cathepsins B and C (*14*), but the supernatant fraction from the mitochondrial preparations inhibited cathepsin B activity. Rademaker and Soons (*26*) found considerable cathepsin A and B activities in the mitochondrial fraction of rat liver and Sawant, Desai, and Tappel (*27*) reported that most of the activity of rat liver lysosomes was due to cathepsin A.

Holt (*19*) has largely discounted the possibility that the esterase activity in lysosomes was cathepsin B or C, on the basis of staining reactions with indoxyl acetate for esterase activity and inhibition by certain chemicals. He concluded that cathepsin D was probably the major

protease responsible for hydrolyzing hemoglobin.

The inability of zinc-ethanol–purified bovine particulate cathepsin to hydrolyze substrates for cathepsin A, B, or C supports the idea that the enzyme from this source responsible for catalyzing hydrolysis of hemoglobin at pH 4.0 is primarily cathepsin D. This preparation was the most active protein obtained and was purified 170-fold as indicated by its high specific activity. The electrophoretic data show that the protein is relatively free from other proteins and even though energy of activation data reveal a high turnover rate, this protein is extremely low in concentration compared to certain other enzymes associated with muscle.

Evidence cited in this report clearly demonstrates the existence of particulate matter having many of the biochemical characteristics of lysosomes from organ tissue studied by de Duve (*9*). However, whether these particles are actually muscle cellular organelles or are due to the presence of certain polymorphonuclear leucocytes is uncertain. Archer and Hirsch (*3*) found that granules from eosinophiles are actually lysosome-like structures and biochemical data accumulated on other macrophages indicate that they contain similar structures (*6*, *7*). Results from preliminary study of staining properties of the muscle particles indicate that they may differ from leucocyte granules, since they are not readily stained by Wright's stain or azures.

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