BBA 46146

LYSOSOMAL HEMOCHROMES AND DIGESTION OF CYTOCHROME *c* BY THE LYSOSOMAL PROTEASE SYSTEM

D. M. HANES AND A. L. TAPPEL

Department of Food Science and Technology, University of California, Davis, Calif. (U.S.A.)

(Received March 12th, 1971)

SUMMARY

1. Difference spectra of Triton WR 1339-filled lysosomes show the presence of unusual hemochromes.

2. Incubation of Triton WR 1339-filled lysosomes with mitochrondria alters the spectral characteristics of mitochondrial cytochromes *c*, c_1 and $a + a_3$ to correspond with the spectral characteristics of the lysosomal hemochrome.

3. Digestion of cytochrome *c* by lysosomes alters the spectral characteristics of the cytochrome. This reaction provides a model that shows how the lysosomal hemochrome could be formed.

4. The pH optimum for reduction of the cytochrome *c* Soret peak by lysosomes is pH 3.6–3.8.

5. No spectral changes were caused by the detergents Triton X-100 or by Triton WR 1339 nor by mitochrondrial and microsomal preparations.

6. Pronase and pepsin digestion of cytochrome *c* produced similar hemochromes. A plot of pH dependence of pronase activity corresponded with a plot of maximum reduction of the cytochrome *c* Soret peak.

7. Digestion of cytochrome *c* and the resulting spectral changes corresponded with the content of cathepsin D in the Triton WR 1339-filled lysosomes and the hydroxylapatite column fractions used as enzyme source.

8. The greater reduction of the Soret peak of cytochrome *c* by lysosomes in the presence of cysteine implicates the participation of the sulfhydryl cathepsins B and C in the digestion process.

9. The absolute spectra of a modified high molecular weight heme compound separated by chromatography from the products of lysosomal digestion of cytochrome *c* were similar to the spectra produced by action of lysosomes and hydroxylapatite fractionated cathepsins on cytochrome *c*.

INTRODUCTION

Lysosomes, a heterogeneous population of subcellular particles, have the ability to degrade other subcellular organelles such as mitochondria and microsomes[1–4]. Electron micrographs clearly show the digestion of these organelles within membrane-bound vacuoles biochemically and microscopically identified as lysosomes. KADENBACH[5] has shown a positive correlation between the half-life of rat cytochrome *c*

and the activity of lysosomal cathepsins in various tissues. This paper describes the presence within lysosomes of pigments which have spectral characteristics unlike any of the known cellular cytochromes. With cytochrome *c* as a model, these studies show the formation of hemochrome products by proteolytic digestion.

MATERIALS

Cytochrome *c* and Triton X-100 were obtained from Sigma Chemical Co., pepsin from Nutritional Biochemical Corp., and pronase from Calbiochem. Triton WR 1339 was purchased from Ruger Chemical Co. Sephadex G-25 and Blue Dextran 2000 were obtained from Pharmacia Fine Chemicals Inc. All other chemicals were of reagent grade.

METHODS

Subcellular fractionations

Triton WR 1339-filled lysosomes were prepared from rat liver as previously described[6]. Subcellular fractions from rat liver were isolated as described by RAGAB *et al.*[7]. Hydroxylapatite column fractions which contained the catheptic enzymes from rat liver were obtained as described by YOUNG *et al.*[8].

Spectral studies

Spectral properties of the hemochrome pigments were measured with a dual-wavelength scanning spectrophotometer (Pheonix Precision Instrument Co.) and a ratio recording spectrophotometer (Beckman DK-2). The spectrophotometers were calibrated with a mercury lamp and with cytochrome *c*. The kinetics of cytochrome *c* degradation were followed by measurements of the decrease of the γ peak of oxidized cytochrome *c* at 410 nm. These reactions were measured with the dual wavelength spectrophotometer with 390 nm as the reference wavelength. For measurement of difference spectra, sodium dithionite was used as the reducing agent.

Chromatography

Lyophilized products of cytochrome *c* digestion were redissolved in 0.3–0.5 ml of 0.1 M sodium acetate buffer (pH 5) which contained 0.2 M NaCl and were applied to a 1.5 cm × 105 cm bed of Sephadex G-25. The column was eluted with the same buffer at a flow rate of 30 ml/h and 1 ml-fractions were collected. The column was standardized with bradykinin (mol. wt., 1060) and with B chain of insulin (mol. wt., 3500). Ninhydrin-positive material in the fractions was analyzed by the method of MOORE AND STEIN[9].

Enzyme determinations

The activities of acid phosphatase and cathepsin D were determined according to the methods of GIANETTO AND DE DUVE[10]; cathepsin A activity by the method of IODICE *et al.*[11]; cathepsin C activity by the method of METRIONE *et al.*[12]; and cathepsin B activity by the method of GREENBAUM AND FRUTON[13], the liberated NH_3 being determined by the method of SELIGSON AND SELIGSON[14]. Protein was measured by the method of MILLER[15].

RESULTS AND DISCUSSION

Spectra and origin of hemochrome pigment

Difference spectra were measured on a number of Triton WR 1339-filled lysosome preparations and unusual hemochrome pigments were found. Fig. 1 shows the difference spectra of these lysosomal hemochromes which are characterized by major peaks in the α region at 562 nm and in the γ region at 428 nm. The absolute spectra have a γ peak at 413 nm for the hemichromes, and a γ peak at 422 nm and an α peak at 562 nm for the hemochromes. The low-temperature spectra of the lysosomal hemochromes have a γ peak at 422 nm, a β peak at 528 nm and an α peak at 561 nm. In comparison with the low temperature spectra of cytochrome *c*, those of the lysosomal hemochromes do not have much sharpening of the α and β peaks.

The use of density-gradient centrifugation in the preparation of Triton WR 1339-filled lysosomes[6] allows good resolution of lysosomal fractions. The lysosomes obtained by this method are freer from contamination by mitochrondria and microsomes than are lysosomes separated by conventional differential centrifugation techniques. This isolation method made possible the identification of the spectral characteristics of the hemochrome pigment of lysosomes, heretofore masked by the cytochromes of mitochondria and microsomes.

In the density gradient the lysosomal fractions are well-separated from the mitochondria and the major part of the microsomes. Analysis of a typical fractionation showed a distinct lysosomal band of 5 ml per 28 ml gradient tube with a protein concentration in the lysosomal band of 0.9 mg protein per ml and a purification of acid phosphatase of 44-fold. The lysosomal fraction had a hemochrome α peak of 562 nm and an absorbance of 0.03. The fractions immediately adjacent to the lysosomal band contained only small amounts of microsomes having an α peak of 557 nm and an absorbance of 0.02 and less. Based upon this evidence it was concluded that microsomal contamination could not account for the lysosomal hemochromes.

Careful measurements of the spectral characteristics of the mitochondrial and microsomal *b* cytochromes indicated that the 562 nm hemochromes found in lysosomes did not correspond with the *b* cytochromes of either of these subcellular fractions

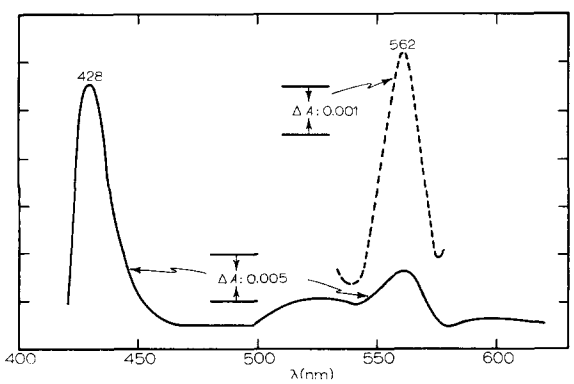

Fig. 1. Difference spectra of lysosomal hemochromes. The liver lysosome preparation had a 44-fold increase in acid phosphatase activity and the spectra were recorded with 1 mg of protein per ml.

In addition, succinate, hydroxybutyrate and ADP were unreactive when tested as substrates for the reduction of lysosomal hemochromes.

Fig. 2 shows the result of the action of lysosomal enzymes on mitochondria. Lysosomes (1 mg of protein per ml) were incubated with mitochondria (2 mg of protein per ml) at pH 5.0. The spectral changes observed after incubation point to an extensive rearrangement of the original protein structure controlling the ligands to the iron and the heme portions of the cytochromes. After 3.5 h of incubation at 37°, the spectral characteristics of cytochromes *c*, c_1 and $a + a_3$, apparent at 0 time, were lost and the α peaks at 553 nm and 560 nm were altered to resemble the 562-nm α peak of the lysosomal hemochromes. The γ region of the spectrum was greatly affected, some 445 nm cytochrome a_3 absorbance being lost after only a short period of incubation and the 428-nm Soret peak being decreased sharply. The spectral changes observed: decreases in the α, β and γ regions of the spectrum and the shifting of the 550-nm (reduced) peak toward the 562-nm peak characteristic of the lysosomal hemo-

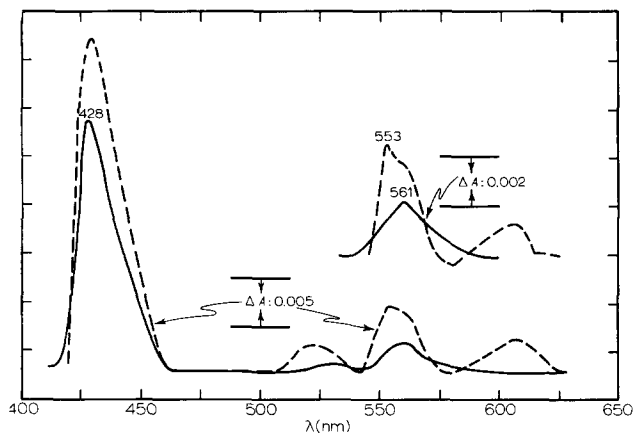

Fig. 2. Difference spectra of the reactants (-------) and products (———) of the digestion of mitochondria by lysosomes. The reaction system contained liver mitochondria, 9 mg of protein, and liver lysosomes, 4 mg of protein, in 4.7 ml of sodium acetate buffer (pH 5.0). After 3.5 h of reaction at 37°, an aliquot was diluted one-half for spectral measurements.

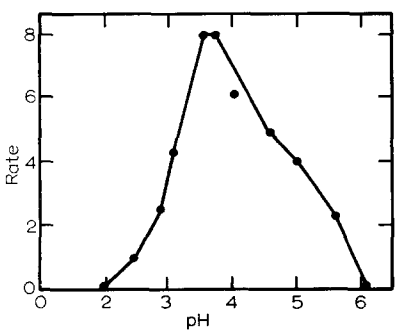

Fig. 3. pH optimum of reduction of the Soret peak of cytochrome *c*. The reaction mixture consisted of 0.5 ml of cytochrome *c* (0.5 mg/ml), 0.3 ml of Triton WR 1339-filled lysosomes (0.3 mg of protein per ml) and 0.2 ml of 0.2 M sodium acetate (pH 2.0–3.4) or 0.1 M sulfate buffer (pH 3.5–6.0). Rate = Δ absorbance/min $\times$ 10^{-3}.

chrome, point to the formation of the pigment by digestion of cellular cytochromes within the lysosomes.

Cytochrome c model system and the effect of pH

A model system in which purified cytochrome *c* was substituted for mitochondria in a reaction system with lysosomes was used to determine the characteristics of cytochrome degradation of the types that might occur in the formation of the lysosomal hemochromes. Cytochrome *c* was chosen to represent a cellular cytochrome.

Fig. 3 shows the effect of pH on the reduction of the Soret peak by lysosomes between pH 2.0 and 6.0. Control samples without enzyme showed that there was no effect of the buffer alone on cytochrome *c* in the pH range tested. The optimum pH for reduction of the Soret peak is seen in Fig. 3 to be pH 3.6–3.8.

The changes seen in the spectra of cytochrome *c* after incubation with lysosomes are similar to the changes seen in the spectra of the mitochondrial cytochromes incubated with lysosomes. Since cytochrome *c* is not the only cytochrome found in the cell, it is unlikely that the spectral characteristics after treatment with lysosomes would be identical with the lysosomal hemochromes found within cells. However, the model system indicates the modification caused by digestion and suggests the possible mode of formation of the cellular lysosomal hemochromes. There are several reports of modified cytochromes, with the process of modification and the resultant spectral characteristics similar to those obtained upon treatment of cytochromes with lysosomal enzymes. ELLIOTT *et al.*[16] have reported the appearance of a pigment, termed mitochrome, which results from aging mitochondrial cytochrome $a + a_3$ at 4° for 14 days. The preparations of cytochrome $a + a_3$ lost their α peak at 605 nm (reduced) and the γ band shifted from 445 nm (reduced) to 442 nm with attendant loss of cytochrome oxidase activity. The 445-nm peak of cytochrome a_3 was also lost upon incubation at 37° with lysosomes and may reflect the same type of shift.

When smooth-surfaced microsomes are digested with proteolytic enzymes or treated with deoxycholate, a hemochrome is obtained which is characterized by absorption peaks at 559 nm, 529–539 nm and 425–426 nm in the reduced form[17]. This hemochrome, when treated with CO in the reduced form, yields a strong band at 420 nm. The native cytochrome before enzymatic or deoxycholate treatment yields a broad peak at 450 nm in the presence of CO.

KEILIN[18] has reported the presence of a pigment, helicorubin, found free in the gastrointestinal tract of land snails and other invertebrates. The absorption spectrum of helicorubin shows sharp peaks at 562.5 nm in the α region and 427.5 nm in the γ region. Helicorubin arises from a cytochrome h, which is modified during the secretory process of the digestive gland. The modified pigment is similar in spectral characteristics to the pigment found in lysosomes.

Effect of detergents on cytochrome c

Triton X-100 (octyl phenoxy polyethoxyethanol), a surface active agent, in concentrations from 1 to 20% and Triton WR 1339 (oxyethylated *tert.*-octyl phenol polymethylene polymer), the non-ionic detergent used in the preparation of lysosomes, in concentrations from 1–35 mg/ml, were tested for their effects on the Soret peak at 410 nm in the absence of lysosomal enzymes. There was no effect on the Soret peak of cytochrome *c* in the presence of Triton X-100 in the concentration range tested.

There was also no significant effect on the Soret peak by Triton WR 1339 until 32–35 mg/ml of the compound were used. These high concentrations were not present in the small amount of lysosomal protein used in the model reaction systems. These results eliminated nonspecific detergent effects as possible causes of the changes seen in the Soret peak.

Effect of mitochondria and microsomes on cytochrome c

Triton WR 1339-filled lysosomes may contain 5–12 %[19] of their total protein as mitochondrial and microsomal contamination, but they are relatively uncontaminated by other cell fractions. To investigate any possible contribution to the effects seen on the Soret peak by mitochondrial or microsomal particles present in the lysosomal fraction, freshly prepared mitochondria and microsomes were incubated with cytochrome *c* at pH 3.6. Mitochondria in concentrations ranging from $0.9 \cdot 10^{-4}$–$9 \cdot 10^{-4}$ mg of protein per ml and microsomes in concentrations ranging from $4.4 \cdot 10^{-3}$–$4.4 \cdot 10^{-3}$ mg of protein per ml were used. There was no effect on the Soret peak of cytochrome *c* by mitochondria or microsomes in the concentration ranges tested. These results eliminated the possibility that the changes observed in the spectrum of cytochrome *c* were caused by subcellular fractions other than lysosomes.

Effect of pronase and pepsin on cytochrome c

The pH optimum for reduction of the Soret peak by lysosomes was shown to be pH 3.6–3.8. Since the pH optimum of cathepsin D, a major proteolytic enzyme of the lysosome, is at pH 3.6[20], the possibility that the reduction of the Soret peak of cytochrome *c* was due to proteolysis was tested with the use of pronase and pepsin as positive controls. An activity curve between pH 3.0 and pH 8.8 for the reduction of the Soret peak was determined with pronase (3 μg/ml) as the enzyme source and cytochrome *c* (0.5 mg/ml) as the substrate; the results are shown in Fig. 4A. A plot of the pH dependence of pronase activity between pH 5.0 and pH 8.4 with cytochrome *c* as substrate is shown in Fig. 4B. Appropriate enzyme and buffer controls were included in both of the experiments. It can be seen from a comparison of the two curves

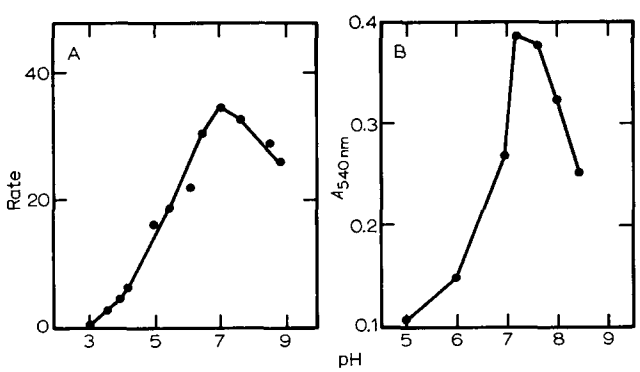

Fig. 4. A. Effect of pH on the reduction of the Soret peak of cytochrome *c*. The reaction mixture was as described in Fig. 3, with pronase (3 μg/ml) replacing the lysosomes. B. pH optimum of pronase with cytochrome *c* as substrate. Pronase (0.3 mg/ml) was incubated with cytochrome *c* (0.25 mg/ml) for 30 min at 37°. Citrate–phosphate buffer (0.2 M) was used from pH 5.0 to 7.6 and 0.2 M Tris–maleate from pH 8.0 to 8.4.

that maximum proteolysis coincides well with maximum reduction of the Soret peak of cytochrome *c*, although the plot of the reduction of the Soret peak is somewhat broader than the plot of proteolysis.

Fig. 5 shows the effects of pronase (0.3 mg/ml, pH 6.8) and pepsin (0.3 mg/ml, pH 3.6) activities on the absolute spectrum of cytochrome *c* (50 μg/ml). The γ peak at 407 nm was shifted to 397 nm with pronase and to 396 nm with pepsin. The loss of the 407 nm peak did not occur when pepsin was reacted with cytochrome *c* at pH 5.0. All incubations were for 10 min at room temperature.

Comparison of enzyme activity and effects on the difference spectra of cytochrome c

Table I gives the protein content, specific activity and purification factor of lysosomal enzymes for two Triton WR 1339-filled lysosome preparations and for six hydroxylapatite column fractions that contained partially purified catheptic enzymes. Conditions for the incubation of the hydroxylapatite column fractions are given in Table I. The lysosome preparations were incubated at 37° with cytochrome *c* (0.5 mg/ml, pH 3.6) for up to 20 h with samples being taken at various intervals for determination of difference spectra. In separate reaction mixtures incubated under the same conditions, cysteine (0.01 M) was added to cytochrome *c* with lysosome Prepara-

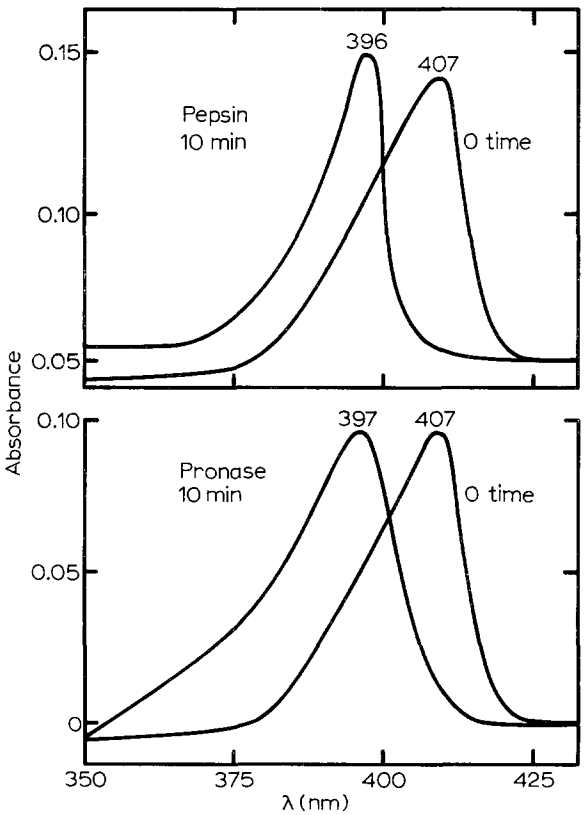

Fig. 5. Effects of pronase and pepsin on the absolute spectrum of cytochrome *c*. Pronase (0.3 mg/ml) buffered at pH 6.8 and pepsin (0.3 mg/ml) buffered at pH 3.6 were reacted with cytochrome *c* (0.05 mg/ml) for 10 min at room temperature.

TABLE I

ENZYME ACTIVITIES OF TRITON WR 1339-FILLED LYSOSOMES AND HYDROXYLAPATITE COLUMN FRACTIONS

Enzyme assays were done as indicated in METHODS. Figures given in parentheses represent the -fold purification of activity over that of the homogenate.

Enzyme source	*Protein (mg/ml)*	*Specific activity (nmoles product formed/mg protein per min)*				
		Acid phosphatase	*Cathepsin A*	*Cathepsin B*	*Cathepsin C*	*Cathepsin D*
Preparation						
I	1.9	148 (7)		21 (4)		54 (10)
II	0.7	245 (10)		35 (8)		97 (11)
Fraction						
1	1.75		474	56	3908	868
2	0.75		1	0.9	88	0
3	0.16		0	0	0	111
4	0.31		46	0	324	397
5	0.23		0	0.8	218	11
6	0.10		0	0	3317	468

tion I. The hydroxylapatite column fractions were selected for their varying ratios of all the cathepsins and they were diluted to equal protein concentration (0.4 mg/ml) before incubation with cytochrome *c* (50 μg/ml).

Fig. 6 shows the effects of the lysosome preparations and the hydroxylapatite column fractions on the difference spectra of cytochrome *c*. In comparing the two preparations (Fig. 6A and 6B), a correlation can be seen between the activities of cathepsins B and D and the effects on the spectra. Preparation I, with relatively low activities of cathepsins B and D, had little effect on the spectrum. The addition of the sulfhydryl group protector cysteine to Preparation I enhanced the effect on the spectrum.

Fig. 6C shows the effect on the spectrum of the different hydroxylapatite fractions. Again a correlation can be seen between enzyme activity and the effect on the spectrum of cytochrome *c*. The effect was more pronounced with Fractions 1 and 3, which contained only cathepsin D or which had high cathepsin D activity, when incubated at the pH optimum of the enzyme. Fractions 2 and 5, low in all of the cathepsin activities, did not affect the spectrum. Fractions 4 and 6, intermediate in activity of the cathepsins, showed a moderate effect on the spectrum. The above results, coupled with the effects of pronase and pepsin in the kinetic and spectral studies, show that the changes seen in the spectrum of cytochrome *c* provide a model study of how the pigment found in lysosomes may result from proteolytic action on the cytochrome molecules. The pH 3.6 optimum and these studies on the effect of varying ratios of catheptic enzymes suggest that cathepsin D would be the primary enzyme responsible for proteolytic action. Activation by cysteine also implicates cathepsins B and C, sulfhydryl activated enzymes, in the reaction.

Effect of hydroxylapatite column fractions on the absolute spectrum of cytochrome c

The hydroxylapatite column fractions that caused the greatest reduction in the peaks of cytochrome *c* as shown by the difference spectra in Fig. 6C caused changes

in the absolute spectra that were the same as those found upon treatment of cytochrome *c* with pronase and pepsin (Fig. 5). The 407-nm (oxidized) peak was lost and a new peak appeared at 398 nm.

Chromatography on Sephadex G-25 of the digestion products of cytochrome c

Cytochrome *c* (20 mg) was digested with Triton WR 1339-filled lysosomes (3 mg) at pH 3.6 for 20, 30 and 50 h at 37°. The reaction mixtures were then lyophilized and chromatographed on a Sephadex G-25 column.

Fig. 7 shows the protein and peptide peaks from the reaction mixture incubated for 50 h. Also shown is the elution pattern of heme-containing compounds as measured by absorbance at 410 nm. Although somewhat broader, the peak of heme-containing

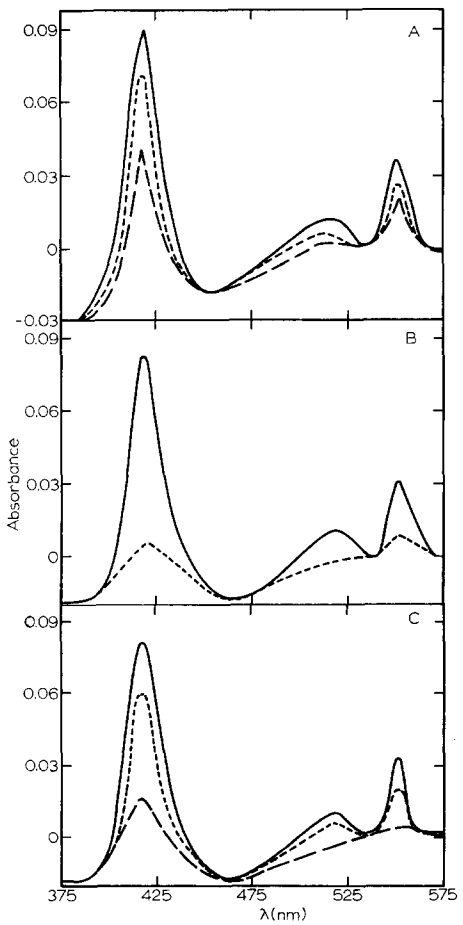

Fig. 6. Effects of Triton WR 1339-filled lysosomes and fractionated cathepsins on the difference spectra of cytochrome *c*. Incubations were done at 37° with 50 μg of cytochrome *c* per ml. A. Preparation I: ———, 0 time, pH 3.6; – – – –, 20 h, pH 3.6; — — —, 20 h, pH 5, 0.01 M cysteine. B. Preparation II: ———, 0 time, pH 3.6; – – – –, 18 h, pH 3.6. C. Hydroxylapatite column fractions: ———, 0 time and after 20 h incubation with either Fraction 2 or with Fraction 5, pH 5, 0.01 M cysteine; – – – –, after 20 h with either Fraction 4 or with Fraction 6, pH 5, 0.01 M cysteine; — — —, after 20 h with either Fraction 1, pH 3.6, or with Fraction 3, pH 3.6, 0.01 M cysteine.

compounds followed the large protein peak that eluted at the beginning of the separation. As the time of incubation was increased from 20 to 50 h, the large protein peak eluting close to the void volume decreased, with a concomitant increase at the end of the separation of the peptide peak that contained smaller molecular weight peptides and amino acids. All colored products formed, as indicated by absorbance at 410 nm, were in the higher molecular weight range (mol. wt., 3500–5000). A smaller molecular weight product, similar to that found for cytochrome *c* digested by pepsin[21] and separated on Sephadex G-25 by PORATH[22], was not found.

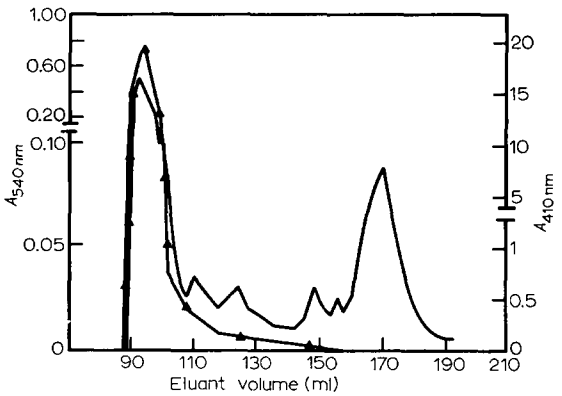

Fig. 7. Chromatography on Sephadex G-25 of the digestion products of lysosomes and cytochrome *c*. Absorbance of ninhydrin-positive products (———) shows the elution pattern of the digestion products. Absorbance at 410 nm (▲—▲) shows the elution pattern of the heme containing compounds.

Cytochrome *c* is not a good substrate for lysosomal cathepsins, possibly because it is a very basic protein that contains many lysine residues. This is undoubtedly one of the reasons that a small molecular weight heme peptide was not formed and could not be found chromatographically. The ease of detection of the pigment within the lysosome may be owing to the slow destruction of the cellular cytochromes with the accumulation of a product that is easily detectable by its spectral properties. Another possible explanation for not detecting a low molecular weight heme peptide could be that such a product was formed but that it polymerized, as has been reported in some cases[23], and that it separated with the higher molecular weight products. Reports[21,24] indicate molecular rearrangements during digestion of cytochrome *c*. Increases and decreases in peak size suggest breaking and reforming of the ligand structure to the heme group and consequent reconstruction of spectral characteristics that are similar to the original cytochrome *c* spectrum. In a complicated digestion system, there may exist many possibilities among protein, peptide and amino acid fragments for the reforming or partial reforming of ligand structures.

The higher molecular weight colored products separated after the void volume were extremely autoxidizable, which suggested lysosomal proteolytic action on the cytochrome *c* molecule. Autoxidizability, noted during the digestion of cytochrome *c* with purified proteases[21], is manifested by a tendency of the reduced spectrum to revert to the oxidized spectrum. Autoxidation did not occur with native cytochrome *c* nor with the chromatographed control cytochrome *c*. Control cytochrome *c*, in-

cubated for 50 h at 37° in the absence of enzyme and then chromatographed, eluted in the void volume as did native unincubated cytochrome *c*.

The absolute spectra of the chromatographed modified high molecular weight colored products of the lysosomal digestion of cytochrome *c* were similar to the spectra produced by action of Triton WR 1339-filled lysosome preparations and the fractionated cathepsins on cytocrhome *c*.

The use of the model system with cytochrome *c* and the digestion by Triton WR 1339-filled lysosomes and partially purified lysosomal cathepsins has demonstrated decreases in the chromophore of cytochrome *c*. These decreases are similar to those found with the digestion of mitochondria by lysosomes and are also found in the fractions separated chromatographically after digestion of cytochrome *c* with lysosomes. Cytochrome *c* was chosen as a convenient model to represent a fairly typical heme compound. Undoubtedly, it accounts for a very small proportion of such types of compounds within the cell that are digested by lysosomes. The combination of studies represented here suggests that the lysosomal hemochromes are digestion products derived from lysosomal enzyme action on all of the cytochromes of the cell. Results with cytochrome *c* demonstrate that small amounts of proteolytic digestion greatly modify its spectral properties in a manner that correlates with what is known of the *in vivo* formation of the hemochromes found in lysosomes.

The numerous electron micrograph studies[2,3,4] and the results of the experiments reported here serve to illustrate the important role played by lysosomes in the process of cellular autophagy. Found in all of the Triton WR 1339-filled lysosome preparations investigated, the unique pigment may be present in steady-state concentrations, representing a marker for the slow continuous breakdown of subcellular particles. By quantitation of the amounts of this pigment present in the lysosomes, a measurement of the extent of normal cellular autophagy can be obtained.

ACKNOWLEDGEMENT

This research was supported by U.S. Public Health Service Research Grant AM 05609 from the National Institute of Arthritis and Metabolic Diseases.

REFERENCES

1 A. L. TAPPEL, P. L. SAWANT AND S. SHIBKO, in A. V. S. DE REUCK AND M. P. CAMERON, *Lysosomes*, Little, Brown and Co., Boston, 1963, p. 78.
2 T. P. ASHFORD AND K. R. PORTER, *J. Cell Biol.*, 12 (1962) 198.
3 A. B. NOVIKOFF AND E. ESSNER, *J. Cell Biol.*, 15 (1962) 140.
4 C. DE DUVE AND R. WATTIAUX, *Ann. Rev. Physiol.*, 28 (1966) 435.
5 B. KADENBACH, *Biochim. Biophys. Acta*, 186 (1969) 399.
6 S. MAHADEVAN AND A. L. TAPPEL, *J. Biol. Chem.*, 242 (1967) 4568.
7 H. RAGAB, C. BECK, C. DILLARD AND A. L. TAPPEL, *Biochim. Biophys. Acta*, 148 (1967) 501.
8 J. O. YOUNG, D. M. HANES AND A. L. TAPPEL, *J. Chromatog.*, 44 (1969) 133.
9 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 211 (1954) 907.
10 R. GIANETTO AND C. DE DUVE, *Biochem. J.*, 59 (1955) 433.
11 A. A. IODICE, V. LEONG AND I. M. WEINSTOCK, *Arch. Biochem. Biophys.*, 117 (1966) 477.
12 R. M. METRIONE, A. G. NEVES AND J. S. FRUTON, *Biochemistry*, 5 (1960) 1597.
13 L. M. GREENBAUM AND J. S. FRUTON, *J. Biol. Chem.*, 226 (1952) 173.
14 D. SELIGSON AND H. SELIGSON, *J. Lab. Clin. Med.*, 38 (1951) 324.
15 G. MILLER, *Anal. Chem.*, 31 (1959) 964.
16 W. B. ELLIOTT, W. C. HULSMAN AND E. C. SLATER, *Biochim. Biophys. Acta*, 33 (1959) 509.

17 T. OMURA AND R. SATO, *J. Biol. Chem.*, 239 (1964) 2379.
18 J. KEILIN, *Biochem. J.*, 64 (1956) 663.
19 F. LEIGHTON, B. POOLE, H. BEAUFAY, P. BAUDHUIN, J. W. COFFEY, S. FOWLER AND C. DE DUVE, *J. Cell Biol.*, 37 (1968) 482.
20 A. J. BARRETT, *Biochem. J.*, 104 (1967) 601.
21 C. L. TSOU, *Biochem. J.*, 49 (1951) 367.
22 J. PORATH, *Biochim. Biophys. Acta*, 39 (1960) 193.
23 A. EHRENBERG AND H. THEORELL, *Nature*, 178 (1955) 158.
24 C. L. TSOU, *Nature*, 164 (1949) 1134.