THE USE OF FORMALDEHYDE-TREATED ^{131}I-ALBUMIN IN THE STUDY OF DIGESTIVE VACUOLES AND SOME PROPERTIES OF THESE PARTICLES FROM MOUSE LIVER

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ABSTRACT

The trichloroacetic acid–soluble radioactivity released during incubation of mouse liver particles containing intravenously injected formaldehyde-treated ^{131}I-albumin consisted almost entirely of ^{131}I-iodotyrosine. The material was shown to be excreted into the medium and was not due to disruption of the particles by acid. Triton X-100 or the absence of sucrose in the medium inhibited hydrolysis of the particle-associated labeled protein. This inhibition was due to disruption of the digestive vacuoles and dilution of the protein and cathepsins in the suspending medium. These results and other experimental evidence strongly suggest that the ^{131}I-albumin–containing liver particles are digestive vacuoles. The results also establish that ^{131}I-albumin may be used to study these vacuoles. High concentrations of sucrose (1 M) inhibited degradation of intraparticulate protein. However, 1 M salts inhibited only the rate of the digestion. Sucrose had an inhibitory effect on a crude cathepsin preparation, and salts stimulated the activity when ^{131}I-albumin was used as substrate. The effect of high sucrose concentrations as an inhibitor of protein hydrolysis within digestive vacuoles was, therefore, most likely due principally to an inhibition of cathepsin activity within the vacuoles. The effect of salt was probably caused by a stimulation of both intra- and extra-particulate cathepsin activities, although 0.5–1.0 M KCl appeared to protect the particles.

INTRODUCTION

The injection of chemically treated or otherwise altered or abnormal proteins (1, 2) or Triton WR-1339 (3) into animals results in an incorporation of the injected material into particles in the liver and kidneys. The material is taken up into micropinocytotic vesicles which later become incorporated into lysosomes (4, 5). This process has been investigated by injecting horse-radish peroxidase which can be localized within cells by histochemical methods (6). Particles (phagosomes) containing blue color due to the action of the injected peroxidase were shown to fuse with others (lysosomes) which were stained red by the action of acid phosphatase (7). The resulting particles have been called phagolysosomes by Straus (7) and digestive vacuoles by de Duve (8).

Formaldehyde-treated ^{131}I-albumin or ^{74}As-arsono-azoalbumin is rapidly cleared from the blood stream and taken up by the liver when injected intravenously into mice (1). Most of the radioactivity is associated with particulate material when the liver is homogenized in 0.25 M sucrose

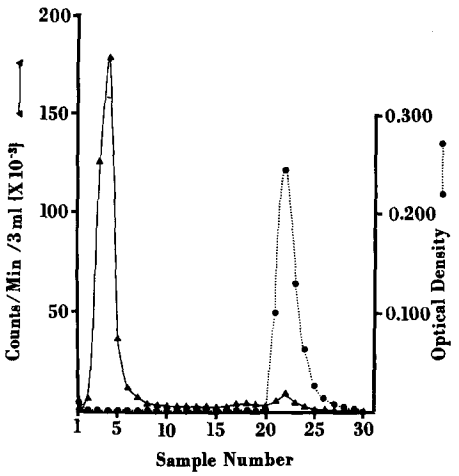

FIGURE 1 Separation of ^{131}I-iodide from tyrosine by ion exchange chromatography on Dowex 50W-x4.

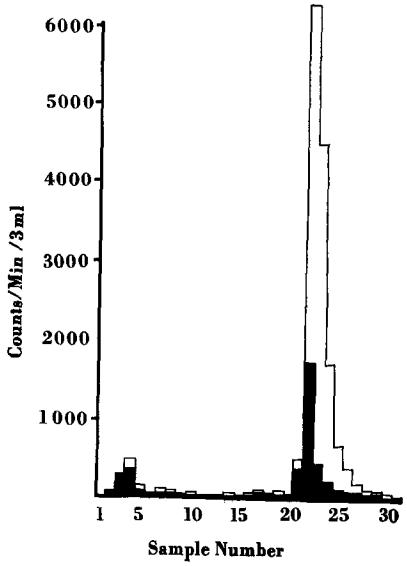

FIGURE 2 Ion exchange chromatography of the acid-soluble material extracted from mouse liver particles before (shaded portions of the bars) and after incubation at 37° (total bars). Two mice were injected with formaldehyde-treated ^{131}I-albumin (0.8 mg, 3.2 × 10^6 counts/min), and the liver particulate material was obtained 30 min after injection as described in Materials and Methods. Total counts in the 0–27,000 *g* liver particles (unwashed): 318,994; total counts in 10 ml of acid-soluble extract before incubation: 16,463; total acid-soluble counts in 10 ml of extract after 1-hr incubation at 37°: 59,014. 3 ml of the extracts were run through the column, and 3-ml samples were collected and counted in a well counter.

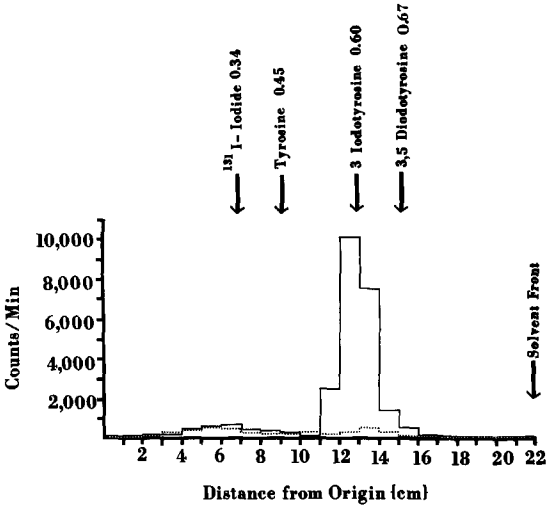

FIGURE 3 Radioactivity profile of a paper chromatogram of the Dowex 50-adsorbed acid-soluble material released during the incubation of ^{131}I-albumin-containing 0–27,000 *g* liver particles. Details are described in Materials and Methods. The Rf's of ^{131}I-iodide, tyrosine, 3-iodotyrosine, and 3,5-diiodotyrosine are indicated on the curve. Dotted line: nonincubated particles.

and centrifuged (1). Changes in distribution of these labeled proteins in various particulate fractions obtained by differential centrifugation are consistent with the results obtained by others indicating that injected macromolecules first appear in small vesicles which coalesce and combine with lysosomes to form digestive vacuoles (5, 7). In addition, 27,000 *g* radioactive particles centrifuged from homogenates of liver from mice injected with formaldehyde-treated ^{131}I-albumin release trichloroacetic acid–soluble radioactivity when incubated at 37° (1, 9). Smaller particles (39,000 *g*) do not release acid-soluble radioactivity. The effect of pH, inhibition by iodoacetamide, stimulation by cysteine, and a requirement for 0.2 M sucrose in the medium suggest that the protein is degraded by cathepsins within digestive vacuoles. The acid-soluble radioactivity may be covalently bound (9).

In the present study, further evidence is presented on the nature of the acid-soluble radioactive material released during incubation of mouse liver particles. Some properties of the intraparticulate digestion process and its relation to the composition of the suspending medium are described.

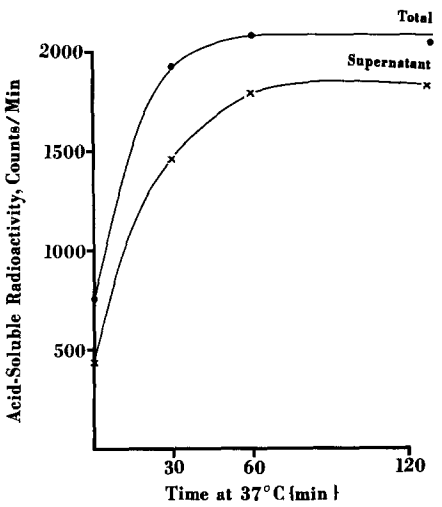

FIGURE 4 Release of acid-soluble radioactivity from ^{131}I-albumin-containing digestive vacuoles. Unwashed particles (0–27,000 *g*) from the liver of a mouse injected with formaldehyde-treated ^{131}I-albumin were incubated at 37° in 0.2 M sucrose at pH 5 (acetate buffer). Two 1-ml samples were removed at the times indicated. One sample was precipitated directly with 1 ml of 10% trichloroacetic acid (curve marked *Total*), and the other was first centrifuged at high speed to remove the particles before precipitation with acid (curve marked *Supernatant*). The trichloroacetic acid supernatant fractions were then counted in a well counter. Total radioactivity in the suspension was 6,300 cpm/ml.

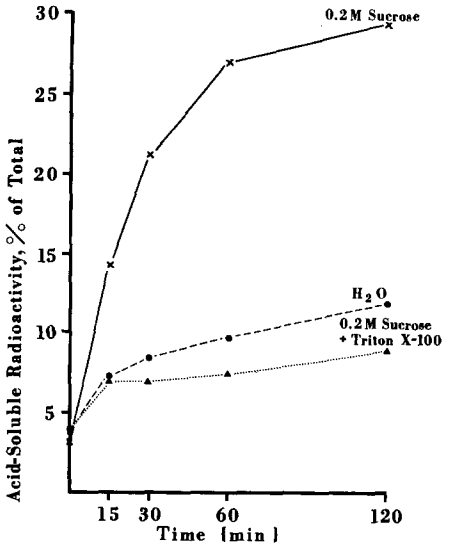

FIGURE 5 The effect of 0.2% Triton X-100 and hypotonicity on the release of acid-soluble radioactivity from liver particles. A mouse was injected with 0.2 ml of formaldehyde-treated ^{131}I-albumin (0.7 mg, 2 × 10^6 cpm) and sacrificed 30 min later. Particles separated at 27,000 *g* from a liver homogenate were suspended in 7 ml of the appropriate solution and incubated at 37° for 2 hr. All samples contained 0.04 M cysteine and 0.02 M acetate buffer (pH 5). Aliquots (1 ml) were removed at intervals and the acid-soluble radioactivity was determined in the supernatant after sedimenting the particles. The suspensions contained the following cpm/ml: 0.2 M sucrose, 9,062; H_2O, 10,415; Triton, 11,023.

MATERIALS AND METHODS

Adult white or C-57 black male mice were used in this investigation. The mice were injected with ^{131}I-albumin (Abbott Laboratories, Oak Ridge, Tenn.) which had been treated with 10% formaldehyde in 0.2 M carbonate buffer, pH 10, for 3 days and then dialyzed. This treatment results in a soluble protein which is removed from the circulation by the liver in which it is rapidly degraded (1). Injections were made into the tail vein with 0.3 to 0.8 mg of labeled protein (usually about 1 μc). Mice were sacrificed 30 min later under ether anesthesia by perfusion with 0.9% NaCl and 5 ml of cold 0.2 M sucrose. The livers were removed quickly and homogenized in 10 ml of cold 0.2 M sucrose using a glass homogenizer and teflon pestle (A. H. Thomas Co., Philadelphia) on a drill press for 1 min at 1100 rpm. The homogenate was then diluted with 10 ml of cold 0.2 M sucrose, for each sample to be incubated, and centrifuged at 27,000 *g* for 10 min. The supernatants were poured off, and the pellets were suspended in the appropriate medium and incubated at 37°. In some experiments, the pellets were resuspended and washed before incubation. 1-ml samples were removed at intervals, added to 1 ml of incubating medium, and centrifuged at 39,000 *g* for 10 min. The supernatant solutions were poured off into counting vials containing 0.5 ml of 25% trichloroacetic acid, mixed, counted, and then centrifuged again to obtain the acid-soluble counts. The acid-soluble counts were subtracted from the total nonsedimentable counts to obtain a measure of the acid-insoluble material released from particles. In some experiments, the samples of particles taken from the incubating medium were precipitated directly with trichloroacetic acid, centrifuged, and the supernatant poured off and counted.

In experiments on the nature of the acid-soluble radioactivity, unwashed liver particles, containing ^{131}I-albumin from two injected mice, were suspended in 50 ml of 0.25 M sucrose buffered with 0.01 M acetate, pH 5. The suspension was divided into two equal parts. Trichloroacetic acid, 50%, (0.1 volume), was added immediately to one part, and the other part was incubated for 1 hr at 37° before addition of the acid. The samples were then centrifuged; the

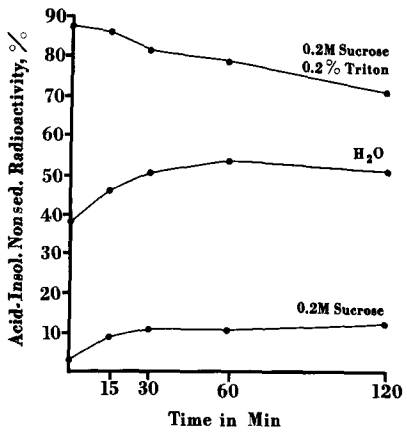

FIGURE 6 Effect of 0.2% Triton X-100 and hypotonicity on release of acid-insoluble, nonsedimentable radioactivity from digestive vacuoles at pH 7.4. Conditions were the same as those described under Fig. 5, except that pH 7.4 Tris-acetate buffer was used. Samples were removed from the suspensions and centrifuged at high speed. The supernatants were poured off into vials containing trichloroacetic acid and counted. They were then centrifuged again to determine the acid-soluble radioactivity. These counts were subtracted from the total counts to obtain the acid-insoluble radioactivity. The suspensions contained the following cpm/ml: sucrose, 10,222; H_2O, 10,401; Triton, 10,242.

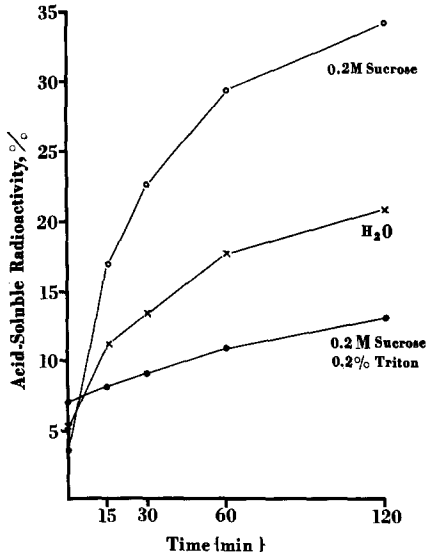

FIGURE 7 Release of acid-soluble radioactivity from the particles described in Fig. 6.

supernatant fractions were poured off, and 3-ml samples were used for ion-exchange column analysis. The remaining solutions were extracted twice with an equal volume of ethyl ether and then treated successively with two 5-ml quantities of 0.1 M acetic acid and water-washed Dowex-50W resin. The resin samples were combined, filtered, and washed with 3–4 volumes of water to remove trichloroacetic acid. No radioactivity was removed by the water washes. The resin was then carefully eluted with three 5-ml quantities of 1 M NH_4OH which removed all the adsorbed radioactivity. The eluate was lyophilized, and the resulting dry powder was dissolved in a small amount of water and chromatographed.

Descending paper partition chromatography of the acid-soluble material released from liver particles was performed on Whatman No. 1 paper using butanol:acetic acid:water (12:3:5) as the solvent (10). Sodium ^{131}I-iodide, tyrosine, 3,5-diiodotyrosine and 3-iodotyrosine were used as references. Tyrosine and tyrosine derivatives were detected on the paper chromatograms by the use of diazotized sulfanilic acid spray, and iodide was detected by oxidation to iodine with periodate and treatment with starch. The chromatograms were cut into 0.5-cm horizontal strips parallel to the solvent fronts, and the strips were counted in a well counter.

A 1- × 12-cm Dowex 50W-x4 column, washed with 0.1 M acetic acid, was used for analysis of the acid-soluble material released during incubation of ^{131}I-albumin-containing particles from mouse livers. A 3-ml sample of the trichloroacetic acid extract was placed on the column which was then eluted with 8 ml of 0.1 M acetic acid followed by gradually increasing pH and sodium ion concentration. The pH gradient elution was performed by dropping 0.125 M NaOH into a closed reservoir containing 50 ml of 0.1 M acetic acid which was mixed and siphoned into the column. Samples (3 ml) were collected, counted in a well counter, and assayed for Folin-Ciocalteau-reacting material according to the Lowry et al. method for protein (11).

RESULTS

A mixture of ^{131}I-iodide and tyrosine in 5% trichloroacetic acid was run through a Dowex 50W-x4 ion exchange resin column to illustrate the separation of these two materials (Fig. 1). Fig. 2 shows the results obtained by running through the column 3-ml samples of the trichloroacetic acid extract from mouse liver particles containing intravenously injected formaldehyde-treated ^{131}I-albumin. Most of the radioactivity was eluted in the same peak with tyrosine (Fig. 1). At zero time (no incubation of the liver particles), approximately 19% of the radioactivity was

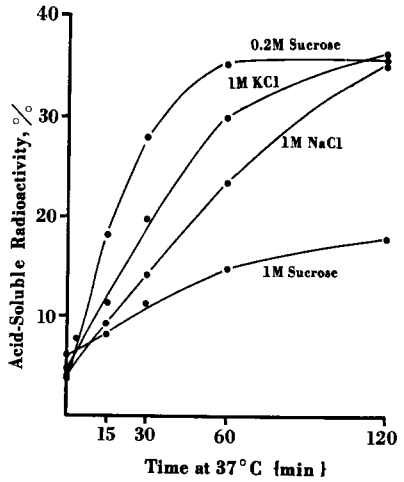

FIGURE 8 Effect of 1 M sucrose, 1 M KCl, and 1 M NaCl on the release of acid-soluble radioactivity into the medium compared with a control of 0.2 M sucrose. Conditions were the same as in Fig. 5. The suspensions contained 4,000–4,500 cpm/ml.

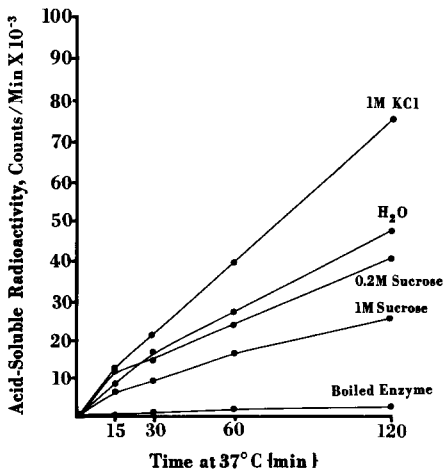

FIGURE 9 Effect of KCl and sucrose on mouse liver cathepsin activity using ^{131}I-albumin as substrate. Livers from 6 mice were homogenized in 0.2 M sucrose, and, after a preliminary low speed centrifugation, the material sedimenting at 27,000 *g* was washed twice in 0.2 M sucrose. The particles were then suspended in distilled water, frozen and thawed 7 times, and 1.5 ml of the preparation was added to equal quantities of the appropriate solution (twice concentrated) containing 1 mg of ^{131}I-albumin (7×10^5 cpm). Each mixture also contained 0.02 M acetate buffer (pH 5) and 0.02 M cysteine. The samples were incubated at 37°; and 0.5 ml aliquots were removed at intervals and the acid-soluble radioactivity determined.

eluted in the first peak. After 1 hr of incubation of the particles at 37°, the first peak increased approximately 18%, but the second increased 4.6-fold. The results show that the increase in acid-soluble radioactivity during incubation or the mouse liver particles occurred in the peak with tyrosine and, therefore, probably consisted of ^{131}I-iodotyrosine. The samples collected from the ion-exchange column shown in Fig. 2 were also assayed for Folin-Ciocalteau–reacting material. Most of the color developed by the phenol reagent occurred in the samples in which the radioactivity was eluted, but some was also collected in the first peak. This also may have been some ^{131}I-labeled organic compound, perhaps deaminated ^{131}I-iodotyrosine or some phenolic derivative of this substance. The peak was not investigated further since it did not constitute an important part of the hydrolytic process occurring during incubation of the particles.

Fig. 3 shows that the acid-soluble radioactive material resulting from the incubation of ^{131}I-albumin–containing particles and adsorbed to Dowex-50 had an Rf identical to that of monoiodotyrosine when chromatographed on Whatman No. 1 paper as described in Materials and Methods.

The addition of trichloroacetic acid to aliquots of particulate suspensions containing digestive vacuoles at various intervals during incubation at 37° results in an increasing amount of radioactivity in the supernatant (1). In order to establish that the acid-soluble material was released during incubation and was not caused by a disruption of the particles by the acid, the suspensions were centrifuged first before precipitation with acid. This procedure was compared with the one previously used in which the acid was added directly to the particulate suspensions (1). The results are shown in Fig. 4. This experiment shows that the radioactive protein fragments were excreted from the particles into the medium. The difference between the two curves in Fig. 4 represents the quantity of acid-soluble radioactivity remaining within the digestive vacuoles during the experiment, since the acid apparently caused the particles to disrupt.

The effect of the nonionic detergent, Triton X-100, and the absence of sucrose in the medium on the digestion process is shown in Fig. 5. It is well known that detergents and the absence of osmotic protective agents such as sucrose cause the

disruption of lysosomes (12, 13). The inhibition of hydrolysis of the particle-associated radioactivity by water and Triton in Fig. 5, therefore, was most likely caused by breakage of the vacuoles with release of their contents and dilution in the medium. However, it was not possible to demonstrate a great amount of acid-insoluble radioactivity released into the supernatant from the samples containing Triton or in the absence of sucrose, under the conditions described in Fig. 5. This release could be shown if the particles were incubated at pH 7.4; this is illustrated in Fig. 6. A slightly alkaline pH appeared to be required for the protein to be released from the broken particles. Fig. 7 shows the release of acid-soluble radioactivity into the supernatant at pH 7.4.

A slow rate of hydrolysis continued in the samples containing water and Triton (Figs. 5 and 7). The extent of this extraparticulate hydrolysis was dependent on the volume in which the particles were suspended. Therefore, a large volume of suspending medium was used to obtain adequate dilution of the vacuole contents to inhibit extensive hydrolysis after breakage of the particles. Suspension of the particles obtained from 1 g liver in about 20 ml was generally sufficient.

High sucrose concentrations (1 M) nearly completely inhibited the degradation of particle-associated radioactive protein. High concentrations of salts slowed the rate of the degradation, but the total quantity of protein hydrolyzed in 1 M KCl or NaCl was usually equal to or greater than that in 0.2 M sucrose. These observations are illustrated in Fig. 8. This experiment was repeated at least four times with the same result: the rate, but not the total quantity, of acid-soluble radioactivity release from the particles was always depressed in the presence of 1 M salt. The results suggest that 1 M KCl had a protective effect on the particles and that sucrose caused them to break. Column chromatography analysis of the acid-soluble radioactivity released from digestive vacuoles in the presence of 1 M KCl produced results identical with those in Fig. 2. High salt concentrations, therefore, did not remove ^{131}I-iodide from the protein in a nonenzymic reaction.

Before a valid conclusion could be drawn concerning the effects of high sucrose and salt concentrations on mouse liver digestive vacuoles, it appeared to be of importance to determine the effect of these agents on cathepsin activity per se.

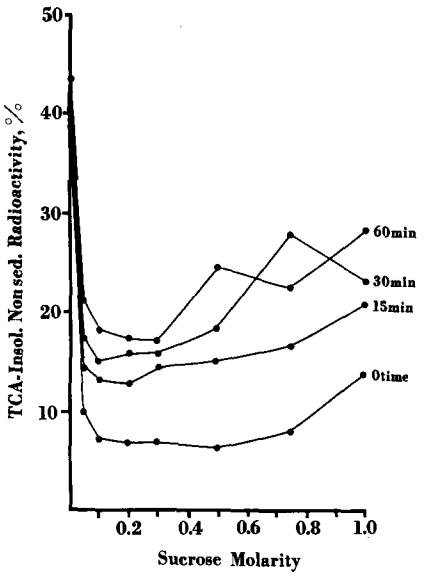

FIGURE 10 Effect of various concentrations of sucrose on the release of nonsedimentable radioactivity from mouse liver particles. 0–27,000 *g* particles were suspended in the media and incubated at 37°. Aliquots were removed at intervals and centrifuged, and the supernatants were counted in a well counter. The media also contained 0.02 M Tris-acetate buffer (pH 7.4) and 0.01 M iodoacetate to inhibit cathepsin activity. Release of acid-soluble radioactivity was negligible. The incubated suspensions contained 4,900–5,700 cpm/ml.

Accordingly, the 27,000*g* fraction from livers of uninjected mice were lysed in a small volume by freezing and thawing in distilled water centrifuged to obtain a crude cathepsin preparation. ^{131}I-albumin was used as a substrate which was incubated at 37° with the enzyme in the presence of 1 M sucrose, 1 M KCl, 1 M NaCl, or 0.2 M sucrose. Samples were removed at intervals and precipitated with trichloroacetic acid to determine the rate of hydrolysis of the labeled protein. The results in Fig. 9 show that salts greatly stimulated cathepsin activity, and that sucrose inhibited the degradation of labeled protein. However, since the initial rate of hydrolysis within digestive vacuoles was considerably higher in the presence of 0.2 M sucrose than in 1 M KCl (Fig. 8), it did not appear likely that sucrose entered the vacuoles in any great quantity. The effects of 1 M sucrose or salt, therefore, could have been due to breakage of the particles and a stimulation of cathepsin activity in the medium by salt and

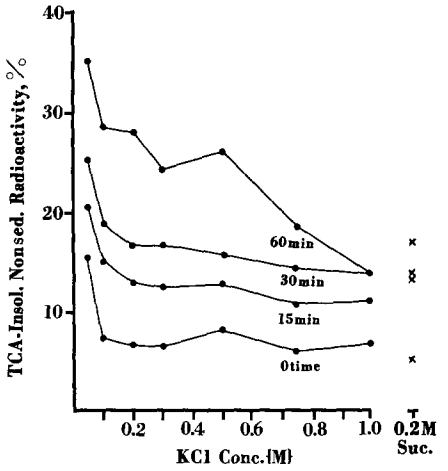

FIGURE 11 Effect of various concentrations of KCl on the release of nonsedimentable radioactivity from mouse liver particles. A control containing 0.2 M sucrose instead of KCl is also shown in the figure (*x*'s). Conditions were the same as described under Fig. 7.

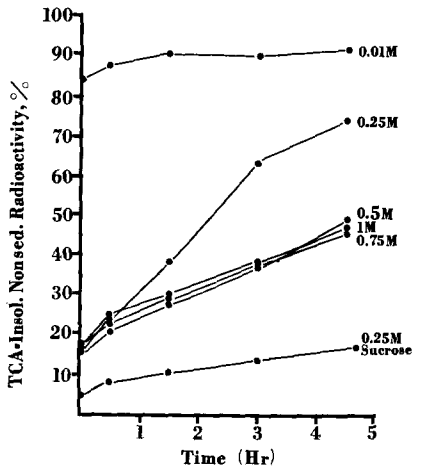

FIGURE 12 Effect of KCl on disruption of mouse liver digestive vacuoles at 0°. Two mice were injected intravenously with 0.15 ml of formaldehyde-treated ^{131}I-albumin (0.6 mg, 10^6 cpm) and sacrificed after 30 min. The perfused livers were homogenized in 0.25 M sucrose, buffered with 0.01 M Tris-acetate (pH 7.3), and centrifuged at low speed (500 *g*) to remove large particles. The supernatant was then centrifuged at 27,000 *g* for 10 min; the particles were resuspended and washed once. The pellet was then suspended in 20 ml of buffered sucrose, and 3 ml aliquots were added to 27 ml of the appropriate solution. Samples were removed at intervals, centrifuged at 39,000 *g* for 10 min, and the supernatant counted. All operations were carried out at 0°. Acid-soluble radioactivity in the samples was negligible. The figures on the curves refer to KCl molarity in the suspensions.

inhibition by sucrose. A measure of the breakage of the particles was necessary to reasonably resolve this problem. It was not possible to obtain an accurate measure of the disruption of digestive vacuoles by the release of intact labeled protein (acid-insoluble radioactivity) in the presence of a high rate of proteolysis within the vacuoles. Therefore, experiments were carried out to determine the release of radioactive material in the presence of various concentrations of salts or sucrose in 0.01 M iodoacetate, an inhibitor of cathepsin activity. The incubations were performed at pH 7.4. The results are shown in Figs. 10 and 11. High sucrose concentrations caused a slightly increased release of radioactive material which was probably not sufficient to account for the inhibitory effect on the hydrolysis of labeled protein in particles. In the experiment illustrated by Fig. 11, 1 M KCl appeared to protect the particles approximately to the same extent as 0.2 M sucrose.

The effect of high KCl concentrations on disruption of digestive vacuoles was also studied at 0°. In the experiment illustrated in Fig. 12, once-washed 27,000 *g* nuclei-free particles were incubated in various concentrations of KCl in an ice bath. Control samples were suspended in 0.25 M sucrose. All samples contained 0.01 M Tris-acetate buffer (pH 7.3). The results show that high concentrations of salt protected the particles but not so effectively as 0.25 M sucrose. However, 0.25–1 M KCl caused a small release of labeled protein at zero time; this may have been caused by a removal of some adsorbed material. KCl, 0.01 M, released nearly 85% of the radioactive protein at zero time.

DISCUSSION

Identification of the acid-soluble radioactivity released from particles containing ^{131}I-albumin, as ^{131}I-iodotyrosine by paper and ion exchange chromatography, clearly establishes the process as a hydrolysis of the labeled protein, rather than a release of labeled iodide or iodine. Nearly all the material released was in the form of iodotyrosine, and, therefore, little or no deiodination occurred in the particulate suspensions. The acid pH optimum of the hydrolytic process and the inhibition by iodoacetamide and stimulation by cysteine (1) also support the conclusion that the labeled protein

was degraded into amino acids or small peptides by proteolytic enzymes in these experiments. Formaldehyde-treated ^{131}I-albumin, therefore, provides a useful, readily available material for the study of uptake and degradation of protein in liver particles.

According to de Duve (8), the presence of a hydrolytic enzyme substrate within a lysosome establishes this particle as a digestive vacuole. In the present study, Triton X-100 and the absence of an osmotic protective agent was shown to inhibit the hydrolysis of particle-associated ^{131}I-albumin. The agents also caused a release of the labeled protein from the particles. Moreover, it has previously been demonstrated that ^{131}I-albumin added to a suspension of the same particles obtained from an uninjected mouse is not hydrolyzed (9). The osmotic properties of the ^{131}I-albumin–containing particles, the effect of Triton, and the inaccessibility of added ^{131}I-albumin to the proteolytic enzymes strongly suggest that the hydrolysis occurs within a membrane-enclosed system. The particles described in this study, therefore, have the physicochemical properties which could be expected of digestive vacuoles (phagolysosomes).

In biochemical studies of lysosomes, hydrolytic enzymes within these particles may be demonstrated only after "activation," i.e. after breakage of the lysosome by certain agents. Demonstration of hydrolytic activity within digestive vacuoles (phagolysosomes), under the conditions used in this study, depends on the presence of protective agents which maintain the integrity of these particles. Close proximity of the enzymes and substrates, such as would be expected in the intact digestive vacuole, is required to demonstrate hydrolytic activity. Disruption of the vacuoles results in release of the enzymes and substrates into the medium in which they are diluted.

Studies on the intraparticulate hydrolysis of labeled protein are complicated by a concomitant thermal destruction of digestive vacuole membranes which releases intact protein and enzyme into the medium. This breakage contributes to the apparent decrease in the rate of proteolysis with time, and it is difficult to determine the relative quantity of radioactivity in the suspensions which is associated with digestive vacuoles. Some of the bound radioactivity may be localized in phagosomes which do not contain hydrolytic enzymes (7). Some labeled protein may also be adsorbed to the surface of particulate material in the suspensions. The effect of pH on the release of acid-insoluble protein by the absence of sucrose or the presence of Triton noted during the course of this study suggests that adsorption is a significant complicating factor. Studies on these questions as well as on the effect of pH and other agents on the integrity of digestive vacuoles have been in progress in this laboratory, and they will be the subject of future publications.

Suspension in dilute buffer (pH 7.4) of mouse liver particulate material containing digestive vacuoles did not release as much labeled protein as treatment with Triton (Fig. 6). The absence of sucrose also did not inhibit the particle-associated hydrolysis as effectively as Triton (Fig. 5). A possible explanation for these observations is that some of the particles were more resistant to osmotic disruption but were broken by the detergent. Another possibility is that Triton solubilized membranes containing adsorbed labeled protein.

The stimulatory effect of salts on hydrolysis of ^{131}I-albumin by cathepsins may have been at least partially responsible for the observed effect on intraparticulate digestion of injected protein. However, it is difficult to explain the inhibition by KCl of the initial rate of hydrolysis in particles. Perhaps some breakage of particles by high salt concentrations and a stimulation of extraparticulate hydrolysis occurred in these experiments. In Fig. 12, 0.5–1.0 M KCl was not so effective a protective agent as 0.25 M sucrose; although, if some of the material released by salt was adsorbed protein, then 0.5–1.0 M salt appeared to be nearly as effective as sucrose, particularly during the first 90 min of incubation.

The effect of 1 M sucrose on intraparticulate hydrolysis of labeled protein can be more easily explained. In addition to the apparent small increased breakage of particles by the high concentrations, some sucrose could have entered the particles, resulting in an inhibition of cathepsin activity.

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