Alterations of Lysosomal Size and Density during Rat Liver Perfusion

SUPPRESSION BY INSULIN AND AMINO ACIDS*

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Perfusion of livers from fed rats for 60 min with a medium devoid of added amino acids and insulin significantly affected lysosomal density as determined from the isopycnic distribution of lysosomal marker enzymes after centrifugation in linear sucrose gradients. When compared with results from unperfused liver, an appreciable quantity of lysosomal enzyme in lysosome-rich samples of control perfused liver was found in gradient fractions of higher sucrose density, forming a distinctly bimodal pattern of distribution. Additions of insulin and amino acids during perfusion completely suppressed the heavier component, resulting in a single enzyme peak which was indistinguishable from that of unperfused liver. A peak of trichloroacetic acid-soluble radioactivity coinciding with the position of the heavier component was observed after prior labeling of livers with L-[2-14C]valine in vivo. This peak was also abolished by the addition of insulin and amino acids.

Electron micrographs of control perfused liver revealed increased numbers of an enlarged, distinctive lysosomal particle. These elements were distributed in the region of Golgi vesicles and each contained one or more sharply demarcated, granular zones resembling glycogen, frequently accompanied by elements of smooth endoplasmic reticulum. No numerical increase in autophagic vacuoles of the type characteristically seen after glucagon treatment was noted. Since general proteolysis is known to be enhanced in the absence of added amino acids and insulin, these changes could signify the existence of a sequestration-mechanism in which certain cytoplasmic elements are taken up and degraded by lysosomal elements in response to the lack of these agents.

Intracellular protein breakdown is known to be enhanced in a wide variety of cells when they are deprived of essential nutrients or growth-promoting agents such as insulin or certain serum factors (1-6). The mechanism of this breakdown is not presently understood, but in mammalian tissues, where much of the intracellular proteolytic activity is located within lysosomes, it is likely that the lysosomal-vacuolar system is involved. Evidence supporting this possibility has been obtained in perfused rat livers (9-11) and in cultured fibroblasts (7). In other studies, lysosomal alterations of a physical nature have been observed in association with changes in the overall rate of proteolysis. For example, work from our laboratory has revealed that both lysosomal osmotic sensitivity (12) and proteolysis (2, 9) spontaneously increase when livers from fed rats are perfused with an unsupplemented control medium and decrease after additions of insulin and amino acids (2, 6, 12). Analogous effects on lysosomal latency have been reported in perfused rat heart (8) and skeletal muscle (13) in response to control perfusion and additions of insulin.

A physical basis for this deprivation-induced enhancement of osmotic and mechanical fragility has not been established, but lysosomal enlargement following the sequestration of intracellular material would be a reasonable possibility. This supposition is based in part on the fact that glucagon, which is a known metabolic antagonist of insulin in liver, also induces autophagic vacuole formation (14-16), enhances lysosomal osmotic and mechanical fragility (17, 18), and stimulates proteolysis (19) in this tissue. While it is difficult for the foregoing deprivation effects are mediated directly by an increase in tissue adenosine 3':5'-monophosphate (20), nevertheless the analogy is a compelling one and it is conceivable that cellular autophagy could be induced in more than one way.

The present investigation was undertaken to obtain more information on the nature of these lysosomal alterations. From the results of prior studies with glucagon (15), changes in density as well as size might be expected in lysosomes that had acquired cytoplasmic material. Thus both parameters were assessed. Density gradient centrifugation revealed a significant increase in the density of lysosomal particles in rat livers perfused with an unsupplemented medium, an effect that was completely prevented by additions of insulin and amino acids. This alteration was associated with a popu-
lation of enlarged lysosomes which appeared to contain glyco-
ogen and smooth endoplasmic reticulum, but which lacked the
typical membranous inclusions, i.e. mitochondria and rough
endoplasmic reticulum of glucagon-induced autophagic vacu-
cles. These and related findings are consistent with the notion
that the lysosomal system is involved in some type of intracel-
lular sequestrational process which is enhanced by amino
d acid and insulin deprivation. The nature of this process is not
presently known.

MATERIALS AND METHODS

Animals—Male rats of the Lewis strain were obtained from
Microbiological Associates and maintained on regular laboratory
chow and water ad libitum. At the time of perfusion, their weight
ranged from 120 to 135 g.

In some experiments, liver protein was labeled in vivo by the
intraperitoneal injection of L-[1-14C]valine (57.6 µCi/µmol, New
England Nuclear Corp.) in 0.5 ml of 0.85% NaCl 17 and 4 h prior to
perfusion. The total dose of isotope was 20 µCi for the experiments
of Table I and 100 µCi for the experiments of Fig. 4.

Liver Perfusion—Livers were cyclically perfused for 60 min in
situ by a method previously detailed (2, 12, 21). The perfusion
medium consisted of a suspension of washed sheep cells in a solution
of Krebs-Ringer bicarbonate buffer and 4.0% bovine plasma albumin
(Fraction V, Armour Pharmaceutical Co.). The cells were washed
twice with 3 volumes of buffer prior to the addition of buffer and
albumin. Care was taken to remove the buffy coat following the
initial centrifugation. The final concentration of red cells averaged
0.27 (v/v) for most of the experiments. A higher red cell concentra-
tion ranged from 45 to 50 ml of which 5 ml were lost as a washout at the
start of perfusion and before the return flow from the liver to the
perfusion flask was established. The perfusate was gassed with 95%
O2, 5% CO2 at a flow of 700 ml/min.

Amino Acid and Insulin Administration—Two amino acid mix-
tures were employed. The first (AA-14) was patterned after an
ovalbumin hydrolysate, except for the omission of leucine, isoleu-
cine, valine, and tyrosine, and was the same as that used in two
previous studies (6, 12). Tyrosine was deleted because of its low
solubility, but its absence was compensated for by doubling the
amount of phenylalanine. The mixture was dissolved in 0.85% NaCl
and was adjusted to 7.4 by the addition of NaOH. It then
was infused continuously into the perfusion medium at a rate of 264
µmol/h after the addition of a priming dose of 132 µmol. Except for
the lack of tyrosine, the second mixture (AA-19) was similar to the
amino acids of plasma and had the following percentage of molar
composition: Ala, 10.3; Arg, 4.4; Asp, 1.0; Cys, 1.5; Gln, 2.8; Gly,
7.0; His, 1.8; Ile, 212; Leu, 3.8; Lys, 8.7; Met, 1.1; Phe, 3.8; Pro, 8.2;
Ser, 13.4; Thr, 6.3; Try, 1.7; Val, 4.7; Asn, 1.8; and Gln, 15.3. Prior
to the administration of this mixture, the pH was adjusted to 7.4
and a single dose of 1.59 mmol in 2 ml of 0.85% NaCl was added to
the perfusion medium at the start of the experiment. This amount
was calculated to raise initial amino acid concentrations to values
10 times normal plasma levels (19).

Trypsin-treated, crystalline zinc pork insulin (Lot 499667, 25
units/mg, Lilly Laboratories) was dissolved in a small volume of
diluted ethylenediamine and then added to a solution of 0.86% NaCl
and 0.5% bovine albumin prior to use. The latter was infused
continuously into the perfusion medium at 2.4 µg/h after the
addition of a 0.8-µg priming dose at the start of perfusion

The conditions required for establishing maximal inhibition of
proteolysis were determined in preliminary control experiments.
Proteolysis was measured in previously labeled perfused livers from
the total release of [14C]valine into a large pool (15 ml) of unlabeled
valine as detailed earlier (21). The results in Table 1 indicate that
50 to 65% inhibition can be obtained with a mixture AA-19 alone or
with either of the mixtures combined with insulin when these
agents are given as specified above. These effects clearly are greater
than the largest suppression obtainable with mixture AA-14 (6) or
insulin (2) alone, and the resultant proteolytic rates are probably
the lowest that can be achieved by physiological agents under these
conditions.

Table I

<table>
<thead>
<tr>
<th>Addition</th>
<th>Hepatic valine release (nmol min⁻¹/100 g rat)</th>
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<tbody>
<tr>
<td>None (7)</td>
<td>239 ± 18</td>
</tr>
<tr>
<td>AA-14 (5)</td>
<td>163 ± 55</td>
</tr>
<tr>
<td>AA-19</td>
<td>104 ± 2</td>
</tr>
<tr>
<td>AA-19 (4 times physiological concentration) (3)</td>
<td>107 ± 19</td>
</tr>
<tr>
<td>AA-19 (10 times) + insulin (5)</td>
<td>109 ± 18</td>
</tr>
<tr>
<td>AA-19 (10 times) + insulin (5)</td>
<td>116 ± 9</td>
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Density Gradient Centrifugation—Segments of liver removed at
the termination of the perfusion experiments and from intact rats
were immediately chilled in iced 0.25 M sucrose containing 1 mM
EDTA adjusted to pH 7.4. Tissue homogenates (1:10, w/v) were
prepared with an all glass hand tissue grinder (Routier) of the type
described by Dounce (22). We have previously established that
turally complete cell disruption with only minimal release of free
lysosomal enzymes can be obtained with four strokes of the A pestle
followed by four strokes of the B pestle (12). A mitochondrial-lysoso-
mal fraction (M + L) then was prepared from 3.6 g of liver following
the fractionation scheme of deDuve (23). The whole homogenate
was spun at 5000 rpm for 5 min and the resultant supernatant was
centrifuged at 11,000 rpm for 20 min, both in a model B-20 Interna-
tional centrifuge equipped with a No. 870 head. The M + L pellet
from the second spin was gently resuspended in 2 ml of the sucrose/
EDTA solution and about 0.5 ml of this suspension was layered on
top of the sucrose gradient prior to centrifugation. The M + L
suspension contained an average of 36% of the total lysosomal en-
zeyme content of the liver.

Linear sucrose gradients were made in Beckman cellnco nitrate
tubes using a Beckman density gradient former. The range of
densities (5') are given in the appropriate figures under "Results." To
check the linearity of these gradients, a small amount of inorganic
phosphate was mixed with the lighter solution used in
making the gradient. Consecutive fractions of the gradient then
were analyzed for inorganic phosphate by the method of Comor
(24). In the initial experiments, gradients were spun in an SW 50L
rotor at 50,000 rpm to equilibrium (60 min) in a Beckman (Spinco
Division) model L3-50 ultracentrifuge. Later an SW 41 rotor, spun
at 41,000 rpm for 75 min, was used. In view of the larger tube size of
the SW 41 rotor, the sample volume was increased proportionally.
With both rotors, equilibration of lysosomal particles was estab-
lished by the lack of movement of the marker enzyme peak after
the time of centrifugation had been doubled.

Fractions were collected by draining the tubes from the bottom.
Trion X-100 then was added to each fraction to give a final
concentration of 0.1% (20). N-Acetyl-β-D-glucosaminidase (β-acetyl-
glucosaminidase) activity was determined essentially as described
by Barrett (26). Modified by reducing the concentration of citrate
buffer according to Woolen et al. (27). Cathepsin D was assayed by a
method outlined by Barrett (26), using 14C-labeled hemoglobin (28)
plus unlabeled hemoglobin at a total concentration of 1%. Protein
was measured by the Lowry procedure (29).

Determination of Radioactivity—In measurements of proteolysis
(Table I), valine and 14C-labeled valine in samples of perfusate were
determined as detailed previously (21). In the experiments of Fig.
7, those fractions from each experiment were pooled and the
separate pooled fractions (1.5 ml) were deproteinized with 5% tri-

chloroacetic acid (final concentration). One-milliliter aliquots of the resultant supernatant were passed over small columns of Dowex 50 for the extraction of amino acids as described earlier (2). The columns were washed with water and then eluted with 5 M NH₄OH. The eluates were dried and the residues each dissolved in 1.25 ml of water. One-milliliter aliquots then were counted in Aquasol (New England Nuclear Corp.) in a Beckman LS-150 liquid scintillation spectrometer. Quenching was corrected for by the use of an external standard.

**Tissue Preparation for Light and Electron Microscopy** Sections from six unperfused livers and from six livers perfused without additions to the medium were prepared as follows. For light microscopy, 2 mm thick sections of liver were fixed in 10% neutral buffered formalin (30), embedded in paraffin, and sectioned at 6 mm thick. Tissue sections were routinely stained with haematoxylin and eosin as well as with the periodic acid-Schiff reaction, with and without diastase digestion (31), for the demonstration of glycogen.

**RESULTS**

**Alterations of Lysosomal Density in Unperfused Rat Livers and in Livers Perfused with and without Insulin and Amino Acids** - Fig. 1 (upper panel) shows sucrose density gradient distributions of ρ-acetylglucosaminidase activity in M + L fractions of unperfused and control perfused rat livers. In unperfused tissue, the enzyme peaked in Fraction 9 at a density of 1.210, a value which agrees closely with a previous estimate of the mean density of rat liver lysosomes (33). Perfusion for 60 min with a standard, unsupplemented control medium moved the peak into denser gradient fractions. Although this shift was small in absolute terms, it was highly reproducible (Table II); comparable results were also obtained with measurements of cathepsin D (data not shown). As shown in Table II, additions of insulin and amino acids (AA-14) during perfusion suppressed the density shift and the resultant peak was intermediate between the unperfused and control peaks.

Of importance was the lack of movement in the distribution of protein (Fig. 1, lower panel). Inasmuch as the main protein peak is derived largely from mitochondria, the lysosomal shift during control perfusion was probably not the result of a generalized increase in organelle density, as might have been produced by a change in particle hydration, for example, but was more likely the consequence of a specific lysosomal alteration.

In order to accommodate larger samples and at the same time to determine whether resolution might be enhanced, subsequent gradient studies were carried out with an SW 41 rotor which has considerably longer tubes than those of the SW 50L rotor. As shown in Fig. 2, the effect of control
than was evident in the foregoing experiments (Fig. 1). In min with (right) and without (left) additions of amino acids (AA-19) dase activity from unperfused livers and from livers perfused for 60
were normalized to a common value which equalled the mean of the total enzyme activity on all the gradients. Number of experiments samples, a significant fraction of enzyme was found in the dense region of the gradient, thus forming a widened, bimodal distribution. The two peaks were separated by six fractions. Since the total liver content of lysosomal enzyme is not changed under these conditions (121, this finding suggests that a substantial quantity of enzyme had shifted from lighter to heavier lysosomes.

In Fig. 3, we plotted the cumulative distribution of β-acetylglucosaminidase activity from data in Fig. 2. Values are means ± S. E.; number of experiments is shown in parentheses.

Electron micrographs of control perfused as compared with unperfused liver revealed a moderate overall increase in the total number of lysosomal profiles. This increase was consistent among individual micrographs and averaged slightly more than 50%. No change in the frequency of peroxisomes was observed. One particular component appeared to be far more numerous in control perfused tissue (compare Figs. 4 and 5). It was larger than the average dense body and contained one or two conspicuous, granular, electron-lucent zones which were sharply demarcated from a zone of moderate electron opacity (inset, Fig. 5). The latter frequently contained one or more electron-opaque cores similar to those normally seen in dense bodies of unperfused liver.

These so-called "perfusion elements" were not commonly found in the vicinity of bile canaliculi, as is the case for dense bodies (Fig. 4), but rather were widely distributed in the cytoplasm, often in a loose circular array around a cluster of Golgi vesicles (Fig. 5). From the rosette-like pattern of granulation, the electron-lucent zone was believed to consist largely of glycogen. Occasionally small vesicles similar to smooth-surfaced endoplasmic reticulum were noted within the granular zones. These vesicular inclusions appeared to be more prevalent in cells that contained smaller than usual amounts of glycogen. Profiles containing clearly identifiable organelle...
FIG. 4 (top). Electron micrograph of unperfused fed rat liver showing portion of a hepatocyte between the nucleus (N) and bile canaliculus (bc). Characteristic dense bodies (db) are evident in the pericanalicular region and particulate glycogen is distributed throughout the cytoplasm. An array of rough endoplasmic reticulum (RER) is seen in the upper half of the micrograph. x 11,000.

FIG. 5 (bottom). Electron micrograph of fed rat liver cyclically perfused for 60 min without additions to the medium. A portion of a parenchymal cell comparable to that seen in Fig. 4 is shown. Note the extensive zone of Golgi vesicles (GOL). Also seen in the Golgi area are numerous large lysosomes (arrows). Most of these enlarged vesicles contain electron-dense material and, in addition, granular zones closely resembling particulate glycogen. Close examination of some of these granular zones (upper center) also reveals the presence of membranous profiles typical of smooth endoplasmic reticulum. A large area of glycogen (GLY) and a portion of a nucleus (N) can also be seen in this micrograph. x 11,000. In the inset some of these perfusion vesicles are seen at higher magnification. The granularity of the material contained within these lysosomes and its similarity to particulate glycogen located in the immediate vicinity of these vesicles are particularly evident in this micrograph. x 17,000.

The effect of control cyclic perfusion on the relative size distribution of lysosomal profiles is depicted in Fig. 6. Most of the images were, of course, noncircular and we arbitrarily chose the longest linear cross-sectional dimension as an index of size. The distribution from unperfused liver formed a single peak at a point slightly less than the mean. The numerical value of this point agrees closely with a previous estimate of the average cross-sectional dimension of hepatic dense bodies (35). By contrast, the corresponding distribution from control perfused liver was bimodal. In addition to the first peak, which coincided with the single peak of unperfused liver, a second was present in a region of about 40% greater linear size. Assuming sphericity, the average volume of the elements comprising the second peak would be about 2.7 times greater than that of the first.

Earlier work has shown that intracellular protein in liver, previously labeled in vivo with $^{14}$C-valine, is taken up in some way by lysosomes and degraded to trichloroacetic acid-soluble products (10, 36). Control experiments showed that the acid-soluble radioactivity associated with lysosomes follows these elements closely during isopycnic centrifugation without exchanging with label in the supernatant (10). Since the density and size alterations in the present study could represent aspects of such a proteolytic process, we investigated the distribution of trichloroacetic acid-soluble radioactivity after density gradient sedimentation of M + L fractions from livers perfused with and without additions of insulin and the AA-19 amino acid mixture. As depicted in Fig. 7, a prominent acid-soluble radioactive peak was observed in Fractions 6 to 7 of control perfused material. Although its position approximated the denser of the two peaks of $\beta$-acetylglucosaminidase activity (Fig. 2), the distribution of radioactivity was not distinctly bimodal. However, a significant alteration in distribution was produced by insulin and amino acid additions (Fig. 7). With treatment, the major peak shifted to Fractions 10 to 11, and the resultant curve coincided closely with the enzyme distribution of similarly treated livers (see Fig. 2). It is important to point out that the values in each fraction were normalized to the total radioactivity associated with particles; thus the amplitude of the peaks have no relationship to rates...
these results may be compared. To minimize differences between livers in overall labeling, the results were normalized to a common
treatment was carried out as in the experiments of Fig. 3, with which
"Materials and Methods." Equilibrium density gradient centrifuga-
from Fractions 1 to 19. Values plotted are means ± S. E.; number of
experiments is shown in parentheses.
additions of amino acids (AA-19) and insulin. Prior to perfusion the
value which represented the average quantity of label recovered
in the unperfused liver distribution is therefore
equivalent to $16 \times 3.18 \times 10^{-4}$ or $0.51 \, \mu m$, a value in close
agreement with the average dimension of dense bodies (35).

size and density alterations can be prevented or, in the case of osmotic sensitiv-
ity, reversed by additions of insulin and amino acids in
association with changes in rates of protein degradation (2, 6,
12) makes these observations particularly relevant to the
question of whether the lysosomal system is directly involved
in the uptake and degradation of endogenous proteins. Exper-
imental support for such a possibility has come from the work
of Poole (7) and Dean (11) and from our own laboratory (37).
Before considering possible explanations for the observed
alterations of lysosomal size and density, some comment
should be made regarding their distribution among the total
population of lysosomal elements. The failure to obtain a bimodal distribution of $\beta$-acetylglucosaminidase in control
perfused livers when density gradient centrifugation was
carried out with the SW 50L rotor was very likely the result
of incomplete particle separation in tubes that were too small
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defined conditions, bimodal curves have been obtained consist-
ently in control perfused livers with the SW 41 rotor. The fact
that the distributions of size (Fig. 6) and density (Fig. 2) were
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gests that conditions present during control perfusion induced
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nents. Proof that the denser elements were, in fact, the
enlarged lysosomes seen on the electron micrographs in this
study has not yet been obtained, but this question could
eventually be verified by electron microscopic examination of
lysosomal particles after density gradient centrifugation.

In our view, the most probable cause for the lysosomal
enlargement observed in this study is the bulk entrapment of
intraacellular substances (cytoplasmic matrix or subcellular
organelles, or both). While cellular injury or the endocytotic
uptake of extracellular material might also be considered,
there is no morphological evidence for the former, and endo-
cytosis in liver is not known to be regulated by insulin and
amino acids. Such an autophagic process, however, would not
necessarily explain the density alteration. Assuming that the
acquired material consisted largely of protein and water at
normal intracellular concentrations, lysosomal density proba-
bly would not increase unless the water associated with
native protein were lost. While this might occur as a conse-
quency of the denaturation of protein immediately after its
uptake, it is likely that protein fragmentation would rapidly
follow, and the overall effect of these two reactions on particle
density would be difficult to predict.

An alternative explanation for the increase in density was
suggested from the electron microscopic appearance of the
enlarged lysosomes (Fig. 5). Most contained granular material
resembling glycogen. The specific gravity of glycogen is known
to be quite high, being of the order of 1.54 in water (38, 39)
and one may presume that its inclusion within the lysosome
would increase its overall density. Since the tubular network
of the smooth endoplasmic reticulum pervades the glycogen
zones (39, 40), these membrane structures and their associated
proteins undoubtedly would be sequestered along with it.
Indeed, evidence for this was obtained in the present study.

Glycogen has been reported as a conspicuous inclusion
within autophagic vacuoles that are formed in rat hepatocytes
during the postabsorptive period (41). It has also been noted
in multivesicular bodies in livers from fed rats (42) and in
autophagic vacuoles of livers from rats with streptozotocin-
induced diabetes (43). In the neonatal rat liver, glycogen is a
major constituent of autophagic vacuoles which are generated
during a period of rapid glycogen depletion within 12 h after

of proteolysis in the livers from which the distributions were
made.

DISCUSSION

The present findings and those from an earlier study (12)
clearly demonstrate that perfusion of the unfasted rat liver
with a standard, unsupplemented medium evokes a number
of physical alterations of the lysosomal system which are
undoubtedly of biological importance. The fact that these
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birth (44). Probably the most extensively studied model for autophagy is in liver that is induced by glucagon (14, 15, 45, 46) or cyclic AMP (16). It is of interest that glucagon has not been reported as a significant component within these autophagocytic vacuoles. However, in most of the glucagon studies, the animals were fasted for 24 h before the experiment, and the content of glucagon was undoubtedly very low at the time glucagon was given. Moreover, the small amounts of glucagon that were initially present would have been depleted rapidly by the glucocorticoidal action of the hormone.

The physiological role of these deprivation-induced lysosomal alterations has not been fully evaluated. Livers of rats are known to lose from 20 to 30% of their protein content during 1 day's fasting (47), and autophagy has been reported to increase dramatically during this time (41). Stereologic studies indicate that, in addition to the well-recognized loss of glycogen, there is a substantial reduction in the endoplasmic reticulum and cytoplasmic matrix (41). It is thus reasonable to conclude that autophagic vacuoles formed in the postsorptive period would contain these elements, a deduction which, in fact, is consistent with our present observations. Lysosomal enlargement and overt autophagy have been observed in livers of rats made diabetic by streptozotocin (43, 48). While it might be supposed that insulin deficiency directly contributed to this alteration, this interpretation is complicated by the fact that glucagon levels increase following streptozotocin administration (43, 49). In the absence of any evidence for a spontaneous rise in cellular cyclic AMP during control perfusion (20), our findings support the view that autophagy is also enhanced by insulin or amino acid deprivation, or both, independently of cyclic AMP.

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