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Regulation of the breakdown rates of biotin-containing proteins in Swiss 3T3-L1 cells

Colin S. CHANDLER and F. John BALLARD*
CSIRO (Australia) Division of Human Nutrition, Kintore Avenue, Adelaide, S.A. 5000, Australia

1. Degradation rate constants for individual biotin-labelled proteins were measured in Swiss 3T3-L1 adipocytes that had been incubated with inhibitors of autophagy or of lysosomal proteolysis. 2. Inhibitory effects produced by 10 mM-3-methyladenine and a combination of 5 mM-NH_4Cl and leupeptin (50 μg/ml) were approximately equal. The inclusion of NH_4Cl did not significantly enhance the responses to 3-methyladenine, suggesting that autophagy was already maximally inhibited. 3. The extent of inhibition by 3-methyladenine or by the NH_4Cl/leupeptin mixture was similar for the cytosolic enzyme acetyl-CoA carboxylase and for the three mitochondrial carboxylases. This inhibition averaged 50%. The breakdown rate of a more-stable 38 kDa biotin-containing mitochondrial protein was more responsive to the inhibitory agents. These results are best explained by mitochondrial proteolysis occurring via a combination of the degradation of whole mitochondria within autophagic vacuoles, supplemented by the selective intramitochondrial breakdown of more labile proteins. 4. A number of intermediate products in the degradation of biotin-containing proteins were detected. Differences in the patterns of radioactivity between these peptides after incubation of cells in the presence of inhibitors of the breakdown process provided evidence that some peptides were produced before autophagy, others as a result of intralysosomal inhibition, while at least one was associated with intramitochondrial proteolysis.

INTRODUCTION

Breakdown of the four biotin-containing proteins characterized in mammalian cells can be measured as the rate of disappearance of radioactivity from each protein after incubation of the cells with labelled biotin. The method is valid because, once biotin has been incorporated into the proteins, the biotinyl–lysine bond is not cleaved until the protein has been completely degraded (Koivusalu *et al.*, 1963; Craft *et al.*, 1985). With this experimental approach applied to normal and transformed human fibroblasts, we demonstrated firstly that the four biotin-containing carboxylases were degraded at characteristic rates and, secondly, that a lysosomal pathway was involved in the breakdown of both the cytosolic acetyl-CoA carboxylase and the three mitochondrial carboxylases (Chandler & Ballard, 1985). However, it was also necessary to implicate intramitochondrial proteolysis in the breakdown of the latter enzymes to account for the heterogeneity of their degradation rates. Moreover, the presence of numerous labelled peptides in the mitochondrial fractions obtained by density-gradient centrifugation of cell extracts suggested that catabolism of biotin-containing proteins had occurred (Chandler & Ballard, 1986).

In the present study we have used inhibitors to quantify the extent to which autophagy, lysosomal proteolysis and non-lysosomal proteolysis contribute to the breakdown of the biotin-labelled proteins as well as to investigate the generation of intermediate products in the degradation process.

MATERIALS AND METHODS

Materials

The insulin used was Actrapid from Novo Industri A/S, Copenhagen, Denmark. 3-Methyladenine was obtained from Fluka AG, Buchs, Switzerland. The sources for other chemicals, cells, culture media and radioisotopes were described previously (Chandler & Ballard, 1985).

Degradation of biotin-labelled proteins

Swiss 3T3-L1 fibroblasts were grown to confluence in 12-place multi-well dishes, differentiated and labelled for 48 h with [^{3}H]biotin as described previously (Chandler & Ballard, 1983), but with the modifications reported by Chandler & Ballard (1986). Subsequently the monolayers were washed twice with Hanks salts and incubated in Dulbecco-modified Eagle's Minimal Essential Medium containing 100 μM-biotin, 2 mM-leucine, 10% (v/v) fetal-bovine serum as well as 60 mg of penicillin G, 100 mg of streptomycin sulphate and 1 mg of fungizone/litre. After 2 h this medium was replaced with 1 ml of a similar medium for control wells and with the indicated combinations of 5 mM-NH_4Cl, 50 μg of leupeptin, 1 μM-insulin or 10 mM-3-methyladenine added to the treatment wells. After incubation at 37 °C in a humidified atmosphere (air/CO_2, 19:1) for 0, 24, 48 or 72 h, the medium was removed and protein precipitated with trichloroacetic acid as described previously (Ballard *et al.*, 1980) to obtain medium amino acid and medium protein fractions. Each monolayer was then washed

* To whom correspondence and reprint requests should be sent.

twice with 1 ml of Hanks salts at 0 °C. The washes were combined, and protein was precipitated after the addition of 1 mg of carrier bovine serum albumin, generating wash amino acid and wash protein fractions. Immediately after the wash procedure, 0.5 ml of 10% (w/v) trichloroacetic acid at 0 °C was added and the cells scraped off and transferred to a centrifuge tube. Two further 0.5 ml portions of 10% trichloroacetic acid were used to effect optimal recovery of cell protein. These combined trichloroacetic acid extracts were centrifuged to obtain cell amino acid and cell protein fractions, the latter being kept at −80 °C before electrophoretic analysis. Any residual protein on the dishes was dissolved in 0.5 M-NaOH containing 0.1% Triton X-100.

The total radioactivity in each well was obtained by summation of the radioactivities in medium protein, medium amino acid, wash protein, wash amino acid, cell protein, cell amino acid and residual protein fractions. Once the radioactivity per well for each biotin-containing protein had been determined after electrophoresis, autoradiography, cutting out the band and scintillation spectrometry (see below), this value was corrected by two factors in order to obtain reliable data. Firstly the radioactivity calculated for a single band per well was multiplied by the sum of the total protein radioactivity in the well (medium, wash, cell plus residual protein) and divided by the cell protein radioactivity for that well to allow for variable amounts of protein dislodged by the washing procedure or left on the dish. Secondly, this value was further corrected to account for differences in biotin incorporation between wells by dividing it by the sum of the radioactivities in all fractions derived from that well (i.e. equal to the radioactivity incorporated at zero time) and multiplying it by an equivalent value from a reference well for that experiment. The corrected radioactivities were used to calculate first-order degradation rate constants ($days^{-1}$).

Two electrophoretic systems were used to determine the radioactivities in specific proteins. For the quantification of all protein bands except those corresponding to propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase, electrophoresis of cell extracts was performed with 6–16% (w/v) acrylamide gradient slab gels (Chandler & Ballard, 1986), whereas 8% (w/v) acrylamide slab gels (Chandler & Ballard, 1985) were used for the separation of the above two enzymes. The procedures for dissociation, preparation of stacking gels, buffer systems, gel staining, drying, autoradiography, cutting out the bands and the measurements of radioactivity were common to both methods and were described previously (Chandler & Ballard, 1985).

Degradation of leucine-labelled cell proteins

The initial procedures used were the same as described for biotin-containing proteins, except for the substitution of [^{3}H]leucine (1 μCi/ml) for labelled biotin. Radioactivities in the monolayers at the completion of the incubation period were determined after dissolution of the cells in 0.5 M-NaOH containing 0.1% Triton X-100 and used to calculate first-order degradation rate constants ($days^{-1}$).

RESULTS AND DISCUSSION

Degradation rate constants for biotin-containing proteins

Biotin-containing proteins obtained from cells immediately after the labelling protocol included one major radioactive band and many minor bands (Fig. 1, lane 2). The major band corresponds to the biotin-containing subunit of pyruvate carboxylase (Chandler & Ballard, 1986) and accounts for approx. 85% of the total biotin incorporation. Substantial radioactivity is also evident in the acetyl-CoA carboxylase band and in the doublet comprising methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase. These last two proteins are adequately separated in the 8% (w/v) acrylamide gel (lanes 3 and 4), although less clearly than found for the equivalent human proteins (Chandler & Ballard, 1985). Many minor radioactive bands can be seen in lanes 1 and 2 (Fig. 1), in confirmation of previous experiments with Swiss 3T3-L1 cells (Chandler & Ballard, 1985, 1986). The relative intensities of these minor bands are not identical with those obtained in our earlier studies because, in the present experiments, monolayers were treated with trichloroacetic acid rather than first scraping the cells from the dishes with water. The acid-quenching procedure was adopted to minimize any proteolytic modifications to the true biotin-containing proteins.

In addition to bands corresponding to all the biotin carboxylases in the 8% acrylamide gels (see Fig. 1, lanes 3 and 4) and to acetyl-CoA carboxylase and pyruvate carboxylase in the gradient gels (see Fig. 1, lanes 1 and 2), one other band had properties not consistent with it being a breakdown product of a biotin carboxylase. Thus

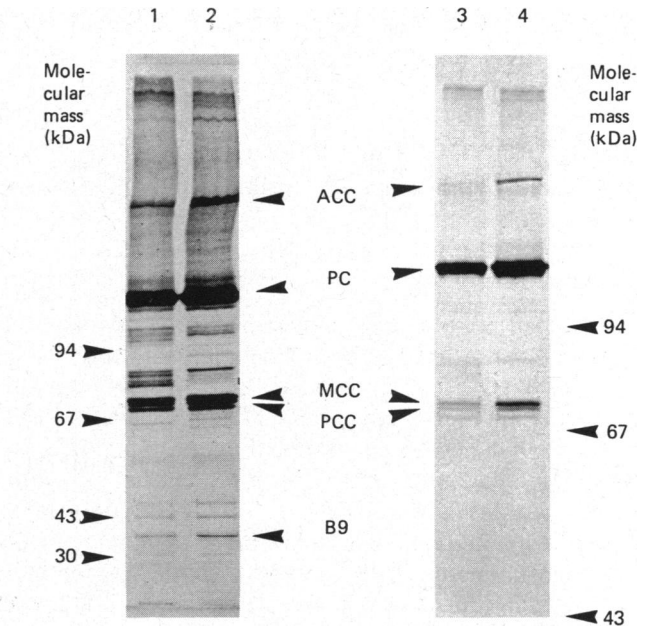

Fig. 1. Autoradiographs of biotin-containing proteins separated on a 6–16% gradient gel (lanes 1 and 2) or a 8% acrylamide gel (lanes 3 and 4)

Equal proportions of cell extracts from each well were analysed. The lanes represent: 2, 4, zero time; 1, 3, after a 3-day degradation period in control medium. The biotin subunits of acetyl-CoA carboxylase (ACC), propionyl-CoA carboxylase (PCC), methylcrotonyl-CoA carboxylase (MCC), pyruvate carboxylase (PC) and band-9 protein (B9) are indicated as are the positions of the molecular-mass markers. The exposure times were 28 days for the gradient gel and 4 days for the 8% acrylamide gel. The pinholes visible alongside some bands were used to assist protein removal from the dried gels.

Table 1. Degradation rate constants for total protein and biotin-containing proteins in two experiments

Values are degradation rate constants (±S.E.M.) determined from protein radioactivities obtained from the numbers of wells given in parentheses. The measurement periods were 0, 1 and 2 days for Expt. 1, as well as for the leucine-labelled total cell protein in Expt. 2, and 0, 1, 2 and 3 days for the biotin-containing proteins in Expt. 2.

Protein	Degradation rate constant ($days^{-1}$)	
	Expt. 1	Expt. 2
Total cell protein	0.397±0.030 (14)	0.376±0.014 (12)
Acetyl-CoA carboxylase	0.494±0.028 (13)	0.322±0.028 (15)
Propionyl-CoA carboxylase	–	0.287±0.042 (11)
Methylcrotonyl-CoA carboxylase	–	0.308±0.034 (11)
Pyruvate carboxylase	0.294±0.033 (13)	0.207±0.019 (15)
Band 9	0.180±0.065 (13)	0.164±0.019 (15)

this 38 kDa protein, termed 'band 9' to be consistent with previous numbering (Chandler & Ballard, 1986), had progressively less radioactivity as the degradation measurement period was extended and never showed radioactivity above the zero-time value even in the presence of proteolytic inhibitors (see below). Moreover, the intensity of band-9 protein in autoradiographs was not increased when labelled cell extracts were incubated *in vitro* (Chandler & Ballard, 1986).

Radioactivity in regions of the dried acrylamide gels that corresponded to the autoradiographic bands for the four biotin carboxylases and band 9 were used to calculate degradation rate constants. In two experiments where degradation was monitored in control incubations, the decreasing order of rate constants was:

Acetyl-CoA carboxylase > pyruvate carboxylase > band 9 (Table 1)

In the second study propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase were degraded with rate constants slightly less than found for acetyl-CoA carboxylase. This study included the use of 8% acrylamide gels and thus allowed adequate quantification of the two closely migrating bands (Table 1). The complete set of data for control wells in the second experiment is shown in Fig. 2. In this experiment each discrete protein showed first-order decay kinetics, although the decay pattern of radioactivity for total cell proteins labelled with [^{3}H]leucine was non-linear, as expected for a protein mixture.

Our finding of a slower degradation rate for pyruvate carboxylase as compared with acetyl-CoA carboxylase is similar to the situation with both diploid and transformed human fibroblasts (Chandler & Ballard, 1985). In that study, however, methylcrotonyl-CoA carboxylase and especially propionyl-CoA carboxylase had longer half-lives than pyruvate carboxylase in each of the cell lines, a situation different from that occurring in mouse Swiss 3T3-L1 cells. If, as proposed previously (Chandler & Ballard, 1985) the differences in degradation rate constants between the three carboxlyases are attributable to different susceptibilities to intramitochondrial proteolysis, we must conclude that mouse pyruvate carboxylase is a relatively poor substitute for the proteinases present in mitochondria.

Effects of inhibitors on degradation rate constants

We have used a panel of inhibitors added at the beginning of the breakdown measurement period in an attempt to determine the nature and site of the degradative process. The breakdown of total cell proteins was also monitored, both as a reference for the biotin-containing proteins as well as to permit comparisons with other investigations. Notwithstanding the generally larger degradation rate constants in the first of the two experiments in Table 1, the relative effects of the inhibitors on total protein breakdown as well as on the breakdown of individual proteins were similar in the two studies. Only data from the second experiment have been presented graphically in Fig. 3. Total protein breakdown was inhibited by approx. 20% in the presence of NH_4Cl, leupeptin, insulin or 3-methyladenine and by approx. 40% when NH_4Cl was added together with leupeptin or 3-methyladenine (Fig. 3). Although the effects of those agents that inhibit lysosomal proteolysis (NH_4Cl, leupeptin), other cysteine proteinases (leupeptin) or autophagy (insulin, 3-methyladenine) are less than reported in some investigations with cultured cells (Ballard, 1987; Seglen, 1987), it should be noted that in the present study the measurements were carried out in the presence of complete culture medium that included 10% fetal-bovine serum. Smaller effects can be expected under such conditions, because the growth factors present will partially inhibit autophagic proteolysis (Ballard, 1987; Seglen, 1987). Serum was included, since preliminary experiments had established that none of the inhibitor combinations led to an increase in the proportion of the biotin radioactivity present in the medium protein fraction. This assessment of protein radioactivity in the medium provides a convenient indicator of cell damage.

Autophagic sequestration in hepatocytes has been reported to be completely inhibited by 10 mM-3-methyladenine (Seglen, 1987). Addition of this compound decreased the degradation rates of all four biotin carboxylases and band 9 by between 45 and 65% (Fig. 3). Although there is no prior evidence that autophagy can be completely prevented by 3-methyladenine in Swiss 3T3-L1 cells, our results imply that the inhibition observed sets the minimum contribution of the autophagic process to overall proteolysis. Moreover, the lack of any additional decrease in degradation rate constants upon inclusion of the lysosomotropic agent ammonia (as NH_4Cl) with 3-methyladenine (Fig. 3) is consistent with an already blocked autophagic/lysosomal system. For most of the biotin-containing proteins, insulin was less effective than 3-methyladenine as an inhibitor of proteolysis, although the relative lack of precision in the determination of degradation rate constants must caution any significance that can be inferred from such differences.

Degradation rates for total cell protein as well as for the individual biotin-containing proteins were inhibited by NH_4Cl and leupeptin, with the effects of these individual agents being generally less than observed with 3-methyladenine (Fig. 3), a result expected for NH_4Cl because the concentration tested was below that required to produce a maximal inhibitory response. With total cell proteins and all biotin proteins except for methyl-

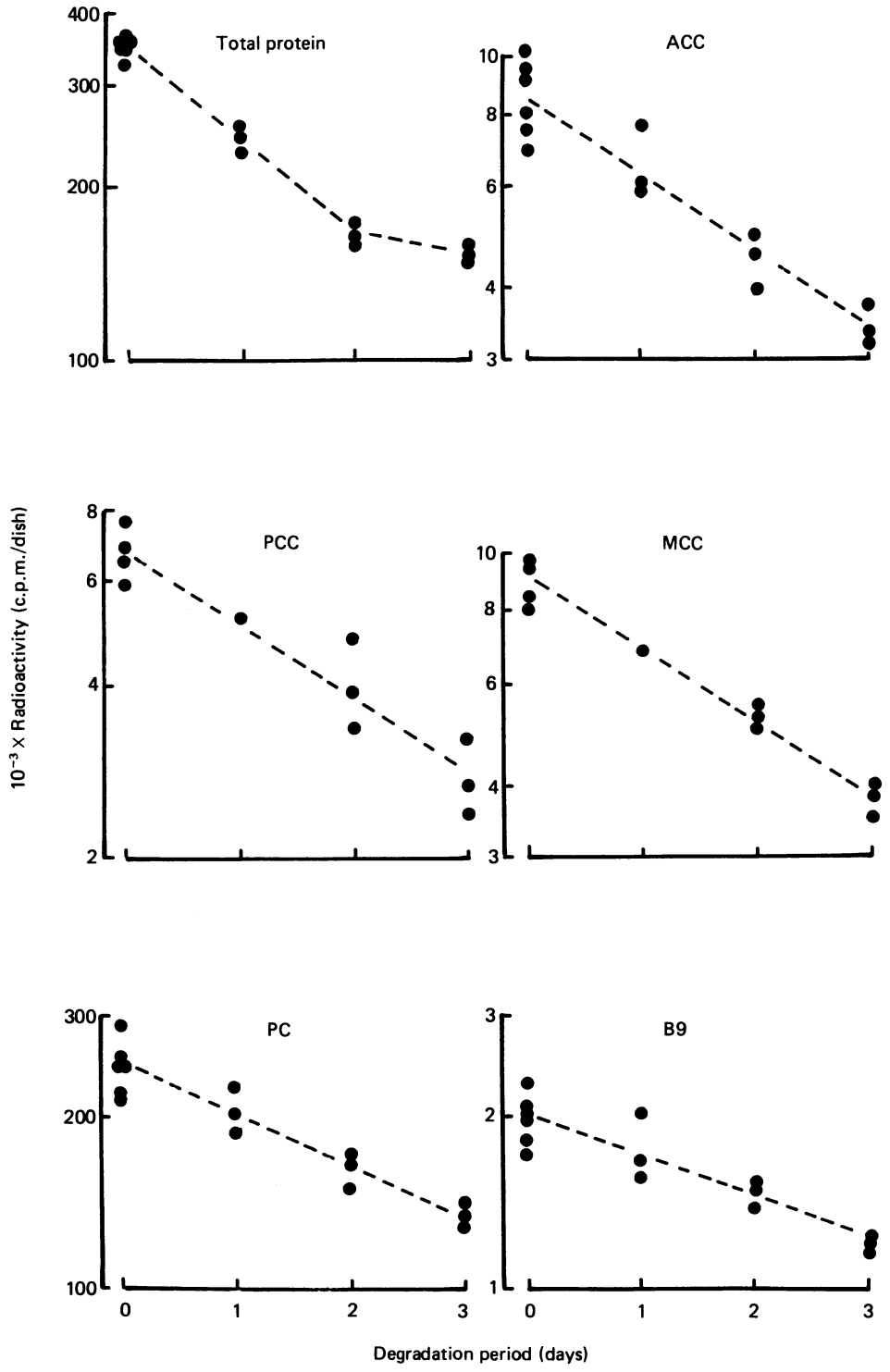

Fig. 2. Degradation of labelled proteins

Cells were labelled with [^{3}H]leucine or [^{3}H]biotin and harvested after the indicated period of incubation in control media. Points represent the radioactivities in individual proteins from single wells. The protein abbreviations are given in the legend to Fig. 1.

crotonyl-CoA carboxylase, the addition of leupeptin plus ammonia inhibited degradation by approximately the same extent as found with 3-methyladenine, thus supporting the tentative conclusion made above that lysosomal proteolysis accounts for about half of the measured proteolysis.

Propionyl-CoA carboxylase, methylcrotonyl-CoA carboxylase and pyruvate carboxylase, as well as band 9, are all localized in the mitochondria of Swiss 3T3 cells (Chandler & Ballard, 1986). It is noteworthy that among these proteins, band 9 has both the slowest degradation rate and is the one that is inhibited to the greatest extent

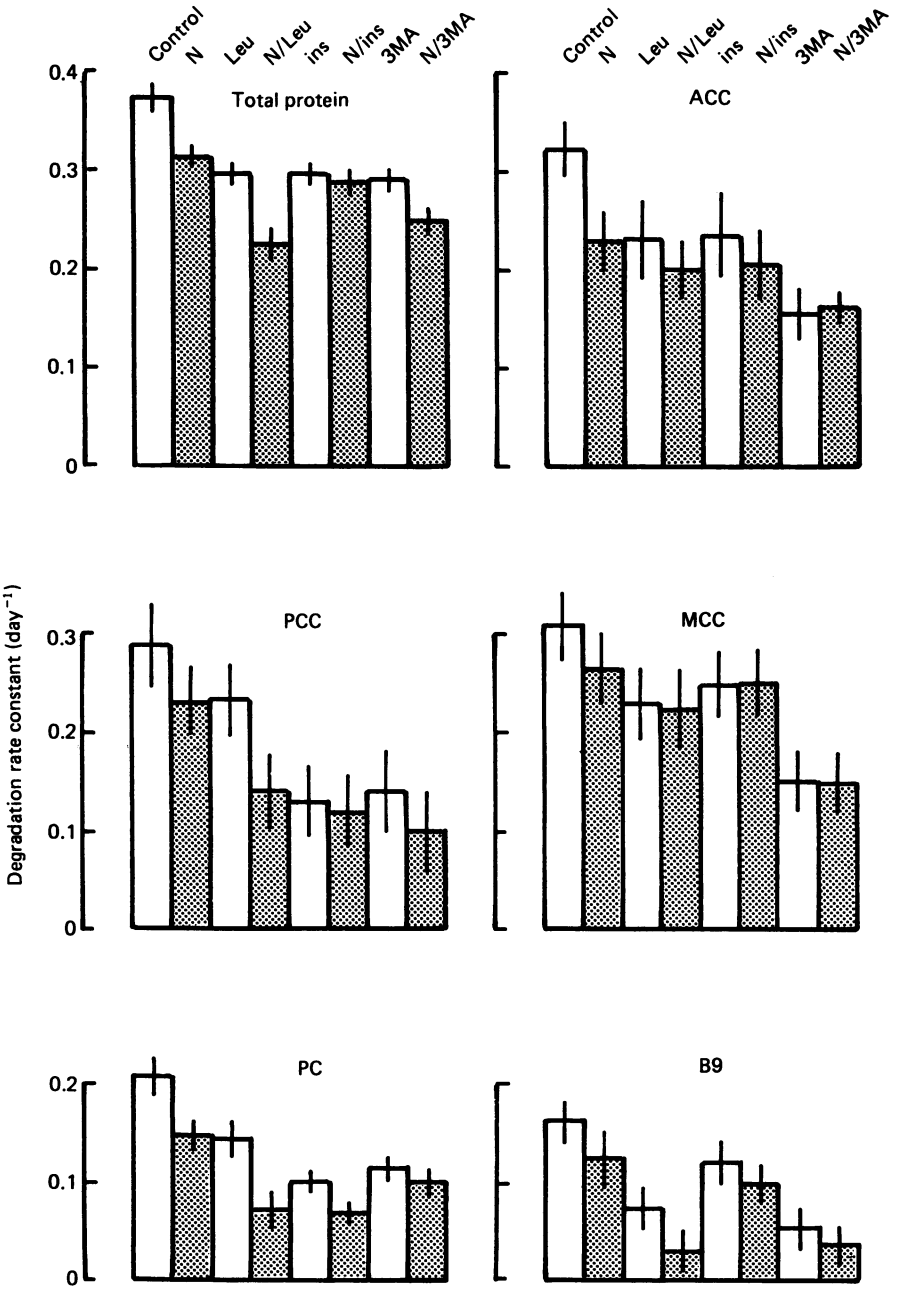

Fig. 3. Degradation rate constants for leucine-labelled total cell protein and the individual biotin-containing proteins

Values were determined from wells harvested after 0-, 1-, 2- and 3-day periods in control incubations, incubations in the presence of 5 mM-NH_4Cl (N), 50 μg of leupeptin/ml (Leu), 1 μM-insulin (ins), 10 mM-3-methyladenine (3MA) or incubations with combinations of NH_4Cl and the other inhibitors. The measurement period was continued for only 2 days with leucine-labelled proteins. Variability is indicated by bars representing S.E.M. The protein abbreviations are given in the legend to Fig. 1.

by either the leupeptin/ammonia or the 3-methyladenine/ammonia mixtures. We interpret these findings as evidence that the more-slowly-degraded protein is predominantly broken down when whole mitochondria are destroyed by autophagic proteolysis. The corollary of this conclusion is that proteins with short half-lives are more susceptible to degradation *in situ* by mitochondrial proteinases. Such proteinases probably account for the large spread of degradation rate constants reported for mitochondrial proteins (Grisolia *et al.*, 1981; Hare & Hodges, 1982).

Generation of degradation products from biotin-containing proteins

Autoradiography after separation of biotin-labelled cell proteins showed complex band patterns, with several changes evident during the degradation period (Fig. 4). Particularly noticeable are the increases with time in band 4B in all treatment wells and the increases in band 13 after leupeptin treatment. Radioactivities in the peptides corresponding to bands 4B, 8, 8B and 13 were quantified from gradient gels at zero time and after 1, 2

and 3 days of the degradation measurement. A similar procedure was adopted for band 5C, except that the radioactivities in this band were obtained from 8% gels, which permitted superior separation of band 5C from propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase. The values at 3 days, are presented, as percentages of the zero-time radioactivity, in Table 2. It should be stressed that the explanations provided in the following paragraphs concerning the generation and removal of each of the bands are based on the assumption that a maximum of three proteolytic processes are involved: (i) autophagy, followed by lysosomal breakdown; (ii) a non-lysosomal, non-mitochondrial, pathway; and (iii) intra-mitochondrial proteolysis. If a more complex combination of proteolytic pathways occurs, such as with the inclusion of lysosomal proteolysis without autophagy, the explanation could differ.

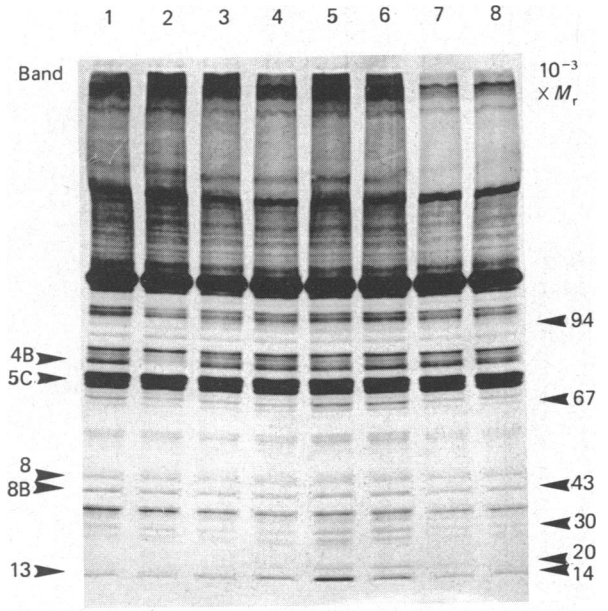

Fig. 4. Autoradiograph of biotin-containing proteins obtained from cells incubated in the presence of different inhibitors

Equal portions of cell extracts were analysed from each well. The lanes represent the following additions during the 2-day degradation period: 1, 5 mM-NH_4Cl plus 10 mM-3-methyladenine; 3, no additions; 4, 5 mM-NH_4Cl; 5, 50 μg of leupeptin/ml; 6, 5 mM-NH_4Cl plus 50 μg of leupeptin/ml; 7, 1 μM-insulin; 8, 5 mM-NH_4Cl plus 1 μM-insulin. Lane 2 represents the 0-day control. The numbered bands are referred to in the text. Other details are given in the legend to Fig. 1.

Radioactivity in band 4B increased 2–4-fold in all wells over the first 24 h period and then remained relatively constant. Since the effect was not altered by the autophagy inhibitors 3-methyladenine or insulin, the accumulation of this peptide probably reflects a proteolytic event independent of autophagy rather than one before or after autophagic sequestration. Moreover, as this peptide is localized in mitochondria (Chandler & Ballard, 1986) and is larger than either methylcrotonyl-CoA carboxylase or propionyl-CoA carboxylase, its generation is presumably via an intramitochondrial cleavage of pyruvate carboxylase.

Radioactivity in band 5C was decreased during incubation of control cultures, but was substantially increased by insulin, and its breakdown was prevented by 3-methyladenine. The lysosomotropic inhibitors leupeptin or ammonia had little effect. Clearly the proteolytic generation of this band must have occurred before the formation of autophagic vacuoles, with the removal of the peptide blocked when autophagy is restricted. The minimal effect of ammonia or leupeptin on band 5C radioactivity suggests that, after autophagy, this peptide is cleaved by a mechanism that is not inhibited by an increased pH or by an inhibition of cysteine proteinases. One of the mitochondrial carboxylases must be the source of this peptide, because it too is mitochondrial (Chandler & Ballard, 1986).

Changes in radioactivity in bands 8 and 8B follow a similar pattern, with an increase, as compared with controls, in the presence of all agents. Such results are best explained by the generation of these peptides in mitochondria before autophagy, with the peptides subsequently degraded within autophagic vacuoles.

A different explanation holds for the changes in band 13, since only leupeptin or possibly ammonia addition led to increases in radioactivity in this band. It seems likely that the peptide and others between 14 kDa and 33 kDa are produced in lysosomes at a step after the autophagic process. Also, the amount of radioactivity that

Table 2. Changes in the radioactivity in peptide bands over the degradation period

Bands are numbered according to Fig. 4. The radioactivity in each band per well was expressed as a percentage relative to the mean radioactivity at zero time. These values are presented as means ± S.E.M. for three wells.

	Radioactivity (%)				
Treatment Band ...	4B	5C	8	8B	13
Control	218±14	74±5	68±4	64±5	99±3
NH_4Cl (5 mM)	210±17	83±2	73±4	74±4	107±1
Leupeptin (50 μg/ml)	230±12	88±5	102±3	120±6	574±16
NH_4Cl/leupeptin	229±15	103±5	115±2	141±11	490±80
Insulin (1 μM)	248±19	158±8	83±1	89±2	87±5
NH_4Cl/insulin	265±27	152±9	87±7	92±7	106±14
3-Methyladenine (10 mM)	274±16	106±7	100±4	115±3	102±6
NH_4Cl/3-methyladenine	292±22	106±7	111±6	118±7	104±12

accumulates in band 13 in the presence of leupeptin over the degradation period is greater than that lost from both methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase. Hence pyruvate carboxylase must be at least a partial source of this peptide. It is interesting that leupeptin alone led to more radioactivity accumulating in band 13 than when ammonia was also present, a difference particularly noticeable on day 1 of the degradation period, when the radioactivities as percentages of zero-time values were 244 and 134 respectively. Perhaps the inclusion of ammonia decreased the production of band-13 precursors by inhibiting lysosomal cathepsins that are not susceptible to leupeptin. This explanation predicts that the cysteine proteinases inhibited by leupeptin account for later proteolytic steps in the breakdown of biotin-containing proteins.

The experimental approach used here to investigate the locations and mechanisms involved in the breakdown of intracellular proteins has had very limited application elsewhere. Breakdown products of aldolase, phosphorylase and phosphofructokinase have been identified by Western blotting and immunochemical reactivity (Reznick *et al.*, 1985; Beynon *et al.*, 1986; Toda & Ohashi, 1986), but the mechanisms responsible for the production of the peptide intermediates are unknown. Microinjection of labelled proteins would seem to provide an ideal method for the study of protein breakdown, provided that the proteins introduced into cells mix completely with their endogenous counterparts. This technique has demonstrated differences between proteins in their susceptibility to autophagic proteolysis (Bigelow *et al.*, 1981; Katznelson & Kulka, 1983; Rote & Rechsteiner, 1983; Slot *et al.*, 1986), the effects of protein modification before microinjection (Backer *et al.*, 1983; Dice *et al.*, 1986; Rogers & Rechsteiner, 1986) and the transfer of microinjected proteins out of the cytosol fraction (Kreis *et al.*, 1979; Rogers & Rechsteiner, 1986; Mayer *et al.*, 1986; Hopgood *et al.*, 1986), but intermediate products in the catabolic process have not yet been investigated.

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