Inhibition of pyruvate carboxylase degradation and total protein breakdown by lysosomotropic agents in 3T3-L1 cells

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(Received 13 September 1982/Accepted 24 November 1982)

1. Exposure to [^3H]biotin during the differentiation of 3T3-L1 cells to adipocytes selectively labelled pyruvate carboxylase (EC 6.4.1.1). A subsequent incubation of labelled cells permitted the measurement of the degradation rate constant of this mitochondrial enzyme. 2. In medium without serum, pyruvate carboxylase was degraded with a half-life of 64 h, considerably longer than that found for average cell protein. The long half-life is commensurate with the enzyme being catabolized when whole mitochondria are destroyed. 3. The breakdown of pyruvate carboxylase was inhibited to a greater extent than the breakdown of total cell protein by insulin, NH_4Cl and inhibitors of lysosomal proteinases, suggesting that the enzyme is degraded by the autophagic lysosomal system of the cell. 4. The above evidence implies that whole mitochondria are degraded in lysosomes, a conclusion that agrees with earlier electron-microscopic evidence showing mitochondria within autophagic vacuoles. 5. A second degradative pathway must be invoked to account for the breakdown of mitochondrial proteins of short half-life.

Between 20% and 50% of the breakdown of protein that has been labelled during a long exposure of cells to a radioactive amino acid is prevented by the incubation of cells in the presence of lysosomal inhibitors (Knowles & Ballard, 1976; Ballard, 1977; Amenta & Sargus, 1979). The degree of inhibition is dependent on the cell type, the control medium used and the lysosomotropic agent being investigated. Nevertheless substantial inhibition can be demonstrated by the addition of insulin or growth factors, which appear to prevent the formation of autophagic vacuoles (Pfeifer, 1977), as well as by proteinase inhibitors or weak bases, which inhibit proteolysis but not the engulfment process (Amenta & Brocher, 1980; Seglen *et al.*, 1981). Cycloheximide and other inhibitors of protein synthesis also decrease rates of protein breakdown (Knowles & Ballard, 1976), possibly by a mechanism similar to that described for insulin.

Although mitochondria are frequently seen inside autophagic vacuoles in electron micrographs of cells undergoing autophagy (Ashford & Porter, 1962; Ericsson, 1969; Arstila *et al.*, 1972; Marzella *et al.*, 1980), there is no direct biochemical evidence that the breakdown of mitochondrial protein is lysosomal. If degradation of mitochondria within lysosomes accounts for a substantial proportion of the breakdown of mitochondrial proteins, the overall

rate of degradation should be regulated in a similar manner to the catabolism of total cell protein. We have approached this hypothesis by measuring the degradation of a normally long-lived mitochondrial protein, pyruvate carboxylase, in 3T3-L1 adipocytes. Our results show that the breakdown of this enzyme is inhibited to a greater extent than the breakdown of total cell proteins by lysosomotropic agents, thus implicating lysosomes in the degradation of pyruvate carboxylase.

Experimental

Materials

3T3-L1 cells were obtained from the American Type Culture Collection, Washington, DC, U.S.A. Foetal-calf serum was purchased from Flow Laboratories, Annandale, N.S.W., Australia. Portions were dialysed exhaustively against biotin-free Dulbecco-modified Eagle's Minimal Essential Medium, and filter sterilized. All media were prepared from analytical-grade components. L-[4,5- ^3H]Leucine (40–60 Ci/mmol) and [8,9- ^3H]biotin (38 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A. Avidin was obtained from Sigma Chemical Co., St. Louis, MO, U.S.A., and CNBr-activated Sepharose 4B from Pharmacia, Uppsala, Sweden. Purified sheep liver pyruvate carboxylase and

purified ^{14}C -labelled chicken liver pyruvate carboxylase were generously provided by Dr. J. C. Wallace, Department of Biochemistry, University of Adelaide. The sources for all other chemicals have been described previously (Hopgood *et al.*, 1977, 1980).

Growth of cells

Cells were subcultured by using a 1:20 split ratio into Costar 24-place multiwell dishes and grown in Dulbecco-modified Eagle's Minimal Essential Medium containing 5% foetal-calf serum and $0.25\mu\text{M}$ -insulin as well as 2 mg of biotin, 60 mg of penicillin G, 100 mg of streptomycin sulphate, 90 mg of gentamycin and 1 mg of fungizone per litre of medium (growth medium); 1 ml of medium was used per well, with changes every second day. For this and all subsequent incubations, the temperature was 37°C and the atmosphere 5% CO_2 in humidified air. When monolayers were confluent, the medium was replaced with a similar medium but containing 0.5 mM -isobutylmethylxanthine in order to initiate differentiation of the cells into adipocytes. After 2 days this medium was replaced with growth medium and the monolayers were left for a further 2–3 days before transfer of the cells to biotin-free medium (see below). At this stage most cells contained a few fat droplets, as reported previously by Green & Kehinde (1974).

Measurement of the degradation of biotinyl-proteins

The apoenzymes corresponding to biotin-containing holoenzymes were induced in 3T3-L1 cells by continuing the differentiation process for a further 48 h in the presence of biotin-free growth medium. $[^3\text{H}]$ Biotin ($0.1\mu\text{Ci/ml}$) was present for the last 42 h. The leucine concentration of this medium was decreased to $100\mu\text{M}$ to facilitate comparison with proteins labelled with $[^3\text{H}]$ leucine, which would be used for measurements of total protein breakdown. During the 48 h period the cells accumulated large amounts of lipid and showed a 4-fold or greater increase in the activity of pyruvate carboxylase. The cell monolayers were subsequently washed twice with Eagle's Minimal Essential Medium and incubated for 2 h in Dulbecco-modified Eagle's Minimal Essential Medium containing $100\mu\text{M}$ -biotin, 2 mM -leucine and 10% foetal-calf serum (chase medium). These chase conditions were chosen to increase the cellular biotin and leucine pools, to ensure that any labile proteins were degraded and to remove any residual insulin.

The degradation medium added to each monolayer was 1 ml of chase medium plus effectors, with serum omitted unless indicated. Degradation of biotin-containing proteins was measured after 6 or 24 h (or otherwise as indicated) as described previously for total cell protein (Ballard *et al.*, 1980a).

Measurement of total protein breakdown

The procedures adopted were exactly as described above for biotin-containing proteins, except that the 48 h preincubation plus labelling period was divided into 32 h preincubation and 16 h labelling in the presence of $1\mu\text{Ci}$ of $[^3\text{H}]$ leucine/ml of medium.

Calculations

Total protein breakdown and the degradation of biotin-containing proteins were calculated as the percentage breakdown of labelled protein, which is 100 times the radioactivity in the trichloroacetic acid-soluble fraction of the medium divided by the sum of the radioactivities in the dissolved cell monolayer (cell proteins), trichloroacetic acid-soluble fraction of the medium (medium amino acids) and trichloroacetic acid-insoluble fraction of the medium (medium protein). Control experiments showed negligible radioactivity in trichloroacetic acid-soluble fractions of the cell monolayer in studies with either total protein or biotin-containing protein. Accordingly the radioactivity in the monolayers after removal of medium could be considered as exclusively cell protein.

Assays for biotin-containing carboxylases

Cells were removed from dishes by scraping in a solution containing 50 mM-Tris, 5 mM- MgSO_4 , 5 mM-ATP, 0.5 mM-EDTA and 0.5% Triton X-100 that had been adjusted to pH 7.0. This extract was homogenized and frozen and thawed twice. Pyruvate carboxylase was assayed as the rate of $\text{NaH}^{14}\text{CO}_3$ incorporation at 37°C , by the method described by Ballard & Hanson (1967). Similar assays were used for the measurement of acetyl-CoA carboxylase and propionyl-CoA carboxylase, but with 10 mM-sodium citrate replacing pyruvate in the former and 1 mM-propionyl-CoA replacing acetyl-CoA in the latter reaction mixture. Malate dehydrogenase (6 units) and NADH (0.75 mM) were included in the pyruvate carboxylase assay to convert oxaloacetate into malate (Mackall & Lane, 1977). Reactions were stopped after 10, 20 and 30 min by the addition of trichloroacetic acid to give a final concentration of 10% (w/v). The inactivated solutions were either gassed with CO_2 or dried at 90°C before the measurement of incorporated radioactivity. Enzyme activities were expressed as nmol of bicarbonate fixed/min per mg of protein.

Polyacrylamide-gel electrophoresis

Cells previously exposed to $[^3\text{H}]$ biotin were removed from dishes by scraping in Dulbecco's phosphate-buffered saline containing 5 mM-EDTA, pH 7.5, collected by centrifugation and dissolved in sodium dodecyl sulphate dissociating solution (Palmiter *et al.*, 1971). Portions of these solutions were

analysed by electrophoresis in either tube or slab gels (Laemmli, 1970). Sheep liver pyruvate carboxylase, ^{14}C -labelled chicken liver pyruvate carboxylase and the Pharmacia high-molecular-weight protein calibration kit were used as reference proteins. The acrylamide concentration was 5%. After electrophoresis, tube gels were stained with Coomassie Blue, and the radioactivity was determined as described previously (Hopgood *et al.*, 1973). Slab gels were stained with Coomassie Blue, photographed and radioactivity was detected by scintillation autoradiography (Bonner & Laskey, 1974).

Protein measurements

The protein content of monolayers dissolved in solutions containing Triton X-100 was determined by an Auto Analyser adaptation of the method described by Dulley & Grieve (1975).

Results and discussion

Identification of the biotin-labelled protein as pyruvate carboxylase

We have noted that deposition of lipid in the 3T3-L1 cell line is accompanied by an increase in the activity of pyruvate carboxylase from 20 nmol/min per mg of protein before the addition of isobutylmethylxanthine to approx. 200 nmol/min per mg at the beginning of the degradation measurements. This change is in accord with published values (Mackall & Lane, 1977; Freytag & Utter, 1980). Measurements on extracts of differentiated cells show propionyl-CoA carboxylase and acetyl-CoA carboxylase activities of 10 and 2 nmol/min per mg of protein respectively. The latter activity is about half that reported by Mackall *et al.* (1976). The expected relative proportions of biotin incorporated into pyruvate carboxylase, propionyl-CoA carboxylase and acetyl-CoA carboxylase can be calculated from the cell contents of the three proteins if it is assumed that all of the enzyme molecules are synthesized *de novo* during differentiation of 3T3-L1 cells, provided that information is available about the specific activity of each protein at purity and the molecular weight of each polypeptide associated with a single biotin molecule. Specific activities of 68, 29 and 26 μmol of bicarbonate fixed/min per mg of protein at purity and at 37°C for pyruvate carboxylase, propionyl-CoA carboxylase and acetyl-CoA carboxylase respectively have been assumed (D. B. Keech, personal communication; Gregolin *et al.*, 1968). Also 1 pmol of biotin has been taken as being associated with 120 000 pg of pyruvate carboxylase, 230 000 pg of acetyl-CoA carboxylase (Wood & Barden, 1977) and 130 000 pg of propionyl-CoA carboxylase, even though the molecular weight of the actual biotin-containing subunit of this last enzyme is 72 000 (Lau

et al., 1979). Use of these values permits the calculation that, for each mg of protein in differentiated 3T3-L1 cells, there should be approx. 24 pmol of biotin in pyruvate carboxylase, 2.6 pmol in propionyl-CoA carboxylase and 0.65 pmol in acetyl-CoA carboxylase.

Electrophoresis of 3T3-L1 cell protein under dissociating conditions after prelabelling with [^3H]-biotin shows two radioactive peaks corresponding to subunit molecular weights of 120 000 and 75 000 (Fig. 1). The former band accounts for 80–85% of the radioactivity applied to the gel and the latter band 10–15%. The peak of higher molecular weight co-electrophoresed with pure sheep liver pyruvate carboxylase (Fig. 1a), a protein with a subunit molecular weight slightly less than that of the mammalian enzyme (J. C. Wallace, personal communication).

Labelled cell lysates were also chromatographed on slab gels and processed for scintillation autoradiography (Bonner & Laskey, 1974). Autoradiograms showed a single band, which corresponded in position and shape to one stained with Coomassie Blue (results not shown). The minor radioactive peak evident on tube gels was not detected. Inclusion of sheep liver pyruvate carboxylase with cell lysate increased the staining intensity of the radioactive band. These electrophoretic results, together with the calculations of the expected incorporation of biotin into the various biotin-containing proteins in 3T3-L1 cells, strongly suggest that the major radioactive band is indeed pyruvate carboxylase. Our results, showing essentially all the biotin radioactivity associated with the pyruvate carboxylase band, agree with earlier investigations by Freytag & Utter (1980), who found that virtually all the radioactivity incorporated after incubation of 3T3-L1 cells with ^{14}C -labelled biotin was precipitated by an anti-(pyruvate carboxylase) antibody. We have subsequently referred to biotin-labelled protein as pyruvate carboxylase.

Pyruvate carboxylase is located exclusively in the mitochondrial matrix in liver (Böttger *et al.*, 1969; Marco *et al.*, 1969) and probably has a similar location in adipose tissue (Hanson & Ballard, 1967). The 10–20-fold induction of pyruvate carboxylase on differentiation of 3T3-L1 cells (Mackall & Lane, 1977; Freytag & Utter, 1980) likewise represents an accumulation of the enzyme within mitochondria, as shown by immunofluorescent studies (Gratzner *et al.*, 1980). The response is selective, since most mitochondrial enzymes increase only slightly during the transition of 3T3-L1 fibroblasts to lipid-containing cells (Mackall & Lane, 1977).

Degradation of pyruvate carboxylase

In order for the release of trichloroacetic acid-soluble radioactivity to be a measure of the

Fig. 1. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of proteins labelled with $[^3\text{H}]$ biotin in 3T3-L1 cells

Labelled protein was prepared by incubating cells in the presence of $[^3\text{H}]$ biotin, dissolved in sodium dodecyl sulphate dissociating solution and mixed with ^{14}C -labelled chicken pyruvate carboxylase before being electrophoresed on 5%-acrylamide tube gels. Gel slices, each 1.5 mm thick, were numbered from the top of the gel. ●, ^3H ; ○, ^{14}C . Further details are given in the Experimental section. (a) Cells at the beginning of the degradation period; (b) cells after 24 h incubation in Dulbecco-modified Eagle's Minimal Essential medium containing 10% foetal-calf serum plus $100\ \mu\text{M}$ unlabelled biotin.

breakdown of pyruvate carboxylase, it is essential that the biotin remains attached to the enzyme until degradation is complete. Biotin is bound in covalent linkage to the ϵ -amino group of a lysine residue as biocytin (Moss & Lane, 1971). Although biocytin can be cleaved by biotinidase to release biotin, this enzyme is unable to act on native or denatured biotin-containing enzymes (Koivusalo *et al.*, 1963).

Fig. 2. Percentage degradation of biotin-labelled protein (○) and leucine-labelled protein (●) in 3T3-L1 cells. After labelling with $[^3\text{H}]$ biotin or $[^3\text{H}]$ leucine, cells were incubated for 3 h before the indicated measurement period. Serum-free medium (chase medium) was used. Further details are given in the Experimental section. Values are means $\pm$ S.E.M. for four determinations at each time.

It is not clear whether partial degradation products of pyruvate carboxylase are substrates for biotinidase or whether free biocytin must first be produced by complete hydrolysis of the protein. This distinction would only become important in the present context if substantial quantities of pyruvate carboxylase breakdown products accumulated within 3T3-L1 cells.

Incubation of prelabelled 3T3-L1 cells for 24 h leads to loss of approx. 20% of the radioactivity from the pyruvate carboxylase band (Fig. 1b). Radioactivity in the minor band also appears to be decreased under these conditions. Essentially all the radioactivity released into the medium in this degradation experiment was trichloroacetic acid-soluble and was retained on an avidin-Sepharese affinity column (Swack *et al.*, 1978), properties that are consistent with the labelled breakdown product being free biotin or biocytin.

The catabolism of biotin-labelled protein in 3T3-L1 cells shows a single first-order rate constant, with a half-life ($t_{1/2}$) of 64 h (Fig. 2). On the other hand, total cell protein labelled with $[^3\text{H}]$ -leucine gives a complex decay curve, as expected for a mixture of proteins with different degradation rate constants.

The addition of millimolar concentrations of leucine to the incubation medium for degradation experiments appears to give adequate chase conditions for several cell lines (Ballard, 1982; Ballard & Francis, 1983), even though other studies indicate lack of equilibration between extracellular amino acids and amino acids attached to tRNA (Airhart *et al.*, 1981; Hildebran *et al.*, 1981; Schneible *et al.*, 1981). The experiment shown in Fig. 3 provides evidence that labelled biotin produced during the degradation of pyruvate carboxylase is released into the medium and is not bound to new apoenzyme. Thus increasing the biotin concentration in the medium does not alter the apparent rate of enzyme breakdown. Whereas reutilization of labelled biotin might have been expected at very low biotin concentrations, some biotin would be present even when none was added at the beginning of the 6 h degradation experiment, because the cells had previously been exposed to 100 μ M-biotin during the 2 h chase period.

Reutilization of labelled biotin released during protein breakdown can also be prevented by the addition of a protein-synthesis inhibitor. The inclusion of 0.1 mM-cycloheximide was found to depress the incorporation of [³H]biotin into pyruvate carboxylase by 97% over the 6 h period used in

Fig. 3. Percentage degradation of pyruvate carboxylase over a 6 h period in the presence of different biotin concentrations

Pyruvate carboxylase degradation was measured after incubating labelled cells in Dulbecco-modified Eagle's Minimal Essential Medium containing 2 mM-leucine in the absence (●) or presence (○) of 0.1 mM-cycloheximide. Further details are given in the Experimental section. Values are means $\pm$ S.E.M. for three determinations at each indicated biotin concentration.

the degradation experiment shown in Fig. 3. The cycloheximide produced a modest inhibition of pyruvate carboxylase catabolism that was not dependent on the concentration of biotin (Fig. 3). We interpret this cycloheximide experiment as evidence that the 100 μ M-biotin normally added during degradation studies was sufficient to prevent reutilization of isotope. Otherwise the addition of cycloheximide should have increased rather than decreased the measured degradation rate.

Regulation of total protein breakdown and pyruvate carboxylase breakdown

The addition of 10% foetal-calf serum or high concentrations of insulin inhibits total protein breakdown in 3T3-L1 cells when the measurements are made over 6 or 24 h (Table 1). These effects are comparable with those reported previously on other cell lines (Ballard *et al.*, 1980b; Ballard, 1982). Although a similar inhibition of pyruvate carboxylase degradation is found after addition of insulin, foetal-calf serum actually increases the degradation rate of the enzyme when the measurement is over 24 h (Table 1). We have noted a stimulation of total protein breakdown by serum in studies lasting longer than 18 h with L6 myoblasts (Ballard, 1982), but the effect observed in Table 1 differs because it is selective for pyruvate carboxylase. One possible explanation of the finding would be the selective enhancement by serum of the viability of biotin-labelled cells as compared with leucine-labelled cells. We routinely measure release of labelled protein into the medium during degradation experiments, because this provides an index of cell loss from the monolayer or cell destruction. In the present series of experiments, this labelled medium protein accounted for approx. 5% and 10% of the total radioactivity in the 6 h and 24 h

Table 1. Effects of insulin and foetal-calf serum on total protein breakdown and the degradation of pyruvate carboxylase

Degradation was measured in chase medium (control) with the indicated additions. Values are means $\pm$ S.E.M. for six determinations. The numbers in parentheses are the percentage changes from control rates of degradation.

Treatment	Incubation period (h)	Percentage degradation of	
		Total protein	Pyruvate carboxylase
Control	6	14.64 $\pm$ 0.20	6.66 $\pm$ 0.27
Insulin (1 μ M)	6	10.87 $\pm$ 0.26 (–26)	5.09 $\pm$ 0.30 (–24)
Serum (10%)	6	10.78 $\pm$ 0.21 (–26)	6.59 $\pm$ 0.60 (–1)
Control	24	40.9 $\pm$ 0.2	21.7 $\pm$ 1.0
Insulin (1 μ M)	24	36.0 $\pm$ 0.6 (–12)	19.2 $\pm$ 1.0 (–12)
Serum (10%)	24	32.1 $\pm$ 0.3 (–22)	24.0 $\pm$ 0.6 (+11)

studies respectively. Since similar proportions were observed for biotin-labelled and leucine-labelled cells, and these were not altered by the addition of serum, it seems unlikely that a relative difference in cell viability could explain the altered response to foetal-calf serum.

The effects of insulin, lysosomotropic agents and cycloheximide have been tested with medium containing 10% foetal-calf serum as the reference control (Table 2). This approach was adopted because, in the absence of serum, cycloheximide leads to a very substantial loss of cell viability after 24 h, with up to 30% of the total radioactivity released into the medium as cell protein. Provided that serum is included, the 3T3-L1 cells show little extra release of labelled proteins in the presence of cycloheximide.

Insulin, leupeptin and NH_4Cl all inhibit the breakdown of pyruvate carboxylase to a greater extent than the breakdown of total protein (Table 2). Similar effects are observed when degradation is measured over 6 h or 24 h. Inhibition by cycloheximide, on the other hand, is considerably greater over the longer measurement period. This progressive increase in the inhibitory effect of cycloheximide has been noted with other cell lines (Amenta *et al.*, 1978; Seglen *et al.*, 1981) and may reflect a combination of a rapid partial inhibition of

autophagy (Kovacs & Kovacs, 1980; Kominami *et al.*, 1982), followed by a gradual decrease in the cell content of lysosomal proteinases as their resynthesis is prevented by the protein-synthesis inhibitor (Amenta *et al.*, 1978).

The concentration-dependence of serum effects on protein breakdown has been examined, as has the response produced by the addition of different NH_4Cl concentrations in the presence of 10% serum (Fig. 4). Foetal-calf serum produces a graded inhibition of the breakdown of total protein and the opposite effect on pyruvate carboxylase breakdown. The NH_4Cl -concentration curves in the presence of 10% foetal-calf serum are similar for both processes, but the percentage effect is considerably greater on the breakdown of pyruvate carboxylase at all concentrations. For example, inclusion of 20 mM- NH_4Cl produces a 70% inhibition of pyruvate carboxylate catabolism and a 45% inhibition of total protein breakdown.

The additivity of leupeptin, NH_4Cl and chymostatin effects has been evaluated on both total protein breakdown and pyruvate carboxylase degradation (Table 3). For these experiments 1% dimethyl sulphoxide was included in each well. At the high concentrations chosen, leupeptin and chymostatin inhibit protein breakdown equally and show no additivity. The catabolism of pyruvate

Table 2. Inhibition of total protein and pyruvate carboxylase degradation by lysosomotropic agents added in the presence of foetal-calf serum

Degradation was measured in chase medium containing 10% foetal-calf serum (control) with the indicated additions. Values are means $\pm$ S.E.M. for six determinations, with the percentage changes from the control rates given in parentheses.

Treatment	Incubation period (h)	Percentage degradation of	
		Total protein	Pyruvate carboxylase
Control (10% serum)	6	10.78 $\pm$ 0.21	6.59 $\pm$ 0.60
Insulin (1 μM)	6	9.53 $\pm$ 0.12 (–12)	5.30 $\pm$ 0.29 (–20)
Leupeptin (50 $\mu\text{g}/\text{ml}$)	6	9.03 $\pm$ 0.19 (–16)	4.85 $\pm$ 0.22 (–26)
NH_4Cl (5 mM)	6	8.48 $\pm$ 0.22 (–21)	2.89 $\pm$ 0.20 (–56)
Cycloheximide (0.1 mM)	6	8.52 $\pm$ 0.11 (–21)	5.04 $\pm$ 0.59 (–24)
Control (10% serum)	24	32.1 $\pm$ 0.3	24.0 $\pm$ 0.6
Insulin (1 μM)	24	29.5 $\pm$ 0.3 (–8)	19.2 $\pm$ 0.4 (–20)
Leupeptin (50 $\mu\text{g}/\text{ml}$)	24	25.3 $\pm$ 0.4 (–21)	15.3 $\pm$ 0.5 (–36)
NH_4Cl (5 mM)	24	23.5 $\pm$ 0.8 (–27)	12.7 $\pm$ 1.8 (–47)
Cycloheximide (0.1 mM)	24	17.6 $\pm$ 0.4 (–45)	11.9 $\pm$ 0.7 (–50)

Fig. 4. Effects of foetal-calf serum and NH_4Cl on the percentage breakdown of total cell proteins (●) and pyruvate carboxylase (○)

Degradation was measured over a 24 h period in 'chase medium' in the presence of the indicated concentrations of foetal-calf serum. NH_4Cl was added to the medium containing 10% serum at concentrations between 1 and 20 mM. Values are means $\pm$ S.E.M. for four determinations. Other details are given in the Experimental section.

Table 3. *Additive effects of lysosomotropic agents on total protein and pyruvate carboxylase degradation*

Degradation was measured over a 24 h period in chase medium containing 10% foetal-calf serum and 1% dimethyl sulphoxide. The concentrations of the additional agents are: leupeptin, 50 µg/ml; chymostatin, 200 µg/ml; NH₄Cl, 5 mM; pepstatin, 50 µg/ml. Values are means ± S.E.M. for four determinations, with the percentage changes from control rates given in parentheses.

Treatment	Percentage degradation of	
	Total protein	Pyruvate carboxylase
Control (10% serum, 1% dimethyl sulphoxide)	31.0 ± 0.7	14.86 ± 0.42
Leupeptin	25.1 ± 0.4 (−19)	9.79 ± 0.29 (−34)
Chymostatin	24.5 ± 0.7 (−21)	9.33 ± 0.33 (−37)
NH ₄ Cl	23.0 ± 0.2 (−26)	6.76 ± 0.33 (−55)
Pepstatin	30.9 ± 0.4 (−1)	13.47 ± 0.35 (−9)
Leupeptin, chymostatin	25.0 ± 0.5 (−19)	9.35 ± 0.39 (−37)
Leupeptin, NH ₄ Cl	20.2 ± 0.5 (−35)	5.40 ± 0.14 (−64)
Chymostatin, NH ₄ Cl	20.2 ± 0.2 (−35)	5.60 ± 0.20 (−62)
Leupeptin, chymostatin, NH ₄ Cl	20.0 ± 0.2 (−36)	4.84 ± 0.17 (−67)
Control minus dimethyl sulphoxide	30.6 ± 0.4 (−2)	20.41 ± 0.82 (+37)

carboxylase is inhibited by a greater amount than observed for total protein breakdown, but again the effects of the two microbial proteinase inhibitors are equal and are not additive. The cathepsin D inhibitor, pepstatin, did not inhibit proteolysis by a significant extent, in accord with investigations on other cell types (Dean, 1975; Knowles & Ballard, 1976). The inability of pepstatin to pass through cell membranes has been proposed to explain the lack of response to this inhibitor (Dean, 1975). NH₄Cl produces a much larger inhibition of pyruvate carboxylase degradation than of total protein breakdown (Tables 2 and 3). The effects on both processes are potentiated by the inclusion of either leupeptin or chymostatin, but little or no additional response occurs when all three inhibitors are present (Table 3).

Dimethyl sulphoxide was included in the control medium used for the experiments described in Table 3 because pepstatin and chymostatin are relatively insoluble in aqueous solutions. However, 1% dimethyl sulphoxide by itself markedly inhibited pyruvate carboxylase breakdown (Table 3). Interestingly, the effect was selective on the mitochondrial protein. Apparently the solvent decreases the accessibility of mitochondria, or at least the mitochondrial protein pyruvate carboxylase, to a protein-breakdown system in the cell.

Insulin, weak bases and the proteinase inhibitors tested here have all been shown to modify rates of protein breakdown by inhibiting the lysosomal degradative system. Thus insulin prevents the formation of autophagic vacuoles (Pfeifer, 1977; Neely *et al.*, 1977), and NH₄Cl destroys the acidic environment of lysosomes required for the action of cathepsins (Ohkuma & Poole, 1978). Leupeptin and chymostatin directly inhibit lysosomal cathepsins B, H and L (Kirschke *et al.*, 1976; Umezawa, 1977), although the action of these inhibitors is not confined to lysosomes. At least part of the effect of cycloheximide on protein breakdown occurs via the inhibition of autophagy (Kovacs & Kovacs, 1980; Kominami *et al.*, 1982). All these agents inhibit the breakdown of pyruvate carboxylase by a greater extent than the breakdown of total proteins in 3T3-L1 cells. Accordingly, we can conclude that a larger proportion of pyruvate carboxylase than of total protein is degraded within the lysosomal system. An important question to resolve is whether the enzyme passes out of mitochondria before its breakdown, or whether pyruvate carboxylase catabolism reflects the breakdown of whole mitochondria. Since we cannot find any evidence for the first possibility, we consider it most likely that pyruvate carboxylase is degraded when intact mitochondria enter autophagic vacuoles, a process frequently observed in electron micrographs (Ashford & Porter, 1962; Ericsson, 1969; Arstila *et al.*, 1972; Marzella *et al.*, 1980).

The half-life of pyruvate carboxylase in 3T3-L1 cells, at 64 h, is considerably longer than the half-life of average cell proteins (see Fig. 2). Pyruvate carboxylase has a half-life of 4.6 days in rat liver, somewhat longer than the 3.8 days observed for total mitochondrial proteins (Weinberg & Utter, 1979, 1980). This longer than average half-life for pyruvate carboxylase in both 3T3-L1 cells and liver is consistent with the relatively greater sensitivity of its catabolism to inhibition by lysosomotropic agents. Thus a number of studies have established that the breakdown of long-lived proteins is much more sensitive to inhibition by weak bases, cathepsin inhibitors or insulin than is the catabolism of short-lived proteins (Knowles *et al.*, 1975; Knowles & Ballard, 1976; Neff *et al.*, 1979; Amenta & Brocher, 1980). Since proteins of the mitochondrial matrix are known to have widely different half-lives, ranging from 70 min for δ -aminolaevulinate synthase (Marver *et al.*, 1966) to several days for many other proteins (Weinberg & Utter, 1979; Grisolia *et al.*, 1981), a process additional to destruction of whole mitochondria must exist. Proteinases have been reported in mitochondrial preparations (Lovaas, 1974; Aoki *et al.*, 1975; Haas *et al.*, 1978), but an unequivocal mitochondrial location for those enzymes has not been established. It is possible that

an intramitochondrial degradative system may be similar to those extralysosomal pathways in which susceptibility of proteins to non-proteolytic events may regulate the subsequent rate of proteolysis. For example, short-lived proteins are selectively degraded by an ATP-dependent process (Etlinger & Goldberg, 1977; Hershko *et al.*, 1979; Wilkinson *et al.*, 1980) which has been shown to be present in liver mitochondria (Desautels & Goldberg, 1982). Nevertheless any system of this type should be considered an adjunct to the basal rate of degradation, which is set by the rate of mitochondrial destruction by autophagy and is characteristic of each cell type and each nutritional, pharmacological or hormonal condition.

We thank Dr. John Wallace, Department of Biochemistry, University of Adelaide, for helpful advice throughout this investigation.

References

- Airhart, J., Arnold, J. A., Bulman, C. A. & Low, R. B. (1981) *Biochim. Biophys. Acta* **653**, 108–117
- Amenta, J. S. & Brocher, S. C. (1980) *J. Cell. Physiol.* **102**, 259–266
- Amenta, J. S. & Sargus, M. J. (1979) *Biochem. J.* **182**, 847–859
- Amenta, J. S., Sargus, M. J. & Baccino, F. M. (1978) *J. Cell. Physiol.* **97**, 267–284
- Aoki, Y., Urata, G., Takaku, F. & Katunuma, N. (1975) *Biochem. Biophys. Res. Commun.* **65**, 567–574
- Arstila, A. V., Shelburne, J. D. & Trump, B. F. (1972) *Lab. Invest.* **27**, 317–323
- Ashford, T. P. & Porter, K. R. (1962) *J. Cell Biol.* **12**, 198–202
- Ballard, F. J. (1977) *Essays Biochem.* **13**, 1–37
- Ballard, F. J. (1982) *Biochem. J.* **208**, 275–287
- Ballard, F. J. & Francis, G. L. (1983) *Biochem. J.* **210**, 243–249
- Ballard, F. J. & Hanson, R. W. (1967) *Biochem. J.* **104**, 866–871
- Ballard, F. J., Wong, S. S. C., Knowles, S. E., Partridge, N. C., Martin, T. J., Wood, C. M. & Gunn, J. M. (1980a) *J. Cell. Physiol.* **105**, 335–346
- Ballard, F. J., Knowles, S. E., Wong, S. S. C., Bodner, J. B., Wood, C. M. & Gunn, J. M. (1980b) *FEBS Lett.* **114**, 209–212
- Bonner, W. M. & Laskey, R. A. (1974) *Eur. J. Biochem.* **46**, 83–88
- Böttger, I., Wieland, O., Brdiczka, D. & Pette, D. (1969) *Eur. J. Biochem.* **8**, 113–119
- Dean, R. T. (1975) *Nature (London)* **257**, 414–416
- Desautels, M. & Goldberg, A. L. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 1869–1873
- Dulley, J. R. & Grieve, P. A. (1975) *Anal. Biochem.* **64**, 136–141
- Ericsson, J. L. (1969) in *Lysosomes in Biology and Pathology*, vol. 2 (Dingle, J. T. & Fell, H. B., eds.), pp. 345–394, North-Holland, London
- Etlinger, J. D. & Goldberg, A. L. (1977) *Proc. Natl. Acad. Sci. U.S.A.* **74**, 54–58
- Freytag, S. O. & Utter, M. F. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1321–1325
- Gratzner, H. G., Ahmad, P. M., Zegadlio, J. & Ahmad, F. (1980) *Cell Biol. Int. Rep.* **4**, 497–508
- Green, H. E. & Kehinde, O. (1974) *Cell* **1**, 113–116
- Gregolin, C., Ryder, E. & Lane, M. D. (1968) *J. Biol. Chem.* **243**, 4227–4235
- Grisolia, S., Timoneda, J., Hernandez-Yago, J., Soler, J., de Arriaga, M. D. & Wallace, R. (1981) *Acta Biol. Med. Germ.* **40**, 1407–1418
- Haas, R., Heinrich, P. C., Tesch, R. & Witt, I. (1978) *Biochem. Biophys. Res. Commun.* **85**, 1039–1046
- Hanson, R. W. & Ballard, F. J. (1967) *Biochem. J.* **105**, 529–536
- Hershko, A., Ciechanover, A. & Rose, I. A. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 3107–3110
- Hildebran, J. N., Airhart, J., Stirewalt, W. S. & Low, R. B. (1981) *Biochem. J.* **198**, 249–258
- Hopgood, M. F., Ballard, F. J., Reshef, L. & Hanson, R. W. (1973) *Biochem. J.* **134**, 445–453
- Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1977) *Biochem. J.* **164**, 399–407
- Hopgood, M. F., Clark, M. G. & Ballard, F. J. (1980) *Biochem. J.* **186**, 71–79
- Kirschke, H., Langner, J., Wiederanders, B., Ansoerge, S., Bohley, P. & Broghammer, U. (1976) *Acta Biol. Med. Germ.* **35**, 285–299
- Knowles, S. E. & Ballard, F. J. (1976) *Biochem. J.* **156**, 609–617
- Knowles, S. E., Gunn, J. M., Hanson, R. W. & Ballard, F. J. (1975) *Biochem. J.* **146**, 595–600
- Koivusalo, M., Elorriaga, C., Kaziro, Y. & Ochoa, S. (1963) *J. Biol. Chem.* **238**, 1038–1042
- Kominami, E., Hashida, E., Khairallah, E. A. & Katunuma, N. (1982) *J. Biol. Chem.* in the press
- Kovacs, A. L. & Kovacs, J. (1980) *Virchows Arch. B* **32**, 97–104
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lau, E. P., Cochran, B. C., Munson, L. & Fall, R. R. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 214–218
- Lovaas, E. (1974) *FEBS Lett.* **45**, 244–247
- Mackall, J. C. & Lane, M. D. (1977) *Biochem. Biophys. Res. Commun.* **79**, 720–725
- Mackall, J. C., Student, A. K., Polakis, S. E. & Lane, M. D. (1976) *J. Biol. Chem.* **251**, 6462–6464
- Marco, R., Sebastian, J. & Sols, A. (1969) *Biochem. Biophys. Res. Commun.* **34**, 725–730
- Marver, H. S., Collins, A., Tschudy, D. P. & Rechcigl, M., Jr. (1966) *J. Biol. Chem.* **241**, 4323–4329
- Marzella, L., Sandberg, P.-O. & Glaumann, H. (1980) *Exp. Cell Res.* **128**, 291–301
- Moss, J. & Lane, M. D. (1971) *Adv. Enzymol.* **35**, 321–442
- Neely, A. N., Cox, J. R., Fortney, J. A., Schworer, C. M. & Mortimore, G. E. (1977) *J. Biol. Chem.* **252**, 6948–6954
- Neff, N. T., DeMartino, G. N. & Goldberg, A. L. (1979) *J. Cell. Physiol.* **101**, 439–458
- Ohkuma, S. & Poole, B. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 3327–3331

- Palmiter, R. D., Oka, Y. & Schimke, R. T. (1971) *J. Biol. Chem.* **246**, 724–737
- Pfeifer, U. (1977) *Acta Biol. Med. Germ.* **36**, 1691–1694
- Schneible, P. A., Airhart, J. & Low, R. B. (1981) *J. Biol. Chem.* **256**, 4888–4894
- Seglen, P. O., Gordon, P. B., Grinde, B., Solheim, A., Kovacs, A. L. & Poli, A. (1981) *Acta Biol. Med. Germ.* **40**, 1587–1598
- Swack, J. A., Zander, G. L. & Utter, M. F. (1978) *Anal. Biochem.* **87**, 114–126
- Umezawa, H. (1977) *Acta Biol. Med. Germ.* **36**, 1899–1915
- Weinberg, M. B. & Utter, M. F. (1979) *J. Biol. Chem.* **254**, 9429–9499
- Weinberg, M. B. & Utter, M. F. (1980) *Biochem. J.* **188**, 601–608
- Wilkinson, K. D., Urban, M. K. & Haas, A. L. (1980) *J. Biol. Chem.* **255**, 7529–7532
- Wood, H. G. & Barden, R. E. (1977) *Annu. Rev. Biochem.* **46**, 385–413