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Distribution and degradation of biotin-containing carboxylases in human cell lines

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1. Incubation of cultured cells with [^{3}H]biotin leads to the labelling of acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase. The biotin-containing subunits of the last two enzymes from rat cell lines are not separated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, but adequate separation is achieved with the enzymes from human cells. 2. Since incorporated biotin is only released upon complete protein breakdown, the loss of radioactivity from gel slices coinciding with fluorograph bands was used to quantify degradation rates for each protein. 3. In HE(39)L diploid human fibroblasts, the degradation rate constants are 0.55, 0.40, 0.31 and 0.19 day^{-1} for acetyl-CoA carboxylase, pyruvate carboxylase, methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase respectively. A similar series of rate constants is found for AG2804 transformed fibroblasts. 4. The degradation rate constants are decreased by 31–67% in the presence of 50 μg of leupeptin/ml plus 5 mM-NH_4Cl. Although the largest percentage effect was noted with the most stable enzyme, propionyl-CoA carboxylase, the absolute change in rate constant produced by the lysosomotropic inhibitors was similar for the three mitochondrial carboxylases, but greater for the cytosolic enzyme acetyl-CoA carboxylase. 5. The heterogeneity in degradation rate constants for the mitochondrial carboxylases indicates that only part of their catabolism can occur via the autophagy-mediated unit destruction of mitochondria. Calculations showed that the autophagy-linked process had degradation rate constants of 0.084 and 0.102 day^{-1} respectively in HE(39)L and AG2804 cells. It accounted for two-thirds of the catabolic rate of propionyl-CoA carboxylase and a lesser proportion for the other enzymes.

INTRODUCTION

The average degradation rate of proteins in cultured cells is decreased by serum, growth factors, amino acids, weak bases, proteolytic inhibitors, microtubule-disrupting agents and inhibitors of protein synthesis. However, unless the cells are exposed to metabolic poisons, the degradation rate in the presence of maximally effective concentrations and combinations of these agents remains substantial, at about half the uninhibited rate achieved in serum-free medium (Ballard, 1977; Amenta & Brocher, 1981; Seglen, 1983). These findings support the hypothesis that only part of the measured extent of protein breakdown can be attributed to inhibition of processes in which lysosomal proteolysis is made limiting by lysosomotropic drugs. The remaining proportion of total protein breakdown is thought to involve either a non-regulatable component of autophagy or events that occur outside the autophagic vacuole system and includes processes, such as the ubiquitin pathway, where the rate of catabolism is largely set by the relative sensitivity of each substrate protein to initiating reactions in proteolysis (Goldberg & St. John, 1976; Ballard, 1977; Neff *et al.*, 1979; Ciechanover *et al.*, 1984).

Little direct information is available on which of the above two types of degradative pathways, the process-limited one of autophagy or the range of non-lysosomal ones, account for the breakdown of an individual protein. It is generally assumed that proteins of very short half-lives are predominantly catabolized by a non-lysosomal pathway, because otherwise selective entry of those proteins into autophagic vacuoles would need to be proposed. A second order of complexity occurs with proteins intrinsic to organelles. Thus, although whole mitochondria have frequently been identified within autophagic vacuoles (Ashford & Porter, 1962; Arstila *et al.*, 1972; Marzella *et al.*, 1980), presumably indicating that all the proteins of those particular mitochondria are targeted for imminent breakdown, some mitochondrial proteins have very short half-lives (Marver *et al.*, 1966; Desautels & Goldberg, 1982) and clearly are degraded independently of total mitochondrial destruction. We have sought an understanding of the mechanism responsible for the degradation of mitochondrial protein by following the turnover of pyruvate carboxylase in 3T3-L1 adipocytes (Chandler & Ballard, 1983). Our experiments have shown that the breakdown of pyruvate carboxylase is markedly decreased by inhibitors of lysosomal proteolysis, suggesting that degradation of this enzyme occurs mostly when whole mitochondria are degraded by lysosomes.

In the present paper we have extended the investigation of pyruvate carboxylase turnover to include the two other biotin-containing carboxylases present in mammalian mitochondria as well as acetyl-CoA carboxylase, a cytosolic enzyme. Our measurements show that the mitochondrial carboxylases are degraded at different rates, with the breakdown of the most stable enzyme, propionyl-CoA carboxylase, occurring predominantly via autophagy.

Abbreviation used: SDS, sodium dodecyl sulphate.

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MATERIALS AND METHODS

Materials

The media used for the growth of cultured cells were either obtained from Gibco, Grand Island, NY, U.S.A., which was also the source of foetal bovine serum, or prepared from analytical-grade chemicals. Penicillin G and streptomycin sulphate were purchased from Glaxo Pty. Ltd., Boronia, Victoria, Australia; gentamycin was from Schering Corp., Kenilworth, NJ, U.S.A., and fungizone was from E. R. Squibb and Sons, Princeton, NJ, U.S.A. $NaH^{14}CO_3$ (50 Ci/mol), L-[4,5-^{3}H]leucine (40–60 Ci/mmol) and [8,9-^{3}H]biotin (30–60 Ci/mmol) were obtained from Amersham Corp., Sydney, Australia. CoA and methylcrotonyl-CoA were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Acetyl-CoA and propionyl-CoA were prepared from CoA and either acetic anhydride or propionic anhydride as described by Stadtman (1957).

The cell lines used were obtained from the following sources: mouse 3T3-L1 fibroblasts and AG2804 transformed human fibroblasts from the American Type Culture Collection, Washington, DC, U.S.A.; rat L6 myoblasts from Dr. J. M. Gunn, Texas A & M University, College Station, TX, U.S.A.; T47D and MCF7 human breast carcinoma cells and BEN bronchial carcinoma cells from Dr. T. J. Martin, Repatriation General Hospital, West Heidelberg, Victoria, Australia; HE(39)L human fibroblasts from Commonwealth Serum Laboratories, Melbourne, Victoria, Australia; SF1967 and SF1972 human skin fibroblasts from Adelaide Children's Hospital, North Adelaide, SA, Australia; rat HTC hepatoma cells from Dr. W. D. Wicks, Department of Biochemistry, University of Tennessee, Knoxville, TN, U.S.A.

Growth of cells

All cell lines were grown as monolayers at 37 °C under an atmosphere of 5% CO_2 in humidified air in Dulbecco-modified Eagle's Minimal Essential Medium containing 1 mg of biotin, 60 mg of penicillin G, 100 mg of streptomycin sulphate, 90 mg of gentamycin and 1 mg of fungizone per litre. Foetal bovine serum was added at a concentration of 10% (v/v) for HE(39)L, SF1967 and SF1972 cells and at 5% for the remaining cell lines. The procedures used for the growth and differentiation of 3T3-L1 cells have been reported previously (Chandler & Ballard, 1983).

Measurement of biotin-containing carboxylase activities

Confluent monolayers in 90 mm Petri dishes were incubated for 48 h in biotin-free Dulbecco-modified Eagle's Minimal Essential Medium containing 10% dialysed foetal bovine serum, and then for 2 h in the same medium but containing 10% foetal bovine serum, 100 μM-biotin and 2 mM-leucine. The monolayers were washed twice with Ca^{2+}- and Mg^{2+}-free Hanks' salts, and the cells were scraped from the dishes in 1.6 ml of a solution containing 50 mM-Tris, 5 mM-ATP, 5 mM-$MgSO_4$, 0.5 mM-EDTA and 0.5% Triton X-100 at pH 7.2. The suspensions were disrupted with a Dounce homogenizer, and the homogenates were used for enzyme measurements.

Pyruvate carboxylase activities were determined essentially as described by Ballard & Hanson (1967), but with the $KH^{14}CO_3$ concentration lowered to 10 mM and the inclusion of 0.7 mM-NADH, 6 μg of malate dehydrogenase and 0.2% Triton X-100. The reaction volume was 250 μl. At the completion of either 5 or 10 min incubation periods at 37 °C, 100 μl portions were added to 20 μl of 50% (w/v) trichloroacetic acid, cooled in ice and centrifuged at 10000 *g* for 2 min. Samples of each supernatant (50 μl) were applied to 18 mm discs of Whatman no. 1 filter paper, dried at 100 °C for 5 min and the radioactivity was measured by liquid-scintillation spectrometry with Beckman Ready-Solv EP scintillant. Malate dehydrogenase and NADH were omitted for the measurements of acetyl-CoA carboxylase, propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase. Other changes were the inclusion of 10 mM-sodium citrate and 1% bovine serum albumin and the omission of pyruvate in the acetyl-CoA carboxylase assay, the omission of acetyl-CoA and pyruvate and the inclusion of 1 mM-propionyl-CoA in the propionyl-CoA carboxylase assay, the omission of acetyl-CoA and pyruvate and the inclusion of 0.4 mM-methylcrotonyl-CoA for the measurement of methylcrotonyl-CoA carboxylase.

The activities of each enzyme were expressed as nmol of bicarbonate incorporated/min (munits) per mg of cell protein after subtraction of blank rates obtained when the CoA derivatives were omitted, except for pyruvate carboxylase, when pyruvate was excluded. The amount of extract was varied so that linear rates of bicarbonate incorporation were obtained over the measurement period.

Identification of [^{3}H]biotin-labelled proteins in cell extracts

Confluent monolayers in 35 mm dishes were labelled for 48 h with [^{3}H]biotin (1 μCi/ml) as described by Chandler & Ballard (1983), washed twice with Hanks' salts and incubated for 2 h in Eagle's Minimal Essential Medium containing 100 μM-biotin, 2 mM-leucine and 10% foetal bovine serum. This medium was discarded and the cells were removed by scraping in three sequential portions of 0.5 ml of water at 0 °C. The combined extract was homogenized at 0 °C in a Dounce homogenizer, and, after removal of a subsample for protein measurement (Lowry *et al.*, 1951), the remainder was freeze-dried. The freeze-dried extract was dissolved in SDS dissociating solution (Palmiter *et al.*, 1971, but containing 2% SDS) so that the protein concentrations were equal, and heated at 95 °C for 10 min. A portion of the dissociated and reduced protein (25 μl) was analysed by electrophoresis using a discontinuous buffer system on an exponential 6–16%-acrylamide gradient gel (O'Farrell, 1975).

For two-dimensional electrophoresis the above protocol was modified by harvesting the cells in 1 ml of lysis buffer (O'Farrell, 1975). The cell lysates were stored at −80 °C until analysed as described by O'Farrell (1975), an 8%-acrylamide gel being used for the second dimension. Staining, drying and fluorography of both one- and two-dimensional gels were carried out as described below.

Degradation of leucine-labelled total cell proteins

The procedures adopted were the same as described previously (Chandler & Ballard, 1983), except that the measurements were carried out in 35 mm Petri dishes in Eagle's Minimal Essential Medium containing 100 μM-biotin, 2 mM-leucine, 1% foetal bovine serum as well as 60 mg of penicillin G, 100 mg of streptomycin sulphate,

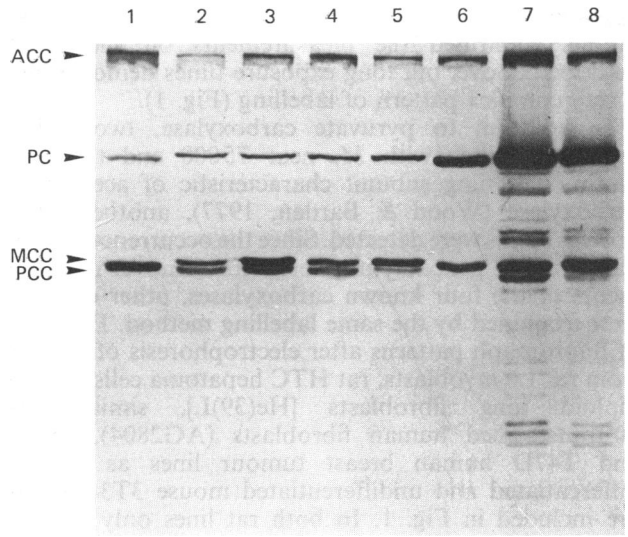

Fig. 1. Fluorographs of SDS/polyacrylamide-gel-electrophoretic separation of biotin-containing carboxylases

Equal amounts of protein from cells labelled in the presence of [^{3}H]biotin were analysed by electrophoresis on SDS/polyacrylamide gradient gels. The positions of the biotin subunits of acetyl-CoA carboxylase (ACC), pyruvate carboxylase (PC), methylcrotonyl-CoA carboxylase (MCC) and propionyl-CoA carboxylase (PCC) are indicated. Lanes 1–8 represent proteins from the following cell lines; 1, L6; 2, HE(39)L; 3, T47D; 4, AG2804; 5, MCF7; 6, HTC; 7, differentiated 3T3-L1; 8, undifferentiated 3T3-L1 cells. The fluorograph was exposed for 18 days.

90 mg of gentamycin and 1 mg of fungizone/litre (chase medium) and inhibitors as indicated.

Degradation of biotin-labelled proteins

Near-confluent cultures in 35 mm Petri dishes were labelled for 48 h as described previously (Chandler & Ballard, 1983) but with the amount of labelled biotin increased to 1 μCi/ml. The monolayers were subsequently washed twice with Hanks' salts and incubated for 2 h in chase medium. The degradation period began after the addition to each dish of 2 ml of chase medium or 2 ml of a similar medium but containing 5 mM-NH_4Cl and 100 μg of leupeptin. After incubation 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. Immediately after the removal of the incubation medium, each monolayer was rapidly washed twice at 0 °C with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (Dulbecco & Vogt, 1954), and the monolayer was removed by scraping in 0.5 ml of water at 0 °C. This extract was combined with two additional 0.5 ml water washes and homogenized in a Dounce homogenizer. Duplicate 0.5 ml samples were frozen immediately in liquid N_2 and freeze-dried for subsequent analysis. Additional 50 μl samples were taken for the measurement of radioactivity.

The fractional degradation rate of total biotin-containing protein was calculated as 100 times the radioactivity in the medium amino acid fraction divided by the sum of the radioactivity in all fractions. It was noted that, even after incubation for 3 days in the presence of lysosomal inhibitors, the radioactivity in the medium protein fraction never exceeded 10% of the total, an indication of good cell viability.

The freeze-dried 0.5 ml portions of cell extract were dissolved in 10 μl portions of SDS dissociating solution (Palmiter *et al.*, 1971, but containing 2% SDS) and heated at 95 °C for 10 min. Samples of this dissociated and reduced protein (25 μl) were applied to 8% acrylamide slab gels and analysed by electrophoresis in the discontinuous buffer system reported by O'Farrell (1975). After 1000 V-h the gels were stained with 0.1% Coomassie Blue R250 in 50% (v/v) methanol/10% (v/v) acetic acid and destained in the same solution but with the dye omitted. Gels were impregnated with diphenyloxazole, dried in a Hoeffer gel-drying apparatus, and fluorography was carried out with Kodak X-omat AR or XAR films (Pulleyblank & Booth, 1981). The position of each radioactive band was identified on the dried gel by alignment with the developed fluorograph, and the appropriate region was cut out and swollen by the addition of 100 μl of water and 200 μl of NCS Solubilizer. Radioactivity in swollen gel portions was determined as described by Hopgood *et al.* (1973) and expressed as the total radioactivity in each carboxylase per dish. These values were corrected to allow for any differences in the amount of cell protein or the extent of biotin incorporation per dish by multiplying the radioactivity in each carboxylase by the sum of the radioactivity in medium protein, medium amino acid and total monolayer

Table 1. Activities of biotin-containing carboxylases in human cell lines

Values are averages of measurements on two dishes and are expressed as nmol of bicarbonate incorporated at 37 °C/min per mg of cell protein.

	Carboxylase			
Cell line	Acetyl-CoA	Pyruvate	Methylcrotonyl-CoA	Propionyl-CoA
AG2804	0.183	0.383	0.256	0.694
HE(39)L	0.062	0.288	0.156	0.637
SF1967	0.052	0.140	0.092	0.300
SF1972	0.049	0.163	0.107	0.464
BEN	0.121	0.988	0.231	0.643
T47D	0.182	0.877	0.807	2.02
MCF7	0.217	0.971	0.324	0.512

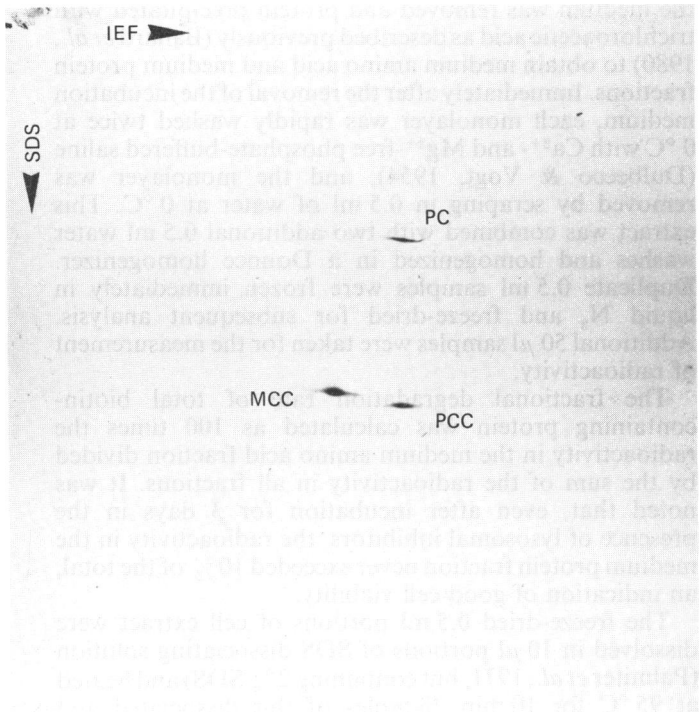

Fig. 2. Fluorograph of two-dimensional-electrophoretic separation of biotin-labelled proteins from AG2804 cells

The directions of isoelectric focusing (IEF) and SDS/polyacrylamide-gel-electrophoretic separation are indicated. The abbreviations are those given in Fig. 1. The fluorograph was exposed for 16 days.

fractions (i.e. total dish radioactivity) from the first zero-time dish and dividing by the total radioactivity in the test dish. The corrected values were used to calculate degradation rate constants ($days^{-1}$).

RESULTS AND DISCUSSION

Identification of biotin-containing carboxylases in cultured cells

Biotin occurs in covalent linkage in four mammalian proteins, acetyl-CoA carboxylase, pyruvate carboxylase, propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase (Wood & Barden, 1977). Once incorporated, the biotin is only released after the complete breakdown of the carboxylase peptide chain to amino acids, since the enzyme responsible, biotinidase, exhibits a high degree of specificity towards biotinyl-lysine but reacts very poorly with peptides containing this biocytin molecule (Koivusalo *et al.*, 1963; Craft & Goss, 1982). Accordingly, the loss of radioactivity from cells prelabelled with biotin can be used as a measure of the average degradation rate of the constituent biotin carboxylases. We first used this approach with differentiated 3T3-L1 adipocytes, in which 80–85% of labelled biotin was incorporated into pyruvate carboxylase (Chandler & Ballard, 1983). Most of the remaining radioactivity occurred in a smaller protein subunit of M_r 75000, the size expected for the biotin-containing subunits of propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase (Wood & Barden, 1977; Lau *et al.*, 1979; Robinson *et al.*, 1983). Fluorographs of 3T3-L1 extracts separated by SDS/polyacrylamide-gel electrophoresis under reducing conditions confirmed the measurements on sliced gels described above, but long exposure times demonstrated a very complex pattern of labelling (Fig. 1).

In addition to pyruvate carboxylase, two poorly separated bands with M_r near 75000 and the large biotin-containing subunit characteristic of acetyl-CoA carboxylase (Wood & Barden, 1977), another 20–25 protein bands were detected. Since the occurrence of these bands would probably interfere with turnover measurements of the four known carboxylases, other cell lines were examined by the same labelling method. Examples of fluorograph patterns after electrophoresis of extracts from rat L6 myoblasts, rat HTC hepatoma cells, human diploid lung fibroblasts [He(39)L], simian-virus-40-transformed human fibroblasts (AG2804), MCF7 and T47D human breast tumour lines as well as differentiated and undifferentiated mouse 3T3-L1 cells are included in Fig. 1. In both rat lines only a single prominent band with a subunit M_r of 75000 was present. Because separation into two bands at this position occurred with the four human lines, these cells were examined further in our attempts to measure the degradation rates of all four carboxylases.

The activities of each carboxylase have been measured in similar extracts to those used for electrophoretic separation (Table 1). In addition to the four human lines analysed in Fig. 1, data on two diploid skin fibroblasts (SF1967, SF1972) and BEN, a bronchial carcinoma line, have been compared. All the human lines tested had measurable activities of the four biotin-containing carboxylases, but with considerable differences in specific activities. The activity differences at least partly reflect the intensity of biotin incorporation into the individual bands. Thus HE(39)L cells had the lowest acetyl-CoA carboxylase activity and the least dense fluorograph band of the four human lines compared in Fig. 1 and Table 1, whereas HE(39)L cells had less pyruvate carboxylase activity and lower fluorograph-band densities than did the two breast tumour lines, T47D and MCF7. Similar comparisons hold for propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase, with the band intensities and activities of these two enzymes being especially pronounced in T47D cells.

It is possible that single bands evident on one-dimensional gels can be resolved into more than one protein spot by two-dimensional electrophoresis. If this occurred with any of the four biotin-containing bands evident in human cell extracts, degradation measurements based on radioactivity changes in regions of the one-dimensional gels would be difficult to interpret. To test for such a situation, AG2804 cell extracts were separated by two-dimensional electrophoresis (Fig. 2). The analysis showed only three spots, corresponding to pyruvate carboxylase, propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase. No spot could be detected at the size expected for the biotin-containing subunit of acetyl-CoA carboxylase, either because this was degraded by endogenous proteinases before isoelectric focusing was complete or because the isoelectric point was outside the separation range of the first-dimensional gel.

Although the four biotin-containing carboxylases have been investigated in liver and other mammalian tissues with respect to intracellular distribution, catalytic mechanism, molecular properties and, to a limited extent,

Table 2. Degradation of total leucine-labelled protein and total biotin-labelled protein

The percentage degradation of total [^{3}H]leucine-labelled and total [^{3}H]biotin-labelled proteins was measured as described in the text. Values are means of measurements on two separate dishes harvested on each day. The numbers in parentheses represent the percentage inhibition of breakdown produced by the inhibitor mixture containing 50 μg of leupeptin/ml and 5 mM-NH_4Cl.

			Percentage degraded after:			
Cell line	Label	Inhibitors	0 days	1 day	2 days	3 days
AG2804	Leucine	−	0.1	28.5	43.7	50.0
		+	0.1	19.3	26.2	28.2
				(32)	(40)	(44)
	Biotin	−	3.7	24.4	38.4	46.0
		+	3.7	15.7	25.1	25.9
				(42)	(38)	(48)
HE(39)L	Leucine	−	0.3	37.7	54.8	64.0
		+	0.3	22.3	32.0	37.5
				(41)	(42)	(42)
	Biotin	−	4.0	34.1	48.6	56.3
		+	4.0	18.7	25.0	30.8
				(51)	(53)	(49)

biological regulation (Moss & Lane, 1971; Wood & Barden, 1977), only pyruvate carboxylase and acetyl-CoA carboxylase have been studied in any detail in cultured cells. These two enzymes catalyse essential reactions in lipogenesis and have been demonstrated at high activities after the differentiation of 3T3-L1 fibroblasts into adipocytes (Mackall *et al.*, 1976; Mackall & Lane, 1977; Gratzner *et al.*, 1980). Likewise acetyl-CoA carboxylase activity is increased in cultured hepatocytes in response to lipogenic stimuli (Kitajima *et al.*, 1975; Fischer & Goodridge, 1978). The activities of pyruvate carboxylase and acetyl-CoA carboxylase in the human cell lines examined here (Table 1) are somewhat lower when expressed per unit of protein than in undifferentiated 3T3-L1 cells, and are typically only 1% of the activities in differentiated 3T3-L1 cells. The highest activities among the human lines were found in T47D cells, a result consistent with the capacity of this breast carcinoma line to synthesize lipid.

Acetyl-CoA carboxylase (Shafrir & Birman, 1981) and the three mitochondrial biotin-containing carboxylases (Bartlett *et al.*, 1981) have been demonstrated in human skin fibroblasts at activities similar to those found in our SF1967 and SF1972 lines. Fibroblasts from patients with a number of inborn errors of metabolism have been shown to be deficient in either one or all of the mitochondrial carboxylases (Bartlett *et al.*, 1981; Robinson *et al.*, 1983), although it is not clear whether the deficiencies limit growth or metabolism of the fibroblasts. In this context HeLa cells cultured in biotin-free medium had a much decreased propionyl-CoA carboxylase activity under conditions where cell viability was unchanged, but after several passages the cells ceased growing (Dakshinamurti & Chalifour, 1981).

Degradation of biotin-containing carboxylases

We chose to investigate the degradation of the carboxylases in HE(39)L human diploid lung fibroblasts and in AG2804 transformed fibroblasts, because, although these two lines have rather low activities of the enzymes, they provide an opportunity to examine whether regulatory controls are qualitatively or quantitatively different between contact-inhibited and transformed cells. Total biotin-labelled protein was degraded by approx. 20% and 30% on the first measurement day in AG2804 and HE(39)L cells respectively (Table 2). On days 2 and 3 the rates were substantially lower, so that first-order plots indicated a mixture of fast and slow rate constants. Nevertheless the percentage inhibition of breakdown by the addition of a lysosomotropic mixture of leupeptin and NH_4Cl was relatively constant for the 3-day measurement period, and was 38–48% in AG2804 cells and 49–53% in HE(39)L cells.

Parallel cultures that were prelabelled with [^{3}H]leucine

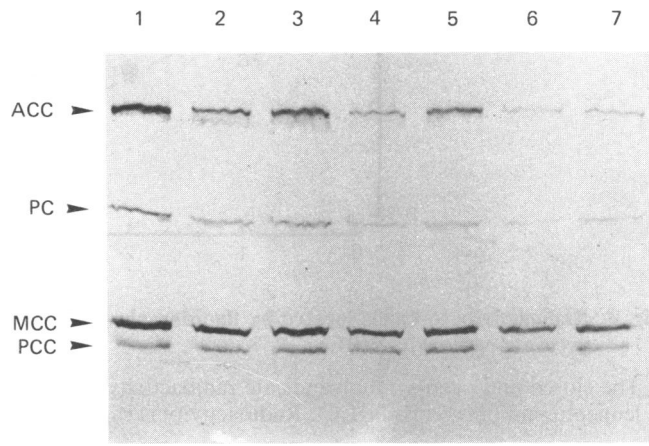

Fig. 3. Fluorographs of [^{3}H]biotin-labelled proteins separated by SDS/polyacrylamide-gel electrophoresis at different times after beginning the degradation measurements with AG2804 cells

Equal volumes of cell lysates from each dish were analysed. The lanes represent: 1, zero time; 2, 4, 6, one, two and three days after incubation in control medium; 3, 5, 7, one, two and three days after incubation in medium containing 50 μg of leupeptin/ml and 5 mM-NH_4Cl. The abbreviations are given in the legend to Fig. 1. The fluorograph was exposed for 21 days.

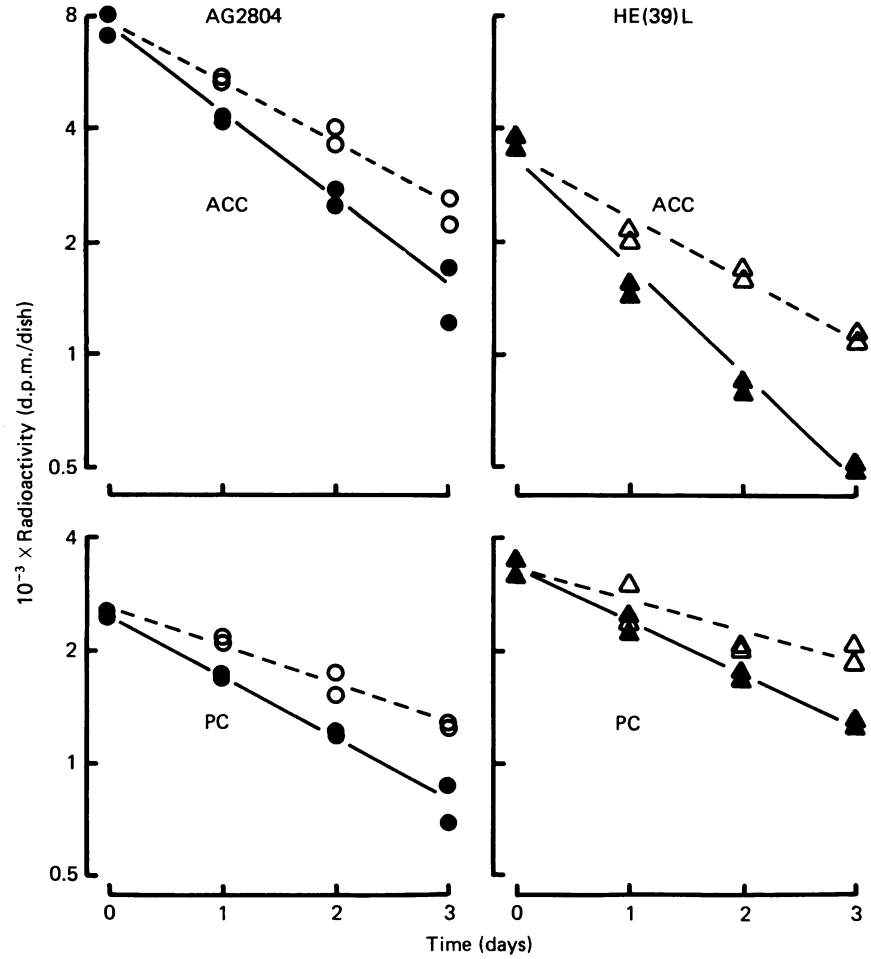

Fig. 4. Radioactivity in bands located by fluorographs similar to those in Fig. 3 and identified as acetyl-CoA carboxylase (ACC) or pyruvate carboxylase (PC)

The closed and open symbols indicate radioactivity from cells incubated in the absence and presence respectively of 50 μg of leupeptin/ml plus 5 mM-NH_4Cl. Radioactivity is calculated as d.p.m./dish, corrected as described in the text.

to measure the degradation of total cell proteins indicated somewhat more rapid average turnover rates than for the total biotin-labelled proteins (Table 2). The relative inhibitory effects produced by the leupeptin/NH_4Cl mixture were lower, and in AG2804 cells increased progressively at longer measurement periods.

The same cell extracts that were used for the measurement of total carboxylase breakdown (Table 2) were fractionated by SDS/polyacrylamide-gel electrophoresis. The gradient gel procedure used in Fig. 1 was replaced by 8%-acrylamide gels to achieve separation of the more-rapidly-migrating methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase, because Robinson *et al.* (1983) had reported good separation of these two enzymes on similar acrylamide gels. Fluorography of the dried gels showed four discrete bands (Fig. 3), with a clearly improved separation of the two smallest biotin-containing peptides (cf. Fig. 1). Loss of radioactivity with time from acetyl-CoA carboxylase is clearly evident, especially in control incubations (cf. lanes 1, 2, 4 and 6, Fig. 3). With the other three enzymes the change in band densities with time can also be seen, although the effects are less pronounced. At each time period and for all four carboxylases, the lanes containing extracts from cultures incubated with leupeptin plus NH_4Cl show denser bands, indicating partial inhibition of enzyme degradation. This assessment was quantified by cutting portions of the dried gels after alignment with the fluorograph bands and determining the radioactivity present.

The radioactivity in the four carboxylases, determined in eight separate culture dishes (two at each time period), gave approximately linear first-order plots (Figs. 4 and 5). Acetyl-CoA carboxylase was degraded in control cultures of AG2804 cells at a slightly slower rate than in HE(39)L cells (Fig. 4, Table 3). However, the lysosomotropic mixture inhibited breakdown more effectively in HE(39)L fibroblasts, so that the degradation rate constants under these conditions were similar for the two cell lines. Pyruvate carboxylase, on the one hand, was degraded more rapidly in AG2804 cells than in HE(39)L cells in both control and inhibited states (Fig. 4, Table 3). Similar relative effects between the two cell lines were also found for methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase (Fig. 5, Table 3), but the percentage decrease in the degradation rate constant by the leupeptin/NH_4Cl mixture was considerably

Table 3. Degradation rate constants (day^{-1}) for individual biotin-containing carboxylases

Values are the degradation rate constants (±S.E.M.) for the data given in Figs. 4 and 5 and calculated in each case from data on eight dishes. The numbers in parentheses represent the percentage decreases in the rate constants produced by incubation of cells in the presence of 50 μg of leupeptin/ml and 5 mM-NH_4Cl.

Carboxylase	Inhibitors	Cell line	
		AG2804	HE(39)L
Acetyl-CoA	−	0.553±0.039	0.664±0.040
	+	0.378±0.024	0.379±0.028
		(32)	(43)
Pyruvate	−	0.399±0.025	0.319±0.015
	+	0.234±0.016	0.189±0.033
		(41)	(41)
Methylcrotonyl-CoA	−	0.311±0.025	0.166±0.010
	+	0.215±0.021	0.083±0.021
		(31)	(50)
Propionyl-CoA	−	0.186±0.028	0.158±0.019
	+	0.075±0.016	0.052±0.024
		(60)	(67)

greater with the last enzyme. Indeed, the inhibitors almost completely suppressed catabolism of propionyl-CoA carboxylase in HE(39)L cells.

The complete experiment shown in Tables 2 and 3 as well as in Figs. 3, 4 and 5 was repeated to establish the reproducibility of the responses. The degradation of leucine-labelled protein was essentially identical between the two series of experiments. However, in the repeat study the catabolism of biotin-labelled proteins was somewhat slower, with 27% and 31% being degraded between days 0 and 2 in the AG2804 and HE(39)L cells respectively (cf. Table 2, where the corresponding values are 35% and 45%). This difference in the breakdown rate of total biotin-containing protein was also reflected in the measurements on the individual carboxylases. Nevertheless, the relative effects of the leupeptin/NH_4Cl inhibitory mixture on all degradation rates were similar between the two studies.

Measurements of degradation rate constants based on the disappearance with time of labelled biotin from a carboxylase band are only valid if the radioactivity is in a single protein and if the biotin cannot be removed except when the whole protein molecule is degraded. Evidence for the former assumption comes from our detection of only four biotin-containing bands by either one- or two-dimensional electrophoresis and from literature reports of biotin-containing proteins in mammals being limited to the four carboxylases studied here. Koivusalo *et al.* (1963) originally noted that biotin cleavage from partial tryptic hydrolysates of propionyl-CoA carboxylase by biotinidase was very much slower than when biocytin was the substrate, a result subsequently confirmed with a range of biocytin-containing peptides (Craft & Goss, 1982). Moreover, Freytag & Utter (1983) demonstrated that the degradation rates of biotin-labelled and leucine-labelled pyruvate carboxylase in 3T3-L1 adipocytes were identical, direct evidence that loss of biotin only occurs on degradation of the enzyme.

In both AG2804 and HE(39)L cells lines the degradation rate constants diminish in the order: acetyl-CoA carboxylase > pyruvate carboxylase > methylcrotonyl-CoA carboxylase > propionyl-CoA carboxylase (Table 3). Furthermore, the relative ability of the ammonia/leupeptin mixture to inhibit breakdown increases proportionally in both cell lines as the degradation rate constants decrease, so that in both cell lines the largest effects are found with propionyl-CoA carboxylase (Table 3). We interpret these findings as evidence for a similarly controlled process acting on protein breakdown in normal and transformed fibroblasts, a process that is directed selectively towards relatively stable proteins. A similar conclusion has been obtained in studies where all cell proteins are labelled. Thus degradation of labile proteins that contain errors in amino acid sequence produced by the incorporation of amino acid analogues is only slightly decreased by the inclusion of lysosomotropic agents as compared with effects on the degradation of normal proteins (Knowles & Ballard, 1976). In addition, the breakdown of normal but short-lived proteins that have been selected by use of a brief labelling protocol is less responsive to inhibitors of autophagy or lysosomal function (Knowles & Ballard, 1976; Seglen *et al.*, 1979; Auteri *et al.*, 1983; Wharton & Hipkiss, 1984). Thus the evidence presented here reinforces the hypothesis that the breakdown of proteins with longer half-lives is more likely to occur via the autophagic/lysosomal system than by substrate-regulated proteolysis.

Implications on the mechanism of mitochondrial proteolysis

The three mitochondrial biotin carboxylases have degradation rate constants that span a 2-fold range (Table 3), a result that is not compatible with mitochondria turning over as units in the two cell lines investigated here. The remaining alternatives are that either proteins must selectively exit from mitochondria for catabolism, or the more labile carboxylases must be degraded by mitochondrial proteinases. Since we have not been able to find support for the first possibility, and there is abundant evidence for the ubiquitous distribution

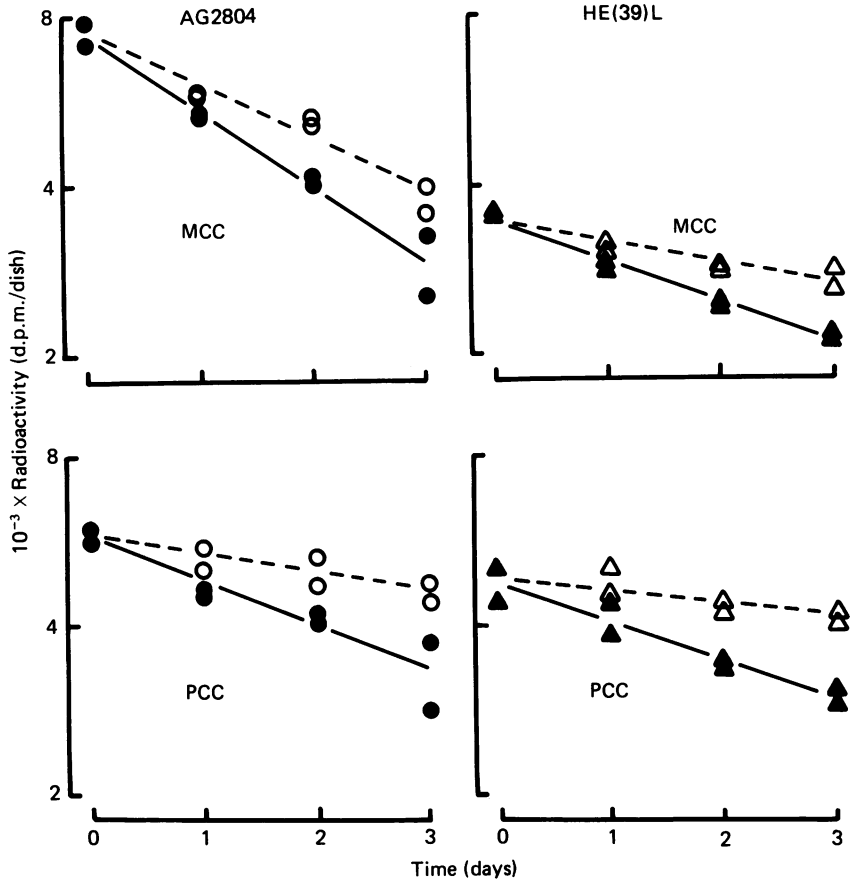

Fig. 5. Radioactivity in bands located by fluorographs similar to those in Fig. 3 and identified as methylcrotonyl-CoA carboxylase (MCC) or propionyl-CoA carboxylase (PCC)

Other details are given in the legend to Fig. 4.

of mitochondrial proteinases (Desautels & Goldberg, 1982, 1985; Rapoport *et al.*, 1982), it seems most likely that such enzymes are involved in the breakdown of the relatively labile mitochondrial proteins.

The proteinase studied by Desautels & Goldberg (1985) is ATP-dependent and shares sufficient properties with the bacterial proteinase La to suggest conservation throughout evolution. Other proteinases have been reported to be present in mitochondria (see, e.g., Haas *et al.*, 1978; Beer *et al.*, 1982), but they have similar properties to known extra-mitochondrial enzymes, so that some doubt exists as to their true cellular location. Although the question of which mitochondrial proteinase is responsible for the catabolism of the more labile carboxylases must await further study, the heterogeneity of mitochondrial protein turnover is not restricted to carboxylases, since mitochondrial inner-membrane proteins and mitochondrial matrix proteins have extremely heterogeneous half-lives (Marver *et al.*, 1966; Grisolia *et al.*, 1981; Hare & Hodges, 1982*a*,*b*).

Whereas the heterogeneity of degradation rates for mitochondrial proteins can be taken as indirect evidence for a functional system of mitochondrial proteolysis, micrographs showing mitochondria within autophagic vacuoles (Ashford & Porter, 1962; Arstila *et al.*, 1972; Marzella *et al.*, 1980) provide conclusive evidence that under some conditions whole mitochondria are degraded as units. The question to resolve, therefore, is the proportion of breakdown that occurs via one or the other degradative pathway. We can obtain one estimate of the autophagy component by subtracting the first-order rate constant obtained in the presence of the lysosomotropic agents from the rate constant without inhibitors. The mean value obtained using all three mitochondrial carboxylases in both the experiment summarized in Table 3 and the repeat experiment is 0.084 day^{-1} for HE(39)L cells and 0.102 day^{-1} for AG2804 cells. These values can only be considered as approximate, because they assume that autophagic proteolysis is totally inhibited by leupeptin plus NH_4Cl and that leupeptin does not inhibit non-autophagic proteolysis. The calculated half-lives of 8.3 and 6.8 days respectively are a measure of the longest possible half-lives for mitochondrial proteins and agree reasonably well with independent estimates of mitochondrial turnover under nutritionally adequate conditions for liver (Pfeifer, 1979; Russell *et al.*, 1980; Lipsky & Pedersen, 1981; Mortimore & Surmacz, 1984) and kidney (Pfeifer & Warmuth-Metz, 1983), although comparable measurements on cultured fibroblasts have not been reported. We can also calculate from Table 3 that about one-third of the pyruvate carboxylase, one-third to one-half of the methylmalonyl-CoA carboxylase and two-thirds of the propionyl-CoA carboxylase are degraded by the autophagic process in HE(39)L and AG2204 cells. Whether the remaining proportion of carboxylase degradation occurs via the ATP-dependent

proteolysis or some other intramitochondrial proteinase cannot be answered at the present time.

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