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Multiple biotin-containing proteins in 3T3-L1 cells

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1. Extracts of 3T3-L1 cells prepared after labelling the monolayer cultures with [^{3}H]biotin contained numerous protein bands that were detected by fluorography of dried SDS/polyacrylamide electrophoresis gels. All labelled proteins in the extracts could be removed by avidin affinity chromatography. 2. The biotin-containing subunits of acetyl-CoA carboxylase, pyruvate carboxylase, methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase, with molecular masses of approx. 220, 120, 75 and 72 kDa respectively, were detected together with minor bands at 100, 85 and 37 kDa that did not appear to be partial degradation products. 3. Additional labelled bands increased in amount during incubation of cell extracts or did not occur in extracts prepared with trichloroacetic acid, 9.5 M-urea or proteolytic inhibitors, and were tentatively classified as partial degradation products. 4. The unknown bands were not removed by incubation of cell monolayers for 24 h, a treatment that gave degradation rate constants of 0.47 day^{-1} for acetyl-CoA carboxylase and 0.28 day^{-1} for pyruvate carboxylase. 5. Upon two-dimensional electrophoresis, pyruvate carboxylase, methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase had isoelectric points of 6.4, 7.2 and 6.4 respectively. Several additional discrete spots with isoelectric points below 6.2 were also present. 6. All the unknown biotin-containing proteins banded with intact mitochondria during density-gradient centrifugation. 7. We conclude that several unknown biotin-containing proteins are present in the mitochondria of 3T3-L1 cells, whereas others are partial breakdown products of mitochondrial proteolysis.

INTRODUCTION

Exposure of 3T3-L1 mouse fibroblasts to [^{3}H]biotin during the period when the cells are differentiating into adipocytes leads to the incorporation of 80–85% of the radioactivity into a single protein, pyruvate carboxylase (EC 6.4.1.1) (Chandler & Ballard, 1983). This result accords with the induction of pyruvate carboxylase to a high activity by the differentiation stimulus (Mackall & Lane, 1977; Freytag & Utter, 1980) as part of a generally increased lipogenic capacity (Green & Kehinde, 1976; Mackall *et al.*, 1976; Kuri-Harcuch & Green, 1977; Reed *et al.*, 1977; Grimaldi *et al.*, 1978; Hu *et al.*, 1983).

Biotin occurs in three other proteins in mammalian cells, acetyl-CoA carboxylase, propionyl-CoA carboxylase and methylcrotonyl-CoA carboxylase, each of which is labelled after incubation of rat, mouse and human cell lines with high-specific-radioactivity [^{3}H]biotin (Chandler & Ballard, 1985). Moreover, fluorographs of SDS/polyacrylamide-gel-electrophoretic separations of cell extracts from biotin-labelled 3T3-L1 cells demonstrated a complex pattern of protein bands, suggesting that additional biotin-containing proteins occurred in the cells (Chandler & Ballard, 1985). In the present paper we have investigated the nature, location and stability of these bands and conclude that, although some are proteolytic breakdown products of the known carboxylases, others probably represent stable cell proteins with unknown functions.

MATERIALS AND METHODS

Growth of cells and labelling of proteins

The conditions for growth, differentiation and labelling were as described by Chandler & Ballard (1983), modified only by the addition of 0.25 μM-dexamethasone during the 2-day differentiation period and the inclusion of 1 μCi instead of 0.1 μCi of [^{3}H]biotin/ml for labelling.

The normal labelling period was 42 h (Chandler & Ballard, 1983). In one experiment (Fig. 4) some monolayers were incubated for the first 38 h in the presence of 10 μg of avidin/ml to prevent biotin incorporation into newly formed apoproteins. Subsequently the cells were thoroughly washed and labelled for 4 h with 1 μCi of [^{3}H]biotin/ml.

For all experiments unless indicated otherwise, cell extracts were obtained after quickly washing the cells twice at 0 °C with Ca^{2+}- and Mg^{2+}-free Hanks' salts by scraping the cells from the 35 mm plastic dishes 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 sub-sample for protein measurement (Lowry *et al.*, 1951), the remainder was used immediately, or frozen in liquid N_2, or freeze-dried.

SDS/polyacrylamide-gel electrophoresis and fluorography

Extracts for analysis by single-dimensional electrophoresis were freeze-dried and subsequently dissolved in dissociating solution (Palmiter *et al.*, 1971, but containing 2% SDS) at 95 °C for 10 min. Portions were analysed by electrophoresis on 6–16% (w/v) acrylamide gradient slab gels with a 4.5% (w/v) acrylamide stacking gel, by using the discontinuous buffer system reported by O'Farrell (1975). Subsequently the gels were stained with Coomassie Blue R250, impregnated with diphenyloxazole, dried, and the radioactivity was detected by fluorography (Chandler & Ballard, 1985). In experiments requiring quantification of radioactivity, the developed fluorograph was repositioned above the dried gel and the desired bands were cut out. They were subsequently swollen by the addition of 100 μl of water and 200 μl of NCS Solubilizer, and the

radioactivity was determined as described by Hopgood *et al.* (1973).

Extracts for two-dimensional electrophoresis, stored at −80 °C, were thawed in the presence of lysis buffer (O'Farrell, 1975) and analysed as described by O'Farrell (1975), with 6–16% (w/v) acrylamide gels for the second dimension. Staining, drying and fluorography were carried out as for single-dimensional gels.

Measurements of degradation of acetyl-CoA carboxylase and pyruvate carboxylase

The procedures used for cell differentiation, labelling and the degradation of the carboxylases were identical with those reported previously (Chandler & Ballard, 1985).

Avidin–Sepharose chromatography

Avidin (4 mg) was coupled to CNBr-activated Sepharose 4B (1 g of dried gel) according to the directions provided by Pharmacia Fine Chemicals. A column containing 0.5 ml of swollen avidin–Sepharose in a 2 ml plastic syringe was prepared and washed with 3 ml of phosphate-buffered saline (10 mM-KH_2PO_4/150 mM-NaCl adjusted with KOH to pH 7.4). To this column was added 300 μl of a supernatant prepared after scraping [^{3}H]biotin-labelled 3T3-L1 cells from a 90 mm Petri dish in phosphate-buffered saline, freeze-drying, reconstituting in 1.5 ml of phosphate-buffered saline and centrifuging at 100000 *g* for 1 h. A further 300 μl of phosphate-buffered saline was added, and the combined eluate was passed through the column ten times to ensure adsorption of biotin-containing material to the immobilized avidin. The eluate from the final pass was collected and analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis, followed by fluorography.

Subcellular distribution of biotin-containing proteins

The method adopted was that reported by Merion & Sly (1983) with minor modifications. Four 90 mm Petri dishes of confluent 3T3-L1 cells were differentiated and labelled with [^{3}H]biotin as described above. The cells were removed from the dishes by scraping in 3 ml of 0.25 M-sucrose containing 1 mM-EDTA (pH 7.0) at 0 °C and homogenized with three strokes of a Dounce homogenizer. Phase-contrast microscopy indicated 90–95% cell breakage. After centrifugation at 800 *g* for 10 min at 4 °C to remove lipid and unbroken cells, 1 ml of the infranatant was layered on to 9 ml of a self-forming Percoll gradient prepared in the homogenizing solution to a density of 1.07 g/ml. The tube was centrifuged at 66000 *g* for 1 h at 4 °C in a type 65 Beckman rotor, and 600 μl fractions were collected by upward-flow displacement with an Isco model 640 fractionator. Triton X-100 was added to each fraction to give a final detergent concentration of 0.5%, and samples were immediately used for enzyme assays or kept at −80 °C for electrophoretic analysis.

Partial purification of methylcrotonyl-CoA carboxylase

Differentiated and [^{3}H]biotin-labelled 3T3-L1 cells in ten 90 mm Petri dishes were harvested by scraping in 20 mM-potassium phosphate containing 0.1 mM-dithiothreitol and 0.1 mM-EDTA at pH 7.0 (final volume 3.6 ml), sonicated and fractionated by centrifugation in the presence of 3% poly(ethylene glycol) as described by Lau *et al.* (1980). This procedure results in the selective precipitation of methylcrotonyl-CoA carboxylase, which is then solubilized in the same potassium phosphate solution but without polyethylene glycol. Supernatant samples from both centrifugation steps were assayed for carboxylase activities and stored at −80 °C for subsequent one- and two-dimensional electrophoretic analysis.

Enzyme assays

Pyruvate carboxylase, acetyl-CoA carboxylase (EC 6.4.1.2), propionyl-CoA carboxylase (EC 6.4.1.3) and methylcrotonyl-CoA carboxylase (EC 6.4.1.4) activities were measured at 37 °C as described by Chandler & Ballard (1985). Glutamate dehydrogenase (EC 1.4.1.2) was assayed at 30 °C as described by Ballard (1971), lactate dehydrogenase (EC 1.1.1.27) at 25 °C as described by Kornberg (1955), and β-*N*-acetylglucosaminidase (EC 3.2.1.30) at 37 °C as described by Plattner & Henning (1975). In each case the unit of activity was defined as the amount of enzyme required to catalyse either the formation of 1 μmol of product or the removal of 1 μmol of substrate/min at the indicated temperature.

Materials

The low-M_r marker kit, CNBr-activated Sepharose 4B and Percoll were obtained from Pharmacia, Uppsala, Sweden. Avidin, polyethylene glycol 6000, phenylmethanesulphonyl fluoride, tosyl-lysylchloromethane (7-amino-1-chloro-3-tosylamidoheptan-2-one; 'TLCK') and lima-bean trypsin inhibitor were from Sigma Chemical Co., St. Louis, MO, U.S.A. Leupeptin was purchased from the Protein Research Foundation, Osaka, Japan. The sources for cells, culture media, radioisotopes and substrates for the carboxylase assays have been described previously (Chandler & Ballard, 1985).

RESULTS AND DISCUSSION

Verification that all [^{3}H]biotin-labelled proteins contain biotin

SDS/polyacrylamide-gel electrophoresis of [^{3}H]biotin-labelled 3T3-L1 cell proteins followed by fluorography has been reported previously to give a very complex protein pattern (Chandler & Ballard, 1985). The addition of avidin to cell extracts before electrophoresis, by the procedure reported by Green (1975), resulted in a decreased migration of all the bands detected by fluorography. Although it is not possible to relate specific bands in the presence of avidin to those evident without the biotin-binding protein, the pattern observed with avidin was consistent with all bands having their subunit molecular masses increased by at least 70 kDa, the molecular mass of avidin.

A second approach to determine whether the labelled proteins truly contained biotin, rather than representing proteins labelled by any impurity in the [^{3}H]biotin, was to adsorb the biotin-containing proteins by passage through an avidin affinity column (Swack *et al.*, 1978). This procedure resulted in the removal of 94% of the radioactivity from the extract and eliminated all fluorography bands except for a small amount of material with a molecular mass of approx. 120 kDa, presumably some pyruvate carboxylase biotin subunit that had not been completely removed by the avidin (cf. lanes 1 and 2, Fig. 1). It is evident from the Coomassie Blue-stained and dried gel that the overall protein pattern was not

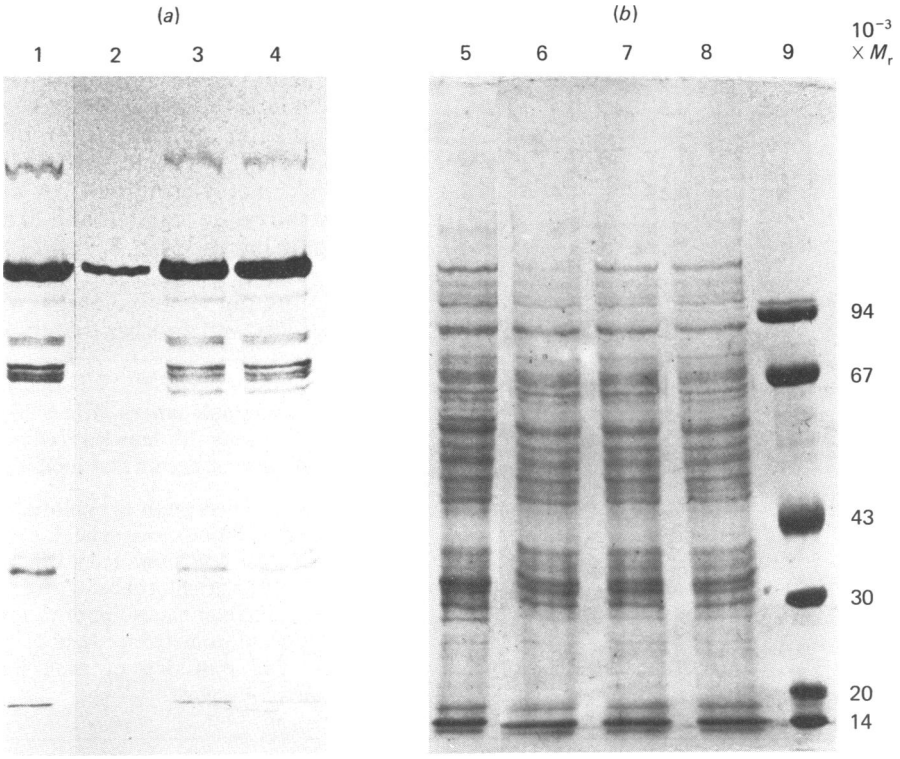


Fig. 1. Effect of passage through an avidin affinity column on the SDS/polyacrylamide-gel-electrophoretic patterns of [^{3}H]biotin-labelled cell extracts

(*a*) Fluorograph pattern (21 days exposure) and (*b*) dried Coomassie-Blue-stained gels after electrophoresis of the following extracts: untreated cell extract (lanes 1, 5); extract passed through an avidin affinity column (lanes 2, 6); extract passed through an avidin affinity column previously saturated with biotin (lanes 3, 7); extract passed through a Sepharose 4B column without prior avidin coupling (lanes 4, 8). Lane 9 contains the molecular-mass markers phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), pancreatic trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa).

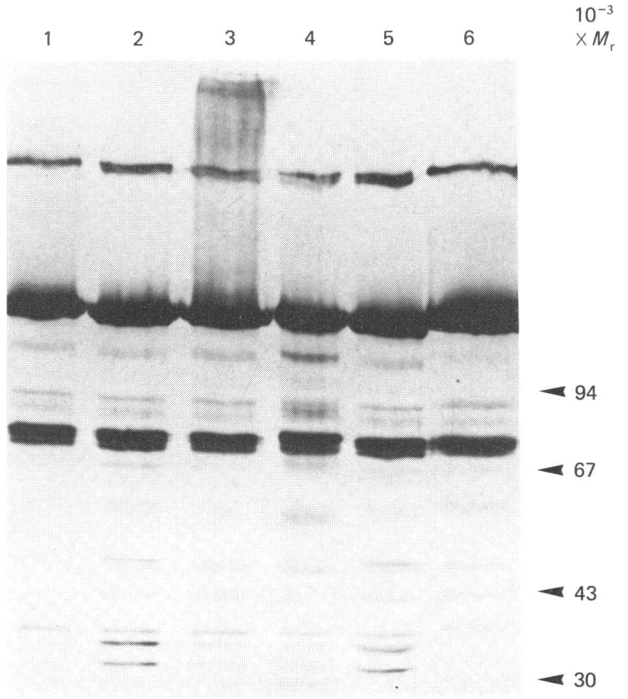
changed by avidin treatment (cf. lanes 5 and 6, Fig. 1). The only difference noted was almost complete removal of the band at 120 kDa after passage of the extract through the affinity column. Control experiments where cell extracts were passed through an avidin column previously saturated with biotin (lanes 3 and 7, Fig. 1) or through a Sepharose 4B column not coupled to avidin (lanes 4 and 8, Fig. 1) demonstrated the specificity of the technique. Both the avidin-addition experiment and that of Fig. 1 establish that the numerous bands found after labelling cells with [^{3}H]biotin represent true biotin-containing proteins.

Possible generation of biotin-containing proteins by proteolysis

Reference to the molecular-mass standards (lane 9, Fig. 1) as well as to myosin heavy chain (205 kDa) suggests that the major labelled proteins have subunit molecular masses of approx. 220, 120, 100, 85 (a doublet), 70–75 (a triplet), 65, 37, 35 and 33 kDa. Many additional bands are found after longer exposure times of the fluorographs (see Figs. 3, 4 and 6). Since the only biotin-containing subunits of proteins in mammalian cells that have been reported previously (Wood & Barden, 1977; Lau *et al.*, 1979; Robinson *et al.*, 1983) are acetyl-CoA carboxylase (220 kDa), pyruvate carboxylase (120 kDa), methylcrotonyl-CoA carboxylase (75 kDa) and propionyl-CoA carboxylase (72 kDa), we have explored the possibility that the extra bands have been generated by proteolysis.

The procedure that we adopted for the electrophoretic analysis of cell extracts involved washing cell monolayers with Hanks' salts, scraping, lysis of the cells with water and homogenization before freezing in liquid N_2 and freeze-drying. Although all solutions were kept at 0 °C and the average time elapsed before freezing was not longer than 3 min, partial hydrolysis of one or more of the known biotin-containing carboxylases may have occurred. The direct addition of trichloroacetic acid to the monolayers to give more rapid fixation decreased the fluorographic intensity of all bands with molecular masses less than 70 kDa, except the 37 kDa protein (cf. lanes 1 and 2, Fig. 2). However, this treatment to prevent proteolysis in cell extracts did not influence the bands with molecular mass above 70 kDa. Essentially similar results were obtained when cells were lysed directly in the presence of 9.5 M-urea (lane 6, Fig. 2). Of the proteolytic inhibitors added during cell harvesting, lima-bean trypsin inhibitor gave equivalent results to those with the water-harvest procedure (cf. lane 5 with lane 2, Fig. 2), whereas phenylmethanesulphonyl fluoride and tosyl-lysylchloromethane (lanes 3 and 4, Fig. 2) produced an intermediate pattern.

A second procedure used to evaluate proteolysis in extracts was to harvest the cells in water at 0 °C and incubate homogenates at 0, 23 and 37 °C for various



periods. Fluorography and quantitation of the radioactivity in individual bands demonstrated different effects of incubation on discrete bands (Fig. 3). Incubations for up to 24 h at 0 °C (Fig. 3) showed no changes or only slight losses in radioactivity from acetyl-CoA carboxylase (band 1), pyruvate carboxylase (band 2) and the combined methylcrotonyl-CoA carboxylase/propionyl-CoA carboxylase region (band 5) as well as the unknown proteins in bands 3, 4, 6, 8, 9 and 10. However, incubation at room temperature divided these unknown bands into two groups, one (bands 3 and 4) which showed increased radioactivity and another (bands 6, 8, 9 and 10) that was

Fig. 2. Fluorograph pattern after SDS/polyacrylamide-gel electrophoresis of labelled cell extracts obtained in the presence of agents that inhibit proteolysis

Cells labelled with [^{3}H]biotin were scraped in the following solutions, dissociated and electrophoresed, and the dried gel was subjected to fluorography for 43 days. Lane 1, 10% trichloroacetic acid; lane 2, water; lane 3, 10 mM-phenylmethanesulphonyl fluoride; lane 4, 1 mM-tosyl-lysylchloromethane; lane 5, lysis buffer (O'Farrell, 1975). The positions of molecular-mass markers are indicated by arrows.

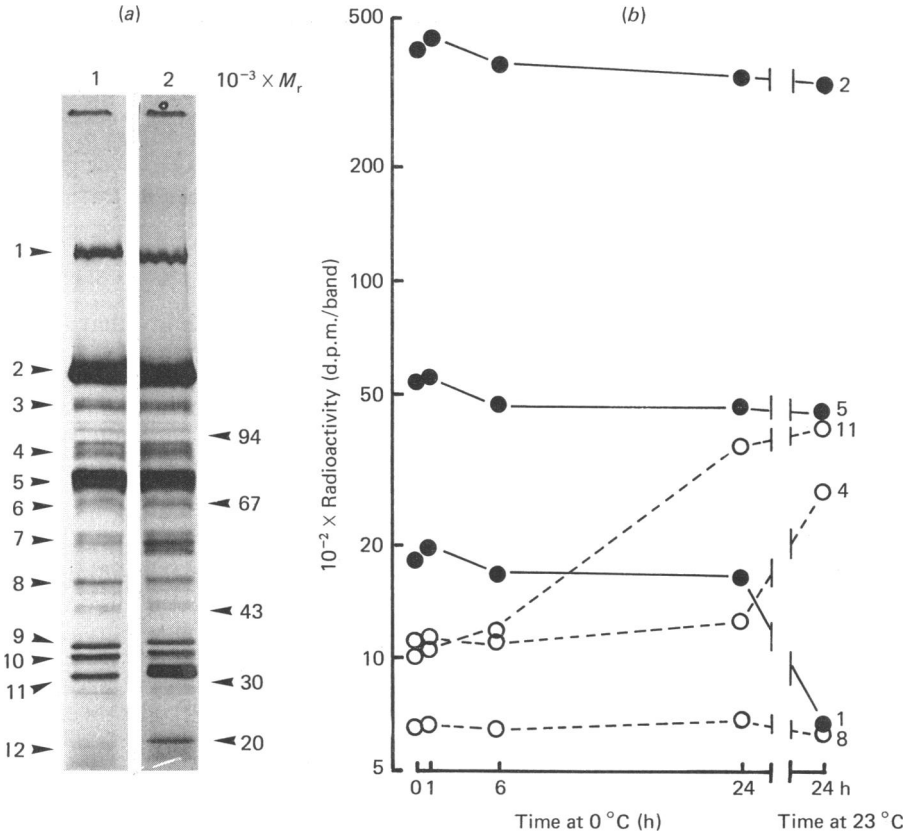


Fig. 3. Fluorography and band radioactivities after SDS/polyacrylamide-gel electrophoresis of labelled cell extracts that had been incubated under different conditions

A single water extract of [^{3}H]biotin-labelled 3T3-L1 cells was incubated for up to 24 h at 0, 23 or 37 °C before electrophoresis and quantification of discrete bands. (*a*) Fluorographs of extracts before incubation (lane 1) and after 24 h incubation at 0 °C (lane 2). The fluorograph was exposed for 14 days. Numbers on the left of the bands are for reference in the text. The positions of molecular-mass markers are indicated on the right of the fluorograph. (*b*) Protein bands located by fluorography of the dried gel were cut out and the radioactivity in each was determined. Band numbers are given alongside the curves showing changes in radioactivity.

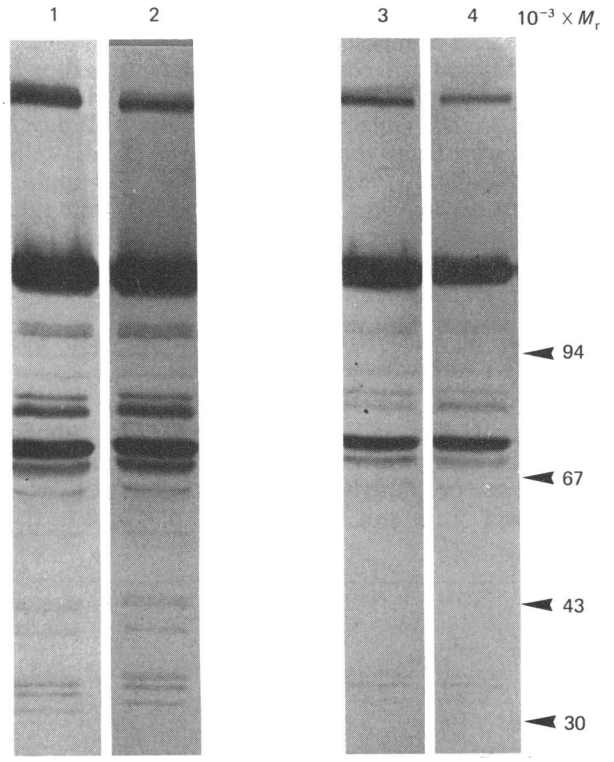


Fig. 4. Effect of short-term labelling and a 24 h chase on the fluorograph pattern of 3T3-L1 cell proteins after SDS/polyacrylamide-gel electrophoresis

Cells were labelled with [^{3}H]biotin for either 42 h (lanes 1, 2) or for 4 h after incubation with avidin for 38 h (lanes 3, 4) before SDS/polyacrylamide-gel electrophoresis of water extracts and fluorography (29 days). The extracts used for lanes 2 and 4 were prepared from cells incubated in chase medium for 24 h after labelling. The positions of molecular-mass markers are indicated.

unchanged or had decreased radioactivity. A third group of unknown proteins showed higher radioactivity on incubation at 0 °C and generally a much greater increase at 23 or 37 °C (bands 7, 11 and 12).

It is likely that bands that increase in fluorographic intensity during incubation of extracts represent proteolytic breakdown products. Moreover, the magnitude of the increases for bands 11 and 12 was so great as to identify them as being derivatives of pyruvate carboxylase, for only this carboxylase band lost sufficient radioactivity. Thus, during 24 h incubation at 23 °C, the radioactivity in pyruvate carboxylase (band 2) was decreased by 9500 d.p.m., whereas that of bands 11 and 12 increased by 3100 and 1900 d.p.m. respectively. Under these same incubation conditions the radioactivity in acetyl-CoA carboxylase fell by only 1200 d.p.m. and that of the dual methylcrotonyl-CoA carboxylase/propionyl-CoA carboxylase band by only 900 d.p.m.

Proteolysis in extracts has been reported as a problem during the isolation of several biotin-containing enzymes, especially liver acetyl-CoA carboxylase and pyruvate carboxylase. Suppression of proteolytic breakdown required the addition of inhibitors such as those used in the experiments described in Fig. 2 (Wood & Barden, 1977).

Band 3, the doublet bands 4 and band 9 neither increased in fluorographic intensity on incubation of extracts at 0 °C (Fig. 3), nor were decreased when proteolysis was prevented with trichloroacetic acid or 9.5 M-urea (Fig. 2). These bands represent 1.4, 1.8 and 1.3% of the total biotin radioactivity respectively in labelled 3T3-L1 cells and total substantially more than the label in acetyl-CoA carboxylase (3% of biotin radioactivity). It seems plausible that these may be previously unreported biotin-containing proteins. Other even less-labelled bands may also qualify as distinct proteins rather than breakdown products.

Changes in biotin-containing proteins during incubation of intact cells

We have tested the stability of labelled biotin proteins within the 3T3-L1 cells by comparing the fluorographic patterns from cells harvested immediately after the labelling period with those incubated for an additional 24 h chase period (Fig. 4). In this Figure lane 1 represents cell proteins at the end of the labelling period and lane 2 cell proteins after the chase. The patterns are almost identical, with perhaps a decrease in acetyl-CoA carboxylase radioactivity during the 24 h incubation. Lanes 3 and 4 are similar to lanes 1 and 2 respectively, except that, instead of the usual 42 h labelling period, the cells were incubated for 38 h in biotin-free medium in the presence of avidin and for only 4 h with [^{3}H]biotin. This procedure results in synthesis of apoenzymes which are rapidly labelled when biotin is added. It has the advantage of a much shorter labelling period and accordingly less time for any putative degradation product to accumulate. Although the intensity of label in each band was decreased to about one-third by the avidin

Table 1. Degradation rate constants of acetyl-CoA carboxylase and pyruvate carboxylase in 3T3-L1 cells

Degradation rate constants (±S.E.M.) were determined from the radioactivities in bands located in dried SDS/polyacrylamide electrophoresis gels by fluorography (Chandler & Ballard, 1985). The values in parentheses are the percentage changes produced by the addition of either NH_4Cl or leupeptin to the incubation medium during the chase period.

	Degradation rate constant (day^{-1})	
Additions	Acetyl-CoA carboxylase	Pyruvate carboxylase
Nil	0.473±0.029	0.278±0.041
10 mM-NH_4Cl	0.401±0.011 (−15%)	0.157±0.061 (−44%)
100 μg of leupeptin/ml	0.500±0.020 (+6%)	0.237±0.041 (−15%)

labelling protocol, the major unknown bands were still present and were similarly unaffected by the 24 h chase.

Since the biotin-containing subunits of acetyl-CoA carboxylase (220 kDa; Wood & Barden, 1977) and pyruvate carboxylase (120 kDa; Wood & Barden, 1977) separated adequately from other labelled proteins after SDS/polyacrylamide-gel electrophoresis, we can measure the degradation rate of these enzymes during the chase period. The respective bands were cut out after fluorography for the quantification of radioactivity. This approach gave degradation rate constants of 0.47 day^{-1} for acetyl-CoA carboxylase and 0.28 day^{-1} for pyruvate carboxylase (Table 1). The value obtained for pyruvate carboxylase is close to that based on decay of total biotin radioactivity in our previous investigation with 3T3-L1 cells, where it was calculated that 85% of the biotin label was present in pyruvate carboxylase (Chandler & Ballard, 1983). The extent of inhibition of pyruvate carboxylase degradation by ammonia was also similar in the two studies, although leupeptin was less effective in the present series of measurements.

The degradation rate constants for pyruvate carboxylase in diploid human fibroblasts and simian-virus-40-transformed human fibroblasts, at 0.32 and 0.40 day^{-1} respectively, were somewhat greater than in 3T3-L1 cells (Chandler & Ballard, 1985). This difference also applies to acetyl-CoA carboxylase, which was degraded with rate constants of 0.66 day^{-1} in diploid fibroblasts and 0.55 day^{-1} in the transformed fibroblasts, as compared with 0.47 day^{-1} in 3T3-L1 cells.

Identification of methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase in cell extracts

Robinson *et al.* (1983) have identified the biotin-containing subunits of 75 and 73 kDa in human fibroblasts as being derived from methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase respectively. Since antisera were not available to distinguish the

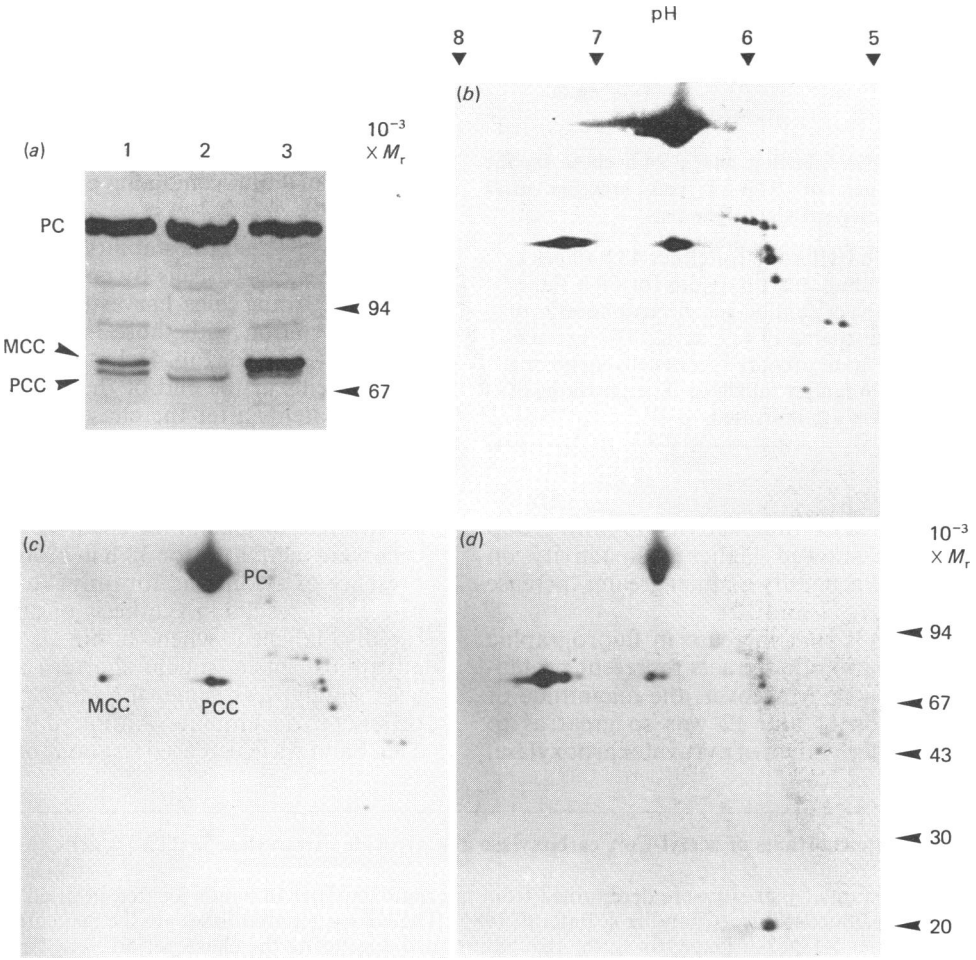


Fig. 5. One- and two-dimensional electrophoresis of [^{3}H]biotin-labelled cell extracts

(*a*) Cell extract (lane 1), the supernatant after treatment of extract with 3% poly(ethylene glycol) (lane 2) and the supernatant after re-extraction of the poly(ethylene glycol) precipitate (lane 3) were analysed by one-dimensional SDS/polyacrylamide-gel electrophoresis followed by a fluorograph exposure of 18 days. Only the region between approx. 140 and 65 kDa is shown, with the position of molecular-mass markers indicated. (*b*), (*c*), (*d*): Two-dimensional electrophoretic analysis of the material analysed in lanes 1, 2 and 3 respectively of (*a*). The pH measured in parallel first-dimension gels is shown on panel (*b*). The migration of molecular-mass markers is indicated in gel (*d*). The spots and bands corresponding to biotin-containing subunits of pyruvate carboxylase (PC), propionyl-CoA carboxylase (PCC) and methylcrotonyl-CoA carboxylase (MCC) are indicated.

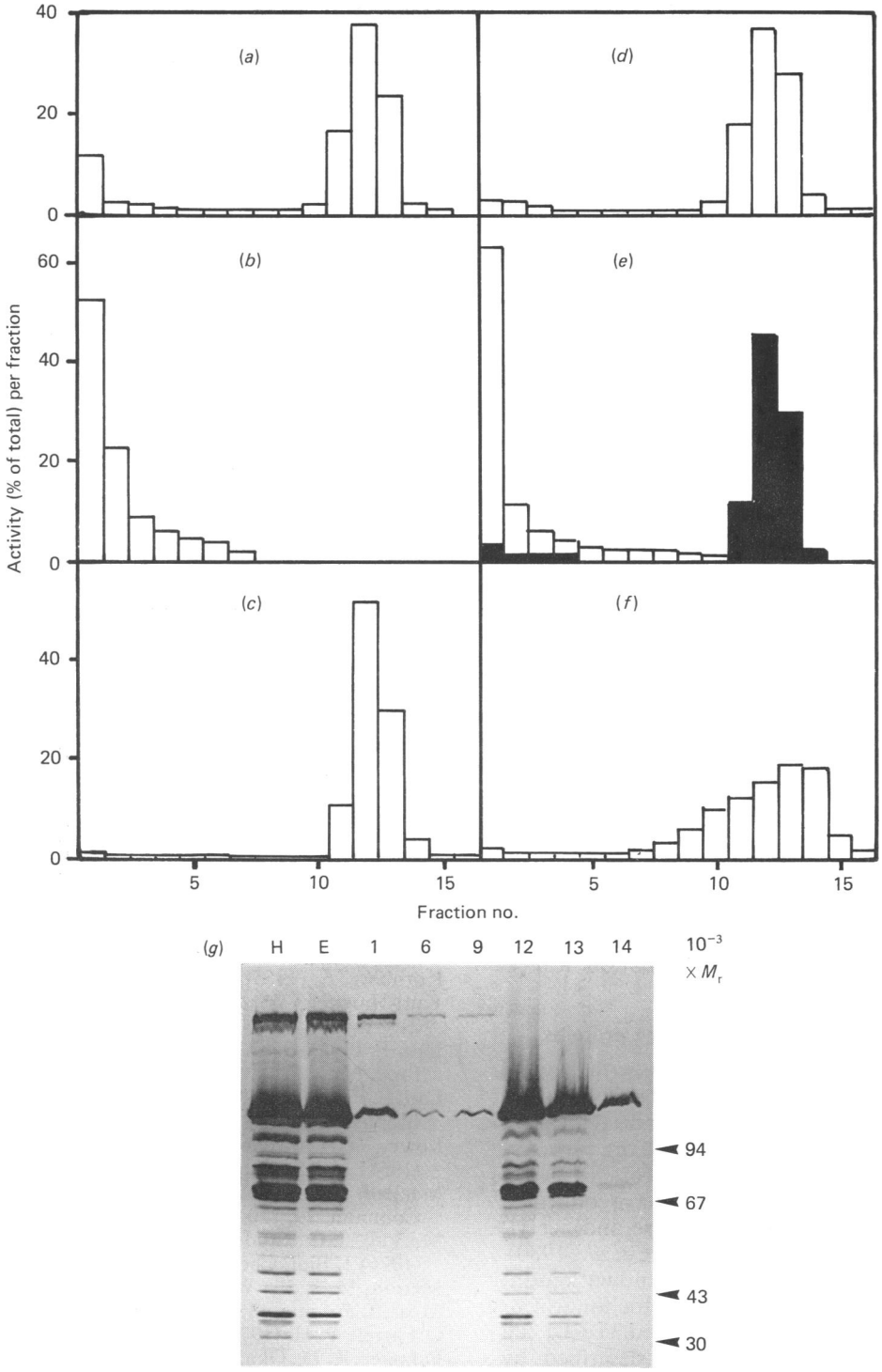


Fig. 6. Intracellular localization of biotin-containing proteins

An extract of [^{3}H]biotin-labelled 3T3-L1 cells was centrifuged in a Percoll gradient as described in the Materials and methods section, and fractions were analysed for (*a*) radioactivity, and for the activities of (*b*) acetyl-CoA carboxylase, (*c*) pyruvate carboxylase, (*d*) propionyl-CoA carboxylase, (*e*) lactate dehydrogenase (□) and glutamate dehydrogenase (■), (*f*) *N*-acetyl-glucosaminidase and (*g*) fluorography after SDS/polyacrylamide-gel electrophoresis of selected fractions. Radioactivity or enzyme activity is indicated as the percentage of recovered material in each 0.6 ml fraction. The fractions were numbered from the top of the gradient. The positions of molecular-mass markers are indicated on the fluorograph, which was exposed for 49 days. The lanes in (*g*) are: H, homogenate; E, extract after centrifugation at 800 *g* for 10 min; 1, 6, 9, 12, 13, 14, samples of the respective gradient fractions.

mobility of these subunits in extracts of mouse 3T3-L1 cells, we used the selective precipitation of methylcrotonyl-CoA carboxylase by poly(ethylene glycol) 6000 to separate the two enzymes. The specific activity of methylcrotonyl-CoA carboxylase after precipitation with poly(ethylene glycol) and solubilization increased from 3.0 to 21.1 munits/mg of protein, whereas propionyl-CoA carboxylase activity was higher, at 6.8 munits/mg of protein in the initial supernatant after poly(ethylene glycol) treatment compared with 3.4 munits/mg of protein in the cell extract. SDS/polyacrylamide-gel-electrophoretic analysis followed by fluorography of cell extract, poly(ethylene glycol) supernatant and solubilized poly(ethylene glycol) precipitate is shown respectively in lanes 1, 2 and 3 of Fig. 5(*a*). Clearly the biotin-containing subunit of methylcrotonyl-CoA carboxylase has the higher molecular mass, in accord with the data reported for human cells (Robinson *et al.*, 1983; Chandler & Ballard, 1985).

The three extracts were also subjected to two-dimensional gel electrophoresis and fluorography (Figs. 5*b*, 5*c* and 5*d*). Fig. 5(*d*), from the poly(ethylene glycol)-precipitated material, shows methylcrotonyl-CoA carboxylase as a double spot focusing near pH 7.2. Only a single spot of much lower intensity is evident at this position after two-dimensional electrophoresis of the first poly(ethylene glycol) supernatant (Fig. 5*c*). All three panels show pyruvate carboxylase as a very dense spot focusing at pH 6.4 and propionyl-CoA carboxylase as a doublet at the same pI. The unidentified spots on the two-dimensional fluorographs focus at isoelectric points below pH 6.2 and are similar in all three extracts, except that a prominent biotin-containing peptide at pI 5.9 and 20 kDa can be seen on electrophoresis of the poly(ethylene glycol) precipitate (Fig. 5*d*). This peptide is probably the same as band 12 (Fig. 3), a derivative of pyruvate carboxylase that accumulates during incubation at 0 °C or higher temperatures.

Intracellular distribution of biotin-containing proteins

Differential centrifugation experiments with several mammalian tissues have established a cytosolic location for acetyl-CoA carboxylase and a mitochondrial location for pyruvate carboxylase, methylcrotonyl-CoA carboxylase and propionyl-CoA carboxylase (Moss & Lane, 1971), and immunohistochemical analyses have confirmed these conclusions for pyruvate carboxylase and acetyl-CoA carboxylase in 3T3-L1 cells (Gratzner *et al.*, 1980). In order to determine whether the unknown biotin-containing proteins are present within mitochondria or are elsewhere in the cell, we have centrifuged 3T3-L1 cell homogenates on a Percoll gradient as described by Merion & Sly (1983) and analysed fractions for radioactivity, pyruvate carboxylase, propionyl-CoA carboxylase, acetyl-CoA carboxylase and marker enzymes (Fig. 6).

Acetyl-CoA carboxylase activity and the expected 220 kDa biotin-containing subunit were found at the top of the Percoll gradient, together with the cytosol marker enzyme lactate dehydrogenase. None of the unknown biotin-containing proteins were present in this fraction (Fig. 6*g*, lane 1) and are thus unlikely to be breakdown products of acetyl-CoA carboxylase. Pyruvate carboxylase, propionyl-CoA carboxylase and all the extra bands detected by fluorography fractionated with mitochondria (compare E with fractions 12 or 13, Fig. 6*g*) as indicated by the marker enzyme glutamate dehydrogenase. With the long fluorograph exposure time of Fig. 6(*g*), some pyruvate carboxylase biotin subunit was detected at the top of the Percoll gradient, presumably as a result of mitochondrial breakage. Neither the microsomal-rich fraction 9 nor the lysosomal-rich fraction 14 contained significant amounts of any unknown biotin-containing protein (Fig. 6*g*). These results provide strong evidence that both unknown distinct biotin-containing proteins and the likely partial proteolytic breakdown products are retained within the mitochondria.

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