Studies on the assay, activity and sedimentation behaviour of acetyl-CoA carboxylase from isolated hepatocytes incubated with insulin or glucagon

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The activity of acetyl-CoA carboxylase, measured in various ways, was studied in 15000g extracts of rat liver hepatocytes and compared with the rate of fatty acid synthesis in intact hepatocytes incubated with insulin or glucagon. Hepatocyte extracts were prepared by disruption of cells with a Dounce homogenizer or by solubilization with 1.5% (v/v) Triton X-100. Sucrose-density-gradient centrifugation demonstrated that the sedimentation coefficient of acetyl-CoA carboxylase from cell extracts was 30–35S, regardless of the conditions of incubation or disruption of hepatocytes. Solubilization of cells with 1.5% Triton X-100 yielded twice as much enzyme activity (measured by [¹⁴C]bicarbonate fixation) in the sucrose-gradient fractions as did cell disruption by the Dounce homogenizer. Analysis by high-performance liquid chromatography of acetyl-CoA carboxylase reaction mixtures showed that [¹⁴C]malonyl-CoA accounted for 10–60% of the total acid-stable radioactivity, depending on the method for disrupting hepatocytes and on the preincubation of the 15000g extract, with or without citrate, before assay. Under conditions in which incubation of cells with insulin or glucagon caused an activation or inhibition, respectively, of acetyl-CoA carboxylase, only 25% of the acid-stable radioactivity was [¹⁴C]malonyl-CoA and enzyme activity was only 13% (control), 16% (insulin), and 57% (glucagon) of the rate of fatty acid synthesis. Under conditions when up to 60% of the acid-stable radioactivity was [¹⁴C]malonyl-CoA and acetyl-CoA carboxylase activity was comparable with the rate of fatty acid synthesis, there was no effect of insulin or glucagon on enzyme activity.

Acetyl-CoA carboxylase (EC 6.4.1.2) catalyses the ATP- and bicarbonate-dependent carboxylation of acetyl-CoA to form malonyl-CoA. The enzyme undergoes polymerization and depolymerization of its filamentous structure (Gregolin *et al.*, 1966a,b). The polymeric form of the enzyme (8 × 10⁶ Da) is active and the protomeric subunit (480000 Da) is inactive (Gregolin *et al.*, 1968a,b; Moss & Lane, 1972). The inactive protomer is converted into the active polymer by incubation with citrate or other carboxylic acids (Martin & Vagelos, 1962; Waite & Wakil, 1962; Vagelos *et al.*, 1963; Matsushashi *et al.*, 1964). It has been suggested that the enzyme exists in the cell as a

polymeric structure (Gregolin *et al.*, 1966a; Meredith & Lane, 1978).

Fatty acid synthesis in isolated rat hepatocytes is enhanced by insulin and inhibited by glucagon. Several studies have shown that acetyl-CoA carboxylase activity, measured in crude cell extracts, correlates with the rate of fatty acid synthesis (Watkins *et al.*, 1977; Geelen *et al.*, 1978; Beynen *et al.*, 1979; Witters *et al.*, 1979). The suggestion has been made that changes in the rate of fatty acid synthesis imposed by insulin or glucagon are due to alterations in the activity of acetyl-CoA carboxylase (Allred & Roehrig, 1973; Watkins *et al.*, 1977; Geelen *et al.*, 1978; Beynen *et al.*, 1979; Witters *et al.*, 1979). The hormonal regulation of hepatic acetyl-CoA carboxylase is thought to occur through changes in the cellular citrate concentration (Watkins *et al.*, 1977), or by a phosphorylation/dephosphorylation mechanism (Carlson &

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Kim, 1973; Allred & Roehrig, 1973; Kim, 1979), or a combination of both (Geelen & Beyen, 1981).

Acetyl-CoA carboxylase activity is routinely measured by the bicarbonate-fixation assay, which involves incubation of the enzyme with [^{14}C]-bicarbonate, acetyl-CoA, MgATP and usually citrate. The reaction is stopped by acid, and reaction mixtures are subsequently dried at 50–70°C. To be quantified, radioactive products of the assay must be both non-volatile and acid-stable. It has been shown that the bicarbonate-fixation assay of acetyl-CoA carboxylase in crude tissue extracts (cf. Allred & Roehrig, 1973; Geelen *et al.*, 1978; Beynen *et al.*, 1979; Witters *et al.*, 1979; Allred & Goodson, 1982) is not a true measure of acetyl-CoA carboxylase activity, because of bicarbonate fixation into compounds other than malonyl-CoA (Davies *et al.*, 1982). Oxaloacetate itself, formed in the assay by the carboxylation of pyruvate, does not contribute to acid-stable radioactivity (Allred & Goodson, 1982).

The aim of the present work was to determine the reliability of the bicarbonate-fixation assay of acetyl-CoA carboxylase in crude hepatocyte extracts as it applies to the hormonal responsiveness of acetyl-CoA carboxylase (Allred & Roehrig, 1973; Geelen *et al.*, 1978; Beynen *et al.*, 1979; Witters *et al.*, 1979; Assimacopoulos-Jeannet *et al.*, 1981).

Parts of this work have appeared in abstract form (Buechler *et al.*, 1981a, 1982).

Experimental

Animals

Male Sprague-Dawley rats (200–250g) were given free access to water and to a standard pellet food.

Chemicals

Acetyl-CoA, ATP, malonyl-CoA, bovine serum albumin (fraction V) and Triton X-100 were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. $\text{NaH}^{14}\text{CO}_3$ (55Ci/mol) was obtained from ICN Chemical and Radioisotope Division, Irvine, CA, U.S.A. $^3\text{H}_2\text{O}$ was purchased from Amersham International, Amersham, Bucks., U.K. Biofluor was obtained from New England Nuclear, Boston, MA, U.S.A. Insulin and glucagon were kindly supplied by Dr. Walter N. Shaw, Lilly Research Laboratories, Indianapolis, IN, U.S.A. All other chemicals were obtained from Fisher Scientific Co., Fair Lawn, NJ, U.S.A.

Isolation and incubation of hepatocytes

Hepatocytes were harvested at 09:00h by the method of Seglen (1976) with the following modifications: the operational temperature was main-

tained at 37°C and 20mM-glucose was added to all buffers used in the isolation, to minimize glycolysis (Harris, 1975). Cell viability as judged by Trypan Blue exclusion was routinely greater than 95%.

Isolated liver cells were incubated in Krebs-Henseleit bicarbonate buffer (1g wet wt. of cells/20ml) supplemented with 10mM-glucose and 3.5% (w/v) bovine serum albumin which had been charcoal-treated (Chen, 1967) and dialysed (Hanson & Ballard, 1968). Incubations were conducted at 37°C in 25ml Erlenmeyer flasks in a metabolic shaker (90 strokes/min). Flasks were continuously gassed with O_2/CO_2 (19:1) during the incubation.

Hormones were freshly dissolved for each experiment. Insulin and glucagon were added to the incubations at final concentrations of 85nM and 10nM respectively.

Measurement of fatty acid synthesis

Hepatocytes were incubated with $^3\text{H}_2\text{O}$ (sp. radioactivity 6.7×10^8 d.p.m./ml). At 30 and 60min, 2ml samples were taken for saponification and extraction of fatty acids as described by Harris (1975). Rates of fatty acid synthesis were calculated between 30 and 60min of incubation.

Acetyl-CoA carboxylase assay

Acetyl-CoA carboxylase activity was assayed at 37°C for 2.0 and 3.0min in crude hepatocyte extracts and in fractions of sucrose-density gradients respectively. Enzyme activity was proportional to time in both assays. Reactions were started by addition of 25 μl of cell extract (0.3–0.5mg of protein) or 25 μl of sucrose-gradient fraction to 225 μl of the assay medium. The assay included: 50mM-potassium phosphate, pH7.0; 3mM-mercaptoethanol; fatty acid-free and dialysed bovine serum albumin (0.4mg/ml); 6mM- MgCl_2 ; 2mM-ATP; 0.5mM-acetyl-CoA; 2mM-citrate; 20mM- KHCO_3 and $\text{NaH}^{14}\text{CO}_3$ (1.5Ci/mol). Alternatively, when enzyme activity was measured in the presence of 20mM-citrate, the MgCl_2 concentration was 20mM. Reactions were stopped by the addition of 25 μl of 6M-HCl and immediately placed on ice. Reaction tubes were centrifuged at 15000g for 1min. Samples (80 μl) were evaporated at 65°C, the dried residue was resuspended in 0.2ml of aq. 50% (v/v) ethanol, and 5ml of Biofluor was added.

Analysis of enzyme reaction mixtures by high-performance liquid chromatography

Malonyl-CoA in enzyme reaction mixtures was analysed as malonate because acid hydrolysis of malonyl-CoA could occur in the HCl-stopped assays. Enzyme reaction mixtures were hydrolysed in 1.3M-KOH for 90min at 65°C. As judged by

t.l.c. and high-performance liquid chromatography, the alkaline hydrolysis of malonyl-CoA was found to be complete. After hydrolysis, samples were acidified to pH 2.0 with HClO_4 and centrifuged at 15000g for 3 min. Samples (155 μl) were analysed for [^{14}C]bicarbonate fixation into malonate, malate and citrate on a Supelco C18 reversed-phase column (4.6 mm $\times$ 250 mm). The mobile phase was 0.2% (v/v) phosphoric acid. The flow rate was 0.5 ml/min. As carriers 53 μg of each organic acid to be separated was added to the samples. Malate, malonate and citrate were eluted as single peaks, with retention times of 8.2, 8.8 and 18.0 min respectively, and were detected by a Waters R401 differential refractometer. Recovery from cell extracts of malate, malonate and citrate was greater than 99% as judged by the recovery of radioactive standards.

Other enzyme assays

At the end of incubation, hepatocytes were disrupted with a Dounce homogenizer or were solubilized by vortex-mixing with 1.5% (v/v) Triton X-100. The activities of lactate dehydrogenase (EC 1.1.1.27) (Bergmeyer & Bernt, 1970), glucose-6-phosphate dehydrogenase (EC 1.1.1.49) (Löhr & Waller, 1970) and glutamate dehydrogenase (EC 1.4.1.2) (Schmidt, 1970) were measured in the 15000g supernatants of these homogenates.

Sucrose-density-gradient centrifugation

Linear gradients containing 7–23% (w/v) sucrose were prepared by the method of Martin & Ames (1961). The 5 ml gradient was slowly formed in a tube designed for a Beckman SW 50.1 rotor and was preheated at 30°C. After careful application of the enzyme sample, the tubes were centrifuged at 45000 rev./min (189000g) at 30°C for 120 min. Gradients were fractionated into 19 fractions, each containing approx. 0.3 ml, by puncturing the bottom of the centrifuge tube. Samples of each fraction were used to measure enzyme activity and sucrose concentration. The linearity of the sucrose gradients was analysed as described by Buechler *et al.* (1981b).

Protein assay

Protein was assayed by the method of Lowry *et al.* (1951), with bovine serum albumin as a standard.

Results

Rates of fatty acid synthesis by isolated hepatocytes measured between 30 and 60 min of incubation were 0.77 ± 0.04 nmol of acetyl units/min per mg of cellular protein (means $\pm$ S.E.M. for three

hepatocyte preparations). In the presence of glucagon (10 nM) and insulin (85 nM) added after 30 min incubation, fatty acid synthesis rates were 0.07 ± 0.01 and 1.23 ± 0.21 nmol of acetyl units/mg of cellular protein respectively.

The activity (as measured by bicarbonate fixation into total acid-stable products) and sedimentation velocity of acetyl-CoA carboxylase were measured in extracts of these hormonally responsive cells. Hepatocytes were disrupted with a Dounce homogenizer or were solubilized by vortex-mixing with 1.5% Triton X-100. The amount of total protein in the 15000g supernatant of the cell homogenate prepared with Triton X-100 was about twice that prepared with the Dounce homogenizer (9.14 and 5.40 mg/ml of supernatant respectively for 11.42 mg of cellular protein). The activity of the cytosolic enzyme lactate dehydrogenase was similar in supernatants (15000g) prepared with Triton X-100 or the Dounce homogenizer (0.65 and 0.70 $\mu\text{mol/min}$ per mg of cellular protein respectively), but Triton X-100 treatment yielded more of the cytosolic enzyme glucose 6-phosphate dehydrogenase (0.98 and 0.36 nmol/min per mg of cellular protein) and of the mitochondrial enzyme glutamate dehydrogenase (1.42 and 0.10 $\mu\text{mol/min}$ per mg of cellular protein) than did the Dounce homogenizer.

Fig. 1 shows that the method of cell disruption

Fig. 1. Effect of different cell-disruption methods on the sedimentation pattern of acetyl-CoA carboxylase. Hepatocytes were disrupted with a Dounce homogenizer (□) or were solubilized with 1.5% Triton X-100 (○) as described in the legend of Table 1. The homogenates were centrifuged at 15000g for 30 s to remove insoluble material. A sample of the supernatant was layered on to a sucrose gradient. Fractions of the sucrose gradient were collected and preincubated for 60 min with 20 mM-citrate. Acetyl-CoA carboxylase was assayed by bicarbonate fixation.

had no effect on the sedimentation velocity of acetyl-CoA carboxylase, but that the activity of the enzyme, measured in sucrose-gradient fractions, was twice as much for the Triton X-100 extract as the activity measured for the Dounce homogenate. The use of Tris/HCl or Tris/acetate rather than potassium phosphate as the homogenization and gradient buffer, in the presence or absence of 20mM-citrate, did not affect the sedimentation velocity of the enzyme (results not shown). Likewise, the presence or absence of NaF (50 or 100mM) and/or EDTA (5 or 10mM) in the homogenization and gradient buffer did not affect enzyme activity in the hepatocyte extract or the sedimentation velocity of the enzyme.

Hepatocytes were incubated for 30min with insulin or glucagon, after which cell extracts were prepared with the Dounce homogenizer. The rate of incorporation of [14 C]bicarbonate into total acid-stable products during the acetyl-CoA carboxylase assay was significantly increased or decreased, respectively (Table 1, condition 3), in these cell extracts. Treatment of the hepatocytes with insulin and glucagon did not alter the sedimentation velocity of the enzyme (results not shown). In addition, enzyme activities measured in sucrose-gradient fractions were identical for control cells and for those incubated with glucagon or

insulin. Sucrose-gradient fractions either were not preincubated or were preincubated for 30min with 20mM-citrate before assay. These results suggest either that hormonal effects on acetyl-CoA carboxylase do not survive the sucrose-gradient centrifugation or that the hormonal effects on total acid-stable products formed from [14 C]bicarbonate do not represent an effect on acetyl-CoA carboxylase. To test the latter possibility we have determined the identity of several acid-stable non-volatile reaction products.

Table 1 compares rates of [14 C]bicarbonate fixation into total acid-stable non-volatile products, malate, malonyl-CoA and citrate during the acetyl-CoA carboxylase assay. The presence of NaF (50 or 100mM) and/or EDTA (5 or 10mM) in the homogenization buffer did not affect [14 C]bicarbonate fixation in these products (results not shown). When cells were solubilized with Triton X-100, rates of [14 C]malonyl-CoA production were not affected by glucagon or insulin, whereas glucagon caused a decrease in the fixation of [14 C]bicarbonate into total acid-stable products, malate and citrate (Table 1, condition 1). Changes in production of [14 C]malate and [14 C]citrate may reflect the observed (Beynen *et al.*, 1979) different amounts of pyruvate in extracts from cells incubated with insulin and glucagon. Preincubation of

Table 1. Incorporation of [14 C]bicarbonate into acid-stable non-volatile products in extracts of isolated hepatocytes. Hepatocytes were incubated for 30min. Subsequently, insulin (85nM) or glucagon (10nM) was added and incubations were continued for an additional 30min. Samples of hepatocytes, representing about 0.25g wet wt., were centrifuged at 100g for 45s at 25°C. The incubation medium was aspirated and 1.25ml of 50mM-potassium phosphate, pH 7.0, 3mM-mercaptoethanol and 0.5mM-EGTA was added to the cell pellet. Cells were either disrupted with a Dounce homogenizer (25 strokes) or solubilized by vortex-mixing with 1.5% (v/v) Triton X-100 for 7s. Homogenates were centrifuged in an Eppendorf Microfuge at 15000g for 30s, and 25 μ l samples of the supernatants were taken for assay of enzyme activity. Where indicated, samples of the supernatants were preincubated for 45min at 37°C with 20mM-citrate plus 20mM-MgCl₂ before assay. Enzyme reaction products were analysed by high-performance liquid chromatography. Results are the means $\pm$ S.E.M. for four determinations. Experimental data are shown for one hepatocyte preparation; the results have been reproduced with other cell preparations.

Condition	Disruption of pelleted cells	Preincubation of supernatant	Additions to cell suspensions	Bicarbonate fixation (nmol/min per mg of protein) into:			
				Total acid-stable products	Malate	Malonyl-CoA*	Citrate
1	Triton X-100	No	None	3.75 $\pm$ 0.10	0.15 $\pm$ 0.01	0.88 $\pm$ 0.09	0.41 $\pm$ 0.03
			Glucagon	2.60 $\pm$ 0.12	0.04 $\pm$ 0.01	0.83 $\pm$ 0.08	0.20 $\pm$ 0.04
			Insulin	3.87 $\pm$ 0.19	0.16 $\pm$ 0.02	0.88 $\pm$ 0.10	0.42 $\pm$ 0.08
2	Triton X-100	Yes	None	5.96 $\pm$ 0.31	<0.01	2.12 $\pm$ 0.25	0.26 $\pm$ 0.03
			Glucagon	4.42 $\pm$ 0.33	<0.01	2.41 $\pm$ 0.18	0.19 $\pm$ 0.04
			Insulin	6.91 $\pm$ 0.51	<0.01	2.90 $\pm$ 0.21	0.67 $\pm$ 0.13
3	Dounce	No	None	0.39 $\pm$ 0.07	0.11 $\pm$ 0.02	0.10 $\pm$ 0.01	0.03 $\pm$ 0.01
			Glucagon	0.15 $\pm$ 0.01	0.06 $\pm$ 0.01	0.04 $\pm$ 0.01	0.01 $\pm$ 0.01
			Insulin	0.71 $\pm$ 0.05	0.13 $\pm$ 0.05	0.20 $\pm$ 0.02	0.08 $\pm$ 0.01
4	Dounce	Yes	None	4.61 $\pm$ 0.24	<0.01	3.11 $\pm$ 0.29	<0.01
			Glucagon	4.95 $\pm$ 0.30	<0.01	3.36 $\pm$ 0.20	<0.01
			Insulin	5.26 $\pm$ 0.36	<0.01	3.40 $\pm$ 0.22	<0.01

* Malonyl-CoA was separated from enzyme reaction mixtures as malonate.

extracts solubilized with Triton X-100 for 45 min with 20 mM-citrate plus 20 mM-MgCl₂ increased the fixation of [¹⁴C]bicarbonate into malonyl-CoA approx. 3-fold (Table 1, condition 2). Glucagon and insulin affected [¹⁴C]bicarbonate fixation into citrate, but did not affect malonyl-CoA formation (Table 1, condition 2).

Homogenization of cells with the Dounce homogenizer as compared with solubilization by Triton X-100 gave much lower rates of [¹⁴C]bicarbonate fixation into acid-stable products and malonyl-CoA when extracts were not preincubated with citrate and Mg²⁺ (Table 1, conditions 1 and 3). Glucagon decreased and insulin increased [¹⁴C]bicarbonate fixation into total acid-stable products, malate, malonyl-CoA and citrate (Table 1, condition 3), but preincubation of extracts with citrate and Mg²⁺ before assay diminished the hormone effects and activated acetyl-CoA carboxylase up to 30-fold (Table 1, condition 4). Of the four assay conditions shown in Table 1, only one (condition 3) indicated that insulin and glucagon affected the rate of production of [¹⁴C]malonyl-CoA.

Discussion

Acetyl-CoA carboxylase activity as measured, without preincubation, by the fixation of bicarbonate into total acid-stable products is much higher in Triton X-100 extracts of hepatocytes than in extracts prepared with the Dounce homogenizer (Table 1, conditions 1 and 3). This difference is most likely due to the presence of mitochondrial enzymes in the Triton X-100 extracts, which is supported by the observed higher glutamate dehydrogenase activity. This contamination probably causes generation of acid-stable products other than malate, malonyl-CoA and citrate, as the sum of radioactivity in these compounds is much smaller than the radioactivity in total acid-stable products (Table 1, condition 1). Furthermore, in the Triton X-100 extracts there was a substantial formation of radioactive citrate, which may be produced from oxaloacetate earlier generated by pyruvate carboxylase (cf. Davies *et al.*, 1982). Although acetyl-CoA carboxylase is not located in the mitochondria, the Triton X-100 method gave higher rates of malonyl-CoA production, as observed without preincubation (Table 1). Possibly Triton X-100 unmask more acetyl-CoA carboxylase, a similar observation being made for glucose-6-phosphate dehydrogenase.

The effect of glucagon on the formation of total acid-stable products with and without preincubation of Triton X-100 extracts of hepatocytes did not reflect the hormonal effect on acetyl-CoA carboxylase, as malonyl-CoA generation was not

affected (Table 1, conditions 1 and 2). Thus the measurement of acetyl-CoA carboxylase by bicarbonate fixation into total acid-stable products in Triton X-100 extracts may give misleading results. This also holds for the measurement in extracts of cells disrupted with a Dounce homogenizer. In these extracts the determination of total acid-stable products did not reflect true acetyl-CoA carboxylase activity in absolute rates either. With extracts prepared with the Dounce homogenizer without preincubation hormonal effects on malonyl-CoA production were seen, which may reflect an effect on acetyl-CoA carboxylase.

The measured activity of acetyl-CoA carboxylase varies for the control condition (Table 1) from 0.10 to 3.11 nmol of [¹⁴C]bicarbonate fixed into malonyl-CoA/min per mg of protein and is dependent both on the method used for hepatocyte disruption and on the preincubation of the enzyme with or without citrate before assay. This range of activity represents a 30-fold difference, and it is unknown where in this range lies the activity of the enzyme which reflects the activity in the cell. In order to measure enzyme activity one has to break up the cell, and this may lead to an erroneous estimation of enzyme activity, as the structural integrity of the cell and the physiological environment of the enzyme are destroyed. In view of these uncertainties, it is difficult to make quantitative comparisons between flux rates in the intact cell and enzyme activities in cell extracts. However, if we do so, the results of Table 1 (condition 1) show that, when acetyl-CoA carboxylase activity is comparable with the rate of fatty acid synthesis (0.77 nmol of acetyl units/min per mg of protein) insulin and glucagon do not bring about a change in enzyme activity. When the measured acetyl-CoA carboxylase activity appears to be hormone-sensitive (Table 1, condition 3), the activity of this enzyme is only 13% (control), 16% (+ insulin) and 57% (+ glucagon) of the rate of fatty acid synthesis *de novo* (see the Results section).

The sedimentation coefficient of acetyl-CoA carboxylase from hepatocyte extracts is 30–35S, irrespective of the method used for hepatocyte disruption (Fig. 1) or of the incubation of the cells with insulin or glucagon. The observation that insulin and glucagon affected acetyl-CoA carboxylase activity (Table 1, condition 3) in the cell homogenate, but not in sucrose-gradient fractions (results not shown), suggests that such changes in apparent enzyme activity are not persistent. The addition of 20 mM-citrate to cell extracts and to sucrose gradients did not affect the sedimentation coefficient of the enzyme, suggesting that acetyl-CoA carboxylase from hepatocyte extracts is fully polymerized.

In conclusion, the activity of acetyl-CoA carb-

oxylase (as measured by malonyl-CoA production) from extracts of isolated hepatocytes can be demonstrated to be sensitive to incubation of intact cells with insulin or glucagon only under specific assay conditions. Under these conditions, the rate of fatty acid synthesis in the intact cell is 2–10 times higher than the measured activity of acetyl-CoA carboxylase. We consider that additional techniques for the assay of acetyl-CoA carboxylase are required to clarify the enzyme's role as a target for short-term insulin and glucagon action.

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