Purification and Some Properties of Acetyl-Coenzyme A Carboxylase from Rabbit Mammary Gland

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1. Acetyl-CoA carboxylase from lactating-rabbit mammary gland was purified to homogeneity by the criterion of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. 2. Use of phosphate buffer throughout the purification gave low recovery of enzyme. Consequently, Tris buffers were used in the extraction and in selected stages of the purification procedure. 3. The purified enzyme had a specific activity of $5.15 \pm 0.3 \mu\text{mol}$ of bicarbonate incorporated/min per mg of protein (mean $\pm$ s.e.m. of five preparations). This represents a purification of 257 ± 16 -fold and a yield of $4.3 \pm 0.13\%$. 4. The kinetic parameters of the purified enzyme were similar to those reported for the enzyme from other tissue sources. 5. The enzyme was assayed by a spectrophotometric assay and by a [^{14}C]bicarbonate-fixation assay. Short incubations were used in the radiochemical assay to avoid substantial loss of [^{14}C]bicarbonate.

Acetyl-CoA carboxylase [acetyl-CoA-CO₂ ligase (ADP-forming), EC 6.4.1.2] catalyses the carboxylation of acetyl-CoA in the biosynthesis of fatty acids *de novo* [see Lane *et al.* (1974) for review]. The enzyme can play a critical role in the acute and chronic regulation of fatty acid synthesis (Numa, 1974).

Acetyl-CoA carboxylase has been highly purified from several animal tissues, including chicken liver (Gregolin *et al.*, 1968a; Majerus & Kilburn, 1969), rat liver (Nakanishi & Numa, 1970; Inoue & Lowenstein, 1972), lactating-rat mammary gland (Miller & Levy, 1969) and bovine adipose tissue (Moss *et al.*, 1972). In most of these reports phosphate buffers were used in the extraction and in the subsequent purification steps. The present report describes the isolation of the purified enzyme from the mammary glands of lactating rabbits. Conventional purification procedures have been used, which are essentially similar to those used to purify the enzyme from other tissues. The present method, however, differs from most others in that Tris buffers were used in the extraction of the enzyme and in selected stages of the purification procedure. Our attempts to prepare the purified enzyme in acceptable yields using phosphate buffers have met with repeated failures.

Materials and Methods

Animals

New Zealand white rabbits were obtained, on the 15th day of lactation, from the Joint Animal Breeding Unit, University of Nottingham School of Agriculture, Sutton Bonington, Leics., U.K.

Materials

NADPH and ATP were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Calcium phosphate gel and dithiothreitol were purchased from BDH Chemicals, Poole, Dorset, U.K. Sepharose 2B was obtained from Pharmacia (G.B.) Ltd., London W.5, U.K., and DEAE-cellulose (Whatman grade DE 23) from W. and R. Balston (Modified Cellulose) Ltd., Maidstone, Kent, U.K. Bovine serum albumin (fraction V, fatty acid-poor) was purchased from Miles Seravac Pty., Maidenhead, Berks., U.K., and glycerol (B.P. grade) was from Hills Pharmaceuticals, Briercliffe, Burnley BB10 2JY, U.K. NaH¹⁴CO₃ was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. CoA was purchased from Boehringer Corp. (London) Ltd., London W5 2TZ, U.K., and sucrose was from May and Baker, Dagenham, Essex, U.K. The other materials used were described by Betts & Mayer (1975). All other chemicals were of A.R. grade. The Natelson microgasometer was purchased from Scientific Industries, Springfield, Mass. 01103, U.S.A.

Preparation and assay of acetyl-CoA

Acetyl-CoA was prepared by the method of Smith *et al.* (1966), and assayed by the method of Chase (1967).

Assay of acetyl-CoA carboxylase

Enzyme activity was determined by the [^{14}C]bicarbonate-fixation assay (Inoue & Lowenstein,

1975), or by a spectrophotometric assay (Numa, 1969).

(i) [^{14}C]Bicarbonate-fixation assay. The assay system contained, in a final volume of 0.4 ml, 100 mM-Tris/HCl buffer, pH 7.5, 20 mM-MgCl₂, 2 mM-ATP, 25 mM-potassium citrate, 0.3 mM-acetyl-CoA, 0.4 mg of bovine serum albumin, 20 mM-NaH $^{14}\text{CO}_3$ (0.4 $\mu\text{Ci}/\mu\text{mol}$) and enzyme (1–2.5 munits). The reaction was started by adding the ATP and NaH $^{14}\text{CO}_3$ (in 0.1 ml) after incubating the other components for 15 min at 37°C in a shaking water bath. Incubations were performed in duplicate and control incubations without acetyl-CoA or without enzyme were also carried out.

After 2 min the reaction was stopped with 0.1 ml of 5M-HClO₄ and the residual [^{14}C]bicarbonate was removed by the method of Smith *et al.* (1966). The radioactivity in a sample of the final supernatant was measured by the method of Manning & Brindley (1972).

Preliminary experiments, designed to measure the loss of bicarbonate during an incubation, involved incubation of the assay medium without enzyme for 30 min at 37°C. At intervals samples were taken for measurement of bicarbonate concentration by using a microgasometer (Natelson, 1951) and for measurement of radioactivity.

(ii) Spectrophotometric assay. This was a modification of the method of Numa (1969), and was used as a routine to monitor the purification of the enzyme. The reactants were the same as in the [^{14}C]bicarbonate-fixation assay, with the addition of NADPH (0.24 mM) and partially purified fatty acid synthetase (more than 50 nmol of NADPH oxidized/min at 30°C).

The reaction mixture was preincubated in the absence of acetyl-CoA carboxylase for 5 min at 30°C, and the reaction was started by the addition of enzyme (2–5 munits), to give a final volume of 1 ml. The reaction was measured at 340 nm at 30°C.

One unit of acetyl-CoA carboxylase activity is that which catalyses the carboxylation of 1 μmol of acetyl-CoA/min.

Partial purification of fatty acid synthetase from mammary gland of lactating rabbit

Fatty acid synthetase was partially purified by a modification of the method of Carey & Dils (1970), in which chromatography on DEAE-cellulose was omitted. The enzyme was assayed at 30°C by the method of Carey & Dils (1970). The specific activity of the enzyme was 0.45 μmol of NADPH oxidized/min per mg of protein.

Purification of acetyl-CoA carboxylase

All operations were performed at 4°C unless otherwise stated.

(1) *Preparation of particle-free supernatant fraction.* Four rabbits (15 days *post partum*) were used for each preparation of the enzyme. The animals were killed by cervical dislocation and the mammary glands (100–150 g fresh wt. per animal) were removed. The particle-free supernatant was prepared by a modification of the method of Betts & Mayer (1975). The tissue was homogenized in 2 vol. of 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 5 mM-KHCO₃, 20% (v/v) glycerol, 0.5 mM-EDTA and 0.2 mM-dithiothreitol.

(2) *Ammonium sulphate fractionation.* Solid (NH₄)₂SO₄ was added slowly, with stirring, to the particle-free supernatant to give a concentration of 176 g/litre of initial volume. After stirring for 30 min the precipitate was collected by centrifugation at 25000 *g*_{av} for 15 min. The supernatant was discarded, and the precipitate was dissolved in 50 mM-potassium phosphate buffer, pH 7.5, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.2 mM-dithiothreitol. The enzyme was dialysed overnight against 30 vol. of the same buffer.

(3) *Calcium phosphate gel fractionation.* The enzyme solution was centrifuged at 25000 *g*_{av} for 15 min to remove any undissolved protein and was then mixed with a suspension of calcium phosphate gel (gel/protein ratio 1.0, w/w). The concentration of potassium phosphate was adjusted to 50 mM by adding 1 M-potassium phosphate buffer, pH 7.5. The gel was removed after 10 min by centrifugation at 2500 *g*_{av} for 10 min and the supernatant was discarded. The gel was washed by resuspension in 50 mM-potassium phosphate buffer, pH 7.5, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.2 mM-dithiothreitol (1 ml/15 mg of calcium phosphate gel). The suspension was centrifuged at 2500 *g*_{av} for 10 min and the supernatant was discarded. The enzyme was eluted by washing the gel twice with 0.25 M-potassium phosphate buffer, pH 7.0, containing 20% (v/v) glycerol, 0.1 mM-EDTA and 0.2 mM-dithiothreitol (1 ml/15 mg of calcium phosphate gel). After centrifugation at 2500 *g*_{av} for 10 min the supernatants were combined and EDTA (0.1 M, previously adjusted to pH 7.0 with KOH) was added to give a final concentration of 0.5 mM.

(4) *Concentration of enzyme solution.* The method of enzyme concentration was based on that of Gregolin *et al.* (1968a). The combined supernatants were dialysed overnight against 25 vol. of 50 mM-potassium phosphate buffer containing 20% (v/v) glycerol, 0.5 mM-EDTA, 0.2 mM-dithiothreitol and 225 g of (NH₄)₂SO₄/litre of dialysis medium (final pH 7.0). The protein precipitate was collected by centrifugation at 25000 *g*_{av} for 15 min, dissolved in 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-NaCl, 20% (v/v) glycerol, 0.5 mM-EDTA, 0.2 mM-dithiothreitol, and dialysed overnight against 30 vol. of the same buffer.

(5) *Chromatography on DEAE-cellulose.* The enzyme solution was applied to a DEAE-cellulose column (2.5 cm × 30 cm) which had been previously equilibrated with 50 mM-Tris/HCl buffer, pH 7.5, containing 50 mM-NaCl, 20% (v/v) glycerol, 0.5 mM-EDTA and 0.2 mM-dithiothreitol. The column was washed with 1 column volume of the same buffer and the enzyme was eluted with a linear gradient (800 ml) of NaCl (50–300 mM) in the same buffer, at a flow rate of 60 ml/h.

(6) *Concentration of enzyme solution.* Fractions (5 ml) containing the highest enzyme activities were pooled and were dialysed overnight against 50 mM-Tris buffer containing 0.2 M-NaCl, 20% (v/v) glycerol, 0.5 mM-EDTA, 0.2 mM-dithiothreitol and 291 g of $(\text{NH}_4)_2\text{SO}_4$ /litre of dialysis medium. The final pH of this solution was adjusted to 7.5 with HCl. The precipitated protein was recovered by centrifugation at 25000 g_{av} for 15 min, then was dissolved in the minimum volume of 50 mM-potassium phosphate buffer, pH 7.0, containing 10% (v/v) glycerol, 0.5 mM-EDTA and 0.2 mM-dithiothreitol. The enzyme was dialysed overnight against 100 vol. of the same buffer.

(7) *Chromatography on Sepharose 2B.* Potassium citrate (0.5 M, previously adjusted to pH 7.0 with HCl) was added to the enzyme solution to give a final concentration of 25 mM. After very gentle mixing the solution was incubated at 30°C for 15 min. The enzyme was applied to the bottom of a Sepharose 2B column (2.5 cm × 60 cm), at room temperature, previously equilibrated with 50 mM-potassium phosphate buffer, pH 7.0, containing 25 mM-potassium citrate, 10% (v/v) glycerol, 0.5 mM-EDTA and 0.2 mM-dithiothreitol. Elution was carried out by pumping upwards through the column the same buffer at a flow rate of 12 ml/h, at room temperature.

(8) *Concentration of enzyme solution.* Fractions (2 ml) with the highest enzymic activity from the Sepharose 2B column were pooled and were dialysed overnight against 100 vol. of 50 mM-Tris/HCl buffer, pH 7.5, containing 0.2 M-NaCl, 10% (v/v) glycerol, 0.5 mM-EDTA, 0.2 mM-dithiothreitol, and then dialysed overnight against 100 vol. of the same buffer containing 361 g of $(\text{NH}_4)_2\text{SO}_4$ /litre of dialysis medium (final pH 7.5).

The precipitate was collected by centrifugation at 25000 g_{av} for 15 min and dissolved in the minimum volume of 50 mM-potassium phosphate buffer, pH 7.0, 20% (v/v) glycerol, 0.5 mM-EDTA and 0.2 mM-dithiothreitol. Potassium citrate (0.5 M, previously adjusted to pH 7.0 with HCl) was added to give a final concentration of 25 mM. The enzyme was dialysed overnight against 100 vol. of 50 mM-potassium phosphate buffer, pH 7.0, containing 25 mM-potassium citrate, 20% (v/v) glycerol, 0.5 mM-EDTA and 0.2 mM-dithiothreitol. The enzyme was frozen in small samples at -20°C and stored for periods up to 1 month with little loss of activity (approx. 10%).

In some preparations there was an indication of some impurity in the enzyme from the Sepharose 2B column as judged by the criterion of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. In these cases the enzyme was further purified by sucrose-density-gradient centrifugation.

(9) *Sucrose-density-gradient centrifugation.* The precipitate from the last concentration step was dissolved in the minimum volume of 50 mM-potassium phosphate buffer, pH 7.0, containing 0.5 mM-EDTA and 0.2 mM-dithiothreitol. Potassium citrate (0.5 M, previously adjusted to pH 7.0 with HCl) was added to give a final concentration of 25 mM, and the enzyme solution was incubated at 30°C for 15 min.

Linear gradients from 5 to 20% (w/v) sucrose in 50 mM-potassium phosphate buffer, pH 7.0, containing 25 mM-potassium citrate, 0.5 mM-EDTA and 0.2 mM-dithiothreitol were prepared at room temperature. Approx. 1 ml of protein solution (containing less than 3 mg of protein) was carefully applied to the top of each gradient to give a total volume of 20 ml. The tubes were centrifuged at 94000 g_{av} for 4 h at 20°C in the MSE Centriscan 75 instrument with an aluminium swing-out rotor (3 × 25 ml). The tubes were pierced and fractions (1.1 ml) containing the highest enzyme activity were pooled, and the enzyme was concentrated and stored as described previously.

Protein determination

Protein at high concentration (5–30 mg/ml) was determined by the biuret method, as described by Hübscher *et al.* (1965). At lower concentrations protein was determined by the method of Lowry *et al.* (1951) or by the method of Warburg & Christian (1941).

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate

Polyacrylamide disc-gel electrophoresis of protein samples, including subsequent staining for protein and destaining, was performed by the method of Betts & Mayer (1975) with 5% (w/v) polyacrylamide gels.

Experimental and Results

Assay of enzyme activity

Both assays gave reproducible results provided that certain precautions were observed.

(i) *Spectrophotometric assay.* The addition of the enzyme was followed by a lag phase (2–3 min) before a linear rate of NADPH oxidation (3–8 min) was observed. This is similar to the results of Waite & Wakil (1962). The duration of the lag phase was decreased, but not abolished, by previous activation

of acetyl-CoA carboxylase by incubation for 15 min at 30°C in the presence of 25 mM-potassium citrate. This residual lag phase may be due to the accumulation of malonyl-CoA. However, the linear rate of NADPH oxidation was not enhanced by previous incubation of the enzyme in the presence of citrate, and therefore this procedure was not included. From these results, it appears that the enzyme is fully activated within 2–3 min in the spectrophotometric assay system at 30°C. The enzyme activity at 30°C in the spectrophotometric assay was approximately half of that obtained at 37°C in the radiochemical assay (Table 1). It is unlikely that this difference was due to product inhibition, since the linear rate of NADPH oxidation remained constant when the amount of fatty acid synthetase was doubled. The difference in enzyme activity was probably due to the temperature difference in the two assays (Nakanishi & Numa, 1970).

(ii) [^{14}C]Bicarbonate-fixation assay. The assay conditions were selected to give linear incorporation of [^{14}C]bicarbonate with respect to time and protein at each stage of purification. Generally, linear rates were observed with 0.2–2 munits of enzyme during an incubation period of up to 3 min. Preliminary experiments showed that a rapid exponential loss of [^{14}C]bicarbonate and of non-radioactive bicarbonate occurred in incubations which contained all components of the assay except the enzyme. Although the specific radioactivity of the [^{14}C]bicarbonate remained constant, this process resulted in the loss of up to 40% of the label in 10 min at 20 mM- NaHCO_3 . Therefore an incubation period of 2 min was used as a routine to decrease the loss of [^{14}C]bicarbonate from the assay medium to approx. 7%. During this time the pH of the assay medium, measured at 37°C, remained constant at pH 7.5.

Purification of enzyme

A typical purification scheme for acetyl-CoA carboxylase is shown in Table 1. The specific activity of the purified enzyme was $5.15 \pm 0.3 \mu\text{mol}$ of bicarbonate incorporated/min per mg of protein (mean $\pm$ S.E.M. for five preparations). This represents a purification of 257 ± 16 -fold and a yield of $4.3 \pm 0.13\%$ compared with the enzyme in the particle-free supernatant.

In the later stages of the purification, considerable care was required to prevent the enzyme undergoing denaturation, which resulted in the formation of stringy precipitates. This tendency is reflected in the low recovery of activity after DEAE-cellulose chromatography (Table 1). The loss of activity during purification was decreased by using mild concentration procedures. This involved dialysis of the enzyme against buffer which contained $(\text{NH}_4)_2\text{SO}_4$ (Gregolin *et al.*, 1968a).

Polymerization of the enzyme by citrate was used

Table 1. Purification of acetyl-CoA carboxylase from mammary gland of lactating rabbits

See the text for details of the purification procedure. n.d., Not determined.

Fraction	Volume (ml)	Protein (mg)	Activity (units)		Specific activity (units/mg of protein)		Purification*	Yield* (%)
			Spectrophotometric assay	Radiochemical assay	Spectrophotometric assay	Radiochemical assay		
1. Particle-free supernatant	850	16784	n.d.	329	n.d.	0.02	1.0	100
2. $(\text{NH}_4)_2\text{SO}_4$ fractionation (176g/litre)	223	1245	92.8	194.4	0.08	0.16	8.0	59
3. Dialysis supernatant	220	984	89.2	182.0	0.09	0.19	9.5	55
4. Calcium phosphate eluate	178	303	61.2	118.1	0.20	0.39	19.5	36
5. DEAE-cellulose eluate	150	27	31.4	69.6	1.16	2.58	129.0	21
6. Sepharose 2B eluate	39	2.72	7.6	14.4	2.78	5.29	264.5	4.4

* Purification and yield are based on data from the radiochemical assay.

during specific stages of the purification which used methods based on separation according to molecular size (e.g. Sepharose 2B chromatography, sucrose-density-gradient centrifugation). In the polymerized state the enzyme is relatively stable and these purification procedures were performed at room temperature.

All buffers contained EDTA, dithiothreitol and glycerol to stabilize the enzyme (Miller & Levy, 1969), except for the buffer used for sucrose density gradients, which did not contain glycerol. In this case the enzyme was stabilized in the polymeric form by the presence of citrate.

Purity of the enzyme

After Sepharose 2B chromatography, samples of the enzyme (5–30 μ g of protein) were analysed by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. Normally this analysis showed one band when stained for protein. Some preparations gave evidence of slight impurity at this stage, and in these cases the enzyme was further purified by sucrose-density-gradient centrifugation. This gave rise to little change in the specific activity of the enzyme, but resulted in the removal of minor contaminants as judged by the criterion of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate.

Kinetics

The kinetics of the purified enzyme were investigated by using the radiochemical assay.

All reactants, except the variable, were present at concentrations which gave maximum enzyme activity, and the assay conditions were selected to give linear rates of incorporation of [14 C]bicarbonate with respect to time and protein. Preliminary investigations indicated that optimum enzyme activity occurred at pH 7.5. The presence of 1 mM-dithiothreitol did not alter the enzyme activity in a 2 min incubation and it was therefore not included in the assay system.

The apparent K_m of the purified enzyme for acetyl-CoA was 4.5×10^{-5} M. Maximum enzyme activity was observed with concentrations above 0.2 mM-acetyl-CoA. Maximum rates of incorporation were obtained with 20–40 mM- NaHCO_3 . The enzyme activity was not measured with NaHCO_3 concentrations greater than 40 mM, since these have been reported to cause enzyme inhibition (Miller & Levy, 1969). The apparent K_m for bicarbonate was 6.4×10^{-3} M.

Optimum rates for enzyme activity were obtained by using 0.5–2 mM-ATP in the presence of 20 mM- MgCl_2 and 25 mM-potassium citrate. Higher concentrations of ATP were inhibitory. With 2 mM-ATP and 25 mM-potassium citrate, maximum activity was observed at 20 mM- MgCl_2 . The activity-concentration

curve was sigmoid and excess of MgCl_2 was inhibitory. With optimum concentration of MnCl_2 (1 mM) the enzyme activity was about 40% of that observed in the presence of the optimum concentration of MgCl_2 (20 mM). Excess of MnCl_2 inhibited the enzyme.

A sigmoid curve was obtained when the concentration of potassium citrate was varied in the presence of 2 mM-ATP and 20 mM- MgCl_2 . Optimum rates were observed with 25 mM-potassium citrate and excess of citrate was inhibitory. For comparative purposes, the kinetic properties of the enzyme in the particle-free supernatant, prepared from lactating mammary gland, were determined under the same conditions and very similar results were obtained.

Discussion

The present method of purification of acetyl-CoA carboxylase differs from most other reports in the choice of buffers used. Several reports on the purification of the enzyme describe the use of phosphate buffers which contain EDTA and a thiol reagent (Gregolin *et al.*, 1968a; Majerus & Kilburn, 1969; Nakanishi & Numa, 1970; Moss *et al.*, 1972; Inoue & Lowenstein, 1972). Under these conditions the chicken liver enzyme is maintained in the polymeric form (Gregolin *et al.*, 1968a), but it is dissociated to the protomeric form in the presence of Tris buffers containing NaCl.

Acetyl-CoA carboxylase is normally distributed between the particle-free supernatant and the microsomal fraction of homogenates of lactating-rabbit mammary gland (Smith *et al.*, 1966). Recent studies have shown that 90% of the enzyme which sediments with the microsomal fraction can be recovered by the use of Tris buffers containing NaCl (S. Clark, J. Knudsen & R. Dils, unpublished work). By contrast, the enzyme does not appear to be recovered from the microsomal fraction in the presence of phosphate buffer. This was confirmed in the present study by preliminary experiments, which indicated that homogenization of the tissue in phosphate buffer which contained glycerol and citrate (Hansen *et al.*, 1971) led to poor recovery of the enzyme in the particle-free supernatant and to extensive loss of activity during dialysis after the $(\text{NH}_4)_2\text{SO}_4$ fractionation. The omission of citrate from this buffer had little effect on the enzyme yield. Consequently, the tissue was extracted in Tris buffer which contained NaCl, which gave better yield of enzyme in the particle-free supernatant, and little activity loss after the $(\text{NH}_4)_2\text{SO}_4$ fractionation. Similarly, poor recovery of the enzyme after DEAE-cellulose chromatography was observed when phosphate buffer was used in the presence or absence of citrate. Again, losses were minimized by using Tris buffers which contained NaCl, giving a recovery of approx. 60%.

In the later stages of the purification, the enzyme

was susceptible to denaturation which resulted in the formation of stringy protein precipitates. Great care was required to minimize mechanical agitation, especially when protein precipitates were redissolved. Gentle concentration procedures which involved dialysis against buffers containing $(\text{NH}_4)_2\text{SO}_4$ were also used to lessen activity losses (Gregolin *et al.*, 1968a).

The specific activity of the purified enzyme is within the range reported for this enzyme purified from other tissues (Gregolin *et al.*, 1966, 1968a; Miller & Levy, 1969; Majerus & Kilburn, 1969; Nakanishi & Numa, 1970). In addition, the kinetics of the enzyme are similar to those reported by other investigators. The apparent K_m for acetyl-CoA ($4.5 \times 10^{-5} \text{ M}$) is within the range reported for the enzyme from chicken liver ($1.6 \times 10^{-5} \text{ M}$; Gregolin *et al.*, 1968a) and from rat liver ($6.6 \times 10^{-5} \text{ M}$; Majerus *et al.*, 1968) and is virtually identical with that reported for the rat mammary-gland enzyme ($5 \times 10^{-5} \text{ M}$; Miller & Levy, 1969). Similarly, the apparent K_m for bicarbonate ($6.4 \times 10^{-3} \text{ M}$) is comparable with that reported for the yeast enzyme ($1.3 \times 10^{-2} \text{ M}$; Matsushashi *et al.*, 1964) and the rat mammary-gland enzyme ($1 \times 10^{-2} \text{ M}$; Miller & Levy, 1969). The sigmoidal responses obtained when the concentrations of MgCl_2 and citrate were varied are similar to those reported for the enzyme from rat mammary gland (Miller & Levy, 1969).

Electrophoresis on 5% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulphate gave a single band when stained for protein. This indicates the homogeneity of the purified enzyme preparation. The four polypeptide subunits of the protomeric form of the enzyme (Guchait *et al.*, 1974) migrate as a single band under the conditions used in the present study. This is similar to the results of Gregolin *et al.* (1968b) for the chicken liver enzyme. By contrast, Inoue & Lowenstein (1972) have reported that the rat liver enzyme may be separated into at least two polypeptide subunit species by polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate. The homogeneity of the purified enzyme was also shown by sucrose-density-gradient centrifugation. This gave a single protein peak, which was exactly coincident with enzyme activity. In preparations where there was evidence of minor impurities, the sucrose-density-gradient centrifugation served as the final purification step.

The $[^{14}\text{C}]$ bicarbonate-fixation assay is widely used to measure the activity of acetyl-CoA carboxylase, and incubations are normally carried out for 5–10 min at 37°C (Gregolin *et al.*, 1968a; Miller & Levy, 1969; Nakanishi & Numa, 1970; Inoue & Lowenstein, 1972; Moss *et al.*, 1972). During this time up to 40% of the bicarbonate has disappeared from the incubation medium. In the present study, incubations were performed for 2 min at 37°C to minimize this loss.

The results emphasize the importance of using short incubation periods for the assay of acetyl-CoA carboxylase by the $[^{14}\text{C}]$ bicarbonate-fixation assay. Alternatively, in longer incubations, it should be established that the bicarbonate concentration at the end of the incubation has remained above the saturation value for the enzyme.

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