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Pyruvate Carboxylase in Lactating Rat and Rabbit Mammary Gland

By BILQUIS GUL and R. DILS
Department of Medical Biochemistry and Pharmacology, University of Birmingham

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1. Pyruvate carboxylase [pyruvate–carbon dioxide ligase (ADP), EC 6.4.1.1] was found in cell-free preparations of lactating rat and rabbit mammary glands, and optimum assay conditions for this enzyme were determined. 2. Subcellular-fractionation studies with marker enzymes showed pyruvate carboxylase to be distributed between the mitochondrial and soluble fractions of lactating rat mammary gland. Evidence is presented that the soluble enzyme is not an artifact due to mitochondrial damage. 3. In contrast, pyruvate carboxylase in lactating rabbit mammary gland is confined to the mitochondrial fraction. 4. The final product of pyruvate carboxylase action in the mitochondrial and particle-free supernatant fractions of lactating rat mammary gland was shown to be citrate. 5. The effects of freeze-drying, ultrasonic treatment and freezing-and-thawing on the specific activity of mitochondrial pyruvate carboxylase were investigated.

Pyruvate carboxylase [pyruvate–carbon dioxide ligase (ADP), EC 6.4.1.1] has been purified from chicken liver (Utter & Keech, 1963; Keech & Utter, 1963) and sheep kidney (Ling & Keech, 1966) and its role in gluconeogenesis investigated in rat liver (Henning, Stumpf, Ohly & Seubert, 1966; Walter, Paetkau & Lardy, 1966) and rat kidney (Mehlman, Walter & Lardy, 1967).

It has been postulated that, in adipose tissue and liver, pyruvate carboxylase has a role in lipogenesis (Wise & Ball, 1964; Ballard & Hanson, 1967) in replenishing intramitochondrial oxaloacetate and hence citrate. The latter then provides extramitochondrial acetyl-CoA. Though lactating mammary gland is known to have a high lipogenic activity (Fritz, 1961; Masoro, 1962), the presence of pyruvate carboxylase has not been reported in this tissue. We therefore investigated the cofactor requirements, the intracellular distribution and some properties of this enzyme in lactating rat and rabbit mammary glands. A short account of this work has already been presented (Gul & Dils, 1968).

MATERIALS

[1,5-$^{14}C_2$]Citric acid monohydrate, $NaH^{14}CO_3$ and [1-^{14}C]*n*-hexadecane were obtained from The Radiochemical Centre, Amersham, Bucks., Triton X-100 was from Lennig Chemicals Ltd., Jarrow, Co. Durham, and 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene were from Nuclear Enterprises Ltd., Edinburgh. Nicotinamide nucleotides, CoA, ATP, monosodium glutamate, sodium pyruvate and 6-phospho-D-gluconate were purchased from Sigma (London) Chemical Co., London, S.W. 6. The dye 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, succinic acid and oxaloacetic acid were obtained from British Drug Houses Ltd., Poole, Dorset. Bovine serum albumin (fraction V) of low fatty acid content was purchased from Pentex Inc., Kankakee, Ill., U.S.A., and avidin from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Dowex 1 (X10; 200–400 mesh) was supplied by Bio-Rad Laboratories, Richmond, Calif., U.S.A., and 2,4-dinitrophenylhydrazine by Hopkin and Williams Ltd., Chadwell Heath, Essex. All other reagents were of A.R. grade.

Animals. Albino rats of the Wistar strain (12–15 days *post partum*) were used. They were fed *ad libitum* on a diet of rat cake (from Heygate and Sons Ltd., Bugbrook Mills, Northampton) and water. Litter size varied between ten and 13. The abdominal and inguinal pairs of mammary glands were used. Albino rabbits of the New Zealand White strain (10–18 days *post partum*) were used; they were given cabbage and a mixture of crushed oats and pellets (4:1, w/w). The pellets were obtained from Walter Brown Ltd., Crewe, Cheshire, and Morning Food Ltd., North-western Mills, Crewe, Cheshire, supplied crushed oats. The litter size varied between two and six.

METHODS

Preparation of subcellular fractions from lactating rat and rabbit mammary glands. Homogenates of mammary gland were prepared in 0·3M- or 0·88M-sucrose by using techniques based on those described by Easter & Dils (1968). After homogenization in 0·3M-sucrose, the nuclear fraction was removed by centrifugation at 1200*g* for 10min., the heavy-mitochondrial fraction at 7500*g* for 10min., and a composite fraction at 15000*g* for 20min. The microsomal fraction was sedimented at 103500*g* for 60min., leaving the PFS.* After homogenization in 0·88M-sucrose, the

* Abbreviation: PFS, particle-free supernatant.

centrifugation scheme used was that of Hogeboom, Schneider & Palade (1948) for the subcellular fractionation of rat liver.

The nuclear and mitochondrial fractions were washed twice in the medium used for homogenization, as described by Easter & Dils (1968). When the percentage distribution of enzymes between subcellular fractions was determined, the nuclear, mitochondrial and composite fractions were washed three times. In all cases, washings were added back to the material still to be fractionated.

All the operations described above were done at 0–4°.

Enzyme assays. All assays were carried out, unless otherwise stated, on fractions stored at 4° for at most 6hr. Unless otherwise stated, assays were carried out in duplicate at 37° on subcellular fractions prepared in 0·3M-sucrose. In all cases, the reaction rate was linear with respect to time and enzyme concentration over the ranges employed in the experiments. All specific activities are expressed as mμmoles of substrate transformed/min./mg. of protein.

(*a*) Succinate dehydrogenase [succinate – 2 - (*p* - iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride oxidoreductase, EC 1.3.99.1] and 6-phosphogluconate dehydrogenase (decarboxylating) [6-phospho-D-gluconate–NADP oxidoreductase (decarboxylating), EC 1.1.1.44] were assayed as described previously, and were regarded as markers for mitochondrial membranes and cell cytoplasm respectively (Smith, Easter & Dils, 1966).

(*b*) Malate dehydrogenase (L-malate–NAD oxidoreductase, EC 1.1.1.37) was assayed by the method of Baldwin & Milligan (1966).

(*c*) Glutamate dehydrogenase [L-glutamate–NAD oxidoreductase (deaminating), EC 1.4.1.2] was assayed by a modification of the method of Strecker (1955). The incubation mixture (total volume 3ml.) contained 50mM-potassium phosphate buffer, pH7·6, 0·4mM-KCN, 33mM-monosodium L-glutamate and 6mM-NAD. This enzyme and malate dehydrogenase were regarded as markers for readily solubilized intramitochondrial material, by analogy with results obtained with mitochondrial preparations from ox heart (Allmann & Bachmann, 1967) and rat liver (Sottocasa, Kuylenstierna, Ernster & Bergstrand, 1967).

(*d*) Pyruvate carboxylase. This was assayed by using an adaptation of the method of Smith *et al.* (1966) for the determination of acetyl-CoA carboxylase [acetyl-CoA–carbon dioxide ligase (ADP), EC 6.4.1.2]. Optimum assay conditions for the enzyme in both the mitochondrial and the PFS fractions were: tris–HCl buffer, pH8·5 (100mM); sodium pyruvate (11mM); acetyl-CoA (0·3mM); ATP (2mM); $NaH^{14}CO_3$ (30mM, 0·166μc/μmole); bovine serum albumin (0·5–1·0mg./ml. and 3·0mg./ml. for the mitochondrial and the PFS fraction respectively). The mitochondrial fraction showed a slightly higher requirement for $MgCl_2$ (10mM) than did the PFS fraction (6mM). Except for ATP (4mM), optimum assay conditions for the enzyme in the homogenate were the same as those for the mitochondrial fraction. The reaction was started by the addition of sodium pyruvate and all incubations were for 15min. in a final volume of 0·5 or 1·0ml. The reaction was stopped with 0·1ml. of 5M-$HClO_4$ and the unchanged $^{14}CO_2$ was removed by flushing four times with 0·1ml. of 0·1M-$KHCO_3$. This reduced the amount of unchanged $^{14}CO_2$ to less than 0·009% of the original [e.g. incubations without added protein had a residual activity of 303±37 disintegrations/min. (mean ±S.D. of six incubations)]. The radioactivity of the product was measured as described by Smith *et al.* (1966). The enzyme was found to have a dependence on added acetyl-CoA, though the incorporation of $H^{14}CO_3^-$ with added acetyl-CoA varied from preparation to preparation. To ensure the measurement of pyruvate- and acetyl-CoA-dependent carboxylation, two control incubations were always used, one without added sodium pyruvate and the other without added acetyl-CoA. The larger of these control values was subtracted from the value for incorporation in the complete system. Duplicate determinations normally agreed to within ±10%, but occasionally a spread of up to 20% was observed.

Ultrasonic treatment. Particulate fractions were treated for 30sec. intervals in an ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., Crawley, Sussex) set at maximum energy output. Fractions were cooled in ice during ultrasonic treatment and samples allowed to cool for 30sec. between these treatments.

Identification of the products of pyruvate carboxylase. Preliminary results showed that the final product of pyruvate carboxylase from rat mammary-gland mitochondrial and PFS fractions was not oxaloacetate. When 2,4-dinitrophenylhydrazones of the reaction products were prepared (Gailiusis, Rinne & Benedict, 1964) and their radioactivity was determined by liquid scintillation with [1-^{14}C]*n*-hexadecane as an internal standard, no radioactivity could be detected.

Ion-exchange chromatography was used to show that the product was, in fact, citrate. A slight modification of the method of Busch, Hurlbert & Potter (1952) was used with a stepwise rather than a gradient elution. The radioactive product from both mitochondrial and PFS fractions was completely eluted from the column in the same fractions, as was radioactive citrate used to standardize the column. The fractions containing the radioactive product were pooled and concentrated, and the enzymic assay described by Hohorst & Rein (1963) was used to confirm the absence of oxaloacetate from these fractions.

Miscellaneous methods. Acetyl-CoA was prepared and assayed as described by Smith, *et al.* (1966) and used within a week. Determinations of protein and avidin were as described by Tame & Dils (1967).

RESULTS

Unless otherwise stated, results apply to subcellular fractions prepared in 0·3M-sucrose.

Subcellular-fractionation studies. Preliminary experiments indicated that pyruvate carboxylase was present in both the particulate and the PFS fractions of cell-free extracts of lactating rat mammary gland, the enzyme in the mitochondrial fraction having the highest specific activity of the particulate fractions. Optimum assay conditions for the enzyme in the mitochondrial and PFS fractions were determined, and are given in the Methods section.

Table 1 shows the subcellular distribution of two marker enzymes, protein and pyruvate carboxylase in fractions of lactating rat mammary gland. Pyruvate carboxylase was found to be confined to the mitochondrial and PFS fractions; activity in

Table 1. *Distribution of marker enzymes, pyruvate carboxylase and protein in lactating rat mammary gland*

Values are for thrice-washed particles (see the Methods section), except for the microsomal fraction, which was unwashed. Results are expressed as means ± S.D. of three preparations. The units of specific activity are mμmoles/min./mg. of protein.

		Succinate dehydrogenase		6-Phosphogluconate dehydrogenase		Pyruvate carboxylase	
Subcellular fraction	Protein (% of full homogenate)	Specific activity	Recovery of full-homogenate activity (%)	Specific activity	Recovery of full-homogenate activity (%)	Specific activity	Recovery of full-homogenate activity (%)
Nuclear	5·7±1·2	2·7±2·8	1·3±0·6	0	0	1·4±0·9	1·3±0·6
Mitochondrial	14±5	76±19	75±21	0	0	12±6	12±2
Composite	15±5	17±10	18±11	18*	1*	4·7±3·3	7·3±3·3
Microsomal	23±3	1·2±0·4	1·5±0·9	27±8	3·7±0·6	0·5±0·4†	0·6±0·5†
PFS	46±1	0	0	364±45	84±5	7·8±3·2	38±8

* In two out of three preparations, no activity could be detected.

† Mean ± absolute deviation, since no activity could be detected in one of the three preparations.

the composite and nuclear fractions could be accounted for by contamination with the mitochondrial fraction. The recovery of pyruvate carboxylase from the full homogenate was consistently low (57–63%). This was not due to the removal of an activator from the mitochondrial fraction during preparation, since in a separate experiment the addition of full homogenate (0·13mg. of protein) to mitochondrial and PFS fractions (0·16 and 0·29mg. of protein respectively) had little effect on the specific activity of the enzyme in these fractions.

The specific activity of pyruvate carboxylase in the mitochondrial fraction varied greatly from preparation to preparation (9·9±8·3mμmoles/min./mg. of protein; mean ± S.D. of eight preparations), as it did in the PFS fraction (8·7±6·8mμmoles/min./mg. of protein; mean ± S.D. of eight preparations). No relationship was observed between the specific activities of the enzyme in the mitochondrial and the PFS fraction.

To determine whether the presence of pyruvate carboxylase in the PFS fraction was due to loss of mitochondrial enzyme during the washing procedure, the washings were assayed for pyruvate carboxylase and marker enzymes (Table 2). The results show that pyruvate carboxylase, unlike the soluble enzyme marker 6-phosphogluconate dehydrogenase, was not readily washed out of the mitochondrial fraction. The washings showed a low activity (both total and specific) for pyruvate carboxylase and succinate dehydrogenase, though the total recovery of the latter was only 69%. The specific activity of particulate pyruvate carboxylase and malate dehydrogenase each showed a 1·6-fold increase in the thrice-washed fraction, though the latter enzyme showed a high overall recovery and a higher proportion released into the washings.

Since 0·88M-sucrose as homogenization medium is reported to minimize morphological damage to mitochondria during preparation (Ziegler, Linnane, Green, Dass & Ris, 1958; Ziegler & Linnane, 1958), subcellular fractionation was repeated with this medium (Table 3*a*). The recovery of pyruvate carboxylase in the PFS fraction (76%) was much higher than was that of glutamate dehydrogenase (21%) in this fraction. The specific activity of pyruvate carboxylase in the PFS fraction was higher than that in the twice-washed mitochondrial fraction, whereas the converse was found for the specific activity of glutamate dehydrogenase. Essentially similar results were obtained with two other preparations, though the overall recovery of glutamate dehydrogenase was erratic. A comparison of Tables 3(*a*) and 3(*b*) shows that there was no difference in distribution of pyruvate carboxylase between the mitochondrial and PFS fractions when 0·3M- and 0·88M-sucrose were used as homogenization media. This also applied to the marker enzymes succinate dehydrogenase and 6-phosphogluconate dehydrogenase, though the recovery of the former was low.

In view of the phenomenon of adsorption of enzymes on to membranes in media of low ionic strength (Green *et al.* 1965; Hultin & Westort, 1966; Hultin, Westort & Southard, 1966) and the observation of Henning *et al.* (1966) that mitochondrial pyruvate carboxylase in rat liver can be solubilized by the use of media of high ionic strength, the following experiment was carried out. A mitochondrial fraction prepared in 0·3M-sucrose was washed once with 0·3M-sucrose containing 0M-, 25M-

Table 2. *Effect of washing on pyruvate carboxylase and marker enzymes of the rat mammary-gland mitochondrial fraction*

All fractionations and washings were carried out with 0·3 M-sucrose. The units of specific activity are mμmoles/min./mg. of protein.

Fraction	Recovery of unwashed-mitochondrial protein (%)	Succinate dehydrogenase		6-Phosphogluconate dehydrogenase		Malate dehydrogenase		Pyruvate carboxylase	
		Specific activity	Recovery of unwashed-mitochondrial activity (%)	Specific activity	Recovery of unwashed-mitochondrial activity (%)	Specific activity	Recovery of unwashed-mitochondrial activity (%)	Specific activity	Recovery of unwashed-mitochondrial activity (%)
Mitochondrial (unwashed)	100	34	100	52	100	190	100	2·8	100
First washing	26	1·3	1·2	143	79	158	24	0·4	3
Second washing	6	1·9	0·3	4·4	0·6	193	6	0	0
Third washing	3	1·0	0·1	0	0	21	3	0·1	0·1
Mitochondrial (thrice-washed)	61	36	67	0	0	305	100	4·5	100

or 100 mM-potassium chloride. The specific activity of the pyruvate carboxylase in the washed pellets was 3·2, 3·2 and 3·9 respectively, no protein being lost into the washings. This indicates that the presence of the mitochondrial enzyme is not due to adsorption of the PFS-fraction enzyme when 0·3 M-sucrose is used as homogenization medium. The results also demonstrate that the enzyme in the mitochondrial fraction cannot be solubilized by media of high ionic strength.

Subcellular-distribution studies with rabbit mammary gland (Table 4) showed the absence of soluble pyruvate carboxylase from this tissue. The recovery of pyruvate carboxylase in the subcellular fractions was better than that observed (Table 3) for rat mammary gland. The activity in the composite fraction (Table 4*a*) was probably due to contamination by the mitochondrial fraction. A comparison of Tables 4(*a*) and 4(*b*) shows that essentially similar distributions of pyruvate carboxylase were obtained with either 0·3 M- or 0·88 M-sucrose as homogenization medium. In both cases the distribution of pyruvate carboxylase in the mitochondrial fraction was similar to that of succinate dehydrogenase. The absence of pyruvate carboxylase from the mitochondria-free fractions of rabbit mammary gland was confirmed with ten separate tissue preparations.

Properties of pyruvate carboxylase. When optimum assay conditions for pyruvate carboxylase were being established, it was noted that for rat mammary gland the mitochondrial fraction showed linearity of reaction rate with amounts of protein up to 0·15 mg./ml. However, the PFS fraction exhibited non-linear reaction with protein with decreased reaction rates at high (> 0·25 mg./ml.) protein concentrations. The addition of fat-free albumin (2–4 mg./ml.) to incubation systems containing PFS protein increased the specific activity of the enzyme threefold and gave a reaction rate that was proportional to protein concentration up to 0·4 mg./ml. The addition of 0·5–1·0 mg. of albumin/ml. to assay mixtures containing the mitochondrial fraction produced a 1·2–1·6-fold increase in the specific activity of the enzyme, though higher concentrations had little stimulatory effect. Fat-free albumin produced no significant increase in the specific activity of the mitochondrial enzyme from rabbit mammary gland.

The optimum pH for pyruvate carboxylase in both the mitochondrial and the PFS fraction from rat mammary gland was pH 8·4–8·7 (Figs. 1*a* and 1*b*). The optimum pH for the enzyme in the mitochondrial fraction from rabbit mammary gland was pH 7·8 (Fig. 1*c*).

Pyruvate carboxylase in both the mitochondrial and the PFS fraction from rat mammary gland and in the mitochondrial fraction from rabbit

Table 3. *Subcellular fractionation of rat mammary gland in* 0·88 M-*sucrose*

All fractionation and washing procedures were carried out with (*a*) 0·88 M- or (*b*) 0·3 M-sucrose. The units of specific activity are mμmoles/min./mg. of protein.

Fraction	Recovery of full-homogenate protein (%)	Succinate dehydrogenase		6-Phosphogluconate dehydrogenase		Glutamate dehydrogenase		Pyruvate carboxylase	
		Specific activity	Recovery of full-homogenate activity (%)	Specific activity	Recovery of full-homogenate activity (%)	Specific activity	Recovery of full-homogenate activity (%)	Specific activity	Recovery of full-homogenate activity (%)
(*a*)									
Full homogenate	100	18	100	181	0	1·6	100	5·5	100
Mitochondrial (twice-washed)	16	73	63	0	0	8·7	88	7·0	20
PFS	40	0	0	350	72	0·9	21	11	76
(*b*)									
Full homogenate	100	14	100	217	100	—	—	11	100
Mitochondrial (twice-washed)	10	61	50	0	0	—	—	19	21
PFS	41	0·5	1	443	83	—	—	18	67

mammary gland was inhibited by avidin. However, incomplete inhibition of the enzyme in the particulate fractions was observed (Fig. 2).

The specific activity of mitochondrial pyruvate carboxylase from rat adipose tissue and chicken liver can be increased by freezing-and-thawing or freeze-drying (Ballard & Hanson, 1967; Utter & Keech, 1963). We have therefore examined this phenomenon with the mitochondrial fraction from rat and rabbit mammary glands. A freshly prepared mitochondrial fraction from rat mammary gland was repeatedly frozen at −20° and thawed after 1 hr. Freezing-and-thawing once caused a 1·5-fold increase in the specific activity of pyruvate carboxylase. A 2·3-fold increase in specific activity occurred after freezing-and-thawing twice, but no further increase resulted when the procedure was repeated a further four times.

In a separate experiment, freezing a mitochondrial fraction at −20° and thawing after 24 hr. caused a 4·9-fold increase in specific activity, which was not increased by repeating the procedure. When portions of a mitochondrial fraction were separately frozen-and-thawed once in this way, the increase in specific activity was found to be consistent. A mitochondrial fraction prepared in 0·88 M-sucrose, however, showed only a slight increase in the specific activity of the enzyme after freezing at −20° and thawing after 24 hr., and subsequent decrease in specific activity when the procedure was repeated (Table 5).

Rapid freezing-and-thawing (twice) of the mitochondrial fraction in solid carbon dioxide–methanol (−78°) produced a 5·4-fold increase in specific activity, which was not increased by further (six times) freezing-and-thawing. A twofold increase in specific activity was observed when a mitochondrial fraction prepared in 0·88 M-sucrose was frozen-and-thawed six times by this procedure (Table 5).

Little change in the specific activity of pyruvate carboxylase in the PFS fraction (prepared in either 0·3 M- or 0·88 M-sucrose) was observed when this fraction was frozen-and-thawed under conditions causing maximum increase in specific activity of the enzyme in the mitochondrial fraction.

The specific activity of pyruvate carboxylase in the mitochondrial fraction of rabbit mammary gland did not increase on freezing-and-thawing (Table 5).

On freeze-drying, a freshly prepared mitochondrial fraction from rat mammary gland showed a 2·3-fold increase in specific activity of pyruvate carboxylase (Table 5). A similar preparation that had been stored for 48 hr. at −20° (causing a 3·5-fold increase in specific activity) showed a twofold increase in specific activity on freeze-drying; another preparation that had been stored for 24 hr. at −20° (2·1-fold increase in specific activity)

Table 4. *Distribution of pyruvate carboxylase, marker enzymes and protein in lactating rabbit mammary gland*

(*a*) Values are for thrice-washed particles prepared in 0·3 M-sucrose. The 'microsomal+PFS' fraction was not separated into its component fractions. Results are expressed as means ± absolute deviation of two preparations. (*b*) Fractionation was carried out in 0·88 M-sucrose. The units of specific activity are mμmoles/min./mg. of protein.

		Succinate dehydrogenase		6-Phosphogluconate dehydrogenase		Pyruvate carboxylase	
Fraction	Recovery of full-homogenate protein (%)	Specific activity	Recovery of full-homogenate activity (%)	Specific activity	Recovery of full-homogenate activity (%)	Specific activity	Recovery of full-homogenate activity (%)
(*a*)							
Nuclear	4±0	5·3±2·6	4±2	0	0	3·0±1·4	2±1
Mitochondrial	14±0	28·5±2·5	80±10	0	0	14·8±9·2	80±19
Composite	19±3	1·3±0·7	5±1	0	0	3·5±3·0	23±17
Microsomal +PFS	58±3	0·1±0·1	2±0	254±47	101±19	0·03±0·02	1±1
(*b*)							
Full homogenate	100	9	100	136	100	8	100
Mitochondrial (twice-washed)	15	28	50	0	0	34	63
PFS	42	0	0	286	90	1	6

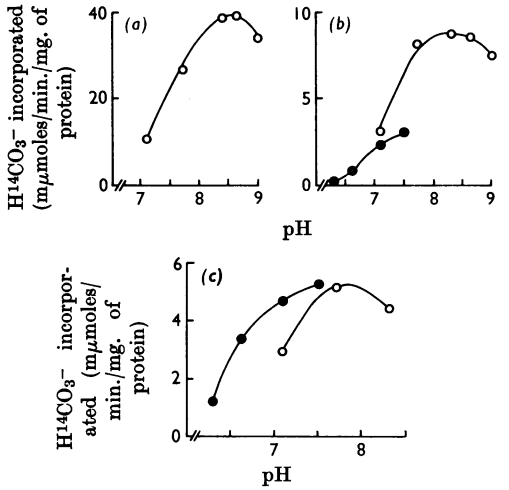

Fig. 1. Dependence of pyruvate carboxylase activity on pH. Optimum assay conditions were used (see the text). (*a*) Rat mammary-gland mitochondrial fraction; (*b*) rat mammary-gland PFS; (*c*) rabbit mammary-gland mitochondrial fraction. ●, Potassium phosphate buffer (0·1 M); ○, tris–HCl buffer (0·1 M). Specific activity is expressed as mμmoles/min./mg. of protein.

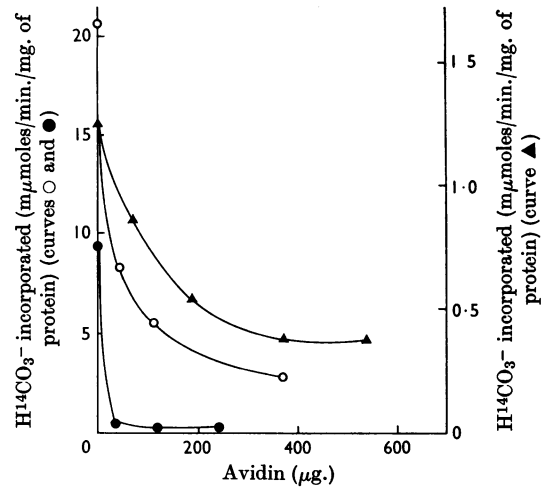

Fig. 2. Inhibition of pyruvate carboxylase by avidin. Optimum assay conditions were used (see the text). ○, Rat mammary-gland mitochondrial fraction; ●, rat mammary-gland PFS fraction; ▲, rabbit mammary-gland mitochondrial fraction.

showed a 77% decrease in specific activity. Freeze-drying of a mitochondrial fraction prepared in 0·88 M-sucrose had little effect on the specific activity of pyruvate carboxylase (Table 5).

With rabbit mammary gland, only a slight (1·3-fold) increase in the specific activity of pyruvate carboxylase was observed when the mitochondrial fraction in 0·3 M-sucrose was freeze-dried, but a 2·6-fold increase was noted when 0·88 M-sucrose was used (Table 5).

Ultrasonic treatment for 1 min. of a freshly prepared mitochondrial fraction from rat mammary

Table 5. *Increase in specific activity of pyruvate carboxylase by freezing-and-thawing, ultrasonic treatment and freeze-drying*

'Concn. of sucrose' refers to the medium used to prepare the mitochondrial fractions. For rabbit mammary gland, two separate preparations of the mitochondrial fraction prepared in 0·3 M-sucrose were used. The units of specific activity are mμmoles/min./mg. of protein.

	Sp. activity of rat mammary-gland mitochondrial fraction		Sp. activity of rabbit mammary-gland mitochondrial fraction	
Concn. of sucrose (M)	0·3	0·88	0·3	0·88
Treatment				
None	1·0	6·5	31·5	1·5
Freezing once at −20°, thawing after 24hr.	4·9	8·1	36·5	2·1
Freezing twice at −20°, thawing after 24hr. each time	5·0	4·9	35·5	1·2
None	1·0	6·5	18·9	1·5
Freezing twice at −78°, thawing after 5min. each time	5·4	—	19·1	—
Freezing six times at −78°, thawing after 5min. each time	5·7	12·3	19·6	2·0
Ultrasonic, 0·5min.	2·8	—	7·2	—
Ultrasonic, 1·0min.	3·6	12·6	4·2	1·2
Ultrasonic, 2·0min.	0·5	—	—	0·9
Freeze-drying	2·3	7·6	24·9	3·9

gland caused a 3·6-fold increase in specific activity of pyruvate carboxylase (Table 5). However, a wide variation in the increase in specific activity of different mitochondrial preparations was observed (1·4–7·0-fold, three preparations), though the increase in specific activity was consistent for a particular preparation. A twofold increase in specific activity occurred with a mitochondrial fraction prepared in 0·88 M-sucrose (Table 5). After storage of a mitochondrial fraction at −20° for 48hr. (3·7-fold increase in specific activity), ultrasonic treatment for 0·5min. caused no increase in specific activity, but a slight decrease was noted after 2min. ultrasonic treatment. Ultrasonic treatment caused no increase in the specific activity of mitochondrial fractions (prepared in either 0·3 M- or 0·88 M-sucrose) from rabbit mammary gland (Table 5).

Pyruvate carboxylase in rat adipose tissue (Ballard & Hanson, 1967) and kidney (Henning *et al.* 1966) is more stable at 23–25° than at 0–5°. We therefore examined the effect of temperature on the activity of pyruvate carboxylase from subcellular fractions of rat mammary gland. Storage of the three freshly prepared fractions at 24 ± 1° for 2hr. caused a 2·0–2·5-fold increase in the specific activity of the enzyme in the mitochondrial fraction but a 32–45% decrease in the specific activity of the enzyme in the PFS fraction.

DISCUSSION

Recently it has been postulated that in adipose and other tissues pyruvate carboxylase has a role in lipogenesis. Since citrate can supply extramitochondrial acetyl-CoA for fatty acid synthesis (Spencer, Corman & Lowenstein, 1964; Spencer & Lowenstein, 1966) in tissues such as rat liver and mammary gland, this could lead to an increased requirement for intramitochondrial oxaloacetate in tissues with high lipogenic activity. Ballard & Hanson (1967) suggested that pyruvate carboxylase replenishes this intramitochondrial oxaloacetate, which can then be converted into citrate by citrate synthase [citrate oxaloacetate-lyase (CoA-acetylating), EC 4.1.3.7] and therefore into extramitochondrial acetyl-CoA and oxaloacetate. Hence the synthesis of oxaloacetate has been suggested (Wise & Ball, 1964; Ballard & Hanson, 1967) to be as important in organs with high lipogenic activity (such as adipose tissue) as it is for gluconeogenesis in liver. Though pyruvate carboxylase has been reported to provide oxaloacetate in rat adipose tissue (Ballard & Hanson, 1967), we report for the first time the presence of this enzyme in lactating rat and rabbit mammary gland, both being tissues with high lipogenic activity.

Pyruvate carboxylase has been shown, by the use of cell-free preparations, to be associated with particulate fractions of a number of tissues. In chicken liver and sheep kidney, subcellular-fractionation studies have shown that pyruvate carboxylase is of highest specific activity in the mitochondrial fraction (Keech & Utter, 1963; Ling & Keech, 1966). In addition to a mitochondrial enzyme, a soluble form of pyruvate carboxylase has been reported in rat liver and kidney (Henning *et al.* 1966) and rat adipose tissue (Ballard & Hanson, 1967). However, in none of these reports were marker enzymes used to characterize the subcellular fractions used.

Our preliminary results indicated the presence of

pyruvate carboxylase in both the mitochondrial and the PFS fraction of rat mammary gland. This was confirmed by using marker enzymes and measuring the total recovery of enzyme activity in the subcellular fractions of the full homogenate (Table 1). The low recovery of pyruvate carboxylase from the full homogenate is difficult to explain, and no comparable data are available for the recovery of the enzyme in other tissues. Nevertheless, the proportions of activity recovered in the various fractions were quite consistent (Table 1).

On repeated washing of the mitochondrial fraction (Table 2), pyruvate carboxylase behaved like succinate dehydrogenase in not being readily removed and unlike 6-phosphogluconate dehydrogenase, an enzyme used to assess contamination by cell cytoplasm. This implies that the pyruvate carboxylase in the mitochondrial fraction is unlikely to be loosely bound to membrane structures. The washing procedure was also monitored by assaying malate dehydrogenase as a marker for readily solubilized intramitochondrial material. Although this enzyme was also found in the PFS fraction, its use as an intramitochondrial-enzyme marker with this tissue was justified for the following reasons. Only 25% of the optimum activity was observed when the enzyme in the PFS fraction was assayed with optimum conditions for the mitochondrial enzyme. Hence at most only 25% of the activity in the unwashed mitochondrial fraction could be due to contamination by the soluble enzyme. Secondly, the washed mitochondrial fraction contained 75% of the recovered malate dehydrogenase but no 6-phosphogluconate dehydrogenase. The results in Table 2 indicate that pyruvate carboxylase cannot be removed readily from the mitochondrial fraction by the procedures used to prepare this fraction. Further evidence to support this comes from the similar distribution of pyruvate carboxylase in the soluble and mitochondrial fractions prepared in 0·88M- and 0·3M-sucrose (Table 3). The presence of glutamate dehydrogenase, which can be used as an indication of mitochondrial damage (de Duve, 1967), in the PFS fraction shows that mitochondrial damage occurs even when 0·88M-sucrose is used as the homogenization medium. This bears out the comments of other workers (e.g. Jones & Gutfreund, 1961) on the difficulty of preparing morphologically intact mitochondria from mammary gland of species other than guinea pig.

From the cumulative evidence presented we conclude that, in lactating rat mammary gland, pyruvate carboxylase has two subcellular sites, one mitochondrial and the other cytoplasmic. The presence of the soluble enzyme cannot be explained by mitochondrial damage and the mitochondrial enzyme cannot be accounted for by adsorption of the soluble enzyme on to mitochondrial material.

In contrast, pyruvate carboxylase in lactating rabbit mammary gland is confined to the mitochondrial fraction (Table 4).

When the optimum conditions for the assay of pyruvate carboxylase in the subcellular fractions of rat and rabbit mammary glands were determined, they were found to be similar to those reported for the enzyme in rat adipose tissue (Ballard & Hanson, 1967) and chicken liver (Utter & Keech, 1963). The pH optimum of 8·5 found for the enzyme in both the mitochondrial and the PFS fraction of rat mammary gland (Fig. 1) was the same as that for the purified enzyme in sheep kidney (Ling & Keech, 1966). The rabbit mammary-gland enzyme had an optimum pH 7·8, as does the purified enzyme from chicken liver (Utter & Keech, 1963). The enzyme in rat adipose tissue was assayed at pH 7·4 by Ballard & Hanson (1967).

Though Ballard & Hanson (1967) assayed pyruvate carboxylase in subcellular fractions prepared in a medium containing 3% of bovine serum albumin, the stimulatory effect on the enzyme of fat-free albumin (see the Results section) has not been previously reported. It is possible that this stimulation and the production of linearity of reaction rate with enzyme protein are due to removal of inhibitors such as fatty acids.

The inhibition by avidin of pyruvate carboxylase in the mitochondrial and PFS fractions of rat mammary gland and in the mitochondrial fraction of rabbit mammary gland (Fig. 2) showed the enzyme to be biotin-dependent, as it is in other tissues investigated (Keech & Utter, 1963; Ling & Keech, 1966; Ballard & Hanson, 1967). The incomplete inhibition of the enzyme in mitochondrial fractions (Fig. 3) may be due in part to avidin not completely penetrating the mitochondria. However, Ling & Keech (1966) reported that even the purified enzyme from sheep kidney was not completely inhibited by avidin.

Though Utter & Keech (1963) found that the product of purified pyruvate carboxylase from chicken liver was oxaloacetate, Ballard & Hanson (1967) showed that citrate was the final product in the PFS fraction of rat adipose tissue. This latter result is to be expected since acetyl-CoA and oxaloacetate would form citrate if citrate synthase (EC 4.1.3.7) were present. Our finding that citrate was the product of pyruvate carboxylase from both the mitochondrial and the PFS fraction of rat mammary gland implies the presence of citrate synthase in both these fractions, though citrate synthase has not been directly assayed in PFS. It is not known if there is a truly soluble citrate synthase in this tissue or whether its apparent presence in the PFS is due to mitochondrial damage.

The results summarized in Table 5 show that for pyruvate carboxylase in the mitochondrial fraction (prepared in 0·3M-sucrose) of rat mammary gland, a maximum and consistent increase in specific activity was achieved by freezing-and-thawing. Ultrasonic treatment and freeze-drying were less effective. This is in contrast with the results of Ballard & Hanson (1967) with mitochondria from rat adipose tissue, where maximum increase in activity of pyruvate carboxylase was effected by freeze-drying but ultrasonic treatment for 1min. inactivated the enzyme (as it did the enzyme in the mitochondrial fraction of rabbit mammary gland; Table 5).

We interpret the increase in specific activity of pyruvate carboxylase in the mitochondrial fraction (prepared in 0·3M- or 0·88M-sucrose) of rat mammary gland by treatments causing damage to mitochondrial structure as further proof of the intramitochondrial location of the enzyme in this tissue. In the treated particles, added cofactors or substrate or both would be more readily available to the enzyme, as suggested by Ziegler & Linnane (1958) from results with ox heart and rat liver mitochondria. However, with the mitochondrial fraction of rabbit mammary gland no increase in the specific activity of the enzyme was observed (Table 5). This could mean that the mitochondria as prepared were already damaged and permeable to substrate and cofactors, or that the enzyme has an intramitochondrial location different from that in rat mammary gland.

Utter & Keech (1963) found that pyruvate carboxylase in chicken liver was only cold-labile when partially purified, though Henning *et al.* (1966) and Ballard & Hanson (1967) found the enzyme to be cold-labile even in crude extracts of rat kidney and adipose tissue. The enzyme in the PFS fraction of rat mammary gland was partially inactivated by storage at 24° for 2hr. This treatment increased the specific activity of the enzyme in the mitochondrial fraction, suggesting that damage to mitochondrial structures occurred during storage at 24°.

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