

## Immunochemical Studies with Soluble and Mitochondrial Pyruvate Carboxylase Activities from Rat Tissues

By F. J. BALLARD, R. W. HANSON AND LEA RESHEF

*Commonwealth Scientific and Industrial Research Organization, Division of Nutritional Biochemistry, Adelaide, S. Austral. 5000, Australia, Fels Research Institute, Temple University Medical School, Philadelphia, Pa. 19140, U.S.A. and Department of Biochemistry, Hadassah Medical School, Jerusalem, Israel*

(Received 27 April 1970)

1. Pyruvate carboxylase (EC 6.4.1.1), purified from rat liver mitochondria to a specific activity of 14 units/mg, was used for the preparation of antibodies in rabbits. 2. Tissue distribution studies showed that pyruvate carboxylase was present in all rat tissues that were tested, with considerable activities both in gluconeogenic tissues such as liver and kidney and in tissues with high rates of lipogenesis such as white adipose tissue, brown adipose tissue, adrenal gland and lactating mammary gland. 3. Immunochemical titration experiments with the specific antibodies showed no differences between the inactivation of pyruvate carboxylase from mitochondrial or soluble fractions of liver, kidney, mammary gland, brown adipose tissue or white adipose tissue. 4. The antibodies were relatively less effective in reactions against pyruvate carboxylase from sheep liver than against the enzyme from rat tissues. 5. Pyruvate carboxylase antibodies did not inactivate either propionyl-CoA carboxylase or acetyl-CoA carboxylase from rat liver. 6. It is concluded that pyruvate carboxylase in lipogenic tissues is similar antigenically to the enzyme in gluconeogenic tissues and that the soluble activities of pyruvate carboxylase detected in many rat tissues do not represent discrete enzymes but are the result of mitochondrial damage during tissue homogenization.

Since the demonstration of pyruvate carboxylase in liver mitochondria (Utter & Keech, 1960), the enzyme has also been found at high specific activities in mitochondria from kidney (Keech & Utter, 1963), white adipose tissue (Ballard & Hanson, 1967*a*; Patel & Hanson, 1970) and mammary gland (Gul & Dils, 1969) and it has been suggested that this enzyme plays an important role in lipogenesis as well as gluconeogenesis (Ballard & Hanson, 1967*a*). Measurements of pyruvate carboxylase in subcellular fractions isolated by differential centrifugation usually show that some activity is associated with the particle-free supernatant or soluble fraction (Henning, Stumpf, Ohly & Seubert, 1966; Ballard & Hanson, 1967*b*; Hanson & Ballard, 1967), but it is not established whether this soluble fraction represents a functional activity of the cell cytosol.

Studies with malate dehydrogenase (Grimm & Doherty, 1961) and phosphoenolpyruvate carboxykinase (Ballard & Hanson, 1969) have shown that the mitochondrial activities of these enzymes are immunochemically distinct from the soluble activities. In this paper we have carefully examined

the distribution of pyruvate carboxylase throughout rat tissues and have used antibodies prepared against the purified enzyme from liver mitochondria to analyse the immunochemical similarities between the various mitochondrial and soluble activities.

### MATERIALS AND METHODS

*Animals.* The rats used were hooded Wistar animals from the Divisional colony or were obtained from the Waite Agricultural Institute of the University of Adelaide. Samples taken for tissue distribution studies were from 200g male rats, except in the cases of mammary gland and brown adipose tissue, which were obtained from rats that had been lactating for 2 days and from newborn rats respectively.

The sheep used were made diabetic by a single intravenous injection of 60mg of alloxan/kg body weight, and were killed 1 week later. The dexamethasone-treated sheep were given five daily intramuscular injections of 0.5mg of dexamethasone/kg body weight and were killed on the fifth day. We thank Dr I. G. Jarrett of the Commonwealth Scientific and Industrial Research Organization for providing these treated animals.

*Chemicals.* DEAE-cellulose used for the purification

studies was Whatman DE 52 microgranular. Fines were removed by washing the cellulose with 50mM-tris-HCl buffer, pH 7.5, containing 0.5mM-EDTA.  $(\text{NH}_4)_2\text{SO}_4$  was an analytical grade from Byproducts and Chemicals Pty. Ltd., Auburn, N.S.W., Australia. This was recrystallized twice before use. ATP and acetyl-CoA were purchased from P-L Biochemicals Inc., Milwaukee, Wis., U.S.A., and  $\text{NaH}^{14}\text{CO}_3$  was from The Radiochemical Centre, Amersham, Bucks., U.K.

**Pyruvate carboxylase assay.** Enzyme activity was measured as described by Ballard & Hanson (1967b) by the fixation of  $^{14}\text{C}$ -labelled bicarbonate. The units of activity are  $\mu\text{mol}$  of bicarbonate fixed/min at  $37^\circ\text{C}$ .

**Propionyl-CoA carboxylase (EC 6.4.1.3) assay.** This enzyme was assayed as described by Lane & Halenz (1962). Bicarbonate fixation in blanks with propionyl-CoA omitted was subtracted from the fixation in the complete assay. The propionyl-CoA, present at a concentration of 1mM in the assay, was prepared from propionic anhydride and CoA by the method of Simon & Shemin (1953). Mitochondrial extracts for propionyl-CoA carboxylase studies were obtained as described in the pyruvate carboxylase purification.

**Acetyl-CoA carboxylase (EC 6.4.1.2) assay.** Soluble fractions of liver homogenates in 0.25M-sucrose were prepared by centrifugation at 100000g for 30min and were used for the measurement of acetyl-CoA carboxylase activity. The enzyme was assayed with the bicarbonate-fixation method as described for pyruvate carboxylase (Ballard & Hanson, 1967b) but with 10mM-citrate replacing pyruvate.

**Purification of pyruvate carboxylase from rat liver mitochondria.** Pyruvate carboxylase has been purified to various degrees of homogeneity from chicken liver (Utter & Keech, 1963), ox liver (Utter *et al.* 1967) and sheep kidney (Ling & Keech, 1966). The methods used are similar and take advantage in particular of  $(\text{NH}_4)_2\text{SO}_4$  precipitations and extractions. The method outlined here is similar in principle to those cited above and only the details that are particularly relevant are described. It was especially important for the antibody experiments that the starting material for the purification of mitochondrial pyruvate carboxylase did not contain any cytosol enzyme.

Liver (100g) from fed rats was homogenized in 400ml of 0.25M-sucrose with a coaxial homogenizer. The homogenate was centrifuged at 1000g for 10min and the supernatant was collected. This was in turn centrifuged at 10000g for 15min and the second supernatant was discarded. The post-mitochondrial particulate fractions were removed from the pellet by swirling with 0.25M-sucrose and were also discarded. The mitochondria were separated from nuclei by a similar process so that most of

the nuclei remained in the centrifuge tube. The mitochondrial fraction was then suspended in 200ml of 0.25M-sucrose, centrifuged for 15min at 10000g and separated from the residual pellet as before. This process was repeated so that the mitochondria had been washed twice. All of these and the following steps were carried out at  $2^\circ\text{C}$ . The washed mitochondria were suspended in a small volume of 0.5mM-EDTA, pH 7.0, and freeze-dried. The freeze-dried mitochondria could be stored dry at  $-20^\circ\text{C}$  for at least 20 days without loss of pyruvate carboxylase activity.

The mitochondria were extracted by homogenization with 100ml of a solution containing 50mM-tris, 5mM- $\text{MgSO}_4$ , 5mM-ATP and 0.5mM-EDTA that had been adjusted to pH 7.0 with acetic acid (Scrutton, Olmsted & Utter, 1969). After homogenization, the pH was readjusted to pH 7.0 with 1M-tris. The suspension was centrifuged at 100000g for 30min to obtain a clear supernatant. Solid  $(\text{NH}_4)_2\text{SO}_4$  was added to this supernatant to bring the  $(\text{NH}_4)_2\text{SO}_4$  concentration to 35% saturation and the precipitate was collected by centrifugation. This precipitate was extracted sequentially with 10ml of solutions that were 33, 31, 29, 25, 23 and 21% saturated with respect to  $(\text{NH}_4)_2\text{SO}_4$  and contained 0.5mM-EDTA at a final pH of 7.0. In all purification trials most of the activity was found in the 25 and 23% extractions, although the relative specific activities in these fractions was quite variable. These two fractions were combined and passed through a Sephadex G-25 column that had been equilibrated with 50mM-tris-HCl buffer containing 0.5mM-EDTA at pH 7.5. The fractions containing protein were placed on a DEAE-cellulose column ( $3\text{cm}^2 \times 15\text{cm}$ ) equilibrated with the tris-EDTA buffer and the protein was eluted with a linear gradient of from 0 to 0.5M-KCl in the same buffer. Those fractions containing pyruvate carboxylase at highest specific activity were combined and  $(\text{NH}_4)_2\text{SO}_4$  was added to give a 50%-saturated solution. Under these conditions the enzyme suspension was stable for at least two months at  $2^\circ\text{C}$ . The complete procedure from extraction of mitochondria to final precipitation of enzyme usually took about 12h. At no stage before this precipitation could the enzyme be left overnight without considerable loss of activity. A typical purification is shown in Table 1.

It is evident from Table 1 that a considerable loss of pyruvate carboxylase occurs during the purification procedure. Although approx. 150 units of activity are discarded in fractions of low specific activity, at least 300 units are unaccounted for. It is possible that this lost activity represents selective loss of a distinct form of the enzyme, but if so, this enzyme would also be of mitochondrial origin.

*Preparation of antibodies to mitochondrial pyruvate*

Table 1. *Purification of pyruvate carboxylase*

Further details of this purification from 100g of rat liver are given in the text.

Fraction	Activity (units)	Protein (mg)	Sp. activity (units/mg of protein)
Mitochondrial extract	586	1680	0.35
25%-satd.-( $\text{NH}_4$ ) <sub>2</sub> SO <sub>4</sub> extract	98	49	2.0
23%-satd.-( $\text{NH}_4$ ) <sub>2</sub> SO <sub>4</sub> extract	142	37	3.8
DEAE cellulose eluate	99	6.9	14.4

*carboxylase*. Enzyme stored as an  $(\text{NH}_4)_2\text{SO}_4$  suspension was centrifuged and approx. 10mg of protein was dissolved in 2ml of 0.9% NaCl. This was mixed with 2ml of complete Freund's adjuvant (Difco) and injected subcutaneously into a rabbit. Additional injections of enzyme without adjuvant were given at monthly intervals. This rabbit, together with a control animal that was injected with adjuvant and 0.9% NaCl, was bled at intervals from an ear vein. The blood (approx. 40ml) was allowed to clot overnight, serum was obtained by centrifugation, and the  $\gamma$ -globulins were precipitated by addition of  $(\text{NH}_4)_2\text{SO}_4$  to 50% saturation. This suspension was centrifuged and the precipitate was washed twice with 50% satd.  $(\text{NH}_4)_2\text{SO}_4$ . The washed precipitate was dissolved in 5ml of 0.9% NaCl and dialysed against 0.9% NaCl. Any precipitate obtained during dialysis was discarded.

*Protein measurements*. Protein was measured by the u.v. method of Warburg & Christian (1941) during the enzyme purifications and as described by Lowry, Rosebrough, Farr & Randall (1951) for tissue distribution studies.

## RESULTS

For all experiments that involve inactivation of enzyme by antibody it is important to be sure that the loss of activity observed is due to a specific effect of the antibody. With pyruvate carboxylase a non-specific loss of activity assumes considerable significance since the enzyme is known to undergo reversible dissociations into subunits (Scrutton & Utter, 1967). Preliminary experiments to conduct antibody-pyruvate carboxylase titrations were completely unsuccessful as the antibody appeared to stabilize the enzyme rather than inactivate it. The problem was caused by the rapid inactivation of pyruvate carboxylase in dilute solution and was found with enzyme from several rat tissues.

Pyruvate carboxylase activity was decreased to 20% or less in 4h when tissue extracts, soluble fractions, freeze-dried mitochondria or purified enzymes were suspended at 2°C or at room temperature in any of the following solutions: 0.25M-sucrose, sucrose buffered with triethanolamine (Henning *et al.* 1966), or a solution containing 0.2M-potassium chloride, 10mM-potassium phosphate, 1mM-EDTA and 0.5mM-dithiothreitol at either pH 6.5 or pH 7.2. Bovine serum albumin was also ineffective in maintaining stability. The addition of glycerol to a final concentration of 50%, as recommended by Böttger, Wieland, Brdiczka & Pette (1969), prevented inactivation, especially if bovine serum albumin was added at 0.5%. However, this solution was viscous and unsuitable for accurate pipetting. The use of a solution containing 50mM-tris, 5mM-ATP, 5mM-MgSO<sub>4</sub> and 0.5mM-EDTA, pH 7.0 (Scrutton *et al.* 1969), stabilized pyruvate carboxylase unless the enzyme was present in very dilute solution. In all the following experiments mitochondria or freeze-dried homogenates were extracted with this buffer and a more concentrated solution was added to soluble fractions to give similar final concentrations. All extracts were kept at room temperature.

The tissue distribution of pyruvate carboxylase is shown in Table 2. High activities are present in liver, kidney, lactating mammary gland and the adrenal glands of adult rats and in the interscapular brown adipose tissue of newborn rats. Significant activities are found in white adipose tissue, brain, heart and testes, and much lower activities in skeletal muscle and intestinal mucosa.

For titration experiments, the antibody and the tissue extracts were mixed and left for 2h at room

Table 2. *Tissue distribution of pyruvate carboxylase*

Tissues were homogenized in 5 vol. (w/v) of 0.25M-sucrose and freeze-dried. The freeze-dried samples were extracted with twice the homogenization volume of a solution containing 50mM-tris, 5mM-ATP, 5mM-MgSO<sub>4</sub> and 0.5mM-EDTA at pH 7.0 and immediately assayed. Blank values in the absence of pyruvate or acetyl-CoA have been subtracted from the total rate of incorporation. In no instance did any of these blanks represent a large increase in radioactivity above the small acid-stable impurity of the NaHCO<sub>3</sub>. Values are means  $\pm$ S.E.M. with the number of animals in parentheses.

Tissue	Activity (units/g of tissue)	Sp. activity (m-units/mg of protein)
Liver	15.5 $\pm$ 2.1 (5)	103 $\pm$ 11 (5)
Kidney	14.1 $\pm$ 1.0 (7)	90 $\pm$ 8 (4)
Brain	0.88 $\pm$ 0.18 (4)	9.7 $\pm$ 2.0 (4)
White adipose	1.92 $\pm$ 0.29 (5)	103 $\pm$ 15 (5)
Brown adipose, newborn animal	4.06 $\pm$ 0.59 (5)	38.0 $\pm$ 5.5 (5)
Adrenal gland	5.30 $\pm$ 1.30 (4)	41 $\pm$ 7 (4)
Lactating mammary gland	5.00 $\pm$ 0.51 (8)	50 $\pm$ 4 (5)
Intestinal mucosa	0.14 $\pm$ 0.05 (4)	1.7 $\pm$ 0.4 (4)
Testis	0.61 $\pm$ 0.04 (4)	9.0 $\pm$ 0.04 (4)
Heart	0.59 $\pm$ 0.09 (4)	4.1 $\pm$ 1.0 (4)
Skeletal muscle	0.12 $\pm$ 0.02 (4)	1.0 $\pm$ 0.2 (4)

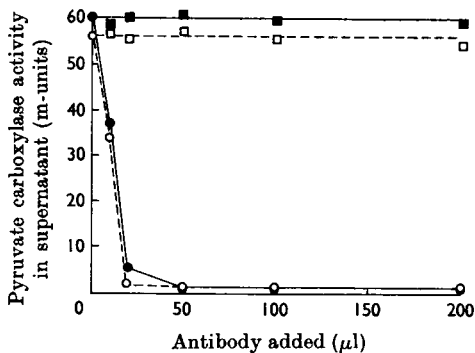


Fig. 1. Inactivation of pyruvate carboxylase from either soluble (○) or mitochondrial (●) fractions of rat liver by increasing amounts of antibody prepared against the mitochondrial enzyme. Titrations of control  $\gamma$ -globulins against soluble (□) and mitochondrial (■) fractions are also shown. Mitochondrial extracts were prepared by extracting 120mg of freeze-dried mitochondria with 5ml of the tris-ATP-MgSO<sub>4</sub>-EDTA solution (Scrutton *et al.* 1969) followed by centrifugation at 100000g for 30min. Supernatant (100 $\mu$ l) was mixed with antibody, water, and sufficient of a more concentrated extraction solution to give a volume of 0.5ml and to bring the concentration of tris, ATP, MgSO<sub>4</sub> and EDTA to 50, 5, 5 and 0.5mM respectively. After standing for 2h at room temperature, the solutions were centrifuged (3000g, 10min) to remove the antibody-antigen precipitate, and the supernatants assayed for pyruvate carboxylase activity. Pyruvate carboxylase in the soluble fraction was obtained by homogenizing 3g of liver with 9ml of 0.25M-sucrose followed by centrifugation at 100000g for 30min. Supernatant (150 $\mu$ l) was mixed with the antibody and the tris-ATP-MgSO<sub>4</sub>-EDTA solution, incubated, centrifuged and assayed as described for the mitochondrial enzyme. The activity remaining in the supernatant after centrifugation of the antibody-antigen precipitate is plotted against the amount of antibody added.

temperature. After centrifugation to remove the antibody-enzyme precipitate, the supernatants were assayed for enzyme activity. Parallel titrations were carried out with control  $\gamma$ -globulin and corrections were made for any loss of activity over the 2h period. It was not possible to overcome the instability difficulties by assaying the solutions before 2h since the precipitation of antibody-enzyme complex was incomplete before this time. An example of a titration experiment with soluble and mitochondrial extracts of liver with increasing amounts of antibody is shown in Fig. 1. Clearly the control  $\gamma$ -globulin did not cause significant enzyme inactivation under these conditions. It can also be seen that the titration of soluble pyruvate carboxylase was identical with that of the mitochondrial enzyme, suggesting that the pyruvate carboxylase found in the two subcellular

fractions is derived from a common pool of antigenically similar enzyme.

Throughout this paper the term cytosol, as originally suggested by Lardy (1965), is applied to the nonparticulate fraction of cells. That fraction obtained in differential centrifugation studies by centrifuging homogenates at 100000g for 30min is called the soluble fraction. This distinction has been made because one purpose of this paper is to determine whether the pyruvate carboxylase activity found in the soluble fraction is representative of a cytosol activity.

A convenient way to quantitate the effectiveness of antibody against different antigens is to measure the amount of enzyme inactivated by a constant portion of antibody. This type of experiment was undertaken by using preparations of soluble pyruvate carboxylase and of particulate pyruvate carboxylase that were isolated from homogenates of liver, kidney, lactating mammary gland, white adipose tissue and brown adipose tissue. The amount of antibody added to each of these fractions was calculated to be insufficient to inactivate all the pyruvate carboxylase present so that the difference between the enzyme activity in the presence and absence of antibody represented that amount of enzyme precipitated by antibody. The results of these experiments (Table 3) show that approximately 60m-units of pyruvate carboxylase were inactivated by the 20 $\mu$ l of antibody used. No statistical differences were detected between the amounts of enzyme inactivated from the various tissue sources.

Although the experiment in Table 3 suggests that no antigenic difference exists between soluble and particulate activities of pyruvate carboxylase, this interpretation might be invalid if the soluble fraction contained two activities, one leached from mitochondria and thus identical with the mitochondrial form, and a second activity that exhibited less inactivation with the antibody and was derived from the cytosol. The error could occur if the activity remaining after addition of antibody was of the second type. This possible contingency is overcome if a reverse type of antibody titration is carried out (Fig. 2). In this experiment the amount of antibody is kept constant while increasing amounts of enzyme are added. An equivalence point is obtained when all the enzyme present is just inactivated by the antibody added. If the soluble fraction contains two distinct activities of pyruvate carboxylase, only one of which is derived from broken mitochondria, whereas the other represents a distinct enzyme that exhibits less cross reaction, then the equivalence point will occur with a smaller amount of added enzyme. Fig. 2 shows that such an interpretation is not possible with the soluble pyruvate carboxylase from rat liver,

Table 3. *Inactivation of pyruvate carboxylase from different tissues by a constant amount of antibody*

Soluble and particulate fractions from different tissues were prepared by homogenization in 0.25M-sucrose followed by centrifugation at 100000g for 30 min. The particles were suspended in 0.25M-sucrose, freeze-dried, and extracted with a solution containing 50mM-tris, 5mM-ATP, 5mM-MgSO<sub>4</sub> and 0.5mM-EDTA at pH7.0. These suspensions were centrifuged at 100000g for 30 min and the clear supernatants were used for antibody precipitation studies. A more concentrated tris-ATP-MgSO<sub>4</sub>-EDTA solution was added to the soluble fraction to attain the same concentrations as for the mitochondrial extracts. Each tissue extract was diluted with tris-ATP-MgSO<sub>4</sub>-EDTA solution to give an approximate pyruvate carboxylase activity of 100m-units/0.5ml. Antibody (20μl) or control γ-globulin (20μl) was added to 0.5ml of each extract and after 2h at room temperature the solutions were centrifuged and the supernatants assayed for pyruvate carboxylase. Values shown are means ±s.e.m. with the number of animals in parentheses. Statistical analysis indicated no significant differences at the 5% probability level.

Tissue	Fraction	Enzyme inactivated/ 20 μl of antibody (m-units)
Liver	Particulate	64.9±5.1 (8)
	Soluble	54.3±3.0 (7)
Kidney	Particulate	62.1±4.1 (8)
	Soluble	59.4±6.9 (8)
Mammary gland	Particulate	69.7±8.4 (6)
	Soluble	59.6±8.1 (7)
Brown adipose tissue	Particulate	60.8±2.7 (4)
	Soluble	56.0±5.5 (4)
White adipose tissue	Particulate	55.3±5.1 (7)
	Soluble	68.1±7.8 (7)

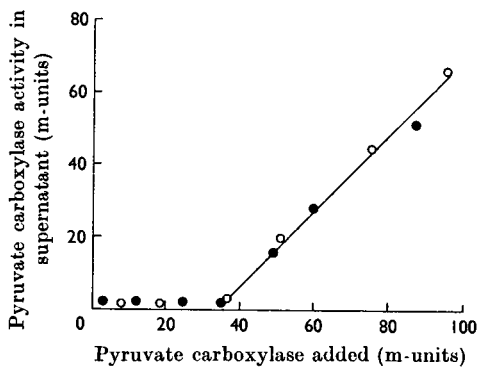


Fig. 2. Titrations of soluble (○) and mitochondrial (●) preparations containing pyruvate carboxylase against 12.5μl of antibody prepared against the mitochondrial enzyme. This experiment was performed in a similar manner to that described in Fig. 1, except that from 20 to 250μl of soluble enzyme and from 5 to 150μl of mitochondrial extract were used. The activity remaining in the supernatant after centrifugation of the antibody-antigen precipitate is plotted against the amount of enzyme added. The antibody used was the same as in the experiment described in Fig. 1.

since this enzyme shows an equivalence point identical with that of the mitochondrial enzyme.

A problem with antibody-antigen titrations that show no differences between tissues is that the antibody is perhaps of rather general specificity

with respect to species or with respect to related antigens. To test this question we have measured pyruvate carboxylase inactivation with enzyme obtained from soluble and mitochondrial fractions of sheep liver. Titration experiments (Fig. 3) indicate that soluble and mitochondrial pyruvate carboxylase activities from livers of diabetic sheep are inactivated to equal extents, but that more antibody is needed to inactivate the sheep enzyme than the rat enzyme. Further experiments of this type with dexamethasone-treated sheep showed comparable results, even though livers from these animals had a much lower activity of pyruvate carboxylase than the diabetic sheep (Filsell, Jarrett, Taylor & Keech, 1969).

In addition to pyruvate carboxylase, several other enzymes in liver catalyse biotin-dependent bicarbonate-fixation reactions. We have compared antibody titrations against two of these enzymes, propionyl-CoA carboxylase and acetyl-CoA carboxylase, with a titration against pyruvate carboxylase, to see whether any antigenic similarity is evident. The results (Fig. 4) indicate that the antibody prepared against pyruvate carboxylase shows no inhibitory action on either acetyl-CoA carboxylase or propionyl-CoA carboxylase.

## DISCUSSION

*Intracellular distribution of pyruvate carboxylase in rat liver.* Some pyruvate carboxylase activity is always obtained in the soluble fraction of liver

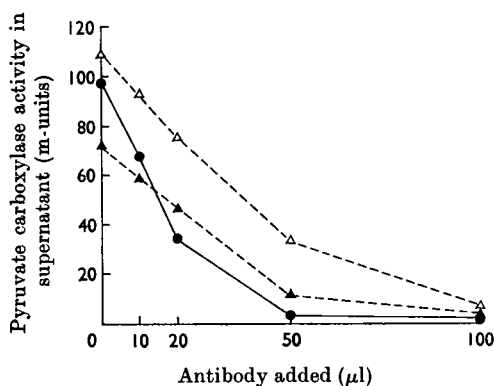


Fig. 3. Inactivation of pyruvate carboxylase from soluble ( $\Delta$ ) or mitochondrial ( $\blacktriangle$ ) fractions of sheep liver or from the mitochondrial ( $\bullet$ ) fraction of rat liver by increasing amounts of antibody prepared against the mitochondrial enzyme of rat liver. Sheep liver was homogenized in 9 vol. (w/v) of 0.25M-sucrose. A portion of this homogenate was centrifuged at 100000g for 30min and some concentrated extraction fluid added to the supernatant to bring the concentrations of tris, ATP,  $MgSO_4$  and EDTA to 50, 5, 5 and 0.5mM respectively. The remaining homogenate was used to prepare mitochondria as described for rat liver in the Materials and Methods section. Freeze-dried mitochondria were extracted as described in Fig. 1. For the antibody titration, 200 $\mu$ l of soluble fraction, 50 $\mu$ l of mitochondrial extract and 150 $\mu$ l of mitochondrial extract from rat liver were used. Other details are described in the legend to Fig. 1. The antibody was the same as used in Fig. 1.

homogenates. Usually the amount is less than 15% of the total activity recovered (Ballard & Hanson, 1967b; Reshef, Hanson & Ballard, 1969; Marco, Sebastián & Sols, 1969), but higher proportions have been reported (Henning *et al.* 1966), especially when the tissue is homogenized in an iso-osmotic solution containing both sucrose and triethanolamine. Although this suggested that part of the pyruvate carboxylase activity was in an outer mitochondrial compartment from which it was released by the salt treatment, careful comparisons of the intracellular distributions of pyruvate carboxylase and of several marker enzymes clearly demonstrated that all the mitochondrial pyruvate carboxylase was localized in the matrix (Marco *et al.* 1969; Böttger *et al.* 1969).

Evidence consistent with a dual location of pyruvate carboxylase in the liver cell is provided by studies of the effects of dietary variations and hormonal treatments. Both Henning *et al.* (1966) and Ballard, Hanson & Kronfeld (1969) have shown that starvation results in an increase in the soluble activity of pyruvate carboxylase in rat liver. How-

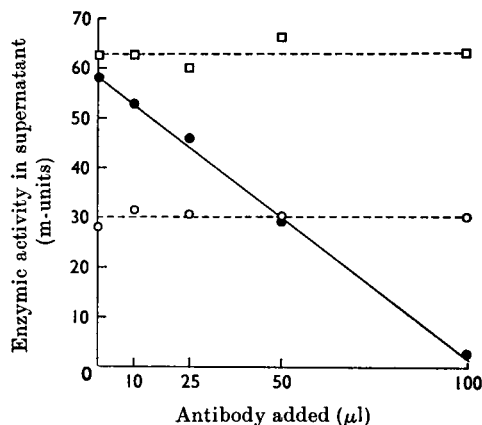


Fig. 4. Titrations of propionyl-CoA carboxylase ( $\square$ ), acetyl-CoA carboxylase ( $\circ$ ) or pyruvate carboxylase ( $\bullet$ ) by increasing amounts of antibody prepared against pyruvate carboxylase. Mitochondrial extracts and the soluble fraction of rat liver were prepared as described in the legend to Fig. 1. For the titration, 100 $\mu$ l of mitochondrial extract was used for pyruvate carboxylase, 400 $\mu$ l of mitochondrial extract was used for propionyl-CoA carboxylase and 400 $\mu$ l of soluble fraction was used for acetyl-CoA carboxylase. Other details are given in the legend to Fig. 1, but the antibody used was of lower potency and was from a different bleeding of the rabbit to that used for the experiments in Figs. 1, 2 and 3.

ever, only the former authors showed an increase in total cellular activity; in the second study the increase in soluble activity was at the expense of the mitochondrial activity. This, together with the knowledge that liver mitochondria became more fragile when the rats had been starved, suggested to Scrutton & Utter (1968) that the increase in soluble pyruvate carboxylase was an artifact of homogenization.

The present immunochemical studies indicate no differences between soluble and mitochondrial activities of pyruvate carboxylase. The antibody-antigen results could be due to a very broad response to the antibody. Thus, little emphasis could be given to the constancy of antibody inactivation if pyruvate carboxylase isolated from other mammalian species were inhibited to an extent equal to that found with enzyme from rat liver. The studies with soluble and mitochondrial fractions of sheep liver indicate, however, that a difference does exist as compared with the rat. The sheep results are particularly noteworthy since in this species the enzyme is especially adaptive to changes in hormonal or dietary conditions (Filsell *et al.* 1969) and the diabetic sheep tested had 60% of the pyruvate carboxylase in the soluble fraction. That this soluble pyruvate carboxylase was anti-

genically indistinguishable from the mitochondrial activity gives further support to the idea that only a single pyruvate carboxylase is present in liver. Further evidence that the pyruvate carboxylase antibody does not have a broad specificity is provided by the results in Fig. 4, which show that propionyl-CoA carboxylase and acetyl-CoA carboxylase are not inactivated, even though these enzymes catalyse reactions that are very similar to the reaction catalysed by pyruvate carboxylase.

The immunochemical experiments and intracellular distribution studies are best interpreted as evidence for a total mitochondrial activity of pyruvate carboxylase. The soluble activity has been obtained by disruption of mitochondria during homogenization. We suggest that pyruvate carboxylase is not, therefore, analogous to phosphoenolpyruvate carboxykinase which is indeed present in both the cytosol and mitochondria of liver from certain mammals (Nordlie & Lardy, 1963; Nordlie, Varricchio & Holten, 1965; Gevers, 1967; Ballard *et al.* 1969; Ballard & Hanson, 1969).

*Physiological role of pyruvate carboxylase.* When pyruvate carboxylase was originally reported in liver (Utter & Keech, 1960) it was thought that the enzyme was uniquely involved in gluconeogenesis. The more recent reports of the presence of the enzyme at high specific activity in both white adipose tissue (Ballard & Hanson, 1967a; Patel & Hanson, 1970; Reshef *et al.* 1969) and lactating mammary gland (Gul & Dils, 1969) together with studies on isolated mitochondria (Patel & Hanson, 1970) and with ruminant tissue (Hanson & Ballard, 1967) suggest that pyruvate carboxylase may play a role in lipogenesis, as well as in gluconeogenesis, by providing oxaloacetate for condensation with acetyl CoA to give citrate, the precursor of acetyl CoA in the cytosol.

One aim of the present study was to test whether the pyruvate carboxylase present in tissues that carry out fatty acid synthesis at a high rate was different antigenically from the enzyme in liver. Preliminary kinetic experiments showed no differences between the reaction in adipose tissue and that in liver (Ballard & Hanson, 1967a). The antibody-antigen precipitation studies in Table 3 are also consistent with the existence of a single pyruvate carboxylase in all tissues tested, so that the same enzyme functions in some tissues in the pathway of gluconeogenesis and in other tissues to supply oxaloacetate for fatty acid synthesis.

It has also been suggested that the cytosol pyruvate carboxylase in lipogenic tissues, if not an artifact, could participate with malate dehydrogenase and 'malic' enzyme to generate NADPH by a cyclic reaction sequence (Ballard & Hanson, 1967a). This latter idea is attractive as there is insufficient NADPH produced by the hexose mono-

phosphate pathway in adipose tissue to support the observed rate of lipogenesis (Flatt & Ball, 1964; Katz, Landau & Bartsch, 1966) and also since all three enzymes would be in the cytosol. The evidence presented in this paper does not support such a role for pyruvate carboxylase since the immunochemical studies are not in agreement with the presence of a distinct cytosol activity of the enzyme. However, this scheme could function with mitochondrial pyruvate carboxylation if either malate or oxaloacetate passed out of the mitochondria for conversion into pyruvate via 'malic' enzyme.

The high activity of pyruvate carboxylase in adrenal glands and in brown adipose tissue reported in this paper further suggest that this enzyme is important for lipogenesis. Lower activities in heart, brain and testes may indicate lipogenic functions of these tissues but more likely it may mean that the enzyme is found in all mammalian tissues where there is a need for oxaloacetate synthesis. Pyruvate carboxylase would thus serve an anaplerotic role.

F. J. B. is a Queen Elizabeth II Fellow.

## REFERENCES

- Ballard, F. J. & Hanson, R. W. (1967a). *J. Lipid Res.* **8**, 72.  
 Ballard, F. J. & Hanson, R. W. (1967b). *Biochem. J.* **104**, 866.  
 Ballard, F. J. & Hanson, R. W. (1969). *J. biol. Chem.* **244**, 5625.  
 Ballard, F. J., Hanson, R. W. & Kronfeld, D. S. (1969). *Fedn Proc. Fedn Am. Socs exp. Biol.* **28**, 218.  
 Böttger, I., Wieland, O., Brdiczka, D. & Pette, D. (1969). *Eur. J. Biochem.* **8**, 113.  
 Filsell, O. H., Jarrett, I. G., Taylor, P. H. & Keech, D. B. (1969). *Biochim. biophys. Acta*, **184**, 54.  
 Flatt, J. P. & Ball, E. G. (1964). *J. biol. Chem.* **239**, 675.  
 Gevers, W. (1967). *Biochem. J.* **103**, 141.  
 Grimm, F. G. & Doherty, D. G. (1961). *J. biol. Chem.* **236**, 1980.  
 Gul, B. & Dils, R. (1969). *Biochem. J.* **111**, 263.  
 Hanson, R. W. & Ballard, F. J. (1967). *Biochem. J.* **105**, 529.  
 Henning, H. V., Stumpf, B., Ohly, B. & Seubert, W. (1966). *Biochem. Z.* **344**, 274.  
 Katz, J., Landau, B. R. & Bartsch, G. E. (1966). *J. biol. Chem.* **241**, 727.  
 Keech, D. B. & Utter, M. F. (1963). *J. biol. Chem.* **238**, 2609.  
 Lane, M. D. & Halenz, D. R. (1962). In *Methods in Enzymology*, vol. 5, p. 576. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.  
 Lardy, H. A. (1965). In *A Symposium on Control of Energy Metabolism*, p. 245. Ed. by Chance, B. New York: Academic Press Inc.  
 Ling, A. & Keech, D. B. (1966). *Enzymologia*, **30**, 367.

- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
- Marco, R., Sebastián, J. & Sols, A. (1969). *Biochem. biophys. Res. Commun.* **34**, 725.
- Nordlie, R. C. & Lardy, H. A. (1963). *J. biol. Chem.* **238**, 2259.
- Nordlie, R. C., Varricchio, F. E. & Holten, D. D. (1965). *Biochim. biophys. Acta*, **97**, 214.
- Patel, M. S. & Hanson, R. W. (1970). *J. biol. Chem.* (in the Press).
- Reshef, L., Hanson, R. W. & Ballard, F. J. (1969). *J. biol. Chem.* **244**, 1944.
- Scrutton, M. C., Olmsted, M. R. & Utter, M. F. (1969). In *Methods in Enzymology*, vol. 13, p. 235. Ed. by Lowenstein, J. New York: Academic Press Inc.
- Scrutton, M. C. & Utter, M. F. (1967). *J. biol. Chem.* **242**, 1723.
- Scrutton, M. C. & Utter, M. F. (1968). *A. Rev. Biochem.* **37**, 249.
- Simon, E. J. & Shemin, D. (1953). *J. Am. chem. Soc.* **75**, 2520.
- Utter, M. F. & Keech, D. B. (1960). *J. biol. Chem.* **235**, 17.
- Utter, M. F. & Keech, D. B. (1963). *J. biol. Chem.* **238**, 2603.
- Utter, M. F., Scrutton, M. C., Young, M. R., Tolbert, B., Wallace, J. C., Irias, J. J. & Valentine, R. C. (1967). *Proc. 7th int. Congr. Biochem., Tokyo*, vol. 2, p. 247.
- Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.