INTRACELLULAR DISTRIBUTION OF PYRUVATE CARBOXYLASE IN LIVERS OF NORMAL AND CORTISOL TREATED RATS

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1. Introduction

All of the enzymes which catalyse the conversion of oxaloacetate to glucose 6-phosphate in gluconeogenesis of rat liver are known to be predominantly localized in the cytosol (for review see ref. [1]). However, the localization of pyruvate carboxylase (PC) which converts pyruvate to oxaloacetate is still not clear. Böttger et al. [2] concluded from their cell fractionation study that at least 97% of the enzyme was mitochondrial whereas the group of Seubert [3, 4], by using a different method, postulated the existence of a physiologically significant extramitochondrial enzyme activity. Both groups used glutamate dehydrogenase (GDH) as a mitochondrial marker enzyme.

The method of Seubert's group involves homogenization of the liver in various media, centrifugation at 70 000 *g* and measurement of pyruvate carboxylase and glutamate dehydrogenase activities in the supernatants. With this method these authors found more than 30% of the total PC activity versus less than 7% of GDH activity in the 70 000 *g* supernatants. The possibility that a differential release of the two enzymes from the mitochondria could be responsible for this result was shown to be unlikely because under various conditions of gradual breakage of isolated mitochondria always equal relative amounts of enzyme activities were liberated [4, 5]. Based on these results, Seubert's group therefore postulated the existence of an extramitochondrial PC activity [3, 4]. In a recent paper, Dugal [5] reported that up to 54% of PC versus only 11% of GDH can be found in the supernatants if the homogenization is prolonged to 11 min.

By using the method and homogenization media of Seubert's group we also found a higher percentage of PC than of GDH activity in the supernatants [6]. In addition we reported that equal percentages of both enzyme activities are found in the supernatants if a phosphate-containing homogenization medium is used. In order to explain the effect of phosphate we proposed that during homogenization with all media equal amounts of both enzymes are released into the cytosol due to breakage of mitochondria, but that the released GDH is stable only in the presence and unstable in the absence of phosphate whereas released PC retains its activity in all media. This conclusion was supported by the fact that GDH added to homogenates could only be recovered in the 70 000 *g* supernatants if phosphate was present whereas in the absence of phosphate 50% to 95% of GDH activity was lost [6]. On the other hand, the recent results of Dugal [5] show that GDH is not destroyed by the homogenization procedure in a phosphate-free medium, because when he added deoxycholate after such a prolonged homogenization, 100% of both enzymes were recovered. Therefore the question remains unanswered as to why the ratio of PC:GDH activities is different in the supernatants of phosphate-containing homogenization media as compared to the phosphate-free media used by Seubert's group.

The results of the present study demonstrate that in the phosphate-free homogenization media substantial amounts of GDH were bound by liver fractions containing mainly microsomes and nuclei and that this GDH activity was released when phosphate was added. These results fully explain the differences in enzyme activities observed in the media with and

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Table 1
The effect of phosphate on the release of glutamate dehydrogenase activity from various liver fractions.

Homogenization media	Liver fraction	GDH-activity released	
		Control	Treated with phosphate
		(μmoles/min/g liver)	
I 0.24 M sucrose, 1 mM EDTA, 1 mM glutathione, 20 mM TEA–HCl pH 7.2	900 *g* Sediment	0.68	5.44
	900 *g* Supernatant	0.81	10.93
	15 000 *g* Sediment	0.40	2.73
	15 000 *g* Supernatant	0.65	8.24
	100 000 *g* Supernatant	0.65	0.70
II 0.28 M sucrose, 1 mM EDTA, 1 mM glutathione, 50 mM sodium acetate	900 *g* Supernatant	0.60	12.53
	100 000 *g* Supernatant	0.60	0.61

Rat livers were homogenized in the media used by Seubert et al. [4] and indicated in the table and then centrifuged at 900 *g* for 8 min. Part of the resulting supernatant was then centrifuged at 15 000 *g* for 10 min. Probes were retained of each liver fraction and their GDH activity was measured as follows: a) *controls:* All 900 *g* and 15 000 *g* 'supernatant' fractions were centrifuged at 100 000 *g* and the GDH activities were then assayed in the resulting supernatants: 'sediment' fractions were resuspended in 10 ml of the respective homogenization media per original gram of liver, then centrifuged at 100 000 *g* and the GDH activities determined in the resulting supernatants; b) *treated with phosphate:* the procedure was the same as with the controls, except that here potassium phosphate solution of pH 7.2 was added to all the respective media (final concentration 50 mM) in which the probes were resuspended. In the case of the liver fractions marked as '100 000 *g* supernatants', no recentrifugations were carried out because these solutions were always clear. The control GDH values of the 15 000 *g* supernatant and 100 000 *g* supernatant therefore always represent the same measurement. When phosphate was added to the 100 000 *g* supernatant, the solution was thoroughly mixed. Times of centrifugation were 8 min for 900 *g*, 10 min for 15 000 *g* and 60 min for 100 000 *g*.

without phosphate and thereby strongly support the concept of an exclusive intramitochondrial localization of PC in rat liver. Furthermore, evidence is presented that cortisol treatment of the rat does not lead to an induction of extramitochondrial PC as has been reported by others [3].

2. Materials and methods

Fed male rats (CFN COBS) from the Tierzucht Institut of the University of Zurich weighing 190 – 240 g were used. PC activity was measured according to Henning et al. [7] except that citrate was determined enzymatically [8]. GDH activity was measured according to Schmidt [9] in the presence of ADP [10].

3. Results and discussion

In the experiments of table 1, two of the phosphate-free homogenization media used by Seubert's group [4] were employed. As can be seen, GDH activity was released from subcellular fractions upon addition of phosphate. The experiments with the triethanolamine buffer-containing medium furthermore show that most GDH activity was bound to the fraction containing mainly microsomes (15 000 *g* supernatant) or nuclei (900 *g* sediment) whereas little enzyme activity was released from the mitochondrial fraction (15 000 *g* sediment). If livers were homogenized in a sucrose–glutathione–EDTA medium, GDH was also released by added phosphate (table 2, expt. 3). On the other hand if acetate was added, even more GDH disappeared whereas the addition of triethanolamine buffer caused no further loss of GDH (table 2). PC activity either remained constant or slightly increased upon addition of the various salts in these experiments.

These results are in good agreement with those published earlier [6] in which soluble GDH and PC were added to homogenate in various media and where the recovery of these enzymes was measured in the resulting supernatants. It was found that about 50% of GDH activity was lost in the presence of triethanolamine buffer and about 95% when sodium acetate was present whereas in the phosphate-containing medium all of the added enzyme activity was recovered. On the other hand none of the added PC disappeared in the various media tested.

Table 2
Effects of various additions to the 900 *g* supernatant fraction on the enzyme activities in the high speed supernatants.

Expt.	Treatment	Enzyme activities in 70 000 *g* supernatant PC	GDH
		(μmoles/min/g liver)	
	None	0.58	1.39
1	Rehomogenized	0.59	1.59
	Rehomogenized after addition of 20 mM TEA–HCl pH 7.2	0.59	1.19
	None	0.35	2.14
2	Rehomogenized	0.43	2.22
	Rehomogenized after addition of 50 mM sodium acetate	0.56	0.43
	None	0.30	2.53
3	Rehomogenized	0.34	2.63
	Rehomogenized after addition of 50 mM potassium phosphate pH 7.2	0.47	15.95

The 900 *g* supernatant fractions were prepared according to Henning et al. [3] in a medium containing 0.3 M sucrose, 1 mM EDTA and 1 mM glutathione. When indicated rehomogenization was carried out in a Potter type homogenizer for 1 min at ice temperature. The supernatant fractions were obtained by centrifugation at 70 000 *g* for 15 min.

The original observation of Seubert's group that a higher percentage of total PC than of GDH activity appears in their supernatant can now be fully explained by the fact that part of the GDH released by the mitochondria during homogenization is bound to subcellular fractions containing mainly microsomes and nuclei. Furthermore the finding that *equal* relative percentages of PC and GDH activities are found in the supernatants of phosphate-containing homogenates [6] provide strong evidence for an exclusive mitochondrial localization of PC in rat liver and argue against the concept of an extramitochondrial PC activity.

In table 3 some results with cortisol-treated rats are summarized. In agreement with Henning et al. [3] and Stormer et al. [11], 20–40% increases in cytosol-PC activity could be observed in livers of cortisol-treated rats when the enzyme activities were measured in the supernatants of phosphate-free homogenates. In the phosphate-containing medium, however, the increases were smaller, and equal relative percentages of GDH and of PC were found in the respective cytosols. On the basis of these results, an induction of an extramitochondrial PC as has been postulated earlier [3] seems unlikely.

After this work was completed, it was shown by

Table 3
Effect of cortisol treatment on the activities of glutamate dehydrogenase and pyruvate carboxylase in rat liver.

Expt.	Homogenization medium	Treatment of animal	Enzyme activities: Total homogenate PC	GDH	Cytosol PC	GDH
			μmoles/min/g liver			
A	I	Fasted (control)	7.6	238	0.87 (12%)	6.36 (3%)
		Fasted + cortisol	8.5	257	1.24 (15%)	6.60 (3%)
	III	Fasted (control)	7.5	250	0.68 (9%)	29.4 (10%)
		Fasted + cortisol	9.0	304	0.78 (9%)	31.8 (12%)
B	I	Fasted (control)	7.3	250	0.86 (12%)	4.15 (2%)
		Fasted + cortisol	8.1	278	1.05 (13%)	6.60 (2%)
	III	Fasted (control)	7.7	258	0.62 (9%)	24.5 (9%)
		Fasted + cortisol	7.8	309	0.71 (8%)	30.0 (10%)

Food was taken away at 10 p.m. and animals were killed at 10 a.m. the following day. When indicated, 5 mg of cortisol (crystalline suspension) per 100 g of body weight was administered i.p. at 4 a.m.; controls received the same volume of physiological saline. For composition of medium I see table 1; medium III contained 0.14 M sucrose and 50 mM potassium phosphate pH 7.2. Extracts of liver homogenates were prepared according to Böttger et al. [2]. Numbers in brackets refer to % of activity of the respective enzymes in the total homogenate.

Godinot and Lardy [12] that labelled glutamate dehydrogenase added to liver homogenates preferentially binds to microsomes, which is in full agreement with our findings. Evidence was also presented showing that a small (< 6%) fraction of the total GDH activity which does not originate from broken mitochondria is bound to microsomes. It was proposed that this microsomal enzyme activity represents newly synthesized enzyme which is to be transported into the mitochondria. If a similar pathway exists for the biosynthesis of PC, the non-mitochondrial fraction should amount to less than 3% according to Böttger et al. [2].

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References

[1] Scrutton, M.C. and Utter, M.F. (1968) Ann. Rev. Biochem. 37, 249–302.
[2] Böttger, I., Wieland, O., Brdiczka, D. and Pette, D. (1969) Eur. J. Biochem. 8, 113–119.
[3] Henning, H.V., Stumpf, B., Ohly, B. and Seubert, W. (1966) Biochem. Z. 344, 274–288.
[4] Seubert, W., Henning, H.V., Schoner, W. and L'Age M. (1968) in: Advances in Enzyme Regulation (by Weber, G.,ed), vol. 6, p. 153–180, Pergamon, New York.
[5] Dugal, S. (1972) FEBS Letters 26, 97–101.
[6] Walter, P. and Anabitarte, M. (1971) FEBS Letters 12, 289–292.
[7] Henning, H.V. and Seubert, W. (1964) Biochem. Z. 340, 160–170.
[8] Walter, P. and Stucki, J.W. (1970) Eur. J. Biochem. 12, 508–519.
[9] Schmidt, E. (1962) in: Methoden der enzymatischen Analyse (Bergmeyer, H.U., ed), p. 752–756, Verlag Chemie, Weinheim.
[10] Williamson, D.H. Lund, P. and Krebs, H.A. (1967) Biochem. J. 103, 514–527.
[11] Störmer, B., Janssen, W., Reinauer, H., Staib, W. and Hollmann, S. (1970) Hoppe-Seylers Z. Physiol. Chem. 351, 296–304.
[12] Godinot, C. and Lardy, H.A. (1973) Biochemistry 12, 2051–2060.