SHORT COMMUNICATIONS

BBA 73125

On the use of microsomal marker enzymes to distinguish the outer membrane of rat liver mitochondria from the microsomes

Several enzymes are generally accepted as marker enzymes for subcellular and submitochondrial fractions, such as glucose-6-phosphatase[1] (EC 3.1.3.9) and NADPH–cytochrome *c* reductase[2] for the microsomes, cytochrome *c* oxidase[2] (EC 1.9.3.1) for the mitochondrial inner membrane, and monoamine oxidase[3] (EC 1.4.3.4) and rotenone-insensitive NADH–cytochrome *c* reductase[2] for the mitochondrial outer membrane.

In a recent publication the use of some microsomal marker enzymes in distinguishing the outer membrane of rat liver mitochondria from the microsomes has been questioned[4]. In the present paper it is pointed out that specific activities alone are not sufficient to draw conclusions about the localization of a microsomal enzyme in the mitochondrial outer membrane, except if the specific activity in isolated mitochondrial outer membranes is significantly higher than in isolated microsomes. In the case of glucose-6-phosphatase and NADPH–cytochrome *c* reductase a several-fold increase in the specific activities has been reported in mitochondrial outer membranes, when compared to whole mitochondria[3,4]. However, the specific activities always remain far below the observed values for isolated microsomes. In this case we want to stress that the pattern of distribution of the total activity over the submitochondrial fractions, after separation of the mitochondria into outer and inner membranes, gives a more accurate picture of the localization of these enzymes than do the observed specific activities in the subfractions.

Fig. 1 shows a representative experiment from a series of 3 experiments. Glucose-6-phosphatase and NADPH–cytochrome *c* reductase are distributed in a different way over microsomal subfractions, isolated by centrifugation of a $27000 \times g_{max}$ (10 min) supernatant of a rat liver homogenate in a continuous sucrose density gradient.

Mitochondria are not present in the $27000 \times g$ supernatant used, so that the relatively high activity of glucose-6-phosphatase in Fractions 3–5 must be derived from microsomal membranes. These membranes have about the same density as mitochondria, when centrifuged in the same way (compare ref. 5). This explains the results shown in Table I, where the specific activities of glucose-6-phosphatase and NADPH–cytochrome *c* reductase in microsomes (P) and heavy mitochondria (M) are compared. Since the enzymes are not homogeneously distributed over the microsomal membranes, the ratio of the specific activities of microsomes to mitochondria (P/M) is not the same. We then tend to conclude that the microsomal contamination of washed mitochondria is due to the presence of "heavy" microsomes, contributing relatively much glucose-6-phosphatase activity.

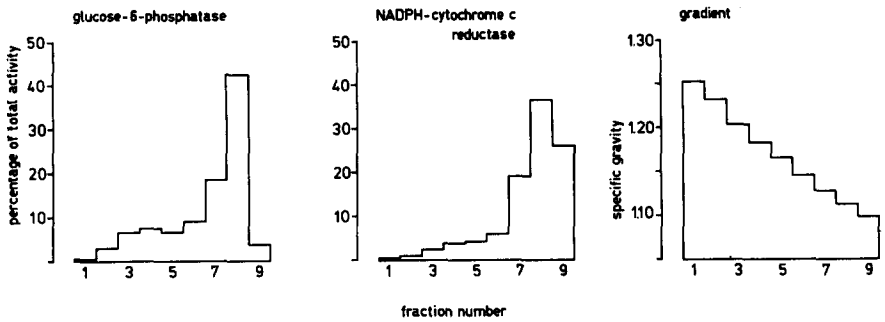

Fig. 1. The distribution of glucose-6-phosphatase and NADPH–cytochrome *c* reductase in fractions from rat liver microsomes, isolated by continuous sucrose density gradient centrifugation. A 20% rat liver homogenate, in 0.25 M sucrose–0.01 M Tris–HCl (pH 7.4), was centrifuged for 10 min at 27000 × g_{max}. 10 ml of the 27000 × *g* supernatant were brought on the gradient. For enzyme assays see the legend to Table I. The percentage of the total activity in Fractions 1–9 is plotted against the fraction No. For further details of gradient fractionation see ref. 5. The recovery of the enzyme activities in the gradient fractions was 81% for glucose-6-phosphatase and 101% for NADPH–cytochrome *c* reductase. The specific activities of the enzymes in isolated microsomes are given in Table I.

TABLE I

SPECIFIC ACTIVITIES OF GLUCOSE-6-PHOSPHATASE AND NADPH–CYTOCHROME *c* REDUCTASE IN MICROSOMES (P) AND HEAVY MITOCHONDRIA (M) ISOLATED FROM RAT LIVER

The subcellular fractions were prepared by differential centrifugation of a rat liver homogenate as described by DE JONG AND HÜLSMANN[7]. NADPH–cytochrome *c* reductase was measured spectrophotometrically according to SOTTOCASA *et al.*[2]. Rotenone (1.5 μM) was present and the reaction was started by the addition of cytochrome *c*. Glucose-6-phosphatase was measured as described by BEAUFAY *et al.*[1]. Enzyme activities were measured at 37°. The means and the standard error of the means of the enzyme activities are given as μmoles of substrate metabolized per mg of protein per h.

Enzyme	*Number of experiments*	*P*	*M*	*P/M*
Glucose-6-phosphatase	4	13.2 ± 3.3	1.40 ± 0.17	9.4
NADPH–cytochrome *c* reductase	4	11.2 ± 1.0	0.52 ± 0.10	21.6

BRUNNER AND BYGRAVE[4] conclude from their experiments with mitochondria washed several times that glucose-6-phosphatase and NADPH–cytochrome *c* reductase are localized not only in microsomes but also in the mitochondrial outer membrane. In order to investigate this further, we determined the distribution of these enzymes over inner and outer mitochondrial membranes. In Fig. 2 a representative experiment out of a series of 5 is shown. As marker enzymes for the inner and outer membranes, cytochrome *c* oxidase and rotenone-insensitive NADH–cytochrome *c* reductase are used, respectively. Glutamate dehydrogenase (EC 1.4.1.2) is used as a marker for the soluble mitochondrial matrix[6]. The mitochondria are subfractionated by a modification[5] of the method of PARSONS *et al.*[9]. The enzymes glucose-6-phosphatase and NADPH–cytochrome *c* reductase have a distribution totally different from the outer membrane marker and the inner membrane matrix markers. This

indicates very strongly that these enzymes (which are both firmly membrane-bound) are not localized in any mitochondrial membrane, but in the microsomes. The different distribution of these microsomal enzymes over the submitochondrial fractions from the gradient can be explained by the observed heterogeneous distribution over the microsomal subfractions as shown in Fig. 1. In Fig. 2 also, relatively more glucose-

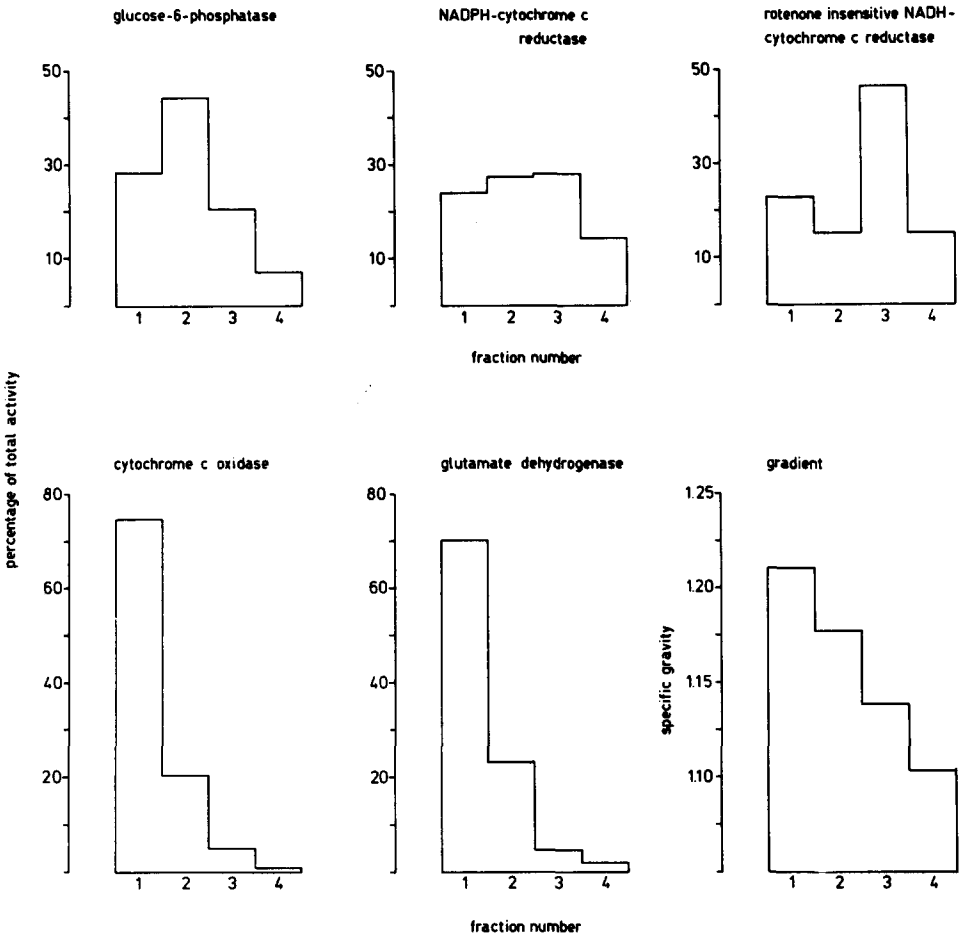

Fig. 2. The distribution of glucose-6-phosphatase, NADPH–cytochrome *c* reductase and some marker enzymes in submitochondrial fractions, isolated from twice-washed rat liver mitochondria. The mitochondria were subfractionated as described before[5], with a minor modification: instead of 9 fractions of 3 ml, 4 fractions of 6 ml were collected. For the estimation of glucose-6-phosphatase and NADPH–cytochrome *c* reductase, see the references given in the legend to Table I. Rotenone-insensitive NADH–cytochrome *c* reductase and cytochrome *c* oxidase were measured according to SOTTOCASA *et al.*[2] and glutamate dehydrogenase according to BEAUFAY *et al.*, as modified by SCHOLTE[8]. Mitochondria and submitochondrial fractions were treated with ultrasonic vibration before incubation (Branson S-75 sonifier; 1 min at 20 kHz). The percentage of the total activity in Fractions 1–4 is plotted against the fraction No. The recovery of the activities of the different enzymes in the gradient fractions varied between 85 and 110%. The specific activities of glucose-6-phosphatase and NADPH–cytochrome *c* reductase in isolated mitochondria are given in Table I. The activities of the marker enzymes in sonicated mitochondria were 18.5, 112 and 13.3 μmoles/mg protein per h for rotenone-insensitive NADH–cytochrome *c* reductase, cytochrome *c* oxidase and glutamate dehydrogenase, respectively. All enzyme activities were measured at 37°.

6-phosphatase is found in the gradient at a site of higher density as compared with NADPH–cytochrome *c* reductase.

The heterogeneous distribution of microsomal marker enzymes over microsomal subfractions as presented here is in agreement with the data reported by DALLNER *et al.*[10,11] and by TATA[12].

It is concluded that glucose-6-phosphatase and NADPH–cytochrome *c* reductase are not endogenous constituents of mitochondrial outer membranes and can therefore be used as marker enzymes for microsomes.

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