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Drug Demethylation by Calcium-aggregated Microsomes Prepared from Rabbit Organs

Demetylacja leków przy użyciu mikrosomów
preparowanych metodą agregacyjną z organów wewnętrznych królika

Деметилирование лекарств при помощи микросомов препарированных методом агрегации внутренних органов кроликов

Isolation of liver microsomes for the study of the *in vitro* metabolism of xenobiotics generally employs a technique of differential centrifugation utilizing an ultracentrifuge. Since this technique is time-consuming and requires an expensive ultracentrifuge, various non-ultracentrifugal methods have occasionally been elaborated for the preparation of the microsomal fractions (8). Among them, a procedure involving the sedimentation of the microsomes after their aggregation in the presence of calcium ions has been widely studied recently and gained acceptance in drug metabolism (1, 3, 5). In comparison with the traditional method, it has the advantage of reducing the preparation time nearly by half, and obviates the need for the ultracentrifuge.

Various authors have reported the preparation of rat hepatic microsomes by means of this calcium aggregation method (see 8). They have found comparable yields of microsomal protein and specific activity of different indicatory enzymes from calcium microsomes and microsomes obtained with the use of ultracentrifuge. On the other hand, the data have also been presented that some enzyme activities of calcium microsomes prepared from other tissues and species are impaired to some extent (2, 8).

The aim of the present report has been to study the application of the calcium aggregation method for the preparation of the enzymatically active microsomes from various rabbit organs, as well as their comparison with microsomes obtained with the use of ultracentrifuge.

MATERIALS AND METHODS

Animals. The experiments were carried out on adult male homebred rabbits of mixed race weighing between 2.0—2.5 kg. They had free access to standard diet and water. Phenobarbital was given as a 0.1% solution in drinking water for 6 days to induce glycogen load, the animal was decapitated, bleeded and the organs (liver, lung, kidney, heart) were excised, rinsed with cold saline and kept in 0.25 M sucrose containing 10 mM Tris-HCl (*pH* 7.4).

Microsomes. Pools of tissue were minced with scissors and homogenized in 4 volumes of sucrose using a teflon and glass Potter-type homogenizer. The homogenate was centrifuged for 15 min at 10,000×g and the resulting fat layer and the precipitate discarded. Each 10,000×g supernatant was divided into 2 equal portions. One portion was taken to prepare microsomes by the traditional method, the other by the calcium aggregation method. For the preparation of the ultracentrifugated microsomes, post-mitochondrial supernatant was centrifuged for 60 min at 105,000×g in a VAC-602 ultracentrifuge (GDR) and the resulting pellet was washed with 0.15 M KCl, containing 10 mM Tris-HCl (*pH* 7.4) and centrifuged again in the same manner. To receive the aggregated microsomes, 10,000×g supernatant was made 10 mM with respect to calcium chloride (*pH* 7.4) and, after 10 min of standing in ice-bath, the resulting precipitate of microsomes centrifuged at 20,000×g for 15 min in K-24 centrifuge (GDR). The microsomes were washed with KCl solution. The pellets were then resuspended in KCl solution in one-fifth volume of the postmitochondrial supernatant. Protein content was estimated with the Folin reagent.

Assays. Demethylation of drugs and hydroxylation of aniline was assayed in the incubation mixture containing in a final volume of 5ml: NADPH — generating system (2.0 μmol $NADP^+$, 25.0 μmol glucose-6-phosphate and 1 unit of glucose-6-phosphate dehydrogenase) Tris-HCl buffer (150 μmol, pH 7.4), $MgCl_2$ (25 μmol) and 5—7 mg of microsomal protein. The demethylation mixture was additionally suplemented with neutralized semicarbazide (25 μmol). After 5 min of preincubation at 37°C in a reciprocating shaker, the reaction was started by adding 150 μl of substrate solution (7.5 μmol). Samples (1 ml) were withdrawn every 5 min, mixed with trichloroacetic acid solution (12.5%, 1.5 ml), centrifuged and the resulting supernatant used for the determination of the product. Demethylation was estimated by measuring the amount of formaldehyde formed (7), while aniline hydroxylation by measuring p-aminofenol (7).

NADH-cytochrome b_5 reductase was determined by measuring its NADH-ferricyanide reductase activity (4). The assay mixture contained in the final volume of 1.5 ml: potassium ferricyanide (1.5 μmol), NADH (0.15 μmol), Tris-HCl buffer, *pH* 7.4 (100 μmol) and 150—300 μg of microsomal protein. The reaction was started at 22°C by the addition of NADH, and the reduction of ferricyanide was measured at 420 nm in a VSU-29 spectrophotometer (GDR). The activity was obtained from the initial rate of decrease in absorbance by using a milimolar extinction coefficient increment of 1.02.

NADPH-cytochrome P-450 reductase was estimated by measuring its NADPH-cytochrome c reductase activity at 550 nm (9). The assay mixture contained in the final volume of 1.5 ml: cytochrome c (0.15 μmol), NADPH (0.15 μmol), phosphate buffer, *pH* 7.4 (250 μmol) and 150—300 μg of microsomal protein. The reaction was initiated by the addition of NADPH. The rate of cytochrome c reduction was calculated using a coefficient of 21.0.

Glucose-6-phophatase activity was evaluated by measuring a liberation of inorganic phosphate. Incubation mixture contained in the total volume of 1 ml: glucose-6-phosphate (20 μmol), Tris-maleate buffer, *pH* 6.5 (100 μmol) and 1.0—1.5 mg of microsomal protein.

RESULTS

Initial experiments were carried out to re-examine the conditions of aggregation of microsomes by calcium ions for the rabbit material. In fact, there are contradictions in literature as to the velocity of centrifugation at which the aggregated microsomes are sedimented completely from the post-mitochondrial supernatant (1, 3), concentrations of calcium salt (3, 6) and additional necessity of magnesium ions for precipitation (2). One more reason for the study was the presence of only a single report about the preparation of calcium microsomes from rabbit material (6).

Figure 1 illustrates precipitation of hepatic microsomes from 10,000× ×g supernatant at different $CaCl_2$ concentrations. We find that 10 mM $CaCl_2$ is sufficient for the proper precipitation of microsomes. $MgCl_2$ increase the sedimentation at lower $CaCl_2$ concentrations, whereas its effect at higher concentrations of calcium salt appeared to be negligible.

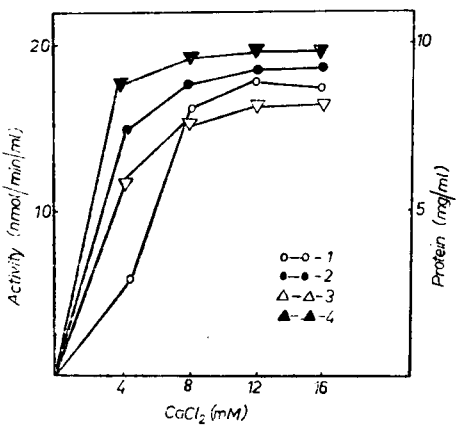

Fig. 1. Effect of Ca^{2+} and Mg^{2+} ions on the aggregation of rabbit hepatic microsomes. Set of 10 ml-samples of the post-mitochondrial supernatant, containing $MgCl_2$ (5 mM) or without this salt, were supplemented with increased amounts of $CaCl_2$, mixed well, kept in the icebath for 10 min, then centrifuged at 20,000×g for 15 min; 1 — protein, without $MgCl_2$, 2 — protein, with $MgCl_2$, 3 — aminopyrine demethylase, without $MgCl_2$, 4 — aminopyrine demethylase, with $MgCl_2$

Centrifugation of the post-mitochondrial supernatant containing 10 mM $CaCl_2$ at 10,00×g for 15 min sedimented most of the microsomes, although occasionally the sediment was not firmly packed. However, 15 min. centrifugation at 20,000×g gave the tight microsomal pellet.

The characteristics of the above calcium microsomes were next compared with the microsomes prepared by ultracentrifugation. At first, stability of the microsomal preparation was examined when it was kept in

the ice-bath. It was found that the half duration times ($t_{1/2}$) of an aminopyrine-demethylase activity were 40.8 hours and 51.6 hours for the calcium and ultracentrifugated preparations respectively. When the preparation was kept at 45°C, the resulting $t_{1/2}$-values of the enzyme activity were 12.2 min for the first preparation and 16.6 min — for the second one.

Table 1 shows the comparison of the protein yield and enzyme activities in the preparation of rabbit hepatic microsomes obtained by the two methods. It is noted that the difference in the protein content between these two preparations is insignificant. The same is also true for the activities of aniline hydroxylase, NADH-ferricyanide reductase and glucose-6-phosphatase. On the other hand, activities of aminopyrine demethylase and NADPH-cytochrome c reductase are slightly lower in the Ca^{2+} microsomes.

In the other series of experiments, microsomes from the extrahepatic organs were prepared by both conventional and calcium methods. As

Table 1. Protein yield and specific activity of enzymes from rabbit hepatic microsomes prepared with conventional and calcium methods

Determination	Methods	
	Conventional	Calcium
Protein *	21.60	19.20
Aminopyrine demethylase **	5.43	4.75
NADH-ferricyanide reductase ***	2.34	2.34
NADPH-cytochrome c reductase ***	43.60	35.60
Glucose-6-phosphate phosphatase ***	49.00	47.50
Aniline hydroxylase **	0.14	0.12

Results of two or three determinations: * mg/g tissue, ** nmol/min/mg protein, *** μmol/min/mg protein.

Table 2. Protein yield and aminopyrine demethylase activity from rabbit extrahepatic microsomes prepared with conventional and calcium methods

Determination	Organ	Methods	
		Conventional	Calcium
Protein *	brain	4.2	3.7
	heart	4.0	2.2
	lung	10.9	9.7
	kidney	9.1	10.7
Aminopyrine demethylase **	brain	0.15	0.19
	heart	0.29	0.27
	lung	0.82	0.75
	kidney	0.74	0.46

Results of two or three determinations: * mg/g tissue, ** μmol/min/mg protein.

shown in Table 2, the yield of microsomal protein from kidney and lung approximates half of the liver content, whereas from heart and brain it is several times lower. Aminopyrine demethylase decreases in the order: lung>kidney>heart>brain, and the values for the lung are about 6--times less. In addition, Ca^{2+}-microsomes from kidney are less active.

Demethylation of some other drugs by the hepatic Ca^{2+}-microsomes is presented in Table 3. The highest values were obtained with aminopyrine, ephedrine and ethylmorphine and the lowest — with diazepam and thioridazine.

Table 3. Drug demethylation by rabbit hepatic microsomes prepared with calcium method

Drugs	Activity	
	μmol/min/mg protein	%
Aminopyrine	4.75	100.0
Ephedrine	3.69	77.7
Ethylmorphine	3.29	69.3
Chlordiazepoxide	1.72	36.2
Pantocaine	1.72	36.2
Papaverine	1.32	27.8
Chinine	1.19	25.1
Chloropromazine	1.19	25.1
Diazepam	1.06	23.3
Thioridazine	0.79	16.6

Results of two determinations: Drugs at the conc. 1.5 mM.

DISCUSSION

According to K a m a t h et al. (3), 8 mM concentration of $CaCl_2$ in rat hepatic post-mitochondrial supernatant brings up the microsomes to sediment well during 10 min centrifugation at 10 000×g. C i n t i et al. (1) have claimed that the use of higher forces, that is 20 000×g or even 27 000×g for 15 min are needed for the complete sedimentation of microsomes. On the other hand L i t t e r s t et al. (6) precipitated microsomes from diluted rabbit post-mitochondrial supernatant at 10 000×g for 10 min in the presence of 32 mM $CaCl_2$. Our findings have established that by using 10 mM $CaCl_2$ in the undiluted post-mitochondrial supernatant it is possible for microsomes to settle down in the form of a tightly packed pellet.

According to L i t t e r s t et al. (6), rabbit hepatic microsomes appeared to be more susceptible to damage by Ca^{2+}-aggregation, since the treatment diminished NADPH-cytochrome c reductase, p-chloro-N-

-methylanine demethylase and biphenyl-4-hydroxylase. These activities were unimpaired in rat and rabbit kidney. Our results indicate that NADPH-cytochrome c reductase and aminopyrine demethylase activities are slightly diminished in Ca^{2+}-microsomes.

In conclusion it can be stated that in spite of the slight diminution of some enzymes in Ca^{2+}-aggregated rabbit microsomes, they are a useful source of mixed function oxidases metabolizing various xenobiotics. The advantage of the method can be especially emphasized when large amounts of tissues are to be processed in a single experiment or if no ultracentrifuge is at our disposal.

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STRESZCZENIE

Opracowano warunki otrzymywania mikrosomów z narządów wewnętrznych królika przy zastosowaniu metody agregowania jonami Ca^{2+}. Uzyskane mikrosomy porównano pod względem zawartości białka oraz aktywności niektórych enzymów metabolizmu leków z mikrosomami izolowanymi za pomocą ultrawirowania.

РЕЗЮМЕ

Обработано условия получения микросомальных препаратов из внутренних органов кролика при помощи скопления ионами Ca^{2+} и сравнено их в отношении содержания белка и активности некоторых ферментов метаболизма лекарств с микросомами изолированными при помощи ультрацентрифугирования.