INTERACTION OF CALCIUM WITH MICROSOMES: A MODIFIED METHOD FOR THE RAPID ISOLATION OF RAT LIVER MICROSOMES

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Summary

The addition of 8 mM Ca^{++} to a dilute post-mitochondrial supernatant of rat liver allows the isolation of the microsomal fraction by centrifugation at 30 xg for 10 minutes. The yield of microsomal protein and phospholipids is comparable to that obtained by centrifuging the untreated supernatant at 105,000 xg for one hour. The composition and functional activities of the membranes isolated by both methods are also similar. This method circumvents the need for an ultracentrifuge and results in a saving of time, particularly when multiple washes are necessary.

Because of the small size of microsomal particles(1), the isolation of the microsomal fraction of the liver requires a centrifugal force of 105,000 xg for 60 minutes(2), and hence is a time consuming operation which utilizes an expensive ultracentrifuge. During attempts to isolate and stabilize plasma membranes in the presence of Ca^{++} ions, the microsomes were noted to bind to this cation, after which they sedimented at a low centrifugal force of 1500 xg, leading to the contamination of plasma membranes(3). This observation formed the basis for a simplification of the conventional procedure for the isolation of hepatic microsomes using the divalent cation, Ca^{++}(4,5). In this communication, we report a further modification of the procedure for the rapid isolation of hepatic microsomes, and compare the characteristics of Ca^{++}-bound microsomes sedimented at a low centrifugal force with microsomes obtained by centrifuging the post-mitochondrial supernatant at 105,000 xg.

Materials and Methods

Sprague-Dawley male rats, weighing 150-200 gm, were fasted overnight (to reduce the amount of hepatic glycogen) and were exsanguinated by decapitation. The livers were excised and washed in ice-cold 0.25 M sucrose. A 20 percent (w/v) liver homogenate in 0.25 M sucrose was centrifuged in a Sorvall SS34

rotor at 12,000 xg for 10 minutes. The supernatant was decanted and centrifuged again in a similar manner, after which it was used for the isolation of microsomes by three methods.

1. Isolation of microsomes at 105,000 xg: The post-12,000 xg supernatant was centrifuged at 105,000 xg for 60 minutes(6) in a Beckman L2-65B ultracentrifuge. The top of the pellet was rinsed with 0.25 M sucrose to remove soluble proteins, after which the entire pellet was resuspended in 0.25 M sucrose.

2. Isolation of Ca^{++}-bound microsomes at 30 xg: The post-12,000 xg supernatant was diluted 1:5 (v/v) with 0.0125 M sucrose, which contained 8 mM $CaCl_2$ (pH 7.5). The solution was stirred for a few seconds and then centrifuged at 30 xg for 10 minutes. The pellet was resuspended as in method 1.

3. Isolation of microsomes at 105,000 xg, followed by Ca^{++}-treatment: The pellet obtained after the centrifugation of the post-12,000 xg supernatant at 105,000 xg for one hour was homogenized in 0.0125 M sucrose, containing 8 mM $CaCl_2$ (pH 7.5), and centrifuged at 30 xg for 10 minutes. The pellet was resuspended as in method 1.

The following measurements and procedures were carried out on the microsomal fraction in each case: total protein(7), lipid extraction(8), phospholipids(9), cholesterol(10), and RNA(11); aniline hydroxylase(12), aminopyrine demethylase(12) and succinic dehydrogenase(13) activities; cytochrome P-450 and cytochrome b_5(14); glucose-6-phosphatase, Na^+-K^+-Mg^{++} stimulated ATPase and 5' nucleotidase(5) activities. The incorporation of P into microsomal membranes was measured by injecting rats with 200 µCi $NaH_2{}^{32}PO_4$ (specific activity 300 mCi per mmole, New England Nuclear Corp.) intraperitoneally one hour before killing. The radioactivity of extracted phospholipids and the total microsomal suspension was determined by counting in a liquid scintillation counter.

The amount of Ca^{++} bound to microsomal protein was also determined either by diluting the 12,000 xg supernatant in the presence of 5 ml 8 mM Ca^{++}

supplemented with 1 μC per ml of $^{45}Ca^{++}$ (14.7 mCi per mg, New England Nuclear Corp.) or by homogenizing the 105,000 xg pellet in 5 ml 8 mM Ca^{++} containing 1 μC per ml of $^{45}Ca^{++}$.

For electron microscopic studies, fresh microsomal preparations were fixed in one percent OsO_4 in phosphate buffer (pH 7.4) for 2 hours. Thin sections of epon embedded material were examined in a Hitachi HS-8 electron microscope.

Results

The results are summarized in Tables 1 and 2. In Table 1 the values are expressed per mg protein, while in Table 2 they refer to gm of wet liver. The values given are the range obtained in four or more experiments.

In all three methods the yields of microsomal protein and phospholipids were comparable. In several experiments the post-12,000 xg supernatant was not diluted, and concentrated Ca^{++} was added. This resulted in lower recoveries even at 1500 xg, possibly because of the higher viscosity of the medium. The composition of the microsomal membranes appeared to be unaffected by the different procedures as evidenced by the similar contents of cholesterol, RNA, cytochrome P-450 and cytochrome b_5. Enzyme activities were also comparable in all preparations, although the activities of the drug metabolizing enzymes were slightly higher in Ca^{++}-bound microsomes sedimented at 30 xg. No significant differences were noted in the specific activities of ^{32}P in the total microsomal suspensions. The specific activity in phospholipids was also about the same; 996, 1018 and 987 counts per minute per μg P respectively in procedures 1,2 and 3.

The specific activity of $^{45}Ca^{++}$ was about 60 percent greater in microsomes obtained by treating the post-12,000 xg supernatant with Ca^{++} than in those sedimented first at 105,000 xg, and then treated with Ca^{++}.

Electron micrographs of all preparations disclosed both smooth and rough vesicles; the latter displayed attached intact ribosomes (Fig. 1).

Discussion

The studies reported here represent a standardization of the rapid

TABLE I

CHARACTERISTICS OF DIFFERENT MICROSOMAL PREPARATIONS PER MG MICROSOMAL PROTEIN

	Ca^{++}-bound Microsomes (30 xg, 10 minutes)	"Conventional" Microsomes (105,000 xg, 1 hour)	"Conventional" Microsomes + Ca^{++}-treatment (30 xg, 10 minutes)
Glucose-6-phosphatase[a]	13.0-15.7	11.0-14.8	10.5-14.2
Na^{+}-K^{+}-Mg^{++} activated ATPase[a]	7.1- 8.5	8.7- 9.1	6.9- 8.3
5'-Nucleotidase[a]	10.1-11.8	9.7-11.8	10.5-10.8
Aminopyrine demethylase[b]	6.1- 6.8	5.8- 6.1	6.2- 6.7
Aniline hydroxylase[b]	0.56-0.69	0.53-0.57	0.53-0.58
Cytochrome P-450[c]	0.68-0.74	0.63-0.76	0.68-0.75
Cytochrome b_5[c]	0.43-0.46	0.45-0.52	0.42-0.48
Succinic dehydrogenase[d]	0.12-0.21	0.31-0.46	0.30-0.34
Phospholipid[e]	0.32-0.37	0.29-0.31	0.30-0.34
Cholesterol[e]	0.025-0.030	0.023-0.029	0.022-0.030
RNA[e]	0.18- 0.22	0.19- 0.23	0.19- 0.21
^{32}P cpm[f]	19400-24000	21600-25000	20600-23200
$^{45}Ca^{++}$ cpm[g]	24400-27500	-	15600-17000

a micromoles Pi liberated per hour
b nano moles HCHO or p-aminophenol formed per minute
c nano moles
d micromoles INT formazan formed per hour
e mg
f Male rats were given 200 µc $NaH_2{}^{32}PO_4$ for 65 minutes before preparing the microsomes.
g Post-mitochondrial supernatant or 105,000 xg microsomal pellet was diluted with 8 mM Ca^{++} containing 1 µc per ml $^{45}Ca^{++}$ and the pellets obtained after centrifugation were washed twice before counting the radioactivity.

TABLE 2
ENZYMATIC ACTIVITIES AND COMPOSITION OF
DIFFERENT MICROSOMAL PREPARATIONS PER GM OF WET LIVER

	Ca^{++}-bound Microsomes	"Conventional" Microsomes	"Conventional" Microsomes + Ca^{++}-treatment
Microsomal yield[a]	30-36	31-36	29-34
Glucose-6-phosphatase[b]	450-475	458-468	398-422
Na^{+}-K^{+}-Mg^{++} activated ATPase[b]	238-256	270-289	201-229
5'-Nucleotidase[b]	348-370	316-340	324-342
Aminopyrine demethylase[c]	194-210	178-190	186-194
Aniline hydroxylase[c]	19.4-21.6	16.8-18.1	16.9-18.6
Cytochrome P-450[d]	22.6-24.1	18.9-22.1	18.6-20.2
Cytochrome b_5[d]	13.9-14.8	14.2-16.4	13.6-15.2
Succinic dehydrogenase[e]	5.1-5.6	8.9-11.3	6.8-9.7
Phospholipid[f]	9.8-11.4	9.1-9.8	9.4-9.7
Cholesterol[f]	0.86-0.92	0.78-0.85	0.80-0.83
RNA[f]	5.9-6.1	6.1-6.4	6.0-6.4

a mg protein
b micromoles Pi liberated per hour
c nano moles HCHO or p-aminophenol formed per minute
d nano moles
e micromoles INT formazan formed per hour
f mg

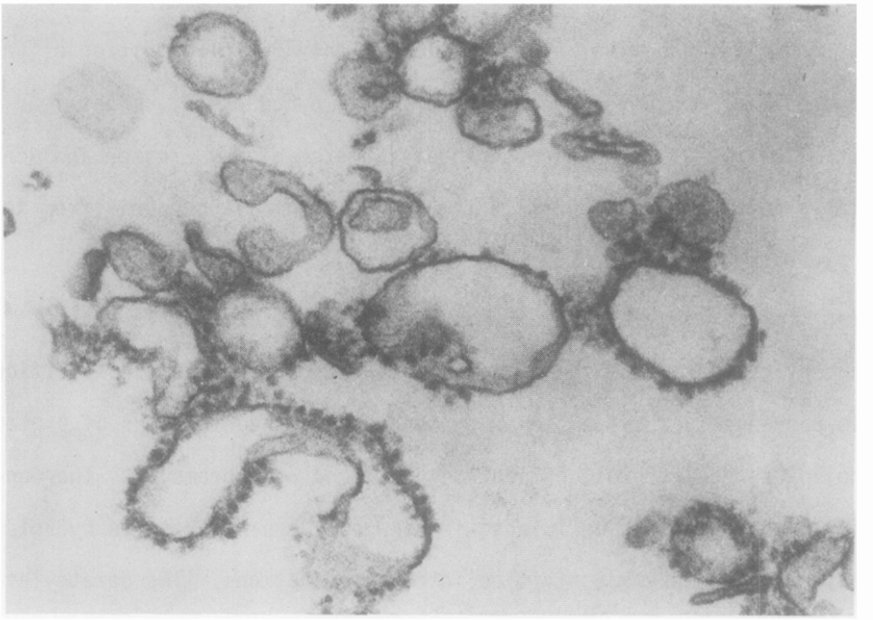

Fig 1. Electron micrograph of Ca^{++}-bound microsomes of rat liver showing smooth vesicles and rough vesicles with attached ribosomes. (X 82,000)

procedure for the isolation of hepatic microsomes using Ca^{++}-binding. This procedure has the advantages of rapidity, simplicity, mild experimental conditions, and the circumvention of a high speed ultracentrifuge, which saves considerable expense. Although a number of divalent ions such as Hg^{++}, Ba^{++}, Cu^{++}, Sr^{++}, Zn^{++}, Fe^{++} and Mg^{++} have the capacity to bind to hepatic microsomes(5), Ca^{++} was the ultimate choice because of its well known physiological functions and importance in cell membrane systems(15).

The dilution of the supernatant from 0.25 to 0.04 M sucrose is necessary for sedimentation of the microsomes at 30 xg. In the original method(4), the tissue homogenizing medium consisted of isotonic sucrose which contained Mg^{++}, K^{+} and Ca^{++} ions. Addition of Mg^{++} alone to a microsomal suspension did not lead to sedimentation at low centrifugal force(4). With the original method, centrifugation at less than 1500 xg resulted in lower recoveries (unpublished observations), presumably because Mg^{++} competes with Ca^{++} for binding sites on the microsomes. In the present method only Ca^{++} was bound to the micro-

somes, resulting in optimal sedimentation at only 30 xg. Addition of Ca^{++} to the homogenizing medium, according to original procedure(4), might lead to the structural disruption of mitochondria, as pointed out by Ernster and Jones(16). Thus the present procedure permits the isolation of mitochondria from the same liver processed for microsomes.

Not only is the yield of Ca^{++}-bound microsomes comparable to that obtained by conventional high speed centrifugation, but their composition is also unchanged, as evidenced by the unchanged concentrations of protein, phospholipids, cholesterol, cytochrome P-450 and cytochrome $\underline{b_5}$. The functional activity also appears to be intact, in view of the similarity in hydrolytic and dehydrogenase enzyme activities in all preparations. The demethylation of aminopyrine (a type I binding substrate(17)) and the hydroxylation of aniline (a type II binding substrate(17)) were actually somewhat augmented in Ca^{++}-bound microsomes, in agreement with the report of others(18). Microsomes derived by the original procedure have been shown to be suitable for the study of drug metabolizing enzyme systems(18,19).

The studies with $^{45}Ca^{++}$ indicate that more Ca^{++} is bound to microsomes when it is added to the post-12,000 xg supernatant, than when added to microsomes obtained at 105,000 xg. This, however, does not affect the recovery after centrifuging the latter at 30 xg for 10 minutes.

The use of Ca^{++} binding for rapid isolation of microsomes has certain advantages over other short methods. Acid precipitation of microsomes(20) requires soluble protein for functional activity, whereas details of functional activity of microsomes precipitated by ethanol(21) are lacking.

The treatment of microsomes isolated at 105,000 xg with Ca^{++}, to make them sediment at 30 xg in 10 minutes, entails additional effort, but has the advantage of retaining the soluble supernatant without the addition of Ca^{++}, which may be important in some circumstances. The soluble protein of the supernatant plays a role in a number of reactions, such as those involving phospholipid transfer between microsomes and mitochondria(22) and ribosomal protein

synthesis(23). Thus where repeated washings of the microsomes are necessary, and the supernatant is required, the addition of Ca^{++} to microsomes sedimented at 105,000 xg may save considerable time.

Schenkman and Cinti(19) described the loss of ribosomes from the endoplasmic reticulum after treatment with Ca^{++}, but in our hands the membranes did not lose their attached ribosomes, as noted in the electron micrographs, and as evidenced by the unchanged content of RNA. The experimental conditions or differences in fixation of microsomes for electron microscopic studies may account for this disparity.

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