Biochemical Pharmacology, Vol. 21, pp. 3249–3256. Pergamon Press, 1972. Printed in Great Britain.

KINETIC PARAMETERS OF DRUG-METABOLIZING ENZYMES IN Ca^{2+}-SEDIMENTED MICROSOMES FROM RAT LIVER*

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(*Received* 13 *March* 1972; *accepted* 7 *July* 1972)

Abstract—Kinetic parameters of five substrates of the mixed function oxidase system were determined to ensure that the mixed function oxidases were unaltered by the Ca^{2+}-sedimented microsomes. Using either of the microsomal preparation procedures, no differences were noted in the V_{max} and K_m for aminopyrine, ethylmorphine and *p*-nitroanisole demethylation, aniline hydroxylation and hexobarbital oxidase. Also, no differences were seen in the NADPH cytochrome P-450 reductase activities. The Ca^{2+}-sedimentation procedure was further simplified to allow microsomal preparation within 1 hr.

SYSTEMATIC attempts to separate cell fractions by differential centrifugation date back to the pioneering studies of Claude[1,2] who first described and named the microsomal fraction of liver. Schneider and Hogeboom[3] modified the procedure for cell fractionation, and today most methods for preparation of hepatic microsomes are based upon their procedure of consecutive increases of centrifugal force.

The conventional procedure in use in our laboratory is modified from an earlier procedure[4] and involves differential centrifugation of a 10–20% liver homogenate (w/v) in 0·25 M sucrose at 2000 rev/min (400 *g*) and 12,000 rev/min (18,000 *g*) for 5 and 10 min, respectively, in a refrigerated centrifuge (Sorvall); these steps remove nuclei plus cell debris and mitochondria. The resultant supernatant is then centrifuged at 30,000 rev/min (105,000 g_{max}) in a Spinco preparative ultracentrifuge for 1 hr. For spectral studies the microsomal pellet obtained is washed by resuspending in 0·15 M KCl (to remove any residual hemoglobin) and recentrifuged for 1 hr at 105,000 *g*. The whole procedure takes 2–3 hr.

In order to accelerate this procedure, advantage was taken[5] of a report by Kamath *et al.*[6] that plasma membranes prepared in the presence of 0·5–5 mM $CaCl_2$ were heavily contaminated with microsomes. Their method[7] was evaluated[8] and found to yield microsomes which appeared, by electron microscopy, to differ slightly from the microsomes prepared in the absence of added calcium ions (ribosomes were stripped from the rough microsomes).[8] The calcium-sedimented microsomes were able to metabolize aminopyrine and aniline at essentially the same rate as the conventionally prepared microsomes.[8] More recently, Kupfer and Levin,[9] using the procedure of

* This work was supported by United States Public Health Service Research Grant GM17021.
† Public Health Service Research Career Development Awardee, GMK4-19,601 of the NIH.

Kamath *et al.*,[7] reported no differences in the demethylation of aminopyrine and *p*-chloro-*N*-methylaniline and the hydroxylation of Δ′-tetrahydrocannibanol between the Ca^{2+}-aggregated and conventionally prepared rat liver microsomes.

In the previous preliminary communications,[7–9] only the presence of enzymic activity was demonstrated. In order to ensure that the mixed function oxidases are not altered by the calcium-augmented sedimentation method for preparing rat liver microsomes, the kinetic constants for the metabolism of several substrates of different classes of microsomal oxidases were measured. The substrates were selected to represent four major oxidative reactions occurring in microsomes, *O*-dealkylation, *N*-dealkylation, aromatic hydroxylation and side chain oxidation. In the process, it was found that our previous method[8] yielded unwieldly volumes; hence, the procedure was further modified.

METHODS

Livers from fed, male Sprague–Dawley rats (250–300 g) are removed, perfused with 0·9% sodium chloride, and homogenized in 0·25 M sucrose; the 10–20% liver homogenate is then differentially centrifuged as shown in Fig. 1 to obtain a post-mitochondrial supernatant (after 12,000 g_{max} × 10 min). Addition of 8 mM $CaCl_2$ allows complete sedimentation of microsomes at 27,000 g_{max} in 15 min. The pellet may then be washed by resuspending in an equal volume of 0·15 M KCl and resedimenting at

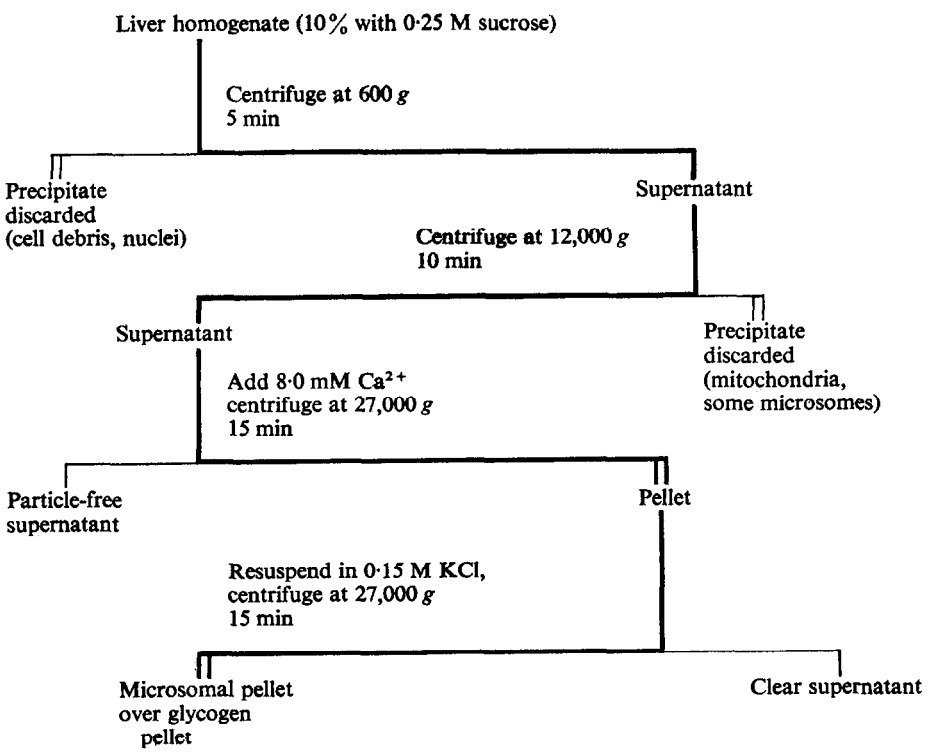

FIG. 1. Schematic representation of procedure for the rapid isolation of microsomes. All steps are carried out at 0–4°.

27,000 g_{max} for 15 min. The resultant pinkish, opalescent pellet of microsomes overlies a small translucent glycogen pellet, from which it is readily separated merely by tapping the test tube. The procedure takes less than 1 hr to yield a microsomal preparation.

Enzyme assays. The *N*-demethylation of aminopyrine and ethylmorphine was estimated by measuring the amount of formaldehyde formed according to the method of Nash.[10] Aniline hydroxylation was determined by measuring *p*-aminophenol formation.[11] Microsomal *O*-dealkylation was determined by the demethylation of *p*-nitroanisole, measuring *p*-nitrophenol by the method of Netter.[12] The hexobarbital metabolism was determined by measuring the disappearance of hexobarbital in the incubation mixture after 30 min by the procedure of Cooper and Brodie.[13]

NADPH cytochrome P-450 reductase activity was determined in an Aminco–Chance dual wavelength/recording spectrophotometer at 450 nm relative to 465 nm, according to Schenkman and Cinti.[14] NADPH cytochrome-*c* reductase was measured by the method of Phillips and Langdon.[15] Cytochromes b_5 and P-450 were assayed by the method of Omura and Sato.[16] Proteins were estimated by the biuret reaction using albumin as a standard. Kinetic constants were obtained by the method of least squares.

RESULTS

By the procedure of Kamath *et al.*,[7] dilution of the 0·25 M sucrose to 0·05 M sucrose with 0·0125 M sucrose, and addition of 8 mM $CaCl_2$ (5 mM $MgCl_2$ and 25 mM KCl are not necessary)[8] allow sedimentation of the 12,000 *g* post-mitochondrial supernatant at 2500 *g* in 10 min due to the lower viscosity and specific gravity. In fact, the microsomes will actually sediment completely while standing in ice for 10 min, but centrifugation is necessary to pack them into a pellet; however, this dilution results in large unwieldy volumes. In the procedure described above (see Methods), the presence of 8 mM $CaCl_2$ in the undiluted 12,000 g_{max} post-mitochondrial supernatant does not cause the microsomes to settle from suspension in the 0·25 M sucrose even after standing for 1 hr in ice. Centrifugation of the undiluted post-mitochondrial supernatant, containing 8 mM $CaCl_2$, at 2500 g_{max} for 10 min loosely sediments about 50 per cent of the microsomes. Centrifugation at 10,000 g_{max} for 10 min sediments most of the microsomes (supernatant slightly turbid), although the pellet is still not firmly packed. At 20,000 g_{max} for 10 min the microsomal pellet is tight, but recentrifugation of the resultant supernatant at higher forces (27,000 g_{max}) will bring down the rest of the microsomes.

Table 1 shows a comparison of the yield and some characteristics of liver microsomes prepared from the same post-mitochondrial supernatant by conventional and rapid (Fig. 1) methods. The yield of microsomal protein per g of liver (starting with 25 g liver from two male, 300-g Sprague–Dawley rats) was the same by both procedures; the yield approximates half of the liver content, as determined by liver slice spectrophotometry.[17] The calcium content of the microsomes before the KCl wash was low, 5 and 0·2 μg/mg protein, in the calcium-sedimented and conventional preparations, respectively, as determined by atomic absorption spectroscopy.

Many substrates of the microsomal mixed function oxidase cause spectral changes when added to liver microsomes.[11] One spectral change, termed type I,[4] has been related directly to mixed function oxidase activity.[18] The other spectral change, termed type II,[4] was shown to be due to formation of a ferrihemochrome.[11] As seen in Table 1,

TABLE 1. COMPARISON OF THE PREPARATIVE PROCEDURES WITH RESPECT TO VARIOUS PARAMETERS CONCERNING DRUG BIOTRANSFORMATION*

Preparation	Milligrams of microsomal protein/per gram of liver	Absorbance				
		Aniline	Hexobarbital	Aminopyrine	Ethylmorphine	*p*-Nitroanisole
Conventional method	20	0·015	0·008	0·005	0·007	0·005
Ca^{2+} method	21	0·016	0·009	0·005	0·006	0·005

* Microsomal protein content in each preparation was 1·0 mg/ml. Absorbance: for aniline, 430 nm minus 500 nm/mg microsomal protein/ml; for hexobarbital, 419 nm minus 500 nm/mg/ml; for ethylmorphine and aminopyrine, 420 nm minus 500 nm/mg/ml; the concentration of aniline was 18 mM, of hexobarbital and *p*-nitroanisole 3·3 mM, and of ethylmorphine and aminopyrine 3·5 and 3·8 mM, respectively; volumes added did not exceed 10 μl. With aniline in the sample cuvette, the addition of hexobarbital to both cuvettes increased the absorbance of aniline to 0·018 using Ca^{2+}-sedimented microsomes and 0·017 microsomes obtained by the conventional method.

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TABLE 2. EFFECT OF THE METHOD OF MICROSOMAL PREPARATION ON THE KINETIC CONSTANTS OF FIVE SUBSTRATES OF THE HEPATIC MICROSOMAL MIXED FUNCTION OXIDASE SYSTEM*

	Ethylmorphine demethylase		Aminopyrine demethylase		Aniline hydroxylase		Hexobarbital oxidase		*p*-Nitroanisole demethylase	
Preparation	V_{max} (nmoles/min/mg)	K_m (mM)	V_{max} (nmoles/min/mg)	K_m (mM)	V_{max} (nmoles/min/mg)	K_m (mM)	V_{max} (nmoles/min/mg)	K_m (mM)	V_{max} (nmoles/min/mg)	K_m (mM)
Conventional method	2·8	0·50	3·4	0·36	0·3	0·08	2·4	0·25	1·6	0·19
Ca^{2+} method	2·8	0·47	3·3	0·35	0·3	0·09	2·5	0·26	1·6	0·18

* The data in the table represent a typical experiment. The experiments were repeated twice with pooled microsomes from two male rats and are consistent with data from other studies.

Table 3. Effect of the method of microsomal preparation on the content of the microsomal hemoproteins, on NADPH cytochrome-*c* reductase activity and on NADPH cytochrome P-450 reductase activity

Preparation	Cytochrome b_5 (nmoles/mg microsomal protein)	Cytochrome P-450 (nmoles/mg microsomal protein)	NADPH cytochrome-*c* reductase* (nmoles/min/mg)	NADPH cytochrome P-450 reductase† (nmoles/min/mg)
Conventional method	0·33	0·56	90	1·92
Ca^{2+} method	0·32	0·60	90	1·91

* 30°.
† 30°, in the absence of added substrate.

no difference was observed in the magnitude of the type I spectral changes elicited by hexobarbital, aminopyrine, ethylmorphine or *p*-nitroanisole addition to the two microsomal preparations. Similarly, the magnitudes of the type II spectral change obtained on addition of aniline to both microsomal preparations were the same. Furthermore, the addition of a type II substrate to the experimental cuvette containing a type I substrate resulted in an increase in the absorption peak of the type II substrate to the same extent with either microsomal preparation (note: the reference cuvette contained only the type I substrate). To obtain a type I spectral change with *p*-nitroanisole, it was necessary to use split-cells because of interference by the yellow color; the absorbance changes were the same with either microsomal preparation.

Enzyme kinetics performed on the microsomal mixed function oxidase indicated that calcium-implemented sedimentation of liver microsomes did not adversely affect the enzyme system (Table 2). The kinetic constants (K_m and V_{max}) obtained from Lineweaver–Burk plots[19] of substrate dependence of aminopyrine, ethylmorphine, and *p*-nitroanisole demethylase, aniline hydroxylase and hexobarbital oxidase did not reveal any differences between the two microsomal preparations.

No difference was observed in the hemoprotein content of the microsomes (Table 3); cytochrome b_5 and cytochrome P-450 looked normal spectrophotometrically. NADPH cytochrome-*c* reductase and NADPH cytochrome P-450 reductase activities also were unaltered by the calcium-sedimentation procedure.

DISCUSSION

These findings indicate that the hepatic microsomal mixed function oxidase and its components do not differ in calcium-sedimented microsomes from those of conventionally prepared microsomes. Several other microsomal enzyme activities (glucose 6-phosphatase, adenosine triphosphatase, 5′-nucleotidase and inosine diphosphatase) have also been found to be unimpaired by the calcium ion.[7] More recently, Kupfer and Levin[9] reported no differences in the rate of demethylation of *p*-chloro-*N*-methylaniline and the rate of hydroxylation of Δ′-tetrahydrocannabinol with the $CaCl_2$-aggregated microsomes or with the normally prepared microsomes.

Since the only structural difference in the microsomes is the absence of ribosomes from the rough microsomes,[8] it would appear that the effect calcium has is to cause an aggregation of microsomes in suspension. This aggregation is more apparent in very dilute sucrose solutions since the lower viscosity does not slow or prevent sedimentation on standing. Of interest is the fact that calcium ions have the opposite effect on another cellular organelle, the nuclei. Normally nuclei tend to aggregate in liver homogenates and to become distorted. The addition of 1·8 mM $CaCl_2$ to the homogenizing medium[20] allows isolation of nuclei free from clumping or adsorbed connective tissue.

The time required from homogenization of liver to final suspension of washed microsomes is about $\frac{3}{4}$ hr, using the method shown in Fig. 1. Using the conventional method of preparation, isolation of the same microsomes would require 2–3 hr, and the use of a preparative ultracentrifuge. The benefit of the described method is at once apparent.

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