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Immobilization of Pig Liver Microsomes

Stability of Cytochrome P-450-Dependent Monooxygenase Activities

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ABSTRACT

Microsomes from pig liver were covalently coupled to Sepharose activated by CNBr and to Sephadex activated by 1,1'-carbonyldiimidazole. Microsomes were also entrapped inside Ca-alginate and κ-carrageenan gels. The concentration of immobilized cytochrome P-450 was determined by CO-difference spectra. The activity of the monooxygenase system was demonstrated by the *N*-demethylation of aminopyrine, the *O*-demethylation of *p*-nitroanisole, and the hydroxylation of perhexiline maleate. Upon immobilization, a 30–40% and a 60–70% decrease in V^{app}_{max} for the *O*- and *N*-demethylations were respectively observed. The V^{app}_{max} values for the hydroxylation of perhexiline maleate were essentially the same for the different immobilized forms and for the freely suspended microsomal cytochrome P-450.

Under storage at 4°C, microsomes entrapped inside κ-carrageenan and Ca-alginate were less stable than the free microsomes, whereas immobilization on CNBr-activated Sepharose improved the

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stability of the hepatic microsomal monooxygenase system at the same temperature. These types of immobilized microsomes have the advantage of being easily recovered and reused for other assays. Finally, microsomes entrapped inside κ-carrageenan or Ca-alginate can be used to follow up the continuous metabolization of *p*-nitroanisole for several hours in a stirred-batch reactor.

Index Entries: Cytochrome P-450; microsomes, entrapment of; microsomes, covalent binding; pig liver.

INTRODUCTION

The oxidative biotransformation of a variety of lipophilic endogeneous and exogeneous compounds is effected by the hepatic microsomal monooxygenase system (*1*), in which cytochrome P-450 is the terminal oxidase.

Immobilization of the hepatic microsomal monooxygenase system has been attempted several times. Schubert et al. (*2*) have immobilized hepatic microsomes by entrapment inside gelatin to construct an enzyme electrode for bioelectrochemical measurement of NADPH. Lehman et al. (*3*) have covalently bound solubilized rabbit liver microsomal cytochrome P-450 to Sepharose-4B activated by CNBr and studied the *O*-demethylation and the subsequent glucuronidation of some lipophilic compounds. In a recent study, rat liver microsomes were entrapped in a synthetic gel, obtained by crosslinking polyacrylamide hydrazide with glyoxal (*4*). However, in almost all cases the cytochrome P-450 activity decreased rapidly, and further investigations are necessary to determine suitable methods for immobilization in order to increase the stability of the hepatic microsomal monooxygenase system.

The field of application of immobilized hepatic microsomes in reactors is in the pioneering stage of development. "Metabolization reactors" could be constructed for the study of drug biotransformation (*5*). Another type of reactors may be developed for the selective hydroxylation of steroids and amines (*6*). The extracorporeal detoxification of blood by metabolization of drug contaminants could also be investigated (*7*).

In the case of immobilization of subcellular fractions and cells, it is not possible to state general rules for the selection of the immobilization method for a given system. The solution to this problem still remains in the domain of experimental verification of the adequacy of various immobilization methods and carriers.

In this paper, we describe the immobilization of microsomes from pig liver by covalent binding onto CNBr-activated Sepharose and 1,1′-carbonyldiimidazole-activated Sephadex. Microsomes were also entrapped inside Ca-alginate and κ-carrageenan and cocrosslinked with an inactive protein by means of glutaraldehyde. Pig liver microsomes were chosen because it has been shown that the metabolism of drugs and other xenobiotics in pig is similar to that in man (*8*). The activity of the

immobilized monooxygenase system was demonstrated by the *N*-demethylation of aminopyrine, the *O*-demethylation of *p*-nitroanisole, and the hydroxylation of perhexiline maleate.

MATERIALS AND METHODS

Chemicals: Sephadex® G-150 and Sepharose®-4B were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Sodium alginate and dithiothreitol (DTT) were obtained from Sigma (St. Louis, Mo, USA). Kappa-carrageenan was from CECA SA (Velizy-France). Aminopyrine (4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolone-5) and *p*-nitroanisole were obtained from Merck (Darmstadt, Germany). Perhexiline maleate [Pexid®, 1,1-dicyclohexyl-2-(2-piperidyl) ethane] was a gift from Merell Toraude (Strasbourg, France). The IPS-600 (*N*-cyclohexyl-3,3-diphenyl propylamine) was a gift from the Institut de Pharmacologie et Médecine Expérimentale (Strasbourg, France). The 1,1'-carbonyldiimidazole (CDI) and cyanogen bromide (CNBr) were purchased from Aldrich Europe (Beerse, Belgium). All other chemicals were commercial products of the highest purity available.

Preparation of Microsomes

Pig liver was rinsed with 10 m*M* phosphate buffer, pH 7.4, containing 9‰ NaCl w/v and 1 m*M* EDTA, then blotted on a filter paper and cut into small slices. Using a Waring Blender homogenizer, the slices were homogenized in 4 vol 50 m*M* phosphate buffer, pH 7.4, containing 250 m*M* saccharose and 10 m*M* EDTA. The homogenate was centrifuged at 1300*g* for 10 min. The supernatant fraction was centrifuged at 10,000*g* for 20 min in a Beckman L5-75 B refrigerated ultracentrifuge.

The pelleted mitochondria were eliminated, and the supernatant was centrifuged at 105,000*g* for 60 min. The microsomal pellets were washed thoroughly with 10 m*M* phosphate buffer, pH 7.4, containing 10 m*M* EDTA and 0.1 m*M* DTT to remove hemoglobin, then centrifuged at 105,000*g* for another 60 min. Microsomes were then suspended in a 100-m*M* potassium phosphate buffer, pH 7.4, containing 1 m*M* EDTA, 0.1 m*M* DTT, and 20% glycerol, and stored at −20°C. The content of cytochrome P-450 was determined by the CO-difference spectra of the reduced form (*9*). The microsomal preparation usually contained 0.4 nmol cytochrome P-450/mg protein.

Immobilization of CNBr Activated Sepharose-4B

Activation of Sepharose-4B was carried out according to the method of Axén et al. (*10*). Sepharose-4B was mixed with an equal vol of water, then CNBr (10% w/v) was added at pH 11.0. The activated Sepharose beads were washed with distilled water to remove unreacted CNBr. The

beads were resuspended in 25 m*M* phosphate buffer, pH 8.3, containing 0.1 m*M* DTT, 1.0 m*M* EDTA, and 20% glycerol (v/v). The microsomal preparation was added to the gel (250 nmol cytochrome P-450/100 mL gel), and the mixture was gently stirred at 4°C overnight. Thereafter, the gel was filtered and washed thoroughly with the same buffer to remove any unreacted microsomes. The beads were resuspended in 25 m*M* phosphate buffer, pH 7.4, containing 0.1 m*M* DTT, 1.0 m*M* EDTA, and 40% glycerol, and stored in the same buffer at 4°C. The concentration of cytochrome P-450/mL gel was also determined by CO-difference spectra (*9*).

Immobilization onto Sephadex G-150 Activated by Carbonyldiimidazole

The activation of Sephadex G-150 by CDI was carried out according to the method of Bethell et al. (*11*), except that the solvent was pure DMF. The activated Sephadex beads were filtered to remove the organic solvent, then resuspended in 25 m*M* phosphate buffer, pH 8.3, containing 0.1 m*M* DTT, 1.0 m*M* EDTA, and 40% glycerol (v/v). The microsomal preparation (50 nmol cytochrome P-450/100 mL gel) was added, and the reaction mixture was stirred at 4°C overnight. Immobilized microsomes were then washed and stored in the same manner described for the immobilization of microsomes on CNBr-activated Sepharose.

Entrapment in Ca-Alginate

Microsomes were entrapped in Ca-alginate, using the method of Kierstan and Bucke (*12*). The microsomal preparation was mixed with 1.2% sodium alginate solution (250 nmol cytochrome P-450/100 mL gel), and the total slurry was slowly extruded through a hypodermic needle into 0.1*M* $CaCl_2$. The pellets formed were filtered and washed with 100 m*M* Tris-buffer, pH 7.4, containing 0.1*M* $CaCl_2$, 1.0 m*M* EDTA, and 0.1 m*M* DTT. No free microsomes should be in the washings. The pellets were suspended again in the same buffer, and the amount of cytochrome P-450/pellet (a) was determined as follows:

$$a = \frac{\text{total amount of cytochrome P-450 used for immobilization}}{\text{total number of pellets obtained}}$$

Entrapment in κ-Carrageenan

A 1.4% κ-carrageenan solution was prepared at 50°C, and the microsomal preparation was mixed in order to obtain a concentration of 71 nmol cytochrome P-450/100 mL gel. A peristaltic pump was used to extrude the slurry mixture through a hypodermic needle into a 0.5*M* KCl solution. The pellets obtained were filtered and stored in 100 m*M* Tris-buffer, pH 7.4, containing 0.5*M* KCl, 1.0 m*M* EDTA, and 0.1 m*M* DTT.

The amount of cytochrome P-450/pellet was determined in a manner similar to that described under entrapment in Ca-alginate.

Cocrosslinking at Microsomes with an Inactive Protein

The cocrosslinking method used was previously described by Broun et al. (*13*): a bovine serum albumin solution (11% w/v) in 20 m*M* phosphate buffer, pH 6.8, containing 0.46% glutaraldehyde, was prepared. After 2 min of prepolymerization, the microsomal preparation was mixed with this preparation at the concentration of 300 nmol cytochrome P-450/100 mL preparation. The reaction mixture was frozen at −20°C for 2 h, then slowly warmed up to 4°C in a refrigerator. After 4 h the copolymer formed had a spongy aspect. It was thoroughly washed to remove any free microsomes.

Measurement of Aminopyrine N*-Demethylase Activity*

N-demethylase activity was assayed according to the method of Yang et al. (*14*). The reaction mixture contained a known amount of immobilized or free microsomes (usually 1 nmol cytochrome P-450), 5.0 m*M* aminopyrine, and the NADPH-generating system, all in 0.1*M* phosphate buffer, at pH 7.4, and the reaction was initiated by the addition of NADP. Incubation was carried out in 5-mL tubes, using a rotatory shaker at 37°C. After 15 min of incubation the reaction was stopped by separating the immobilized microsomes from the unreacted substrate and the demethylated product by filtration or centrifugation. In the case of free microsomes, 20% $ZnSO_4$ solution and a saturated solution of $Ba(OH)_2$ were added, and the turbid mixture was centrifuged to remove the precipitated proteins. Then, 2 mL of the supernatant was mixed with 1 mL of Nash reagent (*15*). The mixture was incubated at 60°C for 30 min, and the amount of formaldehyde produced was calculated from the absorbance at 415 nm. All results are averages of duplicate runs.

Measurement of p*-Nitroanisole* O*-Demethylase Activity*

O-demethylase activity was assayed by measuring the amount of *p*-nitrophenol formed, according to the method of Netter et al. (*16*). The assay procedure was carried out on free or immobilized microsomes in a manner similar to that used for the *N*-demethylase activity determination.

Measurement of Perhexiline Maleate Hydroxylase Activity

To a free- or immobilized-microsomal preparation (1 nmol cytochrome P-450), 100 μL of a solution of the substrate (12.9 m*M* in methanol) and the NADPH-generating system (0.5 m*M* NAD, 0.42 m*M* NADP, 32 m*M* glucose-6-phosphate, and 97 U glucose-6-phosphate dehydrogenase) were added. After incubation at 37°C for 15 min, the re-

action was stopped by addition of 0.6*N* HCl (0.5 mL). Thereafter, 10 μL of 1.5 m*M* internal standard (IPS 600) and 10 mL cyclohexane were added. After shaking and centrifugation, the pH of the aqueous layer was brought to 11. After addition of 10 mL diethyl ether, unmetabolized substrate and metabolites were extracted in the organic layer and evaporated to dryness. The residue obtained was dissolved in 12 mL methanol, transferred into a derivatization vial, and evaporated under nitrogen in a sand bath. One hundred microliters of trifluoroacetic anhydride and 100 μL ethyl acetate were added, and the mixture was incubated at 60°C for 20 min. After evaporation of the derivatization reagents, the residue was dissolved in 400 μL ethylacetate, and 2 μL were injected into a Packard model 429 gas chromatograph, equipped with an N–P thermoionic detector. The glass column was packed with 3% OV-17 on chromosorb W (100–120 mesh).

The operating conditions were as follows: 260°C, detector temperature; 230°C, oven temperature; 250°C, injector temperature; carrier gas nitrogen; and flow rate, 30 mL/min. Chromatograms were recorded on an LTT integrator with an LTT printing system.

The amount of hydroxylated perhexiline maleate was determined from the ratio of the peak surface of the derivatized hydroxylated metabolite to that of the derivatized internal standard.

Measurement of Microsome Release

The release of microsomes from Ca-alginate or κ-carrageenan gels under our storage conditions was checked as follows: entrapped microsomes were stored in 0.1*M* Tris-buffer, pH 7.4, containing 0.1 m*M* DTT, 1 m*M* EDTA, and 0.1*M* $CaCl_2$ or 0.5*M* KCl, at 4°C, for 1 wk. Samples of 300 μL of the storage buffer were taken at different intervals of time, and the protein concentration was determined according to the method of Lowry et al. (*17*), using bovine serum albumin as a standard protein.

Continuous Spectrophotometric Analysis of O-Demethylase Activity in Immobilized Microsomes

The method of continuous spectrophotometric analysis of immobilized enzyme activities was first developed by Mosbach and Mattiasson (*18*).

Continuous metabolization of *p*-nitroanisole, using microsomes entrapped in Ca-alginate or κ-carrageenan, was investigated: entrapped microsomes (corresponding to 10 nmol cytochrome P-450) were suspended in 12 mL of 0.1*M* Tris-buffer, pH 7.4, in a small container, thermostated at 37°C. Then, 30 mL of a 2-m*M* *p*-nitroanisole solution and the NADPH-generating system were added. The reaction was initiated by the addition of NADP. All reagents were mixed and circulated through the pumping system; the flow rate was 15 mL/h and the "dead" vol (i.e., the volume of tubings and flow cell) was minimized to a small fraction of

the total volume. In all experiments, the total volume was 45 mL, whereas the circulating volume was 5 mL. The quantity of *p*-nitrophenol formed was determined, continuously, using a spectrophotometer.

Batchwise Repeated Reactions

The same immobilized-microsomal sample was used several times to measure the *N*- or *O*-demethylase or the hydroxylase activities. The different enzyme assays were carried out under the same experimental conditions mentioned above. The different kinds of immobilized microsomes were incubated with the substrate at 37°C for 15 min and the enzymatic reactions were stopped by centrifugation at 150*g* to separate the immobilized microsomes from the supernatant containing both substrate and metabolites. The recovered immobilized microsomes were washed thoroughly with buffer and used for another assay, whereas the supernatant was used to determine the quantity of metabolites obtained. The same processes (addition of substrate, incubation, centrifugation, and recovery of the immobilized microsomes) were repeated several times until no metabolite could be detected in the supernatant, indicating the complete denaturation of the immobilized microsomes. The maximal amount of *N*-demethylated, *O*-demethylated, or hydroxylated product was calculated from the sum of the respective metabolite formed in the repeated assays.

Free microsomes were assayed for *N*- and *O*-demethylase and hydroxylase activities, for one time only, using the same experimental conditions as that mentioned above for the immobilized microsomes.

RESULTS AND DISCUSSION

In this study, different attempts were made to determine a suitable method of immobilization of microsomes giving the best cytochrome P-450 monooxygenase activities.

The different immobilization methods were compared by determining the cytochrome P-450 immobilization yields (Fig. 1).

We have ascertained that Sephadex and Sepharose gels had no effects on the CO-difference spectra of the reduced form of cytochrome P-450, and, therefore, the concentration of the immobilized enzyme was determined directly on the gels. Covalent attachment of microsomes to Sepharose activated by CNBr results in the retention of 60% of the initial cytochrome P-450, whereas, in the case of immobilization on Sephadex activated by CDI, only 20% of the initial cytochrome P-450 were found in the gel, and, at the same time, an important transformation into the inactive cytochrome P-420 was observed (Fig. 1).

In any event, covalent binding is a very extensively used method of immobilization. In fact, in most cases it provides an immobilized enzyme that is firmly bound to its support (*19*).

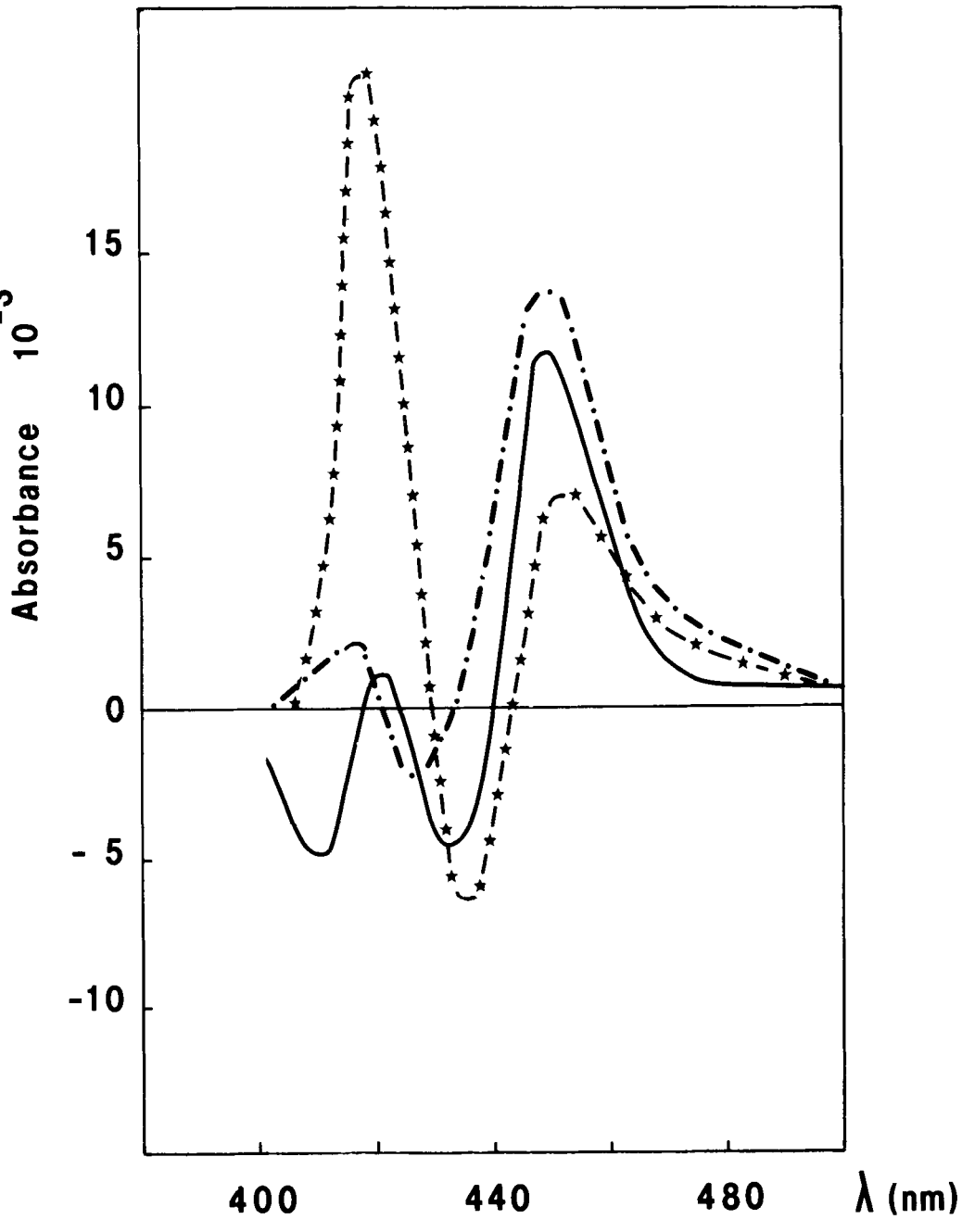

Fig. 1. CO-difference spectra of microsomes immobilized on CNBr-activated Sepharose (—), immobilized on 1,1′-carbonyldiimidazole Sephadex (★) and free microsomes in suspension (·). CO-difference spectra of free microsomes were carried out on a sample of 10 μL microsomal suspension, diluted in 1 mL 0.1*M* phosphate buffer, pH 7.4, 1 m*M* EDTA, 0.1 m*M* DTT, and 20% glycerol; for immobilized microsomes, spectra were recorded on 1 mL gel, prepared as described under Materials and Methods.

Another method of immobilization of microsomes by entrapment inside polymeric gels (Ca-alginate and κ-carrageenan) was also investigated. When microsomes were entrapped in Ca-alginate or κ-carrageenan, because of the quick settling of pellets in the cell, the CO-differences spectra could not be directly recorded. Therefore, the concentration of cytochrome P-450 was estimated by determining the quantity of microsomes remaining in the washings; no free pigment was detected, and a 100% immobilization yield could be expected.

Entrapment is a suitable method for the immobilization of cells and subcellular organelles, allowing a series of enzymatic reactions requiring cofactors to be carried out (*12*). Douglas and Williams have obtained good results for the production of ethanol, using yeast cells entrapped inside Ca-alginate gels (*20*). It has also been shown that Ca-alginate beads possess good mechanical strength (*21*) and good resistance to acidic and basic environments (*22*). An advantage is that entrapment inside K-carrageenan or Ca-alginate can be carried out under very mild conditions, preventing the denaturation of enzymes or cells.

Finally, we tried cocrosslinking with bovine serum albumin and glutaraldehyde, a technique developed by Broun et al. (*13*). This technique showed good results because it provides the protective effect of environmental inactive proteins, together with the stabilizing effect of binding onto polymer matrix (*23,24*).

Microsomes immobilized on Sephadex G-150 activated by CDI, as well as microsomes immobilized by cocrosslinking, showed very weak *N*- and *O*-demethylase activities. We therefore studied and characterized only monooxygenase cytochrome P-450-dependent systems immobilized on CNBr-activated Sepharose-4B and entrapped in κ-carrageenan and Ca-alginate.

Microsomes covalently immobilized on CNBr-activated Sepharose used in the present study contained 0.3 nmol of cytochrome P-450/mL packed gel, whereas microsomes entrapped in κ-carrageenan or Ca-alginate contained approximately 1.0 nmol of cytochrome P-450/100 pellets.

The apparent Michaelis–Menten constants (K_m^{app}) and maximum velocities (V^{app}_{max}) of the *N*-demethylation of aminopyrine, *O*-demethylation of *p*-nitroanisole, and hydroxylation of perhexiline maleate for these three different forms of immobilized microsomes were determined by the Lineweaver and Burk method and compared to those of free microsomes (Table 1).

Whatever the type of immobilization, a 60–70% decrease in V^{app}_{max} of the *N*-demethylation of aminopyrine and a 30–40% decrease in V^{app}_{max} of *O*-demethylation of p-nitroanisole were observed, compared to V^{app}_{max} values of the free microsomes. The V^{app}_{max} values for the hydroxylation of perhexiline maleate by the different immobilized forms of microsomes and the freely suspended one were essentially the same, except for mi-

TABLE 1
Comparison of Aminopyrine *N*-Demethylation, *p*-Nitroanisole *O*-Demethylation, and Perhexiline Maleate Hydroxylation Kinetic Properties of Free or Immobilized Microsomes[a]

	N-demethylase activity		*O*-demethylase activity		Hydroxylase activity	
Preparation	K_m^{app}, m*M*	V_{max}^{app}[b]	K_m^{app}, μm*M*	V_{max}^{app}[b]	K_m^{app}, μm*M*	V_{max}^{app}[c]
Free microsomes	3.88	11.1	30	1.4	10.7	0.028
Immobilized on CNBr-activated Sepharose-4B	5.0	3.8	50	1.00	23.9	0.030
Entrapped in Ca-alginate	4.9	3.32	100	0.85	38.3	0.047
Entrapped in κ-carrageenan	3.2	4.34	36	0.85	27.6	0.026

[a]All regression lines in the Lineweaver–Burk plots were calculated by the method of least squares. Correlation coefficients were always higher than 0.985. K_m and V_{max} presented in the table were the average of three values. In all cases, deviations from the average values do not exceed 5%.

[b]V_{max} expressed in nnmol/min/nmol P-450.

[c]V_{max} expressed in:

$$\frac{\text{surface of the peak of the derivatized hydroxylated metabolite}}{\text{surface of the peak of the derivatized internal standard}} \text{ /min/nmol P-450.}$$

TABLE 2
Effect of Recycling on the Production of Metabolites

Microsomes	Assayed activity	Number of cycles	Cumulated metabolites[a]
Freely suspended	*N*-demethylase	0	166.5[b]
	O-demethylase	0	10.5[b]
	Hydroxylase	0	0.065[c]
Immobilized on CNBr-activated Sepharose	*N*-demethylase	7	210.0[b]
	O-demethylase	7	*48.0*[b]
	Hydroxylase	3	*0.068*[c]
Entrapped in κ-carrageenan	*N*-demethylase	7	300.0[b]
	O-demethylase	7	*40.5*[b]
	Hydroxylase	5	*0.248*[c]
Entrapped in Ca-alginate	*N*-demethylase	5	90.0[b]
	O-demethylase	7	*24.0*[b]
	Hydroxylase	7	*0.220*[c]

[a]Cumulated metabolite: sum of the amount of metabolite formed after several cycles, carried out using the same immobilized microsomes preparation.

[b]Quantity of metabolite expressed in nmol product/nmol P-450.

[c]Quantity of metabolite expressed as $\frac{\text{surface of the peak of the hydroxylated metabolite}}{\text{surface of the peak of the internal standard.}}$

crosomes immobilized on Ca-alginate, for which a higher V^{app}_{max} was observed. Thus, Table 2 shows that, upon immobilization, the hydroxylase and *N*- and *O*-demethylase activities are not equally preserved, probably related to the existence of more than one form of cytochrome P-450 in the microsomal preparation (*25*).

The K_m^{app} values of the different preparations of immobilized microsomes were higher than those obtained for the free microsomes (Table 2). The decrease in the enzyme affinity upon immobilization has been previously observed and discussed in detail by Trevan (*26*) and Goldstein (*27*) and may be a result of chemical changes, steric hindrances in the vicinity of some active sites, and/or diffusional limitations.

Figure 2 shows the effect of immobilization on the stability of microsomes at 4°C. Entrapment of microsomes in κ-carrageenan and Ca-alginate leads to a decrease in the stability of *N*- and *O*-demethylase and hydroxylase. In fact, the increase in the stability upon immobilization is not a general phenomenon. Unchanged or even decreased stability upon immobilization has been reported (*28*). The decrease in the microsomes stability upon entrapment may be explained by ion-pair formation between the polyanion matrix and microsomes. These interactions may stress the tertiary structures of cytochrome P-450 isoenzymes into less stable conformations, and, consequently, may decrease their overall stability (*29*).

We have to mention that the decrease in the immobilized microsomes stability is not a result of the liberation of microsomes from κ-carrageenan or Ca-alginate gels. No released proteins were detected in the buffer solutions containing the entrapped microsomes after storage at 4°C for 1 wk.

Figure 2 also shows that *N*- and *O*-demethylase stability in microsomes were enhanced by covalent binding onto Sepharose activated by CNBr. This could be explained by a limitation of the conformational flexibility caused by multiple crosslinking of cytochrome P-450 to the support, thus creating a more rigid enzyme molecule. Transition to less stable forms would therefore be retarded. Finally, an important loss of the hydroxylase activity of microsomes covalently linked to CNBr-activated Sepharose was observed during the first 2 d of storage. Then the remaining activity was almost stable for another 10 d.

The continuous metabolism of *p*-nitroanisole by microsomes entrapped in Ca-alginate or κ-carrageenan was tested for 3 h. The reaction was carried out in a small container with a temperature control in which the entrapped pellets were suspended in the incubation solution. By means of a peristaltic pump, this solution was continuously circulated into a flow cell in a spectrophotometer to determine the quantity of continuously formed *p*-nitrophenol.

After 3 h of continuous metabolism, 0.9 and 0.7% conversion of the substrate into the corresponding *p*-nitrophenol was obtained, using microsomes entrapped in κ-carrageenan and Ca-alginate, respectively.

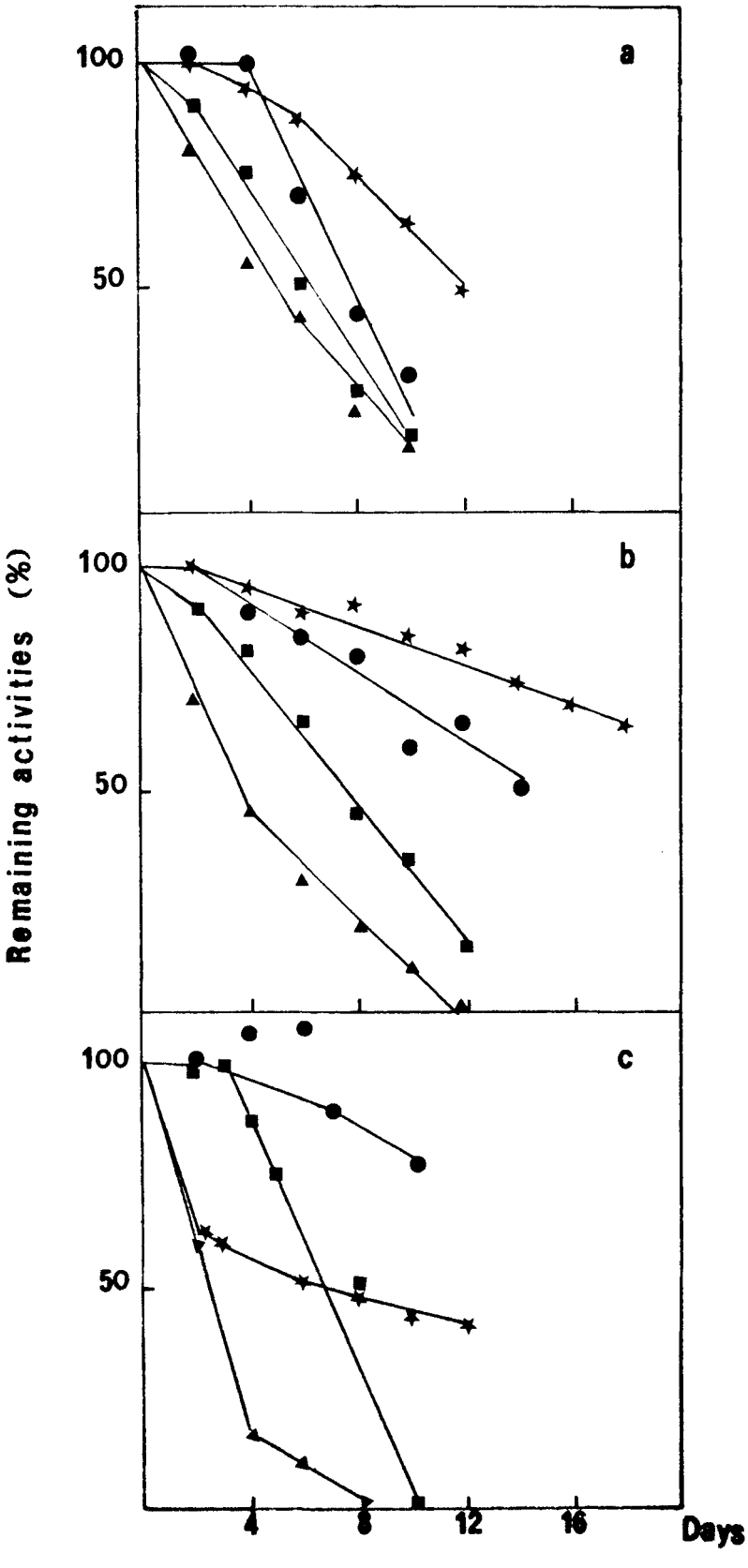

Fig. 2. Stability under storage at 4°C of microsomes, free and bound to various supports: (●) free microsomes; (■) microsomes entrapped in κ-carrageenan; (▼) microsomes entrapped in Ca-alginate; and (★) microsomes covalently bound to CNBr-activated Sepharose. Free microsomes were kept in 0.1*M* phosphate buffer, pH 7.4, containing 0.1 m*M* DTT, 1 m*M* EDTA, and 20% glycerol. Microsomes immobilized on CNBr-activated Sepharose were kept in the same phosphate buffer, containing 40% glycerol. Microsomes entrapped in Ca-alginate were kept in 0.1*M* Tris-buffer, containing 1 m*M* EDTA, 0.1 m*M* DTT, and 0.1*M* $CaCl_2$. Microsomes entrapped in κ-carrageenan were kept in the same Tris-buffer, containing 0.5*M* KCl, instead of 0.1*M* $CaCl_2$. (a) Aminopyrine *N*-demethylase activity; (b) *p*-nitroanisole *O*-demethylase activity; and (c) perhexiline maleate hydroxylase activity.

These conversion values are, respectively, 2.3 and 1.8 times higher than that recorded for the freely suspended microsomes containing approximately the same quantity of cytochrome P-450. In fact, the metabolism of *p*-nitroanisole by microsomes was linear only during the first 15 min. After that time the reaction velocity decreased, and when the reaction ceased, only 0.4% of the substrate had been converted into *p*-nitrophenol (Fig. 3).

Finally, the differently immobilized microsomes were assayed for *N*- and *O*-demethylase and hydroxylase activities in batch reactors in order to test the possibility of reutilizing them several times. The respective efficiency for producing metabolites was determined by comparing the cumulative amount of metabolites produced by each kind of immobilized microsomes.

Table 2 shows that, after having been recycled seven times, microsomes immobilized on Ca-alginate or κ-carrageenan yield approximately three and four times more hydroxylated perhexiline maleate than free

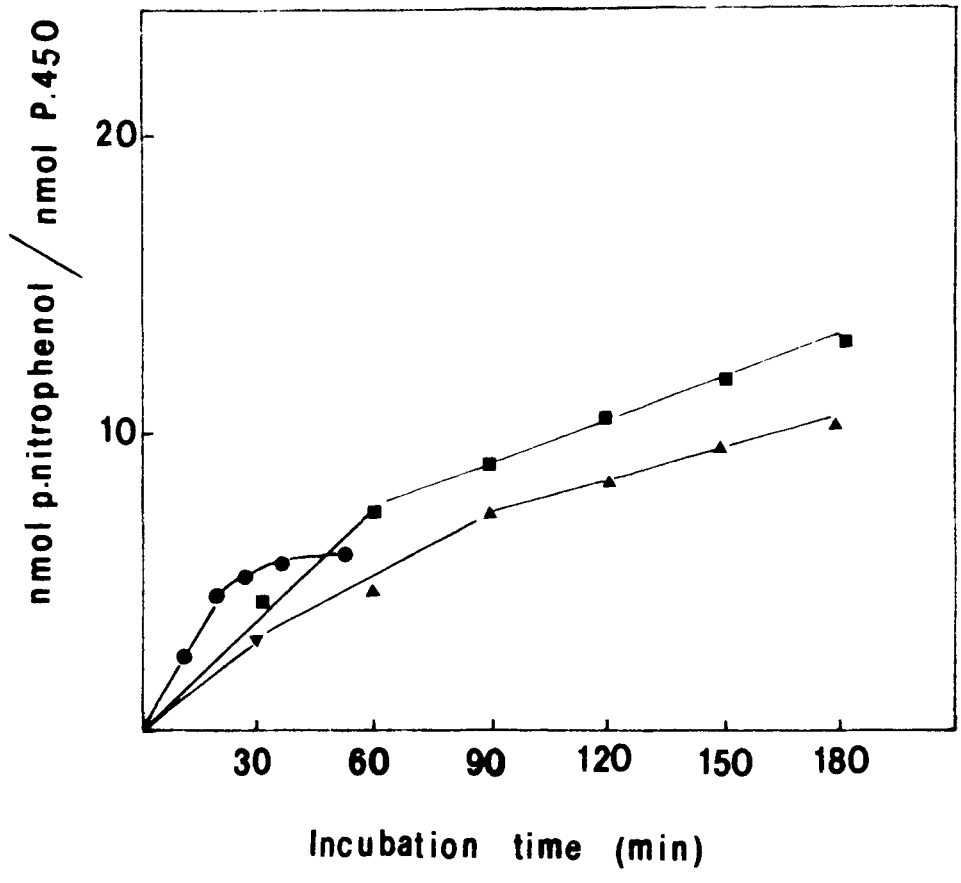

Fig. 3. *O*-demethylation of *p*-nitroanisole by microsomes entrapped in: Ca-alginate (▼); κ-carrageenan (■); and microsomes (★). A stirred-batch reactor with an auxiliary pump and a flow system was used to assay the activity for several hours.

microsomes. The *O*-demethylase activity of the three different forms of immobilized microsomes was stable for six cycles, yielding higher quantities of *p*-nitrophenol than free microsomes. Table 2 also shows that the loss in the *N*-demethylase activity upon immobilization is compensated for by the possibility of using the same immobilized microsomes for several assays. Effectively, high yields of production of metabolites were obtained by the first and second cycles. Thereafter, these yields were decreased, and no metabolites were detected in the last cycle.

In conclusion, five rapid and simple methods were used to immobilize the cytochrome P-450 monooxygenase system of pig hepatic microsomes under mild conditions. In our opinion, the most significant results of the present study are the marked stabilization of microsomes covalently coupled to CNBr-activated Sepharose and the total retention of perhexiline maleate hydroxylation activity, whatever the type of immobilization used. It is also important to mention that these immobilized preparations could be used in a batch reactor for the production of drug metabolites that are normally hardly detected and analyzed in vivo.

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