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STUDIES ON THE MICROSOMAL DRUG-METABOLIZING ENZYME SYSTEM—EFFECT OF ISOOCTANE AND PYRIDINE NUCLEOTIDES*

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Abstract—The extraction of liver microsomes of rat or rabbit with isooctane resulted in a uniform loss of protein, RNA and total lipid. The residual particulate matter metabolized aminopyrine at a reduced rate, whereas the metabolism of acetanilide and aniline was either accelerated or unaffected. The rate of metabolism of *o*-nitroanisole by the isooctane-treated microsomes was essentially unchanged. The extracted microsomes, particularly of the rabbit, were less sensitive to the inhibitory action by SKF 525-A in the metabolism of *o*-nitroanisole. Attempts to prepare a solubilized enzyme system from the isooctane-treated microsomes were not successful. ATP, GTP and various types of pyridine dinucleotides stimulated drug metabolism when they were added to the NADPH generating system containing a catalytic amount of NADP.

MANY OF the reactions concerned with drug metabolism are catalyzed by the microsomal fraction of the liver in the presence of NADPH‡ and molecular oxygen. None of these reactions has been studied in any detail because the enzymes have not been obtained in a soluble form. The solubilization of aniline hydroxylating enzyme was recently reported independently by two groups,[1, 2] but these results have not been confirmed by others. More recently, Nilsson and Johnson[3] reported that reduced NAD was required in addition to reduced NADP and molecular oxygen.

In the present study, the specificity for the requirement of NADH was investigated. Also, the effects of the additional cofactors were studied in four drug metabolic pathways: ring hydroxylation, side-chain oxidation, and *O*- and *N*-dealkylation.

Isooctane was found to remove a considerable amount of material from the microsomes. The nature of the change brought about by isooctane extraction was investigated. Attempts were made to solubilize the extracted microsomes without success.

MATERIALS AND METHODS

Treatment of animals. Twelve adult male New Zealand white rabbits were given sodium phenobarbital (75 mg/kg) i.p. once daily for 3 or 4 days. Twenty-four hr after the last injection, the rabbits were stunned and exsanguinated. The livers were removed

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‡ Abbreviations used: ATP, adenosine triphosphate; GTP, guanosine triphosphate; RNA, tibonucleic acid; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleoride phosphate; NADH and NADPH, reduced forms of NAD and NADP respectively.

and pooled in groups of four and were packaged into 100-g portions, which were kept frozen at −20° until used. The activity against all five substrates was tested with each of the three pooled livers.

Four groups of 8 male Sprague–Dawley rats (200–250 g) were given sodium phenobarbital (75 mg/kg) once daily for 4 days. Twenty-four hr after the last injection, the rats were sacrificed and the livers of each group were pooled for immediate microsome preparation. The activity against all five substrates was tested with each of the three pooled liver preparations.

Preparation of microsomes. Microsomes from the pooled livers of rat and rabbit livers were prepared in an identical manner. The liver was homogenized in 3 volumes of cold 1·15% KCl in a Waring–blendor. The homogenate was centrifuged for 10 min at 10,000 *g* in a refrigerated Servall centrifuge. The resulting supernatant fraction was centrifuged for 30 min in a Spinco model L ultracentrifuge using rotor No. 30 to sediment the microsomes. The postmicrosomal supernatant fraction was purified by one fractionation with solid ammonium sulfate at 80 per cent saturation. The 80 per cent fraction was resuspended in $\frac{2}{3}$ of the original volume of 1·15% KCl and used as a source of glucose 6-phosphate dehydrogenase in the incubations. The sedimented microsomes were thoroughly washed with 1·15% KCl, recentrifuged and taken up in 1 vol. of KCl for each gram of liver. Microsomes upon storage were found to lose activity against some of the substrates more rapidly than against others. Microsomes were therefore routinely stored only overnight at −20° before being incubated or extracted with isooctane.

Isooctane treatment of microsomes. Isooctane-extracted microsomes were prepared by stirring 1 volume of microsomes with $\frac{2}{3}$ vol. of isooctane in an ice bath for 20 min with a magnetic stirrer. The emulsified mixture was centrifuged for 30 min in a Spinco model L centrifuge using rotor No. 30. The organic, lipid and aqueous layers were discarded and the residue was resuspended in $\frac{1}{2}$ the original volume of 1·15% KCt or 0·05 M Tris buffer, pH 8.

Solubilization of microsomes. To fragmentize the microsomes, isooctane-treated microsomes in pH 8 buffer were passed through a French (hydraulic) press. The use of the dilute buffer enhanced the solubilization achieved. The translucent material from the press was centrifuged for 30 min to 2 hr in a Spinco model L centrifuge using rotor No. 40, and the semitransparent supernatant fraction was used as "solubilized" preparation.

Various chemical agents were also investigated for their solubilizing effect on rabbit and rat liver microsomes. An aqueous solution of the solubilizing agent was stirred into the microsomal suspension cooled in an ice bath. After standing in ice for 1 hr, the preparation was centrifuged for 30 min or longer in a Spinco model L centrifuge using rotor No. 40, and the supernatant fraction was used as "solubilized" preparation.

Incubation. The reactions studied were the side-chain oxidation of hexobarbital, hydroxylation of acetanilide and aniline, *O*-dealkylation of *o*-nitroanisole, and *N*-dealkylation of aminopyrine. Incubation mixtures consisted of 200 μmole Tris buffer (pH 8), 40 μmole glucose 6-phosphate, 15 μmole nicotinamide, 20 μmole $MgCl_2$, 0·25 μmole NADP, 0·5 ml of 80% ammonium sulfate fraction of postmicrosomal liver supernatant, 0·5 ml of microsomes or 1·0 ml solubilized preparation and substrate in a final volume of 4·0 ml. The amount of substrate incubated was *o*-nitroanisole, 7

μmole; aniline, aminopyrine and acetanilide, 10 μmole; and hexobarbital, 1 μmole. All incubations also contained 5 μmole NADH unless indicated otherwise. For studying the effects of various nucleotides, 3·5 units (0·025 mg) of glucose 6-phosphate dehydrogenase (Sigma, type V) replaced the postmicrosomal supernatant fraction. Untreated microsomes and freshly prepared isooctane-extracted microsomes were incubated with all five substrates on the same day. The inhibitor, SKF 525-A (β-diethylaminoethyldiphenylpropylacetate), was used at a final concentration of 5×10^{-4} M. Incubations were in air for 30 min in a Dubnoff incubator at 37°.

Assays. The hydroxylated product of acetanilide, *N*-acetyl-*p*-aminophenol, was assayed by the method of Mitoma and Udenfriend.[4] *p*-Aminophenol, the product of aniline hydroxylation, was also assayed by this method without the preliminary hydrolysis step. The cloudiness that appeared in the final color reaction of SKF 525-A-treated samples could be removed by centrifugation. *o*-Nitrophenol, the product of *o*-nitroanisole, was routinely assayed directly in the incubation medium after deproteinizing with 4 ml of 10% TCA. The optical density at 430 mμ was determined after addition of 2 ml of 1 N NaOH to 5 ml of the deproteinized medium. This was a more convenient method of assay than the ether extraction procedure of Netter.[5] The metabolism of hexobarbital was measured by the method of Brodie *et al.*[6] as the amount of hexobarbital remaining after incubation. The *N*-dealkylation of aminopyrine was measured as the amount of 4-aminoantipyrine produced[7]

RNA was determined by the orcinol method[8] with yeast RNA as the standard. Protein was measured by the method of Lowry *et al.*[9] with crystalline bovine albumin as the standard. Lipid was extracted from the microsomes with 2 : 1 chloroform–methanol.[10] For the extraction, a 1-ml aliquot of microsomes was treated with 1 ml of cold 10% TCA and the precipitate was washed twice with 1-ml portions of cold 5% TCA. The residue was homogenized with 5 ml of $CHCl_3$–MeOH by using a glass tissue homogenizer and the resulting extract was centrifuged. The clear supernatant was removed to a 10-ml volumetric flask and the residue was re-extracted with an additional 4 ml of $CHCl_3$–MeOH. The supernatant of the second extraction was added to the volumetric flask and the volume was brought to 10 ml. Total microsomal lipid was determined on the $CHCl_3$–MeOH extract by evaporating a 5-ml aliquot to dryness and weighing the residue. Lipid phosphorus was determined on 0·1-ml aliquots of the $CHCl_3$–MeOH extracts by the modified Bartlett procedure.[11] Data were analyzed by Student's *t*-test with correlated data.*

Effect of isooctane treatment on microsomes. After isooctane treatment, a considerable amount of material was removed from rabbit and rat microsomes and formed a solid layer at the isooctane–water interface upon centrifugation. Rabbit microsomes treated in this manner lost an average of 27 per cent of the protein, lipid, lipid phosphorus and RNA content, whereas rat microsomes lost an average of 37 per cent of these components upon extraction. However, as indicated in Table 1, the proportions of these components within the microsome were not significantly altered by the extraction. Nevertheless, the activity of the microsomes was altered considerably by the treatment, as seen in Tables 2 and 3. Generally, there was an enhancement of the aromatic ring hydroxylating activity and a decrease in the activity against aminopyrine. The metabolism of *o*-nitroanisole was unaffected. Supplementing the extracted

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TABLE 1. EFFECT OF ISOOCTANE EXTRACTION ON PROTEIN, LIPIDS AND RNA CONTENTS OF RAT AND RABBIT LIVER MICROSOMES*

Microsomes	Phospholipid (mg/mg protein)	RNA (mg/mg protein)	Total lipid (mg/mg protein)
Rat			
Untreated	0·52, 0·54	0·08, 0·09	1·31, 1·44
Isooctane-extracted	0·56, 0·50	0·08, 0·09	1·16, 1·38
Rabbit			
Untreated	0·60 ± 0·00	0·11 ± 0·01	1·51 ± 0·09
Isooctane-extracted	0·59 ± 0·04	0·12 ± 0·01	1·32 ± 0·11

* Two samples of pooled rat livers and three samples of pooled rabbit livers were used to prepare the microsomal fractions. The protein loss after isooctane treatment was 34·4 and 39·5 per cent for rat microsomes and 27·2 ± 0·13 per cent for rabbit microsomes.

microsomes with resuspended lipid layer, tetrahydrofolic acid, flavine adenine dinucleotide, coenzyme Q_{10} or vitamin K had no effect on the activity.

Effect of SKF 525-A on microsomes. The effect of the drug metabolism inhibitor, SKF 525-A, on treated and isooctane-extracted microsomes was compared. In general, SKF 525-A inhibited the metabolism of all substrates by both untreated and isooctane-treated microsomes. The only clear exception was observed with rabbit microsomes when o-nitroanisole was the substrate. In this case SKF 525-A showed no inhibition with isooctane-treated microsomes, whereas the untreated microsomes were inhibited.

Effect of nucleotides on the microsome system. As shown in Table 4, the NADPH generating system (glucose 6-phosphate dehydrogenase system) alone catalyzed the *O*-demethylation of *o*-nitroanisole by untreated rat and rabbit microsomes to a limited extent. The addition of ATP increased the amount of *o*-nitrophenol formed and the various forms of the pyridine nucleotides stimulated *O*-demethylation 2- to 3-fold. Doubling the amounts of these compounds did not yield greater amounts of the product. GTP was found to be as effective as ATP. A similar stimulatory effect was obtained with the aniline hydroxylating system of rabbit microsomes upon addition of the pyridine nucleotides. Inorganic phosphate, pyrophosphate, nicotinamide up to 100 μmole, mercaptoethanol and tetrahydrofolic acid did not supplement the NADPH generating system. A phosphodiesterase inhibitor, caffeine, in a final concentration of 10^{-3} M, or the phosphodiesterase substrates, *bis-p*-nitrophenyl-phosphate and *p*-nitrophenyl-thymidine-5′-phosphate (5 μmole each), were also incapable of stimulating the microsome reactions. Hydrolysis of the *p*-nitrophenyl compounds by the microsomes was readily seen after incubation by the formation of *p*-nitrophenol.

The effect of NADH on the activity of rat microsomes is shown in Table 5. The presence of 5 μmole NADH stimulated the activity of the untreated and isooctane-extracted microsomes up to 15-fold. Incubation with 5 μmole NADH without the NADPH generating system did not stimulate the reactions. Optimum activity was achieved only in the presence of both the NADPH generating system and NADH.

Properties of fragmented microsomes. When isooctane-treated microsomes were subjected to sonication or passed through a hydraulic (French) press and then subjected

TABLE 2. EFFECT OF ISOOCTANE TREATMENT ON DRUG-METABOLIZING ACTIVITIES OF RAT MICROSOMES AND ON THE INHIBITORY ACTION OF SKF 525-A

Microsomes	μmoles of product formed or substrate consumed per g microsomal protein									
	o-Nitroanisole		Aniline		Aminopyrine		Acetanilide		Hexobarbital	
	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A
Whole	50·3	22·2	36·2	14·9	30·4	4·3			43·1	14·5
Isooctane-treated	61·1	36·7	73·6	28·4	17·3	5·4			48·8	16·6
Whole	77·0	43·2	44·1	14·0	32·4	3·7	79·6	13·4	79·1	16·9
Isooctane-treated	59·4	55·1	59·9	17·9	8·1	3·0	104·9	35·8	41·3	11·9
Whole	152	81·0	87	36·6	79	6·4	111	37·3	149	18·8
Isooctane-treated	128	95·5	208	46·9	35	10·9	148	49·8	71	31·9
Whole	58	27·2	42	13·1	25	2·8	44	13·8	59	9·6
Isooctane-treated	64	36·8	95	29·1	11	2·8	99	32·2	40	10·4
Significance										
Whole vs. isooctane	NS	<0·005	<0·10	<0·025	<0·05	NS	<0·05	<0·025	NS	NS
SKF 525-A inhibition										
Whole	<0·05		<0·025		<0·10		<0·10		<0·10	
Isooctane	<0·05		<0·10		<0·10		<0·025		<0·001	

TABLE 3. EFFECT OF ISOOCTANE TREATMENT ON DRUG-METABOLIZING ACTIVITIES OF RABBIT MICROSOMES AND ON THE INHIBITORY ACTION OF SKF 525-A

Microsomes	μmoles of product formed or substrate consumed per g microsomal protein									
	o-Nitroanisole		Aniline		Aminopyrine		Acetanilide		Hexobarbital	
	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A
Whole	52·1	24·4	22·6	15·6	26·6	6·2	79·7	48·5		
Isooctane-treated	49·8	46·5	33·5	27·6	9·5	4·4	76·2	66·1		
Whole	68·6	41·6	25·3	16·6	27	6·2	75·2	60·1	25·0	13·2
Isooctane-treated	46·2	43·0	29·7	22·7	4·9	2·9	63·6	49·2	8·8	6·0
Whole	74	45·4	31	18·6	24	6·8	83	56·5	33	4·0
Isooctane-treated	74	67	51	36·4	8	3·9	97	83·6	8	0·2
Whole	141	73·5	38	21·9	13	2·1	89	51·9	50	13·4
Isooctane-treated	128	105	46	32·8	5	2·0	92	64·5	19·5	9·2
Whole	101	46·1			33·3	4·6			73·8	15·0
Isooctane-treated	53·2	51·6			5·8	2·6			19·3	9·4
Significance										
Whole vs. isooctane	NS	<0·05	<0·05	<0·025	<0·005	<0·025	NS	NS	<0·05	<0·01
SKF 525-A inhibition										
Whole	<0·01		<0·025		<0·005		<0·01		<0·05	
Isooctane	NS		<0·025		<0·001		<0·025		<0·025	

to ultracentrifugation, approximately 30 per cent of the original microsomal activity could be obtained in apparently "solubilized" form. The specific activity of this preparation was approximately 15 per cent of that of the original microsomes. The activity of the "solubilized" preparation was preserved on freezing and thawing, but protein was lost from solution in the process.

When native or isooctane-extracted rabbit microsomes were treated with various solubilizing agents, no soluble preparation was obtained that had a specific activity against *o*-nitroanisole or aniline of greater than 25 per cent of the original microsomes. The agents used were Triton-X100 (0·2–2%), sodium deoxycholate (DOC; 0·05–1%), various combinations of DOC and Triton-X100 (0·5–2%), digitonin (0·3%), Triton-N101 (0·5–2%) or Triton-X100 (0·2%) in combination with hyaluronidase, ribonuclease, lysozyme, trypsin or pancreatic lipase. Attempts to enrich the sp. act. of the solubilized preparations by ammonium sulfate fractionation or acetone precipitation were unsuccessful.

DISCUSSION

By using a variety of techniques, several investigators have succeeded in obtaining enzymes in a soluble form from the microsomes. Thus, freezing and thawing[12] and the use of *tert*-amyl alcohol,[13] snake venom[14] and pancreatic lipase[15] solubilized the active components. The application of these techniques in trying to solubilize the drug-metabolizing enzyme system has not been successful. Use of *n*-butanol and

TABLE 4. EFFECT OF COFACTOR ADDITIONS ON THE *O*-DEMETHYLATING ACTIVITY OF UNTREATED MICROSOMES

Incubation	μmoles *o*-Nitrophenol formed per incubation*	
	Rabbit	Rat
Control†	0·37	0·19
+ATP (5 μmole)	0·46	0·47
+GTP (5 μmole)	0·43	0·47
+NAD (5 μmole)	0·99	0·49
+NADH (5 μmole)	1·13	0·61
+NADH (10 μmole)	1·14	0·63
+NADP (5 μmole)	0·91	0·52
+NADPH (5 μmole)	0·97	0·57

* All incubations contained microsomes from 0·5 g of rat or rabbit liver. For other conditions see text.
† NADPH generating system alone.

acetone likewise resulted in an inactivation of the drug-metabolizing enzyme system. In a recent paper, Silverman and Talalay[16] reported that a decrease in the enzymic activity after various treatments of the microsomes was correlated with the conversion of P-450, the carbon monoxide-binding pigment, to the inactive P-420. It would appear that unless one succeeds in solubilizing P-450 in its active form, a soluble preparation of the drug-metabolizing system cannot be obtained.

In the present study, isooctane was found to remove a considerable amount of material from the microsome without damaging the drug-metabolizing system. It appeared that the use of isooctane might possibly aid in releasing the drug-metabolizing protein components from the lipoidal material in the microsomes. Indeed, subject-

ing the isooctane-treated microsome to ultrasonication or the hydraulic press gave rise to a microsomal preparation that did not sediment like the untreated microsomes, but the enzymatic activity was largely lost. This loss in activity could not be reversed by the addition of tissue extracts or known compounds. Furthermore, the apparently solubilized preparation could not be fractionated with ammonium sulfate to yield a preparation with higher specific activity than that of the starting material. The combination of isooctane treatment and a variety of chemical or enzymatic solubilizing agents did not improve the release of drug-metabolizing enzymes from the microsome.

TABLE 5. EFFECT OF NADH ON THE ACTIVITY OF UNTREATED AND ISOOCTANE-TREATED RAT MICROSOMES

	μmoles of product formed per incubation*			
	Untreated		Isooctane-treated	
Substrate	−NADH	+5 μmole NADH	−NADH	+5 μmole NADH
o-Nitroanisole	0·29	0·85	0·24	0·75
Aniline	0·05	0·48	0·15	1·22
Aminopyrine	0·03	0·44	0·02	0·21
Acetanilide	0·28	0·62	0·33	0·87
Hexobarbital	0·55	0·83	0·21	0·42

* All incubations contained the NADPH generating system with 5 mg microsomal protein.

Isooctane was reported to detach the ribosomes from the membrane of rough-surfaced vesicles, although not as effectively as sodium deoxycholate.[17] However, in the present study no enrichment of RNA was observed in the sedimented particulate matters after centrifugation of the isooctane-treated microsomes. Although the nature of the effect of isooctane on microsomes is not clear, it is evident that some changes in the physical nature of the extracted microsomes were produced. Thus, the hydroxylation of aniline by rabbit microsomes and of acetanilide by rat microsomes was enhanced. The metabolism of aminopyrine was decreased and that of *o*-nitroanisol was not affected by isooctane treatment of the microsomes. Also, decrease in sensitivity to SKF 525-A inhibition occurred only in the rabbit with *o*-nitroanisole as the substrate. These data provide additional evidence that drug metabolism through different metabolic pathways is catalyzed by different enzymes.

Under our experimental conditions in which a small amount (0·25 μmole) of NADP is used, the drug-metabolizing activity of the microsomes is greatly stimulated by supplementing the NADP generating system with various pyridine nucleotides. If the amount of NADP is increased to 5 μmole, the stimulation on *o*-nitroanisole metabolism by the addition of NADH is reduced to approximately 20 per cent. A possible explanation for NAD or NADH acting as stimulators under these conditions is that they might protect NADP or NADPH from destruction during incubation. However, nicotinamide, an NADase inhibitor, and a phosphodiesterase inhibitor (caffeine) or its substrates (*bis-p*-nitrophenyl-phosphate and *p*-nitrophenyl-thymidine-5′-phosphate) do not stimulate drug metabolism. Some evidence for the protection of NADP(H) is seen when incubation is carried out for various intervals. The metabolism of *o*-nitroanisole and acetanilide does not proceed much beyond 5 min in the absence of NAD, but in its presence the reaction proceeds beyond 30 min.

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