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PREPARATION AND CHARACTERIZATION OF SUBCELLULAR FRACTIONS FROM THE LIVER OF THE NORTHERN PIKE, *ESOX LUCIUS*

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Abstract—The present study was designed to prepare and characterize subcellular fractions from the liver of the Northern pike (*Esox lucius*), with special emphasis on the preparation of microsomal fractions suitable for studying xenobiotic metabolism. The purity of the different fractions obtained by differential centrifugation, as well as the recovery of different organelles, was determined using both enzyme markers and morphological examination with the electron microscope. Attempts were also made to increase the recovery of fragments of the endoplasmic reticulum in the microsomal fraction. Finally, the subcellular distribution of several drug-metabolizing enzymes (cytochrome P-450, benzpyrene monooxygenase, epoxide hydrolase and glutathione transferases) were determined. With the exception of the subcellular distribution of epoxide hydrolase, the results obtained here resemble closely those reported for rat liver and the microsomal fraction prepared is highly suitable for further studies of drug metabolism in pike liver.

Our aquatic environment is increasingly exposed to a large number of different xenobiotics, including oil, oil products, pesticides and herbicides. At the same time, the tumour incidence in fish is increasing. These facts have led to a growing interest in the biotransformation of xenobiotics by fish and during the past five years a number of different enzyme activities involved in biotransformation have been found in several different species of fish [1–10].

For a number of different reasons we have chosen to study the pathways of xenobiotic metabolism and the relationships between this metabolism and tumourigenesis in the Northern pike (*Esox lucius*). This fish is found in fresh and brackish water throughout the northern hemisphere, is a top predator and does not migrate but resides in a relatively small area. These observations make the Northern pike extremely interesting in connection with suggestions that the induction of drug-metabolizing enzymes in fish might be used as a monitor for certain kinds of environmental pollution [11–14].

In addition, the tumour incidence in this fish is very high in certain waters, e.g. the Baltic Sea and the waters around Ireland and the U.S.A. [15–17]. Indeed, this frequency is more than 20% in certain waters, which is the highest frequency of malignant lymphoma yet reported in any free living verterbrate [16]. Since the tumour incidence in Northern pike seems to be generally proportional to water pollution, this system would seem to provide excellent opportunities for investigating chemical carcinogenesis.

Finally, there are a number of practical advantages to using the Northern pike as an experimental animal. These fish can be kept in an aquarium under controlled conditions for long periods of time. In addition, they are relatively large, so that adequate material for biochemical studies can be obtained from several different organs and tissues from the same fish.

As in mammals, the liver seems in general to be the major site of xenobiotic metabolism in fish [2, 21]. In order to facilitate the biochemical investigation of xenobiotic metabolism and other processes in fish liver, subcellular fractions must be carefully prepared and characterized. In addition, comparison of the subcellular distribution of various activities in pike liver with the corresponding pattern in mammalian liver was expected to support the basic similarity of these tissues, but could also reveal differences. Finally, comparison of the subcellular distributions of various drug-metabolizing enzymes should give hints concerning physiological interactions between these systems in pike liver.

In the present study we have used differential centrifugation to prepare subcellular fractions from pike liver and subsequently characterized these fractions using enzyme markers and electron microscopy. To our knowledge, only one other fish, the rainbow trout [22], has been studied in this way previously.

MATERIALS AND METHODS

Chemicals. [^{3}H]Benzpyrene and 7-[^{3}H]styrene oxide were purchased from the Radiochemical Centre (Amersham, U.K.) and purified as reported previously [19, 20]. Unlabelled styrene oxide was obtained from Schuchardt (Munich, West Germany), 1-chloro-2,4-dinitrobenzene was procured from Merck (Darmstadt, West Germany). All other

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chemicals used were obtained from common commercial sources and were of analytical purity.

Animals. The Northern pike used in this study were purchased from a local fisherman, who caught them in the brackish water of the archipelago outside of Stockholm. The eleven different fish used included seven females and four males weighing between 500 and 3550 g and were 42–76 cm long. All fish were maintained in cold (5–14°) running tapwater for at least one week prior to sacrifice.

Subcellular fractionation. The fish were killed by a hard blow to the head and the liver immediately removed. The gall bladder and bile duct were carefully removed from the liver, since bile is known to inhibit xenobiotic metabolism in fish [2]. All subsequent steps were performed on a refrigerated bench maintained at 0–4° with solutions of the same temperature.

The whole liver was rinsed once with 0.25 M sucrose, minced with a pair of scissors, and placed in a volume of 0.25 M sucrose equal to two times its weight. The sample was then homogenized using 4 up-and-down strokes of a Potter–Elvehjem homogenizer at 440 rpm and the homogenate adjusted thereafter to contain 1 g liver/5 ml. This homogenate and fractions prepared therefrom had a pH of around 7.0. Subsequently, the homogenate was subfractionated using differential centrifugation according to the scheme illustrated in Fig. 1. This fractionation procedure is rather similar to that used routinely in our laboratory for rat liver, with the exception that the high-speed centrifugation must be continued for 150 min for pike liver instead of 60 min. Apparently, the homogenization procedure used breaks the endoplasmic reticulum in pike liver into such small fragments that many of them are not pelleted by 60 min centrifugation.

The data presented here are based on four different experiments, each experiment using the pooled livers of 2 or 3 different fish. In three of these experiments attempts were made to increase the recovery of microsomes from the 7100 g_{av} pellet. The procedure used was to resuspend the 7100 g_{av} pellet in 0.25 M sucrose using four up-and-down strokes by hand in a glass–Teflon homogenizer. This suspension was then used to prepare the 7100 g_{av} pellet and 133000 g_{av} pellet as shown in Fig. 1 and the 133,000 g_{av} pellet obtained was designated microsomes II. The resuspension of the 7100 g_{av} pellet was repeated to obtain microsomes III. For further details on this method, see the report by Eriksson [23].

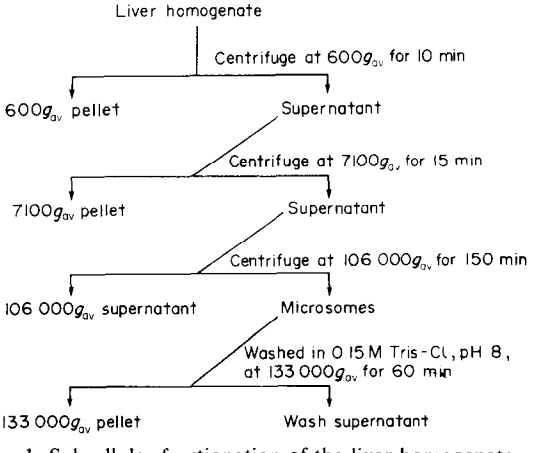

Fig. 1. Subcellular fractionation of the liver homogenate from Northern pike.

Chemical and enzymatic analysis of the subfractions obtained. All assays were carried out on freshly prepared subfractions. Before performing chemical or enzymatic assays on the whole homogenate or on the nuclear or mitochondrial pellets, these fractions were sonicated using a Branson sonicator at setting 4 (7 mA) twice for 30 sec while immersed in an ice bath and with a 1 min cooling interval between sonications. This procedure was utilized to break down existing barriers to enzyme substrates and to facilitate the removal of a representative sample.

DNA (a presumptive marker for the nucleus) [24], cytochrome oxidase (mitochondria) [25], *p*-nitrophenylmannosidase (Golgi) [26], AMPase (plasma membrane) [27], catalase (peroxisomes) [28], β-glycerophosphatase (lysosomes) [29], lactate dehydrogenase (cytosol) (30), NADPH–cytochrome *c* reductase (endoplasmic reticulum) [31], cytochrome P-450 [32], epoxide hydrolase [20], glutathione transferase(s) [33], and protein [36] were all determined using published procedures. Scintillation counting was carried out in a Packard Tri-Carb liquid scintillation spectrometer with Lumagel (C.R. Hintze Trade AB, Lidingö, Sweden) as the scintillation fluid. The external standard ratio procedure was used to correct for quenching. All chemical and enzymatic measurements were performed at least in duplicate and the different values agree with one another to within 10%.

Electron microscopy. To prepare samples for electron microscopy the following procedure was used: an aliquot of the sample was added to 3% glutaraldehyde in 0.1 M cacodylate buffer pH 7.2, with 3.42% sucrose and fixed for 24 hr at 0°. After centrifugation and washing the pellets were fixed with 2% OsO_4 in *S*-collidine for 1–2 hr at 0°. The samples were subsequently dehydrated in ethanol and embedded in Epon 812 for sectioning. All pellets were systematically cut and sectioned near the surface, near the central region, and near the bottom to assure obtaining representative samples.

RESULTS

Figure 2 shows the distribution of various markers upon subcellular fractionation of pike liver. DNA was used as a marker for nuclei, cytochrome oxidase for mitochondria, *p*-nitrophenol-α-mannosidase for the Golgi apparatus, AMPase for the plasma membrane, catalase for peroxisomes, β-glycerophosphatase for lysomes, lactate dehydrogenase for the cytosol, and NADPH–cytochrome *c* reductase (which is the same protein as NADPH–cytochrome P-450 reductase in rat liver) for the endoplasmic reticulum. Even though it is becoming more and more apparent that few of these markers are localized to a single organelle in mammalian liver, the larger part of each is localized to a single organelle and therefore provides a useful indication of the

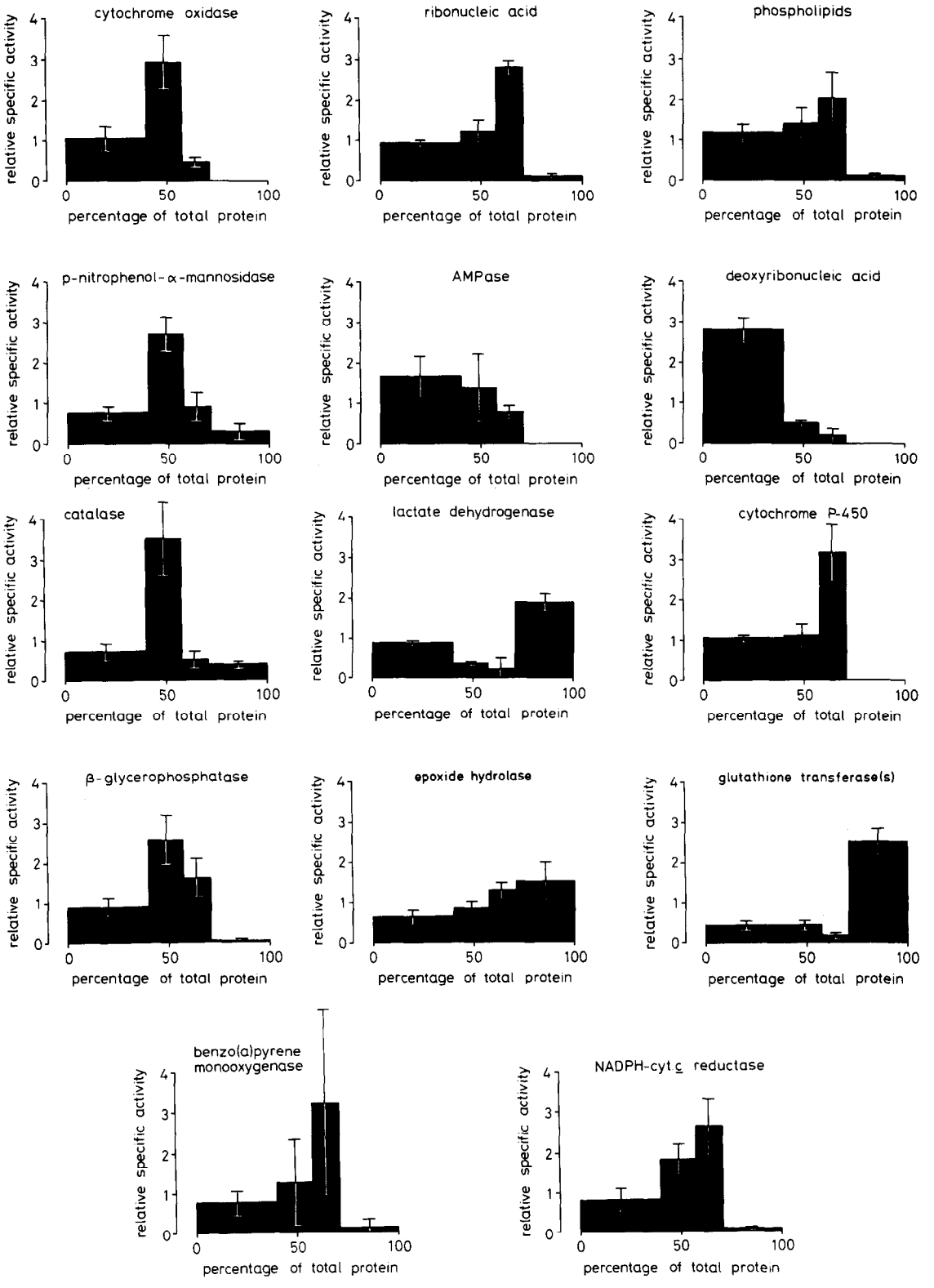

Fig. 2. The distribution of various enzymes and components after subcellular fractionation of pike liver by differential centrifugation. Pike liver was homogenized and subfractionated and the various enzymes and components were assayed as described in Materials and Methods. The second substrate for glutathione transferase was 1-chloro-2,4-dinitrobenzene. The results are presented as deDuve plots [35]. Fractions (left to right) are the 600 g_{av}, 7100 g_{av}, 133,000 g_{av} pellets and 106,000 g_{av} supernatant. Relative specific activity = per cent of the total activity/per cent of the total protein. Total recovery of the different components in the four subfractions ranged generally from 80 to 100% of the corresponding homogenate values.

distribution of these structures. The data are presented as deDuve plots [37].

In general, the pattern illustrated in Fig. 2 is seen to resemble closely the corresponding pattern obtained with rat liver, as well as with the liver from rainbow trout [22]. Markers for mitochondria, the Golgi apparatus, lysosomes, and peroxisomes are all enriched to the greatest extent in the 7100 *g* pellet. More than 50% of the marker for the plasma membrane is recovered in the low-speed 'nuclear' pellet. RNA is most enriched in the microsomal pellet, presumably as a result of the ribosomes bound to the membrane vesicles originating from the endoplasmic reticulum. The microsomal fraction from pike liver also contains 30–35% of the total liver NADPH–cytochrome *c* reductase activity, a value which is somewhat lower than that found for rat liver microsomes [31].

Figure 2 also shows the distribution of the drug-metabolizing components NADPH–cytochrome *c* reductase, cytochrome P-450, benzpyrene monooxygenase (which is catalysed by the cytochrome(s) P-450 system), epoxide hydrolase, and glutathione transferase(s)-activity. As expected, the cytochrome P-450 system is most enriched in the microsomal fraction. The large error limits associated with benzpyrene monooxygenase may reflect the fact that this activity is highly inducible in pike liver [8] and some of the animals used might have been unintentionally induced by xenobiotics in their natural environment.

There are also two surprising aspects of the distribution of drug-metabolizing systems presented in Fig. 2. In the first place 35–40% of the total epoxide hydrolase activity measured with styrene oxide as substrate is recovered in the soluble fraction. The possibility that this activity is associated with small microsomes that do not pellet under the conditions used is rendered unlikely by the use of a long centrifugation time (150 min, see Materials and Methods) and by the observation that only very small amounts of NADPH–cytochrome *c* reductase and cytochrome P-450 are recovered in the soluble fraction. This latter observation also speaks against general solubilization of components of the endoplasmic reticulum during homogenization and/or subfractionation.

The second surprising aspect of these studies is the very high activity of glutathione transferase(s) (with 1-chloro-2,4-dinitrobenzene as second substrate) present in pike liver. This level is more than three times the corresponding level in rat liver. Most of the pike liver glutathione transferase(s) activity is recovered in the soluble fraction (Fig. 2), but the small amount of activity present in the microsomal fraction may arise from an enzyme which is truly localized on the endoplasmic reticulum [38].

Figure 3 consists of electron micrographs of three subcellular fractions prepared from pike liver. The 600 g_{av} pellet contains a number of well preserved nuclei and more or less disrupted nuclear remains. The distribution of DNA (Fig. 2) suggests that virtually all nuclear material is recovered in this fraction.

The 7100 *g* pellet fraction contains a large number of well preserved mitochondria, as well as numerous other membrane profiles which certainly include lysosomes, peroxisomes, fragments of the Golgi apparatus, and vesicles of rough endoplasmic reticulum. The 133,000 g_{av} pellet fraction contains ribosome-coated and smooth vesicles as well as elongated membrane profiles. Only occasional mitochondria and lysosomes are observed. Finally electron micrographs of sedimentable material in the 106,000 *g* supernatant fraction contained very few organelles (not shown).

We also made attempts to increase the recovery of fragments of the endoplasmic reticulum in the

Table 1. Attempts to increase recovery of fragments of the endoplasmic reticulum

Component*	Microsomes I†	Microsomes II†	Microsomes III†
Protein‡	80.6 ± 3.4	14.5 ± 3.7	4.86 ± 1.57
Phospholipid‡	0.952 ± 0.020	1.11 ± 0.11	1.45 ± 0.16
Cytochrome oxidase	0.891 ± 0.048	1.33 ± 0.41	1.86 ± 0.78
p-Nitrophenyl-*α*-mannosidase	0.939 ± 0.192	1.13 ± 0.67	1.46 ± 1.19
AMPase	1.00 ± 0.09	1.29 ± 0.51	1.86 ± 1.42
Catalase	0.710 ± 0.106	2.15 ± 0.48	2.52 ± 0.56
β-Glycerophosphatase	0.849 ± 0.050	1.49 ± 0.41	2.03 ± 0.43
Lactate dehydrogenase	1.05 ± 0.04	0.998 ± 0.117	0.283 ± 0.068
NADPH-cytochrome *c*-reductase	0.855 ± 0.150	1.92 ± 1.32	1.49 ± 0.06
Cytochrome P-450	1.00	1.05	0.880
Benzpyrene monooxygenase	0.932 ± 0.091	1.13 ± 0.24	1.89 ± 0.90
Epoxide hydrolase	0.927 ± 0.034	1.37 ± 0.37	1.31 ± 0.20
Glutathione *S*-transferase	0.939 ± 0.007	1.14 ± 0.25	1.73 ± 0.40

* The values presented for enzyme activities are the relative specific activities ± S.D. (per cent of total microsomal (I + II + III) activity/per cent of total microsomal proteins) of three different determinations, with the exception of cytochrome P-450, which was only determined once.

† See Materials and Methods for the preparation procedure.

‡ mg/g wet weight.

microsomal fraction using a procedure which has been found to increase this recovery 50–100% for rat liver [23]. Microsomes II and III (see Materials and Methods) contained 18 and 6%, respectively, as much protein as was recovered in the original 133,000 g_{av} pellet (microsomes I). It can also be seen from Table 1 that microsomes II and III have higher relative specific activities of all markers utilized than microsomes I, with the exception of lactate dehydrogenase. Thus, microsomes II and III are less contaminated by cytosol and approximately equally contaminated by other organelles as microsomes I. These findings also indicate that microsomes I are a representative sample of the microsomes present in the supernatant after preparation of the 'nuclear' fraction.

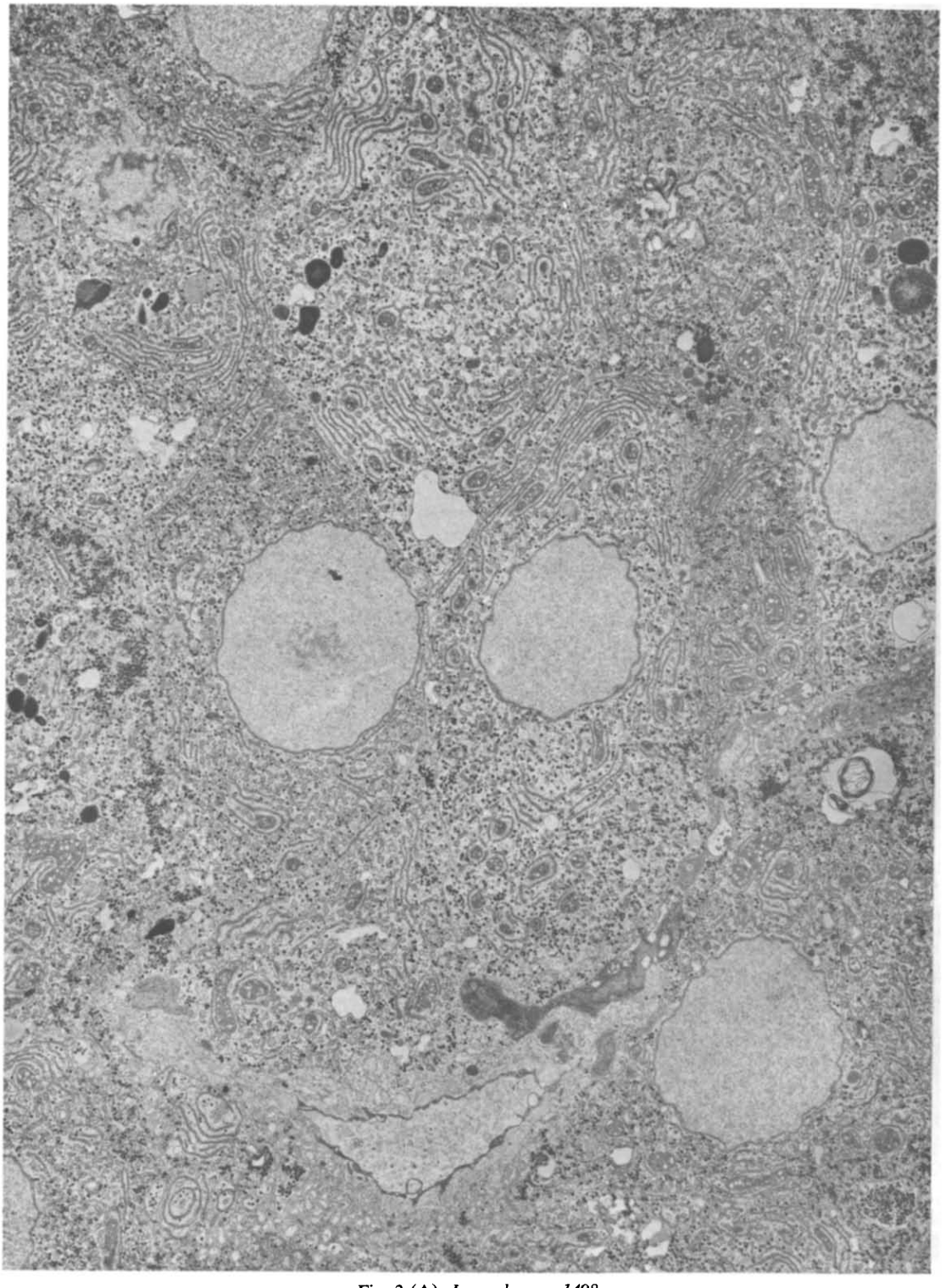

Fig. 3 (A). *Legend on p. 1498.*

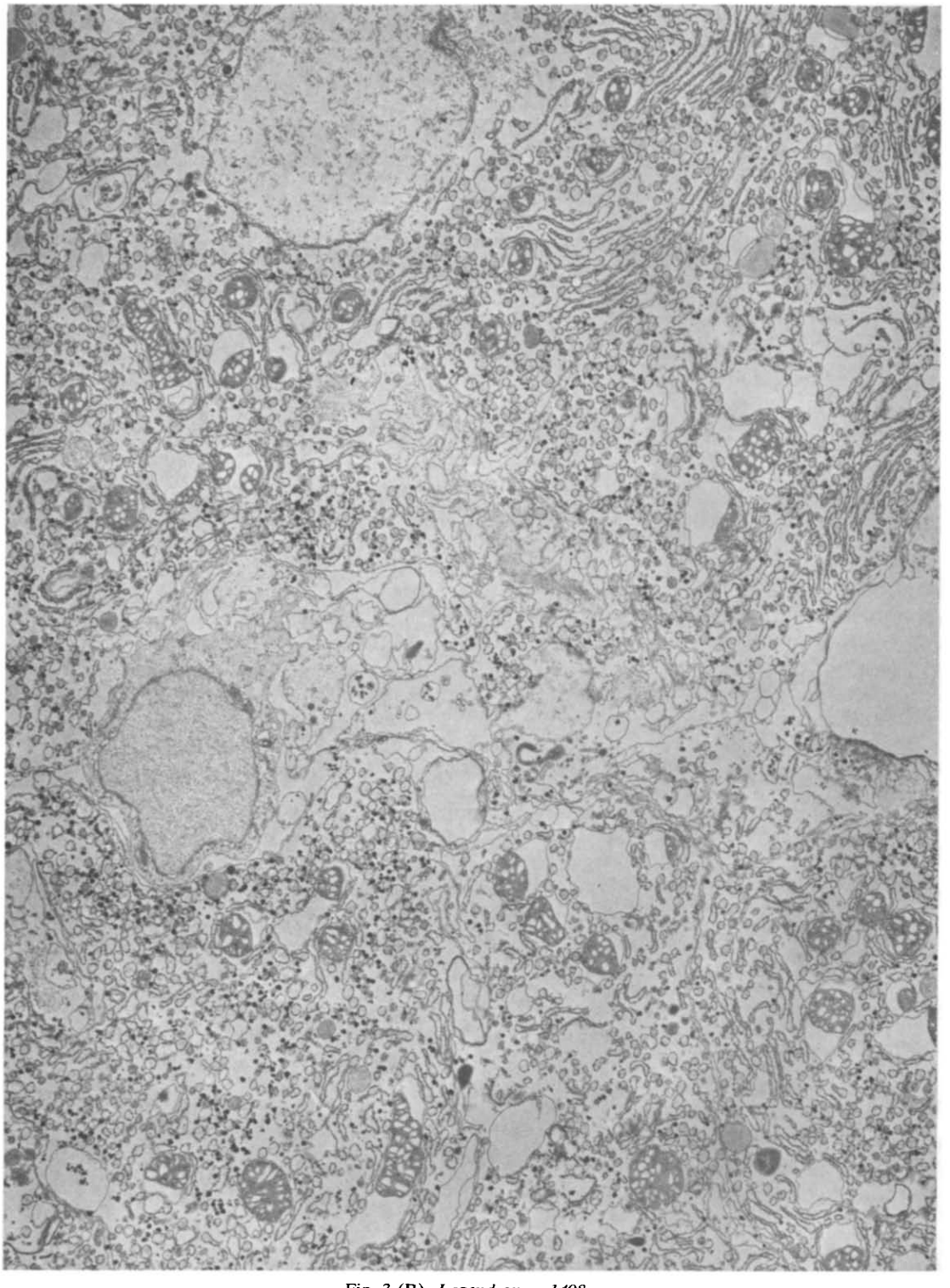

Fig. 3 (B). *Legend on p. 1498.*

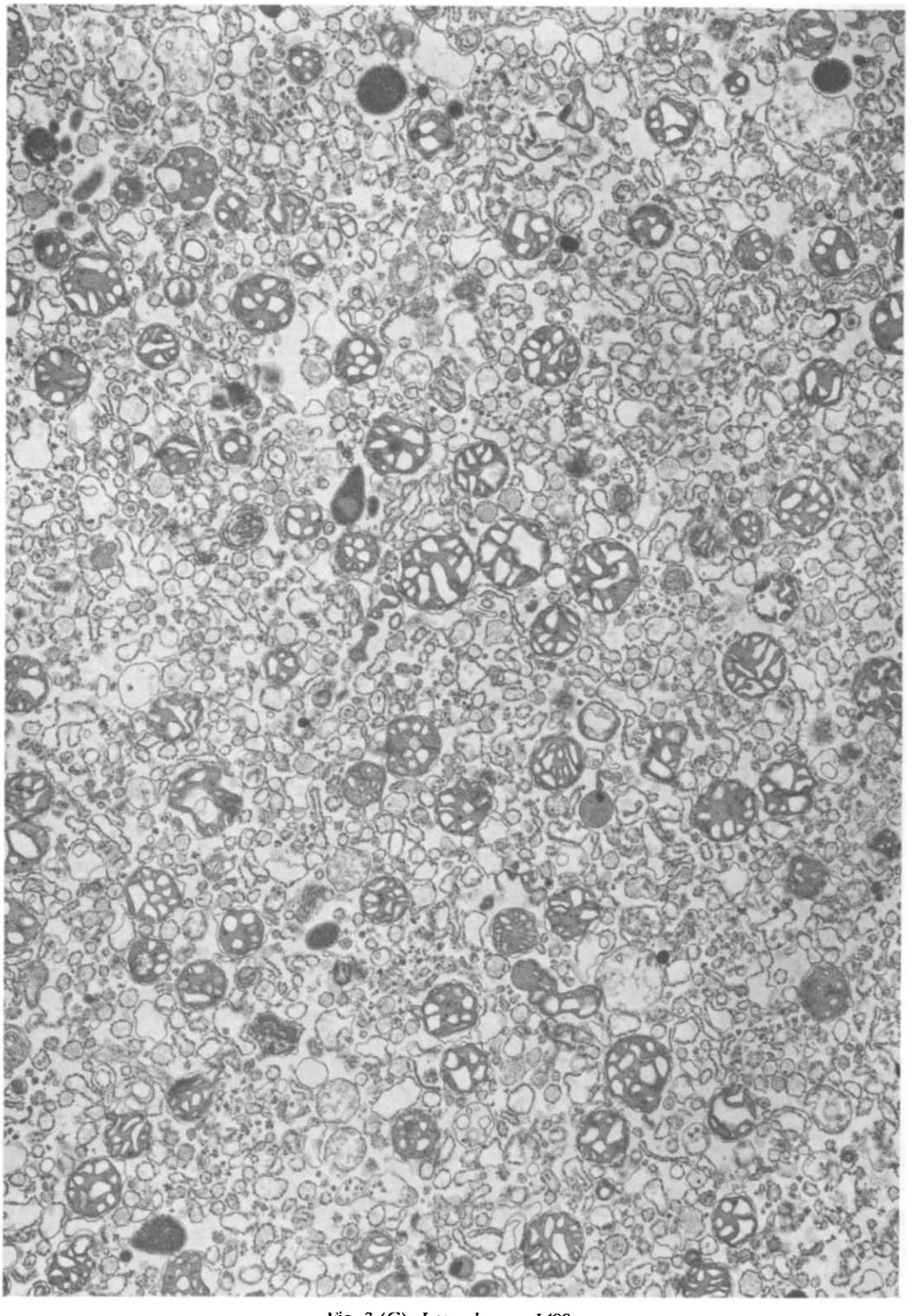

Fig. 3 (C). *Legend on p. 1498.*

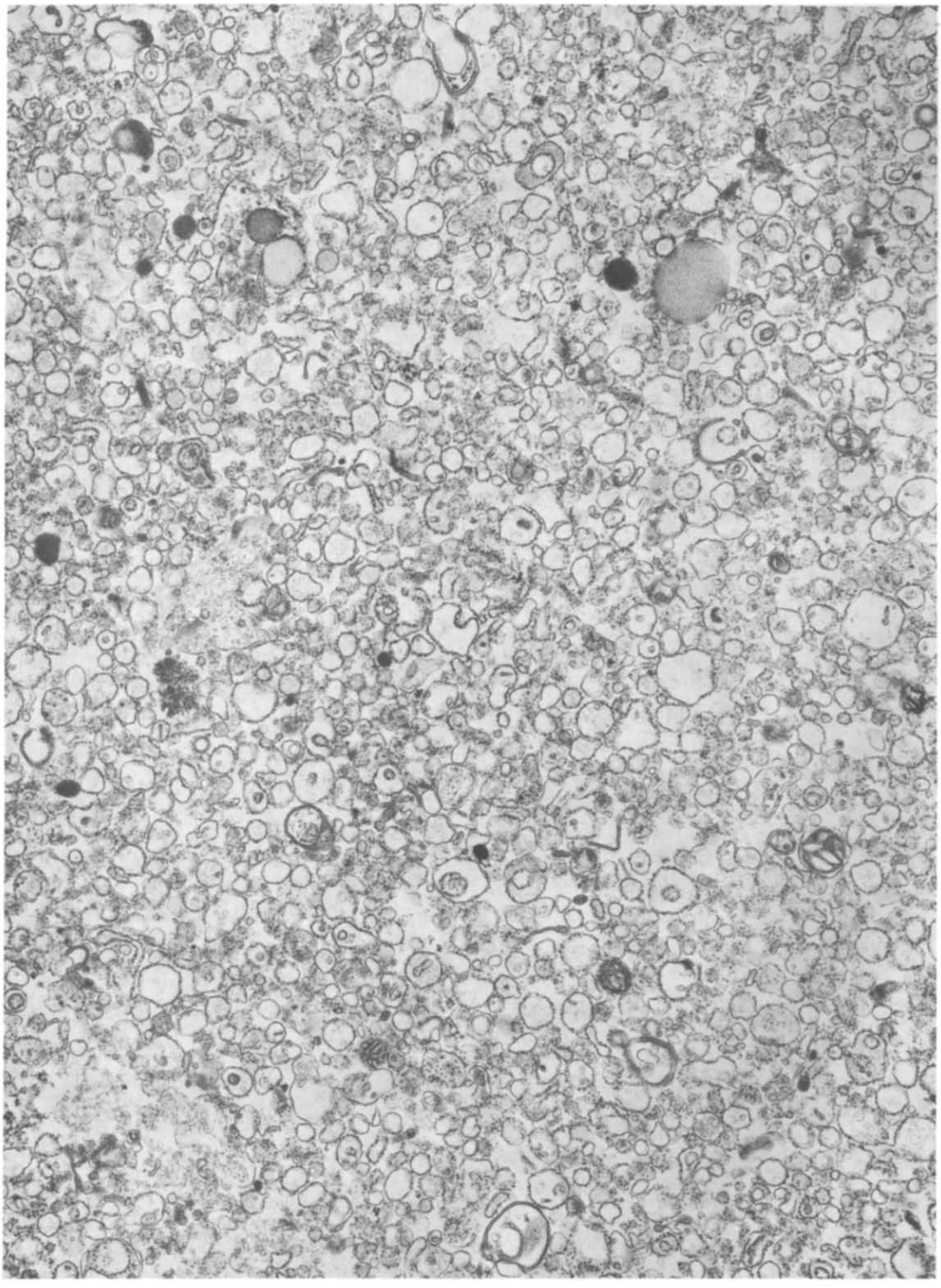

Fig. 3 (D).

Fig. 3. Electron micrographs of three subfractions prepared from pike liver by differential centrifugation. The subfractions were prepared as represented schematically in Fig. 1 and as described in Materials and Methods. (A) Section of whole liver from female pike. Several hepatocytes with large pale nuclei and a moderate amount of glycogen in the cytoplasm. At left a sinusoid with adjoining Kupffer cell (dark), (magnification × 6800). (B) The 600 g_{av} pellet fraction. This fraction contains nuclei, mitochondria, microsomes and glycogen particles (magnification × 9945). (C) The 7100 g_{av} pellet fraction. Numerous mitochondria but also a great number of microsomes and occasional lysosomes can be seen in this fraction (magnification × 12,750). (D) The 133,000 g_{av} pellet fraction. Predominantly microsomal vesicles with occasional mitochondria, lipid droplets and lysosomes (magnification × 17,000).

DISCUSSION

It is clear from the present investigation that the distribution of various presumptive markers in pike liver upon subcellular fractionation by differential centrifugation closely resembles the corresponding distribution of these same markers in rat liver. The one striking exception was the presence of 35–40% of the total epoxide hydrolase activity measured with styrene oxide as substrate in the cytosol fraction (106,000 g_{av} supernatant) from pike liver. It is now clear that mammalian liver also contains cytosolic epoxide hydrolase activity, but not with styrene oxide as substrate [39]. Whether this difference is of any functional importance in the metabolism of xenobiotics remains to be determined. Also of interest is whether both the membrane-bound and soluble epoxide (styrene oxide) hydrolase activities in pike liver are catalysed by the same protein.

Of particular interest here was the subcellular distribution of drug-metabolizing systems. As in rat liver, the cytochrome P-450 system and at least part of the epoxide hydrolase are both localized on the endoplasmic reticulum in pike liver; while most of the glutathione transferse(s) activity and the rest of the epoxide hydrolase are found in the cytoplasm. These localizations may well be of physiological significance with regards to interaction between the various drug-metabolising systems.

Of great interest to us is the high activity of glutathione transferase(s) in pike liver. We have begun isolation of this activity and found that it is catalysed by several proteins which may correspond to the three major isozymes of glutathione transferase(s) found in rat liver [40]. Immunochemical procedures are being employed to determine whether the proteins which catalyse glutathione transferase(s) activity in the liver of the rat and of the pike are immunologically related.

Table 2 documents a comparison between the total microsomal fractions prepared from the livers of the rat and of the pike. It can be seen that recovery of fragments of the endoplasmic reticulum in this fraction from the pike is almost as great as the corresponding recovery in rat. Pike liver microsomes are somewhat more contaminated with mitochondria and lysosomes and much less contaminated with fragments of the plasma membrane than are the rat liver microsomes. We are able to increase the recovery of pike liver microsomes 20% in a simple manner; but this is not a serious problem, since pikes are relatively large animals and the liver constitutes about 0.6–1.9% of their body weight.

Table 2. Comparison of the compositions of the total microsomal fractions from the livers of the rat and the pike

Component	Rat liver‡ microsomes	Pike liver§ microsomes
Protein*	20–25	12
Phospholipid*	6–7	5–6
Markers for:†		
Endoplasmic reticulum	40	35
Mitochondria	0.7	6.5
Plasma membrane	29	9.9
Lysosomes	11	21
Golgi apparatus		13

* mg/g wet weight of tissue.

† Per cent of the total activity found in the original homogenate.

‡ See Ref. 39.

§ Calculated from Fig. 2 in the present study; microsomes I.

Thus, the microsomal fraction prepared here is fully suitable for further studies of drug-metabolizing systems in pike liver. We have already begun characterization of many of the different activities catalyzed by the cytochrome P-450 system using this fraction. These microsomes are also a good starting material for isolation of the components of the cytochrome P-450 system and of epoxide hydrolase. In the future our subfractionation studies and investigation of drug-metabolizing systems will also be extended to other organs of the Northern pike, especially the kidney.

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