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PREPARATION AND CHARACTERIZATION OF SUBCELLULAR FRACTIONS SUITABLE FOR STUDIES OF DRUG METABOLISM FROM THE TRUNK KIDNEY OF THE NORTHERN PIKE (*ESOX LUCIUS*) AND ASSAY OF CERTAIN ENZYMES OF XENOBIOTIC METABOLISM IN THESE SUBFRACTIONS

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Abstract—The present study was designed to prepare and characterize subcellular fractions from the trunk kidney of the Northern pike (*Esox lucius*), with special emphasis on the preparation of a microsomal fraction 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. Finally, the subcellular distributions of several drug-metabolizing enzymes (NADPH-cytochrome *c* reductase, NADH-ferricyanide reductase, glutathione transferase, epoxide hydrolase) were determined. With the exception of NADPH-cytochrome *c* reductase, the subcellular distributions obtained here for drug metabolizing and marker enzymes closely resembled those reported for rat liver. NADPH-cytochrome *c* reductase was apparently partially solubilized here from microsomal vesicles by an endogenous protease, which reduced its usefulness as a marker enzyme and raises questions concerning the measurement of activities catalyzed by the cytochrome P-450 system in these subfractions. In other respects the microsomes and supernatant fraction prepared here from the trunk kidney of the pike seem to be as well suited for investigations of drug metabolism as are the corresponding fractions from rat and pike liver.

Large amounts of virtually all the different xenobiotics with which we pollute our environment end up in our rivers, lakes, and seas. This fact, together with toxic effects and high tumor frequencies in fish living in polluted waters [1], has led to an increasing interest in the fate of xenobiotics in our aquatic environment. Our laboratory has begun a series of investigations on the uptake, distribution, metabolism, and excretion of benzo(a)pyrene and other xenobiotics in the Northern pike (*Esox lucius*).

We have chosen to study this species for a number of different reasons: the Northern pike is a top predator, which means that it ingests xenobiotics accumulated by organisms lower down in the food chain. In addition, the tumor incidence in this fish is very high in certain waters, e.g. the Baltic Sea and waters of certain parts of Ireland and the United States [1–3]. In fact, in some areas 20.9% of the Northern pike have tumors, the highest frequency of malignant lymphoma yet reported in any feral vertebrate [4]. These findings suggest that the Northern pike may be especially sensitive to the carcinogenic effects of different xenobiotics and may therefore be a good model system for studying such effects. Finally, Northern pike spend their entire lives in a relatively restricted geographical area [5], which means that induction of drug-metabolizing enzymes in these fish might be used to monitor certain kinds of pollution in different aqueous environments. There are also practical advantages to using this species, including their large size, ready availability, the ability to determine their sex by external examination [6], and the ease with which they can be maintained in aquaria for long periods of time.

Our earlier studies have demonstrated that Northern pike can be readily used to study the uptake and distribution of benzo(a)pyrene [7, 8], subfractionation of the liver [9] and the pattern of drug-metabolizing enzymes in this organ [10], and the induction of the cytochrome P-450 system and production of mutagenic metabolites via this system [11]. As with mammals, the metabolism of xenobiotics in fish seems to occur to the greatest extent in the liver. However, the mammalian kidney also contains relatively high levels of different drug-metabolizing enzymes. In addition, we have observed that when Northern pike are exposed to benzo(a)pyrene either in their food or in the surrounding water, the levels of benzo(a)pyrene plus metabolites which accumulate in the trunk kidney are comparable to those found in the liver. Moreover, there are certain indications that cells localized in the kidney may be involved in the etiology of

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malignant lymphomas in the Northern pike [12–14]. For these reasons we have chosen to examine drug metabolism in the trunk kidney of Northern pike.

An important first step in such a study is subfractionation of the trunk kidney in order both to obtain preparations suitable for the study of drug-metabolizing enzymes and to ascertain the distribution of these activities in the cell. In the present study we have used differential centrifugation to prepare such subcellular fractions and characterized these fractions using both electron microscopy and enzyme markers. To our knowledge no such study of the trunk kidney of fish has been performed earlier.

MATERIALS AND METHODS

Chemicals. (G-^{3}H)-Benzo(a)pyrene (24 Ci/mmole = 888 GBq/mmole) was purchased from the Radiochemical Centre (Amersham, U.K.) and purified as reported previously [31], while non-radioactive benzo(a)pyrene was bought from Eastman Kodak Company (Rochester, NY, U.S.A.). 1-Chloro-2,4-dinitrobenzene was procured from Merck (Darmstadt, F.R.G.), while NADPH, isocitrate, and trypsin inhibitor (soybean, T-9003) were all obtained from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). *cis*-Stilbene oxide was synthesized by the Synthesis Service (Chemical Centre, Lund, Sweden), while ^{3}H-*cis*-stilbene oxide was synthesized by Dr. Åke Pilotti, Department of Organic Chemistry, University of Stockholm, according to Dr. B. D. Hammock, University of California at Davis (personal communication) and subsequently purified by thin layer chromatography to a purity of >99%. All other chemicals were obtained from common commercial sources and were of analytical purity.

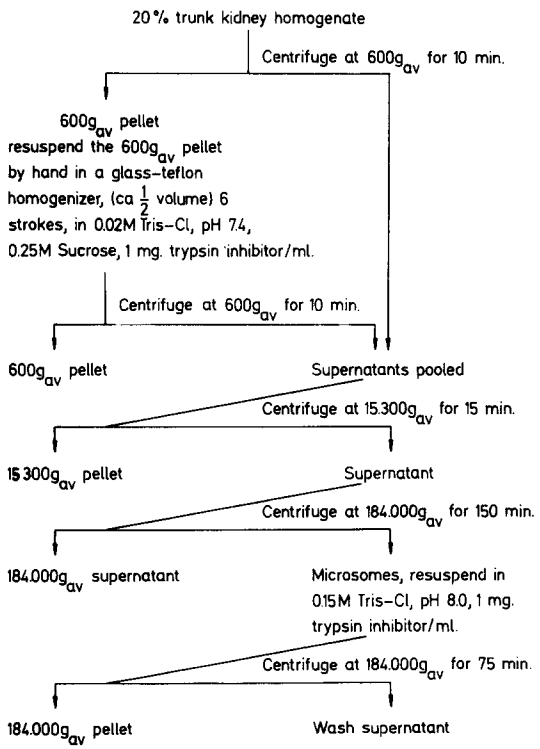


Fig. 1. Subcellular fractionation of the trunk kidney homogenate from Northern pike.

Animals. The Northern pike used in this study were purchased from local fishermen, who caught them in the brackish water of the archipelago outside of Stockholm. The 17 fish used included 11 females and 6 males with an average weight of 2.09 kg (range 0.4–5 kg). The pike were not fed in captivity; however, a starvation period of a few weeks is not uncommon for this animal in its natural environment [15]. All fish were maintained in cold (5–14°) running tap water for at least 6 days before sacrifice.

The rats (Anticimex, Stockholm) used were male Sprague–Dawley weighing 150–180 g. They were fed a standard commercial diet and starved overnight before sacrifice.

Subcellular fractionation. In order to avoid inner bleeding the fish were killed by puncturing the head with a sharp tool and rapidly mincing the brain. The large posterior portion of the trunk kidney is fused and covers the spinal cord completely, while the smaller anterior portion is composed of two slender branches on either side of the spinal cord. In the anterior region of these two branches a diffuse neck segment can be seen. The large mass of tissue posterior to this neck has been designated the trunk kidney and is partially homologous to the mammalian kidney; while the smaller amount of anterior tissue is called the head kidney and is partially homologous to the mammalian adrenal gland [16]. This anatomy corresponds well to the type III kidney in M. Ogawa's classification of the teleostean kidney [17].

Immediately after the fish was killed, the trunk kidney was removed (in pieces, since removing this organ intact was too time-consuming and not necessary for the present study), weighed (an average weight = 0.55% of the body weight was observed), and rinsed in ice-cold 0.25 M sucrose. The tissue was subsequently minced with a pair of scissors, rinsed with sucrose, and the sucrose removed by filtration through gauze. The resulting pieces were placed in a volume of redistilled water at 0° equal in ml to their total weight in grams.

This suspension was immediately homogenized at 0° using 10 up-and-down strokes of a Potter–Elvehjem homogenizer (Arthur H. Thomas, Philadelphia, PA, U.S.A) at 885 rpm. It was found necessary to use a homogenizing vessel with a relatively small clearance (0.15–0.23 mm) and to construct a special pestle with a thick steel rod, since commercially available pestles tend to bend outwards at the speed employed. Immediately after homogenization, which took about 40 sec, sucrose, Tris–Cl, pH 7.4, and trypsin inhibitor were added to give a 20% homogenate containing 0.25 M, 20 mM, and 1 mg/ml, respectively, of these components.

This homogenate was then subfractionated by differential centrifugation with the use of Beckman JA-20 and 50.2 Ti rotors, at 0–4° according to the scheme illustrated in Fig. 1. As was also the case for homogenates for pike liver [9], it was necessary to use relatively high centrifugal force for a relatively long time in order to effectively pellet the microsomes. Resuspension of the initial 600 *g* pellet, centrifu-

gation, and combination of this second 600 *g* supernatant with the first supernatant was found to increase both the recovery of microsomes (about 50%) and of mitochondria (about 100%). We also observed that the 15,300 *g* pellet was composed of a dark lower layer containing 70% of the total fraction protein and a light upper layer containing the rest of the protein. Markers for mitochondria (93% of the cytochrome oxidase) and for lysosomes (78% of the glycerophosphatase) were enriched in the lower layer, while markers for the endoplasmic reticulum (48% of the NADPH-cytochrome *c* reductase and 46% of the epoxide hydrolase) were enriched in the upper layer. The pH values of the fractions prepared as shown in Fig. 1 and resuspended, in the case of the pellets, in 0.25 M sucrose–0.02 M Tris–Cl, pH 7.4–1 mg trypsin inhibitor/ml, were 7.0 for the 600 *g* pellet, 7.1 for the 15,300 *g* pellet, 7.6 for the 184,000 *g* pellet, and 6.8 for the high-speed supernatant.

Chemical and enzyme assays. All assays were performed on freshly prepared subfractions. Before carrying out chemical or enzymatic analysis on the homogenate or on the 600 *g* or 15,300 *g* pellets these fractions were sonicated using a Branson sonicator at setting 4 (7 mA) 4 times for 15 sec while immersed in an ice bath, with a cooling interval of 15 sec between sonications. This procedure was utilized to break down barriers to enzyme substrates and to facilitate removal of a representative sample. However, sonication was not used in the case of cytochrome oxidase determination, since this procedure decreased the measurable activity, presumably by causing formation of everted submitochondrial particles.

DNA [18], cytochrome oxidase [19], *p*-nitrophenyl-α-mannosidase [20], AMPase [21], catalase [22], β-glycerophosphatase [23], lactate dehydrogenase [24], NADPH-cytochrome *c* reductase [25], alkaline phosphatase [26], cytochrome P-450 [27], glutathione transferase activity with 1-chloro-2,4-dinitrobenzene [28], NADH-ferricyanide reductase [29], glucose-6-phosphatase (maximal activity was obtained in the presence of 0.03% DOC) [30], benzo(a)pyrene monooxygenase [31], cytochrome b_5 [32], protease [33], protein (with bovine serum albumin as standard) [34], and inorganic phosphate [35] were all determined according to published procedures. The protein values were corrected for the added trypsin inhibitor. Epoxide hydrolase activity was measured according to Hammock and Gill (manuscript in preparation) in a system containing 0.1 M glycine, pH 9.0, 50 μM ^{3}H-*cis*-stilbene oxide (62,000 dpm) in 1 μl ethanol and sample in a total volume of 0.1 ml. The assay was started by addition of the substrate, run at 37° for 10 min, and stopped by the addition of 0.2 ml *n*-dodecane, followed by vortexing for 30 sec and centrifugation in a desk centrifuge for 15 min to achieve phase separation. With this procedure 92% of the diol product remained in the aqueous phase (the 8% loss of product was routinely corrected for), whereas >99.8% of the remaining substrate was extracted into the organic solvent. In a separate experiment extraction was performed with hexanol to remove both the diol product and remaining substrate from

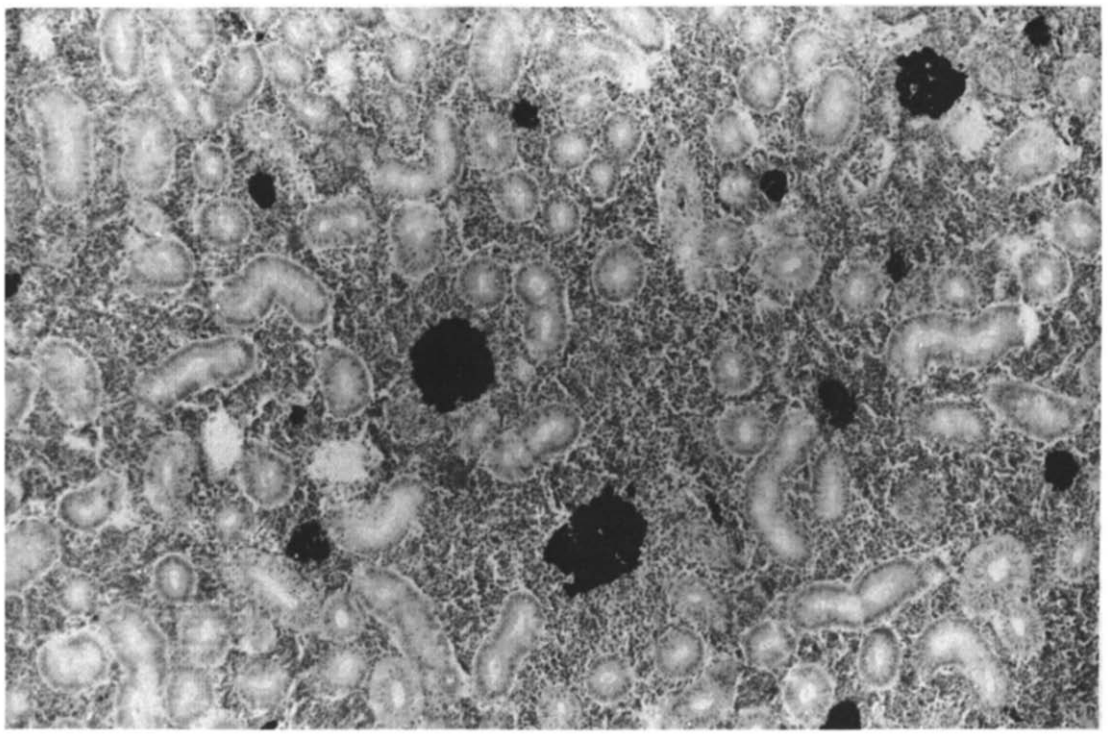


Fig. 2. Light microscopic picture of the trunk kidney of the Northern pike. Tubuli cells, blood-forming tissue and melanomacrophage centers can all be clearly seen. Magnification, 66×.

the aqueous phase. In this case the amount of radioactivity remaining in the aqueous phase was negligible, indicating that the formation of glutathione conjugates with *cis*-stilbene oxide did not interfere here with the assay procedure. Routinely, 50 μl of the aqueous phase was taken for scintillation counting using the external standard ratio procedure to correct for quenching.

All enzyme assays were demonstrated to be linear with time and protein under the conditions used and appropriate background and control incubations were performed. Enzyme determinations were routinely run in duplicate or triplicate and the values for these different samples agreed to within 10%.

deDuve Plots. The distributions of different enzymes among the subcellular fractions were expressed as deDuve Plots [36]. The results from 5 different experiments, in each of which the pooled trunk kidneys from 2 to 5 pikes were used, are shown and the standard deviations indicated by bars in the plots. The sum of the activities of different enzymes in the 600 *g* pellet, the 15,300 *g* pellet, the high-speed pellet and supernatant, and the wash supernatant were 101 ± 20% of the total activities in the original homogenate.

Light microscopy. Small pieces of the trunk kidney were fixed in cold buffered formalin at pH 7.2. Paraffin sections were subsequently stained with hematoxylin-eosin.

Electron microscopy. To prepare samples for electron microscopy the following procedure was used: an aliquot of the sample or a slice was added to 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, with 3.42% sucrose and fixed for 25 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

Light and electron microscopy of slices from the trunk kidney of the Northern pike. In order to determine the types and relative amounts of different cells in the trunk kidney of the Northern pike we performed light and electron microscopy on slices from this organ. The light microscopic studies show that this tissue contains nephrons with glomeruli and tubuli and, interspersed between these nephrons, blood-forming tissue with erythro-, myelo-, and lymphopoesis. The total volumes of the nephron units and the blood-forming tissue are approximately the same, even though the cells of the nephron units are in general larger than the hemopoetic cells and the cells of the blood-forming tissue appear to be more loosely attached to one another than those of the nephron units. In the blood-forming tissue throughout the kidney are located aggregates of granular, darkly stained material (called melanomacrophage centers) which is melanin. A representative picture showing some of these features is shown in Fig. 2.

In Fig. 3 are shown electron micrographs of the different cell types in the trunk kidney of the Northern Pike. Of particular interest here are the following observations: the luminal surface of the tubular epithelial cells is covered with an abundant brush border. These epithelial cells contain numerous large mitochondria in their basal portion and smaller amounts of mitochondria in their luminal half; whereas the cells of the blood-forming tissue contain few mitochondria. Finally, both the epithelial cells and the cells of the blood-forming tissues are seen to be relatively well-equipped with endoplasmic reticulum. The dark particles seen in certain cells of the trunk kidney may be calcium-rich granules [37].

Subcellular distribution of different markers. Figure 4 shows the distribution of various markers upon subcellular fractionation of the trunk kidney of the Northern pike. DNA was used as a marker for nuclei, cytochrome oxidase for mitochondria, *p*-nitrophenyl-α-mannosidase for the Golgi apparatus, AMPase for the plasma membrane in general and alkaline phosphatase for the brush border membrane [38], catalase for peroxisomes, β-glycerophosphatase for lysosomes, and lactate dehydrogenase for the cytosol. Epoxide hydrolase activity with *cis*-stilbene oxide as substrate was found to be the best marker for the endoplasmic reticulum in this study (see below). 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 distribution of these structures.

In general, the distribution of markers shown in Fig. 4 closely resembles the corresponding pattern obtained with rat liver, as well as with the liver from the Northern pike [9] and rainbow trout [39]. Eighty-one percent of the total lactate dehydrogenase activity was recovered in the high-speed supernatant, indicating effective breakage of the cells by the homogenization procedure used. This effective breakage was only achieved by using rather strong disruptive forces (hypotonic homogenization medium and relatively high pestle speed—see the Materials and Methods).

It was of interest to determine whether both cells of the nephrons and cells of the blood-forming tissues were disrupted randomly by our homogenization procedure. The large size of the tubular cells, as well as their tighter association with each other, might favor a selective disruption of these cells. Only these cells contain brush border, to which the alkaline phosphatase activity of the trunk kidney has been localized. In addition, the blood-forming cells contain relatively few mitochondria. We therefore reasoned that the percentages of the alkaline phosphatase and cytochrome oxidase activities which did not sediment in the 600 *g* pellet would indicate the percentage of tubular cells disrupted during the homogenization. These values were 72.1 and 84.5%, respectively, suggesting that approximately 80% of the cells of the nephron units have been disrupted and, consequently, that disruption of the nephron and blood-forming cells during homogenization was essentially random.

Ninety-three percent of the total DNA was localized in the 600 *g* pellet, indicating that this frac-

tion contained virtually all of the nuclei. Cytochrome oxidase was highly enriched (5.7-fold) in the 15,300 *g* pellet, indicating that this was a relatively pure mitochondrial fraction. The 15,300 *g* pellet also contained the highest relative specific activities of markers for the Golgi apparatus and lysosomes. On the other hand, AMPase and alkaline phosphatase were most enriched in the 184,000 *g* pellet, indicating that this microsomal fraction contained relatively large amounts of plasma membrane, a problem which has also been encountered with microsomal fractions from rat kidney [40]. However, judging from the large enrichment of epoxide hydrolase activity in the 184,000 *g* pellet, this fraction was highly enriched in fragments of the endoplasmic reticulum (see also below). Most of the catalase activity was recovered in the low-speed pellet, suggesting either that peroxisomes cosedimented with the nuclei or that catalase is an inappropriate marker for peroxisomes in this tissue.

Since many of the enzymes involved in xenobiotic metabolism are localized in the endoplasmic reticulum, it is of interest to know whether the fragments of this organelle recovered in the 184,000 *g* pellet originated from the tubular cells and/or the blood-forming cells. Since these different cell types were all apparently disrupted to about the same extent by the homogenization procedure employed and since electron microscopy revealed that they contain approximately the same amounts of endoplasmic reticulum, it seems likely that the fragments of endoplasmic reticulum recovered in the 184,000 *g* pellet originated to a similar extent from both the nephron and blood-forming tissues.

Marker enzymes for the endoplasmic reticulum in the trunk kidney of Northern pike. Initially, we attempted to use NADPH-cytochrome P-450 reductase (measured as NADPH-cytochrome *c* reductase) as a marker for the endoplasmic reticulum in the trunk kidney of Northern pike, since this activity is widely used as a marker for the endoplasmic reticulum in mammalian tissues. However, we found that more than one third of the total NADPH-cytochrome *c* reductase activity was recovered in the high-speed supernatant and that much of this activity was removed from the 184,000 *g* pellet during the wash with Tris buffer. These observations led us to suspect that a functional fragment of the NADPH-cytochrome *c* reductase was being solubilized from fragments of the endoplasmic reticulum by a protease present in the homogenate. Indeed, an active segment of the NADPH-cytochrome *c* reductase of rat liver microsomes can be solubilized by trypsin [41, 42].

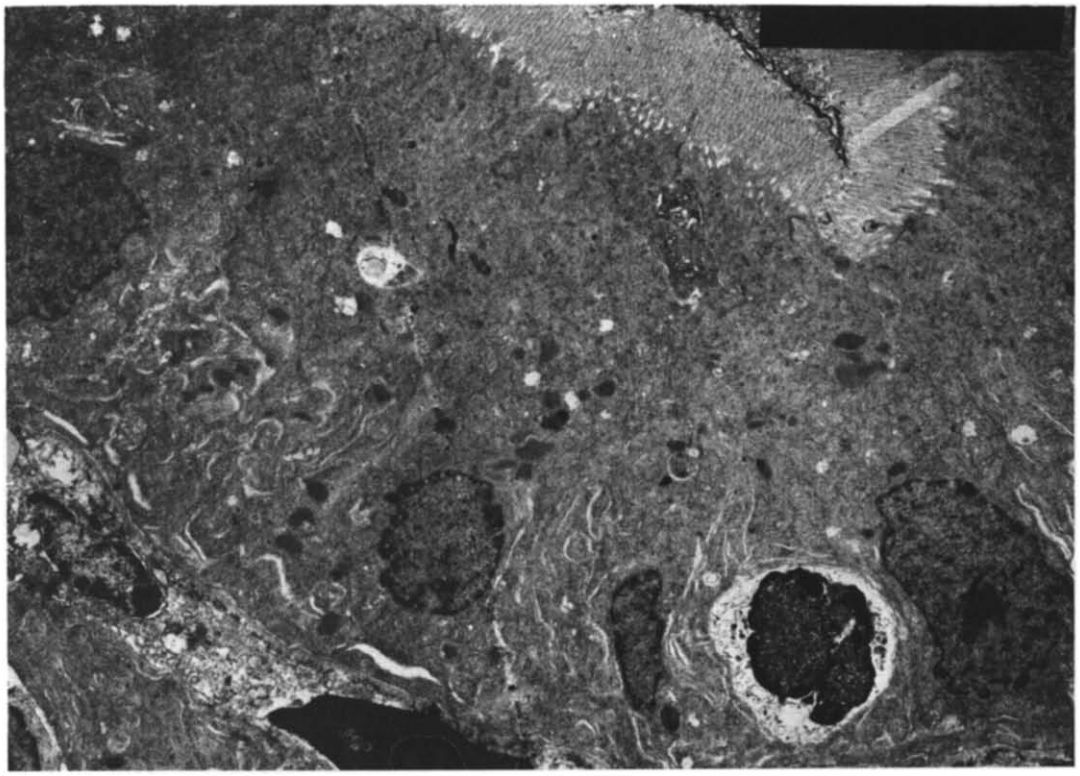
Using an assay for non-specific protease activity [33], we found that the homogenate from the trunk kidney of Northern pike demonstrates protease activity (29.8 ± 4.2 μg tyrosine solubilized/min/mg protein) which is 2.8 times the protease activity in a corresponding homogenate from rat kidney, 3.8 times as much protease activity as a pike liver homogenate, and 8.3 times as much activity as a rat liver homogenate. The specific protease activities of the 600 *g*, 15,300 *g* and 184,000 *g* pellets and the high-speed supernatant from the trunk kidney were 60, 280, 60 and 100%, respectively, of the corresponding activity in the whole homogenate. This activity was extremely temperature dependent, being 69 times greater at 37° than at 0°.

In an attempt to prevent the postulated solubilization of NADPH-cytochrome reductase by protease we added trypsin inhibitor (1 mg/ml final concentration) or phenylmethyl sulfonyl fluoride (50 μM final concentration) to the homogenate immediately after it had been prepared. The phenylmethyl sulfonyl fluoride had no effect, but the trypsin inhibitor increased the specific activity of NADPH-cytochrome *c* reductase in the 184,000 *g* pellet by 100% (to 2.5 times that of the whole homogenate, see Fig. 4) and decreased the amount of this activity recovered in the high-speed supernatant to 20% of the total (see Fig. 4). The addition of larger amounts of trypsin inhibitor (2 or 4 mg/ml) did not have any further effect. Thus, 1 mg trypsin inhibitor/ml, which was found to inhibit the protease activity of the whole homogenate by 25%, was included routinely in the homogenate and in the solutions used in the subfraction procedure.

It should be mentioned here that during the course of solubilization of an active fragment of rat liver microsomal NADPH-cytochrome *c* reductase with trypsin, the total activity of this enzyme increases 35–50% [41]. If the total microsomal NADPH-cytochrome *c* reductase activity in the pike trunk kidney was increased in a similar manner during solubilization of an active fragment by an endogenous protease, then the 20% of the total activity recovered in the high-speed supernatant may actually have represented no more than 13–15% of the microsomal NADPH-cytochrome *c* reductase molecules present. This may be an important consideration when measuring activities catalyzed by the cytochrome P-450 system (in which NADPH-cytochrome *c* reductase is an essential component) in the 184,000 *g* pellet from the trunk kidney of the Northern pike.

We also tried to minimize the solubilization of microsomal NADPH-cytochrome *c* reductase by adding $CaCl_2$ and $MgCl_2$ to the 15,000 *g* supernatant in order to preciptate the microsomes [43] and thereby minimize the time required for their sedimentation (15 min). However, this procedure also had no effect on the level of "soluble" NADPH-cytochrome *c* reductase activity.

The NADPH-cytochrome *c* reductase activity recovered in the high-speed supernatant even after addition of trypsin inhibitor may have been due to continued solubilization of the microsomal enzyme or to an entirely different enzyme. Because of these findings, we felt that NADPH-cytochrome *c* reductase was a somewhat uncertain marker for the endoplasmic reticulum and decided to examine other enzymes which are localized on this organelle in rat liver, namely, glucose-6-phosphatase, NADH-ferricyanide reductase (which is involved in the reduction of cytochrome b_5 and which is less sensitive to solubilization by trypsin than is NADPH-cytochrome P-450 reductase) [41], and epoxide hydrolase activity towards *cis*-stilbene oxide. As can also be seen in Fig. 4, all 3 of these enzymes were enriched in the 184,000 *g* pellet, but epoxide hydrolase activity was enriched in this fraction to a much greater extent that glucose-6-phosphatase and NADH-ferricyanide



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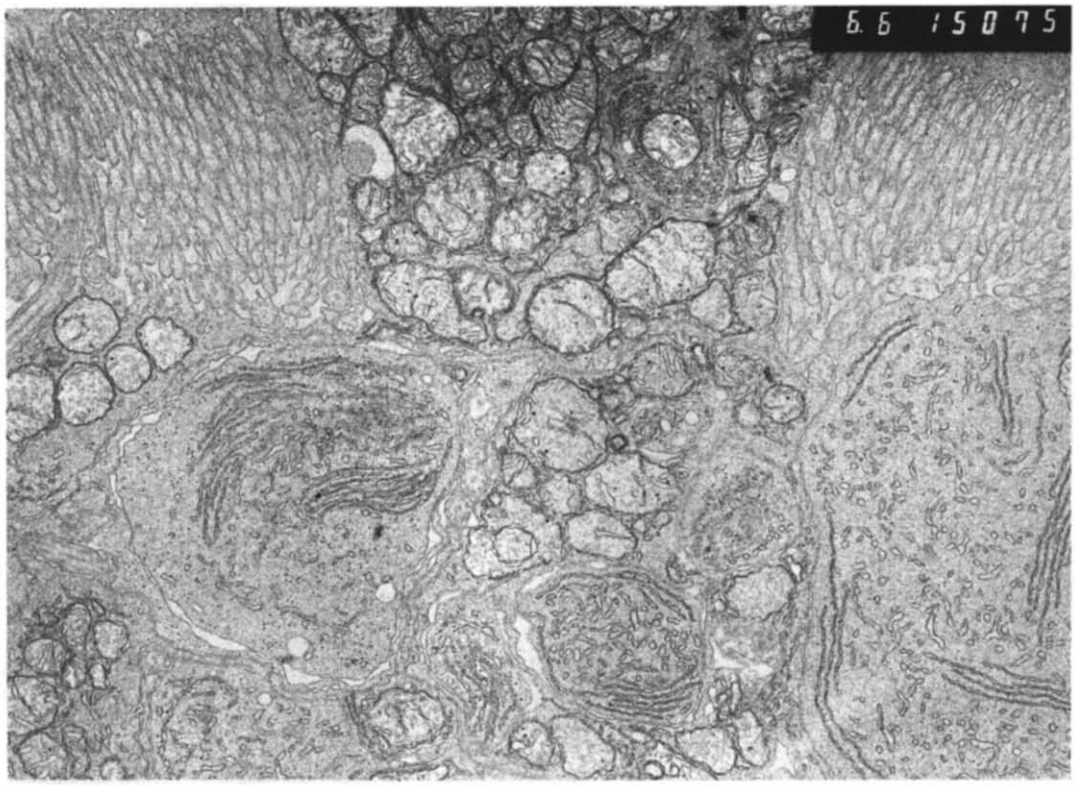

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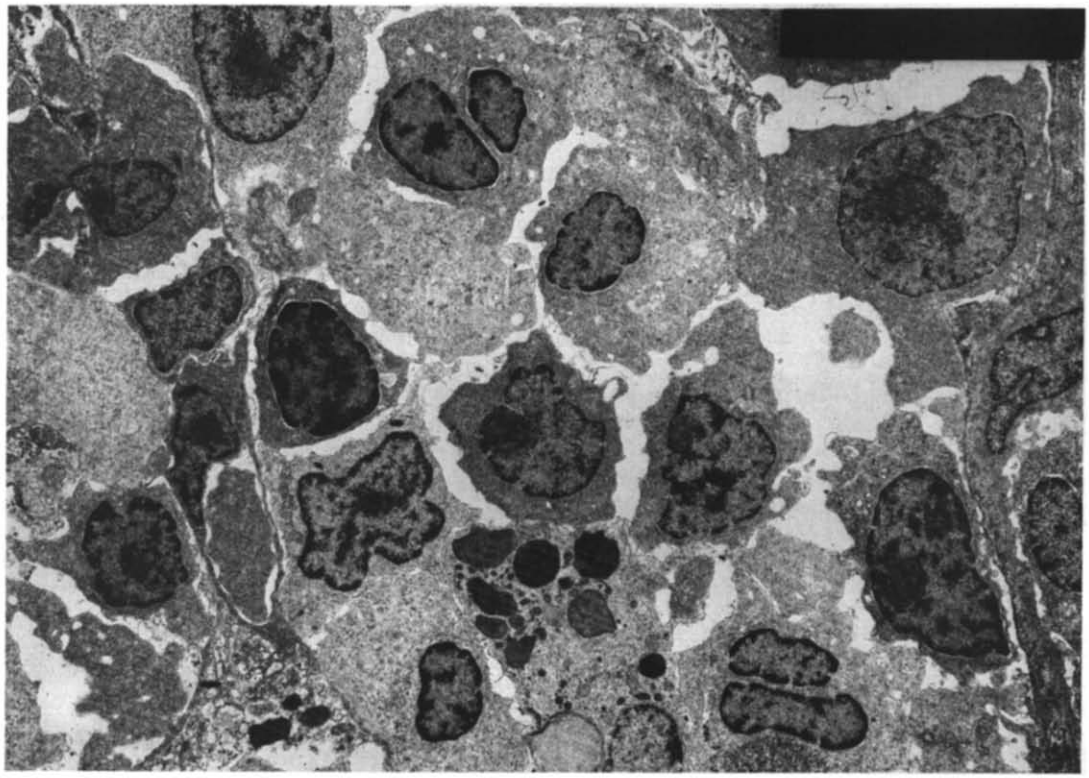

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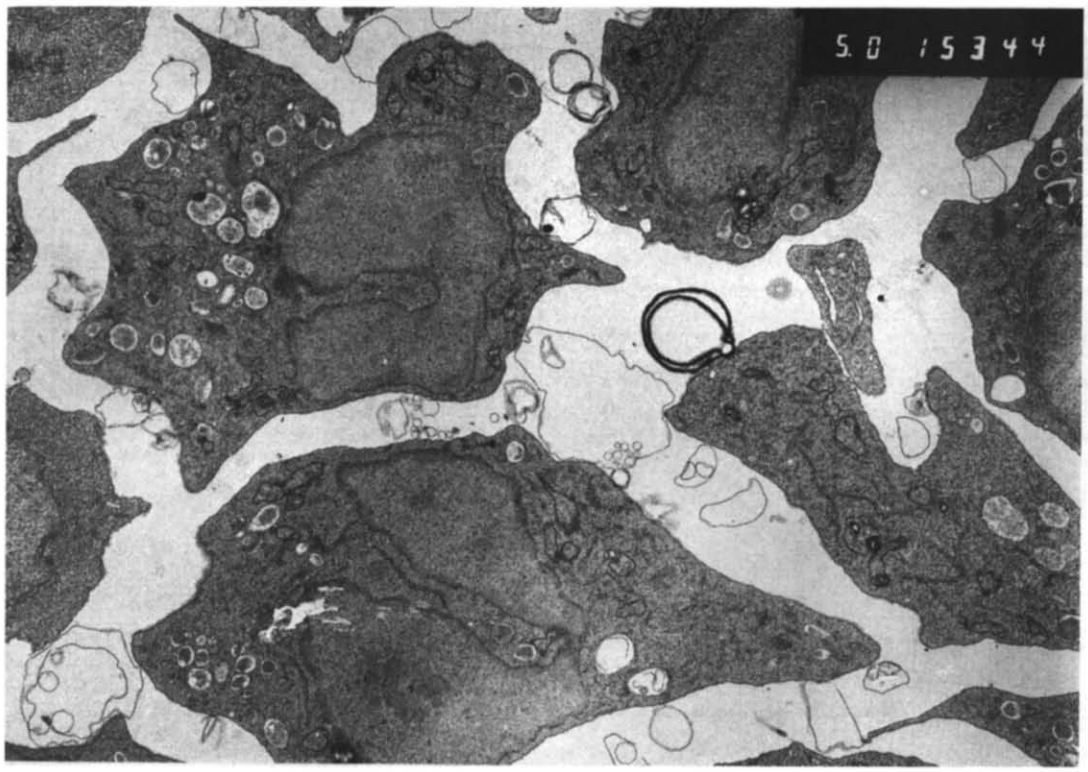
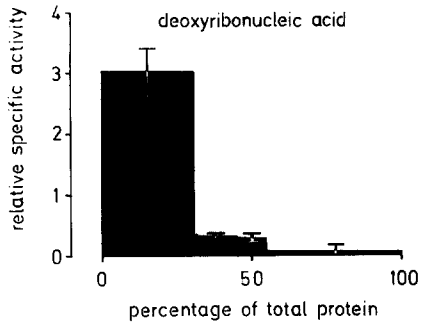


(D)

Fig. 3. Electron micrographs of the trunk kidney of the Northern pike. (A) Survey picture of tubular epithelial cells with brush border on the luminal surface and basal invaginations of the cell membrane. The nuclei are located in the basal portion of the cells. A nucleated red cell can be seen in an interstitial capillary. Magnification, 7200×. (B) Slightly higher magnification of the tubular cells showing numerous mitochondria and extensive endoplasmic reticulum. Magnification, 11,880×. (C) Survey picture of blood-forming tissue, showing numerous immature blood cells. Magnification, 5940×. (D) Higher magnification of a few blood-forming cells. Magnification, 9000×.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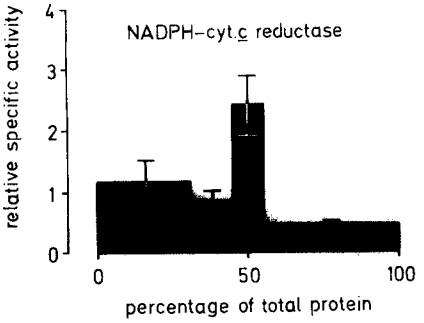
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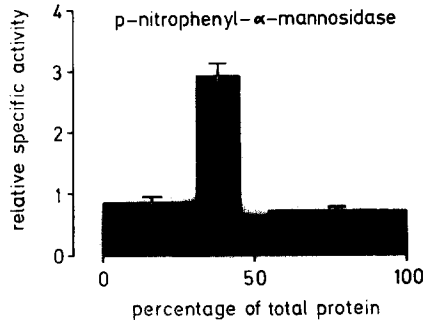
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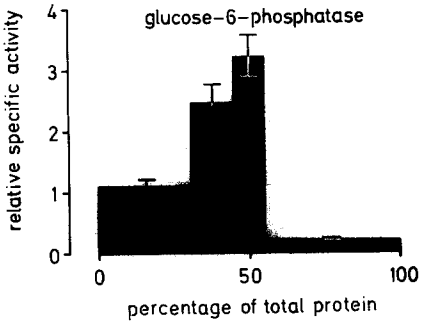
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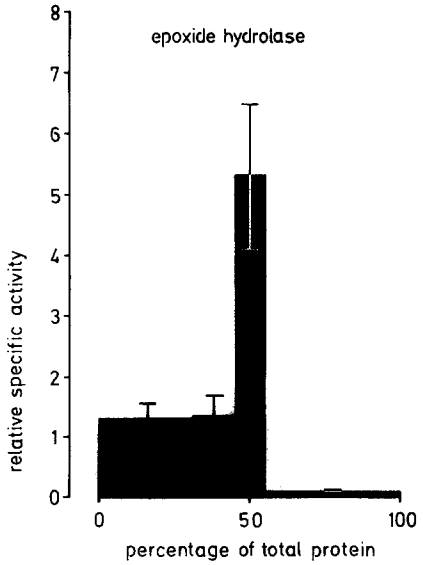
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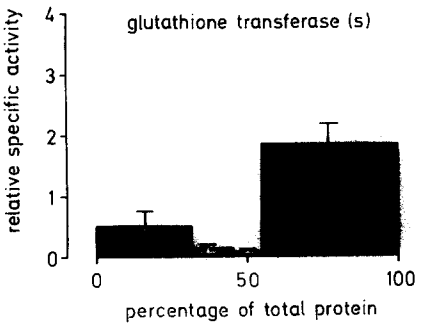
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relative specific activity
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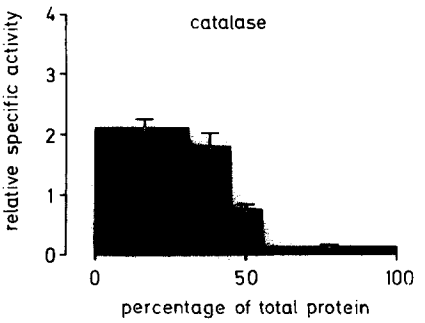
catalase
relative specific activity
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1
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0
50
100
percentage of total protein

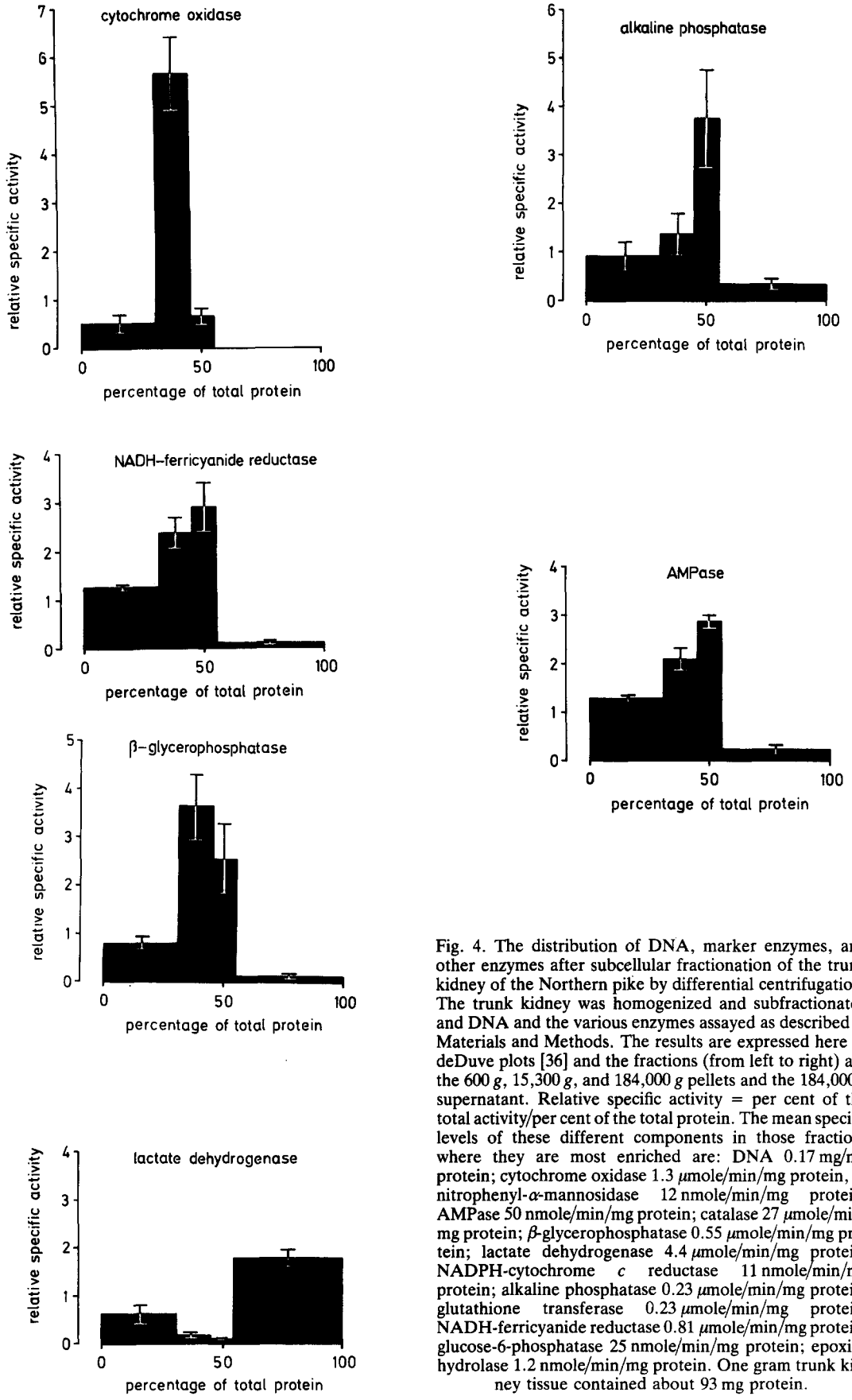

Fig. 4. The distribution of DNA, marker enzymes, and other enzymes after subcellular fractionation of the trunk kidney of the Northern pike by differential centrifugation. The trunk kidney was homogenized and subfractionated and DNA and the various enzymes assayed as described in Materials and Methods. The results are expressed here as deDuve plots [36] and the fractions (from left to right) are the 600 *g*, 15,300 *g*, and 184,000 *g* pellets and the 184,000 *g* supernatant. Relative specific activity = per cent of the total activity/per cent of the total protein. The mean specific levels of these different components in those fractions where they are most enriched are: DNA 0.17 mg/mg protein; cytochrome oxidase 1.3 μmole/min/mg protein, *p*-nitrophenyl-α-mannosidase 12 nmole/min/mg protein; AMPase 50 nmole/min/mg protein; catalase 27 μmole/min/mg protein; β-glycerophosphatase 0.55 μmole/min/mg protein; lactate dehydrogenase 4.4 μmole/min/mg protein; NADPH-cytochrome *c* reductase 11 nmole/min/mg protein; alkaline phosphatase 0.23 μmole/min/mg protein; glutathione transferase 0.23 μmole/min/mg protein; NADH-ferricyanide reductase 0.81 μmole/min/mg protein; glucose-6-phosphatase 25 nmole/min/mg protein; epoxide hydrolase 1.2 nmole/min/mg protein. One gram trunk kidney tissue contained about 93 mg protein.

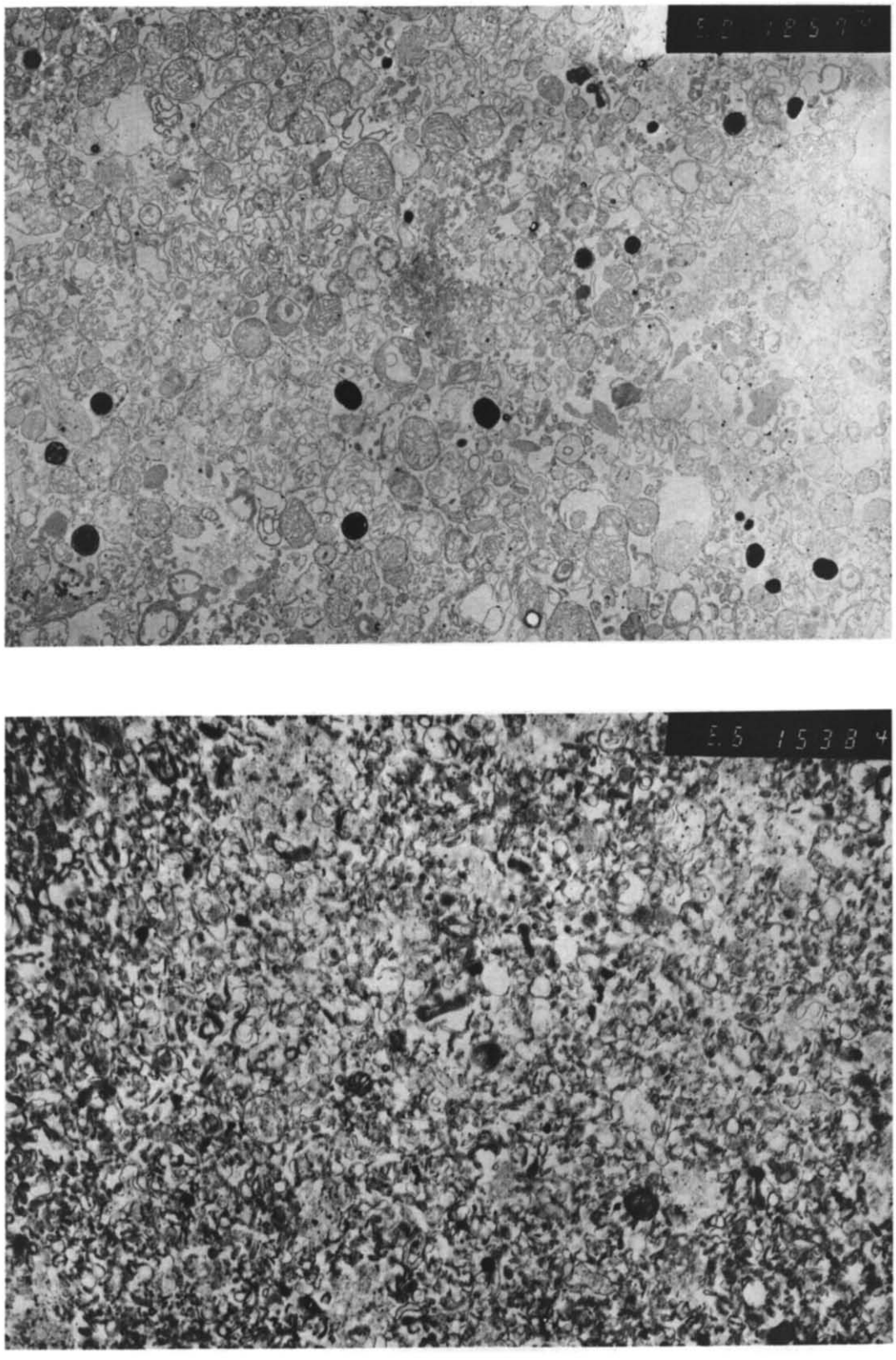

Fig. 5. Electron micrographs of the 15,300 *g* and 184,000 *g* pellets prepared from the trunk kidney of the Northern pike. (A) The 15,300 *g* pellet is seen to contain mainly mitochondria, a number of melanin granules, and some microsomes. Magnification, 9000×. (B) The 184,000 *g* pellet contains mainly smooth microsomes and essentially no mitochondria. Magnification, 11,880×.

Table 1. Comparison of the microsomal fractions from the liver and kidney of the rat and pike

Enzyme or component	Percentage of the total homogenate activity recoved in the microsomal fraction prepared from			
	rat* liver	rat† kidney cortex	pike‡ liver	pike§ trunk kidney
DNA	2	—	2.5	2.8
Markers for mitochondria:				
cytochrome oxidase	—	—	6.5	6.5
succinate-cytochrome *c* reductase	0.7	8	—	—
Markers for lysosomes:				
β-glycerophosphatase	11	7	21	26
Markers for Golgi apparatus:				
p-nitrophenyl-α-mannosidase	—	—	13	6
Markers for peroxisomes:				
catalase	9	—	5.3	7.5
Markers for the plasma membrane:				
AMPase	29	—	9.9	28
alkaline phosphatase	—	—	—	38
Na^+, K^+-ATPase	—	10	—	—
Markers for the endoplasmic reticulum:				
NADPH-cytochrome *c* oxidase	40	34	35	24
glucose-6-phosphatase	40	3	—	32
NADH-ferricyanide reductase	—	—	—	29
epoxide hydrolase	—	—	—	53
IDPase	—	28	—	—
Markers for the cytosol:				
lactate dehydrogenase	—	—	3	1

* From ref. [32].
† From ref. [40].
‡ From ref. [9].
§ From the present study.

reductase. A possible explanation for this finding is given in the Discussion.

Electron microscopy of the subcellular fractions. In order to further assess the purity of the subfractions obtained electron microscopy was performed on the 15,300 *g* and 184,000 *g* pellets. As can be seen in Fig. 5, the first of these pellets consisted mainly of mitochondria, a number of melanin granules, and some microsomes and other smooth vesicles. The high-speed pellet contained mainly smooth vesicles and no mitochondria.

Subcellular distribution of drug-metabolizing enzymes. The present study also provides some information on the subcellular distribution of certain drug-metabolizing enzymes. As can be seen in Fig. 4, NADPH-cytochrome *c* reductase (= NADPH-cytochrome P-450 reductase), NADH-ferricyanide reductase (= NADH-cytochrome b_5 reductase), and epoxide hydrolase activity with *cis*-stilbene oxide were all localized primarily in the 184,000 *g* pellet, which originated chiefly from the endoplasmic reticulum. Glutathione transferase activity toward 1-chloro-2,4-dinitrobenzene was, on the other hand, virtually all soluble.

The trunk kidney of the Northern pike contains melanin in rather large amounts, so that the resuspended 600 *g* and 15,300 *g* pellets were dark brown to black in color. Consequently, it has not yet been possible to measure cytochromes P-450 and b_5 in these fractions. The levels of these cytochromes in the 184,000 *g* pellet, which appeared to be free from melanin, were approximately 0.005–0.080 and 0.031 nmole/mg protein, respectively. Neither of these cytochromes was detected in the high-speed supernatant.

We have also performed preliminary measurements of benzo(a)pyrene monooxygenase activity in the subcellular fractions prepared here from the trunk kidney of the Northern pike. The level of this activity was 370–1100 pmole product/mg protein/min in the 15,300 *g* pellet, which was primarily enriched in mitochondria, and 60–760 pmole product/mg protein/min in the 184,000 *g* pellet, which was most enriched in fragments of the endoplasmic reticulum.

DISCUSSION

It is clear from the present investigation that the distribution of various presumptive markers and of several drug-metabolizing enzymes in the trunk kidney of the Northern pike after subcellular fractionation by differential centrifugation resembles in general the corresponding distribution of these same markers in rat liver. One striking exception to this rule is the distribution of NADPH-cytochrome *c* reductase. Presumed solubilization of an active fragment of this enzyme from the endoplasmic reticulum by endogenous proteases in the trunk kidney, perhaps of lysosomal origin, make it unsuitable for use as a marker enzyme and may also complicate the measurement *in vitro* of xenobiotic metabolism via the cytochrome P-450 system, since NADPH-cytochrome *c* reductase is a component of this system. In a study involving subfractionation of heart tissue from the fish *Stenotomus chrysops* Stegeman and his

coworkers [44] also recovered a large percentage of the total NADPH-cytochrome *c* reductase activity in the high-speed supernatant. Other investigators who prepare subcellular fractions from extrahepatic tissues would, we feel, be well advised to take note of these observations.

We suggest that epoxide hydrolase activity towards *cis*-stilbene oxide is a good alternative marker for the endoplasmic reticulum. This has been found to be the case in the livers of mice, rats, rabbits, hamsters, and guinea pigs (J. Meijer, manuscript in preparation). Epoxide hydrolase activity towards *cis*-stilbene oxide also appears to be a good marker for the endoplasmic reticulum in the trunk kidney of Northern pike. This activity is not solubilized like NADPH-cytochrome *c* reductase, presumably because it is, as in the case of rat liver microsomes [45], deeply buried in the membrane bilayer and inaccessible to proteases. In addition, this activity is more highly enriched in the microsomal (184,000 *g*) pellet than are other presumptive markers for the endoplasmic reticulum (NADPH-cytochrome *c* reductase, NADH-ferricyanide reductase, glucose-6-phosphatase). This observation may mean that epoxide hydrolase activity towards *cis*-stilbene oxide is more exclusively localized in the endoplasmic reticulum than these other enzymes, which have also been found in the outer mitochondrial membrane in rat liver [46, 47].

Since one of our main goals was to prepare fractions suitable for the study of drug-metabolizing systems in the trunk kidney of the Northern pike, the purity and recovery of fragments of the endoplasmic reticulum in our microsomal (184,000 *g*) fraction is of particular interest. In Table 1 the microsomal fractions from rat and pike liver and kidney are compared. It can be seen that the recovery of fragments of endoplasmic reticulum in the microsomal fraction from the pike trunk kidney is at least as good as the corresponding recovery from the other three tissues. Of interest in this respect is the observation that glucose-6-phosphatase activity distributes as do other markers for the endoplasmic reticulum in rat liver and in the trunk kidney of pike, but this enzyme seems to have another distribution in the rat kidney cortex. Microsomes from the trunk kidney of the Northern pike are relatively free of nuclei, mitochondria, Golgi, peroxisomes, and cytosol, but are relatively heavily contaminated with lysosomes and fragments of the plasma membrane—a fact which should be remembered when using this fraction for different studies.

It should also be remembered that the subfractions described here arise from a number of different cell types, a situation which is the rule rather than the exception when working with extrahepatic tissues. However, within the limitations discussed here, the subfractions prepared by our procedure are suitable for further characterization of drug-metabolizing systems in the trunk kidney of the Northern pike as well as for other studies with this tissue. Of particular interest for us at the moment are the question as to whether the cytochrome P-450 system in pike kidney is specialized, as it is in rat kidney, for the hydroxylation of laurate [48] and the possible relationship between xenobiotic metabolism in the trunk kidney in pike and the high frequencies of malignant lymphomas in these animals (see the Introduction).

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