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PREPARATION AND CHARACTERIZATION OF SUBCELLULAR FRACTIONS FROM THE HEAD KIDNEY OF THE NORTHERN PIKE (*ESOX LUCIUS*), WITH PARTICULAR EMPHASIS ON XENOBIOTIC-METABOLIZING ENZYMES

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Abstract—The present study was designed to prepare and characterize subcellular fractions from the head 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 cell components was determined using both enzyme markers and morphological criteria. 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 microsomal fraction prepared here from the pike head kidney seems well-suited for studies of drug metabolism.

The concentrations of many different xenobiotics in our rivers, lakes and oceans are increasing steadily, and the toxic and genotoxic effects of such pollution on aquatic organisms are becoming more and more apparent. Recently, our laboratory has begun a series of investigations on the detoxication of xenobiotics in the Northern pike (*Esox lucius*) [1–4], which we have chosen to study for several different reasons: this animal is a top predator, which means that it ingests xenobiotics accumulated by organisms lower down in the food chain. In addition, the tumor incidence among Northern pike in certain waters is extremely high [5, 6], suggesting that this species might be a good model for studies of chemical carcinogenesis. Moreover, Northern pike spend their entire lives in a relatively restricted geographical area [7], which means that induction of drug-metabolizing enzymes in these fish might be used to monitor certain kinds of water pollution. Finally, there are practical advantages to using this species, including its large size, ready availability, and the ease with which it can be maintained in aquaria for long periods of time.

Our earlier studies have demonstrated that the liver of the Northern pike is actively involved in the metabolism of xenobiotics [1], as is the liver of other fish and mammalian species. However, we have observed using whole-body autoradiography and scintillation counting [8] that when Northern pike are exposed to benzo(*a*)pyrene either in their food or in the surrounding water, relatively high levels of benzo(*a*)pyrene + metabolites accumulate in the head kidney, among other organs. The head kidney of teleost fish is both morphologically and functionally distinct from the trunk kidney, to which it is connected (see also below). The trunk kidney contains nephrons and is responsible, among other things, for the production of urine; while the head kidney seems to be partially homologous, both functionally and morphologically, to the mammalian adrenal gland [9], which is known to have an important role in the metabolism of both steroids and certain xenobiotics [10]. Indeed, altered patterns of steroid biosynthesis in the head kidneys of a teleost fish fed PCB have recently been reported [11]. Finally, there are certain indications that cells localized in the kidneys may be involved in the etiology of malignant lymphomas in the Northern pike [12–14].

For these reasons we decided to investigate xenobiotic metabolism in the head kidney of the Northern pike. An important first step in such a study is subfractionation of this organ 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 subcellular fractions and characterized these fractions with both electron microscopy and enzyme markers. To our knowledge no such investigation of the head kidney of fish has been performed earlier. We have carried out similar subfractionations of the liver [2] and trunk kidney of the Northern pike [15] previously.

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MATERIALS AND METHODS

Chemicals. 1-Chloro-2,4-dinitrobenzene was procured from Merck (Darmstedt, F.R.G.), while NADPH, isocitrate, and trypsin inhibitor (soya bean, T-9003) were all obtained from the Sigma Chemical Co. (St. Louis, MO). *cis*-Stilbene oxide was synthesized by the Synthesis Service (Chemical Centre, Lund, Sweden), while ^{3}H-*cis*-stilbene oxide was synthesized 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.

Animals. Northern pike—11 females and 6 males with an average weight of 2.09 kg—were purchased from local fishermen, who caught them in the brackish water of the archipelago outside Stockholm. All fish were maintained in cold (5–14°) running tapwater without food for at least 6 days before sacrifice. (A starvation period of several weeks is not uncommon for the Northern pike in its natural environment [16]).

Subcellular fractionation. 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 [9]. This anatomy corresponds well to the type III kidney in M. Ogawa's classification of the teleostean kidney [17].

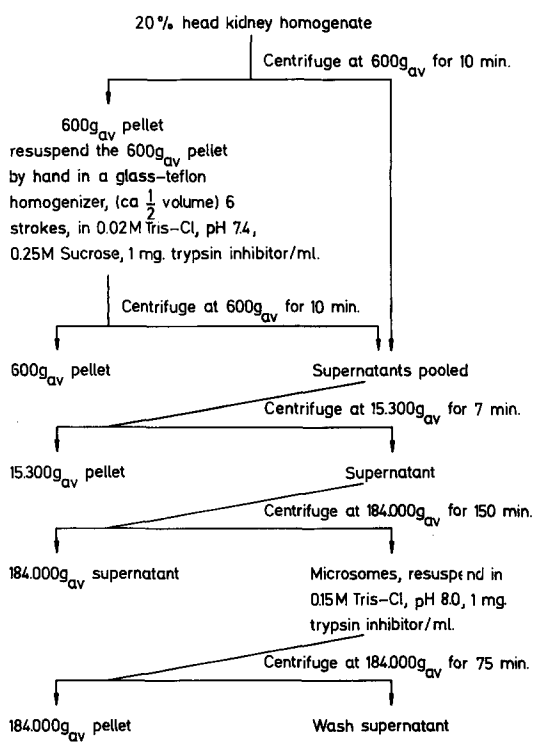


Fig. 1. Subcellular fractionation of the head kidney homogenate from Northern pike.

Immediately after killing the fish, the head kidney was removed, weighed (an average weight = 0.070% of the body weight was observed), and rinsed with ice-cold 0.25 M sucrose. The tissue was subsequently minced with a pair of scissors, rinsed with sucrose, and the fluid removed by filtration through gauze. The resulting tissue fragments were placed in a volume of redistilled water at 0° equal in ml to their total weight in grams.

This suspension was immediately homogenized with 4 up-and-down strokes of a Potter-Elvehjem (Arthur H. Thomas Co., Philadelphia, PA) homogenizer at 885 r.p.m. (0°). 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 25 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 subsequently subfractionated by differential centrifugation at 0–4° according to the scheme illustrated in Fig. 1. As is also the case for homogenates of pike liver and trunk kidney, it was necessary to use relatively high centrifugal force for a relatively long time in order to pellet the microsomes effectively. Resuspension of the initial 600 g_{av} pellet, recentrifugation, and combination of this second 600 g_{av} supernatant with the first supernatant was found to increase both the recovery of microsomes (about 50%) and of mitochondria (about 100%). It was important to wash the microsomal pellet as shown, since this procedure removed as much as 7% of the total activity of the cytosolic marker lactate dehydrogenase and 6% of the total protein, which were adsorbed to the microsomal vesicles but did not originate from the endoplasmic reticulum. The pH values of the fractions prepared and resuspended, in the case of the pellets, in 0.25 M sucrose + 20 mM Tris–Cl, pH 7.4–trypsin inhibitor (1 mg/ml) were 7.1 for the 600 g_{av} pellet, 7.2 for the 15,300 g_{av} pellet, 7.7 for the 184,000 g_{av} pellet, and 7.1 for the high-speed supernatant.

Chemical and enzyme analysis. All assays were performed on freshly prepared subfractions. Before carrying out chemical or enzymatic analysis on the homogenate or on the 600 g_{av} or 15,300 g_{av} pellets, these materials were sonicated with a Branson sonicator at setting 4 (7mA) 4 times for 15 sec while immersed in an ice bath, with a cooling period of 15 sec between sonications. This procedure was used to break down barriers to enzyme substrates and to facilitate removal of a representative sample. However, sonication was not used when cytochrome oxidase was determined, since this activity was decreased by sonication, presumably as a result of

the formation of inside-out submitochondrial particles.

DNA [18], RNA [19], cytochrome oxidase [20], *p*-nitrophenyl-α-mannosidase [21], AMPase [22], catalase [23], β-glycerophosphatase [24], lactate dehydrogenase [25], NADPH-cytochrome *c* reductase [26], cytochrome P-450 [27], epoxide hydrolase activity towards *cis*-stilbene oxide [15], glutathione transferase activity with 1-chloro-2,4-dinitrobenzene [28], NADH-ferricyanide reductase [29], glucose-6-phosphatase (with 0.03% DOC) [30], cytochrome b_5 [31], protease [32], protein (with bovine serum albumin as standard) [33] and inorganic phosphate [34] were all determined according to published procedures. 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 [35]. The results from 5 different experiments, in each of which the pooled head kidney 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 the different enzymes in the $600\,g_{av}$ pellet, the $15{,}300\,g_{av}$ pellet, the $184{,}000\,g_{av}$ pellet, the high-speed supernatant, and the wash supernatant were $104 \pm 24\%$ of the total activities in the original homogenate.

Light microscopy. Small pieces of the head 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 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 from the surface, through the central section, to the bottom to assure obtaining representative samples.

Statisticaly analysis, where indicated in the text, represents the mean ± standard deviation of 5 different experiments.

RESULTS

Light and electron microscopy of slices from the head kidney of the Northern pike

In order to determine the types and relative amounts of different cells in the head kidney we performed light and electron microscopy on slices from this organ. Light microscopy demonstrated the presence of two different types of tissue, one of which is hemopoetic, resembling tissue found in the trunk kidney of the Northern pike [15] and constituting approximately 80% of the head kidney; while the other tissue resembles mammalian adrenal tissue and constitutes the remaining 20% of the head kidney of the pike (Fig. 2; see also the electron micrographs). The hemopoetic tissue is also seen to be much "looser", with fewer connections between individual cells than the other tissue, which may mean that the hemopoetic cells are more difficult to disrupt by usual homogenization procedures.

The electron micrographs reveal that pike head kidney cells homologous to the mammalian adrenal contain large numbers of mitochrondria with a characteristic morphology [36], as do adrenal cortex cells, whereas the blood-forming tissue contains relatively much fewer mitochondria with a more usual appearance (Fig. 3). Since less than 25% of the total cytochrome oxidase activity of the head kidney homogenate is recovered in the nuclear fration (see below), most of the adrenal-like cells must have been disrupted by the homogenization procedure utilized here. Both types of cells in the pike head kidney can be seen to contain endoplasmic reticulum, so that the microsomal ($184{,}000\,g_{av}$) pellet obtained here originated from both tissue types.

Subcellular distribution of different markers

Figure 4 shows the distribution of various markers upon subcellular fractionation of the head 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, 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 the head kidney (see also 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, the liver of Northern pike and rainbow trout, and, in particular, the trunk kidney of the Northern pike. 78% of the total lactate dehydrogenase activity was recovered in the high-speed supernatant, indicating effective breakage of the cells by the homogenization procedure used. Furthermore, 92% of the glutathione transferase activity, which is chiefly soluble in rat [37] and pike liver [2] and in the trunk kidney of Northern pike [15], is also recovered in the high-speed supernatant from the head kidney. 78% of the total DNA is localized in the $600\,g_{av}$ pellet, indicating that this fraction contains most of the nuclei. Cytochrome oxidase is highly enriched in the $15{,}300\,g_{av}$ pellet (10.8-fold), indicating that this is a relatively pure mitochondrial fraction. The $15{,}300\,g_{av}$ pellet also contains the highest relative specific activities of markers for the Golgi apparatus and for lysosomes and of AMPase and glucose-6-phosphatase. Judging from the large enrichment of epoxide hydrolase activity in the $184{,}000\,g_{av}$ pellet, this fraction is highly enriched in fragments of the endoplasmic reticulum (see also below). Most of the catalase activity was recovered in the low speed pellet, suggesting that

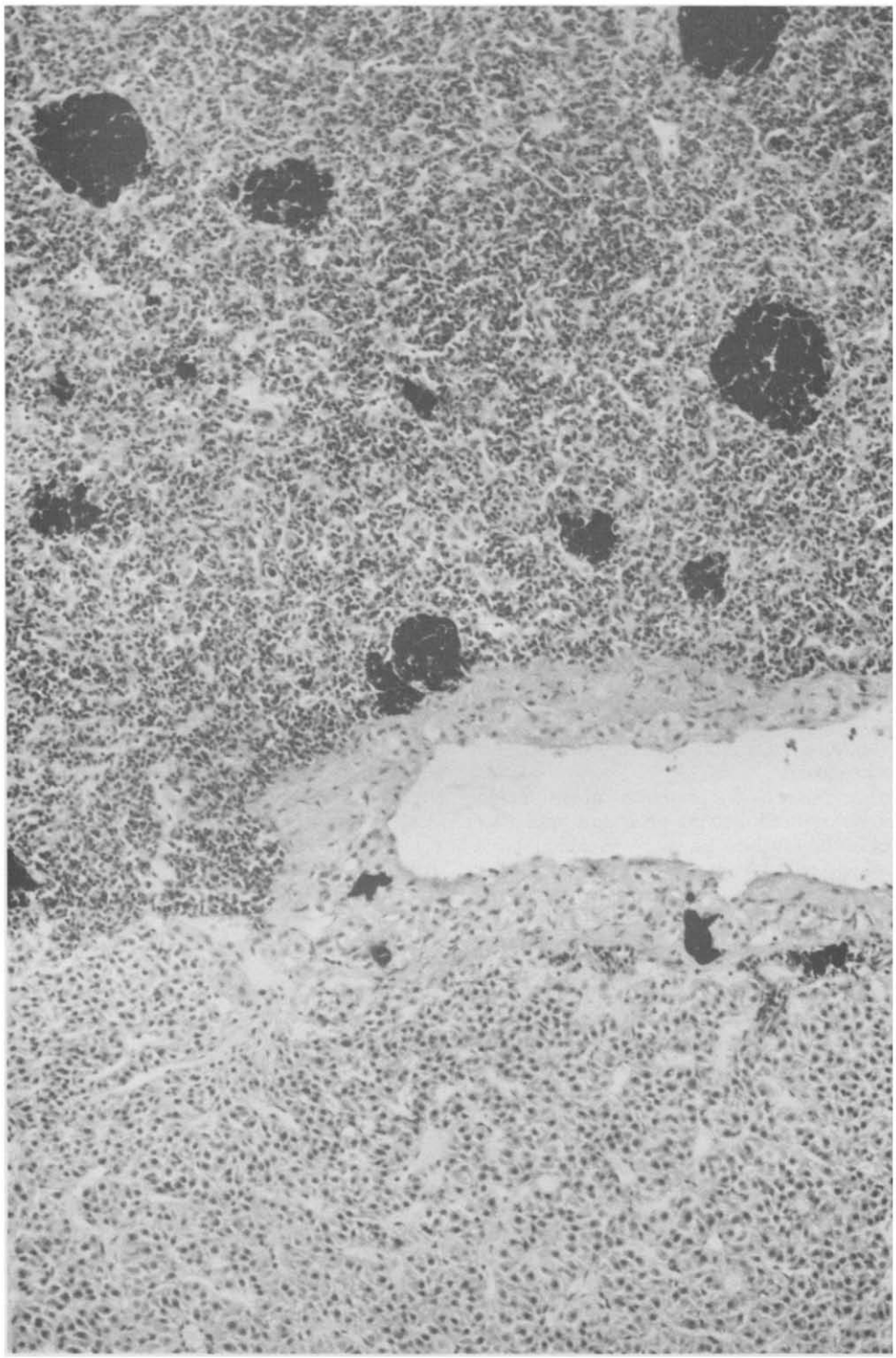


Fig. 2(A).

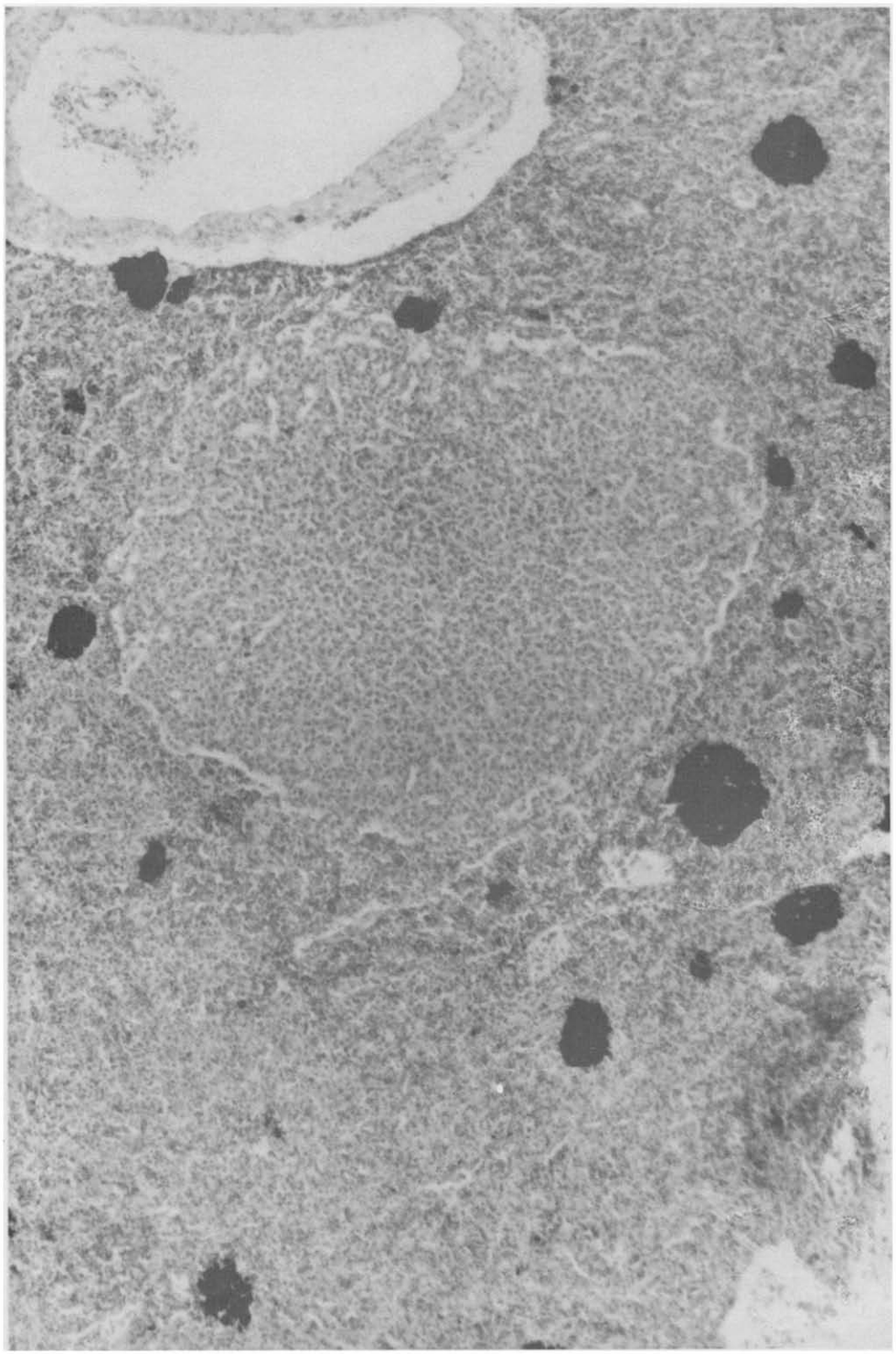


Fig. 2(B).

Fig. 2. Light microscopic picture of the head kidney of the Northern pike. (A) Blood-forming tissue, melanomacrophage centres localized in this hemopoetic tissue (over), and the lighter aggregates of cells homologous with the mammalian adrenal (under), can all be clearly seen. Magnification, 90×. (B) Another view illustrating an island of adrenal tissue in the blood-forming tissue. Magnification 70 ×.

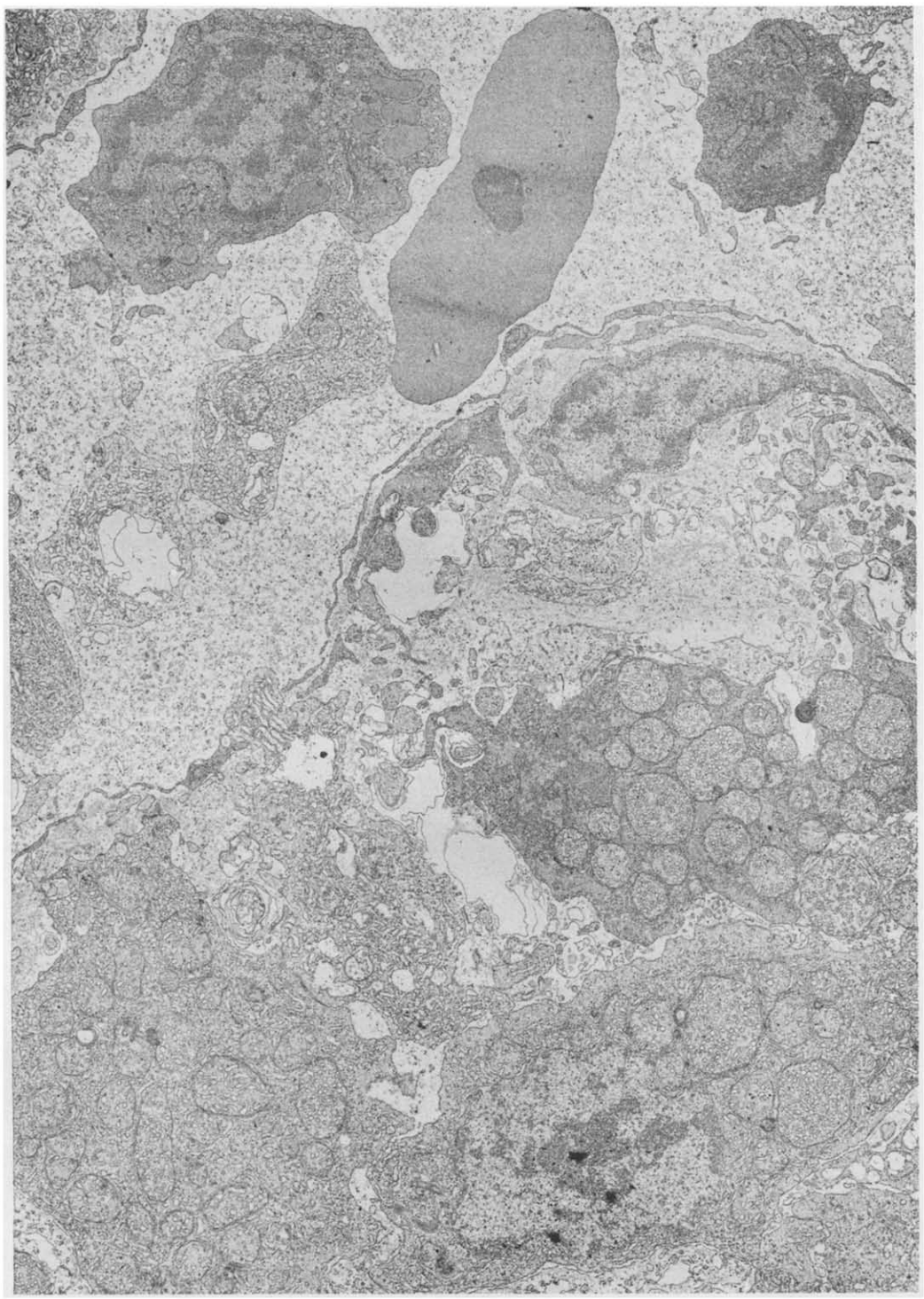


Fig. 3(A).

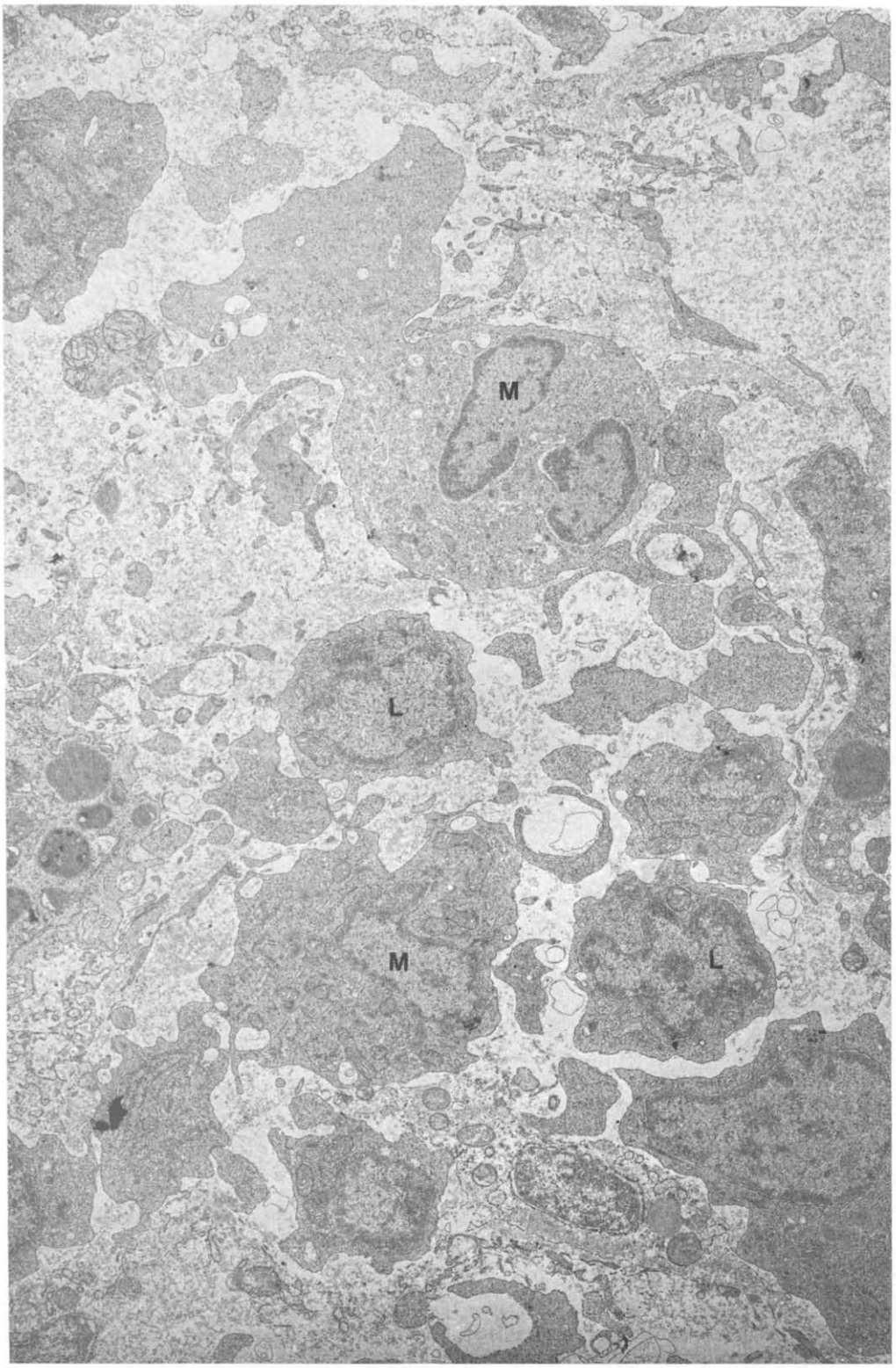


Fig. 3(B).

Fig. 3. Electron micrographs of the head kidney of the Northern pike. (A) To the lower-right in this picture cells homologous to mammalian adrenal cortex cells containing large numbers of mitochondria can be seen. Magnification, 8700 ×. (B) Blood-forming tissue with its "loose" organization, i.e. lack of cell contacts can be seen. Lymphocytes (L) and macrophages (M). Magnification, 6900 ×.

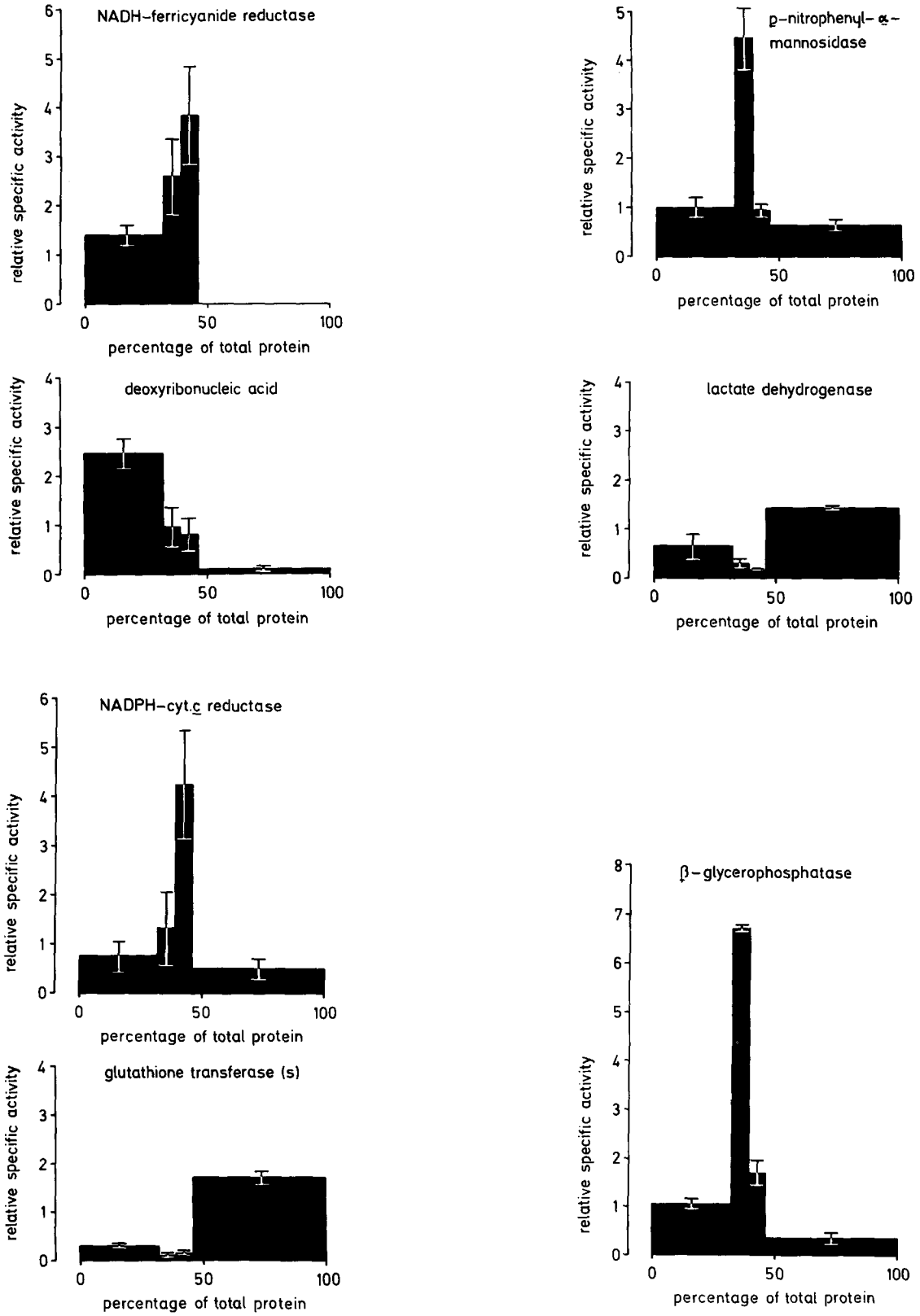
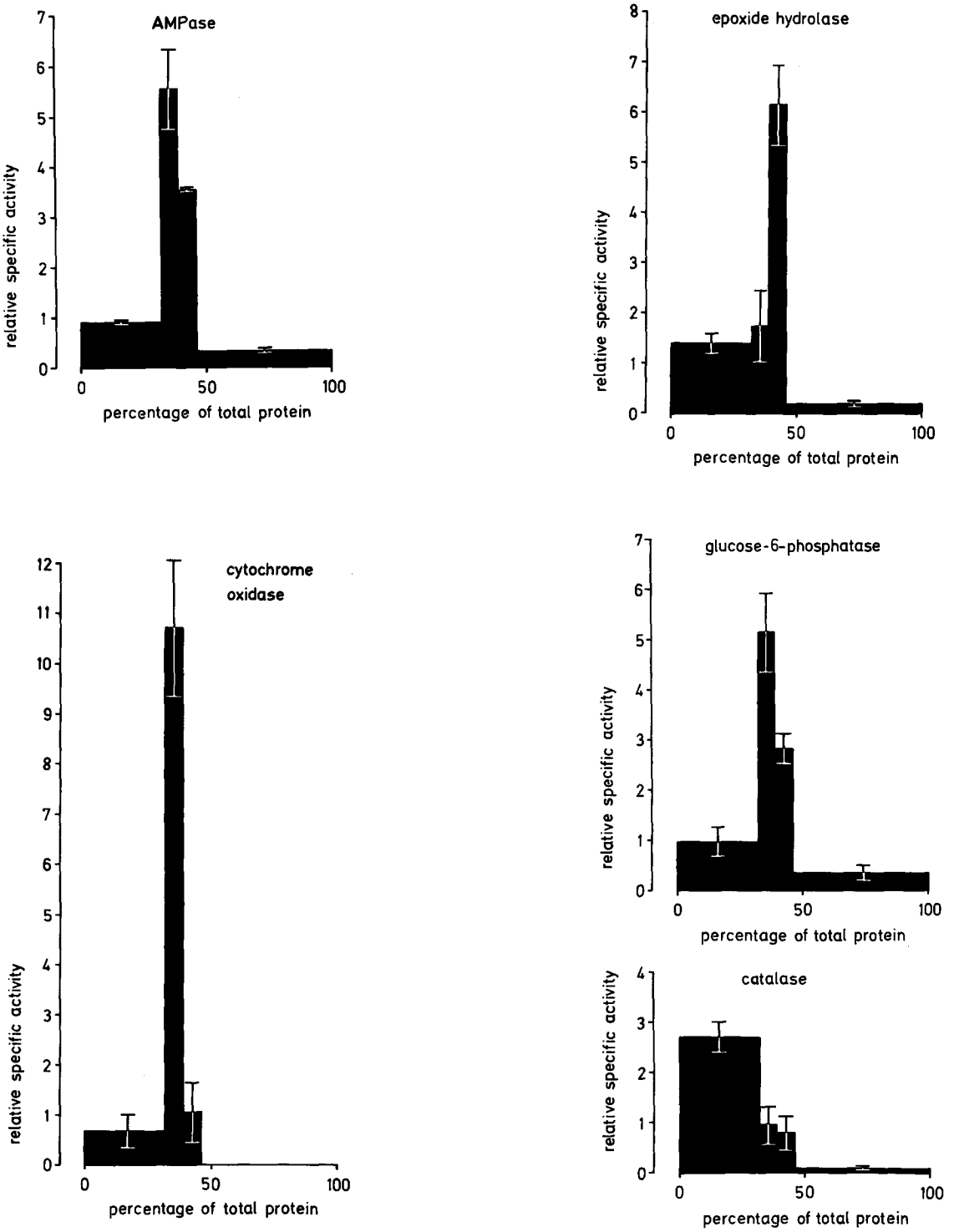


Fig. 4. The distribution of DNA, marker enzymes, and other enzymes after subcellular fraction of the head kidney of the Northern pike by differential centrifugation. The head kidney was homogenized and subfractionated and DNA and the various enzymes assayed as described in the Materials and Methods.



The results are expressed here as DeDuve plots [35] and the fractions (from left to right) are the 600 g_{av}, 15,300 g_{av}, and 184,000 g_{av} pellets and the 184,000 g_{av} 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 0.2 mg/mg protein; cytochrome oxidase 0.83 μmol/min-mg protein; *p*-nitrophenyl-α-mannosidase 12 nmol/min-mg protein; AMPase 51 nmol/min-mg protein; catalase 49 μmol/min-mg protein; β-glycerophosphatase 0.9 μmol/min-mg protein; lactate dehydrogenase 1.8 μmol/min-mg protein; NADPH-cytochrome *c* reductase 24 nmol/min-mg protein; glutathione transferase 35 nmol/min-mg protein; NADH-ferricyanide reductase 0.6 μmol/min-mg protein; glucose-6-phosphatase 44 nmol/min-mg protein; epoxide hydrolase 0.3 nmol/min-mg protein. One gram head kidney tissue contained about 81 mg protein.

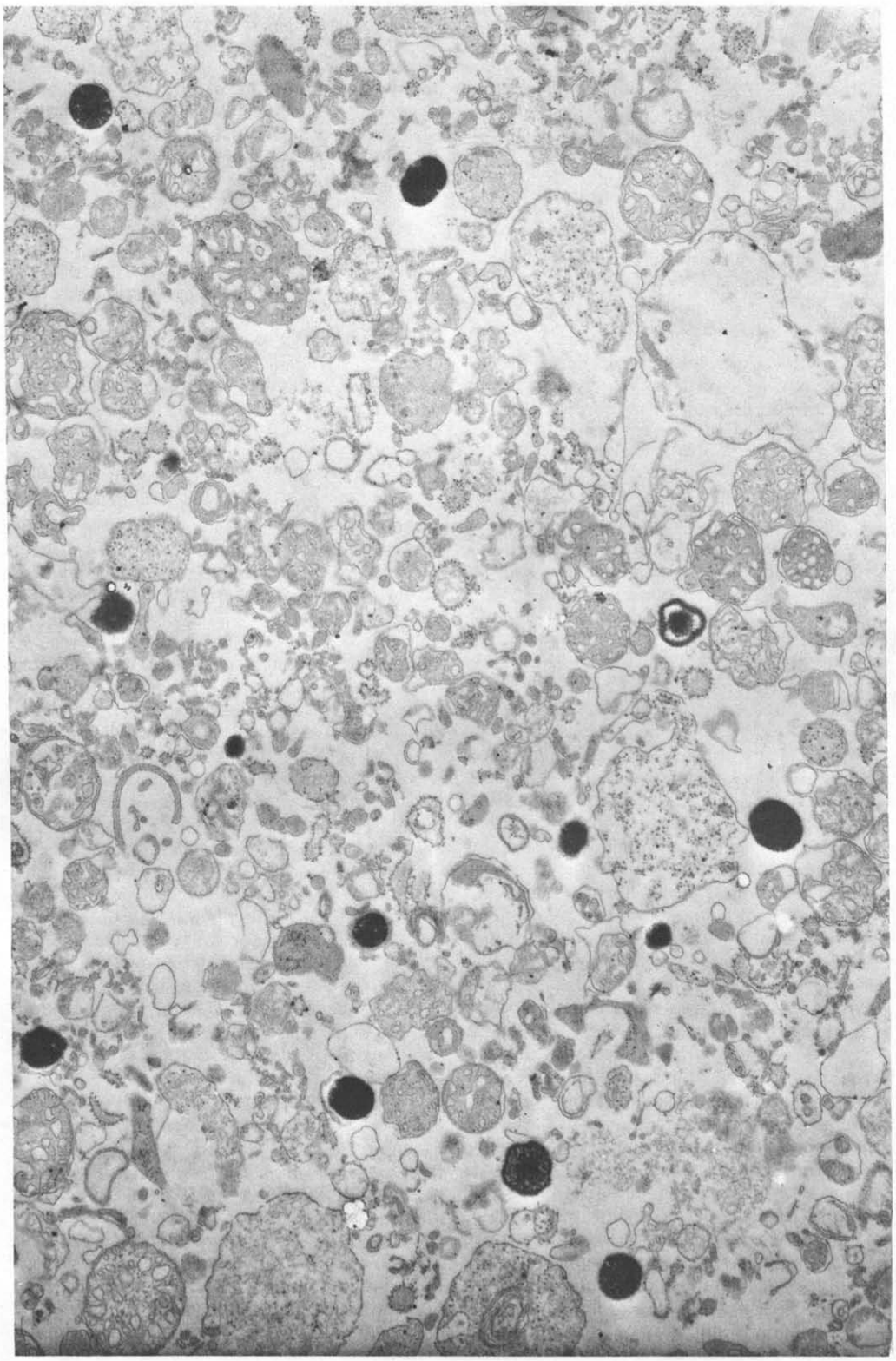


Fig. 5(A).

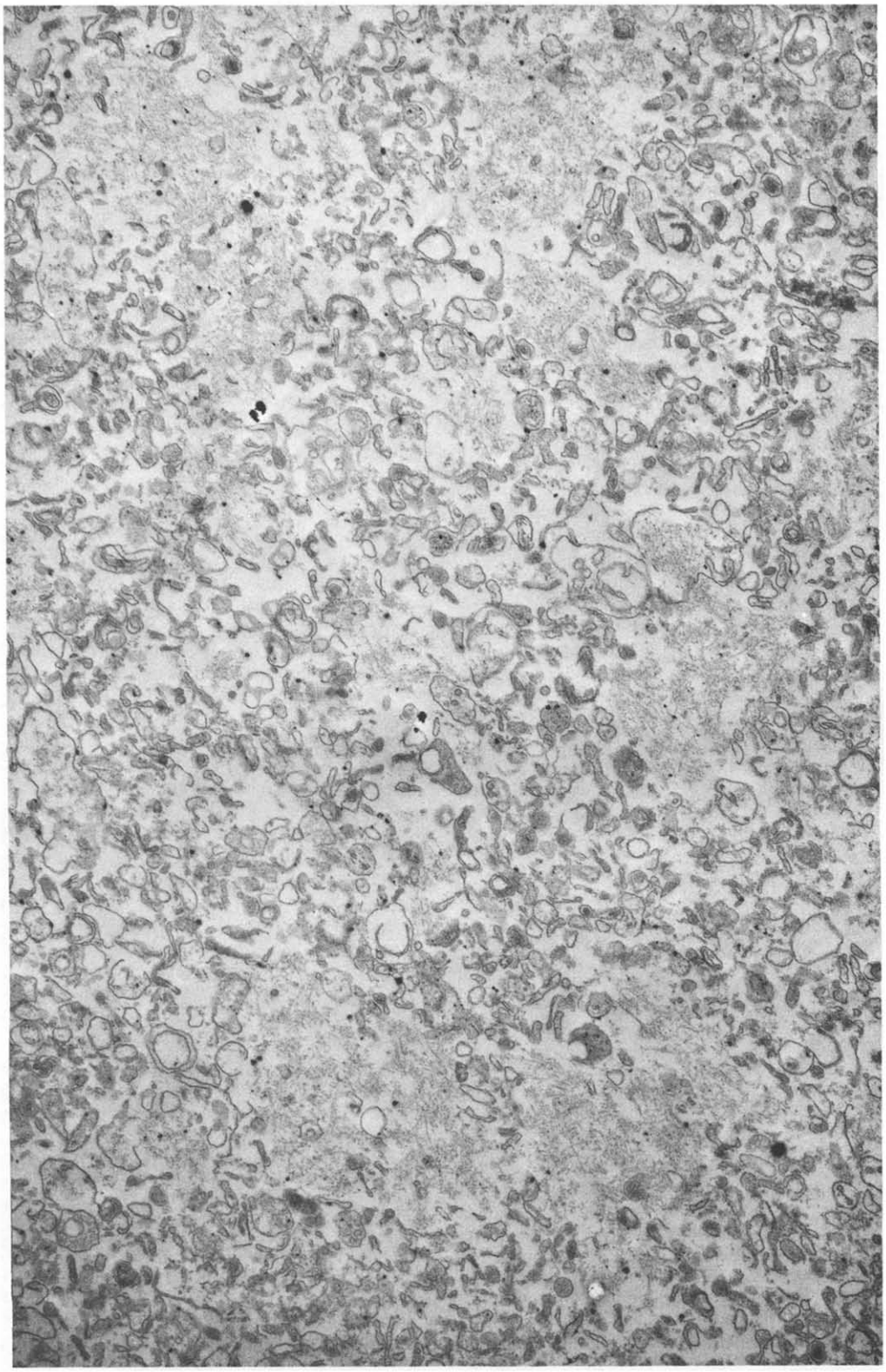


Fig. 5(B).

Fig. 5. Electron micrographis of the 15,300 g_{av} and 184,000 g_{av} pellets prepared from the head kidney of the Northern pike. (A) The 15,300 g_{av} pellet is seen to contain mainly mitochondria, a number of melanin granules, and other membrane profiles. Magnification, 17,500 ×. (B) The 184,000 g_{av} pellet contains no identifiable mitochondria, nuclei, or melanin granules, a large number of smooth membrane vesicles, and some amorphous material. Magnification, 26,600 ×.

peroxisomes cosediment with the nuclei or that this enzyme is not an appropriate marker for peroxisomes in this tissue.

Marker enzymes for the endoplasmic reticulum of the head 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 of the head kidney in Northern pike, since this activity is widely used as a marker for the endoplasmic reticulum in mammalian tissues. However, we found that, as in the case of the trunk kidney [15], a functional portion of this enzyme is apparently solubilized from fragments of the endoplasmic reticulum by a protease present in the homogenate. This protease activity (31.6 ± 5.6 μg tyrosine solubilized/min-mg protein) is about 6 times greater in homogenates of the head kidney than in homogenates of the rat adrenal gland and 72 times greater at 37° than at 0°. Trypsin inhibitor, which inhibits the protease activity about 20%, partially prevents the solubilization of NADPH-cytochrome *c* reductase and was therefore included routinely at a concentration of 1 mg/ml in the homogenate immediately after preparation. (This problem is discussed in more detail in our subcellular fractionation study of the trunk kidney of the Northern pike [15]).

This solubilization rendered NADPH-cytochrome *c* reductase somewhat unreliable as a marker for the endoplasmic reticulum and we decided to examine the distribution of other enzymes which are also localized on this organelle in rat liver—namely, glucose-6-phosphatase, NADH-ferricyanide reductase, and epoxide hydrolase activity towards *cis*-stilbene oxide. As can also be seen in Fig. 4, NADH-ferricyanide reductase and epoxide hydrolase are, like NADPH-cytochrome *c* reductase activity, most enriched in the 184,000 g_{av} pellet, whereas glucose-6-phosphatase is enriched most in the 15,300 g_{av} pellet.

Glucose-6-phosphatase is known to be a good marker for the endoplasmic reticulum only in hepatic tissue and its distribution here may indicate that it is not solely localized on the endoplasmic reticulum of cells in the pike head kidney. Another possibility is that rough endoplasmic reticulum was selectively enriched in the 15,300 g_{av} pellet here and that glucose-6-phosphatase is enriched in this subfraction of the endoplasmic reticulum. In order to test this possibility the distribution of RNA in our subfractions was measured in one experiment. This distribution—1.5-, 0.83-, 2.75- and 0.17-fold enrichment and 52%, 10%, 30% and 7% of the total amount in the 600 g_{av}, 15,300 g_{av} and 184,000 g_{av} pellets and the 184,000 g_{av} supernatant, respectively—closely resembled that seen for NADPH-cytochrome *c* reductase. Thus, the 15,300 g_{av} pellet was not selectively enriched in fragments of the rough endoplasmic reticulum.

Electron microscopy of the subcellular fractions

Electron micrographs of the 15,300 g_{av} and 184,000 g_{av} pellets revealed that the first of these consisted chiefly of mitochondria, but also contained melanin granules and other membrane profiles; whereas the high-speed pellet contained no identifiable mitochondria, nuclei, or melanin granules, a large number of smooth membrane vesicles (the endoplasmic reticulum in the fish adrenal cortex is predominantly free of ribosomes [36]) and some amorphous material (Fig. 5).

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 are all localized primarily in the 184,000 g_{av} pellet, which originates chiefly from the endoplasmic reticulum. Glutathione transferase activity toward 1-chloro-2,4-dinitrobenzene is, on the other hand, virtually all soluble.

The head kidney of the Northern pike contains rather large amounts of melanin, so that the resuspended 600 g_{av} and 15,300 g_{av} pellets are dark brown to black in colour. Consequently, it has not yet been possible to measure cytochromes P-450 and b_5 in these fractions. These cytochromes could not be clearly detected (cytochrome $b_5 \leq$ 9–56 pmole/mg protein and cytochrome P-450 $\leq$ 41 pmole/mg protein) either in 184,000 g_{av} pellet or in the high-speed supernatant, both of which appear to be free of melanin.

DISCUSSION

It is clear from the present investigation that the distribution of various presumptive markers and of several drug-metabolizing enzymes in the head kidney of the Northern pike after subcellular fractionation by differential centrifugation resembles in general the corresponding distribution of these same markers in rat liver and is almost identical to the corresponding pattern observed with the trunk kidney of Northern pike [15]. Both the head and trunk kidneys of the Northern pike differ from rat liver in that a larger portion of the NADPH-cytochrome *c* reductase is recovered in the high-speed supernatant from the pike tissues (presumably due to solubilization from the endoplasmic reticulum by an endogenous protease). The head kidney of the Northern pike differs from both the trunk kidney and the rat liver in that glucose-6-phosphatase is most highly enriched in the 15,300 g_{av} ("mitochondrial") pellet from head kidney tissue. The findings presented here also support our earlier conclusion—based on studies with the livers of mice, rats, rabbits, hamsters, and guinea pigs (J. Meijer, manuscript in preparation); and the trunk kidney of the Northern pike—that epoxide hydrolase activity towards *cis*-stilbene oxide is a good marker for the endoplasmic reticulum.

Since one of the major goals was to prepare fractions suitable for the study of drug-metabolizing systems in the head kidney of the Northern pike, the purity and recovery of fragments of the endoplasmic

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

Enzyme	% of the total homogenate activity recovered in the microsomal fraction prepared from: rat*† liver	pike‡ liver	pike§ trunk kidney	pike‖ head kidney
DNA	2	2.5	2.8	5.6
Markers for mitochondria:				
cytochrome oxidase	2.6	6.5	6.5	7.4
succinate-cytochrome *c* reductase	0.7	—	—	—
Markers for lysosomes:				
β-glycerophosphatase	11	21	26	11.9
Markers for Golgi apparatus:				
p-nitrophenyl-α-mannosidase	—	13	6	6.7
Markers for peroxisomes:				
catalase	9	5.3	7.5	5.6
Markers for the plasma membrane:				
AMPase	29	9.9	28	25
alkaline phosphatase	—	—	38	27
Markers for the endoplasmic reticulum:				
NADPH-cytochrome *c* reductase	40	35	24	30
glucose-6-phosphatase	40	—	32	—
NADH-ferricyanide-reductase	—	—	29	27
epoxide hydrolase	—	—	53	43
Markers for the cytosol:				
lactate dehydrogenase	—	3	1	1

* From ref. 31.
† Cytochrome oxidase from ref. 40.
‡ From ref. 2.
§ From ref. 15.
‖ From the present study.

reticulum in our microsomal (184,000 g_{av}) fraction is of particular interest. In Table 1 the microsomal fractions from rat and pike liver and pike head and trunk kidney are compared. It can be seen that recovery of fragments of the endoplasmic reticulum and contamination by other organelles is approximately the same in all of these microsomal preparations and that the 184,000 g_{av} pellet prepared here seems to be well-suited for studies of drug-metabolizing enzymes localized to the endoplasmic reticulum.

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 obtained with our procedure are suitable for further characterization of drug-metabolizing systems in the head kidney of the Northern pike, as well as for other studies with this tissue. Of central interest for us at present are the relationship between steroid and xenobiotic metabolism in this tissue and the possible relationship between xenobiotic metabolism in the head kidney of the Northern pike and the high frequencies of malignant lymphomas reported in these animals [6, 38, 39].

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