Biochemical Pharmacology, Vol. 26, pp. 1395–1400. Pergamon Press, 1977. Printed in Great Britain.

FRACTIONATION AND SUBCELLULAR LOCALIZATION OF MARKER ENZYMES IN RAINBOW TROUT LIVER*

CHARLES N. STATHAM,† S. PATTY SZYJKA, LAWRENCE A. MENAHAN and JOHN J. LECH‡

Department of Pharmacology, Medical College of Wisconsin, Milwaukee, WI 53233, U.S.A.

(*Received* 5 *August* 1976; *accepted* 10 *December* 1976)

Abstract—The subcellular fractionation of rainbow trout liver homogenates prepared in 0.25 M sucrose was investigated using marker enzymes to assess the homogeneity of the resulting fractions. In addition to the usual mitochondrial and microsomal fractions, an additional fraction was sedimented between 8000 *g* for 10 min and 13,300 *g* for 10 min. Of the four accepted hydrolytic "marker" enzymes for rat liver lysosomes, the high relative specific activity (R.S.A.) of acid phosphatase was indicative of enrichment in this fraction. The R.S.A. patterns of 5′-nucleotidase and alkaline phosphatase indicated that the plasma membranes of fish liver were sedimenting with "nuclear" and microsomal pellets. This latter fraction contained the highest percentage of the total glucose 6-phosphatase, benzopyrene hydroxylase and glucuronyl transferase assayed in the fish liver homogenate before fractionation. The R.S.A. of these same enzymes in the microsomal pellet indicated an enrichment in this fraction relative to other cellular fractions.

In recent years, studies concerning the biotransformation and fate of foreign compounds in fish have become increasingly important in view of the identification of a variety of organic pollutants in the aquatic environment [1, 2]. Although early studies indicated that fish do not metabolize foreign compounds, it has been demonstrated in numerous investigations, both *in vivo* and *in vitro*, that many species of fish have the ability to carry out the biotransformation of xenobiotics [2–7]. The biotransformation reactions which have been described in fish are similar to those which have been extensively studied in mammals and insects and include microsomal hydroxylation, dealkylation, reduction and conjugation [8–11]. As in mammals and insects, the microsomal mixed-function oxidase system in fish is thought to be inducible by certain organic compounds, and increases in the level of benzopyrene hydroxylase have been correlated with water pollution by petroleum components [12–14]. Although techniques for the separation of rat liver subcellular fractions have been extensively studied [15], very little has been done to describe or standardize a method for fractionation of fish liver. In view of the increasing interest in fish liver microsomal enzymes and the fact that there are subtle anatomical differences between trout liver and rat liver [16], a study was undertaken to investigate the optimal conditions for the subcellular fractionation of rainbow trout liver. This report describes the separation and marker enzyme profiles of rainbow trout liver subcellular fractions.

*This work was supported by NIH Grant ES01080 and EPA Grant R803971010.

† Presently a Staff Fellow in the Pharmacology Research Associate Program of the National Institute of General Medical Sciences, National Institutes of Health, Bethesda, MD. 20014.

‡ Recipient of a Research Career Development Award (ES00002).

MATERIALS AND METHODS

Rainbow trout (*Salmo gairdneri*), weighing 80–100 g, were purchased from Kettle Moraine Springs Trout Hatchery, Adell, WI, and they were held in flowing dechlorinated water at 12° with a 12-hr light:dark photo period for a minimum of 1 week prior to use.

All substrates and cofactors needed for the enzyme assays described below were obtained from Sigma Chemical Co., St. Louis, MO, with the exception that 5′-AMP was obtained from PL Biochemicals, Milwaukee, WI, and Fraction V bovine plasma albumin from Rheis Chemical Co., Kankakee, IL. 3-Trifluoromethyl-4-nitrophenol was obtained from Mr. John Howell, U.S. Department of the Interior, Hammond Bay, MI. Radioactive 3-trifluoromethyl-4-nitrophenol (^{14}C ring u.l.), sp. act. 3.7 mCi/m-mole, was obtained from the Mallinckrodt Chemical Co., St. Louis, MO, [7,10-^{14}C]benzo[α]pyrene, sp. act. 51 mCi/m-mole, from Amersham/Searle, Des Plaines, IL, and [8-^{3}H]-5′-AMP, sp. act. 10 Ci/m-mole, from New England Nuclear, Boston MA. Radioactive substrates were purified by thin-layer chromatography before use.

All other chemical reagents and solvents used in the assays described below were of the highest commercial quality available.

Preparation of subcellular fractions. The procedure for the preparation of fish liver subcellular fractions is summarized in Fig. 1. Fish were sacrificed by cervical dislocation. The livers were removed, minced and then subjected to the homogenization and fractionation procedures listed in Fig. 1. One g of fish liver was assumed to have a volume of 1 ml of 0.25 M sucrose and was homogenized in 4 vol. of 0.25 M sucrose. All operations were carried out at 4°. Each particulate fraction was suspended in 0.25 M sucrose in a volume which was $\frac{1}{6}$ of the starting homogenate and repelleted at the same *g* force. This

second supernatant was then combined with the original supernatant obtained for the respective fraction and subjected to the next higher g force to obtain the subsequent fraction. All particulate fractions were finally suspended in a volume of 0.25 M sucrose equal to that of the starting homogenate. All membranous (including starting homogenate) fractions were also sonified with a Branson sonifier cell disruptor model W140 D (20,000 Hz, 40 W) amplitude setting of 6, fitted with a microtip for three (10-sec) periods to give a more homogenous suspension. Between each sonication period, a 30-sec period of cooling at 4° was introduced to prevent rises in temperature.

Assay of enzymatic activities. Succinic dehydrogenase activity was measured using a reaction mixture containing 10 mM phosphate (pH 7.4), 10 mg bovine plasma albumin, 1 mM KCN, and 1 mg horse heart cytochrome c [17] in a final volume of 0.9 ml. After recording the baseline at room temperature, the reaction was initiated by adding 0.1 ml of 50 mM sodium succinate, and the change in absorbance was measured at 550 nm with a 1 cm path in a spectrophotometer (Gilford) with a recorder.

Acid phosphatase was assayed using p-nitrophenyl phosphate as substrate [15]. The β-glucuronidase activity was measured with phenolpthalein glucuronide as substrate [15]. Acid phosphatase, β-glucuronidase and all enzyme assays described below were carried out at 25°. This is considered the optimum temperature for many of the enzymatic activities of fish liver [4] rather than 37°, which is a usual temperature for assaying many of the enzyme activities of mammalian preparations. After the acid phosphatase and β-glucuronidase assays were stopped, respectively, by the addition of 1.0 ml of 2.0 M glycine (pH 10.7) and 1.5 ml of 0.13 M glycine, 60 mM NaCl and 80 mM Na_2CO_3 (pH 10.7), the reaction tubes were centrifuged at approximately 30,000 g for 10 min before reading the absorbance at 420 nm.

N-acetyl-β-glucosaminidase activity was measured utilizing p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate [18]. Similar to the assays described for acid phosphatase and β-glucuronidase, the reaction tubes, after stopping the assay with 2 M NH_4OH (pH 10.7), were centrifuged at approximately 30,000 g for 10 min before reading the absorbance at 420 nm.

Cathepsin D activity [15] was assayed using a final concentration of 0.5% hemoglobin as substrate. After the reaction was stopped with 1 ml of 5% trichloroacetic acid (TCA), the samples were centrifuged at approximately 30,000 g for 10 min and the absorbance was read at 280 nm.

Alkaline phosphatase was assayed by incubating the enzyme preparation in a final volume of 0.5 ml containing 0.1 M ethanolamine (pH 9.5), 1.5 mM sodium fluoride and 5 mM p-nitrophenyl phosphate at 25° for 30 min. After terminating the reaction with 1 ml of 2 M glycine (pH 10.7), the samples were centrifuged at approximately 30,000 g for 10 min before reading the absorbance at 420 nm.

The 5′-nucleotidase assay was carried out at 25° with a reaction mixture containing 50 mM TES [sodium N-tris (hydroxylmethyl) methyl-2-aminoethane sulfonic acid] (pH 7.5), 1 mM EDTA, 5 mM $MgCl_2$ and 0.1 mM [^{3}H]-5′-AMP containing approximately 50,000 cpm in a final volume of 250 μl. After the incubation period (20 min), the reaction was terminated by boiling for 1 min. The [^{3}H]adenosine was then separated from the labeled substrate on a small 4.5-cm column of DEAE-Sephadex A-25, contained within a Pasteur pipette. A similar "pencil" column technique has been described for the separation of labeled adenosine from cyclic AMP, in the assay of cyclic nucleotide phosphodiesterase [19]. The [^{3}H]-5′-AMP, eluted with 3.0 ml of 50 mM Tris–HCl (pH 7.5), was counted in a Packard liquid scintillation spectrometer (model 3310) with 10 ml of a premixed scintillation solution (ScintiVerse, Fisher Scientific Co.). An aliquot of the assay mixture containing [^{3}H]-5′-AMP was counted in 3.0 ml of 50 mM Tris–HCl under exactly the same conditions as the assay samples; thus, no quench correction was necessary.

Rotenone-insensitive NADH or NADPH-cytochrome c reductase was assayed in the presence of 1.5 μM rotenone, and the reduction of cytochrome c at 550 nm was followed [20]. Rotenone for the enzyme assay was prepared by the drop-wise addition of 200 μl of 0.225 M rotenone in dioxane into 10 ml of 10% albumin, and 0.1 ml of this mixture was used in the assay to give a final rotenone concentration of 1.5 μM.

Glucose 6-phosphatase activity was assayed at pH 6.0 by measuring the inorganic phosphate released from glucose 6-phosphate in a reaction mixture containing KF and EDTA to minimize the interference in the assay by acid and alkaline phosphatase activities [21]. After the reaction was stopped with 10% TCA, the samples were centrifuged at approximately 30,000 g for 10 min, and an aliquot of the clear supernatant was assayed for inorganic phosphate with ascorbic acid and ammonium molybdate exactly as described by Ames [22].

UDPGA–glucuronyl transferase (indicated subsequently as glucuronyl transferase) was assayed at 25° with a reaction mixture containing 100 mM sodium phosphate (pH 7.0), 0.2 mM $MgCl_2$, 5 mM saccharo-1,4-lactone, 0.5 mM UDPGA, and 0.5 mM [^{14}C]-3-trifluoromethyl-4-nitrophenol containing approximately 3.0×10^5 cpm in a final volume of 1.0 ml. The reaction was stopped after 10 min with 0.2 ml of 10% TCA, which was followed by the addition of 1.0 ml water to each tube. Extraction of unreacted labeled substrate was done with 4 ml of benzene–ether (1:1, v/v) using a 10-min shaking time with a mechanical shaker after which the upper phase was removed by aspiration. This was repeated an additional two times. An aliquot (0.5 ml) of the lower aqueous phase was counted in 15 ml of ACS scintillation mixture (Amersham/Searle). A reaction tube without added UDPGA was assayed for each cellular function and the radioactivity found under these assay conditions was subtracted from that found in the presence of UDPGA.

The benzopyrene hydroxylase assay, used in the present study, was based on the assay initially described by Hansen and Fouts [23]. The reaction mixture contained 0.1 M Tris–HCl (pH 7.4), 5 mM glucose 6-phosphate, 2 units glucose 6-phosphate dehydrogenase, 1 mM NADP, 5 μM [^{14}C]benzo[α]pyrene containing approximately 10^5 cpm and enzyme in a final volume of 2.5 ml. The reaction was stopped

after 5 min with 1.0 ml of cold acetone and placed in an ice bath (4°). Hexane (5.0 ml) was added to each sample and the mixture was shaken for 20 min. Then 2 N NaOH (2.5 ml) was added to each tube and the shaking was continued for additional 20 min. The samples were then centrifuged at 2500 rev/min with an IEC centrifuge for 10 min, the upper (hexane) layer was aspirated off, and a 1.0-ml aliquot of the aqueous layer was counted in a liquid scintillation spectrometer with 15 ml of ACS scintillation mixture in the presence of 100 μl of glacial acetic acid to minimize chemiluminescense.

Lactic dehydrogenase activity was assayed with a reaction mixture containing 50 mM TES, 1 mM dithiothreitol and 0.2 mM NADH. After recording the baseline, the reaction was initiated with 0.1 ml of 0.1 M pyruvate and the change in absorbance was measured at 340 nm.

Protein was determined by the method of Ross and Schatz [24] using crystalline bovine plasma albumin as the standard.

Expression of enzymatic activity. The enzymatic activity, described throughout the present investigation, has been calculated as relative specific activity, which is the per cent of total activity divided by the per cent of total protein in the respective fraction [15].

Although not included in the data presented, enzymatic or protein analyses were performed on the total homogenate for each respective subcellular distribution carried out. In most instances, the summation of the enzyme activity or protein in the respective subcellular fractions closely approximated that found in the starting homogenate.

RESULTS

Although the scheme described in Fig. 1 was finally adapted to fractionate rainbow trout liver into subcellular fractions, preliminary experiments were carried out in an attempt to optimize conditions for a more definitive separation of "marker" enzymes which are characteristic of the various subcellular components. Initially, a low speed spin at 120 g for 5 min was included in the scheme described in Fig. 1 to remove intact cells etc., but this resulted in variable distribution profiles, particularly for the plasma membrane markers, e.g. alkaline phosphatase and 5'-nucleotidase. Thus, in all the distribution profiles reported in the present work, trout liver homogenates were centrifuged at 600 g for 20 min to obtain the nuclear and cellular debris pellet (Fig. 1).

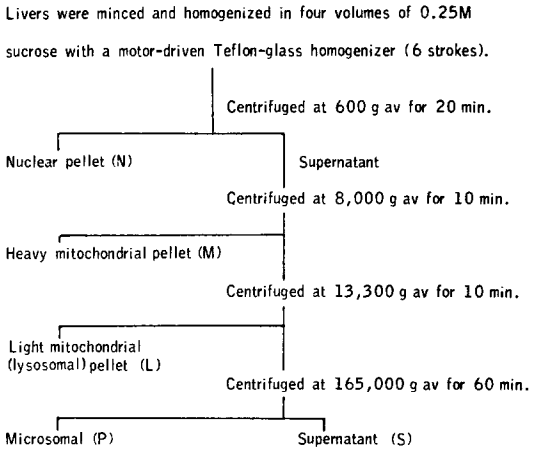

Fig. 1. Scheme for fractionation of rainbow trout liver.

Since the lysosomes are particularly important in protein and lipid degradation in mammalian liver, an attempt was made to optimize centrifugation conditions for a more definitive separation of the hydrolytic enzymes in trout liver homogenates. Lowering of the force of the mitochondrial spin or increasing that of the microsomal spin resulted in a lowered yield (per cent of recovery) of the mitochondrial or microsomal marker enzymes in their respective pellets without a significant increase of the relative specific activity (R.S.A.) of acid phosphatase in the light mitochondrial or lysosomal fraction.

Thus, in the standard fractionation scheme (Fig. 1), the post-mitochondrial fraction was centrifuged at 13,300 g for 10 min to obtain the light mitochondrial or lysosomal pellet. The subcellular distribution of four hydrolytic or lysosomal marker enzymes in trout liver fractionated according to the scheme described in Fig. 1 was studied and the results are reported

Table 1. Distribution of hydrolytic enzymes of rainbow trout liver*

	Nuclear pellet	Mitochondrial pellet	Lysosomal pellet	Microsomal pellet	Supernatant 165,000 g_{av}
Acid phosphatase (6)					
Recovery (%)	14.2	16.2	13.9	45.7	9.3
R.S.A.	0.8	1.3	1.8	2.1	0.2
β-Glucuronidase (4)					
Recovery (%)	25.2	27.2	9.7	15.9	21.9
R.S.A.	1.7	2.3	1.3	0.8	0.5
N-acetyl-β-glucosaminidase (5)					
Recovery (%)	26.7	25.6	10.6	11.5	25.5
R.S.A.	2.1	1.8	1.2	0.6	0.6
Cathepsin D (1)					
Recovery (%)	47.2	14.4	12.8	18.3	7.2
R.S.A.	2.0	1.1	1.9	0.9	0.2

* Figures in parentheses indicate the number of experiments in which the enzymatic analysis was performed and the values shown are averages of the indicated number of experiments. Relative specific activity (R.S.A.) = per cent of total enzymatic activity/per cent of total protein.

Table 2. Enzymatic and protein analysis of subcellular fractions of rainbow trout liver*

	Nuclear pellet	Mitochondrial pellet	Lysosomal pellet	Microsomal pellet	Supernatant 165,000 g_{av}
Alkaline phosphatase (3)					
Recovery (%)	36.2	4.0	3.0	36.5	20.2
R.S.A.	2.0	0.3	0.4	1.7	0.4
5'-Nucleotidase (3)					
Recovery (%)	47.8	6.0	4.1	31.4	10.5
R.S.A.	2.4	0.6	0.6	1.4	0.3
Succinic dehydrogenase (6)					
Recovery (%)	16.2	67.6	16.2	0	0
R.S.A.	0.6	6.5	4.0		
Acid phosphatase (6)					
Recovery (%)	14.2	16.2	13.9	45.7	9.3
R.S.A.	0.8	1.3	1.8	2.1	0.2
Glucose 6-phosphatase (6)					
Recovery (%)	12.1	12.0	10.5	64.5	0.9
R.S.A.	0.6	1.1	1.6	2.9	
Lactate dehydrogenase (3)					
Recovery (%)	7.9	2.0	1.8	2.6	85.6
R.S.A.	0.4	0.2	0.3	0.1	2.2
Protein (8)					
Recovery (%)	20.3	11.8	7.5	22.8	37.5

* Figures in parentheses indicate the number of experiments in which the enzymatic and protein analyses were performed and the values shown are the average of the indicated number of experiments. Relative specific activity (R.S.A.) = per cent of total enzymatic activity/per cent of total protein.

in Table 1. It is clear from these results that there is heterogeneity both in the per cent of recovery and relative specific activity of the four hydrolytic enzymes in the various subcellular fractions. β-Glucuronidase and *N*-acetyl-β-glucosaminidase yielded similar distribution profiles with most of the particulate activity in the nuclear and mitochondrial pellets, but with little enrichment in the lysosomal fraction relative to the mitochondrial fraction. Almost 50 per cent of cathepsin D activity was recovered in the nuclear fraction. The R.S.A. of the enzyme was high and of similar value in both the nuclear and lysosomal pellets, indicating enrichment in both fractions. Acid phosphatase, a hydrolytic enzyme used most often in monitoring lysosomal enrichment in mammalian liver fractionation schemes, yielded R.S.A. values which were similar for both the lysosomal and microsomal pellet, indicating a similar enrichment of this enzyme in both fractions. Since one of the goals of the present work was to explore the distribution profile of certain drug-metabolizing enzymes in trout liver, which are known to reside in the microsomal fraction of mammalian liver, further efforts in delineating a lysosomal fraction in trout liver were not made.

A full enzymatic characterization of subcellular fractions obtained by the scheme outlined in Fig. 1 was then undertaken and these results are summarized in Table 2. Approximately 70 per cent of the succinic dehydrogenase was located in the pellet obtained by centrifuging the post-nuclear supernatant at 8000 *g* for 10 min. The R.S.A. of succinic dehydrogenase of this fraction was 6.5 and the R.S.A. of the lysosomal fraction was 4.0, indicating some contamination of the lysosomal fraction by mitochondria.

Alkaline phosphatase and 5'-nucleotidase, markers of plasma membranes in mammalian liver [25], yielded almost identical distribution profiles. Both the per cent of recovery and R.S.A. values for these enzymes suggest that plasma membranes of trout liver, under the homogenization conditions described, were localized mainly in the nuclear and microsomal pellets (Table 2).

Since one of the goals was to investigate the subcellular distribution profile of certain drug-metabolizing enzymes in trout liver, of particular importance was the distribution of microsomal marker enzymes in subcellular fractions of trout liver prepared according to scheme outlined in Fig. 1. Glucose 6-phosphatase, a gluconeogenic enzyme found in the microsomal fraction of mammalian liver [25], was found to the extent of approximately 65 per cent in the microsomal fraction of trout liver (Tables 2 and 3). There was a definite enrichment of this enzyme in the microsomal fraction as indicated by the high R.S.A. of this fraction.

Microsomal contamination of the high speed supernatant was low, but approximately 10–20 per cent of the 5'-nucleotidase and alkaline phosphatase plasma membrane markers were found in the 165,000 g_{av} supernatant (Table 2). Assay for lactate dehydrogenase indicated that approximately 85 per cent of this enzyme was recovered in the high speed supernatant, indicating minimal contamination of the membranous fractions by cytosolic material.

Since Table 2 is a composite of data accumulated over several experiments, the results from a single subcellular distribution in which all the enzymes and protein content were measured are presented in Fig. 2. A comparison of the relative specific activities of the marker enzymes from this individual fractionation with those presented in Table 2 indicates excellent agreement.

Having characterized the fractionation scheme described in Fig. 1 with marker enzymes, the subcellular distribution of glucuronyl transferase and benzopyrene hydroxylase in trout liver was explored. The

Table 3. Distribution of microsomal enzymes of rainbow trout liver*

	Nuclear pellet	Mitochondrial pellet	Lysosomal pellet	Microsomal pellet	Supernatant 165,000 g_{av}
Glucose 6-phosphatase (6)					
Recovery (%)	12.1	12.0	10.5	64.5	0.9
R.S.A.	0.6	1.1	1.6	2.9	
Rotenone-insensitive cytochrome *c* reductase NADH (3)					
Recovery (%)	14.1	16.0	12.4	57.2	0.2
R.S.A.	0.7	1.4	2.1	2.5	0.1
NADPH (2)					
Recovery (%)	13.4	12.6	15.0	55.2	3.8
R.S.A.	0.7	1.2	3.0	2.2	0.1
Glucuronyl transferase (2)					
Recovery (%)	17.8	9.1	8.6	63.5	0.1
R.S.A.	0.8	0.9	1.4	2.5	1.0
Benzopyrene hydroxylase (2)					
Recovery (%)	15.9	5.2	5.2	63.3	10.5
R.S.A.	0.8	0.5	0.7	2.5	0.3

* Figures in parentheses indicate the number of experiments in which the enzymatic analysis was performed and the values shown are averages of the indicated number of experiments. Relative specific activity (R.S.A.) = per cent of total enzymatic activity/per cent of total protein.

results of several distribution experiments focusing on microsomal enzymes are presented in Table 3. Glucose 6-phosphatase, glucuronyl transferase and benzopyrene hydroxylase had the highest R.S.A. and per cent recovery in the microsomal fraction. Similar distribution profiles and R.S.A. were obtained with rotenone-insensitive cytochrome *c* reductase, another microsomal marker enzyme. However, with NADPH as cofactor, the high R.S.A. of the rotenone-insensitive cytochrome *c* reductase in the lysosomal fraction indicated significant contamination of this fraction by these enzymes. This was not surprising in light of the report by Sottocasa *et al.* [20] that this enzyme is also located in the outer mitochondrial membrane.

In Fig. 3 the results of one such distribution experiment are illustrated. The relative specific activity profiles of glucuronyl transferase and benzopyrene hydroxylase are almost superimposable with that of glucose 6-phosphatase, a microsomal marker in mammalian liver. Recovery of glucuronyl transferase and

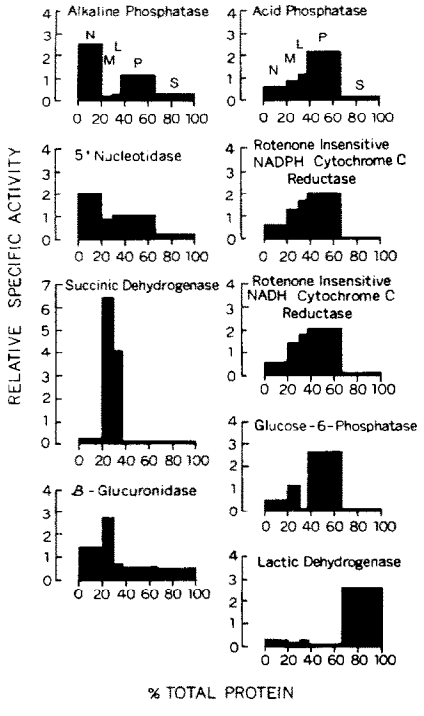

Fig. 2. Distribution of marker enzymes from trout liver.

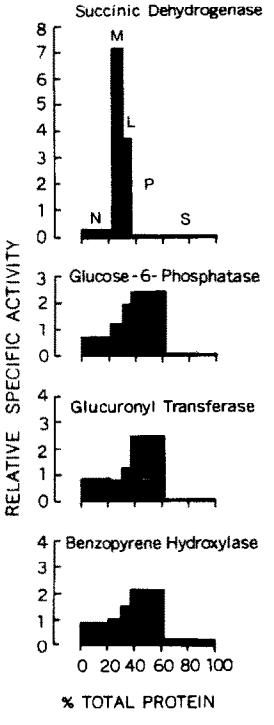

Fig. 3. Distribution of mitochondrial and microsomal marker enzymes from trout liver.

benzopyrene hydroxylase in the microsomal fraction was approximately 70 per cent, which was similar to that found for glucose 6-phosphatase.

DISCUSSION

The studies described in this report have outlined a fractionation scheme for rainbow trout liver and have characterized the resulting fractions with appropriate marker enzymes. The procedure used was arrived at after several trials using different homogenizing media and centrifugal forces. It can be seen from the results that, although a sharp separation of all organelles was not achieved in all cases, the procedure does allow for adequate resolution of the major subcellular organelles. The non-homogeneity of the lysosomal enzymes seen in this study has been observed in other systems and attempts were not made to resolve lysosomes, since the major thrust of this study concerned microsomes.

The R.S.A. and per cent recovery of benzopyrene hydroxylase and glucuronyl transferase were almost identical with these criteria for the microsomal marker, glucose 6-phosphatase. The R.S.A. for rotenone-insensitive NADH and NADPH cytochrome *c* reductase indicates that there was enrichment of this activity in the lysosomal as well as in the microsomal pellet, but these two fractions together contain 70–80 per cent of all microsomal activities studied. The microsomal fraction alone was free of mitochondrial contamination and contained approximately 70 per cent of all microsome markers. Separation of microsomes from plasma membrane has been difficult to achieve in other species, and the relatively high percentage (30–35 per cent) of the plasma membrane markers, alkaline phosphatase and 5′-nucleotidase in the microsomal fraction indicates that this same problem also exists with trout liver. However, the high R.S.A. of the plasma membrane markers in the nuclear pellet indicates the greatest enrichment on a biochemical basis in this latter fraction.

In several studies concerning the metabolism of diazinon [26], aniline [7], aldrin [27] and diethylhexylphthalate [28] by liver subcellular fractions it can be noted that a considerable amount of enzymatic activity resided in the "mitochondrial" or 10,000 *g* pellet. The data presented in this study indicate that, even under carefully controlled fractionation procedures, some microsomes sediment with the mitochondrial fraction, and biotransformation of these compounds by the mitochondrial fraction is most likely associated with contaminating microsomes rather than by mitochondria *per se*.

REFERENCES

1. R. A. Hites and K. Biemann, *Science, N.Y.* **178,** 158 (1972).
2. J. L. Marx, *Science, N.Y.* **186,** 809 (1974).
3. R. H. Adamson, *Fedn Proc.* **26,** 1047 (1967).
4. J. H. Dewaide, in *Metabolism of Xenobiotics*, p. 46. Leijn, Nijmegen (1971).
5. J. L. Ludke, J. R. Gibson and C. I. Lusk, *Toxic. appl. Pharmac.* **21,** 89 (1972).
6. J. J. Lech, *Toxic. appl. Pharmac.* **24,** 114 (1973).
7. R. J. Pohl, J. R. Bend, A. M. Guarino and J. R. Fouts, *Drug Metab. Dispos.* **2,** 545 (1974).
8. D. R. Buhler, *Comp. Biochem. Physiol.* **25,** 223 (1968).
9. L. G. Homsen, I. P. Kapoor and R. L. Metcalf, *Comp. gen. Pharmac.* **3,** 339 (1972).
10. D. R. Buhler and M. E. Rasmusson, *Archs Biochem. Biophys.* **103,** 582 (1968).
11. G. J. Dutton and J. P. Montgomery, *Biochem. J.* **70,** 17P (1958).
12. J. F. Payne and W. R. Penrose, *Bull. envir. Contam. Toxic.* **14,** 112 (1975).
13. J. F. Payne, *Science, N.Y.* **191,** 945 (1976).
14. K. A. Burns, *Comp. Biochem. Physiol.* **53B,** 443 (1976).
15. R. Gianetto and C. DeDuve, *Biochem. J.* **59,** 433 (1955).
16. B. G. Anderson and D. L. Mitchum, in *Atlas of Trout Histology*, p. 16. Wyoming Game and Fish Department, Cheyenne, Wyo. (1974).
17. D. E. Green, S. Mii and P. M. Kohout, *J. biol. Chem.* **217,** 551 (1955).
18. C. Beck, S. Mahadevan, R. Brightwell, C. J. Dillard and A. L. Tappel, *Archs Biochem. Biophys.* **128,** 369 (1968).
19. Y. C. Huang and R. G. Kemp, *Biochemistry* **10,** 2278 (1971).
20. G. L. Sottocasa, B. Kuylenstierna, L. Ernster and A. Bergstrand, *J. Cell. Biol.* **32,** 415 (1967).
21. G. Hübscher and G. R. West, *Nature, Lond.* **205,** 799 (1965).
22. B. N. Ames, in *Methods in Enzymology* (Eds. P. Colowick and N. O. Kaplan), Vol. 8, p. 115. Academic Press, New York (1966).
23. A. R. Hansen and J. R. Fouts, *Chem. Biol. Interact.* **5,** 167 (1972).
24. E. Ross and G. Schatz, *Analyt. Biochem.* **54,** 304 (1973).
25. A. Solyom and E. G. Trams, *Enzyme* **13,** 329 (1972).
26. J. W. Hogan and C. O. Knowles, *Bull. envir. Contam. Toxic.* **8,** 61 (1972).
27. R. H. Stanton and M. A. Q. Khan, *Pestic. Biochem. Physiol.* **3,** 354 (1973).
28. J. E. Carter, D. B. Roll and R. V. Peterson, *Drug Metab. Dispos.* **2,** 341 (1974).