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TÜRKİYE BİLİMSEL ve TEKNİK ARAŞTIRMA KURUMU
TEMEL BİLİMLER ARAŞTIRMA GRUBU

PROJE NO: TBAG-516

ALABALIK KARACİĞER MİKROZOMLARINDAN SİTOKROM P-450'nin KISMEN SAFLAŞTIRILMASI ve KARAKTERİZE EDİLMESİ

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ORTA DOĞU TEKNİK ÜNİVERSİTESİ
BİYOLOJİK BİLİMLER BÖLÜMÜ

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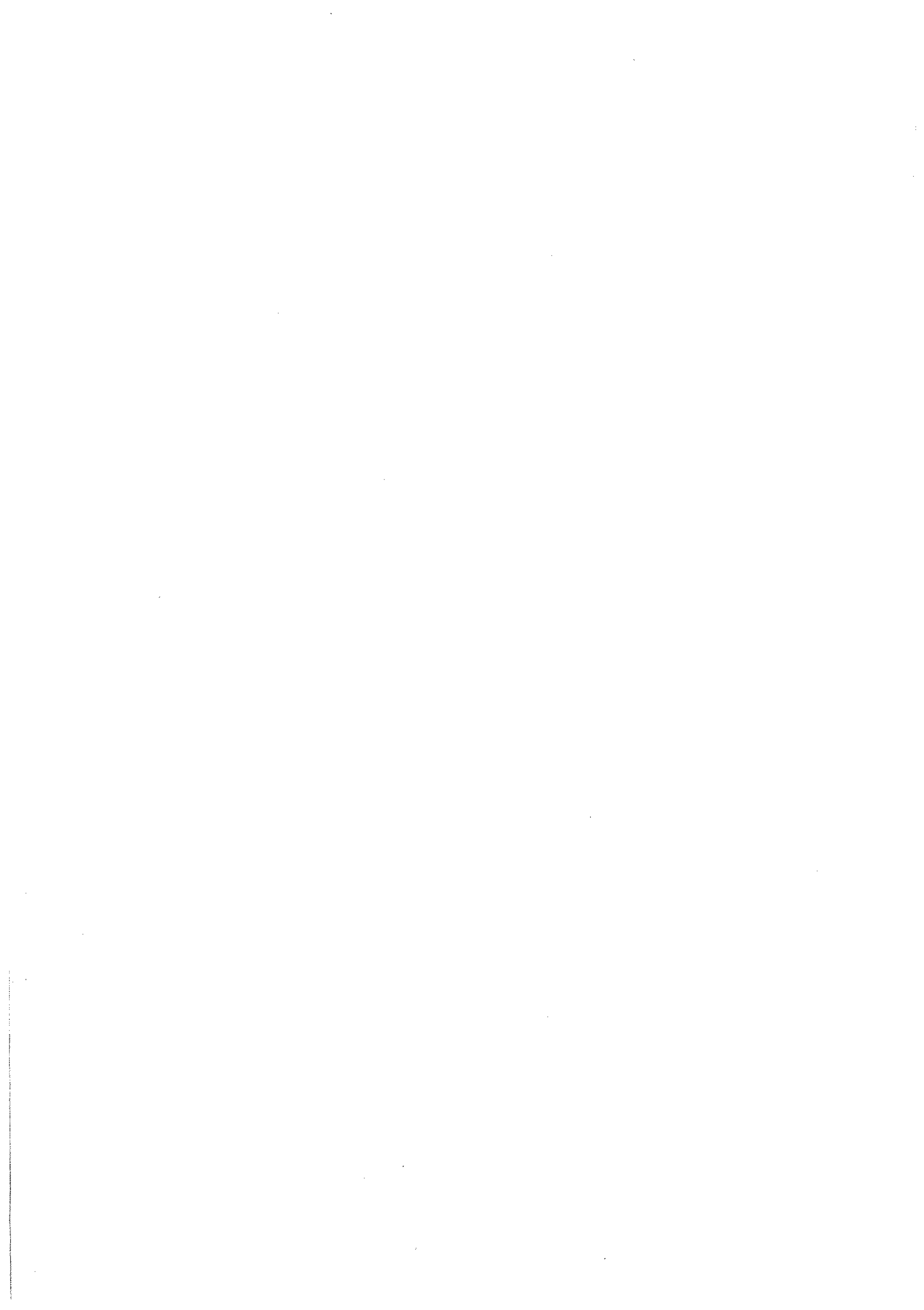
TÜRKİYE
BİLİMSEL VE TEKNİK
ARAŞTIRMA KURUMU
KÜTÜPHANESİ



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ÖZET

ARINÇ, Emel ve ADALI, Orhan. Alabalık karaciğer mikrozomlarından sitokrom P-450'nin kısmen saflaştırılması ve karakterize edilmesi.

Yakın zamana kadar balıklarda, yabancı organik kimyasal maddeleri metabolize eden mikrozomal karışık-fonksiyonlu oksidazlar enzimlerinin bulunmadığına inanılıyordu (Brodie, 1962). Daha sonraki yıllarda yapılan çalışmalar (Chan ve ark'ları, 1967, Dewaide ve Henderson, 1968, Buhler ve Rasmusson, 1968, Pederson ve ark'ları, 1974, Arınç ve ark'ları, 1976) gerek tatlı sularda gerekse denizlerde yaşıyan balıkların karaciğerlerinde sitokrom P-450'ye bağımlı karışık-fonksiyonlu oksidazlar enzimlerinin bulunduğunu gösterdi. Ayrıca anilin 4-hidroksilaz ve etilmorfin N-demetilaz enzimlerinin karışık-fonksiyonlu oksidazlar enzimlerinin bir üyesi olduğu saptandı.

Bu çalışmada, Alabalık, salmo gairdneri, karaciğer sitokrom P-450 özgül miktarı, NADPH'ye bağımlı sitokrom c redüktaz, anilin 4-hidroksilaz ve etilmorfin N-demetilaz özgül aktiviteleri tayin edildi ve sırasıyla 0.16 (N=10) nmol P-450/mg protein, 38 (N=5) ünite/mg protein, 0.04 (N=4) nmol p-aminofenol/dak/mg protein ve 0.174 (N=3) nmol formaldehit/dak/mg protein bulundu.

Alabalık karaciğer mikrozomlarından sitokrom P-450, deterjan sodyum kolat ile çözünürleştirildi. Bundan sonra, DEAE-selüloz kolon kromatografisine tabi tutulan çözünürleştirilmiş balık karaciğeri mikrozomlarından sitokrom P-450-I ve sitokrom P-450-II şeklinde adlandırılan iki ayrı sitokrom P-450 fraksiyonu elde edildi. Sitokrom P-450-I DEAE-selüloz kolonundan %0.3 Emulgen 913 içeren tampon ile ayrılırken, sitokrom P-450-II ise kolondan, kolonun 0.08 M KCl ve %0.3 Emulgen 913 içeren tampon ile elue edilmesiyle ayrıldı. Sitokrom P-450-I ultrafiltrasyona tabi tutularak konsantre edildi ve hidroksil apatit kolonuna uygulanarak mikrozomlara nazaran 11-12 kez daha saflaşmış olarak (özgül miktarı 1.60 nmol P-450/mg protein) %8'lik bir verimle elde edildi. Sitokrom P-450-II ise özgül miktarı 0.55 nmol P-450/mg protein olarak, mikrozomlara nazaran takriben %3'lük bir verimle saflaştırıldı.

Ditiyonit ile indirgenmiş sitokrom P-450-I'in karbon monooksit fark spektrumu 449 nm'de bir tepecik verirken, sitokrom P-450-II'nin aynı şartlardaki spektrumu 451 nm'de bir absorbans gösterdi. Sitokrom P-450-I ve sitokrom P-450-II'nin monomer molekül ağırlıkları sodyum dodesil sülfat jel elektroforez metodu ile tayin edildi ve sırasıyla 56 000 ve 48 500 olarak bulundu. Monomer molekül ağırlıkları ve spektral özellikleri farklı olduğu için, kısmen saflaştırılan sitokrom P-450-I ve sitokrom P-450-II'nin farklı hemoproteinler olduğu kabul edildi.

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SOLUBILIZATION AND PARTIAL PURIFICATION OF TWO FORMS OF CYTOCHROME P-450 FROM TROUT LIVER MICROSOMES

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Running title : Partial purification and characterization of trout liver cytochrome P-450

TÜRKİYE
BİLİMSEL VE TEKNİK
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Abstract-1. Liver microsomal cytochrome P-450 content, NADPH-cytochrome *c* reductase, aniline 4-hydroxylase and ethylmorphine N-demethylase activities of rainbow trout, *Salmo gairdneri*, were found to be 0.16 (N=10) nmol P-450/mg protein, 38 (N=5) units/mg protein, 0.04 (N=4) nmol p-aminophenol/min/mg protein and 0.174 (N=3) nmol formaldehyde/min/mg protein, respectively.

2. Trout liver cytochrome P-450 was solubilized by treatment of microsomes with sodium cholate. Chromatography on DEAE-cellulose column yielded two distinct cytochrome P-450 fractions from solubilized microsomes. Cytochrome P-450-I was eluted with Emulgen 913-containing buffer. Application of 0.08 M KCl in Emulgen 913-containing buffer to the DEAE-cellulose column eluted cytochrome P-450-II fraction. Cytochrome P-450-I was further purified on hydroxylapatite column.

3. CO-difference spectrum of dithionite-reduced cytochrome P-450-I gave a peak at 449 nm while the similar spectrum of cytochrome P-450-II showed a maximum absorbance at 451 nm. Monomer molecular weights of cytochrome P-450-I and cytochrome P-450-II were determined by sodium dodecyl sulfate gel electrophoresis and found to be 56 000 and 48 500, respectively.

INTRODUCTION

Although an early report (Brodie and Maickel, 1962) suggested that fish lacked the ability to oxidatively metabolize foreign compounds, subsequent studies (Chan et al. 1967, Dewaide and Henderson, 1968, Buhler and Rasmusson, 1968, Pederson et al. 1974) have demonstrated that both freshwater and marine fish have cytochrome P-450 dependent mixed-function oxidase (MFO) activity.

The presence of cytochrome P-450, the terminal oxidase of MFO system, in trout liver microsomes was reported as early as 1967 by Chan et al. Later, Pohl et al. (1974) reported the presence of cytochrome P-450 in several species of fish. Ahokas et al. (1975, 1976, 1977) and Elcombe and Lech (1979) quantitatively determined cytochrome P-450 content of trout liver microsomes. Further studies in this field were aimed at the resolution of fish liver microsomal MFO system. As a result of the studies conducted by Arınç et al. (1976) and Bend et al. (1977), skate liver microsomal MFO system was resolved into three components: cytochrome P-450, NADPH-cytochrome c reductase, and lipid. P-450 and NADPH-cytochrome c reductase were partially purified. Benzo(a)-pyrene hydroxylase and 7-ethoxycoumarin deethylase activities of the skate liver microsomes were then reconstituted in the presence of cytochrome P-450,NADPH-cytochrome c reductase and lipid (Arınç, et al. 1976, Bend et al. 1977, Arınç et al. 1978). Recently, Ball, Elmamlouk and Bend (1980) reported the partial purification of two forms of cytochrome P-450 from liver microsomes of little skate pre-treated with 1,2,3,4-dibenzanthracene.

Except for the studies carried out by utilizing skate as a test organism, most of the investigators have been concerned with cytochrome

P-450 content and MFO activities of trout liver microsomes because of its economic and health significance. It has been also established that trout, in particular, are very sensitive to some chemical carcinogens such as aflatoxins and develop hepatomas readily (Sinnhuber et al. 1977). From biochemical and toxicological view point, in vivo studies carried out with chemical carcinogens have clearly demonstrated that different forms of cytochrome P-450 are responsible for the activation and toxicity of these chemical agents (Nebert and Felton, 1976, Wolf et al. 1979). Thus it is important to know whether multiple forms of cytochrome P-450 exist in trout.

However, any information on purification of cytochrome P-450 from trout liver microsomes has not been reported before. This study is carried out to solubilize, partially purify, and characterize cytochrome P-450 from trout liver microsomes. During the purification procedure, two cytochrome P-450 fractions are partially purified. Spectral characteristics and electrophoretic mobilities of these cytochrome P-450s on SDS-polyacrylamide gels are used to distinguish two cytochromes.

TÜRKİYE
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KÜTÜPHANESİ

MATERIALS AND METHODS

Chemicals

p-Aminophenol, formaldehyde, sodium dodecyl sulfate (SDS), 2-mercaptoethanol, horse heart myoglobin were obtained from Fluka A.G., Buchs S.G., Switzerland. Carbon monoxide and aniline (99% pure) were purchased from Fischer Scientific Company, Chemical Manufacturing Division, Fair Lawn, New Jersey, U.S.A.. 3α,7α, 12α-Trihydroxycholanic acid, sodium salt (cholic acid, sodium salt), N,N'-methylene-bisacrylamide (BIS), DL-dithiothreitol (DTT), bovine serum albumin (BSA), horse heart cytochrome c (Type VI), D-glucose-6-phosphate monosodium salt, β-nicotinamide adenine dinucleotide, reduced form (NADPH), yeast D-glucose-6-phosphate dehydrogenase (Type XI), N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES), Coomassie brilliant blue, rabbit muscle phosphorylase a, bovine liver catalase, hog stomach muscle pepsin were purchased from Sigma Chemical Company, Saint Louis, Missouri, U.S.A..

Ethylene diamine tetra acetic acid disodium salt (EDTA), nicotinamide adenine dinucleotide phosphate ($NADP^+$), and acrylamide were obtained from BDH Chemicals Ltd., Poole, England. Glycerol and sodium dithionite ($Na_2S_2O_4$) were the products of E.Merck, Darmstadt, Germany. Emulgen 913 was provided by Kao-Atlas Co. Ltd., Tokyo, Japan. Diethylaminoethyl (DEAE)-cellulose was purchased from Whatman Biochemicals Ltd., Kent, England. N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate, and hydroxylapatite were obtained from Bio-Rad Laboratories, Richmond, California, U.S.A.. Porapak Q was purchased from Waters Associates, Milford, Mass, U.S.A.. All other chemicals were of the analytical grade and were obtained from commercial sources at the highest grade of purity available.

Animals

Fish: Hatchery-reared rainbow trout, Salmo gairdneri irideus Rich which weighed between 90-230 g were obtained from Bozöyük Trout Farm, Turkey. The length of the fish varied between 13 and 32 cm.

Rats: The rats used in the experiments were adult (about 250-300 g) male wistar strain rats on a commercial pellet diet-housed 4 animals per cage.

Rabbits: Rabbits used for comparative experiments were adult (about 2-3 kg) male rabbits housed one animal per cage.

Sheep: Sheep used for comparative studies were 6-18 months old Akkaraman sheep and were slaughtered in the slaughter house.

Preparation of trout liver microsomes

Fish were killed by decapitation. The livers each weighing between 0.9-1.4 g were immediately removed. Following the removal of gall bladders, the livers were placed in plastic bottles containing 0.15 M KCl solution packed in crushed ice. All subsequent steps were carried out at 0-4°C.

In each case, microsomes were prepared from about 30-60 fish livers. The livers were washed in 0.15 M KCl solution several times to remove excess blood. After draining and blotting on a filter paper, the livers were weighed to the nearest 0.1 g and were minced to small pieces with scissors. The resulting tissue mince was homogenized in 0.15 M KCl solution equal to three times the weight of the liver mince, using a Potter-Elvehjem homogenizer coupled with a motor-driven Teflon pestle. Five passes were used for homogenization of the liver mince. After homogenization, the liver homogenate was strained through two layers of cheese-cloth in a Buchner funnel. The homogenate was

centrifuged at 12 000 ·g for 40 min to remove cell debris, nuclei, and mitochondria. Microsomes were sedimented from the supernatant fraction at 133 573 ·g for 60 min in a Beckman L-2 65 B Ultracentrifuge. The supernatant solution was discarded. The tightly packed microsomal pellet was suspended in 0.15 M KCl solution and resedimented at 133 573 ·g for 50 min. The supernatant solution was discarded. The washed microsomal pellet was resuspended in 25% glycerol solution. For each 1 gram of liver, 0.5 ml of 25% glycerol was used. Cytochrome P-450 concentrations, protein content, NADPH dependent cytochrome c reductase, aniline 4-hydroxlase, and ethylmorphine N-dementhylase activities were determined with these freshly made microsomes. About 20-26 mg protein per gram of liver was obtained in microsomal fractions. Additional microsomal suspensions containing 25-45 mg protein per ml were gassed with nitrogen in small plastic bottles and stored at $-20^{O}C$ in a freezer for the partial purification of cytochrome P-450.

Preparation of rat, rabbit and sheep liver microsomes

Rat, rabbit, and sheep liver microsomes were prepared essentially by the same procedure which was used for the preparation of fish microsomes. Details of the procedure was given by Arınç and İşcan (1983).

Partial purification of cytochrome P-450

Solubilization by Sodium Cholate. Cytochrome P-450 was solubilized by treatment of fish microsomes with sodium cholate as described by Arınç and Philpot (1976) with minor modifications: the phosphate buffer concentration was lowered from 100 mM to 80 mM and cholate/protein ratio was increased from 1:1 to 1:1.85. All the procedures were carried out at $0-4^{O}C$.

In a typical preparation, to about 1700 mg of thawed fish liver microsomes (38ml) was added 23.1 ml 1 M potassium phosphate buffer, pH 7.6, 2.9 ml 0.1 M EDTA, 4.52 mg DTT, 50 ml glycerol and the volume of the suspension was made up to 290 ml with distilled water. The resulting microsomal suspension (5.8 mg protein/ml) contained 20% glycerol, 1 mM EDTA, 0.1 mM DTT, and 80 mM potassium phosphate buffer, pH 7.6 in the final concentration. A 15% sodium cholate solution (21 ml) was added dropwise to give a final concentration of 1.85 mg detergent per mg of protein, and the suspension was stirred for 30 min. During the treatment with sodium cholate, the microsomal suspensions became clear. The digested microsomal fraction was then centrifuged at 133 573 g for 90 min and the pellet was discarded.

Column Chromatography on DEAE-Cellulose. The clear, yellow, supernatant solution (296 ml), resulting from centrifugation, was mixed with an equal volume of 20% glycerol solution containing 1 mM EDTA, and 0.1 mM DTT. Then it was applied to a column of DEAE-cellulose (2.1 x 44 cm), previously equilibrated with 40 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA, 0.1 mM DTT, 0.5% sodium cholate, and 20% glycerol (Buffer A). The column was washed with about 680 ml (about 4.5 column volume) of Buffer A, at a flow rate of 50 ml per hour, followed by Buffer A containing 0.3% Emulgen 913 (1050-1150 ml). The column was then eluted with Buffer A containing 0.08 M KCl and 0.3% Emulgen 913. Using this procedure, cytochrome P-450 was eluted in two distinct fractions: one with Buffer A containing 0.3% Emulgen 913 (cytochrome P-450-I), and one with 0.08 M KCl in Buffer A containing 0.3% Emulgen 913 (cytochrome P-450-II). A 600 ml linear KCl gradient (100-600 mM) in Buffer A containing 0.3% Emulgen 913 was then applied to the column. Cytochrome b_5 was eluted with the gradient. An unknown

yellow protein was eluted with the gradient and with 0.6 M KCl solution in Buffer A containing 0.3% Emulgen 913.

Further purification of cytochrome P-450-I

Ultrafiltration. Cytochrome P-450-I fractions from DEAE-cellulose were pooled and concentrated down to 30 ml by Diaflo membrane ultrafiltration by using Amicon XM-100A filter under the nitrogen gas (1.5 atm). Concentrated cytochrome P-450-I was then dialysed overnight against 500 ml of 20 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.25% sodium cholate (Buffer B).

Chromatography on Hydroxylapatite. Columns containing hydroxylapatite were prepared as described by Levin (1962). Cytochrome P-450-I in Buffer B was applied to hydroxylapatite column (2 x 14 cm), previously equilibrated with Buffer B, and the fractions containing cytochrome P-450 were eluted in the void and with Buffer B containing 80 mM, 160 mM, and 300 mM phosphate. A small peak containing the only unknown yellow protein was eluted from the hydroxylapatite column by increasing the phosphate concentration to 600 mM in Buffer B. Cytochrome P-450 fractions were stored in a nitrogen atmosphere at $-5^{o}C$.

Further treatment of cytochrome P-450-II

Ultrafiltration. Since the total content of the pooled cytochrome P-450-II fractions from one DEAE-cellulose column was low, two pooled P-450 fractions from the two separate DEAE-columns were combined and concentrated by using Amicon XM-100A.

Concentrated cytochrome P-450-II preparation was then dialysed overnight against 500 ml of 10 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.25% sodium cholate.

Removal of Excess Detergent. Since cytochrome P-450-II fractions from DEAE-cellulose column contained high concentrations of Emulgen 913, concentrated and dialysed P-450-II samples eluted from DEAE-cellulose column were chromatographed on a column of Porapak Q to remove the excess detergent. The column of Porapak Q (1.2 x 10 cm) was prepared as described by Niederwieser (1974). Porapak Q column was previously equilibrated with 10 mM potassium phosphate buffer, pH 7.6, containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, and 0.25% sodium cholate. The cytochrome P-450-II sample was then applied to the column. Cytochrome P-450-II fraction was eluted in void. A second peak absorbing at 280 nm was eluted from the column by increasing the phosphate concentration to 100 mM. However, this peak did not contain any cytochrome P-450.

Analytical procedures

The protein content of microsomes was determined by the procedure of Lowry et.al. (1951). Crystalline bovine serum albumin was used as a standard. Since glycerol and Emulgen 913 present in the partially purified P-450 fractions interfered with the results, the aliquots from samples were dialysed against distilled water containing 0.05% cholate.

Trout liver microsomal aniline 4-hydroxylase activity was determined by measuring quantity of p-aminophenol (pAP) formed as described by Imai et.al. (1966). The typical assay mixture contained 100 mM HEPES buffer, pH 7.6, 10 mM aniline, 2.5 mM $MgCl_2$, 0.5 mM NADPH generating system consisting of 0.5 mM $NADP^+$, 2.5 mM $MgCl_2$, 2.5 mM glucose 6-phosphate, 1 unit of glucose 6-phosphate dehydrogenase (EC 1.1.1.49, 1 unit of glucose 6-phosphate dehydrogenase reduces 1 μmol of $NADP^+$ per min at 25^oC), 14.2 mM HEPES buffer, pH 7.8 (at 25^oC) and 4-6 mg of microsomal protein in a final volume of 1 ml. The reaction was carried out for

25 min at 25°C since Buhler and Rasmusson (1968) reported that maximum hydroxylation of aniline by trout liver microsomal fractions occured at 25°C.

Trout liver microsomal ethylmorphine N-demethylase activity was determined by measuring the quantity of formaldehyde formed according to the method of Nash (1953) as modified by Cochin and Axelrod (1959). The assay mixture contained 100 mM potassium phosphate buffer, pH 7.8, 15 mM ethylmorphine, 2.5 mM $MgCl_2$, 0.5 mM NADPH generating system, and 4-6mg of microsomal protein in a final volume of 1ml. The reaction was carried out for 15 min at 25°C. Elcombe and Lech (1979) reported that maximum N-demethylation by trout liver microsomal fractions occured at 25-26°C.

NADPH-cytochrome c reductase activity was determined spectrophotometrically at 25°C, with and without 1 mM KCN using the procedure of Masters et al. (1967) on a GSA/Mc Pherson Model EU-707-11 double beam spectrophotometer, equipped with GSA/Mc Pherson Model EU-205-11 strip chart recorder. One unit of reductase was defined as the amount of enzyme catalysing the reduction of 1 nmol cytochrome c under the described conditions.

Cytochrome c reductase activity was determined essentially by the same procedure which was described for the measurement of NADPH dependent cytochrome c reductase activity except that assay medium did not contain NADPH and reaction was started by the addition of the sample to the assay medium.

Cytochrome P-450 was determined by carbon monoxide difference spectra of dithionite-reduced samples on a Cary 17 spectrophotometer using an extinction coefficient of 91 mM^{-1} cm^{-1} for A_{450}-A_{490} (Omura and Sato, 1964a,b). Cytochrome b_5 concentrations were determined

according to the method of Nishibayashi and Sato (1968). The concentration of cytochrome b_5 was estimated from the initial dithionite-reduced minus oxidized difference spectrum using an extinction coefficient of 185 mM cm^{-1} for A_{424}-A_{410}.

SDS-polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed on 3% stacking and 7.5% separating gels in a discontinuous buffer system as described by Laemmli (1970). Phosphorylase a (94 000), BSA (68 000), catalase (60 000), pepsin (35 000), myoglobin (17 200), and cytochrome c (11 700) were used as molecular weight standards. Molecular weight of polypeptide chains were taken from Weber and Osborn (1969).

Separating gels of 7.5% acrylamide with 2.6% crosslinking contained 0.375 M tris-HCl (pH 8.8) and 0.1% SDS. Stacking gel of 3% acrylamide contained 0.125 M tris HCl (pH 6.8) and 0.1% SDS. Both gels were polymerized chemically by the addition of TEMED and ammonium persulphate. The electrode buffer (pH 8.3) contained 0.025 M tris and 0.192 M glycine and 0.1% SDS.

Protein samples were prepared in 0.0625 M tris-HCl buffer (pH 6.8) containing 2% SDS and 10% glycerol in the presence or absence of 2-mercaptoethanol as will be described later.

The gels were subjected to electrophoresis for 40 minutes 2.5 mA/gel at 20°C prior to the application of samples. Electrophoresis was carried out with a current of 2.5 mA per gel until the bromophenol blue marker reached the bottom of the gel.

The gels were simultaneously fixed and stained for protein in a solution containing 0.25% Coomassie blue, 50% methanol, and 7% acetic

acid for 1 hour at room temperature and destained by diffusion of the unbound dye from gels by extensive washing with 30% methanol for 48 hours (Alvares and Siekevitz, 1973). Cytochrome P-450 fractions were stained for peroxidase activity by using benzidine and H_2O_2 as described by Welton and Aust (1974) except that acetate buffer was used in place of citrate buffer.

RESULTS

Some properties of trout liver microsomal enzyme system

Cytochrome P-450 content of different microsomal preparations were found to be 0.07, 0.16, 0.15, 0.16, 0.16, 0.08, 0.12, 0.27, and 0.18 nmol of cytochrome P-450 per mg of microsomal protein. The average specific content was calculated to be 0.16 (N=9) nmol cytochrome P-450 per mg of microsomal protein. The specific content of cytochrome P-450 remained constant for about 3 months when the microsomes were suspended in 25% glycerol and were stored in a nitrogen atmosphere at -20°C.

The carbon monoxide difference spectrum of dithionite reduced trout liver microsomal cytochrome P-450 is shown in Fig. 1. The spectrum shows a peak at 449.8 nm and a shoulder near 423 nm attributable to the presence of cytochrome P-420.

Cytochrome b_5 concentration of trout liver microsomes was determined according to the method of Nishibayashi and Sato (1968) and was found to be 0.06 nmoles b_5 per mg of microsomal protein.

NADPH-cytochrome c reductase, aniline 4-hydroxylase and ethylmorphine N-demethylase activities of rainbow trout liver microsomes were found to be, 38 (N=5) units/mg protein, 0.04 (N=4) nmol pAP/min/mg protein and 0.174 (N=3) nmol formaldehyde/min/mg protein, respectively.

Partial purification of trout liver microsomal cytochrome P-450s

During the solubilization of cytochrome P-450 from trout liver microsomes some problems are encountered. In general, detergent cholate, in ratio of 1 mg cholate:1mg protein is used for the solubilization of cytochrome P-450 from liver and lung microsomes (Levin et al. 1974, Ryan et al. 1975, Philpot et al. 1975, Arınç and Philpot, 1976,

Bend et al. 1977). Thus in the preliminary experiments, trout liver cytochrome P-450 was tried to be solubilized by the same procedures used for solubilization of the rat, (Lu and Levin, 1974, Levin et al. 1974) rabbit, (Lu and Levin, 1974, Philpot and Arınç, 1976) and fish, little skate (Bend et al. 1977) liver microsomal cytochrome P-450s. In the preliminary experiments, trout liver microsomes were diluted to 10 mg protein in 80 mM potassium phosphate buffer, pH 7.6, containing 1 mM EDTA, 0.1 mM DTT, and 20% glycerol. A 10% sodium cholate solution was added to give a final concentration of 1mg of cholate:1mg of protein. Under these conditions only 60-70% of the cytochrome P-450 present in trout liver microsomes were recovered in the solubilized fraction. On the other hand, under the just described conditions about 90-99% of the cytochrome P-450 present in rat, (Lu and Levin, 1974, Levin et al. 1974) rabbit, (Lu and Levin, 1974, Philpot and Arınç, 1976) skate, (Bend et al. 1977) and sheep, (Arınç, 1980) liver microsomes were obtained in solubilized form. Therefore, in the light of the solubilization results obtained from the preliminary experiments, just before the solubilization with detergent sodium cholate, trout liver microsomal protein was lowered to 5.8 mg/ml and cholate:protein ratio was increased to 1.85mg cholate:1mg protein. Under these conditions about 85% of the cytochrome P-450 present in trout liver microsomes were recovered in the solubilized fraction and 15% of cytochrome P-450 which was not solubilized by cholate detected in the pellet. Higher concentrations of cholate was not used due to the denaturation of cytochrome P-450 to cytochrome P-420.

Table 1 summarizes the results obtained during the partial purification of cytochrome P-450s from trout liver microsomes. As seen in Fig.2, chromatography on DEAE-cellulose column yielded two distinct cytochrome P-450 fractions from solubilized microsomes. About 24% of the

total solubilized cytochrome P-450 content was eluted with Buffer A containing 0.3% Emulgen 913 (cytochrome P-450-I). Cytochrome c reductase was partly eluted with cytochrome P-450-I and the greater part of cytochrome c reductase was eluted from the column after cytochrome P-450-I. These fractions reduced the cytochrome c directly without any addition of NADPH and when NADPH was added, the rate of cytochrome c reduction was not affected. Increases in absorbance, seen in Fig.2, starting at about 1850 ml of eluate was essentially due to Emulgen 913 (A max 276 nm). A second cytochrome P-450 fraction, cytochrome P-450-II (about 3.5% of the total solubilized cytochrome P-450) was eluted from DEAE-cellulose column with 0.08 M KCl in Buffer A containing 0.3% Emulgen 913.

Immediately after cytochrome P-450-II, the fractions eluted from the column showed NADPH dependent cytochrome c reductase activity. Cytochrome P-450-II and NADPH-cytochrome c reductase were eluted from the DEAE-cellulose column separately. In the preliminary experiments, when the DEAE-cellulose column was eluted with 0.15 M KCl in Buffer A containing 0.3% Emulgen 913, cytochrome P-450-II and NADPH dependent cytochrome c reductase activity were eluted almost together. By decreasing KCl concentration from 0.15 M to 0.08 M, a good separation of cytochrome P-450-II from NADPH-cytochrome c reductase was accomplished.

A 600 ml linear KCl gradient (100-600 mM) in Buffer A containing 0.3% Emulgen 913 was then applied to the column, some NADPH-cytochrome c reductase, cytochrome b_5 were eluted with the gradient. An unknown yellow protein was eluted with the gradient and with a 300 ml 0.6 M KCl in Buffer A, containing 0.3% Emulgen 913. In a different experiment at this step, column was eluted with 500 ml (in place of 400 ml) 0.08 M KCl in Buffer A containing 0.3% Emulgen 913. Again tailing of the NADPH-cytochrome c reductase activity with the KCl gradient was observed.

Furthermore elution of the column with 1000 ml (in place of 600 ml) linear KCl gradient in Buffer A containing 0.3% Emulgen did not improve the separation of cytochrome b_5 from the yellow protein.

Cytochrome P-450-I and cytochrome P-450-II from the DEAE-cellulose column contained 0.66 nmoles P-450/mg protein and 0.55 nmoles P-450/mg protein and were purified 4.7 and 4 fold with respect to microsomes, respectively.

Cytochrome P-450-I and cytochrome P-450-II fractions from DEAE-cellulose column were pooled and concentrated by Diaflo membrane ultrafiltration by using Amicon XM-100A membrane. By this procedure, concentrated cytochrome P-450-I lost all its cytochrome c reductase activity (see Fig.2) and cytochrome c reductase activity was recovered in the filtrate. Thus, cytochrome P-450-I was separated from the cytochrome c reductase activity. However, the specific content of cytochrome P-450-I was not increased to any detectable value.

Cytochrome P-450-II fraction, concentrated about 5 fold with an Amicon XM-100A filter was then chromatographed on a Porapak Q column to remove the excess non-ionic detergent, Emulgen 913. Cytochrome P-450-II was eluted in the void. The absorbances of the cytochrome P-450-II preparation were measured at 418 nm and 280 nm. The ratio of the absorbance at 418 nm to the absorbance at 280 nm was 1:284 prior to the treatment with Porapak Q and 1:12 following the removal of excess detergent. As seen in Table 1, cytochrome P-450-II fraction contained low levels of P-450, therefore no attempts were made to purify this fraction further.

Cytochrome P-450-I was further purified on hydroxylapatite column. Cytochrome P-450 fractions named as Ia, Ib, Ic and Id were eluted in the void and with Buffer B containing 80 mM, 160 mM, and 300 mM potassium phosphate, respectively (Fig.3). Last fraction (Ie) eluted from the column

with phosphate concentrations of 600 mM did not contain any cytochrome P-450, and its absolute spectrum was the same as yellow protein eluted from DEAE-cellulose column with 100-600 mM KCl gradient. Partially purified cytochrome P-450-Ib (hereafter it will be called as P-450-I) contained 1.60 nmol P-450 per mg of protein and purified about 11.5 fold with the overall yield of 8% (Table 1). Partially purified cytochrome P-450 preparations did not contain any measurable amounts of cytochrome c reductase activity and were essentially free of cytochromes b_5 and P-420. However, significant amounts of cytochrome P-450-I and P-450-II were found to be converted to cytochrome P-420 when these fractions were stored under a nitrogen atmosphere at $-5^{o}C$ for about one week.

CO-difference spectrum of dithionite-reduced cytochrome P-450-I gave a peak at 449 nm while the similar spectrum of cytochrome P-450-II showed a maximum absorbance at 451 nm when the spectra were recorded on the same sheet (Fig.4). Recently, Ball et. al. (1980) reported the partial purification of two spectrally and catalytically different microsomal cytochromes from the livers of the skates pretreated with 1,2,3,4-dibenzanthracene (DBA). CO-difference spectrum of dithionite-reduced DBA-P-450-I showed a maximum absorbance at 448 nm and the same spectrum of DBA-P-450-II had maximal absorption at 451 nm.

SDS-polyacrylamide gel electrophoresis of cytochrome P-450-I and cytochrome P-450-II yielded two major protein bands whose molecular weights were determined to be 59 500, 56 000 and 52 000, 48 500, respectively. Protein scans of the gels containing cytochrome P-450-I and P-450-II are given in Fig.5. Staining of the cytochrome P-450-I and P-450-II containing SDS-polyacrylamide gels for peroxidase activity suggested that cytochrome P-450-I had a monomer molecular weight of 56 000 whereas cytochrome P-450-II had a monomer molecular weight of 48 500.

DISCUSSION

Although an early report (Brodie and Maickel, 1962) suggested that microsomal drug-metabolizing enzymes might be absent in both freshwater and marine species the subsequent studies have demonstrated the presence of varying amounts of MFO activity in some vertabrate species of freshwater and marine origin.

In this study, liver microsomal cytochrome P-450 content, NADPH-cytochrome c reductase, aniline 4-hydroxylase and ethylmorphine N-demethylase activities of rainbow trout (Salmo gairdneri) obtained from Bozöyük, Turkey were determined. The comparison of these values with those reported in the literature for trout liver microsomes is given in Table 2. As early as 1968, Buhler and Rasmusson reported the p-hydroxylation of aniline by trout liver microsomes. Since their values were given as nmol of product per gram of liver per hour, the comparisons could not be made. However, the value of 0.04 nmol pAP/min/mg protein reported in this work compares favorable with the value given for aniline 4-hydroxylase activity by Law and Addison (1981) (See Table 2).

In the present work, ethylmorphine N-demethylase activity of trout liver microsomes was found to be 0.174 nmol formaldehyde/min/mg protein at 25°C (Enzyme activity was negligible at 37°C). As seen in Table 2, ethylmorphine N-demethylase activity was not reported by other groups working with trout liver microsomal MFO system except Elcombe and Lech (1979). The specific activity of the enzyme, 0.77 nmoles formaldehyde/min/mg protein (at 25°C) reported by Elcombe and Lech (1979) was much higher than the value found in this study.

As seen in Table 2, trout liver microsomal cytochrome P-450 levels reported by different laboratories were varied and the highest cytochrome

P-450 levels were obtained in brook trout liver microsomes. On the other hand, NADPH dependent cytochrome c reductase activities of trout liver microsomes reported here was comparible with those reported in literature (See Table 2).

The levels of specific aniline 4-hydroxylase, ethylmorphine N-demethylase, and NADPH-cytochrome c reductase activities and cytochrome P-450 content determined in our laboratory from livers of rabbits, rats and sheep are given in Table 2. These values were much higher than the respected values determined in trout liver microsomes.

Spectral characteristics of cytochrome P-450 in trout liver microsomes were found to be quite similar to those in mammalian liver microsomes. In this study, the CO-difference spectrum of dithionite-reduced rainbow trout, Salmo gairdneri, liver microsomes gave a peak at 449.8 nm. Ahokas et.al. (1977) indicated that λ max of the microsomal carboxyferrocytochrome P-450 of the lake trout, Salmo trutta lacustris, was at 450.6 nm while Elcombe and Lech (1979) showed that the similar spectrum of rainbow trout, Salmo gairdneri gave a maximum absorbance at 449 nm.

During this study, the solubilization and partial purification of cytochrome P-450 from trout liver microsomes were found to be difficult because of problems encountered.

Cytochrome P-450 concentration of trout liver microsomes was found to be approximately 0.16 nmol P-450/mg protein which was so low that hundreds of fish liver (weighing about 1.0-1.2 g) were required in order to start any purification procedure.

Cytochrome P-450 content of the trout liver microsomes decreased drastically (up to 80%) when microsomes were stored in 25% glycerol under the nitrogen gas at $-20^{\circ}C$ in six months. Cytochrome P-450 of

trout liver microsomes were found to be stable for only three months while rabbit, sheep and skate liver microsomal cytochrome P-450 was stable up to 12 months under the nitrogen gas at -20°C.

Following the solubilization of microsomes with cholate, cytochrome P-450 yield was only 85% even though the cholate:protein ratio was increased from 1:1 to 1.85:1 (mg cholate:mg protein). However a similar low yield was reported for solubilized human liver microsomal cytochrome P-450 by Beaune et. al. (1979). When human liver microsomes were treated with cholate to give a final concentration of 1.6 mg cholate/mg of protein, only 68% of the microsomal cytochrome P-450 was recovered in the solubilized fraction (Beaune et. al. 1979).

Chromatography on DEAE-cellulose column yielded two distinct cytochrome P-450 fractions from solubilized trout liver microsomes. Cytochrome P-450-I was eluted with Buffer A containing 0.3% Emulgen 913 and cytochrome P-450-II was eluted from DEAE-cellulose column with 0.08 M KCl in Buffer A containing 0.3% Emulgen 913. The specific activity of cytochrome P-450-II was determined to be 0.55 nmol of P-450 per mg of protein and was purified about 4 fold. Cytochrome P-450-I was further purified by hydroxylapatite column chromatography. The final cytochrome P-450-I preparation contained 1.60 nmol of cytochrome P-450 per mg of protein as compared to 0.14 nmol P-450/mg protein in washed trout liver microsomes. The recovery of cytochrome P-450-I in the partially purified fraction was 8.0% of the amount found in microsomes (Table 1).

CO-difference spectrum of dithionite reduced cytochrome P-450-I gave a peak at 449 nm while the similar spectrum of cytochrome P-450-II showed a maximum absorbance at 451 nm.

Bend et. al. (1977) reported elution of two cytochrome P-450 fractions when solubilized control skate liver microsomes were applied

to DEAE-cellulose column. However, these two skate liver microsomal cytochromes were found to be spectrally similar and CO-difference spectra of both ferro-cytochromes gave a maximum absorbance peak at 450 nm. However, Ball et. al. (1980) obtained two spectrally and catalytically different microsomal cytochromes from the livers of the skates pretreated with 1,2,3,4-dibenzanthracene (DBA). When solubilized liver microsomes of DBA-treated skates were applied to DEAE-cellulose column, 50-60% of the total cytochrome P-450 content was eluted with Emulgen containing buffer (DBA-P-450-I) and its dithionite-reduced CO-difference spectrum showed a maximum absorbance at 448 nm. Application of a KCl gradient to the DEAE-cellulose column eluted a second pool of skate cytochrome P-450 (DBA-P-450-II) whose dithionite reduced CO-difference spectrum had maximal absorption at 451 nm (Ball et. al. 1980).

Monomer molecular weights of cytochrome P-450-I and cytochrome P-450-II were determined by SDS-polyacrylamide gel electrophoresis and were found to be 56 000 and 48 500, respectively. Comparisons of these monomer weights of partially purified trout liver microsomes with those of skate cytochromes could not be made, since the monomer molecular weights of partially purified cytochromes from skate livers were not determined (Arınç et al. 1976, Bend et al. 1977, Ball et al. 1980).

In this study, partially purified trout Liver cytochrome P-450-I and cytochrome P-450-II were found to be distinct hemoproteins since their molecular sizes and spectral characteristics were found to be different. Biocatalytic monooxygenase activity of these two cytochromes could not be determined because of the low concentrations of the cytochrome P-450s in the partially purified fractions. Further work is necessary to accomplish this.

As a conclusion, this study reveals the existence of at least two forms of cytochrome P-450 in trout liver microsomes.

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TABLES

Table 1. Partial purification of cytochrome P-450-I and P-450-II from trout liver microsomes.

Fractions	Total Protein (mg)	CYTOCHROME P-450 Total Content (nmol)	Specific Content (nmol/mg prot)	Purification (fold)	Recovery %
Microsomes	1700	239.4	0.14	1	100
Solubilized microsomes	1500	204.0	0.14	≑	85.2
(DEAE-void)	(403)	(44.3)	(0.11)	-	-
DEAE-P-450-I	75	49.5	0.66	4.7	20.6
DEAE-P-450-II	12.9	7.1	0.55	4.0	3.0
Hydroxylapatite					
Ia	5.6	3.74	0.66	-	-
Ib	11.8	19.00	1.60	11.5	8.0
Ic	3.5	2.25	0.63	-	-
Id	2.4	2.21	1.00	7.1	1.0
yellow protein	2.0	0.0	0.0	-	-

Table 2. A comparison of cytochrome P-450 content, NADPH-cytochrome c reductase, aniline 4-hydroxylase and ethylmorphine N-demethylase activities of liver from various animals.

Animal	P-450 (nmol/mg)	Reductase (U/mg)	Aniline Hydroxylase[a] (nmol pAp/ mg/min)	Ethylmorphine Demethylase[b] (nmol HCOH/ mg/min)
Rabbit [c]	0.80	100	0.65	4.0
Rat [c]	0.60	130	0.32	2.4
Sheep [c]	0.60	120	0.65	5.2
Rainbow Trout[c] (*Salmo gairdneri*)	0.16	38	0.04	0.174
Rainbow Trout [d] (*Salmo gairdneri*)	0.23	N.R	N.R	0.77
Rainbow Trout [e] (*Salmo gairdneri*)	0.10	N.R	N.R	N.R
Rainbow Trout[f] (*Salmo trutta lacustris*)	0.22 [1]	32	N.R	N.R
	0.40 [2]	23	N.R	N.R
Brook Trout [g] (*Salvelinus fontinalis*)	1.00	N.R	0.05	N.R

[a]Hydroxylation of aniline by rainbow trout and mammalian liver microsomes was was performed under the optimum temperature, at 25°C and 37°C, respectively

[b]N-demethylation of ethylmorphine by rainbow trout and mammalian liver microsomes was performed under the optimum temperature at 25°C and 37°C, respectively.

[c]Determined in our laboratory.

[d]Reported by Elcombe and Lech (1979).

[e]Reported by Seppä *et al*. (1981).

[f]1:Reported by Ahokas *et al*. (1975) 2:Reported by Ahokas *et al* (1977).

[g]Reported by Law and Addison (1981)

N.R., not reported.

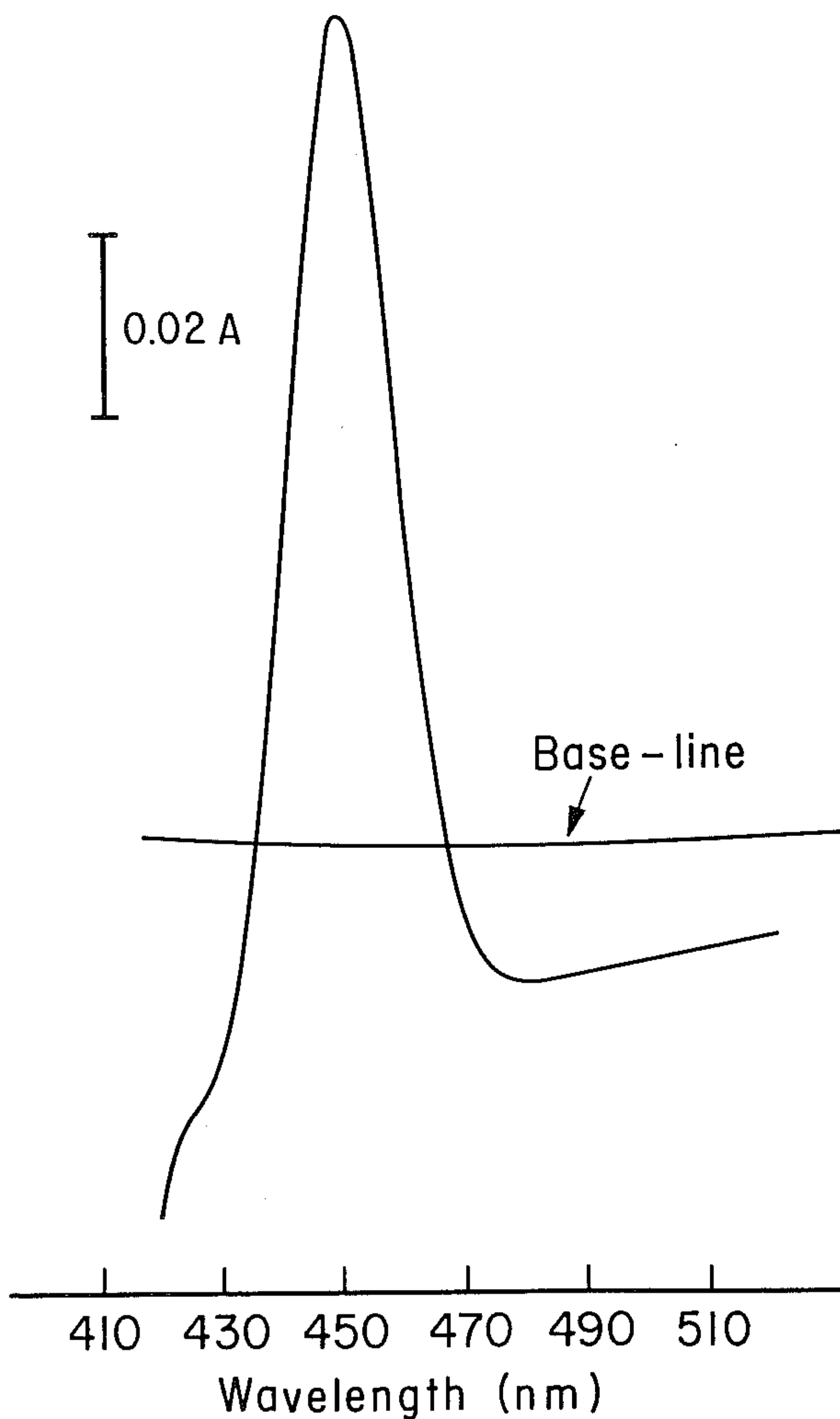

Fig. 1. CO-difference spectrum of dithionite-reduced cytochrome P-450 of rainbow trout liver microsomes. Trout liver microsomes contained 0.2 nmol cytochrome P-450/mg protein.

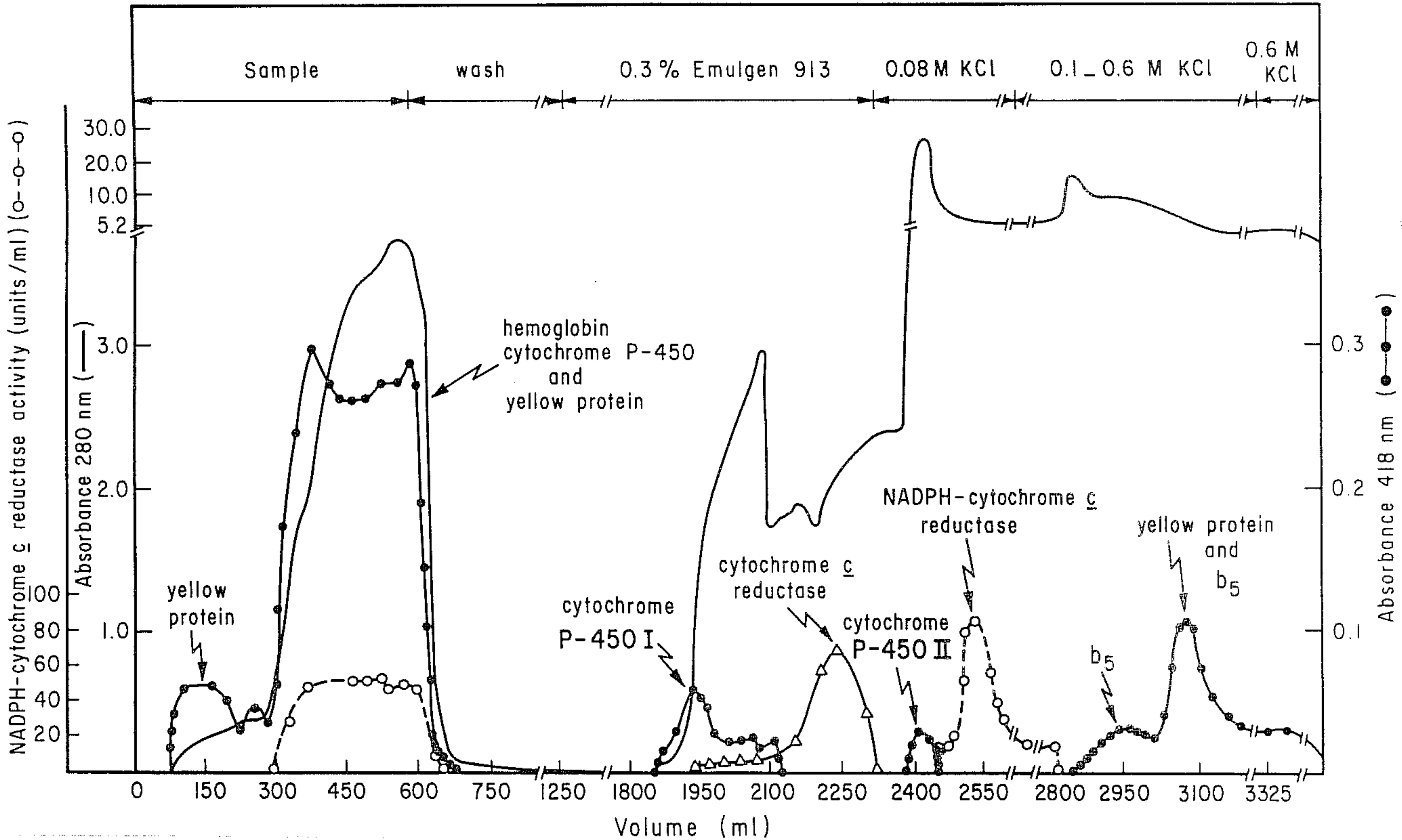

Fig. 2. Elution profile of solubilized P-450 from DEAE-cellulose column (2.1 x 44 cm). Absorbances of the fractions were measured at 280 and 418 nm.

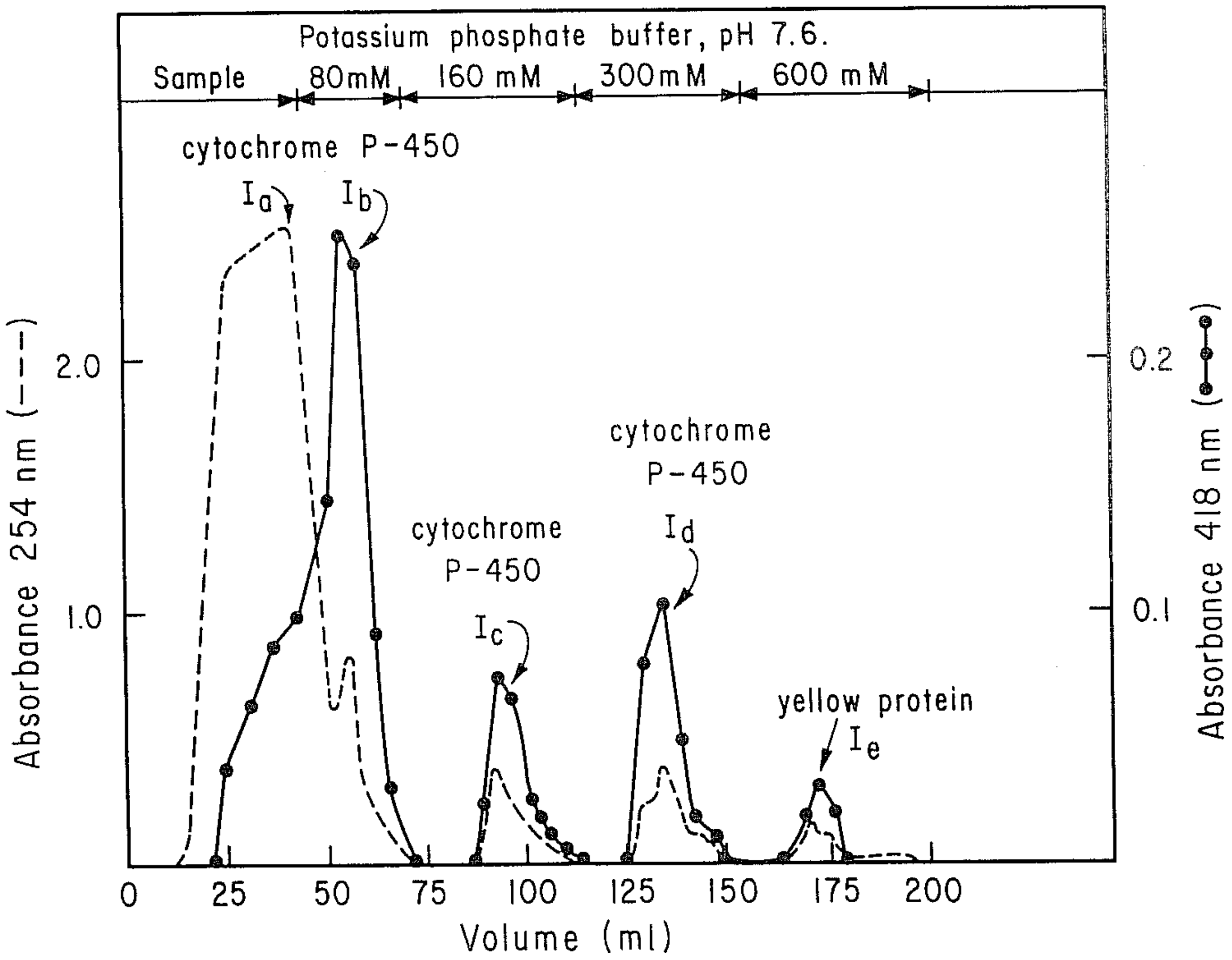

Fig. 3. Elution profile of partially purified P-450 from hydroxylapatite column (2 x 14 cm). Absorbances of the fractions were measured at 254 nm (---) and 418 nm (o---o)

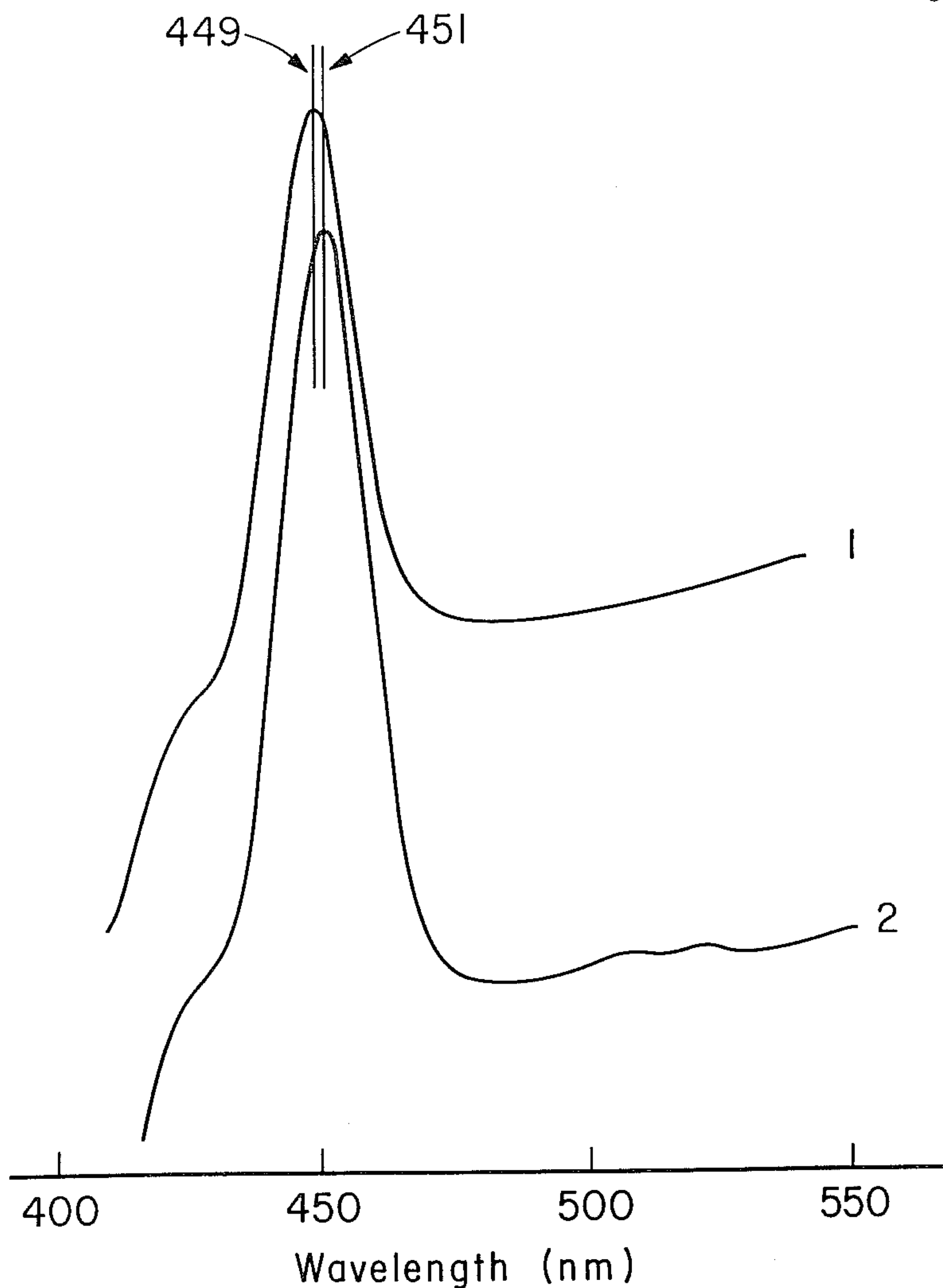

Fig. 4. The CO-difference spectra of dithionite-reduced partially purified P-450-I (1) and P-450-II (2) from trout liver microsomes. P-450-I and P-450-II contained 1.60 and 0.55 nmol of P-450 per mg of protein, respectively.

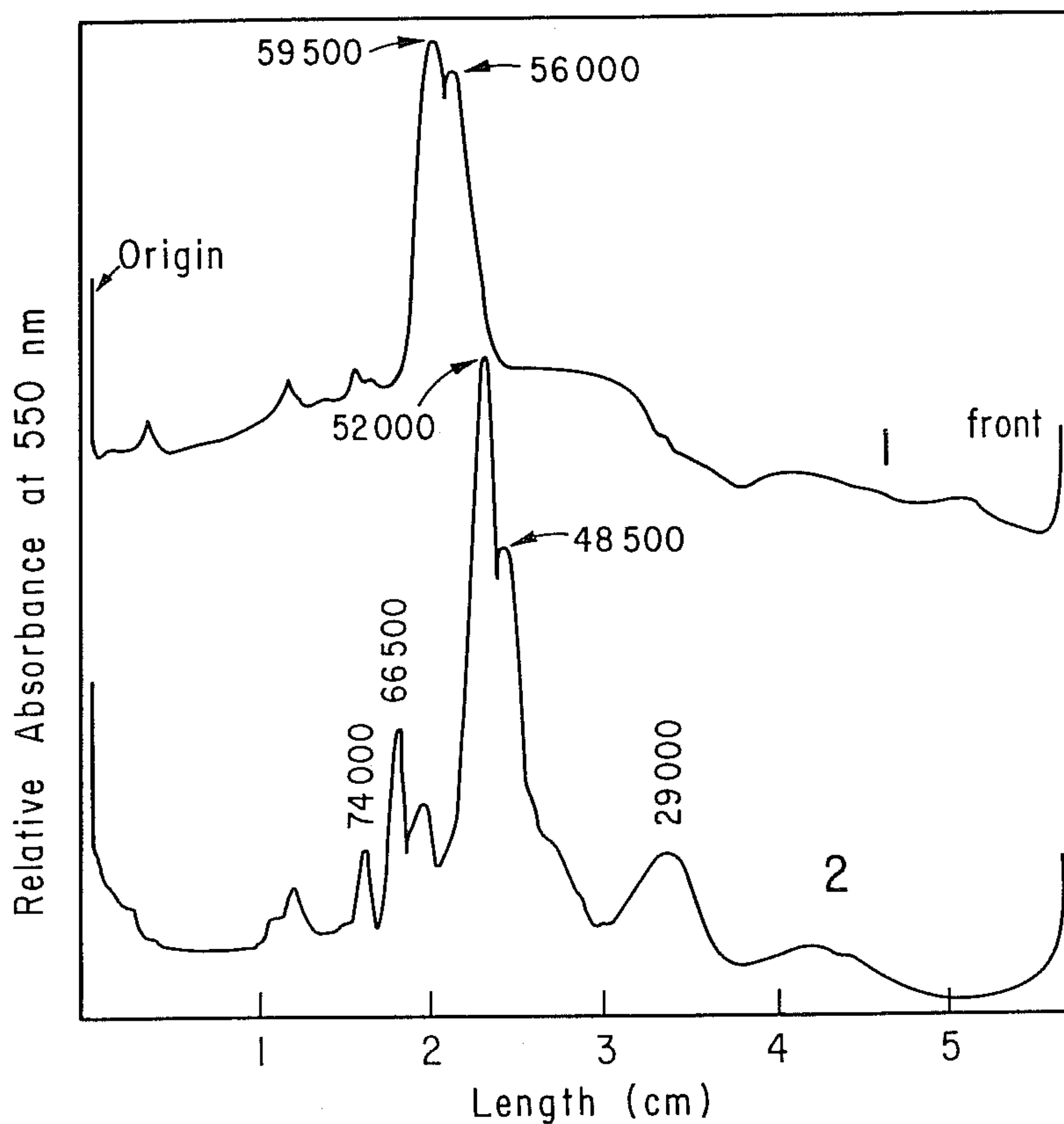

Fig. 5. SDS-polyacrylamide gel electrophoresis protein scans of partially purified trout liver microsomal P-450s. Subsequent to electrophoresis the gels were stained with Coomassie blue and the profiles shown were obtained by scanning the gels at 550 nm.

1: P-450-I (32 μg, 1.60 nmol P-450 per mg of protein).

2: P-450-II (36 μg, 0.55 nmol P-450 per mg of protein).

TÜRKİYE
BİLİMSEL VE TEKNİK
ARAŞTIRMA KURUMU
KÜTÜPHANESİ