Biochimica et Biophysica Acta, 373 (1974) 197–210

BBA 76793

THE EFFECTS OF 3-METHYLCHOLANTHRENE AND PHENOBARBITAL INDUCTION ON THE STRUCTURE OF THE RAT LIVER ENDOPLASMIC RETICULUM*

ANN F. WELTON** and STEVEN D. AUST

Department of Biochemistry, Michigan State University, East Lansing, Mich. 48824 (U.S.A.)

(Received June 24th, 1974)

SUMMARY

Sodium dodecylsulfate-polyacrylamide gel electrophoresis and lactoperoxidase-catalyzed protein radio-iodination were used to compare the structure of the liver endoplasmic reticulum (microsomes) from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats. Prior to iodination, ribosomes and contaminating adsorbed cytoplasmic proteins were removed from the microsomal membrane by washing with 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5. Iodination of membranes was carried out in the presence of butylated hydroxytoluene to prevent the peroxidation of membrane lipids.

The polyacrylamide gel protein and iodination profiles for control microsomes indicated that while many of the minor protein constituents of the membrane incorporated ^{125}I, the major peak of ^{125}I label corresponded to the migration position of major protein constituents having molecular weights of approx. 50 000. Treatment of ^{125}I-labeled control microsomes with trypsin removed ^{125}I from the membrane proteins confirming that lactoperoxidase was iodinating exterior proteins on the membrane. If membranes which were iodinated in the absence of butylated hydroxytoluene were similarly digested with trypsin, most membrane proteins were much more susceptible to trypsin digestion. This suggested that lipid peroxidation, occurring during iodination in the absence of the antioxidant, caused an extensive breakdown in the structure of the membrane.

A comparison of the polyacrylamide gel patterns of the microsomes from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats demonstrated marked changes in both the protein and iodination patterns in the 50 000 molecular weight region of the gels. 3-Methylcholanthrene appeared to induce a protein of slightly higher molecular weight than 50 000 while phenobarbital induced a protein(s) of slightly lower molecular weight. Induction with both compounds increased the amount of ^{125}I incorporated in the 50 000 molecular weight region of polyacrylamide

* Portions of this work were presented at the 57th annual meeting of the Fed. Am. Soc. Exp. Biol., Atlantic City, N.J., in 1973 [1].

** Present address: Section on Membrane Regulation, Laboratory of Nutrition and Endocrinology, National Institute of Arthritis, Metabolism, and Digestive Diseases, Bethesda, Md. 20014, U.S.A.

gels indicating that the induced proteins have an exterior location on the microsomal membrane. The association of the induced proteins with cytochrome P450, the terminal oxidase of the microsomal mixed-function oxidases, is discussed. A model is proposed for the mechanism of insertion of the induced proteins into the microsomal membrane.

INTRODUCTION

Two techniques have recently become available for the study of membrane structure. These are sodium dodecylsulfate-polyacrylamide gel electrophoresis [2] and lactoperoxidase-catalyzed radio-iodination of membrane proteins [3, 4]. Polyacrylamide gel electrophoresis in the presence of sodium dodecylsulfate provides a means by which solubilized membrane proteins can be separated from one another while lactoperoxidase-catalyzed protein iodination provides a mechanism by which only those proteins exposed to the exterior of the membrane can be radio-labeled since this enzyme is impermeable to membranes. In combination, these two techniques can be used to study the spatial arrangement of proteins in a membrane. These techniques have been used to study the structure of many membranes, including the surface membranes of erythrocytes [3–5], blood platelets [6, 7], and other intact cells [8, 9] and the inner mitochondrial membrane [10].

Kreibich et al. [11] have recently shown that these techniques can be used to study the spatial arrangement of proteins in the rough endoplasmic reticulum of rat liver. We too have been interested in the structure of the membrane of this organelle through our study of the microsomal mixed-function oxidase system. It is known that pretreatment of animals with compounds such as phenobarbital or 3-methylcholanthrene induces protein components of the mixed-function oxidase system [12]. Analyses of the protein components of rat liver microsomes by gel electrophoresis has also shown that pretreatment with these compounds induces major microsomal proteins having molecular weights of approx. 50 000 [1, 13–16]. 3-Methylcholanthrene induces a different protein than does phenobarbital [1, 13, 14]. It has been suggested that the induction of these proteins may be related to the induction of cytochrome P450, the terminal oxygen- and substrate-binding cytochrome of the mixed-function oxidase system [13–16]. In this paper we report the use of sodium dodecylsulfate-gel electrophoresis, in combination with lactoperoxidase-catalyzed protein iodination, to determine the spatial position of these proteins in the microsomal membrane.

METHODS AND MATERIALS

Drug pretreatment of animals

Immature male Sprague–Dawley rats weighing between 75 and 100 g were obtained from Spartan Research Animals, Inc., Haslett, Michigan. Animals were pretreated with either phenobarbital (50 mg/kg in saline) by daily intraperitoneal injection 5 days prior to sacrifice or with 3-methylcholanthrene (20 mg/kg in corn oil) by intraperitoneal injection 36 and 24 h prior to sacrifice. Such procedures typically resulted in a 3.0-fold induction of cytochrome P450 by phenobarbital and a 1.5-fold induction of cytochrome P448 by 3-methylcholanthrene. Animals were fasted 18 h before killing by decapitation.

Preparation of microsomal fractions

The total microsomal fraction was isolated by differential centrifugation as previously described [17]. Rough and smooth microsomal subfractions were isolated according to the method of Bergstrand and Dallner [18]. In some cases the isolated membranes were stored by suspension in 0.05 M Tris–HCl, pH 7.5, containing 50 % glycerol to a protein concentration of approx. 50 mg/ml and freezing at −15 °C under N_2 in the presence of 0.01 % butylated hydroxytoluene to prevent lipid peroxidation [19]. Before use the membranes were washed in 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5, by suspension with homogenization to a protein concentration of 1–2 mg/ml and centrifugation at 105 000 × *g* for 90 min. All isolation and washing procedures were carried out at 0–4 °C.

Enzyme assays and analytical methods

Catalase [20], NADPH-cytochrome *c* reductase [21], NADH-ferricyanide reductase [21], and cytochrome b_5 [22] were assayed by previously described techniques. Cytochrome P450 was assayed by CO-difference spectroscopy of dithionite-reduced samples as described by Omura and Sato [22].

Protein was determined by the method of Lowry et al. [23] and standardized with bovine serum albumin using $E_{cm}^{1\%}$ at 280 nm equal to 6.6 [24]. RNA was determined by the method of Munroe and Fleck [25]. Lactoperoxidase concentrations were determined spectrophotometrically using a millimolar extinction coefficient of 114 at 412 nm [4]. H_2O_2 concentrations were determined similarly using a molar extinction coefficient of 72.4 at 230 nm [4].

Iodination procedure

Washed microsomal preparations were suspended to a protein concentration of 0.5 mg/ml in 0.1 M Tris–HCl, pH 7.5 (at 25 °C) containing 10^{-6} M KI (2–5 μCi; ^{125}I/ml), $5.0 \cdot 10^{-7}$ M lactoperoxidase, and 0.0001 % butylated hydroxytoluene. The butylated hydroxytoluene was added to prevent the peroxidation of membrane lipids during the iodination procedure [19]. The iodination was carried out at 25 °C by addition of 5 nmoles H_2O_2/ml at 1-min intervals over a 3-min reaction period. At the end of the 3-min reaction period, the reaction mixture was diluted with cold 0.1 M Tris–HCl buffer, pH 7.5, and centrifuged at 105 000 × *g* for 90 min. The pelleted microsomes were either suspended in buffer for subsequent use or directly prepared for polyacrylamide gel electrophoresis.

Trypsin treatment of iodinated microsomes

^{125}I-labeled microsomes ($5 \cdot 10^6$ cpm/mg microsomal protein) were suspended to 6 mg/ml in 0.05 M Tris, pH 7.5 (at 25 °C) containing 1 mM EDTA and 0.005 % butylated hydroxytoluene. Trypsin (type III from Sigma) was added in a ratio of 10 μg trypsin per mg of microsomal protein and this mixture was incubated under N_2 for 1 h at 25 °C. Rate studies indicated that proteolysis was completed after 1 h of digestion. Soybean trypsin inhibitor was then added in a ratio of 5 μg of trypsin inhibitor per μg of trypsin. The reaction mixture was then diluted 3-fold with 0.05 M Tris, pH 7.5 (at 25 °C) containing 1 mM EDTA and centrifuged at 105 000 × *g* for 90 min. The pellets obtained were immediately resuspended in sodium dodecylsulfate for polyacrylamide gel electrophoresis.

Sodium dodecylsulfate-polyacrylamide gel electrophoresis

The sodium dodecylsulfate-polyacrylamide gel electrophoresis technique of Fairbanks et al. [2] was only slightly modified. Samples (1–5 mg/ml) were prepared in 1 % sodium dodecylsulfate, 5–10 % sucrose, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 40 mM dithiothreitol, and 10 μg pyronin B tracking dye. They were then heated at 100 °C for 15 min and applied to 5.6 % polyacrylamide gels (5 mm × 100 mm) prepared in tubes which had been coated with dichlorodimethylsilane. Electrophoresis was performed with a constant voltage gradient of 5 V/cm. The current ranged between 3 and 4 mA/tube. The running time under these conditions was about 3.5 h. After electrophoresis the gels were placed in 10 % (w/v) trichloroacetic acid overnight. The gels were stained for protein using Coomassie blue [26] and scanned at 550 nm in a Gilford spectrophotometer equipped with a gel-scanning attachment. After scanning for protein, gels of radioactive samples were fractionated using a Savant Autogel Divider and the fractions were counted on a Nuclear Chicago gamma spectrometer to determine ^{125}I distribution. Molecular weight markers β-galactosidase, bovine serum albumin, carbonic anhydrase, alcohol dehydrogenase, trypsin, and ribonuclease A were run in parallel with membrane protein samples. The molecular weights reported in this paper were then calculated from a standard curve plotted using these standards as described by Weber and Osborn [27]. Each value reported is the average of at least three determinations.

Materials

Lactoperoxidase, trypsin (type III), β-galactosidase (grade IV), carbonic anhydrase, alcohol dehydrogenase, ribonuclease A (type III-A), soybean trypsin inhibitor (type I-S), sodium dodecylsulfate, 3-methylcholanthrene and butylated hydroxytoluene were obtained from the Sigma Chemical Co. Bovine serum albumin was purchased from Pentex. All electrophoresis reagents were obtained from Canalco. $Na^{125}I$ (carrier-free) was obtained from New England Nuclear. Phenobarbital was purchased from Merck and Co., Inc. All other reagents were analytical grade.

RESULTS

Comparison of rough and smooth microsomal membranes after removal of ribosomes and adsorbed proteins

The total microsomal fraction is known to be a mixture of rough (ribosome bound) and smooth microsomes and to be contaminated by adsorbed cytoplasmic proteins. Since we were only interested in the membrane proteins, it was desirable to establish a procedure by which ribosomes and contaminating cytoplasmic proteins could be removed from the membranes. Previous studies have indicated that chelating agents are effective in disrupting and removing ribosomes [28–30]. In this work a mixture of 0.3 M sucrose containing 0.1 M sodium pyrophosphate, pH 7.5, was used to remove both ribosomes and adsorbed proteins. As can be seen from Table I, this procedure removed approximately 30 % of the protein and 80 % of the RNA from the membranes. This procedure did not appear to be removing membrane proteins since the specific activity of such membrane-associated proteins as NADPH–cytochrome *c* reductase, NADH–ferricyanide reductase, cytochrome b_5, and cytochrome P450 increased. Furthermore the recoveries of these proteins were nearly 100 %. The

TABLE I

THE LEVELS OF VARIOUS CONSTITUENTS IN THE TOTAL MICROSOMAL FRACTION ISOLATED FROM CONTROL RATS BEFORE AND AFTER WASHING WITH 0.3 M SUCROSE CONTAINING 0.1 M SODIUM PYROPHOSPHATE, pH 7.5

After initially assaying the microsomes, 25 mg of microsomal protein was resuspended to a protein concentration of 1 mg/ml in sucrose containing sodium pyrophosphate. This suspension was centrifuged at 105 000 × *g* for 90 min to pellet the microsomal membrane. The pellet was then resuspended to a protein concentration of 4.5 mg/ml for assay (18.0 mg of protein was found in the pellet resulting in a recovery of 72 % of the original protein).

Constituent	Specific activity		
	Before	After	Recovery (%)
NADPH-cytochrome *c* reductase*	0.160	0.224	102
NADH-ferricyanide reductase*	4.30	5.61	93
Catalase**	97.0	10.2	7.6
Cytochrome b_5***	0.423	0.658	112
Cytochrome 450***	0.809	1.28	114
RNA†	107.0	32.4	21.9

* μmoles of receptor reduced/min per mg protein.
** μmoles of H_2O_2 consumed/min per mg protein.
*** nmoles of cytochrome/mg protein.
† μg of RNA/mg protein.

ability of this washing procedure to remove 90 % of the catalase from the membrane accentuates its effectiveness at removing adsorbed proteins. This was especially important for our studies using lactoperoxidase-catalyzed protein iodination because catalase would interfere with the iodination since H_2O_2 is a substrate for both enzymes.

The sodium dodecylsulfate-polyacrylamide gel electrophoresis profiles of rough and smooth microsomes were similar after washing the membranes with sucrose and sodium pyrophosphate. This can be seen in Fig. 1 which compares the electrophoresis profiles characteristic of rough, smooth, and total microsomes before and after washing. These comparisons were made with several preparations of membranes with very little variance in these profiles from preparation to preparation. The major protein constituents for each type of membrane have molecular weights between 40 000 and 60 000. Unwashed rough microsomes, however, appear to contain several components of molecular weight above 100 000 and below 40 000 which are not present in smooth microsomes. Since Dice and Schimke [31] have reported that the majority of the ribosomal proteins have molecular weights between 15 000 and 34 000 on sodium dodecylsulfate gels, the ribosomes may be the source of the lower molecular weight components in rough microsomes. After washing, the profiles obtained for rough and smooth microsomes are identical and resemble those obtained for the total microsomal fraction after washing. This suggests that the membranes have similar protein compositions after being washed free of ribosomes and adsorbed proteins. Such a conclusion is consistent with several other recent investigations of the protein components of rough and smooth microsomes which have been made using poly-

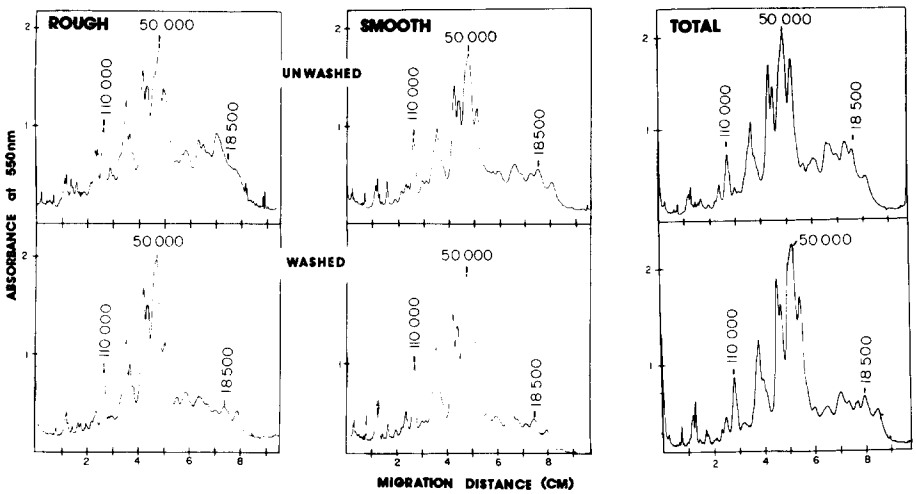

Fig. 1. Sodium dodecylsulfate-polyacrylamide gel electrophoresis protein profiles of rough, smooth and total liver microsomal fractions before and after washing with sucrose containing sodium pyrophosphate. The membrane fractions were isolated from a control rat. The upper scans are of gels run on unwashed membranes while the lower scans are of washed membranes. Between 40 and 45 μg of protein was applied to each gel.

acrylamide gel electrophoresis [16, 32–34]. On this basis we concluded that for these studies it did not appear necessary to subfractionate the total microsome fraction if the membranes were first washed with sucrose containing sodium pyrophosphate.

^{125}I-Labeling pattern of the microsomes from control rats

When washed microsomes from control rats were ^{125}I labeled using lactoperoxidase, as described in Methods and Materials and then electrophoresed on polyacrylamide gels, the ^{125}I incorporation pattern seen in Fig. 2 was obtained. No ^{125}I-

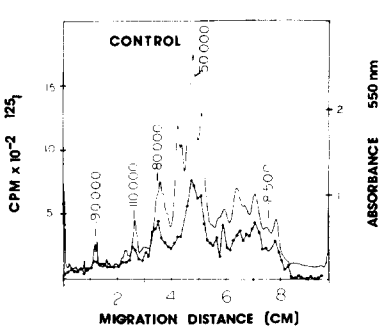

Fig. 2. Sodium dodecylsulfate-polyacrylamide gel electrophoresis protein and ^{125}I-incorporation profiles of the total liver microsomal fraction from a control rat. The smooth line represents the protein profile while the dotted line designates the ^{125}I- incorporation pattern. The membranes were washed with sucrose containing sodium pyrophosphate, iodinated, and prepared for electrophoresis, as described in Methods and Materials. The iodination mixture contained 2 μCi ^{125}I per ml. 40 μg of protein was applied to the gel.

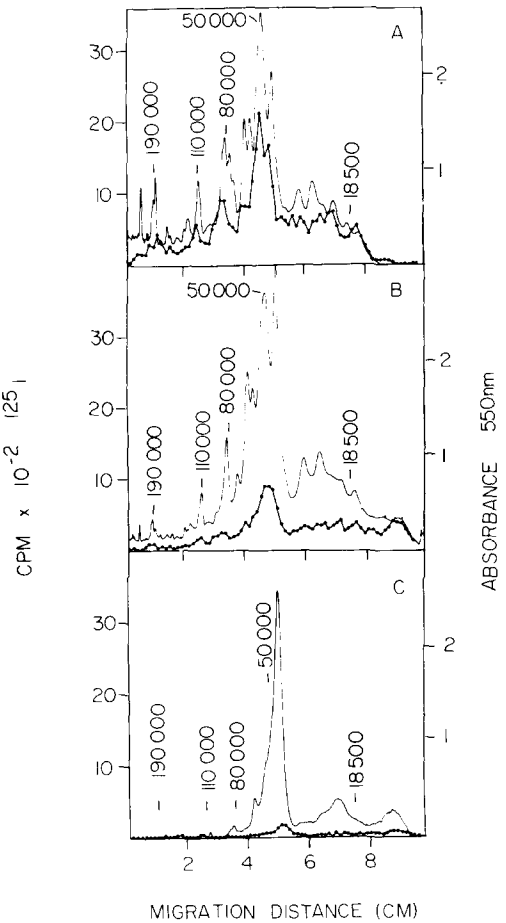

Fig. 3. The effect of trypsin treatment on the sodium dodecylsulfate-polyacrylamide gel electrophoresis protein and ^{125}I-incorporation profiles of the total liver microsomal fraction from a control rat. The smooth line represents the protein profile while the dotted line designates the ^{125}I-incorporation pattern. Microsomal membranes were washed with sucrose containing sodium pyrophosphate and iodinated as described in Methods and Materials. The iodination mixture contained 5 μCi ^{125}I per ml. The membranes were then resuspended to a protein concentration of 6 mg/ml in 0.05 M Tris–HCl, pH 7.5 (at 25 °C) containing 1 mM EDTA and 0.005 % butylated hydroxytoluene. (A) The electrophoresis profiles obtained from ^{125}I-labeled microsomes which were incubated in parallel with the trypsin-treated microsomes; no protease was added to this sample, however. After incubation, the membranes were isolated by centrifugation and resuspended for electrophoresis. 55 μg of protein was applied to the gel. (B) The electrophoresis profiles obtained after the ^{125}I-labeled microsomes were incubated with trypsin, as described in Methods and Materials. After trypsin treatment, the membranes were isolated by centrifugation and resuspended for electrophoresis. 52 μg of protein was applied to the gel. (C) The electrophoresis profiles obtained when microsomes were iodinated in the absence of butylated hydroxytoluene and then subjected to trypsin treatment (in the presence of butylated hydroxytoluene), as described in Methods and Materials. After trypsin treatment the membranes were isolated by centrifugation and resuspended for electrophoresis. 40 μg of protein was applied to the gel.

labeled material was found at the top of the gel indicating that all labeled proteins had entered the gel. It appeared that many of the minor protein constituents of the membrane were ^{125}I labeled. The major peak of ^{125}I incorporation, however, corresponded with the migration position of the major protein components having molecular weights of approx. 50 000. When Kreibich et al. [11] conducted similar studies on rough endoplasmic reticulum, they found a similar pattern except the major radioactivity peak was associated with proteins of less than 30 000 molecular weight. This peak they ascribed to ribosomal proteins. Since our membranes were washed free of ribosomes before ^{125}I labeling, we would not expect to observe such a peak on our gels. Indeed, we observed that the ^{125}I-labeling pattern of rough and smooth microsomes were identical after washing.

It is important in studies such as these [3–11], to establish that lactoperoxidase catalyzes the iodination of only exterior proteins. To demonstrate this property we incubated ^{125}I-labeled microsomes with trypsin. Trypsin has been shown to be impermeable to microsomal membranes but will cleave peptides from the outside of the membrane [35–37]. Thus this protease should be capable of altering the ^{125}I-labeling pattern of microsomes if lactoperoxidase is indeed labeling exterior proteins. As can be seen from Fig. 3, trypsin treatment removed ^{125}I from microsomal proteins indicating that many of the proteins iodinated by lactoperoxidase are also substrates for this protease.

It should be noted that we have previously reported that an antioxidant such as butylated hydroxytoluene should be used during the iodination of liver endoplasmic reticulum membranes to prevent the peroxidation of membrane lipids [19]. To further emphasize the importance of this, Fig. 3c demonstrates the gel protein profile obtained from microsomes iodinated in the absence of butylated hydroxytoluene and then treated with trypsin. It can be seen that if the iodination is not carried out in the presence of butylated hydroxytoluene, lipid peroxidation causes an extensive breakdown in the structure of the membrane as demonstrated by the increased susceptibility of most membrane proteins to trypsin digestion.

Comparison of the sodium dodecylsulfate-polyacrylamide gel protein and iodination profiles of the microsomes from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats

Fig. 4A compares the protein constituents of sucrose and sodium pyrophosphate-washed liver microsomes from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats. Fig. 4B compares the ^{125}I-incorporation patterns of the three types of microsomes. It can be seen that pretreatment of animals with phenobarbital and 3-methylcholanthrene alters the protein profile in the 50 000 molecular region of the gels, however, the two compounds do not cause identical changes. 3-Methylcholanthrene pretreatment appears to induce a protein of slightly higher molecular weight than 50 000 (53 000) while phenobarbital pretreatment induces a protein(s) of slightly lower molecular weight than 50 000. It is difficult to see the actual induction pattern by phenobarbital because of the complexity of the protein pattern in this region of the gels. It is possible that phenobarbital may be inducing more than one protein in this region. In any case, similar changes in the polyacrylamide gel patterns following phenobarbital and 3-methylcholanthrene induction have been previously reported by Alvarez and Siekevitz [13] and by our laboratory [1, 14]. It appears from

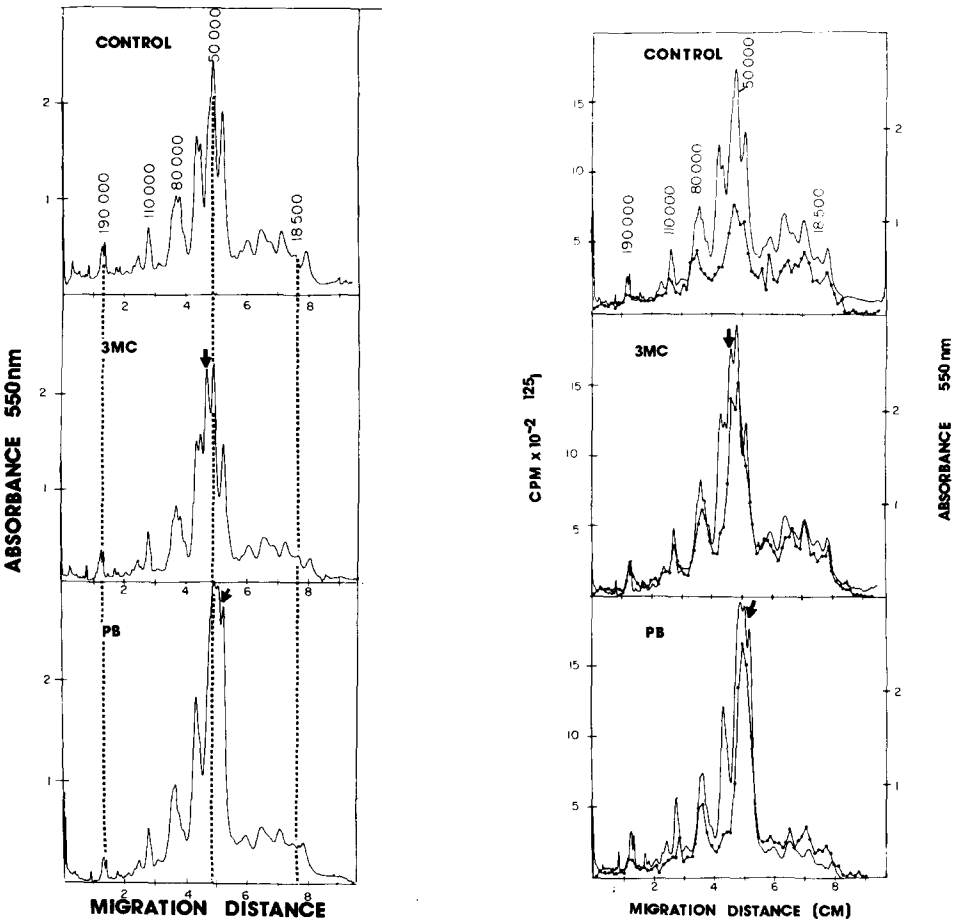

Fig. 4. A comparison of the sodium dodecylsulfate-polyacrylamide gel electrophoresis protein and ^{125}I-incorporation profiles obtained from the liver microsomes of control, phenobarbital-, and 3-methylcholanthrene-pretreated rats. The microsomes were treated with sucrose containing sodium pyrophosphate, iodinated, and prepared for electrophoresis. In each case the iodination mixture contained 2 μCi ^{125}I per ml. As an index of induction, the cytochrome P450 (P448) levels (nmoles/mg microsomal protein) in the three types of microsomes were: control, 1.2; phenobarbital, 3.4; and 3-methylcholanthrene, 1.7. The arrows indicate the positions on the gels of the protein(s) induced by 3-methylcholanthrene and phenobarbital. (A) A comparison of the protein profiles of the three types of microsomes. 40 μg of sample was applied to each gel. (B) A comparison of the protein (smooth line) and ^{125}I-incorporation (dotted line) profiles of the three types of microsomes. 40 μg of sample was applied to each gel.

the ^{125}I-incorporation profiles shown in Fig. 4B, that the microsomes from phenobarbital- and 3-methylcholanthrene-pretreated rats also incorporate more ^{125}I into the proteins of the 50 000 molecular weight region than do control microsomes. Because of the complexity of the protein banding pattern in this region of the gels, a direct comparison of the cpm of ^{125}I/absorbance at 550 nm ratios for the proteins cannot be made. A calculation can be made of the percent of ^{125}I incorporated into microsomal proteins which appears in proteins of 40 000–60 000 molecular weight on

TABLE II

THE PERCENT OF ^{125}I INCORPORATED INTO MICROSOMAL PROTEINS WHICH APPEARS IN PROTEINS OF 40 000–60 000 MOLECULAR WEIGHT ON SODIUM DODECYLSULFATE GELS

Liver microsomes from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats were ^{125}I labeled and electrophoresed on polyacrylamide gels in the presence of sodium dodecylsulfate as described in Methods and Materials. After staining the gels to determine protein distribution, the gels were fractionated for γ counting to determine ^{125}I distribution. The percent of the total cpm recovered from the gel which appeared in fractions from the 40 000–60 000 molecular weight region of the gel was then calculated. The values reported are the average and standard deviations from four experiments.

Type of microsome	%
Control	36.4 ± 2.3
Phenobarbital pretreated	50.5 ± 0.5
3-Methylcholanthrene pretreated	43.3 ± 1.1

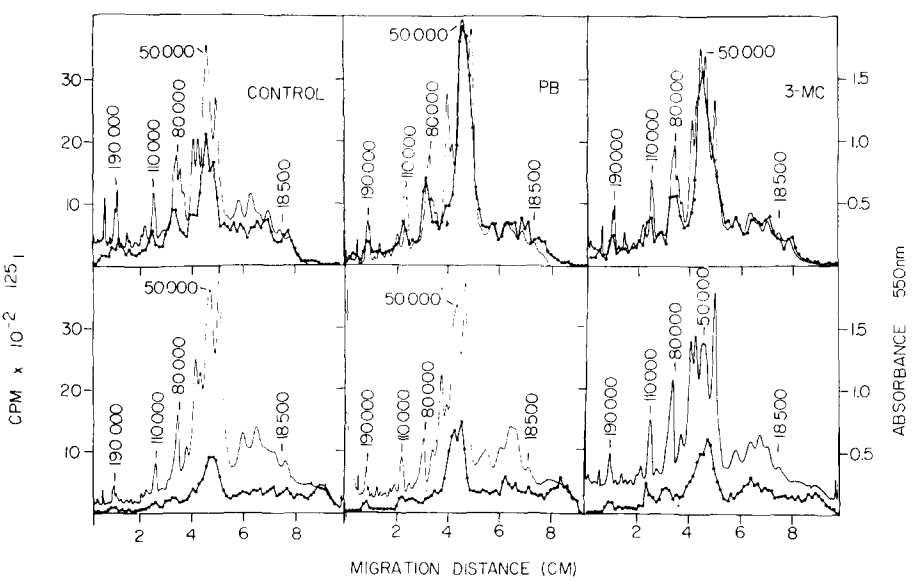

Fig. 5. The effect of trypsin treatment on the sodium dodecylsulfate-polyacrylamide gel electrophoresis protein and ^{125}I-incorporation profiles of the liver microsomes from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats. The membranes were washed with sucrose containing sodium pyrophosphate and iodinated as described in Methods and Materials. In each case the iodination mixtures contained 5 μCi ^{125}I per ml. They were then resuspended to a protein concentration of 6 mg/ml in 0.05 M Tris–HCl, pH 7.5 (at 25 °C) containing 1 mM EDTA and 0.005 % butylated hydroxytoluene. The upper scans are of ^{125}I-labeled microsomes which have been incubated in parallel with the trypsin-treated sample, however without the protease. The lower scans are of ^{125}I-labeled microsomes after treatment with trypsin, as described in Methods and Materials. After incubation the membranes were isolated by centrifugation and resuspended for electrophoresis. In each figure the smooth line denotes the protein profile and the dotted line the ^{125}I pattern. Between 40 and 50 μg of sample was applied to each gel.

gels run of the three types of microsomes, however. This is presented in Table II and by this criterion there is increased ^{125}I incorporation into these proteins in the microsomes of phenobarbital and 3-methylcholanthrene-pretreated rats. Since the induction pattern in the 50 000 molecular weight region of polyacrylamide gels after 3-methylcholanthrene pretreatment of animals is fairly simple, it is possible that the increased incorporation of ^{125}I in this region is due to the induction of the 53 000 molecular weight protein. Because of the complexity of the phenobarbital induction pattern in this region and the resolution limitations involved in fractionating gels to determine the radioactivity profile of proteins of such close molecular weights, it is impossible to determine which of the protein(s) induced by phenobarbital is actually labeled with ^{125}I. It can be concluded, however, that both phenobarbital and 3-methylcholanthrene induce proteins which can be iodinated by lactoperoxidase and, therefore, appear to be located on the exterior of the microsomal membrane. The exterior location of these proteins is also substantiated by another observation. If the three types of microsomes are iodinated and then digested with trypsin, more ^{125}I appears to be removed from the 40 000–60 000 molecular weight proteins of phenobarbital and 3-methylcholanthrene microsomes than from control (Fig. 5). After trypsinization, the percent of ^{125}I which is recovered in the 40 000–60 000 molecular weight region of gels run on the three types of microsomes is nearly identical: control, 35 %; phenobarbital, 38 %; and 3-methylcholanthrene, 37 %. Thus those proteins induced by phenobarbital and 3-methylcholanthrene which are iodinated with lactoperoxidase also appear to be susceptable to proteolysis by trypsin, further confirming their exterior location.

DISCUSSION

Previous studies have shown that pretreatment of animals with phenobarbital and 3-methylcholanthrene induces proteins which migrate in the 50 000 molecular weight region of sodium dodecylsulfate-polyacrylamide gels [13–16]. Different proteins are induced in this region by phenobarbital and 3-methylcholanthrene [1, 13, 14]. In this report we demonstrate that proteins of this molecular weight also incorporated more ^{125}I after phenobarbital and 3-methylcholanthrene induction suggesting that the proteins induced by these compounds are located on the external surface of the microsomal membrane. While the identity of the proteins induced by phenobarbital and 3-methylcholanthrene is unknown, there is a great deal of evidence to suggest that they may be associated with cytochrome P450, the substrate-binding, terminal oxidase of the microsomal mixed-function oxidase system. First of all, although this cytochrome has not been completely purified, it is believed to be a major protein constituent of microsomal membranes [15, 16], and it, or its polypeptide components if it is actually a protein complex as suggested by Autor et al. [39], appears to migrate with proteins of molecular weight 50 000 on gels run in sodium dodecylsulfate [13–16, 40]. Secondly, different forms of this cytochrome appear to be induced by phenobarbital and 3-methylcholanthrene on the basis of spectral [41, 42] and catalytic [43, 44] studies. It is thought that the induction of different forms of this cytochrome accounts for the observed induction of different mixed-function oxidase activities by phenobarbital or 3-methylcholanthrene [43, 44]. Hence it may be that the different 50 000 molecular weight proteins induced by phenobarbital or 3-methylcholanthrene

are different cytochrome P450 hemoproteins or, if cytochrome P450 is a complex, perhaps different "regulatory subunits" of the complex. Indeed, Alvarez and Siekevitz [13] have shown that the different proteins induced by phenobarbital or 3-methylcholanthrene in the 50 000 molecular weight region of polyacrylamide gels co-purify with partially purified cytochrome P450 from these microsomes, suggesting the association of these proteins with cytochrome P450. Furthermore, we have recently reported the resolution of three hemoproteins in the 50 000 molecular weight region of 0.1 % sodium dodecylsulfate gels run on the microsomes from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats [14]. These hemoproteins were identified by staining for heme with benzidine and, in the gel system used for these studies, appeared to have molecular weight of 53 000, 50 000, and 45 000. 3-Methylcholanthrene pretreatment induced the 53 000 molecular weight component while phenobarbital pretreatment induced the 45 000 molecular weight component. This data tends to argue for the existence of multiple cytochrome P450 hemoproteins and the differential induction of the various forms of this cytochrome may account for the changes in the 50 000 molecular weight gel protein profiles reported in this paper. One apparent inconsistency in such speculation, however, is the observation that proteases will not remove this cytochrome from the microsomal membrane [37], while we have observed that the ^{125}I-incorporating proteins induced by phenobarbital or 3-methylcholanthrene are susceptible to trypsin digestion. It has been observed that trypsin treatment of microsomes does convert cytochrome P450 to its P420 form [37], however, and it is possible that this is the result of the degradation of the cytochrome by this protease. Thus the portion of this cytochrome which is iodinated by lactoperoxidase may be released from the microsomal membrane while a very hydrophobic portion, containing the heme, remains bound to the membrane. Obviously, further speculation on the association of cytochrome P450 with the ^{125}I-incorporating proteins induced by phenobarbital or 3-methylcholanthrene will have to await the purification of the cytochrome(s) from microsomes.

Whatever the identity of the proteins induced by phenobarbital or 3-methylcholanthrene in the 50 000 molecular weight region of gels, the data presented in this paper, in conjunction with previously published observation on microsomal proteins, can be used to draw a model of how these induced proteins are incorporated into this membrane. Dehlinger and Schimke [15, 45] have proposed that microsomal proteins are synthesized and degraded as cytoplasmic constituents. Thus, they propose that membrane proteins are actually in a state of equilibrium between associated (membrane bound) and dissociated (cytoplasmic) states. They have also shown that pretreatment of rats with phenobarbital or 3-methylcholanthrene results in the specific increase in the synthesis of 50 000 molecular weight microsomal constituents [15]. If their proposal is correct, increased synthesis of these proteins would have resulted in a larger cytoplasmic pool of these proteins and a shift in the association–dissociation equilibrium to more membrane-bound protein. But if these proteins are synthesized in the cytoplasm, how would they then be inserted into a pre-existing membrane? Since we have shown that the induced proteins appear to be located on the exterior of the membrane (which is the cytoplasmic face) it may be that binding sites for these proteins are present on this side of the membrane. Such sites would be analogous to those already found on the cytoplasmic face of the microsomal membrane for cytochrome b_5 [46]. The binding sites for cytochrome b_5 do not appear to

be saturated in isolated microsomes suggesting that the concentration of this protein in the membrane may be dependent upon the amount available in the cytoplasm for binding. It may be that if such binding sites exist for the proteins induced by phenobarbital and 3-methylcholanthrene, these too are not saturated in control microsomes. In that case the mechanism of insertion of the induced proteins into the membrane would simply be dependent upon the increase in the cytoplasmic level of these proteins which would follow their increased synthesis. To substantiate such a hypothesis, it will be necessary to isolate the proteins in the 50 000 molecular weight region of polyacrylamide gels which are induced by phenobarbital and 3-methylcholanthrene so that the characteristics of their binding to the microsomal membranes from control, phenobarbital-, and 3-methylcholanthrene-pretreated rats can be studied.

ACKNOWLEDGEMENTS

This research was supported by N.S.F. Training Grant GZ-2390, N.S.F. Grant GB-40711 and N.I.H. Grants GM-9699 and GM 21321-01.

REFERENCES

1 Welton, A. F. and Aust, S. D. (1973) Fed. Proc. 32, 665
2 Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617
3 Phillips, D. R. and Morrison, M. (1970) Biochem. Biophys. Res. Commun. 40, 284–289
4 Phillips, D. R. and Morrison, M. (1971) Biochemistry 10, 1766–1771
5 Hubbard, A. L. and Cohn, Z. A. (1972) J. Cell Biol. 55, 390–405
6 Phillips, D. R. (1972) Biochemistry 11, 4582–4588
7 Nachman, R. L., Hubbard, A. and Ferris, B. (1973) J. Biol. Chem. 248, 2928–2936
8 Poduslo, J. F., Greenberg, C. S. and Glick, M. C. (1972) Biochemistry 11, 2616–2621
9 Marchalonis, J. J., Cone, R. E. and Santer, V. (1971) Biochem. J. 124, 921–927
10 Astle, L. and Cooper, C. (1974) Biochemistry 13, 154–160
11 Kreibich, G., Hubbard, A. L. and Sabatini, D. D. (1974) J. Cell Biol. 60, 616–627
12 Ernster, L. and Orrenius, S. (1965) Fed. Proc. 24, 1190–1199
13 Alvarez, A. P. and Siekevitz, P. (1973) Biochem. Biophys. Res. Commun. 54, 923–929
14 Welton, A. F. and Aust, S. D. (1974) Biochem. Biophys. Res. Commun. 56, 898–906
15 Dehlinger, P. J. and Schimke, R. T. (1972) J. Biol. Chem. 247, 1257–1264
16 Siekevitz, P. (1973) J. Supramol. Struct. 1, 471–489
17 Pederson, T. C. and Aust, S. D. (1970) Biochem. Pharmacol. 19, 2221–2230
18 Bergstrand, A. and Dallner, G. (1969) Anal. Biochem. 29, 351–356
19 Welton, A. F. and Aust, S. D. (1972) Biochem. Biophys. Res. Commun. 49, 661–666
20 Beers, Jr, R. F. and Sizer, I. W. (1952) J. Biol. Chem. 195, 133–140
21 Omura, T. and Takesue, S. (1970) J. Biochem. 67, 249–257
22 Omura, T. and Sato, R. (1964) J. Biol. Chem. 239, 2379–2385
23 Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
24 Rutter, W. J. (1967) in Methods in Developmental Biology (Wilt, F. H. and Wessels, N. K., eds), p. 675, Thomas Crowell Co., New York
25 Munroe, H. N. and Fleck, A. (1966) Methods Biochem. Anal. 14, 158–162
26 Johnson, J. C., DeBacker, M. and Beozi, J. A. (1971) J. Biol. Chem. 246, 1222–1232
27 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406–4412
28 Sachs, H. (1958) J. Biol. Chem. 233, 650–656
29 Kuriyama, Y., Omura, T., Siekevitz, P. and Palade, G. E. (1969) J. Biol. Chem. 244, 2017–2026
30 Ragland, W. L., Shires, T. K. and Pitot, H. C. (1971) Biochem. J. 121, 271–278
31 Dice, J. F. and Schimke, R. T. (1972) J. Biol. Chem. 247, 98–111
32 Hinman, N. D. and Phillips, A. H. (1970) Science 170, 1222–1223

33 Weihing, R. R., Manganiello, V. C., Chiu, R. and Phillips, A. H. (1972) Biochemistry 11, 3128–3135
34 Schnaitman, C. A. (1969) Proc. Natl. Acad. Sci. U.S. 63, 412–419
35 Kreibich, G., Debey, P. and Sabatini, D. D. (1973) J. Cell Biol. 58, 436–462
36 Sabatini, D. D. and Blobel, G. (1970) J. Cell Biol. 45, 146–157
37 Ito, A. and Sato, R. (1969) J. Cell Biol. 40, 179–189
38 Welton, A. F., Pederson, T. C., Buege, J. A. and Aust, S. D. (1973) Biochem. Biophys. Res. Commun. 54, 161–167
39 Autor, A. P., Kaschnitz, R. M., Heidema, J. K. and Coon, M. J. (1973) Mol. Pharmacol. 9, 93–104
40 Levin, W., Lu, A. Y. H., Ryan, D., West, S., Kuntzman, R. and Conney, A. H. (1972) Arch. Biochem. Biophys. 153, 543–553
41 Conney, A. H. (1967) Pharmacol. Rev. 19, 317–366
42 Kuntzman, R. (1969) Annu. Rev. Pharmacol. 9, 21–36
43 Lu, A. Y. H., Kuntzman, R., West, S., Jacobson, M. and Conney, A. H. (1972) J. Biol. Chem. 247, 1727–1734
44 Lu, A. Y. H., Levin, W., West, S., Jacobson, M., Ryan, D., Kuntzman, R. and Conney, A. H. (1973) J. Biol. Chem. 248, 456–460
45 Dehlinger, P. J. and Schimke, R. T. (1971) J. Biol. Chem. 246, 2574–2583
46 Strittmatter, P., Rogers, M. J. and Spatz, L. (1972) J. Biol. Chem. 247, 7188–7194