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EFFECTS OF 3-METHYLCHOLANTHRENE ADMINISTRATION ON THE PROTEINS OF ENDOPLASMIC RETICULUM*

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Abstract—The effects of the polycyclic hydrocarbon, 3-methylcholanthrene, have been studied on the proteins of the smooth and rough endoplasmic reticulum. The evidence indicated that 3-methylcholanthrene caused a more rapid increase in the incorporation of ^{14}C-labeled amino acid mixture into these components and a slower degradation of the proteins. The same major protein species incorporated [^{3}H]δ-aminolevulinic acid, a heme precursor, and the ^{14}C-amino acids. Pretreatment of rats with 3-amino-1,2,4-triazole, an inhibitor of heme synthesis, resulted in a reduction in the amount of both the ^{14}C-amino acid and the [^{3}H]δ-aminolevulinic acid incorporation. The evidence was consistent with the hypothesis that a substantial proportion of the microsomal proteins were hemoproteins and that 3-methylcholanthrene administration resulted in marked alterations in the turnover of these components.

THE ENDOPLASMIC reticulum (ER) is a very complex and dynamic membrane system. It consists of many proteins, the rates of synthesis and degradation of which are dependent on diverse factors such as nutrition, physiological and pharmacological stimuli. The kinetics of synthesis and degradation of the proteins of these membranes have been studied both in a normal and in a drug-stimulated state.[1–5] Isotope incorporation techniques have been utilized in determining the turnover, i.e. the overall process of synthesis and degradation, of the total membrane protein, of the "smooth" and "rough" components, and of several specific microsomal proteins.[1–4]

A number of pharmacologic agents, e.g. polycyclic hydrocarbons and barbiturates, exert profound effects upon the microsomal enzymes which are involved in biotransformation reactions (reviewed in refs. 6–8). In this regard, von der Decken and Hultin[9] and Gelboin and Sokoloff[10,11] have shown that the administration of the polycyclic hydrocarbon, 3-methylcholanthrene (3-MC), affects the rate of incorporation of amino acids into microsomal protein. In this manuscript, we report studies on the effects *in vivo* of the polycyclic hydrocarbon on the incorporation of ^{14}C-amino acids and [^{3}H]δ-aminolevulinic acid into the proteins of smooth endoplasmic reticulum (SER) and rough endoplasmic reticulum (RER). The protein components were separated by disc electrophoresis in the presence of urea and a non-ionic detergent. The data from these studies indicate that 3-methylcholanthrene caused a more rapid incorporation of amino acids into these tissues and a slower degradation of the proteins of these fractions.

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MATERIALS AND METHODS

Male Sprague–Dawley rats weighing 70–100 g were used for this study and were routinely starved 24 hr prior to sacrifice. The animals were injected intraperitoneally with either 50 μc of a ^{14}C-amino acid mixture (uniformly labeled, New England Nuclear Corp.; 1·45 mc/mg) or 100 μc of [^{3}H]δ-aminolevulinic acid (New England Nuclear Corp.; 588 mc/m-mole) and were sacrificed at intervals thereafter. Drug-treated rats received 3-methylcholanthrene (Eastman Organic Chemicals) in corn oil, intraperitoneally, at a dosage of 20 mg/kg, 22 hr prior to sacrifice. Control animals were injected with corn oil. The 3-amino-1,2,4-triazole (Sigma) was administered in saline, intraperitoneally, 3 g/kg, 3 hr prior to sacrifice.

Fractions of smooth and rough endoplasmic reticulum were isolated by the method of Dallner *et al.*,[12] utilizing an SW 50 rotor instead of the SW 39 rotor, in a Spinco model L2 ultracentrifuge. Since this modification was a departure from the original method,[12] and cross-contamination [13] was possible, the pellets were examined by electron microscopy. The pellets were fixed in 0·1 M glutaraldehyde in 5% cacodylate buffer, pH 7·4, postfixed in 2% osmium tetroxide, and dehydrated in graded ethyl alcohol solutions. The samples were embedded in a mixture of maraglas (Maraset, Marblett Corp.) and Dow 732 resins (Dow Corp.), thin sections were stained with lead citrate and uranyl acetate, and the stained sections were examined with an RCA EMU-3H electron microscope.

To show further the lack of cross-contamination, labeled SER and RER proteins were isolated in the usual manner from a 3-MC-treated animal that had been injected with the ^{14}C-amino acid mixture. The ^{14}C-labeled SER proteins were then added to a 12,000 *g* supernatant fraction of liver from another 3-MC-treated animal and the SER and RER components were reisolated. Only 20 per cent of the radioactivity was found in the RER fraction.

The ribosomes were removed from RER by the method of Süss *et al.*[14] (The use of the term RER in this manuscript refers to ribosome-free rough endoplasmic reticulum.) The pellets obtained in the final step of the above procedure were then resuspended in a solution of 8 M urea containing 1% Triton X-100 (Rohm & Hass).

Protein concentration was measured by the method of Lowry *et al.*,[15] with bovine serum albumin as standard. A Packard Tri-Carb liquid scintillation spectrometer was used for counting all samples. The scintillation mixture contained 5·5 g 2,5-diphenyloxazole, 0·3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene, 333 ml Triton X-100, and 667 ml toluene. Another scintillation mixture, Scintisol-Complete (Isolab Inc.), was employed in some experiments.

Polyacrylamide gels were prepared according to the formulations provided by Canal Industrial Corp. (Canalco) or by Davis.[16]All solutions were adjusted to 8 M urea and 1% Triton X-100, as suggested by the work of Schneiderman[17] and Kiehn and Holland.[18] A 2·5% solution of acrylamide was employed for separating gel. The gels, in tubes with dimensions 8 × 160 mm, were subjected to electrophoresis at room temperature for 5 hr at 5 mA per gel.

Three sets of gels were prepared for each sample with 0·9 mg protein applied to each gel. One set was stained 15 min in a solution of 1% Buffalo black in 7% acetic acid, and destained by diffusion in 7% acetic acid. Another set of gels was stained for lipoproteins with Oil Red O. A third set was sliced into 1·0 mm discs which were placed in scintillation vials. The slices were solubilized by incubation at 50° with

0·5 ml of 30% hydrogen peroxide, similar to the method of Fishler and Epstein.[19] In some experiments, a 1-ml aliquot of NCS, a protein solubilizer (Amersham/Searle), was added to each vial with 10 ml of scintillation fluid. The vials remained at room temperature for at least 1 hr to dissipate the formed gases. Equivalent results were obtained in other experiments with 10 ml of Scintisol-Complete without the addition of NCS. The efficiency in counting ^{14}C in the gel slices averaged 80 per cent; the efficiency of ^{3}H counting was 20 per cent. We consistently recovered 60–65 per cent of the applied radioactivity from these gels.

RESULTS

Electron micrographs of the subfractions isolated by the above methods are shown in Figs. 1 and 2. Figure 1a shows the SER of the control preparations with very little contaminating RER; Fig. 1b shows RER with little contaminating SER. The RER which had been stripped of ribosomes is shown in Fig. 1c. The "denuding" of the RER is essentially complete as judged by the absence of ribosomes. Figure 2 illustrates the preparations obtained from the livers of MC-treated rats. Again, the SER does not contain a significant amount of contaminating RER (Fig. 2a). However, more ribosome-free membranes were present in the RER preparation (Fig. 2b), but certainly not to the 50 per cent level as reported by Gram *et al.*[20]

Gel electrophoretograms of the solubilized liver proteins of the SER and RER of control and MC-treated rats are presented in Fig. 3. With the use of 8 M urea and 1% Triton, approximately 13–17 bands were observable. This compares favorably with the number of proteins found by Lundkwist and Perlmann,[21] who employed immunological methods, and with the number found by Berkman *et al.*[22] and Schnaitman,[23] using other techniques. Some material which does not migrate is retained at the top of the gel. Following this region, an area was observed which stained only lightly for protein but intensely for lipoprotein. Three major bands were then observed which appeared to represent the bulk of the protein species; many minor bands, not all of which showed up in this photograph, were also apparent. The major protein band patterns were very reproducible within a given sample, but the minor bands varied from preparation to preparation, as was reported by Berkman *et al.*[22]

TABLE 1. PROTEIN CONTENT OF SER AND RER OF LIVER OF CONTROL AND 3-MC-TREATED RATS*

Control (18)† (mg/g liver)		3-MC-treated (16)† (mg/g liver)		
SER	RER	SER	RER	
3·26 ± 0·25		2·69 ± 0·42		$P < 0·25$
	4·85 ± 0·33		6·02 ± 0·60	$P < 0·10$

* Male rats, 70–100 g, were injected intraperitoneally with either corn oil or 3-methylcholanthrene (3-MC), 20 mg/kg, 22 hr previously. The animals were sacrificed, the livers removed, and the SER and RER components were isolated as described in Methods. The results are expressed as milligrams of protein per gram of liver weight ± standard deviation.

† Number of animals.

The effect of administration of 3-methylcholanthrene upon the relative amounts of protein in the SER and RER in liver is indicated in Table 1. The relative amount of SER was unaltered by pretreatment with the drug, in confirmation of the morphological findings of Gram *et al.*[20] The RER fraction, however, was slightly elevated after drug treatment, at a P value $< 0{\cdot}1$.

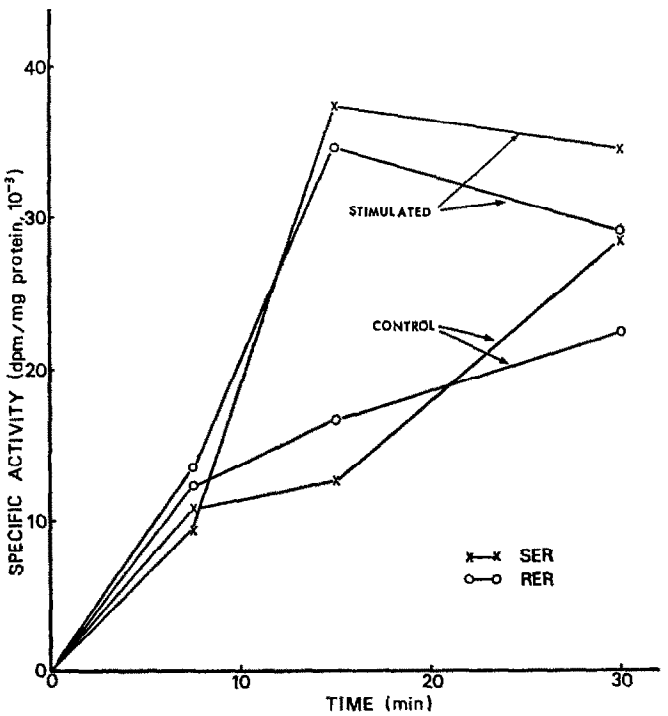

FIG. 4. Effect of 3-MC administration upon incorporation of ^{14}C-amino acids into proteins of smooth and rough ER. Rats were treated with either corn oil or 3-MC, 20 mg/kg, 22 hr prior to the administration of the ^{14}C-amino acid mixture (see Methods). The rats were then sacrificed at the times indicated on the abscissa; the specific activity of the membranal proteins is indicated on the ordinate as dis./min $\times$ 10^{-3}/mg of protein. Each point represents the average of two to four experiments.

The rate of incorporation of the ^{14}C-amino acids into the SER and RER fractions from control and MC-treated rats is shown in Fig. 4. After an incorporation period of 7 min, the specific activity of the RER was slightly higher than that of the SER in both control and drug-treated rats. Fifteen min after administration of the labeled precursors, the specific activity of the control RER was still higher than that of SER. At this time, the incorporation of precursors into SER and RER liver proteins in drug-treated rats was elevated by 3- and 2-fold respectively. Maximum incorporation was achieved by 15–30 min in the drug-treated animals, although no maximum was apparent in the control system for the first 30 min of labeling.*

* It is recognised that changes in the specific activity of the amino acid pools in liver can lead to alterations in the kinetics of incorporation of these precursors in protein. In studies reported elsewhere (M. Yee and E. Bresnick, *Molec. Pharmac.*, in press), we were unable to show any alterations in the pools of the basic amino acids in liver after treatment of rats with 3-methylcholanthrene.

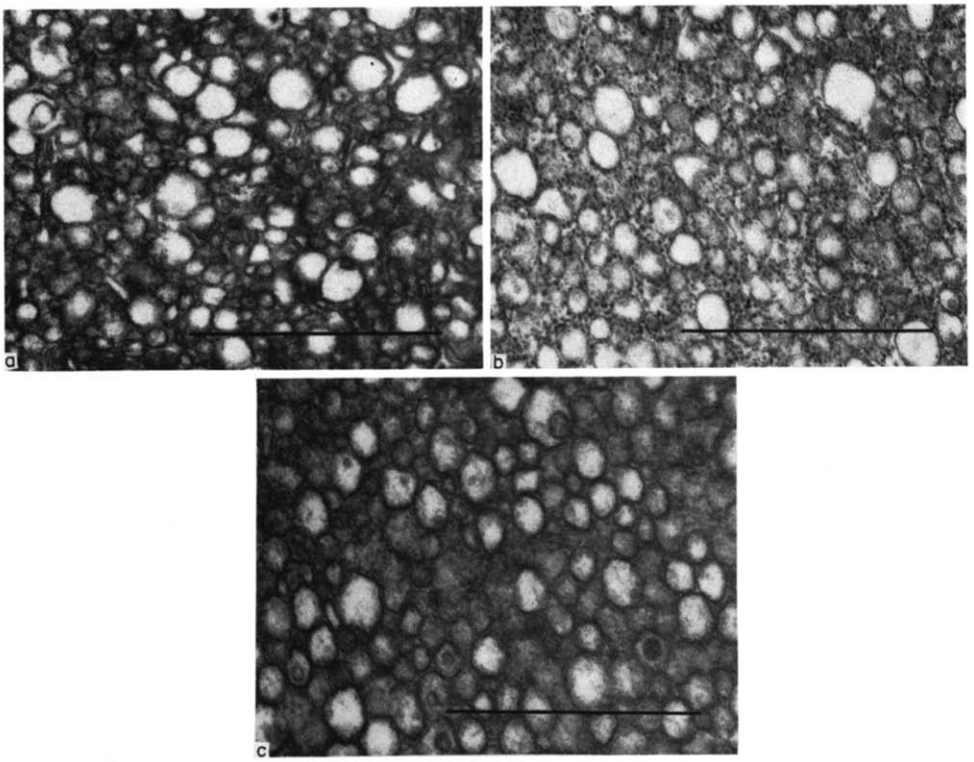

FIG. 1. Electron micrographs of control smooth and rough endoplasmic reticulum (ER) of rat liver. A, smooth ER; B, rough ER; C, rough ER after removal of ribosomes (see Methods). The bar on the electron micrographs represents 0·1 μ.

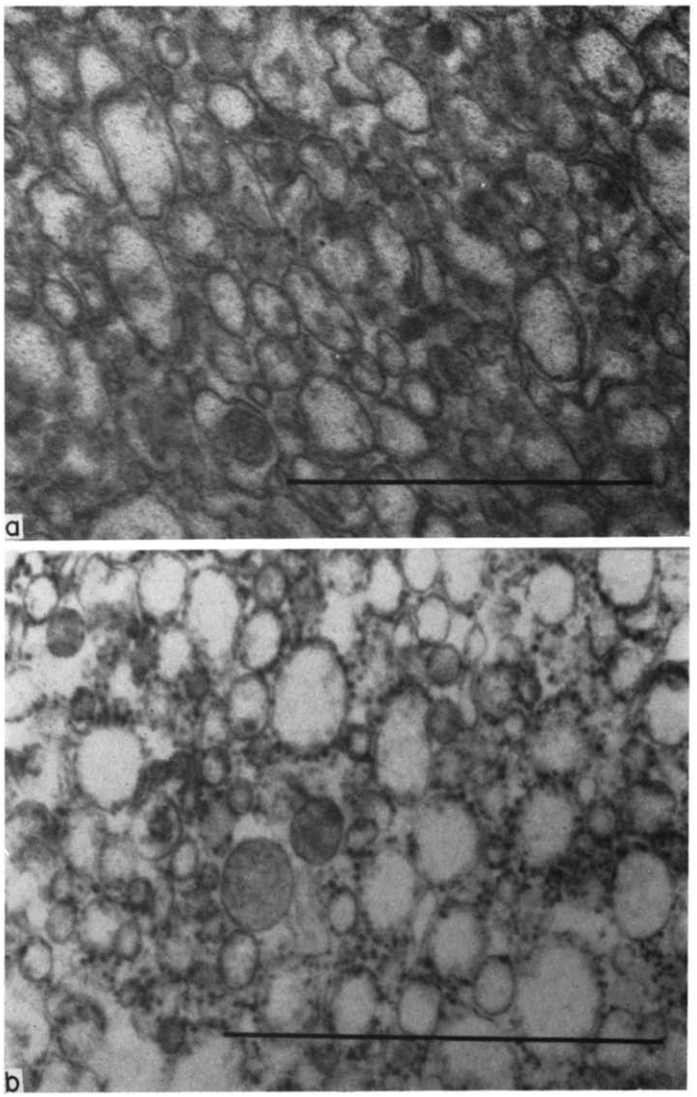

FIG. 2. Electron micrographs of smooth and rough ER from the liver of 3-MC-treated rats. Male rats, 70–100 g, were injected intraperitoneally with 3-MC, 20 mg/kg, and were sacrificed 22 hr later. Details are outlined under Methods. A, smooth ER; B, rough ER. The bar on the electron micrographs represents 0·1 μ.

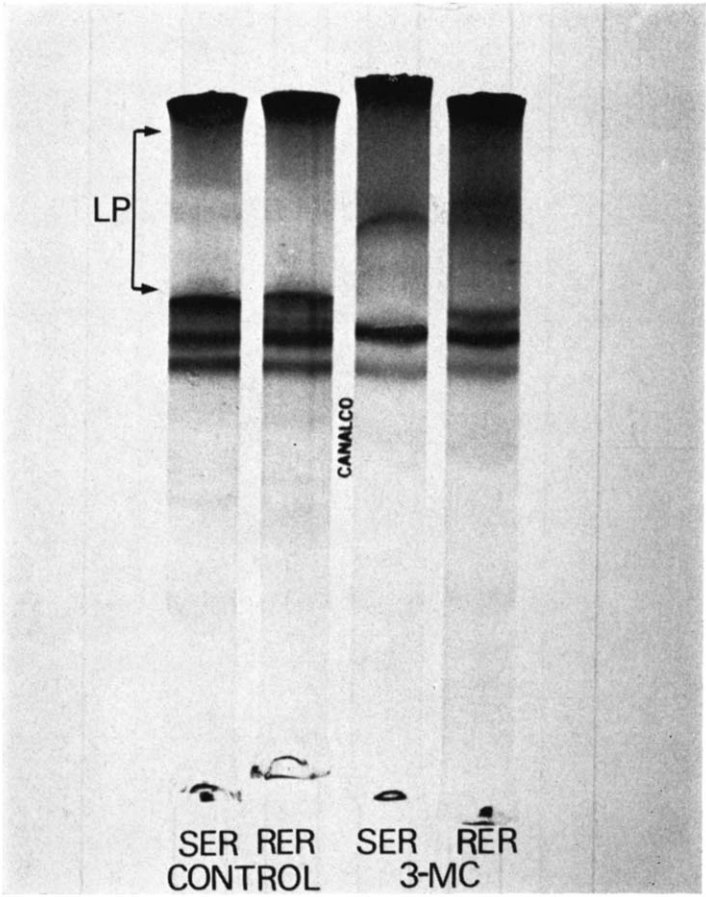

FIG. 3. Disc electrophoretograms of protein fractions from smooth and rough ER. Rats were treated with 3-MC as described in Methods. The livers were removed, the microsomes were separated into the smooth and rough membranal components, and the proteins were "solubilized" as described in Methods. Approximately 900 μg protein in 8 M urea + 1% Triton was applied to each gel and the electrophoresis was conducted as described in Methods. The direction of electrophoresis is from top to bottom. The gels were stained either with Buffalo black (shown in the figure) or with Oil Red O, for lipoprotein. The area of the disc gels which stained positively with Oil Red O is indicated by LP. Although a band of protein appears missing in the profile from the SER of 3-MC-treated rats, this is artifactual due to the composition of the gel.

TABLE 2. DISAPPEARANCE OF ^{14}C-LABEL FROM PROTEINS IN SER AND RER*

Time (hr)	Specific activity (dis./min/mg protein)			
	Control		3-MC-treated	
	SER	RER	SER	RER
5	26,500 ± 2300 (8)*	20,000 ± 2000 (8)	20,000 ± 1700 (3)	16,500 ± 1400 (3)
15	9750 (9500; 10,000)	8500 (7600; 9400)	19,400 ± 2200 (4)	16,000 ± 1900 (4)

* Rats were treated with corn oil or 3-MC as outlined in Table 1. At 5 or 15 hr before sacrifice, animals were injected with 50 μc of a ^{14}C-labeled amino acid mixture. The SER and RER were isolated as described in Methods. The specific activity is expressed as disintegrations per minute per milligram of protein ± standard deviation.

† Number of animals.

The rate of disappearance of the ^{14}C-label from the protein components of SER and RER after drug treatment is shown in Table 2. The first time period was chosen as 5 hr, since Arias *et al.*[2] reported a rapid loss in radioactivity during the first 4 hr, which he attributed to secretion of radioactively labeled serum proteins. At 5 hr after injection of the precursor (Table 2), little change was seen between the control and 3-MC-treated systems. However, after a 15-hr labeling period, the specific activity of the control RER and SER was reduced by 40 per cent, while in the experimental system, the reduction was less than 5 per cent.

The radioactivity profiles of representative gels from control and 3-MC-treated systems, 3 hr after the injection of the ^{14}C precursors, are shown in Fig. 5. Two principal areas of incorporation were apparent, one related to the major bands of the gels (2–4 cm from the top of the gel) and a slower moving area (0·5–2 cm from the top of the gel) which stained weakly with Buffalo black, but strongly with Oil Red O. These areas will be referred to subsequently as the major band and lipoprotein band areas respectively. Variation in the position of the peaks of radioactivity precludes precise statistical quantitative comparisons of these control and 3-MC-treated gels, but it can be seen that 3-MC treatment does cause an increase in the counts per minute in the gel profiles. This change has been observed many times. With the ^{14}C-labeled gels, approximately 87 per cent of the recovered radioactivity is contained within the first 4 cm. The lipoprotein band areas contained 16–23 per cent of the total radioactivity of the gels, while the major band areas contained 20–30 per cent. The protein components, which migrated more rapidly than the major band proteins, did not incorporate a significant amount of radioactivity.

The radioactivity profiles of the gels with proteins labeled with [^{3}H]δ-amino-levulinic acid (ALA) are presented in Fig. 6. The same areas that incorporated the ^{14}C-amino acid label, the lipoprotein band and major band areas, incorporated the heme precursor.

In an attempt to gain additional information about the protein components, an inhibitor of heme synthesis, 3-amino-1,2,4-triazole (AT),[24,25] was administered to rats. In Fig. 7, the radioactivity profiles of only the SER protein components are

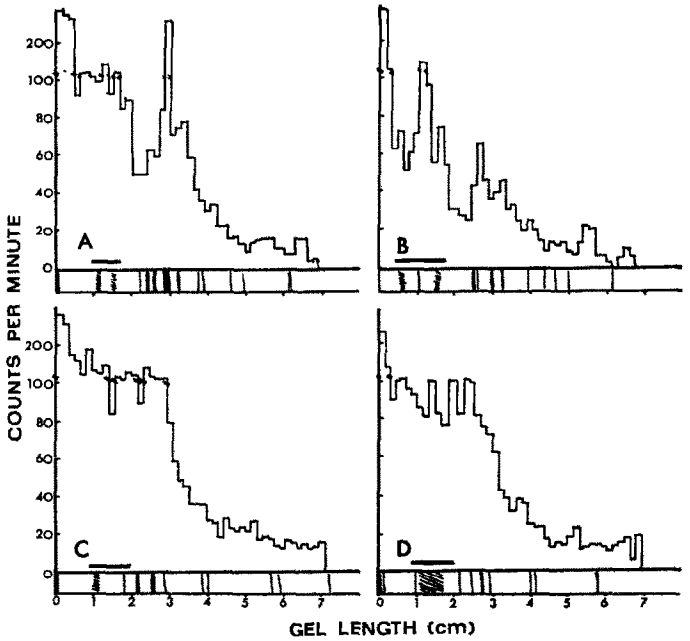

FIG. 5. Profiles of radioactivity of protein components of smooth and rough ER. (See the legend to Fig. 3 and Methods for details.) The ^{14}C-labeled amino acids were injected 3 hr before sacrifice. The direction of electrophoresis is from left to right. The efficiency of counting was uniform across the electrophoretogram. Below each graph is a representation of the pattern of a duplicate gel after staining with Buffalo black. The horizontal bar above the protein bands denotes the area which stained positively for lipoprotein. A, control SER; B, control RER; C, SER from 3-MC-treated rats, 22 hr later; D, RER from 3-MC-treated rats, 22 hr later.

TABLE 3. SPECIFIC ACTIVITY OF FRACTIONS AFTER AMINOTRIAZOLE AT TREATMENT*

Experiment	Isotope	Specific activity (dis./min/mg protein) SER	RER
3-MC	^{14}C-aa	37,300	34,534
3-MC + AT		12,464	18,000
% Decrease		66	47
3-MC	^{3}H-ALA	22,689	24,650
3-MC + AT		2777	4192
% Decrease		88	83

* Rats were injected with 3-MC as described in the legend to Table 1. At 3 hr prior to sacrifice, the AT-treated animals received the drug, 3 g/kg, intraperitoneally. At 15 min prior to sacrifice, the animals received either ^{14}C-amino acid mixture (aa) or ^{3}H-ALA as outlined in Methods.

depicted. The subfractions were isolated from animals that had been pretreated with 3-MC, or with 3-MC and AT, and that had received either ^{14}C-amino acids or ^{3}H-ALA. Note that the inhibitor decreased the radioactivity in the bands uniformly; the reduction was more apparent in the [^{3}H]ALA-labeled samples. The RER gels were

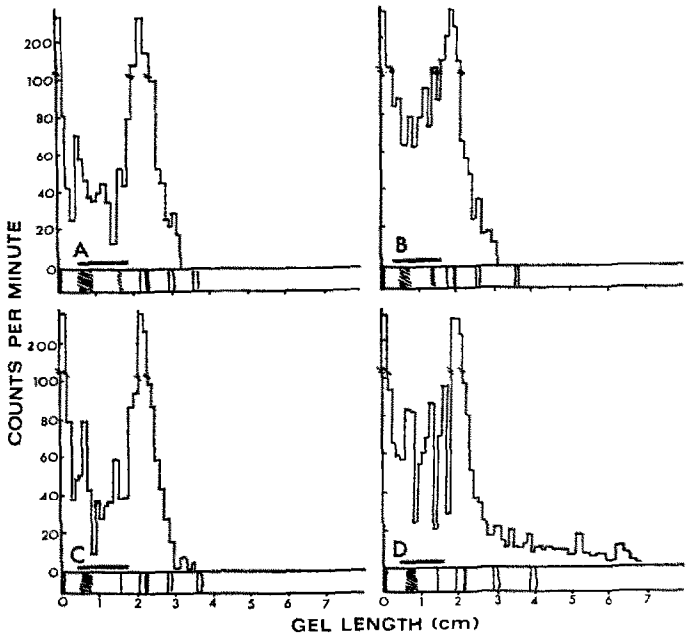

FIG. 6. Profiles of radioactivity of membranal protein components after injection of ^{3}H-ALA. (See the legend to Fig. 5 for details.) The only exception was the injection of ^{3}H-ALA acid into the rats 3 hr prior to sacrifice instead of ^{14}C-amino acids.

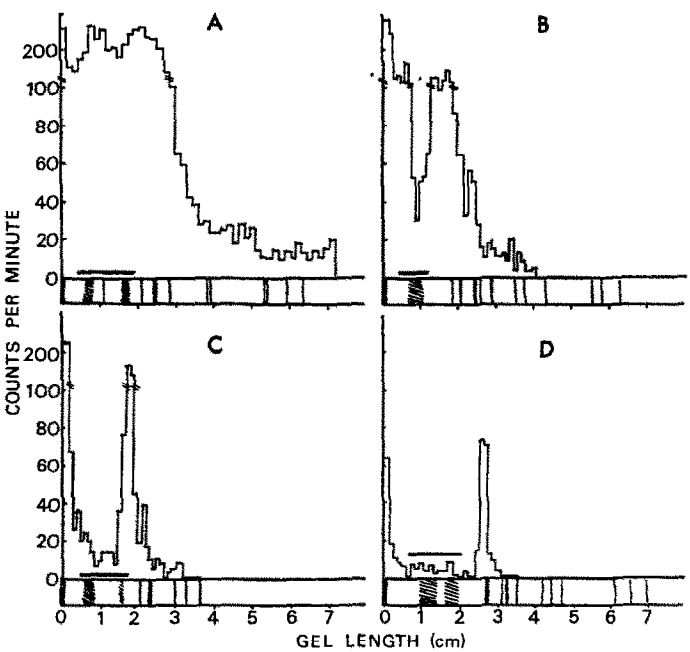

FIG. 7. Profiles of radioactivity of proteins of smooth ER from 3-MC-treated rats with and without aminotriazole (AT) treatment. (See the legend to Fig. 5 for details.) The rats were pretreated with AT 3 hr before sacrifice; the rats were sacrificed 15 min after administration of either ^{14}C-amino acids or ^{3}H-ALA. A, SER from 3-MC-treated rats, labeled with ^{14}C-amino acids; B, SER from 3-MC-treated and AT-treated rats, labeled with ^{14}C-amino acids; C, SER from 3-MC-treated rats labeled with ^{3}H-ALA; D, SER from 3-MC-treated and AT-treated rats, labeled with ^{3}H-ALA.

similar to the SER gels in their response to the aminotriazole. The quantitative aspects of the inhibition are presented in Table 3. The inhibitor reduced the incorporation of ^{14}C-amino acids into SER and RER proteins by 66 and 47 per cent respectively. The incorporation of ^{3}H-ALA into the SER and RER components of the livers from AT-treated rats was reduced by 88 and 83 per cent respectively.

The difference in the AT-induced inhibition of ^{14}C-amino acids and ^{3}H-ALA uptake into ER components is perhaps the result of incorporation of the former into nonheme proteins, the synthesis of which should be unaffected by AT.

DISCUSSION

The morphology of the endoplasmic reticulum serves as a sensitive indicator of the action of a number of pharmacologic agents. For example, the administration of a wide variety of drugs often leads to the development of an enlarged liver and to hypertrophy of the smooth endoplasmic reticulum.[6,26] Furthermore, alterations in the rough endoplasmic reticulum of parenchymal cells represent some of the earliest effects of hepatotoxins and carcinogens in rat liver.[27–31] These alterations are characterized by a breakdown of the lamellar organization of the rough endoplasmic reticulum and the appearance of an increased number of free ribosomes.

After a single injection of 3-methylcholanthrene into rats, the liver does not appear to be substantially enlarged, nor is there any consistent increase in total microsomal protein weight.[32,33] Ichikawa and Yamaro,[34] on the other hand, have reported increases in the protein of both smooth and rough endoplasmic reticulum occurring after 3-MC administration. The results reported here, however, suggest a slight increase in the RER protein per gram of liver tissue 22 hr after the single injection of the polycyclic hydrocarbon. In this regard, Fouts and Rogers[35] observed a small proliferation of the smooth endoplasmic reticulum in liver as a result of 3-MC pretreatment, although in a later work[36] this was not seen. Gram *et al.*[20] found the rough endoplasmic reticulum fraction to be grossly contaminated by smooth membranes. This gross contamination was not seen in the experiments reported in this manuscript.

After the administration of 3-MC, a significant increase in the incorporation of both ^{14}C-amino acids and of ^{3}H-ALA was observed in the fractions of the endoplasmic reticulum. A similar increased incorporation of labeled amino acid into microsomal protein has been reported *in vivo* and *in vitro* by Gelboin *et al.*[10,11] Previously, phenobarbital administration had been shown to produce a profound elevation in incorporation of labeled precursors into microsomal protein.[34,37] Arias *et al.*[2] were also able to demonstrate that the loss of radioactivity from the microsomal proteins occurred in two phases: (1) an initial rapid loss during the first 4 hr, which was presumably associated with the secretion of plasma proteins; and (2) a subsequent slow phase associated with the turnover of membranal proteins. These investigators reported that the retention of the radiolabel in the microsomal protein fraction was dependent to some extent upon the administered amino acid. Thus, a non-reutilized amino acid, i.e. ^{14}C-guanido arginine, gave a shorter $T_{\frac{1}{2}}$ than [^{3}H]leucine. The data reported in Table 3 of this manuscript relate more to the second phase. In this regard, the loss in radioactivity from either the SER or RER was significantly less after administration of polycyclic hydrocarbon. In light of the findings of Arias *et al.*[2] cited above, these data may be interpreted either as indicating a very significant (and almost

complete) reutilization of the labeled amino acids from liver proteins during the experimental time period or a profound effect upon the catabolism of the membranal proteins. The first possibility seems unlikely, since such reutilization of radioactive amino acids would be reflected in the specific activity of the amino acid pools. These pools were previously shown to be unaltered by pretreatment with 3-MC.* A similar interpretation was made by Kuriyama *et al.*[38] with work on phenobarbital-induced enzyme turnover.

The disc electrophoretic technique indicated the presence of three or four major bands of microsomal protein, although approximately 13–17 protein bands were visible. One or more of the main bands appeared to contain hemoproteins, since the same bands incorporated ^{14}C-amino acids and ^{3}H-ALA. Omura and Sato[39] had reported that cytochrome P-450 and cytochrome b_5 were the only hemoproteins present in the microsomal tissue. It had been shown previously that 3-MC administration results in an increase in cytochrome P-450[33,34,40,41] but exerts little effect upon the level of cytochrome b_5.[34] Levin *et al.*[42] have also demonstrated that the majority of ^{3}H-ALA acid label was incorporated into cytochrome P-450.

Garner and McLean[43] have suggested that heme is non-specifically bound to microsomes when labeled heme is mixed with liver homogenates. Such nonspecific binding should, however, result in a more uniform gel pattern and equivalent SER and RER specific activities. The gel patterns show a distinct association with a particular band and the specific activities of the RER were seen to be higher than those of SER in all cases.† Levin *et al.*[42] have reported evidence for an apo-CO-binding hemoprotein and have stated that incorporation of labeled heme into hemoprotein probably does not depend on protein synthesis. A similar finding for cytochrome b_5 has also been reported.[44] Druyan *et al.*,[45] while studying the turnover of heme in rat liver hemoproteins, showed that labeled ALA was a specific precursor for the cytochrome b_5, but not for the apocytochrome b_5. Thus the amount of labeled heme bound in the experiment by Garner and McLean[43] may be related to the amount of apoprotein present.

It had previously been shown[24] that aminotriazole inhibits δ-aminolevulinic acid dehydratase, a key enzyme in heme synthesis. Subsequently, Baron and Tephly[25] reported the blockade of ^{59}Fe incorporation into microsomal heme in AT-treated rats. This compound also prevented the phenobarbital-induced elevation in cytochrome P-450 levels.[41] Since AT was able to affect hemoprotein synthesis without altering protein synthesis in general, Baron and Tephly[25] proposed that the synthesis of heme may be rate-limiting for cytochrome P-450 production.

The data presented herein indicate a 50 per cent reduction of the incorporation of ^{14}C-precursors into microsomal protein of AT-treated, 3-MC-induced rats. Furthermore, over 80 per cent of ^{3}H-ALA incorporation was blocked by AT. The results of Fig. 7 revealed a profound reduction in the incorporation of the heme precursor into many fractions of the disc electrophoretograms, thus suggesting the presence of a large number of AT-sensitive hemoproteins. It would be of interest if these areas correspond to different types of cytochrome P-450.

In summary, the data presented in this manuscript are consistent with the hypothesis that a substantial proportion of microsomal protein is hemoprotein. The

* M. Yee and E. Bresnick, *Molec. Pharmac.*, in press; also, unpublished observations.

† O. Black, Jr., E. T. Cantrell, R. J. Buccino and E. Bresnick, unpublished observations.

administration of 3-MC results in marked alteration in both synthesis and degradation of hemoprotein components of both the smooth and rough endoplasmic reticulum.

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