Induction of Esterases in Various Mouse Tissues by Carcinogens

by

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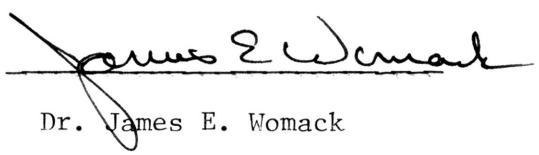

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ABSTRACT

A number of known chemical carcinogens were tested as potential inducers of mouse tissue esterases. Only the nitrosamines, DEN and DPN, altered the electrophoretic profiles of esterases in this study. While nitrosamines had been previously shown to increase esterase levels in mouse plasma, the tissue source and specific genes involved in this increase were not demonstrated. This study indicated that the increased plasma esterase levels were due primarily to increased enzyme production in the liver. More specifically, the product of the ES-11 gene on chromosome 8 was a major contributor to the elevated levels. Closely linked ES-2 and ES-6 genes were not induced by these stimuli. A subcellular study revealed that the increased levels of ES-11 accumulated primarily in the microsomal fraction, probably due to an increased rate of protein synthesis. The time limitations of this study and the difficulty of isolating the product of the ES-11 gene did not allow final verification of this hypothesis.

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INTRODUCTION

In the house mouse, Mus musculus, there are a large number of multiple molecular forms of enzymes known as esterases. The genetics of these enzymes has been studied extensively and genes coding them located on the gene map by using established gene mapping techniques. Twelve esterases have been seperated and identified by differing electrophoretic mobilities and reaction to histochemical staining techniques. These were found to be located on several of the mouse chromesomes, namely chromesome 7, 8, 9, 11, & 14 (1 & 2). Although the chromesomal locations for many of the esterase genes have been determined quite precisely, the exact biological functions of the enzymes are relatively unknown.

Previous experimentation has shown that specific esterases, namely esterase-2 (ES-2) and esterase-6 (ES-6) on chromesome 8, are specifically induced by testosterone (1). This demonstration indicates that specificity exists among the esterases for this particular type of induction. Taking this into account, it seems that the esterases should not be thought of as a non-specific collection of enzymes, but rather a group of closely related enzymes that possibly exhibit a marked functional specificity.

In previous work by Tyndall, et. al. (3), altered plasma esterase profiles were noted after animals were treated with known chemical carcinogens. Increased activity was reported in four bands on the zymogram after exposure to Diethylnitrosamine (DEN), Dipropylnitrosamine (DPN), Dinitrosopiperazine (DNPZ), and Urethane (URTH)(3). This experimentation seems to indicate that esterases may be induced by certain carcinogens;

however, it is likely that the carcinogens listed induce specific esterases rather than all esterases of a particular tissue. Therefore, the specific molecular form and genetic origin of the esterases involved need to be identified. While the altered esterase levels in plasma suggest an increased production, the tissues involved in the induction process were also not noted. By employing more specific biochemical and genetic identification techniques, the specific esterases involved in this induction can be identified and the tissue in which the induction takes place can be determined.

OBJECTIVES

The objectives of these experiments were:

1) to determine the chromesomes and specific genes involved in the induction of esterases by certain chemical carcinogens;
2) to determine in which tissues the induction mechanisms take place;
3) to study other chemical carcinogens as possible esterase inducers.
4) to determine the subcellular location of induced esterases.

MATERIALS AND METHODS

Animals

Inbred strains of mice were used with known profiles of distribution of alleles at esterase loci. The strain utilized in most of the experiments was C57BL/6. This particular strain was used due to its full expression of all esterase genes. One other strain used in surveying the experiment was B6SK, a congenic strain in which several esterase genes from the strain SK have been backcrossed onto C57BL/6. Mice of both sexes were used at approximately 60 days of age.

Carcinogens

The chemical carcinogens used were injected intraperitoneally (IP) over a span of two weeks to allow the induction process to occur. The control animals in each experiment were injected with .01 M phosphate buffer over the same time span. Over the two week period, the total dosages used were as follows: DEN: 4 mg., DPN: 8 mg., 3-methylcholanthrene: 1 mg., Phenobarbital: 4.5 mg.

The phorbol esters, Phorbol myristic acetate (PMA) and Phorbol didecanoate (PDD), were also tested. After a series of toxicity trials, these chemicals were found to not be lethal only if injected subcutaneously. The maximum tolerable dose was determined for both compounds to be .06 mg over a two week span.

Preparation of Samples

After treatment, the mice were killed and tissues were removed.

The blood was heparinized, centrifuged, and plasma was collected. Liver and kidneys were diluted 1:3 with distilled water and homogenized in a glass-Teflon homogenizer. After centrifugation for 30 minutes at 25,000 g and 4°C., the supernatant was collected for further analysis.

Electrophoresis

Starch gel electrophoresis was performed in a Tris-EDTA-borate buffer at pH 8.6. The slots were uniformly loaded at 30 µl of sample per slot and run at 250 V for 17 hours at 4°C. The gel was then sliced horizontally and stained for non-specific esterases (150 ml .025 M phosphate buffer pH 7.0, 0.5 ml alpha-napthyl butyrate dissolved in acetone, and 375 mg Fast BB Blue) for 30 minutes in the dark.

Immunoprecipitation and Radioactive Analysis

Since a highly specific antibody has previously been prepared for ES-2 (Womack, unpublished), a more quantitative test could be performed using immunoprecipitation and incorporation of ^{3}H-Leucine into the mouse's tissues.

After a one week regimen of DEN injections, ^{3}H-Leucine was injected IP and the animals were killed 40 minutes later. Liver samples were extracted, homogenized, and centrifuged in a physiological saline solution. The prepared antibody was added to the supernatant to precipitate out the ES-2 present. The remaining protein was precipitated using trichloroacetic acid (TCA). The two pellets were then dissolved and counted using a liquid scintillation counter. The results were then compared to control animals.

Subcellular Localization

After employing the same experimental regimen as before, the liver supernatant was exposed to differential centrifugation. Samples were run at 800g for 20 minutes to bring down the nuclear pellet which was discarded. The supernatant was then run at 10,000g for 20 minutes spinning down the lysosomal pellet which was saved. The supernatant was spun a final time at 100,000g for 60 minutes and the microsomal pellet and remaining supernatant were saved. The two pellets (lysosomal and microsomal) were suspended in a .01% Triton solution and quick frozen for 30 minutes. After thawing, the samples were centrifuged at 100,000g for 60 minutes and the supernatants were exposed to the aforementioned starch gel electrophoresis technique.

RESULTS AND DISCUSSION

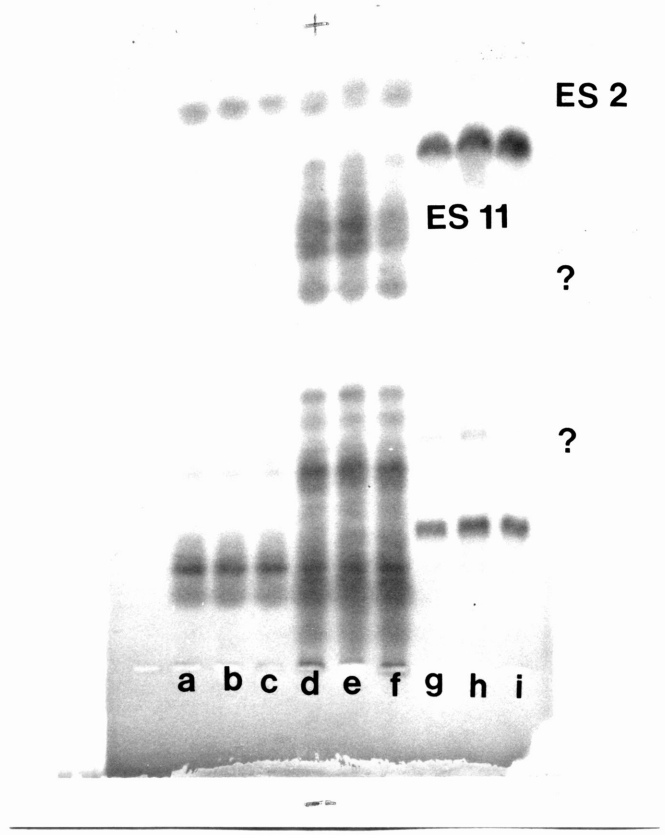
Increased Esterase Bands

Initial experimentation with DEN and DPN displayed the increase in plasma esterase bands described by Tyndall (3); however, only three bands were seen to show a possible increase in plasma with the method utilized (Figure 1). Two of the increased bands are undefined esterases, while the third, known as ES-2, initially appeared to also have a slight increase. While these increases were observed, they were somewhat insignificant when compared to the marked increase in the band known as ES-11 in the liver sample. This increase can readily be attributed to the carcinogen administration since other bands in the liver sample did not show the same effect. The kidney sample did not demonstrate any increases in the induced animals. The presence of the increased esterase production in the liver was anticipated due to its role in detoxification in the body.

Carcinogen Survey

Observable differences in specific esterase bands from the injection of the nitrosamine compounds warranted further exploration of other known chemical carcinogens. In particular, three specific chemicals were selected. 3-methylcholanthrene was selected due to its importance in the Ah complex acivation in the mouse (5) and determination of the esterase's involvement. The phorbol esters, PMA and PDD, having been demonstrated as potent *in vitro* tumor promoters (6), brought up the

FIGURE 1



Zymogram demonstrating Esterases in 3 tissues of the mouse

a: Kidney sample - DPN induced
b: " " - DEN induced
c: " " - control animal
d: Liver sample - DPN induced
e: " " - DEN induced
f: " " - control animal
g: Plasma sample - DPN induced
h: " " - DEN induced
i: " " - control animal

question of whether or not the esterases were intrically involved in detoxification of these large esters. Finally, phenobarbital was studied since it is a known microsomal enzyme inducer in Mus musculus.

After the administration of each chemical and analysis of each animal's tissue samples, no observable differences were noted in any of the esterase bands. These results indicated the specificity of the esterase induction to the nitrosamines and limited the rest of the study to these compounds.

Radioactive Analysis

Since initially ES-2 levels were thought to be increased from nitrosamine administration and a purified antibody to ES-2 was readily available, a more quantitative analysis to determine the role of new protein synthesis in this apparent increase was needed. After incorporation of ^{3}H-Leucine and immunoprecipitation by the ES-2 antibody, samples of the ES-2 pellet and the total protein pellet were counted using a scintillation counter. Ratios of ES-2 to total protein were calculated and comparisons were made between induced and control animals. (Tables 1&2) Results demonstrated that there was no significant increase in the rate of ES-2 synthesis in the induced mice.

Subcellular Localization

By eliminating the possibility of ES-2's involvement with the nitrosamine compounds, the remaining experiments were limited to discussion of the obvious induction of ES-11 in the liver.

Subcellular localizations of liver samples were performed using the differential centrifugation technique described previously to determine

TABLE 1

Counting Results of ^{3}H-Leucine Incorporation
in Control and DEN Induced Animals

Tissue Samples	Total Protein Pellet	ES-2 Pellet	ES-2:Total Protein
Control			
Liver 1	80844	1086.2	.01344
" 2	73414	2086.1	.02842
" 3	35954	1915.0	.05326
Kidney 1	80604	4189.1	.05197
" 2	74234	8337.7	.11232
" 3	82554	10957.2	.13273
Induced			
Liver 1	42614	1307.6	.03068
" 2	53824	2199.2	.04086
" 3	36834	1358.5	.03688
Kidney 1	69844	4144.9	.05924
" 2	66064	8327.1	.12604
" 3	66804	3048.9	.04564

TABLE 2

Arithmetic Means for Control & Induced Samples

Tissue	Control	Induced
Liver	.03171	.03614
Kidney	.09901	.07701

the site of accumulation in the cell. After examination of the zymogram, the most obvious induction of the ES-11 region seems to occur in the microsomal fraction (Figure 2). The band described as ES-2 showed no increases in the induced animals in any of the fractions which supports the previous findings. A band that had not been noted previously showed up in the microsomal fraction of the induced animals only. This band has never been demonstrated in esterase gels before and seems to be unique for the nitrosamine induction in the liver.

Variant ES-11 Strain

In order to prove with greater accuracy that ES-11 was actually being induced with the nitrosamines by the liver microsomes, a variant strain of ES-11 was needed for positive identification. The strain B6SK was utilized due to its electrophoretic shift for ES-11. By performing the same subcellular technique, it was demonstrated that the shifted ES-11 in the B6SK strain was also induced by the nitrosamines in the microsomal fraction (Figure 3). This observation led to positive identification of the esterase ES-11 in the induction process by the nitrosamine compounds, DEN and DPN.

Fig. 2: Subcellular Localization of a Liver Sample

a: Cytoplasmic Enzymes - DEN induced
b: " " - control animal
c & d: Microsomal Fraction - DEN induced
e & f: " " - control animal
g & h: Lysosomal Fraction - DEN induced
i & j: " " - control animal

FIGURE 2

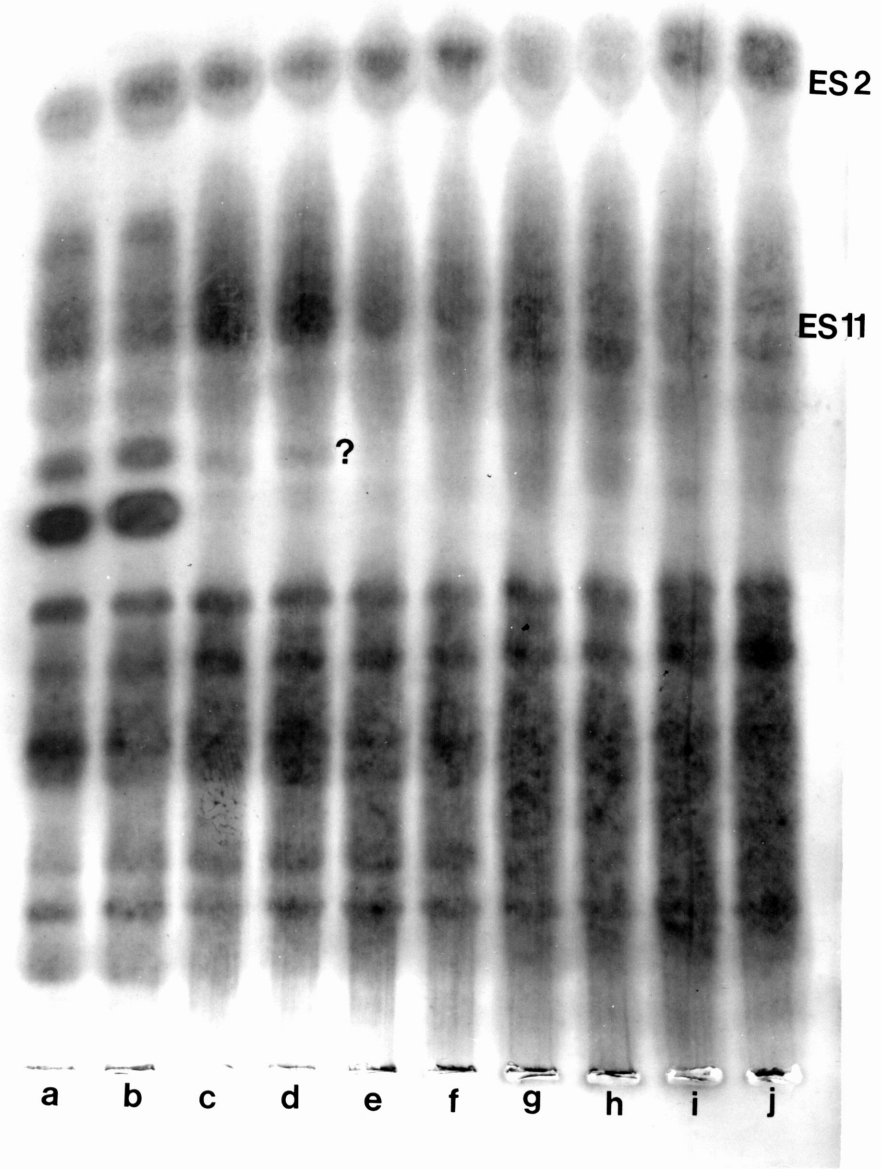
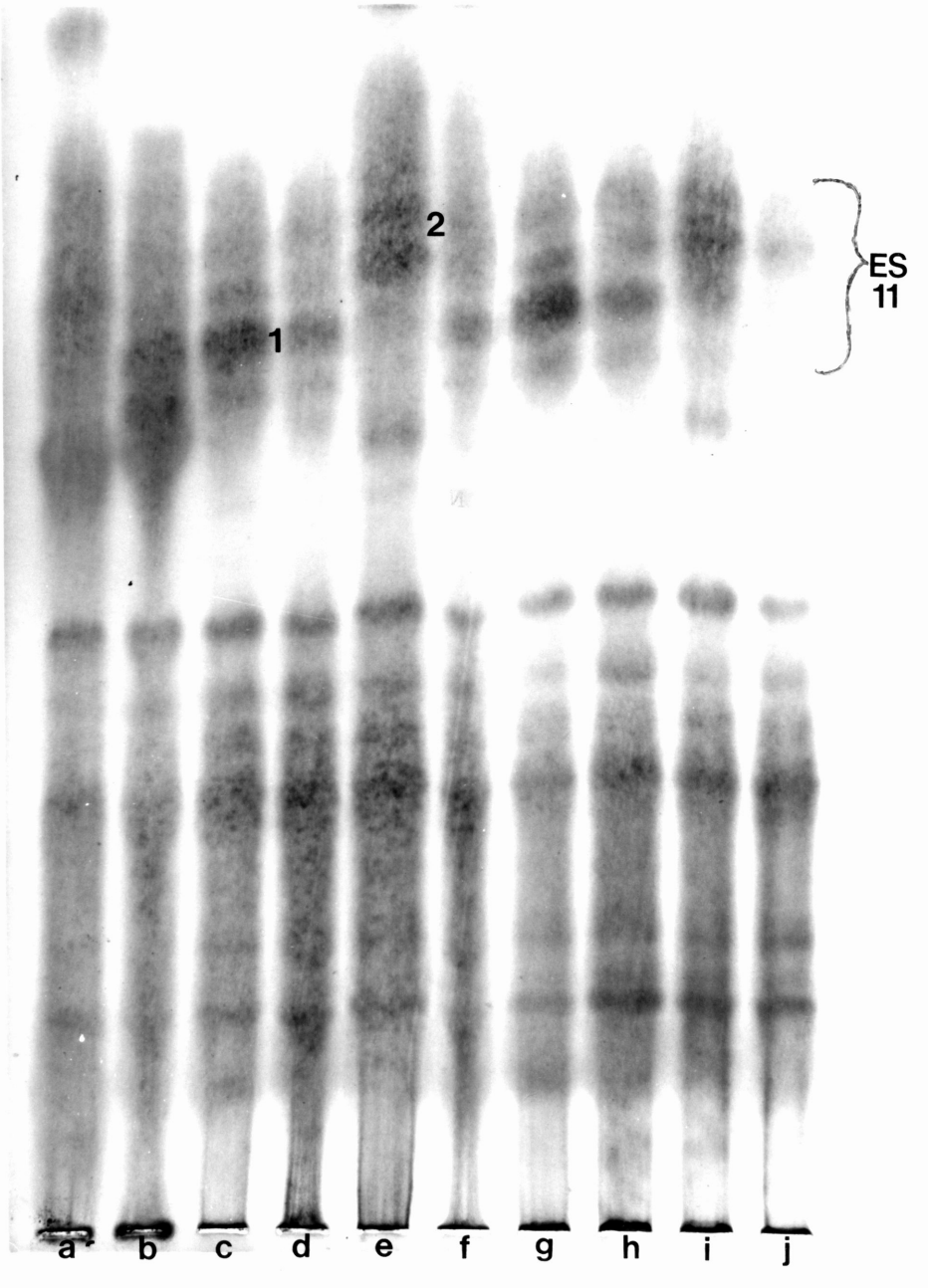


Fig. 3: Subcellular Localization of Liver Samples comparing C57BL/6 and B6SK induced animals.

a: Cytoplasmic Enzymes - DEN induced - C57BL/6
b: " " - " " - B6SK
c: Microsomal Fraction - DEN induced - B6SK
d: " " - control animal - B6SK
e: " " - DEN induced - C57BL/6
f: " " - control animal - C57BL/6
g: Lysosomal Fraction - DEN induced - B6SK
h: " " - control animal - B6SK
i: " " - DEN induced - C57BL/6
j: " " - control animal - C57BL/6

1: Electrophoretic Shift observed in ES-11 in B6SK animals
2: Electrophoretic Shift observed in ES-11 in C57BL/6 animals

FIGURE 3



CONCLUSION

After analysis of known chemical carcinogens, the nitrosamine compounds, DEN and DPN, were shown to cause a definite alteration in esterase profiles. Although these changes were noted in plasma, a more definite change was observed in the liver sample while none was noted in the kidney. These chemicals have a specific effect on the ES-11 esterase whose gene is located on chromesome 8 of Mus musculus. This induction was also noted on an unknown region in the experimental animals only. Closely linked ES-2 and ES-6 genes were not induced by these stimuli. The induction process was found to be mainly occurring in the microsomal fraction of the liver. This increased activity of the microsomes could possibly indicate an increase in the actual rate of protein synthesis for ES-11; however, due to the problem of isolating the ES-11 enzyme, definite proof of this mechanism is difficult to obtain.

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