adrenergic stimulation by isoprenaline. These results are a biochemical explanation for increased beta-adrenergic receptor response of the longitudinal layer found in contractile studies.

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Covalent binding of the proximate carcinogen, 7-hydroxymethyl-12-methylbenz[*a*]anthracene (7-HMBA) to rat liver cytosolic protein via 7-HMBA sulphate

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7 - Hydroxymethyl - 12 - methylbenz[*a*]anthracene (7-HMBA), a potent carcinogen [1] and a major metabolite of 7,12-dimethylbenz[*a*]anthracene (DMBA) in rat liver [2, 3], has very recently been demonstrated to be activated by a dialysed hepatic soluble supernatant fraction (S105) in the presence of a PAPS-generating system to a potent mutagen towards *Salmonella typhimurium* TA 98 [4]. The active metabolite, 7-HMBA sulphate, has been isolated as a sodium salt from the incubation mixture and found to show a potent mutagenicity to the bacteria in the absence of S105 and the PAPS-generating system [4]. Moreover, the bacterial mutagenicity of 7-HMBA or DMBA by a rat liver supernatant fraction (S9) fortified with NADPH- and PAPS-generating systems was much higher than that by S9 fortified with the NADPH-generating system alone [4], strongly suggesting that metabolically formed epoxides of 7-HMBA [5] or DMBA [6] are less important than 7-HMBA sulphate with regard to their mutagenicity.

Sulphotransferase-mediated activation of proximate carcinogens was first proposed by Miller and Miller [7] with *N*-OH-FAA and *N*-OH-MAB, a putative oxidative metabolite of MAB. They have demonstrated that *N*-OH aromatic amine derivatives bind to proteins and nucleic acids in the presence of S105 and PAPS, but failed to detect the active sulphates from the biological systems because of their suggested instability.

7-HMBA sulphate was readily inactivated by S105 in the presence of glutathione to form a non-mutagenic conjugate which was identified as *S*-(12-methylbenz[*a*]anthracene-7-methyl)glutathione [8]. The present investigation deals with the covalent binding of 7-HMBA to S105 protein in the presence of a PAPS-generating system and with the isolation and identification of three carcinogen–amino acid adducts from the protein.

7-HMBA (1 μmole in 1 ml dimethylsulphoxide) was incubated at 37° for 20 min with S105 (35 mg protein, equivalent to 500 mg liver) from male Wistar rats (100–120 g) in the presence of ATP, sodium sulphate (50 μmoles each), magnesium chloride (30 μmoles) and EDTA (1 μmole) in a final volume of 10 ml of 0.1 M phosphate buffer, pH 7.4. Protein was precipitated from the mixture by the addition of an equal volume of 10% trichloracetic acid (TCA), collected by centrifugation, washed successively twice with 5% TCA, acetone and ethanol (10 ml each), dried *in vacuo*, dissolved in 2 N NaOH (2.5 ml), washed with ether, bubbled with nitrogen, and diluted with water (to a final volume of 10 ml). The alkaline solution of the isolated protein showed an intense fluorescence emission spectrum with peak maxima at 408 and 426 nm (relative intensity 1.15 : 1) when irradiated by a 361 nm ray for excitation. The spectrum was superimposable on that recorded with 49.5 nmoles 7-HMBA in the same volume of 0.5 N NaOH. The isolated protein, however, showed no appreciable fluorescence spectrum either when ATP or sodium sulphate was omitted from the incubation mixture or when boiled S105 was used. Data strongly suggest that the hepatic protein

would be covalently modified with the 12-methylbenz[*a*]anthracene-7-methyl moiety of the carcinogen by mediation with cytosolic sulphotransferase.

The reactive metabolite, 7-HMBA sulphate, was isolated as previously reported [4] and determined by ion pair reverse partition HPLC on a Nucleosil $7C_{18}$ ODS column (3.9 mm × 30 cm, 7.5 μ in particle size) in methanol–0.1 M phosphate buffer, pH 7.4 (2:3) containing 2 mM tetrabutylammonium hydroxide at a flow rate of 1 ml/min; under these conditions the sulphate was eluted at 8.4 min as a u.v.-absorbing peak. Data showed that the metabolically formed sulphate ester existed in a protein-bound form rather than a free form; the bound to free ratio was 3.5:1 under the aforementioned incubation conditions.

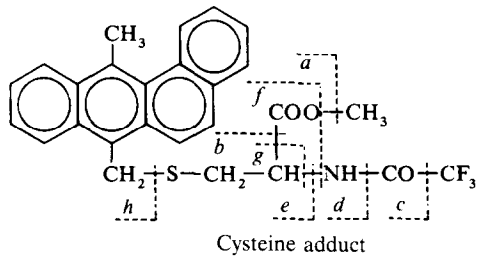
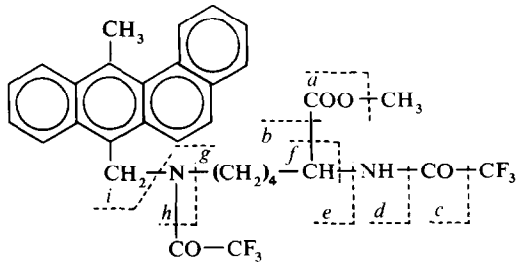
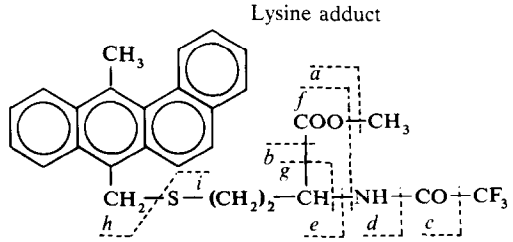
From a large-scale incubation mixture (100 ml) consisting of 7-HMBA, S105 and the PAPS-generating system the S105 protein was precipitated, washed and dried according to the aforementioned procedures. The protein isolated was heated at 100° for 2 hr with 10 N HCl in nitrogen as the gaseous phase. The mixture was concentrated *in vacuo* to dryness, dissolved in 5% ammonia, and poured onto an Amberlite XAD-2 column (1 × 10 cm). The column was washed successively with water (five bed volumes) and 50% (v/v) aqueous methanol (two bed volumes) and eluted with methanol (three bed volumes). The methanolic eluate contained three fluorescent materials appearing at 4.0 (I), 8.3 (II) and 10.2 (III) min in the high-pressure liquid chromatogram obtained on the Nucleosil ODS column in methanol–water (9:1, 0.5 ml/min). Peak materials I, II and III separately eluted from the HPLC column showed single ninhydrin-positive spots at R_f 0, 0.8 and 0.82, respectively, on a cellulose plate (Merck F_{254}) in *n*-butanol–acetic acid–water (4:1:2). Fluorescence emission and u.v. absorption spectra of these three amino acid adducts which were recorded in methanol were not only superimposable on each other but also on the spectrum of 7-HMBA. HPLC data indicated that the acid hydrolysate of the S105 protein contained the adducts in the order of II > I > III.

Adducts II and III were trifluoroacetylated with trifluoroacetic anhydride and then methylated with diazomethane in the standard manner. After being cleaned up on a silica column by HPLC, the trifluoracetyl-methyl derivatives (TFA-Me) of II and III were assigned by MS as the TFA-Me of *S*-(12-methylbenz[*a*]anthracene-7-methyl)cysteine and the di-TFA-mono-Me of *ε-N*-(12-methylbenz[*a*]anthracene-7-methyl)lysine, respectively (Table 1).

The most polar adduct (I) was treated with 10% methanolic sodium hydroxide at 60° for 30 min in nitrogen. A new less polar amino acid adduct was isolated from the reaction mixture by HPLC after treatment with ammonium chloride; the retention time was 8.8 min on the Nucleosil ODS column under the same conditions as used for the separation of the amino acid adducts. The mass spectrum of the TFA-Me of the new adduct indicated it to be *S*-(12-methylbenz[*a*]anthracene-7-methyl)homocysteine (Table 1). Therefore, adduct I was reasonably assigned as *S*-(12-methylbenz[*a*]anthracene-7-methyl)methionine.

Synthetic 7-HMBA sulphate(Na) reacted at a much higher rate with S105 protein at pH 7.4 without any fortifying agents. The maximum covalent binding ratio of the sulphate (0.02–0.5 mM) added to S105 (35 mg protein) was 155 nmoles/mg protein. Amino acid adducts I, II and III were also isolated and identified from the acid hydrolysate

Table 1. Mass spectral data for trifluoroacetyl-methyl derivatives of amino acid adducts from S105 protein treated with 7-HMBA in the presence of the PAPS-generating system

Signal in *m/z* (relative intensity)*		Fragment ion peaks assigned
485 (100, M^+) 470 (13, *a*) 426 (13, *b*) 416 (28, *c*) 388 (13, *d*)	373 (20, *e*) 358 (33, *f*) 314 (25, *g*) 255 (28, *h*) 228 (25)	 Cysteine adduct
606 (100, M^-) 591 (29, *a*) 547 (63, *b*) 537 (36, *c*) 509 (20, *d*) 494 (21, *e*) 468 (18) 435 (50, *f*)	412 (18) 397 (74) 366 (36) 351 (39, *g*) 338 (22) 269 (43, *h*) 255 (45, *i*) 228 (56)	 Lysine adduct
499 (36, M^+) 484 (15, *a*) 440 (22, *b*) 430 (13, *c*) 402 (20, *d*) 387 (30, *e*)	372 (42, *f*) 328 (25, *g*) 255 (100, *h*) 244 (14, *i*) 228 (20)	 Homocysteine adduct (from methionine adduct)

of the 7-HMBA sulphate treated S105 protein in the same manner as applied to the sulphotransferase-mediated binding of 7-HMBA. The synthetic sulphate reacted with L-cysteine, L-methionine and L-lysine at pH 7.4 to yield I, II and III, respectively. It also reacted with L-histidine to yield two ninhydrin-positive adducts. However, they were not found in the acid hydrolysate from S105 protein treated with 7-HMBA sulphate as well as with 7-HMBA in the presence of the PAPS-generating system.

The sulphuric acid ester group of 7-HMBA sulphate appears to behave as a leaving group, so that it may yield a stable 7-methylenecarbonium ion which reacts with the nucleophilic groups of proteins and amino acids. Very recently, both metabolically formed and synthetic sulphates of 7-HMBA and 12-hydroxymethyl-7-methyl-benz[*a*]anthracene (12-HMBA) have been demonstrated to react with DNA in a very similar manner [9]. In addition, sulphates of a series of arylmethanols such as benzyl alcohol, 1- and 2-hydroxymethylnaphthalenes, 1-hydroxy methylpyrene [4], 7-hydroxymethylbenz[*a*]anthracene [10] and 12-HMBA [9] have been shown to be reactive and mutagenic to *Salmonella* while sulphates of methanol, ethanol and *n*-propanol are much less reactive and non-mutagenic [4]. The present study may imply that the reason why DMBA as well as 7-HMBA is a potent adrenonecrogen [11] could be attributable to the facile protein binding of the reactive 7-HMBA sulphate formed in adrenals which are the well-known site of steroid sulphate biogenesis. A study on the metabolism and protein binding of the carcinogens in adrenals is in progress in our laboratory.

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Clofibrate-induced decrease in esterase 1 levels in the serum of the house mouse, *Mus musculus**

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Esterase 1, the so-called albumin esterase, is the principle carboxylesterase (EC 3.1.1.1) isozyme found in the plasma of the house mouse, *Mus musculus* [2]. Concomitant changes in esterase activity and in lipid absorption have been reported by several authors during the past 20 years [3–5]. It is possible that esterase 1 is the chylomicron-associated esterase which is seen in jejunal lymph, and which is transported with triglyceride into the plasma [6]. Clofibrate (chlorophenoxyisobutyrate) has been shown to have strong hypolipidemic effects in animals, including man [7–10]. Here we report the effects of clofibrate feeding upon plasma esterase 1 levels, which indicate another link between lipid levels and this esterase isozyme.

Male and female mice of our experimental line NMRI/Fre.mo-Es-1^f, carrying the allele for esterase 1F (ES-1F), were used in this study. Animals were subject to 12 hr : 12 hr light—dark cycles and had free access to Altromin® pellets and water; the pellets of the experimental group contained 0.5% (w/w) clofibrate. Mice were bled from the tail tips 14 days after the onset of clofibrate feeding. The blood was allowed to clot by standing at room temperature for 30 min, and after centrifugation the supernatant was assayed for total non-specific esterase activity using 4-nitrophenyl hexanoate as substrate. Activities were expressed as units (U)/ml (1 U is defined as 1 μmole of substrate hydrolysed/min at 25° at an initial substrate concentration of 0.13 mmole/l.). Samples of the supernatants were analysed by disc electrophoresis in 7.5% polyacrylamide gels. Levels of esterase 1 protein in the supernatant were assessed by rocket immunoelectrophoresis [11] using antiserum raised against esterase 1. Details of all the above procedures may be found in Otto *et al.* [2].

In female mice, the total serum esterase activity towards 4-nitrophenyl hexanoate fell to about 57% of the control activity following 14 days feeding with clofibrate (Table 1), a statistically significant difference ($P < 0.001$). In male mice, which have a lower control level of serum esterase [2], the results do not show a significant change.

The serum esterases were separated by disc electrophoresis (Fig. 1). The activity of esterase 1, indicated by the staining intensity, was reduced in both males and females following 14 days clofibrate feeding. Activities of other (largely unidentified) esterase bands did not appear to be affected by the treatment.

* This is communication 39 of a research program devoted to the cellular distribution, genetics and regulation of non-specific esterases. The previous paper in the series appeared elsewhere [1].