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Organic solvent soluble sulphate ester conjugates of monohydroxybenzo(a)pyrenes

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Benzo[a]pyrene is a ubiquitous environmental pollutant, which has been shown to occur in exhaust fumes, air, cigarette smoke, soil, water and food [1]. Polycyclic aromatic hydrocarbons such as benzo[a]pyrene produce a wide variety of biological effects including tumour formation in experimental animals and mutagenesis and malignant transformation of cultured cells [1]. Many of these biological effects are mediated via active metabolites. Polycyclic aromatic hydrocarbons are metabolised to several organic solvent soluble metabolites including epoxides, dihydrodiols, diol-epoxides, phenols, quinones and to water soluble conjugates [2, 3]. Recently a high pressure liquid chromatograph system has been developed for the separation of ten benzo[a]pyrene phenols and using this system four different phenols, i.e. 1-hydroxybenzo[a]pyrene, 3-hydroxybenzo[a]pyrene, 7-hydroxybenzo[a]pyrene and 9-hydroxybenzo[a]pyrene have been detected following incubation of rat liver microsomal fractions containing an $NADPH_2$ generating system with benzo[a]pyrene [4]. 3-, 6- and 9-monohydroxybenzo[a]pyrenes are further metabolised by rat liver microsomes *in vitro* [5] and 3-hydroxybenzo[a]pyrene is converted by rat lung microsomes into a metabolite which binds to DNA [6]. Monohydroxybenzo [a]pyrenes are also converted into water soluble glucuronides by a rat liver microsomal system fortified with UDP-glucuronic acid [7]. Recently we have reported that 3-hydroxybenzo[a]pyrene may also be further converted by human, rat and hamster lung cultures into an ethyl acetate-soluble metabolite identified as benzo[a]pyren-3-yl hydrogen sulphate [8]. In this study we report the production of similar ethyl acetate-soluble sulphate conjugates of both 7- and 9-hydroxybenzo[a]pyrene. The route involved is summarized below showing the conversion of 9-hydroxybenzo[a]pyrene into benzo[a]pyren-9-yl hydrogen sulphate.

1. MATERIALS AND METHODS

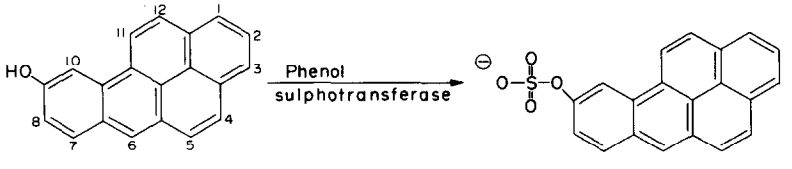
1.1. Materials. Unlabelled or ^{3}H-labelled 9,10-dihydro-9,10-dihydroxybenzo[a]pyrene and 7,8-dihydro-7,8-dihydroxybenzo[a]pyrene were prepared from unlabelled or ^{3}H-labelled benzo[a]pyrene respectively [9]. The ^{3}H-labelled or unlabelled 9-hydroxybenzo[a]pyrene and 7-hydroxybenzo[a]pyrene were prepared by acid hydrolysis of the corresponding ^{3}H-labelled or unlabelled dihydrodiol metabolites. The hydrolysates were extracted with ethyl acetate and the concentrated organic extracts separated by t.l.c. using benzene–ethanol (9:1, v/v). The fluorescent bands migrating with a similar R_f to monohydroxybenzo[a]pyrenes were removed from the plates and their u.v. and fluorescent properties examined. The u.v. spectrum of the 9-hydroxybenzo[a]pyrene obtained by this method was in very good agreement with that reported by Sims [10]. Waterfall and Sims have also reported the acid hydrolysis of the 7,8-diol and the 9,10-diol of benzo[a]pyrene to the corresponding 7- and 9-hydroxy derivatives respectively [11]. Benzo[a]pyren-3-yl hydrogen sulphate was synthesised as previously described [8].

1.2. Lung cultures. Portions of lungs (400–600 mg) from male Wistar albino rats (200–300 g, bred at the University of Surrey) were cultured at 37° for 15–20 hr in 10 ml of Leibovitz L-15 medium with 2 mM L-glutamine and 10% foetal calf serum, penicillin 100 U ml^{-1}, streptomycin 100 $\mu g\ ml^{-1}$ and labelled or unlabelled metabolites [9, 12]. In those experiments with sodium[^{35}S]sulphate, Dulbecco's phosphate buffered saline was used instead of Leibovitz L-15 medium. The medium at the end of the culture was extracted with ethyl acetate and the concentrated organic soluble extracts were separated by t.l.c. in benzene–ethanol (4:1, v/v).

1.3. Enzymic hydrolysis of the products. The major radioactive and fluorescent bands obtained following lung culture of the different phenols were eluted from the t.l.c. plate with spectral grade ethanol (95%), and the fluorescence excitation and emission spectra determined using a Perkin–Elmer MPF-3 fluorescence spectrophotometer. Aliquots were also treated at pH 5.0 for 16–20 hr at 37° with either ketodase (10,000 units, Warner Chilcott Laboratories), aryl suphatase (770 units, type I *Helix pomatia*, Sigma Chemical Co.) in 0.1 M acetate buffer or with 0.1 M acetate buffer alone. The products were extracted with ethyl acetate and the extracts were evaporated to dryness in a rotary evaporator, after having been dried with sodium sulphate. The residue was dissolved in a small volume of ethyl acetate and applied to a t.l.c. plate which was developed in benzene–ethanol (9:1, v/v) and the fluorescent properties of the products examined after elution from the plate.

2. RESULTS AND DISCUSSION

Short-term organ culture of portions of rat lungs with 7-hydroxybenzo[a]pyrene and 9-hydroxybenzo[a]pyrene both resulted in the formation of ethyl acetate-extractable metabolites which migrated with similar R_f values (0.13–0.17 relative to benzo[a]pyrene) to that of benzo[a]-pyren-3-yl hydrogen sulphate in benzene–ethanol (4:1, v/v), and also fluoresced blue under u.v. light (254 nm) [8, 12]. An incubation of [9-^{3}H]hydroxybenzo[a]pyrene with rat lung under similar conditions yielded only one major ethyl acetate-extractable metabolite (M) (Fig. 1), which had similar chromatographic and fluorescent properties to that obtained from unlabelled 9-hydroxybenzo[a]pyrene. The fluorescence excitation and emission spectra of these meta-



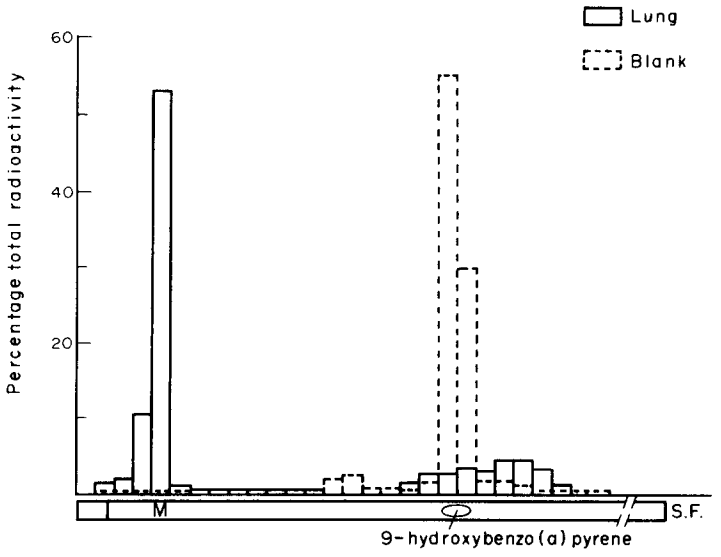


Fig. 1. Ethyl acetate-soluble metabolites from the medium after 16 hr culture of rat lung with [9-^{3}H]hydroxybenzo[a]pyrene. The radioactive products were separated by t.l.c. in a mixture of benzene–ethanol (4:1, v/v). The radioactivity of the products is expressed as a percentage of the total radioactivity on the t.l.c. plate. S.F. indicates solvent front. [9-^{3}H]hydroxybenzo[a]pyrene + lung (——), [9-^{3}H]hydroxybenzo[a]pyrene without lung (– – –). The fluorescent properties of the major metabolite (M) were also examined.

bolites are compared in Figs 2 and 3. The excitation spectra of these metabolites are consistent with their fully aromatic character (Fig. 3), indicating the absence of any diol metabolities. Unlabelled 7-hydroxybenzo[a]pyrene and 9-hydroxybenzo[a]pyrene were incubated with rat lungs in Dulbecco's medium in the presence of sodium[^{35}S]sulphate. The ethyl acetate-soluble products were examined by t.l.c. and the fluorescence characteristics of the material associated with the major radioactive bands determined. The monohydroxybenzo[a]pyrene derivatives each yielded one major radioactive band of a material with a similar R_f and fluorscent properties to those obtained from incubation of the unlabelled metabolites with lung using the Leibovitz L-15 medium. In addition to the main radioactive band, another radioactive band was also observed, but this band was unaltered when the lung was incubated with sodium[^{35}S]sulphate in the absence of any monohydroxybenzo[a]pyrene.

These results suggested that these highly fluorescent ethyl acetate-extractable metabolites were the sulphate

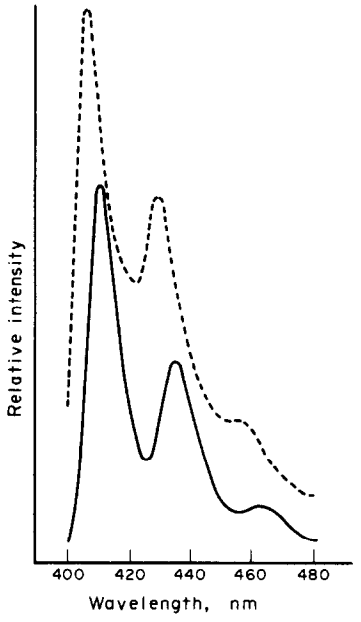


Fig. 2. Uncorrected fluorescence emission spectra of the suspected benzo[a]pyren-9-yl hydrogen sulphate (M) (——) and benzo[a]pyren-7-yl, hydrogen sulphate (· · · · ·) in 95% ethanol. λ_{ex} = 380 nm.

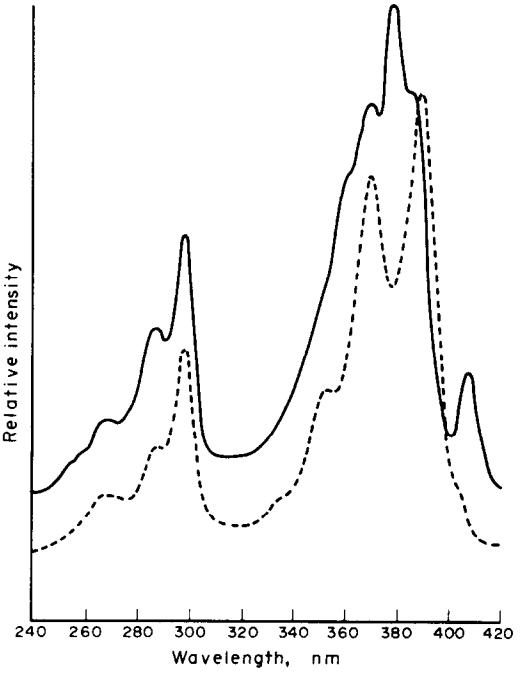


Fig. 3. Uncorrected excitation spectra of the suspected benzo[a]pyren-9-yl hydrogen sulphate (M) (——) and benzo[a]pyren-7-yl hydrogen sulphate (· · · · ·) in 95% ethanol. λ_{em} = 430 nm.

conjugates of their corresponding monohydroxybenzo[a]-pyrene derivatives. This was confirmed by the results of enzymic incubations of the suspected sulphate conjugates of 9- and 7-hydroxybenzo[a]pyrene with sulphatase, when no unchanged conjugates were detected and both compounds yielded products with similar chromatographic and fluorescent properties to the corresponding monohydroxybenzo[a]pyrene derivatives. However, when the suspected sulphate conjugates were incubated with ketodase, unchanged conjugates but not monohydroxybenzo[a]pyrenes were detected. In a recent study when benzo[a]pyrene was cultured with human, hamster or rat lung, benzo[a]-pyren-3-yl hydrogen sulphate was identified as a major ethyl acetate-soluble metabolite by comparison of its fluorescence excitation and emission spectra with those of the synthetic metabolite [8].

Thus monohydroxybenzo[a]pyrenes, one of the major groups of metabolites formed metabolically from benzo-[a]pyrene, are converted into their corresponding ethyl acetate-soluble sulphate esters. These sulphate conjugates, with a mol. wt of approx. 349, which is probably below the threshold for biliary excretion in man [13], would not be expected to be excreted in the bile. The lipophilicity of these conjugates as determined by their solubility in ethyl acetate would also suggest that they will not be readily excreted in the urine. Thus the sulphate conjugates of monohydroxybenzo[a]pyrene derivatives may be retained in man and may require further metabolism before being excreted. If these sulphate esters are further metabolised then the conjugation with sulphate may alter the reactivity of the ring thus changing the orientation of any further metabolism. The increased hydrophilicity of these sulphate conjugates relative to benzo[a]pyrene or their monohydroxybenzo[a]pyrene precursors may also alter the distribution of benzo[a]pyrene related material. The biological importance of the sulphate conjugates of monohydroxybenzo[a]pyrenes and their metabolic products remain to be elucidated.

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Inhibitory effect of xanthines on the incorporation of [^{14}C]valine into proteins and on the secretion of albumin in isolated parenchymal rat liver cells

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Since the observation by Butcher and Sutherland[1] that phosphodiesterases are inhibited by xanthines, these substances have often been added to tissue preparations in order to maintain an increased level of cyclic AMP produced by administration of glucagon or epinephrine. The concentration generally used is 1–5 × 10^{-3} mole l^{-1}. During studies on the role of cyclic AMP in liver protein metabolism we have observed a strongly inhibitory effect of xanthines both on the incorporation of [^{14}C]valine into liver proteins and on the secretion of albumin.

MATERIALS AND METHODS

Female Wistar rats fed *ad lib.* and weighing about 200 g were used. Cells were prepared as described earlier [2]. Aminophylline (theophylline ethylendiamine), caffeine (1,3,7-trimethylxanthine) and theophylline (1,3-dimethylxanthine) were obtained from Sigma, St. Louis, U.S.A. L-[U-^{14}C] valine was from Radiochemical Centre, Amersham, England. Other materials, incubation medium, and incubation were as described [3]. All cell suspensions were preincubated for 20 min. Then the incubations were started by adding [^{14}C]valine (0.25 μC) and xanthine or solvent. The incubation time was 60 min. The incorporation of [^{14}C] valine into liver cell protein (i.e. incorporation into proteins in the cell suspension minus incorporation into proteins in the medium), proteins in the medium and albumin in the medium and also the immunologically measured increase in albumin concentration in the medium were determined as described earlier [2, 3].

RESULTS AND DISCUSSION

Aminophylline, caffeine and theophylline inhibited the incorporation of [^{14}C]valine into liver cell protein with