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BENZO(a)PYREN-3-YL HYDROGEN SULPHATE, A MAJOR ETHYL ACETATE-EXTRACTABLE METABOLITE OF BENZO(a)PYRENE IN HUMAN, HAMSTER AND RAT LUNG CULTURES

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Abstract—Benzo(a)pyrene is metabolised by human bronchial epithelium to ethyl acetate-extractable metabolites which co-chromatograph with 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene, whereas little 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene are formed. Similar results are obtained with human lung except that a major ethyl acetate-soluble metabolite (X) is observed. X has been identified as benzo(a)pyren-3-yl hydrogen sulphate on the basis of enzymic and acid hydrolysis experiments, incorporation of [^{35}S]sulphate and its u.v. and fluorescence spectra which were similar to those of the synthetic metabolite. The u.v. absorption spectrum of benzo(a)pyren-3-yl hydrogen sulphate is comparable with the X_1 metabolite of benzo(a)pyrene, one of the principal metabolites of unestablished identity found in earlier *in vivo* studies. The biological activity of this sulphate ester of 3-hydroxybenzo(a)pyrene is of interest as this metabolite could be extremely persistent in man because of its physico-chemical properties, which may prevent its excretion in the urine and bile.

INTRODUCTION

Polycyclic aromatic hydrocarbons such as benzo(a)pyrene are ubiquitous environmental carcinogens found in the air, food and cigarette smoke as a result of incomplete combustion [1]. In order to exert their carcinogenic action most chemical carcinogens have to be activated [2], thus much work has been devoted to the study of the metabolic fate of these compounds in the body. The first step in the metabolism of polycyclic aromatic hydrocarbons is probably their conversion by a microsomal mixed function oxidase system into reactive epoxides which then undergo one of the following: (i) conversion by the microsomal enzyme epoxide hydrase into the dihydrodiol; (ii) reaction with glutathione to give a conjugate; (iii) spontaneous rearrangement to give a phenol; (iv) attack on a cellular macromolecule which probably initiates the toxic reaction [3].

The dihydrodiols and phenols thus formed may be further metabolised by the microsomal mixed function oxidase system [4, 5] or they may be converted into more polar metabolites by conjugation with endogenous materials such as glucuronic acid and sulphate [6]. In spite of many investigations little is known of the nature and properties of these conjugates of benzo(a) pyrene. Earlier work had shown the conversion of benzo(a) pyrene *in vivo* into four major metabolites X_1, X_2, F_1 and F_2 [7, 8]. F_2 has been identified in these and other studies as 3-hydroxybenzo(a)pyrene. However, the identity of the other metabolites still requires further elucidation.

In a previous study we have reported the presence of a major unidentified ethyl acetate-extractable metabolite (X) formed when rat and hamster lungs were incubated with benzo(a)pyrene [9]. Preliminary results had also shown that X was derived from 3-hydroxybenzo(a)pyrene (II, Fig. 1) rather than any of the other three major primary metabolites, i.e. 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene and 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene [9]. In this study we report the production of X by cultures of human lung with benzo(a)pyrene (I, Fig. 1), X has been identified as benzo(a)pyren-3-yl hydrogen sulphate (i.e. the sulphate ester of 3-hydroxybenzo(a)pyrene) (Fig. 1).

MATERIALS AND METHODS

Materials. Sodium [^{35}S]sulphate (sp. act. 69.7 mCi/m-mole) and [^{3}H]benzo(a)pyrene (sp. act. 5 Ci/m-mole) were obtained from the Radiochemical Centre, Amersham, Bucks., England and the latter diluted with unlabelled benzo(a)pyrene (Aldrich Chemicals) to the appropriate concentration. β-glucuronidase

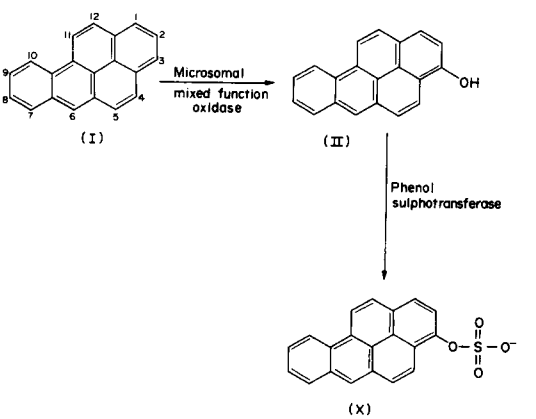

Fig. 1. Formation of benzo(a)pyren-3-yl hydrogen sulphate (X) from benzo(a)pyrene (I) via 3-hydroxybenzo(a)pyrene (II).

(Type H-I, Helix Pomatia), aryl sulphatase (Type H-I, Helix Pomatia) and D-saccharic acid 1,4 lactone were obtained from Sigma Chemical Co. Ketodase was obtained from Warner–Chilcott Laboratories. Leibovitz L-15 medium with L-glutamine and foetal calf serum were supplied by Biocult Laboratories Ltd., Scotland. Glass or aluminium-backed non-fluorescent thin-layer chromatograms (t.l.c.'s), coated with Silica gel G of 0.25-mm thickness, from E. Merck, A-G., Darmstadt, Germany were used. The unlabelled reference compounds 4,5-dihydro-4,5-dihydroxybenzo(a)-pyrene, 7,8-dihydro-7,8-dihydroxy-benzo(a)pyrene, 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene were prepared essentially as described by Sims [10] and their identity confirmed by comparing their u.v. spectra with those reported in the literature.

Preparation of [^3H]*3-hydroxybenzo(a)pyrene.* [^{3}H]3-hydroxybenzo(a)pyrene was prepared by using the 10,000 *g* supernatant fraction from the livers of rats (male Wistar albino rats bred at the University of Surrey) pretreated with 3-methylcholanthrene (20 mg/kg) in corn oil 24 hr prior to sacrifice. The incubation mixture, which consisted of 16 μmoles [^{3}H]benzo(a)pyrene (8 μCi/ml, final concentration), 63 μmoles $NADP^+$, 0.015 moles sodium phosphate buffer (pH 7.4), 834 μmoles glucose-6-phosphate and the equivalent of 19.0 g of liver in a total vol of 150 ml, was incubated in a water bath at 37° for 30 min. After extraction of the incubation mixture twice with equal volumes of ethyl acetate, the organic soluble extracts were pooled, dried with Na_2SO_4 and concentrated to dryness in a rotary evaporator. The residue was dissolved in a small volume of ethyl acetate and applied to a t.l.c. plate and the chromatogram developed in a mixture of benzene–ethanol (9:1, v/v). The bands, with the same R_f value as the authentic 3-hydroxybenzo(a)pyrene standard, were scraped off, extracted with ethyl acetate and purified another twice using t.l.c. plates developed in a mixture of benzene–ethanol (19:1, v/v). The identity of the [^{3}H]-labelled 3-hydroxybenzo(a)pyrene was confirmed by its u.v. spectrum and its mobility and fluorescence characteristics in two solvent systems; benzene–ethanol (9:1, v/v) (A) and benzene–ethanol (19:1, v/v) (B). When chromatographed the [^{3}H]3-hydroxybenzo(a)pyrene gave one major fluorescent and radioactive band which contained $\geqslant$ 96 per cent of the radioactivity.

Synthesis of benzo(a)pyren-3-yl hydrogen sulphate. Chlorosulphonic acid (0.3 g) was added carefully to pyridine (5 ml) and the resulting solution added to 3-hydroxybenzo(a) pyrene, which had been prepared by incubating benzo(a)pyrene with rat liver homogenate [10]. This mixture was heated at 37° for 3 hr, poured into an ice-cold solution of KOH (1 g) in water (20 ml) and extracted with ethyl acetate. The organic soluble extracts were dried with Na_2SO_4 and separated by t.l.c. using solvent systems benzene–ethanol (4:1, v/v) (C), and ethyl acetate–hexane–acetic acid (75:20:5, v/v) (D). The band which fluoresced bright blue in u.v. light (254 nm) and migrated with an $R_f = 0.15$ (relative to benzo(a)pyrene) in both solvent systems, was removed and its u.v. and fluorescent properties examined and compared with the biologically obtained material.

Tissue preparations. Rat and hamster lung and trachea were cultured as previously described [9]. Samples of human lung (53 mg 1.2 g) and stripped bronchial epithelium (22–83 mg), that appeared macroscopically normal after removal at surgery were used. The stripped bronchial epithelium was obtained by holding the bronchial tissue with forceps and easing the epithelium away from the underlying cartilage and supporting tissue with the aid of a scalpel blade. All samples were cultured within 2 hr of removal at surgery 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 either [^{3}H]benzo(a)pyrene (2 μM) or [^{3}H]3-hydroxybenzo(a)pyrene (0.2–1 μM). The samples were incubated for 16–20 hr at 37° and the amounts of ethyl acetate-extractable metabolites determined as described previously [9].

Preparation and examination of metabolite X. For large scale preparations of metabolite X; lungs from 10–16 rats were cultured with unlabelled or [^{3}H]benzo(a)pyrene (2–200 μM) for 15–48 hr. The medium was then extracted with ethyl acetate, dried with Na_2SO_4 and concentrated to dryness in a rotary evaporator. The residue was redissolved in ethyl acetate and purified by t.l.c. using solvent systems (C) and (D). In some experiments after the initial t.l.c. in solvent system (C), the material which co-chromatographed with the synthetic benzo(a)pyren-3-yl hydrogen sulphate was eluted from the plate with ethyl acetate (10 ml), concentrated to dryness in a rotary evaporator, redissolved in a small volume of ethyl acetate, applied to a t.l.c. plate, and the chromatogram developed for 16 cm using solvent system (C). The plate was then removed allowed to dry and rechromatographed in the same solvent system. This procedure was then repeated again. Portions of the major fluorescent and radioactive bands were then eluted from the plate with spectral grade ethanol (2 ml) and their fluorescence excitation and emission spectra examined. U.v. and fluorescence spectra were measured using a Unicam SP 1800 spectrophotometer and a Perkin-Elmer MPF-3 fluorescence spectrophotometer respectively.

Enzymic hydrolysis of X. Aliquots (0.1 or 0.2 ml) of the purified X or [^{3}H]X preparations as prepared above, were incubated overnight at 37°, in a final vol of 0.5 ml–2.0 ml in 0.1 M acetate buffer (pH 5.0) with β-glucuronidase (110 Fishman Units), or ketodase (500–2000 Units of β-glucuronidase), or aryl sulphatase (335–700 Units), to which was added 200 mM D-saccharic acid 1,4 lactone. The products, after extraction with ethyl acetate and separation by t.l.c. in solvent system (C), were visualised under u.v. light, eluted from the plate as described previously and their fluorescence spectra examined. In those experiments using [^{3}H]X, the fluorescent bands were marked, the plate cut into 5-mm segments and the radioactivity determined by liquid scintillation counting as previously described [9].

RESULTS

Metabolism of benzo(a)pyrene by cultures of human lung and bronchial epithelium

[^{3}H]Benzo(a)pyrene was metabolised by human lung cultures to ethyl acetate-extractable metabolites

which co-chromatographed with standard 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene in solvent systems (A) and (C), (Fig. 2a). A major ethyl acetate-extractable metabolite (X) which fluoresced blue under u.v. light and migrated just beyond the origin in solvent system (A) and with an $R_f = 0.15$ (relative to benzo(a)pyrene) in solvent system (C) was also formed (Fig. 2a). Little 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene or 3-hydroxybenzo(a)pyrene was detected (Fig. 2a). The production of metabolites was partially dependent on the weight of lung used for culture. When the weight was between 0.7–1.2 g, the rates of formation of 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene and X were 0.159 (±0.018), 0.057 (±0.005) and 0.133 (±0.016) pmoles g^{-1} min^{-1} respectively, whereas with amounts of lung of 0.15–0.2 g, the corresponding rates for these three metabolites were 1.25 (±0.12), 0.37 (±0.06) and 1.18 (±0.14) pmoles g^{-1} min^{-1} respectively, (all the results are expressed as mean (±S.E.M.) of at least four determinations). The observed decrease in metabolite formation in cultures using larger amounts of lung was partially due to further metabolism of these metabolites as it was also accompanied by a corresponding decrease in ethyl acetate-extractable metabolites. Part of the decrease could also be attributed to the increased uptake of benzo(a)pyrene or metabolites by the larger samples of lung, for example, with lung samples of 1 g only 19 per cent of the initial radioactivity was found in the medium after an 18-hr culture, whereas 52 per cent was found with a sample of 150

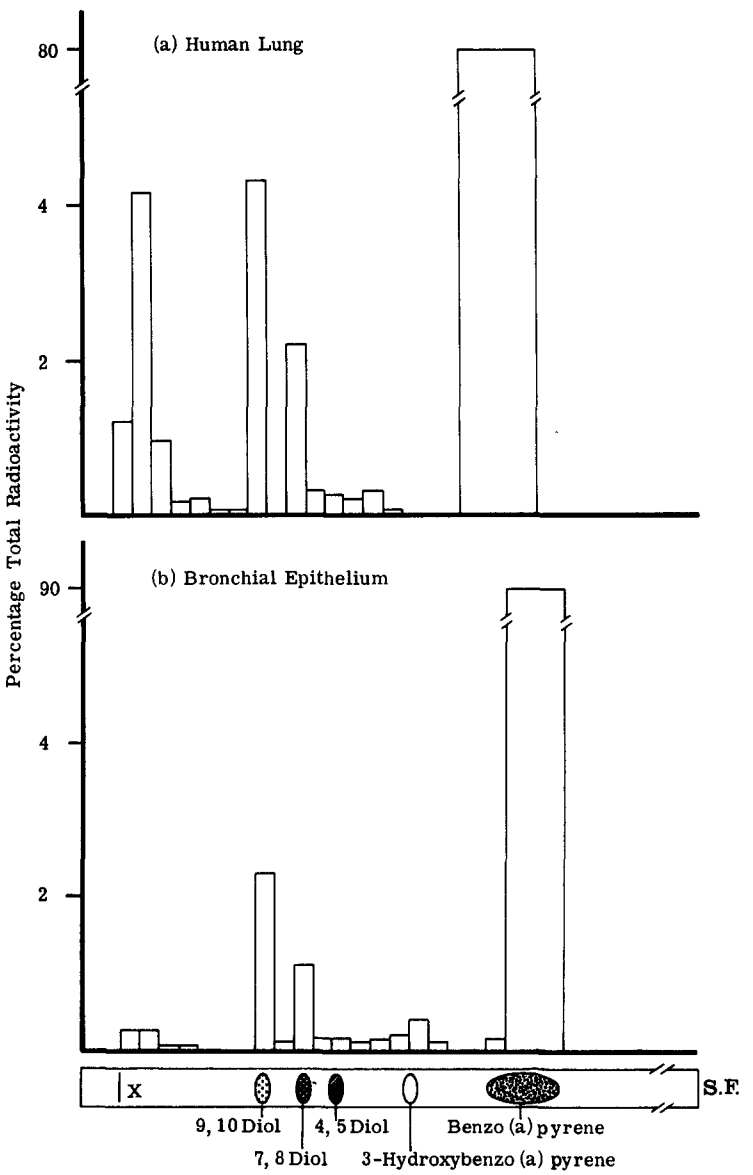

Fig. 2. Ethyl acetate-extractable metabolites from the medium after 16 hr culture with [^{3}H]benzo(a)pyrene (2 μM) of (a) human lung and (b) human bronchial epithelium. The radioactive products were separated by t.l.c. in a mixture of benzene and ethanol (9:1 v/v). The radioactivity of the products is expressed as a percentage of the total radioactivity on the t.l.c. plate. S.F. indicates solvent front.

mg. However, some of the decreased metabolite production could also have been due to inadequate perfusion of the larger specimens of lung.

When stripped human bronchial epithelium was incubated with [^{3}H]benzo(a)pyrene, 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene were again formed (Fig. 2b). Similar to the lung little 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene were formed, but with the bronchial epithelium, in contrast to the lung, little X was detected (Fig. 2b). The rates of formation of 9,10-dihydro-9,10-dihydroxybenzo(a)-pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene by stripped human bronchial epithelium were 2.69 ($\pm$0.31) and 1.42 ($\pm$0.20) pmoles g^{-1} min^{-1} respectively. (Mean $\pm$ S.E.M. of three determinations).

These results are in agreement with our previously reported findings with rat and hamster lung cultures, where benzo(a)pyrene was converted into a major unidentified metabolite (X), with similar fluorescent and chromatographic properties to the major metabolite found in the present studies [9]. When a single hamster or rat lung was incubated with benzo(a)pyrene (2 μM) for 24 hr, 25 and 32 per cent respectively of the ethyl acetate-extractable radioactivity co-chromatographed with X. Formation of X was not detectable when a single rat or hamster trachea was cultured [9]. However, a product with similar chromatographic properties to X was formed, when six rat or hamster tracheas were cultured with benzo(a)pyrene.

Production of X from 3-hydroxybenzo(a)pyrene

When rat, hamster or human lung was cultured with [^{3}H]3-hydroxybenzo(a)pyrene rather than [^{3}H]benzo(a)pyrene a metabolite was also formed with similar fluorescent and chromatographic properties to X in solvent systems (A), (C) and (D) (Fig. 3). On culturing human bronchial epithelium or a single rat or hamster trachea with [^{3}H]3-hydroxybenzo(a)pyrene, under similar conditions, little X was again detected. However, when [^{3}H]3-hydroxybenzo(a)pyrene was incubated with either six hamster or rat tracheas, detectable amounts of X were formed.

Identification of X

(a) *U.v. and fluorescence excitation and emission spectra.* Increased yields of X were obtained by large scale culture of rat lungs with benzo(a)pyrene, as described in Materials and Methods. Following partial purification by t.l.c. either in solvent systems (C) and (D) or by repeated development in solvent system (C) the u.v. and fluorescence excitation and emission spectra of X, 3-hydroxybenzo(a)pyrene and the synthetic benzo(a)pyren-3-yl hydrogen sulphate were compared (Figs. 4, 5 and 6).

The fluorescence emission spectra of the synthetic and biologically formed metabolites were comparable (Fig. 4), and were similar whether human or rat lungs were used to prepare the metabolite. The wavelengths of the two major fluorescence peaks (uncorrected) of

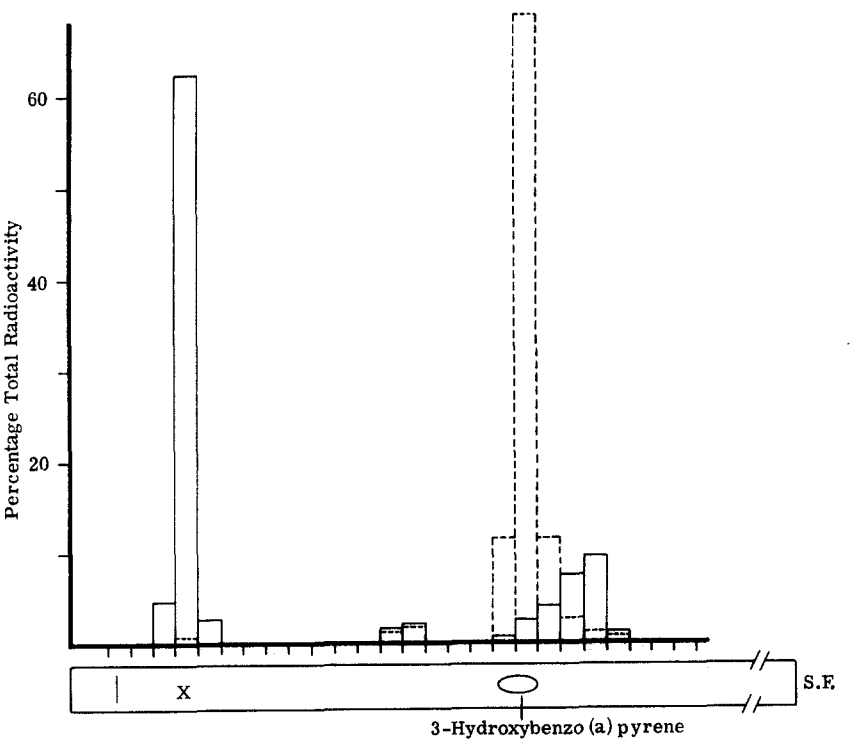

Fig. 3. Ethyl acetate-extractable metabolites from the medium after 16 hr culture of human lung with [^{3}H]3-hydroxybenzo(a)pyrene (0.2 μM). The radioactive products were separated by t.l.c. in a mixture of benzene and ethanol (4:1 v/v). The radioactivity of the products is expressed as a percentage of the total radioactivity on the t.l.c. plate. S.F. indicates solvent front. [^{3}H]3-hydroxybenzo(a)pyrene + lung (——), [^{3}H]3-hydroxybenzo(a)pyrene without lung (– – – –).

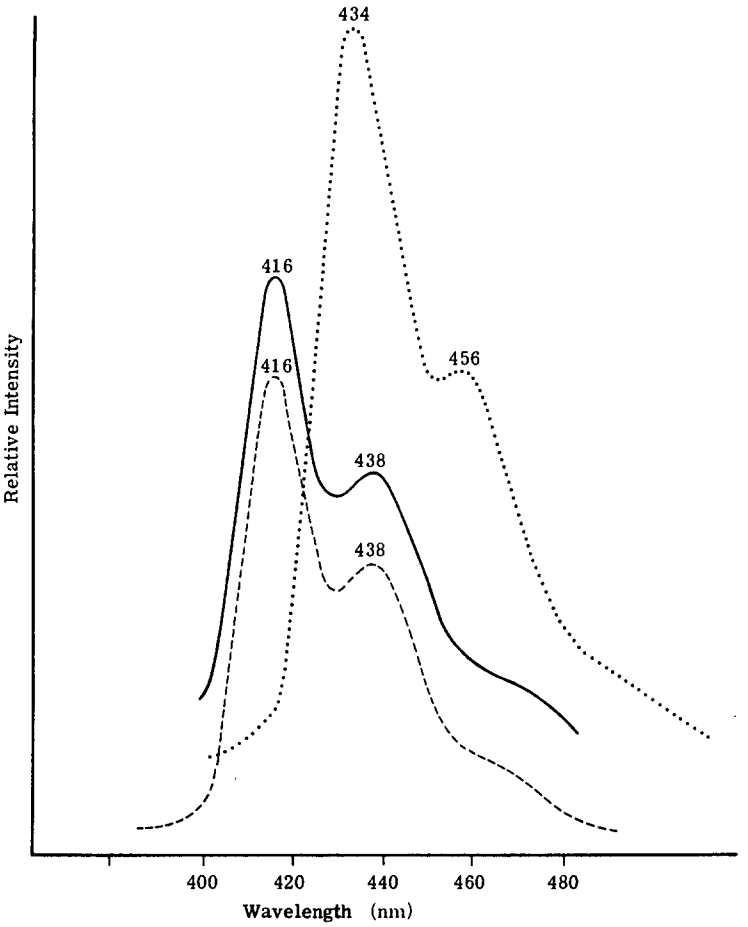

Fig. 4. Uncorrected fluorescence emission spectra of 3-hydroxybenzo(a)pyrene (.....), synthetic benzo(a)-pyren-3-yl hydrogen sulphate (——) and biologically obtained X (– – –) in 95 per cent ethanol. λ_{ex} = 380 nm.

the biologically obtained metabolite with λ_{ex} = 380 nm were 415–416 and 435–439 nm, respectively, whereas those of 3-hydroxybenzo(a)pyrene were shifted to longer wavelengths appearing at 434 and 456–458 nm (Fig. 4). In contrast the fluorescence spectrum of benzo(a)pyrene, when excited at 380 nm, had emission maxima at 404, 428 and 455 nm. Thus the fluorescence spectrum of X appears to be intermediate between that of benzo(a)pyrene and 3-hydroxybenzo(a)pyrene. On addition of alkali the fluorescence spectra of biologically and synthetically prepared metabolites showed no alteration, whereas that of 3-hydroxybenzo(a)pyrene showed a characteristic change with an emission maximum at 522 nm. These results suggested the identity of X as a conjugate of 3-hydroxybenzo(a)pyrene, most probably the sulphate conjugate.

In support of this view the excitation and u.v. absorption spectra of the biologically and synthetically obtained metabolites were very similar, whereas that of 3-hydroxybenzo(a)pyrene was different (Figs. 5 and 6).

The fluorescence excitation spectrum of biologically obtained and synthetic X were almost identical, and were consistent with a fully aromatic system (Fig. 5). Insufficient amounts of biologically derived X were obtained to determine a u.v. spectrum. However, on correcting the fluorescence excitation spectrum of biologically derived X, using the method of Parker [11], the corrected spectrum was almost identical with that of the synthetic benzo(a)pyren-3-yl hydrogen sulphate (manuscript in preparation). The u.v. spectrum of the synthetic metabolite was consistent with the fully aromatic nature of the ring system (Fig. 6).

(b) *Sodium [^{35}S]sulphate incorporation into an X-type metabolite.* In order to confirm that X was a sulphate conjugate, rat lungs were incubated with benzo(a)pyrene (2–20 μM) and sodium [^{35}S]sulphate (80 μCi, 110 μM) for 16 hr using Dulbecco's phosphate-buffered saline and 10% foetal calf serum. Preliminary results using the normal Leibovitz L-15 medium, which contains a high concentration of sulphate (146 mM), showed only a small incorporation of radioactivity into ethyl acetate-extractable products. However, using the modified medium, significant amounts of radioactivity were incorporated into a product which migrated with similar chromatographic and fluorescent properties to X. The radioactivity was split into three bands by developing four times in solvent system (C) (Fig. 7). The radioactive band (F) nearest the origin was also found in lungs incubated with sodium [^{35}S]sulphate in the absence of benzo(a)pyrene, (Fig. 7). The other radioactive bands only occurred when lungs were cultured in the presence of benzo(a)pyrene.

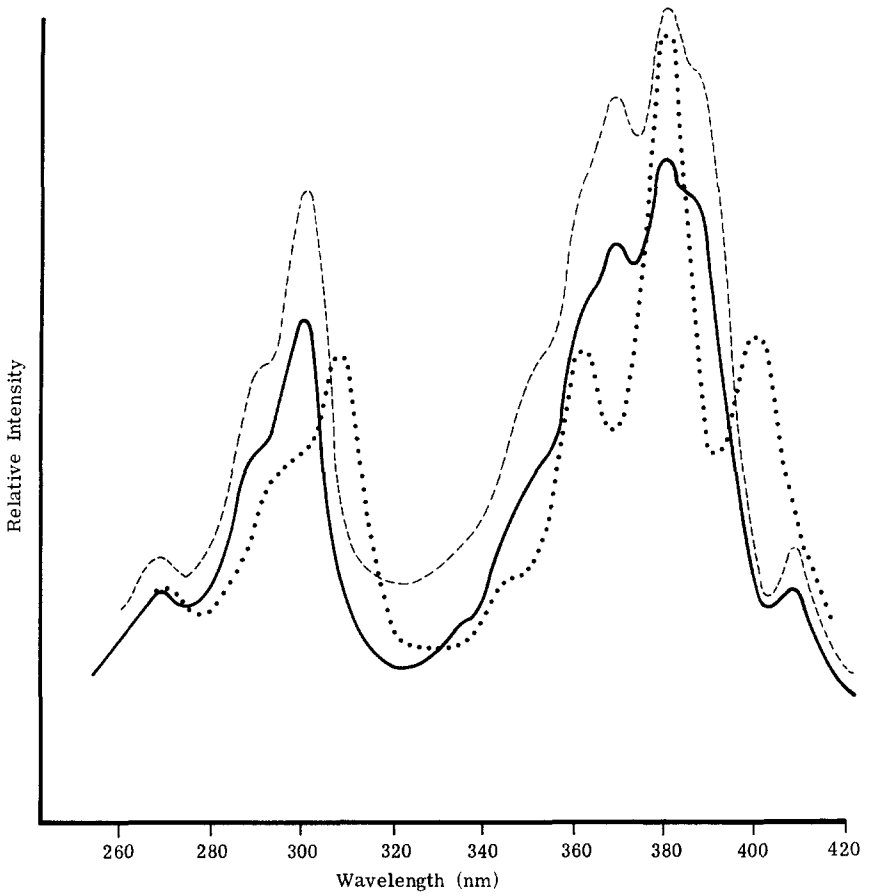

Fig. 5. Uncorrected excitation spectra of 3-hydroxybenzo(a)pyrene (.....), synthetic benzo(a)pyren-3-yl hydrogen sulphate (——), and biologically obtained X (– – –) in 95% ethanol. λ_{em} = 430 nm.

The major radioactive band (X) after elution from the t.l.c. plate with ethyl acetate, concentration to dryness in a rotary evaporator and dissolving in ethanol, had almost identical fluorescent excitation and emission spectra to those of synthetic benzo(a)pyren-3-yl hydrogen sulphate, for example with λ_{ex} = 380 nm the two major fluorescent peaks were at 416 and 438 nm respectively. In contrast to this the small radioactive band (G), which migrated faster than benzo(a)pyren-3-yl hydrogen sulphate (Fig. 7), when eluted from the t.l.c. plate in a similar way and excited at 380 nm had emission maxima at 410, 434 and 461 nm respectively. Preliminary experiments with this metabolite indicate that it is also a sulphate conjugate of a hydroxybenzo(a)pyrene.

(c) *Enzymic hydrolysis of X.* The identification of X as the sulphate conjugate of 3-hydroxybenzo(a)pyrene was further confirmed by the results obtained from enzymic hydrolysis of both biologically derived X and the synthetic conjugate. Aryl sulphatase, with D-saccharic acid-1,4-lactone added to inhibit the β-glucuronidase activity of the preparation, split both compounds to products with chromatographic and fluorescent properties very similar to those of 3-hydroxybenzo(a) pyrene. Incubations of both compounds with ketodase caused neither a breakdown of X nor synthetic benzo(a)pyren-3-yl hydrogen sulphate as witness by the lack of a fluorescent spot on the chromatograms at the R_f of 3-hydroxybenzo(a)pyrene. To confirm this finding, [^{3}H]X was incubated overnight with ketodase, aryl sulphatase or with acetate buffer (pH 5.0). When the hydrolysates were extracted with ethyl acetate and the concentrated extracts chromatographed in solvent system (C), 92, 97 and 34 per cent of the radioactivity migrated with X from the control, ketodase and sulphatase experiments respectively, (Fig. 8). The large loss of radioactivity associated with [^{3}H]X after incubation with sulphatase but not ketodase and the observation that this radioactivity then migrated with the chromatographic properties of 3-hydroxybenzo(a)pyrene (Fig. 8) confirmed the identity of X as the sulphate ester of 3-hydroxybenzo(a)-pyrene. The 34 per cent of radioactivity still co-chromatographing with X after incubation with aryl sulphatase may have been due either to insufficient sulphatase or to the presence of some material not split by the aryl sulphatase. These possibilities could not be distinguished as it was impractical to increase the amounts of aryl sulphatase because of problems of gelling on extraction with ethyl acetate.

(d) *Acid hydrolysis of X.* On gentle warming with 0.1 N HCl, both X and the synthetic benzo(a)pyren-3-yl hydrogen sulphate were converted to a material which co-chromatographed and had similar fluorescence properties to 3-hydroxybenzo(a)pyrene. This further supported the identity of X as the sulphate

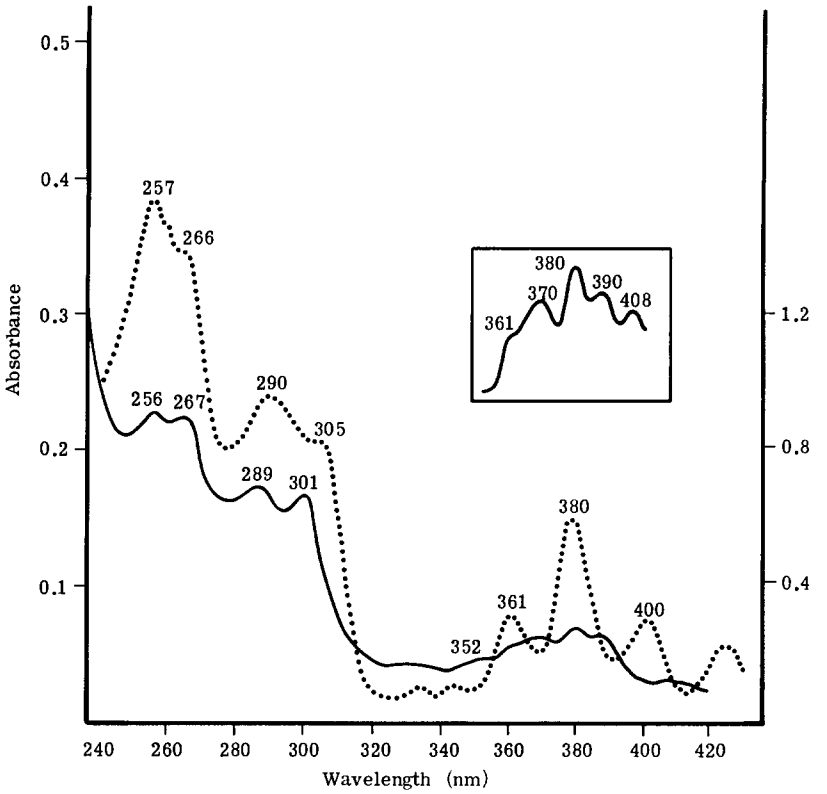

Fig. 6. U.v. spectra of 3-hydroxybenzo(a)pyrene (.....) and synthetic benzo(a)pyren-3-yl hydrogen sulphate (——) in 95% ethanol. The scale on the left hand side and the insert refer to the synthetic metabolite.

conjugate of 3-hydroxybenzo(a)pyrene as Harper has shown that the sulphate conjugate is split under similar conditions, whereas, much stronger acid is required to hydrolyse the glucuronide conjugate of 3-hydroxybenzo(a)pyrene [8].

DISCUSSION

Human bronchial epithelium metabolised benzo(a)-pyrene to ethyl acetate-soluble metabolites which co-chromatographed with authentic samples of 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene and 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene (Fig. 2b). Little 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene, 3-hydroxybenzo(a)pyrene or X was detected (Fig. 2b). These results are similar to those previously obtained with rat and hamster trachea and bronchi [9]. Sims and co-workers have shown the further metabolism of dihydrodiols such as 7,8-dihydro-7,8-dihydroxybenzo-(a)pyrene and 8,9-dihydro-8,9-dihydroxybenz(a)anthracene to the diol-epoxides 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene 9,10-oxide and 8,9-dihydro-8,9-dihydroxybenz(a)anthracene 10-11-oxide respectively [4, 12]. They have also shown that this type of metabolite reacts with DNA in Syrian hamster embryo cells treated with either benzo(a)pyrene or benz-(a)anthracene [4, 13]. These diol-epoxides, but not the parent dihydrodiols, have also been shown to be mutagenic to *S. typhimurium* strain TA 100 without further metabolism [14]. The 7,8-diol in the presence of microsomes reacts with DNA to a 15-fold greater extent than benzo(a)pyrene [15]. These results suggest that the 7,8-diol may be the proximate carcinogen derived from benzo(a)pyrene. Thus the observation that this dihydrodiol is formed by human bronchial epithelium, the portion of the human respiratory tract most susceptible to tumour formation, may be of biological significance. Pal *et al.* have reported the qualitative conversion of benzo(a)pyrene by segments of human bronchus into metabolites with the chromatographic properties of 4,5-diol, 7,8-diol, 9,10-diol and 3-hydroxybenzo(a)pyrene [16].

In this study, in agreement with our previous findings [9], the rate of formation of the dihydrodiols was greater in the bronchial epithelium than in the lung. This may, as we have suggested previously [9], partially explain the different sensitivity of the respiratory tract to carcinogenesis, although many other factors such as larger amounts of further metabolism or binding of the primary metabolites in the lung compared to the bronchus would lead to an underestimation of production of metabolites and a misinterpretation of results. Such conclusions are further complicated by the large variation in the rates of metabolite production with different tissue sizes reported in this study.

Cultures of human lung, when incubated with [^{3}H]benzo(a)pyrene, formed radioactive peaks which co-chromatographed with 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene, 7,8-dihydro-7,8-dihydroxybenzo(a)pyrene and X, but little 4,5-dihydro-4,5-dihydroxybenzo(a)pyrene and 3-hydroxybenzo(a)pyrene were formed (Fig. 2a). These results were similar to those obtained previously with rat and hamster

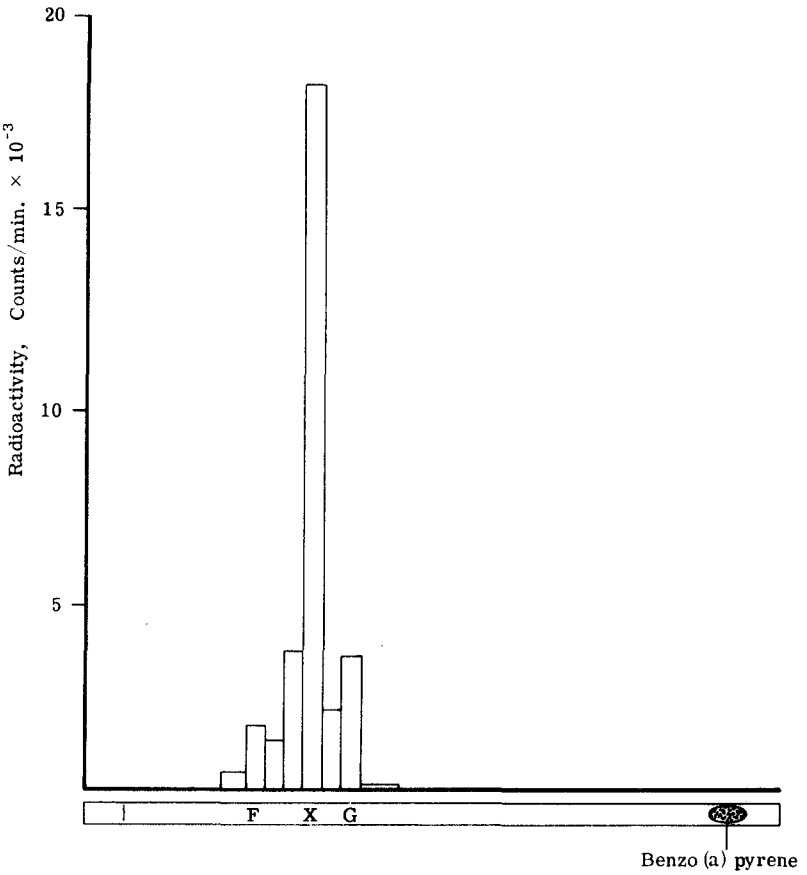

Fig. 7. Incorporation of sodium [^{35}S]sulphate into X. Rat lung was incubated for 16 hr with benzo(a)-pyrene (20 μM) and sodium [^{35}S]sulphate. The ethyl acetate-extractable radioactivity was separated by t.l.c. in a mixture of benzene and ethanol (4:1 v/v), developed four times in one dimension. The fluorescence excitation and emission spectra of metabolites X and G showed they were derived from benzo(a)pyrene. The radioactive band (F) was also observed following incubation of lung with sodium [^{35}S]sulphate.

lungs [9]. X is thus a major ethyl acetate-soluble metabolite formed by human, hamster and rat lung cultures and it is also formed from 3-hydroxybenzo-(a)pyrene (Fig. 3).

X has been identified as the sulphate conjugate of 3-hydroxybenzo(a)pyrene by comparing the results of incubation of the biologically derived material using sulphatase with those using ketodase. Synthesis of benzo(a)pyren-3-yl hydrogen sulphate by sulphation of 3-hydroxybenzo(a)pyrene and comparison of the u.v. absorption, fluorescence excitation and emission spectra of the synthetic and biologically derived materials (Figs. 4, 5 and 6), further supported the identity of X as benzo(a)pyren-3-yl hydrogen sulphate.

Early investigations of the metabolism of benzo(a)-pyrene *in vivo* showed the production of four major derivatives X_1, X_2, F_1 and F_2 [7, 8]. Several studies are in agreement that the identity of F_2 is 3-hydroxy-benzo(a)pyrene [7, 8, 17]. However, much confusion still exists as to the identity of the other metabolites. Sims has proposed that the F_1 metabolite is 9-hydroxybenzo(a)pyrene and arises from the acid decomposition of 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene [18]. Weigert and Mottram suggested the identity of X_1 as 2,3-dihydro-2,3-dihydroxybenzo(a)pyrene which was conjugated at the 3-hydroxyl with an unknown group [19]. However, the u.v. spectrum of X_1, which resembled those of fully aromatic benzo(a)pyrene, with the longest absorption bands showing a bathochromic shift was inconsistent with the proposed conjugated dihydrodiol and it was suggested that X_1 may be a conjugated metabolite of 1-hydroxybenzo(a)pyrene [20]. Harper tentatively identified the X_1 metabolite as the sulphate conjugate of 3-hydroxybenzo(a)pyrene on the basis of its chromatographic behaviour, its instability to acid, its hydrolysis to 3-hydroxybenzo(a)pyrene by takadiastase and synthesis of an ^{35}S-labelled similar metabolite when liver slices were incubated with sodium [^{35}S]sulphate [8]. In a more recent study by Falk and co-workers [17], X_1 was again identified as 3-hydroxybenzo(a)pyrene conjugated with an unknown group. More recently, based on the observation that X_1 is changed into F_1 *in vitro* by treatment with cold alcoholic acid, Arcos and Argus [21] have proposed that X_1 is 9,10-dihydro-9,10-dihydroxybenzo(a)pyrene. The u.v. spectra of X_1 reported by Harper [8] and the u.v. and fluorescence spectra of this metabolite reported by Weigert and Mottram [7] are in close agreement with the corresponding spectra of X repoorted in this study and suggest that X_1 is predominantly the sulphate conjugate of 3-hydroxybenzo(a)pyrene. However because of

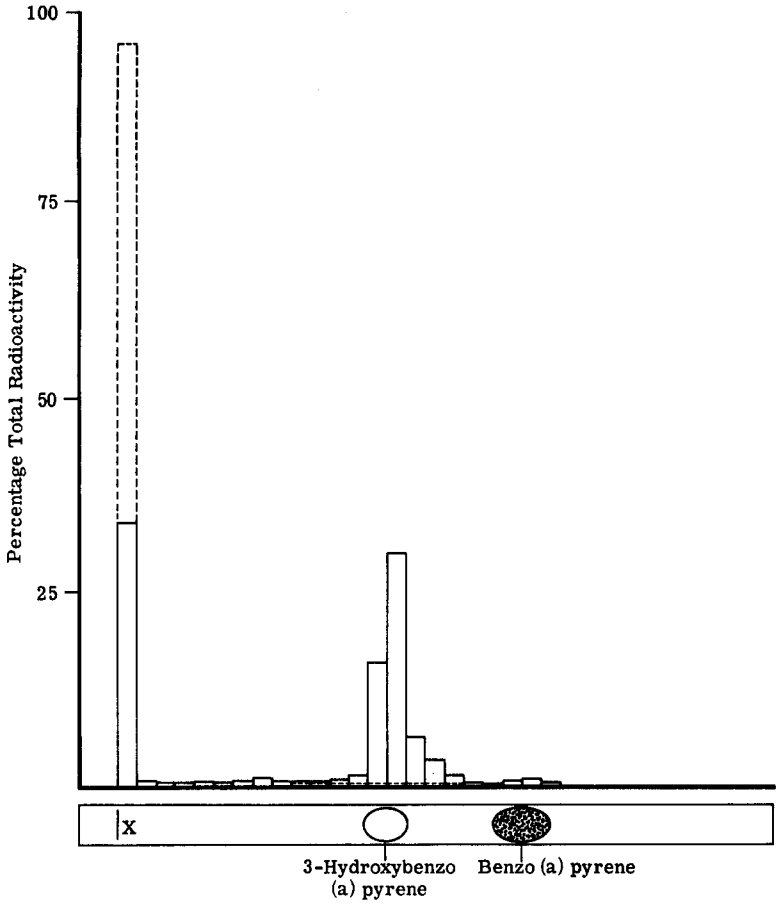

Fig. 8. Ethyl acetate-extractable metabolite pattern after enzymic hydrolysis of [^{3}H]X metabolite, by ketodase (----) or aryl sulphatase (——). The ethyl acetate-extractable metabolites were separated by t.l.c. in a mixture of benzene and ethanol (19:1 v/v). The radioactivity of the products is expressed as a percentage of the total radioactivity on the t.l.c. plate.

the methods of separation used in these earlier studies it is possible that X_1 may have been a mixture of metabolites, e.g. containing other sulphate conjugates of hydroxybenzo(a)pyrene derivatives. Such a mixture for X_1 would appear to be consistent with its spectral and other properties. In the present study the presence of a trace metabolite with very similar chromatographic and fluorescent properties to benzo(a)pyren-3-yl hydrogen sulphate cannot be completely excluded.

Whilst benzo(a)pyren-3-yl hydrogen sulphate was a major ethyl acetate-extractable metabolite formed in lungs, this metabolite was not detected with human bronchial epithelium (Fig. 2b) or by hamster or rat trachea and bronchi [9]. However, when six hamster or rat trachea were incubated with [^{3}H]benzo(a)pyrene or [^{3}H]3-hydroxybenzo(a)pyrene detectable levels of benzo(a)pyren-3-yl hydrogen sulphate were formed. The very low levels of sulphate conjugate formed by bronchial epithelium and trachea may be due either to insufficient formation of 3′-phosphoadenosine 5′-phosphosulphate or to low levels of a phenol sulphotransferase in these areas compared to the lung. The former would seem unlikely because of the synthesis of large amounts of sulphated mucopolysaccharides and glycoproteins in the tracheal and bronchial areas. Some of these differences may however be due to the use of larger samples of lung than those of bronchial epithelium or trachea.

Conjugation of xenobiotics with either glucuronide or sulphate is generally thought of as a detoxication mechanism, converting the compound to a less active, more polar, more readily excretable compound [22]. There are several exceptions to this generalisation and possibly two relevant examples are the conversion of the proximate carcinogen, *N*-hydroxyacetylaminofluorene, to the ultimate carcinogen acetylaminofluorene *N*-sulphate [23] and the formation of certain steroid ester sulphates, which act in the intermediate metabolism of steroids [24].

At the present time it is not known whether the sulphate conjugation of 3-hydroxybenzo(a)pyrene represents a true detoxication or conversion to an active metabolite. However, a consideration of some of the properties of this conjugate, in particular its lipid solubility and molecular weight, suggest the importance of testing its biological activity. Benzo(a)pyren-3-yl hydrogen sulphate appears to be very lipid-soluble as judged by its ease of extractability into ethyl acetate, thus it would not be expected to be excreted in the urine and this appears to be the case as Harper [8] found appreciable amounts of X_1 in

the kidney but only small amounts in the urine. The molecular weight of benzo(a)pyren-3-yl hydrogen sulphate is 349 and thus one would expect it to be excreted into the bile in the rat but not in man where the threshold for biliary excretion is about 500 [25]. Thus the properties of this metabolite suggest its possible retention in man for long periods of time and because of its increased hydrophilicity relative to benzo(a)pyrene and some of the other major ethyl-acetate-extractable metabolites, it could reach targets not normally accessible to these derivatives. It may then be further metabolised by the microsomal mixed function oxidase system or be split by sulphohydrolases present in the tissues acting as a reservoir for 3-hydroxybenzo(a)pyrene which in turn could be activated to a form which binds to DNA [5] or the sulphate conjugate may itself be biologically active.

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