EFFECTS OF CIGARETTE SMOKE CONDENSATES ON CULTURED HUMAN LYMPHOCYTES AND SEPARATION OF BENZO-α-PYRENE METABOLITES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

THESIS

Presented to the Graduate Council of the
North Texas State University in Partial
Fulfillment of the Requirements

For the Degree of

MASTER OF SCIENCE

By

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August, 1979

Ghanayem, Burhan, Effects of Cigarette Smoke Condensates on Cultured Human Lymphocytes and Separation of Benzo-α-pyrene Metabolites by High Pressure Liquid Chromatography. Master of Science (Basic Health Sciences), August 1979, 82 pp., 16 figures, 1 table, bibliography, 91 titles.

Cigarette smoke condensates from all cigarettes tested were found to be potent inducers of AHH enzyme in cultured human lymphocytes and, with the exception of Kent Lights and Carlton CSC's, all were found to be toxic under the experiment conditions. Most of the AHH inducing activity was found in basic and neutral fractions of the 1A1 standard cigarettes.

A radiometric assay of BP metabolites in cultured human lymphocytes was developed in which we were able to separate the primary metabolites and the secondary metabolites from the parent compound (BP) by neutral alumnia HPLC. The primary metabolites were further separated by a selective enzyme hydrolysis and/or reverse phase HPLC.

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CHAPTER I

INTRODUCTION

Chemical carcinogenesis was first discovered in man in 1775 by the British physician Percival Potts, when he correctly identified the high incidence of scrotal cancer as an occupational disease among the chimneysweeps of London. Prolonged exposure to soot and coal tar was assumed to be the cause (52). Chemical carcinogens are non-viral and non-radioactive substances, which have been shown to induce cancer in a wide variety of animals and to cause cellular transformation in cultured tissues (12,21). The known chemical carcinogens comprise a large and structurally diverse group of synthetic and naturally occurring organic and inorganic compounds with various species and tissue selectivities (10,29,30,57).

Epidemiologists consider that 70-90% of human cancer, excluding skin cancers, are caused by environmental chemical pollutants such as polycyclic aromatic hydrocarbons (PAH) (44). Among the chemically induced cancers, lung cancer is one of the leading causes of death and its incidence is increasing dangerously throughout the world. The number of deaths in the United States from lung cancer in 1975 was estimated at 81,000 (13), and if current trends continue, it is expected that 295,000 new cases of lung cancer will occur in the year 2000 (24).

Prospective and retrospective studies from all around the world are in agreement that cigarette smoking is, by far, the most important single contributing factor to the increased risk of developing cancer of the respiratory tract in general, and the lung in particular (24). Statistical evidence from Finland (39), England (18), U.S.A (28), and France (17), and histological evidence through the systemic study of the bronchial epithelium of smokers and nonsmokers (2,3,4), all supported the role of cigarette smoking in the causation of lung cancer.

Accordingly, the Advisory Committee of the U.S. Surgeon General has studied more than 100 prospective and retrospective reports, from more than 15 countries, and summarized the data in the 1964, 1967 and 1972 reports (54,58,59) as follows:

> Cigarette smoking is causally related to lung cancer, and the magnitude of the effect of cigarette smoking outweighs all other factors, with the risk of developing lung cancer increasing with the duration of smoking, is directly proportional to the number of cigarettes smoked per day, and is diminished by discontinued smoking. In comparison with nonsmokers, the average male smokers of cigarettes have approximately a 9-10 fold increased risk of developing lung cancer and heavy smokers at least a 20-fold increased risk. The risk of developing lung cancer for the combined groups of pipe smokers, cigar smokers and pipe and cigar is greater than for nonsmokers, but less than for cigarette smokers. With regard to cancer at other sites, the reports stated that pipe smoking appears to be causally related to lip cancer, and cigarette smoking is a significant factor in the causation of cancer of the larynx.

The type of cigarettes smoked may also influence the risk levels. The risk of lung cancer (squamous cell carcinoma and small cell carcinoma) is less in smokers of filter cigarettes than in those who smoke nonfilter cigarettes (62,63,64) probably due to the lower yields of nicotine and tar in the filter cigarettes.

The following two figures, reproduced from Wynder et al. (63), show that the relative risk of lung cancer correlates with the number of cigarettes smoked per day (Figure 1) and that filter cigarettes are less of a carcinogenic when compared to nonfilter cigarettes (Figure 2).

Figure 1.

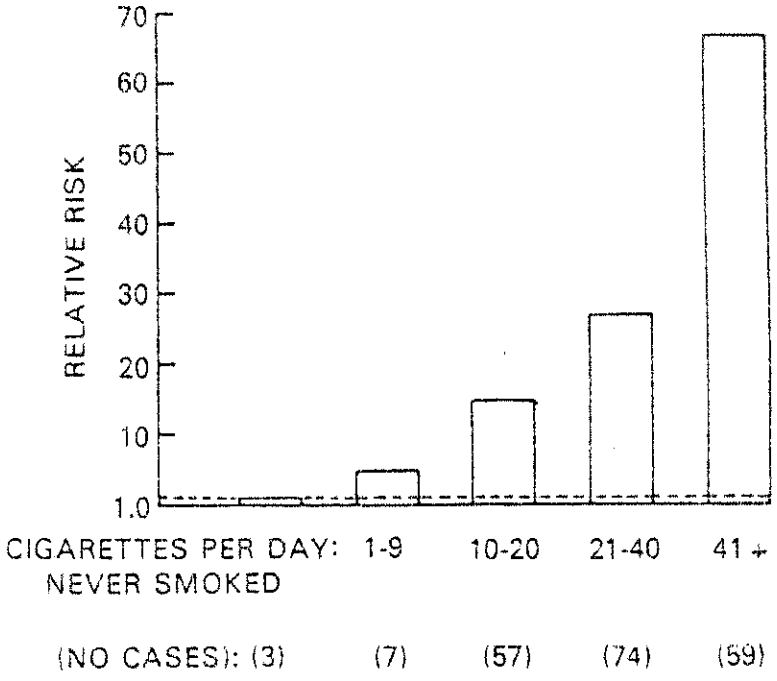
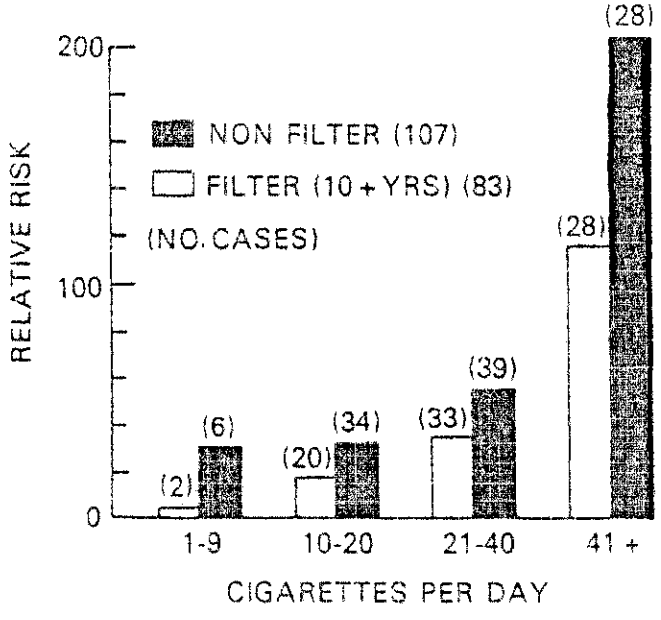


Figure 2.



During tobacco smoking, organic matter burns incompletely resulting in the formation of tobacco smoke. The smoke that emerges from the tobacco product through the mouthpiece during puffing is known as the mainstream smoke. The smoke that comes from the burning cone and from the mouthpiece during puff intermissions is known as the sidestream smoke. Primarily, mainstream smoke is inhaled by a smoker, and hence has gained most of the attention in studying tobacco carcinogenesis.

Tobacco smoke is a two-phase system consisting of a vapor (gas) phase and a disperse (particulate) phase. Cigarette smoke condensate (CSC), which is called tar by some people, is the mixture of smoke constituents (mostly the particulate phase) that is collected by condensation at low temperatures.

The smoke condensate of cigarette, cigar and pipe smoke are carcinogenic to the skin of mice, the bronchial epithelium of dogs and rats and the connective tissue of rats, as demonstrated by more than 100 studies from more than 10 countries (62,31). The dose-response effects observed when CSC is applied to epithelial tissues are quite comparable with those observed with pure carcinogenic compounds (6).

Several investigators have fractionated CSC and bioassayed the main fractions as well as the neutral and weakly acidic subfractions (16,19,32,42,60,62). The CSC of the standard 1A1 low nicotine cigarettes was fractionated by Swain et al. (56) into acidic, basic and neutral fractions which were further divided into 12 subfractions. Those 12 subfractions as well as the original and the reconstituted material have been studied by different investigators (35,38,53).

Polycyclic aromatic hydrocarbons (PAH) which are products of incomplete hydrocarbon combustion are found in polluted city air (20), in smoked food (41), in water and in tobacco smoke (62), where they represent the class of compounds credited with the highest carcinogenic activity (32). Because CSC contains large amounts of PAH, and because many of those PAH's present in CSC are powerful carcinogens in experimental animals, they became logical suspects as the carcinogens causing lung cancer in humans due to cigarette smoking.

Xenobiotics including PAH's are metabolized in the body by a membrane-bound mixed function oxygenase system known as aryl hydrocarbon hydroxylase (AHH) or benzo-a-pyrene hydroxylase. AHH is part of the endoplasmic reticulum and requires NADPH and molecular oxygen to function. This system includes a complex of enzymes with NADPH cytochrome C reductase, cytochrome P-450, cytochrome P-448, a lipid factor and a cytochrome P-450 reductase (50).

AHH has been found in most mammalian tissues including liver, skin, lung, intestine, kidney, placenta, lung alveolar macrophages, monocytes, and lymphocytes (15). With regard to the activity of this enzyme system, it varies in different tissues and is generally very low in most tissues, with the highest activity being located in the liver (15). AHH is inducible by a wide variety of exogenous (PAH, CSC, phenobarbital, pesticides) as well as endogenous (steroidal hormones) compounds.

There is a dose-response relationship between the chemical inducers and the levels of AHH activity, and it is thought by some

that inducibility (the ratio of induced to basal activity) is related to the susceptibility to lung cancer, or may be employed in the assignment of individuals to various AHH genetic groups. Kellerman et al. (36) reported a trimodal distribution of AHH inducibility in the normal human population, with 53% in the low inducibility group, 37% in the intermediate group and 10% in the high inducibility group. Fifty patients with bronchogenic carcinoma were subsequently compared with 46 patients having other types of tumors and with 85 healthy controls (37). They concluded that a person having the intermediate inducibility has a 16 times increased risk and a person having the high inducibility has a 36 times increased risk of developing lung cancer. Other claims of laryngeal carcinoma (5), bronchogenic carcinoma (9), and renal tumors (33) associated with the high AHH-inducibility have also been reported.

AHH activity has been implicated as playing a role in carcinogenesis, teratogenesis, and mutagenesis by PAH and in drug activation and detoxification (1,12,14,23,34,50). Because PAH are found in cigarette smoke and because the incidence of lung cancer parallels the extent and duration of smoking, and the nicotine/tar yield of the cigarettes smoked, AHH possibly plays an important role in the development of cigarette smoke induced cancer.

The procarcinogen benzo-α-pyrene (BP) is a prototypic PAH, which is a universal air pollutant, and it is estimated that 2,000 tons of BP are put into the air of the U.S. each year (44).

The metabolism of BP has been studied in both microsomal (endoplasmic reticulum) preparations and in cells and various organs from several animal species. Polycyclic aromatic hydrocarbons such as BP are generally considered to be carcinogenic in man and animals only after metabolic activation by AHH enzyme to reactive electrophilic intermediates. These are capable of covalent binding to protein and nucleic acids, a step which apparently precedes tumor initiation (7,22,25,51).

BP metabolism is a complex series of reactions, some of which occur simultaneously. BP is initially oxygenated by AHH enzyme to epoxides (26) and/or phenols and quinones (23). These metabolites may be further metabolized to dihydrodiols by hydratases (49). Epoxides and hydroxylated metabolites may also be further metabolized to inactive, water-soluble, urine excretable conjugates of sulfates (11,48) by sulfotransferases, glucuronides (46) by UDP-glucuronyl transferase and glutathione (8,47) by glutathione-S-transferase enzymes. Epoxides may bind to cell macromolecules where they can cause alteration in cell metabolism and possibly induce malignant transformation (27,40,43).

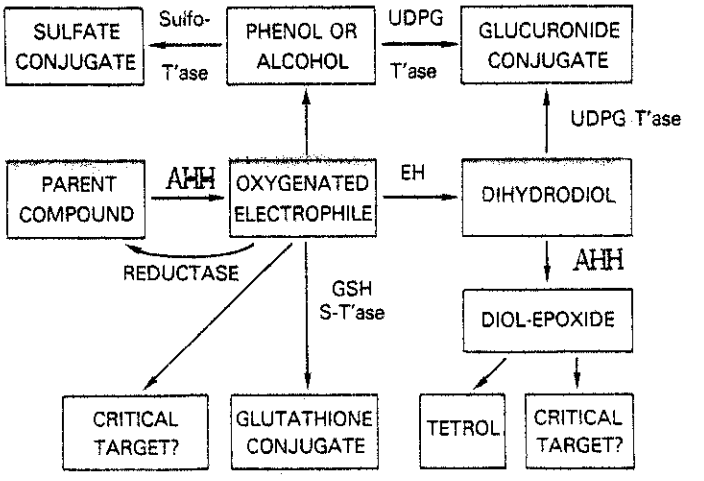


Figure 3.

Generally, the secondary metabolism is a protective mechanism which speeds the excretions of chemicals from the body. An exception is the reoxidation of diols (specifically, 7,8-dihydrodiol) to diol-epoxides which are highly reactive and bind readily to DNA (55). The 7,8-dihydrodiol-9,10-epoxides of BP are considered to be the ultimate carcinogens (61,65) and their carcinogenic activity is apparently related to their characteristic mutagenicity (65). A general scheme of the metabolism of PAH's has been suggested by Nebert et al. (45) and is illustrated in the following Figure (3).

Most investigators have employed animal systems (38,62), animal tissue cultures (53) or bacterial systems (35) to study tobacco carcinogenesis and mutagenesis and to relate that to humans. This study, in part, has employed human tissue (lymphocytes) to measure the AHH induction and cytotoxic action of CSC and CSC fractions by studying the pattern of the dose-response relationship in mitogen stimulated human lymphocytes, between CSC and CSC fractions and AHH activity.

In another part of this study, we devised a means of measuring the primary and secondary metabolite profiles of BP produced by the mitogen stimulated human lymphocytes and effected the separation of the BP conjugates, sulfates, glucuronides and glutathiones from the primary metabolites and parent compound (BP) by the use of HPLC. Use of these methods may provide data on individual persons relative to their rate of activation/deactivation reactions.

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CHAPTER II

MATERIALS AND METHODS

Lymphocytes Isolation and Culturing

Venous blood was withdrawn from human volunteers and was added to one-half volume of sterile heparinized saline solution (0.9% sodium chloride solution containing 10 units/ml heparin sodium salt). Sterile conditions were maintained throughout the cell culture. Twenty to twenty-five ml aliquots of the blood heparinized saline mixture were layered over 8 ml of a ficoll-hypaque solution (6% ficoll, 10% sodium diatrizoate) and centrifuged at 1500 g for 15 minutes. The mononuclear leukocytes (lymphocytes and monocytes) were recovered from the interface between the plasma and the ficoll-hypaque. The lymphocytes were sedimented by centrifugation at 1200 g for 10 minutes. After removing the supernatant, the cell pellet was resuspended in Joklick's modified Minimum Essential Medium (JMEM) (Gibco F-13), containing 10-15% fetal calf serum (Gibco), 1% pokeweed, 1% phytohemagglutinin (Gibco), and 50 units/ml heparin (Sigma). The cell concentration was adjusted to 0.4×10^6 cells/ml. Five ml aliquots were placed into wells of 35 mm costar dishes and the culture medium depth was 4-6 mm. The cell suspensions were

incubated at 37°C in a humidified 3-5% CO_2 atmosphere for 96 hours. Forty-four hours prior to harvest each costar dish, containing six wells, received six different doses (a dose to each well) of each of the tars or the 1A1 CSC fractions in a volume ranging from 6-30 µl of DMSO or acetone: methanol mixture. Six other wells received the inducer 1,2-benzoanthracene (BA) dissolved in 10 µl of methanol to a final concentration of 10 µM. Another six wells received the corresponding solvent at the same volume to serve as control cultures.

Cell Harvest and AHH Assay

The AHH assay was that of Cantrell et al. (2).

The cells were harvested and sedimented by centrifugation at 1000 g then resuspended in 2 ml EMSAD buffer [25 mM 4-(2-hydroxyethyl, 1-1-piperazine propane sulfonic acid)] 0.3 mM magnesium chloride, 125 mM NaCl, 0.1% bovine serum albumin, and 10 mM dextrose] at pH 8.0. The 2 ml EMASD cell suspension was divided into duplicates, and each received 2 µg BP in a volume of 10 µl of methanol, and all were incubated at 37°C for 60 minutes. The reaction was stopped by adding 0.5 ml of 20% neutral formalin with gentle shaking. The cells were then allowed to stand in the dark for 10 minutes for fixation.

Just prior to reading, each tube received one-half ml of 1N NaOH and was mixed vigorously; then the fluorescence of the suspensions was measured with excitation at 460 nm and emission at 522 nm.

Tar Preparation

Ten brands of cigarettes manufactured in the United States were used. The ten brands were Camel, Chesterfield, Marlboro, Marlboro Lights, Kent, Kent Lights, Winston, Winston Lights, True and Carlton.

Ten cigarettes of each brand were smoked by the smoking apparatus shown in Figure (1). Each cigarette was hooked to an aquafilter attached to a glass bubbler immersed in 20 ml of a 1:1 acetone-methanol mixture. The test tube that contained the organic solvent mixture was placed in a conical flask filled with iced water. Vacuum was created inside the flask by closing the syringe valve and drawing back the syringe plunger. The smoke passed into the glass filter and was trapped in the cold acetone-methanol mixture. On opening the valve, the vacuum inside the flask was broken and the cigarette smoking stopped while the plunger was pushed back in. The average puff volume was 35 ml over 2-4 seconds. The cigarettes were smoked to a butt length ranging from 20-25 mm. The tar trapped by the aquafilter was extracted by elution with 20 ml of 1:1 acetone-methanol, and pooled with the other 20 ml that was in the test tube. The 40 ml tar solution was concentrated under nitrogen and each fraction was brought to 20 ml. Ten (10) ml of each fraction was transferred to preweighed vials, dried under nitrogen, and kept in a vacuum dessicator for 48 hours. The vials were removed and weighed and the amount of tar yield per cigarette was determined. Three different concentrations of the remaining 10 ml tar solution was made and used for the AHH induction study.

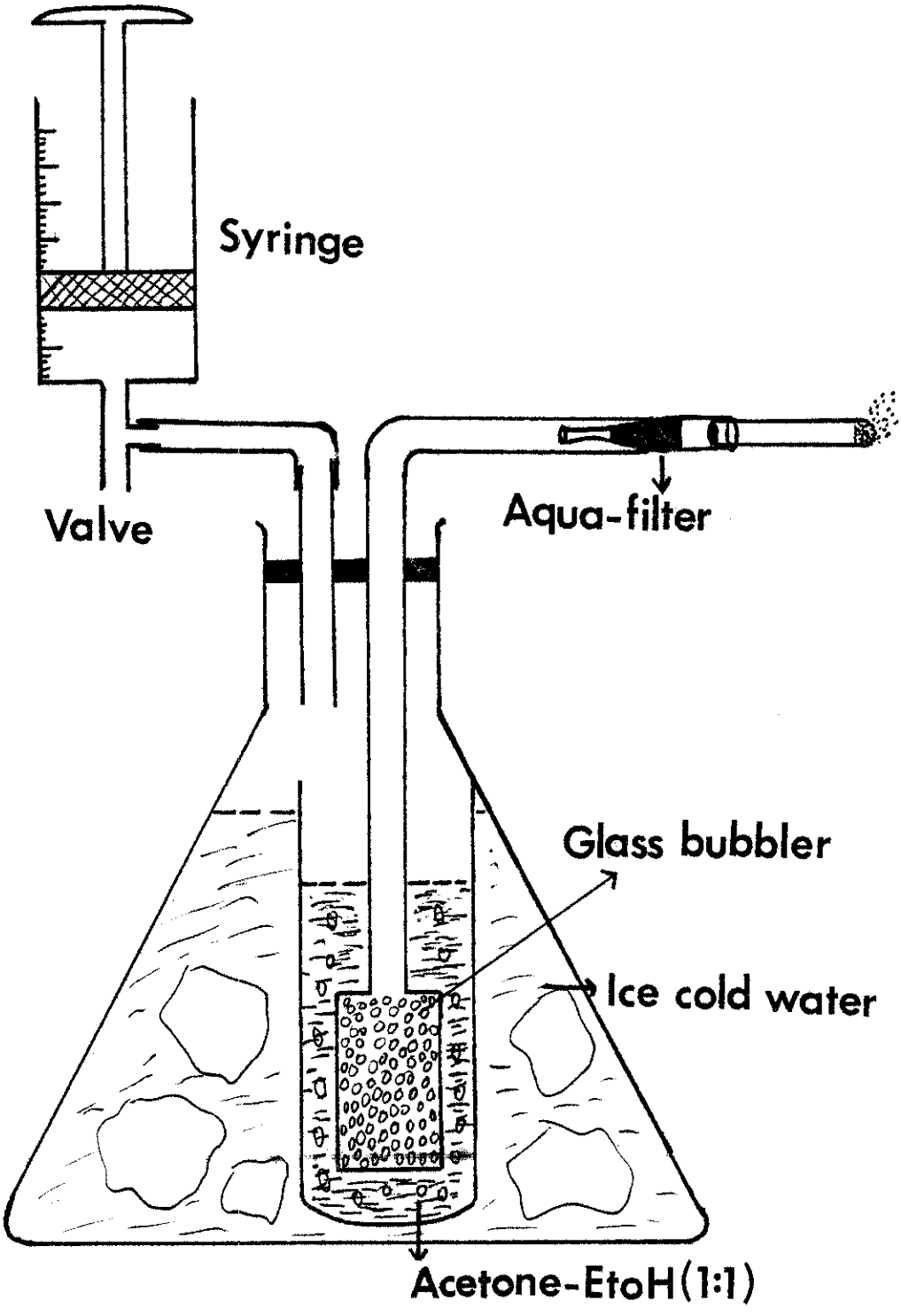
Syringe
Valve
Aqua-filter
Glass bubbler
Ice cold water
Acetone-EtoH(1:1)

Figure 1

The fractions of the CSC from the standard 1A1 cigarettes were generously provided by Dr. Richard E. Kouri (Microbiology Associates, Bethesda, MD). Three different concentrations of each fraction were prepared and kept frozen and later used for the AHH assay.

Assay of BP Secondary Metabolites

The method used was that of Cantrell et al. (3).

Human lymphocytes were isolated and cultured as described under "lymphocytes isolation and culturing". The cultures received BA (10 μM, during the period of culture, other cultures received no BA and served as controls. The BA-induced cells and the control cultures, respectively, were pooled, harvested as mentioned before and resuspended at the end of the 96 hour incubation period in fresh JMEM media at a concentration of 5×10^6 per ml. The cells were divided into 4 aliquots of 1 ml and marked A,B,C and D. Tubes A,B and C received ^{3}H-BP at the start of the reaction and tube D received ^{3}H-BP at the end of the AHH reaction. The amounts of BP and duration of incubation are indicated in the Results section. After the reaction period, each tube received 1 ml of water and the tubes were frozen and thawed twice to lyse the cells and terminate the AHH activity. Additions were made to the tubes as follows: Tube A received 10 μl of a mixture of beta-glucuronidase (0.07 I.U.) and aryl sulfatase (0.1 I.U.) (Calbiochem). Tube B received the enzyme mixture and 0.1 mg D-saccharic acid-1, 4-lactone, an inhibitor of glucuronidase. Tubes C and D received only water. All tubes were

incubated for one hour at 37°C and the hydrolysis then terminated by addition of 5 ml ethyl acetate to each tube with gentle mixing. The tubes were extracted twice with ethyl acetate and the pooled organic phases dried with sodium sulfate, then evaporated under nitrogen. The extracts were brought up to 0.5 ml ethanol and 0.1 ml injected into the high pressure liquid chromatograph for separation of metabolites. A Hibar-II RP-18 reverse phase column was employed as a separation medium. Elution of the column was with a linear 40-90% ethanol in water gradient. Fractions were collected and counted by liquid scintillation spectrometry.

Separation of Both Primary and Secondary Metabolites of BP on Alumina HPLC

The method used was that of Ghanayem et al. (4) which is a modification of the procedure of Autrup et al. (1).

Human lymphocytes were isolated and cultured as mentioned before in Section I, in the presence of 10 μm BA. After 96 hours, the cells were harvested and resuspended in culture medium (JMEM), without FCS or BA at a concentration of 5 x 10^6 per ml. The cells received 72 uCi of ^{3}H-BP (0.75 μg) and incubation was continued for 3 hours. The reaction was stopped by the addition of 3 ml ice-cold ethanol. After allowing the tubes to stand for one hour in a refrigerator, the precipitate was sedimented by centrifugation. The supernatants were transferred to clean tubes and dried under nitrogen. Just prior to chromatography, each residue was redissolved in

2 ml 79% ethanol. A 500 µl aliquot was injected onto the prepacked neutral alumina column for separation. A 4.6 x 250 mm steel HPLC column was dry packed with neutral alumina, Brockmann activity 1, 80-200 mesh size. The column was equilibrated with hexane and the 500 µl sample was injected during a flow rate of 1.2 ml/minute. Immediately the gradient programmer was started so as to form a gradient of hexane to absolute ethanol over 30 minutes. Twenty (20) 2 ml fractions were collected over 34 minutes after which the solvent was changed to water, ten fractions were collected and the solvent was changed to 50 mM ammonium phosphate buffer, pH 3.0. Fifteen (15) fractions were collected and the solvent was changed to 25% formic acid. Fifteen (15) fractions were collected. A total of sixty 2 ml fractions were collected over the 100 minute elution time.

A 200 µl aliquot of each fraction was taken and counted by liquid scintillation spectrometry.

The hexane-alcohol gradient provided resolution of the non-conjugated metabolites into two major peaks (Figure 15 in the Results section). These were Peak A (3 fractions) and Peak B (4 fractions). The two peaks were dried and subjected to reverse phase HPLC on a Dupont Zorbax ODS 4.6 X 250 mm column. The peaks were dissolved in 0.5 ml 70% ethanol, and a 100 µl aliquot injected into the HPLC. Elution was with a 60-100% methanol gradient over 30 minutes with a flow rate of 1 ml/minute. The 200 µl fractions were

collected in minivials and the radioactivity was determined in a Beckman LSC 7000. Identification of some peaks was made by comparison with authentic standards.

CHAPTER BIBLIOGRAPHY

1. Autrup, H. (1978). Separation of water-soluble metabolites of benzo-a-pyrene formed by cultured human colon. Bioch. Pharm. (in press).

2. Cantrell, E., Abreu, M., and Busbee, D. (1976). A simple assay of AHH in cultured human lymphocytes. Biochem. Biophys. Res. Commun. 70:474.

3. Cantrell, E.T., Marshall, M., McLemore, T., Ghanayem, B., and Busbee, D. (1979). Secondary metabolism of benzopyrene in human cells. Western Pharm. Proceedings.

4. Ghanayem, B., Cantrell, E., Busbee, D., Burleson, L. (1979). Separation of both primary and secondary metabolites of benzo-a-pyrene on alumina HPLC chromatography. Abstracts, Texas Pharmacologist's Meeting, May, Abst. #42.

CHAPTER III

RESULTS

Quantitation of CSC in Cigarettes and Effect of Different CSC on AHH in Lymphocytes

The amount of CSC in mg per cigarette was quantitated (Table I).

The effect on AHH activity (BP hydroxylase) of exposure of human lymphocytes to six different doses of CSC from two brands of unfiltered first generation cigarettes (the first generation of manufactured cigarettes to be delivered to the market; they are mostly unfiltered with a high tar and nicotine yield), three brands of filtered second generation cigarettes (the second generation of manufactured cigarettes, mostly filtered, with a comparatively moderate tar and nicotine yield), and five brands of the filtered third generation cigarettes (the third generation cigarettes also called the less harmful cigarettes, mostly have improved filtration systems like air filter and aqua filter with a comparatively low tar and nicotine yield), demonstrates that a maximum induction of 0.54 and 0.50 Pmoles 3-hydroxy beno-a-pyrene (BPOH) per well per minute was reached at 3×10^{4} and 10^{-3} cig./well by Camel and Chesterfield CSC, respectively, (Figure 1). A maximum induction of 0.53, 0.49 and 0.43 Pmoles BP-OH per well per min. was also reached by Marlboro, Winston and Kent CSC's, respectively, at 10^{-3} cig./well dose

(Figure 2). At 10^{-3} cig./well dosage, Winston Lights produced a maximum induction of 0.57 Pmoles per well per min., while Marlboro Lights and True produced their maximal induction of 0.55 and 0.60 Pmoles BP-OH per well per min. at a 3 x 10^{-3} cig./well (Figure 3).

In Figure 1, 2, and 3 and with all CSC's tested, with the exception of Kent Lights and Carlton, toxicity (decline in AHH levels) was noticed at all doses higher than the maximal inducing doses.

Under the same experimental conditions, the enzyme activity of untreated control and BA (10 μm) induced human lymphocytes' response are shown. The activities of control and BA treated cells were 0.25 and 0.80 Pmoles BPOH per well per min., respectively. With all doses, duplicates have been used, and the mean of each duplicate was represented in the figures.

Effect of CSC Subfractions on AHH in Lymphocytes

The effect of the standard 1A1 CSC and its fractions on AHH activity in human lymphocytes was demonstrated in Figures 4-8. Each point represents the mean of two duplicates from each dose.

Whole 1A1 CSC and the reconstituted material demonstrated that the response was proportional to the dose and at all six doses of each of the starting and reconstituted materials neither a maximum inducing nor a toxic dose could be reached or defined within the

limits of solubility (Figure 4). Exposure of cells to the weakly acidic fractions of the 1A1 CSC resulted in a maximum response of 0.18 and 0.19 Pmoles BP-OH per 10^6 cells per min. This was reached at 10^{-1} and 3×10^{-2} mg/well, respectively (Figure 5). Doses higher than 3×10^{-2} produced toxicity by the WA_E (weakly acidic ether soluble fraction) (Figure 5).

The four basic fractions, with the exception of the BW (bases, water soluble) produced an increased response with increasing dose. The $B_I b$ (bases insoluble b) fraction was the most active AHH inducer of all the 1A1 CSC fractions (Figure 6), which had a 0.66 Pmoles BP-OH per 10^6 cells per minute at 3×10^{-1} mg/well dose. From all the basic fractions, BW was the only toxic, and its toxicity was observed at doses higher than 3×10^{-2} mg/well.

The strongly acidic fractions were weakly active with the SA_I fraction (strong acids, insoluble) was the most active (Figure 7). All the strongly acidic fractions were toxic at one dose or another.

Neither a maximum response nor toxic effects were not achieved by any of the neutral fractions rather a direct relationship was observed between the dose and the response at all the dose levels tested. N_{CH} (neutrals, cyclohexane soluble) was the least active of all the neutral fractions (Figure 8).

Estimation of Conjugation by Enzyme Hydrolysis and HPLC

Human lymphocytes incubated for one hour with ^{3}H-BP were found to have approximately 20 milliunits of AHH by expressing radioactivity

in the peak reflecting formation of 3-OH-BP. The metabolite profile was similar to that reported by others for human lymphocytes and the identification of the peaks was assumed to be the same as reported by Holder et al. (2).

Lymphocytes were incubated for 60 minutes with 12 μCi ^{3}H-BP (24 Ci/mM). Figure 9 presents the profiles of tube A which had both aryl sulfatase and beta-glucurondiase enzymes and represents the total of oxidized metabolites, sulfate, and glucuronide. Figure 10 presents the profiles of tube B which reflects the sum of free metabolites plus sulfate only. Figure 11 presents the profiles of tube C which represents only the unconjugated free metabolites.

The amount of radioactivity in the various peaks after hydrolysis is summarized in Figure 12, where 12 μCi ^{3}H-BP was incubated for 60 minutes reaction time. Figure 13 summarizes the results after 12 μCi ^{3}H-BP was incubated for 18 hours reaction time. Figure 14 represents similar data but with 10 μCi of ^{3}H-BP which had been diluted with cold BP to a specific activity of 10.2 μg per 10 μCi (final concentration was 39.6 μm in the tubes).

In Figures 12, 13, and 14, some of the peaks were identified. Four diols were eluted first, followed by one quinone and two phenolic metabolites.

Estimation of Conjugation by LC Separation on Alumina

Cultured human lymphocytes which had been mitogen activated and induced with 10 μm BA for 96 hours were incubated after harvest for

3 hours with 0.75 μg ^{3}H-BP. Figure 15 presents the elution profile. Peak A represents mainly the parent compound and Peak B represents mainly the oxidized metabolites. Both Peak A and Peak B were eluted by the hexane-ETOH gradient.

H_2O eluted the sulfates, phosphate eluted the glucuronides, and formic acid eluted the glutathione conjugates (1).

When fractions of peak A and peak B (from Figure 15) were pooled separately, dried and fractionated by reverse phase HPLC, two different patterns were obtained (Figure 16). The upper profile of peak A shows mostly BP and some small amounts of the oxidized metabolites. The lower profile of peak B shows small amounts of BP and most of the oxidized metabolites.

Figure 1: Log dose-response curves of AHH enzyme by smoke condensate from two brands of the nonfilter first generation cigarettes in cultured human lymphocytes. The lymphocytes were incubated for 96 hours at 2×10^6 cells per 5 ml per well and received the CSC 44 hours prior to harvest. Six different doses of each CSC were added to separate wells. The doses were calculated as the equivalent cigarette (king size) per well. The response was expressed as enzyme activity equivalent to Pmoles BP-OH formed in each well per minute. Solvent-treated control and BA-induced lymphocytes' response are also shown.

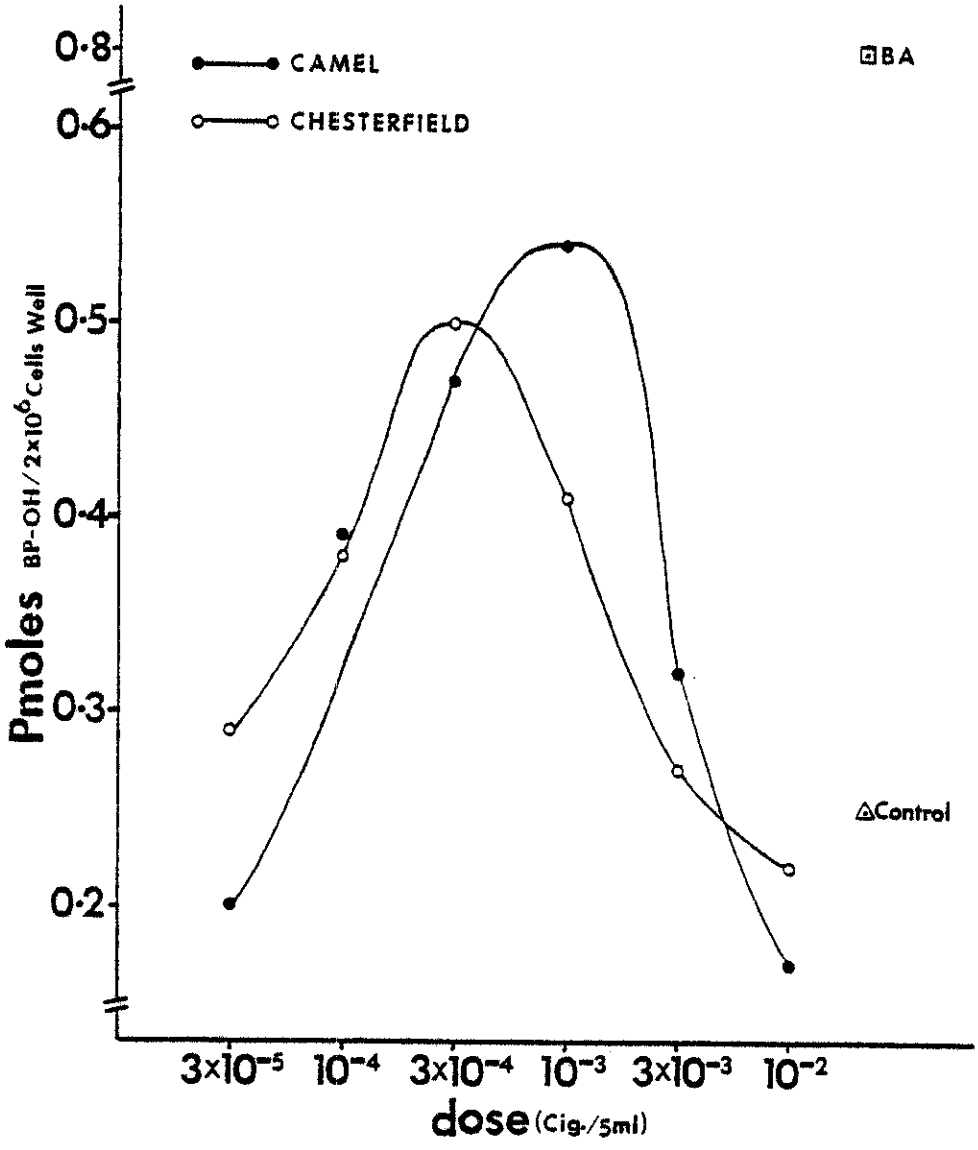
0·8
0·6
0·5
0·4
0·3
0·2
CAMEL
CHESTERFIELD
BA
Control
Pmoles BP-OH/2x10⁶Cells Well
3x10⁻⁵ 10⁻⁴ 3x10⁻⁴ 10⁻³ 3x10⁻³ 10⁻²
dose (Cig./5ml)

Figure 2: Log dose-response curves of AHH enzyme by smoke condensate from three brands of the second generation filter cigarettes in cultured human lymphocytes. The lymphocytes were incubated for 96 hours at 2×10^6 cells per 5 ml per well and received the CSC 44 hours prior to harvest. Six different doses of each CSC were added to separate wells. The doses were calculated as the equivalent cigarette (king size) per well. The response was expressed as enzyme activity equivalent to Pmoles BP-OH formed in each well per minute. Solvent-treated control and BA-induced lymphocytes' response are also shown.

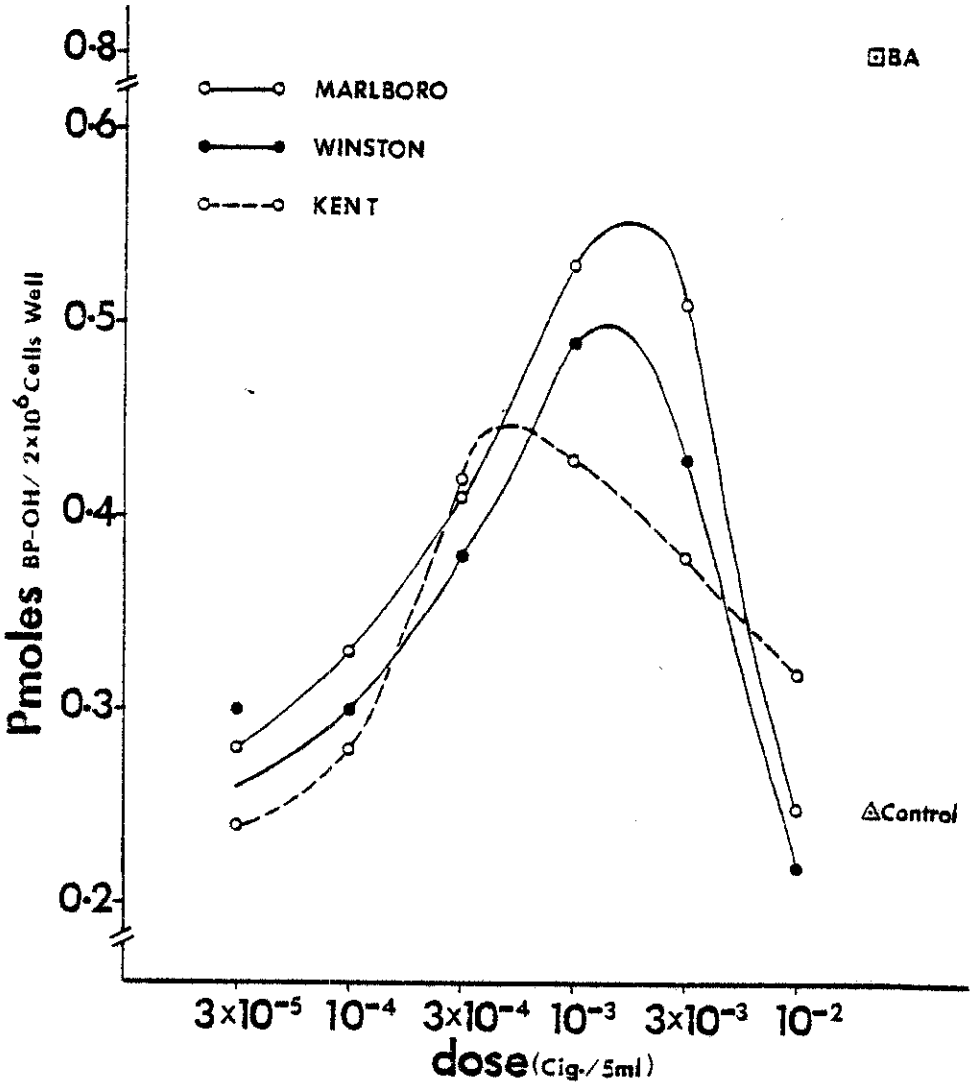
0·8
0·6
0·5
0·4
0·3
0·2
Pmoles BP-OH/ 2×10⁶Cells Well
MARLBORO
WINSTON
KENT
⊡BA
△Control
3×10⁻⁵ 10⁻⁴ 3×10⁻⁴ 10⁻³ 3×10⁻³ 10⁻²
dose(Cig-/5ml)

Figure 3: Log dose-response curves of AHH enzyme by smoke condensate from five brands of the third (modern) generation filter cigarettes in cultured human lymphocytes were incubated for 96 hours at 2 x 10^6 cells per 5 ml per well and received the CSC 44 hours prior to harvest. Six different doses of each CSC were added to separate wells. The doses were calculated as the equivalent cigarette (king size) per well. The response was expressed as enzyme activity equivalent to Pmoles (BP-OH) formed in each well per minute. Solvent-treated control and BA-induced lymphocytes' response are also shown.

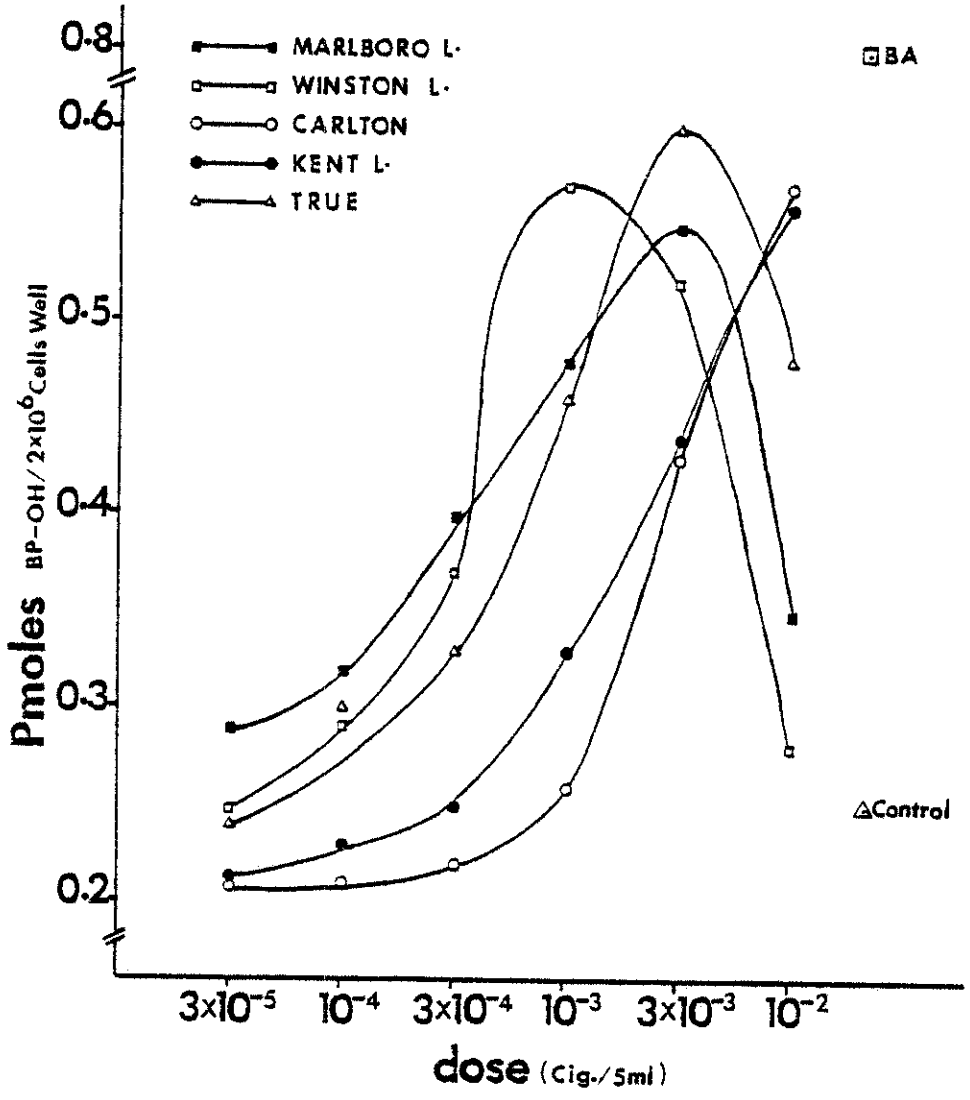
MARLBORO L·
WINSTON L·
CARLTON
KENT L·
TRUE
BA
Control
Pmoles BP-OH/2×10⁶Cells Well
0.8
0.6
0.5
0.4
0.3
0.2
3×10⁻⁵
10⁻⁴
3×10⁻⁴
10⁻³
3×10⁻³
10⁻²
dose (Cig./5ml)

Figure 4: Log dose-response curves of AHH enzyme by the whole smoke condensate and the reconstituted smoke condensate of the 1A1 standard cigarettes in cultured human lymphocytes. The lymphocytes were incubated for 96 hours at 2 x 10^6 cells per 5 ml per well and received the smoke condensates 44 hours prior to harvest. Six different doses of each the starting CSC and the reconstituted material were added to separate wells. The response was expressed as enzyme activity equivalent to Pmoles BP-OH formed in 1 x 10^6 cells per minute. Solvent-treated control and BA-induced lymphocytes' response are also shown.

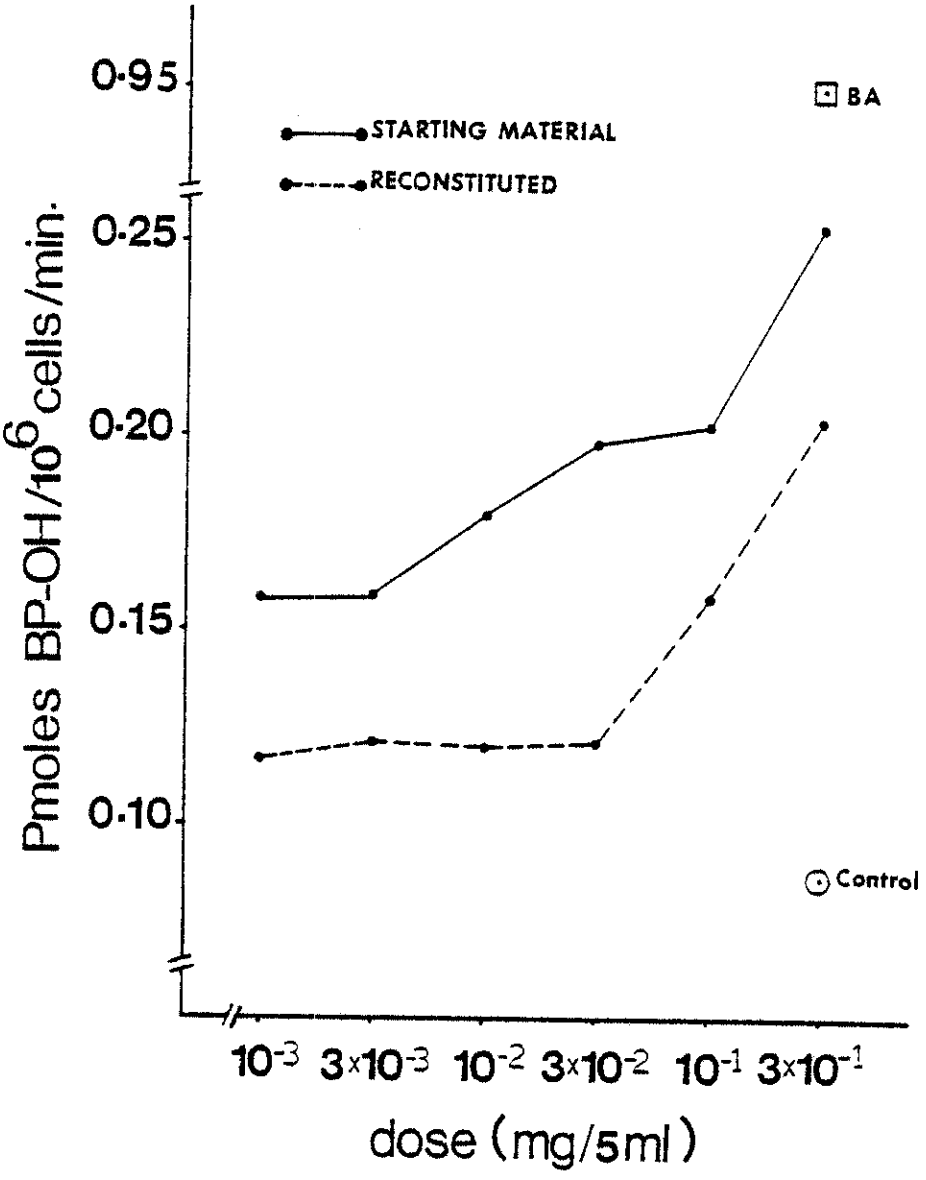
0·95
0·25
0·20
0·15
0·10
Pmoles BP-OH/10^6 cells/min.
STARTING MATERIAL
RECONSTITUTED
BA
Control
10^-3 3x10^-3 10^-2 3x10^-2 10^-1 3x10^-1
dose (mg/5ml)

Figure 5: Log dose-response curves of AHH enzyme by the two weakly acidic fractions of the 1A1 standard cigarettes (WA_E: weakly acidic ether soluble) in cultured human lymphocytes. The lymphocytes were incubated for 96 hours at 2 x 10^6 cells per 5 ml per well and received the CSC fractions 44 hours prior to harvest. Six different doses of each fraction were added to separate wells. The response was expressed as enzyme activity equivalent to Pmoles BP-OH per 1 x 10^6 cells per minute. Solvent-treated control and BA-induced lymphocytes' response are also shown.

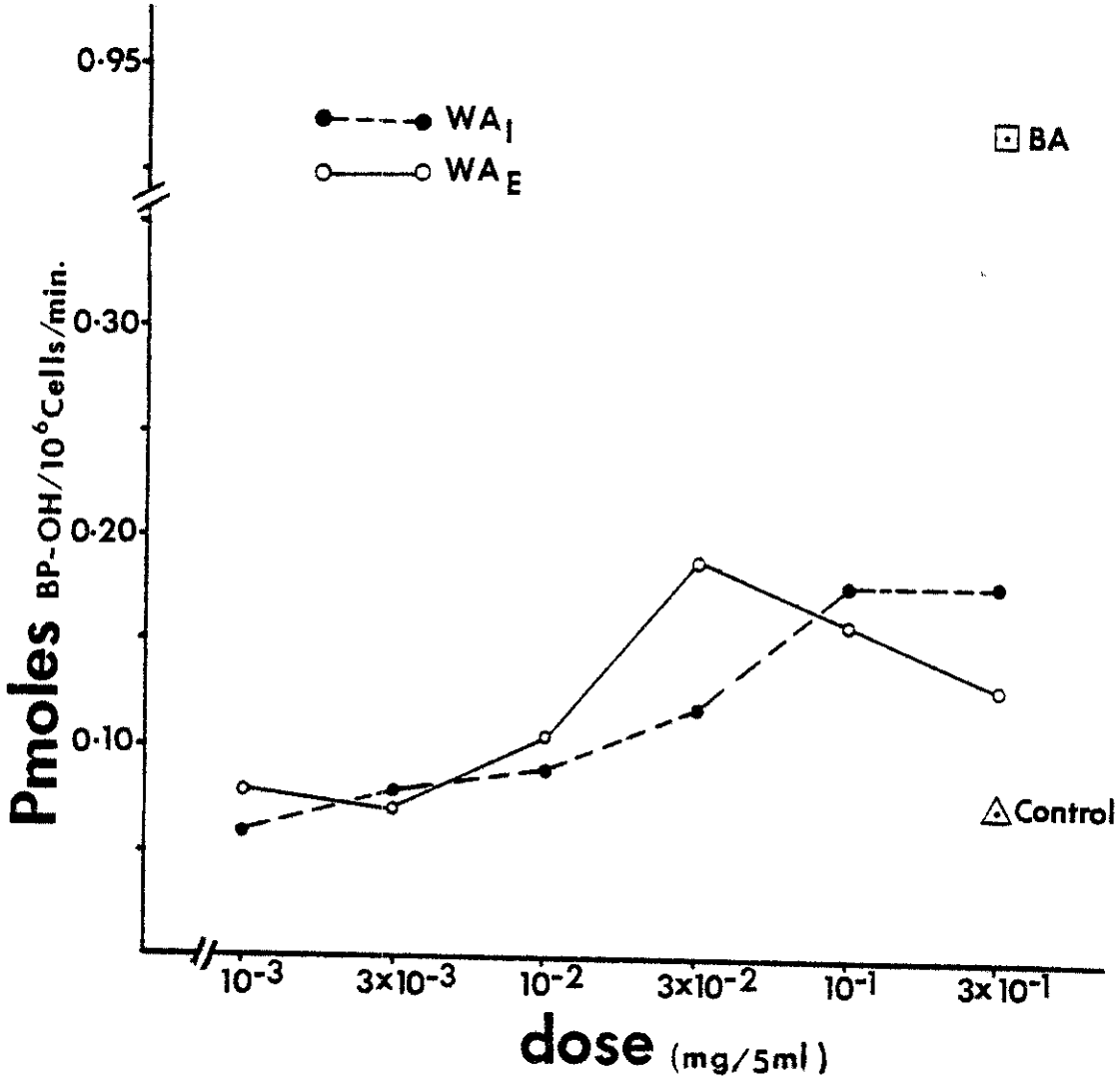
0·95
0·30
0·20
0·10
WA I
WA E
BA
Control
Pmoles BP-OH/10⁶Cells/min.
10⁻³
3x10⁻³
10⁻²
3x10⁻²
10⁻¹
3x10⁻¹
dose (mg/5ml)

Figure 6: Log dose-response curves of AHH enzyme by the four basic fractions of the 1A1 standard cigarettes (B_{Ia}: bases insoluble a, B_{Ib}: bases insoluble b, B_w: bases water soluble and B_e: bases ether soluble) in cultured human lymphocytes. The lymphocytes were incubated for 96 hours at 2×10^6 cells per 5 ml per well and received the CSC fractions 44 hours prior to harvest. Six different doses of each fraction were added to separate wells. The response was expressed as enzyme activity equivalent to Pmoles BP-OH per 1×10^6 cells per minute. Solvent treated control and BA-induced lymphocytes' response are also shown.

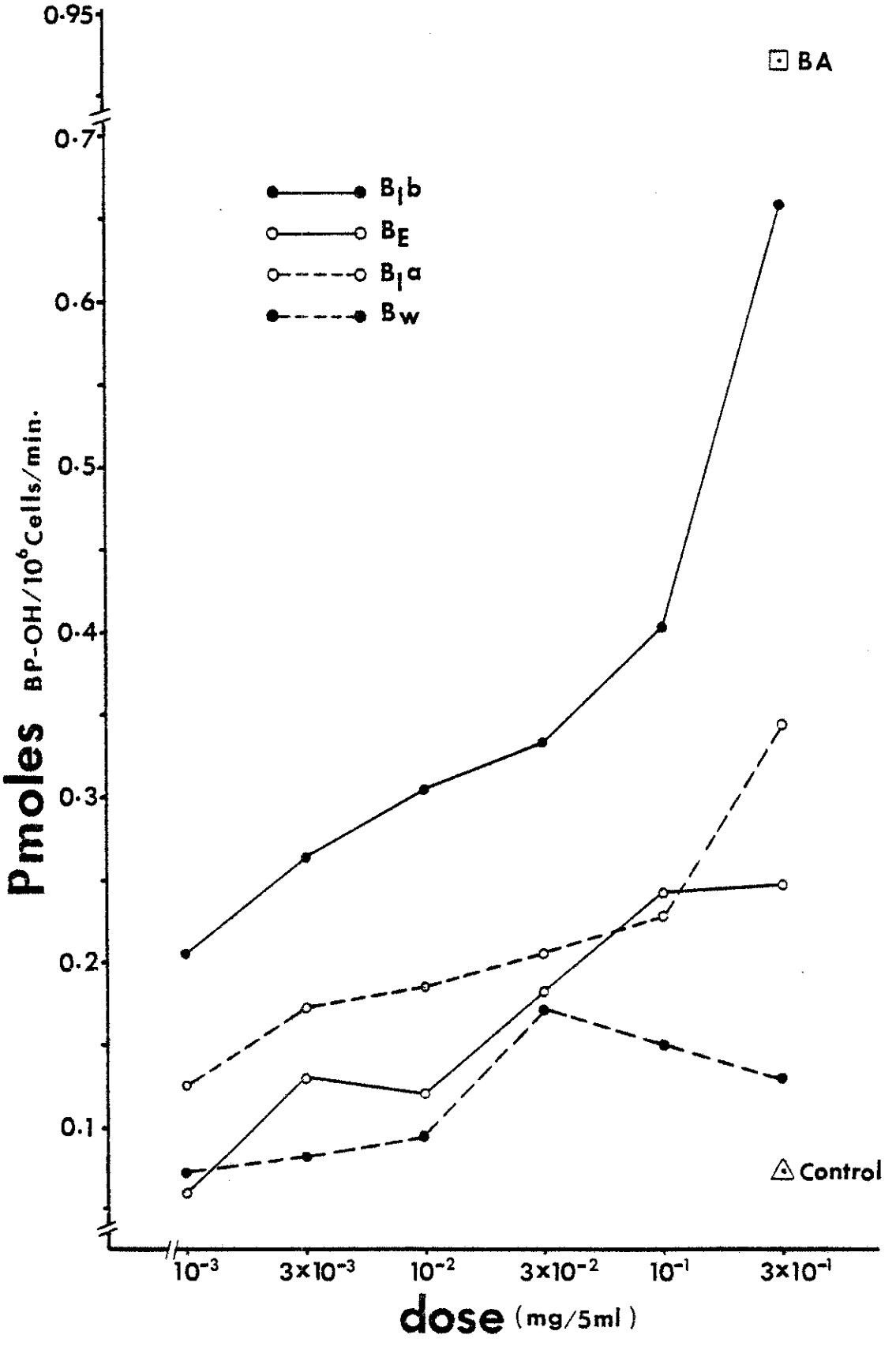
0·95
0·7
0·6
0·5
0·4
0·3
0·2
0·1
Pmoles BP-OH/10⁶Cells/min.
BA
B₁b
B_E
B₁a
B_W
Control
10⁻³
3×10⁻³
10⁻²
3×10⁻²
10⁻¹
3×10⁻¹
dose (mg/5ml)

Figure 7: Log dose-response curves of AHH enzyme by the three strong acidic fractions of the 1A1 standard cigarettes. (SA_I: strong acids, insoluble, SA_e: strong acids, ether soluble, SA_w: strong acids, ether soluble) in cultured human lymphocytes. The lymphocytes were incubated for 96 hours at 2 x 10^6 cells per 5 ml well and received the CSC fractions 44 hours prior to harvest. Six different doses of each fraction were added to separate wells. The response was expressed as enzyme activity equivalent to Pmoles BP-OH per 1 x 10^6 cells per minute. Solvent-treated control and BA-induced response are also shown.

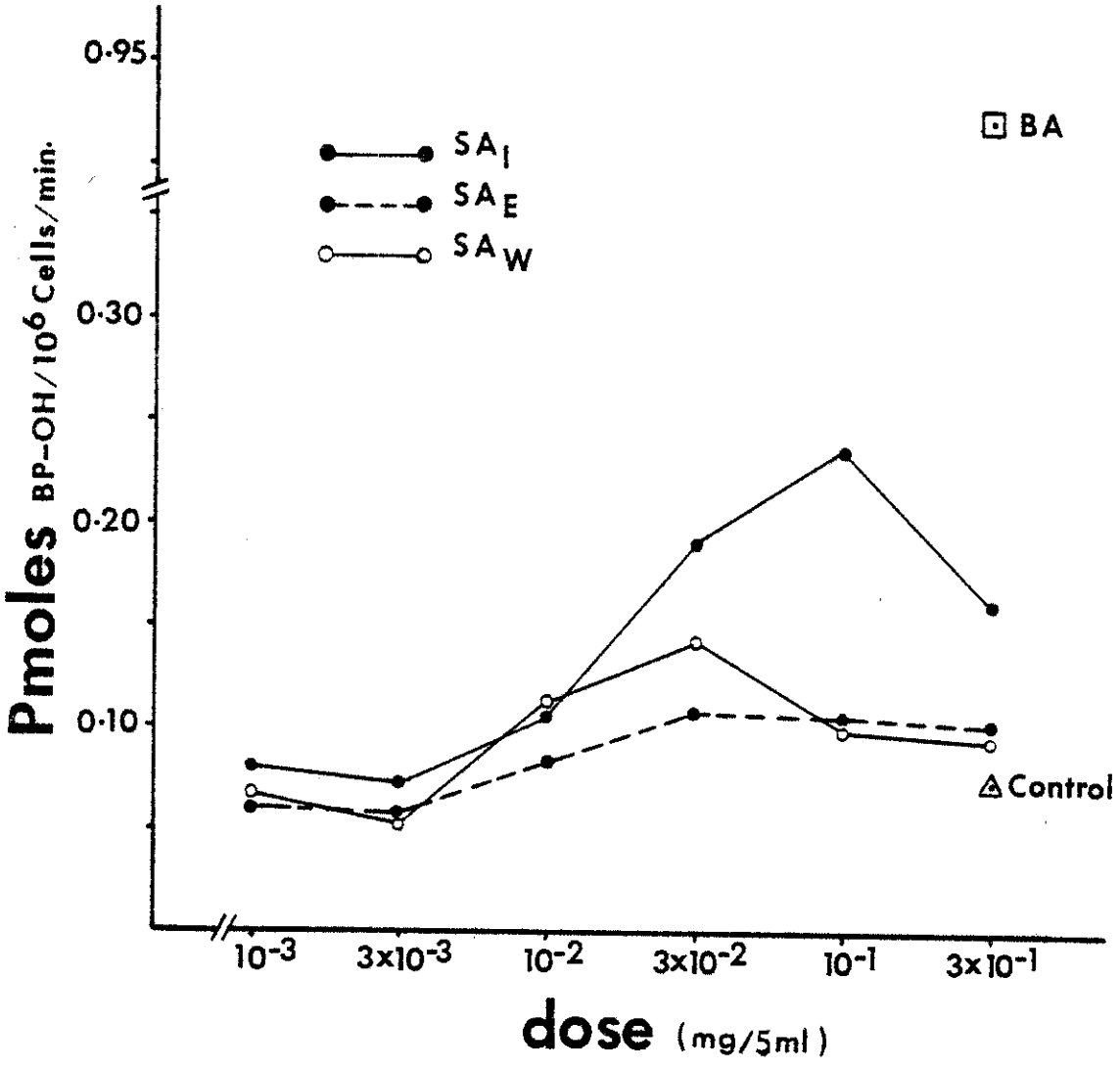
0·95
0·30
0·20
0·10
Pmoles BP-OH/10⁶ Cells/min.
SA I
SA E
SA W
BA
Control
10⁻³
3x10⁻³
10⁻²
3x10⁻²
10⁻¹
3x10⁻¹
dose (mg/5ml)

Figure 8: Log dose-response curves of AHH enzyme by the three neutral fractions of the 1A1 standard cigarettes (N_{meoh}: neutrals methanol soluble, N_{ch}: neutrals cyclohexane soluble, N_{nm}: neutrals nitromethane soluble) in cultured human lymphocytes. The lymphocytes were incubated for 96 hours at 2 x 10^6 cells per 5 ml well and received the CSC fractions 44 hours prior to harvest. Six different doses of each fraction were added to separate wells. The response was expressed as enzyme activity equivalent to Pmoles BP-OH per 1 x 10^6 cells per minute. Solvent-treated control and BA-induced lymphocytes' response are also shown.

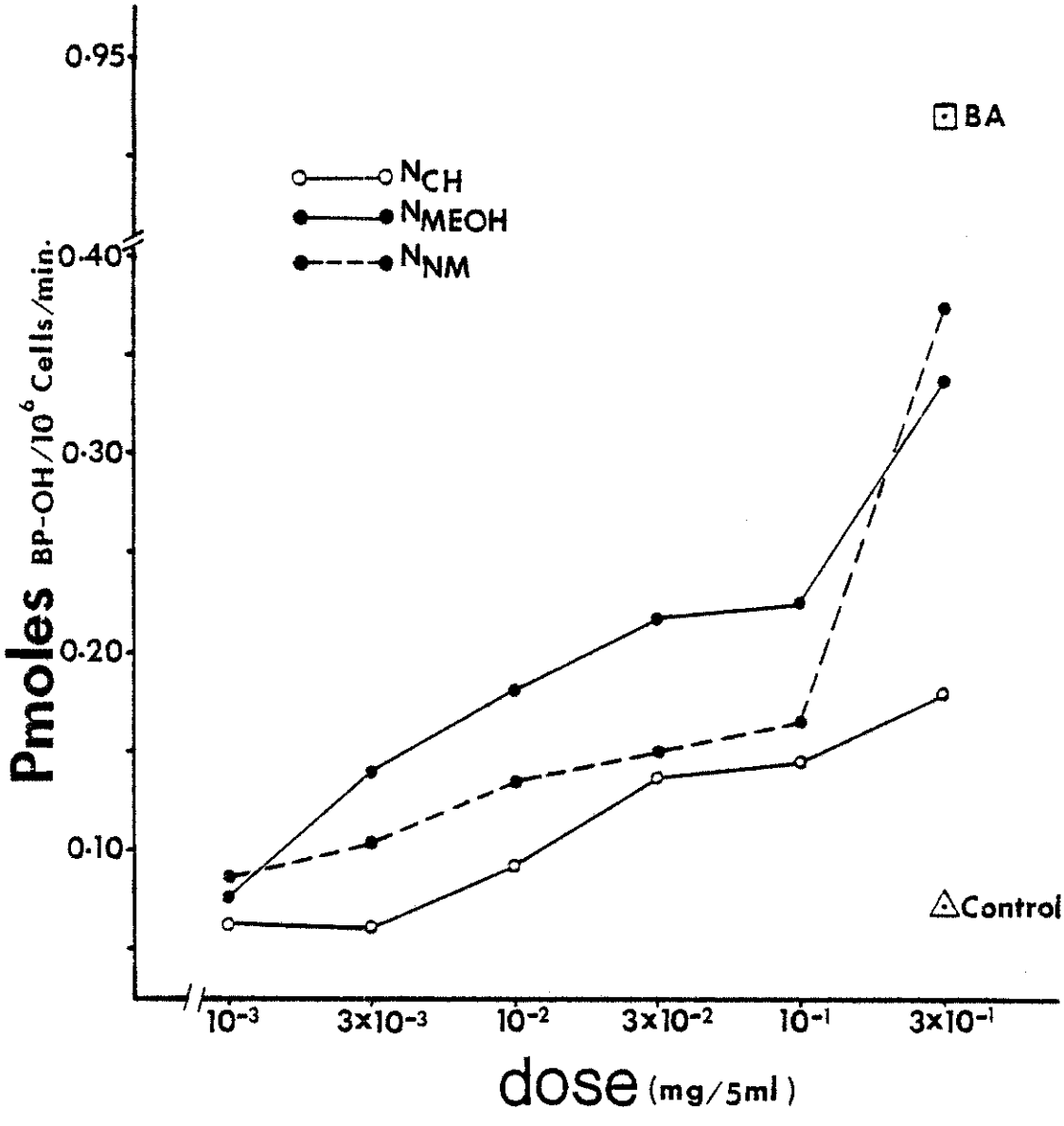
0·95
0·40
0·30
0·20
0·10
Pmoles BP-OH/10^6 Cells/min.
N_CH
N_MEOH
N_NM
BA
Control
10^-3
3x10^-3
10^-2
3x10^-2
10^-1
3x10^-1
dose (mg/5ml)

Figure 9: Representative elution profile of ^{3}H-BP metabolites produced by cultured human lymphocytes. Cells were incubated for 60 minutes at 37°C with 12 μCi (0.5 nmol) ^{3}H-BP per ml of 5 x 10^{6} cells. After 60 minutes of hydrolysis by both aryl sulfatase and beta-glucuronidase enzymes, the dried extracts were separated by HPLC. 0.10 ml sample was injected into a Hibar-II RP-18 column at 40°C and a 40-90% ethanol in water gradient for 30 minutes was used. The flow rate was 1.2 ml/min and 0.2 ml fractions were collected and counted.

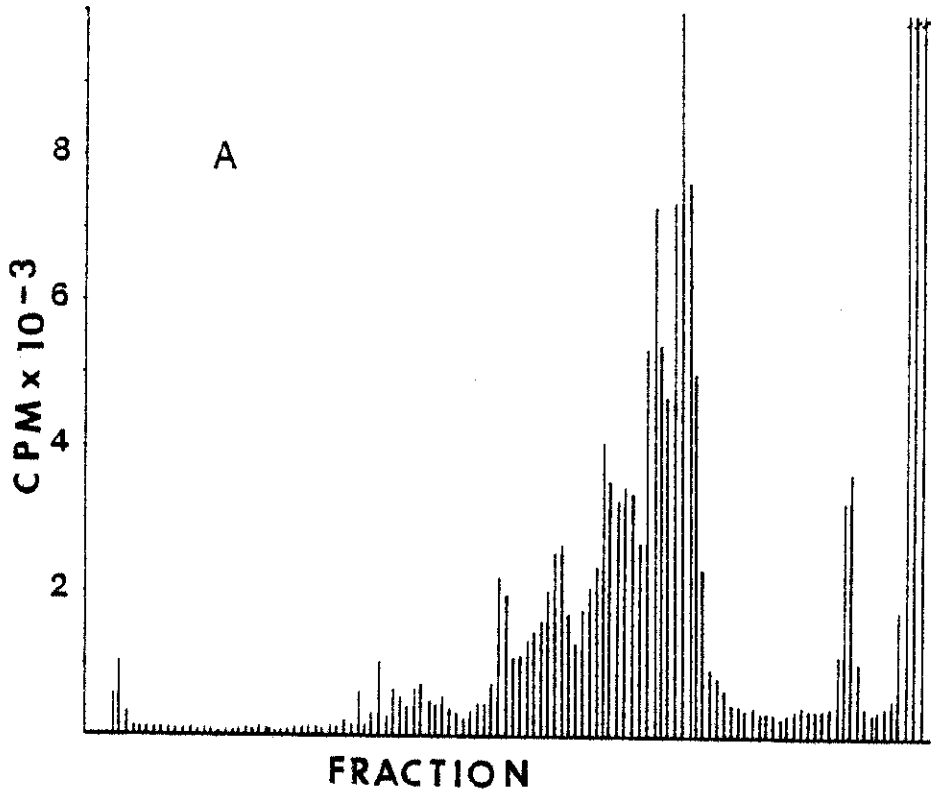
A
8
6
4
2
CPM x 10⁻³
FRACTION

Figure 10: Representative elution profile of ^{3}H-BP metabolites produced by cultured human lymphocytes. Cells were incubated for 60 minutes at 37°C with 12 μCi (0.5 nmol) ^{3}H-BP per ml of 5 x 10^{6} cells. After 60 minutes of hydrolysis by aryl sulfatase enzyme, the dried extracts were separated by HPLC. 0.10 ml sample was injected into a Hibar-II RP-18 column at 40°C and a 40-90% ethanol in water gradient for 30 minutes was used. The flow rate was 1.2 ml/min and 0.2 ml fractions were collected and counted.

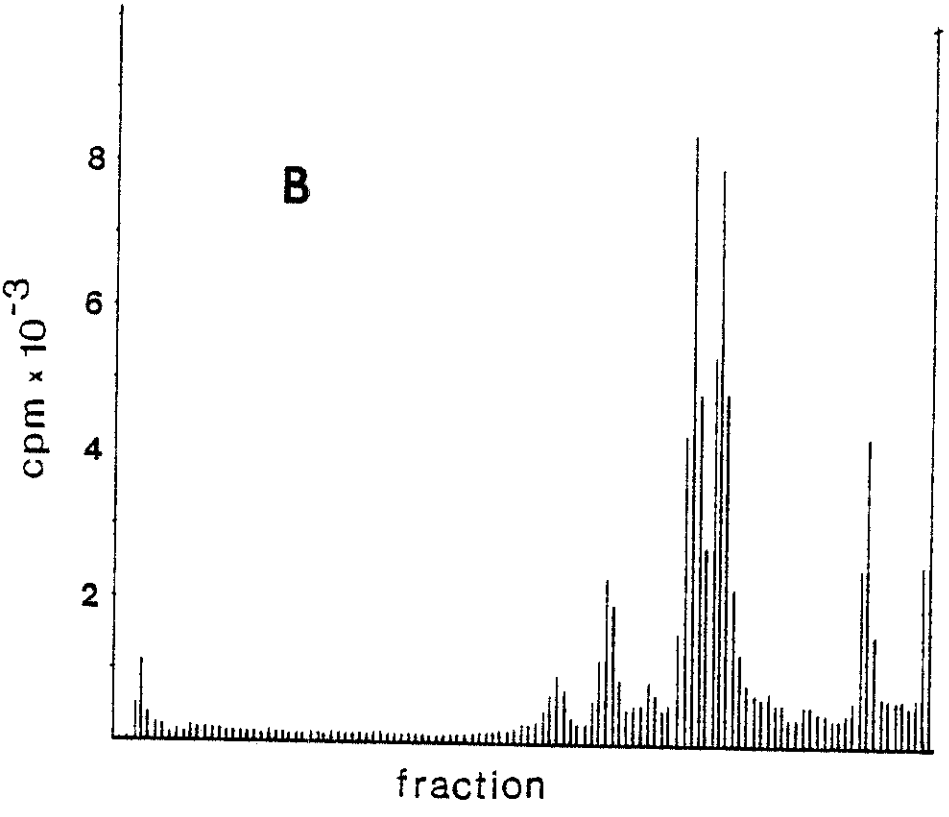
B
8
6
4
2
cpm x 10^-3
fraction

Figure 11: Representative elution profile of ^{3}H-BP metabolites produced by cultured human lymphocytes. Cells were incubated for 60 minutes at 37°C with 12 µCi (0.5 nmol) ^{3}H-BP per ml of 5 x 10^{6} cells. The dried extracts of the nonhydrolysed free metabolites were separated by HPLC. 0.1 ml sample was injected and a Hibar-II RP-18 column at 40°C and a 40-90% ethanol in water gradient for 30 minutes was used. The flow rate was 1.2 ml/min and 0.2 ml fractions were collected and counted.

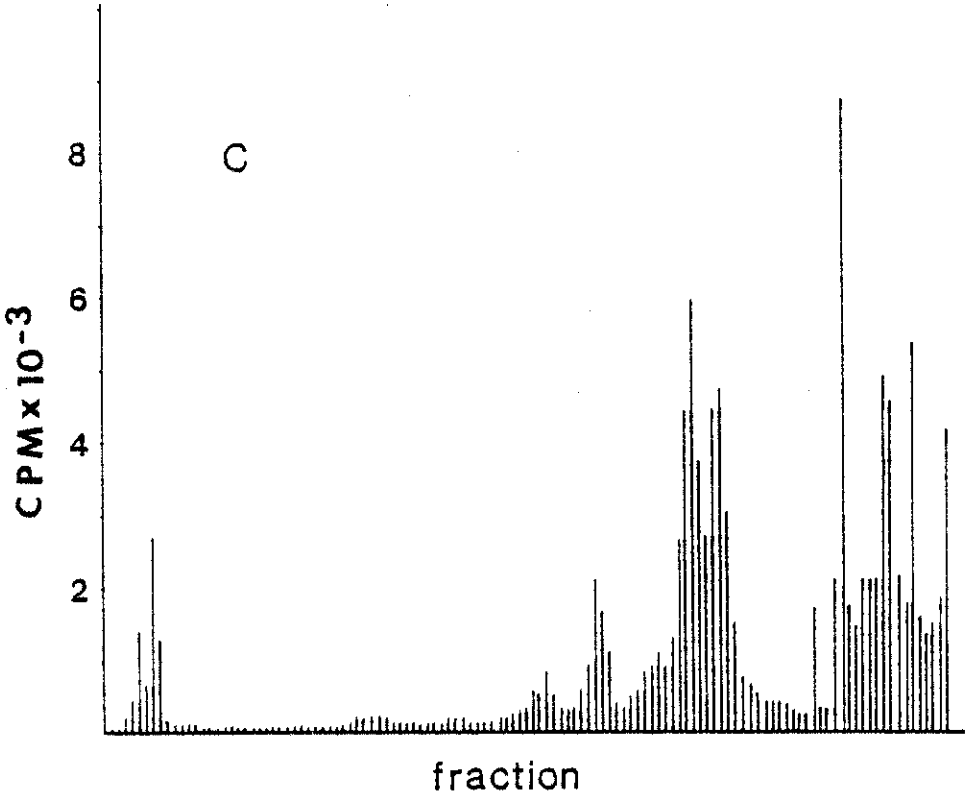
C
8
6
4
2
CPM x 10⁻³
fraction

Figure 12: Summary of amount of radioactivity in various peaks after hydrolysis with beta-glucuronidase or aryl sulfatase in cultured human lymphocytes incubated for 60 minutes at 37°C with 12 μCi (0.5 nmol) ^{3}H-BP per ml of 5 x 10^{6} cells. a) both enzymes; b) both enzymes and D-saccharic acid 1-4 - lactone previously buffered to pH 7.5 (an inhibitor of beta-glucuronidase) c) nonhydrolysed free metabolites. The background plus blank counts are below the ordinate.

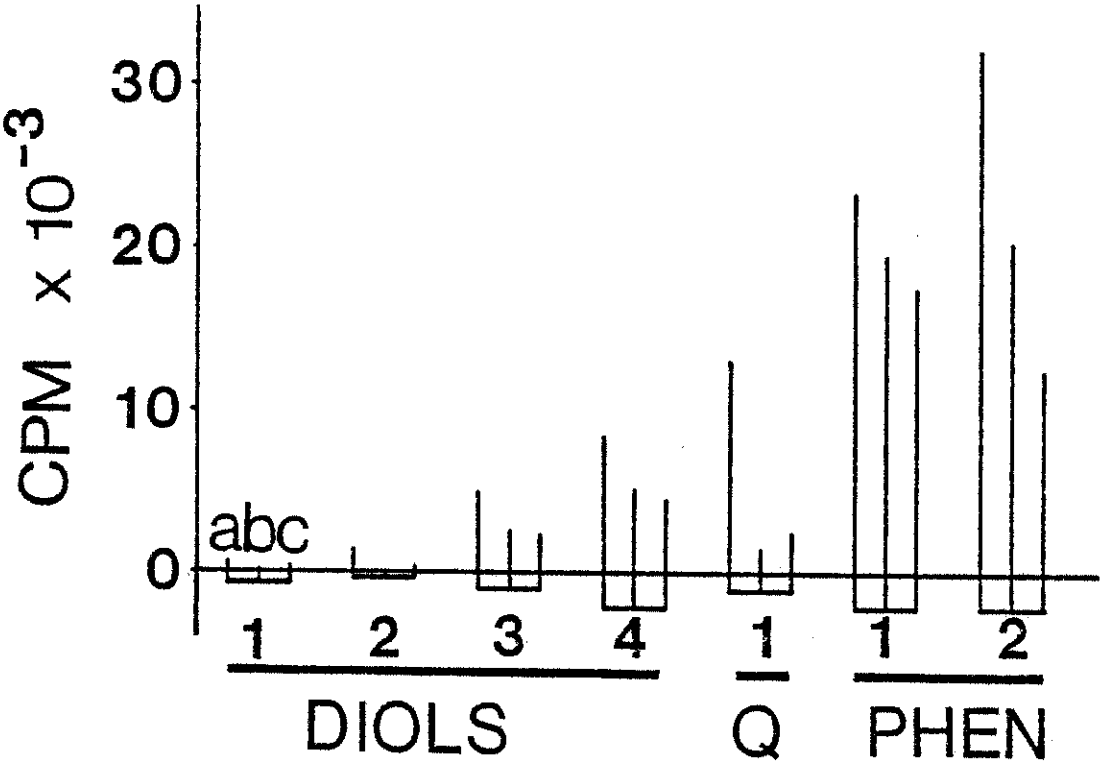
CPM x 10^{-3}
30
20
10
0
abc
1
2
3
4
1
1
2
DIOLS
Q
PHEN

Figure 13: Summary of amount of radioactivity in various peaks after hydrolysis with beta-glucuronidase or aryl sulfatase in cultured human lymphocytes incubated for 18 hours at 37°C with 12 μCi (0.5 nmol) ^{3}H-BP per ml of 4 x 10^{6} cells . a) both enzymes; b) both enzymes and D-saccharic acid 1,4-lactone previously buffered to pH 7.5 (an inhibitor of beta-glucuronidase). c) nonhydrolysed free metabolites. The background plus blank counts are below the ordinate.

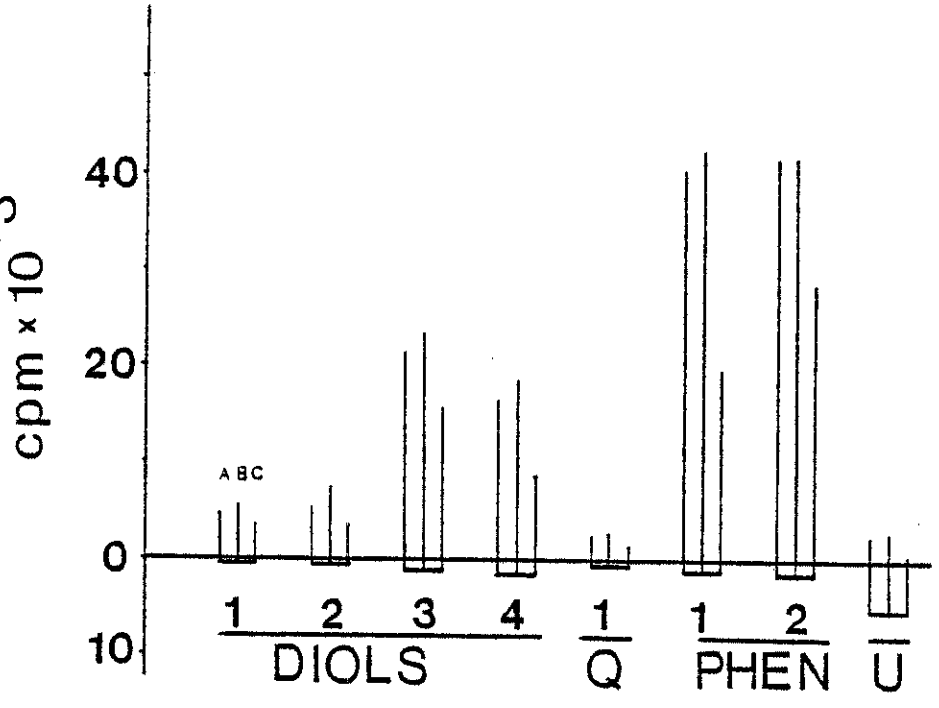
cpm x 10^-3
40
20
0
10
A B C
1 2 3 4
DIOLS
1
Q
1 2
PHEN
U

Figure 14: Summary of amount of radioactivity in various peaks after hydrolysis with beta-glucuronidase or aryl sulfatase in cultured human lymphocytes incubated for 24 hours at 37°C with 10 μCi of ^{3}H-BP which had been diluted with cold BP to a specific activity of 10.2 μg per 10 μCi per ml of 2.5 x 10^{6} cells. a) both enzymes; b) both enzymes and D-saccharic acid 1,4- lactone previously buffered to pH 7.5 (an inhibitor of beta-glucuronidase); c) nonhydrolysed free metabolites. The background plus blank counts are below the ordinate.

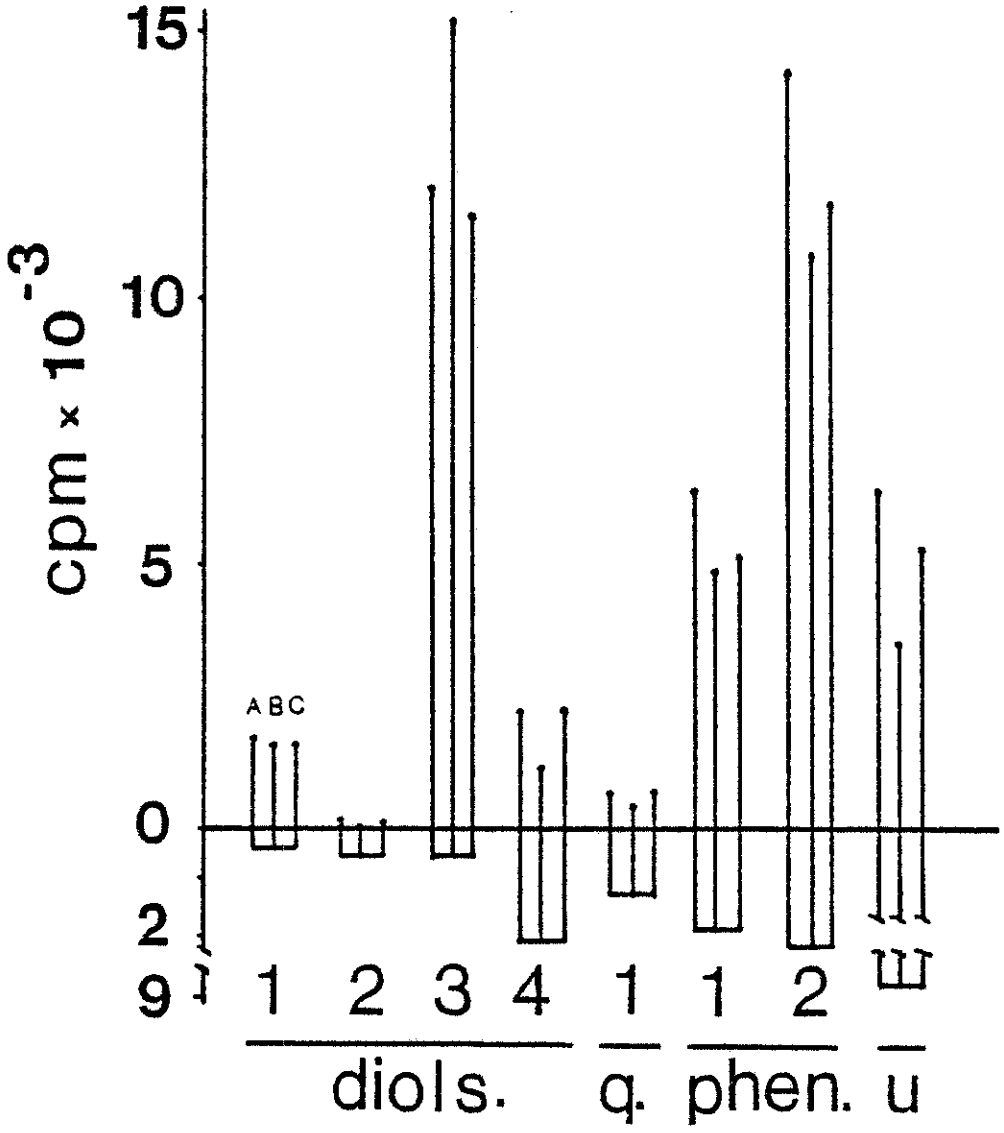
15
10
5
0
2
9
cpm × 10-3
A B C
1 2 3 4 1 1 2
diols. q. phen. u

Figure 15: Profile of separation of ^{3}H-BP metabolites produced by cultured human lymphocytes. The BA induced lymphocytes were incubated for 3 hours at 37°C with 72 µCi ^{3}H-BP (0.75 µGM) at a concentration of 5 x 10^{6} cells per ml. The reaction was stopped by addition of 3 ml ice-cold ethanol. The precipitate was sedimented, and the alcoholic supernatant was taken and dried under nitrogen. The residue was dissolved in 2.00 ml 70% ethanol. 0.5 ml the sample was injected onto a 4.6 x 2.50 nm neutral alumina column during a flow rate of 1.2 ml/min. A linear hexane-ethanol gradient was programmed for 30 minutes. Twenty 2 ml fractions were collected. The solvent was changed to water and another ten fractions were collected after which the solvent was changed to phosphate buffer (0.05M, pH 3.0) . Fifteen fractions were collected then the solvent was changed to 25% formic acid. Fifteen additional fractions were collected. A total of sixty 2 ml fractions were collected and 0.2 ml aliquots were taken and counted.

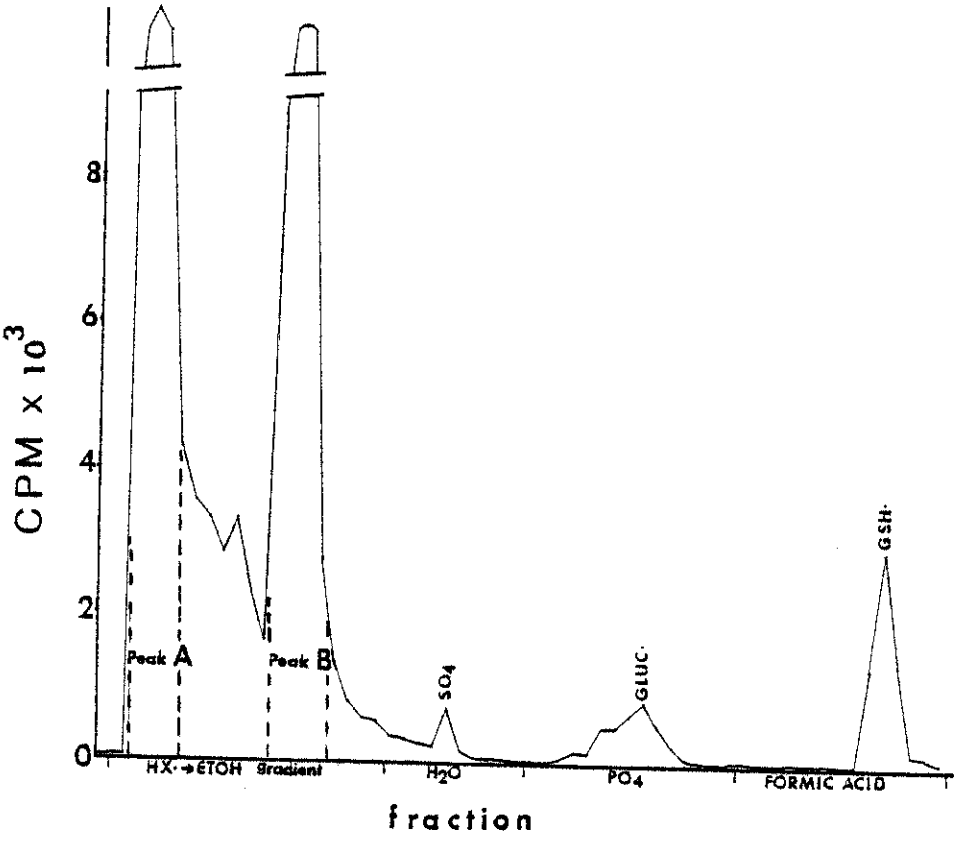
CPM x 10^3
8
6
4
2
0
Peak A
Peak B
SO4
GLUC.
GSH.
HX. → ETOH Gradient
H2O
PO4
FORMIC ACID
fraction

Figure 16: Reverse phase HPLC separation of peaks A and B (non-conjugated metabolites) from the neutral alumina column eluting with hexane-ethanol gradient (Figure 15). Three fractions from peak A and 4 fractions from peak B, respectively, were pooled, dried under nitrogen, and each was dissolved in 0.5 ml 70% ethanol. A 0.10 ml aliquot of each was injected into the HPLC. Elution was with a 60-100% methanol gradient over 30 minutes with a flow rate of 1.0 ml/min; 0.20 ml fractions were collected and counted.

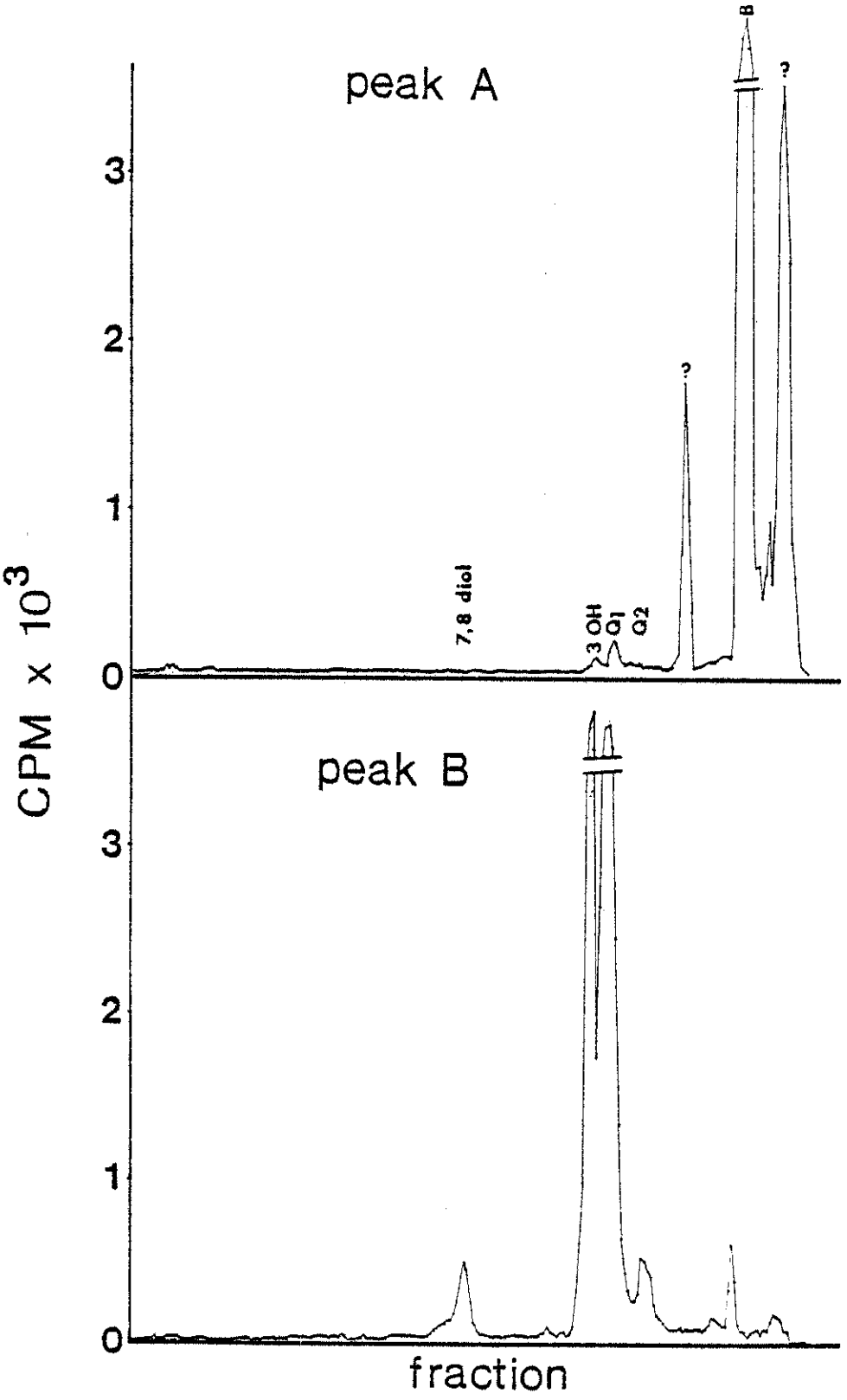
peak A
peak B
CPM x 10^3
fraction
7,8 diol
3 OH
Q1
Q2
?
?
B
0
1
2
3

Table I : Summary of the quantitation of CSC yield of ten brands of cigarettes. Ten ml of the CSC solution (equivalent to 5 cigarettes) was dried under nitrogen in preweighed vials and stored in a vacuum dessicator for 48 hours. The vials were reweighed and the amount of CSC equivalent to five cigarettes and the CSC yield in mg/cig. was calculated.

TABLE I

Brand	Mg. Tar/5 Cig.	Mg. Tar/Cig.
Camel	168.4	33.7
Chesterfield	184.3	36.8
Marlboro	073.4	14.7
Winston	054.2	10.8
Kent	058.2	11.6
Marlboro Lights	052.1	10.4
Winston Lights	076.4	15.3
Kent Lights	005.6	1.12
Carlton	004.7	0.94
True	013.9	2.27

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CHAPTER IV

DISCUSSION

The link between cigarette smoking and lung cancer was established by the results of all prospective and retrospective investigations from many countries. The first experiments to establish tobacco smoke as a carcinogen in laboratory animals were done with CSC (tar) applications to epithelial tissue of mice and rabbits (4,19), which was followed by further confirmations of the same results by a large number of investigations (3,5,17).

Since that time, part of the purpose of the tobacco carcinogenesis' program has been to identify the carcinogenic tobacco smoke components, and once such components have been identified attempts might then be made for their reduction or removal. The identification of carcinogenic tobacco smoke components was positively correlated with the discovery of a lower risk of developing lung cancer in the smokers of filter cigarettes than in the smokers of unfiltered cigarettes (18).

In the present study, the CSC yield of what we call "third generation" cigarettes (the less harmful cigarettes) such as Carlton and Kent Lights was quantitatively lower than the CSC yield of both

the first (unfiltered) and the second generation (poorly filtered) cigarettes. It was also demonstrated that the CSC yield of the filter cigarettes was far lower than the yield of the unfiltered cigarettes. These data, with the results of earlier reports (18) that had attributed the diminished risk of developing lung cancer in the smokers of filter cigarettes to the reduced yield of tar and the suspected association between high AHH inducibility and susceptibility to chemical carcinogenesis, led us to consider the relationship between AHH and the effects of CSC from different cigarettes on mitogen stimulated cultured human lymphocytes. Cultured human lymphocytes responded with increased levels of AHH enzyme upon exposure to cigarette tars from all the brands of cigarettes tested. A dose-response relationship was demonstrated. All the CSC's tested were AHH inducers, but they differed in their potency, efficacy and toxicity. A peak AHH induction (maximum response) was reached at lower doses of CSC's derived from unfiltered cigarettes than CSC's derived from filter cigarettes (the dose was expressed as equivalent cigarettes per well). With the exception of Carlton and Kent Lights, CSC's toxic effects were seen as reduction in enzyme activity per well. Toxic effects were noticed at lower doses of CSC's derived from unfiltered cigarettes than CSC's derived from filter cigarettes. The extreme toxicity of unfiltered cigarettes seemed to attenuate the course of the dose response before maximum enzyme activity was induced. The less harmful cigarettes were good inducers of AHH, with a maximum response reached at very

high doses, and toxicity was comparably low. Carlton and Kent Lights' CSC's were effective inducers, but neither the maximum response nor toxicity was reached by any of the doses tested under our experimental conditions.

The data are consistent with the reports of AHH induction that occurs *in vivo* following cigarette smoking (11,12,13). AHH induction by CSC's in human lymphocytes is also consistent with the observations of Welch *et al.* (16), and Marcotte and Witschi (10) who showed that pulmonary AHH was inducible in rats exposed to regular or marijuana cigarettes' smoke and Kouri *et al.* (8) who showed that B_6 and C3H/fmai mice had responded by increased levels of pulmonary AHH after their exposure to cigarettes' smoke. Our data suggest that the first generation cigarettes (high-tar yield) are highly toxic, which correlates with the high risk of developing lung cancer in the smokers of such cigarettes. The third generation cigarettes (lowest-tar yield) had very low toxicity, which also correlates with the lower risk of developing lung cancer in the smokers of such cigarettes. The data do not address the question of possible relationships between AHH and high risk of developing lung cancer, as all cigarettes were effective inducers of AHH and all cells were derived from the same person.

Cultured human lymphocytes were induced by the whole CSC and the reconstituted CSC derived from the standard 1A1 cigarettes. At any given dose, the starting CSC was more efficacious than the

reconstituted CSC. This can be explained on the basis that some of the actively inducing components of the CSC might be lost during the fractionation processes. This is consistent with the finding of Swain et al. (15) that the reconstitution of the 12 fractions of the 1A1 CSC recovered only 90.8% of the starting CSC activity. Toxicity was not seen by either the starting or the reconstituted materials under the experimental conditions. The data is consistent with the findings of Kouri et al. (8) who described both the starting and the reconstituted 1A1 materials as weak inducers of pulmonary AHH activity in certain strains of mice. It was observed that the starting CSC produced a lower reponse than certain fractions ($B_I b$), which suggests that CSC contains both inducing and inhibitory components.

The weakly acidic fractions (WA_I and WA_E) showed a low efficacy for inducing AHH enzyme in human lymphocytes. In fact, they reduced enzyme activity of AHH at certain dose levels.

According to the findings of other investigators, WA_I only, was found to produce in vitro cellular transformation of the C3H-10T-1/2 cell lines (9). WA_E was found to be an active in vivo tumor promoter (2) and produced in vitro cellular transformation of Swiss mouse cells (14). Both WA_I and WA_E were reported to be weak inducers of AHH in C57/BL6 mouse in vivo (8). Regarding their mutagenic activity, WA_I was reported to be a good mutagenic agent and WA_E to be a weak mutagenic agent in bacteria (7).

All the strongly acidic fractions exhibited an inhibitory action at doses lower than 10^{-2} mg/well and all had a weak activity in inducing AHH in cultured human lymphocytes. All the three fractions were toxic at some dose. The three fractions (SA_I, SA_E, and SA_W) were reported to be weak inhibitors of pulmonary AHH activity in vivo (8). Other investigators reported the three fractions as being devoid of activity in the biological tests that they used (2,7,9,14). With the report of Wynder and Hoffman (17) that a significant tumor promoting activity was detected in the acidic fraction of CSC, it is possible that some of the compounds in WA_I obtained from the 1A1 cigarettes are also present in the WA_E fraction.

The most potent and efficacious of the twelve fractions was the basic fraction B_Ib. B_Ia fraction was a good AHH inducer, B_E was medium in its activity, while B_W was considered an inhibitor and a toxic fraction. Our data is consistent with Kouri et al. (8) who reported both B_Ib and BIa as good inducers of pulmonary AHH in vivo, and with Kier et al. (7) who reported both fractions as mutagenic fractions in Salmonella. B_Ia as good inducers of pulmonary AHH in vivo, and with Kier et al. (7) who reported both fractions as mutagenic dractions in Salmonella. B_Ib was reported to cause in vitro cell transformation in C3H-10T 1/2 (9) and Swiss mouse cells (9) and to cause tumor promotion in C3H-10T-1/2 mouse cells (2). B_E was found to be a tumor promoter (2), a weak inducer

of pulmonary AHH (8), and a weak mutagen in Salmonella (7). B_W was not active in all reported biological assays (2,7,9,14) but was a weak inhibitor of pulmonary AHH (8).

None of the neutral fractions (N_{ch}, N_{nm}, N_{meoh}) was toxic under these experimental conditions. With the exception of N_{ch}, they were potent and efficacious inducers of AHH. This data is consistent with Kouri et al. (8) who described N_{nm} and N_{meoh} as good pulmonary AHH inducers and N_{ch} as a weak inducer. N_{meoh} was a tumor promoter in C3H mouse cells (9) but was devoid of mutagenicity in Salmonella (14). N_{nm} caused in vitro cell transformation in Swiss mouse cells (14) and was devoid of mutagenicity (7).

The good response of AHH activity by certain basic fractions and B_Ib and B_Ia) and by certain neutral fractions (N_{nm} and N_{meoh}) and their reported inhibitory action on BP metabolism by Kouri et al. (8) suggest that these fractions contain certain PAH structurally similar to BP which are capable of inducing AHH and are capable of competitively inhibiting BP metabolism.

In view of the lack of information on the chemical composition of the fractions, any correlation between the data from different tests will be difficult. Until further information about the chemical composition of those fractions and their mechanism of carcinogenesis becomes available, the current data suggest united efforts of the cancer researchers and the tobacco industries to reduce the tar and nicotine yield of cigarettes as an emergent action to face the high risks of lung cancer among smokers. We

have to admit progress has been made in this area, but more work is imperative if we are to achieve the desired purpose and results.

PAH such as BP probably require metabolic activation before they are carcinogenic. Interests have been recently focused on attempts to determine the pathways of activation and to identify the metabolite(s) responsible for initiating carcinogenesis. Since the fluorometric assays of BP metabolism do not measure all the metabolites formed by the enzyme complex, radiometric assays were developed to facilitate quantitation of the total BP metabolism and to quantitate the total activity of the tissues for metabolizing BP. High pressure liquid chromatography (HPLC) has greatly supplanted the conventional column chromatography and thin-layer chromatography (TLC) for separation of BP metabolites. The main advantages of this technique are increased speed of separation and increased resolution. The present study indicates that cultured human lymphocytes have the capacity to metabolize BP to several hydroxy and quinone derivatives. The primary metabolites can be further metabolized by human lymphocytes to conjugates of sulfate, glucuronic acid and glutathione. We were able to separate the primary metabolites (diols, quinones and phenols) by a combination of HPLC and selective enzymatic hydrolysis. By another method, we were able to separate the primary metabolites from the secondary metabolites and the parent compound, BP. The primary metabolite profiles of BP were similar to those reported by others for human lymphocytes and the identity of the peaks were tentatively considered to be the

same as reported by Holder et al. (6). This data suggest that during a short time of reaction where the substrate concentration does not saturate the enzyme complex, a substantial amount of conjugation occurs via glucuronide formation. The phenols were conjugated to an equal extent with glucuronide and sulfate. It also suggested that the primary metabolites and conjugates vary in amount between individuals, and even vary intraindividually depending upon time of incubation and BP concentration.

A revision of a method reported by Autrup et al. (1) led us to separate the conjugates, the primary metabolites nd the parent compound BP by a solvent gradient followed by pH step gradient elution from a neutral alumina column. The advantage of our method over the original method is that we were able to separate the primary metabolites from the parent compound and reduce the total elution volume.

If all of the intermediates of BP are enzymatically produced, then a cell at great risk might either accelerate the production of an activated intermediate (epoxide or diol epoxide) or have a reduced ability to detoxify the activated carcinogen to conjugated, water soluble, urine excretable products. Either action would lead to intracellular accumulation of the active molecular species, with increased probability of reacting with the target site to produce malignant transformation. Conversely, a resistant (or a less susceptible) cell may be characterized by decelerated formation of the activated carcinogen or increased conjugating enzymes' activity yielding water soluble, urine excretable products.

If that assumption were true, then this assay protocol could provide data on a persons relative rate of activation/inactivation reactions, and hence reflect that person's relative risk to develop cancer.

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