CHAPTER III

MODULATION OF MOLECULAR EFFECTS OF BENZO(A)PYRENE BY DIETARY FACTORS

MODULATION OF MOLECULAR EFFECTS OF BENZO(A)PYRENE BY DIETARY FACTORS

3.1. INTRODUCTION

3.1.1. BACKGROUND INFORMATION

The global epidemiological data suggest that majority of human cancers are caused by environmental factors. The ideal control or prevention of human cancer would be to eliminate these factors from our environment. This is impossible to achieve since man is totally dependent on diet, life-style and industrial modernization, all of which contribute to the hazards of cancer. One of the most important areas of cancer research is to find alternate measures to prevent cancer. Nutritional control is one aspect in which serious effort has been directed. In fact, a major advance in cancer research during the past decade has been the development of plausible hypotheses about how diet can affect cancer incidence. These hypotheses are supported by a convergence of evidence from several fields of research. Epidemiological evidences show that prevalence of certain cancers are linked to intake of diet containing high animal fat and protein, high calorie and low fibre (Greenwald and Schreiber 1983). Apart from major food components, it has been known from experimental studies that there are other dietary factors which have definite role in controlling cancer incidence (Bhattacharya 1980). Such basic research has been critical in developing the concept of chemoprevention of cancer.

Evidence that substances produced in nature are capable of strong anticancer activity has been shown for quite some time. Many antitumor compounds of natural origin have been identified (Aszalos and Berdy 1981). Higher plants, in particular, contain an extensive variety of substances, some of which are strong modifiers of chemical carcinogenesis. These substances are of various chemical types and include phenols, indoles, flavones, aromatic isothiocyanates, thiols, coumarins, carotenoids, trace metals, as well as a number of other compounds (Wattenberg 1980; Slaga 1981). Considerable importance has now been attributed to the anticarcinogenic activity of certain agents present naturally in food or added to food and drinks for human consumption (Bhattacharya 1980; Wattenberg, 1980; Slaga 1981; Ames 1983). Especially, natural antioxidants such as α-tocopherol (Newmark and Mergens 1981), ascorbic acid (Mirvish 1981), selenium (Jacobs and Griffin 1981), retinoids (Sporn and Newton 1981) and synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) (Wattenberg 1978; 1980; Slaga 1981) assume importance because of their strong modifying role in chemical carcinogenesis. With BP in particular, several compounds were found to prevent experimental tumor formation in different animal models. The list of these compounds include BHA, BHT, ethoxyquin, coumarin, α-angelicalactone and sulfur containing substances like disulfiram, bisethylxanthogen, 2-chloroallyl diethyldithiocarbamate,

a primary and critical event in the process of carcinogenesis. Any factor which can inhibit this reaction is likely to offer protection against chemical carcinogenesis in exposed organisms. The reaction leading to formation of DNA adduct can be measured both in vivo and in vitro with convenience, reproducibility and speed. This facile in vitro testing procedure also has the advantage to investigate the mechanism of action of a particular agent. Wattenberg (1985) divided the modifiers of carcinogenesis into three broad classes depending on the stage at which they act in the cell. These are: (a) inhibitor of activation of carcinogen, (b) blocking agent of interaction between activated carcinogen and DNA, and (c) suppressor of action of modified target. In vitro tests also can differentiate these actions.

3.1.3. SPECIFIC RELEVANCE TO OTHER WORKS

Anticarcinogenic nature of the modulators chosen for these studies has been either established beyond doubt or is positively implied by several studies. The effect of these factors, however, varied with the carcinogen used, and it deserves emphasis that very few studies have been conducted with BP as carcinogen. It has been found that ascorbic acid affords protection against experimental carcinogenesis induced in animals by several chemicals (Schlegal et al. 1970; Shamberger 1972; Slaga and Bracken 1977; Chan and Fong 1977)

including that by BP (Kallistratos et al. 1983). Ingestion of ascorbic acid also prevents acute hepatotoxicity after administration of carcinogenic precursors (Cardesa et al. 1974; Mirvish 1975; Kamm et al. 1975). Subsequently, it has been established that ascorbic acid inhibits N-nitrosation reaction leading to suppression of formation of carcinogenic N-nitrosamines (Mirvish 1981). Mutation frequency in bacteria induced by carcinogenic N-nitroso compounds has also been found to be reduced significantly by ascorbic acid (Guttenplan 1977). Evidence to date obtained from studies with experimental animals and from human population supports that vitamin A is a natural inhibitor for the onset or development of cancer (Frolik and Roller 1981; Kummet and Meyskens 1983; Mettlin 1983; Basu et al. 1984). In numerous experimental studies vitamin A has been shown to prevent, delay or retard carcinogenesis induced in animals by a variety of chemicals (Newberne and Rogers 1981; Sporn and Newton 1981; Moon et al. 1983). Vitamin A and its analogs, collectively known as retinoids, have therefore, assumed considerable importance today in a scheme of chemoprevention of cancer.

Both ascorbic acid and retinol are nutritional requirements and hence their intake is a necessity and these can be taken in high amounts to a certain extent without inducing any toxicity. Flavonoids, on the other hand, are non-nutritional secondary metabolites of the plant which have

widespread occurrence and are present in vegetarian foods of common consumption (Mabry and Ulubelen 1980; Wollenweber and Dietz 1981). Apart from their wide variety of pharmacological activities (Havsteen 1983), flavonoids have been shown to be antimutagenic to several types of chemicals (Huang et al. 1983; Raj and Morris 1984; Birt et al. 1986). Flavonoids have also displayed anticarcinogenic effect against BP (Wattenberg and Leong 1970; vanDuuren et al. 1971; vanDuuren and Goldschmidt, 1976; Anderson et al. 1983), aflatoxin B_1 (Nixon et al. 1984) and transplantable colon tumors (Plowman et al. 1986).

The anticarcinogenic or modifying effect of substances under investigation can be determined using tumor induction model in experimental animals as has been documented above. This is a long, time-consuming and costly procedure. Before such an effect can be demonstrated with any carcinogen it is important to consider if a particular substance can modify the initial molecular event of that carcinogen. Although retinoids (Sporn and Newton 1981; Frolik and Roller 1981; Yuspa 1983) and flavonoids (Birt et al. 1986) have been shown to have some role in the promotion stage in cancer induction, most inhibitors of carcinogenesis act by interfering at the initiation stage of chemical carcinogenesis (Wattenberg 1985). Therefore, screening of natural factors, particularly food components, for anticarcinogenic activity

through the facile in vitro reaction of DNA adduct formation is considered convenient and fruitful. Additionally, valuable data can be obtained which explain how an anti-carcinogenic substance acts.

3.2. EXPERIMENTAL PROCEDURES

3.2.1. MATERIALS

3.2.1.1. Chemicals:

Ribonuclease-A [E.C. 3.1.27.5 (bovine pancrease)], 3-methylcholanthrene, glucose-6-phosphate, methylbenzethonium hydroxide (1 M in methanol), NADP, NADPH and FMN were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Retinoids were generous gift from Roche Products Ltd., Bombay. Flavonoids were kindly provided by Dr. A.K. Banerjee of Bio-Organic Division of this Research Centre. Grateful appreciation is recorded for Dr. R.G. Harvey of Ben May Laboratory for Cancer Research, Chicago, Il., USA, for his kind gift of synthetic BP-7,8-dihydrodiol and BPDE I. Aroclor was procured from Monsanto Co., St. Louis, MO, U.S.A. L-Ascorbic acid (LAA) was purchased from E. Merck, Darmstad, FRG. For some of the feeding studies LAA was obtained from Sarabhai M. Chemicals, Baroda. 3-Chloroperbenzoic acid was from Riedelde Haen AG, Seelze-Hannover, FRG. Other chemicals used in this study were as mentioned in Chapter II (2.2.1.1).

3.2.1.2. Radioactive chemicals:

[G-^{3}H]Benzo(a)pyrene (sp.act. 1200 or 3700 or 5300 mCi mmol^{-1}) was obtained from Isotope Group of this Research Centre. Other radiochemicals used in this study were as specified in Chapter II (2.2.1.2).

3.2.1.3. Animals:

Animals were as specified in Chapter II (2.2.1.3).

3.2.2. METHODS

3.2.2.1. Treatment of animals:

(a) [^{3}H]Benzo(a)pyrene: Rats were administered i.p. with [^{3}H]BP (sp.act. 64.2 mCi mmol^{-1}) in DMSO, at the dose of 20 mg kg^{-1} body weight, 16 h prior to sacrifice.

(b) L-Ascorbic acid: Two separate routes of administration were followed.

(i) Oral route: Rats were given 2.5% LAA in sweetened drinking water (2% sucrose) for 7 days. Appropriate controls were given only sweetened drinking water.

(ii) Intraperitoneal: LAA (50 mg ml^{-1} in H_2O, pH 5.5) was administered i.p. to rats at the dose of 100 and 300 mg kg^{-1} body weight. The treatment was made either 2 h before sacrifice or 2 h before administration of [^{3}H]BP.

3.2.2.2. Preparation of liver slices:

Livers from rats were collected as specified in Chapter II (2.2.2.1). Sections of 0.2 - 0.4 mm thickness were prepared using a Stadie-Riggs tissue slicer (Arthur H. Thomas Co., Phila., PA, U.S.A.). The slices were suspended

in Krebs-Ringer solution-phosphate buffer [125 mM NaCl, 6 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 0.2% (w/v) D-glucose and 15 mM sodium phosphate buffer (pH 7.4)].

3.2.2.3. Isolation and purification of nuclei from liver:

Nuclei were purified by osmolar method as described in Chapter II (2.2.2.2).

3.2.2.4. Isolation of microsomes from liver:

Liver was homogenized in 3 vol of 0.32 M sucrose-3 mM $MgCl_2$ using Potter-Elvehjem homogenizer and crude nuclear pellet was removed by centrifugation at 800 x g for 10 min. Post-nuclear supernatant was spun at 24,000 x g for 10 min to pellet out mitochondrial fraction and the supernatant was further centrifuged at 110,000 x g_{av} for 1.5 h to obtain purified microsomal fraction. This was suspended in 0.32 M sucrose-1 mM $MgCl_2$ and aliquots were stored frozen at -40^{o} upto 3 months.

For the preparation of induced microsomal enzymes, animals were treated either with 3-methylcholanthrene (dissolved in DMSO and administered i.p. at the dose of 20 mg kg^{-1}, 18 h prior to sacrifice) or with aroclor (dissolved in DMSO and administered i.p. at the dose of 500 mg kg^{-1}, 96 h prior to sacrifice). Microsomes were then prepared by the method described above.

3.2.2.5. Isolation of nuclear DNA:

DNA was isolated from liver nuclei by a procedure adopted from several methods (Marmur 1961; Bernardi 1971; Markov and Arion 1973; Viviani and Lutz 1978). Crude nuclear pellet obtained from 2.5 g liver was washed in 10 ml wash-medium [0.14 M NaCl - 0.02 M EDTA (pH 7.2)] and recovered by centrifugation at 800 x g for 10 min. The washed pellet was suspended in 20 ml lysing medium [1 M NaCl - 0.1 M EDTA (pH 7.2) - 2% SDS (w/v)] and was vigorously shaken at 60° for 10 min. After cooling in ice, it was extracted with equal vol of chloroform : isoamyl alcohol : phenol (24 : 1 : 25) for 10 min at room temperature. Aqueous phase obtained after spinning at 12,000 x g for 10 min was extracted 2x with equal vol of chloroform : isoamyl alcohol (24:1). From the final aqueous phase (chilled in ice), addition of 2.5 vol ethanol (at -20°) separated the DNA which was spooled out on a glass rod. DNA was washed twice in ethanol and was dried in air. It was dissolved in SSC [0.15 M NaCl - 0.015 M Na-citrate, (pH 7.0)] and was treated at 37° for 30 min with RNase A (pre-heated at 80° for 10 min to destroy DNase) at a final concentration of 1 mg ml^{-1}. After cooling in ice, solution was deproteinized by extraction with 0.5 vol phenol mixture [phenol : water : m-cresol (64:16:20) containing 0.1% 8-hydroxyquinoline]. Two more extractions of the aqueous phase were carried out with equal vol of chloroform : isoamyl

alcohol (24:1). From the final aqueous phase, DNA was precipitated with alcohol as specified above and was dissolved in SSC.

3.2.2.6. Isolation of nuclear RNA:

Nuclear RNA was isolated by the method as described in Chapter II (2.2.2.4).

3.2.2.7. In vivo incorporation of [6-^{14}C]orotic acid into hepatic nuclear RNA:

It was carried out according to the method as described in Chapter II (2.2.2.5).

3.2.2.8. In vitro adduct formation between BP and its metabolites with DNA:

(a) With liver slices: Liver slices from 5 g liver were suspended in 15 ml Krebs-Ringer solution-phosphate buffer (pH 7.4) and incubated in air with 0.4 µmol [^{3}H]BP (sp.act. 322.5 mCi mmol^{-1}) at 37^{o} for 2 h with constant shaking. Nuclear fraction and nuclear DNA were prepared from these slices of liver at the end of the incubation. Aliquot of DNA was then used for determination of absorbance at 360 nm and rdioactivity. Adduct formation was expressed as µmol BP metabolite mol^{-1} DNA-P.

(b) With liver nuclei: Adduct formation in vitro between [^{3}H]BP and nuclear DNA was carried out as follows: In a 2 ml reaction mixture containing 15 mM sodium phosphate buffer (pH 7.4), 25 mM EDTA and 1 mg NADPH, nuclei (equivalent of 2 g fresh liver) were reacted with 100 µM [^{3}H]BP (sp.act. 322.5 mCi mmol^{-1}). Microsome from control animals (equivalent of 100 mg fresh liver, ~1 mg protein) was also added to facilitate metabolism of BP. After incubation at 37^o for 30 min, nuclear DNA was isolated. An aliquot of DNA solution was subjected to determination of absorbance at 260 nm and radioactivity. Adduct formation was expressed as µmol BP metabolite mol^{-1} DNA-P.

(c) With liver microsomes: Adduct formation betwen [^{3}H]BP or its metabolites with calf thyumus DNA was carried out according to the method of Gelboin (1969) after suitable modification as follows. Each reaction mixture (2 ml) contained all the components including microsomes as specified for liver nuclear adduct formation assay, except for two components: [^{3}H]BP and nuclei. [^{3}H]BP, 100 µM was added at sp.act. of 54 mCi mmol^{-1} (or at 129 mCi mmol^{-1} in some experiments), and nuclei were replaced by 0.5 mg calf thymus DNA. After incubation at 37^o for 30 min, reaction was stopped by chilling in ice. It was extracted 2x with equal vol of phenol mixture [phenol : water : m-cresol (24:1:25) containing 0.1% 8-hydroxyquinoline and 0.1% SDS]. DNA was separated from final aqueous phase (made

to 3% with potassium acetate) by addition of chilled 2.5 vol ethanol. DNA was washed 3x with cold ethanol and dissolved in 1 ml SSC. An aliquot each was used for measurement of absorbance at 260 nm and radioactivity. Adduct formation was expressed as μmol BP metabolite mol^{-1} DNA-P.

The microsome mediated adduct formation between [^{3}H]BP-7,8-dihydrodiol and DNA was carried out in reaction system identical to the above reaction except that [^{3}H]BP was replaced by 7.7 μM [^{3}H]BP-7,8-dihydrodiol (sp.act. 40.2 mCi $mmol^{-1}$).

(d) Non-enzymatic adduct formation: The adduct formation between [^{3}H]BPDE and DNA was carried out nonenzymatically according to Sims et al. (1974). Each reaction mixture (2 ml) contained 10 mM Tris-HCl (pH 7.5), 0.5 mg calf thymus DNA, 25% (v/v) spectral grade methanol and 4.85 μM [^{3}H]BPDE (sp. act. 36.12 mCi $mmol^{-1}$). DNA was precipitated by ethanol and processed as described in earlier studies.

For the series of experiments to study modulation by retinoids and flavonoids of the microsome mediated adduct formation, volume of reaction mixture was kept at 1 ml instead of 2, and 60 μM [^{3}H]BP (sp.act. 333 mCi $mmol^{-1}$) was reacted with 0.5 mg calf thymus DNA in presence of microsomes and NADPH as mentioned above. After incubation at 37° for 30 min, reaction was stopped by addition of 1 ml chilled 15 mM

sodium phosphate buffer (pH 7.4). Subsequent deproteinization and isolation of DNA was carried out as described above.

3.2.2.9. Preparation and purification of metabolites of BP:

(a) [^{3}H]BP-7,8-dihydrodiol: For large scale metabolic preparation of BP-7,8-dihydrodiol, following procedure of Kinoshita et al. (1973) was used. Liver was homogenized in 4 vol of 0.1 M sodium phosphate buffer (pH 7.4) and homogenate was spun at 800 x g for 10 min. Supernatant was mixed with equal vol of same buffer containing NADP (2 mg ml^{-1}), glucose-6-phosphate (8 mg ml^{-1}) and [^{3}H]BP (1-2 mCi in ethanol). After incubation at 37^o for 1 h, BP and its metabolites were extracted from the reaction mixture by vigorous shaking with equal vol of ethyl acetate. The organic phase was passed through anhydrous sodium sulfate to remove traces of water and was evaporated to dryness. It was dissolved in a small vol of methanol and was applied on 0.25 mm thick layer of silica gel G (20 cm x 20 cm) for t.l.c. Chromatogram was developed with two successive runs of benzene : ethanol (19:1 v/v) according to the published procedures (Waterfall and Sims 1972; Kinoshita et al. 1973; Grover et al. 1974). All the major metabolites were well separated. BP-7,8-dihydrodiol, a bluish-green fluorescent band at $R_f \sim 0.25$ was located just near the violet fluorescent band of BP-9,10-dihydrodiol ($R_f \sim 0.22$) and was eluted from silica gel by triple extraction with methanol. When required, the compound was further purified by t.l.c.

with benzene : ethanol (9:1), a system in which BP-7,8-dihydrodiol has $R_f \sim 0.45$. Its purity was checked by UV spectroscopy (Fig. 3.1) in comparison with standard BP-7,8-dihydrodiol. The product was suitably diluted with similarly prepared cold metabolite to give final sp.act. of 40.2 mCi $mmol^{-1}$.

For determination of metabolic availability of BP-7,8-dihydrodiol, the assay conditions were the same as for the in vitro assay for microsome mediated adduct formation between BP and DNA as described earlier (3.2.2.8) except that DNA was omitted in metabolism assay. At the end of the incubation, mixture was extracted with equal vol of ethyl acetate, which was evaporated to dryness. BP-7,8-dihydrodiol was isolated according to the procedure outlined before and assayed for radioactivity. Results were expressed as radioactivity found in BP-7,8-dihydrodiol band as per cent of total radioactivity,

(b) [^{3}H]BP-7,8-dihydrodiol 9,10-epoxide: BPDE was synthesized chemically from BP-7,8-dihydrodiol by the method of Sims et al. (1974). [^{3}H]BP-7,8-dihydrodiol (17 nmol, sp.act. 36.12 mCi $mmol^{-1}$) dissolved in 0.9 ml chloroform was mixed with 100 µg of 3-chloroperbenzoic acid in 0.1 ml $CHCl_3$. Reaction was allowed to proceed at 4° for 48 h in dark with occasional stirring. The mixture was washed once each with equal vol of 1 mM NaOH and water. Final organic phase was

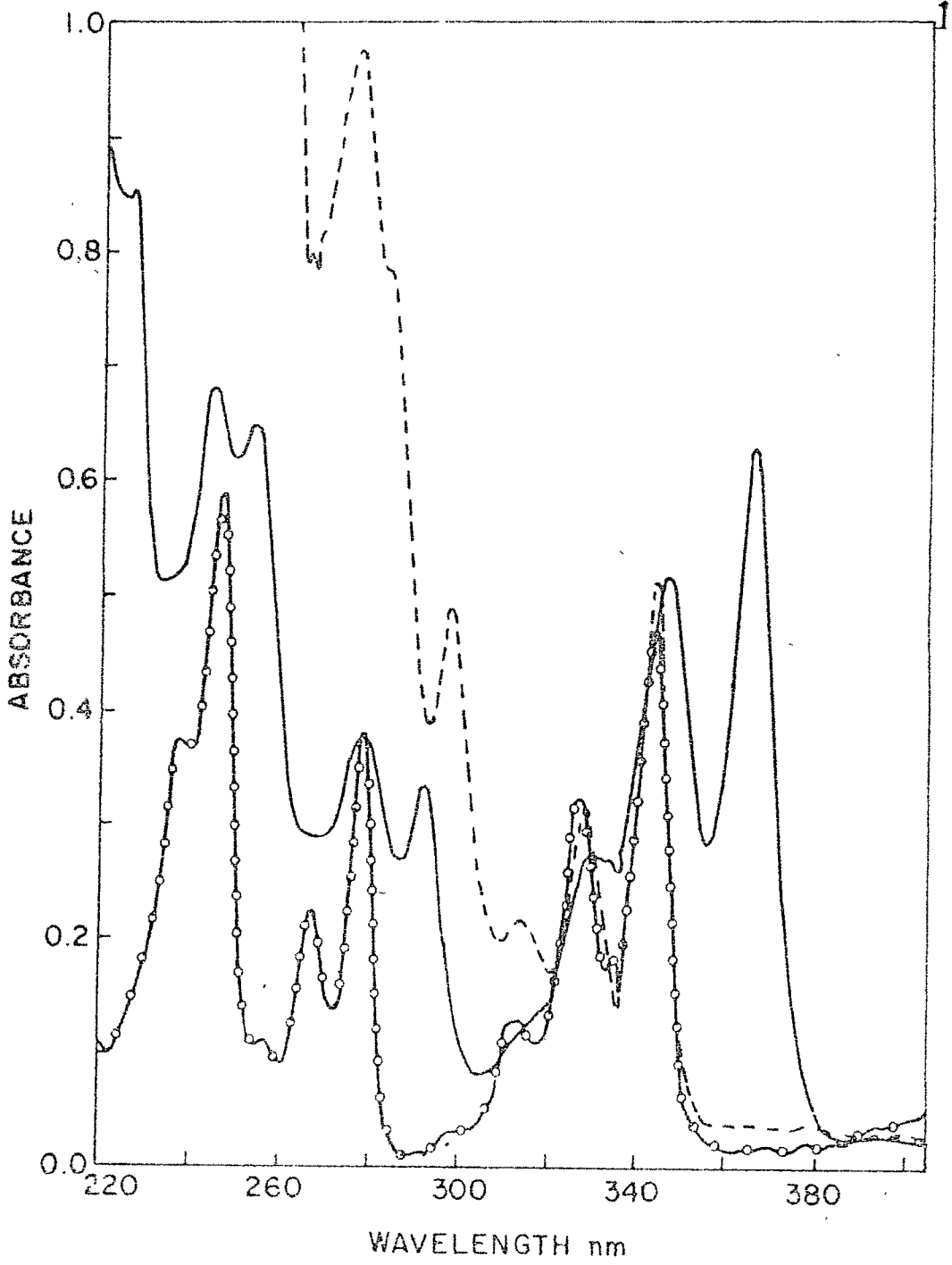


FIG. 3.1. UV-Spectra of purified metabolites of BP:
All the metabolites were dissolved in spectral grade methanol. Solid line (——) refers to BP-7,8-dihydrodiol (13.1 μM); Broken line (---) refers to BP-9,10-dihydrodiol; and broken line with circles (o—o-) refers to BPDE I (9.3 μM). Spectra for BP-7,8-dihydrodiol and BPDE I were found to be superimposable with the authentic samples (curves not shown).

evaporated to dryness and redissolved in methanol. BPDE was purified by t.l.c. on silica gel G using dioxane : cyclohexane (1:1) as developing system. BPDE band observed at $R_f \sim 0.65$ was extracted in methanol and its purity was confirmed by UV spectroscopy in comparison with that of a synthetic BPDE I (Fig. 3.1).

For determination of metabolic availability of BPDE, the reaction conditions were same as that employed for microsome mediated adduct formation between [^{3}H]BP-7,8-dihydrodiol and DNA as described earlier (3.2.2.8), except that DNA was omitted. At the end of the reaction, BP-derivatives were extracted with ethylacetate, evaporated to dryness and redissolved in chloroform. On t.l.c. with dioxane : cyclohexane (1:1) as developing system, unreacted BP-7,8-dihydrodiol was separated from BP-triols and BP-tetraols, which were obtained as a result of hydrolysis of BPDE. The radioactivity associated with these hydrolyzed products expressed as per cent of total radioactivity gave a quantitative measure of metabolic availability of BPDE.

3.2.2.10. Measurement of the rate of disappearance of BPDE I:

Rate of disappearance of BPDE I was studied spectrophotometrically at room temperature according to the method of Huang et al. (1983). In a total vol of 1 ml of aqueous dioxane (9:1) containing 0.1 M sodium perchlorate - 2 mM

Tris-perchlorate buffer (pH 7.0), BPDE I dissolved in dioxane was added at 3 μM (or 4.2 μM) final concentration. The absorbance at 345 nm was monitored in an Aminco DW 2A or a Shimadzu UV/VIS spectrophotometer. Decrease in absorbance at 345 nm was plotted against time.

The absorbance v/s time was also plotted on semi-log paper and by extrapolating the initial linear velocity, time required for absorbance at 345 nm of BPDE I to become 50% of original value ($T_{\frac{1}{2}}$) was determined.

3.2.3. ANALYTICAL PROCEDURES

3.2.3.1. Estimation of DNA:

DNA dissolved in SSC was analyzed spectrophotometrically by measuring absorbance at 260 nm and 280 nm. Ratio of above 1.8 for A_{260} / A_{280} ensured protein free preparation. Molar extinction coefficient of DNA-P was taken as 6600 cm^{-1} at 260 nm.

3.2.3.2. Estimation of RNA:

Spectrophotometric and chemical estimations of RNA were carried out as described in Chapter II (2.2.3.1).

3.2.3.3. Estimation of protein:

Protein concentration of microsomal preparation was determined by the method of Lowry et al. (1951) using crystalline BSA as standard.

3.2.3.4. Radioactivity measurement:

Soft β-emissions from [^{3}H] and [^{14}C] sources were counted in dioxane based scintillation cocktail as described in Chapter II (2.2.3.5). Some [^{3}H]BP metabolite-bound DNA and alkali digests of [^{14}C]RNA were counted directly in the cocktail. When amount of DNA to be counted was large (1-2 mg), it was hydrolyzed with equal vol of 1 M PCA at 70^{o} for 15 min. The sample was cooled, neutralized with 1 M NaOH and centrifuged. The hydrolysate was counted for radioactivity in Triton-toluene cocktail (7 g PPO, 200 mg dimethyl POPOP, 350 ml Triton-X 100 and 650 ml toluene). However, [^{3}H]BP metabolite-bound DNA was routinely analyzed as follows. A 0.5 ml aliquot of DNA solution containing 100 - 200 µg DNA was solubilized with equal vol of methylbenzethonium hydroxide at room temperature for 3 h in the dark. Dioxane based cocktail (10 ml) was added to this and after stabilization of the sample overnight in the dark, radioactivity was counted.

3.3. RESULTS

3.3.1. Effect of L-ascorbic acid in vivo on adduct formation in liver between [^{3}H]BP metabolites and DNA:

Animals were treated i.p. with two doses of LAA (100 or 300 mg kg^{-1} body weight), 2 h before i.p. administration of [^{3}H]BP . Control animals received only [^{3}H]BP. Sixteen hours after administration of [^{3}H]BP, the animals were sacrificed and from their liver nuclear DNA was prepared, estimated and its bound radioactivity was measured after PCA hydrolysis. Adduct formation levels are shown in Fig. 3.2. In hepatic nuclei of normal rats adduct formation was 1.89 µmol mol^{-1} DNA-P. Pretreatment of rats with LAA reduced the in vivo binding of the carcinogen to DNA. The respective inhibition of adduct formation was 60% at 100 mg kg^{-1} and 76% at 300 mg kg^{-1} body weight dose levels.

3.3.2. In vitro adduct formation between [^{3}H]BP-metabolites and DNA, mediated by liver subcellular preparations of rats pretreated with L-ascorbic acid:

Livers from LAA pretreated rats were processed as described earlier (3.2.2.2-4) to obtain liver slices, liver nuclei and microsomes. The capacity of these three subcellular components to mediate adduct formation with DNA (endogenous in the preparations of slices and nuclei, and exogenous for

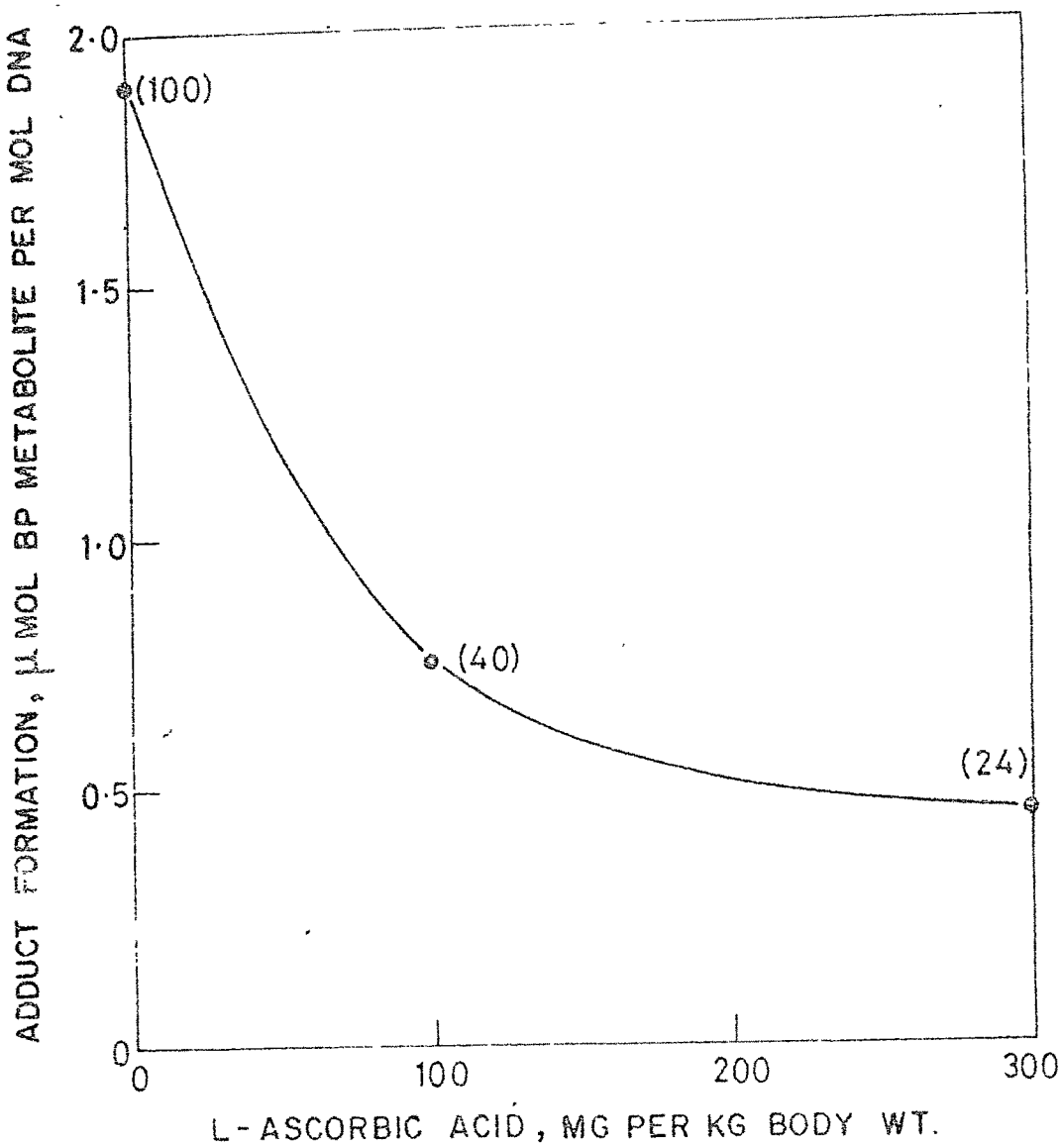


FIG. 3.2. Adduct formation in vivo between BP and hepatic DNA of rats pretreated with L-ascorbic acid:

Adduct formation was determined from the amount of [^{3}H]BP-metabolites covalently bound to DNA isolated from liver nuclei. Each value represents average from 4 rats. Figures in parenthesis are per cent of control value.

microsome assay) was compared with similar components from uninjected control animals. Results are tabulated in Table 3.1.

When [^{3}H]BP was incubated with liver slice preparations from LAA pretreated rats, covalent binding to nuclear DNA was found to be lower as compared to binding observed in liver slices of normal animals. The inhibition was observed to be 18% and 40% at doses of 100 mg kg^{-1} and 300 mg kg^{-1} body weight respectively.

With liver nuclei, 36% inhibition in the adduct formation was observed in preparations from rats pretreated with LAA at 300 mg kg^{-1}. There was no inhibition in preparations from 100 mg kg^{-1} LAA-pretreated animals.

The microsomal preparations from LAA pretreated animals did not show any inhibition in their ability to mediate the adduct formation. The higher activity observed at 100 mg kg^{-1} level was perhaps non-specific with respect to LAA in as much as no further stimulation was observed with a higher dose.

3.3.3. Inhibition by L-ascorbic acid in vitro of microsome mediated adduct formation between [^{3}H]BP and DNA:

The microsome catalyzed adduct formation between [^{3}H]BP and DNA was carried out according to the procedure

TABLE 3.1

In vitro adduct formation mediated by liver subcellular preparations of rats pretreated with L-ascorbic acid

LAA treatment	Adduct formation, μmol (^{3}H)BP-metabolite mol^{-1} DNA P		
	Slices	Nuclei	Microsome
None	2.80 ± 0.15 (100)	2.32 ± 0.20 (100)	28.12 ± 3.1 (100)
100 mg kg^{-1}	2.31 ± 0.20 (82)	2.30 ± 0.24 (99)	34.92 ± 4.5 (124)
300 mg kg^{-1}	1.67 ± 0.22 (60)	1.50 ± 0.25 (64)	26.52 ± 3.9 (94)

Reaction conditions for each assay were as described in Methods (3.2.2.8). Each value represents average from 4 animals with S.D. Figures in parenthesis represent per cent of control of a respective group. Adduct formation in liver slices and nuclei represents adducts with endogenous DNA while in microsome, that with exogenous DNA.

described earlier (3.2.2.8). Microsomes were prepared from 3-methylcholanthrene-induced animals. When LAA was added to this reaction there was dose-related inhibition in the adduct formation (Fig. 3.3). In absence of added LAA, the adduct formation was 70.94 μmol mol^{-1} DNA-P. There was rapid inhibition in adduct formation upto 5 mM LAA (40% at 1 mM and 60% at 5 mM). The adduct formation was less sensitive to increase in concentration of LAA from 5 to 17.5 mM (74% inhibition at 17.5 mM).

3.3.4. Effect of L-ascorbic acid in vitro on relative adduct formation between metabolites of [^{3}H]BP and DNA:

Microsomal enzyme mediated adduct formation of [^{3}H]BP-7,8-dihydrodiol with DNA and nonenzymatic interaction of [^{3}H]BPDE with DNA were carried out according to procedures described (3.2.2.8c & d). The substrate concentrations of BP-7,8-dihydrodiol and BPDE were selected from prior experiments in order to give equal extents of binding to DNA. The average value of adduct formation from several experiments with these metabolites was ~65 μmol mol^{-1} DNA-P. Inhibitory effect of LAA added to these reactions has been depicted in Fig. 3.4. Effect of lower concentrations of LAA on microsome catalyzed adduct formation using [^{3}H]BP as substrate is also represented here for comparison. Using [^{3}H]BP-7,8-dihydrodiol as substrate, the adduct formation was observed to be more

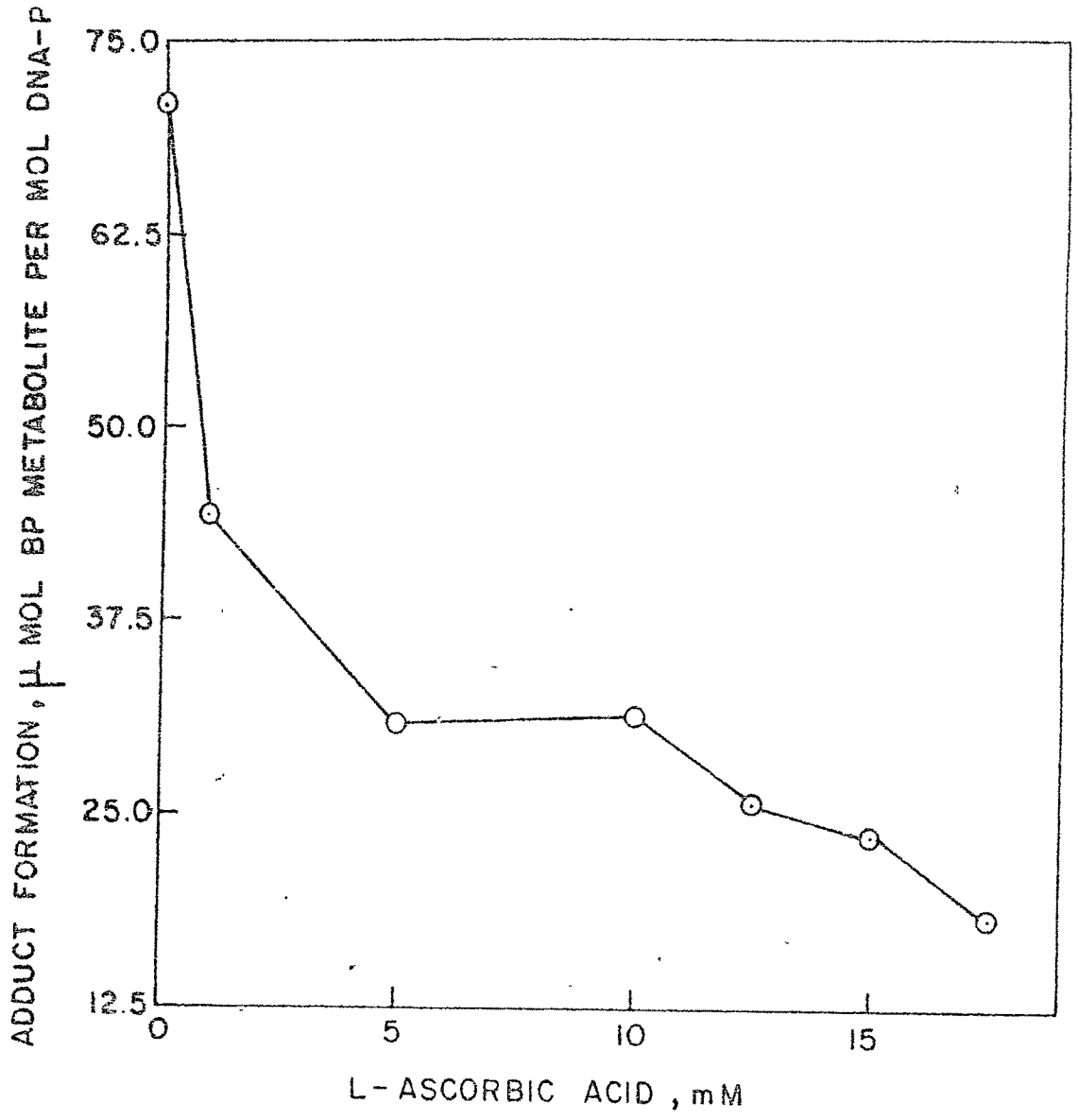


FIG. 3.3. Inhibition by L-ascorbic acid in vitro of adduct formation between BP-metabolites and DNA:

Microsome-mediated adduct formation of [^{3}H]BP with exogenous DNA was measured in reaction mixtures containing different amounts of L-ascorbic acid. Reaction conditions are as described in Methods (3.2.2.8). Each value represents average from three experiments.

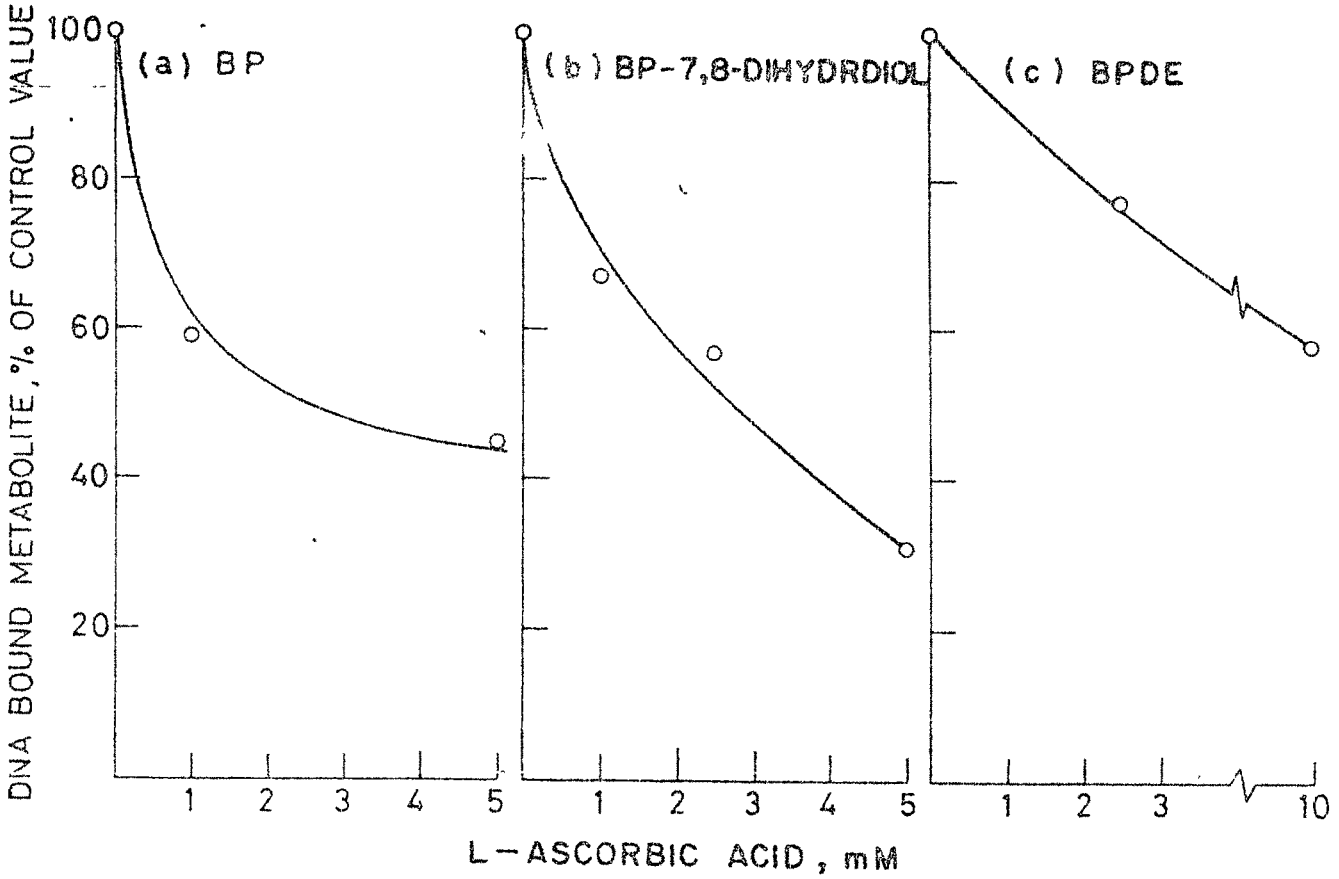


FIG. 3.4. Effect of L-ascorbic acid on relative DNA binding by metabolites of BP:

Microsomal enzymes from 3-methylcholanthrene induced animals were used to mediate adduct formation in a 2 ml assay between DNA (0.5 mg) and either (a) 100 μM [^{3}H]BP (sp.act. 54 mCi mmol^{-1}) or (b) 7.7 μM [^{3}H]BP-7,8-dihydrodiol (sp.act. 40.2 mCi mmol^{-1} as described in Methods (3.2.2.8) Nonenzymatic adduct formation (c) was carried out in 2 ml buffered assay (pH 7.5) between 0.5 mg DNA and 4.85 μM [^{3}H]BPDE (sp.act. 36.12 mCi mmol^{-1}) as described (3.2.2.8). In all three cases average value for control adduct formation from several experiments was observed to be 65 μmol mol^{-1} DNA-P. Each result is average of three experiments. Results are expressed as per cent of respective control.

sensitive to LAA as compared to BP as substrate. There was 33, 45 and 70% inhibition of adduct formation over control at 1, 2.5 and 5 mM concentrations of LAA respectively. LAA could exert its inhibition of adduct formation even between BPDE and DNA in nonenzymatic reaction. The inhibition by LAA was 25 and 42% of control at 2.5 and 10 mM concentrations respectively.

3.3.5. Effect of L-ascorbic acid in vitro on metabolism of BP:

(a) Metabolic availability of BP-7,8-dihydrodiol: Metabolic availability represented an amount of [^{3}H]BP-7,8-dihydrodiol which could be measured in the assay mixture during microsome mediated transformation of [^{3}H]BP in the absence of exogenous DNA (see Methods 3.2.2.9). Addition of LAA at 1 mM final concentration in this reaction, inhibited the amount of BP-7,8-dihydrodiol available at the end of the reaction to the extent of 35% of control (Table 3.2).

(b) Metabolic availability of BPDE: This assay was carried out using microsome mediated conversion of [^{3}H]BP-7,8-dihydrodiol in the absence of DNA (see method 3.2.2.9). Since reaction conditions induce hydrolysis of enzymatically generated BPDE, the mixture of triols and tetraols which would indirectly represent the amount of diol epoxide formed were eluted and counted for radioactivity. When LAA was added to this reaction at 1 mM concentration, it inhibited the availability of BPDE to the extent of 22% of control (Table 3.2).

TABLE 3.2

Effect of L-ascorbic acid on metabolic availability of activated metabolites of BP

Substrate	Metabolite	Availability, pmol per assay		Inhibition %
		- LAA	+ 1 mM LAA	
BP	BP-7,8-dihydrodiol	3397	2211	35
BP-7,8-dihydrodiol	BPDE	339	263	22

Metabolic availability of BP-diol and BPDE were determined in 2 ml assays containing 10 μM (^{3}H)BP (sp. act. 54 mCi mmol^{-1}) or 7.7 μM (^{3}H)-BP-7,8-dihydrodiol (sp. act. 40.2 mCi mmol^{-1}) as described in Methods (3.2.2.9). Each result is average of three experiments.

3.3.6. Alleviating effects of dietary ascorbic acid on BP-induced inhibition of gross transcription in liver:

Gross transcription in liver nuclei, measured as in vivo incorporation of [6-^{14}C]orotic acid into nuclear RNA, was inhibited drastically 2 h after i.p. administration of BP as described in Chapter II (2.3.1). Effect of administration of LAA in drinking water for 7 days on this BP-induced inhibition was studied by methods described earlier (3.2.2.6) and 7). The incorporation of [^{14}C]orotic acid into RNA, expressed as cpm mg^{-1} RNA observed after alkali digestion, was as shown in Table 3.3. Nearly 60% inhibition in gross transcription caused by BP was reduced to 40% inhibition by pretreatment of rats with LAA.

3.3.7. Inhibition by retinoids in vitro of microsome mediated adduct formation between [^{3}H]BP and DNA:

Five retinoids viz. retinol, retinal, retinyl acetate, retinyl palmitate and retinoic acid, each dissolved in ethanol, were tested in adduct formation assay using [^{3}H]BP and DNA as described earlier (3.2.2.8.c). Retinoids were tested at a wide range of concentrations (from 1 to 500 μM). Appropriate solvent control (1% ethanol v/v) resulted in adduct formation of 9.23 ± 0.57 pmol A_{260}^{-1} (mean ± SE from 8 independent observations). Addition of ethanol by itself did not alter the adduct formation beyond 5% of control

TABLE 3.3

Reduction in BP-induced inhibition of gross hepatic transcription by dietary ascorbic acid

Treatment	Incorporation of (^{14}C)orotic acid cpm mg^{-1} RNA	
	No pretreatment	Dietary LAA
DMSO	83,244 (100)	66,412 (100)
BP (2 h)	32,945 (39.6)	40,184 (60.5)

Effect of LAA-pretreatment of animals (7 days in drinking water) on BP-induced inhibition of gross transcription was studied as described (3.3.6). The alkali digest of RNA was subjected to estimation of RNA and radioactivity. Each result is average of two experiments. Figures in parenthesis represent per cent of respective DMSO-control.

without ethanol. Adduct formation in absence of microsome was observed to be less than 5% of value in presence of microsomes. As shown in Fig. 3.5, for most of the retinoids, there was rapid inhibition in adduct formation upto 50 μM concentrations, after which the degree of inhibition was less sensitive to increase in concentrations. The amount of retinoid per assay at which adduct formation was 50% of control value (ID_{50}), was calculated and is shown in Table 3.4. These values are 2.5, 32.5 and 60 nmol for retinal, retinyl acetate and retinyl palmitate respectively. For retinol and retinoic acid ID_{50} was observed to be beyond 500 nmol per assay.

3.3.8. Effect of retinoids on the rate of disappearance of BPDE I:

Rate of disappearance of BPDE I in aqueous dioxane was measured spectrophotometrically as described in Methods (3.2.2.10). In the absence of any added retinoid (10 μL ethanol added as control), the decay in absorbance of BPDE I at 345 nm was very slow as shown in Fig. 3.6. However, when retinoids were added at two different concentrations (50 and 100 μM), there was dose-related acceleration of decrease in absorbance at 345 nm (Fig. 3.6). When these data were plotted on semi-log graph (Fig. 3.7), the time required for A_{345} to become 50% of original absorbance value ($T_{\frac{1}{2}}$) could be determined for each retinoid by extrapolation of initial linear velocity. $T_{\frac{1}{2}}$ was 225 min for control but with retinal,

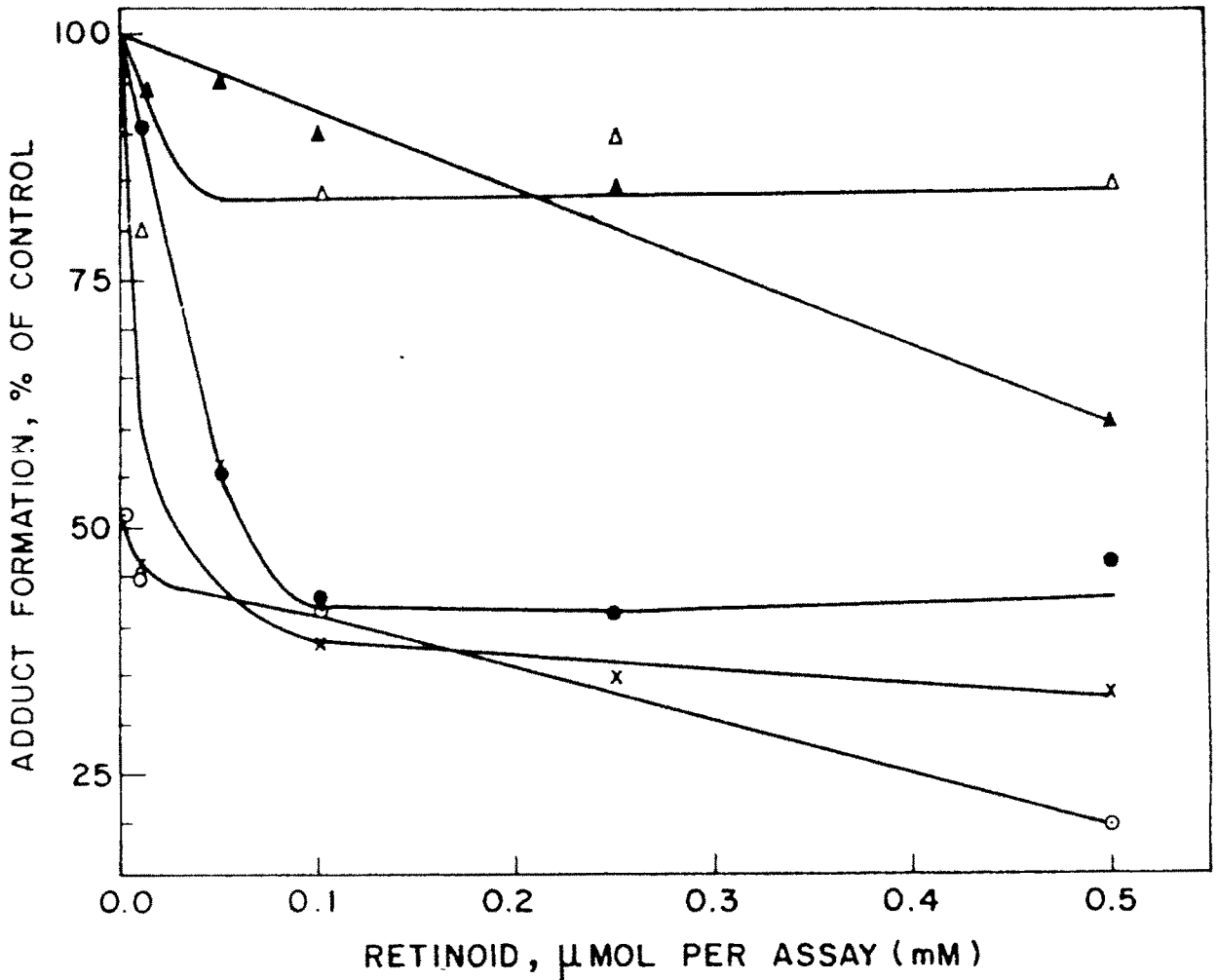
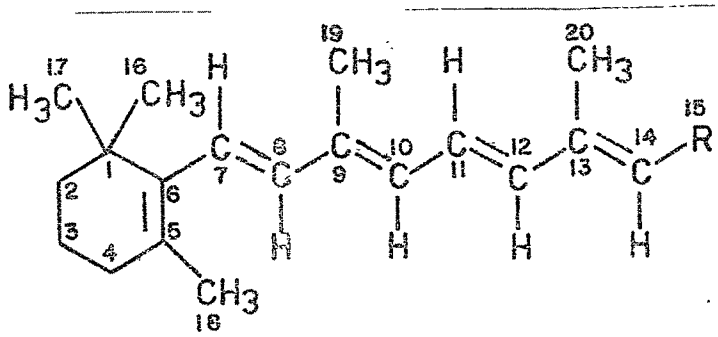


FIG. 3.5. Relative inhibition by retinoids of microsome-mediated adduct formation between [^{3}H]BP and DNA:
Retinoids dissolved in ethanol were added in the adduct formation assay (3.2.2.8) and results were expressed as per cent of control. Adduct formation in absence of retinoid i.e. control containing ethanol (1% v/v) was observed to be 9.23 ± 0.57 (mean ± S.E. pmol A_{260}^{-1} (n = 8). Open circles (o) represent retinal; crosses (x) represent retinyl acetate; closed circles (●) represent retinyl palmitate; open triangles (△) represent retinoic acid; and closed triangles (▲) represent retinol.

TABLE 3.4

ID_{50} Values of different retinoids for BP-DNA adduct formation



Retinoids		ID_{50} (nmol)
Retinal	R = CHO	2.5
Retinyl acetate	R = $CH_2OC(=O)-CH_3$	32.5
Retinyl palmitate	R = $CH_2OC(=O)(CH_2)_{14}CH_3$	60.0
Retinol	R = CH_2OH	>500.0
Retinoic acid	R = COOH	>500.0

The amount of each retinoid required to inhibit in vitro BP-DNA adduct formation by 50% (ID_{50}) was determined from Fig. 3.5.

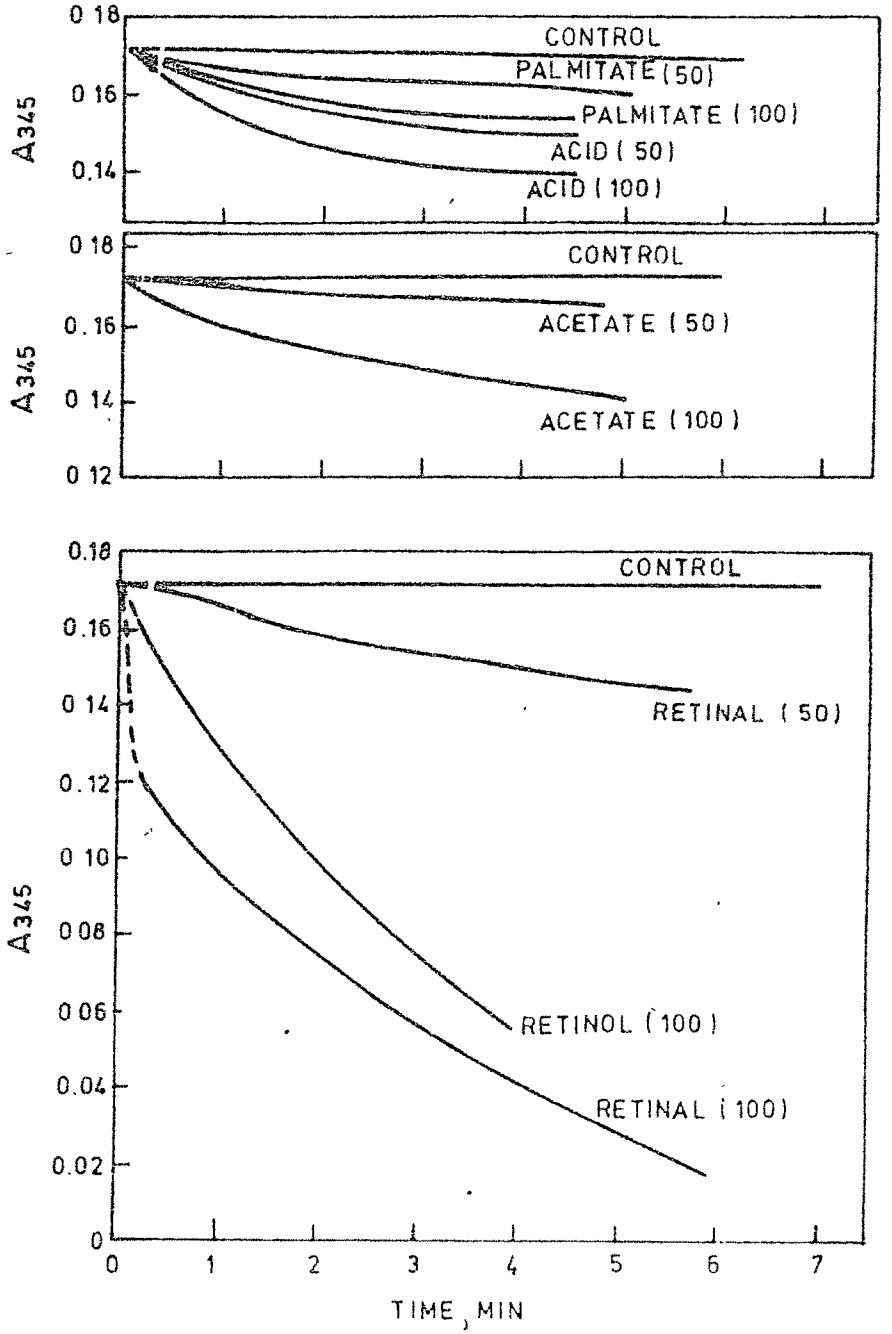


FIG. 3.6. Modulation of the rate of disappearance of BPDE I in presence of retinoids:
The decay of 4.2 μM BPDE I at 345 nm in buffered aqueous dioxane medium was measured spectrophotometrically (3.2.2.10) in presence of each of the retinoids at 50 and 100 nmol per 1 ml assay (concentration shown in parenthesis). The first 15 sec, gap joined by the broken line, was due to time taken for addition and mixing of substrate BPDE I. All observations were taken with appropriate blank which was with retinoid but without BPDE I.

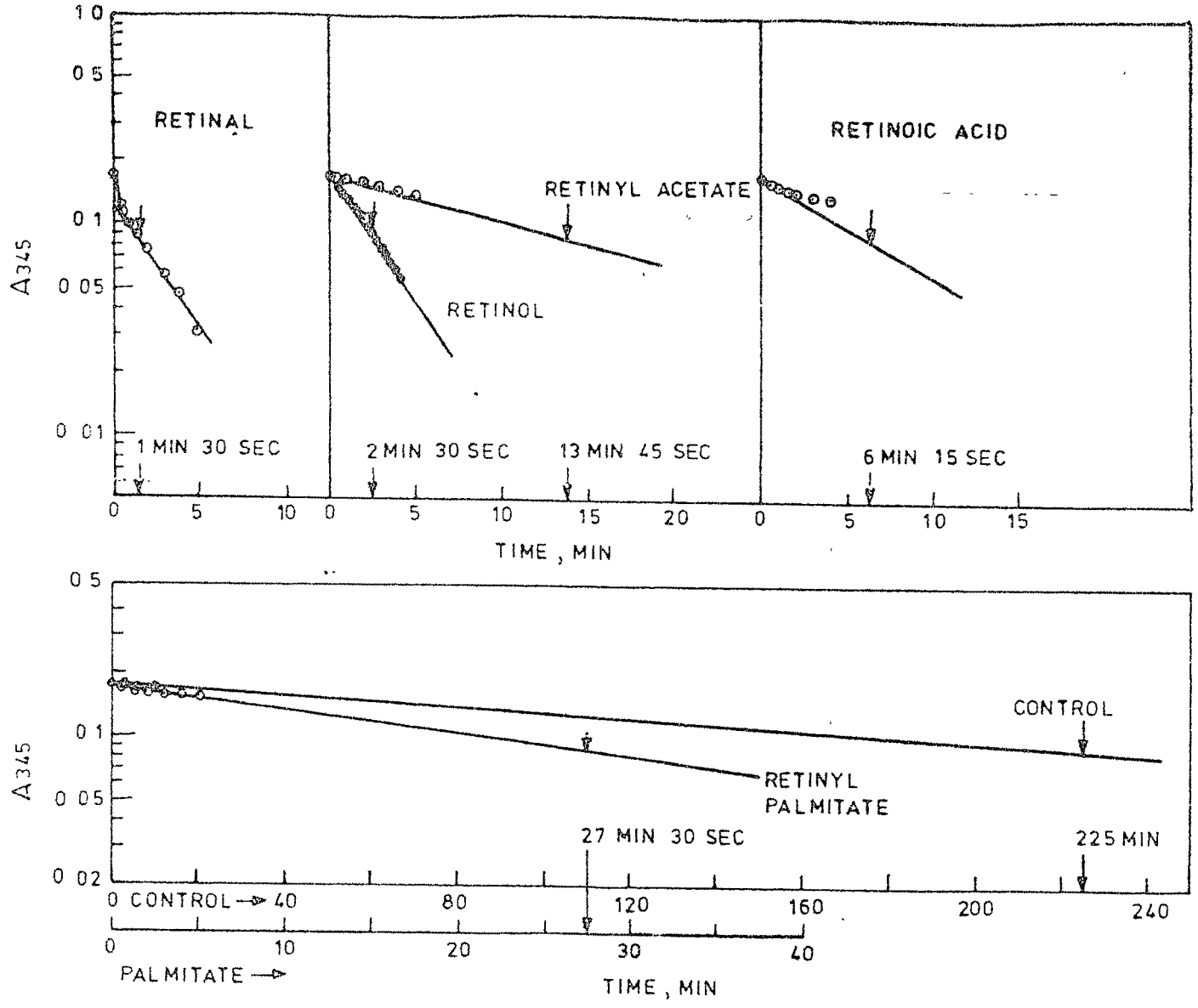


FIG. 3.7. Estimation of $T_{\frac{1}{2}}$ of disappearance of BPDE I in presence of retinoids: The decay in absorbance of BPDE I at 345 nm in presence of 100 µM retinoids (obtained from Fig. 3.6) was plotted on semi-log graph against time. The initial linear velocity was extended to obtain time required for A_{345} to become 50% of original value ($T_{\frac{1}{2}}$).

retinol, retinoic acid, retinyl acetate and retinyl palmitate, $T_{\frac{1}{2}}$ was observed to be 1.50, 2.50, 6.25, 13.75 and 27.50 min respectively.

3.3.9. Modulation by flavonoids and related phenolics of microsome mediated adduct formation between [^{3}H]BP and DNA:

Twenty-six flavonoids and related compounds were screened for their ability to modulate microsome mediated covalent adduct formation between [^{3}H]BP and DNA in in vitro assays as described in Methods (3.2.2.8.c). Results obtained with these compounds studied at two concentration ranges keeping DMSO concentration at 1% v/v are shown in Table 3.5. Polyhydroxy derivatives of flavonoids like robinetin, quercetin and kaempferol showed good inhibitory activity. Morin with five hydroxyl groups, however, showed inhibition only at higher concentration . The inhibition was reduced by methylation of hydroxyl groups as in robinetin tetramethyl ether and quercetin tri- and tetramethyl ether derivatives. Isorhamnetin, with 3'-methoxy substitution was, however, about equally active as the parent compound quercetin. Glycosylation of 3-hydroxyl group of quercetin reduced the activity (e.g. rutin). Flavone, chrysine dimethyl ether and 5-hydroxy-7,8-dimethoxy flavone showed moderate activity. Opening of γ-pyrone ring of flavone nucleus either reduced the activity e.g. flavone

TABLE 3.5

Levels of BP-DNA Adduct formed in vitro in presence of various flavonoids and related compounds

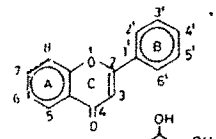
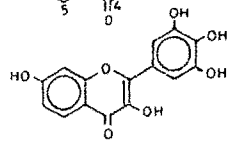
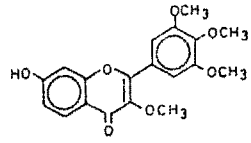
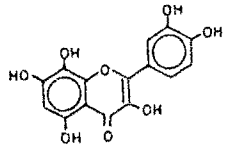
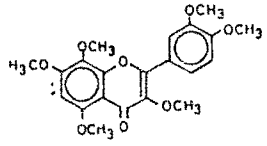
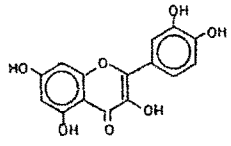
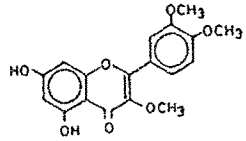
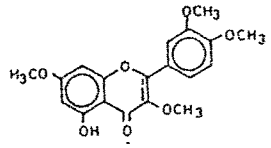
Flavonoid	Structure	Adduct formation (% of control) in presence of flavonoid at 0.1 mM	0.5 mM
1. Flavone		54.7	26.7
2. Robinetin		38.8	26.6
3. Robinetin 3,3',4',5'-tetramethyl ether		51.8	46.3
4. Gossypetin		89.7	82.0
5. Gossypetin hexamethylether		104.2	93.9
6. Quercetin		43.8	32.5
7. Quercetin 3,3',4'-trimethyl ether		60.6	54.2
8. Quercetin 3,7,3',4'-tetramethyl ether		82.9	77.2

TABLE 3.5 (contd.)

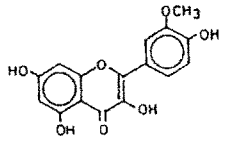
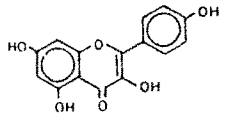
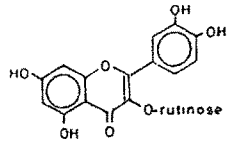
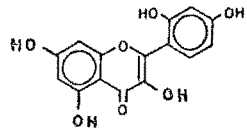
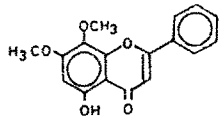
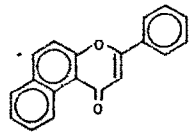
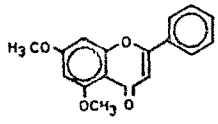
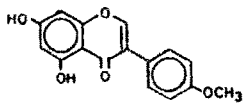
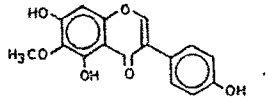
Flavonoid	Structure	Adduct formation (% of control) in presence of flavonoid at 0.1 mM	0.5 mM
9. Isorhamnetin		43.0	38.5
10. Kaempferol		34.7	25.8
11. Rutin		87.0	68.0
12. Morin		77.5	15.2
13. 5-hydroxy 7,8-dimethoxyflavone		75.4	55.3
14. 5,6-benzoflavone		95.3	46.5
15. Chrysine dimethyl ether		68.4	44.7
16. Biochanin-A		95.4	63.0
17. Tectorigenin		52.6	41.7

TABLE 3.5 (contd.)

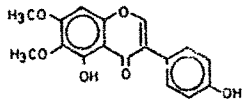
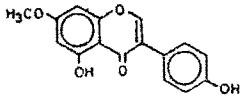
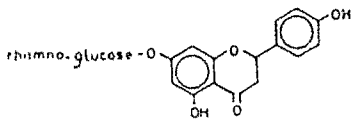
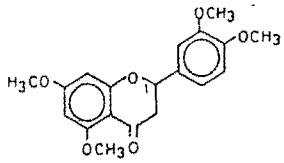
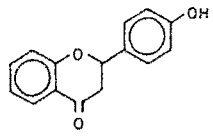
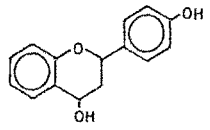
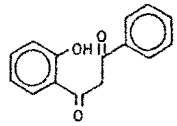
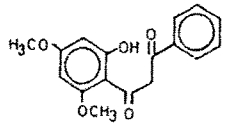
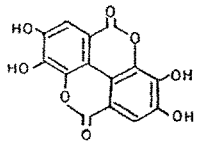
Flavonoid	Structure	Adduct formation (% of control) in presence of flavonoid at 0.1 mM	0.5 mM
18. Tectorigenin 7-methyl ether		90.8	85.5
19. Prunetin		77.9	92.8
20. Naringin		99.6	98.0
21. 5,7,3',4'-tetra-methoxyflavanone		104.2	98.6
22. 4'-hydroxy flavanone		76.4	95.0
23. 4'-hydroxy flavan-4β-ol		116.5	122.5
24. Flavone diketone		85.8	54.6
25. Chrysine dimethyl ether diketone		49.2	60.6
26. Ellagic acid		39.3	17.5

TABLE 3.5 (contd.)

Each flavonoid was dissolved in DMSO at desired concentration and was tested in duplicate for its ability to suppress the BP-DNA adduct formation in vitro as described in Methods (3.2.2.8). Adduct formation was measured as pmol BP bound per A_{260} DNA and expressed as per cent of control. Control value (without added flavonoid) was 3.30 $\pm$ 0.17 pmol/A_{260} (n = 6). Structure of flavone (compound no.1) indicates the numbering system of the flavonoids. Compound nos 2 - 12 are flavonol and derivatives, compound nos. 13 - 15 are flavone derivatives, compound nos. 16 - 19 are isoflavone derivatives, compound no. 23 is a flavan-β-ol derivative and compound nos. 24 and 25 are flavone diketone derivatives. Ellagic acid (compound no. 26) was included for comparison.

diketone or abolished it e.g. chrysine dimethyl ether diketone. Gossypetin with six free hydroxyl groups could not inhibit much, while its hexamethylether was inactive. Benzene substitution at 5,6 position as in 5,6-benzoflavone reduced the activity such that, inhibition was observed only at higher concentration. Among isoflavonoids, biochanin-A and prunetin were not very active, but tectorigenin showed reasonable activity which was once again reduced on methylation of 7-hydroxyl group. Naringin, a flavanone glycoside, as also other flavanones and flavanol were inactive. Ellagic acid, a polyphenolic plant product was found to be very effective inhibitor of BP-DNA adduct formation.

Selected flavonoids were tested from 0.001 - 0.5 mM range in the assay to determine the dose-response of inhibition (Fig. 3.8 and Fig. 3.9) and also to determine ID_{50} (Table 3.6). Robinetin was highly active with ID_{50} of 3.75 nmol per assay, while quercetin, kaempferol and isorhamnetin showed decreasing inhibitory activity with ID_{50} values of 17.5, 40 and 57.5 nmol respectively. Ellagic acid inhibited to a great extent (ID_{50}: 22.5 nmol), while flavone was a very weak inhibitor (ID_{50}: 163 nmol).

For effective flavonoids, it was observed that at very low doses inhibition increased linearly till ID_{50}, after which increase in dose from 0.1 to 0.5 mM rendered the response nonlinear (Fig. 3.8 and Fig. 3.9). This might explain the

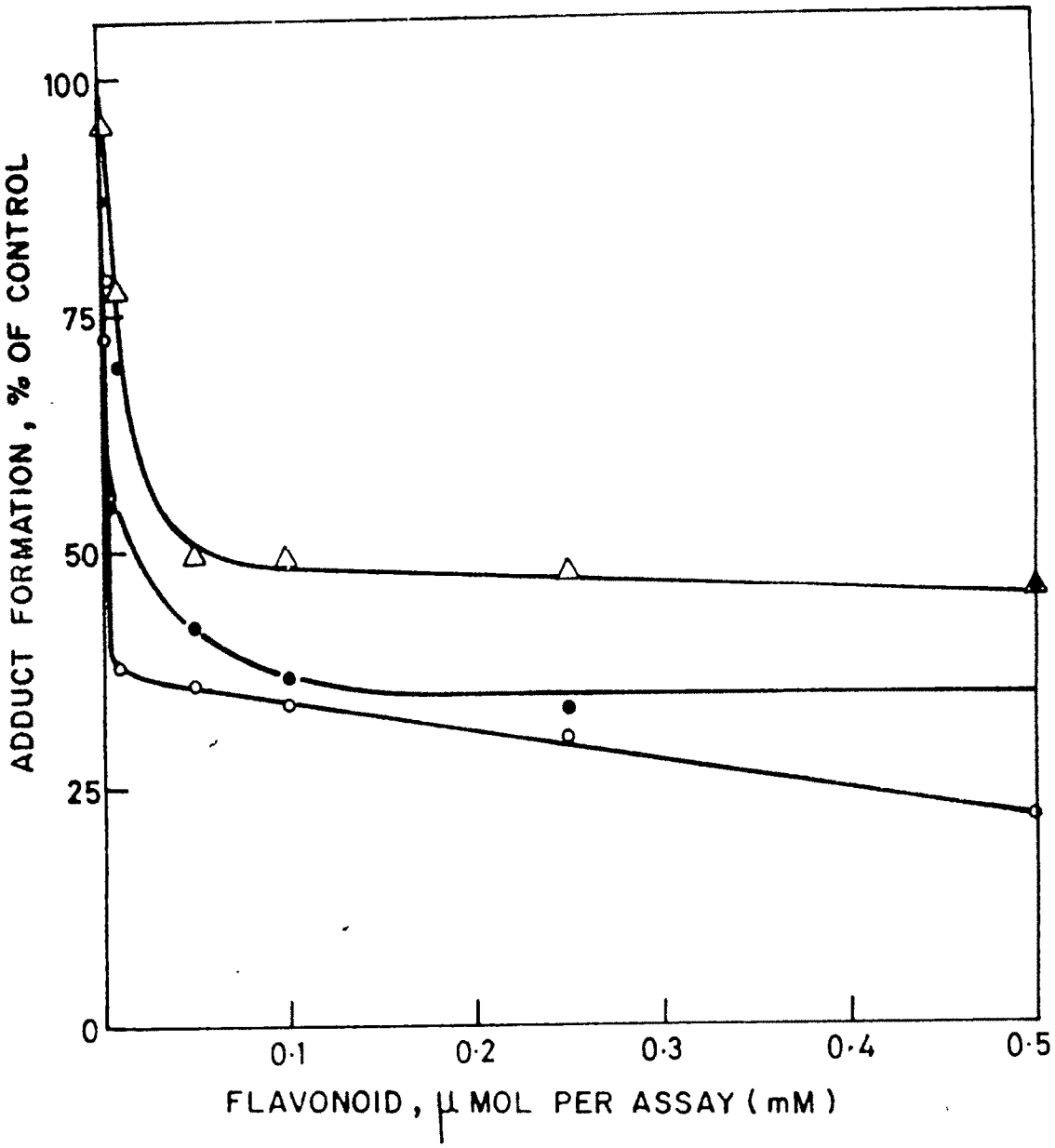


FIG. 3.8: Relative inhibition of microsome-mediated adduct formation between [^{3}H]BP and DNA in vitro by selected flavonoids:

Selected flavonoids dissolved in DMSO were tested at a wide range of concentrations in the adduct formation assay (3.2.2.8). Control adduct formation (in presence of 1% v/v DMSO) was observed to be 3.30 $\pm$ 0.17 (mean $\pm$ S.E.) pmol A_{260}^{-1} (n = 6). Results are expressed as per cent of DMSO control. Open circles (o) represent robinetin; closed circles (•) represent quercetin; and open triangles (△) represent isorhamnetin.

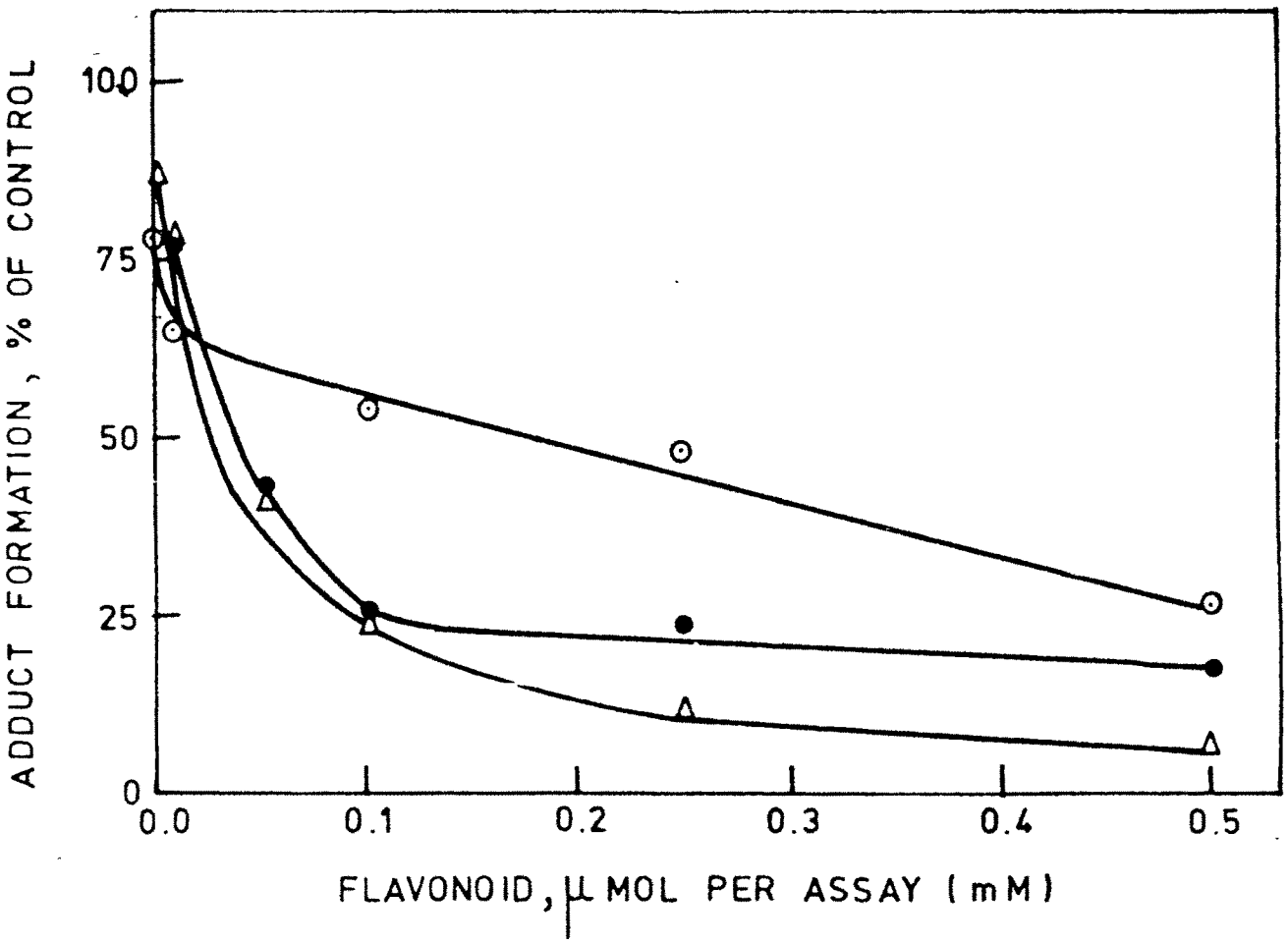


FIG. 3.9. Relative inhibition of microsome mediated adduct formation between [^{3}H]BP and DNA in vitro by selected flavonoids:

Reaction conditions are same as given for Fig. 3.8. Open circles (o) represent flavone; closed circles (●) represent kaempferol; and open triangles (△) represent ellagic acid.

Table 3.6

ID_{50} values of different flavonoids for BP-DNA adduct formation

Flavonoids	ID_{50} (nmol)
Robinetin	3.75
Quercetin	17.50
Kaempferol	40.00
Isorhamnetin	57.50
Flavone	163.00
Ellagic acid	22.50

The amount of each flavonoid required to inhibit in vitro BP-DNA adduct formation by 50% (ID_{50}) was determined from the graph of per cent inhibition plotted against concentration of the flavonoid (Fig. 3.8 and Fig. 3.9). Ellagic acid was included for comparison. Each result is average of three experiments.

apparent discrepancies observed for inhibition at 0.1 mM (Table 3.5) and ID_{50} values (Table 3.6) for compounds with ID_{50} less than 0.1 mM, viz. robinetin (0.00375 mM) and kaempferol (0.04 mM). However, Table 3.5 helps to differentiate between inhibitors and noninhibitors. Those compounds which were either ineffective at both the concentrations or showed spurious inhibition that decreased with increase in concentration were considered as noninhibitors.

3.3.10. Effect of selected flavonoids on metabolic availability of BP-7,8-dihydrodiol:

The metabolic availability of BP-7,8-dihydrodiol was determined by the method already described (3.2.2.9.a). Selected flavonoids at various concentrations (0.1, 0.5 mM and ID_{50} in some cases) were tested for their effects and results are shown in Table 3.7. Robinetin partially inhibited the formation of BP-7,8-dihydrodiol at high concentration. At the ID_{50} dose (3.75 μM), however, it inhibited metabolic availability only by 1%. Isorhamnetin, on the other hand, was found to be active inhibitor at all concentrations tested, showing 31% inhibition at ID_{50} (57.5 μM), 52% and 66% inhibition at 0.1 and 0.5 mM respectively. Flavone and quercetin were moderate inhibitors, while ellagic acid showed doubtful inhibition as it was dose-independent.

TABLE 3.7

Effect of different flavonoids on the metabolic availability of BP-7,8-dihydrodiol

Flavonoids	BP-7,8-dihydrodiol formation (% of total activity) in presence of flavonoids at;		
	ID_{50}	0.1 mM	0.5 mM
Robinetin	6.14 (99.0)	5.16 (83.3)	1.59 (25.6)
Quercetin	5.63 (90.8)	3.91 (63.0)	1.30 (20.9)
Isorhamnetin	4.28 (69.1)	2.98 (48.1)	1.07 (17.2)
Flavone	-	4.14 (66.7)	2.13 (34.3)
Ellagic acid	-	4.08 (65.8)	5.12 (82.5)

BP-7,8-dihydrodiol formation was measured as described in Methods (3.2.2.9.a). ID_{50} for robinetin, quercetin and isorhamnetin were 3.75, 17.5 and 57.5 μM respectively. Control value for BP-7,8-dihydrodiol formation (without added flavonoid) was 6.2% of total activity. Figures in parenthesis indicate per cent of control value. Ellagic acid was included for comparison.

3.3.11. Effect of selected flavonoids on the rate of disappearance of BPDE I:

The rate of disappearance of BPDE I was measured spectrophotometrically in presence of selected flavonoids as described (3.2.2.10). The flavonoids dissolved in DMSO were tested at various concentrations (keeping DMSO at 1% v/v) and results obtained with 50 µM concentration are shown in Fig. 3.10. Robinetin and quercetin greatly accelerated the disappearance of BPDE I while kaempferol and isorhamnetin did this to a lesser extent. Flavone was found to be inactive in this respect. Ellagic acid, a very active compound, and FMN (dissolved in warm water), a very weakly active compound, were added as reference inhibitors for comparison. The projected $T_{\frac{1}{2}}$ values for these compounds were determined as described earlier for retinoids (Fig. 3.7) and these values are given in Table 3.8. It can be seen that in the absence of any added flavonoid, $T_{\frac{1}{2}}$ was 300 min. In presence of robinetin $T_{\frac{1}{2}}$ was brought down drastically to 12 min while with quercetin, isorhamnetin and kaempferol $T_{\frac{1}{2}}$ was 18.3, 22 and 39 min respectively. Flavone was most ineffective showing $T_{\frac{1}{2}}$ of 236 min. Ellagic acid ($T_{\frac{1}{2}}$ = 2.3 min) and FMN ($T_{\frac{1}{2}}$ = 155 min) were added for comparison.

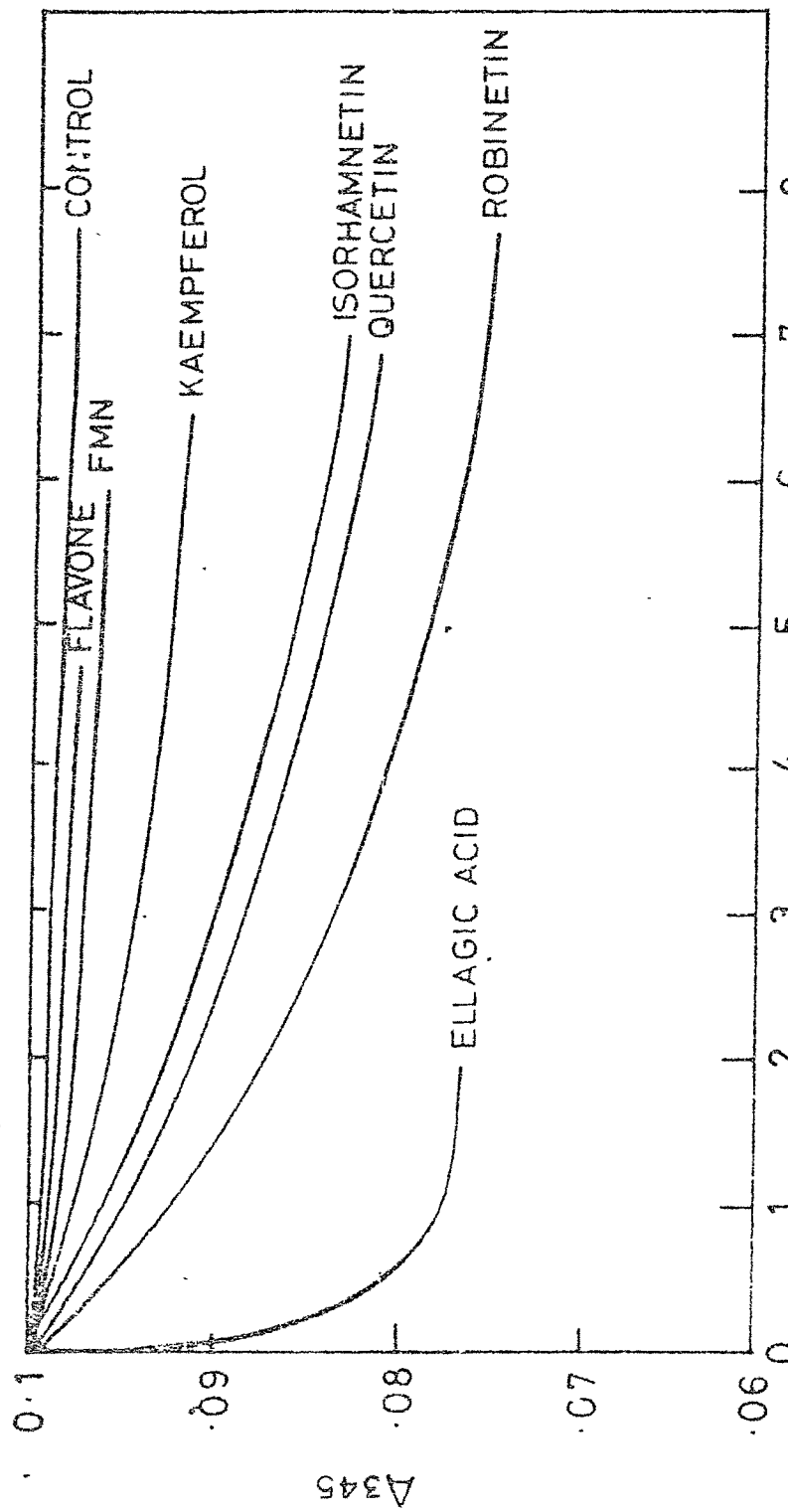


FIG. 3.10. Modulation of the rate of disappearance of BPDE I by selected flavonoids: The solvolysis in buffered aqueous dioxane medium of 3 μM BPDE I was measured spectrophotometrically as described in Methods (3.2.2.10). Each flavonoid was tested at various concentrations and the decay in absorbance in presence of 50 μM flavonoid is represented in the figure. FMN and ellagic acid were added for comparison.

TABLE 3.8

Half-lives for the disappearance of BPDE I in presence of various flavonoids

Flavonoids	$T_{\frac{1}{2}}$ (min)
None	300.00
Flavone	236.00
FMN	155.00
Kaempferol	39.00
Isorhamnetin	22.00
Quercetin	18.25
Robinetin	12.00
Ellagic acid	2.25

Disappearance of BPDE I was measured spectrophotometrically in presence of 50 μM flavonoid as described in Methods (3.2.2.10) and legend of Fig. 3.10. The half-life ($T_{\frac{1}{2}}$) was determined by extending initial linear velocity from graph plotted on semi-log paper. FMN and ellagic acid were included for comparison.

3.4. DISCUSSION

The capacity of ascorbic acid, retinoids and flavonoids to inhibit the adduct formation in vitro between BP and DNA as presented here can be correlated with their ability to do the same in vivo, which may be the basis for their antimutagenic or anticarcinogenic action. The DNA adduct formation has been shown to be modulated by a variety of other agents in vivo as well as in vitro. Antioxidant BHA inhibited the adduct formation in lung as well as in liver (Anderson et al. 1981). Ellagic acid had been shown to be a potent inhibitor of this adduct formation in vitro with calf thymus DNA, in vivo with epidermal DNA (DelTito et al. 1983) and in organ culture (Dixit et al. 1984; Shugart and Kao 1984). Our results with LAA (3.4.1) also show similar positive correlation in vivo and in vitro. Since similarity of adduct formation in vivo and in vitro between BP and DNA has been established (Ch. I), the effectiveness of a modulator in vitro may be a positive indication of its ability to do the same in vivo. Antioxidant ethoxyquin in contrast, was found to increase the adduct formation in vitro (Kahl et al. 1978) but decrease the same in vivo (Kahl 1982). Hence, caution must be exercised in interpreting in vitro results and their application.

Ability of modulators to influence metabolism is another double edged sword which may help or cut both ways.

In intact animal if these substances tend to increase the activity of metabolizing enzyme, it may result in increased detoxification and hence inhibition of carcinogenesis (Wattenberg 1978). Examples of such modulators are β-naphthoflavone, phenobarbital, quercetin and polychlorinated biphenyls. BP and its mono or dihydroxy and quinone derivatives have also been shown to stimulate the metabolizing enzymes and thus inhibit activation of BP or BP-7,8-dihydrodiol (Shen et al. 1979), mutagenicity of BPDE I (Huang et al. 1984) or even the carcinogenicity of other carcinogens (Wattenberg 1978). However this does not always ensure reduced formation of the activated metabolites which may react adversely if the opportunity is made available. A modulator like 7,8-benzoflavone may act as anticancer agent by inhibiting metabolizing enzymes (Wattenberg 1978). However, this too may pose serious threat as detoxification of all xenobiotics may be affected. Ability of the modulator to act as physiological trapping agent, scavenging the ultimate carcinogen away from the target, appears to be a very effective way by which an ideal modulator can modify carcinogenicity of a chemical. It is thus possible that cellular level of these physiological factors will offer control with respect to tissue specificity for the onset of neoplastic response to environmental chemicals. An analysis of the action of individual class of modulators used in these studies is presented in the following paragraphs.

3.4.1. L-Ascorbic acid:

The adduct formation in vivo between [^{3}H]BP and hepatic DNA was severely inhibited due to pretreatment of animals with LAA. The inhibition observed with LAA was dose-related in that at 100 and 300 mg kg^{-1} doses, the adduct formed was inhibited by 60% and 84% of control respectively (Fig. 3.2). LAA was administered 2 h prior to administration of BP and tissues are expected to be flooded at that time with LAA. Therefore, the effect of LAA could be at the level of metabolic activation of BP or at the level of binding of activated metabolite to the target DNA. The adduct formation was monitored 16 h after administration of BP and hence effect of LAA on the excision repair of the adduct cannot be ruled out.

In order to understand the mechanism by which LAA inhibits this adduct formation, in vitro binding study was performed using cellular or subcellular fractions derived from liver of LAA pre-treated animals. When liver slices were reacted with [^{3}H]BP in metabolically conducive medium, there was decreased adduct formation between BP and hepatic DNA in LAA pretreated liver slices. This inhibition by LAA was dose-dependent as at 100 and 300 mg kg^{-1} the inhibition of adduct formation was 18% and 40% respectively (Table 3.1). Two points worth noting are that inhibition in the adduct formation in liver slice system responded better to increase

in dose of LAA than the in vivo adduct formation. The other observation is that the extent of inhibition by LAA in vivo (60%) was more pronounced than the in vitro (18%) inhibition at 100 mg kg^{-1} dose of LAA. Similarly at 300 mg kg^{-1} the inhibition in vivo and in vitro were 84% and 40% respectively. This could be due to ready replenishment of LAA in liver through blood and lymphatic system assuring its continued presence. The amount of LAA in liver at the time of sacrifice is the only LAA that can act in vitro, which in turn may be the reason for better response of slices to increasing dose of LAA. Moreover, LAA is known to induce fragmentation of DNA (Stich et al. 1976) which could induce repair synthesis and removal of adduct.

When nuclei purified from livers of LAA pretreated animals were reacted with [^{3}H]BP, the adduct formation between DNA and BP was inhibited in case of higher dose of LAA (Table 3.1). Nuclei are expected to contain limited amount of LAA, hence those from the lower dose did not show any inhibition of adduct formation. However, microsomes from LAA pretreated animals were not restricted in their ability to mediate adduct formation in vitro between exogenous DNA and BP (Table 3.1). The level of adduct formation was markedly (nearly 10x) higher for microsome-mediated reaction between exogenous DNA and BP than that observed in vivo or with slices and nuclei. This is due to more efficient utilization of the

substrate by the microsomal enzymes and easy accessibility of the activated metabolite to exogenously added DNA. In contrast, in the in situ situation, the activated metabolite has limited access to DNA in regions of chromatin (Jahn and Litman 1979) and the metabolite can also react with macromolecules other than DNA (Ch. I). The slightly higher activity of microsomal preparation observed at 100 mg kg^{-1} body weight level was perhaps non-specific with respect to LAA, in as much as no further stimulation was noted with higher dose of LAA. It is evident that there is a limit to the inhibitory action of ascorbate under both in vivo and in situ situations because the inhibition of the adduct formation is neither linear with dose, nor complete even at high doses. The absence of any inhibition of microsomal activity while there is inhibition at the whole liver cell level indicates that LAA does not have any action at the organelle level. The physical presence of LAA at the site of metabolism and binding is a necessity, because purified microsomal fractions are not expected to contain any LAA, while the whole cells (in slices) and nuclear fractions can have adequate amount of LAA, especially after administration of high doses (Burch 1961).

The physical presence of LAA required to exert its inhibitory effects on the adduct formation between BP and DNA was clearly evident in the ability of LAA to inhibit in vitro the microsome-mediated adduct formation between BP

and exogenous DNA (Fig. 3.3). The limitation of inhibition by LAA becomes apparent in that the increase in concentration of LAA does not linearly increase the inhibition and that even at a high concentration of 17.5 mM, the inhibition is not complete. This is in conformity with the findings under in vivo and in situ situations as stated earlier. The differentiation of the effects of LAA on metabolism of BP and on binding of activated metabolites to DNA was made by stydying both these processes independently in presence of LAA. When BP was used as substrate for binding to DNA, 1 mM LAA brought about 42% inhibition of the aduct formation (Fig. 3.4) while metabolic availability of proximate carcinogen BP 7,8-dihydrodiol in presence of the same concentration of LAA was reduced by a lesser margin (35%) (Table 3.2). Similarly with BP 7,8-dihydrodiol as substrate, 1 mM LAA could bring about 35% inhibition in the adduct formation while 22% inhibition was observed in the formation of BPDE. These observations imply that the inhibitory effect of LAA is partly due to impairment of metabolic activation of BP and also due to interference in binding of activated metabolite with DNA. It is to be noted that at higher concentrations the efficiency of inhibition by LAA is slightly greater during microsome catalyzed binding of BP-7,8-dihydrodiol than that of BP. BP undergoes metabolism via different pathways other than BP-7, 8-dihydrodiol route (Fig. 1.2). It is possible that LAA has also some action on these metabolic steps. The levels of

metabolizing enzymes have been shown to be altered under conditions of deficiency of this vitamin (Zannoni et al. 1978; Peterson et al. 1983) or after its administration (Sikic et al. 1977).

The in vitro effect of LAA on metabolism can hardly be due to its direct effect on the enzyme as LAA is not a known inhibitor of enzyme per se. It can act, however, either at the level of metabolic electron transfer by competing with the carcinogenic substrate for the acceptance of electron or, being an antioxidant, it may reduce the availability of molecular O_2 which is required for the oxidative metabolism of BP. Apart from the fact that inhibition of metabolism by LAA was consistently lower than the inhibition in adduct formation with the same substrates, the observation that LAA could inhibit the adduct formation in direct nonenzymatic interaction between BPDE and DNA (Fig. 3.4) implied that LAA interfered with the binding of activated metabolite to DNA. Our unpublished observation that ascorbate lowers the mutagenic ability of BP in Salmonella/microsome test lends support to this assumption. Guttenplan (1977) derived a similar conclusion from the observation that mutagenesis of a direct acting N-nitroso compound in the S.typhimurium TA 1530 was inhibited by LAA. Although the underlying mechanism of direct interference by LAA in binding of activated metabolite to DNA is not clear, two possible explanations

can be offered. At physiological pH, ascorbic acid exists as an ascorbate anion at C_2 or O^3 positions (Fig. 3.11) of the molecule (Edgar 1974) and can be alkylated at these positions (Jackson and Jones 1965). Therefore LAA can be considered a physiological nucleophile which can interact with the electrophilic BPDE and thus can compete with DNA. The other possibility is that nucleophilic sites on DNA can be bound by LAA, thus affording protection to those sites against attack by electrophilic carcinogen. Jamaluddin et al. (1981) proposed H bonding between oxygen functions at O^2 and O^3 positions of LAA with N-1 and N^2 of G (Fig. 3.12) or N^6 and N-7 of A, which can block availability of these sites for adduct formation with the carcinogen.

Finally the in vivo effect of LAA with regard to BP carcinogenicity cannot be predicted from these observations, although studies with other carcinogens have shown ascorbic acid to have protective role (Schlegal et al. 1970; Shamberger 1972; Slaga and Bracken 1977; Chan and Fong 1977). It is possible that cellular concentration of nucleophiles offers a control with respect to tissue specificity for the onset of carcinogenic response induced by chemicals. In this regard the role of LAA as physiological scavenger seems important. Literature, however, is filled with reports on both the desirable and undesirable effects of ascorbic acid. To cite a few examples, there are studies supporting no ill effects

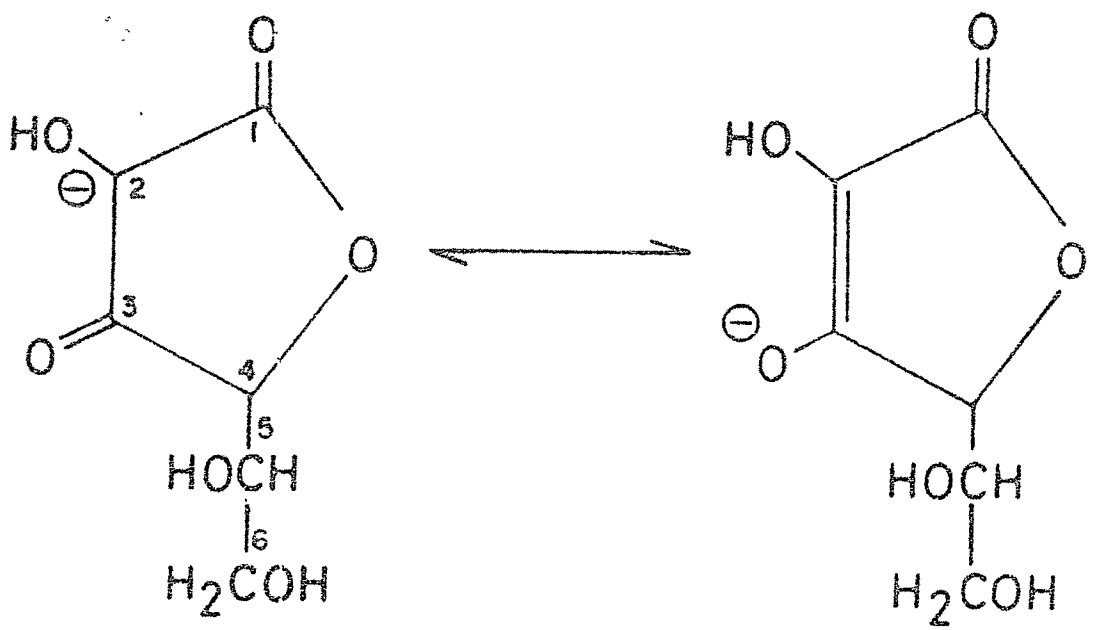


FIG.3.11. Physiological state of L-ascorbic acid:

At physiological pH, L-ascorbic acid and dehydroascorbic acid can form nucleophilic centres at C_2 or O^3 positions of the molecule.

(Edgar 1974).

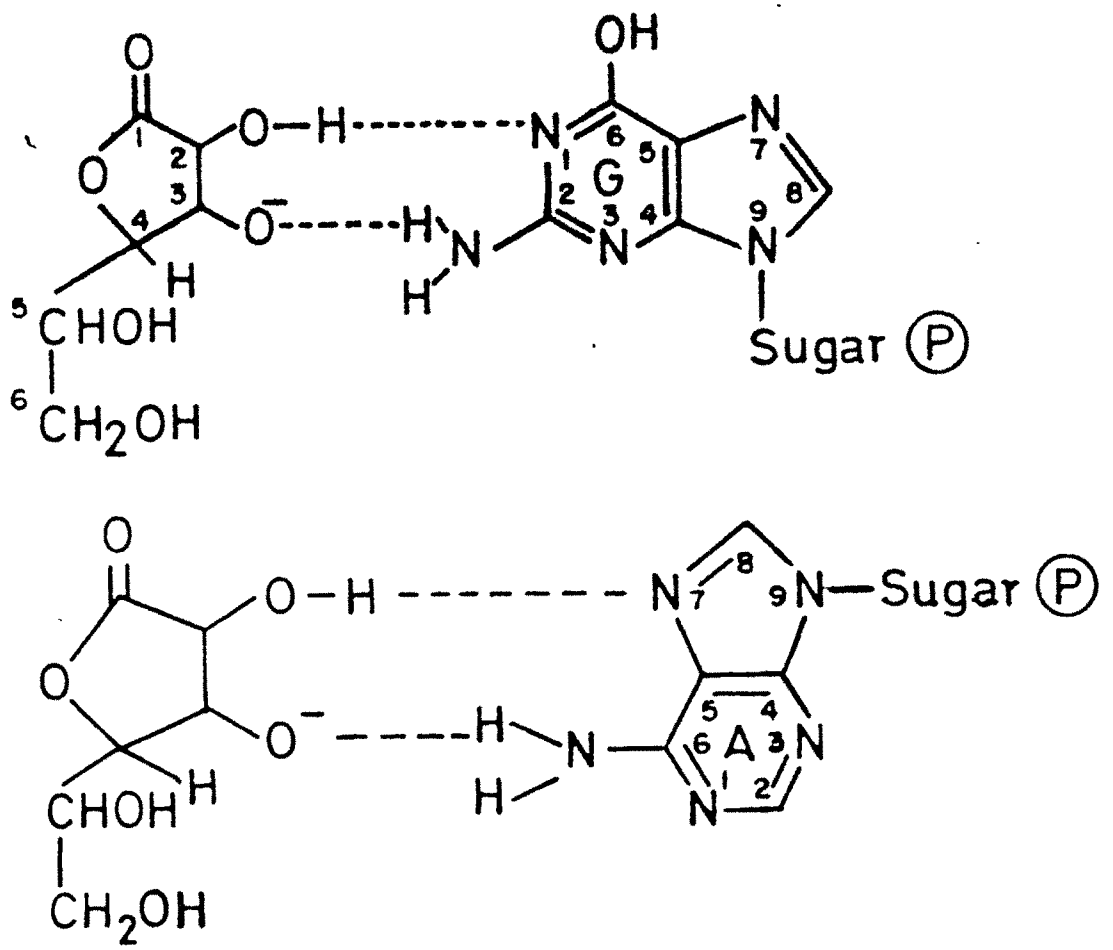


FIG. 3.12. Interaction between L-ascorbic acid and DNA; L-ascorbic acid can form H bonds at O^2 and O^3 positions with N-1 and N^2 of deoxyguanosine or N^6 and N-7 of deoxyadenosine. (Jamaluddin et al. 1981).

in human at large doses of LAA (Pauling 1974; Anderson 1975; Sestili 1983). It was shown to reduce formation of carcinogens like nitroso compounds (Mirvish 1981) and to inhibit toxicity of secondary amines (Cardesa et al. 1974; Kamm et al. 1975). In cell culture it was shown to inhibit transformation and reverse the transformed cells (Benedict et al. 1980). However, it was shown to be mutagenic (Stich et al. 1976), tumor promoter (Fukushima et al. 1983) and these actions were suggested to be due to its ability to hydroxylate G at C_8 position (Kasai and Nishimura 1984). On the other hand, it was observed to be antimutagenic (Shamberger et al. 1973) and was shown to be non-promoter of carcinogenesis (Fukushima et al. 1984; Shirai et al. 1985). It may act as antipromoter by inactivating receptor site for promoters like phorbol ester (Delclos and Blumberg 1982).

In order to put our observations on BP in biochemical perspective regarding effects of LAA, we studied effects of dietary supplementation of LAA on the principal biochemical effect of BP observed by us, i.e. the gross inhibition in hepatic transcription (Ch. II). The incorporation of [^{14}C]orotic acid into alkali-labile RNA was inhibited to the extent of 60% by BP, this inhibition was reduced to 40% by dietary LAA (Table 3.3). Thus LAA could reverse to some extent the inhibitory effects of BP on transcription. Similar instances of reversal of inhibition of transcription by different carcinogens by other dietary and non-dietary

modulators has been shown. Ethionine-induced inhibition in transcription was reversed by adenine (Farber et al. 1974) and S-containing amino acids of the diet could decrease the transcription-inhibiting effects of dimethylnitrosamine (Naslund and von der Decken 1981). Thus ability of LAA to alleviate the inhibitory effect of BP on hepatic transcription may have physiological significance in determining the initial molecular events after exposure to BP.

3.4.2. Retinoids:

The microsome-mediated adduct formation between BP and DNA was observed to be inhibited by some of the retinoids tested. Most notably retinal and the two retinyl esters - acetate and palmitate - actively inhibited the adduct formation, while retinol and retinoic acid were ineffective (Fig. 3.5). The active retinoids exerted most effectively at concentrations less than 0.1 mM in the assay, after which there appeared to be a limitation in their ability to bring about inhibition of the adduct formation. Among the less effective compounds retinol showed poor inhibition but good dose-dependent response upto the highest concentration (0.5 mM). Retinoic acid, on the other hand, was ineffective beyond 0.1 mM concentration. In general, the effective retinoids were able to inhibit the adduct formation at much lower concentration than LAA.

In order to determine whether retinoids act as blocking agents for interaction between ultimate carcinogen and DNA, rate of disappearance of BPDE I at neutral pH was measured in presence of retinoids. BPDE I undergoes spontaneous and acid catalyzed hydrolysis to inactive tetraols (Whalen et al. 1979). Wood et al. (1982a) observed acceleration in hydrolysis of BPDE I in aqueous dioxane at pH 7.0 in presence of FMN. Ferulic and caffeic acids were also shown to aid disappearance of BPDE I, and ellagic acid showed exceptional activity in eliminating BPDE I (Wood et al. 1982b). Retinoids, when tested in this system accelerated the disappearance of BPDE I in concentration-dependent fashion (Fig. 3.6). From $T_{\frac{1}{2}}$, the time required for the absorbance at 345 nm to be reduced by 50%, one gets comparative idea of the ability of different retinoids to accelerate hydrolysis of BPDE I (Fig. 3.7). For the retinoids which were strong inhibitors of the adduct formation, similar pattern of response was observed in their ability to hydrolyse BPDE I i.e. aldehyde > acetate > palmitate. This indicates that inhibitory action of retinoids could emanate from their ability to intercept interaction of carcinogen with DNA. Their action at the level of metabolic activation also can not be ruled out in view of the observation made by Hill and Shih (1974) that retinol in vitro inhibited the formation of reactive metabolites of polycyclic aromatic hydrocarbons capable of binding to

macromolecules. Most surprising was the ability of alcohol and acid to accelerate hydrolysis of BPDE I even though they were ineffective for inhibiting adduct formation. This suggests inability of these retinoids to interact with ultimate carcinogen in the adduct formation assay. This also implies their inability to effectively block the metabolic activation of BP. However, Firozi et al. (1986) observed retinoids to be effective inhibitors of in vitro adduct formation between AFB_1 and DNA and they observed this to be mainly due to their effect on metabolic activation of AFB_1. Thus, the effective retinoids i.e. aldehyde and esters could be acting at two fronts, the level of metabolism and the binding of metabolite to DNA. But the ineffective retinoids i.e. alcohol and acid are either getting inactivated in the binding assay or are getting bound to components of metabolizing system - not responsible for activation of BP. The latter possibility may be stronger in view of the fact that the metabolism of AFB_1 is better with phenobarbital-induced microsomes (predominated by P-450 a and b) than with MC-induced microsomes (predominated by P-450 a and c) and vice versa for metabolism of BP. Further studies are warranted to understand the effect of retinoids on the metabolism of different carcinogens.

3.4.3. Flavonoids and related phenolics:

A variety of flavonoids as observed in these studies have been shown to inhibit the microsome-mediated adduct formation between BP and DNA in vitro. The extent of inhibition varies with different flavonoids. From these results (Table 3.5) certain generalizations can be made, albeit with due caution, regarding the structural features which are contributory towards the inhibitory activity of flavonoids. Flavone nucleus has moderate activity but its integrity is not an absolute requirement because flavone diketone shows weak inhibition ($ID_{50} > 0.5$ mM). Hydroxylation of flavone, in general, enhances the inhibitory activity. Flavonols, having 3-hydroxyl group, are very active. Flavonols like robinetin, quercetin and kaempferol with additional hydroxyl groups show high inhibitory activity. However, the number as well as position of hydroxyl group(s) seem to be important for activity. The hydroxyl groups in 5 and 7 positions of A ring, 3 position of C ring and 3', 4' and 5' positions of B ring, appear to have favourable influence on the inhibitory activity of flavone nucleus. However, there are few exceptions (e.g. morin and gossypetin). The methylation of hydroxyl group generally makes the compound less active or inactive, e.g. robinetin tetramethyl ether and quercetin tri- and tetramethyl ether. Isorhamnetin, a 3'-methylether derivative of quercetin is, however, a good inhibitor with respect to

quercetin. Glycosylation, as in rutin, with 3-O-rutinosyl group on quercetin, also reduces the activity of the parent compound. It appears that the exact role of the number and position of hydroxyl groups can not be very clearly defined from above data, unless studies are carried out with a series of well defined structural derivatives of flavone. Among other structural features, benzoylation of A ring as in 5,6-benzoflavone makes the flavonoid weakly active with respect to moderate activity of flavone. Saturation of 2,3 double bond, as in flavanone, adversely affects the inhibitory activity. 4'-hydroxyflavan with 4-β-hydroxyl group instead of carbonyl group also shows inactivity. Among isoflavonoids, tectorigenin with three hydroxyl groups and a methyl ether group shows moderate activity, which is abolished when hydroxyl group at 7 position is methylated. Another isoflavonoid, prunetin, with two free hydroxyl group and one methoxyl group is also inactive.

The results presented here assume importance in the light of the fact that flavonoids have widespread occurrence in plant kingdom (Mabry and Ulubelen 1980; Wollenweber and Dietz 1981). To the best of our knowledge, very few studies have claimed flavonoids to be carcinogenic to animals. Quercetin and rutin were shown to be weak bladder carcinogens (Boyland et al. 1964). Quercetin was considered to be the carcinogenic component of bracken fern which induced

intestinal and bladder tumors (Pamukcu et al. 1980). However the same authors noted the non-carcinogenicity of quercetin in many other systems (Bryan and Pamukcu 1982). Majority of the studies indicated flavonoids to be non-carcinogenic i.e. neither total carcinogen nor promoter (Hirose et al. 1983). In fact, following studies have clearly shown flavonoids to be anticarcinogenic to BP. Quercetin when used as promoter or inducer with BP, brought about inhibition of skin carcinogenesis (vanDuuren and Goldschmidt 1976). Rutin and morin were shown to be able to decrease BP-induced skin cancer in mice (vanDuuren et al. 1971). 5,6-Benzoflavone displayed good anticancer activity against BP-induced lung and forestomach tumors in mice (Anderson et al. 1983). Wattenberg and Leong (1970), observed 5,6-benzoflavone to be anticarcinogenic to BP-induced pulmonary adenoma and skin cancer. Flavonoids were found to be anticarcinogenic to other carcinogens as well. 5,6-Benzoflavone was observed to afford protection against aflatoxin-B_1-induced carcinogenesis in rainbow trout (Nixon et al. 1984). Indirect studies with cauliflower feeding and reduction in aflatoxin-B_1 carcinogenesis have been recorded by Boyd et al. (1979). In this context, it is worthwhile to note that adduct formation betwen aflatoxin-B_1 and DNA in vitro was shown to be inhibited by many classes of compound including flavonoids viz. 5,6-benzoflavone and 7,8-benzoflavone (Bhattacharya et al. 1984). Some flavonoids were shown to be mutagenic in Ames test systems using

S.typhimurium strains (Bjeldanes and Chang 1977; MacGregor and Jurd 1978; Brown 1980; Nagao et al. 1981). However, in most of the studies with eukaryotic cells flavonoids were found to be non-mutagenic (MacGregor et al. 1983; Carver et al. 1983; van der Hoeven et al. 1984). On the other hand, several flavonoids were found to be antimutagenic to BP and its derivatives like BP-7,8-dihydrodiol and BPDE I in Ames test (Huang et al. 1983).

In our investigation, selected flavonoids have been subjected to further studies in order to differentiate their mode of action. Flavonoids were tested at various doses in adduct formation assay (Figs. 3.8 and 3.9) to determine the dose required to inhibit the adduct formation by 50% (ID_{50}). These values provide a direct and realistic comparison of the inhibitory potential of various flavonoids. In this respect robinetin and quercetin are strong inhibitors, while kaempferol and isorhamnetin are good inhibitors, and flavone is a weak inhibitor (Table 3.6). These selected flavonoids have been tested for their ability to influence metabolic availability of BP-7,8-dihydrodiol (Table 3.7). This assay determines the amount of BP-7,8-dihydrodiol left from the total amount formed, which has not been activated further or detoxified. Thus, it reflects the net outcome of enzymes of activation and detoxification. Flavonoids which are strong inhibitors of adduct formation at low doses (e.g. robinetin

and quercetin) are not active in reducing the availability of BP-7,8-dihydrodiol at these doses. Several studies have shown either stimulation or inhibition of metabolizing enzymes in response to flavonoids (Wattenberg et al. 1968; Wattenberg and Leong 1970; Diamond et al. 1972; Weibel et al. 1976; Havsteen 1983; Nixon et al. 1984). According to present study the ability of flavonoids to inhibit BP-DNA adduct formation does not seem to arise from their ability to reduce the metabolic availability of BP-7,8-dihydrodiol.

In order to determine whether flavonoids act as blocking agents for interaction between ultimate carcinogen and DNA, rate of disappearance of BPDE I at neutral pH has been measured as described earlier for retinoids. It has been observed (Fig. 3.10 and Table 3.8) that flavonoids active in reducing adduct formation accelerate the rate of disappearance of BPDE I to a great extent. It appears, therefore, that flavonoids act as inhibitors of adduct formation between BP and DNA in vitro by virtue of their direct interaction with enzymatically generated BPDE I. Huang et al. (1983) also concluded that the antimutagenic activity of hydroxylated flavonoids resulted from their direct interaction with BPDE I. It is possible that these flavonoids form inactive complexes with BPDE I as suggested for ellagic acid (Sayer et al. 1982) or they may bring about complexation and hydrolysis of BPDE I

as suggested for FMN (Wood et al. 1982a). The capacity of flavonoids to inhibit adduct formation in vitro is expected to reflect on their ability to act as antimutagen and anti-carcinogen.

It is realized that in vitro effect cannot always be taken as a reflection of effectiveness in vivo, where diverse enzymatic influences and biochemical mechanisms operate. Since the carcinogenic action of any chemical is most likely dependent on its reaction with DNA, our in vitro tests based on modulation of either the formation of reactive metabolite or its interaction with DNA must have some relevance in a preliminary programme designed for identification of potentially anticarcinogenic factors. Our knowledge about the mechanism of such intervention may be of considerable help in devising ways and means to fight this most cruel scourge of the mankind. Thus, genetic susceptibility and exposure to chemicals notwithstanding, the nutritional management may be a potentially useful way to minimise human cancer incidence.