Enzymatic Mechanism of Benzo[*a*]pyrene Conversion to Phenols and Diols and an Improved High-Pressure Liquid Chromatographic Separation of Benzo[*a*]pyrene Derivatives†

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ABSTRACT: Benzo[*a*]pyrene is stereospecifically converted in two enzymatic steps by rat liver microsomal mixed-function oxidases and epoxide hydratase to the (−) enantiomers of the *trans*-4,5-, *trans*-7,8-, and *trans*-9,10-diols. The initial mixed-function epoxidation at the 4,5, 7,8, and 9,10 positions occurs at one side of the planar benzo[*a*]pyrene molecule to form epoxide intermediates, and this is followed by a substrate-stereoselective and a product-stereospecific hydration of the epoxide intermediates by epoxide hydratase to the (−)-*trans*-diols. The identification of the diols as optically pure (−)-trans enantiomers was accomplished by high-pressure liquid chromatographic separation of the cis and trans isomers, and of the di-(−)-menthoxyacetates of the (+)- and (−)-trans enantiomers. Incubation of benzo[*a*]pyrene with rat liver microsomes under molecular oxygen-18 and subsequent mass-spectral analyses of the *trans*-diols indicated that each of the *trans*-diols contained one oxygen-18 atom. Oxygen-18 was also found in 3-, 7-, and 9-hydroxybenzo[*a*]pyrenes which were formed by microsomal metabolism of benzo[*a*]pyrene. The diols were treated with acid, and the resulting phenolic products were separated by high-pressure liquid chromatography and analyzed by mass spectrometry. The results indicated that the oxygen-18 of *trans*-4,5-, *trans*-7,8-, and *trans*-9,10-diols were at the 5, 7, and 10 positions, respectively. An improved separation of benzo[*a*]pyrene derivatives by high-pressure liquid chromatography is described which resolves more efficiently the phenols, diols, and quinones than the methods previously reported.

Benzo[*a*]pyrene (BP)[1] is the most commonly studied polycyclic aromatic hydrocarbon of the environment and exerts several biological effects (*Particulate Polycyclic Organic Matter*, National Academy of Sciences, 1972). The biological activities included toxicity, mutagenicity, tumorigenicity, and covalent binding to DNA (Borgen et al., 1973; Daudel et al., 1975; Gelboin, 1969; Grover et al., 1976; Huberman et al., 1976; Jeffrey et al., 1976; King et al., 1976; Koreeda et al., 1976; Levin et al., 1976a,b; Malaveille et al., 1975; Meehan et al., 1976; Newbold and Brookes, 1976; Selkirk et al., 1974b; Sims et al., 1974; Weinstein et al., 1976; Wislocki et al., 1976; Wood et al., 1976; Yang et al., 1977a). BP is converted by the microsomal mixed-function oxidases, epoxide hydratase, and various conjugases to five phenols, three quinones, three diols, several epoxides, and glutathione, glucuronide, and sulfate conjugates of the oxygenated metabolites (Borgen et al., 1973; Cohen et al., 1976; Holder et al., 1974; Kinoshita et al., 1973; Nemoto et al., 1975; Selkirk et al., 1974a,b; Selkirk et al., 1975; Sims and Grover, 1974; Waterfall and Sims, 1972; Yang et al., 1975). Recently, all three diols formed metabolically from BP by rat liver microsomes were reported to be optically active (Yang and Gelboin, 1976b), and the 7,8-diol is an optically pure (−) enantiomer (Yang and Gelboin, 1976b; Yang et al., 1976, 1977e). Recent studies have indicated that the 7,8-diol 9,10-epoxides of BP are very active in the binding to DNA in vitro and to the DNA and RNA of mammalian cells in culture (Daudel et al., 1975; Grover et al., 1976; Jeffrey et al., 1976; King et al., 1976; Sims et al., 1974; Weinstein et al., 1976; Yang et al., 1977a). The reactivities of BP 7,8-diol 9,10-epoxides were found to be due to the carbonium-ion intermediates formed at the C_{10} position (Keller et al., 1976; Yang et al., 1976, 1977c,d).

Malaveille et al. (1975) reported that a BP 7,8-diol 9,10-epoxide synthesized by a peroxyacid oxidation of the 7,8-diol exhibits similar mutagenic activity as the 4,5-epoxide in strains TA 100 of *Salmonella typhimurium*. A subsequent report by Wislocki et al. (1976) showed that the BP diol epoxide II (*r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]-pyrene) was 5 and 20 times more mutagenic than BP 4,5-epoxide in *S. typhimurium* strains TA98 and TA 100, respectively. The same report (Wislocki et al., 1976) also showed that diol epoxide II was 44 times more mutagenic than BP 4,5-epoxide in the cultured Chinese hamster V79 cells. Huberman et al. (1976) reported that diol epoxide I (*r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene) was more mutagenic in cultured Chinese hamster V79 cells than either the diol epoxide II, the 4,5-epoxide, or any of the other BP derivatives tested, including all the known BP metabolites. In the same report (Huberman et al., 1976), evidence was presented to show that the *trans*-7,8-diol, formed metabolically from BP, is converted predominantly to the diol epoxide I, and the racemic *trans*-7,8-diol was highly mutagenic upon further metabolism. Subsequently Yang et al. (1976) reported that a single enantiomer of diol epoxide I was enzymatically and highly stereoselectively formed from the (−)-*trans*-7,8-diol which was enzymatically prepared from BP. The (−)-*trans*-7,8-diol formed enzymatically from BP was found to be optically pure (Yang and Gelboin, 1976b; Yang et al.,

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[1] Abbreviations used are: BP, benzo[*a*]pyrene; HPLC, high-pressure liquid chromatography; THF, tetrahydrofuran; *trans*-7,8-diol, *trans*-7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene; 7,8-epoxide, benzo[*a*]pyrene 7,8-oxide. Other diols and epoxides are similarly abbreviated. Tetrol, tetrahydroxytetrahydrobenzo[*a*]pyrene; triol, trihydroxytetrahydrobenzo[*a*]pyrene; diol epoxide I, *r*-7,*t*-8-dihydroxy-*t*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene; diol epoxide II, *r*-7,*t*-8-dihydroxy-*c*-9,10-oxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene. The "*r*-7" indicates that the substituent at the 7 position is the "reference", the "*t*" and "*c*" indicate that the substituents are trans and cis to the reference substituent, respectively. The (7,10/8,9)tetrol indicates that the C_{10}-OH is cis and C_8-OH and C_9-OH are trans to the reference C_7-OH, respectively. Other tetrols and triols are similarly designated. The 1-hydroxybenzo[*a*]pyrene is abbreviated as 1-OH-BP and other phenols are similarly designated.

1976, 1977c). Similar findings showing the exceptionally high mutagenicity of diol epoxide I was subsequently reported by Newbold and Brookes (1976) and Wood et al. (1976). Other evidence indicating that a single enantiomeric form of diol epoxide I was a metabolic intermediate which bound to nucleic acid was obtained by the analysis of the BP–RNA-bound derivative formed in vivo (Jeffrey et al., 1976; Nakanishi et al., 1977).

As part of our efforts to elucidate the activation and the detoxification pathways of the carcinogenic BP, we present evidence that each of the three *trans*-diols formed metabolically from BP are optically pure (−) enantiomers. Studies of the oxygenation mechanism indicate that both the microsomal mixed-function oxidases and epoxide hydratase are stereospecific in the metabolism of BP to *trans*-diols.

Materials and Methods

Materials. The [^{3}H]BP (5 Ci/mmol) and [^{14}C]BP (25 mCi/mmol) were purchased from Amersham/Searle, Arlington Heights, Ill., and purified by a gravity-flow silica-gel (Bio-SIL A, 100–200 mesh, Bio-Rad Laboratories, Richmond, Calif.) column with benzene as the eluent (Yang et al., 1977a). Synthetic BP derivatives were obtained through National Cancer Institute Contract No. N01-CP-33387 and National Cancer Institute Contract No. NO1-CP-33385. Information on the availability of the synthetic BP derivatives can be obtained from the Manager, Information and Resources Segment, Division of Cancer Cause and Prevention, Room A306, Landow Building, National Cancer Institute, Bethesda, Md. 20014. The partially purified liver epoxide hydratase free of aryl hydrocarbon hydroxylase was prepared from phenobarbital-treated male Sprague Dawley rats according to Lu et al. (1975). Liver microsomes were prepared from untreated and 3-methylcholanthrene-treated male Sprague Dawley rats (Kinoshita et al., 1973). The protein content of the liver microsomes was determined by the method of Lowry et al. (1951) with ribonuclease A (Calbiochem, San Diego, Calif.) as the protein standard. Molecular oxygen-18 (99 atom %) and oxygen-18 water (50 atom %) were purchased from Bio-Rad Laboratories, Richmond, Calif. Radioactive BP metabolites were obtained by incubating radioactive BP with liver microsomes in the presence of NADPH and isolated by HPLC.

HPLC. HPLC was performed on a Spectra-Physics Model 3500 liquid chromatograph fitted with a 6.2 mm i.d. × 25 cm Du Pont "Zorbax" octadecyltrimethoxysilane (ODS) column. The column was eluted with a linear gradient of 60% methanol in water to 100% methanol for 50 min (or for 100 min) at room temperature. The solvent flow rate was 0.8 mL/min and the eluent was monitored at 254 nm. Due to gradual deterioration of the column, several columns purchased in the past year were used during the course of this investigation. Recent ODS columns purchased from Du Pont Instruments (Wilmington, Del.) require a lower initial methanol concentration (60%) to achieve the separation of BP tetrols and triols reported previously (Huberman et al., 1976; Yang et al., 1976).

HPLC Separation of cis- and trans-7,8- and cis- and trans-4,5-Diols. The separation of BP *cis*- and *trans*-7,8-diols was performed with a Spectra-Physics Model 3500 liquid chromatograph fitted either with a Whatman 4.6 mm i.d. × 25 cm octadecyltrimethoxysilane (Partisil ODS-2) column or a Du Pont 6.2 mm i.d. × 25 cm Zorbax ODS column. The column was eluted with a linear gradient of 60% methanol in water to 100% methanol in 100 min at room temperature. The solvent flow rate was 0.8 mL/min.

BP *cis*- and *trans*-4,5-diols were separated on a 6.2 mm i.d. × 25 cm Zorbax ODS column from Du Pont Instruments.

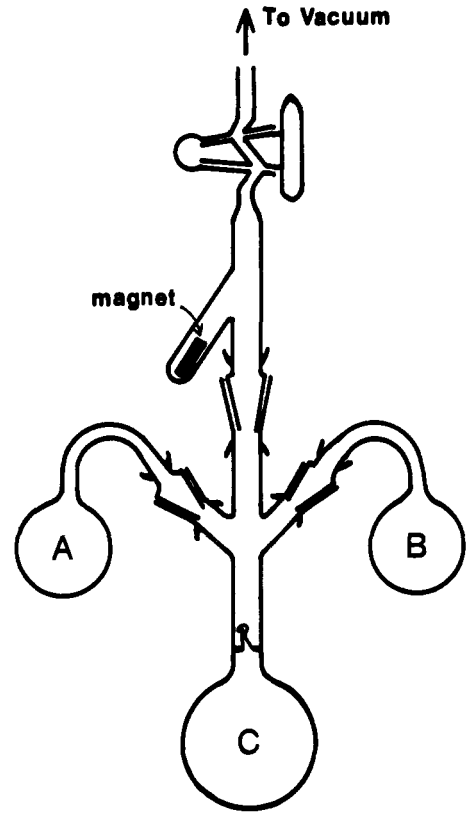

FIGURE 1: Apparatus used to obtain the ^{18}O-containing BP metabolites.

Different columns have been variably effective. The column was eluted with a linear gradient of 65% methanol in water to 100% methanol in 50 min with a solvent flow rate of 0.8 mL/min at room temperature. Recently purchased Du Pont Zorbax ODS columns did not resolve the *cis*- and *trans*-4,5-diols but the *cis*- and *trans*-7,8-diols were resolved.

HPLC Separation of Optical Isomers. The di-(−)-menthoxyacetates of BP (−)- and (+)-*trans*-diols were resolved with a DuPont 6.2 mm i.d. × 25 cm "Zorbax" SIL (silica adsorbent) column on a DuPont Model 830 liquid chromatograph. The column was preequilibrated with methylene chloride or with methylene chloride containing 0.25% (v/v) ethyl acetate. The sample was dissolved in methylene chloride just prior to HPLC analysis. The di-(−)-menthoxyacetates of *trans*-9,10-diol were eluted with methylene chloride and the di-(−)-menthoxyacetates of *trans*-4,5-diol were eluted with methylene chloride containing 0.25% (v/v) ethyl acetate. The solvent flow rate in both cases was 2 mL/min at room temperature. The *trans*-[^{3}H]diols (ca. 0.1 μg) formed metabolically from BP and nonradioactive synthetic (±)-*trans*-diols (50 μg) in 0.1 mL of pyridine were reacted with (−)-menthoxyacetyl chloride (10 mg) for 16 h at room temperature similarly as reported by Cook et al. (1950). One milliliter of water was added to the reaction mixture and was extracted twice with 2 mL of diethyl ether/benzene (1:1, v/v). The organic extract was evaporated to dryness with a gentle stream of nitrogen. The sample was analyzed as described above and the resolution of the optical isomers of the *trans*-7,8-diol has been reported previously (Yang et al., 1977b,e).

In Vitro Incubation of BP under Molecular Oxygen-18. The ^{18}O-containing BP metabolites were obtained with the apparatus shown in Figure 1. Compartment A contains 100 mg of protein equivalent of liver microsomes (in 6 mL of Tris-HCl buffer, pH 7.5) from 3-methylcholanthrene-pretreated male

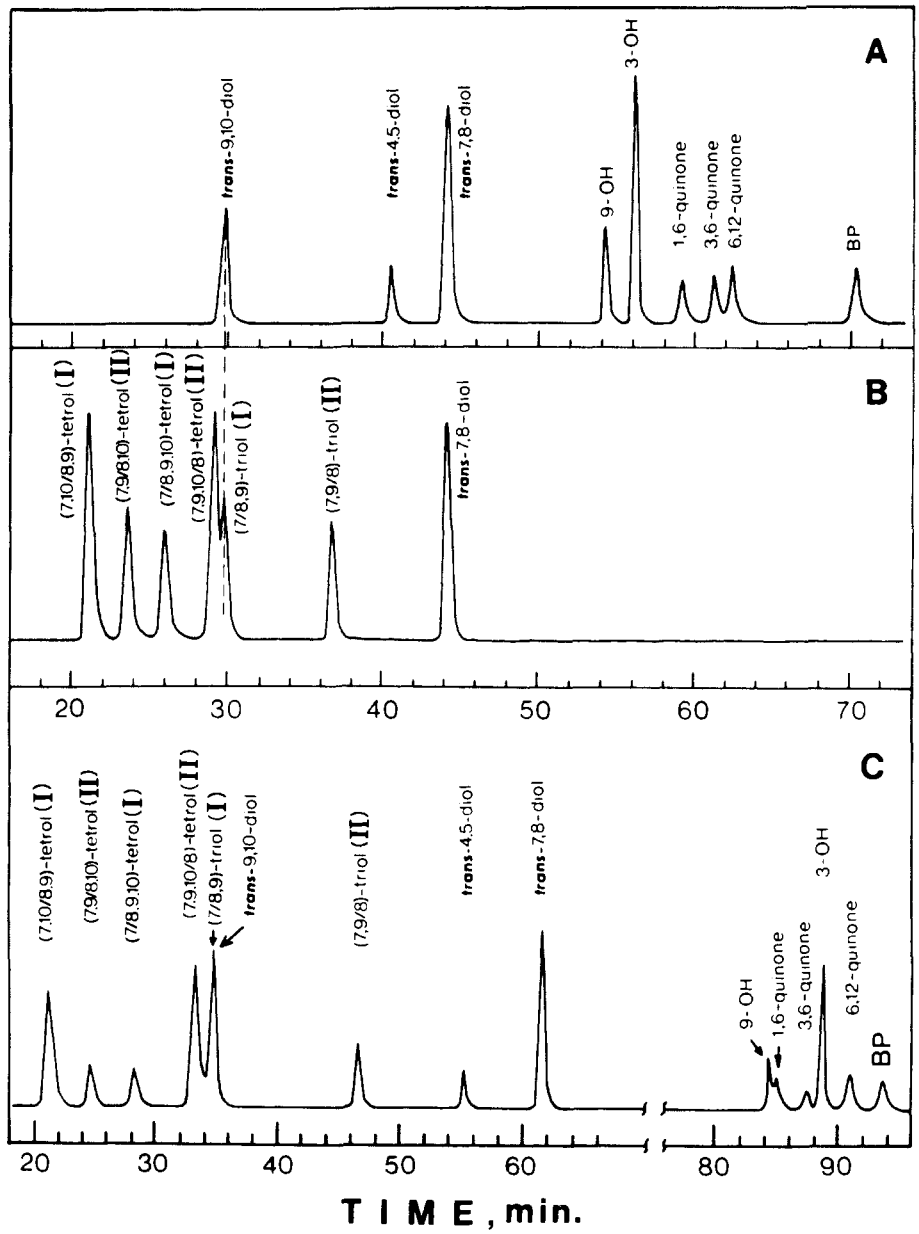

FIGURE 2: HPLC separation of the synthetic BP derivatives. Both A and B were carried out with a linear gradient of 60% methanol in water to 100% methanol in 50 min (100 min in C) with a solvent flow rate of 0.8 mL/min at room temperature on a Du Pont Zorbax ODS (6.2 mm i.d. × 25 cm) column. Retention times of other BP derivatives were established by cochromatography one at a time with those shown in A. The structure of tetrols and triols have been established (Keller et al., 1976; Yang and Gelboin, 1976a,b; Yang et al., 1976, 1977d,e).

Sprague Dawley rats. Compartment B contains 100 mL of buffer (0.05 M Tris-HCl, pH 7.5, 3 mM $MgCl_2$), 100 mg of NADPH, and 2 mg of BP added in 4 mL of methanol. Compartment C contains 250 mL of oxygen-18 gas (99 atom %). The aqueous solutions in compartments A and B and the rest of the glassware were first degassed by a vacuum pump and an on-line cold trap. The residual air in compartments A and B was further removed by vacuum by slightly warming the solutions. After closing the vacuum stopcock, the magnetic bar was dropped to break the seal of compartment C. The solutions in compartments A and B were allowed to mix into compartment C and the mixture was incubated for 1 h at 37 °C. BP and its metabolites were extracted with 100 mL of cold acetone and 200 mL of ethyl acetate. The organic phase was dehydrated with anhydrous magnesium sulfate and evaporated to dryness by reduced pressure, and the residues were redissolved in 1 mL of methanol. BP metabolites were separated by HPLC. About 85% of BP was metabolized under the conditions described above. HPLC analysis indicated that further metabolism of *trans*-7,8-diol (Huberman et al., 1976; Yang et al., 1976) was negligible.

Enzymatic Hydration of BP 4,5-Epoxide and 7,8-Epoxide in Oxygen-18 Water. The racemic 4,5-epoxide or 7,8-epoxide (150 μg in 0.1 mL of THF) was incubated with partially purified epoxide hydratase (0.86 mg of protein in 80 μL of 0.1 M glycine–NaOH buffer, pH 9.0; specific activity 216 pmol of BP 4,5-diol formed per min per mg of protein with BP 4,5-epoxide as the substrate) (Leutz and Gelboin, 1975) in 2 mL of oxygen-18 water (50 atom %) at 37 °C for 1 h under nitrogen atmosphere. The net oxygen-18 content of solvent water in this mixture is therefore 48 atom %. The hydration products (diols) and isomerization products (phenols) were separated by HPLC.

Acid Dehydration of trans-Diols to Phenols. The ^{18}O-containing *trans*-9,10-diol or *trans*-7,8-diol (20 μg) formed metabolically from BP under $^{18}O_2$ was treated at 70 °C for 1

h in 1 mL of H_2O/THF (6:1,v/v) containing 0.5 N HCl. The ^{18}O-containing *trans*-4,5-diol was treated at 100 °C for 6 min in 1 mL of concentrated HCl (Grover et al., 1972). The resulting phenolic products were extracted with 6 mL of ethyl acetate. The ethyl acetate extracts were further washed five times with water and evaporated to dyrness with a gentle stream of nitrogen. The residues were redissolved in methanol for HPLC separation of the phenolic products. The minor phenolic products from the *trans*-7,8-diol and the *trans*-9,10-diol were each purified by repeated HPLC runs until a single chromatographic peak was obtained. 4-OH-BP and 5-OH-BP are separated by 0.4 min on HPLC (see below). The 4-OH-BP was not detected as the acid dehydration product of either *trans*-4,5-diol or *cis*-4,5-diol. Succesive rechromatography of the first third of the 5-OH-BP chromatographic peak failed to detect the presence of 4-OH-BP. Isomerization of racemic 4,5-epoxide also did not yield a detectable amount of 4-OH-BP.

Mass-spectral analysis was performed on a JEOL Model JMS-01SG-2 instrument at 70 eV with a solid probe. Ultraviolet absorption spectra were measured on a Cary 15 spectrophotometer. Optical rotations were measured on a Perkin-Elmer Model 241-MC polarimeter.

Results

HPLC Separation of Synthetic BP Derivatives. A more efficient reversed-phase ODS column and a new gradient system have enabled us to resolve BP derivatives (Figure 2) more efficiently than previously reported (Holder et al., 1974; Selkirk et al., 1974a,b; Yang et al., 1975). The three *trans*-diols and three quinones are well resolved (Figure 2A). The elution order of the phenols and quinones (Figure 2A) are reversed from that previously reported (Holder et al., 1974; Selkirk et al., 1974a,b; Yang et al., 1975) and are also dependent on the gradient conditions (Figure 2A vs. 2C). Due to the variable performance of commercially available prepacked columns, the (7/8,9)triol which eluted between (7/8,9,10)tetrol and (7,9,10/8)tetrol (Yang et al., 1976) is eluted after the (7,9,10/8)tetrol (Figure 2B and 2C). The 7-OH-BP, a nonenzymatic rearrangement product of 7,8-epoxide (Waterfall and Sims, 1972) which eluted with 3-OH-BP (Holder et al., 1974; Yang et al., 1975), is separated from the 9-OH-BP and 3-OH-BP. Under the HPLC conditions of Figure 2A, the retention times of the chromatographic peaks for ten synthetic phenolic derivatives are 54.0 (4-OH-BP and 12-OH-BP), 54.3 (8-OH-BP and 9-OH-BP), 54.4 (5-OH-BP), 54.6 (2-OH-BP), 54.8 (10-OH-BP), 55.5 (7-OH-BP), 56.2 (1-OH-BP and 3-OH-BP) min, respectively. Retention times of the compounds separated on different columns may vary but the relative elution orders are unchanged. Variation of gradient condition improves the separation of some BP derivatives but changes the elution orders of others (Figure 2). For the analysis of metabolites obtained in the in vitro or in vivo incubation of BP, a 50-min linear gradient (Figure 2A) is preferable. However, a 100-min linear gradient (Figure 2C) is a better choice for the study of *trans*-7,8-diol metabolism to diol epoxides (Huberman et al., 1976; Yang, et al., 1976).

The Metabolically Formed 4,5-Diol and 7,8-Diol Are trans Isomers. Under conditions described in Figure 2A, the *cis*- and *trans*-7,8-diols are separated by 1.3 min but one of the earlier purchased Du Pont Zorbax ODS columns did not give separation. However, Figure 3A shows that *cis*- and *trans*-4,5-diols are well resolved. We have also used a Whatman ODS column to resolve the *cis*- and *trans*-7,8-diols (Figure 3B). [^{14}C]-BP-7,8-diol and -4,5-diol, formed metabolically from [^{14}C]BP by liver microsomes from untreated or 3-methylcholanthrene-pretreated rats, each cochromatographed with the synthetic *trans*-diol (Figure 3). Thus, we have established unequivocally that the 4,5-diol and 7,8-diol formed metabolically from BP are trans isomers.

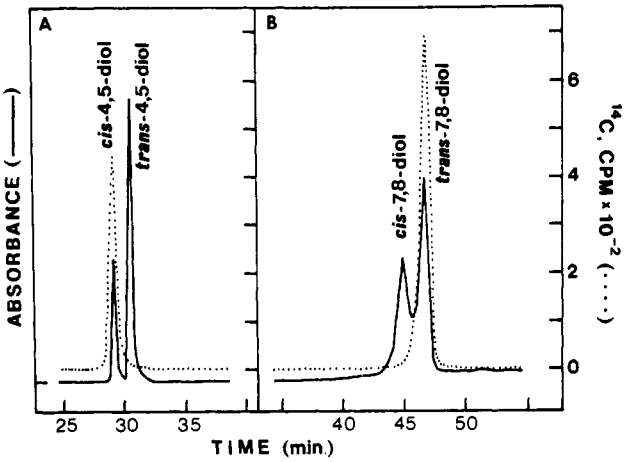

FIGURE 3: HPLC separation of cis and trans isomers of BP 4,5- and 7,8-diols. 4,5-[^{14}C]Diols and 7,8-[^{14}C]diols formed metabolically from [^{14}C]BP with liver microsomes were each cochromatographed with a mixture of synthetic cis and trans isomers. The separation of *cis*- and *trans*-4,5-diols (A) was carried out with a Du Pont Zorbax ODS (6.2 mm i.d. × 25 cm) column purchased early in 1976 with a linear gradient of 65% methanol in water to 100% methanol in 50 min with a solvent flow rate of 0.8 mL/min. The separation of *cis*- and *trans*-7,8-diols (B) was carried out with a Whatman 4.6 mm i.d. × 25 cm Partisil ODS-2 column with a linear gradient of 60% methanol in water to 100% methanol in 100 min with a solvent flow rate of 0.8 mL/min at room temperature.

The 9,10-diol formed metabolically from BP cochromatographed with the synthetic *trans*-9,10-diol under all conditions tested. However, due to the lack of synthetic standard, it is not known whether the *cis*- and the *trans*-9,10-diols can be resolved chromatographically. In a vicinal *cis*-diol test with potassium triacetylosmate (Criegee et al., 1942; Yang and Gelboin, 1976a; Yang et al., 1977d), the 9,10-diol formed metabolically from BP was found to be a trans isomer. The diols formed metabolically from other polycyclic aromatic hydrocarbons with mammalian microsomal enzymes were all found to be trans isomers (Akhtar et al., 1975; Holtzman et al., 1968; Jerina et al., 1970a,b; Miura et al., 1968; Sims and Grover, 1974).

The trans-Diols Formed Metabolically from BP Are Optically Pure (−) Enantiomers. We have developed an HPLC methodology to resolve the di-(−)-menthoxyacetates of the (+) and (−) enantiomers of BP *trans*-7,8-diols (Yang et al., 1977b,e). This method was further improved and used to establish the optical purity of the three *trans*-diols formed metabolically from BP. Under conditions in which the di-(−)-menthoxyacetates of the (+) and (−)-*trans*-diols were completely resolved (Figure 4), we found that the di-(−)-menthoxyacetates of the *trans*-diols formed metabolically from BP were each eluted with the di-(−)-menthoxyacetate of the (−)-*trans*-diols. Since the diols formed metabolically from BP have been previously established to be the (−) enantiomers (Yang and Gelboin, 1976b), we therefore conclude that the *trans*-diols formed metabolically from BP are all optically pure (−) enantiomers.

Metabolism of BP under Molecular Oxygen-18. The *trans*-4,5-, *trans*-7,8-, and *trans*-9,10-diols formed metabolically from BP under $^{18}O_2$ (99 atom %) were each isolated by HPLC. The molecular ions from mass-spectral analysis indicated that each of the *trans*-diols contains greater than 98 atom % of ^{18}O (M^+, *m/e* 288). The results thus indicate that one of

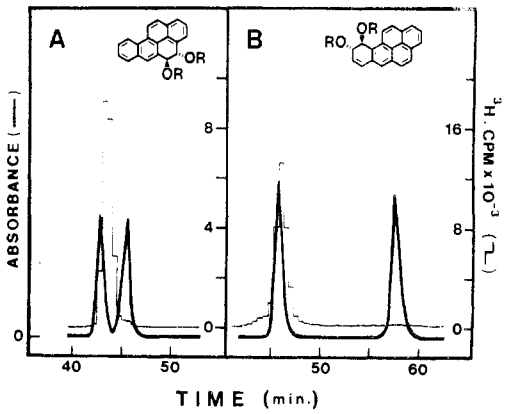

FIGURE 4: HPLC separation of the di-(−)-menthoxyacetates of the (+) and (−) BP *trans*-diols. The metabolically formed *trans*-[^{3}H]diols were each mixed with racemic *trans*-diols and reacted with (−)-menthoxyacetyl chloride and the resulting di-(−)-menthoxyacetates were analyzed by HPLC. The reasons that radioactive and absorption curves do not coincide are due to: (1) slightly delayed fraction collection after UV detection, and (2) attenuated absorption curves due to a large amount of nonradioactive sample and higher detectability of radioactive fractions. Under similar conditions, the *trans*-7,8-diol formed metabolically from BP has previously been reported to be an optically pure (−) enantiomer (Yang et al., 1977e). See Materials and Methods for details.

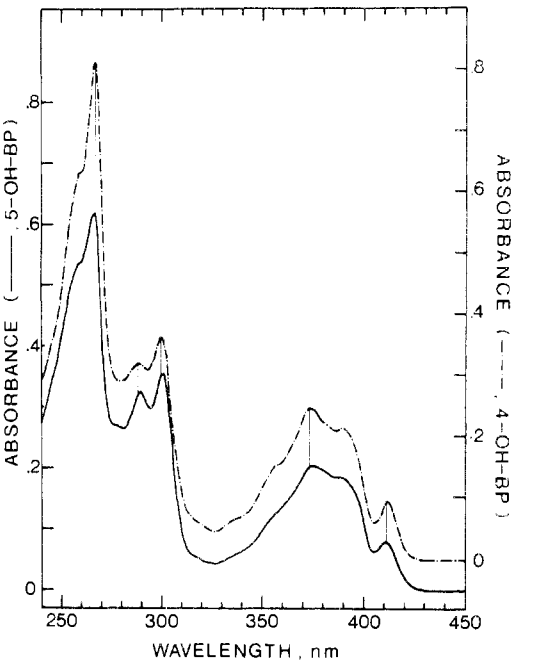

FIGURE 5: Absorption spectra of the synthetic 4-OH-BP (-•-, 3.2 μg/mL) and 5-OH-BP (—, 3.μg/mL) measured in methanol. Molar extinction coefficient ($cm^{-1}M^{-1}$) at 266 nm: 4-OH-BP, 7.25×10^4; 5-OH-BP, 5.32×10^4. Vertical lines indicate differences in absorption maxima.

the oxygen atoms of the *trans*-diols is derived from molecular oxygen. The 3-OH-BP, 7-OH-BP, and 9-OH-BP were also isolated and were found to contain ^{18}O (98 atom %), which indicate that the phenolic oxygens are also derived from molecular oxygen.

Acid Dehydration of the Metabolically Formed ^{18}O-Containing trans-Diols. Each of the three *trans*-diols formed metabolically from BP under $^{18}O_2$ was found to contain one ^{18}O atom. In order to establish the structures of the *trans*-diols, we converted chemically the ^{18}O-containing *trans*-diols to phenolic products by acid dehydration. Since the two possible phenolic products from each of the three *trans*-diols can be separated chromatographically, it is possible to find out which of the phenols contain ^{18}O by mass-spectral analysis of the HPLC-isolated phenols.

Upon acid treatment of the ^{18}O-containing *trans*-4,5-diol, we found that only 5-OH-BP was produced by HPLC analysis. This is in disagreement with the earlier report (Grover et al., 1972) that 4-OH-BP was the acid dehydration product of *trans*-4,5-diol. The UV absorption spectra of 4-OH-BP and 5-OH-BP (Figure 5) are grossly similar. However, close examination indicates that they are not identical (Figure 5). In addition to the differences in retention time on HPLC and several absorption maxima (Figure 5), the molar extinction coefficient of 4-OH-BP at 266 nm is 1.36 times higher than that of 5-OH-BP. The acid dehydration product of *trans*-4,5-diol is identical to the synthetic 5-OH-BP in all the properties examined (i.e., retention times on HPLC and UV absorption spectra). The 5-OH-BP obtained by acid dehydration of the ^{18}O-containing *trans*-4,5-diol was found to contain ^{18}O (98 atom %) by mass-spectral analysis. The results thus establish that the oxygen of C_5-OH of the *trans*-4,5-diol formed metabolically from BP is derived from molecular oxygen (Figure 6A).

Acid dehydration of the ^{18}O-containing *trans*-7,8-diol yielded 7-OH-BP (97%) and 8-OH-BP (3%) which were isolated by HPLC. Mass-spectral analysis revealed quantitative retention of the ^{18}O label in 7-OH-BP (M^+, *m/e* 270; greater than 98 atom %). Thus, it is established that the oxygen of C_7-OH of the *trans*-7,8-diol is derived from molecular oxygen (Figure 6A).

The (−)-*trans*-7,8-diol did not form any 7-OH-BP under the in vitro incubation conditions. Since the 7-^{18}OH-BP was found from the in vitro incubation of BP under $^{18}O_2$, it can be concluded that this 7-^{18}OH-BP was not derived from the (−)-*trans*-7,8-diol but rather derived from the 7,8-epoxide intermediate (Figure 6A).

Acid dehydration of the ^{18}O-containing *trans*-9,10-diol formed metabolically from BP yielded 9-OH-BP (99%) and 10-OH-BP (1%) which were isolated by HPLC. Mass-spectral analysis revealed that the oxygen-18 was exclusively in 10-OH-BP (M^+, *m/e* 270; greater than 83 atom %)[2] and the 9-OH-BP (M^+, *m/e* 268; 100 atom %) did not contain oxygen-18. Thus, the oxygen of C_{10}-OH of the *trans*-9,10-diol is established to be derived from molecular oxygen (Figure 6A).

The 9-OH-BP isolated by HPLC from BP metabolism under $^{18}O_2$ was found to contain ^{18}O. Since the 9-OH-BP obtained from acid dehydration of the ^{18}O-containing *trans*-9,10-diol did not contain ^{18}O, it can be concluded that the 9-^{18}OH-BP produced from BP metabolism under $^{18}O_2$ is not derived from the ^{18}O-containing *trans*-9,10-diol but rather derived from the isomerization of the unstable ^{18}O-containing 9,10-epoxide intermediate (Figure 6A).

Enzymatic Hydration of BP 4,5-Epoxide and 7,8-Epoxide in Oxygen-18 Water with Epoxide Hydratase. Racemic BP 4,5-epoxide and 7,8-epoxide were each incubated in oxygen-18 water (48 atom %) with partially purified epoxide hydratase. The hydration products, *trans*-4,5-diol and *trans*-7,8-diol, were each isolated by HPLC. Analysis of the molecular ions of the mass spectra indicated that the diols contained 48 atom % of ^{18}O which were identical to the ^{18}O content of the aqueous reaction medium used. In addition to the *trans*-4,5-diol, 5-OH-BP (M^+, *m/e* 268; 100 atom %) was also found to form

[2] Interferences in the mass spectra by a small amount of unidentified substances did not allow the demonstration of quantitative retention of ^{18}O label.

from the 4,5-epoxide but the 4-OH-BP was not detected (Figure 6B). The 7-OH-BP (M^+, *m/e* 268; 100 atom %) and the 8-OH-BP in a ratio of approximately 99 to 1 were also formed in addition to the *trans*-7,8-diol in the enzymatic hydration of racemic 7,8-epoxide in oxygen-18 water (Figure 6C). We previously reported (Yang et al., 1977e) that hydration of racemic 7,8-epoxide with partially purified epoxide hydratase yielded a *trans*-7,8-diol containing 86% (−) enantiomer and 14% (+) enantiomer, which indicated that the epoxide hydratase is substrate stereoselective. A large incubation of the racemic epoxide with partially purified epoxide hydratase yielded a *trans*-4,5-diol with a specific rotation ($[\alpha]^{25}_{400}$) of $-62 \pm 8°$ (0.357 mg/mL, methanol). This is compared to the (−)-*trans*-4,5-diol formed metabolically from BP ($[\alpha]^{25}_{400} = -145 \pm 70°$ (0.25 mg/mL, methanol)) which indicates that the *trans*-4,5-diol formed enzymatically from the racemic 4,5-epoxide by the epoxide hydratase contains approximately 71% (−) enantiomers. This result further indicates that the epoxide hydratase is substrate stereoselective.

Upon acid dehydration, the ^{18}O-containing *trans*-4,5-diol lost the ^{18}O label to yield only 5-OH-BP (M^+, *m/e* 268; 92 atom %).[2] The results therefore establish that the C_4-OH of the *trans*-4,5-diol is derived from solvent water (Figure 6B). The results have also established that the ^{18}O label is at the 4 position in both the (+)- and the (−)-*trans*-4,5-diols which are derived enzymatically from the racemic 4,5-epoxide. Therefore, both enantiomers of the racemic 4,5-epoxide are hydrated by the epoxide hydratase to the (+)- and the (−)-*trans*-4,5-diols only by cleavage of the C_4–O bonds. Either one of the two 4,5-epoxide enantiomers is hydrated to a specific enantiomeric form of the *trans*-4,5-diol by cleavage of the C_4–O bond, Therefore, it can be concluded that the epoxide hydratase is product stereospecific.

Acid dehydration of the ^{18}O-containing *trans*-7,8-diol yielded 7-OH-BP (97%) and 8-OH-BP (3%) (Figure 6C). Mass-spectral analysis revealed that the ^{18}O was exclusively in 8-OH-BP, and the 7-OH-BP (M^+, *m/e* 268; 100 atom %) showed no ^{18}O present. The results thus establish that the C_8–OH of both the (+)- and the (−)-*trans*-7,8-diols are derived from solvent water, which indicate that both 7,8-epoxide enantiomers are hydrated only by cleavage of the C_8–O bonds (Figure 6C). The results further demonstrate that the epoxide hydratase is product stereospecific; i.e., optically pure (−)-*trans*-7,8-diol which is formed metabolically from BP is derived from a single enantiomer of the 7,8-epoxide intermediate (Figure 6A).

Discussion

Detailed studies of the *trans*-diols formed enzymatically from BP and BP epoxides by microsomal enzyme systems have established that the *trans*-diols are formed by two consecutive stereospecific reactions (Figure 6). The BP is first oxygenated at one side of the planar molecule by the mixed-function oxidases to form single enantiomers of the 4,5-, 7,8-, and 9,10-epoxide intermediates. This is followed by a product-stereospecific hydration by the microsomal epoxide hydratase to form optically pure (−) enantiomers of the *trans*-4,5-, *trans*-7,8-, and *trans*-9,10-diols. When a racemic epoxide was used as the substrate, the epoxide hydratase was found to be substrate stereoselective and an excess of the (−) enantiomer of the *trans*-diol is formed over the (+) enantiomer (Yang et al., 1977e). The substrate stereoselectivity of the epoxide hydratase was also observed in the study of the enzymatic hydration of other polycyclic aromatic hydrocarbon epoxides (Jerina et al., 1970a,b).

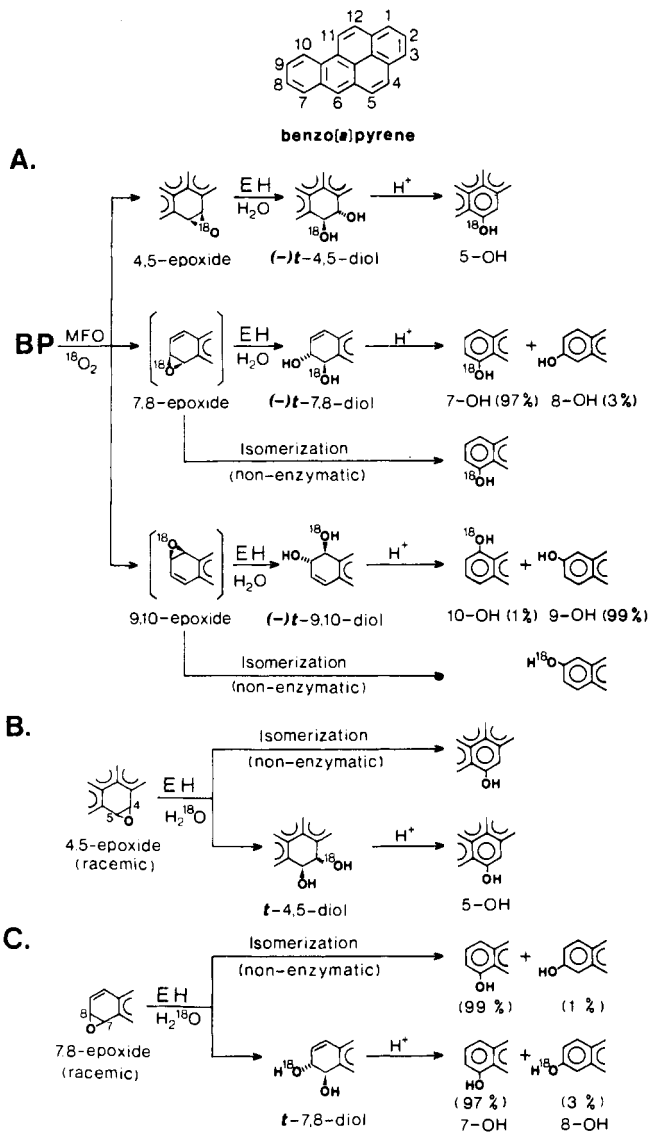

FIGURE 6: Mechanism of enzymatic formation and nonenzymatic acid dehydration of BP *trans*-diols. MFO and EH indicate microsomal mixed-function oxidases and epoxide hydratase, respectively. The *t* abbreviates for *trans*. (A) The (−)-*trans*-diols were obtained by incubation of BP under $^{18}O_2$ with rat liver microsomes and isolated by HPLC. The absolute configuration of the (−)-*trans*-7,8-diol has been reported (Nakanishi et al., 1977) to be 7*R*,8*R*. The absolute configurations of the (−)-*trans*-4,5- and 9,10- diols enzymatically formed from BP are proposed to be 4*S*,5*S* and 9*S*,10*S*, respectively. The triangles and dotted lines indicate that the substituents are toward and away from the viewer, respectively. Epoxides shown in brackets indicate unstable metabolic intermediates. The BP 4,5-epoxide intermediate has been directly detected as a metabolite (Grover et al., 1972; Selkirk et al., 1975). (B and C) The *trans*-4,5- and *trans*-7,8-diols enzymatically obtained from the respective racemic epoxide precursors contain an excess of (−) enantiomers. See Materials and Methods and the text for discussion.

The results summarized in Figure 6 established the positions of the oxygen atoms of the *trans*-diols derived from molecular oxygen or solvent water. The absolute configuration of a BP-RNA adduct formed in vivo by metabolism of BP (Nakanishi et al., 1977) indicated that the absolute configuration of the (−)-*trans*-7,8-diol is 7*R*,8*R* which confirmed our earlier suggestion (Yang and Gelboin, 1976b). Since the (−)-*trans*-7,8-diol is enzymatically and trans stereospecifically derived from the 7,8-epoxide intermediate, it can be deduced that the absolute configuration of the 7,8-epoxide intermediate is 7*R*,8*S* (Figure 6A). We have proposed that all epoxide intermediates, with the absolute configurations indicated in

Figure 6A, are formed at the same side of the planar BP molecule. However, proof of this hypothesis requires the knowledge of the absolute configurations of the *trans*-4,5- and *trans*-9,10-diols.

It was reported (Selkirk et al., 1976) that 1- and 7-OH-BP were found to be the metabolites of BP in addition to 3- and 9-OH-BP. With the column chromatography employed in this report, 7-OH-BP is also separable from the 3- and 9-OH-BP. However, the HPLC methodology described in this report indicated that the 1-OH-BP is cochromatographed with the major phenolic metabolite 3-OH-BP. The resolved 3-, 7-, and 9-OH-BP were all found to contain ^{18}O when BP was metabolized under $^{18}O_2$. The phenolic oxygens of 3-, 7-, and 9-OH-BP are thus derived from molecular oxygen. Whereas the 7- and 9-OH-BP can be accounted for as the rearrangement products of the 7,8- and 9,10-epoxide intermediates (Figure 6), the mechanism of 1- and 3-OH-BP formations are still unknown.

Nonenzymatic isomerization of the racemic 7,8- and 9,10-epoxide yielded 8- and 10-OH-BP as the minor products in addition to the known product 7- and 9-OH-BP, respectively. However, 8- and 10-OH-BP were not detected as metabolites of BP (Selkirk et al., 1976), neither were they detected as rearrangement products of the epoxides (Waterfall and Sims, 1972). We have detected 10-OH-BP as the acid dehydration product of *trans*-9,10-diol and the rearrangement product of 9,10-epoxide. It is possible that 8- and 10-OH-BP are formed from BP metabolism but the amount is below the detectable level. Alternatively, the isomerization to 8- and 10-OH-BP may not be favored when the epoxide intermediates are formed in the microsomal enzyme system.

Both isomerization of 4,5-epoxide and acid dehydration of *trans*-4,5-diol yielded 5-OH-BP. This is contrary to an earlier report (Grover et al., 1972) that 4-OH-BP was the product of acid dehydration of *trans*-4,5-diol. Evidence is presented in this report demonstrating that 5-OH-BP rather than 4-OH-BP is the product formed. Neither 4-OH-BP nor 5-OH-BP was detected as BP metabolite (Selkirk et al., 1976). Apparently, this is due to the stability of the 4,5-epoxide formed metabolically (Grover, et al., 1972; Selkirk et al., 1975). In the microsomal enzyme system, the relatively stable 4,5-epoxide formed from BP is readily hydrated to the 4,5-diol (Yang et al., 1975).

The mechanism of BP metabolism to (−)-*trans*-7,8-diol and subsequently to the reactive 7,8-diol 9,10-epoxides (Huberman et al., 1976; Sims et al., 1974; Thakker et al., 1976; Yang and Gelboin, 1976b; Yang et al., 1976, 1977a,e) by mammalian microsomal enzymes have been established to be at least one of the activation pathways. Elucidation of the alternative major metabolic pathways of BP to the (−)-*trans*-9,10-diol and the (−)-*trans*-4,5-diol further establish the stereospecificity of microsomal mixed-function oxidases and epoxide hydratase (Yang and Gelboin, 1976b; Yang et al., 1977a,e).

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Structural Studies on the Sialic Acid Polysaccharide Antigen of *Escherichia coli* Strain Bos-12†

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ABSTRACT: A polysaccharide, antigenically related to group C meningococcus, has been isolated from *Escherichia coli* strain Bos-12 (016; K92; NM). Like groups B and C meningococcal polysaccharide, the Bos-12 antigen is a pure polymer of sialic acid. ^{13}C NMR studies on the meningococcal group B and C polysaccharides have indicated that the former consists of sialic acid units linked 2→8-α, whereas the latter contains the sialic acid residues linked 2→9-α (Bhattacharjee, A. K., Jennings, H. J., Kenny, C. P., Martin, A., and Smith, I. C. P. (1975), *J. Biol. Chem. 250,* 1926). Comparison of natural abundance ^{13}C NMR spectra of the Bos-12 polysaccharide with group B and C meningococcal polysaccharides established that Bos-12 was either (a) an equimolar mixture of 2→8-α linked sialic acid homopolymers or (b) a 2→8-α/2→9-α heteropolymer. These possibilities were distinguished in the following manner. The fact that Bos-12 polysaccharide precipitated with anti-group C serum but not with anti-group B serum would seem to exclude a. Further, chemical studies (periodate oxidation followed by tritiated $NaBH_4$ reduction) gave saccharides with a radioactive-labeling pattern expected for alternating 2→8-α/2→9-α sialic acid linkages. Bos-12 is thus an 2→8-α/2→9-α heteropolymer.

It has been shown that injection of formaldehyde-treated *Escherichia coli* strain Bos-12 (016; K92; NM) antigen into rabbits induces precipitating meningococcal group C antibodies which fail to react with the group B polysaccharide (Robbins et al., 1975). Preliminary chemical studies revealed, however, that the Bos-12 antigen shares some properties of both the group B and C meningococcal polysaccharides. Both group B and Bos-12 polysaccharides are susceptible to neuraminidase cleavage, whereas the group C polysaccharide is resistant. Unlike the group B polysaccharide, both group C and Bos-12 polysaccharides are susceptible to acid-catalyzed methanolysis (Liu et al., 1971a,b).

^{13}C NMR spectroscopy has, in recent years, been applied to the structural determination of a number of biologically important polysaccharides, including the group B and C meningococcal polysaccharides (Bhattacharjee et al., 1975). These studies have shown that the group B polysaccharide consists of sialic acid units linked 2→8-α, whereas the group C polysaccharides contain the sialic acid residues linked 2→9-α. The present report presents evidence for the structure of the Bos-12 antigen based on ^{13}C NMR spectroscopy and chemical studies.

Experimental Procedure

Materials. The cells of *E. coli* strain Bos-12 were grown for

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