the nucleoprotein occurs at the instant of entry does it become visible in the electron microscope.

The immature form of the virus appears to be unstable, because it is never encountered extracellularly. It thus resembles the "fragile precursor heads" described in the case of T4 bacteriophage (*2*). Finally, the point should be made that maturation of vaccinia virus requires protein synthesis (*7, 15*) in a manner analogous to that encountered in recent studies of the conversion of precursor particles to mature T4 phage (*2*). It is suggested that in the future the "immature" form of the pox viruses be called provirus in keeping with bacteriophage nomenclature.

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Benzo[*a*]pyrene Diol Epoxides as Intermediates in Nucleic Acid Binding in vitro and in vivo

Abstract. *Evidence has been obtained that a specific isomer of a diol epoxide derivative of benzo[a]pyrene, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene, is an intermediate in the binding of benzo[a]pyrene to RNA in cultured bovine bronchial mucosa. An adduct is formed between position 10 of this derivative and the 2-amino group of guanine.*

Two hundred years have elapsed since the British surgeon Sir Percival Pott made the observation that chimney sweepers developed skin cancer because of their exposure to soot (*1*). This astute observation was the first causal association between an environmental (in this case, occupational) agent and human cancer. We now know that some of the carcinogenic principles in soot and coal tars are polycyclic aromatic hydrocarbons (PAH) (*1, 2*). These compounds are widely distributed in the human environment and thus represent major potential human hazards. Perhaps the most extensively studied PAH is benzo[*a*]pyrene (BP) which is emitted into the air of the United States at an estimated rate of about 1300 tons per year (*3*).

It has become an axiom in cancer research that many chemical carcinogens, including BP, are metabolized in vivo to reactive intermediates that become covalently bound to cellular macromolecules (*4, 5*). There is also evidence that this binding is a critical event in the process of carcinogenesis. Some studies have suggested that the major reactive intermediate in vivo in the case of BP is a 7,8-diol-9,10-epoxide metabolite (*6*). Two isomers, **1** and **2** (Fig. 1), of this compound have been synthesized (*7, 8*). In this study we examine the binding in vitro of these two isomers to various nucleic acids, compare the products to adducts of BP and nucleic acids formed intracellularly when bovine bronchial explants were incubated with ^{3}H-labeled BP, and report the structure of one of these products.

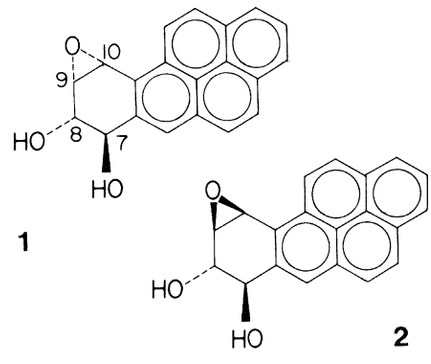

Fig. 1. Structures of compound **1**, (±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene, and compound **2**, (±)-7β,8α-dihydroxy-9β,10β-epoxy-7,8,9,10-tetrahydrobenzo[*a*]pyrene.

Table 1 indicates the binding in vitro of either isomer **1** or isomer **2** to various natural and synthetic nucleic acids. The 7,8,9,10-tetrahydro-BP derivatives have a pyrenelike absorption spectrum with a strong maximum between 340 and 350 nm. The unmodified nucleic acids did not show absorption at 350 nm, whereas the modified polymers showed a distinct absorption peak in this region. Thus, the absorption at 350 nm could be used to estimate the extent of covalent binding of the BP residue to nucleic acids (legend to Table 1). With respect to the synthetic ribonucleotide homopolymers, at *p*H 5 isomer **1** reacted considerably with polyguanylic acid [poly(G)], polyadenylic acid [poly(A)], and polycytidylic acid [poly(C)]. Isomer **2** also reacted with poly(G) and poly(A), and to a lesser extent with poly(C). A somewhat similar pattern of base specificity was seen with synthetic deoxyribonucleotide homopolymers. Little or no reaction was detected with polyinosinic acid [poly(I)], polyuridylic acid [poly(U)], or polydeoxythymidylic acid [poly(dT)]. Extensive reactions were seen with RNA and DNA, which provide all three residues—guanine (G), adenine (A), and cytosine (C)—as substrates. When reactions were performed at *p*H 8.0 with either isomer **1** or **2**, the total extent of modification of nucleic acid was less than at *p*H 5.0 and significant reaction was obtained only with poly(G), RNA, and DNA (Table 1). Separate time course studies indicated that at *p*H 5 isomer **1** or **2** reacted most rapidly with poly(G) and reached a plateau at about 1 hour. Poly(A) reacted somewhat more slowly and reached a plateau at about 3 hours. Poly(C) reacted very slowly. At 3 hours the modification was less than 15 percent that obtained with poly(G) or poly(A), and only after a 26-hour incubation period (Table 1) was there extensive modification of poly(C). Differences in the stabilities of the BP-diol epoxides, as well as the reactivities of the nucleophiles, may account for these results.

Because of the preferential reaction

with guanosine, the remaining studies reported here have been focused on these adducts. The profiles were obtained when the above modified samples of poly(G) were hydrolyzed, and the modified nucleosides were analyzed by high-pressure liquid chromatography (HPLC) (Fig. 2). The products obtained from the reaction with isomer **1** gave three peaks (Fig. 2A). Those from **2** gave four peaks (A,B,C,D) of about equal areas (Fig. 2B); the fourth peak (D) eluted at 67 minutes, but is not shown in Fig. 2B.

To determine the relationship of these materials to compounds formed in vivo, we incubated bovine bronchial explants in a chemically defined medium containing ^{3}H-labeled BP (*9*), and the cellular RNA was extracted and hydrolyzed. The modified nucleosides were isolated on a Sephadex LH-20 column and cochromatographed with guanosine adducts obtained from the reaction in vitro of poly(G) with isomer **1**. Two major radioactive peaks eluted in the same regions as peaks 2 and 3 of the guanosine derivatives of **1** (Fig. 2A). These appear to be mainly guanosine adducts since adenosine adducts derived from the modification of poly(A) with isomer **1** (or **2**) eluted considerably later during HPLC than the guanosine adducts. The first radioactive peak has not been further characterized in this study. To establish that the second radioactive peak and peak 3 were identical, the material eluting between 40 and 45 minutes (Fig. 2A) was pooled and treated with dimethoxypropane in the presence of HCl to form acetonides selectively with vicinal *cis* diols. The ribose moiety of each modified nucleoside reacts at the 2′ and 3′ hydroxyl groups.

Nucleoside adducts resulting from attack of the guanosine residue at position 10 of isomer **1** can form a second acetonide across the 8,9-*cis*-diol group of the BP moiety (*10*). Nucleoside adducts formed from the reaction with isomer **2**, which do not have the 8,9-*cis*-diol configuration, cannot form this additional acetonide and, therefore, should elute much earlier on reverse phase HPLC than those of isomer **1**. Indeed, we found that when applied to a Zorbax ODS (octadecasilyl) column (2.1 mm inside diameter) and eluted with a linear gradient (100 minutes) from 35 to 80 percent methanol in water at 50°C and 3000 pounds per square inch (20.68 megapascals), the acetonide derivatives of the BP-guanosine adducts formed from isomer **1** eluted at 66 and 68 minutes, whereas those from isomer **2** eluted at 38, 41, 51, and 56 minutes. We also found that the radioactive acetonide derivative, which represented the in vivo material, cochromatographed with the diacetonide derivative of the guanosine adduct formed in vitro from isomer **1**, which eluted at 68 minutes. The identity of the latter two materials was further established by cochromatography by HPLC (Zorbax ODS column, inside diameter 7.9 mm, 65 percent methanol in water, 50°C, 2500 pounds per square inch)

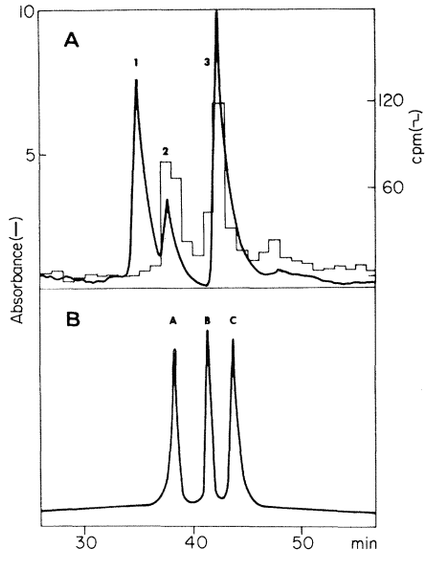

Fig. 2. (A) Bovine mainstem bronchial explants (16 specimens each 1 by 1 cm, from two adult steers) were incubated with [^{3}H]BP (200 μc/ml; 25 c/mole, generally labeled, Amersham/Searle) for 24 hours (*9*). Total cellular RNA was then isolated by a combination of phenol and chloroform extractions, ethanol precipitations, and banding by equilibrium centrifugation in a cesium sulfate density gradient (*17*). This ^{3}H-labeled RNA was mixed with a sample of poly(G) that had been previously modified in vitro with isomer **1**. The mixture was then hydrolyzed with KOH and applied to a LH-20 Sephadex column. The hydrophobic modified nucleotides that eluted with 40 percent methanol were collected and converted to nucleosides with alkaline phosphatase. The latter material was then analyzed by HPLC on a Dupont 830 instrument with a reverse phase Zorbax ODS (2.1 mm inside diameter) column operated at 50°C and 3000 pounds per square inch and eluted with a linear gradient (100 minutes) from 20 to 80 percent methanol in water. Details of these procedures have been described (*11*, *13*). The solid line indicates the elution profile at 280 nm of the nucleosides modified in vitro. The stepped line indicates the radioactivity profile of the modified nucleosides obtained from the bovine bronchial RNA. During the LH-20 fractionation of the in vivo labeled RNA, approximately one-third of the radioactivity was not retained on the column. Separate HPLC studies indicated that this did not represent ^{3}H exchange into unmodified nucleosides. The nature of these additional [^{3}H]BP in vivo derivatives is not known. (B) HPLC profile of modified guanosines obtained from a hydrolyzate of poly(G) that had been reacted with isomer **2**.

of their diacetate derivatives (acetyl residues at the 5′-hydroxyl group of ribose and the 7-hydroxyl group of the BP derivative). There was a slight difference in their retention times (46.0 minutes for the radioactive and 46.8 minutes for the unlabeled material), but this is due to an isotopic separation that we observed during HPLC of other PAH derivatives (*11*). These results provide chromatographic evidence that one of the reactive BP intermediates in vivo, with respect to nucleic acid binding, corresponds to isomer **1**. Direct structural analysis of the in vivo products is not feasible because of the extremely small amounts that are obtained.

Separate studies indicate that peaks 1 and 3 (Fig. 2A) are diastereoisomers (see below). This presumably reflects the fact that isomer **1** is used for the reaction in vitro is a mixture of (+) and (−) forms. Apparently, in vivo one enantiomer, (+) or (−), of **1** is formed and, therefore, the peak 1 product was not seen in the in vivo material. Such stereospecificity has been observed in the metabolism of other PAH's (*12*). Further studies are required to clarify the structure or structures of the ultraviolet absorbing and radioactive materials in the peak 2 region (Fig. 2A).

We have also obtained definitive structural data on the compounds corresponding to peaks 1 and 3 of Fig. 2A. A schematic representation of these structures is given in Fig. 3. The crucial data are summarized below. Complete details of the circular dichroism (CD), proton magnetic resonance (PMR), and mass spectra have been described (*13*).

The absorption spectra of peaks 1 and 3 were identical and had maximum absorption values at 344, 328, 314, 278, 268, and 246 nm, characteristic of a 7,8,9,10-tetrahydro-BP chromophore. Their CD spectra were nearly identical but of opposite sign, indicating a mirror image relationship. This critical CD result, together with the fact that both compounds had identical PMR and mass spectra, indicated that peaks 1 and 3 are diastereoisomers.

During opening of the 9,10-oxide of isomer **1**, guanine could attach to position 10 of BP, generating a hydroxyl group in position 9, or vice versa. For electronic reasons, it is most likely that the guanine residue attaches at position 10. Beland and Harvey showed that the simple nucleophile, *t*-butylmercaptan, reacts with both isomers **1** and **2** at this position (*7*). Guanosine adducts from isomer **1** react with dimethoxypropane to form diacetonides, as described above.

This provides strong evidence for guanine substitution at position 10 since dimethoxypropane could react with the *cis*-8,9-dihydroxy group of the BP residue, but not with a compound in which the *cis* hydroxyl residues were at positions 8 and 10 (*10*). Analysis of the PMR data, based on coupling constants and chemical shifts before and after acetylation, enabled us to assign BP protons 7 to 10 and also provided evidence that the addition of guanine had occurred at position 10. Comparison of the J_{7-8}, J_{8-9}, and J_{9-10} coupling constants of hydration products of isomer **1**, which were either *cis* or *trans* at the 9,10 positions, to the PMR spectra of peaks 1 and 3 suggested that the guanine addition was *trans* rather than *cis*.

The reactions in vitro of **1** and **2** with G, C, and A suggested that the hydrocarbon preferentially attacks the exocyclic amino group of bases, which in the case of guanine is the 2-amino group. More definite evidence that the BP derivative is linked to the 2-amino group of guanine was obtained from the PMR spectra of these adducts, which showed coupling between the 2-amino proton of guanine and the proton at position 10 of BP. High resolution mass spectra also provided evidence that substitution had occurred at the 2-amino group of guanosine since a fragment was obtained with a mass indicating that it represented a BP moiety containing nitrogen. This fragment could result from cleavage of the BP-guanosine adduct between the C-2 and 2-amino group of guanosine (*13*). It may be of significance that in separate structural studies we have found that dimethylbenz[*a*]anthracene-5,6-oxide also reacts in vitro with the 2-amino group of guanosine (*14*). How these PAH-nucleoside structures might distort the conformation and function of the modified nucleic acids remains to be determined (*15*).

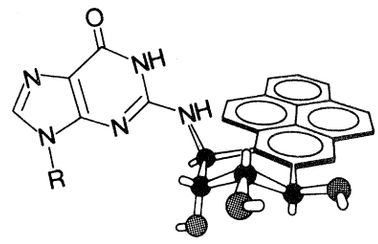

Fig. 3. Schematic representation of the guanosine-BP adduct formed from the reaction of BP-diol epoxide isomer **1** with poly(G). The 2-amino group of guanine is linked to the C-10 position of the BP moiety. The solid black spheres designate carbons 10, 9, 8, and 7 on the saturated ring of BP. The shaded spheres designate OH residues on carbons 9, 8, and 7. The R designates the ribose residue. The guanine residue is *trans* to the 9-OH, and the 9- and 8-OH are *cis* and the 8- and 7-OH are *trans*. The absolute stereochemistry of peaks 1 and 3 and the conformation of the guanine residue are not known and have been drawn arbitrarily. The crucial evidence for this structure is given in the text and in (*13*).

Our in vivo results were obtained with RNA from bovine bronchial mucosa. Separate studies provide evidence that a BP-7,8-diol-9,10-epoxide is also an intermediate in other tissues and species during the covalent binding in vivo of BP to DNA (*6*), although the specific isomer involved had not been previously identified. Our results are consistent with studies indicating that **1** is more mutagenic to mammalian cells than of **2** (*16*). In addition, **1** is apparently formed when rat liver microsomes are incubated with BP-*trans*-7,8-diol (*16*). It appears, therefore, that, in a variety of mammalian systems, **1** is an important metabolite of BP in terms of covalent binding to nucleic acids. We must emphasize, however, that not all of the BP derivatives bound to cellular nucleic acids resemble diol epoxide products. Therefore, additional reactive intermediates of BP apparently exist in vivo and remain to be identified. We have not established that in vivo BP metabolites modify only guanine residues in cellular nucleic acids. Nevertheless, our results, taken together with those of other studies (*6*), provide evidence that BP-7,8-diol-9,10-epoxide is a biologically active metabolite of BP.

Table 1. Binding of BP diol epoxide (**1**) and BP diol epoxide (**2**) to nucleic acids. The indicated polymers (0.1 mg/ml for the deoxyribonucleotide homopolymers and 1 mg/ml for the others) were reacted with an equal concentration of isomer **1** or **2** in 2:1 acetone-water mixtures (1:1 mixtures with DNA and RNA) at *p*H 5 or *p*H 8 (adjusted with KOH) for 24 hours at 37°C. The noncovalently bound material was removed by repeated extractions with ethyl acetate and mixtures of butanol and ethyl acetate, and subsequent precipitations with ethanol. Absorption spectra were then obtained and the extent of binding of hydrocarbon expressed as the ratio of the absorbance at 350 nm to that at the wavelength of maximum absorption ($A_{350}/A_{\lambda max}$). The details of these procedures have been described (*14*).

Polymer	$A_{350}/A_{\lambda max}$			
	*p*H 5		*p*H 8	
	1	**2**	**1**	**2**
RNA	0.178 (4.6)*		0.046 (1.1)	
Poly(G)	0.147 (6.2)	0.111 (4.5)	0.048 (1.7)	0.047 (1.7)
Poly(A)	0.214 (8.1)	0.167 (6.3)	0.006 (0.2)	0.005 (0.2)
Poly(C)	0.211 (4.6)	0.068 (1.4)	0.003 (0.1)	0.004 (0.1)
Poly(U)	0.021 (0.8)	0.010 (0.3)	0.000 (0)	0.000 (0)
Poly(I)	0.034 (1.2)			
DNA	0.235 (8.7)	0.139 (4.2)	0.117 (3.6)	
Poly(dG)	0.060 (2.2)	0.031 (1.1)		
Poly(dA)	0.112 (3.2)	0.038 (1.1)	0.000 (0)	0.000 (0)
Poly(dC)	0.033 (0.9)	0.010 (0.3)	0.000 (0)	0.000 (0)
Poly(dT)	0.000 (0)	0.000 (0)		

*The numbers in parentheses are approximate estimates of the "percent modification"; that is, the percentage of nucleotides in the polymer covalently modified by **1** or **2**. They were calculated from the absorption spectra of the modified polymers, on the assumption that the extinction ϵ_{350} of the modified polymer is the same as the ϵ_{344} of the BP-nucleoside adduct, $2.9 \times 10^4 M^{-1}cm^{-1}$. This value was obtained from a sample of BP-[^{14}C]deoxyguanosine prepared by reacting ^{14}C-labeled deoxyguanosine monophosphate with isomer **1**, purification of the adduct by Sephadex LH-20, conversion to the nucleoside; quantitative absorption measurements were made, and the molarity was calculated from the amount of ^{14}C in the adduct (assuming that in the adduct each residue of deoxyguanosine contains one residue of the BP derivative). The extinctions at 260 nm (ϵ_{260}) of the unmodified polymers are: DNA (denatured calf thymus), 8.5×10^3; RNA (rabbit reticulocyte ribosomal RNA), 6.7×10^3; poly(G), 9×10^3; poly(A), 9.4×10^3; poly(U), 8.9×10^3; poly(C), 5.25×10^3; poly(I), 6.25×10^3; poly(dG), 8.4×10^3; poly(dA), 8.2×10^3; poly(dC), 5.6×10^3; and poly(dT), 8.1×10^3. The percent modification = $100 \times \epsilon_{260} \times (A_{350}/\epsilon_{350}) \times [A_{260} - 0.2(A_{350})]$. The A_{260} and A_{350} are the absorbances of the modified polymer at 260 and 350 nm; ϵ_{260} is the extinction coefficient of the unmodified polymer, and ϵ_{350} is the extinction coefficient of the BP-deoxyguanosine adduct (see above). The absorbance at 260 nm was corrected for the contribution from the BP-diol epoxide derivative by the factor $0.2 \times A_{350}$. These calculations are not corrected for possible changes in absorption associated with the polymer structure.

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Cytoplasmic Aldo-Keto Reductases: A Class of Drug Metabolizing Enzymes

Abstract. *Aldehyde and ketone xenobiotic substances are preferentially reduced to alcohols by cytoplasmic enzymes in mammals. These enzymes are widely distributed in the tissues, have broad substrate specificities, have similar physical-chemical characteristics, and require reduced nicotinamide adenine dinucleotide as cofactor for the reductions. These reductases define a system of detoxification for aldehyde and ketone groups.*

Xenobiotic aldehydes and ketones are metabolized in mammals through both oxidative and reductive pathways (*1, 2*). However, many aliphatic and aromatic ketones and aldehydes yield corresponding alcohol metabolites as prominent excretory products in vivo, and reduction appears to be the favored pathway for these carbonyl group biotransformations (*3–5*). From studies designed to characterize the enzymes catalyzing carbonyl reduction, the aldo-keto reductases are localized to the cellular cytoplasm. These cytoplasmic enzymes show similarities in substrate specificity, tissue distribution, cofactor requirement, *p*H optimum, molecular weight, sulfhydryl sensitivity, noninducibility, and reaction equilibrium. They appear to compose a class.

The enzymatic reduction of several drugs and xenobiotic substances containing the carbonyl group have been studied and described (Table 1). Aromatic aldehydes (such as benzaldehyde) (*6, 7*), aromatic ketones (such as acetophenone) (*8, 9*), aliphatic ketones (such as oxisuran and naloxone) (*10–12*); methyl ketones (such as daunorubicin and warfarin) (*13–15*), a tetralone (such as bunolol) (*16*), and unsaturated ketones (*17*) are substrates of proven cytoplasmic enzyme activities that generate alcohol products.

One characteristic of the few reductases that have been purified and studied is a broad substrate specificity (*6, 7, 9, 14, 20, 21*). The enzymes reduce xenobiotic carbonyls as well as naturally occurring substrates. For example, a purified rat liver aldehyde reductase that reduces several aromatic aldehydes very effectively reduces the methyl ketone daunorubicin and the natural substrate glucuronic acid (*7*). However, absolute substrate specificity is difficult to assess because crude cytoplasmic extracts may contain several reductases. During the purification of rat liver aldehyde reductase, clear separation of the aldehyde reductase and other reductases occurs (*7*), an indication that several enzymes with aldo-keto reducing activity are present in rat liver cytoplasm. In rabbit liver, different cytoplasmic enzymes reduce acetone and acetophenone (*8*) or warfarin, oxisuran, and daunorubicin (*20*). For these reasons, purifications of the cytoplasmic aldo-keto reductases are necessary to resolve the substrate specificity enigma.

The aldo-keto reductases are widely distributed among tissues (Table 1). Although many investigations are limited to liver cytosol reductases, reductase activities occur in several other organ locations (*8, 10, 11, 18*). In fact, the cytosol of every mammalian tissue examined reduces daunorubicin (*13, 14, 19*). Even erythrocytes contain aldo-keto reductases (*8, 13, 17–19*). The wide tissue distribution indicates the large reservoir of catalytic activity that is available.

All the aldo-keto reductases preferentially utilize reduced nicotinamide adenine dinucleotide phosphate (NADPH) as cofactor (Table 1). Purified preparation of rabbit liver oxisuran reductase (*20*), rat liver aldehyde reductase (*14*), and hog kidney aldehyde reductase (*6*), as well as the other unpurified enzymes, require NADPH as cofactor. Only the aliphatic keto reductase activity of crude rat liver cytosol utilizes NADPH and NADH equally (*8*). No other cofactors or prosthetic groups have been identified in these enzymes (*20*).

The *p*H optima of enzymatic carbonyl reductions range between *p*H 6 and *p*H 8 for those enzymes that have been assessed (*6, 7, 9, 14, 16*). As a result, near-maximal enzymatic activities are expressed at the physiological intracellular *p*H range. However, substrate classes with dual *p*H optima have been established for the aldehyde rat liver reductase (*7*).

Since few of these enzymes have been purified, little is known of their physical-chemical characteristics. But for those that have been studied, molecular weights ranging from 30,000 to 40,000 are reported (*14, 20*). Another common feature of the enzymes is the dependence of reductase activity on sulfhydryl groups in the enzyme. *P*-Chloromercuribenzoate, *N*-ethylmaleimide, and other sulfhydryl reagents inhibit these enzymes (*6, 7, 19*).

Unlike microsomal drug metabolizing enzymes, the aldo-keto reductases are not induced or are only slightly affected by phenobarbital pretreatment (*8, 10, 15, 20*). Even prior treatment of rats for 3 days with carboxybenzaldehyde, the favored substrate for the rat liver aldehyde reductase, did not induce that enzyme (*20*).

Although these enzymes are theoretically oxidoreductases, their observed apparent equilibria favor reduction. This is observed in vivo in animals and humans and in vitro with enzyme systems. Reversibility for the oxidative reaction is attainable at low hydrogen ion concentrations (*14*), but some reactions could not be reversed (*11, 16*).