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Microbial Models of Mammalian Metabolism

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An understanding of the metabolism of drugs and other foreign chemicals in mammals has been pursued actively during the past 15 years. Metabolism can include the phenomena of absorption, distribution, and excretion (1). However, in this paper, it will be used synonymously with the more strictly defined term biotransformation. Study of mammalian biotransformations has been stimulated by the fact that useful information on the mechanism of action and/or toxicity of certain compounds can often be obtained.

While there is considerable impetus to perform metabolism studies, several practical problems are encountered including: (*a*) development of analytical methods suitable for the determination of drugs and their metabolites in biological media; (*b*) qualitative and quantitative metabolic differences in various species of animals, especially when attempting to compare lower animals to humans; and (*c*) procurement of sufficient quantities of metabolites for structure elucidation and biological testing. Considerable effort has been expended in solving the first two problems. The refinement of radiochemical techniques (2) and the development of GC (3) and mass spectral (4, 5) methodology have aided significantly in the first case, while recent emphasis on comparative metabolism should ultimately provide solutions to the second difficulty (6, 7).

Until recently, however, there have been relatively few solutions to the problem of preparative synthesis of metabolites. Although direct structural information is often obtainable (through mass spectrometry) on the mere microgram quantities of metabolites usually available from biological specimens, there is no substitute for having larger (gram) quantities for complete structure elucidation and biological evaluation. While organic synthetic methods provide an obvious solution for metabolites of relatively simple drugs, preparation of metabolites of more complex molecules may require vast expenditures in time and effort. Although mammalian tissue preparations (*e.g.*, liver microsomes) occasionally may be utilized in synthesizing preparative quantities of metabolites, there are difficulties related to the stability of such systems (8).

Recently, Smith and Rosazza (9) suggested that it might be possible to define microbial transformation systems that could mimic many biotransformations observed in mammals. As a descriptive name, these systems are called "microbial models of mammalian metabolism." It is important to realize that a microbial transformation system is viewed as a selected group of microorganisms that could be used together to conduct parallel mammalian and microbial xenobiotic metabolism studies. It is unlikely, although not impossible, that a single microorganism can mimic all of the biotransformations conducted by mammalian systems (*e.g.*, liver microsomal preparations). Where metabolites common to both the microbial transfor-

mation system and mammalian systems are observed, the microbial system would be used to obtain sizable quantities (grams) of metabolites *via* routine fermentation scale-up techniques. By this process, difficult-to-synthesize metabolites would be readily obtained for structure elucidation and biological testing. Further advantages of the microbial transformation system, especially with polyfunctional molecules, center about the selectivity and mild conditions with which biotransformations occur.

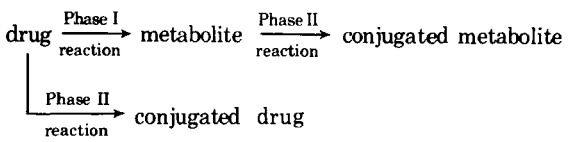
The literature abounds with reports on the metabolism of many types of compounds by microorganisms. These include steroids (10–13), alkaloids (11, 14), antibiotics (15, 16), amino acids (17, 18), and insecticides (19, 20). The general approach taken is one of revealing biogenetic pathways by which compounds are metabolized by one or more microorganisms. The fields of microbial conversions with steroids and antibiotics represent some of the most serious attempts at harnessing the synthetic potential of microorganisms.

Although the literature reveals many types of reactions by microorganisms, many of these are low yielding; few systematic studies on the use of microorganisms for the preparation of metabolites (except steroids and antibiotics) have been made (10–13, 15, 16). The fundamental bases for the selection and use of these microbial systems and their potentialities are the subjects of this paper.

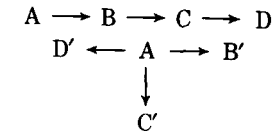
PRINCIPLES OF MAMMALIAN BIOTRANSFORMATIONS

Most drugs or other foreign chemicals entering the mammalian organism are chemically altered. In many instances, changes are effected in a biphasic process. Phase I consists of oxidation, reduction, and hydrolysis reactions, while Phase II involves conjugative reactions (sometimes known as synthetic reactions) between the drug (or metabolite) and common body constituents. These processes are diagrammatically represented in Scheme I. Through biotransformations, drugs are generally converted into more polar compounds which can be readily excreted (primarily *via* the kidney) from the organism. Thus, biotransformation reactions may act as mechanisms for terminating the pharmacological activity of drug substances. Indeed, it has been suggested that in lieu of metabolic transformations, certain drug entities might have biological lifetimes far exceeding those of most animal organisms (21).

In general, biotransformations lead to chemical entities possessing less biological activity and/or toxicity; however, there are exceptions. Particularly with Phase I reactions, cases are now well documented wherein metabolites possessing enhanced activities are produced.



Scheme I—Biotransformations of drugs



Scheme II—Parallel versus *sequential biotransformations of drugs*

Study of the metabolic fate of foreign compounds is complicated by the fact that parallel or sequential biotransformations may occur (Scheme II). Consequently, for some complex molecules (*e.g.*, phenothiazine tranquilizers), tens of metabolites can be formed in mammals (22).

OXIDATIVE TRANSFORMATIONS

Of the three Phase I reactions, oxidative biotransformations are quantitatively of greatest importance. A tremendous explosion has occurred over the past decade regarding the understanding of the mechanisms of oxidation of all types of compounds. In 1962, Hayaishi (23) paid great attention to the gross aspects of oxygen metabolism. Since that time, an understanding of the basic mechanisms of oxygen activation and of the physiological function of the oxygenases has increased (24). New classes of oxygen-activating enzymes appeared during this period, including cytochrome P-450 enzyme systems, flavoprotein oxygenases, and α-ketoglutarate-requiring oxygenases. A greater understanding of superoxide anion, superoxide dismutase, and superoxide-using enzymes also has been gained.

Most oxidative Phase I reactions are catalyzed by cytochrome P-450-linked monooxygenases (Table I), located primarily in the liver of mammals but also occurring in the kidneys, lungs, small intestine, spleen, and steroidogenic organs such as the adrenal cortex, testes, ovary, and placenta (8). Considerable effort has gone into unraveling the electron-transport chain associated with these rather nonspecific enzyme systems (8, 25, 26). Briefly, the oxidized form of cytochrome P-450 (Fe III) complexes with substrate, apparently at a site near the iron atom of the heme protein. The resulting complex undergoes rapid reduction *via* cytochrome P-450 reductase (Fe III reduced to Fe II). This reduction is followed by formation of a ternary reduced cytochrome–substrate–molecular oxygen complex.

After reduction of the oxygen by the cytochrome iron atom (Fe II → Fe III) and further reduction by another electron from an unknown source, the resulting complex internally disproportionates with the transfer of one oxygen to bound substrate and acceptance of two protons by the second oxygen atom. Finally, the complex dissociates into water, oxidized substrate, and oxidized cytochrome P-450. This mechanism (Scheme III) is important because of its apparent similarity to monooxygenases occurring in microorganisms (*vide infra*).

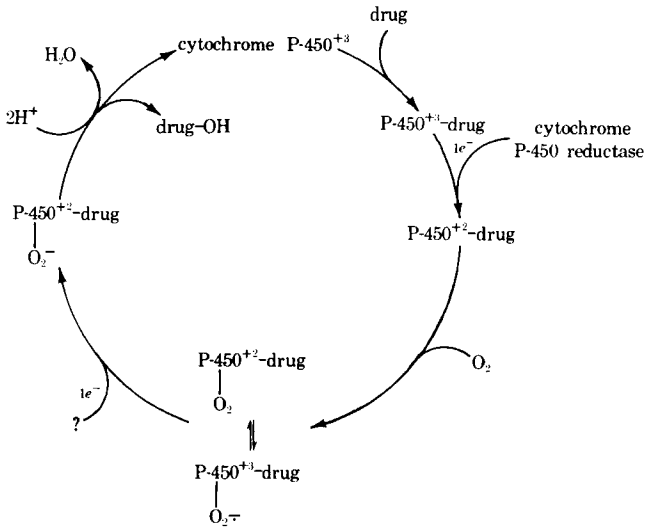
Cytochrome P-450 monooxygenases are believed to mediate aliphatic and aromatic hydroxylations and *N*-, *O*-, and S-dealkylations in mammals. In general, they can be visualized as mediating the attack of sub-

Table I—Mammalian Oxidative Biotransformations

Reaction Type	Substrate	Intermediate	Product
Hydroxylation	Aliphatic and alicyclic compounds	—	Alcohols
	Aromatic compounds	Arene oxides	Phenols
N-Dealkylation	Secondary and tertiary amines	$HOCHRNR_2'$	Aldehyde plus primary or secondary amine
O-Dealkylation	Alkyl–alkyl and alkyl–aryl ethers	HOCHROR'	Aldehyde plus alcohol or phenol
S-Dealkylation	Alkyl–alkyl and alkyl–aryl sulfides	HOCHRSR'	Aldehyde plus thiol or thiophenol
S-Oxidation	Sulfides	—	Sulfoxide
	Sulfoxide	—	Sulfone
N-Oxidation	Primary and secondary amines and amides	—	Hydroxylamine and *N*-hydroxyamides
	Tertiary amines	—	*N*-Oxides
Deamination	Primary and secondary amines	RCHNHR' OH	Ammonia and primary amine plus aldehyde
Dehydrogenation	Primary alcohol	—	Aldehyde
	Secondary alcohol	—	Ketone
	Aldehyde	—	Carboxylic acid
	trans-Dihydrodiol	—	Catechol

strate by an electron-deficient oxygen atom; in most instances, this results in the formation of unstable intermediates that rearrange to the metabolites indicated in Table I. In the case of aliphatic hydroxylations, this process can be thought of as an insertion reaction; in aromatic hydroxylations, arene oxides primarily occur as intermediates (27, 28). In most cases, the hydroxyalkyl intermediates formed during *N*-, *O*-, and S-dealkylations are so unstable that they spontaneously decompose to their corresponding dealkylated metabolites.

S-Oxidation, *N*-hydroxylation (primary and secondary amines), *N*-oxide formation (tertiary amines), and deaminations (primary amines) are mammalian biotransformations (Table I) that occur in a manner similar to that already described. However, these reactions probably involve enzymes other than cytochrome P-450 monooxygenases. All of the oxidative reactions, except oxidative deamination, that occur in mammalian tissues can be duplicated *in vitro* with so-called microsomal fractions (1). The latter arise as microglobular bodies, formed (as artifacts) during disruption of the endoplasmic reticulum of cells.



Scheme III—Oxidation of drugs via cytochrome P-450-linked monooxygenase

Alcohols are oxidized to aldehydes and ketones by a number of oxidoreductases or alcohol dehydrogenases. In this regard, the enzyme of principal importance is (the soluble) liver alcohol dehydrogenase. The coenzyme for this transformation is NAD; the stoichiometry of the reversible reaction involved is shown in Scheme IV.

$$NAD + \text{alcohol} \rightleftharpoons NADH + \text{ketone or aldehyde} + H^+$$

Scheme IV

Because of proton formation, the carbonyl product is favored at higher pH's (optimum = 10). *In vivo*, the conversion of alcohols to aldehydes is favored due to subsequent facile conversion to readily excreted carboxylic acids. Aldehydes are converted to carboxylic acids by a series of soluble enzymes, including aldehyde dehydrogenases, aldehyde oxidase, and xanthine oxidase.

Oxidative deamination of naturally occurring amines is catalyzed by mitochondrial amine oxidases (principally monoamine oxidase). Monoamine oxidase also attacks various exogenous amines.

Enzymes associated with oxidative transformation of xenobiotics appear to exhibit a low degree of substrate specificity; that is, practically all classes of compounds serve as substrates. Within classes of compounds, however, certain directional influences seem to play a major role in determining what position becomes oxidized. For instance, with a given aromatic hydrocarbon, a preponderance of one phenolic metabolite over another is often observed. Such observations pose a dilemma in that it is difficult to discern whether specificities regarding sites of oxidation are qualitative (involving different enzymes) or quantitative (involving different amounts of enzymes) manifestations of enzyme activity. Although considerable research is required to answer such questions, certain generalizations may be noted for given types of oxidative biotransformations.

As a convenient means of representing parallels between microbial and mammalian biotransformations, various reactions that appear to be important

in the metabolism of drug substances will be discussed.

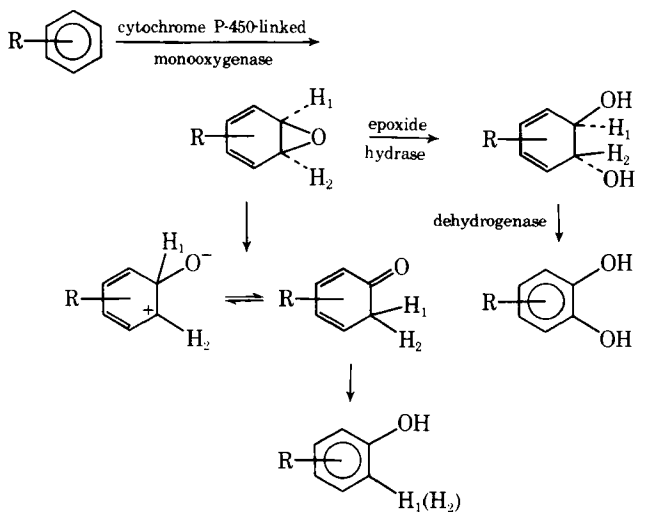
Aromatic Hydroxylations—Aromatic compounds are often converted to phenols in mammalian systems. Patterns of hydroxylations frequently follow the rules of electrophilic substitution reactions. Activated (electron-rich) aromatic rings are readily hydroxylated, whereas deactivated (electron-deficient) aromatic rings are hydroxylated slowly or not at all. Furthermore, hydroxylation occurs at positions predicted by concepts of electrophilic substitution; activated rings [*e.g.*, aniline and anisole (27–30)] are hydroxylated in *ortho*- and *para*-positions, while certain deactivated rings [*e.g.*, benzoic acid and benzamide (30)] are hydroxylated in the *meta*-position. The proportions of *ortho*- to *para*-hydroxylated products generally favor the latter; as expected, in di- and trisubstituted systems, hydroxylation is directed to positions predicted by a summation of substituent effects (29, 31).

Aromatic hydroxylation can be viewed as proceeding by a number of different mechanisms. However, most data are consistent with an oxenoid mechanism involving the intermediacy of an arene oxide and the formation of phenolic metabolites *via* nonenzymatic rearrangement (32). This process is usually accompanied by the so-called NIH shift (27, 28, 33–35) or retention of a substituent at the site of hydroxylation by rearrangement to an adjacent carbon (Scheme V).

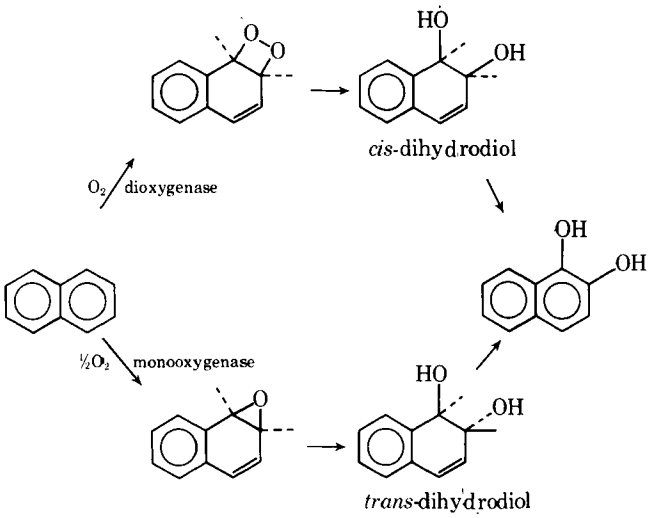
The same enzymes that form arene oxides from aromatic compounds seem to be capable of forming epoxides from isolated and conjugated olefins (32).

Catechols and their conjugates are important metabolites of certain organic compounds [*e.g.*, chlorobenzene (36)]. These metabolites appear to be formed through the action of soluble hepatic dehydrogenases on dihydrodiols rather than by hydroxylation of monophenols (37).

In many biological systems, cytochrome P-450 monooxygenases appear to be linked to epoxyhydrases. Thus, where more stable arene oxides are formed [*e.g.*, naphthalene-1,2-oxide (33, 38)], these may be converted to *trans*-dihydrodiols. When "stable"



Scheme V—Mammalian oxidative biotransformations of aromatic compounds



Scheme VI—Effect of dioxygenase versus *monooxygenase enzymes on naphthalene*

arene oxides are formed, they may participate in alkylation reactions with cellular components. Such processes may be important with respect to the toxicity and/or carcinogenicity of certain aromatic compounds (27, 28).

Lack of a primary isotope effect in enzymatic phenol formation (*e.g.*, deuterated aromatic substrates), demonstration of the NIH shift, and *trans*-dihydrodiol formation are becoming recognized as *prima facie* evidence for the presence of oxenoid monooxygenase activity (27, 28, 39). Such evidence has been useful in differentiating monooxygenases from dioxygenases in microorganisms.

In bacteria, aromatic compounds were traditionally thought to be degraded solely by dioxygenase pathways (40–43) (Scheme VI). A substrate such as naphthalene is converted through the hypothetical cyclic peroxide intermediate to a dihydrodiol and, ultimately, to the catechol derivative. These catechols are further degraded to carbon dioxide and water in bacteria. Jerina *et al.* (44) demonstrated that the reaction was dioxygenase mediated by isolating the *cis*-dihydrodiol of naphthalene, which was characterized by NMR spectral analysis. Prior to this time, a *trans*-dihydrodiol intermediate had been suggested as being predominantly involved in the degradation of naphthalene and other aromatic compounds in microorganisms (44–49). In contrast to this mechanism in microbiological systems, mammalian systems form *trans*-dihydrodiol intermediates *via* a monooxygenase oxidation step; the resulting arene oxide is opened by enzymatic hydration (33, 50, 51).

More recent data indicate that microorganisms may oxidize aromatic compounds like naphthalene through the formation of arene oxide intermediates similar to those obtained in the mammalian system. Cytochrome P-450 enzyme systems have been characterized in *Pseudomonas* species (52), *Claviceps purpurea* (53), and yeasts (54). Additionally, reactions that apparently operate through arene oxides formed in cytochrome P-450 monooxygenase systems from liver occur in fungi (55, 56).

In 1971, Auret *et al.* (55) reported the first occur-

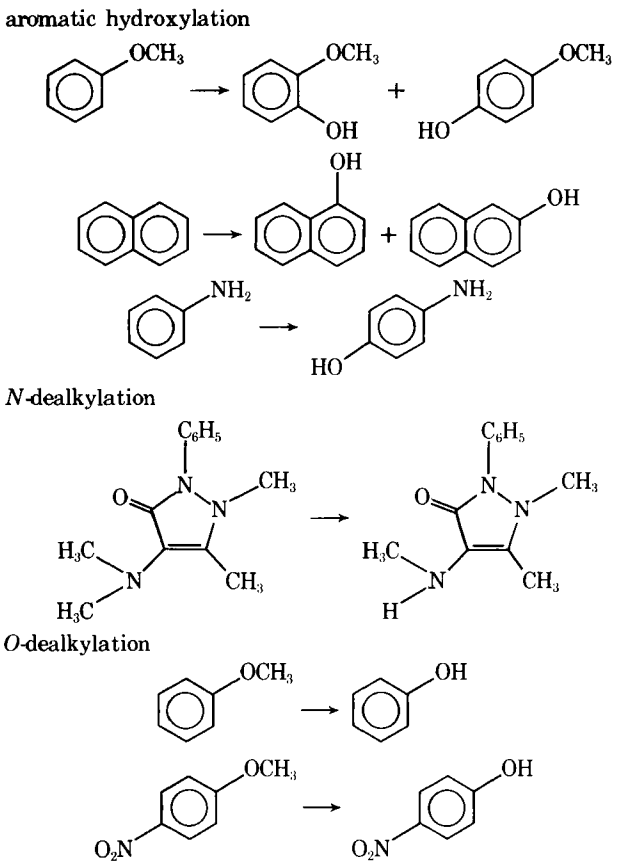
rences of the NIH shift in the hydroxylation of aromatic compounds by fungi. Both 4-D- and 2-D-anisoles were added as substrates to cultures of *Aspergillus niger, Sporotrichum sulfurescens, Cunninghamella elegans, Rhizopus arrhizus, Helicostylum piriforme, Mucor parasiticus, Rhizopus stolonifer, Curvularia falcata,* and *Cunninghamella bainieri,* which displayed the NIH shift during aromatic hydroxylations (55, 56). For *p*-anisole, 69–72% migration and retention of deuterium were observed in the 4-hydroxylated product; 35–57% migration and retention were observed with 2-hydroxylation. The magnitude of the NIH shift observed was consistent with that observed in hydroxylations of anisole by hepatic microsomes (57, 58).

Hydroxylations of these anisole derivatives and other substrates were rationalized on the basis of the involvement of arene oxide intermediates which ultimately gave the phenolic products. Several interesting observations were made on the data obtained in these early experiments:

1. Aryl oxidase enzymes of fungi appear to be similar to monooxygenases of hepatic microsomes, since both show the NIH shift and both have broad substrate specificities.
2. *ortho*-Hydroxylation appears to be predominant in fungi, while *para*-hydroxylation occurs to a greater extent in hepatic systems.
3. Polar substrates seem to oxidize more readily than nonpolar materials in fungi, while the reverse appears to be true in hepatic systems.
4. Arene oxide isomerases similar to those found in plant systems apparently are absent in fungal species.

The importance of this work has been underlined through studies by Ferris *et al.* (56) on the monooxygenase enzyme system in *C. bainieri.* When resting cells were used, monooxygenase activity nearly paralleling that seen in hepatic monooxygenase systems was observed. Naphthalene was converted into a *trans*-dihydrodiol, demonstrating the presence of the enzyme epoxide hydrase. Furthermore, a mixture of α- and β-naphthols was obtained in the ratio of 9:1; this result was very close to that obtained with *in vivo* and *in vitro* mammalian systems (33). Both 2- and 4-hydroxylations of anisole as well as *O*-dealkylation, were observed with this microorganism; however, the ratio of these products was different than that reported in mammalian systems (29). Other transformations reported included *N*-demethylation of aminopyrine, *O*-dealkylation of *p*-nitroanisole, and 4-hydroxylation of aniline (Scheme VII). Ferris *et al.* (56) observed that no other fungal or bacterial system had ever exhibited such a close metabolic parallelism to the drug-metabolizing system of liver microsomes.

In this work, 4-hydroxylation greatly exceeded 2-hydroxylation of anisole. Thus, the conclusion reached by Auret *et al.* (55) that *ortho*-hydroxylation predominates in fungal systems may be faulty, or differences in results obtained by the two groups of workers (55, 56) may be related to the experimental conditions employed by each.



Scheme VII—Transformations affected by C. bainieri (*Ref. 56*)

All previous results that pointed to the similarities in microbial and mammalian enzyme systems represented a foundation for establishing microbial models of mammalian metabolism. Our attention was initially focused on aromatic hydroxylation to test the feasibility of this proposal for three reasons: (*a*) it represents a commonly occurring mammalian biotransformation; (*b*) aryl hydroxylase activity similar to that displayed by mammalian cytochrome P-450 had been demonstrated in several microbial species (53, 55, 56); and (*c*) phenol formation, vis-à-vis arene oxide intermediates, had been implicated in mechanisms of toxicity of certain aromatic compounds (27, 28, 60).

A literature survey indicated that a number of microorganisms possessed aromatic hydroxylating activity (18, 55, 56); 11 microorganisms [*A. niger* (ATCC[1] 9142), *Penicillium chrysogenum* (ATCC[1] 10002), *Cunninghamella blakesleeana* (ATCC[1] 8688a), *Aspergillus ochraceous* (ATCC[1] 1008), *Gliocladium deliquescens* (1086), *Streptomyces* species (1158w), *R. stolonifer* (NRRL[2] 1477), *Curvularia lunata* (NRRL[2] 2178), *Streptomyces rimosus* (ATCC[1] 23955), *C. bainieri* (ATCC[1] 9244), and *H. piriforme* (QM[3] 6945)] were selected as microbial models of mammalian aromatic hydroxylation (9). A selection

[1] ATCC = American Type Culture Collection, Rockville, Md.
[2] NRRL = Northern Regional Research Laboratories, ARS, U.S. Department of Agriculture, Peoria, Ill.
[3] QM = Quartermaster Culture Collection, U.S. Army Laboratories, Natick, Mass.

Table II—Metabolites Formed by Microorganisms

Substrate	Metabolite	Organism(s) Causing Transformation
Acetanilide	2-Hydroxyacetanilide	*A. ochraceous*
	Aniline	*A. niger, Pen. chrysogenum, A. ochraceous, G. deliquescens,* and *S. rimosus*
Acronycine	9-Hydroxy-, 11-hydroxy-, and 9,11-dihydroxy-acronycines	*A. niger, C. blakesleeana, S. rimosus,* and *C. bainieri*
Aniline	Acetanilide	*C. blakesleeana* and *C. bainieri*
	2-Hydroxyacetanilide	*C. blakesleeana, A. ochraceous,* and *C. bainieri*
	4-Hydroxyaniline	*C. bainieri*
Anisole	2-Hydroxyanisole	*C. blakesleeana, A. ochraceous, G. deliquescens, Streptomyces* sp., *R. stolonifer, C. bainieri,* and *H. piriforme*
	4-Hydroxyanisole	*Pen. chrysogenum, C. blakesleeana, A. ochraceous,* and *C. bainieri*
	Phenol	*A. niger, C. blakesleeana, A. ochraceous, Streptomyces* sp., *R. stolonifer, C. bainieri,* and *H. piriforme*
	Hydroquinone	*A. ochraceous*
Benzene	Phenol	*Pen. chrysogenum, C. blakesleeana, G. deliquescens, Streptomyces* sp., and *C. bainieri*
Benzoic acid	2-Hydroxybenzoic acid	*A. niger* and *C. bainieri*
	4-Hydroxybenzoic acid	*Cur. lunata* and *C. bainieri*
	3,4-Dihydroxybenzoic acid	*A. ochraceous*
Biphenyl	2-Hydroxybiphenyl	*A. niger, S. rimosus,* and *H. piriforme*
	4-Hydroxybiphenyl	*A. niger, C. blakesleeana, Streptomyces* sp., *Cur. lunata, C. bainieri,* and *H. piriforme*
	4,4′-Dihydroxybiphenyl	*C. blakesleeana* and *C. bainieri*
Chlorobenzene	2-Hydroxychlorobenzene	*A. ochraceous, G. deliquescens, Streptomyces* sp., *R. stolonifer,* and *C. bainieri*
	4-Hydroxychlorobenzene	*A. ochraceous* and *C. bainieri*
Coumarin	7-Hydroxycoumarin	*G. deliquescens, Streptomyces* sp., *C. bainieri,* and *H. piriforme*
Naphthalene	1-Hydroxynaphthalene	*A. niger, C. blakesleeana, A. ochraceous, Streptomyces* sp. *stolonifer, C. bainieri,* and *H. piriforme*
	2-Hydroxynaphthalene	*A. niger, C. bainieri,* and *H. piriforme*
trans-Stilbene	*trans*-4-Hydroxystilbene	*A. niger, C. blakesleeana, A. ochraceous, Streptomyces* sp., *R. stolonifer,* and *C. bainieri*
	trans-4,4′-Dihydroxy-stilbene	*Streptomyces* sp. and *C. bainieri*
Toluene	2-Hydroxytoluene	*Pen. chrysogenum, A. ochraceous, Streptomyces* sp., *R. stolonifer, C. bainieri,* and *H. piriforme*
	4-Hydroxytoluene	*Pen. chrysogenum, R. stolonifer,* and *C. bainieri*

of aromatic compounds was chosen to be incubated with growing cultures of each microorganism. These included acetanilide, acronycine, aniline, anisole, benzene, benzoic acid, biphenyl, chlorobenzene, coumarin, naphthalene, nitrobenzene, *trans*-stilbene, and toluene. Mammalian systems have been shown to hydroxylate each of these compounds, and this permitted a comparison of mammalian and microbial aromatic hydroxylase activities. Furthermore, these compounds represent the aromatic portions of numerous chemical entities of environmental importance and of drugs.

All of these compounds were incubated with growing cultures of the 11 microorganisms. The results of TLC analyses (61, 62) of the fermentations are summarized in Table II.

The microbial system demonstrated substantial capacity to hydroxylate simple substituted benzenoid compounds. 2- and 4-Hydroxylations were observed in all cases, except with acetanilide where the 2-hydroxyacetanilide product was obtained exclusively. In earlier reported work, Theriault and Longfield (63) showed a *Streptomyces* species to be capable of hydroxylating acetanilide exclusively in the 4-position, while one *Basidiomycete* hydroxylated acetanilide in the 2-position. Hydroxylation at either the 2- or 4-position almost exclusively was observed for microorganisms with anisole, benzoic acid, aniline, and chlorobenzene (9). The microbial metabolites obtained are compared to the mammalian metabolites reported from *in vitro* and *in vivo* metabolism studies in Table III. In general, a good correlation was obtained between the microbial and mammalian metabolizing systems. Nitrobenzene was the only simple substituted benzenoid compound that failed to yield hydroxylated products with the microbial system. The 2-, 3-, and 4-hydroxynitrobenzenes are obtained with mammalian systems.

Additional biotransformations occurred with these substrates. Five microorganisms demonstrated ami-

Table III—Comparison of Metabolites Formed by Microorganisms and Mammalian Systems

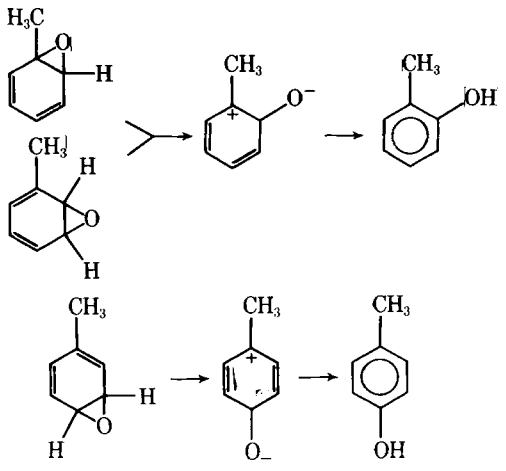
Substrate	Microbial Metabolite[a]	Mammalian Metabolite	
		In Vitro (Reference)	*In Vivo* (Reference)
Acetanilide	2-Hydroxyacetanilide and aniline	2-Hydroxyacetanilide, 4-hydroxyacetanilide, and aniline (57, 64)	4-Hydroxyacetanilide and aniline (65)
Acronycine	9-Hydroxy-, 11-hydroxy-, and 9,11-dihydroxyacronycines	Not reported	9-Hydroxy-, 11-hydroxy-, and 9,11-dihydroxyacronycines (66)
Aniline	Acetanilide, 2-hydroxyacetanilide, and 4-hydroxyaniline	4-Hydroxyaniline (57)	Acetanilide and 2-hydroxy-, 3-hydroxy-, and 4-hydroxyanilines (67)
Anisole	2- and 4-Hydroxyanisoles and phenol	2- and 4-hydroxyanisoles and phenol (64)	2- and 4-Hydroxyanisoles (68)
Benzene	Phenol	Phenol (57)	Phenol (69)
Benzoic acid	2- and 4-Hydroxybenzoic acids and 3,4-dihydroxybenzoic acid	3-Hydroxybenzoic acid (57)	2-, 3-, and 4-Hydroxybenzoic acids (71)
Biphenyl	2- and 4-Hydroxybiphenyls and 4,4′-dihydroxybiphenyl	2- and 4-Hydroxybiphenyls (57, 70)	4-Hydroxy-, 3,4-dihydroxy-, and 4,4′-dihydroxybiphenyls (72)
Chlorobenzene	2- and 4-Hydroxychlorobenzenes	2-, 3-, and 4-Hydroxychlorobenzenes (57)	2-, 3-, and 4-Hydroxychlorobenzenes (73)
Coumarin	7-Hydroxycoumarin	7-Hydroxycoumarin (74)	3- and 7-Hydroxycoumarins [4-, 5-, 6-, and 8-hydroxycoumarins[b] (75, 76)]
Naphthalene	1- and 2-Hydroxynaphthalenes	1- and 2-Hydroxynaphthalenes (33, 59)	1- and 2-Hydroxynaphthalenes (77)
Nitrobenzene	—	4-Hydroxynitrobenzene (57)	2-, 3-, and 4-Hydroxynitrobenzenes and 2-, 3-, and 4-hydroxyanilines (78, 79)
trans-Stilbene	*trans*-4-Hydroxy- and *trans*-4,4′-dihydroxystilbenes	*trans*-4-Hydroxystilbene and *trans*-4,4′-dihydroxystilbene (80)	*trans*-4-Hydroxy-, *trans*-3,4-dihydroxy-[c], and *trans*-4,4′-dihydroxystilbenes (80)
Toluene	2- and 4-Hydroxytoluenes	2- and 4-Hydroxytoluenes and benzyl alcohol (57)	Benzoic acid and conjugates (67)

[a] See Ref. 9. [b] Formed in trace quantities. [c] Presumed metabolite based on characterization of mono-*O*-methyl ethers.

dase activity in removing the acetyl group from acetanilide, a minor mammalian biotransformation. Acetylase activity was observed in four microorganisms with aniline; this pathway is common in mammals. *A. ochraceous* gave good yields of 2-hydroxyacetanilide when incubated with acetanilide as the substrate. In this microorganism, acetylation apparently must precede hydroxylation. This requirement for an amide functional group in the hydroxylation of various substrates was reported by Johnson *et al.* (81) and Fonken *et al.* (82), who have observed that in an extensive series of carbocyclic amines and nitrogen heterocycles an acyl substituent is required on the nitrogen atom for hydroxylation to occur.

With anisole, seven microorganisms caused *O*-demethylation. This reaction was common and occurred with cultures that caused 2-hydroxylation as well. Our results were consistent with those observed by Auret *et al.* (55). *O*-Demethylase activity was reported before, particularly with certain *Pseudomonads* (83, 84) and with *A. niger* (85). With our culture of *A. niger* (9), *O*-demethylase activity was dominant; phenol was the only metabolite of anisole detected. With *A. ochraceous*, greater than 50% conversion of benzoic acid to 3,4-dihydroxybenzoic acid was obtained (9). Microorganisms convert benzoic acid into various hydroxylated products (85–87); in fungi, 3,4-dihydroxybenzoic acid is commonly produced with 3- or 4-hydroxybenzoate serving as intermediates (86, 87).

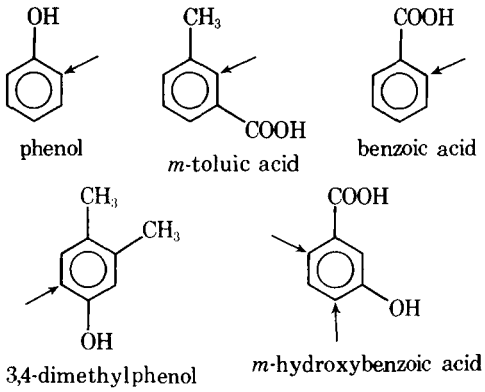
Another interesting, albeit somewhat hidden, result was obtained with the microbial system of Smith and Rosazza (9). Although the number of microorganisms in the study was insufficient to establish a complete trend, ring-activating groups generally resulted in a greater number of microorganisms giving hydroxylated products and provided higher yields. As expected, the products obtained were hydroxylated *ortho* and *para* to ring substituents, a result that may be predicted by considering a composite of the various cationic intermediate forms of possible epoxide ring openings as shown in Scheme VIII (27). The relative reactivities of simple benzenoid compounds (R—C_6H_5) toward microbial hydroxylases follow the order R = OCH_3 > NH_2, CH_3 > H, Cl, $NHCOCH_3$ > NO_2 (9). The highest yields and inci-



Scheme VIII—Conceptualized cationic intermediates associated with ring openings of arene oxides

dences of microbial hydroxylation occurred with anisole, while no hydroxylation was obtained with nitrobenzene as the substrate. Benzoic acid was eliminated from the sequence because it is a more natural substrate for microorganisms.

Omori and Yamada (88) related the position of hydroxylation of several simple aromatic compounds to their electronic structures. The π-electron distributions of various aromatic compounds selected from the literature were calculated using the assumption that microbiological hydroxylation occurs *via* a radial type of mechanism. With phenol, *m*-toluic acid, benzoic acid, 3,4-dimethylphenol, and *m*-hydroxybenzoic acid, good correlation was obtained between the position of hydroxylation [shown by arrows (88)] and the



points of highest calculated frontier electron densities. This work represented a purely theoretical approach to the problem of predicting sites of hydroxylation since no experimental verification of the results was reported. Furthermore, the simple compounds studied render themselves quite readily to simple first-order predictions regarding sites of oxygenation. A more vigorous application of this approach would involve complicated substrates which, after predicting sites of hydroxylation, could be incubated with selected microorganisms.

Excellent correlation between the metabolites obtained in the microbial system of Smith and Rosazza (9) and mammalian *in vitro* and *in vivo* systems was observed with more complicated aromatic compounds such as acronycine, biphenyl, coumarin, naphthalene, and *trans*-stilbene (Table III). A somewhat stronger parallel existed between the *in vitro* mammalian studies and the microbial model system with biphenyl, chlorobenzene, coumarin, and *trans*-stilbene. Table III shows some of the discrepancies in results obtained in *in vitro* and *in vivo* metabolic studies. These findings indicate that intercomparison between *in vivo* and *in vitro* metabolic systems is not always valid and, in fact, may be no more valid than comparisons of metabolic profiles of microorganisms and liver systems.

The production of 9-hydroxyacronycine as a microbial metabolite by *Cunninghamella echinulata* serves as an excellent example of the potential for obtaining gram quantities of difficult-to-synthesize compounds *via* microbial aromatic hydroxylation (61). Acronycine is an antitumor compound, and its metabolism has been studied in humans, rats, pigs, dogs, and mice (66). The major metabolite produced in mammalian systems is 9-hydroxyacronycine, although minor amounts of other metabolites are also observed. In the microbial system (9), acronycine was hydroxylated by four microorganisms in the 9-position. Other metabolites also were detected in small-scale fermentation screening studies (9, 61). In a scaled-up fermentation, 4 g of acronycine was added as the substrate to 7.5 liters of a fully grown culture of *C. echinulata;* a 31% yield of 9-hydroxyacronycine was isolated and fully characterized (61).

Other examples of the use of microbial transformations for the production of hydroxylated metabolites are 5-anilino-1,2,3,4-thiatriazoles (89), fenclozic acid (90), 5-hydroxytryptophol (17), α-methylfluorene-2-acetic acid (91), and levodopa (18).

Mammalian *N*-, *O*-, and S-Dealkylations—Hydroxylation of sp^3 carbon atoms alpha to a nitrogen, oxygen, or sulfur atom generally leads to dealkylation due to the instability and subsequent rearrangement of the C—OH intermediates. The *N*-, *O*-, and S-dealkylations are important biotransformations, because they can significantly alter the lipid solubility and biological potency of drugs.

N-Dealkylation of numerous amines, amides, and imides has been described. Tertiary amines containing dimethylamino functions are often rapidly *N*-demethylated to their corresponding secondary amines, while the latter are much more slowly transformed into primary amines (92). This finding is in accord with the report that *N*-demethylation rates are directly proportional to lipid solubility and inversely proportional to pKa values (93).

The rate of *N*-dealkylation generally seems to decrease as the size and/or complexity of the *N*-alkyl groups increase (94–97). However, Abdel-Monem and Portoghese (98) found that the degree of *N*-dealkylation increased in a series of *N*-substituted normeperidines, going from methyl to ethyl to *n*-propyl, and then progressively decreased with homologs from *n*-butyl to *n*-nonyl. Because of the lack of an α-hydrogen, *N*-*tert*-butyl groups should be resistant to *N*-dealkylation. However, Kamm and coworkers (99, 100) showed that, by a sequence of oxidation reactions (beginning with hydroxylation of the C-methyl group), this alkyl function can be removed *in vivo* and *in vitro*. *N*-De-*tert*-butylation also emphasizes the fact that C-oxidation probably becomes more important as the size of the *N*-alkyl function increases.

A significant degree of stereospecificity has been shown in the *N*-dealkylation of several enantiomeric pairs of drugs. In light of the fact that biological activity often resides in one enantiomer, this observation deserves further attention (101).

Axelrod (102) first reported that *O*-alkyl functions were cleaved in a similar fashion to *N*-alkyl groups. In addition, *para*-substituted ethers are more readily cleaved than their corresponding *ortho*- or *meta*-isomers (102). In 22 *p*-nitrophenyl ethers, the rate of *O*-dealkylation decreased as the size and/or complexity of the *O*-alkyl function increased (103). Creaven *et al.* (104) found a similar relationship for a homologous series of alkoxy biphenyls, although they noted

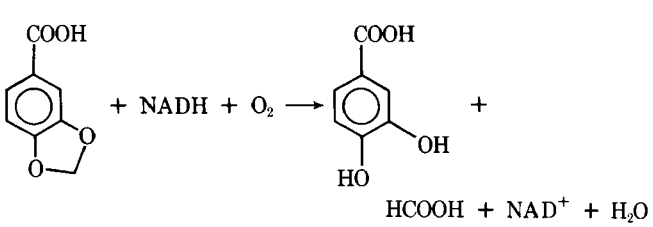
an increase in *O*-dealkylation from methyl to ethyl. Tsukamoto *et al.* (105) reported that *n*-butyl-*p*-nitrophenyl ethers were slowly *O*-dealkylated while ω-1 hydroxylation of the *n*-butyl function predominated in rabbits. The dimethylaminoethyl ether function, often incorporated into drug substances, appears to be quite resistant to *O*-dealkylation *in vitro* and *in vivo* in rats (103).

S-Dealkylation has not been extensively studied, primarily because alkyl–aryl and alkyl–alkyl thioethers are not widely used in drugs. Sulfides embodied in heteroaromatic systems are fairly prevalent among drug entities but are more prone to S-oxidation. The few reported studies indicate that S-demethylation proceeds by a similar mechanism to *N*- and *O*-dealkylation in intact animals and with hepatic microsomal systems (92).

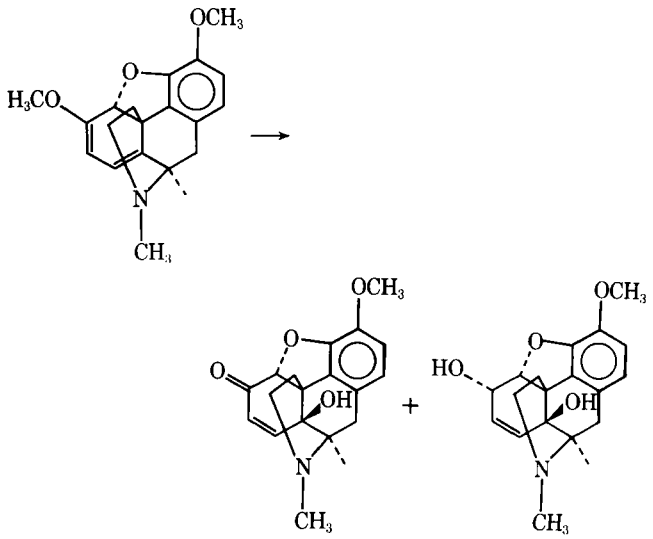
Microbial *O*-Dealkylations—The biodegradation of *O*-alkyl aromatic ethers in bacteria is a monooxygenase-mediated process requiring pyridine nucleotides and oxygen (84). In general, an active form of oxygen attacks the α-carbon of alkyl ethers; the former alkyl group, which exists as an unstable hemiacetal, is easily removed.

Bernhardt *et al.* (84) prepared a cell-free extract and a purified enzyme system from *Pseudomonas putida* capable of *O*-demethylating 4-methoxybenzoate. The purified enzyme system consisted of an iron-flavoprotein and an iron-sulfur protein and was obtained from *P. putida* cells grown on 4-methoxybenzoic acid as the sole carbon source. NADH and oxygen represented cofactor requirements for this enzyme. The purified system acted on many different substrates, but the greatest affinity was observed for 4-methoxybenzoate itself. *para*-Substituted benzoic acid derivatives were the best substrates, while *m*- and *o*-benzoic acids were only slowly demethylated. In this microbial enzyme system, the carboxyl group was an absolute requirement for *O*-demethylation.

Methylenedioxy compounds, which are represented in many natural products, are also converted to their respective phenolic products by *Pseudomonas* species (83). Demethylenation has been reported to occur in NADPH-dependent monooxygenases of rat liver (106). The essential features of the reaction are shown in Scheme IX. Each reaction product was characterized. The catabolism of piperonylic acid by *Pseudomonas fluorescens* appears to be an example of cooxidation in that the methylenedioxy compound did not serve as the sole carbon and energy source and was only degraded when 4-methoxybenzoate (anisic acid) was present. *O*-Dealkylation of various simple methoxylated compounds such as 3-hydroxy-



Scheme IX—Microbial O*-demethylenation of 3,4-methylenedioxybenzoic acid by* Pseudomonas *species (Ref. 83)*



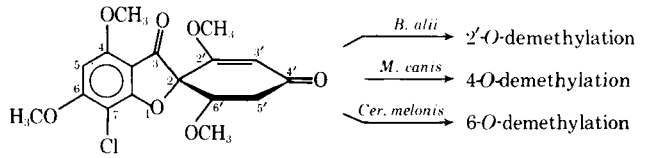
Scheme X—Microbial transformations of thebaine (Refs. 110 and 111)

4-methoxybenzoate (107), anisole (9, 56, 108), and methyl anisate (109) also has been reported in fungi.

Several more complicated examples of *O*-dealkylation have been reported. The alkaloid thebaine undergoes a series of reactions in which the methoxy group is lost (Scheme X). The mechanism by which this reaction occurs is obscure and may involve concerted elimination of the methyl group as a result of hydroxylation (110, 111). The alkaloid aconitine obtained from *Aconitum napellus* (Wolfbane) is also *O*-demethylated by *Streptomyces paucisporogenes* to a compound that is less toxic than the parent alkaloid (112). Boothroyd *et al.* (113) studied the demethylation of griseofulvin by fungi. Several microorganisms were screened for *O*-dealkylase activity; four of these, *Microsporum canis, Alternaria solani, Botrytis alii,* and *Cercosporum melonis,* could metabolize griseofulvin to phenolic products. Each of the three methoxy groups on the griseofulvin molecule could be selectively removed by a different microorganism (Scheme XI). Such selective *O*-demethylase activity has great synthetic utility.

An unusual example of the reverse reaction, *O*-alkylation, was reported (114). With *Lentinus lepideus* surface cultures, *p*-hydroxycinnamic acid was converted into 4-methoxycinnamate in the presence of methionine. This result was confirmed by using ^{14}C-labeled methionine. The methoxy group formed was cleaved by the same fungus when prolonged incubations were conducted.

We have conducted several experiments on microbial *O*-dealkylations, predominantly with methoxylated aporphine and benzylisoquinoline derivatives

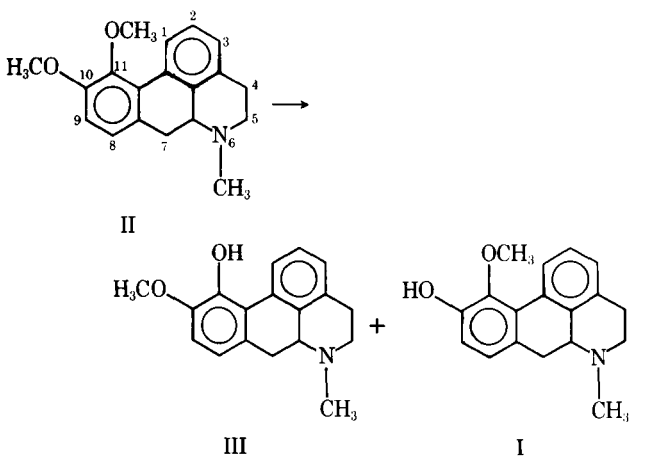


Scheme XI—Microbial O*-demethylation of griseofulvin (Ref. 113)*

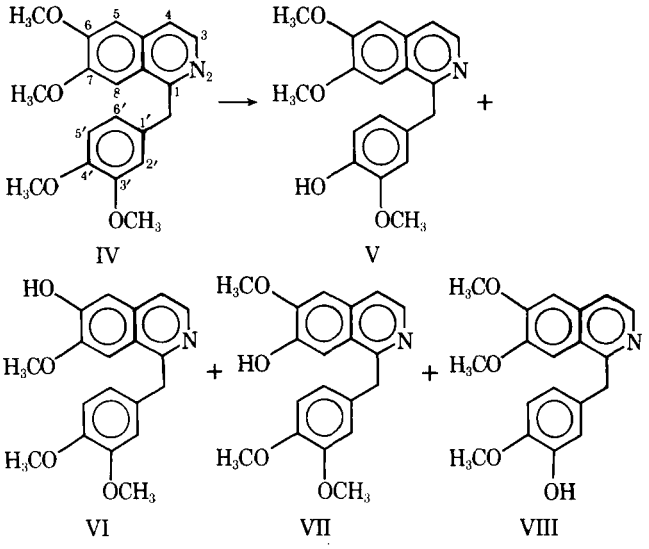
(115). Cannon *et al.* (116) studied the synthesis of the isomeric monomethyl metabolites of apomorphine and metabolism of apomorphine in a catechol-*O*-methyltransferase liver preparation. Considerable difficulty was encountered in the synthesis of one of the possible monomethyl ether isomeric metabolites, isoapocodeine (I). In preliminary experiments, we demonstrated that a *Streptomyces* species converts 10,11-dimethoxyaporphine (II) into an equal mixture of apocodeine (III) and I in about 50% estimated yield (Scheme XII). This process occurs within 24 hr after the substrate is added to growing cultures of the microorganisms.

Two cultures of the microorganisms, *C. blakesleeana* and *C. echinulata,* yielded I as the predominant metabolite, with only traces of III being formed. These results are interesting in view of the results obtained by Cannon *et al.* (116), where the ratio of apocodeine–isoapocodeine obtained in the mammalian liver system was about 75:1. It was rationalized that the formation of isoapocodeine was prohibited due to the sterically hindered hydroxy group at position 11 (Scheme XII). One might expect the reverse reaction (*i.e.,* *O*-dealkylation at position 10) also to be influenced so that isoapocodeine would predominate. Our findings with *C. blakesleeana* and *C. echinulata* (115), which produced mostly isoapocodeine, support the result of Boothroyd *et al.* (113) that selective *O*-dealkylation may be achieved with different microorganisms.

We have also been studying the microbial metabolism of the drug papaverine (IV) (115). Papaverine has been used in treating cerebral and peripheral ischemia associated with arterial spasm (117). The metabolic disposition of papaverine in humans and other mammals has been studied (118). Negligible amounts of the drug are excreted unchanged in urine, indicating that it must be nearly completely metabolized. Only one compound, 4′-*O*-desmethylpapaverine (V), was chemically characterized in the mammalian studies (118). We are developing microbial systems capable of providing gram quantities of all possible *O*-dealkylated isomers, which will be used for structure elucidation and biological testing.



Scheme XII—Microbial O-demethylation of 10,11-dimethoxyaporphine



Scheme XIII—Microbial O-demethylation of papaverine

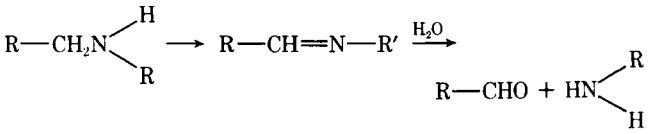
C. echinulata is capable of producing four phenolic metabolites of papaverine (115); two of these have been chromatographically identified as 6-hydroxy-7-methoxy-(3′,4′-dimethoxybenzyl)isoquinoline (VI) and 7-hydroxy-6-methoxy-(3′,4′-dimethoxybenzyl)-isoquinoline (VII) (Scheme XIII). The 3′-hydroxy-4′-methoxybenzyl-6,7-dimethoxyisoquinoline product (VIII) [or its 4′-hydroxy-3′-methoxy isomer (V)] is believed to be the sole product with *C. blakesleeana* (115). Again, this finding demonstrates the selectivity in *O*-dealkylation that may be achieved with microorganisms. Screening of microorganisms is being continued to detect other cultures that might selectively *O*-dealkylate similar systems.

Microbial *N*-Dealkylations—According to Large (119), three major types of pathways are involved in the *N*-dealkylation of amines by microorganisms (Scheme XIV). The oxidases may involve the intermediacy of hydrogen peroxide, while the oxygen of the aldehyde produced comes from water in both the oxidases and dehydrogenases. The third type of reaction is mediated by monooxygenases where molecular oxygen is the source of the oxygen in the aldehyde product.

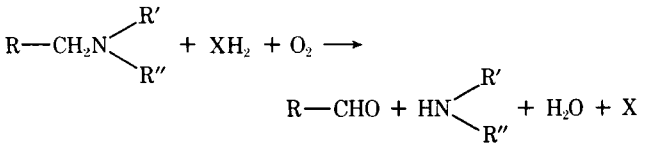
Secondary and tertiary amines are degraded by more than one pathway by bacterial monooxygenases. In *Pseudomonas aminovorans,* trimethylamine is converted into trimethylamine *N*-oxide by an NADPH-dependent tertiary amine monooxygenase enzyme (Scheme XV). The trimethylamine *N*-oxide is then nonoxidatively demethylated by trimethylamine *N*-oxide demethylase to formaldehyde and dimethylamine.

This latter enzyme has been isolated in two bacteria, *P. aminovorans* and a *Bacillus* species (120). This type of *N*-demethylation of an *N*-oxide is apparently not restricted to microbial systems but has been described in the metabolism of tertiary amines by liver microsomal systems (121). Similarities and differences between microsomal and bacterial amine monooxygenases have been compiled (119). The similarities include secondary amines converted to pri-

oxidases and dehydrogenases



monooxygenases

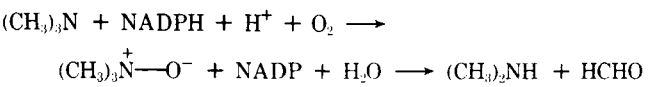


Scheme XIV—Types of microbial N*-dealkylations*

mary amines plus the appropriate aldehyde; tertiary amines converted to amine oxides; tertiary amine oxidation not inhibited by carbon monoxide, cyanide, or proadifen hydrochloride[4]; amine oxide enzymatically converted to secondary amine plus aldehyde; and NADH and NADPH serving as active electron donors.

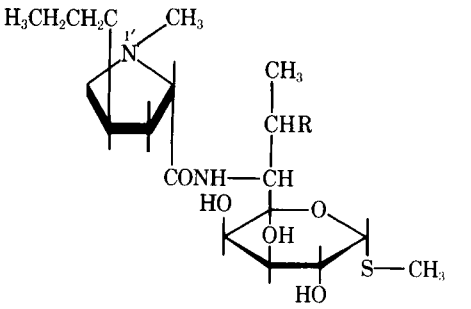
However, in the microsomal system, identical enzymes *N*-dealkylate secondary and tertiary amines, a very wide range of compounds are oxidized, and amine oxide demethylase is inhibited by carbon monoxide, proadifen hydrochloride, and pyridine but not cyanide. In contrast, in the bacterial system, different enzymes affect *N*-dealkylation of secondary and tertiary amines; active substrates must possess one of these structures: CH_3NHR, CH_3CH_2NHR, $(CH_3)_2NR$, or $(CH_3CH_2)_2NR$, where R = C_1 to C_4; and amine oxide demethylase of *P. aminovorans* is inhibited by cyanide but not carbon monoxide, proadiphen hydrochloride, or pyridine. Most importantly, substrate specificity of bacterial *N*-dealkylases appears to be narrower than those of mammalian systems. However, this has not yet been fully explored.

Microbial *N*-dealkylations have been observed in more complicated structures than those already described, although few studies have been conducted concerning the mechanism(s) of these reactions. Clindamycin (IX) and lincomycin (X) are related antibiotics metabolized by various *Streptomyces* species (122, 123). Argoudelis *et al.* (122) produced the 1′-demethylclindamycin compound when clindamycin was added to fermentations of *Streptomyces punipalus*. However, when the compound was metabolized by *Streptomyces armentosus*, primarily the clindamycin sulfoxide was obtained. Other species of microorganisms could convert clindamycin to either the sulfoxide or the 1′-demethylated product or a mixture of both (122). These transformations are significant because the latter two compounds are mammalian metabolites of clindamycin (124), while the *N*-demethylated product is about twice as active as the



Scheme XV—Dealkylation of trimethylamine by P. aminovorans

[4] SK&F 525-A.



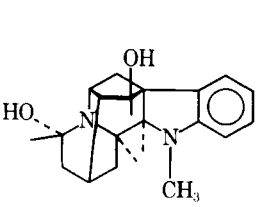
IX: R = Cl
X: R = OH

parent antibiotic. Lincomycin, a structurally related antibiotic, also undergoes *N*-demethylation and sulfoxidation (123). The product in the latter case, however, is approximately one-twentieth as active as the parent antibiotic.

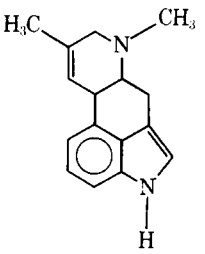
Various compounds that are either drugs or structurally related to classes of drugs are *N*-dealkylated by microorganisms. Bellet and Van Thuong (125) reported the *N*-dealkylation of the dihydroindole alkaloid ajmaline (XI) in 40% yields with a culture of *Streptomyces platensis*. *N*-Demethylation of agroclavine (XII) (126) and certain 6,14-*endo*-ethenotetrahydrothebaine alkaloids (such as XIII) (127) by *C. echinulata* has been reported. Mitscher *et al.* (127) commented on the parallel between the latter *N*-dealkylation and a similar biotransformation observed in mammalian systems.

Tropine (XIV), the basic nucleus of a number of anticholinergic drugs, is *N*-demethylated by *Arthrobacter atropini;* the tranquilizer diazepam (XV) is similarly transformed by *Pellicularia filamentosa* and in 12% yields by *Sp. sulfurescens* (12). This latter transformation again represents a parallel to mammalian metabolism.

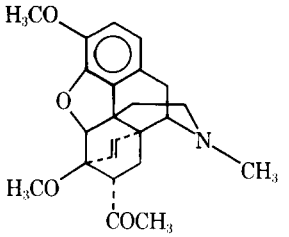
Methamphetamine is *N*-demethylated by the intestinal microflora of guinea pigs. Observing differences in recoveries of metabolites of methamphetamine (XVI) fed to animals, Caldwell and Hawksworth (128) concluded that microflora of the intestine could play a role in degrading the drug. The



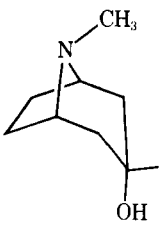
XI



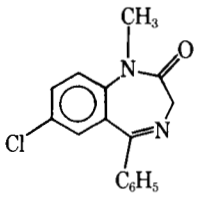
XII



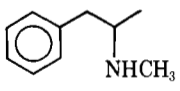
XIII



XIV



XV



XVI

cecal contents of Dunkin–Hartley guinea pigs were removed and incubated with (±)-2-methylamino-1-phenyl-1-^{14}C-propane hydrochloride. Twenty-nine percent of the dose was recovered unchanged while the *N*-dealkylated product, amphetamine, was recovered in 47% yield. An unknown product was also observed. Several genera of human gut bacteria were isolated and examined for their potential to metabolize methamphetamine. In particular, *Lactobacilli, Enterococci,* and *Clostridia* showed the ability to metabolize methamphetamine.

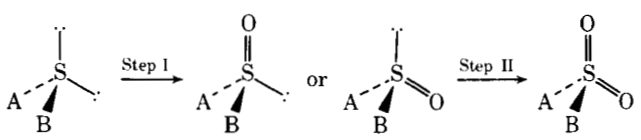
Little is known concerning microbial S-dealkylations. The reported conversion of the thioether group of lincomycin (X) to a hydroxy function by *Streptomyces lincolnensis* (123) might involve S-dealkylation.

S-Oxidations—Sulfides are metabolized to sulfoxides and sulfones in mammals (67) (Table I). No systematic studies have been performed on these biotransformations. The fact that certain sulfide-containing drugs could be converted to optically active sulfoxides (129) appears to have been overlooked. From work with model compounds, there is some reason to believe that low stereoselectivity will be observed with mammalian sulfoxidations (*vide infra*).

Microbiological sulfur oxidations recently have been studied. In general, the oxidation of thioethers (sulfides) proceeds stepwise through the sulfoxide (Step I) to the sulfone derivatives (Step II), as shown in Scheme XVI. In microorganisms, it is possible to stop the reaction at the sulfoxide level, obtaining only small amounts of the sulfone derivative. Two primary advantages exist in using microbiological systems in the oxidation of sulfur-containing compounds:

1. Selective oxidations are possible when other labile groups are found on the substrate molecules.
2. Stereospecificity is obtained to a variable extent, sometimes resulting in sulfoxide products with high degrees of optical purity (12).

One early example of microbial production of optically active sulfoxides was in the isolation of very low yields of (−)-biotin sulfoxide formed as a metabolite of biotin by *A. niger* (130). More recently, Auret *et al.* (131–133) conducted extensive studies related to the microbiological formation of optically pure sulfoxides. *A. niger* cultures and acetone powders obtained from this microorganism produce optically active sulfoxides when unsymmetrical thioethers are added as substrates to fermentations (131). Stereoselectivities ranging from 4 to 100% were observed, depending on the thioethers used as substrates. In the case of *tert*-butyl-*p*-tolyl sulfide, the *R*-(+)-sulfoxide was obtained with a higher optical rotation than ever before recorded.



Scheme XVI—Microbiological oxidation of sulfides

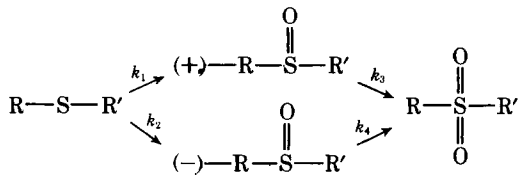
As indicated in Scheme XVI, the absolute configuration of products reflects a demand of various groups for either position A or B. A *p*-tolyl group, for example, prefers position A, while a *tert*-butyl prefers position B. When both substituents were present on the same substrate molecule, optical purities of 94% were obtained (24% yield). Interestingly, with *tert*-butyl-*p*-tolyl sulfide, acetone powders of *A. niger* cells yielded 98–100% optically pure sulfoxide in about 25% yield. With *p*-tolyl sulfides, stereoselectivity in forming sulfoxides depended on the degree of substitution of the α-carbon atom where it was found that alkyl = *tert*-butyl (99%), isopropyl (70%), and *n*-butyl (32%) (average optical purities in percent).

Auret *et al.* (132) extended this work by describing a process whereby optically pure sulfoxides could be obtained from racemic sulfoxide mixtures. When growing cultures of *A. niger* were used, both sulfoxide and sulfone products are obtained, whereas acetone powders seem to be capable of stopping the reaction at the sulfoxide stage. With growing cultures, it was suggested that optical activities observed with sulfoxides could arise partly by further selective oxidation of one or the other sulfoxide intermediate. This proposal is illustrated in Scheme XVII.

To check this hypothesis, racemic mixtures of various sulfoxides were added to growing cultures of *A. niger.* In some instances, the recovered sulfoxides were optically active (132). In addition to substituent effects and selective oxidation of sulfoxides, the influence of species and strain of microorganisms on stereoselectivity of sulfur oxidations was studied (133). Strains originating from the same parent culture yielded sulfoxide products of different configuration and in different yields. This result suggests the importance in reproducing in detail the experimental conditions reported in the literature with this and possibly other biotransformations.

Although a similarity in the pattern of products formed by the hydroxylation of aromatic compounds and other type of reactions by microorganisms and mammalian systems has been noted, stereoselectivity in the oxidation of thioethers to sulfoxide apparently is lower in animals (rat liver microsomes) than in fungal processes. Auret *et al.* (133) incubated *p*-tolyl benzyl sulfide with a rat liver microsomal preparation and obtained a sulfoxide only slightly enriched (1.3% optical purity) in the *R*-enantiomer.

Other examples of the microbial sulfoxidation of drug substances can be cited. Both lincomycin (X) and clindamycin (IX) may be converted into their respective sulfoxide derivatives. *S. lincolnensis* yields lincomycin sulfoxide and 1-demethylthio-1-hydroxylincomycin (123), while *S. armentosus* produces primarily clindamycin sulfoxide (122). *Calonectria de-*



Scheme XVII—Enantiomeric enrichment of sulfoxide racemates; optical purity enhanced when $k_3 > k_4$ *or* $k_4 > k_3$ *(Ref. 132)*

cora converts the steroidal thioether (XVII) into its optically active sulfoxide (134).

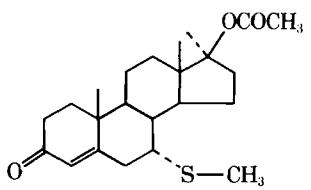
N-Oxidations—Since the *N*-oxidation products of numerous therapeutic agents possess enhanced toxicities, there is considerable interest in this biotransformation (135, 136). The instability and/or low incidence of *N*-oxidized metabolites, however, have hampered such investigation.

In the mammalian system, *N*-oxidation of primary and secondary amines leads to hydroxylamines, while the oxidation of tertiary amines results in *N*-oxides (Table I). These two types of metabolites possess considerably different physicochemical properties. Because certain arylamines are believed to cause methemoglobinemia or tumor formations *via* hydroxylamine metabolites, there has been particular interest in the *N*-oxidation of this class of compounds.

The extent of *N*-oxidation is affected by the nature of the substituent groups on the nitrogen atom. *N*-Methyl- and *N*-ethylaniline are *N*-hydroxylated much faster than aniline. As the alkyl chain length increases, however, the degree of *N*-hydroxylation decreases and the amount of *para*-hydroxylation increases (137). *N*-Hydroxylation is also enhanced in *para*-substituted anilines (136). *ortho*-Substitution seems to inhibit *N*-hydroxylation of *N*-arylamines (135).

N-Hydroxylated metabolites have been reported for *N*-acylated aromatic amines (135), a variety of alicyclic and aliphatic amines (138–141), and the simple carbamate urethan (136). Furthermore, Beckett (140) reported significant stereochemical influences on *N*-hydroxylation *versus* *N*-dealkylation in *N*-benzylamphetamines.

N-Oxides are generally formed as minor metabolites of tertiary amines. Yet, there continues to be considerable interest in these biotransformation products because of their suspected roles in biological activity and/or toxicity of certain drugs (142). *N*-Oxides have been implicated as intermediates in the *N*-dealkylation of tertiary amines, although evidence has been provided to contradict this implication (121, 135, 142). Correlations between structures of tertiary amines and their potential for *N*-oxide formation apparently have not been studied in mammalian systems.

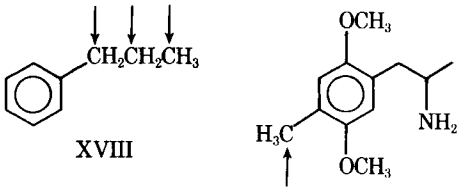


XVII

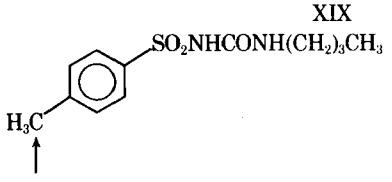
In microorganisms, several examples may be cited in which primary amines are metabolized to their respective nitro analogs. The reaction conducted by *Streptomyces thioluteus* was studied with a series of aromatic amines such as *p*-aminobenzoic acid (143–145). Rates of conversion were slower with *p*-aminophenylacetic acid and *p*-amino-L-phenylalanine, indicating a rather narrow substrate specificity for this reaction. Mechanistically, it was proposed that the primary amino group was converted into a hydroxylamine by the action of an oxygenase and molecular oxygen; this intermediate is subsequently oxidized to the corresponding nitro derivative. Other examples of the amino to nitro oxidation include various aminoimidazoles (146, 147) and the conversion of a chloramphenicol amine precursor to the antibiotic (148).

The production of *N*-oxides by microorganisms has not been widely described, because chemical conversions of tertiary amines to their *N*-oxides may be accomplished with relative ease. Microorganisms were used to make the *N*-oxides of hydroxycodeinone analogs (111) and strychnine and brucine (149). The formation of trimethylamine *N*-oxide by *P. aminovorans* was described earlier (119). Enzymes involved in this microbial oxidation were not fully characterized with regard to substrate selectivity.

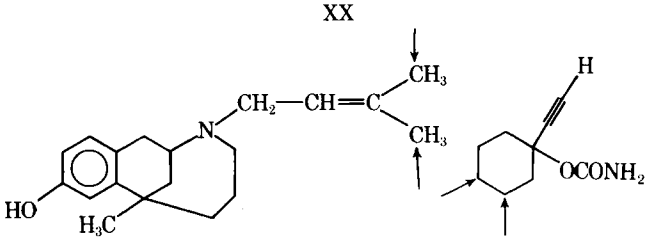
Hydroxylation of Aliphatic Compounds—In mammals, acyclic aliphatic compounds are typically hydroxylated at ω and ω-1 positions. Microorganisms also attack terminal and subterminal carbons in series of alkanes (150, 151). When an aliphatic chain is attached to an aromatic ring, the resulting benzylic position becomes prone to hydroxylation by mammalian (152) and microbial (153) systems. Substrates typically hydroxylated by mammals at aliphatic carbons include *n*-propylbenzene (XVIII) (152), the psychotomimetic 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (XIX) (154), and tolbutamide (XX) (155, 156) (site of hydroxylation indicated by arrows).



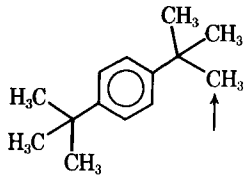
XVIII XIX



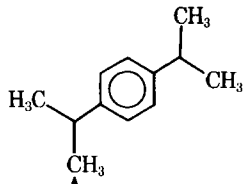
XX



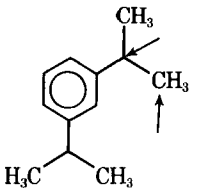
XXIV XXV



XXI



XXII



XXIII

The microbial oxidation of several branched aliphatic substrates (XXI–XXIII) has been systematically studied (153) (site of hydroxylation indicated by arrows). Products obtained with XXII were optically active. By shifting the isopropyl function to a 1,3-orientation on the benzene ring, mixtures of products, some exhibiting optical activity, were obtained (153). In mammalian systems (32), isotopic labeling experiments suggest that alkyl side-chain oxygenations occur by direct insertion of oxygen atoms.

Compounds with allylic carbon atoms, such as pentazocine (XXIV), are hydroxylated in mammalian systems (157). Microbial oxidations of allylic and benzylic positions have been adequately described (12). Ring positions of alicyclic compounds are often oxidized; ethinamate (XXV) (158), for example, is hydroxylated at positions 3 and 4 of the ring. This hydroxylation pattern possesses similarities to the hydroxylation of alicyclic amides by the mold *Sp. sulfurescens* (82).

Oxidative Deamination—Compounds containing the basic structural unit R—CH_2NH_2 are the best substrates for monoamine oxidase. Substitution at the methylene carbon reduces reactivity, while increasing the chain length of an alkyl R group or the complexity of R can reduce reactivity of a substrate with this enzyme (159, 160). On the other hand, monoamine oxidase can accommodate compounds with a variety of aryl—R groups (159). If R is a phenyl group, substitution in the *ortho*-position can substantially decrease deamination (161). In contrast, substitution with electron-withdrawing groups improves substrate susceptibility to enzymatic transformation (161, 162).

Secondary amines can be deaminated by monoamine oxidase, although the reaction rates are substantially slower than with primary amines. Microbial deamination of amine substrates has been demonstrated with tryptamine (163, 164). This type of reaction is much less common in microorganisms than the usual deamination of amino acids, which may occur *via* pyridoxal-mediated transaminases or typical oxidative deamination enzymes.

Oxidation of Alcohols and Aldehydes—Foreign primary alcohols, except methanol, are readily oxidized to aldehydes and finally to carboxylic acids in mammals. Liver alcohol dehydrogenase, the enzyme principally responsible for the initial oxidation reaction, has been extensively studied (165–168). The rate of oxidation increases in the order ethanol < *n*-propanol < *n*-butanol but then decreases. However, higher molecular weight alcohols (*e.g.*, benzyl and furfuryl) are still good substrates for liver alcohol dehydrogenase (166).

With the apparent exception of cyclohexanol (166, 167), secondary alcohols are oxidized substantially slower than primary alcohols. Liver alcohol dehydrogenase appears to have no effect on the following secondary alcohols: 2-propanol, cyclopentanol, methyl phenyl carbinol, 1-indanol, and 1-tetralol (169). McMahon (169) surveyed other mammalian alcohol oxidoreductases that may play a role in the oxidation of xenobiotic alcohols. Most of these, however, seem to have considerably narrower substrate specificities than liver alcohol dehydrogenase and are probably of lesser significance.

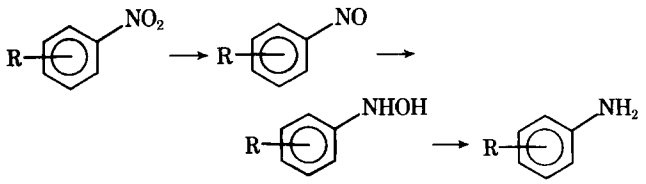
Mammalian aldehyde dehydrogenases oxidize a wide range of aldehydes. While formaldehyde is oxidized at only half the rate of acetaldehyde by liver aldehyde dehydrogenase, higher homologs, including propionaldehyde, butyraldehyde, and isovaleraldehyde, are only slightly poorer substrates than acetaldehyde (170). Substituted benzaldehydes serve as substrates for mammalian aldehyde dehydrogenase (170, 171). Benzaldehyde is the best substrate, while substitution with electron-withdrawing or electron-donating groups decreases activity. A good correlation was observed between log k and Hammett sigma values for this biphasic phenomenon (171).

Several aldehydes have been shown to be substrates for hepatic aldehyde oxidase (172, 173). This enzyme has been described as a hydroxylase (160) rather than a dehydrogenase. Aldehyde oxidase also attacks the α-carbon of a wide variety of *N*-heteroaromatic compounds (*e.g.*, quinoline), causing conversions to their corresponding α-lactam derivatives (173). Xanthine oxidase appears to have similar specificities to those of aldehyde oxidase (169).

Microorganisms commonly oxidize alcohols and aldehydes into their next higher oxidation states (10, 12, 13, 126, 174). These reactions often occur sequentially when a methyl group is first hydroxylated and then converted through the aldehyde to a carboxylic acid derivative. Polyol, terpene, and steroidal alcohol oxidations have been described (13). Various aldehydes are oxidized to carboxylic acids by an aldehyde dehydrogenase from *P. fluorescens* (175).

REDUCTIVE TRANSFORMATIONS

Nitro and Azo Reductions—A number of aromatic nitro compounds are converted to their nitroso, hydroxylamino, or amino derivatives in mammals (Scheme XVIII). This reaction is of considerable importance because of the potential toxicities of these metabolites. It is now generally believed that four to five enzyme systems affect nitro reductions: microsomal enzymes including cytochrome P-450 (176), NADPH-cytochrome c (177), and cytochrome b_5



Scheme XVIII—Bioreduction pathway for aromatic nitro compounds

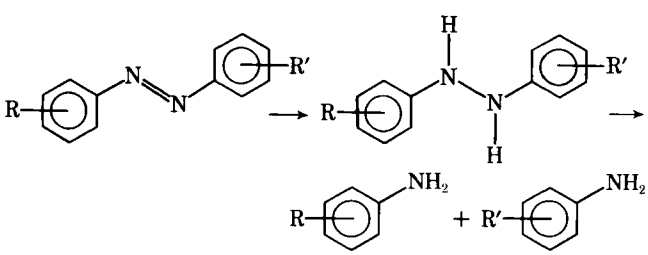
(178) reductases; an unidentified soluble "*p*-nitrobenzoate" reductase (179); and xanthine oxidase (180). All of these enzymes occur in the liver, although unspecified nitroreductase activity has also been detected in kidney, lung, heart, and brain tissue preparations (181).

The extent to which these enzymes reduce nitro compounds *in vivo* can be seriously questioned, because of their dependence on anaerobic conditions and the likely oxidation of proposed intermediates under the oxygen tension in the liver. For these reasons and because of the anaerobic conditions in the gut, bacterial involvement has been implicated in the reduction of aromatic nitro compounds (182). Zachariah and Juchau (183) reported approximately four times greater nitroreductase activity in rat cecal contents than in rat liver preparations.

Numerous aryl nitro compounds are reduced by mammalian and/or bacterial systems (182, 184). However, no systematic study of possible structural correlations has been reported.

The findings with azoreductases in mammals parallel those of nitroreductases. While hepatic azoreductases have been described (184), it seems highly probable that most azo reductions are mediated *in vivo* by gut microorganisms (182, 184, 185). Azo reductions are believed to proceed through hydrazo intermediates (184, 186) and result in the formation of corresponding aromatic amines (Scheme XIX). Because of the production of potentially toxic aromatic amines and the presence of azo linkages in numerous dyestuffs, there is considerable interest in azoreductases. Little seems to be known about possible influences of structural features on the facility with which azo compounds are reduced.

Reductions of Aldehydes and Ketones—Several enzyme systems are responsible for aldehyde and ketone reductions in mammals. Principal among these is liver alcohol dehydrogenase, which reversibly catalyzes reactions with equilibria favoring alcohol formation at physiological pH's (*vide supra*). Simple aliphatic aldehydes are reduced to primary alcohols by liver alcohol dehydrogenase; the activity increases from acetaldehyde through propionaldehyde and is



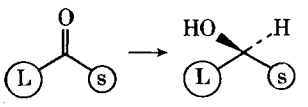
Scheme XIX—Bioreduction pathway for azo compounds

maximum for butyraldehyde (166). As indicated earlier, however, this reaction is of minor importance *in vivo* because of the facile conversion of aldehydes to readily excretable carboxylic acids.

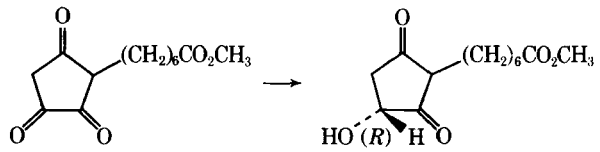
In contrast to the case with aldehydes, reduction of ketones to alcohols by liver alcohol dehydrogenase is an important *in vivo* biotransformation (169). The reduction of cyclic ketones by liver alcohol dehydrogenase has been extensively studied (187, 188). Considering the highly favorable substrate character of cyclohexanone [relative rate of reaction (RRR) = 100], the reduction rates of substituted derivatives are substantially dependent upon stereochemical features. For example, both enantiomers of 2-methylcyclohexanone are poor substrates (RRR = 1–2), while (−)-3-methylcyclohexanone has excellent activity (RRR_{calc} = 102) compared to its (+)-enantiomer (RRR = 0.6). 4-Methylcyclohexanone is a good substrate (RRR = 41), but activity drops dramatically in its more bulky homologs, 4-isopropylcyclohexanone (RRR = 21) and 4-*tert*-butylcyclohexanone (RRR = 2.5). Interesting differences were similarly noted in the reduction of *cis*- and *trans*-2-decalones. Reductions of cyclic ketones are stereoselective, depending on both the position and the absolute configuration of substituents (188). While many six-membered ketones are good substrates for liver alcohol dehydrogenase, cyclopentanone is without activity (169), and adamantanone is essentially inactive (189).

The reduction of ketones by alcohol dehydrogenase from microorganisms leads to the formation of (*S*)-carbinols when the carbonyl is dissymmetrically substituted by a bulky group and a small group (187, 188, 190) (Scheme XX). Presumably, the attack of the carbonyl by the NADH coenzyme in the microorganism is favored on one side only, as has been demonstrated (191) with numerous bi- and tricyclic ketone substrates using a reductase from *Cur. falcata*. On the other hand, alcohols of *R* absolute configuration have been produced by the reduction of compounds such as the triketone (XXVI) in completely stereospecific fashion (192) by *Dipodascus uninucleatus* (Scheme XXI) (193). It has been suggested that the extent of stereospecificity of the reaction product not only depends on interactions of the enzyme–substrate but also on the chirality of the alcohol dehydrogenase of a particular organism (193). Thus, it may be expected that microbial alcohol dehydrogenases capable of producing (*S*)- or (*R*)-alcohol products will be discovered.

In vitro experiments with liver alcohol dehydrogenase are not wholly satisfactory in predicting reductions of aldehydes and ketones in intact animals, because *in vivo* interconversions involving patterns of reduction → oxidation → reduction may occur and additional reductases also may be important in aldehyde and ketone metabolism. The first suggestion is



Scheme XX—Formation of (S)*-carbinol by alcohol dehydrogenase*



Scheme XXI—Formation of (R)-*carbinol by* D. uninucleatus (*Ref. 192*)

demonstrated by the work of Elliott *et al.* (190, 194–196), who showed that cyclohexanones and decalones are converted to their more thermodynamically stable equatorial alcohols in rabbits. This finding is in contrast to the substantial number of axial alcohols produced *in vitro* with liver alcohol dehydrogenase preparations (187, 188).

A number of other aldehyde–ketone reductases have been isolated from mammalian tissues. Similar NADPH-dependent aromatic aldehyde–ketone reductases (A–K reductase) have been isolated from rabbit liver and kidney preparations (169, 197). These enzymes are true reductases in that they do not catalyze the dehydrogenation of carbinols. A relationship similar to that derived for the reduction of benzaldehydes by liver alcohol dehydrogenase (198) was observed for substituted acetophenones and benzaldehydes (197, 199). In addition, a pentanone and adamananone were reduced by kidney A–K reductase (197).

It has been suggested that liver A–K reductase may be the same as the pig liver reductase isolated by Prelog (187). Fraser *et al.* (200) reported the isolation of an α,β-unsaturated ketone reductase from human liver. This enzyme mediates the reduction of either the double bond or carbonyl groups of an α,β-unsaturated ketone and is similar to A–K reductase (197, 199) in that it does not catalyze the reverse reaction (oxidation).

HYDROLYSES

Ester Hydrolyses—Esterases capable of hydrolyzing foreign esters have been found primarily in mammalian plasma, erythrocytes, liver, and kidneys (201). These enzymes include cholinesterase (pseudocholinesterase), carbonic anhydrase, arylesterases, and acetylesterases, which can collectively hydrolyze a vast array of esters. Because of the multiplicity of esterases and significant variation (both qualitative and quantitative) among animal species (67, 201), an understanding of the exact role that ester hydrolyses play in drug metabolism and drug action has been elusive. Some common ester-containing drugs susceptible to ester hydrolysis *in vivo* have been reviewed (201).

Scheline (182) noted that ester hydrolysis may be mediated by intestinal microorganisms. However, in humans this would only be of importance for poorly absorbed drugs (*e.g.*, acetyldigitoxin) capable of reaching the lower intestine. Numerous esterases have been characterized and used in the microbial transformation of steroids (202), and the application of esterases as a synthetic tool in the field of prostaglandin synthesis has been recorded (203). In the latter case, a racemic mixture of esters was stereoselectively hydrolyzed to yield a mixture of the free (*R*)-alcohol and the (*S*)-ester, which could be easily separated.

Amide Hydrolyses—Some amides may serve as substrates, albeit poor ones, of plasma or tissue esterases (67). The prolonged activity of procainamide *versus* procaine can be rationalized in this way. Krisch (204, 205) isolated a microsomal esterase from hog liver which cleaves several amides including acetanilide and lidocaine. In addition, a number of mammalian arylacylamide amidohydrolases have been described as occurring in liver and kidney tissue. These enzymes have been shown to deacylate some acetamido compounds and appear to be especially important in the mechanism of action of sulfonamides (206).

Parke (67) reported that primary amides (*e.g.*, benzamides), hydroxamic acids, hydrazides, and carbamates undergo hydrolysis in mammals, although it is unclear whether all of these are hydrolyzed as the result of enzymatic catalysis. Amide hydrolysis also may be catalyzed by intestinal microorganisms (207). Interestingly, the biotransformation of the sulfamate derivative, cyclamate, to the toxic metabolite, cyclohexylamine, in humans appears to be exclusively mediated by an intestinal microorganism (probably *Enterococci* sp.) (208). Smith and Rosazza (9) observed the hydrolysis of acetanilide in their microbial systems, and amide hydrolyses have been recorded in other microbial systems as well. Particularly well studied is the hydrolysis of amides in several antibiotics by many microorganisms (16).

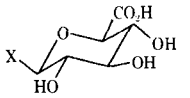
CONJUGATIONS

The major types of Phase II reactions are indicated in Table IV. Most of these biotransformations lead to conjugates of drugs and their metabolites that are more water soluble (and thus more readily excreted). These conjugates often possess less biological activity than the parent drugs. Thus, attempts are rarely made to characterize fully Phase II metabolites. In lieu of typical structure elucidation approaches, structures of conjugates are often inferred from selective hydrolysis reactions (*e.g.*, glucuronidase–sulfatase and chemical cleavages) and characterization of metabolites is done on the "drug-derived" portion of the conjugate.

Two Phase II reactions are exceptional with respect to these generalizations. *N*-Acetylation of amines and methylation of phenols result in more lipid-soluble metabolites that merit evaluation of biological activity. Foreign aliphatic and aromatic primary amines and hydrazides are substrates for a number of *N*-acetyltransferases which occur widely in mammalian species (206, 209–211). These enzymes are usually dependent on acetylcoenzyme A and are distributed in various tissues (67). The influence of substrate structure *versus* formation of *N*-acetyl metabolites is not well understood.

Monomethylation of catechols by the *S*-adenosylmethionine-dependent enzyme, catechol-*O*-methyltransferase, is an important biotransformation of cat-

Table IV—Phase II Biotransformations

Reaction Type	Substrate	Product
Glucuronidation		
	Alcohol	X = R—O
	Phenol	X = aryl—O
	Enol	X = $R'_2C{=}CR{-}O$
	Hydroxylamine	X = $R_2C{=}N{-}O$
	Carboxylic acid	X = RCO_2-
	Primary and secondary amines	X = R_2N-
	Amide	X = RCONR
	Thiol	X = RS
Sulfate conjugation		X′—OSO_3H
	Alcohol	—
	Phenol	Sulfate ester
	Enol	—
	Amine	Sulfamate
Glycine conjugates	Carboxylic acid	$RCONHCH_2CO_2H$
Acetylation	Primary amine	$RNHCOCH_3$
	Hydrazine	$RNHNHCOCH_3$
	Hydrazide	$RCONHNHCOCH_3$
	Sulfonamide	$RSO_2NHCOCH_3$
N-Methylation	Primary, secondary, and tertiary amines	Corresponding secondary, tertiary, and quaternary amines
O-Methylation	Catechol	Monomethyl catechol
	Phenol	Methyl aryl ether

echolamine neurotransmitters and their respective metabolites (212, 213). Catechol-*O*-methyltransferase principally occurs in the liver but is found in kidney, spleen, small intestine, lung, and brain tissue (213, 214). In most instances, the oxygen atoms of a catechol are nonequivalent in terms of electronic and/or steric characteristics. Preferential methylation of one group *versus* another was at one time thought to be a function of relative nucleophilicities (215). However, recent studies indicated that the steric and electronic properties of substituents, as well as the relative lipophilicity of substrate, are equally or more important factors affecting relative isomer formation (*e.g., meta–para* ratios) (216–219). Recently, evidence for mammalian enzymes that methylate monophenols and monomethyl catechols has been presented (220–222). The importance of these biotransformations *in vivo,* however, is still unclear.

An enzyme that occurs in the soluble portion of rabbit lung homogenates has been found to *N*-methylate a variety of foreign primary and secondary aliphatic amines (223, 224). An apparently similar enzyme also has been discovered in rat brain (225). The importance of these enzymes with *in vivo* drug biotransformations has not been established.

Interesting microbial parallels to a number of Phase II reactions have been observed, including the phosphorylation of clindamycin (226) by *Streptomyces coelicolor* and the formation of ribonucleotides of this antibiotic (227). *N*-Acetylation occurs commonly with amines such as tryptamine (and with tryptophan) (17), aniline (9), and other compounds (13, 126). Lysine (228), serine (229, 230), and glycine (231) conjugates of 2,3-dihydroxybenzoic acid are produced by microorganisms. A microbial parallel to mammalian *O*-methylation was previously described in this review in the O-*Dealkylation* section (114).

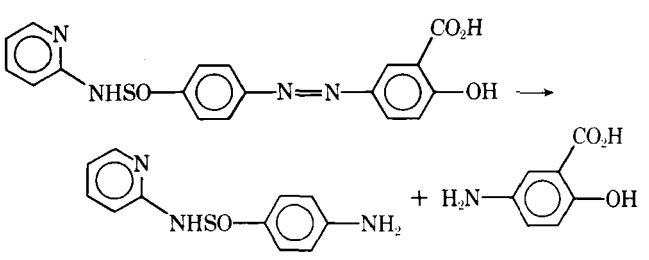
Transformations by Intestinal Microorganisms—Significant interest has been demonstrated recently in the metabolism of drugs by intestinal microorganisms (167, 232, 233). Orally administered drugs, particularly those with low solubility or delayed absorption, pass to the lower portion of the intestinal tract and may be susceptible to biotransformation by the intestinal flora. Additionally, compounds excreted *via* the intestinal route may be susceptible to such biochemical changes. A variety of microorganisms may be found in the intestinal tract.

In a very important study (because of its thoroughness), 113 distinct types of organisms were identified in fecal samples of Japanese–Hawaiians (234). Most fecal organisms are bacteria; however, the nature of the flora may be quite different, depending on such factors as diet, age, health, and use of drugs such as antibiotics, which may destroy the normal GI tract flora. Many types of microbial conversions have been attributed to the intestinal flora, some of which have been described already including *N*-demethylation of methamphetamine (128), reduction of nitro and azo compounds (182), and hydrolysis of esters (182) and amides (208).

Type-reactions carried out by intestinal microorganisms (233) are: hydrolysis of glucuronides, glycosides, ethereal sulfates, amides, glycine conjugates, *N*-acetyl compounds, esters, and sulfamates; C- and *N*-dehydroxylation; decarboxylation; *O*- and *N*-demethylation; dehalogenation; heterocyclic ring fission; reduction of double bonds, nitro groups, azo groups, aldehydes, ketones, alcohols, *N*-oxides, and arsonic acids; aromatization; deamination; nitrosamine formation; acetylation; and esterification.

The mode of action of a drug may be influenced by intestinal flora metabolism (233). For example, it was suggested that when sulfasalazine (salicylazosulfapyridine) (XXVII) (Scheme XXII) (used in ulcerative colitis and other inflammatory diseases of the bowel) is metabolized to its respective components (XXVIII and XXIX), the metabolites were really responsible for the action of the drug. Drug action was ultimately related to the presence of 5-aminosalicylate (salt of XXVIII) in the intestines. When 5-aminosalicylic acid is given orally or parenterally, little of the compound appears in the colon. Thus, sulfasalazine serves as a prodrug and becomes activated only after a microbial conversion step in the intestine.

Other toxicological implications regarding the bio-



Scheme XXII—Microbial transformation of sulfasalazine

transformations of compounds in the GI tract involve the hydrolysis of glucuronides and other conjugates. Many drugs and foreign organic compounds or their metabolites are excreted as glucuronide conjugates and are poorly reabsorbed from the intestines. Bacterial β-glucuronidase activity is high in the intestine; aglycones produced as a result of hydrolytic activity may be reabsorbed and passed again through the liver. For example, glucuronide hydrolysis and reabsorption have been reported with diethylstilbestrol (235) and indomethacin (236).

Microbiological Methodology—Few researchers engaged in mammalian metabolism studies are familiar with microbial transformation experiments, and vice versa. This brief section will provide a simple introduction to some methodology involved in microbial transformation studies. Of course, a review of this scope cannot provide sufficient experimental detail or background to enable the reader to conduct such experiments immediately. Rather, years of experience are required before it becomes possible to deal instinctively with any biological system. Perhaps the ideal situation is one involving the simultaneous efforts of an interdisciplinary team which may include microbiologists (pharmacognosists), analytical medicinal chemists, and pharmacologists.

Microorganisms are convenient sources of the enzymes that mediate reactions like those outlined in the text. Although microbes are capable of conducting single, selective reactions on even the most complex molecule, a host of reactions also can occur simultaneously. It is possible, however, to adjust conditions of microbial experiments to favor formation of a single product or a single type of reaction to increase the yield of a desired metabolite. Typical fermentation conditions that are frequently varied include organic and inorganic medium components, pH, degree of aeration of cultures (usually attained by shaking at different rates), temperature of incubation, and substrate concentration.

Variations in pH, medium composition, and temperature are known to influence the outcome of fermentation experiments. The importance of dissolved oxygen levels for the production of enzymes (237) and on the microbial transformations of steroids (238) has been noted. The time and mode of addition of substrates to fermentations can also influence microbial metabolic profiles (238–244). Several general references detail many aspects of the fermentation process (12, 13, 242, 245). Others dealing with specific types of microbial transformations were cited earlier in this review.

The selection of microorganisms capable of performing a desired chemical transformation may be accomplished in a number of ways. Cultures may be isolated from the soil or from sewage treatment facilities, or they may be obtained in pure form from culture collections. A compilation of sources of microorganisms was published (246), and smaller lists of useful culture collections have been described (10, 12, 13). Microbiologists are often willing to share cultures from the culture collections they maintain. Because cultures from some culture collections are expensive, most workers in the field of microbial transformations maintain collections in their own laboratories (perhaps 100–400 cultures).

Once cultures are on hand, they may be screened for their potential to perform, for example, hydroxylation of aromatic substrates. It is possible to be more discriminating in the choice of microorganisms to conduct a specific type of reaction by taking advantage of the rapidly growing literature in microbial transformations. For success in obtaining active cultures that perform a desired transformation, it is often necessary to combine the "literature" approach and the "random screening" approach.

Several types of microorganisms are used in microbial transformation experiments. These are the bacteria (including the Actinomycetes), yeasts, and filamentous fungi. Growth habits, medium requirements, and culture conditions are generally quite different for each type of microorganism. Some microorganisms are pathogenic and must be handled with extreme care. Most, however, are relatively easy to work with and may be cultivated with little difficulty. Detailed descriptions and taxonomic classifications of various microorganisms have been published (247, 248). Whatever the source of the microorganism, it must be properly identified (*e.g.*, culture collection acquisition number and authentication by suitable authority) and precautions should be taken to prevent contamination with other microorganisms.

To accommodate the diverse growth requirements of different microorganisms, various media have been employed. A large listing of media cross-indexed with various microorganisms is available (249). Media and glassware employed in microbial transformation experiments are usually sterilized in an autoclave (121° for 15 min). After sterilization, solid (agar-containing) medium is caused to solidify into slants in sealed culture tubes. These slants are usually inoculated with various cultures and are incubated for approximately 1 week to obtain fresh and vigorous cultures. The resulting cultures may be maintained in a viable state for long periods (6 months or longer) in a refrigerator at 3–4°. Culture vigor is maintained by periodically transferring stored cultures to fresh slants. Other methods used in the maintenance of culture collections include low temperature storage (from −70 to −198° liquid nitrogen) and lyophilization.

Microbial transformation experiments *per se* are conducted in liquid medium contained in vessels ranging in size from small test tubes to fermentors with several hundred or even thousand gallon capacities. The usual laboratory-scale incubations are carried out in cotton-plugged erlenmeyer flasks containing about one-fifth of their volumes of medium. Table V shows a selection of media used in these kinds of experiments. Note the differences in medium pH used for the cultivation of bacteria and fungi. In general, bacteria grow in media with initial pH values of 7.0 while fungi and yeasts grow well under slightly acidic (pH 4–6) conditions. As a result of the consumption of nutrients and primary metabolic activity, the pH of the medium of most fermentations changes as the cultures grow.

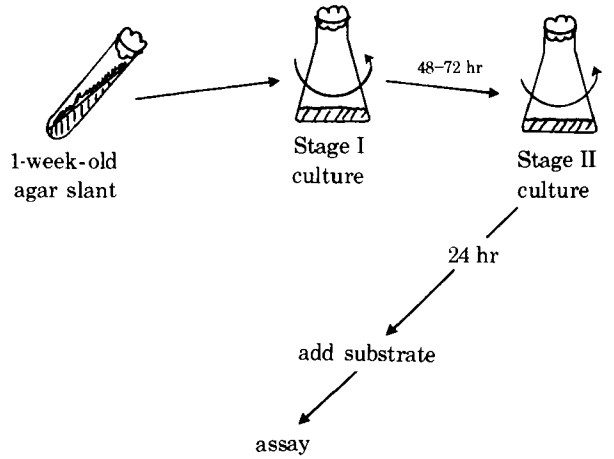
Table V—Various Media Employed in Microbial Transformation Experiments

Research Group	Media Used
Jones *et al.* (250)	Dextrose ($1H_2O$), 30 g; ammonium tartrate, 7.5 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; trace elements, 0.1 g; yeast extract, 1.0 g; and distilled water, 1 liter
	For fungi and streptomycetes, use at pH 5.5; for bacteria, use at pH 7.0
Smith and Rosazza (9) and Betts *et al.* (61)	Dextrose, 20 g; yeast extract, 5 g; soybean meal, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; and distilled water, 1 liter
	Adjust to pH 7.0 with 5 *N* HCl; use for yeasts, fungi, and actinomycetes
Kluepfel and Vezina (251)	Dextrose, 50 g; edamine, 20 g; corn steep liquid, 3 g; and distilled water, 1 liter
	For fungi, use at pH 5.8
	Dextrose, 1.5 g; yeast extract, 1.5 g; peptone, 5.0 g; K_2HPO_4, 3.5 g; and distilled water, 1 liter
	For bacteria, use at pH 7.0
Goodman *et al.* (252)	Dextrose, 20 g; peptone, 5 g; tryptone, 5 g; $CaCO_3$, 2.5 g; and distilled water, 1 liter
	Use for various bacteria

Fermentations are conducted in many ways in the laboratory. Small-scale screening-type fermentations are conducted in 50–125-ml erlenmeyer flasks containing 10–25 ml of medium and are usually incubated with shaking to ensure a rich supply of oxygen for the medium. Larger scale incubations are conducted in large carboys or in stirred fermentors agitated by impellers and aerated by sparging air through the medium. Typical laboratory-scale incubations using stirred fermentors range from 1.5 to 10 liters in volume.

A fermentation scheme is outlined in Scheme XXIII. The description of the fermentation provided is applicable to small- and large-scale experiments and has been generally applied successfully in our laboratory.

Cultures are maintained on Sabouraud–maltose agar slants and are stored in a refrigerator at 4° prior to use.



Scheme XXIII—Typical conditions for conducting microbial transformations (Ref. 9)

Cultures are grown in a soybean meal–glucose medium (Table V).

The surface growth from slants of microorganisms is suspended in 5-ml volumes of sterile soybean meal–glucose medium. This suspension is used to inoculate 25 ml of the soybean meal–glucose medium held in cotton-plugged 125-ml erlenmeyer flasks (Stage I). The Stage I flasks are incubated at 27° on a rotary shaker operating at 250 rpm for 48–72 hr, at which time most cultures are late in the log phase or in the stationary phase of growth. Approximately 3-ml volumes of the actively growing Stage I cultures are transferred to 25 ml of fresh medium held in cotton-plugged 125-ml erlenmeyer flasks (Stage II). After 24 hr of incubation on a rotary shaker, 12.5 mg of substrate dissolved in 0.1 ml of dimethylformamide is added to Stage II flasks. The substrate-containing flasks are incubated for an additional 24–72 hr before being terminated.

The time of addition of substrate to a fermentation, as well as its concentration in the medium, may be critical in microbial transformation experiments. Substrates may be added in concentrated solutions of dimethylformamide, dimethyl sulfoxide, ethanol, acetone, or other solvents to facilitate dispersion in aqueous media. Compounds may be added in water-soluble form, as hydrochloride salts of alkaloids or amino acids, or as sodium or potassium salts of carboxylic acids. In the usual screening experiment, a nominal level of substrate of 0.1–0.5 mg/ml of Stage II fermentation has been successfully employed in our laboratory. Limited success in microbial transformation experiments has been obtained when substrates were added too soon to Stage II cultures. Growth is inhibited, and there is evidence that an optimum time for substrate addition exists with microorganisms.

In screening experiments, analyses are usually performed at two times during the fermentation, 24 and 72 hr after substrate addition. Some microorganisms may completely degrade a substrate early in a fermentation, whereas others may possess inducible enzymes which will only produce metabolites after being in contact with substrates for a period of time. This arbitrary selection of two time periods has worked well in our hands.

Liquid–liquid extraction methods are most widely used in the analysis and isolation of microbial metabolites. Occasionally, for difficult to extract solutes, culture samples have been frozen and lyophilized to facilitate the isolation process. Freeze-dried samples may then be extracted with polar solvents such as alcohols or acetone. In general, it is recommended that the whole culture be examined for metabolites rather than culture filtrates alone because some substrates (and their metabolites) may bind to cellular material. We have observed this to be true both with steroids and with alkaloids such as acronycine. In fact, acronycine and its metabolite, 9-hydroxyacronycine, are easily removed from fermentations by centrifugation of the solids from fermentations of *C. echinulata*

(61). Only traces of acronycine and its metabolite are left in supernates, and the centrifuged solids may be simply extracted with hot ethanol to isolate the relatively insoluble 9-hydroxyacronycine.

CONCLUSIONS

The metabolic fate of drugs and other xenobiotics in mammalian organisms represents an area of intense contemporary interest. Typically, biological systems used to study biotransformations are capable of yielding only minute quantities of metabolites. Recent developments in comparative biochemistry have made it possible to link diverse metabolic systems through similarities in the pathways by which they alter foreign organic compounds. Thus, the potential exists for utilizing microbial metabolic systems to study and possibly predict the metabolic fate of a drug or other foreign compounds in mammals. Furthermore, it should be possible to use such systems to produce preparative quantities of drug metabolites for structure elucidation studies and biological testing.

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RESEARCH ARTICLES

Solvolytic Reactions of Cyclic Anhydrides in Anhydrous Acetic Acid

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Abstract □ The reversible reactions of several cyclic anhydrides with acetic acid to form acetic anhydride and the corresponding dicarboxylic acid, catalyzed by perchloric acid at 25°, were studied. The equilibrium constants, calculated from spectral data, were 4.85×10^{-4}, 1.08×10^{-1}, and 4.6×10^{-1} *M* for succinic, *trans*-1,2-cyclohexanedicarboxylic, and glutaric anhydrides, respectively. Maleic, phthalic, and *cis*-1,2-cyclohexanedicarboxylic anhydrides did not undergo any detectable reaction with acetic acid under these conditions, suggesting still higher stability. The reverse rate constants were found to be relatively independent of the structure of the attacking diacid, while the forward rate constants were found to parallel the equilibrium constants. The rate-determining step for the forward reaction appears to be the breakdown of the tetrahedral intermediate formed by the attack of an acetic acid molecule on the protonated cyclic anhydride.

Keyphrases □ Anhydrides, cyclic—solvolytic reactions in anhydrous acetic acid, rate constants, mechanisms □ Solvolysis of cyclic anhydrides in anhydrous acetic acid—rate constants, mechanisms □ Perchloric acid—catalyst for solvolytic reactions of cyclic anhydrides in anhydrous acetic acid □ Acylation—reversible reactions of cyclic anhydrides in anhydrous acetic acid, rate constants, mechanisms

The chemistry of cyclic and linear anhydrides is of major interest to those concerned with transacylation reactions in both *in vitro* and *in vivo* systems. There is very little doubt that these active chemical species play significant roles in many acyl transfer reactions and in cleavage and formation of polycarboxylic acid derivatives. The present report is concerned with some data obtained on the solvolytic behavior of a series of cyclic anhydrides of varying stability in anhydrous acetic acid. The results indicate relatively strong structural dependency on the ring opening and very little sensitivity to the reverse process.

Anhydrides of dicarboxylic acids have been used in the preparation of hemiesters of water-insoluble