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The Use of Stable Isotopes in Pharmacological Research

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I. Introduction

A. Background

THE use of the stable isotopes of hydrogen, carbon, nitrogen, and oxygen in the life sciences has undergone a dramatic expansion over the past decade, in part due to the increasingly refined technology for the analysis of stable-isotope-labelled compounds, but also as a result of the greater availability of isotopically enriched compounds and the growing demand for the development of nonradioactive tracer techniques for human studies. An historical account of the discovery of the stable isotopes of S, C, N, and H (in that chronological order) during the five-year period from 1927 to 1932 has been given by Klein and coworkers (245) and an excellent survey of the early biological work with deuterium oxide (heavy water), the first stable-isotope-labelled compound to be produced on a commercial scale, is in a review by Blake, Crespi, and Katz (36). Applications of deuterium, carbon-13, and nitrogen-15 were initially devoted almost exclusively to studies of biochemical processes, when major advances were made in our understanding of the dynamics of pathways of intermediary metabolism by workers such as Urey, Rittenberg, and Schoenheimer. However, the growing application in life sciences of tracers labelled with stable isotopes, evident from the literature of the 1930's and early 1940's, was largely overshadowed in the latter part of the 1940's by the introduction of the first radioactive isotope, ^{14}C. The ease with which radioisotopes could be detected and quantified by liquid scintillation counting techniques contrasted sharply with the difficult and tedious procedures necessary for the analysis of stable-isotope-labelled compounds by early mass spectrometers, and consequently the initial growth in stable isotope usage was effectively curtailed. Nevertheless, ex-

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periments such as those performed on the metabolism of barbiturates with ^{15}N-labelled analogs of phenobarbital (301), amytal (302), and barbital itself (303) had demonstrated by 1950 the great potential of stable isotopes for qualitative and quantitative applications in *pharmacological* research.

The first application in the field of toxicology appears to have been a study of the carcinolytic effect of heavy water. Since it was recognized at an early stage that deuterium substitution could affect the kinetics of cellular reactions, it was reasoned that neoplastic tissue and other rapidly proliferating tissues should be particularly susceptible to the inhibitory action of deuterium oxide on cell division (242). Unfortunately, however, while heavy water was found to inhibit tumor growth, the concentrations required were not well tolerated by the host. Extensive studies on the effect of deuterium substitution on the metabolism, toxicology, and pharmacological activity of a wide variety of drugs were performed in the late 1950's and through the following decade (36); the role of molecular oxygen in the metabolism of xenobiotics was discovered in 1955 with the aid of the stable isotope of oxygen, ^{18}O (295); the mechanism of enzyme-catalyzed aromatic hydroxylation reactions (the arene oxide pathway) was revealed by studies carried out in the mid-1960's by Guroff et al. (157–159), who employed substrates labelled at specific positions with deuterium. Investigations such as these demonstrated clearly the potential of stable isotopes for mechanistic applications in pharmacology.

The most significant single event in promoting the development of stable isotope methodology for application to biological problems was the introduction, in the early 1960's, of the technique of combined gas chromatography and mass spectrometry (GC-MS), made possible by the construction of molecule separators of the type described by Ryhage (390) and by Watson and Biemann (479). By the use of GC-MS methods, intact organic molecules could be analyzed directly, without the need for laborious isolation, purification, and, in some cases, chemical degradation to small fragments (e.g. CO_2). The high degree of sensitivity inherent in mass spectrometry, coupled with the excellent chromatographic resolving power of the gas chromatograph, was soon exploited in the development of quantitative assay procedures in which analogs of the compounds of interest, labelled with stable isotopes (usually deuterium), were employed as internal standards (417, 396, 135). This capability for the accurate quantification of compounds at the nanogram level and below led to an explosive growth in the use of stable-isotope-labelled compounds in pharmacological research and a corresponding demand for the increased commercial production of compounds enriched with deuterium, carbon-13, and nitrogen-15. The use of stable isotopes and GC-MS in quantitative work undoubtedly remains the major area of stable isotope application today, when investigations on, e.g. drug pharmacokinetics (either after acute administration or under "steady-state" conditions) and studies of bioavailability (either relative or absolute) with labelled compounds have now become commonplace. Innovative methods, such as the stable-isotope-based "twin ion" or "isotope cluster" technique, have greatly facilitated the detection and identification of drug metabolites in complex biological extracts by mass spectrometry, while mechanistic studies on the formation of chemically reactive drug metabolites and on enzymatic aspects of drug biotransformation rely increasingly on the use of stable-isotope-labelled substrates. The diversity of applications of stable isotopes in pharmacological research may be illustrated by reference to a number of review articles that deal with the use of stable isotopes in pharmacology/clinical pharmacology (254, 136, 154), medicinal chemistry (255, 347, 161), drug metabolism and pharmacokinetics (174, 387, 332), and the life sciences and medicine in general (298, 297, 57). Studies with deuterated drugs have been reviewed thoroughly by Blake et al. (36) for the period up to 1974, while the proceedings of four recent international conferences serve to highlight current developments in this rapidly expanding field (253, 246, 252, 25). A selected bibliography of biomedical and environmental applications of stable isotopes has been published for the period from 1971 to 1978 (247–251), and updates are scheduled to appear biennially. Synthetic procedures for the preparation of deuterium-labelled compounds are found in a useful text by Thomas (449) and a cumulative index for *Journal of Labelled Compounds & Radiopharmaceuticals* vols. 1 to 16, containing a wealth of information on the synthesis of compounds labelled with a variety of isotopes, has been published recently (74). In addition, a valuable compendium of synthetic procedures for the preparation of compounds labelled with the stable isotopes of carbon, nitrogen, and oxygen is now available (364a).

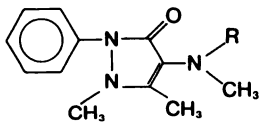
The object of this review is to summarize those areas of pharmacological research in which stable-isotope-labelling techniques have made an important contribution, and also to emphasize those developing applications where stable isotopes are likely to play an important role in future years. The literature in this field is now so extensive that some selection of material for review was unavoidable. An emphasis has been placed, therefore, on more recent work, although leading references to comprehensive treatments of specific topics have been included if available.

B. Analytical Techniques

Although a variety of physicochemical methods have been applied to the determination of compounds labelled with stable isotopes, mass spectrometry in general, and GC-MS in particular, have been by far the most important analytical techniques for use in pharmacological studies involving stable isotopes. Thus, GC-MS provides the investigator with an unrivalled combination of sensitivity, specificity, and versatility of detection. It should be noted, however, that there are two fundamentally

different approaches to the determination of stable isotopic abundance. In the first, an indirect procedure is followed whereby the sample is converted to a gaseous form (e.g. H_2, N_2, CO_2) for subsequent analysis in an isotope ratio mass spectrometer; this technique is characterized by its very high precision of measurement (e.g. 0.01%), but suffers from the disadvantage that relatively large amounts of sample (e.g. 0.1 to 10 mg) are required. The second, much more widely employed technique involves a direct analysis of the compound(s) of interest, normally carried out by GC-MS and frequently based on selected ion monitoring (SIM) procedures; this approach affords considerably poorer precision of isotope ratio determination (1% to 10%) but is specific and can be used for the analysis of very small (<1 ng) samples. The majority of applications of stable isotopes referenced in this review have been carried out in conjunction with mass spectrometric analysis and readers not familiar with the techniques of GC-MS and selected ion monitoring are referred to an introductory text by Watson (478). Many reviews have been published in recent years on the application of mass spectrometry to topics in the biological sciences, many of which emphasize the growing importance of stable isotopes in this field of research. The following examples, published during the period from 1978 to 1980, represent leading articles from which the reader may obtain references to detailed discussions on various aspects of stable isotope usage in pharmacological research. Biennial reviews on mass spectrometry are published both by *Analytical Chemistry* (53, 52) and by the Chemical Society (229); chapters in the latter by Brooks and Middleditch on GC-MS (46) and by Millard on drug metabolism (315) are especially relevant to the topic of this review. Applications of mass spectrometry and stable isotopes in pharmacology have been dealt with in a recent book by Tatematsu et al. (445) and in articles by Jenden (218) and by Draffan et al. (100a, 104), while clinical uses of stable isotopes have been reviewed by Krahmer and McCloskey (262). The use of SIM techniques in pharmacology has been the subject of a commentary by Jenden and Cho (221) and of a book chapter by Ghisalberti (147). Stable isotopes have made a major impact on the development of quantitative applications of mass spectrometry, as illustrated in a book by Millard (314), in articles by Baillie (24), Lehmann and Schulten (273), and McCamish (305), and in the proceedings of an international conference on quantitative mass spectrometry (96). Numerous papers on the uses of stable isotopes in pharmacology appear in the proceedings of the Italian mass spectrometry meetings, held annually (132–134), and in the proceedings of the International Mass Spectrometry Conferences, the last of which was held in Oslo in 1979 (379). Finally, an important treatise on biochemical applications of mass spectrometry, surveying the past decade of developments, has been published recently (474).

The most important analytical technique, other than mass spectrometry, that is used for the study of stable-isotope-labelled compounds in pharmacological research is nuclear magnetic resonance (NMR) spectroscopy. Indeed, the demand for NMR solvents, highly enriched in deuterium, has contributed significantly to the growing demand for, and stimulated commercial production of, compounds labelled with this isotope. Although deuterium does not possess a magnetic moment, its use in conjunction with proton NMR is extremely important in defining the location of deuterium substituents in labelled compounds (174). In this sense, NMR is complementary to mass spectrometry since the precise location of a deuterium atom within a molecule may be difficult or impossible to define on the basis of a mass spectrum alone. As an illustration of the importance of this point, it is likely that the origin of the so-called "NIH shift" (section III C 5), studies on which afforded invaluable information on the mechanism of biological oxidation of aromatic ring systems, would still be obscure were it not for the combined use of deuterium labelling and proton NMR spectroscopy (157–159). Carbon-13 and nitrogen-15 do, however, have magnetic moments and the use of recently developed ^{13}C- and ^{15}N-fourier transform NMR techniques would appear to hold considerable promise for studies in certain areas of pharmacological research (8, 297, 298, 161, 347), notably drug metabolism (174, 410, 59). Poor signal-to-noise characteristics have hitherto delayed the application of ^{15}N NMR to studies of drug metabolism, although ^{13}C NMR is proving to be a popular technique in this area, especially when employed to study the nature of metabolites produced from an analog of the compound of interest, labelled in one or more positions with ^{13}C. Thus, when the spectrum is recorded with proton decoupling, which removes ^{13}C-^{1}H spin-spin coupling, the ^{13}C atoms appear as sharp singlet resonances, the chemical shifts of which may be interpreted in terms of metabolic alteration of the parent drug structure. Examples of this technique are found in studies of the urinary metabolites of [$^{13}C_2$]amitriptyline in the rat and dog (175), in the detection of 4-formylaminoantipyrine (I), a new metabolite of aminopyrine (II) in several mammalian species (355, 356), and in the identification of human urinary metabolites of ^{13}C-labelled BHT, an antioxidant compound (488).



I R = CHO
II R = CH_3

A number of other techniques have been employed to a limited extent for the analysis of compounds labelled with stable isotopes. Infrared (IR) spectroscopy was used by Kubic et al. (267) to detect ^{13}CO as an in vivo metabolite of ^{13}C-labelled dichloromethane in the rat; the absorption bands of carboxyhemoglobin and [^{13}C]carboxyhemoglobin at 1950 cm^{-1} and 1905 cm^{-1}, respectively, served to reveal the presence of ^{13}CO in rat blood and to permit an estimate to be made of the degree of saturation of hemoglobin produced through oxidative metabolism

of the labelled halocarbon. Electron spin resonance (ESR) spectroscopy has been applied to a study of the ^{17}O-enriched oxybenzo[a]pyrene radical produced during incubation of benzo[a]pyrene with rat liver microsomal systems (384); the authors concluded that while the source of oxygen in the radical was atmospheric and that the reaction was mediated by cytochrome P-448 or P-450, the free radical observed in their studies was formed nonenzymatically, possibly via abstraction of a hydrogen atom by molecular oxygen in the solvent used for ESR analysis.

Other methods for stable isotope analysis, such as optical emission spectroscopy and Raman scattering, do not appear to have been used in pharmacological applications (297, 161, 174).

C. *Availability of Labelled Compounds*

As indicated in the foregoing discussion, recent developments in instrumentation for the determination of stable isotope enrichment, coupled with an increased demand for deuterium-labelled NMR solvents of high isotopic purity, are two factors that have greatly stimulated the production of stable-isotope-labelled compounds on a commercial basis. A wide variety of simple organic compounds are now available, labelled with ^{2}H, ^{13}C, ^{15}N, or ^{18}O at enrichments ranging from 90 atom % (^{13}C) to 99.9 atom % (^{2}H), and suitable for incorporation into compounds of pharmacological interest by chemical synthesis. Commonly used synthetic reagents are also available in labelled form, together with a number of multiply labelled compounds (i.e. more than one type of stable nuclide in the same molecule) and a limited number of stable-isotope-labelled drugs. The commercial availability of labelled compounds up to 1975 has been surveyed by Avona and Eck (13) and an excellent account of the processes used in the bulk production of ^{13}C, ^{15}N, ^{17}O, ^{18}O, and ^{34}S has been published in a review by Halliday and Lockhart (161). Synthetic considerations involved in the preparation of compounds labelled with stable isotopes have also been discussed in the latter review, which comments on practical aspects of both chemical and biosynthetic methods.

It should be noted that while much current interest centers on the use of stable isotopes for tracer studies in human subjects on account of the growing concern over the health hazard associated with radioisotopes, certain stable isotopes do contain low levels of their radioactive counterparts. This phenomenon was first reported in the literature by Jordan and May in 1971 (234), who observed that highly enriched deuterium oxide (99.7 atom %) may contain tritium at a specific activity of as high as 10.9 μCi mole^{-1}. These authors pointed out that the enrichment processes for concentrating the stable isotopes of a given element would also concentrate any coexisting naturally occurring radioisotopes of that element, so that deuterium-labelled compounds will contain low levels of tritium, ^{13}C-labelled material will contain ^{14}C, etc. This problem has been avoided in the production of ^{13}C at the Los Alamos Scientific Laboratories by the use of carbon from petrochemical sources that contain a very low level of ^{14}C (254). With ^{15}N and ^{17}O or ^{18}O the problem does not arise since there are no long-lived radioactive isotopes of these elements. In a study of the amounts of radioisotopic contamination in stable-isotope-labelled compounds obtained from commercial sources, Horie et al. (197) found that the ^{3}H and ^{14}C specific activity was extremely low when compared with the daily intake of radioactivity via the diet, and concluded that the short-term administration of stable-isotope-labelled drugs to human subjects should not pose any health hazard from the associated radioactive contamination.

The stable isotopes of greatest interest for pharmacological applications, together with their accurate atomic masses and natural abundance, are listed in table 1.

II. Toxicity

The biological effects of replacing hydrogen with deuterium were investigated within a year after the first published report of the existence of this isotope, when experiments were performed to test the ability of goldfish, earthworms, and protozoa to survive in heavy water that had a deuterium content of 92 atom % excess (446). It is now known that deuterium is fatal to mammals when approximately 30% of the body hydrogen is replaced with the heavy isotope, although toxic effects may be observed at enrichments in excess of 15% (453). It should be noted that long-term (4 months) studies have been carried out in human volunteers who were maintained on $^{2}H_2O$ to give a body water enrichment of 0.5 mole %, with no ill effects (427). An extensive literature exists on the toxicity of deuterium in living systems and the reader is referred to a comprehensive review of the subject by Blake et al. (36). The origin of deuterium toxicity is undoubtedly related to the relatively large isotope effects frequently encountered during the metabolism of deuterium-labelled compounds (section III C 4)

TABLE 1
Some stable isotopes of pharmacological interest

Element	Atomic Mass	Relative Abundance (atom %)
^{1}H	1.00783	99.985
^{2}H	2.01410	0.015
^{12}C	12.00000	98.89
^{13}C	13.00335	1.11
^{14}N	14.00307	99.63
^{15}N	15.00011	0.37
^{16}O	15.99491	99.759
^{17}O	16.99914	0.037
^{18}O	17.99916	0.024
^{32}S	31.97207	95.05
^{34}S	33.96786	4.22
^{35}Cl	34.96885	75.53
^{37}Cl	36.96590	24.47
^{79}Br	78.91834	50.54
^{81}Br	80.91634	49.46

and consequent to the large difference in mass between the protium and deuterium forms of hydrogen. On this basis, one would predict that the toxicity of the heavier stable isotopes, ^{13}C, ^{15}N, ^{18}O, and ^{34}S, would be much less than that of deuterium and this has proved to be the case in the limited number of studies carried out to date. In an experiment designed to study the toxic effects of ^{13}C in mice, Gregg et al. (155) maintained one male and one female weanling mouse on a diet in which the digestible carbon fraction averaged 80 atom % excess ^{13}C. Although one animal apparently was accidentally asphyxiated after 127 days, the second survived normally to 234 days; the ^{13}C tissue content of both mice was found to be 60 atom % excess at the time of death, when neither autopsy nor microscopic examination of the tissues revealed any abnormalities that could be attributed to the high isotope enrichment. In more recent work, Gregg and his associates (425) have studied the effects of ^{13}C incorporation into preimplantation mouse embryos on development both before and after implantation. This combined in vitro and in vivo test system, which was selected on account of its high sensitivity to various embryotoxic treatments, failed to reveal any toxicity from ^{13}C at a tissue enrichment of 20 atom % excess. These findings, taken together with similar observations on the effect of algae and yeast grown in media with ^{13}C labelling at above 90 atom % (297), indicate that there is no discernible biological effect of high levels of ^{13}C enrichment (154). The effect of ^{18}O on the growth and reproduction of mice has been studied by Samuel and coworkers (495), who maintained the animals in atmospheres highly enriched in $^{18}O_2$ or who administered $H_2{}^{18}O$ in the drinking water. Over the course of the 112-day experiment, during which three consecutive generations of mice were born, histological examinations showed that no changes had taken place in any of the organs examined; the results of this study thus indicated that mice can survive and reproduce normally even when 60% of their total body oxygen is replaced by ^{18}O.

III. Applications

A. *Quantitative Applications*

1. Internal Standards. In 1940, Rittenberg and Foster demonstrated that ^{15}N-labelled analogs of the amino acids glycine, glutamic acid, and aspartic acid, and a deuterated variant of the fatty acid palmitic acid, could be employed successfully as internal standards for the corresponding endogenous compounds in assay procedures based on mass spectrometry (385). In addition, the authors pointed out that such stable-isotope-labelled analogs should serve as "ideal" internal standards, in that their physicochemical properties would approximate so closely those of their unlabelled counterparts that resolution of the two species would occur only in the final step of the assay, when labelled and unlabelled forms would be distinguished from one another on the basis of their difference in molecular weight. These pioneering studies established the principle of reverse stable isotope dilution analysis,* a technique that, coupled with later developments in chromatographic methods, made a profound impact on quantitative methodology in the life sciences in general, and on pharmacological research in particular. The methods developed by Rittenberg and coworkers, however, were not widely adopted at first, since they entailed tedious isolation and purification of the compounds of interest (together with their internal standards) from the biological matrix, followed by combustion to a suitable gaseous form (e.g. NH_3 or CO_2) for isotope ratio determination. This situation changed dramatically with the advent of combined GC-MS (390, 479), which permitted organic compounds to be analyzed intact (after conversion to more volatile derivatives where necessary), even when present as minor components of complex biological extracts. Furthermore, the development of "ion-specific detection" (selected ion monitoring) capabilities (163, 436) conferred a high degree of sensitivity on the analytical method, when the mass spectrometer was employed as a very specific and sensitive detection system for the gas chromatograph. Thus, by monitoring the variation with time in the ion current at m/z values characteristic of the compound of interest and its isotopically labelled internal standard, it became feasible to measure accurately the ratio between responses in the selected ion currents with samples at the nanogram level and below. With this information, determination of the absolute amount of the unlabelled compound present in the original sample may be made by reference to a standard curve prepared by measuring peak ratios from differing amounts of the compound of interest with a fixed quantity of internal standard.

Two papers appeared concurrently describing the application of this new technique to the quantitative analysis of compounds of biological interest. In one of these Siekmann et al. (417) reported a method for the determination of estradiol and estrone in human plasma, in which known amounts of the corresponding [6,7-$^2H_2$] analogs were added to plasma samples as internal standards; the lower limit of detection of the two estrogens by this method was in the order of 50 pg injected on-column. The second paper, by Samuelsson, Hamberg, and Sweeley (396), dealt with the assay of nanogram amounts of prostaglandin E_1 (PGE_1) and illustrated a different experimental approach, whereby the internal standard was prepared by reaction of PGE_1 with [$^2H_3$] methoxyamine to give the corresponding deuterated methoxime derivative; in this approach, addition of the labelled standard to the endogenous material takes place

* The convention employed in this review is that when the labelled compound serves as internal standard for its unlabelled counterpart, the assay is referred to as a "reverse" stable isotope dilution procedure. Conversely, when the labelled compound is quantified with the aid of a known amount of the unlabelled species as internal standard, the term "direct" stable isotope dilution analysis is used.

only after extraction and derivatization of the latter, and thus losses that occur during these steps are not accounted for in the assay. It is of some interest to note that whereas stable isotope methodology has not yet found widespread application to quantitative studies in the steroid field, investigations of prostaglandin biosynthesis and metabolism have relied extensively on the use of deuterium-labelled compounds as internal standards for assays based on GC-MS (394, 395). Current interest in this area has centered on the development and application of assays for some of the more recently discovered products of the arachidonic acid cascade and their metabolites, e.g., 2,3-dinor-thromboxane B_2, the major urinary metabolite of thromboxane B_2 in the guinea pig (435), and 6-oxo-$PGF_{1\alpha}$, the stable hydrolysis product of prostacyclin (179, 180). In addition, several prostaglandin analogs of potential therapeutic value have been assayed by using deuterium-labelled internal standards, e.g., 15-methyl-$PGF_{2\alpha}$ (279), 17-phenyl-18,19,20-trinor-$PGF_{2\alpha}$ (279), and 16,16-dimethyl-PGE_2 (426).

In parallel with the development of stable isotope methodology for the quantitative analysis of compounds of endogenous origin, similar procedures evolved rapidly for use with drugs and their metabolites. Following the first reported application of stable-isotope-labelled internal standards for drug analysis, which dealt with the determination of nortriptyline in human plasma (135), increasing numbers of investigators turned to the stable isotope GC-MS approach in view of its unique combination of sensitivity, specificity, and versatility. Today, an impressive array of therapeutic agents and their metabolites has been analyzed by reverse stable isotope dilution procedures (77, 95, 145, 315, 365) and it is undoubtedly true that the continuously growing demand for stable-isotope-labelled internal standards (or synthetic intermediates from which they can be prepared) has contributed significantly to the increased commercial production in recent years of organic compounds enriched in deuterium, carbon-13, nitrogen-15, and oxygen-18.

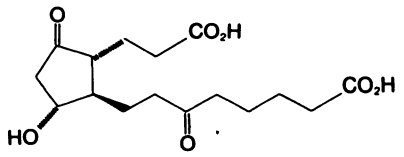
Many of the fundamental aspects of the reverse stable isotope dilution assay technique have been discussed in the text by Millard (314), and several recent papers have dealt with procedures for the treatment of isotope dilution calibration data (72, 372, 404, 407, 408, 504, 53a) and for determining the influence of instrumental variations on the precision of isotope dilution analyses (405). Although there has been considerable debate over the issue as to whether stable-isotope-labelled analogs are indeed superior to other types of internal standards in assay procedures based on GC-MS (314), few investigations have dealt with this question in any depth (67, 145, 269, 448a). In an important study, Markey and coworkers (67) applied variance analysis to the evaluation of precision in selected ion monitoring assays using either stable-isotope-labelled variants or chemically related compounds as internal standards for the tricyclic antidepressant imipramine and its *N*-demethylated metabolite, desipramine. The authors concluded that "stable-isotope-labelled internal standards appear (from this study and others in our laboratory) to produce the lowest variance factors due to long-term, instrumental, and sample manipulation errors." In contrast, Lee and Millard (269) have proposed that "small quantities of a compound are . . . most accurately determined by adding . . . an internal standard giving an ion in common with the compound being measured." The major requirements for this type of internal standard are that it should be a close structural analog of the compound of interest, and that it should be resolved completely from the latter during the gas chromatographic analysis. The advantage of this approach is that the mass spectrometer is tuned to monitor only a single ion during the analysis, with consequent gain in sensitivity and stability. In practice, both stable-isotope-labelled and structural analogs have been employed successfully to measure trace quantities of endogenous or exogenous compounds in samples of biological origin, each approach having its characteristic advantages and disadvantages (314). It is of interest to note in this connection one recent example in which both techniques have been used to quantify the *S*-methylation of cysteine residues in hemoglobin after exposure of rats to the carcinogen methyl methanesulfonate. In preliminary studies, quantification of *S*-methylcysteine, obtained from treatment of globin with 6 M hydrochloric acid, was performed with *S*-[$^2H_3$]methylcysteine as internal standard, and selected ion monitoring GC-MS of the MH^+ ion of the (derivatized) labelled and unlabelled forms with chemical ionization mass spectrometry (117). The analytical method has since been improved through the use of a chiral stationary phase, Chirasil-Val (127, 128), in the GC-MS step (23). In the modified procedure the (unlabelled) D-enantiomer of *S*-methylcysteine is added as internal standard for the *S*-methyl-L-cysteine isolated from hemoglobin; by this means, single ion monitoring of the two enantiomers is made possible, since their *N*-trifluoroacetyl *n*-butyl ester derivatives are completely resolved on the chiral GC column. The authors state that "the use of single ion detection in the assay is three times more sensitive than the use of multiple ion detection with a deuterium-labelled internal standard." It seems likely that this technique will gain in popularity for the assay of optically active compounds, since the enantiomer of a chiral molecule will possess identical physicochemical properties to those of its antipode in a symmetrical environment and therefore will fulfill the requirements of an "ideal" internal standard more closely than can either a structural or stable-isotope-labelled analog. However, it is mandatory to assess experimentally in each case the degree of racemization that occurs during the work-up and gas chromatographic analysis, since this is the principal factor that will determine the feasibility of the method for a given application.

In the development of a GC-MS assay based on the reverse stable isotope dilution principle, several consid-

erations must be taken into account, which may be outlined as follows:

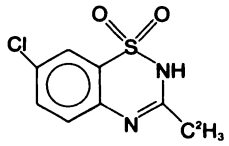
A. The labelled analog should differ in molecular weight from the compound of interest by at least 2, and preferably by 3 or 4 daltons, in order that the m/z value(s) monitored for the internal standard be essentially free of contribution from the unlabelled molecules containing heavy isotopes at the level of their natural abundance (e.g., ^{13}C, natural abundance 1.11%). This requirement is particularly important in compounds that contain chlorine or bromine substituents, due to the high natural abundance of the heavy isotopes of these elements (^{37}Cl, natural abundance 24.47%; ^{81}Br, natural abundance 49.46%). In an assay for the chlorinated antihistamine compound chlorpheniramine, for example, an analog labelled with four atoms of deuterium was employed as internal standard to avoid "crosstalk" between channels (451). It should be noted, however, that internal standards that differ in mass by only 1 dalton have been used successfully, as exemplified by the quantification of 3-phenylpropyl carbamate by use of a [$^{13}C_1$]analog (194) and of 10-hydroxyamitriptyline and 10-hydroxynortriptyline by use of [$^{2}H_1$]variants (143); in the latter case, analysis of the (nonlinear) standard curves was achieved with an iterative computer procedure.

B. The isotopic label(s) must be incorporated at a chemically "stable" position in the molecule, thereby obviating loss of heavy isotope during the analytical procedure. This consideration applies particularly to compounds labelled with deuterium and oxygen-18, where pH-dependent chemical exchange reactions may lead to partial loss of label. A recent example of such a phenomenon is the acid-catalyzed back-exchange of ^{18}O from the carboxyl group of amino acids (334, 335). Interestingly, although no loss of label was observed from the carboxyl group of several [^{18}O]amino acids upon incubation in plasma for three days at 37° C and pH 7.4 (335), the rate of exchange was rapid on incubation with samples of whole blood or cells in tissue culture (70, 334); this finding has been attributed to the possible participation of an amino acid transport system in these cells that catalyzes the formation of a hydrated species of the labelled acid (70). Clearly, therefore, [^{18}O]carboxyl amino acids would be appropriate internal standards for use with plasma samples but would not be appropriate for use with whole blood or suspensions of intact cells. Similarly, deuterium atoms located α to a carbonyl group, while readily introduced synthetically, are susceptible to back-exchange through enolization; compounds labelled in this fashion have been employed as internal standards in certain cases, e.g. in the analysis of 7α-hydroxy-5,11-dioxotetranorprostane-1,16-dioic acid (III), the major urinary metabolite of PGE_1 and PGE_2 in man (460), although stability of label throughout the entire analytical scheme in such cases clearly must be verified prior to use. Less obvious examples of potentially labile sites of ^{2}H substitution have been the deuteromethyl group of



III

[$^{2}H_3$]diazoxide (IV), which underwent partial deuterium exchange on injection of the *N*-methylated derivative into the GC-MS system when methanol was employed as solvent (392), and [3,3,4,4-$^{2}H_4$]$PGF_{2\alpha}$, from which loss of label was noted on prolonged storage in methanolic solution at 0° C, but not at −20° C (76). Certain trideu-



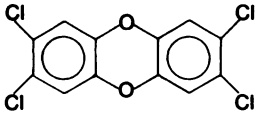
IV

teromethylamines, such as the product formed during pyrolysis gas chromatography of [$^{2}H_3$]propantheline bromide, have been shown to undergo partial exchange of the labelled methyl group when methane (but not ammonia) is used as reagent gas in a chemical ionization (CI) source (123, 124).

C. The internal standard should be labelled to a high degree of isotopic purity, with less than 1% of residual unlabelled molecules, if acceptable "blanks" are to be achieved in the assay. This point is of particular importance if the internal standard is to be added in large excess, when the contribution from the internal standard to the unlabelled channel will determine the lower limit of detection of the assay.

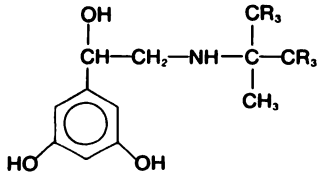
D. The site of labelling must be such that the ion(s) chosen for monitoring retains the heavy atoms. This requirement implies that the major pathways of fragmentation of the compound of interest are understood before synthesis of a labelled analog is undertaken. Should extensive fragmentation occur under conditions of electron impact (EI), "soft" ionization techniques, e.g. chemical ionization (CI), field ionization (FI), field desorption (FD), plasma desorption (PD) (198), or atmospheric pressure ionization (API), may often be employed to obtain prominent molecular ion species. CI/MS has been most widely used in this context, when MH^+ species retain isotopic labels whatever their location in the molecule, and fragmentation of extraneous material present in the sample is also reduced, thus minimizing the risk of nonspecific interference in the channels monitored during GC-MS analysis. It should be noted, however, that although a high proportion of the total ionization in a CI mass spectrum may reside in a single ionic species, e.g. MH^+, this does not necessarily mean that the limit of detection will be lower under CI conditions; a number of factors determine the absolute sensitivity of EI versus CI, including ionization efficiency, and an empirical comparison of the two approaches should be made before one is selected over the other for quantitative purposes (52). Relatively few examples of the use of FI or FD/MS for

quantitative applications have been published (273). Heck and coworkers have reported on the development of sensitive FI/MS assays for the antidepressant imipramine in plasma, in which [$^2H_8$]imipramine served as internal standard (177), and for the hypnotic agent methaqualone in urine, for which a [$^2H_7$] variant was employed for this purpose (383). The determination of the anticancer agent cyclophosphamide by FD/MS has been described by Schulten (409), who employed [$^2H_6$]cyclophosphamide as internal standard. Stable-isotope-labelled analogs would appear to be particularly well suited for use as internal standards in FD work, in view of the often brief and fluctuating ion currents obtained with this type of ionization (273). Finally, API techniques have been employed, together with ^{13}C-labelled internal standards, for the determination of phenytoin in samples of human plasma (203) and for the toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; V) in tissue extracts (319).



V

D. The above considerations regarding positions of labelling versus choice of ionization techniques may be illustrated by reference to two published GC-MS assays for the bronchodilator terbutaline (VI), a [$^2H_6$]analog of which (VII) had been synthesized for use as an internal standard (68, 272). The site of labelling in this compound

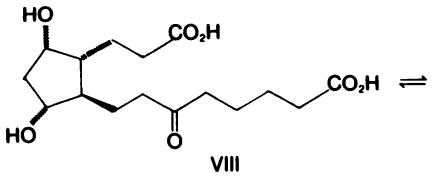


VI R = H

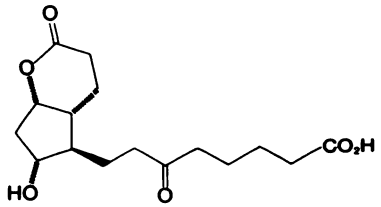
VII R = ^2H

(the *t*-butyl group), while readily accessible from a synthetic standpoint, is not ideal from a mass spectrometric point of view in that fragmentation of the molecule invariably centers around the *N*-*t*Bu function; thus, the EI mass spectra of a variety of derivatives of terbutaline display intense fragment ions at m/z 57, $[C(CH_3)_3]^+$, and m/z 86, $[CH_2 = {}^+NH\text{-}C(CH_3)_3]$, which are shifted to m/z 63 and m/z 92, respectively, in the spectra of the deuterium-labelled compound (68). Selected ion monitoring at such low m/z values is frequently hampered by extensive interference from "chemical background" in the sample and indeed terbutaline could not be quantified in plasma samples by EI/MS in the concentration range of interest (0.5 to 15 ng ml^{-1}) by the reverse stable isotope dilution approach. (An alternative procedure, in which a structural analog was employed, proved successful (68).) In contrast, the *tris*-*O*-TMS derivative of the drug gave an intense MH^+ ion at m/z 442 under CI (methane) conditions, and a GC-MS assay was developed accordingly in which selected ion monitoring of the MH^+ species of the unlabelled and [$^2H_6$]terbutaline derivatives permitted determination of serum levels down to values as low as 0.1 ng ml^{-1} (271, 272). Very recently, Lindberg and coworkers (211) have reported on an analogous procedure for the determination of terbutaline in human plasma, based on the use of GC-MS with ammonia as reagent gas.

An alternative approach to the use of "soft" ionization conditions for obtaining ions suitable for use in reverse stable isotope dilution assay procedures is to prepare derivatives that strongly direct fragmentation under electron impact. One such example is the *t*-butyldimethylsilyl (*t*-BDMS) ether, the mass spectra of which are frequently dominated by intense $[M\text{-}57]^+$ ions, formed through elimination of the *t*-butyl radical from the $M^{+\cdot}$ species. The $[M\text{-}57]^+$ fragment will retain the isotopic label(s) and will therefore be suitable for monitoring purposes. Recent examples of the use of this derivative for quantitative applications have been to the assay of 5α,7α-dihydroxy-11-oxotetranorprostane-1,16-dioic acid (VIII), the major urinary metabolite of $PGF_{1\alpha}$ and $PGF_{2\alpha}$ in man (41), 16,16-dimethyl-PGE_2, an experimental abortifacient agent (426), and the novel steroid 16α-cyano-3β-cyclopentyloxy-5-pregnen-20-one, from which a *t*-BDMS oxime derivative was prepared for GC-MS analysis (146).

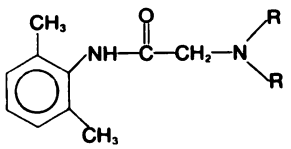


VIII



VIIIa

Despite the desirability of performing selected ion monitoring analyses with ions of relatively high m/z ratio, it should be noted that several assays have been reported in which low mass ions (m/z < 100) have been employed successfully. Lidocaine (IX) and [$^2H_{10}$]lidocaine (XI), for example, have been determined by GC-MS by use of the prominent ions at m/z 86 and 96, respectively, derived from side-chain cleavage (100). In general, however, specificity of GC-MS assays falls off rapidly at low concentrations of the analyte when the mass of the ion(s) being monitored is reduced below m/z 200.



IX R = C_2H_5

X R = $CH_2C^2H_3$

XI R = $C_2{}^2H_5$

E. The actual choice of isotopic label (i.e., ^2H, ^{13}C, ^{15}N, etc.) and its site of incorporation into the internal stan-

dard will be dependent upon all of the above considerations. Deuterium has been most widely used for this purpose, due to its availability at high isotopic purity in a large variety of chemical reagents and synthetic intermediates. Deuterium is also relatively inexpensive and can usually be introduced at multiple sites in a molecule, often by simple exchange reactions (449). The major disadvantage of deuterium lies in its susceptibility to loss during the analytical procedure by way of chemically or thermally mediated back-exchange processes. Oxygen-18, as noted above, suffers from the same drawback. Although the introduction of each atom of ^{18}O is accompanied by an increment of 2 daltons over the corresponding unlabelled material, there have been few examples of ^{18}O-labelled compounds being employed as internal standards (69, 335). In a recent paper, De Leenheer et al. (79) compared [^{18}O]fluorouracil and chlorouracil as internal standards in a GC-MS assay for fluorouracil in human plasma. While both compounds proved to be satisfactory for this purpose, difficulties encountered with the preparation of isotopically pure [^{18}O]fluorouracil led to selection of the chlorinated analog for use in the assay.

Internal standards labelled with ^{13}C or ^{15}N are clearly superior from the standpoint of stability of label, although incorporation of these isotopes at multiple sites in the compound of interest is usually more difficult synthetically and is more expensive than multiple labelling with ^{2}H or ^{18}O. Several assays have been described, however, that have been based on the use of internal standards labelled with multiple atoms of ^{13}C, e.g. [2,4,5-^{13}C]phenytoin has served as internal standard for amobarbital, secobarbital, caffeine, meperidine, and phenobarbital in small (50 to 500 μl) samples of plasma and breast milk (201), [2,4,5-$^{13}C_3$]pentobarbital and [2,4,5-$^{13}C_3$]phenobarbital have been used to quantify the corresponding unlabelled barbiturates in human amniotic fluid (424), a recently developed capillary column GC-MS assay for the β-blocking drug timolol utilizes a [$^{13}C_3$] analog as internal standard (58), and a similar assay, based on the use of API techniques, employs a [$^{13}C_{12}$] variant as internal standard for the determination of trace levels of TCDD (V) in tissue samples (319). An improved procedure for the quantitative determination of clonazepam, based on negative ion CI/MS, employs a multiply labelled species, [^{15}N, $^{18}O_3$]clonazepam, as internal standard (142). This contrasts with an earlier method developed by the same authors in which [^{15}N] clonazepam was used in a positive ion CI/MS assay (317); the singly labelled clonazepam could not be used in the negative ion CI/MS procedure due to the presence of both M^- and $[M - 1]^-$ ions in the negative ion CI/MS spectrum of the drug and its ^{15}N-labelled counterpart. Multiply labelled ([2-^{13}C, 1,3-$^{15}N_2$]) analogs of phenobarbital and its metabolite *p*-hydroxyphenobarbital are now available commercially and have been employed recently as internal standards in a GC-MS assay for the parent compounds in biological fluids (369).

Difficulties encountered in the development of reverse stable isotope dilution assays for polychlorinated compounds, in which broad isotope clusters result from the presence in the unlabelled compound of ^{37}Cl at the level of its natural abundance, have been resolved in the case of TCDD by the preparation of an analog highly enriched in ^{37}Cl and, in addition, labelled with deuterium, [$^{2}H_4$, $^{37}Cl_4$]TCDD (151). By use of this species as internal standard, virtually no cross-contribution was noted in the channels corresponding to unlabelled and labelled TCDD during GC-MS analysis of commercial samples of chlorinated pesticides.

F. In cases where it is not practical to prepare a stable-isotope-labelled analog of the compound of interest as an internal standard, an alternative approach may be adopted in which a labelled derivative may be added to the (previously derivatized) sample. This is the method originally reported by Samuelsson et al. for PGE_1 (396) and, as has been discussed above, suffers from the disadvantage that losses that occur before the labelled compound is added are not accounted for. Such losses, however, may be determined (and thus corrected for) by the additional use of an appropriate radiotracer added at the outset to the biological sample (420). As examples of this approach may be cited methods for the analysis of homovanillic acid in cerebrospinal fluid (420), $PGF_{2\alpha}$ in plasma (148), estrogens in urine (2), and Δ^9-tetrahydrocannabinol in plasma (388).

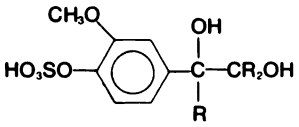
From the foregoing discussion, it will be evident that successful implementation of mass spectrometric assay procedures based on the reverse stable isotope dilution approach requires careful consideration of a number of factors. One aspect, which has been mentioned only briefly above, relates to the synthesis of the required labelled material. Relatively few organic compounds of pharmacological interest, labelled with stable isotopes, are available commercially and internal standards usually have to be synthesized in the investigator's own laboratory. It is this specialized and labor-intensive aspect of work with stable isotopes that contributes most to the high effective cost of isotopically-labelled internal standards when compared with, e.g. structural analogs of the compound of interest. The expense of the necessary analytical instrumentation, together with the skilled personnel required for its operation, are other obvious disadvantages associated with the use of stable isotopes for quantitative applications in pharmacological research. In contrast, assay procedures based on mass spectrometry with reverse stable isotope dilution offer a number of important advantages when compared with most other commonly used analytical techniques. These advantages can be classified under four broad categories as follows:

SENSITIVITY. Mass spectrometry is an inherently sensitive technique and assays based on the reverse stable isotope dilution approach invariably take full advantage of this feature. Reported detection limits for endogeneous or exogeneous compounds in biological samples have

fallen steadily over the years. In 1974, Horning et al. (203) reported a lower limit of detection for phenobarbital of 80 pg injected on-column, although 150 to 200 pg was necessary for work with biological extracts. [$^{13}C_3$]Phenobarbital served as internal standard in the assay, which was based on CI/MS and employed 100 to 200 μl samples of plasma. The following year, Bayne et al. (30) described a method for the determination of scopolamine in plasma and urine, in which a concentration of 50 pg ml^{-1} could be measured by GC-EI/MS, with a 4-ml sample and a [$^2H_3$] analog as internal standard. Recent advances in negative ion CI/MS (208) have led to the development of an assay for melatonin in human plasma that may be used to determine concentrations as low as 1 pg ml^{-1} in 1 ml samples (275); once again, a stable-isotope-labelled analog, [$^2H_4$]melatonin, was selected for use as internal standard in the GC-MS assay, in which the amount of sample actually injected into the instrument was assessed to be as little as 200 fg (200×10^{-15} g). The role of the isotopically labelled internal standard in enhancing the sensitivity of such assays has been hotly debated in recent years and is referred to below under "carrier effect."

SPECIFICITY. The specificity of selected ion monitoring GC-MS assays is exceedingly high and is based on three parameters: 1) the compound being assayed must possess the correct gas chromatographic retention time, 2) maxima in the ion currents at m/z values characteristic of the compound of interest must appear at that retention time, and 3) the relative intensity relationship between these characteristic ions must be the same as that in the mass spectrum of the compound of interest. The internal standard, whether isotopically labelled or a structural analog of the compound of interest, must also fulfill the same requirements. However, minor fluctuations in GC or MS operating conditions that occur during the analysis are much less likely to affect quantitative determinations when a stable-isotope-labelled variant is used, due to the close similarity in retention times between labelled and unlabelled molecules, than is the case with a structural analog where retention times may differ by several minutes.

One example of an assay procedure that employed GC-MS and a stable-isotope-labelled internal standard purely on the grounds of specificity (as opposed to sensitivity) is that reported by Murray et al. (337) for MHPG



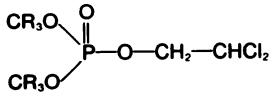
XII R = H
XIII R = ^2H

sulfate (XII), a major urinary metabolite of norepinephrine in man. Concern over the specificity of existing methods for the determination of this conjugate led to the synthesis of a [$^2H_3$] analog of MHPG sulfate (XIII) and to the development of a liquid chromatographic procedure by means of which the sulfate and its internal standard could be isolated from urine, free from unconjugated MHPG and MHPG glucuronide. Furthermore, by means of a novel one-step derivatization reaction (336), MHPG sulfate and its internal standard were converted to their respective *tris*-trifluoroacetate derivatives for GC-MS analysis, without recourse to enzymic hydrolysis procedures that had proven to be unreliable. Application of this highly specific assay to a study of MHPG and its conjugates in samples of human CSF revealed that, contrary to previous reports, MHPG sulfate was not the major form in this fluid; indeed, MHPG sulfate was barely detectable in CSF, while the glucuronide accounted for almost all of the conjugated MHPG (26). Extension of the assay to investigations of stereochemical aspects of MHPG metabolism has recently been made possible by the use of a chiral stationary phase in the GC-MS analysis; with this methodology, it has been shown that urinary excretion of MHPG sulfate in the dog is markedly stereospecific, favoring elimination of the naturally occurring enantiomer (338).

The MHPG sulfate assay cited above appears to be the first in which a stable-isotope-labelled analog of an intact conjugate has been employed for quantitative purposes. Analogous procedures have now been developed for 6-hydroxymelatonin sulfate, a unique urinary metabolite of the putative pineal hormone melatonin, in which deuterium-labelled variants serve as internal standards (122, 448).

Finally, an interesting technique for verification of the specificity of GC-MS analyses has been reported by Miyazaki and coworkers (209). In a study of the profile of bile acids in rat serum, methylation of the extracted and purified bile acids was carried out with an equimolar mixture of methanol and [$^2H_3$]methanol. The resulting esters were then analyzed, after conversion to trimethylsilyl ether derivatives, by selected ion monitoring GC-MS when pairs of ions characteristic of unlabelled and corresponding trideuterated bile acid derivatives were monitored. Any deviation from the expected 1:1 intensity ratio between such pairs of ions was taken as evidence for the presence of interfering components in the biological extract that contributed to the ion current at one or both of the m/z values monitored.

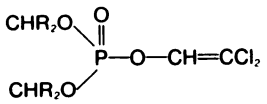
One area of investigation to which stable isotope labelling procedures have made a particularly valuable contribution in terms of specificity has been the study of compounds that are susceptible to chemical transformation during the extraction and/or work-up procedures. This problem may be exemplified by investigations of the organophosphorus compound metrifonate (XIV),



XIV R = H
XV R = ^2H

which has proved effective clinically in the treatment of schistosomiasis. It has been claimed, however, that the pharmacological activity of this drug (a cholinesterase

inhibitor) is not associated with the parent compound, but with its dehydrochlorinated product, dichlorvos (XVI). Since this transformation is known to occur nonenzymatically under both aqueous and anhydrous conditions, investigations of the pharmacokinetics and mode of action of metrifonate must be carried out by techniques that take into account such transformation during the work-up of biological samples. Holmstedt and coworkers (191, 358) have approached this problem by using GC-MS with specifically deuterium-labelled analogs of metrifonate and dichlorvos. [$^2H_6$]Metrifonate (XV) is added to biological samples as an internal stan-



XVI R = H

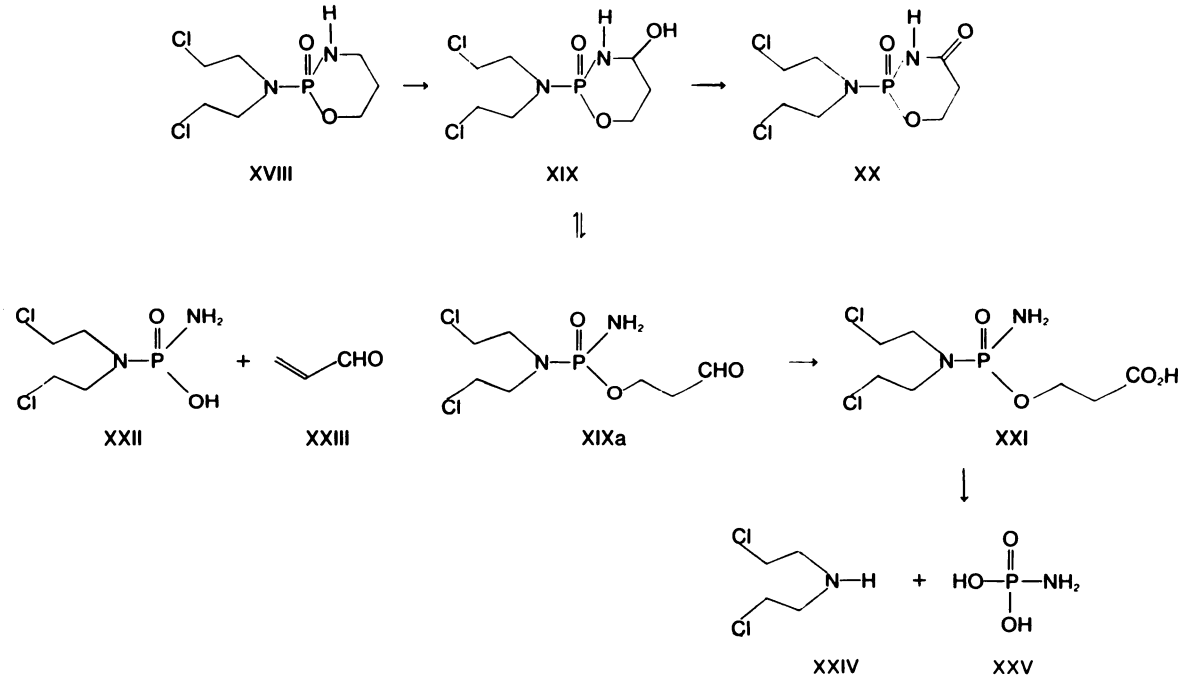
XVII R = ^{2}H

dard for the parent drug, while [$^2H_4$]dichlorvos (XVII) serves as internal standard for the degradation product. During the work-up process, some metrifonate will be converted to dichlorvos, and hence the final extract subjected to GC-MS will contain both unlabelled and [$^2H_6$] dichlorvos, in addition to the tetradeuterated internal standard. From the amount of [$^2H_6$]dichlorvos produced from the known quantity of [$^2H_6$]metrifonate added at the outset, the extent of chemical transformation of metrifonate can be determined and this value can then be used to correct for the *unlabelled* dichlorvos produced from unlabelled drug during the work-up. This approach has been used successfully to study the pharmacokinetics of metrifonate in man (359).

Chemotherapeutic alkylating agents and their metabolites frequently exhibit chemical instability and therefore are best quantified by the use of reverse stable isotope dilution assay procedures. Cyclophosphamide (XVIII), an antineoplastic agent that requires metabolic activation, is one such example (fig. 1). The metabolites 4-oxocyclophosphamide (XX), carboxyphosphamide (XXI), phosphoramide mustard (XXII), and nornitrogen mustard (XXIV) occur in biological fluids at relatively low levels, are highly polar compounds, are difficult to purify, and are believed to decompose easily. The use of stable-isotope-labelled internal standards has permitted the development of specific mass spectrometric assays for these compounds, which would otherwise have been very difficult to achieve (212, 213, 216). A method for the analysis of the chloroethylnitrosourea, BCNU, provides a further example of the use of deuterium-labelled internal standards for the assay of chemically reactive compounds (485).

The prostaglandin F metabolite, 5α,7α-dihydroxy-11-oxotetranorprostane-1,16-dioic acid, illustrates a different analytical problem in that while this compound does not decompose chemically, it exists in aqueous solution as a mixture of two forms, having "open-chain" (VIII) and δ-lactone (VIIIa) structures, respectively. Since the position of the equilibrium between the two species is dependent on pH, any internal standard adopted for use in an assay for this metabolite would have to exhibit similar chemical characteristics. A deuterium-labelled analog of the metabolite was thus adopted as being the "ideal" internal standard in this case and was used to quantify the metabolite in samples of human urine at the 10 ng ml^{-1} level (41).

CARRIER EFFECT. The so-called "carrier effect" refers to the ability of a stable-isotope-labelled analog, usually added to the sample in large excess, to reduce losses of the compound of interest through adsorption processes occurring during extraction, work-up, and derivatization



XVIII XIX XX

XXII XXIII XIXa XXI

XXIV XXV

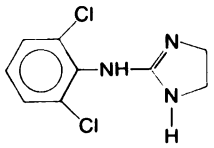
FIG. 1. The metabolism of cyclophosphamide (XVIII).

steps. In their original article on the quantification of PGE_1 by reverse stable isotope dilution GC-MS, Samuelsson et al. (396) employed a large excess of the deuterium-labelled analog to minimize "adsorption and destruction on the column" and thereby to increase the usefulness of the GC-MS approach. The labelled prostaglandin used in this work thus served a dual function, viz, 1) as an internal standard for the quantitative determination of endogeneous PGE_1 and 2) as a "carrier" molecule that acted to minimize losses of the small amounts of the unlabelled species being chromatographed. The term "carrier" rapidly became synonymous with "internal standard" where stable-isotope-labelled analogs were used in quantitative studies, this being especially true in the prostaglandin field. In few cases, however, was any evidence presented to show that a "carrier" effect was indeed operative, although Draffan et al. (101, 103) stated that the hypotensive agent indoramin exhibited such poor chromatographic properties that it could not be analyzed reproducibly by GC-MS carrier-free. Also, a stable-isotope-labelled internal standard was found to be essential for analysis of thyroid hormones at the trace level (371), and a marked improvement was noted in the linearity of standard curves when deuterated chelates were employed as internal standards for various tetraphenylporphyrin metal chelates (93).

In a study by Lee and Millard (269) on levels of $PGF_{2\alpha}$ in human cerebrospinal fluid, $[^2H_4]PGF_{2\alpha}$ was employed as a "carrier" only, and quantification of the endogenous prostaglandin was effected using a structural analog, ω-trinor-16-cyclohexyl $PGF_{2\alpha}$, as internal standard; the latter compound was chosen since it yielded a common fragment ion to unlabelled $PGF_{2\alpha}$, thus permitting the use of single (as opposed to multiple) ion monitoring GC-MS for the assay, with concomitant gain in sensitivity. However, it was observed that the labelled "carrier" was not, in fact, necessary for accurate and precise quantitative measurements of $PGF_{2\alpha}$. This finding was contrary to expectations and raised some question as to the validity of the assumption that isotopically labelled analogs necessarily act as "carriers" in such work. A follow-up study, specifically designed to address this question, showed that in the case of octopamine, no "carrier" effect was evident at either the solvent extraction or GC-MS steps when a deuterium-labelled analog was used (316). Shortly after this work had been published, Haskins et al. (171) added to the controversy over the role of stable-isotope-labelled analogs as "carriers" by demonstrating that when a deuterated analog was used as internal standard for the antidiarrheal agent diphenoxylate, a "carrier" effect was indeed operative and resulted in a sevenfold increase in sensitivity relative to alternative procedures not using the labelled compound. These phenomena were attributed by the authors to serious absorption of diphenoxylate during gas chromatography, a minimum injection of more than 30 ng of the compound being required in order to observe a signal at the detector. An interesting polemic on the uses and functions of deuterated analogs in quantitative mass spectrometry has been published recently by Self (413), in which the results of the above studies are discussed critically.

From the limited information currently available, it would appear that stable-isotope-labelled analogs may, in certain cases, fulfill the role of "carrier" substances for their unlabelled counterparts during one or more steps of the analytical procedure. Whether or not a "carrier" effect does operate must be determined experimentally for each compound of interest. In cases where no such effect can be demonstrated, there would appear to be little justification for the use of a large excess of the labelled species, particularly since "blank" values due to small amounts of residual unlabelled molecules in the internal standard will be greatly magnified if large quantities of the latter are used. Clearly, more experimental information is necessary before this important subject can be well understood.

DIRECT STABLE ISOTOPE DILUTION. As its name implies, this technique refers to the use of an unlabelled compound as internal standard for quantification of a corresponding stable-isotope-labelled analog. Although not widely employed as yet, this approach offers several advantages for quantitative studies of drug metabolism. Thus, use of an appropriate stable-isotope-labelled analog of the drug as metabolic substrate will lead to the formation of corresponding labelled metabolites, which can be quantified by mass spectrometry after addition of known amounts of the unlabelled compounds as internal standards. Since unlabelled metabolites are frequently available from preliminary qualitative studies on metabolism of the drug, only the labelled drug itself need be synthesized for such work; in contrast, the reverse stable isotope dilution method would require the synthesis of labelled analogs of each metabolite to be determined, a substantially greater undertaking. An absolute requirement of the direct stable isotope dilution approach, of course, is that labelled and unlabelled forms of the drug are metabolized in an identical fashion, i.e. there is no isotope effect on metabolism. As an early example of the effective use of this technique may be cited the studies by Baba and colleagues on the metabolism of *l*-ephedrine in man, in which *l*-$[^2H_5]$ephedrine was employed as met-



XXVI

abolic substrate (17, 243). Samples of urine from volunteers given the labelled drug were treated with appropriate quantities of known (unlabelled) metabolites and the resulting mixtures were then processed for quantitative GC-MS analysis. Other instances where this technique has been used include studies of the metabolism in vitro of 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane, a

psychotomimetic amine (138, 507), clonidine (XXVI), a potent antihypertensive agent (27, 102), a number of barbiturates (301–303), and phenytoin (300).

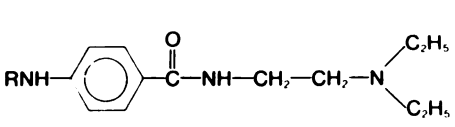
Although multiple deuterium labelling was employed in each of the above investigations, an analog of 3-phenylpropyl carbamate, labelled with a single atom of ^{13}C, has been employed recently to study the metabolic fate of this muscle relaxant in human subjects (196). In this report (196), as in those on *l*-ephedrine metabolism (17, 243), emphasis was placed on the value of administering a labelled form of the drug when known or suspected metabolites are also present as endogeneous components. Thus, both ephedrine and 3-phenylpropyl carbamate are metabolized to benzoic and hippuric acids, of which some 23 mg day^{-1} and 155 mg day^{-1}, respectively, are excreted in the urine without drug administration. The use of a stable-isotope-labelled drug is therefore necessary in such cases where exogeneous and endogeneous products are to be distinguished and quantified; a second labelled variant of the metabolite could ideally be used as an internal standard in such situations.

2. Stable Isotopes in Pharmacokinetic Studies. Requirements for the determination of drug levels in biological fluids for pharmacokinetic studies have traditionally imposed severe demands on the analytical methodology employed for such work in terms of specificity and sensitivity of detection. The latter requirement has become especially important in recent years as a consequence of current trends in the development of highly potent therapeutic agents that are administered in milligram to submilligram doses and give rise to plasma concentrations in the ng ml^{-1} range or below. Quantitative mass spectrometric assay procedures involving the reverse stable isotope dilution approach have made, and no doubt will continue to make, extremely valuable contributions to this area of research. Studies on the pharmacokinetics in man of clonidine (XXVI) effectively illustrate this point, since, without a highly sensitive and specific GC-MS assay method (102), plasma concentrations resulting from normal therapeutic doses (300 μg day^{-1} orally) could not be followed for sufficiently long time periods to provide meaningful kinetic data (92).

In addition to their use as internal standards in assay procedures, however, stable-isotope-labelled compounds have permitted the development of specialized methodology for the study of bioavailability (absolute and relative), steady state kinetics, the origin of drug tolerance, and the differential metabolism of optical isomers of a drug administered as a racemic mixture (332).

BIOAVAILABILITY. In 1975, Atkinson and coworkers (430) introduced a novel technique for the study of the absolute bioavailability in man of *N*-acetylprocainamide (NAPA; XXVII), a pharmacologically active metabolite of the antiarrhythmic agent procainamide (XXVIII). Their procedure entailed the i.v. injection of an analog of NAPA, singly labelled with ^{13}C, at the same time that a capsule of the unlabelled drug was given orally. Plasma levels and urinary excretion of labelled and unlabelled NAPA were then determined by GC-MS, with [$^{2}H_5$] NAPA as internal standard, thereby providing information on the pharmacokinetics of oral and i.v. NAPA in the same subject determined at the same time. Deconvolution of plasma level versus time curves and comparison of the relative amounts of NAPA and [^{13}C]NAPA excreted unchanged in urine over 24 hours provided information on the absolute bioavailability of the oral dose. Interestingly, this study showed not only that there were large interindividual differences in NAPA kinetics, but also that the kinetics were dependent upon the timing of drug administration. The major advantage of this approach to performing bioavailability studies is that it dispenses with the necessity to perform two individual studies in each subject, where an i.v. and an oral test dose are administered on different occasions, separated by a suitable "wash-out" period. In addition to reducing the total number of blood and urine samples by a factor of two (which is clearly more attractive to the subject, to the clinician performing the study, and to the analyst), this stable isotope technique also eliminates the critical assumption in the classical approach that the kinetics of drug absorption, distribution, and elimination remain unchanged in the interval between doses.

In an extension of the method reported in their first paper, Atkinson and coworkers (106) subsequently reported on the kinetics of procainamide and NAPA, determined simultaneously in each of three normal subjects after i.v. administration of a mixture of unlabelled procainamide and [^{13}C]NAPA. This study provided the first definitive comparative data on procainamide and NAPA kinetics in man and showed that the elimination half-life of NAPA was 2.5 times that of procainamide in healthy subjects. The stable isotope methodology employed in this work was central to the design of the study in that it allowed the exogenously administered drug ([^{13}C] NAPA) to be distinguished from the endogenously formed procainamide metabolite (unlabelled NAPA). Although this technique would appear to have widespread applicability to various pharmacokinetic problems, only one other example of its use has been published, in which [^{13}C]NAPA was used to estimate the clearance of NAPA in man via deacetylation to procainamide, without interference from subsequent reacetylation of the metabolite (425a). (This work represented the stable isotope counterpart of an earlier study performed in monkeys by

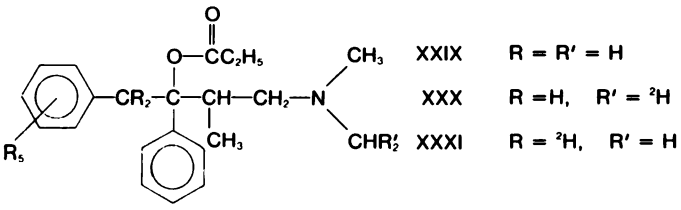


XXVII R = $CH_3—\overset{O}{\overset{\|}{C}}—$

XXVIII R = H—

using [^{14}C]NAPA (98a)). The absolute bioavailability of a novel antidiarrheal agent, SC-27166, has been studied in the rat, beagle dog, and baboon through simultaneous administration of unlabelled and tetradeuterated forms by i.v. and oral routes (170). [$^2H_6$]SC-27166 was employed as internal standard for quantification of each molecular species by GC-MS. Comparison of the results obtained in this study with those carried out independently in the rat and rhesus monkey with a radiolabelled analog of the drug showed good agreement between the two methods. Recently, Eichelbaum et al. (112) have employed the stable isotope approach to determine simultaneously the i.v. and oral pharmacokinetic parameters of verapamil, an antiarrhythmic and antianginal drug that selectively inhibits membrane transport of calcium. The bioavailability of different verapamil tablets has also been determined by this group, who used a solution of deuterated verapamil as the reference formulation (109). It seems likely that the use of stable isotope labelling procedures in studies of absolute bioavailability will increase in the near future, as indicated by recent reports from various laboratories on the development of the necessary analytical methodology for such work (5, 58, 464).

Studies on the relative, as opposed to absolute, bioavailability of, e.g. several different formulations of the same drug, may also be performed effectively by use of a stable-isotope-labelled variant as the reference against which the test unlabelled formulations are compared. This approach was employed by Heck et al. (176) to determine the comparative bioavailabilities of two commercial formulations of imiprimine hydrochloride, in which the test samples were administered as tablets and [$^2H_2$]imipramine was given orally in solution. Levels of labelled and unlabelled imipramine in plasma were then determined by reverse stable isotope dilution analysis, with [$^2H_6$]imipramine as internal standard. The methodology employed in this study is noteworthy in that



XXIX R = R′ = H
XXX R = H, R′ = ^{2}H
XXXI R = ^{2}H, R′ = H

analyses were performed by field ionization mass spectrometry (177). Wolen et al. (494) have also used stable isotopes to study differences in the relative bioavailability of different salt forms of dextropropoxyphene (XXIX) in man; by administering orally mixtures of unlabelled and [$^2H_7$]dextropropoxyphene (XXXI) as their hydrochloride and napsylate salts and measuring the ratio of labelled and unlabelled drug in plasma, it was found that the napsylate salt is absorbed more slowly during a short initial period, although with little effect on the overall blood levels when compared with the hydrochloride salt. The same group later reported on the effect of crystal size on the relative bioavailability of a new anti-inflammatory agent, benoxaprofen. In preliminary animal experiments, dogs were given [$^2H_3$]benoxaprofen as an oral solution together with an equivalent amount of the test (unlabelled) drug in capsule form (493). Analysis of plasma levels of the unlabelled drug relative to the reference deuterated species revealed that the larger of two crystal aggregates studied was less available and was much more variable in bioavailability. Later experiments in human subjects, which were conducted with [$^2H_7$] benoxaprofen as the reference compound, gave a similar result and indicated that while the smaller crystals had an almost complete availability (0.95 to 0.98) relative to the unlabelled form, the larger crystals were much less available (0.41 to 0.46) (492).

While the use of the above stable-isotope-labelling procedures offers considerable advantages over classic techniques for the study of both relative and absolute bioavailability, it is imperative that the bioequivalence of labelled and unlabelled forms of the drug be established before such investigations are conducted. Isotope effects, as will be discussed below, can have a marked influence on the rates of absorption, distribution, and elimination of a drug, particularly where deuterium is employed as the heavy isotope (204, 471). Thus, when a labelled variant is required for bioavailability studies or for any other type of investigation where administration of both labelled and unlabelled compounds is involved, it is important to introduce the isotopic marker at a site(s) remote from positions where metabolic attack is likely to take place. This precaution thus minimizes the risk that primary deuterium isotope effects will render the labelled compound pharmacokinetically distinct from its unlabelled counterpart. A procedure commonly used to verify the absence of an in vivo isotope effect with deuterated compounds is to administer an equimolar mixture of labelled and unlabelled species and to follow the ratio of the two forms in serial plasma samples and urine collections; if a 1:1 ratio is maintained throughout the experiment, the labelled compound can be considered to be pharmacokinetically equivalent to the natural form (18, 33, 108, 310, 323, 433, 492). The use of stable isotopes other than deuterium is clearly preferable for such applications, since isotope effects associated with ^{13}C, ^{15}N, ^{18}O, etc. are very much smaller. With this consideration in mind, [$^{13}C_3$]timolol (58) and [^{13}C, ^{15}N]disopyramide (172) have been synthesized for use in bioavailability studies and have been shown to be kinetically equivalent in vivo to unlabelled timolol and disopyramide, respectively.

An additional factor that may complicate bioavailability studies performed by stable isotope techniques arises where the drug of interest is subject to a saturable "first-pass" effect. Thus, Schmid et al. (401), in an investigation of the pharmacokinetics in humans of methoxsalen (8-methoxypsoralen), found that simultaneous oral administration of [$^2H_3$]methoxsalen in solution with a proprietary brand of the drug in tablet form gave the surprising

result that the methoxsalen tablets exhibited a greater apparent bioavailability than did the solution. After confirming the absence of a deuterium isotope effect for the labelled compound and checking the content uniformity of the tablets employed, the possibility that a "first-pass" effect was responsible was investigated. When solutions containing 20 mg each of unlabelled and deuterated drug were administered some 37 minutes apart, the form contained in the second dose yielded plasma concentrations that were two- to threefold higher than were attained by the form contained in the first. These results were taken to show that methoxsalen is indeed subject to a saturable "first-pass" effect, probably as a result of hepatic, as opposed to gut wall, metabolism. Similarly, studies on the absolute bioavailability of disopyramide phosphate in animals have indicated that the area under the plasma concentration versus time curve for the orally administered form was dependent upon the plasma concentrations generated by the i.v.-administered dose (173); the authors thus concluded that the stable isotope approach is not suitable for bioavailability studies of compounds of this type. A third example of this phenomenon has been described by Wolen et al. (494), who used stable isotope labelling procedures to show that plasma concentrations of *d*-[$^2H_7$]propoxyphene, resulting from oral administration of the drug, could be maintained at higher levels when the *d*- or *l*-form of the drug was coadministered by the same route. Finally, differences in the degree of protein binding may occur with deuterium-labelled drugs, as shown by Thenot et al. (448a), who found that [$^2H_{10}$]phenytoin is protein-bound in human plasma to a significantly greater extent than is the corresponding unlabelled compound.

Although "saturation" phenomena of the types described above may prevent the application of the stable isotope approach to studies of relative or absolute bioavailability, investigations of nonlinear pharmacokinetics can nevertheless benefit from the use of multiple isotopic variants of the drug in question. Thus, Higuchi and Shiobara (181) studied the pharmacokinetics of nicardipine, a potent vasodilator, in dogs by the use of unlabelled and deuterated forms of the drug; in this work, a large dose of the unlabelled compound was given orally in order to produce "saturation" conditions with respect to hepatic metabolism, after which a second dose (oral or i.v.) of [*N*-C$^2H_3$]nicardipine was administered and its kinetics were determined by GC-MS methods. By means of this technique, it was possible to obtain various pharmacokinetic parameters for nicardipine under "saturation" conditions. It is of interest to note that for this drug, no in vivo deuterium isotope effect was observed when [*N*-C$^2H_3$]nicardipine was given, although the corresponding [*N*-$^2H_7$ benzyl]analog exhibited an apparent kinetic isotope effect of $k_H/k_D = 1.37$.

STEADY STATE KINETICS. Stable-isotope-labelled drugs may also be used profitably to investigate differences in pharmacokinetics occurring between acute and chronic administration. This technique, which was proposed originally by McMahon et al. (312), entails administration of a labelled analog of the drug as a single substitute dose during chronic therapy. Analysis of plasma and urine samples for this labelled form thus allow one to study the fate of this "pulse" dose independently of the unlabelled drug being given on a multidose schedule. Application of this procedure to studies of the steady-state pharmacokinetics of propoxyphene (XXIX) in dogs has been reported by Sullivan et al. (432, 433). Dogs treated daily with the unlabelled drug were administered a single "pulse" dose of [$^2H_2$]propoxyphene (XXX) on day 20, when blood levels of the deuterated species were measured in timed samples by selected ion monitoring GC-MS with [$^2H_7$]propoxyphene (XXXI) as internal standard. This study showed that, following an initial rapid equilibrium phase, levels of [$^2H_2$]propoxyphene fell more rapidly than those of the unlabelled drug. The authors inferred from this result the existence of "deep" pools of tissue-bound propoxyphene that exchange very slowly with drug present in the central compartment. A similar technique has been used to study the pharmacokinetics of methadone in opiate-dependent subjects during maintenance therapy (9, 431) and a number of reports have appeared recently on the application of stable isotope methodology to studies of the steady-state kinetics of a variety of anticonvulsant agents, including carbamazepine (33, 110, 111), phenobarbital (150, 237), phenytoin (463), and valproic acid (189, 466).

In an interesting application of the same technique, Baba et al. (16) have studied the origin of the tolerance developed by rats towards the analgesic agent 1-butyryl-4-cinnamylpiperazine during chronic administration of the drug. A dideutero analog was used to develop tolerance and a pentadeutero analog was then administered to investigate pharmacokinetics and metabolism under steady-state conditions. By means of a direct stable isotope dilution approach in which unlabelled drug and its metabolites were employed as internal standards for the corresponding [$^2H_2$] and [$^2H_5$] forms, it was shown that liver and brain tissue from the tolerant rats contained much lower levels of the parent drug than did tissues from the nontolerant animals, whereas the levels of all four metabolites were higher in the tolerant group. On the basis of these results, the authors proposed that development of tolerance to this drug results from an increase in its rate of metabolism due to hepatic enzyme induction. In a related study, Bertilsson et al. (33) have demonstrated that autoinduction of carbamazepine metabolism in children occurs rapidly and is complete within one month after initiation of therapy; it is therefore important to note that single oral-dose kinetics determined before initiation of long-term treatment cannot be used as a basis for predicting steady-state plasma levels of this drug.

3. Differential Metabolism of Optical Isomers. Enantiomeric differences in the metabolism and disposition of

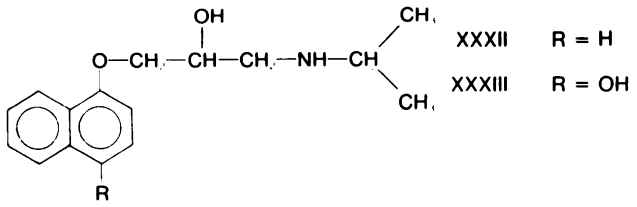
racemic drugs is an area of growing interest and a number of analytical methods have been developed to study the phenomenon. The two most frequently used procedures are: (a) Derivatization of the chiral drug or metabolite with one pure enantiomer of a chiral reagent. The resulting mixture of diastereoisomers is then separated by some chromatographic procedure and the relative amounts of the original enantiomers are determined; when analysis is performed by GC-MS, a stable-isotope-labelled analog of the drug or metabolite may be employed conveniently as an internal standard (137, 296). (b) A "pseudoracemic" mixture of the drug is prepared in which one of the two enantiomers is labelled with a stable isotope (312). In this case, the heavy isotope label becomes a stereochemical marker, and the ratio of labelled to unlabelled molecules provides a direct measure of the optical purity of the compound under study. Where a third isotopic variant of the compound is available, this can be employed as an internal standard for determining the absolute amount of each enantiomer present.

While approach (a) has been employed successfully in a number of applications, e.g. in studies of the stereochemistry of amphetamine metabolism in the rat (137), its use is limited to those compounds that will react readily with one of the chiral derivatizing reagents available commercially. The "pseudoracemate" approach, by contrast, does not suffer from this limitation and may, in certain cases, provide information not obtainable by method (a), e.g. on the stereochemical origin of achiral metabolites of a chiral drug (139, 483).

Several examples of the application of "pseudoracemic" mixtures to studies of stereoselective drug metabolism have now been published. McMahon and Sullivan (310) used an equimolar mixture of *d*-[$^2H_2$]propoxyphene (XXX) and unlabelled *l*-propoxyphene (XXIX) to show that, after oral administration to dogs, more rapid tissue uptake (and disappearance from plasma) occurs with the levo isomer of the drug. Castagnoli and coworkers have made extensive use of "pseudoracemic" mixtures in studies of various aspects of the metabolism in vitro of the psychotomimetic amine 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (138, 483, 506, 507) and of the metabolism in vivo of the antihypertensive agent α-methyldopa (5a, 62); in the latter case, enantioselective uptake of (S)-α-methyldopa into rat brain was demonstrated by the use of a "pseudoracemic" mixture consisting of (S)-[^{13}C]-α-methyldopa and (R)-α-methyldopa.

Differential metabolism of the two enantiomers of the antineoplastic drug cyclophosphamide (XVIII), a dissymmetrical molecule by virtue of the chiral phosphorus atom, has been investigated with the aid of "pseudoracemic" mixtures (82, 84). Studies in the mouse, rabbit, and rat revealed stereoselective metabolism, the pattern of which was species-dependent (215). Early studies on the differential metabolism of cyclophosphamide enantiomers in humans, which were performed by measuring the optical rotation of unchanged cyclophosphamide recovered from urine of patients given the racemic drug, indicated preferential metabolism of the (+)-isomer (83). Recently, however, it has been shown that the measurements of optical rotation on which this conclusion was drawn were subject to interference from endogenous components in the urine extract and that cyclophosphamide recovered from urine of humans given the racemate was, in fact, either racemic or only slightly enriched in the (−)-enantiomer (216). Although the latter study did not employ the "pseudoracemate" approach (^{31}P NMR spectroscopy in the presence of a chiral shift reagent was used), it nevertheless serves to underscore the fact that measurements of optical rotation alone can give erroneous indications of stereoselective metabolism, particularly when the magnitude of the optical rotation of the enantiomers in question is small, as is the case with cyclophosphamide ($[\alpha]_D^{25} \pm 2.3°$).

Stereochemical aspects of the biodisposition of aryloxypropanolamine β-blocking agents have received considerable attention in recent years in view of the fact that, while drugs of this class are employed therapeutically as racemic mixtures, the pure enantiomers often exhibit large differences (10- to 1000-fold) in pharmacological activity. After the development of stable isotope methodology based on the "pseudoracemate" approach, the



XXXII R = H

XXXIII R = OH

metabolic fate of (±)propanolol (XXXII) was investigated in the dog (108, 422, 473, 473a); the results from these studies demonstrated a significantly lower oral bioavailability of (−)-as compared with (+)-propranolol in this species, which appeared to be associated with stereoselective presystemic glucuronidation of (−)-propranolol. It is noteworthy that while the administration of the separate enantiomers of propanolol had been shown previously to result in quite disparate plasma levels of both free and conjugated propranolol (330), such studies do not permit an assessment of the role of the hemodynamic effects of (−)-propranolol in the overall disposition of the two enantiomers. By the use of the "pseudoracemate" technique, on the other hand, it could be demonstrated that the hemodynamic effects caused by (−)-propranolol did not appear to have a significant effect on the stereoselective metabolism of the drug (422). Studies on the aromatic hydroxylation of oxprenolol, another member of the family of aryloxypropanolamine β-blocking agents, have revealed stereoselectivity in the formation of 4′- and 5′-hydroxylated products (50). In this work, rats were administered a "pseudoracemic" mixture comprising (2*R*)-[$^2H_2$]oxprenolol and unlabelled (2*S*)-oxprenolol and the phenolic metabolites excreted in urine were analyzed by GC-MS for deuterium enrich-

ment; hydroxylation at both the 4′ and 5′ positions was shown to occur predominantly for the (2*R*)-enantiomer of the parent drug. It is of some interest, therefore, to note that in the case of propranolol (XXXII), aromatic hydroxylation at the C-4′ position (XXXIII) has been shown to favor the (2*S*)-enantiomer when studied in vivo, although no stereoselectivity could be detected with in vitro systems (377). Such findings indicate that the stereoselectivity in aromatic hydroxylation observed in vivo is primarily a reflection of stereoselectivity in other metabolic pathways and/or tissue distribution.

Further applications of the "pseudoracemate" technique have been to studies on the metabolism and pharmacokinetics of chlorpheniramine, a commonly used antihistamine compound (323, 452), to the metabolism of a new antiarrhythmic agent, drobuline (333), and to the stereoselective disposition of (*R*)- and (*S*)-methadone in man (160, 263). An enantiomeric interaction occurring during the in vitro metabolism of amphetamine has been revealed by the use of "pseudoracemic" mixtures consisting of either (*R*)-[$^2H_3$]amphetamine/(*S*)-amphetamine or (*R*)-amphetamine/(*S*)-[$^2H_3$]amphetamine; in each case, the metabolites *N*-hydroxyamphetamine and 1-phenyl-2-propanol were formed preferentially from the (*S*)-enantiomer of the parent drug, whereas incubations with the pure amphetamine enantiomers gave the opposite result (139). It was concluded from this study that (*S*)-amphetamine or one of its metabolites inhibits the metabolism of the (*R*)-enantiomer. Stereoselectivity in drug-drug interactions may also be investigated by the use of "pseudoracemates" when the effects of one agent on the metabolism of each enantiomer of a second agent can be studied in a single experiment. An example of this type of application is the recent work by Trager and coworkers, who examined the effect of phenylbutazone and seconal on the plasma half-lives of (*R*)- and (*S*)-warfarin in man (205, 361, 362). By administering a "pseudoracemic" mixture of the drug, in which the (*S*)-warfarin enantiomer was labelled with ^{13}C at the C-2 position, together with the use of a reverse stable isotope dilution assay procedure for plasma warfarin in which a [$^2H_5$] analog served as internal standard, it was demonstrated that phenylbutazone increased the half-life of (*S*)-warfarin and decreased the half-life of (*R*)-warfarin, whereas seconal decreased the half-life of both (*R*)- and (*S*)-enantiomers.

In view of the unique advantages of the "pseudoracemate" approach to the study of the biological fate of each enantiomer of a chiral drug in the presence of its antipode, this technique is likely to find widespread application in pharmacological research. Two potential pitfalls of the method should be pointed out, however. First, as noted previously, deuterium isotope effects may influence the pharmacokinetics and metabolism of labelled drugs and therefore if deuterium is to be used for labelling purposes with a "pseudoracemic" mixture, the absence of an isotope effect should first be demonstrated experimentally. For studies in vivo, this may be carried out prior to work with optically resolved material by administration of an equimolar mixture of unlabelled and deuterated material as metabolic substrate; a 1:1 ratio of the two molecular forms of the parent drug in body fluids throughout the experiment would indicate the absence of a deuterium isotope effect on drug absorption, distribution, and elimination. For work in vitro, a common approach is to perform parallel studies in which the two opposite "pseudoracemates" are employed as substrates; the results from incubations with one "pseudoracemic" mixture should mirror exactly those obtained from the second unless deuterium isotope effects operate. Clearly, judicious choice of the position(s) for deuterium labelling is essential if unwanted isotope effects, resulting from metabolic attack at deuterated carbon atoms, are to be avoided. Second, it should be noted that the basis of the "pseudoracemate" approach centers on the use of heavy isotopes as markers of absolute stereochemistry; obviously, if either enantiomer of the compound under study were to undergo inversion of configuration in vivo or in vitro, the procedure would no longer be valid. Several examples of metabolic inversion of configuration at saturated carbon have been reported (235, 264, 480) and it is therefore necessary to establish the absence of such phenomena before proceeding with studies in which "pseudoracemic" mixtures are used. This consideration has been discussed by Jarman et al. (216), who demonstrated that no inversion of configuration takes place at the chiral phosphorus atom of cyclophosphamide (XVIII) after administration of the drug to humans. When the "pseudoracemate" approach is used in conjunction with a chiral derivatizing reagent (see above), any metabolic inversion of configuration is readily detected (62).

4. $^{13}CO_2$ Breath Tests. The principle of the CO_2 breath test is that one manifestation of a metabolic disorder should be an increase or a decrease in the rate at which a specific substrate is oxidized to CO_2 by hepatic enzyme systems. A number of studies have been performed with substrate labelled, at metabolically labile positions, with the radioactive isotope ^{14}C and the exhaled breath monitored for $^{14}CO_2$ content. These investigations have served to establish the general principle of the CO_2 breath test and have been reviewed thoroughly by Glaubitt et al. (149). In view of the health hazard associated with the use of radioisotopes, however, the scope of the $^{14}CO_2$ breath test is necessarily limited and the development of methodology for the substitution of ^{13}C for ^{14}C in breath tests was a natural progression (28, 190, 406). Recently, a stable isotope procedure was described for the assessment of hepatic *N*-demethylase activity in vivo, based on the use of 4-(di[^{13}C]methylamino)antipyrine ([^{13}C]aminopyrine) as metabolic substrate, and was validated by comparison with the established [^{14}C]aminopyrine method (402). The basis of the breath test in this particular example is that aminopyrine (II) undergoes exten-

sive *N*-demethylation in vivo to 4-(monomethyl)-antipyrine (45), the formaldehyde so liberated subsequently enters the one-carbon pool and appears ultimately in exhaled breath as CO_2. Comparison of the time course of exhalation of labelled CO_2 in breath of patients with a metabolic abnormality with that in healthy subjects should therefore provide an index of hepatic *N*-demethylase activity. Although the clinical value of such tests remains to be fully established, Helge and coworkers have successfully employed the $^{13}CO_2$ breath test to show that neonates and infants of epileptic mothers taking primidone (either alone or in combination with phenytoin) exhibited markedly greater *N*-demethylase activity towards [^{13}C]aminopyrine than did controls (339). This finding was taken as evidence for enzyme induction that had occurred during prenatal life. An alternative to the [^{13}C]aminopyrine breath test has been reported recently. [^{13}C]Methacetin is used as metabolic substrate; this latter compound has the important advantage over aminopyrine of exhibiting less toxicity at high dose levels (403).

The value of the $^{13}CO_2$ breath test is that it is both noninvasive and lacks the radiation exposure associated with the use of its $^{14}CO_2$ counterpart. As such, it is ideally suited to investigations in young infants and pregnant women. The major disadvantage, however, is that it is indirect, the metabolic pathway to exhaled $^{13}CO_2$ involving a number of steps including absorption of the labelled drug (when given orally) and a variety of metabolic transformations catalyzed by several distinct enzyme systems. Even if only the rate of oxidative removal of the labelled methyl group were to be affected by the metabolic disorder under investigation, differential induction or repression of different forms of cytochrome P-450 would not be revealed by the $^{13}CO_2$ breath test unless a number of substrates, each of which was metabolized preferentially by one form of the enzyme, were examined in turn. Therefore, from the standpoint of evaluating the capacity of liver enzymes to effect oxidative metabolism of foreign compounds in vivo, the CO_2 breath test must be considered to have limited applicability. Application of the methodology to other areas of clinical research, e.g. studies in children of fat malabsorption (477) and bacterial bile salt deconjugation (423), would appear to hold greater promise for diagnostic purposes.

5. Interindividual Differences in Drug Metabolism. As an approach to the study of interindividual differences in drug metabolism in man, Baty and Robinson (29) have proposed the use of two analgesics, acetanilide and phenacetin, as metabolic substrates. Each compound is metabolized extensively in humans to a common product, acetaminophen, which is formed by aromatic ring hydroxylation of acetanilide and by *O*-deethylation (oxidation at aliphatic carbon) of phenacetin. In their procedure, a mixture of [$^2H_5$]acetanilide and unlabelled phenacetin is administered orally and plasma acetaminophen (free and conjugated) is analyzed for deuterium content by GC-MS. The ratio of unlabelled to [$^2H_4$]acetaminophen thus affords a measure of the relative activities of the enzyme systems responsible for the *O*-deethylation of phenacetin and the *p*-hydroxylation of acetanilide. In preliminary experiments, large interindividual differences in the ratio between the two molecular species of "total" acetaminophen in early plasma samples were observed and were ascribed to interindividual differences in the rates of absorption of the drugs and of the "first-pass" effect to which phenacetin is subject; in contrast, the intraindividual spread of values from repeat experiments was small. The participation of any deuterium isotope effect on the results was excluded by performing a study in which an equimolar mixture of unlabelled and [$^2H_5$] forms of acetanilide was administered and the ratio of acetaminophen to [$^2H_4$]acetaminophen in plasma was found to be unity throughout the experiment.

This technique for the simultaneous study of two types of metabolic transformations in vivo would appear to be useful for investigations of enzyme induction and drug interactions. The authors' statement that "A ratio measurement of the type we have described may serve as an index of a person's oxidative metabolic rate" remains to be established.

6. Stable Isotopes in Studies of the Metabolism of Endogenous Compounds. An increasingly important area of application of stable isotope labelling techniques in pharmacological research is the study of the biosynthesis and metabolism of a variety of endogenous compounds in vivo and the effects of physiological or pharmacological factors on the rates of these processes. In many cases, the use of stable isotopes has been preferred over the more classic radiotracer approach to such work in view of the high degree of specificity attributable to mass spectrometric methods of stable isotope determination, and also since multiple isotopic variants of the same tracer compound can be measured simultaneously. In addition, when stable isotope methodology is used, the procedures are directly applicable to investigations in human subjects to whom the administration of radioactive tracers is severely limited. The group of endogenous compounds that has been most widely investigated by stable isotope techniques comprises the neurotransmitter substances acetylcholine, norepinephrine (noradrenaline), dopamine, serotonin, and γ-aminobutyric acid (GABA). Studies in animals have shown that the rates of synthesis and turnover of neurotransmitters, rather than their levels, reflect changes in functional activity of nerves in both the central nervous system and the periphery. Considerable effort has therefore been directed towards the development of methods by which the biosynthesis and turnover rates of these compounds may be measured directly and which may be applied to studies on the effects of drug administration on the production and degradation of neurotransmitters in vivo.

Tracers labelled with stable isotopes have proved to be particularly valuable for use in turnover studies under "steady-state" conditions, when the pool of endogenous

compound is not perturbed during the experiment (10). A number of workers have developed stable isotope methods for investigations of the acetylcholine system, including Jenden et al., Holmstedt et al., and Costa and coworkers. In each of the methods, a deuterium-labelled analog of choline or phosphoryl choline (e.g. [$^2H_4$]choline) is administered to the animal by injection or infusion and the levels of both the labelled and endogenous choline (XXXIV) and acetyl choline (XXXV) produced are determined in timed blood samples by the use of a reverse stable isotope dilution GC-MS assay procedure; a second deuterium-labelled variant of both choline and acetylcholine (e.g. $^2H_9$) is employed as an internal standard. In the procedure employed by Jenden et al. (224), choline, acetylcholine, and their isotopically labelled variants are recovered from biological media and converted by reaction with sodium benzenethiolate to their corresponding tertiary amines before GC-MS analysis. Selected ion monitoring is carried out, after esterification of choline to its propionyl derivative, at m/z 58 (endogenous choline and acetyl choline), m/z 60 (tetradeutero variants), and m/z 64 (internal standards), when resolution of the choline derivative from acetyl choline is effected by GLC (fig. 2). Applications of this methodology to investigations of acetylcholine biosynthesis and turnover, and to the influence of various pharmacological agents on the kinetics and metabolism of choline and acetylcholine in brain tissue have been reported in a series of publications from Jenden's laboratory (60, 66, 130, 131, 156, 217, 222, 223, 230, 231, 233, 481, 482). In a recent extension of the technique to accommodate a fourth labelled variant, acetylcholine kinetics in mouse brain was investigated through the use of pulse i.v. injections of [$^2H_6$]-, [$^2H_9$]-, and [$^2H_{13}$]choline at 30-second intervals and subsequent measurement of the levels of these labelled species of choline and acetylcholine in brain tissue by GC-MS with the tetradeutero analogs as internal standards (220). Finally, Jenden et al. (219, 225, 232) have investigated the metabolic fate of deanol, the *N*-desmethyl analog of choline, which is believed to cross the blood-brain barrier and to undergo conversion to choline by *N*-methylation in brain tissue. In order to distinguish choline produced endogenously from deanol-derived material, [$^2H_6$]deanol was used as a tracer, thus giving rise to [$^2H_6$]choline and [$^2H_6$]acetylcholine, while different isotopic variants of choline, acetylcholine, and deanol itself were used as internal standards for quantification by GC-MS. Holmstedt and coworkers have used essentially the same methodology for study of choline and acetylcholine (240) and have examined the influence of cholinergic drugs on acetylcholine turnover in brain (238, 239, 281, 358). Cheney, Costa, and others, on the other hand, have studied changes in acetylcholine turnover induced by lidocaine and cocaine (353), kainic acid (502), Δ^9-tetrahydrocannabinol (382), antimuscarinic agents (498), prolactin (501), and selected neuropeptides (499, 500).

Methods aimed at measuring the activity of norepinephrine-based systems have centered mainly on quan-

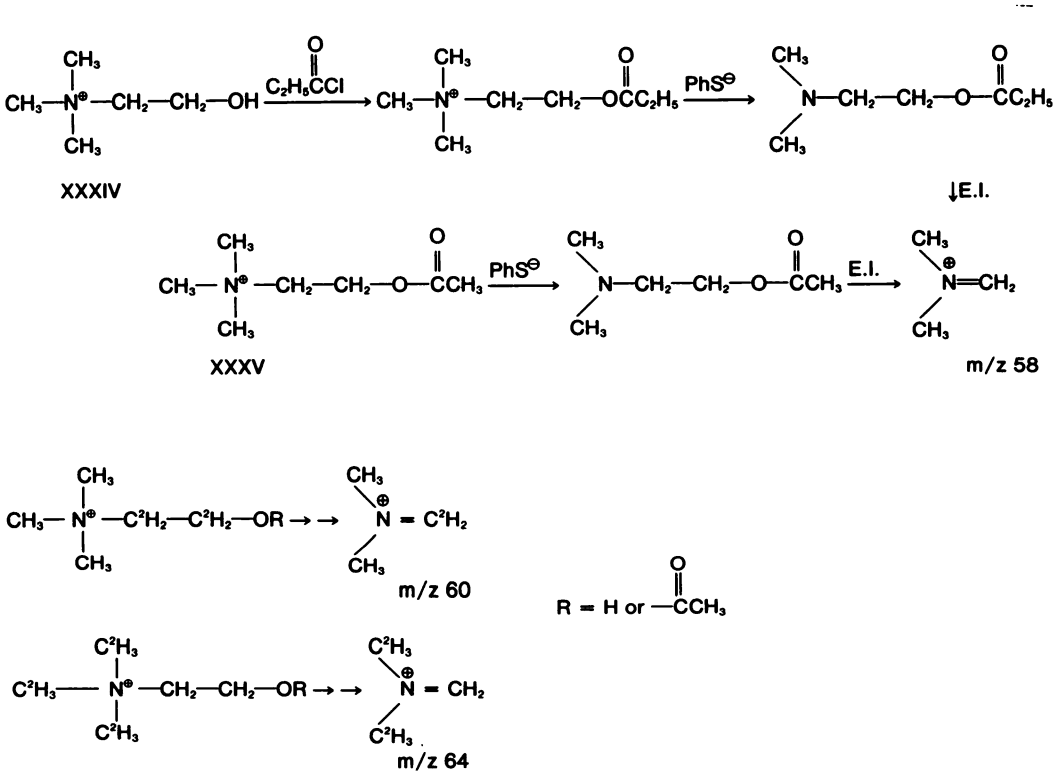
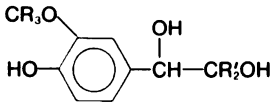


FIG. 2. A. Basis of the analytical method for the determination of choline (XXIV) and acetylcholine (XXV) by GC-MS. The structure of the ion with m/z 58, which represents the base peak in the EI mass spectrum of each compound, is as indicated. B. The consequence of deuterium substitution at specific sites in choline and acetylcholine on the m/z value of the fragment ion used in selected ion monitoring analyses.

titative analyses of its major metabolite, 3-methoxy-4-hydroxyphenylethylene glycol (MHPG; XXXVI). Recent work by Maas, Hattox, and Landis has led to the development of a direct method for studying MHPG production by brain in animals (284, 285) and humans (282).

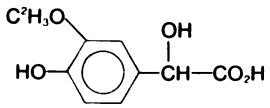


XXXVI R = R′ = H

XXXVII R = H, R′ = ^{2}H

XXXVIII R = ^{2}H, R′ = H

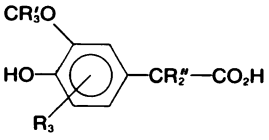
The technique is based on measurements of the venous-arterial difference in MHPG concentration in blood obtained from the internal jugular bulb, coupled with a measure of cerebral blood flow, and uses a reverse stable isotope dilution GC-MS assay approach by which the MHPG is quantified by using [$^2H_2$]MHPG (XXXVII) as internal standard. By means of this approach, it could be demonstrated that the rate of MHPG production in monkey brain is increased by the administration of piperoxan, an α-adrenergic antagonist, and decreased by the administration of clonidine, an α-adrenergic agonist (286). For measurement of whole-body catecholamine turnover, procedures have been developed based on the administration of deuterium-labelled tyrosine and analysis of blood and urinary metabolites (10, 419); protocols using either constant infusion or oral loading with deuterated tyrosine have been reported (10), although in preliminary experiments conducted in the rat, the limited penetration of [$^2H_3$]tyrosine into the brain compartment indicated that it may be difficult to study metabolism in the central nervous system when the labelled amino acid is administered i.v. or orally (419). Preliminary studies have also been performed in which human subjects were given [$^2H_3$]MHPG (XXXVIII) i.v. and the plasma and urinary levels of both endogeneous and labelled MHPG were followed by GC-MS assay methods; in this work, it was found that approximately 40% of the MHPG given by injection was excreted as an oxidation product, [$^2H_3$] vanillylmandelic acid (XXXIX), while the remainder of the dose was recovered mainly as the sulfate conjugate of [$^2H_3$]MHPG (290). In order to clarify the effect of L-



XXXIX

dopa administration on norepinephrine synthesis in vivo, Freed and Murphy (129) performed studies in which unlabelled L-dopa was infused continuously over 19-hour periods to conscious, unrestrained animals, followed by an identical dose of L-[$^2H_3$]dopa. Analysis of the [$^2H_3$] dopamine and [$^2H_3$]norepinephrine production rates under the steady-state conditions achieved in the earlier part of the experiment showed that total dopamine production was proportional to dopa dose, whereas norepinephrine synthesis proceeded at a constant rate that was independent of dopa dose or dopamine concentration and that was similar to the rate of synthesis of endogenous norepinephrine.

Analytical techniques based on the use of stable-isotope-labelled compounds have also been applied to studies of dopamine synthesis and metabolism, when quantitative assays for the principal dopamine metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA; XL) have been performed by selected ion monitoring GC-MS (10, 21, 22, 412). Bacopoulus et al. (22) have investigated the regional distribution of DOPAC and HVA in human and primate brain and



XL R = R′ = R″ = H

XLI R = R′ = H, R″ = ^{2}H

XLII R = R″ = H, R′ = ^{2}H

XLIII R = R″ = ^{2}H, R′ = H.

found that the patterns were very similar in the two species, in contrast to that reported for rat brain. On this basis, these authors suggested that the monkey should be a good model for the human in studies on central dopaminergic function. The same group has also investigated the effect on plasma DOPAC and HVA levels of experimental treatments that altered the function of central dopamine neurons; these studies, which were carried out in the rat, indicated that changes in the concentration of these metabolites in brain tissue are reflected by parallel changes in plasma (21). The techniques developed by Maas et al. (287) for measuring the production rates of catecholamine metabolites by brain in awake animals have also been applied to DOPAC and HVA. In a recent extension of this work to include the serotonin metabolite 5-hydroxyindoleacetic acid (5-HIAA), Maas et al. (283) have provided estimates of dopamine and serotonin synthesis by the awake human brain; in addition, measurement of the urinary excretion of HVA and 5-HIAA during the time when the output of these neurotransmitter metabolites from brain was determined allowed for an estimate to be made of the relative contribution by brain to levels of these urinary metabolites. The effect of the ergot derivative bromocriptine on dopamine metabolism in rat brain has been reported by Markey et al. (291), who administered [methyl-$^2H_3$]methionine i.v. to the animals 30 minutes before decapitation and quantification of the newly synthesized [methyl-$^2H_3$]HVA (XLII) by GC-MS. The results showed that HVA production was decreased by bromocriptine, which is consistent with the known activity of this drug as a dopaminergic agonist. Änggård et al. (10) have measured the turnover rate of HVA in human subjects by administering a single bolus i.v. dose of [$^2H_5$] HVA (XLIII) and following the decay of this species in blood with time; a second isotopic variant, [$^2H_2$]HVA (XLI), was used as internal standard by which levels of both exogenous and endogenous HVA in both blood and urine samples were measured by GC-MS. The results of

this study indicated that renal clearance is not the only mechanism by which HVA is eliminated in man.

Of the stable isotopes commonly used in pharmacological research, deuterium has been by far the most widely employed for studies on the metabolism of endogenous compounds. The catecholamine field is no exception (10, 11) and a number of synthetic procedures have been reported recently for the preparation of deuterium-labelled biogenic amines and their metabolites (292, 293). However, many positions in the structure of catecholamines and their metabolites are unsatisfactory for labelling with deuterium in view of either their susceptibility towards chemical exchange processes in vivo or towards known or potential metabolic transformations. Carbon-13, on the other hand, would appear to be ideal for labelling purposes, as indicated by Castagnoli et al. (5a, 62) for the synthetic catecholamine α-methyldopa, although to date there have been no reports of the use of ^{13}C in studies of the metabolism of endogenous catecholamines. Interestingly, ^{18}O has received considerable attention for tracer studies of catecholamine biosynthesis in vivo since the report by Sedvall and associates (299) on the incorporation of ^{18}O into HVA in rat brain during exposure of the animals to $^{18}O_2$-containing atmospheres. Analysis of the labelling pattern of the [^{18}O]HVA isolated from brain tissue showed that one atom of ^{18}O was incorporated per molecule, at the C-3 position, which indicated that tyrosine was the predominant precursor of brain dopamine. As expected, administration i.p. to the animals of $H_2{}^{18}O$ did not result in incorporation of ^{18}O into brain HVA. These studies thus demonstrated the feasibility of "pulse-labelling" brain dopamine and its metabolites by inhalation of ^{18}O-enriched molecular oxygen, a potentially attractive, noninvasive procedure by which quantitative determination of the turnover of brain dopamine could be assessed in animals and man. Further work by the Swedish group demonstrated the applicability of the technique to studies with nonhuman primates, when the effect of i.v. chlorpromazine administration on dopamine turnover was determined (411). The early work on the use of $^{18}O_2$ in catecholamine research was subsequently confirmed and expanded by Costa et al. (78), who reported that the enrichment of ^{18}O found in dopamine from different nuclei of the same rat brain varied by approximately fourfold, presumably in relation to the turnover rate of dopamine. Recent attempts to develop a kinetic model whereby the rate of dopamine formation in man could be calculated from the rate of ^{18}O incorporation into dopa, dopamine, and their metabolites have revealed that the kinetics of the dopamine labelling in vivo is more complicated than originally anticipated (340). Thus, labelling of dopamine with ^{18}O probably occurs by two different mechanisms, one involving hydroxylation of tyrosine to dopa, followed by decarboxylation of dopa to dopamine, and the other involving hydroxylation of phenylalanine to tyrosine, which then enters the above pathway (141). Although these mechanisms result in the introduction of an atom of ^{18}O at different positions on the aromatic nucleus (C-4 and C-3, respectively), the labelled products cannot be distinguished readily on the basis of their mass spectra. That each of the above hydroxylation processes does indeed operate in vivo is borne out by the experiments of Sedvall et al. (411), in which small amounts of a doubly labelled species of HVA, [$^{18}O_2$]HVA, was detected in the cerebrospinal fluid of a baboon after exposure to $^{18}O_2$. As discussed by Galli et al. (141), "the basic problem which restricts the usefulness of the technique for estimating catecholamine turnover is in interpreting the meaning of the changes of [^{18}O]dopamine and its metabolites after exposure to $^{18}O_2$." This problem does not arise, however, for the monohydroxy indole derivative, serotonin; in this case, $^{18}O_2$ studies should be useful for investigating turnover rates since ^{18}O is incorporated into serotonin at the rate-limiting step in its biosynthesis, the hydroxylation of tryptophan at C-5 (140). Although the putative pineal hormone melatonin (*N*-acetylserotonin) may also be studied by this technique, no reports on the use of $^{18}O_2$ for determining melatonin turnover have appeared to date. A more traditional approach for estimating melatonin turnover, viz quantitative measurement of a unique urinary metabolite, 6-hydroxymelatonin sulfate, has been proposed recently by two groups (122, 448).

The turnover rate of GABA in rat brain has been estimated by ^{13}C-labelling techniques when steady-state concentrations of [$^{13}C_2$]GABA were generated by i.v. infusion of [U-^{13}C]glucose (34). By application of the principles of steady-state kinetics to changes with time in the ^{13}C-enrichment of glutamic acid and GABA, the turnover rates of GABA in different nuclei of brain were calculated. While absolute values for GABA synthesis rates could not be obtained from this study, due to the possibility of recycling of label during the period of infusion, the method was judged to be most useful for investigating how various drugs, e.g. opioids (328), modify the turnover of this neurotransmitter. Stable isotope techniques have also been used to study the relationship between GABA and 2-pyrrolidone, its γ-lactam derivative, in mouse brain (55). After i.v. injection of 2-[3,3,4,4,5,5-$^2H_6$]pyrrolidone into the tail vein, deuterium-labelled GABA was detected in brain, although i.v. injection of [$^2H_6$]GABA itself did not result in labelling of brain GABA pools; these results were taken to indicate that while GABA does not cross the blood-brain barrier, 2-pyrrolidone does so and undergoes hydrolysis to GABA in brain tissue.

Stable isotopes in general, and deuterium in particular, have played a major role in both qualitative and quantitative investigations of the biosynthesis and metabolism of prostaglandins. The effect of nonsteroidal anti-inflammatory agents on inhibiting prostaglandin biosynthesis has been studied by a number of workers, including Hamberg (162), who developed a reverse stable isotope dilution GC-MS assay technique to show that the urinary

excretion of the major metabolite of PGE_2, 7α-hydroxy-5,11-dioxotetranorprostane-1,16-dioic acid (III), is markedly reduced after oral administration of therapeutic doses of indomethacin, aspirin, or sodium salicylate. The "internal" standard used in this work was the *bis*-[*O*-C^2H_3]oxime derivative of the PGE metabolite itself, a similar type of labelled standard to that used in the first GC-MS assay for a prostaglandin, PGE_1 (396). Oates and coworkers (414) adopted a similar assay procedure to demonstrate a significantly greater urinary excretion of the PGE metabolite in patients with hypercalcemia and solid tumors. VandenHeuvel and associates (476), on the other hand, developed a new GC-MS assay for this compound that was based on the use of an analog of the PGE metabolite labelled in the side-chains with both deuterium and tritium as a true internal standard; with this approach, the authors measured the decrease in urinary output of the PGE metabolite in patients with rheumatoid arthritis prior to and during treatment with indomethacin. Similar studies have been carried out on the urinary excretion of the major metabolite of the F series prostaglandins, 5α,7α-dihydroxy-11-oxotetranorprostane-1,16-dioic acid (VIII). In one such investigation, the effects of indomethacin on both whole-body turnover of $PGF_{2\alpha}$ and on the metabolism of $PGF_{2\alpha}$ alone were determined (42, 43); in the latter case, [8,10,10-$^2H_3$]$PGF_{2\alpha}$ was given to human volunteers by i.v. infusion both before and during a course of treatment with indomethacin (200 mg day^{-1}) and excretion of the corresponding deuterium-labelled PGF metabolite was quantified by a direct stable isotope dilution assay procedure. Indomethacin was found to have no detectable effect on the metabolism of the labelled $PGF_{2\alpha}$, whereas the excretion of the endogenous PGF metabolite was reduced markedly by the effect of the drug on prostaglandin biosynthesis. Reports on the effect of drugs on the concentration of endogenous prostaglandins in plasma are relatively rare, although Greén and Svanborg (153) showed recently that administration of the synthetic prostaglandin analog 15-methyl-$PGF_{2\alpha}$, an abortifacient agent, led to an up to 10-fold increase in endogenous production of PGE_2 and $PGF_{2\alpha}$, as determined by reverse stable isotope dilution GC-MS assays of the respective 13,14-dihydro metabolites in plasma. The authors concluded that this effect of 15-methyl-$PGF_{2\alpha}$ probably contributes to the induced uterine activity during the latter part of the abortion process induced by this compound.

[19,19,19-$^2H_3$]Testosterone has been synthesized for use as an internal standard in a GC-MS assay for plasma testosterone (19) and has also been used as a metabolic tracer in humans (20). Thus, after oral administration of 20 mg of the labelled testosterone to two adult male volunteers, peak plasma concentrations were less than 2.5 ng ml^{-1}; [$^2H_3$]testosterone administration did not have any detectable effect on plasma levels or urinary excretion of endogenous testosterone (20). In an extension of this work, the excretion of deuterium-labelled testosterone metabolites in urine was quantified after oral administration of [$^2H_3$]testosterone, when it was found that more than 30% of the administered dose was excreted over a 24-hour period as the glucuronide conjugates of [$^2H_3$]androsterone and [$^2H_3$]etiocholanolone; these findings, considered together with the low plasma levels of the parent compound achieved by oral administration of testosterone, were taken to indicate that this steroid undergoes extensive "first pass" metabolism in the liver (415). Attempts to overcome the low oral bioavailability of testosterone preparations have centered mainly on the use of analogs of the hormone that undergo conversion to testosterone in vivo. One such prodrug is the ester, testosterone undecanoate. In a pharmacokinetic experiment with this compound, de Ridder and Koppens (97) administered to both healthy volunteers and hypogonadal patients an oral 40-mg dose of [2,2-$^2H_2$] testosterone undecanoate and determined the resulting plasma levels of both endogenous testosterone and [$^2H_2$] testosterone by GC-MS; interestingly, a radioactive analog, [1,2-$^3H_2$]testosterone, was used as internal standard for the unlabelled and deuterated compounds.

Further examples of the application of stable isotopes to pharmacological studies on the biosynthesis and metabolism of endogenous compounds have been to investigations on the effect of biguanide on the oxidation of orally administered glucose to CO_2 (270), to the coupling of ethanol oxidation and the biosynthesis of bile acids (85, 86, 489) and tricarboxylic acid cycle intermediates (87), and the effect of ethanol administration on serotonin metabolism (31).

B. Qualitative Applications

1. Isotope Cluster Technique. In the first application of selected ion monitoring GC-MS to studies of drug metabolism, Hammar, Holmstedt, and Ryhage (163) succeeded in identifying chlorpromazine and its mono- and bis-demethylated metabolites in samples of human plasma and red blood cells from patients treated with the drug. As the concentrations of these compounds were too low for full mass spectra to be recorded, identification was based upon the appearance, at appropriate retention times, of maxima in the ion current chromatograms for a few characteristic ions selected for each species. The presence, in both chlorpromazine and its metabolites, of a chlorine substituent in the phenothiazine ring system was especially valuable for this work since each of the ions monitored retained the chlorine atom and hence gave rise to "doublet" peaks, separated by two mass units and derived from the naturally occurring chlorine isotopes, ^{35}Cl and ^{37}Cl (ratio approximately 3:1). Thus, by monitoring both the ^{35}Cl-containing ion and its ^{37}Cl satellite, enhanced specificity of detection was achieved by virtue of the requirement for coincidence in retention times for the peaks in each pair of channels and also as a result of the requirement that the relative intensities of these peaks must be the same as the relative peak heights

for the same ions in the corresponding full mass spectra. The confidence with which the small amounts of chlorpromazine and its metabolites could be identified in this study was thus greatly enhanced by taking advantage of the natural ion doublet exhibited in the mass spectra of chlorine-containing compounds. This feature has since proved to be of great utility in studies of the metabolism of chlorinated drugs when the characteristic $^{35}Cl/^{37}Cl$ isotope cluster immediately draws attention to drug-related components, and is particularly useful in cases where large numbers of mass spectra are being "screened" for the presence of drug metabolites (27). Bromine, which occurs naturally as an approximately 1:1 mixture of two isotopic forms, ^{79}Br and ^{81}Br, also serves as a valuable "marker" for the mass spectrometric recognition of metabolites of brominated drugs (378).

The so-called isotope cluster, ion doublet, or twin ion technique in studies of drug metabolism developed from the above considerations and refers to a procedure first described in 1970 by Morfin et al. (326), in which the substrate under investigation is enriched at a level of approximately 50% with one or more atoms of a suitable heavy isotope. The mass spectrum of the substrate, and all metabolites derived from it, will thus exhibit artificially generated "twin" peaks for the molecular ion and all fragment ions retaining the labelled atoms (fig. 3). By this approach, metabolites of compounds not containing a chlorine or bromine atom may still be readily detected against a complex background of endogenous metabolites by virtue of this conspicuous feature of their mass spectra. Early applications of the isotope cluster technique to metabolic studies centered on investigations of androgen metabolism in vitro when [7-^{2}H]androst-4-ene-3,17-dione and [7β-^{2}H]testosterone were employed as substrates and products of oxidation or ring-A aromatization were identified by GC/MS (326, 40, 116). Concurrently with these studies, Vore and coworkers reported on the metabolism of *N*-*n*-butyl barbital in the rat, in which a 1:1 mixture of the unlabeled drug and its [$^{15}N_2$] analog was administered in order to facilitate identification of metabolites by GC-MS (467, 468). A third group, led by Knapp, Gaffney, and McMahon, explored the potential of the isotope cluster technique for work in drug metabolism and reported on its application to studies on the biotransformation of nortriptyline in the rat (256), human (257), and isolated perfused dog lung (486). By 1973, the isotope cluster technique had become well established in the field of drug metabolism, as illustrated by the elegant work of McMahon, Sullivan, and coworkers on the analgesic drug *d*-propoxyphene (XXIX) (313, 105) and by Prox et al. on LS861, an antiulcerogenic agent, and VK774, an inhibitor of thrombocyte aggregation (378, 505).

From the early work on this technique, a number of principles emerged with respect to the preparation of stable-isotope-labeled substrates for isotope cluster studies.

A. SYNTHESIS OF ISOTOPICALLY ENRICHED MATERIAL. This may be carried out by mixing approximately equimolar amounts of the pure unlabeled compound and an isotopically labeled analog (highly enriched) to give a final sample whose mass spectrum exhibits the desired "twin" peaks with a 1:1 intensity ratio. Alternatively, where the synthetic pathway permits, the heavy isotope is introduced into the substrate from a reagent that is labeled at a level of only 50 atom % excess; by this means,

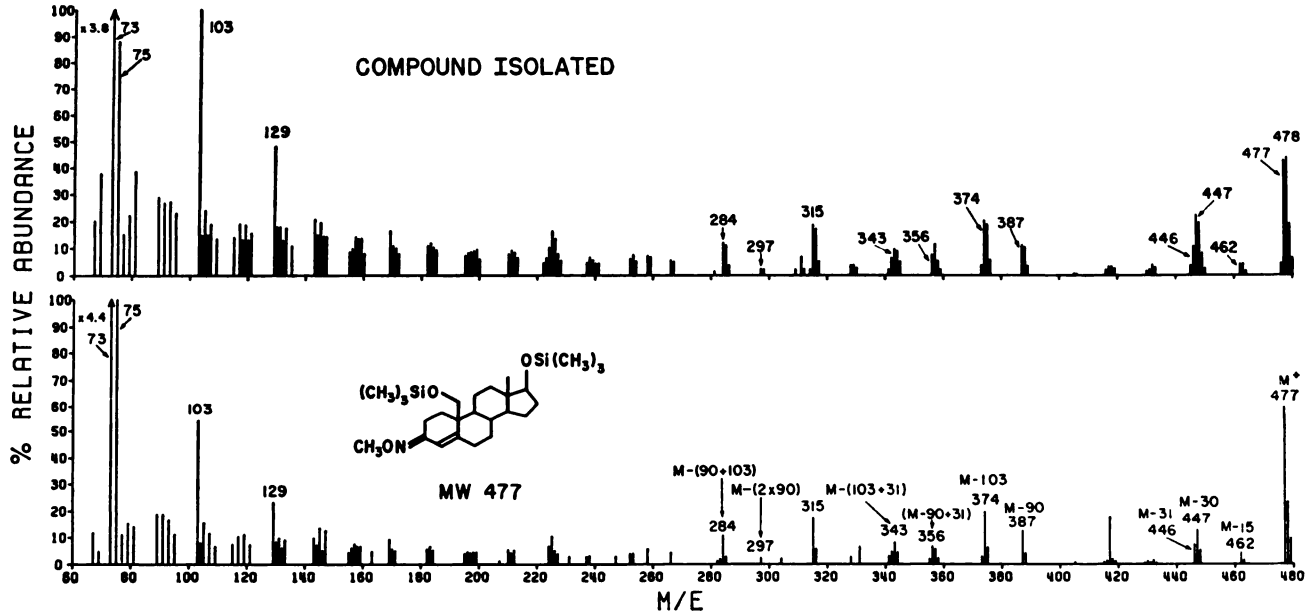


FIG. 3. Use of the isotope cluster technique to identify 19-hydroxytestosterone as a metabolite of testosterone in human placental microsomes. Top: Mass spectrum (70 eV) of the *O*-methyloxime *bis*-trimethylsilylether derivative of the metabolite produced from an equimolar mixture of testosterone and [7β-^{2}H]testosterone. Bottom: Mass spectrum of the corresponding reference compound. Reproduced with permission from W. E. Braselton, Jr., J. C. Orr, L. L. Engel, Anal. Biochem. **53**: 64–85, 1973.

the substrate for isotope cluster studies is obtained directly. While the former approach is used in most cases, the latter has the advantage of reducing significantly the cost of the stable isotopic source, an important consideration in work with the more expensive nuclides ^{13}C, ^{18}O, and ^{34}S.

B. MASS INCREMENT OF LABELED OVER UNLABELED MATERIAL. In their early studies on the metabolism of nortriptyline, Knapp et al. (256) noted that "...the utility of an M, M+1 doublet would be limited by the distortion introduced by natural isotopic contributions," and, for this reason, they employed an analog labeled with three atoms of deuterium. It would appear that the labeled variant should preferably be between two and four mass units heavier than its unlabeled counterpart to optimize the ease of recognition of metabolites by the ion cluster technique. A higher degree of labeling is sometimes found, particularly when multiple deuterium atoms are introduced into the molecule, as exemplified by the use of [$^2H_7$]propoxyphene (XXXI) (313, 105) and [$^2H_9$]carpronium chloride (397). However, of the papers cited in this review, the most frequently used isotope cluster was M, M+1, resulting from the use of either [$^2H_1$] analogs (63, 163, 326, 345, 376, 386, 397), [$^{13}C_1$] analogs (325, 376, 378, 496, 505), or [$^{15}N_1$] variants (258, 324).

C. CHOICE OF ISOTOPE. Dueterium has been, and continues to be, the most widely used heavy isotope for labeling purposes due to its relatively low cost and availability in a wide variety of convenient synthetic reagents, and to the ease with which one or more deuterium atoms generally may be introduced into organic compounds. Unfortunately, deuterium is far from ideal as a marker in metabolic studies for the following reasons:

1. Loss of label may occur due to direct (often unanticipated) oxidation at the site of labeling.

2. Loss of label may occur due to oxidation on the carbon adjacent to the site of labeling if keto-enol tautomerism is thereby introduced.

3. A consequence of 1) above frequently is the manifestation of a primary kinetic isotope effect, which may result in the promotion of an alternative metabolic pathway for the labeled species as compared with that followed by the unlabeled form ("metabolic switching"—see section III C 4).

4. If the substrate is labeled extensively with deuterium and if metabolites are to be analyzed by GC-MS techniques, significant deuterium isotope effects on gas chromatographic mobility may be observed. Thus, particularly when high efficiency glass capillary columns are employed, partial or even total resolution of deuterated and unlabeled species may take place prior to admission of the sample into the mass spectrometer and the object of using the isotope cluster technique may thus be defeated (475). Such isotope effects on GLC mobility also complicate the use of computer programs written to search stored GC-MS data for the presence of "1:1" isotope doublets (56, 71). In contrast, compounds labeled with ^{13}C, ^{15}N, or ^{18}O do not normally exhibit discernible isotope effects on chromatographic mobility and these isotopes are therefore to be favored over deuterium for labeling of metabolic substrates. This point is illustrated effectively in a paper by Miyazaki et al. (324), who showed that on packed column GLC analysis the muscle relaxant mydocalm was not resolved from its [$^{15}N_1$] analog, while a [$^2H_{10}$] variant of the drug eluted considerably ahead of the others.

5. Deuterium (and hydrogen) atoms are prone to rearrangement processes, which may take place either during metabolism (e.g., via the NIH shift—see section III C 5) or mass spectrometric analysis; such processes may complicate the interpretation of results from metabolic studies performed by the isotope cluster technique.

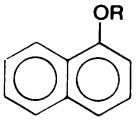
For all of the above reasons, the use of ^{13}C or ^{15}N is strongly favored over deuterium for the preparation of labeled substrates for isotope cluster studies. Indeed, a current trend in this direction is evident from a survey of recent research in which the technique was used (44, 325, 440–442, 444, 494, 496).

D. POSITION OF THE ISOTOPIC LABEL(S). The position at which the isotopic label is introduced is important both from the standpoint of the metabolic transformations that the compound of interest is expected to undergo and of the mass spectrometric properties of the parent compound and anticipated metabolites. As pointed out in the preceding section, judicious selection of labeling site(s) is imperative where deuterium is to be used, in order to avoid either loss of label (256) or untoward isotope effects on metabolism. With ^{13}C or ^{15}N, such effects are much less likely; in fact, it may actually be preferable to introduce a ^{13}C atom at an anticipated site of metabolic oxidation in order to facilitate elucidation of metabolite structure by ^{13}C NMR spectrocopy (175, 488). In all cases where mass spectrometry is to be used for metabolite analysis, however, it is necessary to take into account the fragmentation pattern of the parent compound when selecting positions for label incorporation in order that the label(s) be retained in prominent, high mass ions that exhibit clear isotopic doublets. Thus, β-adrenergic blocking drugs with the general aryloxypropanolamine side-chain may be labeled readily with ^{13}C or ^2H in the terminal *N*-alkyl group. However, under conditions of electron impact, cleavage of the carbon-carbon bond β- to the nitrogen usually predominates to give an intense fragment ion corresponding to $CH_2 = \overset{+}{N}H\text{-}R$. Where R = isopropyl, for example, this ion appears at m/z 72, a region of the spectrum that is highly susceptible to interference from endogenous components present in the sample and therefore not well suited for use in isotope cluster studies. Nevertheless, β-blocking agents labeled with deuterium in the *N*-alkyl group have been employed successfully for metabolic studies, although only when extensive sample "clean-up" procedures are used prior to mass spectrometric analysis (7). Two alternatives to extensive sample purification in this

specific example of aryloxypropanolamines are 1) to employ a derivative that alters the fragmentation of the side-chain and affords an ion with a higher m/z value, e.g. the cyclic dimethylsilyl derivative used in a study of the metabolism of alprenolol by the isotope cluster technique (188), and 2) to perform the mass spectrometric analyses by chemical ionization, rather than by electron impact, when fragmentation is much reduced and intense protonated molecular ions are frequently observed; this approach has been used in isotope cluster studies of the metabolism of propranolol (350).

Finally, it should be noted that where the parent drug and anticipated metabolites have a common structural feature that gives rise to a characteristic fragment ion of high intensity, labeling at this site may provide a convenient method for "screening" complex mixtures by GC-MS for the presence of metabolites that retain this structural unit. This technique was used to advantage by McMahon and coworkers in studies of the metabolism of *d*-propoxyphene (XXIX), the electron impact mass spectrum of which exhibits the tropylium ion, $C_7H_7^+$, at m/z 91. By employing an equimolar mixture of propoxyphene and [benzyl-$^2H_7$]propoxyphene (XXXI) as metabolic substrate, all drug-related products that retained an intact benzyl group could be detected readily by examining spectra from GC-MS analyses for m/z 91/98 doublets (105, 313). Similarly, Baba and coworkers (15, 327) performed computer-based search routines on stored GC-MS data for pairs of ions derived from the labeled cinnamyl moiety of 1-butyryl-4-cinnamylpiperazine and its metabolites.

The isotope cluster technique is suitable for a variety of applications, the most popular of which has been to studies of the "metabolic profile" of drug substances, the conspicuous "twin ions" greatly facilitating the detection of unknown metabolites by mass spectral analysis. Drugs investigated by this approach, in addition to those cited above, include phenoxybenzamine (258), propantheline bromide (470), cinoxacin (494), 3-(2′,4′,5′-triethoxybenzoyl) propionic acid (259, 260), *l*-3-[(dimethylamino)-(*m*-dioxan-5-yl)methyl]pyridine (389), potassium canrenoate (469), suloctidil (63, 386), Δ^9-tetrahydrocannabinol (169), warfarin (376), phencyclidine (496a, 65a, 236a), and enflurane (49). In certain cases, the isotope cluster technique has led to the recognition of new metabolic pathways. Tang, Inaba, and Kalow used equimolar mixtures of unlabelled and [$^{15}N_2$] barbiturates to study the transformation of amobarbital (440, 442) and pentobarbital (441) to polar urinary metabolites of unknown structure; these compounds were initially stated to represent products of *N*-hydroxylation, although they were later shown to be *N*-glucoside conjugates of the parent drugs (443, 444). In an analogous study, Clay, Watkins, and Murphy (71) identified pentose conjugates of the alcohol dehydrogenase inhibitor pyrazole, while the isotope cluster technique was also used by Miyazaki et al. to study a novel metabolic chain elongation of 5-(4′-chloro-*n*-butyl)picolinic acid, an inhibitor of dopamine β-hydroxylase (325). Stable isotope cluster techniques have also proved valuable in the analysis of reactive intermediates in drug metabolism, as illustrated by studies on acetylhydrazine (344, 345) and isopropylhydrazine (345), metabolites of the hydrazine drugs isoniazid and iproniazid, respectively. Mechanistic aspects of drug metabolism may also be investigated by the use of isotope clusters, as was reported by Nelson and Burke for propranolol (XXXII)



XLIV R = —C^2H_2—C^2H(OH)—C^2H_2—NH—$CH(CH_3)_2$

XLV R = —CH_2—CH(OH)—CH_2—NH—$CH(C^2H_3)_2$

XLVI R = —CH_2—CH(OH)—CH_2OH

XLVII R = —CH_2—CH(OH)—CHO

(350); by the use of an equimolar mixture of unlabelled and [side-chain-$^2H_5$]propranolol (XLIV) as substrate, the propranolol-diol (XLVI) formed during incubation with the 9,000 × *g* supernatant fraction of rat liver was found to be a 1:1 mixture of unlabelled and tetradeuterated molecules, thereby indicating an obligatory aldehyde intermediate (XLVII) in the oxidative metabolism of this widely used β-blocking agent.

An interesting variation on the isotope cluster technique is to take advantage of metabolic transformations to generate, from a substrate containing only labelled molecules, a product that is a mixture of labelled and unlabelled species and whose mass spectrum will thus exhibit conspicuous "twin" peaks. Engel and Orr (116) first suggested this approach for studying metabolic oxidation of androst-4-ene-3,17-dione at the C-7 position when they proposed the use of a 1:1 mixture of [7α-^{2}H] and [7β-^{2}H] analogs of the steroid substrate; stereospecific hydroxylation or dehydrogenation involving C-7 would thus generate a product that would be a 1:1 mixture of unlabelled and monodeutero forms and which could thus be searched for by GC-MS analysis. An analogous technique was employed by McMahon and coworkers (313) to detect *N*-demethylated metabolites of *d*-propoxyphene (XXIX) in humans. These authors used a variant of the drug labelled with two atoms of deuterium in one of the two (equivalent) *N*-methyl groups (XXX); thus, while the parent compound was a single molecular species, products of oxidative demethylation could be either labelled or unlabelled, depending on which of the two methyl groups had been removed, and could therefore be detected simply by the appearance of M, M + 2 doublets in their mass spectra. A third example of the usefulness of this general technique is given in a paper by Horie and Baba (195), who studied the human urinary metabolites of 3-phenylpropyl carbamate, a centrally acting muscle relaxant. Since the endogenous compounds benzoic acid and hippuric acid were also expected to be produced during the metabolism of this drug, it was decided to use 3-[phenyl-$^2H_5$]propyl carbamate alone for metabolic work; by this approach, benzoic and hippuric acids isolated from urine were indeed found

to be a mixture of unlabelled and pentadeuterated forms, the mass spectra of which exhibited conspicuous isotopic doublets reflecting the relative contributions to these metabolites from endogenous and exogenous sources.

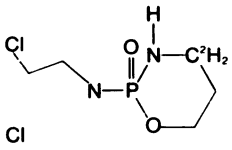
A further technique that has proved to be of value in cerain situations is the use of halogenated (or 50% deuterium-enriched) derivatizing reagents to introduce isotope clusters into the mass spectra of *unlabelled* drug metabolites. Thus, reaction of metabolically generated epoxides with trimethylchlorosilane (TMCS) has been shown to yield trimethylsilyl derivatives of the corresponding chlorohydrin adducts (167a). Since this reaction appears to be specific for the epoxide functionality, and since one chlorine atom is introduced into the molecule per epoxide group, derivatization with TMCS affords a means by which reactive epoxides may be first "trapped" by reaction with the silylating reagent, and subsequently detected by mass spectrometry by virtue of the conspicuous $^{35}Cl/^{37}Cl$ isotope pattern in the mass spectra of the resultant chlorohydrin derivatives. Recent studies on the biotransformation of allylbenzene analogs through the epoxide-diol pathway serve to illustrate the analytical utility of this technique (93a). In general, however, commonly used silylating, acylating, and alkylating reagents lack the required degree of specificity for this approach to the detection of drug metabolites in complex mixtures to be useful; extensive derivatization of endogenous as well as exogenous components of the biological sample usually occurs, and greatly complicates the task of distinguishing, by GC-MS, drug-related compounds from "background" material on the basis of the mass spectral isotope clusters. In contrast, the use of equimolar mixtures of a variety of labelled and unlabelled derivatizing reagents to facilitate the structure elucidation of compounds isolated in relatively *pure* state is a valuable and widely used technique and is dealt with below (section III B 3).

As discussed elsewhere in this review (e.g. section IV), the greatest single disadvantage associated with the use of stable-isotope-labelled compounds for metabolic studies is that no simple technique exists by means of which all labelled components in a given biological sample may be readily detected and quantified. For this reason, the combined use of radioactive and stable isotopes in metabolic studies is growing in popularity and can be expected to find widespread application in situations where the health hazard associated with the use of radioisotopes is not of prime concern. One of the most powerful approaches currently available for investigations of drug metabolism is to use a substrate labelled with both radioactive and stable isotopes, the radiolabel serving as an easily detected marker by means of which *all* drug-related material can be traced during isolation and purification procedures and the stable isotope acting to facilitate recognition and identification of individual metabolites by mass spectrometry (505). Ideally, the stable and radioactive isotopes should be of the same element (e.g. ^{2}H and ^{3}H, ^{13}C and ^{14}C) and should be incorporated at the same position during synthesis of the multiply labelled drug. Although no suitable radioactive isotopes of oxygen or nitrogen exist, this approach could, in principle, be extended to sulfur-containing compounds when ^{34}S (stable) and ^{35}S (radioactive) are employed. As examples of the great utility of this double labelling approach for studies of drug metabolism in animals may be cited papers by Prox and coworkers (378, 505), Wolf et al. (496), Baillie et al. (27), and Baba et al. (15). It should be noted that the use of radioisotopes *alone* for isotope cluster work is possible, although this approach has not proved popular due to the high specific activity necessary for adequate mass spectrometric detection of the isotopically labelled species. However, in recent work on the metabolic fate in the cow of a synthetic prostaglandin analog, cloprostenol, ^{14}C was used to generate isotope doublets by employing [15-^{14}C]cloprostenol with a specific activity of 91.9 μCi mg^{-1} (38, 39).

2. Use of Deuterium Labelling in Defining Sites of Metabolic Attack. Loss or retention of deuterium substituents at specific positions in a molecule can afford valuable information on sites of metabolic attack and is a technique that has been used in studies of drug metabolism for some years. In an investigation of the metabolic fate of *d*-propoxyphene (XXIX) in human subjects, McMahon, Sullivan, and Due (313) employed [benzyl-$^{2}H_7$]propoxyphene (XXXI) as substrate to detect urinary metabolites hydroxylated in the benzyl portion of the molecule; two such compounds, *p*-hydroxypropoxyphene and *p*-hydroxynorpropoxyphene, were readily detected by the presence in the mass spectra of their methylated derivatives of an ion with m/z 127, characteristic of the methoxybenzyl moiety with six deuterium atoms. A similar procedure was employed in subsequent work in laboratory animals (105). Studies on the mechanism of metabolic activation of the antitumor agent cyclophosphamide (fig. 1) have also made use of deuterium-labelling techniques; thus, after incubation with rat liver microsomal preparations of [4,4-$^{2}H_2$]cyclophosphamide (XLVIII), Connors et al. (75) were able to trap an unstable intermediate by the addition of ethanol. Two isomeric ethoxy derivatives were isolated and shown by mass spectrometry to contain only one atom each of deuterium; these results were taken as evidence for the existence of an unstable carbinolamine metabolite, 4-hydroxycyclophosphamide (XIX), which was proposed to form two diasterioisomeric 4-ethoxy derivatives on reaction with ethanol. Although addition of the alcohol to a 4,5-iminium ion, formed via dehydration of 4-hydroxycyclophosphamide, would appear to be a more likely explanation for the production of the ethoxy derivatives, this study nevertheless served to define the site of initial metabolic attack on cyclophosphamide as C-4, and not C-6, a possible alternative.

Tentative evidence for a new metabolic pathway for warfarin, which involved hydroxylation at the benzylic

position, was obtained by Trager and coworkers (376), who subsequently synthesized an analog of the drug labelled in this position with one atom of deuterium.



XLVIII

When this deuterated species was employed as substrate in incubations with rat liver microsomal preparations, the hydroxylated metabolite of interest was found to be unlabelled, thereby confirming the original structural assignment. Metabolic attack at a carbon atom adjacent to a site of deuterium substitution may also, however, give rise to loss of label in situations where tautomeric equilibria are introduced; this phenomenon was used to advantage by Clay, Watkins, and Murphy (71), who were able to assign structures to metabolites of pyrazole, an inhibitor of alcohol dehydrogenase, based on the observed labelling patterns when [$^2H_3$]pyrazole was employed as substrate. Other examples of the use of deuterium-labelling to facilitate identification of sites of metabolic attack have been to studies on the nature of the glutathione conjugate formed during incubation of cambendazole with liver microsomal preparations (496), the assignment of structure to cyanide adducts of metabolically generated reactive intermediates from tertiary amines (186, 354), and the identification of a product of aromatic-ring hydroxylation of the antihypertensive drug, clonidine (27).

Stereochemical aspects of metabolic reactions may also be investigated by the deuterium-labelling technique if one of two stereochemically distinct centers in the substrate is labelled specifically. Thus, McMahon and coworkers (311) employed S(+)-[α-^{2}H]ethylbenzene to show that metabolic hydroxylation of ethylbenzene occurs with retention of configuration at the benzylic carbon. A different approach to the problem of defining stereochemistry of metabolites has been reported recently by Harvey (167) and Harvey and Paton (169), who studied stereochemical requirements for electron impact-induced rearrangement and elimination processes in derivatized cannabinoids. Thus, when the (labelled) metabolites of specifically deuterium-labelled analogs of Δ^1-, Δ^6-, and Δ^7-tetrahydrocannabinol were analyzed by EI/MS, loss or retention of deuterium in certain fragment ions could be used to determine the stereochemistry of substituents in the terpene ring.

It should be noted, however, that retention of deuterium in a hydroxylated metabolite need not necessarily exclude the site originally bearing the label as being the center of metabolic attack; deuterium rearrangements may occur in certain situations, particularly with aromatic ring systems that undergo hydroxylation by way of intermediate arene oxides (see section III C 5). Results obtained from the use of specifically labelled [aromatic-^{2}H] compounds should therefore be interpreted with caution.

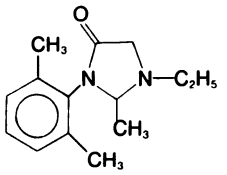
3. The Use of Deuterium-Labelled Reagents to Facilitate Elucidation of Molecular Structure by Mass Spectrometry. Reaction of an unknown compound with both an unlabelled and corresponding deuterium-labelled derivatizing reagent and comparison of the mass spectra of the products so obtained is a useful aid to structure elucidation in studies of drug metabolism and has been used for this purpose for many years. Thus, derivatization with the labelled, as compared with the normal, reagent indicates immediately the number of functional groups that have undergone reaction, while analysis of the mass spectral fragmentation patterns in terms of molecular structure is greatly facilitated by a knowledge of which fragment ions retain certain functional groups. Perhaps the most widely used deuterium-labelled derivatizing reagents are those that donate tri(deuteromethyl)silyl ([$^2H_9$]TMS)groups to reactive -OH, -NH, and -SH functionalities, the labelled silylated products formed exhibiting an increment in molecular weight over their unlabelled counterparts of 9 daltons per TMS group introduced. A recent example of the use of [$^2H_9$]TMS derivatives in the characterization of an unknown drug metabolite is the work by Pohl and associates (294), who used deuterated and unlabelled TMS ether derivatives to identify chloramphenicol alcohol, a product of oxidative dechlorination of the antibiotic chloramphenicol. Analogous procedures may be used for aldehyde- and ketone-containing compounds, which may be converted to the corresponding *O*-methyloxime and *O*-(trideuteromethyl)oxime derivatives by reaction with methoxyamine and trideuteromethoxyamine, respectively. Methylation is used frequently for the mass spectrometric analysis of carboxylic acids, phenols, and amines and deuterated diazomethane or trimethylanilinium hydroxide (27) may be prepared conveniently to afford the corresponding labelled derivatives. Similarly, acetylation may be performed with [$^2H_6$]acetic anhydride. In the case of the latter two reactions, viz methylation and acetylation, the use of deuterium-labelled derivatizing reagents may fulfill a different purpose. Many drugs are known to be metabolized via *O*- and *N*-demethylation or deacetylation processes and therefore derivatization of a mixture of unchanged drug and one or more such metabolites with methylating or acetylating reagents leads to a single product; when a deuterium-labelled derivatizing agent is employed, however, the parent drug and metabolites can be distinguished by mass spectrometric analysis. This approach has been used, for example, in a study of mepyramine metabolites (268).

Reduction of drug metabolites with NaB^2H_4 or $LiAl^2H_4$ has been used in some cases to convert carbonyl-containing metabolites to the corresponding alcohols where the latter are more amenable to characterization; by employing a deuterium-labelled reducing agent, deuterium is incorporated into the product, which would otherwise be

indistinguishable from the same alcohol produced metabolically. Recent examples of the use of this technique are the studies on the metabolism of tetrahydrocannabinol isomers in vivo (167–169) and the glutathione-dependent dechlorination of chloramphenicol in vitro (294).

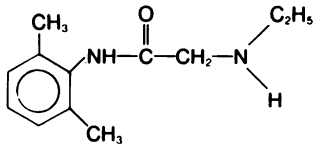
Equimolar mixtures of labelled and unlabelled derivatizing reagents have also been used to facilitate structure elucidation of unknown compounds by mass spectrometry, in much the same sense as in the isotope cluster technique referred to above. Indeed, this application actually predated the isotope cluster technique, being introduced in 1967 for the analysis of amino acid sequences in oligopeptides when derivatization was effected with a 1:1 mixture of acetic anhydride and [$^2H_6$]acetic anhydride (462). A more recent example of the usefulness of this approach for structure elucidation work is the work of Morris et al. (329) on the identification of slow reacting substances from rat basophil leukemia cells.

4. Stable Isotopes in Studies of Drug Interactions. Deuterium labelling techniques have proved to be extremely useful in defining the nature of molecular interactions that may take place as a result of the coadministration of two or more xenobiotics. This application may be illustrated effectively in a study by Nelson, Breck,



XLIX

and Trager (341) on the origin of a cyclic imidazolidinone (XLIX) isolated from the urine of humans and Rhesus monkeys given lidocaine (IX). In order to establish whether this compound was produced via intramolecular cyclization of a metabolically generated carbinolamine derivative, [$^2H_6$]lidocaine (X) was synthesized and administered to a Rhesus monkey. The cyclic metabolite contained only three atoms of deuterium, indicating that it arose in all probability from *N*-deethyllidocaine (L), a known major metabolite of the drug, by condensation



L

with some two-carbon compound. Proof that this second compound was acetaldehyde, formed by ingestion of alcohol, was obtained by an experiment in which lidocaine was coadministered with [$^2H_5$]ethanol; the cyclic metabolite was found to have incorporated deuterium under these conditions, indicating that *N*-deethylation of lidocaine and oxidation of ethanol afforded products that underwent facile condensation to form the imidazolidinone.

A second example of drug interactions involving ethanol is the synergism of chloral hydrate and ethanol in producing a CNS depressant effect. Since trichloroethanol, the reduction product of chloral hydrate, is known to be formed in vivo and to be a more potent CNS depressant agent than chloral hydrate, the synergistic effect of ethanol and chloral hydrate was suspected to be due to an enhancement in the coupled redox reaction for these two compounds. The actual occurrence of such a phenomenon was demonstrated successfully by Wong and Biemann (497), who showed that in rats, coadministration of chloral hydrate and [$^2H_6$]ethanol resulted in the urinary excretion of 2,2,2-trichloro[1-^{2}H]ethanol. Thus, the oxidation of ethanol must be tightly coupled with the reduction of chloral hydrate, the labelled coenzyme (NAD^{2}H) dissociating slowly from the alcohol dehydrogenase relative to the rate of formation of the enzyme-coenzyme-chloral hydrate complex. Further aspects of the enzymology of chloral hydrate reduction in the presence of ethanol have been reported by Shultz and Weiner (416), who showed that the rate of reduction of chloral hydrate and of incorporation of deuterium from [$^2H_6$]ethanol into trichloroethanol were decreased in vitro by the addition of pyrazole and isobutyramide, two inhibitors of alcohol dehydrogenase.

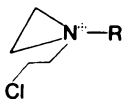
C. Mechanistic Applications

1. Deuterium in Mechanistic Studies. Applications of deuterium labelling to mechanistic problems generally involve studies on either the nature of short-lived, electrophilic intermediates of metabolism or on metabolic processes in which deuterium substitution at specific sites leads to primary or secondary kinetic isotope effects. The latter topic will be discussed separately in section III C 4.

After the discovery in 1962 that the carcinogen dimethylnitrosamine gives rise to alkylated nucleic acids in the liver of rats treated by injection (288), Lijinski et al. investigated the nature of the alkylating species by the use of di[$^2H_3$-methyl]nitrosamine (277). Analysis of hydrolysates of DNA and RNA isolated from the liver of rats given deuterium-labelled nitrosamine resulted in the identification of 7-methylguanine, which was shown to contain three atoms of deuterium. This finding eliminated diazomethane as the reactive methylating intermediate and pointed to some other reactive species that was capable of transferring an intact methyl group to nucleophilic sites on DNA and RNA. The same approach was used subsequently by Sussmuth et al. (434), who showed that *N*-trideuteromethyl-*N'*-nitro-*N*-nitrosoguanidine alkylated DNA in vitro by the transfer of an intact C$^2H_3$-group to the 7-position of guanine; the structure of this modified purine base was established by a combination of NMR spectroscopy and high resolution mass spectrometry. A third category of carcinogenic alkylating agents, the nitrosoureas, was later studied by Lijinski et al. (276), who again showed that an intact methyl group is transferred when one member of the

group, methylnitrosourea, interacts with DNA in vivo. Several substituted nitrosoureas have since found widespread application as chemotherapeutic alkylating agents and their mechanism of action has attracted considerable attention in recent years. 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU; LI), the most popular member of the series, is known to be unstable at physiological pH, decomposing to one or more reactive alkylating agents. Brundrett et al. (48) used two specifically deuterium-labelled analogs, [α-$^2H_4$]- and [β-$^2H_4$]BCNU, to investigate the nature and labelling pattern of these breakdown products in buffer; the results from this study were found to be inconsistent with vinyl-carbonium ion or diazochloroethyl intermediacy, but were consistent with some rearrangement to the 1-chloroethylcarbonium ion and the cyclic chloronium ion. Recently, deuterium labelling and mass spectrometry have been employed by Weinkam and Lin (484) to investigate the reaction sequence of BCNU decomposition and to clarify the origin of some of the products (fig. 4). Thus, when the substrate was an equimolar mixture of unlabelled and [$^2H_8$]BCNU (labelled on the two ethylene groups), it was found that one breakdown product, 2-[(2-chloroethyl)amino]-2-oxazoline (CAO; LIII), became labelled with four, as well as eight, atoms of deuterium; moreover, the relative amount of [$^2H_4$]CAO increased with incubation time, indicating that CAO may be formed by two pathways, directly from BCNU and from cyclization of 1,3-bis(2-chloroethyl)urea (BCU; LII).

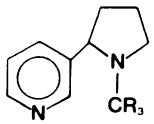
Deuterium-labelling techniques have also been employed profitably in studies of the mechanism of action of the antitumor agent cyclophosphamide. Oxidative metabolism of the heterocyclic ring system, which is a necessary step in the "activation" of this drug (fig. 1), leads to the liberation of acrolein (XXIII), a product that could arise from nonenzymic decomposition of either a 4-hydroxy (XIX) or a 6-hydroxy derivative of cyclophosphamide. In order to distinguish between these alternate pathways, [4,4-$^2H_2$]cyclophosphamide (XLVIII) was synthesized and incubated with rat liver microsomal preparations; the acrolein released was trapped as its 2,4-dinitrophenylhydrazone derivative and shown by mass spectrometry to contain only one atom of deuterium (75). These studies showed that metabolism of [$^2H_2$]cyclophosphamide occurred via initial hydroxylation at C-4 (where one of the two deuterium atoms is lost) rather than at C-6, which would lead ultimately to an analog of acrolein that retained both deuterium labels. Further investigations on two of the cytotoxic metabolites of cyclophosphamide, phosphoramide mustard (XXII) and nornitrogen mustard (XXIV), also employed deuterium-labelling techniques to demonstrate that alkylation of a



LIV

model nucleophile in vitro proceeds via a cyclic aziridinium intermediate (LIV) rather than via a direct S_N2 displacement of the chlorine atom (73).

The use of stable isotopes in toxicological studies of chemically reactive drug metabolites has been reviewed recently by Mitchell and Nelson (318), with examples from studies on isoniazid/acetylhydrazine, iproniazid/isopropylhydrazine, phenacetin, acetaminophen, and furosemide. In the case of acetylhydrazine, deuterium labelling was used to show that metabolism of this compound led to an electrophilic species that retained all three hydrogen atoms of the original acetyl group, and which therefore could not be ketene, $CH_2{=}C{=}O$ (344). Studies on iproniazid and isopropylhydrazine activation, on the other hand, were performed with specifically tritium-labelled substrates, the position of the radiolabel being verified in each case by comparison with the corresponding deuterium-labelled analogs whose structures could be assigned unambiguously (346).The metabolic oxidation of tertiary amines to chemically reactive



LV R = H
LVI R = ^2H

iminium ions has been demonstrated by Castagnoli and coworkers, who employed cyanide ion as an in vitro

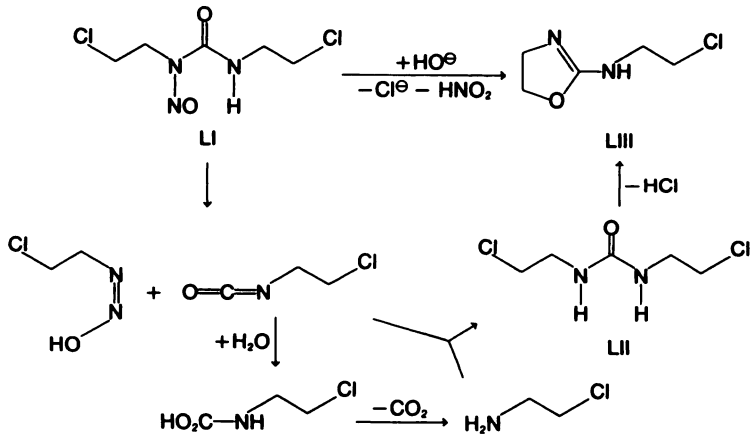
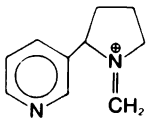


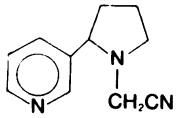
FIG. 4. Pathways for the chemical degradation of BCNU (LI), indicating the dual origin of the oxazoline derivative (LIII) as revealed by deuterium labelling studies.

trapping agent (186, 354). When nicotine (LV) was studied by this technique, evidence was obtained for the in situ generation of an *N*-methyleneiminium species (LVII), formed during the course of metabolic oxidative



LVII

N-demethylation; by using racemic [methyl-$^2H_3$]nicotine (LVI) as substrate in incubations with rabbit liver microsomal preparations, it could be shown that formation of the "trapped" *N*-(cyanomethyl)-nornicotine (LVIII) oc-

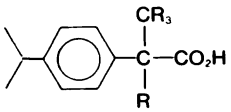


LVIII

curred, at least in part, without prior nitrogen-carbon bond cleavage. In an extension of this work, analogous studies were performed with 1-benzylpyrrolidine as substrate, the results from which suggested that metabolically generated iminium ions are capable of alkylating nucleophilic functionalities present on microsomal macromolecules (186). Finally, the generation of a naturally occurring reactive metabolite, the hydroxyl radical (ȮH), has been studied by a deuterium-labelling procedure (381). In this work, reactions producing ȮH (xanthine/xanthine oxidase or Fe^{2+}/EDTA/H_2O_2) were found to generate methane from dimethylsulfoxide (DMSO); when [$^2H_6$]DMSO was used in place of the unlabelled compound, [$^2H_3$]methane was formed, showing that the $\dot{C}H_3$ radical formed by reaction of ȮH with DMSO does not abstract a proton from a second molecule of DMSO.

An early example of the use of deuterium labelling in mechanistic studies of drug metabolism is that published by Walle et al. (472), who employed [$^2H_6$-isopropyl]propranolol (XLV) to confirm that the isopropylamine detected in the urine of dogs given propranolol was indeed a metabolite of the drug and not an endogenous product of intermediary metabolism. This study demonstrated for the first time that deamination of an *N*-isopropyl compound may occur directly, without prior dealkylation to give the primary amine. A more recent investigation of propranolol metabolism involving a deuterium-labelled analog of the drug has shown that [side-chain-$^2H_5$] propranolol (XLIV) is converted by rat liver 9,000 × *g* supernatant preparations to [$^2H_4$]propranolol-diol, thereby indicating an aldehyde intermediate (XLVII) in the process (350). The results from this latter study are thus in agreement with those of Walle et al. and show that a major pathway for the *N*-dealkylation of propranolol in mammalian system involves initial oxidation at the methylene group adjacent to the side-chain nitrogen atom. In a further example of the usefulness of deuterium-labelled compounds in metabolic studies, Livesey and Anders (280) showed conclusively that the ethylene detected from microsomal incubations of dibromoethane was derived from the halocarbon by the use of dibromo[$^2H_4$]ethane, which yielded [$^2H_4$]ethylene. In vivo deuterium labelling techniques may be used to yield information on the origin of functional groups introduced into xenobiotics from endogenous sources. Thus, in a study of the genesis of the *S*-methyl group in recently detected methylthio metabolites of naphthalene, Horning and coworkers (429) administered [methyl-$^2H_3$]methionine to rats that had been maintained on a methionine-free diet for nine days to deplete endogenous stores of this amino acid; after a single i.p. dose of naphthalene, metabolites were isolated from urine and analyzed by GC-MS. All of the methylthio naphthalene derivatives were found to have incorporated a deuteromethyl group, to the extent of about 40%, and a catechol methyl ether had also become similarly labelled. This study therefore implicated *S*-adenosylmethionine as being the methyl donor in the formation of both methylthio and methylcatechol metabolites of naphthalene.

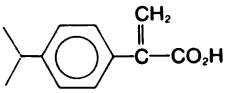
An interesting example of the use of deuterium labelling in mechanistic studies is the work of a group at the Upjohn Company who studied stereochemical aspects of the metabolism of the nonsteroidal anti-inflammatory



LIX R = H

LX R = ^2H

agent ibuprofen (LIX) (480). Following reports that human urinary metabolites of the racemic drug were optically active and that the individual S(+) and R(−) enantiomers of the parent compound were essentially biologically equivalent in vivo, a [$^2H_4$] analog (LX) of the R(−) form was synthesized for administration to human subjects. GC-MS analysis of unchanged drug and metabolites in biological fluids demonstrated clearly that the R(−) enantiomer undergoes facile epimerization to its S(+) antipode through a previously unrecognized enzymatic pathway that allows for inversion of configu-

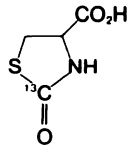


LXI

ration at saturated carbon. The intermediacy of a symmetrical unsaturated intermediate (LXI) in this process was revealed by the conversion of the [$^2H_4$] R(−) isomer to an analog of the S(+) isomer that contained only two atoms of deuterium.

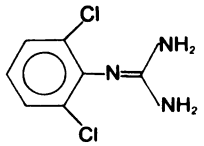
2. Carbon-13 and Nitrogen-15 in Mechanistic Studies. Studies in which the stable isotopes of carbon and nitrogen have been used as tracers in mechanistic studies have been relatively rare compared to those with deuterium and oxygen-18. Anders and coworkers have used ^{13}C-labelled substrates to examine the conversion of haloforms to carbon monoxide; after the demonstration that halocarbons are metabolized to CO in vitro (4) and in vivo (6), the mechanism of the reaction with bromoform was studied with $^{13}CHBr_3$ as substrate (428). When this

labelled species was incubated with rat liver microsomal preparations in the presence of cysteine as a trapping agent, 2-[^{13}C]oxothiazolidine-4-carboxylic acid ([^{13}C] OTZ; LXII) was formed, thereby indicating the existence



LXII

of dibromocarbonyl as an intermediate in the reaction. Additional experiments were also conducted with $^{18}O_2$ and C^2HBr_3, which, taken together with the results from the $^{13}CHBr_3$ work, permitted a detailed mechanism to be proposed for the conversion of haloforms to carbon monoxide. In a recent report from the same group, dichlorocarbonyl (phosgene) was shown to be formed during the aerobic incubation of carbon tetrachloride with hepatic microsomal fractions; $^{13}CCl_4$ was used as substrate and cysteine was again used as a trapping agent (266). Phosgene is also known to arise from the in vitro metabolism of chloroform, as was first shown by Pohl et al. (373), who used ^{18}O labelling techniques and the cysteine trapping method. Labelling with ^{13}C has been used to elucidate the mechanism by which heterocyclic ring cleavage occurs during the metabolism of clonidine (XXVI), the first of a series of imidazoline derivatives to find clinical application as antihypertensive agents (27). Although the larger fragment formed by ring cleavage, 2,6-dichlorophenylguanidine (LXIII), had been characterized previ-



LXIII

ously (187), no information was available as to the fate of the two carbon atoms that formed C-4 and C-5 of the imidazoline ring. In order to distinguish between a mechanism involving liberation of these carbon atoms as one-carbon fragments (e.g. CO_2) or a two-carbon fragment (e.g. glyoxal, CHO-CHO), [4,5-$^{13}C_2$]clonidine was synthesized and used as substrate in metabolic studies. By the use of *o*-phenylenediamine as a trapping agent, it was shown that both glyoxal and its oxidation product, glyoxylic acid, were formed during the metabolism of clonidine and that these compounds each became labelled with two atoms of ^{13}C when [4,5-$^{13}C_2$]clonidine was employed as substrate.

In an application of ^{15}N labelling to mechanistic studies, Cottrell et al. investigated the metabolic fate of dimethyl [$^{15}N_2$]nitrosamine in rat liver 10,000 × *g* supernatant fractions (80). Gaseous $^{15}N_2$ was identified in the headspace from these incubations, but quantitative measurements showed that the rate of $^{15}N_2$ evolution was less than 5% of the rate of formation of either of the carbonaceus products, formaldehyde and methanol. These results are inconsistent with the generally accepted mechanism of dimethylnitrosamine degradation and further studies on quantitative aspects of this problem are thus indicated. The reductive metabolism of nitrous oxide by human and rat intestinal contents has been reported recently by Trudell and coworkers (193), who used $^{15}N_2O$ for incubations with intestinal contents. After removal of excess nitrous oxide, water vapor, and oxygen from the headspace gases at the end of the incubation period, ^{15}N-labelled nitrogen was identified and quantified by isotope ratio mass spectrometry. The authors suggest that bacterial reduction of N_2O to N_2 may proceed through single electron transfer processes with the formation of potentially toxic free radical intermediates. In studies on the mechanism of hydralazine-induced lupus erythematosus, Timbrell and Harland (456) have investigated the possible role of reactive metabolite formation in the genesis of this adverse side effect of hydralazine therapy and have identified hydrazine in the urine of patients treated with the drug. In view of the known instability of hydrazine and derivatives thereof, it was necessary to establish that the urinary hydrazine was not an artefact formed by chemical decomposition in the urine of some unidentified compound. [$^{15}N_2$]Hydrazine was used to resolve this issue and was added in known amounts to samples of urine from a patient who was receiving hydralazine for therapeutic reasons; by measuring the ratio of [$^{15}N_2$]:[$^{14}N_2$] hydrazine in extracts of these samples, it was possible to establish that the unlabelled species was not being produced as an artefact of the sample storage or treatment procedures but was indeed derived from metabolism of hydralazine. The mechanism by which this toxic agent arises from hydralazine, however, remains to be established.

3. Oxygen-18 in Mechanistic Studies. In view of the widespread occurrence of oxidative transformations in the metabolism of both drugs and endogenous substances, it is hardly surprising that tracer experiments with isotopically labelled oxygen outnumber those with ^2H, ^{13}C, or ^{15}N for mechanistic studies. As is the case with nitrogen, no suitably long-lived radioactive isotope exists for oxygen, and one is therefore obliged to use the stable isotopes ^{17}O or ^{18}O. For reasons of availability, isotopic enrichment, cost, and mass increment over ^{16}O, ^{18}O has been the isotope of choice in virtually all applications.

Our present understanding of the reactions catalyzed by mixed function oxidase systems has been, in large part, due to metabolic studies carried out in the presence of $^{18}O_2$ or $H_2{}^{18}O$. In 1955, Mason et al. (295) demonstrated for the first time that the oxygen atom introduced into 3,4-dimethylphenol during metabolism in vitro derived from molecular oxygen, and not from water as had been assumed previously. This pioneering study, in which incubations were carried out under an atmosphere of $^{18}O_2$ or in the presence of $H_2{}^{18}O$, led to greatly renewed interest in the mechanisms of metabolic oxidation, although progress remained slow until the advent of GC-MS technology some nine years later. In a now classic study on the metabolism of naphthalene (LXIV) entitled,

"The Metabolic Products of Naphthalene in Mammalian Systems," Holtzman, Gillette, and Milne (192) carried out in vitro experiments under atmospheres enriched in $^{18}O_2$ and showed, by GC-MS analysis, that the dihydrodiol metabolite, 1,2-dihydro-1,2-dihydroxynaphthalene (LXVI) contained only one atom of ^{18}O and that this labelled oxygen occupied the 1- (or α-) position (fig. 5). The authors inferred that the second oxygen atom derived from water, a conclusion that was verified shortly thereafter by Jerina et al. (227), who used $H_2{}^{18}O$ in incubations with rat liver microsomal preparations. In addition, this latter study resulted in the isolation of the putative arene oxide of naphthalene (LXV) from incubation media and thus provided direct experimental evidence in support of 1,2-naphthalene oxide as being the common obligatory intermediate in the metabolism of naphthalene to the 1,2-dihydrodiol, mercapturic acid derivative (LXVII) and α- (LXVIII) and β-napthol (LXIX). Early studies on the mechanisms of metabolic oxidation at a saturated carbon were performed by McMahon et al. (309), who used $^{18}O_2$ to show that the source of the oxygen atom in microsomal *N*-dealkylation reactions was molecular oxygen; *N*-benzyl-4-phenyl-4-carbethoxypiperidine was the substrate used in this work, while 1-benzyl-4-cyano-4-phenylpiperidine was used in a subsequent study (308), the [^{18}O]benzyldehyde liberated during the course of the reaction in each case being reduced enzymatically in situ to benzyl alcohol in order to minimize any exchange of label with water prior to mass spectrometric analysis. McMahon et al. (311) again used molecular oxygen-18 to show that microsomal oxidation of ethyl benzene occurs via a direct oxygenation of the substrate; stereochemical aspects of the transformation to methyl phenyl carbinol were also investigated in this work, which utilized specifically deuterium-labelled variants of ethyl benzene to show that hydroxylation occurred with retention of configuration at the benzylic carbon. Microsomal 3-hydroxylation of 1,4-benzodiazepines has been investigated by Sadeé et al. (391), who used ^{18}O labelling to show that the principal source of the C-3 hydroxyl oxygen atom was molecular oxygen, thus extending to an imino system the earlier observations of McMahon et al. (309).

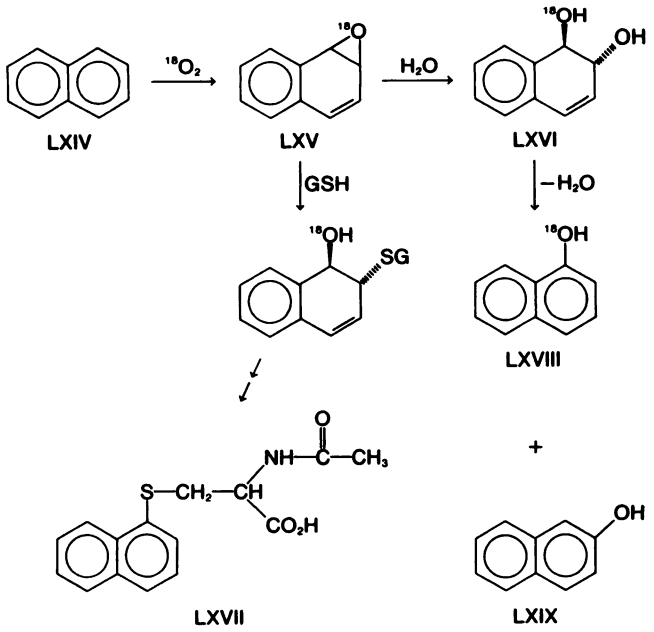


FIG. 5. The metabolism of naphthalene under atmospheres enriched in $^{18}O_2$. GSH, glutathione.

After the above work on oxidation at carbon, a number of papers appeared in which oxygen-18 was applied to studies on the mechanism of oxidation at nitrogen. Schmidt et al. (400) established that the conversion of *p*-thioanisidine to its *N*-hydroxy derivative by rat liver microsomal preparations was accompanied by the introduction of an atom of ^{18}O from an atmosphere of $^{18}O_2$ and was therefore mediated by a mixed-function oxidase enzyme, presumed to be cytochrome P-450; oxidation of *N*-ethylaniline to nitrosobenzene, on the other hand, also gave rise to incorporation of ^{18}O but appeared to be catalyzed by a microsomal flavoprotein. Cytochrome P-450 was implicated in the enzymatic *N*-hydroxylation of 2,4,6-trimethylacetophenone imine to give the corresponding oxime (368). On the basis of this finding, McMahon et al. proposed that the observed formation of oximes from primary amines occurs by a three-step process, viz. α-hydroxylation of the amine to give an unstable carbinolamine, dehydration of the carbinolamine to form an imine and, finally, *N*-oxidation of the imine to afford the oxime. These conclusions suggested that the carbonyl compounds produced during metabolic deamination of amines might arise by two different pathways, one of which involves loss of the elements of NH_3 from the carbinolamine and the other involving hydrolysis of the oxime. In order to distinguish between these alternative mechanisms for the metabolism of *d*-amphetamine to phenylacetone, the Lilly group carried out incubations of the drug with rabbit liver microsomes under an atmosphere of $^{18}O_2$ (367). Incorporation of heavy oxygen into phenylacetone oxime and phenylacetone was found to be 93% to 95% and 25% to 31%, respectively; these figures would suggest that hydrolysis of the oxime (loss of ^{18}O) predominates over cleavage of the carbinolamine (retention of ^{18}O) for this particular substrate, although exchange of label in the [^{18}O]phenylacetone with water of the medium may have contributed to the low measured incorporation of ^{18}O into the ketone (366). The results of this study were verified independently by Hucker et al. (207), who concluded that phenylacetone oxime is an important intermediate in the oxidative deamination of amphetamine by a rabbit liver microsomal system. More recently, the role of a different intermediate, *N*-hydroxy amphetamine, in the deamination process has been investigated by an isotope dilution technique using deuterium-labelled amphetamine (503); this work showed that although phenylacetone can be formed from *N*-hydroxyamphetamine, the latter is not an obligatory intermediate in the process (366). A review of stable-isotope-labelling

procedures employed in the study of mechanistic aspects of amphetamine metabolism has been published by Kammerer et al. (236).

Further studies on the mechanism of metabolic oxidation of aromatic compounds have been reported by Billings and McMahon (35), who employed elegant oxygen-labelling techniques to define the pathway leading from biphenyl to its catechol metabolite, 3,4-dihydroxybiphenyl. By performing incubations with biphenyl under an atmosphere that contained $^{18}O_2$ and $^{16}O_2$ in a 1:1 ratio, analysis of the distribution of labelled molecules in the catechol permitted the authors to distinguish between three distinct pathways for its formation: 1) dehydrogenation of a dihydrodiol produced, in turn, from biphenyl 3,4-arene oxide; 2) formation of a cyclic biphenyl 3,4-endoperoxide and subsequent rearrangement to the catechol; 3) two successive hydroxylations at adjacent carbon atoms. In the event, the last of these pathways was shown to be operative for this substrate. Similar studies with the $^{18}O_2/^{16}O_2$ mixture technique were reported by Murphy et al. (331), who showed that the catechol metabolite of butamoxane was also formed via two consecutive hydroxylations, and by Samuelsson (393), who first used the method to demonstrate the existence of an endoperoxide intermediate in the biosynthesis of prostaglandin E_1 from arachidonic acid.

Recent work on the mechanism of oxygen transfer by prostaglandin hydroperoxidase has shown that an atom of ^{18}O is transferred by the enzyme from [^{18}O]15-hydroperoxyprostaglandin E_2 to a reducing substrate, sulindac sulfide, with the simultaneous formation of [^{18}O]prostaglandin E_2 (107). This is both the first report of oxygen atom addition to reducing substrate from hydroperoxide by the cyclooxygenase-peroxidase complex and of sulfoxide formation via direct oxygen transfer from hydroperoxide to sulfide by any peroxidase. Cytochrome P-450 has been known for some time to support the oxidative metabolism of a variety of xenobiotics under anaerobic conditions if certain organic hydroperoxides are added to the medium, and it has been shown with $H_2{}^{18}O$ that the oxygen atom introduced into the substrate derives from the peroxide and not from water (357). Very recent work on the bacterial $P\text{-}450_{CAM}$ system has clarified the role of lipoic acid, an effector substance necessary for enzyme activity in vitro (421). When incubations were performed under an atmosphere of $^{18}O_2$, one atom of ^{18}O was incorporated into the reaction product (*exo*-5-hydroxycamphor), while the second atom of ^{18}O was found to be present in the carboxyl group of lipoic acid; thus, formation of an intermediate acyl peroxide in the $P\text{-}450_{CAM}$-mediated hydroxylation of camphor was indicated.

Oxygen-18 has been used recently in several investigations of metabolic activation. Thus, as mentioned in the previous subsection, Pohl et al. (373) obtained evidence for the formation of phosgene as a metabolite of chloroform by carrying out microsomal incubations in the presence of $^{18}O_2$, while Kubic and Anders (266) used a similar approach to show that CCl_4 was also metabolized to this reactive intermediate. Similar ^{18}O-labelling techniques were used by Stevens and Anders (428) to demonstrate that bromoform is metabolized to dibromocarbonyl, which can be further converted to carbon monoxide in a glutathione-dependent process. The mechanism by which dihalomethanes are metabolized to carbon monoxide has been studied by Kubic and Anders (265), who showed by ^{18}O-labelling experiments that the oxygen atom of CO derives from molecular oxygen; these workers were thus able to rule out the formation of carbene as an intermediate in the reaction sequence since conversion of this reactive species to CO would necessitate the incorporation of oxygen derived from water. The mechanism of activation of phenacetin to reactive metabolites has received much attention and has been studied thoroughly by Hinson and coworkers (182, 183, 349), who have made extensive use of ^{18}O-labelling procedures. Thus, by performing incubations of phenacetin in the presence of $^{18}O_2$, and by studying the metabolism of [*p*-^{18}O]phenacetin, evidence was obtained for the existence of at least three different pathways by which phenacetin can be converted by liver enzymes to reactive electrophilic intermediates. These are: 1) *O*-deethylation to acetaminophen, which is subsequently activated by cytochrome P-450; 2) direct oxidation to a species tentatively identified as phenacetin-3,4-epoxide; 3) conversion to *N*-hydroxyphenacetin, followed by activation via glucuronide formation. Hinson et al. (184) have also used $^{18}O_2$ to establish the origin of the oxygen atom introduced at the C-3 position during the metabolism of acetaminophen to a catechol metabolite. Two mechanisms for the formation of this compound were considered, viz oxidation of acetaminophen to *N*-acetylimidoquinone, followed by nucleophilic attack by water at C-3, and direct *meta*-hydroxylation of the drug by a mixed-function oxidase system. Incubation of acetaminophen under an atmosphere of $^{18}O_2$ resulted in the formation of 3-[^{18}O]hydroxyacetaminophen, thereby demonstrating the direct hydroxylation pathway. The possible existence of an epoxide intermediate in the oxidative degradation of the imidazolidine ring system of *N,N'*-dimethylclonidine was studied by Baillie and coworkers (91). By carrying out incubations with rat liver microsomal preparations under an atmosphere of $^{18}O_2$, these authors were able to show that the major route of metabolism of this compound involved introduction into the heterocyclic ring of two [^{18}O]hydroxyl groups at adjacent positions to give a *bis*-carbinolamine derivative that underwent spontaneous ring cleavage; thus, metabolism via the epoxide-diol pathway (which would have led to a diol with only one [^{18}O] hydroxyl group) did not represent an important pathway of biotransformation.

Examples of the use of ^{18}O in studies on the mechanism of processes other than metabolic oxidations are investigations of regioselectivity in the enzymatic hydration of *cis*-1,2-disubstituted [^{18}O]epoxides (164) and of enzy-

matic conjugation and hydrolysis of [^{18}O]isoborneol glucuronide (228).

4. Deuterium Isotope Effects. Kinetic deuterium isotope effects have been used widely in studies of the mechanism of enzyme-catalyzed reactions and both theoretical and practical aspects of the subject have been reviewed thoroughly (36, 347, 399, 418, 453, 455, 487). Primary kinetic isotope effects refer to processes in which the bond to deuterium (usually a C-^{2}H bond) is broken during the reaction, and are expressed as the ratio of the specific rate constants, k_H/k_D, for the initial reaction of the protium- and deuterium-labelled compounds, respectively. The origin of the primary deuterium isotope effect lies in the large relative mass difference between ^{1}H and ^{2}H, which results in a difference (1.2 to 1.5 kcal mole^{-1}) between the zero-point energy of a bond to deuterium versus hydrogen. This, in turn, leads to a greater energy requirement for cleavage of a bond to deuterium and hence to isotope effects on the rates of metabolic processes where rupture of a C-^{2}H bond is rate-determining. Wiberg (487) calculated that at 25°C, the maximum primary kinetic deuterium isotope effect should be in the order of 7 to 10, although Aronow (12) has reported the maximum theoretical value to be 18. In multistep enzymatic processes, however, many factors may influence, or "mask," this intrinsic isotope effect and thus observed (or "apparent") kinetic deuterium isotope effects are normally much less than the above figures. Secondary kinetic isotope effects refer to processes in which the bond to deuterium is *not* broken during the reactions; in this case, deuterium substitution in close proximity to the reaction center exerts an effect on the energy of the transition state of the reaction. As would be expected, secondary deuterium isotope effects are normally much smaller than their primary counterparts, although they may be useful in providing information on the geometry of the transition state of metabolic reactions (455). Experimentally, the observation of a large kinetic deuterium isotope effect ($k_H/k_D > 5$) on a given metabolic reaction may be taken as strong evidence that cleavage of the C-H bond in question is rate-limiting in the reaction. Smaller isotope effects ($k_H/k_D < 2$), on the other hand, may be due either to primary or secondary phenomena and cannot be taken to indicate that C-H bond cleavage is the rate-determining step.

The first example of a primary deuterium isotope effect in a microsomal oxygenase system was reported by Elison et al. (114, 115), who compared the rates of *N*-demethylation of morphine and its *N*-deuteromethyl analog. The apparent isotope effect, in this case, was determined to be 1.4 and it was also shown that the labelled drug was bound less tightly to the enzyme, as reflected by the ratio of Michaelis constants ($K^D_m/K^H_m = 1.43$). Furthermore, not only was [$^2H_3$]morphine a less potent analgesic than morphine, but it was a stronger base (pK_a of [$^2H_3$]morphine = 8.17, pK_a of morphine = 8.05). This interesting work thus emphasized some of the differences in physicochemical, as well as pharmacological, properties of deuterium-labelled compounds. Some years after the publication of the morphine studies, Mitoma et al. (321) demonstrated the existence of a primary deuterium isotope effect on the *O*-demethylation of *o*-nitroanisole ($k_H/k_D \sim 2$). These early studies indicated, therefore, that C-H bond breaking was important, and was probably rate-limiting, in the metabolic removal of *N*- and *O*-methyl groups, a finding that is in full agreement with the now accepted common mechanism for *N*- and *O*-dealkylation reactions (307). Subsequently, many other reports of primary deuterium isotope effects in metabolic reactions have appeared. Examples of these are an isotope effect of 1.8 for the hydroxylation of ethylbenzene (311), 1.23 for the *N*-demethylation of ethylmorphine (450), 1.49 for the *N*-deethylation of lidocaine (348), 1.45 for the *N*-demethylation of dimethylaminophenylpropane (1), 1.61 and 1.86 for the *O*-deethylation of phenacetin (144, 342), 1.43 for the 3-hydroxylation of biphenyl (35), 1.46 for the *N*-debenzylation of 1-benzyl-4-cyano-4-phenylpiperidine (308), and 2.0 for the deamination of *l*-amphetamine (125). An unusually large primary deuterium isotope effect of approximately 7 has been measured for the 3-hydroxylation of cotinine in vivo (61, 89).

Early studies on primary deuterium isotope effects in vitro were performed by examining the initial reaction velocities from separate experiments with unlabelled and deuterated substrates. A more accurate technique was developed subsequently, in which a mixture (usually 1:1) of the two molecular species under investigation is used as metabolic substrate and the ratio of rate constants (k_H/k_D) is determined directly by mass spectrometric analysis of the ratio of labelled to unlabelled molecules in the product; this approach does, however, require that the substrate be labelled in two positions, one of which is not susceptible to metabolic attack and which thus leads to a product that can be distinguished from the metabolite formed from the unlabelled substrate. The cotinine study referred to above is illustrative of this approach (89), in which *intermolecular* competition for substrate is generated. More recently, *intramolecular* deuterium isotope effects have been studied, as exemplified by work on the mono-*O*-demethylation of *p*-trideuteromethoxyanisole in rat liver microsomal preparations (126). In this case, competition occurs for metabolism at chemically equivalent positions in the same molecule, one of which is labelled with deuterium and the other is unlabelled. By this approach, the influence of many rate-contributing or rate-limiting steps in the overall enzymatic process is removed and a kinetic isotope effect is obtained that more accurately reflects the true value. Thus, in the above example of *p*-trideuteromethoxyanisole, the primary deuterium isotope effect on *O*-demethylation was found to be 10, while in a similar study on asymmetrically deuterated 1,3-diphenylpropane, Trudell and coworkers (185) obtained a value of $k_H/k_D = 11$. Recent studies by Miwa et al. (322) have

shown that in the *N*-demethylation of *N*,*N*-dimethylphentermine, no *inter*molecular isotope effect could be detected when unlabelled and *N*,*N*-di(trideuteromethyl)phentermine were used as substrates. However, when *N*-methyl,*N*-trideuteromethyl-phentermine was employed alone, an *intra*molecular isotope effect of 1.62 was obtained. The mechanistic implications of these latter findings are discussed in detail by the authors.

As examples of the use of secondary deuterium isotope effects in mechanistic studies may be cited papers on the *N*-deethylation of the antiarrhythmic agent lidocaine (348) and the *O*-deethylation of the analgesic drug phenacetin (144). In the former case, substitution with deuterium on the terminal carbons of each *N*-ethyl group gave rise to a substantial secondary isotope effect (k_H/k_D = 1.52) on *N*-deethylation. In contrast, no secondary isotope effect was detected when the corresponding deuterium-labelled analog of phenacetin was metabolically *O*-deethylated by rabbit liver microsomal preparations. These results thus indicate differences in the precise mechanism of oxidative *O*- and *N*-dealkylation. Secondary deuterium isotope effects were also studied by Hanzlik and Shearer (165), who compared olefin epoxidation by cytochrome P-450 with that obtained chemically with *m*-chloroperbenzoic acid. The results of this investigation showed that in both the enzymatic and chemical epoxidations, the oxygen atom is transferred to the olefin (*p*-phenylstyrene or *p*-methylstyrene) in an asymmetric nonconcerted fashion.

Metabolic hydroxylation of aromatic ring systems do not in general exhibit primary deuterium isotope effects as shown, for example, in the cases of acetanilide (29, 37, 439), phenobarbital (370), and carbamazepine (364). This can be rationalized for compounds metabolized via the arene oxide pathway, since cleavage of a C-O (and not C-H) bond of the intermediate arene oxide is rate-limiting in rearrangement to the phenolic product (241). However, there have been reports of deuterium isotope effects in the *meta*-hydroxylation of certain aromatic substrates, e.g. nitrobenzene, methyl phenyl sulfide, methyl phenyl sufone (457), and biphenyl (35). These findings suggest the existence of alternative (nonarene oxide) pathways, possibly involving a direct hydroxylation mechanism, by which aromatic compounds may be metabolized to phenolic derivatives.

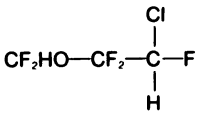
Following the discovery of kinetic deuterium isotope effects on metabolic processes, the effect of deuterium substitution on the pharmacological and toxicological properties of a wide variety of xenobiotics was evaluated. The rationale for such work was to determine whether introduction of deuterium at known (or suspected) sites of metabolic attack could be used to obtain information on rate-determining steps in metabolic processes and on mechanisms of metabolic activation. While much of this work has been reviewed by Blake, Crespi, and Katz (36), the following papers may be cited as examples of the diversity of biologically active compounds studied. Tanabe, Mitoma, and coworkers (437) studied the effect of deuterium substitution on the in vitro oxidation of tolbutamide, a hypoglycemic agent; conversion of [$^2H_3$]tolbutamide to hydroxy[$^2H_2$]methyltolbutamide by the 10,000 × *g* supernatant fraction of rat liver was found to take place at a rate that was only marginally slower than with the unlabelled compound, indicating that carbon-hydrogen bond breaking was not the rate-determining step in this oxidative reaction. A similar conclusion had been reached some six years earlier when the urinary excretion of the carboxylic acid metabolite of tolbutamide was measured in human subjects given the drug, and shown not to differ when the same subjects were given a dose of [$^2H_3$]tolbutamide (274). Tanabe et al. (438) also studied the effect of deuterium substitution at the penultimate (3′) side-chain carbon atom of 5-*n*-butyl-5-ethylbarbituric acid and found that the sleeping time of mice given this labelled drug was approximately double that of mice given the unlabelled equivalent; this pharmacological effect could be correlated with a longer biological half-life of the deuterated drug and a significantly reduced rate of its metabolism in vivo. Both the metabolism and anticonvulsant activity of deuterated *N*-demethyldiazepam, labelled at the C-3 position, differed markedly from that of the unlabelled drug; in this case, deuterium substitution reduced metabolism to oxazepam and hence shortened the duration of anticonvulsant activity in mice from 20 hours to 5 hours (289).

The toxicity of polycyclic aromatic hydrocarbons has received considerable attention in recent years and deuterium-labelling studies have been used to furnish information on mechanisms of metabolic activation. Thus, while an enhancement in carcinogenicity of 7,12-dimethylbenz[a]anthracene resulted from substitution of all hydrogens by deuterium (54), the carcinogenicity of 3-methylcholanthrene was reduced appreciably by selective deuteration at positions 1 and 5 (64); in the latter example, the authors concluded that the 1-carbon atom of the hydrocarbon is a critical binding site to cellular targets in the tumor-initiating process. Several studies have dealt with the effect of deuterium substitution on the carcinogenicity of nitrosamines. Lijinsky and coworkers (244) reported that di(trideuteromethyl)nitrosamine ([$^2H_6$]DMN), when administered chronically to rats, led to a significantly lower incidence of liver tumors than did unlabelled DMN, while Dagani and Archer (88) observed a deuterium isotope effect of 3.8 in the rate of *N*-demethylation of [$^2H_6$]DMN by rat liver microsomal preparations. These findings are consistent with the currently held view of nitrosamine-induced carcinogenesis, in which metabolic activation of the parent compound is required for toxicity and occurs by way of *N*-dealkylation; initial *C*-hydroxylation appears to be rate limiting in this process and hence the observed primary kinetic isotope effect for [$^2H_6$]DMN and the accompanying decrease in its toxicity. 4-Nitrosomorpholine has also been studied by deuterium-labelling techniques, when it was shown

that the incidence of liver tumors in rats could be reduced approximately fivefold by substitution of the α-hydrogens (at positions 3 and 5) with deuterium (278). This same labelled nitrosomorpholine was subsequently found to be five times less mutagenic than its unlabelled counterpart in the *Salmonella typhimurium* TA 1535 test system (65), a result suggestive of a C-H bond α- to the nitrosamino function undergoing cleavage during the rate-limiting step of the activation process. A comparative study has been reported in which the effect of deuterium substitution on the mutagenicity of nitrosamines (which require metabolic activation) and nitrosamides (directly acting mutagens) was evaluated; the results showed that the deuterated nitrosamines, following activation by rat liver enzymes, were less active mutagens than the corresponding unlabelled species, while deuterium substitution had no effect on the mutagenicity of nitrosamides (113). Experiments such as these have proved useful in formulating detailed chemical mechanisms for the production of reactive electrophilic intermediates from carcinogenic and mutagenic precursors.

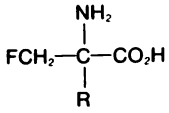
Further examples of the use of deuterium isotope effects in studies of mechanisms of toxicity are investigations of the metabolism of furosemide, a diuretic agent (491), bromoform (6, 428), chloroform (374, 375), dibromoethane (3, 265), dichloromethane (265), and the analgesic drug phenacetin (342). The mechanism of antitumor activity of cyclophosphamide has been studied in detail by Jarman and coworkers (75, 81), who have made extensive use of specifically deuterium-labelled analogs of the drug to define the pathway of metabolic activation. The same group has reported recently on the influence of selective deuteration on the metabolism of the antitumor drug chlorambucil, which is converted by β-oxidation in vivo into phenylacetic mustard, a less effective antitumor agent (120).

Investigations of the hepatic and renal toxicities that may occur as a result of exposure to volatile anesthetics have established that, in the majority of cases, these adverse side effects may be attributed to the formation of reactive electrophilic metabolites and/or the release of fluoride ion, a known nephrotoxin. Specifically deuterium-labelled variants of such drugs have therefore been prepared by a number of workers in order to better define the mechanisms of their metabolism and toxicity. Pohl and coworkers (51), for example, measured the relative rates of defluorination of specifically deuterated analogs



LXX

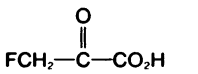
of enflurane (LXX) in order to distinguish between two possible metabolic pathways for the liberation of fluoride ion. Labelling at the chlorofluoromethyl group resulted in significantly lower levels of fluoride ion than were produced from enflurane itself, whereas labelling at the difluoromethyl group had no effect; these results were interpreted to indicate that metabolic attack occurred at the chlorofluoromethyl group, deuterium substitution at which gave rise to a primary kinetic isotope effect and hence a corresponding decrease in fluoride ion formation. The potential therapeutic advantages to be gained by the use of such specifically deuterium-labelled anesthetic agents have received considerable attention in recent years (304). McCarty et al. (306) have studied the effects of substituting deuterium for hydrogen on the metabolism in vivo of a number of volatile anesthetics, including methoxyflurane, halothane, enflurane, chloroform, and two experimental agents, CF_2HOCF_2CFBrH and CF_2HOCF_2 CCl_2H. The authors reported that deuteration altered the metabolism of these compounds, in most cases producing decreases in metabolism, reflecting the introduction of primary deuterium isotope effects on rate-limiting steps in biotransformation. Despite the obvious clinical benefits to be gained by reducing metabolism of drugs to toxic products, much work remains to be done before deuterated volatile anesthetic agents can be considered superior to their unlabelled counterparts for therapeutic use. For example, it is indeed possible, as pointed out by Mazze and Denson (304), that alteration in metabolism by deuterium labelling may lead to the formation of new products with different toxic properties. Nevertheless, this is an important area of research that is likely to expand in the coming years. It is noteworthy that while no deuterium-labelled compound has yet achieved the status of a therapeutically useful drug, a group at the Merck Sharp and Dohme Research Laboratories have reported on a new antibacterial, 3-fluoro-D-alanine (LXXI) and its 2-deutero analog (LXXII) (99,



LXXI R = H

LXXII R = ^2H

261, 458). Intended for use in combination with a prodrug of cycloserine, 2-deutero-3-fluoro-D-alanine exhibited markedly reduced in vivo metabolism and toxicity in comparison with its unlabelled analog. This finding was rationalized in terms of the deuterated compound exhibiting an isotope effect at the initial step of metabolism, the D-amino acid oxidase-mediated conversion of 2-deutero-3-fluoro-D-alanine to 3-fluoropyruvic acid (LXXIII).



LXXIII

Since both labelled and unlabelled forms of the drug possessed the same antibacterial activity, the deuterium isotope effect led to a significantly improved therapeutic index. Some progress has also been made in the design of anticancer agents that exhibit altered metabolism and improved pharmacological properties as a consequence of deuterium substitution at specific sites (215). In addition, deuterium labelling of drugs that are subject to a

significant "first-pass" effect might provide a means of increasing their oral bioavailability, as has been suggested in the case of the antiarrhythmic agent lidocaine (IX) (100), although the practical and economic advantages of such an approach remain to be determined.

One biological consequence of deuterium isotope effects, which was alluded to above, is that by decreasing the rate of one metabolic pathway, the relative amount of drug metabolized along an alternative, competing pathway may be increased markedly. This phenomenon appears first to have been recognized by Mitoma, Dehn, and Tanabe (320), who found that deuterium substitution at different positions in the alkyl side-chain of propyl *p*-nitrophenyl ether led not only to selective inhibition of metabolism at the site in question but also to increased formation of metabolites at other positions. Subsequently, Cox et al. (81) found that deuteration of cyclophosphamide at the 5-position depressed the β-elimination reaction leading to phosphoramide mustard, the cytotoxic metabolite, and shifted the metabolism of the parent drug to alternate pathways that led to inactive products. M.G. Horning et al. (199) encountered a similar phenomenon during studies of the metabolism of deuterated analogs of caffeine ([1-C^2H_3]caffeine and [7-C^2H_3]caffeine) and antipyrine ([*N*-C^2H_3]antipyrine and [3-C^2H_3]antipyrine), two agents that are known to be metabolized by multiple alternate pathways. In these studies, deuterium isotope effects led to "switching" of metabolic pathways when the labelled compounds were studied either in vivo or in vitro, and it was pointed out that in cases where the metabolites themselves possess either different degrees or types of pharmacological activity, the use of drugs labelled at specific positions with deuterium may exhibit quite different types and duration of pharmacological action and metabolic disposition (204). Further examples of "metabolic switching" have been reported recently and include studies on methsuximide (200), various xanthines (202), the anticancer agent 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) (119, 121, 214), and Δ^9-tetrahydrocannabinol (169).

While the foregoing discussion on deuterium isotope effects has been restricted to the altered behavior of deuterium-labelled compounds in biological systems, it should be borne in mind that multiply deuterated compounds frequently exhibit physicochemical properties different from those of their unlabelled counterparts in vitro. The stronger basicity of [$^2H_3$]morphine as compared to unlabelled morphine is one example of this phenomenon (114, 115), although the difference in chromatographic mobility between unlabelled and deuterated compounds is a more widely appreciated effect. Thus, Wilzbach and Riesz (490) first demonstrated the nonequivalence of two isotopic variants, cyclohexane and [$^2H_{12}$]cyclohexane, on GLC, and noted that on a 4-m column of didecylphthalate, the deuterated species eluted first. Bentley et al. (32), by the use of 15- to 25-m narrow bore packed GLC columns, were able to achieve the complete separation of the TMS derivatives of β-glucose from β-[$^2H_7$]glucose; no resolution, however, was obtained with the TMS ethers of glucose and [U-^{13}C]glucose or with the *N*-trifluoroacetyl *n*-butyl esters of glycine and [^{15}N]glycine. With much shorter (7 ft) packed columns, on the other hand, they achieved complete isotopic fractionation of the penta-[$^2H_9$]TMS derivative of glucose from the unlabelled derivative (475). A comparison of the GLC properties of unlabelled and isotopically enriched amino acid TMS derivatives showed that while partial resolution could be observed with deuterium substitution (461), no such fractionation was evident with the ^{13}C-labelled compounds (459). It is of some interest to note that while ^{2}H-containing compounds invariably elute ahead of their unlabelled counterparts on GLC analysis with a variety of stationary phases, Baba et al. (14) observed that the order of elution of *N*-methylpiperidine and deuterated analogs thereof could be reversed on changing from 1.5% OV-1 to 5% dinonylphthalate; on the OV-1 phase, the protio variant eluted ahead of the deutero variant, while the reverse was true with the more polar column. Isotopic fractionation with multiply deuterium-labelled compounds has also been observed to occur on high performance liquid chromatography (HPLC) as exemplified by studies with the novel tetracyclic antimigraine drug Org GC94 (98), with phenytoin (448a), and with prostaglandin E_2 (360). In addition, partial or complete separations of imiprimine from [$^2H_{10}$] imiprimine have been obtained during thin-layer chromatography (TLC) on both silica gel and alumina plates when basic or neutral, but not acidic, solvent systems were used (178). In view of such deuterium isotope effects on chromatographic mobility, analytical procedures in which purification of a mixture of deuterated and unlabelled molecules is effected by TLC or HPLC (e.g. as a "clean-up" step in a quantitative assay based on the reverse stable isotope dilution approach) should be examined carefully to ensure that no fractionation of molecular species has occurred prior to the final determination. Finally, it should be mentioned that deuterium isotope effects have been known for many years in mass spectrometry, where differences (usually small) may be observed in the relative intensities of fragment ions produced under conditions of electron impact. In a recent study on the bioavailability of different salt forms of propoxyphene (XXIX), where chemical ionization conditions were used, Wolen et al. (494) noted a pronounced deuterium isotope effect on the formation of the fragment ions at m/z 131 and 133, derived from unlabelled and [$^2H_7$]propoxyphene (XXXI), respectively. For this reason, it is important that assay procedures based on stable isotope dilution techniques employ standard curves constructed from known mixtures of labelled and deuterated molecules, by which means any isotope effects in the mass spectrometric analysis will automatically be taken into account in the quantitative determination.

5. Molecular Rearrangements. Molecular rearrange-

ments occurring during the course of certain chemical reactions have been recognized for over a hundred years. In many cases, rearrangement is associated with the migration of a hydrogen atom and more recent mechanistic studies on such processes have adopted the use of specific deuterium labelling, together with mass spectrometry and NMR spectroscopy, to better define the origin and ultimate fate of the migrating hydrogen. It is now known that analogous hydrogen rearrangements may occur in biological systems during the course of metabolic transformations, exemplified by the so-called "NIH shift" first reported by Guroff, Daly, and coworkers (159, 158, 157, 226, 90). In the course of developing a radioisotope assay procedure for the *para*-hydroxylation of phenylalanine to tyrosine, these workers (159) found that when [*p*-^{3}H]phenylalanine was used as substrate for the phenylalanine hydroxylase of *Pseudomonas*, little or no tritiated water appeared in the medium although large amounts of tyrosine were produced. Furthermore, this tyrosine retained the tritium label originally present at the position occupied by the newly introduced phenolic —OH group. In order to investigate this unexpected finding further, and to eliminate the possibility that the tritiated phenylalanine was not labelled specifically at the *para*-position, [*p*-^{2}H]phenylalanine was synthesized and its structure was verified by NMR and mass spectrometry. When this stable-isotope-labelled amino acid was employed as substrate for the *Pseudomonas* enzyme, the tyrosine produced was shown unequivocally to have retained a large proportion of the deuterium present in its precursor. Subsequent experiments designed to reveal the location of the deuterium atom in the product showed that migration had occurred from the *para*- to the *meta*-position of the aromatic ring, a finding that permitted the formulation of a reaction mechanism for the enzyme-mediated hydroxylation of phenylalanine (158). These early experiments are summarized in a paper by Guroff et al. (157), who extended their work to a number of other systems where metabolic hydroxylation of aromatic rings occurred and who discussed the possible nature of the electrophilic oxygen species generated by microsomal hydroxylases. Evidence that the NIH shift occurred as a consequence of the rearrangement of an intermediate arene oxide was presented in 1968 by Jerina et al. (226), who demonstrated that synthetic [4-^{2}H]toluene-3,4-oxide (LXXV) rearranged to 4-hydroxy-toluene (LXXVI) with concomitant migration of deuterium to the 3-position. Thus, the origin of the NIH shift and the mechanism by which aromatic ring systems may undergo metabolic hydroxylation were established in an important series of investigations in which deuterium labelling played a key role (90). The mechanism of the arene oxide pathway for the formation of phenolic products from aromatic hydrocarbons is illustrated in figure 6.

As mentioned in the previous section, evidence has been obtained for the existence of an alternative (non-arene oxide) pathway for the hydroxylation of some aromatic systems (457); while little is known about the mechanism involved in this reaction, it appears to occur via direct insertion of oxygen into a C-H bond. Thus, while certain aromatic hydroxylation reactions are not accompanied by an NIH shift (380) and thus presumably do not occur by way of the arene oxide pathway, the majority of systems studied to date do appear to exhibit NIH shifts and are considered to involve intermediate arene oxides (118, 166, 351, 352). It should be noted, however, that the degree of retention of deuterium (or other migrating substituent) varies according to the nature of substituents elsewhere on the ring (214).

Recently, two examples of novel enzyme-mediated rearrangement processes have been reported in which a 1,2-hydride shift occurs during the oxidation of a terminal

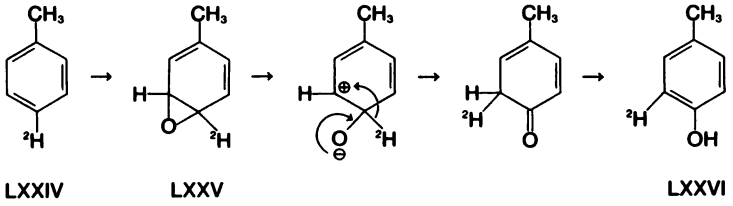


FIG. 6. Metabolic scheme for the conversion of [4-^{2}H]toluene (LXXIV) to 4-hydroxy-[3-^{2}H]toluene (LXXVI) by the arene oxide pathway.

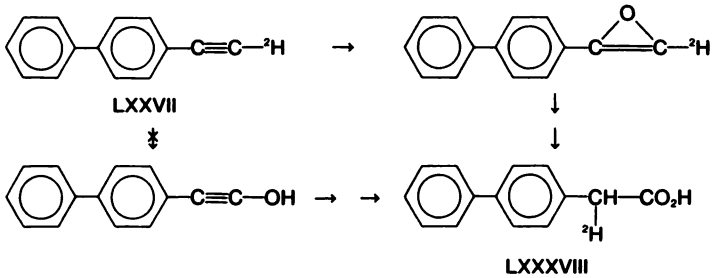


FIG. 7. Metabolism of biphenyl-[^{2}H]acetylene (LXXVII) to biphenyl-[^{2}H]acetic acid (LXXVIII).

olefinic or acetylenic bond. In the first, Delbressine et al. (94) found that after the administration of styrene to rats, a new metabolite, phenaceturic acid, was excreted in the urine. When [$^2H_8$]styrene was administered, however, the metabolite was shown to have retained seven, rather than the expected six atoms of deuterium, indicating that migration of one of the deuteriums on the terminal methylene group to the adjacent benzylic position had taken place; rearrangement of an intermediate styrene oxide to phenylacetaldehyde with concomitant 1,2-hydride shift was proposed as a mechanism to explain the formation of the observed product. In the second example, incubation of 1-[2-^{2}H]biphenylacetylene (LXXVII) with hepatic microsomes from phenobarbital-pretreated rats in the presence of NADPH was found to give 2-[2-^{2}H]biphenylacetic acid (LXXVIII) with essentially quantitative retention of label (363). A 1,2-shift of the acetylenic hydrogen (or of the biphenyl group) thus accompanied the enzymatic oxidation of this substrate, a rearrangement that was also shown to occur on chemical oxidation of the acetylene with *m*-chloroperbenzoic acid. (It has now been shown, by means of ^{13}C-labelling, that it is indeed the terminal hydrogen atom that shifts (363a)). This work constitutes the most direct evidence for enzymatic acetylene oxidation, and establishes that biological oxidation of acetylenes involves initial reaction with the π-bonds rather than insertion of oxygen into the terminal C-H bond (fig. 7). The authors of this paper, Ortiz de Montellano and Kunze of the University of California San Francisco, proposed that by analogy with the oxidative NIH shift of aromatic hydrogens referred to above, this type of rearrangement could be termed the "UC shift."

Finally, deuterium-labelling techniques have also been used to advantage to study hydrogen rearrangements that occur during the nonenzymatic degradation of certain drugs, e.g. the 2-haloethylnitrosourea antitumor agents. In a recent paper, Brundrett (47) studied the effect of pH on the decomposition in aqueous media of specifically deuterium-labelled analogs of BCNU (1,3-bis[2-chloroethyl]-1-nitrosourea); when [β,β'-$^2H_4$]BCNU was used as parent compound, [$^2H_2$]acetaldehyde was formed, the deuterium atoms in which were shown by proton and deuterium NMR spectroscopy to be attached to different carbon atoms. This result indicated that a 1,2-hydride shift had occurred at some stage in the chemical breakdown of the 2-chloromethyl moiety; the participation of a hitherto unknown compound, 4,5-dihydro-1,2,3-oxadiazole, was postulated to account for the experimental observations although direct evidence in support of this pathway remains to be obtained.

IV. Conclusions

The literature cited in this review has been selected to illustrate the great diversity of applications of stable isotopes in pharmacological research and to highlight those areas of more recent interest. Inevitably in any discussion of this subject, comparisons are drawn between stable and radioactive isotopes in terms of their usefulness as tracers, and it is appropriate at this point to summarize the relative merits of the two kinds of isotopic variants. The major advantage associated with stable isotopes is usually stated to be the lack of radiological hazard accompanying their administration to human subjects, especially to children and to women of child-bearing age. Although this consideration may be of overriding importance in the design of certain clinical studies, investigations in which stable-isotope-labelled compounds are given to humans at present represent only a small fraction of the total number of applications in pharmacology as a whole. This situation is likely to change in the near future, however, as growing concern over the health hazard associated with exposure of human subjects to radioactive isotopes dictates that their use should be limited to those situations where administration of radioactivity is clearly beneficial to the *diagnosis* of a patient's condition (245). The use of radioisotopes in human experimental pharmacology and therapeutics, therefore, is likely to face increasingly stringent restrictions in coming years, although in certain situations approval for the use of even low levels of radioactive isotopes in man would be difficult to obtain today. Thus, in the field of drug metabolism, administration of a radioactively labelled tracer to human subjects would be contraindicated where preliminary animal studies had suggested that extensive degradation of the drug occurred to give small fragments that could become incorporated into pathways of intermediary metabolism (454); in this situation, the excretion half-life of radioactivity may well be substantially longer than that of the parent drug or primary metabolites and the health risk of the study increased accordingly (174). The use of a stable-isotope-labelled analog of the drug as tracer, either alone (100) or as an equimolar mixture with the native compound (section III B 1), circumvents this problem although, as will be discussed below, difficulties often arise in the detection of nonradioactive metabolites in biological fluids and excreta.

The use of stable-isotope-labelled compounds in conjunction with analytical techniques based on NMR or mass spectrometry provides the investigator with a highly *specific* means of characterizing metabolites, in contrast to the nonspecific methods employed for the detection of radioisotopes; this feature of stable isotope usage is probably the determining factor in the choice of stable over radioactive tracers for nonclinical applications. In addition, selected ion monitoring GC-MS techniques offer extremely high *sensitivity* of detection that may equal, or even exceed, sensitivities attainable by liquid scintillation counting methods (154). For quantitative work that involves the study of compounds with large body pools and low plasma concentrations, the use of tracers labelled with stable isotopes is often essential (245). In metabolic or pharmacokinetic studies where

unanticipated kinetic isotope effects are encountered, these will be smaller when the tracer is labelled with deuterium rather than with tritium. NMR spectroscopy and, in favorable cases, mass spectrometry can provide accurate assessments of the *position* of a stable isotopic label in a molecule, whereas with the corresponding radiolabelled compound, tedious chemical degradation procedures are normally required. The synthesis and handling of stable isotopes, which do not suffer from the inherent problem of stability, is relatively straightforward and, for tracer studies of nitrogen and oxygen atoms, use of the stable isotopes ^{15}N and ^{17}O or ^{18}O is mandatory since there are no radioactive isotopes of these elements with a sufficiently long half-life to have adequate utility as labels for biological work.

Undoubtedly, the greatest single disadvantage associated with the use of stable isotopes is the lack of a simple, inexpensive, isotope-specific method of detection and quantification comparable to liquid scintillation counting. Stable isotopes have been termed "silent" tracers, their presence in biological matrices not being revealed by the emission of readily detected radiation. In addition, although GC-MS techniques afford an extremely powerful approach to the identification and quantitative analysis of stable-isotope-labelled compounds in complex mixtures, their application involves considerably greater investment of time and effort than is necessary in work with radioisotopes, and for this reason it is preferable that the use of GC-MS and stable isotopes be restricted to problems that yield a large amount of information from a relatively small number of samples. Recent approaches to the former problem, viz the detection of compounds labelled with stable isotopes, have concentrated on metabolites enriched in ^{13}C and have centered on the combustion of biological samples and determination of the ratio of $^{12}CO_2$:$^{13}CO_2$ in the gaseous product by isotope ratio mass spectrometry. While no information is obtained on molecular structure of the labelled compounds present in the original sample, this technique is applicable to excretion/retention "balance" studies in drug metabolism of the type routinely performed with radiolabelled tracers. A convincing demonstration of the potential of this approach has been published by von Unruh et al. (465), who determined the day-to-day variations in the ^{12}C:^{13}C isotopic composition of neutral, acidic, and basic fractions of urine and studied the effect on these basal values of the ingestion of ^{13}C-labelled aspirin; it was shown that a single dose of 23 μg excess carbon-13 to a human subject was sufficient to perturb significantly the isotopic composition of the acidic fraction of urine, which had a mass of 570 mg of C. Difficulties associated with this type of analysis are that highly sophisticated equipment and considerable expertise in sample preparation are required, and that measurements of $^{12}CO_2$:$^{13}CO_2$ ratios in excreta are made against a "background" that fluctuates as a result of dietary variations in ^{13}C intake. An interesting alternative approach to the problem of detecting ^{13}C-labelled metabolites in biological specimens has been developed by Sano et al. (398), who employed catalytic combustion of the effluent from a gas chromatograph to produce CO_2, which was then passed directly to a mass spectrometer for isotope ratio analysis. Gas chromatographic components that were enriched in ^{13}C were detected as a result of the deviation that occurred from the basal $^{12}CO_2$:$^{13}CO_2$ ratio at the retention times of the labelled metabolites (fig. 8). This technique assumes, however, that the labelled components are amenable to gas chromatographic analysis and that appropriate extraction, derivatization, and chromatographic conditions have been employed in order to provide a comprehensive "profile" of all drug-related material in the original sample. Further aspects of this general problem have been discussed in detail by Draffan (100). A similar system to that developed by Sano et al., termed "isotope-ratio-monitoring GC-MS," has been described by Matthews and Hayes (296a). With the aid of a 750°C cupric oxide-packed combustion furnace inserted between the gas chromatographic column outlet and the GC-MS interface, these authors were able to measure nitrogen and carbon isotope ratios down to natural abundance for any organic components that could be resolved gas chromatographically.

Despite the above disadvantages of procedures based on the use of stable isotopes, studies carried out with

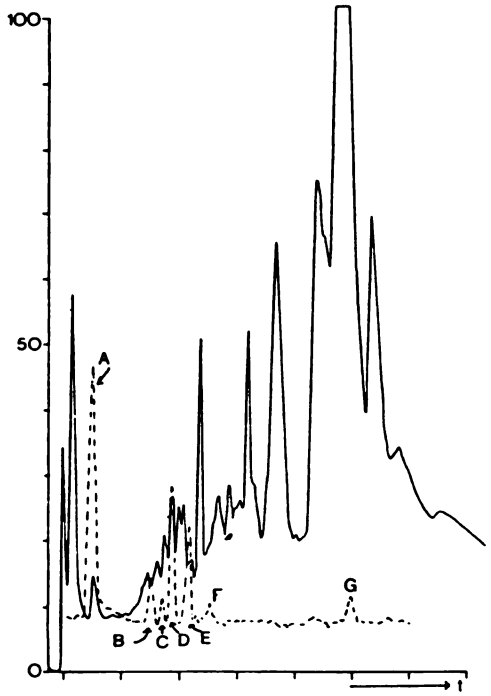


FIG. 8. Detection of ^{13}C-labelled metabolites in urine after administration of [^{13}C]aspirin to a human subject. The effluent from the gas chromatograph is combusted to CO_2 and the ratio of $^{13}CO_2$:$^{12}CO_2$ is determined by mass spectrometry. Solid trace: Gas chromatogram of methylated urinary extract. Broken trace: Ratio of m/z 45 ($^{13}CO_2$): 44 ($^{12}CO_2$) obtained from GC-MS analysis of the same sample. Reproduced with permission from M. Sano, Y. Yotsui, H. Abe, and S. Sasaki, Biomed. Mass Spectrom. 3: 1–3, 1976.

stable-isotope-labelled tracers will undoubtedly continue to increase as the variety of commercially available labelled compounds increases, as the cost of deuterium, carbon-13, nitrogen-15, and oxygen-18 falls in real terms, and as the techniques for the analysis of stable isotopes become more refined and available to a wider spectrum of investigators. In the area of qualitative applications, the use of ^{13}C-NMR spectroscopy in combination with drugs enriched with ^{13}C will undoubtedly become more important in the near future, especially in studies of drug metabolism. Deuterium, which is the most widely employed stable isotope in pharmacological research at the present time (298), is likely to be used with progressively less frequency for in vivo tracer studies, as compared with ^{13}C and ^{15}N, which do not exhibit significant kinetic isotope effects. Thus, one can look to an increase in the use of ^{13}C- or ^{15}N-labelled compounds for "isotope cluster" studies, accompanied by the use of deuterated variants of the compounds of interest as internal standards for quantitative measurements. Current trends towards the development of new therapeutic agents of ever increasing potency have imposed severe demands on the analytical methodology employed for quantitative determination of the resultant low drug concentrations in biological fluids; selected ion monitoring GC-MS techniques, particularly when coupled with the use of stable-isotope-labelled internal standards, are now superior to all alternative assay methods in terms of their unique combination of sensitivity and specificity of detection, and will continue to be widely employed as the basis of analytical procedures that demand a high degree of accuracy and precision. Applications of such methodology to pharmacokinetic studies will probably expand as a result of the growing acceptance of techniques for assessing the relative and absolute bioavailability of different drug formulations by the use of multiple stable-isotope-labelled variants. In the area of mechanistic investigations, the greater availability and reduced cost of the stable isotopes of oxygen will undoubtedly lead to an increase in their use for studies of oxygen activation by the mixed-function oxidase enzyme systems and of detailed aspects of the oxidative pathways of drug metabolism. "Pulse labelling" of endogeneous metabolites through inhalation of $^{18}O_2$-enriched atmospheres appears to hold considerable promise as a convenient, noninvasive approach to studying the in vivo turnover of compounds of pharmacological interest and the effects of external factors on perturbing pathways of intermediary metabolism; although initial enthusiasm over the potential application of this technique to studies in man has been tempered recently by the recognition of the kinetic complexity of certain biosynthetic pathways (e.g. catecholamine formation from tyrosine and phenylalanine), many other metabolic systems appear to be more amenable to study by this type of approach. Oxidative metabolism of nitrogen-containing compounds (e.g. hydrazines, N_2O) frequently leads to the production of toxic or potentially toxic products, the formation and ultimate fate of which can sometimes only be followed by the use of the stable isotope ^{15}N. Thus, hydrazine itself, a known hepatotoxic agent, has been shown recently to be metabolized both in vitro and in vivo to gaseous nitrogen, this end product being detected by the use of [$^{15}N_2$]hydrazine as substrate (343). The origin of the hydrazine detected in the urine of patients given the antihypertensive drug hydralazine (456), and the metabolic fate of the hydrazine moiety of carbidopa, a peripheral decarboxylase inhibitor, would appear to represent problems that could be studied profitably by a similar approach. Labelling of specific endogenous metabolite pools has proved to be a useful means of identifying the source of certain functional groups introduced during the metabolism of foreign compounds, e.g. the *S*-methyl group of methylthio derivatives of naphthalene, which has been shown to derive from methionine (429). Along these lines, labelling with ^{13}C might be employed to reveal the nature of the formylating moiety in the pathway by which certain aromatic amines may be converted to *N*-formyl derivatives (152). The greatly increased interest over recent years in the formation and detoxification of reactive intermediates of drug metabolism has generated a demand for methods by which sulfur-containing conjugates (e.g. glutathione adducts, mercapturic acids, methylthio derivatives) may be studied. In this connection, it is anticipated that the stable isotope ^{34}S will find numerous applications in toxicological research if commercial production of this nuclide can be stimulated and if sulfur-containing amino acids (methionine, cysteine) can be marketed at a realistic cost. Additional demand for ^{34}S may well be generated through studies on the metabolism of sulfur-containing pesticides and related agents that have been shown to undergo interesting enzyme-mediated and spontaneous chemical reactions (447).

The future use of stable-isotope-labelled drugs must be considered to be a more speculative area of application in pharmacological research. The only stable isotope that can be expected to modify significantly the pharmacological or toxicological properties of a drug is deuterium, by virtue of the large primary (or in rare cases secondary) kinetic isotope effects on metabolism observed with this nuclide (204). In no case to date, however, has substitution of deuterium for protium resulted in an analog that has been introduced as a therapeutic agent in its own right (215), although the Merck antibacterial, 2-deutero-3-fluoro-D-alanine (section III C 4), has shown greatest promise in this regard. The recognition that deuterium substitution at a metabolically labile position may not only retard the rate of oxidation at that site but, in cases where *alternative* (as opposed to purely sequential) pathways of metabolism exist for the parent compound, lead to the generation of a quantitatively different metabolic profile (metabolic switching), has led to some concern over the possible toxicological consequences of such modifications. It would appear, therefore, that full toxicology

testing on a potential deuterium-labelled drug would be considered imperative if preliminary disposition studies suggested that metabolic switching had indeed occurred as a result of the heavy isotope substitution. A quite different application of drugs labelled with stable isotopes would be to detect illicit practices such as supplementation in narcotic programs, as has been proposed for use with patients maintained on methadone (206). In this specific case, routine GC-MS analysis of blood samples from patients given maintenance doses of [$^2H_3$]methadone was suggested as a definitive means by which evidence of supplementation with the normal, unlabelled form of the drug could be obtained. Although the authors of this work demonstrated that the unlabelled and deuterated forms of methadone were completely equivalent in animal studies with respect to their rates of adsorption, distribution, and excretion, and that they possessed identical toxicological and pharmacological properties, drugs labelled with ^{13}C or ^{15}N, where no isotope effects on disposition and metabolism would be anticipated, appear to be preferable for this type of application as a general rule. Aside from these two more speculative uses of stable-isotope-labelled drugs, one area of investigation that will continue to rely heavily on the use of metabolic substrates labelled at specific positions with deuterium is the study of mechanistic aspects of biotransformation processes (section III C 1).

Certain authors have stressed that the properties of stable and radioactive isotopes are, in many respects, complementary and that in those situations where the health hazard associated with the use of radioactivity is not of major concern, the simultaneous application of both types of heavy isotopes to a given problem should be the technique of choice in tracer studies (27, 174, 505). Thus, stable isotopes should not be regarded as competitors of their radioactive counterparts, but rather as an additional resource of tracer technology whose unique properties are currently opening up exciting new areas of pharmacological research.

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