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REVIEW ARTICLE

Studies with Deuterated Drugs

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INTRODUCTION

A drug may be defined as an agent used in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans or animals. Included in this category are articles (other than food) used to affect the structure or any function of the body of humans or animals. This definition distinguishes a drug from a chemical. A drug that contains a radioactive nuclide in the molecule is known as a radiopharmaceutical, and several dozens of these are useful clinical agents.

At the present time, there are no drugs on the market that contain deuterium in the molecule or, for that matter, are enriched in any other stable isotope. Numerous deuterated drugs have been obtained by organic synthesis or by biosynthesis. Several of these substances are completely deuterated, some are highly deuterated, and others are partially deuterated, but the majority are deuterated only in specific molecular positions.

Deuterated drugs have proved useful in studying isotope effects, in permitting a better understanding of the mechanism of drug action, and in elucidating metabolic and biosynthetic pathways. Some are simply laboratory curiosities awaiting future study. Heavy water (^2H_2O) itself has been examined for therapeutic applications. This review is concerned with the laboratory application of both heavy water and deuterated drugs.

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Table I—Comparison of Some Physical Properties of Water and Deuterium Oxide[a]

Property	Water	Deuterium Oxide
Melting point	0.00°	3.81°
Boiling point	100.00°	101.72°
Density, d_4^{20}, g/ml	0.9982	1.1056
Temperature of maximum density	3.98°	11.23°
Viscosity, centipoise, 20°	1.005	1.25
Surface tension, dynes/cm, 25°	71.97	71.93
Dielectric constant, 25°	78.54	78.93
Heat of formation, cal/mole, liquid	−68,320	−70,410
Free energy of formation, cal/mole, liquid	−56,690	−58,200
Entropy, eu/mole, liquid	16.75	18.19
Heat of fusion, cal/mole	1,436	1,515
Heat of vaporization, cal/mole, 25°	10,515	10,846
Zero-point energy, cal/mole	13,219	9,664

[a] See Ref. 15.

Biological Effects of Deuterium—Deuterium (^{2}H, D) is the rare, stable heavy isotope of hydrogen (^{1}H). It was discovered (1, 2) in 1932 and was isolated in a high state of purity (3) shortly thereafter. Deuterium occurs in nature to the extent of about 1 part in 6400 and is obtained primarily by isolation of deuterium oxide from ordinary water; its concentration ranges from 0.0156% in sea water to 0.0139% in fresh water. Heavy water is the simplest deuterium-containing compound and differs from ordinary water in many of its properties (Table I). It can be reasonably expected that the mass differences associated with the replacement of deuterium by hydrogen in a molecule will distinctly affect its physical and chemical properties. Such mass differences can also be expected to produce changes in the biological behavior of deuterium compounds.

Extensive deuterium isotope studies were precluded in the 1930's and early 1940's because of the great scarcity of heavy water. Since heavy water is very suitable as a moderator in certain types of nuclear reactors, efficient methods have been developed to isolate heavy water from natural sources. Hence, deuterium (as heavy water) has become a tonnage, high purity, industrial chemical available at low to moderate cost. The ready availability of heavy water to the scientific community as a result of advances in nuclear technology has resulted in a burgeoning interest in the biological effects of deuterium.

The biological implications of deuterium substitution were recognized almost immediately after its discovery. It soon became apparent that extensive replacement of hydrogen by deuterium in biological systems could produce deleterious effects. The early literature for 1932–1950 has been reviewed thoroughly (4). Much of the early work, however, is contradictory and of questionable value, primarily because of poor experimental design forced by inadequate and impure supplies of heavy water and because of the absence of suitable analytical techniques for measuring accurately the deuterium content of biological systems.

A conference under the auspices of the New York Academy of Sciences in 1960 (5) did much to revive and stimulate interest in studies on the biological effects of deuterium. Many of the relevant papers in that monograph will be referred to later in this review. In the same year, Katz published reports (6, 7) that summarized both the early studies in this field and also included new research undertaken during the 1950's, primarily at Argonne National Laboratory.

The physiological effects of deuterium were the subject of a comprehensive monograph (8) in 1963. Flaumenhaft *et al.* (9) reviewed the biological effects of deuterium and focused their comments on isotope effects on the cellular level. They noted specifically the isotope effects on the growth of microorganisms and higher plants in deuterated media and the cytology of deuterated cells.

Katz, Crespi, Blake, and coworkers (10–15) published a series of articles covering various aspects of deuterium isotope effect studies, with special emphasis on applications related to problems of a chemical and biochemical nature. The culturing of various microorganisms in deuterated media, the effect of deuteration on the stability of certain proteins and nucleic acids, and isotope effects in the metabolism of deuterated glucose and mannose by ascites tumor cells were reviewed (10).

Another review (11) covered the effect of deuterium substitution on the conformation of certain biopolymers and deuterium isotope effects in carbohydrate metabolism and in the potentiation of tumor chemotherapy. A third paper (12) described the cultivation of deuterated organisms and their utilization as a practical source of ^{2}H and ^{1}H–^{2}H compounds that are very useful for PMR spectroscopy of biopolymers; it also described the utility of deuterated organisms for following the path of hydrogen in living organisms.

In 1965, Katz (13) presented a comprehensive review of deuterium isotope effects on living organisms and biopolymers and illustrated the power of these methods by a discussion of biogenesis of chlorophyll. In a more recent review, the application of NMR studies to the biosynthesis of the important plant constituents bacteriochlorophyll, chlorophylls *a* and *b*, and the clavine alkaloids was discussed (14). The most recent and perhaps the most comprehensive review of deuterium isotope effects on biological systems was published in 1971 (15). In the present review, principal emphasis will be directed to deuterated drugs and their application.

Deuterium Isotope Effects—The basis for the frequently profound chemical and biological consequences of deuteration merits some discussion. The kinetic isotope effect on the rates of chemical reaction resulting from substitution of deuterium for hydrogen, which must certainly be implicated in the biological effects of deuterium, has received thorough theoretical treatment (16–20). The difference in mass between deuterium and hydrogen causes the vibrational frequencies of carbon, oxygen, and nitrogen bonds to deuterium to have lower frequencies than corresponding bonds to hydrogen. As a result, the

chemical bonds involving ^2H will generally be more stable than those of ^1H.

It has been calculated that the zero-point energy (lowest ground-state vibrational level) for many bonds to deuterium is about 1.2–1.5 kcal/mole smaller than for bonds to hydrogen. The zero-point energy of a bond undergoing chemical reaction reflects the ease with which the molecule is activated from a ground state to the transition state required for bond scission to occur. The more stable deuterium bond requires a greater energy of activation to achieve the transition state; as a consequence, the rate of reaction involving a bond to deuterium is generally slower than that involving a bond to hydrogen. Thus, substitution of deuterium for hydrogen in a chemical bond can affect significantly the rate of bond cleavage and exert marked effects on the relative rates of chemical reactions.

Large isotope effects on reaction rates are apparent where cleavage involves a bond to deuterium at the reaction site. In such instances the effect is referred to as a primary isotope effect and is usually expressed in terms of the ratio of the specific rate constants k_H/k_D. Bigeleisen (21) calculated the maximum possible ratios in specific rate constants for a number of stable and radioactive isotopes (Table II). Wiberg (17) calculated that at 25° the maximum positive primary kinetic isotope effect that can be expected in a chemical reaction involving the breaking of bonds to hydrogen is in the range of 7–10 for C—H, N—H, and O—H *versus* C—D, N—D, and O—D, respectively. For a number of reasons, these maximum ratios are not realized; more generally, values of k_H/k_D are in the range of 2–5.

An observable isotope effect will only be apparent, of course, where the breaking of a C—H or C—D bond is involved in the rate-determining step. In certain acid–base-catalyzed reactions, depending on the mechanism, an inverse isotope effect may take place (22). The pronounced effects on rates of chemical reactions make deuterium isotope studies a particularly useful tool for elucidating the mechanisms of many reactions of biochemical significance (8).

Deuterium atoms in nonexchangeable positions located near, but not at, the reaction center can give rise to secondary isotope effects. While deuterium secondary isotope effects are real and measurable, they are usually much smaller than primary isotope effects. Belleau (23) indicated that the k_H/k_D for secondary isotope effects falls in the range of 1.05–1.25. Secondary isotope effects, although small, may be important in biological systems sensitive to kinetic effects. The physical organic chemist also makes a distinction between solvent isotope effects, where only the isotopic composition of the medium has been altered, and primary and secondary isotope effects resulting from the replacement of hydrogen by deuterium in carbon to hydrogen bonds of organic compounds.

A recent review article (15) pointed out that in living organisms and in many biochemical and physiological systems the distinction between solvent and primary and secondary isotope effects is not always clear. Isotope effects produced by hydrogen atoms that exchange very slowly (if, in fact, they exchange at all) with water, as is the case for most carbon–hydrogen bonds, must be distinguished from those in which exchange occurs very rapidly, as is generally the case for hydrogen atoms bonded to oxygen, sulfur, or nitrogen. Compounds occurring in biological systems will generally have both types of exchangeable hydrogen present. If the isotopic composition of the solvent is altered, this will effect a corresponding change in the isotopic composition of the exchangeable hydrogen in the solute molecules. In living systems, therefore, solvent isotope effects include contributions from both primary and secondary isotope effects.

Table II—Estimated Maximum Possible Rate Constant Ratios at 25° for Various Stable Isotopes

Natural Abundance Isotope	Heavy Isotope	Rate Ratio[a], k_1/k_2
^1H	^2H	18
^1H	^3H	60
^{12}C	^{13}C	1.25
^{12}C	^{14}C	1.5
^{14}N	^{15}N	1.14
^{16}O	^{18}O	1.19
^{32}S	^{35}S	1.05

[a] Rate constant for the light isotope is the numerator.

The compositional or constitutional isotope effects have been defined (15) as those arising from alterations in the isotopic composition of the nonexchangeable hydrogens in the molecule in which all hydrogen sites in nonexchangeable positions are occupied by deuterium. Hybrid constitutional deuterium isotope effects occur when both hydrogen and deuterium are present in nonexchangeable positions. When the isotopic composition of living organisms is changed, as when animals are administered deuterium oxide in the drinking water, the initial effects of deuteration are attributable primarily to a solvent isotope effect. When the deuterium oxide enters the metabolic processes of the organism and is used for the biosynthesis of compounds containing both hydrogen and deuterium, then, in addition to a solvent isotope effect, hybrid constitutional isotope effects are induced.

Finally, in an organism grown in an environment of deuterium free of any hydrogen, constitutional isotope effects become predominant. In the deuteration of living organisms, both water and deuterium oxide are usually present and hybrid isotope effects are most prominent. These hybrid isotope effects are most commonly encountered in living organisms subjected to partial deuteration and are the most difficult isotope effects to interpret at the molecular level.

General Toxicity of Deuterium Oxide—Since deuterium oxide resembles ordinary water so closely, it is natural to speculate on the effects of this apparently small difference in chemical composition on the toxicological properties of the molecule. Concern over the toxicity of heavy water to living organisms was first expressed (24) soon after its discovery. A mouse

fed the equivalent of about 1 g of pure heavy water over a 3-hr period survived the ordeal but showed definite signs of intoxication. Lewis (24) noted that: "The more he [the mouse] drank of the heavy water the thirstier he became." Unfortunately, the experiment had to be terminated because the limited supply of heavy water was exhausted.

An extensive series of studies on the physiological effects of deuterium oxide in mice was next conducted over a 5-year period starting in 1934 (25). The acute lethal dose for deuterium oxide in mice was 5–7 ml/10 g of body weight. This amount was observed to be the lethal dose even when the deuterium oxide was administered as a 50% solution in water. When "concentrated" heavy water was administered orally, a crisis was reached on about the 5th day, at which time the body water became about one-third replaced by deuterium oxide and death generally occurred around the 7th day. However, a mouse ingesting 40% D_2O in its drinking water for a 2-month period showed no apparent ill effects other than retarded weight gain; mice whose body fluids reached 20% deuteration showed no noticeable harmful effects when the heavy water was removed from the diet.

Barbour and Trace (26) fed mice pure (99.5%) deuterium oxide at a dosage rate of 1 ml/10 g of body weight/day. This intake generally proved fatal in 7 days, at which time the mice were deuterated to the extent of 40–50%[1]. Death in these mice was preceded by a characteristic sequence of toxic manifestations.

More recently, Thomson (8) thoroughly reviewed the literature dealing with the physiological and toxicological effects of deuterium oxide in mammals. Katz (7) reported that mice and rats cannot long survive replacement of more than one-third of their body water by deuterium oxide. At the 20% replacement level, rats become hyperexcitable and more aggressive than normal; when the plasma levels approach 30% D_2O, rats frequently convulse when handled. When the body fluids are at the 35% deuteration level, death generally results. Thomson (27) also observed that rats drinking deuterium oxide died when about one-third of their body water was replaced by deuterium oxide. He concluded that the toxic effects were attributable to the summation of a multitude of small changes affecting the rates of enzymatic reactions in the body.

Czajka and Finkel (28) maintained mice on 25% D_2O in the drinking water for up to 280 days without adverse effect on body weight or longevity. In these mice, ingestion of 25% D_2O caused incorporation in the body fluids of about 18–20% deuterium. When the deuterium content of the drinking water was raised to the 25–30% level, fetal viability ceased. Katz *et al.* (29) found that the median survival times for mice deuterated to different deuterium levels ranged from 60 days for mice drinking 40% D_2O to 12 days for mice drinking 75% D_2O. Differences in survival time were related to the rates at which deuteration reached toxic levels and not to differences in deuterium levels *per se*. Most animals died when the body tissue fluid concentration rose to between 30 and 40% D_2O.

Czajka *et al.* (30) studied the toxic manifestations of deuterium in two dogs. One beagle was maintained at 20% D_2O in the body fluids for 50 days, and the other was maintained at the toxic range of 33–35% for a brief period. Deuteration was effected by replacement of ordinary water with deuterium oxide in the food and drink. These dogs (approximately 10 kg) appear to be the largest animals subjected to extensive deuteration. A 30% concentration in the blood plasma appears to be close to the acute danger level for dogs, as it is for mice and rats. Thus, in intact mammals the magnitude of deuterium effect appears on the whole to be quite independent of the size of the organism.

In one report (31) on humans, no ill effects were noted over 4 months when the body water was replaced with deuterium oxide to the extent of 0.5%. In mammals it appears that up to 15% deuterium can be tolerated, but severe toxic effects and possibly death may result at the 30% level and higher. Although toxic effects appear to correlate with the overall level of deuteration, deuterium probably elicits harmful effects in subtle ways even in low concentration.

Incorporation *in vivo* of small amounts of deuterium into sensitive molecules such as enzymes, nucleic acids, and similar important substances possibly could have serious consequences. Knapp and Gaffney (32) pointed out that the small amounts of stable isotopes involved in labeling experiments probably would not cause harmful effects. Nevertheless, they cautioned that it would be wise to label the molecules in molecular positions that are metabolically stable to minimize the extent of distribution throughout the body. The possible toxicity of stable isotopes at low concentrations clearly merits further study.

The ultimate cause of death from deuteration is not clear. Numerous disturbances are observed including renal function impairment, central nervous system (CNS) disturbances, cardiac involvement, enzymic interferences, hormonal imbalances, and glucose metabolism disturbances. All of these factors seem to be involved in the death of the animal, and no single factor appears to be the principal cause of death. Only a few studies have been directed toward finding ways to increase the tolerance of animals to the ravages of high concentrations of deuterium oxide in the body fluids. An extensive study (33) was conducted in which a number of hormones, vitamins, and vitamin mixtures were examined for possible beneficial effects on the ability of mice to survive toxic levels of deuteration. The mice were administered daily injections of hormones, vitamins, or vitamin mixtures while being maintained on 50 or 75% D_2O in the drinking water, but the improvement in survival was marginal at best.

Notwithstanding the damaging effects imposed on animals from high levels of deuteration, deuterium oxide must nevertheless be considered a remarkably nontoxic substance. Few, if any, components of the

[1] Percent deuterium oxide throughout this paper refers to atom percent D in the aqueous fluid. A mixture of water and deuterium oxide is related by a mobile equilibrium: $H_2O + D_2O \rightleftharpoons 2\,HOD$.

body can be replaced to anywhere the same extent as can water by deuterium oxide without the gravest consequences.

Pharmacological and Biological Effects of Deuterium Oxide—Morowitz and Brown (4) reviewed the early physiological studies in this area, and Thomson (8) prepared a comprehensive summary of the literature up to the year 1963.

Heavy water is readily absorbed from the GI tract and rapidly equilibrates with the body fluids. There appears to be no selective excretion of heavy water by the kidney. Pure (99.8 atom % D) heavy water has a taste not too different from distilled water or deaerated water. It is readily administered to animals in their drinking water, and its palatability may be enhanced by addition of traces of sodium chloride. Smith (34) suggested that mice are capable of discriminating between deuterium oxide and water, and he attributed this ability to the formation of the more stable deuterium—oxygen bonds which may be involved in the interaction between the water molecule and "a gustatory receptor site." This study was later challenged (35), since the data were based on only two mice (litter mates)[2].

By the selection of the appropriate deuterium oxide concentration in the drinking water, the equilibrium deuterium oxide concentration in the body fluids can be readily controlled. The course of deuteration has been intensively studied in mice (26, 29, 36) and rats (27). Figure 1 illustrates the rate of deuteration (as reflected by the deuterium content of the urine) as a function of time when varying concentrations of heavy water were administered to mice in their drinking water. An equilibrium state in the body fluids was reached by about the 10th day. With low concentrations of heavy water in the drinking water, the deuterium level in urine reached about 90% that of the ingested water. Where the deuterium oxide content of the drinking water was in the 20–50% range, equilibrium urine values were approached more slowly and were only 70–80% that of the drinking water. Dilution was due primarily to the fact that the food administered to the animal was of ordinary isotopic composition. Similar results are obtained when the course of deuteration is assessed in terms of the deuterium content of the blood serum.

Both mice and rats react similarly to the effects of deuterium ingestion. When the deuterium content of blood serum approaches 20%, signs of hyperirritability are observed and the mice show a tendency to convulse. Their coats become rough and they tend to lose weight rapidly. If the deuterium level is raised to still higher levels, the animals show a pilomotor response and become stuporous, the body temperature drops markedly, and death may occur in a short time. Thomson (27) noted that replacement of the body water by 15% D_2O resulted in signs of hyperactivity in rats. At the 20–25% replacement level, they became hyperexcitable and tended to convulse when stimulated. The animals ceased to groom themselves,

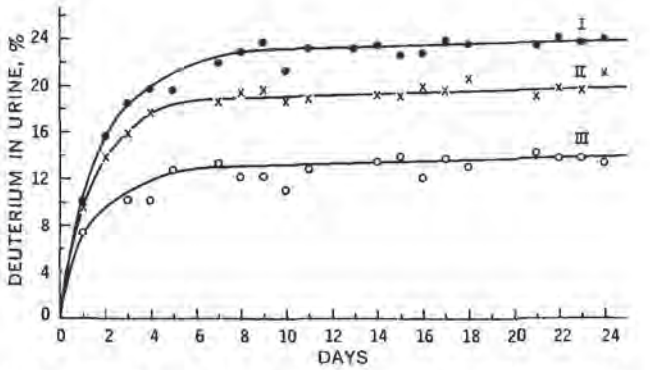


Figure 1—*Course of body fluid deuteration of mice drinking deuterium oxide of various concentrations. Key: I, 30% D_2O; II, 25% D_2O; and III, 15% D_2O. Each point represents a mean of from five to 22 determinations. (Reprinted, with permission, from Ref. 36.)*

ulcers were noted on their paws, and their tails showed signs of necrosis. At the 30% deuterium level, the rats became comatose and refused food; there was a precipitous loss in weight, followed by death due to respiratory depression.

Katz *et al.* (29) found that in mice the brain incorporates deuterium at a slower rate than do the kidneys, liver, and spleen. Lower deuterium incorporation into the brain is probably due to the high lipid content of the brain with its large amount of nonexchangeable hydrogen. Incorporation of deuterium proceeds principally by biosynthesis. The rate at which deuterium is eliminated from the body fluids when deuterium oxide is withdrawn from the body was also studied. The deuterium level of the urine fell rapidly, although appreciable amounts were still detectable after 7 days of withdrawal.

The effects of extensive deuteration on the internal organs of mice and rats were detailed (29, 36), and the effects of deuteration on liver enzyme activity, kidney function, blood constituents, and enzyme systems in rats were discussed (27). Rabinowitz *et al.* (37) studied the effects of heavy water ingestion on hepatic lipogenesis. The physiological effect of extensive deuteration on specific organs and body functions in mice and rats was considered (26, 38).

The physiological consequences of deuteration in two dogs were described (30). (This study was referred to earlier.) Particular attention was paid to alterations in the blood picture and the ECG. Although the two dogs were maintained in a highly deuterated state under different conditions, the changes produced were the same in both dogs. The effects on the blood picture were consistent with those reported in mice (37, 39).

Bachner *et al.* (40) studied the pathological effects of deuteration in mice drinking 75% D_2O. Renal tubular damage was observed as well as destructive changes in the salivary gland ducts. Zunker and McKay (41) also reported kidney damage in mice drinking 75% D_2O. Serious pathological changes were observed in the kidney structure of deuterated mice (42). Thomson (8) reviewed in detail the literature concerning the effects of deuteration on histological

[2] In our experience, mice accustomed to drinking chlorinated water tend to discriminate against deuterium oxide.

changes, on blood and plasma constituents, and on enzyme systems in mammals.

One significant effect of deuteration in mice is on reproductive capacity. Several studies indicated that drinking heavy water produces sterility in mice (43–45). A decrease in litter size and a decrease in the percent of fertile matings were noted when deuterated males were mated with untreated females. Almost complete sterility was produced in male mice who drank 30% D_2O for 6 weeks. Sterile matings resulted from male mice who were returned to ordinary water for at least 2 weeks after a period of deuteration with 30% D_2O. A high incidence of degenerating eggs was found when male mice that were on heavy water for 7 weeks and then were returned to ordinary water for 2 weeks were mated with untreated females. These researchers suggested that when deuterium is substituted for hydrogen, changes are produced in the physical forces that bind macromolecules and that these changes may produce an abnormal genetic material in developing sperm which causes fertilized eggs to degenerate.

Gross and Spindel (46, 47) described in detail the antimitotic activity of deuterium oxide. They attributed abnormalities in the cell division process to viscosity changes caused by changes in the hydrogen bonding properties of deuterated biopolymers. Because heavy water has a pronounced effect on cell division in most organisms, it is not unexpected for rapidly dividing tissues in animals, particularly the reproductive tissues, to be adversely affected by deuterium oxide.

Czajka and Finkel (28) investigated the effect of administration of 25% D_2O in drinking water on the reproductive potential of female mice. Deuteration of the mice was instituted at various times before and after mating to obtain data on the effects on pregnancy and viability of the newborn. Fewer pregnancies were carried to term in deuterated animals, and there was a marked increase in the incidence of wholly nonviable litters along with a decrease in the survival of newborn mice. The ability of female mice to carry through a normal pregnancy and bear viable offsprings was definitely impaired by deuterium. The greatest reduction in fetal viability occurred when the dams were substantially deuterated at the time of fetus implantation (about the 5th day after mating). Fetal viability ceased when the deuterium oxide content of the drinking water exceeded 30%. These untoward effects on reproductive potential were reversed when the female mice were returned to ordinary drinking water.

It was noted that spermatogenesis was essentially normal in deuterated male mice, suggesting that the genetic material in the partially deuterated sperm may be abnormal (48). In another study, prolonged deuteration of mice led to total aspermia, accompanied by the formation of multinucleate giant cells in the testes (49). Hughes and Glass (50) pointed out that the inability of deuterated male mice to produce viable offsprings was due to the failure of the sperm to fertilize the egg and not to the failure of the fertilized egg to develop. The fetuses of deuterated mice were studied, and it was observed that major disturbances occurred in the development of the nervous system (51). The extreme complexity of the effects of deuteration on reproduction was emphasized.

Deuteration in mice at a gradual rate of increase up to one-fifth saturation of the body fluids produced an elevation in body temperature of about 1°, concomitant with an increase in the metabolic rate of about 20% above normal (52). In an earlier study, Barbour (53) noted a lowering of the basal metabolism rate in mice injected subcutaneously with 99% D_2O. Increased levels of deuterium oxide in the body fluids of mice were shown to produce a decrease in metabolic rate and body temperature (26). With a dose of pure deuterium oxide (1.5 ml/10 g of mouse body weight), the temperature of the animals began to fall on the 1st day. With all doses of 50% D_2O, the temperature fall was delayed; with 40% D_2O, the temperature did not begin to fall until the 13th day. A fall in metabolism rate also occurred simultaneously with the fall in temperature.

The effect of heavy water on the heart rate was studied (54). The frequency of pulsation of the excised heart of the frog was slowed in 20% heavy water. The effect of heavy water was similar to that produced by lowering the temperature. This effect was confirmed in a study of ECG changes (55). Barnes and Warren (56) also studied the effects of high deuterium oxide concentrations on the auricles of the turtle heart. They found that 75% heavy water lessened the amplitude of the auricular beat. According to these investigators, the effect of heavy water was equivalent to lowering the body temperature about 5°.

Szczesniak *et al.* (57) determined whether externally applied water can penetrate mammalian skin. Young male rats were immersed for 6–7 hr in 40% heavy water maintained at a temperature of 35°. Blood samples drawn from the pumping heart were analyzed for deuterium content. The analytical data provided evidence that heavy water did penetrate the skin and entered systemic circulation.

The effect of deuterium oxide on wound healing in rats drinking 25% heavy water was studied (58). In these rats the equilibrium deuterium content in the body fluids was 15% deuterium. Skin incisions were made in the abdominal wall, and the effect of deuteration on the healing process was studied. The breaking strength of the wounds after 27 and 47 days of healing was about 40% less in the deuterated animals than in the controls. Cellular population and ground substance were similar in the incisions of both groups. The wounds in the deuterated rats healed abnormally in comparison to the control animals. It was concluded that the untoward effects of deuterium oxide on wound healing were due primarily to the solvation effects of deuterium oxide on developing collagen and that direct incorporation of deuterium into the collagen macromolecule is of minor importance.

Classification of Deuterated Drugs—The number of drugs that have been deuterated has been increasing rapidly. These include compounds contain-

ing a single deuterium atom in a specific molecular position and drugs that are fully deuterated, prepared either by biosynthesis or organic synthesis. Attention has generally been directed to the preparation of the deuterated drug molecule, but the synthesis of intermediate compounds utilized ultimately in the synthesis of a drug has been the object of some research.

The drugs covered in this review are categorized for convenience and the discussion is not based on a strict pharmacological or chemical system. We have made a serious attempt to include all relevant material and apologize for any inadvertent omissions.

HEAVY WATER AS A DRUG

Heavy Water as a Carcinolytic Agent—Deuterium oxide as an agent for controlling tumor growth was one of the earliest therapeutic applications of heavy water to be explored. Because deuteration affects the kinetics of cellular reactions, the consequences of deuteration are more severe the higher the level of metabolic activity. Embryonic tissue and neoplastic tissue are mainly rapidly proliferating tissues and thus should be particularly susceptible to the inhibitory action of deuterium oxide on cell division (36). The hope is that the detrimental effects of deuterium to tumor growth are more severe than the other consequences to the host.

In 1936, Fischer (59) noted that mouse carcinoma cells and fowl sarcoma cells in tissue cultures were unable to grow when the deuterium oxide content of the medium was 50–70%. Early studies (60) indicated that the rate of growth of transplanted lymphosarcoma and mammary carcinoma in mice was reduced to one-half when the animals drank 40% D_2O as compared to mice drinking ordinary water. However, the mice on deuterium oxide died faster because of the toxic side effects of deuterium oxide. Mice with carcinoma drinking 60% D_2O died even sooner than mice on 40% D_2O. Several investigations (61–63) found that mouse and rat tumors were unaffected by heavy water in concentrations as high as 94%.

In more recent studies (36), it was reported that the growth rate of Krebs-2 ascites tumors and P-1534 lymphatic leukemia was reduced in host mice deuterated to a 25% level, which is below the toxic limit for deuteration in mice. The glutamic–oxalacetic transaminase (GOT) determination in the blood serum and in the ascites plasma indicated that there was tumor cell destruction at this level of deuteration.

In a study of Ehrlich ascites tumor in mice, deuterating the mice to 30% increased survival time by about 6 days while deuterating to 40% had no sparing effect, with the toxic effects apparently nullifying any therapeutic advantage (64). It appears to be established that deuteration inhibits tumor growth but does not arrest it at deuterium concentrations tolerated by the host.

Finkel and coworkers (65, 66) observed that the growth of Krebs-2A ascites tumors in mice was depressed when the deuterium oxide levels in the body fluids ranged from 13 to 32%. With increasing deuteration, there was a progressive elevation in serum GOT in tumor-bearing mice and an even greater elevation in the ascites plasma value. These findings suggest that deuteration to high levels leads to tumor cell injury with a release of GOT into the ascites fluid, from where it is subsequently carried into the general circulation and yields elevated serum GOT values.

A combination of deuteration with chemotherapeutic agents may offer beneficial effects in tumor therapy (36), and this possibility was explored (65, 67). A potentiating effect for certain antitumor agents was reported in mice inoculated with solid and ascites Krebs-2 tumors. Fluorouracil was more effective in controlling tumors in mice drinking 30% D_2O than in nondeuterated control mice. Similar results were obtained with mercaptopurine, colchicine, and cyclophosphamide. Additive effects were noted with deuterium oxide and fluorouracil, while a distinct potentiation resulted when deuterium oxide was combined with colchicine or cyclophosphamide. Aminopterin and cyclophosphamide showed an enhanced activity against mouse ascites leukemia in deuterated mice. However, combination therapy was generally accompanied by a shortened survival time; in addition, deuteration may cause an increase in the toxicity of some chemotherapeutic agents.

Katz *et al.* (36) also examined the effect of deuteration on the radiation sensitivity of mice. In some experiments, deuterium oxide produced a slight prolongation of survival time in X-irradiated mice. The effects of deuteration and X-ray treatments on myelogenous leukemia in mice were studied (68). Oral administration of 25% deuterium oxide in drinking water produced a significant prolongation in survival time. X-radiation, in addition to deuteration, gave additive results, but a synergistic effect was not detected. Mice deuterated prior to irradiation were provided some protection against the lethal effects of X-rays.

French *et al.* (69) studied the effect of deuteration in mice infected with ectromelia virus, and they compared the effect on survival time and histopathological characteristics in two groups of infected mice. Mice receiving 30% D_2O in drinking water showed a slight prolongation in survival time and a slight delay in onset of necrosis in the internal organs examined.

The action of nitrogen mustard and deuterium oxide on mitosis and on the nucleic acid content of Ehrlich ascites tumor cells was compared (70). Deuterium oxide only blocks the ability of cells to divide, while nitrogen mustard prevents mitosis irreversibly. The presence of deuterium oxide caused a decrease of desoxyribose bound to purine.

Patients with uterine cervical cancer were treated by local administration of pure heavy water into the tumor and beneficial effects were reported (71). Heavy water also showed a potentiating effect when combined with a chemotherapeutic agent in these studies.

The effects of 30% D_2O administered in the drinking water on a virus-induced mouse leukemia were noted (72). Deuterium was administered in the

drinking water at different time periods after inoculation of the tumor virus. The earlier the initiation of deuterium oxide administration, the higher was the fraction of survivors, suggesting that deuterium oxide may have antileukemia virus activity. Furthermore, 30% D_2O was more beneficial than 15%.

In summary, numerous studies have shown that deuterium does inhibit the growth of tumors but, unfortunately, the concentrations of deuterium required cannot be tolerated by the host.

Heavy Water as Measure of Total Body Water—Heavy water is used for the measurement of total body water in humans by isotope dilution analysis. At the Mayo Clinic (73), this routine diagnostic procedure involves the following basic steps:

1. The patient receives 25 ml of pure deuterium oxide orally.
2. Urine collections are obtained at 2, 3, and 4 hr after administration of the deuterium oxide dose or 2, 4, and 6 hr in patients suspect of fluid retention.
3. Urine is reduced by passage over heated zinc (74).
4. The products of reduction (HD and H_2 gases) are analyzed by a mass spectrometer[3] for ratios of masses 3 and 2. By calibration of the mass spectrometer with known concentrations of HD:H_2, the sample moles percent deuterium oxide "excess" is calculated from the HD:H_2 ratio.
5. The calculation of total body water is then computed from the expression $V_2 = (C_1V_1)/C_2$, where V_2 is the total body water; C_1 and V_1 are the concentration and volume of deuterium oxide administered, respectively; and C_2 is the deuterium content of the urine at equilibrium.
6. Total body water calculated from the last two urine samples are averaged and taken to be the observed total body water. If the values of the last two samples disagree by more than 5%, equilibration is assumed incomplete and an additional specimen is obtained.

The procedure utilized at the Mayo Clinic is based on literature methods (74, 75).

Heavy Water in Neutron Radiography—In recent years, neutron radiography has made possible a number of interesting and potentially useful applications in biomedical research and clinical diagnosis. In certain respects, neutron radiography is similar to X-radiography. However, the two techniques are complementary rather than overlapping; neutron radiographs often reveal information not readily apparent from conventional X-rays.

The subject of neutron radiography was comprehensively treated (76), and various biomedical applications of the procedure were discussed recently (77, 78). The utility of heavy water as a biological contrast agent in slow neutron radiography also was explored (79).

Replacement of a substantial amount of hydrogen with deuterium by exchange with deuterium oxide (the process referred to as deuteration) reduces the attenuation of slow neutrons traversing tissue because the absorption and scattering probabilities of neutrons are much greater for hydrogen than for deuterium. Deuteration is effected by simply contacting the tissue with heavy water, whereby exchange takes place rapidly. The extent of deuteration depends on the chemical nature of the tissue and the number of ionizable or exchangeable hydrogens it possesses. The reader is reminded that hydrogen atoms bonded directly to carbon atoms are not exchangeable, while hydrogen atoms bonded to nitrogen, oxygen, or sulfur are readily exchangeable.

Fatty tissues consist largely of hydrocarbon chains, which contain essentially only nonexchangeable hydrogen. After deuteration, fatty components therefore form an image with high contrast against surrounding muscle, bone, and most body fluids. Fats retain their ^1H content and remain opaque to neutrons. A high level of exchange takes place in muscle tissue, because proteins and carbohydrates have large numbers of exchangeable hydrogen. Since bones are largely inorganic in nature, they are generally radiolucent to neutrons, even without deuteration. The bone marrow does contain variable amounts of connective tissue, proteins, and fats. Extensive deuteration does occur in viable bones, resulting in high contrasts between bones and fatty tissues in neutron radiographs of deuterated sections.

A significant contrast between tumor tissue and surrounding tissue in specimen radiographs was noted (77). Although the specimen was not deuterated in this case, it was suggested that the difference in the neutron absorption pattern may have been due to a higher hydrogen content in the tumor than in normal tissue. In a more recent study (79), tissue slices from an adenocarcinoma of the colon were soaked in heavy water for 24 hr, and comparisons were made among the X-ray, neutron radiograph, and microscopic examinations of the histological section. The neutron radiograph revealed greater contrast between the fat and other tissues. Neutron radiography has been demonstrated to be a unique method for imaging bone marrow directly. The radiograph of a deuterated specimen (a finger soaked in heavy water for 24 hr) showed strong contrast between the radiopaque fatty bone marrow and the radiolucent bone cortex and the connective tissue in the joints.

Heavy water has been employed in a selective imaging technique whereby contrasts were produced by controlled deuteration in localized areas of an animal's body. A heavy water solution containing dextran, glucose, and sodium chloride was infused into both carotid arteries of a living rat over 4 hr. All organs except the brain and aqueous humor of the eye were extensively deuterated. A neutron radiograph of the rat's head revealed high contrast between these organs and the surrounding tissues which became radiolucent to neutrons. When dextran was deleted from the perfusion liquid, the contrast was not observed. Apparently, dextran interferes with the exchange process across the blood–brain and blood–aqueous humor barriers, and this technique may be useful in studying the effect of drugs and other factors on transport across these barriers.

[3] AEI MS 20.

Heavy water as a contrast medium in neutron radiography has limited clinical application at present. It is readily applied to isolated tissue sections by simply soaking the specimen in heavy water. Heavy water may be administered by injection to live research animals within the constraints, of course, of the neutron radiation dose and the toxicity of the heavy water. The thickness of the tissue or organ that may be effectively imaged by neutron radiography increases as the extent of deuteration becomes greater.

Although heavy water in small amounts is generally not toxic to humans or animals, the concentrations required for effective neutron radiography *in vivo* are large and probably beyond the dosage that can be readily tolerated by humans.

Parks *et al.* (79) considered the possibility of effecting deuteration in rats by a single large dose of deuterium oxide administered by intraperitoneal or intravenous injection. They found that rats survived injections of heavy water that did not exceed 40% of the total body fluid weight. This dose resulted in less than 30% deuterium replacement in the body fluids. According to these investigators, this level of deuteration is probably not sufficient for clinical applications of neutron radiography. Even at this level of deuteration, the thickness of the specimen that may be radiographed is only 5 cm.

The extent of deuteration in rats may be increased to 50% by an isolated segment infusion technique. This level of deuteration in the muscles of the hindlimbs did not prove fatal. Similar levels were achieved in the brain of rats by injection of heavy water into the carotid arteries. Parks *et al.* (79) speculated on the possible application of this technique in humans. Deuteration at the 50% level would permit radiography of a limb 7 cm thick while imposing a high, although clinically acceptable, neutron radiation dose. It is questionable whether the risks imposed by extensive deuteration of a human limb would be outweighed by the advantages derived from such a drastic procedure. A review of clinical applications of neutron radiography was presented recently (80).

BARBITURATES

The pharmacological effect of deuterium substitution in butethal[4] (5-*n*-butyl-5-ethylbarbituric acid) was studied (81, 82). 3′-Dideuteriobutethal, which contains two deuterium atoms on carbon 3 of the butyl side chain in the 5-position, was synthesized. The deuterated derivative was compared with the nondeuterated form by intraperitoneal injection in mice and measurement of the duration of induced sleeping time, based on the time from loss to return of the righting reflex. The deuterated barbiturate increased sleeping time better than twofold in comparison with the nondeuterated controls. The biological half-life of the former was about 2.5 times longer than the latter. *In vitro* studies were conducted by incubation with mouse liver microsomes, and an isotope effect of about 1.6 was observed for the *in vitro* rate of hydroxylation of the deuterated compound compared to the nondeuterated molecule.

The major metabolite of butethal is 5-(3-hydroxy-*n*-butyl)-5-ethylbarbituric acid. If the oxidative process involving the penultimate carbon of the side chain of the molecule is the rate-limiting step, replacement of hydrogen with deuterium at the site of oxidation should decrease the rate of oxidation, increase the biological half-life, and prolong the pharmacological activity. Not excluded in these studies is the possibility that deuterated butethal may be less readily metabolized because of a weaker affinity for hepatic microsomal enzymes. Studies with 4′-deuteriobutethal seemed to indicate that the terminal carbon of the side chain may also be involved in the metabolism of butethal.

The effects of deuteration on the activity of 5-(1-methylbutyl)-5-ethylbarbituric acid were investigated (83). The 3′-dideuterio derivative was synthesized, and its anesthetic potency was compared with the ordinary drug in mice. While the onset of sedation was similar in both compounds, the time to peak depression was delayed and the total sleeping time was extended significantly by the deuterated derivative. The biological half-life, determined in dogs, was 1.7–2.0 times greater for the deuterated compound than for the drug of ordinary isotopic composition. The metabolic process and mechanism of action appeared to be the same as those noted for butethal.

The effect of deuterium substitution on the metabolism of phenobarbital in dogs and rats was reported (84). Two deuterated derivatives were studied: one containing a deuterium atom in the *para*-position of the phenyl group at position 5 and the other containing five deuterium atoms in the ethyl group in position 5 in addition to a deuterium atom in the *para*-position of the phenyl group. There was no apparent difference in the rates of disappearance of these compounds and the control from the plasma, which indicated that there was no significant isotope effect. Although the major route of metabolism of phenobarbital in humans, dogs, and rats is through hydroxylation of the *para*-position of the phenyl group, it does not appear that this process is rate limiting. Apparently, biological aromatic hydroxylation lacks a deuterium isotope effect.

Sleep time studies with mice indicated that there were no significant differences in sedative potency among the compounds studied, and there appeared to be no significant difference in the degree of stimulation of drug-metabolizing enzymes. It was also indicated that the 5-ethyl substituent contributes only in a minor way to the sedative activity of barbiturates, perhaps by increasing the lipid solubility of the molecule. There was no apparent alteration in the metabolic pathway of the deuterated drug. Alterations in the pharmacological activity of barbiturates are often dependent on structural changes involving longer and more highly branched alkyl substituents in position 5. Further work based on kinetic isotope studies should be of considerable value in determining the precise effect of molecular changes on the pharmacological function of barbiturates.

[4] Neonal.

The estrogenic activity of α-bromo-α,β-diphenyl-β-pentadeuteriophenylethylene was compared to its nondeuterated analog and found to be considerably less active (85). The pharmacological activity apparently depends on the oxidation to the corresponding bis-*p*-hydroxy derivative, which has a structure similar to diethylstilbestrol. The decrease in activity of the deuterated form is the result of a primary kinetic isotope effect on the oxidation of a C—D bond. The assay procedure (Allen–Doisy test) was based on the induction of estrus in ovariectomized rats. No significant difference was noted between the two compounds in their preoestrus-inducing activity.

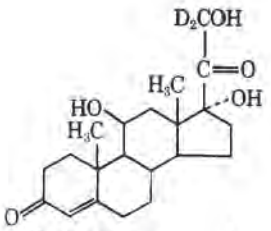
11α-Deutero-17α-hydroxycorticosterone was synthesized, and its biological activity was compared to hydrocortisone (86). In the oral glycogen deposition test in mice and in the oral systemic granuloma assay in rats, the approximate relative potencies of the deuterated derivative compared to hydrocortisone were 0.7 to 1.0 and 1.5, respectively.

The androgenic activity of 3α-deutero-17α-methyl-5α-androstane-3β,17β-diol was compared to the corresponding ^{1}H derivative (87). The 3-hydroxy form is probably androgenically inactive as such and must be oxidized *in vivo* to the keto derivative, which is a potent androgen. If the oxidation to the 3-ketone with the loss of the 3α-hydrogen or 3α-deuterium is the rate-determining step, then the 3α-deutero compound should be oxidized more slowly and therefore exhibit lower androgenic activity than the 3α-hydrogen compound.

This possibility was tested in castrated male rats, and it was found that the ^{1}H compound was 3.36 times more active than the deuterated compound on the basis of the ventral prostate response and 4.65 times more active in the response of the seminal vesicles, demonstrating that oxidation to the keto form is a necessary step for androgenic activity in the rat. This result is interpreted to be due to an *in vivo* isotope effect on the oxidation of the 3-hydroxy group, which involves the cleavage of the 3α-deuterium—carbon bond. No difference was noted in androgenic activity between testosterone and 17α-deuterotestosterone in the castrated rat when administered orally or subcutaneously.

A number of deuterated deaminooxytocin derivatives, variously substituted in the 1β-mercaptopropionic acid position or in the 5-asparagine position were synthesized (88, 89). The biological activity of the deuterated derivatives was compared with deaminooxytocin. Statistical analysis of the data revealed that, within experimental error, all compounds elicited the same vasodepressor and oxytocic activities. It was also shown that deuterated amino acids can be incorporated into peptides, using ordinary synthetic procedures, without significant loss of deuterium.

Pitman *et al.* (90) synthesized hydrocortisone (I) in which two deuterium atoms were incorporated in the C-21 position. Its stability against degradation in aqueous solution under both aerobic and anaerobic conditions was compared with that of hydrocortisone. Under oxidative conditions, the deuterated compound was more stable than the protio form of hydrocortisone. The explanation may be that the reaction mechanism for the degradation involves a rate-determining enolization in the C-17 position of the dihydroxyacetone side chain.



I: hydrocortisone-d_2

Lemieux *et al.* (91) studied the *in vivo* oxidation of the antidiabetic drug tolbutamide (*N*-*p*-toluenesulfonyl-*N'*-*n*-butylurea) to *N*-*p*-carboxybenzenesulfonyl-*N'*-*n*-butylurea. The rate of oxidation in adult human subjects was essentially unaffected by complete deuteration of the methyl group of the toluene residue, as measured by the rate of the excretion of the oxidation product. Tagg *et al.* (92) identified hydroxymethyltolbutamide as the major oxidation product in the urine of rats and rabbits administered tolbutamide, a finding confirmed by Thomas and Ikeda (93). The tolbutamide was shown to be metabolized by liver microsomes in the rat. Only a small isotope effect (H/D = 1.14) was noted in comparing the oxidation rates of tolbutamide containing three deuterium atoms in the methyl group with nondeuterated tolbutamide. The small isotope effect indicates that the breaking of carbon—hydrogen bonds is not the rate-determining step in the oxidative reaction.

The specificity of the pharmacological action of dopamine was examined by substituting deuterium for hydrogen in the alkyl side chain (94). Both α,α'-dideuteriodopamine and β,β'-dideuteriodopamine were synthesized chemically and tested for activity in the dog. No difference in pharmacological activity was noted between the deuterated dopamines and the protio analog, suggesting that this agent acts directly on a dopaminergic receptor without involving the formation of intermediate compounds. If hydroxylation were the rate-determining step involved in the pharmacological activity, then the rate of *in vivo* conversion to norepinephrine would have been reduced due to a primary deuterium isotope effect involving the β-carbon position. If dopamine activity were effected through the monoamine oxidase enzyme, then a comparable effect would have been noted as a result of substitution in the α-carbon position. The absence of these effects supports the concept that dopamine acts directly on a receptor.

Barbour and Dickerson (95) observed the effect of deuterium oxide at 20, 50, and 75% on the hydrolysis of acetylcholine bromide both in the presence and in the absence of cholinesterase. Dog blood serum served as the source of cholinesterase. Deuterium oxide slowed the hydrolysis of acetylcholine to the same extent whether or not enzyme was present. The

rate of hydrolysis was decreased 10.6% in 20% D_2O, 23% in 50% D_2O, and about 33% in 75% D_2O. Apparently, the inhibition of hydrolysis cannot be attributed to poisoning of the enzyme.

Acetylcholine was synthesized with three hydrogen atoms of the acetyl group replaced by deuterium atoms; about a 30% reduction in potency was reported as a result of the deuterium substitution (96).

Belleau (23) synthesized acetylcholine bromide variously substituted with deuterium: Compound A, three deuterium atoms in the acetyl group as synthesized earlier (96); Compound B, two deuterium atoms in the choline part of the molecule alpha to the ester linkage; Compound C, two deuteriums in the choline part beta to the ester linkage; and Compound D, full deuteration of the three methyl groups attached to the quaternary nitrogen. Michaelis constants were obtained for the isomeric deuterated acetylcholine bromides. The respective constants were identical, $4.5 \pm 0.3 \times 10^{-4}$ *M*. Each compound was further tested for cholinergic activity, using the hypotensive response of the dog to intravenous administration before and after treatment with physostigmine. The absence of deuterium isotope effects on the binding of acetylcholine with the esterase enzyme or the cholinergic receptor is at variance with previous observations (96) or a 30% reduction in potency for Compound A.

ANESTHETICS

The anesthetic properties of deuterated chloroform in dogs and mice were studied (97). The exhaled deuterated chloroform had the same isotopic composition as the inhaled chloroform, which indicated that no exchange had taken place. In mice, the response to the anesthetic action of both forms of chloroform was similar. In dogs, the anesthetic syndrome was the same for both compounds. But, on the basis of limited studies, it appeared that deuterated chloroform was somewhat more potent; 20% more ordinary chloroform was required to achieve the same stage of anesthesia as the deuterated form when compared in the same five dogs. It did not appear that chloroform was metabolized to any significant extent.

The anesthetic properties of fully deuterated ethyl ether were also studied (98). The anesthetic syndrome in mice was identical to that for the protio analog. On the basis of mass spectrometric analysis, it was determined that no deuterium exchange had taken place, suggesting that the anesthetic action of ether does not depend on chemical interaction with the biological systems of the body and that it is evoked by the intact molecule involving weak interactions rather than chemical reactions. In an earlier study (99), it was shown that fully deuterated ethylene and acetylene elicited anesthesia in dogs and cats similar to that of ordinary ethylene and acetylene.

The effect of deuterium oxide on the local anesthetic activity of procaine was studied, using the cornea of the guinea pig as the test site (100). The ED_{50} of procaine in deuterium oxide was found to be about one-half of the ED_{50} for procaine in water. No significant difference in the toxicity of procaine was found with either solvent. Stability studies indicated that procaine is more stable in deuterium oxide solutions than in water solutions at comparable pH values. Greater activity of procaine in deuterium oxide was accounted for by the fact that procaine free base is more stable in deuterium oxide solutions and also that a deuterium oxide solution of procaine contains more free base than the corresponding aqueous solution at comparable pH values[5].

The effect of α-deuteration on the metabolism and depressant action of ethanol was studied, using the mouse as the test animal (101). Ethanol and α-deuterioethanol were administered intraperitoneally (4 g/kg). The deuterated compound slowed the rate of metabolism from 794 to 692 mg/kg/hr. Sleeping time was increased from 110 to 170 min for the deuterio compound after the injection of 5 g/kg for both compounds.

ANTIBIOTICS

Shalfer *et al.* (106) examined the effects of deuteration on the growth rate and morphology of the fungi *Penicillium notatum* and *Aspergillus fonsecaeus*. These organisms were grown over 3 months in media containing 10–99% D_2O and protio substrates. Severe growth inhibition was noted at the 50% D_2O level in the *P. notatum* cultures and at the 66% level in the *A. fonsecaeus* cultures. In both fungi, the normal floating-mat type of growth was replaced at the highest deuterium oxide levels by a submerged flocculated type of growth. Pigment production decreased as the deuterium oxide level increased, and sporulation was inhibited in both fungi. *P. notatum* sporulated in media containing 0, 10, and 25% D_2O, while *A. fonsecaeus* sporulated in all but the 99% D_2O medium. Combustion analysis data indicated that the extent of deuterium incorporation into fixed positions in the mycelia was only about 50% that of the deuterium level of the medium, indicative of the ^1H contribution from the ^1H- glucose used in the culture medium. The effect of deuteration on antibiotic production was not investigated in these studies.

Nona *et al.* (107) investigated the effect of deuteration on the growth of *Penicillium janczewskii*, a mold that produces the antifungal antibiotic griseofulvin. The mold was cultured by the shake culture technique and by surface culture in media containing 50, 75, and 99.6% D_2O with protio or deuterio sugars as the carbon source. Griseofulvin titers, pigmentation, and mycelial dry weights were all markedly depressed as the deuterium oxide content of the medium was increased. Corn steep solids and vitamin B complex appeared to enhance both mycelial growth and griseofulvin production in highly deuterated media. In a highly deuterated environment, the mold apparently uses nutrients to support vital metabolic activities and meets only those needs required for

[5] Some literature reports (102–105) have dealt with relating the pD scale and the operational pH in deuterium oxide solutions. Generally, a correction of about 0.40 pH unit is added to the "apparent" pH of a heavy water solution as measured by a conventional glass electrode.

survival. Griseofulvin biosynthesis appears to be nonessential for mold survival and can be depressed without damage to the organism.

A subsequent study (108) found a "replacement culture" technique to be useful for obtaining nonessential metabolites. The mycelial contents of shake cultures grown in a water medium were used as the starting tissue for preparative replacement cultures. After about 1 week's growth, the vegetative tissue was removed from the culture, washed free of water with deuterium oxide, and redispersed in a deuterium oxide medium. When the replacement deuterium oxide medium contained deuterio sugars, fully deuterated griseofulvin was produced; when protio sugars were included in the deuterium oxide medium, the griseofulvin was partially deuterated. In the ^{2}H-cultures, griseofulvin did not accumulate to any appreciable extent before the 15th day of incubation. Therefore, the ^{1}H-mycelium younger than 15 days could be used in a replacement culture without fear of product contamination. This procedure reduced the 50–60 days generally required for maximum antibiotic production by the direct fermentation method to about 30–35 days. No significant increase in antibiotic production was noted by the latter method. The antibiotic was isolated and purified by TLC. PMR (^{1}HMR) spectral analysis indicated complete deuteration in the sample obtained from a fully deuterated replacement medium.

The antifungal activity of the isolated deuterated antibiotic was compared (109) with that of ordinary griseofulvin by the plate assay method, with *Microsporum gypseum* as the test organism, and the deuterated antibiotic was found to be about 10% more active than the protio form. The increased activity of the deuterated griseofulvin may be due to an increased efficiency of action resulting from a primary isotope effect at the target site where the rupture of C—D bonds may be directly involved, or the increased stability of the deuterated molecule may play a role in that the fungus may not be able to metabolize deuterated griseofulvin as readily as it does the protio form. However, a better understanding of the mechanism of antifungal activity of ordinary griseofulvin is needed before the effect of deuterium replacement is more completely understood.

Mohammed *et al.* (110, 111) reported the effects of deuterium oxide on the growth and antibiotic production of 13 low-producing strains of two species of *Penicillium* and three high-producing strains of *P. chrysogenum*. However, the deuterated antibiotic was not isolated in these investigations.

Deuterophenylacetyl-^{15}N-DL-valine was synthesized where 41% of the hydrogen in the benzene ring was replaced with deuterium (112). This compound was used to study the incorporation of phenylacetic acid and valine into the benzylpenicillin molecule during fermentation. It was learned from deuterium analyses that the phenylacetyl moiety was incorporated directly into benzylpenicillin. Very little ^{15}N was found in the molecule, from which it was concluded that the nitrogen of the amide group did not originate from the added labeled valine.

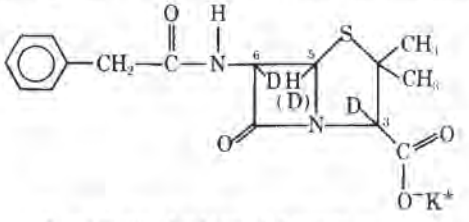
Deuteriobenzylpenicillin, in which the benzene ring contained five deuterium atoms, was utilized as a label for monitoring net penicillin production during fermentation (113).

Deuteriobenzyl-d_7-penicillin was synthesized chemically and used to study the deuterium isotope effect on the biological activity of penicillin (114). The biological activity of the selectively deuterated penicillin was compared to that of the protio form by a turbidimetric assay procedure, using *Staphylococcus aureus* as the test organism. The relative potency (penicillin to deuteriopenicillin) was reported to be 1.25. It was suggested that since the binding of the benzylpenicillin molecule to proteins is apparently mediated by the benzyl moiety and not by the β-lactam-thiazolidine nucleus (115), alteration in the rate and strength of binding may account for decreased bioactivity of the deuterated molecule.

P. chrysogenum (Wisconsin strain 53-414) was cultured in a defined medium containing glucose, acetate, lactate, and phenylacetate as carbon sources and pure deuterium oxide as the solvent (116). Partially deuterated benzylpenicillin was isolated from the culture containing protio substrates and deuterium oxide. The extent of solvent participation in the biosynthesis of penicillin was determined by analysis of the ^{1}HMR spectrum. The culturing procedure involved a modification of the replacement technique, whereby a thick seed culture was produced in a protio medium. The mycelial pad was separated, rinsed with deuterium oxide, and used as an inoculum for the deuterated culture.

Incorporation of deuterium appears almost complete at the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring (II). Only partial incorporation of deuterium (60–70%) at the C-5 position was noted. The deuterium atoms in the C-5 and C-6 positions probably arise during the biosynthesis of cysteine. The deuterium at the C-3 position may arise from the precursor valine or in the final step of biosynthesis involving the thiazolidine ring closure, whereby a deuterium may be incorporated through solvent participation.

A highly deuterated penicillin was obtained (117) by using deuterated substrates in the replacement culture medium. Analysis of the ^{1}HMR spectrum revealed an average deuterium replacement of 89%. Complete deuteration was noted in the phenylacetyl group, at the C-3 position of the thiazolidine ring, and at the C-6 position of the β-lactam ring. Partial ^{2}H incorporation (64%) was noted at the C-5 position of the β-lactam ring and in the methyl groups (77%) at the C-2 position of the thiazolidine ring. When deuterated pyruvate (the precursor for the two meth-



II: partially deuterated benzylpenicillin

yl groups attached to C-2) was included in the medium, the methyl group contained only ^1H. However, when deuterated pyruvate was incorporated in the seed culture medium, deuterium was present in the methyl groups. The methyl groups are apparently preformed in the seed inoculum with little or no additional biosynthesis of methyl groups during fermentation.

The antibiotic potency of highly deuterated benzylpenicillin was compared (118) with the compound of normal isotopic composition by the USP cup-plate assay, with *Sarcina lutea* as the test organism. A relative potency (H/D) of 1.23 was obtained. Thus, as also noted by Laskar and Mrtek (114), deuterium in the penicillin molecule affected the potency adversely. Penicillin acts by inhibiting cell wall formation, possibly at the cross-linking level in mucopeptide synthesis. According to Wise and Park (119), the penicillin molecule fits the active site of a hypothetical transpeptidase enzyme. The β-lactam ring of the penicillin molecule apparently reacts with the enzyme and inactivation results. Penicillin has been shown (120) to be bound irreversibly to a "penicillin-binding component" of bacterial cell walls. The component is presumably the transpeptidase enzyme. The binding strength of deuterated penicillin to the enzyme would be expected to be less than the protio form of penicillin, thus accounting for a decrease in potency.

The deuterium isotope effect on the antibiotic activity of chloramphenicol was studied (121). This antibiotic apparently interferes with the synthesis of proteins essential in cell division (122). The antibiotic may bind irreversibly to an enzyme active center or covalently to an enzyme product used in a subsequent biosynthetic step. Chloramphenicol may act through a hydrogen radical transfer mechanism (123). The benzylic C—H bond is proposed as the point of attack at the receptor site. The antibiotic apparently forms a free radical, which binds covalently to the enzyme. The α-deuterio derivative should possess a lower activity if, in fact, the rate-determining step is a deuterium radical transfer.

α-Deuteriochloramphenicol was synthesized and its antibacterial activity was compared with the protio analog using *Escherichia coli* as the test organism. An isotope effect of 1.27 was obtained based on inhibitory rate constant measurements; *i.e.*, the deuterio form had only 80% of the activity of the protio species. This result is consistent with the hypothesis that the benzylic C—H bond is broken in the rate-determining step and with the concept that the antibiotic blocks an enzyme covalently.

ALKALOIDS

Beckett *et al.* (124) postulated that *N*-dealkylation at the central receptor site is the initial process responsible for morphine analgesia. Since this is an enzymatic oxidative reaction, involving the breaking of C—N and C—H bonds, the rate of demethylation and the pharmacological activity depend on the ease with which these bonds are oxidized.

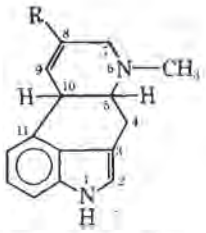
Morphine, in which the *N*-methyl group was completely deuterated, was prepared and then the effect of deuteration on the analgesic potency and enzymatic *N*-demethylation of the molecule was studied (125, 126). A significant lowering in potency in mice (tail-flick method), as well as the LD_{50}, irrespective of the mode of administration was found; no differences in time of onset, maximal effect, or duration of action were detected. The potency ratio (morphine to deuteriomorphine) ranged from 1.56 to 3.00, depending on the mode of administration. Absorption effects were ruled out since similar results were obtained when the drugs were administered intracerebrally and intravenously.

The rate of morphine demethylation was studied *in vitro* by measuring the formaldehyde released when morphine was incubated with rat liver microsomes. An isotope effect (k_H/k_D) of 1.4 was reported. Based on the Michaelis and velocity constants, the observed isotope effect apparently results from a weaker bonding of the morphine molecule to the *N*-demethylating enzyme. The difference in potencies may thus be due to a decreased affinity of the deuterated morphine for the receptor site, assisted by a diminished reaction rate of the bound material.

A series of studies (127–129) examined the biosynthesis of a water-soluble group of clavine alkaloids elaborated by certain strains of *Claviceps purpurea*. Conditions were established for the optimal growth of the *Claviceps* strain 47 A-Tyler in a fully deuterated form (127). At high concentrations of deuterium oxide, saprophytic growth was highly dependent on the nature of the carbon source. This strain of *Claviceps* was well maintained and produced high yields of alkaloids in water on a medium containing a mixture of 5–15% protiomannitol and 0.5% protiosuccinic acid.

At deuterium oxide levels up to 75%, growth and alkaloid production were realized in submerged and mat cultures with 7% protiosuccinic acid as the only carbon source. Reduction of the phosphate content in the nutrient medium stimulated alkaloid production in water cultures but only slightly in deuterium oxide cultures. Complete deuteration of the organism and successful maintenance of the culture were achieved on a low phosphate nutrient medium containing 2% fully deuterated monosaccharides and 0.5% deuteriosuccinic acid plus traces of vitamins. However, at high levels of deuterium oxide, alkaloid production was severely impaired, although the organism grew well.

Further studies (128) used a replacement culture technique for the biosynthesis of highly deuterated clavine alkaloids. Cultures grown in an ordinary hydrogen environment (water and protio substrates) were transferred to a deuterium oxide medium containing only phosphate buffer salts and tryptophan, phenylalanine, succinic acid, and sugars, either in ^1H or ^2H forms. Multimilligram amounts of deuterated alkaloids were obtained from preparative replacement cultures containing phenylalanine and deuteriosuccinic acid as substrates in pure deuterium oxide. The alkaloid fractions consisted of elymoclavine (III)



III: elymoclavine: R = CH_2OH
IV: agroclavine: R = CH_3

with minor amounts of agroclavine (IV) and were isolated by solvent extraction.

No alkaloids were elaborated when deuterated tissue was used in a replacement culture experiment independent of whether the replacement medium was made with water or deuterium oxide. Alkaloid production is apparently a nonessential activity, and organisms that produce this class of compounds do so only under quite specific and probably nonobligatory conditions. As indicated by Mrtek *et al.* (128), a primary effect of deuteration in biological systems appears to be the suppression of nonessential metabolism, which in a way represents an afterthought for the organism. High deuterium oxide concentrations make *Claviceps* use available metabolites for essential nutrition at the expense of alkaloid synthesis, a result similar to that observed by others (107, 116). The replacement culture technique provides a mechanism for circumventing at least some problems encountered in the adaptation of the fungus to a deuterated environment.

The third study of this series (129) provided a detailed magnetic resonance spectral analysis of a number of the clavine alkaloids. The spectra were used to establish the isotopic composition of elymoclavine biosynthesized in deuterium oxide with selected protio substrates. The results indicated a large degree of solvent participation during alkaloid biosynthesis. Alkaloids produced in this way contain both hydrogen and deuterium and are referred to as isotope hybrid compounds. The hydrogen-to-deuterium ratios at the different hydrogen positions in the molecule can be determined from ^{1}HMR data. Elymoclavine obtained from replacement cultures in 99% D_2O with protiosuccinate and protiophenylalanine as substrates was highly deuterated at all positions. The hydrogen atoms at positions 10 and 17 (III) were completely deuterated, and the hydrogens at positions 4, 5, 6, 7, and 9 contained 68–86% deuterium, indicating extensive labilization of carbon-bound hydrogen during biosynthesis.

Blake *et al.* (130) studied the effects of deuteration on peppermint (*Mentha piperita*). This appears to be the first detailed study of the effects of extensive replacement of hydrogen by deuterium on the growth of a higher plant. Cuttings were grown hydroponically in nutrient solutions containing varying concentrations of deuterium oxide. There was a progressive inhibitory effect on growth with an increase in the deuterium oxide content of the nutrient. The effect became particularly pronounced at the 50% level, and growth essentially stopped in 70% D_2O. There was impairment in the ability to flower at all levels of deuterium oxide, which seems to parallel fertility effects in mammals as noted earlier. This probably results because of the high metabolic activity associated with reproduction and the large deuterium kinetic isotope effect that might be expected in such a situation.

A comparison was made between the deuterium content of the water distilled from the deep-frozen plant (labile water), which represents the isotopic composition of the exchangeable hydrogen in the plant, and the deuterium content of the water obtained from the combustion of dried plants, which represents the organically bound exchangeable and nonexchangeable deuterium. The deuterium content of the former was found to be approximately 75% that of the nutrient solution in which the plant was grown, while the latter was about 50% of the deuterium content of the nutrient solution. Thus, the ratio of organically bound to labile deuterium was about 0.66, somewhat higher than that reported for animals (29).

A subsequent study (131) described the histological effects of deuterium. The major effect of deuterium on the growth of peppermint appeared to be inhibition of cell division; in general, the effects were more pronounced in actively growing tissues. The third study of this series (132) reported the effects of certain growth regulators on the growth of mint at different concentrations of deuterium oxide in the nutrient solution. The inhibitory effects of deuterium on the cellular level were not reversed by the plant growth hormones used, but maleic hydrazide, usually considered to be a plant growth inhibitor, stimulated the growth of partially deuterated peppermint plants. The metabolic effects of maleic hydrazide are apparently converted into stimulation because of the contrasted metabolic effects of deuteration.

Although peppermint is not an alkaloid-producing plant, the studies described with mint did suggest the possibility of utilizing an alkaloid-producing plant for the preparation of deuterated or partially deuterated alkaloids by biosynthesis. A series of studies was undertaken involving the growth of the plant *Atropa belladonna* in heavy water. This plant produces atropine and related alkaloids. The general morphological effects of deuteration on belladonna plants grown in nutrient solution containing 30, 50, and 60% D_2O were described (133). The extent of deuterium uptake by the plant, the effect of deuteration on seed germination, and the effect on alkaloid content were examined.

The growth of belladonna was strongly inhibited by deuterium oxide concentrations greater than 50%, and the morphological effects were qualitatively similar to those reported earlier for peppermint plants. In 60% D_2O, the effects were so drastic that the plants were not recognizable as belladonna; plant elongation was slight, only a few pale poorly expanded leaves developed, and there was no indication of inflorescence. In general, with increasing concentrations of deuterium oxide, there was a marked reduction of normal growth patterns; above 50% D_2O in the

medium, there was a loss of reproductive capacity.

Most disappointing, however, was the progressive inhibitory effect on alkaloid production as the deuterium content of the plant increased, a situation not at all unlike that described for the clavine alkaloids (128). In fact, plants grown in 60% D_2O medium did not produce sufficient plant material to permit an analysis for alkaloid content.

A subsequent study in this series described the effects of deuteration on flower, fruit, and seed development in *A. belladonna* grown in high concentrations of deuterium oxide (134). Intercomparison of plants grown through three generations of exposure to heavy water indicated that the most drastic growth inhibition took place during first generation growth in a deuterium environment. After three generations, some adjustment to the deuterium in the environment appeared to occur as the plants showed better growth in 50% D_2O. Blake *et al.* (135) reported that in 50% D_2O, only 75% of belladonna seeds germinated; in concentrations above 50% D_2O, the germination rate was reduced to zero.

A replacement culture technique (128) was used to study the effect of deuteration on alkaloid production in *A. belladonna* (136). Belladonna plants grown to maturity in aqueous (water) nutrient solution were transplanted to nutrient solutions containing 50, 60, 75, and 100% D_2O. The plants placed into a 99.7% D_2O medium showed drastic effects of deuteration within hours, and all plants died in several days. The plants transplanted into 75% D_2O survived about 3 weeks. The 50 and 60% D_2O plants survived the stresses imposed by deuterium and were harvested after a growth period of 7.5 months. Alkaloid production was reduced drastically, ranging from about one-third to one-tenth that of the control plants. The alkaloid content was too small and the amount of plant material collected too little to permit isolation of alkaloid for further study. Although total alkaloid analysis and analysis for atropine and scopolamine content were performed, the isotopic composition of the alkaloid fraction was not determined. Although the time for transplantation was selected when the rate of alkaloid synthesis in the plant was greatest, extremely low alkaloid analyses were obtained for the deuterated plants at harvest. It seems likely that alkaloid production was completely inhibited upon transfer of the plants from normal growth in water to the deuterated nutrient.

All studies reported in this section, much of the work summarized in the section on antibiotics, and some of the investigations covered in the amino acid section were conducted in a series of collaborative efforts between the Chemistry Division of Argonne National Laboratory and the College of Pharmacy of the University of Illinois. These collaborative programs were the subject of a recent article (137).

AMINO ACIDS

The successful culturing of several species of algae in 99.6% D_2O was reported about 15 years ago (138–140), and the details of mass culturing on a continuous basis were published (141) shortly thereafter. The algae are grown autotrophically in deuterium oxide containing inorganic salts, with carbon dioxide as the source of carbon. Under these conditions, all compounds synthesized by the algae are fully deuterated[6].

The identification and isolation of fully deuterated amino acids and monosaccharides in alga hydrolysate were described (142). The isolated amino acids included deuterioalanine, deuterioglutamic acid, deuterioaspartic acid, and deuterioglycine. Deuteriomannose and deuterioglucose were also isolated in greater than gram amounts by chromatography on cellulose, and the amino acids were obtained by passage of the ionic fraction of the hydrolysate through an ion-exchange[7] column. Cohen and Putter (143) reported the preparative separation of most deuterated amino acids present in the protein hydrolysate prepared from the algae *Scenedesmus obliquus* grown in heavy water.

The chemical synthesis of a series of fully deuterated amino acids was described including deuterated glycine (144); deuterated aspartic acid, glutamic acid, asparagine, and glutamine (145, 146); and deuterated phenylalanine (147). One objective of these studies was to prepare highly deuterated derivatives for use in the synthesis of deuterated analogs of oxytocin and vasopressin.

A nonenzymatic transamination method was employed for the preparation of "amino acids" deuterated in the α-position (148). The protio amino acid was heated for 70 hr at 120° with salicylaldehyde in the presence of copper ion in deuterium oxide. The α-substituted deuterio amino acids prepared in this way included glycine, alanine, glutamic acid, valine, isoleucine, aspartic acid, and phenylalanine. α-Deuterioglutamic acid and α,β,β-trideuterioglutamic acid were prepared by enzymatic transamination of glutamic acid with pig heart glutamate–pyruvate aminotransferase and pig heart glutamate–oxaloacetate aminotransferase, respectively (149). α-Deuterioglutamic acid was synthesized by heating the acetylamino acid at 100° in deuterium oxide in the presence of acetic acid-d_1 and acetic anhydride (150).

Guroff *et al.* (151, 152) synthesized phenylalanine with a deuterium atom exclusively in the *para*-position of the phenyl ring by the catalytic reduction of the *para*-bromo derivative of phenylalanine. The *p*-deuteriophenylalanine was used as a substrate for *Pseudomonas* phenylalanine hydroxylase. Hydroxylation of the phenylalanine resulted in the formation of *p*-tyrosine, with the concomitant migration of the deuterium atom to the *meta*-position. Although a significant isotope effect was observed in this study, Guroff and Daly (153), in a subsequent report dealing with the migration of a deuterium atom during phenylalanine hydroxylation, concluded that no isotope effect was observed. The same substrate and enzyme

[6] The term "fully deuterated" and the prefix "deuterio" are used here to indicate that nonexchangeable hydrogen atoms have been replaced with deuterium atoms.

[7] Dowex-50.

system were used in both studies. The authors explained that the discrepancy was apparently caused by the presence of impurities in the deuterated substrate used in the earlier study, which resulted in an overestimation of the actual amount of phenylalanine present. This hydroxylation-induced migration was named the "NIH shift" and was the subject of an extensive review (154). Frequent reference is made to the utilization of deuterated substrates in studies concerned with the elucidation of the mechanism involved in this important phenomenon.

The preparation of specifically labeled or fully deuterated amino acids has provided useful compounds for the synthesis of deuterated drugs, for studying the mechanism of biochemical reactions, and for kinetic isotope studies.

The deuterium kinetic isotope effect in nonenzymatic transamination of alanine was studied with pyridoxal (155). L-Deuterioalanine was isolated from the hydrolysate of the algae *Scenedesmus obliquus* grown in deuterium oxide (142). In the solvent water, the reaction of L-protioalanine with pyridoxal was 2.4 times faster than for L-deuterioalanine, and in deuterium oxide the reaction with L-protioalanine was 2.9 times faster than with L-deuterioalanine. Lin *et al.* (156) determined the isotope effect in the nonenzymatic transamination of L-glutamic acid. L-Deuterioglutamic acid was isolated from deuterated algae as described earlier (142). The reaction with protioglutamic acid was 2.1 times faster than with deuterioglutamic acid. The reaction was found to be general acid–base catalyzed.

Fang *et al.* (157) investigated the kinetic isotope effects in the enzymatic transamination of fully deuterated glutamic acid (isolated from deuterated algae hydrolysate), α-deuterioglutamic acid (synthesized from protioglutamic acid), and 3,3,4,4-d_4-glutamic acid (synthesized from fully deuterated glutamic acid). The glutamate–oxaloacetate aminotransferase enzyme system was employed in the transamination reactions. The two compounds with deuterium in the α-position showed an isotope effect (V_H/V_D) of 1.85, while the compound having a hydrogen in the α-position and deuterium atoms in the β- and γ-positions showed an isotope effect (V_H/V_D) of 1.26. The latter isotope effect was explained in terms of a secondary isotope effect where the deuterium atoms of the molecule decrease the rate of α-hydrogen elimination, the rate-limiting step of the reaction. The data indicated that the bond to the α-carbon and not the bond to the β-carbon is broken during transamination.

L-Deuteriophenylalanine was isolated from the ionic fraction of the cell wall hydrolysate of algae grown in a medium containing 99.6% D_2O (158). The transamination rate of the protio and deuterio analogs to the corresponding analogs of phenylpyruvic acid was compared by using rat brain extract as the enzyme system. The deuterium isotope effect (V_H/V_D) was found to be 1.64.

Partially (37%) deuterated DL-phenylalanine was used to study the metabolic pathways by which phenylalanine is converted to tyrosine (159). A normal patient, a patient with phenylketonuria, and a patient with hyperphenylalaninemia were administered doses of 200 mg/kg of deuterated phenylalanine, and the deuterium content of the excreted aromatic acids was determined. In the healthy subject, the *m*-hydroxyphenylacetic acid produced by metabolism was markedly deuterated, but this was not the case in the patient suffering from phenylketonuria. Thus, *m*-hydroxylation of phenylacetic acid in this disease appears to be blocked. This was also true for the hyperphenylalaninemic patient. No deuterium was incorporated into the metabolites of tyrosine by patients with phenylketonuria and hyperphenylalaninemia. That mandelic acid is a product of phenylalanine metabolism was confirmed in both the healthy as well as the phenylketonuric patient. It was suggested that another metabolic pathway for phenylalanine degradation may exist in patients with hyperphenylalaninemia. Some reservation was expressed about the conclusions arrived at since DL-phenylalanine was used in these studies rather than the naturally occurring L-form.

Methionine containing a deuteriomethyl group was used to study the mechanism of transmethylation in biological systems (160, 161). It was shown clearly that the methyl group is transferred from methionine to choline and creatine in animals. It was further established that the hydrogen atoms of the methyl groups do not exchange with the hydrogen atoms of the body water and that the methyl group is transferred as a unit in the transmethylation process.

Triggle and Moran (162) investigated the kinetic isotope effect involved in the rate of oxidation of α-deuteriophenylglycine and α-deuteriotyrosine by L-aminooxidase. The synthetic procedures yielded 95% deuterium labeling in the α-position. An isotope effect (k_H/k_D) of 1.41 was reported. On the basis of the isotope effect, it was clearly established that removal of an α-hydrogen is rate determining in the enzymatic oxidation of tyrosine and phenylglycine.

Rhodes *et al.* (163) used L-deuterioglutamic acid and L-deuterioaspartic acid, isolated from the ionic fraction of the hydrolysate of deuterated algae, as the starting compounds in the synthesis of 2,6-dioxo-3-phthalimidopiperidine-3,4,4,5,5-d_5 and 2,5-dioxo-3-phthalimidopyrrolidine-3,4,4-d_3. Inasmuch as these compounds are partially deuterated forms of thalidomide and *N*-phthalylaspartimide, respectively, and because both substances elicit sedative and teratogenic activity, the effect of deuterium substitution on these activities can be evaluated. Such studies may provide information about the mechanism by which these agents produce their teratogenic effects.

SYMPATHOMIMETIC AMINES

Deuterium oxide had sympathomimetic activity when substantial amounts of it replaced the body water of mice (164, 165). At the 20% saturation level, the animals showed definite signs of sympathomimetic stimulation including exophthalmos and the pilomotor reaction. It was suggested that deuterium oxide protected the natural sympathetic hormones

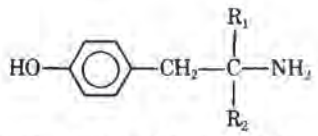
by delaying their disappearance from the body.

Fully deuterated *dl*-epinephrine was synthesized in which 90% of the hydrogen atoms were substituted with deuterium atoms (166). Injection into a cat produced an elevation in blood pressure indistinguishable from that resulting from ordinary epinephrine. It was concluded that deuteration caused little, if any, change in the physiological activity of epinephrine.

The effect of deuterium substitution in certain sympathomimetic amines on their interaction with the enzyme monoamine oxidase was studied (167–169). (The catecholamines, such as epinephrine and norepinephrine, are apparently inactivated primarily through *O*-methylation rather than through attack by monoamine oxidase.) Tyramine was synthesized in which the α-hydrogens were replaced with deuterium atoms. Since tyramine is a good substrate for monoamine oxidase, the rate of oxidation by the enzyme should be decreased in view of the fact that C—D bond cleavage is involved in the reaction. An isotope effect (k_H/k_D) of 2.3 for the oxidation was observed. The magnitude of the pressor response, as noted in cats given barbiturates, was about the same for both compounds, but the duration of effect (pressor area) was twice as prolonged for the dideuterio compound. The magnitude and duration of nictitating membrane contractions were likewise twice as large for the deuterated derivative. No difference in pressor response was noted when *l*-α-dideuterionorepinephrine was compared with *l*-norepinephrine, indicating that at the effector cell level norepinephrine is probably not attacked by monoamine oxidase.

The stereospecific nature of the observed isotope effects was also studied. On the basis of a three-point contact between substrate and enzyme or receptor site, only one enantiomeric form of α-monodeuteriotyramine (V) should be responsible for the isotope effect noted with dideuteriotyramine. Both optical isomers were prepared by enzymic decarboxylation of tyrosine. *In vitro* studies with liver monoamine oxidase showed one optical isomer to be 2.3 times as reactive as the other, a ratio similar to that of the isotope effect noted when the dideuterio compound was compared to the protio form. This clearly demonstrates that the two α-hydrogens in tyramine are not equivalent for the enzyme, and that the observed results are explainable in terms of a three-point contact between substrate and enzyme. The stereospecificity of the isotope effect was also demonstrated from the effects on arterial pressure and nictitating membrane contraction. Belleau *et al.* (167–169) claimed these studies to be the first pharmacological ones in which conclusions about mechanism were based on kinetic isotope effects. These studies have indeed provided significant information on certain aspects of the mechanism of adrenergic activity not readily attainable through more conventional methods.

The effect of deuterium substitution at the carbon alpha to the amine group in the substrate kynuramine on the kinetics of oxidation by monoamine oxidase was examined (169, 170). A primary isotope effect of 2.1 (k_H/k_D) was obtained, which is comparable to the isotope effect reported for the corresponding



V: $R_1 = H$, $R_2 = D$; $R_1 = D$, $R_2 = H$

tyramine compounds as previously noted. Also studied was the effect of deuterium atoms at the carbon beta to the amine group in kynuramine. In this case there was a reduction in the rate of oxidation by monoamine oxidase, with a secondary deuterium isotope effect of 1.18. The ratio of the Michaelis constants for α-d_2-kynuramine to kynuramine (K_m^D/K_m^H) was 3.0, while the Michaelis constant for the β-d_2-kynuramine was identical to that for kynuramine. The α-d_2 compound apparently forms a weaker complex with monoamine oxidase, whereas the β-d_2 compound is bound by the enzyme to about the same extent as kynuramine.

The deuterium isotope effect on the enzymatic deamination of *l*-amphetamine was studied (171). This process appears to be the major metabolic pathway for amphetamine in rabbits, dogs, and humans. Oxidative deamination studies were performed *in vitro* on *l*-amphetamine containing a deuterium on the α-carbon atom by following the substrate concentration in the enzymatic reaction with microsomal enzymes in a rabbit liver homogenate. Relative rates of metabolism of the protio and deuterio forms of amphetamine were determined by simultaneous incubations under identical conditions. The ratio of apparent rate constants (k_H/k_D) based on initial velocities was found to be 2.0 ± 0.3. Since cleavage of a carbon—hydrogen bond is involved in the deamination process and since this step of the reaction is rate limiting, a significant deuterium isotope effect was not unexpected.

Deuterium isotope effects on the metabolism of amphetamine and *N*-substituted amphetamines were studied in humans (172, 173). When the excretion rate of α-deuterio-*d*-amphetamine was compared with that of its protio analog, a small, but not quantitatively determined, deuterium isotope effect was observed. Larger isotope effects were noted in the *dextro*-isomers of the *N*-alkyl-substituted amphetamines than in the *levo*-forms.

Deuterium-labeled amphetamine, *p*-methoxyamphetamine, and α-methyltyramine were synthesized primarily for use as internal standards in GC–mass spectrophotometric procedures for the analysis of amphetamine and its metabolites (174). These compounds were variously labeled with deuterium in the alkyl side chain attached to the phenyl group. Deuterium-labeled phentermine (2-methyl-1-phenylisopropylamine) was synthesized in which the 2-methyl group was fully deuterated (175). The labeled compound was prepared specifically for use in comparative metabolic studies with amphetamine, as suggested in the previous paper. Further reference to the metabolic studies will be made in the section on *Bioanalytical Applications.*

MISCELLANEOUS DEUTERATED COMPOUNDS OF BIOLOGICAL IMPORTANCE

A number of deuterated drugs and related products will be considered here since they do not fit appropriately into any of the categories of drugs already covered.

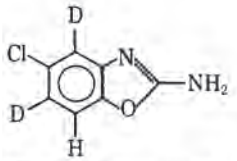
In the section on amino acids, it was noted that some amino acids and monosaccharides were isolated from the hydrolysate of the algae *Scenedesmus obliquus* grown in a medium containing 99.6% D_2O (142). The sugars were fractionated from the nonionic fraction of the hydrolysate on a cellulose chromatographic column. Fully deuterated mannose and glucose were isolated and purified in greater than gram amounts, and some of their properties were studied. These sugars have been incorporated into deuterated nutrient media used for the culturing of organisms that elaborate natural products of therapeutic value. The sugars have been used in mechanistic studies of the biosynthesis of highly deuterated drugs including penicillin (117), griseofulvin (107), clavine alkaloids (127), and riboflavin (176).

Li *et al.* (177) studied the primary and secondary deuterium isotope effects on the rate of mutarotation of fully deuterated glucose and mannose. The decrease in the rate of mutarotation observed in deuterium oxide was attributed to a primary isotope effect arising from the breaking and reforming of O—H and O—D bonds, and a secondary isotope effect was attributed to the C—D bonds in the molecule that were responsible for a further decrease in the rate of mutarotation.

GLC was used to separate fully deuterated from protio carbohydrates (178). Complete separation of glucose and glucose-d_7 was reported. Other carbohydrate mixtures were only partially separated.

A number of fully deuterated fatty acids from *S. obliquus* cultured in heavy water were isolated and characterized (179). The fatty acid composition of the total lipids and of individual lipid classes was determined. The fatty acids, as their methyl esters, were characterized by GLC and mass, IR, and ^{1}HMR spectroscopy. Rohwedder *et al.* (180) determined the composition of the fatty acid fraction obtained from the deuterated algae *S. obliquus* and assigned the IR spectra of the fatty acids. McCloskey *et al.* (181) studied the GC and mass spectrometric properties of fully deuterated fatty acids obtained from the same deuterated algae source.

Zoxazolamine, 2-amino-5-chlorobenzoxazole, is a muscle relaxant metabolized by ring hydroxylation at C-6 to yield 2-amino-5-chloro-6-hydroxybenzoxazole. Dideuteriozoxazolamine-4,6-d_2 (VI) was synthesized, and the ^{1}HMR spectral data were used to establish the exact structure of the compound (182). From *in vivo* studies, it was concluded that there was no isotope effect on the duration of paralysis in rats after drug administration, since the behavior of ordinary and dideuteriozoxazolamine was not significantly different. *In vitro* metabolic studies were conducted with liver microsomes. The deuterated compound was metabolized more slowly than the protio compound (k_H/k_D = 1.4). The isotope effect may be due to a lower affinity of the deuterated compound for the degradative enzyme or to a slower cleavage rate of the C—D bond compared to the C—H bond. No explanation for the absence of a difference in the duration of paralysis between the two forms of zoxazolamine was advanced despite a difference in the *in vitro* degradation rates by the liver microsomes.

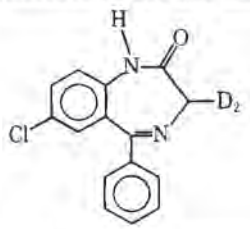


VI: zoxazolamine-4,6-d_2

O-Demethylation is an oxidative reaction catalyzed by the liver microsomes in drug detoxification. The effect produced on the enzymic demethylation of *o*-nitroanisole when the hydrogens of the O—CH_3 group were replaced with deuterium atoms was studied (183). Because the rupture of carbon—hydrogen bonds is involved in the demethylation process, a deuterium isotope effect should be evident if this step in the reaction is rate limiting. On the basis of the rate of formation of the reaction products, a deuterium isotope effect (k_H/k_D) of about 2 was noted. This primary isotope effect could also account for a lowered Michaelis–Menten constant, and a $K_m{}^D/K_m{}^H$ of 0.4 was indeed observed. However, considering the complexity of the enzyme system (liver microsomes), a straightforward interpretation of the change in K_m is not possible.

Marcucci *et al.* (184) investigated the effect of deuteration on the anticonvulsant activity of *N*-demethyldiazepam (7-chloro-1*H*-5-phenyl-1,4-benzodiazepin-2-one), a major metabolite of diazepam with pharmacological properties similar to the parent compound. *In vivo*, *N*-demethyldiazepam is hydroxylated in the C-3 position to form oxazepam, which accumulates in the brain and is responsible for the anticonvulsant activity of the parent compound. *In vitro*, oxidation to the hydroxyl derivative is effected by the liver microsomal system.

Deuterated *N*-demethyldiazepam (VII) was synthesized in which two deuterium atoms were introduced into the C-3 position. If cleavage of the carbon—hydrogen bond in the oxidative process is rate limiting, then deuteration would be expected to affect the metabolism and pharmacological activity in mice. *In vitro* studies revealed that the deuterated form oxidized at a slower rate to form oxazepam than did the protio form of *N*-demethyldiazepam. *In vivo* studies in mice indicated that the anticonvulsant ac-



VII: *N*-demethyldiazepam-d_2

tivity was reduced from 20 to 5 hr for the deuterated compound and that the ED_{50} was increased from 198 μg/kg for the protio compound to 288 μg/kg for the deuterated analog. The decreased activity of the deuterated compound is apparently due to a decreased accumulation of the hydroxylated derivative, oxazepam, in the brain of the mice because of the reduced rate of oxidation of the parent deuterated compound. It was concluded that decreased oxazepam formation in the case of deuterated *N*-demethyldiazepam may arise from one of the following causes: (*a*) a reduced rate of cleavage of the carbon—deuterium bond, (*b*) a lower affinity for the active enzyme site, or (*c*) the formation of a more stable complex with the enzyme.

Hydroxylating enzymes present in liver microsomes play an important role in the detoxification of foreign organic compounds. In the conversion of acetanilide to *p*-hydroxyacetanilide by rabbit liver microsomes, molecular oxygen was incorporated by the microsomes into the hydroxyl group of *p*-hydroxyacetanilide (185). The rate of hydroxylation of pentadeuterioacetanilide was compared to that of ordinary acetanilide to determine whether the breaking of a carbon—hydrogen bond was rate limiting in the microsomal hydroxylation of acetanilide (186). The ratio, k_H/k_D, turned out to be unity, indicating the absence of any deuterium isotope effect and suggesting that cleavage of the carbon—hydrogen bond is not rate limiting. The slow step in the reaction may instead be the incorporation of molecular oxygen in the *para*-position or possibly the fixation of oxygen by the enzyme.

Hydroxylation of *p*-deuterioacetanilide by the hydrolase enzymes in rabbit liver microsomes formed *m*-deuterio-*p*-hydroxyacetanilide (187). The enzyme-catalyzed aromatic hydroxylation appears to be accompanied by a stepwise migration of the group (deuterium in this instance) displaced by the hydroxyl. While a mechanism for the reaction was postulated, possible kinetic isotope effects were not considered.

BIOANALYTICAL APPLICATIONS

Methods—Stable isotopes, particularly deuterium, are proving useful as tracers for drug distribution and metabolism studies. The many advantages of stable isotopes for these purposes include the absence of possible radiation hazards, especially important where children and pregnant women are concerned. Simpler procedures and precautions are required during the synthesis and the general handling of compounds enriched with stable isotopes. In addition to deuterium, ^{13}C, ^{15}N, and ^{18}O are useful stable isotopes for such studies. Deuterium and ^{13}C offer the additional advantage over radioactive tritium and ^{14}C of smaller mass differences as compared to the more abundant natural isotopic form. A smaller mass difference leads in general to a smaller kinetic isotope effect and therefore to a smaller perturbation in the data obtained from tracer experiments. In the case of nitrogen and oxygen, of course, there are no radioactive species of sufficiently long half-life for use as tracers or labels.

A major deterrent to more extensive use of stable isotopes for tracer studies has been the lack of suitable instrumentation that provides for simple, sensitive, specific, and reasonably inexpensive methods of quantitation. These considerations are particularly important where low concentrations of labeled molecules are involved. For radioactive labels, of course, there are convenient, inexpensive, and sensitive methods for detection of emitted radiation; these methods permit detection of labeled drugs or metabolites at the picogram concentration level. Because of concern over possible radiation hazards, however, radioactive isotopes are generally not used for *in vivo* metabolic studies. Radiometric procedures also lack specificity, because the isotope content in a sample is usually measured without regard to the way in which it is incorporated.

The use of stable isotopes for the quantitative determination of compounds can be based on an isotope dilution analysis technique, which is more commonly used with radioactive isotopes. Stable isotopes were first used in this manner by Rittenberg and Foster (188) who employed ^{15}N-labeled amino acids for the determination of glycine, glutamic acid, and aspartic acid in fibrin hydrolysates and deuterium-labeled fatty acid for the determination of palmitic acid in rat fat. Isotope ratios of the isolated and purified compounds were determined by mass spectrographic analysis after combustion.

The development of GC–mass spectrometry–alternating voltage acceleration instrumentation has greatly simplified this procedure and has made possible the application of stable isotope dilution analysis to intact molecules. The need for isolation, purification, and combustion has been largely eliminated. Numerous applications of this technique to the determination of drugs and their metabolites have appeared in the literature in recent years. Some of these applications have already been mentioned, and additional ones will be noted briefly here.

The mass spectrometer is perhaps the most suitable instrument for the detection of stable isotopes. However, complicated separation and purification procedures are usually required for mass spectrometric analysis, and poor yields frequently result. A technique has been developed which combines the high resolving power of GC and the sensitivity and specific ion-detecting ability of the mass spectrometer, thus simplifying considerably the problems encountered previously with mass spectral analysis. GC–mass spectrometry has found wide application in the qualitative identification of drugs (189), especially where small quantities of drugs and metabolites may be involved. The use of stable isotopes in clinical pharmacology, particularly as tracers for drug distribution and metabolism studies, were reviewed (32).

A modification of the GC–mass spectrometry technique, referred to as "mass fragmentography," was introduced in 1968 (190) for the identification of chlorpromazine and some of its metabolites in human blood. The use of a multiple-ion detector accessory

with a gas chromatograph–mass spectrometer permits the mass spectrometer to serve as a sensitive, selective, and molecule-specific GC detector. Molecular species in the picogram range have been detected with this procedure. Specific ions and fragments formed by the compound of interest that elute from the gas chromatograph and enter the mass spectrometer can be conveniently monitored. The mass spectrometer detector selectively monitors only compounds yielding fragments at preselected mass numbers, so the complete mass spectrum need not be scanned.

The use of an alternating voltage accelerator was introduced into the mass spectrometer system, permitting continuous monitoring of the intensity of several ions or fragments (191). Thus, the composition of certain unresolved or partially resolved mixtures in GC effluents can be determined. Two mass numbers were used in determining the composition of a mixture of glucose and glucose-d_7 that was rendered volatile by formation of the penta-*O*-trimethylsilyl derivatives. Samples as small as 0.1 μg could be conveniently analyzed. Mixtures of epiandrosterone and dehydroepiandrosterone were determined with as little as 20 ng of the compounds.

Hammar *et al.* (190) were apparently the first to apply mass fragmentography to the characterization of drugs and their metabolites. They pointed out the extensive possibilities offered, particularly since modern drugs generally have known structures and synthetic analogs containing stable isotope labels are readily available. When mass numbers are employed for continuous monitoring, the peak areas obtained are proportional to the abundance of the fragment in question and are therefore applicable for quantitation. This system of analysis is generally referred to as GC–mass spectrometry–alternating voltage acceleration, and instrumentation with this capability is commercially available.

Several variations in the labeling techniques are utilized. In drug metabolism studies, labeled drug may be added prior to extraction, derivatization, and analysis. This technique of adding a "carrier" compound prior to GC corrects for error introduced as a result of processing tissue samples. This procedure requires the addition of a relatively large quantity of a carrier compound that possesses chemical and physical properties similar to the compound of interest.

Deuterated analogs are ideal carriers. Deuterium is the least expensive of the commonly used stable isotopes and is available in the widest variety of compounds; it is readily introduced into compounds by the usual synthetic methods. Gaffney *et al.* (192) described the use of "internal standards" labeled with stable isotopes that correct for losses of the compound under study during the GLC treatment of the sample. With the mass spectrometer as the detector for the GLC effluent, chemical differences between the compound being determined and the internal standard are not necessary, because mass differences alone are sufficient to discriminate between the compound and the standard.

The ideal internal standard is the actual compound under investigation modified to contain an increased mass by the introduction of a stable isotope in appropriate molecular positions. Quantitation depends on a comparison of the response of the mass spectrometer at two masses, one mass characteristic of the internal standard and the other characteristic of the substance being analyzed. Cho *et al.* (193) noted that since internal standards are isotopic variants of the compound being studied, fragmentation patterns and retention times will be essentially identical.

It is desirable for the standard to have more than one labeled atom. The masses of the fragment ions selected for measurements could therefore differ by perhaps 2 or 3 mass units. Knapp *et al.* (194) developed the technique of using stable isotopes for introducing visually conspicuous mass spectral "isotope clusters" into drug molecules as labels or markers. They prepared trideuterium-labeled nortriptyline, in which the three hydrogen atoms attached to the *N*-methyl group were replaced with deuterium atoms. An M, M + 3 doublet was created by an equimolar mixture of the labeled and nonlabeled drug, and this mixture was used for following the fate of the drug and its metabolic products. The drug and any of its metabolites retaining the labeled moiety showed an M, M + 3 doublet in the mass spectrum for the molecular ion and any fragment ions containing the labeled moiety. The concept of multiple deuterium labeling has been applied in sequence analysis of proteins by mass spectrometry (195, 196).

Applications Involving Deuterated Drugs—Chlorpromazine and several of its metabolites have been identified in human blood (190). Desmethylchlorpromazine and didesmethylchlorpromazine have been identified in plasma and in red blood cells after treatment with β-glucuronidase. 2-Chlorophenothiazinylpropionic acid was identified in plasma after treatment with β-glucuronidase.

The metabolic pathways of deuterated tyrosine and leucine were studied in human subjects (197). Healthy controls and patients suffering from certain metabolic disorders were administered oral doses of 3,5-dideuterio-*l*-tyrosine and *dl*-deuterioleucine (apparently a trideuterio-derivative). The urine was collected and examined for amino acid metabolites using GC–mass spectrometry. In three mentally retarded patients, high concentrations of deuterated hippuric acid and benzoic acid were detected after orally loading the patients with deuterated tyrosine. No isotopic labeling of either acid was found in normal subjects after loading with deuteriotyrosine.

Deuterated 3-hydroxyisovaleric acid was detected in a patient with suspected "sweaty feet syndrome" after oral administration of deuterated leucine. This compound is excreted in increased amounts in patients with the metabolic defect involving leucine degradation (β-hydroxyisovalericacidemia). The significance of stable isotope labeling and GC–mass spectrometry detection technique in studying metabolic pathways *in vivo* was emphasized, particularly in patients with metabolic defects.

Three variously deuterium-substituted amphetamines were synthesized and evaluated for use as internal standards in the GC–mass spectrometry assay for the quantitative determination of amphetamine in biological fluids (193). Because of their desirable GLC and mass spectral properties, the trifluoroacetic acid amide derivatives were used.

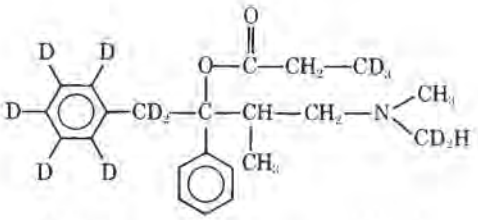
A procedure based on GC–mass spectrometry was developed for the quantitative determination of prostaglandin E_1 (PGE_1) as the methyl ester and *O*-methyl oxime (methoxime) derivative (198). The same derivative was used as a deuterated carrier, except that the methyl of the methoxime contained three deuterium atoms. The ions at *m/e* 470 and 473, produced by electron impact on the effluent, were monitored. The method was sensitive at the nanogram level.

Axen *et al.* (199) reported the GC–mass spectrometry determination of prostaglandins E_2 and $F_{2\alpha}$, which permitted the determination of picomole amounts. The corresponding tetradeuterio (3,3,4,5-d_4) derivatives were synthesized and served as deuterium-labeled carriers. This method is sufficiently sensitive and specific to be useful for the analysis of plasma prostaglandins and for studies involving enzymatic synthesis of prostaglandins *in vitro* from endogenous precursors.

The simultaneous microestimation of choline and acetylcholine was reported using GC–mass spectrometry (200, 201). The corresponding compounds containing nine deuterium atoms in the *N*-methyl groups served as internal standards and carriers. This procedure permitted the quantitative determination of 1 pmole or less of compound.

A very sensitive and specific assay for 5-hydroxyindole-3-acetic acid in human cerebrospinal fluid was reported based on GC–mass spectrometry analysis of the diheptafluorobutyryl methyl ester derivative (202). The two major ions (*m/e* 538 and 597) of the mass spectrum were monitored after elution from the chromatographic column. The dideuterium-labeled 5-hydroxyindole-3-acetic acid was synthesized and served as an internal standard for the quantitation of 5-hydroxyindole-3-acetic acid in cerebrospinal fluid. An analysis required less than 2 min and permitted an accurate determination of 5-hydroxyindole-3-acetic acid in the range of 2–50 ng/ml of cerebrospinal fluid when 2 ml of cerebrospinal fluid was taken for analysis. In the 8–20-ng/ml range, the standard deviation for the method was less than 7%; it was 1–2% at higher levels of 5-hydroxyindole-3-acetic acid.

Deuterated nortriptyline was used as an internal standard for unlabeled nortriptyline (192, 194). In the synthesis of the labeled molecule, two deuterium atoms were introduced into the side chain as $-CHCH_2CD_2NHCH_3$ (the M + 2 amu compound). The trifluoroacetate derivative was prepared by treatment with trifluoroacetic anhydride. Equimolar mixtures of the nortriptylines at the 50–100-pg concentration level were used for producing mass fragmentograms of nortriptyline trifluoroacetate (M = 232) and nortriptyline trifluoroacetate-d_2 (M = 234). Mass fragmentograms of extracts of human plasma samples containing nortriptyline and its deuterated



VIII: propoxyphene (variously labeled)

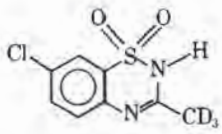
analog were also obtained. Elution and resolution of the two compounds were quite satisfactory.

The stable isotope labeling technique was used to identify the metabolites of nortriptyline in human urine and bile (203). The trideuterium derivative was prepared in which the three deuterium atoms were located in the *N*-methyl position. A single 25-mg oral dose containing an equimolar mixture of the deuterated and unlabeled nortriptylines was administered to adult humans. By mixing stable isotope-labeled and unlabeled drugs, an artificially created isotopic doublet (M, M + 3) was produced, which served as a conspicuous spectral marker for identifying drug metabolites in urine and bile. Urine extracts treated with trifluoroacetic anhydride were examined by GC–mass spectrometry, and the parent drug and any metabolites retaining the labeled moiety were readily identified by the presence of the doublet. Mass fragmentography was successfully employed where metabolites were present in quantities insufficient for ordinary mass spectral scanning.

On the basis of the essentially equal intensity of the artificially created doublet, the following were identified in urine: unchanged nortriptyline and the desmethyl, 10-hydroxy, and 10-hydroxydesmethyl derivatives of nortriptyline. The same compounds were also identified in human bile in the same relative amounts as found in urine. The advantages and further possibilities of the mass fragmentography technique in drug metabolism studies were emphasized. The small dose requirement and the absence of radiation hazards associated with the use of radioactive isotopes make stable isotope labeling particularly attractive for studies involving children and pregnant women.

Clarke and Foltz (204) used morphine, in which the *N*-methyl group contained three deuterium atoms, as a carrier and as an internal standard for determining low morphine levels in human urine by the reverse isotope dilution technique. The deuterated morphine exhibits mass spectral fragments 3 amu higher than those for morphine. The labeled morphine was added to the urine prior to the extraction process, and the morphine content of the urine sample was determined from the ratio of ion current peaks corresponding to the nonlabeled morphine and the added carrier-labeled morphine. The method was sensitive to the nanogram concentration level.

The biotransformation of propoxyphene in humans was studied (205, 206) using variously deuterium labeled forms (VIII) of the drug: (*a*) propoxyphene-d_2, in which one *N*-methyl group was replaced by the $N—CD_2H$ group; (*b*) propoxyphene-d_3, in which the methyl group of the propionyl func-



IX: diazoxide-d_3

tion was replaced by CD_3; and (*c*) propoxyphene-d_7, in which all the hydrogens of the benzyl group were replaced with deuterium. In this study, mixtures of the unlabeled drug and the deuterium-labeled form were used to create isotope clusters in the mass fragmentation patterns. These clusters served as tags for identifying the parent drug and drug metabolites in complex mixtures containing endogenous components such as tissues and body fluids.

The labeled drug was administered orally to human subjects as a 1:1 mixture with unlabeled drug. Urine samples were extracted and the extracts were analyzed by GC–mass spectrometry. Drug metabolites in the mixture were detected by analyzing the fragmentation pattern of each component for the presence of M, M + 2; M, M + 3; and M, M + 7 doublets. Analysis was also performed on the fragmentation patterns obtained from urine extracts of subjects administered only unlabeled or labeled drug. In addition to unmetabolized drug, the following metabolites were identified: norpropoxyphene, dinorpropoxyphene, propoxyphene carbinol, norpropoxyphene carbinol, dinorpropoxyphene carbinol, hydroxypropoxyphene, hydroxynorpropoxyphene, and 6-phenyl-6-benzyl-2-ethyl-5,6-dihydro-5-methyl-4*H*-1,3-oxazine.

The concentration of *N,N*-dimethyltryptamine in human plasma was estimated by stable isotope dilution analysis (207). *N,N*-Dimethyltryptamine is a psychotomimetic agent which, along with related methylated indoleamines, has been identified in the blood and urine of schizophrenics. However, a causative role in mental disease has not been established. Dideuterio-*N,N*-dimethyltryptamine was synthesized in which the two deuterium atoms were located on the carbon alpha to the indole ring. Dideuterio-*N,N*-dimethyltryptamine served as a carrier and was added to the plasma at the beginning of the isolation procedure. It also served as an internal standard in the quantitation step of the GC–mass spectrometry analysis for *N,N*-dimethyltryptamine in the isolate. The sensitivity limit was 0.5 ± 0.2 ng/ml of plasma.

A stable isotope dilution and mass fragmentography assay was used in pharmacokinetic studies with diazoxide, an antihypertensive and hyperglycemic agent (208). Diazoxide-d_3 (IX) was synthesized, in which the three deuterium atoms were located in the methyl group attached to the ring system. This compound served as a carrier and internal standard for determining plasma concentrations of the drug. The sensitivity of the procedure was reported to be about 1 ng/sample. The plasma half-life of the drug ranged between 20 and 53 hr in four adult patients.

Hofmann *et al.* (209) synthesized deuterium-substituted bile acids. A stable isotope dilution technique was developed for determining the bile acid pool size which required only 10 mg of deuterium-labeled bile acid. In patients with cholesterol gallstones, the gallbladder was found to be supersaturated with cholesterol while the amount of circulating bile acids was generally lower than normal. This finding was confirmed in 60 patients with gallstones.

CONCLUSION

An impressive number of drugs have been synthesized or biosynthesized that are variously substituted with deuterium; while they have not found application in drug therapy, they are being used to study the mechanism of drug action, to elucidate metabolic and biosynthetic pathways, and to study kinetic isotopic effects. Deuterated drugs labeled in specific molecular positions are proving invaluable in drug distribution and metabolism studies. Mass fragmentography makes good use of deuterated drugs for the detection of specific ions and molecular fragments representative of metabolic products present in the body fluids at extremely low concentrations.

Heavy water is employed as a routine diagnostic agent for measuring total body water and shows promise as a contrast medium in certain biomedical applications of neutron radiography. Some studies have indicated that heavy water inhibits tumor growth but, unfortunately, the concentrations required are not well tolerated by the host.

The toxicological effects resulting from extensive deuteration in mice, rats, and dogs have been described. In intact mammals the magnitude of deuterium toxicity appears mainly to be independent of the size of the animal. It appears that up to 15% deuterium replacement can be tolerated by mammals, but severe toxic effects and even death result when the level exceeds 30%. The physiological effects of deuteration on numerous specific organs and body functions have been thoroughly studied, including the effects on liver activity, kidney function, blood constituents, enzyme systems, reproductive capacity and cell division, the heart, the nervous system, metabolism, and even the wound-healing process.

A variety of unicellular microorganisms has been cultured in a fully deuterated environment. However, higher plants show a graded response to ^2H_2O, with 60–70% the maximum level tolerated in the nutrient medium. A primary effect of deuteration appears to be suppression of the production of such important natural products as alkaloids and antibiotics. However, the problem has been circumvented to some extent by using replacement culture techniques.

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