If stored in the refrigerator under nitrogen and opened only occasionally to remove samples and subsequently blanketed with nitrogen, no decomposition is observed after several months.

Typical analyses of solid crystalline quingestrone after 5 months' storage are tabulated in Table III.

In solution, quingestrone is stable in the absence of acids, bases, and oxidizing agents, including atmospheric oxygen. It is less stable in chloroform and methanol than in heptane and exhibits best stability in a good grade vegetable oil such as sesame oil. In Table IV, stability data in solvents stored under the indicated conditions are presented. No precaution was taken to exclude air in these studies.

The pharmaceutical dosage form consists of quingestrone dissolved in sesame oil at concentrations of 50, 40, and 25 mg./ml. contained in sealed glass ampuls and/or in sealed, soft gelatin capsules. It is of the utmost importance that the peroxide content of the sesame oil be kept minimal, *i.e.*, less than 50 mcq per ml. as determined by the method of the American Oil Chemists Society (15). As a further precaution, an antioxidant, namely α-tocopherol is added at a concentration of 0.1% of the weight of sesame oil used.

In Table V, stability data are presented for this final dosage form.

SUMMARY

A quantitative paper chromatographic method has been developed which not only permits the determination of quingestrone, but also simultaneously provides a precise method for assaying as little as 1% of the known decomposition products, progesterone and 6-β-hydroxyprogesterone.

Data have been presented to indicate that a solution of the cyclopentyl enol ether of progesterone (quingestrone) in sesame oil of low peroxide content with addition of α-tocopherol and exclusion of atmospheric oxygen represents the best means for preserving the product in a pharmaceutical dosage form.

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Effect of Deuterium Oxide on the Growth of Peppermint (*Mentha piperita* L.) I

Morphological Study

By M. I. BLAKE, F. A. CRANE, R. A. UPHAUS, and J. J. KATZ

The effect of varying concentrations of D_2O on the growth of peppermint plants was studied over a period of at least 50 days. The extent of deuterium uptake is reported. 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.

THE EFFECTS of deuterium on living organisms have attracted the attention of many investigators since this heavy, stable isotope of hydrogen was first discovered in 1933. Early work has been reviewed by Morowitz and Brown (1). Current developments are described by Katz (2) and are the subject of a recent monograph (3). Interest in this general area has been stimulated by the discovery that a considerable variety of organisms can be grown in 99.8% D_2O (4, 5). Since it has proved possible to replace essentially all of the hydrogen in a variety of algae, bacteria, and molds, it becomes of interest to examine the behavior of higher plants in response to isotopic substitution. The effects of deuterium on mice (6), rats (7), and dogs (8) have been reported, but the present paper presents what we believe to be the first detailed study of the effects of extensive replacement of hydrogen by deuterium on the growth of a higher plant.

Since deuterium oxide is now available in adequate quantities and at a moderate cost, it is possible to study the effect of high concentrations of this solvent on the growth of higher plants over an extended period of time. Peppermint plants are ideal for such an investigation since they can

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be conveniently grown from rooted cuttings in an aqueous nutrient solution and require no special attention (9).

The present report describes the effect of varying concentrations of deuterium oxide on the growth of mint plants for a period of at least 50 days, at which time the control plants initiated inflorescences. The extent of deuterium uptake by the plant, as determined by direct analysis, is also reported.

EXPERIMENTAL

Growth Studies.—Cuttings from fully grown mint plants (*Mentha piperita*) were rooted in moist vermiculite. A root system had developed in about 2 weeks. The stem length ranged from 3 to 6 cm. At this time the plants usually bore four to five pairs of small leaves. No sign of lateral shoots was evident. The plants weighed between 1 and 2 Gm. Erlenmeyer (250 ml.) flasks fitted with cork stoppers served as convenient culture flasks. The plant cutting was inserted into a slit cut from the center to the edge through the length of the stopper and narrow enough to hold the cutting erect, the stem extended above the cork and the root system was directed into the flask. The surface of the cork was covered with masking tape to ensure an air tight system and to minimize isotopic exchange with the water vapor in the air.

A concentrated nutrient stock solution was prepared which required a fourfold dilution. This facilitated the preparation of solutions containing

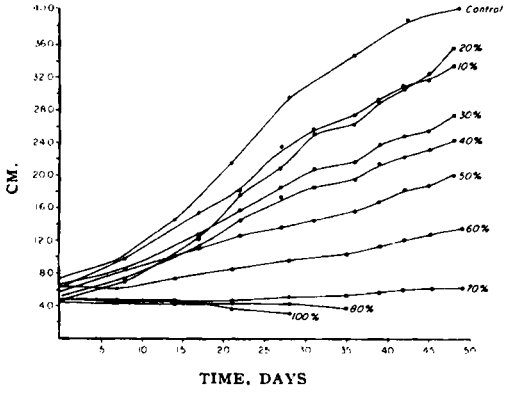

Fig. 1.—Effect of D_2O concentration on growth of stem in peppermint plants.

different concentrations of deuterium oxide. The final nutrient solution had the following composition (Gm./L.): $Ca(NO_3)_2 \cdot 4H_2O$, 1.18; KNO_3, 0.30; KH_2PO_4, 0.14; $MgSO_4 \cdot 7H_2O$, 0.49; $NaNO_3$, 0.60; and FeEDTA, 0.005. The following trace elements were present (p.p.m.): boron; 0.50; manganese, 0.50; zinc, 0.05; copper, 0.01; and molybdenum, 0.01. The deuterium oxide used in this study had an isotopic purity of 99.6% and was distilled from alkaline permanganate in a Barnstead conductivity water apparatus.

Nutrient solution for these studies was prepared by transferring 62.5 ml. of concentrate (1:4) from a buret to a 250-ml. volumetric flask. The volume of deuterium oxide calculated to give the desired D_2O concentration was added from a second buret to the volumetric flask. The flask was then filled to the mark with distilled water. For those experiments in which the deuterium oxide concentration was 80% or above, 4:1 nutrient concentrate was prepared in deuterium oxide. Sufficient water and deuterium oxide were added to give the desired final deuterium oxide concentration. The final nutrient solution was transferred to the culture flask and the cork stopper with plant attached was placed in position. The entire flask was covered tightly with black plastic sheeting to prevent growth of algae in the solution. A 1-ml. sample of nutrient solution was removed periodically, prior to the addition of fresh nutrient. This was done each 7 days in the early stages of plant growth and every 3 or 4 days as the plant elongated more rapidly. The sample was analyzed for deuterium oxide by the method described earlier (10). This served to detect whether the deuterium oxide content of the nutrient solution was being reduced by absorption of H_2O from the atmosphere, or whether isotopic exchange had occurred by other means.

A series of culture flasks was prepared which contained 10, 20, 30, 40, 50, 60, 70, 80, and 100% deuterium oxide in the nutrient solution. Controls were run concurrently. When a sample of solution was removed for deuterium analysis and fresh nutrient was added, the following measurements were made on all plants: stem length from top of cork stopper, lateral shoot length from center of stem, root length, width of root system, and volume of fresh nutrient added. The experiment was terminated after 50 days because the controls went into the flowering stage and normal elongation terminated. The effect of varying degrees of deuteriation on the stem length of the mint plants is shown in Fig. 1. Figures 2 and 3 show photographs of the mint plants prior to termination of the experiment.

Harvesting of Plants.—The plants grown in nutrient solutions ranging in D_2O concentration from 0 to 100% were harvested after 50 days. Those plants in 70% D_2O nutrient were grown as long as 130 days before being harvested. Each plant was divided into leaves, stems, and roots; each portion was weighed. Adequate samples of each plant part were placed in glass vials which were sealed tightly and placed in the deep freeze until analyzed for deuterium content. Samples of the plant parts were killed in Randolph's modified Navashin fluid (11). These were reserved for a histological study to be reported later. The remaining portions of

Fig. 2.—Peppermint plants grown in liquid nutrient solutions containing 0 to 50% D_2O. Plant C is the control.

each plant were combined and steam distilled in a Clevenger distillation apparatus. The volatile oil fraction of each plant was collected and set aside for future study.

Deuterium Analysis.—The deuterium content of the nutrient solution, as previously mentioned, was determined throughout the course of the experiment by analyzing a sample of the medium prior to the addition of fresh nutrient. The harvested plants were analyzed for labile and fixed deuterium. In this context, labile deuterium refers to deuterium present as interstitial and cellular water. Fixed deuterium includes deuterium present in both exchangeable and nonexchangeable portions in the organic components of the plant. A vial, containing leaves, stems, and roots of an experimental plant and which was stored in the deep freeze, was transferred immediately to a distilling tube after removing the cap. Care was taken to avoid exchange of labile and exchangeable deuterium atoms with atmospheric H_2O. The distillation tube was attached to a vacuum line and the labile water was removed from the plant under reduced pressure, condensed in a $-78°$ trap, and transferred to an ampul which was then sealed off. The sample was then analyzed for D_2O content. The dried plant residue was removed from the distillation tube and divided into leaves, stems, and roots. Equal weights of each plant part were placed in a combustion boat. The boat was placed in a combustion apparatus and the sample was burned in oxygen at 700°. The water of combustion was collected in a $-78°$ trap and transferred to an ampul which was then sealed off. The sample was analyzed for deuterium oxide

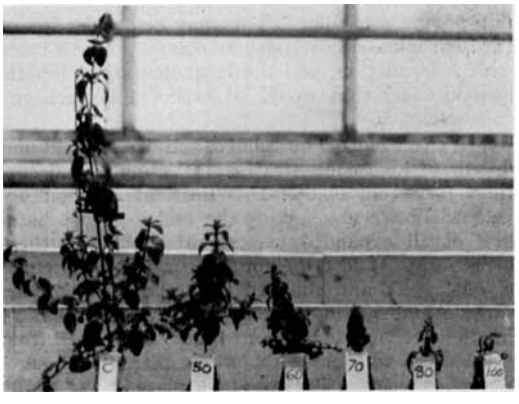

Fig. 3.—Peppermint plants grown in liquid nutrient solutions containing 50 to 100% D_2O.

content. This represents the deuterium in the dry tissues. The data for these analyses are shown in Fig. 4 where the concentration of deuterium in the water and the organic components of the plant are plotted against nutrient D_2O content.

DISCUSSION

The black Mitcham strain of peppermint grown in full nutrient solution by the water culture technique usually develops from a rooted cutting of about 8-cm. length with four pairs of expanded leaves and a weight of about 1.5 Gm., to a mature plant in about 50 days. The mature primary shoot grown on long day photoperiod (14 hours of daylight or longer) generally reaches a height of 35 to 50 cm.; it bears six to eight pairs of long lateral shoots, the lowest of which normally reaches a length approaching that of the primary axis. The leaves, well expanded on the erect stems, elongate to approximately 5 by 8 cm. and are normally bright green.

Elongation of both primary and lateral stems ceases when their apical meristems are stimulated to floral initiation and an inflorescence develops. After flowering occurs, development of secondary lateral shoots takes place at the base of the primary stem (normal ground level of plants grown in soil). These secondary lateral shoots from the same nodes where an erect flowering shoot has arisen are stolons that grow horizontally or pendulous; their leaves remain small and scale-like.

Plants grown on short days (10 hours or less of light photoperiod) do not initiate flowers, are lighter green than long-day plants, and produce many more stolons. When plants are grown for 50 or more days on short photoperiod all of the erect stem apices become stolon-like and even the main apex hangs down and appears as a stolon.

All of the plants described in this work were grown on long-day photoperiod. The natural summer day was extended to 16 hours by a bank of fluorescent and incandescent lamps suspended on an open frame above the plants so that they did not shade during the day, and which provided about 600 f.-c. on the upper parts of the plants during the time the lamps were turned on.

Control plants followed the characteristic growth pattern for peppermint. The major deviation in this experiment from earlier work in this laboratory was that a single plant was grown in a 250-ml. flask, whereas previously several plants (four to eight) were grown in a larger volume of nutrient solution (4 to 12 L.). When plants have 30 or more pairs of leaves they transpire large volumes of water. The nutrient solution had to be replenished or replaced in the 250-ml. Erlenmeyer flasks every 48 to 72 hours in the later phase of the growth cycle. Aside from this problem the peppermint plants grew well in this type of culture flask.

The effect of varying concentrations of deuterium oxide on the elongation of plant is evident in Fig. 1. With the exception of the 20% plant, increasing concentrations of D_2O in the nutrient solution caused a nearly proportionate reduction of elongation growth of the axial shoot. This pattern of elongation was reflected in the absorption of nutrient solution by the roots. The volumes necessary to replenish or replace that taken up for growth or transpiration by the plants were progressively less for each D_2O level. The regular progession of growth shown in Fig. 1 implies limitation of a major factor in the

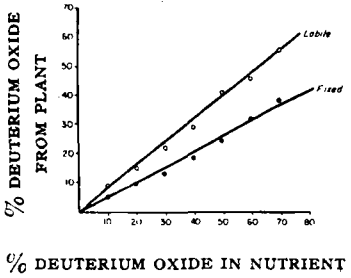

Fig. 4.—Deuterium content of labile water and water of combustion of peppermint plants. Labile, water obtained by distillation; fixed, water obtained by combustion of dried plants.

ability of the plant's primary shoot to elongate under conditions in which rapid elongation did occur in control plants furnished aqueous nutrient solution.

Elongation of the lateral shoots was affected by deuterium in two ways. At lower D_2O concentrations (10 to 40%), the lateral stems grew more nearly like short-day lateral stems. The lateral stems hung recumbent and were elongated more than the corresponding erect lateral branches of the controls. Such stolon-like branches occurred on the controls only as secondary laterals in small numbers after the erect shoots developed inflorescences at their apices. At higher concentrations of D_2O, the pattern of elongation of the lateral shoots was essentially the same as that of the primary shoots. There was a greater reduction in shoot length and greater retardation of leaf development with increase in D_2O concentration. Lateral shoots of the 70% plants were so short that after 50 days of growth they were still difficult to measure. They were overlapped by primary leaves of the main shoot. Both of these growth responses are probably due to the inability of the plants to synthesize adequately their structural, storage, and hormonal materials required for normal growth. The inability of the shoot apices to orient themselves in the erect manner and to stimulate their leaves to elongate to the size of vegetative photosynthesizing leaves reflects a decrease in normal stem orienting hormone. Such lateral shoots, hanging unoriented, elongated to the degree of which they were capable under the constraint of the D_2O concentration on their synthesizing system.

Reduction in elongation of leaf blades and petioles followed the same general pattern as that of the stems. At low D_2O concentrations leaves of the primary shoot elongated nearly as much as the corresponding leaves of the controls. They were held in the normal erect position, were the normal green color, and apparently functioned in a near normal way photosynthetically. At concentrations of 50% D_2O or higher (and in the later phases at lower concentrations), leaves appeared epinastic and recurved downward, clasping the stem or lower leaves, thus making a compact mass that was quite brittle or fragile (Fig. 3, 60 and 70%). The leaves of the plant in 80% D_2O nutrient did not elongate more than a few millimeters, remained epinastic throughout their existence, and eventually became necrotic, brown, and dry.

Fig. 5.—Rooted peppermint cutting after 24 hours in 99.6% D_2O nutrient solution. Brown necrotic areas are outlined in black.

Peppermint commonly develops red or purple coloration under deficiencies of nitrogen, phosphorus, or sulfur. This coloration is due to anthocyanins, water-soluble pigments, which accumulate under conditions in which the normal products of photosynthesis do not continue to build structural or metabolic carbohydrates and proteins. It appears that deuterium oxide interferes with the biochemistry of the plant and exerts its effect on the normal anabolic reactions of the leaf mesophyll by inhibiting the rate of the photosynthetic reactions and ensuing growth. The results are morphological symptoms resembling those of a plant growing under a deficiency of the elements needed for normal growth. Thus, by decreasing the rates in a chain of reactions, the D_2O causes the leaf cells to appear incapable of making normal use of the nitrogen, phosphate, sulfate, and other nutrients that are available to them, even in abundance.

The root system reflected to a lesser degree the same pattern of response to D_2O. At low concentrations the root system was broad, well branched, and nearly as large as that of the controls. Individual roots were turgid, pearl-white, and not noticeably reduced in length. At higher D_2O concentrations the entire amber to brown root system was reduced in extent and appeared shriveled. The ability to absorb water, even from an abundant supply as a liquid nutrient solution, was reduced by D_2O. The total volumes of liquid taken up at higher D_2O concentrations were progressively smaller.

The ability of plants growing on any concentration of D_2O to initiate inflorescence appears to be impaired. Plants were maintained in nutrient solution until the control plants had either initiated inflorescences or had produced full-blown flowers, but in no case were there floral primordia at the apex of any plant grown in D_2O. This seems to parallel the results in animals reported by Hughes and Calvin (12) and Czajka and Finkel (13), whereby substitution of D_2O for a part of the drinking water produced sterility or otherwise affected the reproduction potential in mice. This may be a reflection of the high metabolic activity associated in both cases with reproduction, and the large deuterium kinetic isotope effect that would be expected in such circumstances.

The effect of D_2O on cellular activity is rapid and drastic at high concentrations. Young rooted cuttings placed in 99.6% D_2O nutrient solution developed brown areas along the midrib of the basal half of all expanded leaves. Mesophyll in these areas (Fig. 5) lost its normal turgidity, appeared water-logged, and changed from the normal green to pale amber and then to dark brown within 24 hours. Necrosis of adjacent tissues progressed from these areas to include the whole plant in about 1 week. No elongation ever took place in the plants furnished 99.6% D_2O. Effects on plants furnished 80% D_2O nutrient were similar, but the cycle was accomplished in two to three times the interval of time.

Several plants were maintained at a 70% D_2O level up to 130 days. In these plants the progression of epinastry, necrosis, coloration, and death occurred, as previously described, only after axillary shoots elongated and appeared to grow in a near-normal fashion, but which eventually recapitulated the progression. Then other shoots would continue the sequence and eventually die. This occurred until the apex of the plant became a dense mass of dead primary and lateral shoots. The shoot apex, it would appear, made a valiant effort to achieve the best possible growth it could attain under the constraints of the experimental treatment.

Several cuttings were removed from a plant grown in 50% D_2O nutrient for 40 days. One cutting was transplanted into a culture flask containing 50% D_2O nutrient solution, and the other was placed in a flask containing aqueous nutrient and no D_2O. The former cutting showed no sign of developing a root system and succumbed within 3 to 4 weeks. The latter grew a root system within 2 weeks and continued growing in a normal manner similar to a typical control plant. It was hoped that if a root system would develop in the 50% D_2O medium, it would be possible to propagate a deuteriated plant; and possibly with successive cuttings in constantly increasing concentrations of D_2O a plant could be grown in a highly deuteriated medium. Thus far, this has not been successful, but further work is in progress with this aspect of the investigation.

In Fig. 4 comparison is made between the deuterium content of the water distilled from the deep frozen fresh plants and the water of combustion of the dried plants. The distilled or labile water contains deuterium from water loosely bound by the plant tissues and represents essentially completely exchangeable deuterium. The deuterium content of the labile water from the plant is approximately 75% that of the nutrient solution in which the plant was grown. The water obtained from combustion of the dried plants, representing organically bound exchangeable and nonexchangeable deuterium, contains about 50% of the deuterium content of the nutrient solution. The ratio of organically bound to labile deuterium is about 0.66, somewhat higher than that reported by Katz, *et al.* (6), for certain tissues in mice. This may reflect the nonequilibrium aspects of the situation resulting from the continuous transpiration of water. The active water metabolism and the ensuing fractionation may contribute significantly to the observed isotope effects.

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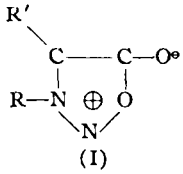
Synthesis of Several Disubstituted Sydnones

By DEVINDRA DHAWAN and LEMONT B. KIER*

A number of new 3,4-dialkylsydnones have been prepared to compare their pharmacological properties with those of a series of 3-alkylsydnones. Both series were similar in their central convulsive activity. The CD_{50} and partition coefficient values of both series were compared. The CD_{50} values for the 3,4-dialkylsydnones were significantly lower than for the 3-alkylsydnones. In addition, the CD_{50} values for the 3,4-dialkylsydnones were close to one another and were independent of the partition coefficient.

PRELIMINARY WORK in this laboratory (1) indicated that 3-*sec*-butylsyndone (I, R = *sec*-Bu, R′ = H)



(I)

was a potent central nervous system stimulant with a particularly stimulating effect on respiration. It did not potentiate acetylcholine, nor did it produce acetylcholinesterase inhibition in these studies. It produced a more favorable respiratory response and blood pressure rise than the same dose of pentylenetetrazole when administered intravenously to a pentobarbitalized dog.

With these preliminary findings in hand, a systematic study of a number of sydnones was initiated with the purpose of further elaborating the pharmacological properties and any structure-activity relationships. In the previous communication from this laboratory (2), a number of 3-alkylsydnones were synthesized. The pharmacological response of these compounds was qualitatively similar to that of 3-*sec*-butylsydnone, but one response in particular—the onset of a charac-

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