tions. This greater complexing tendency probably can be ascribed to the proximity of the polymer and drug molecules in the solutions.

8. *In vivo* evaluation in rats, employing Williamson activity cages, substantiated the results of the dialytic rate studies by showing no appreciable difference in the onset, extent, and duration of sedation of the drug complex with respect to the free drug. *In vivo* studies also demonstrated that the interacted system had a somewhat reduced oral toxicity.

9. The nature of the polymer-drug interaction was elucidated by subjecting the precipitates obtained from solutions containing approximately equal amounts of PVOM and barbiturate to ultraviolet and infrared spectral analyses, melting point determinations, and solubility studies.

10. Results of this research strongly suggest the existence of an association between PVOM and certain barbiturates. It is felt that the association can be ascribed primarily to a dipole–dipole interaction or hydrogen bonding between the polymer and the drug, abetted by secondary attractive forces such as Van der Waals' bonds, spatial arrangement, etc.

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Effects of Heavy Water on *Atropa belladonna*

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The growth of belladonna is strongly inhibited by D_2O concentrations greater than 50 per cent. The alkaloid content decreases progressively as the deuterium level in the plant increases. Germinative ability of seeds containing up to 40 per cent deuterium is unimpaired.

THE EFFECTS of deuterium on algae and other microorganisms have been the subject of numerous investigations (1, 2), but studies of the effects of this isotope on higher plants have been relatively few. The long growth cycles, the relatively large amounts of water lost through transpiration, and the fact that only a few individual plants may be cultured at a time are probably the chief reasons for this situation. Early work concerning deuterium isotope effects on higher plants was concerned often with either short-termed effects or possible effects at very low concentrations of D_2O or effects on some special aspect of plant development, such as germination. Early work has been summarized by Morowitz and Brown (3) and more recently by Thomson (4), while the cytological aspects of deuterium substitution in higher plants have been reviewed by Flaumenhaft *et al.* (5). Recent detailed studies in these laboratories on the growth of the higher plant, *Mentha piperita* L., in heavy water included scrutiny of the morphological (6) and histological (7) changes evoked by various concentrations of D_2O as well as the concurrent effects of various growth regulators (8). These studies provided the first detailed descriptions of the effects of deuterium when present throughout the lifetime of the plant and have encouraged further research with higher plants of pharmaceutical importance. The present communication describes some effects of heavy water on *Atropa belladonna* and includes data on growth, morphology, flowering, and alkaloid content of belladonna plants grown with various concentrations of heavy water in the nutrient medium.

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EXPERIMENTAL

Culture of Plants.—Seeds of soil-grown *A. belladonna* were germinated in moist vermiculite. After a 4-week germination period, seedlings 2 to 4 cm. in length were transferred to liquid media in 1-qt. Mason jars. The standard metal insert cap was replaced with a polyethylene cover having three holes—one for the admission of the plant, the second for an aeration tube, and the third for maintaining the nutrient medium level. Plant stems were supported in the jar cover with sections of cork cut to form an annulus and placed around the stem. Because belladonna grown in liquid nutrient will attain maximum growth only if the roots are aerated, a plastic tube was inserted into the growth medium, and a slow stream of air was bubbled through the solution. Air for aeration first was passed through a gas scrubbing tower filled with an aqueous solution of heavy water of the appropriate concentration. Tightly fitting stoppers and a constant outward flow of air assured no exchange of hydrogen between the moisture of the air and the deuteriated nutrient solution.

Hoagland's medium containing 0, 30, 50, or 60% D_2O was used for plant growth and was prepared as previously described (6). Throughout the period of growth, the level of the solution was maintained near the top of the jar cover by adding, on alternate occasions, fresh nutrient medium or deuteriated solvent of the appropriate concentration. The jars were covered with aluminum foil to inhibit algal growth. Natural north light was supplemented with a movable bank of four 40-w. fluorescent lights. In the initial phases of growth, the light bank was positioned so that cool-white fluorescent lamps provided an intensity of about 500 f.-c. on the leaf surfaces. During the actual experiment, these lights were replaced by Gro-Lux lights (Sylvania) developed especially for horticultural growth. Because of the unique spectral output of these lights, no simple correspondence can be made between ordinary photometer readings and light intensity, compared with regular fluorescent lamps. Plants were grown on a 16-hour long day cycle. As plant growth progressed, the light bank was elevated so as to be just above the topmost leaves. Two control plants (H_2O medium) and two plants at each level of D_2O concentration were used in this study.

Harvesting of Plants.—Controls and plants grown in 30% D_2O were harvested after 135 days, at which time most of the flowers present had been replaced by purple mature berries. Due to decreased growth in highly deuteriated media, plants grown in 50 and 60% D_2O were allowed to grow 188 days, at which time the degree of maturation was comparable to that of controls, judged by inflorescence. At sacrifice, roots were blotted to remove excess liquid, and the plant divided into root, berry, and leaf–stem categories, and each part weighed. A small portion of each plant part (except berry) was stored in a sealed tube and placed in a deep freeze for subsequent deuterium analysis. Plants were air-dried for at least 1 week in a low humidity environment and reweighed to obtain dry weight.

Deuterium Analysis.—All deuterium analyses were carried out by the infrared absorption method described by Crespi and Katz (9). During the course of plant growth, the nutrient media were analyzed to insure that isotopic dilution by atmospheric water did not change the deuterium concentration significantly. Analysis for deuterium was carried out as previously described (6), using vacuum line technique to eliminate isotopic exchange with atmospheric water.

Assay for Total Alkaloids.—Total alkaloid content was determined by the standard methods given in the U.S.P. XVI under belladonna leaf (10) and in the N.F. XI under belladonna root (11).

Germination Studies.—The production of partially deuteriated seeds has never been previously reported. Hence it was of interest to determine the relative germinative capacity of such seeds. Seeds were germinated in vermiculite beds moistened with H_2O–D_2O mixtures; the dishes holding the vermiculite were maintained under bell jars to exclude atmospheric moisture. A current of air, saturated with vapor of a H_2O–D_2O mixture of appropriate concentration, was passed continuously through the jars, thus excluding back diffusion of water-laden ambient air. Seeds were scattered on the vermiculite surface and covered with an additional layer of vermiculite 3–5 mm. deep. During the germination period the containers were exposed to the long daylight cycle of the fluorescent light banks. No scarification of the seed wall or temperature cycling was used to promote germination.

Two groups of seeds—one collected from a control plant and the other from a plant grown in 50% D_2O—were each tested for capacity to germinate in water of the following D_2O concentrations: 0, 50, and 90%. One-hundred seeds were used in each of the six germination beds.

RESULTS AND DISCUSSION

Morphology and General Appearance.—Belladonna plants grown in media containing 30% D_2O developed in a near normal manner. Although slight inhibition in elongation took place, the patterns of development and growth rates were comparable to those of the controls. A moderate increase in stem length during the first month was followed by rapid elongation during the succeeding 2 months. During the fourth month, the flowers present produced fruits which matured to a dark purple color. Plants grown in 50% D_2O attained only 60% of the shoot length of the controls. Their leaves did not elongate to normal size and were irregularly expanded, causing a crinkled appearance. Plants grown in 60% D_2O elongated very little, produced a few pale poorly expanded leaves, and completely lacked any indication of an inflorescence. The marked difference in response of the plants in 50% and 60%, respectively, is shown in Fig. 1. The series is composed of the larger of the two plants grown at each D_2O level. A second series, composed of the smaller plants grown at each D_2O level, presented a graded appearance similar to Fig. 1. There was an appreciable variation in size between the two plants of each set. The ultimate size attained by a given plant is determined by several factors, important among which is the degree of aeration of the root system. Figure 2 shows clearly the effect of aeration on the growth of belladonna in nutrient solutions containing 50% D_2O. The plant whose root system was not aerated produced only eight leaves and did not attain a height exceed-

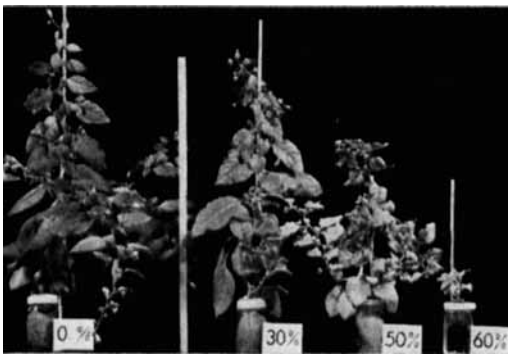

Fig. 1.—Plants of *A. belladonna* L. grown in nutrient solutions containing increasing concentrations of D_2O. The larger of the two plants grown at each concentration is shown in this photograph.

ing 8 in. The root system developed poorly, and the plant showed no indication of inflorescence. The aerated plant developed normally.

High D_2O levels in the nutrient medium affected not only terminal plant size, but also changed the rate at which maturation was reached. Figure 3 shows a berry from a control plant, fully matured, and also an immature berry from a plant grown in 50% D_2O. Both plants are the same chronological age, but a high level of deuteriation has retarded flowering and subsequent fruit development.

Also visible in Fig. 3 is the difference in leaf texture and surface sheen always present in the leaves of deuteriated belladonna. Leaves, especially when small and growing, showed a wrinkled convoluted surface, in particular at the proximal ends. The surfaces appeared shiny or glossy.

Seeds collected from deuteriated plants were smaller than those produced by the controls; the reduction in size was progressive with increasing D_2O content. Based on samples of 200 air-dried seeds each, the average weight per seed was: control, 1.52 mg.; 30% D_2O medium, 1.10 mg.; 50% D_2O medium, 0.70 mg. In addition, fruits from highly deuteriated plants contained a significantly smaller number of seeds. Highly deuteriated seeds often, but not invariably, presented a flattened or concave surface in contrast to the spherical surfaces of control seeds.

The most important generalization concerning growth in increasing concentrations of D_2O probably concerns the marked reduction of normal growth patterns and loss of reproductive capacity which occurs somewhere above 50% D_2O in the medium.

Deuterium Content.—Periodic checks of the nominal deuterium content of the medium showed that the content of deuterium never varied more than 1% from the expressed concentration. After harvest, the deuterium content of the plants was determined by collecting the water obtained from vacuum distillation of the plant and the water resulting from the complete combustion of the dried plant. The D_2O content of the water was determined by the technique referred to earlier. The vacuum-distilled water represents interstitial and cellular water. The water of combustion contains deuterium present in both the exchangeable and non-exchangeable hydrogen positions in the organic components of the plant. Deuterium analyses were carried out on samples of stems and leaves only. Plants grown in 30% D_2O had an interstitial D_2O content of 27.0% and a fixed D_2O content of 24.8%; the corresponding values for plants grown in 50% D_2O were 46.2% and 41.4%. A sample from a plant grown in 60% D_2O indicated an interstitial D_2O content of 55.8%; the small quantity of material used for combustion in this instance did not provide sufficient water to permit analysis of fixed D_2O. Analysis of plants grown in 60% D_2O in subsequent experiments indicated that the amount of fixed deuterium present is a function of the length of time the plant was allowed to grow. Thus, combustion of a young plant growing in 60% D_2O gave a value of 28.8% D_2O for fixed deuterium; combustion of a leaf of a plant several weeks older indicated a value of 35.5% D_2O. Neither of these plants was as

Fig. 3.—Comparison of leaves and fruits of *A. belladonna* grown in aqueous medium and medium containing 50% D_2O. The leaf at top and the dark berry at left were produced on the control (H_2O) plant. The small green berry at right and the leaves surrounding it grew on the 50% D_2O plant.

Fig. 2.—Effect of aeration of belladonna plants grown in nutrient solution containing 50% D_2O. The plant at the right was not aerated during the growth period.

TABLE I.—WEIGHT OF PLANT PARTS AND ALKALOID CONTENT OF BELLADONNA PLANTS[a]

Plant Part	Fresh Wt., Gm.	Dry wt., Gm.	Alkaloid Content, %
Control (H_2O)			
Stems and leaves	110.0	24.3	0.29[b]
Roots	57.4	6.7	0.18
Berries	16.3		
30% D_2O			
Stems and leaves	94.0	16.8	0.23[b]
Roots	32.3	3.1	0.13
Berries	10.3		
50% D_2O			
Stems and leaves	102.3	16.6	0.19[b]
Roots	31.8	4.2	0.10
Berries	8.5		
60% D_2O			
Stems and leaves	16.3	3.1	...
Roots	3.5	0.5	...

[a] The data given are averages for both plants grown at each D_2O concentration. [b] Only the leaves were used in the alkaloid analysis.

mature as those used in the present study. The values of deuterium content given here are not absolute since they are a function of the arbitrary time chosen for harvesting.

Alkaloid Content.—A striking effect of deuteriation on the alkaloid content was the progressive reduction of alkaloid produced as the deuterium content of the plant increased. Per cent total alkaloid is recorded in Table I. Plants grown in 60% D_2O medium did not produce sufficient material to permit quantitative alkaloid analysis by the method employed.

The alkaloid moiety extracted from the plants was fractionated by thin-layer chromatography using silica gel.[1] The developing solvent was 10% ammonia in ethanol. Atropine was identified and isolated from the material of the control plants. The alkaloid fractions derived from 30% and 50% D_2O grown plants yielded partially deuteriated atropine in amounts distinctly smaller than the amount of alkaloid produced by the controls. The alkaloid fractions derived from deuteriated plants showed an additional red component not present in the controls. The nuclear magnetic resonance (NMR) spectra of atropine and the red material suggested that the latter material was structurally similar to atropine. The red material may possibly be an intermediate in atropine synthesis or the result of a blocked or altered biosynthetic pathway, but more detailed studies will be required before the nature of the red substance can be determined.

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Germination Studies.—Belladonna seeds did not germinate in concentrations of D_2O of 50 or 90%, irrespective of whether the seeds originated from H_2O control plants or from plants grown in 50% D_2O. Seeds in vermiculite moistened with H_2O, however, did germinate. The extent of germination of control plants was 82% and that of seeds produced by 50% D_2O-grown plants was 72%. There appears to be no significant difference in the rate of germination in either case, both groups showing about the same numbers of emerging shoots at any given time. Inhibition of germination at 50% D_2O is not consistent with some of the results described by Siegel *et al.* (12). It is apparent from their study, however, that there is a wide variation in species response to germination in deuteriated media. The complete inhibition of germination in concentrations of D_2O as low as 50%, for either control produced seeds or partially deuteriated seeds, stands in contrast with earlier work (13). However, in many of these studies little care was taken to prevent exchange of the D_2O of the germinating medium with atmospheric water and actual D_2O analyses were generally not performed at the termination of the experiment. The present experiments indicate that the inhibitory effect of heavy water on seed germination may be more pronounced than had been previously indicated and suggests that seed mitosis is blocked in the same manner suggested by Gross and Spindel for sea urchin eggs (14).

The effects of increasing concentrations of D_2O on the growth pattern and development of *A. belladonna* are qualitatively similar to those reported earlier (6, 7) for peppermint plants. Above 50% D_2O, a more drastic inhibition of growth was observed in belladonna than with peppermint. This may be correlated with the tendency of belladonna to attain somewhat higher levels of deuteriation than was the case with peppermint.

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