is essential for a pharmaceutical manufacturer to invest in specialized laboratory equipment and skilled personnel. Although constant-temperature baths may be ideal for kinetic studies on a small scale, they are not practical for testing preparations which are packaged in their final containers. This is due to the fact that, in most cases, the packages are of such size that the baths are not of adequate capacity, nor are the packages generally of such a type that can be immersed in a bath. Since the most significant tests, from a practical point of view, must be conducted on the final formulation with due consideration for the influence of the container on stability, space and equipment must be adequate for a large number of samples. For milder conditions of storage, the retention periods will be longer, and space requirements will be greater than for severe conditions of storage. Because rates of change in a property of a tablet preparation are dependent upon environmental conditions, it is essential to obtain equipment capable of operating within narrow limits of temperature, humidity, and light intensity. This will result in an increase in the accuracy of the extrapolated data.

As illustrated in the previous sections of this paper, the stability of tablet formulations can be affected by temperature, light, and humidity. Consequently, it is essential to have adequate equipment available which would permit the performance of stability tests under exaggerated conditions of temperature, humidity, and light. With the availability of such equipment and the application of certain physical chemical principles to the data collected under exaggerated test conditions, it should be possible, in most cases, to predict the chemical and physical stability of tablet dosage forms in a relatively short period of time.

It is hoped that, as a result of the foregoing information, a better insight into the factors that can affect the chemical and physical stability of tablet dosage forms and the utility of employing chemical kinetic principles to assist in predicting the stability under extended shelf conditions from exaggerated test data has been gained.

REFERENCES

(1) Wai, K. N., DeKay, H. G., and Banker, G. S., *J. Pharm. Sci.*, **51**, 1076(1962).
(2) Yamamoto, R., and Kawai, S., *Arch. Pract. Pharm.*, **19** (No. 1), March 1959.
(3) Yamamoto, R., *ibid.*
(4) Bandelin, F. J., and Malesh, W., *J. Am. Pharm. Assoc., Pract. Pharm. Ed.*, **19**, 152(1958).
(5) Nazareth, M. R., and Huyck, C. L., *J. Pharm. Sci.*, **50**, 608(1961).
(6) Ribeiro, D., *et al.*, *J. Am. Pharm. Assoc., Sci. Ed.*, **44**, 226(1955).
(7) Nazareth, M. R., and Huyck, C. L., *J. Pharm. Sci.*, **50**, 620(1961).
(8) Castello, R. A., and Mattocks, A. M., *ibid.*, **51**, 106 (1962).
(9) Goodhart, F. W., Everhard, M. E., and Dickcius, D. A., *ibid.*, **53**, 338(1964).
(10) Carstensen, J. T., *ibid.*, **53**, 839(1964).
(11) Guillory, J. K., and Higuchi, T., *ibid.*, **51**, 100(1962).
(12) Carstensen, J. T., *et al.*, *ibid.*, **53**, 1050(1964)
(13) Lachman, L., *et al.*, *J. Am. Pharm. Assoc., Sci. Ed.*, **49**, 165(1960).
(14) Everhard, M. E., and Goodhart, F. W., *J. Pharm. Sci.*, **52**, 281(1963).
(15) Kubelka, P., *J. Opt. Soc. Am.*, **38**, 448(1948).
(16) Bull, A. W., *J. Pharm. Pharmacol.*, **7**, 806(1955).
(17) Siegel, S., *et al.*, *J. Pharm. Sci.*, **51**, 1068(1962).

Notes

Incorporation of Hydroxyproline-^{14}C into the Principal Alkaloids of *Datura innoxia* Miller

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Datura innoxia **root cultures absorb *dl*-hydroxyproline-2-^{14}C and distribute it to hyoscyamine and scopolamine as well as other alkaloids. The low level of incorporation suggests an indirect route and not a direct incorporation from an exogenous amino acid pool.**

A GREAT deal of interest has been engendered recently in proline–hydroxyproline relationships in animals and plants. The high content of hydroxyproline in collagen and its strikingly reduced quantity in collagen in rheumatoid states in man has been largely responsible for this interest. The wide variance in hyoscyamine–scopolamine ratios in different species of *Datura* has long been of interest and the cause for much study. In view of the obvious proline moiety in the hyoscyamine molecule and the relationship of oxidized hydroxyproline to the scopolamine molecule, attempts were begun some time ago in this laboratory to relate hyoscyamine–proline and scopolamine–hydroxyproline.

Work in this laboratory (1) showed that both growth and alkaloid content were affected in *Datura stramonium* variety *tatula*. More recent work in this laboratory (2) showed that *l*-proline-^{14}C was readily incorporated into the hyoscyamine and scopolamine of both *D. stramonium* var. *tatula* and

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D. innoxia. The *D. stramonium* var. *tatula* roots, which produce primarily hyoscyamine compared to scopolamine, incorporated more radioactive proline into the principal alkaloid. *D. innoxia* roots, which produce primarily scopolamine compared to hyoscyamine, incorporated more radioactive proline into its principal alkaloid. This work suggested that proline is an important compound utilized by these plants in the biosynthesis of alkaloids.

The following questions immediately arose. If free proline is a source of these tropane alkaloids and scopolamine is the oxidized hyoscyamine, is hydroxyproline the precursor of scopolamine? Is scopolamine synthesized directly from hyoscyamine? Can hydroxyproline be directly incorporated into scopolamine?

This investigation was undertaken in an attempt to answer the latter question.

EXPERIMENTAL

Growth and Processing of Root Cultures.—The methods and procedures followed in this portion of the investigation were those developed and utilized by French and Gibson (3). The seeds of *D. innoxia* were obtained from plants grown in the Washington State University pharmacy greenhouse. The sterilization of seeds and the propagation of the 14-day-old root cultures followed the general procedure outlined by Sullivan and Gibson (2). Part of that study utilized uniformly labeled *l*-proline-^{14}C. The study described here utilized 0.001 mg. of *dl*-hydroxyproline-2-^{14}C[1] per 50 ml. of nutrient solution. This is the only tagged hydroxyproline available. It had a specific activity of 18.5 mc./m*M*. Radiation effect on root growth at this level in the concentrations used is considered negligible.

Extraction and Separation of Alkaloids.—The extraction apparatus employed for this procedure was devised by French and Gibson (3). The collection of the extract followed the method of Sullivan and Gibson (2). This latter work utilized 0.001 mg. of *l*-proline-^{14}C (uniformly labeled). To minimize amino acid effect on root growth (2) and to provide comparative root weight data in this study on *dl*-hydroxyproline as compared to *l*-proline, the amounts of amino acids were made the same for both amino acid studies. However, because the *dl*-hydroxyproline used in this study was only labeled at carbon 2, compared to the uniform labeling for the *l*-proline, it was necessary in the work described here to combine roots for the extraction to provide a sufficiently radioactive extract for the purposes of this investigation. Eight combinations of roots were made for extraction to provide various levels of radioactivity to the extracts.

The separation of the alkaloids was accomplished using thin-layer chromatography. The total alkaloid extract was subjected to 5–200-μl. extractions of chloroform. A 25-μl. portion of each chloroform extraction was placed on a thin-layer plate. An $8 \times 8 \times {}^1/_8$-in. glass plate with 0.5-mm. thickness of Silica Gel G[2] was employed. The thin-layer plate was then developed in two directions. The first and second developing phases utilized were, respectively, 10:90 and 15:85 of diethylamine–chloroform. At the conclusion of the developing period, the chromatographic plate was removed and placed in an air circulating oven for 30 min. at 55 to 60°.

Determination of Radioactivity of the Alkaloids.—Autoradiography was employed in this investigation for the determination of the radioactivity of the individual alkaloids. X-ray exposure holders previously described (2) were used. Kodak medical X-ray, tinted, safety base, duplitized, no-screen film was used. Exposure times for the plates varied: 3.5, 5, and 6 months.

RESULTS

D. innoxia roots grown in White's media (4) with added *dl*-hydroxyproline-2-^{14}C appeared as shown by the root in Fig. 1. The autoradiogram resulting from a plate which had 6 months of exposure is shown in Fig. 2. Ideally the radioactivity should have been greater for more distinct definition of spots. However, only this radioactive *dl*-hydroxyproline is available which has only one carbon labeled. Using larger quantities of the amino acid would have resulted in greater root growth inhibition. A compromise was the only recourse.

The dry weight determinations of 44 roots indicated that *D. innoxia* roots grown in 0.001 mg. *dl*-hydroxyproline-2-^{14}C supplemented White's media had a mean dry weight of 6.1 mg. (95% confidence interval 5.6–6.6 mg.). Previous work (2) indicated that these roots grown in just White's media had a mean dry weight of 9.2 mg. (95% confidence interval 8.9–9.5 mg.) and a mean dry weight of 7.0 mg. (95% confidence interval 6.0–8.0 mg.) when grown in 0.001 mg. *l*-proline-^{14}C (uniformly labeled) supplemented White's media. Side roots were consistently shorter in the test cultures.

All autoradiograms thus far developed (6 out of 8) showed incorporation of ^{14}C from *dl*-hydroxyproline into scopolamine. Where the radioactivity

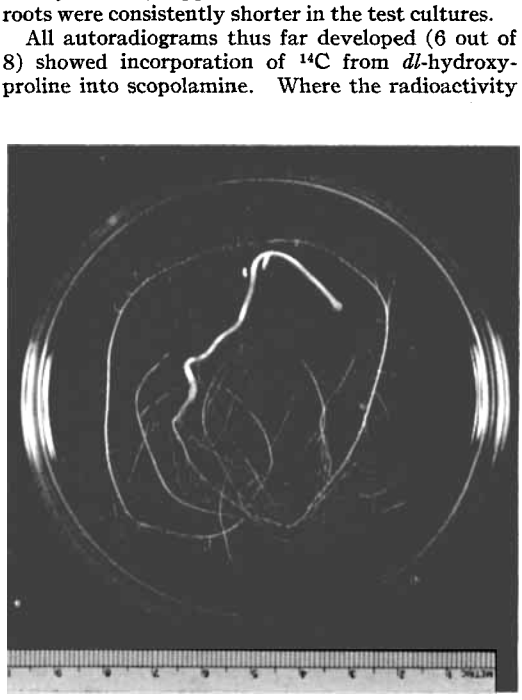

Fig. 1.—*D. innoxia* root culture grown in 0.001 mg. of *dl*-hydroxyproline-2-^{14}C per 50 ml. of solution.

[1] Obtained from Calbiochem, Los Angeles, Calif.
[2] E. Merck AG, Darmstadt, Germany. Obtained from Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.

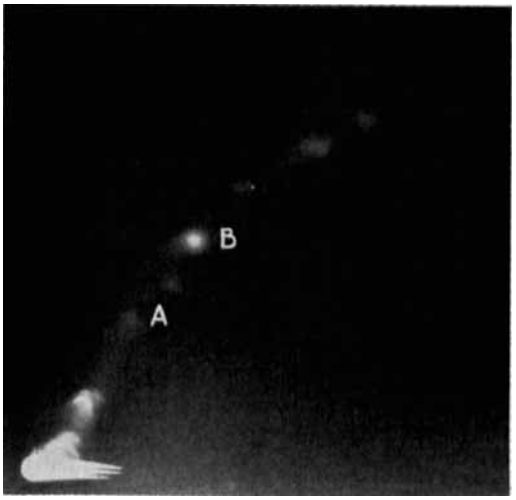

Fig. 2.—Autoradiogram of two-dimensional, thin-layer chromatographic separation of the components of an extract of *D. innoxia* grown in a solution containing radioactive hydroxyproline. Key: *A*, hyoscyamine; *B*, scopolamine.

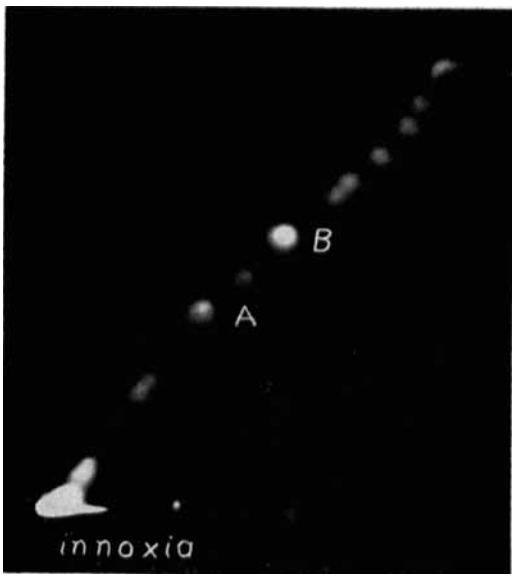

Fig. 3.—Autoradiogram of two-dimensional thin-layer chromatographic separation of the components of an extract of a *D. innoxia* root culture grown in a solution containing radioactive proline. Key: *A*, hyoscyamine; *B*, scopolamine.

was greater and exposure of the films longer, it is obvious, as shown in Fig. 2, that the amino acid is incorporated also into hyoscyamine and a number of other alkaloids which are not identified. This is the same pattern as seen for *l*-proline in Fig. 3 from a previous work (2). Just as for *l*-proline, the ^{14}C of *dl*-hydroxyproline is incorporated in greatest volume into the principal alkaloid of *D. innoxia*, scopolamine.

DISCUSSION

The inhibition of growth caused by the *dl*-hydroxyproline is consistent with the work of Steward *et al.* (5) in carrot phloem explants, in which they suggested that hydroxyproline competed for proline positions in protein molecules.

As illustrated in Figs. 2 and 3, the pattern of distribution into the principal and other alkaloids on the chromatographic plates is similar. It is obvious that hydroxyproline has not been simply converted to scopolamine but has been reduced to form hyoscyamine as well.

Work by Pollard and Steward (6) indicated to them that hydroxyproline-^{14}C when absorbed by cultures of carrot root phloem seemed not to be incorporated into protein as such, but can to a small degree be converted to proline which does go into protein. They concluded that 97% of the hydroxyproline remains as such. They further concluded that the 3% which shows up in protein and hydroxyproline is converted to the latter as part of the protein. This is consistent with a number of other works (7–12). It has been concluded that all of the tissue hydroxyproline comes from proline, whereas very little if any proline stems directly from hydroxyproline.

Previous work (2) and that described here show that ^{14}C from *both* proline and hydroxyproline in the free state when supplied to *D. innoxia* roots are capable of being incorporated into the same alkaloids. Hydroxyproline *is* reduced. If it is not reduced as part of protein, it must be converted to the alkaloids by (*a*) reduction in an extra-protein milieu or (*b*) is extensively catabolized back to such elementary amino acids as glutamic acid which then evolve to proline and hydroxyproline and/or hyoscyamine and scopolamine. There is some evidence that the latter is possible (13).

CONCLUSIONS

When *dl*-hydroxyproline-2-^{14}C is absorbed by *D. innoxia* root cultures, it inhibits their growth and carbon 2 is incorporated into hyoscyamine and scopolamine. It is suggested that the incorporation takes place either in an extra-protein environment or only after extensive degradation and reformation of the molecular structure of the hydroxyproline. The low degree of radioactivity of the alkaloids separated on the TLC plates and the radioactivity of other compounds on the plates suggest that the conversion of hydroxyproline to scopolamine and the other alkaloids is not a direct one from an exogenous pool.

REFERENCES

(1) Gibson, M. R., and Abbott, E. R., *Lloydia*, **26**, 125(1963).
(2) Sullivan, G., and Gibson, M. R., *J. Pharm. Sci.*, **53**, 1058(1964).
(3) French, D. I., and Gibson, M. R., *J. Am. Pharm. Assoc., Sci. Ed.*, **46**, 151(1957).
(4) White, P. R., "A Handbook of Plant Tissue Culture," Ronald Press Co., New York, N. Y., 1943, pp. 90–112.
(5) Steward, F. C., *et al.*, *Biochim. Biophys. Acta*, **28**, 308(1958).
(6) Pollard, J. K., and Steward, F. C., *J. Exptl. Botany*, **10**, 17(1959).
(7) Bernheim, F., and Bernheim, M. L. C., *J. Biol. Chem.*, **96**, 325(1932).
(8) *Ibid.*, **106**, 79(1934).
(9) Weil-Malherbe, H., and Krebs, H. A., *Biochem. J.*, **29**, 2077(1935).
(10) Neber, M., *Z. Physiol. Chem.*, **240**, 70(1936).
(11) Edson, N. L., *Biochem. J.*, **29**, 2489(1935).
(12) Pederson, S., and Lewis, H. B., *J. Biol. Chem.*, **154**, 705(1944).
(13) Stetten, M. R., *ibid.*, **181**, 31(1949).