# Protein–Alkaloid Relationship in *Datura stramonium* var. *tatula*

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**Abstract** ☐ The objective of this research was to find a relationship between protein synthesis and alkaloid synthesis in *Datura stramonium* L. var. *tatula* Torrey, if one existed. Sterile root cultures were grown for 2 weeks in a nutrient solution containing chloramphenicol, cycloheximide, and puromycin. The alkaloids hyoscyamine and scopolamine were separated by TLC and assayed spectrophotometrically. The total nitrogen content was determined by the micro-Kjeldahl method. In the tracer work, the sterile root cultures were grown in the nutrient solution containing L-proline-U-$^{14}C$ and puromycin. The soluble protein content of the radioactive roots was determined by the Lowry method. The incorporation of L-proline-U-$^{14}C$ into alkaloids and trichloroacetic acid-precipitable proteins was determined by the liquid scintillation technique. The growth habits were changed and the weights of the root cultures reduced. Of the three inhibitors tested, only puromycin caused an increase in the hyoscyamine production and in the uptake of proline into it. This is the first relationship demonstrated between protein synthesis and alkaloid synthesis.

**Keyphrases** ☐ *Datura stramonium* L. var. *tatula*—protein synthesis–alkaloid synthesis relationship ☐ Protein–alkaloid syntheses relationship—*Datura stramonium* L. var. *tatula* ☐ Alkaloid–protein syntheses relationship—*Datura stramonium* L. var. *tatula* ☐ L-Proline-U-$^{14}C$—incorporation into alkaloids and trichloroacetic acid-precipitable proteins, *Datura stramonium* L. var. *tatula* ☐ Puromycin inhibition of protein synthesis—*Datura stramonium* L. var. *tatula*

Many years of research have been spent in this laboratory and elsewhere to elucidate the biosynthetic pathway or pathways to alkaloid production in the genus *Datura*. The phytophysiological significance of alkaloids in various plants has only been partly known. There have been conflicting reports in the literature about the origin of alkaloids in plants.

It has long been suggested that there is a relationship between protein and alkaloid biosynthetic mechanisms because of the proved amino acid involvement in both processes (1–4). Schmid and Serrano (5) reported an interesting parallelism between the protein production by the tobacco root and the nicotine content of the whole plant. Using the same plant, Mothes and Englebrecht (6) showed that the low alkaloidal roots were high in protein content.

However, no concrete evidence of a relationship between protein synthesis and alkaloid synthesis has been forthcoming. It has been suggested that alkaloids are the result of protein breakdown, yet their synthesis patterns seem to indicate a correlative relationship. The objective of this research was to find a relationship between alkaloid synthesis and protein synthesis, if one existed.

The plant chosen for this study was *Datura stramonium* var. *tatula*, family Solanaceae, because of earlier research done on the genus *Datura* during the past several years (7–11). *D. stramonium* var. *tatula* produces hyoscyamine and scopolamine as its principal alkaloids, the amount of hyoscyamine being much greater than scopolamine.

Various amino acids have been used to gain clearer and more precise information concerning the production of alkaloids. Glutamic acid, ornithine, proline, and phenylalanine have all been successfully incorporated into hyoscyamine (8, 12–14). Work in this laboratory (11) showed that both growth and alkaloid content were affected by proline in *D. stramonium* var. *tatula*. Later work in this laboratory (8) showed that L-proline-U-$^{14}C$ (U indicates uniformly labeled) was readily incorporated into hyoscyamine and scopolamine of both *D. stramonium* var. *tatula* and *D. innoxia* roots. This work suggested that proline is an important compound utilized by these plants in the biosynthesis of alkaloids. Proline also has been found to stimulate formation of hyoscyamine in *Atropa belladonna* roots (15). In the same plant, Schermeister *et al.* (16) found that proline was present in large amounts and accumulated in roots when the alkaloid concentration declined. These workers also found that neither arginine nor ornithine was present in detectable quantities and concluded that proline was a more direct precursor of tropane alkaloids. Liebisch and Schütte (17) showed that proline-5-$^{14}C$ seems to be stereospecifically incorporated into alkaloids of *D. metel*. It is thus clearly seen that proline plays a dynamic role in the biosynthesis of tropane alkaloids.

L-Proline-U-$^{14}C$ has been incorporated into proteins of various plants, animals, and bacteria. Steward and Pollard (18) found that proline can be directly utilized in protein synthesis. When L-proline-U-$^{14}C$ was supplied to growing cultures, almost all of the radioactivity was found in the proline and hydroxyproline residues of the tissue protein, very little $^{14}C$ being lost as carbon dioxide. Miller and Kempner (19) found that L-proline-U-$^{14}C$ was incorporated into protein by *Candida utilis*. The ready incorporation of carbon from proline into carrot explants was shown by Pollard and Steward (20), who demonstrated that radioactive carbon supplied as proline can be detected in the protein 15 min. after its addition to external solution.

Thus, proline can enter into both proteins and alkaloids. The question arises whether the incorporation of L-proline-U-$^{14}C$ into the principal alkaloids of *D. stramonium* var. *tatula* will increase if the protein synthesis in the root cultures is inhibited by the inhibitors of protein synthesis. If the incorporation of radioactivity into the alkaloids increases after inhibition of protein synthesis, a correlative relationship possibly can be inferred.

The inhibitors chosen for this study were chloramphenicol, cycloheximide, and puromycin. These in-

hibitors were chosen because of their different modes of action in inhibiting the protein synthesis (21).

Chloramphenicol binds specifically to 50S subunits. It inhibits functional attachment of the aminoacyl end of AA-tRNA to the 50S subunits, thus inhibiting transpeptidation, but may also have a direct inhibitory effect on the peptidyltransferase.

Cycloheximide inhibits chain initiation as well as chain elongation by interaction with 60S, but not 50S, ribosomal subunits. It also interferes with chain elongation by inhibiting entry of peptidyl tRNA from A to P sites through interference with release of deacylated tRNA from the donor site.

Puromycin, through its resemblance to the aminoacyl-adenylyl end of AA-tRNA, inhibits protein synthesis by competing with AA-tRNA for this site on 50S subunits of ribosomes. In place of AA-tRNA, it accepts nascent peptides, causing premature release of incomplete polypeptide chains. As a result of these actions, puromycin causes the breakdown of polysomes.

## EXPERIMENTAL

**Growth and Processing of Root Cultures**—The methods and procedures followed in this portion of the investigation were developed and utilized by French and Gibson (7). The seeds of *D. stramonium* var. *tatula* 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, unless otherwise specified, followed the general procedures outlined by Sullivan and Gibson (8).

The basic nutrient solution used for all root cultures (control and experimental) was developed by White (22). The nonradioactive experimental root cultures were grown in three variations which were White's nutrient solution containing: (*a*) 0.8 mcg. of cycloheximide[1]/50 ml., (*b*) 32.35 mcg. of chloramphenicol[2]/50 ml., and (*c*) 1.0 mg. of puromycin dihydrochloride[3]/50 ml. These concentrations were chosen after carrying out a series of experiments with different concentrations of these inhibitors. In these concentrations, approximately 50% reduction in the dry weights of the experimental roots was observed as compared to the control roots. The inhibitors were weighed on glassine paper and placed in the sterile transfer chamber. UV lights were turned on for 15 min. The inhibitors were then transferred quantitatively to the volumetric flasks and the solutions were made to volume with sterilized, distilled, demineralized water. One hundred microliters of these solutions containing the appropriate amounts of inhibitors was added aseptically to the flasks containing 50 ml. of sterilized nutrient solution for the experimental root cultures. The control roots were cultured in 50 ml. of White's nutrient solution.

The control radioactive root cultures were grown in 50 ml. of nutrient solution containing 0.123 $\mu$c. of L-proline-U-$^{14}$C[4]. The experimental root cultures were grown in the nutrient solution containing 0.123 $\mu$c. of L-proline-U-$^{14}$C for 2 weeks and 1.0 mg. of puromycin dihydrochloride for 1 and 2 weeks.

**Extraction and Separation of Alkaloids**—The procedures used for the extraction of alkaloids from the dried root cultures and for the separation of alkaloids were developed by French and Gibson (7) and Sullivan and Gibson (8), respectively. For preliminary work with the nonradioactive roots, these roots were combined in groups of two to three, weighing about 25 mg.; for the extraction of alkaloids from the radioactive roots, each root was extracted separately. The alkaloid extract was collected in a 10-ml. beaker instead of planchet as described by Sullivan and Gibson (8).

The separation of alkaloids was accomplished by two-dimensional TLC on silica gel G[5]. The first and second developing phases utilized were, respectively, 10:90 and 15:85 of diethylamine–chloroform. The total alkaloid extract was subjected to six 200-$\mu$l. extractions of chloroform instead of five. The hyoscyamine and scopolamine spots were removed from the chromatograms according to the method of Rowland and Gibson (10). Hyoscyamine and scopolamine from the silica gel were extracted separately with anhydrous methanol[6] (A.R., spectrophotometric grade) according to the procedures employed by these workers. Fifteen milliliters of anhydrous methanol was used for extraction instead of 10 ml. as used by these workers.

**Assay of Alkaloids**—The alkaloids hyoscyamine and scopolamine were assayed quantitatively using the Vitali-Morin reaction by a spectrophotometric technique developed by French (23) and Rowland and Gibson (10)[7].

**Determination of Radioactivity in Alkaloids**—The method used for the determination of radioactivity ($^{14}$C) in the alkaloids was suggested by the instrument's manufacturer[8]. Hyoscyamine and scopolamine were separated by the TLC method mentioned under *Extraction and Separation of Alkaloids*. The hyoscyamine and scopolamine spots separated were scraped separately with a razor blade and a camel's hair brush into low potassium glass screw-cap scintillation vials[8]. Two milliliters of 1,4-dioxane[6] (A.R., liquid scintillation grade) was added to each vial and the contents were shaken vigorously. The vials were then allowed to stand for 15 min. To each of these vials, 15 ml. of the scintillation solvent, consisting of 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) *p*-bis-2-(5-phenyloxazolyl)benzene in toluene, was added and the contents were shaken well. The vials were then allowed to stand overnight in a scintillation spectrometer[9] at 4°. During this time all the silica gel settled to the bottom of the vials. All samples were counted using this counter. The average of two determinations was reported for each sample activity. Hyoscyamine samples were counted to a $2\sigma$ statistical counting error of less than $\pm 2\%$. Scopolamine samples were counted to a $2\sigma$ statistical counting error of less than $\pm 5\%$. The counter efficiency was determined for each sample by the internal standardization method using toluene-$^{14}$C[8]. The counter efficiency was found to be greater than 87%.

**Determination of Total Nitrogen**—The total nitrogen content of the dried nonradioactive roots was determined by direct Nesslerization of Kjeldahl's digests (24)[10].

**Determination of Protein**—The fresh control and experimental roots grown in L-proline-U-$^{14}$C were individually chopped into small segments with a razor blade and homogenized in 0.05 *M* phosphate buffer (pH 7.2) for 4 min. at full speed in an Omnimixer[11] with a microattachment. The homogenates were then frozen. For protein determination, the homogenates were allowed to thaw at room temperature and then filtered under vacuum through Whatman filter paper. The filtrate was spun in a No. 40 rotor at 40,000 r.p.m. in an ultracentrifuge[12] at 1–2° for 1 hr. The clear supernate was transferred quantitatively to a volumetric flask and made to volume. The protein content of the homogenates was determined by a slight modification of the method of Lowry *et al.* (25) using soybean trypsin inhibitor[13] as the standard protein. The absorbance was read with a 1-cm. light path in a spectrophotometer[14]. The concentration of protein in micrograms was computed from the standard, straight-line curves prepared using soybean trypsin inhibitor. The average of two determinations was reported.

**Determination of Radioactivity in Protein**—The radioactivity ($^{14}$C) in trichloroacetic acid-precipitable protein was determined by a slight modification of the method of Abell *et al.* (26). The root homogenates from the radioactive control and experimental roots

[1] The gift of cycloheximide from The Upjohn Co., Kalamazoo, Mich., is gratefully acknowledged.
[2] The gift of chloramphenicol from Parke Davis & Co., Detroit, Mich., is gratefully acknowledged.
[3] Nutritional Biochemicals Corp., Cleveland, Ohio.
[4] New England Nuclear, Boston, Mass.

[5] E. Merck A.G., Darmstadt, Germany (obtained from Brinkmann Instruments, Inc., Great Neck, Long Island, N. Y.).
[6] J. T. Baker Chemical Co., Phillipsburg, N. J.
[7] The only difference was that the absorbance was measured at 560 nm. with a 1-cm. light path in a Beckman DU spectrophotometer with a Gilford 2000 attachment instead of the percent transmittance being measured in a Beckman DU spectrophotometer.
[8] Packard Instrument Co., Inc., Downers Grove, Ill.
[9] Packard Tri-Carb, model 3320.
[10] This work was done by Mr. S. R. V. Raghavan, Department of Biology, Laval University, Quebec 10e, Quebec, Canada.
[11] Ivan Sorvall Inc., Norwalk, CT 06856
[12] Beckman model L3-40.
[13] Worthington Biochemical Corp., Freehold, N. J.
[14] Beckman DU with a Gilford 2000 attachment.

**Table I**—Summary of Root Growth of *D. stramonium* var. *tatula* under Control and Treated Conditions

| Nutrient Solution | Number of Roots | Mean Weight, mg. | 95% Confidence Interval, mg. |
|---|---|---|---|
| Control | 99 | 28.4 | 26.4–30.4 |
| Chloramphenicol: | | | |
| 6.47 mcg./50 ml. | 49 | 32.1 | 29.7–34.5 |
| 12.94 mcg./50 ml. | 56 | 25.8 | 23.8–27.8 |
| 32.35 mcg./50 ml. | 46 | 16.0 | 14.4–17.4 |
| 64.7 mcg./50 ml. | 63 | 9.7 | 8.6–10.8 |
| Cycloheximide: | | | |
| 0.8 mcg./50 ml. | 46 | 13.9 | 12.1–15.7 |
| 1.6 mcg./50 ml. | 51 | 11.7 | 10.2–13.2 |
| Puromycin dihydrochloride: | | | |
| 0.72 mcg./50 ml. | 46 | 28.0 | 25.6–30.4 |
| 1.44 mcg./50 ml. | 39 | 28.7 | 26.0–31.4 |
| 20 mcg./50 ml. | 28 | 18.0 | 15.6–20.4 |
| 40 mcg./50 ml. | 38 | 19.9 | 18.3–21.5 |
| 200 mcg./50 ml. | 27 | 16.7 | 14.7–18.7 |
| 1.0 mg./50 ml. | 45 | 10.3 | 9.3–11.3 |
| Total number of roots | 633 | — | — |

were treated in the manner described under *Determination of Protein.* To the clear supernate, 1.0 mg. of soybean trypsin inhibitor in aqueous solution was added as a carrier protein. Then an equal volume of 10% (w/v) solution of trichloroacetic acid was added. After 4 hr. at 4°, the precipitated protein was removed by centrifugation at 19,000 r.p.m. in a centrifuge[15] at 2–4°. The trichloroacetic acid-precipitated protein was washed twice with cold 10% (w/v) trichloroacetic acid and twice with 95% ethanol saturated with sodium acetate. The washed precipitated material was taken up in 4 ml. of 0.5 *M* potassium hydroxide in a 15-ml. graduated centrifuge tube. The tube was then placed in a constant-temperature water bath at 55° to solubilize the protein. After cooling, the volume was made up to 4 ml. with 0.5 *M* potassium hydroxide. One milliliter of this solution was pipeted into a glass, screw-cap scintillation vial containing 10 ml. of Aquasol[16]. The contents of the vial were then shaken vigorously and counted to a $2\sigma$ statistical counting error of less than $\pm 1\%$ in the scintillation spectrometer. The average of two determinations was reported for each sample activity measurement. The counter efficiency was determined for each sample by the internal standardization method using toluene-$^{14}$C.

## RESULTS

A total of 931 roots were grown in this investigation. The growth results of the preliminary study of dry weights of both control and the three inhibitors are shown in Table I.

To show growth results under the experimental conditions of puromycin dihydrochloride treatment in the presence of L-proline-U-$^{14}$C, a large number of roots were grown. Sixty-eight control roots grown in a nutrient solution containing 0.123 $\mu$c. of L-proline-U-$^{14}$C for 2 weeks had a mean fresh weight of 226.8 mg. (205.7–247.9 mg., 95% confidence interval), and 38 of these roots had mean dry weights of 23.4 mg. (20.9–25.9 mg., 95% confidence interval). Sixty-six roots which had been grown for a 2-week period in the nutrient solution containing the 0.123 $\mu$c. of L-proline-U-$^{14}$C and 1 mg. of puromycin dihydrochloride for 1 week had a mean fresh weight of 161.8 mg. (149.0–174.6 mg., 95% confidence interval), and 40 of these roots had a mean dry weight of 15.5 mg. (14.0–17.0 mg., 95% confidence interval). Fifty-six roots which had been grown for a 2-week period in the nutrient solution containing the 0.123 $\mu$c. of L-proline-U-$^{14}$C and 1 mg. of puromycin dihydrochloride for 2 weeks had a mean fresh weight of 136.7 mg. (123.1–150.3 mg., 95% confidence interval), and 30 of these roots had a mean dry weight of 12.3 mg. (10.7–13.9 mg., 95% confidence interval).

[15] Servall.
[16] New England Nuclear, Boston, Mass.

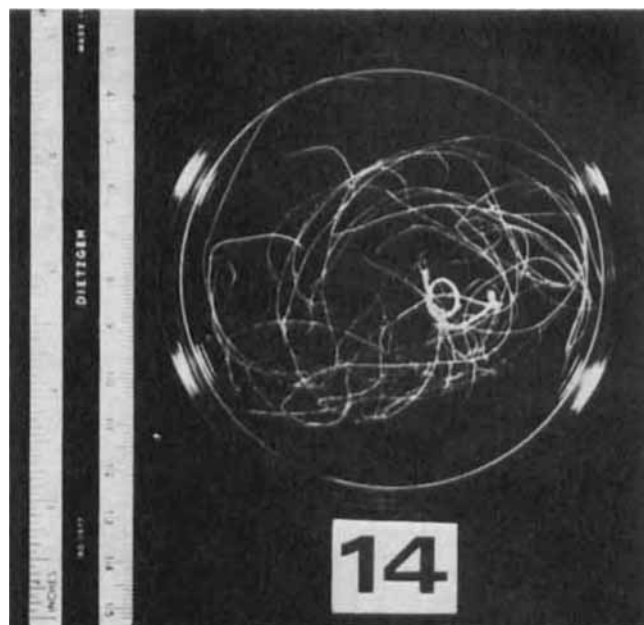


**Figure 1**—D. stramonium *var.* tatula *root culture grown in control nutrient solution.*

The control roots grown in both nonradioactive and radioactive nutrient solution showed a strong primary root with abundant secondary root systems, whereas the puromycin-treated root cultures (Figs. 1 and 2) showed a limited secondary root system. The secondary roots were much thinner as compared to the control roots. Chloramphenicol-treated and cycloheximide-treated root cultures showed a similar trend in growth inhibition; the degree of inhibition, however, was much less as compared to the puromycin-treated roots.

Table I and the results already described in this section indicate that chloramphenicol at a concentration of 32.35 mcg./ml. of the nutrient solution, cycloheximide at a concentration of 0.8 mcg./50 ml. of the nutrient solution, and puromycin at a concentration of 1.0 mg./50 ml. of the nutrient solution produced a statistically significant decrease in growth at the 5% level. The experimental radioactive roots grown in the presence of puromycin also showed a statistically significant decrease in growth with puromycin as

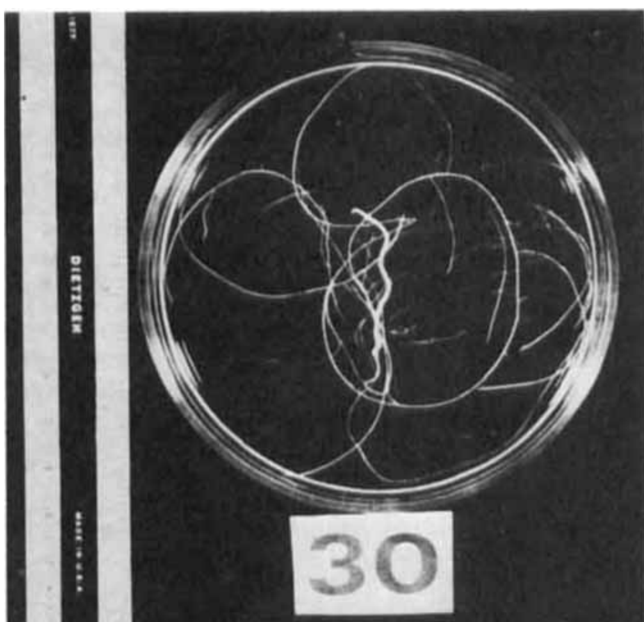


**Figure 2**—D. stramonium *var.* tatula *root culture grown in nutrient solution containing 1.0 mg. of puromycin dihydrochloride.*

Table II—Alkaloid Content[a] of *D. stramonium* var. *tatula* Control and Treated Roots Grown in Nonradioactive Nutrient Solution

| Sample Number | Control | | Chloramphenicol Treated | | Cycloheximide Treated | | Puromycin Treated | |
|---|---|---|---|---|---|---|---|---|
| | Hyoscyamine | Scopolamine | Hyoscyamine | Scopolamine | Hyoscyamine | Scopolamine | Hyoscyamine | Scopolamine |
| 1 | 48.6 | 21.0 | 49.6 | 17.3 | 50.1 | 17.5 | 63.7 | 5.5 |
| 2 | 46.4 | 1.3 | 51.2 | 11.5 | 56.7 | 12.2 | 64.4 | 9.8 |
| 3 | 38.5 | 8.0 | 48.0 | 16.1 | 46.6 | 3.4 | 62.7 | 2.2 |
| 4 | 30.9 | 25.4 | 49.9 | 12.1 | 27.8 | 10.6 | 59.5 | 5.8 |
| 5 | 46.5 | 2.1 | 55.4 | 8.8 | 53.3 | 11.1 | 51.8 | 21.7 |
| 6 | 68.6 | 10.0 | 46.8 | 10.3 | 45.9 | 4.5 | 49.8 | 18.6 |
| 7 | 51.1 | 11.6 | 42.5 | 5.8 | 37.2 | 7.9 | 55.9 | 13.9 |
| 8 | 46.1 | 3.8 | 53.7 | 9.6 | 50.3 | 9.1 | 66.6 | 19.4 |
| 9 | 44.3 | 8.8 | 55.1 | 12.1 | 56.7 | 14.5 | 99.4 | 21.3 |
| 10 | 45.0 | 9.0 | 57.4 | 13.1 | 49.0 | 13.7 | 42.0 | 7.5 |
| 11 | — | — | — | — | — | — | 64.4 | 6.9 |
| 12 | — | — | — | — | — | — | 45.2 | 6.4 |
| 13 | — | — | — | — | — | — | 80.6 | 9.1 |
| 14 | — | — | — | — | — | — | 74.7 | 12.0 |
| Mean | 46.6 | 10.1 | 50.9 | 11.7 | 47.4 | 10.5 | 62.9 | 11.4 |
| 95% Confidence interval | 39.7–53.5 | 4.6–15.6 | 47.7–54.1 | 9.3–14.1 | 41.0–53.8 | 7.4–13.6 | 54.3–71.6 | 4.4–18.4 |

[a] Micrograms per 25 mg. root powder.

compared to the control roots. This decrease is evidenced by nonoverlapping 95% confidence intervals on the average fresh and dry weights of the control and treated roots.

The results of the alkaloidal assays of the control and treated roots are reported in Tables II and III. From Table II, it can be seen that there is no statistically significant difference at the 5% level in alkaloid content (for both hyoscyamine and scopolamine) among the control roots, the chloramphenicol-treated roots, and the cycloheximide-treated roots. However, there is a statistically significant increase at the 5% level in the hyoscyamine content between the control and the puromycin-treated roots as evidenced by the nonoverlapping 95% confidence intervals. A similar trend is seen in alkaloid content (for both hyoscyamine and scopolamine) based upon the 25 mg. root powder from the radioactive roots grown in the presence of puromycin for 1 and 2 weeks (Table III). However, on a single root basis, there is a statistically significant decrease at the 5% level in both hyoscyamine and scopolamine content between the control radioactive roots and the 2-week, puromycin-treated radioactive roots. The radioactive roots grown in the presence of puromycin for the 1st week only show no significant difference at the 5% level in alkaloid content (for both hyoscyamine and scopolamine) on the single root basis.

The results of radioactivity determinations of hyoscyamine and scopolamine are summarized in Table IV. When these results are expressed on a common base of radioactivity as disintegrations per minute per 25 mg. of the dried root powder for comparison, it is seen that puromycin treatment for 1 and 2 weeks causes a statistically significant increase at the 5% level in the uptake of L-proline-U-$^{14}$C as compared to the control. However, on a single root basis, only 1-week treatment with puromycin shows a statistically significant increase at the 5% level in the uptake of L-proline-U-$^{14}$C in both hyoscyamine and scopolamine content as compared to the control.

Table V summarizes the 95% confidence interval data for the total nitrogen content of *D. stramonium* var. *tatula* control and treated roots grown in nonradioactive nutrient solution. The results, on a single root basis, show that there is a statistically significant difference at the 5% level in the total nitrogen content among the control, the chloramphenicol-treated, the cycloheximide-treated, and the puromycin-treated roots. However, when these results are converted to a common base of total nitrogen content per 25 mg. of dried root powder, there is no statistically significant difference at the 5% level in the total nitrogen content of the control and the treated roots.

Table VI summarizes the protein content of the control and experimental roots grown in L-proline-U-$^{14}$C. The results show that there is a statistically significant decrease at the 5% level in the protein content on the single root basis between the control and the 1- and 2- week puromycin-treated roots. When these results are expressed on a common base of protein content per 250 mg. fresh weight, there is no statistically significant difference at the 5% level between the control and the puromycin-treated roots.

The results of radioactivity determinations of trichloroacetic acid-precipitable protein from the control and the puromycin-treated roots are summarized in Table VI. These results show that there is no statistically significant difference at the 5% level in the incorporation of radioactivity into trichloroacetic acid-precipitable protein on the single root basis between the control and the 1- and 2-week, puromycin-treated roots. When these results are expressed on a common base of radioactivity per 250 mg. fresh weight, only 1-week, puromycin-treated roots show a statistically significant increase at the 5% level.

## DISCUSSION

This investigation was undertaken to find the relationship, if any, between alkaloid synthesis and protein synthesis in *D. stramonium* var. *tatula*. The procedure involved the process of isolated tissue culture in which the excised roots of this plant were fed three classical inhibitors of protein synthesis in different concentrations in White's nutrient solution. A series of preliminary experiments with these inhibitors was conducted to find suitable concentrations which would inhibit the growth of the root cultures by approximately 50% as evidenced by the reduction in the dry weight. Puromycin at a concentration of 1.0 mg./50 ml. of the nutrient solution,

Table III—Alkaloid Content of *D. stramonium* var. *tatula* Roots, Control and Puromycin Treated (Mean Values)

| Root Groups | Dry Weight, mg. | Micrograms per Root | | Micrograms per 25 mg. Root Powder | |
|---|---|---|---|---|---|
| | | Hyoscyamine | Scopolamine | Hyoscyamine | Scopolamine |
| Ten control roots grown in nutrient solution containing 0.123 µc. of L-proline-U-$^{14}$C | 22.1 | 31.6 (23.3–39.9)[a] | 7.1 (5.0–9.2) | 35.9 (31.7–40.1) | 8.2 (5.9–10.5) |
| Ten roots grown in nutrient solution containing 0.123 µc. of L-proline-U-$^{14}$C and puromycin dihydrochloride for 1 week | 14.7 | 30.5 (27.3–33.7) | 6.2 (5.7–6.7) | 53.7 (42.3–63.1) | 10.8 (9.2–12.4) |
| Ten roots grown in nutrient solution containing 0.123 µc. of L-proline-U-$^{14}$C and puromycin dihydrochloride for 2 weeks | 8.2 | 16.4 (13.6–19.2) | 3.8 (3.1–4.5) | 49.7 (44.5–54.9) | 11.7 (9.7–13.8) |

[a] 95% confidence interval.

Table IV—Radioactivity of *D. stramonium* var. *tatula* Roots, Control and Puromycin Treated (Mean Values)

| Root Groups | Dry weight, mg. | Disintegrations per Minute per Root: Hyoscyamine | Disintegrations per Minute per Root: Scopolamine | Disintegrations per Minute per 25 mg. Dried Root Powder: Hyoscyamine | Disintegrations per Minute per 25 mg. Dried Root Powder: Scopolamine |
|---|---|---|---|---|---|
| Ten control roots grown in nutrient solution containing 0.123 μc. of L-proline-U-$^{14}$C | 18.8 | 100 (73–127)[a] | 24 (20–30) | 134 (105–163) | 34 (27–41) |
| Ten roots grown in nutrient solution containing 0.123 μc. of L-proline-U-$^{14}$C and puromycin dihydrochloride for 1 week | 15.9 | 388 (330–446) | 67 (43–91) | 636 (541–731) | 104 (71–137) |
| Ten roots grown in nutrient solution containing 0.123 μc. of L-proline-U-$^{14}$C and puromycin dihydrochloride for 2 weeks | 10.2 | 116 (83–149) | 12 (7–17) | 290 (245–335) | 34 (16–52) |

[a] 95% confidence interval.

Table V—Confidence Interval Data for Total Nitrogen Content of *D. stramonium* var. *tatula* Control and Treated Roots Grown in Nonradioactive Nutrient Solution

| Nutrient Solution | Mean Dry Weight, mg. | Total Nitrogen Content per Root, mcg.: Mean | Total Nitrogen Content per Root, mcg.: 95% *CI* | Total Nitrogen Content per 25 mg. Root Powder: Mean | Total Nitrogen Content per 25 mg. Root Powder: 95% *CI* |
|---|---|---|---|---|---|
| Control | 29.0 | 1228.0[a] | 1125.9–1330.0 | 1060.4 | 1015.8–1105.5 |
| Chloramphenicol treated | 18.1 | 720.9[b] | 575.9–865.9 | 988.8 | 931.8–1045.8 |
| Cycloheximide treated | 15.1 | 581.2[b] | 450.9–711.5 | 956.1 | 890.6–1021.6 |
| Puromycin treated | 9.8 | 397.4[c] | 320.2–474.6 | 1011.3 | 978.0–1044.6 |

[a] Mean of nine determinations. [b] Mean of 10 determinations. [c] Mean of 14 determinations.

chloramphenicol at a concentration of 32.35 mcg./50 ml., and cycloheximide at a concentration of 0.8 mcg./50 ml. of the nutrient solution showed a statistically significant reduction at the 5% level in growth as compared to the control. These protein inhibitors, in higher concentrations, showed a much greater inhibition of growth as evidenced by significant reduction in the dry weights; however, these concentrations were not used in this study because the greatly reduced metabolism possibly could have distorted the results. By maintaining the metabolism reasonably near normal, yet producing analytically determinable changes, it was believed that a more accurate picture of metabolic changes could be obtained. Therefore, the inhibitors were used in the concentrations that showed approximately a 50% reduction in root growth. Puromycin at the concentration used in this investigation showed a much greater inhibition of growth as compared to chloramphenicol and cycloheximide. All of these protein inhibitors had a pronounced effect on protein synthesis as evidenced by the decrease in growth.

Chloramphenicol and cycloheximide at the concentrations used showed no statistically significant difference in either hyoscyamine or scopolamine content as compared to the control.

Puromycin at a concentration of 1.0 mg./50 ml., however, did produce a statistically significant increase in the hyoscyamine content of the treated roots as compared to the control roots; the scopolamine content remained unaffected.

No definite conclusions can be derived about the scopolamine content from this investigation. Because of the lower content of this alkaloid, there was greater variability in the analyses. Therefore, the subsequent discussion will concentrate on hyoscyamine only.

On a single root basis, these inhibitors did show a statistically significant decrease at the 5% level in the total nitrogen content (as determined by the micro-Kjeldahl method) of the experimental roots as compared to the control roots. These results are indicative of the fact that these inhibitors did impair the protein biosynthesis machinery of the roots. When these results are converted to a common base of total nitrogen per 25 mg. root powder, there is no significant difference between the control and experimental roots.

From Table II, it is seen clearly that only puromycin shows a significant difference in the hyoscyamine content as compared to the control. Therefore, it was thought advisable to concentrate future work with the radioactive proline only in the presence of puromycin to achieve the objectives of this research, *i.e.*, to determine protein–alkaloid relationships.

Two sets of experiments were performed with puromycin and L-proline-U-$^{14}$C. In the first experiment the root cultures were exposed to puromycin for the 1st week; in the second the root cultures were exposed for 2 weeks. L-Proline-U-$^{14}$C was supplied for both weeks in these experiments. The results of the alkaloidal assays showed that there was a significant decrease in both the hyoscyamine and scopolamine content in the case of 2-week, puromycin-treated roots when the results were expressed on the per root basis. This may be explained by the fact that the overall metabolism in the roots was slowed down as evidenced by the reduction in the dry and fresh weights. However, when these results are converted to a common base of alkaloid content per 25 mg. dried root powder, both 1- and 2-week treatment with puromycin showed an increase in the hyoscyamine content. A similar increase in the L-proline-U-$^{14}$C

Table VI—Protein Content and Radioactivity of Protein of *D. stramonium* var. *tatula* Roots, Control and Puromycin Treated (Mean Values)

| Root Groups | Fresh Weight, mg. | Protein Content, mcg.: Per Root | Protein Content, mcg.: Per 250 mg. Fresh Weight, mg. | Fresh Weight, mg. | Disintegrations per Minute in Trichloroacetic Acid-Precipitable Protein: Per Root | Disintegrations per Minute in Trichloroacetic Acid-Precipitable Protein: Per 250 mg. Fresh Weight |
|---|---|---|---|---|---|---|
| Ten control roots grown in nutrient solution containing 0.123 μc. of L-proline-U-$^{14}$C | 219.1 | 1136.9 (954.1–1319.7)[a] | 1335.2 (1222.1–1448.3) | 237.5 | 14,670 (12,025–17,315) | 16,222 (12,972–19,472) |
| Ten roots grown in nutrient solution containing 0.123 μc. of L-proline-U-$^{14}$C and puromycin dihydrochloride for 1 week | 154.9 | 814.4 (643.9–984.9) | 1341.9 (1229.2–1454.6) | 131.6 | 13,189 (11,247–15,131) | 26,377 (22,384–30,370) |
| Ten roots grown in nutrient solution containing 0.123 μc. of L-proline-U-$^{14}$C and puromycin dihydrochloride for 2 weeks | 141.2 | 655.5 (527.5–783.7) | 1162.4 (1067.8–1257.0) | 143.8 | 10,794 (8,602–12,986) | 19,486 (14,500–24,472) |

[a] 95% confidence interval.

**Table VII**—Incorporation of L-Proline-U-$^{14}C$ into Hyoscyamine of *D. stramonium* var. *tatula* Roots Grown under Control and Treated Conditions

| Particulars | Disintegration per Millimole of Hyoscyamine Per Root | Per 25.0 mg. Root Powder |
|---|---|---|
| Control | $1.076 \times 10^6$ | $1.080 \times 10^6$ |
| One week, puromycin treated | $3.403 \times 10^6$ | $3.427 \times 10^6$ |
| Two weeks, puromycin treated | $1.645 \times 10^6$ | $1.689 \times 10^6$ |

incorporation into hyoscyamine was also seen in these roots. Table VII shows this increase more clearly when the data are expressed as disintegrations per minute per millimole of hyoscyamine. Thus, it can be seen that puromycin does increase the hyoscyamine content and incorporation of L-proline-U-$^{14}C$ into it.

There does not seem to be any significant change in the protein content (as determined by Lowry's method) of the puromycin-treated roots as compared to the control. This may be attributable to the secondary effect associated with puromycin, which consists of a breakdown of polysomes due to an acceleration of the ribosomes along the mRNA and subsequent release of the ribosomes from the polysomes (27–29). The proteins probably are constantly turning over and during this process the proline may be diverted to alkaloid synthesis. On a single root basis, however, there is a decrease in the protein content, which seems reasonable since both the dry weights and fresh weights are considerably reduced.

The incorporation of L-proline-U-$^{14}C$ into trichloroacetic acid-precipitable protein is not affected by either the 1- or 2-week treatment with puromycin. When the results are expressed on the 250-mg. fresh weight basis, there is a statistically significant increase in the incorporation of radioactivity into trichloroacetic acid-precipitable protein only in 1-week, puromycin-treated roots. Similar results were obtained by Stenesh and Shen (30), who worked on *Bacillus licheniformis* and *Bacillus stearothermophilus* 10. They showed that, under certain conditions, puromycin can actually lead to a stimulation of amino acid incorporation in cell-free systems. Whether stimulation or inhibition is observed depends on the concentration of puromycin, the temperature of incubation, the concentration of tRNA, and the age of the subcellular fraction.

The greater increase in the incorporation of L-proline-U-$^{14}C$ into hyoscyamine in the 1-week, puromycin-treated roots as compared to the 2-week, treated roots may be explained on the basis of increased enzyme activity of the key enzymes involved in hyoscyamine formation in the first case, since the inhibition was removed in the 2nd week of growth.

## CONCLUSIONS

1. Chloramphenicol, cycloheximide, and puromycin, under the conditions of this investigation, inhibit the growth of isolated root cultures of *D. stramonium* var. *tatula* as evidenced by the decrease in the dry and fresh weights. Growth habits of the cultures are also changed.
2. At the concentrations used for the three inhibitors tested, the puromycin-treated roots show a statistically significant increase in the hyoscyamine content and in the uptake of L-proline-U-$^{14}C$ into hyoscyamine by the isolated root cultures. Total protein appears unaffected.
3. Hyoscyamine production in *D. stramonium* var. *tatula* is increased by the use of puromycin in isolated root cultures. This is the first direct relationship demonstrated between protein synthesis and alkaloid production.

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