Incorporation of ^{14}C from Acetate and Mevalonate into Rishitin and Steroid Glycoalkaloids by Potato Tuber Slices Inoculated with Phytophthora infestans

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ABSTRACT

The accumulation of steroid glycoalkaloids and rishitin in potato tubers after cutting or cutting and inoculation with *Phytophthora infestans* appears due to de novo synthesis via the acetate-mevalonate pathway. The branch point in the synthetic pathway which leads to rishitin or the steroid glycoalkaloids appears to be after mevalonate. Uninoculated slices incorporated more ^{14}C from acetate and mevalonate into steroid glycoalkaloids than did slices inoculated with *P. infestans*. Slices inoculated with an incompatible race incorporated less isotope than those inoculated with a compatible race. Incorporation into rishitin was greatest with inoculated slices and most marked with slices inoculated with the incompatible race.

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Many compounds accumulate in the surface tissue of potato tubers infected by an incompatible race of *Phytophthora infestans* (Mont.) De Bary (4). Two of these compounds, the terpenoids rishitin and phytuberin (8, 10, 11) are believed to play a role in "R" gene resistance and their structure suggests they are synthesized via the acetate-mevalonate pathway. The steroid alkaloids are pseudoalkaloids which are considered simple derivatives of nitrogen-free steroid constituents. Their biosynthesis in both animals and

plants appears to utilize the acetate-mevalonate pathway (1). Guseva et al. (3) reported a significant incorporation of mevalonic acid-2-^{14}C and acetate-1-^{14}C into the steroid glycoalkaloids of potato seedlings, though this incorporation was much less than the incorporation into cholesterol from the same precursors in rat liver (2, 7). Incorporation of cholesterol-4-^{14}C into solanidine has been reported (9), and recently, Ripperger et al. (5) showed that lanosterol and cycloartenol were precursers of Solanum alkaloids. The content of solanine is very low in peeled healthy potato tubers. It markedly increases at the site of injury when tubers are cut, and the increase is markedly suppressed after inoculation with *P. infestans* (6).

The purpose of this investigation was to help ascertain whether: (i) acetate and mevalonate are precursors for steroid glycoalkaloids and rishitin in potato tubers; (ii) accumulation of steroid glycoalkaloids after cutting and rishitin after inoculation involve de novo synthesis; (iii) inoculation with *P. infestans* reduces incorporation of acetate and mevalonate into steroid glycoalkaloids and increases incorporation into rishitin (especially following inoculation with an incompatible race); (iv) the branch for rishitin biosynthesis in the acetate mevalonate pathway occurs at or before mevalonate; (v) suppression of steroid glycoalkaloid accumulation after inoculation occurs concomitantly with rishitin accumulation.

MATERIALS AND METHODS.–The sodium salt of acetate-1-^{14}C (Calatomic, specific activity 25 mc/mmole) was diluted with water and used in experiments. DL-Mevalonic acid-2-^{14}C (Calatomic, specific activity 2.25 mc/mmole) was obtained in the form of the dibenzylethylenediamine (DBED) salt. DBED was liberated by adding excess sodium bicarbonate to an aqueous solution of the commercial salt and removed by extraction twice with ether. Residual ether in the aqueous solution was removed with a stream of nitrogen, the solution was neutralized with 0.1 N HCl, and the mevalonic acid was ready for experiments.

Preliminary investigation of acetate incorporation into rishitin.–Spore suspensions were prepared as described (6). Kennebec potato tuber slices (ca. 1-cm thick) were inoculated with a spore suspension of race 4 (incompatible) of *P. infestans* and incubated in moistened petri dishes at 18 C for 48 hr. Fifty μc of acetate-1-^{14}C were dissolved in 20 ml sterile deionized water and 2 ml of solution/petri dish was evenly applied on the upper surface of the slices. The top and second mm of tissue from slices in two dishes were harvested 6, 12, 24, and 30 hr after addition of isotope. Approximately 20 g of tissue was obtained at each time interval, and it was immediately frozen and lyophilized. One g of the dried tissue was extracted with methanol in a microsoxhlet for 10 hr. The resulting extract was evaporated to dryness under reduced pressure in a rotary evaporator, and the residue was partitioned between 25 ml each of chloroform and 0.2 M aqueous acetic acid. The chloroform layer was separated, washed with 20 ml of deionized water and evaporated to dryness. The dry residue was washed into a 2-ml volumetric flask with methanol and the volume of the solution was adjusted with methanol. Two hundred μl of each sample, equivalent to 0.1 g dried tissue or 0.45 g fresh tissue, were applied on a channel of a 20 X20 cm silica gel (250 μ) thin-layer plate (Analtech, Inc.). The plate was developed with cyclohexane:ethyl acetate (1:1, v/v). Rishitin was detected by spraying the plate with vanillin-sulfuric acid reagent (3 g of vanillin and 0.5 ml of concd. H_2SO_4 added to 100 ml methanol) and heating at 120 C. The colored gel containing rishitin was scraped off with a razor blade and mixed with 15 ml of dioxane scintillation fluid in a scintillation vial. Radioactivity was measured with a Beckman CPM 100 liquid scintillation counter. Rishitin was the only compound detected in the rishitin fraction by two-dimensional thin-layer chromatography (TLC) and gas-liquid chromatography using two different liquid phases.

Isotope incorporations in cut and inoculated tissues.–Kennebec tuber slices were inoculated with a heavy spore suspension (ca. 4 X 10^5 zoospores/ml) of Race 4 (incompatible) or 1.2.4 (compatible) of *P. infestans* or covered with a film of deionized water. After incubation for 6 or 40 hr, acetate-1-^{14}C (1.8 X 10^6 cpm/petri dish containing 6-8 slices) or mevalonic acid-2-^{14}C (4.4 X 10^5 cpm/petri dish containing 6-8 slices) was applied uniformly to slice surfaces. The top mm layer of the slices was removed 7, 14 and 21 hr after isotope treatment and extracted immediately with chloroform:acetic acid: methanol (50:5:45, v/v). The extract was evaporated to dryness on a rotary evaporator and rishitin and steroid glycoalkaloids were separated by shaking the dried residue with equal volumes of chloroform and 0.2 M acetic acid; the steroid glycoalkaloids were in the aqueous acidic fraction, whereas rishitin was in the chloroform layer. The steroid glycoalkaloids were precipitated with concd. ammonium hydroxide solution (6) and purified twice by reprecipitation. Only the four steroid glycoalkaloids found in aged Kennebec slices were detected in this fraction when examined by TLC using five different solvent systems. Rishitin was isolated by TLC on silica gel as described earlier. Radioactivity in the steroid glycoalkaloid and rishitin fractions was counted with a liquid scintillation counter as described earlier.

RESULTS.–Measurement of the distribution of radioactivity after TLC of extracts of slices 6 hr and 12 hr after isotope application (54 hr and 60 hr after inoculation with Race 4 of *P. infestans*) showed that rishitin coincided with the largest zone of radioactivity on the TLC plate with the exception of the origin. After TLC, the band containing rishitin was free of acetate or mevalonate. Almost 3% of the total ^{14}C applied as acetate was incorporated into the rishitin fraction of the top mm 12 hr after application of isotope and 60 hr after inoculation with Race 4 (Table 1). Total incorporation was less thereafter though previous studies demonstrated that the total amount of rishitin was still increasing (6). A similar pattern was found in the second mm which was not

directly in contact with the fungus although necrosis was often visible in this layer of tissue.

The maximum incorporation of ^{14}C from mevalonate into the rishitin fraction was 8.5% 14 hr after application of the isotope and 54 hr after inoculation with Race 4 (incompatible) (Table 2). This was approximately three times the maximum percent incorporation for labeled acetate. The maximum incorporation of ^{14}C from acetate and mevalonate into the rishitin fraction after inoculation with race 1.2.4 (compatible) was 1.2% and 2.2%, respectively. The incorporation of ^{14}C from acetate and mevalonate into steroid glycoalkaloids was greater in slices aged for 40 hr as compared to those aged for 6 hr, and incorporation of ^{14}C was greater from mevalonate than acetate (Table 3). Inoculation with *P. infestans* reduced incorporation and the effect was most marked with the incompatible race and tissue treated with ^{14}C mevalonate.

DISCUSSION.—The magnitude of the incorporation of ^{14}C from acetate and mevalonate into rishitin and steroid glycoalkaloids suggests their synthesis involves the acetate-mevalonate pathway and occurs de novo. The greater efficiency of incorporation of ^{14}C from mevalonate than acetate is consistent with the fact that mevalonate appears after acetate in the biosynthetic pathway. It also may reflect greater competition for acetate by the tricarboxylic acid cycle, fatty acid synthesis and other metabolic functions. The maximum incorporation of ^{14}C from acetate is 11%, not 3%, when only the fractionof label "fixed" in the topmm of tissue is used as the basis for calculation (Table 1). The maximum incorporation of mevalonate (Table 2) is 17%, not 8.5%, when compensation is made for the fact that racemic mevalonate was applied but only one of its stereoisomers is biologically active. The greater accumulation of rishitin (6) in slices inoculated with incompatible vs. compatible races of *P. infestans* is consistent with the isotope

TABLE 1. Distribution of radioactivity at intervals after application of acetate-1-^{14}C to Kennebec tuber slices inoculated with *Phytophthora infestans* Race 4

Hr after isotope application[a]	Distribution of radioactivity (cpm × 10^{-4}/g dry wt) Total nonvolatile[b]	Methanol-$CHCl_3$ soluble	Rishitin fraction[c]	Rishitin fraction(%)[d]
6	130(26)[e]	47(12.8)	8.8(3.7)	1.76(0.74)
12	138(40)	64(18.5)	14.7(5.7)	2.94(1.14)
24	100(37)	42(15.5)	8.6(3.6)	1.72(0.72)
30	103(37)	50(18.2)	6.6(3.3)	1.32(0.66)

[a]Isotope applied 48 hr after inoculation with Race 4 (incompatible).
[b]Dry tissue was mix .l with scintillation fluid 10 hr before measurement.
[c]Silica gel scraped from TLC.
[d]Based on the total radioactivity applied to the slices (1.1×10^7 cpm/petri dish/2.2 g dry wt top or second mm of slice).
[e]Figures outside parentheses for top mm, whereas figures in parentheses are for the second mm.

TABLE 2. Incorporation of isotope from acetate-1-^{14}C and mevalonate-2-^{14}C into rishitin fractions in Kennebec potato tuber slices treated with water or inoculated with *Phytophthora infestans*

Isotope added[a]	Hr after isotope added	Incorp into rishitin fraction (%) Water	Race 1.2.4[b]	Race 4[b]
Acetate-1-^{14}C	7	0.50	0.48	2.33
	14	0.41	1.23	2.90
	21	0.33	1.00	2.20[c]
Mevalonate-2-^{14}C	7	0.15	0.65	2.1
	14	0.36	1.53	8.5
	21	0.38	2.20	7.4[d]

[a]Isotope added 40 hr after cutting or cutting and inoculation.
[b]Slices inoculated with *P. infestans* Race 1.2.4 (compatible) or Race 4 (incompatible) immediately after cutting.
[c]Accumulated 55 μg rishitin/g dry wt top mm.
[d]Accumulated 50 μg rishitin/g dry wt top mm.

TABLE 3. Incorporation of isotope from acetate-1-^{14}C and mevalonate-2-^{14}C into steroid glycoalkaloids of Kennebec potato tuber slices treated with water or inoculated with *Phytophthora infestans*

Isotope added	Time (hr)		Incorp into steroid glycoalkaloid fraction (%)[d]		
	Incubation[a]	Isotope added[b]	Water	Race 1.2.4[c]	Race 4[c]
Acetate-1-^{14}C	6	7	0.14	0.14	0.11
		14	0.22	0.18	0.19
		21	0.33	0.12	0.17
	40	7	0.73	0.12	0.15
		14	1.28	0.47	0.12
		21	1.43	0.49	0.12
Mevalonate-2-^{14}C	6	7	0.38	0.35	0.40
		14	1.57	0.54	---[e]
		21	2.38	---[e]	0.43
	40	7	2.70	0.52	0.54
		14	3.84	1.03	0.45
		21	4.50	2.02	0.52

[a]Hr after inoculation with fungus prior to isotope application.
[b]Hr after addition of isotope.
[c]Race 1.2.4 = compatible interaction; Race 4 = incompatible.
[d]Based on total isotope applied.
[e]Data not available.

incorporation data as is the suppression of steroid glycoalkaloid accumulation in slices inoculated with the fungus. The suppression of steroid glycoalkaloid accumulation and reduction of isotope incorporation is greatest in slices inoculated with the incompatible race. The decrease in percent incorporation of ^{14}C from acetate and mevalonate into rishitin after reaching a maximum suggests rishitin is not a stable end product.

LITERATURE CITED

1. BLOCH, K. 1965. The biological synthesis of cholesterol. Science 150:19-28.
2. BUCHER, N. L. R. 1953. The formation of radioactive cholesterol and fatty acids from C^{14}-labeled acetate by rat liver homogenates. J. Amer. Chem. Soc. 75:498.
3. GUSEVA, A. R., V. A. PASESHNICHENKO, & M. G. BORIKHINA. 1961. Synthesis of radioactive mevalonic acid and its use in the study of the biosynthesis of steroid glycoalkaloids from Solanum. Biochemistry (USSR) 26:631-635.
4. LOCCI, R., & J. KUĆ. 1967. Steroid alkaloids as compounds produced by potato tubers under stress. Phytopathology 57:1272-1273.
5. RIPPERGER, H., W. MORITZ, & K. SCHREIBER. 1971. Biosynthesis of Solanum alkaloids from cycloartenol or lanosterol. Phytochemistry 10:2699-2704.
6. SHIH, M., J. KUĆ, & E. B. WILLIAMS. 1972. Suppression of steroid glycoalkaloid accumulation as related to rishitin accumulation in potato tuber. Phytopathology 63:821-826.
7. TAVORMINA, P. A., M. H. GIBBS, & J. W. HUFF. 1956. The utilization of β-hydroxy-β-methyl-δ-valerolactone in cholesterol biosynthesis. J. Amer. Chem. Soc. 78:4498-4499.
8. TOMIYAMA, K., N. ISHIZAKA, N. SATO, T. MASAMUNE & N. KATSUI. 1968. "Rishitin," a phytoalexin-like substance. Its role in the defence reaction of potato tubers to infection. p. 287-292. *In* T. Hirai [ed.]. Biochemical Regulation in Diseased Plants or Injury. Kyoritsu Printing Co., Tokyo.
9. TSCHESCHE, R., & H. HULPKE. 1967. Biosynthesis of steroid derivatives in plants. VIII. Biogenesis of solanidine from cholesterol. Z. Naturforsch. 22b:791.
10. VARNS, J. L., & J. KUĆ. 1971. Suppression of rishitin and phytuberin accumulation and hypersensitive response in potato by compatible races of Phytophthora infestans. Phytopathology 61:178-181.
11. VARNS, J. L., J. KUĆ, & E. B. WILLIAMS. 1971. Terpenoid accumulation as a biochemical response of the potato tuber to Phytophthora infestans. Phytopathology 61:174-177.