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PRECURSORS OF RICININE IN THE CASTOR BEAN PLANT

TREVOR ROBINSON
Department of Biochemistry, University of Massachusetts, Amherst, MA 01003, U.S.A.

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Key Word Index—*Ricinus communis*; Euphorbiaceae; castor bean; ricinine; alkaloid biosynthesis.

Abstract—Isotopic tracer experiments confirmed that glycerol and succinic acid are good precursors of the pyridine ring of ricinine in castor bean plants. Tritium from C-2 was lost from tritiated glycerol while tritium from C-1 was retained. Thus a derivative of dihydroxyacetone is likely to be intermediate. By simultaneous feeding of glycerol-1-(3)-[^{3}H] and succinic acid-2(3)-[^{14}C], it was hoped to find precursors of ricinine containing both labels, but none could be found. There was no evidence for the appearance of labeled quinolinic acid, which is presumed to be a precursor of ricinine.

INTRODUCTION

Many previous results have demonstrated that the pyridine alkaloid ricinine of castor bean plants (*Ricinus communis* L.) is derived from nicotinic acid or other participants of the pyridine nucleotide cycle. The immediate precursor of compounds in this cycle is quinolinic acid, which is apparently simultaneously decarboxylated and glycosylated to make nicotinic acid monocucleotide. Further, it is known that quinolinic acid is derived by condensation of a C_3 fragment related to glycerol and a C_4 fragment related to succinic acid or aspartic acid [1, 2]. Aspects of this pathway that remain unknown are: (1) the exact components of the $C_3 + C_4 \rightarrow C_7$ reaction, (2) the nature of the reactions leading from members of the pyridine nucleotide cycle to ricinine, (3) whether there is any accumulation of quinolinic acid.

The experiments described here were intended to clarify these questions. The overall design involved feeding to castor bean plants variously tritiated glycerols and succinic acid-2,3-[^{14}C] either simultaneously, or separately to paired plants. Ricinine was then isolated, degraded and the ratio of ^{3}H/^{14}C in its pyridine ring determined. Any precursors of ricinine would require a ratio equal to or higher than this, since the pathway requires a loss of ^{3}H relative to ^{14}C (Scheme 1). Plant extracts were fractionated chromatographically in order to isolate compounds for determination of their isotopic ratios. In addition an attempt was made to detect quinolinic acid to see whether it was present with an isotopic ratio appropriate to its presumed role as a precursor.

RESULTS AND DISCUSSION

The data from several feeding experiments are shown in Table 1. Results of the degradation to locate the labeled atoms are given in Table 2. The label present in the ring plus cyano group of ricinine can be calculated by difference.

PC fractionation of *iso*-PrOH and aqueous extracts resulted in *ca* 10 unidentified radioactive spots containing both ^{3}H and ^{14}C; ratios of cpm varied widely. However, these data are not presented since no spot showed a ratio compatible with its being a precursor of the ricinine isolated in the same experiment. Such a precursor should have a ^{3}H/^{14}C ratio equal to or not more than 3 times the ratio found in the ricinine.

The ion exchange separation also revealed no components with a ^{3}H/^{14}C ratio that would suggest a role as a ricinine precursor. More specifically, although a standard quinolinic acid chromatographed as a sharp peak at about the middle of the gradient, the plant extracts showed neither ultraviolet absorption nor radioactivity at this position. Analysis of field-grown plants by a similar method indicated the presence of quinolinic acid, but it was not rigorously identified [3].

The excellent incorporations of label from glycerol-2-[^{14}C] into ricinine (Experiments 1A, 2A and 3) merely confirm the findings of others that glycerol is a good precursor of the pyridine ring. The data of Table 2 show

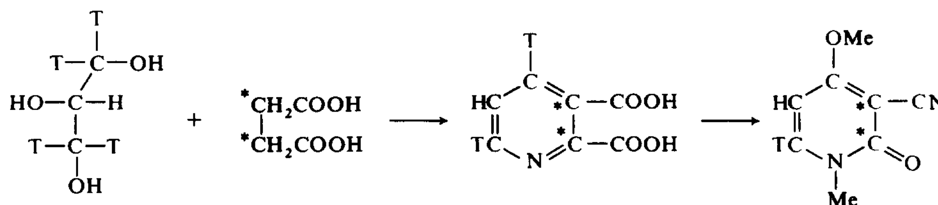

Fig. 1. Outline of pathway from glycerol plus succinic acid through quinolinic acid to ricinine. T denotes tritium as it would be distributed starting with glycerol-1(3)-[^{3}H]. Asterisks denote the location of ^{14}C starting with succinic acid-2(3):[^{14}C].

Table 1. Incorporation of label from various precursors into ricinine

Experiment	Compound fed	cpm supplied*	No. of plants	Age of plants (days)	Duration of exposure (hr)	Ricinine isolated (mg)	cpm in ricinine	Sp act of ricinine (cpm/mg)	Percentage incorporation of label	Ratio of $^3H/^{14}C$ in precursors	Ratio of $^3H/^{14}C$ in product
1A	Glycerol-2-[^{14}C]	3.84×10^7	8	37	24	38	4.56×10^4	1200	0.119	6.35	0.134
1B	Glycerol-2-[^3H]	2.44×10^8	8	37	24	19	6.12×10^3	323	0.0025		
2A	Glycerol-2-[^{14}C]	2.20×10^7	8	29	48	19.1	8×10^4	4190	0.364	14.8	4.69
2B	Glycerol-1(3)-[^3H]	3.26×10^8	8	29	48	24.4	3.75×10^5	15400	0.115		
3	Glycerol-2-[^{14}C]	8.45×10^6	5	132	6	10.9	5.5×10^3	505	0.065	—	-
4	Succinic acid-2(3)-[^{14}C] plus	4.78×10^7	7	56	18	19.6	4.7×10^5	24000	0.98	7.62	0.817
	glycerol-1(3)-[^3H]	3.64×10^8					3.84×10^5	17600	0.11		

* Of the total amount supplied *ca* 98 % was taken up by the plants.

that the label is in the ring rather than in the methyl groups. Experiment 3 is noteworthy for the incorporation obtained in a relatively brief time. The results with tritiated glycerol are important for suggesting the pathway followed by the glycerol unit. In the case of the pyridine ring of nicotine it has been shown [4] that D-glyceraldehyde is a more efficient precursor than glycerol. This result suggested that C-1 of glyceraldehyde-3-phosphate condenses with the amino group of aspartic acid to initiate pyridine ring formation. The expected pathway to aldehyde from glycerol would have as intermediates glyceryl-1-phosphate and dihydroxyacetone phosphate and would require loss of the hydrogen at C-2. The results of Experiment 1 argue for this pathway since only 2 % of the ^3H (from C-2) as the ^{14}C-2 itself reaches the ricinine in parallel experiments. The most likely explanation for this difference is a 2-keto intermediate. Experiment 2, in contrast, showed that ^3H from the C-1 or C-2 position was incorporated into ricinine. The ratio of 3.2 between ^{14}C and ^3H incorporation also fits the pathway shown in Fig. 1, in which it is evident that 3/4 of the ^3H from C-1 and C-3 should be lost in the ricinine. Chandler and Gholson [4] reported that when glycerol-1(3)-[^3H] was supplied to cell-free extracts from *E. coli*, which do incorporate label from glycerol-1-[^{14}C] into quinolinic acid, no ^3H was incorporated. This bacterial pathway, therefore, seems to differ from that in higher plants. In the former, it is probable that the glycerol is degraded to formate and that *N*-formyl-L-aspartic acid is the precursor of quinolinic acid [6, 7].

The data of Table 2, which are important to establish that very little label in the isolated ricinine was found in its methyl groups, are also of some interest in showing that glycerol is a better precursor of methyl groups than are C-2 or C-3 of succinic acid. This result would be expected from the close relationship of triose derivatives to serine and glycine as compared to the metabolic distance of succinate from these methyl group precursors. Yang and Waller [8] found a lower (0.13 %) total incorporation into ricinine than reported here in plants exposed to glycerol-2-[^{14}C] for 96 hr but a much higher fraction of the total was present in the methyl groups (about 1/5 in each).

The failure to identify any intermediates between the compounds fed and ricinine, in particular the apparent absence of labeled quinolinic acid, was a disappointing aspect of these experiments. Nevertheless, the technique used seems worthy of attention and of exploitation in other pathways where some product is derived from condensation of two unknown fragments. In the case of ricinine, the conclusion must be that no C_7 intermediates are allowed to accumulate, that whatever the rate-limiting step is, it must occur before the $C_3 + C_4$ condensation step. Alternatively, intermediates may be enzyme-bound or otherwise sequestered. Members of the pyridine nucleotide cycle might be expected to show a higher $^3H/^{14}C$ ratio than the ricinine for which they are precursors since their ribose moieties could readily be derived from glycerol-1(3)-[^3H] with retention of ^3H. Such nucleotides could have been among the high $^3H/^{14}C$ spots detected on chromatograms. If so, though, the failure to find substances with a $^3H/^{14}C$ ratio like that of the ricinine would indicate that freeing of the ricinine from a nucleotide derivative is probably a very late step in its biosynthesis. This late appearance of free ricinine was proposed long ago by Leete and Leitz [9] and was also suggested by the work of Waller *et al.* [10].

EXPERIMENTAL

Glycerol-2-[^{14}C], glycerol-2-[^3H], and succinic acid 2(3)-[^{14}C] were obtained from New England Nuclear. Glycerol-1(3)-[^3H] was obtained from Amersham/Searle. These substrates were checked for the presence of radioactive contaminants by PC and scanning as described below. Purification was found to be unnecessary. The castor bean plants were Hale variety from seed supplied by the U.S. Department of Agriculture, Texas Agriculture Experiment Station.

Seeds were planted in soil and germinated in the dark at 30°, then grown in an environmental chamber at 35° with 14 hr light and 10 hr darkness. Fluorescent light was supplemented with low intensity incandescent lamps. At times (indicated under Results) plants were fed labeled compounds by cotton wicks inserted through the stems [11]. Ricinine was extracted from the plants and purified by the method of ref. [12]. In Expts 3 and 4, the residue from $CHCl_3$ extraction was extracted

Table 2. Distribution of label in ricinine isolated from isotopic feedings

Compound fed	Total cpm in sample	cpm in *O*-methyl	% in *O*-methyl	cpm in *N*-methyl	% in *N*-methyl
Glycerol-2-[^{14}C]	3.12×10^5	1.15×10^4	3.68	1.77×10^4	5.68
Glycerol-1(3)-[^3H]	3.55×10^5	1.12×10^4	3.13	—	—
Glycerol-1(3)-[^3H]	3.04×10^5	1.4×10^4	4.62	8.85×10^3	2.92
Succinic acid-2(3)-[^{14}C]	8.14×10^5	3.55×10^3	0.43	1.98×10^3	0.244

further, first with *iso*-PrOH–H_2O (3:1) and finally with H_2O. Fractions obtained from purification of the ricinine-containing $CHCl_3$ extract were also saved for characterization of other radioactive constituents.

Isolated ricinine, after sublimation to constant sp. act. and determination of its $^3H/^{14}C$ ratio, was degraded by the method of ref. [13] in order to distinguish ^3H in Me groups from ^3H on the ring.

2D ascending PC used Whatman No. 1 paper with *iso*-PrOH–NH_4OH–H_2O, 13:1:6 in the first direction and *iso*-PrOH–HOAc–H_2O 13:3.3:3.7 in the second direction. Direct scanning of radioactivity was carried out with a radiochromatogram scanner after cutting the paper into strips. To differentiate between ^3H and ^{14}C the strips were scanned by detectors with and without a covering of polyvinylidine chloride food wrap ('Saran'). This shielding completely eliminated counts from ^3H with negligible effect on counts from ^{14}C. However, because of the great difference in efficiency of counting the 2 isotopes (*ca* 1% vs. *ca* 50%) this method of differentiation was adequate only to exclude from further consideration spots with very high ratios of $^3H/^{14}C$. For more accurate determination of ratios, radioactive zones were cut from the paper, eluted with 95% EtOH, the EtOH evapd in a stream of N_2 and 15 ml of Aquasol (New England Nuclear) added for scintillation counting.

The *iso*-PrOH–H_2O extract was diluted with H_2O and subdivided into cations, anions and neutral materials by the use of Amberlite IR-120 and Dowex-1 ion exchange columns. The 3 fractions were lyophilized. The anion and cation fractions were separately chromatographed on Dowex-1 after redissolving them in 0.075 M NH_4OAc, pH 7.5. After washing the column with 0.1 M NH_4OAc and H_2O, adsorbed material was eluted by a linear gradient of 0.05–0.5 N HCl followed by N HCl. Fractions (6 ml) were collected and any that contained radioactivity identified by streaking 20 μl aliquots across paper strips that were run through the scanner. Accurate determinations of $^3H/^{14}C$ ratios were made by counting 20 μl aliquots in the scintillation counter. UV absorption of the column effluent was also monitored.

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