MEVALONIC ACID AS A PRECURSOR IN THE BIOGENESIS OF DIGITOXIGENIN

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(±)-Mevalonic acid-2-^{14}C has been injected into a growing plant of *Digitalis lanata* L. to test if this acid serves as a precursor in the biosynthesis of cardenolides. After nine days a portion of the leaves was extracted and the constituents submitted to paper chromatography followed by autoradiography. The carbon 14 was detected in the lipid fraction and in the cardiac glycoside spots, but mostly in an area containing an as yet unidentified compound. Of the two glycosides forming the major glycoside area, one was predominant and, as after Mannich hydrolysis and chromatography, glucose, digitoxose and digitoxigenin were identified, this corresponds to lanatoside A. The aglycone fraction contained the radioactivity.

SINCE Kennedy's review[1] on the discovery of mevalonic acid lactone (3-hydroxy-3-methyl-pentane-5-lactone) and its possible role in sterol synthesis, a great deal of interest has been directed to this subject. Tavormina, Gibbs and Huff[2] demonstrated that mevalonic acid is converted efficiently to cholesterol by cell-free liver preparations and found that as much as 43·4 per cent of the radioisotope from (±)-mevalonic-2-^{14}C acid could be recovered as cholesterol after incubation with homogenates of rat liver. Tavormina and Gibbs further showed[3] that, in the utilisation of mevalonic acid to form cholesterol, the carboxyl group is lost during the conversion since the isotope labelling of the resulting cholesterol was negligible when carboxyl-labelled mevalonic acid was used, the radioactivity in these experiments being recovered mainly as carbon dioxide. The evidence indicates that mevalonic acid is the direct source of the isoprenoid units in squalene and sterol[4].

As the structures of the cardenolides are chemically closely related to the sterols, it appears likely that they also should derive from mevalonic acid. To test this hypothesis, mevalonic acid-2-^{14}C was fed to *Digitalis lanata* and allowed to metabolise for 9 days, and the selective incorporation of the radioactive carbon into steroids, including the main cardioactive glycoside, studied.

EXPERIMENTAL

Materials, Reagents and Procedures

Reference materials. Digitoxin U.S.P. reference standard and digoxin U.S.P. reference standard. *Paper.* Whatman No. 1, cut to 16 cm. × 57 cm. *X-ray film.* Kodak medical X-ray film, no-screen.

Solvent systems. For chromatography of sugars and glycosides, solvent system 1: butanol: glacial acetic acid: water (4 : 1 : 1). Solvent system 2, for glycosides: toluene: butanol (3 : 1) saturated with formamide, according to Satch and others[5].

Chromatographic procedures. All paper-chromatographic runs were carried out by the descending technique. In the case of solvent system 2 the filter paper was impregnated with formamide by passing the paper through a solution of formamide and acetone (1 : 4) and then allowing the acetone to evaporate.

Reagents. Mevalonic acid-2-^{14}C, from Tracerlab, Inc. (1·22 mc./mM of free mevalonic acid). Radiologically pure. The trichloracetic acid reagent used for the detection of cardiac glycosides was prepared as described by Jensen and Tennoe[6] modified to the extent that sodium hypochlorite was substituted for chloramine.

The *p*-anisidine hydrochloride reagent used for the detection of sugars was prepared as described by Bliss and Ramstad[7].

Preparation and Extraction of Plant Material

A volume of 0·15 ml. of (±)-mevalonic acid-2-^{14}C, representing 3·3 mg. of mevalonic acid having an activity of 15 μc, was injected into the leaf petiole and the stem of a young plant of *Digitalis lanata* by placing with a hypodermic syringe small drops of the dissolved acid on the petiole and stem and puncturing the veins of the tissue with the needle. Due to negative pressure within the plant, the fluid was sucked in and more solution added on the same area before it dried. As shown by means of a Geiger-Müller tube, the radioactivity spread throughout the whole leaf blade in the course of minutes; in the course of hours or days, it spread throughout the entire plant. After 9 days, 1·47 g. (fresh weight) of leaf was harvested from the injected plant. The leaf material was ground in a mortar with 95 per cent ethanol until a slurry was obtained. The liquid was filtered through cotton into a medicine dropper. This extraction was repeated three more times. The extracts were combined and concentrated at room temperature.

Chromatography of Initial Extractive

The concentrated extract was streaked on a piece of chromatographic paper at a distance of 13 cm. from one end (streak = 6541 c.p.m.). The streaked paper was chromatographed for 16 hours with solvent system 1. The paper chromatogram was allowed to dry thoroughly at room temperature and was then placed on X-ray film for a 45-hour exposure. The developed X-ray film showed the presence of four main radioactive areas on the chromatogram (Table I).

TABLE I

MAIN RADIOACTIVE AREAS

Area designation	Nature	R_F value
1	Cardiac glycosides	0·26
2	Unidentified compound, being the most radioactive	0·35
3	Mevalonic acid	0·65
4	Chlorophyll-sterol area	0·85–0·90

A 1·5 cm. strip was cut from the long side of the paper chromatogram and examined for the presence of cardenolides by means of the trichloracetic acid reagent spray. One of the major radioactive areas (R_F, 0·26; 2·5 cm. wide) as well as several minor radioactive areas, gave positive cardenolide reaction with trichloracetic acid reagent. As judged from their R_F values, none of the glycosides gave indication of being a secondary glycoside such as digitoxin or gitoxin.

Elution and Rechromatography of Area 1

Area 1 (major cardiac glycoside area) was cut from the chromatogram and eluted for 48 hours with 50 per cent ethanol. The eluate was concentrated at room temperature and spotted on two chromatographic papers. One paper was chromatographed by use of solvent system 1 (initial spot: 723 c.p.m.). The second paper was chromatographed with solvent system 2 (initial spot: 741 c.p.m.). The results are given in Table II.

Identification of the Major Glycoside in the Eluate and Demonstration that the Labelled Carbon of Mevalonic Acid is Present Only in the Alygcone

Mannich hydrolysis. As indicated in Table II, the radioactive cardiac glycoside area (area 1) consisted of two radioactive cardenolides. The low R_F indicated that they were primary glycosides such as lanatosides A, B or C. In each case, the glycoside having the lower R_F was the major glycoside. It was subjected to a Mannich hydrolysis. The procedure for the hydrolysis was essentially as described by Bliss and Ramstad[7] modified in that the reaction was carried out at 55° for 3 days instead of 21 days at room temperature. The materials subjected to the hydrolysis were: digitoxin U.S.P. reference standard, 3 mg.; digoxin U.S.P. reference standard, 3 mg.; and an aliquot of the eluate from area 1. At the end of the hydrolysis the solvent (acetone) was evaporated from each tube and a volume of 0·5 ml. of water and 0·5 ml. of chloroform was added and the tubes shaken.

TABLE II

RESULTS OF CHROMATOGRAPHY OF ELUATE FROM AREA 1

Solvent system	R_F of cardenolide spot*	Colour under ultra-violet light after treatment with trichloracetic acid reagent
1	0·02 (major spot)	Cream
	0·10	Light blue
2	0·14 (major spot)	Cream
	0·16	Light blue

* Each of the spots were radioactive as established by a 113-hour exposure to X-ray film.

The Sugars

The aqueous layers from the Mannich hydrolysis were drawn off and allowed to concentrate at room temperature. The residue was spotted on Whatman No. 1 paper and chromatographed with solvent system 1. At

the completion of the chromatography the dried chromatograms were sprayed with the *p*-anisidine hydrochloride reagent and heated for 10 minutes at 80–85° and then examined under ultra-violet light. The results are given in Table III.

TABLE III

RESULTS OF CHROMATOGRAPHY OF SUGARS FROM MANNICH HYDROLYSATE

Material hydrolysed	R_F value	Colour under ultra-violet light after treatment with *p*-anisidine hydrochloride reagent
Digitalis lanata		
Eluate from area 1 ..	0·08 (glucose)	Vivid yellow
	0·17 (digitoxose)	Reddish brown
Digitoxin	0·15 (digitoxose)	Reddish brown
Digoxin	0·16 (digitoxose)	Reddish brown

The two sugars in the eluate from area 1 of the *Digitalis lanata* experiment were identified as glucose and digitoxose from their R_F values and colour reactions with the reagent. Glucose and digitoxose constitute the sugar portion of the major glycosides of *Digitalis lanata.*

The paper chromatogram of the sugars from area 1 was exposed to X-ray film for a period of 21 days. Upon development of the film no radioactivity was noted to reside in the sugar spots.

The Aglycone

The chloroform layers of the Mannich hydrolysis were concentrated and each were spotted on formamide-treated paper. To the chloroform solution of the eluate from area 1 was added a chloroform solution of digitoxigenin. The paper was then chromatographed by use of solvent system 2. The chromatograms were treated with the trichloracetic acid reagent and then inspected under ultra-violet light. The results are given in Table IV.

TABLE IV

RESULTS OF THE CHROMATOGRAPHY OF CHLOROFORM LAYER FROM MANNICH HYDROLYSATE

Material hydrolysed	R_F values	Colour in ultra-violet light after treatment with trichloracetic acid reagent
Digoxin	0·72 (digoxigenin)	Cream
	0·78 (anhydro form of digoxigenin)	Blue
Digitoxin	0·63 (digitoxigenin)	Yellow
	0·82 (anhydro form of digitoxigenin)	Yellow
Eluate from area 1 *Digitalis lanata* ..	0·62 (digitoxigenin)	Yellow

The chromatogram of the eluate from area 1 was exposed to X-ray film for a period of 21 days. The outline of the radioactive area on the chromatogram matched exactly the area of the aglycone as outlined after treatment with trichloracetic acid reagent.

DISCUSSION

The label showed up in the lipidic fraction (sterols) and in the cardiac glycoside spots. The greatest amount of radioactivity was present in

a yet unidentified compound. The major radioactive glycoside area consisted of two glycosides, one of which was predominant. The predominant glycoside, upon Mannich hydrolysis, and chromatography furnished digitoxose, glucose and digitoxigenin and thus corresponds to lanatoside A, the major cardioactive glycoside of *Digitalis lanata*[8]. The aglycone contained all the activity of the glycoside; digitoxose and glucose contained no detectable radioactivity.

The hypothesis that mevalonic acid serves as a precursor in the biosynthesis of the cardiac glycosides has found justification in the experiment with *Digitalis lanata*. That mevalonic acid follows a direct path into the steroid cardenolides and not after the label has entered the general metabolism is supported by the observation that common cell metabolites were not found labelled and also from the fact that the sugar portion of the glycoside was unlabelled.

References

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