Biosynthesis of Plant Sterols. Conversion of Cholesterol to Pregnenolone in *Digitalis purpurea*[1]

In this paper we report that cholesterol is metabolized by *Digitalis purpurea* to several products, among them pregnenolone. Since TSCHESCHE and LILIENWEISS[2] have shown that pregnenolone is transformed by the plant into digitoxigenin, it follows that cholesterol *may* serve as a precursor of steroidal butenolides.

It is at present assumed that the biosyntheses of sterols in plants and in animals proceed by similar routes. This supposition implies that the primary plant sterol would be cholesterol, or an analogous triterpene, which would then be metabolized to the various sterols found in the plant. Hence, for example in *Digitalis purpurea*, cholesterol or its analog would be the precursor of sterols with a butenolide moiety[3]. LEETE et al.[4] have demonstrated that digitoxigenin can be biosynthesized from both 2-C^{14}-mevalonic acid (MVA) and 1-C^{14} acetic acid. However, carbons 22 and 23 in digitoxigenin biosynthesized from 2-C^{14}-MVA did not originate from the administered precursor, while on the other hand, the product obtained from 1-C^{14}-acetate contained C^{14} at C-23. These observations support the view that the initial step in the conversion of cholesterol to butenolides could be cleavage between C-20 and C-22 to yield pregnenolone or a similar C_{21}-20-ketone[4,5]. Subsequent addition of a 2 carbon unit, probably of acetate, could give, after appropriate biological transformations, sterols with a butenolide moiety[4,5]. For the theory to be tenable, we considered it important to determine whether indeed the plant has the capacity to metabolize cholesterol to pregnenolone and/or other C_{21}-C-20-ketones.

A sample[6] of 4-C^{14}-cholesterol (approx. 10 mg; $4 \cdot 10^8$ dpm) was divided into equal portions and each dissolved in 0.5 ml diglyme. The solutions were fed through leaves to 2 plants[7]. After 18 days the whole plants were removed from soil, cleaned, and rapidly dried at 120 °C. The extraction and preliminary fractionation of the crude extract ($2 \cdot 10^7$ dpm) was carried out according to the method described by GISVOLD[8]. The fraction consisting of glycosides, aglycones and other sterols contained about $2.7 \cdot 10^6$ dpm, and was hydrolyzed with aqueous methanolic hydrochloric acid.

To an aliquot (10%) of the above fraction, cold pregnenolone (40 μg) was added and the mixture was then chromatographed sequentially[9] as outlined in Table I. In the first chromatography several radioactive peaks were observed which are being investigated. The majority of radioactivity in the second chromatography was located in front of pregnenolone[10]. The radioactivity scans of chromatography 3 and 4 showed single symmetrical peaks which coincided with the pregnenolone spots.

A portion of the radioactive pregnenolone recovered from chromatography 4, approx. $2.5 \cdot 10^3$ dpm, was diluted with cold pregnenolone. The diluted material was twice crystallized from ethyl acetate and each crop counted. The twice crystallized solid was then acetylated and the product crystallized from methanol. 2 crops were taken and counted.

2 isolation experiments of pregnenolone from the extract were performed and the results are summarized in Table II.

Table I. Chromatographic isolation of radioactive pregnenolone from an aliquot (10%) of the metabolites

Chromatographic system (temperature 25 °C)	Rf	Radioactivity[a] in pregnenolone zone · 10^3 dpm
1. Propylene glycol-2% toluene/hexane	0.27	44.1
2. Hexane-methanol-water 100:70:30	0.68	7.0
3. Heptane-methanol-water 500:475:25	0.4	4.3
4. Propylene-glycol-4% toluene/hexane	0.37	2.9

[a] Corrections were made for the fractions removed for counting and color reactions.

Table II. Specific activity in dpm · 10^3 of pregnenolone and pregnenolone acetate

Sample	Experiment I		Experiment II	
	per mg	per m*M*	per mg	per m*M*
1st crystallization Crop 1	0.128	40.5	0.043	13.6
1st crystallization Crop 2			0.042	13.3
2nd crystallization Crop 1	0.128	40.5		
Acetate Crop 1	0.108	39.5	0.038	13.6
Acetate Crop 2	0.108	39.5	0.038	13.6

The counting was carried out in a Packard Instrument Co. Model 314 scintillation counter. The samples were dissolved in 16 ml of a solution of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg *p-bis*-[2-(5-phenyloxazolyl)]-benzene per 1000 ml. The samples were counted until at least $6 \cdot 10^3$ counts were recorded.

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[5] R. TSCHESCHE, Angew. Chem. *73*, 727 (1961); Fortschr. Chem. org. Nat. Stoffe *12*, 131 (1955).

[6] The sample appeared homogeneous and gave a single radioactive spot when tested in several thin layer and paper chromatographic systems.

[7] The feeding was carried out in the greenhouse of Harvard University, Cambridge, Massachusetts. On each of 2 large 2-year-old plants the top of a nearly full-grown leaf was cut off and its basal part folded and inserted into the solution. When active transpiration had caused the solutions to be taken up, more diglyme (0.2 ml) was added to the vials and fed via a different leaf. Upon completion of the feeding, the plants were freely watered. The feeding leaves died after about 48 h, but by that time the radioactivity had become distributed over other parts of the plant, especially in the root-crown.

[8] O. GISVOLD, J. Pharmac. Sci. *52*, 83 (1963).

[9] In all chromatograms pregnenolone was detected by its color test with phosphomolybdic acid on a 1.5 mm strip removed from the center of the chromatogram. Only the radioactive zones coinciding with pregnenolone, by color detection, were cut out and further processed.

[10] The Rf value of this product was 0.78, while pregnenolone showed an Rf value of 0.68.

It is apparent that *Digitalis purpurea* can utilize cholesterol and converts it to several metabolites. The important intermediate pregnenolone constitutes about 1% ($2.9 \cdot 10^4$ dpm) of the total metabolites ($2.7 \cdot 10^6$ dpm). This indicates a minimum 0.005–0.01% conversion of the administered cholesterol to pregnenolone.

Since the pregnenolone was characterized by dilution and recrystallization to constant specific activity of the free alcohol, as well as of the acetate, it was desired to provide additional evidence for its identity. With this in mind a third isolation experiment was carried out as above and the obtained pregnenolone ($20.9 \cdot 10^3$ dpm/mM) was converted to its acetate ($20.7 \cdot 10^3$ dpm/mM). This acetate was then submitted to Baeyer-Villiger oxidation as previously described by us[11]. Upon saponification of the recovered product the known 3β,5α,6β,17β-tetrahydroxy-5α-androstane was obtained[11] which showed a specific activity of $20.9 \cdot 10^3$ dpm/mM. This transformation corroborates fully the identity of the isolated metabolite.

The evidence presented tends to support the hypothesis that cholesterol or a closely related triterpene are the 'primary' sterols formed in the plant. It seems also that pregnenolone, or a related C_{21}-20-ketone may play an important role in the biosynthesis of butenolides[12]. In any event this constitutes proof that cholesterol may serve as a precursor in the synthesis of plant sterols[13,14].

Résumé. Un échantillon purifié de cholestérol-4-C^{14} a été absorbé par des feuilles de 2 plantes de *Digitalis purpurea*. Après 18 jours on a extrait les plantes et séparé les composés radioactifs. Environ 1% des métabolites globales a été constaté en forme de pregnénolone. Ainsi le cholestérol peut agir comme précurseur des stérols végétaux.

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Worcester Foundation for Experimental Biology, Shrewsbury and Harvard University, Cambridge (Massachusetts, USA), April 18, 1966.

[11] E. Caspi, D. O. Lewis, D. M. Piatak, K. V. Thimann, and A. Winter, J. Am. chem. Soc. *87*, 3224 (1965).

[12] R. D. Bennet and E. Heftmann, Science *149*, 653 (1965) have recently demonstrated the conversion of pregnenolone to progesterone in *Holarrhena floribunda*.

[13] Dr. R. D. Bennett and Mr. E. Heftman informed us that they have also isolated pregnenolone upon administration of cholesterol to *Haplopappas heterophyllus* (Phytochemistry, in press).

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Ceruloplasmin in Human Semen

Several enzymes have been detected in human semen: phosphatases[1,2], lactic dehydrogenase[3], transaminases[4–6] and nucleoside phosphorylase[7].

The presence of ceruloplasmin was suspected by us, since this fluid contains copper[8] and is very rich in mucoproteins[9]. Adrenaline oxidase has also been detected in human prostatic gland and seminal vesicle[1]. As ceruloplasmin is an oxidase for this amine[10], we thought it of interest to study its activity in human semen.

Material and methods. Semen samples from 21 men were used throughout this study and several tests were made to establish criteria of normality (sperm concentration, motility and vitality, volume of ejaculate, percentile quantity of abnormal forms). Men were classified into 3 groups: 5 normospermic (spermatozoa number between 56 and 150 millions/ml), 11 oligospermic (between 0.5 and 40 millions/ml) and 5 azoospermic.

Ceruloplasmin was determined enzymatically by the method of Ravin[11] and values were expressed in μg/100 ml.

Results. The results obtained are presented in the Table.

Normospermic semen showed very low values of ceruloplasmin (594 ± 87 μg/100 ml) as compared with dog semen (average 190 mg/100 ml). In this animal, copper values are also much more elevated than in humans (average 814 μg/100 ml)[4].

Condition	No. of cases	Spermatozoa number (millions/ml)	Ceruloplasmin (μg/100 ml)[a]
Normospermic	5	56–150	594 ± 87
Oligospermic	11	0.5–40	504 ± 199
Azoospermic	5	0	242 ± 55

[a] Mean $\pm$ standard deviation.

A statistically significant decrease of ceruloplasmin activity was observed in azoospermic samples in relation to normospermic ones: azoospermic/normospermic: $t = 3.417$, $P < 0.01$. Decrease for oligospermia was not statistically significant: $t = 0.415$, $P > 0.5$.

Résumé. La céruloplasmine a été déterminée dans le liquide spermatique de l'homme. On a trouvé des valeurs basses pour cette enzyme et une diminution significative au point de vue statistique a été observée dans les cas d'azoospermie.

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