Biosynthesis of Plant Sterols. VII. The Possible Operation of Several Routes in the Biosynthesis of Cardenolides from Cholesterol*

J. A. F. Wickramasinghe, P. C. Hirsch, S. M. Munavalli, and E. Caspi

ABSTRACT: The significance of the biosynthetic pathway, cholesterol → 20α-hydroxy cholesterol → pregnenolone → progesterone → cardenolides, in plants has been examined by the simultaneous administration of a mixture of [7-^{3}H]20α-hydroxycholesterol and [4-^{14}C]cholesterol (^{3}H/^{14}C ratio 8.68) to a *Digitalis lanata* plant. The *in vivo* transformation of the administered steroids to cardenolides was observed. In addition, [7-^{3}H]progesterone totally devoid of ^{14}C was isolated. The variations in the ^{3}H/^{14}C ratios of the cardenolides, digitoxigenin (6.74), digoxigenin (1.72), and gitoxigenin (2.58), may be viewed as being indicative of the operation of several biosynthetic routes. Apparently, the route involving progesterone is not the most significant pathway. These deductions are based on the assumption that the pool sizes and the relative rates of penetration to the active sites, of the two administered precursors, are comparable.

The biosynthesis of cholesterol from mevalonic acid in *Digitalis purpurea* (Jacobsen and Frey, 1967) has been demonstrated. The metabolism of cholesterol to pregnenolone (II) (Chart I) (Caspi *et al.*, 1966) and the incorporation of the latter to cardenolides has been proved (Tschesche and Lilienweiss, 1964; Tschesche and Brassat, 1966; Caspi and Lewis, 1967). Subsequently, the conversion of pregnenolone (II) into progesterone (III) (Caspi and Lewis, 1967) and of the latter into cardenolides was demonstrated in *Digitalis lanata* (Caspi and Lewis, 1967; Bennet *et al.*, 1968). The sequence of steps from pregnenolone to the C_{23} products requires the intermediacy of a 3-ketone, presumably of progesterone (Caspi and Hornby, 1968).

The degradation of the C_{27} precursor to C_{21} intermediates in the plant has been considered to be analogous to that incident in animals (Heftmann, 1967; Frantz and Schroepfer, 1967). Consequently, the sequence cholesterol → 20α-hydroxycholesterol (I) → pregnenolone (II) → progesterone (III) → cardenolides was implied to be the operating pathway in the biosynthesis of cardiac aglycones. The present work was undertaken with a view to examine the validity of this assumption.

The rationale of our approach was to administer a mixture of [4-^{14}C]cholesterol and [7-^{3}H]20α-hydroxycholesterol to a *D. lanata* plant and to evaluate the relative incorporation of the two tracers into cardenolides.

Experimental Section

Chromatography. Silica gel (Merck HF_{264}) was used for thin-layer chromatography in the indicated solvent systems. Strips (40 cm long) of Whatman paper No. 1 were used for paper chromatography. In the case of Bush systems, the papers were pretreated prior to development (Caspi and Lewis, 1967; Bush and Crowshaw, 1965).

The chromatographically homogeneous precursors and products were further checked for purity and identity by cocrystallization to constant specific activity and constant T/^{14}C ratios.

Counting. Counting was carried out in a Nuclear-Chicago automatic liquid scintillation counter, Model Mark I. The samples were dissolved in 15 ml of a scintillator solution of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis[2-(5-phenyloxazolyl)]benzene per 1000 ml.

Preparation of [7-^{3}H]20α-Hydroxycholesterol (Petrow and Stuart-Webb, 1956). A solution of [7-^{3}H]pregnenolone acetate (18.8 mCi) in benzene was added dropwise to excess of isohexylmagnesium bromide in ether, at 0°. The mixture was distilled until the distillate temperature reached 78° and gently refluxed thereafter for 3 hr. After cooling, the mixture was decomposed with ammonium chloride solution, and the product was isolated with benzene. The crude product was fractionated on thin-layer chromatography (silica gel; benzene–ethyl acetate (9:1)). The zone corresponding to 20α-hydroxycholesterol (I) was extracted, and the extract (15.7 mCi) was purified by chromatography on paper, in ligroin–toluene methanol–water (33:17:40:10, v/v) for 3.5 hr.

Administration of the Mixture of [7-^{3}H]20α-Hydroxycholesterol and [4-^{14}C]Cholesterol to D. lanata. The administration was carried out in early autumn. The coating of leaf wax was removed from the upper surfaces of the leaves by wiping gently with cotton wool moistened with ethyl acetate. A solution of the radioactive precursors, [7-^{3}H]20α-hydroxycholesterol (12.58×10^8 dpm of ^{3}H) and [4-^{14}C]cholesterol (1.45×10^8 dpm of

* From the Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545. *Received March 14, 1968.* The work was supported by Grants HE 10566 from the National Institute of Health and by a Career Program Award CA K3-16614 from the National Cancer Institute.

^{14}C) ($^{3}H/^{14}C$ ratio 8.68) in acetone (0.15 ml), was then deposited on to the leaves using a glass rod. After evaporation of the acetone, the leaves were sprayed with a 10% solution of silicon oil D.C. 200 in ligroin (bp 60–90°) (Caspi and Lewis, 1967). The plant was illuminated with light of 12,000 lm/m² for 16 hr each day and watered as necessary. The washings of the administration vial and glass rod were applied in the same manner 6 days later.

The Processing of the D. lanata Plant. After 5 weeks, the plant was harvested; the leaves were rinsed with ethyl acetate to remove unabsorbed radioactive materials and processed (Caspi and Lewis, 1967; von Euw and Reichstein, 1964). The leaves and stem were macerated with a razor blade. To the macerate was added 50 ml of water and 3 drops of toluene. This was stored at 23° for 40 hr. Then 20 ml of ethanol was added and the mixture was warmed to 70° and filtered. The residue was suspended in aqueous ethanol (50%;60 ml) and homogenized in a Waring blender. The resulting homogenate was again warmed to 70° and filtered. The residue was extracted seven times more in the same manner using 60-ml portions of aqueous ethanol. The concentration of the ethanol in the aqueous ethanol portions was increased stepwise from 55 to 90%. The volume of the combined aqueous alcoholic extracts was reduced *in vacuo* to about 30 ml and then treated with a suspension of $Pb(OH)_2$ (from 5 g of $Pb(OAc)_2 \cdot 3H_2O$) in ethanol (30 ml). The mixture was shaken vigorously for 10 min and filtered through Celite. The clear brownish filtrate was slightly acidified with dilute H_2SO_4 to pH 6 and concentrated *in vacuo* to 25 ml. The pH was checked at intervals and maintained at 6. The white precipitate was filtered, and the filtrate was extracted with three 50-ml portions of ligroin (bp 60–90). The combined ligroin extracts were washed twice with 75% aqueous ethanol, dried, and evaporated.

The aqueous phase and the above aqueous alcholic washings were combined and concentrated *in vacuo* to about 50 ml. This was then extracted with five 50-ml portions of chloroform. The combined chloroform extracts were washed with water (10 ml), 2 N NaOH (10 ml), and water (10 ml). The extract was then dried and evaporated.

The aqueous phase was combined with the first aqueous washing, and the total was extracted with three 50-ml portions of a mixture of chloroform–ethanol(2:1). The combined organic phase was washed with water (10 ml), 2 N NaOH (10 ml), and water (10 ml). The extract was then dried and evaporated.

The aqueous phase was combined with the first aqueous washing and the whole was concentrated *in vacuo* to about 35 ml; anhydrous Na_2SO_4 (3.5 g) was added and extracted with four 50-ml portions of a mixture of chloroform–ethanol (3:2). The combined organic phase was washed with 20 ml of half-saturated aqueous sodium sulfate, 2 N NaOH, and 20 ml of half-saturated aqueous sodium sulfate. The extract was dried and evaporated. The distribution of the isotopes in the various extracts is given in Table I.

Isolation of Digitoxigenin (IVa). An aliquot of the chloroform extract (20%) was dissolved in methanol (4 ml) and treated with 0.1 N H_2SO_4 (4 ml). The mixture was refluxed under nitrogen for 20 min. The methanol was removed *in vacuo*, and the aqueous residue was heated at 70° for a further 20 min. After cooling, the

CHART I

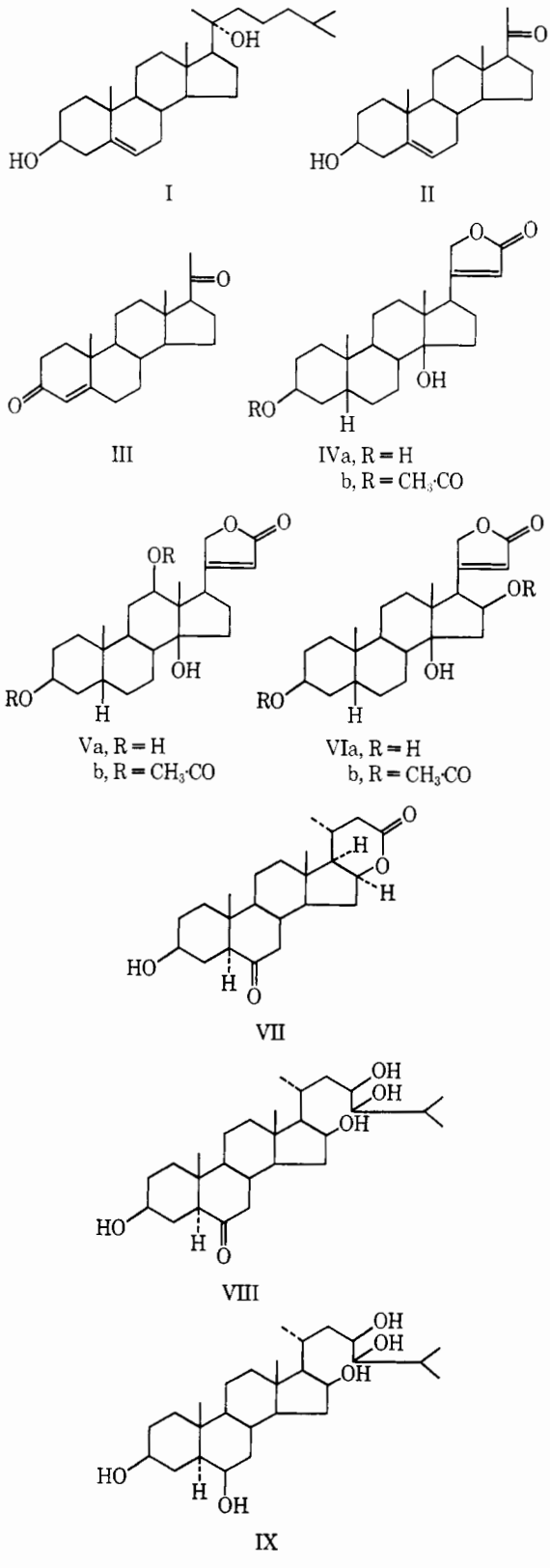

TABLE I: Distribution of Tracers in Extracts of *D. lanata* Plant after the Administration of [7-^{3}H]20α-Hydroxycholesterol (12.58 × 10^8 dpm) and [4-^{14}C]Cholesterol (1.45 × 10^8 dpm) (^{3}H/^{14}C ratio 8.68).

Extract	Radioactivity (× 10^7 dpm) ^{3}H	^{14}C	^{3}H/^{14}C Ratio
Leaf washings	10.85	1.41	7.7
Ligroin extract	0.44	0.065	6.78
Chloroform extract	7.71	0.50	15.38
Chloroform–ethanol (2:1)	1.25	0.06	19.58
Chloroform–ethanol (3:2)	1.54	0.04	35.0
Percentage recovery in sterol fractions[a]	8.7	4.6	

[a] Corrected for the unabsorbed radioactivity.

TABLE II: The ^{3}H/^{14}C Ratios of Digitoxigenin, Digoxigenin, and Gitoxigenin Recovered after the Administration of [7-^{3}H]20α-Hydroxycholesterol (12.58 × 10^8 dpm) and [4-^{14}C]Cholesterol (1.45 × 10^8 dpm) (^{3}H/^{14}C ratio 8.68) to a *D. lanata* Plant.

	^{3}H/^{14}C Ratio
Digitoxigenin	
1st crystallization	7.51
2nd crystallization	6.76
3rd crystallization	6.74
Digitoxigenin acetate	
1st crystallization	6.77
Digoxigenin	
1st crystallization	1.25
2nd crystallization	1.72
Digoxigenin diacetate	1.76
Gitoxigenin	
1st crystallization	2.53
2nd crystallization	2.58
Gitoxigenin diacetate	2.47

mixture was diluted to 30 ml and extracted with four 25-ml portions of ethyl acetate. The combined ethyl acetate extracts were washed twice with 15-ml aliquots of ice-cold water, once with 15 ml of ice-cold 2 N NaOH, and finally twice with 15-ml aliquots of ice-cold water. The extract was then dried and evaporated. To the hydrolyzed aglycones, nonradioactive digitoxigen (5 mg) was added and the mixture was fractionated on tlc (silica gel; benzene–ethyl acetate (1:4)). The digitoxigenin was located under ultraviolet light, and the area of radioactivity corresponding to this zone was extracted. The extract was then purified by continuous tlc (3 hr) (silica gel, benzene–methanol (50:1)). A single radioactive symmetrical peak coinciding with the zone of digitoxigenin was observed. The eluate of the zone was diluted with nonradioactive material (40 mg) and crystallized several times from ethyl acetate until the ^{3}H/^{14}C ratio was constant (Table II). On the basis of the ^{3}H and ^{14}C present in the digitoxigenin cocrystallized to constant specific activity and constant ratio, the digitoxigenin in the total chloroform extract contained 2.52 × 10^5 dpm of ^{14}C (equivalent to the incorporation of 0.174% of ^{14}C) and 1.7 × 10^6 dpm of ^{3}H (equivalent to the incorporation of 0.135% of ^{3}H). These results have been corrected for the amounts of the isotopes left in the vial from which the steroids were administered and for those found in the leaf washings.

Digitoxigenin Acetate (IVb). A sample of the diluted [^{3}H-^{14}C]digitoxigenin (11 mg) was converted into the 3β-acetate by treatment with acetic anhydride in pyridine. After a conventional work-up the acetate was crystallized from ethyl acetate and counted (Table II).

Isolation of Digoxigenin (Va) and Gitoxigenin (VIa). A portion of the hydrolysate of the chloroform extract (40%) was chromatographed on tlc (silica gel, benzene–ethyl acetate (1:4)). The radioactive zone posterior to the digitoxigenin zone was extracted and submitted to paper chromatography in ligroin (bp 90–120°)–toluene–*t*-butyl alcohol–methyl alcohol–water (5:5:3:4:6) for 6 hr. The digoxigenin and gitoxigenin zones were located by their color reaction with phosphomolybdic acid, and the areas of radioactivity corresponding to these zones were extracted separately. The two extracts were purified by sequential chromatography in the following systems: (i) paper chromatography in isopropyl ether–benzene–methanol–water (1:1:1:1) for 5 hr, (ii) thin-layer chromatography (silica gel, ethyl acetate–methanol (98:2), developed twice), and (iii) paper chromatography in system i for 5 hr. The purified digoxigenin zone contained 2.8 × 10^4 dpm of ^{14}C (equivalent to the incorporation of *ca.* 0.02% ^{14}C) and 4.9 × 10^4 dpm of ^{3}H (equivalent to the incorporation of *ca.* 0.004% ^{3}H). The purified gitoxigenin zone contained 3.7 × 10^4 dpm of ^{14}C (equivalent to the incorporation of *ca.* 0.03% ^{14}C) and 9.6 × 10^4 dpm of ^{3}H (equivalent to the incorporation of *ca.* 0.008% ^{3}H). The purified digoxigenin and gitoxigenin zones were mixed with nonradioactive digoxigenin (10 mg) and gitoxigenin (10 mg), respectively, and crystallized several times. The ^{3}H/^{14}C ratios are summarized in Table II.

Diacetates of Digoxigenin (Vb) and Gitoxigenin (VIb). Samples of the diluted [^{3}H-^{14}C]digoxigenin and gitoxigenin were acetylated and processed in an identical manner. The sample was dissolved in acetic anhydride pyridine (1:1) and stored for 16 hr at ambient temperature. After a conventional work-up, the recovered product was submitted to thin-layer chromatography (ethyl acetate–methanol (98:2)). The chromatographically homogeneous diacetate was counted (Table II).

Isolation of [7-^{3}H]Progesterone (III) from the Plant Extract. A portion of the hydrolysate of the chloroform extract (20%) was chromatographed on thin-layer chro-

matography (benzene–ethyl acetate (1:4)). The radioactive zone anterior to the digitoxigenin zone was extracted and submitted to continuous thin-layer chromatography (benzene–methanol (99:1)) for 7 hr. The progesterone zone was located under ultraviolet light, and the area of radioactivity corresponding to this zone was extracted. The extract was purified by chromatography on paper, in the system heptane–methanol–water (20:19:1, v/v) for 3.5 hr. The chromatogram was scanned for radioactivity, and the progesterone zone was located under ultraviolet light. A single radioactive symmetrical peak coinciding with the zone of progesterone was observed. The eluate of this zone, which contained only tritium (no ^{14}C), was mixed with nonradioactive progesterone (15 mg) and crystallized to constant specific activity (Table III).

TABLE III: The Specific Activity of [7-^{3}H]Progesterone[a] Isolated after the Administration of the Mixture of [7-^{3}H]20α-Hydroxycholesterol and [4-^{14}C]Cholesterol (a) to a *D. lanata* Plant and (b) to a Preparation of Bovine Adrenal Mitochondria.

	Isolation I ^{3}H (dpm/mg)	Isolation II ^{3}H (dpm/mg)
(a) Progesterone isolated from the plant[b]		
1st crystallization	1550	6318
2nd crystallization	1413	6060
3rd crystallization	1246	5632
4th crystallization	1192	5523
Mother liquor 4th crystallization	1176	5508
(b) Progesterone isolated from the adrenal incubation[b]		
1st crystallization	5018	
2nd crystallization	4848	
3rd crystallization	4837	
4th crystallization	4882	
Mother liquor 4th crystallization	4936	

[a] The progesterone was devoid of ^{14}C. [b] For the method of isolation, see Experimental Section.

The isolation of progesterone was repeated on another portion of the hydrolysate of the chloroform extract (40%). The extract of the progesterone zone from thin-layer chromatography (benzene–ethyl acetate (1:4)) was purified by sequential chromatography in the following systems: (i) thin-layer chromatography (dichloromethane–methanol (97:3), (ii) paper chromatography in heptane–methanol–water (20:19:1, v/v) for 3.5 hr, and (iii) thin-layer chromatography (benzene–methanol (97:3)). The purified progesterone zone containing only tritium (no ^{14}C) was mixed with nonradioactive progesterone (30 mg) and crystallized to constant specific activity (Table III).

Incubation of the Mixture of [7-^{3}H]20α-Hydroxycholesterol and [4-^{14}C]Cholesterol with a Bovine Adrenal Mitochondrial Preparation. Bovine adrenal mitochondrial acetone powder (170 mg) (Raggatt and Whitehouse, 1966) was suspended in supplemented sucrose (13 ml) (Halkerston *et al.*, 1961) and allowed to stand for 1 hr in ice. The mitochondria were resuspended by homogenization and centrifuged at 600*g* for 15 min. To the supernatant (6 ml) was added a cofactor solution (5 ml) containing (−)-malic acid (10.1 mg), ATP[1] (19 mg), β-NAD (21 mg), and 3,5-cyclic AMP (7 mg) in phosphate buffer (pH 7.4; 10 ml). This was incubated with the mixture of [7-^{3}H]20α-hydroxycholesterol (6.15×10^{6} dpm) and [4-^{14}C]cholesterol (6.7×10^{5} dpm) ($^{3}H/^{14}C$ ratio 9.14) in dimethylformamide (0.2 ml), containing 3β-hydroxy-Δ^{5}-cholenic acid (374 μg) for 3 hr at 37°. The incubate was then extracted several times with diethyl ether–benzene (4:1, v/v). The combined extracts were dried and evaporated. The residue was fractionated on thin-layer chromatography (dichloromethane–methanol (97:3); developed twice). The progesterone and pregnenolone zones were located under ultraviolet light, and the areas of radioactivity corresponding to these zones were extracted. The two extracts were purified separately by sequential chromatography in the following systems: (i) paper chromatography in heptane–methanol–water (20:19:1, v/v) for 3.5 hr and (ii) thin-layer chromatography (benzene–ethyl acetate (1:4)). The purified progesterone zone, which contained only tritium (no ^{14}C), was mixed with nonradioactive progesterone (25 mg) and crystallized to constant specific activity (Table III). Purification of the suspected pregnenolone zone from preliminary fractionation revealed the absence of any significant amount of radioactive pregnenolone.

Results and Discussion

Prior to the use of the mixture of [4-^{14}C]cholesterol and [7-^{3}H]20α-hydroxycholesterol in the plant experiment, we wished to evaluate the viability of the 20α-hydroxycholesterol (I) toward adrenal tissue. With this in view, the mixture of precursors was incubated with a bovine adrenal mitochondrial preparation (Raggatt and Whitehouse, 1966), and the produced progesterone (III) was isolated. The progesterone contained tritium only, indicating the preferential cleavage of the 20α-hydroxycholesterol.

Five weeks after the administration of the mixture of [7-^{3}H]20α-hydroxycholesterol and [4-^{14}C]cholesterol ($^{3}H/^{14}C$ ratio 8.68), the *D. lanata* plant was harvested and processed, in the manner described earlier (Caspi and Lewis, 1967; Caspi and Hornby, 1968; von Euw and Reichstein, 1964). The distribution of the two isotopes in the various extracts was determined (Table I). Con-

[1] Abbreviations are listed in *Biochemistry 5*, 1445 (1966).

siderable variations in the distribution were observed, but this is attributed to the limitations of the experimental design. The difficulties encountered in the interpretation of the results from experiments of this nature have been pointed out in previous papers (Caspi *et al.*, 1968; Burstein and Dorfman, 1962; Burstein and Bhavnani, 1967). Hence, the conclusions drawn from the present studies are subject to such errors incurred. We wish to stress, therefore, that the arguments presented and the deductions made below are valid, *only if there are no* significant differences in pool sizes and in the relative rates of penetration of the administered precursors, to the active sites.

Digitoxigenin (IVa), digoxigenin (Va), and gitoxigenin (VIa) were isolated from the chloroform extract. The $^{3}H/^{14}C$ ratios were: digitoxigenin, 6.74; digoxigenin, 1.72; and gitoxigenin, 2.58. It is evident at first sight that the isotopic ratios of the investigated cardenolides differ considerably within themselves and are significantly lower than that of the administered mixture of precursors (8.68). A corollary of these results is that the route involving 20α-hydroxycholesterol is less "significant" than the pathways originating from cholesterol. While the contribution of the route *via* 20α-hydroxycholesterol appears to be still relatively important in the biosynthesis of digitoxigenin (IVa), its relative merit was much less in the biosynthesis of digoxigenin (Va) and gitoxigenin (VIa). Hence, it appears that the biosynthesis of these three cardenolides from cholesterol proceeds by different routes. Furthermore, these routes do not involve a single common intermediate (*e.g.*, pregnenolone), as originally thought. Consequently it may be concluded that the route cholesterol → 20α-hydroxycholesterol (I) → pregnenolone (II) → progesterone (III) → cardenolides (or its variations, *e.g.*, *via* cholestenone to III) is neither the sole, nor the most important, pathway operating in the biosynthesis of cardenolides. This view was corroborated by the isolation of [7-^{3}H]progesterone totally devoid of ^{14}C, from the plant. From the absence of ^{14}C in the isolated progesterone, it may be inferred that the 20α-hydroxycholesterol isomer, which is the precursor of pregnenolone and progesterone in adrenal tissue, is also the precursor of these C_{21} steroids in the plant. The argument for the possible biosynthetic involvement of 20β-hydroxycholesterol cannot be sustained in view of the already-mentioned absence of ^{14}C in the isolated progesterone. This deduction would be valid provided that the 20β-hydroxylation process is not "totally" inhibited by the administered 20α-hydroxycholesterol.

It was indicated earlier that the administration of two different steroids to the plant provides a complex biological model. Results of such experiments may be influenced by differences in the relative rates of absorption, translocation, etc., of the exogenously supplied substrates, to the site of biosynthesis. It is possible that the biosynthesis of the three cardenolides may take place in several parts of the plant. However, the available evidence appears to indicate that the leaves are a major area of formation of cardenolides. Some support for this view is provided by the isolation of *de novo* biosynthesized (IVa, Va, and VIa) from excised *D. lanata* leaves, after the administration of either [7-^{3}H]pregnenolone or [7-^{3}H]progesterone (Caspi and Lewis, 1967; see also Tschesche and Brassat, 1965). In addition, in the present experiments, the progesterone isolated after the administration of the [4-^{14}C]cholesterol and [7-^{3}H]-20α-hydroxycholesterol contained only tritium, being devoid of ^{14}C. In contrast the $T/^{14}C$ ratios of the cardenolides indicate a preferential incorporation of ^{14}C from the [4-^{14}C]cholesterol.

Our results may be interpreted in two ways. It is possible that the observed large variations of the $T/^{14}C$ ratios of the products are the consequence of *differences in the penetration of the precursors to the sites of biosynthesis*. On the other hand, this may be the consequence of the operation of different pathways leading to the three cardenolides.

Fundamentally, two basic pathways from cholesterol to cardenolides in *D. lanata* may be envisaged. The route supported by present experimental evidence entails the degradation of the C_{27} steroid to a C_{21} intermediate, which subsequently adds on the equivalent of a two carbon unit, to yield the C_{23} products. Alternatively, the scission of the side chain of cholesterol between C-23 and C-24, and the incorporation *in toto* of the resulting C_{23} moiety into cardenolides, may be visualized. Our observations indicate that the route cholesterol → pregnenolene (II) → cardenolides is not the most "significant" pathway. Consequently, if indeed the biosynthesis of cardenolides requires the obligatory formation of a C_{21} intermediate, then it is likely that structural modifications of the cholesterol nucleus, *e.g.*, saturation of the C-5 double bond and/or hydroxylation, precede the side-chain cleavage. The feasibility of a pathway involving the cleavage of the cholesterol side chain between C-23 and C-24 is indicated by the recent isolation of the C_{23}-steroidal lactone, chiogralactone (VII) (Takeda *et al.*, 1965) together with chiograsterols A (VIII) and B (IX) (Takeda *et al.*, 1968), from the steroidal components of *Chionographis japonica*. Experiments are in progress at present to evaluate the merits of these possibilities.

References

Bennett, R. D., Sauer, H. H., and Heftmann, E. (1968), *Phytochemistry 7*, 41.

Burstein, Sh., and Bhavnani, B. (1967), *Endocrinology 8*, 351.

Burstein, Sh., and Dorfman, R. I. (1962), *Acta Endocrinol. 40*, 188.

Bush, I. E., and Croshaw, K. (1965), *J. Chromatog. 19*, 114.

Caspi, E., and Hornby, G. M. (1968), *Phytochemistry 7*, 423.

Caspi, E., and Lewis, D. O. (1967), *Science 156*, 519.

Caspi, E., and Lewis, D. O. (1968), *Phytochemistry 7*, 7332.

Caspi, E., Lewis, D. O., Piatak, D. M., Thimann, K. V., and Winter, A. (1966), *Experientia 22*, 506.

Caspi, E., Wickramasinghe, J. A. F., and Lewis, D. O.

(1968), *Biochem. J. 108*, 499.
Frantz, I. D., and Schroepfer, Jr., G. J. (1967), *Ann. Rev. Biochem. 36*, 691.
Halkerston, I. D. K., Eichhorn, J., and Hechter, O. (1961), *J. Biol. Chem. 236*, 374.
Heftmann, E. (1967), *Lloydia 30*, 209.
Jacobsen, G. M., and Frey, M. J. (1967), *J. Am. Chem. Soc. 89*, 3338.
Petrow, V., and Stuart-Webb, I. A. (1956), *J. Chem. Soc.*, 4675.
Raggatt, P. R., and Whitehouse, M. W. (1966), *Biochem. J. 101*, 819.
Takeda, K., Minato, H., and Shimaoka, A. (1968), *Chem. Commun. 2*, 104.
Takeda, K., Shimaoka, A., Iwasaki, M., and Minato, H. (1965), *Chem. Pharm. Bull. (Tokyo), 13*, 691.
Tschesche, R., and Brassat, B. (1965), *Z. Naturforsch. 20b*, 707.
Tschesche, R., and Brassat, B. (1966), *Z. Naturforsch. 21b*, 894.
Tschesche, R., and Lilienweiss, G. (1964), *Z. Naturforsch. 19b*, 226.
von Euw, J., and Reichstein, T. (1964), *Helv. Chim. Acta 47*, 711.

Self-Associating Systems. I. Multinomial Theory for Ideal Systems*

Moisés Derechin

ABSTRACT: A general expression for the calculation of the equilibrium constants of self-associating ideal systems has been established. The derivations make use of the multinomial theorem. This general expression is applicable to associating systems of any degree of polymerization irrespective of whether the weight-, M_w or the number-average, M_n, molecular weight is used in the calculations. Using this expression, the equilibrium constants, from K_1 to K_4, for self-associating systems have been derived.

Many substances in solution behave as self-associating systems. The biological activity of some macromolecules, *e.g.*, subunit-type enzymes, appears to be closely related to their state of aggregation. The need to clarify some aspects of this relation in some of the systems currently under study in our laboratory was the main stimulus for this work. A theory will be presented that will allow by means of a single general expression the analysis of experiments that supply molecular weight averages, irrespective of whether these are either the weight average or the number average. A fundamental step in this work is the use of the multinomial theorem (see Parzen, 1960) and for this reason it is designated as the multinomial theory.

Theoretical

We are going to examine self-associating reactions of the type

$$nP_1 \rightleftarrows a_2P_2 + a_3P_3 + \ldots + a_mP_m, \text{ with } \sum_{i=2}^{m} ia_i = n \quad (1)$$

It is assumed that all species participating in the self-associating reaction have the same partial specific volume ($\bar{v}_1 = \bar{v}_2 = \ldots = \bar{v}$), the same refractive index increment [$(dn/dc_1)_{T,P} = (dn/dc_2)_{T,P} = \ldots = (dn/dc)_{T,P}$], and that the activity coefficient of each associating species can be represented as

$$\ln y_i = iBM_1c + \text{higher powers in } c \quad (2)$$

where B is the virial coefficient, M_1 is the molecular weight of the monomer, and c is the total solute concentration. The higher powers in c are neglected. In the particular case $i = 1$, it is

$$\ln y_1 = BM_1c \quad (2a)$$

For ideal systems, $BM_1 = 0$. Using eq 2 and 2a and the nomenclature of Adams and Fujita (1963), the condition for chemical equilibrium can be stated in terms of the equilibrium constants, K_i, as

$$c_i = K_i \frac{y_1^i}{y_i} c_1^i = K_i c_1, \qquad i = 1, 2, \ldots \quad (3)$$

Consequently, $K_1 = 1$. Also, since

$$c = \sum_{i=1}^{m} c_i, \qquad i = 1, 2, \ldots \quad (4b)$$

* From the Molecular Enzymology Unit, Departments of Biochemistry and Biochemical Pharmacology, and the Center for Theoretical Biology, State University of New York, Buffalo, New York. *Received April 16, 1968.*