QUARTERLY REVIEWS

BIOSYNTHESIS OF STEROLS, STEROIDS, AND TERPENOIDS. PART II. PHYTOSTEROLS, TERPENES, AND THE PHYSIOLOGICALLY ACTIVE STEROIDS

By R. B. CLAYTON

(DEPARTMENT OF PSYCHIATRY, STANFORD UNIVERSITY SCHOOL OF MEDICINE PALO ALTO, CALIFORNIA)

The Biosynthesis of Phytosterols and Tetracyclic Triterpenes

THE steps involved in the generation of the isoprene units of terpenoid compounds and in the biosynthesis of cholesterol have been described in Part I.* There is good experimental evidence that the pathways of biosynthesis of ergosterol (LXXVII)[157–159] and of the C_{31} tetracyclic triterpene, eburicoic acid (LXXVIII)[160–161] involve the same intermediates that are involved in cholesterol biosynthesis at least as far as the first tetracyclic cyclisation product of squalene. Indeed, the isolation of cholesterol from red algae[162] and, more recently, from the potato plant[163] demonstrates the presence of the whole enzymic mechanism in certain plant tissues, for the synthesis of this characteristically "animal" sterol. The origin of the "extra" carbon atoms substituted at $C_{(24)}$ in side chains of plant and fungal sterols and triterpenes, and the structure of the steroid or triterpene acceptor molecule in the alkylation are problems of particular interest.

The origin of the $C_{(28)}$-methyl carbon of ergosterol in the one-carbon pool (labelling from formate) was demonstrated by Danielsson and Bloch[164] who interpreted their results to exclude the methylation of squalene before

* Part I, Clayton, *Quart. Rev.*, 1965, **19**, 168 (preceding Review). References in Part II which appeared in Part I are as follows: [12]Samuels, "Metabolic Pathways", ed. Greenberg, Academic Press, New York, 1960, p. 471. [14]Bergström, Danielsson, and Samuelsson, "Lipid Metabolism", ed. Bloch, Wiley Sons, New York, 1960, p. 291. [15]Danielsson, "Advances in Lipid Research," ed. Paoletti and Kritchevsky, Vol. 1, Academic Press, New York, 1963, p. 335. [43]Bernfeld, "The Biogenisis of Natural Compounds", Pergamon Press, New York, 1963. [75]Popják, Goodman, Cornforth, Cornforth, and Ryhage, *J. Biol. Chem.*, 1961, **236**, 1934. [107]Arigoni, CIBA Foundation Symposium: Biosynthesis of Terpenes and Sterols, ed. Wolstenholme and O'Connor, Little, Brown, and Co., Boston, 1959. [108]Birch and Smith, ref. 107, p. 245. [109]Birch, Kocor, Sheppard, and Winter, *J.*, 1962, 1502.

[157] Hanahan and Wakil, *J. Amer. Chem. Soc.*, 1963, **75**, 273.

[158] Dauben and Hutton, *J. Amer. Chem. Soc.*, 1956, **78**, 2647.

[159] Dauben, Hutton, and Boswell, *J. Amer. Chem. Soc.*, 1959, **81**, 403.

[160] Dauben, Ban, and Richards, *J. Amer. Chem. Soc.*, 1957, **79**, 968.

[161] Dauben and Richards, *J. Amer. Chem. Soc.*, 1956, **78**, 5329.

[162] Tsuda, Agaki, Kishida, Hayatsu, and Saka, *Chem. Pharm. Bull., Tokyo*, 1958, **6**, 724.

[163] Johnson, Bennet, and Heftmann, *Science*, 1963, **140**, 198.

[164] Danielsson and Bloch, *J. Amer. Chem. Soc.*, 1957, **79**, 500.

its cyclisation. The methylene group of eburicoic acid has also been shown by Dauben and his co-workers to be derived from a one-carbon source and not from acetate.[159,165]

More detailed studies by Alexander *et al.*[166] of the origin of $C_{(28)}$ in ergosterol have implicated methionine as the methyl donor, since incorporation of the labelled methyl group from this source was more efficient than from formate and took place without change of the $^{14}C/^{3}H$ ratio from [^{14}C,^{3}H]methyl-labelled methionine. Subsequently, *S*-adenosylmethionine was shown to be a more effective methyl donor than methionine[167].

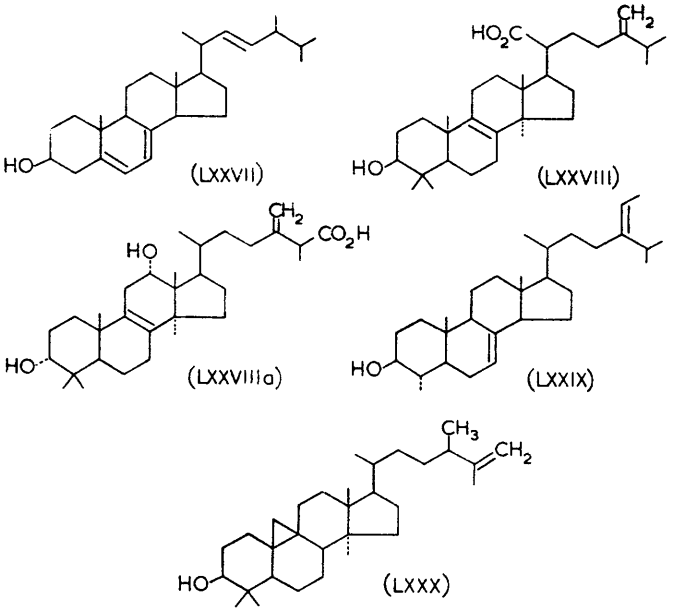
The conclusion was drawn by Alexander *et al.*,[168] on the basis of their double-labelling experiment, that no loss of hydrogen from the methyl group of methionine occurred during its incorporation as $C_{(28)}$ of ergosterol. This conclusion is open to question on the grounds that the isotope effect would favour selective loss of hydrogen rather than tritium, from the migrating methyl group. Lederer and his co-workers[168] have recently re-examined the methylation process in this light, using [$^{2}H_3$]methyl-labelled methionine as the methyl donor for ergosterol synthesis in a methionine-less stain of *Neurospora crassa*. Mass spectrometry of the ergosterol isolated from the organism indicated the incorporation of only two deuterium atoms per molecule.

The introduction of the $C_{(28)}$ carbon atom of ergosterol by transfer from methionine was the first reported example of *C*-methylation from this source, but similar *C*-methylations have since been shown to occur in the formation of the cyclopropane ring in cyclopropane fatty acids,[169–171] and the branching methyl group of tuberculostearic acid (10-methylstearic acid)[172] and in other microbiological processes.[173,174] Experiments with CD_3-methionine[175] have shown that in tuberculostearic acid, as in ergosterol, the incorporation of the methyl group entails an exchange of one hydrogen atom. The acceptor molecule in this reaction is an unsaturated fatty acid derivative in which the ethylenic linkage is the acceptor site.

There is so far little biochemical evidence to indicate the structure of the biosynthetic intermediate which undergoes the alkylation reaction at $C_{(24)}$. There is evidence[176] for the conversion of lanosterol but not zymosterol into ergosterol in yeast and the structure of citrostadienol

[165] Dauben, Fonken, and Boswell, *J. Amer. Chem. Soc.*, 1957, **79**, 1000.
[166] Alexander, Gold, and Schwenk, *J. Amer. Chem. Soc.*, 1957, **79**, 2967.
[167] Parks, *J. Amer. Chem. Soc.*, 1958, **80**, 2023.
[168] Lederer, *Experientia*, 1964, **20**, 473.
[169] O'Leary, *J. Bacteriol.*, 1959, **78**, 709.
[170] Liu and Hofmann, *Biochemistry*, 1962, **1**, 189.
[171] Zalkin, Law, and Goldfine, *J. Biol. Chem.*, 1963, **238**, 1242.
[172] Lennarz, Scheuerbrandt, and Bloch, *J. Biol. Chem.*, 1962, **237**, 664.
[173] Schwenk, Alexander, Gold, and Stevens, *J. Biol. Chem.*, 1958, **233**, 1211.
[174] Birch, English, Massey-Westrop, Slaytor, and Smith, *J.*, 1958, 365.
[175] Jaureguiberry, Law, McCloskey and Lederer, *Compt. rend.*, 1964, **258**, 3587.
[176] Schwenk and Alexander, *Arch. Biochem. Biophys.*, 1958, **76**, 65.

(LXXIX),[177,178] isolated from citrus fruits, suggests that in these higher plant tissues also the acceptor molecule is a Δ^{24}-sterol preceding zymosterol in the biosynthetic pathway. The small degree of conversion of cholesterol into 24-methylenecholesterol in clams[179] could be attributable to the desaturation of cholesterol, possibly to desmosterol, by these organisms.[180] The occurrence of such compounds as the polyporenic



acids (LXXVIIIa),[181,182] eburicoic acid (LXXVIII),[183] and cyclolaudenol (LXXX),[184] which contain some variant of the lanosterol structure with addition of a carbon atom at $C_{(24)}$, suggests that lanosterol itself may be the acceptor in these cases. The structure of cyclolaudenol, with an unsaturation in the 25,26-position, suggests that the methylation reaction involves the formation of a carbonium ion intermediate for which several possible routes of stabilisation may be available depending upon the characteristics of the enzyme involved. Hypothetical schemes are shown in Fig. 19 to account for the formation of sterol and triterpene side chains of the 24-methylene (LXXXI), 24-methyl (LXXXII), and 25-methylene (LXXXIII) types and the cyclopropane fatty acids (LXXXIV). It is now known that methionine provides both carbon atoms of the ethyl group

[177] Mazur, Weizmann and Sondheimer, *J. Amer. Chem. Soc.*, 1958, **80**, 1007, 6293.
[178] Mazur and Sondheimer, *J. Amer. Chem. Soc.*, 1958, **80**, 6296.
[179] Fagerlund and Idler, *Canad. J. Biochem. Physiol.*, 1961, **39**, 1347.
[180] Fagerlund and Idler, *Canad. J. Biochem. Physiol.*, 1961, **39**, 505.
[181] Roth, Saucy, Anliker, Jeger, and Heusser, *Helv. Chim. Acta*, 1953, **36**, 1908.
[182] Halsall and Hodges, *J.*, 1954, 2385.
[183] Holker, Powell, Robertson, Simes, Wright, and Gascoigne, *J.*, 1953, 2422.
[184] Henry, Irvine, and Spring, *J.*, 1955, 1607.

substituted at $C_{(24)}$ in the C_{29}-sterols, β-sitosterol (LXXXVII),[185,186] and spinasterol (LXXXVIII)[187] and of the ethylidene group in fucosterol (LXXXIX).[168] It seems that a 24-methylene substituent (LXXXI) may either remain as such (*e.g.*, 24-methylenecholesterol)[188,189], be reduced to 24-methyl (ergosterol type), or be remethylated, giving a 24-ethylidene-sterol (LXXXV) (fucosterol type) which, in turn, may be reduced to the 24-ethyl derivative (LXXXVI) (sitosterol type). A similar scheme has been suggested by Castle *et al.*[185] and several possible mechanisms of these reactions are discussed by Lederer.[168]

The recent findings concerning the origin of the 24-ethyl group in the C_{29} sterols offer a new possibility of studying the mechanism of these methyl group-transfer reactions since the simple mechanisms shown in

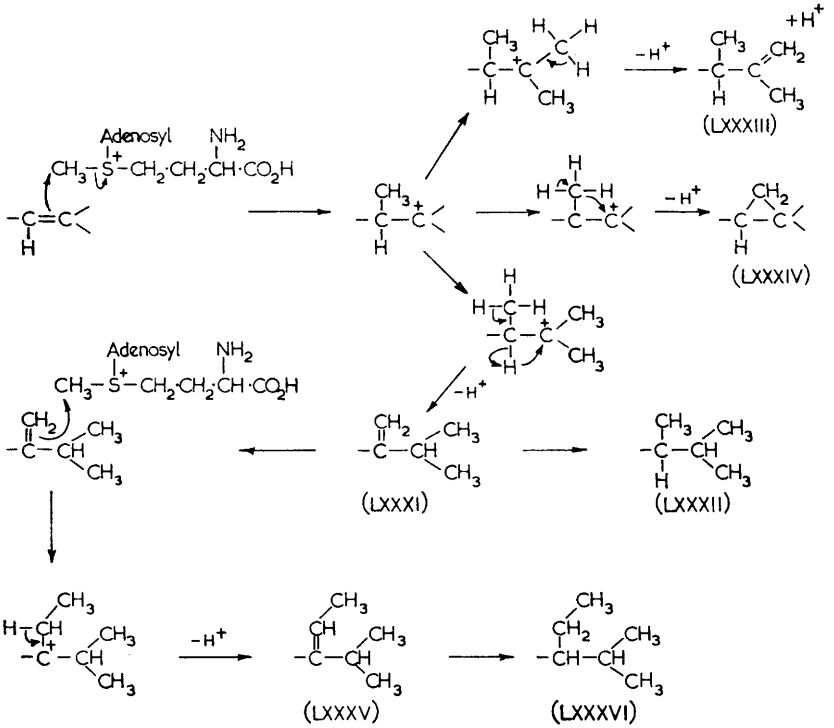
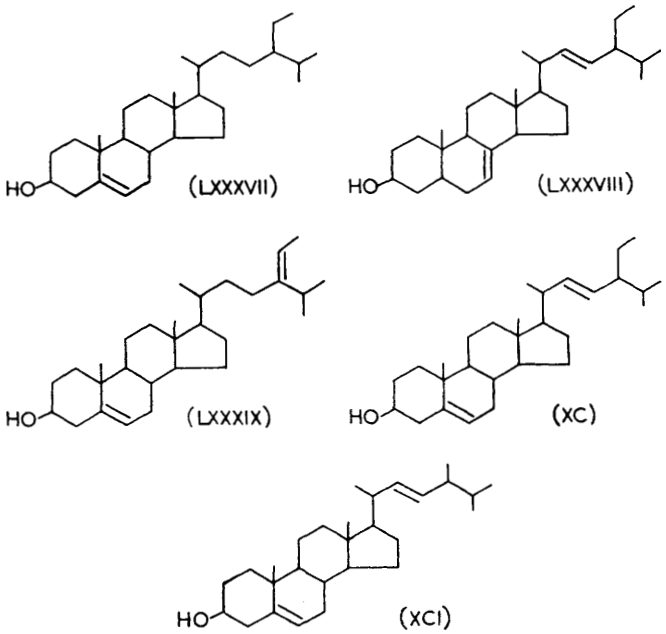


FIG. 19. Possible mechanisms of methylation of ethylenic bonds leading to various sterol side-chains and cyclopropanes.

Fig. 19 should result in transfer of CD_3 without loss of deuterium in the case of the C_{29} sterols and also in cyclolaudenol (LXXX).

[185] Castle, Blondin, and Nes, *J. Amer. Chem. Soc.*, 1963, **85**, 3306.
[186] Nicholas and Moriarty, *Fed. Proc.*, 1963, **22**, 529.
[187] Bader, Gulielmetti, and Arigoni, *Proc. Chem. Soc.*, 1964, 16.
[188] Barbier, Hügel, and Lederer, *Bull. Soc. Chem. Biol.*, 1960, **42**, 91.
[189] Idler and Fagerlund, *J. Amer. Chem. Soc.*, 1955, **77**, 4142.

The mechanism of introduction of the Δ^{22}-bond in such compounds as ergosterol, stigmasterol (XC), and brassicasterol (XCI) has not been investigated, but studies of the time course of incorporation of mevalonic



acid into β-sistosterol and stigmasterol in the potato plant suggest that the former sterol may be a precursor of the latter.[190] On the other hand, the possibility that $\Delta^{22,24(28)}$ compound is a companion of ergosterol in yeast[191] suggests that desaturation at $C_{(22)}$ may occur before reduction of the $\Delta^{24(28)}$-bond. It seems likely that the biochemistry of the side-chain transformations in plants varies not only among species, but in different tissues of a given species, and in the same tissues at different phases of growth.

Biogenesis of the Physiologically Active Steroids

Following the early experiments of Bloch and his co-workers in which deuterated cholesterol was shown to be converted *in vivo* into cholic acid[192] and urinary pregnanediol,[193] the main outlines of the biosynthesis of the steroid hormones and bile acids have been clarified. It seems unlikely, on

[190] Johnson, Heftmann, and Houghland, *Arch. Biochem. Biophys.*, 1964, **104**, 102.
[191] Breivik, Owades, and Light, *J. Org. Chem.*, 1954, **19**, 1734.
[192] Bloch, Berg, and Rittenberg, *J. Biol. Chem.*, 1943, **149**, 511.
[193] Bloch, *J. Biol. Chem.*, 1945, **157**, 661.

the basis of evidence now available,[194,195] that there is any significant contribution to their formation by pathways not involving cholesterol though the possibility of contributions from precursors such as desmosterol appears to exist.[196] Some aspects of steroid hormone and bile acid formation that have been the subject of recent investigations will be discussed below.

The Steroid Hormones: Cleavage of the Side Chain of Cholesterol.— Since evidence began to accumulate that this was probably a key point of action of pituitary adrenocorticotrophic hormone (ACTH) in stimulating adrenal steroid hormone output,[197,198] considerable interest has centred on the exact mechanism of conversion of the iso-octyl side chain of cholesterol into the two carbon, 20-oxo-structure found in the steroids of the pregnane group. Although the mechanism of action of ACTH is still not clarified, the steps leading from cholesterol to pregnenolone, the parent C_{20} steroid, are now known in some detail.

Earlier reports of the fission of the cholesterol side chain by adrenal

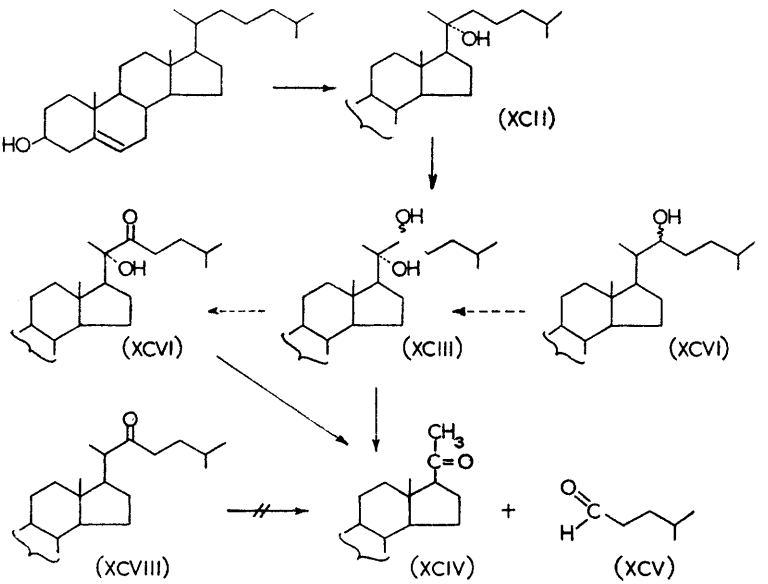


FIG. 20. Biosynthesis of pregnenolene. Demonstrated conversions: possible conversions -----> , no conversion: —//—> .

[194] Caspi, Dorfman, Khan, Rosenfeld, and Schmid, *J. Biol. Chem.*, 1962, **237**, 2085.
[195] Werbin and Chaikoff, *Arch. Biochem. Biophys.*, 1961, **93**, 476.
[196] Goodman, Avigan, and Wilson, *J. Clin. Invest.*, 1962, **41**, 2135.
[197] Hayano, Saba, Dorfman, and Hechter, *Recent Progr. Hormone Res.*, 1956, **12**, 79.
[198] Tchen, 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 543.

enzymes to yield pregnenolone and isocaproic acid,[199,200] directed attention to the metabolism of cholesterol derivatives oxygentated at either or both $C_{(20)}$ and $C_{(22)}$ (Fig. 20). The initial cleavage product representing the side-chain carbon atoms is now known to be isocaproic aldehyde[201] which is only subsequently converted into the acid. The initial oxidation product appears to be the 20α-hydroxy-compound (XCII).[202] This is further converted into the 20α,22ξ-diol (XCIII) which undergoes oxidative cleavage to pregnenolone (XCIV) and isocaproic aldehyde (XCV).[203] This may not be the exclusive pathway, however, since it has also been shown[204] that 22-hydroxycholesterol (XCVI) serves as a better substrate for the cleavage enzyme system than cholesterol itself. It is not clear whether the 20α,22ξ-dihydroxy-derivative is oxidised to the 22-ketone before fission of the 20–22 bond, but the 20α-hydroxy-22-ketone (XCVII) is an effective substrate for the cleavage enzyme system, though 22-oxocholesterol (XCVIII), unlike 22-hydroxycholesterol (XCVI), is not metabolised.

These oxidative steps have been studied in soluble adrenal enzyme systems; they require oxygen and utilise either NADH or NADPH, though optimally the latter is utilised.[198,205] A requirement for ferrous ion is also reported.[198] Their mechanism is no doubt similar to that of other mixed function oxidase reactions that are responsible for the various stereospecific hydroxylations of the steroid hormones and bile acids.[206]

The same pathway of cleavage of the cholesterol side chain has been established[207] in the testis in which the production of the androgenic C_{19} steroids takes place predominantly by removal of the side chain from a C_{21} 20-keto-steroid.[208] A proposed alternative pathway of formation of C_{19} steroids *via* 3β,17α,20α-trihydroxycholest-5-ene[208] could not be demonstrated.[209] The conversion of 20α-hydroxycholesterol into pregnenolone and progesterone in enzyme preparations from corpus luteum has also been observed.[210] The derivation of the œstrogens from these C_{21} precursors by way of the C_{19} androgenic steroids has been reviewed in detail by Breuer.[211]

A recent development in the study of cholesterol catabolism in the adrenal has been the recognition that cholesteryl sulphate may serve as the substrate in the cleavage reaction. The finding that dehydroepiandrosterone

[199] Lynn, Staple, and Gurin, *J. Amer. Chem. Soc.*, 1954, **76,** 4048.
[200] Staple, Lynn, and Gurin, *J. Biol. Chem.*, 1956, **219,** 845.
[201] Constantopoulos and Tchen, *J. Biol. Chem.*, 1961, **236,** 65.
[202] Shimizu, Hayano, Gut, and Dorfman, *J. Biol. Chem.*, 1961, **236,** 695.
[203] Shimizu, Gut, and Dorfman, *J. Biol. Chem.*, 1962, **237,** 699.
[204] Chaudhari, Harada, Shimizu, Gut, and Dorfman, *J. Biol. Chem.*, 1962, **237,** 703.
[205] Halkerston, Eichorn, and Hechter, *J. Biol. Chem.*, 1961, **236,** 374.
[206] Hayano, in Hayaishi, "Oxygenases," Academic Press, New York, 1962, p. 225.
[207] Toren, Menon, Forchielli, and Dorfman, *Steroids*, 1964, **3,** 381.
[208] Dorfman, Forchielli, and Gut, *Recent Progr. Hormone Res.*, 1963, **19,** 251.
[209] Shimizu, *Biochemistry, Tokyo*, 1964, **56,** 201.
[210] Hall and Koritz, *Biochemistry*, 1964, **3,** 129.
[211] Breuer, *Vitamins and Hormones*, 1962, **20,** 285.

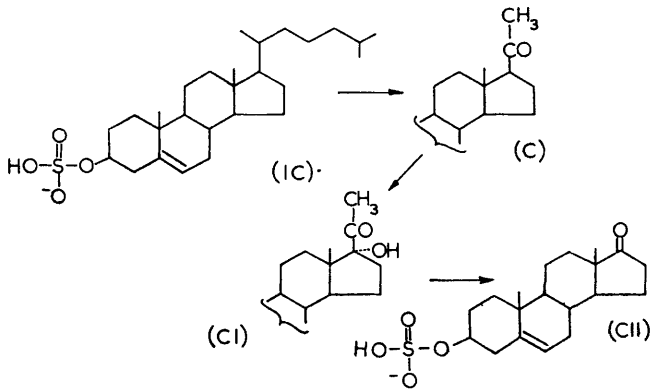


FIG. 21. Conversion of cholesteryl sulphate to dehydroisoandrosterone sulphate.

sulphate (Fig. 21, CII) may be a primary secretory product of the adrenal[212] prompted a study of the metabolism of cholesteryl sulphate (IC), doubly labelled with ^{14}C and ^{32}S. When perfused through an adrenal tumour, this compound yielded dehydroepiandrosterone sulphate containing the labelled atoms in essentially unchanged ratios.[213] Pregnenolone sulphate (C) has also been shown to yield 17α-hydroxypregnenolone sulphate (CI)[214] and dehydroepiandrosterone sulphate (CII)[215] in adrenal preparations *in vitro*. Cholesteryl sulphate has recently been isolated from normal bovine adrenal tissue,[216] though apparently it is present in very low concentrations (*ca.* 1·5 mg. per kg.). Until data as to its rate of turnover and the relative efficiencies of metabolism of free cholesterol and cholesteryl sulphate are available, the significance of these observations remains uncertain.

Further Metabolism of Pregnenolone in the Adrenal.—Largely as a result of early studies using adrenal perfusion or *in vitro* incubation, in which various substrates were tested for their capacity to undergo hydroxylation to more highly oxygenated corticosteroids, it was concluded that pregnenolone (Fig. 22) (XCIV) was oxidised to progesterone (CIII) before further hydroxylation.[217] The preferred order of hydroxylation of progesterone according to these earlier experiments was $C_{(17)}$, $C_{(21)}$, $C_{(11)}$ with a branching of pathways occurring, depending on whether the initial attack was at $C_{(17)}$ (CIV) [leading to cortisol (CV)] or at $C_{(21)}$ [leading to corticosterone (CVI)]. Recent experiments may call for some modification of this scheme.

[212] Vande Wiele, MacDonald, Gurpide, and Lieberman, *Recent Progr. Hormone Res.*, 1963, **19**, 275.
[213] Roberts, Bandi, Calvin, Drucker, and Lieberman, *J. Amer. Chem. Soc.*, 1964, **86**, 958.
[214] Calvin and Lieberman, *Biochemistry*, 1964, **3**, 259.
[215] Calvin, Vande Wiele, and Lieberman, *Biochemistry*, 1963, **2**, 648.
[216] Drayer, Roberts, Bandi, and Lieberman, *J. Biol. Chem.*, 1964, **239**, PC 3112.
[217] Hechter and Pincus, *Physiol. Rev.*, 1954, **34**, 459.

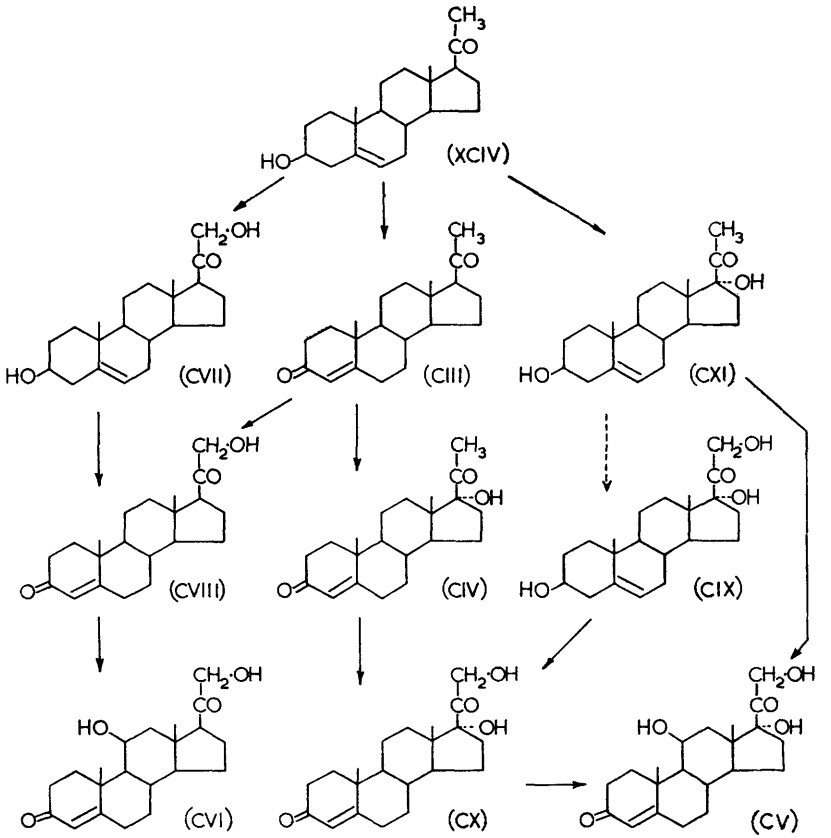


FIG. 22. Relationships of pregnenolene and progesterone in the biosynthesis of some corticosteroids.

Berliner *et al.*[218] showed that adrenal enzymes could catalyse the oxidation of the 3β-hydroxy-Δ^5-moeity to the Δ^4-3-ketone in steroids that had undergone prior hydroxylation at $C_{(17)}$ and $C_{(21)}$. Thus, 3β,21-dihydroxy-Δ^5-pregnen-20-one (CVII) yielded deoxycorticosterone (CVIII), and 3β,17α,21-trihydroxy-Δ^5-pregnen-20-one (CIX) gave 11-deoxycortisol (CX). Weliky and Engel[219] further found that when [4-^{14}C]progesterone (CIII) and [7α-^{3}H]17α-hydroxypregnenolone (CXI) were incubated together in an adrenal tumour homogenate, the latter steroid was converted into cortisol more efficiently (63%) than the former (17%). These authors, citing other work in support, suggest that the major route to cortisol may lie through 17α-hydroxypregnenolone rather than through progesterone.

[218] Berliner, Cazes, and Nabors, *J. Biol. Chem.*, 1962, **237**, 2478.
[219] Weliky and Engel, *J. Biol. Chem.*, 1962, **237**, 2089.

Other evidence consistent with this view has been adduced together with the demonstration that pregnenolone may be converted in the adrenal into 21-hydroxypregnenolone.[220] Hence, 17α-hydroxylation of pregnenolone is not a prerequisite for 21-hydroxylation. An evaluation of the relative importance of the "new" and "old" metabolic pathways *in vivo* or even in normal tissue *in vitro* remains to be made. The pathway *via* 17α-hydroxypregnenolone would account for the formation of urinary 3β,17α,20α-trihydroxy-Δ^5-pregnene.[221]

Levy *et al.*[222] have reported the isolation of 17α-hydroxypregnenolone as a metabolite of cholesterol perfused through the bovine adrenal under conditions in which 11α-hydroxylation is pharmacologically inhibited. These workers also record the remarkable observation that in this system Δ^4-androstene-3,17-dione (CXII) was converted in part into 11-deoxycortisol, *i.e.*, a C_{19} steroid was converted into a C_{21} steroid. This suggests that under certain conditions the conversion of a 17α-hydroxy-20-ketone into the 17-ketone may be reversed and recalls an earlier, though unconfirmed, report by Hechter[223] that ^{14}C labelled dehydroepiandrosterone (CXIII) appeared to be converted into cortisol or some similar steroid. Another recent instance of the reversal of a steroid metabolic transformation previously considered to be irreversible is the demonstration by Ward and Engel[224] of the reversibility of the enzymic conversion of Δ^5-3β-hydroxy-steroids into their Δ^4-3 keto-derivatives. In the experiments reported (Fig. 23) Δ^4-androstene-3,17-dione (CXII) was converted by a

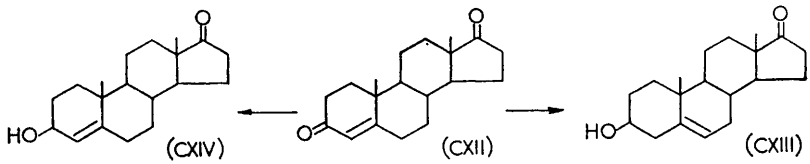


FIG. 23. Reversibility of Δ^5–3β–OH—Δ^4–3–keto–steroid transformation.

microsomal enzyme preparation from the sheep adrenal, into both 3β-hydroxy-Δ^5-androsten-17-one (CXIII) and 3β-hydroxy-Δ^4-androsten-17-one (CXIV).

In recent years a number of genetically determined defects in adrenal steroid hydroxylation mechanism have been recognised.[12,225,226] These defects may involve any of the three positions 11β, 17α, or 21 and give rise to characteristic clinical symptoms, in some cases fatal. When $C_{(11)}$ or

[220] Sharma and Dorfman, *Biochemistry*, 1964, **3**, 1094.
[221] Fotherby, *Biochem. J.*, 1958, **69**, 596.
[222] Levy, Cha, and Carlo, 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 584.
[223] Hechter, CIBA Foundation Colloquia in Endocrinology, London, Churchill, 1953, vol. 7, p. 161.
[224] Ward and Engel, *J. Biol. Chem.*, 1964, **239**, PC 3604.
[225] Bongiovanni and Root, *New Engl. J. Med.*, 1963, **268**, 1283.
[226] Wilkins, *Amer. J. Clin. Nutr.*, 1961, **9**, 661.

$C_{(21)}$ hydroxylations are defective, cortisol output is seriously impaired and feed-back control of ACTH production fails, with consequent hyperplasia of the adrenal gland. The major route of metabolism of 17α-hydroxy-20-keto-steroids then becomes their direct conversion into C_{19} androgenic steroids by cleavage of the side chain. Hence the prevalance of severe virilising symptoms in these conditions. An interesting contribution to the understanding of these diseases is the finding[227,228] that several C_{19} steroids whose output from the adrenal is thus enhanced are themselves inhibitors of hydroxylation at both $C_{(11)}$ and $C_{(21)}$. A curious situation is therefore indicated, in which the end product of the primary defect serves progressively to exacerbate the defect.

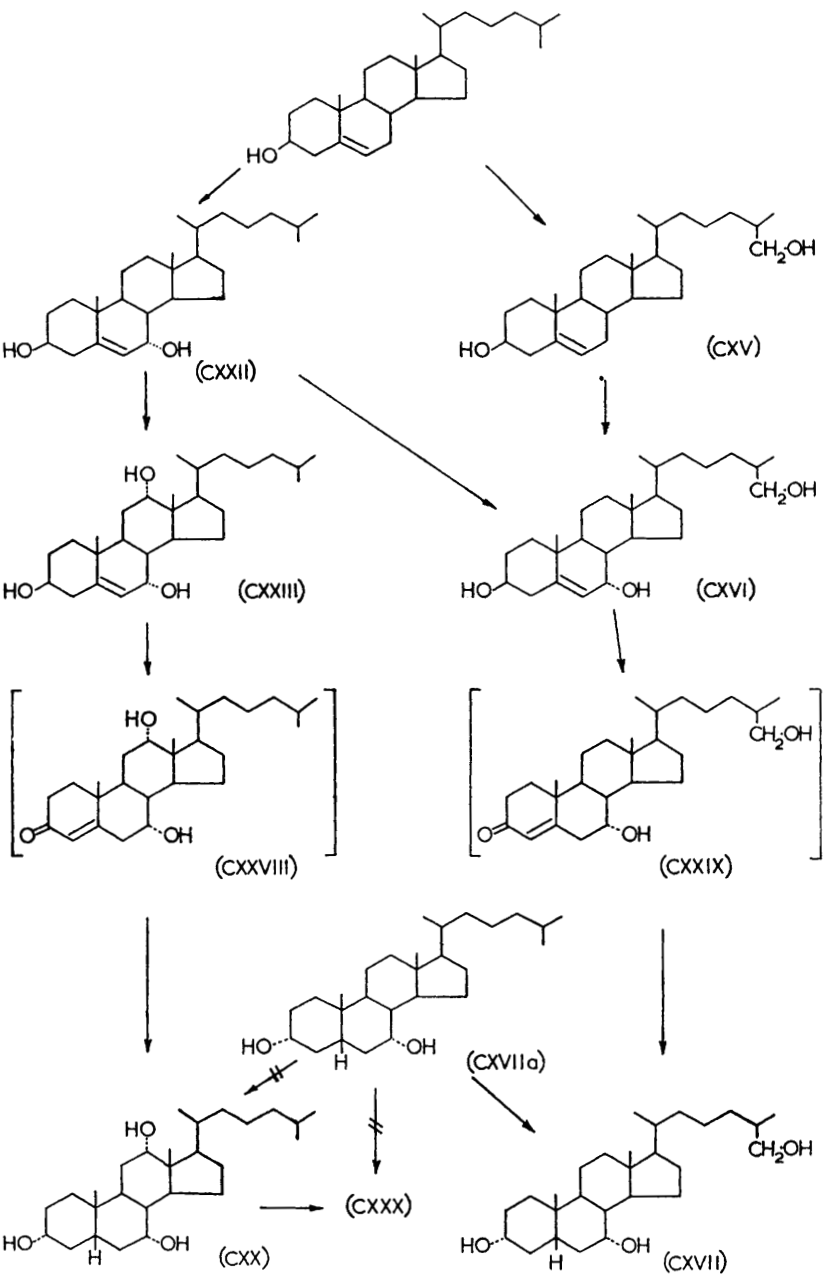
A more physiologically appropriate instance of this type of "feed-back" control is the recently reported inhibition of the conversion of cholesterol into 20α-hydroxycholesterol by pregnenolone.[229] This effect may well be significant in the normal homeostatic control of the conversion of cholesterol to the adrenal steroid hormones.

Bile Acid Formation.—The biochemistry of bile acid formation has recently been authoritatively reviewed in all its aspects by Danielsson.[15] This field owes its somewhat confusing complexity to several factors. The composition of the bile acid mixture of normal bile depends not only upon the action of liver enzyme systems which yield a "primary" secretory product, but also upon the action of intestinal micro-organisms which convert a proportion of the secreted bile acids into derivatives, some of which are resorbed from the intestine to be "re-secreted" in the bile, either in the same or in modified form. Further, no satisfactory *in vitro* system has so far been devised which would permit a systematic analysis of the enzymic steps leading from cholesterol to cholic acid and there is evidence that, just as in some other aspects of sterol and steroid biosynthesis that have been discussed, pathways of biosynthesis of bile acids are to some extent flexible. Finally, there are marked inter-species differences with respect to the types and proportions of different steroid constituents of the bile which, however, are of considerable interest from the evolutionary point of view.

We shall here be concerned in detail with only a few aspects of the formation and metabolism of cholic, chenodeoxycholic, and deoxycholic acids.

Sequence of Events in the Formation of Cholic Acid.—Intraperitoneal administration of labelled hypothetical intermediates between cholesterol and cholic acid to rats with cannulated bile ducts, thereby obviating most of the problems due to activities of intestinal micro-organisms, has been one of the most fruitful techniques to be applied in this field. Much of the evidence for the pathways outlined in Fig. 24 comes from experiments

[227] Sharma, Forchielli, and Dorfman, *J. Biol. Chem.*, 1963, **238**, 572.
[228] Sharma and Dorfman, *Biochemistry*, 1964, **3**, 1093.
[229] Koritz and Hall, *Biochemistry*, 1964, **3**, 1298.



(Continued on facing page).

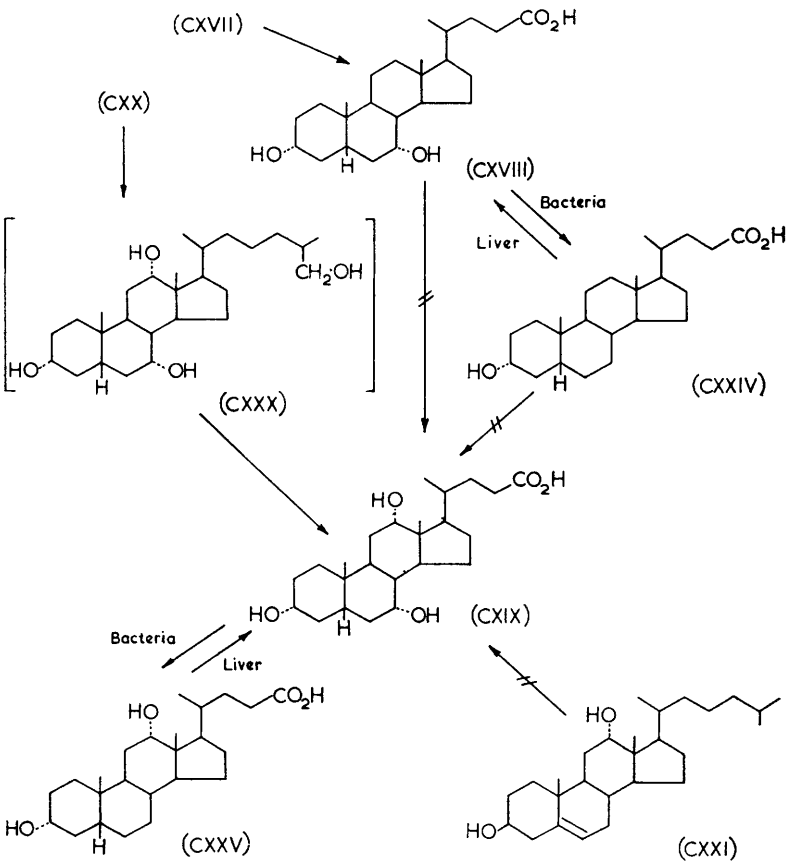
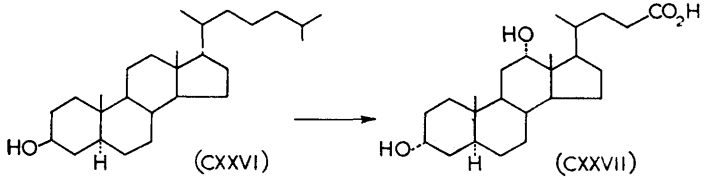


FIG. 24. Pathways of bile acid formation.

of this type. Work before 1957 indicated that the nuclear transformations preceded scission of the side chain[14,15] and the inversion of the $C_{(3)}$-hydroxyl group and saturation of the Δ^5-bond took place after hydroxylation at $C_{(7)}$[230,231] and possibly after hydroxylation at $C_{(12)}$.[232] In a recent study[233] coprostane-3α,7α-diol (CXVIIa) was not hydroxylated at $C_{(12)}$ in a mouse liver preparation *in vitro*, but was converted into the corresponding 3α,7α,26-triol (CXVII). Hydroxylation at $C_{(26)}$ probably constitutes the first step in degradation of the side chain,[234] and since 26-hydroxycholesterol (CXV), cholest-5-ene-3β,7α,26-triol (CXVI), and coprostane-3α,7α,26-triol (CXVII) were good precursors of chenodeoxycholic acid

[230] Lindstedt, *Acta Chem. Scand.*, 1957, **11**, 417.
[231] Danielsson and Einarsson, *Acta Chem. Scand.*, 1964, **18**, 831.
[232] Danielsson, *Acta Chem. Scand.*, 1962, **16**, 1534.
[233] Berseus and Danielsson, *Acta Chem. Scand.*, 1963, **17**, 1293.
[234] Frederickson, *J. Biol. Chem.*, 1956, **222**, 109.



(XCVIII) but not of cholic acid (CXIX), which, however, was formed from coprostane-3α,7α,12α-triol (CXX),[235] it seems clear that 12-hydroxylation must occur before any attack on the side chain. Further support for this conclusion comes from the recent demonstration of the enzymic conversion of cholesterol into coprostane-3α,7α,12α-triol (CXX) in rat liver.[236] Since in the rabbit cholic acid is formed only in poor yield from 12α-hydroxycholesterol (CXXI), but in good yield from 7α-hydroxycholesterol (CXXII),[232] hydroxylation at $C_{(12)}$ probably follows that at $C_{(7)}$. The foregoing observations, together with others to be discussed below, strongly suggest that the major route to cholic acid is as depicted on the left-hand side of Fig. 24.

If attack on the side chain is initiated before 12α-hydroxylation the latter is evidently excluded and, clearly, the specificity of the enzyme systems responsible for side-chain oxidation and hydroxylation at $C_{(7)}$ must be much lower than that of the 12α-hydroxylase. Thus, lithocholic acid (CXXIV) is metabolised in the rat to several products[237] including chenodeoxycholic acid and compounds hydroxylated at both $C_{(6)}$ and $C_{(7)}$, but 12α-hydroxy-products are absent. On the other hand deoxycholic acid (CXXV) is converted into cholic acid.[238] It is interesting, in view of this, that a bile acid formed from cholestan-3β-ol (CXXVI) in the rabbit is now identified[239] as 3α,12α-dihydroxy-5α-cholanic acid (CXXVII), suggesting that the specificity of the 12α-hydroxylase is influenced considerably by the stereochemistry of the junction of rings A and B. The specificity of this enzyme is also subject to important species differences, since chenodeoxycholic acid is in part converted into cholic acid in the python[240] and in the chicken.[241]

Inversion of the 3β-Hydroxyl Group and Saturation of the Δ^5-Bond.— The precise stage at which the characteristic structural feature of the bile acid series: a 3α-hydroxyl group with a *cis*-fused A/B ring junction, is introduced into the molecule, has not yet been determined, though it seems likely that it follows the completion of the nuclear hydroxylation

[235] Danielsson and Kazuno, *Acta Chem. Scand.*, 1964, **18**, 1157.
[236] Mendelsohn and Staple, *Biochemistry*, 1963, **2**, 577.
[237] Thomas, Hsia, Matschiner, Doisy, Elliot, Thayer, and Doisy, *J. Biol. Chem.*, 1964, **239**, 102.
[238] Bergstrom, Rottenberg, and Sjoval, *Z. physiol. Chem.*, 1953, **295**, 278.
[239] Hofmann and Mosbach, *J. Biol. Chem.*, 1964, **239**, 2813.
[240] Bergstrom, Danielsson, and Kazuno, *J. Biol. Chem.*, 1960, **235**, 983.
[241] Ahlberg, Ziboh, Sonders, and Hsia, *Fed. Proc.*, 1961, **20**, 283.

steps.[15] Several aspects of the changes involving the 3-hydroxyl group and the Δ^5-bond of cholesterol are, however, now clarified as a result of the efforts of Samuelsson and his co-workers. It was shown that the saturation of the Δ^5-bond involved stereospecific addition of hydrogen in the 5β- and 6β-positions[242] and further details of the reaction have now been elucidated by means of experiments with [3α-^{3}H, 4-^{14}C]- and [4β-^{3}H, ^{14}C]-cholesterols.[243] Loss of 3α-^{3}H occurred during conversion into bile acids but a significant fraction of the 4β-^{3}H was retained. In chenodeoxycholic acid isolated from the bile in these experiments, about 25% of the 4β-^{3}H was still present and by administration of this material to another bile-cannulated rat so that it was metabolised further to a 6β-hydroxylated derivative, it was shown that most of the ^{3}H that had been retained was located in the 6β-position. The conclusion was drawn that the inversion of the 3-hydroxyl group takes place *via* an intermediate 3-ketone and that reduction of the Δ^5-bond is preceded by its migration to the Δ^4-position (CXXVIII and CXXIX). In the course of this migration an appreciable part of the β-hydrogen at $C_{(4)}$ migrates to the axial 6β-position. As the authors point out, these observations are similar to those of Wang *et al.*[244] with a bacterial Δ^5–Δ^4 isomerase, but conflict with those of Werbin and Chaikoff[245] who reported complete loss of 4β-^{3}H from cholesterol during cortisol biosynthesis. While it seems likely that the efficient enzymic isomerisation and reduction of the double bond are facilitated by the formation of a keto-group at $C_{(3)}$, the epimerisation of the 3β-hydroxyl group does not depend upon the concomitant changes involving the double bond, as is evident from the formation of 3α,12α-dihydroxycholanic acid (CXXVII) from cholestanol (CXXVI) and the conversion of coprostanol into various bile acids of usual structure all having 3α-hydroxyl groups.[246]

Degradation of the Side-Chain—Staple and his co-workers[247] demonstrated the conversion of 3α,7α,12α-trihydroxy-5β-cholestan-26-oic acid into cholic acid and have since studied the fate of the three terminal carbon atoms of [26-^{14}C]3α,7α,12α-trihydroxy-5β-cholestane (Fig. 25, CXX) in detail.[248] A preparation from rat liver converted this compound *in vitro* into the corresponding 3α,7α,12α,26-tetraol (CXXX) and the 3α,7α,12α,26-carboxylic acid (CXXXI), both of which were isolated and identified. Carbon atoms, 25, 26, and 27 were found to be split off as propionic acid labelled in $C_{(1)}$ and $C_{(3)}$ and evidence for the formation of the coenzyme A derivative (CXXXVII) of this fragment was obtained. The authors suggested the pathway shown involving the coenzyme-A

[242] Samuelsson, *J. Biol. Chem.*, 1959, **234**, 2852.
[243] Green and Samuelsson, *J. Biol. Chem.*, 1964, **239**, 2804.
[244] Wang, Kawahara, and Talalay, *J. Biol. Chem.*, 1963, **238**, 576.
[245] Werbin and Chaikoff, *Biochim. Biophys. Acta*, 1963, **71**, 471.
[246] Bell, Elliott, and Doisy, 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 564.
[247] Briggs, Whitehouse, and Staple, *J. Biol. Chem.*, 1961, **236**, 688.
[248] Suld, Staple, and Gurin, *J. Biol. Chem.*, 1962, **237**, 338.

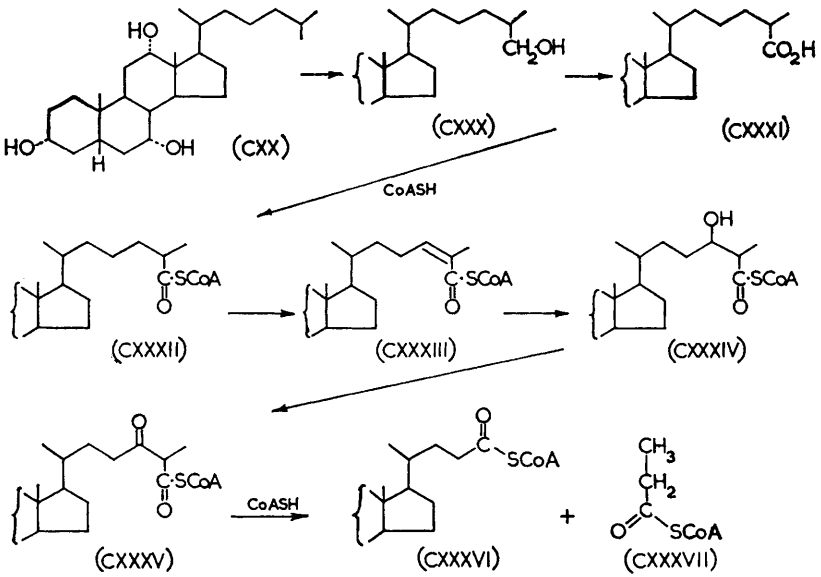


FIG. 25. Formation of the bile acid side-chain.

derivatives (CXXXII—CXXXV) by analogy with the well established pathway of fatty acid degradation. They pointed out that the final thiolytic cleavage reaction would leave the cholic acid residue in the form of its CoA ester and hence ready for conjugation with glycine or taurine.[249] This suggested route of formation of cholic acid is consistent with the views of Haslewood[250,251] concerning the evolutionary significance of the bile acids of different species. According to this author the primary cholesterol metabolites of bile have evolved from $C_{(27)}$-hydroxylated products, more closely related to cholesterol, to the "modern" $C_{(24)}$-acidic products whose present-day pathway of formation still recapitulates certain features of the evolutionary trend. Thus, 3α,7α,12α-trihydroxycoprostanic acid (CXXXI), the predominant bile acid of certain reptiles and amphibians, was suggested[252] as a likely precursor of cholic acid in higher organisms. Apart from the evidence cited above which supports this concept, it is further strengthened by the recent isolation of 3α,7α,12α-trihydroxycoprostanic acid from human bile.[253,254] On the other hand, there is also good evidence that several C_{27} and C_{26} polyhydroxy-steroids found in lower vertebrates

[249] Elliott, *Biochem. J.*, 1956, **62**, 427, 433.
[250] Haslewood, in "Comparative Biochemistry," eds. Florkin and Mason, Academic Press, New York, 1962, vol. III, p. 205.
[251] Haslewood, 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 539.
[252] Haslewood, *Physiol. Rev.*, 1955, **35**, 178.
[253] Staple and Rabinowitz, *Biochim. Biophys. Acta*, 1962, **59**, 735.
[254] Carrey and Haslewood, *J. Biol. Chem.*, 1963, **238**, PC 855.

should be regarded as deviants from the major biosynthetic (and evolutionary) pathway to the bile acids of the mammals.[235]

Some experimental observations that are not consistent with the proposed scheme of side-chain degradation are discussed in detail by Danielsson.[15] It will suffice here to note briefly that some evidence has been reported for the cleavage of the three terminal carbon atoms in the form of acetone.[255,256] 25-Hydroxycholesterol was tentatively identified as a metabolite of cholesterol *in vitro*,[234] though this was not confirmed.[25,257] These observations suggest a cleavage mechanism involving a 24,25-glycol analogous to that by which pregnenolone is formed, but so far little evidence has been forthcoming to indicate that this is more than a minor pathway. The accumulation of desmosterol in the liver as a result of inhibition of the 24,25-reductase by triparanol has been discussed in a previous section. It seems most probable that under these conditions desmosterol is oxidised[196] to bile acid mixtures similar to those formed normally from cholesterol.[258] Evidence for the conversion of desmosterol into its 26-hydroxylated derivative in mouse liver homogenates has recently been described.[259] On further oxidation this compound could yield the $\alpha\beta$-unsaturated carboxylic acid which is presumed to be an intermediate in the normal pathway from cholesterol.

Formation of Deoxycholic Acid.—The foregoing discussion of the sequence of hydroxylations implies that any normally formed bile acid having a 12α-hydroxyl group should also have a 7α-hydroxyl group, yet deoxycholic acid (CXXV) and lithocholic acid (CXXIV) are well known constituents of the bile of many species. The presence of these constituents in the bile is due to the action of intestinal bacteria in removing the 7α-hydroxyl group from cholic and chenodeoxycholic acids. The mechanism of this dehydroxylation has been studied by Samuelsson[260] and is discussed in full by Bergström *et al.*[14] Although no intermediate in the process has been isolated, ingenious experiments with ^{3}H-labelled substrates indicate that the 7α-hydroxyl group is initially eliminated together with the *trans*-6β-hydrogen, and that the resulting Δ^6-bond is then reduced by *trans*-addition of hydrogen in the 6α- and 7β-positions. The micro-organism(s) responsible for these changes remains unidentified, but in a recent study[261] of the metabolism of cholic acid by the ærobic soil bacterium *Corynebacterium simplex* several transformation products were isolated, including 7α,12α-dihydroxy-3-oxochol-4-enic acid, 12α-hydroxy-3-oxochola-4,6-dienoic acid, and 12α-hydroxy-3-oxochol-4-enoic acid. These results

[255] Whitehouse, Rabinowitz, Staple, and Gurin, *Biochim. Biophys. Acta*, 1960, **37**, 382.
[256] Whitehouse, Staple, and Gurin, *J. Biol. Chem.*, 1961, **236**, 68.
[257] Danielsson, *Arkiv Kemi*, 1961, **17**, 373.
[258] Goodman, Avigan, and Wilson, *J. Clin. Invest.*, 1962, **41**, 962.
[259] Danielsson and Johansson, *Acta Chem. Scand.*, 1964, **18**, 788.
[260] Samuelsson, *J. Biol. Chem.*, 1960, **235**, 361.
[261] Hayakawa and Samuelsson, *J. Biol. Chem.*, 1964, **239**, 94.

suggest that in *C. simplex* the elimination of the 7α-hydroxyl group may also involve the formation and subsequent saturation of a Δ^6-bond.

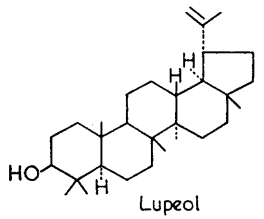
Biosynthesis of Other Classes of Terpenoid

The discovery of mevalonic acid, and its recognition as a specific precursor of the isoprene unit, have enormously accelerated the study of the biogenesis of most of the major classes of terpenoid compound. These include the carotenoids, rubber, certain alkaloids, the ubiquinones, the mono-, sesqui-, di-, and tri-terpenes of plants, as well as various fungal metabolites of partial terpenoid structure. An adequate review of these extensive areas in an article of this type is clearly impossible. A useful comprehensive survey[43] gives good coverage of most of them up to 1962. All that will be attempted here will be to indicate some features of interest in the development of these various fields.

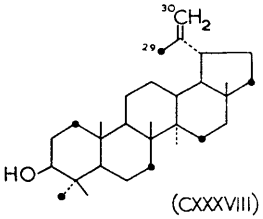
Cyclic Terpenes.—Ruzicka[262] has recently reviewed the field of biogenesis of cyclic terpenes of all the major classes, with emphasis on testing the hypothetical mechanisms of their formation by the use of labelled biological precursors. Many of the topics mentioned below are fully discussed in that review.

Pentacyclic Triterpenes.—Experiments proving the retention of identity by the isopropylidene carbon atoms of squalene in ring A of soyasapogenol have already been noted.[107] Ruzicka[262] has now discussed an experiment designed to demonstrate that in lupeol (cf. Fig. 14, Part I) the terminal methyl groups of squalene distal to the point of initiation of cyclisation similarly retain their stereospecific individuality. The experiment also serves as a test of the postulated stereoelectronic mechanism of squalene cyclisation in the formation of this type of compound.

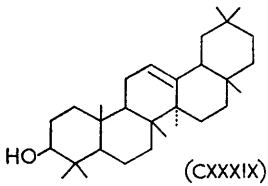
Lupeol biosynthesised from [2-^{14}C]mevalonate was predicted to have the labelling distribution shown (CXXXVIII) and in complete accord with the



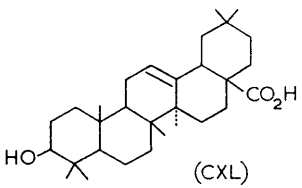
Lupeol



(CXXXVIII)



(CXXXIX)



(CXL)

[262] Ruzicka *Pure Appl. Chem.*, 1963, **6**, 493.

theory, the methylene group ($C_{(30)}$) on oxidation with osmium tetroxide yielded formaldehyde that was devoid of radioactivity, while the terminal methyl group ($C_{(29)}$) of the resulting ketone contained one-sixth of the total radioactivity of the starting material. Studies, with more biological objectives, of the incorporation of mevalonate into β-amyrin (CXXXIX)[263] and oleanolic acid (CXL)[264] have also been reported and have indicated interesting differences between the efficiences of incorporation of the precursor into sterol and terpenoid products.

Diterpenes.—As discussed above, rosenonolactone (LIV) and gibberellic acid (LIII) also retain the steric individuality of the *gem.*-substituted carbon $C_{(15)}$ and $C_{(16)}$.[107,108] Gibberellic acid biosynthesised from

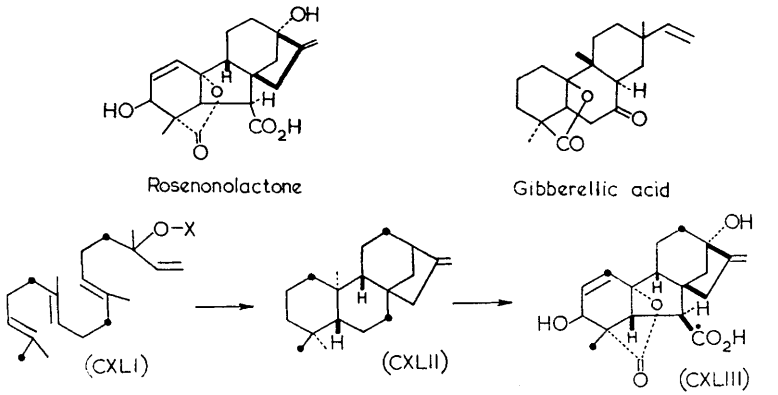


FIG. 26. Biosynthesis of gibberellic acid: labelling distribution from [2-^{14}C]mevalonic acid.

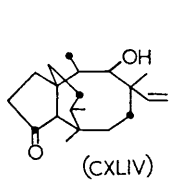
[2-^{14}C]mevalonic acid[265] (Fig. 26) was labelled as in (CXLIII) in accord with its possible derivation from geranyl linaloöl (CXLI) *via* kaurene (CXLII) with contraction of ring B as shown. If, as has recently been implied,[266] kaurene is converted intact into gibberellic acid, the hydroxylation that occurs at $C_{(3)}$ in the latter compound is unusual in that it must be unrelated to the initiation of cyclisation. Results suggestive of the formation of gibberellic from all-*trans*-geranyl geranyl pyrophosphate have been reported.[267] Geranyl linaloyl pyrophosphate could, of course, be an intermediate in this transformation.

The diterpene mutilin, biosynthesised from [2-^{14}C]mevalonate, has the labelling distribution shown (CXLIV) which is also rationalised on the basis of its derivation from geranyl linaloöl (Arigoni, quoted in ref. 262). The failure of sclareol (CXLV) to incorporate the label of [2-^{14}C]meval-

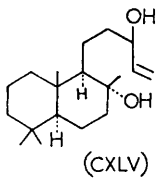
[263] Baisted and Nes, *J. Biol. Chem.*, 1963, **238**, 1947.
[264] Nicholas, *J. Biol. Chem.*, 1962, **237**, 1481.
[265] Birch, Richards, and Smith, *Proc. Chem. Soc.*, 1958, 192.
[266] Cross, Galt, and Hanson, *J.*, 1963, 2944.
[267] West, Dennis, Upper, and Lew, 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 601.

onic acid in *Salvia officinalis*[264,268] as in other similar cases, may have been due to the failure of the precursor to reach the appropriate enzymic site. It is noteworthy that labelled CO_2 was readily incorporated into the diterpene.[268]

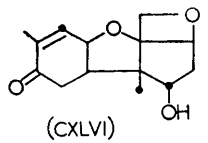
Sesquiterpenes.—Jones and Lowe[269] have used [2-^{14}C]mevalonic acid as a precursor of trichothecolone (CXLVI) and have rationalised its formation from farnesyl pyrophosphate on the basis of the resultant labelling pattern. The stereochemical aspects of this transformation and its theoretical relation to the formation of bisabolene (CXLVII) are discussed in detail by Ruzicka.[262] The distribution of label from [1-^{14}C]acetate in carotol (CXLVIII), a sesquiterpene of carrot seed, permitted a discrimination between two possible modes of cyclisation of the farnesyl residue



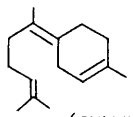
(CXLIV)



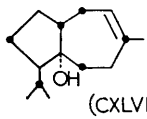
(CXLV)



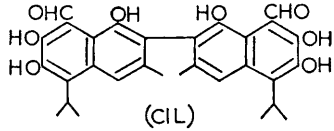
(CXLVI)



(CXLVII)



(CXLVIII)



(CIL)

in the course of its biosynthesis.[270] No such distinction could be made, however, in a study[271] of the labelling of the symmetrically united sesquiterpenoid fragments of gossypol (CIL).

Monoterpenes.—[2-^{14}C]Mevalonic acid has been used in the study[272] (Fig. 27) of the biosynthesis of the monoterpenes, β-pinene (CL) and thujone (CLI). Oxidation of β-pinene to norpinic acid (CLII) and of thujone to the diketone (CLIII) resulted in each case in retention of ^{14}C and thus supported the derivation of both terpenes from geranyl pyrophosphate *via* the respective intermediates (CLIV and CLV) with similar skeletal structures but different charge distributions. An ingenious demonstration of the retention of stereochemical individuality by the superficially identical terminal *gem.*-dimethyl groups in an open-chain terpenoid structure has been reported by Birch *et al.*[109] (Fig. 28). The structure in

[268] Nicholas, *Biochim. Biophys. Acta*, 1964, **84**, 80.
[269] Jones and Lowe, *J.*, 1960, 3959.
[270] Souček, *Coll Czech. Chem. Comm.*, 1962, **27**, 2929.
[271] Heinstein, Smith, and Tove, *J. Biol. Chem.*, 1962, **237**, 2643.
[272] Sandermann and Schweer, *Tetrahedron Letters*, 1962, 257, 259.

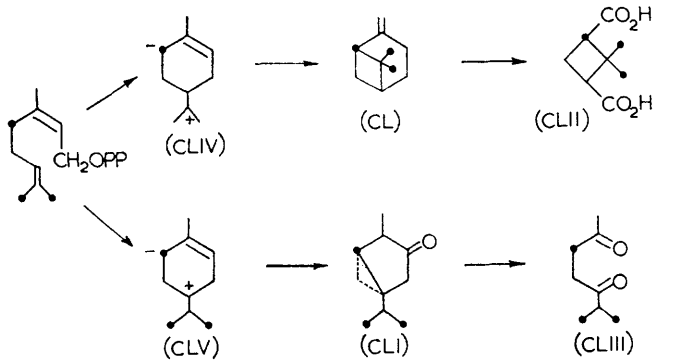


FIG. 27. Biosynthesis of β-pinene and thujone.

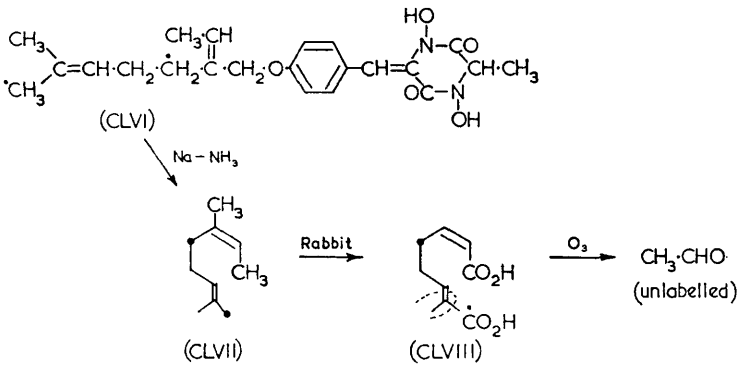


FIG. 28. Retention of steric individuality in isopropylidene group methyl carbons of mycelianamide.

question is the monoterpenoid chain of mycelianamide. This compound was biologically labelled as shown (CLVI) from [2-^{14}C]mevalonic acid and the terpenoid chain (CLVII) cleaved by treatment with sodium in liquid ammonia. The resulting hydrocarbon was administered to a rabbit and recovered from the urine in the form of its dicarboxylic acid metabolite (CLVIII). Ozonolysis of this material yielded acetaldehyde that was free from radioactivity, indicating that enzymic attack on one of the *gem.*-dimethyl groups had been confined to that which was labelled.

In contrast to this and the other examples that have been cited, of the retention of steric individuality by the *gem.*-dimethyl groups of terpenoid isopropylidene structures, Yeowell and Schmid[273] (Fig. 29) have reported evidence for a randomisation of the isopropylidene methyl carbons of citronellal (CLIX) in the course of its presumed conversion *via* iridodial

[273] Yeowell and Schmid, *Experientia*, 1964, **15**, 251.

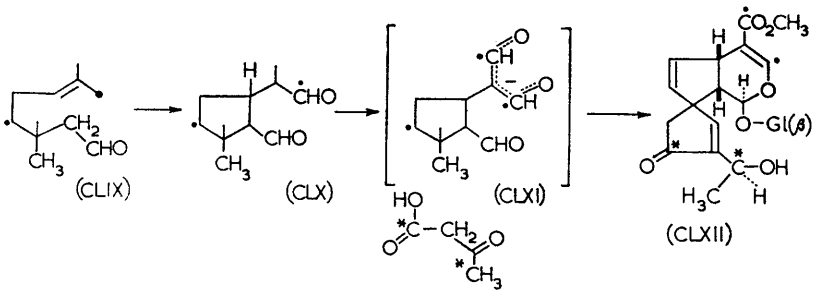


FIG. 29. Biogenesis of plumieride aglycone.
• = label from [2-^{14}C]mevalonate,
* = label from [1-^{14}C]acetate,
Gl = glucose.

(CLX) into the plant glucoside, plumieride (CLXII). Comparative studies of the distribution of label from [1-^{14}C]acetate and [2-^{14}C]mevalonate respectively led the authors to postulate the union of acetoacetate with an intermediate of the type (CLXI) in which the isopropylidene carbons becomes indistinguishable from each other.

Carotenoids.[274,275]—The biogenesis of the carbon skeleton of lycopersene (CLXV) and related C_{40} acyclic carotenoids (Fig. 30) clearly presents a problem analogous to that of squalene biosynthesis but involving tail-to-tail condensation of two C_{20} (geranylgeranyl) units rather than the two C_{15} (farnesyl) units as in squalene. The terminal structure of the open-chain carotenoid hydrocarbons is appropriate for the formation of the mono- and di-cyclic carotenes and zanthins, by what is apparently a proton-initiated mechanism.

The utilisation of mevalonic acid in carotenoid synthesis in a variety of organisms has been demonstrated by several workers[276–279] and there is evidence for the utilisation of mevalonic pyrophosphate,[280] farnesyl pyrophosphate, and isopentenyl pyrophosphate.[281,282] The enzymic synthesis of geranylgeranyl pyrophosphate (CLXIII) has been reported to occur in yeast,[283] and has recently been studied in detail in carrot root and pig liver enzyme preparations[284] and in the yellow bacterium *Micrococcus lysodeikticus*.[285] These various enzyme systems, which apparently

[274] Olson, *J. Lipid Res.*, 1964, **5**, 281.
[275] Chichester and Nakayama, ref. 43, p. 475.
[276] Purcell, Thompson, and Bonner, *J. Biol. Chem.*, 1959, **234**, 1081.
[277] Steele and Gurin, *J. Biol. Chem.*, 1960, **235**, 2778.
[278] Braithwaite and Goodwin, *Biochem. J.*, 1960, **76**, 194.
[279] Yokoyama, Nakayama, and Chichester, *J. Biol. Chem.*, 1962, **237**, 681.
[280] Suzue, *Bull. Chem. Soc., Japan*, 1964, **37**, 613.
[281] Yamamoto, Yokoyama, Simpson, Nakayama, and Chichester, *Nature*, 1961, **191**, 1299.
[282] Beeler, Anderson, and Porter, *Arch. Biochem. Biophys.*, 1963, **103**, 26.
[283] Grob, Kirschner, and Lynen, *Chimia (Switz.)*, 1961, **15**, 308.
[284] Nandi and Porter, *Arch. Biochem. Biophys.*, 1964, **105**, 7.
[285] Kandutsch, Paulus, Levin, and Bloch, *J. Biol. Chem.*, 1964, **239**, 2507.

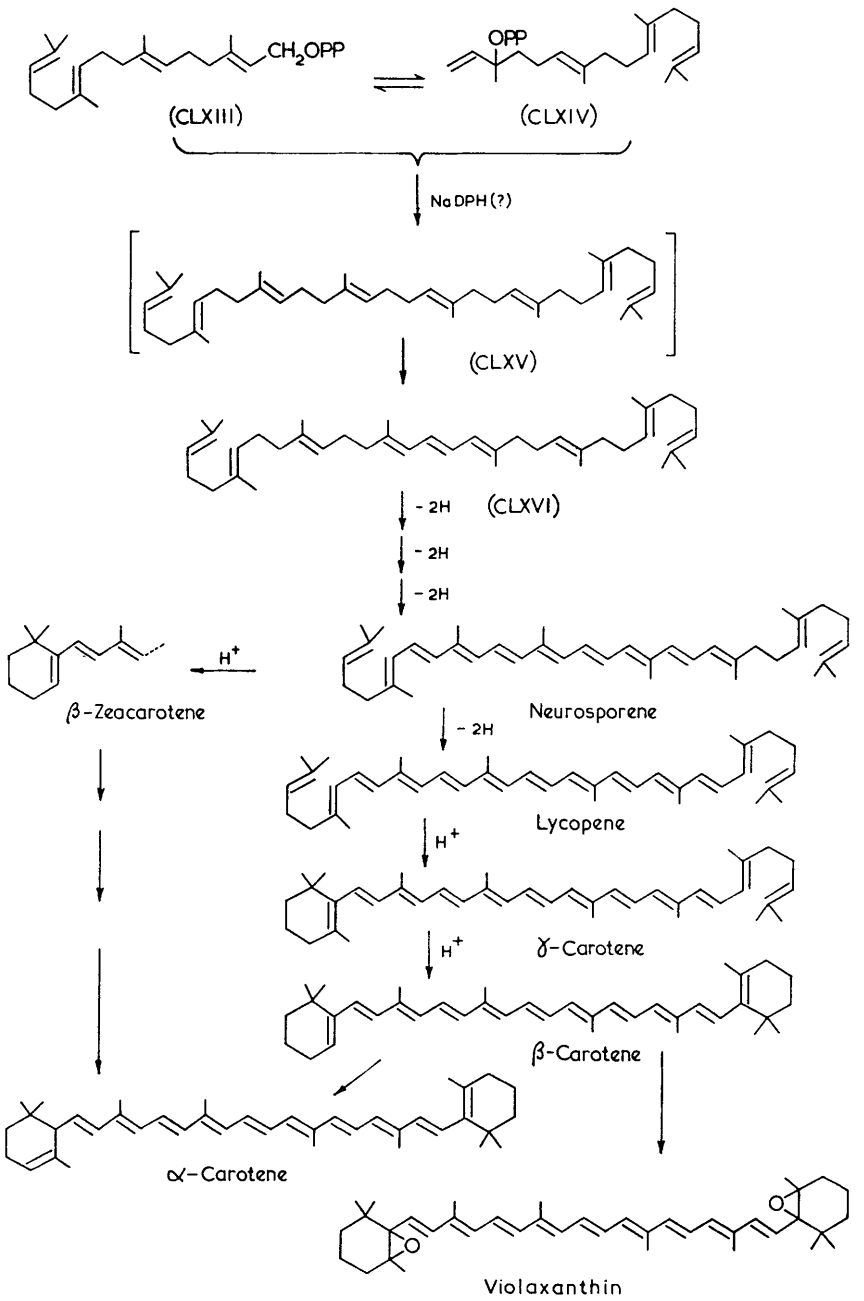


FIG. 30. Biogenesis of carotenoids.

have quite similar characteristics, synthesise geranylgeranyl pyrophosphate from isopentenyl pyrophosphate and C_5, C_{10}, and C_{15} allyl pyrophosphates. The mechanism of condensation of geranylgeranyl pyrophosphate to form the C_{40} structure is at present unknown. In the enzymic synthesis of geranylgeranyl pyrophosphate, the formation of small amounts of geranyllinaloöl (shown as pyrophosphate, CLXIV) was noted.[284,285] This material is the C_{20} analogue of nerolidol which, in the form of the pyrophosphate (XL, Fig. 12), is a hypothetical participant in the farnesyl pyrophosphate coupling reaction according to one possible mechanism[75] (cf. Fig. 12, Part I), but since this type of structure readily arises by acid-catalysed isomerisation from the farnesol type, its appearance in these experiments may not be significant. In any case, the analogy between the biosynthesis of carotenoids and of squalene cannot be pressed too far on the basis of evidence available at present, since conflicting results have been obtained in the search for the first-formed C_{40} compound in carotenoid biosynthesis. Grob and his co-workers[283,286] presented results which implicate lycopersene as the immediate product of coupling of two geranylgeranyl units in a reaction, which, like the formation of squalene, requires NADPH. Others,[287,288] however, have obtained evidence for phytoene (CLXVI) as the primary C_{40} product and, in keeping with this less reduced structure, no NADPH requirement could be demonstrated.

Despite the uncertainty as to the coupling mechanism there seems no doubt that either lycopersene or phytoene is the "parent" carotenoid from which all others arise by progressive desaturation, cyclisation, and oxidation reactions. Evidence for the pathways shown in Fig. 30 comes from a wide variety of biochemical, kinetic, and comparative structural studies that are reviewed by Olson.[274]

Some characteristics of the bacterial geranylgeranyl pyrophosphate synthetase, which has been obtained in a relatively high state of purity,[285] suggest that it may be a single protein which catalyses the stepwise elongation of an enzyme-bound terpenoid pyrophosphate chain by addition of isopentenyl pyrophosphate units. This type of enzyme system might be expected to participate in the biosynthesis of a variety of compounds having extended terpenoid chains, such as rubber, the ubiquinones, and the vitamins of the E and K groups.

Rubber.—In rubber biosynthesis[289] acetate is incorporated *via* mevalonic acid, and an enzyme system from *Hevea brasilienis* converts isopentenyl pyrophosphate into rubber with high efficiency.[290] There is also evidence that dimethylallyl pyrophosphate is a "starter" for the polymerisation.[291] The *cis*-ethylenic bonds are a distinctive feature of natural rubber and it

[286] Grob, ref. 107, p. 267.
[287] Mercer, Davies, and Goodwin, *Biochem. J.*, 1963, **87**, 317.
[288] Anderson and Porter, *Arch. Biochem. Biophys.*, 1962, **97**, 509.
[289] Bonner, ref. 43, p. 727.
[290] Archer, Ayrey, Cockbain, and McSweeney, *Nature*, 1961, **189**, 663.
[291] Archer, Audley, Cockbain, and McSweeney, *Biochem. J.*, 1963, **89**, 565.

has been shown by experiments with stereospecifically labelled [4-^{3}H]-mevalonate, that they arise as a result of steric specificity of the polyisoprenoid synthetase enzyme. Rubber incorporated ^{3}H from 4-*S*-[4-$^3H_1$]-mevalonate but not from 4*R*-[4-^{3}H]mevalonate which, on the other hand, yielded ^{3}H-labelled *trans-trans*-farnesyl pyrophosphate in the same enzyme preparation.[292] The stereochemical course of rubber biosynthesis can therefore be represented as in Fig. 31. The extension of the rubber polymer chain takes place in a particulate fraction of latex.[291] Ribonuclease is reported to destroy the enzymic activity, suggesting that ribonucleic acid (RNA) contained in these particles plays some rôle in the maintenance of enzymic activity.[293]

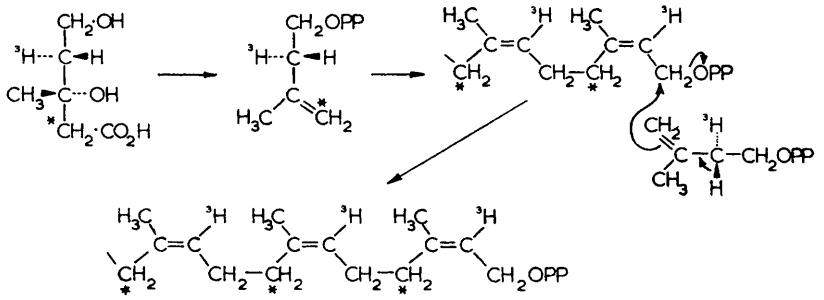


FIG. 31. Absolute stereochemistry of polymerisation in rubber formation from 4-*S*-[4-^{3}H]mevalonate. * indicates hypothetical label distribution from [2-^{14}C]mevalonate.

Ubiquinones.—These compounds, of general structure (Fig. 32, CLXVII) all contain polyisoprenoid chains of varying lengths, bound to a substituted *p*-quinone nucleus. Their biosynthesis is still far from fully understood. A major problem is the low level of incorporation of labelled precursors, and the evident difficulty of obtaining *in vitro* systems suitable for systematic enzymic analysis.

Important aspects of the biochemistry of the ubiquinones, including both physiological function and biosynthesis, are covered in a CIBA Foundation Symposium.[294] The most reasonable hypothesis to account for the formation of these compounds visualises the attachment of the preformed isoprenoid chain (presumably reacting in the form of the pyrophosphate) to the quinone ring or its precursor.[295–299] In keeping with this

[292] Cockbain and Popják, quoted in Samuelsson and Goodman, *Biochem. Biophys. Chem. Comm.*, 1963, **11**, 125.
[293] McMullen, 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 586.
[294] CIBA Foundation Symposium on Quinones in Electron Transport, eds. Wolstenholme and O'Connor, Churchill, London, 1961.
[295] Martius, ref. 294, p. 312.
[296] Wiss, Gloor, and Weber, ref. 294, p. 264.
[297] Birch, ref. 294, p. 233.
[298] Lynen, ref. 294, p. 244.
[299] Olson, Dialameh, and Bentley, ref. 294, p. 284.

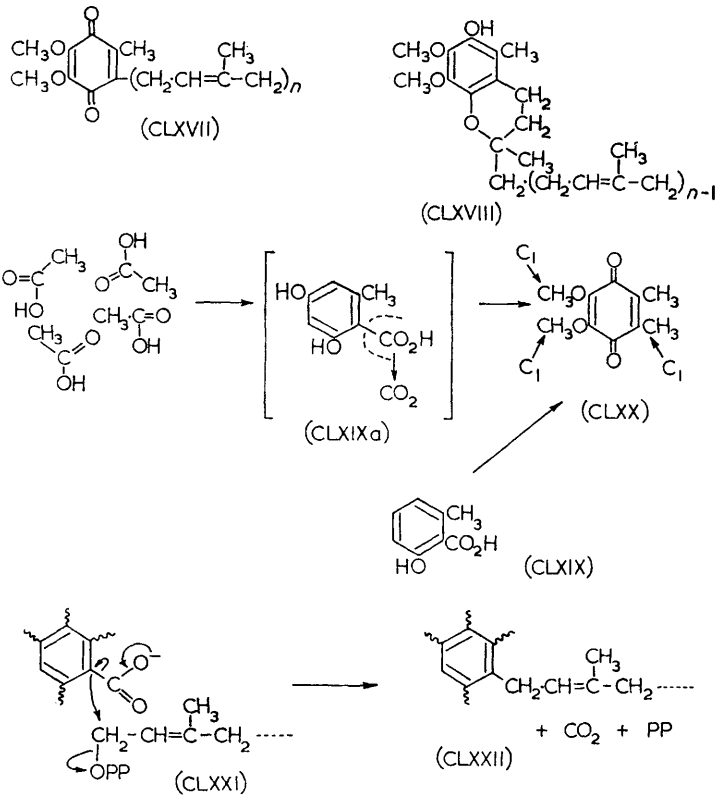


FIG. 32. Ubiquinones and some possible biogenetic relationships.

concept, Martius[25] described the conversion of tritium-labelled 2,3-dimethoxy-5-methylbenzoquinone (CoQ_0) into CoQ_4 (CLXVII, n = 4) by rat liver homogenates to which geranylgeranyl pyrophosphate was added. Some synthesis of CoQ_9 and CoQ_{10} was also observed simultaneously. CoQ_9 (CLXVII, n = 9), which apparently is the predominant form of coenzyme Q in the rat,[299-301] was not formed from CoQ_6 (CLXVII, n = 6)[302] but results consistent with the shortening of the isoprenoid chain have been reported.[302,303] Although Wiss *et al.*[296] were able to demonstrate incorporation of labelled mevalonate into the ubiquinone polyisoprenoid chain *in vivo*, with a labelling pattern consistent with that found in other isoprenoid systems, they could not confirm the utilisation of the preformed quinone nucleus as described by Martius,[295] nor was

[300] Gloor and Wiss, *Biochem. Biophys. Res. Comm.*, 1960, **2**, 222.
[301] Lawson, Mercer, Glover, and Morton, *Biochem. J.*, 1960, **74**, 38P.
[302] Rudney and Sugimura, ref. 294, p. 211.
[303] Threlfall and Glover, *Biochem. J.*, 1962, **82**, 14P.

mevalonate utilised in a rat liver homogenate.[304] More recently, however, the incorporation of mevalonate into ubiquinone in a rat liver homogenate has been demonstrated.[305]

The structure of the acceptor molecule to which the isoprenoid chain becomes attached is still uncertain. Olson *et al.*[306] confirm the finding of Wiss *et al.*[296] that the preformed quinone nucleus is apparently not utilized. The absence of known biosynthetic pathways for the formation of aromatic compounds (other than œstrogens) in mammals has directed attention to the possible rôle of phenylalanine and tyrosine as precursors of the quinone moiety. While earlier attempts to test this possibility gave negative results,[296] it now seems clear that these essential amino-acids can provide the quinone moiety of the ubiquinones in the rat.[307] When unlabelled *p*-hydroxybenzoate was administered simultaneously with the labelled amino-acids, the labelling of the quinone ring was virtually abolished, suggesting that *p*-hydroxybenzoate may be an intermediate. Benzoate labelled in the ring was poorly incorporated and the label from carboxyl-labelled benzoate was entirely lost. Further studies by Olson *et al.*[306] indicate that 2,4-dihydroxybenzoate and 3,4-dihydroxybenzoate may be precursors of the quinone moiety. The *C*-methyl group of the quinone ring and also the *O*-methyl groups were derived from the 1-carbon pool.[307]

The above results of Olson and his co-workers in the rat are similar in many respects to those reported by Rudney and Parson for the biosynthesis of CoQ_{10} by *Rhodospirillum rubrum*. These authors observed the incorporation of *p*-hydroxybenzaldehyde into the quinone moiety with loss of the aldehydic carbon.[308] *p*-Hydroxybenzoic acid was also utilised[309] and the *C*-methyl group of the CoQ ring was derived from methionine.[308] Rhodoquinone, which differs from ubiquinone only in that one phenolic hydroxyl group is unmethylated, is probably not a precursor of ubiquinone.[309]

Birch and his co-workers have suggested an analogy between the biosynthesis of the ubiquinones and the mould metabolite aurantiogliocladin (CLXX). This compound is apparently synthesised from four acetate molecules with loss of the carboxyl carbon of one of them, and introduction of one *C*-methyl and two *O*-methyl carbons from the 1-carbon pool.[310,297] The intact incorporation of 6-methylsalicylic acid (CLXIX) was reported, but orsellinic acid (CLXIXa), originally suggested as an intermediate,[310] was not utilised.[297] The significance of these results for

[304] Wiss, Gloor, and Weber, *Amer. J. Clin. Nutr.*, 1961, **9**, 27.
[305] Green, Diplock, Bunyan, and McHale, *Biochim. Biophys. Acta*, 1963, **78**, 739.
[306] Olson, Dialameh, Aiyar, Ramsey, Riegl, and Bentley, Proc. 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 434.
[307] Olson, Bentley, Aiyar, Dialameh, Gold, Ramsey, and Springer, *J. Biol. Chem.*, 1963, **238**, PC 3146.
[308] Rudney and Parsons, *J. Biol. Chem.*, 1963, **238**, PC 3137.
[309] Parson and Rudney, Proc. 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 434.
[310] Birch, Fryer, and Smith, *Proc. Chem. Soc.*, 1958, 343.

ubiquinone synthesis is unclear. However, the observed introduction of the *C*-methyl group from the 1-carbon pool in both the mammal[307] and a mould[308] together with other evidence[306] makes it unlikely that orsellinic acid is a precursor in these systems.

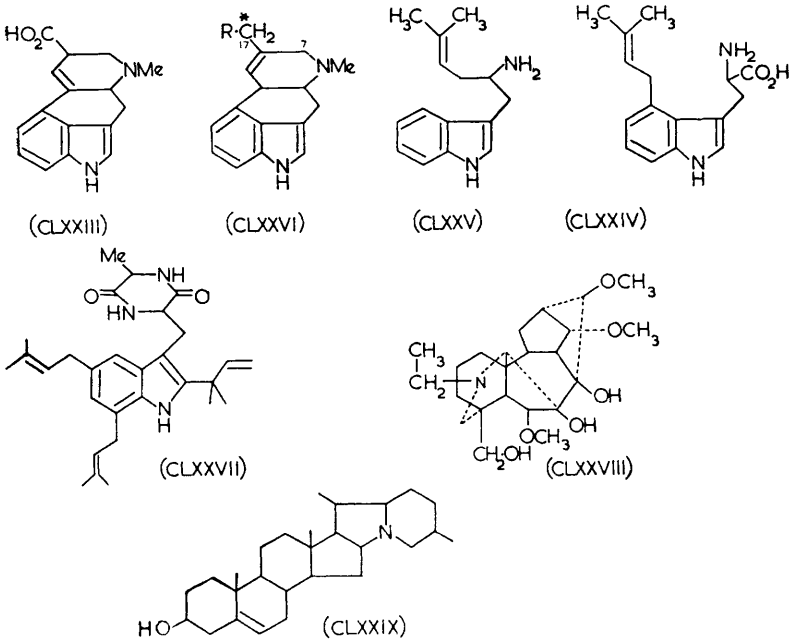
The decarboxylation indicated by these various studies could be coupled with the attachment of the polyisoprenoid chain in an enzymic S_N2 reaction (CLXXI—CLXXII). An analogous coupled decarboxylation–methylation process may be involved in the introduction of one of the *C*-methyl groups of aurantiogliacladin.

Considerable interest centres around the inter-relationship of the ubiquinones and cyclised derivatives of the ubichromenol type (CLXVIII). Although various lines of evidence[311–313] have suggested that ubichromenol may be an artifact of isolation or storage, evidence for its biosynthesis from ubiquinone or from some common precursor has been presented.[305] The possible participation of ubichromenol derivatives in the generation of high-energy phosphate in the process of oxidative phosphorylation has been discussed by several authors.[168,314–317]

Terpenoid Alkaloids.—Several classes of alkaloid have carbon skeletons that are totally or partially isoprenoid. Excellent sources of references up to 1962 are the reviews by Leete[318] and Battersby.[319]

It is now clear that the ergot alkaloids related to lysergic acid (CLXXIII) originate, as suggested by Mothes *et al.*[320] by condensation between (presumably) dimethylallyl pyrophosphate and tryptophan. Thus, in agroclavine (CLXXVI, R = H) and elmyoclavine (CLXXVI, R = OH) the bulk of the label of [2-^{14}C]mevalonic acid is found[321] in the branch carbon, $C_{(17)}$, as shown, though some labelling was present in $C_{(7)}$. Controversy now surrounds the question of how the isoprene and tryptophan fragments become united, in particular whether the dimethylallyl substituent becomes attached initially to $C_{(4)}$ of the tryptophan nucleus (CLXXIV) or to the 2-carbon of the alanyl side chain (CLXXV). Weygand *et al.*[322] have recently discussed a variety of suggested mechanisms and have described experiments to compare the utilisation of their suggested intermediate (CLXXV) with that of the 4-dimethylallyl intermediate (CLXXIV) suggested by Pleininger *et al.*[323] who have also reported similar experi-

[311] Draper and Csallany, *Biochem. Biophys. Res. Comm.*, 1960, **2**, 307.
[312] Links, *Biochim. Biophys. Acta*, 1960, **38**, 193.
[313] Stevenson, Henning, and Morton, *Biochem. J.*, 1964, **89**, 58P.
[314] Folkers, Shunk, Linn, Trenner, Wolf, Hoffman, Page, and Koniuszy, ref. 294, p. 100.
[315] Clark and Todd, ref. 294, p. 190.
[316] Slater, Colpa-Boonstra, and Links, ref. 294, p. 161.
[317] Moore and Folkers, *J. Amer. Chem. Soc.*, 1964, **86**, 3393.
[318] Leete, ref. 43, p. 739.
[319] Battersby, *Quart. Rev.*, 1961, **15**, 259.
[320] Mothes, Weygand, Groger, and Grisebach, *Z. Naturforsch.*, 1958, **13b**, 41.
[321] Battacharji, Birch, Brack, Hofmann, Kobel, Smith, Smith, and Winter, *J.*, 1962, 421.
[322] Weygand, Floss, Mothes, Groger, and Mothes, *Z. Naturforsch.*, 1964, **19b**, 202.
[323] Pleininger, Fischer, and Leide, *Angew. Chem.*, 1962, **74**, 430.



ments.[324] The suggested precursors were supplied to growing *Claviceps* cultures, either singly or simultaneously, with the two compounds differently labelled with ^{14}C and ^{3}H. Elymoclavin (CLXXVI, R = OH) was isolated and the relative efficiency of incorporation of the test substances was assayed. Both groups of workers agreed that the 4-dimethylallyl derivative (CLXXIV) was the better precursor, a finding which supports the proposals of Battacharji *et al.*,[321] but doubts were expressed as to the real significance of the results and the rôle that degradative reactions might have played in making the labelled materials available for alkaloid synthesis. The testing of doubly-labelled precursors with (for example) ^{14}C in the dimethylallyl chain and ^{3}H in the alanine chain, with comparison of labelling ratios in the starting material and in the product, might answer such doubts. The biological substitution of a dimethylallyl residue in the aromatic ring of tryptophan presumably has an analogy in the biosynthesis of the mould metabolite echinulin (CLXXVII) for which tryptophan is a precursor.[325]

The uncertainties that may arise in assessing the utilisation of a precursor by intact plant tissues is illustrated by the recent reports of non-utilisation[326] and utilisation (with low efficiency)[327] of labelled mevalonate in the biosynthesis of the *Delphinium* alkaloid, lycoctinine (CLXXVIII).

[324] Pleininger, Fischer, and Leide, *Annalen*, 1964, **672**, 223.
[325] Birch and Farrar, *J.*, 1963, 4277.
[326] Herbert and Kirby, *Tetrahedron Letters*, 1963, **23**, 1505.
[327] Benn and May, *Experientia*, 1964, **20**, 252.

The incorporation of mevalonate into *Solanum* alkaloids, such as solanidine (CLXXIX) in which the carbon skeleton is identical with that of cholesterol, has also been reported.[328] That cholesterol itself may indeed be a precursor of these alkaloids should now be seriously considered in view of its proven occurrence in the same plant tissues.[163] An intermediary rôle of cholesterol in the biogenesis of sapogenins has been suggested on the basis of studies with ^{14}C-labelled precursors in *Dioscorea* plants.[329]

Conclusion

It happens that while these paragraphs were being written, the 1964 Nobel prizes for medicine were being awarded to Konrad Bloch and Fodor Lynen for their independent contributions to the fundamental understanding of the biosynthesis of terpenoids and other lipids. In attempting to assemble material for this Review, it has become all too apparent how vast are the implications of this new knowledge of terpene biosynthesis for so many different areas of biology and biochemistry. It would be impossible, except in an extensive monograph, to cover in detail all of the topics that have been touched upon, and even then an author with the requisite chemical and biochemical expertise in all of these fields would be hard, if not impossible, to find. Moreover, the rate at which fundamental understanding of terpenoid biosynthesis is now being utilised in further explorations would outdate such a monograph even before it left the printing press.

One of the most striking features of the area reviewed is the extensive interaction that has occurred between the "chemical" and "biochemical" disciplines. In the context of historical development many examples have been cited and in terms of recent contributions the extraordinary success of the joint work of Cornforth and Popjàk is pre-eminent. Such interdisciplinary contributions will no doubt become more frequent, since much work especially in relation to the biogenesis of alkaloids and mould products poses interesting problems calling for study at an enzymic level. Moreover, in the light of rapidly increasing knowledge of protein structure, comparative studies of some of the enzymes responsible for the formation of closely related cyclic terpenoids may provide new insight into the detailed structures of the active sites of these enzymes and consequently into their mechanisms of action.

Finally, the unerring accuracy with which organic chemical theory has been able to predict the course of many biological transformations in the terpene series poses fascinating, but at present unanswerable, questions concerning biochemical evolution, for in no area of biochemistry is it more obvious that enzymes exploit the inherent potential reactivities of their substrates.

This Review was written, during tenure of an Established Investigatorship of the American Heart Association.

[328] Coseva and Paseschnichenko, Proc. 5th International Congress of Biochemistry, Moscow, 1961, vol. 7, 287–293, Pergamon, 1963.

[329] Bennett and Heftmann, Proc. 6th International Congress of Biochemistry, New York, 1964 (Abstracts), p. 565.