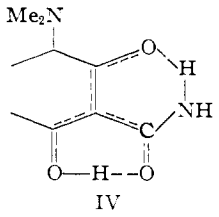
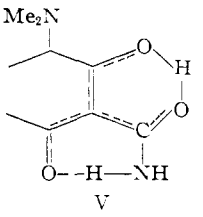
A. Comparable degradation studies on tetracycline and epitetracycline confirm this spectral evidence in establishing the area involved.

In group A only two possibilities exist for isomerization. These include epimerization at C.4 and enol tautomerism involving such structures as IV and V in which the strongly chelated carboxamide group could assume different orientations.



IV



V

The latter possibility can be excluded since the epimers give isomeric nitrile derivatives, 10-benzenesulfonyltetracylinonitrile $((\alpha)^{25}D$ (1% in dimethylformamide) $-470°$, *Anal.* Calcd. for $C_{23}H_{26}N_2O_9S$: C, 59.36; H, 4.62; N, 4.94. Found: C, 59.46; H, 5.02; N, 4.64) and 10-benzenesulfonylepitetracyclinonitrile $((\alpha)^{25}D$ (same solvent) $-431°$, *Anal.* C, 59.83; H, 4.89; N, 5.19) on treatment with benzenesulfonyl chloride in pyridine solutions.[6] The nitrile derivatives show absorption differences similar to those observed with the parent epimeric amides (*cf.* Fig. 2).

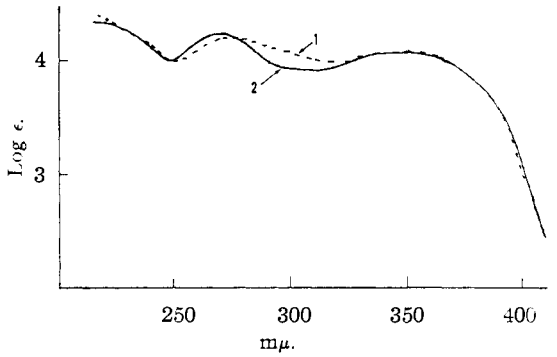


Fig. 2.—Ultraviolet absorption spectra in 0.01 *N* methanolic HCl: 1, - - - 10-benzenesulfonyltetracyclinonitrile; 2, ——— 10-benzenesulfonylepitetracyclinonitrile.

Further confirmation of the epimerization reaction is found in a study of the properties of desdimethylaminotetracycline[6] (m.p. 210–215° (dec.) $(\alpha)^{25}D$ (0.5% in methanol, 0.1 *N* in HCl) $-260°$, *Anal.* H, 4.72; N, 3.47). This compound, differing from tetracycline only in that the $-NMe_2$ is replaced by H (thus removing the asymmetry at C.4), undergoes no change when subjected to conditions which rapidly epimerize either tetracycline or epitetracycline.

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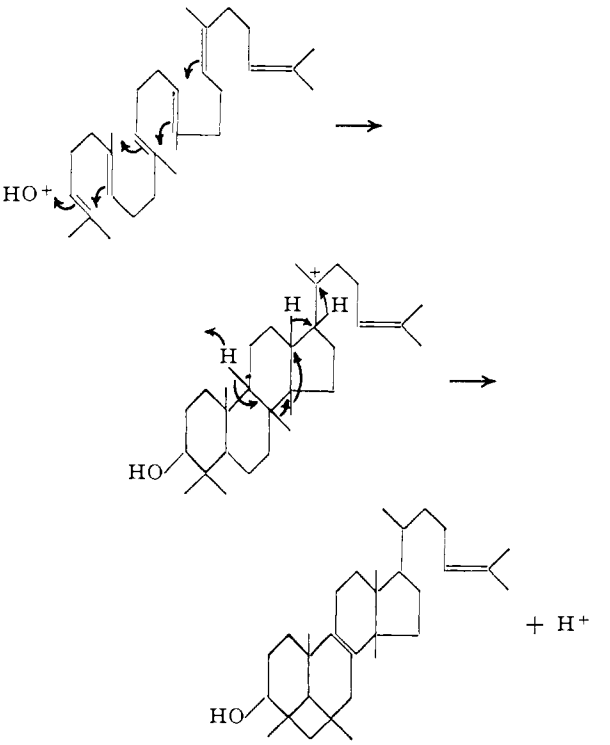
(6) Reference 5 includes a discussion of similar products from chlorotetracycline and oxytetracycline.

ON THE MECHANISM OF CYCLIZATION OF SQUALENE[1]

Sir:

The transformation of squalene to lanosterol[2] and of lanosterol to cholesterol[3] has been reported recently. In order to rationalize the biogenetic relation between squalene and the steroids, and the origin of lanosterol in particular, it had earlier been proposed[4] that in the cyclization process the triterpenoid chain assumes the folded form shown in Fig. 1.[5] In their comprehensive theoretical treatment of the mechanism of steroid and triterpene



biogenesis, Ruzicka[6] and Eschenmoser, *et al.*,[7] arrive at the important conclusion that the transformation of squalene to lanosterol is a concerted or non-stop process, *i.e.*, it occurs without formation of stabilized intermediates. In their formulation the cyclization is initiated by the attack of an hypothetical electrophilic OH^+. We now wish to present the results of studies which provide strong experimental evidence in support of a concerted reaction mechanism. Moreover, it is demonstrated that molecular oxygen is involved in this oxidative cyclization.

In the first series of experiments, squalene was incubated with liver homogenate in the presence of

(1) Supported by grants-in-aid from the National Science Foundation and the Life Insurance Medical Research Fund.

(2) T. T. Tchen and K. Bloch, THIS JOURNAL, **77**, 6085 (1955).

(3) R. B. Clayton and K. Bloch, *J. Biol. Chem.*, **218**, 305 and 319 (1956).

(4) R. B. Woodward and K. Bloch, THIS JOURNAL, **75**, 2023 (1953); W. G. Dauben, *et al.*, *ibid.*, **75**, 3038 (1953).

(5) Since the limited space does not permit a discussion of the stereochemistry of the cyclization process, the configuration of the methyl groups and of the hydrogens is not indicated in Figure 1.

(6) L. Ruzicka, *Experientia*, **9**, 359 (1953).

(7) A. Eschenmoser, L. Ruzicka, O. Jeger and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).

D_2O. The data in Table I show that lanosterol was formed without incorporation of deuterium from the medium and that it contained less than 5% of the value calculated for the incorporation of one atom of D per molecule of sterol synthesized.

TABLE I

Expt.	% of D in medium	Homogenate Ml.	Homogenate Mg.	Lanosterol formed,[a] % of added squalene	Atom % excess D Calcd.[b]	Atom % excess D Found
1	63	250	4.47	7.2	0.114	0.005
2	39	53	1.27	8.6	.040	− .003
3	42	62	0.84	5.4	.038	.001

[a] Calculated from C^{14} conversion values. [b] Calculated for lanosterol diluted with carrier and for incorporation of 1 proton from the medium per molecule of lanosterol formed and assuming a reaction rate for H^+ 5.5 times that for D^+.

Therefore, in the over-all process, no C–H bonds are formed with participation of protons from an external source. The formation of unsaturated intermediates, either partially cyclized or tetracyclic, by proton elimination from transitory carbonium ions can be ruled out because subsequent conversion to lanosterol would of necessity involve the uptake of D from the reaction medium. Therefore hydride shifts must be responsible for the transport of methyl groups to the positions in which they appear in lanosterol. Furthermore the failure of protons to enter into the cyclization precludes the addition of water to the squalene chain as a participating reaction and hence OH^- cannot be the source of the 3β hydroxy group of lanosterol. In corroboration of this conclusion it has been found that O^{18} from H_2O^{18} is not incorporated into lanosterol. On the other hand, when squalene is cyclized in an atmosphere of $O_2{}^{18}$, heavy oxygen is found in lanosterol (Table II). Activated oxygen must therefore be the oxidant as well as the initiating agent for the cyclization.[8]

The rat liver enzyme appears to be specific for the conversion of squalene to sterols since lanosterol is the only product in the system described. Similar but not identical enzymes must exist in plants to catalyze the cyclization of squalene to the various tetracyclic and pentacyclic triterpenes. For the enzyme system described here, the first of a general type, we wish to propose the name *squalene oxidocyclase I*.

(8) We have considered as an alternative that the cyclization is initiated by a stereospecific addition of proton to the carbon atom which becomes C_3 in the steroid ring system and that the same hydrogen is subsequently replaced by OH in an oxidative step. Neither the deuterium nor the O^{18} data rule out this possibility. However, in this event, a tetracyclic hydrocarbon might be expected to be an intermediary product and to accumulate under anaerobic conditions. The search for a metabolite of squalene having these properties has been negative.

TABLE II

Expt.	Atom % excess O^{18}	Homogenate, ml.	Lanosterol formed[a] mg.	Lanosterol formed[a] % of squalene	Atom % excess O^{18} in lanosterol Calcd.[b]	Atom % excess O^{18} in lanosterol Found
$O_2{}^{18}$	10.9	210	0.44	0.48	0.134	0.070
H_2O^{18}	0.9	210	2.30	4.1	.085	− .002

[a] Calculated from C^{14} conversion value. [b] Calculated for lanosterol after addition of carrier and on the assumption that the oxygen in the lanosterol formed derives from oxygen gas and from water in the first and second experiments, respectively.

Experimental.—In the experiments with D_2O or H_2O^{18}, the livers from 100 g. male rats were homogenized in a Waring Blendor for 20 seconds with a minimum amount of 0.1 *M* phosphate buffer of *p*H 7.4 and aged at room temperature for 10 minutes. Approximately two volumes of ice-cold phosphate buffer in D_2O or H_2O^{18} (0.1 *M* *p*H 7.4) was added and the mixture homogenized again for 20 seconds. After centrifugation for 10 minutes at 700 × *g* the supernatant was decanted and 2.5 mg. of nicotinamide added per ml. of homogenate. In the experiments with O^{18} gas, the experimental vessels were evacuated and flushed with helium three times and incubated in an oxygen (O^{18} enriched)-helium atmosphere. The preparation of C^{14} squalene and the isolation of lanosterol have been described.[1,2] Incubations were carried out for 3 hours at 38° in a Dubnoff shaker. Lanosterol was analyzed for O^{18} as described by Doering and Dorfman.[9]

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(9) W. von E. Doering and E. Dorfman, THIS JOURNAL, **75**, 5595 (1953).