physical property. For these reasons, we have elected to study the mass spectrum of its decomposition products as a function of time.

The apparatus consisted of a Bendix time-of-flight mass spectrometer with a silica sample tube mounted axially "upstream" from the ionizing electron beam. A pinhole (diameter about 40 μ, in mica) approximately 1 mm. from the electron beam separated the two chambers. About 8 in. beyond the spectrometer the sample tube joined a conventional vacuum system. The sample tube was also equipped, through a buffer chamber, with a silicone rubber septum for injection of samples. A flash tube, Tesla-triggered, could be placed next to the sample tube. The tube was supplied by a 1.5-μf. capacitor charged to 12 kv.; the time constant of the capacitor was 1 μsec.

The oscilloscope output of the mass spectrometer was fed into half of a Type G differential preamplifier in a Tektronix 533 oscilloscope. The other side of the preamplifier received as its signal a single slow (2 or 5 msec.) sawtooth, triggered by the discharge of the flash lamp. The oscilloscope itself was triggered by the spectrometer at the beginning of each of its very rapid sweeps of the mass spectrum. The repetition frequency was adjusted to 20 kc. Thus, the oscilloscope showed a continual display of superposed mass spectra on a single base line, *except* for the 2 or 5 msec. following the flash discharge. During this interval, each sweep was displaced vertically from that preceding it, so that a sequence of 20 or more individual mass spectra could be observed. A conventional Tektronix camera with Polaroid back recorded the spectra. Signal-to-noise ratio was typically 3 or 4 to 1 (or more) for major peaks.

Samples (0.5 to 2.5 mg.) of benzenediazonium 2-carboxylate were injected in water or occasionally methanol solution, the solvent was pumped away, the pure solid was flashed, and the time-resolved mass spectrum was recorded. The spectrometer background could be recorded before photolysis by triggering the sawtooth manually; mass spectra of products after photolysis and calibration spectra of known substances were obtained in the same way.

The mass spectra show a very distinct evolution. There first occurs an interval of 200–300 μsec. during which only the background is present. This level is ordinarily the oscilloscope line width except at masses 18 and 28, which are slightly above the line width. The delay is due to the distance between the solid precursor and the pinhole, and to any induction period in decomposition. Then, within the 50 μsec. time resolution of our system, *masses 28, 44, and 76 appear simultaneously, at or near their maximum intensities.* Mass 152 appears at the same time but at an intensity less than one-fourth of its maximum. There are, naturally, several other peaks, which depend on the ionizing voltage. Most of pattern thereafter also depends on the ionizing voltage and on the sample size. Mass 152 most frequently rises to approach its maximum intensity about 200–250 μsec. after the first appearance of masses 28, 44, and 76; this maximum persists for more than the duration of the sawtooth sweep. The rise time of 152 is closely matched by the disappearance time of 76, usually 250–300 μsec. but occasionally longer.

It proved very helpful to compare mass spectra obtained with different ionizing voltages. Spectra at 50–55 v. show many masses between 76 and 152; mass 76 decays to a nonzero intensity. Comparisons showed that mass 76 is a cracking product of mass 152. Other peaks, especially 26, 50, 51, and 61–64, decrease to background in the same time that 76 goes to its limiting value. With an ionizing energy of 42 v., the intensities of all masses between 76 and 152 were reduced almost to background, while 76 and 152 remained relatively high. At 35 v., the only peaks which cannot be attributed to background, N_2, or CO_2 are 152, 77, 76, and, less intense, 50 and a trace of 26.

The cracking pattern associated with mass 76, determined at 50-v. ionizing energy, from the first 200 μsec. after appearance for masses above 18 is as follows (relative intensities in parentheses): 25 (2), 26 (8–9), 27 (4–8), 37 (3), 38 (6), 39 (10), 40 (3), 50 (9–4), 51 (4–3), 52 (2), 61 (2), 62 (6), 63 (2), 64 (1), 74 (3), 75 (4), 76 (9), and traces of 77 and 78.

At this point we can consider the foregoing results in light of the evidence cited in the second paragraph. We infer that the peak at mass 152 is due to biphenylene, and that masses 28 and 44 are due to N_2 and CO_2, respectively. We infer also that the biphenylene, which appears much more slowly than diffusion alone would indicate, is formed as a gas. We infer from the rates of appearance of 152 and disappearance of 76 that the former is a product of dimerization of the latter. We infer, finally, that mass 76 is due to the transient benzyne molecule, and that no other species besides nitrogen, carbon dioxide, biphenylene, and benzyne are present in significant concentration in the gas phase.

The length of time in which the benzyne mass is observable is the same, within experimental uncertainty, as that during which a transient continuous absorption spectrum is detectable.[4] This agreement, together with the absence of other transient parent masses, is strong confirming evidence for the assignment of the spectrum to benzyne.

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Endogenous Formation of $\Delta^{5,7,24}$-Cholestatrien-3β-ol[1]

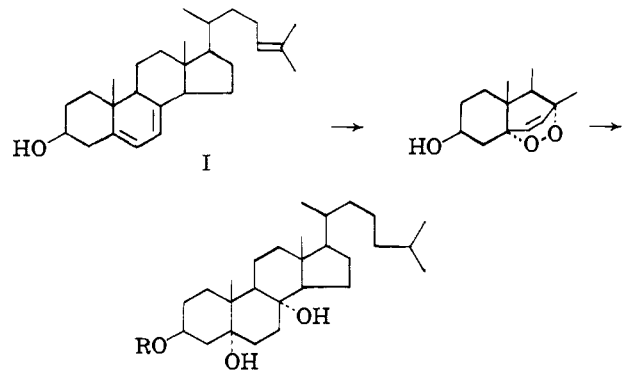
Sir:

The over-all mechanism of cholesterol biosynthesis is known and the reaction sequence between mevalonate

(1) Agents Affecting Lipid Metabolism. Part X. Part IX: M. Kraml, J. F. Bagli, and D. Dvornik, *Biochem. Biophys. Res. Commun.*, **15**, 455 (1964).

and lanosterol is well established.[2] Despite the fact that a number of lanosterol derivatives have been isolated from, or their presence suggested in, cholesterogenic tissues,[3] uncertainty still exists about the normal sequence of enzymatic reactions involved in the conversion of lanosterol to cholesterol.[4] Insufficient information about the size and lifetime of the available pool of an intermediate sterol permits only tentative deduction as to its place in the metabolic chain of cholesterol biosynthesis. A useful method of elucidating intermediate steps in metabolic processes is based on the interference with one of the enzymes by a specific inhibitor which causes accumulation of its substrate. Thus it may be possible to isolate and to characterize an intermediate substrate even though normally it may be present in too small a quantity to detect.[5] Using this approach we have recently established[6] the often suggested possibility[3i,7] that 7-dehydrocholesterol is an intermediate on the major pathway of hepatic cholesterol synthesis. Evidence, secured by the same method, of endogenous formation of $\Delta^{5,7,24}$-cholestatrien-3β-ol (I) is reported herewith.

The molecular changes involved in the biosynthesis of cholesterol from lanosterol are of two types, nuclear and peripheral. The nuclear transformations comprise the removal of three methyl groups (the *gem*-dimethyl at C-4 and the angular methyl at C-14) and a "shift"[3a] of the $\Delta^{8,9}$-bond to position 5,6. The peripheral change is the saturation of the Δ^{24}-bond. Available experimental data indicate that the enzyme system producing changes in the sterol nucleus can act on all cholesterol precursors independently of the oxidation level of their 24,25-bond[8] and, furthermore, that the enzyme(s) affecting saturation of the side chain can act on any intermediate sterol regardless of the state of its nucleus.[9] The stage at which an intermediate will undergo the (peripheral) Δ^{24}-bond saturation may differ from tissue to tissue,[10] thus reflecting a tissue-specific pattern of enzymes[11] involved in the transformation of lanosterol to cholesterol. In accordance, inhibition of the enzymes causing side-chain reduction must lead to repletion of the product of all nuclear changes in the lanosterol molecule, *viz.*, 24-dehydrocholesterol. On the other hand, inhibition of any enzyme involved in the sequence of nuclear transformations of lanosterol must cause accumulation of the C-24-saturated intermediate which is the substrate of the inhibited enzyme. Both possibilities have been borne out experimentally. Agents which prevent saturation of the (peripheral) Δ^{24}-bond cause accumulation of 24-dehydrocholesterol in laboratory animals, *e.g.*, triparanol,[12] 3β-(β-dimethylaminoethoxy)-Δ^5-androsten-17-one,[13] 22,25-diazacholestanol,[14] etc. AY-9944,[15] on the other hand, shown to inhibit the reduction of the (nuclear) Δ^7-bond,[6,16] causes accumulation of 7-dehydrocholesterol (II).[17] Consequently, simultaneous administration to laboratory animals of triparanol and of AY-9944 should cause accumulation of the intermediate $\Delta^{5,7,24}$-cholestatrien-3β-ol (I), whose role as possible precursor of II was first considered by Johnston and Bloch[3a] and whose presence was surmised in the small intestine of a guinea pig fed with triparanol.[3j]



III

The postulate was confirmed experimentally as follows. The liver of a pig fed with triparanol and AY-9944[18] contained "fast-acting" sterol(s)[19] which showed absorption bands in the ultraviolet typical of steroid homoannular dienes.[7b,20] To facilitate the isolation[21,22] the homoannular diene system was con-

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(18) A daily dose of 150 μmoles/kg. of triparanol and of 75 μmoles/kg. of AY-9944 was given orally over a period of 14 days. At the conclusion of the feeding period the liver (750 g.) was homogenized in ethanol–ether, the extract hydrolyzed, and the neutral fraction (3.29 g.) separated.

(19) Color development in the Liebermann–Burchard reaction after 1.5 min. [*cf.* P. R. Moore and C. A. Bauman, *J. Biol. Chem.*, **195**, 615 (1952)]. Expressed as II, the concentration was 133 mg. %. The cholesterol content was 36 mg. %. The liver of another pig of same age, given orally, 75 μmoles/kg. of AY-9944 for 4 weeks, contained 139 mg. % of "fast-acting" sterols and 64 mg. % of cholesterol. The liver of a control pig contained 281 mg. % cholesterol but no "fast-acting" material.

(20) *Cf.* L. Dorfman, *Chem. Rev.*, **53**, 47 (1953). Expressed as II, the concentration was 155 mg. %.

(21) Isolation of II is troublesome due to unrewarding chromatographic separation [see, for example, P. D. Klein and P. A. Szczepanik, *J. Lipid Res.*, **3**, 460 (1962)] and its proneness to peroxide formation[6] [*cf.* A. A. Kandutsch, *J. Biol. Chem.*, **237**, 358 (1962)].

verted to a stable transannular peroxide[23,24] which was isolated, characterized, and identified as the 5,8-peroxide of $\Delta^{5,7,24}$-cholestatrien-3β-ol, m.p. 138–139°, $[\alpha]_D$ −4.3° ($CHCl_3$), $[\phi]$ −18[25]; n.m.r., τ 9.10, 9.17 (C-18, C-19, and C-21 methyls); 8.28, 8.36 (C-25 vinyl methyls); 3.51, 3.80 (C-6 and C-7 vinyl protons); 4.96 (C-24 vinyl proton). *Anal.* Found: C, 77.93; H, 10.17, in agreement with $C_{27}H_{42}O_3$. Structure was adduced by hydrogenation with PtO_2 as catalyst to the cholestane-3β,5α,8α-triol (III, R = H),[26] m.p. 185–187°, $[\alpha]_D$ −21.4° (pyridine), $[\phi]$ −88. *Anal.* Found: C, 77.20; H, 11.43, in agreement with $C_{27}H_{48}O_3$. The triol (III) proved to be identical with an authentic sample prepared from II by photosensitized transannular addition of oxygen[23] followed by hydrogenation.

Our findings reflect the role of 7-dehydrocholesterol as intermediate on the major pathway of hepatic cholesterol synthesis[6] and indicate that $\Delta^{5,7,24}$-cholestatrien-3β-ol (I) is a precursor of 7-dehydrocholesterol and of 24-dehydrocholesterol and is not a metabolite of the latter.[3j] However, the quantitative importance of I as an obligatory intermediate on the normal pathway of cholesterol biosynthesis depends on the degree of side-chain reduction occurring at any one point in the normal pathway.[4]

Acknowledgment.—We thank Dr. E. Greselin for the animal work and Drs. G. Schilling and G. Papineau-Couture for the gas–liquid chromatographic data.

(22) Attempts to isolate pure I from livers of 30 rats treated with triparanol and AY-9944 were not successful. However, gas–liquid chromatography of the hydrolyzed neutral fraction indicated the presence of a peak with retention time corresponding to that calculated for I by the method of R. B. Clayton, *Biochemistry*, 1, 357 (1962). This finding lends support to the recent report on the probable presence of I in rat liver homogenates [M. E. Dempsey, J. D. Seaton, M. G. Sanford, and R. W. Trockman, *Federation Proc.*, **23**, 425 (1964)].

(23) F. Schenck, K. Buchholz, and O. Wiese, *Ber.*, **69**, 2696 (1936). The peroxide (0.8 g.) was separated from cholesterol by chromatography on florisil and purified by "thick" layer chromatography.

(24) It is interesting to note that the same procedure, applied to the brain of the same pig, has led to the isolation of the peroxide of II, thus suggesting inability of triparanol to cross the blood–brain barrier.

(25) Molecular rotation [*cf.* P. M. Jones and W. Klyne, *J. Chem. Soc.*, 871 (1960)].

(26) The corresponding acetate (III, R = CH_3CO) exhibited m.p. 160–162°, $[\alpha]_D$ −41.2° ($CHCl_3$), $[\phi]$ −190; n.m.r., τ 4.96 (carbinolic proton). *Anal.* Found: C, 75.09; H, 10.81, in agreement with $C_{32}H_{50}O_4$.

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The Vertical Ionization Potentials of Phenyl and Phenoxy Radicals

Sir:

Recent measurements[1] of the appearance potentials of $C_6H_5^+$ ions from phenyl halides lead to $\Delta H_f(C_6H_5^+)$ = 288 kcal./mole. This value is significantly lower than earlier values of ~300 kcal./mole given by the appearance potentials of $C_6H_5^+$ ions from benzene and toluene[2–4] and phenyl halides.[4] From $\Delta H_f(C_6H_5)$ = 70 kcal./mole[5,6] and $\Delta H_f(C_6H_5^+)$ = 288 kcal./mole Majer and Patrick obtained 9.4 v. for the ionization potential of the phenyl radical. This result is much lower than earlier values of $I(C_6H_5)$ = 9.9 v.[2–4] based on $\Delta H_f(C_6H_5^+)$ = 300 kcal./mole, and is close to the ionization potential of benzene itself, 9.25 v.[7,8]

The vertical ionization potential of the phenyl radical has been recently measured in this laboratory by electron impact ionization of phenyl radicals generated by the thermal decomposition of azobenzene at 800° in a reactor coupled to a mass spectrometer. In addition to the phenyl radical, benzene and biphenyl were produced. The apparatus and the method of evaluating the vertical ionization potential by comparison with a rare gas standard have been described.[9] The average of six determinations gives $I_{vert}(C_6H_5)$ = 9.20 v., in reasonable agreement with Majer and Patrick's value.[1] $I_{vert}(C_6H_6)$ measured in the same apparatus was 9.50 v., *i.e.*, 0.25 v. higher than the adiabatic value.[7] Since it may reasonably be expected that the adiabatic ionization potential of phenyl radical will be lower than the vertical value of 9.2 v. by a roughly similar amount, it appears that $I(C_6H_5)$ is less than $I(C_6H_6)$ by about 0.3 v.

An upper limit for $\Delta H_f(C_6H_5^+)$ can be obtained from the present result, using the relationship

$$\Delta H_f(C_6H_5^+) \leq I_{vert}(C_6H_5) + \Delta H_f(C_6H_5)$$

Although $\Delta H_f(C_6H_5)$ is generally taken at 70 kcal./mole[5,6] the bond stretching frequency in benzene suggests a slightly higher value, 73 kcal./mole.[10] Taking this value and $I_{vert}(C_6H_5)$ = 9.20 v. (212 kcal./mole), $\Delta H_f(C_6H_5^+) \leq$ 285 kcal./mole, in close agreement with Majer and Patrick's value of 288 kcal./mole (which is also an upper limit). This result clearly shows that the appearance potentials for $C_6H_5^+$ ions from aromatic hydrocarbons include considerable excitational energy. This is consistent with labeling experiments[11] which show that the formation of $C_6H_5^+$ ions from toluene and other aromatic hydrocarbons is accompanied by extensive reshuffling of H atoms, and perhaps even the loss of the phenyl structure. On the other hand, the agreement of the upper limit for $\Delta H_f(C_6H_5^+)$ derived from the present result with that obtained from the dissociative ionization of phenyl halides[1] indicates that $C_6H_5^+$ ions from phenyl halides have probably retained the phenyl configuration.

The measurement of the vertical ionization potential of the phenoxy radical C_6H_5O was carried out in the same apparatus. The radicals were produced by the thermal decomposition of allyl phenyl ether. The phenoxy radical has previously been detected by flash photolysis in absorption[12] and in the thermal decomposition of anisole by mass spectrometry.[13] In the thermal decomposition of allyl phenyl ether, which decomposes at a much lower temperature than anisole, a good yield of phenoxy radicals was obtained. Comparison of the ionization efficiency curve for phenoxy radical (mass 93) with that of a Kr standard gave

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