hydroxyquinone as identified by m.p. and mixed m.p. determinations.

Action of Amines on 2,5-Dimethoxybenzoquinone.—A mixture of 0.3 g. of the quinone and few drops of ethylamine, butylamine, benzylamine or aniline was refluxed in about 30 ml. of alcohol on the water-bath till the quinone has disappeared (about 20 minutes, except for aniline which required about 5 hours). The reaction mixture was cooled and the deposited crystals were collected and washed with alcohol. The yield was almost quantitative with all the amines.

Ethylamine gave 2,5-bis-(ethylamino)-benzoquinone, brilliant crimson crystals from benzene, m.p. 210°, undepressed with a sample prepared from benzoquinone and ethylamine.[8]

Butylamine gave bronze crystals of 2,5-bis-(butylamino)-benzoquinone, m.p. and mixed m.p. 160°.

Benzylamine gave deep red glistening plates of 2,5-bis-(benzylamino)-benzoquinone, m.p. 252°; with concentrated sulfuric acid, an orange color is produced.

Anal. Calcd. for $C_{20}H_{18}O_2N_2$: C, 75.45; H, 5.70; N, 8.80. Found: C, 75.12; H, 5.43; N, 8.57.

With aniline the product was 2,5-bis-(anilinoamino)-quinone,[9] does not melt up to 350°.

(8) M. Martynoff and G. Tsatsas, *Bull. soc. chim., France*, 52 (1947).

Anal. Calcd. for $C_{18}H_{14}O_2N_2$: C, 74.47; H, 4.86; N, 9.65. Found: C, 74.05; H, 4.97; N, 9.43.

Photochemical Reaction between 2,5-Dimethoxybenzoquinone and Benzylamine.—Two-tenths gram of 2,5-dimethoxyquinone was suspended in 50 ml. of dry benzene containing a few drops of benzylamine in a closed tube filled with nitrogen. When the tube was exposed to sunlight for three days (March), the quinone gradually disappeared and a red crystalline material was formed. The product was filtered and recrystallized from glacial acetic acid in deep red shining crystals, m.p. 252°, yield 0.22 g. A mixed m.p. with 2,5-bis-(benzylamino)-benzoquinone prepared above was undepressed.

Reaction between 2,5-Bis-(benzylamino)-1,4-benzoquinone and Benzaldehyde.—Three-tenths gram of the bisaminoquinone was refluxed with few drops of benzaldehyde in absolute alcohol and in the presence of piperidine as a catalyst. After seven hours the reaction mixture was cooled and the product was collected. It was proved to be unchanged material by m.p. and mixed m.p. determinations.

ABASSIA, CAIRO, EGYPT

(9) Comp. A. W. Hofmann, *J. Chem. Soc.*, 145 (1863); H. and W. Suida, *Ann.*, **416**, 113 (1918).

[CONTRIBUTION FROM THE CHEMICAL LABORATORY, UNIVERSITY OF CALIFORNIA]

The Biosynthesis of the Triterpene, Eburicoic Acid: The Utilization of Methyl-labeled Acetate[1,2]

BY WILLIAM G. DAUBEN, YOSHIO BAN AND JOHN H. RICHARDS[3]

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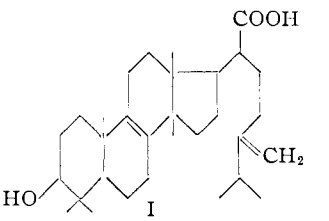
The C_{31}-triterpene, eburicoic acid, has been biosynthesized by allowing *P. sulfureus* to grow on a medium containing methyl-labeled acetate. By degradation of the acid, it has been shown that the carboxyl carbon, C_{21}, and the two methyl groups, C_{30} and C_{31}, of the *gem*-dimethyl group of ring A are derived from the methyl of acetate. Such a distribution is predicted on the basis of the squalene hypothesis of a "universal" biosynthetic mechanism leading to both the triterpenes and the sterols. It also was found that the extra carbon atom, C_{28}, found on C_{24} of the side-chain is not derived from acetate.

During the past few years, much insight has been gained into the mechanism of biosynthesis of the perhydrocyclopentanophenanthrene nucleus and one of the important results has been the establishment of the same or very similar pathway of formation of the steroids and triterpenes.[1-6]

Recently, it was shown in this Laboratory[1] that the tetracyclic triterpene eburicoic acid (I) could be obtained readily in labeled form by allowing the fungus, *P. sulfureus*, to grow on a labeled media. Using carboxyl-labeled acetate as the marked precursor, it was found that this moiety was used as a two-carbon unit, that C_4 of ring A and C_{11} and C_{12} of ring C were derived from the carboxyl of acetate and that the *gem*-dimethyl group of ring A, containing C_{30} and C_{31}, the carboxyl group, C_{21}, and the extra methylenic group, C_{28}, were not derived from the carboxyl of acetate. The location of the labeled atoms as well as the unlabeled atoms are those predicted on the basis of the squalene hypothesis.



I

Although the absence of radioactivity at certain positions in a molecule which has been derived from a carboxyl-labeled acetate strongly suggests that they are derived from the methyl of acetate, it is, indeed, worthy to establish this point by direct experiment. Accordingly, the fungus, *P. sulfureus*, was grown on a standard medium[1,7] which contained methyl-labeled acetate. After processing the dried mycelium in the usual fashion, it was found that the ether-extractable material, which is principally eburicoic acid, amounted to 53% of the dry weight of the mycelium. From this value and the specific activity of the crude extract, it can be calculated that 5.1% of the methyl-labeled acetate was incorporated into ether-extractable substances (mainly eburicoic acid). This incorporation can be compared with a value of 2.3% found with carboxyl-

(1) For the previous paper in this series, see W. G. Dauben and J. H. Richards, THIS JOURNAL, **78**, 5329 (1956).

(2) This work was supported, in part, by grant No. AT(11-1)-34, Project No. 16, U.S. Atomic Energy Commission.

(3) National Science Foundation Predoctoral Fellow, 1954-1955.

(4) For an excellent review of the pertinent work, see J. W. Cornforth, *Rev. Pure Appl. Chem.*, **4**, 286 (1954); C. Popják, *Roy. Inst. Chem.*, Lecture No. 2, 1955.

(5) R. B. Woodward and K. Bloch, THIS JOURNAL, **75**, 2023 (1953); W. G. Dauben, S. Abraham, S. Hotta, I. L. Chaikoff, H. L. Bradlow and A. H. Soloway, *ibid.*, **75**, 3038 (1953).

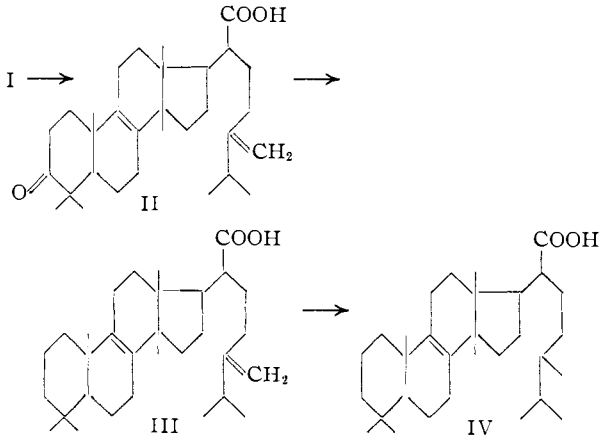
(6) A. Eschenmoser, L. Ruzicka, O. Jeger and D. Arigoni, *Helv. Chim. Acta*, **38**, 1890 (1955).

(7) W. G. Dauben and J. H. Richards, *Chemistry and Industry*, 94 (1955).

labeled acetate. On the basis of the fact that 18 of the carbon atoms of eburicoic acid should be derived from the methyl of acetate as compared to 12 from the carboxyl of acetate and on the basis that the randomization of label which occurs when methyl-labeled acetate passes through the tricarboxylic acid cycle[8] would bring about a more efficient utilization of the label, the higher value is in line with expectation.

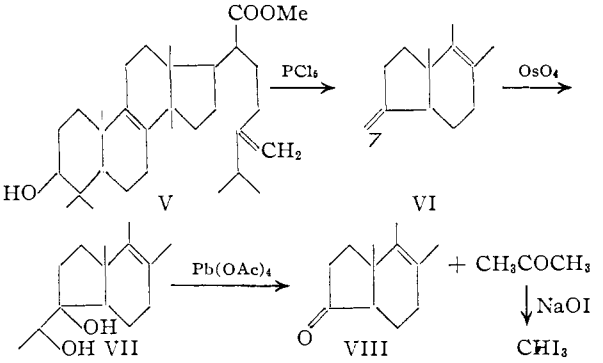
The eburicoic acid so derived from methyl-labeled acetate was degraded essentially along the lines presented in the earlier paper.[1] The first position studied was the methylene group at C_{24} on the side-chain. Upon ozonization of the methyl 3-acetoxy-eburic-8,24(28)-diene-21-oate, the methylenic carbon (C_{28}) was obtained as formaldehyde and the remainder of the molecule, after further oxidation with chromic acid, isolated as methyl 3-acetoxy-eburic-28-nor-7,11,24-trione-8-ene-21-oate. The formaldehyde so obtained was not radioactive. Thus, it can be concluded that this extra carbon whose progenitor is not predicted by the squalene hypothesis is not derived from either the methyl or the carboxyl of acetate. Such a result implies it is derived from a one-carbon unit and it will be of importance to determine the exact precursor of this extra carbon atom.

The next group studied was the carboxyl of eburicoic acid. In the earlier work using material derived from carboxyl-labeled acetate,[1] a Schmidt reaction employing eburicoic acid directly yielded CO_2 which was devoid of radioactivity. Due to the low solubility of the acid in the reaction medium, the reaction had to be run under quite vigorous conditions. In order to circumvent this difficulty, the hydroxyl and methylenic groups were removed. Eburicoic acid (I) was oxidized under



Oppenauer conditions to eburic-8,24(28)-diene-3-one-21-oic acid (II) and then reduced *via* the Wolff–Kishner reaction to eburic-8,24(28)-diene-21-oic acid (III).[9] The acid III was hydrogenated to eburic-8-ene-21-oic acid (IV) which was decarboxylated by reaction with hydrazoic acid. The acid possessed a specific activity of 78 cts./min./mg. C and the evolved CO_2 possessed a specific activity of 102 cts./min./mg. C, or 76% of the 31/18 ratio of the squalene hypothesis. A lower specific activity than the maximum was expected since, as previously mentioned, methyl-labeled acetate in going through the tricarboxylic acid cycle becomes randomized into a doubly-labeled moiety. Hence, the carboxyl group of eburicoic acid is derived from a methyl group of acetate. This demonstration, for the first time, of a specific methyl to carboxyl oxidation at this position is of importance since in some pentacyclic triterpenes (*ex.* oleanolic acid) the carbon atom which is biosynthetically equivalent to C_{21} of eburicoic acid is also found as a carboxyl group. The finding of a methyl group of acetate as a precursor of a carboxyl group calls attention to the danger of speculating as to the biosynthetic origin of a carbon atom on the basis of its oxidation state.

The next point of interest in eburicoic acid was to ascertain, with certainty, the biosynthetic source of the *gem*-dimethyl group (C_{30} and C_{31}) of ring A. The eburicoic acid obtained from methyl-labeled acetate was degraded by the retropinacol procedure described earlier[1] and the sequence of reactions is shown below. Starting with the diol VII of a specific activity of 90 cts./min./mg. C, the acetone obtained possessed a specific activity of 95 cts./min./mg. C and the iodoform derived from the acetone possessed a specific activity of 115 cts./min./mg. C. From these values it follows that the carbonyl carbon of acetone possessed a specific activity of 55 cts./min./mg. C. These results demonstrate that the carbon atoms C_{30} and C_{31} are derived from the methyl of acetate as predicted by the squalene hypothesis. Furthermore, the specific activity of these methyl-derived carbons is only 72% of that expected if no randomization had occurred. This value is to be compared to the 76% value found for the C_{21}-carboxyl carbon. Such an agreement substantiates the assumption that the lower specific activity is due to randomization and that the activity found is solely derived from the carbon atoms investigated and not due to other side-reactions.



Having obtained the actual specific activity of carbons in eburicoic acid derived from the methyl and the carboxyl of acetate, it is possible to evaluate the extent of the randomization which had occurred in the precursor before its utilization for eburicoic acid synthesis. The percentage randomization, as calculated, indicates the percentage of the total acetate employed in eburicoic acid syn-

(8) J. W. Cornforth, G. D. Hunter and G. Popják, *Biochem. J.*, **54**, 597 (1953).

(9) R. M. Gascoigne, J. S. E. Holker, B. J. Ralph and A. Robertson, *J. Chem. Soc.*, 2346 (1951).

thesis which had passed *once* through the tricarboxylic acid cycle.[10] From the activities obtained, it is found that the acetate was 60% randomized. Considering the length of the incubation period, *i.e.*, 4 months, such a value is quite low as compared to values of 32–64% randomization[5,8] obtained in liver slice experiments of 3-hour duration.

The results of degradation experiments performed on eburicoic acid derived from labeled acetate show that carbons 4, 11 and 12 are derived from the carboxyl of acetate and that carbons 21, 30 and 31 from the methyl of acetate. These data are in accord with those predicted on the basis of the squalene hypothesis and support the concept of a "universal" biosynthetic mechanism leading both to the triterpenes and the steroids. Furthermore, it is seen that the extra carbon found on the side-chain of eburicoic acid, and which is also found in ergosterol, is not derived from acetate. It should be re-emphasized that such data do not unequivocally demand squalene itself as a *direct* precursor of these groups of compounds.[11] The findings with eburicoic acid when coupled with those reported for ergosterol[12] with respect to the source of carbons 11 and 12 of ring C, however, suggest that if squalene is, indeed, not the *direct* precursor of triterpenes and steroids, the precursor is very closely related to it.

Experimental[13]

Growth and Isolation.—A potato broth (1000 ml.) containing 3% glucose and 82.0 mg. (1 mmole) of methyl-labeled sodium acetate with a specific activity of 2 mc./mmole was subjected to the same operations described for the previous growth experiments.[1] The dried mycelium (6.1 g.) was ground and then extracted in a Soxhlet apparatus first with petroleum ether (60–90°) for two hours and then with ether for 28 hours. The ether extract yielded 5.24 g. (53%) of crude eburicoic acid. A portion of the material was purified as the free acid and the remainder was acetylated and methylated prior to purification. The ester acetate possessed a specific activity of 9200 cts./min./mg. C and was essentially free from the 7,9(11)-dienic contaminant.

Ozonolysis of Methyl 3-Acetoxyeburic-8,24(28)-diene-21-oate.—Ozonolysis of the ester (95 mg., 0.18 mmole, s.a. 4500 dis./min./mg. C) in 10 ml. of glacial acetic acid followed by treatment as previously described[1] yielded 35 mg. (67%) of recrystallized formaldehyde dimedon derivative, m.p. 190.2–191.0°, s.a. 10 dis./min./mg. C.[14] The organic residue after oxidation with chromic acid followed by chromatography and recrystallization afforded 30 mg. (30%) of methyl 3-acetoxyeburic-28-nor-7,11,24-trione-8-ene-21-oate, m.p. 194.0–195.6°, ϵ^{271}_{max} 8700, s.a. 4300 dis./min./mg. C.

Eburic-8,24(28)-diene-3-one-21-oic Acid (II).—A mixture of C^{14}-3-hydroxyeburic-8,24(28)-diene-21-oic acid (2.330 g., 4.95 mmoles), cyclohexanone (5.85 ml., 56.5 mmoles), aluminum *t*-butoxide (6.13 g., 24.9 mmoles) and dioxane (17.6 ml.) was heated under reflux for 6.5 hours, cooled and poured into 45 ml. of 2 *N* sulfuric acid. The dioxane and excess cyclohexanone were removed by steam distillation and the residual solid recrystallized from ethanol to yield eburic-8,24(28)-diene-3-one-21-oic acid (1.524 g., 65.6%), m.p. 225–226° (lit.[9] 226–227°). By recrystallization of the second and third crops an additional 435 mg. (18.7%) of keto-acid was obtained, m.p. 225–226°.

Eburic-8,24(28)-diene-21-oic Acid (III).—A mixture of 1.634 g. (3.49 mmoles) of C^{14}-eburic-8,24(28)-diene-3-one-21-oic acid (II), 0.54 ml. (17 mmoles) of 100% hydrazine, 0.87 g. (15.6 mmoles) of potassium hydroxide and 5.4 ml. of anhydrous diethylene glycol was heated under reflux for 1.5 hours. After removal of the excess hydrazine by distillation, the reaction mixture was kept at 205° for 4.5 hours, cooled, diluted with 5 ml. of water and acidified with 7.5 ml. of 6 *N* hydrochloric acid. The solid was filtered and recrystallized from ethanol; yield 1.008 g. (69%), m.p. 252–253° (lit.[9] 252–253°).

Eburic-8-ene-21-oic Acid (IV).—A solution of eburic-8,24-(28)-diene-21-oic acid (508 mg., 1.12 mmoles) in 250 ml. of ethanol was hydrogenated at room temperature and atmospheric pressure in the presence of 500 mg. of 5% palladium–charcoal catalyst. One mole equivalent of hydrogen was absorbed in 70 minutes. A similar hydrogenation was conducted with 400 mg. (0.88 mmole) of the acid and the materials combined. After filtration of the catalyst and distillation of the solvent, the resulting white solid (m.p. 268–270°) was dissolved in 3 ml. of chloroform and poured onto a column (2.5 × 26 cm.) containing 80 g. of Fluorex XXS which had been prepared in pentane. Elution of this column with pentane–ether (1:1) yielded a main fraction of 700 mg. which was recrystallized from ethanol to give 596 mg. (65%), m.p. 270–271°, $[\alpha]^{29}_{D}$ +52.5° (*c* 3.0 in pyridine).

Anal. Calcd. for $C_{31}H_{52}O_2$ (456.73): C, 81.52; H, 11.48. Found: C, 81.65; H, 11.27.

Carbon Dioxide from Carbon 21 of Eburic-8-ene-21-oic Acid (IV).—A solution of 208 mg. (0.45 mmole) of C^{14}-eburic-8-ene-21-oic acid (s.a. 78 cts./min./mg. C) in 8 ml. of C.P. chloroform was cooled to 0° and 4 ml. of 100% sulfuric acid was added. The mixture was stirred magnetically and the flask swept with a slow stream of carbon dioxide-free dry nitrogen. Sodium azide (100 mg., 1.54 mmoles, recrystallized from aq. ethanol) was added in one portion and the mixture first allowed to come to room temperature, kept at that temperature for 30 minutes and then warmed to 50–65° for 3 hours. The mixture was cooled to room temperature and swept with nitrogen for an additional 4 hours. All effluent gases were first passed through a solution of 5% potassium permanganate and then through 1 *N* carbon dioxide-free sodium hydroxide. The barium carbonate was precipitated in the usual fashion with ammonium chloride and barium chloride, filtered, washed with water, methanol and ether and dried at 90° under reduced pressure; yield 83.0 mg. (92%), s.a. 102 cts./min./mg. C.

Methyl 3-(2′-Hydroxy-2′-propyl)-3-hydroxyeburic-A-nor-8-ene-21-oate (VII).—The compound was prepared in the fashion described previously,[1] m.p. 157–158° (lit.[1] 159–160°), s.a. 90 cts./min./mg. C.

Methyl Eburic-A-nor-3-one-8-ene-21-oate (VIII) and Acetone.—The diol VII (1.567 g., 3.13 mmoles) was allowed to react with lead tetraacetate in glacial acetic acid as described previously. The yield of mercury–acetone complex was 2.168 g. (60%, assuming 20 mg. of solid complex is equivalent to 1 mg. of acetone), s.a. 99 cts./min./mg. C. The nor-ketone VIII, in the form of the 2,4-dinitrophenylhydrazone, was obtained in a yield of 64% (1.250 g.), m.p. 225–226° dec. (lit.[1] 225–226° dec.), s.a. 75 cts./min./mg. C.

The mercury–acetone complex (1.965 g.) was decomposed with 3 *N* hydrochloric acid and the acetone distilled into 500 ml. of 1 *N* sodium hydroxide. To this solution was added 50 ml. of 0.5 *M* potassium triiodide solution and the mixture warmed on a steam-bath for 10 minutes. The iodoform was filtered and recrystallized from methanol, yield 235 mg., m.p. 118–119° (lit.[1] 119°), s.a. 115 cts./min./mg. C.

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(10) Percentage randomization = $(2A/A + B)\cdot 100$, where A is the specific activity of a carbon derived from the carboxyl of acetate and B is the specific activity of a carbon derived from the methyl of acetate.

(11) L. M. Corwin, L. J. Schroeder and W. G. McCullough, THIS JOURNAL, **78**, 1373 (1956), and references therein cited.

(12) W. G. Dauben and T. W. Hutton, *ibid.*, **78**, 2647 (1956).

(13) All analyses were performed by the Microanalytical Laboratory, Department of Chemistry and Chemical Engineering, University of California, Berkeley. The radioactivity was determined as described previously [W. G. Dauben, J. C. Reid and P. E. Yankwich, *Anal. Chem.*, **19**, 828 (1947)].

(14) These activities were determined on a vibrating reed electrometer through the courtesy of Prof. J. D. Roberts, California Institute of Technology.