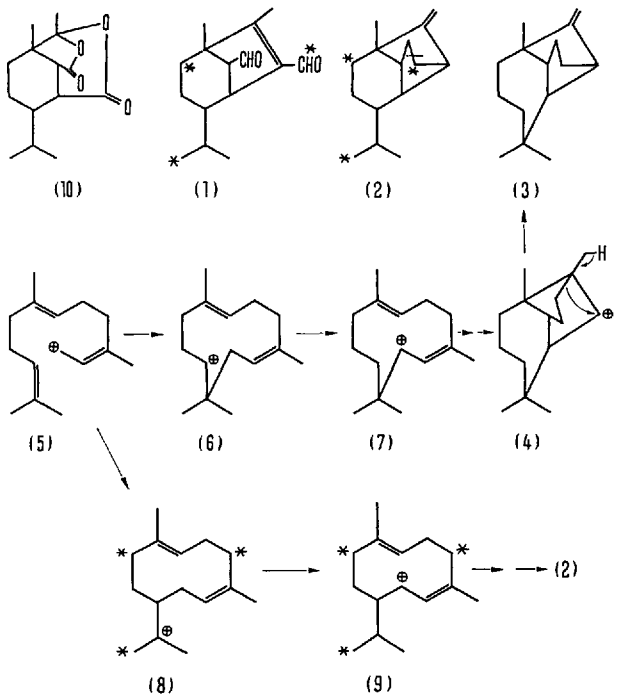


sation as discussed below) by oxidative cleavage at the point indicated. Cleavage of this type has already been suggested in monoterpenoids[5].

If such were the case the unsaturated aldehyde carbon (1, asterisked) should be derived from C_2 of a mevalonic acid unit and not from the 3' methyl. The structure of this intermediate (2) resembles that of longifolene (3) the biogenesis of which has also not been elucidated. It should be noted that, if the proposal of HENDRICKSON is correct[4], the final stage in longifolene genesis (5) → (6) → (7) → (4) → (3) is a Wagner-Meerwein rearrangement (4, see arrows). However, even if a parallel transformation should occur in helminthosporal genesis the predictions with regards the aldehyde labelling would still be valid.

Helminthosporium sativum was cultured as previously described[1], *DL*-mevalonic acid labelled with carbon-14 at C_2 (100 μC) being added, in methanol, after 3 days growth. The dialdehyde was isolated after 7 days, and converted directly to the stable monocarboxylic acid (1, carboxyl for unasterisked aldehyde) which was crystallised to constant activity (which, after dilution with inactive acid, was 1.88×10^{-4} μC mg[6]) and melting point. Ozonolysis then gave the dilactone (10)[2] in which only the asterisked aldehyde in (1) is lost. This material, also crystallised to constant m.p., had 62% of the original activity, in good agreement with the proposed loss of one of the three radioactive carbons.

If helminthosporal follows a similar pathway to that proposed for longifolene by HENDRICKSON the ion (8)[3,4] must be converted into (9) the subsequent steps being parallel to those for longifolene. No direct evidence of the possibility of such a transformation is available, but it is noteworthy that a number of sesquiterpenoids are known, in which cyclisation evidently proceeds with cationic attack on the isopropylidene group, which do not have the expected double-bond equivalent (derived from the tertiary cation) in the isopropyl side chain. In these cases an oxygen function is present, however, at the α-position. These include carotol[7], acorone[8], junenol[9] and the verbesinols[10].

Transfer by two successive 1:2 shifts or through cyclopropane intermediates are both conceivable[11]. The Hendrickson scheme for longifolene has very recently received support[12].

Zusammenfassung. Die Biogenese des Pilzstoffes Helminthosporal wurde untersucht, indem der Pilz bei Anwesenheit von Mevalonsäure, mit C_2 mit Kohlenstoff-14 markiert, gezüchtet wurde. Es wird gezeigt, dass das Dialdehyd aus einem tricyclischen Vorläufer durch oxidative Spaltung entsteht.

P. DE MAYO, J. R. ROBINSON,
E. Y. SPENCER, and R. W. WHITE

Department of Chemistry, University of Western Ontario, and Research Institute, Canada Department of Agriculture, London (Canada), May 7, 1962.

[5] H. C. BEYERMAN et al., Bull. Soc. chim. France *1961*, 1812.
[6] Radio-assays were made by combustion to CO_2 followed by ion chamber measurement using a vibrating-reed electrometer.
[7] L. H. ZALKOW, E. J. EISENBRAUN, and J. N. SHOOLERY, J. org. Chem. *26*, 981 (1961). – V. SYKORA, L. NOVOTNY, and F. ŠORM, Tetrahedron Letters No. *14*, 24 (1959).
[8] V. SYKORA, V. HEROUT, J. PLIVA, and F. ŠORM, Coll. Czech. Chem. Comm. *23*, 1072 (1958).
[9] O. MOTL, V. HEROUT, and F. ŠORM, Coll. Czech. Chem. Comm. *22*, 785 (1957). – S. C. BHATTACHARYYA, A. S. RAO, and A. M. SHALIGRAM, Chem. and Ind. (London) *1960*, 469.
[10] P. D. GARDNER, G. J. PARK, and C. C. ALBERS, J. Amer. chem. Soc. *83*, 1511 (1961).
[11] The authors would like to thank Mr. L. G. CRAWFORD for growing the organism and isolating the crude toxin.
[12] W. SANDERMANN and K. BRUNS, Tetrahedron Letters, 261 (1962).

Distribution of Tritium in WILZBACH Treated Cholesterol[1]

Biological experiments requiring comparatively large amounts of radioactive material suggested the preparation of tritiated cholesterol according to WILZBACH[2]. However, since NYSTROM and SUNKO[3] have shown that cholesterol-H^3 prepared in this manner is contaminated with small amounts of cholestane-3-ol-H^3 by addition of tritium to the double bond, the elimination of this impurity became necessary. This procedure could be carried out indeed as indicated below.

The availability of pure cholesterol-H^3 seemed to make worthwhile an investigation of the distribution of tritium in this molecule especially in view of the interesting results

[1] This work was supported by an Institutional Grant No. P-108A of the American Cancer Society and by a Grant No. G-7477 of the National Science Foundation. The authors are grateful to Dr. M. GUT of this Institute for the WILZBACH treatment of cholesterol.
[2] K. E. WILZBACH, J. Amer. chem. Soc. *79*, 1013 (1957).
[3] R. F. NYSTROM and D. E. SUNKO, *Atomlight* (New England Nuclear Corp., Jan. 1959).

1 Experiment No.	2 Compound	3 Counts $\times 10^5$ per min/mg	4 Counts $\times 10^{11}$ per min/mmole	5 %	6 % Loss of radioactivity	7 Position of carbon atoms from which H^3 is lost
0	Cholesterol-H^3	8.23	3.18	100	—	
1	5α,6β-dibromocholestane-3-ol-H^3	5.24	2.86	90.0	10.0	6^1
2	Cholesterol-H^3 acetate	6.96	2.98	93.8	6.2	3^1
3	Cholesterol-H^3 acetate boiled with Zn + acetic acid	6.95	2.97	93.6	6.4	3^1
4	Cholesterol-H^3 acetate hydrolyzed with KOH	7.66	2.96	93.1	6.9	3^2
5	Dehydroepiandrosterone-H^3 acetate	5.46	1.80	56.7	43.3	3^1, 6^1, 17–27^{18}
6	Dehydroepiandrosterone-H^3	5.80	1.67	52.6	47.4	3^1, 6^1, 16^2, 17–27^{18}
7	Δ^5-Cholestene-3-one-H^3	7.34	2.81	88.7	11.3	3^2
8	Δ^4-Cholestene-3-one-H^3	6.62	2.54	80.0	20.0	2^2, 3^2, 4^2, 6^1
9	Δ^4-Cholestene-3-one-H^3	6.05	2.33	73.1	26.9	2^2, 3^2, 4^2, 6^1
10	Δ^4-Cholestene-3,6-dione-H^3	5.90	2.35	73.9	26.1	2^2, 3^2, 4^2, 6^1

obtained by FUKUSHIMA and GALLAGHER[4] in the deuteration of cholesterol by platinum catalysed exchange.

For tritiation commercial cholesterol (Wilson) was purified first by treatment with anhydrous oxalic acid in ethylacetate according to MIESCHER and KAEGI[5] and then by bromination as described by FIESER[6]. This material and its acetate was tritiated in 5 g batches with 15 and 20 c of tritium for 15 and 10 days respectively. For stabilization each sample was boiled for 5 h with 20% methanolic KOH and then recrystallized from methanol-ether. Only the first fraction was used for further purification. In agreement with NYSTROM and SUNKO[3] it contained only about 10% of the total radioactivity but amounted to 80–90% of the total weight. Its specific activity was 17.3×10^6 counts/min/mg. Purification of this material was carried out by bromination according to [6]. The ether solution obtained after debromination of the crystalline 5α,6β-dibromocholestane-3-ol-H^3 was evaporated to a small volume and directly used to repeat the bromination. Altogether six such brominations and debrominations were necessary to prepare a cholesterol-H^3 which would not lose count after further bromination. At this point all three fractions isolated in recrystallizations of the material derived from the crystalline dibromo-compound as well as two fractions from the bromination mother liquors had essentially the same specific activity of 9.9×10^6 counts/min/mg.

For the experiments on the distribution of tritium in the molecule this pure cholesterol-H^3 was diluted ten times with cholesterol which had been purified by treatment with oxalic acid and bromination as indicated above. Its specific activity was now 8.2×10^5 counts/min/mg. The following experiments (same numbers as in Table) were carried out with this material. (1) Boiling with 20% alkali in methanol for 5 h and recrystallization from methanol-ether. (2) Bromination; the 5α,6β-dibromocholestane-3-ol was washed with methanol, but not recrystallized. (3) Acetylation in pyridine with acetic anhydride and crystallization from methanol. (4) This acetate was boiled with zinc and acetic acid for 3 h, precipitated and washed with water and crystallized from methanol-ether. (5) Acetylation, dibromination and oxydation with chromic acid according to SCHWENK et al.[7] afforded the semicarbazone of dehydroepiandrosterone-H^3 acetate, from which the latter compound was obtained following the directions of HERSHBERG[8]. (6) Dehydroepiandrosterone-H^3 was prepared from this acetate by alkaline hydrolysis and crystallization from methanol. (7) Δ^5-cholestene-3-one-H^3 was obtained according to RUZICKA and BOSSHARD[9]. (8) This substance was rearranged to Δ^4-cholestene-3-one-H^3 (FIESER[10]). (9) Δ^4-cholestene-3-one-H^3 made by a Meerwein-Ponndorf reaction was purified by treatment with Girard-T reagent and recrystallized from methanol. (10) Δ^4-cholestene-3,6-dione-H^3 was obtained by oxidation with sodium dichromate (FIESER[11]). All substances were dried over P_2O_5 *in vacuo* for at least 24 h before counting. For each experiment two samples of substance were weighed on a microbalance and solutions prepared in benzene. At least two, but in several instances more different samples were counted in a scintillation counter.

The Table presents the results of these experiments. The figures in column 7 give the positions of carbon atoms in the molecule of cholesterol-H^3 from which tritium atoms are apparently abstracted in the studied reactions and the subscripts give the probable number of tritium atoms involved.

The total loss of radioactivity in the preparation of dehydroepiandrosterone-H^3 and Δ^4-cholestene-3,6-dione-H^3 calculated from experiment 6 and the mean of experiments 8, 9, 10 is 71.7%. From this must be deducted 16.9%, calculated from experiments 1 and 4, because the H^3-atoms on carbon 3 and 6 appear in both sets of experiments. Total loss of radioactivity is therefore 54.8% involving 27 H^3-atoms on carbon atoms 2, 3, 4, 6, 16 and 17 to 27.

These results differ considerably from the findings of FUKUSHIMA and GALLAGHER[4]. They found that in the platinum catalyzed deuteration of cholesterol more than 97% of the incorporated deuterium was concentrated in the isopropyl group of the side chain and the carbon atoms around the hydroxyl-carrying carbon atom 3. The here reported experiments show a remarkably even distribution of tritium throughout the molecule of cholesterol-H^3. The 27 tritium atoms attached to carbon atoms 2, 3, 4, 6, 16 and 17 to 27 which are involved in the reactions studied form 58.7% of the total hydrogen content of the molecule, a figure which compares well with the *de facto* found 54.8% as calculated above from the %-loss figures in column 6 of the Table. One tritium atom thus accounts for 6.44×10^3

[4] D. K. FUKUSHIMA and T. F. GALLAGHER, J. biol. Chem. *198*, 861 (1952).
[5] K. MIESCHER and H. KAEGI, Helv. chim. Acta *24*, 986 (1941).
[6] L. F. FIESER, J. Amer. chem. Soc. *75*, 5421 (1953).
[7] E. SCHWENK, N. T. WERTHESSEN, and A. F. COLTON, Arch. Biochem. and Biophys. *48*, 322 (1954).
[8] E. B. HERSHBERG, J. org. Chem. *13*, 542 (1948).
[9] L. RUZICKA and W. BOSSHARD, Helv. chim. Acta *20*, 244 (1937).
[10] L. F. FIESER, *Organic Synthesis* (John Wiley & Sons Inc., New York 1955), vol. 35, p. 46.
[11] L. F. FIESER, *Organic Synthesis* (John Wiley & Sons Inc., New York 1955), vol. 35, p. 36.

counts while it should theoretically contribute 6.91×10^3 counts. This good agreement between found and calculated values shows that tritium is evenly distributed throughout the molecule of cholesterol-H^3 prepared by WILZBACH's method.

Zusammenfassung. Es wird gezeigt, dass Cholesterin-H^3, nach Behandlung von besonders gereinigtem Handelscholesterin mit Tritium entsprechend der Vorschrift von WILZBACH, bei sechsmal wiederholter Bromierung nach FIESER frei von Cholestanol-H^3 erhalten wird. In so gereinigtem Cholesterin-H^3 ist der schwere Wasserstoff gleichmässig über alle wasserstofftragenden Kohlenstoffatome verteilt.

E. SCHWENK and ERZSEBET JOACHIM

Worcester Foundation for Experimental Biology, Shrewsbury (Mass., U.S.A.), May 7, 1962.

The Mucopeptide Turnover in the Cell Walls of Growing Cultures of *Bacillus megaterium* KM

A protein turnover of approximately 1%/h takes place in the cells of growing bacilli[1] while, under the same conditions, the proteins in cells of Gram-negative bacteria remain stabile and disintegrate only in a medium lacking nitrogen[2]. Certain changes in the cell walls occur in growing cultures of *Bacillus megaterium* KM and are responsible for their increased sensitivity to lysozyme and the release of part of the mucopeptides[3]. Investigations were carried out in order to ascertain whether the changes in the mucopeptidic moiety of the cell walls cause the release of their components into a pool. In measuring the velocity of the turnover, the circumstance was taken into account that the cells of *Bacillus megaterium* to a certain degree decarboxylate diaminopimelic acid to lysine which is utilized for the protein synthesis[4]. In the experiment, the cell walls of the culture were prelabelled with ^{14}C diaminopimelic acid (DAP)[5]. The culture was grown on C agar with 1% lactose, 0.1% peptone and 0.03 μC ^{14}C-DAP/ml. Lysine was added to the medium in order to restrict the utilization of degradation products of DAP for protein synthesis. The fully grown culture was washed and incubated for 2 h in C medium, containing 0.5% glucose. As a result, the content of ^{14}C DAP and its products in pool were reduced to 7%. Having been centrifuged and washed, the cells were cultivated for another 6 h in fresh C medium (400 ml of medium, containing 0.5 mg dry weight of cells/ml at 32°C and 0.3 l/min aeration).

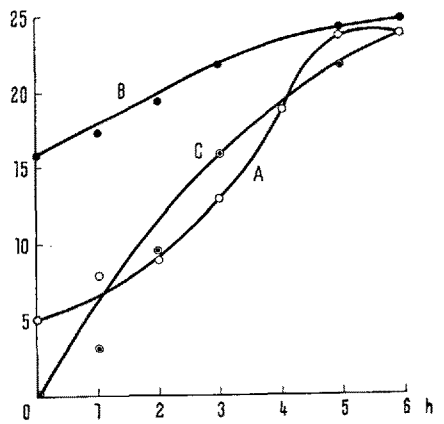
In intervals of 1 h, samples of the suspension (10 ml) were collected and the β-galactosidase established[6] in the medium and in the cells. Another sample of the medium, containing 40 mg dry weight, was used to determine the radioactivity of the proteins and mucopeptides. Trichloracetic acid (TCA) was added to a concentration of 5%. Nucleic acids were removed from the material by extraction with hot TCA, while the lipids were removed by extraction with alcohol, ether and acetone. The protein and mucopeptidic fractions were then isolated[7]. The samples, containing 0.2–0.4 mg dry weight/ml, were counted with the Friesecke-Hoepfner counter in the atmosphere of methane. Employing the KOCH-LEVY method[8], the rate of mucopeptide degradation was derived from the increase in the percentage of radioactivity in the protein moiety.

In our case the probability coefficient (q) for the decarboxylation of the released diaminopimelic acid and its incorporation into the proteins in the form of lysine was, on the average, equal to 0.44 (Table). As indicated in our illustration, a considerable cell wall turnover occurs in multiplying cells (Figure), equalling a velocity of 4%/h and a half-time of 17.3 h. Provided that 7% of the radioactivity present in the cell pool at the beginning of the experiment are fully utilized during the growth, the rate of turnover drops to approximately 3%/h, while the half-time of the mucopeptidic moiety of the cell wall increases to 22.6 h. Since during 6 h of cultivation no measurable amount of β-galactosidase was released from the cells, and consequently, the cells did not die during the experiment,

Incorporation of ^{14}C from DAP into the cells of *Bacillus megaterium*

Min	Radioactivity in the proteins	Radioactivity in the cell wall	q
30	5263 cpm	7565 cpm	0.41
60	8930 cpm	11268 cpm	0.44
90	14618 cpm	15120 cpm	0.49

The washed culture was incubated in C/G medium (1.6 mg/ml), containing 0.03 μC ^{14}C DAP/ml. The cells were fractionized by the PARK-HANCOCK method[7].



The mucopeptide turnover in a growing culture of *Bacillus megaterium*. A: growth expressed in mg of dry weight/10 ml. B: % ^{14}C in the protein fraction. C: % of turnover of the mucopeptidic component in the cell wall.

1 C. R. URBÁ, Biochem. J. *71*, 513 (1959).
2 J. MANDELSTAM, Biochem. J. *69*, 110 (1958).
3 J. CHALOUPKA, P. KŘEČKOVÁ, and L. ŘÍHOVÁ, Folia microbiol., in press.
4 J. CHALOUPKA and K. VEREŠ, Z. allg. Mikrobiol. *1*, 325 (1961).
5 J. CHALOUPKA and K. VEREŠ, Exper. *17*, 562 (1961).
6 H. W. RICKENBERG, Biochim. biophys. Acta *35*, 122 (1959).
7 J. T. PARK and R. HANCOCK, J. gen. Microbiol. *22*, 249 (1960).
8 A. L. KOCH and H. R. LEVY, J. biol. Chem. *217*, 949 (1955).