nol inhibition with *T. pyriformis* mating Type II(6) while squalene effectively reversed this inhibition with *Ochromonas*(2), also needs an explanation. The fact that fatty acids reverse triparanol-inhibition of growth of *Tetrahymena*(1-5) implies that a more fundamental process is affected. Kidder and Dewey(20) have suggested unconjugated pteridines as cofactors in the cyclization of squalene and in the desaturation of long-chain fatty acids. It is entirely possible that the changes reported here and previously, describing alterations in fatty acid composition(5), are brought about through an effect of triparanol on enzyme systems involving pteridines or other cofactors which mediate the above processes.

Summary. In triparanol-inhibited cultures of *T. pyriformis* (strains W, H and S), squalene is greatly increased in amount and tetrahymanol is decreased. In contrast, squalene appears in low concentration in uninhibited cultures. It appears that squalene might be converted directly to the pentacyclic compound, tetrahymanol, and that the cyclization of squalene is blocked by triparanol. Cholesterol was not identified in control or inhibited cultures and appears neither to be a sterol of major importance in this organism, nor an intermediate in the synthesis of tetrahymanol. Methods of purification and identification of tetrahymanol are given.

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Studies in Valine Biosynthesis VII. An Enzymatic Clue in *Penicillium chrysogenum* and Other Organisms.* (30066)

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Penicillium chrysogenum and many other higher fungi can grow on ammonium salts as the main nitrogen source(1) and thus must have the enzymatic capacity to synthesize all of their amino acids. However, the pathway of valine and isoleucine formation has

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not been fully elucidated in this genus. Certain patterns of amino acid metabolism, as demonstrated by radioisotope studies in *Penicillium digitatum*(2), are similar to other microorganisms. Since C^{14}-labeled valine(3, 4 and earlier papers of these authors) and S^{35}-labeled cystine are incorporated into the penicillin molecule and since speculation on possible mechanisms of valine and penicillin biosynthesis have appeared(5,6), an experimental inquiry into the pathway of valine biogenesis in *P. chrysogenum* was undertaken. Similar incorporation of labeled valine and cystine into the structurally related antibiotic, cephalsporin C, has recently been reported(7,8,9).

The steps in valine and isoleucine biosynthesis have been elucidated thoroughly in *Neurospora crassa, Escherichia coli* and *Saccharomyces cerevisiae*(10,11). A reaction in the middle of these biosynthetic steps is the dehydration of α,β-dihydroxyisovaleric acid to α-ketoisovaleric acid, the immediate precursor of valine. The enzyme for this reaction, dihydroxyacid dehydratase, has a widespread distribution in biological systems that can grow on ammonium salts as the main nitrogen source—8 bacteria and 3 yeasts(11), a green alga and 29 higher plants(12). Herein is reported the presence of the dehydratase in 2 actinomycetes and 4 more fungi, including *P. chrysogenum* and *Cephalosporium* sp.

Materials and methods. Penicillium WIS Q-176, ATCC No. 10002, was grown in 4 liter Erlenmeyer flasks on a medium (1) of glucose—40 g per liter, ammonium acetate—10 g per liter and inorganic salts for 24 to 40 hours at 23° on a rotary shaker. Prior to leaving the exponential phase of growth (6.0 mg dried mycelium per ml; generation time of 8 hours; 3% inoculum volume), the mycelium was harvested by straining through several layers of cheesecloth; after washing twice with distilled water and squeezing to remove excess water, the pad was promptly frozen with dry ice. Frozen mycelium of *Aspergillus niger,* ATCC No. 11394 and *Streptomyces aureofaciens,* NRRL No. 2209 were a significant gift from Dr. B. L. Hutchings, Lederle Laboratories, American Cyanamid Co., Pearl River, N. Y. Frozen *Nocardia corallina,* MB 276, was a valuable donation from Dr. David Hendlin, Merck, Sharp and Dohme Research Laboratories, Merck and Co., Inc., Rahway, N. J. *A. niger*(13), *S. aureofaciens*(14) and *N. corallina*(15) have the synthetic capacities that will permit their growth on chemically defined media with ammonium salts as the main nitrogen source. The batches of *Cephalosporium,* C.M.I. 49, 137, mutant No. 8650, grown on defined medium and harvested in the exponential phase were a much appreciated gift from Dr. E. P. Abraham, Sir William Dunn School of Pathology, University of Oxford, England. Fresh baker's yeast and mushrooms, a basidiomycetous fungi, were procured from a local source.

The above mycelia were ruptured in several ways due to the known difficulty in extraction of enzymes. These extracts were then analyzed for dihydroxyacid dehydratase activity and protein concentration. These procedures, along with the determination of the keto acid product and source of synthetic substrates, are outlined in the legend of Table I and have been previously described (11).

Results. To find effective conditions to rupture these spongy mycelia, the same batch of each organism was used for the various rupture procedures described in Table I. Unless stated otherwise, all procedures were conducted in a cold room (3°) or in an ice bath; cold potassium phosphate buffer, 0.10 M, pH 7.4, was routinely used. For sonic oscillation (16), 10 g of frozen mycelia were thawed in the presence of 40 ml of buffer. This 1:5 wet weight/volume (w/v) suspension was treated in a 250 watt 10-kc ultrasonic oscillator (Raytheon Manufacturing Co., Waltham, Mass.) at 2-4° for 10, 20, 30 and 40 minutes. By use of a circulating —4° cooling solution, the contents of the cup stayed at 2 to 4° during the longer treatment periods. This extract and others to be described were routinely centrifuged at 18,000 $\times$ *g* for 30 minutes. The supernatant fluids were stored at —20° for up to one week until assay. Only the time for sonic treatment which gave the highest activity is reported in Table I. Similar procedures were used for sonication of a 20% w/v suspension of baker's yeast cake, but with the additional observation

that use of 0.10 M $(NH_4)_2HPO_4$ gave higher specific activities than 0.10 M solutions of potassium phosphate buffer, pH 7.4, K_2HPO_4 or $NaHCO_3$.

The mycelia were also ruptured by mechanical abrasion(16,17,18). Frozen mycelia (20 g) were placed in a 50 ml stainless steel cup with 40 ml buffer and 40 g cold micro glass beads (Superbrite, 0.2 mm diam., Minnesota Mining and Manufacturing Co., St. Paul, Minn.). After surrounding the cup with crushed ice, cells were homogenized for 2, 5, and 10 minutes of high speed homogenization (Virtis Co., Gardiner, N. Y.). The observed internal temperature at 10 minutes was 12 to 14°. After cooling, centrifuging and assaying the extract, the data was corrected to the constant ratio of 1:5 w/v. Table I contains the data for the rupture time with the highest specific activity. For *S. cerevisiae,* the micro glass beads were a more effective abrasive than powdered glass (Fisher Scientific Co., St. Louis, Mo.), alumina A-301 (Aluminum Co. of America, Chemicals Division, Pittsburgh, Pa.) or Hyflo Super Cel (Johns-Manville Corp., New York)(16).

Acetone powders of the mycelia were prepared in the usual manner at −20°(16).

TABLE I. Distribution of Dehydratase Activity in Microorganisms.

Cell-free microbial extracts were prepared as described in text. These extracts, usually 0.1 to 0.3 ml, were incubated with 0.10 M Tris (hydroxymethyl) aminomethane·HCl buffer, pH 7.4, 0.02 M $MgCl_2$ and 0.02 M DL-α,β-dihydroxyisovaleric acid in a final volume of 1.0 ml. After incubation at 37° for 30 minutes in triplicate tubes, the reaction was terminated by addition of trichloroacetic acid and protein was removed by centrifugation. Keto acid concentration of the supernatant was determined and corrected for traces of apparent keto acid in extract and substrate blanks. These dehydratase units (μmoles of keto acid formed per 30 min) were calculated to express the enzyme's concentration in constant terms (units per 0.3 ml of supernatant of a 1:5 w/v microbial extract), except where noted by an asterisk.

Class, order and genus	Method of rupture	Dehydratase conc (units/.3 ml)	Protein conc (mg/ml)	Specific activity (units/mg protein)
Schizomycetes				
Actinomycetales				
Nocardia corallina	Sonic oscillation 40 min	.29	15.3	.06
	Glass bead grinding 10 min	.13	11.1	.04
Streptomyces aureofaciens	Sonic oscillation 40 min	.30	4.0	.25
" "	Glass bead grinding 10 min	.22	2.2	.33
	Acetone powder	.39*	2.2	.59
Ascomycete				
Saccharomyces cerevisiae	Sonic oscillation 40 min, $(NH_4)_2HPO_4$	1.92	16.0	.40
" "	Sonic oscillation 40 min, buffer	.95	14.5	.22
" "	Glass bead grinding 10 min	.72	16.2	.15
" "	Dry ice grinding	1.22	25.0	.16
" "	Hydraulic pressure cell, 3 passes	1.90	24.5	.26
" "	Extraction of dried cells	4.41*	49.4	.30
" "	Liquid nitrogen freezing	.56	8.0	.23
" "	Toluene autolysis	.60	6.3	.32
Basidiomycete				
Psalliota campestris	Homogenization	.07	8.6	.03
Fungi Imperfecti				
Penicillium chrysogenum	Sonic oscillation 30 min	.14	2.4	.19
" "	Glass bead grinding 5 min	.12	2.4	.17
" "	Acetone powder	.37*	10.0	.12
" "	Dry ice grinding	.08	2.4	.11
" "	Hydraulic pressure cell, 3 passes	.04	1.6	.08
	Sand grinding in a mortar	.05	2.0	.08
Aspergillus niger	Sonic oscillation 40 min	.16	2.6	.20
" "	Glass bead grinding 10 min	.26	2.2	.39
" "	Acetone powder	.21*	2.8	.25
" "	Dry ice grinding	.11	2.0	.18
Cephalosporium C.M.I. 49,137	Sonic oscillation	1.12	4.9	.76
	Hydraulic pressure cell, 2 passes	.88	2.6	1.13

After testing several variables with *A. niger,* the best conditions found were to mix one part of acetone powder by weight with 19 volumes of buffer in a Vir Tis homogenizer and continue the extraction for an hour at 2° and centrifuge.

Several organisms were ground with dry ice, a procedure that has been used with *N. crassa* and other molds. After comparison of several conditions with *A. niger,* the best result was obtained by mixing 20 g frozen mycelia, 80 g cold micro glass beads and excess dry ice in a cold stainless steel Waring blendor jar. At several minute intervals, the mycelia on the walls of the jar were scraped down with a spatula and more dry ice added. When a total of 15 minutes of grinding was reached, the dry ice was allowed to sublime away, and 40 to 80 ml buffer were added. After a brief homogenization and extraction for 30 minutes in the cold the extract was centrifuged and assayed.

A precooled French high pressure cell (American Instrument Co., Silver Spring, Md.) and Wabash hydraulic press were used for the rupture of 20% w/v suspensions of *S. cerevisiae, P. chrysogenum* and *Cephalosporium* sp. at 10,000 psi. The specific activities of suspensions that had been passed through three times were several fold greater than those from one pass.

Baker's yeast was also air-dried(16) and extracted with H_2O, or 0.10 M solutions of K_2HPO_4, $(NH_4)_2HPO_4$ or $NaHCO_3$ under various conditions of time and temperature. The most effective means for preparing a potent extract was to stir the dried yeast for 6 hours at 37° with 3 vol of 0.10 M $NaHCO_3$. An intermediate specific activity was found upon freezing the yeast cake with liquid nitrogen(16), allowing the nitrogen to evaporate, adjusting the pH to 8.3-8.5 with NH_4OH and stirring with an equal volume of H_2O for 2 days in the cold; other solvents and extraction times gave lower activities. The autolysis of yeast by warming with toluene at 38°(16), standing for 3 hours at 25° and lysis with an equal vol of H_2O for 16 hours at 3° gave higher specific activities than similar extraction with several buffers.

Frozen mushrooms were homogenized in a stainless steel Waring blendor with 4 vol of cold buffer for 5 minutes. The suspension was clarified by straining through cheesecloth and centrifuging. The data in Table I indicate that the best rupture procedure, of those tested, was sonic oscillation for *N. corallina* and *P. chysogenum,* grinding with glass beads for *A. niger,* acetone powder for *S. aureofaciens,* and pressure change for *Cephalosporium* species. The release of the dehydratase from *S. cerevisiae* was effectively accomplished by sonic oscillation, toluene plasmolysis or extraction of dried yeast cells. The 4 fungal extracts tested also catalyzed keto acid formation from α,β-dihydroxy-β-methyl-n-valeric acid, the intermediate in isoleucine synthesis, though at a slower rate than α,β-dihydroxyisovaleric acid. In addition, the information in Table I establishes the presence of the dihydroxyacid dehydratase in 6 new microorganisms.

Discussion. The earliest evidence for the pathway of valine biosynthesis in *P. chrysogenum* is the observation of Stevens and De Long(4) that acetate-1-C^{14} was incorporated into mycelial valine, which was labeled predominantly but not exclusively in the carboxyl group, and into the penicillamine carboxyl group of penicillin. In both experiments addition of valine to the medium reduced the incorporation of acetate-1-C^{14} into the valine group(4). Although similar isotope incorporation patterns have been previously found in *S. cerevisiae, T. utilis,* and *A. aerogenes* (references cited in 10 and 11), conclusive evidence for a given pathway must depend on detection of the enzymes therein. In *N. crassa, E. coli* and *S. cerevisiae*(10) these enzymes are the α-acetolactate formation enzyme, reductoisomerase, dihydroxyacid dehydratase and transaminase. Thus the combined dehydratase and isotopic evidence confirms earlier speculations on the valine pathway(5), differentiates between 3 proposed pathways(6) and is consistent with the conclusion that the valine formation proceeds by the same pathway in *P. chrysogenum* as in other microbial systems.

At first glance, one earlier experiment might militate against such a hypothesis. Stevens and De Long(4) reported that addition of α-ketoisovaleric acid, α,β-dihydroxyisovaleric acid, β-hydroxy-DL-valine or β,β′-

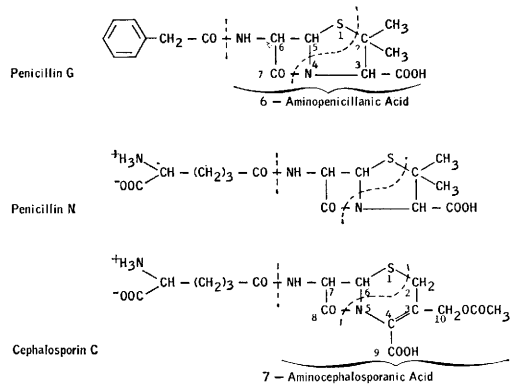

FIG. 1. Structures of 3 antibiotics showing their constituent biosynthetic groups.

dimethylacrylic acid (rearranged in order of descending effectiveness) did not appreciably reduce the incorporation of valine-1-C^{14} into penicillin between the 63-72 hours of fermentation, and stated that these compounds were not intermediates between valine and penicillin. Another possible, though not stated, deduction for this mild reduction of valine incorporation is that the α-ketoisovaleric acid and α,β-dihydroxyisovaleric acid are endogenous precursors of valine. Since penicillin synthesis occurs in the late stages of fermentation(1,5,6), isotopic experiments of mycelial valine at early time intervals should clarify these relationships.

The new antibiotic, cephalosporin C, contains the moieties of α-aminoadipic acid, cysteine, a C_5 fragment which yields valine upon hydrogenolysis and acetoxy group(8)(Fig. 1). It has a fused β-lactam-dihydrothiazine ring, instead of the fused β-lactam-thiazolidine ring of penicillin. Since radioactive valine (7,9), cystine(7) and α-aminoadipic acid are incorporated into cephalosporin C in the anticipated position and since δ-(aminoadipoyl) cysteinylvaline has been isolated from the mycelium of *P. chrysogenum*(19), the two antibiotics may be synthesized by pathways which have features in common(8). In a procedure similar to that in penicillin biosynthesis(4), Abraham and associates found that when using a complex medium, acetate-1-C^{14} was incorporated primarily into the aminoadipoyl and the acetoxy group of cephalosporin C and to a much lesser extent into the isovaleryl group(20). To speculate, the acetate incorporation into the isovaleryl part might have been greater on a defined salts medium with the avoidance of end product inhibition and/or repression of valine neogenesis. The labelled cystine was also incorporated to a minor extent in a pattern consistent with the intermediates of pyruvate and α-acetolactate(7); in collaboration with Dr. E. P. Abraham, the present *Cephalosporium* extract was found to contain cysteine desulfhydrase(7). Studies of the nutritional factors affecting cephalosporin C synthesis have been undertaken(21,22), but do not lead to definitive conclusions of the biosynthetic pathway due to permeability questions and metabolic control mechanisms.

Several other observations are consistent with the findings in Table I. Findings related to *S. aureofaciens* were the secretion of isoleucine by *Streptomyces rimosus* when grown in media containing threonine or α-ketobutyrate(23). The formation of α-ketoisovaleric acid (dimethyl pyruvic acid) and pyruvic acid has been observed with preformed mycelial felts of *A. niger* on Hida's medium of NH_4Cl, K_2HPO_4, Na_2SO_3 and a carbon source(24). As addition of acetate to a glycerol medium increased the production of ketoisovaleric acid, these authors postulated a 10-step pathway. On the other hand it might be proposed that after oxidation of glycerol to pyruvate, the presence of acetate (or its enzymatically active form) enhances the $C_2 + C_3$ condensation to yield α-acetolactic acid, a known early intermediate in valine biosynthesis(7). Rearrangement, reduction and dehydration would yield α-ketoisovaleric acid. Therefore the present enzymatic formation of α-ketoisovaleric acid by *A. niger* extracts suggests a new explanation for this older observation.

Thus the dihydroxyacid dehydratase has been found in specific representatives of the phylum, Thallophyta, including both alga (*i.e., Chlorella pyrenoidosa*(12)) and fungi. The data in Table I have widened its known distribution to now include the following classes of fungi: Schizomycetes—both Eubacteriales(11) and Actinomycetales, Ascomycetes—3 members(11), Basidiomycete and Fungi Imperfecti. While the Basidiomycetes are considered to be saprophytic, this attribute must be restricted to nutrients other

than valine and isoleucine. The presence of the dehydratase in many members of the phylum Spermatophyta has also been demonstrated(12). Therefore the last 2 steps of dehydration and transamination in valine and isoleucine biosynthesis are identical in these diverse aerobic biological systems. By contrast the pathway of ornithine, lysine and tryptophan formation in molds differs from that in bacteria.

Conclusions. A dihydroxyacid dehydratase has been detected in extracts of 2 Actinomycetes, 3 Ascomycetes, a Basidiomycete and 3 Fungi Imperfecti, and provides a clue for the specific pathway of valine and isoleucine biosynthesis in the organisms studied. Some methods of extraction of the dehydratase were compared. This enzymatic evidence complements earlier isotopic studies on the route of formation of the valine moiety in the ring of penicillin and cephalosporin C.

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