3-Ethylenedioxy-5-pregnene-11β,20α-triol 20-Acetate (VI).—The mixture of C_{20}-epimers remaining from the isolation of II was acetylated in acetic anhydride–pyridine (100°, 12 minutes) and gave 900 mg. of a crystalline mixture. Recrystallization from acetone–ether, acetone and ether provided 198 mg. of VI melting at 177–180° with partial re-solidification and melting at 200°; $\lambda_{max}^{CHCl_3}$ 2.75, 2.85, 2.92, 5.78 μ; $[\alpha]^{22}D$ −56 ± 2° (*c* 1.07, $CHCl_3$).

Anal. Calcd. for $C_{26}H_{40}O_6$: C, 68.77; H, 9.24. Found: C, 68.72; H, 9.15.

Acid hydrolysis (*p*-toluenesulfonic acid in acetone) of the mother liquors from which VI was separated, gave, after chromatography on acid-washed alumina, 350 mg. of the 20β-acetate IV and 140 mg. of the 20α-acetate VII.

4-Pregnene-11β,17α,20α-triol-3-one 20-Acetate (VII).—A solution of VI (97 mg.) in 2.5 ml. of acetone containing 25 mg. of *p*-toluenesulfonic acid was boiled for 15 minutes, diluted with water and the crystalline product was collected; 83 mg., m.p. 208–220°. Recrystallization from methanol, acetone and ethyl acetate provided pure VII melting at 224–226°; λ_{max} 2.9, 5.77, 6.05, 6.19 μ; $[\alpha]^{22}D$ +79 ± 2° (*c* 1.01, $CHCl_3$).

Anal. Calcd. for $C_{23}H_{34}O_5$: C, 70.74; H, 8.98. Found: C, 70.71; H, 8.20.

4-Pregnene-11β,17α,20α-triol-3-one (VIII).—To a solution of 148 mg. of VII, m.p. 215–223°, in 8 ml. of methanol was added 4 ml. of 1 *N* potassium carbonate solution. After 16 hours at room temperature the reaction mixture was treated with water and the product was extracted with chloroform. The dried extract was evaporated to dryness and the crystalline residue was recrystallized from ether; yield 112 mg., m.p. 190–193°. Pure VIII was obtained by recrystallization from acetone–petroleum ether; m.p. 193–194°; λ_{max} 242 mμ, ϵ_{mol} 15,900; λ_{max} 2.9–2.95, 6.08 μ; $[\alpha]^{23}D$ +107 ± 2° (*c* 0.550, $CHCl_3$).

Anal. Calcd. for $C_{21}H_{32}O_4$: C, 72.38; H, 9.26. Found: C, 72.11; H, 9.22.

A subsequent saponification of VII yielded VIII with a melting point of 210–214°. A sample which melted at 193–194° was converted to the higher-melting form by seeding a super-saturated solution.

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Occurrence of Choline Sulfate in *Penicillium Chrysogenum*[1]

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The appearance of a note by de Flines[2] prompts us to report more quantitative data obtained earlier on the occurrence of "cyclic" choline sulfate in the mycelium of *Penicillium chrysogenum* (Wis 48-701), and on the utilization of this compound as a source of sulfur for the biosynthesis of penicillin.[3]

Using a mutant strain of *Neurospora crassa*[4] which shows a growth response to choline sulfate as well as to free choline, it was demonstrated that culture filtrates of *P. chrysogenum* grown on a purified medium with S^{35}-labeled sodium sulfate as the sole source of sulfur contained negligible amounts of choline or its derivative, while autolysates of the washed mycelium showed significant choline activity. Choline sulfate was determined quantitatively by addition of synthetic choline sulfate to the autolysates, reisolation and determination of the specific activity of the isolated material. The results indicate approximately 0.2 g. of choline sulfate per 100 g. of dry mycelium.

Hockenhull[5] reported a high content of ethereal sulfate in the mycelium of *P. chrysogenum* (Q-176) and suggested that this material may "act as a reservoir of sulfur for the organism." To test specifically whether added choline sulfate is utilized by the mold, the compound was synthesized from S^{35}-labeled sulfuric acid by the method of Schmidt and Wagner.[6] When added to the culture medium as the sole source of sulfur, the compound supported good growth of the mold. When, however, it was tested against inorganic sulfate in competitive utilization experiments,[7] it was found that the latter was utilized almost exclusively as the source of sulfur for the biosynthesis of penicillin.

Experimental

Procedures for culture of the mold, assay for penicillin, determination of radioactivity, etc., have been previously described.[7] After 72 hours fermentation, the mycelium was collected, washed, a portion retained for moisture determination, and the remainder allowed to autolyse.[8] To the autolysate was added a known amount of choline sulfate. After treatment of the solution with Neuberg's reagent,[8] choline sulfate was reisolated by precipitation with phosphotungstic acid and decomposition of the phosphotungstate,[8] or by direct crystallization from the autolysate after passage through cation-exchange (Amberlite IR-112) and anion-exchange (Amberlite XE-75) columns. In either procedure, the recovered choline sulfate was recrystallized from 95% ethanol to constant radioactivity. Specific activities of the samples indicated a content of 0.2 g. of choline sulfate per 100 g. of dry mycelium. This must be considered a minimum value for the intact mycelium, since it was not determined whether any destruction of choline sulfate occurred during autolysis.

S^{35}-Labeled choline sulfate was prepared from choline chloride and sulfuric acid by the general procedure of Schmidt and Wagner.[6] A solution of 2 g. of dried choline chloride in 5 ml. of concentrated sulfuric acid was heated at

TABLE I

COMPARISON OF UTILIZATION OF SULFATE WITH CHOLINE SULFATE FOR PENICILLIN BIOSYNTHESIS

Compd. added[a]	Yield of penicillin, units/ml. 70 hr.	120 hr.	Radioactivity in extracted penicillin, c.p.s./100 units 70 hr.	120 hr.
S^{35}-Labeled Na_2SO_4	150	275	5.6	5.8
S^{35}-Labeled Na_2SO_4	140	275	5.2	5.1
S^{35}-Labeled Na_2SO_4 + choline sulfate	110	400	4.9	3.6
S^{35}-Labeled Na_2SO_4 + choline sulfate	130	300	5.8	4.7
Na_2SO_4 + S^{35}-labeled choline sulfate	110	325	0.3	0.03
Na_2SO_4 + S^{35}-labeled choline sulfate	140	350	0.3	0.1

[a] Each compound was added in an amount equivalent to 10.9 mg. S. The total radioactivity of added sodium sulfate was 11,200 c.p.s. and of added choline sulfate 1,900 c.p.s. by the counting procedure employed.

(1) This work was supported in part by a grant from Eli Lilly and Company, Indianapolis.

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90–100° for 4.5 hours, 40 ml. of absolute ethanol added, and the mixture cooled overnight at 10°. The product was collected and recrystallized repeatedly from 80% ethanol; the yield was 0.8 g. (30%).

In preliminary experiments, it was shown that choline sulfate supported good growth of *P. chrysogenum* when added to the culture medium as the sole source of sulfur. Choline sulfate was then compared with sodium sulfate as a source of sulfur for penicillin biosynthesis. Experiments were performed in which equimolar amounts of each compound were added to the medium, but in which one compound only was labeled with S^{35}. Determinations of the specific activity of the penicillin formed provided a measure of the extent to which each of the two compounds had been utilized. It can be estimated from the data of Table I that 80–90% or the penicillin sulfur is derived from inorganic sulfate.

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Saponin from Ladino Clover (*Trifolium repens*)[1,2]

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Saponins isolated[4] from water extracts of alfalfa have been shown to cause typical symptoms of bloat in ruminants.[5] These results suggested that other bloat-producing forages be examined for similar compounds.

Experiments with water extracts of ladino clover (*Trifolium repens*), designed to isolate the saponin as the cholesterol addition product as had been done with alfalfa, showed that the reaction of cholesterol with the water extract yielded only small amounts of cholesteride. However, extraction of either green or dry material with ethanol–water solutions yielded a mixture from which a saponin could be crystallized readily; this proved to be a mixed calcium–magnesium salt of at least three saponins. Hydrolysis of the mixture yielded glucose, galactose, xylose and rhamnose plus a mixture of neutral sapogenins. Chromatographic separation of the sapogenins furnished soyasapogenol B and soyasapogenol C; traces of a third substance, possibly soyasapogenol A, also were encountered. It would appear, therefore, that these sapogenins may be more common among plants than had hitherto been suspected.

Experimental[6]

Isolation of Saponin.—Freshly cut ladino clover (150 kg.) was immersed in 360 liters of 95% alcohol for 2 days, the mixture was filtered and the filtrate was concentrated to about 30 liters. The cooled solution along with some dark green insoluble lipid fraction was transferred to large separatory funnels. The residue was transferred with diethyl ether, followed by a small quantity of water. The funnels were inverted gently several times (vigorous shaking gave emulsions) until most of the chlorophyll and lipid material were in solution. After about 30 minutes the aqueous layer was drained. The saponin crystallized spontaneously in shimmering micro-plates. The ether layer then was washed repeatedly with small quantities of water and the water solutions were combined and allowed to stand for a length of time to obtain additional crops of crystalline saponin. The aqueous phase was centrifuged and the crystalline saponin was washed in centrifuge tubes several times with small quantities of water. The saponin finally was washed with acetone until no more colored material was removed. This yielded white, crystalline saponin (70 g. after drying *in vacuo* at 60°), representing 0.23% of the dry weight of ladino clover.[7]

This material after recrystallization from aqueous methanol and drying *in vacuo* at 100°, decomposed at *ca.* 225°, $[\alpha]^{26}D$ −3.3° (CH_3OH).

Anal. Found: C, 59.5; H, 8.37; N, 0.02; sulfated ash, 6.70. The ash contained calcium and magnesium equivalent to 1.65 and 0.15%, respectively, of the saponin.

Dowex-50 Treated Saponin.—The mixed calcium–magnesium salts of saponin (2 g.) were dissolved in 1800 ml. of 50% ethanol and poured through a column containing 50 g. of Dowex-50 resin. The filtrate was concentrated to 100 ml., and the free acids of the saponin were filtered, washed with water, and dried at 100° in a vacuum; yield 1.93 g.

Anal. Found: C, 61.2; H, 8.39; sulfated ash, 0.06; av. equiv. wt., 963.

When the saponin was hydrolyzed, neutral sapogenins, several sugars and an unknown acid were liberated. The sugars were identified by paper chromatography as glucose, galactose, xylose and rhamnose. The acid has not been identified, but paper chromatograms indicate that it is not galacturonic, glucuronic or gluconic acid.

Properties of Ladino Clover Saponin.—The saponin preparation was poorly soluble in water (0.33 g./liter), moderately soluble in 50% alcohol, and slightly soluble in higher concentrations of alcohol. It did not hemolyze red blood cells at a concentration of 1% and did not kill fish (*Lebistes reticulatus*) in a saturated aqueous solution. In the dry crystalline state it gave a red color with sulfuric acid. When tested with isolated strips of rabbit intestine, ladino saponin in a concentration of 10 mg./50 ml. of saline bath caused an abrupt increase in tonus, a rapid loss in peristalsis, and early damage to the tissue.

Isolation of Sapogenins.—A mixture of 10 g. of saponin, 600 ml. of 50% ethanol and 20 ml. of concentrated sulfuric acid was refluxed for 68 hours, cooled, filtered and the solid was washed free of acid and dried. This material then was dissolved in 800 ml. of ether and decolorized with charcoal. Two recrystallizations from methanol–chloroform yielded 1.28 g. of sapogenins (m.p. *ca.* 235°) and an additional 0.7 g. could be secured from the original filtrate by concentration and ether extraction. The sapogenin mixture was soluble in the common organic solvents (*e.g.*, chloroform, ether, alcohol) and gave a red color with sulfuric acid and the Liebermann–Burchard reagent and a pale yellow color with tetranitromethane. Attempts to titrate the sapogenins with alkali indicated that no acidic groups were present.

The sapogenins (20 g.) dissolved in 1 liter of benzene were chromatographed on a column (36 mm. × 48 cm.) of deactivated alumina[8] and eluted with increasing concentrations of methanol in benzene. Three definite fractions (m.p. 239–241°, 258–260° and 318–320°) were eluted with, respectively, 0.5, 2.5 and 100% of methanol. These melting points are in reasonable agreement (*cf.* Table I) with those reported[9,10] for soyasapogenols C, B and A isolated from soya beans. Soyasapogenol B (*ca.* 75%) and soyasapogenol C (*ca.* 20%) were identified unambiguously as shown below, while no additional work was done with the presumed soyasapogenol A which was present only in trace quantities.

(1) This note is to be considered Paper XVIII in the series "Terpenoids" from Wayne University.

(2) Article not copyrighted.

(3) Wayne University, Detroit, Michigan.

(4) E. D. Walter, G. R. Van Atta, C. R. Thompson and W. D. Maclay, THIS JOURNAL, **76**, 2271 (1954).

(5) I. L. Lindahl, A. C. Cook, R. E. Davis and W. D. Maclay, *Science*, **119**, 157 (1954).

(6) Melting points were determined on the Kofler block. Unless noted otherwise, rotations were measured in chloroform solution.

(7) Crystalline saponin also was isolated in small quantity from alfalfa by the same procedure. This saponin appeared to be identical with that from ladino clover as indicated by X-ray diffraction studies, infrared absorption, and by microscopic examination of the crystals. Potter and Kummerow (*Science*, **120**, 224 (1954)) isolated a saponin preparation from alfalfa which they found yielded at least three triterpenoid sapogenins, one of which was identical with soyasapogenol B.

(8) Alorco alumina grade F-20 was deactivated by shaking 300 g. of alumina with hexane containing 12 ml. of 10% aqueous acetic acid for 3 hours.

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