tion were spotted upon Vaseline-impregnated Whatman No. 1 paper and chromatographed. They gave zones with R_F values of 0.25, 0.18, and 0.19, respectively.

Catalytic Hydrogenation of an Original Preparation of Vitamin K from M. phlei.—A 2.4-mg sample of an original preparation from *M. phlei* was catalytically hydrogenated. A sample of the product, in the quinone form, equivalent to 300 μg of the preparation before reduction, was spotted upon Vaseline-impregnated Whatman No. 1 paper. Samples of the corresponding products from vitamin $K_9(H)$ and $K_{2(50)}$ were also placed upon the paper. The paper chromatogram was developed (descending) for 13 days with glacial acetic acid saturated with Vaseline as solvent. A mixture of the vitamin $K_{2(50)}$ and $K_9(H)$ reduction products gave zones 3.6 cm and 7.4 cm, respectively, from the origin. The product from the original preparation from *M. phlei* gave a slightly elongated zone 7.0 cm from the origin.

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Enzymatic Formation of Testololactone*

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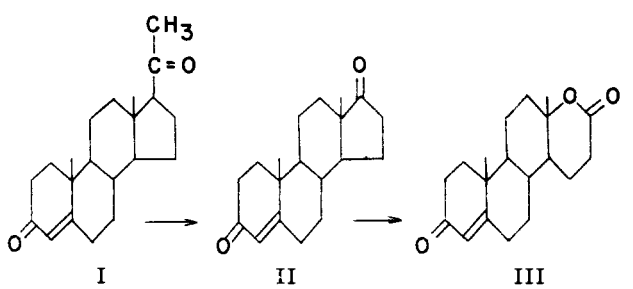
The conversion of testosterone to testololactone has been studied in soluble and partially purified enzyme systems derived from *Penicillium lilacinum*. This transformation is carried out by two steroid-induced enzyme systems: a diphosphopyridine nucleotide–linked 17β-hydroxysteroid dehydrogenase that catalyzes the reversible interconversion of testosterone and 4-androstene-3,17-dione; and a lactonizing enzyme system that converts 4-androstene-3,17-dione to testololactone. The lactonization reaction shows an absolute requirement for reduced triphosphopyridine nucleotide and for molecular oxygen. Tracer studies with oxygen^{18} have shown that the single oxygen atom incorporated during the formation of the lactone is derived from molecular oxygen and is located in the ethereal and not the carbonyl group linked to C-17.

In 1953 two groups of workers (Fried *et al.*, 1953; Peterson *et al.*, 1953) reported that several species of *Penicillia* and *Aspergilli* converted various C_{19} and C_{21} steroids to testololactone (III), a six-membered steroid ring D lactone. The formation of testololactone was discovered incidental to certain other microbiological oxidations, particularly the degradation of the side-chain of C_{21} steroids and the introduction of a Δ^1-double bond into ring A. In a typical reaction, progesterone (I) was converted via 4-androstene-3,17-dione (II) to testololactone (III).



I II III

The testololactone formed during the course of these reactions was identical to a compound prepared by Jacobsen (1947) by the peracetic acid oxidation of a 17-ketosteroid. In 1953, the precise chemical structure of testololactone and related steroid lactones was uncertain, and it was not known whether the lactone oxygen atom was located between C-13 and C-17, or between C-16 and C-17. This question was subsequently resolved unequivocally in favor of the structure shown in formula III (Wendler *et al.*, 1955; Murray *et al.*, 1956).

The discovery of the microbiological conversion of 4-androstene-3,17-dione to testololactone in high yield offered the interesting opportunity of studying the mechanism of a novel biochemical reaction. The possibility was also considered that lactonization of the steroid ring D might constitute a general pathway for the microbial degradation of the steroid skeleton. Recent work by Bradshaw *et al.* (1959) and by Conrad *et al.* (1961, 1962) has demonstrated that the microbial oxidation of camphor proceeds via the lactonization of cyclic ketones. There are chemical similarities between the lactonizations of steroids and camphor, and these transformations are formally analogous to the Baeyer-Villiger (1899) reaction.

This paper describes the conversion of testosterone

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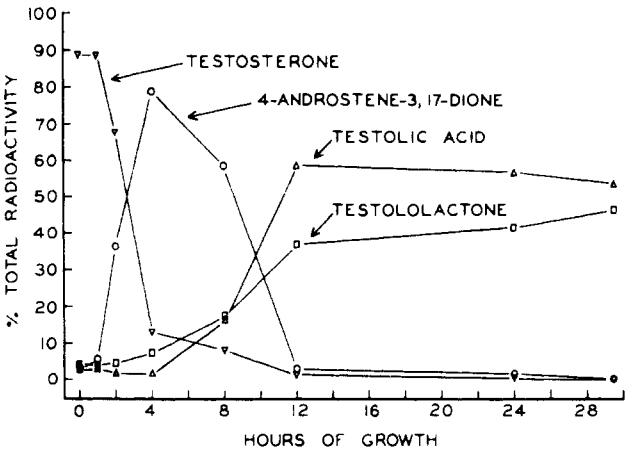

FIG. 1. Time course of the conversion of testosterone-4-C^{14} to testololactone and other products by a growing culture of *Penicillium lilacinum*. 100 mg of testosterone-4-C^{14} (approximately 1 μc) in 2 ml acetone was added to 1000 ml of a 12-hour growth of *Penicillium lilacinum*. Ten-ml aliquots of the culture were removed at the times indicated. Each sample was extracted with methylene dichloride (3 × 10 ml) and the extracts were evaporated to dryness and redissolved in 0.1 ml of methanol; 0.04-ml aliquots were chromatographed on paper. The positions of the ultraviolet absorbing steroids were located, and the chromatogram was cut transversely into 5-cm strips. Each strip was eluted with three 4-ml portions of CH_2Cl_2, and the extract was transferred to a scintillation counter vial, evaporated to dryness, and its radioactivity determined. The distribution of radioactivity at each time interval is expressed as a percentage of the total radioactivity recovered in each time sample. There was complete correspondence between the location of the ultraviolet absorbing material and the radioactivity.

to testololactone via 4-androstene-3,17-dione by growing cultures of *Penicillium lilacinum* (Thom) and by partially purified enzymes derived from this fungus. Two soluble steroid-induced enzyme systems are involved in these reactions: A DPN-linked 17β-hydroxysteroid dehydrogenase which interconverts testosterone and 4-androstene-3,17-dione; and a lactone-forming enzyme system which catalyzes the conversion of 4-androstene-3,17-dione to testololactone.

Results and Discussion

The enzyme systems responsible for the steroid transformations were extracted from the lyophilized mycelium by homogenization in a medium (*p*H 7.8) composed of 0.1 M Tris chloride and 0.02 M EDTA, with the aid of a Potter-Elvejhem homogenizer. This homogenate was centrifuged at 30,000 × *g* for 20 minutes; the supernantant fluid was decanted, filtered through cheesecloth, and processed further as described below. The 17β-hydroxysteroid dehydrogenase and the lactonizing enzyme system were undiminished in activity in the supernatant fluid when crude extracts were centrifuged at 105,000 × *g* for one hour. Both enzyme systems are induced by the addition of testosterone or 4-androstene-3,17-dione to the growing cultures. The maximum specific activity of the lactone forming enzyme system was about sixty times higher than the uninduced level, whereas the hydroxysteroid dehydrogenase was increased more than 200-fold.

17β-Hydroxysteroid Dehydrogenase.—Crude extracts prepared in this manner reduced DPN in the presence of testosterone. The 17β-hydroxysteroid dehydrogenase was purified by the following successive steps: removal of nucleic acids by treatment with protamine sulfate (1 mg of protamine sulfate for 2 mg of protein); fractionation with ammonium sulfate (0.4–0.5 saturation), dialysis, fractionation with acetone at −20° (40–50%), dialysis, and chromatography on ECTEOLA-cellulose (adsorption from 0.005 M Tris, 0.001 M EDTA, *p*H 7.8, and elution with a linear gradient to 0.5 M KCl in the same buffered medium). These procedures achieved a 50-fold purification, and gave a preparation which reduced 9.0 μmoles DPN per minute per milligram of protein under the conditions of assay. The product of the reaction was identified as 4-androstene-3,17-dione by paper chromatography. At *p*H 6.0 DPNH was oxidized by 4-androstene-3,17-dione in the presence of partially purified preparations of the 17β-hydroxysteroid dehydrogenase. These results are consistent with the following stoichiometry:

$$\text{Testosterone} + \text{DPN}^+ \rightleftharpoons \text{4-androstene-3,17-dione} + \text{DPNH} + \text{H}^+$$

In addition to testosterone, these enzyme preparations oxidize a variety of other 17β-hydroxysteroids, such as 17β-estradiol, 17β-hydroxy-5α-androstan-3-one, and 17β-hydroxy-5β-androstan-3-one. Estriol (3, 16α, 17β-trihydroxy-1, 3, 5(10)-estratriene) is not oxidized, and in contrast to the (3 and 17)β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* (Talalay and Marcus, 1956), the enzyme derived from *P. lilacinum* is inert toward 3β-hydroxysteroids.

The 17β-hydroxysteroid dehydrogenase of *P. lilacinum* can utilize DPN as well as certain of its analogs, *e.g.* 3-acetylpyridine adenine dinucleotide, pyridine aldehyde adenine dinucleotide, and thionicotinamide adenine dinucleotide, as hydrogen acceptors, but is inert toward TPN and the hypoxanthine analogs of DPN and TPN in the oxidation of testosterone.

Conversion of Testosterone to Testololactone.—The time course and intermediates of the conversion of testosterone to testololactone by growing cultures of *P. lilacinum* were analyzed by paper chromatography with the aid of testosterone-4-C^{14} as substrate. It was observed that 4-androstene-3,17-dione was the earliest product, and that testosterone was converted almost completely to the 4-androstene-3,17-dione before the appearance of significant amounts of testololactone (Fig. 1). Concomitant with the formation of the lactone, a highly polar compound was detected in approximately equivalent amounts. This material was identified as testolic acid (the hydroxy acid formed upon hydrolysis of testololactone) by its facile conversion to testololactone under acidic or anhydrous conditions. Whether the primary oxidation product of 4-androstene-3,17-dione is testololactone or testolic acid remains uncertain, since no satisfactory method was found for measuring these compounds simultaneously without causing their interconversion.

The lactonization reaction was assayed with the aid of 4-androstene-3,17-dione-4-C^{14} as substrate. Since testololactone is a saponifiable lipid, whereas 4-androstene-3,17-dione is not, the substrate could be separated from the product by extracting the aqueous reaction mixture with organic solvents, from basic and from acidic solutions respectively. This procedure (see Table I) provided a sensitive, rapid, and precise assay for the lactonization reaction.

The lactone-forming enzyme system was purified from crude extracts of the lyophilized mycelium of *P. lilacinum*, grown in the presence of testosterone, by isoelectric precipitation at *p*H 5.5 and fractionation between 0.4 and 0.6 saturation of ammonium sulfate at *p*H 7.0. These enzyme preparations were stable for several months when left as ammonium sulfate slurries

at −20°, but rapidly lost activity in solution. The initial extracts had activities in the range of 15 to 80 mμmoles testololactone formed per hour per mg of protein under the conditions of the assay (Table I). The purification procedure gave a 5 to 10-fold increase in specific activity. These preparations contain an active TPN-linked glucose-6-phosphate dehydrogenase.

TABLE I

FORMATION OF TESTOLOLACTONE IN THE PRESENCE AND ABSENCE OF O_2

Each Thunberg tube contained 100 μg of 4-androstene-3,17-dione-4-C^{14} (8,300 cpm) dissolved in 0.1 ml of 50% aqueous methanol (placed in the side-arm), 250 μmoles of Tris buffer, *p*H 7.8, 10 μmoles of glucose-6-phosphate, 1.5 μmoles of TPN^+, and 8.6 mg of protein from a 40 to 60% ammonium sulfate fraction of an extract prepared from lyophilized *P. lilacinum*, in a final volume of 2.9 ml. The tubes for the anaerobic experiments were evacuated twice and filled with oxygen-free nitrogen gas after each evacuation. The aerobic vessels were likewise evacuated, but air was readmitted. The reaction was initiated by tipping the contents of the side-arm into the main vessel. After incubation at 30° for 1 hour, 0.2 ml of 12 N HCl was introduced into each tube to stop the reaction. The contents of the tubes were transferred to 50 ml capacity Maizel-Gerson vessels with the aid of 3 ml of acetone. The radioactive steroids were extracted with 7 ml of benzene. The organic phase was removed, pooled with a second extract of acetone and benzene, and reduced to dryness by evaporation in a boiling water bath. The steroids were redissolved in 1 ml of acetone, saponified with 2.5 ml of 0.16 N NaOH at room temperature for 5 minutes, and extracted with 4 ml of benzene. The extraction with acetone and benzene was performed twice more, and the pooled extracts containing the nonsaponifiable steroids were evaporated to dryness. The aqueous phase was acidified with 0.1 ml of 12 N HCl and extracted twice with 1 ml of acetone and 4 ml of benzene. These extracts, containing the saponifiable steroids, were likewise pooled and evaporated to dryness. Radioactivity was determined in an automatic Packard Tri-Carb Scintillation Spectrometer.

Assay Conditions	Saponifiable Radioactivity (cpm)	Nonsaponifiable Radioactivity (cpm)
Anaerobic	49	8209
	74	8222
Aerobic	1380	6912
	1348	7130
Zero time	77	8194

The formation of testololactone was a strictly aerobic reaction (Table I). Partially purified preparations of the lactone-forming enzyme system also showed an absolute requirement for TPNH which could be satisfied by a system for continuously generating TPNH or by the direct addition of TPNH (Table II). The oxidized form of triphosphopyridine nucleotide and both the oxidized and reduced forms of diphosphopyridine nucleotide were completely inactive in this respect.

A number of properties of the lactone-forming enzyme system were examined in an effort to elucidate its mechanism. The reaction was found to be insensitive to catalase and was unaffected by the addition of hydrogen peroxide or of a hydrogen peroxide-generating system (glucose and glucose oxidase). The following substances were added (final concentration shown in parentheses) to the standard assay system and found to be without significant effect on the rate of formation of testololactone: $MnCl_2$ (3.3 mM), $MgCl_2$ (3.3 mM), $FeCl_3$ (0.5 mM), $FeSO_4$ (1 mM), $CaCl_2$ (0.1 mM), $CoCl_2$ (1 mM), $AlNH_4(SO_4)_2$ (1 mM), EDTA (17 mM), α,α′-dipyridyl (1 mM), 1,10-phenanthroline (1 mM), cupferron (1 mM), KCN (2 mM), amethopterin (0.1 mM), aminopterin (1 mM), glutathione (10 mM), and ascorbic acid (1 mM).

TABLE II

PYRIDINE NUCLEOTIDE REQUIREMENT FOR THE ENZYMATIC FORMATION OF TESTOLOLACTONE

In addition to the components listed below, each Maizel-Gerson vessel contained in a final volume of 3 ml: 250 μmoles of Tris buffer, *p*H 7.85, and 100 μg of 4-androstene-3,17-dione-4-C^{14} (8,400 cpm) in 0.1 ml of 50% aqueous methanol. The reaction was initiated by the addition of 5.5 mg of protein from a 40 to 60% ammonium sulfate fraction derived from an extract of lyophilized *P. lilacinum*. After incubation at 38° for 1 hour, the reaction was arrested by the addition of 0.2 ml of 12 N HCl. The saponifiable and nonsaponifiable radioactive steroids were measured as described in Table I.

Additions	Saponifiable Radioactivity (cpm)	Nonsaponifiable Radioactivity (cpm)
Enzyme only	56	8515
	75	8512
+ 1.5 μmoles TPN^+	86	8435
	69	8317
+ 10 μmoles G-6-P	56	8295
	35	8467
+ 1.5 μmoles TPN^+ + 10 μmoles G-6-P	899	7498
	765	7440
+ 2.0 μmoles TPNH	526	8141
	400	7995
Zero time controls	52	8296
	32	8282

Addition of $CuSO_4$ (20 μM) or $ZnSO_4$ (100 μM) to the reaction vessel inhibited the formation of testololactone, and addition of *p*-mercuriphenylsulfonic acid (1 mM) suppressed the formation of testololactone, when the reaction system contained 0.67 mM TPNH. The requirements for oxygen and for TPNH in the conversion of 4-androstene-3,17-dione to testololactone are consistent with the following stoichiometry:

$$\text{4-androstene-3,17-dione} + \text{TPNH} + O_2 + H^+ \longrightarrow \text{testololactone} + \text{TPN}^+ + H_2O$$

Oxygen^{18} *Experiments.*—Testololactone contains one oxygen atom in addition to the two present in 4-androstene-3,17-dione. The strictly aerobic nature of the reaction suggested that the additional oxygen atom was derived from molecular oxygen, but did not specify its site of incorporation, *i.e.*, whether this oxygen atom is the ethereal oxygen atom linking C-13 and C-17, or the carbonyl oxygen atom at C-17. Experiments to clarify these questions were conducted with oxygen^{18}. Conversion of 4-androstene-3,17-dione to testololactone by a growing culture of *P. lilacinum* was carried out in an atmosphere of oxygen enriched with oxygen^{18}, obtained by electrolysis of H_2O^{18}. When the conversion was at least 90% complete the testololactone was isolated from the culture medium, purified, and analyzed for oxygen^{18} content. The testololactone was found to contain the equivalent of 0.78 atom of oxygen^{18}. The discrepancy between this value and the anticipated 1.0 atom of oxygen per molecule is probably to be ascribed to analytical difficulties discussed below.

Attention was then turned to determining the location of the oxygen atom introduced during the course of microbiological lactonization. Since no simple method was available for degrading the steroid so as to permit analysis of each of the three oxygen atoms of

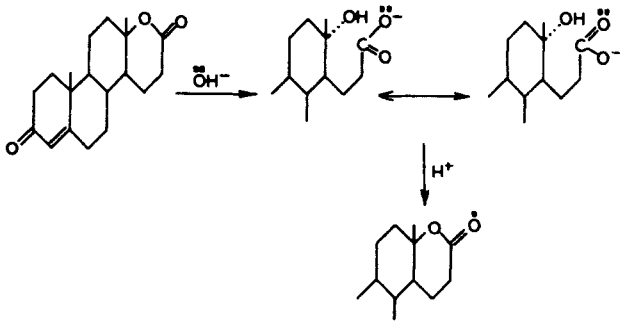

FIGURE 2

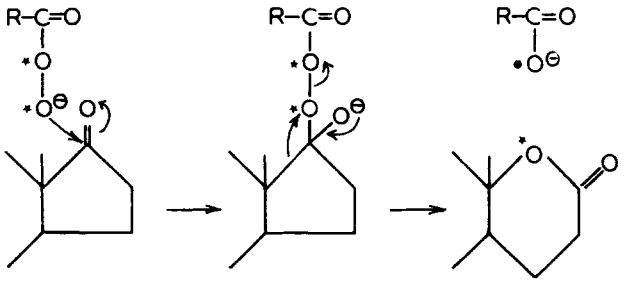

FIG. 3.—Mechanism of the peracid oxidation of a 17-keto-steroid to form a ring D lactone.

testololactone separately, the site of incorporation of the oxygen[18] was established by determining the exchangeability of this label with H_2O (Table III).

The testololactone was dissolved in methanol, hydrolyzed with aqueous base, acidified, and permitted to equilibrate. The steroid was then reisolated and analyzed for its oxygen[18] content. It may be assumed that the hydrolysis of testololactone occurs with acyl oxygen fission (Long and Friedman, 1950), and that in the anionic form of the hydroxy acid the two oxygen atoms of the carboxylate ion become equivalent but do not undergo further exchange with the medium (Bunton and Spatcher, 1956). Consequently, upon hydrolysis in base and subsequent relactonization of testololactone, one-half of the oxygen of the carbonyl group at C-17 could be expected to exchange with the medium in the manner shown in Figure 2.

Equilibration in strong acid may lead to further exchange of the carbonyl groups at C-3 and C-17. The 13α tertiary hydroxyl group of the hydroxy acid may be assumed to be nonexchangeable in base, but would be subject to exchange in acid *via* the formation of a carbonium ion. However, this process would be expected to be much slower than re-formation of the lactone for the following reasons: (1) in general, the rate of δ-lactone formation is relatively rapid compared to the formation of a carbonium ion from a tertiary alcohol; (2) the presence of the steroid ring system should increase the rate of lactonization by restricting the number of conformations available to the hydroxy acid and should decrease the rate of carbonium ion formation due to strain thus imposed on ring C. Therefore, of the three oxygen atoms present in testololactone, only the one between C-13 and C-17 should be resistant to exchange, since in base, the tertiary alcohol does not exchange, and in acid, closure of the lactone ring occurs more rapidly than exchange of the hydroxyl group. Table III presents the results of the O^{18} exchange between testololactone and H_2O which may be summarized as follows. Under conditions which resulted in the exchange of the equivalent of one atom of oxygen between water and testololactone (experiment 2) the microbiologically incorporated oxygen was completely resistant to exchange (experiment 1). Thus, the atom of oxygen incorporated into testololactone by *P. lilacinum* is the ethereal oxygen atom between C-13 and C-17. The equivalent of one atom of oxygen[18] was exchanged between the steroid and water, and this isotope is distributed between the C-3 and C-17 carbonyl oxygen atoms.

TABLE III

EXCHANGE OF OXYGEN[18] BETWEEN WATER AND TESTOLOLACTONE

The conditions for exchange were as follows: To a solution of 15 mg of testololactone in 10 ml of anhydrous methanol were added 5 ml of 0.2 N NaOH. After standing one hour at 25°, the solution was acidified with 0.15 ml of 12 N HCl and permitted to equilibrate at 32° for 1 week. The solution was neutralized by the addition of 0.2 ml of 4 N NaOH and evaporated to dryness on a rotatory evaporator (20–35°). The residue was extracted two times with 1 ml of methanol, and the extracts were combined and dried at room temperature. This material was transferred to a microsublimation tube. After 2 days at 145° and less than 1 μ Hg pressure, the sublimed testololactone was removed and converted by sealed tube combustion to CO_2 (Rittenberg and Ponticorvo, 1956). A mixture of 5 mg of testololactone and 200 mg of $HgCl_2$ was heated for 7 hours at 530°. Masses 44 and 46 were measured in a Consolidated Electrodynamic Mass Spectrometer. The atoms O^{18} incorporated into testololactone were calculated on the basis of a 3-fold dilution.

Experiment	Atoms % Excess O^{18}			Atoms O^{18} Incorporated into Testololactone
	Water Used for Equilibration	Testololactone		
		Before Equilibration	After Equilibration	
1	0.00	0.370[a]	0.392	0.07
2	1.38	0.00	0.451	0.98
3	1.38	0.370[a]	0.848	1.04

[a] The preparation of this material is described in the section on Experimental Procedure.

The demonstration that in the enzymatic lactonization reaction molecular oxygen enters exclusively the ring oxygen is in accordance with the suggestion of Fried *et al.* (1953) and is consistent with the mechanism of the chemical Baeyer-Villiger reaction in which a peroxydic species (the peracid) attacks the C-17 ketone to give an addition compound which rearranges by a 1,2-shift to give as products the lactone and the normal acid derived from the peracid (Fig. 3). In agreement with this mechanism, studies of the chemical lactonization with the aid of oxygen[18] as tracer have shown that the ethereal oxygen atom is derived from one of the peroxy atoms of the peracid and that this oxygen atom does not exchange with the carbonyl oxygen atoms of the ketone, the lactone, the peracid, and the normal acid or with the oxygen atoms of water of the medium (Bunton *et al.*, 1956; Doering and Dorfman, 1953; Hassal, 1957).

EXPERIMENTAL PROCEDURES

Materials.—All solvents were reagent grade and were redistilled. Toluene used for scintillation counting and formamide used for paper chromatography were not distilled. Pyridine nucleotides, their analogs, and sodium glucose-6-phosphate were purchased from Pabst Laboratories, Milwaukee, Wis., or Sigma Chemical Company, Saint Louis, Mo. Testosterone-4-C^{14} (2 μc per μmole) was a product of the Isotope Specialties Company, Los Angeles, Calif. 4-Androstene-3,17-dione-4-C^{14} was prepared from testosterone-4-C^{14} by

enzymatic oxidation with the purified 17β-hydroxysteroid dehydrogenase of *Pseudomonas testosteroni* (Talalay and Marcus, 1956).

Determinations of Radioactivity.—Radioactive steroids were evaporated to dryness in counting vessels and dissolved in 15 ml of a mixture containing 4 g of diphenyl oxazole and 50 mg of 1,4-bis-2(5-phenyloxazolyl)-benzene per liter of toluene. The samples were counted in an automatic Packard Tri-Carb Liquid Scintillation Spectrometer. Appropriate corrections for background and for counting efficiency were introduced.

Paper Chromatography.—Descending chromatograms were run on Whatman No. 43 filter paper (15 × 57 cm) at room temperature in a system of the type described by Zaffaroni (Zaffaroni and Burton, 1951). The paper was impregnated with a mixture of equal volumes of methanol and formamide and developed with benzene-hexane (1:1) saturated with formamide. The solvent front was permitted to descend to the lower edge of the paper (2.25–2.75 hours). Chromatograms were examined with an ultraviolet scanner (Haines, 1952).

Growth of Cultures and Preparation of Enzymes.—Cultures of *P. lilacinum* (Thom) (A.T.C.C. 10114) were grown in a medium containing the following ingredients: 10 g of glucose, 1 g of $(NH_4)_2HPO_4$, 1 g of $(NH_4)H_2PO_4$, 2 g of KH_2PO_4, and 10 ml of a trace element solution[1] per liter of distilled water. Growth of the cultures was carried out in flat-bottomed flasks with large surface area to volume ratio (Corning Glass Works No. 4420) which were agitated on a reciprocating horizontal shaker at 30°. Culture flasks (1000 ml) were inoculated by the addition of 25 ml of a starter culture which had been finely dispersed by treatment for one minute in a small size Waring Blendor jar. Cultures were induced by addition of 300 mg of testosterone or 4-androstene-3,17-dione (suspended in 3 ml of water by sonic oscillation) approximately 20 hours after inoculation with mycelium. In order to obtain maximum enzymatic activity for lactone formation, it is necessary to have substrate present in the growth medium at all times, and, accordingly, further additions of steroid were made periodically. After a total of 50 hours of growth, the mycelium was collected by filtration on cheesecloth, washed with water, pressed between filter papers, and lyophilized. The brittle cake was powdered and stored at −20°. The enzymatic activities in this state were stable for more than one year.

Conversion of 4-Androstene-3,17-dione to Testololactone in an Atmosphere of Oxygen[18].—The conversion was carried out in a 2.5-liter culture flask (Corning No. 4420) equipped with a ground glass joint at the neck and with inlet and outlet tubes permitting a continuous flow of oxygen over the surface of the culture fluid. The flask was fitted with a small port closed with a rubber ampul seal for the introduction and removal of liquid by means of a syringe and a hypodermic needle. Oxygen was generated from a U-tube electrolysis cell provided with platinum electrodes and containing H_2O^{18} (1.42 atoms % excess) and H_2SO_4 as electrolyte. The generated oxygen was passed through a trap immersed in a dry ice–acetone bath prior to its entry into the growth flask. The flask (containing 500 ml growth medium) was agitated reciprocally and maintained at 30°. The system was first flushed with N_2 and was then equilibrated thoroughly (14 hours) with the oxygen generated from electrolysis of H_2O^{18} at the rate of 300 ml per hour (1.45 amperes at 11.5 volts). Two hundred fifty mg 4-androstene-3,17-dione in 8 ml acetone was added and the flask was inoculated with a starter culture of *Penicillium lilacinum*. Every 6 hours, an aliquot of the culture was removed, its *p*H was determined, and the steroids were extracted with a mixture of acetone and benzene (40:60). The extracts were evaporated and chromatographed on paper, and the intensities of the ultraviolet-absorbing regions corresponding to 4-androstene-3,17-dione and testololactone were noted. Thirty hours after inoculation, the *p*H of the culture had fallen from an initial *p*H of 6.25 to 3.45. Addition of $(NH_4)_2HPO_4$ raised the *p*H to 6.40. At the end of 55 hours of growth (*p*H 5.55), the conversion of steroid appeared to be at least 90% complete.

The mycelium was separated from the culture medium by filtration on cheesecloth, and was washed with water and acetone. The culture medium and washings were combined and extracted with a mixture of acetone and benzene (1:4). The organic extracts were reduced to dryness on a rotatory evaporator under reduced pressure. The steroid crystallized on standing at room temperature. It was recrystallized from a mixture of hexane and acetone and sublimed (< 1 μ Hg, 120–142°). A total of 210 mg of testololactone was isolated. The material was chromatographically pure and had a m.p. of 211–212° (corr.).

Anal. Calcd. for $C_{19}H_{26}O_3$: C-75.46%, H-8.67%. Found: C-75.40%, H-8.74%.

The oxygen[18] content of the gas passing through the growth vessel was determined at various time intervals during the experiment. Direct analysis of masses 32 and 34 gave values between 1.40 and 1.42 atoms% excess oxygen[18]. Samples of testololactone (5 mg) were combusted to CO_2 with 200 mg $HgCl_2$ in sealed tubes for 7 hours at 530° (Rittenberg and Ponticorvo, 1956). The CO_2 was analyzed for relative abundance of masses 44 and 46. The best duplicate analyses gave 0.777 and 0.782 atoms oxygen[18] per molecule. A number of reasons for the apparent incorporation of only 0.78 atom rather than 1.0 atom of oxygen[18] were considered. The most probable explanation for this discrepancy appears to lie in the analytical procedure. An inherent assumption in the determination is that either the steroid is completely burned to CO_2 or that the CO_2 which is analyzed is derived to an equal extent from all three oxygen atoms of testololactone. Preliminary studies of conditions of the combustion have indicated that the requirements for complete combustion or equal contribution from the three oxygen atoms have probably not been met.

[1] The trace element solution had the following composition: 20 g of $MgSO_4 \cdot 7H_2O$, 1 g of NaCl, 0.5 g of $ZnSO_4 \cdot 7H_2O$, 0.5 g of $MnSO_4 \cdot 3H_2O$, 0.05 g of $CuSO_4 \cdot 5H_2O$, and 10 ml of 0.1 N H_2SO_4 per liter.

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