313. S.Mukharji, R.Sharma, and O.Vig, J.Chem.Soc., 4770 (1958).
314. R.Koehler, L.Goodman, J.DeGraw, and B.Baker, J.Amer. Chem.Soc., 80, 5779 (1958).
315. King Sheng and Chi-Yi Hsing, Acta Sci.Sinica, 5, 455 (1956); Chem.Zentr., 9879 (1959).
316. P.McCloskey, J.Chem.Soc., 4732 (1958).
317. D.Banerjee and P.Shafer, J.Amer.Chem.Soc., 72, 1931 (1950).
318. E.Horning, M.Horning, M.Fish, and M.Rutenberg, J. Amer.Chem.Soc., 74, 773 (1952).
319. E.Bergmann and J.Szmuszkovicz, J.Amer.Chem.Soc., 75, 3226 (1953).
320. R.Burnell and W.Taylor, J.Chem.Soc., 3486 (1954).
321. P.Talukdar and P.Bagchi, Sci.and Culture, 18, 503 (1953).
322. P.Taludkar and P.Bagchi, J.Org.Chem., 20, 21 (1955).
323. P.Talukdar and P.Bagchi, J.Org.Chem., 20, 25 (1955).
324. W.Wildmann, J.Amer.Chem.Soc., 80, 2567 (1958).
325. M.Rubin and H.Wishinsky, J.Amer.Chem.Soc., 68, 828 (1946).
326. R.Meltzer, A.Lewis, J.Volpe, and D.Lustgarten, J.Org. Chem., 25, 712 (1960).
327. W.Hahn, Roczniki Chem., 33, 1501 (1959).
328. A.Campbell and I.Stevens, J.Chem.Soc., 959 (1956).
329. H.Irie, Y.Tsuda, and S.Uyeo, J.Chem.Soc., 1446 (1959).
330. U.Schmidt, Chem.Ber., 92, 1171 (1959).
331. R.Frank and J.McPherson, J.Amer.Chem.Soc., 71, 1387 (1949).
332. H.Zahn and P.Schäfer, Chem.Ber., 92, 736 (1959).
333. S.Sugasawa and S.Ushioda, Tetrahedron, 5, 48 (1959).
334. L.Cohen and B.Witkop, J.Amer.Chem.Soc., 77, 6595 (1955).
335. E.Bergmann and R.Corett, J.Org.Chem., 23, 1507 (1958).
336. F.Mann and A.Tetlow, J.Chem.Soc., 3352 (1957).
337. H.Bruson and T.Reiner, J.Amer.Chem.Soc., 66, 56 (1944).
338. J.Clements and L.Rice, J.Org.Chem., 24, 1958 (1959).
339. E.Bergmann and R.Corett, J.Org.Chem., 21, 107 (1956).
340. N.Sugimoto and S.Ohshiro, Tetrahedron, 8, 296 (1960).
341. J.Nemec, U.S.P. 2839538 (1958); Chem.Zentr., 9400 (1959).
342. T.Kametani, W.Taub, and D.Ginsburg, Bull.Chem.Soc. Japan, 31, 857 (1958); Ref.Zhur.Khim., 78614 (1959).
343. A.P.Terent'ev and A.N.Kost, Vestnik Moskov.Gos.Univ., No.12, 71 (1950).
344. E.Van Tamelen, T.Spencer, D.Allen, and R.Orvis, J. Amer.Chem.Soc., 81, 6341 (1959).
345. R.Welcher, G.Johnson, and V.Wystrach, J.Amer.Chem. Soc., 82, 4438 (1960).
346. F.Mann and A.Wilkinson, J.Chem.Soc., 3336 (1957).
347. F.Mann and I.Millar, J.Chem.Soc., 4453 (1952).
348. F.Poppelsdorf and R.Myerly, J.Org.Chem., 26, 131 (1961).
349. H.Beyer and K.Leverenz, Chem.Ber., 94, 407 (1961).
350. P.Scheiner and W.Vaughan, J.Org.Chem., 26, 1923 (1961).
351. T.Kasturi, Indian Inst.Sci., Golden Jubilee Research Vol., 40 (1961); Chem.Abs., 55, 23371 (1961).
352. J.Cavalla, J.Davoll, M.Dean, C.Franklin, D.Temple, J.Wax, and C.Winder, J.Medicin Pharmaceut.Chem., 4, 1 (1961); Chem.Abs., 55, 23488 (1961).
353. R.Angier and W.Curran, J.Org.Chem., 26, 1891 (1961).
354. S.Takagi and S.Uyeo, J.Chem.Soc., 4350 (1961).
355. C.Grob and O.Weissbach, Helv.Chim.Acta, 44, 1736 (1961).
356. G.Casini, S.Gulinelli, and M.Tontodonati, Ann.Chim. (France), 50, 1207 (1960).
357. M.Ishidate, Y.Sakurai, and I.Aiko, Chem.Pharm.Bull. (Tokyo), 8, 732 (1960); Chem.Abs., 55, 18574 (1961).
358. J.Lynn, J.Org.Chem., 26, 2119 (1961).
359. F.Bruderlein, H.Bruderlein, H.Favre, R.Lapierre, and Y.Lefebvre, Canad.J.Chem., 38, 2085 (1960).
360. H.Stetter, I.Krüger-Hansen, and M.Rizk, Chem.Ber., 94, 2702 (1961).
361. R.Adams, S.Miyano, and M.Nair, J.Amer.Chem.Soc., 83, 3323 (1961).
362. S.Baldwin, J.Org.Chem., 26, 3280 (1961).
363. J.Cavella and J.Davoll, B.P. 862513 (1961); Chem.Abs., 55, 19950 (1961).
364. K.Sennewald, A.Gotz, and G.Kallrath, German P. 1081008 (1961); Chem.Abs., 55, 13338 (1961).
365. K.Sennewald, A.Gotz, and G.Kallrath, German P. 1089754 (1960); Chem.Abs., 55, 17536 (1961).
366. H.Cripps, U.S.P. 2914541 (1959).
367. V.F.Vasil'eva, V.G.Yashunskii, and M.N.Shchukina, Zhur. Obshch.Khim., 31, 1501 (1961).
368. V.I.Gunar and S.I.Zav'yalov, Dokl.Akad.Nauk SSSR, 139, 367 (1961).
369. N.P.Shusherina, R.Ya.Levina, and Huan Hua-min, Zhur. Obshch.Khim., 31, 3477 (1961).

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MICROBIOLOGICAL REACTIONS OF STEROIDS

L. M. Kogan

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I. INTRODUCTION

Hardly anyone has to be convinced nowadays of the importance of microorganisms for the development and sustenance of life on earth. Nor do chemists have to be told of the significance of microbiological processes in present-day industry. The mere mention of antibiotics is sufficient.

The present review deals with an extremely narrow field of the metabolic activity of microorganisms, which has, nonetheless, attracted the attention of chemists all over the world.

This review of the reactions of steroids induced by microorganisms is not exhaustive. Its purpose is to emphasize the possibilities for the cooperation of chemists and microbiologists, without going into too many details.

Transformation of a substrate is caused by enzymes produced by the metabolic activity of a microorganism. Microbiological reactions such as alcoholic and lactic fermentation have been in use since time immemorial. The correlation of these processes with the metabolic activity of living organisms was first observed by Schwann in the thirties of the last century. Pasteur termed the microbiological reactions accompanied by gas evolution as "fermentations", but this term was later extended to other microbiological processes.

The capacity of certain microorganisms to induce various transformations in steroids was noticed more than 20 years ago. The earliest work, by Mamoli and Vercellone[1,2,3], dealt with the hydrogenation of an oxo-group conjugated with a double bond, or with the dehydrogenation of a secondary hydroxyl group. In 1948 Kramli and Horvath[4] first succeeded in introducing a hydroxyl group into a steroid molecule by a microbiological method. It was not, however, until after Peterson[5] had obtained 10% 11α-hydroxyprogesterone (I) by incubating progesterone (II) with a *Rhizopus nigricans* culture that microbiological transformations of steroids awakened widespread interest.

Microbiological steroid reactions are extremely diverse in character. They include introduction of hydroxyl groups, formation of oxo-groups, formation and cleavage of epoxides, hydrogenation, dehydrogenation, etc. In spite of this diversity, techniques employed in carrying out microbiological reactions are fairly uniform. In experiments where a large quantity of the substance is either not required or is not available trial runs are carried out in flasks mounted on agitators, several flasks being usually set up in series. Most often 750–1000 ml flasks are used with 100–200 mg steroid. For steroid amounts of the order of 1–2 g glass fermenters of 10–20 litre capacity are used, provided with stirrers, thermometers, devices for filling, emptying, and sampling, heat-exchange elements, and, when needed, with an air bubbler. In industry and in pilot plants steel apparatus is usually employed, fitted with similar devices.

The procedure used in the glass fermenter is essentially the same as that used in flasks or the steel apparatus (this does not mean, however, that the reaction in the flask can completely model that in the fermenter). The fully equipped fermenter is sterilised, and filled with a sterile culture medium, which has been inoculated by the microorganism under sterile conditions. Special attention must be paid to the observance of strictly sterile conditions throughout all the operations. Depending on the experiment, sterilisation is carried out in an autoclave at 110°–120°, by working in boxes illuminated with bactericidal lamps, or by carrying out filling, sampling, etc. in the flame of a gas burner. After the addition of the inoculated culture medium (usually transferred by means of compressed air), a definite period of time is required for the development of the culture (from several hours to several days). The steroid is introduced in solution in an organic solvent, *e.g.* acetone, alcohol, propylene glycol, or dimethylformamide. A suspension of the steroid, obtained by precipitation with water of an ethanol or acetone solution, is sometimes advantageous as far as the yields are concerned. Reaction is carried out at a temperature requisite for the normal growth and metabolic activity of the microorganism, mostly at 24°–33°. The course of the reaction is followed by assay using either paper chromatography or microbiological methods. "Thin layer chromatography", on an adsorbent on glass plates, without a binding agent, is widely used in following the course of microbiological reactions of steroids[6,7]. By this method steroids can be separated and detected in 30–45 min, in contrast to several hours needed by paper chromatography.

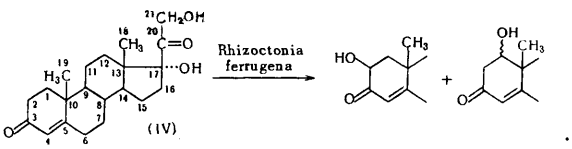
At the end of the process the reaction mixture is extracted by an organic solvent immiscible with water, usually chloroform, dichloroethane, ethyl acetate, methylene chloride, and sometimes ether. In some cases only the filtered reaction liquid need be extracted. For complete separation of steroids, extraction is sometimes preceded by homogenisation of the reaction mixture. The extract is concentrated *in vacuo* and the steroid isolated from it either by crystallisation or chromatography.

According to a recent paper, microbiological steroid reactions can be carried out by passing the steroid solution through a layer of the microorganism culture on a carrier[8]. This method has possibilities as a continuous industrial process.

II. VARIOUS TYPES OF MICROBIOLOGICAL STEROID REACTIONS

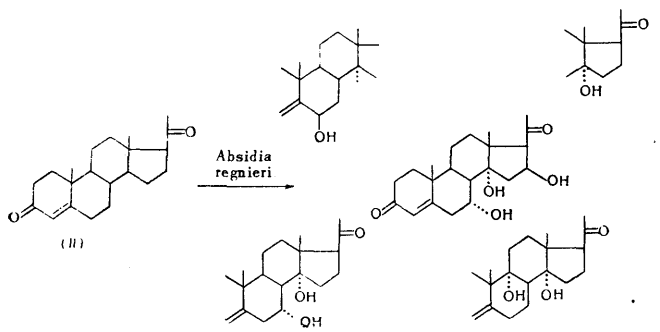
1. Introduction of a Hydroxyl Group

Introduction of a hydroxyl group at carbons 1 and 2 of the steroid molecule was carried out on various steroid substrates, *e.g.* androstenedione[9] (III), Reichstein's substance S [10] (IV), 9α-fluorohydrocortisone[11] (V), and other steroids, by incubation with microorganisms such as *Streptomyces*, *Penicillium*, *Rhizoctonia*, and *Sclerotinia*.

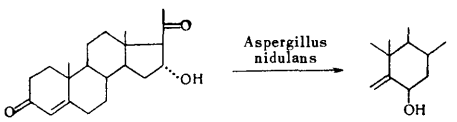


In all cases examined both 1α- and 2β-isomers were obtained. The yield of the 1- and 2-hydroxy derivatives rarely exceeds 10%. Hydroxylation in the 1- and 2-positions is usually complicated by various side-reactions, which make difficult the isolation and purification of the reaction products. Most often 1- and 2-hydroxylations proceed concomitantly with hydroxylations at C_{11} and C_{15}, and with the reduction of the oxo-group at C_{20}.[12]

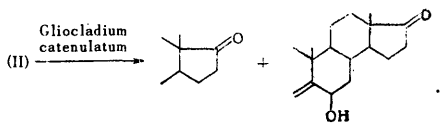
Hydroxylation in the 6β-position, mostly induced by fungi, is also seldom completely selective. For example, incubation of progesterone (II) with *Absidia regnieri* culture[13] yields five products:



6β-Monohydroxylation only occurs if the other possible position for the hydroxyl group is already taken[14]:



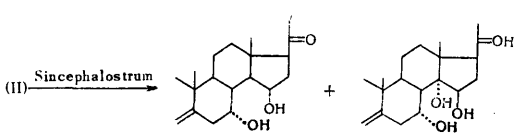
Peterson[16] reported a case where the 6β-hydroxylation was accompanied by oxidative degradation of the side-chain:



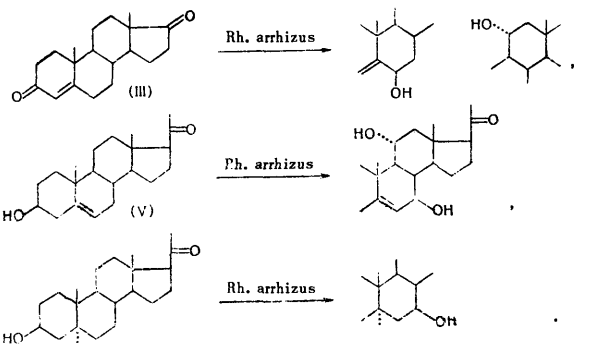
The preparation of 30% 6β,17α,21-trihydroxypregn-4--ene-3,20-dione by incubation of Reichstein's substance S (IV) with *Bacillus cereus* has recently been reported[15].

For all substrates undergoing 6β-hydroxylation the presence of the 4-ene-3-oxo-grouping is a characteristic feature, the nature of the side-chain at C_{17} not being of decisive importance. 6β-Hydroxy-derivatives were also obtained from compounds such as progesterone (II) and androstenedione (III).[13,16]

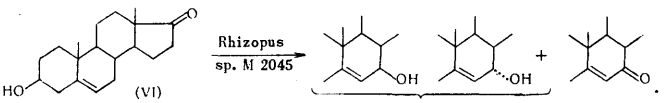
Microorganisms which induce 7α-hydroxylation are *Cephalosporium*[17], *Helmintosporium*[18], *Proactinomyces*[19], and *Sincephalostrum*[20].



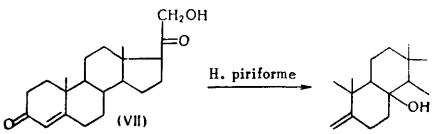
The course of hydroxylation is in certain cases predetermined by the structure of rings A and B of the steroid nucleus. Thus, the mould *Rhizopus arrhizus*, which induces 11α- and 6β-hydroxylations in 4-ene-3-oxo-steroids, *e.g.* androstenedione (III),[21] hydroxylates pregnenolone (V) in positions 7β and 11α, and compounds of the allopregnane series in the 7β-position[22,23]:



On incubation of 3β-hydroxyandrost-5-en-17-one (VI) with a species of *Rhizopus* (M2045), Dodson[24] isolated from the reaction products a molecular compound of the 7α- and 7β-hydroxy-derivatives:

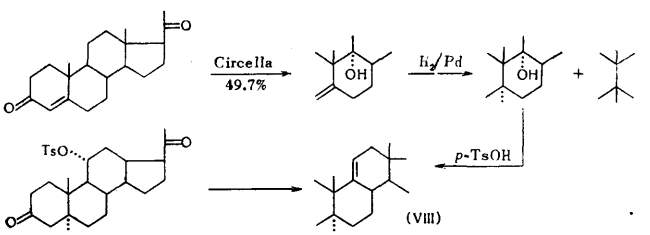


A tertiary hydroxyl group was introduced at C_8 of deoxycorticosterone (VIII) by the action of *Helicostylum piriforme*[21]:



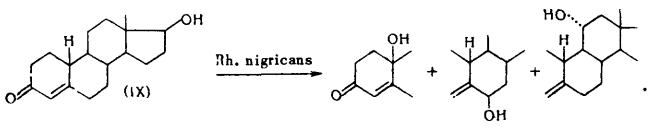
8-Hydroxylation can be carried out by microorganisms such as *Curvularia pallescens*[25], *Mucor parasiticus*[26], etc.

Schubert and collaborators[27] obtained 9α-hydroxyprogesterone using a *Circella* culture. Its structure was established by subsequent hydrogenation and dehydration into the known pregn-9(11)-ene-3,20-dione (VIII):



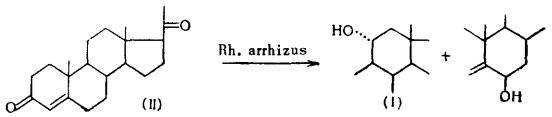
Hydroxylation of the tertiary carbon atom in the 10-position occurs on incubation of 19-nortestosterone (IX) with a

species of *Rh.nigricans*, the microorganism which usually hydroxylates the 11α - and 6β-positions[28]:

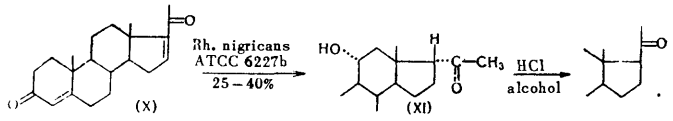


Djerassi's group[29], however, reported 11α-monohydroxylation of 19-norprogesterone in 70% yield by means of a species of *Rh.nigricans*.

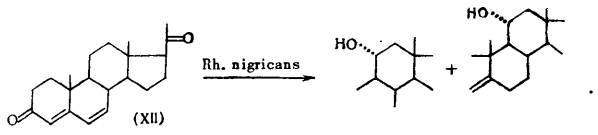
The microbiological preparation of 11α-hydroxyprogesterone (I) in 1952 by Peterson opened up a simple way for the introduction of an oxygen-containing functional group at the 11-position of steroids. This is of great importance for preparing corticosteroids difficult to obtain by chemical methods.



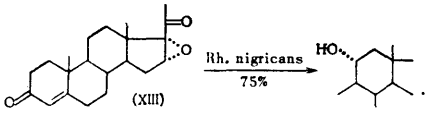
Incubation of progesterone with *Rh.nigricans* gave 95% of 11α-hydroxyprogesterone[30]. This oxidation, however, does not always proceed unidirectionally, 11α-hydroxylation often being accompanied by 6β-,[15,31] 17α-,[32,33] and 21-hydroxylations[34]. Incubation of Δ[16]-dehydroprogesterone (X) with *Rh.nigricans* gave 11α-hydroxy-17-isoprogesterone (XI):[35]



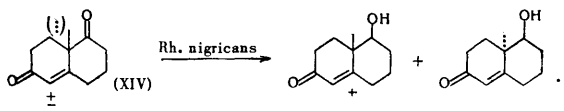
On incubation of Δ[6]-dehydroprogesterone (XII) with a species of *Rh.nigricans*, partial hydrogenation of the Δ[6]-double bond took place[36]:



Hydroxylation of pregnane-3,20-dione and allopregnane-3,20-dione with *Rh.nigricans* gave the corresponding 11α-hydroxy-derivatives in 40% and 25% yields, respectively[37]. Ercoli[38] and Peterson[39] and their collaborators carried out the fermentative hydroxylation of 16α,17α-epoxyprogesterone (XIII) in 75% yield:

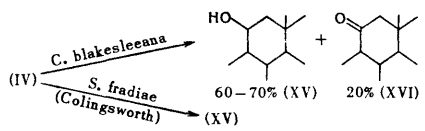


Hydroxylation of steroids by *Rh.nigricans* is an excellent example of the combination of specificity and diversity of action of microorganisms. Incubation with *Rh.nigricans* induces primarily hydroxylation in position 11 of steroid substrates such as progesterone (II), Δ[6]-dehydroprogesterone (XII), 16α,17α-epoxyprogesterone (XIII), etc. But 11α-hydroxylase is not the only enzyme formed by *Rh.nigricans*. Prolog and Acklin[40] showed that *Rh.nigricans* can reduce substrates such as Δ[4]-methyloctaline-3,8-dione (XIV):



It has been reported[41] that during the incubation of 4-methyltestosterone the methyl group in position 4 hinders the introduction of the hydroxyl group at carbon 6, with the result that 7β- and 11α-hydroxy-derivatives are chiefly formed, with very small amounts of 7α- and 6β-hydroxy-derivatives. Simultaneous formation of 11α- and 15β-hydroxy-derivatives has been described by Muir and Dodson[42]. Meister and Weintraub[43] obtained both 11α-hydroxytestosterone and testosterone with *Sporotrichum epigaem*. 11α-Hydroxylation has been effected by incubating steroids with *Condiobolus sp.*, *Basidiobolus ranarum*[44], and *Metarrhizium anisopliae*[45].

Even more important is the introduction of the 11β-hydroxy-group into the steroid molecule, since it is known to be responsible for the physiological activity of corticosteroids (hydrocortisone, corticosterone). Colingsworth and his collaborators[46] were the first to carry out this hydroxylation, although with very small yield:

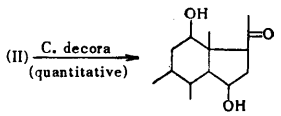


Shull and Kita[47] obtained 41%, and Dulaney[48] 50% of hydrocortisone (XV) by incubating Reichstein's substance S (IV) with *Curvularia lunata*. Connel[49] increased the yield of hydrocortisone to 60–70% by incubating compound S with *Cunninghamella blakesleeana*, obtaining at the same time 20% of cortisone (XVI).

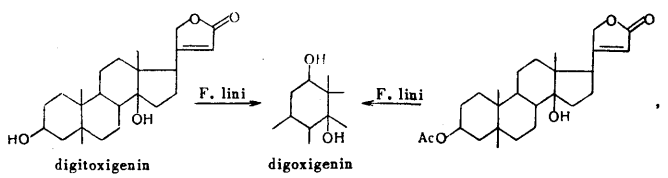
It has been shown that the yield of hydrocortisone can be increased by changing the culture medium, *e.g.* by adding trioleates[50], phenol[49], and other substances.

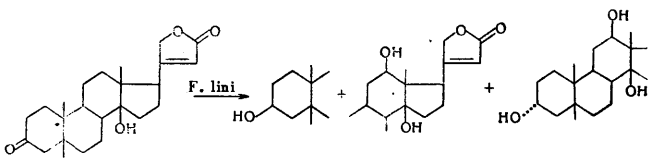
A number of communications appeared on the 11β-hydroxylation by means of other microorganisms. Davisson[51] patented the preparation of 70% of hydrocortisone (XVI) from Reichstein's substance S (IV) by means of *Stachilidium theobromae*. Thoma and his collaborators[52] obtained prednisolone (XVII) from 17α,21-dihydroxypregna-1,4-diene-3,20-dione using *Coniothyrium helleborine*[52], and Sardinas and Shull[53] carried out 11β-hydroxylation with *Spondiocladium australe*, *S. xylogenum*, as well as *Epicoccum*. The 11β-hydroxylation is often accompanied by hydroxylation in position 14α,[47] and the reduction of the 20-oxo-group[54]. Schmidt-Thome[55] obtained hydrocortisone (XV) and epihydrocortisone (XVIII) in equal amounts by incubating compound S with *Absidia glauca*.

Schubert and his collaborators[56] carried out 12β-hydroxylation of progesterone (II) using *Calonectria decora*:

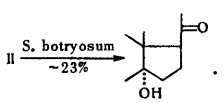


In their investigation of the effect of microorganisms on cardiac glycosides and aglycones Gibler and Tamm[57,58] established that *Fusarium lini* contains at least four enzymic systems, namely hydroxylase, capable of hydroxylating the 12β- and 15α-positions, esterase, capable of saponifying acetyl groups, hydrogenase, and β-glycosidase:

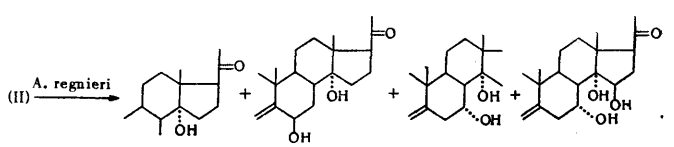




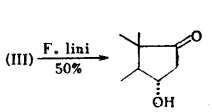
14α-Hydroxylation can be induced by microorganisms such as *Stemphilium botryosum*, *Curvularia lunata*, and *C.blakesleeana*[59]:



The preparation of 14α-hydroxyprogesterone[60] by means of *Bacillus cereus* is one of the comparatively few examples of hydroxylations carried out by bacteria. On incubating progesterone (II) with *Absidia regnieri*, Shirasaka[61] obtained a mixture of reaction products:

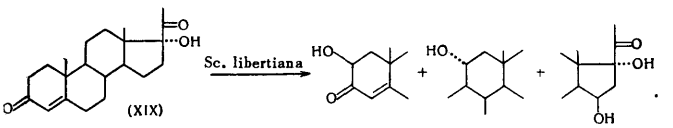


15α-Hydroxylation can also be effected by various microorganisms, most important being *Fusarium*[62,63] and *Penicillium*[64]:



There are comparatively few references to side reactions during the 15α-hydroxylation. On incubating substance S (IV) with a *Spicaria* species, Bernstein and his collaborators[65] obtained the corresponding 15β-hydroxy--derivative. Incubation of substance S (IV) with *Bacillus megatherium* gave 50% of 15β,17α,21-trihydroxypregn-4--ene-3,20-dione[66]. Incubation of progesterone gave a smaller yield of 15β-hydroxyprogesterone. An attempt to introduce the 15β-hydroxy-group into prednisone and prednisolone using this method was unsuccessful, hydrogenation of the double bond in the 1-position taking place instead[66].

A group of Japanese chemists[67] isolated 15β-hydroxy--derivatives from a complex mixture of products obtained on incubation of 17α-hydroxyprogesterone (XIX) with Sclerotinia libertiana:

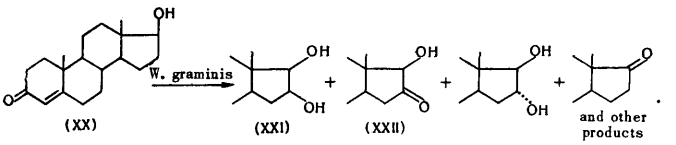


The important 16α-hydroxylation was effected using various species of *Actinomycete*. Perlman and his collaborators[68] obtained 30–40% of 16α-hydroxyprogesterone by incubating progesterone with the *Streptomycete* species MD2428. Incubation of 9α-fluorohydrocortisone with *Streptomyces roseochromogenus* gave 50% of 9α-fluoro--16α-hydroxyhydrocortisone[69].

11β,16α-Dihydroxy-9α-fluoroandrost-4-ene-3,17-dione was produced by *St.roseochromogenus*[70]. 16α-Hydroxylations induced by moulds *Pestalotia funerea*[71] and *Didymella vodakii*[25] have also been reported.

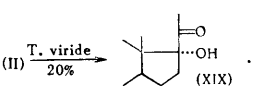
Despite the intensive search for microbial cultures capable of inducing steroid reactions, the microbiological introduction of the 16β-hydroxy-group was not discovered till 1960.[72]

An elaborate chromatographic technique applied to products obtained by incubation of testosterone (XX) with a *Wojnowicia graminis* (CBS) culture revealed ten products, including 16β-hydroxytestosterone (XXI) and 16-oxotestosterone (XXII):

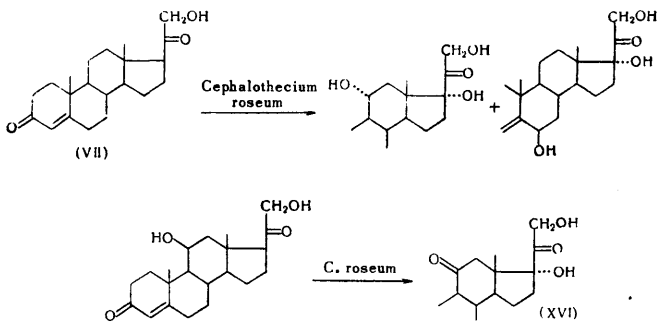


It had been reported earlier[73,74] that *W.graminis* can introduce a hydroxyl group in the 21-position of pregnene steroids.

The important 17α-hydroxylation has so far only been achieved using three microorganisms. *Trichothecium roseum*,[75], *Trichoderma viride*[76], and *Gliocladium*[77].

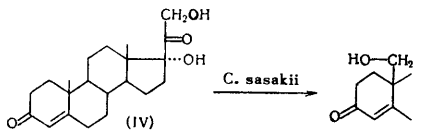


In other cases 17α-hydroxylation proceeds with concomitant 11α- and 6β-hydroxylations[33,76], and the oxidation of the hydroxyl group in position 11.[76]



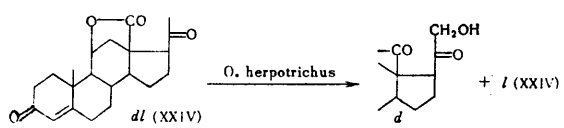
The Ciba group patented the preparation of 18-hydroxy--steroids by the action of fungi on 13-methylsteroids[78].

Hydroxylation in position 19 has been reported by Japanese workers[79]. They prepared 17α,19,21-trihydroxypregn--4-ene-3,20-dione (XXIII) by incubating compound (IV) with *Cotricium sasakii*:



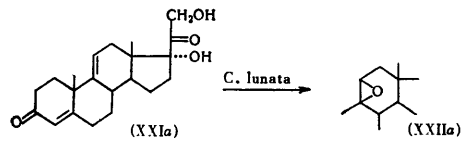
The same group carried out the 19-hydroxylation of hydrocortisone and 11-epihydrocortisone.

Hydroxylation in position 21, first effected by Wettstein's group[75], often proceeds exclusively. It can be induced by microorganisms such as *Aspergillus niger*[80], and the *Sphaeroidaceae*, *Hendersonia*[77], and *Wojnowicia*[81]. Vischer, Schmidlin, and Wettstein[82] used microbiological hydroxylation to separate the racemate of *dl*-11β-hydroxypregn-4-ene--3,20-dione-18-carboxylic acid (18→11)lactone (XXIV). Incubation of racemate (XXIV) with an *Ophiobolus herpotrichus* resulted in the 21-hydroxylation of the steroid with the natural configuration, while the *l*-form remained unchanged:

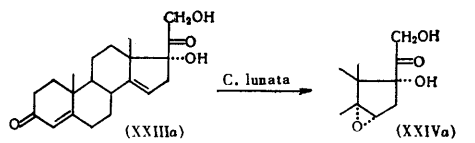


2. Formation and Opening of an Epoxide Ring

Closely related to microbiological hydroxylation is the microbiological formation of epoxide rings. The method was elaborated by Bloom and Shull[83] who, on incubation of 17α,21-dihydroxypregna-4,9(11)-diene-3,20-dione (XXI*a*) with *Curvularia lunata*, known to induce 11β-hydroxylation, or with *C. blakesleeana*, obtained 17α,21-dihydroxy-9β,11β--epoxypregn-4-ene-3,20-dione (XXII*a*):



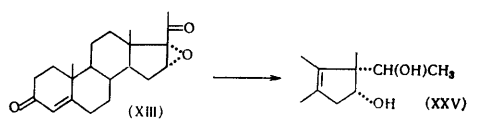
The same species was used for transforming 17α,21--dihydroxypregna-4,14(15)-diene-3,20-dione (XXIII*a*) into 17α,21-dihydroxy-14α,15α-epoxypregn-4-ene-3,20-dione (XXIV*a*):



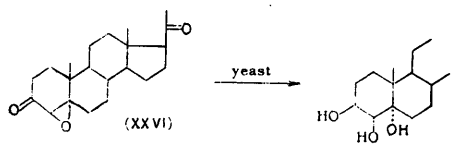
No epoxide was formed when the same substrate was incubated either with microorganisms which normally induce 11α-hydroxylation or those which are incapable of hydroxylating position 14. Perlman *et al.*[84] failed to obtain an epoxide by incubating pregna-4,16-diene-3,20-dione with an *Actinomycete* (which normally introduces a hydroxyl at 16α). As noted earlier, incubation of androst-5-en-3β--ol-17-one with *Rhizopus*[24] and of Δ^{16}-dehydroprogesterone with *Rh. nigricans*[35] also failed to produce epoxides. Perlman considers that microbiological epoxide formation can take place in steroids containing isolated double bonds, by means of microorganisms which are capable of introducing axial hydroxyl groups in positions corresponding to those taken up by the epoxide.

Quite recently Czech workers[85] found that the incubation of 3β-hydroxy-B-norandrost-5-en-17-one with *Rh. nigricans* yields 3β-hydroxy-5ξ,6ξ-epoxy-B-norandrostan-17--one and 3β,11α-dihydroxy-5ξ,6ξ-epoxy-B-norandrostan--17-one, together with 11α- and 6β-hydroxy-derivatives.

Epoxide rings can also be opened by microbiological reactions. Using yeast, Camerino and Modelli[86,87] opened the epoxide ring in 16α,17α-epoxyprogesterone (XIII). At the same time, rearrangement into 16α,20α-dihydroxy--17β-methyl-18-norpregna-4,13-dien-3-one (XXV) occurred:



In 1959 Camerino[86] succeeded in opening the epoxide ring in 4,5-epoxyprogesterone (XXVI), in 65% yield. Reduction of the 3-oxo-group took place simultaneously:

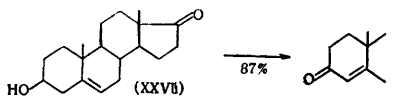


3. Introduction of an Oxo-Group

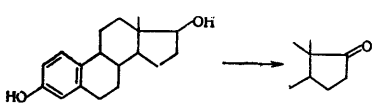
Enzymic transformations of steroids sometimes include the direct introduction of an oxo-group. For instance, hydroxylation of Reichstein's substance S with *C.blakesleeana*[49] also yields 20% of cortisone. If hydrocortisone is used as substrate, there is no formation of cortisone[89]. As already mentioned[24], an oxoderivative was also isolated after incubation of 3β-hydroxyandrost-4-en-17-one with *Rhizopus*. All this can be taken as evidence that in certain cases the formation of an oxo-group does not proceed *via* oxidation of a hydroxyl group, but by direct oxidation of a methylene group. Direct introduction is, however, not the only way of the microbiological formation of an oxo-group. Eroshin[90] showed that incubation of hydrocortisone (XV) with a *Cunninghamella elegans* culture yields cortisone (XVI), while under the same conditions Reichstein's substance S (IV) gives a mixture of hydrocortisone, epihydrocortisone, and cortisone. On incubating prednisolone (XVII) and epihydrocortisone with *C.elegans*, Eroshin found no such transformation products.

4. Oxidation of a Hydroxyl Group

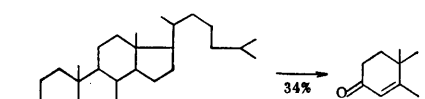
In 1937 Mamoli and Vercellone[1] published a paper on the oxidation (in Milan) of the 3β-hydroxy-group in dehydroepiandrosterone (XXVII) with yeast. When, however, Mamoli attempted to repeat this experiment in Berlin, using the local yeast, no oxidation could be detected. He established that the dehydrogenation of the hydroxyl group is carried out by aerobic bacteria, which were contaminating the Milan yeast[3].



Hanč and his collaborators[91] carried out a selective dehydrogenation of the 17β-epimer of oestradiol by means of *Actinomycete* species:

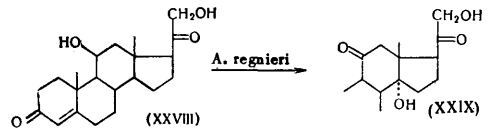


Turfitt[92,93] described the microbiological dehydrogenation of various steroids, including the oxidation of cholesterol (XXXV) in 34% yield,



and of testosterone, to yield 47% androstenedione, by means of *Proactinomyces erythropolis*.

Microbiological oxidation of the 3β-hydroxy-group is concomitant with the shift of the double bond from ring B into ring A. Incubation of corticosterone (XXVIII) with *Absidia regnieri*[94] gave 14α,21-dihydroxypregn-4-ene-3,11,20-trione (XXIX):

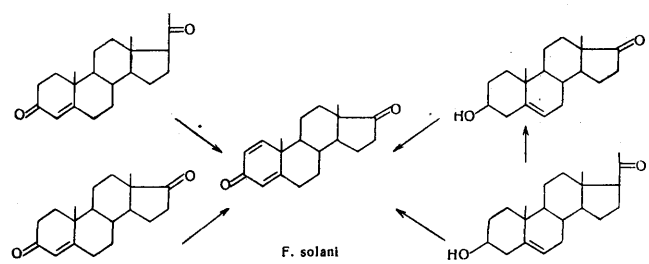


No transformation products were detected on incubation of epicorticosterone and hydrocortisone.

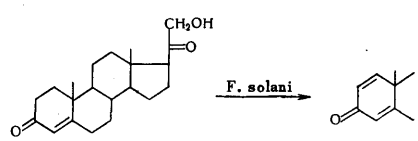
Oxidation of hydroxyl groups in positions 7 and 12 has also been described[95].

5. Dehydrogenation of the Steroid Skeleton

The discovery by Vischer and Wettstein[96] of microbiological dehydrogenation by the *Fusarium* fungi was followed by a number of papers and patents dealing with the microbiological dehydrogenation of steroids in the 1,2-position. On incubation of the pregnane steroids, a simultaneous oxidative degradation of the side-chain occurred:



Vischer *et al.*[97] carried out the dehydrogenation of pregn-4-enes with *F.solani* and *Calonectria decora*, without the concomitant degradation of the side-chain:

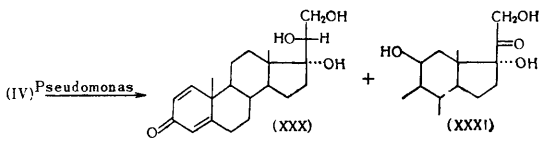


According to a Japanese patent[98], incubation of progesterone (II) with *F.solani* yields 30–50% of Δ^1-dehydroprogesterone and 30–40% of androsta-1,4-diene-3,17-dione. Degradation of the side-chain does not take place on incubation with *F.solani* if the substrates contain a dihydroxyacetone radical in position 17.[99]

Skryabin and his group[100,101] incubated cortisone (XVI) and hydrocortisone (XV) with a species of *Mycobacterium* and obtained 75–80% of prednisone and prednisolone, respectively.

Of special interest was the preparation of prednisolone from Reichstein's substance S (IV) by a group of Japanese workers. They first described the incubation of (IV) with a species *Pseudomonas* bacteria, which yielded a mixture

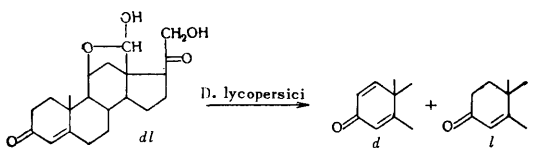
of 17α,20β,21-trihydroxypregna-1,4-dien-3-one (XXX) and 11β,17α,21-trihydroxypregn-4-ene-3,20-dione (XXXI), the latter in very small yield only[54].



They also obtained 30% of prednisolone (XVII) from Reichstein's substance S using a *Pseudomonas* mutant obtained by ultra-violet irradiation[102]. This example shows that the formation of enzymic systems in microorganisms has a certain flexibility, and that the methods of preparing mutant strains has a great future and is worthy of a systematic study.

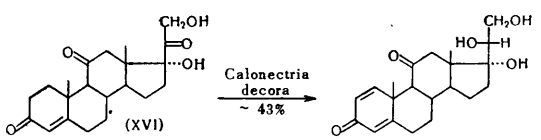
It has already been indicated that unfortunately in many cases microorganisms form not one, but several enzymic systems, giving rise to different reaction products, or attacking simultaneously several points in the steroid molecule. In the last example, however, the combination of Δ^1-dehydrogenase and 11β-hydroxylase was most advantageous, allowing a one-stage conversion of substance S to prednisolone.

According to the paper of Vischer *et al.* mentioned above[82], incubation of the racemate of aldosterone with *Didymella lycopersici* results in the dehydrogenation of the compound of natural configration only:

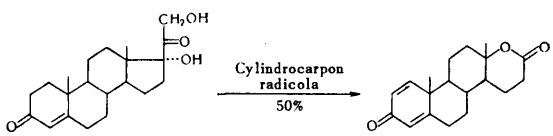


Charney and his group[103] prepared 95% of prednisone by incubating cortisone with *Corynebacterium simplex*.

The 1,2-dehydrogenation is often accompanied by the reduction of a 20-oxo-group to a 20β-hydroxy-group[104]:

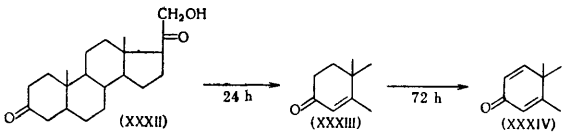


It is interesting that oxidative degradation of the D ring, yielding a lactone, often proceeds simultaneously with dehydrogenation[105]:



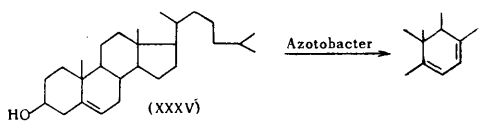
Incubation of 2α,17β-dihydroxyandrost-4-en-3-one[106] with *Bacillus sphericus* (ATCC 7055) gave 2α-hydroxyandrosta-1,4-diene-3,17-dione. To make certain that the last compound is not an artefact, the same substrate was incubated with a denatured culture of *B.sphericus*; it was fully recovered after incubation.

There are cases of microbial steroid dehydrogenation occurring at other positions in the molecule. On incubating 21-hydroxypregnane-3,20-dione (XXXII) with *Protaminobacter alboflavum*, Shull[107] obtained 21-hydroxypregn-4-ene-3,20-dione (XXXIII), and on longer incubation, 21-hydroxypregna-1,4-diene-3,20-dione (XXXIV):



On incubating 3β-hydroxyandrost-5-en-17-one with *F.solani*, Vischer and Wettstein[96] obtained androsta-1,4-diene-3,17-dione.

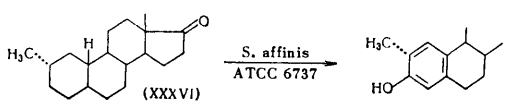
The introduction of a double bond in position 7 of cholesterol (XXXV) by incubation with a species of *Azotobacter*, carried out by Horvath and Kramli[108], was the first case of microbiological introduction of a double bond into a steroid molecule:



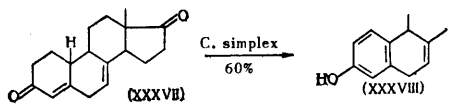
Peterson and his collaborators[109] showed that incubation of pregnane-3,11,20-trione and allopregnane-3,11,20-trione with *Septomyxa affinis* (ATCC 6737) gives, respectively, 5–10% and 40–45% of derivatives dehydrogenated in the 1,2-position. This is yet another clear example of the different behaviour of *cis*- and *trans*-isomers of condensed ring systems in microbiological reactions. The same work dealt with incubation of 17β-hydroxy-19-norandrost-4-en-3-one with *S.affinis*, which yielded 55% of oestrone and 12% of oestradiol. The absence in this case of an angular methyl group at position 10 resulted in the aromatisation of ring A.

On incubating Δ^4-3-oxo-19-norsteroids with *Corynebacterium simplex*, Kushinskii[110] obtained an aromatic A ring. A small yield of a compound containing a hydroxyl group in position 1 or 2 was also found among the reaction products. This may well indicate that dehydrogenation proceeds *via* hydroxylation and subsequent dehydration.

Peterson and his collaborators[111] found that aromatisation of 2α-methyl-19-norandrostan-17-one (XXXVI) with *Septomyxa affinis*, was accompanied by hydroxylation:



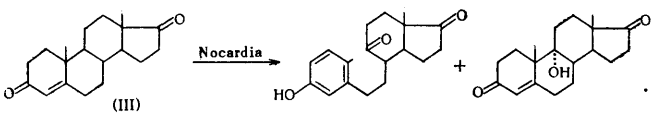
Djerassi and collaborators[112] incubated 19-nor androstan-4,7-diene-3,17-dione (XXXVII) with *C.simplex*, and obtained 60% of equilin (XXXVIII):



(XXXVII) (XXXVIII)

On incubating 19-norhydrocortisone with *Corynebacterium simplex* (ATCC 6946), Herzog and Nobile[113] obtained the corresponding compound with an aromatic A ring.

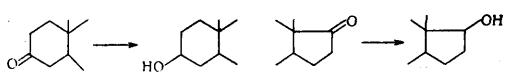
A condition for the microbiological aromatisation of ring A in steroids seems to be the absence of an angular methyl group at position 10. Nonetheless, aromatisation occurs simultaneously with the cleavage of the bond between carbons 9 and 10 when androstenedione (III) is incubated with *Nocardia*, apparently *via* a 9α-hydroxylation stage[114]:



(III)

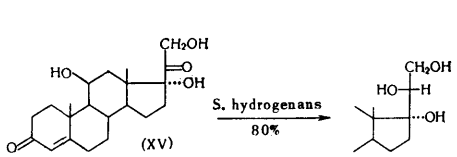
6. Reduction

Microbiological reduction of steroids is also of widespread occurrence, and in certain cases it has a great practical significance. As early as the thirties Mamoli and Vercellone[1,2,115,116] carried out the following transformations of the C_{19} steroids with yeast:



In their study of the microbiological transformations of androstenedione (III) with *Trichomonas gallinae* (*Protozoa*) Sebek and Michaels[117] established that incubation under aerobic conditions induces the oxidation of the hydroxyl group, while under anaerobic conditions it results in the reduction of the oxo-group at C_{17}. Incubation of 3β-hydroxyandrost-5-en-17-one acetate with *Penicillium notatum* gave 79% of testosterone[118].

Many microorganisms are capable of the stereospecific reduction of the 20-oxo-group in various steroid substrates. Schmidt-Thome[55] reduced selectively the 20-oxo-group of hydrocortisone (XV) in 80% yield with a *Streptomyces* species from the soil (*S.hydrogenans*):

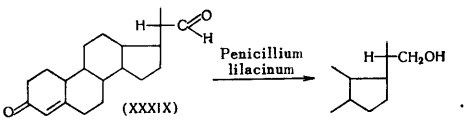


(XV)

Later[119] Schmidt-Thome established the specificity of the action of *S.hydrogenans* upon the dihydroxyacetone group, since the incubation of progesterone (II) and deoxycorticosterone (VII) resulted in no change. This specificity of action on the dihydroxyacetone group in the 17-position has not been found in any other microbial species. Peterson[21] reduced the 20-oxo-group in deoxycorticosterone by incubation with a *Streptomyces* species.

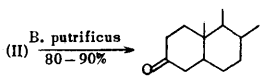
The capacity to reduce the 20-oxo-group to form the 20β-hydroxy-group is characteristic of many species of *Actinomycete*[120]. Many other microorganisms, for instance *Curvularia lunata*[181], *Corynebacterium simplex*[122], *Pseudomonas*[123], and *Mycobacterium*[124,125], are also capable of reducing the 20-oxo-group.

Peterson[126] reduced the aldehyde group in the 22-position of bisnorchol-4-ene-3,22-dione (XXXIX):



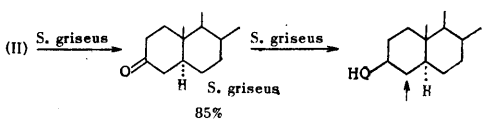
(XXXIX)

Microorganisms can also carry out the hydrogenation of double bonds. Hydrogenation of the Δ⁴-double bond in various steroids has been effected in high yields with *Bacillus putrificus*[127]:



(II)

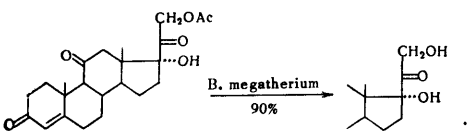
Weisz and his collaborators[34] noticed that incubation of deoxycorticosterone with *Ophiobolus herpotrichus* may induce the hydrogenation of the double bond. Using *Streptomyces griseus*, Vischer and Wettstein recently[128] carried out a stepwise reduction of a Δ⁴-3-oxo-steroid, first into an unsaturated ketone, and then into an alcohol.



(II)

7. Hydrolysis

The microbiological saponification of acetyl groups usually proceeds readily. It can be effected, for instance, by means of *Flavobacterium dehydrogenans*[129], or, as in Suvorov's laboratory, using *Bacillus megatherium*, which is specific in the deacetylation of 21-acetates[130]:



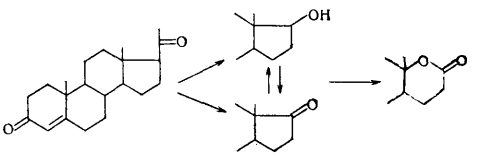
In many cases deacetylation proceeds alongside the main reaction characteristic of the given microorganism. Zetsche[131] showed that during incubation of the 21-acetate

derived from Reichstein's substance S with *Curvularia lunata* the rate of deacetylation exceeds the rate of hydroxylation of substance S, and that only the saponified diol is capable of undergoing hydroxylation. This is consistent with the results of Eroshin and Krasil'nikov[90] who incubated hydrocortisone 21-acetate with *Cunninghamella elegans*.

Certain microorganisms produce enzymes capable of hydrolysing steroid saponins to sapogenins and sugar[132].

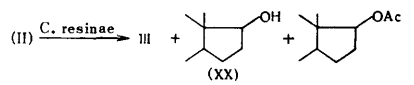
8. Cleavage of Carbon–Carbon Bonds

It has already been mentioned[105] that certain microbiological reactions, especially dehydrogenation, may be accompanied by the modification of the steroid D ring to a lactone. Microbiological formation of lactones can also proceed independently. Čapek and his collaborators[133] used microorganisms such as *Aspergillus orizae*, *A. chevalieri*, *Penicillium citrium*, etc. to carry out progesterone transformations according to the following scheme:



Microbiological degradation of the side chain in pregnane steroids, resulting in a 17-oxo- or 17β-hydroxy-group, is quite common. By incubating progesterone (II) with *Cylindrocephalum aureum* QM 610, Shull and Routien[134] obtained a mixture of androstenedione (III) and testosterone (XX). On incubating progesterone with *Neocosmospora vasinfecta*, they obtained 69% of testosterone[135]. Several other microorganisms are capable of inducing such degradation of the side chain[136].

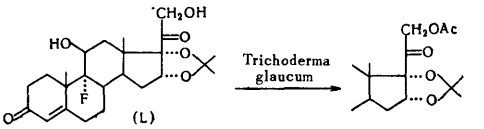
A paper appeared quite recently on the microbiological formation of steroid 17β-acetates. Incubation of progesterone with *Cladosporium resinae*[137] gave androstenedione, testosterone, and testosterone acetate:



Under the same conditions testosterone does not form testosterone acetate. Using [21-^{14}C]progesterone, it was shown that the formation of testosterone acetate is in fact an intermediate step in the degradation of the side chain.

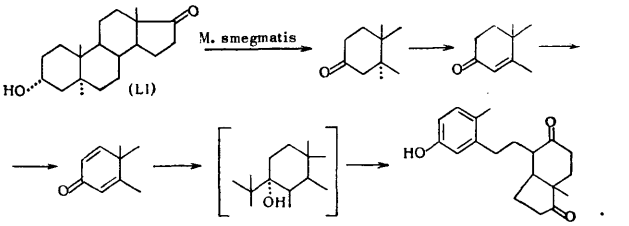
This path of the microbiological formation of acetates is obviously not the only one possible. Incubation of androstenedione (III) with *Saccharomyces fragilis* (ATCC 100222)[138] resulted in a mixture of testosterone and testosterone acetate. In this case it is difficult to guess the source of carbon used in the formation of the acetoxy-group.

The 21-acetates were isolated in high yields after incubation of 16α,17α-isopropylidenedioxysteroids with *Trichoderma glaucum* (Lederle No. Z-636)[139]. The yield of the corresponding 21-acetate obtained from 11β,21-dihydroxy-16α,17α-isopropylidenedioxy-9α-fluoropregn-4-ene-3,20-dione (L) was 70%:



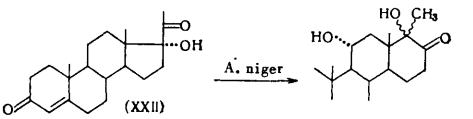
Incubation of steroids not containing the 16α,17α-isopropylidenedioxy-group with the same culture gave no acetylation products.

Among papers describing the cleavage of a carbon–carbon bond in the steroid skeleton that of Schubert and his collaborators[140] is worthy of note. On the basis of their kinetic data, they suggested the following scheme for the transformations of 3β-hydroxyallopregnan-17-one (LI) induced by *Mycobacterium smegmatis*:

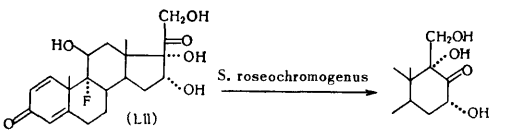


This mechanism for the opening of the B ring of the steroid skeleton has been confirmed by the work of Dodson and Muir[114,141,142].

The isolation[31] of a D-homosteroid after the incubation of 17α-hydroxyprogesterone (XXII) with *Aspergillus niger* is rather curious:



No less so is the formation of a D-homosteroid on incubation of triamcinolone (LII) with *Streptomyces roseochromogenus*:



Goodman and Smith[144,145] recently proved that D-homo-isomerisation in this case is caused by an excess of iron ions in the incubation medium, and not by the biological processes involved.

III. SOME ASPECTS OF THE MECHANISMS INVOLVED IN MICROBIOLOGICAL REACTIONS OF STEROIDS

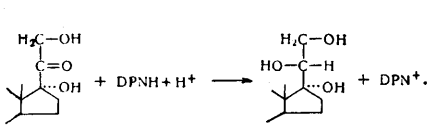
There is no doubt whatsoever that the microbiological transformations of steroids are caused by the enzymic systems produced by the microorganisms. Such systems contain several enzymes, each being responsible for one

definite step in the reaction. In certain cases the enzymes involved have been obtained in a reasonably pure state. The Talalay team is carrying on a systematic study of enzymes from the soil bacterium *Pseudomonas testosteroni*, which is capable of oxidising testosterone (XXVI) to androstenedione (III)[146]. They isolated 3β-, and 17β-dehydrogenases[147], and Δ^1- and Δ^4-5α-dehydrogenases[148] of high purity. In 1960 a paper appeared reporting the crystallisation of Δ^5-3-oxo- and Δ^4-3-oxo-isomerases[149].

The reason why microorganisms cause transformations in the steroids they come in contact with appears incomprehensible at first sight. In the overwhelming majority of cases microorganisms do not require steroids as food and they are perfectly capable of carrying out their metabolic functions in the absence of steroids. It is true that certain microorganisms contain and metabolise sterols[150], but their formation has no connection with the enzymes involved in steroid transformations. Vischer and Wettstein[151] showed that the ergosterol isolated from 11α-hydroxylating fungi of the *Rhizopus* species does not contain any 11-hydroxy--derivatives. Work done in recent years has thrown some light on this subject. Capek[152,153] established that the introduction of steroids, *e.g.* progesterone, into a culture of *Rh. nigricans* growing in a nutrient medium suspends the growth and respiration of the microorganism during the transformation of the progesterone into 11α-hydroxyprogesterone, while the introduction of 11α-hydroxyprogesterone has no such effect. Lester[154] showed that the growth of *Neocosmospora crassa* is inhibited by the addition of deoxycorticosterone into the culture medium. Maxwell and his collaborators[155] found that the addition of steroids (androstenedione, deoxycorticosterone, progesterone, and androstanedione), in a concentration of 0.13 mg ml^{-1}, inhibits the growth of *Saccharomyces fragilis*. Androstenedione is thereby transformed into testosterone acetate. It could be therefore that certain steroids act as poisons for a given microbial species, which subsequently adapt themselves by producing enzymes capable of catalysing the transformation of the steroids into a non-toxic form. There are also indications that the microorganism absorbs the steroid and discharges its transformation products into the culture medium[146,151].

The work of Talalay[147], Peterson[156], and some others has shown the adaptive character of the formation of enzymes capable of transforming steroids.

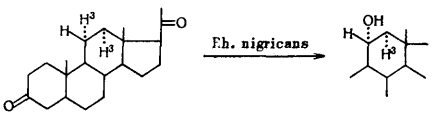
A great deal of interesting information which would help in explaining the enzymic mechanism of the biological steroid transformations could be expected from studies of the "fine" structure of enzymic reactions, the role of cofactors, and so on. Unfortunately, consideration of such work, *e.g.* Refs. 157 and 158, is outside the scope of this review. For detailed information about this subject, the reader is referred to the interesting review by Vischer and Wettstein[99]; only a few most recent communications will be considered here. Hübener and Lehmann[159] established that the reduction of 20-oxo-steroids by the action of an enzyme isolated from *Streptomyces hydrogenans* requires the presence of diphosphopyridine nucleotide and proceeds as follows:



A stoichiometeric amount of DPN is thereby oxidised.

It is remarkable that 2,4-dinitrophenol, which acts as an inhibitor of the most diverse processes in a living organism (processes associated with energy transfer), has no effect, even in large concentrations, on the hydroxylation of progesterone induced by *Curvularia lunata*[160].

In their study of the 7α-, 11α-, and 11β-hydroxylations of labelled steroids Peterson and his collaborators[161,162] showed that the Walden inversion did not take place in the process, and that the hydroxyl group assumed the position of the hydrogen atom, thus leaving the configuration unchanged:

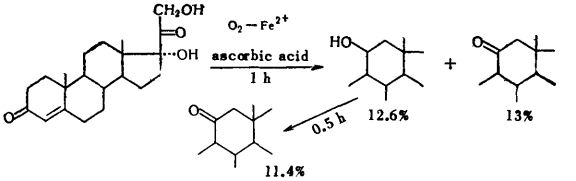


Schmidt-Thome and his collaborators[163] found that the addition of small amounts of a *Rh. nigricans* culture to the culture medium during the dehydrogenation of corticosteroids with *Bacillus subtilis* increases the yield of Δ^1-dehydro--derivatives, although *Rh. nigricans* itself is incapable of inducing dehydrogenation. It was also found that the addition of small amounts of certain steroids favours dehydrogenation. Murray[164] found that the addition of 0.1 mg of progesterone per 20 mg of cortisone undergoing dehydrogenation with *Septomuxa affinis* had a favourable effect on the reaction. The additives may possibly play a role of cofactors catalysing the hydrogen transfer in the substrate–enzyme system.

Zetsche[165] made a thorough investigation of steroid hydroxylations with 15 strains of *Curvularia*, and showed by experiments with filtered mycelial mass that the native liquid of *Curvularia* does not contain steroid hydroxylases. From a study of the effect of the addition of NaCN, NaN_3, 2,4-dinitrophenol, and NaF, he concluded that hydroxylation with *Curvularia* requires enzymes containing heavy metals, capable of catalysing the transfer of molecular oxygen to the steroid molecule. Hydroxylation requires Fe^{2+} ions, while Cu^{2+} ions are hardly needed at all.

From the difference in the effect on hydroxylation of various vitamins such as nicotinamide, pantothenyl alcohol, and vitamins B_1, B_2, and B_6, Zetsche concludes that hydroxylation is catalysed by flavin enzymes. According to him, the strongly lipophilic steroids block the active cell centres of the microorganism, and as hydroxylation lowers the lipophilic nature of the steroid, it consequently serves as a protective function of the microorganism. Hydroxylases from *Curvularia* are not produced by adaptation to steroids, and Zetsche accounts for their presence by the fact that under natural conditions the microorganism has to protect itself from similar, although non-steroid, lipophilic material.

A brief mention may be made of the interesting oxidation in systems modelling the multienzyme systems of microorganisms. A system consisting of ascorbic acid, iron sulphate, and molecular oxygen in pH 7.2 phosphate buffer, at 37°, acts upon Reichstein's substance S by inducing stereospecific hydroxylation, similar to the biological one, followed by the oxidation of the hydroxyl to an oxo-group[166]:



In concluding this section, it must be emphasised that, although microorganisms are capable of effecting a wide variety of transformations of various substrates, in certain cases they also display high specificity. Incubation of Reichstein's substance S with *Bacillus megatherium* yields 50% of the 15β-hydroxy-derivative, although such derivatives could not be obtained from prednisone and prednisolone[66]. *Absidia regnieri* cultures oxidise the 11β-hydroxy--group and introduce a 14α-hydroxy-group into corticosterone, but are without effect on epicorticosterone and hydrocortisone[95]. *Synceyphalostrum racemosum* induces hydroxylation of progesterone at the 7α-, 14α-, and 15β--positions, but has no effect on hydrocortisone and 6β-, 11α-dihydroxyprogesterone even on prolonged incubation[20]. These are only a few from a long list of such examples.

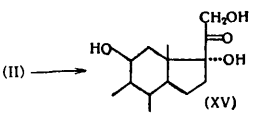
IV. APPLICATION OF MICROBIOLOGICAL REACTIONS

It is evident from this brief survey of the microbiological steroid transformations that there are prospects of many such reactions being used in the steroid hormone industry. Many are already being used as intermediate stages in the production of hormones. In the Soviet Union 11α-hydroxyprogesterone is obtained on a semiindustrial scale by incubation of progesterone with *Rh. nigricans*[167]. Microbiological 11β-hydroxilation is of great industrial importance, since introduction of an oxygen-containing functional group in the 11-position by chemical means is extremely complicated. Hydroxylation of steroids in positions 16α and 21, and dehydrogenation in the 1,2-position, makes it possible to obtain highly active hormone preparations. "Chemical" dehydrogenation by means of SeO_2 yields hormones contaminated with selenium, which rules out their clinical application. Microbial dehydrogenation competes with the chemical reaction not only in this respect, but also as regards yield.

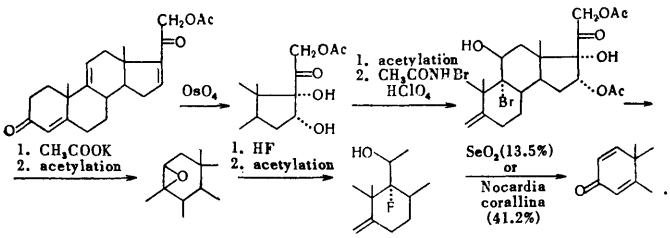
Industrial application of microbiological reactions is partly limited by the need to use large amounts of solvents to separate steroids from the fermentation broth, but this could be overcome by the development of a technological process for their recovery.

Of great interest to industry are reactions with mixed microbial cultures, which are able to induce hydroxylations at different positions of the molecule in one stage.

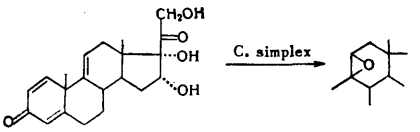
On incubating progesterone with three microbial species simultaneously, *i.e. Wojnowicia graminis*, *Trichoderma viridae*, and *Curvularia lunata*, McAleer[168] obtained hydrocortisone:



The significance of microbiological reactions is not limited to their industrial application. In the last few years they have often been used by chemists in synthetic projects in cases when they increased the yield of a particular stage, or helped to reduce the number of stages. In their synthesis of 16α-hydroxy-9α-fluoro-steroids Bernstein and his collaborators[169] chose microbiological dehydrogenation in preference to dehydrogenation by SeO_2, thus increasing the yield of that stage from 13.5 to 41.2%:



On incubating $\Delta^{9(11)}$-steroids with *Corynebacterium simplex*, they obtained the 9β,11β-epoxide:



This is only one of many examples of this kind.

Microbiological reactions are very important in establishing the structure of new natural and synthetic steroids. For instance, Fried[170] demonstrated the structure of 16α--hydroxyprogesterone by its conversion to 16α-hydroxytestosterone, after the microbiological degradation of the side chain. To prove the structure of 14α-hydroxyprogesterone, it was converted by a microbial reaction to 14α-hydroxyandrost-4-en-3,17-dione. Neher and Wettstein[171] employed microbial reactions for establishing the structure of compounds isolated from adrenal steroids, *e.g.* 6β-hydroxycorticosterone, 19-hydroxycorticosterone, etc. Talalay and Hurlock used purified steroid dehydrogenases for analytical assay of steroids in urea[172].

1. L.Mamoli and A.Vercellone, Ber., 70, 470 (1937).
2. L.Mamoli and A.Vercellone, Z.phys.Chem.(Leipzig), 245, 93 (1937).
3. L.Mamoli and A.Vercellone, Ber., 71, 1686 (1938).
4. A.Kramli and J.Horvath, Nature, 162, 619 (1948).
5. D.H.Peterson and H.C.Murray, J.Amer.Chem.Soc., 74, 1871 (1952).
6. E.A.Mistryukov, Coll.Czech.Chem.Comm., 26, 2071 (1961).
7. V.Černy, J.Joska, and L.Labler, Coll.Czech.Chem.Comm., 26, 1658 (1961).
8. G.Wix and K.Albrecht, Nature, 183, 1279 (1959).
9. R.M.Dodson, A.H.Goldkamp, and R.D.Muir, J.Amer.Chem. Soc., 82, 4026 (1960).
10. G.Greenspan, C.P.Schaffner, W.Charney, H.L.Herzog, and E.B.Hershberg, J.Amer.Chem.Soc., 79, 3922 (1957).
11. W.J.McAleer, M.A.Kozlowski, T.H.Stoudt, and J.M.Chemerda, J.Org.Chem., 23, 508 (1958).
12. M.Shirasaka and M.Tsuruta, Arch.Biochem.Biophys., 87, 338 (1960).
13. K.Tanabe, R.Hayashi, R.Takasaki, and M.Shirasaka, Chem. and Pharm.Bull.(Japan), 7, 811 (1959).
14. J.Fried, R.W.Thoma, D.Perlman, J.E.Herz, and A.Borman, Recent Progr.Hormone Res., XI, 149 (1955).
15. S.Shinichi, T.Masako, S.Makoto, and N.Michikazu, Arch. Biochem.Biophys., 80, 383 (1959).

16. D.H.Peterson, S.H.Eppstein, P.D.Meister, H.C.Murrey, H.M.Leigh, A.Weintraub, and L.M.Reineke, J.Amer.Chem. Soc., 75, 5768 (1953).
17. S.Bernstein, W.S.Allen, M.Heller, R.H.Lenhard, L.S. Feldman, and R.H.Blank, J.Org.Chem., 24, 286 (1959).
18. W.J.McAleer, M.A.Kozlowski, T.H.Stoudt, and J.M.Chemerda, J.Org.Chem., 23, 985 (1958).
19. A.Kramli and J.Horvath, Nature, 163, 219 (1949).
20. T.Asai, T.Tanaki, E.Ohki, T.Matsuhisa, Y.Takeda, and T.Juni, J.Agric.Chem.Soc.Japan, 33, 985 (1959).
21. S.H.Eppstein, P.D.Meistre, H.C.Murray, and D.H. Peterson, Vitamins and Hormones, 14, 359 (1956).
22. F.W.Kahnt and P.D.Meystre, Experientia, 8, 422 (1952).
23. H.C.Murray and D.H.Peterson, U.S.P. 2602769 (1952).
24. R.M.Dodson, R.T.Nicholson, and P.D.Muir, J.Amer.Chem. Soc., 81, 6295 (1959).
25. S.H.Eppstein, Experientia, 11, 465 (1955).
26. D.Stone, M.Hayano, R.J.Dorfman, O.Hechter, C.R.Robinson, and C.Djerassi, J.Amer.Chem.Soc., 77, 3926 (1955).
27. A.Schubert, D.Onken, R.Siebert, and K.Heller, Chem.Ber., 91, 2549 (1958).
28. H.C.Murray and D.H.Peterson, U.S.P. 2888384 (26th May, 1959); Chem.Abs., 53, 20143a (1959).
29. A.Bowers, C.Casas-Campilo, and C.Djerassi, Tetrahedron, 2, 165 (1958).
30. D.H.Peterson, H.C.Murray, S.H.Eppstein, L.M.Reineke, A.Weintraub, P.D.Meister, and H.M.Leigh, J.Amer.Chem. Soc., 74, 5933 (1952).
31. J.Fried, R.W.Thoma, J.R.Gerke, J.E.Herz, M.N.Donin, and D.Perlman, J.Amer.Chem.Soc., 74, 3962 (1952).
32. E.L.Dulaney, W.J.McAleer, H.R.Barkemeyer, and C.Hlavak, Appl.Microbiology, 3, 372 (1955).
33. P.D.Meister, L.M.Reineke, R.C.Meeks, H.C.Murray, S.Eppstein, H.M.Leigh, A.Weintraub, and D.H.Peterson, J.Amer.Chem.Soc., 76, 4050 (1954).
34. E.Weisz, G.Wix, and M.Bodansky, Naturwiss., 43, 39 (1956).
35. P.D.Meister, D.H.Peterson, H.C.Murray, S.H.Eppstein, L.M.Reineke, A.Weintraub, and H.M.Leigh, J.Amer. Chem.Soc., 75, 55 (1953).
36. D.H.Peterson, A.H.Nathan, P.D.Meister, S.H.Eppstein, H.C.Murray, A.Weintraub, L.M.Reineke, and H.M.Leigh, J.Amer.Chem.Soc., 75, 419 (1953).
37. S.H.Eppstein, D.H.Peterson, H.M.Leigh, H.C.Murray, A.Weintraub, L.M.Reineke, and P.D.Meister, J.Amer. Chem.Soc., 75, 421 (1953).
38. A.Ercoli, P.de Ruggieri, and D.Della Morte, Gazzetta, 85, 628 (1955).
39. D.H.Peterson, P.D.Meister, A.Weintraub, L.M.Reineke, S.H.Eppstein, H.C.Murray, and H.M.Leigh, J.Amer. Chem.Soc., 77, 4428 (1955).
40. V.Prelog and W.Acklin, Helv.Chim.Acta, 39, 748 (1956).
41. D.N.Kirk, V.Petrow, and M.H.Williamson, J.Chem.Soc., 3872 (1960).
42. R.D.Muir and R.M.Dodson, U.S.P. 2823170 (11th Feb., 1958).
43. P.D.Meister and A.Weintraub, U.S.P. 2877162 (10th Mar., 1959).
44. A.Weintraub and P.D.Meister, U.S.P. 2879206 (24th Mar., 1959).
45. Kurosawa Juichiro, J.Agric.Chem.Soc.Japan, 32, 515 (1958).
46. D.R.Colingsworth, M.P.Brunner, and W.J.Haines, J.Amer. Chem.Soc., 74, 2381 (1952).
47. G.M.Shull and D.A.Kita, J.Amer.Chem.Soc., 77, 763 (1955).
48. E.L.Dulaney and E.O.Stapley, Appl.Microbiology, 7, 276 (1959).
49. K.Mann, F.Hanson, P.Connel, H.V.Anderson, M.P.Brunner, and J.N.Karnemaat, Appl.Microbiology, 3, 14 (1955).
50. P.W.O'Connel, K.M.Mann, and E.D.Nielson, Appl.Microbiology, 3, 16 (1955).
51. J.W.Davisson, U.S.P. 2830937 (1958).
52. R.W.Thoma, J.R.Gerke, and J.Fried, U.S.P. 2793163 (21st May, 1957).
53. I.L.Sardinas and G.M.Shull, U.S.P. 2876160 (1959).
54. H.Nawa, M.Uchibayashi, R.Takeda, J.Nakanishi, T.Kusaska, J.Terumichi, M.Uchida, M.Katsumata, K.Yoshino, and H.Fujitani, Tetrahedron, 4, 201 (1958).
55. J.Schmidt-Thome, Angew.Chem., 69, 238 (1959).
56. A.Schubert, G.Langbein, and R.Siebert, Chem.Ber., 90, 2576 (1957).
57. A.Gibler and Ch.Tamm, Helv.Chim.Acta, 41, 297 (1958).
58. A.Gibler and Ch.Tamm, Chimia (Switz.), 13, 116 (1959).
59. N.Masamoto and H.Hikoichi, Pharm.Soc.Japan, 78, 1256 (1958).
60. J.Fried, R.W.Thoma, D.Perlman, J.E.Herz, and A.Borman, Recent.Progr.Hormone Res., 11, 149 (1955).
61. M.Shirasaka, R.Hayashi, and M.Tsuruta, Bull.Agric.Chem, Soc.Japan, 23, 244 (1959).
62. A.Gibler and Ch.Tamm, Helv.Chim.Acta, 41, 301 (1958).
63. H.C.Murray and P.D.Meister, U.S.P. 2889255 (2nd June, 1959).
64. J.Fried, R.W.Thoma, D.Perlman, and J.R.Gerke, U.S.P. 2879280 (24th Mar., 1959).
65. S.Bernstein, L.I.Feldman, W.S.Allen, R.H.Blank, and C.E.Linden, Chem.and Ind., 111 (1956).
66. H.L.Herzog, M.J.Gentles, W.Charney, D.Sutter, E.Townleu, M.Yudinz, P.Kabasakalian, and E.B.Hershberg, J.Org.Chem., 24, 691 (1959).
67. K.Tanabe, R.Takasaki, T.Hayashi, and M.Shirasaka, Chem.and Pharm.Bull.(Japan), 7, 804 (1959).
68. D.Perlman, E.O'Brien, A.R.Bayem, and R.B.Greenfield, J.Bact., 69, 347 (1955).
69. R.W.Thoma, J.Fried, S.Bonanno, and P.Grabowich, J. Amer.Chem.Soc., 79, 4818 (1957).
70. S.Bernstein, R.H.Lenhard, N.E.Rigler, and M.A.Darken, J.Org.Chem., 25, 297 (1960).
71. J.Fried, D.Perlman, A.F.Langlykke, and E.O.Titus, U.S.P. 2872381 (3rd Feb., 1959).
72. H.L.Herzog, M.J.Gentles, A.B.Coscarelli, A.Basch, M.E.A.Zeitz, and W.Charney, J.Org.Chem., 25, 2177 (1960).
73. E.L.Dulaney and W.J.McAleer, U.S.P. 2819200 (7th Jan., 1958).
74. W.McAleer and E.L.Dulaney, U.S.P. 2875132 (24th Feb., 1959).
75. Ch.Meystre, E.Vischer, and A.Wettstein, Helv.Chim.Acta, 37, 1548 (1954).
76. E.L.Dulaney and W.J.McAleer, U.S.P. 2863806 (27th Dec., 1955).
77. W.J.McAleer and E.L.Dulaney, U.S.P. 2831798 (22nd April, 1958).
78. Swiss P. 330669, quoted in Chimia (Switz.), 13, 36 (1959).
79. M.Nishikawa and H.Hagiwara, Chem.and Pharm.Bull. (Japan), 6, 226 (1958).
80. A.Zaffaroni, C.Casas-Campillo, F.Cordobe, and G.Rosenkranz, Experientia, 11, 219 (1955).
81. W.McAleer and E.L.Dulaney, U.S.P. 2875132 (24th Feb., 1959); Chem.Abs., 53, 16223a (1959).
82. F.Vischer, J.Schmidlin, and A.Wettstein, Experientia, 12, 50 (1956).
83. B.M.Bloom and G.M.Shull, J.Amer.Chem.Soc., 77, 5767 (1955).
84. D.Perlman, E.Titus, and J.Fried, J.Amer.Chem.Soc., 74, 2126 (1952).
85. Z.Procházka, J.Faikoš, J.Joška, and F.Šorm, Coll.Czech. Chem.Comm., 26, 2068 (1961).

86. B.Camerino and R.Modelli, Gazzetta, 86, 1219 (1956).
87. B.Camerino and R.Modelli, Gazzetta, 86, 1226 (1956).
88. B.Camerino and R.Sciaky, Gazzetta, 89, 654 (1959).
89. Unpublished, quoted in Vitamins and Hormones, 14, 359 (1956).
90. V.K.Eroshin and N.A.Krasil'nikov, Dokl.Akad.Nauk SSSR, 137, 968 (1961).
91. O.Hanč, E.Jirat, A.Čapek, and M.Tadra, Chem.Listy, 51, 1950 (1957).
92. G.E.Turfitt, Biochem.J., 38, 492 (1944).
93. G.E.Turfitt, Biochem.J., 40, 79 (1946).
94. M.Shirasaka, R.Takasaki, and M.Suruta, Nature, 186, 390 (1960).
95. W.M.Hoehn, L.N.Schmidt, and H.B.Hughes, J.Biol.Chem., 152, 59 (1944).
96. E.Vischer and A.Wettstein, Experientia, 9, 371 (1953).
97. E.Vischer, C.Meystre, and A.Wettstein, Helv.Chim.Acta, 38, 835 (1955).
98. M.Nisikawa, T.Hasegawa, S.Noguti, and H.Hagiwara, Jap. P. 528 (9th Feb., 1959).
99. E.Vischer and A.Wettstein, Adv.Enzymol., 20, 263 (1958).
100. N.A.Krasil'nikov, G.K.Skryabin, I.V.Aseeva, and L.O. Korsunskaya, Dokl.Akad.Nauk SSSR, 128, 1063 (1959).
101. N.A.Krasil'nikov, G.K.Skryabin, I.V.Aseeva, and L.O. Korsunskaya, Dokl.Akad.Nauk SSSR, 128, 863 (1959).
102. R.Takeda, J.Nakanishi, J.Terumichi, N.Uchida, M.Katsumata, M.Uchibayashi, and H.Nawa, Tetrahedron Letters, 4, 17 (1959).
103. W.Charney, Lecture at the Institute of Microbiology of the Academy of Sciences of the USSR; quoted by A.G.Baichikov, A.S.Barmenkov, and V.K.Eroshin, Med.Prom.SSSR, No.6, 15 (1959).
104. S.A.Szpilfogel, P.A.Van Hemert, and M.S.de Winter, Rec. Trav.Chim., 75, 1227 (1956).
105. J.Fried, R.W.Thoma, and A.Klingsberg, J.Amer.Chem. Soc., 75, 5764 (1953).
106. C.Gual, S.R.Stitch, M.Gut, and R.J.Dorfman, J.Org. Chem., 24, 418 (1959).
107. G.M.Shull, U.S.P. 2876171 (3rd Mar., 1959).
108. J.Horvath and A.Kramli, Nature, 160, 639 (1947).
109. O.K.Sebek, L.M.Reineke, D.H.Peterson, R.C.Meeks, P.D.Meister, S.H.Eppstein, J.P.Rosselet, A.Weintraub, and H.C.Murray, Chem.and Ind., 391 (1958).
110. S.Kushinski, J.Biol.Chem., 230, 31 (1958).
111. D.H.Peterson, L.M.Reineke, H.C.Murray, and O.K. Sebek, Chem.and Ind., 1301 (1960).
112. J.A.Zderic, A.Bowers, H.Carpio, and C.Djerassi, J. Amer.Chem.Soc., 80, 2596 (1958).
113. H.L.Herzog and A.Nobile, U.S.P. 2928850.
114. R.M.Dodson and R.D.Muir, J.Amer.Chem.Soc., 80, 6148 (1958).
115. A.Vercellone and L.Mamoli, Z.phys.Chem., 248, 277 (1937).
116. L.Mamoli, U.S.P. 2186906 (9th Jan., 1940).
117. O.K.Sebek and R.M.Michaels, Nature, 179, 210 (1957).
118. O.Hanč, A.Čapek, and M.Tadra, Czech P. 86728 (15th July, 1957); Ref.Zhur.Khim., 43881 P (1960).
119. F.Lindner, R.Junk, G.Nesemann, and J.Schmidt-Thome, Z.phys.Chem.(Leipzig), 313, 117 (1958).
120. L.M.Kogan, M.S.Oranskaya, N.N.Suvorov, G.K.Skryabin, and I.V.Torgov, Izv.Akad.Nauk SSSR, Otd.Khim.Nauk, 302 (1962).
121. D.Could, J.Havsky, R.Gutenkunst, and E.B.Hershberg, J.Org.Chem., 22, 829 (1957).
122. L.L.Smith, J.J.Garbarini, J.J.Goodman, M.Marx, and H.Mendelson, J.Amer.Chem.Soc., 82, 1437 (1960).
123. M.Uchibayashi, Chem.and Pharm.Bull.(Japan), 8, 112 (1960).
124. N.N.Suvorov, V.M.Novikova, L.V.Sokolova, and N.F. Kovylkina, Med.Prom.SSSR, No.1, 22 (1960).
125. L.B.Sokolova, V.M.Ryzhkova, G.K.Skryabin, and N.N. Suvorov, Med.Prom.SSSR, No.11, 29 (1961).
126. Unpublished, quoted in "Perspectives and Horizons in Microbiology", Rutgers University Press, 1955, p.121.
127. L.Mamoli, R.Koch, and H.Teschen, Z.phys.Chem., 261, 287 (1939).
128. E.Vischer and A.Wettstein, Experientia, 16, 355 (1960).
129. D.H.Gould, E.B.Hershberg, and E.Schapiro, U.S.P. 2864835 (16th Dec., 1958).
130. N.N.Suvorov, L.V.Sokolova, V.M.Ryzhkova, and D.M. Zaikina, Dokl.Akad.Nauk SSSR, 132, 1325 (1960).
131. K.Zetsche, Naturwiss., 48, 407 (1961).
132. R.Thoma and J.Fried, U.S.P. 2793162 (1957).
133. A.Čapek, O.Hanč, K.Maček, M.Tadra, and Riedl-Tůmová, Naturwiss., 43, 471 (1956).
134. G.M.Shull and J.B.Routien, U.S.P. 2866737 (30th Dec., 1958).
135. G.M.Shull and J.B.Routien, U.S.P. 2872380 (3rd Feb., 1959).
136. Swiss P. 363380/1 (1959).
137. G.S.Fonken, H.C.Murray, and L.M.Reineke, J.Amer. Chem.Soc., 82, 5507 (1960).
138. J.S.McGuire, E.S.Maxwell, and G.M.Tomkins, Biochim. Biophys.Acta, 45, 312 (1950).
139. C.E.Holmlund, L.J.Feldman, N.E.Rigler, B.E.Nielsen, and R.H.Evans, J.Amer.Chem.Soc., 83, 2586 (1961).
140. K.Schubert, K.H.Böhme, and C.Hörhold, Naturforsch., 15b, 584 (1960).
141. R.M.Dodson and R.D.Muir, J.Amer.Chem.Soc., 83, 4627 (1961).
142. R.M.Dodson and R.D.Muir, J.Amer.Chem.Soc., 83, 4631 (1961).
143. L.L.Smith, M.Marx, J.J.Garbarini, T.Foell, V.E.Origoni, and J.J.Goodman, J.Amer.Chem.Soc., 82, 4616 (1960).
144. J.J.Goodman and L.L.Smith, Appl.Microbiology, 8, 363 (1960).
145. J.J.Goodman and L.L.Smith, Appl.Microbiology, 9, 372 (1961).
146. P.Talalay, M.M.Dobson, and D.T.Tapley, Nature, 170, 620 (1952).
147. P.Talalay *et al.*, Cancer, 10, 738 (1957).
148. H.R.Levy and P.Talalay, J.Biol.Chem., 234, 2014 (1959).
149. F.S.Kawahara and P.Talalay, J.Biol.Chem., 235, PC I (1960).
150. A.Fiertel and H.P.Klein, J.Bact., 78, 738 (1959).
151. E.Vischer and A.Wettstein, Unpublished, quoted in Adv. Enzymol., 20, 264 (1958).
152. A.Čapek, H.Pavlů, and O.Hanč, Folia biol.(Československ.) 4, 337 (1958).
153. A.Čapek, H.Pavlů, and O.Hanč, Naturwiss., 45, 89 (1958).
154. G.Lester, D.Stone, and O.Hechter, Arch.Biochem.Biophys., 75, 196 (1958).
155. F.S.Maxwell, J.S.McGuire, and G.M.Tomkins, J.Bact., 80, 1 (1960).
156. D.Perlman, M.J.Weistein, and G.E.Peterson, Canad.J. Microbiol., 3, 841 (1957).
157. B.Hurlock and P.Talalay, J.Biol.Chem., 233, 886 (1958).
158. P.Talalay and V.S.Wang, Biochim.Biophys.Acta, 18, 300 (1955).
159. H.J.Hübener and C.O.Lehmann, Z.phys.Chem.(Leipzig), 313, 124 (1958).
160. K.Zetsche, Naturwiss., 47, 232 (1960).
161. M.Hayano, M.Gut, R.J.Dorfman, O.K.Sebek, and D.H. Peterson, J.Amer.Chem.Soc., 80, 2336 (1958).
162. E.J.Corey, G.A.Gregoriou, and D.H.Peterson, J.Amer. Chem.Soc., 80, 2338 (1958).

163. F.Lindner, R.Junk, H.Keh, G.Nesemann, and J.Schmidt--Thome, Naturwiss., 43, 39 (1956).
164. H.Murray, U.S.P. 2902411 (1959); Chem.Abs., 54, 1660a (1960).
165. K.Zetsche, Arch.Microbiology, 38, 237 (1961).
166. A.Revol, C.Nofre, and A.Cier, Compt.rend., 247, 2486 (1958).
167. A.G.Timofeeva, A.S.Barmenkov, M.V.Fedotova, Med. Prom.SSSR, No.7, 23 (1957).
168. W.J.McAleer, U.S.P. 2875132 (1959); Chem.Abs., 53, 16223a (1959).
169. S.Bernstein, R.H.Lenhard, W.S.Allen, M.Heller, R.Littel, S.M.Stolar, L.Feldman, and R.Blank, J.Amer. Chem.Soc., 81, 1689 (1959).
170. J.Fred, R.W.Thoma, F.F.Sabo, and P.Grabowicz, Unpublished, quoted in Ref.151.
171. R.Neher and A.Wettstein, Helv.Chim.Acta, 39, 2062 (1956).
172. B.Hurlock and P.Talalay, Proc.Soc.Exp.Biol.Med., 93, 560 (1956).

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NUCLEAR MAGNETIC RESONANCE IN HIGH POLYMERS

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I. INTRODUCTION

The method of nuclear magnetic resonance (NMR) was applied to polymers shortly after its discovery[1,2]. Since reliable and comparatively simple NMR spectrometers have become available in many countries, the number of experiments in this field is increasing rapidly.

The NMR method is especially valuable in its application to the investigation of stereospecific polymers and in the study of microstructure and molecular motion in polymers[3-6].

The purpose of the present review is to discuss the main trends in the investigation of high polymers by means of NMR. We assume that the reader is familiar with the principles of the method which is described in books[7-13] and in review articles published here[14-20] and abroad[21-24]. Reviews discussing the application of the NMR method to polymers have also been published[25-36].

In discussing NMR phenomena in polymers we must keep in mind certain special features of structure and properties of polymeric substances. There are two structural units in polymers, namely chain molecules and repeat (monomeric) units; "superstructures" can also be formed[37,38].

In the crystalline polymers there are non-ordered non-crystalline regions, non-crystallised packets, so that it is possible to have a "gaslike" structure with the chain centres forming crystalline domains with random orientation[39]. In glasslike polymers individual structural units may retain freedom of reorientation down to very low temperatures. A polymer in a viscoelastic state may be considered glasslike as far as whole chain molecules are concerned but liquid-like if only individual units are considered. Obviously the spectrum of a solid polymer will then be like that of a crystal or a liquid, depending on experimental conditions.

A very important property of polymers, which has to be taken into account when discussing the NMR data, is the complex nature of their relaxation processes. Dielectric and dynamic-mechanical measurements show that polymers do not have a single correlation frequency of molecular motion but rather a distribution of frequencies which in certain cases may be very broad. Therefore, the simple theory of nuclear magnetic relaxation, whose applicability to small molecule substances has been confirmed in many cases, may be applied to polymers only with reservations; sometimes this simple theory is not even in qualitative agreement with experiment.

The results obtained for the longitudinal, T_1, and the transverse, T_2, relaxation times in polydimethylsiloxane[40]