$\Delta^{9(11)}$-Pregnene-3,20-dione 3,20-Bis-(ethylene Ketal) (VII).—The alcohol prepared above was treated with 10 ml. of phosphorus oxychloride in 60 ml. of pyridine at room temperature for 60 hours.

It was worked up by the method described for the preparation of the 11-deuterated isomer V. The crude $\Delta^{9(11)}$-pregnene-3,20-dione 3,20-bis-(ethylene ketal) weighed 4.28 g.

$\Delta^{9(11)}$-Pregnene-3,20-dione.—Most of the crude olefin VII prepared above (4.1 g.) was mixed with 100 ml. of acetic acid and 8 ml. of water and heated on a steam-bath for 45 minutes. Most of the acid was then distilled off *in vacuo* and the residue was taken up in ether–methylene chloride, washed with 5% aqueous sulfuric acid (to ensure removal of even traces of pyridine if any were still present), 5% aqueous sodium hydroxide and water, dried over magnesium sulfate and the solution was evaporated to dryness to give crude $\Delta^{9(11)}$-pregnene-3,20-dione (3.22 g.).

$\Delta^{9(11)}$-Pregnene-3,20-diol (IX).—The dione prepared above was reduced with 0.87 g. of lithium aluminum hydride in 400 ml. of ether at reflux for 2 hours. The reaction mixture was worked up as usual, to give a quantitative yield of crude $\Delta^{9(11)}$-pregnene-3,20-diol (actually a mixture of epimeric alcohols).

Catalytic Reduction of $\Delta^{9(11)}$-Pregnene-3,20-diol with Deuterium.—The diol IX was dissolved in 85 ml. of acetic acid-*d* and was hydrogenated with deuterium gas in the presence of 1.2 g. of platinum oxide (Baker, lot no. 8369-2) with shaking for 20 hours. The solution was transferred to another flask and fresh catalyst (1.0 g.) was added. The hydrogenation was continued for 72 hours and again fresh catalyst (0.51 g.) was added and shaking was continued for 60 more hours. The uptake of deuterium had slowed down very much at the end of this period. Most of the acetic acid-*d* was then distilled off *in vacuo* and the residue was taken up in methylene chloride–ether. It was washed with aqueous sodium hydroxide and water, dried over magnesium sulfate and evaporated to dryness. A positive tetranitromethane test and spectroscopic data indicated that the reduction had not proceeded to completion (*ca.* 80–85% completed).

The partly acetylated product obtained was deacetylated (all but 0.65 g.) with 0.4 g. of lithium aluminum hydride in 1.8 liters of ether. It was worked up as usual to give 2.31 g. of the deuterated pregnane-diol.

Pregnane-3,20-dione-9α,11α,12α-d_3.—Part of the deuterated pregnane-diol prepared above (1.7 g.) was mixed with 280 ml. of benzene cooled to 7°. A solution of 4.0 g. of chromic oxide and 4.0 g. of sodium dichromate dihydrate in 48 ml. of water and 10 ml. of acetic acid was cooled to 7° and was added to the steroid. The two-phase system was stirred at that temperature for 2 hours and then at 14° for an additional 3 hours. The organic layer was then separated, was washed successively with water, aqueous sodium hydroxide, water, and was evaporated to dryness to give crude product (1.61 g.). This product was chromatographed through 190 g. of acid-washed alumina (Merck) (column diameter, 2.5 cm.) suspended in cyclohexane. Benzene–ether (29:1) was the eluent used. Three main products were obtained. The best fractions of the first product melted at 149–152° and showed no C–D absorption. This product was unreduced $\Delta^{9(11)}$-pregnene-3,20-dione [lit.[3] m.p. 148–150°, m.p. 153–155° (Kofler)] and weighed *ca.* 0.19 g. The second product was deuterated pregnane-3,20-dione. About 0.4 g. of essentially pure dione was obtained (m.p. 117–120°) in addition to several intermediate fractions. The over-all yield from pregnane-3,11,20-trione was 16%. The dione was crystallized from cyclohexane and sublimed to give 0.24 g. of pure compound, m.p. 119–120.5°.

The deuterium content of the deuterated pregnane-dione XV obtained was 2.83 atoms deuterium/molecule. The theoretical maximum deuterium content should be 2 atoms per molecule. The excess (0.83 atom/molecule) is due to incorporation of deuterium at the 12-position in accord with previous observations,[4] and the product is formulated as pregnane-3,20-dione-9α,11α-d_2-12α-$d_{0.83}$ (X).

URBANA, ILL.

[CONTRIBUTION FROM THE NOYES CHEMICAL LABORATORY, UNIVERSITY OF ILLINOIS]

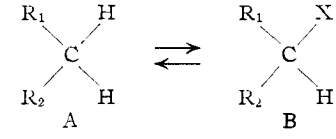
Stereospecific Syntheses of the 7-Deuterio- and 7-Tritiocholesterols. The Mechanism of Enzyme-catalyzed Hydroxylation at a Saturated Carbon Atom

BY E. J. COREY AND GEORGE A. GREGORIOU[1]

RECEIVED NOVEMBER 22, 1958

Cholesterol-7-α-*t* and cholesterol-7-α-*d* have been synthesized stereospecifically by the sequence: 7α-bromo-6-ketocholestanyl acetate → 7α-^{n}H-6-ketocholestanyl acetate (Zn–^{n}HOAc) → 7α-^{n}H-6β-hydroxycholestanyl acetate ($NaBH_4$) → 7α-^{n}H-cholesterol ($POCl_3$–C_5H_5N, followed by $LiAlH_4$). Cholesterol-7β-*t* and cholesterol-7β-*d* have been prepared by a similar process starting with 5α,7β-$^{n}H_2$-7α-bromo-6-ketocholestanyl acetate using unlabeled acetic acid in the debromination step. The facts presently available regarding the mechanism of enzyme-catalyzed hydroxylation at a saturated carbon atom are summarized and a substitution mechanism consistent with these is proposed.

The mechanism of enzyme-catalyzed hydroxylation at a saturated carbon atom[2] has been of particular interest because of the utility of this process for the controlled oxygenation of steroids and because of the fact that many oxygenated natural products are produced in this way. Since the stereochemistry of this reaction is an intrinsic feature of mechanism which can be determined by use of hydrogen isotope, as discussed previously for the general transformation A ⇄ B,[3] such studies have been carried out in these laboratories with a number of steroids. For the specific case of the 7α-hydroxylation reaction which occurs during the biosynthesis of cholic acid from cholesterol it was necessary to obtain cholesterol stereospecifically labeled in the 7α- and 7β-positions by hydrogen isotope and as a result two different syntheses were devised. One of these has already



been outlined in brief in a previous report on the bioöxidation studies using the 7-labeled cholesterols.[4] The first part of this paper deals with the details of this synthetic work and the latter part is concerned with the mechanistic implications of the results of the enzymatic studies.

The more successful and practical synthesis of the two 7-deuterio- and 7-tritio-cholesterols followed

(1) Alfred P. Sloan Foundation Fellow 1956-1958.

(2) For a recent review see P. Talalay, *Physiol. Rev.*, **37**, 362 (1957).

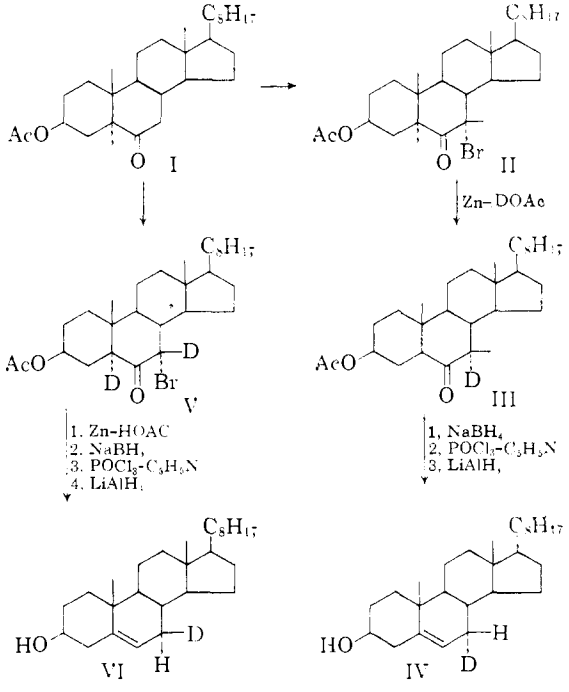
(3) E. J. Corey, M. G. Howell, A. Boston, R. L. Young and R. A. Sneen, THIS JOURNAL, **78**, 5036 (1956).

(4) S. Bergstrom, S. Lindstedt, B. Samuelsson, E. J. Corey and G. Gregoriou, *ibid.*, **80**, 2337 (1958).

the general plan used previously for the synthesis of epimeric hydrogen labeled steroids: (1) selection of a suitable stereospecific process for fixing the orientation of the heavy isotope, (2) synthesis of the epimeric deuterium compounds by use of the same reactions but with a different sequence for the introduction of deuterium and hydrogen, (3) application of infrared analysis to confirm the orientation of deuterium and to estimate stereochemical purity of the epimeric deuterated products and (4) synthesis of the epimeric tritium compounds by the procedures developed for the deuterium analogs.[5] The reaction used to fix the orientation of isotope in this route is somewhat unusual because it is not one of the standard stereospecific methods which depend on nucleophilic displacement with inversion or on strong steric control, but is based instead on the stereospecificity recently observed in the ketonization of steroid enols in acetic acid.[6] It was found in the case of this reaction that attachment of hydrogen to carbon in the axial orientation is greatly favored over the alternative equatorial orientation, even with the operation of an adverse steric effect, and that stereoelectronic effects are dominant. Axial protonation of a steroid Δ^6-en-7-ol by acetic acid at C_6 proceeds at least nine times as fast as equatorial protonation despite strong steric retardation. It was anticipated, therefore, that protonation of a Δ^6-en-6-ol at C_7 by axial (α) approach of hydrogen would prevail overwhelmingly since discriminatory steric influences are essentially absent.

The synthesis of cholesterol-7α-*d*-proceeded as follows. Cholesterol was converted to 6-ketocholestanyl acetate (I) which was brominated to give the 7α-bromo derivative II.[7] The bromoketone II was transformed into 7α-deuterio-6-ketocholestanyl acetate (III) *via* the enol by treatment with zinc–acetic acid-*d*.[8] Reduction of the 6-keto group in III by sodium borohydride gave the corresponding 6β-hydroxy derivative which was dehydrated to cholesteryl acetate by phosphorus oxychloride–pyridine. Very pure cholesterol-7α-*d* (IV) was obtained by treatment of the labeled acetate with lithium aluminum hydride (73% overall yield from the bromoketone II). The product contained 0.97 ± 0.03 atom deuterium/molecule and manifested C–D stretching absorption at 2102 and 2127 cm.$^{-1}$.

The first step in the preparation of cholesterol-7β-*d* from I was the exchange of α-hydrogens for deuterium in ether–acetic acid-*d* solution containing a small amount of deuterium bromide (generated by addition of bromine) to give the trideuterio ketone. When the exchange was complete as determined by infrared analysis of samples withdrawn from the reaction mixture, bromine was added to form 7α-bromo-6-ketocholestanyl acetate 5α,7β-d_2. This was converted to cholesterol-7β-*d*(VI) by successive treatment with zinc–acetic acid, sodium borohydride, phosphorus oxychloride–pyridine and lithium aluminum hydride as mentioned above. The cholesterol-7β-*d* so obtained possessed 0.97 ± 0.03 atom deuterium/molecule and showed infrared absorption due to C–D stretching at 2147 and 2160 cm.$^{-1}$.



The infrared spectra of the 7α- and 7β-deuteriocholesterols were sufficiently different so that the stereospecificity of isotope orientation could be calculated quite closely. In fact, there was no evidence of any contamination of either deuterio compound by the other and the isotope labeling was calculated conservatively as 97 ± 3% stereospecific using optical densities. Each isomer shows appreciable absorption in a region where the other shows none.

The method described for the synthesis of the two deuterated cholesterols was also employed for the synthesis of cholesterol-7α-*t* and cholesterol-7β-*t* and, indeed, a great advantage of this synthesis is the ready adaptability to tritium labeling. Acetic acid-*t* with an activity of *ca.* 1.7 millicuries/mmole, (prepared from acetic anhydride and tritiated water) was used instead of acetic acid-*d*. In all other respects the two procedures were identical except for the experimental technique which was somewhat different (*e.g.*, transfer of liquids *in vacuo*) for the preparation of the radioactive compounds.

The isotope orientation in the tritiated cholesterols should be as specific as in the deuterated compounds (97 ± 3%) since the same step, protonation of the Δ^6-enol, determines stereochemistry in a way which does not involve discrimination between isotopes. In this connection it should be noted that the attachment of *both* deuterium and hydrogen to the Δ^6-enol takes place with complete stereospecificity within experimental error.

(5) Since even tritium-labeled compounds of the highest available activity contain only a minute fraction of tritiated molecules, spectroscopic analysis is not feasible and prior synthesis and examination of the deuterated analogs is desirable.

(6) E. J. Corey and R. A. Sneen, THIS JOURNAL, **78**, 6269 (1956).

(7) E. J. Corey, *ibid.*, **76**, 175 (1954).

(8) This configuration of deuterium is indicated by the independent synthesis described below as well as on the basis of the preference for axial protonation demonstrated previously (ref. 6).

Although stereospecificity of isotope orientation in the 7-tritiated cholesterols was assured, it was anticipated that the problem of contamination of these materials by Δ^6-cholestenol might be serious. A small amount of the Δ^6-olefin was expected to result from the dehydration of 6β-hydroxycholestanyl acetate in addition to the main product, the Δ^5-olefin. In the preparation of cholesterol-7α-*t*, the presence of a small amount of Δ^6-olefin is of no concern in hydroxylation studies since this impurity would not be radioactive (assuming the Δ^6-olefin is formed by *trans* diaxial elimination involving the 6β-hydroxyl and 7α-hydrogen or hydrogen isotope). However, in the preparation of cholesterol-7β-*t* the material subjected to dehydration was necessarily a mixture of 3β-acetoxycholestan-6β-ol-7α-*t* and 3β-acetoxycholestan-6β-ol-5α-*t* in roughly equal amounts, much less 3β-acetoxycholestan-6β-ol-5α,7α-t_2 and mainly unlabeled 3β-acetoxycholestan-6β-ol. Dehydration of 3β-acetoxycholestan-6β-ol-5α-*t* to give 3β-acetoxycholest-6-en-5α-*t* instead of the Δ^5-isomer, cholesteryl acetate, should be favored by a factor corresponding to the kinetic isotope effect k_H/k_T for elimination, possibly as large as 30, because formation of Δ^6-olefin involves loss of 7α-hydrogen whereas formation of Δ^5-olefin involves loss of 5α-tritium. Thus, there might be a greater amount of Δ^6-olefin present in the preparation of cholesterol-7β-*t* and, moreover, it would be a radioactive impurity. Accordingly, dilution experiments were carried out with the radioactive cholesterol-7β-*t* using 3β-hydroxycholest-6-ene (see Experimental) and it was found that less than 12% (and probably much less) of the activity of the cholesterol-7β-*t* preparation was due to the Δ^6-isomer. Purification of the cholesterol-7β-*t* was accomplished by way of the dibromo derivative after admixture with inactive 3β-hydroxycholest-6-ene.

A less satisfactory but more conventional route for the synthesis of the 7-labeled cholesterols involved the replacement of bromine in the previously described 7α- and 7β-bromocholesteryl benzoates by hydrogen isotope using labeled lithium aluminum hydride as the reagent. The configurations of these bromides have recently been derived from X-ray diffraction studies[9] and it now seems definite that the more levorotatory epimer possesses the 7α-configuration,[10] contrary to the previous assignment.[11] In our experience the photobromination method[11] is rather unsatisfactory as a route to either of the 7-bromo derivatives of cholesteryl benzoate and there is at present no acceptable route to the pure 7β-epimer. The procedure of the Swiss workers for the preparation of the 7β-epimer yielded material with properties similar to those reported[11] [m.p. 114–115°, $[\alpha]^{20}D$ + 20°] but which actually proved to be impure. It was found that considerable further purification could be effected, but only with substantial material loss, and that purified 7β-bromocholesteryl benzoate melting at 125–125.5° and having $[\alpha]D$ + 120° could be obtained. The less soluble 7α-epimer was prepared from crude 7β-epimer by the epimerization method used previously[11] and the constants of the purified product were in agreement with those reported[11] [m.p. 144.5–145°, $[\alpha]D$ −199°].[12] A more convenient route to the 7α-bromide was found to be *via* 7-keto cholesteryl benzoate by reduction with sodium borohydride to 7β-hydroxycholesteryl benzoate (65% yield) followed by reaction with hydrogen bromide in ether at low temperatures (60% yield).

The reduction of 7α-bromocholesteryl benzoate by lithium aluminum deuteride proved to be quite unsatisfactory as a method for preparing 7-deuterated cholesterol. The reaction product was a mixture of substances which afforded only a low yield of impure cholesterol-7β-*d*. The isomeric Δ^6-compound was also formed and could be isolated after extensive crystallization. The infrared spectrum of the cholesterol which was isolated showed C–D stretching absorption at 2147 and 2160 cm.$^{-1}$ in agreement with that of the 7β-deuterio compound prepared by the sequence described in the previous part.[13]

In contrast, the reduction of 7β-bromocholesteryl benzoate by lithium aluminum deuteride proceeded well and gave a 65% yield of 7α-deuteriocholesterol which displayed peaks in the C–D stretching region at 2102 and 2127 cm.$^{-1}$ corresponding to the cholesterol-7α-*d* obtained by the alternate method described above. The stereospecific formation of the epimeric 7-deuteriocholesterols from the epimeric 7α-bromocholesterols indicates clearly that substitution by deuterium occurs with inversion of configuration and attention should be focused on these results as conclusive evidence for the assignment of isotope orientation.[14]

Nature of the Enzymatic Hydroxylation Reaction.—As reported by us[4] 7α-hydroxylation of cholesterol proceeds with loss of the 7α-hydrogen in cholic acid synthesis, *i.e.*, with retention of configuration. It has also been found that 11α- and 11β-hydroxylation occur with re-

(9) W. Nowacki, H. Bürki and G. F. Bonsma, *Chimia*, **10**, 254 (1956).

(10) See also F. Hunziker, F. X. Müllner, K. G. Reuteler and H. Schaltegger, *Helv. Chim. Acta*, **38**, 1316 (1955), and K. Tsuda, K. Arima and R. Hayatsu, THIS JOURNAL, **76**, 2933 (1954).

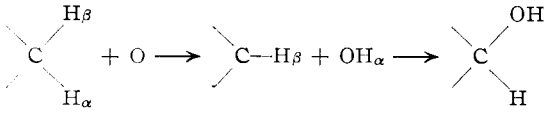
(11) H. Schaltegger and F. X. Müllner, *Helv. Chim. Acta*, **34**, 1096 (1951).

(12) The composition of the mixture obtained by equilibration of 7α- and 7β-bromocholesteryl benzoates has been calculated[11] as 70% β- and 30% α-epimer based on the erroneous value of $[\alpha]D$ +20° for the 7β-epimer. From our data it would seem that the equilibrium constant is approximately one.

(13) The reduction of 7α-bromocholesteryl benzoate by lithium aluminum deuteride has been investigated by D. K. Fukushima, S. Lieberman and B. Praetz [THIS JOURNAL, **72**, 5205 (1950)] who also noted that only a low yield of impure cholesterol could be obtained. These workers also prepared cholesterol-7-*d* by reduction of 7α-bromocholesteryl benzoate with "deuterized" Raney nickel and with deuterium over 5% palladium-on-calcium carbonate. By infrared analysis of the original samples, obtained through the courtesy of Dr. Fukushima, we have found that these deuterated cholesterols are mixtures of the 7α- and 7β-epimers.

(14) Previous work in this Laboratory (ref. 3) had shown that the reduction of cholesteryl tosylate by lithium aluminum deuteride in ether gives 3β-deuteriocholestane and 6-deuterio-3:5-cyclocholestane, products indicative of an intermediate 3:5-cyclocholesteryl cation. Consequently, it is of interest that the reduction of the epimeric 7-bromocholesteryl benzoates by lithium aluminum deuteride does not lead to the same mixture of deuterated products as expected from the intermediacy of a free allylic cation. It would seem that in the case of the 7-bromides, attack by hydride is either concerted with ionization of bromide or else it occurs on an ion-pair intermediate in which the bromide ion maintains orientation with respect to the α- and β-sides of the steroid.

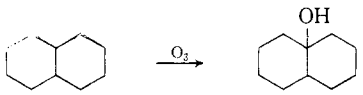
tention,[15,16] an indication that this may generally be the predominant stereochemical course of biohydroxylation at a saturated carbon atom. In this regard it is noteworthy that 11β-hydroxylation occurs with retention despite the fact that steric effects discriminate strongly against this course relative to a process such as



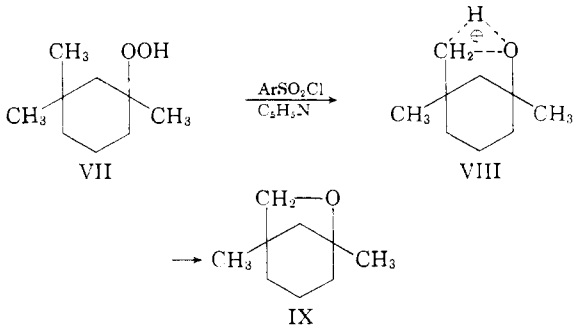
which proceeds with over-all inversion.

It has been shown previously[17] that microörganisms which effect steroid hydroxylation at a specific saturated carbon atom often produce 1,2-epoxides from steroids in which that carbon is part of an olefinic linkage, and it has been pointed out that the same enzyme may be involved in both processes. As a result the enzymatic reagent has been compared to a peroxide or peracid and has been considered as an electrophilic, non-radical species. Furthermore, it has been established that the hydroxylation process does not involve hydration of an olefinic intermediate, but direct incorporation of molecular oxygen into the reagent and thence into the steroid, and that metal ions and TPNH are involved,[2] providing additional evidence for a peroxidic intermediate.

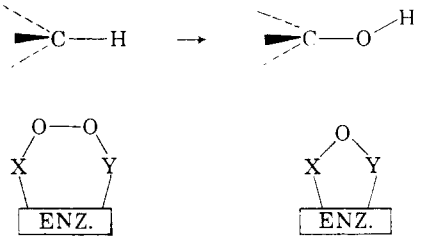
There are only two examples of non-radical replacement of alkyl hydrogen by oxygen in chemical systems which can be regarded as especially germane to steroid hydroxylation. Both involve electrophilic oxygen. The first of these is the reaction of *cis*- and *trans*-decalin with ozone to give *cis*- and *trans*-9-hydroxydecalin, respectively, a stereospecific substitution proceeding with retention of configuration.[18] The second is the recently discovered



conversion of VII to IX, an electrophilic substitution reaction which probably involves a cationic transition state of structure VIII.[19]



The assemblage of all these data into a hypothetical scheme for steroid hydroxylation leads to the formulation.



It is also possible that a proton is involved catalytically, although one is not shown in this scheme. In addition, X and Y might be metal ions.

Experimental[20]

7β-Hydroxycholesteryl Benzoate.—A mixture of 14.0 g. of 7-ketocholesteryl benzoate, 1 l. of methanol and 600 ml. of ethanol was treated with 10 g. of sodium borohydride with vigorous stirring for 45 minutes. The insoluble ketone went slowly into solution and when most of it had dissolved, the crystalline product precipitated out. An additional 4.0 g. of sodium borohydride was added and the mixture was stirred for 30 minutes. The excess of the borohydride was decomposed with acetic acid and the product was collected after the addition of 200 ml. of water and cooling. The crude alcohol (12.4 g.), m.p. 185–189°, gave, on crystallization from cyclohexane–hexane, 9.1 g. (65%) of 7β-hydroxycholesteryl benzoate, m.p. 191–192° (lit.[21] m.p. 192°).

7β-Bromocholesteryl Benzoate.—A solution of 14.7 g. of bromine in 600 ml. of dry carbon disulfide (distilled from phosphorus pentoxide) was added to a vigorously stirred solution of 45 g. of cholesteryl benzoate in 1.1 liter of dry carbon disulfide (in a 3-liter three-necked flask) maintained under aspirator-vacuum and illuminated with two 200-watt lamps and two No. 2 photoflood lamps. The reaction was instantaneous as indicated by the very rapid decoloration of the bromine solution upon contact with the reaction mixture. The temperature of the solution was between 30 and 35° during the addition which required a period of 7 minutes. The remainder of the hydrogen bromide and the carbon disulfide was then evaporated under reduced pressure, care being exercised to maintain low temperature (5°). The residue, a light brown oil, was dissolved in 45 ml. of low boiling petroleum ether and stored immediately at −5° for 12 hours. The crystals were collected, washed with petroleum ether and vacuum-dried.

Polar solvents could not be used for crystallization because of epimerization[11] to the 7α-isomer. Crystallization from hexane was found to be unsatisfactory. Purification could be effected, however, by shaking the product (after grinding) with a limited amount of hexane for 30 minutes and collecting the material not dissolved. Crystallization from hexane accomplished by dissolution in a large volume of the solvent followed by concentration (*in vacuo*) was used only as the last step of the purification procedure.

The crude 7β-bromocholesteryl benzoate obtained from the photobromination reaction weighed 34.8 g., m.p. 109–114° dec., $[\alpha]_D$ −33° (*c* 1.8, benzene) [lit.[11] m.p. 114–115° dec., $[\alpha]^{20}_D$ +20 (*c* 2.15, benzene)]. Shaking the crude product with hexane two times and recrystallizing it from the same solvent gave 7β-bromocholesteryl benzoate, m.p. 115–116°, $[\alpha]_D$ +24°, in agreement with the reported values. However, further purification, by the method mentioned above, resulted in gradual rise of both the melting point and the specific rotation of the compound. It finally gave pure 7β-bromocholesteryl benzoate, m.p. 125–125.5°, $[\alpha]_D$ +120° (*c* 1.9, benzene).

7α-Bromocholesteryl Benzoate. Procedure A.—A solution of 3.3 g. of partially purified 7β-bromocholesteryl benzoate, m.p. 115–116°, in 3.2 ml. of chloroform was treated

(15) M. Hayano, M. Gut, R. I. Dorfman, O. K. Sebek and D. H. Peterson, THIS JOURNAL, **80**, 2336 (1958).

(16) E. J. Corey, G. A. Gregoriou and D. H. Peterson, *ibid.*, **80**, 2338 (1958).

(17) B. M. Bloom and G. M. Shull, *ibid.*, **77**, 5767 (1955).

(18) J. R. Durland and H. Adkins, *ibid.*, **61**, 429 (1939).

(19) E. J. Corey and R. W. White, *ibid.*, **80**, 6686 (1958).

(20) We are indebted to Dr. R. F. Nystrom for assistance in the tritium work and to Mr. J. Nemeth for the deuterium analyses [accuracy, ±0.03%, "falling-drop" method of A. S. Keston *et al.*, *J. Biol. Chem.*, **122**, 227 (1942)]. The infrared spectra of deuterated steroids in the C–D stretching region were obtained using a Perkin–Elmer single-beam, double-pass spectrometer fitted with a lithium fluoride prism [accuracy, ±1 cm.$^{-1}$, see ref. 3].

(21) H. J. Eckhardt, *Ber.*, **71**, 461, 469 (1938).

slowly with 7 ml. of acetone. Epimerization was rapid and the rapid isomerization of IV to V resulted in the precipitation of crystalline 7α-bromo epimer which was collected (after standing at 0° for 6 hours), m.p. 138.5–140°, $[\alpha]_D$ −188° (benzene), wt. 2.15 g. Two crystallizations from hexane gave 7α-bromocholesteryl benzoate with a constant m.p. of 144.5–145.5° (slow decomposition), $[\alpha]_D$ −199° (benzene) (lit.[11] m.p. 143–144°, $[\alpha]^{20}_D$ −156°). That the rotation of −199° is that of the pure compound was confirmed by stirring a 0.20-g. sample of the pure product with 5 ml. of hexane and measuring the rotation of the residue which did not dissolve as well as that of the product obtained by crystallization of the mother liquor. Both materials exhibited the same specific rotation, $[\alpha]_D$ −199° (benzene).

The specific rotation is a better criterion of the purity of these bromides than their melting point which varies with the rate of heating. Apparently, isomerization to the epimeric bromide sets in (along with decomposition) as soon as traces of the sample start softening, which results in a low melting point.

Procedure B.—Ether (5 ml.) was saturated with hydrogen bromide at 0° and was then cooled in a Dry Ice–acetone-bath after dilution with 45 ml. of dry ether. To the cold solution was added 1.2 g. of 7β-hydroxycholesteryl benzoate, m.p. 190.5–191°, and the mixture was stirred at −80° for 15 minutes. The temperature was allowed to rise slowly (30 minutes) to −10°. The solution was then concentrated in the same reaction flask to dryness *in vacuo* (0.1 mm.). Care was exercised to keep the temperature low (below −10°) and to avoid contact with air while the product was still in solution or wet. The crude product was crystallized from about 14 ml. of hexane to give 0.8 g. (60%) of 7α-bromocholesteryl benzoate, m.p. 139–140.5°, $[\alpha]_D$ −197° (benzene). The specific rotation of this product remained essentially unchanged on further purification. Mixed melting point with the bromide prepared by procedure A was undepressed.

Cholesterol-7α-*d* (IV) from 7β-Bromocholesteryl Benzoate.—A solution of 0.95 g. of 7β-bromocholesteryl benzoate (partially purified, $[\alpha]_D$ +35°) in 25 ml. of dry ether was added dropwise to a stirred solution (under nitrogen) of 0.9 g. of lithium aluminum deuteride in 40 ml. of dry ether maintained at 10°. The addition was completed in 1 hour and the mixture was allowed to warm up to room temperature and stored for 18 hours. The product was worked up in the usual way and gave, after one crystallization from hexane–low boiling petroleum ether, 0.45 g. (65%) of cholesterol 7α-*d*, m.p. 145–147°, infrared max. at 2102 and 2127 cm.$^{-1}$ plus two weak peaks at 2147 and 2160 cm.$^{-1}$, which indicate contamination with some of the epimer. This is not surprising in view of the fact that the bromosteroid used ($[\alpha]_D$ +35°) was contaminated with the 7α-bromide. The extent of contamination of the deuteriocholesterol, however, is small in spite of the fact that the bromide used could contain as much as 25% of the epimer, probably because reduction of the 7α-epimer gives only a poor yield of cholesterol.

Cholesterol-7β-*d* (VI) from 7α-Bromocholesteryl Benzoate.—A solution of 1.25 g. of 7α-bromocholesteryl benzoate in 30 ml. of ether was added to a stirred solution (under nitrogen) of 0.4 g. of lithium aluminum deuteride in 35 ml. of ether maintained at 0°. The addition was completed in 1 hour and 30 minutes and the mixture was allowed to stand at 0° for 2 hours and then was stored at room temperature for 2 days. The product was worked up in the usual way and after a number of crystallizations from hexane and from alcohol, a low yield of impure cholesterol-7β-*d* was obtained, m.p. 138.5–140.5° (after drying at 80° under 0.1 mm.), infrared max. at 2147 and 2160 cm.$^{-1}$.

3β-Acetoxy-7α-bromocholestan-6-one (II).—A solution containing 0.730 g. of bromine in 9 ml. of acetic acid was added dropwise to a solution of 2.00 g. of 3β-acetoxycholestan-6-one[22] (I) (m.p. 130–130.5°) in 30 ml. of dry ether and 5 ml. of acetic acid at reflux with stirring. The addition was completed in 10 minutes and the resulting solution was maintained at reflux for 26 hours. The ether was evaporated under reduced pressure and the acetic acid solution was diluted to 20 ml. (with acetic acid).

Seeding with the bromoketone followed by the gradual addition of 2 ml. of water gave 1.72 g. of crude product, m.p. 137–143°. Recrystallization from acetic acid–water gave 1.19 g. (51%) of 3β-acetoxy-7α-bromocholestan-6-one (II), m.p. 148.0–148.5° (lit.[23] m.p. 144–145°).

3β-Acetoxycholestan-6-one-7α-*d* (III).—A solution of 0.78 g. of the bromoketone II in 25 ml. of ether and 4 ml. of acetic acid-*d* cooled to 15° was treated with 2.2 g. of zinc dust. The reaction mixture was stirred and allowed to warm up to room temperature (one hour) where it was maintained for an additional 8 hours. It was filtered, washed with 5% aqueous sodium carbonate, then with water, dried over magnesium sulfate, filtered and concentrated to dryness *in vacuo*. The crude 3β-acetoxycholestan-6-one-7α-*d* (III) obtained had m.p. 129.5–130.5° (m.p. of pure material 130.5–131°) (infrared max., 2138 cm.$^{-1}$, one broad band).

3β-Acetoxycholestan-6β-ol-7α-*d*.—All but 0.020 g. of the crude deuterioketone III obtained above was treated with 0.18 g. of sodium borohydride in 30 ml. of methanol and the mixture was stirred at room temperature for 20 minutes. The rapid dissolution of the ketone was followed by the appearance of a crystalline precipitate. Acetic acid was added to decompose the excess of sodium borohydride and crystallization was induced by the slow addition of 3.5 ml. of water. Filtration gave 0.550 g. of crystalline acetoxy alcohol, m.p. 157.5–158.5° infrared max., 2140 cm.$^{-1}$, one broad band). Pure material melts at 159–159.5°, but the reported[24] melting point is considerably lower, 141–142°. However, further identification was obtained by hydrolysis to cholestan-3β,6β-diol, m.p. 190–191°. The hydrolysis was accomplished in excellent yield by the use of lithium aluminum hydride in contrast to basic hydrolysis which resulted in impure product. Further identification of the acetoxy alcohol was obtained by repetition of the catalytic reduction (platinum oxide in ethanol solution) of 3β-acetoxycholestan-6-one reported[24] as giving 3β-acetoxycholestan-6β-ol, m.p. 141–142°. This reduction gave in our hands the higher melting product, m.p. 159–159.5°.

Cholesteryl-7α-*d* Acetate.—A solution of 0.515 g. of crude 6β-alcohol in 4.5 ml. of dry pyridine was treated with 1.8 ml. of phosphorus oxychloride and the mixture was allowed to stand at room temperature for 24 hours. It was decomposed by pouring slowly into a mixture of 4% hydrochloric acid, ether and ice. The ether layer was washed successively with water, 5% aqueous sodium bicarbonate and water. Drying over magnesium sulfate and then concentration to dryness gave 0.500 g. of crude product, m.p. 108–113°, which was recrystallized from 5 ml. of 95% ethanol to give 0.40 g. of cholesteryl-7α-*d* acetate, m.p. 114–115°. The mother liquor gave a second crop, m.p. 112–113.5°, wt. 0.035 g.

Cholesterol-7α-*d* (IV).—A solution of 0.385 g. of cholesteryl-7α-*d* acetate and 0.20 g. of lithium aluminum hydride in 70 ml. of dry ether was stirred for 1 hour and 15 minutes at room temperature. The excess hydride was decomposed with ethanol and the mixture was washed successively with 4% hydrochloric acid, water, 5% aqueous sodium bicarbonate and with water. Drying over magnesium sulfate followed by concentration to dryness (*in vacuo*) and crystallization from ethanol–water gave very pure cholesterol-7α-*d* (0.333 g., 96% yield), m.p. 149.8–150°. The deuterium content of the product was 0.97 atom/molecule. It exhibited infrared absorption maxima in the C–D stretching region at 2102 and 2127 cm.$^{-1}$. The deuterium orientation was found to be stereospecific within the analytical sensitivity of ±3%. It follows that the deuterium labeling was 97 ± 3% stereospecific. The over-all yield of cholesterol-7α-*d* based on 3β-acetoxy-7α-bromocholestan-6-one was 73%.

3β-Acetoxycholestan-6-one-5α,7α,7β-d_3.—A solution of 1.300 g. of 3β-acetoxycholestan-6-one in 22 ml. of dry ether and 4.5 ml. of acetic acid-*d* was treated with 0.027 g. of bromine, source of a catalytic amount of hydrogen bromide, and was heated to reflux for 48 hours. Samples were drawn out at various intervals and the infrared spectrum of the recovered 3β-acetoxycholestan-6-one was obtained for each sample in the C–D stretching region. Comparison of these spectra suggested that exchange of the hydrogen atoms, alpha to the 6-keto group, with deuterium was complete after 21 hours at reflux but not after 6 hours.

(22) R. M. Dodson and B. Riegel, *J. Org. Chem.*, **13**, 424 (1948).

(23) I. M. Heilbron, *et al.*, *J. Chem. Soc.*, 801 (1937).

(24) Pl. A. Plattner and W. Lang, *Helv. Chim. Acta*, **27**, 1872 (1944).

3β-Acetoxy-7α-bromocholestan-6-one-5α,7β-d_2 (V).—The trideuterioketone prepared by exchange as described above, was brominated in the medium in which it was prepared by the addition of a solution of bromine in acetic acid-*d* under conditions identical with those employed for the bromination of the undeuterated ketone. The crude product was crystallized from acetic acid–water (by moderate heating (50°) to effect dissolution in glacial acetic acid followed by cooling and slow addition of water after the crystalline product started precipitating) containing traces of sodium acetate (to neutralize any hydrogen bromide formed, thus ensuring against possible exchange of deuterium with hydrogen from the solvent). The bromoketone exhibited infrared absorption maxima at 2136 (weak) and at 2168 cm.$^{-1}$ (very weak shoulder).

Cholesterol-7β-*d* (VI) was prepared from the bromoketone V by the same sequence of reactions by which cholesterol-7α-*d* was prepared from the undeuterated bromoketone II. The only difference was that undeuterated acetic acid was used in the zinc–acetic acid reduction of V. Sodium borohydride reduction of this reduction product gave 3β-acetoxycholestan-6β-ol-5α,7β-d_2 (infrared max., strong at 2166, weaker at 2106, weakest at 2151 cm.$^{-1}$). The yields obtained were similar to those of the former series and the cholesterol-7β-*d* prepared melted at 149–149.3° and had a deuterium content of 0.97 atom/molecule. It exhibited two infrared absorption maxima in the C–D stretching region at 2147 and 2160 cm.$^{-1}$. The deuterium labeling was stereospecific to the extent of 97 ± 3%.

Acetic Acid-*t*.—Acetic anhydride (1.414 g., 13.87 mmole), 0.206 g. (11.44 mmole) of water and 0.05 g. (2.78 mmole) of tritiated water with an activity of *ca.* 50 millicuries were distilled using a high vacuum line into a 12-ml. breakseal tube. The tube was sealed and placed in a bath at 50° which resulted in a rapid hydrolysis of the anhydride as indicated by the formation of a homogeneous solution. After two hours at that temperature, the acetic acid-*t* was distilled into a 5-ml. calibrated tube from which aliquots could be removed and measured by distillation into a 0.5-ml. calibrated tube.

3β-Acetoxycholestan-6-one-7α-*t*.—An amount of 0.55 g. of 3β-acetoxy-7α-bromo-cholestan-6-one was placed in a 25-ml. round-bottom flask sealed to a condenser and was dried in the high vacuum line. It was dissolved in 16 ml. of ether, distilled directly into the reaction flask from lithium aluminum hydride. Similarly, 0.42 ml. of acetic acid-*t* was distilled into the solution which was then magnetically stirred (under nitrogen atmosphere) with 1.0 g. of zinc dust (dried at 80° under 0.1 mm. for 4 hours). Ether was added and the mixture was filtered into 60 ml. of 5% aqueous sodium carbonate. The ether layer was washed once more with sodium carbonate and then three times with water. It was dried over magnesium sulfate and then concentrated to dryness (*in vacuo*) to crude 3β-acetoxycholestan-6-one-7α-*t*, m.p. 129.5–130.2°, wt. 0.408 g.

3β-Acetoxycholestan-6β-ol-7α-*t*.—All of the crude product obtained above was treated with 0.28 g. of sodium borohydride in 19 ml. of methanol and the mixture was stirred at room temperature for 50 minutes. Acetic acid was added to decompose the excess of sodium borohydride and crystallization of the product from the reaction mixture was induced by the slow addition of 2.5 ml. of water. Filtration gave 0.375 g. of crystalline acetoxy alcohol, m.p. 157.0–157.8°.

Cholesteryl-7α-*t* Acetate.—The crude acetoxy alcohol described above was dissolved in 4 ml. of dry pyridine (distilled from calcium hydride) and was treated with 1.8 ml. of phosphorus oxychloride. The heat of reaction was dissipated by placing the reaction flask in a bath at 15° for 3 minutes. It was then allowed to stand at room temperature for 21 hours. Ether was added and it was poured slowly into a mixture of 4% hydrochloric acid, ether and ice. The ether layer was washed successively with 4% hydrochloric acid, water, 5% aqueous sodium bicarbonate and water. Drying over magnesium sulfate followed by concentration to dryness *in vacuo* gave crude product, m.p. 111–114°, which after crystallization from 4.5 ml. of absolute ethanol plus 0.3 ml. of water gave cholesteryl-7α-*t* acetate, m.p. 113.7–114.8°.

Cholesterol-7α-*t*.—A solution of the cholesteryl-7α-*t* acetate prepared above and of 0.19 g. of lithium aluminum hydride in 40 ml. of dry ether was stirred for 1 hour and 30 minutes at room temperature and was then worked up in the usual way. The crude product melted at 148.3–149.4°. Crystallization from 4 ml. of ethanol plus 0.6 ml. of water gave 0.237 g. of cholesterol-7α-*t*, m.p. 149.2–149.5°.

3β-Acetoxy-7α-bromocholestan-6-one-5α,7β-t_2.—A solution of 0.800 g. of 6-ketocholestanyl acetate in 6.5 ml. of dry ether (distilled directly into the reaction flask in a vacuum line) and 0.48 ml. of acetic acid-*t* was treated with 0.019 g. of bromine. The solution was refluxed for three days and was then brominated at reflux by the dropwise addition of a solution of 0.28 g. of bromine in 0.4 ml. of acetic acid-*t*. The addition was completed in 8 minutes. The solution was maintained at reflux for 28 hours, and was then worked up. To recover the tritiated acetic acid undiluted, the volatile constituents of the reaction mixture were removed by distillation in the vacuum line. The solid residue was dissolved in ether, filtered (cotton plug) and crystallized from 20 ml. of acetic acid and 1.15 ml. of water to give 0.50 g. of the tritiated bromoketone, m.p. 144.5–146.5°. Recrystallization from acetic acid–water brought the melting point to 147.5–148.3°, wt. 0.385 g.

3β-Acetoxycholestan-6-one-5α,7β-t_2.—The tritiated bromoketone prepared above was dissolved in 12 ml. of ether and 1.3 ml. of acetic acid and was treated with 1.1 g. of zinc dust. The mixture was stirred for 4 hours and was worked up as in the preparation of the dideuterioketone to give crude 3β-acetoxycholestan-6-one-5α,7β-t_2, m.p. 129.5–130.3°.

Cholesterol-7β-*t*.—The tritiated ketone prepared above was submitted to the same sequence of reactions as described in going from 3β-acetoxycholestan-6-one-7α-*t* to cholesterol-7α-*t*. It gave cholesterol-7β-*t*, m.p. 148–148.8°, wt. 0.140 g.

3β-Acetoxy-Δ^6-cholestene.—3β-Acetoxy-7α-bromocholestan-6-one (1.175 g., m.p. 147.5–148.5°) was added to a solution of 0.42 g. of sodium borohydride in 25 ml. of ethanol and the mixture was stirred for 25 minutes. The bromoketone went rapidly (3 minutes) into solution as the reaction proceeded, indicating that the reduction was very rapid. Acetic acid was added to decompose the excess borohydride, followed by water added to turbidity. The product began coming out of solution as an oil but after the slow addition of some absolute ethanol so as to form an upper layer of pure alcohol, a needle-like crystalline product appeared at the interphase. Stirring, followed by the addition of more water, gave a crystalline product; 0.995 g. (85% yield) of crude material, m.p. 142–150°, which was not characterized further.

The crude product (0.950 g. of it) prepared above was mixed with 80 ml. of acetic acid and 8.0 g. of zinc dust in a flask equipped with a reflux condenser and a magnetic stirrer, It was immersed in an oil-bath preheated to 160°. The stirred mixture went rapidly into reflux and was allowed to react at that temperature for 15 minutes while an additional three 0.2-g. portions of zinc dust were added. At the end of this period the reaction mixture was filtered, while hot, and the zinc was washed with 50 ml. of hot acetic acid. It was cooled to room temperature and water was added to turbidity. The crystalline product which appeared was collected after the addition of an additional 23 ml. of water and cooling. The product was recrystallized from 95% ethanol to give 0.66 g. (85%, over-all 72%) of 3β-acetoxy-Δ^6-cholestene, m.p. 108.6–109° (lit.[6] m.p. 106–107.5°).

The method described above was used for the synthesis of 3β-acetoxy-Δ^6-cholestene-6-*d* (infrared max., strong and sharp at 2248 cm.$^{-1}$, weak shoulder at 2220 cm.$^{-1}$). Sodium borodeuteride, instead of the unlabeled sodium borohydride, was employed in the reduction of the starting bromoketone.

Δ^6-Cholestenol.—A solution of 0.58 g. of 3β-acetoxy-Δ^6-cholestene and 0.18 g. of lithium aluminum hydride in 45 ml. of dry ether was refluxed for 30 minutes. The product was worked up in the usual way and recrystallized from alcohol to give 0.48 g. (92%) of Δ^6-cholestenol, m.p. 119–124°, $[\alpha]^{25}D$ −94° (*c* 1.2, chloroform) (lit.[25] m.p. 129–131°, $[\alpha]D$ −81° chloroform). The melting point of this alcohol could not be used as a criterion of purity in view of the fact that it varied *irregularly* between 118 and 130° depending on the solvent from which it was crystallized and on the way it was dried (vacuum alone or heating under vacuum).

(25) O. Wintersteiner and M. Moore, THIS JOURNAL, **72**, 1923 (1950).

The optical rotation of the product and the high purity of the Δ^6-cholestenyl acetate used along with our experience that the lithium aluminum hydride deacetylation of steroid acetates gives a very pure product, suggested that the Δ^6-cholestenol prepared was a pure compound.

Dilution Experiments with Cholesterol-7β-t.—In order to determine whether part of the radioactivity of the cholesterol-7β-t synthesized above was due to tritium-containing Δ^6-cholestenol (specifically, Δ^6-cholestenol-5α-t), a small amount (0.0101 g.) of cholesterol-7β-t was mixed with Δ^6-cholestenol (0.3447 g.) and the acetate of the mixture was submitted to purification to remove the 7β-tritiocholesteryl acetate. The acetate was prepared by dissolving the mixture in 4 ml. of pyridine, treating it with 1 ml. of acetic anhydride for 36 hours at 42°, and working up the product in the usual way. The activity of the material was determined at the various purification stages. The data obtained are: cholesterol-7β-t had an activity of 3550×10^6 d.p.m./mmole[26] (disintegrations per minute/millimole). The mixture of the two alcohols should have an activity of 101×10^6 d.p.m./mmole. The Δ^6-cholestenyl acetate obtained after one crystallization from 10 ml. of 95% ethanol had an activity of 94.2×10^6 (the same units, d.p.m./mmole, are used throughout). Subsequent crystallizations were carried out in absolute alcohol. A second crystallization gave a product with an activity of 63.6×10^6, three more crystallizations gave a product with an activity of 25.2×10^6. Addition of 9.5 mg. of cholesteryl acetate to the 188 mg. of the product and two more crystallizations from alcohol gave Δ^6-cholestenyl acetate with an activity of 12.2×10^6.

The above data indicate that of the activity 101×10^6, less than 12.2×10^6 is due to tritiated Δ^6-cholestenol. In fact, the trend observed in these data suggests that continued purification would have lowered this limit even further.

Purification of Cholesterol-7β-t from Traces of Δ^6-Cholestenol-5α-t.—A mixture of 0.054 g. of cholesterol-7β-t (m.p. 148–148.8°) and 0.015 g. of Δ^6-cholestenol was dissolved in 0.35 ml. of ether and 1 ml. of acetic acid. The solution was allowed to stand for 10 minutes and an additional 1.65 ml. of acetic acid was added. It was cooled to 18° and was treated with 0.29 ml. of a bromine solution in acetic acid containing some sodium acetate (0.68 g. of bromine and 0.31 g. of sodium acetate in 6 ml. of acetic acid). The reaction mixture was placed in a bath at 3° and, after 4 minutes, the crystalline cholesterol-7β-t dibromide which had precipitated out was collected by filtration and was washed with cold acetic acid.

The crude dibromide was stirred with 1 g. of zinc dust and 0.1 ml. of acetic acid in 25 ml. of ether for 1 hour and 25 minutes. The reaction mixture was worked up in the usual way to give 0.035 g. of crude alcohol, m.p. 145–149°, which on crystallization from ethanol–water gave 0.024 g. of cholesterol-7β-t, m.p. 148–149°.

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(26) 1 millicurie is equivalent to 22.2×10^8 d.p.m.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, PURDUE UNIVERSITY]

Alkaline Degradation of Periodate-oxidized Starch[1]

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RECEIVED DECEMBER 6, 1958

Periodate-oxidized whole corn starch is treated with oxygen-free sodium hydroxide solution and with lime-water at room temperature. The degradation products are mainly acids, among which glycolic, DL-2,4-dihydroxybutyric and formic acids predominate. Carbon dioxide also is produced and a Cannizzaro rearrangement product is detected. The mechanism of the degradation reactions is discussed.

For some time it has been recognized that oxidized polysaccharides containing carbonyl groups readily undergo depolymerization in alkali with formation of acidic products. This effect is of importance in the industrial processing of both cellulose and starch. In particular, the chemical behavior of periodate oxystarch is of current interest because methods for producing this modified starch have become available at reduced cost through improved regeneration of periodate.[2]

Alkali-lability of periodate oxystarch often has been noted[3-5] and the formation of acids during alkaline degradation has been attributed to Cannizzaro rearrangements.[4,5] However, recent work[6] on monosaccharide analogs of periodate oxidation products of starch and cellulose have indicated that the alkaline degradation proceeds predominantly by way of a β-alkoxycarbonyl elimination at C5 of the original D-glucose unit. This has been confirmed in the case of periodate oxycellulose,[7] which yields glycolic and DL-2,4-dihydroxybutyric acids as the major acidic products of alkaline degradation.

This is a report on the major acidic products which result from treatment of periodate-oxidized whole starch with sodium hydroxide or lime-water. Glycolic, DL-2,4-dihydroxybutyric and formic acids are identified as the predominant acidic products in both short-term and long-term alkali treatments, and their approximate relative yields under various conditions are shown in Table I.

It is concluded that the degradation is similar to that occurring with periodate oxycellulose and takes place predominantly as shown (I → V + VI). The 1→6 links of the amylopectin component would be expected to have little effect on the final products since these linkages become part of a glyoxal hemiacetal structure in the intermediate III and this type of structure is known to be alkali-labile. The α-alkoxycarbonyl group in intermediate II is known to inhibit further alkaline rearrangements under normal circumstances,[8] but in the completely oxidized starch, the group R is equivalent to intermediate IV. The degradation of IV as shown will therefore remove the α-al-

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(8) J. Kenner and G. N. Richards, *ibid.*, 2921 (1956).