Chemical Actions of Ionising Radiations in Solution. Part XII. The Action of X-Rays on Some Steroids in Organic Solvents.*

By BRYAN COLEBY, MAX KELLER, and JOSEPH WEISS.

[Reprint Order No. 4535.]

When irradiated by *X*-rays, cholestane, cholest-2-ene, cholest-5-ene, and cholestan-3-one suffer little change. Cholesterol in methanol gives 3β-hydroxycholest-5-en-7-one, cholest-5-ene-3β : 7β-diol and cholestane-3β : 5α : 6β-triol; in acetone and dioxan cholesterol α- and β-oxide (5α : 6α-epoxycholestan-3β-ol and 5β : 6β-epoxycoprostan-3β-ol) and cholest-5-ene-3β : 7β-diol are formed. Cholesteryl acetate in acetic anhydride gives cholesteryl acetate α- and β-oxide. A mixture of cholesteryl acetate α- and β-oxide in aqueous acetic acid gives 3β-acetoxycholestane-5α : 6β-diol. Of cholesteryl benzoate α- and β-oxide in methanol, acetone, or dioxan, only the α-form is attacked to give 3β-benzoyloxy-cholestane-5α : 6β-diol; in methanol some of the α-form is converted into the β-oxide. Cholest-4-en-3-one in methanol gives 6β-hydroxycholest-4-en-3-one. Hydroxylation in the 6-position presumably also occurs when progesterone is irradiated in methanol, but only the isomerisation product, *allo*pregnane-3 : 6 : 20-trione, could be isolated in a pure condition. Cortisone acetate, when irradiated in methanol or aqueous acetic acid, undergoes some hydrolysis in addition to chemical change.

EARLIER papers of this series (Keller and Weiss, *J.*, 1950, 2709; 1951, 25, 1247) reported the effect of *X*-radiation on solutions of some steroids in water, dilute alkali, and aqueous acetic acid. Because of the general insolubility of most steroids in aqueous solvents it was necessary, in the case of cholesterol, for example, to use the succinyl half-ester in order to effect solution. Before the products could be separated chromatographically this ester group had to be removed by hydrolysis, and operations of this kind might make it difficult to isolate any relatively labile products. It was therefore decided to study the use of organic solvents in which the steroid itself was soluble. An additional justification for this step was the earlier observation that, in some cases, irradiation in glacial acetic acid and in aqueous solution gave essentially very similar products; also, use of organic solvents led to cleaner products and we were often able to account for 95—100% of the starting material. Further, yields from acetone solutions were comparatively high.

In these experiments, in particular as in some of our earlier ones, our main concern has been to isolate and characterise the products, and to this end we have employed large doses of *X*-rays (205 kv; 15 ma; 15 hr.). The solvents used were methanol, acetone, dioxan, acetic acid, and acetic anhydride. Little is known about the primary processes occurring during irradiation of these solvents with high-energy radiations. Prevost-Bernas, Chapiro, Cousin, Landler, and Magat (*Discuss. Faraday Soc.*, 1952, **12**, 98) attempted to use the stable free radical, diphenylpicrylhydrazyl, to obtain information about the free radicals formed during irradiation: it was possible, to some extent, to "count" the number of free radicals by this method, but, to date, there has been no indication of their identity. In methanol it is probable that hydroxyl radicals can be

* Part XI, *J.*, 1953, 3091.

formed according to the process, $CH_3{\cdot}OH \longrightarrow CH_3 + OH$; we have not observed any effects so far to indicate that the alternative reaction, $CH_3{\cdot}OH \longrightarrow CH_3O + H$, occurs, although such a process is by no means excluded. For the other solvents interpretation of the primary processes is less easy and no attempt at this is made here.

To gain some idea of the mode of attack on the steroid molecule, we irradiated solutions of cholestane and of some steroids possessing only one simple functional group. After long irradiation of cholestane, cholest-2- and -5-ene, or cholestan-3-one in methanol, 92—98% of the starting material was recovered, the other products being unidentified oils and, in two cases, minute quantities of unidentified crystals. Thus, the steroid skeleton is fairly stable under our conditions. However, when two functional groups are present, the attack becomes more significant, but only at positions which are known to be sensitive to ordinary chemical reagents. Previous work has shown that Fenton's reagent (Clemo, Keller, and Weiss, *J.*, 1950, 3470) and *X*-irradiation of aqueous solutions of cholesterol derivatives (Keller and Weiss, *J.*, 1950, 2709) give 7-oxocholesterol and cholestane-3β : 5α : 6β-triol. In methanol, the same products were isolated, together with 7β-hydroxycholesterol. Isolation of the diol provides some evidence for the mechanism previously suggested (*J.*, 1950, 2709) for the formation of 7-oxocholesterol. The 7-hydroxy-compound was also isolated after irradiation of cholesterol in acetone and dioxan, where the formation of cholesterol α- and β-oxide was observed but not that of the 7-ketone or the *trans*-triol. The *trans*-triol might have been formed *via* the α- and β-oxide : this reaction could occur since the *trans*-triol was obtained when the α-oxide was irradiated in methanol, acetone, dioxan, or aqueous acetic acid, but it is so slow that the epoxide cannot be the main intermediate in the formation of the *trans*-triol in methanol and aqueous acetic acid. The mechanism previously suggested (*J.*, 1950, 2709) is more probable.

Methanol was chosen as a solvent for our work with cholest-4-en-3-one, progesterone, and cortisone acetate, because in this solvent some similarity to irradiation of aqueous solutions has been noted. From cholest-4-en-3-one small amounts of 6β-hydroxycholest-4-en-3-one were isolated, together with unidentified oils (cf. Weiss, Ciba Foundn. Coll. Endocrin., 1953, **7**, 142). This 6-hydroxy-compound is easily transformed into cholestane-3 : 6-dione which has been isolated from pigs' testes (Prelog, Tagmann, Lieberman, and Ruzicka, *Helv. Chim. Acta*, 1947, **30**, 1080). Progesterone did not give the 6-hydroxy-compound, but the isomerisation product, *allo*pregnane-3 : 6 : 20-trione, was characterised. Hydroxylation in the 6-position is known to occur biologically (Eppstein, Meister, Peterson, Murray, Leigh, Lyttle, Reineke, and Weintrub, *J. Amer. Chem. Soc.*, 1953, **75**, 408). Cortisone acetate was less affected by irradiation in methanol or acetic acid, only the starting material (87—92%) and some oil being isolated. The position of this oil on the chromatographic column and its behaviour in the sulphuric acid chromogen test (Zaffaroni, *J. Amer. Chem. Soc.*, 1950, **72**, 3828) indicated that it was a mixture of cortisone and its acetate, but the more sensitive methods of paper chromatography (Bush, *Biochem. J.*, 1952, **50**, 370) showed several distinct substances to be present : these have not yet been identified.

Experimental

Irradiation was effected as described by Keller and Weiss (*J.*, 1950, 2709; 1951, 25). M. p.s were taken on a Kofler block. Alumina used for chromatography was from Savory and Moore (London), standardised according to Brockmann; "acid-washed alumina" was prepared by digesting this alumina with 18% hydrochloric acid at 80—90° for 2 hr., washing it extensively with water, then with methanol, and drying it at 130° for 2 hr.

Results are shown in the Table. The steroid solution (usually 200 ml.) was irradiated (205 kv; 15 ma) for, usually, 15 hr. The solvent was then removed under reduced pressure and the residual solids were dried in a vacuum and examined by elution chromatography (Reichstein and Shoppee, *Discuss. Faraday Soc.*, 1949, **7**, 305); the ratio, steroid : alumina : volume of each fraction of eluant, was 1 g. : 30 g. : 100 ml. All the solvents were purified by distillation, light petroleum (b. p. 60—80°) and benzene over sodium hydroxide, ether and chloroform over calcium chloride, and methanol over magnesium methoxide. Identities were established by crystallisation, m. p. and mixed m. p., and the positions on the chromatographic column. Additional work on the products is recorded in the following notes.

TABLE

Substance irradiated	Solvent, concn. (%)	Products *	Yield † (%)	Eluting solvents ‡	Note
Cholestane	MeOH, 0·15	S.M.	98	Pet	1
		Oil	6	Pet–C_6H_6; C_6H_6–Et_2O	
Cholest-2-ene	,,	S.M.	97	Pet	2
		Oil	5	Pet–C_6H_6; C_6H_6–Et_2O	
		M. p. 219—225°	0·5	Et_2O	
Cholest-5-ene	,,	S.M.	93	Pet	3
		Oil	8	Pet–C_6H_6; C_6H_6–Et_2O	
		M. p. 190—216°	0·5	Et_2O	
Cholestan-3-one	MeOH, 0·25	S.M.	96	Pet; Pet–C_6H_6	
		Oil	5	Mixt. C_6H_6, Et_2O, $CHCl_3$	
Cholesterol	,,	S.M.	86	C_6H_6–Et_2O (9 : 1)	4
		3β-Hydroxycholest-5-en-7-one	5	,, (4 : 1)	
		Cholest-5-ene-3β : 7β-diol	4·5	$CHCl_3$; $CHCl_3$–MeOH (49 : 1)	
		Cholestane-3β : 5α : 6β-triol			
		M. p. 168—173°	4·5	$CHCl_3$–MeOH (24 : 1)	
,,	$COMe_2$, 0·28	Oil (*A*)	2	C_6H_6–Et_2O (9 : 1)	5
		S.M.	63	,, (4 : 1)	
		5α : 6α-Epoxycholestan-3β-ol	23	,, (3 : 7)	
		5β : 6β-Epoxycoprostan-3β-ol			
		Cholest-5-ene-3β : 7β-diol	7	Et_2O–$CHCl_3$ (1 : 1); $CHCl_3$	
		Oil (*B*)	4	$CHCl_3$–MeOH	
,,	Dioxan, 0·33	S.M.	84	C_6H_6–Et_2O (9 : 1)	6
		5α : 6α-Epoxycholestan-3β-ol	3	,, (4 : 1)	
		5β : 6β-Epoxycoprostan-3β-ol	4		
		Oil (*A*)	5	,, (7 : 3; 3 : 2)	
		Cholest-5-ene-3β : 7β-diol	4	,, (1 : 1; 3 : 7)	
		Oil (*B*)	13	Mixt. Et_2O, $CHCl_3$, MeOH	
Cholesteryl acetate	Ac_2O, 0·23	S.M.	76	Pet–C_6H_6 (4 : 1; 7 : 3)	7
		3β-Acetoxy-5β : 6β-epoxycoprostane	13	,, (13 : 7; 1 : 1)	
		Cholesteryl acetate α- and β-oxides	6	,, (2 : 3)	
		3β-Acetoxy-5α : 6α-epoxycholestane	2	,, (3 : 7; 1 : 9)	
Cholesterol α- + β-oxide	90% AcOH, 0·07	After acetyln. :			8
		3β-Acetoxy-5β : 6β-epoxycoprostane	19	C_6H_6	
		3β-Acetoxy-5α : 6α-epoxycholestane	14	C_6H_6–Et_2O (9 : 1; 4 : 1)	
		Oil	16	,, (7 : 3; 1 : 1)	
		3β : 6β-Diacetoxycholestan-5α-ol	44	Et_2O	
3β-Benzoyloxy-5α : 6α-epoxycholestane	MeOH, 0·14	Cholesteryl benzoate α- and β-oxide	10	Pet–C_6H_6 (7 : 3)	9
		S.M.	82	,, (3 : 2); C_6H_6	
		3β-Benzoyloxycholestane-5α : 6β-diol	7	C_6H_6–Et_2O (2 : 3)	
,, ,,	$COMe_2$, 0·14	S.M.	89	Pet–C_6H_6; C_6H_6	10
		3β-Benzoyloxycholestane-5α : 6β-diol	9	C_6H_6–Et_2O (2 : 3)	
		Cholestane-3β : 5α : 6β-triol	1	$CHCl_3$–MeOH (19 : 1)	
,, ,,	Dioxan, 0·17	S.M.	94	Pet–C_6H_6 (4 : 1); C_6H_6	11
		3β-Benzoyloxycholestane-5α : 6β-diol	6	C_6H_6–Et_2O (3 : 2)	
		Oil	7	$CHCl_3$–MeOH (19 : 1)	

TABLE—(*Continued.*)

Substance irradiated	Solvent, concn. (%)	Products *	Yield † (%)	Eluting solvents ‡	Note
3β-Benzoyloxy-5β : 6β-epoxycoprostane	MeOH, 0·14	S.M.	91	Pet–C_6H_6 (4 : 1; 1 : 4)	12
		M. p. 144—151°	4	C_6H_6–Et_2O (4 : 1)	
		Oil	2	,, (2 : 3)	
,, ,,	$COMe_2$, 0·14	S.M.	95	Pet–C_6H_6 (4 : 1; 3 : 7)	11
		Oil	1·5	$CHCl_3$–MeOH (19 : 1)	
,, ,,	Dioxan, 0·17	S.M.	98	Pet–C_6H_6 (9 : 1; 3 : 7)	11
		Oil	3	$CHCl_3$–MeOH (15 : 1)	
Cholest-4-en-3-one	MeOH, 0·25	S.M.	95	C_6H_6	13
		Oil	2	C_6H_6–Et_2O (3 : 1)	
Cholest-4-en-3-one	MeOH, 0·25	6β-Hydroxycholest-4-en-3-one	4	C_6H_6–Et_2O (1 : 1)	
		Oil	3	$CHCl_3$–Et_2O	
Progesterone	MeOH, 0·23	S.M.	96	C_6H_6–Et_2O	14
		M. p. 156—210°	0·12	Et_2O–$CHCl_3$ (4 : 1; 7 : 3)	
Cortisone acetate	MeOH, 0·1	S.M.	87	C_6H_6–Et_2O (1 : 4); Et_2O–$CHCl_3$ (2 : 3; 1 : 4)	15
		Oil (?cortisone and its acetate)	8	$CHCl_3$; $CHCl_3$–MeOH (19 : 1)	
,, ,,	85% AcOH, 0·1	S.M.	92	C_6H_6–Et_2O; Et_2O–$CHCl_3$	16
		Oil (?cortisone and its acetate)	8	$CHCl_3$; $CHCl_3$–MeOH (19 : 1)	

* S.M. = starting material; un-named products were not identified.
† Crude wt.-%. ‡ Pet = light petroleum.

Notes.—1. The positions of the oils on the chromatographic column were consistent with the introduction of one hydroxyl or keto-group.

2. The oils were similar to those obtained from cholestane. The compound, m. p. 219—225° (from methanol), had a position on the column which indicated introduction of two oxygen atoms.

3. As 2.

4. The identity of the oxocholestenone was established by its position on the column, mixed m. p., and preparation of its acetate, m. p. 158—160°. The mixture of cholestenediol and cholestanetriol was collected from a few irradiation experiments and separated chromatographically on a small column of alumina. Elution with ether–chloroform (1 : 1) gave, first, 7β-hydroxycholesterol, m. p. 148—156° (from methanol), and then a substance, m. p. 215—224° (from methanol), mainly cholestane-3β : 5α : 6β-triol. Identity of the diol was shown by the blue coloration with antimony trichloride in chloroform (specific for 7-hydroxy-Δ^5-steroids; Barr, Heilbron, Parry, and Spring, *J.*, 1936, 1437), and by preparation of the dibenzoate (benzoyl chloride–pyridine; chromatography), m. p. and mixed m. p. 171—174° (cf. Schoenheimer and Evans, *J. Biol. Chem.*, 1936, **114**, 276). None of the 7α-hydroxy-compound was isolated, either before or after benzoylation. The m. p. of the cholestanetriol is lower than that quoted in the literature (239°) but its identity was confirmed by preparation of the 3 : 6-diacetate (acetic anhydride–pyridine), m. p. and mixed m. p. 167—168°. The unidentified compound, m. p. 168—173° (Found, on a sample dried at 60°/high vac. for 5 hr.: C, 77·46; H, 10·9. Calc. for $C_{27}H_{46}O_3$: C, 77·5; H, 11·0%), had a position on the column indicating the presence of three hydroxyl groups.

5. The acetone was purified by refluxing and distillation over potassium permanganate, followed by redistillation over calcium chloride. Irradiation was for 14 hr. The oil (*A*) gave poorly defined crystals, m. p. 55—67°, from methanol–ether. It is not impure cholest-4-en-3-one, since the semicarbazone prepared by Casanova and Reichstein's method (*Helv. Chim. Acta*, 1950, **33**, 417) had m. p. 202—218°, whereas that from cholestenone melts at 234—235° (Jones, Wilkinson, and Kerlogue, *J.*, 1942, 391). The mixture of cholesterol α- and β-oxide had m. p. 100—126° and was separated chromatographically after benzoylation (pyridine–benzoyl chloride; room temp.; 16 hr.). Chromatography on alumina and elution with light petroleum–benzene (4 : 1) gave 3β-benzoyloxy-5β : 6β-epoxycoprostane, m. p. and mixed m. p. 167—170°

(from methanol–ether), $[\alpha]_D^{17}$ +20·3° ± 4° (*c* = 1·40 in $CHCl_3$) (Baxter and Spring, *J.*, 1943, 613, give $[\alpha]_D^{20}$ +16°) (Found: C, 80·5; H, 10·0. Calc. for $C_{35}H_{50}O_3$: C, 80·6; H, 9·9%). Further elution with light petroleum–benzene (1 : 1) gave 3β-benzoyloxy-5α : 6α-epoxycholestane, m. p. and mixed m. p. 168—169·5° (from methanol–ether). The ratio of β-oxide : α-oxide was about 65 : 35. The identity of the 7β-hydroxycholesterol was confirmed as in Note 4. Again, no 7α-hydroxycholesterol could be isolated. Since the yield of cholesterol epoxides is high (in one experiment irradiation for 25 hr. gave a 42% yield of the oxides) secondary attack to give the *trans*-triol almost certainly occurs, but no crystalline acetate or benzoate could be prepared from the oil (*B*).

6. Dioxan was purified according to Eigenberger's method (*J. pr. Chem.*, 1931, **130**, 75; quoted by Weissberger and Proskauer, "Organic Solvents," Oxford Univ. Press, 1939, p. 139). The cholesterol oxides, as obtained initially after chromatographic separation of the irradiation products, were present in a mixture with some unchanged cholesterol; the amounts of each form of the oxide and unchanged cholesterol (as shown in the Table) were ascertained after benzoylation and quantitative chromatographic separation, as in Note 5. The cholestenediol was identified as in Note 4. In the unesterified form, 7-hydroxycholesterols are very difficult to crystallise, owing to the formation of solvates (cf., *e.g.*, Wintersteiner and Ritzmann, *J. Biol. Chem.*, 1940, **136**, 696), which explains the great variation in the m. p.s of samples isolated. No crystalline derivatives could be prepared from the oils (*A*) and (*B*). Some (*B*) (~2%, eluted with ether) gave a blue colour with antimony trichloride in chloroform, indicating the presence of 7-hydroxycholesterol.

7. The m. p.s of the acetates of cholesterol and both of its epoxides are fairly close, but mixed m. p.s sufficed to indicate the identity of the products. The β-oxide acetate was analysed (dried in a high vacuum) (Found: C, 78·6; H, 11·2. Calc. for $C_{29}H_{48}O_3$: C, 78·3; H, 10·9%) and had $[\alpha]_D^{18}$ −1·2° ± 3° (*c*, 0·974 in $CHCl_3$). The identity of cholesteryl acetate α-oxide, $[\alpha]_D^{20}$ −44·2° ± 3° (*c*, 0·732 in $CHCl_3$), was confirmed by preparation of the free sterol : 50 mg. of the acetate were refluxed with methanol (8 ml.) and 0·5N-sodium hydroxide (1·5 ml.) for 2 hr. Water precipitated the sterol which, when washed, dried, and crystallised from alcohol, had m. p. and mixed m. p. 132—134°.

8. The irradiated compound was a 1 : 1 mixture of cholesteryl acetate α- and β-oxide. During vacuum-evaporation of the solvent the temperature was kept below 35°. The crude product was acetylated (pyridine–acetic anhydride) before chromatographic separation.

9. 3β-Benzoyloxy-5α : 6α-epoxycholestane and -5β : 6β-epoxycoprostane were prepared by Spring and Swain's method (*J.*, 1943, 613) but separation was achieved chromatographically by a method similar to that of Barton and Miller (*J. Amer. Chem. Soc.*, 1950, **72**, 370). The solution was irradiated for 5 hr. The mixture of the cholesteryl benzoate α- and β-oxide, separated from the irradiation products, gave a blue thermoluminescence on melting, characteristic of this mixture when one or other form is present in excess. The β-form must be formed during the irradiation since the starting material was chromatographically homogenous.

10. The solution was irradiated for 5 hr. The 3β-benzoyloxycholestane-5α : 6β-diol was analysed (Found: C, 78·1; H, 10·2. Calc. for $C_{35}H_{52}O_4$: C, 77·9; H, 9·9%).

11. The solution was irradiated for 5 hr. Acid-washed alumina was used for the chromatographic separation.

12. The solution was irradiated for 5 hr. On recrystallisation from methanol–ether, the unidentified compound had m. p. 114—156°, but it could not be obtained in a form sufficiently pure for analysis. Its m. p. was depressed by 10—15° by admixture with cholesteryl benzoate α- or β-oxide.

13. The unidentified oils obtained in this irradiation all had the characteristic absorption of a 3-keto-Δ^4-steroid at 240 mμ. The identity of the 6β-hydroxycholestenone was confirmed by preparation of the semicarbazone in methanol at room temperature, chromatography in chloroform on alumina, and crystallisation from methanol; it had m. p. 213—216° (Ellis and Petrow, *J.*, 1939, 1078, give m. p. 221°). 6β-Hydroxycholest-4-en-3-one (10 mg.) was refluxed for 30 min. with 95% ethanol (5 ml.) containing 5 drops of concentrated hydrochloric acid. Partial evaporation followed by the addition of water gave crude cholestane-3 : 6-dione which was dried, dissolved in a few ml. of chloroform, and filtered through alumina. Evaporation followed by crystallisation from ether–light petroleum gave needles, m. p. 167—170·5° (Butenandt and Schram, *Ber.*, 1936, **69**, 2289, give m. p. 169°). The crystals showed negligible light absorption in methanol at 240 mμ. A specimen for analysis was recrystallised from acetone–ether and dried under high vacuum over phosphoric oxide for several hours at 120° (m. p. 170—171°) (Found: C, 80·5; H, 11·1. Calc. for $C_{27}H_{44}O_2$: C, 80·9; H, 11·1%), $[\alpha]_D^{20}$ 15° ± 3° (*c*, 1·418 in $CHCl_3$).

The dioxime of the diketone was prepared in boiling aqueous ethanol and crystallised from ether–hexane as needles, m. p. 205—208° (Ruzicka, Bosshard, Fischer, and Wirz, *Helv. Chim. Acta*, 1936, **19**, 1147, give m. p. 208—210°).

14. The solution was irradiated for 30 hr. Repeating the experiment several times gave 6 mg. of the compound, m. p. 156—210°. Further crystallisation did not improve the m. p. The material was recovered unchanged after attempted acetylation at room, or at higher, temperature. The compound was then refluxed with absolute alcohol (2 ml.) and concentrated hydrochloric acid (3 drops) for 30 min. Water was added and the precipitate centrifuged, washed with water, and dried. The yellow powder obtained was dissolved in chloroform and filtered through a small amount of alumina. The chloroform was removed and the colourless residue crystallised from acetone–ether to give short prisms (1·3 mg.), m. p. 226—230°, not depressed on admixture with *allo*pregnane-3 : 6 : 20-trione, kindly supplied by Winthrop-Stearns, Inc., Rensellaer, N.Y.

15. Acid-washed alumina was used for the chromatographic separation. The oil had ultra-violet absorption characteristic of a 3-keto-Δ^4-steroid, and when the oil had been treated with concentrated sulphuric acid its absorption (Zaffaroni, *J. Amer. Chem. Soc.*, 1950, **72**, 3828) was indistinguishable from that given by cortisone or its acetate. When this oil was run on a paper chromatogram (Bush, *Biochem. J.*, 1952, **50**, 370) testing with aqueous sodium hydroxide (specific for 3-keto-Δ^4-steroids) showed several spots, one of which moved at the same speed as cortisone acetate and one at the same speed as cortisone, three being more polar than cortisone.

16. Temperatures were below 40° during evaporation of solvent. Remarks as in Note 15 apply.

We gratefully acknowledge financial assistance from the Medical Research Council in support of this work, and two of us (B. C. and M. K.) are indebted to the Medical Research Council for the award of maintenance grants.

KING'S COLLEGE, UNIVERSITY OF DURHAM,
NEWCASTLE-ON-TYNE, 1.

[*Received, July 29th*, 1953.]