THE EFFECT OF ULTRA-VIOLET IRRADIATION ON CARDENOLIDES

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Solutions of digoxin, digoxigenin and digitoxigenin in ethanol were subjected to ultra-violet irradiation in the absence of oxygen. Chromatographic analysis showed that the starting material was transformed into several other compounds some of which may be intermediate in the reaction. Cardiotonic activity was absent from the irradiated material. There was no longer the characteristic unsaturated lactone ring of the cardenolides after irradiation. Upon analysis a crystalline compound isolated from irradiated digoxigenin corresponded to the original molecule with an added molecule of water.

IT is a pharmacopoeial recommendation[1] that cardiac glycosides should be stored in the absence of light and it has also been noted in these laboratories that digitalis extracts left exposed to light show decreased cardiac activity. These observations suggested an examination of the effect of ultra-violet irradiation upon these drugs and the present paper describes some preliminary observations.

Recently developed paper chromatographic methods have enabled the progress of such changes in the molecule to be followed by observing the decrease in concentration of the starting material with time of irradiation and the appearance of new spots upon the chromatogram corresponding to the irradiation products as they are formed.

The method consisted of the irradiation of dilute solutions of various cardenolides in 90 per cent ethanol by immersing a quartz lamp into the solution, the whole being maintained at or close to 0°. Oxygen is excluded from the system by bubbling nitrogen slowly through the alcoholic solution, and samples are removed at intervals for chromatographic study.

The first compound studied was a pure (chromatographically homogenous) sample of digoxin.

In preliminary experiments with a long time of irradiation it was found that a marked decrease of digoxin occurred after 20 to 30 minutes of irradiation, and with a solution of 50 mg. in 100 ml. of solvent the loss was clearly detectable after 15 minutes.

Parallel with the fall in digoxin three or possibly four new compounds were seen on the chromatograms. These were designated Digoxin Irradiation products A, B, C and D in order of increasing R_F values on the chromatograms. Two of these, DIA and DIB which were much more polar and stayed nearer the starting line formed the main products of the reaction. The quantities of these materials increased as irradiation continued whereas a third compound DIC, travelled more closely with digoxin and could be detected only after approximately half the digoxin had been changed. The amount of this material also increased progressively

and made it difficult to determine the point at which all the digoxin had disappeared.

From several such experiments it appeared that, under the conditions employed, all the digoxin was transformed in 5 to 6 hours and half was converted after 1 to 2 hours. When irradiation was further extended to 20 to 24 hours the material DIA decreased as also did DIC, whereas material DIB remained unaffected or even increased slightly in amount.

In addition to these three products it was often possible to detect a further one or two spots placed between DIA and DIB on the chromatogram but the concentration of these materials was low and they further decreased with prolonged irradiation.

A sample of digoxin which was irradiated in this way for 24 hours was tested for cardiotonic activity using the isolated papillary muscle of the cat as described by Cattell and Gold[2]. With digoxin a marked positive inotropic effect is observed on this preparation with concentrations of 1 in 10^7 to 2 in 10^7 but with the irradiated solution no effect at all could be detected when the concentration (equivalent to the digoxin originally present) was increased to 1 in 10^5. It is therefore considered that the irradiation products were cardiotonic.

Attempts were made to isolate the reaction products and to characterise them as crystalline substances but great difficulty was encountered. Upon working up the irradiated solution a waxy material was obtained which was partly soluble in water and could not be obtained in crystalline form by repeated solution in different solvents. Column chromatography on diatomaceous earth also failed to yield a crystallisable substance but from certain strongly polar fractions of the eluate a white noncrystalline powder was obtained which was homogenous and very nearly pure when tested by paper chromatography. This corresponded to the material DIB.

This substance on analysis gave an empirical figure which corresponded to digoxin with one molecule of water added. Since this material has not been obtained crystalline the melting point was indefinite and we would not regard it as a pure compound.

To simplify the problem the aglycones digoxigenin and digitoxigenin were also irradiated as it was considered that the absence of the sugar moiety might make the production of crystallisable materials possible.

The common feature of the irradiation of all the three substances was the production of compounds showing increased polarity, the loss of pharmacological activity and the loss of a positive reaction to the Raymond test indicating that the unsaturated lactone ring is no longer present after irradiation.

In the case of digitoxigenin three compounds were formed which were all more polar than the starting material and these were designated TGA, TGB and TGC in order of ascending R_F values. The intensity of the spots TGA and TGC increased until all the digitoxigenin was transformed (4 to 5 hours) whereas TGB reached a maximum after $1\frac{1}{2}$ to 2 hours irradiation and then decreased quickly indicating that this material is itself sensitive to ultra-violet irradiation and may be an intermediate product.

Efforts to isolate these reaction products in pure form by fractional crystallisation or partition column chromatography have not so far been successful and only the two most polar substances have been separated from the remaining reaction mixture.

The behaviour of digoxigenin upon irradiation which is, in general, characteristic of all the three cardenolides studied is illustrated in Figure 1.

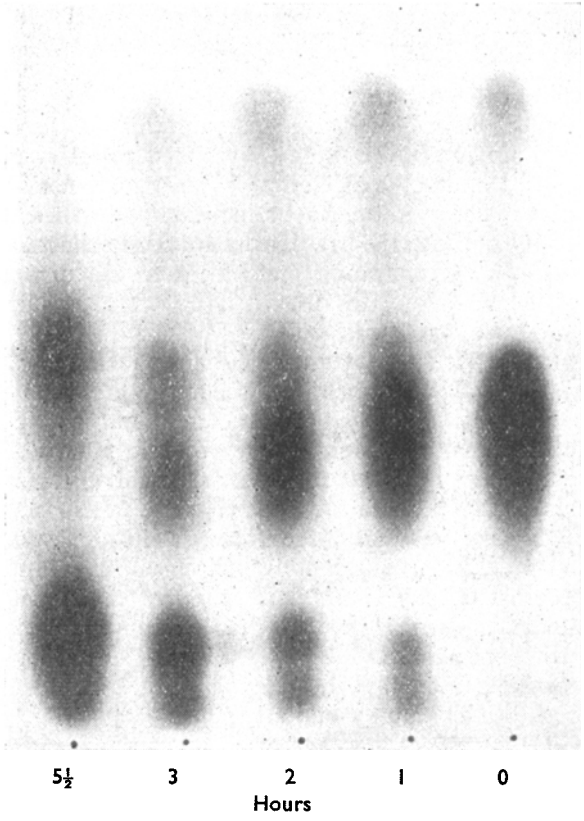

FIG. 1. Ultra-violet fluorescence chromatogram showing the change at various times in composition of digoxigenin solution by irradiation.

Three compounds are formed as the result of the irradiation, two of which are more polar than digoxigenin and stay near the starting line. The third travels slightly ahead but is seen clearly only when the digoxigenin spot is greatly reduced, after 3 or 5½ hours in the figure. It will be seen that practically none of the original material remains after 5½ hours.

The separation of the reaction products of digoxigenin have met with partial success. A total of 750 mg. was subjected to irradiation and the reaction materials collected. This material was separated by repeated chromatography on partition columns followed by several recrystallisations and finally a substance corresponding to compound B was

obtained. This crystallised well from chloroform-ethyl acetate and approximately 30 mg. was obtained with a melting point 238 to 240°. Analysis corresponded to $C_{23}H_{36}O_6$ and indicated a molecule of digoxigenin with an added molecule of water. The Raymond test was negative so that we might presume this product of irradiation was formed either by opening the lactone ring coupled perhaps with isomerisation at the double bond or by the addition of the elements of water across the double bond of the unsaturated lactone ring.

We are at present engaged in a further characterisation of this compound and in the isolation of the other reaction products of the irradiated mixture.

Experimental

A solution of the cardenolide dissolved in 90 per cent ethanol, purified for spectrophotometry, was irradiated.

The alcoholic solution was placed in a cylindrical vessel approximately 48 mm. in diameter and 150 mm. high. The vessel was surrounded with coolant maintained at −5° and the alcoholic solution stirred and kept free of oxygen by bubbling through it a slow stream of nitrogen, previously washed by passage through Fieser's solution, and then a wash bottle containing 90 per cent ethanol. The nitrogen was allowed to flow for an hour to rid the solution of dissolved oxygen before irradiation was commenced. The lamp was a U-shaped Hanovia low pressure quartz lamp approximately 25 mm. wide and 150 mm. high, running at 300 V. 30 m.A. This was vertically mounted and immersed in the solution. After introducing the solution the opening of the container was tightly plugged with cotton wool to prevent the diffusion of air into the container which was kept under a slight positive pressure by the constant stream of nitrogen. No difficulty was experienced in keeping the temperature of the solution within the limits ±2° by adjusting the bath temperature to approximately 5° below the temperature of the irradiated solution.

Digoxin

The first experiments were made with 100 ml. of a solution of 50 mg. digoxin in 90 per cent ethanol irradiated for 12 to 18 hours. In later experiments the concentration of digoxin was increased to 100 mg./100 ml. solvent and the irradiation time reduced to 6 to 7 hours at 0° to +4°. To isolate the reaction product the alcoholic solution was treated with 2 to 3 ml. of a concentrated solution of sodium bicarbonate and the solvent was removed under reduced pressure at 40 to 45°. Upon repeated trituration with small quantities of water the product turned brittle and powdery and could be separated by filtration or centrifuging. The composition of this material differed very little from the original irradiation mixture. Efforts to purify it by extraction or crystallisation from various solvents were not successful. The material was very soluble in ethanol and methanol, and partly soluble in ether. A further quantity of material was found to have dissolved in the aqueous extract mentioned above from which it could be largely recovered by salting out with NaCl; this fraction also failed to yield a crystalline compound.

Encouraged by the good separations achieved on paper, the material obtained in the several previous irradiation experiments (620 mg.) was mixed and submitted to partition chromatography using a column 26 mm. in diameter and packed to a height of 150 mm. with 35g. of diatomaceous earth (purified "Hyflo") mixed with an equal weight of water saturated with benzene. The solvents used for elution and the fractions obtained are those shown in Table I.

TABLE I

FRACTIONATION OF IRRADIATED DIGOXIN BY PARTITION COLUMN CHROMATOGRAPHY

Fraction No.	Solvent used (100 ml.)	mg. recovered	Composition by paper chromatogram
1	Benzene	47	mixture
2	,,	20	,,
3	Benzene : chloroform 9 : 1	22	1 spot
4	8 : 2	21	
5	7 : 3	18	1 spot different from 3
6	6 : 4	27	2 spots
7	1 : 1	36	1 spot
8	3 : 7	60	,,
9	2 : 8	54	2 spots } the main corresponds to
10	Benzene : chloroform : ethyl acetate 2 : 75 : 0·5	64	,, } the spot of fraction 5
11	2 : 7 : 1	44	,,
12	2 : 6 : 2	15	,,
13	2 : 6 : 3	8	
14	1 : 6 : 3	6	
15	Chloroform : ethyl acetate 7·5 : 2·5	6	
16	Chloroform : ethyl acetate : methanol 7·5 : 2·5 : 0·5	21	3 spots

Some more material was recovered by eluting the diatomaceous earth with hot chloroform—methanol mixture 1 : 1. The material of fraction 8 was purified by reprecipitation from a solution in chloroform by light petroleum. It represented then a white fine non-crystalline powder which melted over a range of 165 to 170° with previous sintering at approximately 155°. This substance gave a positive Keller-Kiliani and negative Raymond test. It was homogenous when chromatographed on paper and analysed C, 61·9; H, 8·7 per cent, calculated for $C_{41}H_{66}O_{15}$ C, 61·6; H, 8·3.

Fraction 7 when purified as above gave an amorphous powder melting in a range 165 to 170° with preceding sintering at approximately 150°. On paper chromatography it showed the presence of a trace of a more polar component. It gave the following analytical figures: C, 62·4; H, 8·8 per cent.

The material from fractions 9 to 12 was treated together by reprecipitation from methanol and water but no homogenous compounds were isolated from the different fractions.

Digoxigenin

In a preliminary experiment, using 100 mg. digoxigenin in 100 ml. ethanol (90 per cent) (purified and freed from acids and aldehydes) it was shown that after an irradiation of 6 hours practically all the starting material had been converted into secondary products which comprised three main components. Based on this experience 669 mg. of digoxigenin

dissolved in 140 ml. ethanol 90 per cent were irradiated for 6 hours and the solution was evaporated under reduced pressure at a temperature below 45°. The residual viscous oil was combined with the corresponding material from the pilot experiment and dissolved in ethanol; this solution was taken up into 4 g. of diatomaceous earth. After removing the solvent the dry mixture in the diatomaceous earth was placed on a column (28 mm. diameter) packed with 35 g. of diatomaceous earth mixed with 35 ml. of water previously saturated with benzene. The column was eluted with 100 ml. portions of solvent and the fractions collected as shown in Table II.

TABLE II

FRACTIONATION OF IRRADIATED DIGOXIGENIN BY PARTITION COLUMN CHROMATOGRAPHY

Fraction No.	Solvent composition	mg.	Substance composition by paper chromatography
1	Benzene	3·5	3 spots
2	,,	6·0	,,
3	Benzene : chloroform 9·5 : 0·5	7·0	,,
4	9 : 1	9·5	,,
5	8 : 2	16·3	2 main spots and 1 smaller one
6	7 : 3	16·1	,, ,,
7	1 : 1	35·0	,, ,,
8	2 : 8	118·1	2 main spots and trace of a third
9	Chloroform	131·5	1 main spot, 1 small and a trace of a third
10	Chloroform : ethyl acetate 9·25 : 0·25	79·8	1 main spot, 1 very small and a trace of a third
11	9 : 1	166·9	1 main spot and trace of a second
12	8 : 2	104·7	,, ,,
13	6 : 4	38·7	1 main spot and a small second
14	Chloroform : ethyl acetate + methanol 6 : 4 ½ per cent	18·9	
15	6 : 4 1 ,,	11·6	
16	6 : 4 3 ,,	7·1	
17	6 : 4 5 ,,	4·4	
18	6 : 4 5 ,,	4·7	

The material from fractions 11 and 12 together totalling 266 mg. was rechromatographed on a column as described before and 22 fractions using 50 ml. solvent of increased polarity were collected.

Fractions 15 to 19 eluted with chloroform containing increasing proportions of ethyl acetate (1 to 5 per cent) carried the bulk of material (140 mg.) and showed on the chromatogram that they consisted mainly of one compound contaminated by a slight admixture of two other components.

This material was rechromatographed as before using 30 ml. of similar solvents for each fraction.

Three corresponding fractions eluted with chloroform containing 1 to 3 per cent ethyl acetate yielded 37·5, 28·8 and 19·2 mg. of material. The material from these fractions was twice recrystallised separately from a mixture of chloroform and ethyl acetate. From the middle fraction a crystalline compound showing a sharp m.p. 238 to 240° was recovered and gave the following analytical figures: C, 66·65, 66·8; H, 8·8, 8·8 per cent, for $C_{23}H_{36}O_6$ required C, 67·3; H, 8·9 per cent. The specific rotation $[\alpha]_D = +22·1°$ (*c*, 1·14 per cent in ethanol).

From the third fraction a compound was recovered which melted less sharply at 238 to 241° with previous sintering at approximately 234°. It analysed for C, 66·2; H, 8·9 per cent.

All these compounds gave a negative Raymond test but a blue fluorescent spot with trichloroacetic acid in the reaction of Svendsen and Jensen.

References

1. U.S. Pharmacopeia XV.
2. Cattell and Gold, *J. Pharmacol.*, 1938, **62**, 116.