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PROCESS OF PREPARATION OF STEROL OXIDATION PRODUCTS CONTAINING TWO CARBONYL GROUPS

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The oxidation of cholesterol, whose double bond is temporarily protected, was described by several authors already. This method of oxidation and the subsequent restoration of the double bound yielded cholestenone. In quite an analogous manner, sitosterol was oxydised to sitostenone. No record was found, however, showing, that diketones can be produced and separated from the raw oxydation mixture of such or similar sterols. I have now found that sterols or sterol derivatives containing at least one free hydroxyl groups whose carbon atom configuration between the carbon atoms 1–22, both inclusive, is identical with the corresponding carbon atom configurations of either cholesterol, or allo-cholesterol or epicholesterol, if treated—under temporary protection of the double bond—with oxidants able to split aliphatic chains, yield oxidation-mixtures which after restoring of the double bond, contain oxidation products having two carbonyl groups; these products can be obtained either in form of highly enriched extracts or in pure crystalline state.

As starting materials protected temporarily in the double bond the dibromo-derivatives of sterols, e. g. in case of cholesterol dibromo-cholesterol may be used. As oxidants permanganates, such as potassium permanganate, or chromic acid or chromates can be employed. It is of advantage if the oxidation is carried out in presence of mineral acids as, if so, the temperature can be kept lower than in the absence of those.

The valuable products of the present process are obtained by removing the acid substances from the oxidized starting material, subsequent restoring of the double bond, followed by elimination of starting material oxidized only in position 3, that is to say, e. g. in case of dibromo-cholesterol of cholestenone. The remaining prepurified product contains hormonally active oxydation products, such as androstendione and corpus luteum hormone which can be enriched in extracts suitable for practical purposes and, furthermore, can also be isolated in crystalline form.

From the crude reaction product obtained by oxidation I remove first the acid substances reestablish then the double bond and remove then starting material oxidized only in position 3—e. g. in case of dibromo-cholesterol the cholestenone—by recrystallization from acetone, or in some other way, e. g. by distribution between water-miscible and water-immiscible solvents such as methyl alcohol and petrol ether, in which case the bulk of the cholestenone will pass into the petrol ether layer. The product thus freed from the greater part of cholestenone is then subjected either directly or after removal of constituents less soluble in methyl alcohol to a chromatographical selection, e. g. in benzene solution using aluminum oxide as absorbent. The sterol oxidation products containing two carbonyl groups pass the aluminiumoxyde column unaffected and remain in the filtrate. Further enrichment was achieved by extracting the petrol ether solution of the residue of this filtrate with aqueous 90 per cent methyl alcohol, thus collecting the greater part of the oxidation products containing two carbonyl groups in the methyl alcohol layer.

In order to obtain the hormones in crystalline state, the benzene filtrate, which passed the aluminium oxide column is evaporated and the resulting residue is dissolved in petrol ether. This solution is subjected to a repeated chromatographical selection, preferably on aluminium oxide and—as the case might be—in the presence of a suitable dye as e. g. sudan-III, which acts as an indicator. In this case, however, the hormones are absorbed by and remain in the aluminium oxide column; the distribution of the products containing two carbonyl groups is marked by the indicator used. The top-layers of the column will contain androstendione practically free from corpus luteum hormone, while the lower layers will contain this hormone contaminated with androstendione. After separating the different layers of the column each layer containing the hormone and the hormone-mixture are extracted separately by ether, methyl alcohol or alcohol. Further purification can be effected by evaporating the ethereal or alcoholic solutions thus obtained, and by distribution of the respective residues between aqueous methyl alcohol and petrol ether, in course of which the water-content of the methyl alcohol is gradually increased, the two hormones are collected in the methyl alcohol layer.

These methods of distribution and of chromatographical selection, repeated several times if necessary, yield concentrates which contain the hormones practically free from each another; from these concentrates the hormones may be isolated—eventually after foregoing distillation in high vacuô—in pure crystalline form.

The products prepared from the top and from the lower layers of the chromatographical column may be freed from the indicator and subjected to a repeated chromatographical selection, eventually after foregoing purification by the distribution method mentioned above. This second chromato-

graphic selection is preferably performed with a benzene-petrol ether solution of the hormones and with aluminium oxide as adsorbent. The first batches of the solution passing the column contain corpus luteum hormone, whereas the later ones and the column itself render androstendione.

The fractions, obtained by the separation methods described further above, containing the compounds with two carbonyl groups, can also be brought into reaction with ketone-reagents, e. g. with semicarbazide. The ketone-derivatives thus obtained are subjected to fractionating crystallization, e. g. in case of semicarbazones to repeated recrystallization from alcohol, by which the mixture can be separated into several fractions enriched in compounds containing two carbonyl groups, these fractions being now more separated from each other. From these ketone-derivatives fractions the ketones are subsequently regenerated and, if necessary, distilled in high vacuô.

The above mentioned refining processes may be employed in other sequences; the single stages may be repeated or combined according to need, by which sterol oxidation products containing two carbonyl groups are obtained in form of enriched extracts or of pure crystalline state.

Side-fractions obtained in course of the refinement process not quite freed from sterol oxidation products containing two carbonyl groups may be collected and re-introduced into the next batch at a suitable stage of the purification process.

The details of the new processes are explained by the following examples:

Examples

1. One kg of cholesterol is dissolved in 14 kg of carbon-tetrachloride and brominated with 0.42 kg. of bromine dissolved in 1.5 kg. of carbon-tetrachloride (Solution "A").

In a mixture of 9 kgs of glacial acetic acid and of 1 kg. of water 2.57 kgs of chromic acid (chromium trioxide) are dissolved by heating (Solution "B").

Into a mixture of 8.46 kgs of carbon-tetrachloride, 19 kgs of glacial acetic acid and 3.53 kgs of concentrated sulphuric acid the solutions "A" and "B" are introduced, under energetic cooling and stirring, in such a way that the velocity of addition of both solutions should be the same; the volumina of the added solutions should be about 2 litres per hour and the rise of temperature should not exceed 10°C. After further stirring for another half an hour 1.4 kg of methyl alcohol is added in small portions and stirring is continued for a period of 2 hours. Subsequently 3.3 kgs of sodium acetate are introduced and stirred for 30 minutes. The solution which should show a neutral reaction against congo-paper, an acid reaction against lithmus and a positive test with potassium iodide starch paper, is evaporated in vacuum at a temperature not exceeding 65°C. The resulting residue is redissolved in warm water and extracted several times with benzene. The united benzene-extracts are evaporated and washed subsequently with water, 10 per cent sodium hydroxide solution, diluted sulphuric acid and again with water until neutral reaction, dried over anhydrous sodium sulphate and evaporated in vacuô in the presence of a little zinc dust.

The remaining neutral bromide-product is dissolved in glacial acetic acid of the same weight and de-brominated under cooling and stirring by zinc dust of the same weight. The addition of zinc dust depends on the time and on the cooling. It lasts about one hour and a half, during which the temperature of the mixture is kept at about 30°C and is allowed to rise to 35–40°C only when the reaction is nearly over. When the total amount of zinc dust is introduced, glacial acetic acid is added, the weight of which being the same as that used originally for dissolving the bromine-product, and stirring is continued for 80 minutes at 50°C. Then the mixture is cooled to 15–20°C, diluted with benzene under continued stirring and the resulting solution washed—after removal of the solid parts—with water diluted sodium hydroxide solution and again with water until the reaction becomes neutral. The benzene solution, after being dried over anhydrous sodium sulphate, is evaporated in vacuô and the resulting residue is dried at a pressure of 1 mm at 70°C.

In order to remove cholestenone, the residue is recrystallized from acetone of the same weight. The mother-liquor of the cholestenone crystals is freed from the solvent in vacuô and yields 200 grams of a residue which is subsequently redissolved in methyl alcohol of the same weight and chilled to −12°C under stirring. Two layers are formed. The upper layer is removed by decantation and the one below is extracted 3–4 times with methyl alcohol. The methyl alcoholic solutions are united and evaporated in vacuô until constance of weight. The residue weighs about 100 grams and exhibits strong activity in the corpus luteum test.

This said residue (X) can be further purified e. g. by chromatographical analysis. One-hundred grms of this products are dissolved in benzene and the solution made to pass a tube filled with 1 kgs of aluminium oxide prepared sec. Brockmann and the tube is washed out subsequently with benzene. The resulting benzene filtrate is evaporated, the residue thus obtained redissolved in petrol ether and re-chromatographed on aluminium oxide. This time the diketones remain in the column absorbed. If a small amount of sudan-III is added to the original petrol ether solution, the division of the column, after the chromatogram has been developed, provides no difficulty. The content of the tube, beginning from the top down to the bottom of a well marked dark red colour ring, is taken out and subjected to further treatment. In the upper third part of the column is androstendione present, practically free from corpus luteum hormone, whereas in the lower two third parts corpus luteum hormone is found contaminated with androstendione. These parts of the column are separated from each other, and from each the adsorbed products are eluted by several extractions with boiling methyl alcohol (ethyl alcohol or ether). The elutes are evaporated to a small volumen, the dye-stuff is removed by active charcoal and cholestandione is precipitated by chilling and, if necessary, by seeding. The mother-liquor thus obtained from the elute of the upper part of the column is evaporated; it is rich in androstendion. The mother-liquor similarly obtained from the elute of the lower two thirds of the column is evaporated; it contains androstendion and corpus luteum hormone in a concentrated state.

2. One kgr of cholesterol, dissolved in 27 kgrs of carbon-tetrachloride, is brominated with 0.42 kgr of bromine, dissolved in 1.6 gr of carbon-tetrachloride. The resulting solution of dibromo-cholesterol is energetically stirred or shaken with 41 litres of a 5 per cent aqueous solution of potas-

sium permanganate and with a mixture of 1.05 kgr of concentrated sulphuric acid and of 0.6 kgr of water, at 25–30°. When the aqueous layer becomes decolorized, 2.1 kgr of finely powdered potassium permanganate is added in small portions and the stirring or shaking is maintained until the aqueous layer becomes again decolorized. At this point 2.5 kgrs of sodium bisulphite are introduced in order to remove manganese dioxide, after which the carbon-tetrachloride layer is separated and the aqueous layer is repeatedly extracted with carbon-tetrachloride. The united carbon-tetrachloride solutions are washed with sodium hydroxide solution and subsequently with water and evaporated in vacuô in the presence of a little calcium carbonate. The resulting residue is debrominated according to the description given in Example 1.

In order to remove cholestenone the debrominated product is dissolved in petrol ether of about 5 times its weight and extracted 6–7 times with 90 per cent methyl alcohol of the same weight. Cholestenone is collected in the petrol ether layer.

The aqueous methyl alcohol solutions are evaporated until a resinic substance precipitates, which together with the solution is extracted 3 times with benzene; the benzene solutions are washed with water in order to remove the last traces of methyl alcohol. The benzene solutions yield on evaporation about 100 grms of a residue of high corpus luteum hormone activity which can still be increased by way of chromatography. This is realized by redissolving the residue in benzene of 5 times of its weight and made to pass a tube filled with aluminium oxide of 15 times of its weight and washing the column with benzene. The benzenic solution yields on evaporation about 60 grms of a straw yellow resin, which is redissolved in a mixture of 300 cc. of petrol ether and of 90 cc. of methyl alcohol and mixed under shaking with 10 cc. of water, in order to effect further enrichment. The petrol ether layer separates and is extracted in subsequence 4 times aqueous 90 per cent methyl alcohol using 100 cc. on each occasion. The methyl alcoholic fractions are united and evaporated. The resulting residue is taken up in benzene and washed with water in order to remove last traces of methyl alcohol. On evaporation 10 grms of a straw coloured resin is obtained which in the physiological test exhibits a very high corpus luteum hormone activity. This product is an excellent starting material for isolation of the diketones in crystallized state.

3. The procedure is essentially the same as described in Example 1 as far as obtaining the products readily soluble in methyl alcohol. Further purification was effected in the following way:

One-hundred grms of the product (X) readily soluble in methyl alcohol were dissolved in a mixture of 500 cc. of petrol ether and of 90 cc. of methyl alcohol and distributed between these two solvents by addition of 10 cc. of water under thorough shaking. The petrol ether layer thus separating was extracted 6 times with 90 per cent methyl alcohol using 100 cc. of the latter each times. The methyl alcohol extracts were united and evaporated and yield 60 grms of a straw coloured resin which was subjected in subsequence to chromatographical selection.

The benzene solution of this resin was made to pass a tube filled with Brockmann's aluminium oxide and the column was washed with benzene. The filtrate was concentrated to a smaller volumen and again chromatographed in a tube filled with aluminium oxide. Thus the benzene filtrate yields 20 grms of a residue when evaporated.

This residue was redissolved in 100 cc. of petrol ether, 0.25 grm. of sudan-III was added, after which it was made to pass a tube filled with 400 grms. of Brockmann's aluminium oxide the column being washed subsequently with petrol ether. After the chromatogram is fully developed the column is divided according to the description given in Example 1. The lower two third layers were extracted with methyl alcohol. The extract yields on evaporation 6.5 grms. of a residue showing strong corpus luteum hormone activity in the physiological test. The layer of the upper third part of the column contain a product which exhibits strong activity in the vesicular test.

4. The product obtained according to the process described in Example 1 was subjected to a further selection in the following way:

Eighteen grms. of the product obtained from the lower two third parts of the petrol ether chromatogram of Example 1 were dissolved in 800 cc. of absolute alcohol and heated for two hours on a steam-bath with semicarbazide acetate, prepared from 21.3 grms. of semicarbazide hydrochloride and 26 grms. of sodium acetate. During this procedure the mixture was concentrated to about 400 cc. After allowed to stand for 24 hours in the ice-chest, the crystals of crude semicarbazone were filtered by suction triturated with hot water and, subsequently, after drying, with ether. Nine grms. of a product were obtained, decomposing at 215–219° C.

From this semicarbazone 7.4 grms. were recrystallized in 300 cc. of boiling alcohol, yielding 4.7 grms. of a substance which decomposes at 225° C. This product, if recrystallized from 1000 cc. of boiling alcohol and allowed to stand at 0° C. for a longer period, yields a product which weighs 1.5 grms. and decomposes above 300° C. The mother-liquor of these crystals contain another product which decomposes at 221° C.

The substance of the decomposition-point of above 300° C. was heated with 40 cc. of 96 per cent alcohol and 5 cc. of pyruvic acid for 2 hours, in course of which a clear solution is obtained. This was poured into diluted alkali and extracted with ether. The ethereal extract was washed subsequently 3 times with sodium hydroxide solution, with diluted sulphuric acid and, finally, with water until neutral reaction. Evaporation of the ether gave 1.3 grm. of a substance which was distilled at a mercury pressure of 0.001 millimetre from an air-bath of 165–170° C. temperature. The distillate, which was recrystallized from a mixture of ether and petrol ether, is corpus luteum hormone.

The semicarbazone-fraction of the decomposition-point 221° C., obtained as mentioned above by evaporation of the alcoholic mother-liquor, yield on fission of the semicarbazone and on subsequent distillation in high vacuô crystalline androstendione.

5. The product obtained by the process described in Example 3 from the lower two third of the chromatogram can be re-purified in the following way:

From this substance 6.5 grms. were dissolved in a mixture of 60 cc. of methyl alcohol and of 60 cc. of petrol ether and in small portions and under shaking 6.65 cc. of water were added. To the methyl alcohol layer thus separating 60 cc.

of petrol ether and, while shaking carefully, 8.35 cc. of water were added. The methyl alcohol layer obtained in this way was mixed again with 60 cc. of petrol ether and with 10.75 cc. of water, shaken thoroughly and then the petrol ether layer was removed. The methyl alcohol solution yields 2.02 grms. of a substance possessing a specific rotation of $(\alpha)_D+109°$ dissolved in absolute alcohol (Fraction "A").

The united petrol ether solutions yield 4.68 grms. of dry residue which were dissolved in a mixture of 100 cc. of petrol ether and of 90 cc. of methyl alcohol and distributed by addition of 10 cc. of water while shaking. The separating methyl alcohol layer was mixed with 100 cc. of petrol ether and afterwards 12.5 cc. of water were added in portions. The methyl alcohol layer again separates and was mixed again with 100 cc. of petrol ether and with 16 cc. of water this latter being added in small portions under shaking. The methyl alcohol contains a residue possessing the specific rotation of $(\alpha)_D+104°$ (Fraction "B").

The re-united petrol ether solutions obtained while preparing fraction "B" contain 3.98 grms. of a product which was subjected to the like treatment described above. The resulting methyl alcoholic solutions render 0.69 grm. of a substance of the specific rotation of $(\alpha)_D+81.2°$ (Fraction "C").

The fractions "A", "B" and "C" are united (3.38 grms.) and re-dissolved in a mixture of 10 cc. of benzene and of 30 cc. of petrol ether and the solution is made to pass a tube filled with 60 grms. of Brockmann's aluminium oxide that was previously soaked with benzene and petrol ether in the ratio 1:3. The chromatogram was developed by washing with the same solvent-mixture and the dry residue respectively the concentration of the passing solution was continually controlled. The concentration increases at the beginning, after which it falls rapidly and later decreases very slowly. From the passed solution nine fractions were taken, 15 cc. each, and evaporated.

The first fraction after being evaporated shows no tendency of crystallization; the residue of the second and the third fractions crystallize from an ether-petrol ether mixture and yield corpus luteum hormone. The fourth fraction still contains corpus luteum hormone but also androstendione. The fifth to ninth fractions render crystalline androstendione. However, the bulk of the androstendione remains absorbed in the column and is to be eluted with ether and recrystallized from the same solvent.

The last elutions with ether contain also a by-product, melting at 217°C.

6. The product obtained according to the process described in Example 3 from the lower two third parts of the chromatogram can be purified in the following way:

From this substance 6.5 grms are dissolved in 180 cc. of alcohol and boiled for 2 hours with semi-carbazide acetate that was prepared from 7.7 grms of semicarbazide hydrochloride and 9.5 grms of sodium acetate, meanwhile concentrated to 130 cc. and allowed to crystallize over night. The crystals are filtered by suction, extracted with boiling water, then dried and finally washed with ether. In this way 5.6 grms of a semicarbazone are obtained of the decomposition-point of 275–280°C. This semicarbazone of 5.6 grms are suspended in 75 cc. of alcohol, 10 cc. of pyruvic acid are added and the mixture is boiled for three hours and a half, in course of which clear solution is obtained. In subsequence the mixture was poured into 200 cc. of a 10 per cent solution of potassium carbonate, diluted with water and extracted with ether. The ethereal extracts are washed with diluted solutions of potassium carbonate, with diluted sulphuric acid and with water until neutral reaction was reached and finally evaporated. The product thus obtained weighs 3.4 grms, showing a specific rotation of $(\alpha)_D+98°$.

In a mixture of benzene and petrol ether of the ratio 1:3 3.4 grms of this product are dissolved and chromatographed in a tube containing 75 grms of Brockmann's aluminium oxide. The concentration of the solution passing the column increases at the beginning but decreases considerably later on. The filtrate renders 1.02 grm of a residue which gives rise to a substance of the melting-point 126–127° if recrystallized from a mixture of ether and petrol ether. The mother-liquor of these crystals and the ethereal elutions of the column give 2.14 grms of a product which yields, on repeated chromatographical analysis as used in example 5 for purifying the united fractions A, B and C and recrystallization, corpus luteum hormone and androstendione.

The experimental conditions of the examples given above may be varied in several instances. For instance, instead of cholesterol also other sterols e. g. allocholesterol, epicholesterol, sitosterol and the likes may be used as a starting material.

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