acid also increases on storage it is not yet possible to say in which form the vitamin is primarily synthesized.

The nature of the changes underlying the phenomenon is still obscure. The evidence strongly suggests, but does not absolutely prove, that the vitamin is formed from a chemically related precursor during storage. The increase of vitamin C in an apple of about 22 g. during 7 months of storage was found to be equivalent to about 2 mg. of ascorbic acid, the total content reaching a level of about 7 mg. per fruit. Some years the increase is somewhat greater (cf. Fig. 1). On the other hand, the total vitamin content per apple picked normally in October of the same year was about 24 mg. The total quantity of the vitamin present in these immature fruits was therefore very much less than that found in a Bramley's Seedling apple, picked at the usual time. If it be assumed that the precursor in young apples is fully utilized during storage, the extra vitamin found in the apple matured on the tree must in consequence have either been synthesized in the fruit from a precursor supplied from other parts of the plant as the apple developed or, more probably, it must have reached the fruit in a preformed state. Even if the latter were the case, the process of formation of the vitamin may be similar to that occurring during the storage of the young apple. These and other points which arise cannot be settled on the basis of existing knowledge and the information so far obtained is insufficient to shape a comprehensive hypothesis. Nevertheless the data presented here are consistent enough to serve as a basis for a more detailed inquiry, the results of which might eventually throw some light on the physiological function of *l*-ascorbic acid not only in the plant but also indirectly in the animal organism.

SUMMARY

Vitamin C is synthesized in Bramley's Seedling apples during storage at 3°. The capacity for this synthesis diminishes with the age of the fruit.

We should like to express our thanks to Dr R. G. Hatton, Director of East Malling Research Station, for facilities placed at our disposal during the years 1939–42 and to Mr T. N. Hoblyn for statistical guidance in the work.

REFERENCE

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Metabolism of Steroids

4. KETONIC ACIDS DERIVED FROM CHOLIC ACID

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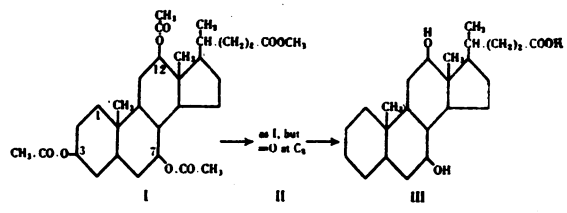
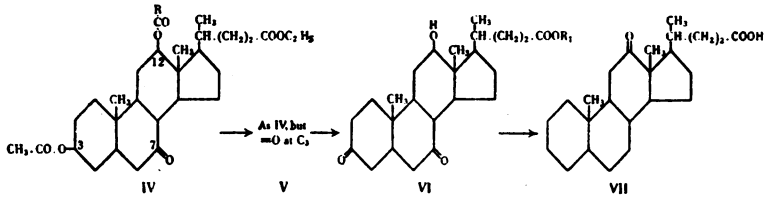
It was shown by Schmidt, Hughes, Green & Cooper (1942) that cholic acid is converted by *Alcaligenes faecalis* into a mixture of ketonic acids, one of which is certainly dehydrocholic acid. Moreover, the many experiments of Japanese workers on the administration of bile acids and their derivatives to several species of animal suggest that ketonic bile acids may be of importance in animal metabolism. It has therefore seemed of interest to complete the series of compounds derived by oxidation of the secondary hydroxyl groups of cholic acid to carbonyl.

With the steric configuration of cholic acid, six hydroxy-keto acids are theoretically possible. Of these, three are listed by Sobotka (1938), and the remaining three are 7:12-dihydroxy-3-keto- (Sihn, 1938), 3:12-dihydroxy-7-keto- (Gallagher & Long, 1943; Haslewood, 1943), and 12-*hydroxy*-3:7-*diketocholanic acid*, now described. The preparation of this last acid depended on a method used by Reichstein and his collaborators (e.g. Lardon & Reichstein, 1943), by which C_3 steroid esters are 'alcoholyzed' by acidified alcohols to give the corresponding C_3 hydroxy compounds. Thus, methyl triacetyl cholate (I) was converted by methanol-HCl, followed by chromic acid oxidation, to *methyl* 7:12-*diacetoxy*-3-*keto-cholanate* (II), which was identified by conversion to 7:12-dihydroxy-cholanic acid (III).



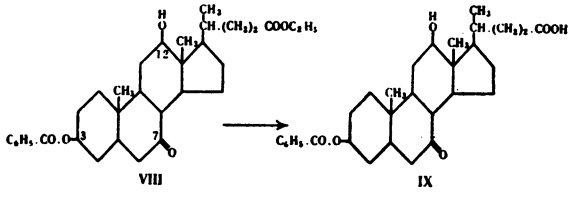
I II III

Similarly, acid alcoholic treatment, followed by oxidation, of *ethyl* 12-(p-*nitro*)-*benzoyloxy*-3-*acetoxy*-7-*keto-cholanate* (IV, $R=C_6H_4NO_2$) and of *ethyl* 12-(3′:5′-*dinitro*)-*benzoyloxy*-3-*acetoxy*-7-*keto-cholanate* (IV, $R=C_6H_3(NO_2)_2$) gave the corresponding 3:7-diketo-compounds (V). Conditions were not found in which the 3:5-*dinitro-benzoate* (V, $R=C_6H_3(NO_2)_2$), as prepared, could be hydrolyzed without the formation of intensely coloured products, but the p-*nitro-benzoate* (V, $R=C_6H_4NO_2$) was hydrolyzed in appropriate conditions to a crude acid which was re-esterified to give *ethyl* 12-*hydroxy*-3:7-*diketo-cholanate* (VI, $R_1=C_2H_5$). This ester was converted to the required 12-*hydroxy*-3:7-*diketo-cholanic acid* (VI, $R_1=H$). It was also oxidized to ethyl dehydrocholate, and reduced, with saponification, to a product which on chromic oxidation gave a high yield of 12-keto-cholanic acid (VII).



IV V VI VII

The 3-*mono-benzoyl* (VIII, m.p. 139°), like the 3-*mono-acetyl* derivative of ethyl 3:12-dihydroxy-7-keto-cholanate, was easily made: when heated with dilute aqueous-alcoholic K_2CO_3, it was partly converted into a substance (m.p. 250°) which is probably 12-*hydroxy*-3-*benzoyloxy*-7-*keto-cholanic acid* (IX).



VIII IX

EXPERIMENTAL

All melting-points are uncorrected. Elementary micro-analyses were by Dr H. Nisbet and Mr A. T. Macdonald, Edinburgh. *Abbreviations*: H-test = Hammarsten's HCl test, as described (Haslewood, 1943); l.p. = light petroleum (b.p. 40–60°); 20% CrO_3 = a solution made by dissolving 20 g. of CrO_3 in the minimal amount of water and making up to 100 ml. with acetic acid.

Ethyl 3:12-*dihydroxy*-7-*keto cholanate* (improved method of preparation). Cholic acid (1 part) was oxidized and the product purified as described (Haslewood, 1943), except that water (70 parts) and NaCl (15 parts) were used for the first precipitation. The final product, precipitated with 2N-H_2SO_4 (10 parts) and NaCl (7·5 parts), was collected after 16 hr., washed with water, and dried *in vacuo* over H_2SO_4. This crude product could be used directly for reduction to deoxycholic acid. It (25 g.) was heated under reflux for 2 hr. with ethanol (75 ml.) containing H_2SO_4 (7·5 ml.). An ether extract of the cooled diluted mixture was washed with water, ammonia, and water, dried ($CaCl_2$) and evaporated. The residue, twice crystallized from 90% (v/v) methanol in water, finally gave colourless prisms (9·5 g.) which, after drying at 60–70°, melted at 155–157°. A further crop could be obtained from the mother-liquors. 3:12-Dihydroxy-7-keto-cholanic acid, from this ester, had m.p. c. 80° from aqueous acid (Haslewood, 1943) or, after drying, m.p. 196–197°, from ethyl acetate (Gallagher & Long, 1943).

Ethyl 12-*hydroxy*-3-*acetoxy*-7-*keto-cholanate*. A solution of the above ester (20 g.) in dry benzene (80 ml.) with pyridine (20 ml.) was cooled in ice and treated gradually, with shaking, with a 2:1 (v/v) mixture (40 ml.) of dry benzene and acetyl chloride. After 2½ hr. at 0°, the mixture was diluted with water, and ether extracted. The extract, washed with dilute HCl, ammonia, and water, and dried ($CaCl_2$), was evaporated and the residue crystallized by adding l.p., collected, washed with l.p., and dried at 60–70°. (Yield, 18·6 g.; m.p. 142–145°.) Recrystallized from l.p./benzene, the ester formed glistening white leaflets, m.p. 147–148°; H-test, yellow. (Found: C, 70·5; H, 9·6. Calc. for $C_{28}H_{44}O_6$: C, 70·6; H, 9·3%.) This substance (0·1 g.) in acetic acid (1 ml.) was treated at 20° with 20% CrO_3 (0·5 ml.). After 15 min., water was added, and the precipitated solid collected, washed, dissolved in benzene and the solution dried ($CaCl_2$). Evaporation of the filtered benzene gave *ethyl* 3-*acetoxy*-7:12-*diketo-cholanate*, which crystallized from l.p./benzene as white leaflets, m.p. 145–147°; H-test, negative. (Found: C, 70·4; H, 8·9. Calc. for $C_{28}H_{42}O_6$: C, 70·8; H, 8·9%.) This ester was hydrolyzed with alkali to 3-hydroxy-7:12-diketo-cholanic acid.

Ethyl 12-(p-*nitro*)-*benzoyloxy*-3-*acetoxy*-7-*keto-cholanate* (IV, $R=C_6H_4NO_2$). A mixture of ethyl 12-hydroxy-3-acetoxy-7-keto-cholanate (10 g., m.p. 142–145°) in pyridine (50 ml.) with freshly made *p*-nitro-benzoyl chloride (10 g.) was allowed to stand at 20° for 2 hr. and then heated at 100° for 1 hr. The product was diluted with aqueous HCl and the precipitated solid collected, washed, and stirred with aqueous K_2CO_3. The undissolved material was collected, washed, and twice crystallized from 90% (v/v) methanol in water. (Yield, 8·4 g.; m.p. 155–157°.) Recrystallization from l.p./ether gave small white needles, m.p. 159–160°; H-test, negative. (Found: C, 67·8; H, 7·9; N, 2·5. Calc. for $C_{35}H_{47}O_9N$: C, 67·2; H, 7·6; N, 2·2%.)

Ethyl 12-(p-*nitro*)-*benzoyloxy*-3:7-*diketo-cholanate* (V, $R=C_6H_4NO_2$). The above ester (2 g.), ethanol (20 ml.), and 10N-HCl (4 ml.) were boiled together under reflux for 3 hr. Cold water (4–5 vol.) was then added and the product left overnight. The precipitated solid was collected, washed

with water, and dissolved in acetic acid (20 ml.). To the solution was added 20% CrO_3 (2 ml.), and, after 30 min., water (4–5 vol.). After 24 hr., the solid was collected, well washed, and dissolved in benzene, which was then dried ($CaCl_2$), filtered, and evaporated. The residue crystallized from 90% (v/v) methanol in water in small needles. Yield, 0·8 g.; m.p., 153–156°, raised to 160–161° by recrystallization from l.p./benzene and l.p./ether. (Found: C, 68·7; H, 7·4; N, 2·6. Calc. for $C_{33}H_{43}O_8N$: C, 68·2; H, 7·5; N, 2·4%.)

Ethyl 12-*hydroxy*-3:7-*diketo-cholanate* (VI, $R_1 = C_2H_5$). The above ester (2·6 g.; m.p. 153–156°) was added to a fresh solution of KOH (2·6 g.) in methanol (260 ml.) and the mixture boiled under reflux for 3 hr. The cooled solution was acidified with acetic acid and evaporated to dryness, finally *in vacuo*. To the residue was added ethanol (25 ml.) containing H_2SO_4 (2·5 ml.) and the mixture was refluxed for 2 hr. and then diluted with water and excess $NaHCO_3$. Undissolved solid was collected after 24 hr., washed with water, and dissolved in ethanol. The residue from evaporation of the filtered solution was washed with l.p. and crystallized from ethanol/water (charcoal), when it gave long colourless needles (1·5 g.), m.p. 168–169°, not raised by recrystallization or sublimation at 220°/0·1 mm. (Found: C, 72·6; H, 9·1. Calc. for $C_{26}H_{40}O_5$: C, 72·2; H, 9·3%.) This ester gave a clear yellow in the H-test, and a strongly positive reaction when mixed with alkali and an alcoholic solution of purified *m*-dinitrobenzene, which had been freed from the *o*-isomer with glucose and alkali.

A solution of the above ester (50 mg.) in acetic acid (0·5 ml.) was left for 15 min. with 20% CrO_3 (0·1 ml.). Dilution with water gave a solid which was collected, washed, and crystallized from dilute ethanol, giving long needles (40 mg.), m.p. 218–220°, undepressed by authentic ethyl dehydrocholate. Another 50 mg. of ester were converted in the usual way to the 3:5-dinitro-benzoyl ester, m.p. 200–202°, undepressed by V ($R = C_6H_3(NO_2)_2$).

Ethyl 12-hydroxy-3:7-diketo-cholanate (0·2 g.), alcoholic sodium ethoxide (from 0·2 g. of Na in 4 ml. ethanol), and hydrazine hydrate (0·2 ml. of 95–99%) were heated in a sealed metal bomb at 195–210° for 4 hr. The contents of the cooled bomb were washed out with water and the acidified mixture gave a solid product which, however, could be made to yield only 4–5 mg. of crystals, m.p. *c.* 140°. The main product was dissolved in acetic acid (1 ml.) and treated in the cold with 20% CrO_3 (0·2 ml.). Almost at once, the whole mixture appeared to crystallize, and dilution with water, followed by recrystallization of the precipitate from dilute ethanol, gave white leaflets (0·1 g.) whose m.p. was raised by one recrystallization to 183–184°, undepressed by authentic 12-keto-cholanic acid. (Found: C, 77·1; H, 10·2. Calc. for $C_{24}H_{38}O_3$: C, 76·95; H, 10·2%.)

12-*Hydroxy*-3:7-*diketo-cholanic acid* (VI, R_1 = H). A mixture of ethyl 12-hydroxy-3:7-diketo-cholanate (0·5 g.), acetone (5 ml.), water (5 ml.), and 10N-HCl (0·5 ml.) was refluxed for 5 hr. Acetone was removed by evaporation and the remaining aqueous product diluted to *c.* 25 ml. with water and excess $NaHCO_3$. After a thorough shaking, with warming, the solution was left for some hours, filtered from a little insoluble material, and the clear filtrate acidified with HCl and saturated with NaCl. After 16 hr., the solid product was collected, washed, and dissolved in acetone, which was filtered and evaporated. The residue readily crystallized with a little ether, yielding white needles (0·3 g.). These could be recrystallized from chloroform-ether, and 12-*hydroxy*-3:7-*diketo-cholanic acid* was finally obtained as white needles, m.p. 165–166°, with an apparent further change in viscosity at 175°. This melting-point was consistently obtained and was unaffected by recrystallization. The acid slowly crystallized after precipitation by acidification of an aqueous alkaline solution. It gave similar colour reactions to those of the above ethyl ester: with H_2SO_4 it gave a yellow colour and in the Liebermann-Burchard test a reddish orange. (Found: C, 70·9; H, 8·9. Calc. for $C_{24}H_{36}O_5$: C, 71·3; H, 9·0%.)

Ethyl 12-(3′:5′-*dinitro*)-*benzoyloxy*-3-*acetoxy*-7-*keto-cholanate* (IV, $R = C_6H_3(NO_2)_2$) was obtained in a manner exactly analogous to the preparation of the *p*-nitro-derivative. Yield, 0·75 g. from 1·3 g. of ethyl 12-hydroxy-3-acetoxy-7-keto cholanate. The new ester crystallized readily from 90% (v/v) methanol in water in long almost colourless needles, m.p. 171–172°. (Found: C, 62·6; H, 7·4; N, 4·5. Calc. for $C_{35}H_{46}O_{11}N_2$: C, 62·7; H, 6·9; N, 4·2%.)

Ethyl 12-(3′:5′-*dinitro*)-*benzoyloxy*-3:7-*diketo-cholanate* (V, $R = C_6H_3(NO_2)_2$) was prepared from the above substance in the same way as the corresponding *p*-nitro-derivative. It separated from dilute ethanol or ethanol in almost colourless needles, m.p. 203–204°. (Found: C, 63·1; H, 6·7; N, 4·7. Calc. for $C_{33}H_{42}O_{10}N_2$: C, 63·3; H, 6·7; N, 4·5%.)

Ethyl 12-*hydroxy*-3-*benzoyloxy*-7-*keto-cholanate* (VIII). Ethyl 3:12-dihydroxy-7-keto-cholanate (1 g.) was dissolved in a mixture of dry benzene (4 ml.) and pyridine (1 ml.). To the solution, cooled to 15°, 2 ml. of a mixture of dry benzene (3 ml.) and benzoyl chloride (2 ml.) were added slowly, with agitation. After 4 hr. at 16–18°, the product was diluted with water and ether extracted. The extract was washed with dilute HCl, ammonia, and water, dried ($CaCl_2$), and evaporated. The residue formed crystals at once with l.p. and this product (1 g.) was recrystallized from l.p./benzene, giving colourless leaflets, m.p. 138–139°; H-test, negative. (Found: C, 73·35; H, 8·6. Calc. for $C_{38}H_{46}O_6$: C, 73·6; H, 8·6%.) This ester (0·2 g.) was oxidized in acetic acid (2 ml.) with 20% CrO_3 (0·2 ml.), and the product purified as in the case of ethyl 3-acetoxy-7:12-diketo cholanate. Thus, *ethyl* 3-*benzoyloxy*-7:12-*diketo-cholanate* gave colourless needles, m.p. 167–168°. (Found: C, 74·4; H, 8·8. Calc. for $C_{33}H_{44}O_6$: C, 74·0; H, 8·3%.) On alkaline hydrolysis, this substance gave 3-hydroxy-7:12-diketo-cholanic acid.

12-*Hydroxy*-3-*benzoyloxy*-7-*keto-cholanic acid* (IX). Substance (VIII) (1 g.) was refluxed for 1·5 hr. with a mixture of ethanol (20 ml.), and aqueous K_2CO_3 (10 ml. of a solution containing 2 g./100 ml.). The mixture was then diluted with water (4–5 vol.), acidified with HCl, and left for 20 hr. The liquor was decanted through a filter and the residual semi-solid mass washed with water and aqueous $NaHCO_3$. The undissolved bulk of the material was collected, washed, and dissolved in ethanol. Evaporation of this solution, finally *in vacuo*, left a residue which crystallized at once with ether, with which it was washed. Recrystallization from dilute ethanol gave needles (0·25 g.), which from l.p./ethanol had m.p. 250–251° (decomp.). (Found: C, 73·4; H, 8·5. Calc. for $C_{31}H_{42}O_6$: C, 72·9; H, 8·2%.) This substance gave a negative H-test: it dissolved in dilute alkalies, including ammonia solution.

Methyl 7:12-*diacetoxy*-3-*keto-cholanate* (II). Methyl triacetyl cholate (1 g., H-test negative), methanol (20 ml.) and 10N-HCl (1 ml.) were refluxed together for 2 hr. After addition of water (4–5 vol.), the cold mixture was kept for 24 hr., when the liquor was decanted and the residual gum

washed with water. This gum gave an H-test showing yellow → greenish-violet (dichroic) colours. It was dissolved in acetic acid (10 ml.) and the cold solution treated with 20% CrO_3 (1 ml.). After 15 min. it was diluted: the partly crystalline precipitate was collected after 2–3 hr., washed, and recrystallized from dilute ethanol. Yield, 0·55 g. of needles, m.p. 185–188°, raised to 190–191° by recrystallization from l.p./benzene and ethyl acetate. (Found: C, 69·3; H, 8·8. Calc. for $C_{29}H_{44}O_7$: C, 69·0; H, 8·8%.) H-test, negative.

This ester (0·3 g.) was heated in a sealed metal bomb for 4 hr. at 200–210° with a solution of Na (0·1 g.) in ethanol (2 ml.) with hydrazine hydrate (0·3 ml. of 95–99%). Acidification of the diluted contents of the cooled bomb precipitated a solid which separated from dilute ethanol as long white needles (0·15 g.) of 7:12-dihydroxy-cholanic acid (III), m.p. 205°.

SUMMARY

1. The series of six possible acids obtainable by the oxidation to carbonyl of one or two secondary hydroxyl groups in natural cholic acid has been completed by the preparation of 12-hydroxy-3:7-diketo-cholanic acid.

2. New derivatives of this acid, of 3:12-dihydroxy-7-keto-, 7:12-dihydroxy-3-keto-, and 3-hydroxy-7:12-diketo-cholanic acids are described; methods for obtaining such substances have been further explored.

The author thanks Allen and Hanburys Ltd. for a gift of cholic acid.

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A Growth Factor for *C. diphtheriae* Present in Liver

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In studies on the nutritional requirements of *C. diphtheriae*, Evans, Handley & Happold (1939) found that a basal medium of known composition including nicotinic acid, pimelic acid and β-alanine (Mueller, 1937 *a*, *b*; Mueller & Cohen, 1937) sufficed for the growth of certain exacting *gravis* strains only with the further addition of certain liver fractions soluble in amyl alcohol, which were themselves replaceable by pantothenic acid. It was shown that these more exacting *gravis* strains, in contradistinction to other strains, were unable to effect the synthesis of pantothenic acid from β-alanine and thus required the complete molecule in their nutrient medium. Subsequently it became apparent that there existed strains, of *intermedius* and *gravis* types, which failed to grow when inoculated lightly into the pantothenate medium but which did so readily upon the further addition of liver concentrates. Representatives of both *intermedius* and *gravis* types have now been used as test organisms and it has become apparent that the new factor or factors required are identical for both the exacting *intermedius* and *gravis* strains. With a *gravis* strain of *C. diphtheriae*, sub-type Dundee, growth occurs in the form of a pellicle, the medium below remaining clear. The pellicle form lends itself to rapid visual estimation suitable for the routine measurement of the growth response to various addenda. We have utilized the growth response of the Dundee organism as the criterion of the presence of active material in liver preparations, and have attempted to concentrate the active substance required by this class of exacting strain (preliminary report: Chattaway, Happold & Sandford, 1943).

EXPERIMENTAL

Inocula. The standard *gravis* strain used throughout was isolated by Prof. Tulloch of Dundee. All stock cultures were maintained on Loeffler medium in the ice-chest. Prior to inoculation of test medium the strains were grown overnight on chocolate agar, after which a small amount of the culture was introduced into approximately 5 ml. of sterile distilled water so that a turbidity was just discernible under the meniscus. After thorough distribution by shaking, inoculations were made with a loopful of the weak suspension.

Basal medium: l-tryptophan, 0·2 g.; *dl*-cystine, 0·2 g.; *dl*-phenylalanine, 0·1 g.; glycine (synthetic), 0·5 g.; *dl*-methionine, 0·1 g.; *dl*-valine, 0·2 g.; *dl*-glutamic acid HCl,