Biochem. J. (1969) **115**, 249
Printed in Great Britain

Microbiological Degradation of Bile Acids

RING A CLEAVAGE AND 7α,12α-DEHYDROXYLATION OF CHOLIC ACID BY *ARTHROBACTER SIMPLEX*

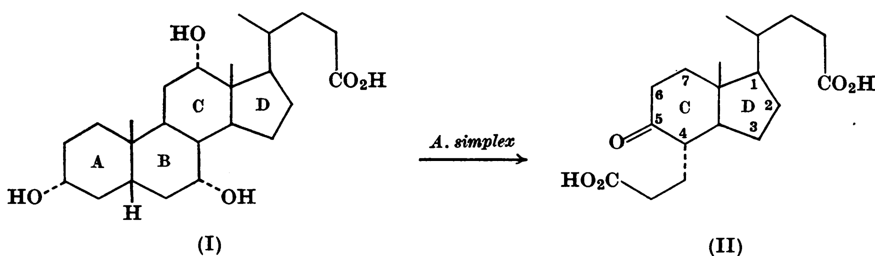
By SHOHEI HAYAKAWA, YOSHIKO KANEMATSU AND TAKASHI FUJIWARA
Shionogi Research Laboratory, Shionogi and Co. Ltd., Fukushima-ku, Osaka, Japan

(*Received* 2 *June* 1969)

The metabolism of cholic acid by *Arthrobacter simplex* was investigated. This organism effected both ring A cleavage and elimination of the hydroxyl groups at C-7 and C-12 and gave a new metabolite, (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid, which was isolated and identified through its partial synthesis. A degradative pathway of cholic acid into this metabolite is tentatively proposed, and the possibility that the proposed pathway could be extended to the cholic acid degradation by other micro-organisms besides *A. simplex* is discussed. The possibility that the observed reactions *in vitro* could occur during the metabolism of bile acids *in vivo* is considered.

An important pathway for the elimination of cholest-5-en-3β-ol (cholesterol) from the body is the conversion into bile acids, which are excreted as a complex mixture in the faeces. It is well established that the complexity of this mixture is a consequence of the extensive transformation of the primary bile acids induced by the intestinal flora (Bergström, Danielsson & Samuelsson, 1960; Haslewood, 1967, pp. 51–71; Danielsson & Tchen, 1968). Many of the investigations described in these reviews suggest that efforts directed at the isolation and identification of the responsible micro-organisms and their transformation products might provide significant information on the metabolism of cholesterol and bile acids. Although the micro-organisms that we have used have not been isolated directly from the intestine, we have studied the microbiological degradation of bile acids *in vitro* as a practical working model for the prediction of possible intermediates in the metabolism of bile acids in the intestine; an outline of our earlier work has been already reported (Hayakawa, Fujii, Saburi & Eguchi, 1957; Hayakawa, Saburi, Tamaki & Hoshijima, 1958*b*). During our studies we found that *Arthrobacter* (*Corynebacterium*) *simplex*, cultured in a medium containing 3α,7α,12α-trihydroxy-5β-cholanoic acid (cholic acid) as the sole carbon source, produces a variety of metabolites (Hayakawa & Samuelsson, 1964). Our continued interest in defining the intermediates and reaction sequence involved in the degradation of bile acids has prompted us to continue our studies on the catabolism of cholic acid by this organism.

The present paper deals with the isolation and identification of a new metabolite of cholic acid (I) by *A. simplex* (Scheme 1), (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid (II); a preliminary note of this work has already appeared (Hayakawa, Kanematsu & Fujiwara, 1967).



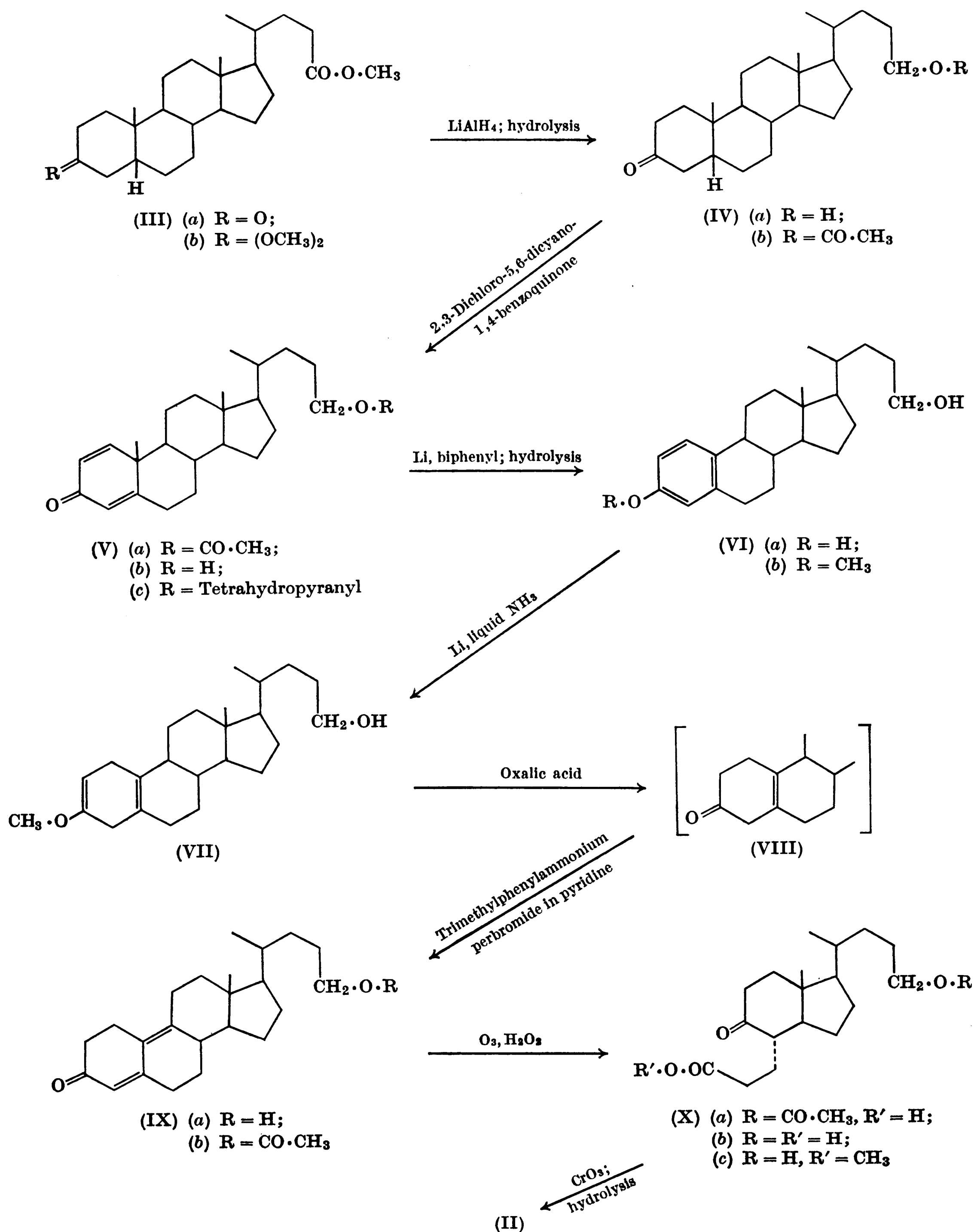
Scheme 1.

RESULTS

When the cholate concentration in a medium was 0·2%, *A. simplex* was able to metabolize cholic acid (I) to a variety of transformation products having the steroid skeleton and some unidentified products (Hayakawa & Samuelsson, 1964). Further work on one of the unidentified products (the upper spot of that of the products IV and V in Fig. 1 of Hayakawa & Samuelsson, 1964) has been prevented by lack of material. With a cholate concentration of 0·1%, however, this unidentified product accumulated as a major metabolite (approx. 40% yield) in the incubation mixture during a 2-day incubation period. The accumulation of this product was also observed in parallel with a disappearance of the metabolites isolated and identified previously (the products I, II, III, IV and V in Fig. 1 of Hayakawa & Samuelsson, 1964), which appeared in the incubation mixture in an early stage of the incubation.

The new metabolite was isolated as crystals. The elementary analysis was consistent with $C_{18}H_{28}O_5$ and the non-aqueous titration gave a neutralization equivalent of 330·3 as dicarboxylic acids. The i.r. spectrum showed the presence of a carboxylic group at ~2700–~3150 and 1703 cm.$^{-1}$. The analysis of the methyl ester was consistent with $C_{20}H_{32}O_5$ and its i.r. spectrum showed the presence of both six-membered ring carbonyl and ester carbonyl groups at 1705 and 1730 cm.$^{-1}$ respectively. The n.m.r. spectrum showed bands at δ 0·93 (superimposable doublet; 3H; one *sec.*-methyl group), 0·99 (singlet; 3H; one *tert.*-methyl group) and 3·66 (singlet; 6H; two ester methyl groups) p.p.m. From its source and these physical data, the structure of this metabolite was assigned as (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid (II). It was surprising that the assigned structure did not possess the two hydroxyl groups at C-7 and C-12 of the original cholic acid molecule. If the assigned structure is correct, it could be expected that 3α-hydroxy-5β-cholanoic acid (lithocholic acid), which contains only one hydroxyl group, at C-3, might be converted into the same metabolite (II) by the same organism, *A. simplex*. Therefore we examined the degradation of lithocholic acid by this organism and obtained compound (II) as a major metabolite, as expected. This result further substantiated the assigned structure given above. Since these results are only indicative, however, we sought a more definitive proof of the structure by partial synthesis (Scheme 2).

Lithocholic acid was converted into the known compound methyl 3-oxo-5β-cholanoate (IIIa) (Hoehn & Mason, 1940; Fieser & Ettorre, 1953; Savin & Volovel'skii, 1966). The ester (IIIa) was converted into the dimethyl ketal (III*b*). The ketal (III*b*) was subjected to reduction with lithium aluminium hydride followed by deketalization to yield 24-hydroxy-5β-cholan-3-one (IV*a*). The acetate (IV*b*) was treated with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (cf. Muller, Martel & Huynh, 1961) to yield 24-acetoxychola-1,4-dien-3-one (V*a*), which was purified with the use of Girard T reagent (Girard & Sandulesco, 1936) by the procedure of Oughton & Stephenson (1957). Hydrolysis of the dienone acetate (V*a*) to the free alcohol (V*b*) and its conversion into the tetrahydropyranyl derivative (V*c*) followed by a reductive aromatization method for $\Delta^{1,4}$-3-oxo steroids reported by Dryden, Webber & Wieczorek (1964) resulted in the formation of the tetrahydropyranyl derivative of 19-norchola-1,3,5(10)-triene-3,24-diol (VI*a*). After hydrolysis, the phenol (VI*a*) was treated with alkaline dimethyl sulphate and the resulting ether (VI*b*) was submitted to the modified Birch reduction (Wilds & Nelson, 1953) to yield 3-methoxy-19-norchola-2,5(10)-dien-24-ol (VII). Its i.r. spectrum exhibited bands at 1668 and 1698 cm.$^{-1}$, characteristic of a non-conjugated dihydroanisole ring (Stork, 1951). The structure of compound (VII) was confirmed through its conversion into 24-hydroxy-19-norchol-4-en-3-one by treatment with hydrochloric acid (cf. Wilds & Nelson, 1953). The enol ether (VII) was treated with oxalic acid by the method of Wilds & Nelson (1953) to obtain 24-hydroxy-19-norchol-5(10)-en-3-one (VIII). However, the purification of compound (VIII) presented certain difficulties and compound (VII) was converted into 24-hydroxy-19-norchola-4,9-dien-3-one (IX*a*) without the isolation of compound (VIII). A conversion of compound (VIII) into compound (IX*a*) was carried out essentially as reported by Perelman, Farkas, Fornefeld, Kraay & Rapala (1960) with the use of trimethylphenylammonium perbromide (Marquet *et al.* 1961) instead of pyridinium bromide perbromide. The presence of a $\Delta^{4,9}$-3-oxo structure in compound (IX*a*) was demonstrated by the u.v.-absorption maximum at 306·5 nm. (ϵ 21400), and by αβ, γδ-unsaturated ketone and C=C stretching bands at 1649 and 1609 cm.$^{-1}$ respectively in the i.r. spectrum (cf. Perelman *et al.* 1960). The dienone (IX*a*) was converted into the acetate (IX*b*) with cold acetic anhydride–pyridine, which with ozone and hydrogen peroxide gave 3-{1β-[(1*R*)-4-acetoxy-1-methylbutyl]-3aα-hexahydro-7aβ-methyl-5-oxoindan-4α-yl}propionic acid (X*a*). Compound (X*a*) was treated with aqueous alkali to yield the hydroxy-oxo acid (X*b*), which with diazomethane gave methyl 3-{1β-[(1*R*)-4-hydroxy-1-methylbutyl]-3aα-hexahydro-7aβ-methyl-5-oxoindan-4α-yl}-propionate (X*c*). Attempts at crystallization of compounds (X*a*), (X*b*) and (X*c*) failed, but their i.r. spectra showed reasonable absorption bands.



Scheme 2.

Oxidation of compound (Xc) with chromic anhydride in acetic acid followed by hydrolysis resulted in the formation of (4*R*)-4-[4α-(2-carboxyethyl)-3aα-hexahydro-7aβ-methyl-5-oxoindan-1β-yl]valeric acid (II). Identity of compound (II) with a sample obtained from growing cultures was established by mixed melting point and by comparison of the optical rotation and the i.r. spectrum. This sequence of reactions conclusively established the structure of the product (II), including its stereochemistry.

EXPERIMENTAL

Materials, micro-organism and culture methods. Cholic acid, other materials and the micro-organism were as described by Hayakawa & Samuelsson (1964). Light petroleum refers to the fraction of b.p. 40–60°. The organism, *Arthrobacter simplex*, was cultured in a medium adjusted to pH 7–7·4 with aq. M-NaOH and containing (g./l.): $(NH_4)_2SO_4$, 2; KH_2PO_4, 1; $MgSO_4,7H_2O$, 0·5; KCl, 0·5; $FeSO_4,7H_2O$, 0·01; yeast extract (Difco Laboratories, Detroit, Mich., U.S.A.), 0·5; sodium cholate, 1. The inoculated flasks were incubated at 28° on a reciprocal shaker. To follow the course of the degradation of cholic acid, a sample of the incubation mixture was separated from the flasks under sterile conditions, acidified with dil. HCl to pH approx. 2 and extracted with ethyl acetate. The extract was examined by t.l.c. in cyclohexane–ethyl acetate–acetic acid (5:5:1, by vol.) as described below. When the result of t.l.c. indicated the complete degradation of cholic acid and its metabolites, which had been isolated and identified by Hayakawa & Samuelsson (1964), the incubation was stopped and the growing cultures were used for the isolation of the product (II) as described below. The incubation was usually continued for 48hr. to obtain a better yield of the product. In continuing an additional incubation, the product (II) was gradually degraded without the apparent accumulation of further degradative intermediates.

Chromatography. Column chromatography was carried out on alumina (Woelm neutral, grade I–III; M. Woelm, Eschwege, Germany), silica gel (M. Woelm) and silicic acid (Mallinckrodt 2847; Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.).

Dry-column chromatography was carried out essentially as reported by Loev & Snader (1965) with the use of a seamless cellulose tubing and silica gel (see above) containing 10% (w/w) of silica gel HF_{254} (nach Stahl; E. Merck A.-G., Darmstadt, Germany).

T.l.c. was carried out on silica gel GF_{254} (see above HF_{254}). Spots were rendered visible by spraying with conc. H_2SO_4 followed by heating at approx. 180° or by using a u.v. (253·7nm.) scanner (cf. Eneroth, 1963; Hofmann, 1964).

Physical measurements. All melting points were taken on a Kofler block apparatus and were uncorrected. The u.v.-absorption spectra were measured in ethanol solution on a Hitachi model ESP-2 spectrometer. Unless otherwise stated, optical rotations and i.r.-absorption spectra were determined in chloroform solution on a Perkin–Elmer model 141 polarimeter and a Nihonbunko model IR-S spectrometer respectively. The n.m.r. spectra were measured in deuterochloroform on a Varian Associates A-60 spectrometer at room temperature. The chemical shifts (δ) in p.p.m. are measured relative to tetramethylsilane internal standard and are reported to the nearest 0·01 p.p.m. The coupling constants, in cyc./sec., are reported to the nearest 0·5cyc./sec.

Isolation of the degradation product. (*a*) From cholic acid (I). The incubation mixture, which was judged by the result of t.l.c. as described above, was centrifuged in a Servall type RC-2 automatic super-speed refrigerated centrifuge (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.) at 5860*g* for 30min. to remove the cells. The cell-free supernatant was concentrated *in vacuo* to about one-tenth of its original volume, acidified with dil. HCl and extracted well with ethyl acetate. The extract was washed with saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* at room temperature to give a mixture of products. In a typical run, 1g. of sodium cholate gave 450mg. of the degradative products, which was recrystallized from acetone to yield (4*R*)-4-[4α-(2-*carboxyethyl*)-3aα-*hexahydro*-7aβ-*methyl*-5-*oxoindan*-1β-*yl*]*valeric acid* (II) as prisms (320mg.), m.p. 165–167°. An analytical sample, further recrystallized from methanol, had m.p. 169–170°, $[\alpha]_D^{27}$ +23·9±2° (*c* 1·035 in ethanol) and i.r. maxima (Nujol) at ~2700–~3150 (OH of CO_2H) and 1703 (C=O and C=O of CO_2H) cm.$^{-1}$ [Found: C, 66·7; H, 8·7. $C_{18}H_{28}O_5$ requires C, 66·6; H, 8·7%; equiv.wt. (two CO_2H groups), 330·3].

The *methyl ester*, prepared with ethereal diazomethane, which crystallized from ether–light petroleum to yield *methyl* (4*R*)-4-[3aα-*hexahydro*-4α-(2-*methoxycarbonylethyl*)-7aβ-*methyl*-5-*oxoindan*-1β-*yl*]*valerate* in plates, m.p. 39–40°, $[\alpha]_D^{23}$ +21·7±2° (*c* 1·030 in ethanol), i.r. maxima at 1730 (ester) and 1705 (C=O) cm.$^{-1}$ and n.m.r. absorptions at approx. 0·93 (3H; superimposable doublet; *J* approx. 5cyc./sec.; CH_3 in the side chain), 0·99 (3H; singlet; 7aβ-CH_3) and 3·66 (6H; singlet; $2CO_2\cdot Me$) p.p.m. (Found: C, 68·3; H, 9·2. $C_{20}H_{32}O_5$ requires C, 68·2; H, 9·2%).

(*b*) From lithocholic acid. The incubation of lithocholic acid with *A. simplex* and the isolation of its degradation products were done in a manner similar to that described above and the acid (II) was obtained. Identity of this acid with a sample obtained from the above cholic acid degradation was established by mixed melting point and by comparison of the optical rotation and the i.r. spectrum.

Partial synthesis of compound (II)

Oxidation of methyl lithocholate. A solution of the ester (98g.) in acetone (2·5l.) was treated with Beckmann reagent (194g.), which consists of $K_2Cr_2O_7$ (60g.), conc. H_2SO_4 (80g.) and water (270ml.) (Beckmann, 1889), at 3–5° during over a period of 1·5hr. The mixture was stirred for a further 1hr. at the same temperature. A small volume of water was added to precipitate a greenish slurry and the slurry was removed by filtration. The filtrate was diluted with water and the precipitated white crystals were collected by filtration, washed with water and dried. A solution of the completely dried product in benzene (2l.) was filtered through alumina (50g., grade I) to remove a small amount of polar impurities. The filtrate was evaporated *in vacuo* to a small volume and diluted with light petroleum to yield methyl 3-oxo-5β-cholanoate (IIIa) as needles (83g.), m.p. 120·5–121°, $[\alpha]_D^{27}$ +34·1±3° (*c* 0·742 in ethanol) and i.r.

maxima at 1729 (ester) and 1708 (C=O) cm.$^{-1}$ (Found: C, 77·6; H, 10·5. Calc. for $C_{25}H_{40}O_3$: C, 77·3; H, 10·4%). Hoehn & Mason (1940), Fieser & Ettorre (1953) and Savin & Volovel'skii (1966) give m.p. 128–128·5°, m.p. 119–120° and m.p. 126–128° respectively for this compound.

Ketalization of methyl 3-*oxo*-5β-*cholanoate* (IIIa). A suspension of the ester (82g.) in methanol (700ml.) containing toluene-*p*-sulphonic acid (2g.) was heated for approx. 5min. under reflux until the solids were dissolved. The colourless crystalline solid, which separated out on cooling, was filtered and recrystallized from methanol to yield *methyl* 3-*oxo*-5β-*cholanoate dimethyl ketal* (IIIb) as plates (72g.), m.p. 100·5–102°, $[\alpha]_D^{25}$ +26·5±2° (*c* 1·043) and i.r. maxima at 1732 (ester) and 1098 and 1048 (ether) cm.$^{-1}$ (Found: C, 74·9; H, 10·8. $C_{27}H_{46}O_4$ requires C, 74·6; H, 10·7%).

Lithium aluminium hydride reduction of the dimethyl ketal (IIIb). A solution of the ketal (50·6g.) in anhydrous ether (300ml.) was added dropwise to a solution of $LiAlH_4$ (19g.) in anhydrous ether (500ml.), with stirring. The mixture was heated for 2hr. under reflux, cooled to room temperature and carefully treated with ethyl acetate (200ml.) and 6M-HCl (500ml.). The organic layer was separated and the aqueous layer was extracted with ether. The combined ether extract was washed with aq. 5% (w/v) $NaHCO_3$ and then with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield a mixture of products (52·1g.). The mixture was dissolved in aq. 80% (v/v) acetic acid (500ml.) without further purification and heated in a boiling-water bath for 1hr. The solvents were completely evaporated *in vacuo*. The residue was dissolved in methanolic 5% (w/v) KOH (500ml.) and heated for 1hr. under reflux. The mixture was neutralized with dil. acetic acid to pH approx. 8 and as much solvent as possible was evaporated *in vacuo*. The white crystals that separated out on adding water to the concentrate were filtered. The crystals were dissolved in ether–benzene (1:1, v/v) (1l.). The solution was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to dryness. Recrystallization from acetone–light petroleum gave 24-*hydroxy*-5β-*cholan*-3-*one* (IVa) as prisms (28·67g.), m.p. 113–114°. A further 2g. was recovered from the mother liquor by alumina (grade III) chromatography. Further recrystallization from acetone–light petroleum led to an analytical sample, m.p. 115·5–116·5°, $[\alpha]_D^{22}$ +34·1±2° (*c* 0·946) and i.r. maxima at 3440 and 3600 (OH) and 1706 (C=O) cm.$^{-1}$ (Found: C, 80·0; H, 11·2. $C_{24}H_{40}O_2$ requires C, 79·9; H, 11·2%).

The *acetate* (IVb), prepared with acetic anhydride–pyridine (1hr. in a boiling-water bath), crystallized from light petroleum in prisms, m.p. 81·5–83°, $[\alpha]_D^{24}$ +32·2±2° (*c* 0·989) and i.r. maxima at 1730 (C=O of acetate) and 1711 (C=O) cm.$^{-1}$ (Found: C, 77·7; H, 10·6. $C_{26}H_{42}O_3$ requires C, 77·6; H, 10·5%).

2,3-*Dichloro*-5,6-*dicyano*-1,4-*benzoquinone dehydrogenation of the acetate* (IVb). A mixture of the acetate (31·35g.) and 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (43·8g.) in anhydrous dioxan (1·1l.) was heated for 15hr. under reflux. The solid that was precipitated on cooling was filtered off and the filtrate was evaporated *in vacuo* to dryness. The residue was dissolved in ether (1·5l.), washed with aq. 5% (w/v) Na_2CO_3 and then water, dried over anhydrous Na_2SO_4 and the solvent was evaporated *in vacuo* to yield a semi-crystalline solid (24·55g.). The solid was dissolved in anhydrous ethanol (300ml.) containing acetic acid (30ml.) and Girard T reagent (5g.) and heated for 30min. under reflux. The reaction mixture was cooled to approx. 5°, neutralized with aq. 2% (w/v) $NaHCO_3$ to pH 6·8 and extracted with ether. The extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to yield an oily product (20·01g.). The residue was dissolved in methanolic 5% (w/v) KOH (200ml.) and heated for 40min. under reflux. After neutralization with dil. acetic acid, as much solvent as possible was evaporated off *in vacuo*. The white crystals that were precipitated on adding water to the concentrate were filtered, washed with water and dried. Recrystallization from methanol–water gave a crystalline solid (13·97g.), m.p. 136–137·5°. Repeated recrystallizations from acetone–ether afforded an analytical sample of 24-*hydroxychola*-1,4-*dien*-3-*one* (Vb), m.p. 138·5°, $[\alpha]_D^{24}$+26·8±5° (*c* 0·269), i.r. maxima at 3600 and 3450 (OH), 1657 (cross-conjugated C=O) and 1620 and 1606 (C=C) cm.$^{-1}$ and $\lambda_{max.}$ 246nm. (ε 15400) (Found: C, 81·1; H, 10·3. $C_{24}H_{36}O_2$ requires C, 80·9; H, 10·2%). The u.v.-absorption spectrum, which exhibits a very weak peak at approx. 300nm., indicated that this sample contains a $\Delta^{1,4,6}$-3-oxo compound, which might be 24-hydroxychola-1,4,6-trien-3-one, but the impurity could not be completely eliminated.

The *acetate* (Va), prepared with acetic anhydride–pyridine (16hr. at room temperature), crystallized from acetone in rods, m.p. 93–94°, $[\alpha]_D^{24.5}$+19·1±2° (*c* 1·040), i.r. maxima at 1725 (C=O of acetate), 1658 (cross conjugated C=O) and 1620 and 1604 (C=C) cm.$^{-1}$ and $\lambda_{max.}$ 246·5nm. (ε 14900) (Found: C, 78·5; H, 9·6. $C_{26}H_{38}O_3$ requires C, 78·4; H, 9·6%). The impurity, which shows an absorption at approx. 300nm. (ε 250) as described above, still remained in this sample, but the amount would be less than 2% (w/w) for its low ε value.

Reductive aromatization of the $\Delta^{1,4}$-3-*oxo compound* (Vb). A suspension of the dienone (20·54g.) in a mixture of anhydrous tetrahydrofuran (100ml.) and dihydropyran (50ml.) was treated with phosphoryl chloride (0·5ml.), with cooling in ice and with stirring, and the solids were completely dissolved within a few minutes. After the stirring was continued for a further 1·5hr. at room temperature, the mixture was poured into a large volume of aq. saturated $NaHCO_3$ and thoroughly extracted with ether. The organic layer was washed with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give a mixture of products, which was chromatographed on an alumina (600g., grade II) column. Elution with benzene–light petroleum (2:3, v/v) and benzene gave 24-*tetrahydropyranyloxychola*-1,4-*dien*-3-*one* (Vc) as an oil (20·27g.). A solution of biphenyl (14·32g.) and diphenylmethane (9·21ml.) in freshly distilled tetrahydrofuran (190ml.) was heated under reflux in an atmosphere of N_2 with lithium metal (1·521g.) for 30min. with stirring. The resulting dark-green solution was cooled to room temperature and to this a solution of the above tetrahydropyranyl ether (14·58g.) in freshly distilled tetrahydrofuran (105ml.) was added dropwise with stirring. The mixture was heated for 15min. under reflux and then cooled in ice. All these treatments were carried out in an atmosphere of N_2. The excess of lithium metal was destroyed by the careful addition of methanol (100ml.). The mixture was acidified with a mixture of water (170ml.) and conc. HCl (50ml.), heated for 15min. under reflux and steam-distilled. The precipitated yellowish powder was filtered, washed well with water and

dried to yield a mixture of products (13·28g.). Recrystallization from methanol–ether gave 19-*norchola*-1,3,5(10)-*triene*-3,24-*diol* (VI*a*) as plates (5·693g., m.p. 229–230·5°; 1·866g., m.p. 226–228·5°). Further purification by silica-gel chromatography with methanol–chloroform (1:99, v/v) as eluent led to an analytical sample, m.p. 231–231·5°, $[\alpha]_D^{24.5}+75{\cdot}6\pm4°$ [*c* 0·492 in chloroform–methanol (1:1, v/v)], i.r. maxima (Nujol) at 3330 and 3135 (OH) and 1621, 1589 and 1498 (C_6H_5) cm.$^{-1}$ and $\lambda_{max.}$ 281·5 and 287 nm. (shoulder) (ϵ 2050 and 1880 respectively) (Found: C, 80·4; H, 10·1. $C_{23}H_{34}O_2$ requires C, 80·7; H, 10·0%).

Methylation of the phenol (VI*a*) *and the modified Birch reduction of the ether* (VI*b*). A suspension of the phenol (12·03g.) in methanol (180ml.) was treated alternately with small portions of aq. 40% (w/v) KOH (720ml.) and dimethyl sulphate (360ml.) at 60–65° with stirring. The stirring was continued for a further 1hr. at approx. 70°. The mixture was cooled to room temperature, diluted with water and extracted with ether. The extract was washed with water, dried over anhydrous Na_2SO_4 and evaporated to yield a gum (12·54g.), which was chromatographed on alumina (270g., grade III). Elution with benzene–light petroleum (1:4, v/v) and benzene–light petroleum (1:1, v/v), on recrystallization from acetone, gave 3-*methoxy*-19-*norchola*-1,3,5(10)-*trien*-24-*ol* (VI*b*) as prisms (7·687g.), m.p. 120–122·5°, $[\alpha]_D^{25}+75{\cdot}8\pm2°$ (*c* 1·066), i.r. maxima at 3600 and 3440 (OH) and 1611, 1576 and 1499 (C_6H_5) cm.$^{-1}$ and $\lambda_{max.}$ 278·5 and 287·5nm. (shoulder) (ϵ 2040 and 1890 respectively) (Found: C, 80·7; H, 10·2. $C_{24}H_{36}O_2$ requires C, 80·9; H, 10·2%).

A solution of compound (VI*b*) (2·496g.) in a mixture of 2-methylpropan-2-ol (30ml.) and tetrahydrofuran (30ml.) was added dropwise to distilled liquid NH_3 (60ml.) during a period of 7min., with stirring. To the stirred solution was added lithium metal (834mg.) during a period of 15min. and the stirring was continued for a further 4hr. The mixture was treated with methanol (38ml.) to destroy the excess of lithium and kept overnight at room temperature. The mixture, resulting from spontaneous evaporation of the NH_3, was further concentrated *in vacuo* at room temperature to approx. 30ml. The crystalline solid that was precipitated on adding water (50ml.) to the concentrate was filtered, washed with water and dried to yield a powder (2·413g.), which was recrystallized from ether to give 3-*methoxy*-19-*norchola*-2,5(10)-*dien*-24-*ol* (VII) as prisms (1·484g., m.p. 139–142°; 648mg., m.p. 135–140°). Further recrystallization from acetone gave an analytical sample, m.p. 142–144°, $[\alpha]_D^{24.5}+116{\cdot}8\pm2°$ (*c* 1·063) and i.r. maxima at 3580 and 3450 (OH) and 1698 and 1668 (unconjugated dihydroanisole ring) cm.$^{-1}$ (Found: C, 80·4; H, 10·8. $C_{24}H_{38}O_2$ requires C, 80·4; H, 10·7%). The u.v.-absorption spectrum [278·5 (ϵ 27) and 287·5nm. (ϵ 25)] indicated that this sample contains 1·3% (w/w) of the unchanged starting material (VI*b*), but attempts to decontaminate it were unsuccessful.

Hydrolysis and isomerization of the enol ether (VII) *into an αβ-unsaturated ketone*. A solution of the enol ether (70mg.) in a mixture of methanol (8ml.) and 10% (w/w) HCl (2ml.) was heated at 60–65° for 20min. Water was added, and the solution was saturated with NaCl and extracted with ether. The ether extract was successively washed with water, aq. 5% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give 24-*hydroxy*-19-*norchol*-4-*en*-3-*one*, which crystallized from acetone in plates (42mg.), m.p. 135–136°, $[\alpha]_D^{23}+47{\cdot}4\pm0{\cdot}5°$ (*c* 0·886 in ethanol), i.r. maxima at 3580 and 3436 (OH), 1661 (αβ-unsaturated C=O) and 1618 (C=C) cm.$^{-1}$ and $\lambda_{max.}$ 241·5nm. (ϵ 14600) (Found: C, 80·2; H, 10·6. $C_{23}H_{36}O_2$ requires C, 80·2; H, 10·5%).

Hydrolysis and bromination–dehydrobromination of the enol ether (VII). A mixture of the enol ether (3·61g.) in methanol (360ml.) and oxalic acid dihydrate (5g.) in water (40ml.) was kept at room temperature for 40min. The mixture was diluted with a large volume of water, saturated with NaCl and extracted with ether. The extract was washed with aq. 5% (w/v) $NaHCO_3$ and then with water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give crude 24-*hydroxy*-19-*norchol*-5(10)-*en*-3-*one* (VIII) as an oil (3·54g.). The oil was dissolved in pyridine (90ml.) and chilled to approx. −3° to −7°. A portion of trimethylphenylammonium perbromide (3·891g.) was gradually added to the chilled solution over a period of 20min., with stirring. The orange mixture, which was stirred at the same temperature for a further 1·5hr. and then kept overnight at room temperature, was diluted with water and extracted with ether. The extract was successively washed with dil. HCl, water, aq. 5% (w/v) $NaHCO_3$ and water, dried over anhydrous Na_2SO_4 and evaporated *in vacuo*. The residue (3·1g.) was triturated with ether–light petroleum to give an amorphous powder (1·997g.) and a gum (1·103g.). A fraction of the powder was divided into two parts (approx. 2:1, w/w) and each part was chromatographed on two dry columns [silica gel (400g.), 65cm. × 3cm.; silica gel (200g.), 59cm. × 1·8cm.] respectively (see under 'Chromatography' above). Distinct separation of two yellowish fluorescent zones, which consist of the impurities as detected by the ultraviolet scanner, was achieved by developing with cyclohexane–ethyl acetate (2:1, v/v) saturated with water. The wide zone that lies between the above fluorescent zones was cut off with a razor and extracted with ether. The extract, on recrystallization from ether, gave crystals (1·268g.) with m.p. above 102°. A fraction of the above gum was treated by a similar dry-column chromatography and gave a crystalline solid (224mg., m.p. 102–105°). These crystalline fractions were further recrystallized from methanol to afford 24-*hydroxy*-19-*norchola*-4,9-*dien*-3-*one* (IX*a*) as prisms (1·207g.), m.p. 105–107°, $[\alpha]_D^{24}-263{\cdot}5\pm2°$ (*c* 1·093), i.r. maxima at 3580 and 3445 (OH), 1649 (αβ, γδ-unsaturated C=O) and 1609 (C=C) cm.$^{-1}$ and $\lambda_{max.}$ 306·5nm. (ϵ 21400) (Found: C, 80·6; H, 9·99. $C_{23}H_{34}O_2$ requires C, 80·7; H, 10·0%).

The *acetate* (IX*b*), prepared with acetic anhydride–pyridine (16hr. at room temperature), crystallized from ether in prisms, m.p. 106°, $[\alpha]_D^{24}-244{\cdot}8\pm2°$ (*c* 1·030), i.r. maxima at 1725 (acetate), 1645 (αβ, γδ-unsaturated C=O) and 1606 (C=C) cm.$^{-1}$ and $\lambda_{max.}$ 306·5nm. (ϵ 21500) (Found: C, 77·98; H, 9·5. $C_{25}H_{36}O_3$ requires C, 78·1; H, 9·4%).

Ozonolysis of the dienone acetate (IX*b*). The acetate (362mg.) in ethyl acetate (30ml.) containing acetic acid (3ml.) was ozonized with a stream of 3% (w/w) ozonized O_2 at 0–3° for 20min. Water (10ml.) was added and the mixture was stirred for 1hr. at the same temperature. Then 1·8ml. of 30% (w/w) H_2O_2 was added and the mixture was kept overnight in a refrigerator. The mixture was diluted with water and extracted with ether. The ethereal solution was extracted several times with aq. 5% (w/v) $NaHCO_3$ and the combined extract was washed with ether to remove

contaminated neutral materials. The alkaline extract was acidified with dil. HCl and extracted with ether to recover an acidic fraction. Evaporation of the solvents gave crude 3-{1β-[(1*R*)-4-*acetoxy*-1-*methylbutyl*]-3aα-*hexahydro*-7aβ-*methyl*-5-*oxoindan*-4α-*yl*}*propionic acid* (X*a*) as an oil (233mg.), which showed i.r. bands (film) at ~2600–~3200 (OH of CO_2H), 1730 and 1240 (acetate) and 1705 (C═O and C═O of CO_2H) cm.$^{-1}$ The oily acetate was treated with aq. M-KOH (1·5ml.) at 70–80° for 30min. After the mixture was extracted with ether to remove contaminated neutral materials, it was acidified with dil. HCl, saturated with NaCl and extracted with ether. The extract was washed with aq. saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give an oil (212mg.), which was chromatographed on silicic acid. Elution with methanol–dichloromethane (1:99, v/v) gave a gum (123mg.), which is believed to be 3-{1β-[(1*R*)-4-*hydroxy*-1-*methylbutyl*]-3aα-*hexahydro*-7aβ-*methyl*-5-*oxoindan*-4α-*yl*}*propionic acid* (X*b*). It ran as one spot on t.l.c. in cyclohexane–ethyl acetate–acetic acid (5:5:1, by vol.), but this acid could not be induced to crystallize.

Methylation, oxidation and hydrolysis of the hydroxy-oxo acid (X*b*). The *methyl ester* (X*c*), prepared with ethereal diazomethane from the hydroxy-oxo acid, gave an oil and showed i.r. bands (film) at 3426 (OH), 1734 (ester) and 1706 (C═O) cm.$^{-1}$. The ester (120mg.) in acetic acid (2·3ml.) was treated with a solution of CrO_3 (38mg.) in 0·34ml. of 80% (v/v) acetic acid under cooling at 0–5° for 1hr. After the mixture had been kept overnight at room temperature, methanol was added and the solution was concentrated *in vacuo*, diluted with water and extracted with ether. The extract was partitioned into a neutral fraction (37mg.) and an acidic fraction (83mg.) by the usual $NaHCO_3$ extraction procedure, which was described above in the ozonolysis of compound (IX*b*). The neutral fraction, which mainly consists of starting material by its i.r. spectrum and t.l.c. in iso-octane–ethyl acetate–acetic acid (50:50:1, by vol.), was not further treated. The acidic fraction was chromatographed on silicic acid (3·3g.). Elution with dichloromethane gave a gum (76mg.), which is believed to be (4*R*)-4-[4α-(2-*methoxycarbonylethyl*)-3aα-*hexahydro*-7aβ-*methyl*-5-*oxoindan*-1β-*yl*]*valeric acid*. This half-ester in aq. M-KOH (2ml.) was heated at approx. 60° for 1hr. The solution was acidified with dil. HCl, saturated with NaCl and extracted several times with ether. The extract was washed with aq. saturated NaCl, dried over anhydrous Na_2SO_4 and evaporated *in vacuo* to give a gum, which was chromatographed on silicic acid. Elution with methanol–dichloromethane (1:99, v/v) and methanol–dichloromethane (1:49, v/v) gave (4*R*)-4-[4α-(2-*carboxyethyl*)-3aα-*hexahydro*-7aβ-*methyl*-5-*oxoindan*-1β-*yl*]*valeric acid* (II), which crystallized from acetone–light petroleum in prisms (24mg.), m.p. 166–167·5°, $[\alpha]_D^{26}+24{\cdot}0\pm4°$ (*c* 0·542 in ethanol) and i.r. maxima (Nujol) at ~2700–~3150 (OH of CO_2H) and 1703 (C═O and C═O of CO_2H) cm.$^{-1}$ (Found: C, 66·3; H, 8·6. $C_{18}H_{28}O_5$ requires C, 66·6; H, 8·7%). Identity with the product obtained from growing cultures was established by mixed melting point and by comparison of the optical rotation and the i.r. spectrum.

DISCUSSION

The results of the above chemical investigations show that, in the degradative sequence, the original stereochemistry of the cholic acid molecule remains undisturbed and two hydroxyl groups at C-7 and C-12 in the original molecule are eliminated. Although the 7α-dehydroxylation of cholic acid by *A. simplex* has already been discussed by Hayakawa & Samuelsson (1964), the present experiment demonstrates that the 12α-dehydroxylation also occurs in the degradative sequence of cholic acid by the same organism. However, where the 12α-dehydroxylation step is located in the degradative sequence of cholic acid, especially before or after the ring A cleavage, has not yet been confirmed.

In our earlier work (Hayakawa *et al.* 1957, 1958*b*) we demonstrated that some micro-organisms, which were capable of utilizing cholic acid as the sole carbon source, could convert this acid into several metabolites containing a Δ^4-3-oxo or a $\Delta^{4,6}$-3-oxo structure. From the present knowledge on the microbiological degradation of steroids (cf. Sih & Whitlock, 1968; Coulter & Talalay, 1968) it might be presumed that some of the metabolites that we described earlier had served as the precursors of the 9,10-seco and 4,5:9,10-diseco compounds, which are further metabolized to perhydroindane-carboxylic acids such as compound (II). It seems likely from our results obtained so far that the degradative sequence by all micro-organisms that can utilize cholic acid as the sole carbon source probably proceeds through either of the following two pathways: cholic acid → 7α-hydroxy-3-oxo C_{24} acids → 7α-hydroxy-Δ^4-3-oxo C_{24} acids → $\Delta^{4,6}$-3-oxo C_{24} acids → Δ^4-3-oxo C_{24} acids → $\Delta^{1,4}$-3-oxo or 9α-hydroxy-Δ^4-3-oxo C_{24} acids → 9,10-seco C_{24} acids → perhydroindane C_{18} acids → further degradation, or cholic acid → 7α-hydroxy-Δ^4-3-oxo C_{24} acids → 7α-hydroxy Δ^4-3-oxo C_{22} acids → further degradation. In earlier work (Hayakawa, Saburi & Tamaki, 1958*a*) we reported that one of the cholic acid utilizers, *Streptomyces rubescens*, was able to convert cholic acid into metabolites having a Δ^4-3-oxo or a $\Delta^{4,6}$-3-oxo structure and unidentified products. In continuing our earlier work, we have found that one of the unidentified products with m.p. 162–164° is compound (II) itself and also that this organism is capable of converting cholic acid into various nitrogen-containing derivatives of C_{18}, C_{16} and C_{13} perhydroindane-carboxylic acids besides compound (II) (Hayakawa, Hashimoto & Onaka, 1969). This result provides support for the presence of one of the above postulated general pathways.

There is no evidence as yet to indicate ring-opening or side-chain-shortening of bile acids during their intestinal passage, and the occurrence of Δ^4-3-oxo bile acids in mammals has not yet been demonstrated. Nevertheless, the occurrence of 3-oxochola-4,6-dienoic acid (probably in bile as 7ξ-hydroxy-3-oxochol-4-enoic acid) in the bile of

domestic fowl has been shown (Haslewood, 1967, p. 8), and it has been reported that 3-oxo and Δ^4-3-oxo bile acids are probable intermediates in the microbiological transformation of $3\alpha,12\alpha$-dihydroxy-5β-cholanoic acid (deoxycholic acid) into $3\alpha,12\alpha$-dihydroxy-5α-cholanoic acid (allodeoxycholic acid) both in the rabbit (Danielsson, Kallner & Sjövall, 1963) and in the rat (Kallner, 1967*a*,*b*). Further, Nakada *et al.* (1968) have proposed that Δ^4-3-oxo bile acid(s) can be formed in the rat liver as intermediates in the bile acid formation. It is therefore conceivable that, in certain animals, Δ^4-3-oxo bile acids in limited amounts might be present in the liver (bile) or in the intestine (or both) as the normal metabolites of cholesterol or the microbiological artifacts of the primary bile acids (or both). Although the Δ^4-3-oxo bile acids are formed *in vivo* in such a way, it is not yet established whether or not they are submitted in the intestine to the following reactions by micro-organisms: shortening of the side chain, ring-opening of the steroid skeleton, formation of nitrogenous compounds or amino acid conjugation (Hayakawa, Fujiwara & Tsuchikawa, 1968), which have been observed in our experiments *in vitro*.

We thank the members of the analytical and the physicochemical departments of this laboratory for analytical and optical data.

REFERENCES

Beckmann, E. (1889). *Liebigs Ann.* **250**, 325.
Bergström, S., Danielsson, H. & Samuelsson, B. (1960). In *Lipide Metabolism*. p. 291. Ed. by Bloch, K. New York: John Wiley and Sons Inc.
Coulter, A. W. & Talalay, P. (1968). *J. biol. Chem.* **243**, 3238.
Danielsson, H., Kallner, A. & Sjövall, J. (1963). *J. biol. Chem.* **238**, 3846.
Danielsson, H. & Tchen, T. T. (1968). In *Metabolic Pathways*, vol. 2, p. 117. Ed. by Greenberg, D. M. New York: Academic Press Inc.
Dryden, H. L., jun., Webber, G. M. & Wieczorek, J. J. (1964). *J. Amer. chem. Soc.* **86**, 742.
Eneroth, P. (1963). *J. Lipid Res.* **4**, 11.
Fieser, L. F. & Ettorre, R. (1953). *J. Amer. chem. Soc.* **75**, 1700.
Girard, A. & Sandulesco, G. (1936). *Helv. chim. Acta*, **19**, 1095.
Haslewood, G. A. D. (1967). *Bile Salts*. London: Methuen and Co. Ltd.
Hayakawa, S., Fujii, T., Saburi, Y. & Eguchi, T. (1957). *Nature, Lond.*, **179**, 537.
Hayakawa, S., Fujiwara, T. & Tsuchikawa, H. (1968). *Nature, Lond.*, **219**, 1160.
Hayakawa, S., Hashimoto, S. & Onaka, T. (1969). *Lipids*, **4**, 224.
Hayakawa, S., Kanematsu, Y. & Fujiwara, T. (1967). *Nature, Lond.*, **214**, 520.
Hayakawa, S., Saburi, Y. & Tamaki, K. (1958*a*). *J. Biochem., Tokyo*, **45**, 419.
Hayakawa, S., Saburi, Y., Tamaki, K. & Hoshijima, H. (1958*b*). *Nature, Lond.*, **181**, 906.
Hayakawa, S. & Samuelsson, B. (1964). *J. biol. Chem.* **239**, 94.
Hoehn, W. M. & Mason, H. L. (1940). *J. Amer. chem. Soc.* **62**, 569.
Hofmann, A. F. (1964). In *New Biochemical Separations*, p. 261. Ed. by Morris, L. J. & James, A. T. London: Van Nostrand Co. Inc.
Kallner, A. (1967*a*). *Acta chem. scand.* **21**, 87.
Kallner, A. (1967*b*). *Acta chem. scand.* **21**, 315.
Loev, B. & Snader, K. M. (1965). *Chem. & Ind.* p. 15.
Marquet, A., Dvolaitzky, M., Kagan, H. B., Mamlok, L., Quannes, C. & Jacques, J. (1961). *Bull. Soc. chim. Fr.* p. 1822.
Muller, G., Martel, J. & Huynh, C. (1961). *Bull. Soc. chim. Fr.* p. 2000.
Nakada, F., Oshio, R., Sasaki, S., Yamasaki, H., Yamaga, N. & Yamasaki, K. (1968). *J. Biochem., Tokyo*, **64**, 495.
Oughton, G. & Stephenson, L. (1957). *Brit. Patent* 788307.
Perelman, M., Farkas, E., Fornefeld, E. J., Kraay, R. J. & Rapala, R. T. (1960). *J. Amer. chem. Soc.* **82**, 2402.
Savin, B. M. & Volovelškii, L. N. (1966). *J. gen. Chem. U.S.S.R.* **36**, 2103.
Sih, C. J. & Whitlock, H. W., jun. (1968). *Annu. Rev. Biochem.* **37**, 680.
Stork, G. (1951). *J. Amer. chem. Soc.* **73**, 504.
Wilds, A. L. & Nelson, N. A. (1953). *J. Amer. chem. Soc.* **75**, 5366.