101. A DIETARY FACTOR CONCERNED IN COPROSTEROL FORMATION

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In spite of the progress made in recent years in the chemistry of cholesterol, little is known about its metabolism, so that even the mechanism of its biological conversion into coprosterol remains still unexplained. The excretory origin of coprosterol, taken in conjunction with the fact that it differs from cholesterol in elementary composition merely by two additional hydrogen atoms, obviously led the early investigators to the assumption that hydrogenation of cholesterol was effected by intestinal bacterial action.[1] There is indeed convincing experimental evidence that coprosterol originates in the large intestine, but there is equally conclusive proof that, even under the most favourable conditions, intestinal bacteria are unable to hydrogenate cholesterol *in vitro* (for recent unsuccessful attempts see Schoenheimer *et al.* [1930] and Dam [1934]). Moreover, direct hydrogenation to coprosterol, a *cis*-decalin derivative, is inherently improbable, since under all known conditions of hydrogenation cholesterol yields only the isomeric dihydrocholesterol (β-cholestanol), a *trans*-decalin derivative.

We suggested some time ago [Rosenheim & Starling, 1933] that coprosterol may arise in the intestine not as a product of direct hydrogenation of cholesterol itself, but through that of an intermediary oxidation product, the ketone cholestenone. The primary oxidation product of cholesterol, the α-glycol *cis*-3:4-Δ^5-cholestenediol, easily furnishes cholestenone by loss of water, the reaction involving a shift of the ethylenic linkage from C_5 to C_4 and thereby enabling a transition to the *cis*-decalin derivative coprosterol on hydrogenation. In feeding experiments with these oxidation products we found that they were excreted as coprosterol [Rosenheim & Webster, 1935; Rosenheim & Starling, 1937]. Similar results were obtained by Schoenheimer *et al.* [1935] and by Anchel & Schoenheimer [1938], who made use of the ingenious device of 'labelling' cholestenone with deuterium.

We have since attacked the problem from another side by investigating a hitherto neglected incidental observation, made over 30 years ago, which suggests a relationship between diet and coprosterol formation. In their study of cholesterol metabolism Dorée & Gardner [1908] kept a dog on various mixed diets of meat and vegetables. Cholesterol only was excreted by the dog under these conditions. On account of its large cholesterol content brain seemed to be a convenient medium for the administration of cholesterol, and Dorée & Gardner found, in a single experiment with raw brain as the exclusive diet, that the faecal sterols consisted mainly of coprosterol. No explanation for this remarkable result was available at the time and the experiment was not repeated.

[1] For the sake of historical accuracy it should be noted that the excretory origin of coprosterol was first recognized by W. Marcet, F.R.S. [1857; 1860], a physician at Westminster Hospital. The method used by Marcet for its isolation was essentially the same as that employed by later workers, and the melting point, 96°, of his 'excretin' is identical with that given for coprosterol by Bondzinsky [1896], who is usually credited with the discovery.

In a preliminary experiment we kept two dogs on a diet of raw brain, but had to discontinue the experiment as the animals suffered severely from diarrhoea, in spite of a liberal supply of bones as roughage. A second attempt with two dogs of a different breed had the same result, the dogs rapidly losing weight. When we used rats, however, as experimental animals and replaced raw brain by cooked brain mixed with bone meal, they excreted large quantities of coprosterol. As much as 40 g. of fairly pure coprosterol, M.P. 95–96°, were obtained in 4 days from 100 rats. Possibly the feeding of cooked brain to dogs would give similar results, but for various reasons we continued the work with rats on a synthetic diet.

On the sterol-free standard diet the rats excreted only traces of coprosterol. When cholesterol to the extent of 1–2 % of the diet, dissolved in lard and mixed with the diet, was given, it was excreted unchanged. On replacing part of the cholesterol by cooked brain, however, large amounts of coprosterol appeared in the faeces. It became thus evident that brain contains a substance or factor which conditions the conversion of cholesterol taken by the mouth into faecal coprosterol. A cholesterol-free brain powder was subsequently prepared by exhaustively extracting minced brain of ox or sheep with cold acetone. On giving this powder, mixed with the diet containing 1–1·5 % cholesterol, the rats excreted coprosterol to the extent of 70–90 % of the total faecal sterols. Systematic experiments then showed that the factor is thermostable, insoluble in water, acetone, ether or petrol, but soluble in hot 85 % alcohol. It was present in the 'white matter' of Thudichum, which is deposited when hot alcoholic brain extracts are cooled, and which consists of the cerebrosides phrenosin, kerasin and nervon, the phosphatide sphingomyelin etc.

The disentanglement of the individual constituents of 'white matter' is laborious and their preparation in a pure condition and in sufficient amounts for feeding experiments requires larger supplies of 'white matter' than we had available. Although our preparations of phrenosin, the most abundant of the cerebrosides, were not analytically pure, they showed a high degree of activity. Fortunately we had at our disposal a specimen of pure phrenosin, prepared more than 60 years ago by its discoverer Thudichum [see Rosenheim, 1930]. When Thudichum's phrenosin, in amounts proportional to that contained in the brain powder, was added to the standard diet plus cholesterol, the rats excreted coprosterol to the extent of over 80 % of the faecal sterols. In a single experiment Thudichum's kerasin was inactive, as also were lecithin, kephalin and choline.

A preliminary survey of the distribution of the factor showed that brain was the richest of all the organs examined and that pancreas, kidney and liver contained it in much smaller amounts and in decreasing order. It is present in egg yolk, but absent from muscle (horse flesh) and from milk. The distribution of the factor thus seems to run parallel with the cerebroside content of the various organs and foods examined.

Experimental

Rats of the Institute strain of Wistar rats and of the average weight of 200 g. were used. Groups of 3 to 6 rats, with the same number as controls, served for each experiment. The animals were housed in cages with coarse wire-grid bottoms which allowed the faeces to drop on to blotting paper below. The rats were kept on the diet for a few days before the start of the experimental period, which usually lasted for 7 days. The faeces were collected during the experimental period only, no quantitative sterol balances being attempted.

The standard diet consisted of

Casein (Glaxo C)	20 parts	+20 mg. cod liver oil per rat, per day
Rice starch	72 ,,	
Yeast powder (B.D.H.)	6 ,,	
Salt mixture	2 ,,	

supplemented with 15 g. of lard per 100 g. of the diet. Cholesterol, 1–1·5 % of the diet, was dissolved in the lard and incorporated with the diet. The average food consumption per rat, per day was 10–12 g.

For analysis the faeces, collected daily and preserved in 40 % KOH, were worked up by Fex's [1920] method and saponification was completed by treating the ether extracts with sodium ethoxide for 24 hr. Total sterols were determined gravimetrically by the digitonin method and saturated sterols by Schoenheimer's [1930] method. Coprosterol was recovered from the saturated digitonides by the pyridine-ether method.

Coprosterol excretion on brain diet. A series of experiments was carried out in which rats were kept exclusively on a diet of cooked sheep's brain. The brain was steamed for ½ hr. in a steam cooker, the supernatant fluid decanted and the remaining mass thoroughly mixed with bone meal (4 g. to 100 g. of fresh brain). The diet was prepared daily and readily taken by the animals, 10–15 g. being consumed per rat, per day. The brain diet, given for relatively short periods, did not produce any signs of ill health, but the animals lost slightly in weight.

In a typical experiment 12 rats received 200 g. of brain per day for 7 days and excreted 20·5 g. of sterols, of which 17·0 g. (82·9 %) were saturated sterols. The unsaponifiable portion of the ethereal extract was fractionally recrystallized from methyl alcohol and yielded 12·5 g. colourless needles of coprosterol, M.P. 100–101°, $[\alpha]_D^{20°}$ +23·5°. A mixed melting point with authentic coprosterol, prepared by catalytic reduction of cholestenone [Ruzicka *et al.* 1934], showed no depression. The benzoate, obtained by treatment of coprosterol with benzoyl chloride in pyridine, had M.P. 124–125°, and the acetate, M.P. 89–90°. A small quantity (0·4 g.) of impure cholesterol, M.P. 138–140°, was isolated from one of the fractions.

In a large scale experiment 100 rats consumed 5·5 kg. of brain in 4 days. They excreted 56·5 g. of sterols, of which 85·6 % were saturated sterols. The coprosterol excretion per rat, per day was 120 mg. The unsaponifiable fraction of the ether extract, worked up in the usual way, yielded 41·4 g. of crude coprosterol, M.P. 96–97°, and 0·5 g. of cholesterol, M.P. 146–147°.

The bulkiness of the faeces of rats on a brain diet was a constant feature in these experiments. The increase in volume and weight is partly due to undigested neurokeratin, present in brain to the extent of 15–20 %, and partly to the excretion of cleavage products of certain brain constituents (see below). Since the increased 'roughage' of the diet might have favoured coprosterol formation, control experiments were made in which cellulose (filter paper), bone meal or calcium phosphate was added to the diet together with cholesterol. The results of these experiments showed that 'roughage' had no effect on coprosterol excretion (see Table 1). An increase in the amount of 'unsaponifiable' ran parallel with the increase in weight of the faeces of rats on a brain diet. This increase was traced to the presence of a substance crystallizing in sphaerocrystals, which was isolated in considerable amounts (*ca.* 20 g.) from the ethereal extracts before or after saponification. The sphaerocrystals are readily soluble in hot methyl alcohol, acetone and ethyl acetate, and are easily separated from coprosterol owing to their insolubility in chloroform and petrol. In its melting point, 87–89°,

and properties the substance resembles lignoceryl sphingosin, a partial cleavage product of kerasin, which occurs in liver [Thannhauser & Fraenkel, 1931], lung and spleen [Tropp, 1935] and has also been found in serum in a case of hypercholesterolaemia [Schoenheimer, 1933]. In view of its possible bearing on the mechanism of coprosterol formation the substance is being further investigated.

Coprosterol excretion on the standard diet etc. The amount of sterols excreted on the standard diet was found to be so minute that a large number of rats had to be used in order to obtain reliable quantitative results. When 23 rats were kept for 7 days on the diet, they excreted 1·02 g. sterols of which 0·47 g. (46 %) consisted of saturated sterols. From the digitonide obtained after bromination in alcoholic solution, coprosterol, M.P. 96–97°, was isolated by the pyridine-ether method, the average amount per rat, per day being only 3 mg.

The effect of the addition of cholesterol to the standard diet was investigated by using a batch of 60 rats, in groups of 6 rats each, which served also as controls in other experiments. Cholesterol, 1 g. per day for each group, was incorporated in the diet. The total sterol excretion amounted to 38·3 g. of which 29·8 g., or nearly 80 %, consisted of cholesterol, which was readily isolated in typical crystals, M.P. 148–149°. Coprosterol was obtained from the saturated digitonides as above described, the average daily excretion per rat, 18 mg., being somewhat higher than on the standard diet alone.

Preparation of cholesterol-free brain powder etc. Minced ox brain (12 kg.) was treated at room temperature for 24 hr. with acetone (10 l.), filtered through linen on large Büchner funnels and the extraction repeated 5 times. After the 6th extraction and filtration the mass was freed from acetone in evacuated desiccators and finely ground in a mill. Four more extractions of the powder with 5 l. acetone each were made, the last extract yielding only traces of cholesterol. The final product, a cream-coloured powder, weighed 2 kg. Crude cholesterol (350 g.) was obtained from the acetone extracts. Cholesterol-free powders of pancreas, liver and egg yolk were prepared by the same technique.

Brain powder free from cholesterol and unsaturated phosphatides (lecithins and kephalins) was prepared by extracting minced brain (12 kg.) as above

Table 1

				Cholesterol intake		Sterol excretion				
						Total		Coprosterol		
Supplement	Per rat, per day g.	No. of rats	Days	Total g.	Per rat, per day mg.	g.	Per rat, per day mg.	g.	Per rat, per day mg.	% of total
Brain powder (ox) (cholesterol-free)	1·6	3	7	3·4	160	2·52	120	1·86	89	74
,,	1·5	3	7	3·4	160	2·51	120	2·22	105	89
Brain powder (sheep)	1·6	3	7	3·4	160	2·40	115	1·77	84	74
Brain powder (ox) (cholesterol and lecithin etc. free)	1·6	6	7	5·8	138	2·81	67	2·29	55	83
,,	1·0	3	21	9·1	145	6·48	102	5·67	90	88
Pancreas powder (cholesterol-free)	2·0	3	7	3·0	145	1·50	71	0·73	35	49
Egg-yolk powder (cholesterol-free)	2·0	3	7	3·0	145	1·71	81	0·78	37	45
Kidney (steamed)	3·5	6	7	7·0	167	4·07	95	1·35	32	33
Liver powder (cholesterol-free)	2·5	6	7	6·9	165	3·00	71	0·82	20	28
Horse flesh (steamed)	5·0	6	7	7·0	167	3·62	86	0·53	13	15
Cellulose	0·8	6	4	3·9	167	1·94	81	0·30	13	16

5 times with acetone, followed by immediate treatment with 10 l. ether at $\pm 0°$. The intermediate drying procedure was omitted in order to avoid oxidation of the unsaturated phosphatides. After 5 ether extractions, the last with 7·5 l., had been made, the mass was freed from ether and passed through a mill. The ether extracts deposited a small amount of 'white matter' on standing at $-4°$ which was mixed with the remaining brain powder. Petrol, B.P. 40–60°, was successfully used several times in place of ether, but its use was given up after it had been found on one occasion that the brain powder had been rendered inactive.

Some of the results of feeding rats on the standard diet with these powders together with cholesterol are recorded in Table 1.

The strikingly large conversion of cholesterol into coprosterol by rats on a diet containing brain powders is demonstrated by these results and contrasts strongly with the low coprosterol excretion on a meat diet (horse flesh), which is of the same order as that of the controls (see above).

Preparation of 'white matter', phrenosin etc. After the removal of cholesterol and unsaturated phosphatides, the finely ground brain powder (500 g.) was treated with 85 % alcohol (2·5 l.) for 1 hr. in a water bath at 45–50°, filtered and the extraction repeated three times. On cooling to room temperature the extracts deposited 'white matter' which was obtained as a fine white powder after filtering and drying in a desiccator. Details of feeding experiments with two of these preparations and the cerebrosides isolated from them are given in Table 2.

Phrenosin. The cerebroside fraction of the 'white matter' was separated from sphingomyelin by means of pyridine [Rosenheim, 1914]. The phrenosin fraction, still containing kerasin after fractionation from acetone at 36°, was further purified by two recrystallizations from mixtures of chloroform-acetic acid (2 : 3). The specimens of phrenosin so obtained were still contaminated with appreciable amounts of kerasin, as shown by the selenite plate test [Rosenheim, 1914], but contained only traces of sphingomyelin. Lack of material prevented further purification. The rats received daily doses of 150–300 mg. intimately mixed with the diet.

Thudichum's phrenosin. The following description of its preparation was recorded in Thudichum's own handwriting on the label of the bottle:

'From *Human Cerebrin Mixture* which had been exhausted with cold ether and then passed treatment with PbAc.NH_3 in spirit solution. This is *Phrenosin* which had been recrystallized from spirit only a great number of times, filtering at 28°, thus separating all Kerasin, until in the filtrate no more of the latter appeared. This portion deposited above 28° throughout and is probably free from kerasin—moist with spirit. In the last filtrate from kerasin $CdCl_2$ produced no more precipitate.'

The bottle had been so efficiently stoppered that its contents were still 'moist with spirit' on opening. The white lumps of phrenosin (60 g.) were ground up with spirit (600 ml.), warmed to 36°, kept at this temperature for 24 hr. and filtered. The alcoholic filtrate remained clear on cooling to $-4°$ and did not contain any kerasin. The white precipitate was washed with alcohol, acetone and ether and was finally obtained as a fine powder. The substance had M.P. 210–212°, $[\alpha]_D^{20°} + 4·55°$; $[\alpha]_{5461}^{20°} + 5·0°$ in pyridine ($c = 5·800$, $l = 4$ dm.). The selenite-plate test showed complete absence of kerasin. On hydrolysis by the technique described by Rosenheim [1913] phrenosinic acid, sphingosine and galactose were obtained in the usual amounts. Judged by modern standards therefore, this specimen of Thudichum's phrenosin is as pure as any prepared since by later investigators.

Thudichum's kerasin had also been obtained from human brain, no details of its preparation appearing on the label. The white product was fractionally crystallized from 10 vol. chloroform-acetic acid mixture (2 : 3). The fraction depositing below 28° had the characteristic properties of kerasin, setting to a translucent jelly from solvents on cooling, waxy when dry, but easily powdered to a white powder, M.P. 185°. In contrast to phrenosin the substance is laevo-rotatory in pyridine solution: $[\alpha]_D^{19°} -3{\cdot}11°$; $[\alpha]_{5461}^{19°} -4{\cdot}0°$ ($c=5{\cdot}190$; $l=4$ dm.). The selenite-plate test showed sphaerocrystals exclusively of the typical kerasin type. Only one experiment could be carried out with this preparation, which gave a decisively negative result.

Various preparations of more or less pure sphingomyelin were obtained from the 'white matter' during the pyridine fractionation process. They sufficed for only three feeding tests which gave inconclusive results. One specimen which contained a considerable amount of cerebrosides gave as much as 90% coprosterol in doses of 250 mg. per rat, per day, whilst another much purer specimen, in doses of 150 mg., proved to be inactive. Of the final cleavage products of the cerebrosides, galactose had no influence on coprosterol excretion. Only a few experiments have so far been made with the fatty acid components of phrenosin and kerasin. Phrenosinic acid, M.P. 102–103°, in doses of 150 mg. per day, yielded 58% coprosterol in one experiment, but its ethyl ester, M.P. 65–66°, was inactive in doses of 180 mg. Presumably the ester was not hydrolysed by the rat organism, as the fatty acid contents of the faeces showed an abnormally high figure in this case. The ethyl ester of lignoceric acid, M.P. 56–57°, was equally inactive. The basic component of phrenosin, sphingosine, could not be tested since the animals consistently refused to eat the diet containing either the free base or the sulphate. Choline hydrochloride had no effect on coprosterol formation in daily doses of 100 mg.

Table 2

				Cholesterol intake		Sterol excretion				
						Total		Coprosterol		
Supplement	Per rat, per day mg.	No. of rats	Days	Total g.	Per rat, per day mg.	g.	Per rat, per day mg.	g.	Per rat, per day mg.	% of total
White matter	330	3	7	3·3	157	2·01	96	1·75	84	87
,,	150	3	7	3·2	152	2·01	96	1·62	77	80
Phrenosin	300	3	7	3·1	148	1·67	79	1·38	66	83
,,	150	3	7	3·4	162	2·31	110	1·19	57	52
Thudichum's phrenosin	300	3	7	3·4	162	2·32	110	1·86	89	81
,,	330	3	7	3·4	162	2·13	101	1·75	83	82
,,	300	3	20	12·0	200	6·32	105	5·15	86	82
,,	165	3	7	3·4	162	2·21	105	1·13	54	51

The results recorded in Table 2 clearly show that the dietetic factor is soluble in hot 85% alcohol and is contained in the 'white matter' deposited on cooling the extracts. A tenfold concentration of the factor in the 'white matter' had been effected, daily doses of 150 mg. of the latter being as active as 1·5 g. of the cholesterol-free brain powder. Phrenosin is also highly active, but it is evident that it does not account for the whole activity of the 'white matter', since both Thudichum's and our own preparations of phrenosin had appreciably less effect on coprosterol excretion in daily doses of 150 mg. than the same doses of 'white matter'.

Discussion

Vague statements frequently occur in the literature regarding cholesterol metabolism, suggesting that the excretion of coprosterol is connected in some undefined manner with the kind of diet consumed. The experimental evidence now available shows that the presence in the diet of a definite chemical substance, or substances, is essential for the functioning of the mechanism concerned in coprosterol formation and a firm basis is thus provided for further investigation.

Meanwhile, existing views can be considerably clarified. Thus it becomes increasingly clear that the belief in the reducing activity of intestinal bacteria as the sole causative agent in coprosterol formation can no longer be upheld. For in numerous feeding experiments with cholesterol under conditions in which there was no reason to doubt that the usual bacterial flora was at work, we found that practically the whole of the cholesterol of the food was excreted unchanged so long as the factor was missing. It might, indeed, be suggested in explanation of this fact that a different type of bacterial flora develops under the influence of the factor. No evidence for this was obtained, however, in any of the cases in which the animals excreting coprosterol ceased to do so when the factor was withdrawn or resumed the excretion when the factor was added to the diet. Müller's [1900] early and somewhat primitive experiment demonstrating the now well-established fact that cholesterol only is excreted by adults on a milk diet, is traditionally quoted in support of the suggestion that the reduction to coprosterol is effected by certain bacteria. It would be more difficult to explain on these lines the fact that cholesterol, and not coprosterol, is the main constituent of the faecal sterols in animals receiving an exclusive diet of such a generally adequate bacterial nutrient as lean meat [see Table 1; Dorée & Gardner, 1908; Ammundsen, 1936]. Both observations find a more immediate explanation in the fact that cerebrosides are absent from both milk and muscle.

At this preliminary stage of the enquiry it would be premature to assume that the cerebrosides, and phrenosin in particular, are the only active constituents of 'white matter'. Nor is experimental evidence as yet available to supply an interpretation for the mode of action of the factor which would make its connexion with the cholestenone mechanism intelligible. The relatively large amount of active substance required to convert a given amount of cholesterol into coprosterol suggests a stoichiometric relationship between the two substances rather than a catalytic or vitamin-like activity of the factor. It may be suggested that the metabolism of the cerebrosides is interlocked with that of cholesterol esters and fats by a process of exchange-esterification of the kind envisaged by Sobotka [1938] as being concerned in the transport of fatty acids. Subsequent interaction of lipolytic and oxidative enzymes would then lead to the intermediate formation of cholestenone and its final hydrogenation to coprosterol in the intestine. This suggestion is supported by experiment in so far as we found that, in the absence of fat from the standard diet, phrenosin was unable to effect coprosterol formation and cholesterol only was excreted.

Arising out of the consideration that coprosterol excretion in man and omnivorous animals might be of the nature of a protective mechanism, enabling the organism to rid itself of the excess of cholesterol supplied in the food, it was of interest to investigate the possible inhibitory effect of phrenosin on cholesterol infiltration of the liver and the production of 'fatty livers' which result from prolonged cholesterol administration. A test of this possibility gave a completely negative answer. In a series of experiments in which rats were kept for 3 weeks on diets containing 2 % cholesterol with the addition of brain powder

in some cases and phrenosin in others, it was found that brain powder completely prevented 'fatty liver' formation, but that with phrenosin in the diet, the fat and cholesterol content of the livers was as high as that of the livers of the control animals. The brain powder was free from cholesterol and lecithins, but rich in sphingomyelin. The choline content of the latter was clearly responsible for the preventive or curative effect of the brain powder, since the same result was produced by the administration of a sphingomyelin preparation together with cholesterol.

Summary

A dietetic factor is contained in brain and other organs, but not in muscle and milk, which is essential for the conversion of cholesterol into coprosterol by the animal organism.

The factor is thermostable, insoluble in water, acetone, ether or petrol, but soluble in hot 85 % alcohol.

Thudichum's 'white matter', deposited on cooling alcoholic extracts of brain powder (free from cholesterol and unsaturated phosphatides), is rich in the factor.

A specimen of Thudichum's original phrenosin from human brain, as well as other preparations of phrenosin from ox brain, effected a conversion of cholesterol into coprosterol to the extent of 80 % of the total faecal sterols.

REFERENCES

Ammundsen (1936). *Biochem. Z.* **284**, 313.
Anchel & Schoenheimer (1938). *J. biol. Chem.* **125**, 23.
Bondzinsky (1896). *Ber. dtsch. chem. Ges.* **29**, 476.
Dam (1934). *Biochem. J.* **28**, 820.
Dorée & Gardner (1908). *Proc. roy. Soc.* B, **80**, 227.
Fex (1920). *Biochem. Z.* **104**, 134.
Marcet (1857). *Philos. Trans.* **147**, 403.
—— (1860). *Ann. Chim.* (*Phys.*), **59**, 91.
Müller (1900). *Hoppe-Seyl. Z.* **29**, 129.
Rosenheim (1913). *Biochem. J.* **7**, 610.
—— (1914). *Biochem. J.* **8**, 110.
—— (1930). *Chem. and Ind.* **48**, 238.
—— & Starling (1933). *Chem. and Ind.* **52**, 1056.
—— —— (1937). *J. chem. Soc.* p. 377.
—— & Webster (1935). *Nature, Lond.*, **136**, 474.
Ruzicka, Brüngger, Eichenberger & Meyer (1934). *Helv. chim. Acta*, **17**, 1415.
Schoenheimer (1930). *Hoppe-Seyl. Z.* **192**, 77.
—— (1933). *Z. klin. Med.* **123**, 749.
—— Behring, Hummel & Schindel (1930). *Hoppe-Seyl. Z.* **192**, 73.
—— Rittenberg & Graf (1935). *J. biol. Chem.* **111**, 183.
Sobotka (1938). The Chemistry of the Steroids, p. 83. Baltimore.
Thannhauser & Fraenkel (1931). *Hoppe-Seyl. Z.* **203**, 183.
Tropp (1935). *Hoppe-Seyl. Z.* **237**, 178.