CXVI. THE FORMATION OF COPROSTEROL IN THE INTESTINE[1].

II. THE ACTION OF INTESTINAL BACTERIA ON CHOLESTEROL.

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ALTHOUGH various investigators [Bondzynski and Humnicki, 1896–97; Schönheimer *et al.*, 1930; Beumer and Bischoff, 1930] had reported the failure of attempts to convert cholesterol into coprosterol by bacteria *in vitro*, it was stated by Bischoff [1930, 1, 2] that the degree of saturation of faecal sterols increases when faeces are putrefied. This result, obtained by applying the iodine method of Bürger and Winterseel [1929] to sterols isolated from their digitonides by extraction with xylene, points to the probability that coprosterol can be formed by bacterial action.

An easier method is now available for the determination of total saturated sterols in the presence of unsaturated sterols [Schönheimer, 1930], and a method for the determination of dihydrocholesterol and coprosterol in a mixture has been described in the preceding paper. With the aid of these methods I have been able to confirm Bischoff's statement and to show that under the conditions of intestinal putrefaction cholesterol is converted not into dihydrocholesterol but into coprosterol.

Most of the putrefaction experiments were conducted quantitatively, and it was found that the total sterol was constant during putrefaction, indicating that intestinal bacteria are not able to destroy cholesterol or coprosterol.

EXPERIMENTAL.

The putrefaction experiments were generally carried out in an atmosphere of nitrogen or hydrogen although some were performed under aerobic conditions. When the putrefaction was conducted in the absence of oxygen, water or mercury seals were employed; under aerobic conditions the vessels were closed merely with a cotton plug. (In the aerobic experiments the processes in the inner mass of the faeces were most probably anaerobic because of the slow diffusion of gases from the surface.)

It was noted that in putrefaction under hydrogen the gas disappeared in amounts greatly exceeding those corresponding to the hydrogenation of the cholesterol.

The faeces were rubbed up to a uniform consistency in a mortar and weighed out with an accuracy of about 50 mg. Unless otherwise stated in the tables, 10 g. of faeces were employed in each experiment. The unsaponifiable matter

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was isolated, and the saturated sterols were determined according to the method of Schönheimer [1930]. Only when the degree of saturation was very small (when cholesterol was added to the faeces or with a milk diet) did cholesterol dibromide crystallise from the alcohol on addition of bromine. In general 30–100 mg. of digitonide were obtained; only with low degrees of saturation were the amounts smaller than this, in which case the accuracy of the results was correspondingly influenced.

1. *Putrefaction of faeces without additions.*

Table I.

		Total sterols in 10 g.				
		Before incubation		After incubation		
	Diet	Amount mg.	Saturation %	Amount mg.	Saturation %	Incubation
1. Adult (H. D.)	Mixed	94·8	91·6	92	92·6	Aerobic, 49 hrs., 40°
2. ,, (S)	,,	63·0	78·6	65	88	In H_2, 7 days, 38°
3. ,, (corpse)*	,,	75·2	80·0	77·8	87·5	In H_2, 10 days, 38°
4. ,, (E. J.)	Milk†	59·8	69·6	61·8	79·5	In H_2, 48 hrs., 37°
5. ,, (I. D.)	Milk‡	85·2	2·5	85·3	1·8	In H_2, 67 hrs., 38·5°
6. Infant (8 days old)	Milk	81·2	8·5	80·2	9·1	Aerobic, 48 hrs., 37°

* Colon contents.
† 4 litres daily, beginning 4 days before experiment.
‡ 1 litre milk, 0·5 litre 30 % cream, 1 zwieback, daily, beginning 4 days before experiment.
p_H of the faeces = 8·1 (quinhydrone) (a few days earlier the faeces were acid).

In none of the experiments was a decrease observed in the total sterols during putrefaction, the difference lying within the limits of error. In Exps. 2, 3 and 4 hydrogenation of the sterols is clearly evident. No hydrogenation occurred in Exp. 1, which may be ascribed to the fact that the initial degree of saturation was very high (92 %). These experiments confirm the observation of Müller [1900] that the degree of saturation of the sterols falls on a milk diet. In cases of extremely low initial saturation (Exps. 5 and 6) no hydrogenation occurs during putrefaction.

2. *Putrefaction of the contents of the jejunum and ileum.*

The amount of cholesterol hydrogenated during putrefaction of colon contents and faeces even in the positive experiments could be only small, for the major part of the sterols existed at the outset in the form of coprosterol. An experiment (No. 7, not reported in the Tables) was accordingly undertaken with the united contents of the jejunum and ileum, in which hydrogenation has, as a rule, proceeded only to a slight extent. The material employed was obtained from the individual whose colon contents were examined in Exp. 3 (Table I). On analysis 10 g. of the fresh material showed 23·6 mg. of total sterols of which 2·5 % was saturated; after incubation for 10 days at 38° in H_2 the total sterols were 24·8 mg. with 2·6 % saturation. The hydrogenation of sterols thus appears to be associated with the contents of the large intestine.

3. *Putrefaction of faeces with added cholesterol.*

As the cholesterol for these experiments had to be in a form that could be attacked by bacteria, crystalline material was unsuitable. Egg-yolk, known to be rich in cholesterol, was employed; or cholesterol was either brought into

solution in bile acids and fatty acids or employed in the form of the water-soluble ester salts synthesised by Schönheimer and Breusch [1932]. In later experiments (Tables III and IV) colloidal cholesterol solutions secured from the firm of von Heyden were also employed. All added solutions were neutralised to litmus.

Table II.

		Substance	State	Total sterols in 10 g. Amount mg.	Total sterols in 10 g. Saturation %	Incubation
8.	Adult (H. D.)	35 g. faeces + 15 g. egg-yolk	Fresh	120	61·4	—
			Incub.	120	64·9	Aerobic, 48 hrs., 37°
9.	,, (E. J.)	100 g. faeces + 18 g. egg-yolk	Fresh	93·4	69·3	—
			Incub.	93·5	85·4	In H_2, 49 hrs., 39–43°
10.	,, (S)	8 g. faeces + 10 ml. sol. A	Fresh	—	33·2	—
			Incub.	—	36·1	In H_2, 4 hrs., 37°
			,,	—	34·4	In H_2, 47 hrs., 37°
11.	,, (corpse)	60 g. colon content + sol. B	Fresh	207	60·7	—
			Incub.	204·5	74·7	In H_2, 18 days, 37°
12.	,, (S)	8 g. faeces + 1·5 ml. sol. C	Fresh	115·2	43·8	—
			Incub.	114	50·8	In H_2, 7 days, 38°
			,,	117	52·3	In N_2, 7 days, 38°
			,,	113	43·8	Sterilised in boiling water-bath, then 7 days at 38° in H_2
13.	,, (S)	20 g. faeces + 3 ml. sol. D	Fresh	—	45	—
			Incub.	—	58·4	In H_2, 9 days, 37°

All were on mixed diet.
Solution A. 6·2 g. sodium desoxycholate, 1 g. sodium stearate and 0·35 g. cholesterol in 30 ml. water.
,, B. 1·2 g. sodium salt of monocholesterylbutanetetracarboxylic acid in 8 ml. water. The whole solution was added.
,, C. 1·5 g. sodium salt of monocholesterylbutanetetracarboxylic acid in 25 ml. water.
,, D. 0·6 g. sodium salt of monoallocholesterylbutanetetracarboxylic acid in 6·5 ml. water.

Cholesterol in egg-yolk or in the form of soluble preparations was only slightly hydrogenated, the increase in saturated sterols being little more than that obtained without the addition of cholesterol.

A parallel experiment in which allocholesterol was added in the form of its soluble acid ester (Exp. 13) was also carried out. The failure of an attempt to convert allocholesterol into coprosterol by putrefaction has been reported by Beumer and Bischoff [1930]; these authors however had heated the allocholesterol before the experiment, a procedure which may have converted it into cholesterol. The soluble allocholesterol ester was not converted any more rapidly than the analogous cholesterol ester. The addition of large quantities of bile acids and fatty acids appears to diminish the extent of hydrogenation.

4. *Effect of formates on putrefaction.*

Sodium formate, which is an efficient hydrogen donator for *B. coli* and other bacteria, was without influence.

In Exp. 14 the rather extensive hydrogenation was not increased by formate. Essentially the same result was obtained in Exp. 15, in which it was found that cholesterol betaine ester was practically not hydrogenated.

Table III.

	Substance	State	Saturation of sterols %	Incubation
14.	9 g. faeces + colloidal cholesterol solution	Fresh	62·7	—
		Incub.	96·5	In N_2, 6 days, 38°
		,,	92·5	Ditto, with 3 ml. 5 % H.COONa
15.	3 g. faeces + 86 mg. cholesterylbetaine ester hydrochloride	Fresh	22·9	—
		Incub.	23·5	Aerobic, 12 days, 38°
		,,	27·7	Ditto, with 3 ml. 5 % H.COONa

Subjects were adults on mixed diet.

5. *Attempted hydrogenation with pure bacterial cultures*[1].

Table IV.

		Saturation of sterols %
16.	Human bile (gall bladder), 170 ml. + 17 ml. 5 % H.COONa + 4·9 g. *B. coli*. Incubated aerobically for 10 days	4·0
17.	Brain hash (human) 62 g. + 6·2 ml. 5 % H.COONa inoculated with *B. coli*. Incubated *in vacuo* 5 days	1·9
18.	Brain hash (human) 37 g. + 3·6 ml. 5 % H.COONa, inoculated with *B. coli*. Incubated *in vacuo* 5 days	0·3
19.	Colloidal cholesterol in protein (von Heyden) 1 g. + 4 ml. water + 700 mg. *B. coli*. Incubated *in vacuo* 12 days	5·7
20.	Colloidal cholesterol in protein, 1 g. + 5 ml. 5 % H.COONa + 1·16 g. *B. coli*. Incubated *in vacuo* 12 days	0·6
21.	Colloidal cholesterol solution (von Heyden), 5 ml. + 20 ml. nutrient bouillon with 10 % glucose, inoculated with *Enterococcus* Type B. Incubated aerobically 2 days	*

* The amount was too small to weigh.

The action of pure bacterial cultures was, in spite of the large amounts of bacteria employed, barely perceptible, and certainly considerably less than in putrefaction of faeces.

6. *Nature of the saturated sterols formed during putrefaction.*

The digitonin-precipitable, saturated sterols formed from cholesterol can consist only of coprosterol or dihydrocholesterol, so that the isolation of the saturated sterol formed by putrefaction cannot be accomplished by methods at present available. In the previous communication however a method has been described for determining which of the two sterols has been formed. Since relatively large quantities of sterol are necessary for the solubility determinations, the digitonides from Exps. 2 and 12 were employed. Both experiments involved the same sample of stool, of which a large quantity was available. In Exp. 12 soluble cholesterol ester was added. The appropriate combined digitonides from Exps. 2 and 12 were united for the determination of sterols before and after putrefaction, respectively. During putrefaction 11–13 % of saturated sterols had formed; if these had consisted of dihydrocholesterol, the solubility of the digitonides in methyl alcohol would have been considerably lower than before putrefaction, whereas if coprosterol had formed, the solubility would barely have changed. Actually the solubilities were 42·0 and 43·4 mg. in 2 ml.

[1] Thanks are due to Miss R. Marget for valuable help in the bacteriological work.

of methyl alcohol respectively, before and after putrefaction. If dihydrocholesterol had been the sole product of hydrogenation, the solubility of the sterol-digitonide after putrefaction would have been about 37 mg.

Discussion.

The observation that cholesterol and coprosterol are not degraded by intestinal bacteria is of importance in the interpretation of cholesterol balance experiments. It is therefore possible to ascribe the destruction of cholesterol in the body [Dam, 1931; Page and Menschick, 1932; Menschick and Page, 1933; Schönheimer and Breusch, 1933] to a process of intermediary metabolism rather than to the agency of intestinal bacteria.

The present results confirm those of Bischoff [1930, 1, 2] in indicating that the amount of saturated sterols increases, but they differ from them quantitatively. In one case Bischoff found an increase from 12 to 66 %; I have never observed so large a change. The values found by Bischoff for the degree of saturation of the sterols of normal human faeces are rather low in comparison with the observations of Schönheimer and von Behring [1930] and those here reported which were found by the gravimetric method of Schönheimer. This discrepancy may perhaps be explained by some technical error in the determinations of Bischoff—an interpretation supported by the statement of Bischoff that a coprosterol with a melting-point of 97° employed by him was precipitable by digitonin only to the extent of 60 %.

Although hydrogenation took place only to a small extent on putrefaction, its occurrence was conclusively established in almost every experiment with normal faeces. It failed to occur only when the initial degree of saturation was extremely high (Exp. 1), and in some of the milk diet experiments in which the initial saturation was extremely low. Complete hydrogenation of unsaturated sterol was never attained, even on long incubation.

Added cholesterol, whether in the form of egg-yolk, as soluble ester or in colloidal solution, remained largely unattacked by the bacteria. It thus seems possible that the cholesterol exists in the colon in some unknown form which is especially susceptible to bacterial action.

The hydrogenation is certainly a bacterial process. It does not take place after sterilisation (Exp. 12), and it is associated particularly with the contents of the colon, for it takes place neither in the small intestine nor on the inoculation of cholesterol-containing organs or soluble cholesterol preparations with bacterial cultures. It may be supposed that either the optimum conditions for the growth of the appropriate bacteria can be obtained only in the colonic secretions, or these secretions contain special hydrogen donators. Sodium formate can apparently not be employed by the bacteria for the formation of coprosterol. Better results might perhaps be obtained by the use of sterile colon excretions as nutrient medium, but such material was not available.

The identity of the causative bacteria could not be determined.

The proof, by solubility determinations of the digitonides, that the hydrogenated sterol is not dihydrocholesterol but coprosterol establishes the fact, long suspected, that it is coprosterol which is formed by bacterial hydrogenation of cholesterol.

Summary.

In the putrefaction of faeces the degree of saturation of the sterols rises owing to formation of coprosterol from cholesterol. The total amount of sterol remains constant.

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