Conversion of Cholesterol into Coprosterol by Bacteria *in vitro*

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SUMMARY: Certain anaerobic bacteria from human faeces were found to be able to hydrogenate cholesterol *n vitro*; some also decreased to a marked degree the total amount of sterols in the incubation mixture. Other organisms did not hydrogenate or decrease the amount of sterols under the conditions chosen; among these were; *Clostridium welchii*, *C. sporogenes*, *Bacterium bifidum*, various streptococci and micrococci, *Escherichia coli*, *Aerobacter aerogenes*. A 'germ-free' filtrate from human faeces was inactive. In a series of incubated samples, where the Tschugaeff reaction was applied to the non-saponifiable matter, an orange colour developed, suggestive of the presence of lathosterol.

That hydrogenation of cholesterol can be carried out with human faeces or colon contents *in vitro* was demonstrated by Dam (1934*b*). At the same time it was shown that faeces heated at 100° for 20 min. failed to hydrogenate cholesterol. Certain bacteria in pure culture, such as *Escherichia coli* and a type B enterococcus were inactive. Later, Rosenfeld, Fukushima, Hellman & Gallagher (1954*a, b*) succeeded in converting cholesterol into coprosterol with human faeces *in vitro*. Rosenheim & Webster (1943) found that the bacteriostatic agent succinylsulphathiazole inhibited coprosterol formation *in vivo* in rats, but they also observed inhibition by the amoebicidal agent *p*-carbamino-phenylarsonic acid which did not suppress the coliform flora. They therefore questioned the participation of bacteria in the process. The present work shows that cholesterol can be converted into coprosterol by some anaerobic bacteria from human faeces, whereas other such bacteria are inactive in this respect.

METHODS

Composition of media. Suspensions of brain were used as cholesterol-containing substrate. Rosenheim & Webster (1941) were of the opinion that cerebroside such as those in brain are necessary for the hydrogenation of cholesterol in the intestine. However one of us (Dam, 1934*a*) found that, in man, faecal cholesterol was hydrogenated to more than 80 % without ingestion of cerebroside. Further, Rosenfeld *et al.* (1954*a, b*) used substrates without added cerebroside in their *in vitro* experiments. The reason why we used brain suspensions as substrate is that such substrates are an easy means of providing a colloidal suspension of cholesterol which at the same time contains nutrients for bacteria. Desiccated hog or ox brain (2.5 or 5 g. portions) were stirred with tap water to make 5 % (w/v) suspensions. In some cases, 1 % (w/v) peptone and 0.1 % (w/v) glucose were also added. Undissolved

matter was not filtered off. Sterilization was carried out for 30 min. at 100° (in live steam) on two consecutive days and for 30 min. at 120° on the third day. After the second heating 0.1 % (w/v) of cysteine hydrochloride was added. In every experiment the contents of all flasks were adjusted to contain the same amount of the substrate.

Flasks inoculated with clostridia were pasteurized at 80° for 7 min. after addition of the clostridia, but before the addition of non-sporing bacteria.

After inoculation as described in Table 1 the mixtures were incubated *in vacuo* in the dark at 37° for 7–8 days. After incubation the contents of each flask were saponified with an equal volume of 60 % (w/w) KOH on a steam bath for 2–3 hr. on 2 or more days until all bulky material had been dissolved. The non-saponifiable matter was shaken out with ether three times, the extract dried with anhydrous sodium sulphate, filtered, evaporated to dryness and the residue taken up in 25 ml. chloroform. The amount of dry matter in the chloroform solution was determined.

As a preliminary the Liebermann–Burchard reaction was carried out on all samples of the chloroform-soluble material. On some samples (Table 1, Exp. 4) Tschugaeff's reaction (see Hanel & Dam, 1955), was also used. Thereafter samples were taken up in 96 % (v/v) ethanol in water and the total amount of sterols determined by precipitation with digitonin (1 %, w/v, in 96 % ethanol). The determination with digitonin was repeated after adjustment of the concentration of the sterol as found by the preliminary determination, to 0.5 % (w/v), with a 50 % excess of digitonin.

The amount of saturated sterol was determined in another sample by the bromine-digitonin procedure of Schönheimer (1930) with the slight modification that, when possible, the final determination was made with an ethanolic (96 %) solution containing 0.5 % (w/v) of saturated sterol.

In cases where a degree of hydrogenation above 90 % was found, the saturated sterol was liberated from the digitonides by the pyridine-ether method (Schönheimer & Dam, 1933), recrystallized from 96 % ethanol and identified by crystal form, melting-point and melting-point of its mixture with a sample of coprosterol previously prepared from dog faeces.

RESULTS

The results are presented in Table 1. Expt. 1 shows (as found earlier: Dam, 1934*b*), that it is possible to hydrogenate cholesterol by incubation with human faeces *in vitro*. The same appears from Expt. 2*b* and *c*. In cases where the incubation had resulted in hydrogenation of the sterols, the non-saponifiable matter was oily or largely oily, whereas it was crystalline in cases where hydrogenation of sterols had not occurred. Expt. 2*f* shows that incubation with a 'germ-free' filtrate (i.e. free from dead organisms also) from the same faeces did not result in hydrogenation of cholesterol. Neither was hydrogenation obtained with a mixture of certain bacteria from the same faeces with or without addition of 'germ free' filtrate (Expt. 2*d*, *e*). The inactive bacteria were various streptococci, micrococci, *Escherichia coli*, *Aerobacter aerogenes* and *Bacterium bifidum*.

Table 1

Inoculum	No. of days incubated at 37°	Total sterol (mg.)	Saturation of sterols (%)	Non-saponifiable material	
				mg.	Appearance
Expt. 1. 10% dried hog brain in water, total vol. 100 ml.					
(a) Human faeces 12.5 g. sterilized 120°, 15 min.	7	405	13.5	575	Crystalline
(b) Human faeces, 12.5 g.	7	350	80	690	Oily
Expt. 2. 5% dried hog brain +0.1% cysteine hydrochloride in water, total vol. 50 ml.					
(a) No addition	8	201	1.8	268	Crystalline
(b) 6 g. human faeces sterilized 120°, 15 min.	8	214	18	320	Crystalline
(c) 6 g. human faeces	8	198	82	340	Oily
(d) streptococci, micrococci, coli, aerogenes, bifidum from faeces of same person as above	8	199	18	305	Crystalline
(e) Bacteria as in (d) + 'germ-free' filtrate* from 6 g. human faeces	8	196	15.5	298	Crystalline
(f) 'Germ-free' filtrate* from 6 g. human faeces	8	196	4.0	290	Crystalline
Expt. 3. 5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 50 ml. (pH 7.2)					
(a) No addition	8	220	2.0	270	Crystalline
(b) <i>Clostridium welchii</i>	8	249	1.5	310	Crystalline
(c) <i>C. sporogenes</i>	8	258	2.0	306	Crystalline
(d) Anaerobic bacteria from human faeces†	8	136	89	385	Oily
(e) Anaerobic bacterium no. 103 from human faeces	8	229	2.2	252	Crystalline
(f) <i>B. bifidum</i> A9,‡ <i>B. bifidum</i> K1§	8	225	2.7	270	Crystalline
(g) Anaerobic bacteria nos. 101, 102, 106, 107 from human faeces	8	187	1.8	232	Crystalline
(h) Anaerobic bacterium no. 108 from human faeces	8	226	3.0	250	Crystalline
5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 50 ml. (pH 7.2) + 1% peptone + 0.1% glucose					
(i) Anaerobic bacteria from human faeces	8	26	80	315	Oily
Expt. 4. 5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 100 ml. (pH 7.2)					
(a) No addition	8	465	2.1	570	Crystalline
(b) Clostridia nos. 3 and 7 from hamster faeces	8	465	5.0	620	Crystalline
(c) Clostridia M2 + H2 from human faeces	8	478	2.4	680	Crystalline
(d) Anaerobic bacteria from human faeces as in Expt. 3 <i>d</i>	8	105	77.0	738	Oily
(e) Anaerobic bacteria from human faeces as in Expt. 3 <i>i</i>	8	114	79.5	730	Oily
(f) Clostridia as in <i>b</i> and <i>c</i> + 16 different bacteria from human faeces	8	493	4.8	688	Crystalline
(g) Clostridia as in <i>c</i> + bacteria nos. 120, 121, 122 and 123 from human faeces	8	488	2.3	660	Crystalline
(h) Anaerobic bacteria tolerating 1 mg. Cu in 10 ml. substrate¶	8	267	93	773	Oily
(i) Bacteria as in <i>c</i> + <i>h</i>	8	260	93	800	Oily
(j) Culture as in <i>d</i> stored at 15°, 6 months	8	442	92	760	Oily
5% dried ox brain +0.1% cysteine hydrochloride in water, total vol. 100 ml. (pH 7.2) + 1% peptone +0.1% glucose					
(k) Anaerobic bacteria from human faeces as in Expt. 3 <i>i</i>	8	61.5	87	698	Oily

* The 'germ-free' filtrate from faeces was prepared by grinding 45 g. faeces with 90 g. water, centrifuging the mixture and passing the supernatant fluid through a sterile Seitz filter by suction.

† 5 g. faeces were inoculated into 25 g. 10% ox-brain extract +0.1% cysteine HCl, and incubated *in vacuo* at 37° for 2 days; thereafter 0.5 ml. of the mixture was inoculated into another 25 g. portion of the same substrate, incubated *in vacuo* as above; thereafter the same procedure was repeated 3 times.

‡ Isolated from faeces of an old and presumably normal person.

§ Isolated from infant less than six months of age.

¶ As note † but +1% peptone +0.1% glucose in substrate.

¶ This mixture of anaerobic bacteria was obtained by inoculating the bacteria used in Expt. 3d into a 10% ox-brain extract to which had been added CuSO₄ in an amount corresponding to 1 mg. Cu/10 ml. medium. They were re-inoculated once into a similar substrate before use.

Expt. 3 shows that *Clostridium welchii* and *C. sporogenes*, two strains of *Bacterium bifidum*, and some anaerobic bacteria from human faeces did not hydrogenate cholesterol under the conditions of the experiment, whereas certain anaerobic bacteria from human faeces gave a high degree of hydrogenation (Expt. 3*d, i*). These latter bacteria also decreased the total amount of sterol in the incubation mixture, especially when peptone and glucose had been added (Expt. 3*i*). In Expt. 4*d*, considerable hydrogenation of cholesterol was obtained by the same anaerobic bacteria as in Expt. 3*d*. Expt. 4*e, k* show that these same bacteria also hydrogenated cholesterol, whether or not peptone and glucose had been added. When these anaerobic bacteria were grown in a medium containing 1 mg. Cu/10 ml. their ability to hydrogenate cholesterol remained intact.

Expt. 4*d* was repeated after the culture had been stored at 15° for 6 months. The results (Expt. 4*j*) show that the ability to hydrogenate cholesterol had been retained, whereas the ability to convert cholesterol to non-digtonin precipitable substances was largely lost.

Several clostridia and bacteroides from human faeces did not hydrogenate (Expt. 4*b, c, f, g*).

The digitonides from the Expt. 4*h, i* and *j* were dissolved in pyridine and decomposed with ethyl ether according to Schönheimer & Dam (1933), digitonides from *h* and *i* being pooled. After recrystallization from 96% ethanol the sterol obtained from (*h* + *i*) melted at 98–100°, that from *j* at 99–101°. In both cases the crystals showed the needle form characteristic of coprosterol and showed no depression of melting-point after being mixed with a sample of coprosterol obtained from dog faeces. Attempts are being made to isolate, from the mixtures which were found active, purified cultures of organisms capable of hydrogenating cholesterol. In these mixtures *Bacteroides* spp. and pleuropneumonia-like organisms were especially evident.

In Expt. 4, where the Tschugaeff reaction was used, it was found that samples *c, d, e, h*, and *k* gave an orange colour, suggesting the presence of lathosterol (Δ^7 -cholestenol; cf. Hanel & Dam, 1955). This recalls the finding of lathosterol in rat faeces reported by Wells, Coleman & Bauman (1955).

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