BILIRUBIN AND UROBILINS IN GERMFREE, EX-GERMFREE, AND CONVENTIONAL* RATS‡

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It seems to be a well established fact that bilirubin is reduced to urobilins by the intestinal microbial flora. (The term urobilins is used to mean the *group* of reduction products of bilirubin: the levorotatory stercobilin, the inactive urobilin, and the dextrorotatory dehydrourobilin, or their chromogens.) Researches upon this subject are reviewed by Lemberg and Legge (1), Watson (2, 3), and With (4). Very little is known, however, concerning the kind of microorganisms involved. Another problem is whether a production of urobilins also occurs outside the intestinal tract. Baumgärtel (5) is of the opinion that urobilinogen is produced by liver enzymes, whereas stercobilinogen is formed by bacteria. After a survey of the experimental evidence Watson (3), however, stated "if the liver participates in urobilinogen formation, it must be to a very minor and unimportant extent and that in general the intestinal origin of the urobilinogen group is by far the most significant."

If an extraintestinal formation of urobilin occurs urobilin must be formed also in germfree animals. The excretion of bilirubin and urobilin have therefore been determined in germfree and conventional rats. The findings in the germfree animals have also been compared with those of the same animals after contamination with full intestinal flora from conventional animals or with strains of bacteria isolated from the intestinal tract of humans or conventional rats.

Material and Methods

A. Germfree and Bacteriological Technique.—The germfree rats of the 4th or higher generations were reared according to the technique by Gustafsson (6, 7) and given a semisynthetic diet (7) and water *ad libitum*. The diet was sterilized with saturated steam at 121°C. for 20 minutes. Conventional rats of the same strain were kept in the animal room and given the same sterilized diet as the germfree rats. Adult rats of both sexes were studied. Urine and feces were collected every 24 hours in metabolism cages.

* The expression "conventional" refers to normal animals, raised and maintained under ordinary conditions, without any attempt at altering their indigenous microbial flora.

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For the study of the effect of full or partial bacterial intestinal flora germfree animals were infected in five different ways. (*a*) Rats were transferred from the germfree apparatuses and given orally 0.1 ml. of a suspension in water of feces from the conventional rats. (*b*) As it was known that germfree rats develop a defective intestinal flora if transferred to a laboratory with no other animals (Gustafsson, 8) a group of germfree rats was treated in this way. (*c*) To study the influence of intestinal spore formers some germfree rats were given cecal contents from conventional rats, which material was diluted with equal parts of water and heated to 80°C. for 5 minutes. (*d*) Germfree animals were infected with anaerobic bacterial strains isolated from the feces of the rats which became urobilin-positive after contamination according to (*a*) and (*b*). (*e*) Germfree rats were contaminated with known intestinal bacteria. In this latter group the following bacteria of human origin were studied: *Clostridium welchii* type A, *Streptococcus fecalis*, *Lactobacillus acidophilus*, *Proteus vulgaris*, a strain of *Escherichia coli* (G 14), isolated from the feces of the conventional rats, and some accidental contaminants of the species *Bacillus subtilis*, *Sarcina*, and *Mucor*. The rats in the series (*c*), (*d*), and (*e*) were kept in the apparatuses during the contamination experiments.

TABLE I

Tests for Urobilins in Feces from Germfree and from Conventional Rats

	No. of animals	No. of determinations	Urobilins in feces	
			Negative	Positive
Germfree	25	30	30	0
Conventional	14	25	0	25

The isolation of the anaerobic strains used under (*d*) above was performed on blood agar plates in hydrogen + carbon dioxide (5 per cent) atmosphere using platinum to remove the last traces of oxygen.

2. Chemical Methods.—The Schlesinger reaction (9) was used as a test for *urobilins*. These compounds were also determined as chromogens according to Schwartz *et al.* (10) after reduction with ferrous sulfate. The urobilins were also determined as hydrochlorides in methanol, according to Legge (11). *Bilirubin* was determined with a modification of the method of Malloy and Evelyn (12). The colour of the oxidized feces extract was determined spectrophotometrically with a blank containing an extract of the same sample of feces but without H_2O_2. To prevent oxidation of bilirubin in the blank, ascorbic acid was added. Schmidt's $HgCl_2$-test was also used for detecting urobilins and bilirubin (13).

RESULTS

When the Schlesinger, Schmidt, and Ehrlich aldehyde tests for urobilin were applied all samples of feces from the germfree rats were negative (Table I), whereas these tests in the conventional rats all were strongly positive. The presence of urobilin in the feces from conventional rats was confirmed by the method of Legge. The tests for urobilin in urine were negative both in the germfree and conventional rats.

When the amounts of urobilins were determined with the Ehrlich aldehyde reaction (10) the values given in Table II were found. The production of uro-

bilins was calculated as micromol stercobilinogen per 24 hours per 1000 *gm.* body weight. The 9 conventional rats investigated produced on an average 3.4 micromol urobilins, while the feces from germfree rats in accordance with the qualitative tests showed zero values. In germfree rats the bilirubin content in feces was significantly higher than in conventional rats (Table III).

When germfree rats were given "full flora," *i.e.*, a suspension of feces from conventional rats, urobilins appeared in feces on the 2nd or 3rd day after the contamination. The production of urobilins was already on the 3rd day of about the same magnitude as in conventional rats (Table IV).

TABLE II

Urobilins in Feces from Germfree and from Conventional Rats

Calculated as micromol stercobilinogen per kilogram body weight per 24 hours.

	No. of animals	No. of determinations	Micromol urobilins	
			Mean	Range
Germfree	3	3	0.0	0.0–0.0
Conventional	9	15	3.4	2.1–5.0

TABLE III

Bilirubin in Feces from Germfree and from Conventional Rats

Calculated as micromol bilirubin per kilogram body weight per 24 hours.

	No. of animals	No. of determinations	Bilirubin	
			Mean	Range
Germfree	7	13	4.68	(2.93–7.80)
Conventional	8	10	1.56	(0.92–2.65)

A weak positive urobilin reaction was observed in feces 1 or 2 days after 9 germfree rats were transferred to a laboratory with no conventional animals. 1 to 5 days later a strong positive urobilin reaction was observed and at the same time the Obermayer test for indican in the urine became positive.

When germfree rats were contaminated with cecal contents from conventional rats heated to 80°C. for 5 minutes the urobilin reaction became positive in 3 different experiments. Bacteriological examination of the feces revealed a large number of spore formers. The attempts to isolate the active organisms from this mixture failed, however.

One of the exgermfree animals which became strongly positive in urobilin after the transfer to the laboratory harboured a great number of spore-forming rods. This *Clostridium*-like organism (G 62) was isolated and given to germfree

animals in a series of 12 contamination experiments. On the 2nd or 3rd day after these contaminations the tests for urobilins in feces were positive. The quantity of urobilins produced in 24 hours was, however, low in comparison with the production in conventional animals. When germfree animals were again infected with the same strain of *Clostridium* (G 62) and with a strain of *E. coli* (G 14) the animals produced more urobilins than the rats infected with

TABLE IV

Urobilins in Feces from Exgermfree Rats Contaminated with Feces from Conventional Rats

Calculated as micromol stercobilinogen per kilogram body weight per 24 hours.

Animal No.	Days after contamination								
	1 + 2	3 + 4	5 + 6	7 + 8	9 + 10	11 + 12	13 + 14	25 + 26	27 + 28
1	0.08	1.53	1.78	1.53	1.49	1.78	1.50	0.97	1.23
2	1.62	2.02	3.30						
3	2.55	2.58	1.97						
4	2.11	1.73	2.56						

TABLE V

Urobilins in Feces from Exgermfree Rats Contaminated with Clostridium (G 62) or with Clostridium (G 62) + E. coli (G 14)

Urobilins calculated as micromol stercobilinogen per kilogram body weight per 24 hours.

Animal No.	Contaminated with	Days after contamination					
		3–6	7–10	11–14	15–18	112–115	325
5	*Clostridium* (G 62)	0.12	0.11	0.11	0.34		
6	" "	0.17	0.08	0.11	0.32		
7	" "	0.27	0.15	0.34	0.25	0.72	0.25
8	*Clostridium* (G 62) + *E. coli* (G 14)	0.52	0.86	0.77	1.34		
9	" "	0.30	0.37	0.91	0.89		
10	" "	0.82	0.79	0.99	0.94		

Clostridium (G 62) only, as is seen from Table V. But still on the 15th to 18th day after infection the content of urobilins in feces was only about the third of that produced by conventional rats.

A number of known bacteria strains were also tested and proved to have no effect on the urobilin formation. The tested bacteria were: *Clostridium welchii* A alone or in combination with strains of *E. coli* of human origin, *E. coli* alone, *Enterococcus* alone or in combination with *E. coli* of rat origin (G 14), *Clostridium sporogenes* alone, *Lactobacillus acidophilus* alone, *Proteus vulgaris* alone, strains of *B. subtilis*, *Sarcina*, and *Mucor*.

It has not been possible to definitely classify the urobilin-producing strain (G 62) isolated in these studies. It is an anaerobic non-motile Gram-labile rod with oval, terminal endospores that causes distension of the bacterial body (Fig. 1). The organism grows on 10 per cent blood agar plates in round, convex, smooth colonies without pigment formation. It showed no saccharolytic or proteolytic activity with conventional substrates. The strain of *E. coli* (G 14) has been classified as 04:K3:H?[1]

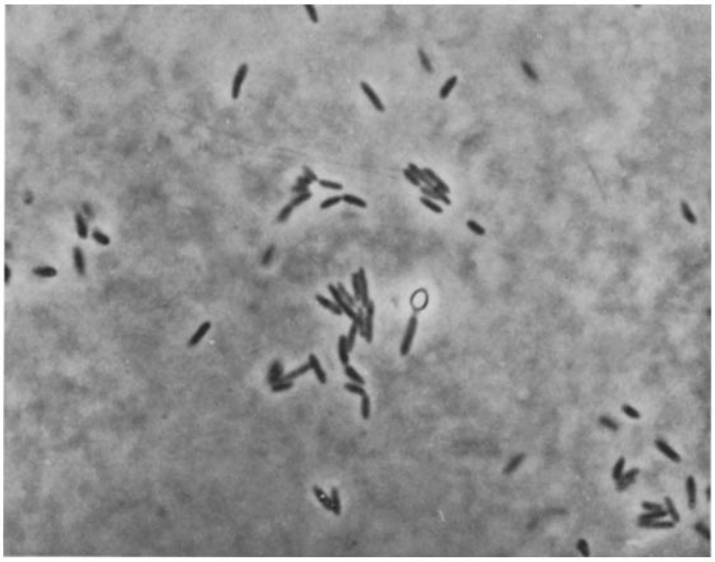

FIG. 1. Living bilirubin converting *Clostridium* (our No. G 62) in broth. Phase contrast. × 1200.

DISCUSSION

As no urobilins are present in feces and urine of germfree rats the present investigation confirms the concept that bilirubin is reduced to urobilins exclusively by the intestinal flora and that no extra intestinal urobilin formation occurs in the rat. This is in accordance with Watson (2, 3). It is also elucidating that the amount of bilirubin excreted in the feces of germfree animals is almost the same as the sum of bilirubin and urobilins excreted in conventional rats (Tables II and III). The conclusions above are further strengthened by the fact that urobilin formation in germfree animals can be evoked by contamination with intestinal flora from conventional animals and even with a single

[1] We are greatly indebted to Drs. Ørskov and Lautrop of the State Serum Institute in Copenhagen for these data.

strain of intestinal bacteria from such animals. This organism seems to be of the genus *Clostridium* and there is evidence that its effect was increased by the presence of a strain of *E. coli*. The nature of this relationship is the topic of current *in vitro* studies. It is interesting to note that Kämmerer and Miller (14) isolated anaerobic Gram-positive spore formers which *in vitro* converted bilirubin to urobilins when *E. coli* was added to the substrate. Passini and Czaczkes (15) stated that bacteria which according to given data must have been of the genus *Clostridium*, produced urobilins from bilirubin. Baumgärtel (16) on the other hand claimed that the effect on the bilirubin produced by fecal mixtures depended on the presence of the dehydrogenases of *E. coli*.

In this paper no distinction according to Watson and Weimer (17) has been made between the levorotatory stercobilin, the inactive urobilin, and the dextrorotatory dehydrourobilin, or their chromogens. The possibility exists that single members of this complex might be formed by single strains of the intestinal flora only. This in turn might be the reason that the *Clostridium* in our experiments never was able to bring the "urobilin" formation up to the values of the conventional animals. Problems of this type can be further elucidated in monocontamination experiments with germfree animals.

SUMMARY

No urobilinogens are present in the feces or urine of germfree rats. After contamination of germfree animals with feces from conventional animals the exgermfree rats produced urobilins to the same extent as conventional animals on the same diet. The negative urobilin test turned positive in germfree animals infected with a single *Clostridium*-like microorganism isolated from the intestinal contents of rats with urobilins in the feces. The output increased in these monoinfected animals after superinfection with a strain of *E. coli* but never reached the values of conventional animals.

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