Efficiency of digestion in germ-free and conventional rabbits

By T. YOSHIDA,* J. R. PLEASANTS, B. S. REDDY AND B. S. WOSTMANN

Lobund Laboratory, Department of Microbiology, University of Notre Dame, Notre Dame, Indiana, USA

(*Received* 17 *April* 1968—*Accepted* 1 *July* 1968)

1. Germ-free (GF) and conventional (CV) rabbits wearing collars to prevent coprophagy were fed an autoclaved diet with added cellulose. Their faecal excretion was analysed to determine nutrient digestibility.

2. Clearly distinguishable hard faeces were excreted by the GF rabbit only if the diet contained at least 15 % cellulose. Unlike CV rabbits, the GF rabbits did not consume their soft faeces even when permitted to do so. Soft faeces made up a larger proportion of the total output of GF than of CV rabbits. Food intake and total dry-matter excretion per kg body-weight were similar in both groups.

3. Although digestibility of dry matter was similar in the two groups, in the GF rabbits there was a higher digestibility of crude fat and true protein and a lower digestibility of crude fibre and nitrogen-free extract. GF rabbits excreted a higher percentage of ingested calcium and phosphorus in the urine than did CV rabbits.

4. The results suggest that intestinal microbes, even without the enhancing effect of coprophagy, aid in the digestion of carbohydrate by rabbits. The greater faecal excretion of crude fat and true protein by CV rabbits could result from poorer digestion and absorption, but could also represent nutrients synthesized by microbes from simpler materials. The re-ingestion of faecal crude fat and true protein might therefore improve the quality of the total nutrient intake. The results suggest ways of assuring an adequate dietary intake by GF rabbits in the absence of contributions from an intestinal microflora.

It has been reported that rabbits excrete both hard and soft faeces with virtually complete consumption of the soft type (Morot, 1882; Madsen, 1939; Taylor, 1939, 1940, 1941; Eden 1940*a*; Southern, 1940, 1942; Olsen & Madsen, 1944; Harder, 1949; Thacker & Brandt, 1955; Kandatsu, Yoshihara & Yoshida, 1959). The soft faeces reportedly are rich in nitrogen, B vitamins, and most of the dietary minerals (Eden, 1940*b*; Olcese, Pearson & Schweigert, 1948; Harder, 1949; Scheunert & Zimmermann, 1952; Kulwich, Struglia & Pearson, 1953; Huang, Ulrich & McCay, 1954; Herndon & Hove, 1955; Thacker & Brandt, 1955; Kandatsu *et al.* 1959). Since faeces contain products of the metabolism of intestinal micro-organisms, e.g. amino acids and B vitamins, rabbits obtain essential nutrients in possibly important quantities by consuming faeces containing bacterial products. Thus, under certain conditions rabbits prevented from practising coprophagy might become deficient in certain nutrients.

Germ-free (GF) rabbits grow normally and reproduce on natural-type diet L-478 (Reddy, Pleasants, Zimmerman & Wostmann, 1965), suggesting that a diet can be adequate for rabbits without any supplement of known or unknown nutrients provided by microbial synthesis. Microbes might, however, contribute in some way

* Present address: Tachikawa College of Tokyo, Tachikawa, Japan.

to the availability or digestibility of nutrients already present in the diet, since the same authors (Reddy *et al.* 1965) found that a diet (L-477) containing mostly inorganic iron produced anaemia in GF rabbits although it was adequate for normal haemoglobin formation in conventional (CV) rabbits. Except for this one mineral, no comparison has been made of the digestibility of nutrients in GF and CV rabbits.

Only a few studies have been reported on the digestibility of the major nutrients in other GF species (Luckey, 1963; Evrard, Hoet, Eyssen, Charlier & Sacquet, 1964). Stern, Huković & Fukarek (1955) reported that in CV rabbits treated orally with antibiotic the rise in blood sugar after a meal of oats was slower than normal. This suggested that the rabbit as a species might have become relatively deficient in carbohydrases after it had evolved an elaborate interrelationship with its intestinal microbiota. A comparison of the digestibility of the major nutrients in GF and CV rabbits could help to explain the role of micro-organisms in the nutritional economy of this species.

The present study was therefore undertaken (1) to determine if the GF rabbit excretes two kinds of faeces, and whether it re-ingests any of its faeces; (2) to determine by analysis of faeces the apparent digestibility of various major nutrients; (3) to determine if GF rabbits produce adequate quantities of the carbohydrases needed for the digestion of starch and disaccharides; (4) to indicate a possible role for coprophagy and microbial activity in the nutrition of the rabbit; and (5) to find possible ways o improving the diets of GF rabbits.

Preliminary observations showed that GF rabbits fed diet L-478 excreted no faecal pellets (hard faeces) but only very moist faeces which were not typical soft faeces. When, in preliminary trials, an additional 15% (on air-dry basis) of non-nutritive fibre was added to diet L-478 clearly distinguishable hard and soft faeces were produced; the soft faeces were still not typical soft faeces, but resembled the very soft formless faeces produced on low-fibre diet L-478. The experimental results obtained with the high-fibre diet (L-478-E 1) are described below.

EXPERIMENTAL

Animals. Because of the scarcity of GF rabbits, three GF and three CV rabbits were used. Each group contained one male and two females of the Dutch strain, 1–3 years of age. One of the CV rabbits was from the Lobund CV colony, one was from a commercial supplier, and one had been GF for 1·5 years and was used for digestibility studies 5 months after it had been removed from its GF isolator to CV laboratory quarters. Since all the CV rabbits in our colony were heavier than the GF rabbits, it was not possible to match weights between groups, but weights were similar within groups. The GF rabbits averaged 1·26 kg and the CV rabbits 1·91 kg. It is possible that some of the differences between the two groups in nutrient utilization could have been due to differences in body-weight, but this seems unlikely in view of the similarities in nutrient digestibility reported by other authors for rabbits differing widely in body-weight.

Housing. The GF rabbits were housed in metal isolators, of the type described by

Reyniers (1959), with floors of 1·3 cm wire-mesh. CV animals were kept in the animal house in cages with floors of 1·3 cm wire-mesh. Each type of cage provided about 0·5 m^2 of floor space. One week before each metabolism study, the GF rabbits were moved into a metabolism cage contained in a plastic isolator similar to that described by Trexler (1959). However, the entire bottom of the isolator was of stainless steel, as described by Pleasants, Zimmerman, Reddy & Wostmann (1963). After completion of their metabolism test, the GF rabbits were moved back into their normal isolators, and CV rabbits were then placed in the same metabolism cages in the same plastic isolator except that it was no longer GF. The isolator was kept in a temperature-controlled room (23°) for all studies.

Table 1. *Composition of diet after sterilization*

(Mean values and standard errors for two batches)

Moisture (% of fresh diet)		24·91 ± 1·43
Crude ash	(% of dry matter)	5·43 ± 0·20
Crude fat		4·92 ± 0·14
Crude protein		26·13 ± 1·53
Crude fibre		16·07 ± 0·18
Nitrogen-free extract		47·46 ± 1·69
Calcium		0·665 ± 0·025
Phosphorus		0·493 ± 0·003
True protein		23·23 ± 1·45
Ratio, true protein: crude protein (expressed as %)		88·9 ± 0·4

Diet. All GF and CV animals were maintained on autoclaved diet L-478-E 1 prepared by General Biochemicals, Inc., Chagrin Falls, Ohio. This diet has the same composition as diet L-478 (Reddy *et al.* 1965), but contains an additional 15%, on an air dry basis, of cellulose powder (non-nutritive fibre, General Biochemicals, Inc.). After addition of 22% water, the diet was compressed into pellets, 0·6 cm in diameter and about 1 cm long. It was kept refrigerated until it was sterilized at 121° for 25 min. Both diet and distilled water were given *ad lib.* The crude composition of the diet is shown in Table 1.

Apparent digestibility. All animals were kept in metabolism cages as described above. To prevent coprophagy, each rabbit was fitted with a rigid plastic collar as described by Kametaka (1967). The collar was 3 mm thick, 25 cm in outside diameter, and 4·5–5 cm in inside diameter; it weighed 190 g. The diet was renewed at 13.00 h each day. The animals, with collars attached, were first fed the diet for a 6-day adjustment period. During the subsequent 6 days, the amounts of food eaten were determined, and the faeces and urine were collected daily. For analysis, the diet and the faeces were dried and ground to pass a 20-mesh screen.

Chemical analysis. The official methods of the Association of Official Agricultural Chemists (1960) were used for determination of moisture, crude ash, crude protein, crude fibre, crude fat and N-free extract (NFE). True protein was determined as N

precipitated by 7·5% trichloroacetic acid (Kandatsu & Yasui, 1955). Calcium was measured by the method of Ingols & Murray (1949) as modified by Kubo & Tsutsumi (1951). Phosphorus was determined by the method of Allen (1940).

Because of the small number of rabbits in each group, the estimate of standard error given in the tables has been calculated from the pooled mean square from both groups together. The differences between the two groups have been analysed for significance by Student's *t*-test. In Table 3, where hard and soft faeces from the same animals have been compared, paired sample *t*-tests were used. Interactions between microbial status and type of faeces were tested for significance by analysis of variance (*F*-test).

Determination of intestinal enzymes. After the experimental period the animals were anaesthetized with ether and bled from the heart until dead. The entire small intestine, large intestine and caecum were removed immediately and their contents collected separately. The homogenates of intestine were prepared as described previously (Reddy & Wostmann, 1966) and the contents were homogenized similarly. After centrifugation the supernatant fluid was analysed for amylase and dextranase activities. All preparative procedures were carried out in a cold room at 4°.

The activities of α-glucosidase, β-fructofuranosidase, trehalose-1-glucohydrolase, β-galactosidase, and β-glucosidase in the wall of the small intestine were determined by the methods described previously (Reddy & Wostmann, 1966). Amylase activities in the small intestine, large intestine, and caecum, and dextranase activity in the small intestine were determined by the methods of Dahlqvist (1962, 1963). The protein contents of the supernatant fractions obtained from the homogenates of the wall of the small intestine were measured by the method of Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

As shown in Table 1, the autoclaved diet contained 25% moisture immediately before feeding. However, it contained only 21·5% moisture after standing for 24 h in the feeding dishes within the isolator. This indicates that a drying environment existed in the isolator. It must therefore be assumed that the faeces and urine also underwent some loss of moisture during the 24 h collection period.

Table 2 summarizes the intake of diet and the excretion of faeces by GF and CV rabbits. When average food consumption was expressed per kg body-weight, the two groups were found to be eating the same amount of diet. This finding agrees with what has been reported for the guinea-pig by Newton & DeWitt (1961) and for the rat by Luckey (1963). The intake of diet by rabbits in which coprophagy was not prevented was not determined, because Thacker & Brandt (1955) had reported that prevention of coprophagy had little influence on food consumption by rabbits.

Each of the three GF rabbits excreted more soft faeces than hard faeces. The two CV rabbits which had been exposed to microbes since birth excreted more hard than soft faeces, but the CV rabbit which had been GF in its early life excreted more soft than hard faeces. Therefore, a significant difference in proportions of hard and soft faeces between the two groups could be demonstrated only when results for this

formerly GF rabbit were excluded from the results in the CV group. The resulting average ratio of hard to soft faeces in the CV group then resembled closely the ratios reported for CV rabbits by Eden (1940*a*), Kulwich *et al.* (1953), Huang *et al.* (1954), and Thacker & Brandt (1955). It appears, therefore, that typical CV rabbits excrete more hard than soft faeces, whereas GF rabbits excrete more soft than hard faeces.

In GF rabbits the excretion of both types of faeces was determined both with and without a collar to prevent coprophagy. Table 2 shows that the results were almost the same. This demonstrates that the GF rabbits did not ingest their soft faeces even when they were physically capable of doing so. The soft faeces of GF rabbits were different in appearance from soft faeces of CV rabbits but were roughly similar in composition (see Table 3 for details).

Table 2. *Intake of diet and excretion of faeces by conventional and germ-free rabbits*

(Mean values and standard errors; g dry matter/kg body-weight per day for three rabbits)

	Conventional: wearing collar		Germ-free: Wearing collar	Germ-free: Not wearing collar	Estimate of SE
Intake of diet	29·0	—	27·0	—	±2·41
	A*	B*			
Excretion of faeces					
Total	10·80	10·75	11·27	11·20	±1·13
Hard	6·70	8·12†	3·48	3·69	±1·10
Soft	4·10	2·63†	7·79	7·51	±1·37

* 'A' group included two conventional rabbits and one conventionalized rabbit which had been removed from a germ-free isolator 5 months before the experiment. When it was found that this rabbit still showed the germ-free pattern of excretion, the values for the two conventional rabbits (group 'B') were averaged and proved significantly different from the values for the germ-free animals.

† Difference from germ-free significant at $P < 0{\cdot}05$.

The results of analysing hard and soft faeces, and caecal contents of CV and GF rabbits for moisture, crude ash, crude fat, crude protein, crude fibre, true protein, calcium and phosphorus are given in Table 3. The results for CV rabbit faeces were similar to those already reported in several papers (Eden, 1940*b*; Huang *et al.* 1954; Thacker & Brandt, 1955; Herndon & Hove, 1955; Kandatsu *et al.* 1959). Faecal composition in the one CV rabbit which had formerly been GF was almost the same as in the two other CV rabbits.

In both GF and CV rabbits the soft faeces had higher contents of moisture, crude protein, and non-protein nitrogen (NPN), a lower crude-fibre content, and a lower ratio of true protein to crude protein than hard faeces. In CV rabbits, however, the soft faeces contained more crude ash, P, and true protein than the hard faeces; the differences were not significant in GF rabbits. In the GF rabbits there was a lower Ca content in soft faeces than in hard faeces. Nevertheless, analysis of variance showed significant interactions between microbial status and type of faeces only for moisture, crude fibre, true protein, NPN, and the ratio of true protein to crude protein.

Table 3. *Composition of hard and soft faeces and caecal contents in conventional and germ-free rabbits* ¶*

(Mean values with estimated standard errors averaged over germ-free and conventional groups)

Constituent	Hard faeces CV	Hard faeces GF	Hard faeces SE	Soft faeces CV	Soft faeces GF	Soft faeces SE	Caecal contents CV	Caecal contents GF†	Caecal contents SE
				% in fresh matter					
Moisture	17·54‡‖	29·88§	±2·95	44·71‖	74·27	±4·05	74·34‖	85·17	±0·87
				% in dry matter					
Crude ash	6·20‡	5·80	±0·41	7·71	6·71	±0·41	8·63	6·36	±0·80
Crude fat	1·40‖	0·93	±0·11	1·29‖	0·74	±0·056	0·96	0·91	±0·14
Crude protein	20·26‡	13·25§	±2·15	39·65‖	21·82	±2·55	42·19	26·48	±5·3
Crude fibre	47·40‡	44·02§	±1·65	26·43	33·55	±2·43	24·43	27·18	±4·95
Nitrogen-free extract	24·75‖	36·00	±1·10	24·91‖	37·18	±0·88	23·80	39·07	±4·15
True protein	18·33‡‖	7·10	±2·05	31·65‖	6·67	±2·19	27·93‖	7·85	±2·50
Non-protein N	0·31‡‖	0·99§	±0·056	1·28‖	2·43	±0·10	2·28	2·98	±0·23
Calcium	1·18‖	0·92§	±0·055	0·96	0·71	±0·093	0·82‖	0·48	±0·03
Phosphorus	1·05‡	0·95	±0·085	1·41‖	0·89	±0·044	1·44‖	0·55	±0·059
True protein × 100 / crude protein	90·5‡‖	53·6§	±1·35	79·8‖	30·6	±2·36	66·2‖	29·6	±3·56

* All rabbits wore collars to prevent coprophagy. Three animals per group unless otherwise noted.

†Two rabbits in this group.

‡Difference from conventional soft faeces significant at $P < 0·05$ using paired sample *t* test.

§ Difference from germ-free soft faeces significant at $P < 0·05$ using paired sample *t* test.

‖ Difference from germ-free significant at $P < 0·05$.

¶ Interaction was tested between status (germ-free and conventional) and faeces (hard and soft) using analysis of variance. Interaction was significant ($P < 0·05$) for moisture, crude fibre, true protein, non-protein N, and (true protein/crude protein) × 100.

Both types of faeces and caecal contents from GF rabbits contained more moisture than those from CV rabbits. The difference in moisture content between caecal contents and soft faeces was not meaningful under the conditions of this experiment because the faeces were exposed to a drying atmosphere in the isolator for periods of up to 24 h before collection. The much higher moisture content found in caecal contents of GF rabbits than in those of CV rabbits agrees with observations (Wynngate, Horton & Forbes, 1958; Wostmann & Bruckner-Kardoss, 1959; Luckey, 1963) in other species reared germ-free.

Although slightly less similarity in composition was found between caecal contents and soft faeces in GF than in CV rabbits, the similarity was still much greater than that between caecal contents and hard faeces. In CV rabbits caecal contents differed significantly from soft faeces only in the ratio of true protein to crude protein. In GF

Table 4. *Apparent digestibility* (%) *of diet in conventional and germ-free rabbits**

(Mean values and estimate of standard errors for three rabbits)

Dietary constituent	Conventional	Germ-free	Estimate of SE
Dry matter	63·0	58·3	±1·2
Crude ash	53·2	52·3	±2·5
Crude fat	89·5	93·4†	±0·3
Crude protein	61·4	71·0	±3·7
Nitrogen-free extract	80·5	66·5†	±1·3
Crude fibre	8·5	3·6†	±1·2
True protein	63·1	88·4†	±3·8
Calcium	38·1	53·1†	±3·0
Phosphorus	9·4	23·5†	±2·5

* All the rabbits wore collars to prevent coprophagy.
† Difference from conventional significant at $P < 0{\cdot}05$.

rabbits caecal contents differed significantly from soft faeces in crude fat and P levels. These findings thus confirm, in a more defined system, the assumption of Huang *et al.* (1954) and of Yoshihara & Kandatsu (1960) that soft faeces represent caecal contents which have passed very rapidly through the colon.

On a dry-matter basis, caecal contents of GF rabbits were lower than those of CV rabbits in true protein, Ca, P, and the ratio of true protein to crude protein. When combined hard and soft faeces of the GF rabbit were compared with those of the CV rabbit on a dry-matter basis, the former were lower in crude fat, crude protein, true protein, Ca, P, and the ratio of true protein to crude protein than the corresponding types of the latter. The lower levels of these various nutrients in faeces of GF rabbits can be partly explained by their higher percentage of NFE. However, NPN was higher in faeces from GF rabbits.

Coefficients of apparent digestibility in CV and GF rabbits, in all of which coprophagy was prevented, are presented in Table 4. The values for CV rabbits were approximately similar to those obtained on a different pelleted diet (Yoshida, 1967), although a higher digestibility of crude fat and NFE and a lower digestibility of crude fibre were found in the present experiment. The latter difference might be due to the larger amount of pure cellulose in the present diet, since Thacker & Brandt (1955)

observed that the digestibility of pure cellulose is markedly lower than that of the crude fibre in roughage.

The coefficient of apparent digestibility of dry matter did not reveal a statistically significant difference between the CV and GF groups. However, its lower coefficient for GF rabbits is in line with the observations that apparent digestibility of dry matter is decreased in rats under GF conditions (Luckey, 1963). In view of the small number of animals involved, the possibility that a real difference exists should not be dismissed simply because $P > 0.05$ (actual value = 0·065). Further investigation is needed.

Table 5. *Intake and excretion of calcium and phosphorus in conventional and germ-free rabbits**

(Mean values and estimate of standard errors for three rabbits)

	Intake and excretion (mg/kg body-weight per day)			Excretion as % of intake		
	Conventional	Germ-free	Estimate of SE	Conventional	Germ-free	Estimate of SE
Ca intake	194	186	±10·2	100	100	
Ca excretion						
Total	186	177	±12·4	96	95	±1·5
In urine	65	90	±8·9	34	48†	±2·8
In faeces						
Total	121	87†	±8·0	62	47†	±3·2
Hard	79	33	±15·0	41	18	±6·7
Soft	42	54	±14·2	21	29	±7·1
P intake	143	134	±10·4	100	100	
P excretion						
Total	136	127	±7·7	95	95	±2·0
In urine	6	25†	±4·5	4	19†	±3·2
In faeces						
Total	130	102	±7·9	91	76†	±2·9
Hard	72	33	±15·0	50	25	±9·8
Soft	58	69	±12·5	41	51	±11·7

* All the rabbits wore collars to prevent coprophagy.
† Difference from conventional significant at $P < 0.05$.

A higher digestibility of crude fat, true protein, Ca and P, and a lower digestibility of NFE and crude fibre were found in GF rabbits as compared with CV rabbits (Table 4). The higher digestibility of crude fat in the GF rabbit parallels observations of Luckey (1963) and Evrard *et al.* (1964) in rats. The higher coefficient of digestibility for Ca in GF rabbits corresponds with similar findings by Edwards & Boyd (1963) in the chicken.

The results showed a slight digestion of crude fibre by the GF rabbits. However, the disappearance of this small amount of crude fibre might result from its becoming trapped in the enlarged caecum of the GF rabbit, which Reddy *et al.* (1965) found to contain 3·6 times as much contents as the caecum of an average CV rabbit when both were fed diet L-478.

Table 5 shows faecal and urinary excretions of Ca and P. For both Ca and P, the

faecal excretion was less and the urinary excretion greater in the GF rabbits than in the CV rabbits. However, the differences were statistically significant only for faecal Ca and urinary P. The differences appear more clearly when the two forms of excretion are expressed as percentages of intake. The renal excretion of both Ca and P was then significantly higher, and the faecal excretion significantly lower in the GF rabbits.

Table 6. *Carbohydrases in the intestinal wall of germ-free and conventional rabbits**

Enzyme	Activity†	
	Conventional	Germ-free
Maltase (α-glucosidase)	16·4 (13·4, 18·4)	35·7 (32·7, 38·7)
Invertase (β-fructofuranosidase)	4·76 (4·13, 5·39)	9·44 (8·28, 10·6)
Trehalase (trehalose-1-glucohydrolase)	2·72 (2·61, 2·81)	6·57 (6·51, 6·63)
Lactase (β-galactosidase)	0·19 (0·14, 0·24)	0·46 (0·36, 0·57)
Cellobiase (β-glucosidase)	0·07 (0·05, 0·09)	0·22 (0·20, 0·24)
Amylase	55·8 (50·5, 61·0)	63·4 (49·1, 77·8)
Dextranase	1·26 (1·00, 1·53)	1·44 (1·13, 1·76)

* Two animals per group. The individual values are given in parentheses beneath the mean values.
† Amylase and dextranase activities are expressed as μmoles maltose liberated/30 min per mg protein. Disaccharidase activities are expressed as μmoles disaccharide hydrolysed/60 min per mg protein.

Table 7. *Amylase activity in the intestinal contents of germ-free and conventional rabbits**

	Activity†	
	Conventional	Germ-free
Small intestine	7·37 (5·89, 8·84)	8·37 (8·08, 8·66)
Caecum	0·60 (0·36, 0·85)	2·55 (2·35, 2·75)
Large intestine	0·33 (0·25, 0·41)	1·69 (1·47, 1·91)

* Two animals per group. The individual values are given in parentheses beneath the mean values.
† m-moles maltose liberated/30 min per g fresh contents.

Gustafsson & Norman (1962) found that the GF rat excreted in its urine five times as much Ca but only one-third as much P as did the CV rat. Thus, the microbial flora affects P excretion differently in the two species.

Intestinal carbohydrase activities are shown in Tables 6 and 7. Table 6 shows that α-glucosidase, β-fructofuranosidase, trehalose-1-glucohydrolase, β-galactosidase and β-glucosidase activities were higher in the wall of the small intestine of GF rabbits compared to CV rabbits, confirming the observations of Reddy & Wostmann (1966) in rats. However, amylase and dextranase activities in the small intestine of GF and

CV rabbits were similar. When the contents were analysed, however (Table 7), the amylase activities in the contents of the large intestine and caecum of GF rabbits were higher than in the CV animal. Enzyme levels in the contents of the small intestine did not differ between GF and CV rabbits.

DISCUSSION

Patterns of excretion of hard and soft faeces. GF rabbits excreted both hard and soft faeces, although hard faeces were not recognized as such until the fibre level in the diet had been raised above that previously fed. Therefore the mechanism bringing about an alternation of the two types of faeces is not dependent on the presence of a living intestinal microflora. However, the mechanism was influenced by the microflora since GF rabbits excreted a higher proportion of their faeces as soft faeces than did the CV rabbits. It is surprising that one CV rabbit removed from the GF unit after 1·5 years of GF life continued to excrete soft and hard faeces in the same ratio as GF rabbits. This would suggest that the pattern had become fixed in some way, or that the rabbit had not acquired, even after 5 months in a laboratory environment, those components of the intestinal flora which could influence its excretion pattern.

Although soft faeces were excreted by the GF rabbits, they were not consumed. Therefore, coprophagy may depend on the presence of certain bacterial products in the faeces. The soft faeces of the GF rabbits had no conspicuous odour, in contrast to those of the CV rabbits. Wagner (1958) found indole in the faeces of CV rats, mice, and chickens, but none in the faeces of the same species when GF. Other bacterial products with a strong flavour or odour, possibly volatile fatty acids and amines, may be similarly absent or low in faeces of GF animals.

The pattern displayed by the GF rabbit, namely excretion of large amounts of soft faeces without re-ingestion, makes more difficult an interpretation of the part played in CV rabbits by the ingestion of soft faeces. Thacker & Brandt (1955) found that in rabbits prevented from practising coprophagy digestibility of dry matter was lower than in rabbits permitted coprophagy. The practice of coprophagy could have a number of useful results: (1) Re-cycling of still undigested food nutrients exposes them a second time to the normal sequence of digestive enzyme activity. (2) Coprophagy enables the rabbit to benefit from nutrients produced or made more available by microbial activity in the lower gut. Wostmann & Knight (1961) found no absorption of bacterially produced thiamine in the caecum and colon of the rat. (3) Coprophagy maintains a high population density of beneficial micro-organisms in the gut, aiding digestion in the entire gastro-intestinal tract (Griffiths & Davies, 1963). (4) Coprophagy becomes a necessity for efficient digestion once the pattern of soft faeces excretion has evolved in this species, since soft faeces have not been exposed to the full absorptive capacity of the colon (Huang *et al.* 1954; Yoshihara & Kandatsu, 1960) and are higher than hard faeces in N and ash content. Thus a rabbit prevented from consuming its soft faeces is more handicapped in the absorption of nutrients than it would be if rabbits had never developed the pattern of soft faeces excretion.

An evaluation of the relative roles of these mechanisms in producing the effects of

coprophagy cannot be made until the GF rabbit can be induced to consume its own soft faeces. Then the contribution of microbes and the contribution of re-cycling as such can be separated and compared. Meanwhile, however, the contribution made by intestinal microbes in the absence of coprophagy has been estimated in this experiment by comparing CV and GF rabbits in both of which coprophagy was prevented. It is recognized that in a rabbit wearing a collar neither the animal's own digestive system nor its intestinal microflora can make a maximum contribution to the digestion of nutrients, for the reasons stated in the foregoing paragraph. Nevertheless, the important role of microbial activity in the lower intestine can be inferred from the significant interactions (footnote to Table 3) found between differences in microbial status and differences between hard and soft faeces. Significant interactions were found for the levels of moisture, crude fibre, true protein, NPN and (true protein/crude protein) × 100.

Digestibility of nutrients. The comparison of CV and GF rabbits in which coprophagy was prevented showed a definite effect of intestinal microbes on the digestibility of the major nutrients. The apparent digestibility of NFE was 80·5% in the CV rabbit and only 66·5% in the GF rabbit. Coprophagy would have raised the CV level even higher, according to Thacker & Brandt (1955). Griffiths & Davies (1963) found that conversion of certain carbohydrates into lactates by the resident gastro-intestinal flora improved NFE digestibility. Coprophagy further enhanced this conversion by maintaining larger populations of the lactate-forming bacteria throughout the digestive tract. Their findings and ours would agree with those of Stern *et al.* (1955), who reported that antibiotics given orally slowed the digestion of starch in CV rabbits. Nevertheless, the role of bacteria in the digestion of starch by the rabbit is only an enhancing one, since Table 6 shows that rabbits do not lack any of the important carbohydrases. In fact, disaccharidase levels in the mucosa of GF rabbits were higher than those in the mucosa of CV rabbits, paralleling the observations of Reddy & Wostmann (1966) in the rat. Amylase concentrations were also higher in the lumen of the caecum and colon in the GF as compared to the CV rabbit (Table 7). However, Baker, Nasr, Morrice & Bruce (1950) found that starch granules broke down more rapidly when surrounded by living bacteria than when merely suspended in amylase. Bacterial breakdown of hemicellulose might also have improved NFE digestibility in the CV rabbit.

The apparent digestibility of protein was adversely affected by the presence of intestinal microflora when coprophagy was prevented. Apparent digestibility of crude protein was higher, though not significantly so, in the GF rabbits (71% *v.* 61%). This difference occurred despite the apparently greater ability of the colon of the CV rabbit to utilize true protein. If we accept the postulate of Huang *et al.* (1954) and of Yoshihara & Kandatsu (1960) that hard faeces have remained much longer in the colon than soft faeces, then the difference in composition between hard and soft faeces is an index of the colon's absorptive capacity. In CV rabbits the true protein content dropped from 32% in soft faeces to 18% in hard faeces; for GF rabbits the content of true protein was 7% in both types of faeces, suggesting that true protein as such is not absorbed by the colon. Its removal by the colon of the CV rabbit would therefore suggest that the

microflora had converted it into absorbable forms of N. Nevertheless, this conversion only partly compensated for the high content of true protein in soft faeces of CV rabbits. Though this high content could indicate poorer digestion of nutrient protein in the CV rabbit, it is more probable that it represents microbial protein presumably synthesized from various non-protein sources. Griffiths & Davies (1963) found that 81% of the protein in soft faeces was contained in microbial cells. Whereas soft faeces of GF rabbits had only one-fifth as high a percentage of true protein as the soft faeces of CV rabbits (Table 3), they had nearly twice as high a level of NPN. Furthermore, in the hard faeces of GF rabbits the concentration of NPN was about three times that found in the hard faeces of CV rabbits (Table 3). These differences suggest a multiple role for intestinal micro-organisms in protein digestion. They may convert NPN into protein N in the caecum and may then convert some of this protein N, during its passage through the caecum, into NPN which is more absorbable than the original NPN, or of higher nutritive value or both.

Caecal contents of GF rabbits had a lower total N content but a higher NPN content than those of CV rabbits, although differences were not significant. In rats, however, the total N content of caecal contents is higher in GF than in CV animals (Levenson & Tennant, 1963; Evrard *et al.* 1964). Combe, Penot, Charlier & Sacquet (1965) and Lepkovsky, Furuta, Ozone, Koike & Wagner (1966) also found higher levels of NPN in the caecal contents of GF rats as compared to CV rats; the former authors found higher levels of amino acids in the caecal content of GF rats; they also found some urea in the contents of GF rats, though there was none in those of the CV rat. Lindstedt, Lindstedt & Gustafsson (1965) found much higher levels of NPN in the form of mucin derivatives in the caecum of the GF as compared to the CV rat. The form in which NPN occurs in the caecum and faeces of the GF rabbit remains to be investigated. Most, but not all, of it appears to be absorbed as the caecal contents pass through the colon.

The comparison of GF and CV faeces suggests considerable synthesis of NPN into microbial protein by the intestinal microflora. The resulting protein could have higher nutritive value for the rabbit than the nitrogen sources from which it was synthesized. Although many of the proteins of rabbit feeds have a low lysine content (Block & Weiss, 1956), micro-organisms have a relatively high lysine content (Anderson, Rhodes, Nelson, Shekleton, Barreto & Arnold, 1958; Bigwood, 1963). Such enhancement of the biological value of dietary N by microbial synthesis would be analogous to that occurring in ruminants (McNaught, Smith, Henry & Kon, 1950), and would at least partly explain why prevention of coprophagy reduces protein utilization in the rabbit (Thacker & Brandt, 1955).

The provision of foods rich in lysine (soya-bean meal and lactalbumin) to the GF rabbits of this experiment would tend to compensate for loss of lysine during autoclaving of the diet (Rice & Beuk, 1953) and for the absence of microbially produced lysine. High levels of B vitamins were also included in the diet to compensate for losses during sterilization and for the absence of the B vitamins made available to the CV rabbit by coprophagy (Olcese *et al.* 1948; Scheunert & Zimmerman, 1952; Kulwich *et al.* 1953).

The presence of bacteria adversely affected the apparent digestibility of crude fat in rabbits in which coprophagy was prevented. This agrees with the findings of Evrard *et al.* (1964) in rats. These authors have discussed the possibility that micro-organisms diminish the absorption of alimentary fat by modifying the intestinal mucosa (Sprinz, 1962) or by altering bile salts within the intestinal lumen (Dawson & Isselbacher, 1960). On the other hand, Sperry (1929) found 40% of the faecal crude fat to be contained in the bacterial cells. It is possible that some of the faecal crude fat was synthesized by the intestinal flora from simpler nutrients. Such synthesis would cause a decrease in the apparent digestibility of fat by CV animals even though the alimentary fat had actually been well digested. In CV rabbits permitted coprophagy, such synthesis could provide a gain in the quantity or quality of fat digested.

The lower apparent digestibility of crude fat and protein in CV rabbits could also be caused, at least partly, by a more rapid sloughing of the intestinal mucosa in the CV animal. This has not been studied in the rabbit, but the rate of mucosal sloughing in the CV mouse is twice that observed in the GF mouse (Abrams, Bauer & Sprinz, 1963).

Although there was no difference in the apparent digestibility of crude ash between the two groups, GF rabbits showed increased digestibility of Ca in comparison with CV rabbits. The increased intestinal absorption of Ca required increased renal excretion by the GF rabbit. That this increased absorption of Ca by the GF animal is not necessarily advantageous is shown by the finding of Gustafsson & Norman (1962) that in GF rats given a semi-purified diet the renal excretion of Ca was very high; the GF rats developed urinary calculi, whereas CV rats did not. Miyakawa (1963) has also reported more Ca deposits in the kidneys of GF Swiss mice than in those of CV mice. Reyniers & Sacksteder (1958) found soft tissue calcification in GF C3H mice but not in CV controls when a semi-purified diet was fed to both.

Practical application of our results to the formulation of diets for GF rabbits. The results of our experiment suggest some practical conclusions about diets for GF rabbits. The carbohydrate content of the diet described here might well be decreased, since NFE was less well digested by the GF rabbit. The place of carbohydrate in the diet could be taken by fat, since GF rabbits digested fat well at the 5% level in diet L-478-E 1, and Thacker (1956) has already shown that diets containing 10–25% fat produce greater body-weight gains in CV rabbits than diets containing only 5% fat. The fact that the GF animal does not obtain any microbial protein suggests that the diet should continue to contain animal as well as vegetable protein or at least a vegetable protein high in lysine. The increased Ca absorption in GF animals in general suggests a reduction in the Ca content of diets prepared for germ-free rabbits.

Our findings indicate that a diet of grain and soya-bean meal supplemented with animal protein and extra vitamins and minerals is adequate for GF rabbits, but the digestibility of its carbohydrate is enhanced by the presence of an intestinal microflora even when the rabbits are not permitted coprophagy. The results also suggest an active role of microbes in altering the forms of N available to the host. On certain types of diet such contributions, further enhanced by coprophagy, could be of critical importance in the rabbit's nutritional economy.

The authors are grateful to Mr H. Flis and to Mrs F. Ceuterick for their care of the experimental animals. This study was supported specifically by grant number HDO 0855 of the National Institutes of Health, USA, and generally by the Office of Naval Research NONR 1623 (04), and by the University of Notre Dame, USA.

REFERENCES

Abrams, G. D., Bauer, H. & Sprinz, H. (1963). *Lab. Invest.* **12**, 355.
Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Anderson, R. F., Rhodes, R. A., Nelson, G. E. N., Shekleton, M. C., Barreto, A. Jr & Arnold, M. (1958). *J. Bact.* **76**, 131.
Association of Official Agricultural Chemists (1960). *Official Methods of Analysis*, 9th ed. Washington, D.C.: Association of Official Agricultural Chemists
Baker, F., Nasr, H., Morrice, F. & Bruce, J. (1950). *J. Path. Bact.* **62**, 617.
Bigwood, E. J. (1963). *C. r. Rech. Inst. Encour. scient. Ind. Agric.* no. 30, p. 142.
Block, R. J. & Weiss, K. W. (1956). *Amino Acid Handbook: Methods and Results of Protein Analysis.* Springfield, Illinois: Charles C. Thomas.
Combe, É., Penot, É., Charlier, H. & Sacquet, E. (1965). *Annls Biol. anim. Biochim. Biophys.* **5**, 189.
Dahlqvist, A. (1962). *Scand. J. clin. Lab. Invest.* **4**, 145.
Dahlqvist, A. (1963). *Biochim J.* **86**, 72.
Dawson, A. M. & Isselbacher, K. J. (1960). *J. clin. Invest.* **39**, 730.
Eden, A. (1940*a*). *Nature, Lond.* **145**, 36.
Eden, A. (1940*b*). *Nature, Lond.* **145**, 628.
Edwards, H. M. Jr & Boyd, F. M. (1963). *Poult. Sci.* **42**, 1030.
Evrard, E., Hoet, P. O., Eyssen, H., Charlier, H. & Sacquet, E. (1964). *Br. J. exp. Path.* **45**, 409.
Griffiths, M. & Davies, D. (1963). *J. Nutr.* **80**, 171.
Gustafsson, B. E. & Norman, A. (1962). *J. exp. Med.* **116**, 273.
Harder, W. (1949). *Verh. dt. Zool. Mainz.* **2**, 95.
Herndon, J. F. & Hove, E. L. (1955). *J. Nutr.* **57**, 261.
Huang, T. C., Ulrich, H. E. & McCay, C. M. (1954). *J. Nutr.* **54**, 621.
Ingols, R. S. & Murray, P. E. (1949). *Analyt. Chem.* **21**, 525.
Kametaka, M. (1967). *Agric. biol. Chem.* **31**, 616.
Kandatsu, M. & Yasui, T. (1955). *J. agric. chem. Soc. Japan* **25**, 27.
Kandatsu, M., Yoshihara, I. & Yoshida, T. (1959). *Jap. J. zootech. Sci.* **29**, 365.
Kubo, S. & Tsutsumi, C. (1951). *Shokuryo Kenkyujo Hokoku* **5**, 171.
Kulwich, R., Struglia, L. & Pearson, P. B. (1953). *J. Nutr.* **49**, 639.
Lepkovsky, S., Furuta, F., Ozone, K., Koike, T. & Wagner, M. (1966). *Br. J. Nutr.* **20**, 257.
Levenson, S. M. & Tennant, B. (1963). *Fedn Proc. Fedn Am. Socs exp. Biol.* **22**, 109.
Lindstedt, G., Lindstedt, S. & Gustafsson, B. E. (1965). *J. exp. Med.* **121**, 201.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Luckey, T. D. (1963). *Germfree Life and Gnotobiology.* New York and London: Academic Press, Inc.
McNaught, M. L., Smith, J. A. B., Henry, K. M. & Kon, S. K. (1950). *Biochem. J.* **46**, 32.
Madsen, H. (1939). *Nature, Lond.* **143**, 981.
Miyakawa, M. (1963). *Mukindobutsu* (*Germfree Animals*). Tokyo: Ishiyakushuppan.
Morot, C. (1882). *Mém. Soc. cent. Med. vet.* **12**, Ser. 1.
Newton, W. L. & DeWitt, W. B. (1961). *J. Nutr.* **75**, 145.
Olcese, O., Pearson, P. B. & Schweigert, B. S. (1948). *J. Nutr.* **35**, 577.
Olsen, H. M. & Madsen, H. (1944) *Vidensk, Meddr. dansk. natur. Foren.* **107**, 37.
Pleasants, J. R., Zimmerman, D. R., Reddy, B. S. & Wostmann, B. S. (1963). In *Proceedings of the Gnotobiote Workshop and Symposium*, p. 36. Columbus, Ohio: Ohio State University.
Reddy, B. S., Pleasants, J. R., Zimmerman, D. R. & Wostmann, B. S. (1965). *J. Nutr.* **87**, 189.
Reddy, B. S. & Wostmann, B. S. (1966). *Archs Biochem. Biophys.* **113**, 609.
Reyniers, J. A. (1959). *Ann. N.Y. Acad. Sci.* **78**, 47.
Reyniers, J. A. & Sacksteder, M. R. (1958). *Proc. Anim. Care Panel* **8**, 41.
Rice, E. E. & Beuk, J. F. (1953). In *Advances in Food Research.* Vol. 4, p. 233. [E. M. Mrak and G. F. Stewart, editors.] New York, N.Y.: Academic Press Inc.
Scheunert, A. & Zimmermann, K. (1952). *Arch. f. Tierernahr.* **3**, 217.
Southern, H. N. (1940). *Nature, Lond.* **145**, 262.
Southern, H. H. (1942). *Nature, Lond.* **149**, 553.
Sperry, W. M. (1929). *J. biol. Chem.* **81**, 299.

Sprinz, H. (1962). *Fedn Proc. Fedn Am. Socs exp. Biol.* **21**, 57.
Stern, P., Huković, S. & Fukarek, V. (1955). *Gastroenterologia* **83**, 349.
Taylor, E. L. (1939). *Nature, Lond.* **143**, 982.
Taylor, E. L. (1940). *Vet. Rec.* **52**, 259.
Taylor, E. L. (1941). *Proc. zool. Soc., Lond.* A **110**, 519.
Thacker, E. J. (1956). *J. Nutr.* **58**, 243.
Thacker, E. J. & Brandt, C. S. (1955). *J. Nutr.* **55**, 375.
Trexler, P. C. (1959). *Ann. N.Y. Acad. Sci.* **78**, 29.
Wagner, M. (1958). *Bact. Proc.* **11**, 88.
Wostmann, B. S. & Bruckner-Kardoss, E. (1959). *Am. J. Physiol.* **197**, 1345.
Wostmann, B. S. & Knight, P. L. (1961). *J. Nutr.* **74**, 103.
Wynngate, A. E., Horton, R. E. & Forbes, M. (1958). *Germfree Animal Studies*, p. 93. (University of Pennsylvania Project, Walter Reed Army Institute of Research, Walter Reed Army Medical Center.)
Yoshida, T. (1967). *Jap. J. zootech. Sci.* **38**, 370.
Yoshihara, T. & Kandatsu, M. (1960). *Bull. agric. chem. Soc. Japan* **24**, 543.

Printed in Great Britain