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Effect of High Fat Diets on Intestinal Microflora and Serum Cholesterol in Rats

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ABSTRACT

GRABER, C. D. (Baylor University College of Medicine, Houston, Tex.), R. M. O'NEAL, AND E. R. RABIN. Effect of high fat diets on intestinal microflora and serum cholesterol in rats. J. Bacteriol. **89**:47-51. 1965.—Differential bacterial counts of feces and total plasma cholesterol determinations were performed on 60 Wistar rats fed several high lipid diets for a period of approximately 6 months. Fecal flora remained relatively stable irrespective of diet, but cholesterol levels rose in animals fed butter and sodium cholate. The six most commonly cultured organisms in all diets were enterococci, *Proteus*, lactobacilli, *Escherichia coli*, *Staphylococcus aureus*, and other micrococci. No enteric pathogens which did not ferment lactose or fermented it slowly were grown. Fungi and yeasts were rare. Aerobes generally outnumbered anaerobes in proportions which were sometimes as high as 300:1. *Clostridium perfringens* isolations were consistently high in animals given the butter diets, particularly when sodium cholate was added. Evidence is reviewed which indicates that this organism may play a role in bile salt and cholesterol metabolism. This experiment would seem to demonstrate that differences in plasma-cholesterol levels among the various dietary groups of rats were the result of dietary factors rather than alteration in intestinal flora.

It has been surmised for some time that intestinal microflora markedly influence synthesis, interconversions, and excretion of bile acids and their precursor, cholesterol, but only in the past few years has this been inferentially confirmed by germ-free animal experimentation (Wostmann and Wiech, 1961). In vitro and in vivo work aimed at delimiting the bacteria involved has been scant. The impression remains, however, that enterococci and, to some extent, clostridia participate in hydrolysis of conjugated bile acids (Norman and Grubb, 1955). These bacteria also may reduce cholesterol to nonabsorbable coprostanol, and cause conjugation of lithocholic acid, restricting its reabsorbability (Bergstrom, 1962).

Norman and Grubb (1955), by use of media containing C^{14}-labeled conjugated bile acid and chromatographic separation methods, were able to show hydrolysis of taurocholic and glycocholic acid by several species of clostridia and enterococci, but by no other anaerobes or aerobes. Similarly, Gustaffson et al. (1957) found that monocontamination of axenic rats with *Clostridium perfringens* resulted in some splitting of the cholic acid molecule, whereas other aerobes and anaerobes failed.

The purpose of our study was to determine the effect of the constituents of high lipid diets, such as are commonly fed in the production of experimental atherosclerosis, on the types and number of intestinal microflora of rats, and how, in turn, this might affect blood-cholesterol levels in these animals. Of particular interest was how the addition of bile salts to the high lipid diets would affect microflora. Because few organisms are known to be capable of growing in high bile salt concentrations, it seemed that this capability might indicate those prospering as the organisms principally involved in cholesterol and bile salt excretion under these conditions.

MATERIALS AND METHODS

Sixty male Wistar albino rats, with an average weight of 80 g, were initially equilibrated on laboratory pellets (Purina) for 2 weeks, and randomly assigned in lots of 20 to three basic diets (diets 2, 3, 4 in Table 1), all of which were a modification of the "infarct-producing" diet of Hartroft and Thomas (1957). The animals, individually caged, were fed these diets *ad libitum* for 54 days, after which the butter- and corn oil-fed animals were given 5% cholesterol as supplement (diets 5 and 6). The basal rats were maintained unchanged on diet 2 for the length of the experiment, 149 days. Rats were continued on the cholesterol-supplemented diet for 36 days. These rats then additionally received 2% sodium cholate (diets 7 and 8), and were fed this diet for 59 days. At the end of this time, all animals (basal, butter, and corn oil) were returned for 15 days to the pellet

TABLE 1. *Constituents of eight basic diets* fed rats*

Constituent	Basal no. 2	Corn oil no. 3	Butter no. 4
	%	%	%
Casein	20.0	20.0	20.0
Sucrose	65.8	27.8	27.8
Corn oil	2.0	40.0	—
Butter	—	—	40.0
Vitamins, salt mix filler	12.2	12.2	12.2

* Diet 1 = pellet; diet 5 = diet 3 plus 5% cholesterol less 5% sucrose; diet 6 = diet 4 plus 5% cholesterol less 5% sucrose; diet 7 = diet 5 plus 2% sodium cholate less 2% sucrose; diet 8 = diet 6 plus 2% sodium cholate less 2% sucrose.

diet (switch-back animals). None of the animals received thiouracil as a basic component of the Hartroft-Thomas diet.

Daily, a complete aerobic and anaerobic cultural profile was performed on the feces of one animal from each group, and an animal from each group was bled for total cholesterol estimation. The method employed was that of Searcy and Berquist (1960) which requires only 40 μliters of plasma, so that blood loss was small. During the course of the experiment, at least eight samplings of feces and 13 cholesterol determinations were performed on each animal.

Cultural methods. Two freshly excreted fecal pellets were caught in 5 ml of Gall's holding medium, immediately triturated, and decimal dilutions carried out to 10^{-7} in Gall's dilution fluid (Gall and Helvey, 1963). A 4-mm biconvex loopful of inoculum (0.02 ml) was spread thoroughly over the surfaces of 21 plates of media from the various dilutions. The media utilized included three blood plates (7% sheep blood) for total aerobic count, three Phenylethyl Alcohol (BBL) plates (7% sheep blood) for aerobes other than *Proteus*, two LBS (BBL) plates for lactobacilli, two Staphylococcus Medium No. 110 (Difco) plates for staphylococci, two KF Streptococcus agar (Difco) plates for enterococci, and three MacConkey (Difco) plates for gram-negative organisms. Two blood plates were used for anaerobic total counts plus two plates of kanamycin-vancomycin-blood (KVB) agar or laked blood agar (Finegold, Miller, and Posnick, 1964) for *Bacteroides*. A plate of Omata's (1959) medium was used for fusiform bacteria, and a Nagler plate fortified with neomycin (Lowbury and Lilly, 1955) for *C. perfringens* isolation. Initially, SS media (Difco) was used in an attempt to isolate gram-negative bacteria which did not ferment lactose or fermented it slowly, but, when it became apparent that such organisms were rare, this medium was discontinued and MacConkey Agar was used instead to cultivate all Enterobacteriaceae. Litman (BBL) agar for recovery of fungi and yeast was used for only a short period, because of the paucity of these organisms.

All aerobic plates were incubated at 37 C for 24 hr followed by incubation for 12 hr at room temperature. The LBS plates were incubated in a candle-jar atmosphere of 10% CO_2. Anaerobes were grown in either a Torbal or Brewer jar in which H_2 was burned to exhaust atmospheric O_2. All anaerobic plates were incubated at 37 C for 72 hr.

Identification procedure. The selective media used provided means for ready recognition of most of the organisms, thereby reducing the biochemical tests required for more definitive identification. The Staphylococcus Medium No. 110 grew both *Staphylococcus aureus* and other micrococcal colonies which were subsequently differentiated on coagulase-mannitol agar (Difco). The LBS medium grew only lactobacilli; no species differentiation was made of this genus. Enterococci produced red colonies on the KF media and were easily enumerated. MacConkey agar was found to be superior to EMB (Difco) for inhibiting *Proteus*, and, therefore, counts of coliform organisms were made on this medium. The Phenylethyl Alcohol plates served as a double check for counts of micrococci and enterococci. Hemolytic colonies of gram-positive bacilli growing on blood-agar under anaerobiosis, with or without spores, which failed to grow on aerobic-plate transfer were labeled unidentified clostridia. Other obligatory anaerobic colonies were considered *Bacteroides* if they were nonsporulating gram-negative bacilli that grew on KVB or laked blood medium, but failed to grow on aerobic transfer. In some instances, highly pleomorphic filamentous, branching, and coccoid forms were observed which retained gram-positivity. These were also considered to be *Bacteroides*. Omata's medium was not highly successful in growing the fusobacteria. Far too often, gram-negative organisms, principally coliform, overgrew the entire surface of this medium. Nagler plates fortified with 100 μg of neomycin grew large colonies with opalescent zones which were judged to be *C. perfringens* if no spores were found on the smear. Final identification of some organisms required IMViC reactions, or other biochemical tests, or both. For this work, the PathoTec (Warner Chilcott, Morris Plains, N.J.) impregnated filter papers were used for rapid bacterial identification.

RESULTS

Table 2 lists the types and number of bacteria recovered from rats placed on the eight different diets, and the average of total plasma-cholesterol values obtained. Average cholesterol values rose in the corn oil and butter groups concomitant with addition of dietary cholesterol and bile salts. The highest average value was 452 mg/100 ml in the butter-bile salt group.

Fecal flora was relatively stable, irrespective of diet. No general or sweeping changes occurred in the intestinal microflora of the animals, nor did

TABLE 2. *Intestinal microflora, total cholesterol, and weight of 60 rats fed eight diets for various periods indicated*

Organism	Pellet		Basal	Corn oil	Butter	Corn oil + cholesterol	Butter + cholesterol	Corn oil-cholesterol-bile salts	Butter-cholesterol-bile salts
	Only	Switch-back							
Enterococci	1.2×10^8 *	8.5×10^7	9.3×10^7	8.4×10^7	4.9×10^7	1.3×10^8	7.4×10^7	2×10^7	2.9×10^7
Proteus sp.	5.9×10^7	4.3×10^7	9.1×10^7	2.3×10^7	4×10^7	5.4×10^7	7.7×10^7	8.8×10^7	8.3×10^7
Micrococci	2×10^7	2.4×10^7	9×10^6	6×10^5	2×10^6	6×10^6	7×10^6	2.1×10^7	2.9×10^7
Lactobacilli	1.4×10^7	2×10^7	7×10^6	1.1×10^7	1.7×10^7	8×10^6	3×10^6	2.2×10^5	9×10^5
Escherichia coli	3×10^6	1.4×10^7	8×10^6	3×10^7	3.8×10^7	5×10^7	3.7×10^7	5.9×10^7	5.8×10^7
Staphylococcus aureus	3×10^6	2×10^7	1.7×10^7	3×10^6	7×10^6	1.6×10^7	1.3×10^7	2×10^7	1.3×10^7
Pseudomonas aeruginosa	6×10^5	5×10^5	3×10^5	3.7×10^4	8.3×10^4	2×10^5	10^5	7×10^5	1.5×10^6
Klebsiella-Aerobacter	—	10^5	2×10^6	7×10^5	6×10^5	3×10^6	6×10^4	1.2×10^7	1.5×10^7
Corynebacterium sp.	—	7×10^4	2×10^5	2×10^4	—	—	—	—	—
Geotrichum sp.	—	4×10^5	9×10^4	6×10^4	4×10^5	2×10^5	6×10^5	5×10^5	3.5×10^5
Bacteroides sp.	3×10^6	3×10^6	5.4×10^7	2.5×10^5	5×10^5	8×10^5	2×10^6	9×10^5	5.5×10^5
Clostridium perfringens	—	2×10^5	10^4	10^5	7×10^4	2×10^6	4×10^5	4×10^5	2×10^6
Clostridia (unidentified)	10^5	—	2×10^4	1.5×10^5	—	—	2×10^4	10^5	3×10^5
Streptococcus (anaerobic)	—	—	5×10^4	8×10^4	3×10^4	—		4×10^4	3×10^8
Days on diet	14	15	149	54	54	36	36	59	59
Cholesterol (mg/100 ml)	121	286	150	143	159	163	182	294	452
Weight (g)	370	386	382	330	331	420	450	398	374

* Log viable cells per gram of wet stool. Figure is average value for all rats on a particular diet.

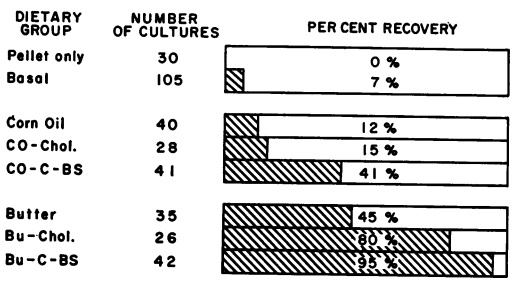

FIG. 1. *Effect of various dietary components on percentage of cultural recovery of Clostridium perfringens. High recovery rate of C. perfringens is evident in diets containing butter and butter with cholesterol or bile salts.*

any organism, with one possible exception, emerge which was peculiar to a particular diet. Counts were lower generally for all organisms than anticipated, with aerobes outnumbering anaerobes.

Enterococci and *Proteus* proved to be the most numerous, exceeding 50 million in each diet. *S. aureus* and other micrococci were next most common. In all dietary groups, counts of these organisms remained in the millions as did counts for the lactobacilli and *Escherichia coli*. There appeared to be no gradual reduction in counts of *E. coli* with age as reported by Smith and Crabb (1961). No pathogens which did not ferment lactose, such as *Salmonella* and *Shigella*, were recovered. No organisms which fermented lactose slowly (*Paracolobactrum* sp.) were grown. Fungi and true yeasts were virtually nonrecoverable. *Geotrichum* was found in numbers above 10,000 in all diets, except in the pellet diet used for initial equilibration. The ratio of principal aerobes to anaerobes varied for the diets from 300:1 in the pellet to as small as 2:1 for the basal diet. This is illustrated in the first and third columns of Table 2; enterococci and *Proteus* outnumber *Bacteroides* and unidentified clostridia by a ratio of 300 to 1 (pellet diet), but the same organisms only outnumber *Bacteroides* by a ratio of 2 to 1 (basal diet).

Of interest is the recovery of *C. perfringens* in the groups fed high lipid diets with cholesterol and bile salts. Table 2 shows only a nominal increase in numbers of this organism for rats fed corn oil and butter, but does not reflect the consistently high rate of recovery for these diets (Fig. 1). *C. perfringens* was never recovered from the rats continuously fed pellets. This organism also was isolated only twice from the feces of switch-back animals, and then only immediately after these animals were changed from the butter and sodium cholate diet; subsequently no *C. perfringens* was grown from these animals cultured daily for 30 days. In the basal-diet group, recoveries of *C. perfringens* were very rare. Corn oil- and butter-fed rats, however, consistently yielded this organism, although the number was not great. In only 2 of 42 cultures was *C. perfringens* missing in rats fed butter, cholesterol, and sodium cholate. Isolations of the organism from such diets occurred so consistently that it was quite easy to identify the dietary group by looking at the fecal cultures on Nagler plates. The butter used was not a source of *C. perfringens*, as it was repeatedly negative for this organism.

DISCUSSION

Considerable quantitative inaccuracies are to be expected in any attempt to study the bacteria in material as bacteriologically complex as feces. However, the counts made daily on our rats were quite consistent and followed a definite pattern, so that there is justification for feeling that the comparative occurrence of fecal bacteria, for a period as short as 6 months, in rats on different diets was being reasonably charted.

The wide difference in ratio of aerobes to anaerobes found for the rats fed the pellet diet (300:1) as opposed to those fed basal diet (2:1) was not exactly unexpected, but was different from the results of Zubrzycki and Spaulding (1962), who reported a preponderance of *Bacteroides* in humans. Porter and Rettger (1940) found only small numbers of gram-negative, nonsporulating anaerobes and clostridia in the cecum of rats fed various diets. Even with a combination of better selective media and use of an enrichment menstruum (Gall's medium), calculated to keep the fecal pellet at the proper Eh prior to culturing, our recovery of anaerobic organisms in the pellet group was not high. Our results agree with those of Porter and Rettger (1940) who found the "normal" bacterial fecal flora of the white rat to be comparatively simple, comprised mainly of enterococci, lactobacilli, and two gram-negative organisms, *E. coli* and *Proteus*. *S. aureus* and other micrococci, however, were frequently recovered by us, and species of *Bacteroides* and *Geotrichum* were regularly encountered; these organisms should possibly be considered part of the commensal flora.

These commensals remained fairly stable with diet changes and time. *E. coli* did not diminish in numbers with aging of the animal as Smith and Crabb (1961) reported. Enterococci, believed by some investigators (Norman and Grubb, 1955) to be mainly responsible for deconjugating bile acids, were recovered from our rats in almost the same number, irrespective of diet. Thus, it is difficult to ascribe a role for this organism in plasma-cholesterol fluctuation as it occurred here.

In contrast to the reports of other investigators

is our high number of aerobic micrococci and *S. aureus* isolations from all dietary groups. Smith and Crabb (1961) were unable to recover any *S. aureus* from the feces of calves, lambs, piglets, young rabbits, dogs, cats, or mice, and they concluded that this organism was simply indigenous to man. Our staphylococcal organism fermented mannitol, was coagulase-positive, and otherwise was found to be a typical *S. aureus* strain. The cages used for our rats permitted coprophagy, and, thus, it is possible that *S. aureus* ingestion by the animals could have occurred and been fostered by the high fat diets. On the other hand, feces from pellet-fed rats also provided high counts of *S. aureus*.

Most interesting to us, because of its possible role in bile acid and cholesterol metabolism, was the recovery of *C. perfringens*. This organism was never present in the feces of any of the rats fed pellets; it was found only twice in switchback animals, probably as a transient organism still subsisting from the recent high-fat dietary regimen. In the feces of basal diet rats, recoveries also were low. Rats fed high-fat diets with cholesterol and sodium cholate, however, yielded *C. perfringens* consistently. Recovery of this organism in some groups was almost 100%. Perhaps this was related to the bile salt content of the diet. Bile salts are commonly employed in bacteriological media to inhibit certain gram-positive and gram-negative bacteria, and *C. perfringens* is known to grow well in concentrations exceeding 40 mg/ml of taurocholic acid (Norman and Grubb, 1955). Bile salts were not entirely responsible for the preponderance of *C. perfringens* (Fig. 1). Recoveries of this organism from butterfed rats exceeded isolations from those on corn oil and sodium cholate. Butyric acid was found by Bergeim et al. (1941) to be toxic for a great number of aerobic and anaerobic organisms, but did not inhibit growth of lactobacilli, *E. coli*, *Proteus*, and *C. perfringens*. A combination of bile salts and the butyric acid present in the butter was probably conjointly responsible for the consistent number of *C. perfringens* recoveries. (Bile salts, also known to selectively enhance enterococci recoveries, caused the lowest counts in our dietary groups for these organisms.)

It is possible that the elevated plasma-cholesterol levels, obtained in several of the groups fed high fat diets, might have been even more elevated had *C. perfringens* not been present in the gut, converting exogenous cholesterol to unabsorbable coprostanol. Study of this mechanism is currently being planned in vitro and in axenic marmosets monocontaminated with *C. perfringens*.

In these experiments, one must conclude, however, that the differences in blood-cholesterol levels among the various dietary groups of animals were probably the result of dietary factors. These same dietary factors presumably altered intestinal anaerobic flora while affecting aerobic flora only slightly. No effect of changes in bacterial flora on blood-cholesterol levels can be ascertained from our results.

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