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Acetate Utilization and Butyryl Coenzyme A (CoA):Acetate-CoA Transferase in Butyrate-Producing Bacteria from the Human Large Intestine

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Seven strains of *Roseburia* sp., *Faecalibacterium prausnitzii*, and *Coprococcus* sp. from the human gut that produce high levels of butyric acid in vitro were studied with respect to key butyrate pathway enzymes and fermentation patterns. Strains of *Roseburia* sp. and *F. prausnitzii* possessed butyryl coenzyme A (CoA):acetate-CoA transferase and acetate kinase activities, but butyrate kinase activity was not detectable either in growing or in stationary-phase cultures. Although unable to use acetate as a sole source of energy, these strains showed net utilization of acetate during growth on glucose. In contrast, *Coprococcus* sp. strain L2-50 is a net producer of acetate and possessed detectable butyrate kinase, acetate kinase, and butyryl-CoA:acetate-CoA transferase activities. These results demonstrate that different functionally distinct groups of butyrate-producing bacteria are present in the human large intestine.

There is currently much interest in optimizing the health-protective activities of the colonic microflora and in preventing gastrointestinal disorders through control of the diet (4, 15, 16, 17). The anaerobic microbial communities of the gut are exceedingly complex, however, being characterized by high cell densities and strain diversity and a significant degree of interspecies cross-feeding and microbial interaction (9). Recent molecular studies have demonstrated that up to 75% of the 16S ribosomal DNA (rDNA) sequence diversity is apparently unrelated to known bacterial species (13, 25). The challenge now must be to identify and culture new bacterial species that contribute to key aspects of anaerobic metabolism in the gut.

The main products of microbial fermentation in the large intestine can vary significantly in their relative concentrations and production rates depending on diet and site of production (27, 28), with typical ratios in feces being around 3:1:1 acetate-propionate-butyrate (6, 7). Butyrate has a particularly important role as the preferred energy source for the colonic epithelium and a proposed role in providing protection against colon cancer and colitis (1, 4, 5). Butyrate can reduce gut mucosal inflammation by inhibiting the activity of the transcription factor NF-κB, which in turn mediates the effects of proinflammatory cytokines (14). Barcenilla et al. (2) recently isolated a range of butyrate-producing strains from human feces. Most (>80%) of the strains that produced high concentrations (>10 mM) of butyrate in batch culture in vitro have now been identified by 16S rDNA sequencing and phenotypic tests as species of the gram-positive anaerobes *Roseburia* (10) and *Faecalibacterium* (previously *Fusobacterium*) (11). Many of the *Roseburia* isolates (Table 1) were found to belong to a new species, *Roseburia intestinalis* (10). Nothing is currently known about the enzymology or physiology of butyrate production by any of these species.

Butyrate is normally formed from two molecules of acetyl coenzyme A (CoA), yielding acetoacetyl-CoA, which is then converted to butyryl-CoA (reviewed in reference 8). Thereafter, butyryl-CoA may yield butyrate via butyrate kinase, as in some strains of ruminal *Butyrivibrio fibrisolvens* (8) or via butyryl-CoA:acetate-CoA transferase (Fig. 1). In the latter reaction butyryl-CoA is exchanged with exogenously derived acetate to yield acetyl-CoA and butyrate. Diez-Gonzalez et al. (8) suggested the existence of two distinct metabolic types, differing in acetate utilization and lactate formation, among butyrate-producing *B. fibrisolvens* strains from the rumen, apparently correlating with the possession of either butyrate kinase or butyryl-CoA:acetate-CoA transferase. Here we report metabolic and enzymological studies of strains belonging to four species of butyrate-producing bacteria from human feces, *Coprococcus* sp., *Roseburia* sp., *R. intestinalis*, and *Faecalibacterium prausnitzii.*

Butyrate-producing isolates. The seven strains studied are human fecal isolates that were found to produce at least 10 mM butyrate in vitro on first isolation (2). Based on 16S rDNA sequences and phenotypic tests, strains L1-82, L1-952, and L1-8151 belong to the newly defined species *R. intestinalis* (10). Strains A2-181 and A2-183 appear to belong to another new species, not yet named, that is 95% related in rDNA sequence to *Roseburia cecicola* and will be referred to here as *Roseburia* sp. Strain L2-50 is a representative of *Coprococcus* sp. (2), and strain A2-165 belongs to the new genus *Faecalibacterium* (previously *Fusobacterium*) *prausnitzii* (11).

Growth requirements and media. The strains studied here were originally isolated from anaerobic roll tubes with M2GSC (20) medium containing 30% clarified rumen fluid, as described by Barcenilla et al. (2). All seven strains were, however, able to grow in yeast extract-Casitone-fatty acids (YCFA) medium lacking rumen fluid provided that a mixture of sources of

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TABLE 1. Effects of SCFA on growth rates of fecal bacteria on YCFAGSC medium

Species	Strain	Specific growth rate (h^{-1}) on[a]:		
		Complete medium with SCFA	Medium with all SCFA omitted	Medium with all SCFA except acetate omitted
Coprococcus sp.	L2-50	0.34 ± 0.06	0.15 ± 0.01	0.17 ± 0.01
F. prausnitzii	A2-165	0.21 ± 0.03	0.09 ± 0.0	0.32 ± 0.04
Roseburia sp.	A2-181	0.37 ± 0.02	0.22 ± 0.0	0.39 ± 0.02
Roseburia sp.	A2-183	0.42 ± 0.04	0.31 ± 0.021	0.35 ± 0.01
R. intestinalis	L1-82	0.35 ± 0.02	0.37 ± 0.02	0.43 ± 0.01
R. intestinalis	L1-952	0.44 ± 0.05	0.46 ± 0.03	0.48 ± 0.02
R. intestinalis	L1-8151	0.39 ± 0	0.40 ± 0.05	0.41 ± 0.02

[a] SCFA concentrations were 33 mM acetate, 9 mM propionate, 1.2 mM iso-butyrate, 1.0 mM iso-valerate, and 1.0 mM valerate. Growth rate values (μ) are averages of results of three replicates ± standard deviations.

carbon and fatty acids (SCFA) (giving final concentrations of 33 mM acetate, 9 mM propionate, 1.2 mM iso-butyrate, 1.0 mM iso-valerate, and 1.0 mM valerate) was added (Table 1). Medium YCFAGSC contains the following ingredients (per 100 ml): 1 g of Casitone; 0.25 g of yeast extract; 0.2 g each of glucose, starch, and cellobiose; 0.4 g of $NaHCO_3$; 0.1 g of cysteine; 0.045 g of K_2HPO_4; 0.045 g of KH_2PO_4; 0.09 g of NaCl; 0.009 g of $MgSO_4 \cdot 7H_2O$; 0.009 g of $CaCl_2$; 0.1 mg of resazurin; 1 mg of hemin; 1 μg of biotin; 1 μg of cobalamin; 3 μg of *p*-amino benzoic acid; 5 μg of folic acid; 15 μg of pyridoxamine; 5 μg of thiamine; and 5 μg of riboflavin. Thiamine and riboflavin were added to the medium as filter-sterilized solutions after being autoclaved (121°C, 15 min). Media were prepared and maintained under O_2-free CO_2 by the methods referred to in reference 2.

No growth occurred when fermentable carbohydrates were omitted from YCFAGSC medium (data not shown). Omitting all SCFA almost completely prevented the growth of *Coprococcus* sp. strain L2-50 and *F. prausnitzii* A2-165 and reduced the growth of *Roseburia* sp. strains A2-181 and A2-183 (Table 1). Addition of acetate as the sole SCFA restored or stimulated the growth of all strains except *Coprococcus* sp. strain L2-50. Strain L2-50 is therefore assumed to require one or more other SCFA for growth. *F. prausnitzii* A2-165 grows poorly on medium that does not contain acetate, and this requirement is likely to account for its previously observed dependence on rumen fluid (21).

Fermentation product analysis and fermentation balances. Analysis of fermentation products demonstrated net utilization of acetate by the five *Roseburia* strains and by *F. prausnitzii* A2-165 when they were grown for 24 h in YCFAG medium (YCFAGSC medium without cellobiose or soluble starch but

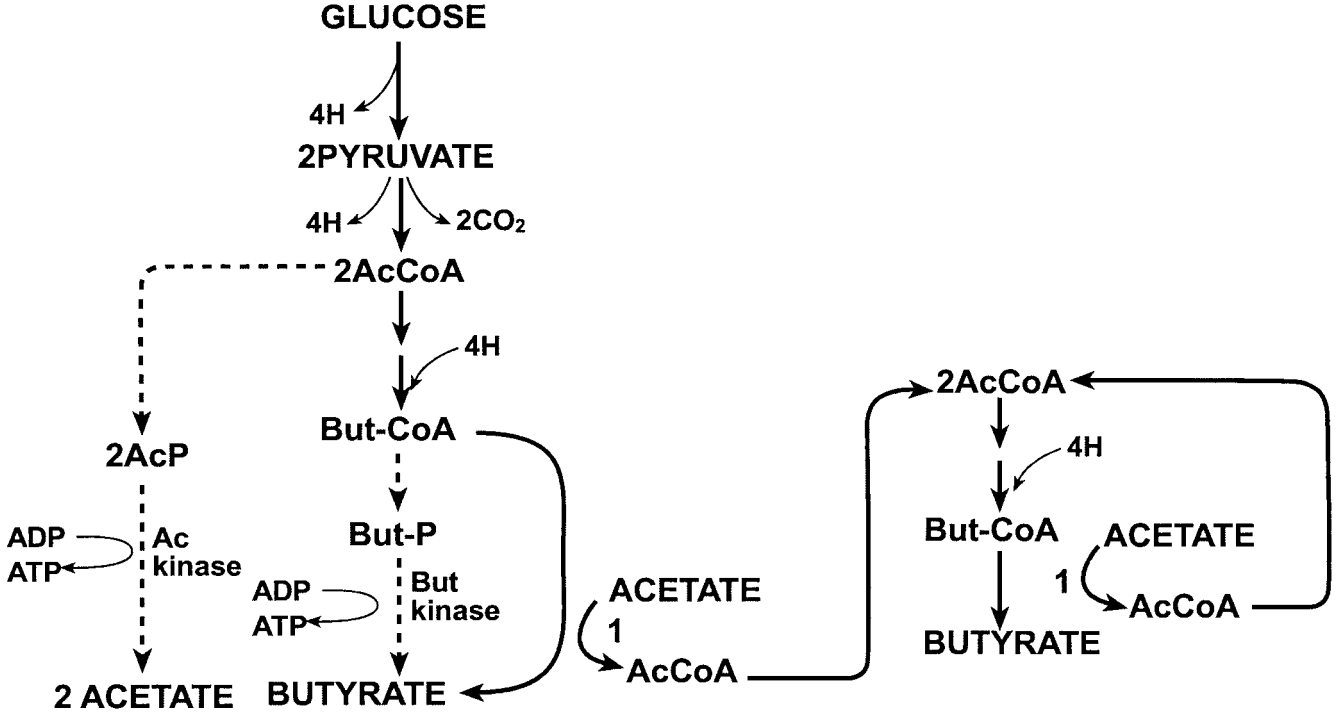

FIG. 1. Pathways for acetate and butyrate formation from glucose and glucose plus exogenous acetate. 1, butyryl-CoA:acetyl-CoA transferase; AcP, acetyl phosphate; But, butyryl or butyrate.

TABLE 2. Acidic fermentation products formed by butyrate-producing bacteria isolated from human feces on YCFAG[33] medium[a]

Species	Strain	Acid final concn (mM)[b]			
		Formate	Acetate	Butyrate	Lactate
Coprococcus sp.	L2-50	16.26 ± 1.22	3.08 ± 2.64	6.19 ± 0.06	1.25 ± 0.22
F. prausnitzii	A2-165	9.66 ± 1.55	−9.55 ± 1.12	10.45 ± 1.53	0.46 ± 0.10
Roseburia sp.	A2-181	6.95 ± 1.43	−10.57 ± 1.87	11.01 ± 1.03	0.23 ± 0.09
Roseburia sp.	A2-183	7.56 ± 0.12	−6.09 ± 1.13	11.42 ± 0.59	1.16 ± 0.13
R. intestinalis	L1-82	7.04 ± 0.32	−7.57 ± 0.60	10.92 ± 0.18	1.40 ± 0.02
R. intestinalis	L1-952	6.88 ± 0.81	−8.38 ± 1.80	10.44 ± 0.39	1.69 ± 0.39
R. intestinalis	L1-8151	8.37 ± 0.78	−3.04 ± 0.48	11.59 ± 0.49	1.01 ± 0.32

[a] YCFAG[33] medium is YCFA containing 33 mM acetate.
[b] Negative values indicate acetate utilization. Data are the averages of results from three separate incubations ± standard deviations.

with glucose at 10 mM) (Table 2). All the strains produced butyrate, various amounts of formate, and relatively small amounts of lactate. The level of production of butyrate in these experiments was slightly lower, especially for *Coprococcus* sp. strain L2-50, than that previously reported for the same strains when they were grown on the rumen fluid containing M2GSC medium (2).

In order to estimate carbon and electron recoveries, we performed additional experiments in which the medium was prepared and maintained under O_2-free N_2 in place of CO_2 and $NaHCO_3$ was omitted. In order to avoid possible contributions from energy sources other than glucose, Casitone was omitted and the concentration of yeast extract was reduced to 0.2% (wt/vol) (medium YFAG). *F. prausnitzii* A2-165, however, was found to require yeast extract and Casitone, and this strain was therefore grown on YCFAG. Carbon and electron recoveries were calculated with reference to appropriate control cultures lacking glucose. Acid production was determined by capillary gas chromatography (GC) following conversion to *t*-butyldimethylsilyl derivatives (22). H_2 production was analyzed by packed-column GC (24). Carbon dioxide production was determined in these experiments by isotope dilution. Three replicate 24-h incubations were stopped by the addition of 1 ml of concentrated orthophosphoric acid through a gas-tight septum. One tube of each pair was used to determine the natural abundance of the CO_2, and to the other tube 100 μl of

TABLE 3. Recoveries of carbon and electrons for four butyrate-producing strains grown in batch culture

Strain and/or parameter[a]	No. of carbons or electrons[b] or concn of substrate consumed		No. of carbons or electrons or concn of product formed[c]							Recovery (%)[d]
	Glucose	Acetate	Formate	Acetate	Butyrate	Lactate	H_2	Actual CO_2	Theoretical CO_2	
No. of carbons/mol	6	2	1	2	4	3	0	1	1	
No. of electrons/mol	24	8	2	8	20	12	2	0	0	
C. eutactus L2-50										
Concn (mM)	10		17.1	3.9	7.0	0.9	3.5	1.1	0.8	
Carbon (mM)	60		17.1	7.8	28.0	2.7		1.1	0.8	94.0
Electrons	240		34.2	31.2	140	10.8	7	0	0	93.0
F. prausnitzii A2-165										
Concn (mM)	10	5.2	5.8		10.9	0.9	0	12	16	
Carbon (mM)	60	10.4	5.8		43.6	2.7		12	16	96.7
Electrons	240	41.6	11.6		218	10.8	0	0	0	85.4
Roseburia sp. A2-183										
Concn (mM)	10	10.2	3.4		12.7	0.9	5.5	16.2	22.0	
Carbon (mM)	60	20.4	3.4		50.8	2.7		16.2	22.0	98.1
Electrons	240	81.6	6.8		254.0	10.8	11.0	0	0	87.9
R. intestinalis L1-82										
Concn (mM)	10	8.7	2.3		12.7	0.9	10.4	12.1	23.1	
Carbon (mM)	60	17.4	2.3		50.8	2.7		12.1	23.1	101.9
Electrons	240	69.6	4.6		254.0	10.8	20.8	0	0	93.7

[a] For *F. prausnitzii* strain A2-165, we used medium YCFAG[60], which contains 10 mM glucose and 60 mM acetate. For the other strains, the medium was modified by omitting the Casitone and reducing the yeast extract content to 0.2% to minimize the presence of non-carbohydrate C. All incubations were under O_2-free N_2, and bicarbonate was omitted from the medium. *C. eutactus*, *Coprococcus eutactus*.
[b] References to electrons indicate electrons released upon formal oxidation to CO_2.
[c] Data are averages of values from three separate fermentations.
[d] Total number of carbons or electrons recovered from the products formed from the substrates, expressed as a percentage and calculated from the theoretical CO_2 values.

TABLE 4. Enzymatic activities of butyrate-producing strains harvested in exponential and stationary phases

Species	Strain	Enzyme activity (μmol^{-1} mg of protein^{-1} min^{-1})[a]					
		Acetate kinase		Butyrate kinase		Butyryl-CoA:acetate-CoA transferase	
		E	S	E	S	E	S
Coprococcus sp.	L2-50	0.67 ± 0.13	0.15 ± 0.07	0.82 ± 0.39	0.11 ± 0.05	118.39 ± 5.02	10.8 ± 4.29
F. prausnitzii	A2-165	0.05 ± 0.00	1.49 ± 0.11	<0.01	<0.01	18.64 ± 7.07	19.0 ± 0.0
Roseburia sp.	A2-181	2.29 ± 0.21	2.85 ± 0.65	<0.01	<0.01	15.58 ± 5.98	2.79 ± 1.58
Roseburia sp.	A2-183	2.03 ± 0.34	0.61 ± 0.08	<0.01	<0.01	38.95 ± 3.40	9.1 ± 0.27
R. intestinalis	L1-82	1.82 ± 0.02	1.61 ± 0.63	<0.01	<0.01	25.50 ± 0.71	8.9 ± 1.17
R. intestinalis	L1-952	2.32 ± 0.02	3.52 ± 0.83	<0.01	<0.01	17.45 ± 2.52	1.32 ± 0.0
R. intestinalis	L1-8151	1.76 ± 0.02	1.31 ± 0.03	<0.01	<0.01	9.24 ± 1.48	2.05 ± 0.49

[a] Data are the means of results of duplicated assays of two separate cultures. ± indicates the range of values. E, exponential phase; S, stationary phase.

2.5 mM $NaH^{13}CO_3$ (98 atom%; Isotec Inc., Miamisburg, Ohio) was added by injection. All tubes were then shaken at 100°C for 15 min, and then 0.5 ml of the hot headspace gas was removed into a gas-tight syringe and injected into a continuous-flow gas isotope mass spectrometer (Tracermass 20-20; Europa Scientific, Crewe, Cheshire, United Kingdom). From the increase in enrichment (from ion intensities at *m/z* 44 and 45) above natural abundance and the amount of $NaH^{13}CO_3$ added, the total amount of CO_2 in the tube was calculated. The amount of CO_2 obtained in the uninoculated medium was subtracted to yield the quantity of CO_2 produced. Theoretical CO_2 values calculated according to the method discussed in reference 29 were in general higher than the measured values (Table 3).

Carbon recoveries were determined using the theoretical CO_2 values, as the direct measurements appear likely to be underestimated due to incomplete recovery of CO_2 in the growth medium. On this basis, carbon recoveries ranged from 94 to 102% (Table 3). Strains were screened for ethanol and butanol production by GC, but no traces of these products were found. The electron balance for *F. prausnitzii* A2-165, which gave the lowest carbon recoveries and which did not produce H_2, suggested that the products were comparatively oxidized (85.4%). Whether or not the fermentation by these bacteria was accompanied by changes in the redox balance of the (highly reduced) medium was not examined.

Strain distribution of key butyrate synthetic activities. For the determination of butyrate pathway enzymes, strains were grown on M2GSC medium (80 ml) for 6 to 8 h (mid-exponential phase) and for 20 h (stationary phase) and the bacterial cell pellets were harvested by centrifugation (10,000 × *g* for 10 min) at 4°C. The cells were washed in 50 mM phosphate buffer containing 0.05% cysteine-HCl, centrifuged as described above, and resuspended in 4 ml of the same buffer. The cells were disrupted by sonication (MSE Soniprep setting 150 for 3 min). The disrupted cells were centrifuged at 10,000 × *g* for 5 min, and the cell debris was discarded; the extracts were used for determination of enzyme activities. Acetate kinase and butyrate kinase activities were determined by a colorimetric assay (23). Butyryl-CoA:acetate-CoA transferase activity was measured by the method of Barker et al. (3). The protein contents of the extracts were measured after boiling the extracts in alkali (18).

Significant butyrate kinase activity was detected in only one of the seven strains (*Coprococcus* sp. strain L2-50) (Table 4). Butyryl-CoA:acetate-CoA transferase activity, however, was detected in all seven strains in both the exponential and stationary phase, demonstrating that this is a potentially important route for butyrate synthesis among human fecal isolates. *Coprococcus* sp. strain L2-50 possessed the highest butyryl-CoA:acetate-CoA transferase activity in exponentially growing cells. Activities for this enzyme were 2.5- to 12-fold higher in exponential- than in stationary-phase cells in six of the seven strains, the exception being *F. prausnitzii* A2-165, for which activities were similar at the two growth stages.

Conclusions. The butyrate-producing strains studied here represent four distinct species of gram-positive anaerobes that belong to the *Clostridium coccoides* (*Roseburia* sp., *Coprococcus* sp.) and *Clostridium leptum* (*F. prausnitzii*) clusters of gram-positive bacteria. Bacteria belonging to these clusters are dominant members of the human intestinal flora, but they are frequently underrepresented among cultured isolates (12, 25, 29). *F. prausnitzii* may represent up to 15% of the human fecal microflora (26). Although first recovered using a complex rumen fluid-based medium (2), all four species can be grown on simpler rumen fluid-free media supplemented with short-chain fatty acids. *F. prausnitzii* shows a strong requirement for acetate, and *Coprococcus* sp. strain L2-50 apparently requires an SCFA other than acetate for growth. The difficulty in cultivating many human gut anaerobes may therefore often be overcome simply through supplying essential nutrients that are normally available by cross-feeding from other bacteria in the complex ecosystem in vivo.

Little work has been done previously on the enzymes that contribute to butyrate synthesis in commensal bacteria from the human colon. Butyrate kinase was detected here in only one strain, *Coprococcus* sp. strain L2-50. On the other hand, butyryl-CoA:acetate-CoA transferase activity was detected in all seven isolates studied here and thus provides the only known route for butyrate synthesis in the *Roseburia* sp. and *F. prausnitzii* strains.

These findings reveal a clear metabolic difference between the species studied. *Roseburia* sp. and *F. prausnitzii* strains show net acetate utilization and possess butyryl-CoA:acetate-CoA transferase but apparently lack butyrate kinase. On the other hand, *Coprococcus* sp. strain L2-50 produces acetate and possesses both butyrate kinase and butyryl-CoA transferase. At this stage we cannot say whether the net consumption or

production of acetate is in general correlated with a lack of butyrate kinase activity. The behavior of these human fecal strains, however, recalls that of a subdivision noted by Diez-Gonzalez et al. (8) among rumen *Butyrivibrio* spp., although the two *B. fibrisolvens* strain groupings were reported to possess either butyrate kinase or butyryl-CoA:acetate-CoA transferase but not both activities. It is possible that additional patterns of butyrate metabolism exist among human fecal isolates, but this question must await a much wider strain survey. Interestingly, almost all (95%) of the strains isolated by Barcenilla et al. (2) that showed net acetate utilization proved to be butyrate producers, although only 50% of the butyrate producers showed net acetate consumption. This suggests a very strong link between acetate utilization and butyrate production in this group of human colonic bacteria. In strains of *B. fibrisolvens* that possess butyryl-CoA:acetate-CoA transferase, Diez-Gonzalez et al. (8) showed that increasing the acetate concentration favored butyrate production and shifted product ratios. It will be of interest to establish whether or not this effect occurs also in *F. prausnitzii* and *Roseburia* sp.

From the still-limited evidence available, it appears that *F. prausnitzii*, *Roseburia* sp., and related strains may account for a significant proportion of the more active butyrate-producing strains in the human colon (2, 13, 26). If so, we would predict that much of the butyrate carbon may be generated from acetate through the CoA transferase route in vivo, consistent with the findings of Miller and Wolin (19). It has also been suggested that utilization by butyrate producers of lactate produced by other species, such as bifidobacteria and lactobacilli, might be significant in vivo (15). None of the strains studied here were able to utilize lactate as a sole carbon and energy source, but we have not excluded the possibility that lactate can be utilized by strains growing on carbohydrates.

Given the current interest in dietary approaches for stimulating butyrate synthesis, especially in the distal large intestine, it is vital to understand the microbiology associated with butyrate synthesis and the relationships between substrate supply and product formation. Further work on metabolite flow in vivo and on the prevalence of *Roseburia* sp., *Coprococcus* sp., and *F. prausnitzii* strains, in particular, in different individuals and in response to diet appears desirable.

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