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Phenylacetic Acid Stimulation of Cellulose Digestion By *Ruminococcus albus* 8

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The rate of cellulose digestion by *Ruminococcus albus* 8 grown on a defined medium could be increased by adding a minimum of 6.6% (vol/vol) rumen fluid. Strain 8 was grown on half this concentration, and the culture medium before and after growth was analyzed by gas chromatography-mass spectrometry to determine which components of the rumen fluid were used. Phenylacetic acid was identified as the component needed to make the defined medium nutritionally equivalent to one supplemented with rumen fluid. [^{14}C]phenylacetic acid fed to cultures of strain 8 was primarily incorporated into protein. Hydrolysis of protein samples and separation of the resulting amino acids showed that only phenylalanine was labeled. The results indicate that cellulose digestion by strain 8 was probably limited by phenylalanine biosynthesis in our previously reported medium. The data obtained on the utilization of other rumen fluid components, as well as on the production of metabolites, illustrate the potential usefulness of this method in formulating defined media to simulate those in nature.

We have previously reported 3-phenylpropanoic acid (PPA) as the nutrient accounting for most of the stimulatory effect of rumen fluid on the rate of growth and cellulose digestion by *Ruminococcus albus* 8 (10). In a chemically defined medium containing PPA, the time required for a 1% inoculum to digest 0.3% pebble-milled cellulose was 2 days, as compared with 2 weeks in medium without PPA. The addition of rumen fluid to this medium decreased the time to about 1 day, an indication that rumen fluid contained an additional unidentified nutrient(s).

The minimal amount of rumen fluid required for stimulation of the culture was determined. The components in it were analyzed by gas chromatography-mass spectroscopy, and their concentrations before and after growth of strain 8 were compared. The identified growth factor, phenylacetic acid (PAA), was fed to strain 8 and was recovered exclusively as phenylalanine.

MATERIALS AND METHODS

Culture procedures. *R. albus* 8 was maintained by daily transfer of a 1% inoculum into 6 ml of 0.15% pebble-milled cellulose broth prepared as described previously (10), except that 2.5×10^{-5} M PPA, isobutyric acid, and 2-methylbutyric acid were included, and *n*-valeric and isovaleric acids were eliminated. *R. albus* 8 has been maintained on this defined medium for over a year with no diminution in the rate of cellulose hydrolysis.

Cultures grown for gas chromatographic-mass spectroscopic (GC/MS) analysis contained 48 ml of this medium (with additions where noted) in 125-ml Wheaton serum bottles stoppered with Bellco rubber stoppers held with crimped aluminum caps.

One-liter cultures of defined medium containing 2.2 μM [1-^{14}C]PAA (Pathfinder Laboratories) at a specific activity of 10^7 dpm/μmol were inoculated with 6-ml cultures of *R. albus* 8 grown for 24 h for the tracer studies described below.

Preparation of rumen fluid. Rumen fluid was obtained in 1-liter batches from an alfalfa hay-fed Jersey heifer provided with a rumen fistula. The rumen fluid was immediately centrifuged at 25,000 × *g* for 30 min at 4°C under CO_2 in Teflon-lined stainless steel centrifuge bottles equipped with rubber gasket seals (Servall, Inc.). The supernatant was filtered through an Amicon PM-10 membrane in an Amicon model 202 stirred ultrafiltration cell under 20 lb/in^2 of CO_2. The filtrate was collected under CO_2 in a 250-ml Pyrex flask which was stoppered and stored at 4°C.

When rumen fluid was required for supplementation of defined media, it was filter sterilized by passage through a 0.22-μm filter (type GSWP, Whatman, Inc.) and stored under CO_2 in an autoclaved Bellco screw-cap tube (16 by 125 mm) stoppered with a number 00 butyl rubber stopper. Additions of sterile anaerobic rumen fluid and test compounds and inoculation of cultures was accomplished anaerobically with disposable plastic syringes (Monoject, Inc.).

Determination of the minimal amount of rumen fluid required for enhancement of growth of *R. albus* 8. A series of tubes containing 6 ml of 0.15% pebble-milled cellulose in defined medium supplemented with increasing amounts of rumen fluid was inoculated with 0.10 ml of a ⅓ dilution of a 16-h culture of *R. albus* 8 and was incubated at 37°C. Cellulose digestion was routinely measured visually by the decrease in the volume of pebble-milled cellulose at the bottom of the undisturbed tube. We have previously shown this to

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be proportional to the dry-weight decrease (10). The minimal concentration of rumen fluid for maximal stimulation of growth was determined, and a concentration half as great was utilized to supplement 48-ml cultures grown for GC-MS analysis.

Processing and extractions of culture. Cultures (48 ml) supplemented with 3.3% (vol/vol) rumen fluid were prepared in pairs, with one member of each pair inoculated with 0.1 ml of a 24-h culture of *R. albus* 8. Both the inoculated and uninoculated cultures were incubated at 37°C until the cellulose in the inoculated culture was digested (ca. 50 h). The cultures were then centrifuged at 9,600 × *g* for 10 min at 4°C in 30-ml acid-washed Corex tubes. The supernatant was acidified to pH 2 with 1 M H_2SO_4 and extracted successively with 40, 20, and 15 ml of diethyl ether (USP; Mallinckrodt, Inc.). The ether extracts were pooled and evaporated to a volume of 10 ml with a stream of N_2.

Preparation of samples for GC-MS analysis. Diazomethane dissolved in ether was prepared from Diazald (Aldrich Chemical Co.) by distillation from an alkaline ethereal solution, using the basic procedure supplied by the manufacturer. It was stored in Teflon-lined screw-cap tubes in the freezer at −10°C. Methyl esters were prepared from the 10-ml concentrated ether extracts by adding enough diazomethane to color the solution yellow for at least 15 min. The methylated extracts were then evaporated to 0.5 ml with a stream of N_2 in a Kolmer graduated centrifuge tube (Corning Glass Works).

GC-MS analyses. The methyl esters from inoculated and uninoculated (control) cultures supplemented with rumen fluid were analyzed by gas chromatography-mass spectroscopy, and the profiles were compared. A 2.0-μl sample of the concentrated methylated extract was injected into a Finnegan 3200 gas chromatograph-mass spectrograph equipped with a 30-m SE-54 fused silica capillary column (J & W Scientific, Inc.). The carrier gas was helium at a flow rate of 28 cm/s. The vaporized sample was split 30:1, and the temperature was increased from 50 to 300°C at a rate of 6°C min. Eluting compounds were ionized at 70 eV to obtain mass spectra, which were computer matched against a library of mass spectral data for preliminary identification.

Confirmation of identity and quantity was based on the injection of methylated standards containing 2 mg of the following compounds per ml: isobutyric acid, *n*-butyric acid, and 3-methylbutyric acid (Eastman Kodak Co.); *n*-valeric acid, *n*-hexanoic acid, PAA, octanedioic acid, nonanedioic acid, and α-keto-β-methyl-*n*-valeric acid (Sigma Chemical Co.); cyclohexanecarboxylic acid and PPA (Aldrich); and 2-methylbutyric acid (K & K Laboratories, Inc.). Identification of *R. albus* fatty acids was aided by a reference standard consisting of 22 methyl esters of bacterial fatty acids (Supelco, Inc.).

Isolation and hydrolysis of protein. One-liter cultures were centrifuged at 25,000 × *g* for 45 min at 4°C in stainless steel centrifuge bottles to remove the cells, and the protein in the supernatant was concentrated to dryness over an Amicon PM-10 membrane in an Amicon model 202 stirred ultrafiltration cell under 20 lb/in^2 of N_2. The protein was washed three times in the ultrafiltration cell by the addition of 100 ml of water, followed each time by concentration to dryness. The protein was taken up in a minimal volume of water (ca. 5 ml), and its concentration was measured by the binding of Coomassie blue dye (5). Samples were hydrolyzed in 6 N HCl under N_2 at 121°C for 24 h and were stored at −10°C.

Amino acid separation and detection. Samples of hydrolyzed protein were analyzed on a Glenco amino acid analyzer described previously by Lefevre and Rucker (11). A 100-μg sample was evaporated to dryness with a stream of N_2 and dissolved in 200 μl of 0.2 M sodium citrate (pH 3.19), and a 50-μg aliquot was applied to a jacketed column (0.325 by 60 cm) of Aminex A-9 resin (Bio-Rad Laboratories) equilibrated with 0.2 M sodium citrate (pH 3.19) at 55°C. A stepwise elution with 0.2 M sodium citrate (pH 3.19, 20 min), 0.2 M sodium citrate (pH 4.25, 35 min), 0.35 M sodium citrate (pH 6.7, 20 min), and 0.35 M sodium citrate (pH 7.9) containing 1 M NaCl (70 min) at a flow rate of 12 ml/hr was used to separate the amino acids. The column was regenerated by washing with 0.2 M NaOH for 20 min and then reequilibrated with a 45-min wash of 0.2 M sodium citrate (pH 3.19).

The effluent from the column was passed through either a Glenco RC-1 ninhydrin reaction coil equipped with a Glenco 57V dual absorbance monitor for estimation of ninhydrin-reactive nitrogen or a Flo-one model HP radioactive flow detector (Radiomatic Instruments and Chemical Co.). A Hewlett-Packard model 3390 integrator-printer aided in interpreting results. Citrate buffers and the ninhydrin reagent used in the amino acid analysis were from Pierce Chemical Co. The [^{14}C]phenylalanine used as a standard was a gift from Eric E. Conn, University of California, Davis.

RESULTS

Cellulose digestion by *R. albus* 8 in the PPA-containing defined medium was stimulated not only by rumen fluid but also by an ether extract of acidified rumen fluid. To determine the minimal amount of rumen fluid giving this effect, 6-ml cultures containing defined medium plus 25 μM PPA were supplemented with increasing amounts of rumen fluid, inoculated with a 24-h culture of *R. albus* 8, and grown for 15 h. As little as 0.4 ml of rumen fluid per 6-ml culture (6.6%, vol/vol) was fully effective.

To maximize the use of the stimulatory nutrient(s) in the inoculated culture as compared with that in the control, the concentration of rumen fluid was reduced to 3.3% in two 48-ml cultures. Both the inoculated and uninoculated cultures were incubated until the cellulose in the inoculated culture was digested. They then were processed and analyzed by gas chromatography-mass spectroscopy as described above (Fig. 1). The identities of many of the peaks are listed in Table 1.

Figure 1 shows PAA (peak 13) in the control but not in the inoculated culture. In addition, the peaks representing isobutyric acid (peak 3), 2-methylbutyric acid (peak 5), butyric acid (peak 4), and PPA (peak 16) decreased in the inoculated culture. In addition, Fig. 1B shows peaks for

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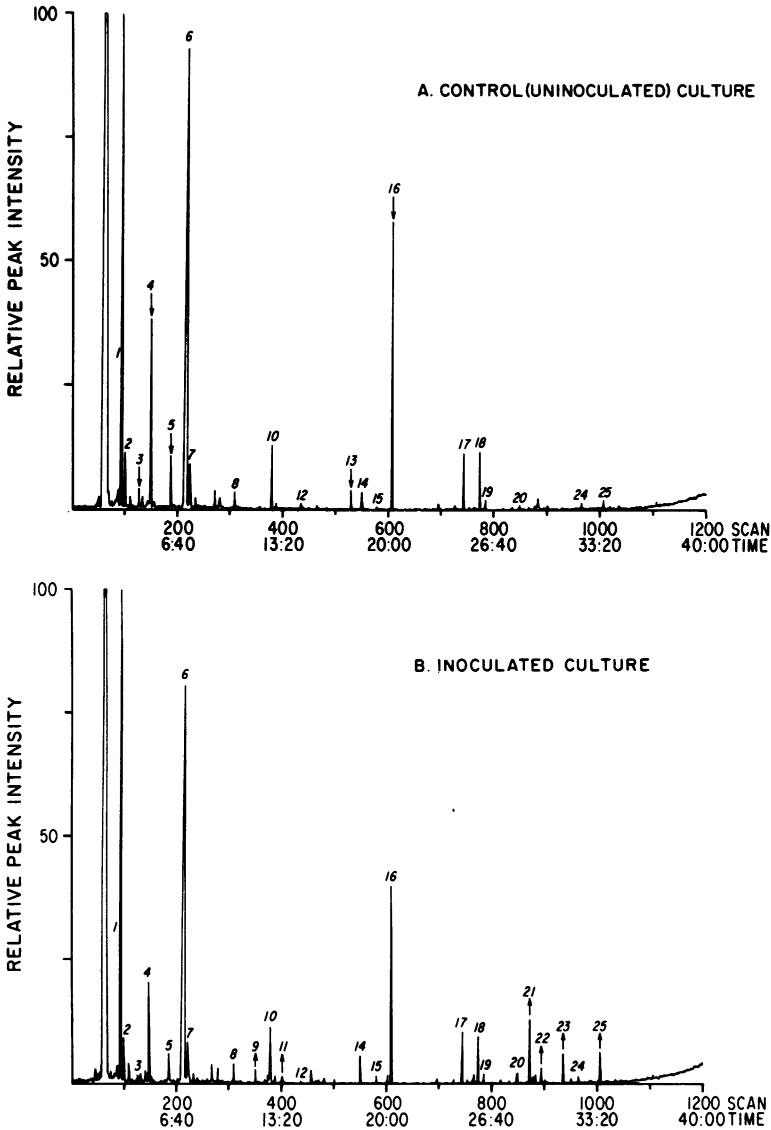

FIG. 1. Results of GC-MS analysis of the methyl esters. (A) Profile from the control (uninoculated) culture. Compounds utilized by *R. albus* 8 are indicated by an arrow (↓). (B) Profile from the inoculated culture. Compounds produced by *R. albus* 8 are indicated by an arrow (↑). Numbers refer to the peaks as listed in Table 1.

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tetradecanoic acid (peak 22), hexadecanoic acid (peak 25), two unidentified long-chain fatty acids (peaks 21 and 23), 2-keto-3-methylpentanoic acid (peak 9), and a trace of succinic acid (peak 11) not observed in Fig. 1A.

The rates of cellulose digestion in cultures supplemented with PAA, butyric acid, and rumen fluid were compared (Fig. 2). PAA and rumen fluid were equally effective, and all of the cellulose was digested in 24 h. PAA was effective only when PPA was present. Omission of PAA increased the time for cellulose digestion to almost 2 days, whereas omission of PPA almost stopped growth, with all of the cellulose being digested only after 2 weeks.

Figure 3 shows the undigested cellulose in a series of tubes supplemented with increasing concentrations of PAA. A concentration of 5 μM was required for maximal cellulose digestion. Several samples of acidified rumen fluid, exhaustively extracted with ether, methylated, and analyzed by gas chromatography-mass spectroscopy, contained between 56 and 87 μM PAA.

TABLE 1. Identification of peaks shown in Fig. 1[a]

Peak no.	Compound
1	Acetic acid
2	Propanoic acid
3	Isobutyric acid
4	Butyric acid
5	2-Methylbutyric acid and 3-methylbutyric acid
6	Unknown
7	Pentanoic acid
8	Hexanoic acid
9	2-Keto-3-methylpentanoic acid
10	2-(2-Ethoxyethoxy)ethanol
11	Succinic acid
12	Cyclohexanecarboxylic acid
13	PAA
14	2-Hydroxybenzoic acid
15	Benzothiazole
16	PPA
17	Butylatedmethoxytoluene
18	Butylatedhydroxytoluene
19	Nonanedioic acid
20	2-(Methylthio)benzothiazole
21	Unidentified fatty acid
22	Tetradecanoic acid
23	Unidentified fatty acid
24	Dodecanedioic acid
25	Hexadecanoic acid

[a] Detected as the methyl esters. Numbers refer to Fig. 1.

Analysis of a 1-liter culture of *R. albus* 8 grown on defined medium supplemented with 2.2 μM [^{14}C]PAA (specific activity, 10^7 dpm/μmol) gave the results shown in Table 2. More than 90% of the label was incorporated into protein, with essentially no labeling of lipids or CO_2. A small percentage of the label (<4%) was not assimilated.

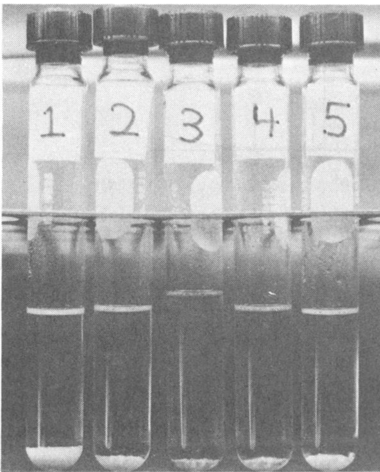

FIG. 2. Supplementation of defined medium with rumen fluid, PAA, or butyric acid. All tubes contained 6.0 ml of defined medium with 25 μM PPA. Tube 3 also contained 0.4 ml of rumen fluid; tube 4, 5.0 μM PAA; and tube 5, 25 μM butyrate. Tube 1 was uninoculated.

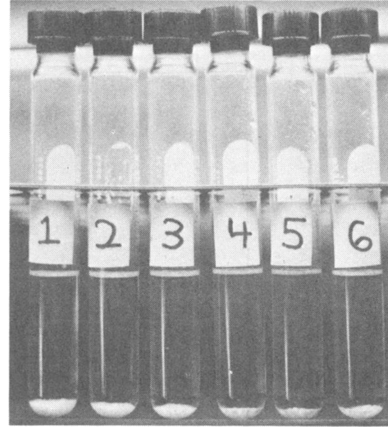

FIG. 3. Effect of PAA concentration on cellulose digestion by *R. albus* 8. All tubes contained 6.0 ml of defined medium with 25 μM PPA. Tubes 1 through 6 were supplemented with 0, 1.0, 2.0, 5.0, 10.0, and 20.0 μM PAA, respectively.

Separation by high-pressure liquid chromatography of the amino acids resulting from hydrolysis of a sample of extracellular protein gave the results shown in Fig. 4. Figure 4A shows the amino acid pattern from 50 μg of extracellular protein when the column effluent was reacted with ninhydrin and read at 570 nm. Figure 4B shows the distribution of the ^{14}C label in an identical sample analyzed for radioactivity. The ^{14}C peak coincides exactly with that for phenylalanine. A third 50-μg sample of protein supplemented with standard [^{14}C]phenylalanine also gave a single radioactive peak. The error in reproducibility of retention times for analytical runs on the instrument described was 0.3 to 0.5%.

The effect of PPA concentration on the degree

TABLE 2. Incorporation of [^{14}C]PAA into *R. albus* 8 fractions

Fraction	Total dpm[a]	Label (%)
Total protein	20,161,700	91.6
Culture gas	8,400	<0.1
Not utilized[b]	861,700	3.9

[a] Determined on a Beckman LS31458 liquid scintillation counter, using external standard channels ratio for efficiency determination.

[b] Petroleum ether-extractable disintegrations per minute from the ultrafiltrate acidified to pH 2 with H_2SO_4.

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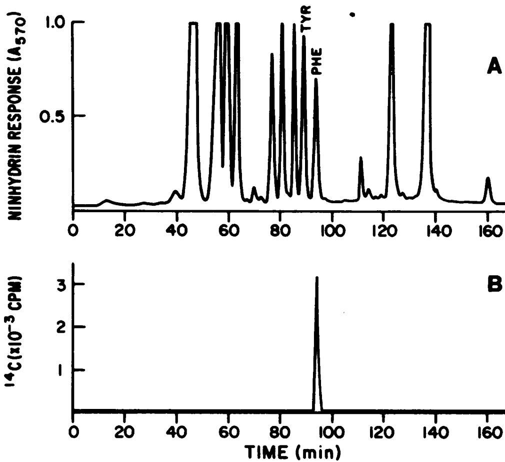

FIG. 4. Amino acids in the hydrolyzate of extracellular protein from *R. albus* 8 labeled with [^{14}C]PAA. (A) Elution profile at 570 nm obtained from a 50-μg sample when the column effluent was reacted with ninhydrin. Tyrosine and phenylalanine peaks were indicated. (B) Identical 50-μg sample analyzed for ^{14}C content with radioactive flow detector.

of incorporation of [^{14}C]PAA into phenylalanine is shown in Table 3. There was no significant difference in the specific activity of the resultant phenylalanine in cultures with and without PPA. This is additional evidence that PPA does not participate in the formation of phenylalanine. The increased extracellular protein in the culture without PPA may have been due to increased cell lysis.

DISCUSSION

The results of this study indicate the potential usefulness of gas chromatography-mass spectroscopy in identifying organic nutrilites in culture media. The method described is sufficiently sensitive that utilization of relatively small amounts of the nonvolatile nutrient acids PPA and PAA could be accurately measured, and the decrease in the volatile acids (isobutyric acid, 2-methylbutyric acid, and butyric acid) could be detected. Four of these five rumen fluid components removed by *R. albus* 8 have now proved to be essential or stimulatory nutrients, and a defined medium including them supports a growth rate equal to that achieved in their natural milieu.

Both isobutyric and 2-methylbutyric acids are essential nutrients for *R. albus* 8 (10), as well as for strain RAM (9). They may function as precursors for valine and isoleucine in a reductive carboxylation pathway (2) or perhaps be involved in the synthesis of higher lipids (4). Both isobutyric and 2-methylbutyric acids have been shown to be growth factors for several other rumen organisms (3, 6, 8).

The peak areas in Fig. 1 show an 18% decrease in the PPA peak for the inoculated culture relative to the control. This decrease indicates a utilization of 4.7 μmol of PPA per liter, in fair agreement with the 3.0 μmol/liter concentration previously reported as sufficient to achieve maximal rates of cellulose digestion (10).

The rumen fluid used to supplement the cultures shown in Fig. 1 contained 87 μM PAA. The minimal stimulatory level of 6.6% rumen fluid then corresponds to 5.8 μM PAA, which agrees well with the 5.0 μM PAA found optimal for totally defined medium (Fig. 3).

The only additional rumen fluid component utilized by *R. albus* 8 was butyric acid. Supplementation of the defined medium with butyrate did not affect the rate of growth or cellulose digestion. Butyrate may be utilized by *R. albus* 8 for the synthesis of higher lipids. It is of interest that except for butyrate, identified growth factors were the only rumen fluid components removed from the medium by *R. albus* 8. It is unlikely that additional organic acids stimulating growth of *R. albus* 8 will be found in rumen fluid.

PAA has been previously identified in rumen fluid, and Scott et al. (13) have suggested that it is created from the action of ruminal organisms on dietary phenylalanine. The results presented here (Fig. 4 and Table 2) indicate that *R. albus* 8 utilizes PAA for phenylalanine biosynthesis. Allison (1) has previously demonstrated phenylalanine biosynthesis from PAA in pure cultures of both *Ruminococcus flavefaciens* and *Bacteriodes succinogenes*, but did not find it in *R. albus* 7 (2). However, both *R. albus* 7 and 30 were shown to convert indole-3-acetic acid to tryptophan (M. J. Allison and I. M. Robinson, Biochem. J. **102p**:36, 1967). Metabolic differences among various strains of *R. albus* may be expected, owing to the great variability of nutritional requirements reported for this organism (7).

Several acids may be produced by *R. albus* 8 when it is grown on either defined or rumen

TABLE 3. Effect of PPA concentration on [^{14}C]PAA incorporation[a]

Culture	[PPA]	Extracellular protein (mg)	Sp act of protein (dpm/mg)
1	75 nM[b]	30.3	147,400
2	25 μM	24.3	156,100

[a] PAA concentration, 2.2 μM.
[b] From carryover in 6-ml inoculum.

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fluid-containing medium. In addition to 2-keto-3-methylpentanoic acid (peak 9, Fig. 1), we have often observed variable quantities of the α-hydroxy and α-keto acids corresponding to the carbon skeletons of valine, leucine, isoleucine, and glutamate.

The results of this study show the potential usefulness of GC-MS analyses in determining or improving microbiological culture media. In addition, information on metabolites produced by different organisms alone or in coculture may be obtained.

A limitation of the method is that the materials must be volatile or convertible to volatile derivatives, but they cannot be too volatile. It is not possible to recover quantitatively the esters of acetate and propanoate. They are lost owing to unfavorable partitioning between the aqueous and organic phases and also during evaporation of the diethyl ether.

Several extraneous peaks may result from the preparative treatment. Peak 10 in Fig. 1 is due to 2-(2-ethyoxyethoxy)ethanol (diethylene glycol monoethyl ether). A certain amount of this compound distills over with the diazomethane and is added to the acids during the preparation of the methyl esters. Peak 15 has been identified as benzothiazole. Peak 20 represents 2-(methylthio)benzothiazole, which probably results from a reaction between diazomethane and 2-mercaptobenzothiazole. These latter two compounds leach out of the rubber plungers of the disposable syringes used in this study (12). Peak 18 is due to 2,6-bis(*tert*-butyl)-4-methylphenol, better known as butylated hydroxytoluene. This compound is added to diethyl ether by the manufacturer to prevent the accumulation of peroxides during storage. Peak 17 represents butylated hydroxytoluene with a methylated phenolic hydroxyl group. Methylation of phenolic hydroxyl groups by diazomethane has been discussed previously by Williams (14) and may be avoided under suitable conditions. None of these peaks posed any significant problems in this study.

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