Isolation and Properties of a Phospholipid-hydrolysing Bacterium from Ovine Rumen Fluid

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SUMMARY

A bacterium which can rapidly degrade phospholipids has been isolated from the ovine rumen and tentatively identified as a non-cellulolytic strain of *Butyrivibrio fibrisolvens*. When the organism was grown in batch culture the phospholipase activity appeared during the late exponential phase, peaked 4 to 8 h after maximum culture turbidity had been reached, and then rapidly disappeared. Activity was associated both with the whole cells and the cell-free culture supernatant.

Phosphatidylcholine was degraded via lysolecithin to produce free fatty acids and glycerylphosphorylcholine, indicating phospholipase A+B activity. The activity was stimulated by the addition to the incubation medium of oleic acid or sodium dodecylsulphate. There was no requirement for divalent cations, but the presence of cysteine at 10^{-2} M stimulated the activity many-fold even though the incubation was performed under an oxygen-free gas phase. Phosphatidylethanolamine and phosphatidylinositol were also attacked. The possible role of the organism in bringing about the degradation in the rumen of plant phospholipids in the sheep's diet is discussed.

INTRODUCTION

Early studies showed that ovine rumen contents rapidly hydrolysed a ^{32}P -labelled lecithin substrate added in the pure liquid crystalline form (Dawson, 1959). The degradative pathway was primarily, but not exclusively, through a deacylation producing initially glycerylphosphorylcholine which was subsequently decomposed to glycerophosphoric acid and choline. More recently the rapid decomposition of lecithin in plant materials fed to sheep without ciliated protozoa in their rumen has been demonstrated (Dawson & Kemp, 1969*a*), as well as the catabolism of lecithin in totally-labelled ^{14}C -grass introduced into the normal rumen (Dawson & Hemington, 1974*a*).

It is possible that a certain proportion of dietary plant lipids may be decomposed in the rumen by lipolytic enzymes present in the leaves themselves, rather than by micro-organisms (Dawson & Hemington, 1974*a*; Faruque, Jarvis & Hawke, 1974). However, the pathway of lecithin degradation which was observed to proceed via glycerylphosphorylcholine does not fit in with the action of the main phospholipid-hydrolysing enzyme found in leaves, phospholipase D. In addition, ruminant saliva contains a powerful inhibitor of plant phospholipase D (Dawson & Hemington, 1974*b*) so that little activity is present in the bolus delivered to the rumen.

We have, therefore, investigated the ability of individual rumen bacteria to hydrolyse exogenous phospholipids to see if such an activity is a widespread property of many different species of bacteria or is confined to certain organisms. This has resulted in the isolation of

a *Butyrivibrio* sp. which is especially active in completely deacylating diacyl phosphoglycerides. The characteristics of the organism and the properties of the enzyme are described.

METHODS

Animals. Clun-Forest sheep were rumen-fistulated by Dr P. Kemp and maintained on hay-chaff (800 g) and crushed oats (200 g) fed once daily, or on pasture. Rumen contents were removed by aspiration and strained through muslin to remove coarse food particles. Sheep with defaunated rumens were prepared by adapting the method of Abou Akkada *et al.* (1968) to sheep (Dawson & Kemp, 1969*a*).

Labelled substrates. ^{32}P -labelled phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol were prepared biosynthetically using *Saccharomyces cerevisiae* (Bangham & Dawson, 1960; Hauser & Dawson, 1967). The phospholipids were initially separated on alumina columns as described, the phosphatidylcholine being removed with chloroform-methanol (1:1, v/v) and phosphatidylethanolamine plus phosphatidylinositol with chloroform-ethanol-water (2:5:2, by vol.). The former phospholipid was purified by silicic acid column chromatography, the latter two were separated and purified by preparative t.l.c. using two solvent systems (Coleman, Kemp & Dawson, 1971). They were located by autoradiography after each run.

Phospholipase assay. Phospholipase activity was assayed by incubating ^{32}P -phospholipid at 39 °C and under oxygen-free CO_2 with the appropriate bacterial suspension, together with added cofactors, in a total volume of 5 ml. The yeast phospholipid suspension was obtained by drying down suitable samples of ^{32}P -phospholipid and non-radioactive yeast phospholipid from chloroform solution, and shaking the residue with buffer and a single glass bead in a vortex mixer. It was added to give a final concentration of 5 to 12 $\mu\text{g P/ml}$. The lecithin suspension was sometimes ultrasonicated for 10 min in a KB 80/1 ultrasonic bath (Kerry, Basildon, Essex) before being added to the incubation medium. The incubation was terminated by adding 80 ml chloroform-methanol (2:1, v/v), and after standing for 30 min the single-phase mixture was shaken with 0.2 vol. water. On centrifuging, a lower, chloroform-rich phase separated, containing unchanged substrate, fatty acids and lysolecithin, while the upper aqueous phase contained any ^{32}P water-soluble decomposition products. Radioactivity in the upper and lower phases was determined by evaporating 10 ml portions to dryness and oxidizing the residues with 1.2 ml perchloric acid (72 %, w/v) on a shielded digestion rack. After the addition of 8.3 ml water to each digested sample the Cerenkov radiation emitted (Lanchli, 1969) was monitored on a Nuclear Chicago liquid scintillation counter (Unilux 11).

Determinations of the nature and quantity of the reaction products. Phosphate esters liberated when various phospholipid substrates were incubated with bacterial suspensions were examined in the upper aqueous phase (0.5 to 1.5 ml) prepared as above by paper ionophoresis and chromatography (Dawson, 1956; Sheltawy & Dawson, 1969). Lipid hydrolysis products were detected using thin-layer chromatography on Kieselgel F_{254} plates (Merck). The plates were first run in diethylether to remove pigments and apolar lipids to the solvent front and then in chloroform-methanol- NH_3 (sp. gr. 0.880)- H_2O (110:50:3:5, by vol.) to separate the phospholipids. Phosphorus-containing lipids were detected by autoradiography or the spray reagent of Vaskovsky & Kostetsky (1968).

^{32}P -radioactivity was determined by carefully digesting either the cut-out paper spots or the scraped-off silica gel spots with perchloric acid (1.2 ml). The digest was diluted to 9.3 ml with water and counted by Cerenkov radiation in a Nuclear Chicago liquid scintillation

counter (Unilux 11). A portion of this solution was then removed for micro-determination of phosphorus as described by Bartlett (1959).

Isolation of bacteria from rumen fluid. Isolations were achieved by the use of highly-reduced media capable of supporting the growth of obligately-anaerobic, rumen bacteria. The preparation, distribution, inoculation and incubation of such media were all carried out by the open-tube technique under an atmosphere of sterile oxygen-free carbon dioxide (Hungate, 1969; Latham & Sharpe, 1971). Although a number of different media and techniques were used in an attempt to isolate organisms possessing considerable phospholipase activity, the most active bacterial isolates were obtained using the selective medium described by Hobson & Mann (1971). This medium contained emulsified linseed oil droplets as the only energy source. Rumen fluid was removed from a pasture-fed sheep and centrifuged at 17400 g for 20 min to remove large micro-organisms. The supernatant containing the smaller bacteria, which initial experiments had shown to be a rich source of phospholipase activity, was diluted serially using the complete medium minus linseed oil as diluting fluid. Portions (0.5 ml) of each dilution were used to inoculate roll bottles containing 5 ml of medium prepared as described by Hobson & Mann (1971). After incubation at 30 °C, colonies were picked from the medium in the manner described by Hungate (1969) and Latham & Sharpe (1971) and inoculated into broths of the above medium and a non-selective medium containing rumen fluid and a number of different carbon sources (medium 2; Hobson, 1969). Phospholipase activity of the isolates was scanned by growing them in the non-selective medium containing a portion of sonicated ³²P-yeast lecithin (prepared as above) at a final concentration of 5 µg P/ml broth. After a period of incubation well in excess of that required to produce maximum turbidity, the tubes were assessed for lecithin breakdown as described under Phospholipase assay. Isolates possessing phospholipase activity were maintained on slopes of the non-selective medium containing agar (2 %, w/v) and 0.5 % (w/v) glucose. The inoculated slopes were incubated for 48 h at 39 °C and then stored at 3 °C for 4 weeks, when they were again subcultured. The purity of isolates was checked by examining gross morphology of successive subcultures by Gram-stained smears. Contaminated cultures were purified by rediluting them in roll tubes and picking single colonies for culture in broth.

On the basis of these tests, a Gram-negative rod designated LM8/IB was selected for further examination since it consistently hydrolysed 80 to 100 % of a lecithin substrate under the test conditions.

Culture conditions used for examining phospholipase activity. The organism LM8/IB was grown in non-selective liquid medium 2 (Hobson, 1969) modified so that the rumen fluid was replaced by a mixture of volatile fatty acids and haemin at levels which, according to Caldwell & Bryant (1966), simulated the concentration of these substances in a medium containing 20 % (v/v) rumen fluid. The fermentable sugar was glucose (0.5 %, w/v); the required low redox potential was achieved by including 0.1 % (w/v) cysteine-HCl and the buffering capacity was increased by adding 1 % (w/v) NaHCO₃. The medium (200 ml) was prepared in 250 ml Erlenmeyer flasks with optically-matched side arms, and inoculated from a culture which had been grown to maximum turbidity and stored at 3 °C for 3 h before use. The flask was incubated at 39 °C in an orbital incubator (Gallenkamp Ltd). Growth was measured by tipping some of the bacterial suspension into the side arm and assessing the turbidity in an EEL portable colorimeter (Evans Electroselenium Ltd), at 530 nm. Standard curves relating the turbidity of liquid cultures of the organism LM8/IB to bacterial N and dry weight were linear over the ranges 0 to 0.12 mg N/ml and 0 to 1.0 mg dry weight/ml, respectively. Portions (5 ml) of the bacterial suspension were removed

aseptically, centrifuged at 2000 *g* for 10 min and the supernatant removed and stored under CO₂. The bacteria were washed by suspending in cysteine-containing buffer and centrifuging, and then resuspended in 5 ml of the same buffer for incubation with the phospholipid substrate (see above).

Identification of isolated bacterium. The biochemical tests employed for identification purposes were based on those described by Hobson (1969) with the basal medium 2 being varied in composition according to the requirements of each test. Single carbohydrates (0.2 %, w/v) or starch (0.5 %, w/v) were added to the medium without agar. Cellulose digestion tests were carried out according to the two procedures described by Hobson & Mann (1971). Volatile fatty acid production was measured in medium 2 devoid of agar, rumen fluid and all carbohydrates except glucose (0.5 %, w/v). Volatile fatty acid utilization was measured in the same medium to which rumen fluid had been added. Volatile fatty acids were analysed by g.l.c. after growth for 48 h at 39 °C. A portion of the culture medium was mixed with 20 % (v/v) of 6 M-HCl and 5 µl applied to a column of 20 % (w/w) Tween 80 coated on 100 to 120 mesh Gas-chrom Z (Applied Science Laboratories). Separation was carried out at 140 °C with argon as carrier gas (30 to 40 ml/min) in a Pye Unicam series 104 chromatograph with a flame ionization detector. Ethanol and hydrogen production were measured in the complete medium 2 minus agar after growth of the organism at 39 °C for 24 h. Ethanol was determined by g.l.c. as above, except that a column of Chromosorb 102 (Phase Separations Ltd) was used and a temperature of 145 °C. A sample of the gas phase was injected into a Pye Unicam series 104 chromatograph fitted with a Katharometer head. The column packing used was molecular sieve (MS 5A; Pye Unicam Ltd), with N₂ at a flow rate of 40 ml/min as the carrier gas and 18 °C as the operating temperature. On such columns, H₂, CH₄ and O₂ are readily separated although CO₂ is irreversibly absorbed.

RESULTS

In initial experiments it became apparent that the phospholipase activity present in consecutive, similar batch cultures of the organism LM8/1B was extremely variable. Eventually it was found that to obtain more or less reproducible levels of activity, it was necessary to assay the enzyme at a specific stage of growth and also to supplement the incubation medium with an activator such as free fatty acid or sodium dodecylsulphate. In addition, all attempts to store the active washed bacteria or culture supernatant at low temperatures or with enzyme stabilizers such as glycerol failed to prevent a rapid fall-off in enzymic activity, which meant that for each enzyme experiment it was necessary to grow the organism and use the culture immediately.

Relationship between growth phase and activity

Figure 1 shows a typical development of the enzymic activity responsible for phosphatidylcholine degradation. Such results, which were reproduced in essential detail in a number of similar experiments, showed that the enzymic activity became apparent only during the late exponential phase of growth. It appeared to rise towards a maximum about 4 to 8 h after the maximum culture turbidity had been reached, by which time the turbidity and the bacterial nitrogen/ml of culture were decreasing, indicating that autolysis was probably occurring. Additional confirmation of this was obtained by electron microscopy, which showed that 4 h after maximum culture turbidity 10 % of the cells were degenerating or were already disrupted. Enzymic activity decreased to almost negligible levels within 20 h of the culture reaching its maximum turbidity. The greater part of the activity was associated

Fig. 1. Relationship between growth phase and phospholipase activity for a shake-flask culture. (a) and (b) show the results of two typical experiments. Cells from 5 ml portions of culture were harvested by centrifuging at 2000 *g* for 10 min at various stages during the growth of a shake-flask culture of LM8/1B, and were washed once with 0.1 M-NaOH-dimethylglutaric acid buffer pH 6.8 containing cysteine (10^{-2} M) and EDTA (10^{-3} M), before being resuspended to 4 ml volume in the same buffer. After equilibration at 39 °C under a CO_2 gas phase, the cells were transferred to tubes containing yeast [^{32}P]phosphatidylcholine (2 μmol), potassium oleate (2 μmol) and a glass bead. Each tube was gassed with O_2 -free CO_2 , vortexed for 30 s to disperse the substrate and incubated at 39 °C for 2.5 h. Phospholipase activity of: ■, whole culture; ○, washed cells; ▲, cell-free culture supernatant; ●, turbidity.

with the whole bacteria but there was always a small, but variable, amount of activity found in the supernatant. This latter activity could not be sedimented by centrifugation (30 min at 96000 *g*) so it would appear to be truly soluble and not associated with distinct fragments of bacterial membrane. Many attempts were made to liberate soluble enzyme from late exponential phase cells but none of these were successful. The methods tried included ultrasonic disruption, passage through a French pressure cell, and the lysis of both growing and autolysing cells, either by suspension in a suitable buffer and re-incubation at the growth temperature or by the addition of butanol (Hayaishi & Kornberg, 1954) followed by re-incubation. As a result of the instability of the enzyme and the difficulty in solubilizing the bulk of the activity from the bacteria, subsequent experiments were confined to washed cells harvested from cultures 4 h after the attainment of maximum turbidity and these were incubated with substrate as quickly as possible (up to 20 min after washing).

Nature of the enzymic products formed from phosphatidylcholine

The major water-soluble ^{32}P -labelled product formed by the breakdown of ^{32}P -phosphatidylcholine co-chromatographed on paper with a glycerylphosphorylcholine standard in two solvent systems: phenol saturated with water-acetic acid-ethanol (100:10:12, by vol.), R_F 0.93, and ethanol-ammonia (sp. gr. 0.880)- H_2O (7:3:1, by vol.), R_F 0.54. No phosphorylcholine or glycerophosphoric acid could be detected, although a small percentage of the total water-soluble radioactivity was present as inorganic P. Lipid-soluble ^{32}P -radio-

activity examined by t.l.c. resided chiefly in unchanged phosphatidylcholine (R_F 0.22), although a small proportion of a slower moving spot (R_F 0.06), co-chromatographing with a lysolecithin marker, was always present.

Breakdown of phosphatidylethanolamine and phosphatidylinositol

Both ^{32}P -labelled phosphatidylethanolamine and phosphatidylinositol were broken down to water-soluble products when incubated with the washed, whole-cell fraction from a stationary-phase batch culture. The breakdown of phosphatidylethanolamine was never complete; for example, in a typical experiment with $10\text{ }\mu\text{g}$ phosphatidylethanolamine P/ml and $0.57\text{ mg dry wt cells/ml}$, 20 % of the lipid P had been rendered water-soluble after 1 h and this had only increased to 26 % after 3 h. Somewhat more substantial breakdown (75 % in 1 h) was obtained in incubations in which the cell to phospholipid concentration ratio was increased threefold. Paper chromatography of the water-soluble ^{32}P -products in phenol saturated with H_2O -ethanol-acetic acid (100:12:10, by vol.) showed that initially the major water-soluble product was ninhydrin-reacting and co-chromatographed with a glycerylphosphorylethanolamine standard (R_F 0.64). Subsequently glycerophosphoric acid (R_F 0.29) and inorganic phosphate (R_F 0.14) appeared in the fraction, indicating that liberated glycerylphosphorylethanolamine was being further hydrolysed by the actions of phosphodiesterase and phosphomonoesterase. In occasional incubations, there also appeared a minor water-soluble ^{32}P -spot in the region of phosphorylethanolamine. The mechanism of its formation is unknown, although phosphorylethanolamine has been identified as a minor product in the degradation of phosphatidylethanolamine by a rumen ciliate protozoon, *Entodinium caudatum* (Coleman *et al.* 1971). Thin-layer chromatography of the lipid fraction obtained after incubation with the present organism indicated that although lyso-phosphatidylethanolamine had been formed there were also a variety of *N*-acylated products formed by transacylation.

In further experiments [^{32}P]phosphatidylinositol was incubated with LM8/1B cells for 4 h. Only 8 % of the substrate was broken down into water-soluble ^{32}P , and the product appeared to be entirely inorganic phosphate. No lipid component apart from the original substrate could be detected in the chloroform-soluble fraction.

Stimulating effect of fatty acid and sodium dodecylsulphate on enzymic activity

Because of the reported activation of phospholipase A from various sources by fatty acid (Epstein & Shapiro, 1959; Subbaiah & Ganguly, 1970; Waite *et al.* 1969) and sodium dodecylsulphate (Bangham & Dawson, 1959; Dawson & Hauser, 1967), these substances were tested in the present system. The addition of potassium oleate to the incubation medium produced a 7- to 10-fold stimulation of lecithin breakdown. The liberation of both lyso-lecithin and glycerylphosphorylcholine reached maximum values when the oleate was added in approximately equal molar amount to that of the substrate: higher ratios caused inhibition (Fig. 2). The breakdown of phosphatidylinositol was stimulated by equimolar oleate only twofold while that of phosphatidylethanolamine was 50 % inhibited.

Sodium dodecylsulphate added at a level of $0.5\text{ }\mu\text{mol}/\mu\text{mol}$ phosphatidylcholine substrate was a potent activator of phospholipid breakdown (8- to 10-fold): higher concentrations caused an inhibition of the activity. With a detergent:substrate molar ratio of 5, negligible activity was observed.

Fig. 2

Fig. 3

Fig. 2. Effect of varying concentrations of potassium oleate on phospholipase activity. For each incubation, cells from 10 ml portions of culture (5.7 mg dry wt cells) were harvested by centrifugation 4.25 h after maximum culture turbidity and washed once with 0.1 M-Na₂HPO₄-NaH₂PO₄ buffer pH 6.8 containing cysteine (10⁻¹ M) and EDTA (10⁻³ M) before being resuspended to 4 ml volume in the same buffer. After equilibration at 39 °C, the suspensions were transferred to tubes containing yeast [³²P]phosphatidylcholine (2 µmol) dispersed by vortexing for 30 s in 1 ml buffer to which had been added varying amounts of potassium oleate. Incubations were for 3 h at 39 °C under a gas phase of O₂-free CO₂. ■, Lysolecithin³² released; ▲, water-soluble phosphorus released; ●, lysolecithin + water-soluble phosphorus.

Fig. 3. Effect of cysteine and oxygen on phospholipase activity. For each time-course study, cells from 20 ml culture (17 mg dry wt cells) were harvested by centrifugation 4 h after maximum culture turbidity, and washed once in the appropriate buffer plus the additions described below before being resuspended to 16 ml volume in the same buffer. After equilibration at 39 °C, cell suspensions were transferred to tubes containing yeast [³²P]phosphatidylcholine (240 µg P) which had been dispersed in 4 ml of 0.1 M-dimethylglutaric acid buffer pH 6.8 with 10⁻³ M-EDTA and cysteine added as indicated. Incubations were carried out at 39 °C with the indicated gas passing through continuously. Samples (1 ml) were withdrawn and treated with 20 volumes of chloroform-methanol (2:1, v/v). ▲, Air and cysteine (10⁻² M); ■, O₂-free N₂; ●, O₂-free N₂ and cysteine (10⁻² M); ○, CO₂-N₂ (1:19, v/v) and cysteine (10⁻² M).

Effect of other possible co-factors

Calcium, which is an activator for many types of phospholipase activity, did not stimulate the hydrolysis of phosphatidylcholine when added at a concentration of 10⁻³ M. The same result was found with 10⁻³ M-ferrous chloride. Cysteine at a concentration of 10⁻² M produced an appreciable stimulation of activity (Fig. 3). During a 2 h incubation, the stimulated rate of hydrolysis was not affected by varying the added cysteine concentration over the range 5 × 10⁻¹ to 1 × 10⁻⁴ M. The hydrolysis of phosphatidylcholine was very slow if a stream

Fig. 4. Effect of pH on activity towards (a) phosphatidylcholine and (b) phosphatidylethanolamine. For each incubation, cells from 10 ml portions of culture (7.1 mg dry wt cells for incubations in dimethylglutaric acid buffer and 8.8 mg dry wt cells for incubations in tris and phosphate buffers) were harvested by centrifugation 4.5 h after reaching maximum culture turbidity and were washed once with the appropriate buffer which contained cysteine (2×10^{-2} M) and EDTA (10^{-3} M) before being resuspended to 4 ml volume in the same buffer. After equilibration at 39°C , the cells were transferred to tubes containing either [^{32}P]phosphatidylcholine ($1.05 \mu\text{mol}$) plus sodium dodecylsulphate ($1.0 \mu\text{mol}$) or [^{32}P]phosphatidylethanolamine ($0.97 \mu\text{mol}$) alone, and dispersed in 1 ml buffer by vortexing for 30 s with a glass bead. Incubations were carried out at 39°C for 1.5 h under a gas phase of O_2 -free CO_2 . ●, 0.1 M-NaOH-dimethylglutaric acid buffers; ■, 0.1 M- Na_2HPO_4 - NaH_2PO_4 buffers; ▲, 0.1 M-tris-HCl buffers.

of air rather than N_2 or CO_2 - N_2 (1:19, v/v) was passed through the incubation medium, even though cysteine was present (Fig. 3). The hydrolysis stimulated by 10^{-2} M-cysteine was not inhibited by the addition of 10^{-3} M-EDTA, again suggesting that divalent metal ions are not an essential component of the system.

Effect of pH

The hydrolysis of phosphatidylcholine in a series of buffers of differing pH seemed to vary not only with the pH but also with the buffer composition (Fig. 4). Activity appeared to be maximal at a pH above 8 but since the hydrolysis had been stimulated by the addition of sodium dodecylsulphate in these experiments, it is possible that the true optimum was somewhat lower. Quarles & Dawson (1969) found that although sodium dodecylsulphate stimulated phospholipase D, the pH optimum was shifted 1.5 pH units higher than in the non-stimulated hydrolysis. The non-stimulated hydrolysis of phosphatidylcholine proved to be too slow for a determination of the optimum to be undertaken. However, using phosphatidylethanolamine as substrate, where no activation was required, the pH optimum appeared to be in the region of 6.5 to 7.5 (Fig. 4), a pH similar to that in the rumen.

Identification of LM8/1B

The organism was an anaerobic Gram-negative, capsulated rod (1 to $2 \times 0.5 \mu\text{m}$). Motility was not obvious under shake-flask batch culture conditions, but was consistently present in young cultures grown statically in a liquid medium containing 0.05 % (w/v) glucose as the only fermentable sugar, and was shown by electron microscopy to be by virtue of a single

polar flagellum. In exponential-phase, shake-flask cultures individual cells were predominantly straight with rounded ends and tended to form filaments of three or more cells, but in stationary-phase batch cultures there were no filaments and the majority of individual cells had a pronounced curvature. The isolate was not capable of using cysteine, polypeptides or water-soluble material from autolysed yeast as its sole sources of fermentable carbon since it did not grow in the basal medium, but it grew vigorously in the presence of 20 out of a total of 26 different carbohydrates. Cellulose was not digested. The principal fermentation products were butyric acid, ethanol and hydrogen. When the organism was cultured in liquid medium (Hobson, 1969; medium 2) containing rumen fluid and 0.5 % (w/v) glucose, butyric acid levels increased 6 to 10 times that in uninoculated medium, and up to 25 % of the acetic acid present in the medium was utilized. In liquid medium 2 containing 0.5 % (w/v) glucose but no rumen fluid, the isolate grew less vigorously but produced similar levels of butyric acid and about half as much acetic acid, on a molar concentration basis. There was no evidence of any absolute nutritional requirement for substances contained in rumen fluid, but growth was noticeably more vigorous in the presence of the fluid or a mixture of volatile fatty acids at concentrations similar to those in the rumen fluid. The results obtained for various identification tests were compared with the characteristics of known rumen bacterial genera as listed by Bryant (1959, 1963) and Hungate (1966). The isolate was similar to *Butyrivibrio fibrisolvens* with respect to 40 characteristics. As a consequence it was tentatively identified as a non-cellulolytic strain of *B. fibrisolvens*.

DISCUSSION

We obtained no evidence that the ability to degrade exogenous phospholipids rapidly is a widespread property among differing species of rumen bacteria. Although more than 200 isolations were made from the rumen contents of both normal and defaunated sheep on different feeding regimes, screening of these isolates for the ability to degrade added yeast [^{32}P]-lecithin under batch culture conditions revealed that only three isolates were really active in this respect. This paper has been concerned with only one of these isolates, but preliminary tests indicated that all three are similar, if not identical. This is in conformity with the limited distribution of rumen bacterial species which can hydrolyse triglycerides (Hobson & Mann, 1961) and hydrogenate unsaturated fatty acids (Dawson & Kemp, 1969b). The organism contained a phospholipase active against both phosphatidylcholine and phosphatidylethanolamine, the main phospholipids present in the herbage diet of a ruminant (Roughan & Batt, 1969), and had properties which allowed it tentatively to be identified as a strain of *Butyrivibrio fibrisolvens*, a bacterium which commonly occurs in both ovine and bovine rumens. The property of degrading phospholipids appears not to be characteristic of all rumen *Butyrivibrio* species since other isolates (donated by Mr R. W. White and Dr P. N. Hobson) were completely inactive. A decision as to the usual numbers of the present bacterium in the rumen and its importance in rumen metabolism must depend on further work.

The phospholipase A activity present in the organism produces a deacylation of the phospholipid substrate, initially liberating a lysophospholipid and a free fatty acid. The rapid appearance of a water-soluble glycerylphosphorylated derivative and minimal accumulation of lysophospholipid implies that an active lysophospholipase is also present. Phospholipase A associated with a dominant lysophospholipase has been frequently described in the membranes of other bacteria, e.g. *Mycobacterium phlei* (Ono & Nojima, 1969). *Serratia plymuthicum* (Hayaishi & Kornberg, 1954) and *Escherichia coli* (Proulx & Van Deenen,

1967; Shibuya & Maruo, 1971). We could not determine whether the present phospholipase A is specific for the fatty acid at the 1 or 2 glycerol carbons of the phosphoglyceride molecule, because of the minimal appearance of lysophospholipid and the difficulty in separating phospholipase A from lysophospholipase activity due to the instability of the enzyme preparation. Sometimes both phospholipases (A_1 and A_2) can be present in the same organism, although it is not certain whether two enzymes or a single enzyme lacking in specificity is involved (Doi & Nojima, 1973).

The present phospholipase activity shows a number of characteristics which are unusual when compared with bacterial deacylating systems previously studied. The marked activation caused by cysteine is exceptional and is possibly connected with the necessity of providing a low redox potential to prevent oxygen poisoning of the enzymes. Little enzymic activity was observed when the incubation with substrate was carried out under aerobic conditions. A slight activation of a lysolecithin deacylating enzyme in *Vibrio cholera* by glutathione and cysteine has been reported (Chattergee & Mitra, 1962) but the present effect is of an entirely different magnitude.

Most types of bacterial phospholipase A previously studied had an absolute requirement for Ca^{2+} ions. However, the present enzyme is not stimulated by addition of this divalent cation or inhibited by the addition of EDTA. In this respect it is similar to the soluble enzyme produced by *Bacillus megaterium* (Raybin, Bertsch & Kornberg, 1972). The latter enzyme is also similar to the present system in that it is greatly stimulated by the presence of anionic detergents when it is attacking a zwitterionic phospholipid. It therefore seems likely that the present enzyme system prefers a substrate-water interface which contains an excess of negative charges. This would also explain the stimulatory effect of oleic acid which is likely to enter the substrate particles and present orientated and ionized carboxylic acid groups at the interface. The lack of stimulatory action of oleic acid using a phosphatidylethanolamine substrate could possibly be due to the surface of the phosphatidylethanolamine particles already having a negative charge at a neutral pH due to deprotonization of some of the amino groups (Dawson, 1963). [Many phospholipid-metabolizing enzymes have a very precise requirement for the Zeta potential appertaining on the surface of the substrate (Dawson, 1973).

Few attempts have been made previously to equate phospholipase activity with the stage of bacterial growth, although Chattergee & Mitra (1962) found that the lysolecithinase of *V. cholera* appeared in the late exponential phase and was maintained through 30 h of stationary phase. The development of the present enzyme in shake-flask batch cultures appears to be specifically associated with autolysis of the organism, as shown by the decrease in the turbidity of the culture and by electron microscopy. The association of enzymic activity with a comparatively short phase of the growth of the organism does not preclude it playing a physiological role in degrading dietary phospholipid in a rumen environment. Hobson (1970) has indicated that the death and lysis of rumen bacterial cells, which frequently follows the cessation of rapid growth in batch cultures *in vitro*, almost certainly occurs *in vivo* also. In a similar context, Jarvis (1968) found that *Streptococcus bovis* and a species of *Butyrivibrio* were susceptible to lysis when placed in rumen contents, so it would seem that lysis could result in the unmasking of phospholipase activity *in vivo*.

Although both the intact cells and the soluble enzyme can degrade phospholipids, it is possible that the latter rapidly disappears in the rumen because active proteolytic enzymes are known to degrade certain soluble proteins added to the rumen (Mangan, 1972). A number of experiments in the present investigation have shown that ovine rumen fluid centrifuged to remove all bacteria is virtually devoid of phospholipase activity. However,

even with a short half-life the soluble enzyme could still be physiologically active. The hydrolysis of dietary triglycerides in the rumen may, in part at least, be brought about by an extracellular lipase secreted by *Anaerovibrio lipolytica* (Henderson, 1971), yet rumen fluid supernatant seems to be totally devoid of the enzyme (Garton, Lough & Vioque, 1961).

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