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In vitro lipolysis of triglycerides by bovine rumen microorganisms

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IN VITRO LIPOLYSIS OF TRIGLYCERIDES

BY BOVINE RUMEN MICROORGANISMS

by

Franklin Delano Hill

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INTRODUCTION

A major portion of the diet of the adult bovine often is derived from forages. Of particular importance are the leaves of forage crops (grasses and legumes), the dry matter of which contain 4-6% lipids made up of glycerides, waxes, sterols, and phospholipids together with the salts of phosphatidic acids. In fact, the bulk of the fatty acids in the ingested forage is in the form of various glycerides.

Although no precise information is available, it has been estimated that a cow eating 100 lb. of grass or legume daily will ingest approximately 360 g. of glycerides. Even much greater amounts are obtained from other rations such as those used in stall feeding of milk cows and in the fattening of beef animals. Lipids are present in feed-stuffs such as hay, silage, and in concentrates which may contain products of both vegetable and animal origin and often contain added fats or oils. Thus, the amount of dietary triglycerides consumed by the bovine is quite significant.

In addition to the above dietary sources, various fats or oils (i.e. lard, soybean, peanut, olive, tallow, whale and corn) have been given to ruminants for both the prevention and treatment of bloat. Although they have been used successfully in many instances, their effects have been for relatively short duration only.

Though studies have been made on the effect of fats and oils on milk yield, on blood lipids, and on nutrient digestion, the fate of the glycerides in the rumen of the bovine has not been established. Also, there is no clear explanation for the "short-time" effect of fats or oils in the cure and prevention of bloat. These observations have led to the present investigation with the objectives (a) to determine if there is degradation of triglycerides in the rumen of the bovine, and if so, (b) to study the rate at which this action occurs, (c) to separate and determine the products of this lipolysis, (d) to study the effects of various physical and chemical factors upon the rate of breakdown, and (e) to determine the origin of the enzymes involved.

LITERATURE REVIEW

Rumen Fermentation

Although a great deal of information has been written
about rumen microbial fermentation of carbohydrates and
proteins, relatively little is known at present about the
breakdown of fats in the rumen. Several excellent articles
on carbohydrate and protein fermentations have recently
appeared in the literature (1-5). Moreover, these subjects
recently have been reviewed by Oxford (6) and by Eadie (7).
These aspects of rumen digestion will not be discussed in
the present report.

Attention was first directed to the influence of the
rumen on lipids in 1951. Reiser (8) found that when lin-
seed oil was incubated in vitro with sheep rumen contents a
marked reduction in linolenic acid content of the oil took
place. He attributed this finding to hydrogenation by the
rumen bacteria. Subsequently, Willey et al. (9) found that
steers on a diet containing 5% cottonseed oil had a depot
fat containing a higher content of saturated acids and a
lower content of oleic acid than that of control animals
which received the basal low-fat diet only. Saponification
of rumen contents of goats on diets containing 10% cotton-
seed oil or 10% linseed oil gave fatty acids whose iodine

values indicated that extensive hydrogenation of the ingested lipids had taken place (10).

Shorland et al. (11) reported that linolenic acid was effectively hydrogenated; over 50% of that in pasture glycerides was converted into stearic acid in the rumen. Trans-isomers of unsaturated acids also were formed in the rumen. This latter observation substantiated an earlier report by Hartman et al. (12) that trans-unsaturated fatty acids were characteristic of ruminant deposit fats and were present in the rumen contents of sheep though none was detected in the lipids of ingested pasture.

In a detailed study of hydrogenation, Shorland et al. (13) incubated oleic, linoleic and linolenic acids (cis-isomers of plant origin) singly with sheep rumen contents for 2 days in CO_2 atmosphere. With each, about 20% of the original acid was fully saturated to yield stearic acid, and trans-isomers accounted for 17, 48, and 60%, respectively, of oleic, linoleic and linolenic acids. From both linoleic and linolenic acids, positional isomers of the former were produced which included isomers in which the double bonds were in a conjugated state and which were apparently resistant to further hydrogenation.

Another illustration of the resistance of double bonds to simple saturation by hydrogen was the finding that the carotene content of the rumen did not change during these

incubation experiments. As the authors discussed in detail, it is interesting to note that the positional and geometrical isomers that result from the microbial hydrogenation of unsaturated fatty acids are similar to those which are produced during industrial hardening of lipids by hydrogenation.

Recently, Wright (14) made a study of the type of microorganism involved in the hydrogenation of unsaturated lipids within the rumen. Washed suspensions of protozoa incubated in an acetate-bicarbonate-phosphate buffer with clover starch effectively hydrogenated both linseed oil and linoleic acid. Hydrogenation of chloroplast lipids by the protozoa was observed also. Preliminary experiments using washed rumen bacteria indicated that they did not contribute a great deal to the hydrogenation occurring in the rumen.

Subsequently, Wright (15) re-examined his work with the rumen bacteria and found considerable hydrogenation of. chloroplast lipids by bacteria in rumen juice from which the protozoa and plant material had been removed by centrifugation (1000 x g for 5 min.). Wright suggested that the negative finding previously obtained with washed bacteria was presumably the result of either harmful effects caused by handling and preparation of the suspensions or the presence of some factor in rumen fluid that facilitates hydrogenation.

In 1958, Garton et al. (16) found, in experiments in which 1 g. of linseed oil was incubated with 100 ml. of

sheep rumen contents, that not only did hydrogenation take place, as indicated by a fall in iodine value of the lipid, but lipolysis also occurred. More than 75% of the total lipid recovered at the end of incubation was in the form of free higher fatty acids and, of the lipids initially present in the rumen contents alone, 50-60% represented free higher fatty acids.

Garton et al. (16) also incubated 1 g. linseed oil anaerobically with 100 ml. of rumen contents obtained from a fistulated sheep 4 hours after feeding hay and concentrates and with the same rumen contents previously heated in a boiling water bath. Calculations showed 60% liberation of the esterified fatty acid residues from the oil. No lipolysis was observed in the heated flask. Extensive lipolysis of ingested glycerides in the intact sheep was found on examination of the rumen contents of an animal that was fed several months on a diet of hay and concentrates to which 40 g. of linseed oil were added daily. At slaughter, 7 hours after the last feed, 92% of the rumen lipids consisted of free, partially hydrogenated, higher fatty acids. No lipolytic activity was detected in the mixed saliva of the adult sheep and it was presumed that rumen microorganisms were responsible for production of the potent lipase.

More recently, Garton et al. (17) incubated 1 g. of various oils with 100 ml. of sheep rumen contents and re-

ported hydrolysis of 95, 70, and 40% respectively of lin-
seed oil (iodine value 181.1), olive oil (iodine value 80.4)
and cocoa butter (iodine value 39.9). From the results the
authors suggested that perhaps the extent of hydrolysis in
the rumen of the sheep was related to the degree of unsat-
uration of their components.

The fermentation of glycerol by rumen contents of sheep
was studied by Johns (18) who reported that it gave rise
almost entirely to propionic acid. In the living animal the
rate of fermentation was not high since almost 50% of a 180
g. dose was present in the rumen 7 hours after it had been
administered. Similar results were obtained in vitro.

Use of Oils in Treatment and Prevention of Bloat

For many years oils have been used as foam inhibitors
in the prevention of bloat. In a study of foams in 1934,
Berkman and Egloff (19) mentioned the capacity of certain
oils to serve as inhibitors. Johns (20) and Reid and Johns
(21) found that administration of vegetable oils (peanut,
olive, soybean), whale oil and emulsified tallow to cattle
before grazing on bloat-producing red clover protected these
animals from bloating for a short time. The oils were fed
in different levels (30 g. to 750 ml.) but no amount used
gave protection for periods of more than a few hours. Even
though mixing the oils with grain prolonged its effect

slightly, doses fed in the morning did not extend protection through the following morning.

During recent years a number of publications have appeared in which several methods have been used in administering oils to animals. Spraying bloat provocative pasture with oil before grazing (22, 23) has proven effective. Other methods consisted of treatment of drinking water with oils (23-25) and the addition of oils to various feed-stuffs (23, 26, 27).

Several workers have used oils effectively in the relief of bloat. Reid and Johns (21) found the following oils (at levels of between 50 and 300 ml.) to be highly effective in the treatment of serious bloat: vegetable oils (peanut, olive, soybean, and linseed), turpentine, emulsified tallow, whale oil, cream, lanolin, liquid paraffins, and paraffin-wax emulsions. By drenching with the oils, deflation of the rumen was usually complete within 30 minutes. Though no significant variations were found between the effectiveness of the different oils or between the dose rates, peanut oil and emulsified tallow appeared to be the most desirable and were without undesirable side effects. In addition to the oils given above, corn oil (28) and vegetable oil (Wesson oil) (29) have been shown to promptly reduce intraruminal pressures in bloated animals. The work of Boda and coworkers

(30) has substantiated the effectiveness of the oils in treating bloat.

Because of the brief residual effect of fats in controlling bloat (a period of less than 4 hours after administration) their use in bloat prophylaxis under pasture conditions has been limited.

Characterization of Some Lipases

Among the enzymes which hydrolyze lipids are the water soluble lipases which hydrolyze the water insoluble fat molecules. The close contact between enzyme and substrate occurs at the water-fat interface. By emulsification this area between water and oil phases is greatly increased and allows lipolysis to proceed at a speed that is known to occur in the gut, even though substrate and enzyme are in different phases.

Most evidence indicates that the mechanism involved in splitting ester linkages in triglycerides is different from that of the esters of monohydric alcohols. In addition to catalyzing the breakdown of glycerides, lipases hydrolyze simple esters of long chain fatty acids (31). On the other hand, esterases accelerate the splitting of simple esters but are ineffective on the ester linkages of triglycerides.

Although it appears that under certain conditions fat hydrolysis occurs to a minor extent in the stomach, it is not

clear whether this action is due to lipase originating in the stomach mucosa or to regurgitated pancreatic lipase from the small intestine. Hull and Keeton (32) found lipolytic activity in neutral secretions of Pavlov accessory stomachs in dogs. Willstatter and Memmen (33) suggested that lipase is secreted in an active form by the stomach withhighest concentration in the region of the cardia. The greatest activity occurred under conditions of alkalinity.

The pH of the stomach contents of monogastric animals is acidic and thus is unfavorable for the action of reported gastric lipases. Such low pH values are unfavorable for proper emulsification which so greatly increases the activity of most fat hydrolyzing enzymes. Everett (34) states that even in the infant, where the pH better approximates that of the optimum of both pancreatic and gastric lipases, less than 5% of fat is hydrolyzed in the stomach.

Regurgitation of intestinal contents may aid lipolysis, both by furnishing lipase and by producing a reaction of gastric contents near the optimum pH of the enzyme. While the gastric digestion of fats in monogastric animals is of very minor importance in itself, any free fatty acids produced would be of some importance in accelerating the rate of emulsification of the lipids upon entering the small intestine.

Pancreatic lipase (steapsin) is the most important enzyme involved in the digestion of neutral fat. In 1856, Claude Bernard (35) recognized the ability of pancreatic juice to produce emulsions and to hydrolyze fats. Pavlov (36) reported at length on the lipase present in the pancreas of the dog. The enzyme has been shown to be activated by bile, bile salts, egg albumin, soaps, saponin, and alcohols. Willstatter and co-worker (37) state that such non-specific activating agents exert their effect by affording an expecially efficient adsorption environment which facilitates contact between a water soluble enzyme and the water insoluble substrate. Glick and King (38, 39) have shown that the activating effect of a compound on lipase is proportional to the lowering in the surface tension which it produces.

Pancreatic lipase, although soluble in water, is quite unstable in aqueous solution (40). The enzyme acts most rapidly at 40°C. and at a pH between 7 and 8. It is most effective in causing hydrolysis of triglycerides and attacks diglycerides and monoglycerides to a progressively lesser degree. The mechanism of action of pancreatic lipase has recently been studied in detail by Borgstrom (41, 42) and by Mattson and Beck (43).

The intestinal walls have been shown to produce a lipase which, like pancreatic lipase, is activated by bile. This activity has been demonstrated in depancreatized dogs with

Thiry-Vella fistulas (44).

The lipolytic activity of the serum has been ascribed in part to the present of a lipase and an esterase. Hiruma (45) demonstrated the pancreatic origin of the lipase by observing an increase in blood lipase upon ligation of the pancreatic duct. Cherry and Crandall (46) also noted an increase in lipase with no concomitant rise in esterase upon ligation of the duct. The enzyme showed a marked decrease after pancreatectomy of dogs (47). Blood esterase is believed to be produced in the liver.

In experiments published in 1938, Mattick and Kay (48) found lipase activity in all samples of fresh cow's milk examined. The enzyme, called tributyrinase because of its activity on tributyrin, was shown to be quite sensitive to sunlight. This finding was later confirmed in 1946 by Kay (49).

That plants possess lipase activity was first reported by Green (50) in 1890 when he discovered such activity in germinating seeds of the castor bean plant (Ricinus communis), and also in rapes (Brassica napus annua and Brassica napus oleifera). The enzyme has been shown to be a true lipase instead of a zymogen and, although with little action on lower esters (51), it is stated to have both lipase and esterase properties (52). Willstatter and Waldschmidt-Leitz (52) found the enzyme to have a pH optimum between 4.7 and

5.0 and a temperature optimum of 35°C. In the absence of
substrate the enzyme activity was destroyed at 65°C. but in
the presence of substrate it resisted destruction at much
higher temperatures.

Lipases also have been reported in sunflower seeds (53)
and from preparations of papain (54). Singer and Hofstee (55)
obtained a soluble lipase from wheat germ which acted on
water soluble simple esters as well as the glycerides, showing
an optimum temperature of 38°C. and an optimum pH between 6.8
to 7.4.

It has long been known that microorganisms produce
lipase, and its activity has been demonstrated repeatedly
in both molds and bacteria. In 1897, Camus (56) found that
filtrates of Penicillium glaucum possessed weak lipolytic
activity on monobutyrin. This was verified in the same year
by Gerard (57), and in 1903 Garnier (58) noticed that fil-
trates of Aspergillus glaucus had appreciable lipase activity.

A recent study of lipase in the saprophytic fungus
(Aspergillus niger) and in green cheese mold (Penicillium
roqueforti) has been recorded by Shipe (59). Both enzymes
hydrolyzed tricaprylin, tricaproin, tributyrin, and tri-
propionin. Ramakrishnan and Banerjee (60) reported on the
lipase of Penicillium chrysogenum which also possessed syn-
thetic activity. Fiore and Nord (61) found an intracellular
lipase in growths of the saprophytic "imperfect" fungus that

causes flax wilt (Fusarium lini).

Extracellular lipase which retained its activity independent of formed cells was obtained from the botulinus bacillus (Clostridium botulinum) (62). In 1949, Lubert et al. (63), after studying the lipolytic activity of several microorganisms, found that Pseudomonas fluorescens had the greatest activity among those studied. In 1941, Castell and Garrard (64) studied the lipase of 40 pure cultures of bacteria. Rosenfield (65) observed the ability of sulfate-reducing bacteria to carry out anaerobic lipolysis.

A large number of papers have appeared in the literature concerning lipolytic activity of microorganisms, especially that of bacteria, involved in producing rancidity in dairy products. Among these are the works of Hammer and his colleagues (66, 67) who found that the lipolytic bacterium of Pseudomonas-Achromobacter groups cause flavor defects in butter. Nashif and Nelson (68) made a detailed study of the lipase action of Pseudomonas fragi on oils from various natural products. Goldman and Rayman (69) made an intensive investigation of hydrolysis of fats by bacteria of the Pseudomonas genus. Lipolytic powers of different strains and species were found to vary widely in both rate and extent of hydrolysis. Susceptibility of different fats to microbial hydrolysis appeared dependent upon particle size of the fat and not upon the composition of the component triglycerides.

Recently, Kushner (70) prepared cell free lipase from
Bacillus cereus.

Although no reports on lipase activity by specific
rumen microorganisms are available, work by Garton and
associates (16, 17) suggests that lipase activity in the
rumen of sheep causes substantial hydrolysis of triglycerides.

GENERAL EXPERIMENTAL METHODS

The experimental work performed in this investigation consisted of (a) collection of rumen ingesta rrom fistulated dairy animals, (b) incubation of the rumen liquid, and various microbial preparations obtained from it, with triglycerides, (c) measuring the extent of hydrolysis, and (d) analysis of the fatty acids and glycerides resulting from lipolysis of the triglycerides.

Experimental Animals

The experimental animals (Table 1) were from the Iowa State University dairy herd and were fitted with rumen fistulas. All were physiologically normal with functional rumens.

Animals on two types of rations served as the source of rumen ingesta. One group received a feed-lot ration consisting of corn, oats, soybean oil meal, bran, bone meal, and salt. Approximately two pounds of the mixture were fed twice daily at 6:30 AM and 4:30 PM. Good quality alfalfa hay and fresh water were available ad libitum. The animals were kept in individual stalls in one wing of the Dairy Cattle Nutrition Laboratory at the University dairy farm, except during warm days when they were kept as a group in an outside lot.

Table 1. Experimental animals

Animal no.	Breed	Sex	Date of birth	Ration at sampling
4491	Holstein	Male	6-21-57	Alfalfa pasture
4507	Holstein	Male	7-7-57	Alfalfa pasture
4524	Holstein	Male	8-21-57	Feed lot
4723	Ayrshire	Male	10-19-57	Alfalfa pasture
4728	Brown Swiss	Male	11-1-58	Alfalfa pasture
4772	Holstein	Male	2-24-59	Alfalfa pasture
4777	Brown Swiss	Female[a]	3-2-59	Alfalfa pasture
4799	Holstein	Male	4-27-59	Feed lot

[a]Freemartin

During the spring and summer months (mid-May through mid-September) the animals which constituted the second group were on a pasture bloat experiment. A small amount of concentrate was fed to part of the animals prior to grazing alfalfa from 7 to 10 AM and from 4 to 7 PM. At other times the animals were confined to dry lot with access to only fresh water.

Sampling and Sample Treatment

The rumen ingesta were obtained at 6-6:30 AM., except during diurnal studies, after an overnight fast and before feeding. The contents were obtained via fistula from the center portion of the rumen; a special effort was made to obtain the ingesta from the same location at each sampling. It was strained immediately through four layers of surgical gauze and poured into a vacuum bottle which previously contained warm water (approximately 40°C.). These operations were performed as rapidly as possible to prevent cooling and unnecessary exposure of the rumen liquid to atmospheric conditions.

In most experiments this rumen liquid was used as the source of microbial lipase. In the laboratory it was transferred to a large, preheated flask and, while stirring vigorously, 10 ml. aliquots were transferred to 50 ml. Erlenmeyer flasks containing substrate.

Various microbial preparations obtained from the rumen liquid also were employed for studying lipase activity. A typical experiment consisted of centrifuging the juice at 25,000 x g in a Sharples Super centrifuge and testing the supernatant for hydrolytic activity. Supernatant and re-suspended residue from centrifugation at lower speeds also were assayed. The residue was resuspended in a synthetic medium commonly employed (71) for assaying cellulose digestion by rumen bacteria.

Substrates

The substrates used for assaying the various fractions of rumen liquid for lypolytic activity were soybean oil and a mixture of soybean oil and C^{14}-carboxyl labelled tripalmitin. The soybean oil (bleached, refined, with a saponification number of 193.9) was furnished by Durkee Famous Foods, Chicago, Illinois. Less than 5 drops of 0.04N KOH in methanol were required to titrate a 0.5 g. sample, dissolved in ether-Skellysolve B (2:3), to the phenophthalein end point.

The radioactive tripalmitin was obtained from the Volk Radio-chemical Company, Chicago, Illinois. The material was alkaline washed (72) to remove fatty acids and any water soluble impurities.

Incubation

Incubation was performed at 38-39°C. in a Dubnoff Metabolic Shaking Incubator. The apparatus was fitted with two stainless steel racks with a total capacity of twenty 50 ml. flasks. One large cover, for incubation of all flasks under a single gaseous environment, and two smaller covers each of which covered 10 flasks, for incubation under different gases simultaneously, were provided. In most experiments incubation was carried out under an environment of CO_2; however, enzymic activity under nitrogen and air atmospheres also were investigated.

Analytical Procedures

Following incubation, 10 ml. of 95% ethanol were mixed with the flask contents and the flask was stoppered until extraction.

Extraction and titration

A Mojonnier procedure, similar to that used by Nashif and Nelson (68), was employed for extraction of lipids. The contents of the flasks were acidified with 25% sulfuric acid and transferred to a Mojonnier flask. After extracting twice with 30 ml. of ether-Skellysolve B (2:3), an additional 5 ml. of 95% ethanol were shaken with the aqueous layer in the Mojonnier flask. The layer was then extracted a third

time with 30 ml. of ether mixture.

The extracts of the samples containing soybean oil as the sole substrate were titrated with approximately 0.04 N KOH in methanol, using as indicator 10 drops of a 1% phenolphthalein in ethanol. Blanks, rumen liquid without added substrate, were incubated, extracted, and titrated in a similar fashion.

Separation and determination of neutral fat and fatty acids

Samples containing C^{14}-carboxyl tripalmitin were separated into fatty acid and neutral fat fractions by the method of Borgstrom (72). The combined extracts were concentrated to about 30 ml. under vacuum at 50°C. This concentrate was transferred with Skellysolve B to a 250 ml. separatory funnel.

Fifty ml. of alkaline ethanol solution, consisting of 50 parts 95% ethanol, 5 parts 1N potassium hydroxide, 44 parts distilled water, and 1 part 1% thymol blue solution in ethanol, were shaken with the Skellysolve B solution. The lower layer was drawn off into a second separatory funnel containing 30 ml. Skellysolve B and the extraction repeated.

The alcoholic layer was transferred to a third funnel, containing 30 ml. Skellysolve B, and acidified with a 1:1 dilution of hydrochloric acid. After shaking, the acidified alcoholic layer was transferred to a forth separatory funnel

and extracted again with 30 ml. Skellysolve B. The lower
layer was then discarded.

The extraction procedure was repeated from funnels 1
through 4 with two additional 50 ml. portions of the alkaline
ethanol solution.

The Skellysolve extracts in funnels three and four, con-
taining fatty acids, were combined and dried over anhydrous
sodium sulfate. After concentrating to 10 ml., a 0.2 ml.
aliquot was transferred to an aluminum planchet (3/32 x $1\frac{1}{4}$
inch). The Skellysolve was evaporated under heat lamp and
the radioactivity in the planchet was counted. A Nuclear-
Chicago Model M-5 manual sample changer with Model D-47 gas
flow counter was used. At least 1000 counts were recorded
for each sample.

The Skellysolve extracts in funnels 1 and 2, which con-
tained neutral fat, were combined, dried, concentrated and
counted in the same manner as outlined above for contents of
funnels 3 and 4.

Paper chromatography

Chromatography of the long chain fatty acids was
essentially by the procedure of Buchanan (73). Whatman No.
1 chromatography paper was cut into strips $7\frac{1}{4}$ x 24 inches.
A spotting line was drawn 3 inches from the leading edge.
Starting one inch below this line, six parallel slits ($\frac{1}{4}$ x

20 inch), separated from each other by one inch, were cut out. Thus, the strips remained fused at the bottom and top.

The paper was passed through a solution of water-washed USP heavy mineral oil in USP ethyl ether (7 g. per 100 ml.) and allowed to air dry in a hood. One tenth ml. of a solution of fatty acid in USP chloroform, approximately 0.1 g. per ml. and containing about 25,000 cpm, was streaked across the spotting line. For reference, solutions of lauric, myristic, palmitic, oleic and stearic acids (10 g. perml. of each individual acid) were applied to a strip on each chromatographic sheet. After the solvent had evaporated from the band, the paper was heated in an oven at 85-90°C. for 5 minutes to fuse the fatty acids into the paper.

Two developer solvent systems were employed. The developer for the saturated fatty acids in the present of large amounts of unsaturated fatty acids consisted of glacial acetic acid, 88% formic acid and 30% hydrogen peroxide (6:1:1). This solution was prepared just prior to use. In the chromatography of a mixture of unsaturated and saturated fatty acids on the same paper the developer was acetic acid-water (85:15).

The chromatograms were developed for 16 hours by the descending technique in 12 x 24 inch glass jars which were kept at 37°C. in a large incubator. After removal from the jars, the papers were air dried in a hood.

The strip containing the reference acids was, separated from the strips containing the radioactive fatty acids. It was immersed for 15 minutes in a 0.1% solution of mercuric acetate containing 0.5 ml. of glacial acetic acid per l. Excess mercuric acetate was removed by washing the chromatogram in running tap water for 45 minutes. After air drying the chromatogram was sprayed with a 0.2% solution of s-di-phenylcarbazide in 95% ethanol which produced purple colored bands at the site of the acids.

The strips containing the C^{14}-labelled free fatty acids were scanned for radioactivity with a Nuclear-Chicago C-100A Actigraph II with a Nuclear-Chicago Model 1620A scaler. The activity was recorded simultaneously in graph form by a Rectilinear Recording Milliammeter (Texas Instruments Incorporated) connected to the Actigraph II.

Column chromatography

The glycerides were separated into mono, di- and tri-glycerides by column chromatography (74). The reagents, with methods of purification are given below:

Ligroin (Skellysolve A) was shaken with sulfuric acid and washed with water. After drying over calcium chloride it was distilled.

Isooctane, 99.5%, was purified in the above manner listed for Skellysolve A.

Isopropyl ether was purified by washing with ferrous sulfate solution to remove peroxides. After washing with water and sodium sulfate, it was distilled.

Reagent grade anhydrous ethyl ether was used without
purification.
Absolute ethanol was distilled over magnesium.
Benzene, thiophene-free, was redistilled.
Silica gel, Davison No. 12 (200 mesh), was used as
supplied from commercial source.

Two borosilicate glass chromatographic columns, 45 cm.
long and 20 mm. in diameter, were each packed uniformly with
25 g. of silica gel. After filling, isooctane was percolated
through the columns until the silica gel was completely
wetted.

Approximately 0.2 g. of glyceride mixture, dissolved in
10 ml. of 70% benzene in isooctane, was added to one of the
chromatographic columns.

The eluant employed had the following composition:

Fractions	Composition
0-6	16% isopropyl ether in isooctane
7-12	100% isopropyl ether
13-18	70% ethyl ether in isooctane
19-25	20% ethanol in isopropyl ether

The fractions were collected in 10 ml. portions and then
transferred to previously weighed beakers. Two tenths ml. of
each fraction was transferred to aluminum planchets for
counting.

The remainder of the eluted solvent was allowed to
evaporate spontaneously and the residue in the beakers was
determined by weighing. Blank determinations were made on
the second column, using the same solvents.

RESULTS AND DISCUSSION

The Extraction Procedures

Many methods of measuring lipase activity have been
employed. One of the most popular of the older methods con-
sisted of titrating the entire test substrate after a given
reaction period. Inherent errors due to the interference of
buffers, the existence of fat and aqueous phases, and the
formation of soaps by the liberated fatty acids, especially
when the pH of the test substrate was on the alkaline side,
were unavoidable. More recent methods include measuring mano-
metrically the CO_2 liberated by the fatty acids produced by
incubating substrate and enzyme in bicarbonate solution.
Assays involving some type of extraction procedure have been
most useful. In some, the total lipids are extracted and the
fatty acids titrated in presence of neutral fat; whereas in
others the two fractions are separated and the fatty acid
fraction analyzed separately, as by titrating, weighing or
measuring radioactivity.

In the course of preliminary investigations, it was
observed that various extraction, separation, and weighing
procedures described in the literature were very tedious and
often very time consuming. A modification of the extraction
procedure used by Nashif and Nelson (68), in which acidifi-
ication of the test substrate to a pH at which all fatty

acids were in free form before extraction, was superior to most others for this investigation from a number of standpoints. By employing Mojonnier butterfat extraction flasks, smaller samples could be employed with reproducible results. Several extractions could be performed simultaneously and a number of determinations carried out in a short time. Unlike most weighing procedures, the results are available almost immediately and with a minimum of transfers and other steps which are often laborious and result in the introduction of errors.

In preliminary observations with the extraction-titration procedure; it was found that by using only 10 ml. volumes of the ether mixture (ethyl ether:Skellysolve B, 2:3) that very often rather stable emulsions were formed with the rumen liquid. By shifting to a larger volume, 30 ml., only a few emulsions were encountered and those formed were easily broken by adding a small amount of 95% ethanol and shaking the flask to cause thorough mixing. By carrying out three extractions the few emulsions encountered were without detrimental effects on the reproducibility of the results. Although the extraction procedure is by no means perfect, since some of the lower fatty acids are only partially recovered (68), the soybean oil used contained such low percentages of these fatty acids (75) as to render this discrepancy rather insignificant.

As originally found by Nashif and Nelson (68), employing two 10 ml. portions of the ether mixture for extracting of lipids in bacterial cultures and by this author in extracting rumen liquid with three 30 ml. fractions of the ether mixture (Table 2), more than 97% of added fatty acids higher than caprylic were accounted for by titration.

Table 2. Recovery of fatty acids added to rumen liquid

| Trial | Percent recovery of fatty acids | | | |
	Lauric	Myristic	Oleic	Stearic
1	97.8	99.0	97.4	99.2
2	97.2	98.4	97.1	98.6
3	--	--	--	97.5
4	--	--	--	98.4
Average	97.5	98.7	97.3	99.7

The alkaline ethanol extraction procedure of Borgstrom (72), for separation of fatty acids and neutral fat, was employed in some of the trials in which analysis of the neutral fat fraction was performed. These were mainly those in which C^{14}-carboxyl labelled tripalmitin added to soybean oil was used as substrate.

This procedure was used to determine the recovery of lauric, myristic, oleic, and stearic acids when added at

levels up to 0.5 g. to 1 g. soybean oil. In these trials
the fatty acids and soybean oil were weighed, dissolved in
30 ml. of Skellysolve B, and the solutions were transferred
to a 250 ml. separatory funnel. The extractions were carried
out according to the procedure listed under GENERAL EXPERI-
MENTAL METHODS.

Recovery of the soybean oil and fatty acids by this
technique, as determined by weighing, is summarized in Table
3. In all trials excellent recovery of both fatty acids and
soybean oil was obtained.

The combination of the Mojonnier extraction procedure
and alkaline-ethanol extraction was tested for recovery of
added lipids. In this study the oil and fatty acids were
weighed into Mojonnier flasks, 10 ml. of rumen liquid were
added and the lipids were extracted with three 30 ml. portions
of the ether-Skellysolve B mixture. Flasks containing only
rumen liquid served as blanks. The ether extracts of some of
the samples were concentrated under vacuum to about 50 ml.
and transferred to separatory funnels. Following alkaline
ethanol extraction the fatty acid and neutral fat fractions
were transferred to weighed beakers, the solvent evaporated
and the residue weighed. After making allowance for blank
determinations the percent recoveries (90 to 99%) of the two
fractions were found to be similar to those given in Table 3.

In another recovery experiment, fatty acids in the
ether extracts were titrated with 0.04N KOH in methanol and
then the ether solutions were treated as described above
(concentration, alkaline ethanol extraction, evaporation of
solvent and weighing). In four trials wherein extracts were
titrated before the alkaline ethanol separations, the average
percent recovery of soybean oil was only 79.4%. The recovery
of the fatty acids was better than that of the soybean oil
and varied between 87 and 98%. Moreover, the results of

Table 3. Recovery of fatty acids and soybean oil added to
rumen liquid

	Percent recovery of fatty acids and soybean oil				
Trial	Lauric	Myristic	Oleic	Stearic	Soybean oil
1	95.8	96.3	98.9	99.1	96.6
2	98.1	97.6	97.5	98.7	98.4
3	--	--	98.3	97.9	97.2
4	--	--	--	98.0	96.4

duplicate determinations did not agree. Evidently, the
introduction of methanol interferred with the subsequent
extraction. Thus, it was found that determination of the
fatty acid by both measuring radioactivity and titrating the

same sample led to results which were non-reproducible.
Because of this fact duplicate assays (titration and radio-
activity measurement) were not performed on the same sample.

Hydrolysis of Soybean Oil by Rumen Liquid

In the early phases of this work it was desirable to
determine if there was lipase activity within the rumen. To
accomplish this goal 1.0 g. of soybean oil was incubated with
100 ml. rumen liquid for 24 hours at 38°C. To inactivate
any enzyme which might have been present, aliquots of the
rumen liquid were heated to 90°C. for 30 minutes before addi-
tion of substrate and incubation for 24 hours at 38°C. An-
other portion of this liquid was centrifuged 30 minutes at
12,000 r.p.m. and the supernatant was incubated, as outlined
above, to investigate the possibility that the enzyme was
extracellular and independent of living organisms. Following
extraction of the lipids, the neutral fat and fatty acids
were separated and the fractions were weighed. The results
of this trial are given in Table 4.

Neither the heated nor the centrifuged liquid exhibited
lipase activity, whereas part of the fat incubated with un-
treated liquid definitely showed some hydrolysis to fatty
acids. Centrifugation of rumen liquid decreased levels of
both the extractable neutral fat and fatty acids. Apparently
part of the lipids which were extracted from untreated rumen

32

Table 4. Hydrolysis of soybean oil by rumen liquid

Rumen liquid treatment	Soybean oil	Recovery (g.)	
		Neutral fat	Fatty acids
None	None	0.3397	0.1670
None	1.0 g.	0.8627	0.5345
Heated	None	0.2388	0.1695
Heated	1.0 g.	1.3422	0.1367
Centrifuged	None	0.1031	0.0243
Centrifuged	1.0 g.	1.1358	0.0146

liquid remained with the residue during centrifugation. Thus, it appears that unlike the lipase production by many other microorganisms (56, 57, 58, 68) the lipase of the bovine rumen is not an extracellular enzyme but is in some manner concerned with the internal metabolism of living microorganisms.

Since lipase activity was assayed by two different methods, it was desirable to compare results from both procedures using the same enzyme preparation. For this purpose C^{14}-labelled tripalmitin (61,500 c/m) was added to incubation flasks containing 0.1, 0.2, 0.3, and 0.4 g. soybean oil. Flasks containing the same levels of soybean oil only, also were prepared. Ten ml. rumen liquid from animal No. 4799,

which was on the feed-lot ration, were added to each flask and all flasks were incubated for 24 hours at 38°C.

Percent hydrolysis of the soybean oil as determined by extraction and titration is given in Table 5. The results of duplicate samples are averaged in the column at the right. The weight of substrate added and the volume of base required for titration are also listed.

Percent hydrolysis of substrate as measured by level of radioactivity in the fatty acid fraction is given in Table 6. The first two samples contained no soybean oil. Total recovery of neutral fat and fatty acids is given in the column to the extreme right.

The results obtained from the two different procedures are summarized in Figure 1. Curve A represents percent hydrolysis of substrate obtained by titration of fatty acids, while Curve B gives values obtained by counting the radioactivity in the fatty acid fraction.

Hydrolysis as measured by titration (Curve A) averaged about 15% higher than that measured by counting the C^{14} activity. The discrepancy in the results of the two methods is probably due mainly to two factors: (a) a slower attack on the solid, saturated tripalmitin molecule, although an attempt was made to have the solid dissolved or dispersed throughout the soybean oil, and (b) absorption of the β-radiation from C^{14}-labelled palmitic acid by the oily fatty

Table 5. Hydrolysis of various levels of soybean oil by rumen liquid

Trial	Soybean oil (g.)	0.0470N KOH (ml.)	Hydrolysis %	Average %
1	--	27.35	--	--
2	--	27.45	--	--
3	0.1063	35.55	92.4	--
4	0.1013	34.75	87.1	90.1
5	0.2075	40.65	77.0	--
6	0.2053	40.82	78.9	78.0
7	0.3017	46.85	77.7	--
8	0.3041	46.66	76.3	77.0
9	0.4023	49.05	64.9	--
10	0.4027	49.60	66.5	65.7

Table 6. Hydrolysis by rumen liquid of C^{14}-labelled tripalmitin mixed with various levels of soybean oil

Trial	Soybean oil (g.)	Fatty acids		Neutral		Total % Recovery of C^{14} activity
		Activity (c/m)	Recovery (%)	Activity (c/m)	Recovery (%)	
1	--	45,615	74.1	6,370	10.4	84.5
2	--	47,360	76.9	7,035	11.4	88.3
3	0.1069	49,435	80.3	2,800	4.6	84.9
4	0.1121	47,320	76.9	2,035	3.3	80.2
5	0.2058	41,480	67.4	6,580	10.7	78.1
6	0.2059	43,230	70.3	7,915	12.9	83.2
7	0.3034	30,365	49.4	11,460	18.6	68.0
8	0.3076	33,350	54.2	14,460	23.5	77.7
9	0.4055	27,615	44.9	18,355	29.8	74.7
10	0.4029	28,510	46.3	18,800	30.6	76.9

Figure 1. Hydrolysis of various levels of soybean oil as measured by titration (Curve A) and by counting C^{14} activity (Curve B)
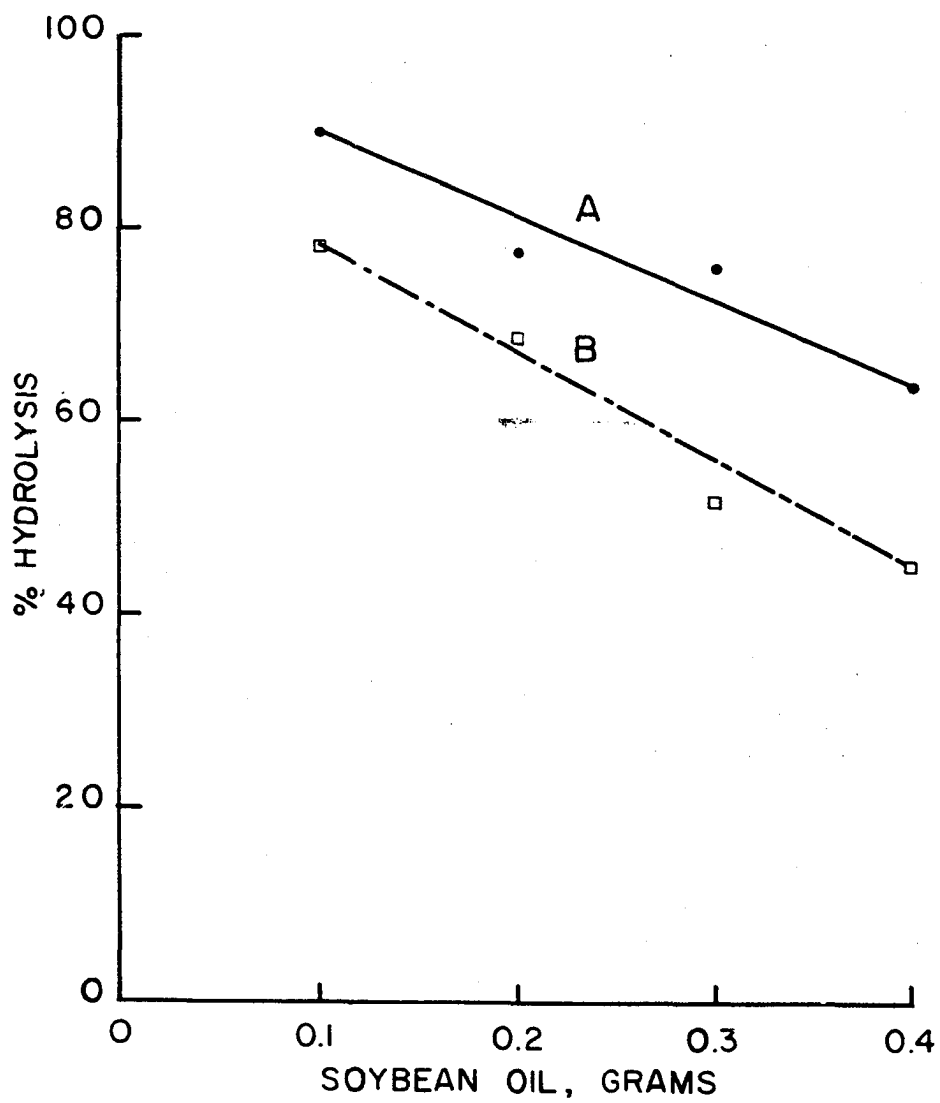
acid material liberated by hydrolysis of the soybean oil.
Support for the latter explanation is indicated by the fact
that the smaller the level of soybean oil substrate the
closer the curves approach each other. Since the total per-
cent recovery of radioactive materials at the 0.1 g. soybean
oil level (Table 6) is less than the percent fatty acids
obtained by titration, at this same soybean oil level, this
factor (absorption of β -radiation) could not alone account
for the differences obtained in the two methods.

That the first explanation probably accounts for part
of the discrepancy is supported by the findings of Garton et
al. (17) that the extent of hydrolysis of several substrates
by sheep rumen contents was related to the degree of un-
saturation of their components. It is also possible that
rumen microorganisms utilize very small amounts of fatty
acids and that saturated acids may be used in preference to
their unsaturated analogues.

The maximum total recovery of radioactive material was
obtained when no soybean oil was added, and as the level of
oil increased the total recovery decreased. This indicates
that one or more of three factors is/are responsible.
Firstly, radiation absorption becomes a factor. A second
explanation is that fatty acids may be utilized to a limited
extent. Finally, large amounts of monoglycerides may be

produced and not completely extracted into the Skellysolve layers.

Factors Affecting Rumen Lipase Activity

Time of incubation

The effect of time of incubation upon the appearance of free, higher fatty acids is summarized in Figure 2. The ingesta liquid was obtained on 3 different days over a 13 day period from animal No. 4507 on alfalfa pasture. The pH of the liquids at the various samplings were 6.65 (6-29-59), 6.45 (7-7-59) and 6.45 (7-10-59). All samples contained 0.6 g. soybean oil and the incubation time at 38°C. ranged from 4 to 36 hours. Each point represents the average of duplicate titrations.

During the first 4 hours an increase in the amount of acids appeared in the blanks of two of the trials. Although the exact reason for this increase is not known, two alternate proposals are advanced. It is possible that in addition to hydrolysis of added substrate, hydrolysis of the plant lipid (primarily chloroplast lipid) also occurred. The second possible origin of the fatty acids is from rumen micro-organisms. Protozoa have been seen to ingest bacteria (76) and it is possible that protozoal lipase attacks lipids present in the bacterial cell.

As the time of incubation increased there was a definite decrease in the titratable fatty acids in extracts of the blanks. Since this investigation will show later that oleic and palmitic acids are not utilized to any major extent

Figure 2. The effect of time of incubation upon the appear-
          ance of fatty acids in blanks and in samples con-
          taining soybean oil
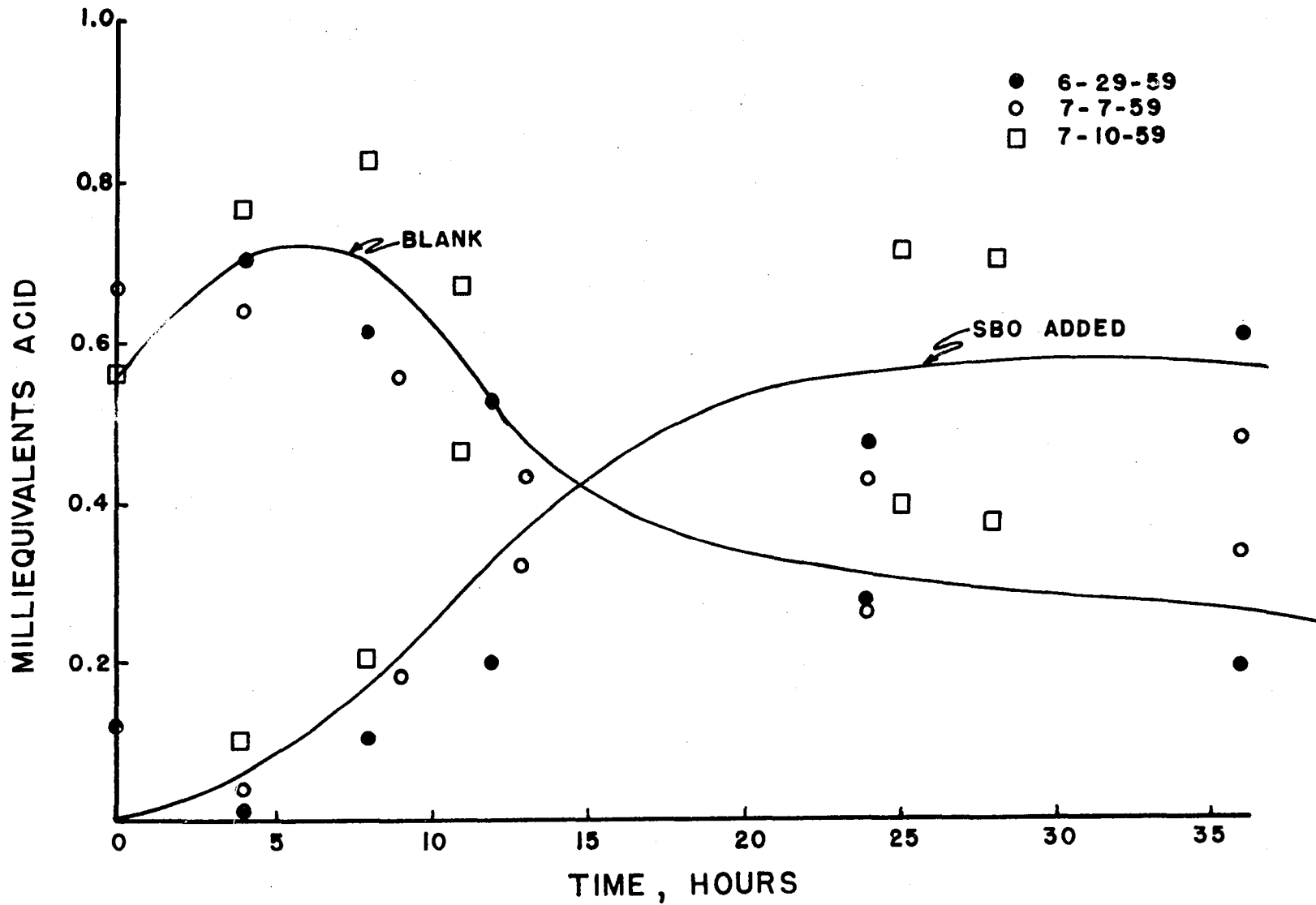
during incubation with rumen liquid, the nature of the change observed above is not understood. However, it is possible that rumen microorganisms use or metabolize higher fatty acids other than palmitic or oleic.

The lipase activity seems to be essentially linear between 4 and 18 hours. This is indicated more clearly by observing individual experiments. It is felt that the initial lag period observed in most experiments was due to establishment of environmental conditions favorable to enzyme action, such as anaerobic conditions or emulsification of substrate. After a 24-hour period the production of fatty acids leveled off. This is probably due to any of the following factors: dying of the microorganisms, inhibition of the lipase by inactivation, or the appearance of toxic substances in the reaction medium.

There was little change in either the titratable acidity of the blanks or the hydrolysis of soybean oil after 24 hours. A reaction period of 24 hours at 38°C. was chosen to compare different lipase preparations even though this portion of the curve deviated considerably from a straight line function.

The effect of time of incubation under CO_2 upon the hydrolysis of two levels of soybean oil is presented in Figure 3. Rumen ingesta were obtained from animal No. 4799 in the morning before it consumed dry feed. Ten ml. of the strained liquid were incubated with 0.1 g. soybean oil to

Figure 3.   The effect of time of incubation upon hydrolysis
            of C^{14}-tripalmitin mixed with 0.1 g. soybean oil
            (Curve A) and upon the hydrolysis of 0.2 g. soy-
            bean oil as determined by titration (Curve B)
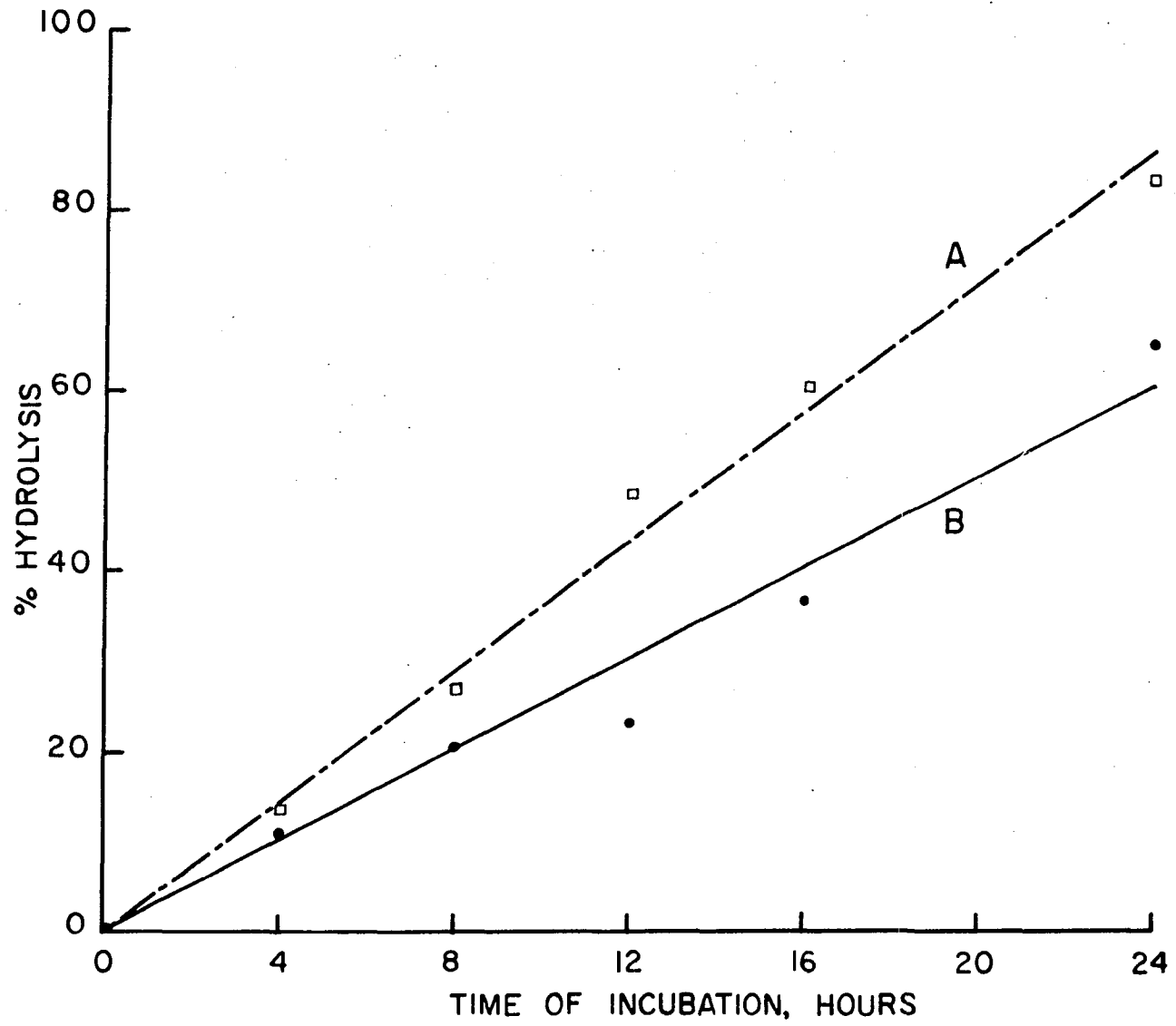
which C^{14}-labelled tripalmitin (61,500 c/m) was added.

Results obtained by counting the fatty acids produced are

given by Curve A of Figure 3. Another series was performed

in which the same ingesta were incubated with 0.2 g. soybean

oil and the extent of hydrolysis calculated from titration

data. This series is summarized in Curve B of Figure 3.

It is noted that even though different levels of neutral

fat were used in the two series and that the assay methods

were different, the degree of breakdown increased linearly

with time throughout the 24 hours, of observation. It is

probable that a plateau, as observed previously, would have

occurred beyond the 24 hour period. The only difference

between this trial carried out under CO_2 and the earlier one

carried out without CO_2 was the short lag period in the

latter. This lag period was probably due to establishment

of anaerobic (CO_2) conditions resulting from fermentations

by the microorganisms. That certain rumen protozoa produce

much CO_2 endogenously has been shown (77, 78) and it is

likely that a short time was needed in the earlier experi-

ment to produce this gas and to establish conditions for

maximum lipase activity.

C^{14}-Labelled palmitic acid   In the trials in which

radioactive tripalmitin was added to soybean oil as sub-

strate (Curve A, Figure 3) the fatty acids obtained after

12 and 24 hours incubation were separated by paper chromato-

graphy according to the procedure of Buchanan (73) described under GENERAL EXPERIMENTAL METHODS. The acids represented, respectively, 49 and 84% of the added radioactivity.

Scanning the strips revealed only a single peak subsequent to development both with the oxidizing and non-oxidizing developers. Since no faster moving components were observed under oxidative conditions, it is assumed that no dehydrogenation or oxidation of the hydrolyzed product was obtained. Furthermore, the acid was not converted to higher or smaller analogues containing the C^{14} label since any formed would have appeared as different peaks on the chromatograms. Although several investigations (8-16) have confirmed the fact that hydrogenation of various unsaturated compounds takes place in the rumen, no researchers have reported desaturation of saturated compounds. These limited observations reported herein indicate that no major dehydrogenation of palmitic acid occurs in the rumen and that the carboxyl group of the acid does not appear in other higher fatty acids upon incubation of the triglyceride with rumen liquid.

Glyceride recovery   The relative concentrations of tri-, di- and monoglycerides remaining in the neutral fat fractions after various incubation times were determined. The recovered neutral fat after 4, 12, and 16 hours incubation (Curve A, Figure 3), represented, respectively, 78.1,

55.0, and 43.1% of the added radioactivity and was separated by column chromatography (74).

The results obtained by counting radioactivity and weighing the residue of each fraction are summarized in Figures 4, 5, and 6. There is rather good agreement between both measurements. The peaks were obtained in the same fractions reported by Ravin et al. (74) who obtained quantitative separation of the glycerides of stearic acid. These workers found triglyceride in fractions 6 through 12, diglyceride in fractions 13 through 18 and monoglyceride in fractions 19 through 25. Highest concentrations of tri-, di- and monoglycerides were obtained in fractions 9, 16, and 23, respectively. In the present investigation if it is assumed that the different glycerides were eluted from the column in the same sequence, then the highest peak (fraction 9-10 represents triglyceride, the second (fraction 16) diglyceride and the third (fraction 22-23) monoglyceride. In the case of the C^{14} activity curve, these correspond to labelled tripalmitin, dipalmitin and monopalmitin, respectively.

An estimate of the relative amount of each of the components was obtained by drawing a perpendicular at the lowest part of the trough between the peaks and cutting out and weighing the separate curves. A summary of the relative concentrations is presented in Table 7.

Figure 4. Chromographic separation of glycerides remaining
after 4 hours incubation of soybean oil con-
taining C^{14}-labelled tripalimitin with rumen
liquid

Figure 5. Chromatographic separation of glycerides remaining
after 12 hours of incubation of soybean oil con-
taining C^{14}-labelled tripalmitin with rumen liquid
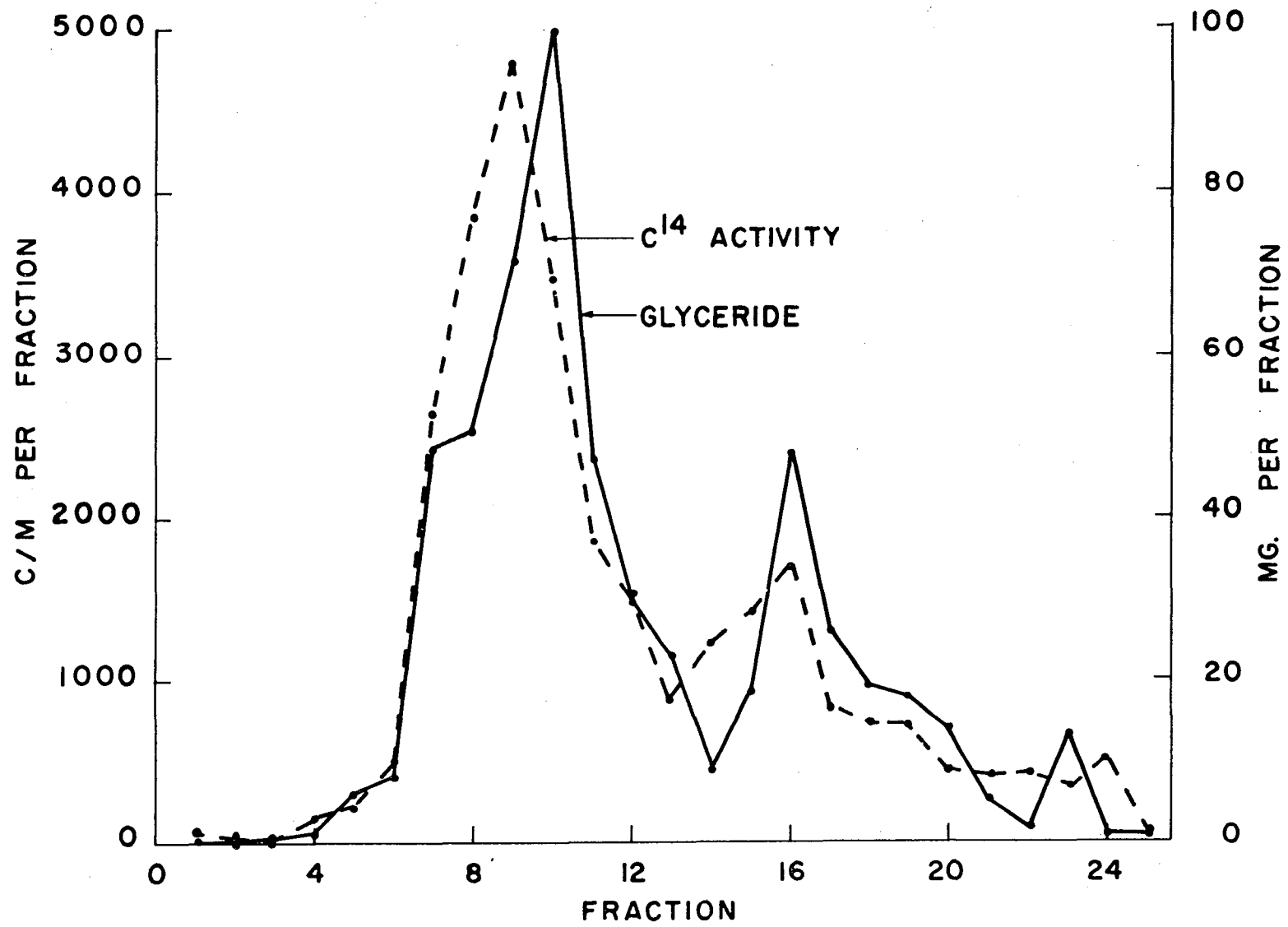
Figure 6. Chromatographic separation of glycerides remaining after 16 hours of incubation of soybean oil containing C^{14}-labelled tripalmitin with rumen liquid

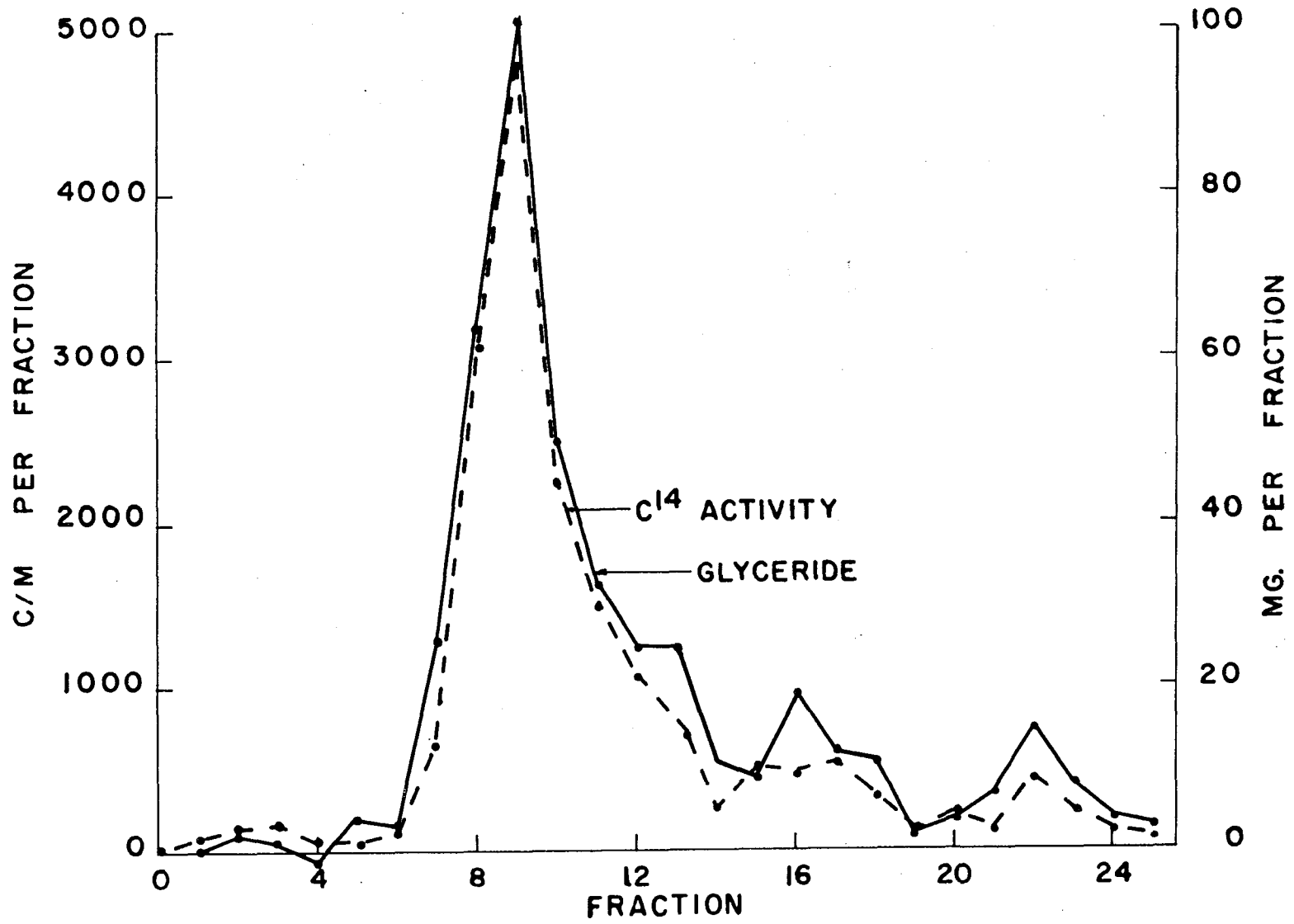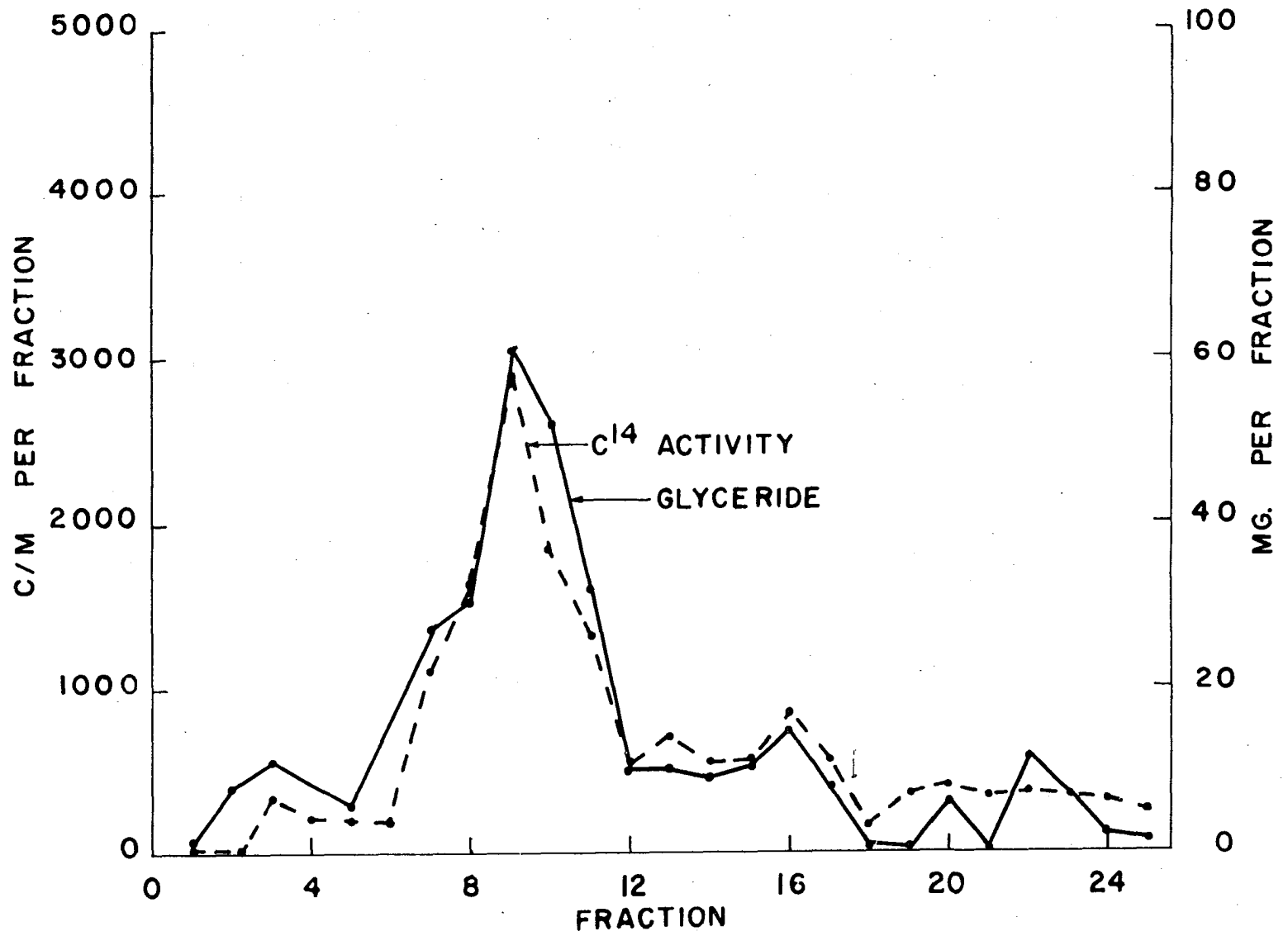Even after 16 hours of incubation the bulk of the un-
hydrolyzed glyceride was triglyceride. Relatively low con-
centrations of di- and monoglyceride were observed throughout
the incubation studies. Only at 4 hours of reaction did more
than 15% of the total fractions appear as diglyceride and
only in one case did more than 10% of the material consist
of monoglyceride. After 12 hours incubation an unidentified
component appeared in fractions 1 through 4.

The fact that the high relative concentration of tri-
glycerides did not change significantly during the observa-
tions is unlike the change found during the action of pan-
creatic lipase on triglycerides (41, 43). With the extra-
cellular enzyme a rapid decrease in triglyceride with a
corresponding increase in di- and monoglycerides occurred
during the initial reaction period. Subsequently, the di-
glyceride concentration decreased with a further rise in the
monoglyceride percentage.

The results of this investigation support the fact that
the microorganisms attack the triglyceride molecule with the
concomitant release of only very small quantities of the di-
and mono-components. This implies that the hydrolysis of a
molecule is mainly complete while the lipase activity is ex-
hibited. In intracellular lipolysis the conditions may be
more favorable for complete breakdown of triglycerides to
glycerol and fatty acids than the one or two step process

Table 7. Relative concentration of glycerides after incubation of rumen liquid with soybean oil (containing C^{14}-labelled tripalmitin)

Time of incubation (hours)	Determination	Glyceride Concentration (%)			Unidentified fraction (%)
		Tri-	Di-	Mono-	
4	C^{14} activity	67.4	25.6	7.0	--
	Glyceride (wt.)	70.3	26.4	3.3	--
12	C^{14} activity	81.2	10.5	7.8	0.7
	Glyceride (wt.)	77.0	12.8	9.4	0.8
16	C^{14} activity	65.7	14.9	14.7	4.7
	Glyceride (wt.)	69.8	12.3	8.2	8.9

with the release of di- and triglycerides as observed with
extracellular enzymes.

Oleic acid recovery   Previous reported findings in
this investigation indicate considerable hydrolysis of
glycerides in the rumen of the bovine. To determine whether
the microorganisms utilize appreciable quantities of released
fatty acid, the rumen liquid was incubated with oleic acid
and the amount recovered by extraction was measured by titra-
tion.

Rumen liquid, from steer No. 4507 on alfalfa pasture,
with a pH of 5.8 was incubated for various lengths of time
with oleic acid (0.1 g. per 10 ml.) and the extracted lipids
were titrated. The percent recovery of acid at the different
times is summarized in Table 8.

Table 8. The recovery of oleic acid added to rumen liquid

Trial	Incubation time (hours)	% Recovery
1	4	78.2
2	8	83.7
3	12	101.7
4	24	97.7

After 4 hours incubation the quantity of fatty acid
recovered was least. Subsequently, the recovery increased
and was approximately 100% at 12 and 24 hours of incubation.

These observations suggest that the microorganisms of the
rumen liquid removed or utilized in some way part of this
acid during the 4- and 8-hour incubation periods. It is
proposed that the low recovery may be caused by the transfer
of the fatty acid molecule to the surface or interior of the
microorganism and the incorporation of the acid into glyc-
erides by esterification. Subsequently, hydrolysis of the
microbial lipids could release the fatty acid, resulting in
good recovery of fatty acid equivalent to that added before
incubation. While the oleic acid molecule is within or in
contact with the microorganisms it is probable that some
change occurs and that the released fatty acid is different
than the original compound. That rumen microorganisms,
bacteria and protozoa, are able to alter unsaturated acids
has been reported recently by Wright (14, 15) wherein he
observed hydrogenation of linoleic acid. The fact that
rumen microorganisms are able to bring about this change
indicates that the fatty acid molecule probably comes in
contact with the organisms. It is postulated that while
this change occurs the fatty acid is in esterified form or
in some way is unextractable as fatty acid by the organic
extraction procedure.

pH

In an effort to determine the pH at which in vitro
lipolysis was most rapid it was considered desirable to

control pH by buffering the rumen liquid with some substance normally found within the rumen. In early studies several unsuccessful attempts were made to control pH by the addition of sodium carbonate. After six hours of reaction time the pH had usually approached its original value. Later, buffering with phosphate proved more reliable, but in most cases it was observed that the final pH of samples containing substrate was somewhat lower than the initial adjusted value, probably due to the effect of the fatty acids produced. The change in pH values for blanks was less pronounced.

Two trials on the effect of pH on the observed lipase activity will be presented. In the first trial, rumen liquid, obtained at 9:30 AM from animal No. 4491 on alfalfa pasture, was employed as enzyme solution. To 500 ml. of the rumen liquid (pH 5.9) was added 3 g. of KH_2PO_4. The pH decreased to 5.6 and aliquots, for 24-hour incubation with 0.6 g. soybean oil, were taken at this value and at values 6.0, 6.5, 6.9, and 7.3 obtained by addition of a concentrated solution of Na_2HPO_4. The degree of hydrolysis obtained at the various pH levels is summarized in Table 9.

Although only a small amount of activity was observed, there was increased breakdown of substrate with increase in pH throughout the pH range employed. Due to the low activity of the liquid in this trial, it was repeated with rumen liquid obtained before feeding from a different animal and

the lipase activity was tested over a wider range of pH

values.

Table 9. The effect of pH upon in vitro hydrolysis of soy-
bean oil by rumen microorganisms

pH	% Hydrolysis
5.6	3.8
6.0	5.2
6.5	6.2
6.9	8.4
7.3	9.9

In the second trial, the pH of the rumen liquid from

animal No. 4723, on alfalfa pasture, was 6.2 before addition

of the KH_2PO_4 and fell immediately to 5.9 upon dissolution

of the monobasic salt in the liquid to give a 0.1M solution.

Aliquots having pH values 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5

were prepared by addition of NaOH. At each pH level, with

the exceptions of pH 6.0 and 8.5, 10 ml. aliquots of rumen

liquid were transferred to duplicate flasks containing 0.5 g.

soybean oil and two containing no substrate to serve as

blanks. At pH values 6.0 and 8.5, only single determinations

were performed on each (blank and sample with substrate).

After 12 hours incubation the lipids were extracted and

the fatty acids were titrated. The percent hydrolysis at

different pH values is summarized in Table 10.

Table 10. The effect of pH upon the hydrolysis of soybean
oil in rumen liquid

| Beginning pH | pH after incubation | | % Hydrolysis |
	Blanks	Samples with SBO[a]	
6.0	6.0	6.6	40.9
6.5	6.5	6.1	38.8
7.0	6.7	6.3	47.5
7.5	6.8	6.5	45.9
8.0	7.0	6.5	45.0
8.5	7.0	6.5	41.8

[a]Soybean oil

The pH values changed slightly during incubation.
Duplicate samples had the same final pH. Apparently due to
the effect of fatty acid production by hydrolysis of soybean
oil, the final pH of samples containing substrate was lower
(except for the sample with initial pH 6.0) than that of the
blanks without oil.

High lipase activity was observed throughout the pH
range studied. Maximum activity was observed at pH 7.0 and
it appears that the most desirable value would be somewhere
between 6.8 and 7.4. As shown in previous trials, the
enzyme responsible for this action is not extracellular and
thus the pH level at which maximum hydrolysis occurred was
probably the result of a number of factors and not necessarily
the optimum for a cell-free preparation which would contain

the lipase. There may be many factors which might influence
the rate at which microorganisms perform and the balance of
the several factors need not necessarily be the pH optimum
of the enzyme. It also is possible that rumen liquid ob-
tained under different conditions show maximal activity at
slightly different pH values. This aspect of the problem
needs further study.

Level of substrate

The extent of hydrolysis of various levels of substrate
by rumen microorganisms in rumen liquid obtained from animals
on different days was investigated. The ingesta were obtained
at 6:30 AM from two animals on alfalfa pasture. Male steer
No. 4723 (two trials) and female No. 4777 (one trial) were
used. The percent hydrolysis of the various levels of sub-
strate is summarized in Figure 7. Curves A and B represent
hydrolysis of soybean oil by rumen liquid obtained on
different days from animal No. 4723. Points on Curve C
represent percent hydrolysis of soybean oil by rumen liquid
from animal No. 4777. The higher fatty acids in blanks for
Curves A, B, and C required, respectively, 1.42, 1.05, and
1.84 milliequivalents of base for titration.

If the first point on Curve A is disregarded then its
slope is similar to those in Figure 1. These are of the
type obtained under ideal conditions for percent activity

Figure 7. Hydrolysis of various levels of soybean oil by
different samples of rumen liquid. Curves A
and B represent hydrolysis by rumen liquid from
the same animal on different days and Curve C
shows hydrolysis by rumen liquid from a different
animal
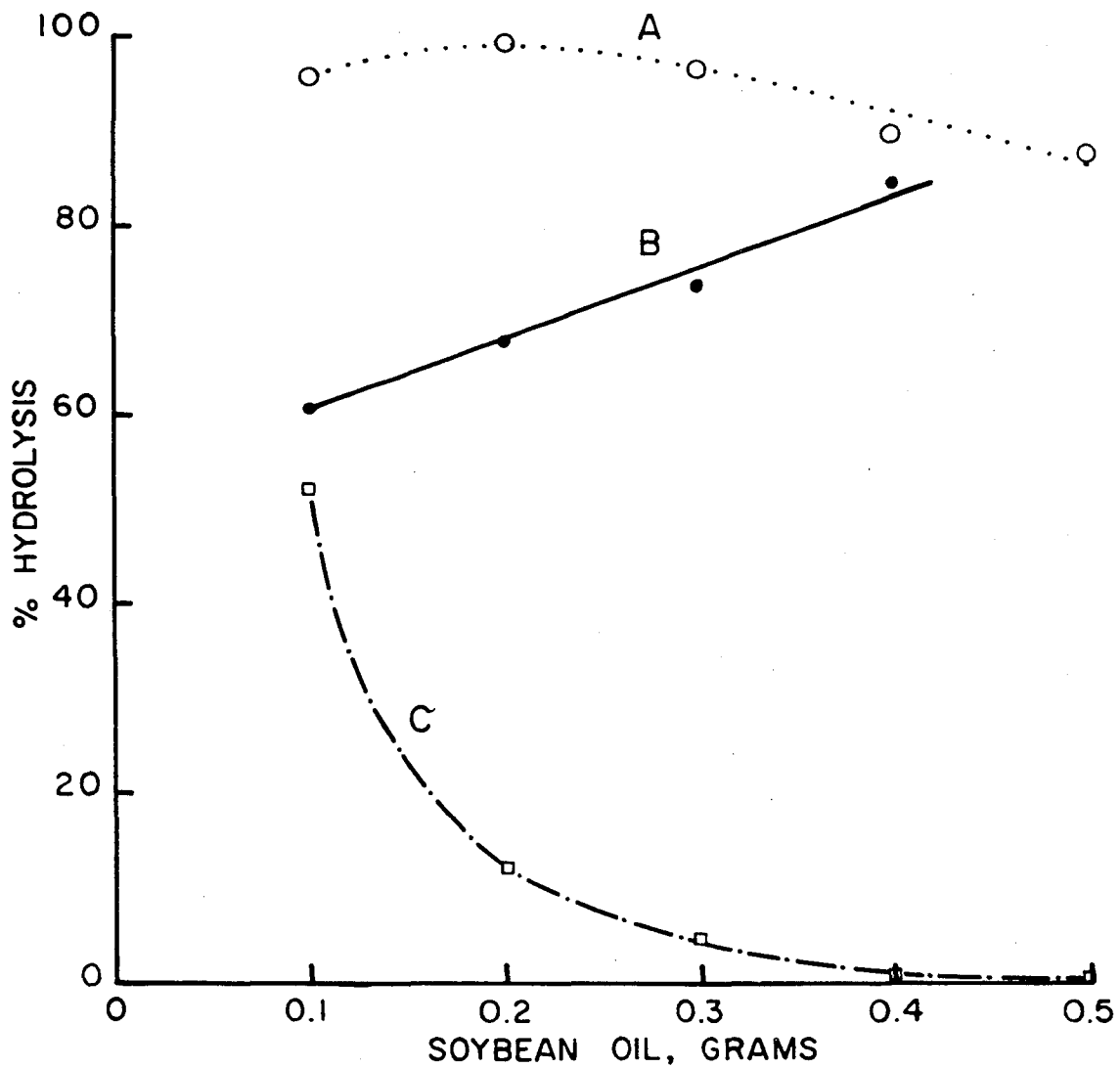
with increase in substrate concentration.

Curve B, which has a positive slope, is similar to the
type expected in enzyme induction. Although this is the
only case observed in which percent hydrolysis increased
with increase in substrate concentration, in a number of
trials the hydrolysis of radioactive tripalmitin was less
than that of the combination of the same amount of radio-
active material and 0.1 g. of soybean oil. This latter
observation, however, was attributed to the fact that in the
combination of substrates the small amount of solid radio-
active material was dissolved or dispersed throughout the
liquid substrate and in such a form was more liable to
enzymatic attack. Another factor considered was the con-
centration of calcium salts of higher fatty acids which have
been shown to activate other lipases (79).

It is probable that this hypothesis did not contribut
greatly because the higher fatty acid content of rumen
liquid was high (1.05 milliequivalents) and should have been
ample to cause enzyme activation.

Since the conditions under which the enzyme acts in
such a complex mixture are so complicated, it is felt that
no definite conclusions can be drawn from Curve B.

Curve C (Figure 7) also represents a complicated con-
dition for which a direct explanation is lacking. It is
noted that more hydrolysis occurred in the 0.1 g. samples

than in any others. At the 0.4 and 0.5 g. levels of soybean oil, essentially no hydrolysis was observed. Excluding the fact that other data in this investigation indicated the contrary, it would appear that soybean oil inhibited lipase activity in this trial. However, it is more probable that the substrate inhibited the production of other essentials, possibly carbohydrate or protein or some of their metabolic products, which the living microorganism must have for continued life and in order to hydrolyze the fat molecule. It follows then that in other observations of the degree of hydrolysis with varying substrate concentrations that this essential was present in adequate amounts. Utilization of fatty acids produced is not considered a major factor in the shape of Curve C. There are, without doubt, other postulations which one could advance but the true explanation will come to light only after further study of the observed complications.

Time of ingesta sampling

Since rumen liquid obtained at certain times of the day often showed considerably more activity than at others, it seemed desirable to study the diurnal variation in lipase activity of the ingesta.

Experiment 1   Samples of ingesta were obtained early in the morning before feeding (6:30 AM), immediately upon

conclusion of alfalfa grazing (9:30 AM), before consuming
a small amount of grain in the mid-afternoon (3:30 PM), upon
conclusion of the afternoon grazing (6:45 PM), and, finally,
later at night (9:45 PM).

Animal No. 4507 was employed as the source of ingesta
samples for two trials which were separated by a period of
9 days. Three sets of determinations were made on the rumen
liquid at each sampling time. These included (a) the free,
higher fatty acid content of samples before incubation, (b)
the free, higher fatty acid content of rumen liquid without
added soybean oil after 24 hours of incubation, and (c) the
free, higher fatty acids produced by incubation of 10 ml.
of rumen liquid with approximately 0.6 g. of soybean oil for
a 24-hour period. The pH of each sample of rumen liquid also
was measured.

The results of the two trials are given in Tables 11
and 12 and the average values are presented in Figure 8.
From the tables it is seen that even though pH values of
samples, taken at corresponding times on different days, were
slightly different the trends in these values were the same
for the two trials. High pH readings were recorded at 6:30
AM and 3:30 PM, while the lowest value observed in both
trials was at 9:45 PM. At the higher pH values the difference
between the samples without oil (blanks with and without 24
hours incubation) was greatest. Thus, 24 hours of incuba-

Figure 8. Diurnal effect on lipase activity in the rumen
liquid and on the level of free, higher fatty
acids in rumen liquid at the time of sampling
(blank, no incubation) and in rumen liquid after
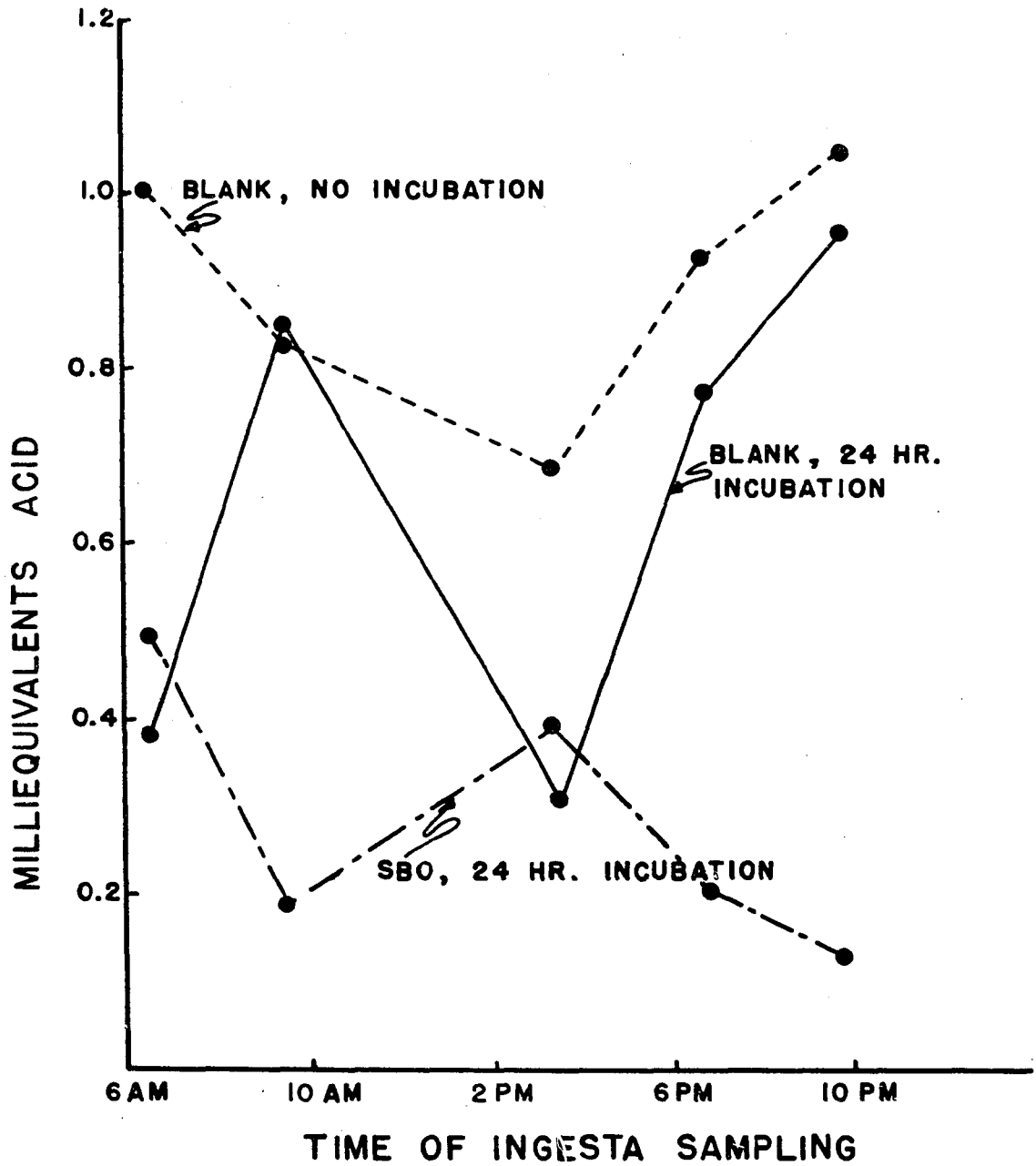incubation for 24 hours

Table 11. Effect of time of ingesta sampling on pH and
titration values for extracted free, higher
fatty acids from fresh rumen liquid

Time of sampling	Trial	pH	0.04116N KOH (ml.)
6:30 AM	1	6.4	27.04 26.38
	2	6.1	21.82 21.32
9:30 AM	1	6.2	18.06 18.04
	2	5.4	21.91 21.96
3:30 PM	1	6.5	12.80 12.92
	2	5.8	19.87 20.20
6:45 PM	1	6.0	19.55 18.80
	2	5.6	25.32 25.68
9:45 PM	1	5.8	22.14 22.45
	2	5.3	30.16 31.11

tion of rumen liquid with high initial pH resulted in a

marked reduction in fatty acid content. Incubation of

liquid with lower pH values had only slight effects upon

titrations. An inverse relationship was observed between

Table 12. Effect of time of ingesta sampling on titration
values for extracted free, higher fatty acids from
rumen liquid incubated 24 hours in the absence
or presence of soybean oil

Time of sampling	Trial	Soybean oil (g.)	0.04116N KOH (ml.)
6:30 AM	1	--	8.25
		--	9.15
		0.6413	22.18
		0.6793	23.40
	2	--	9.27
		--	10.00
		0.6127	19.15
		0.6334	19.60
9:30 AM	1	--	21.39
		--	20.89
		0.6228	24.81
		0.6269	24.02
	2	--	20.12
		--	20.27
		0.6179	26.85
		0.6215	24.91
3:30 PM	1	--	7.25
		--	6.46
		0.6213	17.95
		0.6286	17.62
	2	--	9.03
		--	7.36
		0.6235	15.88
		0.6177	16.18
6:45 PM	1	--	10.27
		--	10.21

Table 12. (Continued)

Time of sampling	Trial	Soybean oil (g.)	0.04116N KOH (ml.)
		0.6118	17.38
		0.6061	19.15
	2	--	26.92
		--	26.11
		0.6424	27.68
		0.6176	28.69
9:45 PM	1	--	15.32
		--	14.90
		0.6218	20.47
		0.6343	20.68
	2	--	31.03
		--	30.42
		0.6075	30.92
		0.6263	32.42

pH and free, higher fatty acid content of the blanks after 24 hours incubation.

The fatty acid content of the rumen liquid (Figure 8) was high in the early morning samples; it decreased pro-gressively to a low at mid-afternoon (3:30 PM) and then in-creased to a high at 9:45 PM. The number of milliequivalents of acid produced from soybean oil were highest in the ingesta liquid obtained at 6:30 AM and 3:30 PM.

Table 13 presents average values for the degree of hydrolysis of soybean oil and the corresponding average pH

Table 13. The pH and percent hydrolysis of soybean oil by rumen liquid sampled at various times during the day

	Time of ingesta sampling				
	6:30 AM	9:30 AM	3:30 PM	6:45 PM	9:45 PM
Initial pH	6.30	5.90	6.20	5.82	5.58
% Hydrolysis	22.1	8.6	18.0	9.3	6.0

of the rumen liquid at the different sampling times. The lipase activity varied directly with the observed pH of the rumen liquid. It was observed that samples obtained early in the morning and at mid-afternoon prior to feeding had highest pH and also possessed the greatest lipase activity. At other times the enzymic action obtained was appreciably smaller.

Experiment 2   A diurnal study of rumen lipase activity was also performed with rumen fluid from animal No. 4799 on the feed-lot ration. The ingesta was obtained at the same times as that employed in the previous trials from animals on alfalfa pasture. The rumen liquid was incubated under CO_2 for 24 hours in flasks containing 0.2 g. soybean oil and C^{14}-labelled tripalmitin (61,500 c/m) and the release of fatty acids was determined by counting the radioactivity.

The results of this experiment are given in Table 14. All determinations were done in duplicate and only average values are presented. As with animals grazing alfalfa pasture the rumen lipase activity in feed-lot animals vary diurnally, the highest activity occurring before feedings (both in the morning and at mid-afternoon).

Table 14. The effect of time of sampling upon in vitro hydrolysis of triglycerides

Time of sampling	Fatty acids (c/m)	% Hydrolysis
6:30 AM	43702	71.0
9:30 AM	12360	20.1
3:30 PM	45843	74.5
6:45 PM	12313	20.0
9:45 PM	13138	21.4

Experiment 3   Other diurnal studies in which samples were obtained more frequently than those in Experiments 1 and 2 are presented in Figure 9. Curves A and B represent percent hydrolysis of 0.1 g. soybean oil by 10 ml. rumen liquid obtained from animal No. 4799 on the feed-lot ration. The liquid used in Curve A was obtained on May 2, 1960 and that for Curve B 29 days later. In both trials soybean oil was mixed with radioactive tripalmitin (61,500 c/m) and the degree of hydrolysis was obtained by counting the radioactive

Figure 9.   Diurnal effect on lipase activity in rumen liquid
            from animals on the feed-lot ration (Curves A and
            B) and on alfalfa pasture (Curve C)
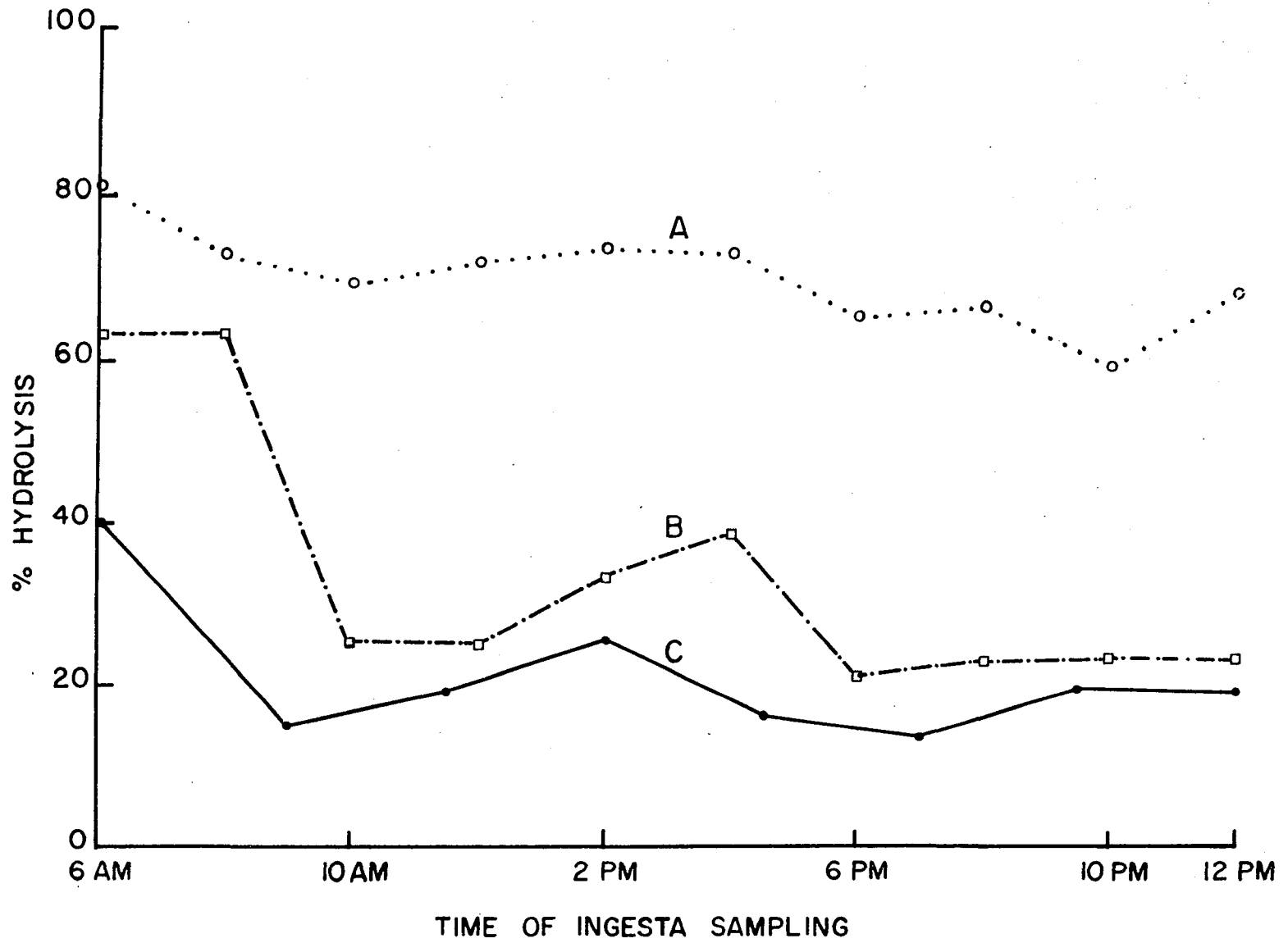
fatty acid fraction liberated by the enzyme. In the first trial the animal was maintained throughout the day in a separate stall and was fed normally, including high quality alfalfa hay ad libitum. The animal failed to consume all of its concentrate mixture; feed was present in the trough throughout the sampling period. In the second trial the animal was outside among a group of animals on the same feeding regime but in this case the concentrates were consumed immediately by all animals including the fistulated animal from which the ingesta was obtained.

There was a great deal of difference in the degree of hydrolysis, represented by the two curves, and there was more variation in Curve B than in Curve A. The only known difference in the animal on the two sampling dates was the rate and amount of concentrates consumed. It appears that concentrates or dry feeds reduce the lipase activity within the rumen, perhaps by dilution. This decrease in microbial lipase activity also could be related in part to a lowering in pH, as was discussed previously. After an overnight fast the difference in enzyme activity was less noticeable.

Curve C shows the percent hydrolysis of soybean oil by rumen liquid from animal No. 4777 for which green alfalfa pasture served as the only source of food. Ten ml. of rumen liquid were incubated with 0.5 g. soybean oil and the degree of hydrolysis of the oil was calculated from the amount of

standard base required to titrate the acids liberated. Highest values of activity were present in samples obtained before the morning grazing and also prior to afternoon grazing.

From these observations it appears that although the consumption of concentrate decreases the rate of hydrolysis of glycerides, a decrease also occurs at approximately the same times in the rumen content of animals receiving only alfalfa by grazing. Although the inhibitory effect of the latter is not as pronounced as that of concentrates, it is significant and should be considered in any studies of rumen microbial lipase. The extent to which dilution of rumen liquid, resulting from consumption of feeds, affects lipase activity needs clarification.

Gaseous environment

An experiment was designed to test the effects of incubation under air, carbon dioxide and nitrogen upon rumen lipase activity. Two samples of rumen liquid, from animal No. 4723 on alfalfa pasture, were obtained over a 3 day interval. In the first trial two samples of this liquid were incubated with and without 0.5 g. soybean oil under anaerobic (CO_2) conditions. Duplicate samples were investigated simultaneously under atmospheric conditions (that is, without any gas supplied by external source). During the second trial

the effects of incubation under carbon dioxide and nitrogen were compared.

In the first trial the average hydrolysis under CO_2 was 24% while the same enzyme preparation under normal atmospheric conditions (without gas control) hydrolyzed the oil to the extent of 25%.

In the second trial the average hydrolysis under CO_2 was 72% whereas the same lipase solution under nitrogen was effective in hydrolyzing only 24% of the same substrate.

From these observations it seems that exogenous CO_2 is not a major essential in in vitro lipolysis, using rumen liquid as the source of microbial lipase. However, this does not necessarily imply that the organisms do not require anaerobic conditions but that external provision of CO_2 is not important. It is likely that the rumen microorganisms produced an ample supply of CO_2 and thus a favorable anaerobic condition for lipolysis resulted.

In the second trial the lipase activity of a different rumen liquid sample, under identical conditions of CO_2, was much more effective than in the first case. Nitrogen decreased lipolysis by more than three fold. Thus, even though air contains a high percent of N_2, it was not inhibitory to lipase action as was observed when the reaction was performed under nitrogen alone. Assuming that intraruminal gasses affect the activity of the lipase within the rumen, then

under conditions in which CO_2 comprises the major fraction of rumen gases, triglyceride hydrolysis should be more extensive, other things being equal, than in those cases in which higher levels of nitrogen are present. Further study is needed before definite conclusions can be drawn.

The rumen of animals consuming rations which produce high levels of CO_2 and contain less nitrogen would be expected to have more lipase activity than the rumen of animals on diets wherein relatively high levels of nitrogen occur. Olson (80) has shown that the rumen of animals on legumes contains considerably more carbon dioxide and less nitrogen than that of animals eating corn or grass forages.

Location of Lipase Activity

Separation of microorganisms

To determine the type or types of microorganisms responsible for lipase activity in rumen liquid, various centrifugation studies were performed. Since typical protozoa are larger than most bacteria, most protozoa and feed particles can be removed from the lighter bacteria by differential centrifugation. Once the feed particles were removed, the state of emulsification of the added soybean oil probably would be a factor in the rate at which the microorganisms attacked the substrate. The following experiment was performed to test this hypothesis.

Rumen liquid (350 ml.) from animal No. 4723, on alfalfa pasture, was centrifuged at 2,000 r.p.m. for 3 minutes in an International Inclosed Superspeed centrifuge. Ten ml. aliquots of the supernatant and of the residue resuspended in 100 ml. of synthetic medium (71), both with and without sodium taurocholate (0.3%), were incubated with 0.5 g. soybean oil under anaerobic (CO_2) conditions for 24 hours. Aliquots (10 ml.) of the original rumen liquid with added soybean oil (0.5 g.) were incubated in a similar manner. Lipase activity was measured by titration.

The enzyme activity of each fraction is summarized in Table 15. The data represent the average of duplicate determinations on each fraction.

The supernatant was practically devoid of lipase action; less than 1% hydrolysis of the soybean oil occurred in the untreated supernatant. The addition of sodium taurocholate to the extent of 0.3% increased this breakdown to less than 3.5% compared to more than 26% by the rumen liquid before centrifugation. Resuspension of the residue in a volume of synthetic medium less than one-third that of rumen liquid from which the residue was obtained resulted in a solution which was almost three times as active as the untreated rumen liquid. However, the addition of the emulsifying agent, sodium taurocholate, to the extent of only 0.3% had a marked inhibitory effect upon the activity of this microbial and feed

Table 15. Hydrolysis of soybean oil by various fractions of
          rumen liquid

Lipase preparation	% Hydrolysis
Untreated rumen liquid	26.7
Supernatant	0.9
Supernatant + taurocholate (0.3%)	3.4
Resuspended residue	77.4
Resuspended residue + taurocholate (0.3%)	26.3

particle suspension.

The results indicate that the lipase is not extracellular
and that the microorganisms in rumen liquid responsible for
the hydrolysis of soybean oil were centrifuged from the major
portion of bacteria. It is possible, however, that large
bacteria or those adsorbed onto the feed particles, could be
the source of lipase activity. The very slight increase in
breakdown of oil obtained by addition of sodium taurocholate
to the supernatant was apparently due to an increase in
emulsification of the oil and subsequent hydrolysis by the
contributing organisms remaining in this fraction after
centrifugation. That the lipolytic capacity of the super-
natant was of necessity small is substantiated by the fact
that the major portion of the enzyme activity of the untreated
liquid was transferred to the residue during centrifugation.

Further centrifugation studies were made with rumen
liquid from animal No. 4799 on the feed-lot ration. Centri-

fugation of 100 ml. of rumen liquid was performed at 2,000 r.p.m. for 5 minutes and the residue was resuspended in 50 ml. of synthetic medium after washing twice with 0.85% saline solution at 38°C. Untreated rumen liquid, supernatant, and resuspended residue with and without $CaCl_2$ (0.01M) were incubated with 0.2 g. soybean oil (to which C^{14}-labelled tripalmitin, 61,500 c/m, was added) for 24 hours at 38°C.

The percent of the added C^{14} activity recovered in the neutral fat and fatty acid fractions are given in Table 16.

Table 16. The effect of various fractions of rumen liquid upon hydrolysis of C^{14}-carboxyl tripalmitin mixed with soybean oil

	% Recovery	
Lipase preparation	Neutral fat	Fatty acids
Untreated rumen liquid	23.4	67.0
Supernatant	80.3	8.7
Resuspended residue	5.1	83.7
Resuspended residue + $CaCl_2$(0.01M)	6.2	79.8

The results were essentially the same as those obtained previously. Virtually no lipase action was found in the supernatant, whereas an appreciable amount of the added radioactive material was hydrolyzed to fatty acids by the resuspended residue. Addition of $CaCl_2$ (0.01M) had little effect on this activity. The total recovery of C^{14} activity

ranged from 86.0 to 90.4% in this experiment. The fate of the activity not recovered was not determined.

Use of microbial inhibitors

To study the type or types of microorganisms involved in the lipolysis of the triglycerides and also to determine substances which might have some control on the lipase action, the effect of several compounds (mostly antibiotics) was studied. Ingesta from animal No. 4723, a steer highly susceptible to bloat and whose rumen liquid had always exhibited relatively high lipase activity, was employed. The compounds whose inhibition was studied included procaine penicillin G, neomycin, erythromycin, streptomycin, chloramphenicol, tylosin and mannose. These compounds were dissolved in a small amount of water and added to the rumen liquid to give a 0.003% solution in the case of antibiotics and a 0.5% solution of mannose. The substrate consisted of 0.5 g. soybean oil and the degree of activity was estimated by titration of the extracted fatty acids after incubation for 24 hours at 38°C.

The results (average of duplicate samples) are given in Tables 17 and 18.

Penicillin, mannose, erythromycin, and tylosin were very effective inhibitors; all decreased enzymic activity by more than 50%. Streptomycin decreased activity by 49% and chloram-

Table 17. The effect of penicillin, neomycin and mannose
         upon rumen microbial lipase activity

Compound added	% Hydrolysis of soybean oil
None	74.4
Neomycin	66.8
Penicillin	25.8
Mannose	9.8
Neomycin + penicillin	23.0

Table 18. The effect of various antibiotics upon rumen
         microbial lipase activity

Antibiotic	% Hydrolysis of soybean oil
None	98.8
Erythromycin	37.6
Streptomycin	49.0
Chloramphenicol	68.0
Tylosin	37.4

phenicol by 30%. Neomycin was least effective of the sub-
stances tested and mannose possessed the greatest inhibitory
effect. The effects of neomycin, penicillin, streptomycin,
and mannose upon rumen microorganisms have been discussed by
Oxford (81).

Neomycin, which has previously been shown to be partic-
ularly effective in killing rumen Gram-negative bacteria, was
ineffective in checkingrumen lipase activity in this inves-

tigation. This finding indicates that Gram-negative bacteria are not directly involved in the hydrolysis of glycerides within the rumen or that the particular organisms are resistant to this antibiotic.

The degree of inhibition by penicillin indicates that either Gram-positive bacteria are the source of the rumen lipase or that they are in some way indirectly connected with its production. This latter factor could be due to their production of some extracellular dietary essential for other bacteria or to the fact that intact microorganisms may serve as a source of food for the rumen protozoa. The combination of neomycin and penicillin did not decrease the enzyme activity appreciably below that caused by penicillin alone. This substantiates the observation that neomycin is ineffective in reducing rumen lipase activity.

It is known that streptomycin is distinctly toxic to some rumen protozoa (oligotrich ciliates) (82) and non-toxic to others (holotrichs) (77). In the present study this antibiotic was quite effective in decreasing rumen lipase action on soybean oil. These observations suggest that the oligotrich ciliates contain a lipase capable of glyceride hydrolysis or that the antibiotic is detrimental to some species of bacteria which are essential to protozoal lipase function.

The purpose of addition of mannose was to kill and dis-

integrate the holotrich ciliates (83). The high inhibitory effect observed by this compound upon the enzyme activity quite clearly associated these protozoa with lipolysis of glycerides by rumen microflora. The relatively low activity found probably was due to the time lag before its full effect was exhibited. It is realized, however, that these observations are not conclusive evidence that the holotrich ciliates are the only microorganisms involved in lipase activity, but it is almost certain that they possess some lipase or are necessary for its production elsewhere.

The purpose of testing the other antibiotics (Table 18) was to determine the possible relationship between inhibition of fat hydrolysis in the rumen and bloat prevention by antibiotics. The ability of different antibiotics to prevent bloat in ruminants on legume pasture has been studied in detail at this University. An excellent report and discussion on this subject has been made recently by Johnson (25). Penicillin, erythromycin, and tylosin were highly effective for periods of several days but more lasting protection was obtained by using several antibiotics in rotation or in combination. Chloramphenicol and streptomycin were somewhat less effective. As seen in Tables 17 and 18, penicillin, erythromycin and tylosin are good lipase inhibitors, whereas chloramphenicol and streptomycin are not such potent inhibitors. These limited observations suggest that

those microorganisms which in some way take part in bloat also may contribute to the lipase action in the bovine rumen. It should be recognized, however, that the level of antibiotic added in the lipase inhibitor studies was considerably higher than that commonly employed in the prevention of bloat in animals grazing alfalfa.

GENERAL DISCUSSION

The first indication that the rumen possessed lipase activity was advanced by Garton et al. (16) in 1958. These workers in a study primarily concerned with hydrogenation, found more than 75% conversion of linseed oil and tung oil to free, higher fatty acids when 1 g. of the fat was incubated in vitro, anaerobically, with 100 ml. of sheep rumen contents. A subsequent investigation (17) which involved studies with olive oil and cocoa butter at the same level (1 g. per 100 ml. rumen contents) substantiated the earlier in vitro hydrolysis work. That the hydrolysis was enzymatic was demonstrated by comparison of hydrolysis by heated and unheated liquids.

This author has extended these findings by observing lipase activity ranging from low to very high levels with rumen liquid obtained from fistulated steers. Levels of substrate up to five times that employed by Garton's group under similar conditions were hydrolyzed to about the same percent as that found originally using rumen liquid from sheep. In most cases ingesta from animals on feed-lot ration had approximately the same lipase activity as Garton and associates observed with ingesta from sheep on a hay-concentrate diet. However, the rumen contents of animals grazing alfalfa pasture often exhibited several times the

the lipase activity observed with rumen liquid of animals consuming concentrates and alfalfa hay.

Preliminary studies performed with ingesta from different animals showed that there was a great deal of difference among animals and also within the same animal on different days. Limited observations with animals on alfalfa pasture indicated that those which were more susceptible to bloat often had considerably higher activity than the non-bloaters. However, more work is needed in this area before any definite conclusions can be drawn.

Diurnal studies indicated changes in lipase activity during the day. A decrease in enzyme activity, which was accompanied by a decrease in pH, was observed after feed consumption. It has been reported that the mean concentration of dividing protozoa in the rumen of the sheep has been correlated with changes in ruminal pH (84, 85). Thus, it is probable that the low lipase activity observed at the low pH values in the diurnal studies also was due to lower concentrations of microorganisms responsible for lipase activity. Other factors which may account for the level of lipase activity include dilution of the active organisms by feeds, water, and saliva, and the presence of toxic substances, inhibitors and activators. The effects of these factors upon rumen lipase activity need study. The large variations in lipase activity, within days, and between days, stress the

necessity for using caution in obtaining rumen liquid for lipase activity studies and in comparing work performed with animals under different conditions.

In most in vitro studies of rumen fermentation, anaerobic conditions have been maintained by gassing with carbon dioxide, nitrogen or a mixture of the two in various proportions. In view of the present findings, the extent to which the gas affects the reactions being studied should be determined prior to in vitro investigations in which whole rumen liquid is employed as the source of enzyme. This is of special importance since there is much carbon dioxide produced endogenously (77, 78) and the need for a high partial pressure of other anaerobic gas may be unimportant or its presence actually have detrimental effects upon the metabolic transformations under consideration. Although lipase activity in the rumen of animals on several different diets has not been thoroughly investigated, it is likely that results of such studies will show that the relative levels of active microorganisms present, as influenced by such factors as the partial pressures of ruminal gases, substrates present and other factors, will determine the extent of lipase activity.

The physiological state (appetite, hormonal balance, nervous condition, etc.) of the animal also probably affects rumen metabolic activity, including that of the microorganisms which are involved in fat hydrolysis.

Although the investigation of the type of microorganism responsible for lipase activity was not conclusive, the results indicated that possibly three types of organisms are involved. Results obtained using streptomycin, which kills oligotrichs but is non toxic to the holotrichs (77), implicates the former or bacteria which are resistant to the antibiotic. The fact that penicillin causes a considerable reduction in lipase activity and also inhibits most rumen Gram-positive bacteria (81) indicates that this group of microorganisms may be involved in lipolysis to a certain extent. Finally, the almost complete inhibition of enzyme activity by the addition of mannose to the incubation medium implicates the holotrichs. Studies with neomycin indicate that the Gram-negative bacteria are not the contributing organisms. Thus, the evidence indicated that possibly three different groups of microorganisms, oligothichs, holotrichs, and Gram negative bacteria are contributory to rumen lipase activity. It is possible, however, that only one of these is directly concerned, and the others may contribute activators or other essentials needed by the microorganism directly involved in hydrolysis. Triglyceride hydrolysis may depend upon a synergisteric relationship between the different microorganisms.

Attempts to obtain lipid hydrolysis during cultivation of protozoa isolated from other components of the liquid were

unsuccessful. Although in most trials the organisms died after several hours of incubation, in others where some were active after 24 hours of incubation, there was no appreciable enzyme activity.

Further in vitro experiments with rumen protozoa and bacteria in the presence of various feed nitrients and controlled media are needed. Of special importance would be a study of the contribution that different microorganisms make to one another and other factors which govern rumen fermentations. The present study suggests that in vitro hydrolysis of triglycerides in rumen liquid is brought about under complex conditions to which the existence and metabolic activity of three different classes of organisms affect the occurrence and activity of the lipase.

Unlike in the stomach of monogastric animals in which no appreciable hydrolysis of triglycerides occurs, a high degree of lipolysis has been observed in studies with rumen liquid. This process may have significant effects upon other metabolic transformations within the rumen and subsequent parts of the digestive tract.

A number of workers have shown that the digestibility of various feed stuffs in ruminants, especially that of cellulose, is significantly decreased by the addition of various fats and fatty acids (86, 87, 88). It is possible that these adverse effects are due to the presence of con-

siderable quantities of fatty acids formed by hydrolysis of fat. An interesting finding in this connection is that growth of Gram-negative and Gram-positive bacteria is inhibited by higher fatty acids (89). Also, Camien and Dunn (90) have discussed growth inhibition of certain bacteria due to antimetabolic effects of saturated fatty acids. Thus, it appears that fatty acids formed from fats in the rumen exert, either by their emulsification properties or their inhibitory effects upon rumen microorganisms, a pronounced effect upon the physiology of the rumen.

SUMMARY

In vitro lipase activity in bovine rumen liquid has been studied with three assay methods. Production of fatty acids from soybean oil and C^{14}-carboxyl tripalmitin mixed with soybean oil was measured by extraction-weighing, extraction-titration, and tracer techniques.

A considerable range in lipase activity was found in the in vitro studies. In some trials 10 ml. of rumen liquid hydrolyzed 0.5 g. soybean oil almost to completion within a 24-hour period. In others, hydrolysis values as low as 5 percent were observed. Heating the liquid to 90°C. for 30 minutes completely destroyed lipase activity.

Hydrolysis under CO_2 increased almost linearly the first 24 hours; subsequently, a plateau occurred. A short lag period (approximately 4 hours) was observed in trials performed without gassing.

The glycerides remaining after various periods of incubation consisted mainly of triglyceride; relatively low levels of di- and monoglycerides were found. No detectable degradation of palmitic acid produced from tripalmitin was noted. However, low titration values for added oleic acid were obtained after 4 and 8 hours of incubation; after 12 hours approximately 100% of added acid was indicated in the extracts.

Hydrolysis of soybean oil occurred over a wide pH range. Maximal activity was obtained between pH values of 6.8 and 7.4 when the pH of the liquid was adjusted with phosphate buffer (0.1M).

The level of lipase activity varied diurnally in samples of ingesta liquid obtained from fistulated animals on both feed-lot and pasture. In both cases high levels were observed before morning and mid-afternoon feedings. Good correlation between enzymatic activity and high pH values was found.

Large differences in enzyme levels were observed among different animals and in the same animal on different days. Samples from animals on pasture exhibited more hydrolysis than did those from animals on the feed-lot ration. Ingesta liquid from animals more susceptible to bloat generally had higher levels of lipase activity than liquid from other animals. Antibiotics which decrease bloat severity of animals grazing alfalfa pasture also decreased the lipase activity of the rumen liquid.

Approximately the same degree of lipase activity was observed in samples gassed with CO_2 and those not gassed; nitrogen gassing depressed activity considerably.

Centrifugation studies indicated the source of lipase activity was not extracellular and was easily centrifuged from the liquid. Limited observations with antibiotics

indicated that possibly three types of rumen microorganisms (holotrichs, oligotrichs and Gram-positive bacteria) are associated with the enzyme activity.

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