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STUDIES ON SALIVARY LIPASE IN YOUNG RUMINANTS*

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Introduction

During 1954, during the course of an investigation on the abomasal secretion of young dairy calves, a sample of milk collected from an oesophageal fistula in a sham-fed calf was taken to determine its pH value. As the pH of the milk before feeding was 6·7 and that of the particular calf's saliva 8·4, a reading of approximately 7·0 to 7·2 in the milk collected from the oesophagus was expected. Contrary to expectation, a drop in the pH to 6·4 within five minutes and to 6·0 within 20 minutes after drinking was noted. Various tests were then carried out and it was finally established that a salivary lipase was responsible for this phenomenon.

Subsequently the saliva of young lambs and kids, as well as of adult bovines' sheep and goats was tested for lipolytic activity. The saliva of young lambs and kids had the same action on milk as that of calves, but no lipolytic activity could be demonstrated in the saliva of any of the adults tested. Further tests indicated that the lipase disappears from the saliva of calves at the age of approximately three months. It has also been established that the palatine salivary glands are responsible for the secretion of the lipolytic enzyme.

The only reference found to this lipolytic action of calf saliva is the following paragraph by Dukes (1947): "*Pregastric Changes in Milk Drunk by Calves.*—Experiments by Wise, Miller and Anderson indicate that milk drunk by calves undergoes modification, apparently of a digestive nature, before it reaches the stomach. The calves were sham-fed by causing the ingested milk to run out of an esophageal fistula or out of a flexible tube inserted into the cardiac orifice via a rumen fistula. Among the changes noted in the sham-fed milk were decrease in pH, increase in lipolytic activity, and increase in rate of coagulation by rennin. It is suggested that the salivary or esophageal glands may secrete a lipolytic enzyme."

Identification of the Source of Acid formed in Milk during Drinking by Calves

As stated above milk (pH = 6·7) drunk by a calf and collected from the oesophagus became more acid (pH = 6·0) within 20 minutes. Various possible causes for this phenomenon were considered, e.g. the formation of lactic acid, amino acids or fatty acids by enzymes and bacterial action, although the change appeared too rapid for the last possibility to be tenable. The following preliminary experiment was therefore conducted.

Method: Two young Friesland calves No. 7461 and 7474, aged 13 and five days respectively were operated on under chloral hydrate anaesthesia. An incision of approximately 5 cm long was made just below and parallel to the left external jugular vein, about halfway up the neck. By blunt dissection a longitudinal slit was then made through the cutaneous and sterno-cephalic muscles through which the oesophagus was drawn to the surface. A semicircular notch with a radius of approximately 6 mm was cut from the skin on both sides of both ends of the incision. The oesophagus was severed and the two ends sutured to the edges of the notches in the skin so that the mucous membrane of the osephagus was continuous with the epidermis. The skin wound between the two oesophageal openings was sutured in the normal way. The calves were fed by allowing them to drink their normal volume of milk which was then collected from the anterior oesophageal fistula and subsequently poured down the posterior oesophageal fistula through a funnel and rubber tubing. Plate 1 shows such a calf drinking from a bucket while the milk squirts from the oesophageal fistula.

PLATE 1.—A calf provided with oesophageal fistulae drinking milk from a bucket.

Saliva emerged as a frothy mass from the anterior oesophageal opening during acts of swallowing. Samples could be collected in glass beakers without difficulty.

To determine the source of the acid found in milk drunk by calves, samples of saliva from these two calves were incubated with each of the following " substrates ": 4 per cent lactose solution; 3·5 per cent fat-free casein solution; fresh cow's milk; fresh cow's milk with Merthiolate (1: 10,000) as bacteriostatic; fresh cream from cow's milk and fresh skimmed milk. Four test tubes were filled with 10 ml of each of the " substrates " and preheated in a water bath at 37° C for five minutes. Then 0·3 ml of the saliva samples were added to the four tubes, viz. saliva from calf 7461 to the one, saliva from calf 7474 to the second and inactivated saliva from the same calves (heated in boiling water bath for ten minutes) to the remaining two tubes of each of the substrates.

Immediately after mixing the saliva with the substrates, the pH of all the mixtures were measured with a pH-meter. They were then incubated in the water bath at 37° C and pH readings were again recorded after 30 and 60 minutes incubation.

Results: The pH of the saliva samples from calves 7461 and 7474 were found to be 8·6 and 8·7 respectively. The various pH readings obtained from the above-mentioned procedures are represented in Table 1.

TABLE 1.—*pH changes in various substrates due to the action of calf saliva*

Substrate	Time incubated (minutes)	pH Values			
		Calf 7461		Calf 7474	
		Saliva	Inactivated saliva	Saliva	Inactivated saliva
Lactose.....	0	8·05	8·1	8·05	8·1
	30	8·05	8·05	8·1	8·1
	60	8·1	8·1	8·05	8·1
Casein.....	0	7·6	7·6	7·6	7·6
	30	7·6	7·6	7·6	7·6
	60	7·6	7·6	7·6	7·6
Cow's milk.....	0	6·7	6·7	6·7	6·7
	30	6·3	6·7	6·2	6·7
	60	6·0	6·7	6·0	6·65
Milk with Merthiolate...	0	6·7	6·7	6·7	6·7
	30	6·3	6·7	6·2	6·7
	60	6·0	6·7	6·0	6·7
Cream.....	0	6·7	6·7	6·7	6·7
	30	5·9	6·7	5·7	6·75
	60	5·8	6·7	5·7	6·7
Skim milk.....	0	6·7	6·7	6·7	6·7
	30	6·6	6·7	6·7	6·65
	60	6·6	6·65	6·6	6·7

Conclusions: It is quite clear from the table that both saliva samples caused the formation of acid with only three of the substrates used, viz. fresh cow's milk, fresh cow's milk with Merthiolate and cream. These three substrates were the only ones containing fat. No acid was formed in any of the substrates with inactivated saliva. All the tubes in which acid was formed smelt of butyric acid while no such odour could be detected in any of the other tubes.

It was therefore concluded that a lipase in the saliva of these two calves was responsible for the drop in pH of the milk.

PROPERTIES OF THE SALIVARY LIPASE OF CALVES

(*a*) *Its action on various triglycerides*

One of the first questions that arose was, which fats are attacked by the lipase and what fatty acids are formed from milk fat? According to Achaya & Hilditch (1950) a wide variety of fatty acids is present in the glycerides of milk fat. Table 2 gives the proportions of these fatty acids in the fat of the milk from British dairy cattle.

TABLE 2.—*Proportions of fatty acids in milk fat of British dairy cows (Achaya & Hilditch,* 1950)

Proportions of fatty acids in mols. per cent									
Saturated fatty acids								Unsaturated	
C4	C6	C8	C10	C12	C14	C16	C18	Oleic	Others
10	4	2	3	2	8	24	11	29	7

The possibility therefore existed that the lipase could be capable of liberating only some or all of these fatty acids from milk.

Various methods were tried to isolate the long and short chain fatty acids simultaneously from a sample of creamy milk to which they were added, but all proved to be unsuccessful. Other methods had therefore to be resorted to to solve this problem.

Experiment No. 1

The activity of the salivary lipase was tested on a few pure triglycerides to obtain some indication of its activity on fats containing fatty acids differing in chain length.

Method: Ten per cent w/v emulsions of triacetin, tributyrin, tricaproin, tripalmitin, tristearin and triolein in water were prepared with 0·2 per cent purified albumen as emulsifying agent. Four samples (4·5 ml) of each of these emulsions were placed in test tubes to which were added, 0·5 ml of fresh calf saliva diluted with an equal volume of saline, 0·5 ml of the same saliva diluted to eight times its volume with saline and 0·5 ml of each of the two saliva dilutions after heating in a boiling water bath for ten minutes, respectively. The saliva was collected from an oesophageal fistula from a 22-day old Brown Swiss calf (Saliva Sample A). The tubes were then placed in a water bath at 37° C with continuous mechanical agitation for 30 minutes. Immediately after removal from the water bath the tubes were packed in ice and titrated as soon as possible with 0·025 N NaOH with phenolphthalein as indicator. It had been found that this enzyme is inactive below 10° C (see later) and cooling of the tubes in ice was considered a practical method of stopping lipolytic action.

To control the findings in this experiment, it was repeated eight days later. Fresh emulsions were prepared and fresh saliva (Saliva Sample B) was collected from the same calf but this time it was used undiluted and at a 1 in 2 dilution. The incubation was prolonged to 60 minutes.

Results: The difference in the volume of alkali used to neutralize the substrate with active saliva and that used to neutralize the substrate with heated saliva represented the acid formed through enzyme action. The abovementioned two saliva samples produced the amounts of acids given in Table 3 from 0·45 gm of the triglycerides tested.

TABLE 3.—*Micromols of acid formed by salivary lipase from various synthetic triglycerides*

Substrate	Micromols of acid formed from 0·45 gm triglyceride			
	Saliva A (1: 2)	Saliva A (1: 8)	Saliva B	Saliva B (1:2)
Triacetin*	2·5	Traces	10	3
Tributyrin*	217	62	636	338
Tricaproin†	0	0	0	0
Tripalmitin*	0	0	0	0
Tristearin*	0	0	0	0
Triolein*	0	0	0	Traces?

* Sigma grade
† Prepared by Dr. J. M. M. Brown, Section of Physiology, Onderstepoort

The titration values are given in Table 11 (see Appendix).

Conclusions: Of the triglycerides tested, the salivary lipase of calves was capable of hydrolyzing only tributyrin (glycerol tributyrate) at a significant rate.

Experiment No. 2

After discovering that only short chain fatty acids, mainly butyric acid, were liberated from their respective triglycerides by the enzyme, it was decided to attempt the isolation of these fatty acids from milk after incubation with saliva.

Method: A sample of creamy milk was divided into five portions of 10 ml each. One of these was incubated with calf saliva at 37° C for 60 minutes, another with inactivated saliva as control, and ten drops of butyric acid, valeric acid and caproic acid respectively were added to the remaining three samples. Thereafter all the samples were treated similarly, viz. concentrated NH_4OH was added drop by drop to the mixtures while kept at 5° C till they just turned to a permanent pink with phenolphthalein. Two parts of acetone (adjusted to pH 9 with concentrated NH_4OH) were then added to each part of milk mixture to precipitate the proteins which were then filtered off. The remaining fat in the filtrates was extracted with chloroform and the acetone/water fractions concentrated by evaporation in a current of air from an electric blower. The concentrated fractions were spotted on Whatman No. 1 paper and chromatographed in a system of ammonia-saturated butanol (n-Butanol: 1·5 N NH_4OH = 1: 1) by means of the ascending technique. A watery solution of 0·05 per cent bromophenol blue, acidified with 0·2 per cent citric acid, was used as spray (Block, Durrum & Zweig, 1958). Ammonium esters of fatty acids show as purple spots on a yellow background. An ammoniated propanol system (n-Propanol: concentrated NH_4OH = 7: 3 v/v) was also tried, but did not separate the ammonium salts satisfactorily.

Results: The paper chromatogram is represented by Fig. 1. As can be seen, the butyric, valeric and caproic acids moved at different R_F rates. The spot produced by the acid formed in milk by salivary lipase corresponded to the butyric acid spot and had the same R_F value. Apart from a faint spot, caused by an unknown substance in all the samples and which moved with the solvent front, no other spots appeared in the control sample.

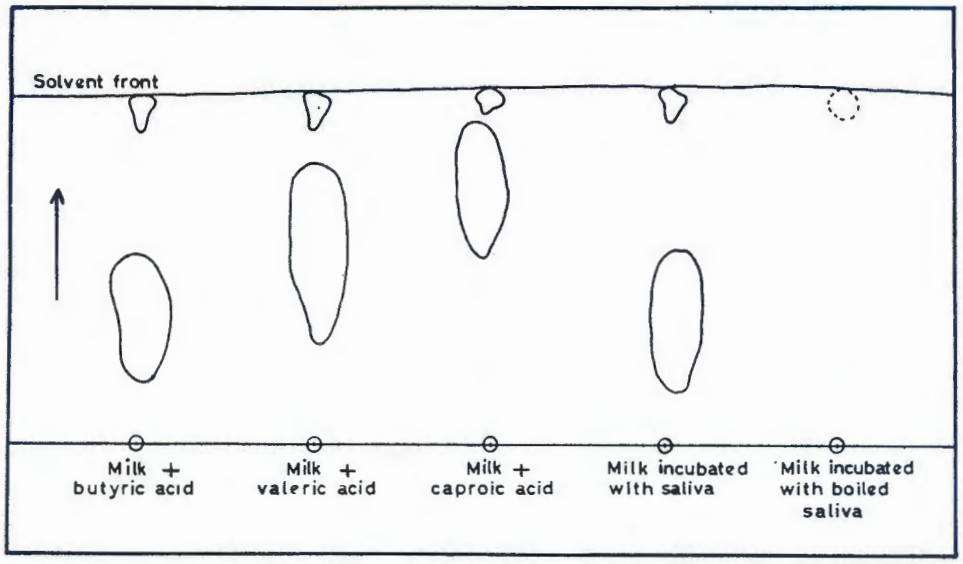

FIG. 1.—Paper chromatogram showing the relation between butyric acid and the acid formed from milk fat by calf saliva

Conclusion: The acid produced in milk by the action of salivary lipase is butyric acid. It appears that the lipase is not capable of liberating other fatty acids from their glycerides in milk.

(*b*) *The effect of pH on the activity of the salivary lipase*

The effect of various pH values of the substrate on the enzyme action was tested as follows:—

Method: Citrate and phosphate buffers were prepared to obtain a pH range from approximately pH 2 to pH 8 according to the method described by King & Wootton (1956) (see Table 12 in Appendix). These two buffers were selected because both phosphate and citrate are normally present in milk, the natural substrate of the enzyme. Unfortunately no suitable single type of buffer could be used and it was expected that the two types of buffers might produce different results. They were, therefore, selected so that some overlapping of pH values would occur.

Two small Erlenmeyer flasks with 4·5 ml of emulsified tributyrin substrate each were now prepared with each of the nine buffer solutions. Every flask contained 0·45 ml of tributyrin, 0·9 ml of a 1 per cent purified albumin solution and 3·15 ml of buffer solution. The actual pH values of the final substrates as measured with a pH-meter differed slightly from those expected for the pure buffer solutions (see Table 12).

Fresh calf saliva collected from the oesophagus of a 27-day old Brown Swiss calf was used as enzyme. Of this saliva 0·5 ml was added to one series of substrate suspensions while 0·5 ml of the same saliva, after heating in a boiling water bath for ten minutes, was added to the other series as controls. All 18 flasks were now placed in a water bath fitted with a mechanical shaker and incubated at 37° C for 30 minutes with continuous agitation.

After removal from the water bath the flasks were immediately packed in ice to stop enzyme action. The mixtures were then titrated with 0·025 N NaOH and phenolphthalein as indicator.

Results: The difference between the titration values of the test and control mixtures at any given pH was regarded as being equivalent to the acid formed by the enzyme. Full details of the results of the titrations are given in Table 13 (Appendix). By plotting the acid produced against pH, the graph shown in Fig. 2 was obtained. From this graph it can be seen that enzyme action was completely inhibited at pH 2·4 with the citrate buffer and on the alkaline side at pH 7·8 with the phosphate buffer.

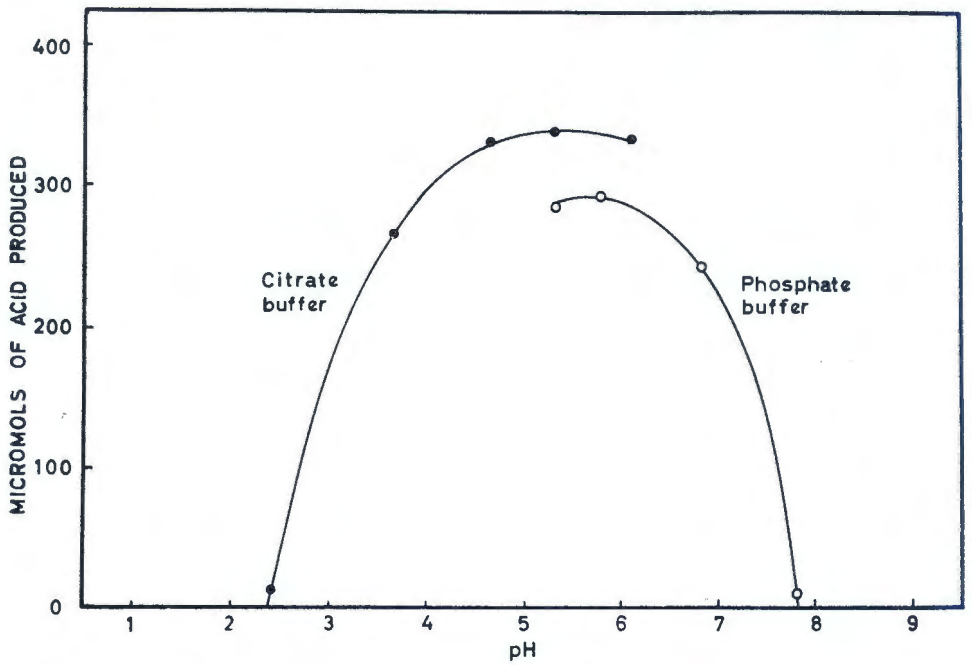

FIG. 2.—The effect of pH on the action of salivary lipase on tributyrin substrate

Conclusions: The optimal pH for the action of salivary lipase on tributyrin is between pH 4·5 and 6·0. The enzyme action was completely inhibited below pH 2·4 and above pH 7·8.

(*c*) *Determination of the optimum temperature for salivary lipolytic action*

An enzyme has its specific temperature range for optimal activity. Below and above this it is less active and, being a protein, is usually destroyed by a temperature exceeding 60° C. The optimum temperature for this lipase was determined as follows:—

Method: Twenty samples of 4·5 ml of milk substrate were prepared. Fifteen of these were used to determine the lipolytic action at various temperatures while five served as controls and were incubated with heat-inactivated saliva at five different temperatures for 30 minutes. To obtain the desired temperature levels the substrate

enzyme systems were either placed in a warm water bath, kept at room temperature, placed in cold water or put into a refrigerator. To each, 4·5 ml of substrate 0·5 ml of fresh calf saliva was added, shaken well and then kept at the desired temperature for 30 minutes.

Titration with 0·025 N NaOH, and phenolphthalein as indicator, was done immediately after the 30 minutes were over and the net acid formed was calculated as in the previous experiment.

Results: Full details of the results of titration are given in Table 14 (Appendix)· By plotting the acid formed from milk by the salivary lipase against temperature the graph presented in Fig. 3 was obtained. From this curve it is evident that the activity of the enzyme is completely inhibited below 11° C and above 58° C. The activity was maximal at 39° C.

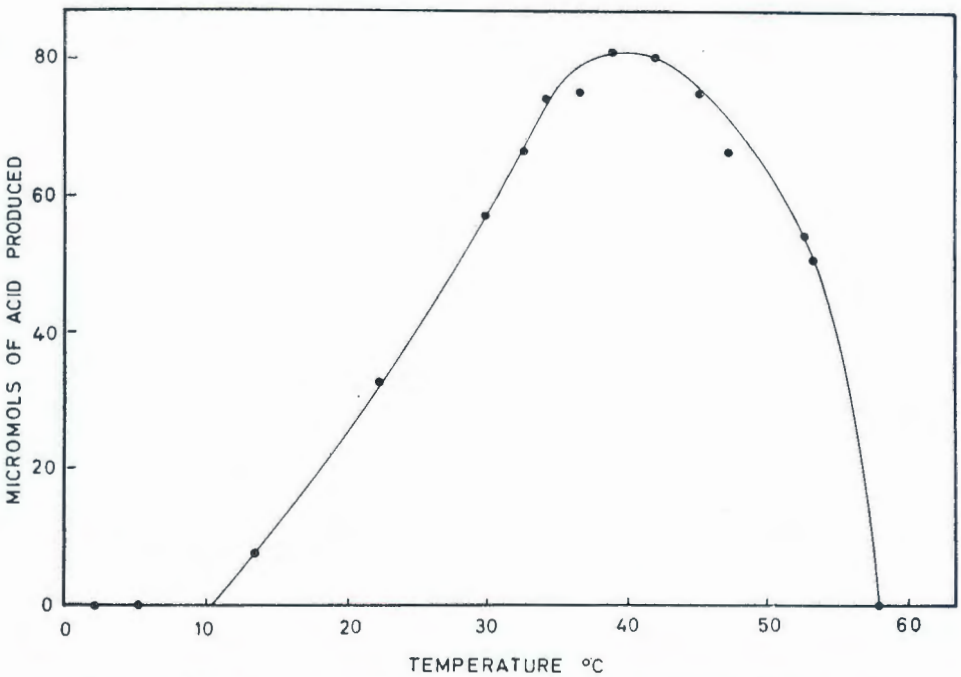

FIG. 3.—The effect of temperature on the activity of salivary lipase (milk substrate)

Conclusions: The optimal temperature for the salivary lipase of calves is 39° C· The activity of this enzyme is completely inhibited below 11° C and above 58° C.

(d) Estimation of enzyme concentration

Before studying the effects of various factors on the secretion of the lipase by young ruminants it was found necessary to establish some method of estimation of enzyme concentration. According to Bier (1955) a surprisingly large number of widely different procedures is reported for the determination of the activity of various lipase preparations. The most frequently employed procedures are based on the titrimetic determination of the acids liberated by the action of the enzyme. Emulsified olive oil was most commonly used as substrate but many other esters

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and triglycerides, natural or synthetic, were utilized (Bier, 1955). As emulsifying agents albumin (Hawk, Oser & Summerson, 1954), sodium oleate (Willstätter, Waldschmidt-Leitz & Memmen, 1923), gum arabic (Fodor, 1946) and gum acacia (Crandall & Cherry, 1931) have been employed.

Although milk fat is the natural substrate, it was felt that its composition and pH were not sufficiently constant for assaying the enzyme. As tributyrin was hydrolized well by the lipase (*vide supra*) it was decided to use it as substrate. Albumin, a component of the natural substrate, was selected as emulsifying agent. The digestion was allowed to continue for 30 minutes at 37° C and the enzyme action was inhibited by rapid cooling to below 5° C.

Method: Serial twofold dilutions of fresh calf saliva collected from a 20-day old calf, were prepared in distilled water up to 1 in 256. Duplicate samples of the dilutions were inactivated by heating in a boiling water bath for ten minutes. The substrate emulsion was prepared by shaking up 10 gm of tributyrin with 20 ml of a 1 per cent purified albumin solution and distilled water added to 100 ml. Of this 4·5 ml were added to each of 18 small Erlenmeyer flasks. To nine of these 0·5 ml of the saliva solutions were added and 0·5 ml of the inactivated saliva solutions to the remaining nine. All the flasks were put into a warm water bath at 37° C and continuously agitated mechanically. Thirty minutes later they were removed and placed into a dish with ice and water. Titration with 0·025 N NaOH, using phenolphthalein as indicator was carried out as soon as possible. A similar experiment was repeated the same day with only the 1 in 1, 1 in 2, 1 in 4 and 1 in 8 dilutions of saliva.

Results: Full details of the titration results are shown in Table 15 (see Appendix). By plotting the micromols of acid liberated from the tributyrin by the enzyme against the dilution on double log scale, the graph in Fig. 4 was obtained.

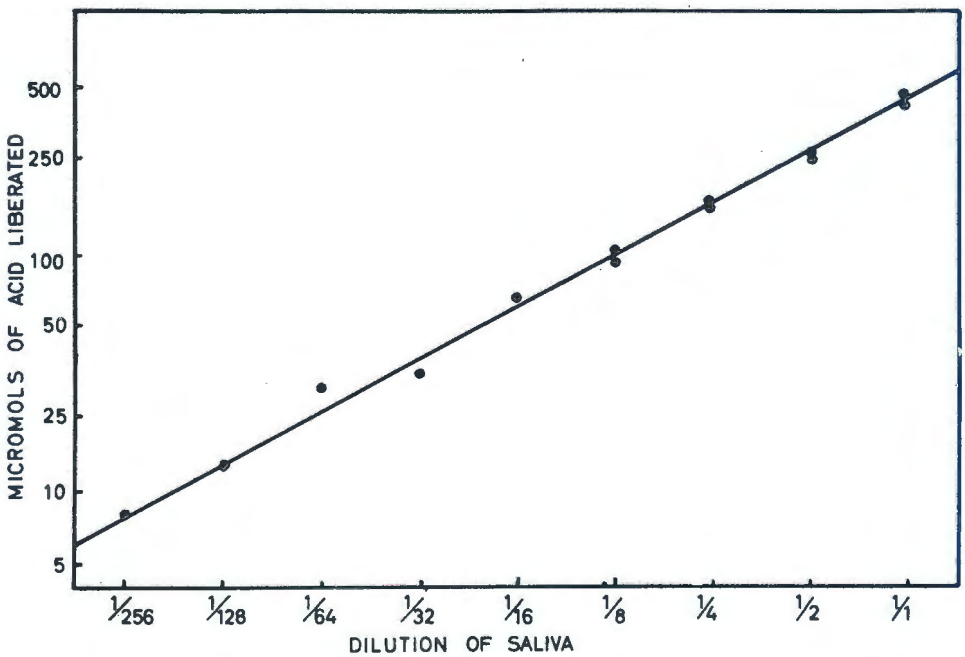

FIG. 4.—A comparison between dilution and activity of salivary lipase

Conclusions: From Fig. 4 it is evident that there is a direct relationship between the concentration of enzyme employed and the micromols of acid produced under standard conditions. The amount of acid so produced can therefore be used as an indication of the concentration of lipase in any given saliva sample.

(*e*) *A comparison between milk and tributyrin as substrate for salivary lipase*

Since a number of the early experiments with calves, lambs and kids had been done with milk as the substrate, a comparison had to be drawn between milk and tributyrin as substrates to be able to compare the results. Because the amount of tributyrin immediately obtainable was limited, only a few determinations of enzyme activity were done on it as substrate.

Method: Saliva was collected from an oesophageal fistula of a 19-day old calf by allowing it to suck the collector's finger. Serial two-fold dilutions from 1 in 1 to 1 in 16 in distilled water were prepared. Duplicate samples of each were inactivated by heating in a boiling water bath for 10 minutes. Fresh cow's milk and 10 per cent w/v tributyrin emulsion (prepared as above) were used as substrates. The individual dilutions of saliva and the inactivated saliva samples were added to each of the two substrates in the proportion of 0·5 ml of saliva to 4·5 ml of substrate. After mixing, they were incubated in a warm water bath at 37°C with continuous agitation for 30 minutes. Immediately after incubation, enzyme action was inhibited by cooling in ice water. All samples were titrated as soon as possible with 0·025 N NaOH with phenolphthalein as indicator.

Results: The results are shown in Table 16 (Appendix). By plotting the net acid liberated from the two substrates by each dilution of saliva against one another the graph presented in Fig. 5 was obtained.

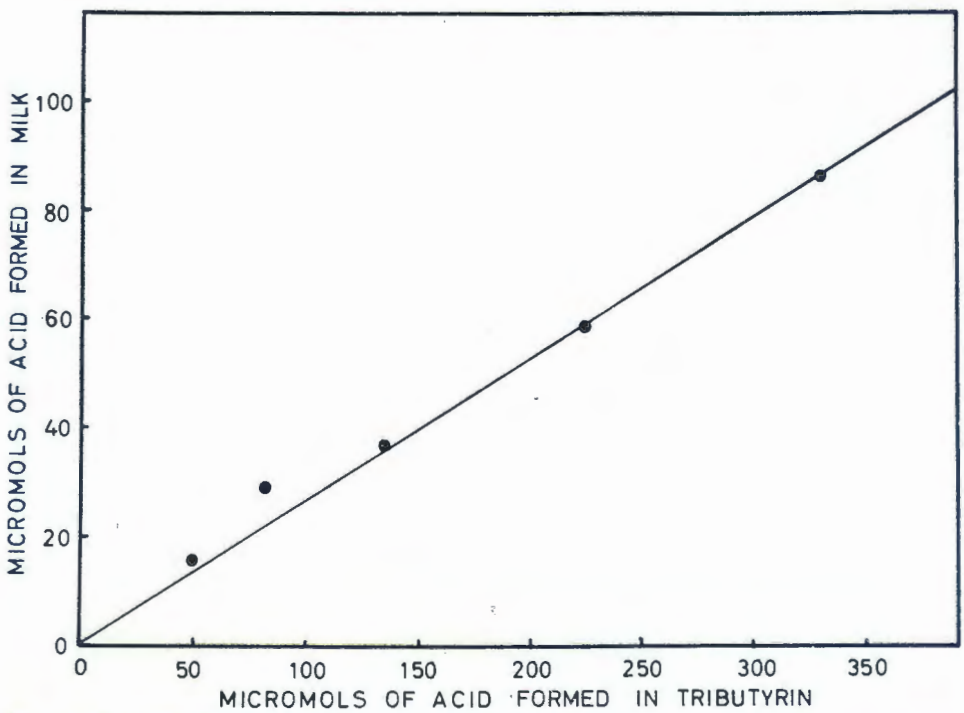

FIG. 5.—A comparison between the activity of salivary lipase on milk and tribyturin substrate

Conclusions: Since a direct correlation between the acid formed by salivary lipase from milk and tributyrin substrates was obtained, it was concluded that the micromols of acid produced in milk substrate could be interpreted in terms of those obtained from tributyrin substrate by using this graph.

Factors influencing the Secretion of Salivary Lipase by Young Ruminants

(*a*) *The difference in lipolytic activity of saliva from young and adult ruminants*

When the presence of a salivary lipase in the saliva of calves was established, the question arose as to whether it was also present in the saliva of adult cattle. The following experiments were then carried out.

(i) *A comparison between lipolytic activity of the saliva of a calf and an adult bovine*

Method: Saliva was collected from an adult Friesland cow after subcutaneous injection of carbamylcholine chloride (0·012 mgm/Kg body weight) by withdrawing her tongue and allowing the saliva to flow into a glass beaker. Saliva was also collected from a 50-day old Friesland calf in the same way. A portion of each of the two samples was inactivated in a boiling water bath for ten minutes. Of each of these saliva and inactivated saliva samples 0·3 ml were added to 10 ml of fresh cow's milk and to cream. The pH readings of the mixtures were taken immediately after mixing and again after 30 and 60 minutes incubation in a water bath at 37° C.

Results: The pH values obtained can best be given in tabular form (Table 4). The pH of the milk, calf's saliva and cow's saliva was found to be 6·7, 8·8 and 8·1 respectively.

Table 4.—*Effect of incubation of cow's and calf's saliva with milk and cream on the pH of the mixtures*

Origin of saliva	Substrate	pH Values		
		Before incubation	30 min incubation	60 min incubation
Calf	Milk	6·7	6·3	6·3
Calf	Cream	6·7	5·9	5·85
Cow	Milk	6·7	6·7	6·7
Cow	Cream	6·7	6·7	6·7
Calf-inactivated	Milk	6·7	6·7	6·7
Calf-inactivated	Cream	6·7	6·7	6·7
Cow-inactivated	Milk	6·7	6·7	6·7
Cow-inactivated	Cream	6·7	6·7	6·7

Conclusions: A 50-day old calf secreted lipase in its saliva whereas an adult cow did not.

(ii) *A comparison between lipolytic activity of the saliva of kids and adult goats*

It was felt that the method of collecting saliva used in the previous experiment was not completely satisfactory. As anaesthesia is more difficult in bovines than in goats, it was decided to collect saliva from a goat ewe and her twin kids under anaesthesia, and to compare the lipolytic activity of the samples so obtained.

Method: A goat ewe and one of her 20-day old twin kids (No. 1) were anaesthetized by the intravenous administration of thiopentone sodium B.P. A plug of cotton wool covered with cheese cloth was inserted into the oesophagus of each to prevent regurgitation of ruminal contents. They were then injected subcutaneously with 0·022 mgm carbamylcholine chloride per Kg bodyweight to stimulate salivary flow. A thin polyethylene tube was inserted into both parotid ducts of the ewe to allow the parotid secretion and the rest of the saliva to be collected separately. The saliva was collected in glass beakers as it flowed from the mouth. A fistula was produced in the oesophagus of kid No. 2 as described for calves (*vide supra*). Saliva from this kid was collected from the oesophageal fistula, also after the injection of carbamylcholine.

Five millilitres of each of these salivary samples were added to 100 ml of fresh cow's milk, incubated for 30 minutes at 37° C and titrated with 0·1 N NaOH in the same way as previously described. As controls, heated saliva samples were used.

The experiment was repeated on the same animals seven days later.

Results: The values obtained with titration are given in Table 5.

TABLE 5.—*The acid liberated from* 100 *ml of milk by* 5 *ml of saliva collected from a goat ewe and her twin kids*

Saliva sample	0·1 N NaOH used for titration in ml		
	Saliva	Inactivated saliva	Difference (Acid formed)
First expt.			
Ewe-parotid	17·2	17·3	— 0·1
Ewe-exclud. parotid	17·5	17·4	0·1
Kid No. 1—mixed	48·0	17·6	30·4
Kid No. 2—mixed	50·6	17·5	33·1
Second expt.			
Ewe-parotid	17·3	17·7	— 0·4
Ewe-exclud. parotid	17·5	17·8	— 0·3
Kid No. 1—mixed	50·3	17·7	32·6
Kid No. 2—mixed	50·6	17·8	32·8

Conclusions: The saliva of young goat kids contained a salivary lipase. The lipase was not present in the saliva of an adult goat. No difference in lipolytic activity could be determined in the saliva samples collected from the mouth of one kid under anaesthesia and the saliva of its twin collected from an oesophageal fistula.

(iii) *A comparison between the lipolytic activity of the saliva of an adult sheep and a lamb*

It was decided to establish whether the salivary lipase was also present in lamb's saliva and absent in the saliva of adult sheep.

Method: Saliva was collected from the mouth of a six-tooth Merino wether by allowing it to chew the end of a piece of rubber tubing. The free end of the tube was held over a glass beaker into which the saliva flowed through the tube. Lamb saliva was collected through an oesophageal fistula from a 15-day old Merino lamb. A portion of each sample was inactivated by heating.

One ml of each of the saliva and inactivated saliva samples was added to 10 ml of fresh cow's milk and incubated for 30 minutes at 37° C. Titration with 0·1 N NaOH was carried out as described in the previous experiments.

Results: The differences between the amounts of NaOH used on the untreated and inactivated saliva samples were zero in the case of the adult sheep and 3·4 ml in those from the lamb.

Conclusions: The saliva of a lamb showed lipolytic activity when incubated with milk. No such activity could be detected in the saliva of a six-tooth Merino wether.

(*b*) *The decrease in salivary lipolytic activity with increasing age*

As salivary lipolytic activity had been shown to be present in young ruminants and absent in adults, it was decided to investigate the decline of this factor with age.

(i) *Calves*

Method: The saliva samples were collected from three calves which were kept together in the same pen. They had access to lucerne hay and were given whole milk from a bucket twice a day. The calves were anaesthetized with intravenous thiopentone sodium and given a subcutaneous injection of 2·5 mg Prostigmine (Roche) per 100 lb body weight to stimulate salivary flow. A plug was inserted into the oesophagus to prevent ruminal regurgitation. The saliva flowing from the mouth was collected in glass beakers from the sixth to the fourteenth minute after prostigmine administration. Samples were assayed by using 4·5 ml of tributyrin substrate for every 0·5 ml of saliva as described (*vide supra*).

Results: A comparison between the age of the calf and the potency of its salivary lipase is drawn in Table 6.

TABLE 6.—*The effect of age of the calf on the lipolytic activity of its saliva*

No. of calf	Breed	Age in days	Micromols of acid produced
3431	Friesland	2	161
		78	0
3324	Friesland	15	120
		22	75
		62	38
3258	Jersey	65	38
		105	0

Conclusions: Since the acid produced is an indication of enzyme concentration it can be concluded that the potency of salivary lipase of calves decreased with increasing age. No lipase was found in the saliva of calves of 78 days or older.

(ii) *Goat kids*

Saliva samples from a pair of hand-reared goat twins, collected from time to time in a similar way, were used to determine the decline in lipolytic potency with age.

Method: The saliva samples were collected in the same way as described in the preceding experiment with calves except that 0·022 mgm carbamylcholine chloride per Kg body weight was administered subcutaneously to stimulate salivary secretion. Estimation of the lipolytic potency of the saliva samples was done by incubating 0·5 ml of saliva with 4·5 ml of fresh cow's milk and determining the titratable acidity.

Results: The lipolytic potency of the saliva from the twin kids can be compared to their ages, as shown in Table 7.

TABLE 7.—*The effect of age of twin goat kids on the lipolytic activity of their saliva*

No. of kid	Age in days	Micromols of acid produced
89927	14	326
	18	323
	47	138
	57	154
	83	1
89928	20	318
	42	210
	47	198
	57	148
	83	2

Conclusions: With increasing age the lipolytic activity of goat kid saliva decreased. The potencies observed at the age of 83 days were insignificant.

(*c*) *The effect of the ration on the potency of salivary lipase in calves*

The impression was gained that the lipolytic activity of the saliva of calves declined rapidly when they started to consume more roughage and meal in relation to milk, i.e. when the fat content of their total rations decreased and the roughage increased. To determine the effect of the fat level of the ration on the secretion of salivary lipase, calves were fed on the low fat diet and the potency of their salivary lipase was compared to that of a calf fed whole milk.

Method: Four Friesland bull calves received colostrum for the first five days and then milk substitute (mainly reconstituted skimmed milk powder), lucerne hay and calf meal pellets. Very little of the hay and pellets was consumed during the first three weeks. The pellets were manufactured from degerminated maize meal, solvent extracted oil cake meals, wheaten bran, lucerne meal, dicalcium phosphate and salt and were therefore relatively fat free. A fifth Friesland bull calf was raised on whole milk (5 lb twice per day) and lucerne hay *ad lib.* Saliva was obtained from the calves under thiopentone sodium anaesthesia and 0·022 mgm carbamylcholine per Kg body weight to stimulate salivary secretion.

The lipolytic activity was estimated as before, using milk as substrate.

Results: The potency values obtained are listed in Table 8.

TABLE 8.—*The lipolytic activity of saliva collected from calves receiving different rations*

No. of calf	Diet	Age in days	Micromols of acid produced by 0·5 ml saliva from 4·5 ml milk
1	Skimmilk + hay + pellets.....	63	2·0
2	,, ,, ...	50	0
3	,, ,, ...	22	1·5
4	,, ,, ...	21	0·5
5	Whole milk + hay.....	38	254

Discussion and conclusions: The calf raised on whole milk secreted saliva with a high lipolytic potency after the administration of carbamylcholine. The saliva of the four calves on the relatively fat free diet, on the other hand, showed very little or no lipolytic activity. The age of the control calf (No. 5) was very close to the average of the other four. The influence of the age factor can therefore be ignored in this instance. It was shown that saliva, secreted after the administration of carbamylcholine, has a higher lipolytic activity than that secreted after other forms of stimulation (see next experiment). It is therefore doubtful whether the four calves could have secreted saliva with a higher lipase level under other circumstances.

When one considers the fact that 2 micromols of acid produced represent a titration value of only 0·08 ml of 0·025 N NaOH and that the colour change in milk cannot be estimated accurately, then the potencies obtained in the saliva of the four experimental calves should all be considered as being insignificant. The high potency value of the salivary lipase obtained in the control calf is fully representative of calves raised on whole milk and one control animal was therefore considered to be sufficient.

(*d*) *The effect of various methods of stimulation of salivary secretion on the lipolytic activity of saliva*

It has been observed during the course of the investigations that the lipolytic potency of saliva samples collected during rest from an oesophageal fistula was much lower than that collected while the calf sucked a nipple. Carbamylcholine administration caused a more rapid flow of saliva with a high lipase content while stimulation by anticholinesterases, e.g. neostigmine, gave similar, but less marked, results.

The following experiments indicated these differences:—

(i) *The difference in potency of salivary lipase secreted by a calf during rest, while sucking and after carbamylcholine administration*

Method: Saliva was collected for five minutes from the oesophageal fistula of a 32-day old Friesland calf and from a 14-day old goat kid before their morning milk ration and while they were standing at rest. The calf was then allowed to suck from a teat-bucket (Grosskopf, 1959), and the kid from a baby's bottle. They were accustomed to drinking their milk in this way and sucked vigorously. Saliva was collected during five minute periods while sucking.

The calf and the kid were then injected subcutaneously with carbamylcholine chloride (0·022 mgm/Kg) and after ten minutes saliva was again collected for a further five minute period. Some saliva dribbled from the mouth of the calf and was not included in the sample.

The lipolytic potency of all the samples was determined as described, using 4·5 ml of milk as substrate. The volumes of the individual samples obtained from the calf were also determined.

Results: The results are given in Table 9.

TABLE 9.—*The effect of sucking and the administration of a cholinergic drug on saliva volume and lipase potency*

Time of collection of saliva	Calf		Goat kid
	Vol. in ml 5 min	Micromols of acid formed	Micromols of acid formed
During rest.....	7·5	18	254
During sucking.....	16·5	221	302
After carbamylcholine.....	17·5 plus	254	326

Conclusions: Sucking on a teat stimulated salivary flow in the calf and caused an increase in lipolytic activity of the saliva of both animals. The administration of carbamylcholine was followed by a further increase in volume in the calf and lipase content in both. The saliva of the kid had a higher lipolytic potency than that of the calf, even when the difference in age is taken into account.

(ii) *A comparison between the effect of cholinergic and anticholinesterase drugs on salivary lipase secretion in a calf*

Method: A six weeks old Friesland bull calf with an oesophageal fistula was injected subcutaneously on alternate days with carbamylcholine chloride and neostigmine methyl sulphate. The dose of carbamylcholine used was 0·022 mgm per Kg and that of neostigmine 0·055 mgm per Kg body weight. Saliva was collected as it escaped from the oesophageal fistula for a five minute period, starting ten minutes after administration of the drug. The lipolytic activity of the saliva was estimated as before by determining the acid formed during incubation of 4·5 ml of milk with 0·5 ml of saliva.

Results: The acid produced in milk by the salivary lipase as secreted after administration of the two drugs is given in Table 10.

Conclusions: From this one experiment it would appear that the subcutaneous injection of 0·022 mgm of carbamylcholine chloride per Kg of body weight is a more powerful stimulant to the secretion of salivary lipase than 0·055 mgm Neostigmine methyl sulphate per Kg.

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TABLE 10.—*The effect of carbamylcholine and neostigmine on salivary lipase secretion in a calf*

Saliva collected after administration of	Date	Micromols of acid formed from 4·5 ml of milk by 0·5 ml of saliva at 37° C during 30 minutes
Carbamylcholine	25.1.55	183
Neostigmine	26.1.55	125
Carbamylcholine	27.1.55	174
Neostigmine	28.1.55	134

(iii) *The effect of feeding milk from the open bucket or through a teat on the salivary lipase secretion of a calf*

It has been shown that the feeding of milk to calves from an open bucket did not stimulate the secretion of abomasal enzymes as readily as the feeding through a teat (Grosskopf, 1959). It was expected that these two methods of feeding should have similar effects on salivary lipase secretion.

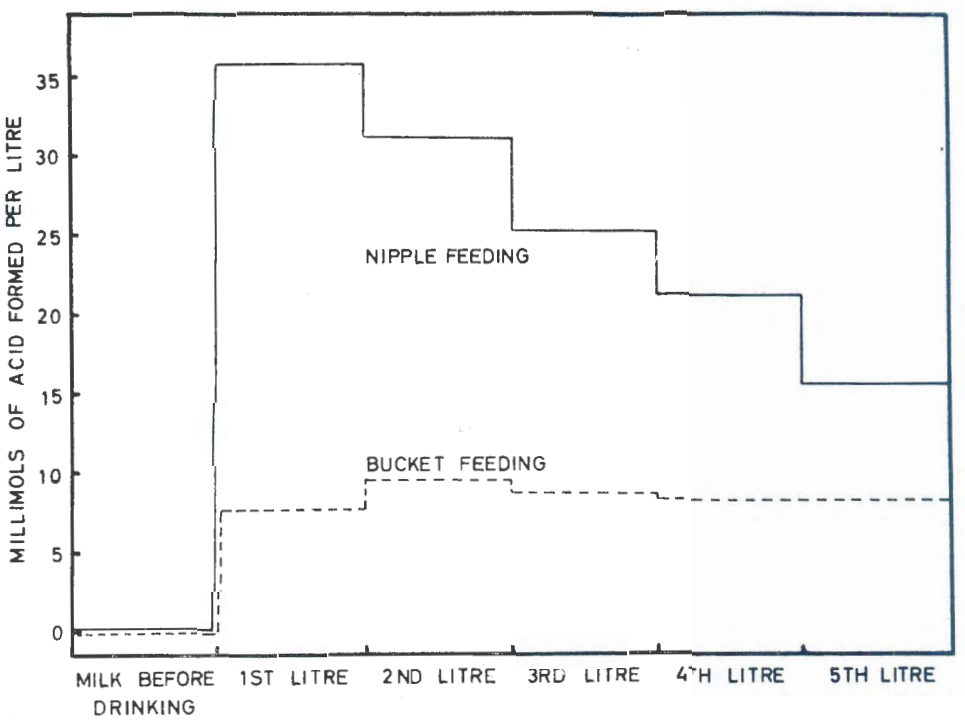

FIG. 6.—The difference between the effects of nipple and bucket feeding on the acid formation in five successive litres of milk drunk by a calf

Method: A 59-day old calf with an oesophageal fistula, fed only on whole milk since birth, was given five litres of milk in separate litre volumes to drink from an open bucket. The milk was preheated to body temperature. The milk squirted from the oesophageal fistula (see Plate 1) and was collected in litre quantities as it escaped. The time taken to drink each litre was recorded. Immediately after collection, samples of the separate litre volumes were cooled in a dish with ice and water to stop further enzyme action. The pH of each sample was determined prior to cooling and the titratable acid of a 100 ml sample of each measured as soon as possible. As the milk appeared to have a higher viscosity after than before drinking, the consistency of the different samples was determined according to the method described by Weiss (1953) for ruminal contents. The same determinations were done on a sample of the milk not drunk by the calf.

The same procedures were followed the next day but the calf was given the milk from a teat bucket.

Results: The results are shown in Tables 17 and 18 (see Appendix) and are plotted graphically in Fig. 6, 7 and 8.

Very close to 30 seconds were taken to drink each of the litre quantities offered in succession from the bucket. On the other hand, it took the calf between 175 and 189 seconds to finish the individual litre quantities of milk through the teat.

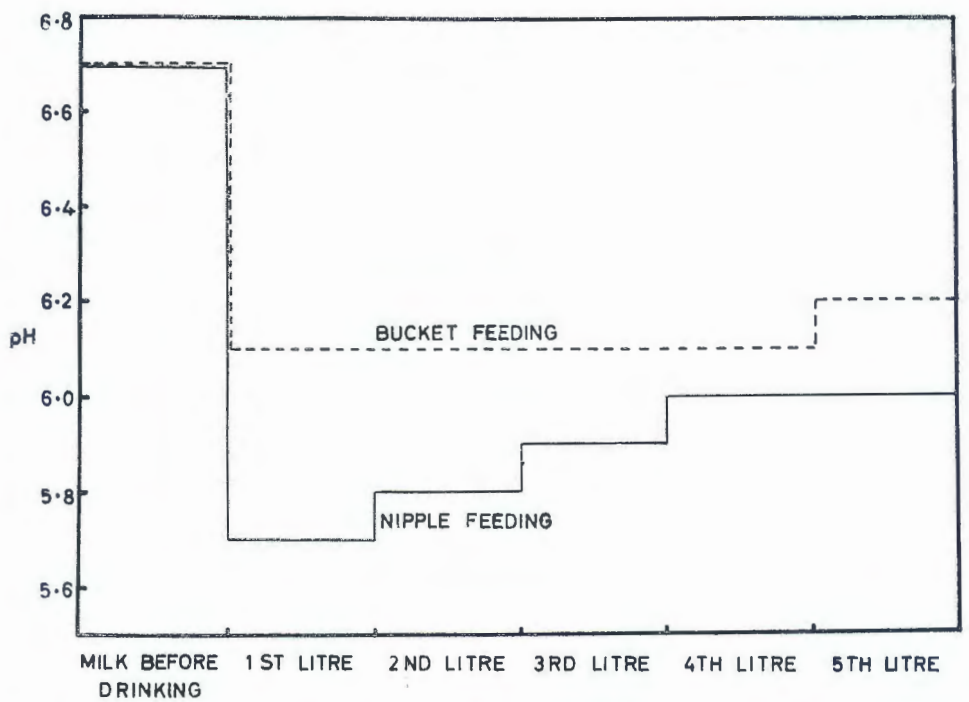

FIG. 7.—The difference between the effects of nipple and bucket feeding on the pH in five successive litres of milk drunk by a calf

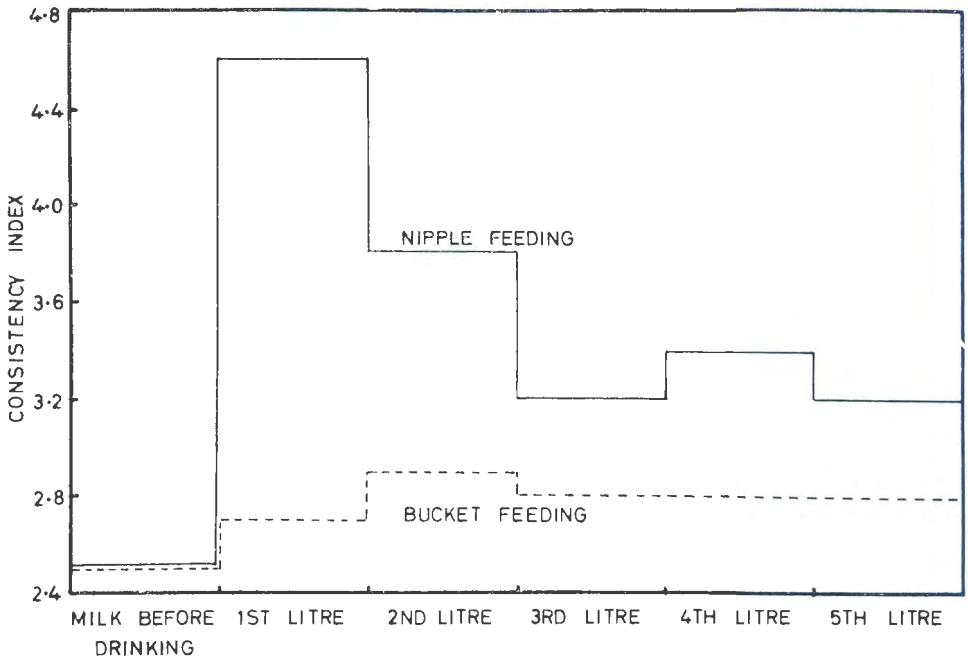

FIG. 8.—The difference in viscosity of five successive litres of milk drunk by a calf from an open bucket or from a nipple

Conclusions: Teat feeding stimulated the secretion of salivary lipase more than did bucket feeding. It took the calf six times as long to drink 5 litres of milk from the teat as from a bucket. The viscosity of the milk was increased in both instances but more so in teat feeding.

A certain amount of secretory fatigue occurred during the drinking of the successive litre quantities. This gradual decline in lipase secretion was more evident with teat feeding than with bucket feeding, but even so, more lipase was secreted during the drinking of the fifth litre of milk from the teat than during the drinking of the first litre from the bucket.

THE GLANDS RESPONSIBLE FOR THE SECRETION OF SALIVARY LIPASE

Several attempts to isolate the lipase from the salivary glands of young ruminants failed. Suspensions and extracts of the parotid, mandibular, buccal and sublingual glands from calves, lambs and goat kids, extirpated immediately after slaughter, gave only negative results when incubated individually or in combinations with cream or milk. A seven-day old lamb was then injected with carbamylcholine and slaughtered five minutes later. On dissection of its head an accumulation of a mucous secretion was noticed on the ventral side of the soft palate and it was decided to include the palatine glands (*glandulae veli palatini*) in the test. These glands proved to be responsible for the secretion of the lipase.

Method: A seven-day old Merino lamb was injected subcutaneously with 0·25 mgm of carbamylcholine chloride and slaughtered five minutes later. The parotid, mandibular, sublingual, dorsal and ventral buccal and palatine glands were extirpated and cut into small sections. To each gm of gland 1 ml of saline was added and the glands ground with a pestle and mortar. The dorsal and ventral buccal glands were minced together. The fibrous particles were removed and 0·5 gm of the more fluid extracts was added individually to 5 ml of cream and incubated in a water bath at 37° C for one hour. Similar mixtures were prepared with heated gland extracts. Two mixtures, viz. mandibular plus parotid and mandibular plus palatine glands were also incubated with milk. For these 0·25 gm of each gland extract was used.

Immediately after incubation the mixtures were titrated with 0·025 N NaOH using phenolphthalein as indicator. The smell of the mixtures was noted before titration.

Results: The titration values obtained are given in Table 19 (see Appendix). As can be seen from this table, acid was only formed in the two mixtures containing palatine gland extract. These two mixtures had a strong butyric acid-like smell after incubation.

Conclusions: The palatine salivary glands are responsible for the secretion of the salivary lipase in young ruminants.

General Discussion and Conclusions

A salivary lipase is excreted by the palatine salivary glands of young calves, lambs and goat kids. It hydrolyzes butyric acid esters of glycerol by splitting off butyric acid. The reaction occurs fairly rapidly as proved by the fact that milk escaping from an oesophageal fistula is already more acid than before drinking. The optimum pH for the enzyme is lower than generally accepted for most lipases. It hydrolyzes tributyrin best at pH 4·5 to 6·0 and is only inhibited completely at a pH of less than 2·4. The pH of the abomasum of young calves after a milk meal has been found to average 3·5 (unpublished data) and it should therefore be possible for the lipase to continue its action in the abomasum for some time after ingestion. The enzyme was found to be most active at temperatures ranging from 37° to 42·5° C.

Serial dilutions of the lipase produced different amounts of acid in the same substrate. When the acid so formed was plotted logarithmically against the different dilutions a straight line could be produced. The amount of acid liberated from a standard substrate could therefore be used as an indication of the potency of the lipase.

No lipase could be detected in the saliva of adult ruminants. In an experiment where saliva was collected from a few calves at varying intervals, it was found that the lipase decreased with increasing age and that it was no longer present in calves older than 77 days. Apparently the type of ration has a marked influence on the secretion of the lipase by calves. In a group of calves raised on a diet low in fat and induced to consume roughage at an early age, only insignificant amounts of lipase could be found to be secreted by calves as young as 21 days old. On the other hand, a calf raised on whole milk only, secreted saliva with an unusually high lipolytic potency at the age of 62 days. It is therefore possible that a high fat intake may

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stimulate the secretion of the salivary lipase or that the consumption of roughages tends to inhibit its secretion. The impression was gained that the lipolytic activity of the saliva of calves fed on whole milk decreased rapidly as they consumed greater proportions of roughage. Since it is well known that roughage has a marked influence on salivary secretion (Clark & Weiss, 1952) it is possible that it also plays some role in the secretion of this salivary lipase.

The secretion of the lipase is stimulated by the drinking of milk when a greater volume of saliva with a higher enzyme content is secreted. Drinking from a teat acts as a better stimulus than drinking from a bucket. As milk drunk by calves coagulates better with rennin than undrunk milk (Dukes, 1947) and nipple feeding stimulates the secretion of rennin more than bucket feeding (Grosskopf, 1959), it is possible that these factors may be jointly responsible for the finer coagula of milk found in the abomasum of calves fed through nipples. The slower calves drink their milk, the more acid is produced from it by salivary lipase. On one occasion a weak calf drank its milk very slowly the day after the operation. The milk collected from the oesophageal fistula was titrated with alkali about half an hour after collection and was found to contain the high level of 80·6 millimols of acid per litre more than the milk before drinking.

Cholinergic drugs markedly stimulated the secretion of the salivary lipase by causing an increased flow of saliva with a higher lipolytic potency. Anticholinesterases had a similar effect but to a lesser degree.

Secretory fatigue could be demonstrated where calves were given excessive volumes of milk to drink. Under normal condition where calves are seldom fed more than 2·5 litres of milk during any one meal this should be of little significance.

As ruminants are normally dependent on rations with relatively low fat levels they are not particularly well adapted to digest fats. In the young, however, their diets may contain as much as 38 per cent of fat or more on a dry matter basis and they should be equipped to make full use of it. It is therefore thought that this salivary lipase serves as a supplement to the pancreatic and intestinal lipase during the stage of high fat intake.

Summary

1. The presence of a lipase in the saliva of young calves, lambs and goat kids has been established.

2. The lipase acts only on triglycerides containing butyrate groups.

3. The optimum temperature for the activity of the salivary lipase lies between 37 and 42·5° C. It is inhibited completely by temperatures below 11° C and above 58° C.

4. The optimum pH for the enzyme was found to be between pH 4·5 and 6·0. It was completely inhibited by pH below 2·4 and above 7·8.

5. A direct relationship exists between the potency of the lipase and the amount of acid it will produce in suitable substrates.

6. The lipolytic potency of the saliva of young calves and goats has been shown to decrease with increasing age. It decreased more rapidly in calves fed high roughage and low fat rations than in calves fed on whole milk only. In calves fed by the conventional methods, the enzyme disappeared from the saliva during the third month of life.

7. The secretion of the enzyme is stimulated by the calf sucking a teat or drinking milk. Sucking milk from a teat acts as a better stimulus than drinking milk from a bucket. Slower intake also stimulates lipase secretion.

8. The injection of cholinergic drugs caused increased flow of saliva with a higher lipase content. Anticholinesterases also stimulated its secretion but to a lesser degree.

9. The salivary lipase is secreted by the palatine salivary glands (*glandulae veli palatini*).

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APPENDIX

TABLE 11.—*Determination of the acid formed by salivary lipase from various triglycerides*

4·5 ml of substrate	0·5 ml of saliva sample	ml of 0·025 N NaOH used for titration		Net acid produced (micromols)
		Active saliva	Boiled saliva	
Triacetin	Saliva A (1: 2)	0·24	0·14	2·5
Tributyrin	,,	8·96	0·28	217
Tricaproin	,,	0·14	0·15	0
Tripalmitin	,,	0·09	0·10	0
Tristearin	,,	0·09	0·12	0
Triolein	,,	0·48	0·49	0
Triacetin	Saliva A (1: 8)	0·16	0·12	0·05
Tributyrin	,,	2·73	0·24	62
Tricaproin	,,	0·12	0·11	0
Tripalmitin	,,	0·09	0·08	0
Tristearin	,,	0·09	0·09	0
Triolein	,,	0·40	0·38	0
Triacetin	Saliva B	0·62	0·24	9·5
Tributyrin	,,	26·22	0·80	636
Tricaproin	,,	0·23	0·22	0
Tripalmitin	,,	0·20	0·19	0
Tristearin	,,	0·58	0·56	0
Triolein	,,	0·93	0·90	0
Triacetin	Saliva B (1: 2)	0·35	0·22	3
Tributyrin	,,	14·13	0·62	338
Tricaproin	,,	0·20	0·20	0
Tripalmitin	,,	0·17	0·18	0
Tristearin	,,	0·47	0·52	0
Triolein	,,	0·84	0·78	1·5

TABLE 12.—*Citrate and phosphate buffers prepared*

(*a*) *Citrate buffers*

0·1M Sodium citrate (ml)	0·1N HCl (ml)	0·1N NaOH (ml)	Theoretical pH	pH of substrate as measured
3·33	6·67	—	2·27	2·42
5·0	5·0	—	3·69	3·68
8·0	2·0	—	4·65	4·62
8·0	—	2·0	5·31	5·31
5·5	—	4·5	6·34	6·13

TABLE 12 (*contd.*)

(*b*) *Phosphate buffers*

0·1M Na_2HPO_4	0·1M KH_2PO_4	Theoretical pH	pH of substrate as measured
0·25	9·75	5·29	5·33
1·0	9·0	5·91	5·80
5·0	5·0	6·81	6·86
9·5	0·5	8·04	7·81

TABLE 13.—*Results of titration of buffered tributyrin emulsions after incubation with calf saliva*

pH of substrate	ml of 0·025 N NaOH used for titration		Net acid produced in micromols
	Active saliva	Boiled saliva	
2·42	13·2	12·8	10
3·68	23·4	12·7	268
4·62	25·8	12·6	330
5·31	22·3	8·8	338
6·13	15·2	1·9	333
5·33	24·2	12·8	285
5·80	24·2	12·5	293
6·86	16·3	6·4	243
7·81	1·6	1·2	10

TABLE 14.—*Effect of temperature on lipolytic activity of saliva*

Temperature of incubation	ml of 0·025 N NaOH used for titration	
	4·5 ml milk + 0·5 ml saliva	4·5 milk + 0·5 ml boiled saliva
2° C.	4·34	—
5° C.	4·34	—
13·5° C.	4·65	—
22° C.	5·64	4·34
30° C.	6·64	—
32·5° C.	7·01	—
34° C.	7·30	—
36·5° C.	7·34	4·33
39° C.	7·57	—
42° C.	7·54	—
45° C.	7·34	4·32
47° C.	7·00	—
52·5° C.	6·50	—
53° C.	6·37	4·35
58° C.	4·36	—
Average	—	4·34

TABLE 15.—*The micromols of acid formed by serial dilutions of calf saliva from tributyrin substrate*

Dilution of saliva	ml of 0·025 N NaOH used for titration		Net acid produced (micromols)
	Active saliva	Boiled saliva	
Undiluted	18·57	0·29	457
1 in 2	10·83	0·27	264
1 in 4	7·0	0·26	168
1 in 8	4·42	0·25	104
1 in 16	2·83	0·24	65
1 in 32	1·48	0·23	31
1 in 64	1·40	0·22	27
1 in 128	0·74	0·22	13
1 in 256	0·50	0·19	8
Undiluted	16·98	0·30	417
1 in 2	10·47	0·26	255
1 in 4	6·63	0·25	160
1 in 8	3·94	0·26	92

TABLE 16.—*A comparison between the amounts of acid produced by salivary lipase in milk or tributyrin substrates*

Dilution of saliva	ml of 0·025 N NaOH used for titration				Micromols of acid produced	
	Milk + saliva	Milk + boiled saliva	Tributyrin + saliva	Tributyrin + boiled saliva	Milk	Tributyrin
Undiluted	7·07	3·58	13·54	0·33	87	330
1 in 2	5·91	3·56	9·28	0·31	59	224
1 in 4	5·02	3·54	5·71	0·30	37	135
1 in 8	4·70	3·54	3·54	0·28	29	82
1 in 16	4·15	3·52	2·25	0·27	16	50

TABLE 17.—*Changes in pH, titratable acidity and consistency of five successive litres of milk drunk by a calf from an open bucket*

No. of milk sample	Volume of sample (ml)	Time taken to consume (sec)	Milk collected from oesophagus			
			pH	0·1 N NaOH used for titration of 100 ml (ml)	Net acid produced in 100 ml (micromols)	Consistency index
1	1000	27	6·1	26·0	760	2·7
2	1000	29	6·1	27·8	940	2·9
3	1000	30	6·1	27·0	860	2·8
4	1000	30	6·1	26·8	840	2·8
5	1000	33	6·2	26·8	840	2·8

Milk before drinking: pH..... 6·7
Titratable acid..... 1,840 micromols/100 ml
Consistency index..... 2·5

TABLE 18.—*Changes in pH, titratable acidity and consistency of five successive litres of milk drunk by a calf from a nipple*

No. of milk sample	Volume of sample (ml)	Time taken to consume (sec)	Milk collected from oesophagus			
			pH	0·1 N NaOH used for titration of 100 ml (ml)	Net acid produced in 100 ml (micromols)	Consistency index
1.....	1000	169	5·7	54·0	3570	4·6
2.....	1000	171	5·8	49·5	3120	3·8
3.....	1000	174	5·9	43·3	2500	3·2
4.....	1000	180	6·0	40·3	2100	3·4
5.....	1000	193	6·0	33·6	1530	3·2

Milk before drinking: pH..... 6·7
Titratable acid..... 1830 micromols/100 ml
Consistency index..... 2·5

TABLE 19.—*The acid produced by incubating extracts from the different salivary glands of a lamb with cream*

Extract prepared from	0·025 N NaOH used for titration (ml)		Net acid produced (micromols)	Smell
	Fresh extract	Heated extract		
Parotid.....	4·3	4·3	—	—
Mandibular.....	4·1	4·1	—	—
Buccal.....	4·1	4·2	—	—
Sublingual.....	3·9	4·0	—	—
Palatine.....	16·2	4·5	293	Butyric acid
Mandib. + Parotid....	4·2	4·1	—	—
Mandib. + Palat.....	14·8	4·3	263	Butyric acid