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LACTOSE CRYSTALLIZATION IN DRY PRODUCTS OF MILK. I. A METHOD FOR ESTIMATING THE DEGREE OF CRYSTALLIZATION

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In continuing the study (2) on the state of lactose in dry products of milk, a different approach to determine the degree of crystallization of lactose has been investigated. In a saturated solution of lactose the *alpha* and *beta* forms of lactose are in equilibrium with one another and with the crystalline α-lactose hydrate solid phase. Crystalline α-lactose hydrate when added to such a solution will not dissolve. On the other hand, amorphous or "glass" lactose, which is a supersaturated solution of lactose, will dissolve because this essentially amounts to diluting the lactose "glass." Likewise, crystalline β-lactose will dissolve because of its greater solubility in water than lactose hydrate (5). Therefore, when a dry product of milk, which may contain α- and β-lactose in either the amorphous or crystalline state or both, is added to a saturated solution of lactose, only crystalline α-lactose hydrate will remain undissolved. If the suspension is centrifuged quickly to prevent any appreciable seeding or crystallization from occurring, then, by analyzing the supernatant liquid for the amount of lactose which has gone into solution and also for the total lactose in the dry sample, the degree of crystallization can be calculated.

METHOD

A solution of lactose, saturated at 25° C., was prepared by dissolving exactly 22.8 g. of lactose hydrate (c.p.) in 100.0 g. of distilled water. The solution was covered with a thin layer of toluene and allowed to stand at least 24 hr. before use. Exactly 25.00 g. of the saturated solution were weighed into a 50-ml. Pyrex centrifuge tube and the content brought to the temperature of 25° C. in a constant-temperature water bath. An accurately weighed quantity of dry product of milk (5.00 g. for nonfat dry milk solids or 3.5 g. for dry whey solids) was quickly added to the saturated solution. The tube was tightly stoppered with a rubber stopper, shaken in the water bath for 1 min. at the rate of about two short excursions per second and then immediately centrifuged for 2 min. at about 1,500 r.p.m. The supernatant liquid was carefully decanted into a dry test tube. Total solids were determined on the supernatant liquid using the

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A.O.A.C. method for fluid milk (1). Lactose in the supernatant liquid was determined by the Hinton-Macara method (3) by accurately weighing an aliquot of 3.5 to 4.0 g. into a 100-ml. volumetric flask and diluting to volume after precipitating the proteins with 5.00 ml. of precipitating reagent. Twenty-five ml. of the clear filtrate were oxidized with the chloramine-T reagent.

From the per cent of water in the supernatant liquid, as calculated from the total solids content, the lactose concentration can be expressed as grams of lactose monohydrate per 100 g. of water. The per cent of the total lactose that is in the crystalline α-hydrate form may be calculated as follows:

Crystalline lactose hydrate (as per cent of total lactose) =

$$100 - \frac{20.4\ (C - 22.8)\ 100}{WL}.$$

C is the concentration of lactose in the supernatant liquid in grams per 100 g. of water, W is the weight of dry sample used, and L is the per cent of lactose in the dry milk. The factor 20.4 represents the number of grams of water in 25 g. of the saturated lactose solution.

RESULTS AND DISCUSSION

The accuracy of the present method for the determination of crystalline α-lactose hydrate is dependent, to a large extent, upon the amount of crystallization that may take place when the dry milk product is shaken with the saturated lactose solution. Except in the case where all of the lactose is present as the crystalline α-hydrate, the resultant solution is supersaturated to various degrees with respect to lactose. In the absence of nuclei of α-lactose hydrate, spontaneous crystallization probably will not occur, since the degree of supersaturation is well below the super-solubility of lactose (7). However, with products containing both α-lactose hydrate and amorphous lactose, crystallization may take place in the 1-min. shaking period and during part of the centrifuging period. Therefore, a series of experiments was conducted to investigate this factor. Crystalline α-lactose hydrate powder (c.p.), crystalline β-anhydride (c.p.) and a spray-process nonfat dry milk solids in which the lactose is amorphous were used to prepare mixtures of varying concentrations of crystalline α-hydrate to correspond to different degrees of crystallization of the lactose. Each mixture contained a total of about 2.5 g. of lactose, approximately the same quantity as present in the amount of dry milk sample used for analysis. The concentration of β-lactose was maintained at the equilibrium value and assumed to be unaltered until all of the α-lactose has been crystallized. This assumption is reasonable, since the rate of crystallization of α-lactose in dry products of milk probably is much faster than the rate of conversion of *beta* to *alpha* form. The mixtures were analyzed by the method described above. The amount of lactose which dissolved in the saturated solution is compared with the theoretical soluble lactose, which is the sum of the β-lactose and the lactose contributed by the nonfat dry milk solids. Data are presented in table 1.

It may be noted that lactose hydrate (mixture 1) does not dissolve in the saturated solution. For mixture 7 containing only 5.00 g. of nonfat dry milk

solids 97 to 99 per cent of the total lactose dissolved, indicating little or no crystallization. Mixture 6 consisting of 1.0 g. of crystalline α-hydrate and 1.5 g. of β-lactose shows slight crystallization, since only 94 to 96 per cent of the β-lactose actually dissolved. On the other hand, for mixtures containing crystalline α-lactose hydrate, β-anhydride and amorphous lactose, which is an equilibrium mixture of α- and β-lactose, recoveries are not as good, a fact which definitely indicates crystallization of the α-lactose from solution. The lower degree of crystallization in the case of mixture 6 may be attributed to the fact that the β-lactose must undergo mutarotation to the *alpha* form before crystallization can take place.

TABLE 1

Results of seeding experiments using crystalline α-lactose hydrate, spray process nonfat dry milk solids (ndms) and β-lactose

Mixture	Composition			Lactose soluble (as g. lactose hydrate/100 g. H_2O)				Recovery in trial:		
	Lactose · H_2O	ndms	Beta	Theoretical	Found in trial:			1	2	3
					1	2	3			
	(*g.*)	(*g.*)	(*g.*)					(%)	(%)	(%)
1	2.5	0	0	0	0	0	0	.....	.....	.....
2	0.2	4.0	0.3	11.6	10.8	10.8	10.6	93	93	91
3	0.4	3.0	0.6	10.6	9.8	9.1	.....	93	86	.....
4	0.6	2.0	0.9	9.60	9.6	8.6	8.8	100	90	92
5	0.8	1.0	1.2	8.72	8.0	8.1	7.8	92	93	89
6	1.0	0	1.5	7.59	7.3	7.1	7.2	96	94	95
7	0	5.0	0	12.6	12.4	12.2	12.2	99	97	97

In an effort to minimize this seeding effect, various experimental conditions were studied. A working temperature of 39° C. as well as doubling the amount of each component was investigated, but no improvement was found. Also studied was the effect of addition of a dye, crystal violet. Leighton and Peters (7) found that crystal violet was one of the six dyes out of 39 studied that have a retarding effect on lactose crystallization. The concentration of the dye used was not reported by the authors. Accordingly, a fairly strong solution (0.5 per cent) was prepared in a saturated lactose solution and different aliquots of this diluted with saturated lactose solution to give a total of 25.0 g. The results obtained indicate no retarding action on crystallization.

The method has been applied to a study of the degree of lactose crystallization in various dry products of milk. Troy and Sharp (8) have previously used the seeding test of Hudson and Brown (6) and examination by means of a polarizing microscope to study the state of lactose in dry milks. These tests show only the presence or absence of lactose hydrate crystals but give no indication of the quantity present. Data in table 2 show the degrees of crystallization of lactose in nonfat dry milk solids and dry whey solids as determined by the present method. Nonfat dry milk solids samples 1 to 6 inclusive, were relatively fresh products containing less than 4 per cent moisture. Samples 7 to 11, inclusive, which had been stored in paper bags under high relative humidity, had

moisture contents of 8 to 9 per cent. For the samples of dry whey solids the α- and β-lactose contents were determined by the solubility method (3).

In all samples of spray- and roller-process nonfat dry milk solids of low moisture content and in dry whey solids samples 1 to 3, inclusive, the lactose is almost entirely in the amorphous state. In these products the ratio of β- to α-lactose is about 1.6 to 1, as determined by the solubility method (3). These results confirm the observations of Troy and Sharp (8). In the samples of

TABLE 2

Degree of crystallization of lactose in nonfat dry milk solids and dry whey solids

Sample	% of total lactose cryst'd as *alpha* hydrate	*Alpha-beta* lactose (as % total lactose)	
		Alpha	*Beta*
	Nonfat dry milk solids		
1 (spray)	5.2	.....	.....
2 (spray)	3.5	.....	.....
3 (spray)	4.2	.....	.....
4 (roller)	2.6, 1.1	.....	.....
5 (roller)	1.4	.....	.....
6 (roller)	0.5	.....	.....
7	39.6	.....	.....
8	49.4	.....	.....
9	51.6	.....	.....
10	63.2	.....	.....
11	50.6	.....	.....
	Dry whey solids		
1	6.7	38.0	62.0
2	4.1, 4.1	37.4	62.6
3	5.8, 5.8	40.4	59.6
4	70.0, 69.5	71.5	28.5
5	70.0, 70.0	69.9	30.1
6	91.1	89.0	11.0
7	87.2	86.8	13.2
8	82.6	82.7	17.3
9	76.0	77.2	22.8
10	78.3	79.8	20.2
11	83.6	84.1	15.9

nonfat dry milk solids of high moisture content the lactose is crystallized to the extent of from 39.6 to 63.2 per cent. In the remaining samples of dry whey solids in which crystallization of the lactose was induced during processing to reduce the hygroscopicity of the final product, the amount of crystalline α-lactose hydrate varies from 69.7 to 91.1 per cent of the total lactose. These values agree closely with the total α-lactose as determined by the solubility method (3). This fact indicates that the α-lactose in these samples has been completely crystallized and that the rate of crystallization of α-lactose is greater than the rate of isomerization of the *beta* to the *alpha* in the later stage of crystallization. For this type of dry milk product a determination of the amount of crystalline α-lactose hydrate also would indirectly give the β-lactose content.

Application of this method to the study of the effects of moisture and 95 per cent ethyl alcohol on lactose crystallization will be described in another paper.

SUMMARY

A method is described for estimating the amount of crystalline α-lactose hydrate in dry products of milk. It is based upon the fact that crystalline α-lactose hydrate is insoluble in a saturated lactose solution, while amorphous lactose and crystalline β-anhydride are soluble. The simultaneous presence of crystalline lactose hydrate and amorphous lactose decreases somewhat the accuracy of the method due to crystallization.

It has been shown by this method that the lactose is non-crystalline in fresh nonfat dry milk solids of low moisture content and also in some dry whey solids. For nonfat dry milk solids which had been allowed to absorb considerable amounts of moisture at high relative humidities and for dry whey solids in which crystallization had been induced during manufacturing the method shows a large percentage of crystalline α-lactose hydrate.

REFERENCES

(1) Association of Official Agricultural Chemists. *Official and Tentative Methods of Analysis.* 6th ed. p. 207. Washington, D. C. 1945.

(2) Choi, R. P., O'Malley, C. M., and Fairbanks, B. W. Moisture Studies on Dry Products of Milk. II. Estimating Water of Crystallization of Alpha Lactose in Dry Whey Solids. J. Dairy Sci., **31**: 619–625. 1948.

(3) Choi, R. P., Tatter, C. W., O'Malley, C. M., and Fairbanks, B. W. A Solubility Method for the Determination of Alpha and Beta Lactose in Dry Products of Milk. J. Dairy Sci., **32**: 391–397. 1949.

(4) Hinton, C. L., and Macara, T. The Determination of Aldose Sugar by Means of Chloramine-T with Special Reference to the Analysis of Milk Products. Analyst, **52**: 668–688. 1927.

(5) Hudson, C. S. Further Studies on the Forms of Milk Sugar. J. Am. Chem. Soc., **30**: 1767–1783. 1908.

(6) Hudson, C. S., and Brown, F. C. The Heat of Solution of the Three Forms of Milk Sugar. J. Am. Chem. Soc., **30**: 960–971. 1908.

(7) Leighton, A., and Peter, P. N. Factors Influencing the Crystallization of Lactose. Proc. World's Dairy Congress, **1**: 477–485. 1923.

(8) Troy, H. C., and Sharp, P. F. Alpha and Beta Lactose in Some Milk Products. J. Dairy Sci., **13**: 140–157. 1930.

LACTOSE CRYSTALLIZATION IN DRY PRODUCTS OF MILK. II. THE EFFECTS OF MOISTURE AND ALCOHOL

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In dry products of milk manufactured by the ordinary spray and roller processes experimental evidence (2, 6) indicates that the lactose is amorphous. Upon absorption of sufficient moisture, crystallization and mutarotation takes place. The amount of moisture that must be present before lactose can crystallize as the monohydrate has not been investigated adequately, presumably because of the lack of a quantitative method for measuring lactose crystallization.

Hauser (3) and Hauser and Hering (4) in their studies of the relation of moisture and heat of wetting of dry whole milk found a break in the curve at about 3 per cent moisture. They interpreted this to be the point at which the lactose becomes completely hydrated. Lampitt and Bushill (5) found considerable differences between spray- and roller-process dry whole milk in the amount of fat extractable by organic solvents. They believed that the lactose in the roller-process product is crystalline and that in the spray product is amorphous. In the latter product absorption of moisture either from a humid atmosphere or from 95 per cent ethyl alcohol causes the lactose to crystallize and frees the fat from its impermeable coating of amorphous lactose. The stage when the fat becomes "free" was believed to coincide with the crystallization of lactose. The moisture content necessary to liberate the fat, termed the "critical moisture content," was found to be 8.6 to 9.2 per cent for normal dry whole milk and higher for products of lower fat content.

With the development of a method for estimating crystalline α-lactose hydrate (2), the effect of moisture and 95 per cent ethyl alcohol on lactose crystallization in dry products of milk can be easily studied.

LACTOSE CRYSTALLIZATION AT DIFFERENT MOISTURE LEVELS

Samples of spray- and roller-process nonfat dry milk solids and spray-process dry whole milk of varying moisture contents were prepared by allowing approximately 4 lb. of each product to absorb moisture from a stream of warm air saturated with water vapor. To prevent local saturation, the milk solids were given constant mechanical mixing in a small rotary mixer during the addition of moisture. The Karl Fischer method (1) was used to follow moisture absorption. As soon as the desired moisture content had been reached, a sample was collected and stored in an air-tight container at room temperature, which varied from 20 to 25° C. Several samples of each product covering the range from low to high moisture content were prepared in this manner. These samples were analyzed at known time intervals for crystalline α-lactose hydrate. Results for

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spray-process nonfat dry milk solids, roller-process nonfat dry milk solids and spray-process dry whole milk are presented in tables 1, 2 and 3, respectively.

From the results obtained, there appears to be a "critical" moisture level for each product above which crystallization of the lactose takes place readily and extensively. For spray-process nonfat dry milk solids (table 1) at moisture content of 7.5 per cent or below, less than 10 per cent of the lactose crystallized, even after 30 days of storage. Above this level the degree of crystallization

TABLE 1

Results on lactose crystallization in spray-process nonfat dry milk solids at different moisture levels

Days stored	Lactose crystallized at different moisture levels (as % of total lactose)						
	4.7	7.1	7.5	9.0	9.8	11.3	13.6
0	0.9	2.1	1.0	13.5	22.4	41.8	61.4
1	2.1	6.3	3.5	20.4	32.9	55.0	63.3
2	1.0	.....	1.5	24.5	32.0	60.4	66.9
3	4.9	2.9	1.0	21.4	33.7	.....	.....
6	2.5	7.9	9.1	22.0	43.0	60.4	66.9
9	.....	.....	6.6	29.6	47.6	60.4	66.9
14	.....	5.3	3.5	33.0	53.5	66.5	67.7
30	3.2	7.1	8.5	41.5	61.2	67.4	66.9

TABLE 2

Results on lactose crystallization in roller-process nonfat dry milk solids at different moisture levels

Days stored	Lactose crystallized at different moisture levels (as % of total lactose)					
	3.2	5.6	7.4	9.8	11.7	14.2
0	1.0	0	0	34.0	62.6	74.4
4	.....	8.2	9.5	50.5	76.0	80.8
14	.....	4.2	9.3	61.7	76.0	79.0
30	1.0	6.0	9.3	68.3	79.5	79.9

TABLE 3

Results on lactose crystallization in spray-process dry whole milk at different moisture levels

Days stored	Lactose crystallized at different moisture levels (as % of total lactose)						
	2.7	4.9	6.1	8.7	10.3	12.2	13.6
0	6.2	4.5	7.5	45.9	59.5	72.2	72.7
4	.....	.....	.....	63.3	67.8	73.5	78.9
14	7.3	4.5	7.5	62.3	67.8	73.5	77.7
28	6.2	9.0	7.5	64.7	67.8	73.5	73.5

increased with moisture content until at 13.6 per cent approximately 67 per cent of the lactose has crystallized. Similarly, for roller-process nonfat dry milk solids (table 2) and spray-process dry whole milk (table 3) very little lactose crystallization took place at and below 7.4 and 6.1 per cent, respectively. When the extent of lactose crystallization was plotted against moisure content S-shape curves were obtained which indicated a "critical" moisture level of about 6.5 to 7.0 per cent for spray-process dry whole milk and about 7.5 to 8.0 per cent for

both spray- and roller-process nonfat dry milk solids. Apparently, the "critical" moisture level must be reached before the lactose molecules can crystallize. As the moisture content is raised above this point crystallization proceeds rapidly and extensively. It is interesting to note that both spray- and roller-process nonfat dry milk solids, which differ considerably in regard to the degree of dispersibility of their proteins but which have essentially the same lactose content, possess almost the same "critical" moisture level. On the other hand, spray-process dry whole milk with a lower lactose content shows a lower "critical" moisture level. These results indicate that the "critical" moisture level may depend on the amorphous lactose content of the product.

The rate of lactose crystallization at different moisture levels also is worth noting. For each product very little crystallization took place even after 30 days when the moisture content was below the "critical" level. Above that, the rate of crystallization increased with moisture content. For example, for spray-process nonfat dry milk solids (table 1) at 11.3 and 13.6 per cent moisture, the maximum extent of crystallization was approached after 13 and 2 days, respectively, while at 9.0 and 9.8 per cent, crystallization was still progressing after 30 days. Similar observations can be made from the data presented for the other two products.

LACTOSE CRYSTALLIZATION IN ALCOHOL

Crystallization of the lactose of spray-process dry whole milk in 95 per cent ethyl alcohol next was studied in relation to fat liberation. In a series of experiments, varying volumes of 95 per cent ethanol (25, 30, and 35 ml.) and of anhydrous methanol (25 and 30 ml.), as well as a mixture of 25 ml. 95 per cent ethanol and 1 ml. water, were added to 25-g. portions of a spray-process dry whole milk and the mixtures allowed to stand for 30 min. at room temperature. The mixtures, which caked during this period, then were broken up with a stirring rod. The alcohol was evaporated under vacuum at 60 to 65° C. Moisture, solubility index (1), "free" fat and degree of lactose crystallization were determined. Two methods were used for determining free or extractable fat. Method 1 was similar to that used by Lampitt and Bushill (5) and is as follows: Two g. of the treated dry whole milk sample were allowed to stand at room temperature in 100 ml. of carbon tetrachloride for 18 hr. with occasional shaking. The mixture then was filtered and the residue washed twice with about 10-ml. portions of carbon tetrachloride. The quantity of fat extracted was determined by evaporating the combined filtrate and washings in an aluminum dish and weighing the dried residue. In method 2, one g. of dry whole milk was extracted with 100 ml. of ethyl ether in a Soxhlet extractor for 1 hr. The solvent was evaporated and the residue weighed as in method 1. Data are presented in table 4.

The results indicate that very little of the lactose is crystallized as the α-hydrate from treatment with 95 per cent ethanol. Similar results were obtained with methanol, which, because of its low water content (0.5 per cent), would not be expected to promote the formation of crystalline α-lactose hydrate. Addition of more water to 95 per cent ethanol, however, increased the amount

of crystalline α-lactose hydrate formed. On the other hand, solubility index, which indicates the degree of insolubility of the dry milk, and extractable fat were greatly affected by both methanol and 95 per cent ethanol. The solubility index increased from the original value of 0.1 ml. to 10.0 ml. Likewise, whereas only about 30 per cent of the fat was extractable in the original whole milk sample, after alcoholic treatment the fat became almost quantitatively extractable with the organic solvents.

These results seem to indicate that the freeing of the fat by treatment with 95 per cent alcohol probably is not caused by crystallizaion of the lactose as the monohydrate, although it is possible that methanol and 95 per cent ethanol may bring about other physical changes in the lactose "glass" of dry whole milk which may aid in the liberation of the fat. Moreover, the large difference between spray- and roller-process dry whole milk with respect to the free fat

TABLE 4

The effects of methanol and 95% ethanol on the extractable fat, solubility index and lactose crystallization of spray-process dry whole milk

Alcohol added	Final moisture	Extractable fat[a]		Solubility Index	Crystalline α lactose hydrate (as % of total lactose)
		Method 1	Method 2		
(*ml./25 g. dry milk*)	(%)	(%)	(%)	(*ml.*)	
0	2.1	8.0	8.5	0.1	5.2
25 ethanol	1.7	24.8	25.3	9.0	8.2
30 ethanol	1.5	25.0	25.6	9.0	8.4
35 ethanol	1.6	25.0	25.5	9.0	8.4
25 ethanol + 1 ml H_2O	2.3	24.8	25.0	10.0	28.5
25 methanol	1.4	25.4	25.7	10.0	4.3
30 methanol	1.6	25.2	25.6	10.0	5.0

[a] Total fat by the Mojonnier method—26.5%.

content also cannot be attributed to difference in the degree of lactose crystallization, since analyses of several roller-process dry whole milk showed that the lactose in this type of product also is amorphous. It may be reasonable to suggest that fat liberation is due to coagulation of the milk proteins. This would explain the fact that fat in roller-process dry whole milk is quantitatively extractable, since coagulation of the proteins during processing destroys the colloidal properties of the proteins to form a protective membrane around the fat. Similarly, 95 per cent ethanol, methanol or high moisture content will liberate the fat in spray-process dry whole milk, because each of these causes extensive coagulation of the milk proteins as evidenced by the high solubility indices.

SUMMARY

The effects of moisture and 95 per cent ethanol on the crystallization of lactose in dry products of milk have been studied. Results indicated a "critical" moisture level of about 6.5 to 7.0 per cent for spray-process dry whole milk and 7.5 to 8.0 per cent for both spray- and roller-process nonfat dry milk solids. Below

these critical moisture levels, very little crystallization took place, while, above these levels, crystallization of the lactose proceeded rapidly and extensively.

For spray-process dry whole milk, it has been found that treatment with methanol and 95 per cent ethanol caused very little of the lactose to crystallize as the α-monohydrate. Increasing the water content of the alcohol increased crystallization. It is suggested that the freeing of the fat in spray-process dry whole milk by 95 per cent ethanol results from coagulation of the milk proteins and not from crystallization of the lactose as the monohydrate.

REFERENCES

(1) American Dry Milk Institute, Inc. *The Grading of Dry Whole Milk and Sanitary and Quality Standards.* Chicago, Illinois. 1947.

(2) Choi, R. P., Tatter, C. W., and O'Malley, C. M. Lactose Crystallization in Dry Products of Milk. I. A Method for Estimating the Degree of Crystallization. J. Dairy Sci., **34**: 845–849. 1951.

(3) Hauser, E. A. Eine neue Methode der Bestimmung des Feuchtigkeitgehaltes von Milchpulver und es Zustandes seiner kolloidalen Bestandteile. Proc. World's Dairy Congress, **2**: 1154–1156. 1923.

(4) Hauser, E. A., and Hering, H. Reaction thermodynamiques chez les poudres de lait. Leur emploi pour le dosage rapide de leur teneur en eau. Lait, **4**: 388–394. 1924.

(5) Lampitt, L. H., and Bushill, J. H. The Physico-chemical Constitution of Spray-dried Milk Powder. J. Soc. Chem. Ind., **50**: 45T. 1931.

(6) Troy, H. C., and Sharp, P. F. Alpha and Beta Lactose in Some Milk Products. J. Dairy Sci., **13**: 140–157. 1930.

STUDIES ON MILK FEVER IN DAIRY COWS. III. FURTHER STUDIES ON THE EFFECT OF VITAMIN D ON SOME OF THE BLOOD CHANGES AT PARTURITION AND THE COMPOSITION OF COLOSTRUM IN NORMAL AND MILK–FEVER COWS

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The effects of feeding, respectively, one and two million units of vitamin D in the form of irradiated dry yeast[3] daily for 1 mo. before parturition on the changes in blood Ca, P, Mg, phosphatase and vitamin D in normal and milk-fever cows were described in a previous report (3). Data were included showing the changes in the blood serum protein and plasma carotene, vitamin A and ascorbic acid at parturition in these cows. The composition of colostrum secreted by a small number of normal and milk-fever cows fed vitamin D at these two levels also was shown.

No marked effect on the blood picture could be attributed to vitamin D feeding except a slight prepartum increase in serum Ca and P when two million units of vitamin D were fed daily along with a high-mineral grain ration containing 5 per cent steamed bonemeal. This was accompanied by a slight decrease in serum Mg. However, the small prepartum increase in serum Ca and P had disappeared within 12 hr. postpartum in normal as well as milk-fever cows.

Vitamin D assays of the blood showed that when one million units of vitamin D were fed daily the blood level was about double the normal level at parturition and about four times normal when two million units were fed for 1 mo. prepartum.

Despite the relatively high blood vitamin D levels in the treated cows, no lowering of milk fever incidence was observed (2). It was reasoned that if vitamin D is to be effective in milk fever prevention it must exert sufficient calcemic effect to maintain a normal blood Ca level through the critical 18- to 24-hr. postpartum period. Therefore, it was decided to try still larger amounts of vitamin D with the aim of attaining this objective.

RESULTS AND DISCUSSION

From September, 1945, until April, 1946, there were 18 available mature Jersey cows due to freshen in the Main and Pasture Farm Experiment Station herds. These were divided into two groups. One group served as controls and the other group was fed five million units of vitamin D daily by capsule in the

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[1] Now located at the U. S. D. A., Bureau of Animal Industry, Animal Disease Station, Beltsville, Md.

[2] Now Associate Director, Ohio Agricultural Experiment Station.

[3] This work was supported and the irradiated yeast provided by Standard Brands, Inc., to whom the authors herewith extend grateful appreciation.

TABLE 1

The effect of feeding 5 million units of vitamin D daily for 2 wk. prepartum and 3 days postpartum on some of the blood changes in normal and milk-fever cows

Group	No. of cows	Blood constituent	Mg. % of blood constituents at:					
			2 wk. pre-fresh	12 hr. pre-fresh	12 hr. post-fresh	Milk fever	3-day post-fresh	1 wk. post-fresh
Normal parturitions								
Control	6	Ca	10.73 ± 0.62	9.90 ± 0.68	9.44 ± 1.51	.....	9.94 ± 0.92	10.41 ± 1.10
Vit. D-fed	5	Ca	10.94 ± 0.32	11.97 ± 0.72	9.34 ± 1.26	.....	9.08 ± 1.32	11.13 ± 0.80
Control	6	P	6.09 ± 0.62	6.78 ± 1.91	4.66 ± 1.06	.....	5.88 ± 1.59	5.12 ± 0.89
Vit. D-fed	5	P	6.05 ± 0.52	7.72 ± 0.65	5.15 ± 1.25	.....	6.36 ± 1.80	5.61 ± 0.74
Control	6	Mg	2.80 ± 0.27	3.19 ± 0.58	3.13 ± 0.54	.....	2.41 ± 0.89	2.41 ± 0.33
Vit. D-fed	5	Mg	2.50 ± 0.24	2.54 ± 0.01	3.39 ± 0.69	.....	3.02 ± 1.06	2.24 ± 0.98
Milk-fever parturitions								
Control	1	Ca	8.94	.....	6.50	4.30	9.30	11.20
Vit. D-fed	4	Ca	10.79 ± 0.82	11.96 ± 1.17	9.51 ± 1.66	4.66 ± 1.11	8.71 ± 1.45	10.00 ± 0.90
Control	1	P	4.33	.....	1.50	1.68	4.60	3.91
Vit. D-fed	4	P	6.01 ± 1.26	8.86 ± 1.39	5.81 ± 0.95	3.02 ± 0.98	7.79 ± 3.40	6.63 ± 1.08
Control	1	Mg	2.84	.....	3.24	3.40	2.24	2.58
Vit. D-fed	4	Mg	2.48 ± 0.15	2.28 ± 0.18	2.54 ± 0.71	2.89 ± 0.69	2.27 ± 1.02	1.79 ± 0.30

form of irradiated ergosterol in oil (Viosterol[4] containing one million units of vitamin D per gram) beginning 2 wk. prior to the due date and continuing until 3 days postpartum. This concentrated source of vitamin D was selected because of the high unitage required. (More potent irradiated yeast is now available which had not been manufactured at the time of this experiment.)

The same high-mineral ration containing 5 per cent steamed bonemeal previously described (3) was fed at the rate of 6 lb. daily beginning 8 wk. before the due date and continuing for 1 wk. after parturition.

Blood samples were drawn for analyses of Ca, P, Mg, vitamin A, carotene and vitamin D 2 wk. and with 12 hr. before parturition and with 12 hr., 3 days and 7 days postpartum. If milk fever developed blood samples were drawn for analyses before treatment.

TABLE 2

The effect of feeding 5 million units of vitamin D daily for 2 wk. prepartum and for 3 days post partum on the vitamin D content of dried whole blood

Group	No. of cows	Mg. dried blood fed	% Healing 2 wk. pre-fresh	Mg. dried blood fed	% Healing 12 hr. pre-fresh	Mg. dried blood fed	% Healing 12 hr. post-fresh	Mg. dried blood fed	% Healing milk fever	Mg. dried blood fed	% Healing 1 wk. post-fresh
Normal parturitions											
Control	6	1,000	50	1,000	46	1,000	49	1,000	.....	1,000	48
Vit. D-fed	3	1,000	39	100	.....	100	.....[a]	100	.....	100	.....[a]
Milk-fever parturitions											
Control	1	1,000	46	1,000	50	1,000	.....	1,000	38	1,000	50
Vit. D-fed	4	1,000	38	100	39	100	39	100	38	100	41

[a] These 12 hr. post-fresh and 1 wk. post-fresh samples failed to give a satisfactory response in 2 trials. No explanation is apparent.

Before the calf nursed, the cows were milked out and a sample of colostrum was obtained for determination of the specific gravity, total solids and the ash, fat, total protein, carotene and vitamin A content. The methods of analyses were similar to those previously described (3).

In the control group three of the nine cows developed milk fever. Complete blood data were obtained on only one of these milk-fever cases. In the vitamin D-fed group four of the nine cows developed milk fever. Complete blood data were obtained on all four of these cases. The results of the blood analyses are shown in tables 1, 2 and 3. The composition of the colostrum of normal and milk-fever cows, both controls and vitamin D-fed, is indicated in table 4. The Ca content of a number of the colostrum ash samples was determined in order to check on possible differences in the per cent of Ca in the ash of colostrum secreted by normal and milk-fever cows and also cows fed various amounts of vitamin D (table 5).

The amount of colostrum obtained at each of the first two postpartum milk-

[4] Kindly supplied by the R. P. Scherer Corp., Gelatin Products Division, Detroit 13, Mich.

TABLE 3

Changes in the plasma carotene, vitamin A, and ascorbic acid content of Jersey cows' blood in normal and milk-fever parturitions

Group	Blood constituent	No. of cows	2 wk. pre-fresh	12 hr. pre-fresh	12 hr. post-fresh	Milk fever	1 wk. post-fresh
Normal	Carotene (γ %)	10	417	340	336	.....	314
Milk-fever	. .	4	318	243	213	203	177
Normal	Vitamin (γ %)	10	19.1	10.9	9.80	.....	12.50
Milk-fever	. .	4	20.5	12.3	8.40	7.40	5.90
Normal	Ascorbic Acid (*mg.* %)	10	.51	.44	.46	.....	.44
Milk-fever	. .	4	.49	.51	.49	.47	.29*

* This value is abnormally low due to one cow which had an extremely low plasma ascorbic acid level (.04 mg. %).

ings was average for 47 normally freshening Jersey cows and 39 milk-fever cows (table 6). Calves were left with their dams for 3 days as a regular managerial practice during most of this period, so that the colostrum weights obtained represent in most cases the amount of colostrum secreted minus the amount taken by the calf. This variable is believed to be reasonably constant considering the relatively large numbers of cows included.

The feeding of five million units of vitamin D daily in the form of Viosterol for 2 wk. prepartum resulted in an increase in serum Ca and P (table 1). The increase in serum Ca was greater than that resulting from either one or two million units fed daily for 1 mo. prepartum as previously reported (3).

Although the increases in serum Ca and P were found, in general, to be related to the dosage, it is of interest to note (figures 1 and 2) that the prepartum increases in serum Ca and P are nullified within approximately 12 hr. postpartum in both normal and milk fever parturitions. Thus, on the basis of the blood picture, it is not surprising that four of the nine treated cows developed milk fever.

The serum Mg (table 1) followed much the same changes as those previously

TABLE 4

The composition of colostrum from normal and milk-fever Jersey cows fed 5 million units of vitamin D as compared to their controls

Group	No. of cows	Units of vitamin D fed daily	Specific gravity	Total solids (%)	Ash (%)	Fat (%)	Total protein (%)	Carotene (%)	Vit. A (%)
			Normal parturitions						
Control	6	None	1.054	18.8	1.05	4.2	11.7	172	93
Vit. D-fed	4	5,000,000	1.069	23.8	1.24	4.3	15.8	251	214
Av.	...	.....	1.060	20.7	1.13	4.3	13.3	204	141
			Milk-fever parturitions						
Control	1	None	1.072	25.4	1.79	4.1	18.6	347	121
Vit. D-fed	4	5,000,000	1.058	22.1	1.15	4.8	14.1	175	110
Av.	...	.....	1.061	22.7	1.28	4.7	15.0	209	112

TABLE 5

A comparison of the calcium content of colostrum from normal and milk-fever Jersey cows fed various amounts of vitamin D

Group	No. of cows	% Ash	% Ca in Ash[c]	% Ca in colostrum
		Normal parturitions		
Control	6	1.09	16.5	0.182
Fed 1 million units of D[a]	3	1.07	19.2	0.202
Fed 2 million units of D[a]	3	1.05	18.9	0.200
Fed 5 million units of D[b]	4	1.24	17.2	0.216
Av.		1.12	17.6	0.198
		Milk-fever parturitions		
Control	1	1.15	18.0	0.207
Fed 1 million units of D[a]	2	1.03	16.9	0.167
Fed 2 million units of D[a]	0	.....	.....	.....
Fed 5 million units of D[b]	3	1.16	16.8	0.195
Av.		1.12	17.0	0.188

[a] Fed vitamin D for 1 mo. prepartum, as described in previous report (3).
[b] Fed vitamin D for 2 wk. prepartum.
[c] The % Ca in the ash of a composite sample of normal milk from the Pasture Farm Jersey Herd taken just before the pasture season was 16.8%. Normal Jersey milk contains an average of 0.118 % Ca.

reported (3) except that an unexplainably high level was maintained as compared to previous years (figure 3). The role of serum Mg in the symptomatology of milk fever has been discussed previously (3, 4).

Vitamin D assays of the blood indicated that cows fed five million units of vitamin D daily for 2 wk. prepartum had approximately ten times the vitamin D in their blood at parturition as the control cows (table 2). Previous results (3) had shown respectively two and four times the normal blood vitamin D level when one and two million units were fed daily for 1 mo. prepartum.

As shown in table 3, the changes in plasma carotene, vitamin A and ascorbic acid were somewhat similar to those previously reported (3). The higher carotene level in 1944–45 probably was due to the feeding of higher quality roughage which resulted in a higher level of both carotene and vitamin A during and after the period of parturition. It will be noted that the vitamin A level was extremely low postpartum in the four milk-fever cows.

TABLE 6

A comparison of the amount of colostrum produced by normal and milk-fever cows.

No. of cows	Lb. of colostrum[a] first milking	Lb. of colostrum[a] second milking
	Normal parturitions	
47	7.9 ± 6.5	7.2 ± 4.7
	Milk-fever parturitions	
39	8.1 ± 6.0	7.3 ± 5.1

[a] The colostrum weights in most cases are exclusive of the amount taken by the calf.

Table 4 shows the composition of the colostrum of control and vitamin D-fed cows which freshened normally and those that developed milk fever. No consistent difference in colostrum composition could be attributed to feeding five million units of vitamin D nor was there any marked difference between the colostrum of normal and milk fever cows. This is in agreement with previous results where one and two million units of vitamin D were fed (3).

The greater decrease in blood Ca at parturition in normally freshening cows with intact udders than in mastectomized cows, as shown by Niedermeier *et al.* (5), indicates that mammary secretion doubtless is responsible for the major drain on blood Ca at parturition.

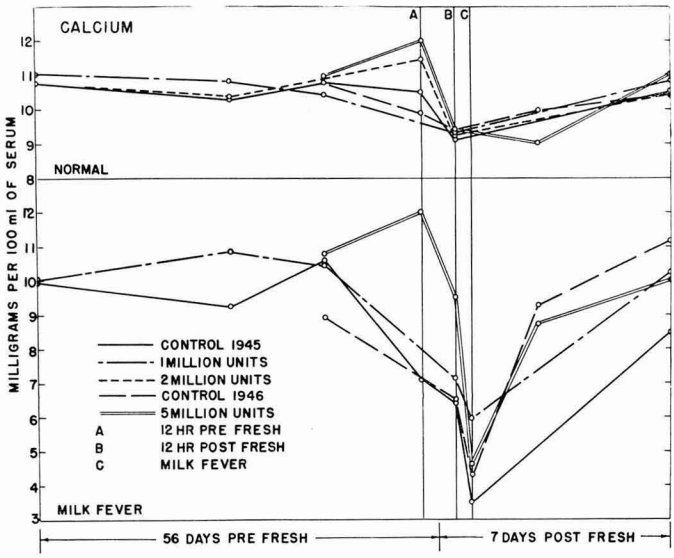

FIG. 1. Changes in the pre- and postpartum blood serum Ca in normal and milk-fever cows fed, respectively, one, two and five million units of vitamin D daily. In 1945, vitamin D was fed for 1 mo. prepartum as previously described, (table 7 of reference (3)). In 1946 vitamin D feeding was begun 2 wk. prepartum (table 1).

However, no difference in the per cent ash of colostrum was found between normal and milk-fever cows or among cows fed various amounts of vitamin D (table 4 and also table 9 (3)). Furthermore, the percentage of Ca in colostrum and colostrum ash is practically the same between normal and milk-fever cows regardless of the amount of vitamin D administered (table 5). The question therefore is logically raised as to whether cows that develop milk fever may secrete more colostrum than normally freshening cows and thus be subject to a greater drain on their blood Ca at parturition. It is a common belief that heavy producers are more subject to milk fever. A comparison, therefore, was made of the colostrum secreted by 47 normally freshening cows and 39 milk-fever cows (table 6). No difference in the amount of colostrum secreted at the first and

second milkings was found between cows that freshened normally and those that developed milk fever. The work of Smith *et al.* (7) also is of interest in this connection, as these workers have shown that neither premilking nor partial prepartum milking lowered milk fever incidence.

Since there appears to be no greater drain on the blood Ca for colostrum secretion in milk-fever cows than in normally freshening cows, it seems logical to assume that, while colostrum secretion probably precipitates the attack, the fundamental difference lies in relative ability to mobilize Ca from the tissue reserves. This may be simply a difference in the rapidity and/or degree of parathyroid response to meet the sudden demand for blood Ca at parturition. Other

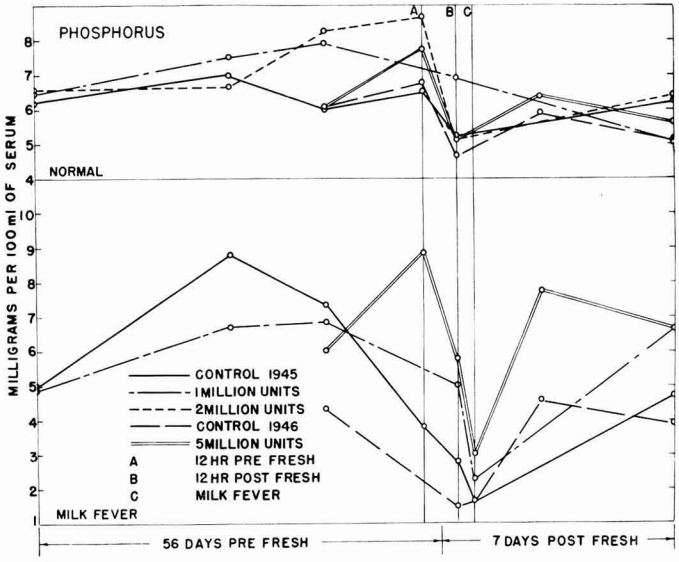

FIG. 2. Changes in the pre- and postpartum blood serum phosphorous in normal and milk fever cows fed, respectively, one, two and five million units of vitamin D daily. In 1945, vitamin D was fed for 1 mo. prepartum as previously described (table 7 of reference (3)). In 1946 vitamin D feeding was begun 2 wk. prepartum (table 1).

possibilities are that sufficient parathyroid hormone is secreted but is rendered temporarily inactive by some metabolic condition in the tissues, as discussed in a recent review (4), or that there is a lack of readily available tissue stores of Ca.

Further evidence that the primary difficulty in milk fever is the temporary failure of the blood Ca regulatory mechanism is to be found in a study of the lapse of time between parturition and the first and second attacks in cases where relapses occur (table 7). It seems significant that the time between parturition and the first attack is approximately the same as the time between the first treatment and the second attack or relapse. After the usual Ca injection at the first attack, the blood Ca immediately rises to approximately 17 mg. per 100 ml. This is followed by a rapid decline which levels off somewhere near the normal

level (figure 1 of reference 3). However, if the post-treatment fall in blood Ca is not checked by recovery of the blood Ca regulatory mechanism, the blood Ca continues to fall until a relapse is precipitated. Therefore, the difference between those cows which recover after one treatment and those that have a relapse may be due to a difference in the time required for them to recover their ability to mobilize Ca. This may or may not be dependent upon the injected Ca. In other words, the injected Ca may only serve to offset the drain of mammary

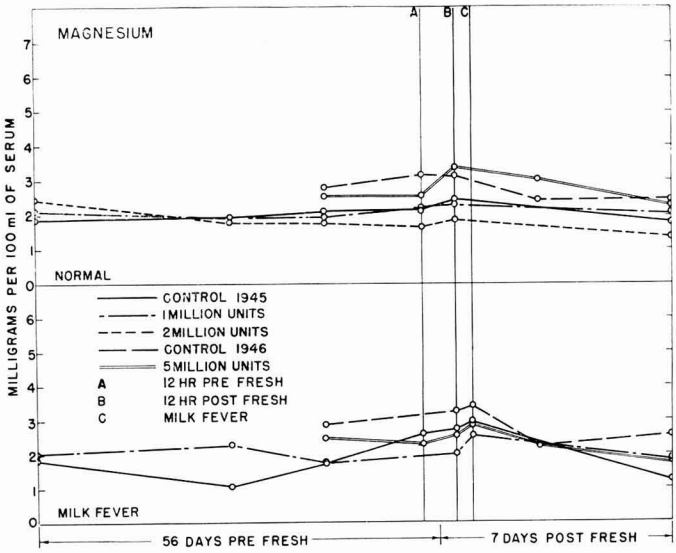

FIG. 3. Changes in the pre- and postpartum blood serum magnesium in normal and milk-fever cows fed, respectively, one, two and five million units of vitamin D daily. In 1945 vitamin D was fed for 1 mo. prepartum as previously described (table 7 of reference (3)). In 1946 vitamin D feeding was begun 2 wk. prepartum (table 1).

secretion until the blood Ca regulatory mechanism has a chance to take over that function. If this does not occur soon enough, a relapse results and another Ca injection is required.

When udder inflation with air is used as the treatment, mammary secretion is stopped, thereby ending the drain on the blood Ca. The disappearance of milk fever symptoms apparently is due to the subsequent recovery of the cow's own blood Ca regulatory mechanism which may require several hours. According to data presented by Niedermeier and Smith (6), re-absorption of Ca into the blood from the mammary gland due to the air pressure in the udder doubtless hastens the disappearance of the symptoms.

Campbell and Turner (1) have shown that vitamin D administration causes a decrease in the animal's own parathyroid activity when fed for long periods of time. It is reasoned, therefore, that the failure to obtain sufficient calcemic effect at parturition from our vitamin D feeding to prevent milk fever may have been

due to either too low a dosage or too long a feeding period with its associated prolonged elevated blood Ca level which may have caused a suppression of the cow's own parathyroid activity. Thus, it seems that if vitamin D is to be used as a milk fever preventive, the dosage must be sufficiently large and must be timed in such a way as to supplement but not suppress parathyroid activity at parturition.

Experiments are now in progress in which much larger amounts of vitamin D are being fed daily, limiting the dosage to a few days prepartum so that the peak

TABLE 7

The lapse of time between parturition and milk fever in Jersey cows

Year	No. cases milk fever	Attack (hr. postpartum)	No. of relapses	Relapse (hr. post-treatment)
1941	2	14.0 ± 2.5	0	.....
1942	11	20.0 ± 7.8	4	17.0 ± 5.3
1943	5	33.0 ± 13.3	1	29.0 ± 0.0
1944	8	20.0 ± 5.1	1	26.0 ± 0.0
1945	4	19.0 ± 7.8	0	.....
1946	6	22.0 ± 4.6	2	24.0 ± 0.0
	36	21.7 ± 7.1	8	21.4 ± 2.7

of blood Ca occurs as near to parturition as possible with the aim of supplementing without suppressing parathyroid activity. The results to date based on blood changes in Ca, P and Mg and on the incidence of milk fever have been most encouraging and will be published in a later communication.

SUMMARY

Eighteen mature Jersey cows were divided into two groups of nine cows eash. One group was fed five million units of vitamin D in the form of Viosterol daily for 2 wk. prepartum and for 3 days postpartum. The other group served as controls. All cows were fed a high mineral ration containing 5 per cent steamed bonemeal at the rate of 6 lb. daily beginning 8 wk. before the due date and continuing for 1 wk. postpartum. Blood samples were drawn for analyses two weeks prepartum, within 12 hr. both pre- and postpartum and 1 wk. postpartum.

An increase in serum Ca and P resulted prior to parturition which was greater than when one and two million units had been fed previously. However, the increase was nullified within 12 hr. postpartum or before the usual time for milk fever to occur, which was found to average 21.7 ± 7.1 hr. postpartum in 36 cases. The upward trend of serum Mg at parturition was similar to changes previously observed. Four of the nine vitamin D-fed cows and three of the controls developed milk fever.

The vitamin D content of the blood of cows fed five million units daily was found to be about ten times higher than that of the control cows. The changes in plasma carotene, vitamin A and ascorbic acid were somewhat similar to those previously reported.

Data are presented showing that milk-fever cows secrete no more colostrum

than normally freshening cows, nor does the colostrum of milk-fever cows have a higher percentage of ash or Ca than normally freshening cows. Thus, no greater drain on blood Ca due to mammary secretion is experienced by milk-fever cows than cows that freshen normally. It also was shown that the average time between the first treatment and the relapse, when one occurred, was approximately the same as that between parturition and the first attack. These observations are considered evidence that milk fever is primarily caused by the failure of the blood Ca regulatory mechanism (probably the parathyroid glands) to mobilize blood Ca rapidly enough to meet even the normal drain on blood Ca at parturition. Thus recovery is characterized by the return to normal of the blood Ca regulatory mechanism and may or may not be influenced by the injected Ca which serves to eliminate the symptoms by elevating the blood Ca.

It is reasoned that the failure to obtain sufficient calcemic effect at parturition from vitamin D feeding to prevent milk fever may have been due, at least in part, to the suppression of the cow's own parathyroid activity resulting from too long a feeding period with its associated prolonged elevated blood Ca. Also, the dosage may have been too low. Experiments are now in progress in which larger dosages of vitamin D are being fed for shorter periods of time prepartum with the aim of supplementing without suppressing parathyroid activity.

REFERENCES

(1) CAMPBELL, I. L., AND TURNER, C. W. The Relationship of the Endocrine System to the Regulation of Calcium Metabolism. Mo. Agr. Expt. Sta. Bull. 352. 1943.

(2) HIBBS, J. W., KRAUSS, W. E., MONROE, C. F., AND SUTTON, T. S. Studies on Milk Fever in Dairy Cows. I. The Possible Role of Vitamin D in Milk Fever. J. Dairy Sci., 29: 617–623. 1946.

(3) HIBBS, J. W., KRAUSS, W. E., POUNDEN, W. D., .MONROE, C. F., AND SUTTON, T. S. Studies on Milk Fever in Dairy Cows. II. The Effect of Vitamin D on Some of the Blood Changes in Normal and Milk Fever Cows at Parturition. J. Dairy Sci., 29: 767–782. 1946.

(4) HIBBS, J. W. Milk Fever (Parturient Paresis) in Dairy Cows—A Review. J. Dairy Sci., 33: 758–789. 1950.

(5) NIEDERMEIER, R. P., SMITH, V. R., AND WHITEHAIR, C. K. Parturient Paresis. III. A Study of Various Blood Constituents at Parturition in Mastectomized Cows. J. Dairy Sci., 32: 927–934. 1949.

(6) NIEDERMEIER, R. P., AND SMITH, V. R. Parturient Paresis. IV. The Effect of Udder Inflation upon Blood Levels of Calcium, Magnesium and Phosphorous in Cows with Parturient Paresis. J. Dairy Sci., 33: 38–42. 1950.

(7) SMITH, V. R., AND BLOSSER, T. H. Parturient Paresis. I. The Incidence of Parturient Paresis and Changes in the Total Blood Serum Calcium at Parturition in Prepartum Milked Cows. J. Dairy Sci., 30: 861–866. 1947.

(8) SMITH, V. R., NIEDERMEIER, R. P., AND HANSEN, R. G. Parturient Paresis. II. The Effect of Partial Versus Complete Milking upon the Total Blood Serum Calcium of Dairy Cows at Parturition. J. Dairy Sci., 31: 173–177. 1948.

ETHYLENEDIAMINE TETRASODIUMACETATE USED IN DETERGENCY[1]

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Previous washing studies published by the Michigan Agricultural Experiment Station (4) show the detergency effects of various detergent ingredients as determined by washing milk-filmed glass panes. In those studies outstandingly high detergency resulted from use of high levels of condensed phosphate and wetting agent chemicals. The condensed phosphates provided the obvious benefit of calcium sequestration that caused a softening and dissolving action on fixed milk films while also softening the water of the washing solution. A recently developed product of organic origin, ethylenediamine tetrasodiumacetate,[2] has demonstrated outstanding qualities as a water softening agent.

Ethylenediamine tetrasodiumacetate possesses the property of tying up water hardness minerals in a manner akin to sequestration performed by the inorganic condensed phosphate salts. These chelating properties have been studied by Schwarzenback and Ackermann (8, 9). The product has been used primarily for water softening, as shown by Martell and Bersworth (7), although Hilfer (3) has used it with soaps and Diehl *et al.* (2) used it to determine water hardness. Claims for its use in detergency have been made by the Bersworth Chemical Co. (1), under whose trade mark the product is known as Versene. However, no previous experimental data are available that show the detergency qualities of ethylenediamine tetrasodiumacetate.

Laboratory washing tests therefore were made, by methods previously explored, that would establish relative detergency values of this product when such proven products as condensed phosphates and other detergent products were used for comparison in washing trials.

EXPERIMENTAL

Washing tests were made by comparing the washing quality of EDTSA with polyphosphate under conditions of equal quantities of these water conditioning salts and wetting agent supplement. Preliminary trials indicated that high detergency would be secured when they were tested against air-dried raw milk films; consequently, two types of more resistant film also were used. These consisted of air-dried film preparations treated with alkaline washing solution with subsequent drying in a hot air oven, and air-dried films that were rinsed in 250 ppm. solution of sodium hypochlorite. Observations made during studies of milking machine washing by Jensen and Bortree (5) and on milk can cleanli-

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[2] For the sake of convenience EDTSA will be used throughout the text in place of ethylenediamine tetrasodiumacetate.

ness by Jensen and Waterson (6) had indicated that milk films of somewhat similar nature were poorly removed by normal washing practices.

In these experiments, a double strength, B-type glass was cut into 3-in. squares. All the panes were washed clean, rinsed with distilled water and allowed to dry at room temperature. They were tested for cleanliness by determining the per cent light that was transmitted when placing them in the filter position of a Cenco Sheard Photelometer that had been adjusted to give 100 per cent readings on a clean glass pane reserved for a standard.

Well mixed, fresh, whole, raw milk held at a temperature of 40 to 50° F. was used for the preparation of the milk films.

The air-dried films were prepared by twice immersing the glass panes in the milk to produce a uniform milk film. The panes were placed on metal frames at an approximate angle of 45° to drain and dry at room temperature for 15-min. or more. Each pane similarly milk-coated following the mechanical washing treatment was used over a series of 10 washings.

After air drying milk films as described above, panes were placed in an oven at 180 to 185° F. for a 15-min. period. They then were removed and each pane was dipped in a 0.3 per cent solution of a detergent consisting of 49 per cent Na_3PO_4, 49 per cent Na_2CO_3 and 2 per cent Nacconal. The panes were not agitated to remove the entire milk film, but were immersed only sufficiently to remove the soluble portion of the milk film. After drying, the panes again were placed in the oven for 15-min. This sequence was followed until each set of panes had been immersed in the milk five times and in the detergent solution four times, each followed by a 15-min. oven-drying period.

Air-dried milk films were immersed in a 250 ppm. sodium hypochlorite solution, made up from liquid sodium hypochloride. The panes were allowed to dry for a 15-min. period. Five similar applications of milk and four of chlorine solution were made on panes between each washing with the mechanical washing apparatus. Freshly prepared chlorine solutions were used for each immersion.

All panes were washed with the mechanical washing apparatus that has been described by Jensen (4). The panes were impelled through different detergent solutions at a rate of 45 oscillations per minute, for 1 min. washing time, preceded by 1 min. of soaking.

Pre-rinsing and after-rinsing, with respect to washing in detergent solutions, consisted of impelling the panes five complete oscillations through rinsing mediums at a distance of 3 in. in a manner to force the water against the face of the glass. Pre-rinsing was done in mediums held at 60° F., washing at 120° F. and after-rinsing at 150° F.

Following drying of the panes, they were measured for cleanliness by determining the average percentage of light transmitted when each corner area of a pane was placed in the filter position of a Cenco-Sheard spectrophotometer. Photometer readings were made with the light entrance slit adjusted at 2 mm., the exit slit at 20 mm. and a 400 mμ light wave length. After a 15 to 20 min. warming up period, the galvanometer was adjusted for "*zero* per cent reading"

with no light transmitted and for "*100* per cent reading" with light transmitted through a clean glass pane used throughout as a standardizing control.

The water used for washing in these studies contained a total hardness of 370 to 400 ppm., calculated as $CaCo_3$, determined by the versenate method (2).

Three polyphosphates were used, tripoly-, tetra- and hexameta-. These are designated, respectively, as *a*, *b* and *c*.

Detergent strength. Washing solutions were made to contain 0.3 per cent detergent, except wetting agent only was used at 0.2 per cent concentration.

RESULTS

Washing effect of detergents containing ethylenediamine tetrasodiumacetate and/or condensed phosphate salts and wetting agent. The detergency property of EDTSA was compared with that secured with (a) sodium tripolyphosphate (b) sodium tetraphosphate and (c) sodium hexametaphosphate, when these products were used with equal quantities of wetting agent. Ten machine washings were made of specially prepared milk films. Photometer readings were made following each washing.

TABLE 1

The influence of using various condensed phosphate salts and/or EDTSA in combination with wetting agent in washability of variously applied milk-coated glass panes

Detergent Composition (%)			Percent transmissibility of light through panes when milk films were:		
EDTSA	Condensed phosphates[a]	W.A.	Air-dried	Oven-dried	Milk-chlorine
	90a	10	98	74	94
	90b	10	98	66	87
	90c	10	97	78	97
90		10	99	96	68
	80a	20	98	96	68
	80b	20	98	96	82
	80c	20	99	74	98
80		20	97	89	96
40	40a	20	96	96	91
40	40b	20	98	93	94
40	40c	20	98	89	99

[a] a = sodium tripolyphosphate; b = sodium tetraphosphate; c = sodium hexometaphosphate.

The average photometer readings of the air-dried, oven-dried and milk-chlorine panes, washed with different combinations of EDTSA, condensed phosphates and wetting agent are shown in table 1. All the air-dried films were effectively removed with these detergent combinations. The oven-dried and milk-chlorine films were removed less effectively than the air-dried films. However, high film removal was secured when compared with that from using alkaline detergents (table 2).

The oven-dried films were not removed as well with condensed phosphate-wetting agent detergent as with EDTSA wetting agent detergent in the 90:10 combination, but when these detergents were used in 80:20 concentration ratio, the *a* and *b* condensed phosphate detergent solutions produced better film removal than did solutions containing EDTSA.

When washing the milk-chlorine films, the EDTSA detergent solutions produced somewhat higher film removal than the phosphate wetting agent solutions. Photometer readings also tended to be higher as phosphates were used in the order *c, b, a,* thus indicating that sequestering or chelating properties are important functions in the removal of resistant milk films.

Washing quality of EDTSA and/or condensed phosphate salts in combination with wetting agent and alkaline salts. Washing tests were made on the three types of films using detergents containing either Na_3PO_4, Na_2SiO_3, or $NaHCO_3$ as 40 per cent of the detergent ingredient. The balance consisted of either 40 per cent EDTSA or condensed phosphate *a, b,* or *c* and 20 per cent wetting agent. The results of the washings are shown in table 2, where the

TABLE 2

The influence of EDTSA and/or condensed phosphate salts with wetting agent on the detergency of various alkaline salts

Detergent[a] added to basic alkali (%)	Percentage light transmissibility after repeated washings when the films were:		
	Air-dried	Oven-dried	Milk-chlorine
Trisodium phosphate (40%)			
EDTSA (40) WA (20)	95	24	20
Phosphate a (40) WA (20)	90	24	47
Phosphate b (40) WA (20)	95	28	27
Phosphate c (40) WA (20)	89	25	29
Sodium metasilicate (40%)			
EDTSA (40) WA (20)	87	29	52
Phosphate a (40) WA (20)	84	32	40
Phosphate b (40) WA (20)	84	31	27
Phosphate c (40) WA (20)	83	33	37
Sodium bicarbonate (40%)			
EDTSA (40) WA (20)	95	20	18
Phosphate a (40) WA (20)	91	23	25
Phosphate b (40) WA (20)	94	30	31
Phosphate c (40) WA (20)	93	27	30

[a] a = sodium tripolyphosphate; b = sodium tetraphosphate; c = sodium hexometaphosphate.

average of 10 washings for each series are presented. There was a marked decrease in film removal when the alkaline salts were used to replace either condensed phosphates or EDTSA (table 1). This was especially marked when the oven-heated and milk-chlorine films were washed.

Essentially equal film removal was secured when Na_3PO_4 or $NaHCO_3$ was used as the alkaline constituent in washing air-dried films. The highest average percentage light transmission for both was 95, while the lowest reading for the $NaHCO_3$ detergent was 91 and for the Na_3PO_4 detergent 89. When Na_2SiO_3 was used, the highest average secured was 87, the lowest 83.

Somewhat higher film removal was secured when working oven-heated or milk-chlorine films with Na_2SiO_3 as the detergent constituent than with Na_3PO_4 or $NaHCO_3$.

EDTSA gave greater film removal than condensed phosphate salts in the

detergent combinations when washing air-dried films. The reverse tended to be the result when oven-heated and chlorine-milk films were washed. There was no consistent or appreciable difference in the washing results secured with the three condensed phosphate salts.

The influence of milk dilution on washability of detergents that contain high amounts of EDTSA and/or condensed phosphate salts and wetting agent.

The amount of milk product that enters the wash water can be expected to lower its washing quality, because of its diluting effect and because of adding organic matter. Milk contains comparatively high quantities of Ca that will lower the chelating and sequestering properties of EDTSA and condensed phosphates. Washing trials therefore were made to determine the influence on detergency when milk was added to washing solutions containing high levels of EDTSA and/or condensed phosphate. Air-dried milk films were used and detergents were compounded to contain "90–10" and "80–20" condensed phos-

TABLE 3

The influence of adding 10 per cent whole milk to different detergent solutions consisting of wetting agent, condensed phosphate and/or EDTSA

Detergent composition		Milk added (% of solution)	Percentage transmissibility after 10 repeated washings
1. Wetting agent,	(10)	0	98.6
condensed phosphate	(90)	10	94.4
2. Wetting agent,	(20)	0	99.6
condensed phosphate	(80)	10	97.3
3. Wetting agent,	(10)	0	98.7
condensed phosphate	(70)	10	96.4
EDTSA	(20)		
4. Wetting agent,	(20)	0	99.7
condensed phosphate	(60)	10	96.9
EDTSA	(20)		
5. Wetting agent,	(10)	0	99.6
condensed phosphate	(45)	10	96.2
EDTSA	(45)		
6. Wetting agent,	(20)	0	100
condensed phosphate	(40)	10	96.3
EDTSA	(40)		

phate and wetting agent; "20–70–10," "20–60–20," "45–45–10" and "40–40–20" of EDTSA, hexametaphosphate and wetting agent. Each washing solution was made to contain 0.3 per cent detergent and the solutions were varied with respect to milk content so that 10 per cent whole raw milk was added to each washing solution over a series of ten washings, while similar washings were made without milk being used.

The average photometer readings of ten washings are shown (table 3). While there was a decrease in washing efficiency due to milk in the detergent solutions, the washing quality of this class of detergent remained high. The lowest reading (94.4) followed washing with 90–10 condensed phosphate-wetting agent solution to which 10 per cent whole milk had been added. The best washing when milk was added was had by the detergent that contained 20 per cent wetting agent and 80 per cent condensed phosphate.

There was very little difference in the washing quality of detergent solutions to which milk was added, with respect to using either EDTSA or condensed phosphate. They can be assumed to be of practically equal value.

Influence of various types of pre-rinses on the washability of milk films. Washing operations generally are conducted by first rinsing the article to be washed (pre-rinsing) then follow washing in detergent solutions and rinsing after washing (after-rinsing). Earlier studies (4) had shown that the manner of rinsing affects the cleanliness attained. Further washing trials therefore were made to determine comparative detergency that would be secured when various rinsing media, including EDTSA, were used either as pre-rinse only, after-rinse only, or when both pre- and after-rinsings were used. A series of ten washings was conducted using each rinsing medium shown in table 4 in the different stages

TABLE 4
Influence of various types of rinses on the washability of milk-filmed glass panes

Rinsing medium	Time of rinsing	Transmissibility of light through films (%)[a] when washed with solution of	
		Wetting agent	"75–25"
Water	Before	84	98
	After	85	98
	Before and after	60	96
EDTSA	Before	99	99
	After	95	99
	Before and after	93	98
Condensed phosphate + Wetting agent "75–25"	Before	92	99
	After	88	99
	Before and after	87	99
EDTSA + Wetting agent	Before	95	100

[a] Average of photometer readings of 10 successive rinsings and washings.

of washing. Two kinds of detergents were used, one a wetting agent and the other a "75–25" combination of condensed phosphate and wetting agent. Air-dried films were washed. The averages of the photometer readings of ten consecutive washings under each condition of rinsing and washing are shown in table 4.

When the detergent was used that consisted of condensed phosphate and wetting agent in the "75 : 25" ratio all washing results were so high that there was little difference in the manner or the kind of rinsing that was used. There was a noticeable lowering in film removal when water was used for both pre-rinsing and for after-rinsing; however, the loss in detergency was not serious. These results are in line with those reported previously (4).

When wetting agent was used as detergent, a marked difference in film removal was secured, depending on the nature of pre-rinsing solutions. With water as the rinsing medium, the detergency secured as a result of pre-rinsing or after-rinsing was practically the same (average photometer readings 84 and 85). When used for both pre- and after-rinsings, the average of photometer readings

dropped to 60. Higher detergency values were secured when rinsings were made with EDTSA, EDTSA combined with wetting agent or "75–25" condensed phosphate-wetting agent, all used in 0.1 per cent solution. The best washing result using wetting agent detergent (99) was secured when EDTSA in 0.1 per cent solution was used for pre-rinsing, and without applying a rinse after washing. When the rinse followed washing, a slight decrease in washability occurred (95). The lowest washing resulted for the EDTSA rinsing series when both pre- and after-rinsing was applied. Condensed phosphate-wetting agent, "75–25," used for rinsing ahead of washing in wetting agent detergent also produced high detergency (92), although significantly less than EDTSA (99) or when EDTSA plus wetting agent was used (95). A lower washing value (88) was also secured with this detergent when the panes were rinsed following washing and still lower (87) when both pre- and after-rinsing was applied.

DISCUSSION

The chelating properties of EDTSA appeared to contribute the same qualities to detergents as are produced by the sequestering properties of condensed phosphates. Both were of practically equal value in causing removal of milk film when they were used in combination with a wetting agent. EDTSA in combination with wetting agent was somewhat more consistent in the removal of the tougher oven-dried and milk-chlorine films than were the condensed phosphate-wetting agent combinations.

There appeared to be some benefit to detergency, when measured against all the films used, by combining the properties of condensed phosphates and EDTSA with wetting agent to produce a detergent with these ingredients in the ratio of 40:40:20. However, condensed phosphate-wetting agent combinations produce solutions that have low alkalinity and, as such, can be used in washing operations where the milk constituents are not affected by alkalinity. EDTSA detergent solutions at 0.3 per cent concentration have pH ranging from 9 to 9.92, while the pH of the condensed phosphate detergent groups ranged from 7.59 to 9.0, depending on the polyphosphate selected. From the observation of flush-washing cream separators, following the separating of raw milk, a pH in the washing solution in excess of 8.3 produces excessive sliminess.

There was some indication that the EDTSA solutions were more corrosive than the condensed phosphate solutions. This is being investigated further.

Fairly satisfactory washing results were secured when air-dried films were washed in the usual type of alkaline detergent solutions that also contained condensed phosphate or EDTSA and wetting agent. Poor washability was secured when those detergents were used on the oven-heated and milk-chlorine films. Obviously some decrease in milk film removal results when the chelating or sequestering quality of washing solutions are lowered. The differences presented by these washing results likely will not be as noticeable when scrubbing action is applied to washing. Where washing is done without scrubbing, such as in can washing or in flush-washing units and pipe lines, the detergency properties

that are produced by EDTSA and/or condensed phosphates and wetting agent are greatly needed.

The value of using a detergent that retains high washing quality in spite of containing milk ingredients in substantial quantity is obvious. The mineral-sequestering properties of detergents containing high proportions of such ingredients apparently make this possible, but a sufficient amount of wetting agent also is needed to supplant that used up on the milk ingredients. This was demonstrated by the increase in detergency that occurred when 20 per cent wetting agent was used with 80 per cent condensed phosphate in place of the 90–10 composition detergent.

Rinsing and its result on the transmissibility of washed glass panes were effected by the nature of the rinse solution, when wetting agent only was used as the detergent. Rinsing both before and after washing caused the lowest washing results with all the rinsing media, but the lowest photometer readings occurred when water was used as the rinsing medium. Under conditions of washing where only a pre-rinse is used, followed by washing in a detergent solution, it is believed that a film of wetting agent detergent remains that resists the adherence of the milk to which the pane is exposed in the preparation of air-dried milk films. Consequently, such panes may be expected to retain less milk upon filming and should be washed more completely free than ones more heavily coated.

Earlier studies (4) had shown better washing results when no water pre-rinse was used before washing with wetting agent-detergent solution. These results were substantiated in this study, for when the after-rinse only was used, the washing value was higher than when the panes were rinsed both before and after washing.

EDTSA appeared to be practically equal to the polyphosphate salts in the detergency tests applied in this study. It is not unlikely that the product might show an advantage over polyphosphates where washing is applied at temperatures above 140° F., the optimum for polyphosphates. At present, EDTSA commands a price that makes it uneconomical to use for detergent combinations when polyphosphates are available.

CONCLUSIONS

Ethylenediamine tetrasodiumacetate was compared with condensed phosphate and alkaline salt detergents for washing quality against milk films that were air-dried, oven-dried and chlorine-treated. The ethylenediamine tetrasodiumacetate and condensed phosphate salts contributed practically equal detergency properties. Alkaline salt replacement for either condensed phosphate or ethylenediamine tetrasodiumacetate lowered film removal properties. Both salts in combination with wetting agent produced high detergency quality against air-dried films when the detergent solution contained 10 per cent added whole milk.

When the rinsing mediums included ethylenediamine tetrasodiumacetate, higher detergency was secured than when condensed phosphate salts were used, using wetting agent as detergent.

REFERENCES

(1) BERSWORTH CHEMICAL CO. The Modern Chelating Agent, Versene. Bersworth Chem. Co., Framingham, Mass., Tech. Bull. 1. 24 pp. 1949.

(2) DIEHL, H., GOETZ, C. A., AND HACH, C. C. The Versenate Titration for Total Hardness. J. Am. Water Works Assoc., **42** (1): 40–48. 1950.

(3) HILFER, H. Chelating Agents. Drug and Cosmetic Ind., **64** (1): 40–41. 1949.

(4) JENSEN, J. M. Measuring Detergency Functions as Affected by Various Detergents and Procedures Against Milk Films by Application of a Mechanical Washing Apparatus. J. Dairy Sci., **29**: 453–463. 1946.

(5) JENSEN, J. M., AND BORTREE, A. L. Storage and Treatment of Milking Machines Inflations under Farm Conditions. J. Dairy Sci., **29**: 231–239. 1946.

(6) JENSEN, J. M., AND WATERSON, J. A Method for Measuring the Cleanliness of Milk Cans. J. Milk Food Technol., **13**: 332–335. 1950.

(7) MARTELL, A. E., AND BERSWORTH, F. C. Amino Acids as Chelating Agents. Proc. Toilet Goods Assoc., Sci. Sect., **10**: 10–13. 1948.

(8) SCHWARZENBACH, G., AND ACKERMANN, H. Komplexone V. Die Athylenediamin-tetraessigsaure. Helv. Chim. Acta, **30**: 1798–1804. 1947.

(9) SCHWARZENBACH, G., AND ACKERMANN, H. Komplexone XII. Die Homologen der Athylenediamin-tetraessigsaure und ihre Erdalkalkomplexe. Helv. Chim. Acta, **31**: 1029–1048. 1948.

THE EQUIVALENCE OF PANTOTHENIC ACID AND p-AMINOBENZOIC ACID FOR GROWTH OF *BACTERIUM LINENS*

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The factors involved in the associative action between certain yeasts and *Bacterium linens* during the ripening of limburger-type cheese have been the subject of recent investigations. Iya and Frazier (4) reported that media which had supported growth of a yeast supplied necessary factors for the growth of *B. linens*. Recently, Purko *et al.* (10) have shown that several yeasts isolated from limburger cheese secrete pantothenic acid, niacin, riboflavin and biotin during growth. These investigators also found that *B. linens* grows as a result of this vitamin production. In this connection, Burkholder *et al.* (2) previously had reported that one strain of *B. linens* requires pantothenic acid for growth in a synthetic medium. In a study of the vitamin requirements of twenty-five strains of *B. linens*, Meyer (8) observed that eight strains required pantothenic acid for growth. In addition, six strains appeared to have a "non-specific" vitamin requirement. The latter organisms were incapable of growing in a vitamin-free medium, but grew when eight B-vitamins were present. However, good growth was obtained when any one of the vitamins was omitted. Thus, the omission of a single vitamin did not demonstrate a specific requirement.

Further nutritional studies with a strain of *B. linens* exhibiting the "non-specific" vitamin requirement have been undertaken in order to elucidate the nature of this vitamin interaction. It has been found that this behavior is due to the ability of the organism to grow when either p-aminobenzoic acid (PAB) or pantothenic acid (PA) is present. Several observations have been reported which indicate a biochemical interrelationship between PAB and folic acid (5, 9) and between PAB and amino acids (7, 11) as well as purine and pyrimidine bases (3, 6), but the nutritional equivalence of PAB and pantothenate for the growth of bacteria appears to be unknown.

METHODS

Bacterium linens, strain 456, isolated from limburger cheese by Meyer (8) using the method of Albert *et al.* (1) was selected as representative of the "non-specific" group. The inoculum was grown with aeration at room temperature ($27 \pm 3°$ C.) for 24 hr. in a medium composed of 1 per cent of yeast extract, 1 per cent of tryptone, 0.5 per cent of dipotassium phosphate and 0.5 per cent of glucose. The cells were washed twice with sterile distilled water and resuspended in sterile distilled water to an optical density of 0.1 when measured as described below. One drop of this suspension was used to inoculate each tube.

The composition of the basal medium and the supplements is shown in table 1.

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The constituents for 100 ml. of medium were dissolved in 71.5 ml. of distilled water, 5-ml. aliquots of which were dispensed in 18-mm. test tubes. Water and supplements were added to each tube to a total volume of 7 ml. The tubes were plugged with aluminum foil-coated rubber stoppers equipped with cotton-plugged, glass-venting tubes and sterilized by autoclaving. After inoculation, the stoppers were sealed to the glass with collodion, the tubes inclined at an angle of approximately 37 degrees from horizontal and aerated by shaking (120 1.378 in. strokes per min.) at room temperature for 90 hr. Since *B. linens* is an obligate aerobe, growth conditions are required which will furnish O_2 without excessive evapora-

TABLE 1

Composition of the medium

Basal medium		Vitamin supplements	
	(*mg./7 ml.*)		(*γ/7 ml.*)
Casein hydrolysate, enzymatic	70.0	Thiamin HCl	2.0
Glucose	280.0	Riboflavin	2.0
L-Tryptophane	1.40	Pyridoxine HCl	2.0
L-Cystine	2.80	Ca pantothenate	2.0
Adenine sulfate	0.14	Niacin	2.0
Guanine HCl	0.14	Biotin	0.015
Uracil	0.14	Pteroylglutamic acid	0.5
Salts A[a]	0.07 ml.	Inositol	2.0
Salts B[a]	0.07 ml.	Choline HCl	0.5
pH	6.8	p-Aminobenzoic acid	0.1
		Vitamin B_{12}	0.01

[a] Salts A: Dissolve 25 g. K_2HPO_4 and 25 g. KH_2PO_4 in 250 ml. H_2O. Salts B: Dissolve 10 g. $MgSO_4 \cdot 7H_2O$, 0.5 g. NaCl, 0.5 g. $FeSO_4 \cdot 7H_2O$ and 0.5 g. $MnSO_4 \cdot 4H_2O$ in 250 ml. H_2O.

tion. With this arrangement, satisfactory aeration was obtained without loss of volume. In addition, growth could be estimated turbidimetrically at desired intervals. The turbidity measurements were made in an Evelyn colorimeter equipped with a no. 660 filter and expressed as optical density, which is the ratio of the intensity of the incident light (I_O) to that of the transmitted light (I_T). Thus, for the Evelyn colorimeter which is graduated in per cent transmission, $I_O = 100\%$ and I_T = the galvanometer reading. Thus, optical density = 2-log of the galvanometer reading. The inoculated tube before incubation was taken as zero optical density.

RESULTS

Vitamin dependence was tested by adding single vitamins to the basal medium in concentrations shown in table 1. The results shown in table 2 clearly indicate that growth occurred when either PAB or PA was present and that other vitamins were ineffective in promoting growth.

To investigate further this finding, media containing either PAB or PA and also containing both vitamins were inoculated and the course of growth followed turbidimetrically. The results of a typical experiment are shown in fig. 1. Growth occurred when either vitamin was added, although the rate and extent of growth were different with each supplement. In the presence of PA (20 γ per 7 ml.), the culture grew rapidly after a lag of about 20 hr. and the amount of

growth was about one-half that obtained with a mixture of PAB and PA. An increase in PA concentration from 20 to 80 γ per 7 ml. caused only a slight increase in the amount of growth. When only PAB (0.1 γ per 7 ml.) was added,

TABLE 2

Vitamin requirement of B. linens, strain 456. Composition of the basal medium and vitamin concentrations are as shown in table 1. Cultures were incubated with aeration for 90 hr. at room temperature (27 ± 3° C.)

Addition to basal medium	Optical density × 10
None	0.132
Ca pentothenate	4.32
p-Aminobenzoic acid	2.84
Thiamin HCl	0.088
Riboflavin	0.132
Pyridoxine HCl	0.088
Biotin	0.223
Pteroylglutamic acid	0.177
Choline HCl	0.132
Inositol	0.132
Niacin	0.132
All vitamins	7.46

detectable growth occurred after about 60 hr. With an optimum PAB concentration (0.4 γ per 7 ml.), the turbidity increased rapidly after 20 hr. and leveled off at about two-thirds that obtained with both vitamins present. No growth was obtained in the basal medium.

The possibility exists that the growth was due to the presence of traces of PA, PAB or other factors contributed by the inoculum. To test this possibility, serial transfers were made from each of the above media into the same media for four successive subcultures. If growth factors from the inoculum were an im-

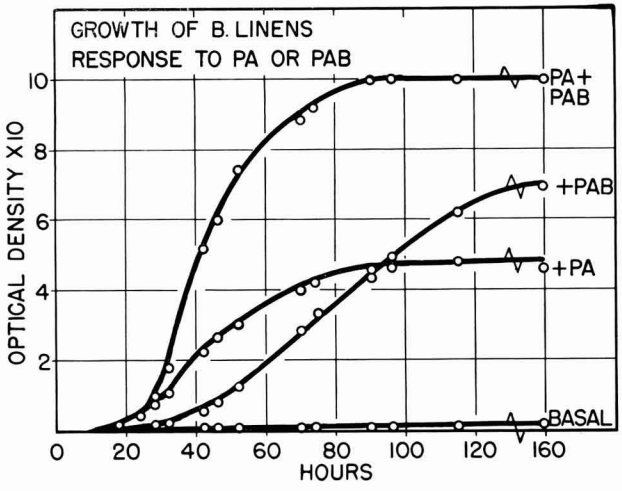

FIG. 1. Growth of *B. linens* with p-aminobenzoic acid (PAB) or pantothenic acid (PA). Conditions as in table 2 except 20 γ of PA and 0.4 γ of PAB per 7 ml.

portant factor in these results, progressively less growth would be expected in each subculture due to dilution. However, the results were the same as shown in fig. 1 in each transfer. Also, two different sources of PA and PAB have been used with the same results, thus lessening the possibility of vitamin contamination.

The addition of niacin, thiamin, pyridoxine, pteroylglutamc acid and riboflavin, singly or in combination in the amounts shown in table 1, did not alter the results shown in fig. 1. However, as shown in fig. 2, the addition of biotin to media containing either PA or PAB increased both the rate and amount of growth. In the presence of an optimum PAB concentration (0.4 γ per 7 ml.) the lag period was not altered. However, when PAB was limiting (0.1 γ per 7 ml.), biotin decreased the lag period from 60 to 20 hr. In contrast to these observations, biotin had little effect when both vitamins were present. Biotin appears to be stimulatory rather than required, since serial transfers in the vitamin-free

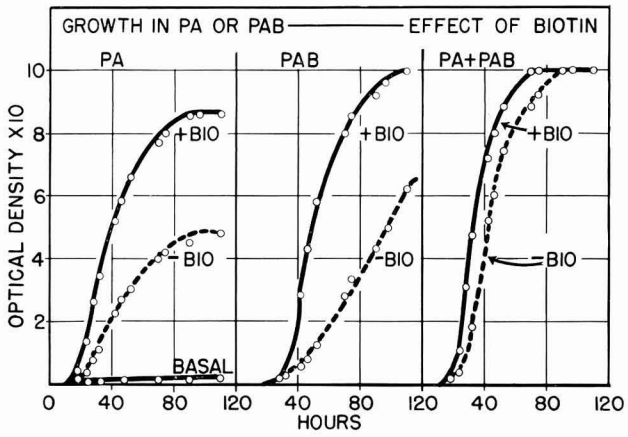

FIG. 2. Effect of biotin upon the growth of *B. linens*. Conditions as in fig. 1 except as indicated.

medium supplemented with PAB or PA grew without added biotin. In the event that biotin were required, its concentration in the inoculum and the medium would be so low as to limit the amount of growth. However, maximal growth was obtained in its absence (fig. 1). In addition, an amino acid mixture has been successfully substituted for casein hydrolysate in an otherwise biotin-free medium, thereby eliminating that source of biotin (and other growth factors) as a contaminant.

DISCUSSION

In line with the general hypothesis that a vitamin not required for growth is synthesized during growth, the observations reported herein indicate that *B. linens* may effect a synthesis of each vitamin under specific conditions. Further, it appears that either PAB is involved in the synthesis of PA or that PA functions in the synthesis of PAB. The role of biotin in these processes is not apparent. However, growth cannot be initiated by the addition of biotin alone,

nor is it required for growth in the presence of PA or PAB. Thus, the possibility exists that the rate of biotin synthesis is limiting in the presence of either PA or PAB. The fact that biotin plus PA and biotin plus PAB produces growth roughly equivalent to that obtained with PA plus PAB suggests that biotin may function in the synthesis of the omitted vitamin. Further experiments designed to test the possibilities are in progress.

The increased rate and amount of growth obtained with mixtures of PA, PAB and biotin indicate that many of the vitamins which are synthesized by the yeasts of limburger cheese (10) may be important factors in the yeast-*B. linens* associative action and in cheese ripening, even though all of the vitamins are not actually required for the growth of *B. linens*.

SUMMARY

B. linens, strain 456, grows in a semi-synthetic medium supplemented with either pantothenic acid or p-aminobenzoic acid. Biotin is not required for growth, but greatly increases the rate and amount of growth in the presence of PA or PAB.

REFERENCES

(1) ALBERT, J. O., LONG, H. F., AND HAMMER, B. W. Classification of the Organisms Important in Dairy Products. IV. *Bacterium linens*. Iowa State Agr. Expt. Sta. Research Bull., 328. 1944.

(2) BURKHOLDER, P. R., COLLIER, J., AND MOYER, D. Synthesis of Vitamins by Microorganisms in Relation to Vitamin Content of Fancy Cheeses. Food Research, **8**: 314–322. 1943.

(3) CUTTS, N. S., AND RAINBOW, C. Nutrition of a Strain of Brewer's Yeast Requiring p-aminobenzoic Acid. Nature, **164**: 234–235. 1949.

(4) IYA, K. K., AND FRAZIER, W. C. The Yeast in Surface Smear of Brick Cheese. J. Dairy Sci., **32**: 475–476. 1949.

(5) JUKES, T. H., AND STOKSTAD, E. L. R. Pteroylglutamic Acid and Related Compounds. Physiological Revs., **28**: 51–106. 1948.

(6) LAMPEN, J. O., AND JONES, M. J. The Growth-promoting and Antisulfonamide Activity of p-aminobenzoic Acid, Pteroylglutamic Acid and Related Compounds for *Lactobacillus arabinosus* and *Streptobacterium plantarum*. J. Biol. Chem., **170**: 133–146. 1947.

(7) LAMPEN, J. O., ROEPKE, R. R., AND JONES, M. J. The Replacement of p-aminobenzoic Acid in the Growth of a Mutant Strain of *Escherichia coli*. J. Biol. Chem., **164**: 789–790. 1946.

(8) MEYER, R. I. A Study of Some of the B-vitamin Requirements of Non-sporeforming, Peptonizing, Rod-shaped Bacteria Isolated from the Surface of Limburger Cheese. M. S. Thesis. Univ. of Ill. 1949.

(9) NIMMO-SMITH, R. H., LASCELLES, J., AND WOODS, D. D. Synthesis of "Folic Acid" by *Streptobacterium plantarum* and its Inhibition by Sulfonamides. Brit. J. Exptl. Path., **29**: 264–281. 1948.

(10) PURKO, M., NELSON, W. O., AND WOOD, W. A. The Associative Action between Certain Yeasts and *Bacterium linens*. J. Dairy Sci., **34**: 699–705. 1951.

(11) WOODS, D. D. Biochemical Significance of the Competition between p-aminobenzoic Acid and the Sulphonamides. Annals of the New York Academy of Science, **52**: 1199–1211. 1950.

ASSOCIATIVE GROWTH OF *STEPTOCOCCUS LACTIS AND AEROBACTER AEROGENES* IN MILK[1, 2]

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One purpose of the use of *Streptococcus lactis* cultures as starters for cheese is to inhibit the growth of undesirable bacteria, such as the gas formers, during the making process. *Aerobacter aerogenes,* one of the worst of the gas formers, often is the cause of "early gas" in cheese and seems hard to suppress in cheese made from raw milk. Therefore, a study has been made of the associative growth of *S. lactis* and *A. aerogenes* in milk under different conditions, in an attempt to learn how effective the lactic starter may be in holding down growth of the gas former and the production of gas.

METHODS

Cultures. The cultures of *S. lactis* and *A. aerogenes* were from the collection of this Department and were checked for purity and authenticity. Stock cultures were carried in skimmilk, with periodic transfer, incubation at 30° C. and storage in the refrigerator.

Preparation of inocula. Growth curves of the two cultures growing in skimmilk at 30° C. indicated numbers of the organisms at the beginning of the maximum stationary phase when they were used as inocula. In most experiments about one million *S. lactis* cells were added per milliliter of skimmilk, approximating the numbers added to milk as cheese starter, and the numbers of *A. aerogenes* cells were varied so as to give about the rod-to-coccus ratio desired. In one experiment, a ratio of 1:4,900 was maintained but the numbers of cells of each species were increased a hundred fold.

Enumeration of viable cells. Since both species of bacteria tend to chain or clump to some extent, a colony count is misleading. Therefore, plate counts were adjusted for the average chain length of the bacteria in each sample as determined microscopically. Quintuplicate plates were poured with the carrot-liver agar of Garey *et al.* (3) and incubated for 18 to 24 hr. at 30° C. for *A. aerogenes* and 48 hr. for *S. lactis.* In the preparation of samples for plating, an equal amount of sterile 2 per cent sodium citrate solution was mixed with the milk sample to break up pieces of curd and the clumps of bacteria.

Skimmilk medium. Growth of the two species alone and together was studied in skimmilk which had been freshly autoclaved in 10- and 25-ml. lots. Incubation was in thermostatically controlled water baths with less than 0.1° F. varia-

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tion in temperature. At least four trials were made under each set of conditions.

Determination of acidity. Titratable acidities expressed as lactic acid and pH values were determined by conventional methods. Total volatile acidities were estimated by the method of Hammer and Sherwood (4) and expressed as milliliters of 0.1 *N* volatile acid per 10 ml. of sample.

Determination of liberated gas. Liberated or free gas was measured by an apparatus similar to that used by Cranston (2) and Bartholomew (1). A 50-ml. burette was attached by one end to a reservoir by means of rubber tubing and at the other end to the culture tube. A two-holed rubber stopper in the culture tube permitted insertion of glass tubing for connection with the burette, and a small funnel for introduction of small amounts of concentrated H_2SO_4. The burette and reservoir contained a solution of 20 per cent Na_2SO_4 and 5 per cent H_2SO_4, as recommended in Methods of the Chemists of the U. S. Steel Corporation (5). CO_2 and H_2 have negligible solubility in this solution. All volumes were measured after the levels had been equalized and were corrected to standard conditions of 0° C. and 760 mm. of pressure.

Preparation of small lots of cheddar-like cheese. To study the associative growth of the two organisms in cheese curd, small lots of cheddar cheese were made by methods simulating those used in cheese factories. About 2 gal. of fresh milk were pasteurized at 160° F. for 16 sec. and cooled rapidly to 86° F. The starter used contained such numbers of *S. lactis* and *A. aerogenes* that the desired ratio of these organisms in the milk was obtained and over 1 per cent of *S. lactis* culture was added. When the titratable acidity of the milk had reached 0.15 per cent, 0.6 ml. of rennet extract was diluted to 20 ml. with cold water and added to the milk at 86° F. Because the cheese was "slow," allowances had to be made in methods of cutting and cooking the curd. Whey was drawn off when it showed an acidity of 0.12 to 0.13 per cent. The curd (25 g.) was transferred to large culture tubes which were connected to the apparatus for measurement of total evolved gas and were incubated at 30 or 37° C. for different periods.

RESULTS

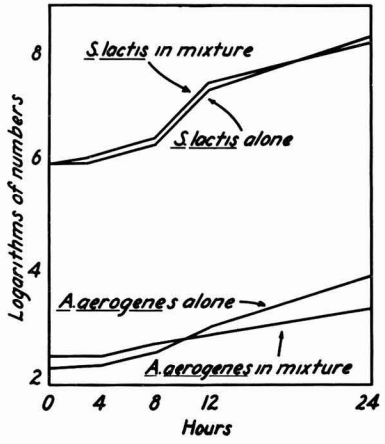
Inclusion of all of the data would make this report overlong. Therefore, only one example is presented of changes in pH, and titratable acidities have been omitted. Since changes in volatile acidities parallel those on gas, only one example is given. Growth and activities of the bacteria were followed for 72 or 96 hr. when incubation was at 20° C., but data for only the first 24 hr. are given.

Associative growth at 20° C.

When the initial inoculum consisted of *A. aerogenes* and *S. lactis* in the ratio of 1:3,900, the growth of the gas former was inhibited during the entire growth period of 72 hr. (figure 1). By the end of 3 days there were 780 times as many *A. aerogenes* organisms in the pure culture as in the one growing with *S. lactis* and four times as many after 24 hr. The growth curves for *S. lactis,* however, were practically identical in pure and in mixed cultures. Measurable amounts

of gas were not produced during the first 24 hr., and only small amounts by 96 hr., when there was about five times as much gas from the pure culture of *A. aerogenes* as from the mixed culture. Changes in acidity of the mixed culture were almost identical with those in the pure culture of *S. lactis*. Amounts of volatile acid were inconsiderable, although slightly more was produced by the pure culture of the gas former by 96 hr.

When the ratio of rod to coccus in the inoculum was 1 : 12, both *A. aerogenes* and *S. lactis* grew equally well in pure culture and together at 20° C. for 24 hr., with a slight inhibition of the gas former in the mixed culture thereafter (figure 2). Changes in acidity of the mixed culture were practically the same as those



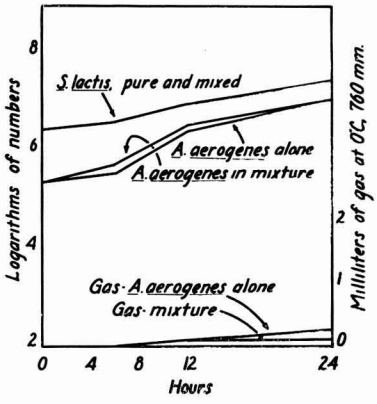


FIG. 1. Growth of *A. aerogenes* and of *S. lactis* separately and together in milk at 20° C., with an original rod: coccus ratio of 1:3,900 in the mixed culture. No measurable gas from *S. lactis* and little from *A. aerogenes*.

FIG. 2. Growth and gas production of *A. aerogenes* and of *S. lactis* separately and together in milk at 20° C., with an original rod: coccus ratio of 1:12 in the mixed culture. No measurable amounts of gas from *S. lactis* alone.

of a pure culture of *S. lactis*. Comparatively small amounts of volatile acid and gas were produced at 20° C., but there was some suppression of production of these volatile products after the first 12 hr. in the mixed culture.

Associative growth at 30° C.

When *A. aerogenes* and *S. lactis* were grown together in milk at 30° C., with an original rod-to-coccus ratio of 1 : 4,600 (figure 3), the growth of *A. aerogenes* was slightly inhibited during the first 12 hr. *S. lactis* grew as well as or better than in pure culture. Gas formation was not inhibited markedly until after the first 12 hr. Results on volatile acidity (figure 4) show that its production was suppressed more than was the evolution of gas and formation of acid was slowed down by the growth of the gas former along with *S. lactis*.

A rod-to-coccus ratio of 1 : 10 in the inoculum with incubation at 30° C. (figure 5) demonstrated that *A. aerogenes* and *S. lactis* grew practically as well

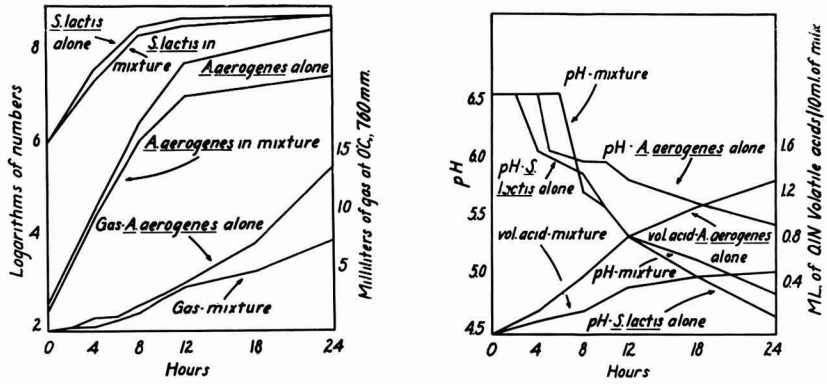


FIG. 3. Growth and gas production of *A. aerogenes* and of *S. lactis* separately and together at 30° C., with an original rod: coccus ratio of 1:4,600 in the mixed culture. A total of only 0.08 ml. of gas was produced by *S. lactis* in 24 hr.

FIG. 4. Changes in pH and in volatile acidity of *A. aerogenes* and of *S. lactis* separately and together in milk at 30° C., with an original rod: coccus ratio of 1:4,600 in the mixed culture. *S. lactis* did not produce measurable amounts of volatile acid.

in mixed as in pure culture, but that gas formation by *A. aerogenes* was inhibited appreciably throughout 24 hr. of incubation of the mixed culture. Results not shown demonstrated that production of acid was slower in the mixed culture after 12 hr. than in the pure culture of *S. lactis* and that the production of volatile acid was inhibited much as was gas formation. Gas production was suppressed to only a slight extent when a ratio of 1:4,900 was maintained but

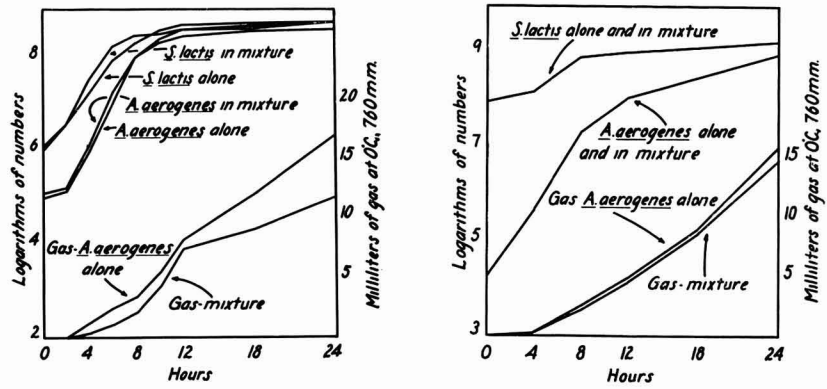


FIG. 5. Growth and gas production of *A. aerogenes* and of *S. lactis* separately and together in milk at 30° C., with an original rod: coccus ratio of 1:10 in the mixed culture. Negligible amounts of gas from *S. lactis* alone.

FIG. 6. Growth and gas production of *S. lactis* and of *A. aerogenes* separately and together in milk at 30° C., with an original rod: coccus ratio of 1:4,900 and 100 times the usual inoculum.

the inocula were increased one hundred fold (figure 6), and both organisms grew as well in mixed as in pure culture.

Associative growth at 37° C.

When the ratio of *A. aerogenes* to *S. lactis* in the inoculum was 1:5,300 and incubation was at 37° C., both organisms grew about equally well in mixed and in pure culture (figure 7). Gas formation was slower in the mixed culture than

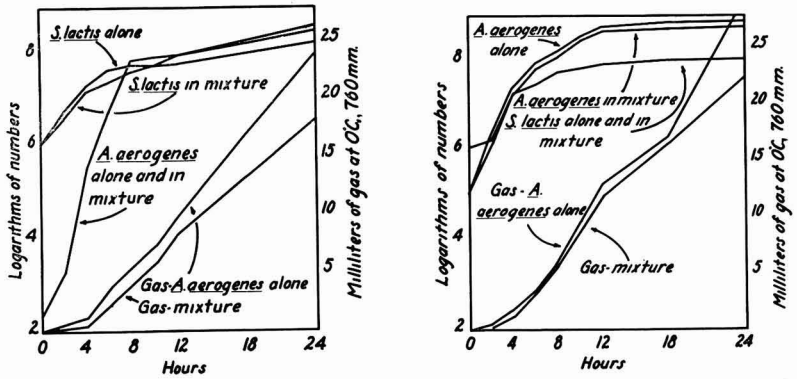


FIG. 7. Growth and gas production of *A. aerogenes* and of *S. lactis* separately and together in milk at 37° C., with an original rod: coccus ratio of 1:5,300 in the mixed culture. Negligible amounts of gas from *S. lactis* alone.

FIG. 8. Growth and gas production of *A. aerogenes* and of *S. lactis* separately and together in milk at 37° C., with an original rod: coccus ratio of 1:10. Negligible amounts of gas from *S. lactis* alone.

in the pure culture of *A. aerogenes* throughout 24 hr. of incubation. Formation of volatile acid by *A. aerogenes* was suppressed by the *S. lactis* bacteria after 8 hr. (not shown in figure), but total acidity was about the same in the mixed culture as in the pure culture of *S. lactis* throughout.

When the ratio of *A. aerogenes* to *S. lactis* in the inoculum was 1:10 and incubation was at 37° C., the organisms grew about equally well in mixed and in pure culture (figure 8) and gas production was only a little slower in the mixed culture than in the pure culture of *A. aerogenes*. Results not shown in the figure indicated a suppression of formation of volatile acid after the eighth hour, so that there was 80 per cent as much volatile acid in the mixed culture as in the *A. aerogenes* culture after 24 hr.

Gas production in cheddar cheese curd

When cheddar-like cheese curd was prepared from pasteurized milk inoculated with different proportions of *A. aerogenes* and *S. lactis* and incubated at 30 or 37° C., gas production was as shown in table 1. Slightly more *S. lactis* culture was added than is customary in cheese making. Gas was produced at a faster rate and in greater amounts at 37 than at 30° C. and increasing numbers of *A. aerogenes* organisms in the starter led to increased gas production. Even

when there were 10,000 times as many *S. lactis* as *A. aerogenes* organisms in the inoculum and the latter were only about 200 per milliliter in the inoculated milk, appreciable amounts of gas were produced.

TABLE 1

Total gas production by S. lactis and A. aerogenes in cheddar cheese-like curd at 30° and 37° C. with rod: coccus ratios of 1:10, 1:5,000 and 1:10,000 in the original inoculum

Ratio rod: coccus	1:10			1:5,000			1:10,000		
Elapsed time (hr.)	0	8	24	0	8	24	0	8	24
Incubation temp. (°C.)	(*ml. of gas*)[a]			(*ml. of gas*)			(*ml. of gas*)		
30	0	2.44	6.65	0	0.09	2.56	0	0.09	0.46
37	0	2.73	11.81	0	1.00	4.02	0	0.46	2.72

[a] All gas volumes corrected to 0° C. and 760 mm. of pressure.

DISCUSSION

Results with *A. aerogenes* and *S. lactis* in autoclaved skimmilk do not necessarily reflect what would happen in raw or in pasteurized milk, but the experiment on cheddar-like curd from pasteurized whole milk gave results similar to those in autoclaved skimmilk. It might be argued that increasing the heat treatment would destroy more of the accessory food substances necessary for *S. lactis* and thus give *A. aerogenes* a better chance. As a matter of fact, however, experiments in these laboratories have indicated that autoclaved whole milk from individual cows gives better growth and acid production by *S. lactis*, than will milk heated at 80° C. for 10 min., and this in turn is better than pasteurized milk.

The inhibition of growth and metabolism of *A. aerogenes* by *S. thermophilus* at 37° C. was observed by Bartholomew (1) to become most marked when the pH had dropped to about 5.15. In the present experiments the growth of *A. aerogenes* was inhibited appreciably only when the original ratio of rod to coccus was 1:4,600 and incubation was at 30° C. Gas formation was suppressed the most at this temperature, although it was reduced some at 37 and 20° C. Any relationship of acidity caused by *S. lactis* and inhibition of gas production was not obvious. When original numbers of *A. aerogenes* organisms were low, large numbers of *S. lactis* were effective in reducing the production of gas and volatile acid, but when a large inoculum of the gas former was used, even 5,000 times as many *S. lactis* bacteria had little effect.

S. lactis was most effective in suppressing *A. aerogenes* at 30° C., which is about the optimum temperature for the streptococcus, and was less effective at 20° C. At 37° C., the best temperature for *A. aerogenes*, *S. lactis* had little effect on the growth and activity of the gas former, and it would be likely that 40° C. would be still more in favor of *A. aerogenes*. Therefore, the temperatures used in cheese making would be advantageous to *A. aerogenes*.

At 30° C. there seemed to be some stimulation of the growth of *S. lactis* by growth of *A. aerogenes* but little or none at 37 or 20° C.

It is apparent that the cheese maker cannot rely much on a pure *S. lactis*

starter holding down growth and production of gas and volatile acid by *A. aerogenes* and that he should try to obtain milk in which these gas formers are low in numbers or absent. The starter used by most American makers is a mixture of lactic streptococci and *Leuconostoc* species (aroma formers), and it has been observed that lactic acid is produced faster in the vat by this mixed culture than by most pure culture strains of *S. lactis*. It is possible that the mixed culture would be able to hold down *A. aerogenes* better than would a pure culture of *S. lactis* and this combination should be tried. Adequate pasteurization of milk for cheese and prevention of recontamination will, of course, eliminate trouble with *A. aerogenes*.

SUMMARY

Streptococcus lactis was added to sterilized milk at about the level used in cheese making and *Aerobacter aerogenes* added in different proportions. Growth of both kinds of bacteria in pure culture and together was followed at 20, 30 and 37° C., together with increases in acidity, volatile acidity and gas. Tests also were made on cheddar-like cheese curd.

At 20° C. *S. lactis* grew with *A. aerogenes* as if the gas former were not present and *A. aerogenes* was suppressed only slightly. Amounts of gas and volatile acids produced at this temperature were negligible.

At 30° C., growth of *S. lactis* was stimulated some by the growth of *A. aerogenes*. The growth of *A. aerogenes* was suppressed when the ratio of rod to coccus in the inoculum was 1 : 4,600 but was affected but little when the ratio was 1 : 10. Production of gas and volatile acid was suppressed, especially after 12 hr., in the presence of *S. lactis*.

At 30° C., when 100 times the usual inoculum of *S. lactis* was used and *A. aerogenes* added to make the ratio of rod to coccus 1 : 4,900, growth of each organism in the mixed culture was the same as in pure culture and production of gas and volatile acid was not affected appreciably.

At 37° C., growth of both *A. aerogenes* and *S. lactis* in the mixture was like that in pure culture. Production of gas and volatile acid was inhibited more when the rod-to-coccus ratio in the inoculum was 1 : 5,300 than when it was 1 : 10, but the suppression was not great in either instance.

Cheddar cheese-like curd from pasteurized milk inoculated with *A. aerogenes* and *S. lactis* in different proportions and incubated at 30 or 37° C. showed that gas was produced at a faster rate and in greater amounts at 37 than at 30° C.; increasing numbers of *A. aerogenes* in the starter led to increased gas production. Large numbers of *S. lactis* bacteria were unable to prevent gas formation by small numbers of *A. aerogenes*.

It is concluded that the effect of *S. lactis* on growth and production of gas and volatile acid by *A. aerogenes* decreases as the temperature is increased from 30 to 37° C. and even large numbers of *S. lactis* do not suppress small numbers of *A. aerogenes* to any great extent when growing in autoclaved skimmilk.

REFERENCES

(1) BARTHOLOMEW, J. W. Association of Aerobacilli and Other Gas Formers with *Streptococcus thermophilus* in Milk. Ph. D. Thesis, University of Wisconsin. 1944.

(2) CRANSTON, J. A. Studies in Gas Production by Bacteria. I. Apparatus for Measurement of the Rate of Gas Production. Biochem. J., **24**: 525–528. 1930.

(3) GAREY, J. C., FOSTER, E. M., AND FRAZIER, W. C. The Bacteriology of Brick Cheese. I. Growth and Activity of Starter Bacteria during Manufacture. J. Dairy Sci., **24**: 1015–1025. 1941.

(4) HAMMER, B. W., AND SHERWOOD, F. F. The Volatile Acids Produced by Starters and by Organisms Isolated from Them. Iowa Agr. Expt. Sta. Research Bull. 80. 1923.

(5) *Methods of the Chemists of the United States Steel Corporation for the Sampling and Analysis of Gases.* 3rd ed. Carnegie Steel Co., Pittsburgh, Pa. 1927.

BACTERIA ASSOCIATED WITH A GELATINOUS OR SLIMY CURD DEFECT OF COTTAGE CHEESE[1]

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Cottage cheese plants in various parts of the United States in recent years have reported difficulty with a gelatinous curd defect in both creamed and uncreamed curd. The defect was first described in 1949 (2), but all available evidence indicates that it has been widespread and frequent, and losses due to this type of spoilage have been extensive for several years.

The defect may develop in 24 hr. during refrigerated storage or may be delayed for several days. Creamed curd usually has shown more frequent and rapid spoilage than uncreamed. The defect is characterized by formation of a thick, gelatinous, slimy film around each curd particle. In one form of the defect the gelatinous film has been whitish or yellowish in color and somewhat translucent. This form may be accompanied by little change in odor, and in other cases a fruity odor may be apparent. In still another form of the defect the film surrounding the curd particles may be yellowish or tinged with brown, extensive proteolysis may occur, the odor may be rotten or putrid and slightly rancid and the flavor quite bitter.

Terms that have been used in the trade to refer to the curd defect include: gelatinous, slimy, glassy, scummy, tapioca and fruity. Such terms usually indicating changes in physical appearance actually may refer only to the final, late stages of the spoilage. A number of off-flavors such as fruity, rancid or bitter may precede the formation of the gelatinous film around the curd particle.

Studies carried out several years ago in the Midwest and more recently on the West Coast suggested certain bacteria as causative agents. This paper presents a number of observations made during a study of the bacteriology of the defect.

EXPERIMENTAL

Microscopic examination of defective curd on different occasions always revealed large numbers of gram-negative rods in addition to the normal lactic streptococcus flora. Plating on both veal infusion and tryptone-glucose-skimmilk agar resulted in numerous colonies of gram-negative rods. Representative types were picked into litmus milk and the cultures segregated into four groups on basis of change in reaction in the milk after 5 days at 15° C. First attempts to reproduce the typical gelatinous defect with representative isolated cultures by inoculating normal creamed and uncreamed cottage cheese curd were unsuccessful. Later, the inoculation was repeated in uncreamed curd that had been soaked and washed a number of times with sterile alkaline tap water to raise the pH of the curd. Within 48-hr. storage at 15° C. following this treatment, a slimy film

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developed over each curd particle in some lots of the inoculated cheese. The defect produced experimentally appeared in two general forms that coincided with observations on the spoilage of commercial cheese.

The cultures that produced the defect experimentally were selected for fur-

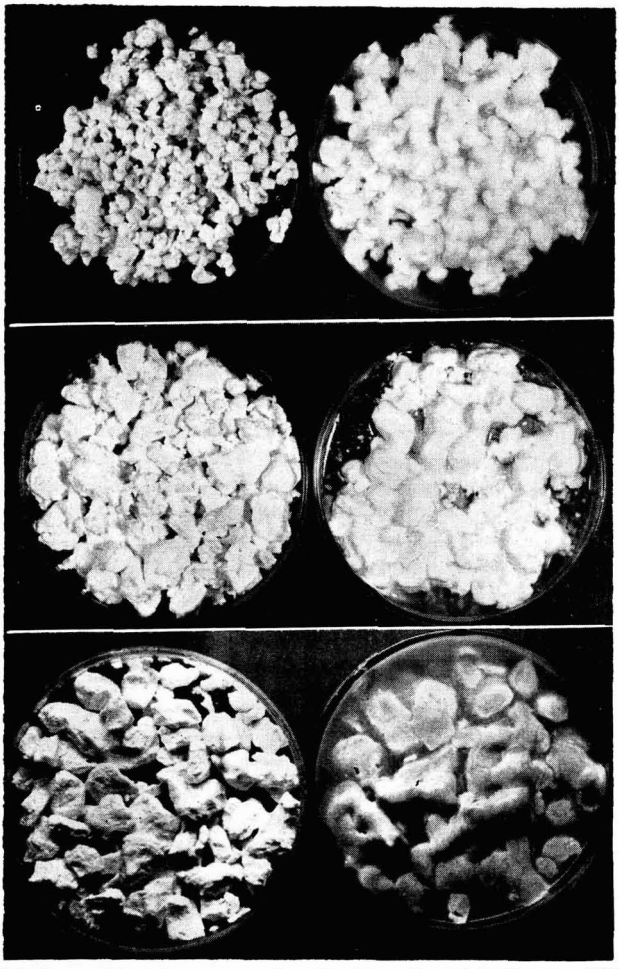

FIG. 1. Gelatinous defect in uncreamed curd. Top, *A. metalcaligenes.* Middle, *P. fragi.* Bottom, *P. viscosa.* Uninoculated control plates on left.

ther study and identification. A study of morphological and cultural characteristics revealed two species capable of producing the defect. The first of these produced the above described yellowish or brownish slime (fig. 1) and extensive proteolysis accompanied by a slightly rancid, rotten or putrid odor and a bitter flavor. Its characteristics coincided exactly with the description of *Pseudomonas viscosa* (1).

The second organism produced a somewhat translucent, slightly yellowish gelatinous film around each curd particle with no pronounced change in odor. Its characteristics coincided in every detail with those of *Alcaligenes metalcaligenes* (1). Some commercial lots of spoiled cheese had demonstrated a fruity odor. This led to inoculation of experimental cheese with *Pseudomonas fragi*. It also proved capable of producing the defect in "overwashed curd." *P. fragi* caused a defect similar to that of *A. metalcaligenes* (fig. 1) and, in addition, produced a distinct fruity odor and slightly rancid flavor in the cheese curd.

It was observed that the greenish-yellow pigment produced by *P. viscosa* on cottage cheese fluoresced strikingly under long-wave ultraviolet light (Mineralight or Black Light) in a darkened room (fig. 2). Tiny specks representing beginning spoilage of cheese by the organism could be detected easily in this manner in keeping quality tests before they were readily visible in ordinary light.

Source of spoilage organisms. Since the above observations, other outbreaks

FIG. 2. Defect produced by *P. viscosa* in creamed curd. Photographed in dark room with long wave ultra-violet light (Mineralight). Control plate on left.

of the gelatinous or slimy defect have yielded the above organisms from raw milk, spoiled cheese, water supplies and from milkstone deposits on cottage cheese vats or packaging equipment. In one instance *A. metalcaligenes* was isolated from a plant water supply and *P. viscosa* from the city water supply entering the same plant. Chlorination of the wash water supply, use of special cleaning procedures to remove milkstone deposits and stringent hypochlorite sanitization have greatly reduced incidence of the outbreaks. There is definite evidence that plant water lines may develop deposits of grit and slime that protect the spoilage bacteria, and these must be cleaned out before water chlorination can be practiced successfully. Creaming mixtures contaminated from poorly cleaned and sanitized equipment such as cans have been another source of the organisms.

Significance of pH and salt content of cheese. The observation that a higher pH favored growth of spoilage organisms both in creamed and uncreamed curd

led to trials to determine relative growth at different pH levels. Uncreamed, unsalted curd was ground and sufficient dilute lactic acid or Na_2CO_3 solution added to adjust the pH to 4.6, 4.8, 5.0, 5.2 and 5.4. Adjusted curd was allowed to stand for 1 hr. and readjusted to the desired pH if necessary. The curd next was drained and pressed into small flakes to approximate its original moisture content and physical appearance. Respective portions in sterile petri dishes then were inoculated with a 1 to 1,000 dilution of a 72-hr. milk culture of different strains of the three bacterial species isolated. Samples were incubated at about 15° C. and examined daily. The results in table 1 indicate *P. viscosa* to be the most acid-tolerant. It produced the defect in some trials at pH 5.0 but not at pH 4.8. *A. metalcaligenes* and *P. fragi* grew in the uncreamed curd at pH 5.2 but not at 5.0. Another observation of interest was that a stock strain of *Alcaligenes viscosus* closely resembling *A. metalcaligenes* in most characteristics was unable to produce the defect in three trials under the same conditions at pH 5.0, 5.2 or 5.4. A stock strain of *Pseudomonas fluorescens* very similar to *P. viscosa* in growth characteristics likewise failed to produce the defect in three trials at pH 5.0, 5.2 and 5.4.

TABLE 1

Effect of pH of uncreamed, unsalted cottage cheese on development of gelatinous curd defect during storage at 15° C. for 72 hr.

Species	Strain	Extent of defect at pH:			
		4.8	5.0	5.2	5.4
A. metalcaligenes	2	–	–	+	+++
	3	–	–	++	++
	5	–	–	++	+++
	6	–	–	+	+++
	11	–	–	+	++
P. viscosa	2	–	±	+++	+++
	3	–	±	+	+++
	10	–	±	+	+++
P. fragi	2	–	–	+	++
	3	–	–	++	+++
	7	–	–	++	+++

Data in this table represent a composite of 5 different trials. – indicates no growth; ± growth only in some trials; + slight growth; ++ moderate growth; +++ abundant growth.

The pH values of a number of dry curd samples of commercial cottage cheese from two plants were checked to determine increase in pH during creaming. Curd cut at 0.55 to 0.58 per cent titratable acidity showed pH values after washing and draining ranging from 4.65 to 4.75. Following creaming, the same samples ranged from pH 4.85 to 5.05. The average increase in pH of curd due to creaming was in the range of 0.2 to 0.35. However, creamed cottage cheese samples from other companies have shown pH values in the range of 5.2 to 5.3. It also was observed in these studies that when the uncreamed curd was washed sufficiently with alkaline water to raise the pH to approximately 5.5 to 5.6, a translucent, gelatinous layer immediately developed around each curd particle.

Effect of salt content in creamed curd was studied to determine whether a

slight increase in normal salt content of commercial cottage cheese would restrain growth of spoilage organisms. Cottage cheese curd and pasteurized cream were adjusted to desired pH levels before mixing. After mixing curd and cream, salt contents of different samples were adjusted to 0 and 1.0 per cent, respectively, in the samples at pH 4.8; to 0, 1.0, 1.5, 2.0, 2.5, and 3.0 per cent in the samples at pH 5.0; and to 0, 2.0, 2.5, and 3.0 in the samples at pH 5.2. The adjusted samples were inoculated with strains of each of the three spoilage bacteria. Results shown in table 2 indicate that *P. viscosa* is the most salt-tolerant of the three species. *P. fragi* appeared to be the least salt tolerant. All three species grew in 2 per cent salt at pH 5.2.

Heat resistance. Ten-ml. quantities of sterile milk in screw-cap vials were inoculated with 0.01 ml. of a 64-hr., 25° C. milk culture of the respective organism and pasteurized in a water bath at 61.7° C. (143° F.) for 30 min. Plate counts

TABLE 2

Effect of salt and pH level on extent of gelatinous curd development in creamed cottage cheese at 15° C. for 72 hr.

Species	Strain	Extent of defect at different pH and salt levels										
		pH 4.8		pH 5.0						pH 5.2		
		No salt	1%	No salt	1.0%	1.5%	2.0%	2.5%	3.0%	2.0%	2.5%	3.0%
A. metalcaligenes	2	–[a]	–	–	–	–	–	–	–	++	+	–
A. metalcaligenes	3	–	–	–	–	–	–	–	–	++	+	–
P. viscosa	3	–	–	±	±	±	±	–	–	+++	++	+
P. fragi	3	–	–	–	–	–	–	–	–	+	–	–

[a] Data in this table represent a composite of 5 trials. – indicates no growth; ± growth only in some trials; + slight growth; ++ moderate growth; +++ abundant growth.

of inoculated tubes ranged from about 1,000,000 to 5,000,000 per milliliter before pasteurization. After pasteurizing, the tubes were cooled immediately to 21° C. and incubated at that temperature for 1 wk., after which they were examined microscopically and subcultured for any other evidence of growth in the milk. Four trials with five strains of *A. metalcaligenes*, four of *P. viscosa* and one strain of *P. fragi* indicated all cells destroyed in every instance by the pasteurization.

DISCUSSION

The organisms capable of producing the gelatinous curd defect are typical soil and water types. The two most logical sources from which they might enter the plant are the water supply, either plant or municipal, and raw milk. There is evidence that they may gain entrance through excessive contamination of equipment with dust in dry areas of the country. Fresh vegetables incorporated in cottage cheese represent another source of *P. viscosa* and possibly of other spoilage organisms, and again point to the soil and water as reservoirs of these types. An interesting substantiation of the characteristics of *A. metalcaligenes* was observed. A strain of this species isolated from bull semen in an independent investigation proved capable of producing a marked gelatinous film when inoculated

onto cottage cheese curd. However, a strain of *A. viscosus* closely related to *A. metalcaligenes* in many respects was unable to produce the defect. Likewise a strain of *P. fluorescens* closely related to *P. viscosa* was unable to produce the defect. This however does not preclude the possibility that there are strains of *P. fluorescens* and perhaps other species of bacteria not yet isolated from defective cheese that are capable of producing a gelatinous curd defect.

Isolation of causative organisms from equipment such as cottage cheese vats emphasized the fact that special cleaning procedures may be necessary for such equipment. The high protein content of the curd and high cooking temperatures employed develop a film that sometimes cannot be removed by conventional cleaning procedures. Use of stainless steel sponges and other metal cleaning devices to overcome the cleaning difficulty only aggravate the problem by producing deep abrasions that collect additional deposit. Stainless steel curd rakes and similar devices also produce objectionable abrasions. In some instances even sanitization with 200 ppm. hypochlorite has been unable to penetrate to spoilage organisms in milkstone deposits on vats.

Data on effect of pH on growth suggest pH control as a means of avoiding the defect. As a result, acidification or culture inoculation of creaming mixture, ripening milk to higher acidities before cutting, use of acidified wash water and fewer washings all have been employed to produce a lower pH in the final product. Results indicate that a pH below 5.0 is necessary for consistent control of the defect, and the result of an unusually low pH usually is decreased consumer acceptance of the product. Therefore, the only certain method of preventing sporadic outbreaks is to eliminate the causative organisms by proper pasteurization of milk and cream, chlorination of the wash water and thorough cleaning and sanitization of equipment. The trend toward a more bland-flavored cottage cheese with lower ripening acidity, more thorough washing of the curd and higher final pH may explain part of the increased difficulty with this defect in recent years. Another factor may be centralization of cottage cheese plants with consequent longer marketing time required.

At pH 5.2, salt levels required to prevent growth of the three spoilage organisms studied are too high to be employed under practical conditions. Consumer complaints usually occur when salt content of creamed cheese reaches 1.5 per cent. Again elimination of causative bacteria appears the only sure means of preventing the defect. These studies have been limited to a few strains of causative bacteria. It is possible that effect of pH and salt may vary slightly with other strains of the three species studied and with variations in method of manufacture and composition of commercial cheese. Creaming of commercial cheese appears to encourage development of the defect due in part to the increase in pH of curd attending the creaming operation.

Heat resistance trials indicate that in moderate numbers the three spoilage organisms studied should be destroyed by conventional pasteurization treatment for cottage cheese milk. It is possible that contamination of milk with large clumps or massive numbers of organisms may result in survival of occasional cells.

However, most evidence suggests that spoilage organisms entering a plant in the raw milk or cream supply may circumvent the pasteurizer via such means as common washing facilities for raw and pasteurized milk equipment and also by dust and transfer on hands and clothes of workers.

SUMMARY

Three species of bacteria, *A. metalcaligenes, P. viscosa* and *P. fragi,* have been shown to be responsible for a gelatinous or slimy curd defect of cottage cheese. The defect may be accompanied by a distinct fruity odor and flavor or in some instances a rotten or putrid or slightly rancid odor and bitter flavor.

The principal sources from which the organisms enter the product are believed to be the water used to wash the curd and also improperly cleaned and sanitized manufacturing equipment.

Limited control of the defect may be attained by regulation of the pH of the final product; however, preventing entrance of causative bacteria into the pasteurized milk, cheese curd, creaming mixture or final product appears to be the only consistent, effective means of preventing it.

Data indicate that in moderate numbers all of the three species of causative bacteria should be destroyed by proper pasteurization of the cheese milk.

REFERENCES

(1) Breed, R. S., Murray, E. G. D., and Hitchens, A. P. *Bergey's Manual of Determinative Bacteriology.* 6th ed. The Williams and Wilkins Co., Baltimore. 1948.

(2) Elliker, P. R. *Practical Dairy Bacteriology.* McGraw-Hill Book Co., Inc., New York. 1949. p. 223.

RELATION OF DIFFERENT NUMBERS OF BACTERIOPHAGE AND BACTERIA TO POPULATION CHANGES AND ACID PRODUCTION

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Action of bacteriophage is one of the principal causes for failure in the production of lactic acid during the manufacture of certain dairy products, especially cheddar and cottage cheese. Rotation of cultures, strict sanitization of equipment and the use of procedures which minimize the number of bacteriophage particles that may gain entrance into milk and milk products from the air have been emphasized as means of keeping down the concentration of bacteriophage (6, 12). However, the level to which contamination with bacteriophage must be reduced to permit a given amount of lactic starter to coagulate milk normally has not been established.

Whitehead and Hunter (11) observed that increasing the inoculum to about 1.0 to 1.6 per cent permitted one additional propagation of some cultures before evident bacteriophage action. Anderson and Meanwell (2) indicated that retarded development of acid frequently could be overcome by using 4 per cent starter. However, Turner (10) added about 10 particles per milliliter of bacteriophage F68 to concentrations of about 1,000, 10,000 and 1,000,000 *Streptococcus lactis* IP5 bacteria per milliliter and found that the larger numbers of bacteria decreased the time required for mass lysis.

Recent investigations of Cherry and Watson (4) and Overcast et al. (9) have explained in part the occasional failure of seemingly normal cultrues. Cherry and Watson, working with *S. lactis* 122,4 and its homologous bacteriophage, suggested pH 5.0 as a critical level below which lysis does not occur. Overcast studied the influence of pH on five *S. lactis*-bacteriophage combinations and found that rates of proliferation of the bacteria and bacteriophage were in most cases greatly reduced at pH 5.0 and below. In general, bacteriophage proliferation and bacterial growth were affected similarly at low pH. However, with combinations 712-F56 and 122,1-F43, bacterial growth occurred at pH levels low enough to prevent proliferation of bacteriophage. These results suggested that starter bacteria may lower the pH rapidly enough to prevent mass lysis when the bacteriophage population is small.

This laboratory recently has encountered starter failures in the manufacture of cottage and cheddar cheese. In some cases the bacteriophage seemed to result from contaminated vats and equipment, and in others the cultures seemed to have been contaminated. This investigation has been undertaken to clarify further the effects of contamination with small numbers of bacteriophage on population changes and acid production of lactic streptococci and to determine the advantage of using large amounts of lactic culture as starter.

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METHODS

Cultures of *Streptococcus lactis* and *Streptococcus cremoris* were propagated in litmus milk at 32° C. Fresh cultures were used for each experiment. When 0.5 per cent culture was used as inoculum, cultures were diluted 1 : 3 in sterile distilled water to facilitate measurement of the inoculum. Undiluted culture was used when the amount of inoculum was either 4 or 8 per cent culture.

Strains of bacteriophage were maintained as whey filtrates. A series of 10-fold dilutions of each filtrate was prepared, using 100-ml. quantities of sterile skimmilk. Dilutions containing the correct numbers of bacteriophage per milliliter for use as inoculum were kept in a refrigerator and used for subsequent experiments. This procedure was considered to give more adequate control of the numbers of bacteriophage used than would have been the case had dilutions been prepared from the undiluted filtrate each day. Dilutions were such that about 1 per cent of diluted filtrate was required as inoculum for the skimmilk used in experiments.

Except in one case in which 5 l. of skimmilk were used with combination W2-F24, experiments were run using 300-ml. quantities of sterile skimmilk in 500-ml. bottles. After bottles of skimmilk were allowed to reach 32° C. in a water bath, they were inoculated with culture and diluted filtrate. A period of 5 min. was allowed before making initial determinations of titratable acidity and of the numbers of bacteria and bacteriophage. Subsequent determinations were made, usually at hourly intervals. Duplicate experiments were run, and the experimental values obtained for the two experiments were averaged.

Bacteriophage titers were estimated in litmus milk by the modification of the limiting active dilution technique of Nelson *et al.* (8) used by Collins *et al.* (5). This modification involved making 100-fold dilutions of the material to be tested in sterile distilled water and dispensing 1-ml. and 0.1-ml. quantities of each dilution into triplicate tubes of litmus milk. The tubes then were inoculated with one drop of a 1 to 8 dilution of freshly coagulated culture of susceptible bacteria, agitated and incubated at 32° C. for 15 to 16 hr. The last tubes that did not show the typical acid, reduction or coagulation of the control (litmus milk inoculated with one drop of the diluted susceptible bacteria) were used for computing the most probable number of bacteriophage particles per milliliter by consulting a probability table (3). Thus, it was considered that only one particle of bacteriophage was necessary to result in mass lysis of the bacteria added to a tube of litmus milk. This report shows that under certain circumstances a considerable number of particles were necessary to result in abnormal development of bacteria. However, a study of the data indicates that under the standardized conditions used for determining a titer in litmus milk inoculated with about 0.07 per cent culture, the presence of only one particle per tube gives ample bacteriophage inoculum to result in mass lysis. Furthermore, Turner (10) found that bacteriophage titers determined by the limiting dilution method were approximately equal to those found with the plaque count method.

Although use of triplicate tubes of litmus milk rather than one tube decreases error in the limiting dilution method and makes it possible to express bacterio-

phage titers in terms of most probable numbers, the use of such numbers should not be understood to indicate that values obtained by this method are exact. Most probable numbers obtained by the limiting dilution method are considered only approximately accurate. Since duplicate experiments were run, the average values reported represent results of a total of 6 tubes per dilution.

Dilutions used for determining bacteriophage titers also served for determining bacterial populations. These were determined at 32° C. by the standard plate count (1) on tryptone-glucose-beef extract-milk agar.

RESULTS

The effects of adding different numbers of bacteriophage particles to 0.5 per cent culture in skimmilk were determined. This amount of culture resulted in an initial bacterial count of about 10 million per milliliter. The titer of bacteriophage particles added was varied from as high as about 5 thousand per milliliter to as low as approximately one or two particles per 300 ml. of skimmilk for

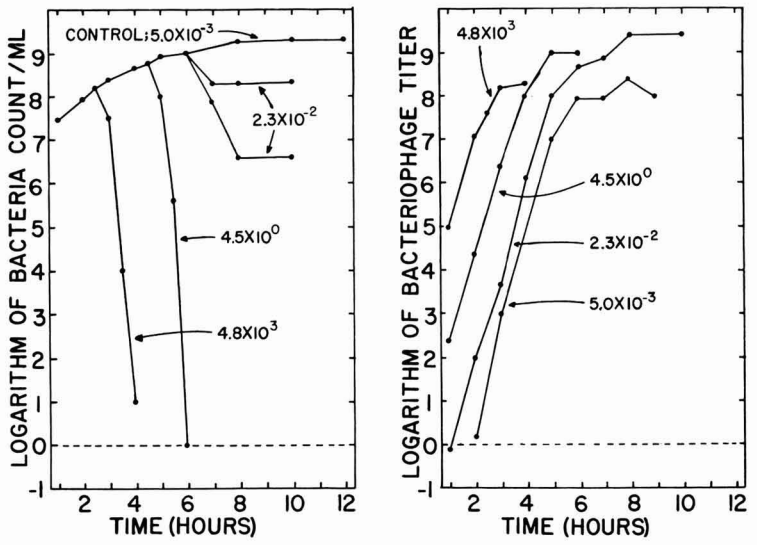

FIG. 1. Population changes resulting from combination of different numbers of bacteriophage F54 and 0.5 per cent culture of *Streptococcus lactis* 565. Curves are identified by the most probable number of bacteriophage particles per milliliter at 0 hr. Values of 2.3×10^{-2} and 5.0×10^{-3} were obtained by calculation.

cultures 565 and E8 and two or three particles per 5 l. of skimmilk for culture W2. Before mass lysis, evidenced by enormous reduction in the numbers of surviving bacteria, plate counts of surviving bacteria were not detectibly different from plate counts of bacteria growing normally in the absence of bacteriophage. It has been shown previously by Turner (10) that the normal and survival growth curves are not significantly different before mass lysis. Thus, results obtained for the initial portions of the survival growth curves were averaged with the

results obtained in the absence of bacteriophage, and the average values were used to represent normal growth of the bacteria. For all cases in which mass lysis occurred, the results following mass lysis were plotted.

The logarithms of counts of bacteria and of bacteriophage titers per milliliter are given in figure 1 for *S. lactis* 565 and its homologous strain of bacteriophage. Mass lysis occurred between 2.5 and 3 hr. with the addition of 4.8×10^{3} particles per milliliter of bacteriophage F54, and between 4.5 and 5 hr. with approximately 4.5 particles per milliliter. However, with the addition of 2.3×10^{-2} particles per milliliter, the counts of surviving bacteria following reductions between 6 and 7 hr. were considerably greater than those usually encountered following mass lysis. Considerable variation in the counts of bacteria surviving the action of bacteriophage occurred in duplicate and in repeated experiments, but the method used did not indicate differences in the numbers of bacteriophage. Duplicate results of plate counts, rather than average values, were plotted for this case in figure 1. When the number of bacteriophage particles added was decreased to 5.0×10^{-3} per milliliter, the survival curve remained the same as the normal growth curve throughout the 12 hr. experiment, and increases in the counts of bacteria and bacteriophage per milliliter were negligible after coagulation of the skimmilk between 6 and 7 hr.

TABLE 1

Effect on acidity production of adding different numbers of bacteriophage F54 to 0.5 per cent culture of Streptococcus lactis 565

Most probable no. of bacteriophage per ml. at 0 hr.	Approximate time of mass lysis	Per cent titratable acidity at:		
		0 hr.	7 hr.	12 hr.
	(*hr.*)			
4.8×10^{3}	2.5 – 3	0.21	0.24	.....
4.5×10^{0}	4.5 – 5	0.20	0.32	.....
4.5×10^{-1}	5 – 6	0.21	0.34	.....
2.3×10^{-2}	6 – 7[a]	0.21	0.52	0.70
5.0×10^{-3}	.....	0.20	0.54	0.78
Control	.....	0.20	0.53	0.76

[a] Numbers of surviving bacteria were greater than those usually encountered after mass lysis.

The rate of bacteriophage multiplication was not changed by adding smaller numbers of bacteriophage. Except in those cases in which mass lysis did not occur, differences in time required for mass lysis appeared to result entirely from the longer periods of time required for the smaller numbers of bacteriophage to equal the number of bacterial cells.

Titratable acidity values at 0 hr. and at either 6 or 12 hr., depending upon whether or not mass lysis of the bacteria occurred, and the hour at which mass lysis occurred with the different levels of bacteriophage are given in table 1. Cultures with added bacteriophage developed acidity prior to mass lysis as rapidly as did those without bacteriophage. After mass lysis, the rate of acid production decreased and usually there were no increases in titratable acidity after approximately 1 hr. In cases of the addition of 2.3×10^{-2} bacteriophage particles

per milliliter, coagulation occurred between 6 and 7 hr. at about the same time as the occurrence of decreases in the counts of surviving bacteria. That the counts of surviving bacteria were greater than those usually found following mass lysis probably was due to the high acidity during the time at which mass lysis normally would have occurred. Titratable acidity values at 8 through 12 hr. were only slightly less than those of cultures growing in the absence of bacteriophage. The addition of 5.0×10^{-3} bacteriophage particles per milliliter resulted in acidity values as high as those of cultures containing no bacteriophage.

The experiment described below was conducted to determine whether failure of mass lysis to occur in cases of the addition of small numbers of bacteriophage was related to high acidity. Three bottles of skimmilk were inoculated with 0.5 per cent culture of *S. lactis* 565. About 5.0×10^{-3} particles per milliliter of bac-

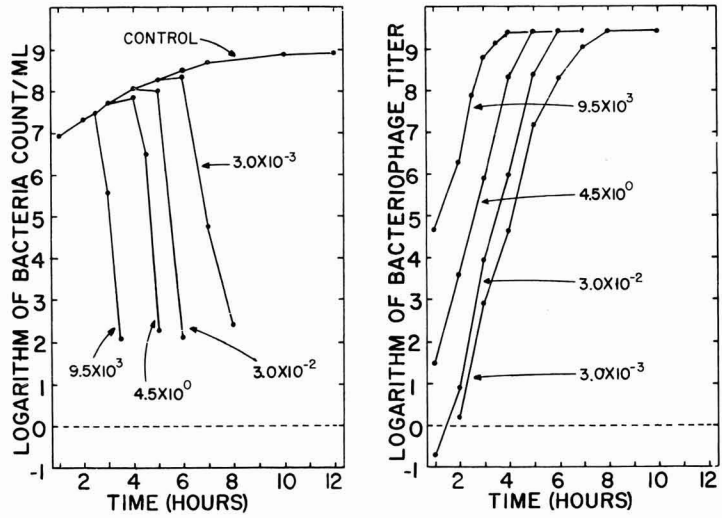

FIG. 2. Population changes resulting from combination of different numbers of bacteriophage F63 and 0.5 per cent culture of *Streptococcus cremoris* E8. Curves are identified by the most probable number of bacteriophage particles per milliliter at 0 hr. Values of 3.0×10^{-2} and 3.0×10^{-3} were obtained by calculation.

teriophage F54 were inoculated into one bottle, and NaOH was added at intervals to maintain a titratable acidity of less than 0.30 per cent. The other two bottles were inoculated with 2.5×10^{5} bacteriophage per milliliter. Enough sterile 10 per cent lactic acid to result in immediate coagulation and a titratable acidity of 0.56 per cent was added to one bottle, and the other was coagulated by means of rennet and $CaCl_2$.

Maintenance of low acidity enabled 5.0×10^{-3} bacteriophage particles per milliliter to cause mass lysis of 0.5 per cent culture between 8 and 10 hr. The plate count at 10 hr. was 10 per milliliter. With 2.5×10^{5} bacteriophage particles per milliliter, coagulation with lactic acid prevented mass lysis during 7 hr., but

coagulation with rennet and calcium chloride did not prevent mass lysis from occurring after an incubation period of about 2 hr.

In view of the importance of acidity in determining whether a small number of bacteriophage will result in mass lysis, a comparatively slow acid producing culture, *Streptococcus cremoris* E8, and its homologous strain of bacteriophage were used in experiments similar to those reported in figure 1 for combination 565-F54. Results with *S. cremoris* E8 and bacteriophage F63 (figure 2) differed from those with combination 565-F54 in that bacteriophage titers for F63 were higher than those for F54, with approximately the same bacteriophage count at 0 hr. Mass lysis occurred even with the smallest number of F63 used. The titratable acidity values with E8 (table 2) were considerably lower than with 565, and results indicated that much smaller numbers of bacteriophage F63 could be used without permitting coagulation before mass lysis when 0.5 per cent culture was used as inoculum.

Of the five strains of bacteriophage used by Overcast et al. (9), only F24 multiplied at pH as low as 4.8. Since our culture of W2 produced lactic acid in skim-

TABLE 2

Effect on acidity production of adding different numbers of bacteriophage F63 to 0.5 per cent culture of Streptococcus cremoris E8

Most probable no. of bacteriophage per ml. at 0 hr.	Approximate time of mass lysis	Per cent titratable acidity at:		
		0 hr.	7 hr.	12 hr.
	(*hr.*)			
4.5×10^{3}	2.5 – 3	0.21	0.23	.....
4.5×10^{0}	4	0.20	0.25	.....
3.0×10^{-2}	5	0.21	0.28	.....
3.0×10^{-3}	6	0.21	0.31	0.32
Control	–	0.21	0.33	0.52

milk at approximately the same rate as *S. lactis* 565, experiments were run with combination W2-F24 to determine whether mass lysis would fail to occur with about the same number of added bacteriophage as found with combination 565-F54. Results with W2-F24 were similar to those with 565–F54 except that mass lysis of W2 occurred with a lower number of bacteriophage. With the addition of 3.0×10^{-3} particles per milliliter, reductions in numbers of surviving bacteria occurred in duplicate trials at about 7 hr., similar to results with 2.3×10^{-2} particles per milliliter of F54. However, when the number of particles of F24 added was decreased to 5.0×10^{-4} per milliliter by increasing the volume of skim-milk to 5 l., mass lysis did not occur; results of plate counts and titratable acidity determinations throughout the 12 hr. incubation period were equal to those of a culture which contained no bacteriophage.

Increases in the amount of culture used as inoculum has been found to give slight protection against failure in acid production due to bacteriophage (11). However, a determination of the advantage of using larger amounts of culture required consideration of the effect on proximity of bacteriophage to bacteria. In plant difficulty and in experiment the number of bacteria almost invariably

is much greater than the number of bacteriophage. Thus, increases in the number of bacteria undoubtedly increase the chances of contact between bacteria and bacteriophage. Increased chances of contact would be expected to influence the rate of adsorption and consequently the rate of bacteriophage multiplication. Turner (10) found that mass lysis required 7, 6, and 4 hr. when 10 bacteriophage particles were added to 1,000, 10,000 and 1,000,000 bacteria. Recently, Mull (7) reported appreciable increases in the rate of bacteriophage multiplication when a constant number of about 25 bacteriophage particles per milliliter were added to concentrations of 300, 15,000 and 500,000 bacteria per milliliter.

The following experiment was run to determine the effects of adding 0.5, 4 and 8 per cent culture of *S. lactis* 565 to approximately 25 particles per milliliter of bacteriophage F54. These amounts of culture were larger than those which

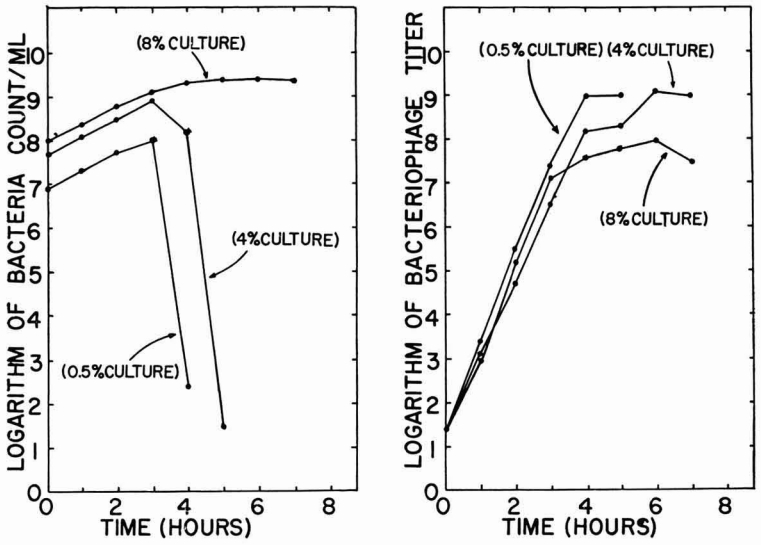

FIG. 3. Population changes resulting from combination of 0.5, 4 and 8 per cent culture of *Streptococcus lactis* 565 and a most probable number of 25 particles per milliliter of bacteriophage F54.

have been reported and more nearly comparable to the amounts used in the manufacture of dairy products. The average values for duplicate trials are plotted in figure 3. Differences in the number of bacteria did not seem to change the rate of bacteriophage multiplication. The rate was approximately the same for 0.5, 4 and 8 per cent culture until after 3 hr. After mass lysis of 0.5 and 4 per cent culture, the bacteriophage multiplied less rapidly, probably due to the lack of susceptible bacteria. With 8 per cent culture, coagulation occurred between 3 and 4 hr. Although mass lysis did not occur, the rate of bacteriophage multiplication decreased after 3 hr. Eight per cent culture of 565 was capable of apparently normal growth after addition of about 5,000 times as many bacterio-

phage as had been found possible with 0.5 per cent culture (figure 1). This probably was due to the earlier presence of larger amounts of lactic acid which retarded earlier the multiplication of bacteriophage.

Experiments were run to determine the incidence of mass lysis upon the addition of 25 bacteriophage particles per milliliter to 4 and 8 per cent culture of 3 other relatively fast acid-producing strains of lactic streptococci. The bacteria-bacteriophage combinations were W2-F24, 712-F56 and Hl, 10-PF10.

Mass lysis occurred after incubation of 4 per cent culture for about 4 to 5 hr.; coagulation did not occur during 7 hr. for any strains of bacteria used except *S. lactis* 712. With this culture the titratable acidity was 0.52 per cent, the plate count was 2.0×10^8 per milliliter, and coagulation had occurred at 5 hr. Although the results indicated 9.5×10^8 bacteriophage particles per milliliter at 5, 6 and 7 hr., the plate count at 7 hr. was 4.0×10^7, and the titratable acidity had increased to 0.62 per cent. Mass lysis did not occur with any of the 3 strains of bacteria when 8 per cent culture was used as inoculum (table 3). Coagulation

TABLE 3

Changes in populations of host bacteria after the addition of about 25 particles of homologous bacteriophage per ml. to 8 per cent culture in skimmilk

Hours incubation at 32° C.	Plate count/ml. for:			
	S. lactis 565	*S. lactis* W2	*S. lactis* 712	*S. cremoris* Hl–10
1	2.4×10^8	3.1×10^8	2.0×10^8	1.2×10^8
2	6.4×10^8	6.1×10^8	1.3×10^8	2.2×10^8
3	1.4×10^9	1.3×10^9	1.1×10^9	3.2×10^8
4	1.9×10^{9a}	1.5×10^{9a}	1.5×10^{9a}	5.5×10^8
5	2.7×10^9	1.8×10^9	1.4×10^9	3.8×10^{8a}
6	2.6×10^9	1.6×10^9	1.8×10^9	5.0×10^8
7	2.3×10^9	1.3×10^9	6.9×10^8	6.1×10^8

[a] Coagulation had occurred at this hour.

had occurred at either 4 or 5 hr., and titratable acidity values continued to increase throughout the 7 hr. incubation periods. At 7 hr., the titratable acidity values were 0.84 per cent for culture W2, 0.82 per cent for 712 and 0.64 per cent for Hl,10. The maximum titers of bacteriophage per milliliter were 9.5×10^6 at 4 hr. for F56, 2.5×10^7 at 5 hr. for PF10, and 2.5×10^8 at 5 hr. for F24.

DISCUSSION

Addition of approximately five particles of bacteriophage F54 per liter or approximately two or three particles of bacteriophage F24 per 5 l. of skimmilk resulted in normal plate counts and in normal development of titratable acidity by 0.5 per cent culture of *Streptococcus lactis* strains 565 and W2. However, the use of only slightly greater numbers of bacteriophage resulted in mass lysis of the host bacteria. These results indicate the need for using extreme care to avoid contamination in propagating mother and bulk starters and emphasize that extreme caution should be employed to sanitize equipment adequately just before the manufacture of products requiring lactic starter. It is evident that normal coagulation of a starter does not necessarily imply that it is free of bac-

teriophage. Although the addition of five particles of F54 per liter and 2 or 3 particles of F24 per 5 l. resulted in normal coagulation with 0.5 per cent culture for inoculum and normal development of bacteria following coagulation, the most probable numbers of bacteriophage after about 5 hr. were in each case about 10 million or more per milliliter. Had these seemingly "normal" cultures been used as starter, failure in the development of acidity would have resulted.

Neither the addition of different numbers of bacteriophage to 0.5 per cent culture (figures 1 and 2) nor the addition of 0.5, 4 and 8 per cent culture to a constant number of bacteriophage (figure 3) resulted in significantly different rates of bacteriophage multiplication. Changes in the number of bacteriophage added to a constant and much larger number of bacterial cells would not be expected to alter appreciably the chances of contact between bacteria and bacteriophage. It is not surprising that where mass lysis occurred, the time required for lysis was determined by the time required for the different numbers of bacteriophage, multiplying at a common rate, to equal the number of bacteria. However, increases in the number of bacteria undoubtedly increased the chances of contact. Increases in chances of contact were expected to increase the rate of adsorption and, consequently, increase the rate of multiplication of bacteriophage. But with these comparatively large numbers of bacteria, the rate of adsorption seems to have been at or near a maximum, permitting an essentially maximum rate of multiplication which was relatively unchanged by differences in number of bacteria.

Use of 8 per cent starter resulted in normal acid development and apparently normal growth of three duifferent strains of *S. lactis* and one strain of *S. cremoris* in the presence of approximately 25 particles of homologous bacteriophage per milliliter (figure 3 and table 3). With 4 per cent starter, only one strain, *S. lactis* 712, gave coagulation. Combination 712-F56 represents a case in which the bacteria are capable of causing coagulation in the presence of larger numbers of bacteriophage than with the other combinations studied. This possibly could be extended to include the five combinations studied by Overcast et al. (9). He found that of five cultures studied, *S. lactis* 712 multiplied most rapidly at pH 5.0. However, bacteriophage F56 increased very poorly at pH 5.0.

Use of large amounts of starter in cases where this is practical may be advantageous in helping to overcome the possible inadequacy of even diligent sanitization practices. However, in view of the large numbers of bacteriophage that may be present even in the absence of starter failure, it would seem extremely difficult to reduce the numbers of contaminating bacteriophage on the second day to that of the first. Thus, the use of large amounts of starter appears inadequate as a method for controlling bacteriophage unless combined with careful application of sanitization practices and rotational use of unrelated starters.

SUMMARY

The action of bacteriophage did not result in mass lysis of host bacteria where coagulation of skimmilk occurred before the population of bacteriophage became large enough to cause mass lysis. Experiments indicated that the high acidity at

the time of and after coagulation retarded the multiplication of bacteriophage and prevented mass lysis following the addition of small numbers.

Addition of about 5.0×10^{-3} particles per milliliter of bacteriophage F54 or about 5.0×10^{-4} particles per milliliter of bacteriophage F24 to 0.5 per cent culture of host bacteria in skimmilk resulted in normal coagulation, normal development of acidity, and normal bacterial populations. However, in the absence of evident bacteriophage action, the population of bacteriophage was 10 million or greater per milliliter in each case after about 5 hr.

The addition of as small numbers of bacteriophage as approximately 4.5×10^{-1} per milliliter of F54, 2.5×10^{-2} per milliliter of F24, or 3.0×10^{-3} per milliliter of F63 resulted in mass lysis and prevented coagulation of skimmilk by 0.5 per cent culture of host bacteria.

The rate of multiplication of bacteriophage was not appreciably changed by adding different numbers of bacteriophage to 0.5 per cent culture. For those cases in which mass lysis occurred, differences in length of time required for the different numbers of bacteriophage, multiplying at a common rate, to equal the number of bacteria appeared to determine the time required for mass lysis.

Adding 0.5, 4 and 8 per cent culture to a constant number of bacteriophage did not change the rate of bacteriophage multiplication. With these large numbers of bacteria the rate of bacteriophage multiplication appeared to be at or near a maximum which was relatively unchanged by differences in number of host bacteria.

With one combination, *S. lactis* 712 and bacteriophage F56, mass lysis failed to occur with addition of 4 per cent culture and about 25 bacteriophage particles per milliliter. Mass lysis did not occur when 8 per cent culture and 25 particles per milliliter of bacteriophage were used with four bacteria-bacteriophage combinations. For *S. lactis* strains 565 and W2, this number was about 5 thousand and 50 thousand times greater, respectively, than the largest numbers of bacteriophage which had been found to permit normal growth of 0.5 per cent culture.

REFERENCES

(1) American Public Health Associan. *Standard Methods for the Examination of Dairy Products*, 9th ed. Am. Pub. Health Assn., New York, N. Y. 1928.

(2) Anderson, E. B., and Meanwell, L. J. The Problem of Bacteriophage in Cheese Making. I. Observations and Investigations on Slow Acid Production. J. Dairy Research, **13**: 58–72. 1942.

(3) Buchanan, R. E., and Fulmer, E. I. *Physiology and Biochemistry of Bacteria*, Vol. I, p. 11. Williams and Wilkins Co., Baltimore, Md. 1928.

(4) Cherry, W. B., and Watson, D. W. The *Streptococcus lactis* Host-virus System. I. Factors Influencing Quantitative Measurement of the Virus. J. Bact., **58**: 601–610. 1949.

(5) Collins, E. B., Nelson, F. E., and Parmelee, C. E. The Relation of Calcium and Other Constituents of a Defined Medium to Proliferation of Lactic Streptococcus Bacteriophage. J. Bact., **60**: 533–542. 1950.

(6) Hunter, G. J. E. The Influence of Bacteriophage on the Cheese Making Process. J. Dairy Research, **13**: 294–301. 1944.

(7) Mull, L. E. Factors Influencing Organism-Bacteriophage Populations. Unpublished Ph.D. Thesis, Iowa State College Library, Ames, Iowa. 1950.

(8) NELSON, F. E., HARRIMAN, H. A., AND HAMMER, B. W. Slow Acid Production in Butter Cultures. Iowa Agr. Expt. Sta. Research Bull. 256. 1939.

(9) OVERCAST, W. W., NELSON, F. E., AND PARMELEE, C. E. Influence of pH on Proliferation of Lactic Streptococcus Bacteriophage. J. Bact., **61**: 87–95. 1951.

(10) TURNER, G. E. Dynamics of the *Streptococcus lactis* Bacteriophage Relationship. Unpublished Ph.D. Thesis, Iowa State College Library, Ames, Iowa. 1948.

(11) WHITEHEAD, H. R., AND HUNTER, G. J. E. Starter Cultures of Cheese Manufacture. Maintenance of Acid-producing Activity in Cultures of Lactic Streptococci. J. Dairy Research, **10**: 120–132. 1939.

(12) WHITEHEAD, H. R., AND HUNTER, G. J. E. Bacteriophage Infection in Cheese Manufacture. J. Dairy Research, **14**: 64–80. 1945.

THE EFFECT OF SUBSTITUTING COLOSTRUM FOR WHOLE MILK IN THE RATION OF DAIRY CALVES

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The importance of colostrum to the new-born animal long has been recognized. Ehrlich (2) found that colostrum, from mice immunized against the toxic proteins ricin and albin, transmitted specific antibodies that were absorbed by the nursing young. Sato *et al.* (17) found, in all normal parturitions, that bovine colostrum always contained agglutinins. Howe (6) showed that the blood serum of the new-born calf acquires specific protein fractions upon the ingestion of colostrum. Hansen and Phillips (5), in their electrophoretic studies, observed an immediate increase in the blood serum globulin of calves following the ingestion of colostrum or the colostrum pseudo-globulin during the first 24 hr. of life. When calves were reared without access to colostrum, the blood serum protein fractions did not approach normal values until the animals were about 8 wk. of age.

In addition to providing the calf with immunizing proteins, the colostrum gives the calf a large supply of some of the minerals, essential vitamins and other nutrients. Parrish *et al.* (14) found that the specific gravity and the total solids, solids non-fat, total protein and ash content of the colostrum decrease rapidly during the first six milkings. Garrett and Overman (3) found that the Ca, Mg, Na, P and Cl content of colostrum is high at parturition and during the early hours of lactation, but rather rapid decline toward a fairly constant level soon sets in as the milk becomes normal. Investigations by Pearson and Darnell (15) and Sutton *et al.* (19) showed that colostrum contains two to three times as much riboflavin as milk secreted later in the lactation period. Pearson and Darnell (15) found that colostrum contained twice as much thiamine as did milk. No difference was found between colostrum and whole milk in its nicotinic acid content. Work by numerous investigators (4, 9, 13, 16, 18, 19) reveals that colostrum contains several times more vitamin A and carotene than does whole milk secreted by the same animals later in lactation.

Investigations by Moore and Berry (10), Nezvesky *et al.* (12) and Sutton *et al.* (18) showed that the blood plasma vitamin A and carotene of the new-born calf were quite low. After ingestion of colostrum, there was a several-fold increase in vitamin A and carotene blood levels. Work by Sutton and Esh (20) showed that the tryptophan level in the blood plasma of the new-born calf was quite low and that after the ingestion of colostrum there was a marked increase. Kaeser and Sutton (8) found that when the available colostrum was substituted for the whole milk in the ration, calves receiving colostrum maintained higher levels of blood plasma vitamin A and carotene, made more rapid weight gains, especially during the first 6 wk., and were superior in physical appearance.

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Allen (1) fed only colostrum to nine calves for all or most of the milk-feeding period. Neither scouring nor serious digestive upsets occurred.

Since on most dairy farms the new-born calf is unable to consume all of the colostrum produced, it often is discarded. This is a very wasteful practice, especially since vitamin A and other nutrients are very essential to the health and growth of the very young calf.

This experiment was inaugurated to determine whether colostrum could be fed in place of whole milk in the ration of calves, whether it possesses any particular growth-promoting properties not contained in whole milk, and to study its effect on the health of calves.

EXPERIMENTAL PROCEDURE

Forty-seven heifer calves of the Jersey and Holstein breeds, which were born from March, 1946, through October, 1948, were used in this experiment. During the first 3 days after birth, all calves were allowed to suckle their dams. Three days after birth they were divided into two experimental groups which will be referred to as the colostrum group and the whole-milk group. Twenty-one calves were assigned to the colostrum group and received colostrum in place of whole milk until they became 60 days of age. Twenty-six calves were assigned to the whole-milk group and received whole milk until they reached 60 days of age.

The amount of whole milk fed was adjusted every 10 days. The Jersey calves received whole milk for the six 10-day periods at the rates of 6.0, 7.0, 8.0, 7.0, 5,0 and 4.0 lb. daily for each consecutive 10-day period. Holstein calves in the same group received 8,0, 9.0, 8.0, 6.0, 4.0 and 2.0 lb. daily. On the average, 357 lb. of whole milk were fed. The whole milk was produced by cows on winter rations.

The amount of colostrum fed was based on its dry matter content, in order to equalize the energy intake of the colostrum group with that of the whole-milk group. The colostrum was collected as the cows freshened from the herd at Beltsville, and was stored in 10-gal. cans and frozen until fed. Twenty-three 10-gal. batches of colostrum were collected and fed in the experiment. The colostrum group received on an average 268 lb. of colostrum, or the same amount of dry matter from this source as the whole-milk group obtained from whole milk.

Vitamin A and carotene determinations were made on each batch of colostrum, and similar determinations were made monthly on the whole milk as fed. All calves received U. S. no. 1 alfalfa hay and a grain mixture made up of 30 lb. of corn meal, 25 lb. of linseed oil meal, 25 lb. of soybean oil meal, 20 lb. of wheat bran, and 1 lb. of salt.

Weekly vitamin A and carotene analyses were made on the blood plasma of all calves until they reached 120 days of age. The condition of the feces was observed daily. The number of days each calf scoured or produced soft feces was recorded. Sulfaguanidine and sulfathiazole were used to control scours. The calves were weighed every 10 days.

RESULTS AND DISCUSSION

The vitamin A and carotene potencies of the colostrum milk were quite variable. The concentration of vitamin A in the different batches ranged from 25.8 γ per 100 ml. to 213.4 γ per 100 ml. The carotene potency ranged from 17.3 γ per 100 ml. to 213.4 γ per 100 ml. The concentration of vitamin A in the whole milk ranged from 12.56 γ per 100 ml. to 44.77 γ per 100 ml. The carotene in whole milk ranged from 4.12 γ per 100 ml. to 41.3 γ per 100 ml.

The vitamin A concentration of the blood plasma of the two groups of calves is shown in table 1. For the first 7-day period, the initial vitamin A values were

TABLE 1

Average amount of carotene and vitamin A in the blood plasma of dairy calves receiving colostrum and whole milk

Age	Carotene		Vitamin A	
	Whole-milk group	Colostrum group	Whole-milk group	Colostrum group
(*d.*)	(γ/*100 ml.*)	(γ/*100 ml.*)	(γ/*100 ml.*)	(γ/*100 ml.*)
1– 7	(14)[a] 28.7 ± 3.8	(16) 48.0 ± 6.6	(15) 8.6 ± 0.59	(15) 11.2 ± 1.01
8– 14	(20) 31.2 ± 3.6	(20) 96.0 ± 11.7	(20) 7.9 ± 0.36	(20) 12.7 ± 0.94
15– 21	(20) 30.0 ± 2.6	(20) 131.2 ± 14.6	(19) 7.0 ± 0.48	(20) 13.8 ± 1.10
22– 28	(19) 34.9 ± 4.9	(20) 133.0 ± 19.3	(17) 6.6 ± 0.36	(21) 12.7 ± 0.52
29– 35	(21) 29.8 ± 3.6	(20) 100.1 ± 15.1	(21) 5.9 ± 0.39	(20) 12.1 ± 0.67
36– 42	(20) 38.5 ± 7.9	(20) 87.1 ± 14.2	(20) 6.2 ± 0.49	(20) 12.4 ± 0.67
43– 49	(21) 57.4 ± 8.7	(21) 72.0 ± 11.7	(21) 6.5 ± 0.46	(21) 12.0 ± 0.55
50– 56	(21) 63.1 ± 8.4	(21) 67.6 ± 11.1	(20) 6.1 ± 0.49	(20) 11.3 ± 0.58
57– 63	(20) 77.9 ± 18.5	(19) 65.4 ± 10.4	(20) 8.0 ± 0.81	(20) 12.0 ± 0.65
64– 70	(20) 111.9 ± 17.7	(19) 87.6 ± 17.8	(21) 9.3 ± 0.87	(18) 11.7 ± 0.85
71– 77	(21) 144.3 ± 21.8	(21) 108.3 ± 16.8	(21) 10.2 ± 0.83	(20) 12.5 ± 0.74
78– 84	(20) 137.3 ± 17.4	(19) 128.3 ± 18.5	(19) 10.6 ± 0.92	(19) 13.0 ± 0.87
85– 91	(21) 154.0 ± 19.0	(18) 133.8 ± 23.5	(21) 12.0 ± 1.07	(20) 13.3 ± 0.96
92– 98	(20) 183.2 ± 15.5	(21) 142.3 ± 19.8	(19) 12.2 ± 0.76	(20) 13.6 ± 1.07
99–106	(21) 199.0 ± 16.0	(21) 143.9 ± 20.0	(21) 13.0 ± 0.76	(21) 14.4 ± 0.81
107–113	(21) 220.3 ± 16.6	(20) 165.8 ± 19.9	(20) 14.1 ± 0.69	(21) 15.1 ± 0.83
114–120	(20) 215.9 ± 18.7	(20) 190.3 ± 17.8	(21) 15.3 ± 0.83	(20) 15.4 ± 0.92

[a] Figures in parentheses indicate the number of determinations.

higher for the colostrum group than for the whole-milk group. This is due to the fact that the blood samples were not obtained until the latter part of the 7-day period, since blood samples were not drawn until after the calves were removed from the cow and placed in the calf barn. Thus, the effect of colostrum feeding was evident in the first 7-day period. The values remained at about the same levels for the first 3 mo. of the calf's life.

In the case of the whole-milk group, there was a decline in the plasma vitamin A values to about 60 days of age and then a rise. From birth to 35 days of age, these calves apparently were being depleted of their small reserve store of vitamin A which was built up during the 3-day period that they nursed their dams. At 35 days of age, the vitamin A level in their blood plasma was within the deficient range as judged by older calves (12). At 60 days of age the vitamin A level in the blood plasma started to rise and continued to rise through the remaining portion of the experimental period. This coincided with the period in which there was a large increase in the daily hay consumption. At

120 days of age, the plasma vitamin A concentrations of both groups were practically equal.

The calves in the whole-milk group received on the average the equivalent to 2,641 I.U. of vitamin A per day in the whole milk when carotene and vitamin A were expressed in terms of I.U. of vitamin A. For the colostrum group, the average daily intake of vitamin A and carotene, expressed in terms of I.U. of vitamin A, was 15,401 I.U. per day. The plasma vitamin A values of the colostrum group are higher than the values reported by Jacobson *et al.* (7) for calves receiving 50,000 I.U. of supplemental vitamin A (in cod-liver oil) per day to 50 days of age. Judging these results from the blood plasma picture, it would appear that vitamin A from colostrum is more efficiently utilized than vitamin A from cod-liver oil.

Table 1 also shows that there was a wide variation in the carotene blood plasma level at different ages within the groups, as well as large differences between the two groups at the same age. Considering both groups to 35 days of age, the calves in the colostrum group had from two to four times more carotene in their blood plasma than the calves in the whole-milk group. Since the average carotene potency of colostrum milk was ten times greater than that of the whole milk, the total carotene intake of the calves in the colostrum group was several times

TABLE 2

Average number of days the calves scoured and produced soft feces

	Jersey		Holstein	
	Whole-milk	Colostrum	Whole-milk	Colostrum
	(*d.*)	(*d.*)	(*d.*)	(*d.*)
Scours	3.3	6.1	4.1	2.8
Soft feces	4.8	7.6	3.3	7.8

greater than that of the calves in the whole-milk group. The feeding of colostrum provided an excellent way of increasing carotene intake of young calves to maintain a high concentration of carotene in the blood plasma.

From 30 to 60 days of age, there was a decline in the plasma carotene level of the colostrum group, which probably was a reflection of the reduction in the amount of colostrum fed during this period. While hay was the other source of carotene, the intake was not enough to compensate for the decreased intake of colostrum during this period and, as a result, daily carotene intake was reduced. During the same interval, the plasma carotene level of the whole-milk group showed a rather steady increase. The gradual increase in hay intake during this period apparently was responsible. The increased hay consumption (and the accompanying increase in carotene intake) more than compensated for the reduction of carotene from the milk source, which was initially low in carotene. However, the plasma carotene levels of the calves in the colostrum group were always higher than those of the calves in the whole-milk group for the first 70 days.

Table 2 shows the average number of days the calves scoured during the first 60 days and also the average number of days the feces were abnormal. The

results indicate that there was a greater incidence of scours among the calves in the colostrum group. The calves in this group produced feces that were abnormal or soft for longer periods than in the case of the calves in the whole-milk group. Apparently the extra antibodies or immunizing fractions which are contained in colostrum did not prevent scours. However, the colostrum as fed was not always of the best quality, because it was necessary to keep each can thawed out during the period of its use. This may account for increased occurence of scours in the colostrum group. It also will be noted that the Jersey calves scoured more often than the Holstein calves.

Mortality undoubtedly is a better measure of the value of the extra immunizing fractions in colostrum. None of the calves receiving the colostrum died, while 5 out of the 26 calves receiving the whole milk died. One of the five died of an ear infection at 15 days of age. Two calves died the third day, and two died at 8 and 14 days of age, respectively. The latter two calves had scoured the day before they died. While colostrum did not prevent scours in the calves, it may have helped them to resist the bacteria associated with scours and other infections.

Weight gains also can be used as a criterion in determining the general health of the calf. Table 3 shows that the weight gains of the colostrum group closely

TABLE 3

Average live weight of the two groups of calves

Age	Jersey		Holstein	
	Whole-milk	Colostrum	Whole-milk	Colostrum
(*d.*)				
0	(15)[a] 53.3 ± 1.9	(16) 57.4 ± 2.1	(6) 90.5 ± 5.7	(5) 88.6 ± 5.2
30	(15) 69.3 ± 1.8	(15) 71.3 ± 0.9	(6) 111.5 ± 2.1	(5) 110.8 ± 5.9
60	(15) 92.8 ± 2.8	(16) 93.3 ± 1.4	(5) 149.6 ± 4.4	(5) 148.8 ± 6.9
90	(15) 116.5 ± 4.4	(16) 118.3 ± 2.6	(6) 201.5 ± 2.6	(3) 195.7 ± 9.7
120	(15) 152.4 ± 5.4	(16) 154.1 ± 3.6	(6) 252.3 ± 3.1	(5) 235.6 ± 16.3
365	(15) 461.6 ± 16.5	(16) 456.0 ± 10.6	(6) 637.3 ± 14.2	(5) 610.8 ± 27.5

[a] Figures in parentheses indicate number of determinations.

paralleled those of the whole-milk group. This would indicate that colostrum milk, when fed on the same dry matter basis as whole milk, could be used successfully to replace whole milk in the ration of the dairy calf. This would result in a considerable saving of whole milk for salable purposes. The results also show that colostrum does not contain any special growth-promoting properties over and above whole milk, when both are fed on an equal dry-matter basis.

CONCLUSION

Calves fed colostrum milk until 60 days of age had much higher vitamin A and carotene blood levels than calves fed whole milk.

The weight gains of these two groups of calves were about the same. This would indicate that colostrum, when fed on the same dry matter basis as whole milk, can be used to replace whole milk.

The extra antibodies consumed by the colostrum-fed calves did not prevent the occurrence of scours but seemed to aid in the prevention of fatal infections.

REFERENCES

(1) ALLEN, N. N., DICKEY, H. C., AND FOOTE, M. W. Colostrum Milk for Calf Feeding. I. Stored Colostrum as a Substitute for Marketable Milk for Feeding Dairy Calves. II. The Effect of Cold Storage on the Vitamin A and Carotene Content of Colostrum Milk. Vt. Agr. Expt. Sta. Bull. 544. 1948.

(2) EHRLICH, P. Ueber Immunitat durch Vererbung und Säugung. Z. Hyg. Infektionskrankh., **12**: 183–203. 1892.

(3) GARRETT, O. F., AND OVERMAN, O. R. Mineral Composition of Colostral Milk. J. Dairy Sci., **23**: 13–17. 1940.

(4) HANSEN, R. G., PHILLIPS, P. H., AND SMITH, V. R. Colostrum Milk and its Vitamin A Content. J. Dairy Sci., **29**: 809–814. 1946.

(5) HANSEN, R. G., AND PHILLIPS, P. H. Studies on Protein from Bovine Colostrum. I. Electrophoretic Studies on the Blood Serum Proteins of Colostrum-free Calves and of Calves Fed Colostrum at Various Ages. J. Biol. Chem., **171**: 1, 223–227. 1947.

(6) HOWE, P. E. An Effect of the Ingestion of Colostrum upon the Composition of the Blood of New-born Calves. J. Biol. Chem., **49**: 115–118. 1921.

(7) JACOBSON, W. C., CONVERSE, H. T., AND MOORE, L. A. Effects of Vitamin A and Carotene Intake on Depletion Time of Young Dairy Calves. J. Dairy Sci., **32**: 418–428. 1949.

(8) KAESER, H. E., AND SUTTON, T. S. Beneficial Effect and Economic Importance of Using All Colostrum Produced in Calf Raising. J. Dairy Sci., **31**: 523–532. 1948.

(9) KRAMER, M. M., BAIR, MARGARET D., KUNERTH, BERNICE L., AND RIDDELL, W. H. The Vitamin A Value of Colostrum and Milk of Four Cows Determined by the Single Feeding Method. J. Agr. Research, **56**: 227–232. 1938.

(10) MOORE, L. A., AND BERRY, M. H. Effect of Colostrum on the Vitamin A and Carotene Content of Blood Plasma of New-born Calves. J. Dairy Sci., **27**: 867–873. 1944.

(11) MOORE, L. A., SYKES, J. F., JACOBSON, W. C., AND WISEMAN, H. G. Carotene Requirements for Guernsey and Jersey Calves as Determined by Spinal Fluid Pressure. J. Dairy Sci., **31**: 533–538. 1948.

(12) NEZVESKY, L., EATON, H. D., JOHNSON, R. E., MATTERSON, L. D., BLISS, C. L., AND SPIELMAN, A. A. The Effect of Vitamin A from Prenatal Storage and from Ingestion of Colostrum on the Neonatal Calf. J. Dairy Sci., **33**: 315–323. 1950.

(13) PARRISH, D. B., WISE, G. H., ATKESON, F. W., AND HUGHES, J. S. Properties of the Colostrum of the Dairy Cow. III. Several Factors Affecting Vitamin A and Carotenoid Content. J. Dairy Sci., **32**: 209–221. 1949.

(14) PARRISH, D. B., WISE, G. H., HUGHES, J. S., AND ATKESON, F. W. Properties of the Colostrum of the Dairy Cow. V. Yield, Specific Gravity and Concentrations of Total Solids and its Various Compounds of Colostrum and Early Milk. J. Dairy Sci., **33**: 457–465. 1950.

(15) PEARSON, P. B., AND DARNELL, A. L. The Thiamine, Riboflavin, Nicotinic Acid and Pantothenic Acid Content of Colostrum and Milk of the Cow and Ewe. J. Nutrition, **31**: 51–58. 1946.

(16) RAY SARKAR, B. C. Carotene and Vitamin A in the Colostrum of Cows of Typical Indian Breeds. J. Dairy Sci., **31**: 479–487. 1948.

(17) SATO, M., OGURA, K., AND IKEJIMA, H. Agglutinin Complement and Chemical Composition of the Colostrum of the Cow. J. Dairy Sci., **14**: 156–172. 1931.

(18) SUTTON, T. S., AND KAESER, H. E. Some Physiological Effects of Extending the Colostrum Feeding Period of Dairy Calves. J. Dairy Sci., **29**: 13–26. 1946.

(19) SUTTON, T. S., WARNER, R. G., AND KAESER, H. E. The Concentration and Output of Carotenoid Pigments, Vitamin A and Riboflavin in the Colostrum and Milk of Dairy Cows. J. Dairy Sci., **30**: 927–932. 1947.

(20) SUTTON, T. S., AND ESH, G. C. The Nutrition of the Newborn Dairy Calf. I. Changes in the Tryptophan Content of the Blood Plasma Following Birth and the Ingestion of Colostrum. J. Dairy Sci., **31**: 183–187. 1948.

THE INFLUENCE OF ENERGY INTAKE ON THE NITROGEN RETENTION OF GROWING CALVES

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The relationship of caloric intake to the efficiency of protein utilization has been studied in the dog, rat, mouse and man (1, 2, 3, 8). These studies showed that a restriction in the caloric intake results in a decreased efficiency of protein utilization in both adult and growing animals. In most cases the restricted caloric intake was considerably below the level required for normal maintenance or growth. For example, Allison *et al.* (1) found that in adult dogs the utilization of dietary protein, as measured by nitrogen balance, was not affected until the caloric intake was reduced to 50 per cent or less of the normal allowance.

Less is known of the effect of an increase in caloric intake above that considered adequate for normal maintenance or growth. While studying the protein requirements of growing dairy calves (Lofgreen *et al.*, 5), data were obtained on the effect of an increased energy intake at a constant protein level on nitrogen utilization. This paper presents the results of such studies. The term "nitrogen utilization" is used herein to refer to nitrogen retained as determined in balance studies.

EXPERIMENTAL PROCEDURES

Eighteen Holstein heifer calves weighing approximately 150 lb. and averaging 9 wk. of age were divided at random into four groups and placed upon four dietary treatments as indicated in table 1. The low levels of energy and of protein were the recommended allowances of total digestible nutrients (TDN) and digestible crude protein of the 1936 Morrison feeding standard (7). The high energy level was the Morrison standard plus 15 per cent and the high protein level was an average of 160 per cent of the protein allowance. The bases of these levels have been discussed elsewhere (Lofgreen *et al.*, 5).

The hay fed was good quality alfalfa, clover or early-cut timothy. From 150 to 250 lb. body weight a commercial calf starter containing approximately 16 per cent digestible protein was fed. Beginning at 200 lb. a gradual change from the calf starter to a more simple growing mixture was made, the change being complete at 250 lb. The proper level of protein was maintained by supplementing the starter or growing mixture with various proportions of blood flour, casein, soybean meal and linseed meal. The quality of the protein in the rations of ruminants usually is considered to be of relatively minor importance in comparison to non-ruminant animals (7). It is possible, however, that there may be conditions under which the utilization of protein by the ruminant may be influenced by its dietary source (5). Because of this possibility, it was

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TABLE 1

Dietary treatment of animals

Group	No. of animals	Treatment
1	5	Low energy, low protein
2	4	High energy, low protein
3	5	Low energy, high protein
4	4	High energy, high protein

thought that a mixture of high protein supplements may provide a protein source of higher quality than a single supplement.

To furnish the higher energy level, the total feed allowance was increased while maintaining the protein intake constant by the proper reduction in the concentration of protein in the concentrate mixture. This resulted in a 20 per cent increase in the non-nitrogenous TDN intake for the high energy groups.

Nitrogen balances were determined at the weights of 150, 200, 250, and 300 lb. A constant dietary intake was maintained for a period of at least 7 days prior to collection of mixed urine and feces. The collection periods were of 5 days duration during which formaldehyde and alcohol were added to prevent nitrogen loss and the excreta were removed from the pans two or three times daily. The use of formaldehyde was based upon the work of French (4) and the alcohol was used to prevent the excreta from freezing. The 5-day composite was diluted to a known volume and samples taken for nitrogen analysis. In order to determine the digestible protein intake, digestion trials were conducted on male calves from which feces could be collected separately.

RESULTS AND DISCUSSION

A summary of the results of this study is presented in table 2. It is apparent that a 20 per cent increase in the non-nitrogenous TDN brought about a marked increase in the efficiency of utilization of the nitrogen available above maintenance requirements when the calves were on the low protein intake. This

TABLE 2

The effect of energy intake on nitrogen retention

	Low Protein		High protein	
	Low energy	High energy	Low energy	High energy
Nitrogen apparently digested (*g./d.*)	41.0	39.4	75.1	71.2
Apparently digested nitrogen above maintenance needs[a] (*g./d.*)	26.5	24.9	60.6	56.7
Non-nitrogenous TDN consumed (*lb./d.*)	3.2	3.9	3.0	3.6
Nitrogen retained (*g./d.*)	15.6	19.6	22.9	20.3
Nitrogen retained (% of consumed nitrogen)	25.8	31.6	24.4	21.5
Nitrogen retained (% of apparently digested nitrogen above maintenance needs)	58.8	78.7	37.8	35.8

[a] Apparently digested nitrogen minus the maintenance requirement which is calculated using 43.5 g. of apparently digestible nitrogen (0.6 lb. apparently digestible protein) as the daily maintenance requirement of a 1,000 lb. animal and assuming that the requirement varies with the 0.75 power of body weight.

increase was statistically significant. When the animals were consuming the high protein level, an increase in the energy intake brought about no increase in nitrogen retention.

There was no significant difference in the weight gains made on the two protein levels at the low energy intake, the average for all calves being 1.2 lb. per day. The calves on the higher energy level gained an average of 1.4 lb. per day with no significant difference in the gains on the two protein levels.

It is well known that if the non-nitrogenous nutrient intake of an animal is below that needed for maintenance, protein is used as a source of energy. In the growing animal the ability to utilize absorbed nitrogen for tissue building is limited by the rate at which the body is able to synthesize these protein tissues. If the energy intake is above the maintenance requirement but insufficient to permit maximum growth and the protein supply is sufficient to meet the needs of tissue synthesis, the rate of growth and nitrogen retention will be limited by the energy intake. Any excess protein above that used to synthesize the protein tissue formed will be used for energy. Under such conditions an increase in non-nitrogenous TDN intake clearly would bring about a faster rate of gain and an increased efficiency of utilization (for tissue synthesis) of the protein in the ration. If the energy intake remained constant, one would not expect an increased protein intake to increase the nitrogen retention. The excess protein would merely be used to furnish energy. In the case of the calves on the low energy intake, however, an increase in protein intake brought about a significant increase in nitrogen retained despite the lowered efficiency. A possible explanation of this happening could be that the low protein level was not sufficient to meet the needs of the potential rate of protein synthesis permitted by the growth on the low energy level; an increase in protein, while maintaining the TDN constant, permitted the maximum rate of synthesis of protein tissue to be achieved. The gain then would be made up of a higher proportion of protein than that of the low protein calves which gained at the same rate. Werner (8) found that in adult man in negative nitrogen balance due to caloric restriction an increase in nitrogen intake while maintaining the low caloric level would restore nitrogen balance. The increase in gain and efficiency of nitrogen retention brought about by the increased non-nitrogenous TDN intake on the low protein level could be due to the sparing effect of carbohydrate on urinary nitrogen excretion (6) rather than a simple correcting of a deficient energy intake on a ration adequate in protein.

As shown in figure 1, the increase in efficiency of protein utilization due to an increase in energy intake on the low protein level was most marked at 150, 200 and 250 lb. At 300 lb. the increase was only slight. This small increase probably was due to the fact that the non-nitrogenous TDN increase was only 11 per cent at this weight, while for the 150, 200 and 250 lb. weights the increases amounted to 29, 20 and 30 per cent, respectively.

These results with young dairy calves confirm the observations made on other species that the efficiency of protein utilization as measured by nitrogen balance, is markedly affected by energy intake. Furthermore, at the energy and protein

levels fed, an increase in energy level does not increase the efficiency if the animal is on a high protein intake. The observations show the importance of liberal feeding of growing calves in order to take advantage of the increased efficiency of protein utilization. The data further indicate the importance of maintaining an adequate energy intake in studies of quantitative protein requirements and in studies of the nutritive quality of proteins.

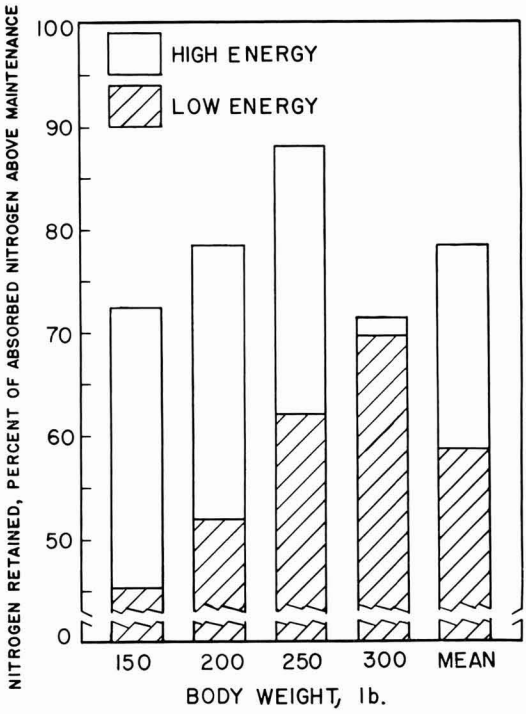

FIG. 1. The efficiency of nitrogen utilization at different weights on the low protein intake.

SUMMARY

A study was conducted with young dairy calves on the effect of energy intake on the efficiency of protein utilization as measured by nitrogen balance.

It was shown that on a low protein intake an increase in non-nitrogenous TDN consumption resulted in a marked increase in the retention of the nitrogen available for growth.

The findings with the young growing calf are in accord with the results with the adult dog, rat, mouse and man and growing mice and rats.

REFERENCES

(1) ALLISON, J. B., ANDERSON, J. A. AND SEELEY, R. D. The Determination of the Nitrogen Balance Index in Normal and Hypoproteinemic Dogs. Ann. N. Y. Acad. Sci., **47**: 245–271. 1946.

(2) BARNES, R. H., AND BOSSHARDT, D. K. The Evaluation of Protein Quality in the Normal Animal. Ann. N. Y. Acad. Sci., **47**: 273–296. 1946.

(3) BOSSHARDT, D. K., PAUL, W., O'DOHERTY, K., AND BARNES, R. H. The Influence of Caloric Intake on the Growth Utilization of Dietary Proteins. J. Nutrition, **32**: 641–651. 1946.

(4) FRENCH, R. B. The Use of Preservatives to Prevent Loss of Nitrogen from Cow Excreta during the Day of Collection. J. Agr. Research, **41**: 503–506. 1930.

(5) LOFGREEN, G. P., LOOSLI, J. K., AND MAYNARD, L. A. Comparative Study of Conventional Protein Allowances and Theoretical Requirements of Growing Holstein Heifers. J. Animal Sci., **10**: 171–183. 1951.

(6) LUSK, G. *The Science of Nutrition.* 4th ed. W. B. Saunders Co., Philadelphia and London. 1928.

(7) MORRISON, F. B. *Feeds and Feeding.* 20th ed. Morrison Publishing Co., Ithaca, N. Y. 1936.

(8) WERNER, S. C. Some Effects upon Nitrogen Balance of the Independent Variation of Protein and Calories in Man. J. Clin. Invest., **27**: 561. 1948.

FACTORS AFFECTING THE ANTIRACHITIC ACTIVITY OF ALFALFA AND ITS ABILITY TO PREVENT RICKETS IN YOUNG CALVES

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Green plants have been shown to be low in antirachitic activity and it has been assumed that artificially dried roughages contain little or no vitamin D. The antirachitic activity of artificially dehydrated alfalfa was found to be nil or too small to measure (33, 34, 35) and to be increased by exposure to sunshine after the green alfalfa was cut for hay (3, 33, 34, 35, 39). In fact, an inverse relation between the carotene and vitamin D content of alfalfa has been demonstrated (30, 34, 39).

There also is evidence that some roughages possess antirachitic activity without being exposed to solar radiation while drying (2, 5, 19, 23, 25, 29, 38, 39). Several investigators found no direct relation between the vitamin D content of the hay crop and the amount of sunshine it received during the curing process (19, 25, 29, 39).

Hodgson and Knott (19) found that green pasture herbage had definite calcifying activity for rats, the amount being equal to that found in the same material cured in the sun for 15 hr. Voltz and Kirsch (38) found antirachitic activity in English ryegrass, even when it was grown in total darkness on garden soil or on a nutrient medium. However, Hess and Weinstock (17) found no activity in the young wheat plant grown in darkness, but when grown in light and artificially irradiated while growing, it was found to have definite antirachitic activity. They also found no antirachitic activity in lettuce until after it was cut and artificially irradiated. Mellanby and Killick (23) found some antirachitic activity in summer-grown grass, dandelion leaves, and carrot and turnip greens, but none in cabbage. Green summer-grown spinach was reported to have slight antirachitic activity for the rat but no activity was found in spinach grown under tinted glass or in that grown during the fall, winter or spring months (5).

Recently, three different investigators (25, 29, 30, 42) presented data showing that the vitamin D content of the hay crop was not always correlated with the amount of sunshine it received during the curing process. The evidence indicates, as has been mentioned previously by this laboratory (25) and by others (40), that factors other than the amount of sunshine the roughage receives during curing may influence its final antirachitic activity. Since these other factors have not been investigated heretofore, one purpose of the experiments reported in this paper was to investigate their nature.

It is well known that the young ruminant requires some form of vitamin D for proper growth and bone formation. It has been well established that both sunshine (1, 7, 11, 13, 15, 31, 32, 40) and the usual field-cured roughages (3, 4, 16, 21, 26, 31, 40, 41) are able to furnish the antirachitic factor to the young ruminant. Under usual conditions, these sources have supplied sufficient

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amounts of the factor to prevent rachitic symptoms in young calves. Several experiments (13, 15, 26, 31) have shown that calves kept without access to sunshine and fed the usual amounts of field-cured roughages develop normally without any evidence of rickets. On the other hand, a number of investigators have reported the occurrence of rachitic conditions in young calves (9, 16, 18, 32) and in young sheep (8, 9, 10) that were reared under usual farm conditions. In most of these investigations the presence of rickets was confirmed and the symptoms were alleviated by vitamin D-active supplements.

It is realized that the antirachitic activity of sunshine is seasonally very low in some areas and that hays have been noted to be inconsistent in their antirachitic activity. The increase in the use of legume silage, artificially dried hays and barn-cured hays makes it desirable to have an understanding of the factors affecting the amount of antirachitic activity furnished by these feeds. The antirachitic activity of one crop of wilted alfalfa silage and mow-dried alfalfa was reported by this station to be sufficient to cure rachitic symptoms in 4-mo.-old rachitic calves when the roughage was fed at the accepted minimum rate (25).

The antirachitic activity of alfalfa hays dried without exposure to the sun, and of alfalfa silage, both of which have been commonly thought to contain very little or no vitamin D, has been investigated in this present study. The effect of stage of maturity on the antirachitic activity of the hay also has been investigated.

PLAN OF EXPERIMENT AND METHODS

The experiment was designed to determine (a) whether the stage of maturity at which alfalfa is cut affects the vitamin D activity of the hay and, if so, to evaluate some factors that may be responsible for any change in activity; and (b) to determine if artificially dried alfalfa or alfalfa silage will prevent rachitic symptoms in calves when it is their only source of vitamin D.

An alfalfa field was divided into three plots and each plot was cut at a different stage of maturity. One plot was cut on August 6, 1947, when the alfalfa was in the bud stage, *i.e.*, before any appreciable number of blossoms appeared. The second plot was cut at the half-bloom or hay stage on August 18. The third plot was cut at the mature or seed stage on September 3.

Immediately after the alfalfa was cut, it was placed on a barn drier and dried with heated air forced through the hay. When dry, it was baled and stored in a hay loft until needed as feed for the calves. A sample for chemical and vitamin D analysis was obtained by boring the bales with a hay-bale borer. The sample representing each stage of maturity was ground and stored in the dark in an amber reagent bottle until used for determination of its vitamin D content, which was performed by using the curative line test technique with rats (37).

Samples also were taken from each of the three plots immediately before cutting, and the plants in these samples were separated by hand picking into three portions. One portion consisted of leaves and stem portions that were entirely dead and either dry or partially dry. Another portion consisted of leaves whose surface area was more than half dead or yellow from insect injury

or other causes. The dead leaves from samples of the three plots were combined into one sample, and the half-dead leaves were similarly combined, the remainder of the plant being discarded.

A third portion consisted of alfalfa leaves that were entirely green. Since there were no entirely green leaves on any of the alfalfa plants harvested at the three stages of maturity, it was necessary to pick the top leaves and stems from very young alfalfa plants. Therefore, leaves on the next growth of alfalfa before it was 2 wk. old were used for this portion.

The samples of the three portions or parts of the alfalfa plant were artificially dried without exposure to sunlight in the laboratory, ground and stored in the dark in amber reagent bottles until mixed with the rat diet for vitamin D assay.

The antirachitic activity of forage obtained from the three stages of alfalfa also was tested with calves. Alfalfa hay cut in the bud, half-bloom and mature stages was fed to calves in groups 1, 2 and 3, respectively. Calves in group 4 were fed a good field-cured alfalfa hay from a commercial source. Group 5, the positive control group, received dried beet pulp as its only roughage and viosterol supplying 3,000 I.U. of vitamin D per cwt. per day by capsule. Group 6, the negative control group, received dried beet pulp and no vitamin D. The calves in group 7 were fed alfalfa silage made from a different crop without preservative as their only roughage.

All calves in groups 1 to 6 were Jersey males. They received colostrum for 3 days, then whole milk for the next 7 days. At 10 days of age, they were changed to skimmilk and offered a grain mixture and one of the experimental roughages. The grain mixture consisted of 40 parts ground corn, 30 parts wheat bran, 20 parts soybean meal, 10 parts linseed meal, 1 part iodized salt and 2 parts ground limestone. The hay or beet pulp was limited to 1.5 lb. per cwt. after this rate of consumption was reached, which occurred when the calves were about 3 mo. of age. The mature-stage alfalfa was not eaten readily by the young calves because of its advanced stage of maturity; neither was some of the bud-stage alfalfa because it was baled and stored before it was sufficiently dry. All these calves received skimmilk until they were 120 days of age, but the average amount was about 6 lb. daily from 90 to 120 days of age. After 3 to 4 mo. of age, the T.D.N. intake of all calves was limited to Morrison's standard.

All calves in groups 1 to 6 received a supply of carotene (100 γ per lb. per day) which was fed by capsule. This was discontinued to groups 1, 2, 3 and 4 after the fifth month, when the alfalfa consumed supplied sufficient carotene. A mineral mixture composed of 64 per cent limestone and 36 per cent bonemeal was fed to the animals receiving beet pulp in order that their Ca and P intakes would approximate those of the calves receiving alfalfa.

The two calves in group 7, which were crossbred male calves, were fed alfalfa silage, a grain mixture and whole milk until they were 50 and 60 days of age, respectively, after which no milk was fed. The grain mixture was composed of 60 parts ground corn, 40 parts wheat bran, 50 parts soybean meal, 50 parts linseed meal and 2 parts iodized salt. The two calves were fed this grain mixture

until they were 94 and 137 days of age, respectively, after which they were fed the same mixture as calves in groups 1 to 6. They were fed alfalfa silage at the rate of 1.5 lb. of hay-equivalent per cwt. daily.

All calves were kept in a darkened barn into which no sunshine was admitted. They were kept in individual stalls bedded with wood shavings, but occasionally they were turned into an inside alleyway where they could excercise and their movements could be observed. They were weighed every 10 days. The amount of feed fed and refused was recorded every day.

Plasma levels of inorganic P, Ca and serum phosphatase were determined bi-weekly, beginning when the calves were about 30 days of age (occasionally 3 wk. elapsed between determinations) (25). Hemoglobin, plasma vitamin A and carotene levels also were determined frequently.

When the calves were 8 mo. old they were slaughtered, a post-mortem examination was made and the weight of several organs was recorded. The negative control calves (group 6) were slaughtered when movement became difficult and before they began to decrease in body weight.

One calf in group 1 was slaughtered at 167 days of age, when the supply of bud-stage hay became exhausted. Calf #542 was started in group 2 but was transferred to group 3 at 120 days of age, when the supply of half-bloom stage alfalfa was exhausted.

At time of slaughter, the 8th and 9th ribs were obtained, then cleaned and dried. The distal 10 per cent was broken into four parts and then extracted with hot ethanol, followed by petroleum ether. The ash content was determined on the fat-free bone after ignition at 600° C.

Antirachitic activity of the forages was evaluated by the following criteria: (a) Growth and general appearance of the calves, and their freedom from visible rachitic symptoms during the experiment; (b) values and trends in Ca, P and phosphatase levels in the blood; (c) roentgenograms of the distal epiphyseal cartilage area of the ulna; (d) the ash content of the rib ends; and (e) post-mortem examination of the bones for evidence of rickets.

RESULTS

Antirachitic values of hay crops for calves. The growth of all calves was within the normal range up to 60 days of age, as indicated in table 1. In fact, most negative and positive control calves grew at a more rapid rate than those consuming alfalfa hay, probably because they consumed dried pulp more readily than the others consumed hay at the younger ages. The two crossbred calves fed alfalfa silage, grain and whole milk made the greatest gains in body weight of all young calves. After 2 mo. of age, the growth rate of the negative control group decreased rapidly. At about this time, the clinical data indicated that these calves had a slight degree of rickets, and visible symptoms began to appear at about 3 mo. of age. Progressive changes indicative of rickets were shown by the following symptoms: (a) Bowing of the front legs and the characteristic straightness of the pastern, (b) stiffness and shortness of steps when walking, (c) anorexia, (d) reluctance to move about or stand up while in their pen, (e) increased respiration rate, especially when handled, (f) decrease in depth of

TABLE 1

Individual data on the experimental calves and results of some criteria used to evaluate rickets

Group no. and roughage	Calf	Age at slaughter	Birth wt.	Daily gain 0–60 d.	Aver. total daily gain	Feed consumption				Kidney as % of body wt.	Bone ash	Ulnar epiphyseal cartilage width	Evidence of stiffness and visible symptoms	Evidence of rickets at post-mortem examination
						Grain	Roughage	Skim-milk	Minerals					
	(*no.*)	(*d.*)	(*lb.*)	(*lb.*)	(*lb.*)	(*lb.*)	(*lb.*)	(*lb.*)	(*g.*)	(%)	(%)	(*mm.*)		
Group 1	2728	244	69	0.67	1.06	632.5	551.8	1117.0	.....	0.571	46.10	0.68	none	none
Bud	2732	242	45	0.87	1.19	588.4	514.0	1122.0	.....	0.455	45.77	1.16	"	"
stage	540	234	61	0.53	1.15	556.5	464.0	1084.0	.....	0.336	50.80	1.84	"	"
	545	167	66	0.73	1.17	325.8	288.3	1136.0	.....	0.326	52.77	1.86	"	"
Av.		222	60	0.70	1.14	525.8	454.5	1115.0	.....	0.422	48.86	1.23[a]		
Group 2	533	245	68	0.59	1.26	625.6	609.8	1117.4	.....	0.302	55.27	0.58	none	none
Hay or half-	2734	242	54	0.85	1.24	586.1	589.0	1192.5	.....	0.300	54.97	1.68	"	"
bloom stage	542	.....	54	0.73	.....	.....	.....	.....	.....	.....	.....	.....		
Av.		244	59	0.72	1.25	605.8	649.4	1155.0	.....	0.301	55.12	1.13		
Group 3	531	244	56	0.82	1.14	623.3	492.4	1115.0	.....	0.317	52.73	0.71	none	none
Seed or	536	242	66	0.65	1.04	559.9	514.9	1213.5	.....	0.333	52.30	1.20	"	"
mature	541	240	56	0.73	1.29	583.2	538.2	1070.0	.....	0.301	50.50	0.46	"	"
stage	542	240	54	.....	1.25	617.6	499.4	1080.0	.....	0.296	52.67	0.84	"	"
Av.		242	58	0.73	1.18	596.0	511.2	1119.6	.....	0.312	52.05	0.80		
Group 4	538	245	72	0.60	1.14	619.7	554.8	1216.0	.....	0.339	50.80	1.42	none	none
Field-	2731	251	56	0.52	1.00	572.8	439.6	1209.5	.....	0.340	52.34	1.54	"	"
cured	2744	237	56	0.55	1.38	637.3	538.3	1055.5	.....	0.284	54.80	0.00	"	"
Av.		244	61	0.56	1.17	609.9	510.9	1160.3	.....	0.321	52.65	0.99		
Group 5	544	241	71	0.70	1.38	559.7	598.3[b]	1083.0	3270.0	0.324	51.97	0.20	none	none
Positive	2729	243	51	0.98	1.60	512.8	671.9[b]	1153.0	2901.4	0.299	53.18	0.92	"	"
controls	2735	240	63	0.83	1.45	467.1	631.9[b]	1129.0	2938.8	.....	54.33	0.80	"	"
Av.		241	62	0.84	1.48	513.2	634.0	1122.0	3036.7	0.312	53.16	0.64		
Group 6	535	169	66	0.75	0.88	169.7	192.5[b]	1177.5	1939.0	.....	28.35	.....	yes	yes
Negative	539	182	75	0.75	0.86	250.3	291.7[b]	1262.5	1839.0	0.437	26.04	6.04	"	"
controls	2739	119	44	0.85	0.74	76.7	68.3[b]	1095.5	446.0	0.591	32.96	8.86	"	"
Av.		157	62	0.78	0.83	165.6	184.2	1178.5	1408.0	0.514	29.12	7.45		
Group 7	X352	282	65	1.21	1.35	880.3	1802.6[c]	385.0[d]	.....	.....	57.83	.....	none	none
Alfalfa silage	X355	240	91	1.08	1.29	673.1	1580.4[c]	431.0[d]	.....	.....	56.36	.....	"	"
Av.		261	78	1.15	1.32	776.7	1691.5	408.0			57.10			

[a] Average of only the first 3 animals in this group.
[b] Dried beet pulp.
[c] Alfalfa silage.
[d] Whole milk.

body and a round appearance of the body, mainly due to increased rib curvature and (g) beading, or enlargement of the costo-chondral junction of the ribs. Only one calf exhibited tetanic convulsions during this experiment.

The data in table 2 show the trends of the periodic blood samples in Ca, inorganic P and serum phosphatase. The values were averaged for each calf, and

TABLE 2

Average calcium, phosphorus and phosphatase values by monthly periods of the experimental groups

Roughage	Group no.	Age in days							
		20–30	31–60	61–90	91–120	121–150	151–180	181–210	211–240 ±
		Plasma-serum calcium (mg. %)							
Bud	1	10.2[a]	9.87	9.33	10.23	11.07	10.77	10.38	10.26
Half-bloom	2	.....	9.78	10.20	11.11	10.01	10.16	9.95	10.24
Mature	3	.....	9.36	9.62	10.65	10.87	10.83	10.40	10.68
Field-cured	4	9.48	10.39	11.36	10.36	10.34	11.00	10.90	11.24
Positive control	5	9.3[a]	10.43	10.54	10.97	10.76	10.34	11.01	11.04
Negative control	6	10.65	9.76	7.95	6.58	6.55	6.74	7.21[a]	.....
Silage	7	.....	10.1[a]	10.18[a]	10.72	10.65	10.43	10.17	10.18[a]
		Plasma inorganic phosphorus (mg. %)							
Bud	1	7.15[a]	7.20	6.94	7.19	8.48	8.02	6.83	7.00
Half-bloom	2	.....	7.68	7.65	8.58	7.95	8.65	9.30	9.29
Mature	3	.....	6.22	7.33	7.50	7.84	8.45	9.05	8.78
Field-cured	4	7.9	6.99	8.00	7.74	9.20	9.07	9.57	8.63
Positive control	5	7.8[a]	7.86	8.50	8.63	8.52	8.64	8.35	8.44
Negative control	6	9.0	7.67	7.67	6.75	7.54	6.9	5.27[a]	.....
Silage	7	.....	5.90[a]	7.46[a]	8.05	7.33	7.80	8.07	8.65[a]
		Serum phosphatase (Bodansky units)							
Bud	1	6.9[a]	10.1	16.4	12.3	7.9	8.3	10.1	10.7
Half-bloom	2	.....	9.6	10.7	8.8	8.2	7.7	7.5	6.9
Mature	3	.....	10.7	15.2	10.0	7.7	6.9	7.1	6.7
Field-cured	4	5.0	6.8	10.0	10.2	9.9	7.6	7.5	7.4
Positive control	5	6.4[a]	9.6	12.1	10.1	8.2	10.5	10.0	8.7
Negative control	6	4.7	10.2	20.9	23.6	23.2	26.9	30.7[a]	.....
Silage	7	.....	7.0[a]	6.2[a]	6.9	6.7	6.6	7.4	6.6[a]

[a] Value given is for only one animal. All others are averages of all animals in the group.

then for each group by monthly ages of the calves. The negative controls (group 6) showed a decreased plasma Ca level before 60 days of age, which continued to decrease for the next 2 mo. and became stabilized at about 6.5 mg. per cent. The calves fed vitamin D, field-cured alfalfa hay or alfalfa silage (groups 4, 5 and 7) maintained plasma Ca levels at normal values throughout the experimental period. Some, but not all, calves in groups 1, 2 and 3 showed slightly

decreased levels of plasma Ca between 30 and 90 days of age, but all were normal after this time.

Until about 120 days of age the plasma inorganic P levels of all groups were within the range of normal values expected for calves of this age. At this time, the negative control calves started to show a drop in the level of plasma inorganic P and the values continued below those of the other groups. During the seventh and eighth months, the calves fed bud-stage alfalfa also showed this tendency.

The serum phosphatase values of the calves in the negative control group were much higher than those of the other groups after 60 days of age. The individual phosphatase values indicate that the negative control calves had clinical rickets, beginning when they were about 50 to 60 days of age. The individual values for serum phosphatase also indicate that some of the calves in groups 1, 2, 3, 4 and 5 had values that might be indicative of clinical rickets when they were 60 to 90 days of age. After this age, the values were practically the same as those of calves fed an ample amount of vitamin D (group 5) or those of calves raised under normal conditions in this herd. Phosphatase values above 11 units during the third or fourth months were found in two calves of both groups 1 and 3 and in one calf each in groups 2, 4 and 5, and also in one calf each in groups 1 and 5 during their seventh month. Phosphatase values for the calves fed alfalfa silage were never above 8.0 units at any time during the experimental period.

The alfalfa consumption of some of these calves was low until after their third month. The average roughage consumption of all calves receiving alfalfa was 0.3, 0.9, 1.9, 2.7, 3.3, 3.9 and 4.4 lb. daily for the second to eighth months, respectively. The phosphatase values probably would not have increased if the young calves had eaten more alfalfa during their first 3 mo. As mentioned later, the calculated vitamin D intake was below the accepted requirement for many of these calves during their first 3 mo.

The last section of table 1 shows the data on other measures of rachitic conditions. At slaughter time, only the calves in group 5 had enlarged rib ends and skeletal bones that appeared softer than normal. These calves also had enlarged kidneys, as did two calves in group 1. The ash content of the distal 10 per cent of the ribs of the negative control calves was very much lower than that of the other calves. Two calves fed bud-stage alfalfa (group 1) had bone ash values of less than 50 per cent, but all other calves had values above this, and the range was from 50.5 to 57.8 per cent. Calves in groups 2, 3, 4 and 5 had practically the same amount of ash in their rib bones.

Roentgenograms of the distal epiphyseal cartilage area of the ulna were taken periodically of most calves and terminally of all calves except 535. The technique for this procedure and its evaluation has been mentioned previously (2, 36) and the average width of the epiphyseal cartilage at the end of the experiment is given in table 1. Calves in the negative control group had much wider cartilage areas than those found in the other calves. The cartilage width had actually increased during the experiment in the rachitic calves, but showed a normal pattern of decreasing with age in all other calves. Terminally, the

average cartilage width of calves fed viosterol (group 5) was less than the values for the groups fed the alfalfa hays, and those in group 1 were wider than in any other group receiving alfalfa. However, there was considerable individual variation. The significance of small differences in width of the ulnar epiphyseal cartilage at 8 mo. of age has not been ascertained. One calf in group 1 was slaughtered at 167 days of age and the value for this calf cannot be compared directly with the other calves at 240 days of age.

The Ca and P intakes of the calves were calculated, using the determined values for the grain and the alfalfa hays and published values for dried beet pulp and skimmilk. They were averaged by monthly ages and the group aver-

TABLE 3

Average calcium and phosphorus intakes by monthly ages (mg./lb./day)

	Age in days							
	11–30	31–60	61–90	91–120	121–150	151–180	181–210	211–240
Calcium								
Group 1	79.2	149.4	161.0	172.3	162.1	161.8	157.0	156.9
" 2	96.7	157.9	200.6	190.3	168.4	169.8	164.0	160.9
" 3	91.4	140.4	163.2	156.5	150.2	151.7	150.7	149.6
" 4	76.6	130.4	159.4	162.8	159.3	157.2	157.7	139.0
" 5	83.7	117.8	137.2	127.8	112.2	110.7	105.7	101.8
" 6	79.7	122.1	123.8	133.2	128.4	123.8	.....	.....
Phosphorus								
Group 1	65.5	114.3	115.4	103.6	91.8	91.7	87.8	86.1
" 2	79.1	108.7	119.3	99.5	86.8	87.4	84.1	79.5
" 3	74.3	111.0	115.4	96.4	86.9	86.6	84.6	82.0
" 4	60.4	104.7	120.6	99.9	82.7	80.7	79.9	72.1
" 5	65.4	86.2	93.9	73.9	53.8	51.9	48.5	43.4
" 6	62.8	91.7	82.0	70.1	57.7	50.0	.....	.....
Ca : P ratio								
Group 1	1.2 : 1.0	1.3 : 1.0	1.4 : 1.0	1.7 : 1.0	1.8 : 1.0	1.8 : 1.0	1.8 : 1.0	1.8 : 1.0
" 2	1.2	1.5	1.7	1.9	1.9	1.9	1.9	2.0
" 3	1.2	1.3	1.4	1.6	1.7	1.8	1.8	1.8
" 4	1.3	1.2	1.3	1.6	1.9	1.9	2.0	1.9
" 5	1.3	1.4	1.5	1.7	2.1	2.1	2.2	2.3
" 6	1.3	1.3	1.5	1.9	2.2	2.5	.....	.....

ages are presented in table 3. Ca and P intakes of all calves were equal to or above the intakes recommended by the Committee on Animal Nutrition of the National Research Council (22). The Committee recommended a Ca intake that varies from 80 to 35 mg. per pound at 50 to 400 lb. of body weight and a P intake that varies from 60 to 27.5 mg. per pound at 50 to 400 lb., respectively. The intakes of the alfalfa-fed calves were somewhat higher than those of the calves fed dried beet pulp. Evidently not enough of the mineral mixture was fed with the beet pulp to provide as much Ca and P intake as the alfalfa hays.

The Ca : P ratios are also given in table 3. The ratio from 11 to 90 days varied from 1.2 to 1.7 for all calves. The ratio from 91 to 240 days ranged from 1.6 to 2.0 for the groups consuming alfalfa and from 1.7 to 2.5 for the positive and negative control groups.

Factors affecting vitamin D content of hays. The vitamin D content of the various portions of the alfalfa plant was found to be strikingly different as determined by the rat assay method. The vitamin D content of the dead leaves was found to be 2,700 International Units (I.U.) per pound and that of the half-dead or yellowed leaves was 295 I.U. per pound. The vitamin D content of entirely green leaves and top portions of stem was too low to be measured on two separate assays. However, calculations from these assays give the vitamin D content as less than 33 I.U. per pound. These figures indicate that the dead material on the alfalfa plant as it stands in the field is sufficiently potent to affect the vitamin D potency of the entire plant.

The percentages of dead leaves and half-dead leaves on the alfalfa plant as it was cut at the three stages of maturity are given in table 4. The vitamin D

TABLE 4

Percentage of dead and half-dead leaves on the alfalfa plant at different stages of maturity, and the vitamin D content of the alfalfa plant and the other crops used in this experiment

Stage of maturity	Dead leaves and stems[a]	Half-dead leaves[b]	Vit. D. content of entire crop
	(%)	(%)	(*I.U./lb.*)
Bud stage	2.4	7.1	96
Half-bloom or hay stage	2.9	3.5	145
Seed or mature stage	6.5	3.1	291
Field-cured alfalfa	.....	.....	740
Wilted alfalfa silage	.....	.....	393[c]

[a] Vitamin D content 2,700 I.U./lb.
[b] Vitamin D content 290 I.U./lb.
[c] Expressed in hay equivalent

content of the hays used in this experiment also is given in table 4. The vitamin D content of the plant was shown to increase from 96 I.U. per pound at the bud stage to 291 I.U. per pound at the mature stage, and the proportion of dead leaves on the standing plant increased from 2.4 per cent at the bud stage to 6.5 per cent at the mature stage.

DISCUSSION

The increase in the amount of high vitamin D potency dead material on the plant as it matures is a probable explanation for a large part of the increase in the vitamin D activity of the plant as it matures. The amount of high potency dead material harvested in the hay crop also may be an explanation for some of the variability noted in the vitamin D content of some hay crops. It may help explain the higher amount of vitamin D found in alfalfa silage when compared to that found in barn-dried alfalfa when both were harvested from the same field (25). When the hay crop is harvested as silage, the dead material does not become dry and probably adheres to the plant until it is put in the silo, whereas when the crop is harvested by the barn-drying method the dead leaves probably are dry enough to shatter and drop from the plant before it reaches the barn. It is possible that the maturity of the crop and the proportion of dead material could modify or mitigate the effect that sunlight has on the vita-

min D activity of the plant after it has been cut, and thus offer an explanation for some of the discrepancies noted in published values.

Our finding of no antirachitic activity in absolutely green alfalfa is in agreement with the previous observations of others (33, 34, 35). Several investigators (5, 19, 23, 29, 38, 42) have reported that some calcifying activity or rickets-healing activity is present in green material. This latter group of investigators used principally grasses or garden crops for their source of green material. Possibly there may be some fundamental difference in the antirachitic properties of green growing legumes and grasses.

Under growing conditions at Beltsville, alfalfa was shown to have considerable vitamin D activity as it stands in the field; this activity probably is due to the irradiation received by the dead and injured portions of the plant. It is probable that under optimum growing conditions there may be somewhat smaller amounts of dead portions than are found on the plants at Beltsville and under such conditions the vitamin D activity of the standing plant may be decreased accordingly. This situation and the effect of limestone in the grain ration are presently being investigated at Beltsville.

The results of feeding these alfalfa hays to calves as their only source of vitamin D indicate that all these specially harvested hays protected the calves from gross or visible signs of rickets. However, the clinical data (slightly increased phosphatase values, slightly decreased bone ash and slightly wider ulnar epiphyseal cartilage) indicate that, as an average, the calves receiving bud-stage alfalfa probably had a mild degree of rickets, which indicates that the average vitamin D intake of this group was near the borderline in meeting the requirement of young calves. The calves fed the other alfalfa hays or silage were adequately protected from rickets (except a few calves at 2 to 3 mo. of age), as indicated by all diagnostic methods employed. Furthermore, this experiment furnished a more drastic test of the antirachitic adequacy of these forages than would be furnished by conditions on most farms, where the calves usually have some exposure to sunlight and the hays usually have more exposure to sunshine than the hays used in this experiment. The above statements presuppose the fact that calves are fed hay at the rate of at least 1.5 lb. per cwt.

Other investigators (30) have compared barn-cured and field-cured alfalfa hays as sources of vitamin D for growing dairy heifers and concluded that both were adequate, but they also fed some green grass or silage which, according to their own (29, 30) and other data (4, 23, 38), contain variable amounts of antirachitic activity.

When the calves consumed the alfalfa hays at the rate of 1.5 lb. per cwt. daily, their calculated vitamin D intakes in groups 1, 2, 3, 4, 5, 6 and 7 were 144, 218, 437, 1,110, 3,000, 0 and 590 I.U. per day per cwt., respectively. During their first 3 mo., this intake of alfalfa (and consequently vitamin D) was not attained. The vitamin D requirement of young calves was found to be 300 I.U. per cwt. by Bechdel *et al.* (2), and 350 to 450 was found to be adequate by Huffman and Duncan (20). The requirement of young sheep was found to be slightly lower (180–250 I.U. per cwt. per day) than the figures cited for calves (1, 6). The

calculated vitamin D intake of calves fed bud-stage and half-bloom stage alfalfa did not meet the accepted requirement, yet none of these calves showed any visible rachitic symptoms and only some of the calves fed bud-stage alfalfa had slight clinical evidence of rickets.

Calves in group 6, which, after 10 days of age, received no vitamin D, made normal body weight gains and were free of visible or clinical rickets until they were about 60 days of age. This indicates that calves have a store of antirachitic agent at 10 days of age or that their requirement is very small. Most investigators have not found rickets in calves during their first 2 mo. of age. At this age calves raised according to accepted practical methods would have access to some sunshine and would be eating sufficient amounts of good alfalfa to protect them from rickets. Young lambs also have been found to become depleted of vitamin D stores after 6 to 8 wk. on a deficient diet (6).

Only during the last few years have serum phosphatase measurements by the Bodansky method been used for the detection of rickets in young animals (6, 18, 25, 27, 28, 30). In this experiment and others carried out at Beltsville, a close relationship between the degree of rickets and the increase in serum phosphatase values has been noted. Recently, serum phosphatase measurements have been proposed as an assay method for vitamin D, using chicks (28). When plasma phosphatase by the Kay method has been used, the results have not been as conclusive (1, 32). Serum phosphatase measurements have been used routinely to show evidence of rickets in young dogs and children (12, 14, 24). In rachitic calves, the increase in serum phosphatase and the decrease in serum Ca occurred at about the same time. However, the increase in serum phosphatase values was greater than the decrease in plasma and/or serum Ca values.

SUMMARY

The antirachitic activity of an alfalfa crop was shown to increase with the stage of maturity. Dead leaves on the alfalfa plant were shown to be very high in vitamin D content and their amount on the plant increased with the stage of maturity of the plant. The stage of maturity and its content of dead material or insect-injured areas with their high vitamin D content may help explain some of the apparent discrepancies that have been noted in the vitamin D activity of alfalfa hays. Alfalfa leaves and top stem portions that were entirely green were devoid of vitamin D activity.

Alfalfa cut at three stages of maturity and dried without further exposure to sunlight was found to protect young calves from visible symptoms of rickets when it supplied their only source of vitamin D but some clinical evidence of a rachitic tendency was noted in some calves fed alfalfa cut at the bud stage. Several criteria were used to detect rachitic tendencies in calves. They were plasma Ca, plasma inorganic P, serum phosphatase, roentgenograms of the distal epiphyseal cartilage of the ulna, ash content of ribs, visible and gross symptoms and a complete post-mortem examination. The results of this experiment indicate that calves reared under accepted practical conditions should not require an additional source of vitamin D providing they consume at least 1.5 lb. per cwt. of alfalfa or its equivalent as alfalfa silage and receive some access to sunshine.

REFERENCES

(1) ANDREWS, E. D., AND CUNNINGHAM, I. J. The Vitamin D Requirement of the Sheep. New Zealand J. Sci. Technol. **27**: 223–230. 1945.

(2) BECHDEL, S. I., HILSTON, N. W., GUERRANT, N. B., AND DUTCHER, R. A. The Vitamin D Requirement of Dairy Calves. Pa. Agr. Expt. Sta. Bull. 364. 1938.

(3) BECHDEL, S. I., LANDSBURG, K. G., AND HILL, O. J. Rickets in Calves. Pa. Agr. Expt. Sta. Bull. 291. 1933.

(4) BECHTEL, H. E., HUFFMAN, C. F., DUNCAN, C. W., AND HUPPERT, C. A. Vitamin D Studies in Cattle. IV. Corn Silage as a Source of Vitamin D for Dairy Cattle. J. Dairy Sci., **19**: 359–372. 1936.

(5) CHICK, H., AND ROSCOE, M. H. The Antirachitic Value of Fresh Spinach. Biochem. J. **20**: 137–152. 1926.

(6) DUCKWORTH, J., GODDEN, W., AND THOMSON, W. The Relation between Rates of Growth and Rickets in Sheep on Diets Deficient in Vitamin D. J. Agr. Sci., **33**: 190–196 1943.

(7) DUNCAN, C. W., AND HUFFMAN, C. F. Vitamin D Studies in Cattle. III. Influence of Solar Ultraviolet Radiation upon the Blood Chemistry and Mineral Metabolism of Dairy Calves. J. Dairy Sci., **19**: 291–303. 1936.

(8) EWER, T. K. Rachitogenicity of Green Oats. Nature, **166**: 732–733. 1950.

(9) EWER, T. K. Rickets in Sheep: Field Trials in East Anglia. Vet. Record, **62**: 603–606. 1950.

(10) FITCH, L. W. N., AND EWER, T. K. The Value of Vitamin D and Bone-flour in the Prevention of Rickets in Sheep in New Zealand. Australian Vet. J., **20**: 220–226. 1944.

(11) FRANKLIN, M. C., REID, R. L., AND JOHNSTONE, I. L. Studies on Dietary and Other Factors Affecting the Serum-Calcium Levels of Sheep. Parts I to VI. Council for Scientific and Industrial Research Bull. 240. 1948.

(12) FREEMAN, S., AND MCCLEAN, F. C. Experimental Rickets. Blood and Plasma Changes in Puppies Receiving a Diet Very Low in Phosphorus, with and without Vitamin D. Arch. Path., **32**: 387–408. 1941.

(13) GALLUP, W. D., AND KUHLMAN, A. H. Sunlight in Oklahoma as an Important Factor in the Prevention of Rickets and the Maintenance of Normal Calcium and Phosphorus Metabolism. Proc. Oklahoma Acad. Sci. for 1941, **22**: 11–15. 1942.

(14) GRAY, J. D., AND CARTER, F. S. The Early Diagnosis of Rickets. Arch. Diseases Childhood, **24**: 189–194. 1949.

(15) GULLICKSON, T. W., AND ECKLES, C. H. The Relation of Sunlight to Growth and Development of Calves. J. Dairy Sci., **10**: 87–94. 1927.

(16) GULLICKSON, T. W., PALMER, L. S., AND BOYD, W. L. A Rickets-like Disease in Young Cattle. Minn. Agr. Expt. Sta. Tech. Bull. 105. 1935.

(17) HESS, A. F., AND WEINSTOCK, M. Antirachitic Properties Imparted to Inert Fluids and to Green Vegetables by Ultraviolet Radiation. J. Biol. Chem., **62**: 301–313. 1924.

(18) HIBBS, J. W., KRAUSS, W. E., MONROE, C. F., AND POUNDEN, W. D. A Report on the Occurrence of Rickets in Calves under Farm Conditions. J. Dairy Sci., **28**: 525–529. 1945.

(19) HODGSON, R. E., AND KNOTT, J. C. The Calcifying Properties of Green Artificially Dried and Sun-cured Pasture Herbage. J. Agr. Research, **48**: 439–446. 1934.

(20) HUFFMAN, C. F., AND DUNCAN, C. W. A Preliminary Study of the Vitamin D Requirement of Calves when Fed Natural Milks as the Source of this Vitamin. J. Dairy Sci., **18**: 432. 1935.

(21) HUFFMAN, C. F., AND DUNCAN, C. W. Vitamin D Studies in Cattle. I. The Antirachitic Value of Hay in the Ration of the Dairy Cattle. J. Dairy Sci., **18**: 511–526. 1935.

(22) LOOSLI, J. K., HUFFMAN, C. F., PETERSON, W. E., AND PHILLIPS, P. H. Recommended Nutrient Allowances for Domestic Animals. No. 3. Recommended Nutrient Allow-

ances for Dairy Cattle. National Research Council Bull. 1945.

(23) MELLANBY, M., AND KILLICK, E. M. CXIII. A Preliminary Study of Factors Influencing Calcification Processes in the Rabbit. Biochem. J., **20**: 902–926. 1926.

(24) MIGUEL, E. J. Alkaline Blood Phosphatase in Rachitic Uruguayan Children. Anales asoc quim y farm Uruguay, **50**: 63–69. 1948.

(25) MOORE, L. A., THOMAS, J. W., JACOBSON, W. C., MELIN, C. G., AND SHEPHERD, J. B. Comparative Antirachitic Value of Field-cured Hay, Barn-dried Hay, and Wilted Grass Silage for Growing Dairy Calves. J. Dairy Sci., **31**: 489–499. 1948.

(26) MORRISON, F. B., AND RUPEL, I. W. Do Calves Need Sunlight? Wisc. Sta. Bull. 396. 1927.

(27) MOTZOK, I. Studies on the Plasma Phosphatase of Normal and Rachitic Chicks. 2. Relationship between Plasma Phosphatase and the Phosphatases of Bone, Kidney, Liver, and Intestinal Mucosa. Biochem. J., **47**: 193–196. 1950.

(28) MOTZOK, I. Studies on the Plasma Phosphatase of Normal and Rachitic Chicks. 3. The Assay of Antirachitic Preparations by a Method Based on Determination of Plasma Phosphatase Activity. Biochem. J., **47**: 196–199. 1950.

(29) NEWLANDER, J. A. Vitamin D Content of Roughages. J. Dairy Sci., **31**: 455–460. 1948.

(30) NEWLANDER, J. A., JONES, C. H., AND FOOTE, M. W. Barn-cured and Field-cured Hays as Sources of Vitamin D and Carotene (Vitamin A) for Dairy Cattle from Birth to First Freshening. Vermont Agr. Expt. Sta. Bull. 561. 1950.

(31) OLSON, T. M. The Effect of Sunlight on the Growth, Production and Reproduction of Dairy Cattle. So. Dak. Agr. Expt. Sta. Bull. 319. 1938.

(32) RUPEL, I. W., BOHSTEDT, G., AND HART, E. B. Vitamin D in the Nutrition of the Dairy Calf. Wisc. Agr. Expt. Sta. Research Bull. 115. 1933.

(33) RUSSELL, W. C. The Effect of the Curing Process upon the Vitamin A and D Content of Alfalfa. J. Biol. Chem., **85**: 289–297. 1929.

(34) SMITH, M. C., AND BRIGGS, I. A. The Antirachitic Value of Alfalfa as Affected by Exposure to Sunshine in the Curing Process. J. Agr. Research, **46**: 235–240. 1933.

(35) STEENBOCK, H., HART, E. B., ELVEHJEM, C. A., AND KLETZIEN, S. W. F. Dietary Factors Influencing Calcium Assimilation. VI. The Antirachitic Properties of Hays as Related to Climatic Conditions with some Observations on the Effect of Irradiation with Ultraviolet Light. J. Biol. Chem., **66**: 425–440. 1925.

(36) THOMAS, J. W. The Use of X-ray Photographs in the Detection of Rickets in Calves. BDIM-Inf-95. June 1950. J. Dairy Sci., **33**: 387. 1950.

(37) U. S. PHARMACOPOEIA XII. Mack Printing Co., Easton, Pa. 1942.

(38) VOLTZ, W., AND KIRSCH, W. Der Nachweis des Antirachitischen Faktors bei Gras, das im Dunkeln auf einer Kunstlichen Nahrlosung Gewachsen ist. II. Biochem. Zeit., **193**: 281–284. 1928.

(39) WALLIS, G. C. Vitamins D and A in Alfalfa Hay. So. Dak. Agr. Expt. Sta. Circular 53. 1944.

(40) WALLIS, G. C. Vitamin D Deficiency in Dairy Cows. Symptoms, Causes and Treatment. So. Dak. Agr. Expt. Sta. Bull. 372. Rev. 1946.

(41) WALLIS, G. C., PALMER, L. S., AND GULLICKSON, T. W. The Relation of Vitamin D to Calcium and Phosphorus Retention in Cattle as Shown by Balance Trials. J. Dairy Sci., **18**: 213–228. 1935.

(42) WALLIS, G. C., SMITH, C. A., FISHMAN, R. H. *et al.* The Vitamin D Content of Roughages. J. Dairy Sci., **32**: 715. 1949, and mimeographed material presented at Am. Dairy Sci. Assoc. meetings, 1949.

THE EFFECT OF ORALLY ADMINISTERED MOLYBDENUM ON GROWTH, SPERMATOGENESIS AND TESTES HISTOLOGY OF YOUNG DAIRY BULLS

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Since evidence was first presented (9) that excess molybdenum was responsible for certain disorders of ruminants, there has been considerable extension of knowledge concerning the toxicity of this element and its compounds (1, 2, 4, 8, 10, 15). The toxic action of molybdenum has been shown to be alleviated when additional copper is given to the animal (3, 4, 5, 10, 11, 12, 13). It also has been shown that the relative levels of copper and molybdenum in the diet are the principal factors involved in molybdenum toxicity (3, 4, 5, 11). Actually, symptoms of molybdenum toxicity are hard to differentiate from symptoms of copper deficiency (3, 4, 11, 17). Some of these symptoms appear to be similar to some rachitic symptoms, especially the reported increase in serum phosphatase, leg stiffness, and growth inhibition in young calves (2, 3, 4, 5, 6).

More recently the possibility that molybdenum may be a factor in bovine hyperkeratosis or X-disease has been mentioned (7, 16). Also, cows affected with symptoms of molybdenum toxicity or copper deficiency conceive with difficulty, and various other breeding troubles have been mentioned as symptoms (1, 2, 5).

This experiment was planned in order to explore the possibility that large oral doses of molybdenum might produce the X-disease (hyperkeratosis) syndrome. Simultaneously, the effects of orally administered sodium molybdate on sexual and bone development were studied.

PROCEDURE

Two male Holstein calves were fed C. P. $Na_2MoO_4 \cdot 2H_2O$ for 129 days, beginning when they were 190 and 217 days of age. The salt was given daily by capsule and the amount increased as the experiment progressed. From the first to the 37th day of the experiment, the calves were fed 2.6 g. of the salt daily; from 38 to 46 days, 4.0 g.; from 47 to 56 days, 2.8 g.; from 57 to 74 days, 4.0 g.; and from 75 to 129 days, 5.0 g. At the beginning of the experiment, the two calves were fed 7.1 and 8.0 lb., respectively, of a grain mixture composed of ground corn, oats, wheat bran, soybean meal and salt, and 1.0 lb. of good quality alfalfa hay daily. As the calves grew, the hay allowance gradually was increased to 4.8 lb. per day at the end of the experiment and the grain was increased to 7.5 and 8.5 lb., respectively. Their total digestible nutrient consumption was approximately equal to their requirement. The calves were slaughtered on the 129th day of the experiment, when a complete post-mortem examination was performed and specimens of several organs were preserved in 10 per cent formalin for histological examinations.

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RESULTS AND DISCUSSION

The molybdenum intake and the body weight gains before and after initiating molybdenum feeding are averaged by monthly intervals and are shown in table 1. The calves gained at a slower rate after Na_2MoO_4 was fed, and this finding is in agreement with that reported by Cunningham (4). At the beginning, a mild chronic diarrhea occurred in calf 721 for 1 wk., and in calf 2963 for 2 to 3 wk. The diarrhea did not persist after this time, nor was it as severe as reported by other investigators (1, 2, 4, 10).

Calf 2963 became stiff and walked as if in pain after molybdate had been fed for only 2 wk. However, after a few days the calf's motions improved but at intervals throughout the experiment this calf would relapse and exhibit a marked degree of stiffness. The other calf (721) became slightly stiff with no impairment of its motions, although both calves were slightly lethargic throughout the

TABLE 1

Body weight changes and molybdenum intake of calves

	Calf no. 721	Calf no. 2963
Average daily gain in body weight (lb./day)		
90–60 d. before Mo feeding	2.2	1.9
60–30 " " " "	2.2	2.1
30–0 " " " "	3.2	3.0
0–30 " after " "	1.1	1.8
30–60 " " " "	1.6	0.0
60–90 " " " "	1.5	2.3
90–129 " " " "	0.9	0.4
Body weight at beginning of Mo feeding (*lb.*)	494	427
Mo fed (mg./kg. body weight)		
0–30 days	4.11	4.77
30–60 "	4.99	5.94
60–90 "	6.53	7.62
90–129 "	6.84	7.84
Mo fed (ppm. of the dry matter consumed)		
0–30 days	263	302
30–60 "	262	303
60–90 "	347	365
90–129 "	375	411

experiment. This stiffness or bone involvement has not been mentioned in other experiments where molybdenum has been administered to calves (2, 10), although it has been noted in cases of cattle grazing on molybdenum-toxic pastures (2, 4, 5).

The black hair on calf 2963 began to be replaced with white or grey hair 6 wk. after molybdenum feeding was initiated. The change started on the ears and gradually developed in all the black haired areas, giving them a grey appearance. This phenomenon of black areas turning grey or rusty has been reported previously with calves on molybdenum-toxic pastures (2, 9) and with a calf fed molybdenum (2) and with copper-deficient rabbits (15) and sheep (12).

Seven periodic blood analyses for calcium, inorganic phosphorus and phosphatase were made on the two calves. Individual values for plasma calcium varied from 9.6 to 12.7 mg. per cent, plasma inorganic phosphorus varied from

6.1 to 10 mg. per cent and serum phosphatase varied from 6.0 to 9.2 Bodansky units. All values were within the normal range for calves of this age and showed no definite trend during the experiment. Cunningham (4) found normal blood levels of calcium and phosphorus in calves fed molybdenum, while other investigators have reported an increase in phosphatase values of copper-deficient cattle (5, 6). The copper intake of the two calves in this experiment was not determined and, therefore, the actual molybdenum-copper relationship was not known.

Anemia has been reported in calves fed molybdenum (2, 4). Lower-than-normal hemoglobin and hematocrit values were found in the two calves in this experiment. Hemoglobin levels before molybdenum was fed were 10.2 and 9.3, respectively, for nos. 721 and 2963; after 81 days of feeding, the respective levels were 8.7 and 7.7. At this time, hematocrit values were 25.9 and 28.6 per cent, respectively. Increasing the roughage intake of these calves may have alleviated some of the molybdenum toxicity symptoms, since dry roughage has been reported to have this effect when fed in addition to grazing on toxic pastures (1, 2, 10).

The most marked symptom was the lack of libido exhibited by these male Holstein animals when molybdenum was fed. At no time during the period of molybdenum feeding did they show any sign of libido or sex interest, even though they were occasionally allowed to run with cows and stilbestrol-injected heifers. Normal male Holstein animals at a similar age show a considerable degree of sex interest, but these two were entirely devoid of such. Normal male dairy calves of this age and fed similar rations have sperm in the majority of seminiferous tubules and are capable of reproduction (18).

A histological examination of the testes showed that in animal 721 with the less advanced stage of poisoning the seminiferous tubules were in various stages of degeneration. All the tubules were devoid of sperm or spermatids. In some tubules, the degeneration had progressed to a stage where only a single row of atrophied spermatogonial cells remained. Some tubules were collapsed and clumped into a mass in the center of the lumen and most other tubules had cellular debris in them. The less damaged tubules still showed a thickened basement membrane. The interstitial cells also were in the process of degeneration but some areas were still intact.

In the case of animal 2963, where the poisoning was more advanced, the degeneration of the tubules was more complete than noted in animal 721. Most tubules showed only a bare outline of a single row of highly vacuolated spermatogonial cells with pycnotic nuclei and the Sertoli cells appeared to be in a process of degeneration. The basement membrane was less thickened and the interstitial cells had almost entirely disappeared. It appears that the effect of the poisoning was a gradual disappearance of spermatogenetic and interstitial tissue, the spermatogenic tissue possibly being first attacked and a wave of disintegration progressing from the center of the tubule outward. The changes which occurred in the testes are shown in fig. 1.

The histological findings in the testes offer an explanation for the lack of libido exhibited by the two calves.

The damage to testicular tissue by feeding high levels of molybdenum indi-

cates that it would be interesting to know if it would have a similar or any effect on the ovary. It is possible that this information, on the male and female, would help explain the reduced breeding efficiency noted in molybdenum-toxic areas (1) or in copper-deficient areas (5). Whether similar changes would occur in the testes of other species when fed excess molybdenum would be of interest. Intakes of molybdenum that can damage the cells of the testes to the extent found in this study offer a new technique that could be used in future hormone and cancer studies involving these cells. Whether the damage found was reparable or permanent also would be of interest, but it seems probable that the damage to the testes would be irreparable.

When the calves were autopsied, there were marked cartilagenous erosions in the metatarsal joints of both calves. Calf 2963 had a similar condition in one metacarpal joint. In one rear hock joint of calf 2963, there was a solid union

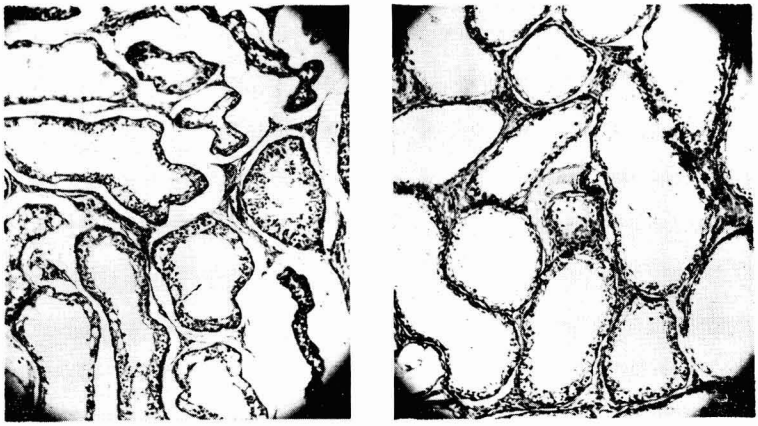

FIG. 1. Photomicrographs of testes from 721 (left) and 2963 (right), showing marked general degeneration of tubular and intestitial cells caused by feeding molybdenum. (X 55).

of articulating surfaces to the point of ankylosis and fusion. It broke away from the head of the bone when an attempt was made to examine the joint. All leg joints showed a greater degree of erosion and irregular surfaces than do the joints of normal animals of this age.

Although were some gross abnormalities in the bone joints, the amount of ash in rib ends was approximately normal. The ash in the distal 10 per cent of the rib ends was 50.6 and 55.8 per cent, respectively, for calves 721 and 2963. Normal calves of this age have 53.0 to 62 per cent ash in rib ends (14). Administered molybdenum has been found to be retained to the largest degree in bone (2, 9). No other rachitic symptoms except stiffness appeared in these two calves, probably because they were too old when molybdenum feeding was initiated. There were no other abnormalities noted during autopsy than those mentioned in the text.

In both calves, the prescapular and inguinal lymph nodes were enlarged and endematous but not inflamed. Others (3) found that when labeled molybdenum

was administered, the intestinal lymph nodes had a very high concentration of the administered molybdenum.

It is unlikely that the ration fed was deficient in copper. Similar rations have been fed to numerous young dairy animals of both sexes with no interference with normal development (18).

The symptoms of molybdenum toxicity and of induced copper deficiency, caused by feeding excess molybdenum are similar and at the present time are considered distinguishable only when the intakes of each element are known (1, 2, 4, 5, 8, 10, 11). Therefore, it cannot be definitely stated whether the results noted in this experiment were due to the toxicity of the molybdenum or due to a deranged metabolism of copper caused by feeding of the large excess of molybdenum.

No gross symptoms similar to X-disease (hyperkeratosis) symptoms (7, 16) were noted in these calves. Routine histologic examination of liver, gall bladder, kidney, pancreas, intestine, abomasum, skin and oral mucosa showed no evidence for the presence of X-disease.[1] It seems probable from this experiment and others (7) that molybdenum is not the causative agent in X-disease, although larger than normal amounts of this element have been found in the tissues of some affected animals (16).

SUMMARY

Sodium molybdate was fed to two Holstein male calves. In general, the usual symptoms of molybdenum poisoning consisting of mild diarrhea, decreased body weight gains, anemia and greying of the black hair areas were observed. In addition, lameness and joint involvement were found. The most striking and heretofore unnoted symptom was the lack of sexual interest or libido exhibited by these two animals. Histological examination of the testes showed marked damage to interstitial cells and germinal epithelium with little or no spermatogenesis present. Further investigations of these effects and the possibility of using molybdenum as a tool in experiments involving the testes and its products are indicated.

REFERENCES

(1) Barshad, I. Molybdenum Content of Pasture Plants in Relation to Toxicity to Cattle. Soil Sci., **66**: 187–195. 1948.

(2) Britton, J. W., and Goss, H. Chronic Molybdenum Poisoning in Cattle. J. Am. Vet. Med. Assoc., **108**: 176–178. 1946.

(3) Comar, C. L., Singer, L., and Davis, G. K. Molybdenum Metabolism with Interrelationships with Copper and Phosphorus. J. Biol. Chem., **180**: 913–922. 1949.

(4) Cunningham, I. J. Copper and Molybdenum in Relation to Diseases of Cattle and Sheep in New Zealand. Pp. 246–273. Copper Metabolism, A Symposium on Animal, Plant and Soil Relationships. Johns Hopkins Press. 1950.

(5) Davis, G. K. Influence of Copper on the Metabolism of Phosphorus and Molybdenum. Pp. 216–229. Copper Metabolism, A Symposium on Animal, Plant and Soil Relationships. Johns Hopkins Press. 1950.

(6) Davis, G. K., and Hannan, H. Copper Metabolism with Relation to Alkaline Blood Phosphatase and Blood Ascorbic Acid. J. Animal Sci. **6**: 484. 1947.

[1] The authors wish to thank Dr. H. R. Seibold, Pathological Division, Bureau of Animal Industry, U. S. D. A. for the histological preparation of these tissues and the interpretation of the observations.

(7) EVANS, H. J., AND BEAR, F. E. Molybdenum in Relation to Plant and Animal Life. Proceedings of the Johns Hopkins University Conference on X-disease in Cattle. Pp. 5–11. 1949.

(8) FAIRHILL, L. T., DUNN, R. C., SHARPLESS, N. E., AND PRITCHARD, E. A. Toxicity of Molybdenum. U. S. Public Health Service Bull. 293, 36 pp. 1945.

(9) FERGUSON, W. S., LEWIS, A. H., AND WATSON, S. J. Action of Molybdenum in Nutrition of Milking Cattle. Nature, **141**: 553. 1938.

(10) FERGUSON, W. S., LEWIS, A. H., AND WATSON, S. J. The Teart Pastures of Somerset. I. The Cause and Cure of Teartness. J. of Agr. Sci., **33**: 44–51. 1943.

(11) GRAY, L. F., AND ELLIS, G. H. Some Interrelationships of Copper, Molybdenum, Zinc and Lead in the Nutrition of the Rat. J. Nutrition, **40**: 441–452. 1950.

(12) MARSTON, H. R. Problems Associated with Copper-deficiency in Ruminants. Pp. 230–245. Copper Metabolism, A Symposium on Animal, Plant and Soil Relationships. Johns Hopkins Press. 1950.

(13) NEILANDS, J. B., STRONG, F. M., AND ELVEHJEM, C. A. Molybdenum in the Nutrition of the Rat. J. Biol. Chem., **172**: 431–439. 1948.

(14) MOORE, L. A., THOMAS, J. W., JACOBSON, W. C., MELIN, C. G., AND SHEPHERD, J. B. Comparative Antirachitic Value of Field-cured Hay, Barn-dried Hay, and Wilted Grass Silage for Growing Dairy Calves. J. Dairy Sci., **31**: 489–499. 1948.

(15) ROBINSON, W. O., AND EDGINGTON, G. Toxic Aspect of Molybdenum in Vegetation. Soil Sci., **66**: 197–198. 1947.

(16) ROBINSON, W. O., LEE, A. M., ELLIS, N. R., DAVIS, R. E., JOHNSON, A. G., AND MOORE, L. A. Report of the U. S. Department of Agriculture including the Summary of Their Preliminary Survey of X-disease in Cattle. Proceedings of the Johns Hopkins University Conference on X-disease in Cattle. Pp. 14–20. 1949.

(17) SMITH, S. E., AND ELLIS, G. H. Copper Deficiency in Rabbits. Achromotrichia, Alopecia and Dermatosis. Arch. Biochem., **15**: 81–88. 1947.

(18) SYKES, J. F., THOMAS, J. W., AND MOORE, L. A. Unpublished. 1948–1950.

HYDROLYTIC RANCIDITY IN MILK. III. TRIBUTYRINASE DETERMINATION AS A MEASURE OF LIPASE

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Tributyrin has been used as substrate in several methods for the determination of lipolytic enzymes in milk, but little attention has been given to establishing whether tributyrin hydrolysis provides a reliable measure of lipase. In this paper two procedures are described, one for the detemination of tributyrinase based on measurement of tributyrin hydrolysis, and one for estimation of lipase by measurement of milkfat hydrolysis. The terms "tributyrinase" and "lipase" are used to indicate the substrate hydrolyzed, but is it not intended that they prejudice a conclusion as to whether the enzymes involved are separate entities. Results illustrating the correlation between tributyrinase and lipase determinations and the similarity of pH activity curves for the enzymes measured by the two methods provide greater justification for the use of tributyrinase determinations as a measure of lipase in milk.

The literature dealing with the determination of lipase in milk has been reviewed by others (2, 3, 7, 8). Mattick and Kay (4), Peterson *et al.* (5) and Reder (6) described methods for the determination of tributryinase in which conditions for lipolysis were standardized more completely than in most procedures used previously. A tributyrinase determination which is essentially a modification of those described by Mattick and Kay, and Peterson *et al.* was developed for use in this study (1) and recently has been re-examined and modified (9).

The pH optimum for tributyrinase at 37 to 40° C. has been reported as 8.5 to 8.7 (1, 4, 5, 9), and for lipase at 3 to 4° C. as 8.4 to 8.6 (8).

PROCEDURE

Determination of tributyrinase. The milk sample to be tested was centrifuged to remove the fat, 2 ml. of the resulting skimmilk were pipetted into a test tube containing 5 ml. borate buffer (6.23 g. boric acid and 50 ml. 1 *N* NaOH per liter) and adjusted, if necessary, to pH 8.7, using 0.1 *N* NaOH or 0.1 *M* phosphoric acid. The buffered milk was placed in a water bath at 37° C. and allowed to reach temperature, 0.6 ml. of tributyrin was added and the mixture emulsified by shaking vigorously for 5 sec. The tube was returned to the water bath and held without agitation. At the end of a 30-min. hydrolysis period, 5 ml. of 1 *M* phosphoric acid were added to stop hydrolysis and permit the subsequent extraction of the liberated butyric acid, mixed by inverting twice, and the tube transferred to another water bath at approximately 20° C. After allowing 2 or 3 min. for temperature adjustment, 10 ml. of ether were added and the mixture shaken vigorously for 5 sec. The ether separated rapidly after

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this extraction, but the tube was allowed to stand approximately 5 min. to permit small water droplets to settle out of the ether layer. A 5-ml. aliquot of the clear ether was transferred by means of a pipette to a 50-ml. Erlenmeyer flask containing 10 ml. of titration mixture (50 per cent ethanol containing 25 mg. cresol red per liter) and titrated with 0.025 *N* NaOH to a faint orange color. The hydrolysis was expressed as the number of milliliters of 0.025 *N* base required for the titration, corrected by deducting the titer for a blank determination obtained by following the above procedure except that the hydrolysis period was omitted, *i.e.*, the phosphoric acid was added immediately after emulsifying the tributyrin. This value for the hydrolysis was used as the measure of tributyrinase.

Determination of lipase. A procedure similar in principle to the tributyrinase determination, but employing a 20 per cent milkfat emulsion as substrate was used for the determination of lipase. The substrate emulsion was prepared by pasteurizing a mixture of milkfat and skimmilk at 74° C. for 15 min., homogenizing twice at 2,500 and 1,500 p.s.i. and cooling. The sample to be tested was centrifuged, 15 ml. of the skimmilk were mixed with 5 ml. of substrate emulsion and the recombined milk containing 5 per cent milkfat was buffered at pH 8.7 by addition of 50 ml. of borate buffer. The buffered recombined milk was held at 10° C. for 24 hr. and shaken gently three times during this period. The procedure followed for determining the blank in the tributyrinase method was applied to a 7-ml. aliquot at the beginning and end of the 24-hr. period and the difference was used as the measure of hydrolysis of milkfat. This hydrolysis value was assumed to be proportional to the lipase concentration.

A correction was made for possible production by bacterial action of ether-soluble acids other than fatty acids. This was done by substracting from the value for hydrolysis of milkfat a blank value obtained by holding separately the skimmilk and fat emulsion (both buffered) for the 24-hr. period and then mixing them in the required proportions immediately before making the determination. Hydrolysis by bacterial lipase was considered insignificant because standard plate counts made on the buffered milk samples at the end of the 24-hr. period in no case exceeded 2,000 per milliliter.

RESULTS

Relation between tributyrinase and lipase. Both tributyrinase and lipase determinations were made on milks of individual cows selected to show a reasonably wide range of tributyrinase concentrations. Tributyrinase determinations were applied to the buffered recombined milk at the beginning and end of the 24-hr. hydrolysis period of the lipase determination and averaged. Thus, the tributyrinase results represent averages of two determinations, but the lipase results were obtained by single determinations. Figure 1 presents data from three experiments.

In experiments 1 and 2 the buffered recombined milk was not agitated during the hydrolysis period of the lipase determination and the rate of lipolysis de-

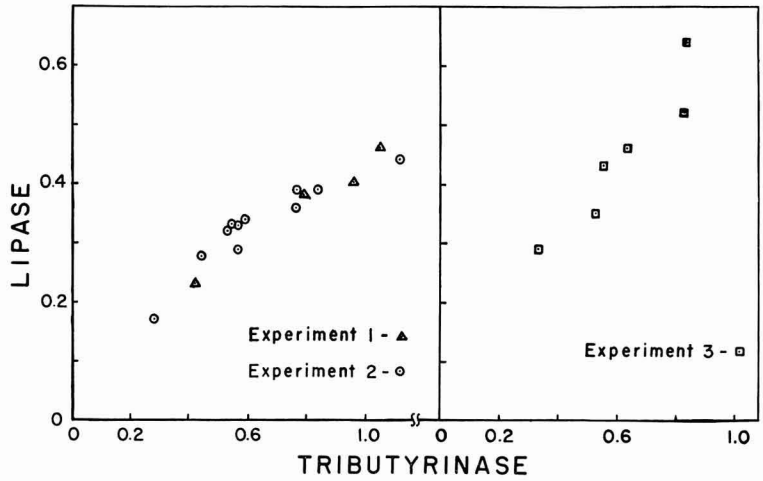

FIG. 1. Relation between tributyrinase and lipase in three experiments. Agitation of the reaction mixture during the hydrolysis period was omitted in experiments 1 and 2. Results are expressed as hydrolysis in milliliters of 0.025 *N* base.

creased after appreciable hydrolysis had occurred. In experiment 3 agitation of the reaction mixture three times during the 24-hr. hydrolysis period increased lipolysis and maintained a more uniform hydrolysis rate.

The coefficients of correlation between the tributyrinase and lipase results for experiments 1, 2 and 3 were 0.984, 0.923 and 0.941, respectively. These coefficients are significant at approximately the 1 per cent level for experiment 1, and at less than the 1 per cent level for experiments 2 and 3.

pH optima of tributyrinase and lipase. The activity at different pH's of tributyrinase at 37 and 10° C. and of lipase at 10° C. is illustrated in figure 2.

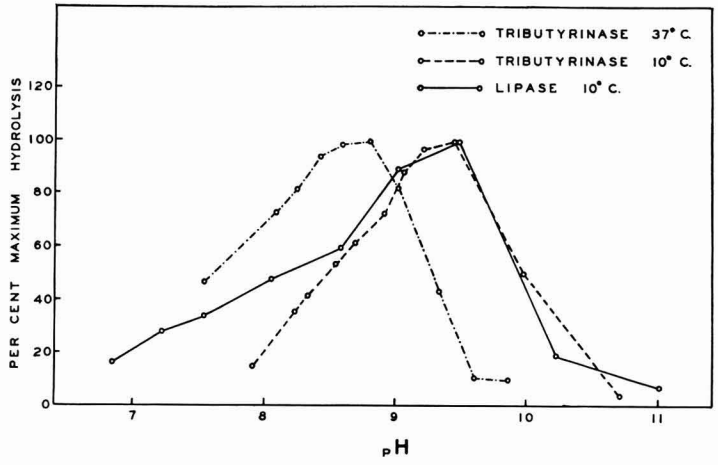

FIG. 2. Activity at different pH values of tributyrinase at 37 and 10° C. and of lipase at 10° C.

The skimmilk was buffered with a series of 0.1 *M* borate buffers which gave the desired range of pH. The pH values reported are averages of determinations at the beginning and end of the hydrolysis periods. In determining activity of tributyrinase at 10° C., a 2-hr. hydrolysis period was used. At 10° C. tributyrinase and lipase had the same pH optimum (about 9.5). It is possible that the change in pH optimum for tributyrinase with temperature may have resulted from a decrease in rate of inactivation of the enzyme at the lower temperature.

DISCUSSION

Some early lipase determinations using milkfat as substrate did not provide adequate standardization of variables other than lipase concentration which influence lipolysis. Enzymatic reactions are known to depend upon such factors as concentration of enzyme, temperature, pH, presence of certain activators, inhibitors or coenzymes, composition of the substrate, amount of substrate and area and condition of substrate surface. The procedures followed in most reliable lipase determinations provide for standardization of all such factors except the lipase concentration (preferably to optimum conditions for the enzyme), permit hydrolysis for a fixed time and use the amount of lipolysis as the measure of lipase.

The desirability of milkfat as the substrate for lipase determinations is recognized. However, the use of milkfat introduces difficulties in eliminating variations in the composition of the substrate, size distribution of the emulsion and nature of the substrate surface. The long hydrolysis period necessary to produce accurately measurable lipolysis also is a disadvantage. Tributyrin is less subject to the above limitations because it is available in relatively pure form, its solubility assures adequate dispersion and it is hydrolyzed more rapidly than milkfat. Chemically, tributyrin meets the definition of a fat even though it is a smaller molecule than the triglycerides of milkfat.

A limitation of the tributyrinase determination described above is that it does not eliminate the possible variable influence of accelerators or inhibitors present in milk samples. For example, low concentrations of $CaCl_2$ consistently caused some increase in hydrolysis when added at the beginning of the hydrolysis period. Variations in the concentration of calcium and perhaps other activators or inhibitors in milk may influence the results but probably do not introduce a serious error in the tributyrinase determination.

The authors consider that the correlation of results of the tributyrinase and lipase determinations depicted in figure 1 and the coincidence of the pH optima shown in figure 2 indicate that for some research purposes the tributyrinase determination may be useful as a measure of lipase in milk. A logical explanation for these results is that the two determinations measure the same enzyme or enzymes, but the data reported are insufficient to justify a definite conclusion on this point.

SUMMARY

Procedures for the determination of tributyrinase and lipase are described.

Data showing the correlation between tributyrinase and lipase determinations applied to the milk of individual cows are presented.

At 10° C. the pH optimum for both tributyrinase and lipase was about 9.5.

The correlation of results of tributyrinase and lipase determinations and the coincidence of the pH optima provide greater confidence in the use of the tributyrinase determination as a measure of lipase in milk.

ACKNOWLEDGMENT

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REFERENCES

(1) DUNKLEY, W. L. Unpublished data. 1946.

(2) HERRINGTON, B. L. Lipase in Dairy Products with Special Reference to its Effect on Flavors and its Control. Milk Ind. Foundation, 43rd. Ann. Conv. Lab. Sect. Proc., pp. 30–45. 1950.

(3) HILEMAN, J. L., AND COURTNEY, E. Seasonal Variations in the Lipase Content of Milk. J. Dairy Sci., **18**: 247–257. 1935.

(4) MATTICK, E. C. V., AND KAY, H. D. A Lipase (Tributyrinase) of Cows' Milk. I. Occurrence, Method of Estimation and Relationship to Lactation Cycle. J. Dairy Research, **9**: 58–71. 1938.

(5) PETERSON, M. H., JOHNSON, M. J., AND PRICE, W. V. Determination of Milk Lipase. J. Dairy Sci., **26**: 233–240. 1943.

(6) REDER, RUTH. Chemical Composition and Properties of Normal and Rancid Jersey Milk. III. Titratable Acidity, Hydrogen-ion Concentration and Lipase Content. J. Dairy Sci., **21**: 369–377. 1938.

(7) RICE, F. E., AND MARKLEY, A. L. Proof of the Presence of Lipase in Milk. J. Dairy Sci., **5**: 64–82. 1922.

(8) ROAHEN, D. C., AND SOMMER, H. H. Lipolytic Activity in Milk and Cream. J. Dairy Sci., **23**: 831–841. 1940.

(9) SMITH, L. M., LUBERT, D. J., AND THORNTON, H. R. Estimation of Lipase in Dairy Products. I. An Extraction-titration Method for Estimation of Milk Lipase. Can. J. Research, F, **27**: 483–490. 1949.

HYDROLYTIC RANCIDITY IN MILK. IV. RELATION BETWEEN TRIBUTYRINASE AND LIPOLYSIS

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This paper reports an investigation of the relation between both spontaneous and temperature-induced lipolysis and the concentration of tributyrinase in the milks of individual cows throughout their lactation periods. One of the purposes of this study was to provide additional information regarding factors which determine whether milk becomes rancid.

Some investigators who have reported that the lipase content of milk is highest in late lactation and in winter have based their conclusions on what might be considered measurements of lipolysis rather than lipase, since variables other than lipase concentration may have been involved. Results reported by Mattick and Kay (7), Pfeffer *et al.* (8), Reder (9) and Roahen and Sommer (10) indicate that lipolytic enzymes in milk do not increase in winter or in late lactation.

Tarassuk and Henderson (11) found that the development of spontaneous rancidity may be prevented by mixing susceptible milk with about four times its volume of normal milk within 1 hr. after milking.

Tarassuk and Jack (12) reported that the lipase of naturally lipolytically active milk adsorbs irreversibly on the fat globules when milk is cooled. Observations of Kelly (6) and Roahen and Sommer (10) are consistent with this report.

EXPERIMENTAL

The method for the tributyrinase determination used in this study was the same in principle as that described previously (3) but there were minor differences in procedure (1). These differences do not influence interpretation of the results. Tributyrinase determinations and surface tension measurements (2) were applied at weekly intervals to morning milk samples from 10 to 15 cows of three breeds (Holstein, Jersey and Shorthorn) over a 2.5-yr. period. The data for tributyrinase and surface tension measurements for six selected, but typical, lactation periods are plotted in figures 1 to 6. The surface tension scale has been inverted in these graphs to facilitate visual estimation of correlation between tributyrinase and surface tension.

In view of the report that naturally lipolytically active lipase adsorbs on the fat globules when milk is cooled (12), tributyrinase determinations were applied to skimmilk separated from warm milk immediately after milking and from the same milk after it had been held at 2° C. for 4 hr. Cooling the milk did not result in a decrease in tributyrinase in the skimmilk. This was true for both normal milk and milk which developed rancidity spontaneously. Adsorption of lipolytic enzyme on the fat in cooled milk apparently was not a factor influencing results of the tributyrinase determination.

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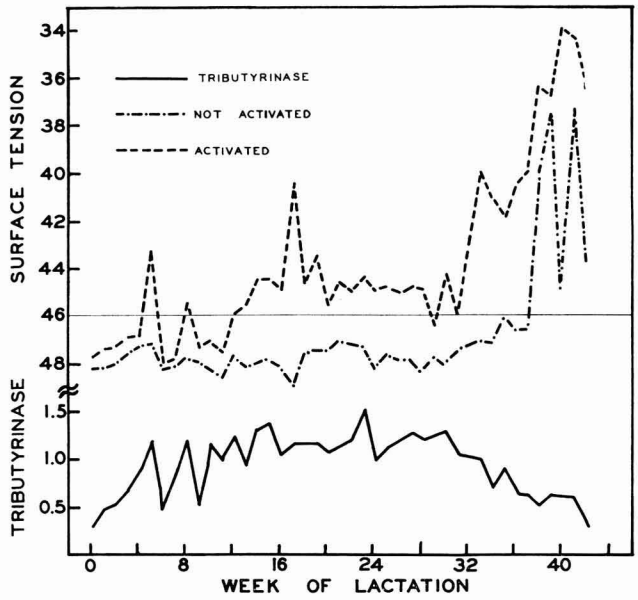

FIG. 1. Tributyrinase and surface tension of milk samples taken at weekly intervals during Polly's lactation of June, 1945, to April, 1946. Tributyrinase results are expressed as hydrolysis in milliliters of 0.025 *N* base and surface tension in dynes per centimeter.

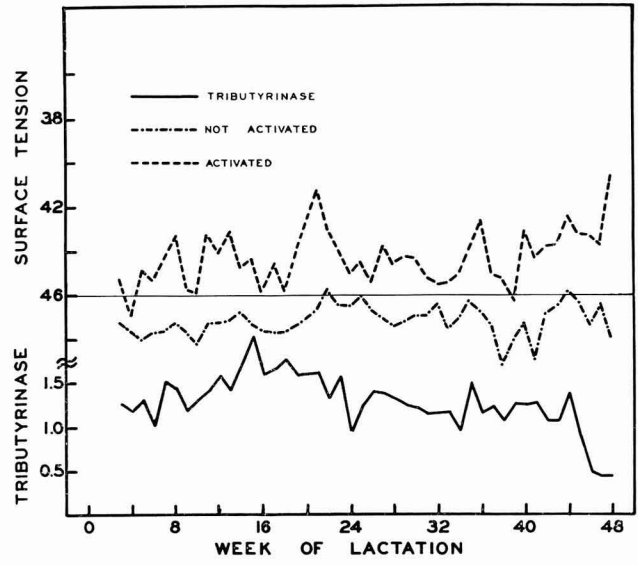

FIG. 2. Tributyrinase and surface tension of milk samples taken at weekly intervals during Poppy's lactation of February, 1946, to January, 1947. Tributyrinase results are expressed as hydrolysis in milliliters of 0.025 *N* base and surface tension in dynes per centimeter.

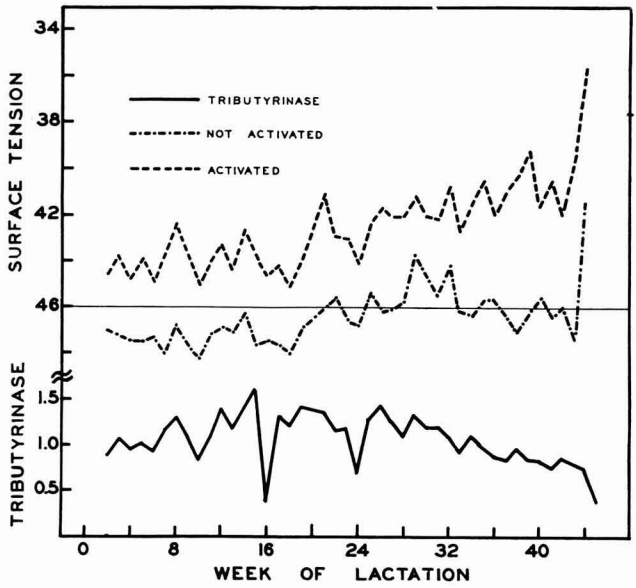

FIG. 3. Tributyrinase and surface tension of milk samples taken at weekly intervals during Omieron's lactation of February, 1946, to January, 1947. Tributyrinase results are expressed as hydrolysis in milliliters of 0.025 *N* base and surface tension in dynes per centimeter.

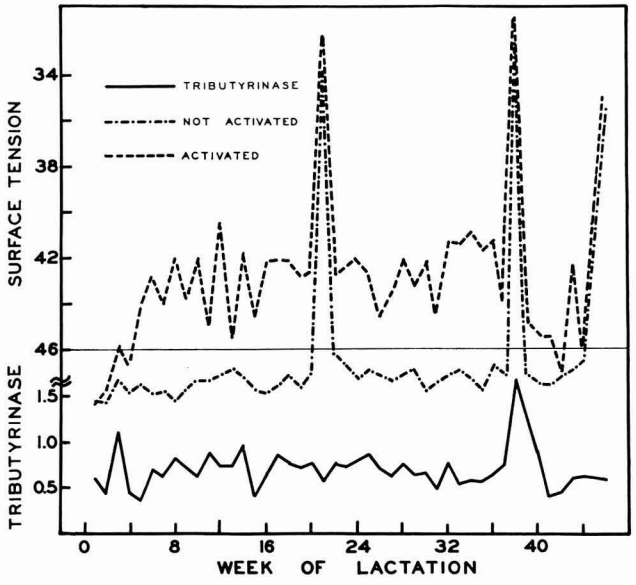

FIG. 4. Tributyrinase and surface tension of milk samples taken at weekly intervals during Maxine's lactation of August, 1945, to July, 1946. Tributyrinase results are expressed as hydrolysis in milliliters of 0.025 *N* base and surface tension in dynes per centimeter.

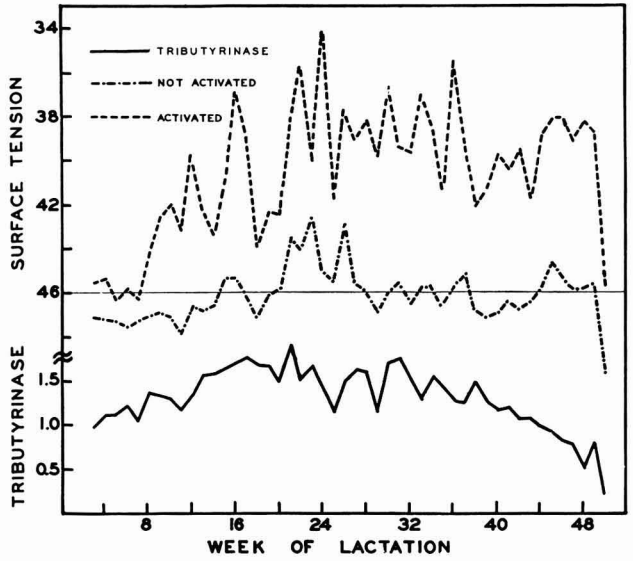

FIG. 5. Tributyrinase and surface tension of milk samples taken at weekly intervals during Frieda's lactation of February, 1946, to February, 1947. Tributyrinase results are expressed as hydrolysis in milliliters of 0.025 *N* base and surface tension in dynes per centimeter.

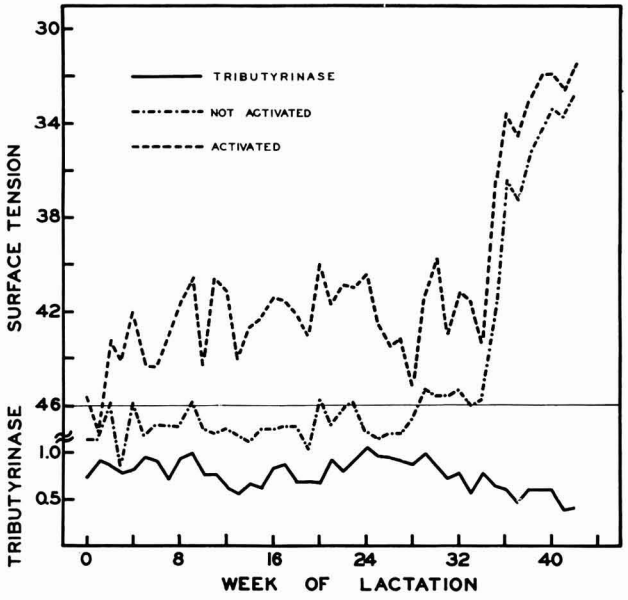

FIG. 6. Tributyrinase and surface tension of milk samples taken at weekly intervals during Lucy's lactation of December, 1945, to October, 1946. Tributyrinase results are expressed as hydrolysis in milliliters of 0.025 *N* base and surface tension in dynes per centimeter.

DISCUSSION

In figure 4 the three marked decreases in surface tension occurred the day following injection of the cow with a pituitrin-stilbestrol mixture (4). In only one case was this decrease accompanied by an increase in tributyrinase.

Most of the data showed little or no correlation between tributyrinase and lipolysis. However, in some cases the concordance of tributyrinase and surface tension of activated milk samples indicated that the enzyme concentration might have been an important factor limiting induced lipolysis during the early part of the lactation period. To illustrate this point, data for the first 12 wk. (17 wk. in figure 7A) of the lactation periods included in figures 1 to 6 are plotted as

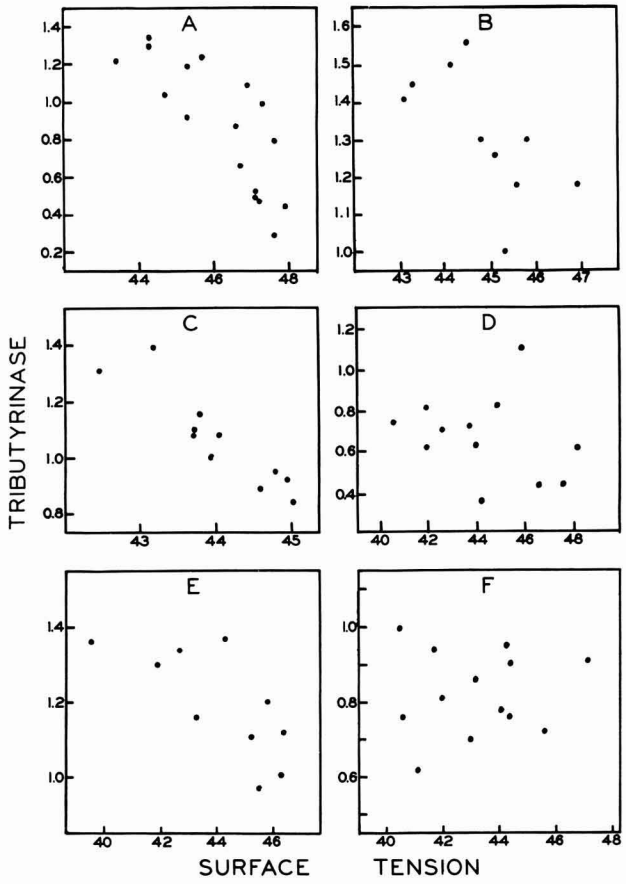

FIG. 7. Scatter diagrams illustrating correlation between tributyrinase and surface tension of temperature-activated milk samples taken during the first 12 wk. (17 in A) of lactations for which data are plotted in figs. 1 to 6. Tributyrinase is expressed as hydrolysis in milliliters of 0.025 *N* base and surface tension in dynes per centimeter. A—Polly, B—Poppy, C—Omieron, D—Maxine, E—Frieda, F—Lucy.

scatter diagrams in figure 7. During the first 17 wk. of Polly's lactation (figure 7A), correlation is evident between tributyrinase and induced lipolysis. Induced rancidity did not develop when the tributyrinase was low. However, some samples showing relatively high tributyrinase did not develop rancidity, probably because some factor other than tributyrinase limited the lipolysis in these cases. In spite of this indication that a factor or factors in addition to enzyme concentration was involved in limiting lipolysis in these samples, the coefficient of correlation between tributyrinase and surface tension for these data is -0.80. This value is significant at less than the 1 per cent level. The results plotted in figure 7C have a correlation coefficient of -0.92, which also is significant at less than the 1 per cent level. The coefficients for the data in figure 7B and E are -0.65 and -0.72 respectively, both of which are significant at the 5 per cent level, but not at the 1 per cent level. The data in figure 7D and F have correlation coefficients of -0.24 and $+0.11$, neither of which is significant. On the basis of the assumption that the tributyrinase measurements reflect variations in lipase content, these results indicate that during the early part of some lactation periods the amount of lipase in milk is a factor limiting the development of rancidity induced by temperature-activation.

For milk produced after the first 3 or 4 mo. of lactation, there was no positive correlation between tributyrinase and either spontaneous or temperature-induced lipolysis. These results indicate that the lipolytic enzymes measured by the tributyrinase determination were not an important factor limiting the lipolysis during the latter part of the lactation periods.

In most of the lactations for which complete data were obtained, the tributyrinase was higher in the middle of the period than at the beginning or end. No seasonal variations were observed.

Results of this investigation are consistent with many reported elsewhere in indicating that the amount of lipase present usually is not the principal factor determining whether milk goes rancid. Since rancidity can be induced in most (perhaps all) milks by treatments such as homogenization, agitation and controlled temperature fluctuations, a reasonable assumption is that sufficient lipase is present to promote the lipolysis. In the authors' opinion the condition of the surface of the fat appears to be the most important factor determining whether milk develops rancidity.

Activation treatments such as temperature-fluctuation, agitation and homogenization generally are considered to depend on changes in the substrate for their effectiveness. Consequently, the term "activation of lipolysis" is preferable to "activation of lipase" since the latter designation implies that it is the enzyme, not the substrate, whch is involved in the changes produced by activation. Furthermore, since the basis for differentiating spontaneous and induced rancidity is whether lipolysis occurs without activation, the authors question the advisability of using the term "naturally active lipase" until it definitely is established that this lipase differs from that of milk which requires activation to induce lipolysis.

Herrington (5) discussed some contradictory results in the literature which may be attributed to differences in the properties of lipases in milk. In view of experimental evidence available at present, explanations based on variations in the substrate or on the action of an inhibitor also are tenable in accounting for certain characteristics of hydrolytic rancidity. The finding that spontaneous rancidity can be inhibited by mixing susceptible milk with about four times its volume of non-susceptible milk (11) could result from the presence of a lipolysis inhibitor in normal milk which is deficient in susceptible milk. Such an inhibitor might prevent lipolysis by preferential adsorption on the substrate to block the enzyme rather than by acting on the lipase itself. This mechanism for inhibition of lipolysis also would account for activation by agitation and homogenization, since these treatments disrupt the protected fat surface.

SUMMARY

Spontaneous and temperature-induced lipolysis, measured by surface tension changes, and tributyrinase were determined at weekly intervals in milk from 10 to 15 cows over a 2.5-yr. period.

In the early part of some lactation periods there appeared to be a correlation between tributyrinase and temperature-induced lipolysis. For milk produced after the first 3 or 4 mo. of lactation, there was no positive correlation between tributyrinase and either spontaneous or temperature-induced lipolysis.

The tributyrinase determinations showed a trend downward toward the end of lactation, but no seasonal variations were observed.

The importance of lipase concentration and conditions at the surface of the fat globules in determining whether milk develops rancidity is discussed.

ACKNOWLEDGMENTS

J. E. Bowstead, Department of Animal Science, provided the milk samples used in this investigation. This study was supported by a grant from the Committee on Agricultural Research Grants of the University of Alberta.

REFERENCES

(1) Dunkley, W. L. Unpublished data. 1946.
(2) Dunkley, W. L. Hydrolytic Rancidity in Milk. I. Surface Tension and Fat Acidity as Measures of Rancidity. J. Dairy Sci., **34**: 515–520. 1951.
(3) Dunkley, W. L., and Smith, L. M. Hydrolytic Rancidity in Milk. III. Tributyrinase Determination as a Measure of Lipase. J. Dairy Sci., **34**: 935–939. 1951.
(4) Fredeen, H., Bowstead, J. E., Dunkley, W. L., and Smith, L. M. Hydrolytic Rancidity in Milk. II. Some Management and Environmental Factors Influencing Lipolysis. J. Dairy Sci., **34**: 521–528. 1951.
(5) Herrington, B. L. Lipase in Dairy Products with Special Reference to its Effect on Flavors and its Control. Milk Ind. Foundation, 43rd Ann. Conv., Lab. Sect. Proc., pp. 30–45. 1950.
(6) Kelly, P. L. Milk Lipase Activity: A Method for its Determination, and its Relationship to the Estrual Cycle. J. Dairy Sci., **28**: 803–820. 1945.
(7) Mattick, E. C. V., and Kay, H. D. A Lipase (Tributyrinase) of Cows' Milk. I. Occurrence, Method of Estimation and Relationship to Lactation Cycle. J. Dairy Research, **9**: 58–71. 1938.

(8) PFEFFER, J. C., JACKSON, H. C., AND WECKEL, K. G. Observations on the Lipase Activity in Cow's Milk. J. Dairy Sci., **21**: A143. 1938.

(9) REDER, RUTH. Chemical Composition and Properties of Normal and Rancid Jersey Milk. III. Titratable Acidity, Hydrogen-ion Concentration and Lipase Content. J. Dairy Sci., **21**: 369–377. 1938.

(10) ROAHEN, D. C., AND SOMMER, H. H. Lipolytic Activity in Milk and Cream. J. Dairy Sci., **23**: 831–841. 1940.

(11) TARASSUK, N. P., AND HENDERSON, J. L. Prevention of Development of Hydrolytic Rancidity in Milk. J. Dairy Sci., **25**: 801–806. 1942.

(12) TARASSUK, N. P., AND JACK, E. L. Lipolytic Flavors of Milk. Milk Plant Monthly, **38** (10): 48. 1949.

JOURNAL OF DAIRY SCIENCE

ABSTRACTS OF LITERATURE

Prepared in cooperation with the
International Association of Ice Cream Manufacturers
and the Milk Industry Foundation

ANIMAL DISEASES

W. D. POUNDEN, SECTION EDITOR

485. The vaccination of pregnant cattle with strain 19 Br. abortus vaccine during an outbreak of brucellosis in a dairy herd. A. McDIARMID, Agr. Research Council, Field Station, Compton, Berkshire. Vet. Record, **63:** 15: 265–268. Apr., 1951.

A newly infected herd of 42 Ayrshire and Friesian cows and heifers was vaccinated with S. 19 vaccine. Average period of gestation was 5.7 mo., with a range of 12–236 d., and all animals either were negative or reacting at 1 : 10 when vaccinated. At parturition, cultures were prepared from colostrum, cotyledons and stomach contents of all dead calves, and strains recovered were classified. Agglutination titres were determined at vaccination, 3 wk. after, 3 mo. after and at parturition. Gestation period ranged from 172–296 d., with 36 live calves produced from the 42 pregnant animals. Five were infected with *Br. abortus* at calving, of which 4 showed infection from field strains and 1 apparently with S. 19. Author states that results of this field trial justify vaccination of pregnant cattle when dealing with a severe outbreak of contagious abortion in a dairy herd.

R. P. Niedermeier

486. The ring test for Brucellosis in herd management. J. S. BRYAN, Walker-Grodon Lab., Plainsboro, N. J. J. Milk & Food Technol., **14:** 109–110, 121. May–June, 1951.

A comparison of the ring test with the blood test for detecting brucellosis was made. Cows showing a positive blood test also were positive by the ring test, but those strongly positive by ring test are not always positive by blood test.

Heifers vaccinated as calves seldom retain a blood titre and as a rule do not give a positive ring test when they are brought into milk production.

The ring test will probably never replace the agglutination method. However, the test can serve as an adjunct to the blood test in a good herd management program. H. H. Weiser

487. Mastitus in practice. 1. Epizootiology and bacteriology. J. BERGER, Alderley Edge, Cheshire, and J. Francis, Imperial Chemical Inds., Ltd., Wilmslow, Cheshire. Vet. Record, **63:** 16: 283–293. Apr., 1951.

This study involved a 3-yr. period during which all mastitis cases treated by the senior author were reported, and when possible milk samples were taken before and after treatment for bacteriological examination. The cow population involved was estimated to be 2,397, including Shorthorns, Ayrshires and Friesians. Data are summarized to show incidence of mastitis, relationship to calving, age of cows affected with mastitis, association of injury with mastitis, seasonal incidence and types of bacteria isolated.

R. P. Neidermeier

488. New methods of treating ketosis—a preliminary report. R. B. JOHNSON, Livestock Sanitary Service Lab., College Park, Md. North Am. Vet., **32,** 5: 327–332. May, 1951.

Four clinical cases and 2 subclinical cases of ketosis were treated by various methods, and the milk production, plasma glucose level and plasma ketone levels were followed with each treatment. Treatments included massive intravenous glucose injection, a combination intravenous and subcutaneous and glucose injection, oral sugar administration and oral administration of glycerin and tripropionin. The author states that the use of glycerin shows considerable promise.

R. P. Niedermeier

BUTTER

O. F. HUNZIKER, SECTION EDITOR

489. The acidity of cream affecting the flavour of cream and butter (Preliminary report). H. MULDER and J. H. B. KLEIKAMP. Netherlands Milk & Dairy J., **1,** 4: 225–231. Oct., 1947.

Cream was acidified with HCl to various pH levels and the flavor of the fat and the butter was checked for the "trainy" defect. (This flavor is known as fishiness in this country but in the opinion of the authors it is not quite correct. The authors feel that trainy flavor is between the fishy and oily flavors.) The defect was strongest at pH 2.55. If the pH was lowered still further, the fat had more of a tallowy flavor. The addition of 20 mg/l. of hydroquinone prevented the oxidation defects at pH levels of 4.80–0.71. The "trainy" defect did not occur in homogenized butterfat dispersed in skimmilk. However, in artificial cream prepared by emulsifying butterfat in buttermilk from sweet cream the defect was again prevalent at pH 2.80. The "trainy" defect seems to be associated with the surface layers of the fats. W. W. Overcast

490. The influence of the churning temperature on the consistency of butter. H. MULDER. Netherlands Milk & Dairy J., **1,** 4: 232–234. Oct., 1947.

Churning cream at 9° C. gave a harder butter than churning at 15° C. The hardness of the butter was estimated by the method of Kruisheer and Den Herder after the butter had set.

W. W. Overcast

491. The influence of the fat content of cream on the consistency and the water content of butter. H. MULDER. Netherlands Milk & Dairy J., **1,** 4: 235–237. Oct., 1947.

The fat content of the cream had no influence upon the hardness of the butter. Butter churned from low-testing cream (9%) had a higher moisture content and retained more of the buttermilk than butter churned from average test cream. Fat losses also were greater in churning the low-testing cream. W. W. Overcast

CHEESE

A. C. DAHLBERG, SECTION EDITOR

492. An early gas defect in cheese caused by yeasts. T. E. GALESLOOT. Netherlands Milk & Dairy J., **1,** 4: 238–243. Oct., 1947.

Lactase-fermenting yeasts were found to be the cause of early gas in raw milk cheese containing either KNO_3 or $KClO_3$ to prevent gas due to the coliform organisms. The glucose-fermenting yeasts were not capable of producing this defect. Pasteurization of the milk and adequate care of the starters readily eliminated the difficulty.

W. W. Overcast

493. Handling cheese. B. F. DAVIS (assignor to Wingfoot Corp.). U. S. Patent 2,558,673. 5 claims. June 26, 1951. Official Gaz. U. S. Pat. Office, **647,** 4: 1266. 1951.

Blocks of cheese are wrapped in air-impervious oil-resistant moisture-proof material and pressed to squeeze out all the air between the cheese and wrapper. After partial curing, the cheese may be cut into smaller blocks and each piece again wrapped and pressed and packed tightly in boxes for final curing and for sale to the retail consumer.

R. Whitaker

494. Preparation of blue-veined cheese. F. E. NELSON and I. I. PETERS (assignors to Iowa State College Research Foundation). U. S. Patent 2,560,182. 8 claims. July 10, 1951. Official Gaz. U. S. Pat. Office, **648,** 2: 546. 1951.

Prior to setting pasteurized milk in the usual manner for making blue cheese, the milk is subjected to lipolytic action resulting from the addition of lipase obtained from a culture of *Mycotorula lipolytica.* R. Whitaker

495. Détermination des teneurs en matière sèche, ne calcium et en phosphore des principaux fromages francais á pâte molle (Determination of the amounts of drymatter, calcium and phosphorous in the principal soft French cheeses). L. RANDOIN and C. VATINEL. Lait, **31,** 303–304: 113–121. Mar.–Apr., 1951.

Analytical data on the Ca, P and moisture content of certain soft varieties of cheese are presented. A number of these were found to be superior in Ca and P content and their consumption is recommended for reasons of good nutrition.

S. Patton

496. Process for the treatment of a soft cheese product. J. A. KILLENGREEN. U. S. Patent 2,558,256. 5 claims. June 26, 1951. Official Gaz. U. S. Pat. Office, **647,** 4: 1153. 1951.

A cheese dessert is prepared by heating skim-milk to such an extent that its rennet coagulability is impaired, then setting the cooled milk with starter and rennet for several hours to form a finely flocculated curd. After whey has been pressed out, the curd is homogenized and blended with whipped cream or other fat emulsion to form a foamy dessert of predetermined fat content. R. Whitaker

CONDENSED AND DRIED MILKS; BY-PRODUCTS

F. J. DOAN, SECTION EDITOR

497. Package for whey concentrate and other dairy products. N. L. SIMMONS. U. S. Patent 2,557,576. 2 claims. June 19, 1951. Official Gaz. U. S. Pat. Office, **647,** 3: 893. 1951.

A cylindrical cardboard with flat ends, containing an acid resistant flexible moisture proof resinous bag for plastic dairy products such as whey concentrate, animal feeds, etc., is described.

R. Whitaker

498. Process for producing a reducing sugar and milk product. P. F. SHARP (assignor to Golden State Co.) U. S. Patent 2,558,528 7 claims. June 26, 1951. Official Gaz. U. S. Pat. Office, **647,** 4: 1227. 1951.

A blend of milk and a reducing sugar having a total solids content of at least 25% is heated above 212°F. to produce a product suitable for improving the consistency of baked goods, ice cream, etc. R. Whitaker

Also see abs. no. 509.

DAIRY BACTERIOLOGY

P. R. ELLIKER, SECTION EDITOR

499. The bacteriological grading of milk by means of the resazurin test. J. G. REVALLIER-WARFFEMIUS. Netherlands Milk & Dairy J., **1,** 3: 152–165. July, 1947.

A comparison of the methylene blue test and the 1-hr. resazurin test is presented. The methylene blue test is preferred over the resazurin test as an indirect measure of the keeping quality. The resazurin test is more sensitive to the reducing power of leucocytes and tissue cells. The presence of large numbers of leucocytes due to mastitis is accompanied by an abnormal chemical composition of the milk, often causing retarded clotting; the detection of milk of this quality may be important for cheese making and the resazurin test was especially recommended for it. Contradictions between the 2 tests ran from 14-30%. A closer correlation between the resazurin and methylene blue tests could be obtained if the

milk was stored at 2-3°C. for 24 hr. before running the test. W. W. Overcast

500. Synthetic culture media for reference use in dairy bacteriology. M. J. Pelczar and J. H. Brown, Univ. of Maryland, College Park. J. Milk & Food Technol., **14:** 90–91, 97. May–June, 1951.

A synthetic agar medium was compared to the standard TGEM agar in determining the bacterial counts of several pasteurized milk samples. The results were comparable if the incubation period of the synthetic medium was extended to 96 hr. for S_4 agar and 72 hr. for S_4 agar plus sodium ethyl oxalacetate in comparison with 48 hr. for TGEM medium.

The authors believe they have a reproducible synthetic medium in dry form which may serve as a reference standard in which different lots of peptone media could be evaluated. H. H. Weiser

501. A comparative study of six agars proposed for bacterial plate counts of milk. Vivian Pessin, Dept. of Health, New York City, and Luther Black, U. S. Pub. Health Service, Cincinnati, O. J. Milk & Food Technol., **14:** 98–102. May–June, 1951.

A comparative study was made in 10 different laboratories on the bacterial count of 505 milk samples using standard agar, agar plus skimmilk and 4 agar media without skimmilk. The new medium with milk was comparable to be present standard culture medium, while 4 without milk showed 8% greater bacterial count.

Participating laboratories reported that the agars without skimmilk were superior to those with milk in regard to freedom from precipitate, cleanness of background and size of colonies. H. H. Weiser

502. Influence of penicillin in milk on total and coliform bacteria plate counts. H. H. Wilkowske and W. H. Krienke, Fla. Agr. Expt. Sta., Gainesville. J. Milk & Food Technol., **14:** 92–94. May–June, 1951.

No appreciable effect on the coliform count was noted when 10 units of penicillin/ml. were added to milk. It was suggested that the coliform organisms may produce "penicillinase". When 1.0 unit of penicillin/ml. was added to milk and held at 10° C. for 72 hr., no significant increase was noted in the plate count. The control samples showed a marked increase in the total counts. The authors contend that penicillin has a preservative property in milk, although its use is not recommended. H. H. Weiser

503. Action inhibitrice de la pénicilline sur les lactobacilles utilisés dans la fabrication du gruyère (Inhibiting action of penicillin on the lactobacilli utilized in the manufacture of Gruyere cheese). J. Auclair. Lait, **31,** 303–304: 121–134. Mar.–Apr., 1951.

Levels of penicillin up to 0.3 unit/ml. of milk did not materially inhibit acid development by 2 stock cultures of *L. helveticus,* but such levels drastically reduced acid development by 2 cultures of *L. lactis.* Some adaption of these cultures to the presence of penicillin was found possible but never to the extent or normal acid production. Use of penicillinase to inactivate penicillin was studied under both laboratory and cheese manufacturing conditions. A qualitative method for detecting penicillin in milk based on inactivation by penicillinase is presented. S. Patton

504. Die Bedeutung der Wuchsstoffe im Stoffwechsel der Milchsäurebakterien (The significance of growth factors in the metabolism of lactic acid bacteria). E. F. Möller. Milchwissenschaft, **5,** 9: 313–316; 10: 359–362. Sept., Oct., 1950.

The group of lactic acid bacteria lends itself well for the study of the significance of growth factors in bacterial metabolism. The author discusses the influence of growth factors upon lactic acid bacteria on the basis of published information under the following headings: (a) Lactic acid formation; (b) conversion of pyruvic acid within the cycle of carbohydrate metabolism; (c) conversion of pyruvic acid within the cycle of protein metabolism; (d) anabolism and catabolism of complex amino acids; (e) folic acid and B_{12} in metabolism of lactic acid bacteria. 73 references are listed. I. Peters

505. Response of lactic acid bacteria to amino acid derivatives. II. Glycine. R. B. Malin, M. N. Camien and M. S. Dunn, Univ. of Calif., Los Angeles. Arch. Biochem. & Biophysics, **32,** 1: 106–112. June, 1951.

The microbiological response of 5 glycine-requiring lactic acid bacteria toward 25 glycine-containing substances was tested over a wide range of concentration and at several incubation times from 18–229 hr. All 5 dipeptides and the tripeptide, L-leucyl-glycylglycine, were more active than glycine toward at least 1 organism. None of the bacteria showed significant responses toward glutathione and the dimethyl, trimethyl, benzoylglycyl and benzenesulfonylglycyl N-substituted glycine derivatives. Hippuric acid had a relatively high activity toward all of the bacteria. Under some conditions lactic acid bacteria may utilize dipeptides directly. H. J. Peppler

506. Cathode rays recommended for food, drug sterilizing. Anonymous. Chem. Eng. News, **29,** 28: 2818–2819. 1951.

This is a brief report of papers presented at the Annual Meeting of the Institute of Food Technologists, June, 1951, on cathode ray sterilization, preservation with antibiotics, loss of riboflavin in bread and new methods for heat sterilization. Samples of milk subjected to cathode rays were said to be unchanged in flavor after irradiation and after storage for at least 1 mo. in a refrigerator. B. H. Webb

507. Occurrence of D-amino acids in some natural materials. C. M. Stevens, P. E. Halpern and R. P. Gigger, State College of Wash., Pullman. J. Biol. Chem., **190,** 2: 705–710. June, 1951.

D-Amino acids were found in acid hydrolysates of cells of *Lactobacillus arabinosus* and *Bacillus*

brevis to the extent of 8–12% of the total amino acids. In contrast, casein hydrolysate contains 0.02% D-amino acids, *Penicillium chrysogenum*, 0.01%, and *Torulopsis utilis*, 0.08%.

H. J. Peppler

Also see abs. no. 492.

DAIRY CHEMISTRY

H. H. SOMMER, SECTION EDITOR

508. Effect of pH on the denaturation of β-lactoglobulin and its dodecyl sulfate derivative. M. L. GROVES, N. J. HIPP and T. L. MCMEEKIN. Eastern Reg. Research Lab. U. S. D. A., Philadelphia 18. J. Am. Chem. Soc., **73,** 6: 2790–2793. June, 1951.

β-Lactoglobulin denatures unimolecularly in both veronal and borate buffer solutions more alkaline than pH 8. The rate of denaturation of β-lactoglobulin, as measured by the increase in optical rotation, is inversely proportional to the 1.1 power of the hydrogen ion concentration at 3 and 25° C. β-Lactoglobulin containing 2 equivalents of dodecyl sulfate denatures more slowly than the normal substance. A comparison of the effects of pH, heat, urea, guanidine-HCl and dodecyl sulfate on the stability of β-lactoglobulin is given. H. J. Peppler

509. Determination of alpha- and beta-lactose in dry products of milk from rates of crystallization. R. P. CHOI, C. W. TATTER and C. M. O'MALLEY, Am. Dry Milk Inst., Inc., Chicago 1, Ill. Anal. Chem., **23,** 6: 888–890. 1951.

The increase in the concentration of lactose that results from the addition of dry milk to a continuously agitated saturated solution of lactose containing crystalline α hydrate is determined and calculated as the β-lactose of the sample. The α-lactose may be obtained as the difference between the total lactose and β-lactose. Data are presented showing the α- and β-lactose content of some dry products of milk. The ratio of β- to α-lactose in dried skimmilk is similar to that found in fresh fluid milk. In some samples of dried whey, α-lactose predominated, indicating that crystallization of lactose was induced during processing. B. H. Webb

510. Le dosage de l'enzyme de Schardinger à l'aide de la réduction des nitrates. I. Teneur en enzyme du lait de femme (Determination of Schardinger's enzyme by means of the reduction of nitrates. I. Quantity of enzyme in mother's milk). L. M. BURUIANA and E. GIRANIOU. Lait, **31,** 303–304: 134–150. Mar.–Apr., 1951.

Schardinger's enzyme was measured by its capacity to reduce nitrates to nitrites in the presence of benzaldehyde. The resulting nitrite formation then was followed by color development with naphthylamine-sulfanilic acid reagent. The method was thoroughly studied and is presented in detail.

Results of the investigation indicate that mother's milk contains only traces of the enzyme, and in 25% of the cases no activity was found. However, colostrum always contains the enzyme and it is a richer source than normal milk. A number of other factors were studied with regard to their effect on enzyme concentration and activity. S. Patton

511. The condition and mutual relationships of calcium caseinate and calcium phosphate in milk. M. G. TER HORST. Netherlands Milk & Dairy J., **1,** 3: 137–151. July, 1947.

A review article of 16 papers with some recalculating of results for comparison is presented. W. W. Overcast

512. Studies on the structure of nucleic acids. II. Investigation of pentose nucleic acid and enzyme-resistant residue. L. F. CAVALIERI, S. E. KERR and A. ANGELOS, Sloan-Kettering Inst. for Cancer Research, New York 25. J. Am. Chem. Soc., **73,** 6: 2566–2578. June, 1951.

The enzymes ribonuclease and acid-phosphatase were employed as tools for the study of the structure of pentose nucleic acid from beef pancreas and yeast nucleic acid. Quantitative determinations of the rosaniline-binding process, together with pH and periodate titrations, were carried out on the parent nucleic acid and on fractions resulting from the action of ribonuclease and riconuclease followed by acid phosphatase. From the interaction of rosaniline with the various nucleic acid samples it was established that the binding sites involve the phosphoric acid groups, of which about 13% are available for binding. A similarity of intrinsic binding constants and n values of the nucleic acids studied indicates a similarity of backbone structure. Ultraviolet and infrared absorption spectra of both parent and enzyme-treated nucleic acids are alike and cannot effectively serve as a means of identification. X-ray powder patterns indicate that some of the residues remaining after the action of ribonuclease and acid-phosphatase are partially crystalline. H. J. Peppler

513. Crystalline trypsin inhibitor from colostrum. M. LASKOWSKI, JR. and M. LASKOWSKI, Marquette Univ., Milwaukee, Wis. J. Biol. Chem., **190,** 2: 563–573. June, 1951.

Bovine and human colostrum contain significant amounts of trypsin inhibitor. One ml. of 1st-day bovine colostrum inhibited from 120–600 γ trypsin. Less inhibitor was found in human colostrum; of 10 samples analysed, the greatest amount of trypsin inhibited by 1 ml. of colostrum was 60 γ. This discovery of marked trypsin inhibition by colostrum offers an explanation for the transmission of immune globulins to the circulation of the new-born. The crystalline inhibitor has been isolated from bovine colostrum, and its properties are briefly described. H. J. Peppler

514. Improved procedure for extraction of DDT in milk. H. D. MANN and R. H. CARTER, Bureau of Entomology and Plant Quarantine, U. S. D. A., Beltsville, Md. Anal. Chem., **23,** 6: 929–930. 1951.

The milk to be analyzed 1st is coagulated with acetic acid. The fat rises to the top and is extracted with chloroform after the addition of a buffer salt to prevent the formation of an emul-

sion. The Schechter and Carter procedure then is followed. The modified method reduces by one-third the time formerly required to make the determination. The successive treatments of the Milk with ethyl alcohol, ethyl ether and petroleum ether are eliminated. The new procedure gives as good recovery as the older method.

B. H. Webb

515. Gauging the toxicity of chemicals in foods. B. L. OSER, Food Research Labs., Inc., Long Island City, N. Y. Chem. Eng. News, **29,** 28: 2808–2812. 1951.

A general discussion under such topics as the criteria for the use of chemicals, the natural occurrence of chemicals in foods, the harmfulness of such substances, the determination of toxicity and the role of the Food and Drug Administration in the development and enforcement of standards is presented. B. H. Webb

Aso see abs. no. 495.

DAIRY ENGINEERING

A. W. FARRALL, SECTION EDITOR

516. Ein neues Ultraviolett-Milch-Vitaminisiergerät (A new milk irradiation apparatus). English summary. E. SAUTER. Milchwissenschaft, **5,** 9: 296–299. Sept., 1950.

A new apparatus is described which is suitable for irradiating milk by means of a low pressure mercury gas lamp. This lamp emits 80% of its light in wavelength of 254 mμ. The flowing milk is irradiated consecutively in 12 stainless steel tubes 2 m. long. Advantages of this apparatus are given as follows: (a) Milk is irradiated in the absence of O_2, thus no formation of O_3; (b) the apparatus is so constructed that practically all the light waves emitted by the lamp are used in irradiating the milk. Illustrations and diagrams are presented. I. Peters

517. Rotary can washer. W. S. CAMPBELL (assignor to Cherry Burrell Corp.). U. S. Patent 2,558,818. 7 claims. July 3, 1951. Official Gaz. U. S. Pat. Office, **648,** 1: 103. 1951.

Structural details are given covering a rotary-type washer for milk cans and lids.

R. Whitaker

518. Freezer. L. S. MARANZ (assignor to Freeze King Corp.). U. S. Patent 2,558,449. 7 claims. June 26, 1951. Official Gaz. U. S. Pat. Office, **647,** 4. 1205. 1951.

A horizontal-type freezer for making soft ice cream, frozen custard, etc., is described. A mix supply inlet is located at the front end of the freezer. Mix, automatically and proportionately fed into the freezer when the finished product is withdrawn, passes through the hollow shaft, containing a worm-type conveyor, entering a compartment at the back of the freezer where it is whipped and partially frozen. From this it passes into the main freezing chamber where it is whipped and frozen to the desired overrun and temperature. R. Whitaker

HERD MANAGEMENT

H. A. HERMAN, SECTION EDITOR

519. Milking machine pulsator. S. P. WALL (assignor to Rite-Way Products Co.). U. S. Patent 2,559,035. 14 claims. July 3, 1951. Official Gaz. U. S. Pat. Office, **648,** 1: 163. 1951.

A device for causing pulsations in the vacuum supply to a milking machine is described.

R. Whitaker

520. Control unit for milking machines. A. G. PERKINS. U. S. Patent 2,558,152. 14 claims. June 26, 1951. Official Gaz. U. S. Pat. Office, **647,** 4: 1126. 1951.

A device is described which stops a milking machine when the milk flow from the cow ceases.

R. Whitaker

521. Milking machine rinser. E. REDIN. U. S. Patent 2,558,628. 8 claims. June 26, 1951. Official Gaz. U. S. Pat. Office, **647,** 4: 1254. 1951.

A device for cleaning the teat cups and Milk tubes of milking machines is described.

R. Whitaker

522. Milker pail cover with fluid trap chamber. F. G. HUDSON (assignor to International Harvester Co.). U. S. Patent 2,555,543. 1 claim. June 5, 1951. Official Gaz. U. S. Pat. Office, **647,** 1: 146. 1951.

A trap is built in the cover of the milk reservoir on a milking machine to prevent milk from entering the suction line. R. Whitaker

523. Milking truck. C. I. YOUNG. U. S. Patent 2,560,059. 2 claims. July 10, 1951. Official Gaz. U. S. Pat. Office, **648,** 2: 512. 1951.

A hand truck for use in a barn is described, consisting of a platform for carrying pails and cans of milk and a bracket for holding scales and record sheets for recording milk production from each cow. R. Whitaker

524. Mobile milk cooler. M. P. BANNISTER and J. F. PFEIFER. U. S. Patent 2,557,252. 1 claim. June 19, 1951. Official Gaz. U. S. Pat. Office, **647,** 3: 807. 1951.

A hand truck consisting of an insulated tank surrounded by cooling coils is described. A compressor beneath the tank supplies refrigeration. Milk direct from the cow is poured into the receiving inlet where it is directed against the walls of the tank by a baffle arrangement to facilitate cooling. R. Whitaker

525. Calf weaner. W. KUES. U. S. Patent 2,559,018. 4 claims. July 3, 1951. Official Gaz. U. S. Pat. Office, **648,** 1: 159. 1951.

A device for attaching to the head of a calf to discourage sucking by the calf is described.

R. Whitaker

526. Cow stanchion construction. M. REIFSNYDER. U. S. Patent 2,557,688. 13 claims. June 19, 1951. Official Gaz. U. S. Pat. Office, **647,** 3: 922. 1951.

Details are given covering construction of a row of stanchions of the chain type featuring a

barrier which when in the raised position prevents the cows from eating. R. Whitaker

ICE CREAM

C. D. DAHLE, SECTION EDITOR

527. Corn syrup solids in ice cream. R. L. LLOYD, American Maize Prod. Co., New York. Sou. Dairy Prod. J., **48,** 6: 116–117, 120, 122–123. Dec., 1950.

Corn syrup is made in a range of 24–64 dextrose equivalent (D. E.). In the higher part of the range the proportion of low molecular weight sugars is greater. Corn syrup solids is spray-dried corn syrup and is available in the range of 24–42 D. E. The drying process is similar to that for spray-dried milk.

Sugar imparts to ice cream palatability, smoothness of texture and food value. It increases specific gravity and viscosity and depresses the freezing point. Sucrose is the cheapest sweetener. Mixtures of sugars are preferable for smoothness. Dextrose-sucrose mixes have less viscosity than all-sucrose mixes. The freezing point is lowered more by the lower molecular weight sugars. Corn syrup solids lower the freezing point less than sucrose or dextrose. Because of their being less sweet than sucrose, they may be used in greater amounts, resulting in smoother texture and chewier body. The solids content of ice cream can be increased at less cost than with serum solids and without danger of a salty taste. Reductions of sucrose in all-sucrose mixes from 14–15% to 10–12% and the addition of 4–5% corn syrup solids is recommended. F. E. Bennett

528. Marking cartons. A successful method of preparing neat legible cartons for your retailers. B. BEANE, Borden Co., Pittsburgh, Pa. Sou. Dairy Prod. J., **49,** 2: 48, 117. Feb., 1951.

A method of marking 2.5- and 5-gal. ice cream cans with 3 2-in. diameter rollers, 3 in. in length is described. Two rollers into which are inserted 5/8-in. letter rubber stamps designating the flavor and code date are mounted, 1 on each side of the conveyor and operated on a weak spring. The 3rd roller is placed over the can on an adjustable shaft.

A model 240 multigraph printer using a metal slug which impregnates the word and ink into the paper marks the flavor on 4 sides of cartons of any size up to 0.5-gal. at the rate of 5,400 cartons/hr. F. W. Bennett

529. Sanitary jacket for ice cream cones and method of manufacture. C. C. HUITT and W. L. PARROTT. U. S. Patent 2,557,602. 7 claims. June 19, 1951. Official Gaz. U. S. Pat. Office, **647,** 3: 900. 1951.

An ice cream cone fitted with a conical heat sealed thin plastic sanitary jacket is described. R. Whitaker

530. Method and apparatus for preparing frozen confections. E. F. BURTON. U. S. Patent 2,557,-813. 15 claims. June 19, 1951. Official Gaz. U. S. Pat. Office, **647,** 3: 955. 1951.

A small ice cream freezer is described. R. Whitaker

531. Frozen confection and method of making same. C. H. MINSTER. U. S. Patent 2,558,453. 12 claims. June 26, 1951. Official Gaz. U. S. Pat. Office, **647,** 4: 1206. 1951.

A mix consisting of non-fat milk solids, suitably sweetened, stabilized and flavored is frozen and whipped to yield a smooth porous product resembling marshmallow. R. Whitaker

532. Confection-making apparatus. M. B. RASMUSSON (assignor to Vitafreze Equipment Inc.). U. S. Patent 2,559,463. 7 claims. July 3, 1951. Official Gaz. U. S. Pat. Office, **648,** 1: 277. 1951.

A conveyor system for automatically coating frozen stick confections is described. R. Whitaker

533. Ice cream dispenser. R. L. ARTHUR. U. S. Patent 2,559,840. 7 claims. July 10, 1951. Official Gaz. U. S. Pat. Office, **648,** 2: 456. 1951.

Ice cream in cylindrical bulk containers is removed from the container and placed in a refrigerated cylinder. A piston forces the ice cream down against the bottom of the cylinder in which a pivoted outlet valve is located. A measured quantity of ice cream is dispensed through the valve as desired. R. Whitaker

534. Ice cream dispenser. T. R. TESIERO. U. S. Patent 2,558,887. 7 claims. July 3, 1951. Official Gaz. U. S. Pat. Office, **648,** 1: 122. 1951.

Bulk ice cream is dispensed into packages, cups, cones, dishes, etc., in this device, consisting of a piston which pushes the ice cream through an outlet valve. R. Whitaker

535. Ice cream and frozen confection freezer and dispenser. A. J. TACCHELLA (assignor to Steady-Flow Freezer Co.). U. S. Patent 2,559,-032. 26 claims. July 3,1951. Official Gaz. U. S. Pat. Office, **648,** 1: 162. 1951.

A freezer with automatic controls for continuously making soft ice cream, frozen custard, etc., for immedate consumption is decribed. R. Whitaker

MILK AND CREAM

P. H. TRACY, SECTION EDITOR

536. Die u. v.-Bestrahlung der Milch (Irradiation of milk). English summary. K. SCHEER. Milchwissenschaft, **5,** 9: 292–296. Sept., 1950.

On the basis of published articles and personal experiences with irradiation of milk the author makes the following statements: Irradiation of milk by ultra-violet light at a wavelength of 250–300 mμ changes 7-dehydrocholesterin to vit. D_3. Proper irradiation will increase the vit. D_3 content of whole milk (1–2 γ/1.) 20–25 times. The vit. D_3 content of irradiated milk varies little throughout the year. The skimmilk portion of irradiated whole milk retains about 20% of vit. D_3, the vitamin being attached to albumin, globulin and casein.

Properly irradiated whole milk contains about 25 γ vit. D_3/1. (Steenback's method about 3 γ/1.). Infants require 7–10 γ/d. and grownups 3–4 γ/d. Irradiated milk is twice as effective as

non-irradiated milk containing an equal amount of added pure vit. D_3, and 15 times as effective as an equal amount of pure vit. D_2. I. Peters

537. Gewinnung und Abgabe homogenisierter, pasteurisierter und vitaminisierter Kindermilch im Rahmen der städtischen Milchversorgung (Production and delivery of homogenized, pasteurized and vitaminized milk for children in the city milk supply area). English summary. G. Schneider, H. Wittmann and E. Vogel. Milchwissenschaft, **5,** 9: 289–292. Sept.,1950.

This report deals with the successful establishment of a superior milk for infant feeding in the city of Nürnberg, Germany. The above milk, produced by T.B.-free cows, is standardized to 3.4% fat, fortified with vit. D_2 to 1000 I.U./1., preheated to 60° C., homogenized at 2400–2800 lb./in.^{2}, pasteurized at 85° C., cooled to 5° C. and bottled in brown glass bottles. Although this milk sells for more than the regular milk (25 pfg. as against 22 pfg./0.5 1.), the volume sold has risen from 1,709 units in Jan., 1950, to 6,114 units in July, 1950. I. Peters

538. Für Nürnberg's Kinder eine Milch aus tuberkulosefreien überwachten Rinderbeständen (Tuberculosis-free milk for Nürnberg's children). English summary. G. Beck. Milchwissenschaft, **5,** 9: 287–289. Sept., 1950.

A considerable improvement was brought about in the raw milk supply of the 35,000 producers in the Nürnberg milk shed through the combined efforts of the city milk plant (Bavarian Milk Supply Co.) and the regional veterinarians. The improvement was brought about by establishing price differentials for milk from T.B.-free herds as well as for clean milk low in bacterial count. The milk was further improved for infant feeding and for expectant mothers by raising the fat content from 2.5–3.0%, by homogenizing the pasteurized milk and by the addition of vit. D_2 to a level of 1000 I.U./1. I. Peters

539. Methods of preparing cream for frozen storage. H. C. Olson, Okla. A. & M. Coll., Stillwater. Sou. Dairy Prod. J., **49,** 2: 122, 124–126. Feb., 1951.

The following recommendations for preparing cream for frozen storage are made: Store fresh cream free from developed acidity, eliminating metal contamination. Pasteurize at 170° F. for 20–30 min. or 180° F. for 5 min. or longer. Standardize and condense to produce cream containing 40% fat and 20% serum solids.

F. W. Bennett

Also see abs. no 516.

MILK SECRETION

V. R. SMITH, SECTION EDITOR

540. Der quantitative Verlauf der Milchbildung (The quantitative course of milk secretion). English Summary. L. Eisenreich and U. Mennicke. Milchwissenschaft, **5,** 9: 310–313; 10: 362–364. Sept.; Oct., 1950.

By following the same experimental methods described earlier (*ibid,* 5: 140. 1950.), the authors were able to show that with cows in advanced stages of lactation and pregnancy milk secretion is a factor of time, primarily. However, when time intervals between milkings exceeded 20 hr., the succeeding milk yield was considerably lower than expected. The authors believe that milk production is a function of time but that the rate of production is governed by oestrogenic hormones within the animal. I. Peters

541. The use of an iron stain for the study of alveolar development in the mouse mammary gland. H. E. Rawlinson, Univ. of Alberta, Edmonton. Can. J. Research, **28,** 1: 1–4. Feb., 1950.

A method is described of using whole mounts of the mouse mammary gland with Gömöri's potassium ferrocyanide-HCl mixture for staining Fe. Because Fe accumulates in the epithelial cells in a granular form, most of the mammary gland tree of the mature nonlactating female mouse can be shown clearly. The staining reaction is quick and uniform, muscle can be clearly differentiated in microdissection and alveolar development can be estimated from the amount of Fe retention in the nonlactating gland. (Author's abs.)

O. R. Irvine

NUTRITIVE VALUE OF DAIRY PRODUCTS

R. JENNESS, SECTION EDITOR

542. Homogenisierte und D-vitaminisierte Kinder-Vorzugsmilch (Homogenized and vitamin D fortified milk preferred for infant feeding). English summary. A. Adam. Milchwissenschaft, **5,** 9: 283–286. Sept., 1950.

The use of homogenized, vitamin D-fortified milk (standardized to 1000 I.U./1. by the addition of vit. D_2 or D_3) by 270 infants over a period of 9 mo. proved the above milk to be superior to nonhomogenized, nonfortified milk. The soft curd formed by acid coagulation of the homogenized milk offered special advantages in the preparation of cultured milk for patients. The antirachitic effect of the fortified milk was excellent throughout the whole period, which included the fall and winter months. I. Peters

543. Milch in der Rachitis-Prophylaxe (Milk in prophylaxis of rickets). English summary. E. Auhagen and W. Grab. Milchwissenschaft, **5,** 9: 299–304. Sept. ,1950.

The authors recommend fortification of milk by the addition of pure vit. D_3, rather than by irradiation with ultra-violet light. The reasons are as follows: (a) The standard level (Germany) of 1000 I.U. of vit D_3/1. cannot be obtained by irradiating milk. (b) Variations in provitamin in milk result in variation in final concentration of vit. D_3 in irradiated milk. (c) There is no possibility of changing milk flavor, whereas irradiation to the extent of obtaining 400 I.U./1. (min. regulation by doctors) may result in off-flavors. (d) Toxic by-products, such as toxisterin$_3$ and suprasterine$_3$, which may form during irradiation process, are not present. (e) Changes in other vitamins and in proteins in milk are not possible. (f) Contamination of milk by passing it through

equipment that is difficult to clean is avoided. (g) Skimmilk may be fortified to the desired level. (h) The fortification is simple and easy to perform and more economical than irradiation.
I. Peters

544. Rolmilch oder pasteurisierte Milch für Säugling-und Kinderernährung (Raw or pasteurized milk for infant feeding). English summary. F. TRENDTEL. Milchwissenschaft, **5,** 9: 308–310. Sept., 1950

Use of raw *versus* variously heat-treated milk for infant feeding is discussed. The author feels that a closer collaboration is desirable among the child specialist, local health official, veterinarian and milk producer in order to obtain a higher quality milk which can be advocated for infant feeding.
I. Peters

545. Milchkonzentrate und Pulvermilchpräparate in der Sauglingsernährung und ihre Vitaminisierung mit Vitamin D (Milk concentrates and milk powder preparations fortified with vitamin D for infant feeding). English summary. E. MULLER. Milchwissenschaft, **5,** 9: 304–307; 10: 353–359. Sept., Oct., 1950.

The author compares infant feeding practises in Germany with those in U.S.A. He attributes the much higher infant mortality rate in Germany in children below 2 mo. of age (18–20% as against 1.8–2.0% in U.S.A.) to the extensive use of canned milks in U.S.A. Milk modified for infant feeding should form a soft curd, such as is the case in evaporated and sweetened condensed milk. He classifies all infant foods into 3 main groups: Group 1: "Complete" or "humanized milk" prepared in a manner to resemble mothers' milk. Group 2: Per each kg. of body weight feed 3.5 g. cows' milk protein, 3–1.5 g. fat and 12–14 g. non-milk carbohydrate (in addition to lactose in milk). Group 3: Acidified, low-fat cows' milk, such as 1.5% fat cultured buttermilk, natural or dried.
I. Peters

Also see abs. no. 516.

SANITATION AND CLEANSING

K. G. WECKEL, SECTION EDITOR

546. Dairy plant design and its relation to sanitation. G. W. PUTNAM and A. H. WAKEMAN, Creamery Pkg. Mfg. Co., Chicago. Sou. Dairy Prod. J., **49,** 1: 32, 104–105. Jan., 1951.

Consultation with federal, state and city health departments will help the designer in laying out a plant to meet modern sanitary requirements. Good housekeeping is not a luxury; the best housekeepers operate the most profitable and satisfactory plants. In designing an effort should be made to make the clean-up job efficient, convenient and pleasant. Consideration should be given to the effects of larger capacity equipment, convenience of arrangement, lighting, ventilation and safe, sanitary and well-drained floors. Layout should give a continous flow of products with a minimum of sanitary piping. Permanent sanitary piping installations, where approved, will eliminate much labor in clean-up operations. Adequate lighting may lessen damage to equipment during cleaning. A pitch of 0.25 in./ft. should be provided in dairy plant floors.
F. W. Bennett

547. A time and motion study in the cleaning of dairy plant equipment. W. E. SCHIFFERMILLER, Mich. State Coll., E. Lansing. Sou. Dairy Prod. J., **49,** 2: 25, 102–106. Feb., 1951.

A time and motion study on cleaning operations was made in 3 commercial fluid milk plants. The total routine cleaning time was distributed as follows: assembly and disassembly, 38–44%; rinse, 10–16%; washing exteriors, 4–8%; washing milk contact surfaces, 30–33%; preparing cleaning solutions, 5–8%; and getting and returning hose, 2–3%. Permanent glass sanitary pipe lines, improved designs of equipment for easy assembly and disassembly, pipe and fitting racks and small parts tables, power-driven solution-fed brushes for pipe and fittings, a hot water generator with nozzle-end shut-off valves, central cleaning solution tank, convenient general plant layout and definite efficient cleaning methods based upon a time and motion study are suggestions for most efficient cleaning operations.
F. W. Bennett

548. The relation of soil film buildup and low surface wetting properties to plastic and china surfaces. G. J. HUCKER, A. J. EMERY and E. WINKEL, N. Y. Agr. Expt. Sta., Geneva. J. Milk & Food Technol., **14:** 95–97. May–June, 1951.

Film buildup accumulates more rapidly on plastic than on china table service. Soiled plastic after several washes cleans much easier than cleaned plastic. Chinaware seems to retain the soil film more firmly than plastic.
H. H. Weiser

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