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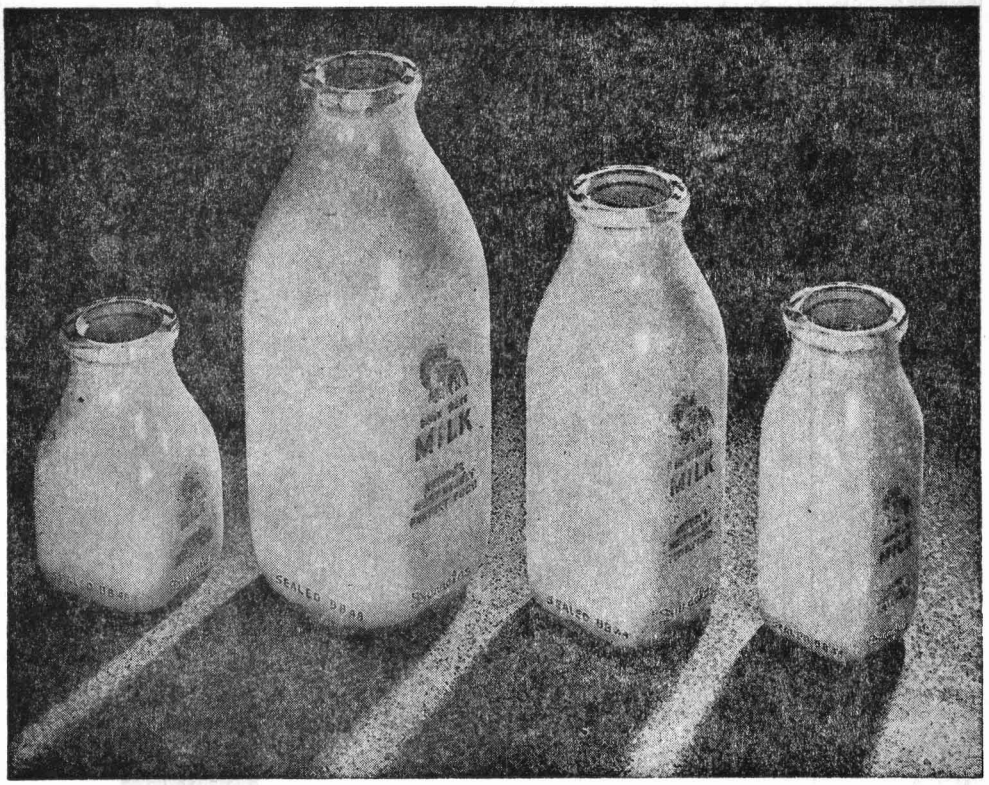

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THE USE OF UREA IN RATIONS FOR DAIRY COWS UNDER HAWAIIAN CONDITIONS*

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Research conducted at experiment stations in the United States (1, 7, 18) and in other countries (3, 17) has demonstrated that urea has considerable value as a substitute for high-protein concentrates in rations for lactating cows. The results have been so satisfactory, in fact, that the Association of American Feed Control Officials have approved its inclusion in feeds for ruminants, and it has been widely used.

Early work conducted at the Hawaii Agricultural Experiment Station (21) showed that urea could be utilized by dairy heifers for growth. Balance studies conducted by Harris, Work, and Henke (6) with four steers indicated that the nitrogen from urea was as digestible as that from soybean oil meal but that its biological value was lower. Trials were also initiated to determine its usefulness in rations for dairy cows under Hawaiian conditions. Because of the shortage of ordinary high-protein concentrates, this project assumed considerable importance during the war. In two double-reversal trials using a total of twenty cows, significantly lower milk production was obtained when urea replaced part of the soybean oil meal in the control ration (11). The results of four more recent trials with lactating cows are reported in this paper. The first two were designed to give more conclusive information on the value of this synthetic product. One additional trial measured the value of urea fed at two different levels. Another determined the effect of large amounts of molasses upon the utilization of urea.

GENERAL PLAN OF TRIALS

The trials reported herein were of the double change-over type as designed by Cochran and associates (2), and the cows were allotted to the groups in each trial in accordance with the plan outlined by them. The cows were all of the Holstein-Friesian breed, and each had one or more previous

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lactations. With five slightly deviating exceptions, the experimental periods began at least one month after calving and ended not later than the eighteenth week of pregnancy. The equalized-feeding method devised by Lucas (14) was followed with some modification due to the group feeding of roughage. When not being milked, the cows were in vegetation-free paddocks where chopped Napier grass was fed. Because the number of paddocks available was limited, the cows receiving a given concentrate mixture

TABLE 1

The rations

Composition figures are from actual analyses. (All figures in percentages)

Concentrate mixture	Trials I and II			Trial III		
	Basal	Basal plus urea	Control	2.5% urea	1.25% urea	Control
Cane molasses	25.0	25.0	25.0	25.0	25.0	25.0
Pineapple bran	30.0	27.5	30.0	36.5	36.5	36.5
Soybean oil meal	3.5	3.5	21.0	4.0	12.75	21.5
Corn or barley	39.5	39.5	22.0	.....	.....	.....
Barley	.....	.....	.....	30.0	22.5	15.0
Urea (Uramon)	.....	2.5	.....	2.5	1.25	.....
Salt	1.0	1.0	1.0	1.0	1.0	1.0
Bone char	1.0	1.0	1.0	1.0	1.0	1.0
Total crude protein equivalent	7.4	13.2	13.4	12.8	12.2	12.6
Total digestible nutrients	66.8	64.6	65.7	61.1	61.1	64.2
Ether extract	1.5	1.3	1.8	0.9	1.2	1.6

	Trial IV			
	No urea		Urea	
	Molasses	No molasses	Molasses	No molasses
Cane molasses	25.0	.....	25.0	.....
Pineapple bran	30.0	55.0	28.0	53.0
Soybean oil meal	21.0	22.0	7.0	8.0
Barley	22.0	21.0	36.0	35.0
Urea (Uramon)	.....	.....	2.0	2.0
Salt	1.0	1.0	1.0	1.0
Bone char	1.0	1.0	1.0	1.0
Total crude protein equivalent	13.5	13.7	14.0	12.7
Total digestible nutrients	71.6	74.4	70.8	72.7
Ether extract	3.6	3.1	3.1	2.8

were placed together; thus only the total roughage consumption of the cows receiving a given mixture could be controlled and measured.

The concentrate mixtures, shown in table 1, were typical of those fed by dairymen in the Territory in that they contained large amounts of cane molasses and pineapple bran. The latter feed, also known as dried pineapple pulp, is a by-product of the pineapple industry. It consists of the pulp remaining after juice is extracted from the fruit, and also of the outer shell and the core. The soybean oil meal contained about 42 per cent

total crude protein. The urea was a commercial product called "Uramon." In the initial trial, corn made up the cereal portion of the concentrate mixture. Since corn became unavailable later, barley was used throughout the remaining trials.

The Napier grass was fed in equal amounts to all lots in a given trial and in amounts such that practically all would be consumed. Concentrates were fed in quantities to supply considerably more total digestible nutrients than is recommended by Morrison (16) for good cows under usual conditions. Such heavy feeding is commonly practiced in the tropics and is undoubtedly justified because of the high price of milk in relation to feed costs (12) and because of the apparent need of cows in the tropics and subtropics for greater intakes of energy to maintain reasonable production. The total digestible protein in the control rations was calculated to supply somewhat more than Morrison recommends for good cows under usual conditions. The control and urea rations were computed to supply the same amounts of total crude protein equivalent. The term "protein equivalent" refers to the nitrogen in the ration multiplied by 6.25.

The chemical compositions of the feeds and the nutrient intake data presented are based upon analyses of the feeds in question. In calculating digestible nutrients from the proximate analyses, the coefficients for apparent digestibility given by Morrison (16) were used for soybean oil meal, corn, and barley. The coefficients determined by Work (20) were used for pineapple bran and Napier grass.

The milk yields have been converted to the same energy equivalent of 4 per cent fat-corrected milk (F.C.M.) by Gaines' formula (5). The data from the first week of each period were omitted to minimize carry-over effects from one period to another. Statistical analyses were carried out in accordance with the methods outlined by Cochran and associates (2) and Snedecor (19).

TRIALS I AND II. UREA PARTIALLY UTILIZED BY DAIRY COWS

The procedures in the first two trials were identical with two exceptions. In the first trial corn was used, and the three experimental periods were each three weeks in length. In the second trial barley replaced corn, and the length of each period was four weeks. The data have been combined and are presented together. The purpose of these trials was to determine if urea was utilized by cows receiving typical Hawaiian rations and if the production of cows receiving part of their nitrogen from urea would compare favorably with cows receiving all from plant sources.

Accordingly, at a given time two of the six cows in each trial received a basal ration which would supply inadequate protein. Two received the basal plus enough urea to raise the protein equivalent in the ration to the level required. Two received the control ration which consisted of the basal

plus soybean oil meal and which contained required amounts of protein. The results are presented in table 2.

During the last period in the first trial one cow receiving the urea ration went "off feed" to such an extent that she had to be removed from the experiment. For statistical analysis the missing data for this cow were estimated. For convenience in statistical interpretation of the milk yields, the mean which included the estimated value is presented in the table. An estimated value for this cow is also included in the average for concentrate consumption. These means and those calculated without the estimated values were not essentially different.

TABLE 2

Results of trials I and II. Average daily milk yields and feed and nutrient intakes of cows receiving the basal (low protein) ration, the basal plus urea, and the control ration (basal plus soybean oil meal)

Ration	Basal	Basal plus urea	Control
Cow periods, No.	12	12	12
4% F.C.M. yields, lbs.	20.0	21.6	22.8
Concentrate consumed, lbs.	16.5	16.3	16.5
Napier grass consumed, lbs.	56.0	59.0	59.0
T.D.N. required, lbs.*	15.3	15.7	16.2
T.D.N. consumed, lbs.	17.9	17.8	18.2
Digestible protein required, lbs.*	1.70	1.77	1.84
Total non-urea digestible protein consumed, lbs.	1.09	1.03	1.99
Total crude protein equivalent consumed, lbs.	1.83	2.81	2.89
Urea total crude protein equivalent consumed, lbs.	.....	1.08	.....
Urea consumed, lbs.	.....	0.41	.....
Least difference between 4% F.C.M. means required for probability of			
5%		0.95	
1%		1.40	

* According to Morrison (16) for good cows under usual conditions.

There was a highly significant difference (probability less than 1 per cent) in 4 per cent F.C.M. yields of the cows when receiving the basal ration and when receiving either of the other two rations. These data demonstrated that urea was utilized for milk production. The difference in F.C.M. yields of the cows when receiving the urea and the control rations was significant (probability less than 5 per cent), indicating that the nitrogen from urea was not utilized as efficiently as that from soybean oil meal.

TRIAL III. COMPARISON OF RATIONS CONTAINING TWO LEVELS OF UREA WITH A NON-UREA RATION

In the two previous trials the urea ration contained 2.5 per cent urea in the concentrate mixture. This amount of urea supplied over one-third of the total intake of total crude protein equivalent. It was thought that this

large amount might account for the lower production of the cows when receiving the urea ration than when receiving the control ration. A third trial with six cows was, therefore, planned to compare a ration containing 2.5 per cent urea in the concentrate mixture with one containing 1.25 per cent urea and with a control ration containing soybean oil meal as the only protein supplement. The experimental periods were four weeks in length. The results are presented in table 3.

TABLE 3

Results of trial III. Average daily milk yields and feed and nutrient intakes of cows receiving concentrate mixtures containing 2.5 per cent, 1.25 per cent and no urea (control ration)

Ration	2.5% urea	1.25% urea	Control
Cow periods, No.	6	6	6
4% F.C.M. yield, lbs.	26.5	27.1	28.9
Concentrate consumed, lbs.	19.1	18.9	19.0
Napier grass consumed, lbs.	63.0	63.0	62.0
T.D.N. required, lbs.	17.9	18.1	18.7
T.D.N. consumed, lbs.	19.8	19.7	20.2
Digestible protein required, lbs.	2.06	2.09	2.18
Total non-urea digestible protein consumed, lbs.	1.30	1.73	2.31
Total crude protein equivalent consumed, lbs.	3.47	3.34	3.41
Urea total crude protein equivalent consumed, lbs.	1.26	0.63	.....
Urea consumed, lbs.	0.48	0.24	.....
Least difference required between 4% F.C.M. means for probability of			
5%		0.73	
1%		1.21	

Although there was no significant difference between the milk yields of the cows when receiving the two urea rations, there was a highly significant difference between their yields when receiving either of the two urea levels on the one hand and no urea on the other. Consequently, it would appear that the feeding of large amounts in the preceding trials was not the main cause of the low production when receiving urea.

TRIAL IV. EFFECT OF LARGE AMOUNTS OF MOLASSES ON UREA UTILIZATION

Since cane molasses made up 25 per cent of the concentrate mixtures in the previous experiments, it was thought such high levels might be the cause of the comparatively low milk production of the cows when receiving the urea rations. Rupel and associates (18) have shown that levels of corn molasses up to 10 per cent do not cause detrimental effects. Other work at the same station (15) has demonstrated, however, that molasses as the only source of readily available carbohydrate in the ration did not enable satisfactory urea utilization. The next experiment at the Hawaii Station was, therefore, designed to determine if the large amounts of molasses fed

in Hawaii may, likewise, cause poor utilization even if other sources of carbohydrate are contained in the concentrate mixture.

Eight cows were divided into two groups: one to receive 25 per cent cane molasses in the concentrate mixture, and one to receive no molasses. Each of these two groups was, in turn, divided into two subgroups: one to receive 2 per cent urea in the concentrate mixture and the other none. This trial was, therefore, a factorial experiment. Each period was three weeks in length. The results are presented in table 4.

TABLE 4

Results of trial IV. Average daily milk yields and feed and nutrient intakes of cows receiving rations with and without urea and with and without molasses in a factorial experiment

Ration	No urea		Urea	
	Molasses	No molasses	Molasses	No molasses
Cow periods, No.	8	8	8	8
4% F.C.M. yield, lbs.	21.0	22.4	21.2	21.3
Concentrate consumed, lbs.	16.0	16.1	16.0	15.5
Napier grass consumed, lbs.	71.0	69.0	72.0	71.0
T.D.N. required, lbs.	14.8	15.3	14.9	14.9
T.D.N. consumed, lbs.	22.2	22.4	22.3	22.0
Digestible protein required, lbs.	1.69	1.76	1.69	1.70
Total non-urea digestible protein consumed, lbs.	2.07	2.07	1.42	1.22
Total crude protein equivalent consumed, lbs.	3.02	3.05	3.12	2.83
Urea total crude protein equivalent consumed, lbs.	.....	.....	0.84	0.82
Urea consumed, lbs.	.....	.....	0.32	0.31

When receiving the urea-no-molasses ration one cow refused part of her feed for over a week. She, nevertheless, continued to maintain her anticipated milk flow. This fact, along with the unaccountably low protein content of the concentrate mixture, accounts for the low average feed and protein intake of the cows when receiving this ration.

There were no significant differences in milk yields when the cows were receiving the different rations. Contrary to the expectation that the molasses might be detrimental to maximum urea utilization, the trend of the data was actually in the direction to indicate that molasses may promote the utilization of urea. It is interesting to note that the over-all trend was in favor of the non-urea rations when compared with the urea rations. This was in agreement with the previous trials.

DISCUSSION

The results obtained from these trials are in agreement with other data collected at this station. In two double-reversal trials previously mentioned, Henke and associates (11) obtained significantly higher milk production

from control rations than from rations containing urea. In addition, Henke (8) has alternately in three-week periods fed a urea and a non-urea ration to a group of cows. At first 2.5 per cent urea was fed, and later 1.5 per cent. These studies extended over intervals of fifty-seven and fifty-one weeks, respectively. Cows were occasionally added to or removed from the group. When receiving the control rations the milk production was 6.7 per cent higher than when receiving the 2.5 per cent urea, and 1.7 per cent higher than when receiving the 1.5 per cent urea ration.

In the four trials reported in this paper, the differences in favor of the non-urea rations containing adequate protein are greater on a percentage basis than those reported by most other workers. It is interesting to note, however, that Archibald (1) when using the short period, double-reversal type of experiment obtained greater differences than when using the continuous, long-period type. Such results might suggest that cows may need some time to become adapted to the utilization of urea, probably through changes in the flora of the rumen, and that long-period trials would, therefore, give a better measure of its value. Most workers using the continuous type of trial, including Archibald (1), Rupel and associates (18), and Hastings (7), report slight differences. On the other hand, Ehrenburg and associates (3), also using a continuous-type trial, reported a difference of similar magnitude on a percentage basis and in the same direction as those obtained in the double-reversal trials conducted at the Hawaii station. Owen and associates (17), using periods of varying lengths, but with most being thirty-six days or less, obtained no appreciable difference. It is not at all conclusive, therefore, that long-period trials necessarily should give more reliable results than short-period ones, at least enough so to compensate for the greater precision in measuring small differences possible with the reversal type. The continuous trial of the nature of that run at Wisconsin (18) is, however, undoubtedly valuable in determining the effects of feeding urea throughout a complete lactation.

In the four trials reported in this paper the mean 4 per cent F.C.M. yield of the cows when receiving 2.0 or 2.5 per cent urea in the concentrate mixtures can be compared with the mean yield when receiving soybean oil meal as the only high-protein supplement at required levels. The probability of the difference being due to chance alone is less than one-tenth of 1 per cent as determined from the variance ratio tables of Fisher and Yates (4).

Because some feed refusals occurred during these trials, there were some differences in nutrient intakes between the treatments being compared. For this reason, the 4 per cent F.C.M. yields were adjusted to the same total crude protein equivalent and total nutrient intakes by means of covariance. These adjustments made no appreciable changes in the results or in their interpretation. In these analyses an accurate regression coefficient for milk yield on nutrient intake could not, however, be determined,

for the practice of holding the cows receiving a given concentrate mixture in one paddock where the roughage was fed, did not permit a measure of individual roughage consumption. Only average figures for each paddock were used. Adjusting the milk yields for carry-over effects from one period to another according to the method outlined by Cochran and associates (2) also made no appreciable changes in the results.

The rations fed in these trials are, as previously mentioned, typical of those fed in the Territory. The fat contents of the concentrate mixtures, as can be seen by inspection of table 1, were in most cases below 2 per cent, considerably lower than the amounts found necessary for maximum production in the Cornell studies (13). Limited work by Henke and associates (9, 10) has indicated that an increase in 4 per cent F.C.M. production may result from adding fat to low-fat rations similar to those used as controls in these trials. The fat contents of the rations also decreased with the addition of urea. It is, therefore, possible that the effect of differences in fat contents was being measured as well as the effect of urea feeding. In urea studies with similar rations but with the fat adjusted close to 4 per cent by use of coconut oil meal, Henke and associates (11), nevertheless, obtained differences which were significant but of a lower magnitude on a percentage basis than those reported in this paper. To determine the part that fat has played in the results of these urea studies will require further investigation. There is always the possibility that conditions peculiar to Hawaii, such as the all-year-round feeding of low-protein soilage crops or the climate, may directly or indirectly be responsible for the relatively poor results obtained when feeding urea at this station.

Although, as mentioned previously, two cows had digestive disturbances when receiving the urea rations, there were feed refusals by cows receiving other feeds as well; so it cannot be definitely said that urea would cause more trouble in this respect than if it had not been fed.

In any event, at least under Hawaiian conditions, the dairyman apparently must expect some drop in milk production when urea on an equal total crude protein equivalent basis is substituted for part of the high-protein supplement in the ration. This fact, along with the relative costs of feeds, must, then, be taken into consideration when determining the economy of feeding urea. One could not afford to feed it unless cereal grains or other high-energy feeds are considerably cheaper than high-protein feeds or unless the latter are unavailable.

SUMMARY

Four double change-over urea-feeding trials involving twenty-six Holstein-Friesian cows in eighty-six cow-periods are described. The conclusions are as follows:

1. The dairy cow is able to utilize nitrogen from urea in the production of milk, but apparently not as efficiently as the nitrogen derived entirely from plant sources.

2. Less milk was obtained when urea was fed whether the daily intake was 0.48 pound or 0.24 pound. These amounts provided 36 per cent and 19 per cent, respectively, of the total crude protein equivalent intake.

3. The feeding of cane molasses in amounts to make up 25 per cent of the concentrate mixture had no detrimental effect upon the synthesis of protein from urea in the rumen.

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MOISTURE STUDIES IN DRY PRODUCTS OF MILK. I. KINETICS OF THE DEHYDRATION OF ALPHA LACTOSE HYDRATE IN TOLUENE

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In connection with our studies on the toluol distillation method of determining moisture in dry milk products (1), we had occasions to determine the moisture desorption-time curve of pure lactose monohydrate. It was found that the rate of moisture removal follows quite well the unimolecular kinetics expression,

$$k = \frac{1}{t} \ln \frac{a}{(a-x)},$$

where "k" is the rate constant; "a," the initial moisture content (5.0%); and "x" the amount of water removed in time "t." As lactose monohydrate is the most difficult constituent in dry milk products to dehydrate (3), it was thought interesting to determine the magnitude of the rate constant and to study some of the factors, such as particle size and rate of distillation, which affect its rate of dehydration, since the results of such a study might throw some light on the conditions necessary for quantitative removal of water from dry milk products by the toluol distillation method.

EXPERIMENTAL PROCEDURE

The apparatus, which consists of a 300-ml. Erlenmeyer flask, a Liebig condenser, a Precision electric heater (type RH), and a moisture trap graduated from 0 to 4 ml. in 0.1-ml. divisions, was the same as that used in this laboratory for the routine determination of moisture (1), except that a motor-driven and mercury-sealed stirrer was inserted to help agitate the sample. The toluene used was of C.P. grade with zero blank for moisture. The lactose monohydrate was Baker's C.P. powder, containing 5.0% water as determined by the Karl Fischer method (4).

Fifty grams of lactose monohydrate of the desired particle size as determined by U. S. Standard sieves were weighed into a 300-ml. Erlenmeyer flask and quickly covered with 100 ml. of toluene. After attaching the flask with sample to the apparatus, enough toluene was added through the top of the condenser to fill the moisture trap. Stirring was then started at a rate of about 3,400 r.p.m. The rate of condensation was adjusted to give two to three drops per second. After the first appearance of moisture in the trap, the moisture collected was read at several time intervals. This was multiplied by two to convert to per cent of moisture desorbed. Droplets which failed to settle out were dislodged by means of a stiff long wire prior

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to each reading. The logarithm of the difference between the initial moisture, which is 5.0 per cent, and that determined at each time interval was then plotted against the corresponding time in minutes. The slope of the best straight line through the points, when multiplied by the conversion factor 2.303 gives the rate constant per minute.

RESULTS AND DISCUSSION

In figure 1 are shown some of the dehydration curves of different particle sizes of lactose monohydrate. As might be expected from the general influence of surface area on the rate of evaporation, the effect of decrease in particle size is to increase the rate of dehydration. Furthermore, by assuming the particles to be spherical and the rate constant to be a function of

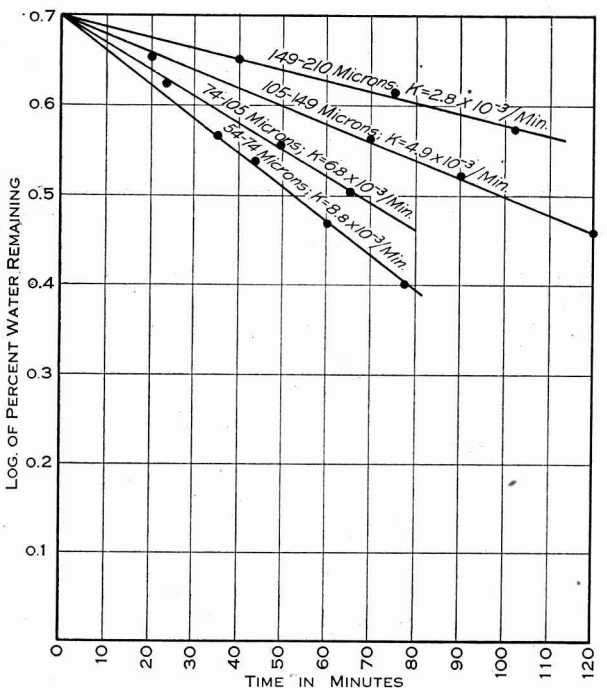

FIG. 1. Rates of dehydration of lactose monohydrate of different particle sizes.

the total surface area, which per unit weight, is the product of the number of particles and the surface area per particle, it can be shown mathematically that the rate constant "k" varies inversely with the first power of the average diameter "d." The quantity "d" is calculated by taking the arithmetical mean of the lowest and the highest sieve sizes of each series. The

experimental data, when plotted as in figure 2 gives rise to a straight line, expressed by the equation, $k = \frac{0.565}{d}$, in excellent agreement with the above calculation.

The magnitude of the rate constants is also interesting. Since the reaction is unimolecular, the rate constant has the dimension per unit time. Therefore at a rate constant of 2.8×10^{-3}/min., or 0.168/hour, as in the case of particle sizes 149–210 microns, 16.8 per cent of the total amount of lactose hydrate present at any given time is dehydrated per hour. For particle sizes 54–74 microns, 53 per cent of the total lactose monohydrate is dehydrated per hour. It is apparent that an adequate toluol distillation time for the determination of moisture in dry products of milk is dependent

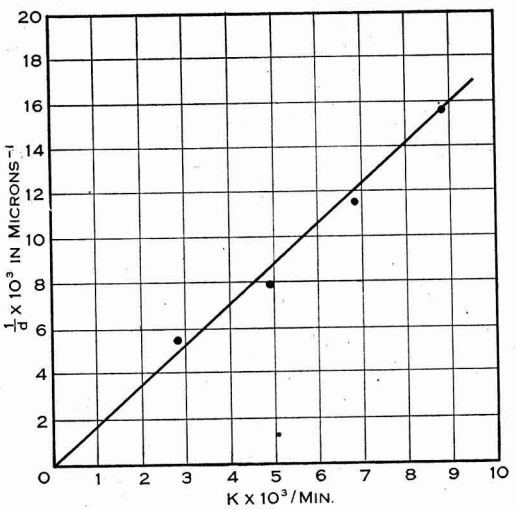

FIG. 2. Relation of particle diameter to the rate constant for the dehydration of lactose monohydrate.

upon the particle size and the amount of lactose hydrate, and thus cannot be arbitrarily established. For this reason the method of the American Dry Milk Institute (1) provides for one hour distillation and additional fifteen-minute distillation periods until constant moisture values over a fifteen-minute period are obtained. The slow rate of dehydration necessitates three hours distillation time for the quantitative determination of moisture in most dry whey solids, and may also explain why moisture results for some dry milk products obtained by the toluol distillation method, using one hour distillation are lower than those obtained by the Karl Fischer method as found in this Laboratory and by Heinemann (2) in his recent paper.

The effect of distillation rate on the rate of dehydration was next studied with the aim of finding whether there exists a rate of distillation above which

the velocity of dehydration is constant. Results of such studies, using lactose monohydrate of particle sizes under 149 microns in diameter, are shown in figure 3. The last three points of the curve taken in the order of increasing distillation rate represent the maximum temperature of the hot plate (about 400° C.) in combination with stirring rates of 3,400, 4,400 and 5,800 r.p.m., respectively. It can be seen that above a distillation rate of 6 ml. per minute, or approximately 2 drops per second, the velocity of dehydration is independent of the rate of distillation. This result indicates that the rate of 3 drops per second as specified by the American Dry Milk Institute (1) for the determination of moisture in dry milk products is adequate.

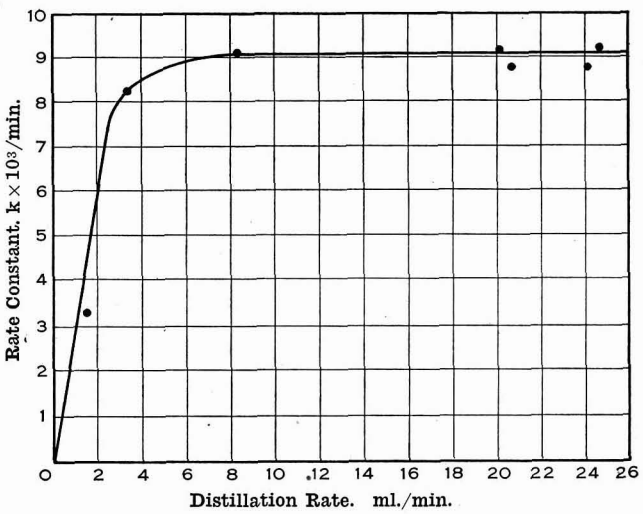

FIG. 3. Effect of distillation rate on the velocity of dehydration of lactose monohydrate.

The results of this investigation may possibly be applied to the determination of lactose hydrate in some dry milk products such as dry whey, since, in these products, surface moisture and moisture bound by proteins are easily desorbed (3), so that a logarithmic plot of the total moisture present at any time "*t*" against the time should theoretically result in a curve composed of two distinct portions. The first steeper portion represents the dehydration of lactose hydrate and the other loosely bound forms of moisture, while the second portion which should be linear, represents the dehydration of lactose monohydrate alone. Therefore, extrapolation of the second portion of the curve to zero time will give the initial amount of water bound as the hydrate of lactose. This value when divided by 0.050 should yield the per cent of lactose monohydrate in the sample. This possibility is being investigated.

SUMMARY

The dehydration of lactose monohydrate in boiling toluene is found to be a first order reaction, the rate constant of which varies with particle size in accordance with the equation $kd = 0.565$, where "k" is the rate constant in min.$^{-1}$ and "d" is the average diameter of the particle in microns. The effect of the magnitude of the rate constant on the length of distillation period necessary for the quantitative determination of moisture for dry milk products by the toluol distillation method is discussed. The rate of distillation has some influence on the velocity of dehydration of lactose monohydrate, but above a distillation rate of 6 ml. per minute any further increase in the distillation rate has no effect on the rate of dehydration.

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COLORIMETRIC DETERMINATION OF AMMONIA IN MILK AND DRY PRODUCTS OF MILK

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In the course of our study of proteolysis in dry products of milk, we needed a rapid and sensitive method for determining ammonia. The small amount of ammonia present in fresh fluid milk and in dry milk products makes accurate determination a matter of some difficulty. The various methods have been reviewed by Perkins (3), many of which leave much to be desired from the standpoints of accuracy and convenience. Preliminary experiments in this Laboratory showed that the permutit adsorption method of Folin and Bell (2) does not give satisfactory recovery of added ammonia either in milk or in deproteinized milk filtrate. This was also found to be true by Perkins (3). Nessler's reagent is too sensitive to volatile constituents from rubber when using steam distillation. However, the reactions of ammonia with alkaline phenol and sodium hypochlorite solution have been used by Van Slyke and Hiller (6) and by Borsook (1) with some success. Recently, Russell (5) has improved the sensitivity and reproducibility of the method with the result that as little as 0.5 microgram of ammonia may be conveniently determined. This improved method forms the basis of the following procedure in which milk is deproteinized with sodium tungstate in sulfuric acid solution and the filtrate steam distilled with magnesium hydroxide to liberate the ammonia.

Reagents: 1. Sodium Tungstate (10%). 100 grams of sodium tungstate are dissolved in 900 ml. of distilled water.

2. Sulfuric Acid ($\frac{1}{2}$ N). To a liter volumetric flask containing about 300 ml. of water, 13.6 ml. of concentrated sulfuric acid low in ammonia are added and the volume diluted to 1 liter.

3. Sulfuric Acid (0.01 N). One part by volume of 0.5 N H_2SO_4 is diluted with 49 parts by volume of water.

4. Magnesium Hydroxide (8%). Eighty grams of magnesium oxide (C.P.) are stirred into 600 ml. of water. The content is boiled for 5 minutes. After cooling the solution is made up to 1 liter.

5. Alkaline Phenol Reagent. Twenty-five grams of crystalline phenol are mixed with a small amount of water. To this 54 ml. of 5 N sodium hydroxide are added with stirring. After making up to 100 ml., the solution is preserved in a brown bottle at refrigeration temperature.

6. Hypochlorite Solution. Twenty-five grams of calcium hypochlorite (U.S.P.) are dissolved in 300 ml. of hot water. To this, 135 ml. of potassium carbonate solution (20%) are added. The mixture is heated to 90° C., cooled,

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and made up to 500 ml. A small volume of solution is filtered and the filtrate examined for Ca++ by adding more potassium carbonate solution. If a positive test is obtained more carbonate is added until no Ca++ is present in the filtrate. The mixture is then filtered and stored in small brown bottles in the refrigerator. The solution must be water clear and contains from 1.3 to .4 grams of free chlorine per 100 ml. as determined by iodometric titration. The strength of this reagent is checked occasionally.

7. Managanese Sulfate (0.003 M).

Procedure: Ten grams of the dry milk product are added to 93 ml. of distilled water and shaken vigorously to bring the solids into uniform colloidal suspension. The final volume is close to 100 ml., since the partial specific volume of all dry products of milk is about 0.7 ml. Ten milliliters of this reconstituted milk are taken for analysis. The proteins are precipitated by means of 10 ml. of 10 per cent sodium tungstate and 20 ml. of $\frac{1}{2}$ N

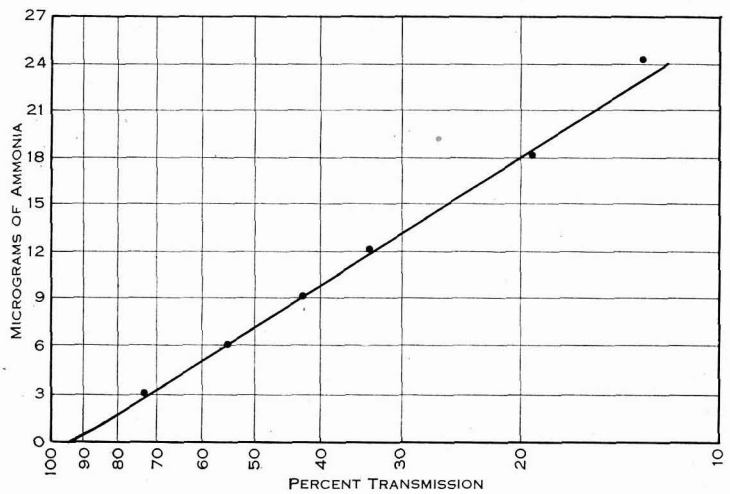

FIG. 1. Calibration curve.

sulfuric acid, added in the order named. A twenty-five milliliter portion of the filtrate representing 0.625 grams of the sample, or a smaller aliquot if the sample is high in ammonia, is steam distilled in a Pregl apparatus, using 10 ml. of 8 per cent magnesium hydroxide to liberate the ammonia, 5 ml. of 0.01 N sulfuric acid as the absorbing agent, and a distilling time of seven minutes.

The distillate is transferred to a 50 ml. volumetric flask and diluted to the mark. An aliquot of 1.0 to 5.0 ml. containing from 2 to 20 micrograms of ammonia is pipetted into a 15-ml. centrifuge tube kept in an ice bath. A drop of manganese salt solution is added to the sample, followed by 1 ml. of phenol reagent and 1 ml. of hypochlorite solution. After gently mixing the content by means of a stirring rod, the tube is immediately placed in a

boiling water bath for 5 minutes. The sample is removed from the bath, stirred vigorously, and allowed to cool. When cooled, the volume is made up to 10 ml. The blue color developed is read in a Pfaltz and Bauer photoelectric colorimeter, using matched test tubes and a filter having maximum absorption at 6100 Å. The quantity of ammonia corresponding to the observed transmission is estimated from a curve (Fig. 1), previously calibrated with varying concentrations of ammonium sulfate. A blank containing equivalent amounts of all reagents is also run. The total amount of ammonia calculated in milligrams per 100 grams of sample is obtained by means of the following expression:

$$\frac{100V_0'Y}{WV_x} = \text{mg. ammonia/100 grams}$$

V_0' = total volume of distillate
V_x = volume of distillate taken for color development
Y = (mg. ammonia in sample) – (mg. ammonia in blank)
W = Grams of sample by aliquot of filtrate taken for distillation.

RESULTS AND DISCUSSION

Steam distillation has been successfully employed in many micro-Kjeldahl procedures for isolating ammonia, and has the advantage of a short distillation time. Since in this procedure a weaker base, magnesium hydroxide, is used instead of the usual sodium or potassium hydroxide, it was necessary to determine the time of distillation needed for quantitative removal of ammonia from the deproteinized filtrate. Results summarized in table 1 for a sample of dry whey solids using a 10 ml. aliquot, and similar data on other dry products of milk not shown here, indicate 7 minutes distillation to be sufficient. It may be pointed out that the dry whey sample used above has the highest ammonia content yet encountered and that the 10 ml. aliquot of the deproteinized filtrate contains almost 500 micrograms of ammonia, an amount seldom found in even a 25 ml. aliquot from most dry products of milk.

Several recovery experiments were also run by adding known quantities of ammonia to a sample of reconstituted dry buttermilk. Results presented in table 2 show that over 95% of the added ammonia was recovered in each case.

TABLE 1

Effect of varying the time of distillation on the amount of ammonia liberated

Distillation time (Mins.)	NH_3 liberated (Mgs./100 gms.)
3	149
5	197
7	198
8	198

TABLE 2

Recovery of ammonia added to reconstituted dry buttermilk

NH_3 added (Micrograms/ml.)	NH_3 found (Micrograms/ml.)	Recovery of added NH_3 (Micrograms/ml.)
0	4.5	0
5.0	9.7	5.2
10.0	14.4	9.9
15.0	19.9	15.4
30.0	33.5	29.0

Several dry products of milk were examined for ammonia by the above procedure. Of the large number of each analyzed, dry whole milk contained from 3 to 7 mgs./100 gms. and nonfat dry milk solids from 4 to 9 mgs./100 gms. The ammonia content of dry buttermilk solids ranged from 3 to 82 mgs./100 gms. with most of the samples containing less than 15 mgs./100 gms. Dry whey solids, in general, shows high ammonia content, the range being from 20 to 198 mgs./100 gms. Of the several samples of commercial fluid whole milk examined, the ammonia varies from 0.30 to 1.30 mgs./100 ml., values which are of the same order of magnitude as those reported by previous workers (3, 4).

In order to gain some ideas about the reproducibility of the procedure, ten separate determinations were run on the same sample of pasteurized milk. The data are presented in table 3. A statistical analysis of the results yielded an average deviation from the mean of 0.016 mg./100 ml. and a standard deviation of 0.026 mg./100 ml., indicating satisfactory reproducibility.

TABLE 3

Reproducibility of the method

Trial	Mg. NH_3/100 ml. milk	Deviation (d) from average
1	0.66	0.04
2	0.70	0.00
3	0.70	0.00
4	0.75	0.05
5	0.70	0.00
6	0.70	0.00
7	0.73	0.03
8	0.66	0.00
9	0.70	0.00
10	0.70	0.00
Average	0.70	0.016

Standard Deviation = 0.026 mg./100ml.

SUMMARY

A colorimetric method is described for the determination of ammonia in fluid milk and in dry products of milk based upon the reaction of ammonia with alkaline phenol and sodium hypochlorite. A statistical analysis of

data for one sample of fluid milk shows an average deviation from the mean of 0.016 mg./100 ml. and a standard deviation of 0.026 mg./100 ml., indicating satisfactory reproducibility.

The ammonia content of some dry milk products and fluid milk varies considerably with dry whey solids having the highest ammonia content.

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THE RELATIONSHIP OF THE GROWTH OF ALL BACTERIA AND COLIFORM BACTERIA IN PASTEURIZED MILK HELD AT REFRIGERATION TEMPERATURES*

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A recent study on the keeping quality of pasteurized milk has been published by Dahlberg (1, 2) in which total bacterial counts and coliform counts were made on 108 different lots of pasteurized milk collected in July–August, 1945, and October, 1944. The pasteurized milk was obtained from six milk companies in the New York Metropolitan area that processed about 800,000 quarts daily. The milk was held for four days at three different refrigeration temperatures. The bacterial counts were made on standard agar and desoxycholate agar using the procedure outlined in the 1941 edition of *Standard Methods for the Examination of Dairy Products,* published by the American Public Health Association. The data gave an opportunity to compare the rate of growth of all bacteria and coliform bacteria as they occur in a pasteurized milk supply of excellent quality.

It has been customary to teach that the lactic acid bacteria grow more rapidly than the coliform bacteria at cold temperatures. However, in raw milk Sherman (3) found that "our studies at various temperatures have shown that the colon count in milk generally increases more rapidly than the total count during the presouring stage, which includes the whole period of interest in the fluid milk industry." The temperatures employed were 50°, 59°, 70° and 86° F. Morris (4) found that coliform cultures grew more rapidly in pasteurized than in raw milk due to the bactericidal substance naturally occurring in raw milk.

RESULTS

The data, table 1, include all milks for total bacterial counts and each count represents 54 different bottles of milk. The coliform counts were averaged for those milks which were coliform positive so the initial counts in October include only about 6 per cent of the samples and in July–August about 35 per cent. However, after storage at 55–60° F. for 4 days from 73 to 88 per cent of the milks were coliform positive. If all milks had been included irrespective of the presence of coliform bacteria, all coliform counts would have been lower.

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* *Acknowledgments:* Several of the leading milk companies in the New York–New Jersey Metropolitan area assisted in this study by providing the required samples of milk, by providing necessary laboratory facilities, and through research grants to Cornell University. The author is indebted to Eleanor Brusten, Marion Goldberg, Eva Schiffer, and Maria Gonzalez for making the bacteriological analyses.

TABLE 1

The total bacterial count and the coliform count on 108 lots of pasteurized milk in 1620 quart bottles collected at 6 New York Metropolitan plants on 3 consecutive weeks in July–August and in October, and stored at 3 different temperatures

Days stored	Bacterial counts *					
	35–40° F.		45–50° F.		55–60° F.	
	Total	Coliform	Total	Coliform	Total	Coliform
	October					
0	14,000	2	13,000	3	13,000	2
1	12,000	1	12,000	3	11,000	3
2	12,000	2	13,000	19	133,000	3,000
3	8,000	2	16,000	397	2,100,000	373,000
4	9,000	4	180,000	10,000	34,900,000	1,870,000
	July–August					
0	13,000	3	15,000	2	19,000	5
1	18,000	5	19,000	10	77,000	343
2	14,000	11	27,000	57	444,000	66,000
3	13,000	9	84,000	4,000	5,400,000	565,000
4	11,000	123	335,000	295,000	11,900,000	6,000,000

* The total bacterial count was the standard plate count and the coliform count was determined in desoxycholate agar according to the 1941 edition of Standard Methods for the Examination of Dairy Products published by the American Public Health Association.

It will be noted, table 1, that the total bacterial counts tended to decrease during storage for 4 days at 35–40° F., but coliform counts at 35–40° F. remained constant in cool weather and actually increased slightly in warm

TABLE 2

The growth of all bacteria and coliform bacteria in pasteurized milk expressed as percentages of the total count that were coliform bacteria

Days stored	Percentages of total counts that were coliform		
	35–40° F.	45–50° F.	55–60° F.
	October		
0	0.01	0.02	0.01
1	0.01	0.02	0.02
2	0.01	0.14	2.25
3	0.02	2.48	17.76
4	0.03	5.55	5.36
	July–August		
0	0.02	0.01	0.02
1	0.03	0.05	0.44
2	0.08	0.21	14.86
3	0.07	4.76	10.27
4	1.12	88.05	50.42

weather. There was no increase in the percentages of all bacteria which were coliform types in cool weather but the percentages increased from 0.02 to 1.12 in warm weather, as shown in table 2.

At 45–50° F. the coliform bacteria increased more rapidly than the total count. After 4 days the coliform bacteria represented 5.55 per cent of the total count in October and 88 per cent in July and August. The much greater relative growth of coliform bacteria at 45–50° F. is illustrated by figure 1. The actual numbers of coliform bacteria were less than a half million per ml. after 4 days.

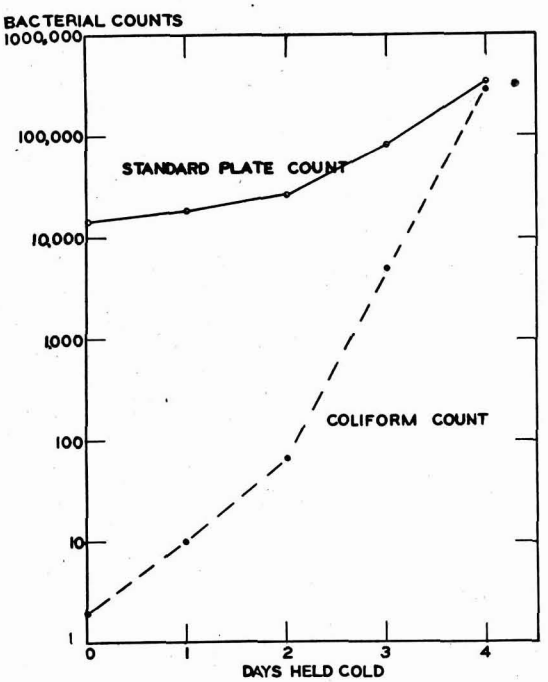

FIG. 1. The standard plate count for total bacteria and the coliform bacteria count on desoxycholate agar on 54 lots of milk collected in July–August and stored at 45–50° F.

The growth of coliform and all bacteria was rapid at 55–60° F. and even the number of coliform bacteria exceeded a million after 4 days. At this temperature bacteria other than coliform grew rapidly also. The coliform bacteria finally represented 5.36 per cent of the total bacteria in October and 50.42 per cent in July and August.

At all temperatures of storage the growth of coliform bacteria was more rapid in the warm summer months than it was during the cool month of October. This was true of total numbers of coliform bacteria and of the percentage of the total bacteria which were coliform.

DISCUSSION

As this study progressed it was observed that most of the bottles of milk which had been stored at 55–60° F. for 4 or 7 days showed gas in the cream layer. Sometimes in coagulated milk the gas was also evident in the skim milk. This observation substantiated the high coliform count obtained in milk held at so warm a temperature. It has been known for years that pasteurized milk often does not give a good lactic acid fermentation. The rapid growth of coliform bacteria, especially at 45–50° F., was a contributing factor toward abnormal fermentation as other bacteria grew so slowly at this temperature.

There are two possible explanations for the more rapid growth of coliform bacteria in pasteurized milk. It may be that under exactly comparable conditions the coliform bacteria multiply more rapidly than all other types of bacteria present in milk. In pasteurized milk, however, the coliform bacteria present are recontaminants which have not been subjected to the heat treatment and which are actively growing bacteria, especially in warm weather. All other bacteria in pasteurized milk are dormant as they have been subjected to refrigeration in the raw milk and growing strains have been destroyed by pasteurization.

The growth of coliform bacteria at all refrigeration temperatures including slight growth at 35–40° F. emphasizes the importance of making coliform tests only on freshly pasteurized milk if such tests are to be interpreted as indicating recontamination after pasteurization. Furthermore, such tests should be made promptly on well-iced samples. These facts are recognized but not always adhered to.

SUMMARY

The coliform bacteria in pasteurized market milk held at refrigeration temperatures increased more rapidly in numbers than the total count. In the freshly pasteurized milk less than 0.02 per cent of the total bacteria were coliform types. At 35–40° F. the percentage of the total bacteria which were coliform did not increase in October, but in July and August increased to 1.12 per cent in 4 days. After storage for 4 days at 45–50° F. and at 55–60° F. the coliform bacteria constituted about 5 per cent of the total count in October. During July and August the coliform count became 88 per cent of the total count after 4 days' storage at 45–50° F. and 50 per cent at 55–60° F. The coliform bacteria grew more rapidly in warm weather than in cool weather.

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THE EFFECT OF PREGNANCY ON THE BODY WEIGHT OF DAIRY COWS*

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Growth curves have been developed for all of the principal dairy breeds of cattle. Growth is usually measured by the height at withers and the weight of the animals. While the height at withers is not affected greatly by pregnancy, the weight of the animal may increase considerably. After a heifer is bred the weight curve takes an upward turn from the non-pregnant growth curve and is no longer a measure of growth alone but includes also the weight of the fetus and accompanying material including the fetal membrane and the amniotic fluid. After the heifer freshens, the weight curve declines to a new basic level and for a short period may be slightly below this level, especially if the cow is a heavy producer. The cycle is repeated for each pregnancy with the weight curve attaining a higher basic level until the cow is mature and this level becomes static.

Several studies (1, 2, 3, 4) have been made to determine how much of the gain during gestation is due to growth and how much can be ascribed to pregnancy, and to correct the growth curve which is turned awry when the animal becomes pregnant. The results of most of these studies, however, were based on small numbers of cows with consecutive pregnancies. All of these studies showed increases in body weights, month to month, from service to parturition, with the weight curves showing rather sharp upturns toward the middle and latter parts of the gestation period and at parturition the curves took sudden drops.

Bartlett (1) studied the increase due to pregnancy of grade dairy Shorthorn cattle over a five-year period, using 16 animals as the maximum number. He found that there was a progressive gain in weight, month by month, with a sharp upturn toward the end. The loss of weight at parturition varied from 154 to 190 pounds with an average of 170 pounds or 12.2 per cent loss of weight.

Moseley, Stuart, and Graves (3) took weights on 53 Holstein cows at an average age of 5 years and 3 months. There was an increase of weight from service to parturition of 285 pounds with the weight curve showing a sharp upturn during the middle and latter part of the gestation period. Ninety-four per cent of the gain occurred during the last five months of the period. The average weight of the calves was 97 pounds and the weight of the placenta and amniotic fluid was 58 lbs.

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Morgan and Davis (2) made the most extensive study so far reported. This involved all breeds and 656 separate gestations. They found that there was a progressive increase in weight from conception to parturition. During the first gestation these gains were approximately equal each month throughout this entire gestation period whereas during succeeding gestations the monthly gains proceeded rather evenly for the first four months only, and then the curve of gain started to bend upward and continued thus until parturition. The gain in weight during the first pregnancy was greater than in succeeding pregnancies for each breed and was approximately $\frac{1}{3}$ the initial weight of the animal. For succeeding pregnancies the gain was only about $\frac{1}{7}$ the weight of the cow. The loss due to calving was about $\frac{1}{8}$ the initial weight of the cow, irrespective of age.

Willard (4) has given a table for Holstein heifers which includes additions to be added to the normal weight in calculating weight for pregnant animals. He took the differences between the weight of animals before and after calving as representing the gains during pregnancy.

EXPERIMENTAL

At the West Virginia Agricultural Experiment Station a study was made of the actual body weights (taken monthly) of 56 normal Ayrshire females during their first three pregnancies. The average age at first calving was 32 months; at the time of second calving, 46 months; and at the third calving, 59 months.

Table 1 shows the mean weights by months for the three different pregnancies. This table shows that there was a progressive gain in weight from pregnancy to parturition with a sharp decline at pregnancy.

The animals increased about 34.5 per cent of their initial weight during the first pregnancy; 20.5 per cent during the second pregnancy; and 19.0 per cent during the third pregnancy.

The average monthly gains and total gain for the period during each of

TABLE 1

Average body weights of 56 Ayrshire females during pregnancy

Month of pregnancy	First pregnancy	Second pregnancy	Third pregnancy
	wt. lbs.	*wt. lbs.*	*wt. lbs.*
Service date	810	1003	1068
1	840	1003	1075
2	861	1014	1079
3	883	1028	1085
4	912	1042	1096
5	950	1062	1122
6	987	1096	1156
7	1024	1138	1197
8	1057	1157	1224
9	1091	1206	1270
Month after calving	991	1078	1128

the three gestation periods are given in table 2. During the first gestation period the animals increased in weight at a faster and more uniform rate than in the later periods. Approximately 50 per cent of the gain was made during the first five months of the first pregnancy while in the second and third pregnancies gains of only 29 and 27 per cent, respectively, were made. The total gain during the first pregnancy was 281 pounds while the gains for the second and third pregnancies were 203 and 202 pounds, respectively. This difference in increase was probably due to the fact that the younger animals were growing faster during this period and also the fact that they were not lactating.

The average gain during the eighth month of pregnancy was less than in the months immediately preceding and following. In the older cows this

TABLE 2.

Average monthly gains in body weight of 56 pregnant Ayrshires

Month of pregnancy	First pregnancy gain		Second pregnancy gain		Third pregnancy gain	
	For month	For period	For month	For period	For month	For period
	lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*	*lbs.*
1	30	30	0	0	7	7
2	21	51	11	11	4	11
3	22	73	14	25	6	17
4	29	102	14	39	11	28
5	38	140	20	59	26	54
6	37	177	34	93	34	88
7	37	214	42	135	41	129
8	33	247	19	154	27	156
9	34	281	49	203	46	202

might be explained through the management procedure of giving each cow a two-month dry period. Often the need arises to reduce feed consumption during the early part of the dry period. The difference in the first-calf heifers was probably not significant.

In order to determine the gain in weight due to pregnancy, a growth curve is necessary to indicate the growth of open animals in comparison with the curve of the pregnant animals. As open animals were not available, a curve was drawn using the average weights of the animals at one month before they were bred as the normal weight of the animals at that time. The weights of the pregnant animals were then plotted and the difference noted as shown in table 3 and in figure 1. When the gains, attributed to pregnancy only, were considered it was found that as heifers they actually did not make as much total gain as a result of pregnancy as they did when older; *i.e.*, only 142 pounds as compared to 148 pounds during the second pregnancy and 162 pounds during the third pregnancy. The weights of the calves for the first pregnancy averaged 70.4 pounds; for the second, 74.6 pounds; and for the third, 74.8 pounds.

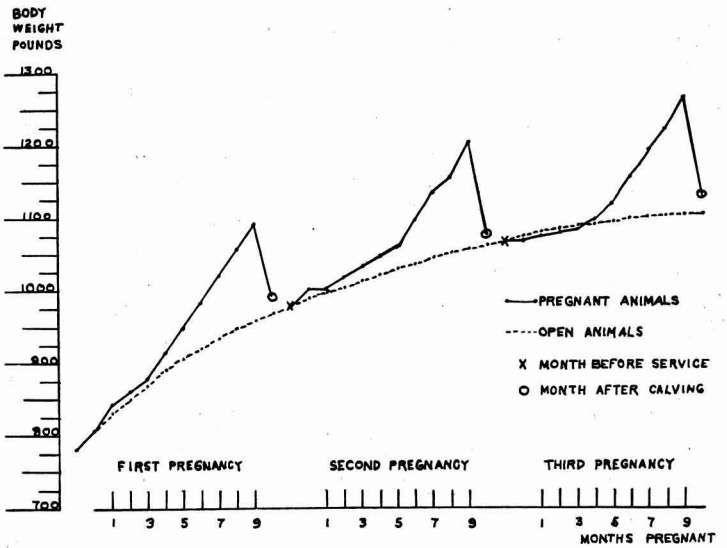

FIG. 1. Effect of pregnancy on body weight of Ayrshires.

DISCUSSION

The analysis of the data would indicate that approximately 50 per cent of the gain in weight of first-calf heifers can be attributed to permanent growth and the other 50 per cent to products of conception. The average permanent gain in weight for the first-calf heifers was about 16 pounds per month, the gain being faster during the early months of gestation. The slower growth of the animal during the latter months of pregnancy can be attributed to the larger nutritive demands of the fetus.

TABLE 3

Increase of body weights due to pregnancy

Month of pregnancy	First pregnancy			Second pregnancy			Third pregnancy		
	Gain per mo.	Total gain	Per cent inc.	Gain per mo.	Total gain	Per cent inc.	Gain per mo.	Total gain	Per cent inc.
	lbs.	*lbs.*		*lbs.*	*lbs.*		*lbs.*	*lbs.*	
1	10	10	1.2	4	4	0.4	−3	−3	−0.3
2	1	11	1.3	2	6	0.7	−1	−4	−0.4
3	3	14	1.7	6	12	1.3	1	−3	−0.3
4	11	25	3.1	6	18	1.8	7	4	0.4
5	21	46	5.7	12	30	3.0	22	26	2.7
6	21	67	8.3	27	57	5.7	30	56	5.3
7	23	90	11.1	35	92	9.3	37	93	8.8
8	20	110	13.5	13	105	10.6	25	118	11.1
9	32	142	17.5	43	148	14.9	44	162	15.7

The amount of weight which was ascribed to permanent growth during the second pregnancy was only about 27 per cent and for the third pregnancy, 20 per cent of the total gain in weight. In both the second and third this permanent gain in weight was evenly distributed throughout the gestation period. The gains attributed to conception were in general made during the middle and latter parts of the gestation period.

SUMMARY

1. A study was made of the effect of pregnancy on the body weights of 56 Ayrshire cows during the first three periods of gestation.

2. The body weights showed a definite pattern of increase as the stage of pregnancy advanced.

3. The gain in weight over and above normal weights was not great before the fifth month of pregnancy. From 75 per cent to 85 per cent of the gain came in the last four months of pregnancy.

4. The gain in weight from month to month was much more uniform with first-calf heifers than with the older animals. This was probably because the heifers were still growing and not producing milk.

5. The average monthly gains made during the eighth month of pregnancy was less than in the months immediately preceding and following.

6. The animals in the last month of pregnancy were on the average 151 pounds heavier than open heifers at the same age. During the first pregnancy they were 142 pounds heavier; during the second pregnancy, 148 pounds heavier; and during the third pregnancy, 162 pounds heavier.

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STUDIES ON THE CHEMICAL COMPOSITION OF CALF BLOOD*

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A study, conducted by the Department of Dairy Husbandry of this Experiment Station, pertaining to the raising of dairy herd replacements on dry calf rations, offered an excellent opportunity for obtaining a considerable amount of data concerning the values for several blood constituents of young calves. The components studied were carotene, ascorbic acid, nicotinic acid, calcium, phosphorus, glucose, cholesterol, and non-protein nitrogen; and the results of these analyses are presented in this report.

EXPERIMENTAL

Experimental animals. Guernsey, Jersey, and Holstein calves under five months of age were used in this study. They were maintained in individual pens for the duration of the experiment. Whole milk was fed in limited amounts for six to eight weeks, and a dry calf ration and mixed hay were added to the dietary as soon as the animals would consume them. The calves used in obtaining the following data made weight gains within the usual range and were apparently normal in all respects.

Methods of analysis. Carotene was determined spectrophotometrically at a wave-length of 450 mμ, after extraction as described by Yudkin (13), while ascorbic acid was measured according to the method of Mindlin and Butler (9). Non-protein nitrogen was determined by the method of Koch and McMeekin (8), sugar by the method of Folin and Wu (5), inorganic phosphorus according to Fiske and Subbarow (4), and analyses for calcium were carried out by the Clark-Collip procedure (3). Values for plasma cholesterol were obtained essentially as described by Sackett (11), while nicotinic acid determinations were carried out according to a method previously reported from the Chemical Laboratory of this Experiment Station (12) after extraction as described by Friedemann and Barborka (6). Final measurements of the above blood constituents, with the exception of carotene, which was determined spectrophotometrically, and calcium, which was determined titrimetrically, were carried out by use of a Klett-Summerson photoelectric colorimeter.

RESULTS AND DISCUSSION

A significant difference, with respect to plasma carotene was found be-

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TABLE 1

Carotene and ascorbic acid values
(Holstein calves)

Constituents determined	Number of calves	Number of determinations	Amount in 100 ml. plasma *		
			High	Low	Average
Birth to 4 weeks					
Carotene	12	20	95.0	7.0	32.8
Ascorbic acid	12	18	0.73	0.22	0.41
5 to 8 weeks					
Carotene	12	21	37.0	10.0	20.2
Ascorbic acid	13	23	0.59	0.30	0.45
9 to 14 weeks					
Carotene	9	24	41.0	16.0	30.0
Ascorbic acid	9	23	0.66	0.26	0.42
15 to 23 weeks					
Carotene	11	26	82.0	16.0	44.0
Ascorbic acid	11	27	0.52	0.26	0.43

* Carotene expressed in micrograms; ascorbic acid expressed in milligrams.

tween results obtained with the Holstein calves and those obtained with the other two breeds. Furthermore, a preliminary study indicated a possible correlation between this factor and vitamin C. Therefore, two tables have been prepared to show the results for these two blood constituents, table 1 including the Holstein calves only and table 2 including the Jerseys and Guernseys. Since the remaining data did not show such a significant difference, the information presented in tables 3 and 4 represents results obtained with all of the animals used. As it seemed that the data would be more informative if they showed the values for different ages, these tables have been divided into four age ranges covering the period from birth to 23 weeks of age. No attempt has been made to show seasonal variations.

TABLE 2

Carotene and ascorbic acid values
(Jersey and Guernsey calves)

Constituents determined	Number of calves	Number of determinations	Amount in 100 ml. plasma *		
			High	Low	Average
Birth to 4 weeks					
Carotene	6	8	54.0	12.0	26.9
Ascorbic acid	5	7	0.64	0.30	0.40
5 to 8 weeks					
Carotene	13	17	74.0	8.0	36.0
Ascorbic acid	12	15	1.00	0.18	0.41
9 to 14 weeks					
Carotene	11	26	180.0	21.0	61.0
Ascorbic acid	11	27	0.99	0.16	0.45
15 to 23 weeks					
Carotene	12	37	144.0	21.0	64.5
Ascorbic acid	12	36	0.99	0.22	0.48

* Carotene expressed in micrograms; ascorbic acid expressed in milligrams.

However, it should be stated that the values shown resulted from analysis of blood samples collected during all seasons of the year.

In tables 1 and 2, values for carotene and ascorbic acid are presented. For these constituents the figures shown in the columns headed "high" and "low" are apparently true extremes, since such values were found in only a relatively small number of samples. The averages, as would be expected, show a much greater constancy, and also clearly show the considerably lower values obtained with the Holsteins, particularly for plasma carotene. From these average values, it is seen that the blood carotene increases but slightly, or even decreases, during the first few weeks, undoubtedly due to the using up of reserves furnished by colostrum, and then steadily increases as hay consumption increases. The ascorbic acid values show no particular correlation with age, the averages remaining quite constant. The values found were slightly higher than those previously reported by Bortree, Huffmann, and Duncan (1) but the finding of wide variations is in agreement with their results. Furthermore, it appears that breed differences with respect to this factor are not significant for calves of the age range studied.

TABLE 3

*Calcium, phosphorus, and glucose values**

Constituents determined	Number of calves	Number of determinations	High	Low	Average
			mg. per 100 ml.		
Birth to 4 weeks					
Calcium	12	21	13.0	10.9	12.12
Phosphorus	12	22	7.0	5.1	6.18
Glucose	11	20	150.0	77.7	110.00
5 to 8 weeks					
Calcium	17	24	12.8	10.0	11.68
Phosphorus	17	29	7.9	5.0	6.46
Glucose	16	28	113.0	60.8	89.70
9 to 14 weeks					
Calcium	12	23	12.0	9.7	11.03
Phosphorus	12	33	8.5	4.5	6.05
Glucose	12	30	93.5	64.3	75.70
15 to 23 weeks					
Calcium	15	38	12.6	9.8	11.03
Phosphorus	14	43	7.9	5.1	6.53
Glucose	15	44	86.0	60.0	74.20

* Calcium was determined on serum; phosphorus and glucose on whole blood.

There does not appear to be any correlation between age and blood calcium and phosphorus values (table 3), over the particular age range studied. The averages found for both of these constituents are, however, slightly higher than those reported for older animals by Kennedy, Anderson, Bechdel, and Shigley (7). Blood glucose, however, does show a definite decrease with age, and the data of table 3 upon which this statement is based agree exceedingly well with the findings of these workers (7).

The cholesterol values, seen in table 4, are of considerable interest since those of the greatest magnitude were found in the youngest age group, rather

closely paralleling the results found for glucose, while the values found for the older calves are of the order of those which have been reported by Boyd (2) for cows. It is a rather well established fact that blood cholesterol values are affected by various factors; however, it is not yet possible to state the fundamental reason for such changes. Muller (10) in 1929 stated that the blood cholesterol level is "controlled by some substance which is present in varying amounts in animal organs." Possibly this "controlling factor" is of particular significance in the early life of the calf. In this same table are presented the values found for non-protein nitrogen and for nicotinic acid. The former, although slightly lower, are in good agreement with similar values found for older animals in a study previously

TABLE 4

*Cholesterol, nicotinic acid, and non-protein nitrogen values**

Constituents determined	Number of calves	Number of determinations	High	Low	Average
			mg. per 100 ml.		
Birth to 4 weeks					
Cholesterol (total)	14	24	180.0	87.0	130.0
Nicotinic acid	7	9	1.48	0.39	0.98
N. P. N.	11	20	42.4	23.0	29.40
5 to 8 weeks					
Cholesterol (total)	24	42	158.0	86.0	124.5
Nicotinic acid	14	21	1.42	0.19	0.81
N. P. N.	16	29	40.0	24.0	29.00
9 to 14 weeks					
Cholesterol (total)	20	49	186.0	71.0	108.3
Nicotinic acid	12	30	1.21	0.18	0.86
N. P. N.	12	30	36.7	22.0	31.40
15 to 23 weeks					
Cholesterol (total)	16	51	143.0	82.0	108.8
Nicotinic acid	9	14	1.26	0.40	0.79
N. P. N.	15	42	34.8	23.0	29.70

* Cholesterol was determined on plasma; nicotinic acid and NPN on whole blood.

cited (7). As for the nicotinic acid results, extensive data are lacking in the literature with which to make comparisons. Hence, these values, although resulting from too few determinations to warrant any definite conclusions, can, when supplemented by further investigations, be of considerable significance in the establishment of what might be considered the normal range of values for this vitamin in the blood of calves.

SUMMARY

Data resulting from a large number of analyses for several blood constituents of normal Jersey, Guernsey, and Holstein calves have been presented. The results, tabulated in the form of ranges of values found, show what might be considered as normal values for calves of the age groups studied. An attempt was made, not to show the variations found in single animals, but rather to show average values for a fairly large number of

animals. Correlation of the data with age has been made by presenting the results in four divisions, covering the period from birth to 23 weeks of age.

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THE EFFECT OF VITAMIN A DEFICIENCY ON REPRODUCTION IN DAIRY BULLS [1]

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Degenerative changes in the seminiferous tubules in laboratory animals resulting from vitamin A deficiency have been observed by numerous workers and reviewed by Mason (8). The need for vitamin A in maintaining normal reproductive functions in bulls is less well understood. Guilbert and Hart (3), Thorp and associates (15), Sutton and associates (14) and Erb and associates (2) have reported varying degrees of degeneration of the germinal epithelium in vitamin A deficient young bulls. With biopsies Moore (11) has shown varying degrees of damage of the germinal epithelium of young bulls when fed carotene at a level that just prevented blindness. A five- to six-fold increase in carotene intake promoted considerable repair of the damaged epithelium. Hart (4), Madsen and associates working in the Bureau of Animal Industry, United States Department of Agriculture (12), and Erb and associates (2) have reported that sperm production in vitamin A deficient bulls can be restored by adding this vitamin to the ration; this indicates that the damaged germinal tissue is reparable. Madsen and associates (12) observed that bulls of the beef breeds lose sex drive and produce semen of poor quality when developing avitaminosis A. Upon carotene therapy the breeding ability returned fairly rapidly but the quality of the semen improved more slowly. Jones and Haag (5) reported that two bulls made permanently blind as a result of vitamin A deficiency at six to eight months of age later in life produced semen that was apparently normal and one of them bred and probably settled three or four cows.

Additional information was needed on the actual fertility of bulls maintained on rations poor or deficient in vitamin A and to determine if an insufficiency of this vitamin might be a cause of low breeding efficiency. Furthermore there were no data available on the possible relation between various reproductive phenomena and atrophy of the anterior pituitary which frequently occurs in vitamin A deficiency in the bovine (Madsen *et al.*, 7). The present report gives data on the reproductive performance of dairy bulls raised and maintained on rations containing varying amounts

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of vitamin A and carotene that produced vitamin A deficiency at different ages; and of bulls maintained on rations containing different amounts of supplemental carotene after the development of typical vitamin A deficiency.

PROCEDURE

Six Holstein bulls, five Jersey bulls, and one crossbred Red Danish × Jersey bull were used in these experiments over the period from July 1941 to January 1946. Each bull was fed colostrum for 3 days after birth and skim milk to 6 months of age. A concentrate mixture was fed, which consisted of 30 per cent white corn or barley, 20 per cent wheat bran, 25 per cent soybean meal, and 25 per cent linseed meal, plus 2 per cent bonemeal and 1 per cent salt. This mixture contained about 0.5 microgram carotene per gram. Timothy hay low in carotene content was fed as soon as the calves would eat it. Sufficient nutrients were offered to enable the animals to make normal growth when adequate carotene or vitamin A was supplied. In no instance was more than 8.5 pounds of hay fed daily. In order to induce vitamin A deficiency in the bulls at various ages and yet prevent them from succumbing completely from this deficiency, the ration was supplemented with varied amounts of cod-liver oil for varied lengths of time. Cod-liver oil containing 600 micrograms of vitamin A per gram was used. In several instances the bulls were given carotene supplement either as alfalfa meal or as carotene in cottonseed oil after the appearance of marked symptoms of vitamin A deficiency, such as blindness, loss of appetite, diarrhea, and muscular incoordination.

Each lot of hay was analyzed for carotene before it was fed as were also a number of samples of the concentrate mixture in the early phase of the experiment. The method of analysis was similar to that described in an earlier report from this laboratory (16) except that the carotene portion was filtered through an absorption column of magnesium carbonate.

The bulls were observed daily for the usual gross symptoms of vitamin A deficiency. The occurrence of total blindness, which was usually associated with fits and muscular incoordination, indicated advanced deficiency. At infrequent intervals the carotene and vitamin A content was determined for the blood plasma and for the livers of some of the bulls at autopsy. In the first part of the experiment the blood analyses were conducted by the method described by Davis and Madsen (1). Later a modification of this method was used in which the carotene and vitamin A were extracted from the plasma by the method of Davis and Madsen (1) after which the carotene was treated as described previously (16). The vitamin A determination was made by extracting the non-saponifiable matter with Skellysolve F, bringing to dryness and then dissolving in chloroform for analysis by the antimony trichloride color development at 620 millimicrons. Toward the end of the experiment a few samples of blood were analyzed

for carotene and vitamin A by the method described by Moore (10). The carotene content of the liver was determined by the same method as was used with the feed (16); the vitamin A determination on liver was made from the alcohol extract which had been diluted with an equal volume of 10 per cent sodium chloride and then extracted by Skellysolve F. The latter extract was brought to dryness in vacuo and saponified. The non-saponifiable matter was extracted in Skellysolve F, brought to dryness in vacuo, and then dissolved in chloroform for analysis by the antimony trichloride color development at 620 millimicrons.

As the bulls reached 10 to 12 months of age they were given the opportunity to mount a cow and serve an artificial vagina. (In this paper, for sake of brevity, the term "breeding" is used to denote the act of mounting and ejaculating into an artificial vagina.) In some instances bulls showed interest but were too weak or uncertain of themselves to mount. With few exceptions attempts were made to collect semen from each bull once a week. Two ejaculates were collected when they were obtainable. In some cases collections were made more often while in others collections were not obtained weekly. If a bull showed no interest in the cow he was judged to be without normal breeding ability. When bulls would not serve the artificial vagina, semen frequently was obtained by massage according to the technique developed by Miller and Evans (9).

After the semen samples were collected they were brought to the laboratory without delay and were rated for motility, volume, pH, and sperm concentration according to the methods outlined by Lambert and McKenzie (6). Specimens of diluted semen were dried on glass slides, left unstained and examined for percentage of normal sperm—a total of 200 being counted for each production interval of 30 days for some bulls and 60 days in the case of others. Each sample of semen was stored undiluted at 4° C. and the motility after 48 hours was recorded. Semen from the bulls was used to test its fertilizing capacity as often as estrus cows were available.

As each bull finished the experiment an autopsy examination was made. Samples of various tissues were prepared and examined histologically.

RESULTS

The carotene content of the roughage and the amounts of vitamin A or carotene supplements and the ages at which the supplements were given are shown in table 1. Table 2 gives the length of time each bull was on the experiment, the age when deficiency symptoms first appeared, and the age when the first semen was obtained. The live weights of the bulls, compared with the normal growth curves for Holstein and Jersey bulls reported by Ragsdale (13), are shown in figure 1.

All except H–2106, H–126, and J–314 were decidely under-weight after the first 3 or 4 months. These three bulls began falling below the normal

at from 10 to 12 months of age. The Red Danish × Jersey hybrid (X–132) cannot be compared with a normal but his growth curve also leveled off at about this age. Bulls H–2106, H–126, J–314, J–2324, and X–132 were more than 90 per cent of normal in weight at 10 and 13 months of age when they started to breed (table 2). These bulls received more vitamin A supplement or received it for a longer time than the others.

TABLE 1

Carotene content of the roughage and vitamin A or carotene supplement fed

Bull No.	Weighted average carotene content of roughage	Supplement fed at different ages (micrograms per day) * †						
		1	2	3	4	5	6	7
	meg./g.							
H–2106	1.7	2–490 5426	.....	.....	.....	.....	.....	.....
H–126	1.7	2–180 6000	575–637 8033‡	638–997 10986§	.....	.....	.....	.....
H–130	1.7	32–80 1686	95–109 264	166–178 325	235–246 600	273–341 1020	373–503 591	504–694 600
H–245	1.9	2–111 1278	199–210 600	237–305 1108	337–467 600	468–646 300	.....	.....
H–2174	2.2	2–130 1662	171–175 600	183–192 600	248–468 300	.....	.....	.....
H–256	1.8	2–114 1333	153–162 966	223–380 600	.....	.....	.....	.....
J–314	1.6	2–90 7400	481–821 7176‖	.....	.....	.....	.....	.....
J–470	2.1	2–125 1301	424–540 300	577–653 3600‖	654–713 7200‖	.....	.....	.....
J-2311	2.3	2–125 1360	213–222 300	306–393 600	394–597 300	651–835 5400‖	836–936 10800‖	937–1065 75000‖
J-2320	2.2	2–133 1091	158–161 750	333–454 300	.....	.....	.....	.....
J-2324	1.4	2–244 1312	.....	.....	.....	.....	.....	.....
X–132	1.8	2–261 1190	543–905 8000‖	.....	.....	.....	.....	.....

* Unless otherwise indicated the supplement is vitamin A in cod-liver oil.

† In each column the top figure is the age in days when the bull was fed the supplement and the bottom figure is the amount fed.

‡ Carotene mixed in cottonseed oil and injected subcutaneously.

§ Carotene fed mixed in cottonseed oil.

‖ Carotene fed in the form of alfalfa meal.

The six animals that became blind before 300 days of age did not start to breed at the expected 10 to 12 months of age nor did J–470 even though he was not totally blind until 420 days old. The first semen was taken by massage from H–245, H–130, H–2174, J–470, J–2311, and J–2320 after it was apparent they would not breed. No semen was obtained from H–256

before his death at 380 days. They were all 77 per cent, or less, of the expected weight at the time the first sample was taken. These seven bulls received the least vitamin A supplement up to this time.

The bulls that started to breed at the expected age were not blind and two of them (H–2106 and J–2324) retained their breeding ability long after total blindness had occurred. Bulls H–126 and J–314 failed to breed shortly after going blind, possibly in part due to weakness and incoordination in the rear legs. Bull X–132 bred very irregularly soon after going blind and semen had to be taken from him by massage. These three

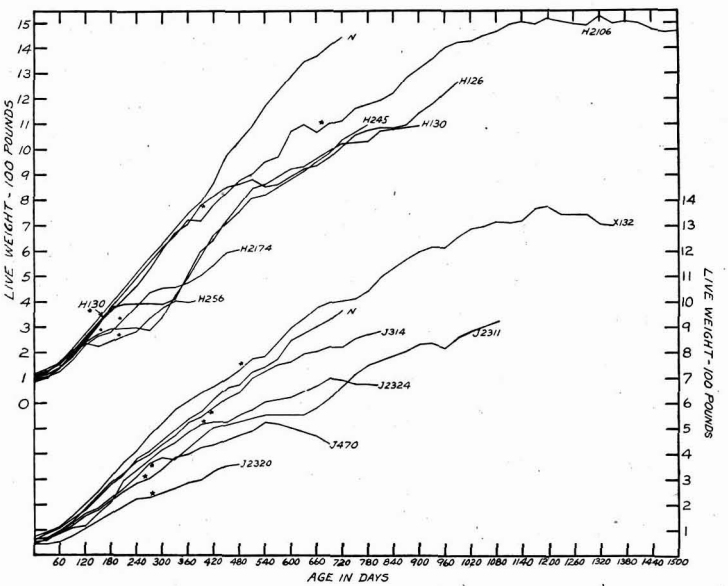

FIG. 1. Growth curves of bulls compared with respective breed average. *N* Normal growth curves for Holstein and Jersey bulls for first two years as published by Missouri station (13). X–132 is a Dane-Jersey cross. Asterisk indicates the age at which total blindness occurred.

animals regained their breeding ability following carotene supplementation although they remained blind.

In addition to the small amount of carotene consumed in the hay (5.9 micrograms per pound weight) by H–126, daily subcutaneous injections of 8,033 micrograms of carotene (9.3 micrograms per pound weight) for 62 days followed by daily feedings of 10,986 micrograms of carotene enabled him to resume breeding 83 days after the first injection. J–314 was consuming about 6.0 micrograms of carotene per pound weight daily from the roughage and when 7,176 micrograms of carotene (11.1 micrograms per pound weight) was added to his daily ration he started to breed again within

16 days. Adding 8,000 micrograms of carotene (10.2 micrograms per pound weight) to the 6.9 micrograms carotene per pound of weight daily that X–132 was taking in the hay shortly after he had become a very irregular breeder caused him to start breeding almost immediately. After about a year the carotene supplement was stopped but he continued to breed even though he again became very weak.

Bull J–2311 did not breed until he was 651 days of age, which was 115 days after 5,400 micrograms of carotene (8.4 micrograms per pound weight) had been added to the 5.5 micrograms per pound weight that he

TABLE 2

Ages of bulls at end of experiment, when total blindness occurred and when semen was first obtained and the per cent of normal weight when blindness occurred and semen was first collected

Bull No.	Age at end of experiment	Age when vitamin A deficiency appeared		Age when semen first obtained	Per cent of expected weight when	
		First fit	Totally blind		Totally blind	When first semen obtained
	days	*days*	*days*	*days*	%	%
H–2106	1517	676	685	306	78	100
H–126	997	430	396	317	99	101
H–130	911	151	152	456*	94	74
H–245	801	134	156	433*	80	77
H–2174	486	174	205	470*	74	68
H–256	380	225	222	.....	51	.....
J–314	821	.....	420	364	95	99
J–470	708	208	420†	505*	70	70
J–2311	1095	508	279	556*	90	76
J–2320	496	295	276	477*	56	54
J–2324	828	93	425‡	395	84	91
X–132	1347	450	481	363	.....	.....

* Obtained by massage.
† Blind in one eye at 234 days of age.
‡ Blind in one eye at 281 days of age.

was receiving in the hay. Semen had been previously obtained however by massage. H–245 was fed the same deficient basal ration as the other bulls but as shown in table 1 a small amount of vitamin A in addition, which was thought necessary for sustained life, was fed as cod-liver oil from 337 to 646 days of age. He gave semen only by massage from 433 days to 607 days of age after which he started to breed. From 433 days until the cod-liver oil was discontinued the amount of vitamin A fed averaged 348 micrograms daily or less than 0.5 microgram per pound weight. Bulls H–130, H–2174, J–470, and J–2320 failed entirely to breed. However a few samples of semen were obtained from each by massage. Apparently the small amount of vitamin A fed in addition to the carotene that these three bulls received in the basal ration (see table 1) after the first sample of semen was obtained by massage was not sufficient to enable them to breed.

The blood plasma vitamin A and carotene of some of the animals determined at various times are given in table 3. The bulls that were maintained vitamin A deficient after becoming blind (H–2106, H–130, H–245, and J–2324) showed progressive decreases in the plasma vitamin A and carotene. Bulls H–126, J–314, J–470, J–2311, and X–132 supplemented with carotene after developing avitaminosis A showed increases in the

TABLE 3

Vitamin A and carotene content of blood plasma and livers

Bull No.	Vitamin A and carotene content of blood plasma at different ages (micrograms per 100 cc. plasma) * †									Vitamin A and carotene in liver *
	1	2	3	4	5	6	7	8	9	
										mg./g.
	490	609	681	1106	1175‡	1190‡	1474§	1495§	1509§	1517
H–2106	15	7	9	8	7	6	2	3	3	0.004
	18	35	25	23	15	12	5	5	2	0.005
	479	506	590	605	637	665	725	796	997	.....
H–126	5	4	6	6	15	11	12	18	16	.....
	11	11	18	19	29	46	53	56	63	.....
	685‡	720‡	754‡	911‡	.....	.....	.....	.....	.....	911
H–130	6	5	2	2	.....	.....	.....	.....	.....	0.02
	14	9	7	.....	.....	.....	.....	.....	.....	0.08
	669‡	712‡	801‡	.....	.....	.....	.....	.....	.....	801
H–245	3	2	1	.....	.....	.....	.....	.....	.....	0.08
	6	7	7	.....	.....	.....	.....	.....	.....	0.12
	402	462	537	592	.....	.....	.....	.....	.....	.....
J–314	8	6	8	13	.....	.....	.....	.....	.....	.....
	14	10	33	44	.....	.....	.....	.....	.....	.....
	560‡	601‡	632‡	708‡	.....	.....	.....	.....	.....	708
J–470	5	6	6	11	.....	.....	.....	.....	.....	0.42
	10	18	32	73	.....	.....	.....	.....	.....	1.16
	635‡	664‡	682‡	775‡	804‡	945§	970§	985§	.....	.....
J–2311	5	7	7	8	5	12	17	24	.....	.....
	4	15	14	15	11	34	74	119	.....	.....
	481‡	725‡	748§	767§	.....	.....	.....	.....	.....	.....
J–2324	7	3	2	2	.....	.....	.....	.....	.....	.....
	12	8	6	7	.....	.....	.....	.....	.....	.....
	463	523	583	653	1078	1174‡	1347‡	.....	.....	1347
X–132	8	6	12	11	13	3	5	.....	.....	0.03
	11	9	41	37	22	8	4	.....	.....	0.05

* In each column the top figure is the age of the bull, the middle figure the vitamin A content and the bottom figure the carotene content.

† Unless otherwise indicated the vitamin A and carotene was determined by Dr. L. L. Madsen according to the Davis and Madsen procedure (1).

‡ Vitamin A and carotene were determined by the procedure herein described.

§ Vitamin A and carotene were determined by Dr. L. A. Moore (10).

plasma vitamin A and carotene content. Accompanying this increase was an increase in feed consumption, weight, and, except for J–470, in the ability to breed. The improvement in feed consumption and weight may have contributed much to the increased breeding ability. On the other hand, when carotene was added to the ration of J–470 the daily feed

allowance was kept constant. His appetite become ravenous, he lost weight, and his ability to breed showed no improvement even though the plasma vitamin A and carotene increased. He remained in a very weak condition.

The bulls that were in advanced vitamin A deficiency when slaughtered had practically no vitamin A or carotene in their livers (table 3). The vitamin A and carotene content of the liver of J–470 was very low even though the carotene consumed in hay and carotene supplement was increased from 1,900 micrograms daily to 5,500 micrograms for 77 days, then

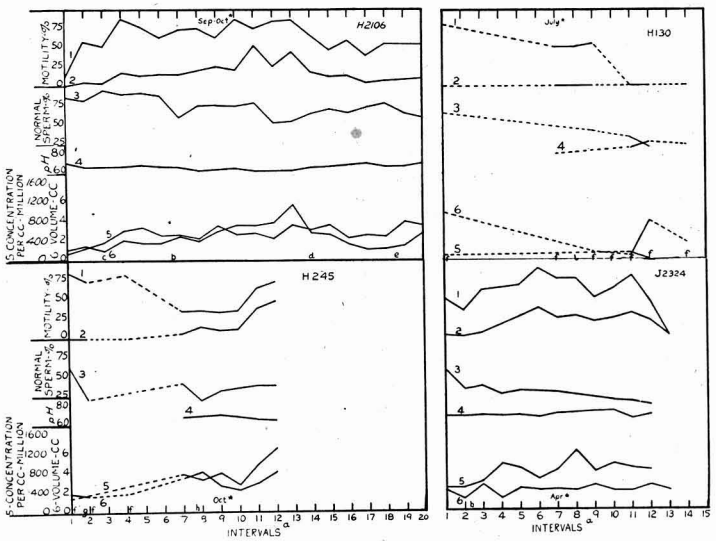

FIG. 2. Characteristics of semen. 1. Average per cent initial motility. 2. Average motility after storage. 3. Average per cent normal sperm. 4. Average pH. 5. Average number of sperm per cubic centimeter. 6. Average volume per ejaculate in cubic centimeters. *a*. Each interval represents the average for individual ejaculates obtained from the time semen was first obtained through successive 60-day periods for H–2106 and 30-day periods for the other bulls (dotted lines indicate that no samples were obtained during successive intervals). *b*. Total blindness occurred. *c, h* and *i*. Vitamin A supplement discontinued. *d*. Semen collections increased to three times weekly. *e*. 300 cubic centimeters mineral oil daily added to ration. *f*. Semen samples obtained by massage. *g*. Vitamin A supplement reduced from 600 to 300 micrograms daily. Asterisk indicates month or months during which samples representing this average were collected.

to 9,100 micrograms for an additional 60 days. This represented an increase of from 3.7 to 10.6, then to 20.0 micrograms per pound weight.

Figure 2 gives the pertinent data concerning the semen produced beginning with the first sample obtained either by the artificial vagina or by massage by four of the bulls (H–2106, J–2324, H–245, and H–130) that were not supplemented with carotene. Insufficient semen was obtained from

TABLE 4

Condition of anterior pituitary of bulls at autopsy

Bull No.	Description of pituitary
H–2106	Small cyst; AP about one-half size and not greatly damaged.
H–126	Large cyst; AP damaged but not extensively.
H–130	Very large cyst; AP little more than a shell, extensive pressure atrophy; probably not more than 10 per cent of normal tissue remaining.
H-245	,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,, ,,
H–2174	Medium sized cyst; AP showed little tissue damage.
H–256	Small cyst; AP showed small portion of tissue damaged.
J–314	,, ,, ,, ,, ,, ,, ,, ,, ,,
J–2320	,, ,, ,, ,, ,, ,, ,, ,, ,,
J–2324	,, ,, ,, ,, ,, ,, ,, ,, ,,
J–470	Large cyst; AP approximately 65 per cent destroyed.
X–132	Large cyst; AP little more than a shell, probably only 20 per cent of normal tissue remaining.

H–2174, J–2320, and H–256 for graphic analysis. Table 4 gives a description of the condition of the pituitaries and figure 3 shows photomicrographs of the condition of the testes of these bulls when slaughtered. Table 5 gives the fertility records.

TABLE 5

Fertility of the bulls

Intervals *	First figure is the number of times semen was used and second figure the number of conceptions during each 30-day interval								
	H–2106†	X–132†	H–126†	H–130	H–245	J–314	J–470	J–2311	J–2324
1	0	0	0	0	2–0	0	0	0	5–2
2	0	0‡	0‡	0	3–1	0‡	4–1	3–1	0‡
3	1–1	1–0	1–0	0	0	0	3–1§	2–0	2–1
4	0	1–0	2–2	0	2–2	0	0	0	0
5	3–2	0§	0§	0	0	1–1§	0	0	0
6	4–2	0	0	0	0	0	0	0	1–0
7	2–2	1–1	1–1	0	0	0	0	1–0	0
8	0	0	1–0	0	1–0	0	.....	0	0
9	1–0	0	2–0	2–0	1–0	0	.....	1–0	0
10	2–2	4–0‖	2–0	0	2–1	0	.....	1–1§	0
11	1–1	6–3	4–3	0	1–1	0	.....	1–0	0
12	1–1	0	0	0	1–1	0	.....	0	0
13	0	0	.....	0	.....	0	.....	0	.....
14	4–2	0	.....	0	.....	0	.....	0	.....
15	6–2	0	.....	.....	.....	0	.....	0	.....
16	2–0	1–0	.....	.....	.....	.....	.....	0	.....
17	1–0	.....	.....	.....	.....	.....	.....	1–1	.....
18	3–1	.....	.....	.....	.....	.....	.....	.....	.....
19	3–1	.....	.....	.....	.....	.....	.....	.....	.....
20	4–2	.....	.....	.....	.....	.....	.....	.....	.....

* Intervals conform with semen production periods shown in figures 2 and 4.
† Sixty-day intervals.
‡ Bull went blind in this period.
§ Supplemental carotene started in this period.
‖ Supplemental carotene was discontinued at end of this period.

The average motility of the freshly collected semen from H–2106 increased steadily during the first eight months (four 60-day intervals) but as the bull developed vitamin A deficiency (685 days) this trend did not continue. The motility after storage followed the same general trend but at no time was the keeping quality good. The percentage of normal sperm was above 80 before blindness occurred but thereafter it was usually below this figure and decreased to less than 50 per cent during some intervals. The pH of the semen varied between 6.3 and 7.2 with a gradual tendency toward alkalinity after the number of collections per week was increased. The number of sperm per cubic centimeter increased gradually during the first five intervals (306 to 606 days) and then fluctuated between 400 and 800 million per cubic centimeter showing no definite trend. At 1,095 days of age semen collections were increased from two to three times per week with two ejaculates being taken each time in an unsuccessful effort to exhaust his semen production. The volume of semen gradually rose through the first 13 intervals but after the number of collections per week was increased the volume and motility decreased. When he was 1,380 days old and until slaughtered, 300 grams of mineral oil was fed daily to reduce absorption of the small amount of carotene in the feed. In addition five grams of yeast daily was fed as a source of vitamin D. This bull had a small pituitary cyst (table 4) and the germinal epithelium was markedly degenerated (fig. 3, *a*) when he was slaughtered at a little over four years of age. The semen was used just before blindness occurred and throughout the following long period when he was becoming progressively more deficient (table 5). In all, 38 cows were inseminated and 19 of them conceived, the last one within 30 days before the bull was killed.

Semen was collected from J–2324 for about 13 months, beginning about 30 days before he became blind. His semen showed an irregular increase in initial and storage motility, concentration, and pH over the first eleven 30-day intervals (figure 2). During the last two intervals the initial and storage motility dropped sharply. He was very weak from vitamin A deficiency during this time. The volume averaged below 2.5 cubic centimeters throughout. The percentage of normal sperm decreased steadily and was very low at all times. This bull had a medium sized pituitary cyst that had produced some damage to the anterior lobe tissue (table 4) and there was marked degeneration in the tubules of the testes (fig. 3, *d*). His semen was used to inseminate five cows in the month preceding blindness and two of them conceived. It was used three times after blindness occurred and one of the cows become pregnant.

The semen from H–245, obtained by massage, was very low in concentration and contained a high percentage of abnormal sperm. The motility of these samples was high but this was not maintained after storage. The semen collected after he started breeding improved steadily in initial

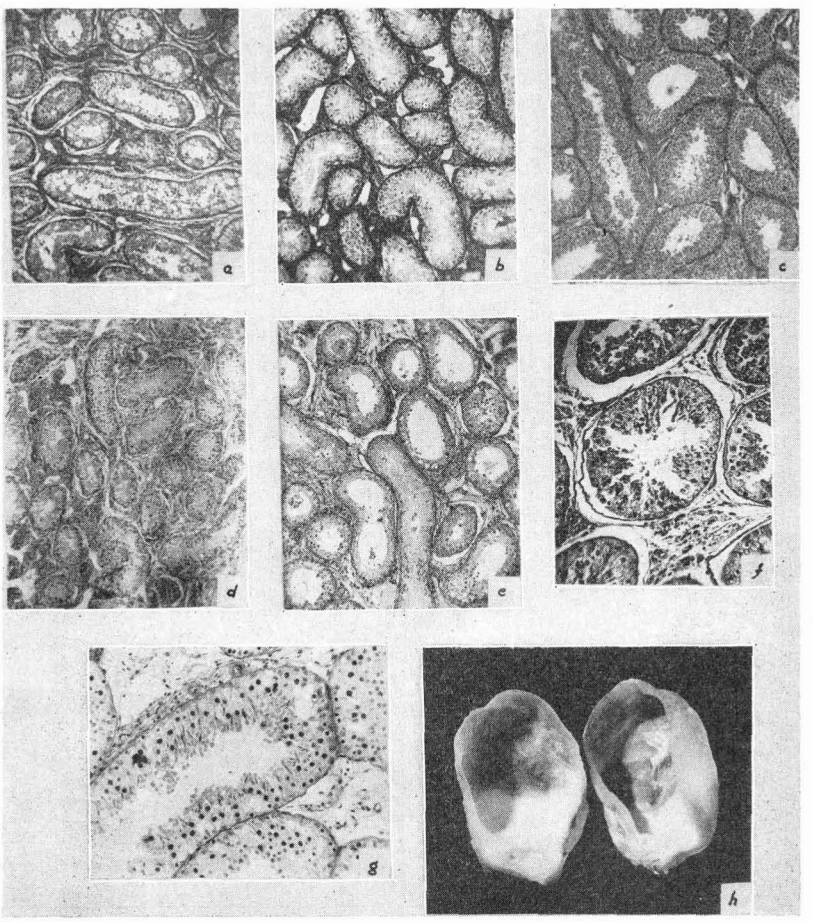

FIG. 3. Photomicrographs of section of testes cut at 7–8 micra and stained with Delafield's Haematoxylin. *a.* H–2106 at autopsy. This animal, sacrificed at a little more than four years of age, was proved fertile about a month before this specimen was obtained. The final stages of depletion were hastened by the addition of mineral oil to his ration. × 75. *b.* H–130 at autopsy. He also received mineral oil during the final months. His anterior pituitary was severely damaged (see *h* below). × 75. *c.* H–245 at autopsy. His pituitary was damaged as severely as H–130 and he had marked vitamin deficiency symptoms. H–245, however, was not given mineral oil toward the end of the experiment. Note the remarkable preservation of the germinal epithelium. × 75. *d.* J–2324 at autopsy. Note the decrease in the diameter of the tubules. × 75. *e.* J–2320 at autopsy. An occasional sperm could be found in the lumen of the tubules. × 75. *f.* H–2174 at autopsy. Early stages in degeneration of the germinal epithelium. × 210. *g.* H–256 at autopsy. Note the vacuolation in the otherwise fairly normal germinal epithelium. × 210. *h.* H–130. Little more than a "shell" remains of the pituitary gland as a result of the formation of the cyst. At the lower end of the photograph is the posterior lobe which was not as damaged as the anterior.

and storage motility, concentration, and percentage of normal sperm. The pituitary weighted 18.5 grams and contained a large cyst. The germinal tissue however contained a high percentage of apparently normal cells and numerous sperm could be seen in the lumen (fig. 3, *c*). As shown in table 5, semen obtained by massage was used to inseminate seven cows and three of them became pregnant. Later when the bull was serving the artificial vagina his semen was used six times and three cows were settled.

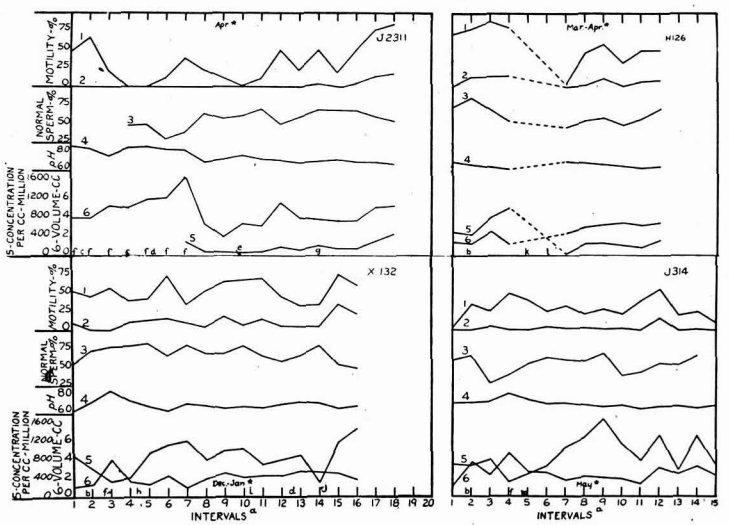

FIG. 4. Characteristics of semen. 1. Average per cent initial motility. 2. Average motility after storage. 3. Average per cent normal sperm. 4. Average pH. 5. Average number of sperm per cubic centimeter. 6. Average volume per ejaculate in cubic centimeters. *a*. Each interval represents the average for individual ejaculates obtained from the time semen was first obtained through successive 60-day periods for bulls H–126 and X–132 and for 30-day periods for bulls J–314 and J–2311 (dotted lines indicate that no samples were obtained during successive intervals). *b*. Total blindness occurred. *c*. Vitamin A supplement discontinued. *d*, *h*, *l*, and *m*. Supplemental carotene added to daily ration. *e* and *g*. Carotene supplement increased. *f*. Semen samples obtained by massage. *j*. Carotene supplement discontinued. *k*. Carotene supplement injected subcutaneously. Asterisk indicates month or months during which samples representing this average were collected.

Although H–130 received a little more cod-liver oil and for a little longer time than did H–245 after going blind, semen was obtained from him only by massage. It contained a small number of sperm, only a few of which were motile (fig. 2). The pituitary of this bull was damaged extensively (table 4 and fig. 3, *h*) as was the germinal epithelium (fig. 3, *b*). His semen was used twice in the ninth interval but neither time successfully.

Only a few samples of semen were obtained from H–2174 and J–2320

and these were by massage. Neither animal was tested for fertility. Both had pituitary cysts which had damaged the anterior lobe tissue (table 4) and they had considerable degeneration of the germinal epithelium (fig. 3, *f* and *e*, respectively).

Bull H–256 was considerably undersized for his age and semen was never obtained from him by either method before his death from pneumonia at 380 days. He had a small pituitary cyst at the time of death (table 4) but the germinal layer (fig. 3, *g*) shows only a small amount of degeneration and a few sperm were apparent in the lumen.

Bulls H–126, X–132, J–314, J–2311 and J–470 make up the group that received supplemental carotene after blindness had developed (see figs. 4 and 5, and tables 4 and 5).

The semen collected from H–126 during the time he was developing vitamin A deficiency gradually improved in motility and concentration while the per cent normal sperm and pH decreased. The volume of semen averaged only 1.5 to 2.5 cubic centimeters. The semen produced after supplemental carotene was added in general increased in motility, percentage of normal sperm, and volume. However, the initial and storage motility did not improve and the concentration remained very low. The pH gradually changed from about 7.1 to 6.4. Autopsy examination revealed a large pituitary cyst (table 4). A biopsy specimen of the testes, taken shortly after blindness developed and during the time when he would not breed, showed some germinal tissue degeneration (fig. 5, *b*); while after carotene feeding, as shown in figure 5, *c*, this tissue was improved but not entirely recovered. His semen was used to inseminate three cows between the time he went blind and the time he no longer would breed and two of them were settled (table 5). Ten cows were inseminated after he had resumed breeding following carotene supplementation and four of them became pregnant.

The semen obtained from X–132 both by breeding and by massage before carotene supplementation contained sperm which were poor in motility, low in concentration, and high in pH (fig. 4). After carotene was added and the bull resumed breeding the concentration increased (but not to a normal level) and the initial and storage motility improved slightly. There was little change in the volume, percentage of normal sperm, and pH. When the carotene supplement was discontinued the initial motility decreased, the pH increased, and the storage motility and volume showed no change. Increasing the collections from two to three times per week produced a slight decrease in concentration and an increase in percentage of normal sperm. When 15,000 units of pregnant mare serum were given subcutaneously during a period of 24 days, beginning with the fourteenth interval, a sharp rise occurred in the initial and storage motility, concentration, and percentage of normal sperm. The increased motility did

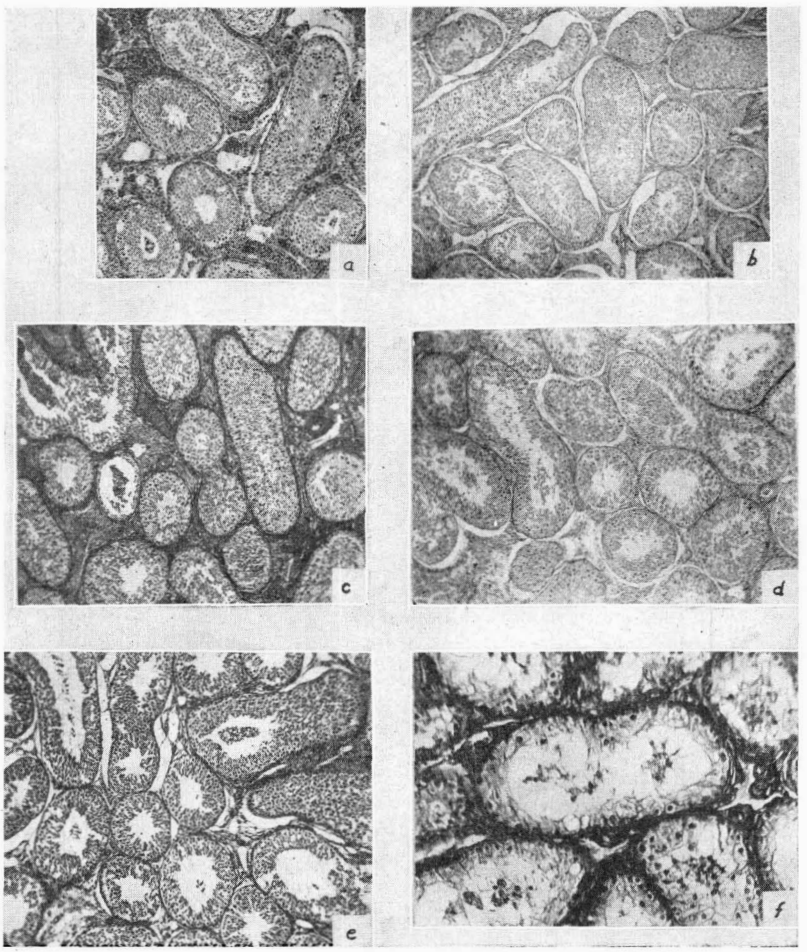

FIG. 5. Photomicrographs of section of testes cut at 7–8 micra and stained with Delafield's Haematoxylin. *a.* Normal mature bull. × 75. *b.* Biopsy of H–126 shortly after blindness occurred on a vitamin A deficient ration. At this time he was too weak to breed and no massage samples of semen were collected. From the appearance of the testes at this time he was probably producing many normal sperm. × 75. *c.* H–126 at autopsy. The general, although not complete improvement following limited carotene therapy may be seen. × 75. *d.* J–314 at autopsy. This animal also was sacrificed after a period of carotene therapy. Notice the normal appearance of much of the germinal epithelium. × 75. *e.* X–132 at autopsy. This animal was sacrificed after a second depletion period. Note the fairly normal condition of the germinal epithelium and sperm are present in the lumen of the tubules. × 75. *f.* J–470 at autopsy. This animal received limited carotene therapy but his energy intake was held down. Note the complete absence of spermatogenesis. × 210.

not persist long after the treatment was stopped but the concentration remained high to the end of the experiment. This bull had a pituitary cyst (table 4) and the germinal tissue showed some degeneration (fig. 5, *e*). The semen was used during his first depletion to inseminate two cows but without success. Later, during the carotene-feeding period and the second depletion, his semen was used to inseminate 12 cows and four became pregnant.

The semen produced by J–314 (fig. 4) throughout the experiment was of very poor initial motility and had practically no motility after storage. During no interval were the sperm more than 70 per cent normal. The pH of the semen was more than 7.0 during the first seven intervals and between 6.5 and 7.0 thereafter. The concentration of sperm varied between 3.5 and 4 hundred million per cubic centimeter until supplemental carotene was given, after which it increased to over 16 hundred million in the ninth interval, then ranged between 6 and 12 hundred million. This bull had only a small pituitary cyst (table 4) and the germinal epithelium showed some irregular areas of damaged tissue (fig. 5, *d*). The semen was used only once in the fifth interval, 24 days after carotene was added to the ration, and the cow became pregnant.

The sperm obtained from J–2311 by massage varied in initial motility from sample to sample and they did not keep well in storage. Although the concentration was not determined it was extremely low and less than 50 per cent of the sperm appeared normal. During this time his fertility was tested with six estrus cows, only one of which conceived. The semen produced after he started to breed following carotene supplementation was very low in concentration and the motility varied considerably. It did not store well and the pH ranged between 7.0 and 7.2. There was a gradual increase in the percentage of normal sperm as the amount of carotene fed increased (table 1). Six cows were inseminated with his semen after he started to serve the vagina and three of them settled.

While J–470 yielded semen by massage occasionally, not enough was obtained for graphic analysis. When he was slaughtered a pituitary cyst of medium size was found and, as shown in figure 5, *f*, marked degeneration had occurred in the germinal epithelium which carotene therapy apparently had not corrected in the absence of an adequate feed intake. Seven cows were inseminated with his semen while he was in advanced vitamin A deficiency and shortly before carotene was fed. Of these seven, two became pregnant.

Microscopic examination of preparations obtained at autopsy of several organs and tissues from the bulls including the adrenals, thyroid and kidneys, muscle and small intestines, showed no pathological changes. Scrapings of the epithelium of the trachea obtained at autopsy of the bulls in the most advanced stages of vitamin A deficiency (H–2106, H–130, H–245 and

J–2324) showed active ciliated cells in all cases. Changes in the bony structure of the head including constriction of the optic foramen was noted in the bulls, and particularly in H–130 and H–245 where the bony pocket encasing the pituitary body was much enlarged, probably caused by pressure from the cystic gland. The skull of H–130 particularly was spongy and the bone brittle.

DISCUSSION

One important effect of a marked vitamin A deficiency on the reproduction function of dairy bulls is the inability to mount and deliver semen. The extent to which this function is impaired or retarded depends somewhat on the age at which deficiency occurs and the severity of the deficiency. The bulls that were given enough vitamin A to make satisfactory growth up to the age when they were expected to start breeding in most instances did so and continued to breed for long periods even though marked deficiency symptoms appeared. Those developing avitaminosis A at about the time they started to breed, frequently failed to breed shortly after going blind. However, they bred following the addition of small amounts of supplemental carotene to the basal ration (8 to 11 micrograms per pound daily bringing the total daily consumption to between 14 and 20 micrograms per pound). This confirms the work of Hart (4) and Madsen *et al.* (12). The bulls that showed marked deficiency before they were 8 or 9 months of age did not start to breed at the expected age, although when supplemental carotene was added at older ages dramatic improvement occurred. The fact that the bulls could not see apparently was not the cause of their lack of desire or ability to breed, since three of the blind bulls continued to breed for a long time while remaining on the deficient ration and four others also bred when the ration was supplemented with carotene even though they remained blind.

The occurrence of a pituitary cyst with its accompanying damage to the anterior lobe may be the cause of failure in the development of breeding ability at the expected age because of a reduced secretion of pituitary hormones. Bulls that had the largest cysts or the greatest damage to the anterior lobe generally had been the poorest breeders and the most retarded. However H–245 having the largest cyst, had started to breed at a late age but bred regularly after he started. Also, the bulls that had pituitary cysts and that had not bred developed this function when fed supplemental carotene, although the cyst persisted. Sutton *et al.* (14) also observed gross pituitary damage and some evidence of histological changes in this gland. Inanition and impaired growth accompanying advanced vitamin A deficiency may be at least partly responsible for breeding failure. Weakness, incoordination, and a feeling of uncertainty in mounting may also be contributing factors to this failure. When J–470 was given carotene

without increasing his feed intake there was no improvement in breeding ability.

The seminal fluids obtained by either method from vitamin A deficient bulls invariably contained sperm. In most instances the sperm were motile. The sperm did not maintain motility after storage, were low in concentration, and a high percentage of them were abnormal in shape. The pH of the semen tended to increase in very advanced stages of vitamin A deficiency. The semen, although of apparently poor quality, was frequently successfully used to settle cows. Bulls were proved fertile while in advanced deficiency when showing blindness, incoordination, weakness, diarrhea, and edematous joints, low blood plasma carotene and vitamin A and, at autopsy, cystic pituitaries and degenerated testicles. Calves born to normally fed cows bred to these vitamin A deficient bulls delivered calves that were normal in every respect.

It is apparent from these results that gross vitamin A deficiency symptoms such as impaired eyesight and incoordination, etc., are apt to appear before any serious impairment in a bull's reproductive performance occurs. Under practical feeding conditions these symptoms are not often encountered. The quality of the roughage used in these experiments to produce vitamin A deficiency was poorer than that usually available on most farms.

Even though a number of the vitamin A deficient bulls in these experiments were fertile their semen was low in concentration, contained a high percentage of abnormal sperm, and lacked staying qualities—factors that may limit the bulls' usefulness, particularly in artificial insemination associations. The minimum level of vitamin A feeding that will permit bulls to produce high quality semen over long periods of time remains to be determined.

SUMMARY

Typical deficiency symptoms (total blindness, incoordination, emaciation, and extremely low blood plasma carotene and vitamin A values) were made to appear in 12 dairy bulls at different ages by adjusting the vitamin A intake.

Seven of the animals (all that were tested more than twice) were proved fertile by artificially inseminated cows. The semen used was usually obtained in an artificial vagina but it had to be collected by massage from the animals that would not mount. These bulls were also fertile.

Generally the semen was low in concentration, had a high percentage of abnormal sperm, a high pH, and did not store well.

When gross vitamin A deficiency symptoms appeared before the expected breeding age, the bulls failed to breed; when symptoms appeared at about the expected breeding age, the bulls frequently failed to breed

shortly thereafter; but when the deficiency became apparent after they had started breeding, this capacity was maintained.

At autopsy all the bulls were found to have cystic pituitary glands. The degree of damage to the anterior lobe varied, probably less than one-fifth normal tissue remaining in four bulls,—three of which incidentally were among those proved fertile. The animals exhibiting avitaminosis A earliest in life generally showed the greatest damage to this gland.

The epithelium of the seminiferous tubules was found to have undergone degeneration—some extensively. This epithelium, the anterior lobe of the pituitary gland, and the bones of the skull were the only tissues of several examined that had become altered.

Four of the bulls were fed rations supplemented with small amounts of carotene (8 to 11 micrograms per pound) after the appearance of avitaminosis A. This caused them to start breeding but the quality of the semen generally was not substantially improved. The testes of these animals were generally in better condition at autopsy than those of animal that did not get supplemented rations—giving evidence that carotene had brought about repair.

The germinal epithelium of one vitamin A deficient bull whose ration was supplemented but whose feed consumption was restricted was badly damaged, indicating failure to regenerate.

It is likely that gross vitamin A deficiency symptoms will appear before severe impairment to a bull's reproductive performance will occur.

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METHODS OF TESTING 80 PER CENT CREAM FOR BUTTERFAT

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In recent years, the process of manufacturing cream containing about 80 per cent butterfat has become commercially practical. Since no work has been published relative to the fat testing of this product, the following report is presented to suggest a procedure for purposes of control and billing "80 per cent cream."

Conditions which complicate the determination of butterfat in 80 per cent cream are: (a) obtaining a representative sample, (b) large amount of butterfat present, (c) rapidity of evaporation of moisture. (d) degree of accuracy required, and (e) speed of analysis required for control purposes.

Since the processes now in use for the production of 80 per cent cream are largely continuous, it is obvious that condition (a) above depends largely upon the uniformity of the separation process. If there is a wide variation in the fat test of the 80 per cent cream in a given lot, more samples are required to make a composite which is representative of that lot.

The buyer also must obtain a composite sample which is representative. His sampling may be complicated by the fact that the product is frozen and by his inability to sample containers at uniformly distributed intervals throughout the lot.

The large amount of butterfat in the cream presents the same difficulties encountered in testing butter. In extraction procedures, for example, it is necessary to use great care to insure complete extraction of the fat. In weighing the sample it is necessary to weigh more accurately than for a product containing a small amount of butterfat.

In order to mix properly a sample of 80 per cent cream, the temperature is such that moisture evaporates quickly. Samples held in partly filled jars or in loosely covered jars may lose as much as one per cent moisture in four hours.

Since 80 per cent cream is sold on the basis of fat content and since large amounts of butterfat are represented by a single test, it is apparent that the test should be within 0.2 per cent of the true amount of butterfat represented. This requirement automatically eliminates the Babcock test from consideration.

For control purposes the most desirable test is one which will yield results of the desired degree of accuracy in less than ten minutes. This condition eliminates the possibility of the Mojonnier test to be used as a

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control test, even though it was considered to be the test likely to be most accurate.

MOJONNIER METHOD FOR 80 PER CENT CREAM

The results of Herreid *et al.* (2) show that the efficiency of recovery of pure dry butteroil by the Mojonnier method using two extractions on 32 samples ranged from 98.4833 to 100.2123 per cent with an average of 99.5143 per cent. Assuming a fat content of a cream sample to be 80.00 per cent, these results would indicate a recovery of from 78.79 to 80.17 per cent with an average of 79.611 per cent. In view of this a similar study was made of the Mojonnier method using pure dry butteroil. The results of this study showed that in order to obtain an average of 100.00 per cent recovery, it was necessary to make the following modifications:

The Mojonnier extraction flask was suspended from the stirrup of the analytical balance, weighed, and a one-gram sample of 80 per cent cream injected into the flask with a 2-cc. syringe (without needle) avoiding contact of the syringe with the flask. The flask was quickly weighed again, the increase in weight being the weight of the sample. Water, ammonia, and alcohol were added and the contents mixed without stoppering the flasks. This procedure greatly reduced the hazard of blowing out fat. The extraction time was increased to one minute of vigorous shaking. Neoprene stoppers were used. Thirty-second centrifugings were found sufficient. Three extractions were found to be necessary instead of two as in the standard Mojonnier method. Blank determinations were made on each batch of reagents used. All determinations were made in duplicate and those not agreeing by 0.1 per cent were re-run. All Mojonnier tests reported (except where noted in tables 1 and 2) were run according to the above technic.

KOHMAN METHOD FOR CHILLED 80 PER CENT CREAM

Overman and Okimoto (4) compared the Kohman method with the Mojonnier method on eight samples of butter. Their results averaged 81.851 per cent fat for the Mojonnier and 81.825 per cent for the Kohman method.

The method followed in this report is the same as approved by the committee of the American Dairy Science Association (1). When this method was applied to chilled 80 per cent cream it was noticed that the curd (milk-solids-not-fat) averaged slightly more than 10 per cent of the moisture. Because the Kohman method for fat in 80 per cent cream took more than twenty minutes, it was decided to study a method of arriving at the fat content by determining the moisture content of the 80 per cent cream.

KOHMAN CALCULATED METHOD FOR CHILLED 80 PER CENT CREAM

In early experiments, the Kohman method (1) as used for butter

moisture was followed (except that the cup was cooled in water and carefully dried before weighing) and ten per cent of the moisture was added to the moisture and this sum subtracted from 100 to obtain the per cent fat. For example, if the Kohman moisture were 16.5 per cent, 10 per cent of this is 1.65 per cent. One hundred per cent minus the sum of 16.5 per cent and 1.65 per cent (or 18.15 per cent) is 81.85 per cent butterfat.

Later work (5) showed that more accurate results could be obtained by determining the exact percentage of the moisture which the curd represented. This percentage of the moisture was called the "factor" and was obtained as follows:

$$\text{Factor} = \frac{100 - (\%\ \text{Kohman moisture} + \%\ \text{Mojonnier fat})}{\%\ \text{Kohman moisture}}$$

In practice, this factor was determined weekly on a composite sample on one lot. Since slight variations were found, tables were prepared so that with a given factor the Kohman moisture could be read directly as per cent butterfat.

The practice at this plant has been to consider 500 packages of 80 per cent cream as one lot. As these packages were filled every tenth package was sampled and tested by the Kohman Calculated Method for fat. After 500 packages were filled the lot was completed and the 50 tests run during the lot were averaged. This average test was used for billing purposes. Once a week a composite sample was made up from every twentieth can. This composite sample was tempered at 100–110° F., well mixed with a malted milk mixer, and tested for fat by the Mojonnier method using three extractions. All necessary precautions were observed during the sampling and mixing operations to reduce to a minimum the loss of moisture from the sample. The Kohman moisture was also run

TABLE 1

Recovery of pure fat by the Mojonnier method

	Wt. sample	% Recovery
Using two extractions	0.5194	99.96
	0.6866	100.01
	0.7958	99.89
	0.9367	99.82
	1.4389	99.86
	1.5949	99.80
	1.6780	99.83
	1.7130	99.66
	1.7630	99.62
	1.8429	99.58
	2.2948	99.57
Using three extractions	0.9835	100.05
	1.2167	99.98
	1.9749	100.00
	2.0036	99.94

TABLE 2

*Comparison of standard Kohman and standard Mojonnier methods when applied to 80 per cent cream**

Sample No.	Kohman			Mojonnier Fat
	Moisture	Curd	Fat	
1	18.60	1.90	79.50	79.34
2	19.38	2.15	78.47	78.13
3	17.75	1.70	80.55	80.72
4	16.68	1.60	81.72	81.45
5	15.78	1.60	82.62	82.63
6	18.38	2.17	79.45	79.63
7	18.20	1.80	80.00	80.10
8	17.33	1.77	80.90	80.74
9	17.20	1.95	80.85	81.09
10	17.73	1.97	80.30	80.33
Average	.....	.....	80.438	80.416

* All tests run in duplicate and averaged.

on this same sample and the factor to be used for the following week was calculated using the formula given above.

TABLE 3

*Variations in "factor" as determined on various composite samples of extra heavy sweet cream**

Sample No.	Mojonnier fat	Kohman moisture	Culculated factor
1	79.66	18.45	0.1024
2	78.94	19.05	0.1050
3	79.46	18.60	0.1044
4	78.70	19.25	0.1049
5	79.65	18.40	0.1059
6	80.24	17.80	0.1101
7	80.40	17.80	0.1010
8	79.72	18.40	0.1022
9	79.92	18.20	0.1033
10	80.25	17.90	0.1028
11	79.30	18.80	0.1012
12	79.02	18.90	0.1101
13	80.12	17.90	0.1101
14	79.03	19.00	0.1037
15	79.79	18.40	0.0984
16	81.70	16.60	0.1063
17	81.00	17.30	0.0983
18	80.30	17.85	0.1036
19	79.75	18.55	0.0916
20	81.01	17.30	0.0976
21	79.64	18.50	0.1005
22	80.58	17.55	0.1065
23	79.69	18.60	0.0919
24	80.15	18.10	0.0967
25	79.45	18.70	0.0989
26	79.41	18.55	0.1100
27	80.49	17.85	0.0929
28	80.52	17.70	0.1006
29	80.31	17.90	0.1000
30	81.08	17.15	0.1032
Average	.....	.....	0.10214

* All determinations run in duplicate and averaged.

TABLE 4

*Comparison of Mojonnier fat test of composite sample and average of calculated Kohman fat of 50 individual samples making up composite sample**

Sample Number	1 Composite (Moj. Fat Test)	2 Ave. of 50 tests by Kohman Calc. Method	3 Difference (Col. 1 – Col. 2)
1	80.68	80.48	0.20
2	79.36	79.32	0.04
3	80.29	80.52	– 0.23
4	80.75	80.77	– 0.01
5	81.24	81.22	0.02
6	80.20	80.33	– 0.13
7	80.11	79.94	0.17
8	80.95	80.91	0.04
9	80.39	80.23	0.16
10	80.58	80.70	– 0.12
11	79.83	79.76	0.05
12	79.38	79.32	0.06
13	81.02	80.95	0.07
14	80.38	80.64	– 0.26
15	80.60	80.79	– 0.19
16	80.78	80.83	– 0.06
17	80.73	80.57	0.16
18	80.68	80.76	– 0.10
19	80.97	81.08	– 0.13
20	81.34	81.15	0.19
21	81.55	81.33	0.22
22	81.19	81.00	0.19
23	81.10	81.10	0.00
24	80.70	80.63	0.07
25	80.94	81.02	– 0.08
26	80.48	80.40	0.08
27	79.92	79.90	0.02
28	80.45	80.52	– 0.07
29	81.10	81.25	– 0.15
30	80.50	80.52	– 0.02
31	80.65	80.37	0.28
32	80.10	80.26	– 0.16
33	80.07	80.08	– 0.01
34	79.79	79.98	– 0.19
35	81.19	80.95	0.21
36	81.10	80.93	0.17
37	82.07	81.79	0.28
38	82.00	82.09	– 0.09
39	80.50	80.75	– 0.25
40	82.42	82.29	0.13
41	80.80	80.61	0.19
42	80.74	80.58	0.16
43	81.46	81.69	– 0.23
44	81.38	81.13	0.25
45	81.08	81.12	– 0.04
46	80.30	80.27	0.03
47	80.48	80.25	0.23
48	80.38	80.16	0.22
49	79.41	79.39	0.03
50	80.15	80.18	– 0.03
Average	80.6854	80.6562	0.0292

* All determinations run in duplicate and averaged.

RESULTS

Table 1 gives the percentage recovery of pure dry butterfat when two extractions are used and when three extractions are used. Good recovery was obtained when less than 1 gram of butter was used but the per cent recovery dropped as the weight of the sample was increased when only two extractions were made. Best results were obtained when one or two grams of butterfat were extracted three times.

The accuracy of the Kohman method for 80 per cent cream is given in table 2. The fat test of 10 samples averaged 80.438 per cent by the Kohman and 80.416 per cent by the standard Mojonnier method.

Table 3 gives the variations in the factor that may be expected over a period of a year. The factor ranged from 0.0916 to 0.1101 with an average of 0.10214. On the basis of 18.3 per cent moisture in a given sample of 80 per cent cream, this variation would yield a Kohman calculated fat test of from 79.72 to 80.02 per cent. This range of 0.3 per cent represents the degree of accuracy that may be expected by testing an individual sample of 80 per cent cream for fat by the Kohman calculated method.

Table 4 gives a comparison of the Mojonnier fat test on a composite sample of a lot with the average Kohman calculated fat test of fifty samples of cream from the same lot. The Mojonnier test of the composite averaged 80.6854 per cent fat, the average of 50 tests made by the Kohman calculated method on the same lots was 80.6562 per cent, and the Mojonnier was from 0.26 per cent lower to 0.28 per cent higher than the Kohman calculated method.

CONCLUSIONS

The Mojonnier Method using three extractions was considered to be the most accurate method for testing 80 per cent cream.

On ten samples of 80 per cent cream, the Kohman Method averaged 80.438 per cent fat and the Mojonnier Method averaged 80.416 per cent fat.

A rapid control test, suitable for billing purposes, was described as the Kohman Calculated Method for chilled 80 per cent cream.

The average test of 50 lots of 80 per cent cream tested by the Kohman Calculated Method was 80.6562 per cent and the average test of a composite sample of each lot run by the Mojonnier Method was 80.6854 per cent.

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FERTILITY OF BULL SEMEN DILUTED AT 1:100

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The studies to determine the optimum rate of dilution of bull semen, which have been underway for several years in our laboratory (1, 2), have been continued. The earlier reports dealt with dilutions, in the first case of as high as 1 part of semen to 16 parts of the yolk-citrate diluent (1), and, in the second case, with dilution rates as high as 1:50 (2). No statistically significant differences in fertility among the dilution rates were found in either experiment. The results of the two experiments differed in total over-all fertility level. However, this was attributed not to the dilution rates used but to differences in the fertility of the bulls available for study.

In the present experiment the dilution potential has been pushed farther towards the supposed inevitable point were reduced efficiency of conception might be expected (2). However, in this as in the previous studies no obvious effect of the lower number of spermatozoa used in insemination has manifested itself. While these investigations are part of a plan to attempt to establish the minimum number of bull spermatozoa required for efficient fertility, the practical importance of these investigations for artificial breeding units is obvious. Therefore, the results, though not answering the fundamental problem, are being presented here for use by others.

EXPERIMENTAL

The experiment was conducted in cooperation with the New York Artificial Breeders' Cooperative, Inc. A total of 19 bulls were made available, 11 Holstein-Friesians and 8 Guernseys. The precautions and methods used in the earlier investigations (1, 2) have been continued. However, as the earlier results had indicated that the precautions taken to standardize the use of all treatments rigorously within each collection period were not necessary the Latin square design previously used was abandoned. In its place a randomized block design was used. Each bull was considered as a block, and for each of 4 semen collections, spaced about 10 days apart, dilution rates were assigned at random. The dilution rates used were 1:40, 1:60, 1:80, and 1 part of semen to 100 parts of yolk-citrate diluent.

The effectiveness of this method for assuring uniform semen for the various dilutions is indicated from the average data for each dilution rate given in table 1. A statistical analysis of the semen characteristics showed highly significant differences between bulls, but no significant differences between dilution rates for the percentage of motile spermatozoa on initial examination, for concentration of spermatozoa per mm.^{3}, or for methylene blue reduction time.

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TABLE 1

Average data for semen used at each dilution rate

	Ratio of semen to diluter			
	1:40	1:60	1:80	1:100
Initial motility, %	78.4	79.4	79.4	79.4
Concentration, 1000's/mm.^{3}	1,286	1,273	1,364	1,299
Methylene blue reduction time, minutes	5.1	4.8	4.6	4.7

Each diluted semen sample was shipped to 61 different inseminators. These men used 1.0 ml. of the diluted semen for each insemination and each morning examined the semen for motility before using it for insemination that day. Few inseminations were made with fresh semen. The bulk of the inseminations were made on the second, third, and fourth days following collection.

RESULTS

The methods used for determining the results of insemination were discussed earlier (1, 2). The 5-month non-returns to service were considered as conceptions. The complete results are given in table 2. The highest average efficiency of conception was obtained with the highest dilution rate, 1:100. However, the small differences shown between dilution rates were not significant either when the percentage of conceptions for each ejaculate was used for the analysis of variance, or when the analysis of covariance using services as the independent variable and conceptions as the dependent variable was made. The average number of spermatozoa used for insemination at each of the dilution rates was as follows: 1:40, 31,361,000 spermatozoa; 1:60, 20,865,000; 1:80, 16,836,000; and 1:100, 12,861,000 spermatozoa per 1.0 ml. diluted semen.

The statistical analysis indicated highly significant differences between

TABLE 2

Fertility of the diluted semen by dilution rates

	Ratio of semen to diluter				Total	Non-returns %
	1:40	1:60	1:80	1:100		
Total services	1331	1386	1501	1547	5765	.....
Non-returns after breeding						
Avg. 45 days	914	938	1038	1086	3976	69.0
Avg. 75 days	807	827	923	966	3523	61.1
Avg. 105 days	760	772	868	914	3314	57.5
Avg. 135 days	740	738	836	885	3199	55.5
Avg. 165 days	722	725	820	870	3137	54.4
5-month non-returns, %	54.2	52.3	54.6	56.2	.....	.....

the fertility levels of the bulls. This result was to be expected as bulls vary in their inherent level of fertility. Also, there was a highly significant interaction between bulls and dilution rates. This interaction showed no trend, but was a result of the variations in semen quality among the 4 samples from each bull. A careful study of the results from individual bulls shows no tendency for any one of them to decrease in fertility consistently with each dilution rate.

The results of this experiment show that bull semen of satisfactory quality may be diluted as high as 1 : 100, and that less than 0.01 ml. of semen in the 1.0 ml. of diluted material used for insemination, containing an average of about 13 millions of spermatozoa, will give results as satisfactory as lower rates of dilution. The conditions to which the diluted semen were subjected were as rigorous as are generally found in artificial breeding units. These conditions involve shipping of semen to all inseminators and competition for use by the inseminator between the semen in this experiment with other less dilute semen from other bulls shipped on the same days.

The data in table 2 is arranged to show the numbers of cows that are apparently "settled" an average of 45, 75, 105, 135, and 165 days after insemination. In the right hand column of the table the percentage of apparent conceptions after each interval of time is shown. These data show an important fact observed in all records studied in this work. Apparently many artificially inseminated cows show delayed return to heat. The results reported here are typical of many studied by the author. Whether or not the cows showing delayed estrus after insemination actually conceived, or abortion resulted is not known. Nor is it clear whether or not these results are actually different than those found in natural service. Detailed evidence on this question would be desirable.

SUMMARY

In an investigation involving 5,765 inseminations no difference in fertility was found between dilution rates of 1 : 40, 1 : 60, 1 : 80, or 1 part of bull semen to 100 parts of the egg yolk-citrate diluent. At the highest rate of dilution on the average 12,836,000 spermatozoa were introduced in each 1.0 ml. of diluted semen used for insemination.

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FROZEN HOMOGENIZED MILK. 1. EFFECT OF FREEZING AND STORAGE TEMPERATURE ON THE PHYSICAL CHARACTERISTICS OF HOMOGENIZED MILK

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During World War II frozen homogenized milk was used to supply fresh milk to patients on hospital ships. Usually this milk was very acceptable when thawed after having been stored in a frozen condition for 3 months and frequent reports indicated that its acceptability was good after 6 to 9 months of storage. Instances occurred, however, where the milk was unacceptable when thawed after storage for less than 3 months. In these instances either a separation of the caseinate system had occurred or the milk had developed an oxidized flavor. No adequate explanation was available for these conditions, since the milk apparently was processed, frozen, and stored in the same manner as the milk that was acceptable.

To determine why some milk was acceptable after prolonged storage in a frozen state, while similar milk apparently treated in the same manner was unacceptable after a short storage period in a frozen state, experiments were conducted at the Veterinary Laboratory, Medical Department Professional Service Schools, Army Medical Center, Washington, D. C., to study the effect of freezing and storage temperatures on the physical, chemical, and bacteriological characteristics of homogenized milk.

A large proportion of the information reported in the literature on the effect of freezing on milk is based on observations with unhomogenized milk. However, Cvitl (1) reported that the tendency to separate, of almost all the milk constituents under the same experimental conditions, is clearly less and the fluctuations from the average values smaller, in homogenized milk than in normal milk. Grayson (2) reported that homogenized milk packaged and frozen under vacuum remained of excellent quality, and that comparison with fresh milk indicated there was very little, if any, change in the frozen product. Webb and Hall (3) state that slow freezing of milk or cream caused a gradual precipitation of the caseinate system and an immediate destruction of the fat emulsion. Thurston (4) states that homogenization, prolonged agitation at low temperature, and freezing and thawing of milk reduce or eliminate its susceptibility to oxidized flavor development. Bell (5) reported that homogenization prevents the formation of an undesirable layer of fat on the surface of condensed milk that

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has been frozen and aids in retarding changes which are indicated by an oxidized flavor. Roadhouse and Henderson (6) reported that pasteurized homogenized milk frozen in cans vacuum sealed under 23 inches of vacuum at 25° F. with agitation and stored at −5° F. for 6 weeks had a good flavor, but not quite equal to the product when fresh; and Trout (7) states that a slightly greater percentage of homogenized than unhomogenized milk froze in a given period at 0° F.

PROCEDURE

The milk used in this work was obtained from a commercial dairy in the Washington, D. C., area where it was packaged in quart containers. An effort was made to obtain milk of uniform quality by selecting cases of milk as they came from the filler. The milk had a butterfat content slightly under 4 per cent. It was properly homogenized, as shown by the test for homogenized milk recommended in the United States Public Health Service Milk Ordinance and Code (1939).

The milk was frozen in the original containers and stored in constant-temperature freezers. The frozen milk was thawed at room temperature. The visual appearance of the milk when thawed was noted and the degree of separation was determined by pouring 50-cc. portions from the quart samples, after thoroughly mixing the sample, into conical tubes and centrifuging. This procedure was the same as that used for determining the solubility index of dried milk and is referred to as sediment in this paper. Flavor determinations were made, after the milk was completely thawed out, by a panel of three men all of whom had had previous experience in milk judging.

EXPERIMENTAL RESULTS

Freezing and storing at constant temperature. To determine the effect of freezing and storing milk at a constant temperature, milk was frozen and stored at −10° C. (14° F.), −32.8° C. (−27° F.), and −40° C. (−40° F.). Table 1 shows the results obtained.

Table 1 indicates that freezing and storage at constant temperature can be applied to homogenized milk satisfactorily. For long storage periods, however, very low temperatures should be used. Samples held at −10° C. showed a slightly flat flavor at the end of 21 days, whereas, samples held at −32.8° C. and −40° C. were normal for at least 115 days. Of the 14 samples held at these two lower temperatures one had a slightly flat flavor and one had a slightly oxidized flavor. No reason was found for these changes.

Freezing homogenized milk and storing at lower temperatures. To determine whether milk could be frozen at one temperature and stored at a lower one, four samples of homogenized milk were frozen and stored for 9 days at −10° C. (14° F.). Then two of the samples were moved to

storage at −32.8° C. (−27° F.) and two to storage at −40° C. (−40° F.). Two other samples were frozen and stored for 4 days at −32.8° C. (−27° F.) and then moved to storage at −40° C. (−40° F.). Results are shown in table 2.

Table 2 shows that in all instances the appearance remained normal. This was confirmed by low sediment readings. No abnormal flavors other than garlic were noted. Section B of table 2 shows that these samples also had a normal appearance and flavor when thawed at the end of 58 and 89 days respectively. Table 2, therefore, indicates there are no physical

TABLE 1

Effect of storing frozen homogenized milk at constant temperature

No. of samples	Total storage time (days)	Appearance when thawed	Flavor	Sediment (cc.)
		(A) Frozen and held at −10° C.		
2	21	Normal	Slightly flat	.....
1	58	Normal	Flat	0.45
2	89	Slight separation	Flat	1.50
3	100	Separated	.....	6.00
		(B) Frozen and held at −32.8° C.		
1	30	Normal	Normal	.....
1	40	Normal	Normal	.....
1	58	Normal	Normal	0.08
1	89	Normal	Slightly flat	0.03
2	100	Normal	.....	0.08
6	115	Normal	Normal	0.10
		(C) Frozen and held at −40° C.		
1	51	Normal	Normal	0.10
1	82	Normal	Slightly oxidized	0.03

effects on homogenized milk frozen at one temperature and moved to a lower temperature.

Freezing homogenized milk and storing at higher temperatures. To further determine the effect of temperature changes on the physical characteristics of frozen homogenized milk, samples were frozen and held at one temperature and then stored at a higher temperature. Results are shown in table 3.

Table 3 indicates that homogenized milk should not be frozen and held at one temperature and then stored at a higher temperature. The only exception in table 3 was the milk frozen and stored at −40° C. for 1 day and then moved to −32.8° C., which was still normal at the end of 82 days. This may be attributed to the small difference between the initial

TABLE 2

Effect of storing frozen homogenized milk at a temperature lower than the initial freezing and holding temperature

No. of samples	Subsequent temperature	Subsequent storage time (days)	Appearance when thawed	Flavor	Sediment (cc.)
(A) Frozen and held for 9 days at −10° C.					
1	−40° C.	49	Normal	Very slight garlic*	0.15
1	−40° C.	80	Normal	Very slight garlic*	0.08
1	−32.8° C.	49	Normal	Trace garlic*	0.10
1	−32.8° C.	80	Normal	Very slight garlic*	0.03
(B) Frozen and held for 4 days at −32.8° C.					
1	−40° C.	54	Normal	Normal	0.08
1	−40° C.	85	Normal	Normal	0.08

* These samples were taken at a time when garlic was prevalent in the area and in view of the other samples it seems apparent that the garlic flavor existed in the milk prior to freezing.

TABLE 3

Effect of storing frozen homogenized milk at a temperature higher than the initial freezing and holding temperature

No. of samples	Subsequent temperature	Subsequent storage time (days)	Appearance when thawed	Flavor	Sediment (cc.)
(A) Frozen and held for 1 day at −40° C.					
1	−32.8° C.	50	Normal	Normal	0.08
1	−32.8° C.	81	Normal	Normal	0.03
1	−10° C.	50	Normal	Oxidized	0.50
1	−10° C.	81	Separated	Oxidized	4.00
(B) Frozen and held for 5 days at −32.8° C.					
1	−10° C.	15	Normal	.....	.....
1	−10° C.	46	Normal	Flat	0.45
2	−10° C.	77	Separated	Oxidized	3.25
1	−3° C.	15	Slightly separated	.....	.....
1	−3° C.	100	Separated	Flat	1.6
(C) Frozen and held for 13 days at −32.8° C.					
1	−3° C.	100	Separated	Flat	1.5

freezing and storage temperature and the subsequent storage temperature. In those samples where the temperature difference was great, there were flavor changes. The flavor became either flat or oxidized. For the two samples frozen and stored at – 40° C. for 1 day and moved to – 10° C. the appearance was normal for at least 51 days, but had separated to the extent of 4.00 cc. of sediment in 82 days. Similar results were obtained when samples were frozen at – 32.8° C. for 5 days and moved to storage at – 10° C. However, samples stored at – 3° C. (26.6° F.) following freezing and storage at – 32.8° C. for 5 days showed a slight separation in 20 days. The sediment readings on the two samples held for 100 days at this temperature do not fully indicate the separation that took place in this milk. No explanation can be given for this but the sediment in these samples was in the form of very fine particles instead of being flaky as it was in the other samples.

Effect of exposing frozen homogenized milk to room temperature. To simulate conditions of transferring frozen milk from land storage to ship storage, samples were exposed for various lengths of time at room temperature, *i.e.*, 23° C. (73.8° F.). Table 4 shows the effect of freezing and holding homogenized milk at different temperatures for different lengths of time, exposing it to room temperature for 30 minutes to 4 hours, then storing it at – 10° C.

Table 4, A, B, C, indicates as does table 1 that – 10° C. is too high a temperature for freezing and storing homogenized milk over long periods of time. Homogenized milk frozen and stored at this temperature and exposed to room temperature during its storage period was separated when thawed. The amount of separation, as confirmed by sediment readings, increased with the increase in exposure time and with the increase in the storage period. A comparison of the sediment readings obtained from frozen homogenized milk held at a constant temperature of – 10° C. (table 1) with those obtained where the milk was held at this temperature but exposed to room temperature (table 4, A, B, C,) shows that exposure to room temperature increased the degree of separation. Where the samples were stored for 58 days at a constant temperature (table 1) there was a sediment reading of 0.45 cc. as compared to 0.70, 2.30, 0.75 and 3.60 cc. where the milk was exposed to room temperature for ½ hour, 1 hour, 2 hours, and 4 hours, respectively (table 4). After 89 days storage the sediment reading increased from 1.50 cc. (table 1) to 2.75, 3.00, 3.50 and 9.50 cc. when exposed for ½ hour, 1 hour, 2 hours, and 4 hours, respectively (table 4). Likewise after 100 days storage the sediment readings increased from 6.00 cc. (table 1) to 9.00, 11.00, 13.00, and 12.00 cc. when exposed for ½ hour, 1 hour, 2 hours, and 4 hours, respectively (table 4).

Table 4, D, shows that homogenized milk frozen and stored at – 32.8° C. for 5 days before exposure to room temperature, with subsequent storage at

TABLE 4

Effect of exposing frozen homogenized milk to room temperature and returning to storage

No. of samples	Length of exposure (hours)	Total storage time (days)	Appearance when thawed	Flavor	Sediment (cc.)
(A) Frozen and held at −10° C. for 9 days, exposed to room temperature and then stored at −10° C.					
1	½	58	Slight separation	Flat	0.70
2	½	89	Separated	Flat	2.75
1	1	58	Separated	Flat	2.30
1	1	89	Separated	Flat	3.00
1	2	58	Slight separation	Flat	0.75
1	2	89	Separated	Flat	3.50
1	4	58	Separated	Flat	3.60
2	4	89	Separated	Flat	9.50
(B) Frozen and held at −10° C. for 21 days, exposed to room temperature, and then stored at −10° C.					
1	½	100	Separated	.....	9.00
1	1	100	Separated	.....	11.00
1	2	100	Separated	.....	13.00
(C) Frozen and held at −10° C. for 30 days, exposed to room temperature, and then stored at −10° C.					
1	4	100	Separated	.....	12.00
(D) Frozen and stored at −32.8° C. for 5 days, exposed to room temperature, and then stored at −10° C.					
1	½	51	Normal	Slightly oxidized	0.15
2	½	82	Separated	Slightly oxidized	2.00
1	1	51	Normal	Slightly oxidized	0.10
1	1	82	Slight separation	Slightly oxidized	1.30
1	2	51	Normal	Slightly oxidized	0.50
1	2	82	Separated	Slightly oxidized	1.80
1	4	51	Separated	Slightly oxidized	4.20
2	4	82	Separated	Slightly oxidized	2.50
(E) Frozen and stored at −32.8° C. for 5 days, then stored at −10° C. for 20 days, exposed to room temperature, and then stored at −10° C.					
1	½	104	Separated	.....	12.00
1	1	104	Separated	.....	13.00
1	2	104	Separated	.....	13.00

– 10° C., when thawed was normal in appearance for exposure up to 2 hours and storage time up to 51 days. The milk exposed to room temperature for 4 hours, however, was separated. The flavor of the samples including those that were normal in appearance was slightly oxidized. A comparison of table 4, D, with table 3, B, indicates that where homogenized milk is frozen and held at one temperature and then stored at a higher temperature, exposing it to room temperature does not greatly increase the degree of separation over that caused by a change in storage temperature but does affect the flavor of the milk.

Table 4, E, shows that samples which were frozen and stored at – 32.8° C. for 5 days, then stored at – 10° C. for 20 days before exposure to room temperature, and then stored at – 10° C. for an additional 79 days, were badly separated after thawing and all sediment readings were very high.

SUMMARY

Homogenized milk remained normal when frozen and stored at a constant temperature. The length of time it remained normal in the frozen state depended on the freezing and storage temperature.

Homogenized milk frozen and stored at – 10° C. (14° F.) had a slightly flat flavor when it was thawed at the end of 21 days. At the end of 89 days the flavor was definitely flat and slight separation had occurred. Homogenized milk frozen and stored at – 32.8° C. (– 27° F.) remained normal for 115 days and there were indications that it would remain normal for a longer period. There were also indications that homogenized milk frozen and stored at a still lower temperature, *i.e.*, – 40° C. (– 40° F.) would remain normal longer than milk frozen and stored at – 32.8° C. (– 27° F.).

Homogenized milk frozen and held at one temperature was moved to a lower temperature without causing any abnormalities to develop, but when it was moved to a higher temperature it became abnormal in appearance and flavor. Homogenized milk frozen at – 32.8° C. (– 27° F.) and subsequently stored at a lower temperature, *i.e.*, – 40° C. (– 40° F.), was still normal when thawed at the end of 89 days of total storage time. However, milk frozen at – 40° C. (– 40° F.) and then stored at a higher temperature, *i.e.*, – 10° C. (14° F.), was abnormal both in appearance and flavor when thawed if the total storage time had exceeded 50 days.

When frozen homogenized milk was exposed to room temperature for ½ hour, or more, at any time during its storage period with subsequent storage at a temperature higher than the initial storage temperature, its flavor when thawed was either flat or oxidized even though the milk was normal in appearance.

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THE PLACENTAL TRANSMISSION AND FETAL STORAGE OF VITAMIN A AND CAROTENE IN THE BOVINE

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The literature records several references (1, 3, 5, 7) that state there is little or no reserve of vitamin A in the newborn calf. However, it is known that a lack of vitamin A or its precursor, carotene, in the pregnant cow's diet may result in a stillborn, blind, or weak calf. Obviously, a sufficiency of this vitamin must traverse the placental barrier to provide for the normal fetal development. However, the factors involved in the placental transmission and fetal storage of vitamin A have not been explored. The results of Braun and Carle (2), although complicated by pathological conditions, suggest that the vitamin A content of the bovine fetal liver may be influenced by the maternal diet.

The relationship of the quantitative and qualitative factors of the maternal diet to the postnatal health of the newborn calf has received only limited attention. As a part of a broad project to investigate this relationship, data have been obtained regarding the effect of the prepartum diet on carotene and vitamin A storage in the newborn calf.

EXPERIMENTAL

Twenty-nine pregnant Holstein and four pregnant Guernsey heifers with similar management history were divided into four dietary groups approximately 60 days prior to calving. Each animal received a 12 per cent protein fitting ration composed mainly of cereal grains. The diets of the four groups in addition to the fitting ration were made up as follows: low carotene diet (S) of wheat straw, normal dry cow diet (N) of U. S. No. 1 timothy-clover mixed hay and corn silage, high carotene diet (C) of the same roughages as the N diet supplemented with one million I.U. daily of carotene, and a high vitamin A diet (A) of the same roughages as the N diet supplemented with one million I.U. daily of vitamin A in shark-liver oil. The carotene supplement was a commercial concentrate[1] containing 50,000 U.S.P. units per gram derived from vegetable sources. The vitamin A supplement was a high potency shark-liver oil[2] containing 41,000 I.U. per gram. The carotene content of the mixed hay and the corn silage as fed was 11.6 and 5.0 micrograms per gram, respectively. Unfortunately a shortage of labor made it impractical to record daily feed intakes.

Plasma carotene and vitamin A analyses were made on three successive days at the beginning of the experimental period (60 days before calving)

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[1] Purchased from General Biochemicals, Inc., Chagrin Falls, Ohio.

[2] Supplied by National Oil Products Co., Patterson, N. J.

and again about 18 days prior to the calving date. Because of the large daily variation it was believed that the average of three consecutive days would be more representative of the plasma levels. The blood plasma of the newborn calves was analyzed for vitamin A and carotene prior to colostrum ingestion. The method of Kimble (6) was used to determine plasma vitamin A and carotene. Several calves from the cows in each dietary group were killed at birth and liver storage of vitamin A and carotene determined according to the extraction procedure of Davies (4).

RESULTS

Plasma carotene and vitamin A of the pregnant cows. Table 1 shows the plasma carotene and vitamin A of the pregnant cows at approximately 60 days and 18 days before calving. The mean values accompanied by their standard errors are presented for each dietary group. Although there was considerable individual variation, the means for carotene and vitamin A were quite high at the beginning of the experimental feeding period.

The mean differences between the 60-day and 18-day values are a measure of the effects of the different dietary regimens on the plasma carotene and vitamin A. It does not seem likely that the usual gestational decrease in plasma carotene and vitamin A was a factor since this effect has been reported (8, 12, 13) to occur mostly during the two weeks prior to parturition. It is not known whether large daily intakes of vitamin A will prevent this decline. However, Lund (10) reported that the daily administration of 25,000 I.U. of vitamin A to pregnant women was ineffective.

The mean plasma carotene and vitamin A of group S was significantly lower than the means of the other groups 18 days before calving. The decline in plasma carotene and vitamin A of the N group is typical of the usual trend when cows are placed on barn feeding following a period on pasture. It should be pointed out that all of the experimental animals were on pasture previous to the start of the experiment. Oral administration of one million I.U. of carotene daily to the C group maintained the plasma carotene level, but did not prevent a slight decline in vitamin A over the 42-day period. It is of interest that the mean plasma vitamin A at 18 days before calving was highest for group A and lowest for group S. The difference between these means was highly significant. The difference between the other means was not significant statistically. It seems clear from these results that in maintaining the plasma levels of carotene and vitamin A of the dry cow, there is no advantage in supplementing a good practical ration with additional carotene or vitamin A. However, these results show the value of feeding the dry cow a ration of natural feedstuffs high in carotene.

Plasma carotene and vitamin A of the newborn calf. The data in table 2 show the relationship of the prepartum diet of the cow to the plasma carotene and vitamin A of the newborn calf. No significant differences were observed

TABLE 1

The maternal plasma carotene and vitamin A 60 days and 18 days before calving

Animal number	Plasma carotene			Plasma vitamin A		
	60 days	18 days	Change	60 days	18 days	Change
Low carotene diet (S)						
	γ/100 ml.	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*
R–26	851	138	−713	36	17	−19
3	484	101	−383	23	19	− 4
R–86	879	91	−788	37	21	−16
8	647	101	−546	32	17	−15
12	385	65	−320	42	15	−27
13*	1406*	569*	−837*	27*	16*	−11*
17	429	112	−317	29	17	−12
R–95	473	82	−391	34	19	−15
24	304	45	−259	21	17	− 4
Mean	557 ± 66	92 ± 9	−465 ± 69	32 ± 0.8	17 ± 0.6	−14 ± 0.3
Normal diet (N)						
	γ/100 ml.	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*
1	537	297	−240	40	18	−22
7	669	336	−333	35	45	+10
14	235	195	− 40	52	30	−22
R–74	846	624	−222	41	45	+ 4
23	406	239	−167	33	25	− 8
34	.....	.....	.....	.....	.....	.....
Mean	539 ± 105	338 ± 76	−200 ± 48	40 ± 3.9	32 ± 5	−8 ± 7
Normal diet plus carotene (C)						
	γ/100 ml.	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*
4	520	511	− 9	37	25	−12
5	409	408	− 1	36	33	− 3
9	596	488	−108	29	23	− 6
10	764	276	−488	27	31	+ 4
18	301	352	+ 51	28	22	− 6
29*	618*	474*	−144*	42*	52*	+10*
30*	364*	1313*	+949*	24*	18*	− 6*
31	173	284	+111	43	33	−10
32	254	276	+ 22	36	32	− 4
33	217	243	+ 26	38	34	− 4
Mean	404 ± 65	355 ± 36	− 50 ± 66	34 ± 2	29 ± 2	− 5 ± 2
Normal diet plus vitamin A (A)						
	γ/100 ml.	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*	*γ/100 ml.*
2	708	217	−491	30	25	− 5
R–64	1067	284	−783	44	27	−17
6	356	282	− 74	29	39	+10
16	204	291	+ 87	28	32	+ 4
20*	962*	544*	−418*	39*	45*	+ 6*
22	142	142	0	39	43	+ 4
26	303	155	−148	37	50	+13
27	373	106	−267	28	40	+12
Mean	450 ± 124	211 ± 29	−239 ± 116	33 ± 2	36 ± 10	+ 3 ± 4

* Denotes Guernsey cows not considered in average.

in the mean plasma carotene of newborn calves from the four dietary groups. The mean of group A was the highest while the mean of group C was the lowest. There was no correlation between the levels of maternal and new-

TABLE 2

The effect of maternal diet upon the plasma carotene and vitamin A of the newborn calf

Dam's number	Plasma of newborn calf	
	Carotene	Vitamin A
Low carotene diet (S)		
	γ/100 ml.	*γ/100 ml.*
R–26	4.5	5.1
3	0.8	1.0
8	1.5	1.4
12	1.5	2.6
13	6.0*	2.9*
17	1.9	0.6
R–95	1.7	3.1
24	0.8	1.5
Mean	1.8 ± 0.5	2.2 ± 0.6
Normal diet (N)		
	γ/100 ml.	*γ/100 ml.*
1	1.6	1.5
7	1.5	1.5
14	1.7	1.6
R–74	0.8	3.3
34	1.0	2.5
Mean	1.3 ± 0.2	2.1 ± 0.4
Normal diet plus carotene (C)		
	γ/100 ml.	*γ/100 ml.*
4	1.5	3.9
5	0.8	5.2
9	1.0	8.1
10	1.5	5.2
18	1.6	4.1
29	0.8*	0.8*
31	1.0	3.0
32	1.5	0.8
33	0.0	2.8
Mean	1.1 ± 0.4	4.6 ± 0.8
Normal diet plus vitamin A (A)		
	γ/100 ml.	*γ/100 ml.*
2	6.7	9.3
6	1.5	20.0
16	2.7	7.3
20	2.2*	6.6*
22	1.2	8.3
26	0.0	6.7
27	1.0	4.2
Mean	2.2 ± 1.0	9.3 ± 2.2

* Guernsey omitted from average.

TABLE 3

The effect of maternal diet upon the fetal storage of carotene and vitamin A in the liver of the newborn calf

Dam's number	Birth weight of calf	Weight of calf's liver	Liver storage				Total I.U. in liver‡
			Carotene	Vitamin A	Total carotene	Total vitamin A	
Low carotene diet (S)							
	lbs.	*gms.*†	γ/*gm.*†	γ/*gm.*†	γ	γ	
3	100	666	0.014	0.18	9.32	119.88	492
13	60*	575*	0.32*	0.085*	184.00*	48.88*	502*
17	78	720	0.10	0.08	72.00	57.60	432
R–95	105	856	0.13	0.019	111.28	16.26	256
Mean	94	750 ± 61	0.081 ± .03	0.093 ± .047	64.2 ± 29.7	64.5 ± 49.2	393 ± 71.9
Normal diet (N)							
	lbs.	*gms.*†	γ/*gm.*†	γ/*gm.*†	γ	γ	
7	87	675	0.136	0.33	91.80	222.75	1,044
R–74	122	940	0.140	1.74	131.60	1,635.60	6,760
23	88	733	0.090	0.096	65.97	70.37	390
34	78	707	0.107	0.39	75.65	275.73	1,226
Mean	94	764 ± 59	0.118 ± .013	0.64 ± .37	91.2 ± 14.4	551.1 ± 364.5	2355 ± 1481
Normal diet plus carotene (C)							
	lbs.	*gms.*†	γ/*gm.*†	γ/*gm.*†	γ	γ	
4	93	653	0.085	0.22	55.51	143.66	663
29	67*	490*	0.21*	0.22*	102.90*	107.80*	602*
30	76*	430*	0.43*	0.14*	184.90*	60.20*	548*
31	97	850	0.24	0.49	204.00	416.50	2,005
32	94	846	0.52	0.36	439.92	304.56	1,948
33	87	655	0.19	0.18	124.45	117.90	655
Mean	93	687 ± 77	0.26 ± .04	0.31 ± .08	205.9 ± 83.7	245.6 ± 70.4	1,319 ± 380
Normal diet plus vitamin A (A)							
	lbs.	*gms.*†	γ/*gm.*†	γ/*gm.*†	γ	γ	
6	100	810	0.14	25.45	113.40	20,614.50	85,011
16	102	729	0.11	4.31	80.19	3,141.99	12,705
20	65*	419*	0.22*	25.42*	92.18*	10,650.98*	43,874*
22	110	766	0.11	43.50	84.26	33,321.00	133,374
26	105	988	0.57	44.70	563.16	44,163.60	177,735
27	77	640	0.14	30.10	89.60	19,264.00	77,060
Mean	99	787 ± 56	0.21 ± .09	29.61 ± 7.35	186.1 ± 94.4	24,101.1 ± 6936	97,177 ± 881

* Guernsey omitted from average. † Wet basis. ‡ Assuming 0.6 micrograms carotene = one I.U. of vitamin A.

born plasma carotene, nor was there any demonstrable relation of maternal diet to the plasma carotene of the newborn.

The mean plasma vitamin A values of groups S and N are comparable to the values for normal calves reported by Moore and Berry (11). However, the mean value for group C was twice as great as for groups S and N, and the mean for group A was fourfold greater than normal. Statistically significant differences were found between the mean of the C or A groups and the means of the other groups. There was no correlation between the plasma A and carotene of the newborn calf.

According to Lund (10), similar studies with humans gave somewhat different results. He reported that fetal plasma levels of vitamin A were completely independent of maternal levels and could not be elevated even when the mother was given enormous doses (330,000 I.U.) of vitamin A daily before delivery. However, he reported that fetal carotene values were directly related to those of the mother and to the prenatal diet. In contrast, the results reported herein indicate that only the vitamin A in the plasma of the newborn calf may be elevated by the prenatal diet of the cow.

Fetal liver storage of carotene and vitamin A. Table 3 shows the individual carotene and vitamin A content per gram of liver in the newborn calf for each of the four diets, the total micrograms of carotene and vitamin A, and the total vitamin A potency per liver.

The effects of maternal diet on fetal liver storage of carotene and vitamin A were quite evident. Carotenoids were present in significant amounts in the livers of all calves contrary to the reports of other investigations. While the amount per gram of liver tissue is a measure of concentration, it does not represent a true picture of the carotene reserves. It should be noted that the mean total liver storage of carotenoids in terms of micrograms was 64.2 for group S, 91.2 for group N, 205.9 for group C, and 186.1 for group A. These results show that carotene is not only stored in the bovine fetal liver, but that the amount stored is in direct relation to the carotene content of the maternal diet. The considerable variation observed among individual calves emphasizes the importance of making several observations.

The effects of the cow's prepartum diet on the storage of vitamin A in the liver of her newborn calf are shown in table 3. For the low carotene (S) group, the mean value was 0.093 micrograms per gram of fresh liver tissue while the mean for the normal diet (N) group was 0.64. The reason that the mean value of 0.31 for the carotene-supplemented (C) group was lower than the N group was due to the large values obtained for the calf from cow R–74. Placental transmission and fetal storage was tremendously greater in the calves of group A from the vitamin A supplemented dams. The mean for this group was 29.61 micrograms per gram. Total liver reserves of vitamin A, *per se,* were 64.5 for group S, 551.1 for group N, 245.6 for group C and 24,101.1 micrograms for group A.

In the final column of table 3 we have listed under the heading of total I.U.[3] the vitamin A reserves in the liver of the newborn calves. The mean values were 393 for group S, 2,355 for group N, 1,319 for group C, and 97,177 for group A. Mention should be made of the fact that the calf from cow R–95 was blind at birth. Since this calf had a total liver storage of only 256 I.U., the blindness may have been due to insufficient vitamin A. If we assume complete utilization and a daily requirement of 10,000 I.U. per 100 pounds of body weight as suggested by Lewis and Wilson (9), the calves from the vitamin A supplemented cows were born with a storage equal to ten days' requirement of vitamin A.

No differences were observed in the mean birth weights of the calves, nor was there any relation between body weight and weight of the fresh liver.

DISCUSSION

It is worthy of note that the daily addition of a million I.U. of vitamin A to the usual dry cow ration either depressed or did not prevent a marked decline in the plasma carotene of the cows in group A. Additional data are being obtained on this problem and on the effect of diet on the gestational decrease of maternal plasma vitamin A.

Apparently the plasma carotene of the newborn calf is independent of the maternal plasma level and diet. This inference does not preclude placental transfer of carotene as indicated by the increased liver storage of carotene in the newborn calves of group C. These calves had slightly less vitamin A storage in the liver, yet their plasma vitamin A was higher than normal newborn calves. These facts together with the increased maternal values for plasma carotene and no apparent increase in plasma vitamin A are indicative of some fetal synthesis of vitamin A.

The variation of the results among the individual cases subjected to fairly uniform treatment suggests that factors in addition to diet are involved in the placental transmission and fetal storage of vitamin A and carotene. Although this variation tends to nullify the statistical differences among the means, the importance of the results is not diminished.

To our knowledge the data here presented represent the first clear demonstration that, in the normal bovine, the prepartum diet may influence markedly the level of both vitamin A and carotene in the plasma and liver of the new-born calf. In contrast to the present commonly accepted viewpoint, it has been shown that calves need not be deficient in vitamin A at birth. The question warrants further study as to whether or not feeding extra vitamin A to the pregnant cow will benefit the newborn calf. Clearly it would seem more economical to feed vitamin A directly to the newborn calf unless increasing the fetal reserve can be shown to give superior performance.

[3] Assuming 0.6 micrograms carotene = one I.U. of vitamin A.

SUMMARY

Four different rations were fed to 29 Holstein and 4 Guernsey heifers during the last 60 days of their gestation periods. The rations were: wheat straw plus a concentrate mixture, a normal fitting ration of concentrates, hay and corn silage, a normal ration plus one million I.U. of carotene daily, and the normal ration plus one million I.U. of vitamin A daily.

No significant differences were observed in the mean plasma carotene of the newborn calves from the four dietary groups. However, the plasma vitamin A of the newborn calves from the carotene supplemented cows was twice that of the normal group, while the vitamin A supplemented group showed a fourfold increase.

Carotenoids were present in significant amounts in the livers of all newborn calves and varied directly with the carotene content of the maternal prepartum diet.

The addition of one million I.U. of vitamin A daily to the normal ration of pregnant cows resulted in an average total fetal liver storage of 97,177 I.U. of vitamin A.

Evidence has been presented that the prepartum diet of the normal bovine may influence markedly the vitamin A and carotene reserves of the newborn calf.

Addendum—

Since the completion of the study presented herein, Wise and associates (14) have reported limited data indicating that the addition of vitamin A to the late gestation diet of the cow augmented significantly the vitamin A concenration in the blood and livers of the newborn calf.

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DIFFERENTIAL NITROGEN RETENTION FROM CASEIN, LACTALBUMIN, AND SOY PROTEIN, AND HYDROLYSATES THEREFROM[1]

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Protein utilization by the body involves three important features: the inherent amino acid content of the protein, the degree to which its components may be absorbed and the retention and utilization after absorption. Methods for the integration and evaluation of these features include determination of the amino acids by analytical methods, determination of digestibility, and nitrogen retention with experimental animals.

From a theoretical point of view a protein hydrolysate should limit variations in digestibility. Assuming that the method of preparation has not destroyed essential amino acids the first phase of digestion may be considered to have been accomplished before ingestion. Conservation in the efficiency of protein metabolism through the expedient of predigestion does not assume, however, superiority of retention and utilization because of increased absorption.

The hydrolytic process is essentially one of degradation, therefore, a protein hydrolysate may not necessarily be the full equivalent of the starting material. Destruction of tryptophane by acid hydrolysis and the destruction of cystine and methionine by alkali are well known. Our immediate interest, however, concerns enzymatic hydrolysis simulating in principle, protein cleavage within the body. The literature has not revealed extensive studies designed to show the relative biological efficiency of natural proteins and their hydrolysates. Aside from a more rapid and possibly greater degree of absorption, there is no reason to presume that a hydrolysate has a greater biological value than the protein from which it was derived. Inasmuch as our unpublished data have shown that the amino acids resulting from the primary enzymatic cleavage of protein may be degradated to their fatty acid components by further enzymatic action, we have undertaken the present comparative study using three common proteins and their primary hydrolysates in recognition of the possibility of a destructive action depending upon the method of preparation. The proteins used for this study were casein, lactalbumin and a protein derived from soy bean. Each of these products could be readily prepared in purified form suitable for the biological studies and efficient preparation of the hydrolysate.

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EXPERIMENTAL

The protein in each instance was prepared in a manner designed to yield a product dispersible in water at a pH below neutrality. The casein as used in the experimental diets as well as that used for the enzymatic treatment was a purified di-calcium caseinate containing 1.72 per cent of bound calcium. At no time was the protein subjected to an alkalinity greater than pH 7.05 or an acidity stronger than pH 4.6. For the preparation of the hydrolysate a 10 per cent concentration of the dispersed caseinate was subjected to the action of a proteolytic enzyme for about 3 hours. The amino nitrogen as measured by formol titration was 28.4 per cent.

For the lactalbumin studies two unhydrolyzed products were used in comparison with the hydrolysate. One was a thoroughly washed, insoluble, iso-electric lactalbumin and the other a dispersible sodium lactalbuminate. This was prepared from the iso-electric lactalbumin by dispersing the insoluble product in weak alkali under controlled time and temperature conditions designed to minimize the destructive effect of the alkali. Upon completion of the dispersion the alkalinity was reduced to pH 6.8–6.9. An 8 per cent concentration of this dispersion was subjected to the same proteolytic enzyme employed for the preparation of the casein hydrolysate for a period of 4 hours. The hydrolysate contained 31.8 per cent amino nitrogen.

The soy protein was derived from a high grade of defatted soy meal by initial precipitation of the protein at pH 5.3. This precipitate subsequently subjected to a plurality of washings was then dispersed in hydrochloric acid at pH about 1.9–2.0. The protein in colloidal solution was precipitated at pH 4.3 and subjected to a series of washings while progressively increasing the pH of the suspension to 5.1. An 8 per cent concentration of the suspended protein was partially dispersed at pH 5.8 and subjected to the action of the hydrolytic enzyme for a period of 6 hours. The hydrolysate contained 32.2 per cent amino nitrogen.

According to the manufacturer the proteins of the soy meal used for the isolation of the protein used in this study are slightly, if at all, denatured by heat; "the protein is as undenatured as the protein of raw soy beans with a water solubility of about 80 per cent." Soy protein similarly prepared from the same starting material has been shown to cause a 10-gram gain per week in white rats when comprising 16 per cent of the ration supplemented with choline (6).

The determination of the biological merits of the various proteins and hydrolysates involved the primary features of methods proposed by Mitchell (3), Mitchell and Carman (4), Cannon (2), and Allison and Anderson (1) with adaptations to meet the requirements of this particular study.

Mature white rats averaging in excess of 200 grams were furnished a depletion ration consisting of the following: Salt mixture, No. 40 (5), 4 parts; hydrogenated vegetable oil (Crisco), 3 parts; cod-liver oil, 2 parts;

Cellu flour, 4 parts; dextrin, 50 parts; lactalbumin, 1.5 parts; and sucrose, 35.5 parts. The following vitamin factors were provided as a 2.5 ml. supplement daily: Thiamin, 12.5 mcgs.; riboflavin, 10.0 mcgs.; pyridoxine, 10.0 mcgs.; calcium pantothenate, 50.0 mcgs.; and choline, 10.0 milligrams. The ration contained 0.193 per cent nitrogen. The animals were maintained on this diet for a period of 2½ to 3 months during which period loss of weight averaged approximately 25 per cent while maintaining a slightly positive daily nitrogen balance based upon urinary excretion only, averaging 2.3 milligrams. Following this extended depletion period the lactalbumin was eliminated from the ration and the animals continued on a nitrogen-free (0.068 per cent nitrogen) ration and the vitamin supplements for a period of 7 days. During this final depletion period the animals lost on an average of 8.8 per cent of their weight following the low nitrogen ration and an average loss of 32 per cent of their original weight. The slightly positive urinary nitrogen balance shifted to a negative balance averaging 21.7 milligrams per day.

At the end of the last depletion period the test proteins and hydrolysates were incorporated in the "nitrogen-free" ration at comparable nitrogen levels without regard to protein equivalency. A series of nitrogen levels varying from 0.193 to 2.173 per cent were provided during a continuous sequence of 4-day test periods at each level, the vitamin supplements remaining the same. The rations were fed *ad lib* in guarded containers preventing scattering and food consumption records were obtained daily. Four to six animals were used for each test material.

The animals were maintained in individual metal and screened bottom cages. Urine and feces were collected daily on filter paper. The droppings were immediately dried to constant weight and composited for each rat for each four-day period. The urine absorbed on the filter paper and that which may have adhered to the removeable trays was recovered daily by three extractions with 2 per cent boiling sulphuric acid. In order to follow closely the effect of each test material, nitrogen determinations were made on the urine daily for each animal in most instances and for all animals through the transition period from a negative nitrogen balance to a well-established positive balance.

All nitrogen determinations were made by a semimicro method involving digestion in Folin tubes and subsequent distillation with circulating steam under slight pressure.

The plan of procedure permits the calculation of biological values of the test materials according to Mitchell (3) wherein endogenous fecal and urinary nitrogen are calculated from the values obtained during the per-test period on the nitrogen-free ration and again following the test period; such values for each product at each of the nitrogen levels are shown in table 1. It will be noted that in all instances the higher biological values are shown

at the three lower nitrogen percentage levels decreasing progressively at the higher levels. This relationship indicates optimum efficiency of nitrogen utilization at the lower intake levels. The range of variations noted may be due, in part at least, to the calculated endogenous urinary nitrogen value. Our data indicate that the endogenous urinary nitrogen may vary depending upon the quality of the protein, especially under experimental conditions involving an extended depletion period. For example, striking evidence of variation in excretion of endogenous nitrogen was consistently evident in the case of the lactalbumin feedings. The endogenous urinary nitrogen as determined from the nitrogen-free ration was in all cases higher and in some instances twice as high as the total urinary nitrogen excreted when lactalbumin was included in the ration, especially at the lower or medium per-

TABLE 1

Biological values of lactalbumin, casein, and soy protein, and their hydrolysates at different nitrogen intake levels

% Nitrogen in ration	Biological value						
	Lactalb.	Dispersed lactalb.	Dispersed lactalb. hydrolysate	Casein	Casein hydrolysate	Soy protein	Soy protein hydrolysate
0.193	83.4	100.0	96.7	92.4	92.7	78.8	69.0
0.386	100.0	100.0	96.6	91.2	99.2	71.4	74.6
0.773	100.0	99.9	98.9	90.1	89.9	68.7	68.4
1.165	100.0	87.5	92.5	80.6	85.6	54.0	62.0
1.584	89.8	82.5	79.2	76.6	72.3	51.6	55.8
2.162	76.6	47.4	70.1	60.0	64.6	43.6	47.8

centage levels. This relationship may arise from varying degrees of depletion of the dispensable protein reserve, labile protein reserve or possibly indispensable fixed body protein according to the concept of Whipple (7), since food intake during the standardization periods and the test periods at the lower nitrogen intake levels was substantially iso-caloric and adequate for basal metabolism requirements. For illustration, the calculated caloric requirement during the standardization periods averaged 25.66 calories per day per animal for the group receiving lactalbumin; the actual caloric intake was 33.08 calories per day. During the lactalbumin test periods at the lower nitrogen levels, the caloric requirement averaged 24.68 calories per day; the caloric intake was 32.66 calories per day.

In view of the above we have presented the data correlating nitrogen balance with absorbed nitrogen on a cumulative basis, no attempt being made to calculate the endogenous increment of the urinary nitrogen. These values are recorded in table 2 and figure 1. The nitrogen balance correlated with absorbed nitrogen assumes a striking linear relationship similar to that shown by Allison and Anderson (1). Also, the graph clearly shows the

TABLE 2

Establishment and degree of positive nitrogen balance caused by lactalbumin, casein and soy protein and their hydrolysates
(Grams/sq. m./day—cumulative basis)

Test material	% nitrogen in ration	% absorbed	Amount absorbed	Amount retained
			gm.	*gm.*
None	0.068	.....	0.2300	0.6841 –
Lactalb.	0.193	64.75	0.7860	0.2557 –
"	0.386	79.41	1.2710	0.2537
"	0.777	94.14	3.1353	2.0187
"	1.166	96.91	6.4598	4.7028
"	1.586	96.89	10.6518	7.8965
"	2.151	95.06	15.2778	10.5190
None	0.068	.....	0.1348	0.0927 –
Dispersed lactalb.	0.198	94.37	0.7491	0.0272 –
" "	0.389	95.17	1.3908	0.3099
" "	0.774	96.56	3.4054	1.8449
" "	1.168	95.67	5.6202	3.5627
" "	1.584	95.30	9.1868	5.9338
" "	2.163	95.99	12.7533	8.6694
None	0.068	.....	0.1956	0.7826 –
Lactalb. hydrolysate	0.193	75.08	0.8059	0.2176 –
" "	0.386	90.25	1.2311	0.1224
" "	0.771	96.51	3.3498	1.5148
" "	1.160	96.36	6.4885	3.6597
" "	1.590	95.49	10.2677	6.5481
" "	2.164	95.61	14.3976	8.4251
None	0.068	.....	0.2482	0.7909 –
Casein	0.193	88.61	0.7832	0.2773 –
"	0.386	88.48	1.4055	0.0510 –
"	0.774	96.01	3.3506	1.3120
"	1.154	97.66	6.7934	3.2764
"	1.593	98.23	12.5479	6.8392
"	2.171	95.69	17.9786	10.6189
None	0.068	.....	0.2700	0.7315 –
Casein hydrolysate	0.193	80.81	0.9885	0.2795 –
" "	0.386	83.77	1.3667	0.1856 –
" "	0.773	90.12	2.9563	0.8107
" "	1.165	95.00	5.7399	2.5013
" "	1.584	95.39	8.9637	4.2433
" "	2.162	95.74	15.0303	7.6375
None	0.068	.....	0.1429	0.9598 –
Soy protein	0.193	93.30	0.6988	0.1584 –
" "	0.386	96.45	1.4096	0.0558 –
" "	0.773	96.56	3.0823	0.6213
" "	1.163	96.31	5.5650	1.5636
" "	1.582	98.08	8.9643	3.0676
" "	2.164	96.99	12.9224	4.7472
None	0.068	.....	0.3050	0.7602 –
Soy protein hydrolysate	0.193	72.46	0.9842	0.5589 –
" " "	0.386	84.91	1.4784	0.0499 –
" " "	0.770	93.59	2.9785	0.4747
" " "	1.153	95.86	5.3699	1.5954
" " "	1.576	96.44	8.7165	2.6308
" " "	2.173	95.31	13.6188	4.6058

comparative nitrogen retention from the various proteins, and from the individual proteins and their respective hydrolysates. This type of evidence correlated with the biological value as usually calculated seems to yield more comprehensive information regarding the test material than is possible from the determination of the biological value resulting from a single feeding level. These data show that the lactalbumin and its hydrolysate are measurably better than casein or its hydrolysate, both of these proteins being superior to the soy protein; also, the protein appears to be slightly better than its hydrolysate in each instance.

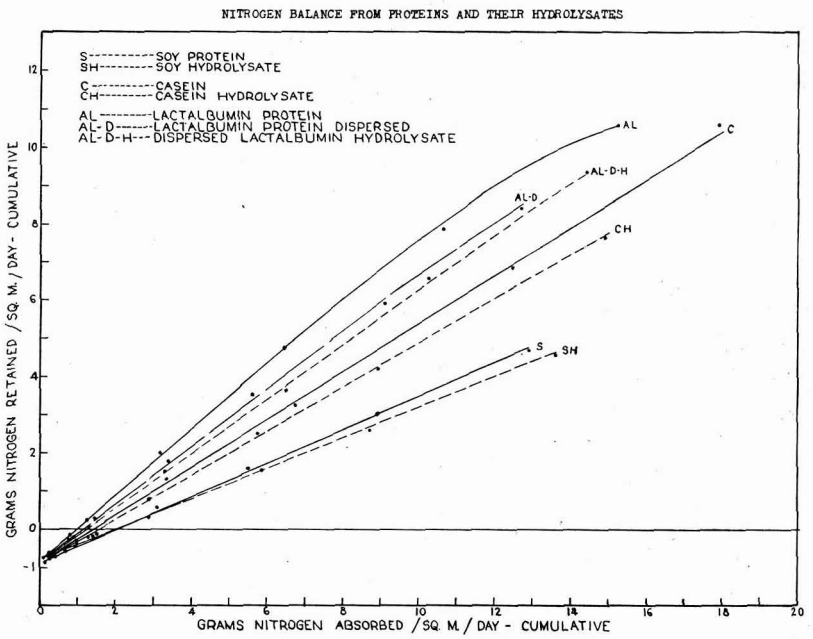

FIG. 1. Nitrogen balance from proteins and their hydrolysates.

Hemoglobin determinations and blood counts were made at the beginning and at the end of the 24-day test period. In all cases (table 3) there is an increase in both the hemoglobin and blood count. The data are not adequate for demonstrating a clear and conclusive differentiation between the different proteins and their respective hydrolysates.

Further evidence concerning the relative merits of the proteins and their hydrolysates is shown by the difference in nitrogen intake necessary to bring the animals to a state of equilibrium following the negative nitrogen balance existing at the end of the depletion period. Table 4 shows these values calculated as cumulative nitrogen intake per square meter of body area. The

TABLE 3

Hemoglobin and blood count regeneration as affected by the test proteins and hydrolysates during the 24-day test period

Material	Start of test period		End of test period	
	Hemoglobin	Blood count	Hemoglobin	Blood count
	gm./100 ml.	*cu. mm.*	*gm./100 ml.*	*cu. mm.*
Lactalb.	10.2	7,610,000	10.4	8,770,000
Dispersed lactalb.	9.9	7,550,000	10.9	8,130,000
Lactalb. hydrolysate	8.4	6,260,000	10.8	6,770,000
Casein	7.0	6,910,000	9.8	8,170,000
Casein hydrolysate	9.2	6,175,000	10.7	8,520,000
Soy protein	9.8	8,140,000	11.2	9,730,000
Soy protein hydrolysate	7.9	5,457,000	9.8	6,850,000

relationship between individual proteins and between the protein and its hydrolysate remains the same as shown by the curves in figure 1.

It is not presumed that analytical values are the only criteria for determining the relative merits of protein material. Weight gain is the most commonly used method for evaluating proteins, both qualitatively and quantitatively. Figure 2 correlates the loss or gain in weight with nitrogen retention for the entire 24-day test period involving the six different nitrogen intake levels. The graphs therefore, represent the overall results from the test products in overcoming the deficit in body nitrogen reserves and for reestablishment of weight gain. Lactalbumin and casein are shown to be superior to the soy protein. Gains of 10 to 12 or 15 grams per four-day period at the higher intake levels were not uncommon from the milk proteins after body reserves had been replenished at the lower intake levels.

Since high reserves of body protein are known to reduce nitrogen loss following hemorrhagic or traumatic shock, the influence of the three hydrolysates and a dispersible casein were used in a comparable manner designed to determine their effectiveness in reducing urinary nitrogen loss following severe shock. Four groups of mature rats were placed on the nitrogen-free ration previously described until a negative nitrogen balance

TABLE 4

Nitrogen intake required to establish equilibrium following the nitrogen depletion period

Material	Nitrogen balance at end of depletion	Nitrogen intake to establish equilibrium	
	gm./sq. m./day	*gm./sq. m.*	*gm./rat*
Lactalb.	0.6841 –	2.981	0.0954
Lactalb. hydrolysate	0.9826 –	3.203	0.1086
Casein	0.7909 –	5.400	0.1647
Casein hydrolysate	0.7315 –	6.456	0.1672
Soy protein	0.9598 –	3.780	0.1739
Soy protein hydrolysate	0.7602 –	8.994	0.2401

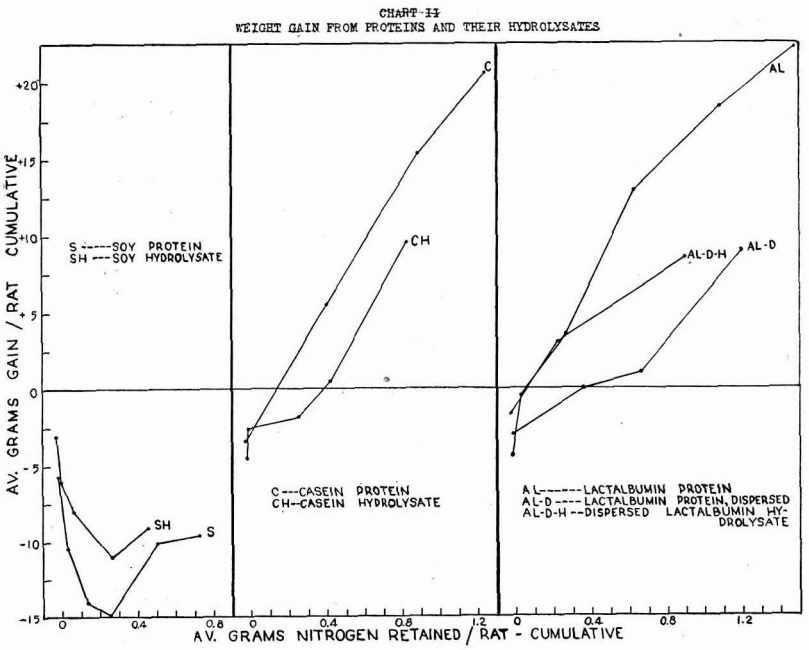

FIG. 2. Weight gain from proteins and their hydrolysates.

was well established. Two ml. of blood were withdrawn from the heart by hypodermic syringe and replaced with the test material dissolved in 2.0 ml. of physiological salt solution. The injection carried 15.9 mg. of nitrogen. The entire operation involved about five minutes. Since the object was to determine the comparative effectiveness of the test materials in reducing nitrogen loss, the urinary excretion was determined for the 48-hour period prior to injection and for a similar period following injection. As might be expected the highest percentage increase resulted following injection of the unhydrolyzed protein (table 5). The nitrogen excretion following injection

TABLE 5

Comparative loss of nitrogen before and after hemorrhagic shock and injection of protein hydrolysates

Material	Loss during 48 hr. before shock	Loss during 48 hr. after shock	% increase
	mg.	*mg.*	
Lactalb. hydrolysate	79.2	110.1	39.0
Casein hydrolysate	99.6	148.6	49.2
Dispersed casein	85.4	244.2	185.9
Soy protein hydrolysate	72.3	153.2	110.5

of the hydrolysates was substantially lower; the lactalbumin hydrolysate being most effective in reducing nitrogen loss, the soy hydrolysate being least effective, with the casein hydrolysate in an intermediate position approaching the effectiveness of the lactalbumin. Rate of nitrogen excretion for all groups returned to the pre-injection level after 5–7 days on the nitrogen-free ration.

SUMMARY

The various data illustrating different methods for the biological evaluation of the protein lead, generally speaking, to the same conclusions. The evidence clearly shows the superiority of lactalbumin over casein and the superiority of both casein and lactalbumin over soy protein. If a relative value of 100 was assigned to the lactalbumin, the value for the casein would be about 80 and that of the soy protein between 50 and 60. These general relationships have been indicated by published data from time to time, although the magnitude of the differences have been subject to variations depending upon the particular methods used.

Reference has been made to the linear relationship between nitrogen balance and absorbed nitrogen wherein variations in the slopes of the curves are of value as a means for determining the relative or absolute merits of the test materials. Such evidence shows that in each instance the original protein is slightly superior to its hydrolysate in respect to maintenance of a positive nitrogen balance. This relationship, however, is not consistently shown by the calculated biological values (table 1). The relatively greater difference between the iso-electric albumin and its hydrolysate may be accounted for by the intermediate step of peptization before hydrolysis. The difference between the peptized lactalbumin and the hydrolysate prepared direct therefrom is not of substantial magnitude.

Two significant deductions may be drawn from the data as a whole. First, appropriate preparation of protein hydrolysates may offer a means of extracting unavailable or indigestible protein from sources not recognized as suitable food products. Second, the mere stipulation of a given percentage of protein in foods or feed stuffs is inadequate in describing their protein value. For instance, in using the nitrogen balance data as a basis of interpretation the soy protein has only about 50 per cent the value of the lactalbumin and about 60 per cent the value of casein; the other data are in substantial agreement. From the standpoint of protein conservation and food economy it is seemingly apparent that as a particular protein more nearly approaches the ideal a commensurate reduction in the amount of dietary protein could be made. The physiological aspects of the lower protein intake may also be of importance from the standpoint of health because the data indicate that a large proportion of the nitrogen absorbed from inferior protein is excreted without commensurately contributing to physiological functions.

The average survival period of the groups of animals used in these investigations when furnished the nitrogen-free diet following the test period tends to indicate a significant effect of the previous protein intake. The average survival time for the lactalbumin group was about 30 per cent longer than for the soy protein group, and the survival time for the casein group was about 24 per cent longer than for the soy protein group.

One may presume that the variations observed are due to an imbalance or deficiency of amino acids, or possibly to an absence of physiologically important linkages in the inferior protein molecule. If evidence should clearly disclose that the former condition is the cause of the differences, the fortification and enhancement of a deficient protein by appropriate synthetic amino acids in proper balance should prove to be of importance from the standpoint of health and economy and conservation of natural food protein.

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STUDIES OF THE EFFECT OF HEAT ON MILK DIALYSATES

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About twenty years ago Grindrod (3) patented a process for dialyzing unsterilized, evaporated milk, and claimed that the removal thereby of 10 per cent of the salts resulted in a greatly improved flavor and color. He indicated that the calcium, citrate, and phosphate ions in excess were detrimental to flavor and color, although his evidence to prove this point was not conclusive. Kass and Palmer (5) showed that citrates and phosphates catalyze the color development or caramelization reaction but they did not study flavor. The claims of Grindrod for flavor improvement prompted us to investigate further the relation of dialysis to heated flavor development.

In checking Grindrod's claims it was found that dialyzing forewarmed or evaporated milk did result in a decreased flavor when the milk was sterilized. However, when the milk was reconstituted with respect to eight known constituents, for which analysis had been made, no improvement in flavor resulted when the milk was sterilized. When raw milk was dialyzed and then forewarmed and evaporated an improved flavor resulted even though the milk had been reconstituted with respect to the known eight constituents. These results indicated that unheated milk contains an unidentified substance which is dialyzable and which contributes to the flavor development of sterilized evaporated milk. These results also indicate that if the milk has been heated before dialysis this substance is not removed by dialysis. In the latter case, however, the salt balance of the milk must be restored before the unknown substance will develop its flavor upon sterilization.

Aging experiments on these dialyzed (reconstituted) samples demonstrated that any flavor improvement was temporary in effect. In fact dialyzed evaporated milk became tallowy in storage whereas milk dialyzed prior to any heat treatment showed about the same degree of aged flavor as the control. The commercial impracticality of using such a procedure for improving the flavor, and the fact that only temporary effects were gained, were, however, not sufficient reasons to justify abandoning the approach of dialysis studies toward learning more about the cause and source of heated milk flavor and aroma.

The dialysis experiments demonstrated that some unrecognized substance of low molecular weight was being removed from milk by dialysis which contributed to the flavor of heated milk. It was discovered that the dialysate from raw milk when heated to 80°–100° C. for 10 minutes did produce a

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cooked milk flavor and odor, though not the typical sulphide aroma from heat-coagulated proteins. This was an unexpected finding and, to our knowledge, has not been reported in the literature dealing with cooked milk flavor. (For a review of the literature on cooked milk flavor the reader is referred to the work of Gould and Sommer (2).) Therefore an extensive effort was made to examine the milk dialysate to determine the source of the cooked flavor and aroma.

EXPERIMENTAL RESULTS

Preliminary fractionation attempts. Initial attempts were made to evaporate the dialysate to dryness under vacuum and at low temperature. It was hoped the residue could then be fractionated through the use of various solvents. This procedure was soon abandoned because of the large volumes of dialysate involved and because the high concentration of lactose and salts in the residue made fractionation difficult. Selective precipitation from the dialysate seemed to be a more reasonable approach.

The addition of $BaCl_2$ to the dialysate from raw milk, following removal of the phosphates at pH 8.5 with $CaCl_2$, forms a precipitate which, upon removal, prevents any cooked odor from appearing in the supernatant liquid upon heating. The barium precipitate upon analysis was shown to consist of barium sulphate and the barium salt of some organic compound. When the organic portion of the precipitate was dissolved and heated, it had a fatty odor but not that of heated milk. Apparently the compound had been destroyed in isolation.

A series of fourteen chemical reagents (mostly salts of heavy metals) of varied properties were prepared in M/1 concentrations for the purpose of attempting to precipitate the cooked flavor precursor from the dialysate without destroying it chemically. Of this whole series it was found that only $HgCl_2$ at pH 8.0 was effective in removing the substance, and fortunately upon regeneration of the precipitate with H_2S and heating the eluate, the cooked aroma was obtained. Considerable experimentation with various salts at varying pH was carried out for the purpose of removing impurities prior to the mercury precipitation. Too high alkalinity (above pH 8.5) was definitely detrimental but it appeared that high acidity in dilute aqueous solution was not harmful even after a week at room temperature.

A working procedure for gross isolation was adopted whereby fresh raw whole milk was dialyzed in cellophane casings against an equal volume of water for 24 hours in the refrigerator. The dialysate was warmed to room temperature and 30 ml. of M/1 $CaCl_2$ were added per liter of dialysate. The pH was adjusted to 8.0 to remove the phosphates, which settled from the supernatant liquid permitting decantation and centrifuging. 100 ml. of a 0.2 M $HgCl_2$ solution were added per liter of supernatant liquid and the pH was adjusted to 7.8 to 8.0. A heavy yellow precipitate settled rapidly and was filtered. This precipitate was washed from the filter paper and sus-

pended in a minimum of water. H_2S was bubbled into the solution while being shaken mechanically for about 30 minutes. The black mercury sulphide was filtered off and H_2S was removed by evacuation and slight evaporation of the solution. The pH of this Hg-free solution was approximately 2.0 probably due to the HCl present from $HgCl_2$ used for precipitation. This solution has always given the cooked aroma in repeated isolation experiments.

Many fractionation experiments and analyses have been carried out using this Hg-free solution as the starting material. A preliminary analysis of this Hg eluate gave the results shown in tables 1 and 2.

TABLE 1

Quantitative analysis of Hg eluate; 100 ml. from 5 gallons of dialysate

Constituent	Concentration	
	mg. per cent	*per cent of solids*
1. Total solids	420.0	100.0
2. Total nitrogen	55.0	13.1
3. Amino nitrogen	11.0	2.62
4. Reducing material calculated as glucose	26.5	6.3
5. Phosphorus	0.0	0.0
6. Sulphur	0.0	0.0
7. Arginine	3.4	0.8
8. Tyrosine	4.2	1.0
9. Creatine and creatinine	17.6	4.2

TABLE 2

Qualitative tests on Hg eluate

Chemical Test	Result
1. Pentose	Questionable
2. Tryptophane	Present
3. Histidine	Present
4. Aldehyde (with fuchsin-sulphurous acid)	Absent
5. Acetyl-glucosamine	Absent
6. Na-nitroprusside and KCN	Absent
7. Labile S with hot alk. $PbAc_2$	Absent
8. Biuret	Absent
9. Phenylhydrazine ppt.	Absent
10. Semicarbazone ppt.	Absent

It is concluded that the solution represents a mixture of substances highly nitrogenous in character and having a high reducing power. Some of the reducing power probably is due to contaminating lactose. The absence of P and S is highly significant and limits the field of suspected compounds considerably.

Since the Hg eluate undoubtedly represented a mixture of substances, various means of fractionation were tried. $PbAc_2$ was found to remove a large proportion of the nitrogenous and highly reducing materials which did not show cooked aroma upon regeneration with H_2S and heating. A white

crystalline fraction characterized by hexagonal plates was obtained from this Pb eluate. The total amount of cooked aroma substance seemed to decrease with the Pb treatment and it was finally concluded that a degradation accompanied the treatment. Therefore it was abandoned.

An attempt was made to utilize the synthetic resins Amberlite IR–4 and IR–100 for purifying and separating the cooked flavor producing substance from impurities. Block (1) successfully separated the basic amino acids from protein hydrolysates by use of these resins. It was found that the cooked flavor substance could be absorbed from solution by the IR–100 after a preliminary treatment with IR–4 but no reagent was discovered which would elute the desired material from the resin. The possibility also exists that our substance was destroyed in the treatment thus accounting for the failure to elute.

The most promising method of fractionation of the mercury eluate seemed to be that of evaporating under high vacuum and low temperature, removal of crystalline fractions or precipitates as they formed, and continued evaporation to dryness, testing all precipitates for cooked flavor by solution and heating. The cooked flavor producing substance remains in solution until practically all the H_2O has been evaporated and after removal of two or three yellow crystalline or granular fractions possessing no cooked flavor properties. The final liquid was first thought to be a heavy viscous yellow oil but actually it was a syrup since it was found that it could be dried by continued evacuation with the Hy-Vac pump. It dried on the walls of the flask as a white crystalline material, interspersed with red-yellow streaks. Strong vapors of HCl were present in the flask and accounted for the high acidity of the mercury eluate.

From a solubility basis it was found that the dry crystalline material is most soluble in water, slightly soluble in methyl alcohol, and less soluble in ethyl alcohol. It is insoluble in acetone, dioxane, petroleum, and ethyl ether, chloroform and isobutyl alcohol. A further purification was attempted on the basis of differential solubility.

The highly viscous syrup prior to removal of the last traces of water was diluted with an equal volume of methyl alcohol. A white crystalline fraction separated rapidly which was found to be almost cubical in shape. This fraction dissolved upon the addition of more methyl alcohol. Acetone caused a white sticky precipitate which contracted into a ball. This was centrifuged off and it was found upon dissolving in water and heating that the acetone insoluble portion possessed the cooked flavor.

When the syrup was completely dried and a minimum of methyl alcohol added, a white crystalline fraction similar to the above separated. The addition of more alcohol caused it to dissolve and upon evaporation of this solution it became darker in color, changing from red-yellow to red-black. The dry material was rather brown in color. Resolution in methyl alcohol and

evaporation after standing a few hours caused a considerable darkening in color—deep cherry red color. Upon drying it became blacker and changed to a tarry mass. It was noted that acetone after treating with dry HCl had similar properties, changing from yellow-green to red, due to the formation of phorone (4). In this respect our concentrate has a property in common with acetone. A loss of power to produce cooked flavor accompanied the color-changing phenomenon noted above. This loss seemed considerably more rapid in acid methyl alcohol in the absence of water than in the former case where water was used to dissolve the dry material and alcohol used to crystallize. Considerable material was lost in this manner before the conditions for stability were established.

Revised methods of isolation of cooked flavor precursor. Fourteen batches of dialysate varying from 5 to 25 gallons each were prepared during the next period of investigation. In all cases a volume of water was used approximately equal to that of the milk. Skim milk was employed satisfactorily for some of the 25-gallon batches. A tank $8' \times 1' \times 1'$ was constructed for large-scale operations, which minimized the labor of filling and tying the cellophane tubes. It was found possible to utilize the dialyzing tubing several times by washing thoroughly and heating in boiling water to destroy bacteria.

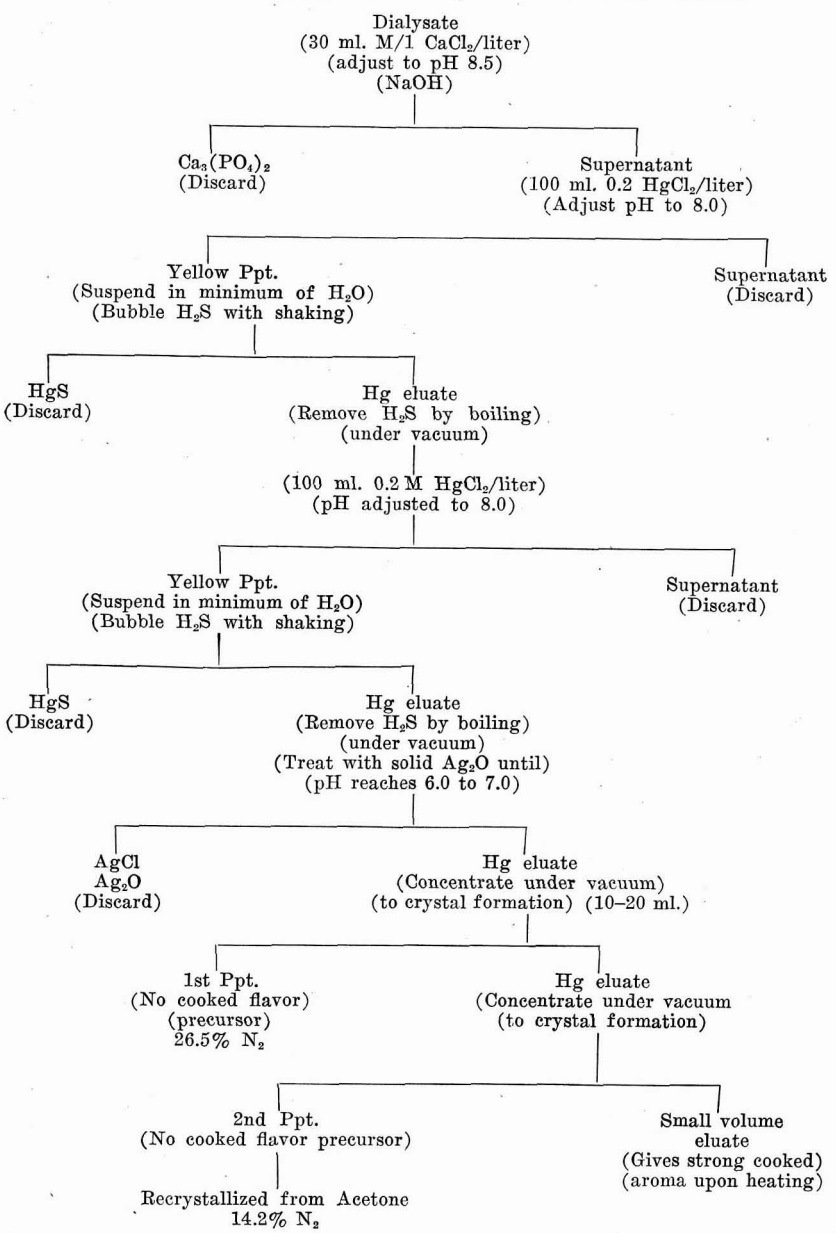
Some additions were made to the isolation procedure. A second mercury precipitation and regeneration was found to be advantageous for removal of lactose. Neutralization of the second mercury eluate with AgO to pH 6.0 to 7.0 followed by filtration of AgCl removed excess HCl which had caused destruction of the cooked flavor precursor, especially when organic solvents were employed for subsequent fractionation.

Various crystalline fractions were isolated from this solution by concentration under vacuum and by means of organic solvents. These fractions were subjected to analyses and recrystallizations with the ultimate hope of identifying them.

Properties of fractions. It was recognized that the substance isolated by the adopted isolation procedure consisted of a mixture of substances as evidenced by variable nitrogen contents and reducing values. Attempts to fractionate this substance resulted in impure fractions some of which were crystalline and possessed varying degrees of cooked aroma precursor as evidenced by heating 10 mg. dissolved in 10 ml. H_2O. The results indicated that the cooked aroma precursor was carried along in these fractions as an impurity and could produce a strong cooked aroma when present in concentrations lower than 1 mg./ml. Later work revealed that 0.2 mg./ml. was sufficient to give a strong cooked aroma and amounts less than 0.2 mg./ml. could be detected.

It was noted that the cooked flavor substance was only slightly soluble in neutral methyl alcohol. Therefore repeated attempts at purification

MILK DIALYZED AGAINST AN EQUAL VOLUME OF WATER



using this organic solvent were tried. Unfortunately an inactivation or destruction appeared to accompany repeated treatment with methyl alcohol. It was reasoned that if a trace of formaldehyde were present in the alcohol it could react with the amino groups in the cooked flavor substance and thus account for the loss of active substance. Even though freshly purified methyl alcohol was used there resulted a destruction of the cooked flavor substance. Therefore fractional crystallization from neutral aqueous medium was resorted to and found to cause less destruction than did the methyl alcohol.

Six lots of dialysate were subjected to this fractional crystallization treatment. A crystalline fraction of rods separated at 10–20-cc. volume (from 5 gal. dialysate) and was filtered off. This fraction (recrystallized) had 26.5 per cent N_2 and did not produce cooked aroma on heating. A strong Schiff test was given for this fraction whereas a negative test was obtained in those fractions showing strong cooked aroma.

Further evaporation of the supernatant liquid caused a needle or rosette fraction to crystallize. This fraction was first thought to be the cooked aroma substance, but when care was taken to remove all the supernatant liquid by suction, it likewise was found to be inactive. The cooked aroma substance still remained in the supernatant liquid. This needle-rosette fraction crystallized from cold 25 per cent acetone solution readily. It was found to contain approximately 14.2 per cent N_2.

The volume of solution remaining after removal of the two crystalline fractions was exceedingly small, thus preventing further fractional crystallization attempts. Further evaporation of the supernatant liquid resulted in a sticky, hygroscopic non-crystalline mass. Larger initial volumes of dialysate will be necessary before this last fraction can be obtained in quantities large enough for analysis.

Identification. Even though the cooked flavor precursor has not been isolated in the pure state attempts have been made to identify the substance in the impure mixture.

It has been concluded that the reducing power found in original samples (table 1) is due to contaminating lactose and not a property of the active substance. In all cases where a highly active substance has been purified a rather high but variable nitrogen content has been found. Two contaminating crystalline fractions have been isolated and recrystallized which have high nitrogen contents. Large amounts of these fractions remaining in the active substance may be misleading us with regard to nitrogen content.

The possibility that the two crystalline fractions might be inactive degradation products of an active parent substance has been considered. It has always appeared that we suffered a continual loss of active substance in our isolation attempts. This loss corresponds to a chemical degradation with time rather than a manipulative loss which might accompany inefficient

separation by precipitation or elution. The two crystalline fractions have been heated together at varying proportions of concentration with no cooked aroma resulting, thus eliminating the possibility of an interaction of the two compounds to produce the cooked aroma.

Attempts to identify the cooked aroma substance by heating a few milligrams of various chemicals dissolved in water or 5 per cent lactose solution have failed. To date 46 chemical compounds of known structure, which might be suspected as being the cooked flavor precursor, have been tried but not one has had an aroma similar to that of our unknown substance. Types of compounds tried have been amino acids, vitamins, purine bases, amines and substituted amines and a few miscellaneous compounds. Apparently the final solution of the problem will appear only upon isolation and identification of the particular compound in question.

DISCUSSION

After much of this work had been carried out it became apparent that other methods of procedure would have to be employed to prevent the large degradative losses of active substance. A re-evaluation of the effect of pH on stability showed that acidity was far more destructive than originally believed. Neutral solutions were the most stable, so any new attempts at isolation should be based on this consideration. Possibly a less acid mercury compound such as $HgAc_2$ or the use of a less acid regenerating agent such as Na_2S or $(NH_4)_2S$ could be used satisfactorily.

The cooked flavor precursor may be any one of a multiple of nitrogenous products in milk. A consideration of all the properties and reactions of the cooked flavor precursor as well as the analysis of some of the compounds isolated which might be considered as degradation products suggest the possibility of phospholipids and cerebrosides of milk (6) being the compounds involved. They are in general slightly soluble in water, non-crystalline, should dialyze through cellophane membranes because of their surface activity and their low molecular weight of about 800, they form compounds with $HgCl_2$, are precipitable with acetone and are labile compounds to acid, alkali and heat. The acid regeneration treatment of the mercury precipitate could cleave the molecule such that the phosphorus remained insoluble, but the remaining portion of the molecule could be eluted and still give rise to the cooked aroma upon heating. Of course there is no phosphorus present in cerebrosides so this explanation is not necessary for this compound. They all contain nitrogen residues and fatty acids; cerebrosides contain the carbohydrate galactose, a reducing sugar which we could have erroneously thought to be lactose.

The needle or rosette fraction with a nitrogen content of 14.2 per cent agrees fairly well with either trimethylamine hydrochloride or amino ethanol hydrochloride (theoretical N for both = 14.7 per cent). The rod-shaped frac-

tion showing a nitrogen content of 26.5 per cent might be ammonium chloride (theoretical N = 26.2 per cent) except that its solubility in water is far too low.

SUMMARY AND CONCLUSIONS

Dialysis experiments on raw milk demonstrated that some unrecognized substance of low molecular weight could be removed, which contributed to the flavor of heated milk.

It was discovered that the dialysate from raw milk when heated to 80°–100° C. produced a heated milk flavor and odor, though not the typical sulphide aroma obtained from heat-coagulated proteins. The flavor and aroma of heated milk appears to be derived from two sources—the heat coagulable proteins and a dialysate factor.

The addition of a barium salt prevented the production of a cooked aroma upon heating. Because it was not found possible to regenerate the cooked aroma precursor from the barium precipitate, it was concluded that the desired compound had been destroyed.

Of fourteen other precipitating agents tried, only $HgCl_2$ at pH 8.0 was effective in removing the cooked flavor precursor from the dialysate. Cooked aroma was obtained by heating the eluate from the H_2S regenerated precipitate after complete removal of residual H_2S.

Some of the properties of the partially purified cooked flavor precursor were established, significant of which are its solubility in water, lesser solubility in methyl alcohol, slight solubility in ethyl alcohol and insolubility in other solvents such as acetone, ethers and chloroform. There is no P or S present in the compound. It is unstable in aqueous alkaline solutions, but somewhat stable in aqueous acid solution. It is very unstable in acid methyl alcohol solution free from water, showing a reaction similar to that of acetone in the presence of dry HCl.

Fourteen batches of dialysate varying from 5 to 25 gallons each were used for the purification of the cooked aroma precursor by revised methods. A second mercury precipitation and the use of Ag_2O to remove excess HCl improved the isolation procedure.

Two crystalline fractions have been isolated and recrystallized from the purified cooked flavor concentrate, neither of which is the desired cooked flavor precursor. A rod fraction having 26.5 per cent nitrogen and a needle-rosette fraction with 14.2 per cent nitrogen have been obtained in good quantity and recrystallized with the ultimate hope of identifying them.

The cooked flavor substance has not been isolated in its pure crystalline state. Slow inactivation or destruction with all techniques tried to date has made the problem difficult.

Of 46 chemical compounds of known structure which might be suspected as the cooked flavor precursor, not one produced an odor resembling that of the unknown substance when heated in aqueous solution.

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ABSTRACTS OF LITERATURE

BOOK REVIEWS

351. Currents in Biochemical Research. EDITED BY DAVID E. GREEN. 486 pages. $5.00. Interscience Publishers, Inc., 215 Fourth Ave., New York 3, N. Y.

"*Currents in Biochemical Research* represents an attempt by some thirty research workers to describe in as simple language as possible the important developments in their own fields and to speculate a little on the most likely paths of future progress. The aim of these essays has been to excite the imagination and to provide glimpses of some of the fascinating horizons of biochemical research."

The subjects treated and the authors of these essays are as follows: (1) The Gene and Biochemistry, G. W. Beadle; (2) Viruses, W. M. Stanley; (3) Photosynthesis and the Production of Organic Matter on Earth, H. Gaffron; (4) The Bacterial Cell, René J. Dubos; (5) The Nutrition and Biochemistry of Plants, D. R. Hoagland; (6) Biological Significance of Vitamins, C. A. Elvehjem; (7) Some Aspects of Vitamin Research, Karl Folkers; (8) Quantitative Analysis in Biochemistry, Donald D. VanSlyke; (9) Enzymic Hydrolysis and Synthesis of Peptide Bonds, Joseph S. Fruton; (10) Metabolic Process Patterns, Fritz Lipmann; (11) Biochemistry from the Standpoint of Enzymes, David E. Green; (12) Enzymic Mechanisms of Carbon Dioxide Assimilation, Severo Ochoa; (13) Hormones, B. A. Houssay; (14) Fundamentals of Oxidation and Reduction, Leonor Michaelis; (15) Mesomeric Concepts in the Biological Sciences, Herman M. Kalckar; (16) Viscometry in Biochemical Investigations, Max A. Lauffer; (17) Isotope Technique in the Study of Intermediary Metabolism, D. Rittenberg and David Shemin; (18) Mucolytic Enzymes, Karl Meyer; (19) Some Aspects of Intermediary Metabolism, Konrad Bloch; (20) The Steroid Hormones, Gregory Pincus; (21) Plant Hormones and the Analysis of Growth, Kenneth V. Thimann; (22) Chemical Mechanism of Nervous Action, David Nachmansohn; (23) Some Aspects of Biochemical Antagonism, D. W. Wolley; (24) Chemotherapy: Applied Cytochemistry, Rollin D. Hotchkiss; (25) Biochemical Aspects of Pharmacology, Arnold D. Welch and Ernest Bueding; (26) Some Biochemical Problems Posed by a Disease of Muscle, Charles L. Hoagland; (27) Physiology and Biochemistry, Surgeon Captain C. H. Best; (28) X-Ray Diffraction and the Study of Fibrous Proteins, I. Frankuchen and H. Mark; (29) Immunochemistry, Michael Heidelberger; (30) Social Aspects of Nutrition, W. H. Sebrell; (31) Organization and Support of Science in the United States, L. C. Dunn. T.S.S.

352. Annual Review of Biochemistry, Vol. XV, 1946. EDITED BY J. MURRAY LUCK. 616 pages plus author and subject indices. $5.00. Annual Reviews Inc., Stanford University P. O., California.

Again Annual Reviews Inc. brings us up to date in the biochemical field with Volume XV of Annual Review of Biochemistry. The subjects covered and the authors are as follows: (1) Biological Oxidations and Reductions, K. A. C. Elliott; (2) Non-Oxidative Enzymes, A. M. Wynne; (3) Plant Carbohydrates, S. Peat; (4) The Chemistry of the Lipids, J. B. Brown; (5) The Chemistry of the Proteins and Amino Acids, T. L. McMeekin and R. C. Warner; (6) The Chemistry of the Steroids, T. Reichstein and H. Reich; (7) Carbohydrate Metabolism, C. F. Cori and G. T. Cori; (8) Fat Metabolism, W. C. Stadie; (9) The Metabolism of Proteins and Amino Acids, D. Rittenberg and D. Shemin; (10) The Vitamins, R. A. Dutcher and N. B. Guerrant; (11) The Chemistry of the Hormones, H. Selye and H. Jensen; (12) The Biochemistry of Teeth, H. M. Leicester; (13) Growth Factors for Microorganisms, E. E. Snell; (14) Photosynthesis, C. S. French; (15) The Respiration of Plants, W. O. James; (16) The Biochemistry of Yeast, C. Neuberg; (17) Bacterial Metabolism, H. A. Barker and M. Doudoroff; (18) Immunochemistry, E. A. Kabat; Organic Insecticides, W. M. Hoskins and R. Craig; The Viruses, N. W. Pirie; Inactivation and Detoxication of Pressor Amines, W. H. Hartung.

The increasing tempo of activity in the field of biochemistry makes the Annual Review a must in the library of the teacher and research worker. The present volume maintains the high level of those that have previously appeared. T.S.S.

353. Enzymes and Their Rôle in Wheat Technology. EDITED BY J. ANSEL ANDERSON. Published for the American Association of Cereal Chemists by Interscience Publishers, Inc., New York. 1946. 371 pages, 11 chapters, indexes.

This is the first of a proposed series of monographs sponsored by the American Association of Cereal Chemists on subjects of immediate interest to cereal chemists. Enzymes play a very important part in cereal technology. This monograph is restricted principally to those of greatest interest to cereal chemists. It includes the amylases, proteases, lipases, oxidases and the fermentation enzymes. For each of these are twin chapters; the first covering a broad review of existing knowledge of the class, including both plant and animal enzymes, the second a discussion of the rôle of the particular enzymes in wheat technology. This monograph involves, therefore, a review of the knowledge extant on the properties of the enzymes, and the application of this knowledge to utilization of cereals, particularly wheat, in milling, baking and fermentation processes. The increasing diversity of

processes within the dairy industry includes those in which enzymes play an important part, viz., malt products, protein derivatives, cheese, and ferment products. This book is to be highly recommended to the student, and to the dairy research laboratory for the fundamental and practical knowledge it provides. Various chapters written by cereal chemists include: an excellent review of the general chemistry of enzymes; amylases, and their applications in milling and baking technology; esterases, their rôle in milling and baking; oxidizing enzyme systems, their occurrence and effects in wheat and flour; proteases, their rôle in baking; the mechanism of alcoholic fermentation, yeast fermentation. K.G.W.

BUTTER

354. Bacteriology of Butter. VIII. Salt Distribution in Butter and Its Effect on Bacterial Growth. W. H. HOECKER AND B. W. HAMMER. Iowa State College Res. Bul. 339. 1945.

Micromethods were used to determine the moisture and salt contents of butter. It was found that the moisture and salt contents became more and more uniform as working progressed and their distribution was uniform in well-worked normal butter. Generally, in mottled butter, the light-colored portions contained the least salt and always the smallest droplets of water.

When butter was first salted and lightly worked, the water droplets in the original butter granules were free from salt. It is believed that even in well-worked butter there were some of these droplets without salt. Moisture and salt distribution may be made less uniform by printing.

When organisms causing butter spoilage were added to churned butter before working, the presence of 1.5% salt slowed down their growth and the rate of spoilage. However, when these organisms were added to the cream just before churning, the results varied. When the butter was poorly worked spoilage was often more rapid as the moisture in the original butter granules had no salt. As working progressed the salt distribution and keeping quality were improved for fewer water droplets were free of salt. A.C.D.

355. German Continuous Butter Churn. Jour. Milk Technol., *9*, No. 3: 143. May–June, 1946.

A continuous butter-making machine was captured in Germany by the Quartermaster Corps Intelligence Team. The machine was designed to produce 1500 pounds of butter per hour, and was manufactured by Roth Moelkerei Maschinenfabrik in Stuttgart.

The machine will be tested by the Research Committee of the American Butter Institute under contract with the Quartermaster Corps Food and Container Institute. Dr. H. A. Ruehe, Department of Dairy Husbandry, University of Illinois, will supervise the experiments in the Beatrice Creamery Company plant at Champaign, Illinois.

The results will be reported to the Quartermaster Corps and turned over to the Office of the Publication Board for general release to all interested persons. H.H.W.

CHEMISTRY

356. The Fatty Acids of Human Milk Fat. J. B. BROWN AND BETTY M. ORIANS, Laboratory of Physiological Chemistry, Ohio State University, Columbus, Ohio. Arch. Biochem., *9*, No. 2: 201–219. 1946.

The relatively large volume of 52 liters of human milk, yielding 1300 grams of fat, was used for this investigation. The component fatty acids were separated by the ester fractionation procedure followed by low-temperature crystallization. Details of the method as well as six separate charts outlining each step are given. A comparative table of fatty acid values reported by the principle previous investigators as well as the present work is presented. The authors conclude that "no evidence could be found for the presence of more than traces of acids below C_{10}" and—"the amount of linolenic acid is negligible. The finding of dienoic acids of series above C_{18}, . . . was confirmed, and in addition evidence was presented for the presence of high molecular weight trienoic acids. This specimen of fat is much more similar to human body fat than it is to a typical milk fat." A.O.C.

357. The Distribution Pattern of Fatty Acids in Glycerides of Milk Fat. E. L. JACK, J. L. HENDERSON, AND E. B. HINSHAW (From the Division of Dairy Industry, University of California, Davis). Jour. Biol. Chem., *162*, No. 1: 119–128. 1946.

Milk fat (neither the source nor the season of the year are given) was dissolved in Skellysolve A and fractionated by freezing out at various temperatures (for detailed description see JOUR. DAIRY SCIENCE, *28:* 65–78, Jan., 1945). These various fractions, as well as the methyl esters obtained from each, were analyzed. The molar percentage of the various fatty acids, which include the C_4 to C_{22} both saturated and unsaturated, are listed for each fraction and are compared with similar values on the whole fat.

Considerable discussion is devoted to the probable distribution pattern of the various fatty acids in the glycerides and evidence is given to show that it "tends more nearly to the pattern of *widest possible distribution* than to the pattern of *random distribution.*" Some interesting hypothetical patterns are compared with the experimental data. A.O.C.

FOOD VALUE OF DAIRY PRODUCTS

358. The Effect of Fat on the Utilization of Galactose by the Albino Rat. R. P. GEYER, R. K. BOUTWELL, C. A. ELVEHJEM, AND E. B. HART,

Department of Biochemistry, College of Agriculture, University of Wisconsin, Madison. Jour. Biol. Chem., *162*, No. 2: 251–259. 1946.

In previous work the Wisconsin workers reported that when whole milk was fed to rats, calves and pigs rather than skim milk as the sole diet there was less galactose excreted in the urine than on the non-fat diet. Other investigators later concluded that fat as such had no influence upon the utilization of galactose. The work reported here is a more detailed investigation relative to the influence of fat on galactose utilization when skim milk or synthetic rations containing lactose or galactose were fed to rats. It corroborates their previous work and substantiates the statement that "not only various fats such as butter fat, cocoanut oil, etc., were effective, but also that the fatty acid fraction was the active component of the fat." It was shown that only the even-numbered fatty acids having more than 10 carbon atoms could function in lowering the urinary galactose loss, and that glucose was found to be slightly effective with some of the animals. They further concluded that "Fat increased the utilization of galactose by the rat when either lactose or galactose is ingested. This phenomenon occurs on milk or synthetic type ration." A.O.C.

MILK

359. Quality Control of Milk as It Enters and Leaves the Plant. E. A. CRAWFORD, Dept. of Health, New York City. 19th Ann. Rpt. N. Y. State Assoc. Milk Sanit., p. 67. 1945.

First physical examination of milk is the odor. "Off odors" are usually due to feed, excessive bacterial content, and mastitis-infected udders. A reddish tinge in color indicates mastitis. The suction type sediment test is now preferred. The strainer dipper shows both excessive sediment and flakes. The temperature of the milk is obtained approximately by the hand on the outside of the can.

Plate counts on producers' milk are made by some plants. The direct microscopic count is probably best on milk with counts over 150,000 per ml. The Department of Health requires that plants using high-temperature short-time pasteurizers shall make weekly thermoduric counts on each source of milk supply. If three consecutive counts are high, then thermoduric counts must be made on each producer's milk. Producers' milk with thermoduric counts over 20,000 per ml. must be corrected. High thermoduric counts are generally caused by milking machines, milk stone, re-used strainer cloths, and open can seams.

Farm inspection is important. Recently cryoscopic tests on producers' milk have shown evidence of adulteration by watering in 10% of the samples.

At the raw milk shipping plant it is important to promptly cool the milk

to 36° F. or below and run directly into well-cleaned and sterilized tanks which should be nearly filled with milk. A.C.D.

360. Control of Milk Watering. PAUL CORASH, Dept. of Health, New York City, 19th Ann. Rpt. N. Y. State Assoc. Milk Sanit., p. 53. 1945.

Tests on retail milk in New York City show 0.6 to 1.8% to be sub-standard in butterfat or total solids in the past 5 years. Recently, the Department of Health began checking producers' milk for watering by determination of freezing points. Pure milk has a freezing point varying from −0.53 to −0.56° C. Night's and morning's milk for the same herd have the same freezing point. When the results of the test were to be confirmed, samples of milk were obtained from observed milkings on the farm. Tests should not be made on samples with increased acidity.

Rather striking evidence of watering milk was secured in some instances. This method of detecting watering was found to be very useful. A.C.D.

361. Water Supplies for Milk Plants and Dairy Farms. F. N. THOMSON, N. Y. State Dept. of Health, Albany, N. Y. 19th Ann. Rpt. N. Y. State Assoc. Milk Sanit., p. 7, 1945.

It seems quite unnecessary to emphasize the need for an ample supply of pure, safe water for a milk plant or dairy farm. There are certain difficulties with pollution that are sometimes experienced. In appraising the source of water it is necessary to consider the surroundings, the development, and the results of laboratory tests.

Although 100 to 200 feet distance between pollution and the water source is usually ample, there are some noted exceptions. In a limestone area the sewage from a milk plant polluted a spring 1½ miles away. Sometimes leaky sewers have been found to pollute wells. Many farm wells do not have satisfactory protection against surface contamination draining back into the well. A.C.D.

362. The Lack of Preservative Action of Surface-active Cationic Germicides in Milk. ADRIEN S. DUBOIS AND DIANA D. DIBBLEE, Onyx Oil and Chemical Co., Jersey City, N. J. Jour. Bact., *51,* No. 3: 406. March, 1946. Abs. Proc. of Local Branches.

"The bacterial count of raw milk was not affected by the presence of 1:500 to 1:25,000 dilutions of alkyldimethylbenzyl-ammonium chloride, upon incubation at either 10, 20, or 37° F. However, 1:500 and 1:1,000 dilutions of the germicide caused an immediate, appreciable reduction in the initial count of the milk. Less acid was produced in the treated milk during incubation. This was especially noticeable with the higher concentrations of the germicide.

"The lower acidity in the treated milk is assumed to be due to the inhibition of the gram-positive acid-forming organisms by the alkyldimethylbenzyl-ammonium chloride. The gram-negative rods are not inhibited and this accounts for the high bacterial counts. Qualitative evidence for this was obtained by identification of the organisms growing under the various conditions of test.

"Concentrations of surface-active cationic germicides varying from 1:500 to 1:20,000 can easily be determined in milk by titration with duponol PC in the presence of bromphenol blue." D.P.G.

MISCELLANEOUS

363. Rodent Control in Food Processing Plants. DONALD H. LEWIS, U. S. Fish and Wildlife Service, Ithaca, N. Y. 19th Ann. Report N. Y. State Assoc. Milk Sanit., p. 37. 1945.

Information is presented on the habits of rats and the damage which they cause. Modern rat control consists of four distinct methods, namely: (1) sanitation or elimination of food, (2) ratproofing buildings and elimination of exterior harborages, (3) killing by traps, poison, and fumigants, and (4) maintenance of the first three conditions. It was pointed out that red squill is the only rat poison that is not toxic to man and most domestic animals.

A.C.D.

364. Insect Control with DDT in Dairy and Milk Plants. CAPT. R. S. TAGGART, Sanitary Corps (R), U. S. Army, Washington, D. C. 19th Ann. Rept. N. Y. State Assoc. Milk Sanit., p. 21. 1945.

DDT was first synthesized in 1874 by a German chemist, Othman Zeidler. In 1939, Paul Muller in Switzerland was looking for an insecticide for the J. R. Geigy Company to control the Colorado beetle and he discovered the phenomenal powers of DDT. The U. S. Bureau of Entomology and Plant Quarantine, in 1942, tested numerous insecticides for war purposes and enthusiastically endorsed DDT.

The ordinary housefly and stable fly are killed by DDT within 30 minutes or more. The spray is not a repellent. The spray needs 3 to 5 per cent DDT and surfaces need good coverage. Tests show one spraying in early June and one in early August will keep barns free of flies. The residual effect lasts 1 or 2 months. The use of this spray does not reduce the need for other good sanitary measures. The spray is effective only against flies and not against eggs or larvae.

A 10% DDT mixture in talc will destroy cattle lice. This powder is effective against the American and German cockroaches but the results are not as spectacular or as complete as against flies. This powder is effective against fleas.

DDT may be safely handled with reasonable precautions. When fed in large quantities it kills animals and in regular small amounts it causes chronic poisonings. In oils it will be absorbed through the skin. It may be applied safely as a dust on animals. As a liquid to secure a residual effect, it should be sprayed in oil or as a water emulsion as a wet spray, but not as a fog. It may be applied with a brush. About 200 mg. of DDT are required per square foot of sprayed surface. A.C.D.

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