THE SOURCE OF THE ACIDITY OF FRESH MILK

by

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The Source of the Acidity in Fresh Milk.

The nature of the acidity of milk and milk products has long claimed the attention of workers in the field of Dairy Science. Distinct differences in the titratable acidities of different milks have been reported by practically all investigators. Recent advances in Physical Chemistry have made possible the application of the fundamentals of acid-base equilibria to an otherwise confusing problem.

This problem is of immense practical importance. The acidity of milk plays an important part in the processing of many dairy products. The manufacture of cheese is controlled largely by the development of appropriate acidities. In buttermaking acidity is an important factor, both in the grading of cream and in proper neutralizing of the cream prior to churning. A test for acidity is considered essential in the purchasing of raw fluid milk and forms a basis for the grading of the incoming milk supply of many leading milk plants, condenseries and cheese-making establishments.

The use of the acidity test as a means of grading milk implies several assumptions of doubtful accuracy. In creamery practice, it is assumed that the normal average acidity of mixed fresh milk varies from 0.14% to 0.16% calculated as lactic acid. It is further assumed that milk which is over 0.18% acidity is not acceptable for bottling purposes, since the increased acidity may be due to bacterial fermentation. Such milk would be of poor keeping quality. In normal practice these assumptions may seem justified, yet there has been reported evidence which tends to contradict them, either as a whole or in part.

An instance of this nature was noted by the writer in January 1930. A considerable quantity of high grade Jersey milk was in danger of rejection by a local creamery, since it showed an acidity of 0.20%. Subsequent investigations by Professor R.W.Brown, of the Department of Dairy Husbandry, Manitoba Agricultural College, and the writer, showed that the milk was produced under ideal conditions and that the apparently high acidity was present in the milk when freshly drawn. The milk was used in the usual manner by the creamery, and showed no indication of possessing a poor keeping quality. If strict adherence to the common practice of rejecting milk over 0.18% acidity is to be maintained, there is a possibility of penalizing unjustly the milk producer and causing him to

suffer direct financial loss.

It is of interest then to know what constituent or constituents are responsible for the acidity of fresh milk. Various investigators have reported on the influence of single constituents in determining this property. Some have even prepared artificial milks employing suspected constituents in an endeavour to correlate their acidity with that of the natural product. As far as the writer is aware, no one investigator has analyzed a series of milks for all of these possible "acid constituents". It was proposed therefore, to perform such a series of analyses and to make a statistical study of the values so obtained.

Historical.

Various methods have been used to determine quantitatively the acid content of milk; the results have been reported in varied terms. A brief discussion in this connection may be of value in order that results obtained by different investigators may be comparable.

Soxhlet (1873) made the first quantitative determination of the acid content of milk. His method consisted of titrating 100 c.c. of milk with 0.25 N. sodium hydroxide, using 2 ccs. of a 2% alcoholic solution of phenolphthalein as the indicator. The number of cubic centimeters of alkali necessary for neutralization were reported as "degrees".

In France, reporting results in terms of Dornic degrees has been favoured. In this method, 100 ccs. of milk are titrated with 0.1 N. sodium hydroxide, using phenolphthalein indicator; the number of cubic centimeters of alkali necessary constitute the number of Dornic degrees.

Generally, however, it has been found convenient to calculate results in terms of lactic acid. At present, the accepted method is to titrate a 9 cc. sample of milk with 0.1 N. sodium hydroxide, using 2 drops of a 1% alcoholic solution of phenolphthalein as an indicator. The results are converted to terms of lactic acid by multiplying the number of cubic centimeters required for neutralization by 0.1. While it is recognized that this method does not give the actual amount of lactic acid present, especially in the case of fresh milk, it has been found practical and convenient in everyday creamery operations.

Marked variations are introduced into the results by the addition of varying amounts of water to the sample being titrated. Sommer and Menos (1931) have shown that the dilution of cow's milk lowers the titratable acidity appreciably. This explains some of the low values for acidity reported by earlier workers, especially since the Standard Methods of Milk Analysis, Fifth Edition (1927) advised the addition of water to the sample being titrated.

Variations in Acidity.

While it is generally accepted in creamery practice that the average acidity calculated as lactic acid of normal mixed herd fresh milk ranges from 0.14% to 0.16%, wide variations have been noted in the case of milk from individual cows. Henkel (1907), on results gathered from 10,000 acidity tests of milk from individual cows, states the range of acidity to be from 0.1237% to 0.2025%. Wider variations have been noted, as:
Somer and Hart (1919); 0.102%-0.257% (86 individual samples),
Rice and Markley (1924); 0.086%-0.229% ("large number of samples")
Sherwood and Hammer (1926); 0.10%-0.25% (335 samples).

The average acidity of mixed herd milk is generally low. Goulding, Mackintosh and Mattick (1932) state that the acidity of samples taken weekly over a three year period of the mixed milk from 10 Shorthorn cows, ranged from 0.14% to 0.18% with a yearly average of 0.16%. Henkel (1907) reports herd milk of 70 cows as varying from 0.143% to 0.16% during the course of a year. The generally accepted opinion of many investigators and practical creamery operators is summed up in the statement of Sommer (1935) that "the expected acidity of fresh milk at milk plants may be regarded as 0.145% to 0.165%".

Factors Influencing Acidity.

Houston (1931) lists as factors which may influence the acidity as well as the composition of milk, the following: health of the cow, feed, age, breed, stage of lactation, interval of milking and even different quarters of the udder. Furthermore, composition and acidity may vary during the course of a single milking. This agrees with the work of other investigators, differences being expressed more in regard to the extent of variation than to the particular factor or factors involved.

Attempts have been made to alter the acidity of the milk by the feeding of organic and inorganic acids with the normal ration. These have met with little success. Duncombe (1924) fed lactic, butyric, acetic, and phosphoric acids. Sommer and Hart (1921) included sulphuric acid in the ration to the extent of 120 ccs. concentrated H_2SO_4 daily for six days. In neither instance was the acidity of the milk altered.

It might be expected that the acidity would be affected by the normal ration the animal receives. Evidence which shows a definite change in acidity due to any certain type or combination of feed, is lacking.

There appear to be conflicting opinions regarding the influence of the stage of lactation on the acidity of milk.

It is agreed generally that colostrum is high in acidity and that this acidity rapidly decreases to a lower level which is characteristic for the individual cow as was shown by Henkel (1907). Recently Mattick and Hallett (1929) and Houston (1931) have published data by which it is definitely shown that the acidity is highest at the beginning of lactation and lowest at the end. Houston's findings are the results of over 2500 tests, representing 39 complete and 38 incomplete lactations. A composite graph prepared from these results shows the acidity to drop rapidly during the first eight weeks of lactation and to remain fairly uniform until the seventh week before lactation ceases. In the latter seven weeks, a noticeable drop in acidity occurs.

Variations in acidity of the milk from different quarters of the udder of the individual cow have been reported by Inglis (1903), Fitch and Copeland (1924), Rice and Markley 1929) and Keiferle, Schwaibold and Hackman (1925). Mattick and Hallett (1929) state that on the whole, the acidities of the milk from the two hind quarters seem to be similar and also those of the two front quarters. There were distinct differences in acidity between the milk of the fore and hind quarters.

It has been found that certain infections of the udder, namely, mastitis, cause a lowering in acidity of the milk from the infected quarter. The lowering may be as great as 0.08% to 0.10% (Sommer-1935). This fact is used in the clinical

detection of the infection. Rosell (1933) states that when 40 ccs. of fresh milk, not secreted during the last period of lactation, is neutralized by less than 6 ccs. of a 0.1 N. sodium hydroxide solution, one may with a certainty of approximately 98%, diagnose the milk as being the product of a mastitic udder.

Constituents responsible for Acidity.

Soxhlet and Henkel (1888) in an investigation of the organic acids present in milk, were unable to detect the presence of lactic acid in normal fresh milk. This has been verified by other investigators, chiefly Van Slyke and Bosworth (1918). Citric acid in the free form or as citrate was discovered as a normal constituent of cows' milk by Soxhlet and Henkel (1888) and confirmed by Sommer and Hart (1918). Since lactic acid is not the acid responsible for the acidic nature of fresh milk, various investigators have attempted to explain the phenomenon on the basis of other constituents. Van Slyke and Bosworth (1914) state that the acidity of fresh milk is due to acid phosphates of the type MH_2PO_4, and that, in the course of titration with sodium hydroxide, the salt $CaHPO_4$ is precipitated. The precipitated salt then hydrolyzes to $Ca(OH)_2$ and H_3PO_4. A further step is the formation of insoluble $Ca_3(PO_4)_2$ by the action of the $Ca(OH)_2$ on the mono- and di-calcium phosphates, leaving H_3PO_4 to react with a further quantity of sodium hydroxide.

High total solids and a high ash content have been associated with high titratable acidity of fresh milk by Rice (1919) and McInerey (1920). Rice and Markley (1924) as a result of the analysis of thirteen samples, together with data obtained from other investigators, formed the constituents into hypothetical combinations and arrived at the conclusion that the casein contributes about one-half of the total titratable acidity; citrates, carbon dioxide and lact-albumin a small portion, and the phosphates contribute the balance. They also advance the hypothesis that a condition of equilibrium exists between the citric acid, phosphoric acid, casein and the bases, therefore the acidity due to any one of these depends upon its relation to the others.

The mutual effect of the hydrogen, calcium, phosphate and citrate ions on one another was investigated by Whittier (1929). It was shown that the precipitation of $Ca_3(PO_4)_2$ was prevented by the presence of sufficient citrate ions. This appears to counteract in part the hypothesis suggested by Van Slyke and Bosworth (1914)and Sommer and Menos (1931). Sommer (1935) concludes that the acidity of fresh milk is due principally to the phosphates and proteins in milk and to a lesser extent to the carbon dioxide and citrates.

In attacking the problem from a different angle, Wiley (1935) stresses the point that previous investigators

have not defined the effect of various buffers, or supported their work by direct experimental evidence, stating results merely in terms of hypothetical combinations of milk constituents. He prepared the various buffers in concentrations approximately equal to those in which they appear in milk and studied their buffer effects over different $_pH$ ranges. He found the effect of increasing concentration of the buffer constituents on titration with sodium hydroxide solution between $_pH$ of 6.6 to 8.0 to be as follows:

phosphates increase titration,
citrates increase titration,
calcium slightly decreases titration,
casein slightly increases titration.

The conclusion of his work "emphasized the interdependence of the buffers, phosphate, citrate, casein, and calcium".

Heinemann(1919) states that the milk of most mammals reacts acid to rosalic acid and phenolphthalein, amphoteric to litmus and alkaline to dimethyl orange. This statement is an expres- of the reaction of these particular indicators at the pH of milk. The phrase "amphoteric to litmus" is open to criticism, since the color change of litmus acts between a $_pH$ of 4.5 to $_pH$ 8.3. Reported values for the $_pH$ of fresh milk show considerable variation. The "Fundamentals of Dairy Science, Second Edition, 1935, list values ranging from $_pH$ 6. 4 to $_pH$ 7.2. It is also pointed out that variations

in method of measurement of $_pH$, in source and nature of sample and other factors, are responsible for the wide range of values reported. Tentatively, it is suggested that a $_pH$ of 6.6 be taken as the average. Tapernoux (1928) shows that there is no close relationship between titratable acidity and $_pH$ except when acidity is developed by lactic fermentation.

Experimental.

I. Source of Samples.

Through the courtesy of Mr. N.N.Smith of the R.Smith Company, Winnipeg, it was possible to secure samples of milk from a herd of thoroughbred Jersey cows. The farm, situated near Bird's Hill, Manitoba, is under careful management with respect to feed, health of animals, milking conditions, etc., removing much uncertainty with regard to otherwise uncontrolled factors in the production and care of the milk.

It was felt that the milk from this herd would be well suited to a study of the nature of acidity since earlier work showed the general herd average acidity to be higher than normal. Milk from individual cows had been found to vary from 0.14% to 0.27% acidity, thus affording a wide range of acidities for investigation.

In each case, samples consisted of a quart of milk

taken from the mixed complete milking of the individual cow, capped to prevent loss of carbon dioxide and kept at icebox temperatures until analyzed. The analysis for carbon dioxide, titration of acidity and pH determinations were made on the fresh milk within three hours of milking. Samples were then preserved by the addition of 1 cubic centimeter of a 36% solution of formaldehyde and held in the icebox for further analysis.

No attempt was made to select for study milk from cows in any particular stage of lactation. There were from 30 to 40 cows milking during the winter and samples were taken at random. In the preliminary study, all samples were of the 4 p.m. milking. Samples numbering 1 to 30 were from the morning milking.

II. Buffer Capacity of the Milk.

A preliminary study of the buffer capacity of samples of milk of different acidities was made in the expectation that some indication of the constituents responsible for the buffering would be obtained. Four samples of milk were selected giving a range of titratable acidity of from 0.18% to 0.22%.

Data for the buffer curves were obtained by adding 0.1 N. NaOH in 1 cc. portions to a 50 cc. sample of the milk. After each addition, the sample was stirred, temperature

noted and a $_pH$ measurement made. A second 50 cc. sample of the same milk was similarly treated, using 0.1 N. H_2SO_4 in place of the sodium hydroxide. Buffer curves for each of the four samples were plotted employing the amounts of acid or alkali added as the abscissae and the $_pH$ values as ordinates.

Measurements of $_pH$ were made potentiometrically using the quinhydrone-saturated KCl-calomel electrodes. From observed E.M.F. and temperature readings, the $_pH$ values were calculated by application of the formula:

$$pH = \frac{Eq - E\ cal. - E}{.0001983\ T},$$

where, Eq = Potential between quinhydrone electrode and hydrogen electrode.

E cal. = Potential between the calomel electrode and the normal hydrogen electrode, and

E = observed E.M.F.

$Eq = 0.7175 - 0.00077\ t$ (Clark).

so

$t^oC.$	Eq.	E cal.(saturated KCl)	0.0001983 T
18	0.7044	0.251	0.0577
20	0.7029	0.250	0.0581
25	0.6992	0.2458	0.0591
30	0.6955	0.242	0.0601
35	0.6198	0.238	0.0611

Data obtained.

Results are indicated in Tables I and IA and are reproduced graphically in Figure I. From a cursory examination of the buffer curves, it was noted (1) that the milk of highest titratable acidity showed the greatest buffer effect, and (2) since the buffer effect was indicated in the alkaline and acid range of $_pH$, an amphoteric buffer might be suspected. This would suggest the proteins or some buffer constituent or constituents capable of exerting an effect in both directions.

In order to obtain sufficient data for a statistical correlation study between the titratable acidity and possible buffer constituents, the analysis of thirty samples of milk from individual cows was undertaken for the following constituents:

(1) Titratable Acidity,

(2) Free Carbon Dioxide,

(3) Carbonates,

(4) Citric acid and Citrates,

(5) Casein,

(6) Albumin and Lactoglobulin,

(7) Inorganic Phosphates.

These include all of the substances which have been held by various workers to be the cause of acidity of fresh milk.

Table I. $_{p}H$ values obtained on the addition of measured amounts of 0.1 N. NaOH to 50 ccs. of milk.

0.1 N NaOH	Sample A 0.18% Acidity.			Sample B 0.195% Acidity.		
ccs added	Temp. °C	E.M.F. Volts	pH	Temp. °C	E.M.F. Volts	pH
0.0	22.0	.0720	6.512	22.5	0.705	6.529
1.0	23.0	.0650	6.609	23.0	.0640	6.630
2.0	23.0	.0571	6.744	23.0	.0568	6.752
3.0	22.5	.0488	6.897	23.0	.0490	6.885
4.0	22.5	.0408	7.031	23.0	.0416	7.011
5.0	22.5	.0319	7.186	23.0	.0329	7.160
6.0	22.5	.0231	7.336	23.0	.0242	7.308
7.0	----	-----	-----	23.0	.0151	7.463
7.1	22.5	.0115	7.534	----	-----	-----
8.0	22.5	.0012	7.709	23.0	.0048	7.638
8.25	----	-----	-----	23.0	.0023	7.681
8.4	----	-----	-----	23.0	.0013	7.698
9.0	----	-----	-----	----	-----	-----

Table I. $_{p}H$ values obtained on the addition of measured amounts of 0.1 N. NaOH to 50 ccs. of milk. (cont'd.)

0.1 N. NaOH	Sample C. 0.215% Acidity.			Sample D. 0.22% Acidity.		
ccs added	Temp. °C	E.M.F. Volts.	pH	Temp. °C	E.M.F. Volts.	pH
0.0	21.0	.0758	6.468	22.0	.0706	6.583
1.0	20.5	.0696	6.585	22.5	.0635	6.648
2.0	20.5	.0617	6.721	22.5	.0565	6.767
3.0	20.5	.0551	6.852	22.5	.0496	6.885
4.0	20.5	.0472	6.953	22.5	.0423	7.010
5.0	20.5	.0394	7.104	22.5	.0353	7.112
6.0	20.5	.0312	7.245	22.5	.0278	7.257
7.0	20.5	.0219	7.405	22.5	.0198	7.394
7.1	----	-----	-----	----	-----	-----
8.0	20.5	.0121	7.573	22.5	.0111	7.511
8.25	----	-----	-----	----	-----	-----
8.4	-----	- ---	-----	----	-----	-----
9.0	20.5	.0005	7.773	22.5	.0020	7.667

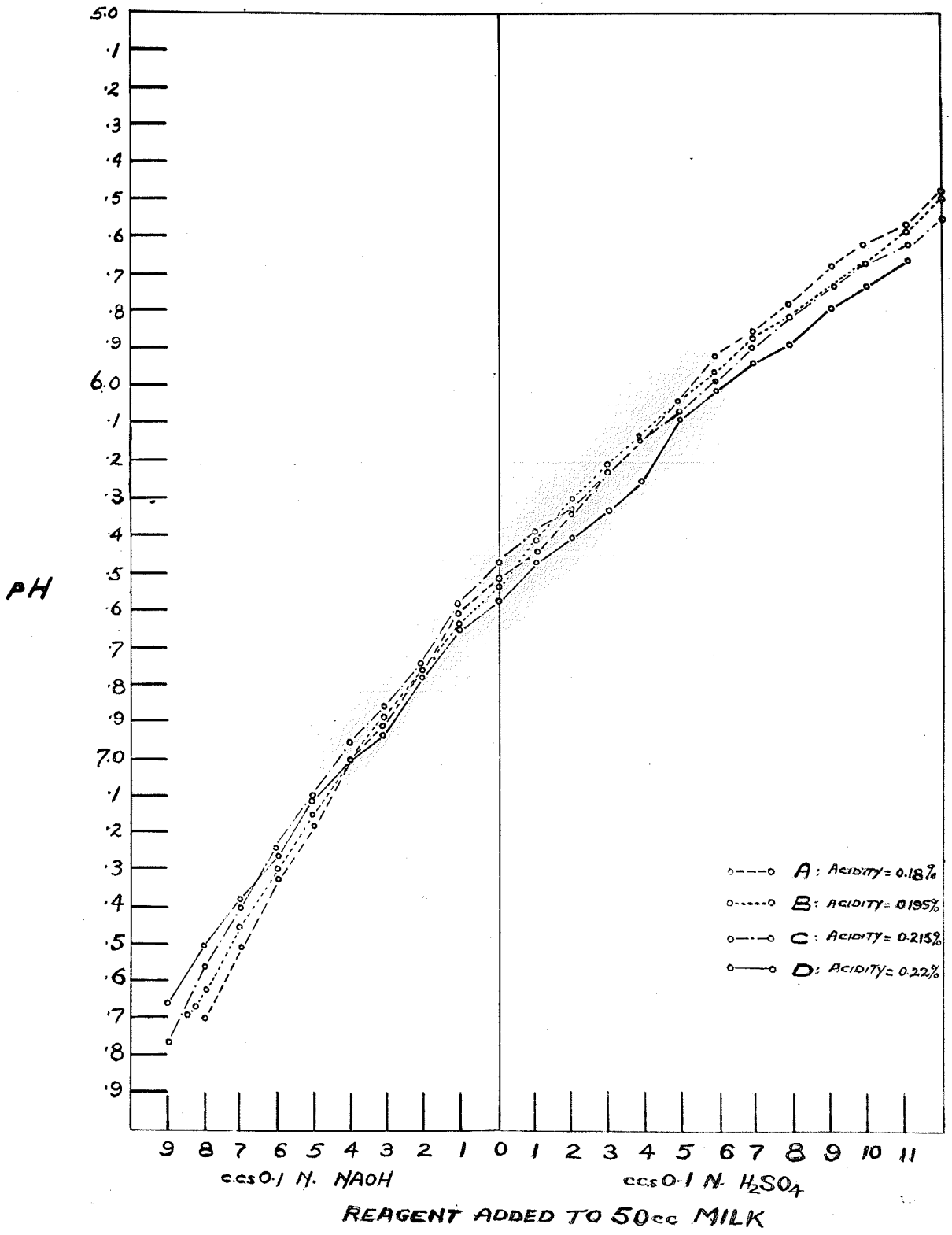
Table IA. $_{p}H$ values obtained on the addition of measured amounts of 0.1 N. H_2SO_4 to 50 ccs. of milk.

0.1 N. H_2SO_4	Sample A 0.18% acidity.			Sample B. 0.195% acidity.		
ccs. added	Temp. °C.	E.M.F. Volts.	$_{p}H$	Temp. °C	E.M.F. Volts	$_{p}H$
0.0	21.5	.0708	6.544	23.0	.0696	6.534
1.0	21.5	.0768	6.441	24.0	.0755	6.414
2.0	21.0	.0830	6.346	24.0	.0817	6.308
3.0	21.5	.0886	6.239	24.0	.0870	6.219
4.0	21.5	.0948	6.133	24.0	.0925	6.125
5.0	21.5	.1012	6.023	24.0	.0978	6.035
6.0	21.5	.1062	5.910	24.0	.1025	5.955
7.0	21.5	.1118	5.842	24.0	.1077	5.867
8.0	21.5	.1169	5.785	24.0	.1113	5.806
9.0	21.5	.1210	5.684	24.0	.1154	5.736
10.0	21.5	.1249	5.618	24.0	.1198	5.662
11.0	21.5	.1289	5.580	24.0	.1245	5.582
12.0	21.5	.1328	5.482	24.0	.1276	5.496

Table IA. $_{p}H$ values obtained on the addition of measured amounts of 0.1 N. H_2SO_4 to 50 cc. of milk.

0.1 N. H_2SO_4	Sample C. 0.215% acidity.			Sample D. 0.22% acidity.		
ccs. added	Temp. °C	E.M.F. Volts.	$_{p}H$	Temp. °C.	E.M.F. Volts.	$_{p}H$
0.0	20.5	.0758	6.479	21.0	.0690	6.585
1.0	20.0	.0813	6.395	21.5	.0748	6.476
2.0	20.0	.0848	6.335	21.5	.0783	6.416
3.0	20.0	.0908	6.240	21.5	.0829	6.337
4.0	20.0	.0961	6.141	21.5	.0884	6.243
5.0	20.0	.1009	6.067	22.0	.0980	6.068
6.0	20.0	.058	5.974	22.0	.1022	5.996
7.0	20.0	.1114	5.877	22.0	.1061	5.929
8.0	20.0	.1157	5.803	22.0	.1088	5.883
9.0	20.0	.1191	5.745	22.0	.1143	5.789
10.0	20.0	.1232	5.674	22.0	.1181	5.724
11.0	20.0	.1269	5.611	22.0	.1208	5.678
12.0	20.0	.1301	5.555	----	-----	-----

FIGURE I — BUFFER CURVES



III. Analysis of Milk.

Methods:

(1) Titratable Acidity.

A 50 cc. sample of milk was titrated with 0.1 N. sodium hydroxide, using 10 drops of a 1% alcoholic solution of phenolphthalein as the indicator. Results are expressed in terms of percent lactic acid. Bureau of Standards glassware was used in making all titrations.

(2) Free Carbon Dioxide.

It was decided to make an adaptation of the method indicated by Faulk (1914) for the determination of carbon dioxide in inorganic substances, since a review of available literature showed no method suitable for the determination of CO_2 in milk.

The method, in brief, consists of releasing the carbon dioxide from milk by boiling, passing the gases through drying agents and collecting the carbon dioxide in a caustic potash absorption bulb. Figure II represents a diagram of the principal features of the apparatus used.

Details of the Apparatus:

A represents a 300 cc. Erlenmeyer flask fitted with a two-hole stopper carrying a short condenser, B, of the bulb type, and a round separatory funnel, C, of 200 cc. capacity.

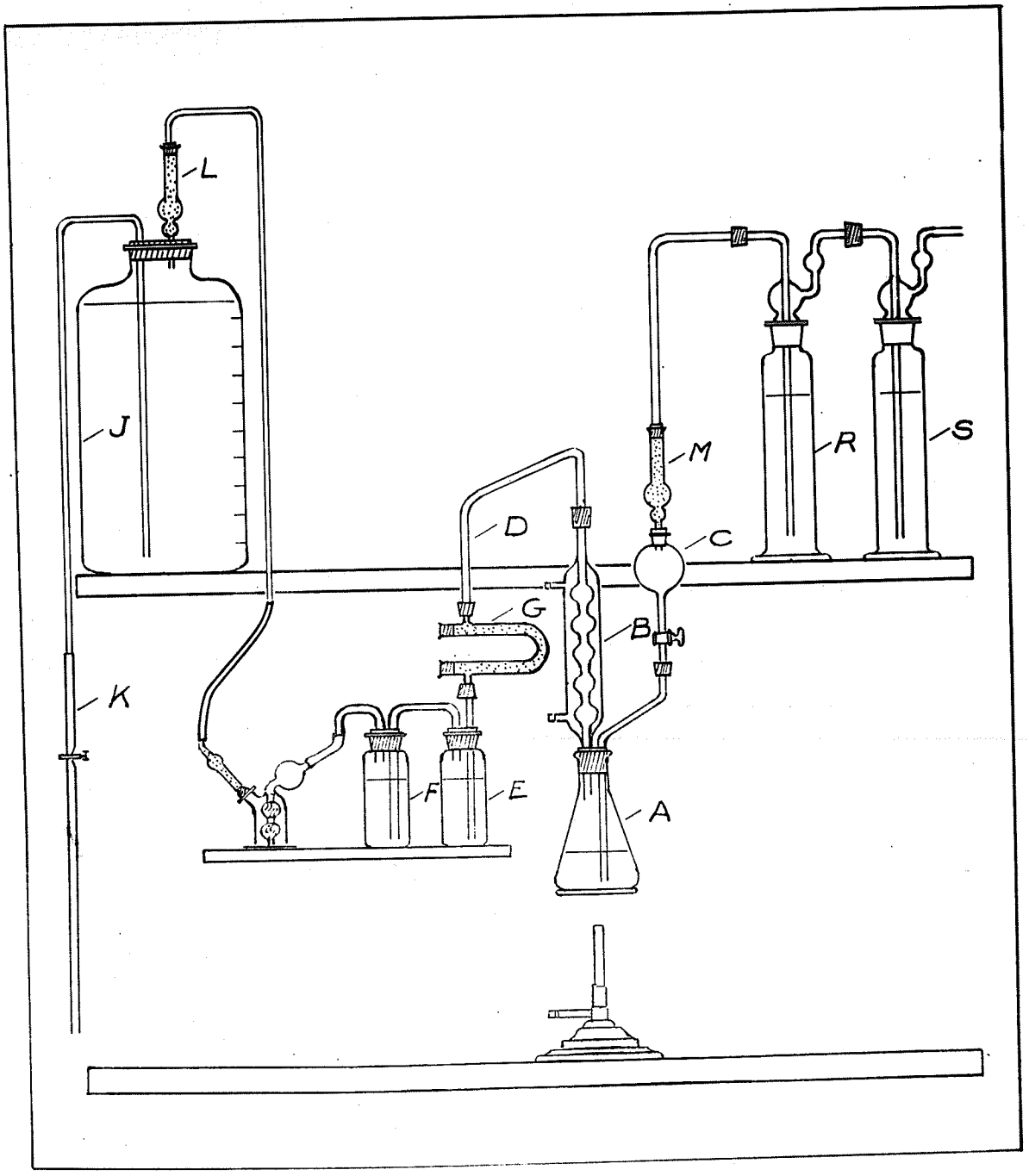
The glass tube, D, carries the evolved gases to the bottles E and F, which are half-filled with concentrated sulphuric acid. The acid serves to remove water which may have escaped from the condenser B.

From F, the gases pass through a 6-inch U-tube G, filled with granular anhydrous calcium chloride, into a Geissler absorption bulb filled with a solution of KOH, which was prepared by dissolving the solid in twice its weight of water.

An aspirator J, for drawing air through the apparatus was prepared by fitting a 10 litre bottle with a syphon tube K. The aspirator was placed upon the shelf of the desk. It was drained by means of a rubber tube K, fitted with a screw pinch-cock for regulating the rate of flow. The volume of air aspirated was measured by referring to 1-litre graduations marked on the side of the aspirator. A guard tube L, filled in its lower half with granular calcium chloride and in the upper half with soda-lime, is connected to the absorption bulb by a length of soft rubber tubing.

The separatory funnel C, is fitted with a guard tube M filled with granular calcium chloride. When air is aspirated through the apparatus, it passes through two large wash bottles R and S, containing concentrated KOH solution, passes the guard tube M, and enters the separatory funnel C.

FIGURE II



Solid rubber corks were used to make all joints.

Determination of Free Carbon Dioxide.

Several preliminary trials were made to determine the conditions under which operation of the apparatus would give consistently reproducible results. Before commencing a determination, it was found necessary to disconnect the absorption bulb and aspirate at least 4 litres of CO_2 -free air through the apparatus. Boiling the milk for thirty minutes was found sufficient to obtain constant results from a 200 cc. sample. Excessive foaming of the milk was an early defect in procedure, but this was corrected by the addition of approximately a half of a cubic centimeter of capryl alcohol to the sample before boiling. Blank determinations run on boiled distilled water and the above-mentioned amount of capryl-alcohol gave no increase in weight of the absorption bulb.

The procedure adopted was to first disconnect the absorption bulb and aspirate 4 litres of air through the apparatus. The previously weighed absorption bulb was then placed in the train and a 200 cc. sample of milk introduced into the Erlenmeyer flask through the separatory funnel. Half a cubic centimeter of capryl alcohol is also added at this stage. Replacing the guard tube on the separatory funnel, the milk is then heated, at first gently then more vigorously, to the boiling point. After boiling for thirty

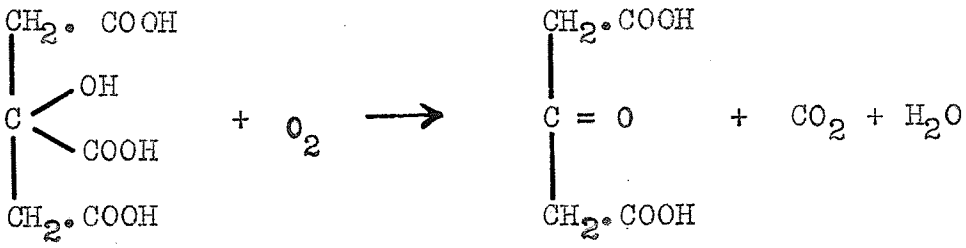
minutes, the aspirator is connected and two litres of air drawn through the apparatus. The rate of aspiration is regulated to be slow at first and then increased, the complete aspiration taking about thirty minutes. The absorption bulb is then disconnected, cooled and weighed, using the method of tares. The increased weight due to CO_2 is converted and reported in terms of percent by volume.

3. Carbonates, determined as free Carbon Dioxide.

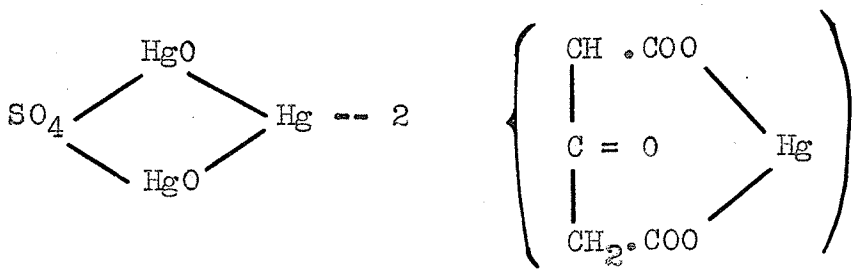
The absorption bulb used in the CO_2 determination is replaced in the absorption train. By means of the separatory funnel, 20 ccs. of a 20% lactic acid solution are introduced into the milk previously freed of CO_2 by boiling in the CO_2 determination. The milk and acid are boiled together for thirty minutes, 2 litres of air aspirated, the absorption bulb disconnected, cooled and re-weighed. The increase in weight will give the amount of CO_2 liberated from carbonates by the lactic acid.

4. The Determination of Citric Acid.

The method employed for the determination of citric acid was that of Beau's (1904) modification of the method proposed by Denige (1900). This method consists of oxydizing citric acid to acetone-dicarboxylic acid with potassium permanganate, as:



The acetone-dicarboxylic acid is converted, in the presence of mercuric sulphate, to a white precipitate of a mercury-sulfo-acetone-dicarboxylic acid complex of constant composition, corresponding to the formula:



Molecular weight : 1416.

This reaction is not given by malic, tartaric, succinic or lactic acids. It has been pointed out by Allen (1931) that it is doubtful whether the reaction is really quantitative since acetone-dicarboxylic acid breaks down to acetone and CO_2 even at low temperatures. However, Sherwood and Hammer (1926) claim the method is more accurate than that of Stahre (1895).

Beau's Method, based on Denige's Reaction.

Mercuric sulphate solution is prepared by mixing 50 grams of red oxide of mercury with 400 to 500 ccs. of dis-

tilled water in a litre flask, and then gradually adding H_2SO_4 sp.gr. .66° Baume, until the oxide is dissolved (about 75 cc. of H_2SO_4). Make up to the litre, boil, and filter.

Fifty ccs. of milk were put into a 200 cc. graduate and 75 cc. distilled water added. 50 cc. of the $HgSO_4$ solution was then added, the mixture shaken and made up to the 200 cc. mark with distilled water. The casein, etc. which precipitated was filtered out on a Buchner funnel.

One hundred cc. of the filtrate were heated to boiling in a flask and then oxidized with a one percent solution of $KMnO_4$, added drop by drop. A yellowish-white precipitate was formed which, on the addition of a slight excess of $KMnO_4$ solution, became brownish in color and settled out. From 5 to 10 cc. of $KMnO_4$ solution were usually required and a slight excess, indicated by the brownish color, was removed by boiling the liquid again and adding H_2O_2 drop by drop, until the precipitate became white.

When cool the precipitate was collected on a suction filter and washed with distilled water until the addition of $BaCl_2$ solution gave no reaction for sulphates.

The precipitate and filter paper were then transferred to a 600 cc. Erlenmeyer flask, 10 cc. of concentrated HCl added and the solution heated on a water bath to the temperature of boiling water; 100 cc. of distilled water were added and the temperature again raised to boiling. The

solution was cooled and filtered through a Buchner funnel, the flask rinsed and finally, the filter was washed with distilled water.

The resultant clear solution was transferred to an Erlenmeyer flask, made alkaline with 20 cc. of concentrated NH_4OH, and 10 cc. of a solution of KCN (13 grams to the litre) run in. Titration with 0.1 N. $AgNO_3$ was made, using 10 drops of a 10% solution of KI as the indicator. A slight cloudiness which persisted on shaking was taken as the end-point. Previously, 10 cc. of the KCN solution was titrated in the same manner, requiring a volume about equal to the $AgNO_3$ solution. By difference, the quantity of $AgNO_3$ corresponding to the mercury of the precipitate was obtained.

To facilitate calculations, Beau prepared a table which gives directly in centigrams per litre, the citric acid content of the milk under examination, from the 0.1 N. $AgNO_3$ equivalent. The table is reproduced in the appendix as Table A, and is applicable only when the method outlined is followed in detail.

5. Determination of Casein.

Moir (1931) has made a thorough review of the determination of milk proteins and compared the accuracy of existing methods. It was decided, therefore, to follow his proposed new method. The method was found convenient in use and gave close agreement of duplicate determinations.

Details of the method are as follows:

Ten cubic centimeters of the well mixed sample were pipetted into a previously weighed covered beaker of 100 cc. capacity and weighed again quickly. The sample was diluted with 50 cc. of distilled water warmed to 40° - 42°C., 1.5 cc. of 10% acetic acid added and the milk stirred gently. After standing for 20 minutes, 4.5 cc. of 0.25 N. sodium acetate solution was added, the milk stirred gently and allowed to stand for one hour. The separated casein was removed on a Buchner funnel, using a No. 42 Whatman filter paper. The precipitate was then washed with distilled water and, together with filter paper, transferred to a Kjeldahl flask. The beaker was cleaned out immediately by adding water and successive portions of the sulphuric acid required for the Kjeldahl digestion. The stirring rod and inside of the beaker were wiped with a small piece of filter paper which was added to the contents of the digestion flask. Casein nitrogen was then determined by Kjeldahl's method and casein computed by using the factor 6.38 x N.

6. Determination of Albumin and Lactoglobulin.

The procedure adopted was that proposed by Moir for the combined determination of these proteins.

To the filtrate obtained from the previous isoelectric precipitation of the casein, sufficient trichloracetic acid was added to make the final concentration approximately 4 percent. The mixture was heated on the water bath at the

temperature of boiling water for half an hour. On cooling it was filtered and washed with a 1% solution of trichloracetic acid. The precipitate and filter paper were placed in a digestion flask and nitrogen determined by Kjeldahl's method. From the amount of nitrogen the combined albumin and lacto-globulin was found by using the factor 6.38.

7. Inorganic Phosphates.

The determination of inorganic phosphates was made by a modification of the method of Fiske and Subarrow (1925) for the colorimetric determination of phosphorus. Designed primarily for the estimation of inorganic phosphates in urine and blood, the method, according to the authors, may be applied to any biological material with proper attention to detail.

In this method phospho-molybdic acid is reduced to a blue substance by 1-2-4 amino-naphthol-sulfonic acid. Reduction was rapid when milk serum was used and the blue coloration intense and permanent. Reagents used in the method were:

Ammonium Molybdate:-

A 2½% solution prepared by dissolving 25 grams of ammonium molybdate in 200 cc. of distilled water; added to a litre volumetric flask containing 500 cc. of 5 N. H_2SO_4 and the volume made up to the mark with distilled water.

0.25% Amino-naphthol-sulfonic Acid:-

0.5 grams of recrystallized technical amino-naphthol-sulfonic acid dissolved in 195 cc. of 15% sodium bisulphite and 5 cc. of 20% sodium bisulphite added. This solution should be freshly prepared as needed.

Standard Phosphate:

A standard solution containing 0.4 mg of Phosphorus to each 5 cc. prepared by dissolving 0.3509 grams of pure mono-potassium phosphate in distilled water, adding 10 cc. of 10 N. H_2SO_4 and making up to a litre.

In applying Fiske and Subarrow's method to the determination of the inorganic phosphates of milk, it was decided to use as a serum, the filtrate from the precipitation of albumin and lactoglobulin in the determination made according to Moir's method. To exclude the possibility of phosphate contamination due to the reagents used in precipitating the proteins, blank determinations were run on all reagents. These gave negative phosphate reactions. The dilution of the serum necessary to give sharp reading on the colorimeter was determined by making several preliminary trials with varied amounts of serum.

Details of the procedure adopted are as follows: the filtrate obtained from the isoelectric precipitation of proteins from a weighed sample of milk by Moir's method, was

made up to 100 cc. with distilled water. Into a 100 cc. volumetric flask, 10 cc. of the serum were pipetted, 10 cc. of the 2½% ammonium molybdate solution added and the solution diluted with 20 cc. of distilled water. To effect reduction, 4 cc. of the amino-naphthol-sulfonic acid were added and the volume made up to 100 cc. with distilled water. The solution was then allowed to stand over-night in order that equilibrium might be attained.

A standard solution for comparison was prepared by adding 10 cc. of the molybdate solution and 4 cc. of the reducing agent to 10 cc. of the standard phosphate solution in a 100 cc. volumetric flask, making the volume up to 100 cc. with distilled water. This gave a solution containing 0.8 mg. of phosphorus for comparison purposes. With the standard set at a height of 20.0mm., comparisons were sharp and distinct.

Comparisons of unknown and standard solutions were made with the colorimeter and results calculated as inorganic phosphate per 100 grams of milk in terms of inorganic phosphorus.

Results of analysis.

Results of analysis obtained by the methods outlined, of thirty samples of fresh milk from individual Jersey cows, are shown in Table II. With the exception of free

carbon dioxide and carbonate, all determinations were made in duplicate and average values reported.

Since the results were intended for a statistical study, only constituents customarily reported in terms of percentage were calculated on that basis.

Table II.

Results of Analysis of Milk for Suspected Buffer Constituents.

Sample Number	Titratable Acidity.	Free Carbon Dioxide	Carbonate as free Carbon Dioxide	Citric Acid and Citrates as Citric Acid.
	%	% by Vol.	% by Vol.	Centigrams per litre
1	.180	5.51	0.53	28.0
2	.190	6.22	1.12	26.5
3	.154	6.09	0.63	69.5
4	.183	5.89	0.48	35.5
5.	.165	7.19	0.58	44.5
6	.169	5.17	0.86	45.7
7	.140	8.13	0.96	53.0
8	.158	7.62	0.51	32.5
9	.176	7.09	0.86	12.0
10	.151	5.45	0.68	14.7
11	.176	7.67	0.96	59.0
12	.176	5.66	0.56	51.5
13.	.169	3.72	0.56	42.7
15	.187	5.40	0.61	50.0

Table II (cont'd)

Results of Analysis of Milk for Suspected Buffer Constituents.

Sample Number	Casein	Albumin and Lactoglobulin	Inorganic Phosphates as Phosphorus.
	%	%	Mg. P per 100 grams.
1	2.44	0.53	67.9
2	1.81	0.53	71.4
3	2.48	0.44	61.9
4	3.14	0.47	64.3
5	2.38	0.65	66.2
6	2.52	0.54	60.0
7	2.60	0.57	61.7
8	2.51	0.60	64.3
9	2.79	0.70	62.7
10	2.82	0.48	63.5
11	3.24	0.46	65.9
12	3.34	0.54	56.5
13	3.21	0.56	63.7
14	2.50	0.75	64.8
15	3.38	0.66	67.1

Table II (cont'd)

Results of Analysis of Milk for Suspected Buffer Constituents.

Sample Number	Titratable Acidity	Free Carbon Dioxide	Carbonate as free Carbon Dioxide	Citric Acid and Citrates as Citric Acid.
	%	% by Vol.	% by Vol.	Centigrams per litre
16	.187	4.94	0.73	32.5
17	.183	5.12	0.86	40.0
18	.205	4.18	0.68	26.5
19	.174	5.51	0.53	62.0
20	.193	8.27	0.74	19.0
21	.160	7.73	0.86	60.5
22	.162	6.12	0.74	59.0
23	.187	6.89	0.76	32.5
24	.140	8.45	0.20	56.0
25	.165	9.48	0.69	48.5
26	.190	7.61	0.43	26.5
27	.153	6.43	0.76	47.2
28	.165	7.55	0.66	50.0
29	.173	7.95	0.84	54.5
30	.155	7.10	0.94	48.7

Table II (cont'd)

Results of Analysis of Milk for Suspected Buffer Constituents.

Sample Number	Casein	Albumin and Lactoglobulin	Inorganic Phosphates as Phosphorus.
	%	%	Mg. P per 100 grams.
16	3.36	0.66	72.6
17	3.02	0.57	65.7
18	3.56	0.69	71.6
19	3.06	0.52	62.0
20	3.64	0.65	72.3
21	2.59	0.50	55.9
22	3.07	0.70	54.2
23	2.93	0.50	69.9
24	2.57	0.53	56.3
25	2.92	0.67	59.0
26	2.26	0.50	67.3
27	2.57	0.60	57.2
28	3.00	0.57	60.9
29	2.76	0.54	61.7
30	2.68	0.60	55.1

Statistical Analysis.

Employing the data of Table II, the average and standard deviation for each constituent was computed by the usual method and results shown in Table III.

Correlation coefficients were obtained by the formula given by Fisher (1925) using as variables

1. Titratable Acidity,
2. Free Carbon Dioxide,
3. Carbonate as Carbon Dioxide,
4. Citric Acid,
5. Casein,
6. Albumin and Lactoglobulin,
7. Inorganic Phosphates as Phosphorus.

These are shown in Table IV and were applied to formulae adapted from the formulae given by Fisher for the partial correlation coefficients of six variables. Results are indicated in Table V.

Table III.

Average and Standard Deviation of Constituents.

Constituent.	Average.	Standard Deviation.
Titratable Acidity	0.1716	.015702
Free Carbon Dioxide	6.4986	1.368265
Carbonates as Free Carbon Dioxide	0.7010	.185909
Citric Acid	42.3833	14.499703
Casein	2.8383	.410577
Albumin and Lactoglobulin	0.5760	.080233
Inorganic Phosphates as Phosphorus	63.4533	5.174800

Table IV.

Correlation Coefficients between pairs of Constituents.

Constituents.	Correlation Coefficients.
Acidity and Free Carbon Dioxide	- .3786
Acidity and Carbonates	+ .0863
Acidity and Citric Acid	- .4669
Acidity and Casein	+ .0365
Acidity and Albumin and Lactoglobulin	+ .0217
Acidity and Inorganic phosphates	+ .7412
Free Carbon Dioxide and Carbonates	+ .0469
Free Carbon Dioxide and Citric Acid	+ .1527
Free Carbon Dioxide and Casein	- .2251
Free Carbon Dioxide and Albumin and Lactoglobulin	- .0870
Free Carbon Dioxide and Inorganic Phosphates	- .2621
Carbonates and Citric Acid	- .0336
Carbonates and Casein	- .1108
Carbonates and Albumin and Lactoglobulin	+ .1017
Carbonates and Inorganic Phosphates	+ .0724
Citric Acid and Casein	- .0044
Citric Acid and Albumin and Lactoglobulin	- .2157
Citric Acid and Inorganic Phosphates	- .6070
Casein and Albumin and Lactoglobulin	+ .2420
Casein and Inorganic Phosphates	+ .1704
Albumin and Lactoglobulin and Phosphates	+ .1342

Table V.

Partial correlation coefficients for titratable acidity with a single constituent, the other five constituents being held constant.

With	Correlation Coefficient	p
Free Carbon Dioxide	- .3238	greater than 0.1
Carbonates as Free Carbon Dioxide	+ .0647	greater than 0.1
Citric acid and Citrates	- .0351	greater than 0.1
Casein	- .1589	greater than 0.1
Albumin and Lactoglobulin	- .1109	greater than 0.1
Inorganic Phosphate as Phosphorus	+ .6423	less than 0.01

p is the probability that values obtained are the result of random sampling and is taken from Table VA in Fisher's "Statistical Methods for Research Workers," (1925).

Discussion.

In the titration of fresh milk to determine its titratable acidity, using 0.1 N. NaOH and phenolphthalein, all that is obtained is an expression of the amount of the alkali necessary to shift the acid-base equilibrium from the original state of equilibrium to that of the $_{p}H$ in which phenolphthalein shows a color. There is no relationship between the $_{p}H$ of fresh milk and titratable acidity such as can be expressed in a simple acid-base system. This fact is evident in a physical sense, since the samples of milk under examination were all sweet to taste and smell, whereas, if there were such a relationship, the samples of high titratable acidity would have been noticeably sour.

The titratable acidity in the case of normal fresh milk therefore, is a measure of the buffer capacity. Buffer capacity may be defined as the capacity of the milk to withstand the addition of acid or alkali in producing changes of $_{p}H$. Van Slyk (1922) adopted the differential ratio $\frac{d\ B}{d_{p}H}$, that is the ratio of change in normality of acid or base to the corresponding change in $_{p}H$ when the change in $_{p}H$ is infinitely small, as the buffer index. The buffer index of a solution is not a constant value but changes over different ranges of $_{p}H$.

In attempting to correlate titratable acidity to buffer constituents, the fact of ionic association of the constituents must not be overlooked. An example of this was shown by Whittier (1929) in that the solubility of $Ca_3(PO_4)_2$ in milk is affected by the amount of citrates present. More recently, Wiley (1935) demonstrated the pronounced effect of calcium ions on the buffer effect of the phosphates.

Referring to Table V it will be seen that the partial correlation coefficient between titratable acidity and free carbon dioxide is of the negative form and does not reach a significant value at the 5% point. The value, however, is greater than that for some of the other variables, and the negative aspect might be interpreted on the basis that a solution of high acidity would contain less carbon dioxide under similar conditions than one of low acidity.

The partial correlation coefficients of acidity-carbonate, acidity-citric acid, acidity-casein and acidity-albumin and lactoglobulin, were too low to be considered significant.

The value of + .6423 obtained as the partial correlation coefficient of acidity and inorganic phosphate with other constituents held at a constant level, is significant. The probability of chance happening in this case is less than .01 and the positive value indicates a direct relationship.

That this relationship is linear to some extent is shown in Fig. III, in which the frequency grouping of titratable acidity and inorganic P for the 30 samples is shown. Since the inorganic phosphate gave the greatest partial correlation coefficient with titratable acidity and shows a direct linear relationship, it is suggested that this constituent may be regarded as the principal constituent responsible for titratable acidity of the various milks examined.

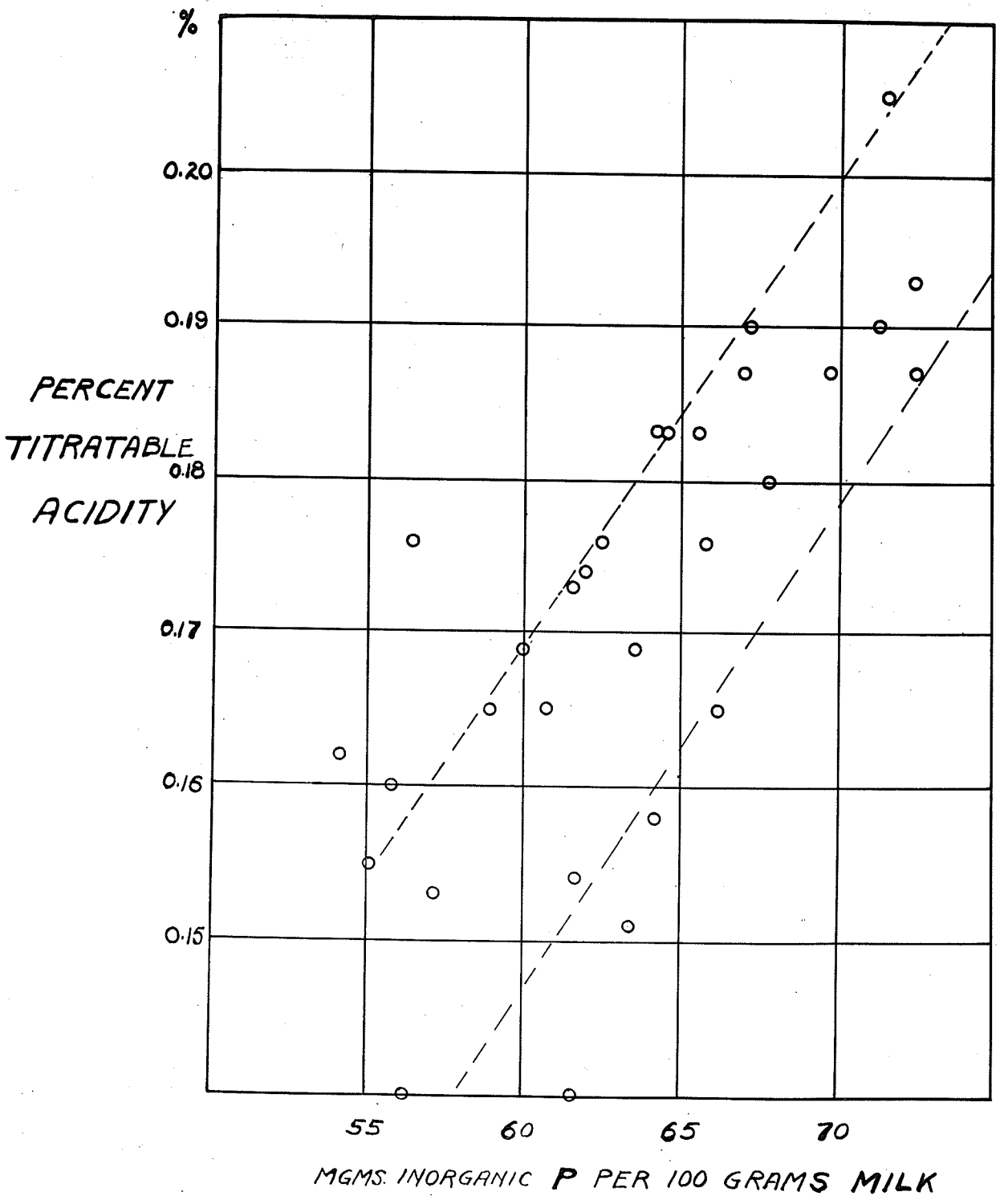
Extending this suggestion to the particular case of high titratable acidity of milk from Jersey cows as compared to milk from other breeds, an application of the data of other workers is of assistance. Watson (1931) reports Jersey milk to have a greater buffer capacity than Holstein milk in the pH zone of 5.0 to 6.0. This is significant since Clark and Whittier (1935) state that calcium phosphate in milk exerts its maximum buffering effect in the neighborhood of pH 5.5. Also, Graham and Kay (1933) report an influence of breed on the phosphorus content of milk. They found the Jerseys to be highest in total phosphorus, acid-soluble phosphorus and ester phosphorus. Taken in conjunction with the direct linear relationship already shown, these findings would indicate the phosphates of Jersey milk to be responsible for the high average titratable acidity.

Similarly the titratable acidity of fresh milk from

other breeds may be caused by the same constituent. Although only Jersey milk was used as a source of samples, the range of titratable acidity extended from 0.14% to 0.205% and investigations have shown instances of individual cows of other breeds covering the same range.

It is not suggested that the acidic nature of fresh milk is entirely due to the phosphate content. Sufficient work has been accomplished by investigators to indicate that an ionic equilibrium of other constituents exists. Thus while the buffer curves obtained in the preliminary investigation show the milk of high titratable acidity to buffer to a greater extent over a wide range of $_{p}H$ than milk of low titratable acidity, this "amphoteric buffering" cannot be attributed solely to phosphates alone. The fact of changes in equilibrium over a wide range of $_{p}H$ by the addition of acid or alkali may be the net result of the combination of buffer effects of the various constituents, each acting solely or in combination at different stages of $_{p}H$ concentration. Results of this experiment would, however, suggest the phosphates as the principal cause of the differences in titratable acidity as determined by titration with sodium hydroxide, using phenolphthalein as an indicator.

FIGURE III.


%
0.20
0.19
PERCENT
TITRATABLE
0.18
ACIDITY
0.17
0.16
0.15
55
60
65
70
MGMS. INORGANIC P PER 100 GRAMS MILK

Summary and Conclusions.

1. A method is indicated for the determination of Free Carbon Dioxide in fresh milk.

2. The method shown is extended to the estimation of Carbonates, which are reported as Carbon Dioxide.

3. Partial correlation coefficients of the results of analyses for the suspected buffer constituents, viz., Free Carbon Dioxide, Carbonates as Carbon Dioxide, Citric Acid and Citrates, Casein, Albumin and Lactoglobulin combined, and Inorganic phosphates as Inorganic P., with titratable Acidity, gave significant values only in the case of the Inorganic Phosphates.

4. It is shown graphically that the relationship between the Inorganic Phosphates and Titratable Acidity, is approximately linear.

5. A suggestion is offered that the Inorganic Phosphate content is the principal cause of the acidity of fresh milk as determined by titration with sodium hydroxide and phenolphthalein, and also that this constituent is responsible for the variations in titratable acidity of different samples of fresh milk.

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APPENDIX.

Table A.

Prepared by Beau (1904) to give the citric acid content of milk, in centigrams per litre, corresponding to the $AgNO_3$ equivalent. To be applicable, Beau's method must be followed exactly.

CITRIC ACID CONTENT IN CENTIGRAMS PER LITRE.

ccs. 0.1 $AgNO_3$	0.0	0.1	0.2	0.3	0.4
0	0.0	3.0	6.0	9.0	12.0
1	29.0	32.5	38.5	41.5	41.5
2	59.0	62.0	65.0	68.0	71.0
3	88.5	91.5	94.5	97.5	100.5
4	118.0	121.0	124.0	127.0	130.0
5	147.5	150.5	153.5	156.5	159.5
6	178.0	181.0	184.0	187.0	191.0
7	210.0	213.0	216.0	219.0	223.0
8	242.0	245.0	248.0	251.0	255.0
9	273.5	276.5	279.5	282.5	285.5
10	305.5	307.5	311.5	314.5	317.5

APPENDIX.

Table A (cont'd).

Prepared by Beau (1904) to give the Citric acid content of milk, in centigrams per litre, corresponding to the $AgNO_3$ equivalent. To be applicable, Beau's method must be followed exactly.

CITRIC ACID CONTENT IN CENTIGRAMS PER LITRE.

ccs. 0.1 $AgNO_3$	0.5	0.6	0.7	0.8	0.9
0	15.0	17.5	20.5	23.5	26.5
1	44.5	47.0	50.0	53.0	56.0
2	74.0	76.5	79.5	82.5	85.5
3	103.5	106.0	109.0	112.0	115.0
4	133.0	135.5	138.5	141.5	144.5
5	162.5	165.0	169.0	172.0	175.0
6	194.0	197.0	200.0	203.0	207.0
7	226.0	229.0	232.0	235.0	239.0
8	258.0	261.0	264.0	267.0	270.0
9	289.5	292.5	295.5	298.5	301.5
10	321.5	325.0	328.0	331.0	334.5