ELECTRONIC DETECTION OF ABNORMAL MILK

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ABSTRACT

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By

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Milk conductivity was measured using an in-line electrodeless conductivity cell. Overall correlation analysis revealed a positive correlation ($p < 0.01$) between conductivity and somatic cell numbers ($r = 0.25$). Milk conductivity was also significantly correlated ($p < 0.01$) with milk constituents including sodium ($r = 0.60$), potassium ($r = -0.28$) and lactose ($r = -0.56$). These constituents were markedly influenced by lactation variables. Milk sodium concentration (mg/100 ml) increased linearly ($p < 0.01$) from 43.3 for two year olds to 62.5 for cows older than six years. In contrast, milk potassium (mg/100 ml) and lactose (%) decreased linearly ($p < 0.01$) from 146.2 and 5.0 for two year old cows to 136.0 and 4.6 for cows greater than six years, respectively. Milk yield markedly influenced sodium, potassium and lactose concentration. Sodium concentration (mg/100 ml) decreased with increasing milk production from 81.2 for yields less than 10 lbs to 51.8 for yields greater than 30 lbs. Conversely, milk lactose and potassium concentration increased linearly ($p < 0.01$) with increased

milk yield. It is concluded that changes in milk constituents due to physiological factors influenced milk conductivity more markedly than those associated with inflammation. As a result, the relationship of conductivity to milk somatic cells is too low to be useful in detection of abnormal milk.

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INTRODUCTION

Inflammation of the mammary gland is commonly referred to as mastitis, a term derived from the Greek work "mastos" meaning breast and the suffix "itis" meaning inflammation of.

Despite five decades of active research, mastitis continues to be a major problem to the dairy industry. Estimated annual losses due to mastitis range up to five million dollars in the United States (National Mastitis Council Inc., 1965).

Mastitis has been difficult to eradicate for many reasons, foremost among these are the numerous causative agents as well as a variety of predisposing factors. Prerequisite to the control of any disease is the availability of accurate, dependable and economical methods of detection. There are a number of tests for mastitis referred to as screening tests. Most of these are based either directly or indirectly on the somatic cell content of milk. Somatic cells increase in milk during udder inflammation and constitute one part of the animal's natural defense mechanism for combatting diseases. Other changes in milk that occur concurrently with udder inflammation are increased albumin, globulin, sodium, chloride and decreased lactose, potassium, magnesium, calcium and phosphates. Increase in sodium and

chloride results from the tendency of the mammary gland to maintain isotonicity with the blood regardless of the state of lactose synthesis. When lactose synthesis is impaired as is common in mastitis, the osmotic deficiency is compensated by increases in sodium and chloride (Webb and Johnson, 1965).

At least two basic laboratory tests for inflammation have been developed utilizing increased milk chloride. One test is based upon determination of chloride content by titration of milk, while the second method is based on the chloride to lactose ratio. This latter method is known as the "Kuestler Test," which provides a chloride-lactose number that can be compared to normal values. Several investigators have attempted to measure the change in milk electrolyte concentration by electrical conductivity. This would provide a quick and convenient method for the early detection of udder inflammation. However, such problems as electrode polarization, temperature variation and accumulation of milk on electrodes have obscured the results of such attempts.

The purpose of this study was to investigate the possibility of developing a screening test for mastitis based on electrical conductivity of milk using an electrodeless conductivity cell.

REVIEW OF LITERATURE

I. Electrical Conductivity of Milk

Electrical conductivity is a physical property of milk. A solution containing electrolytes (i.e., acids, bases and salts) exhibits electrical resistance, which is measured in terms of a specific resistance defined as the resistance in ohms offered by one cubic centimeter of a substance to the passage of electricity, the current being perpendicular to two parallel faces. The reciprocal of the specific resistance is the specific conductance or conductivity expressed as $ohms^{-1}$ cm^{-1} or mhos/cm. In a solution containing only electrolytes, conductivity is a function of ionic concentration. However, in heterogenous solutions such as milk the dispersed fat and colloidal substances decrease conductivity by obstructing migration of ions.

Cows with normal udders will secrete milk with nearly identical conductivities usually ranging from 45 to 48 x 10^{-4} mho, while cows with inflamed udders secrete milk with higher conductivities (Webb and Johnson, 1965). The foregoing observation together with results of individual investigations have led a number of investigators to advocate the use of electrical conductivity as a potential screening

test for mastitis. Foremost among these are Davis et al. (1943), Malcolm et al. (1942), McCulloch (1940), Krenn (1932) and Shulz (1956). Malcolm et al. (1942) noted the electrical conductivity of milk varies with stage of lactation, therefore, factors such as calving date and daily milk yield should be considered in using electrical conductivity for diagnostic purposes. Davis (1947) suggested the difference in conductivity between the four quarter samples of individual cows could be used successfully as an indicator of inflammation. But Jones (1949) evaluated the same method and failed to find good agreement between conductivity and direct somatic cell counts, the usual indicator of udder inflammation. However, his milk samples were stored at room temperature for 24 to 28 hours before analysis, which according to Verma (1950) can significantly influence ionic equilibrium, and hence, electrical conductivity. Other investigators (Little et al., 1968; and Postle and Blobel, 1965) have failed to recommend electrical conductivity as a screening test for mastitis, mainly because of its low correlation with direct microscopic cell counts. Little et al. (1968) found a correlation of 0.40 between the conductivity of quarter milk samples and direct microscopic cell count, while Postle and Blobel (1965) obtained a correlation coefficient of 0.24 between conductivity of bulk milk samples and direct microscopic cell count. Both groups of investigators recognized

electrical conductivity as a quick, objective and repeatable test for mastitis, but Little et al. (1968) contended that although there is an increase in chloride content and therefore increased conductivity during the inflammatory reaction, there would appear to be no base-line upon which to judge such changes.

Costle and Shelburn (1919) attribute 50 to 80% of the conductivity of milk to its chloride ion content. Similarly, Montefredine (1942) reported that only the chloride content of milk was correlated with its electrical conductivity. Other workers (Jackson and Rothera, 1914) demonstrated a reciprocal relationship between electric conductivity and percentage milk lactose, but only within stage of lactation. Pickerton and Peters (1958) observed an inverse relationship between conductivity and milk lactose, however, Barry and Rowland (1953) suggests this relationship results from the inverse relationship between lactose and chloride content. Pickerton and Peters (1958) also found electrical conductivity at 37°C to range between 5.04 and 5.63 millimhos per centimeter (m. mhos/cm) for normal milk, and 5.95 to 6.86 m. mhos/cm for milk from inflamed quarters. Shulz and Sydow (1957) reported wide variations for conductivity of milk free of chloride ions, but did not report the cause of such variations.

Most of the early work on milk conductivity has been summarized in reviews by Gerber (1926), and Shulz (1956). Early investigators were mainly interested in developing a

rapid test for adulterated milk, but also noted the potentials for diagnosis of udder inflammation. The electrode type of conductivity device has been used for most studies on milk conductivity. This has been a primary limiting factor in the use of electrical conductivity as a screening test. The electrodes become contaminated with colloidal materials during one measurement, resulting in reduced accuracy of succeeding measurements. Prentice (1962) described a method for cleaning the electrodes, but this is far too complex a procedure for in-line measurements.

In recent years the dairy industry has been subjected to increased demands in terms of efficiency of production. This increased efficiency dictates the necessity to develop a simple, efficient and economical method for early detection and treatment of mastitis. With the advent of more sophisticated electronic devices the electrodeless conductivity cell has become available, and this has created renewed interest in the use of electrical conductivity as a means of achieving the above mentioned goals. An electrodeless cell for measuring conductivity of fluids was described by Fielden (1952), Gupta and Hills (1965) and Sperry (1965). It was also used by McPhillips and Snow (1958) to follow the acid production of steptococcus lactis in milk, and more recently by Gerrish and Bickert (1970) in an automatic milking machine detacher.

II. Normal Composition of Milk

Milk is a characteristic secreation of the mammary gland of all mammals. Because of its function namely, nourishment of the young it is a complex that must supply nutrients, minerals and vitamins in the proper form, kind and amount (Henderson, 1971). Interest in the composition of milk stems largely from its use as human food, since the nutritive value of milk depends on its composition. Jenness and Patton (1959) stated that prior to 1850, the composition of milk was known only to the extent that it contained fat, sugar, proteins and minerals. However, numerous studies during the past century have revealed the presence of a wide array of constituents within each of these classes.

Milk from all mammalian species contains the same constituents but in varying amounts (Henderson, 1971). An average gross composition of normal cow's milk would be as follows: water, 87%; fat, 3.9%; lactose, 4.9%; proteins, 3.5% and ash or minerals, 0.7% (Watt and Merrill, 1963). Milk fat, lactose and some of the proteins (i.e., caseins, B-lactoglobulin and α-lactalbumin) are characteristic of milk and synthesized only in the mammary gland (Jenness and Patton, 1959).

Armstrong (1959) observed that although there is a voluminous literature on the normal composition of milk,

one is handicapped in attempting to analyse and integrate the data due to a number of limiting factors namely:

(1) A paucity of detailed information especially regarding mineral constituents.

(2) Testing methods: some information is from laboratory research, presumably conducted under controlled conditions, whereas other information represents data gathered from herd testing association records.

(3) Variations in preservation of samples prior to analysis.

(4) Variation in analytical procedures.

In some cases the quantity of data appears to be sufficient to establish meaningful averages for gross composition; whereas, in other cases, the data are lacking in quantity and uniformity (Armstrong, 1959).

1. Mineral Constituents

The mineral constituents of milk are those constituents which contain only inorganic elements in their ions (Allen, 1931). Schalm et al. (1971) reported that calcium, phosphorus, potassium, sodium, sulfur, chlorine and magnesium are the major mineral constituents in milk. Together with the minor mineral constituents these minerals account for 0.6 to 0.8% of milk by weight (Henderson, 1971). The secretory cells of the mammary gland cannot produce minerals, therefore, all the minerals in milk are supplied by the blood.

Although the minerals in milk are derived from the blood, it is not known whether they are absorbed in proportion

to their concentration in blood, or if mechanisms exist for selective uptake. There is some evidence (Azimov et al., 1962; Knutsson, 1964a, 1964b, and Mackenzie and Lascelles, 1965b) that mammary epithelial cells can discharge minerals into either blood or milk, suggesting the presence of an active transport mechanism. No attempt will be made to elaborate on all the mineral constitutents of milk in this review. It is the author's intention to mention only those constituents that are directly concerned with this study namely, sodium and potassium.

a. Sodium

Sodium is one of the major mineral constituents of milk. Together with lactose, potassium and chloride, it maintains the osmotic equilibrium of milk (Rook and Wheelock, 1967). Jenness and Patton (1959) reported the sodium concentration of milk to be lower than that of the blood. This difference in sodium concentration between blood and milk was reported to be greatest at the beginning of lactation, but the difference narrows as the lactation period advances (Barry and Rowland, 1953). Barthe and Dufilho (1927) reported that the sodium concentration of milk of healthy cows was never greater than 50 mg per 100 ml. But in a later report they (Barthe and Dufilho, 1928) suggested that the sodium content of cow's milk increased as the lactation period advanced, indicating that higher values might be realized. Comparing milk of 20 cows from the

Shorthorn and Guernsey breeds Jones and Davies (1935) found sodium concentration to range from 39.2 to 139.2 mg per 100 ml depending on stage of lactation. Macy et al. (1953) reported a mean value of 58 mg per 100 ml for the sodium concentration of normal cow's milk, while Jenness and Patton (1959) reported a mean concentration of 50 mg per 100 ml.

b. Potassium

Potassium is a physiologically important ion in the soluble phase of normal milk. Like sodium, it is a major mineral constituent which is involved in osmotic equilibrium in milk (Rook and Wheelock, 1967). But unlike sodium its concentration in milk is much higher than that in blood (Barry and Rowland, 1953). Studies by Rook and Wood (1959) indicated that healthy cows had the ability to produce milk with a constant potassium concentration throughout the first four to five months of lactation including the period of transition from colostrum to normal milk when changes in other constituents were large. However, significant variations between areas in the United States (Ward, 1963) and areas within California (Nickerson, 1960) have been reported. Barry and Rowland (1953) indicated that the potassium concentration of milk decreased to approach the concentration of blood as lactation period advanced. This is especially true during the last two months of lactation. In a study of the mineral elements characteristic of the soluble phase

of cow's milk, Barry and Rowland (1953) found that a negative linear relationship existed between potassium and sodium and potassium and chloride, while a positive linear relationship was found to exist between potassium and lactose. Macy et al. (1953) reported a mean concentration of 141 mg per 100 ml for the potassium content of normal cow's milk.

2. Lactose

The characteristic carbohydrate of normal milk is the disaccharide, lactose, which is synthesized only in the mammary gland. Lactose exists in two stereoisomeric forms, i.e., alpha and beta. Each form is composed of one molecule of D-glucose and one molecule of D-galactose. In cow's milk as in all aqueous solutions both forms are present with the equilibrium mixture consisting of 1 part alpha to 1.65 parts beta (Ling et al., 1961).

First records of lactose isolation date back to Bartolettus (1633), who isolated it from whey. However, it was Scheele (1780) who proved that lactose is a true sugar, and listed it as a constituent of milk. Rook and Wheelock (1967) reported that lactose accounted for a major part of the osmotic pressure of milk, and increased lactose concentration caused an influx of water and a decrease in sodium and chloride concentration in milk. The secretion of lactose is not constant throughout lactation. Unlike milk fat and protein which tend to increase as lactation

advances, lactose production decreases steadily throughout lactation (Schalm et al., 1971). The lactose content of normal cow's milk ranges from 4.4 to 5.2% accounting for about 52% of the solids-not-fat content of milk (Nickerson, 1965).

III. Udder Inflammation and Its Influence on Milk Composition

1. General

Inflammation is the final phase of a proposed three-phase concept (i.e., invasion, infection and inflammation) of udder infection (Murphy, 1947). Little et al. (1968) reported that the effect of udder inflammation on the composition of milk was dependent upon severity of the inflammatory response. On the basis of severity, inflammation may be classified as clinical inflammation characterized grossly by swelling, heat, redness, pain and impaired function, or as subclinical which is the existence of inflammation in the absence of overt clinical signs (Schalm et al., 1971). Herd surveys, pathologic changes seen in infected glands and results of transmission experiments leave little doubt that bacterial infection is the primary cause of udder inflammation. Predisposing factors such as teat patency, age, chilling, feeding and hygiene are considered secondary factors because they contribute to udder inflammation primarily by decreasing resistance to bacterial

infection or by causing development of clinical inflammation in quarters harboring infectious organisms (Plastridge, 1958).

Udder inflammation may be caused by more than twenty different types of pathogenic bacteria, plus rickettsia, yeasts, fungi and viruses. However, of these microorganisms, four gram-positive bacteria (i.e., streptococcus agalactiae, streptococcus dysgalatiae, streptococcus uberis and staphylococcus aureus) account for 97% of all udder infections (Roberts, 1967). Plastridge (1958) observed that these organisms usually caused chronic inflammation, with changes in milk composition and loss of milk yield, with or without appearance of clinical symptoms. Actually it is not the bacteria per se, but rather their toxins that cause an inflammatory response. The purpose of the inflammatory response is to destroy or neutralize the irritant, repair tissue and return the gland to its normal function (Schalm et al., 1971).

Rook (1961) reported that inflammation of the mammary gland modified milk composition by altering the permeability of the udder tissue and by impairing the ability of the secretory tissue to synthesize milk constituents. Characteristic alteration in milk composition associated with these changes are decreased lactose and potassium content, with compensatory increase in sodium and chloride (Barry and Rowland, 1953; Munch-Petersen, 1938, McDowall,

1945 and Wheelock et al., 1966). Also an increase in globulin content, and to a lesser extent, increased serum albumin and proteoses, and decreased casein content have been reported (Rowland, 1938). Rook (1961) also observed that, although changes in composition are generally more marked when an inflamed quarter showed clinical symptoms of disease (i.e., mastitis), attempts to relate their extent to the degree and incidence of infection have not been successful. This he claimed might be due to the numerous organisms capable of attacking udder tissue, and because the presence of pathogenic bacteria in milk is not necessarily associated with extensive damage to udder tissue. Laing and Malcolm (1956) and Van Rensburg (1947) have also demonstrated that mammary gland inflammation could occur in the absence of pathogenic bacteria in the milk or udder tissue.

2. Effect of Udder Inflammation on:

a. Sodium and Potassium Content

Bitman et al. (1963) suggested that variations in sodium and potassium content in milk indicated inflammatory edema. McDowall (1945) observed an increased sodium chloride content in milk from inflamed quarters. More recently Barry and Rowland (1953) and Wheelock et al. (1966) reported increased sodium and decreased potassium in milk from inflamed udders. Barry and Rowland (1953) indicated that the changes in these constituents in the inflamed udder

were similar to changes observed during late lactation. They hypothesized that the increased sodium and decreased potassium are due to the mixing of milk secreted by the mammary epithelial cells, with a diluent in which the concentrations of these ions are approximately the same as in blood serum. The observations of Davis (1933) and Preskett and Folley (1933) in which they considered milk of low solids-not-fat to be made up of a true milk fraction, and a diluting fraction somewhat similar to a transudate of lymph serum origin, lends credence to this hypothesis. Rook (1967) reported changes in the sodium and potassium concentration in milk from inflamed quarters almost invariably occurred in association with reduction in milk volume. He suggested that although sodium is present in increased concentration, it is usually secreted in reduced amounts, except for short periods after development of bacterial infection (Wheelock, Rook, Neave and Dodd, 1966), in the period following extended milking interval (Wheelock, Rook, Dodd and Griffin, 1966) and after administration of oxytocin (Wheelock et al., 1965d).

b. Lactose

Numerous investigators (Barry and Rowland, 1953; Munch-Petersen, 1938; McDowall, 1945; and Wheelock et al., 1966) reported that there was a decreased lactose content in milk from inflamed udders. Wheelock et al. (1966) observed that the reduction in lactose concentration was

closely related to the severity of the clinical symptoms of inflammation. Since osmotic equilibrium between milk and blood was maintained, Rook (1961) and Rook and Wheelock (1967) suggested that the observed decrease in lactose content in milk from inflamed udder quarters was compensatory to the increased sodium and chloride content of such milk. This increase in sodium and chloride might be due to decreased lactose synthesis by the injured epithelial cells which would therefore require more sodium and chloride to maintain osmotic equilibrium. The changes in milk lactose concentration following udder inflammation were similar to changes observed in late lactation (Barry and Rowland, 1953), and also when milk was allowed to accumulate in the mammary gland (Wheelock et al., 1965b and Wheelock et al., 1966).

Some investigators (Wheelock et al., 1965b and Wheelock et al., 1966) suggested that the reduction in lactose concentration occurred as a result of resorption into the blood and excretion in the urine, while Rook and Wheelock (1967) claimed that it might occur both from impairment of lactose synthesis and partial resorption.

c. Milk Somatic Cells

Inflammation is characterized by the accumulation of neutrophilic leucocytes and humoral substances in the area of injury. In udder inflammation these substances pass into the milk (Schalm et al., 1971). A number of

investigators (Anderson, 1946; Little, 1940; McLeod and Anderson, 1952; McEwen and Cooper, 1947; Murphy, 1943 and Murphy and Stuart, 1953), observed that milk from normal quarters rarely contained more than 500,000 leucocytes per ml, while the milk from inflamed quarters usually had leucocytes in excess of this number.

Hughes (1954) showed that a high somatic cell count in milk was associated with inflamed quarters. McFarlane et al. (1949) and Chu (1949) concluded that high cell counts were an indication of mastitis. Furthermore, it has been demonstrated by many workers such as Branum and Newbould (1961), Jensen (1957), Leidl and Schalm (1961), Leidl et al. (1961) and Schalm (1959), that the California Mastitis Test and the Milk Quality Test for udder inflammation are indirect measures of the concentration of leucocytes in milk.

Blackburn and Macadam (1954) and Blackburn et al. (1955) reported that there are two major types of cells in milk; polymorphs, which provide an indication of the extent of acute lobular inflammation, and epithelial cells which reflect the extent of post inflammatory involution. Waite and Blackburn (1957) suggested that for animals with subclinical inflammation throughout the major part of their lactation, there was an association between total cell counts in milk and changes in milk composition. They considered that milk with a total cell count of less than 100,000 per ml, did not indicate subclinical mastitis.

With cell count increasing to 500,000 per ml, they observed a progressive reduction in solids-not-fat and lactose. Macadam (1958) concluded that the proportion of granulocytes usually exceeded 70% of the total cells in milk from acutely inflamed quarters, whereas it was usually less than 40% during mammary involution. Likewise, Blackburn et al. (1955) indicated that milk samples with a low total cell count generally contained less than 45% granulocytes. They suggested that differential cell count is a valuable criterion to confirm conclusions drawn from results of total cell counts, especially where doubts arise owing to the presence of bacteria in milk.

IV. Other Factors Affecting Milk Composition

1. Age and Lactation Number

Under commercial conditions a progressive decrease in the concentration of lactose and potassium, and an increase in sodium with increasing lactation number have been reported (Rensburg, 1947; Waite et al., 1956; Politiek, 1956; Vanschoubrock, 1963; and Vanschoubrock et al., 1964). Rook and Campling (1965) reported that the major constituents of milk secreted during the period from the fifth to the twenty-second week of lactation decreased with lactation number. Decreased solids-not-fat due mainly to reduction in lactose concentration up to the fifth lactation have been reported (Bailey, 1952a; Waite et al., 1956; and Wilcox et al., 1959).

Rook and Wheelock (1967) indicated that the extent to which these observed changes in milk composition are directly attributable to age is uncertain. They suggested that bacterial infection of the udder, the incidence of which tends to increase with age might be expected to contribute to these changes. Results from studies of Rook and Campling (1965) in which the effects of udder infection were almost completely excluded showed that changes in lactose content for three animals from the first to the third lactation were -0.09, ± 0.00 and -0.08%. However, O'Donvan et al. (1960) observed an average decrease of 0.1% in solids-not-fat due mainly to decreased lactose concentration, between consecutive infection free lactations. This latter observation would seem to suggest that there might be a specific effect related to age.

2. Stage of Lactation

The composition of cow's milk changes considerably with the progress of lactation. The greatest changes are reported to occur at the beginning, and at the end of the lactation period (Jenness and Patten, 1959). Some of the first recorded studies of milk composition (Richmond, 1899 and Crowther and Ruston, 1911) established that the lactose content, although exceptionally low in colostrum, is at a maximum early in lactation, and tends to decrease as the lactation period progresses (Rook and Wheelock, 1967). Rook and Campling (1965) reported that lactose content

reached a maximum in forty-five days, decreased slowly until about 165 days after calving, after which the decline was more rapid. They also reported that the lactose content of milk from cows in their first lactation decreased much more slowly with advancing lactation than that of milk from older cows.

Converse changes in the concentration of sodium were reported by Richmond (1899). More recently, numerous investigators (Azarme, 1938; Bonnier et al., 1946; Waite et al., 1956 and Voigtländer, 1963) have confirmed the general trends, and have demonstrated that changes in potassium content generally follow those of lactose (Rook and Campling, 1965).

MATERIALS AND METHODS

1. Conductivity Cell

The electrodeless conductivity cell used to measure the electrical conductivity of milk samples was a modification of the Gupta and Hills device (1965) as modified by Gerrish and Bickert (1970). It was used in conjunction with: (1) a Signal Conditioner Model 300-D (Daytronic Co., Dayton, Ohio) including an amplifier indicator with a Type 71 differential transformer input module, modified for use with the electrodeless conductivity cell and (2) a Speedomax-G two pen Recorder (Leeds and Northrup, North Wales, Pennsylvania).

The electrodeless conductivity cell (Figure I) consists of two transformers. The first transformer comprises a thirty-four turn primary winding (labeled input), and a single secondary turn of milk in a nonconducting tube. This single turn of milk acts also as a one-turn primary winding for a second transformer, which has a thirty-four turn secondary winding (output). Both primary and secondary windings are wound around a toroidal core of laminated magnetic material such as might be found in current transformers.

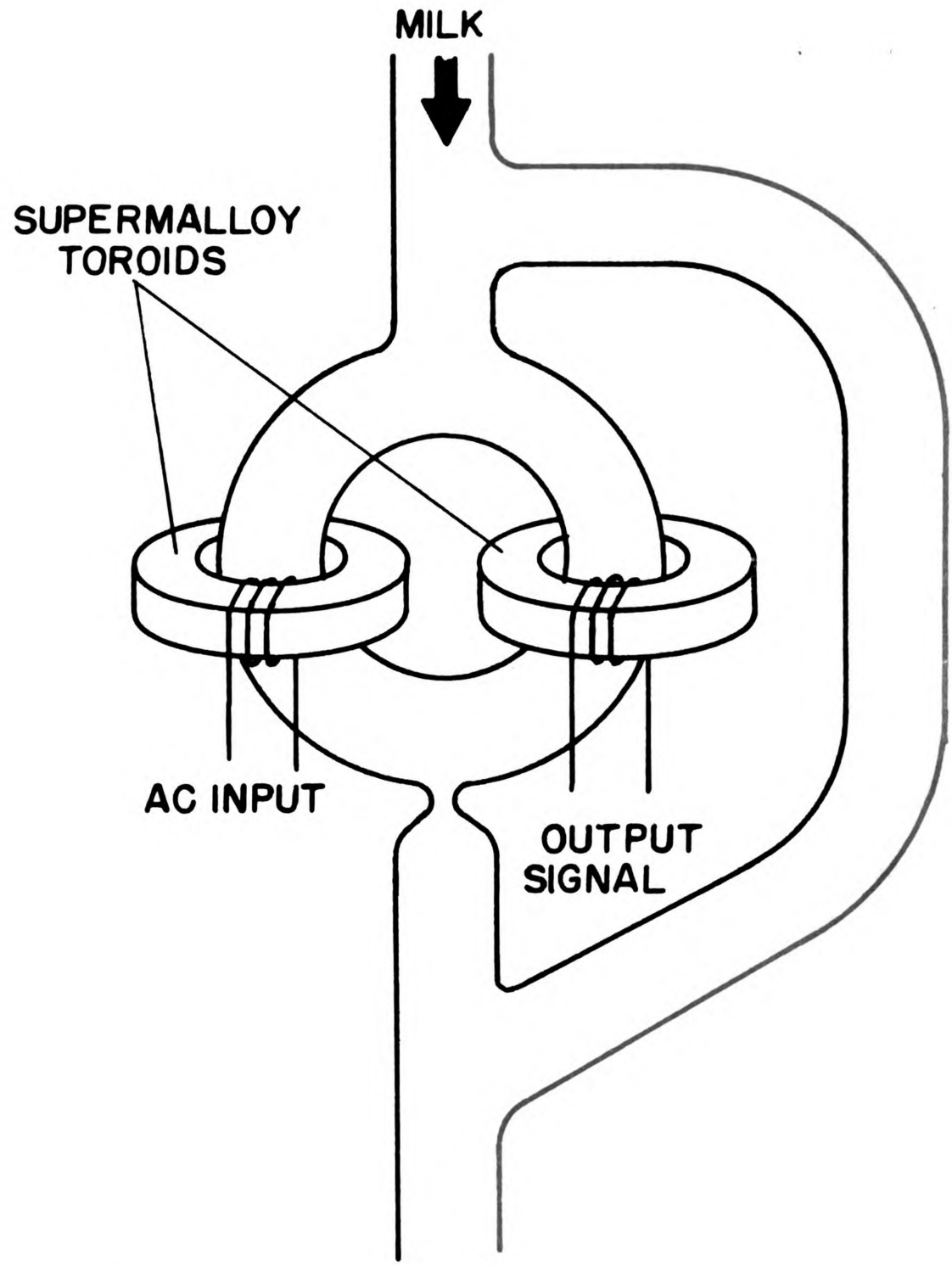

Figure 1. An In-line Electrodeless Conductivity Cell.

Supermalloy, an alloy with high magnetic permeability at low magnetizing force, was used as the core material for the second transformer.

When the flow rate of milk entering the milk loop is greater than that which can pass through a 2.25 millimeter orifice at the bottom of the milk loop, the milk loop fills and excess milk is diverted through a by-pass tube. An alternating input voltage will cause a small alternating current to flow in the continuous loop of milk. If this current is sufficient to provide a magnetizing current for the second transformer core, an output signal will be obtained. This output signal was amplified and transferred to the recorder so that a measurement in terms of peak height was obtainable. The electrical conductivity of each milk sample was determined by measuring peak height and converting to conductivity using a standard chart. This chart was prepared by first determining the cell constant, using a standard 0.05M sodium chloride solution, and then drawing a calibration curve using a resistance substitution box.

2. Methodology

a. Sodium and Potassium Analysis

Within 60 minutes of collecting daily milk samples, duplicate 2 ml aliquots of each milk sample were placed in 16 ml polypropylene centrifuge tubes (Sorvall, Newtown, Conn.). Eight ml of 20% Trichloroacetic acid (Mallinckrodt

Chemical Works, St. Louis) was added to the contents of each tube, mixed, allowed to stand for 10 minutes, after which it was centrifuged at 6000 x g for 5 minutes.

Following centrifugation duplicate 1 ml aliquots of the trichloroacetic acid supernatant were transferred into 15 ml Pyrex Brand tubes (Scientific Prod., Evanston, Ill.) containing 10 ml of deionized water and fitted with crew caps. A water blank consisting of 10 ml of deionized water was prepared daily. All samples were stored at 5C until analysed by atomic flame emission spectrophotometry, using a Jarrel-Ash Model 82-516 Spectrophotometer (Fisher Scientific Co., Fairlawn, N.J.) equipped with Hecto total consumption burner. The concentration of each substance was determined by comparing the unknown measurement with standard quantities of sodium and potassium. Working solutions for standard curves of 40, 30, 20, 15, 10 and 5 mg/liter for sodium and 40, 30, 20 and 10 mg/liter for potassium were made up from stock solutions (500 mg/liter) of sodium and potassium as per Table 1.

b. Lactose Analysis

Lactose analysis was carried out by modification of the chloramine-T method of Hinton and Macara (1927). Duplicate 2 ml quantities of each milk sample were added to 50 ml polypropylene centrifuge tubes and the protein and fat precipitated by the addition of 8 ml tungstic acid

Table 1. Working Solutions for Standard Sodium and Potassium Curves

Working Solution (mg/litre)	Stock Solution (ml)	10% T.C.A. (ml)	Deionized Water (ml)
40	8.0	6.82	85.18
30	6.0	6.82	87.18
20	4.0	6.82	89.18
15	3.0	6.82	90.18
10	2.0	6.82	91.18
5	1.0	6.82	92.18

reagent (Appendix I). Following precipitation, duplicate 10 ml samples of the supernatant were pipetted into 125 ml erylenmeyer flasks. To each flask 5 ml of potassium iodide and 20 ml of N-chloro-p-toluenesulfanamide (Chloramine-T); (Matheson, Coleman and Bell Inc., Norwood, Cincinnati) were added, after which each flask was stoppered with parafilm (American Can Co., Neenah, Wisconsin) and stored in the dark for 1.5 hr at room temperature. A water blank consisting of 10 ml of double distilled water was carried through the analysis as control. At the end of the specified period 5 ml of 1N hydrochloric acid was added to each flask and the unreacted chloramine-T measured by titration of the liberated iodide with 0.04N sodium thiosulfate using 1% soluble starch as an indicator. The difference between the chloramine-T in the blank solution and that remaining in the sample analysed is equivalent to the lactose content in the sample.

c. Direct Microscopic Somatic Cell Count Procedure

The technique of Direct Microscopic Somatic Cell Count is recommended for estimation of the number of nucleated somatic cells in milk samples. It is a modification of the technique described by Prescott and Breed (1910).

Within 30 minutes of obtaining milk samples duplicate smears were prepared for each milk sample by transferring 0.01 ml of milk to a precleaned microscope slide using a 10-lambda pipette. Between samples the pipette was washed

in detergent, and rinsed in distilled water and acetone. The 0.01 ml of milk was spread over a circular area of one square centimeter, air-dried for 24 hr, and stained in Newman's Stain (Appendix 2A) according to the procedure outlined in Appendix 2B. Cover slips were mounted with the aid of Permount (Fisher Scientific Co., Fairlawn, N.J.) and then cleaned with Xylene (Merck and Co. Inc., Rahway, N.J.). Microscopic counting of nucleated somatic cells was carried out on a Leitz Ortholux compound microscope (E. Leitz Inc., New York, N.Y.) using an oil-immersion objective of 54X magnification and 10X eyepiece. The unit area of the milk film counted was a strip, the width of which was defined by the distance between two parallel lines on a special eyepiece reticle and the length being the diameter of the milk film. Estimation of the cellular concentration in a milk sample was based on the counts of two mutually perpendicular strips on each of duplicate milk films.

3. Experimental Procedure

The study reported here involved electrical conductivity measurements and collection of 295 milk samples over a six month period. Samples were obtained from 94 Holstein cows of various ages and stages of lactation. Prior to the start of the experiment the electrodeless conductivity cell (Figure I) together with accompanying electronics and Recorder were installed in a double eight Herringbone milking parlor at the Michigan State University

dairy barn. The conductivity cell was attached to a single milking machine, and conductivity measurements were obtained for quarter composite samples. Thereafter, milk in the conductivity cell was collected via an orifice at the bottom of the milk loop. The milk in the by-pass tube was allowed to run out, and only milk coming from the milk loop was collected. This method of sampling ensured that only milk from which a conductivity measurement had been taken was included in the sample.

Samples were collected in 150 ml glass bottles during the first minute of milking. These samples were transported to the laboratory soon after collection and were analysed for lactose, sodium, potassium and total somatic cells.

RESULTS

1. General

Overall correlation analysis for 295 milk samples revealed that milk conductivity was positively correlated ($p < 0.01$) with age and lactation number (Table 2). Similarly, sodium concentration was correlated ($p < 0.01$) with age, lactation number and milk yield. In contrast, correlation between lactose and age and lactose and lactation number were negative ($p < 0.01$). Potassium was negatively correlated ($p < 0.01$) with stage of lactation, but positively correlated ($p < 0.01$) with milk yield. The relationships between direct microscopic somatic cell count and individual lactation variables were low and did not approach significance ($p > 0.05$).

Further correlations between individual parameters measured are shown in Table 3. The correlations between conductivity and sodium ($r = 0.60$) and conductivity and direct microscopic somatic cell count ($r = 0.25$) were positive and highly significant ($p < 0.01$). Conversely, highly significant ($p < 0.01$) negative correlations were found to exist between conductivity and potassium and lactose respectively. When samples were classified as normal (cell concentration $< 5 \times 10^5$) or abnormal (cell

Table 2. Correlations between Lactation Variables and Milk Constituents or Conductivity[a]

	Age (Yrs)	Lactation No.	Lactation Stage (Days)	Milk[b] (lbs)
Sodium	0.26**	0.23**	0.19**	-0.21**
Potassium	-0.12	-0.10	-0.24**	0.29**
Lactose	-0.27**	-0.25**	-0.03	0.13
Conductivity	0.28**	0.30**	0.02	0.04
DMSCC[c]	0.11	0.11	-0.04	-0.11

[a] n = 295

[b] Yield in lbs at sampling time

[c] Direct Microscopic Somatic Cell Count

** r significant at $p < 0.01$

Table 3. Correlations between Milk Constituents and Conductivity[a]

	Potassium	Lactose	Conductivity	DMSCC[b]
Sodium	-0.24**	-0.63**	0.60**	0.33**
Potassium	1.00	0.37**	-0.28**	-0.11
Lactose		1.00	-0.56**	-0.27**
Conductivity			1.00	0.25**

[a] n = 295

[b] Direct Microscopic Somatic Cell Count

** r significant at $p < 0.01$

concentration > 5 x 10^5) the correlation between conductivity and direct microscopic cell count for 53 abnormal samples was 0.24 which was significant at the 10% level.

Compared to direct microscopic somatic cell count, conductivity measurements were less variable, and more reproducible. For a total of 284 samples the mean somatic cell count x10^4 was 44.1 ± 6.8 and individual counts ranged from 0.9 to 1436 x 10^4. The mean conductivity value was 6.5 ± 0.1 millimhos per centimeter (m.mhos/cm), with individual values ranging from 4.6 to 9.8 m.mhos/cm. There was however, considerable variation between conductivity measurements and somatic cell counts for many samples. Conductivity values as high as 9.1 m.mhos/cm were found where somatic cell counts were less than 10 x 10^4 cells/ml, whereas for cell counts 50 x 10^4 cells/ml and greater, 12 samples or 22.6% of the samples had conductivity values below 6.0 m.mhos/cm. Comparison of means (± standard errors) for several milk constituents and conductivity of samples classified as normal or abnormal based on somatic cell counts are shown in Table 4. Mean conductivity for samples having somatic cell concentration of > 50 x 10^4 cells/ml was significantly higher ($p < 0.01$) than the mean value for samples with < 50 x 10^4 cells/ml. The mean sodium concentration of high cell milk was 74.1 mg/100 ml which was larger ($p < 0.01$) than the comparable mean for low cell milk (53.7 mg/100 ml). In contrast, mean potassium

Table 4. Mean Sodium, Potassium, Lactose and Conductivity Values of Normal[a] and Abnormal[b] Milk

Somatic Cells per ml	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Lactose (%)	Conductivity (m.mhos/cm)
$<5 \times 10^5$[c]	53.7±1.3	144.6±1.5	4.8±0.0	6.4±0.1
$>5 \times 10^5$[d]	74.1±4.0	131.7±3.9	4.5±0.1	7.0±0.2

[a] Normal Milk = $< 5 \times 10^5$ somatic cells/ml

[b] Abnormal Milk = $> 5 \times 10^5$ somatic cells/ml

[c] $n = 231$

[d] $n = 53$

(131.7 mg/100 ml) and lactose 4.5% concentration of high cell milk was less ($p < 0.01$) than comparable values for low cell milk (144.6 and 4.8 respectively).

2. Influence of Individual Lactation Variables on Milk Constituents and Conductivity

a. Age

The effect of age on milk constituents and conductivity is shown in Table 5. Mean sodium concentration increased linearly ($p < 0.01$) with increase in age from 43.3 mg/100 ml in milk from two year olds to 62.5 mg/100 ml in milk from cows greater than age six. Similarly, mean milk conductivity values increased linearly ($p < 0.01$) with age in cows two to six years. On the other hand mean milk lactose and potassium concentration decreased linearly ($p < 0.01$) with increase in age. The effect of age on somatic cell concentration was not significant ($p > 0.05$).

b. Lactation Number

Mean sodium concentration increased linearly ($p < 0.01$) with increasing numbers of lactations (Table 6). Milk sodium concentration increased from 43.1 mg/100 ml for milk samples from cows in their first lactation to 66.7 mg/100 ml for milk samples from cows in their fifth lactation. Like sodium, mean conductivity increased linearly ($p < 0.01$) from 6.0 m.mhos/cm during the first lactation to 7.4 m.mhos/cm during the fifth lactation. Conversely, mean lactose concentration decreased linearly ($p < 0.01$) as number of

Table 5. Milk Sodium, Potassium, Lactose and Somatic Cell Concentration and Electrical Conductivity--Effect of Age

Age (Yrs)	No. of Samples	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Lactose (%)	Conductivity (m.mhos/cm)	DMSCC[1] (x10^4/ml)
2	30	43.3[a]	146.2[a]	5.0	6.2[a]	43[a]
3	60	46.1[a]	144.8[a]	4.9	6.0[a]	25[a]
4	56	59.1[b]	144.9[a]	4.7[a]	6.4	38[a]
5	48	63.1[b]	137.9[a]	4.6[a]	6.7[b]	46[a]
6	53	64.8[b]	141.4[a]	4.6[a]	6.8[b]	94[a]
>6	48	62.5[b]	135.6[a]	4.6[a]	6.8[b]	38[a]
Overall Variance		252	488	0.37	0.07	161

[1]Direct Microscopic Somatic Cell Count

[a,b]Values in a column having the same superscript are not different ($p > 0.05$).

Table 6. Milk Sodium, Potassium, Lactose and Somatic Cell Concentrations, and Electrical Conductivity--Effect of Lactation Number

Lactation No.	No. of Samples	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Lactose (%)	Conductivity (m.mhos/cm)	DMSCC[1] ($x10^4$/ml)
1	72	43.1	$145.7^{a,b}$	5.0^{a}	6.0	33^{a}
2	59	55.7^{a}	146.9^{a}	4.7^{a}	6.4^{a}	31^{a}
3	57	65.0^{b}	134.2^{b}	4.6^{a}	6.6^{a}	50^{a}
4	48	65.9^{b}	$140.2^{a,b}$	4.6^{a}	6.8^{a}	64^{a}
5	28	66.7^{b}	$143.4^{a,b}$	4.5^{a}	7.4	33^{a}
>5	31	55.3^{a}	$137.3^{a,b}$	4.7^{a}	6.6^{a}	43^{a}
Overall Variance		228	475	0.31	0.71	180

[1]Direct Microscopic Somatic Cell Count

^{a,b}Values in a column having the same superscript are not different ($p > 0.05$).

lactations increased. Lactose concentration decreased from 5.0% for samples from cows in their first lactation to 4.5% for milk from cows in their fifth lactation. Milk potassium and somatic cell concentrations were not significantly ($p > 0.05$) influenced by lactation numbers.

c. Stage of Lactation

The effect of stage of lactation on milk constituents and conductivity is shown in Table 7. Mean sodium concentration increased linearly ($p < 0.01$) with advancing lactation. Mean milk potassium concentration on the other hand decreased linearly ($p < 0.01$) with advance in stage of lactation. Milk conductivity, lactose and direct somatic cell count were not significantly ($p > 0.05$) influenced by stage of lactation.

d. Milk Yield

Mean milk sodium concentration decreased linearly ($p < 0.01$) with increase in milk yield. Sodium concentration decreased from 81.2 mg/100 ml for yields up to 10 lbs to 51.8 mg/100 ml for yields greater than 30 lbs (Table 8). In contrast, mean lactose and potassium concentration increased linearly ($p < 0.01$) with increase in milk yield. Lactose concentration increased from 4.2% for yields up to 10 lbs to 4.7% for yields greater than 30 lbs, while potassium concentration increased from 105.7 mg/100 ml for the former group to 149.4 mg/100 ml for the latter. Neither

Table 7. Milk Sodium, Potassium, Lactose and Somatic Cell Concentration and Electrical Conductivity--Effect of Stage of Lactation

Stage of Lactation (Days)	No. of Samples	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Lactose (%)	Conductivity (m.mhos/cm)	DMSCC[1] (x10^4/ml)
100	68	52.8[a]	144.2	4.7	6.5[a]	52[a]
200	86	55.1[a]	151.1	4.8[a]	6.6[a]	58[a]
300	96	55.9[a]	136.8[a]	4.8[a]	6.3[a]	21[a]
>300	45	69.8	130.7[a]	4.6	6.8	43[a]
Overall Variance		302	425	0.45	0.09	174

[1]Direct Microscopic Somatic Cell Count

[a]Values in a column having the same superscript are not different ($p > 0.05$).

Table 8. Milk Sodium, Potassium, Lactose and Somatic Cell Concentration and Electrical Conductivity--Effect of Milk Yield

Milk Yield (lbs)	No. of Samples	Sodium (mg/100 ml)	Potassium (mg/100 ml)	Lactose (%)	Conductivity (m.mhos/cm)	DMSCC[1] (x10^4/ml)
1-10	12	81.2	105.7	4.2	7.1	64[a]
11-20	114	59.2[a]	137.3[a]	4.7[a]	6.5[a]	46[a]
21-30	129	54.5[a]	146.6[a]	4.8[a]	6.5[a]	32[a]
>30	35	51.8[a]	149.4[a]	4.7[a]	6.6[a]	44[a]
Overall Variance		307	391	0.49	0.08	181

[1]Direct Microscopic Somatic Cell Count

[a]Values in a column having the same superscript are not different ($p > 0.05$).

milk conductivity nor direct somatic cell counts were significantly ($p > 0.05$) influenced by milk yield.

GENERAL DISCUSSION

In spite of its poor reproducibility, the direct microscopic somatic cell count is usually used as a standard to which other indirect screening tests for abnormal milk are compared (Stryndaka and Thornton, 1937; Postle and Blobel, 1965). Davis and MacDonald (1953) and Whittlestone and Palmer-Jones (1944) demonstrated a positive correlation between cell counts and electrical conductivity of milk. Davis et al., 1943; Malcolm et al., 1942 and McCulloch (1940) recommended using electrical conductivity as a diagnostic tool for mastitis. In contrast, Postle and Blobel (1965) and Little et al. (1968) rejected electrical conductivity as a screening test for abnormal milk because of the low correlations they observed between milk conductivity and direct somatic cell counts of quarter and bulk milk samples. Results of the present study showed a significant positive correlation ($r = 0.25$) between direct microscopic somatic cell count and electrical conductivity for composite quarter milk samples, collected within the first minute of milking. This correlation compares favorably with that ($r = 0.24$) reported by Postle and Blobel (1965). The correlation between conductivity and direct microscopic

somatic cell count was not increased when only samples judged to be abnormal by direct cell count (i.e., greater than 500,000 cells/ml) were included in the correlation ($r = 0.24$). Postle and Blobel (1965) explained the low correlation observed in their study in relation to the relatively high coefficient of variability associated with the technique for estimation of somatic cells in milk. The low correlation obtained in this study emphasizes the inadequacy of somatic cell counts as an indicator of abnormal milk. Since the milk samples studied were quarter composites, dilution of abnormal milk with normal milk could account for the low correlation. Perhaps individual quarter measurements would yield a higher correlation. The high correlations between conductivity and milk constituents other than somatic cell indicates conductivity is importantly related to changes in milk that might be considered physiological and which are at least not highly correlated with changes in somatic cells. On the basis of other correlations obtained in this study, it would also appear that lactation variables exert greater influence on milk composition than inflammation.

Little *et al.* (1968) reported a wide range of conductivity measurements for any given level of direct somatic cell count. They found conductivity values for 501 foremilk samples to range from 4 to 10 millimhos (m.mhos). Horrall (1933) also observed a wide range of conductivities

for any given level of cell counts. None of these investigators attempted to explain the cause of the variation. In contrast Pickerton and Peters (1958) reported definite ranges of conductivity values for normal and abnormal quarter milk samples. The range of conductivity values reported herein are well within the range reported by Little et al. (1968). Similarly, the wide range of conductivities observed for any given level of cell counts confirms the observations of Little et al. (1968) and Horrall (1933), and appears to be due to normal lactation variation among animals. Malcolm et al. (1942) discussed the value of cell count and electrical conductivity as a criteria of bovine mastitis and indicated that better results are obtained with foremilk samples than with bulk samples. They suggested that samples having conductivity readings higher than 4.9 m.mhos or somatic cells greater than 5×10^5 to be abnormal. If these values were applied to the results of the present study, only four samples or 1.4% would be diagnosed as normal on the basis of conductivity; whereas 109 or 36% of the samples would be normal based on somatic cell counts. The significant positive correlation observed in this study between milk sodium and conductivity seemed feasible, since milk conductivity is known to be a direct function of milk electrolyte concentration. Results reported herein are in good agreement with those of Pickerton and Peters (1958), who reported an inverse relationship between milk lactose

and conductivity. Barry and Rowland (1953) suggested that the inverse relationship between lactose and conductivity results from the inverse relationship between milk chloride and lactose.

Jenness and Patton (1959) reported that sodium concentration of cow's milk is lower than that of blood, while milk potassium concentration was higher than that of blood. These differences in ionic composition of milk and blood were greatest at the beginning of lactation, but later the ionic composition of milk was more like that of blood (Barry and Rowland, 1953). These investigators also indicated that milk lactose decreased as lactation advanced, and that milk samples from cows with mastitis showed changes in lactose and ionic composition similar to those observed for advanced lactation. Results of the present investigation support those of Rook and Wheelock (1967), who suggested that within animal effects such as cow age, lactation number, and udder inflammation all decreased milk lactose and potassium concentration, but increased sodium concentration. These relationships seemed to be consistent, since milk cell count is reported by numerous investigators (Schalm, 1959; Hughes, 1954 and McFarlane et al., 1949) to be an index of udder inflammation, and both sodium and somatic cell counts are known to increase with udder inflammation, while lactose is known to decrease. The results reported here appear to be consistent with the reports of

Rook and Campling (1965), who observed that changes in milk potassium usually parallel those of milk lactose.

The nonsignificant correlations observed for direct microscopic somatic cell count with age, lactation number, stage of lactation and milk yield may be as a result of dilution of samples, since the samples analysed in this study were composite quarter samples. Also, the low correlations may be due to the fact that the majority of the animals sampled were around their mid-lactation stage. The positive relationship between lactose and potassium and the negative relationships between lactose and sodium and potassium and sodium concentration of milk reported herein agree with the results of Barry and Rowland (1953). In general, the changes in milk constituents and conductivity with age, lactation number, stage of lactation and milk yield reported here are in good agreement with results of Rook and Wheelock (1967), Waite et al. (1956) and Barry and Rowland (1953). These changes in milk constituents and conductivity appear to be associated with aging effects on the secretory tissues of the udder, which may cause increased permeability of the mammary tissues. This, according to Legates (1960) may reflect udder deterioration, either as a result of increasing incidence of mastitis or as slight physical damage with age. Van Rensberg (1947) explained changes in milk composition with age on the basis of physiological wear and tear. With increased permeability of the

mammary tissues regardless of cause, there is thought to be a partial resorption of milk lactose and an assumed mixing of milk within the udder with a transudate of blood serum, resulting in increased milk sodium and decreased milk lactose and potassium (Davis, 1933; Preskett and Folley, 1933 and Barry and Rowland, 1953).

Although a number of significant correlations were observed in this study, they are not high enough to use as a prediction tool. In addition, the results would seem to indicate that electrical conductivity as a screening test for mastitis could prove useful, if the investigation is extended to individual quarter sampling. This would eliminate dilution of samples which appeared to be a major contributing factor to the poor correlation found between direct microscopic cell count and electrical conductivity for the composite quarter samples studied.

SUMMARY AND CONCLUSION

Since bovine mastitis continues to be a major problem to the dairy industry, there is a need for a more effective and efficient method of detection. In this study, an attempt was made to develop an in-line electronic screening test for abnormal milk, based on electrical conductivity. The principle involved is that milk secreted by normal udders have almost identical conductivities, but with udder inflammation there is an influx of sodium and chloride ions into the mammary gland. Because electrical conductivity is a function of milk electrolyte concentration there is usually an increase in milk conductivity with udder inflammation. Electrical conductivity measurements were obtained for a total of 295 quarter composite milk samples over a six month period, using an electrodeless conductivity cell. These samples were further analysed for sodium, potassium, lactose and somatic cell content.

Milk conductivity increased linearly (6.2 to 6.8 m.mhos/cm) in cows two to six years old, and from the first to the fifth lactation (6.0 to 7.4 m.mhos/cm). In contrast, milk conductivity decreased linearly with increase in milk yield from 7.1 m.mhos/cm for yields up to 10 lbs to 6.6 m.mhos/cm

for yields greater than 30 lbs. Changes in milk conductivity appeared to be associated with changes in milk sodium as sodium concentration was shown to increase linearly with increase in age and lactation number; while it decreased linearly with increase in milk yield. Overall correlation analysis revealed a significant and positive correlation between milk conductivity and direct somatic cell count ($r = 0.25$). Milk conductivity was also significantly correlated with milk constituents including sodium ($r = 0.60$), potassium ($r = -0.28$) and lactose ($r = -0.56$). These constituents were markedly influenced by lactation variables namely age, lactation number, stage of lactation and milk yield.

Direct relationships were found to exist between lactose and potassium, whereas inverse relationships were found between lactose and sodium and potassium and sodium concentration of milk. No significant relationships were found between direct somatic cell count and age, lactation number, stage of lactation or milk yield. Conductivity measurements showed less variation than direct somatic cell count and appeared to be more reproducible. It would appear that changes in milk constituents due to physiological factors exerted greater influence on milk conductivity than those associated with inflammation. Although a number of significant correlations were obtained there were not high enough to be used for prediction purposes.

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APPENDICES

APPENDIX I

TUNGSTIC ACID REAGENT USED FOR PRECIPITATION OF PROTEIN AND FAT IN LACTOSE ANALYSIS

1. Dissolve 7 g of sodium tungstate (Mallinckrodt Chemical Works, St. Louis, Mo.) in 870 ml double glass distilled water.

2. Add 1 ml orthophosphoric acid (88%) (Mallinckrodt Chemical Works, St. Louis, Mo.) and 70 ml of IN sulfuric acid.

APPENDIX 2

A. NEWMAN'S STAIN FOR MILK SMEARS

1. Dissolve 2 g of Methylene blue (Sigma Chemical Co., St. Louis, Mo.) in 60 ml (95%) warm alcohol.
2. Add 40 ml of Xylene (Merck and Co. Inc., Rahway, N.J.) and 6 ml of glacial Acetic acid (Mallinckrodt Chemical Works, St. Louis, Mo.).
3. Filter through Whatman No. 1 filter paper.
4. Store in tightly stoppered bottle--at room temperature.

B. STAINING PROCEDURE

1. Immerse slides in Newman's stain for 5 minutes.
2. Remove wash and rinse in separate containers of tap water.
3. Place slides on level surface to air-dry.