INVESTIGATION OF THE OXIDATION-REDUCTION POTENTIAL OF RUMEN CONTENTS

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By

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A THESIS

Submitted to the School of Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Dairy

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The oxidation-reduction potential of rumen contents removed from several cows at three times during the day indicates that a close relationship exists between E_h and metabolic rate. Titration of samples, from which the large feed particles had been removed by centrifugation, with acid and a strong oxidizing agent, respectively, indicated that a linear relationship exists between E_h and pH and that the metabolic rate of the microflora is reflected by the amounts of reducing substances found. Very little of the reducing capacity of acidified rumen contents was found to be associated with the bacterial cells indicating that extreme care must be taken to maintain the proper redox potential when the rumen microflora are separated from their natural environment if a normal inocula is desired. The titration curve for rumen contents indicates that there is no large amount of any one reductive substance present that is responsible for the potential observed in rumen contents.

The only rumen function that seemed to be affected by alteration of the reductive characteristics of rumen fluid was gas production. Additives employed were DPPD, methylene blue, cysteine and thioglycollate. The explanation suggested for decreases in the per cent of methane produced was

that the potential may have been stabilized at a level higher than the optimum level for methane producing bacteria. Addition of oxygen affected neither the redox potential nor the gas, volatile fatty acid or ammonia production of rumen contents incubated *in vitro*. Methylene blue did not affect gas production in cultures of rumen flora that were stabilized with formate as their sole carbon source.

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INTRODUCTION

Oxidation-reduction systems play an intimate and essential part in the processes of life. In fact, life could be defined as a continuous oxidation-reduction reaction. The end products of rumen fermentation arise as a result of the interaction of many oxidation-reduction systems. Very little is known about these systems and the environment in which they act.

The purpose of this investigation is to study the oxidation-reduction potential which exists in the rumen, the stability of this potential and the effect alterations of the potential have upon several rumen end products.

REVIEW OF LITERATURE

Explanation of Oxidation-Reduction Potentials

Taken in the general sense, oxidation occurs whenever an atom loses electrons, while reduction takes place when an atom gains electrons. The oxidation-reduction (redox) potential measures the tendency to take on or lose electrons on a relative scale. The basis of the scale is the standard hydrogen electrode which is arbitrarily assigned a value of zero. Biological convention assigns a positive value to compounds that are oxidized by hydrogen (Hewitt, 1950). Giese (1957) indicates that there are four variables affecting the measured potential (E_h): the ratio of the concentrations of the oxidized and reduced forms of a compound, the hydrogen ion concentration, the valence change involved and the temperature. This is summarized in Peter's equation (West and Todd, 1957):

$$E_h = E_o + \frac{RT}{nF} \ln \frac{[\text{oxidant}]}{[\text{reductant}]} + \frac{RT}{F} \ln [H^+]$$

where: R = the gas constant (8.315 joules per degree)

T = the absolute temperature

n = valency change involved

F = the Faraday (96,000 coulombs)

E_o = normal potential when [oxidant] = [reductant] and $[H^+] = 1$

E_h = observed potential

at 30° when n = 1

$$E_h = E_o + .06 \log_{10} \frac{[\text{oxidant}]}{[\text{reductant}]} - 0.06\ pH$$

Pardee (1954) states that every compound may be considered to have a definite amount of free energy (F) stored in it under any set of conditions, mostly as the energy of bonds holding the atoms together. He further defines the change in free energy (ΔF) as the difference between the free energies of one mole of reactant and its products under standard conditions. E_h is proportional to the maximum work potential of each electron transferred between the hydrogen electrode and the redox system under study (Clark, 1948). Thus, it is possible to estimate the work available from the reaction of one mole by subtracting the E_h value of the reactant from the E_h value of the products and converting to ΔF according to the equation:

$$\Delta F = -nF\ (E_{hp} - E_{hr})$$

where:

ΔF = change in free energy or the work available from the reaction

n = no. of equivalents per mole

F = Farraday (23,068 cal. per volt equivalent)

E_{hp} = E_h of the product

E_{hr} = E_h of reactants

In order for a reaction to go spontaneously ΔF must be negative; i.e. the products must possess less free energy

than the reactants. When ΔF of a reaction is zero the reaction tends to go equally in both directions and the reactants and products are at equilibrium. ΔF and subsequently E_h can also be calculated from thermodynamic data (Pardee, 1954).

The most direct method of determining the normal potentials (E_o) of redox systems is the electrometric measurement of equimolar mixtures of the two components (oxidant and reductant), while the more convenient method and in some cases the only feasible one is afforded by potentiometric titration of the reduced component (Ryklan and Schmidt, 1944). Some systems have so far resisted attempts to make them amenable to electrical study while others can only be studied by means of mediators that are electromotively active (Clark, 1948; West and Todd, 1957).

The standard hydrogen electrode is difficult to maintain. Therefore, in practice a reference electrode that has a known potential relative to the hydrogen electrode is employed (Clark, 1948). Some commonly used reference electrodes are the calomel, the quinhydrone, and the silver: silver chloride (Hewitt, 1950). The potential of the calomel electrode varies with the concentration of KCl used in its manufacture. Hewitt (1950) finds the 3.5 normal KCl electrode most satisfactory for measurements in biological systems because of its low temperature coefficient and

stability. The quinhydrone electrode is very sensitive to pH changes and must be prepared fresh daily (Clark, 1948). The silver: silver chloride electrode is becoming popular and has a lower potential than the calomel (Hewitt, 1950).

The electromotive force between the reference electrode and a oxidation-reduction system can be measured with any volt meter with sufficient sensitivity. An inert electrode is necessary to carry electrons from the redox system to the volt meter (Clark, 1948). Hewitt (1950) recommends the bright platinum electrode for measurements in biological systems since it is readily prepared and cleaned, and yields steady, reproducible readings. Longsworth and MacInnes (1935) prefer the gold electrode for investigations of bacterial cultures. Lepper and Martin (1931) report that the pure graphite electrode is unsuitable for continual use and that electrodes of Iriduim can be used successfully.

The electrical circuit between the reference electrode and the solution of reactants must be completed without permitting the two systems to mix. The liquid: liquid junction typified by the salt bridge made of KCl saturated water and 2% agar performed very satisfactorily for Clark (1928) and Hewitt (1930).

Complete descriptions of apparatus suitable for the continual measurement of E_h in bacterial cultures and of E_o in organic redox systems appear in Hewitt (1930, 1950), Clark (1928) and Ryklan and Schmidt (1944).

Compounds that change color when they are transformed from the oxidized to the reduced state can be used as E_h indicators in much the same manner in which weak acids and bases that change color when they become ionized can be employed as pH indicators. Clark and his collaborators (1928) have characterized a considerable number of dyes which behave as oxidation-reduction indicators. This data has been compiled in tabular form by Hewitt (1950). With the help of Clark's data it is possible to measure the E_h of biological systems providing, of course, that the color change is observable in the system under investigation. Dyes as indicators of E_h are not in widespread use due to difficulty in the interpretation of data obtained.

Ball and Chen warned in 1933 that: "Results obtained by means of oxidation-reduction indicators on unstable systems or on biological material where such systems are present, must be interpreted with great care." Hewitt (1950) attaches significance to the poising or capacity factor in biological systems; i.e. the concentrations of oxidants and reductants that make up the observed potential. Indicators measure the existing E_h and not the capacity of a system to remain at that E_h. Biological systems are under constant change and an indicator that equilibrates slowly makes it nearly impossible to determine electrode potentials at different periods (Hewitt, 1950). The indicator dyes are reversible oxidation-

reduction systems (Clark, 1928) and as a result of this property along with other properties such as basicity are able to act as catalysts (Barron and Hoffman, 1930; Schott and Borsook, 1933; Green et al., 1934; Greville and Stern, 1935; Young, 1937) and as inhibitors (Perdrau and Todd, 1933; Quastel and Wheatley, 1931; Chambers et al., 1931).

Some Biologically Active O-R Systems

In this chapter attention is directed chiefly toward a discussion of the potentiometric behavior and biological significance of the sulfhydryl compounds, coenzyme I, the flavoproteins, ascorbic acid, the tocopherals, methylene blue and several commercial antioxidants.

Although a considerable amount of experimental work has been done on the estimation of the oxidation potentials of sulphydryl compounds the subject is still in a state of controversy (Ryklan and Schmidt, 1944). Dixon and Quastel (1923) measured the potentials of solutions of cysteine and glutathione with their corresponding disulfides. They found that the potentials were independent of the relative concentrations calculated from the relation $\pi = \pi_o - \frac{RT}{F} \ln [RSH] + \frac{RT}{F} \ln [H^+]$ where π is the observed potential, π_o is the normal reduction potential and the other notations have their usual significance. This relation does not describe a system that is reversible in the thermodynamic sense but suggests

a change in free energy without a corresponding change in the state or properties of the system (Ryklan and Schmidt, 1944).

Kendall and Nord (1926) reported that in the presence of small amounts of oxygen an active addition product was formed with the sulfhydryl compound whereby the system acquires the power to reduce indigo carmen. They concluded that in the presence of the hypothetical addition compound the system was reversible. It has been noted that Kendall and Nord (1926) failed to report a stoichiometric relationship to support their conclusion (Dixon and Tunnicliffe, 1927; Michaelis and Flexner, 1928).

Potentiometric titration of cysteine with iodine, potassium dichromate and potassium iodate yielded variable results but the titration curves resembled those of reversible redox systems. This evidence led Williams and Drissen (1930) to postulate that the potentials observed depended not only upon the presence of cysteine and hydrogen ions but also upon the presence of an intermediate oxidation product. This view was supported by the results of Fischer (1930). Other workers who investigated the behavior of the sulfhydryl: disulfide couple employed dyes, different electrodes and thermal data with divergent results (Ghosh et al., 1932; Green, 1933; Ghosh and Gangula, 1934; and Borsook et al., 1937b). Ryklan and Schmidt (1944) pointed out that Dixon and Quastel (1923), Kendall and Nord (1926), and Dixon and Tunnicliffe (1927)

failed to set up the correct conditions for a reversible couple and that the methods of calculation employed by Williams and Drissen (1930) were erroneous. Ryklan and Schmidt (1944) found that the potential was dependent upon the concentration of cysteine, cystine and hydrogen ion. During potentiometric titrations, addition of cysteic acid caused a large increase in potential thus excluding the possibility that this compound might be the intermediate oxidation product postulated by Williams and Drissen (1930). Mixtures of cysteine and cystine did not establish equilibrium at the platinum electrode when pure solutions were employed but equilibrium was quickly reached when potassium iodide was used as a mediator (Ryklan and Schmidt, 1944). The normal redox potentials (E_o) reported by Ryklan and Schmidt (1944) are:

RSH Component	E_o in volts
thioglycollic acid	.27
cysteine	.27
thiolhistidine	.32
monothioethyleneglycol	.35
ergothioneine	.36
glutathione	.45

These workers suggested that the sulfhydryl groups of proteins probably have a potential equal to or larger than that of glutathione, in view of the large potential of glutathione

in relation to the simpler compounds and the fact that cysteine activates protein sulfhydryl groups.

The importance of active sulfhydryl groups, present in the enzymes themselves or belonging to substances associated with the substrates has long been recognized (Lugg, 1945). An extensive list of enzyme systems that require an active sulfhydryl group for activity was compiled by Elliot (1946). Many of the systems on the list were investigated by Barron and Singer (1945). These workers noted that enzyme systems inactivated by p-chloromercuribenzoate and iodoacetamide were reactivated by glutathione. This led these workers to suggest that the main function of glutathione is the continuous reactivation of sulfhydryl enzymes; a view supported by Elliot (1946) but contrary to the results reported by Ames and Elvehjem (1945). A more complete discussion of the biological significance of glutathione is given by Colowick (1954).

The flavoproteins are complex proteins composed of one of the isoalloxazine nucleotides derived from the vitamin, riboflavin, in combination with a specific protein (Hellerman, 1947). Early workers noted the accumulation of an intermediate oxidation product that was red in acid solutions and green in neutral solutions of lactoflavin held at low temperatures. These workers concluded from the characteristics of the titration curve of this flavoprotein that the colored form was a semiquinone (Kuhn and Wagner-Jauregg, 1934;

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Michaelis et al., 1936; and Klemperer et al., 1937). The electrode potential of riboflavin in neutral solutions was reported to be -0.208 millivolts (Michaelis et al., 1936). Kuhn and Boulanger (1936) found that phosphorylation of riboflavin did not affect the potential appreciably but that when the phospho-riboflavin-protein complex was formed the potential rose to -0.060 volts at pH 7.

Measurement of the redox potential of diphosphopyridine nucleotide (DPN or Coenzyme I) with various dyes indicated the potential was -0.26 volts at pH 7.2 (Ball, 1939). A different value was calculated from thermal and free energy data (Borsook, 1940). Burton and Wilson (1953) calculated that the potential of DPN was -0.320 volts at pH 7. Their calculations were justified by Rodkey (1955) who reported that within the pH range from 6.5 to 10.5 the potential of solutions of coenzyme I are described by the relation:

$$E_h = -0.1071 + 0.0301 \log \frac{[DPN]}{[DPNH]} - 0.0301 \text{ pH}$$

The stoichiometry of the titrations carried out by Rodkey (1955) indicated the following reaction for the DPN couple: $DPNH \rightleftharpoons DPN^+ + 2\ominus + H^+$. He reported that the potential of the system did not change in the presence of increasing levels of xanthine oxidase.

The significance of the flavins and phosphopyridine nucleotides as components of the general scheme of aerobic oxidation is well recognized. Excellent reviews of recent

advances in the study of biological oxidations appear annually in "Annual Review of Biochemistry." Clark (1948) presents a comprehensive list of the oxidation-reduction potentials of many thermodynamically reversible systems illustrating the possibilities for the stepwise release of energy through the progressive increase in the oxidation level of electrons removed from metabolites. Burton and Wilson (1953) calculated the changes of free energy associated with the exchange of hydrogen between several known hydrogen carriers.

Despite the mass of data relating to the effects of ascorbic acid in enzyme systems and intact organisms, nothing certain is known of the exact function of this vitamin aside from the effects of its reducing properties (Snell and Metzler, 1956). Early attempts to measure the redox potential of ascorbic acid were largely unsuccessful. Green (1933) reported that the potential was due to the concentration of the reductant alone. Borsook (1933) noted that in solutions with a pH higher than 5.75 the oxidized form was unstable, while Fruton (1934) reported that the potential of ascorbic acid in neutral solutions was -0.081 volts. The oxidation of ascorbic acid in three separate steps, the first step being reversible when the system was measured colorimetrically, was reported by Borsook (1937). Employing methylene blue, indigotetrasulfonate and thionine as mediators,

Ball (1937) succeeded in characterizing the electromotively sluggish ascorbic acid system. He reported that the normal potential was + 0.390 volts; that the slope of the E_h/pH curve changed from 0.06 to 0.03 at pH 4 remaining at 0.03 slope up to pH 8.6; and that the half-life of dehydroascorbic acid at pH 7.25 and 38° C. was 2 minutes. He concluded that dehydroascorbic acid could not exist in tissue. The function of ascorbic acid in plant growth was investigated by Virtanen and Hausen (1949). These workers found that growth was stunted in the presence of too little or too much vitamin C and that normal growth occured when this vitamin function of ascorbic acid in plants was to regulate the redox potential at an optimum level. Koser and Thomas (1955) noted that the requirement for cysteine was eliminated in certain strains of Lactobaccili when ascorbic acid was added to the media. The stimulatory effect that ascorbic acid had upon the action of the enzyme homogentisicase was traced to protection of the ferrous ion required by the enzyme (LaDu, 1952; Schepartz, 1953; Greenberg and Rinehart, 1955; Crandall, 1955; and LaDu and Greenberg, 1957). Ascorbic acid also protects or aids in the mobilization of the ferrous ion required by aconitase and is therefore, necessary for the efficient operation of the Krebs cycle (Takeda and Hara, 1955).

Antioxidants have been defined as substances which inhibit the autoxidation of fats in contact with molecular oxygen (Dam, 1957). Clark and Geissman (1949) discussed the

practical applications of physiological antioxidants and suggested several mechanisms by which these compounds may act. Many antioxidants act as simple hydrogen donors. That is, they prevent undesirable oxidations because they are preferentially oxidized (Weissberger, 1948; Greenberg and Rinehart, 1955; Crandall, 1955; Hickman and Harris, 1946; Greenberg et al., 1957). Some antioxidants interfere with the mechanisms of fat oxidation (Michaelis, 1948; Weissberger, 1948), while others inhibit "undesirable" enzymes (Seifter, 1948; Fieser, 1948). Seifter (1948) and Nason and Lehman (1955) have reported cases where antioxidants promote desirable oxidations.

The tocopherals (vitamin E) and redox substances which have similar physiological properties have been the subject of extensive research. The remainder of this chapter will be concerned with a discussion of the characteristics of these physiological antioxidants, and their mode of action.

Michaelis (1948) suggested that the antioxidant activity of hydroquinones depended not only upon reversible oxidation to their respective quinone but also upon the reversible formation of an intermediate semi-quinone. Evidence that the rate of semi-quinone formation depended upon the partial pressure of oxygen and the hydrogen ion concentration was presented by Weissberger (1948). He also noted that cysteine, ascorbic acid and thioglycollate depressed the oxidation of

hydroquinone until they themselves were completely oxidized. Michaelis and Wollman (1949) observed the free radical or semiquinone form of vitamin E at the temperature of liquid air (indicated by the color development). A reversible quinone-semiquinone-hydroquinone couple was proposed by Mackenzie (1949). Seifter (1948) found that no correlation existed between the chemical properties of hydroquinones or diamines and their toxicity. Investigation of the antimalarial action of hydroquinones indicated that their effectiveness was due to the ability of their side chains to combine with proteins rather than their redox characteristics (Fieser, 1948). Ames and Harris (1948) reported that alpha-tocopheral phosphate inhibited all the enzymes it was tested on and does not act as an antioxidant.

The effectiveness of certain non-tocopheral antioxidants in preventing or delaying the appearance of the signs of vitamin E deficiency in various species have been demonstrated. The development of exudative diathesis in chicks was prevented by the administration of nordihydroguaiaretic acid (NDGA), disulfram (tetraethylthiuram disulfide), methylene blue, and to some extent by ascorbic acid (Dam, 1957). Encephalomalacia did not develop in E-deficient chicks when NDGA; ascorbic acid; methylene blue; diphenyl-p-phenylenediamine (DPPD); 2, 6-di-t-butyl-4-methoxy phenol (BHT); or 1, 2-dihydro-2, 2, 4-trimethyl-6-ethoxyquinoline (Santoquin) was

administered (Dam, 1957; Singsen, 1955). Several commercial antioxidants and redox dyes prevented reabsorbtion gestation and muscular dystrophy in the rat (Dam, 1957; Moore et al., 1954). Blaxter et al. (1953) reported that methylene blue protected against muscular dystrophy in calves induced by feeding cod liver oil but noted that the effect may have been due to a direct protective action of methylene blue against the toxicity of polyethenoic acids of cod liver oil rather than a relationship with vitamin E. Booker et al. (1954) proposed that the increased uptake of oxygen observed when animals treated with antioxidants were subjected to stress was due to protection of the sulfhydryl groups of enzymes necessary for normal metabolism. Vitamin E deficient rats had less resistance to the effects of oxygen under high pressure than did rats treated with alpha-tocopheral, glutathione or methylene blue (Taylor, 1958). DPPD failed to prevent exudative diathesis in chicks fed on diets essentially devoid of vitamin E. Scott et al. (1955) concluded from this data that the effect of DPPD was due to protection of the vitamin E existing in the tissue. Shull et al. (1957) observed that the protective action of antioxidants against muscular dystrophy in guinea-pigs disappeared during long term experiments. These workers concluded that antioxidants are only effective while there is some vitamin E present in the tissue. Draper et al. (1958) studied the effectiveness

of DPPD, BHT, and methylene blue in preventing and curing reabsorbtion-gestation in rats fed E-deficient diets for prolonged lengths of time. They found that DPPD and methylene blue were effective but BHT was not. They suggested that DPPD either participated directly in some physiological function essential to reproduction and normally assumed by vitamin E or activated residual amounts of E that were otherwise inadequate. Greenberg et al. (1957) and Tucker et al. (1957) reported that tocopheral, ascorbic acid and DPPD enhanced the therapeutic value of iron in the treatment of anemia in milk fed rats. Although not identifying a tocopherol as a participant in the oxidative transport chain, Nason and Lehman (1955) did implicate a tocopheral or some closely related lipid soluble co-factor as a participant somewhere between DPN and cytochrome C. Several antioxidants including DPPD did not have this activity. Limited work on the effect of the level of vitamin E in the diet of dairy cattle indicated that increasing levels of vitamin E in the diet result in increasing levels of vitamin A in milk (Worker and McGillivray, 1957; Hickman and Harris, 1946). Several antioxidants fed at low levels increased vitamin A and carotene retention in dairy calves but higher levels had the opposite effect (Pirchner et al., 1957; DeLuca et al., 1956).

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Pathways of Carbohydrate Metabolism in the Rumen

The fermentative and oxidative pathways leading to pyruvate, acetaldelyde, triosephosphate and other intermediates of carbohydrate metabolism in microorganisms were reviewed by Gunsalus, Horecker and Wood (1955). In this chapter attention is directed chiefly toward a discussion of the pathways leading from metabolic intermediates to volatile fatty acids, CO_2 and methane.

Werkman and Wood (1942) proposed two mechanisms for the formation of propionic acid from pyruvic acid:

A. $HOOCCOCH_3 \xrightleftharpoons{+\ CO_2} HOOCCOCH_2COOH \xrightleftharpoons{+\ 2H} HOOCCHOHCH_2COOH$
pyruvic acid — oxalacetic acid — malic acid

$\xrightleftharpoons{-H_2O} HOOCCH{=}CHCOOH \xrightleftharpoons{+\ 2H} HOOCCH_2CH_2COOH \xrightleftharpoons{-\ CO_2}$
fumaric acid — succinic acid

$HOOCCH_2CH_3 + CO_2$
propionic acid

B. $HOOCCOCH_3 \xrightleftharpoons{+\ H_2} HOOCCHOHCH_3 \xrightleftharpoons{-\ H_2O} HOOCCH{=}CH_2 \xrightleftharpoons{H_2}$
pyruvic acid — lactic acid — acrylate

$HOOCCH_2CH_3 \longrightarrow HOOCCH_3 + CO_2$
propionic acid — acetic acid

Elsden (1945) reported the conversion of lactic acid to acetic acid, propionic acid and CO_2 by a non-cellulytic Propionibacterium isolated from the rumen of sheep. A similar organism isolated from enrichment cultures of rumen bacteria by Johns (1949) seemed to form propionic acid by a reaction sequence similar to (A) proposed by Werkman and Wood (1942). The

optimum pH for the decarboxylation of succinic acid by this organism was 5-5.2 while at pH 6.8 the reaction proceeded very slowly. Delwiche (1948), Johns (1951a) and Johns (1951b) demonstrated the quantitative decarboxylation of succinic acid by Propionibacterium pentosaceum and Veillonella gazogenes. Washed suspensions of rumen bacteria formed succinic acid as an intermediate during cellulose digestion and decarboxylated added succinate with quantitative yields of propionate (Elsden and Sijpesteign, 1950; Sijpesteign and Elsden, 1952). Delwiche et al. (1956) investigated the mechanism of succinate decarboxylation while Wood et al. (1956) demonstrated the formation of acetic acid from propionate.

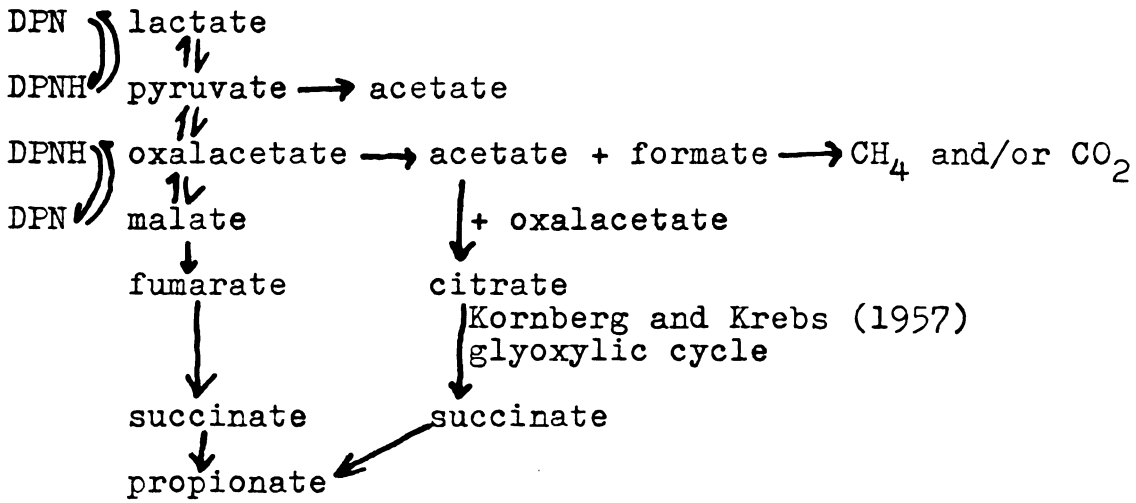
Cardon and Barker (1947) reported that Clostridium propionicum converted lactate, pyruvate, and acrylate to acetic and propionic acid. Although he was unable to find acrylate in solution, Johns (1952) postulated that Cl. propionicum produced propionic acid by the reaction sequence B proposed by Werkman and Wood (1942) on the basis that washed suspensions of this organism did not attack either succinate, malate, or fumarate. Cell free extracts from the rumen organism LC-1 converted mixtures of acrylate and lactate to hydrogen, carbon dioxide, acetate, and propionate. The reaction was not as simple as reaction B since the coupled oxidation-reduction involving lactate and acrylate yielded

some pyruvate which extracts from this same organism were able to convert to hydrogen, carbon dioxide and acetate (Walker and Todd, 1958; Todd and Walker, 1958).

The condensation of two acetate molecules to form succinic acid and hydrogen by Aerobacter indologenes P. pentosaceum, and E. coli was demonstrated (Slade and Werkman, 1943; Carson, 1948; Kalnitsky et al., 1943). Krampitz et al. (1943) reported the enzymatic fixation of carbon dioxide and pyruvate to form oxalacetate. The phosphoroclastic split of pyruvate to acetate and formate was demonstrated by Utter et al. (1945).

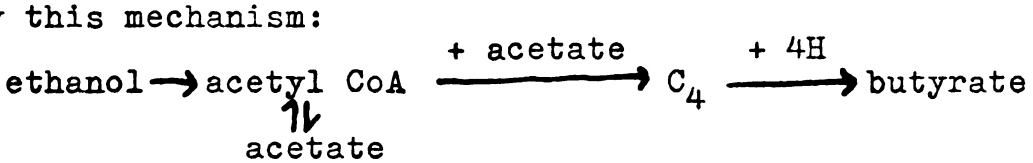
The large number of propionibacterium found in the rumen led Gutierrez (1953) to conclude that they are important in acetate and propionate formation. Barker and Lipmann (1944) investigated the dependence of the propionic acid fermentation upon the hydrogen balance or oxidative state of the substrate and found that glycerol, the reduced substrate, yielded a higher proportion of propionate than did pyruvate, the highly oxidized substrate. Gall et al. (1953) reported increased levels of lactic acid in the rumen of cows fed a high grain diet. A high correlation was found between the amountsof lactate and propionate found in the rumen (Waldo and Schultz, 1956). Barnet and Dow (1957) found that pyruvate yielded more acetate than propionate whereas propionate was the chief acid derived from lactate.

Alpha-ketoglutarate was utilized slowly to form acetate. These workers suggested that the following reactions occur in the rumen:



Hershberger et al. (1956) postulated that an acetate to propionate ratio of 2 to 1 was necessary to maintain a proper hydrogen balance. This postulate did not account for other fermentation end products such as carbon dioxide and methane.

Clostridium kluyveri derives its energy solely from the conversion of 2-carbon compounds to 4 or 6-carbon compounds. Enzyme preparations from this organism carry out the reaction by this mechanism:



(Barker et al., 1945; Stadtman and Barker, 1949; Stadtman, 1952). Rumen organisms synthesize valerate from propionate

and acetate while butyrate and caproate arise from acetate (Gray et al., 1951; Gray et al., 1952). LC, an organism isolated from the sheep's rumen, carried out the following reactions:

$$\text{pyruvate} \xrightarrow[-CO_2]{CoA} \text{acetyl CoA} + 2H^+ + 2\bar{e}$$

$$\text{acetyl CoA} + 4H^+ + 4\bar{e} + \begin{matrix} \text{acetate} \longrightarrow \text{butyrate} \\ \text{propionate} \longrightarrow \text{valerate} \\ \text{butyrate} \longrightarrow \text{caproate} \end{matrix} + CoA + H_2O$$

(Elsden and Lewis, 1953).

Gray and Pilgrim (1952) demonstrated the production of butyric acid from proteins. El-Shazly (1952a) found a high correlation between ammonia production and increased levels of isobutyric, isovaleric, and higher fatty acids formed during rumen fermentation and concluded that the volatile fatty acids arise from protein as well as from carbohydrate. Further experiments carried out with casein hydrolysate as substrate for washed suspensions of rumen organisms substantiated this conclusion (El-Shazly, 1952b). Sirotnak et al. (1954) described the reductive deamination of aspartic acid to form succinic acid and the subsequent decarboxylation of succinate to form propionate and carbon dioxide. The conversion of leucine to isovalerate was investigated by Otakaki et al. (1955).

Considerable progress has been made in the study of rumen methanogenesis over the past few years. Mylroie and

Hungate (1954) isolated Methanobacterium formicicum from sewage sludge. This organism utilized no carbon source other than formate, or hydrogen and carbon dioxide. This same group of workers later reported that rumen microorganisms produced carbon dioxide, hydrogen and methane from formate and methane from carbon dioxide and hydrogen. C^{14} labeled formate was quantitatively converted to carbon dioxide which in turn could be reduced to methane:

$$HCOOH \longrightarrow CO_2 + H_2$$

$$CO_2 + 2H_2 \longrightarrow CH_4$$

(Carrol and Hungate, 1955). Washed suspensions of rumen organisms have the ability to reduce carbon dioxide to methane (McNeill and Doetsch, 1956; McNeill and Jacobson, 1955) as does Methanobaccillus omelianski (Pine, 1958). Stabilized cultures of rumen bacteria were established that were able to utilize formate as their sole source of energy (Nelson et al., 1956). These cultures produced carbon dioxide and methane in theoretical amounts according to the equation:

$$4HCOOH \longrightarrow CH_4 + 3CO_2 + 2H_2O$$

Cultures stabilized to formate adapted very slowly to changes in substrate (Opperman et al., 1957a).

Pine and Barker (1956) investigated the pathway of hydrogen in the acetate fermentation and found that the methyl

carbon goes to methane with its hydrogens intact:

$$CH_3COOH + HX \xrightarrow{-2H} CH_3X + CO_2$$
$$\xrightarrow{+2H} CH_4 + HX$$

(where X is an unknown compound). Opperman et al. (1957b) established stabilized cultures which could utilize acetic, butyric and valeric acid but could not establish a culture which could utilize propionic acid. The ratios of gas produced from these substrates indicated the following reactions:

$$CH_3COOH \longrightarrow CH_4 + CO_2$$

$$2CH_3(CH_2)_2COOH + CO_2 + 2H_2O \longrightarrow 4CH_3COOH + CH_4$$

$$2CH_3(CH_2)_3COOH + 4H_2O \longrightarrow 2CH_3CH_2COOH + 2CH_3COOH + 4H_2$$

Cultures stabilized to butyrate or valerate utilized acetate readily. Propionate produced in the valerate cultures was not utilized but remained as an end product (Nelson et al., 1957).

In Vitro Techniques

Many in vitro techniques for the study of the biochemistry and microbiology of the rumen have been developed in recent years. The principal methods used by most investigators may be roughly classified into:

(a) Pure culture studies

(b) Centrifuged cells

(c) Whole rumen liquor diluted or undiluted (artificial rumen)

(d) Stabilized cultures.

Pure cultures allow one to study the metabolic activities of the predominant bacterial species of the rumen (Anonymous, 1958). Hungate (1957) investigated changes in the microbial population in the rumen of cattle fed a constant ration. He made direct microscopic and culture counts on samples obtained by stomach tube and identified Streptococcus bovis, Ruminococcus albus, R. flavefaciens, Cl. lochheadii, Bacteroides succinogenes, and Cl. Longisporum. Samples withdrawn with the stomach tube were mixed and diluted 1:10 with a salt solution in equilibrium with 5% carbon dioxide in a screw top bottle. Samples were taken to the laboratory where air was again displaced with a N_2 - CO_2 mixture. Further dilutions were made and innoculated into rumen fluid cellulose agar and feed extract agar. Strep. bovis were observed after incubation at 39° C. for 24 hours. Total colony counts were made 10 days after innoculation and cellulytic colonies in rumen cellulose agar were examined after 20 days. Strep. bovis was isolated by picking colonies and subculturing in an agar medium containing inorganic salts, rumen fluid and starch. Other colonies were subcultured in tubes of rumen fluid cellulose agar. Great care was taken to maintain anaerobiosis. The fermentative characteristics of the various

isolates were investigated by innoculation into tubes containing various fermentable carbohydrates. Other workers have investigated the nutritional requirements of organisms in pure culture (Bryant and Burkey, 1953; Bryant and Doetsch, 1954; Chu and Bryant, 1958; Hungate, 1947; Hungate, 1950). The fermentative mechanisms of pure cultures have also been investigated (Elsden, 1945; Johns, 1952; Delwiche et al., 1956; Walker and Todd, 1958; Todd and Walker, 1958).

The washed suspension technique was developed as a means for studying the whole rumen population in a simplified system. The first worker to utilize this system employed a technique which might more correctly be called the centrifuged unwashed cell technique (Marston, 1948).

The desire to study the metabolism of the whole rumen flora in a system uncomplicated by presence of a myriad of substrates and end products led to the development of the centrifuged cell technique which has been modified by various workers to suit their particular needs. Marston (1948) removed the coarse feed particles from cheese-cloth strained rumen fluid by slow centrifugation. The microorganisms were separated from the resulting supernatant in a high speed centrifuge and suspended in a phosphate buffer. The suspended cells were then put into a 3.5 liter hermatically sealed glass pot containing 2.5 L. of a salt solution and a source of cellulose. Anaerobiosis was maintained by passing a

continuous stream of nitrogen into the fermentation vessel. The pH was adjusted back to 6.8 at regular intervals by adding 0.5 M Sodium Carbonate. Elsden and Sijpesteijn (1950), Sijpesteijn and Elsden (1952) and Bentley et al., (1954) used unwashed cells prepared by similar techniques to investigate the end products of cellulose digestion and the requirements of the rumen flora. Considerable amounts of Substrate and unidentified factors stimulatory to cellulose digestion were carried over into the fermentation vessel when unwashed centrifuged cells were used. Cheng et al. (1955) reduced the amount of carry-over by washing the centrifuged cells before adding them to the fermentation flask. Doetsch et al. (1955) modified the washed cell method still further by preincubation to deplete reserves within the cells so that little or no activity would occur in the absence of added substrate.

Three main types of artificial rumen systems have been described: (1) Undiluted or slightly diluted rumen liquor incubated in all-glass impermeable systems (Pearson and Smith, 1943); (2) Whole rumen fluid diluted to about half strength with a mineral solution (Burroughs et al., 1950); and (3) Whole undiluted rumen liquor incubated with substrate in a semipermeable container surrounded by a mineral solution (Louw et al., 1949). Pearson and Smith (1943) who studied protein metabolism in whole rumen fluid claimed that the microflora remained normal for 2 - 4 hours in their impermeable

glass system. Burroughs et al. (1950) attempted to prevent alteration in the microflora due to accumulation of end products by diluting rumen fluid before and during the fermentation while Louw et al. (1949) allowed the end products to diffuse out of his semipermeable fermentation vessel, into a salt solution. The system of Louw et al. (1949) was modified by Wasserman et al. (1952) and Huhtanen et al. (1954). Several biological and chemical criteria for establishing the validity of artificial rumen studies have been proposed by Warner (1956).

A method for establishing stabilized enrichment cultures of rumen bacteria was developed by Opperman, Nelson, and Brown (1957) for studying methane and carbon dioxide formation from various substrates. Cheese-cloth strained rumen fluid was added to an equal volume of salt solution. Formate, acetate, butyrate or valerate was added to the fermentation vessel daily as the sole source of carbon. Only organisms which could utilize this substrate (directly or symbiotically) survived after a period of several weeks. When the yield and constitution of the gas produced remained consistent for several days the cultures were considered stabilized.

Redox Potentials in Bacterial Systems

Perhaps the greatest difficulty in dealing with biological systems generally and with bacterial populations in

particular arises from the fact that we do not have rigidly defined static systems possessing fixed characteristics. The redox potentials observed in bacterial populations reflect to some extent the dynamic aspects of this phenomenon we call life. This chapter deals with the significance of redox potentials observed in bacteriological systems.

According to Gillespie (1920) measurements of the redox potential in bacterial cultures gave very characteristic curves representing the lag, logarithmic growth, and death phases in the culture. Coulter and Issacs (1929) proposed that the rapid drop in potential observed during the logarithmic growth phase was due to the disappearance of oxygen from the media and the stability of this low potential during the death phase resulted from the release of reductive substances from the dead cells. This explanation was discredited by Hewitt (1950) as an attempt to explain a cause by listing the symptoms. The oxygen deficit arises as a result of the metabolic activities of the multiplying organisms. In the absence of oxygen all available hydrogen acceptors are utilized by the organisms in order that metabolism may continue. Thus the reduction of intercellular oxidizing systems and the surrounding media which results in low redox potentials are symptoms of the dynamic equilibrium that exists between cellular enzymes and medium constituents (Hewitt, 1950).

The oxidation-reduction potential observed in bacterial cultures is due to the presence of many electromotively active compounds in various states of oxidation. When these compounds are present in minute quantities or are predominantly in the oxidized or reduced state the potential is usually unstable. When large quantities of these compounds are present, considerable oxidation or reduction must take place before the observed potential will vary. As a general rule, the potentials of bacterial cultures tend to vary over a range of ± 30 millivolts (Hewitt, 1950). The reducing properties of aerobes generally range from about +0.4 volts to -0.2 volts while anaerobes are capable of reducing the potential from about +0.05 volts to about -0.4 volts. These reducing properties are a manifestation of the characteristics of the enzyme systems that the various organisms possess. That is, aerobes are capable of metabolizing in a highly oxidative media because they possess a cytochrome system while anaerobes depend largely upon enzymes which cannot metabolize normally until the media is partially reduced. (Aubel, Rosenberg, and Grunberg, 1946; Hewitt, 1950).

Very little information is available concerning the redox potential of rumen contents. The potential observed in the <u>in vitro</u> system of Marston (1948) was in the vicinity of -0.380 volts at pH 7 with rapid fluctuations of ± 30 millivolts. The potential of rumen contents treated with various

levels of aureomycin and pennicilin ranged from -0.193 volts to -0.288 volts (Horn, Snapp and Gall, 1955). Broberg (1957a, b,c and 1958) reported his measurements of rumen potential as rH in an attempt to simplify his data. Hewitt (1950) severly criticized the use of the term rH when referring to biological systems "Since these are systems of unknown constitution and incompletely studied properties, the relation between E_h and pH cannot be defined and hence no term containing both factors can be obtained in our present state of knowledge, whilst the use of rH makes a tactic assumption that there is a known simple relationship between the E_h and pH of the system. For this and other reasons, Clark rightly insists that the use of the term rH, originally introduced by himself, should be discontinued." (Hewitt, 1950). The relationship between rH, pH and E_h at 30° C. is represented by the equation: $E_h = .03(rH - 2pH)$ (Hewitt, 1950).

Broberg's results were converted to E_h by inserting his rH and pH values into the above formula. This conversion was made in order to facilitate a comparison of his experimental results with those reported by other investigators. Measurements of the potential in untreated rumen contents taken from eight healthy bovines while they were in the stable, after they had been on pasture for two days and after three months on pasture were not significantly different (Broberg, 1957a). Samples taken from fistulated sheep on a normal ration twenty

to thirty minutes after concentrate feeding had an rH of 6.9 ± 0.09 (E_h of -0.14V at pH 5.75) while samples taken from these same sheep later in the day had an rH of 8.3 ± 0.06 (E_h of -0.15V at pH 6.7) (Broberg, 1957a). Acidification of rumen contents under both oxygen and carbon dioxide atmospheres showed a characteristic curve until the pH dropped below five. Thereafter the ability of rumen contents to maintain a constant redox potential was lost and a continuous change toward higher values could be observed in the presence of oxygen (Broberg, 1957c). The redox potential of rumen contents from sick animals was very stabile except in a few cases of complete anorexia for extended periods, due to acute overeating of grain and in a number of bloat cases (Broberg, 1957b). The author suggested, in view of the wide variations in potential observed in the bloat cases, that enzymatic processes other than the normal govern the course of events in a number of bloat cases. Large amounts of oxygen bubbled through rumen contents failed to cause more than a temporary rise in potential *in vivo*. No correlation was noted between carbon dioxide production and oxygen consumption *in vitro* (Broberg, 1958).

PART I

THE REDOX POTENTIAL OF BOVINE RUMEN CONTENTS

Procedure

Three cows, T3, T25 and K139, fitted with the screw cap, plastic fistula plugs described by Hentschl et al. (1954) were used in this experiment. T3 and K139, respectively, were maintained on hay rations of 20 pounds of timothy daily and 20 pounds of first cutting alfalfa daily. T25 was fed 10 pounds of first cutting alfalfa hay and 10 pounds of a grain mixture which was 77.5% ground shelled corn, 20% soybean oil meal, 1% calcium carbonate, 1% dicalcium phosphate, 0.5% trace mineral salt and contained 10,000,000 international units of vitamin A and 90,000 international units of vitamin D per ton. The grain mixture was 15.8% crude protein. All animals received 50 grams of trace mineral salt daily. The experimental rations were fed once daily at 7 A.M. for at least three weeks before the investigation was initiated. The animals had free access to water at all times.

Samples of rumen fluid were collected from each animal at 6:00 A.M., 10:30 A.M. and 6:00 P.M. No animal was sampled more than once daily in order to eliminate the possibility of a carry over effect in the second sample. Samples were strained through a double layer of cheese cloth into an Erlenmeyer flask that had been flushed with nitrogen beforehand.

In order to facilitate the determinations the samples were separated into several fractions by centrifugation. The large feed particles (fraction C) were separated from strained rumen fluid (fraction A) in an International Centrifuge running at 200 x G for 3 minutes. The resulting supernatant fluid (fraction B) was then subjected to a force of 27,600 x G for 15 minutes in a Servall centrifuge type SS-1. The supernatant from this treatment was designated as fraction D and the residual as fraction E.

The measurements of the initial E_h and pH were made on a Beckman, Model K, automatic titrator. A glass electrode and a fiber junction saturated calomel electrode were used to determine the pH, while a bright platinum electrode and a liquid junction saturated calomel electrode connected to the sample through a salt bridge made of KCl saturated water and 2 per cent agar were used for measurements of electromotive force. All electrodes were checked daily against standard solutions. The use of the salt bridge was deemed necessary due to the marked instability of the calomel electrode when placed in rumen fluid for the prolonged lengths of time required for measurements of electromotive force. All measurements were conducted under a continuous flow of nitrogen and one drop of 0.002 per cent methylene blue was added to each sample to act as a mediator. The rumen fluid was constantly stirred to insure a measurement of the whole

system. Potentiometric titrations were carried out on the same equipment under the same circumstances with the exception that fraction B was used instead of fraction A. Ceric Sulphate was used as the oxidizing agent for most of the determinations of reducing capacity. Similar values were obtained when potassium dichromate was used but the titrations took much longer. In view of the fact an actively metabolizing system such as rumen fluid continually produces reducing substances, it was necessary to kill the organisms in order to determine the reducing capacity at a given instant. When ceric sulphate was used as titrant this was accomplished by adjusting the pH to approximately 1, the pH of the ceric sulphate solution. In cases where dichromate was used the system was killed by the addition of 2 milliliters of saturated mercuric chloride solution. The relationship between pH and E_h was determined by using 50 per cent sulphuric acid as the titrant and measuring the subsequent changes in the pH and E_h of the sample. All determinations were made in duplicate. While the first titration was being carried out the duplicate was kept in a water bath at 40° C. The hay-grain extract was prepared by adding 30 grams of ground alfalfa hay and 10 grams of cornmeal to 1000 ml. of water and allowing the mixture to stand overnight in a refrigerator.

Results

The data from the titrations carried out with sulphuric acid as titrant appear in table 1. This data was coded by setting the initial pH and E_h of each titration at 0.2 and -10 millivolts respectively and adjusting the other values

Table 1

Relationship Between E_h and pH

Titrations											
1		2		3		4		5		6	
pH	EMF[a]	pH	EMF	pH	EMF	pH	EMF	pH	EMF	pH	EMF
6.2	-260	6.8	-300	6.6	-280	7.2	-380	7.0	-300	7.2	-270
5.0	-200	6.0	-250	5.3	-200	5.9	-300	5.8	-250	6.3	-200
4.3	-150	5.1	-200	4.5	-150	5.1	-250	5.1	-200	5.2	-150
3.4	-100	4.2	-150	3.7	-100	4.2	-200	4.2	-150	5.0	-100
2.4	- 40	3.6	-100	2.6	- 50	3.5	-150	3.4	-100	4.0	- 50
2.2	+ 30	2.6	- 50	1.8	0	2.5	-100	2.4	- 50	2.8	0
		1.9	0			1.8	- 50	1.5	0		
						1.0	0				

Regression coefficient b = 0.0595 volts

Standard error = $\pm$0.0061 volts

[a]EMF expressed in millivolts potential between calomel electrode and rumen fluid.

accordingly. The regression coefficient, b, calculated by the method of least squares was -0.06 volts and the standard error (deviation about the regression line) calculated by the method of Snedecor (1946) was $\pm$ 0.006 volts. The relationship between the pH and E_h of rumen fluid can be expressed by the relation $E_h = E_o - 0.06pH$ where E_h is the potential in volts at any pH; E_o is the potential in volts and 0.06 is the regression coefficient.

The E_h of the water extracts of alfalfa hay and cornmeal was + 0.250 volts at pH 7. Data on the titrations of the various fractions of rumen fluid with ceric sulphate appear in table 2. In view of the potential observed in the water extracts of hay and cornmeal all titrations reported were

Table 2

Proportion of Reducing Substances Present in The Various Fractions of Rumen Fluid at 10:30 A.M.

Fraction		A	B	C	D	E
Average milliliters per fraction		50	36	14	34	2
Milliequivalents reducing substance per fraction	1.	1.95	0.90	1.00	0.70	0.15
	2.	2.20	1.10	1.00	0.90	0.20
	3.	2.40	1.10	1.20	0.80	0.15
	4.	2.00	1.05	.90	0.80	0.25
	Mean	2.14	1.04	1.03	0.80	0.19
Per cent of total		100.0	47.6	47.2	37.5	8.9

carried only up to a potential of + 0.200 volts at pH 7 to eliminate the possibility that the titration values would be influenced by the amount of water soluble feed constituents present. Fraction A was taken as representing the total amount of reducing substances present and set at 100 per cent. Fractions B, C, D, and E contained 47.6, 47.2, 37.5 and 8.9 per cent of the reducing substances, respectively.

The amounts of reducing compounds found in fraction B at three different times of day in cows on three different rations are presented in table 3. The values here represent the amounts of reducing substances with a potential below + 0.225 volts at pH 7.

Table 3

Potentiometric Titrations of Fraction B

Cows	Time		
	6:00 A. M.	10:30 A. M.	6:00 P. M.
T-25	1.55[a]	2.65	1.64
T-3	1.25	2.33	1.75
K139	1.78	2.21	2.01

[a]Mean of 4 values presented in Appendix Table 1 expressed as milliequiv. reducing substance/50 ml. fraction B.

The method for analysis of variance in multiple classifications presented by Snedecor (1946) was applied to this data.

Ration, time, and the ration by time interaction were designated as sources of variance. The differences in titration values with time of day were highly significant at the 0.01 level of confidence, the amount of reducing substance being highest at 10:30 A.M. The interaction between ration and time was also significant ($P = 0.01$) due to the relatively high amounts of reducing components present in samples from T25 at 10:30 A.M. and the continual presence of moderate amounts of reducing compounds in the hay fed cows.

The values in table 4 represent the E_h and pH of strained rumen fluid collected at three times of day from the same three cows. The corrected values presented were adjusted for pH according to the formula: $E_h = E_o - 0.06pH$.

This data was analyzed in the same manner that was described for the titrations presented earlier. The effect of time of day upon the uncorrected E_h values approached significance at the 0.05 confidence level. When the E_h values were corrected to pH 7 the effect of time of day upon the E_h was significant at the 0.01 level of confidence. The E_h was always lowest at 10:30 A.M. Ration did not influence the E_h.

Table 4

E_h and pH of fraction A

Time	6 A.M.			10:30 A.M.			6 P.M.		
Cow	E_h[a]	pH	E_h[b]	E_h[a]	pH	E_h[b]	E_h[a]	pH	E_h[b]
T3	-125	7.3	-107	-155	6.4	-191	- 75	7.0	- 75
	- 65	7.1	- 59	-145	6.3	-187	-125	7.3	-107
T25	-125	7.2	-113	-135	6.4	-171	- 95	7.2	- 83
	-135	7.1	-129	-155	6.7	-173	- 15	7.1	- 9
K139	-105	7.4	- 81	-165	6.8	-177	- 95	6.2	-143
	- 35	7.2	- 23	- 85	6.2	-133	-155	7.4	-131

[a]Uncorrected E_h.
[b]E_h corrected to pH 7.0.

PART II

STABILITY AND IMPORTANCE OF THE REDOX POTENTIAL IN RUMEN CONTENTS

Procedure

Inoculum from the same cows described in part I were used for the in vitro fermentations in this phase of the experiment. The cows were sampled in the same manner as was previously described. Measurements of E_h and pH and the titrations were carried out as described in part I.

The methylene blue and DPPD used as additives in these fermentations were in the oxidized form while the cysteine, thioglycolate and ascorbic acid were used in their reduced forms.

The short term fermentations were carried out in 300 milliliter Erlenmeyer flasks in a water bath held at 40° C. The flasks were closed with rubber stoppers with two outlets. The first outlet was connected to a calibrated gas burette while the second outlet was kept closed except in the fermentations to which oxygen was added. In this case the second outlet was attached to the oxygen source. In the fermentations where DPPD was the additive, 5 grams of cornmean and 5 grams of ground alfalfa hay were added to the flask before the fermentation was started. The other

fermentations were carried out without added substrate. The fermentation systems were thoroughly flushed with nitrogen before 250 milliliters of fresh rumen fluid were added and then the system was flushed again. The first 25 milliliters of gas produced were discarded. All additives used in these fermentations were added beforehand with the exception of oxygen gas which was added in small amounts throughout the fermentation time. The fermentation mixtures were shaken every twenty minutes, with the exception that during the runs where oxygen was added all flasks were shaken almost continually. At the end of each fermentation, the gas collecting system was closed off and the ferment subjected to a force of 2,700 x g in a centrifuge for 5 minutes. Fifty milliliters of the resulting supernatant were refrigerated in a bottle containing 1 milliliter of 50 per cent (V/V) sulphuric acid for further determinations. The gas collected was analyzed for carbon dioxide, methane and oxygen as quickly as possible in a Burrell portable type gas Analyzer, model 39. Cultures of rumen organisms were stabilized to formic acid using the technique outlined by Opperman et al. (1957). Gas from cultures stabilized late in the experimental period was analyzed for carbon dioxide in the Burrel analyzer previously mentioned while amounts of methane and hydrogen were determined in the Cenco Vapor Phase Analyzer with a silica gel column. Nitrogen flowing at 26.6 milliliters per minute was used as

the carrier gas. The temperature was 100° C. Hydrogen and methane peaks were identified from their retention times. The amounts of methane per sample were determined from a standard curve and hydrogen by difference.

Volatile fatty acids were determined by the direct chromatographic technique described by Keeney (1955) with the exception that petroleum ether (B.P. 65-110) was used instead of hexane and the valeric acid was not separated from the higher acids.

Ammonia was determined by the permutite method described by Hawk et al. (1954) with modifications. Four ml. of rumen fluid were added to a 100 ml. volumetric flask containing 2 grams of amberlite 1R-120H. The mixture was allowed to stand several minutes before the rumen fluid was decanted from the flask and the resin washed with distilled water. Two ml. of 10 per cent sodium hydroxide solution were added and the flask was allowed to stand again. After the addition of 75 ml. of water to the mixture, 10 ml. of Nessler's reagent were added and five minutes allowed for color development before diluting to 100 ml. Two drops of Gum Ghatti solution were added before the Nessler's reagent to prevent clouding. The optical density of the samples was then determined at 490 mu in a Beckman B Spectrophotometer. A standard curve was prepared in a similar manner using aqueous ammonium sulfate solutions.

Statistical analysis of data obtained in this phase of the experiment was carried out according to the methods suggested by Snedecor (1946) for the analysis of variance in experiments with multiple classifications. The only sources of variance considered were treatment differences and differences due to day to day variation in the innoculum.

Results

DPPD (diphenyl-para-phenylenediamine) lowered methane production *in vitro* but the effect was not significant at the 0.05 level of confidence (Appendix Table 2). No effect upon volatile fatty acid or ammonia production was noted. Although the addition of cysteine and thioglycollate seemed to result in a lower potential at the end of the fermentation, the probability of such an effect only approached significance at the 0.05 confidence level (table 5). These additives did lower methane production significantly (P = 0.05) (table 6). The effect was most notable when cysteine was added. The difference between thioglycollate and cysteine was not significant (P = 0.05). It should be noted that some cysteine was utilized by the organisms as shown by the lower titration values at the end of the fermentation. No effect upon volatile fatty acid and ammonia production was noted (Appendix Table 3).

Table 5

Effect of Thioglycollate and Cysteine Upon E_h

	Control		Thioglycolate		Cysteine	
	Run #1	Run #2	Run #1	Run #2	Run #1	Run #2
E_h at zero time	-180[a]	-200	-200	-290	-200	-320
pH at zero time	6.8	6.4	6.7	6.4	6.7	6.4
Reducing substance at zero time	3.48[b]	3.32	6.60	6.64	12.4	12.6
E_h at end of fermentation	+ 10	-210	-100	-300	-220	-310
pH at end of fermentation	5.3	6.3	5.2	6.3	5.3	6.4
Reducing substances at end of fermentation	3.12	3.32	6.68	6.60	11.3	12.0

[a]Millivolts.

[b]Milliequivalents/50 ml. fraction B.

Table 6

Effect of Thioglycollate and Cysteine Upon Gas Production

		Ml. CO_2		Ml. CH_4		Total Gas		% CO_2	
		Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
Control:	1	70.5	145.3	54.5	69.7	125	215	56.4	67.6
	2	73.1	144.1	56.9	65.9	130	210	56.2	68.6
Thioglycollate:									
	1	78.8	147.0	51.2	63.0	130	210	60.6	70.0
	2	76.7	162.9	53.3	62.1	130	225	59.0	72.4
	3	75.4	159.6	54.6	70.4	130	230	58.0	69.4
Cysteine:	1	79.6	164.7	50.4	60.3	130	225	61.2	73.2
	2	86.8	165.6	53.2	64.4	140	230	62.0	72.0
	3	79.2	152.3	50.8	57.7	130	210	60.9	72.5

The data in table 7 indicate that both methylene blue and ascorbic acid caused slight increases in the potential. Very little of the ascorbic acid remained at the end of the fermentation (table 7). Methylene blue caused a very significant increase in the per cent of carbon dioxide produced (table 7) but did not alter volatile fatty acid or ammonia production (Appendix Table 4). Results from fermentations to which catalytic amounts of methylene blue and oxygen were added are presented in table 8. None of these treatments caused a significant change in potential although the values

Table 7

Effect of Methylene Blue and Ascorbic Acid

Upon E_h and Gas Production

	Controls		MB			Ascorbic		
	Repl. 1	Repl. 2	Repl. 1	Repl. 2	Repl. 3	Repl. 1	Repl. 2	Repl. 3
Start E_h	-125[a]	-125	-55	-55	-55	-65	-65	-65
Start pH	7.0	7.0	6.6	6.6	6.6	6.7	6.7	6.7
Milliequiv. of additive	0	0	8.8	8.8	8.8	9.0	9.0	9.0
End E_h	-115	-115	-75	-75	-75	-65	-65	-65
End pH	6.4	6.4	6.3	6.3	6.3	6.2	6.2	6.2
Milliequiv. additive remaining	0	0	8.7	8.8	8.8	0	0	0
Ml. CO_2	46.9	46.6	66.3	64.0	69.4	66.5	109.0	98.7
Ml. CH_4	22.1	22.4	17.9	17.2	18.4	27.0	41.9	37.4
% CO_2	68.0	67.5	78.7	77.5	79.0	71.4	72.2	72.5

[a]Millivolts.

Table 8

Effect of Methylene Blue and Oxygen

Upon In Vitro Fermentations

	Control		MB[a]		MB[a] + O_2		O_2	
	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2	Run 1	Run 2
E_h at zero time	-65[b]	-55	-65	-55	-65	-55	-65	-55
pH at zero time	6.8	6.7	6.8	6.7	6.8	6.7	6.8	6.7
E_h at end of fermentation	-85	-105	-55	-75	-85	-85	-55	-105
pH at end of fermentation	6.2	6.2	6.1	6.2	6.2	6.2	6.1	6.1
ml. CO_2 produced	153.5	215.5	152.1	231.1	169.7	231.5	172.2	238.8
ml. CH_4 produced	72.1	87.5	72.0	85.5	45.5	78.0	51.4	98.1
Total ml. gas produced	225.6	303.0	224.1	316.6	215.2	309.5	223.6	336.9
% CO_2	68.0	71.1	67.9	73.0	79.0	74.8	77.0	73.6
ml. O_2 utilized	--	--	--	--	36.0	28.0	31.8	30.5
% O_2 in gas	1.3	1.5	1.0	1.0	9.0	15.0	9.0	13.7
Acetic acid[c]	7.37[c]	7.00	7.34	7.09	6.95	7.24	7.16	7.45
Propionic acid	1.92	1.63	1.93	1.60	1.92	1.62	1.79	1.64
Butyric acid	1.70	1.77	1.71	1.83	1.73	1.62	1.76	1.76
Higher acids	.57	.58	.53	.59	.53	.54	.63	.55
Ammonia nitrogen[d]	41.2	38.4	40.8	39.1	40.2	38.0	41.0	42.0

[a] 3 milligrams/250 ml. rumen fluid.

[b] Millivolts.

[c] Millimoles acid/100 ml. rumen fluid.

[d] Milligrams ammonia nitrogen/100 ml. rumen fluid.

did seem lower in the controls than in the flasks to which methylene blue was added in the absence of oxygen. Despite the rather notable increase in carbon dioxide production when oxygen was present the difference was not statistically significant ($P = <0.05$) in any case.

Cultures stabilized to formate produced theoretical amounts of carbon dioxide and methane ($3CO_2$ to $1CH_4$) whether methylene blue was present or not (Appendix Table 5). Considerable amounts of hydrogen (up to 5 per cent of total gas) were produced by two of the cultures during the second week of the stabilization period but this could not be attributed to methylene blue since only one of the cultures contained the compound. Early attempts to stabilize cultures to formate were unsuccessful in the respect that a 1 to 1 ratio of carbon dioxide to methane production persisted for prolonged lengths of time and the ratio was very stable (Appendix Table 5). The techniques used to stabilize all cultures were identical except that the cultures producing a 1 to 1 ratio were from samples of rumen contents collected at 10:30 A.M. while the cultures which produced theoretical yields were from samples collected at 6:00 P.M.

DISCUSSION

The linear relationship between E_h and pH shown in table 1 is not in agreement with the results of Broberg (1957a) who found no consistent relationship between E_h and pH when he acidified rumen contents either by stages or abruptly. A possible explanation for the difference is that Broberg worked with a living population while dead rumen bacteria were used in this experiment. Broberg reported that when the pH was within the physiological range 10 to 15 minutes passed before the system came to equilibrium. When the organisms are dead equilibrium is reached very quickly. These facts indicate that the effect of pH upon E_h is modified by the living organisms. The equation reported above is known to be true only in the case of dead rumen contents and may not rigorously hold in the case of a living system. In most cases, therefore, results, in this experiment, were only adjusted according to this equation when dead rumen contents were under investigation. The one exception, in table 4, when this author took the liberty of applying the correction to living rumen contents is, perhaps, not justified but the correction was very convenient for the purposes of discussion.

The results of the titration of the various centrifugally separated fractions of rumen fluid reported in table 2 show

that only 8.9 per cent of the reductive substances found in rumen fluid are directly associated with the bacterial cells. This result indicates the terrific ability that microorganisms have to affect their environment. The significant increase in the amounts of reductive substances found in fraction B several hours after feeding (table 3) and the significant decrease in potential at the same time, illustrate the capacity of the rapidly metabolizing rumen population to utilize the hydrogen acceptors in their environment. These results indicate that the potentiometric characteristics of rumen contents can be used as an indicator of metabolic rate in the same manner that pH measurements are now used.

The significant interaction between ration and time (table 3) correlates very well with the accepted fact that concentrates are digested more quickly than roughages. The cow on a ration of concentrate and roughage had a high level of reduced component several hours after feeding with comparatively low amounts later in the day. Conversely, the cows that received roughage alone maintained intermediate amounts of reductive component throughout the day.

The low poising capacity associated with the bacterial cells (fraction D) indicates that extreme care must be taken to maintain reducing conditions when the pure culture, washed and centrifuged cell techniques are employed. Several

workers have already noted that some reactions proceed very slowly or not at all when these techniques are used unless proper reducing conditions are maintained (El-Shazly, 1952a; Hungate, 1957; Gilroy and Heuter, 1957; Chu and Bryant, 1958). Smith and Hungate (1958) reported that Methanobacterium ruminantium cultured better at E_h levels below those produced when cysteine is used as the reducing agent. Carson (1948) has reported the dependence of the propionic acid fermentation upon the reductive state of the substrate.

The identities of the compounds which heretofore have been referred to as reducing substances are mostly unknown. The titration curve of rumen fluid from approximately -0.250 volts to +0.200 volts is almost a straight line indicating that there is no large amount of any one substance present that is responsible for the potential observed in rumen contents but rather a large number of compounds present in various states of oxidation whose normal potentials represent a continual gradation from very low to moderately high values. The following data taken from West and Todd (1957) presents the normal potentials of many common biological redox systems.

System	E_h in volts	pH
Cytochrome A	+0.29	7.4
Cytochrome C	+0.27	7.0
Cytochrome B	-0.04	7.4
Flavoprotein (yellow enzyme)	-0.059	7.0

System	E_h in volts	pH
Lactate-pyruvate system	-0.186	7.0
Riboflavin	-0.208	7.0
Cystine-cysteine	-0.227	7.15
Glutathione	-0.233	7.15
Coenzyme I	-0.325	7.4

The E_h values reported in this table represent the potentials of these compounds when the concentration of the oxidized form equals the concentration of the reduced form. Redox substances are analogous to pH buffers and must be subjected to considerable oxidation or reduction before their potentials vary substantially from the normal. The cytochromes would exist in an almost totally reduced condition in the rumen. In view of the function of the cytochrome system in aerobic metabolism one would expect that any oxygen present would be rapidly utilized. Flavoproteins, also, would be in a highly reduced state in the rumen while the lactate-pyruvate, cystine-cysteine, and glutathione systems could range from a highly reduced to a highly oxidized state depending upon the metabolic rate of the rumen contents. Coenzyme I would be at least one-half oxidized most of the time indicating that reactions requiring this coenzyme would not be impaired to any great extent in the rumen even at the highest metabolic rates.

The amounts of reducing substances found in rumen contents are not very high, at least, not high enough to be totally responsible for the stabile conditions that exist in the rumen. For example, it would take only 5 equivalents of a strong oxidizing agent to raise the potential of 175 pounds of rumen contents to +0.200 volts if the titration values were the only stabilizing factor in rumen fluid. A potential of +0.200 volts is inconsistent with normal anaerobic metabolism. The second part of this experiment was initiated to investigate other factors that contribute to the stability of the rumen environment. The several additives used in this phase of the experiment were chosen because they represented various potential ranges and the potentiometric behavior was known.

DPPD was chosen because its behavior resembles that of the tocopherols and it is or was commonly used as a stabilizer for vitamin A and carotene. Although the exact normal potential of DPPD is unknown and attempts to measure it were unsuccessful it was assumed that the potential falls between +0.100V and +0.300V and that DPPD should act as a hydrogen acceptor. Results obtained with DPPD as an additive were very inconclusive, probably due to the low solubility of this compound in water.

Cysteine and thioglycolate did not affect the potential of the rumen contents substantially. This result was expected since their normal potentials are approximately equal to the

E_h of untreated rumen contents (table 5). The objective in adding these compounds was the stabilization of the potential and alteration of the characteristics of the titration curve. The only notable effect was a decrease in methane production and an increase in carbon dioxide production. This result is hard to rationalize since both were added in the reduced state and therefore could not be acting simply as hydrogen acceptors. A possible explanation is that these additives poised the system above the optimum E_h level of Methanobacterium ruminantium. Smith and Hungate (1958) reported that this organism subcultured much better when the E_h of the media was lower than the potential produced when cysteine was used as reducing agent.

The choice of ascorbic acid as an additive was unfortunate since according to Ball (1937) the life of this compound in neutral solutions is very limited. This was the case in rumen fluid since none of the ascorbic acid remained at the end of the fermentation. Since the ascorbic acid disappeared and its exact fate or its length of life in the rumen fluid is unknown, no explanation for the increase in CO_2 production can be offered.

Methylene blue is known to act as a hydrogen acceptor in biological systems. The significant decrease in methane production found was, therefore, expected when comparatively large amounts of methylene blue were added (table 7). Even

though the hydrogen accepting capacity of these large amounts of methylene blue were sufficient to account for the decrease in methane production several factors indicated that this may not have been the only effect of methylene blue. The decrease in methane production, for example, was not large enough to explain the increase in carbon dioxide formation. The methylene blue was added in the oxidized state but was reduced quickly and the total amount added could be accounted for by titration differences almost immediately. After the initial saturation it is doubtful whether or not the effect could have been due simply to hydrogen acceptance. In view of these observations further investigation of this additive seemed advisable.

The effect of catalytic amounts of methylene blue alone and in the presence of oxygen was investigated (table 8). No significant differences in gas composition were found despite the notable affect of oxygen upon CO_2 production. A very slight tendency for methylene blue to affect gas production was still noticeable so the effect of this compound upon the gas production of cultures stabilized to formate was investigated. No apparent affect was noted. No explanation for the cultures which produced carbon dioxide and methane in a 1 to 1 ratio can be offered. The effect of large amounts of methylene blue upon gas production may be attributed to its hydrogen accepting capacity or to an

alteration in the conditions required by the methane producing organism reported by Smith and Hungate (1958). Neither of these affects would occur in stabilized cultures due to the long lengths of time involved.

The failure of substantial amounts of oxygen to alter the redox potential of rumen samples and the minor effect of this gas upon the end products of fermentation (Table 8, and Broberg, 1957c) lends support to the hypothesis that the potential of rumen contents is very stable. Although the rumen fermentation is mainly anaerobic in nature, it is commonly agreed that many facultative organisms are present. It is upon the presence of these organisms that an explanation for the stability of the potential in the presence of a strong oxidizing agent such as oxygen must be based. The presence of oxygen in microbiological media will inhibit the growth of anaerobes but if a facultative organism such as E. coli is added along with the anaerobe both organisms grow well. The E. coli utilize the oxygen in the media and lower the potential to a level where the anaerobe can also metabolize. The rumen is a somewhat more complex case than the one considered above but the situation is the same when the rumen contents become saturated with oxygen. This function which is fulfilled by the facultative organisms is a symptom of the dynamic equilibrium which exists in the rumen and the capacity of the rumen population to control its environment.

SUMMARY

The oxidation-reduction potential of rumen contents removed from several cows at three times during the day indicates that a close relationship exists between E_h and metabolic rate. Titration of samples, from which the large feed particles had been removed by centrifugation, with acid and a strong oxidizing agent, respectively, indicated that a linear relationship exists between E_h and pH and that the metabolic rate of the microflora is reflected by the amounts of reducing substances found. Very little of the reducing capacity of dead rumen contents was found to be associated with the bacterial cells indicating that extreme care must be taken to maintain the proper redox potential when the rumen microflora are separated from their natural environment if a normal innocula is desired. The titration curve for rumen contents indicates that there is no large amount of any one reductive substance present that is responsible for the potential observed in rumen contents.

The only rumen function that seemed to be affected by alteration of the reductive characteristics of rumen fluid was gas production. Additives employed were DPPD, methylene blue, cysteine and thioglycollate. The explanation suggested for decreases in the per cent of methane produced was that the potential may have been stabilized at a level higher

than the optimum level for methane producing bacteria. Addition of oxygen affected neither the redox potential nor the gas, volatile fatty acid or ammonia production of rumen contents incubated *in vitro*. Methylene blue did not affect gas production in cultures of rumen bacteria that were stabilized with formate as their sole carbon source.

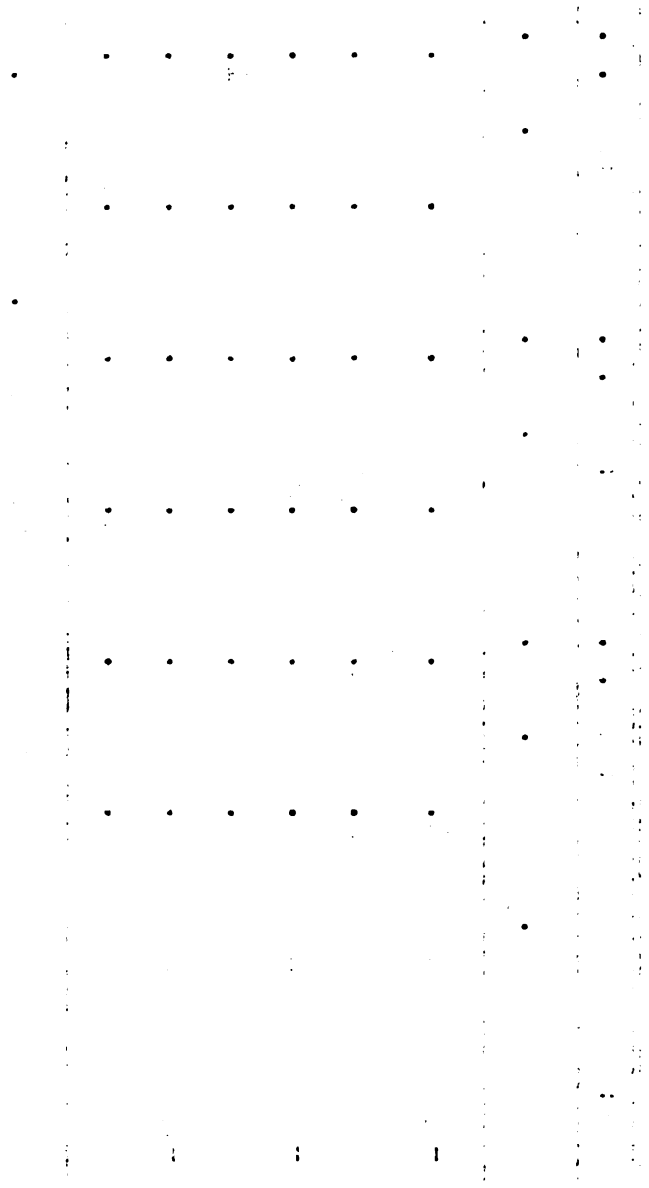
APPENDIX

Appendix Table 1

Potentiometric titrations of fraction B

Time:		6:00 A.M.		10:30 A.M.		6:00 P.M.	
Cow	Repl.	Dupl.	tit.	Dupl.	tit.	Dupl.	tit.
T-25	1	1.51[a]	1.47	2.60	2.70	1.75	1.71
	2	1.58	1.63	2.63	2.70	1.58	1.53
T-3	1	1.20	1.18	2.25	2.30	1.75	1.77
	2	1.30	1.32	2.38	2.40	1.70	1.77
K-139	1	2.00	1.96	2.22	2.25	2.05	2.02
	2	1.60	1.58	2.18	2.20	2.00	1.96

[a]Milliequivalents of reducing substances/50 ml. fraction B.



Appendix Table 2

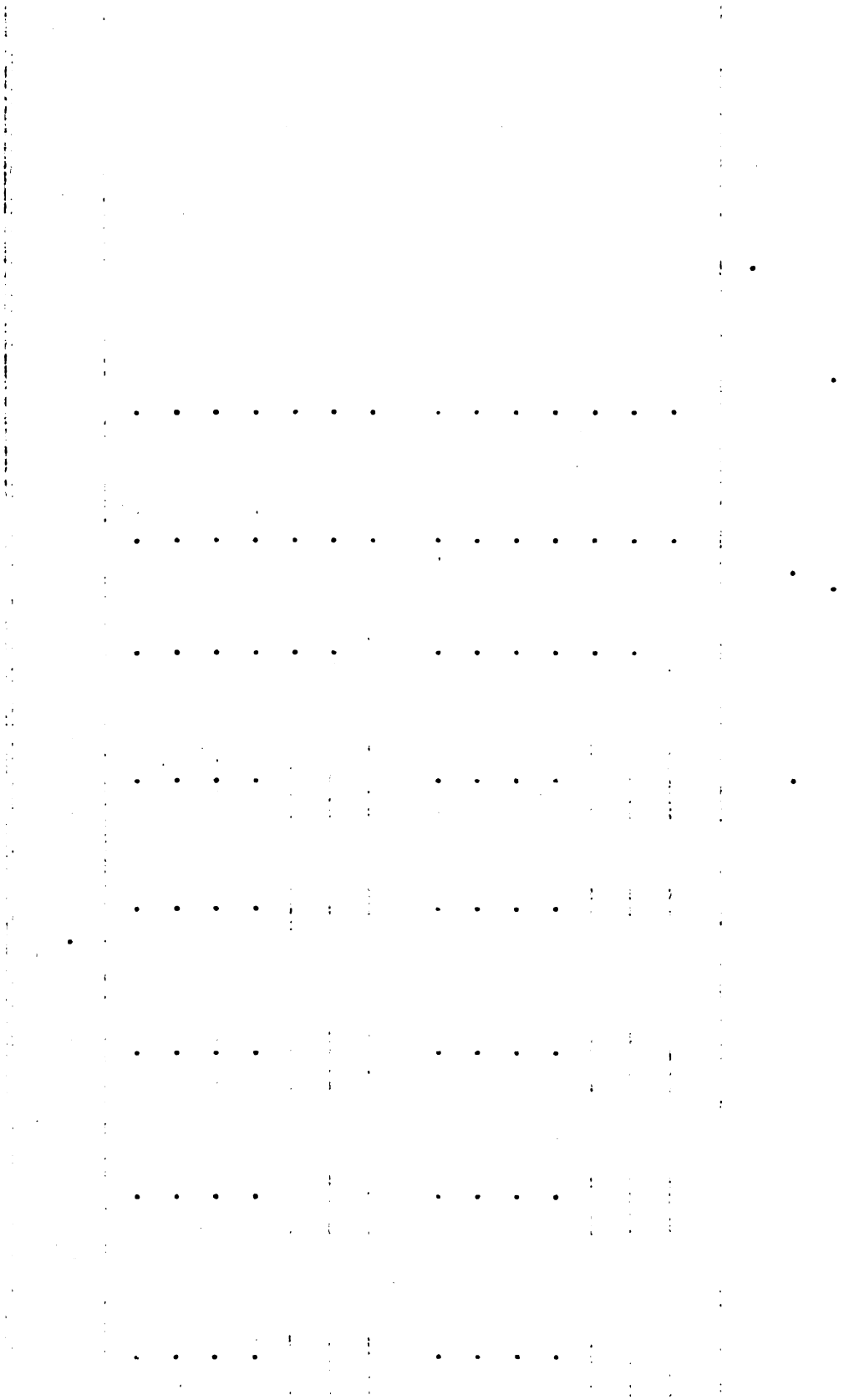
Effect of DPPD upon end products

		Total gas production[a]	% CO_2	% CH_4	NH_4[b]	Volatile Fatty Acids[c] Acetic	Prop.	Butyric	Valeric	Higher
DPPD	1	358	80.1	19.9	37.2	12.1	3.18	2.49	0.50	0.05
	2	328	80.3	19.7	39.8	11.85	3.22	2.65	0.50	0.05
	3	325	77.0	23.0	38.7	11.19	3.04	1.86	1.01	0.04
	4	315	82.6	17.4	35.4	12.18	3.63	1.99	6.75	0.05
	5	390	82.1	17.9	41.6	-----	----	----	----	----
	6	390	81.3	18.7	40.1	-----	----	----	----	----
	7	220	75.8	24.2	----	-----	----	----	----	----
Control	1	385	77.9	22.1	37.5	12.01	3.20	2.53	0.37	0.05
	2	340	80.9	19.1	40.1	11.85	3.25	2.65	0.48	0.05
	3	325	76.2	23.8	36.9	11.00	3.00	2.43	4.60	0.05
	4	305	82.2	17.8	35.6	12.30	3.62	2.15	0.65	0.05
	5	395	78.8	21.2	43.0	-----	----	----	----	----
	6	395	79.2	20.8	39.0	-----	----	----	----	----
	7	200	77.8	22.2	----	-----	----	----	----	----

[a]Milliters/flask.

[b]Milligrams ammonia nitrogen/100 ml. rumen fluid.

[c]Millimoles acid/100 ml. rumen fluid.



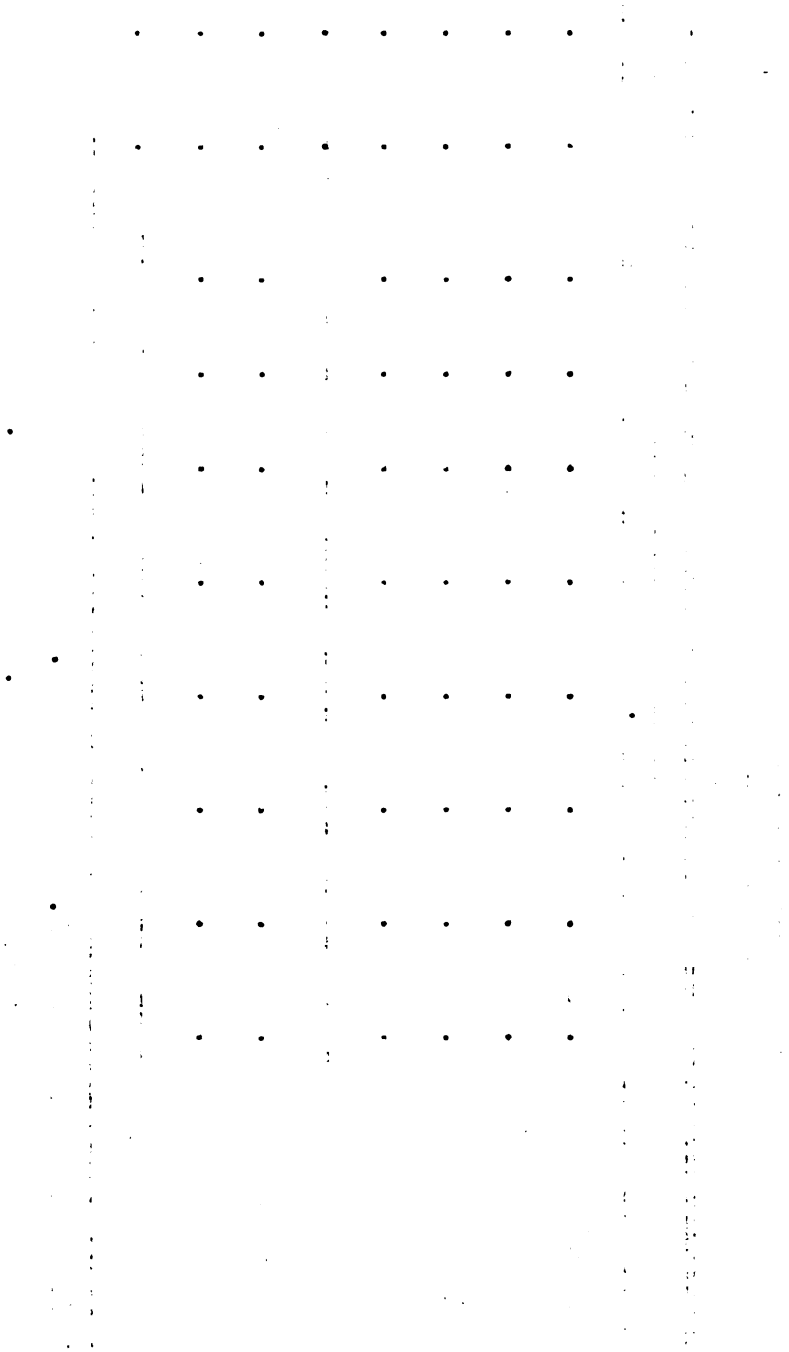
Appendix Table 3

Effect of thioglycollate and cysteine upon production of the volatile fatty acids and ammonia

		Volatile Fatty Acids[1]								Ammonia[2]	
		acetic		prop.		butyric		higher		nitrogen	
Control	1	8.26	7.21	2.00	1.81	2.01	1.50	.79	.89	38.1	42.1
	2	8.25	6.92	2.03	1.81	2.01	1.56	.91	.58	38.4	41.5
Thioglycollate	1	8.31	7.00	2.07	1.85	2.05	1.60	.63	.71	37.5	40.0
	2	8.26	7.11	2.05	1.76	2.09	1.60	.82	.52	38.3	41.8
	3	----	----	----	----	----	----	----	---	37.8	41.5
Cysteine	1	8.29	6.89	2.03	1.78	2.08	1.60	.77	.58	38.1	42.0
	2	8.26	6.95	2.07	1.73	2.11	1.71	.82	.69	38.0	41.7
	3	----	----	----	----	----	----	---	---	37.2	41.9

[1]Millimoles acid/100 ml. rumen fluid.

[2]Milligrams ammonia nitrogen/100 ml. rumen fluid.



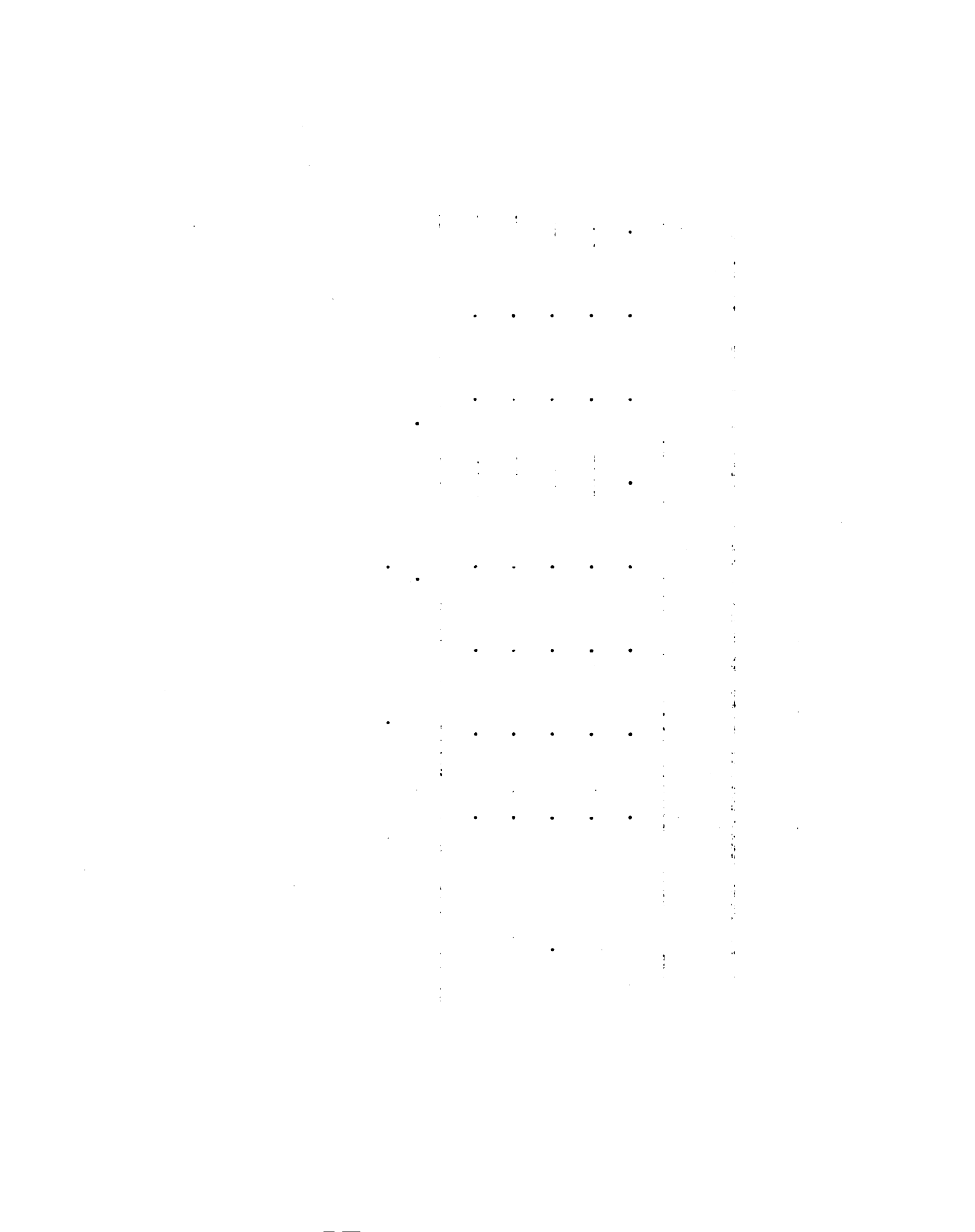
Appendix Table 4

Effect of Methylene Blue and Ascorbic Acid Upon Volatile Fatty Acids and Ammonia

	Controls		Methylene blue			Ascorbic Acid		
	1	2	1	2	3	1	2	3
Ammonia N[a]	35.6	34.8	35.0	34.6	36.1	35.2	35.6	35.0
Acetic[b]	6.86	6.63	6.71	6.76	----	6.79	6.54	---
Prop.	1.74	1.59	1.60	1.63	----	1.57	1.68	---
Butyric	1.90	1.55	1.64	1.68	----	1.75	1.63	---
Higher	.79	.60	.59	.55	----	.50	.54	---

[a]Milligrams ammonia nitrogen/100 ml. rumen fluid.

[b]Millimoles acid/100 ml. rumen fluid.



Appendix Table 5

Changes in Gas Composition During Culture Stabilization[a]

	incubation	Composition controls			methylene blue		
	(days)	CO_2[b]	CH_4	H_2[c]	CO_2	CH_4	H_2
Trial I	16	18	48				
	27	35	60				
	40	44	56				
	45	47	52				
Trial II	12	25	60		24	61	
	18	63	28		64	27	
	21	65	30	5	65	30	5
	25	69	27	4	68	28	4
	26	68	28	4	69	28	3
	28	70	27	3	69	29	2

[a]Formate sole carbon source.

[b]Per cent of total gas represents mean value for two cultures.

[c]Calculated by difference.

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