A STUDY OF GASEOUS EXCHANGES IN ENSILAGE

by

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I. INTRODUCTION

The main objective of herbage conservation is to preserve as efficiently as possible the nutrients accumulated by forage crops during their growth. Hay making is the standard technique used where weather conditions are favourable. In this process, crops are cut at an advanced stage of maturity, and severe losses may occur as a result of the leaching of nutrients by rain during field curing. Respiration losses may be heavy. Mechanical losses due to the shattering of leaves during harvesting are difficult to avoid.

Frequently, it is desired to take advantage of the higher protein content and higher available energy level of the young growing plants. A protection against adverse weather conditions may also be desired. Silage making is one of the best methods available for such purposes. Silage is essentially the result of a fermentation by microorganisms acting on the fermentable carbohydrates in the ensiled material to produce acids such as lactic and acetic acids. When sufficient acid is produced the microorganisms are destroyed by the product of their own metabolism and what is termed "stable" silage is produced. This occurs when the acidity of silage is in the vicinity of 4.0 pH units.

Basically there are three different methods of making silage. The most commonly used method may be termed the natural process. Crops rich in carbohydrates are ensiled without any previous treatment, or if poor in carbohydrates some additive such as

molasses is used. In this process bacterial fermentation is enhanced and if lactic acid organisms develop satisfactorily, a low pH is attained rapidly.

A second process relies on sterilization of the crop at ensiling time. The acidity of the mass is increased by the addition of mineral acid until it is between pH 3.0 and 4.0. This is the process developed by Virtanen (1933). It is also possible to use organic acids such as formic and lactic acids, sodium metabisulfite or SO_2 gas to achieve a certain degree of sterilization. In this process the action of plant enzymes and of most bacterial enzymes is decreased by the acid condition prevalent in the silage mass.

A third process, based on wilting of the crop in the field until its moisture content is between 40 and 70 per cent, is gaining more and more popularity where weather conditions permit its use. In this case, pH stays high and there is little loss of dry matter during conservation. An explanation of the good keeping quality of this silage has been proposed by Wieringa (1958). He showed that the increase of osmotic pressure in wilted silage decreased the pH tolerance of butyric acid bacteria. The low quantity of extracellular moisture present in silage made from wilted grass is probably a limiting factor in the fermentation since, according to Ruschmann (1939), fermentation takes place in the moisture outside the plant cells rather than inside the cells. This would explain the delay in bacterial multiplication observed by Stirling (1951) in wilted silage.

The three silage-making methods described above have this in common, that carbon dioxide is formed in the first few days

after ensiling through the continuation of the respiration of plant cells and during a longer period as a result of bacterial fermentation. The extent of the production of carbon dioxide from the two sources is dependent on the degree of success of the method used for ensiling.

The production of carbon dioxide and other gases in ensilage is one of the two ways by which ensiled dry matter can be lost during storage (the second way is by effluent loss). Virtanen (1946) has used carbon dioxide production as an index of material losses in ensilage. He stated that carbon dioxide evolution is also a measure of the extent of harmful fermentations, since the formation of volatile acids is always accompanied by carbon dioxide evolution.

The present study of gaseous exchanges in ensilage deals with the transformations occurring to some substrates during ensilage. The possibility of CO_2 fixation during conservation is investigated. In view of the lack of a method readily adaptable to the measurement of radioactivity in individual volatile and non-volatile acids, Part I of this work was devoted to that subject.

II. REVIEW OF LITERATURE

1. Gas evolution in ensilage

Few studies have been conducted on the nature and quantity of the gases formed in ensilage. Virtanen (1947) in the course of the development of the ensilage process named after him, collected total gases evolved from laboratory silos and, after analyses of the gases, concluded that only carbon dioxide was formed, at any rate in silage made from acidified plant material. His studies showed that carbon dioxide is formed in large quantities in ordinary silage in the first ten days of ensilage and at a decreasing rate afterwards. Carbon dioxide evolution was reduced fivefold in silage acidified with mineral acids and ceased almost completely after about 40 days. Total carbon dioxide evolution ranged between 100 and 140 g/kg of dry matter for silage made without additives or with molasses added. It was only 20 to 40 g/kg in A.I.V. silage kept for the same period of time.

Further studies on the nature of the gases produced during ensilage were prompted by reports of frequent cases of a mysterious pulmonary illness caused by toxic gases produced in recently filled silos (Lowry and Schuman, 1956; Grayson, 1956). It was shown by Peterson and coworkers (1958) that the gases present in laboratory silage at the peak of gas evolution were, by order of decreasing importance, carbon dioxide (CO_2), nitrogen (N_2), nitric oxide (NO), nitrous oxide (N_2O), nitrogen dioxide (NO_2), oxygen (O_2) and argon (A). Obviously some of those gases were atmospheric gases which had not yet been

displaced or utilized in the fermentation. Nitrate nitrogen in the ensiled forage was shown to be the main source of oxides of nitrogen, while amino acids had a contribution only one-tenth of that of nitrates. Wang and Burris (1960) studied the composition of gas mixtures recovered at the level of 5-foot from the bottom of 12 x 40-foot tower silos. In general, the changes were as follows: 5 hours after ensiling, oxygen had decreased from 21 per cent (percentage composition in atmosphere) to less than 1 per cent. In the same interval, carbon dioxide had increased from a trace to 25 per cent. With time, carbon dioxide displaced other gases to the extent that it contributed close to 90 per cent of the total gases in a sample taken 65 hours after ensiling. The only other gas present in significant quantity at that time was N_2. Isotopic studies indicated that this gas, present in large quantities in atmospheric air (ca 78 per cent), was also formed during conservation. Nitrogen gas was liberated by the reduction of nitrates and also, in small quantities, by the reaction of amino acids with nitrous acid (Van Slyke reaction) (Peterson _et al._, 1958). The oxides of nitrogen formed during ensilage (Wang and Burris, 1960) were: NO reaching a peak (9 per cent by volume in one experiment with corn silage and 3 per cent in a second experiment with the same crop) 25 to 35 hours after ensiling and disappearing after about 50 hours; N_2O present in quantities below 5 per cent in the initial phase of the experiment and decreasing to 1 per cent at 65 hours; NO_2 present in minimal amounts and arising from the spontaneous oxydation of NO in the presence of oxygen.

The virtually complete disappearance of oxygen was also observed by Langston _et al_. (1958) at Beltsville. In air-tight silos, no oxygen remained 5 hours after sealing the silos. In unsealed and loosely packed silos, oxygen was not detected at sampling locations lower than 6 inches from the silage surface after storage for 14 or 39 days.

In Minnesota, Lessard (1959) measured the rate of evolution of gases from 5-pound laboratory silos which had been filled with lucerne at 3 moisture levels. Although the nature of the gases was not determined it may be assumed safely from the results of Wang and Burris (1960) that most of the gas was carbon dioxide, except maybe for the gas originally entrapped which was displaced by carbon dioxide during the early period after ensiling. In the study, NO and NO_2 had been removed by bubbling the gas through α-naphthylamine-sulfanilic acid re-agent in the presence of oxygen. In silage made from the fresh, untreated crop, carbon dioxide was evolved at a high rate immediately after ensiling and this evolution ceased almost completely after 10 hours of ensilage. A new peak of gas evolution was reached at 20 hours. Carbon dioxide evolution again decreased at 2½ days and continued at a low rate for 12 days. After this, gas evolution increased again and continued at an irregular rate for several weeks. In the lucerne crop wilted to 37 per cent dry matter content, the same initial high gas evolution was observed and it ceased at about the same time. However, the second peak in gas evolution was observed at 35 hours rather than at 20 as in the fresh crop. After 8 days, all gas evolution had ceased.

In a third part of the crop where the dry matter content was raised to 45 per cent by a longer period of wilting, the same initial gas evolution was observed and it was almost over after 25 hours.

In the two wilted silages, it was noted that, not only gas was no longer evolved after 8 days, but a negative pressure was developed. It was hypothesized that carbon dioxide was fixed during ensilage.

Further indications were obtained by the author (unpublished) that CO_2 may be fixed during ensilage. In experimenting with plastic sleeves of a capacity of 35 tons as a means of silage storage according to the technique described by Larrabee and Sprague (1957), we observed that the sealed bags would become inflated a few minutes after sealing; internal gas pressure appeared to reach a maximum 2 days after filling. A slow deflation occurring afterwards brought internal pressures to near atmospheric, one week after filling. A few days later the plastic was laying flat against the side of the silage pile. Larrabbe and Sprague (1957) had made similar observations with the only difference that inflation continued for a longer period; this may be explained by the fact that they used a low dry-matter crop for ensiling, while we used a wilted crop and thereby reduced gas evolution. Larrabbe and Sprague (1957) attributed the deflation to the slow exit of carbon dioxide through the plastic film. According to manufacturers' specifications, the plastic (polyvinyl chloride, 0.008-inch thick) is permeable to carbon dioxide but not to

oxygen. While this explanation could account for some deflation, it could not account for the removal of the whole of the gas. In fact the plastic film was adhering to the silage pile as firmly as if gases had been removed from the pile with a vacuum pump.

Added to the above is the frequently made observation that in air-tight storage, if there is no seepage loss, the dry matter loss is usually very low (Briggs, 1957; Hodgson *et al.*, 1958; Gordon *et al.*, 1961). The possibility exists that the carbon dioxide evolved, if not lost immediately, could be neutralized during storage.

2. Carbon dioxide fixation by higher plants and bacteria

Non-photosynthetic fixation of carbon dioxide has been shown to occur in many plants and bacterial systems. There is, for example, the much-studied crassulacean metabolism in which organic acids increase during the dark and decrease during light (Vickery and Pucher, 1940). The formation of organic acids was shown to be linearly related to the concentration of CO_2 by Bonner and Bonner (1948). By the use of ^{14}C-labelled carbon dioxide, incorporation of CO_2 into organic acids was demonstrated conclusively (Thurlow and Bonner, 1948; Varner and Burrell, 1950).

In higher plants other than the succulents, dark carbon dioxide fixation was first reported in barley roots by Laties (1949). The majority of $^{14}CO_2$ was found in malate (Poel, 1953; Jacobson, 1955; Graf and Aronoff, 1955). An enzyme system capable of incorporating carbon dioxide has been found in the

roots, the leaves and the stems of a large number of plants (Jackson and Coleman, 1959).

Carbon dioxide fixation by heterotrophic bacteria has been demonstrated by Wood and Werkman (1936). They noted that *Propionibacterium pentosaceum* fermented glycerol to yield carbon compounds in excess of the quantity of organic carbon supplied. The carbon increase was found to come from the carbonate in the medium. Further evidence for CO_2 fixation was obtained by the use of radioactive tracers (Carson and Ruben, 1940). The tagged carbon in CO_2 was found in the carboxyl groups of succinic and propionic acids. The same reaction was observed in *E. coli* (Elsden, 1938) and in many other bacteria.

There is a large number of reactions through which carbon dioxide can be fixed non-photosynthetically. Wood and Werkman (1936) postulated that carbon dioxide was condensed with pyruvate to yield oxaloacetate. This is known as the Wood-Werkman reaction and is accomplished by the enzyme oxaloacetic decarboxylase. The enzyme exists both in bacteria and in plant (Gollub and Vennesland, 1947), but the equilibrium of this reaction favours decarboxylation rather than fixation of CO_2 (Burton and Krebs, 1953; Tchen *et al.*, 1955). It is doubtful, therefore, that fixation of CO_2 by this reaction would occur in significant amounts. A second enzyme, the malic enzyme, is capable of fixing carbon dioxide to pyruvate to yield malate (Conn *et al.*, 1949; Ochoa, 1951). This reaction is mediated by triphosphopyridine nucleotide (TPN or NAD) and is reversible.

Oxaloacetate could be formed from malate with the aid of malic dehydrogenase. Again the equilibrium of this reaction favours decarboxylation. The most likely reaction for entry of CO_2 into malate and oxaloacetate, in plants at any rate, is the enzymatic synthesis of oxaloacetate from phosphoenolpyruvate and carbon dioxide (Jackson and Coleman, 1959). The enzyme phosphoenolpyruvate carboxylase is the responsible agent. This third reaction, unlike the previous two mentioned, is not dependent on a high CO_2 pressure. A few other pathways of fixation have been demonstrated. Woods (1936) showed that formic acid is synthesized from CO_2 by _E. coli_ by combination with molecular hydrogen. The enzyme hydrogenlyase is involved in the reaction. Hydrogenlyase activity is dependent on the partial pressure of both CO_2 and H_2. Alpha-oxoglutarate is also a possible acceptor of CO_2 resulting in the formation of oxalsuccinate which can be removed as isocitrate. This has been shown to occur in parsley roots (Vennesland _et al._, 1947). The equilibrium constant is again strongly on the side of decarboxylation and carbon dioxide fixation would require the availability of an energy source. The energy could be supplied by coupling with reactions producing reduced TPN. Nutting and Carson (1952) working with _E. coli_ demonstrated the condensation of carbon dioxide (or formate) with acetate to yield lactic acid.

It is evident from the above that there is a variety of mechanisms for the entry of CO_2 into organic compounds both in plants and bacteria. The following acids have been reported as the immediate condensation products: formic acid, lactic acid,

oxaloacetic acid, malic acid and oxalosuccinic acid. Depending on environmental conditions, these acids can undergo different transformations. Oxaloacetic acid can be removed as aspartic acid by a transamination reaction, and become part of bacterial proteins. Similarly, lactic acid can go to alanine via pyruvic acid. The accumulation of succinic acid in many bacterial systems can be explained by the Wood and Werkman (1936) reaction or some modification of it. If CO_2 is fixed by phospho-enolpyruvate into oxaloacetate, this acid is first converted to malate by the enzyme malic dehydrogenase. Fumarate is formed by fumarase and succinate results by the action of succinic dehydrogenase. If CO_2 is fixed directly into malate, the step from oxaloacetate to malate is omitted.

3. Organic acids in forage crops

The organic acids are an important group of compounds in plants in view of their role in plant respiration. Respiration occurs by way of the citric acid cycle. All the organic acids in the Krebs cycle have been found in plants. Some are found in large quantities in all tissues, others are present only in trace amounts. From the standpoint of plant physiology, an acid in low concentration may be more important than an acid present in large quantities. Accumulation of an acid in the plant indicates that the mechanism for the formation of the acid is more active than the system responsible for its removal, or even that no mechanism exists for its removal. Low concentrations of an acid may be indicative of the large number of processes

responsible for further metabolism. The oxoacids such as pyruvate, oxaloacetate and alpha-oxoglutarate are good examples of this group. They may be dissimilated via the citric acid cycle, may become amino acids by transamination reactions and are important steps in the formation of a variety of other compounds.

In forage crops, the organic acids account for a proportion of the dry matter varying from 2 to 6 per cent for grasses (Fauconneau and Jarrige, 1954), and from 6 to 8 per cent for legumes. Factors such as stage of growth of the plant, type of fertilization have their influence on acid composition (Playne, 1964) but those will not be considered here. Differences between the various parts of the plant are also outside the scope of this investigation.

Malic and citric acids have been found in practically all forage crops. In perennial ryegrass they have been found to account for 60 to 75 per cent of the organic acidity (Hirst and Ramstad, 1957; Fauconneau, 1958), malic acid by itself accounting for about 50 per cent. Quinic acid was the only other acid found in significant quantities. A similar picture was found for the other grasses, such as orchard grass and meadow fescue (Fauconneau, 1960). In addition, succinic acid has been found in small amounts (Hirst and Ramstad, 1957). Other acids found in grasses but not determined quantitatively include shikimic, pyrrolidonecarboxylic, malonic, fumaric (Hirst and Ramstad, 1957), chlorogenic (Hulme and Richardson, 1954), aconitic and tricarballylic acids (Nelson and Hasselbring, 1931; Nelson and Mottern, 1931).

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Among the legumes, only lucerne has been studied to any extent. Like in grasses, malic acid was the dominant acid (Fauconneau, 1958, 1960). In one report, however, it was exceeded by succinic acid (Richardson and Hulme, 1957). It is likely that succinic acid was an artifact produced by the method of preparation of the sample (Fauconneau, 1960). The other major acids were citric, malonic and quinic and shikimic acids (Fauconneau, 1960). Richardson and Hulme (1957) found those same acids, but could not detect citric acid. In addition, they found glyceric, fumaric, citramalic, alpha-oxoglutaric, glycollic and pyrrolidonecarboxylic acids. This last acid was probably an antifact due to the extraction treatment. In other studies (Pierce and Appleman, 1943; Lessard _et al._, 1961), oxalic acid was also found. This acid was retained on the anion exchange columns used by the authors mentioned previously (Palmer, 1955). Oxo-acids and volatile acids have been reported only infrequently in grasses and legumes. They would be lost by evaporation or decomposed by the separation methods usually employed. However, the presence of acetic and lactic acids has been noted in a few instances (Langston _et al._, 1958; Lessard _et al._, 1961).

Most of the acids reported in plants have a known role to play in plant respiration in connection with the operation of the Krebs cycle in plants. A few of the reactions taking place have been mentioned earlier in connection with the review of the mechanisms capable of fixing carbon dioxide. In respiration, the action of the mechanism is reversed and usually

decarboxylations occur rather than carbon dioxide fixation.

The role of a few of the acids in plants is not so well known. Malonic acid is an inhibitor of the enzyme succinic dehydrogenase and as such would block the operation of the cycle. Glycerate, glycollate and oxalate are not part of the Krebs cycle. Apparently oxalic acid is precipitated as the calcium salt immediately after it is formed and its participation in further reactions is very limited. Quinic and shikimic acids have been found to be precursors in the biosynthesis of aromatic amino acids in microorganisms (Davis, 1955). The two acids appear to have the same role in higher plants (Weinstein _et al._, 1961). Shikimic acid is also a precursor of polyphenolic compounds (Hathway, 1956), of lignin (Brown and Neish, 1955), of rutin (Underhill _et al._, 1957) and of vitamin K_2 (Cox and Gibson, 1966).

4. The ensilage process

The importance of the organic acid content of a crop being ensiled comes from the fact that, together with soluble carbohydrates and amino acids, they form the substrate which is being acted upon during silage conservation. A second consideration of importance is that they may act as buffers to prevent the lowering of pH when lactic and volatile acids are formed.

There has been a long-standing controversy regarding the agent or agents responsible for the changes during ensilage. As early as 1900, Babcock and Russell conducted an investigation on the problem and concluded that the increase in acidity and heat production in silage were the result of intramolecular

respiration. Russell (1908) also decided that plant cells and enzymes were the primary factors in silage fermentation and that bacteria had a minor role. Bacteria and yeasts (Esten and Mason, 1912), bacteria alone (Hunter and Bushnell, (1916), bacteria and plant enzymes (Lamb, 1917) were in turn attributed the responsibility for the changes during ensilage. Hunter (1921) made a review of the status of the question and after a series of experiments concluded that: production of acids was due to microorganisms; yeasts had little influence on the fermentation except during the first few days; plant enzymes were chiefly responsible for the hydrolysis of proteins with the formation of amino nitrogen; both plant enzymes and bacteria were responsible for ammonia formation.

Some of the analytical techniques used by Hunter can be criticized when compared with present-day methods. For example, the total acidity measurements consisted of a straight titration of the grass or silage extract with standard barium hydroxide to the phenolphthalein end-point. Since the pH of forage plants is usually in the vicinity of 6 units, while that of successful silage is about 4, the two values cannot be compared as they are not on the same basis. Similarly, volatile acidity measurements obtained by distillation without a prior acidification of the material gave a poor measure of the volatile acidity. Nevertheless, many of the conclusions reached by Hunter (1921) have been confirmed.

(a) Role of plant enzymes

Mabbitt (1951) using aseptically-grown grass has been shown

that plant enzymes are responsible for the breakdown of proteins to amino acids during ensilage. Ammonia production also resulted. This could have been due to deamination of amino acids but it is likely that it arose at least partly by reduction of nitrate to ammonia. According to Nason (1956) such a reduction is accomplished readily by plant tissues.

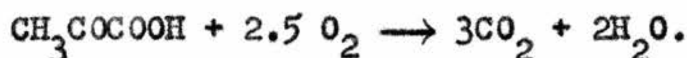
The studies of Wang and Burris (1960), also using aseptic "silage", have shown that carbon dioxide accumulation in bacteria-free silage resembled the pattern of carbon dioxide accumulation in a normal silage fermentation. In addition, nitric oxide was formed from the nitrates and the content of nitrogen increased with time indicating that the plants have the ability to reduce NO to N_2. From the studies of Wang and Burris (1960), it can be concluded that plant enzymes have an important role in the production of gases in the silo. Peterson _et al_. (1958) had arrived at an opposite conclusion from experiments where bacterial growth was inhibited by the addition of chloroform and toluene. However, on examination of their results, it is seen that only one gas sampling was taken in each experiment and only carbon dioxide and nitrogen dioxide were measured. Also, in one of the two experiments reported, the production of the two gases was not inhibited by the bacteriostatic agents employed.

The role of plant enzymes has been shown also by Playne _et al_. (1966). Those authors studied the changes in organic acids in bacteria-free grass ensiled as such, or inoculated with strains of streptococci or lactobacilli. After 10 days of storage, the pH of all silages was higher than at ensiling. The

main changes in the microbe-free silages were an increase in acetic and succinic acids and a decrease in malic, glycollic and two unidentified acids. Those changes are not unlike those occurring in ordinary silages, and indicate that plant enzymes contribute substantially in the process of silage production. The main differences with an ordinary silage fermentation of similar duration were that lactic acid was not produced and that malic acid was not so completely dissimilated. The sparing of malic acid observed in the experiment when only plant enzymes were acting might not be so large in farm silos. In the laboratory silos used the oxygen was quickly consumed and prevented from re-entering. Consequently, the Krebs cycle, an aerobic process, became inoperative in a short time. In large silos, where usually air exclusion is not so complete, a larger proportion of the Krebs cycle acids would disappear.

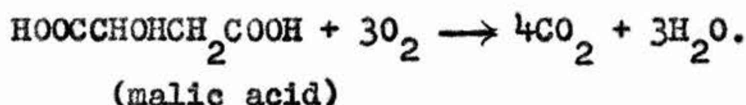
Further indications of the importance of plant enzymes in silage production can be derived from a consideration of the changes taking place in the hemicellulose fraction during conservation. Macpherson _et al_. (1957) found that the content of water-soluble carbohydrates was considerably higher in silage that had been preserved with sodium metabisulfite than it was in the original crop. This is explained by the presence in plants of enzymes capable of hydrolysing hemicellulose to produce pentose sugars (Dewar _et al_., 1963). This plant enzymic activity might account for the release of sugars in proportions as high as 3 per cent of the dry matter. Lactic acid bacteria were unable to use hemicellulose as an energy source (Dewar _et al_., 1963).

As shown above, the role of plant enzymes in silage production is partly a wastage of nutrients and partly a preparative role to prepare a suitable substrate for micro-organisms. The wastage occurs during the first few hours after ensiling and is due mostly to aerobic respiration. The simplified overall mechanism for the oxidation of pyruvic acid via the Krebs cycle can be represented as follows:



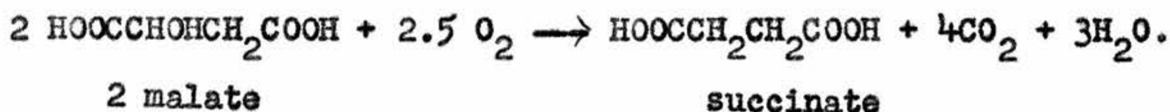
$$CH_3COCOOH + 2.5\ O_2 \longrightarrow 3CO_2 + 2H_2O.$$

Since in the absence of a bacterial fermentation malic acid is probably the main source of pyruvic acid, at the same time as being the source of the oxaloacetate which is combined with acetyl-Co A to form citric acid, the equation can be rewritten as follows:



$$\underset{\text{(malic acid)}}{HOOCCHOHCH_2COOH} + 3O_2 \longrightarrow 4CO_2 + 3H_2O.$$

The above simplified equation is true only if the dissimilation of malic acid goes through a complete cycle. However, Playne (1964) showed that an increase of succinic acid was caused by plant enzymes. This suggests that the enzyme succinic dehydrogenase was not operative in that system, or that it was not operating fast enough to remove all of the succinate formed. The inhibition of that enzyme may have been caused by malonic acid. Assuming that the enzyme was completely inhibited, the following reaction could then be expected:



$$\underset{\text{2 malate}}{2\ HOOCCHOHCH_2COOH} + 2.5\ O_2 \longrightarrow \underset{\text{succinate}}{HOOCCH_2CH_2COOH} + 4CO_2 + 3H_2O.$$

In the two microbe-free silages in Playne's (1964) study, the quantity of succinate formed was almost exactly one-half of the malate which had disappeared. The rest of the destroyed malate was accounted for by the acetic acid produced.

From data presented by Lessard (1959) it has been calculated that about 0.3 per cent of the original dry matter was evolved as CO_2 in the 5 hours following ensilage. This is an underestimate. Since malic acid is present as a salt in plant material, the degradation of two moles of malate results in the liberation of two cations. Those cations are immediately used for the neutralization of the carbon dioxide evolved and, two moles of bicarbonate result. Furthermore, as stated by Umbreit, an equilibrium becomes established between CO_2 gas and CO_2 dissolved as carbonic acid. The formation of ammonia (Mabbitt, 1951) from amino acid degradation or from nitrates is an additional source of cations for bicarbonate formation. It follows that, of the 4 moles of carbon dioxide formed during the degradation of 2 moles of malate less than 1 mole is evolved as gas. The dry matter lost as CO_2 in the initial 5 hours of ensilage was therefore of the order of 1.2 to 1.5 per cent of the dry matter.

Plant enzymes are also capable of hydrolysing proteins and hemicellulose. They play a major role in breaking down proteins. There are indications that they may be responsible for further metabolism of amino acids. For instance, Macpherson (1959) found that gamma-aminobutyric acid accumulated in large quantities in silage and microbe-free grass, arising from glutamic acid.

Evidence suggesting that the decomposition of amino acids proceeds down to the ammonia stage has been presented by Hunter (1921) and Mabbitt (1951). But in both cases, at least some of the ammonia resulted from the reduction of nitrates and the extent of amino acid decomposition by plant enzymes was probably not important.

(b) Role of bacteria in silage conservation

In well-made silage, the main role expected of bacteria is to produce lactic acid in sufficient quantity to lower the pH of silage to about 4.0. When this pH is attained quickly the action of plant enzymes and of proteolytic bacteria is stopped allowing silage to keep well (Virtanen, 1933, 1947).

In practice, in all silage fermentations there is a large number of compounds being formed by the action of microorganisms besides lactic acid. The evolution of carbon dioxide and other gases has been mentioned previously. Langston *et al.* (1958) have shown that acetic acid increased during conservation in all silages irrespective of their quality. Succinic acid increased also, but at least some of the production was due to plant enzymes. Other typical products of silage fermentation are butyric, propionic and formic acids. Iso-butyric, iso-valeric, valeric, iso-caproic and caproic acids have been found in poorly-preserved silages (Lessard *et al.*, 1961; Jones and Fricker, 1962; Morgan and Pereira, 1962A). In addition, Morgan and Pereira (1962A, 1962B) found that for every volatile acid detected in silage the corresponding alcohol and aldehyde were present. Ester combinations of most of the volatile acids and

alcohols were also found or suspected to be present.

The bacteriological changes during silage conservation have been adequately reviewed by Gibson _et al._ (1958) and by Gibson and Stirling (1959), and many of the important silage bacteria have been characterized more fully in recent years (Whittenbury, 1961; Lennard, 1964; Playne, 1964).

It has been pointed out by Gibson _et al._ (1958) that lactic acid bacteria are scarce in fresh plant material and that the initial bacterial population tends to disappear quickly during ensilage. The typical silage organisms then proceed to multiply rapidly. The period of multiplication has been found to last for only a few days (Gibson _et al._, 1958) in ordinary silage. In wilted silage, however, the period of multiplication is longer (Stirling, 1951). The more severe the wilting, the longer is the period of multiplication (Gouet _et al._, 1965). Judging by the rate of evolution of silage gases, such as nitric oxide (Wang and Burris, 1960) and carbon dioxide (Lessard, 1959), it would appear that bacteria start exerting a significant effect 5 to 10 hours after ensiling. In wilted silage the initiation of bacterial fermentation is delayed to possibly one day after ensiling.

It is likely that the changes in the period prior to the initiation of bacterial fermentation have a determining influence on the course of the fermentation and the quality of the conserved product. Many crops are low in fermentable carbohydrates. As noted earlier, one of the effects of plant enzymes is to convert malic acid and other acids of the Krebs cycle into carbon dioxide

and water. According to Whittenburg (1961) and Lennard (1964) many of the silage bacteria are able to utilize malic and citric acid. Phillips et al. (1956) have noted that for some lactic acid bacteria, the malic/lactic conversion may be almost quantitative. Citric acid, on the other hand, was converted to lactic and acetic acids. It follows, therefore, that in carbohydrate-poor crops, the prevention of the destruction of organic acids caused by plant respiration would make available to the bacteria a larger quantity of fermentable substances. This might be enough to make the difference between a good conservation and a poor conservation of silage.

In general, forage crops do contain sufficient soluble carbohydrates for producing the 3 to 5 per cent of lactic acid theoretically required to produce "stable" silage (McDonald and Henderson, 1962). In the conversion of citrate to lactate and acetate by bacteria, there is little change in the neutralizing power since the third carboxyl-group is mostly present in the free-acid form at the pH of grass. The malate/lactate conversion results in the liberation of one cation which will need to be compensated for by acid production. This increase in neutralizing power resulting from the dissimilation of malate and citrate by bacteria is considerably smaller than the increase resulting from the complete decarboxylation of the acids in plant respiration.

It has been shown that the dissimilation of malate and citrate is not always so simple as described above, depending on the types of lactic acid bacteria present (Whittenburg, 1964).

However, the rise in pH, production of carbon dioxide and of neutral products observed by Whittenbury (1962) can all be explained by the reactions mentioned above.

It is suggested that one of the most important factors for successful ensilage is the rapid removal of oxygen and its complete exclusion afterwards. It may be noted "en passant" that a method of removing oxygen from ensiled forage has been made available recently (Doutre, 1964). The method employs vacuum and seems to be of easier adaptation than previous vacuum techniques.

In the present review on the action of bacteria in silage fermentation, we have covered only a few points which have not been stressed sufficiently in previous publications. More detailed accounts of bacterial fermentations in silage have been published by Barnett (1954), by Watson and Nash (1960) and by Playne (1964).

5. The determination of organic acids, volatile and non-volatile in plant material

From the previous review it has become abundantly clear that organic acids are some of the most important compounds which influence silage conservation. Some are formed while others disappear in ensilage. In the present work, it was desired to determine both volatile and non-volatile acids; the measurement of radioactivity in the various acids was a second problem which we were faced with, since we wanted to take advantage of the great resolution power of liquid scintillation counting. It was thought originally that the

Wiseman and Irvin (1957) method, which has become a standard method in silage analysis, could provide the separation of the volatile acids and of lactic and succinic acids. An ion-exchange method such as used by Hirst and Ramstad (1957) could then have been used for non-volatile acids. After several attempts it was found that the Wiseman and Irvin (1957) method did not separate lactic and succinic acids, at least under our conditions. This led to a re-examination of the methods of organic acid analysis.

Six main techniques have been used for separating organic acids. Prior to the advent of chromatography, organic acids were determined either by fractional distillation after esterification (Nelson and Mottern, 1931; Nelson and Hasselbring, 1931) by fractional precipitation of the salts (Klein, 1932), or by specific chemical methods (Pucher _et al._, 1934). The chromatographic techniques which have been used for measuring organic acids are ion-exchange chromatography (Busch _et al._, 1952; Hulme, 1951), paper chromatography (Lugg and Overell, 1948), gas liquid chromatography (James and Martin, 1952), column chromatography on silica gel (Isherwood, 1946), or on celite (Phares _et al._, 1952), and thin-layer chromatography (Ting and Dugger, 1965).

Paper and thin-layer chromatography are considered as qualitative or only roughly quantitative techniques. Gas-liquid chromatography would be an attractive method, and so far it has been used for separating free volatile acids of silage (Barnett, 1954) and methyl esters of volatile acids

and lactic and succinic acids (Lessard _et al_., 1961). The methyl esters of other non-volatile acids have also been separated (Kuksis and Vishwakarma, 1963; Luke _et al_., 1963; Rumsey _et al_., 1964), but the method has not been adapted for quantitative studies. Interesting separations of the non-volatile organic acids have been obtained by Busch _et al_. (1952), Palmer (1955) and Hulme and Wooltorton (1958) when using a strongly basic anion exchange resin in the formate or acetate form. However, the technique has a few drawbacks, such as the necessity of removing water and formic or acetic acids by evaporation before titration. In the process, slightly volatile or labile acids may be lost or decomposed unless special precautions are taken. Glycollic, glyceric and lactic acids are among those. Oxalic acid is not eluted from the column. Volatile acids are completely lost. A few acids emerge together and have to be separated in some other way. Claims are made that the resin has a higher capacity (at least tenfold) than silica gel resins. In fact, however, in all the studies reviewed by the author, the quantities of acids applied to columns of anion-exchangers have been lower than or equivalent to what is generally applied on silica gel columns (1.0-1.5 mequiv). Chromatography on celite columns has been successful in separating volatile acids and lactic and succinic acids (Wiseman and Irvin, 1957) and the use of an internal indicator permitted to follow visually the development of the column. However, after many others, we have been unable to obtain the separation of lactic and succinic acid using that method. It is not possible,

using an internal indicator to separate acids which are eluted after lactic acid. Chromatography of non-volatile acids on celite has been successful (Phares _et al._, 1952).

No doubt, the method partition chromatography on silica gel columns introduced by Isherwood (1946) has been the most widely used and it seemed to be the method most likely to give satisfactory results in our work. That the original method was not entirely satisfactory is evident from the number of modifications which have been published.

None of the available silica gel methods was satisfactory for our purposes. It appeared useful to make a review of the successive modifications, in order to incorporate the most desirable features in a method adapted to the determination of organic acids from silage and to the efficient counting of the radioactivity of ^{14}C-labelled acids. The method evolved will be presented in the experimental section of this thesis.

6. The separation of organic acids by silica gel chromatography

In silica gel chromatography, the separation of organic acids is due to differences in distribution between two phases. The stationary phase is water, held within the silica gel particles. The mobile phase is a mixture of organic solvents of different polarity. The acids go from the stationary phase to the mobile phase at different times according to the polarity of the eluting solvent and are separated in this way. Fractions of equal volume are collected for the determination of individual acids.

(a) The stationary phase

Two processes can take place during chromatography on silica gel; some compounds may be retained by the silica gel itself by a phenomenon of adsorption or the compounds may be retained only in the water for which silica gel is a support. A partition between the aqueous phase and an organic phase is then obtained. In silica gel chromatography of organic acids, it is necessary to eliminate the adsorptive properties of silica gel. The method of preparation of the silica gel and the composition of the aqueous phase are the main steps in the elimination of the adsorptive properties of silica gel.

Isherwood (1946) prepared silica gel from sodium silicate (water glass) by precipitation with 10N HCl and his method of preparation was followed by numerous workers (Marshall et al., 1949; Neish, 1949; Frohman et al., 1951; Ladd and Nossal, 1954; DeKock and Morrison, 1958). After curing for two to three weeks, the silica acid obtained was washed again with 10N HCl, then with water, ethyl alcohol and ether ("En passant", the proper term for the compound used for organic and chromatography is silicic acid and not silica gel. As the two terms have been used interchangeably in the literature and commercially, we will continue using the term silica gel, which is the most frequently used). Some variability was often obtained in the chromatographic properties of the silica gel prepared by different workers. In later studies the Mallinckrodt silicic acid, 100 mesh, prepared according to Ramsey and Patterson (1948) has been used as such (Marvel and Rands, 1950; Scott, 1955), or

after removal of the smaller particles (Bulen _et al._, 1952; Zbinovsky and Burris, 1954; Ramsey, 1963; Wall _et al._, 1961) or after washing with HCl (Wager and Isherwood, 1961). The removal of the smaller particles is desirable in order to increase the flow rate through the column and it does not modify the chromatographic properties of the silica gel. It is achieved by repeated suspensions of the silica gel in water, followed by decantations, until about one-third of the silica gel has been removed (Bulen _et al._, 1952) or by suspending 250 grams of silica gel in one litre of water and discarding the supernatant portion after allowing to settle for 4 minutes (Ramsey, 1963). The silica gel was then dried at 110°C for 24 hours and stored over concentrated sulphuric acid. The washing with acid is recommended since, apparently the various batches of Mallinckrodt silica gel do not have all the same chromatographic properties. Washing with HCl eliminates cations and adsorptive properties which would cause retention of some acids in the gel or would produce unsymmetrical peaks for some acids (Wager, personal communication). Resnik _et al._ (1955), using silica gel prepared by Davison Chemical Corporation have also found an acid-washing necessary and Hathway (1958) has treated silica gel twice with boiling 2N hydrochloric for 2 hours, stirring the slurry continuously during the boiling.

The aqueous phase of the column is usually a dilute mineral acid in order to repress ionization of the acids on the column. The most widely used is 0.5N sulphuric acid, although concentrations have varied in the range from 0.05N (Kinnory _et al._, 1955)

to 4.7N (Frohman *et al.*, 1951). Hydrochloric acid has also been used (Neish, 1949) in order to prevent destruction of formic acid. A 0.1N H_2SO_4 solution in water-glycerol (1:1) has been recommended as the stationary phase for specialized separation of pairs of acids such as malonic and trans-aconitic acid (DeKock and Morrison, 1958). Finally, Marvel and Rands (1950) have used water as the stationary phase. It is generally considered, however, that the addition of mineral acid is useful to prevent adsorption of strong organic acids such as formic and oxalic acids. In general, sulphuric acid is to be preferred as it is eluted from silica gel columns later than hydrochloric or phosphoric acids and causes less interference in the elution of the more strongly retained organic acids. No improvement of separation has been obtained by increasing the concentration of sulphuric acid over 0.5N or by decreasing it.

The solvent mixtures used to develop silica gel columns have to be saturated with the same liquid used as a stationary phase. This is done by shaking the two liquids in a separating funnel, removing the aqueous layer after allowing time for the two layers to separate and filtering the solvent layer through a filter paper to remove suspended droplets. By this method there is always a small quantity of mineral acid in the eluate and allowance has to be made for it in the determination of organic acids. If the solvent mixtures were used without first saturating with the liquid in the stationary phase, the silica gel would soon lose its water of hydration and be useless for effecting partition. If, on the other hand, water only were

used as saturating agent, part of the mineral acid would be removed with the solvent and a phenomenon of adsorption rather than partition would take place.

A certain quantity of aqueous phase mixed with a given quantity of silica gel constitutes the stationary phase in the column. It is generally recommended to add as much of the aqueous phase as the silica gel will adsorb without sticking to the walls of the container. The quantity of aqueous phase to be added depends on the source of the silica gel. Some methods of preparation yield a silica gel which can adsorb almost double its weight of water (Neish, 1949); others prepared silica gel which could adsorb its own weight of water without becoming moist (Isherwood, 1946); the Mallinckrodt silica gel has adsorption capacity for only about 70 per cent of its weight. Although small variations in the degree of hydration of silica gel would have little effect on the partition properties of a column, it is recommended to use always the same quantity of aqueous phase in order to obtain reproducible results.

The weight of silica gel utilized to prepare a column has varied according to the method employed, to the capacity of the silica gel for adsorbing water and also to the scale of the separation attempted. The quantity of aqueous phase used to prepare a column is more important than the quantity of silica gel, since the separation is based on a partition of the acids between water and solvents (Isherwood, 1946). For example, 3 to 5.5 ml of aqueous phase have usually been used for the

separation of about one milliequivalent of total acids. Depending on the origin of silica gel (see above), the quantity used varied between 3 and 10 grams. When it is desired to separate larger quantities of acids, it is possible to scale up the preparation by using a column of larger diameter with a larger quantity of silica gel.

In chromatography, the aim is to obtain an efficient separation between acids and to obtain individual acids in sharp peaks, with low eluant volumes. It is logical to expect that the longer the column, the more efficient is the separation between acids. Mader (1954) has shown that this is a fact. However, the increased efficiency of separation of the longer columns was also accompanied by flattened peaks and high eluant volumes. It is therefore necessary to compromise between efficiency of resolution and sharp peaks. Both cannot be obtained at the same time.

(b) Transfer of organic acids to an analysing column

Various methods have been used to transfer organic acids to a silica gel column.

1. The acids are dissolved in one or two ml of a mixture of t-amyl alcohol and chloroform (Isherwood, 1946; Marshall *et al.*, 1949) or of n-butanol-chloroform (Marvel and Rands, 1950) and this is placed on top of the column.
2. The sample is dissolved in a small volume of aqueous phase and mixed with 0.5 g to 1.0 g of silica gel. The silica gel is transferred to the top of a prepared

column as a free-flowing powder (DeKock and Morrison, 1958; Wager and Isherwood, 1961) or as a slurry in chloroform (Bulen et al., 1952). When the sample is introduced in a powdery form, it is slurried directly in the column. In one case (Wager and Isherwood, 1961), the sodium salts of the acids were added to the column in silica gel and acidification took place directly in the column.

3. The sample is dissolved in a small volume of aqueous phase and introduced with a pipette to the top of a silica gel column made deficient in aqueous phase (Zbinovsky and Burris, 1954).
4. The non-volatile acids are adsorbed in a disc of blotting paper, the disc is dried and placed on top of the analysing column (Zbinovsky and Burris, 1954; Kinnory et al., 1955).
5. The acids are adsorbed in a disc of blotting paper which has been placed on top of the analysing column prior to addition of the sample. If the acids are added in the salt form, acidification can take place directly in the column (Wall et al., 1961).

The ideal method of introduction of samples is the introduction to the top of the analysing column. The use of mixtures of solvents is not satisfactory in view of the difference in solubility of the various carboxylic acids. Some loss of the volatile acids is possible when the sample is mixed with silica gel. Direct addition of the acid solution to the silica gel in

the column is suitable only when very small quantities of solution are added. The disc method seems to offer the largest number of possibilities and is especially advantageous when volatile acids have to be separated, since hydrolysis of acids can take place inside the column and losses are very small (Zbinovsky and Burris, 1954).

(c) The mobile phase

In general, a mixture of an alcohol and chloroform constitutes the mobile phase in silica gel chromatography of organic acids. The following alcohols have been used: n-butanol (Isherwood, 1946; Marshall et al., 1949 and 1952; Bulen et al., 1952; Wager and Isherwood, 1961); n-amyl alcohol (Donaldson et al., 1952); t-butyl alcohol (Resnik et al., 1955); t-amyl alcohol (Marshall et al., 1949; Frohman et al., 1951; Wall et al., 1961); and mixtures of n-amyl alcohol and t-amyl alcohol (Marshall et al., 1952; Ladd and Nossal, 1954). In some procedures, methyl isobutyl ketone (Hulme, 1952) and ether (Phares et al., 1952) have been used without admixture with other solvents. Chloroform has been replaced by benzene (Neish, 1949; Bulen et al., 1952) or tetrachloroethylene (Wager and Isherwood, 1961) for special separations.

The use of n-butanol in mixture with butanol has been criticized on the ground that esterification of strong carboxylic acids, particularly oxalic acid, is liable to occur under the acid conditions of the column (Bulen et al., 1952). There is no danger of esterification if n-butanol is replaced with t-amyl alcohol or t-butyl alcohol. According to Neish (1949) esterification is also avoided if benzene is used as the non-polar solvent

instead of chloroform. The use of ketones instead of alcohols has been suggested (Scott, 1955), but the separations obtained have not been as efficient as with alcohols.

In fermentation studies it is particularly important to obtain an efficient separation of individual volatile acids and lactic acid from other compounds. Wager and Isherwood (1961) have found that losses of 5 to 10 per cent of acetic acid may occur with chloroform-butanol mixtures during elution. This was due to the rapid evaporation of chloroform and acetic acid at the tip of the analysing column and in the receiving tube. They corrected the situation by replacing chloroform by a less volatile solvent, in the occurrence tetrachloroethylene. Chloroform has a boiling point of 61°C while tetrachloroethylene boils at 121°C. Benzene, boiling point 80°C, would also eliminate most of the loss if titrations were carried out without delay.

Lactic and succinic acids are not separated with the mixture n-butanol-chloroform. However, they are separated with an n-butanol-benzene solvent (Neish, 1949). The ether-benzene mixture can also be used for the same purpose (Kinnory *et al.*, 1955). This last solvent is less convenient for the elution of malic and citric acids. Ramsey (1963) has separated lactic and succinic acids using t-butyl alcohol-chloroform, but Bove and Raveux (1957) did not get a separation between the two acids using the same solvents. Wall *et al.* (1961) used t-amyl alcohol-chloroform with success for separating the two acids, but Bulen *et al.* (1952) failed to obtain a separation. It appears that t-butyl and t-amyl alcohol, in mixture with chloroform, might

effect a separation between the two acids when the lactic acid concentration is low, but results might be less satisfactory in fermentation products where lactic acid is dominant.

(d) The elution of organic acids

The elution of organic acids can be accomplished by the use of solvent mixtures in which the concentration of the more polar solvent is increased stepwise. It is the process which has been used by most workers. It is flexible and solvent composition can be varied according to the acid composition of the sample, in order to obtain an improved separation of the most important acids. The main drawback of the method is the need to follow elution closely in order to avoid getting air into the column when one of the solvent mixtures has been exhausted. Another disadvantage is the impossibility to continue elution overnight, so that 2 days are required for one sample.

Instead of a stepwise increase of the polar solvent obtained by premixing various concentrations of polar to non-polar solvents, it is possible to make use of a continuously modified solvent in which the concentration of the polar solvent is gradually increased. Donaldson *et al*. (1952) have described an apparatus for continuous gradient elution. It consists essentially of a mixing chamber, in which is placed a given quantity of the non-polar solvent, and a reservoir containing the polar solvent. In operation, the two solvents are mixed automatically owing to density differences, and a continuous gradient is produced. Various types of mixing chambers have been utilized (Wager and Isherwood, 1961; DeKock and Morrison,

1959; Donaldson _et al._, 1952). A theoretical discussion of the relation between the geometry of the mixing apparatus and the elution curve has been presented by Bock and Ling (1954).

The main advantages of a continuous gradient system are the avoidance of the spurious peaks caused by a sudden increase in concentration of the polar solvent, and the ease of operation. Difficulties have been encountered in equilibrating continuously changing solvent mixtures with the aqueous phase (Roberts and Mason, 1956; Wager and Isherwood, 1961; Dajani and Orten, 1958). This was apparently due to the type of mixing chambers used since Wall _et al._ (1961) did not report any such difficulties. In the gradient elution systems used to date, there is a certain rigidity in the elution schedule. Once the system has been started, the rate of change in polarity is fixed permanently and it is not possible to change it to suit the material under study. It should be possible, by appropriate changes in design, to add more flexibility to a system of continuous gradient elution (see experimental section of this thesis).

The rate of passage of solvent mixtures through silica gel columns does not seem to be critical to obtain good separations of organic acids, since rates varying between 0.5 ml/min. and 5 ml/min. have been used with apparently equivalent results. However, esterification of some acids could occur if a solvent containing a high proportion of butanol were passed through a column at a slow rate. The main methods of increasing flow rate are: the removal of the finest particles of silica gel, the application of external pressure (compressed air or nitrogen)

on top of the column, the increase in height of the column of liquid over the column.

Fractions of 2 to 5 ml are collected usually.

(e) Determination of eluted acids

Individual fractions are titrated using 0.005N or 0.01N sodium hydroxide to the end point of an indicator such as thymol blue (Isherwood, 1946), phenol red (Neish, 1949), or phenolphthalein (Wager and Isherwood, 1961). A stream of CO_2-free air is bubbled through the liquid, and at the end of the titration the tube is shaken vigorously, avoiding the formation of an emulsion. When t-butyl alcohol is the polar solvent, it is possible to remove chloroform by evaporation before titration (Resnik et al., 1955). The presence of only one phase makes the titration easier. However, this method is not satisfactory with volatile acids.

The easiest method is titration in the presence of ethanol (Wager and Isherwood, 1961). Again only one phase is present, and there is no necessity to remove one of the solvents. The occurrence of emulsions is also avoided.

Special methods of determination, in addition to titration, are available for groups of acids. Aromatic acids, usually too low in plant material for titrimetric determinations, can be analysed by the sensitive method of ultraviolet absorption (Sondheimer, 1957; Wall et al., 1961). Measurements are taken at 260 and 320 millimicrons in a spectrophotometer. The same technique also measures fumaric and trans-aconitic acids. The polycarboxylic acids can be measured by a fluorimetric method

(Frohman and Orten, 1953) after formation of fluorescent derivatives in presence of resorcinol. The method is of particular value if isocitric acid and nitric acid are present in the same plant. Both acids are eluted at the same time from the column in some methods, and thus it is possible to distinguish between organic and inorganic acidity. Some qualitative information can also be gained from the color of the fluorescence.

It is possible to obtain reproducible elution curves from chromatographic columns when the conditions of operation are similar. In practice, there is variation of a few millilitres in elution from one run to another. Nevertheless, the place of elution of an acid gives some information on its identity. It is always necessary to resort to other tests to make sure that the acid measured is the one acid expected and that it is not mixed with some other acid having the same elution volume. Paper chromatography in many solvents or thin layer chromatography, followed by specific reactions can be sufficient characterization. In some cases it may be necessary to isolate, purify unknown acids and to determine their physico-chemical constants and those of derivatives in order to obtain a positive identification.

III. OBJECT OF STUDY

The present study deals with the production and utilization of carbon dioxide in ensilage. Importance was attached to organic acids in view of the abundance of plant acids in forage crops, of their disappearance during ensilage, and of the prominent role of some acids as accumulation products or metabolic intermediates in fermentations. Radioactive carbon was used as a tracer.

Problems were encountered in the separation of organic acids and in the determination of the respective quantity of ^{14}C-labelling in each organic acid. Experiments designed to overcome those difficulties will be described. For sake of clarity, the experimental work on techniques for separation of organic acids and measurement of radioactivity therein will be reported as a separate entity.

The two parts of the thesis are:

Part 1. The determination of organic acids.

Part 2. Carbon dioxide exchanges in ensilage.

IV. EXPERIMENTAL

PART I. THE DETERMINATION OF ORGANIC ACIDS

Originally it was thought that a combination of ion exchange chromatography and partition chromatography such as used by Hirst and Ramstad (1957) would provide the desired separation of the organic acids in grass and silage. Only, the colorimetric method of determination of lactic acid used by those authors was not satisfactory for our purposes. On the other hand, the method of Wiseman and Irvin (1957) using partition chromatography on celite columns was reported to provide a separation of the volatile fatty acids, and of lactic and succinic acids. It should therefore replace with advantage the method of partition chromatography on silica gel columns used by Hirst and Ramstad (1957) for volatile acids. The method was followed in all its details, but the separation between succinic and lactic acids was not as good as claimed by the originators. Various refinements of the technique were attempted. Further purification of the solvents used (hexane and acetone) did not improve the separation. Changes in the extent to which the column was buffered were not more successful. The conclusion was reached that the method was not capable of separating lactic and succinic acids.

With the availability of an automatic fraction collector, it became possible to use a column without an internal indicator. In a recent publication, Wall *et al*. (1961) showed a successful separation of lactic and succinic acids as well as a good resolution of many other acids. Attempts to duplicate the

separation, using acid mixtures similar to those encountered in silage failed to produce a satisfactory separation of lactic and succinic acids (Figure 1).

After several attempts, the procedure described below was adopted.

A. DETERMINATION OF ORGANIC ACIDS BY SILICA GEL CHROMATOGRAPHY

1. REAGENTS -

Silica gel - Mallinckrodt's A.R. silicic acid, 100 mesh, was used. It was pretreated by boiling twice with 2N hydrochloric acid for 2 hours each time, according to Hathway (1958). Only the coarsest fraction was used. One third of the finest particles was removed by repeated suspensions and decantations (Bulen et al., 1952). After treatment, the silica gel was washed with distilled water until free of chloride, dried for 24 hours at 110°C and stored in a desiccator over H_2SO_4.

Solvents -

All solvents used were of the purest grade available. Chloroform was washed twice with distilled water to remove the ethyl alcohol preservative. Ethanol was redistilled from solid sodium hydroxide.

Indicator solution -

A quantity of 0.10 g phenolphthalein was dissolved in 500 ml absolute ethanol and the solution was titrated to a light pink. Fifteen ml of 0.01N NaOH were then added. The indicator was therefore 0.003N with respect to NaOH.

Standard solutions of organic acids -

Solutions of organic acids were prepared individually

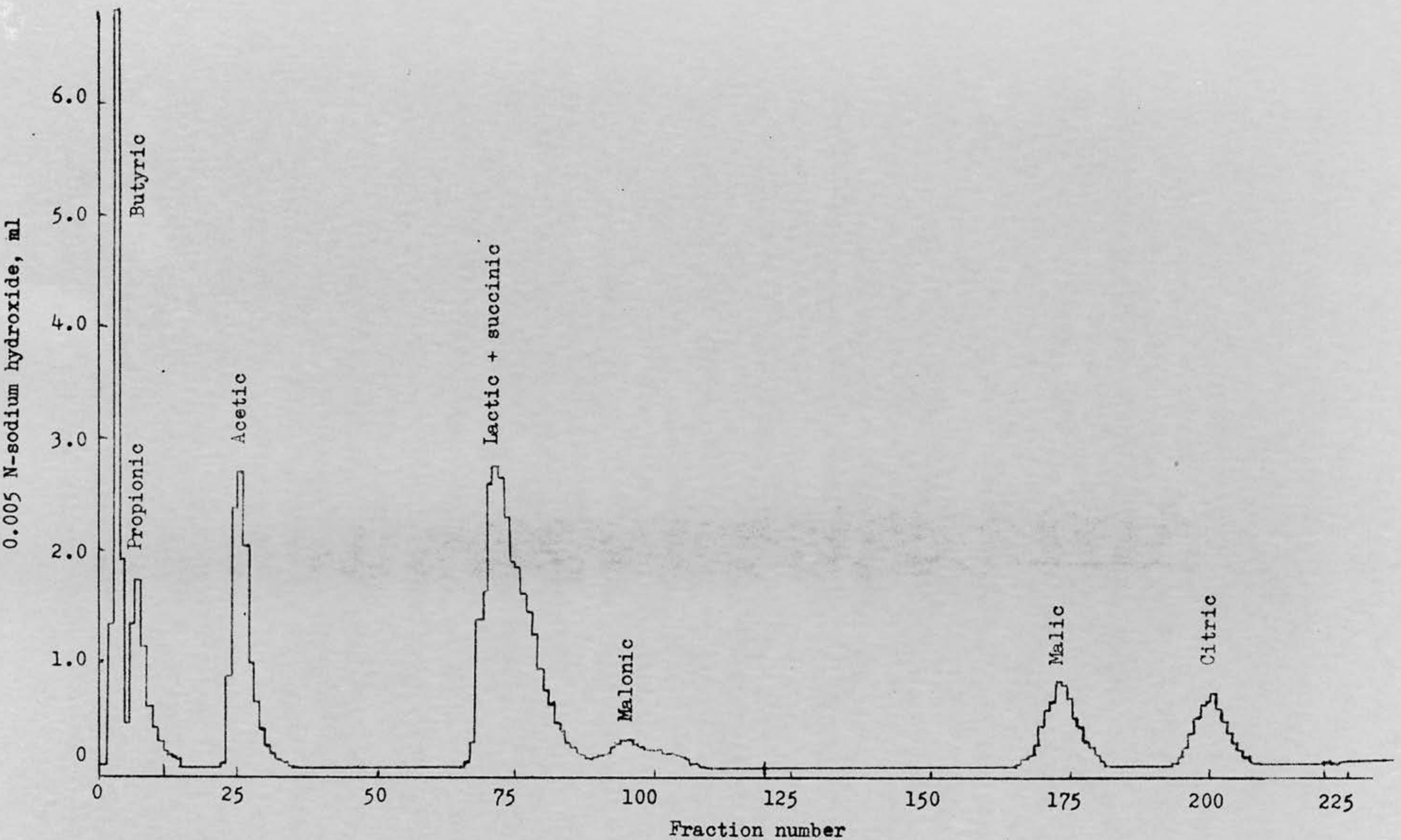


FIGURE 1. Separation of a synthetic mixture of organic acids using the method of Wall et al. (1961). Note the poor separation between butyric and propionic acids and the lack of separation between lactic and succinic acids (Fractions of 3.3 ml).
Elution: up to fraction 12, pure chloroform; between fractions 12 and 120, gradient of chloroform → 20% t-amyl alcohol in chloroform; beyond fraction 120, gradient of 20% t/amyl alcohol in chloroform → 60% t-amyl alcohol in chloroform.

and accurately titrated before mixing. Acids were of analytical reagent grade and were not purified further before use.

Sodium hydroxide, 0.01N in 80 per cent ethanol - 2400 ml of CO_2-free ethanol was mixed with 540 ml CO_2-free water and 60 ml of 0.5N sodium hydroxide. This solution was accurately titrated and stored in an aspirator bottle attached to the burette used for titrating fractions. The solution in both the aspirator bottle and the burette was protected against contact with atmospheric carbon dioxide.

2. APPARATUS

Gradient elution apparatus (Figure 2), composed of the following parts:

(a) Chromatographic column - It was a tube of 1.3 cm, inside diameter, fitted at the upper end with a B.29 ground-glass joint for connection to the gradient elution apparatus and at the lower extremity with a fritted-glass disc to support the column packing and a stopcock to control the rate of flow of the eluate.

(b) Mixing chamber - A 32.5 x 5 cm tube fitted with a B.24 joint at the upper end, a side-arm just below the 360 ml mark and a stopcock at the lower extremity. The side-arm was made of two parts joined by a ball-and-socket joint. The part free from the mixing chamber was a capillary tubing with stopcock and terminating with a B.29 inner joint with air vent.

(c) Reservoir - The reservoir was a 1-litre separatory funnel fitted with a piece of glass tubing extending to the bottom of the mixing chamber. Reservoir and mixing chamber were

A - B.24 GROUND-GLASS JOINT

B - 1 LITRE FLASK

C - TEFLON STOPCOCKS

D - B.24 GROUND-GLASS JOINT

E - TUBE 5 x 32.5 CM, VOLUME 360 ML. TO LINE

F - BALL AND SOCKET JOINT

G - B.29 GROUND-GLASS JOINT

H - 1.3 CM. I.D. CHROMATOGRAPHIC COLUMN

I - CAPILLARY TUBING

J - AIR VENTS

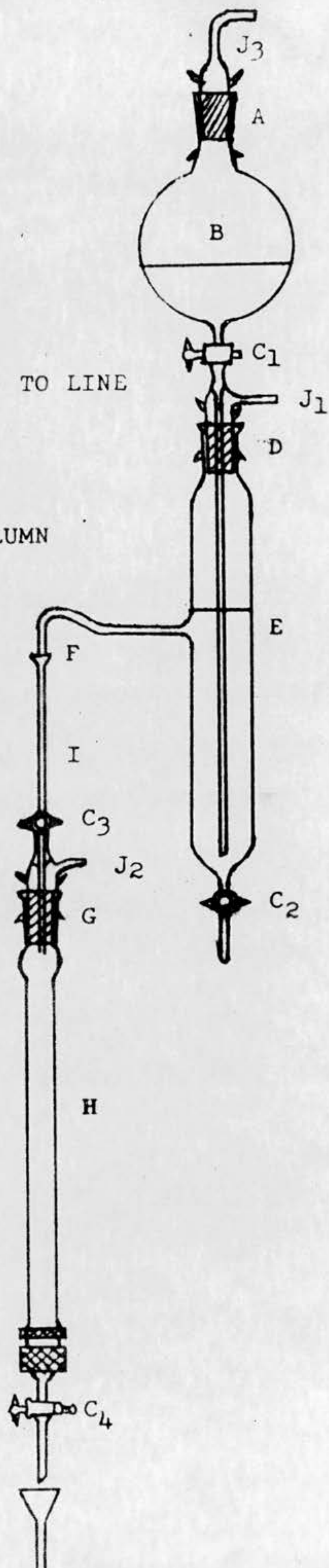


FIGURE 2. Gradient elution apparatus

held together by a B.24 joint. An air vent was provided above the joint.

Fraction collector -

A RadiRac automatic fraction collector equipped with a volumetric siphoning device was used. Water, instead of the eluate from the column, was measured with the syphon and the apparatus was used as a timed-flow system.

3. TECHNIQUE

Eluants -

The eluting solvents or solvent mixtures were equilibrated before use with approximately one-tenth of their volume of 0.5N sulfuric acid. After the two phases were separated, the organic phase was filtered through a coarse filter paper to remove suspended aqueous droplets.

The following pairs were used to produce the desired gradients:

Gradient I: benzene, 100 per cent and butanol-benzene, 20:80.

Gradient II: t-amyl alcohol-chloroform, 20:80, and t-amyl alcohol-chloroform, 60:40.

These two gradients have been the most useful for the separation of the organic acids in plant material and silage. Variations may be employed to separate pairs of important acids in some special applications. We have used the following:

Gradient IIA: t-amyl alcohol-chloroform, 5:95, and t-amyl alcohol-chloroform, 15:85, the latter being followed by t-amyl alcohol-chloroform, 60:40.

Preparation of analytical column -

The preparation of a good column is one of the most important steps for a satisfactory separation of organic acids. For this reason the mode of preparation used is described in detail.

A quantity of 10.0 g of dry silica gel was weighed in a 150 ml beaker, and 6.0 ml of 0.5N sulfuric acid were added. The powder was thoroughly mixed by working it against the walls of the beaker with a spatula. Sufficient benzene was added to make a thin slurry.

A disc of coarse filter paper was placed above the fritted glass disc, the stopcock was closed and 15 ml of benzene was poured into the column. The slurry of silica gel and benzene was poured in and any visible air bubble was immediately removed using a flat-end tamping rod with the end slightly smaller than the inside diameter of the tube. The stopcock was opened and the gel was allowed to settle. The stopcock was closed when only about 5 ml of benzene was left above the column packing. The beaker was rinsed with a few ml of benzene and the rinsings were used to dislodge the gel particles adhering to the walls of the chromatographic tube. The remaining particles were removed with tissue paper. A pad of filter paper cut to fit snugly into the tube was placed over the packing, care being taken not to entrap air under the pad. With 1.3 cm columns, it was convenient to use No. 740-E (Schleicher and Schuell Co.) discs of adsorbent paper "for the assay of Penicillin and other antibacterial substances". Each disc is of the exact size required

and adsorbs evenly a minimum of 0.08 ml of water. A pad of 8 discs was satisfactory for the size of sample used. The column was compressed with a tamping rod down to a mark etched in the glass, for more reproducibility between successive determinations, and the benzene left on top of the pad was rapidly poured out.

Transfer of acids to the analyzing column -

The standard acid mixture used in the calibration procedure contained the acids in the free form. On the other hand, the method was to be applied to plant and silage extracts where the salts were present. In order to use a similar procedure for both cases, the sequence for sample application was as follows: 0.1 ml of distilled water was added to the surface of the pad of filter paper, then 0.4 ml of standard acid solution and 0.1 ml of distilled water (the composition of the standard acid mixture is shown in Table 1). This was allowed to penetrate into the pad of filter paper and was washed in with two 2-ml portions of benzene. Three ml of benzene were placed above the pad of filter paper and the column was connected with the gradient elution apparatus (Figure 2) above the fraction collector.

The elution of acids -

Gradient I was first used for the elution. The following sequence of operations is recommended for starting gradient elution.

Introduce 360 ml of benzene in the reservoir (B), stopcock C_1 being closed. Close stopcocks C_2 and C_3 but leave opened air vent J_1. Make sure the ball-and-socket joint F is firmly

secured. Open stopcock C_1 and allow benzene to drain into the mixing chamber. Close stopcock C_1 when the level of the liquid has reached it. Close air vent J_1 with a piece of plastic tubing and a spring clip. Introduce 360 ml of butanol-benzene (20:80, v/v) into the reservoir (B). Fill the side-arm of the mixing chamber by opening stopcock C_3 and close the stopcock immediately. Close air vent J_2 with a piece of plastic tubing and a screw clip. Start operation of the column by opening stopcock C_4, C_3 and C_1 in rapid succession. A continuous gradient is produced.

When the reservoir (B) is empty, close stopcocks C_4 and C_3 and remove the solvent left in the mixing chamber by opening stopcock C_2. Repeat the same procedure with the solvent mixture of gradient II, 360 ml of 20 per cent t-amyl alcohol in chloroform being placed in the mixing chamber and 450 ml of 60 per cent t-amyl alcohol in chloroform going into reservoir (B).

The rate of flow of eluents through a chromatographic column prepared as described above was about 1 ml per minute. This is a satisfactory rate, but it could be increased, if desired, to 2 or 3 ml without change in efficiency by application of pressure through air vents J_1, J_2 and J_3. The rate of flow of 1 ml/min. allowed the change of solvent to take place within the day the column was prepared, if prepared in the morning. Elution with gradient II was continued, unattended, overnight.

Determination of acids -

One ml of the alcoholic phenolphthalein solution was added

to each 4-ml fraction which was then titrated with 0.01N sodium hydroxide in 80 per cent alcohol. The slight alkalinity of the indicator solution was sufficient to neutralize the inorganic acidity in the initial stage of the fractionation. With the increase in butanol in the eluting liquid it became necessary to subtract a blank from the titration value of each fraction.

Results -

The column described above can separate up to 1.5 milliequivalents of total carboxylic acidity and is sensitive to as little as 1 microequivalent of acid, especially in the initial stage of the fractionation. In late fractions, 2 microequivalents are required for detection. In recovery experiments (Table 1) where 0.4 ml of a synthetic solution containing most of the silage acids and a few plant acids was separated by the solvent systems described above, good recoveries were obtained for most acids. Formic and oxalic acids were the most difficult acids to recover quantitatively; nevertheless, recoveries of above 90 per cent were always obtained. Sharp peaks were obtained for all acids. In the beginning of development, three fractions contained the whole of valeric acid. However, the number of fractions necessary to elute other acids increased considerably, especially toward the end of the elution with the t-amyl alcohol-chloroform gradient. It was possible to prepare columns with similar characteristics from one run to the other. However, the fractions in which the acids were collected varied to a slight extent depending on concentration and various other

TABLE 1

Performance of chromatographic column
(0.4 ml portions of standard solutions of acid were used)

	Concentration of individual acids in standard solution (mequiv/ml)	Fractions with acids	Average blank value of peak (ml of 0.01 N-NaOH)	Recovery of acids (average of 4 runs) %
(Benzene/butanol eluant) (3.5 ml fractions)				
n-Valeric	0.1	4-6	0.01	98.9
n-Butyric	0.2	8-13	0.01	100.1
Propionic	0.2	15-21	0.01	99.0
Acetic	0.6	27-35	0.02	98.4
Formic	0.1	39-48	0.02	92.5
Succinic	0.2	70-77	0.03	99.3
Lactic	0.6	82-98	0.03	101.1
(Chloroform/t-amyl alcohol eluant) (4.0-ml fractions)				
Oxalic	0.1	106-121	0.04	91.2
Malic	0.6	128-142	0.06	98.3
Citric	0.3	144-158	0.10	98.0

factors including the temperature of the room. Therefore, the fraction numbers presented are shown as an approximation only. The blank values obtained when a column was run under the same conditions but with the sample replaced by 0.5N sulfuric acid were low at the beginning of column development but increased in later stages of the elution.

An example of the analytical possibilities of the chromatographic system described is shown in Figure 3. In this experiment, the column was developed with the following solvent schedule: 360 ml of benzene was introduced in the mixing chamber and 45 ml of benzene in the reservoir. Valeric and butyric acids were eluted with this solvent. After exhaustion of the benzene, gradient I was started by adding 280 ml of 20 per cent butanol in benzene to the reservoir. This was followed by gradient IIA consisting of 360 ml of 5 per cent t-amyl alcohol in chloroform in the mixing chamber and 100 ml of 15 per cent t-amyl alcohol in chloroform in the reservoir. When the reservoir emptied, it was filled with 450 ml of 60 per cent t-amyl alcohol in chloroform and elution was continued as before.

The use of pure benzene as the eluant prior to gradient elution was necessary to separate butyric and valeric acids. The introduction of gradient IIA allowed a complete separation between lactic and malonic acids. The presence of a double peak for lactic acid was due to the fact that this acid was on the point of being eluted when the eluants were changed. A gradient from 10 per cent t-amyl alcohol to 20 per cent t-amyl alcohol gave some separation between lactic and malonic acids,

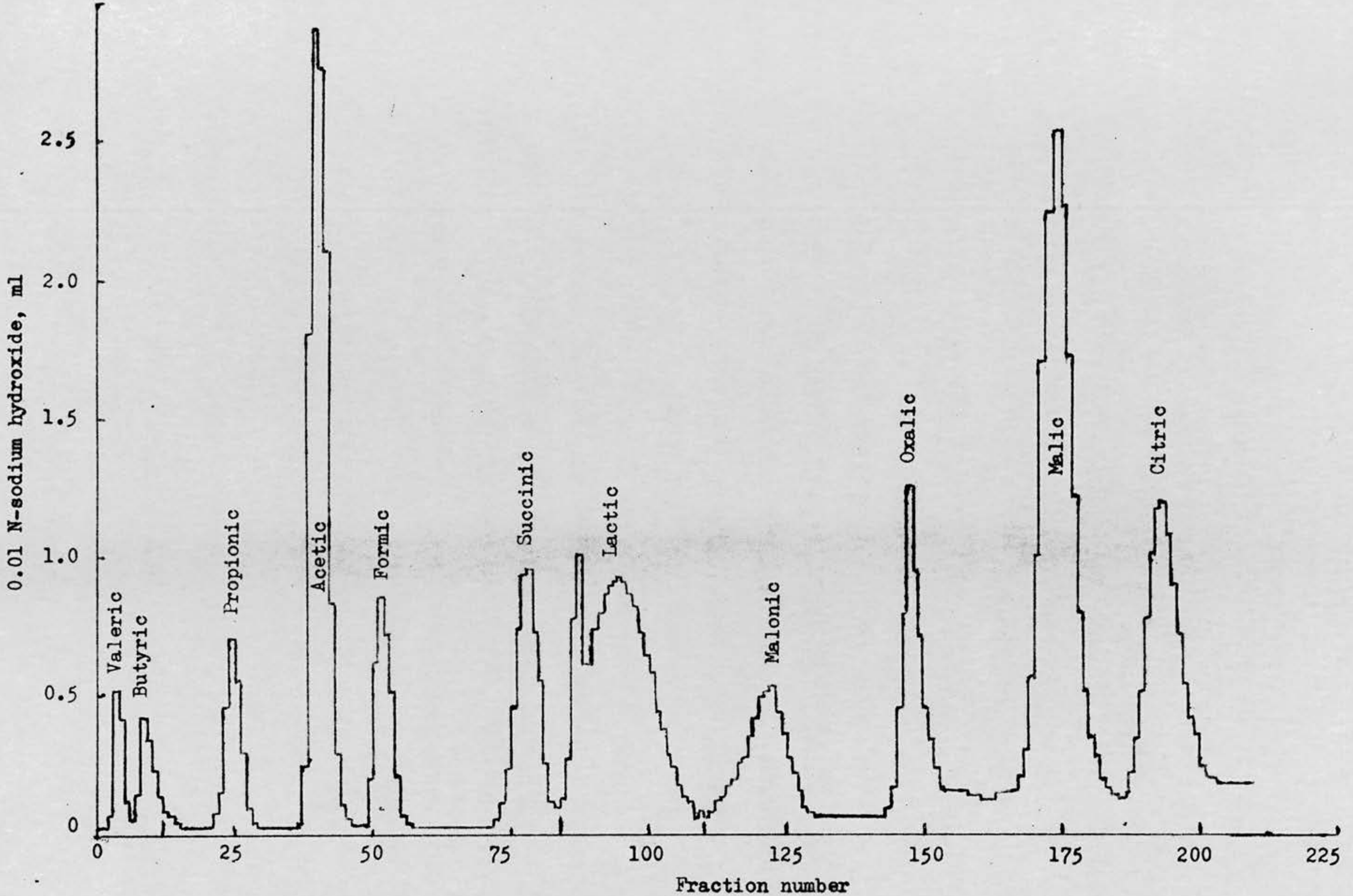


FIGURE 3. Separation of a synthetic mixture of organic acids by improved method. Valeric, butyric and propionic acids as well as succinic, lactic and malonic acids are separated (fraction of 4.0 ml).
Elution: up to fraction 12 pure benzene; between fractions 12 and 84 gradient of benzene → 20% butanol in benzene (285 ml); between fractions 85 and 110, gradient of 5% t-amyl alcohol in chloroform → 15% t-amyl alcohol in chloroform; beyond fraction 110, gradient → 60% t-amyl alcohol in chloroform.

but the separation was not as good as with the 5 to 15 per cent t-amyl alcohol solvent.

B. APPLICATIONS OF THE METHOD TO BIOLOGICAL MATERIALS

The organic acid composition of many plant materials and silages has been determined using the method described previously. An additional problem was encountered in this determination; the extraction methods utilized by various workers were not always reliable. Ether extraction (Ramsey, 1963) of the acidified plant material is a relatively safe method from the standpoint of changes occurring during the extraction. However, the low solubility of the more polar acids such as citric acid make it necessary to prolong extraction for extended periods of time. Since glyceric acid is insoluble in ether it would not be extracted even after a long extraction period. Extraction with ethyl alcohol has been used extensively. The main disadvantage of this solvent is that it cannot be used in acidic medium because of the possibility of esterification of some organic acids. On the other hand, many salts of organic acids are insoluble in ethanolic solutions. Most salts of organic acids are at least slightly soluble in water. The free acids are all soluble enough in water for extraction in a small volume of water. This was the basis for a method of extraction of volatile fatty acids and lactic and succinic acids proposed by Wiseman and Irvin (1957). In view of the simplicity of the method, it was decided to test whether or not this extraction method could be extended to other organic acids. Since the ethanol extraction procedure is almost a standard method with non-volatile acids it

was used for comparison purposes.

1. Extraction methods - application to red clover tissues

Samples for analysis -

Red clover was used for testing the efficiency of the two extraction methods. The samples were obtained from the 1963 crop grown on the Langhill Farm of the University of Edinburgh. The red clover was cut when the crop was just beginning to bloom. The crop was mainly red clover and the few grasses present were separated by hand. A sample was oven-dried for dry matter determination. The dry matter content was 14.5 per cent.

Extraction with alcohol and water -

A 20-gram sample was rapidly killed by dropping it into 100 ml of boiling ethanol and boiling was continued for 3 minutes. A globe condenser on top of the beaker prevented loss of volatile acids. The alcohol extract was decanted and saved. The residue was macerated with cold 80 per cent ethanol in a high speed blender. The macerate was filtered and the residue was reextracted twice with 80 per cent ethanol and then with distilled water, with finally two water washings. The alcohol extract was concentrated to a syrup after titration to pH 8.5. The concentrated solution was filtered, the residue was washed with distilled water and the filtrate was mixed with the aqueous extracts. The combined extract was purified by ion-exchange chromatography. It was first passed through a 10-ml column of Amberlite IR-120 (16-50 mesh) in the free-acid form to remove cations and amino acids. The column was washed with 200 ml of distilled water, precautions being taken to prevent the eluate

from the column coming into contact with atmospheric CO_2. An aliquot of the eluate was titrated to determine total anions and discarded. The remainder of the solution was passed through a second 12-ml column of Amberlite IR-45 in the hydroxyl form. The extract was followed by 150 ml of CO_2-free distilled water. The acids were eluted with 300 ml of 4N aqueous ammonia. Ammonia was removed and the ammonium salts were concentrated to a small volume in a rotary evaporator at a temperature lower than 40°C. The concentrate was desiccated over phosphorus pentoxide in a vacuum desiccator. The salts were dissolved in sufficient water to make a total volume of 2 ml for application to the analytical column.

Extraction with dilute mineral acid -

Dilute mineral acid was the extractant in the second method (Wiseman and Irvin, 1957). A 20-gram sample of finely chopped material of same origin as in the extraction previously described was placed in a small wide-mouthed bottle and covered with 20 ml of 0.3N-sulfuric acid. A crystal of thymol was added as a preservative. The material was tamped to ensure intimate contact of the acid with the plant material. The bottle was capped and stored for a week in a refrigerator. During storage the contents were mixed and compressed on two occasions. The liquid was squeezed out of the bottle into a centrifuge tube. After centrifugation, 15 ml of the supernatant liquid was thoroughly mixed with 23 grams of silicic acid purified as described previously (p. 42). The silicic acid was quickly covered with benzene and slurried in order to prevent evaporation of volatile acids from

the large surface area of the gel. The slurry was poured into a previously prepared column. The column was a 3-cm glass tube fitted with a sintered glass disc and a stopcock at the lower extremity. It contained 4 grams of silicic acid saturated with 2.5 ml of distilled water and slurried with benzene. A 5-cm layer of benzene had been left above the silicic acid. With the stopcock open, the column was packed with pressure from a nitrogen tank and the benzene level was brought down to the level of the gel. The wall of the column was cleaned with a glass wool plug which was left on top of the gel. Acids were eluted with 450 ml of a 60 per cent v/v solution of butanol in benzene which had been saturated with 0.5N sulphuric acid. A few ml of distilled water were added to the contents of the separatory funnel and the acids were neutralized to the cresol red end point with 0.1N-sodium hydroxide. The aqueous layer was removed and the solvent was washed twice with water. The combined aqueous fractions were concentrated to a small volume in a rotary evaporator and were taken to dryness over phosphorus pentoxide and pellets of sodium hydroxide in a vacuum desiccator. The salts were dissolved in water and the solution made to 1 ml for application to the analytical column.

Transfer of acids to the analytical column -

The samples were transferred to the analytical column the same way as described previously (p. 48).

Gradient elution -

Same as described (p. 48), using gradients I and II. Details of the use of gradient I in combination with IIA were

worked out only after this experiment was conducted.

Calculations -

With the alcohol extraction, followed by aqueous extractions, the calculations are straightforward, provided the dry matter content of the herbage material is known. Calculations of the acid concentration after the extraction with mineral acid are more complicated. The success of the extraction depends on the assumption that the relative concentration of organic acids in the extracting liquid is the same as in the tissues of the plant. For this equilibrium to become established, the sample is allowed to stand in a refrigerator for one week and the material is stirred and compressed occasionally. After this period of time, if the extraction has been successful, any aliquot of the liquid should contain an equivalent proportion of each acid. Thus, if 20 grams of herbage material containing 80 per cent moisture is extracted with 20 ml of dilute mineral acid, each ml of liquid, after equilibration, should contain 1/36 of the total acids present in 4 grams of dry matter, or, 1/9 of the acids in one gram of D.M. If 15 ml of solution is used for the purification step, we are working with 15/9 of the acids in one gram of D.M. When the concentrated salt solution obtained after the purification step is made to 1.0 ml and 0.4 ml is transferred to the analytical column, the titration value for each acid represents 6/9 of the acids in one gram of D.M.

Multiplying by 100 converts the titration value to mequiv/ 100 g D.M., a convenient scale for expressing the organic acidity of plant material. In practice, a conversion factor (K) is

calculated for each sample separated, and all titration values, after correction for the blank, are multiplied by this factor. The formula for the calculation of K is the following:

$$K = \frac{N \times \left[V_1 + (W \times M)\right] \times V_2 \times 100}{V_3 \times V_4 \times W \times D}$$

Where:

N is the normality of the NaOH used for titrating the fractions,

V_1 is the volume of mineral acid added to the sample,

W is the fresh weight of the sample,

M is the moisture content of the sample expressed as a fraction of 100,

V_2 is the volume of the concentrated solution of salt obtained after the purification step,

V_3 is the volume of extract used for the purification procedure,

V_4 is the volume of concentrated solution of salt transferred to the analytical column, and

D is the dry matter content of the sample expressed as a fraction of 100.

Identification of acids -

Fractions from each peak were pooled and taken to dryness in a rotary evaporator. Salts were dissolved in a small quantity of water, cations were removed by shaking with Amberlite IR-120 (H^+) resin, and the resulting solution was spotted on chromatographic paper. Where volatile acids were suspected, a few drops

of concentrated ammonia were added to the solution of acids before spotting. Standard acids were spotted alongside the acids to be identified.

Paper chromatography -

Uni-dimensional paper chromatography was used in most cases. Each sample was assayed in solvents (a) and (b) below:

(a) Propanol: ammonia, 60:40 (Isherwood and Hanes, 1953);

(b) Butanol: formic acid:water, 120:20:20 (Cheftel et al., 1953).

In addition, solvent (c) was used for suspected volatile acids.

(c) Propanol: ammonia, 70:30 (Isherwood and Hanes, 1953).

With this last solvent, the chromatographic paper was exposed to ammonia vapors before the samples were spotted. Suitable indicator solutions were used as general location reagents. Specific colour reactions, when available, were used for confirmation of the identification. This was judged sufficient for acids which were known to occur in the type of material utilized.

Results -

One of the main difficulties in the determination of organic acids from plant tissues is their extraction. The purification of the extract is also cumbersome. The usual method of extraction with 80 per cent ethanol (Wager and Isherwood, 1961; Weinstein and Laurencot, 1958), improved by following it with an aqueous extraction (Fauconneau, 1958), was tested against an acid extraction method.

Results are shown in Table 2 and a histogram of the distribution of acid-extracted organic acids from red clover is shown in Figure 4. The method of preparation of the different reagents used is shown in Appendix I. Using a combination of silica gel chromatography and paper chromatography, a minimum of 19 acids were detected and the most abundant ones have been identified. One acid, (peak 16) accounted for more than 37 per cent of the acids titrated.

As shown in Figure 4, the unidentified acid was eluted after citric acid, in the approximate position of shikimic, tartaric and glyceric acids. The acid was extracted from the appropriate fractions with dilute ammonia and concentrated. After purification on Whatman 3mm papers using n-propanol:ammonia (60:40) solvent, the acid was reacted on paper chromatograms with the following reagents: (1) sodium metaperiodate followed by sodium nitroprusside and piperazine (Cartwright and Roberts, 1955); (2) ammoniacal silver nitrate (Brown, 1951). In reaction (1) a white spot against the faint mauve background was produced. After the ammoniacal silver nitrate spraying followed by development at room temperature, a yellow spot with a purple edge appeared. Reaction (2) is characteristic of hydroxy acids while reaction (1) indicates the presence of a glycol group. Rf values in three solvents were the same as for authentic glyceric acid. When the acid was precipitated as the calcium salt, it had the same melting point (138°C) and infrared spectrum as an authentic sample of calcium d-glycerate (MacGregor, unpublished). On the basis of these tests, the acid was identified as glyceric acid.

TABLE 2

The organic acids of red clover (mequiv/100 g dry matter) determined by silica gel chromatography, after two different methods of extraction

Peak No.	Methods of extraction: Dilute acid extraction (mequiv/100 g)	Methods of extraction: Alcohol extraction followed by water extraction (mequiv/100 g)	Identification
1	trace	0.00	Unidentified
2	trace	trace	Unidentified
3	trace	trace	Unidentified
4	trace	0.00	Unidentified
5	trace	0.00	Unidentified
6	5.44	1.31	Acetic acid
7	7.20	2.29	Fumaric acid
8	trace	0.00	Unidentified
9	0.84	1.04	Succinic acid
10	11.37	11.13	Malonic acid, trace of lactic acid, trace of 2 unidentified acids
11	1.45	0.90	Oxalic acid
12	trace	trace	Glycolic acid, trace of 2 unidentified acids
13	trace	0.0	Unidentified
14	20.51	21.00	Malic acid
15	7.05	5.66	Citric acid
16	34.00	34.77	Glyceric acid, trace of 1 unidentified acid
Total anions		153.8	
Acids determined	89.6	68.8	
Acids extracted	100.5		

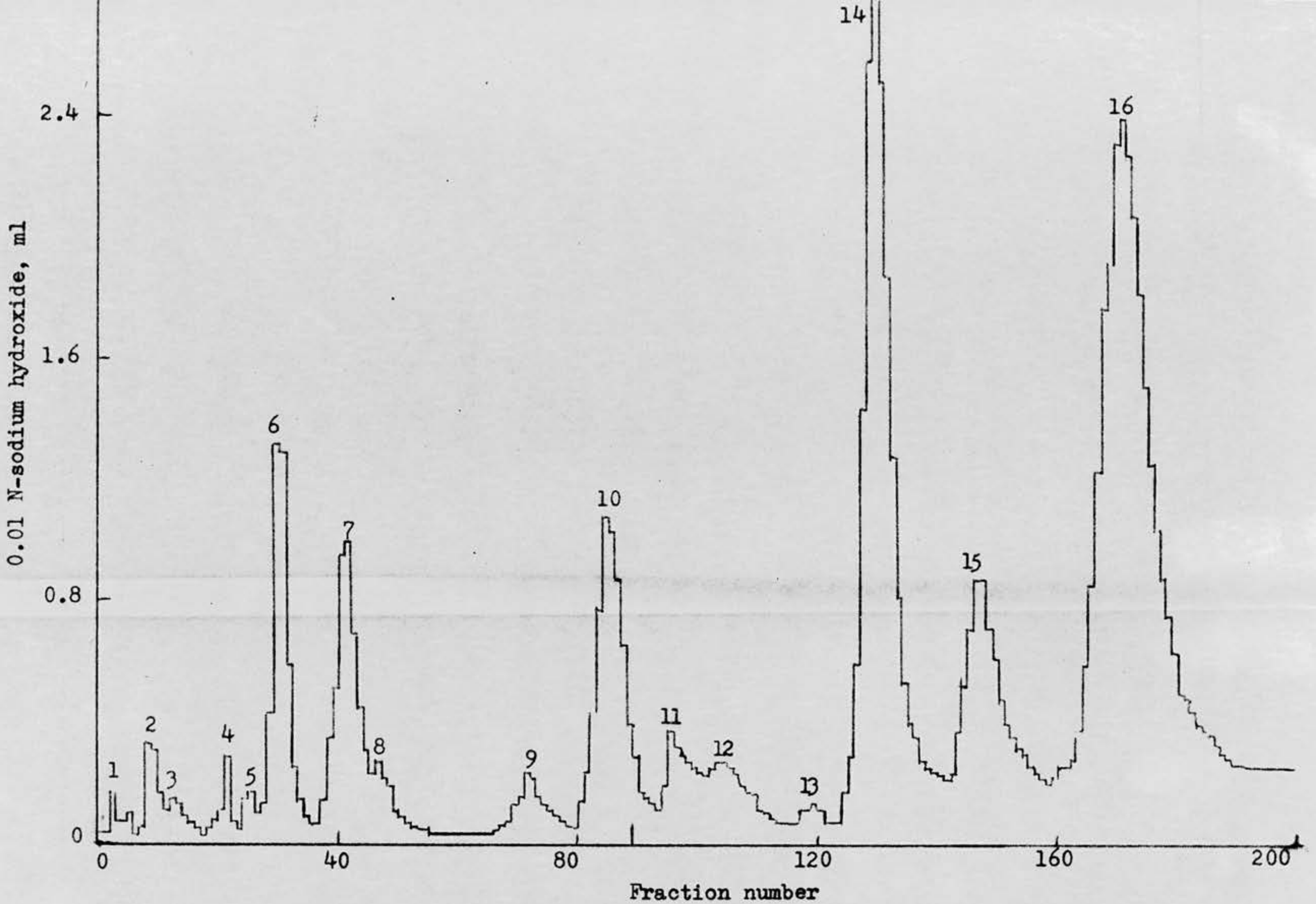


FIGURE 4. Histogram of distribution of acids from red clover using the acid extraction procedure (for key to peak numbers see Table 2).
Elution: up to fraction 88, gradient of benzene → 20% butanol in benzene beyond fraction 88, gradient of 20% t-amyl alcohol in chloroform → 60% t-amyl alcohol in chloroform.

The next acid in order of magnitude was malic acid. Malonic, fumaric, citric and acetic acids were important components. Oxalic, glycolic and lactic acids also appeared. Traces of other acids (less than 0.4 me/100 g) were also detected, but not identified. Quantities present were too small for further tests and for accurate titration.

The two methods of extraction and purification yielded similar results for the three major acids. However, results were not similar for citric, oxalic and fumaric acids. The acid extraction showed a superiority for the determination of those acids. Many compounds present as traces in the acid extraction procedure were completely absent in the alcohol-followed-by-water extraction. Acetic acid was present in larger quantities in the acid extracted sample.

In addition to the silver nitrate reagent (Brown, 1951), useful for the identification of lactic, glycolic and glyceric acids, and the Cartwright and Roberts (1955) reagent, for glyceric acid, the other spray reagents used were: Potassium ferrocyanide, followed by ferric ammonium sulfate (Martin, 1955) for the detection of fumaric, succinic and oxalic acids; diazotized p-nitroaniline followed by a sodium carbonate spray (Swain, 1953) for the identification of malonic acid (Wall _et al_., 1961); p-dimethylaminobenzaldehyde in acetic anhydride (Smith, 1958) for detection of citric acid. In addition, the chromatograms were examined under ultraviolet light before spraying to detect absorption by aromatic acids and unsaturated acids such as fumaric and aconitic acids. The semicarbazide spray of

Magasanik and Umbarger (1950) was also used to test for the possible presence of oxo-acids. Details of the preparation of the reagents and limits of usefulness of these tests are given in Appendix I.

Spot tests (Feigl, 1960) were also used for the identification of acetic, malic and citric acids.

2. Application to silages made from red clover

In the experiment just reported, it was found that the acid extraction method was more efficient for the extraction of organic acids than the usual alcohol extraction, even when the latter was improved by successive aqueous extractions. However, the increase in acetic acid in the acid extraction was unexpected. In this experiment, the acetic acid content was determined after extraction by the two methods using red clover and red clover silages as test materials. The content of individual organic acids was also determined.

Materials and Methods -

Red clover (Trifolium pratense) was cut on two consecutive days and ensiled after wilting for 24 hours. The first cutting (August 13, 1962) was from the Langhill farm and was wilted under good weather conditions. The second cutting (August 14, 1962) was from Boghall and was subjected to rain during most of the wilting period. Two silos were filled with each crop and an inoculum of Pediococcus acidilactici was added in pressure spray to one of the silos during filling.

The silos used in this experiment have been described previously (McDonald et al., 1960). They had a capacity of

more than 500 kilograms of herbage.

The organic acids were determined as described (p. 41-50) after extraction with dilute mineral acid (p. 56). Separate extractions with alcohol and water were also used for the determination of acetic acid only (p. 55).

Dry matter contents of silages were determined by the toluene distillation technique of Dewar and McDonald (1961).

Other analytical techniques used have been described by McDonald *et al*. (1960).

Results -

The organic acid composition of the fresh clover and of the inoculated and control silages is shown in Table 3. The organic acid content of the crop when ensiled after a period of wilting of 24 hours was generally higher for the crop cut on August 14 than for the crop cut on August 13. Malic and glyceric acid contents were the only exceptions. The effect of inoculation was not clear, but a few observations can still be made. Although the butyric and propionic acid contents were low in all silages, they were generally lower in the inoculated silages. The reverse was true for lactic and formic acids. The major acids, namely malic, citric and glyceric acids, virtually disappeared during fermentation, although some sparing of malic and citric acids was observed in one of the control silages.

Again, the extraction with dilute mineral acid resulted in an overestimate of acetic acid concentration in one of the fresh clover samples. For silage, the two methods of extraction yielded similar results.

TABLE 3

Organic acid content of wilted red clover and of red clover silages with or without inoculation with Pediococcus acidilactici (m.equiv/100 g D.M.)

	Red Clover					
	as ensiled		as removed from silos			
Acid	Cut Aug. 13	Cut Aug. 14	Control	Inoculated	Control	Inoculated
	A and B	C and D	A	B	C	D
Valeric	nil	nil	0.08	0.14	0.09	0.06
Butyric	nil	nil	1.38	0.14	0.95	0.22
Propionic	nil	nil	0.65	0.44	0.63	0.48
Acetic*	0.51	6.98	28.77	36.86	47.56	44.59
Acetic	0.49	8.31	27.40	35.21	48.22	46.00
Formic	nil	nil	1.41	2.00	3.29	6.64
Fumaric	0.32	3.43	nil	trace	nil	trace
Succinic	0.90	2.21	1.71	2.47	3.60	3.09
Aconitic	1.02	3.54	nil	nil	3.30	nil
Malonic	7.51	14.58	N.D.**	N.D.**	N.D.**	N.D.**
Lactic	nil	trace	100.10	118.27	86.02	88.50
Oxalic	1.75	3.04	1.49	1.28	1.81	1.03
Malic	16.34	17.19	0.09	0.18	4.23	0.30
Citric	8.91	18.69	0.92	0.78	2.44	0.12
Glyceric	17.83	21.80	1.45	1.87	0.30	0.13
TOTAL	54.26	82.76	138.06	169.77	155.05	145.16

*Acetic acid after extraction with 80 per cent ethyl alcohol and then water.

**Malonic acid was present but was not separated from lactic acid.

The identity of the acids was verified by paper chromatography and various spray reagents as described previously and by specific tests. Besides the acids shown in Table 3, the following acids were shown to be present: glycolic and cis-aconitic acids appeared in the oxalic acid peak; glycolic acid was not found in the silages, but traces of cis-aconitic were still present. Shikimic acid appeared in small quantity in the glyceric acid peak, both in the fresh clover and in the silages. Four unidentified acids were present in red clover in the position of elution of propionic acid. Those were probably aromatic acids. They were not detected in silages. Formic acid was not present in the fresh clover.

Quinic acid would not be eluted from the silica gel columns used in this experiment.

The chemical composition of the fresh clover and of the silages as well as the dry matter losses in the silages are shown in Table 4.

The data show that the wilting treatment was not equally successful in the two crops of clover. In one case, the dry matter content was increased to over 22 per cent while in the second crop (C and D), the dry matter content at ensiling was only 14 per cent. This was reflected in the effluent losses of the silages. About 10 per cent of the dry matter was lost from silos C and D while only 1 per cent was lost from silos A and B. The two crops ensiled were of different composition. Clovers A and B had a lower crude protein content but higher crude fibre, sugars (water soluble) and cellulose than B and D.

TABLE 4

Composition of clovers and silages (per cent of dry matter) and dry matter losses in the various ensilages

	Clover (A and B)	Clover (C and D)	Silage A	Silage B	Silage C	Silage D
Dry matter	22.45	14.19	21.52	21.56	17.76	17.73
Crude Protein	18.65	21.22	19.71	19.01	21.86	21.45
Crude Fibre	23.99	18.51	26.59	25.46	21.02	21.10
Sugars	11.26	6.00	1.40	1.15	0.38	0.35
Fructosans	0.96	1.19	0.11	0.10	0.11	0.12
Cellulose	27.75	24.24	29.74	27.83	27.42	26.78
pH	6.1	6.0	4.04	4.06	3.98	4.15
D.M. losses (total)			7.53	8.08	13.29	15.69
In effluent			0.82	1.12	9.55	10.80
Other			6.71	6.96	3.74	4.89

Some fructosans (about 1 per cent) were found in both crops, probably arising from the contamination of the clover with ryegrass.

Fermentation losses were higher in the crop ensiled at the higher dry matter content. There was a tendency for increased effluent losses and fermentation losses in the inoculated silages. All silages were of good quality with pH values ranging from 3.98 to 4.15.

C. MEASUREMENT OF ^{14}C-ACTIVITY IN ORGANIC ACIDS

The measurement of radioactivity in samples labelled with ^{14}C isotopes has been done routinely with end-window Geiger-Muller tubes (Wager, 1963) or gas-flow Geiger-Muller tubes (Weinstein and Laurencot, 1958). The end-window G.M. tube has a low counting efficiency: the gas-flow G.M. tube permits counting with as high an efficiency as liquid scintillation counting. However, if samples are easy to prepare for G.M. counting, special precautions have to be taken to prevent exchange of radioactive material with atmospheric CO_2 in the case of ^{14}C-carbonates and volatile acids.

In liquid scintillation counting, the main factors conducive to high counting efficiency are, besides the choice of a proper apparatus and the use of proper settings, the choice of an optimum combination of solvents, scintillator and wavelength shifter, the absence of quenching substances in the scintillator solution or the sample, and the use of optimum ratio of scintillator solution and sample (Guinn, 1958).

A liquid scintillator is composed of three essential ingredients: a solvent, a scintillator and a wavelength shifter. The alkylbenzenes such as toluene and xylene are the best liquid scintillation solvents (Kallmann and Furst, 1959). Benzene is also a good solvent (Guinn, 1958). In view of the insolubility of organic acids in benzene or other aromatic hydrocarbons a secondary solvent has to be used to dissolve them. This secondary solvent should not act as a quencher. Ethers and esters reduce the counting efficiency by about 25 per cent when mixed in equal volume with the scintillator solution (Guinn, 1958). With alcohols, the efficiency is reduced further. Organic acids are themselves quenching agents, and consequently a phenomenon of self-absorption could be expected when organic acids at a high concentration are being counted. Chlorinated compounds are severe quenchers. For example, 1 ml of chloroform in 25 ml of liquid scintillator lowers the efficiency of counting by 42 per cent (Guinn, 1958). In the present study the fact that a benzene-based solvent was used with increasing concentrations of butanol (a mixture of a good solvent with a moderate quencher in low concentration) opened the possibility of counting ^{14}C-organic acids as they were eluted from the column, without further preparation.

1. Calibration of the counting apparatus

A standard solution was prepared by dissolving 98.0 milligrams of ^{14}C-n-hexadecane of known activity (1.07 microcurie/g) in 50 ml of NE 213 scintillator. This scintillator solution is based on xylene, contains naphthalene, activators and 1,4-bis-

(2-(5-(phenyloxazolyl))-benzene) as spectrum shifter and is supplied by Nuclear Enterprises Ltd.

The counting apparatus was an Ekco Scintillation Counter, Type N664A, coupled with an Ekco Automatic Scaler, Type N530G (Ekco Electronics Ltd.).

Results -

A volume of 0.5 ml of the standard solution was mixed with 5 ml of NE213 scintillator in a scintillation vial and first counted at the settings recommended by the manufacturers for the photomultiplier used (Technical Instructions of Ekco Electronic Equipment for Scintillation Counter Type N664A). Those are: amplifier gain of 250, a 15 volts discriminator bias and a photomultiplier voltage of 1090V. Under those conditions, a reading of 16.1 counts per second was obtained. A background reading of 0.41 c/s was obtained when 5 ml of NE213 scintillator solution was counted. Since the standard counted contained 1.049 millimicrocuries, this was an efficiency of 40.5 per cent. The manufacturers claimed a 60 per cent efficiency with a background of 0.42 c/s under those conditions.

It was, therefore, evident that a new calibration of the apparatus was necessary.

According to Loevinger and Berman (1951), the optimum working conditions of a counter are obtained when the sensitivity is increased until R_s^2/R_b, the ratio of the square of the net sample rate to the background rate is a maximum. The sensitivity so obtained is independent of the sample used and is the highest which is advantageous for all sample strengths.

The gain was set at 1000 and the discriminator bias setting was varied by increments of 5 volts over each photomultiplier voltage tested. The photomultiplier voltage was tested by 50 volt increments. From the results the ratio R_s^2/R_b was calculated. Part of the results were plotted in Figure 5. It is apparent that the sensitivity was at a maximum at a discriminator bias of 15 volts, and at a photomultiplier voltage of about 1000 volts. The discriminator bias was, therefore, set at 15 volts and the photomultiplier voltage (H.V.) was varied by 10 volt increments in the vicinity of 1000 (Figure 6). The effect of varying amplifier gain is also shown in the same Figure 6.

From those results, it was decided to use the following settings: discriminator bias, 15 volts; photomultiplier voltage (H.V.), 1000 volts; amplifier gain, 1000. At those settings, the efficiency was 66.6 per cent and the background 0.375 counts/second.

It is evident from Figures 5 and 6 that other instrument settings would be almost equally satisfactory for counting. For example, a decrease of the amplifier gain to 250 would make it possible to use a photomultiplier voltage setting of 1200 volts and the efficiency would be only slightly decreased. However, it is always desirable to count at as low a voltage as possible in order to obtain a longer life of the photomultiplier tube. To each discriminator bias setting corresponds an optimum high voltage setting. The settings selected were such that a slight drift of the high voltage or of the discriminator bias would cause a minimum change of the counting rate. It may be

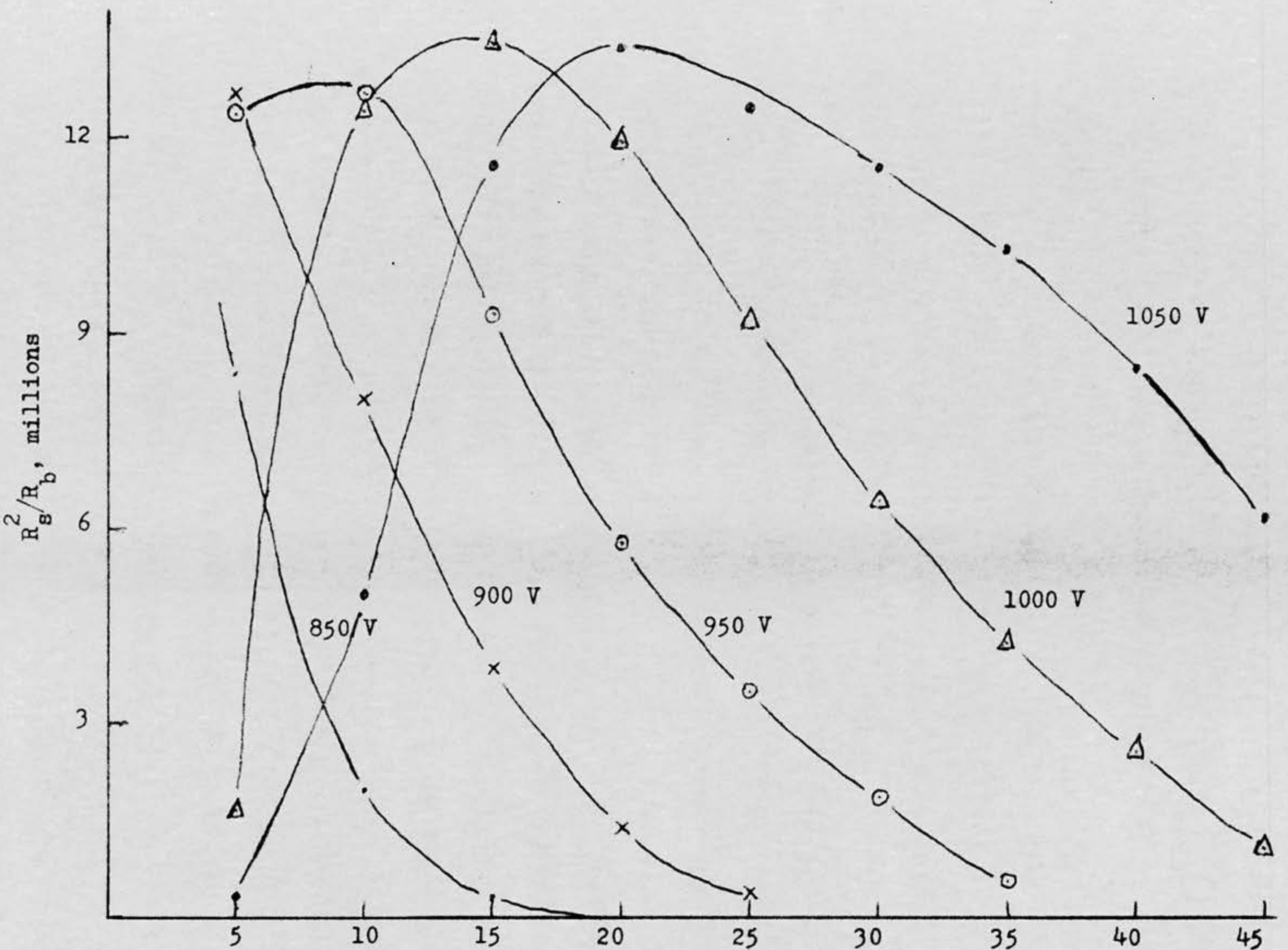


FIGURE 5. The effect of varying discriminator bias and high voltage on counting sensitivity. Counting sensitivity is maximum when R_s^2/R_b, the ratio of the square of the net sample counting rate over the counting rate of background is at a maximum.

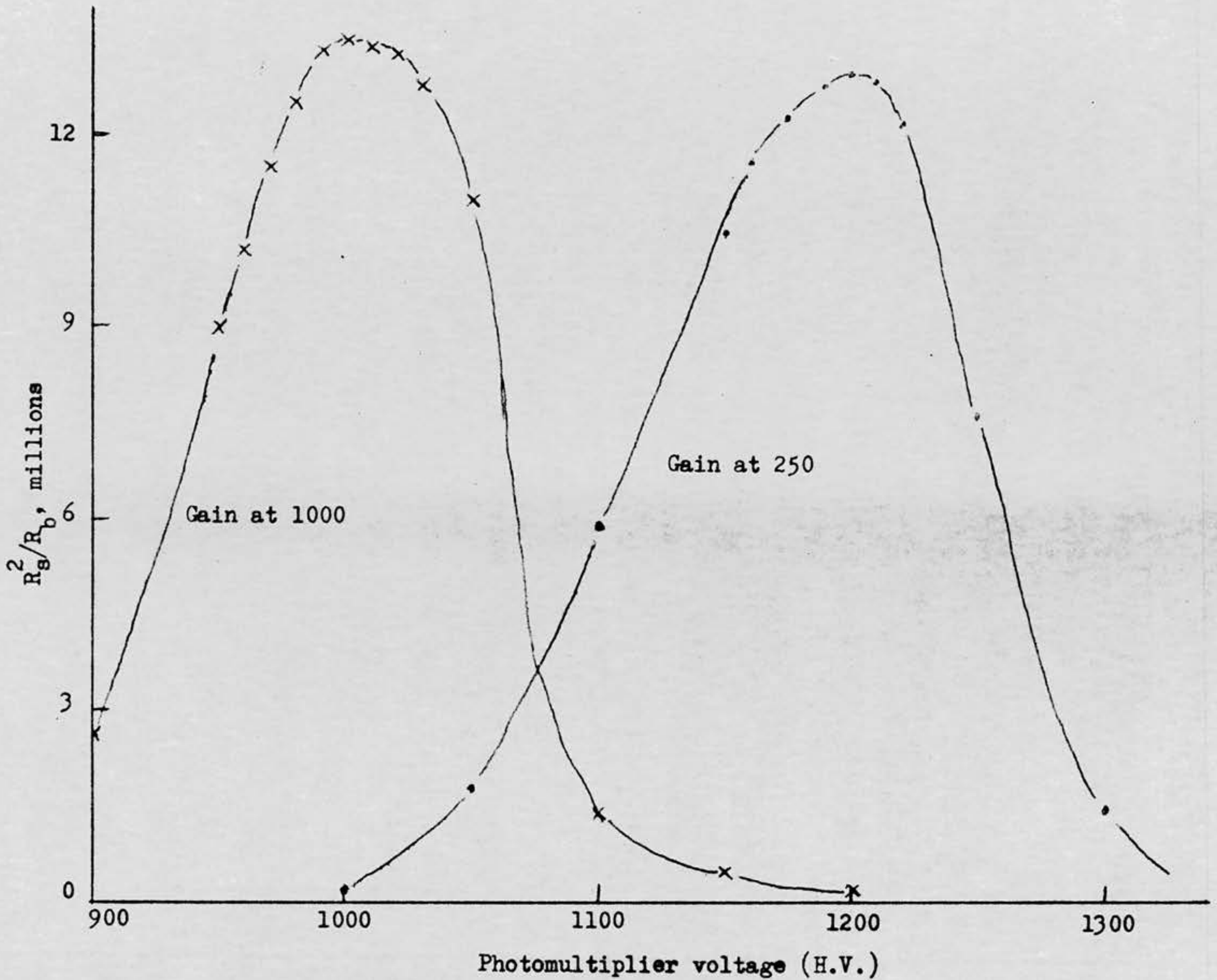


FIGURE 6. The effect of gain and high voltage on counting sensitivity. (Discriminator bias set at 15 volts.)

noted that the settings maximizing the R_s^2/R_b value does not correspond to the maximum counting efficiency. For example, at a photomultiplier voltage of 1000 volts, discriminator bias of 10 volts and amplifier gain at 1000, an efficiency of counting of over 75 per cent is attainable with samples containing high quantities of labelled material. However, excessive time would be required for counting weak samples with precision.

2. The efficiency of ^{14}C-counting in organic acids eluted from a silica gel column

A silica gel column was prepared as described previously and a standard mixture of organic acids such as used for calibration (Table 1) was introduced by the usual method. Gradient I (benzene-butanol) was used for the elution. Alternate fractions from the column were titrated to ascertain the position of elution of the acids. Fractions corresponding to the top of each peak were selected for measurement of the efficiency of counting. The samples were degassed by bubbling nitrogen gas through for 30 seconds. An accurately measured volume of 0.5 ml of the selected fractions was taken and blended with volumes of 2.0 and 5.0 ml of NE213 scintillator. The scintillator was deoxygenated by bubbling with nitrogen gas for 5 minutes immediately before use. To each fraction was added a standard number of counts from a ^{14}C source of n-hexadecance in benzene. Samples were similarly prepared but without a ^{14}C source for background counting. Gradient II (chloroform-t-amyl alcohol) was used for the second part of the elution. Samples for counting were prepared in a similar way as for Gradient I.

Results -

The effect of adding 0.5 ml of effluent from the chromatographic column on counting efficiency and background count is shown in Table 5, for selected samples corresponding to the top fraction of the peak of each of the major silage acids. The effect of scintillator volume on counting efficiency and background is also shown.

TABLE 5

Effect of volume of NE213 scintillator and of increasing butanol concentration in the effluent from a silica gel column on counting efficiency and background count

Elution peak, acid	Fraction No.	Background count per second vol. of scintillator		Counting efficiency scintillator volume	
		2 ml	5 ml	2 ml	5 ml
Valeric	5	0.350	0.363	67.2	66.7
Butyric	11	0.343	0.383	67.6	67.0
Propionic	18	0.341	0.366	67.7	66.3
Acetic	31	0.358	0.377	66.0	66.6
Formic	43	0.349	0.377	66.9	66.7
Succinic	73	0.356	0.365	66.5	66.0
Lactic	89	0.361	0.375	67.9	65.2
Average		0.351	0.372	67.11	66.36
L.S.D.		0.009		0.75	

The smaller volume of scintillator gave significantly smaller background counting rate and higher efficiency. It was evident that the solvent from the first stage of the elution (Gradient I) could be used over the whole range for determining

the radioactivity in organic acids. With the t-amyl alcohol-chloroform gradient (figures not shown), the efficiency of counting was decreased by about 95 per cent, on account of quenching due to chloroform.

The following procedure was then tested: 1-ml aliquots of fractions from the malic and citric acid peaks were dried in a water bath at 45°C while a stream of nitrogen continuously agitated the surface of the liquid. The residue was dissolved in 1.0 ml of a 0.5 M solution of Primene in methanol (Radin and Fried, 1958) and 0.5 ml was blended with 2 ml of NE213 scintillator and counted. When a standard number of counts was added in 0.5 ml of the scintillator, the counting efficiency of the malic acid peak, was similar to that of the benzene-butanol fractions. More difficulties were experienced with fractions from the citric acid peak. Recoveries for added counts were somewhat erratic and varied from 80 to 90 per cent of the usual. The variation could not be satisfactorily explained. It may have been due to some residue from the t-amyl alcohol-chloroform solvent, to the higher blank value due to the elution of some sulfuric acid or to some other factor. The variation observed in recoveries of activity in the citric acid peak suggested the necessity of using an internal standard for counting this acid (Hayes, 1956).

There was no evidence of any quenching caused by either butanol or the acid being counted. This could be expected since the quantity of butanol present in 0.5 ml of eluate was never greater than 0.07 ml; at the time of lactic acid elution, the

butanol concentration is about 13 per cent. The quantity of acid in fractions from any peak is always lower than 0.5 milli-equivalents; the maximum quantity to be expected in a sample is, therefore, 0.01 gram, a negligible quantity.

D. APPLICATION OF THE LIQUID SCINTILLATION TECHNIQUE TO THE COUNTING OF ORGANIC ACIDS FROM SILAGE AFTER SEPARATION BY SILICA GEL CHROMATOGRAPHY

1. The fate of ^{14}C-sucrose in ensilage

The liquid scintillation counting technique was tested on an actual silage sample supplied by Professor W. O. Brown of Queens University, Belfast. The conditions of ensiling and sampling have been described (Brown, 1962) but they are repeated here for convenience. A lot of 10 kilograms of fresh young grass was chopped to 1-inch lengths and compressed by hand into a silo. Two hundred ml of a solution of equal parts by volume of molasses and water were thoroughly mixed with the grass before compaction into the silos, representing an addition of 113 g of molasses. The molasses solution added contained 75 microcuries of generally labelled ^{14}C-sucrose (specific activity 20 mc/mM). The contents of the silo were covered by placing a weight of 112 lb on a sheet of hardboard which covered the surface of the grass completely. The silo was kept in a room at 30°C for 4 weeks.

The sample was extracted for organic acid determination by shaking 200 g of fresh silage with 400 ml of water for 2 hours. After filtration, a 50-ml aliquot was titrated to pH 8.5, concentrated in a rotary evaporator at 45°C, and made to 5 ml. A 0.4-ml sample was introduced in a column prepared as in previous experiments, being preceded and followed by 0.1 ml

10N H_2SO_4. Gradients I and II were used to elute the acids. Fractions of about 4.0 ml were collected. Titrations and samplings for radioactivity counting were performed without delay in order to avoid loss of volatile acids. Alternate fractions were first titrated in order to ascertain the position of the peak of individual acids. A minimum of two fractions from each peak were counted. They were deoxygenated with nitrogen gas. An accurately measured volume of 0.5 ml of eluate was taken before titration and mixed with 2 ml of NE213 liquid scintillator (deoxygenated). After preparation each vial was kept in the dark until counted and 15 minutes more of dark adaptation were allowed after the sample was placed in the scintillation counter. Each sample was counted until the standard deviation was reduced to 1 per cent, i.e., a minimum of 10,000 counts. Background was counted using fractions eluted between two peaks.

For fractions eluted with Gradient II (t-amyl alcohol chloroform), 1.0 ml of the fraction was evaporated by keeping test tubes in a water-bath at 45°C and blowing a stream of nitrogen on the surface of the liquid. The acid was then dissolved in 1.0 ml of a 0.5 M methanolic solution of Primene. A volume of 0.5 ml was blended with 2 ml of NE213 scintillator for counting. It was found, however, that 1 ml of the solution was not sufficient for titrating with precision the peaks of malic and citric acid, since those acids disappear to a large extent during ensilage. On the other hand, the peaks being flat and broad, it was possible to estimate with accuracy the

acid content of a fraction by interpolating between the fraction previous to the one to be counted and the one following it. It was, therefore, possible to evaporate a whole fraction, to dissolve the acid in 1 ml of Primene and to count 0.5 ml of the resulting solution. This procedure was adopted.

Titrations of the fractions used for radioactivity measurement were performed using 1.00 ml of the fractions or the whole fractions depending on the acid concentration in the fractions. When 1 ml was sufficient to give a titration value of 1 ml or more, using 0.005 N sodium hydroxide in 80 per cent ethanol, the titer was used in conjunction with the counts for calculating the specific activity. The residue of the fractions was also titrated for calculation of the total acidity in the peak. When the titer of a 1-ml aliquot was less than 1 ml, the residue after sampling for radioactivity measurement was accurately measured and titrated. This titer was then used for specific activity calculations.

The total activity of individual acids was calculated from the average specific activity of the two fractions counted and the total titer of the acid.

Results -

The concentration of organic acids in the sample investigated is shown in Table 6, together with the results of titrations and radioactivity measurements in fractions utilized for the calculation of total activity in the specified acids. Acetic acid was the main acid found, lactic acid coming second.

TABLE 6

Partition of ^{14}C-activity in organic acids extracted from a silage preserved with ^{14}C-sucrose and molasses (concentration and activity expressed on a dry matter basis)

Acid	Concentration		Fraction counted		Specific activity		Total activity	
	m.equiv/100 g	g/100 g	d/s/ml	µ.equiv/ml	d/s/µ.equiv	Average	d/s/g	µmc/g
Butyric	9.14	0.81	0.669 0.465	0.818 0.565	0.818 0.822	0.820	74.95	2.026
Acetic	69.85	4.19	1.893 1.335	5.71 4.33	0.332 0.308	0.320	220.64	5.965
Succinic	9.36	0.46	nil* trace**	0.402 0.986	- -	-		
Lactic	46.04	4.15	0.639 3.410	1.68 10.06	0.380 0.339	0.359	165.28	4.467
Malic	11.12	0.75	nil nil	1.05 1.95	-	-		
Citric	14.04	0.90	0.667 0.746	1.64 0.703	0.407 1.061	0.734	nil	nil
Unidentified	7.33	-	nil nil	0.401 0.606				

*Nil indicates that the ratio of the sample count rate plus background count rate to the background count rate was lower than 1.1.

**Trace indicates that the ratio was between 1.1 and 1.3.

A small quantity of butyric, succinic, malic and citric acids and an unidentified acid were also present. In general, each fraction used for counting contained enough acid for a titration value of 1.0 ml of 0.005 N sodium hydroxide in either 1 ml of the fraction or the measured residue after sampling for ^{14}C measurement. It was therefore possible to carry three significant figures in the titration values. It would have been preferable to use the whole residue after sampling for ^{14}C counting for obtaining the acid concentration in the fraction. This was not convenient since the fraction size varied from one tube to the other.

The count rate in all fractions was low and it was necessary to count for excessive periods of time in order to obtain any precision. Most of the radioactivity was in acetic, lactic and butyric acids. Traces were also present in succinic acid. The specific activity calculated from two distinct fractions in each acid peak was in close agreement for all acids except citric acid. It is apparent that there was no radioactivity in citric acid and that the counts recorded arose from the ^{14}C sucrose added or from one of its decomposition products. Sugars can be eluted slowly from a silica gel column toward the end of an elution when the alcohol concentration in the eluant is high.

A relatively high specific activity was recorded for butyric acid compared with lactic and acetic acids. A total activity of 5.26 micromillicuries per gram of dry matter was accounted for by organic acids.

2. Other applications of the technique - elution coincidence

In the analyses of ^{14}C-tagged organic acids eluted from a silica gel column, it would be possible to pool appropriate fractions and to determine the acid concentration and the activity therein by only one titration and one counting. Above, we have titrated each fraction independently and determined the activity on two of those fractions. This was considered a minimum. Other compounds may be eluted in approximately the same position as the acid being measured, as was the case in the citric acid peak, and the activity attributed to the acid may arise from those compounds. When two fractions are used as above, a wide difference in the specific activity calculated is an indication of contamination. When the specific activity of the acids is high enough, or when a scintillation counter with an automatic sample changer is available, it is possible to count the activity in all fractions from a peak. Any contamination is readily detected by calculation of specific activity or by plotting on a same graph the titration values and the radioactivity of the fractions. The technique, known as elution coincidence, was used by Benson et al. (1952) and Daus et al. (1952). This technique was used in the course of experiments to be reported in a later part of this thesis. An illustration is presented below.

Results -

Figure 7 shows the elution curve of succinic and lactic acids. A plot of the disintegration rate of the fractions (measured on a 0.5 ml aliquot) was superimposed on the same

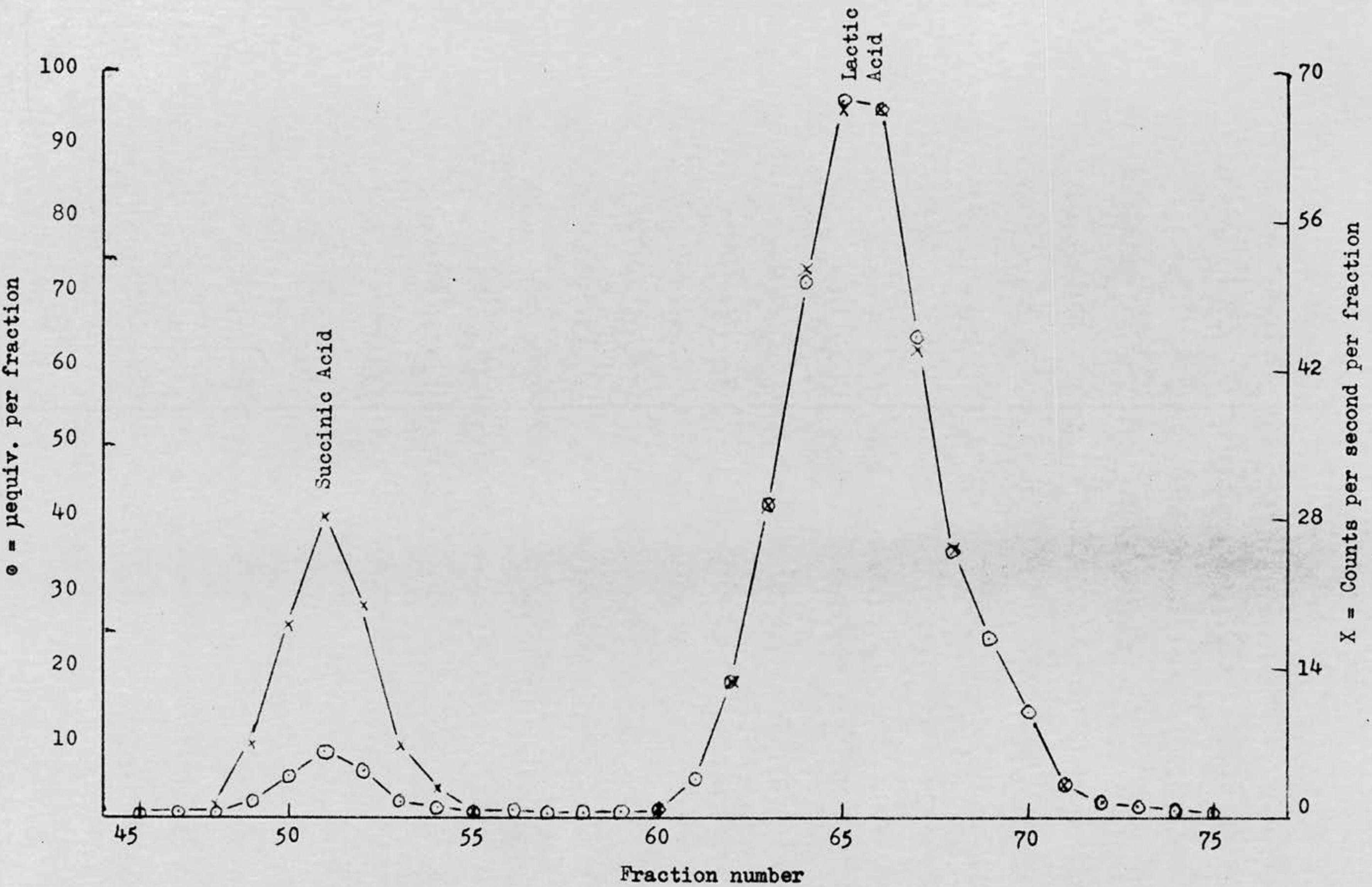


FIGURE 7. Coincidence elution curve showing the separation of succinic and lactic acids in a silage sample. The activity curve and the concentration curve of lactic acid coincide almost exactly.

graph. It is seen that at the scale used, titration values and disintegration rate coincide almost exactly for lactic acid. By changing one of the scales of measurement for succinic acid, the same coincidence could be shown to apply to succinic acid.

The specific activity of the two acids, calculated from each fraction, is shown below (Table 7).

TABLE 7

The specific activity of succinic and lactic acid determined independently from several fractions in their respective elution peak

	Specific activity		
Fraction No.	Succinic acid c/s/μ-equiv.	Fraction No.	Lactic acid c/s/μ-equiv.
49	6.304	62	1.394
50	6.384	63	1.358
51	6.312	64	1.440
52	6.440	65	1.378
53	6.330	66	1.388
		67	1.360
		68	1.404
Mean ± S.D.	6.354 ± 0.0573		1.389 ± 0.0282

The precision of the estimate of specific activity is dependent on both the radioactivity counting and the titration. The error in the two measurements is additive. From the equation of the standard curve, it is known that in a large series of

measurements 68 per cent of the readings will differ from the mean by less than one standard deviation, and 95 per cent will differ by less than twice the standard deviation. In the case of succinic acid, this means that the specific activity calculated from a single fraction will have 95 chances out of 100 of being within 1.8 per cent of the true specific activity. The specific activity of lactic acid was estimated with slightly less precision (within 4 per cent). From the above it can be concluded that, provided the sample is counted with sufficient accuracy and provided the titration value is high enough (over 1.00 ml), one measurement of the specific activity from each peak would be sufficient. The main purpose of taking two or more measurements in a single peak is to guard against contamination.

V. DISCUSSION

1. Methods of extraction and purification

In the separation of organic acids from plant materials, the most frequently employed method has been the extraction with 70 or 80 per cent ethanol, especially when a preliminary fractionation of the extract into organic acids, amino-acids and neutral materials was desired (Hulme, 1951; Weinstein and Laurencot, 1958; Wager and Isherwood, 1961). The use of inorganic acid is made difficult by the necessity of removing the swamping acid which would interfere with subsequent chromatography. However, Fauconneau (1958) fractionating his extracts on Dowex-1 anion exchangers, has found it possible to extract the acids from plant material with dilute hydrochloric or perchloric acid, removing the inorganic acid by precipitation as the potassium perchlorate at 0° or taking the extract to dryness repeatedly. The removal of perchloric acid by precipitation was not satisfactory for silica gel chromatography since the traces of perchloric acid left were eluted with malic acid and subsequent fractions. Hydrochloric acid volatilization was not satisfactory either because of the resulting loss of volatile acids. Fauconneau (1958) has also shown that an aqueous extraction after the alcoholic extraction was a more efficient extraction method than with alcohol alone. The method of extraction with dilute sulphuric acid presented above has been evolved from Isherwood's (1946) extraction method for organic acids in fruits. A similar method has been used for silage

acids by Wiseman and Irvin (1957) and by Wilson and Tilley (1964). For ease of concentration it was necessary to keep the volume of dilute sulphuric acid to a minimum. On the other hand, too concentrated a sulphuric acid solution might result in the production of artifacts. Sulphuric acid at a concentration of 0.3 N has been used in the present study since there is only a slight excess after salt splitting in the plant material. For use in plants containing less organic acids than clover, such as the grasses, a 0.2 N solution might be sufficient. In silage, 0.6 N-sulphuric acid would be required to reduce the pH to 2.0.

In confirmation of the work of Fauconneau (1958) and Roux and Lesaint (1959) we have found that ethanol and water extraction do not extract all the organic acids from plant material. The insoluble calcium citrate and calcium oxalate were not completely extracted by ethanol even when followed by aqueous extractions. This would suggest that figures reported in the literature for those two acids are frequently an under-estimate of the true value. Many acids, which were present in trace amounts with the dilute acid extraction, were completely absent in the alcohol extract. It is likely that they were retained on the ion exchangers. Similar retention of aromatic acids has been noted by Wall _et al_. (1961). Fumaric acid was probably also retained on the ion exchangers (Carrol, 1955). It is difficult to explain why acetic acid was present in smaller quantities in the alcohol + water extracts. However, a difference was found only for the samples of fresh grass. The

two methods of extraction yielded similar results for silage samples. An artifact is probably produced when fresh grass is extracted with mineral acid from some compound which is absent in ensilage. Losses were also possible through volatilization, even if all precautions were taken to avoid that.

It can therefore be concluded that the method of extracting acids from plant material with 0.3 N-sulphuric acid was clearly satisfactory and can replace with advantage the usual alcohol extraction. The use of silica gel for purification had an added advantage. It separated the organic acids from inorganic anions, which, when present, are partly eluted with citric acid and other acids eluted after it. In our sample, the difference between the total anions as measured by titration of an aliquot of the eluate from the Amberlite IR120 resin and the organic anions eluted from the silica gel column was 53.3 mequiv./100 g. (Table III). This would indicate that about one-third of the anions were inorganic anions.

It was also found that the purification of the extract on the silica gel column was much easier and more convenient than on ion-exchange resins. The use of benzene/butanol as the eluant instead of the usual chloroform/butanol (Isherwood, 1946; Wager and Isherwood, 1961) was found more convenient, as removal of the water layer and washings after titration can be done without removal of the solvent layer.

2. Chromatographic separations on silica gel

The technique employed resembled that used by Wall *et al.* (1961), but many improvements were made on the gradient elution

apparatus utilized and improved resolution of some acids was obtained.

Three main changes were made in the design of the apparatus. The rubber stopper joining the mixing chamber to the column was replaced by a ground glass joint. The rubber stopper was gradually decomposed by vapors of benzene and had to be replaced often. In addition, the fact of having a column with an enlarged top permitted easier filling of the column. The rigidity of the glass apparatus was a disadvantage in Wall's system, and breaking of the side arm of the mixing chamber could result. The inclusion of a flexible joint in the side-arm prevented this. One added disadvantage of Wall's apparatus was the necessity of taking it apart for emptying after each elution. This was even more inconvenient with the two gradients used in our experiments, since it meant disassembling the apparatus completely in the middle of an elution. The addition of a stopcock at the bottom of the mixing chamber permitted a greater flexibility in the use of the apparatus.

The procedure and the solvent systems utilized by Wall *et al.* (1961) permitted the separation of a very wide range of acids. However, a greater resolving power was desired in our experiments, especially because of the difficulty of separating lactic acid, particularly abundant in silage, from succinic acid. The replacement of the chloroform-based eluant by a benzene-based eluant (Neish, 1949) in the first phase of the elution provided a separation between those two acids. In addition, it was possible to separate valeric from butyric

acid and even caproic and valeric acids (Ramsey, 1963). In some cases, it may be desired to separate lactic and malonic acids. This is possible on the same column when gradient IIA is used immediately after succinic acid is eluted. Bulen et al. (1952) had to use a second column to separate lactic and zuccinic acids. Malonic acid was not tested in their experiments. Pyruvic, fumaric, glutaric and formic acids were not separated in the system used. However, since glutaric and formic acids are not usually found in forage crops, and since pyruvic acid is lost during sample preparation, this was not considered to be a serious weakness of the technique. In silage, formic acid may be present, but fumaric acid would be metabolized during fermentation. If a separation is necessary, it can be done according to Bulen et al. (1952) on a second column of silica gel.

In some techniques of continuous gradient elution, problems have been encountered for the equilibration of the eluting solvents with 0.5 N sulfuric acid. Wager and Isherwood (1961) have solved the problem by inserting, between the mixing vessel and the analytical column, an additional column containing silica gel and a layer of chloroform on which was floated a 30-cm layer of the equilibrating liquid. No such cumbersome device was necessary in our experiments. All solvent solutions were equilibrated before use. When the alcohol-rich solvent from the reservoir was mixed with the solvent poor in alcohol, aqueous droplets were formed. When the eluant was benzene-based, the droplets accumulated at the bottom of the mixing

chamber and did not interfere with the operation of the column. In the case of the chloroform-based solvents, the droplets accumulated on top of the solvent layer of the mixing chamber. For that reason it was particularly important that the level of solvent in the mixing chamber be above the side-arm. The curve in the side-arm also retained some precipitated droplets.

The continuous gradient-elution apparatus has the further advantage that it can be left unattended for long periods of time. If the reservoir empties after elution with benzene/butanol, the column does not dry immediately.

3. Rapid method of detection

The method as described is not quite rapid enough for routine use. However, for fermentation studies where the interest might be only in the volatile acids, lactic and succinic acids, the method may be shortened considerably, provided the acid concentration of the extract is high enough. The purification and concentration steps are omitted. Two ml of the properly acidified extract are mixed with 3 g of the silica gel and the resulting powder is added to the top of the column (Wilson and Tilley, 1964) over a filter paper. It is then slurried with a small quantity of benzene. A second filter paper is placed on top of the sample and the column is developed as before, omitting the t-amyl alcohol/chloroform solvent. The titration procedure is also shortened by adding to each tube before collection 1 ml of an alcoholic cresol red solution made 0.0005 N with sodium hydroxide. The appropriate fractions are pooled before titration. Under such conditions four columns can

be developed simultaneously in one day using a four-row, 240-tube fraction collector. Columns can also be re-used by scraping off the adsorbent used to add the sample.

4. Organic acids in red clover

The presence of malic, malonic, oxalic, glycollic, lactic, fumaric, succinic and acetic acids in red clover has been established. The presence of most of these acids in other forage plants had been reported previously by Fauconneau (1958, 1960), by Hulme and Richardson (1954) and by others, but to our knowledge, it is the first time they are reported in red clover. In addition to these acids, another acid was found, which is the major acid of red clover. Playne and McDonald (1966) tentatively identified the acid as glyceric acid. The identification was confirmed by the determination of the melting point and the infrared spectrum of the calcium salt of the unknown acid (MacGregor, 1965). Both determinations gave results identical with those obtained with an authentic sample of calcium D-glycerate. Evidence is accumulating of the widespread occurrence of glyceric acid in the plant world. So far the acid has been found in cress seedlings (Isherwood et al., 1953), in tobacco leaves (Palmer, 1956), in broad bean leaves (Morrison and DeKock, 1959) and in field bean leaves (Coic and Lesaint, 1960). In forage crops, Hulme and Richardson (1954) found glyceric acid (acid B in their paper) in grass but ascribed its presence to an artifact formed during passage of the extract through anion exchange resins (Richardson and Hulme, 1954). It is more likely that it was really glyceric acid since

their sample contained malonic acid, an indication of the contamination of the grass with some legumes. Glyceric acid was absent in a sample of pure ryegrass. Richardson and Hulme (1957) found that glyceric acid was a minor constituent of lucerne. Playne and McDonald (1966) found large amounts of glyceric acid in white clover. The evidence accumulated to date indicates that glyceric acid is present in many legumes, but is absent in grasses.

In addition to the above, not less than nine unidentified acids have been found, but they were not present in sufficient quantity for characterization. Quinic acid was not determined in the present work. It would not be detected in the present elution system as it would be masked by the high blank value. A more suitable method of separation for quinic acid would be chromatography on a strongly basic anion-exchanger in the acetate or formate form (Fauconneau, 1959; Richardson and Hulme, 1957). The presence of quinic acid has been reported in most agricultural plants (Fauconneau, 1959; Hulme and Richardson, 1954; Richardson and Hulme, 1957).

5. Changes in organic acid content during ensilage of wilted red clover

The two crops ensiled were of different composition and this may have been due to the origin of the crops and to the changes during wilting. The presence of some ryegrass may also have influenced one treatment more than the other. The organic acid composition of the fresh crop resembled the composition of the fresh red clover reported in Table 2. However,

the amount of glyceric acid was much lower than in the previous experiment. Citric acid was unexpectedly high in one of the samples (C and D). There are indications that the wilting treatment may have decreased the acid content of crop A and B more than that of crop C and D. A drastic reduction of the organic acid content of red clover has been shown to occur as a result of wilting for 48 hours (Playne, 1964). The fact that the wilting was unsuccessful in red clover A and B may explain the difference in acid content between these two crops. Both crops contained aconitic in both the cis- and trans-form. The trans-aconitic probably arose from cis-aconitic during chromatography. Such a transformation has been demonstrated by Coic *et al*. (1961).

All silages were of good quality with pH values ranging from 3.98 to 4.15 and lactic acid ranging from 7.7 per cent to 10.2 per cent of the dry matter. Valeric, butyric and propionic acids were very low, at 0.1 per cent or less of the dry matter. That the silages were all of good quality is somewhat surprising, since legumes ensiled without wilting or preservatives are generally considered difficult to preserve. Although wilted, the two crops ensiled still had a high moisture content. In other experiments in Edinburgh (McDonald *et al*., 1965) using fresh or wilted clover crops, silages of equally satisfactory quality were produced. It appears that red clover contains adequate sources of fermentable materials. In this experiment the fresh herbage contained 11.3 per cent and 6.0 per cent sugar and the lower quantity was sufficient to produce good

silage. Other factors in the fermentation medium, such as amino acid and organic acid content may compensate for the low sugar content.

The inoculum of Pediococcus acidilactici did not seem to affect the course of the fermentation to a large extent although a slight increase in lactic acid and a slight decrease in butyric acid were observed in the inoculated silages. The major acids, glyceric, citric, and malic, were almost completely metabolized during fermentation. It may be purely coincidental but the difference in citric acid content of the ensiled crops resulted in an almost equivalent difference in acetic acid content in the inoculated silages. This gives support to the findings of Phillips et al. (1956) that citric acid was converted by lactic acid bacteria to lactic and acetic acids.

Total dry matter losses were lower in the silages at the higher dry matter level. This was due to the low effluent losses in those silos. However, losses from other causes were almost double those in the other silos. Some of the difference may be explained by the oxidation and the surface spoilage taking place as a result of the lesser compaction of the drier silages.

One point brought forward in the course of these experiments and others is that it is dangerous to use column chromatography without other confirmation of the identity of the acid determined. For example, if the Wiseman and Irvin (1957) method is used for fresh grass, legumes and silage, fumaric acid may be mistaken for formic acid, malonic for lactic acid

and acids eluted early from the column for butyric or acetic acid.

6. Scintillation counting

As shown in Table 5, the benzene/butanol solvent system was satisfactory for direct addition to the scintillator. When counting of fractions was performed on samples containing radioactivity, and an internal standard was added to verify efficiency, quantitative recoveries were obtained. This has also been verified with fractions from the t-amyl alcohol/chloroform system after evaporation of the solvents and addition of Primene to dissolve the acids. It was found preferable, however, to use an internal standard for counting acids eluted late in the fractionation. In confirmation of the results of Stitch (1959) and of Brown and Badman (1961) we have found that maximum counting efficiencies and minimum background counting rate are attainable at low volumes of scintillator (2 ml) with sources soluble in toluene or xylene. The efficiency obtained with benzene or with benzene/butanol for counting were similar to those reported by Stitch (1959) but were not as high as those reported by Brown and Badman (1961).

A useful technique known as coincidence elution (Daus et al., 1952; Benson et al., 1952) can be used with radioactive samples. The titration value of every fraction of a peak and the counts per fraction are plotted on a same graph. If the two curves coincide (using appropriate scales) at all points, this is evidence of absence of contamination. An illustration was

presented (Figure 8). Usually it is sufficient to take radioactivity measurements on two fractions of each peak, in order to obtain two independent measurements of the specific activity. The eluate from a chromatographic column has been used directly for scintillation counting by Satter _et al_. (1964) using the Wiseman and Irvin (1957) method. In view of the quenching by acetone, an internal standard had to be used with each sample. Only one measurement of radioactivity was taken for each acid.

7. Fate of ^{14}C-sucrose in ensilage

Brown (1962) reported that the dry matter content of a silage preserved with molasses and containing ^{14}C-sucrose as a tracer passed from 22.5 to 17.7 per cent during ensilage and that 5.5 per cent of the added radioactivity was recovered in silage effluent. The silage itself still contained 88.1 per cent of the added ^{14}C. The pH of silage was 4.95.

In the extract which we fractionated (Table 6) we found that acetic and lactic acids each accounted for 4.2 per cent of the dry matter. While the high lactate content indicated that a desirable fermentation had taken place in the initial stages of ensilage, the presence of butyric acid, together with the high pH, left some doubt on the keeping quality of this silage. It is generally considered that butyric acid is the result of a secondary fermentation of the lactate already present in the silage by anaerobic sporeformers such as the _Clostridia_. Usually, those bacteria do not show an increase in ensilage until 10 to 15 days after ensiling (Allen and Harrison, 1937; Allen _et al_., 1937) or even 30 days after

ensiling (Gouet et al., 1965). Since the conservation period of the silage was only 28 days the presence of butyric acid at the level of 0.8 per cent of the dry matter was significant.

Malic and citric acids still accounted for 1.65 per cent of the dry matter after 28 days of conservation. This is unusual since malic and citric acids usually disappear in the first week of ensilage, as shown by Hirst and Ramstad (1959), by Playne (1964) and by the author in an experiment reported in a previous part of this thesis. However, in none of the above experiments was a molasses additive used. It appears that some bacteria utilize sugars preferentially to malate or citrate when an ample supply is available. Playne (1964) in an experiment with pure cultures showed that *Streptococcus faecalis* did not utilize malate to any extent in the presence of glucose, but utilized it entirely in its absence. *Streptococcus faecium*, on the other hand, could utilize malate under both circumstances, but the utilization was enhanced by the presence of glucose.

A consideration of the specific activity of silage acids brings out the point that in the present experiment, the specific activity of butyric acid was more than twice the specific activity of lactic or acetic acids. The difference in specific activity per milliequivalent of acid between acetic acid and butyric acid could be explained partly by the difference in the number of carbon atoms in the molecules and partly by the multiplicity of pathways available for the formation of acetic acid from sources other than sucrose. The difference

in specific activity between lactic and butyric acids is not so easy to explain. The difference in the number of carbon atoms explains only a small part of the difference. It could be speculated that lactic acid arose from malate or citrate after butyrate was formed. However, as mentioned earlier, malate and citrate are usually dissimilated in the early days of ensilage. Hemicellulose breakdown and the resulting pentose formation are due to plant enzymes (Dewar et al., 1963) and would also be expected in the early stages of ensilage. The possibility that the butyric acid peak was contaminated by non-acidic compounds cannot be completely excluded, since the purification step was omitted in the preparation of the extract. However, the possibility is remote in view of the very close agreement between the two independent determinations of the specific activity.

A summary of the recovery of radioactivity added as ^{14}C-sucrose is presented in Table 8. The results of Brown (1962) (recalculated) and our own have been used in the preparation of the table.

There is an apparent dissimilarity between the results presented in the table and those shown by Brown (1962). This is due to the fact that the data were calculated on a dry matter basis, while Brown's results were calculated on a fresh weight basis.

At first glance it appears impossible that the silage had a higher specific activity than the grass at ensiling. The anomaly is possibly due to the method of determination of the

TABLE 8

The recovery of ^{14}C activity of sucrose after ensilage for 28 days

Constituent	Specific activity (μmc/g D.M.)	Total activity (μc)	Percentage of total activity Added	In silage
In:				
Herbage (calculated)	32.120**	75.0**		
Out:				
Silage	37.836**	66.1**	88.1**	
Acetic acid	5.963	10.4	13.9	15.7
Lactic acid	4.467	7.8	10.4	11.8
Butyric acid	2.026	3.5	4.7	5.3
Succinic acid	trace	trace		
Effluent	7.414* **	4.1**	5.5**	

*Specific activity of effluent is expressed on the basis of effluent weight, not on a dry matter basis.

**Data recalculated from Brown (1962).

dry matter content. The dry matter of silage was determined by oven drying without taking into account the loss of volatiles on drying. Those losses may be very serious as shown by McDonald and Dewar (1960) and Minson and Lancaster (1963). In silage at a pH of 4.95 as in the present instance, about 40 per cent of the acetic acid is in the free acid form (pKa = 4.76) and consequently is lost on drying. In view of the high acetic acid value of this silage, a loss as high as

1.7 per cent of the dry matter could be expected. A further loss of 0.4 per cent is expected for butyric acid. Since lactic acid is also volatile to some extent, an additional source of error is possible but it would not be serious at this pH. This would lead to an underestimate of the dry matter content of silage, and in turn to an overestimate of the dry matter losses. That this is the case is shown by the fact that only 6.4 per cent of the ^{14}C-sucrose is unaccounted for, while the dry matter losses were calculated to be above 25 per cent (including effluent losses). Losses of this order are unexpected after such a short storage period.

The data in Table 8 were recalculated using 19.8 as the dry matter content, and the results are shown in Table 9. Since the acid concentration in the silage was also influenced by the correction for dry matter, the corrected results are also shown.

It is evident that the correction for dry matter did not account for all the substances lost in drying. Other sources of error which were not taken into account were: the presence of other volatiles such as esters of organic acids (Morgan and Pereira, 1962A, 1962B); the presence of salts of volatile acids (acetic) and volatile bases. For example, ammonium acetate would decompose to acetic acid and ammonia on heating. No further correction will be made as there is no basis other than speculative, on which they could be made. It can be seen that a change of 2.1 in the percentage of dry matter has brought the specific activity of silage to a more realistic value.

TABLE 9

The recovery of ^{14}C activity of sucrose after ensilage for 28 days, corrected for losses in the determination of dry matter by oven drying

Constituent	Acid concentration* (m.equiv/100 g)	Specific activity (μmc/g)	Total activity (μc)	Percentage of total activity added	in silage
In:					
Herbage		32.120	75.0		
Out:					
Silage		32.95	64.4	85.9	
Acetic	61.97	5.290	10.3	13.7	15.9
Lactic	40.85	3.963	7.7	10.3	12.0
Butyric	8.11	1.797	3.5	4.7	5.5
Succinic	8.30	trace	-	-	-
Malic	9.87	nil	nil	nil	nil
Citric	12.46	nil	nil	nil	nil
Unidentified	6.50	nil	nil	nil	nil
Effluent		7.414**	4.1	5.5	

*On a dry matter basis.
**Per gram of effluent.

Similarly, the calculated loss of dry matter has been brought down from over 25 per cent to 16.3 per cent, while the ^{14}C loss is still 8.6 per cent.

The data indicate that some gaseous losses occurred during storage. Those losses were probably of the order of 10 per cent and arose mostly from the utilization of carbohydrates with CO_2

as one of the end products. A further 5.5 per cent of the dry matter was lost as effluent. Of the radioactivity remaining in the silage, 33.4 per cent was recovered as organic acids, mainly acetic and lactic acid. No work was done to determine whether the residual activity was still in the carbohydrate fraction or had been metabolized to form bacterial proteins and lipids. However, the radioactivity measured in the citric acid peak indicates that a proportion of the sucrose added was still in the carbohydrate fraction.

VI. EXPERIMENTAL

PART II. CARBON DIOXIDE EXCHANGES IN ENSILAGE

A. CARBON DIOXIDE FIXATION IN ENSILAGE

In the review of literature of this thesis, the reasons which led us to suspect a possible fixation of carbon dioxide in ensilage have been given in detail and they will not be repeated here. In biochemical studies on ensilage, an experimenter is always faced with a decision as to whether work should be done with pure strains of bacteria in well-defined media or with a heterogeneous mixture of organisms such as present in silage and in not-so-well-defined media such as grass. Any bacteriologist will, of course, decide for pure strains of bacteria in well-defined media, on the ground that elucidation of the main pathways of metabolism of silage bacteria will yield the basic information needed for an understanding of the biochemical happenings in ensilage. This is not denied, but it is also well known that bacteria can deviate from their normal fermentative behaviour when placed in an unusual environment. Since silage is a dynamic environment, since the nutrient composition of grass varies according to locality, weather and soil conditions, etc., and since the bacterial flora of grass is varied, if scarce, it becomes evident that a very large number of screening tests will be necessary before sufficient information has accumulated to explain the biochemical changes taking place in ensilage. The possibility that a succession of organisms (each preparing the

medium for the next one) may be necessary in successful silage making militates in favour of using grass with its natural flora of microorganisms.

We have followed the natural course of using grass and bacteria from grass for our studies, realizing that results from one experiment may not be applicable to the next one, and that many questions may be left unanswered.

Another problem is attendant with the use of grass in conjunction with isotopic carbon; if $^{14}CO_2$ is used, photosynthesis may be responsible for incorporation of some of the tracer into organic compounds; if other ^{14}C-compounds are used, they may be metabolized by plant enzymes. However, this last possibility is not a serious problem since the natural compound in the plant is subjected to the same reactions resulting from plant enzymes.

1. Preliminary experiment on CO_2 fixation

Materials and methods -

In a preliminary experiment carbon dioxide of low specific activity was used. The $^{14}CO_2$, the respiration product of laboratory animals injected with ^{14}C-cholesterol, was collected in sodium hydroxide. The bicarbonate was decomposed by adding 1N H_2SO_4 and the liberated CO_2 was trapped over water acidified to a pH of 2.0. A filtering flask contained the bicarbonate solution and a separatory funnel was used to introduce the acid. The flask and the funnel were connected by a rubber stopper. Nitrogen was used to displace oxygen in the void space of the

the filtering flask before acid addition. The liberated carbon dioxide was trapped over acidified water in an aspirator bottle, a second aspirator bottle being used to create a vacuum for collection of the gas. Acid addition was discontinued when the radioactive solution was acidic to methyl orange indicator. Two liters of CO_2 were collected.

Perennial ryegrass cut at a young stage of growth was used for the experiment. The grass was chopped with scissors and 60 grams of it were packed into a 19 x 3 cm test-tube. After filling, the tube was sealed with a mercury valve, wrapped in aluminum foil and incubated at 30°C in a water bath for 9 days. Circulation of the radioactive CO_2 through the silage was started after 2 days of ensilage and was continued for 7 days. The first 2 days of ensilage were skipped in order to avoid excessive dilution of the radioactive carbon dioxide with carbon dioxide from silage. The test-tube silo and the arrangement for circulating carbon dioxide are shown diagrammatically in Figure 8. The carbon dioxide, after circulation through the silo, was collected in N-sodium hydroxide contained in a gas washing bottle. The sodium hydroxide in the trap was replaced every other day.

After 9 days of incubation, nitrogen gas was circulated through the silage for 3 minutes to remove free carbon dioxide and the test-tube was emptied.

Analytical techniques -

Fifteen grams of silage were placed in a small bottle and covered with 15 ml of 0.6 N H_2SO_4. A crystal of thymol was

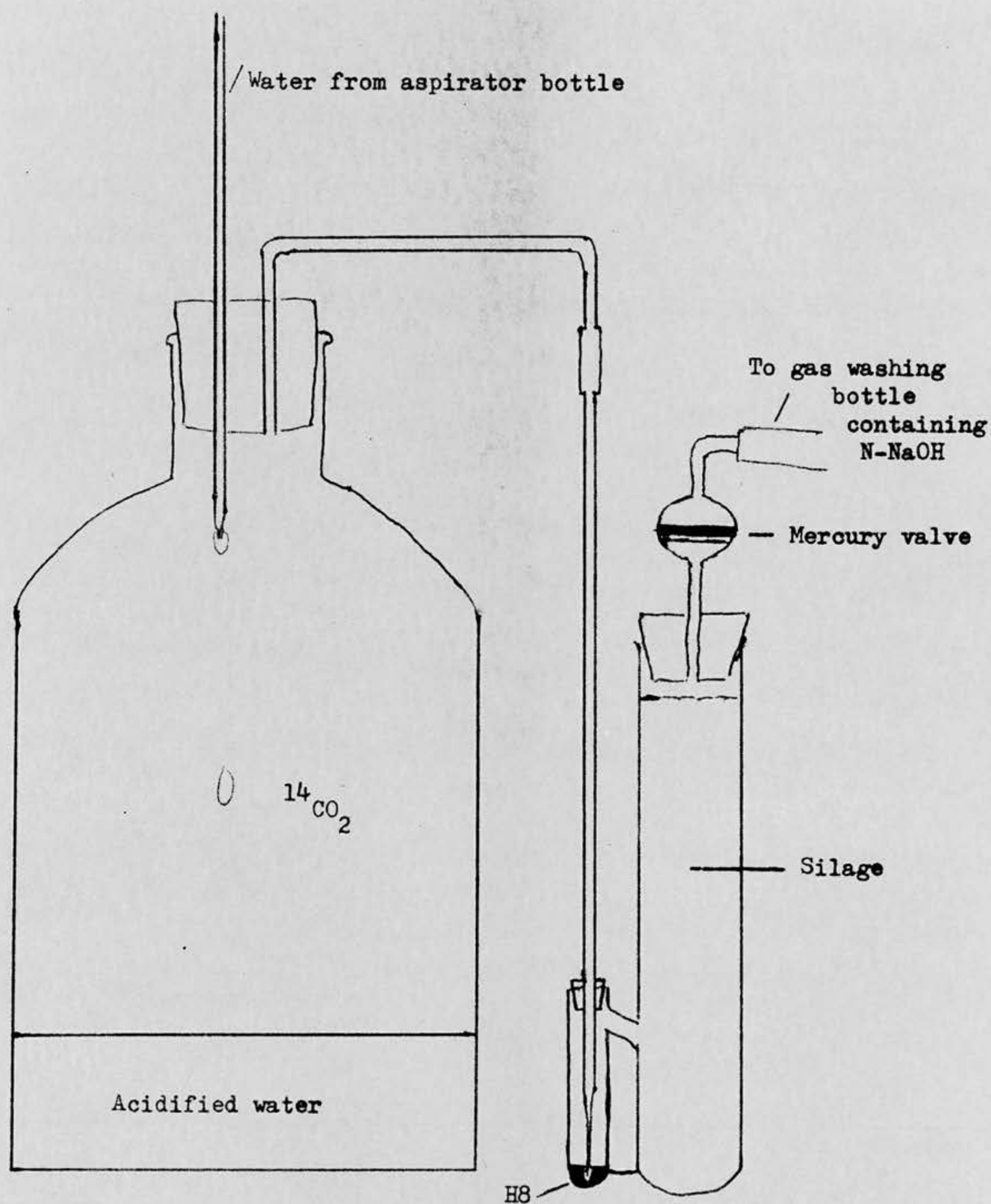


FIGURE 8. Arrangement for circulating dilute $^{14}CO_2$ in laboratory silo.

added and the sample was stored in a refrigerator for 1 week. An extract was obtained as described by Wiseman and Irvin (1959) and a fractionation of organic acids was effected following the method of the same authors.

The initial dry matter content of the grass was determined by oven drying and the moisture content of the silage by toluene distillation (Dewar and McDonald, 1961).

Twenty grams of silage were macerated with 100 ml of distilled water and pH was determined on the resulting solution using a glass electrode.

Radioactivity measurements -

The fractions from the celite column were titrated, evaporated to dryness and taken up in 1 ml of water. They were subsequently counted in 15 ml of NE220, a dioxane-based scintillator, using 0.1 ml of the salt solution. An internal standard was used to correct for counting efficiency.

Results -

The results of a fractionation of organic acids according to Wiseman and Irvin (1957) are shown in Table 10.

TABLE 10

Organic acids and activity measurements of a silage exposed to $^{14}CO_2$ gas for 7 days

Acid	Concentration (mequiv/100g D.M.)	Specific activity (d/s/mequiv)	Total activity (d/s/g D.M.)
Butyric	1.47	nil	-
Propionic	0.81	trace	-
Acetic	20.15	nil	-
Succinic and lactic	45.84	356	163

In 9 days, the pH of the silage decreased from 6.21 to 5.46. The main acids formed during the period were acetic, lactic and/or succinic acids. Some radioactivity was measured in the peak containing succinic and lactic acids, and a trace was found in propionic acid.

Two additional fractionations were attempted using the same original extract. Instead of relying on the change of color of the indicator, fractions of 15 ml were collected, titrated individually and measured for radioactivity. It was hoped that the specific activity and the concentration of lactic or succinic acid could be measured by this procedure. Results were negative. This last failure of the Wiseman and Irvin method (1957), after the failures at separating unlabelled acids, led us to undertake additional work on methods of determination of organic acids. The procedure evolved has been described in Part I of this thesis.

2. Main experiment on carbon dioxide fixation in ensilage

Materials -

The material used was cocksfoot (*Dactylis glomerata*) cut at the leafy stage, before heading. Weeds and clovers were removed. The grass was cut to 1-inch lengths, packed tightly into 19 x 3 cm test-tubes holding about 65 grams, and sealed with a mercury valve (Figure 9). Aluminum foil was wrapped around the tubes. These laboratory "silos" were incubated in a water bath at 30°C.

After 24 hours of incubation, one silo was emptied for chemical analysis. The other two silos were injected with

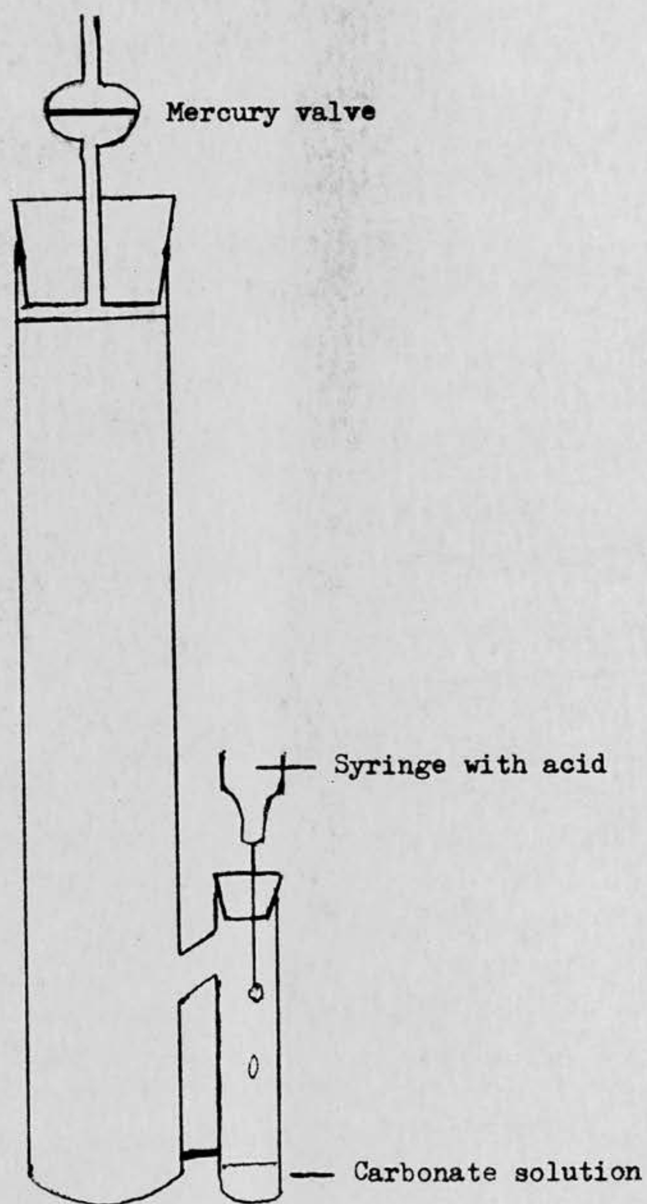


FIGURE 9. Liberation of $^{14}CO_2$ gas in laboratory silo.

radioactive $^{14}CO_2$ gas in the following way: a quantity of 0.855 mg of sodium ^{14}C-carbonate at a specific activity of 5.650 millicuries per milliequivalent (obtained from Radiochemical Center, Amersham) was dissolved in 0.5 ml of water and injected with a syringe into the side tube of the silos through a rubber stopper (Figure 9). A slight vacuum had been created in the silos before adding the carbonate solution. More vacuum was then applied. Carbon dioxide was liberated by the addition of 0.1 ml of N perchloric acid into the side tube.

Times of exposure to $^{14}CO_2$ for the silos were 7 days and 4 months.

Analytical methods -

Organic acids were extracted from 15 grams of silage with 15 ml of 0.6 N sulfuric acid, the time of contact of the acid with silage being 7 days. The extracts were purified through large size columns of silica gel as described previously. The methods of fractionation and activity counting have also been described.

For radioactivity measurements of free amino acids, 15-gram samples were rapidly killed with boiling 80 per cent ethyl alcohol. The alcohol was decanted and the residue was macerated with two lots of 150 ml of 80 per cent ethanol. The extracts were pooled and concentrated to a syrup in a rotary evaporator at 40°C. The concentrate was taken up in distilled water and amino acids were adsorbed onto an Amberlite IR-120 (H) column. The column was washed with distilled water to remove organic acids and neutrals. Amino acids were eluted with 100 ml of 4 N

ammonium hydroxide. Excess ammonia and water were removed in a rotary evaporator at 40°C and the concentrate was taken to dryness over phosphorus pentoxide in a vacuum desiccator. The residue was dissolved in 0.8 ml of Formamide (Nuclear Enterprises Ltd., Edinburgh) and made to 1 ml. A volume of 0.1 ml of the Formamide-amino acid solution was then added to 15 ml of NE220 scintillator and counted. An internal standard was used to measure efficiency.

The dry matter content of the silages was determined by toluene distillation (Dewar and McDonald, 1961), using 20 grams of silage.

Results -

The organic acid content and pH values of the silages after 1 day, 8 days and 4 months of incubation is shown in Table 11.

The pH values indicated that fermentation was rapid. After 1 day, pH was down to 5.07, a decrease of 1 pH unit. At the end of 4 months, it was down to 4.0. The distribution of organic acids was indicative of a normal fermentation. In 1 day, there was a virtual disappearance of malic and citric acids. Lactic and acetic acids were the only two acids present in significant proportions. In the 8-day silage, butyric and propionic acids appeared, while acetic, lactic and succinic acids increased considerably. In the 4-month silage, propionic acid accounted for 1.6 per cent of the dry matter, while butyric acid was still very low. Acetic acid was in higher concentration than lactic acid.

TABLE 11

The organic acid content and pH values of silages made from cocksfoot and incubated for different periods of time (m.equiv/100 g D.M.)

Acid	Incubation period		
	1 day	8 days	4 months
pH	5.07	4.42	4.01
Butyric	nil	2.03	3.86
Propionic	nil	1.25	21.40
Acetic	12.57	98.64	115.88
Fumaric	0.81	nil	nil
Formic	nil	trace	1.16
Succinic	2.93	4.68	5.18
Lactic	31.85	78.24	92.40
Malic	1.56	nil	nil
Citric	1.00	trace	trace

Table 12 shows the specific activity of organic acids in the 4-month silage, as well as the total activity in the silo. The activity in the 8-day silage is not reported. The samples were prepared and mixed with a scintillator, but could not be counted immediately on account of a breakdown of the scintillation counter. When they were measured, 2 weeks later, the background count rate measured on samples collected between peaks was variable. Counting of radioactivity on the two fractions from the same peak gave widely differing specific

TABLE 12

Specific activity and total activity in organic acids and free amino acids of a cocksfoot silage incubated for 4 months in presence of $^{14}CO_2$

Acid	Specific activity (d/s/m.equiv)	Total activity in the silo (mμc)
Butyric	trace	trace
Propionic	1,110.0	11.7
Acetic	20.4	6.45
Formic	nil	nil
Succinic	9,417.0	133.0
Lactic	2,073.0	523.0
Malic	nil	nil
Citric	nil	nil
Amino acids	-	27.9
Total incorporation		702.0

activity. The data were therefore considered to be unreliable and were discarded. The highest specific activity in the 4-month silage was found in succinic acid, followed by lactic and propionic acids. A low specific activity was also found in acetic acid. Formic acid did not contain any labelled carbon. Lactic acid showed the highest incorporation followed by succinic acid. Small quantities were also found in acetic and propionic acids. The fraction containing the free amino

acids also contained small quantities of ^{14}C. A total of 702.0 millimicrocuries were recovered as organic acids or free amino acids. A quantity of 90.9 microcuries had been added. The incorporation was therefore of the order of 0.77 per cent.

B. THE DISSIMILATION OF MALATE, CITRATE AND GLUCOSE DURING ENSILAGE

Materials and methods -

Perennial ryegrass 523 (*Lolium perenne*) was used. The grass was cut at a young, leafy stage of growth on July 25, 1963, and brought to the laboratory without delay. Weeds were removed by hand.

The grass was divided into lots of 50 grams and spread in plastic trays of suitable dimensions. The trays were covered with paper and kept in the dark for 10 hours.

After this interval, radioactive materials were added. The three tracers used were malate, citrate and glucose. They were obtained from the Radiochemical Center, Amersham.

Quantities of 1.19 mg of uniformly labelled l-malic acid-^{14}C and 1.09 mg of dl-citric acid-1,5-^{14}C were dissolved in sterile distilled water, neutralized with 0.20 ml and 0.14 ml of 0.1 N sodium hydroxide respectively, and final volumes were made to 10 ml. Each stock solution contained 0.1 millicurie.

One ml of radioactive solution was used for each tray of grass. Ten microcuries of each acid went into each silo.

Glucose-^{14}C(V) was similarly diluted and applied at the rate of 8 microcuries per silo.

The herbage was thinly spread on the bottom of the plastic trays and the radioactive substances were applied by means of a small brush. The grass was ensiled immediately after application of the radioactive tracers. Silos were sealed with a mercury valve, wrapped in aluminum foil, and placed in a water bath at 30°C.

Additional silos of similar size, one for each tracer, were filled and incubated. They were prepared especially to allow for collection of radioactive CO_2 at intervals during the fermentation. They were test-tubes fitted at the bottom with capillary glass tubing (Figure 10). The capillary tubing was connected by rubber tubing with a nitrogen tank. The mercury valve was connected with a gas washing bottle containing 40 ml of 1.0 N sodium hydroxide. Dispersion of the gas in the alkali solution was ensured by a fritted glass disk at the end of the inlet tube. During storage the gas inside these silos was displaced at regular intervals by running nitrogen through for 3 minutes at a slow rate. The sodium hydroxide solution was replaced after each gas displacement and kept for radioactivity measurements.

Activity of ^{14}C-carbon dioxide

Carbon dioxide was adsorbed in sodium hydroxide and was therefore present as a bicarbonate, or a carbonate. Since dioxane is miscible with as much as 20 per cent of its volume of water, it was first tried to dissolve 1 ml of the solution in 5 ml of NE220 scintillator, a dioxane-based scintillator. The bicarbonate precipitated immediately and deposited on the

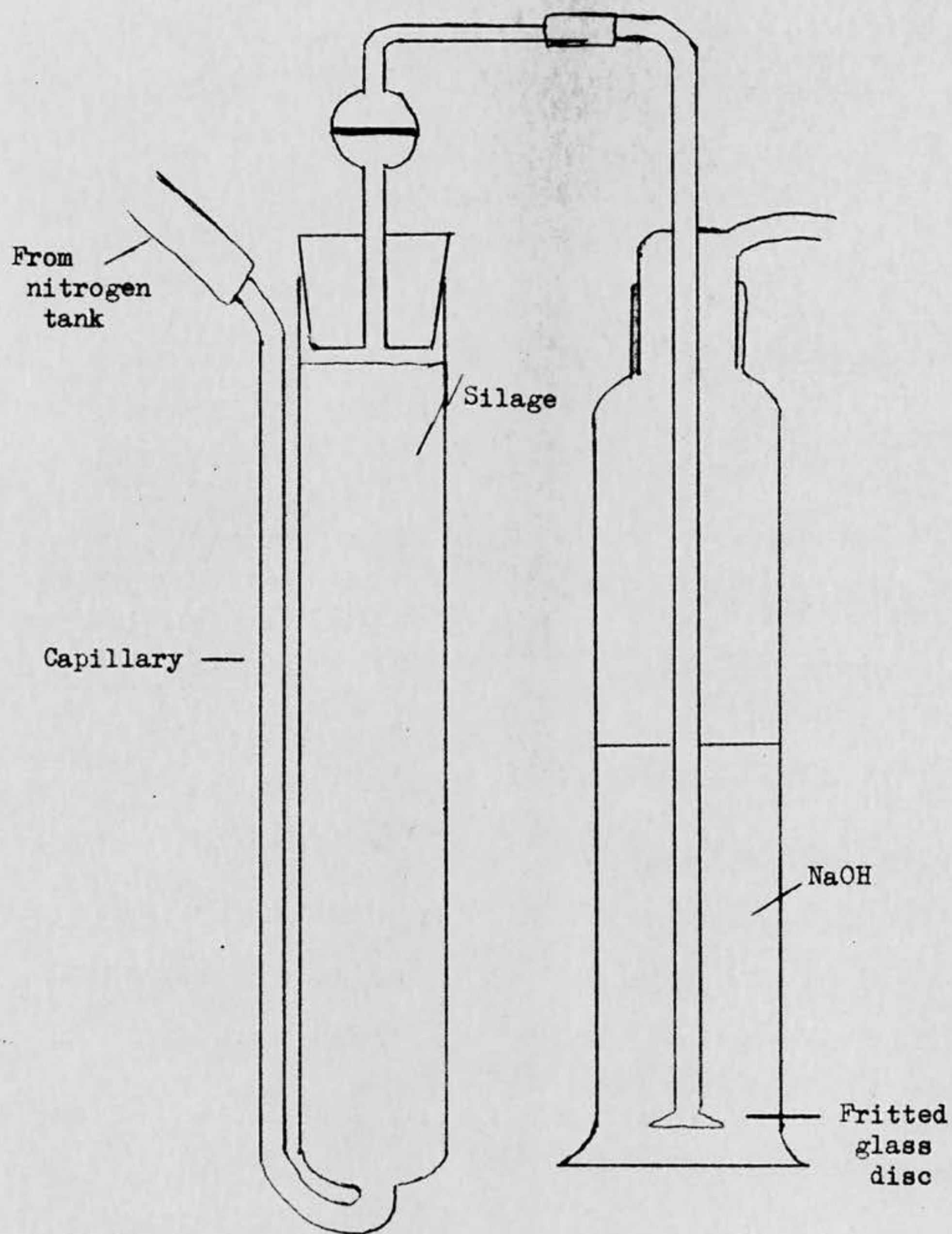


FIGURE 10. Apparatus for the collection of $^{14}CO_2$ arising from the dissimilation of labelled malate, citrate and glucose.

bottom of the counting vessel. Solution of 1 ml of the bicarbonate-sodium hydroxide solution with 15 ml of scintillator produced the same results. Although dioxane-based scintillators are miscible with water, it was evident that they could hold only small quantities of organic compounds in solution. The same observation was made by Takahashi <u>et al</u>. (1963) working with leucine. In their experiments, a volume of 10 ml of a dioxane-based scintillator could hold only 1 mg of leucine. The volume of scintillator was therefore kept to the maximum (15 ml) and decreasing quantities of radioactive solutions were tried. Using samples in which the quantity of bicarbonate was likely to be highest, it was found that no precipitation occurred when only 0.1 ml of radioactive solution was mixed with 15 ml of scintillator. A one to ten dilution of the solution and the use of 0.1 ml of the diluted solution did not improve counting efficiency. Since the use of 0.1 ml of solution and 15 ml of NE220 scintillator gave reproducible counts, the procedure was adopted. Using internal standards, it was found that counting efficiency was 43.6 per cent and background counts were 0.93 counts per second. In the conditions of this experiment we did not observe the large spurious counting component due to alkalinity reported by Brown and Badman (1961), working with Hyamine and NE213 scintillator. The main disadvantage of the technique used was the necessity of long counting times if low activity was present.

Other convenient techniques which have been used with success for trapping and counting $^{14}CO_2$ have been: the direct

trapping of $^{14}CO_2$ in 1 M Hyamine hydroxide after initial moisture removal by passage through a cold trap (Fredrickson and Ono, 1958); the trapping of CO_2 in barium hydroxide and the counting of the carbonate as a suspension in thixotropic gelling agents such as Thixcin (Nathan _et al_., 1958) or Cab-O-Sil (Ott _et al_., 1959) using toluene or xylene-based scintillators.

Activity in ^{14}C-organic acids

Extraction, purification, fractionation and radioactivity counting in organic acids were as described previously. The activity in the non-volatile organic acids was determined only for the 31-day samples.

Results -

Carbon dioxide production

The production of labelled carbon dioxide from malic acid, citric acid and glucose was measured at 3-hour intervals during the first 2 days and at less frequent intervals afterwards. The cumulative production of this gas is shown in Figure 11. In this figure, the data for glucose have been adjusted for equal initial radioactivity as for malic and citric acids by dividing the data from each measurement by 0.8. It is shown that gas production was high in the first few hours after ensiling and decreased afterwards. Gas production was highest for citric acid, was intermediate for glucose and was lowest for malic acid.

In Figure 12 is shown the rate of production of labelled carbon dioxide for each of the three silos. These data were obtained by dividing the activity at each sampling by the number

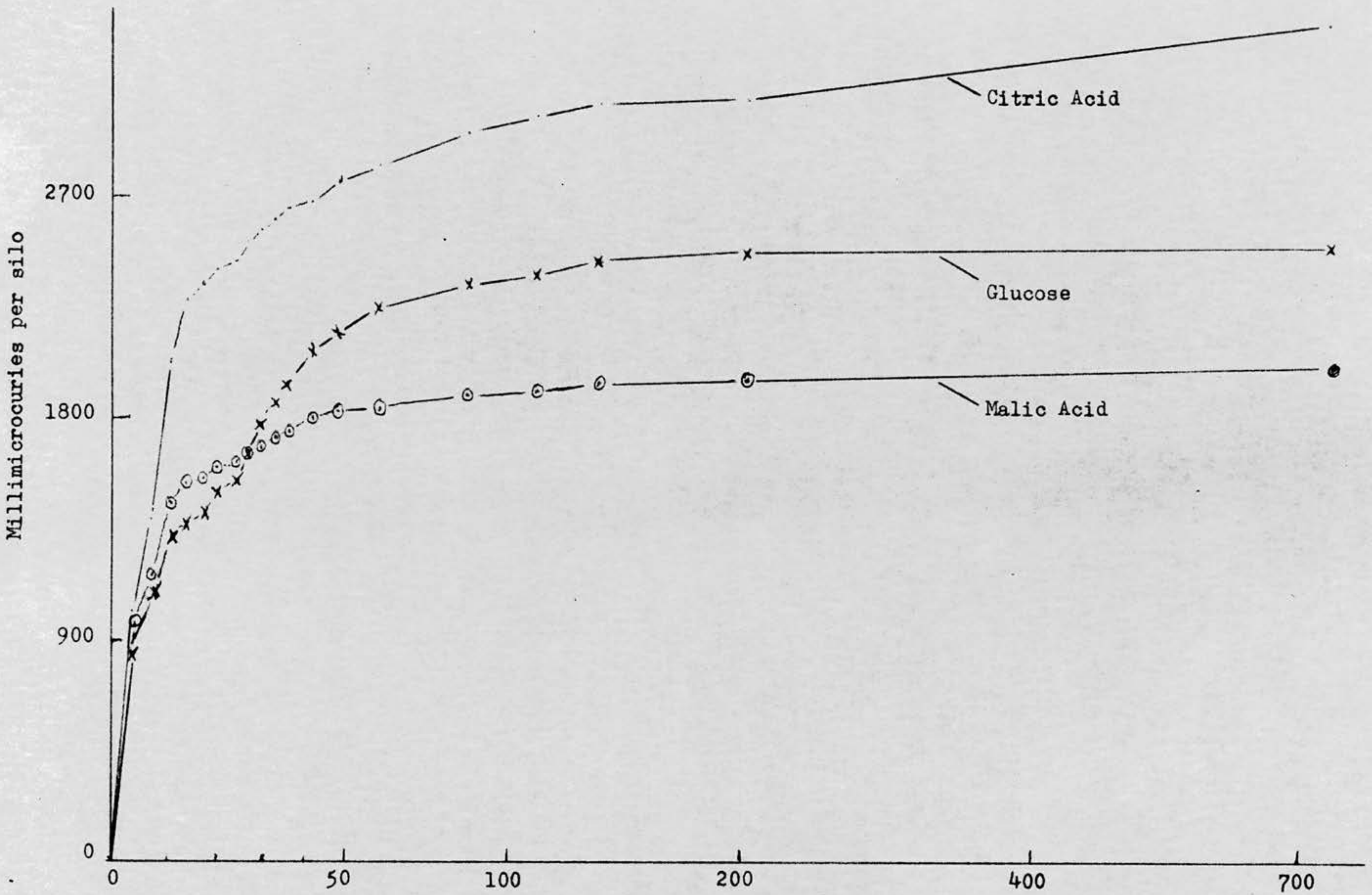


FIGURE 11. Cumulative production of $^{14}CO_2$ in ensilage made from perennial ryegrass and ensiled ^{14}C-labelled citrate, malate or glucose (time shown on a logarithmic scale).

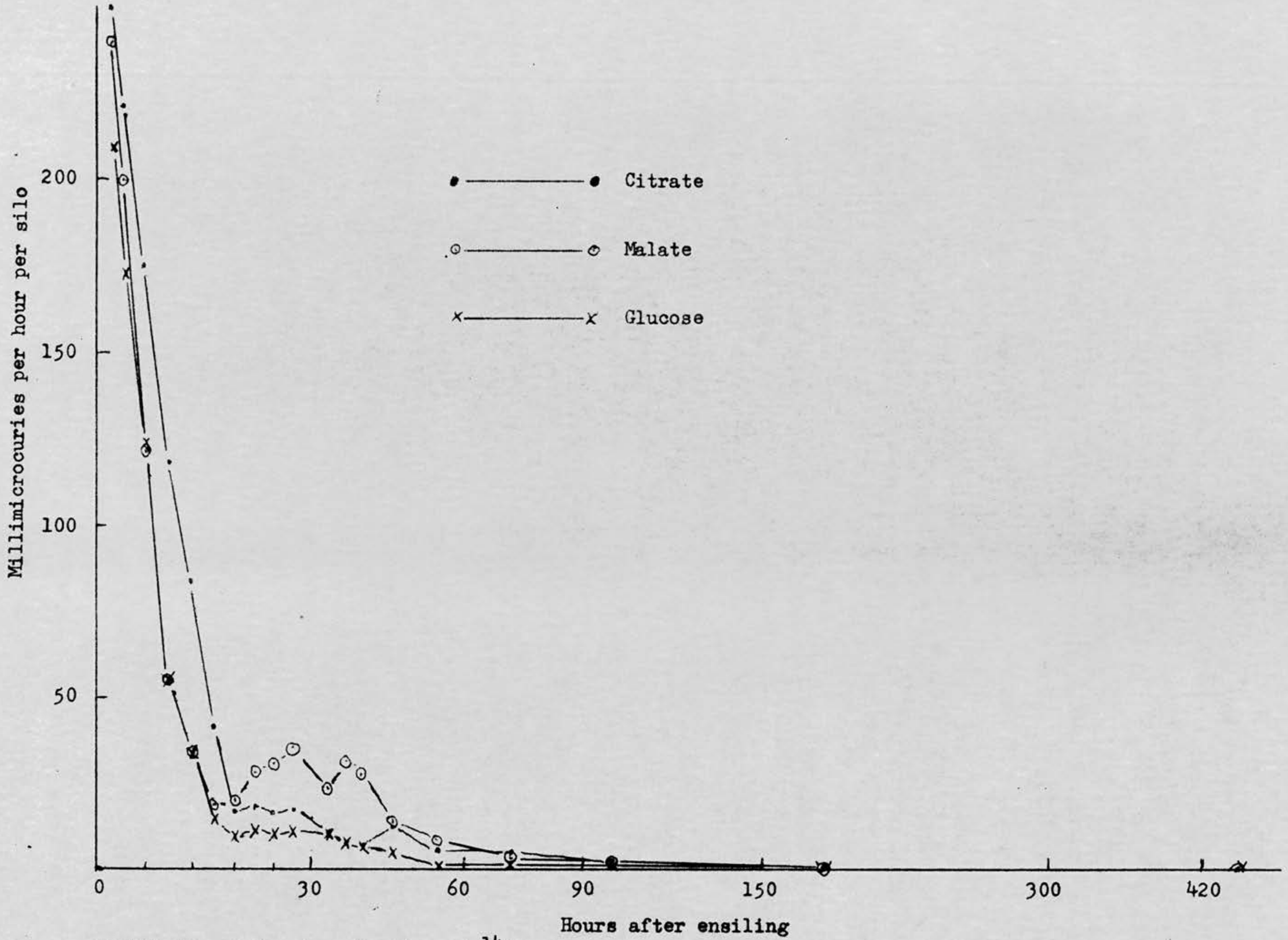


FIGURE 12. Rate of production of $^{14}CO_2$ in ensilage made from perennial ryegrass and ensiled with ^{14}C-labelled citrate, malate or glucose (time shown on a logarithmic scale).

of hours between samplings. In plotting, the calculated rate of $^{14}CO_2$ production was placed at mid-point of sampling intervals. The method of moving averages was used in order to obtain a smoother curve and to avoid jagged lines due to sampling variations. The first sampling data were plotted as such; the second data were obtained by adding samplings 1, 2 and 3 and dividing by 3; the third data were the average of samplings 2, 3 and 4; and so on until sampling 13 when the use of longer sampling intervals prevented the use of this method. Rates for sample 13 and following samples were plotted as observed. Measurements and sampling times from which the data in Figures 11 and 12 have been calculated are shown in Appendix 2. Figure 12 shows that $^{14}CO_2$ production was highest for the three treatments in the period immediately following ensiling. There was a sharp decrease at each subsequent sampling until the 17-hour sampling when a low was obtained for all three treatments. Production of $^{14}CO_2$ was maintained at approximately the same low rate for silos in which ^{14}C-malate and ^{14}C-citrate acids had been added up to the 54-hour sample and was negligible afterwards. In the silo to which ^{14}C-glucose had been added there was the same initial decrease in $^{14}CO_2$ production during the first 15 hours, but after a short lag, $^{14}CO_2$ production increased again to reach a new peak 25 hours after ensiling. After 54 hours, CO_2 production became negligible as in the other two silos.

Chemical composition of the grass and silages

The chemical composition of the grass when cut was as follows: dry matter, 21.1 per cent; water-soluble sugars, 9.7 per cent; water-soluble fructosans, 6.5 per cent; total water-soluble carbohydrates, 16.2 per cent; and total nitrogen, 2.7 per cent. The above values are expressed on a dry-matter basis, except for dry-matter content, which is expressed as a percentage of fresh weight. The grass was not analysed again when ensiled, except for dry-matter content which was 21.0 per cent. Analysis of a 31-day silage prepared in the same way as the main silage but without added radioactivity indicated that soluble nitrogen was 69.4 per cent of total nitrogen. Volatile nitrogen was 19.1 per cent of soluble nitrogen and 13.3 per cent of total nitrogen.

The pH value and organic acid composition of the grass as ensiled and of silage after different incubation periods is shown in Table 13. The main acids in the original grass were malic and citric acids. Fumaric, acetic, succinic and four unidentified acids were also present in low quantities. During incubation, malic and citric acids disappeared gradually. Succinic, acetic, butyric and lactic acids increased. Fumaric acid was absent in the 31-day silages. In general, the changes in the two sets of silos were similar, but there were a few exceptions. For example, the lactic acid content was higher and the butyric acid content, lower for the 31-day silage to which malate had been added than for the corresponding silage to which citrate had been added. In both silages, however, the

TABLE 13

The pH value and organic acid composition of silage made from perennial ryegrass after different ensilage periods (m.equiv/100 g D.M.)

	Time after ensiling						
	Original grass	Citrate added			Malate added		
Acid		24 hr	48 hr	31 days	24 hr	48 hr	31 days
pH	6.01	6.23	5.96	5.05	6.18	6.03	4.96
Butyric	nil	nil	nil	22.82	nil	nil	12.67
Propionic	nil	nil	nil	3.84	nil	nil	1.61
Acetic	3.86	12.56	22.03	27.23	12.29	14.67	19.94
Fumaric	3.90	1.32	0.86	nil	0.96	1.05	nil
Formic	nil	2.10	4.31	2.58	3.70	1.21	4.35
Succinic	2.55	8.89	24.05	41.35	10.03	24.83	36.56
Lactic	nil	10.52	14.06	17.51	10.70	13.57	27.38
Unknown A	0.25	0.31	0.59	0.34	0.26	0.36	2.58
Unknown B	4.06	4.36	5.12	0.47	3.83	1.02	1.66
Unknown C	3.76	2.82		2.10	2.20	1.24	3.47
Malic	18.20	13.04	11.20	0.41	15.68	5.80	1.91
Citric	12.93	9.27	8.96	2.49	7.07	5.74	3.23
Unknown D	4.10	4.07	3.31	2.41	1.99	3.10	3.31

lactic acid content was low, while the succinic acid content was high. The simultaneous presence of fumaric and formic acids in the silage samples (indicated by paper chromatography with propanol-ammonia (70:30) (Hanes and Isherwood, 1953) necessitated the recourse to a separation on a second silica gel column. The fractions of the peak were pooled after titration and evaporated. The salts were taken up in water and dried. The salts were dissolved in 0.5 ml of 0.5 N sulfuric acid, mixed with 0.8 grams of silicic acid, slurried with chloroform and applied on a column of silica gel prepared as previously, except that chloroform was the mobile phase. Elution was then accomplished with a solvent composed of t-amyl alcohol (8 per cent) and chloroform (92 per cent). Under those conditions fumaric acid was eluted ahead of formic acid.

In addition to the usual acids in grass or silage, four unidentified acids were observed. As they were present in low concentration, they were not investigated further. In general, they tended to decrease during incubation. Their position in the elution schedule is shown in Figure 13.

Specific activity of organic acids

The specific activity of organic acids is shown in Table 14 for silages to which labelled malate or citrate had been added. In silages to which labelled citrate had been added, the specific activity was highest in succinic and fumaric acids in the 24-hour silage and decreased as fermentation progressed. The specific activity in acetic acid was low in the 24-hour

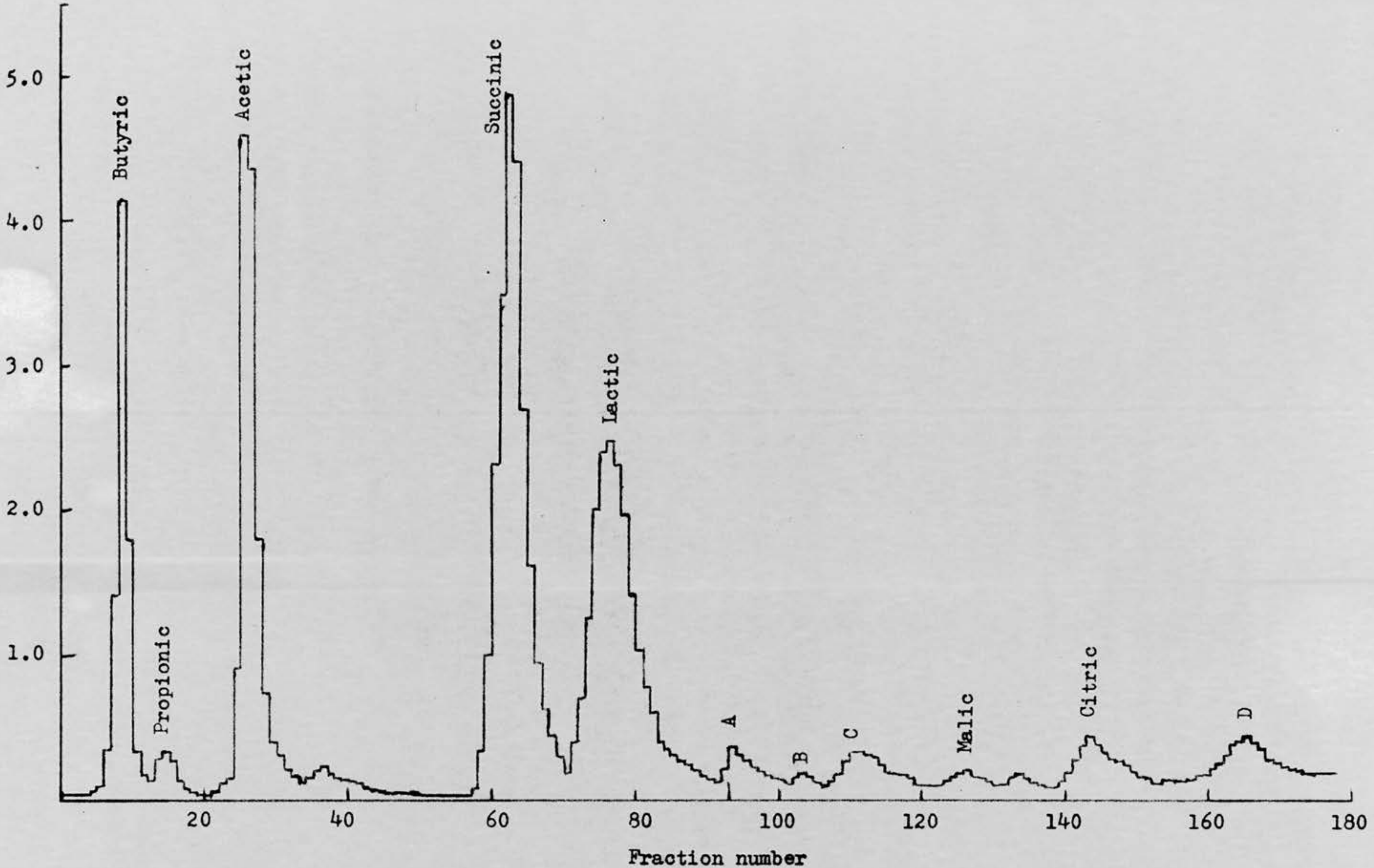


FIGURE 13. Histogram of distribution of acids from ryegrass silage ensiled for 31 days with tagged malic acid. Fractions of 4 ml.
Elution: with gradients I and II.

TABLE 14

Specific activity of organic acids in ensilage made from perennial ryegrass after different ensilage periods (d/s/m.equiv)

Acid	Time after ensiling							
	Original grass (calculated)	^{14}C-citrate added			Original grass (calculated)	^{14}C-malate added		
		24 hr	48 hr	31 days		24 hr	48 hr	31 days
Butyric	-	nil	nil	1,180	-	nil	nil	1,620
Propionic	-	nil	nil	nil	-	nil	nil	5,830
Acetic	-	350	975	1,030	-	2,250	905	690
Fumaric	-	2,030	680	nil	-	9,440	4,680	nil
Formic	-	nil	nil	nil	-	nil	nil	trace
Succinic	-	9,800	5,330	3,980	-	60,900	39,400	41,200
Lactic	-	1,000	1,630	815	-	20,400	6,620	5,150
Malic				trace	203,300			5,580
Citric	286,200			124,000	-			1,150
A				1,500	-			nil
B				nil	-			1,830
C				2,110	-			710
D				2,400	-			295

sample and increased subsequently. In lactic acid, there was a maximum in the 48-hour sample and a subsequent decrease. The specific activity of citric acid was only about half of the original in the 31-day sample. Butyric acid was detected only in the 31-day silage. No activity was measured in propionic or formic acid. There was a relatively high activity remaining in three unidentified acids after 31 days of incubation.

The samples to which ^{14}C-malate had been added also showed the highest specific activity in succinic acid in the 24-hour silage. The activity decreased in the 48-hour silage and showed little change afterwards. The activity in lactic acid, only one-third of that in succinic acid in the 24-hour sample, decreased in the 48-hour sample and again in the 31-day sample. The activity in fumaric acid decreased in the 48-day sample. This acid was not detected in the 31-day sample. The specific activity of acetic acid decreased in the course of the fermentation. Propionic and butyric acid appeared only in the 31-day sample and both acids contained radioactivity. The activity in malic acid was only about 2.5 per cent of the original in the 31-day sample. Citric acid as well as three unidentified acids contained some radioactivity in the final sample.

Distribution of total ^{14}C-activity among organic acids

Table 15 shows the partition of ^{14}C-activity among organic acids in the various silages. In the citrate silages, incorporation was mostly in succinic acid, and the quantity increased with each sampling. There was some incorporation in lactic

TABLE 15

Partition of ^{14}C-activity among organic acids in silages made from perennial ryegrass after different ensilage periods (millimicrocuries per silo)

Acid	Time after ensiling							
	Original grass (calculated)	^{14}C-citrate added			Original grass (calculated)	^{14}C-malate added		
		24 hr	48 hr	31 days		24 hr	48 hr	31 days
Butyric	-	nil	nil	64.2	-	nil	nil	48.0
Propionic	-	nil	nil	nil	-	nil	nil	21.8
Acetic	-	11.0	54.1	67.1	-	69.1	34.2	32.4
Fumaric	-	6.9	7.4	nil	-	22.7	12.6	nil
Formic	-	nil	nil	nil	-	nil	nil	trace
Succinic	-	221	324	393	-	1526	2515	3530
Lactic	-	26.7	57.6	34.1	-	545	228	330
Malic	-			trace	10,000			25.0
Citric	10,000			738	-			8.7
A	-			1.2	-			nil
B	-			nil	-			7.0
C	-			10.6	-			5.8
D				12.5				2.3
Sub-total	10,000			1321	10,000			4011
CO_2	-	2445	2761	3427	-	1626	1829	2026
Total	10,000			4748	10,000			6037

acid in the 24-hour sample and the quantity doubled in the 48-hour sample. However, there was a decrease in the 31-day sample. The amount of tracer in acetic acid increased as fermentation progressed. Butyric acid contained a significant quantity of ^{14}C in the 31-day sample. The residual citric acid contained about 7 per cent of the added labelled carbon. There was little ^{14}C-activity in the unidentified acids. A total of 47.5 per cent of the activity added at ensiling time was recovered in organic acids and carbon dioxide.

Of the activity added as malate a large proportion was recovered as succinic acid. About 15 per cent of the malate had been converted to succinate in the 24-hour sample. After 48 hours and 31 days this value had increased to 25 and 35 per cent respectively. In the same periods, the activity in lactic acid was only 5.5, 2.3 and 3.3 per cent respectively. The activity in acetate decreased as incubation progressed. Only a very small percentage of the added malate remained after 31 days. Butyric and propionic acids contained some ^{14}C-labelled carbon in the 31-day sample. The quantity of ^{14}C in citric acid and the unidentified acids was low. In all, 60.4 per cent of the radioactive carbon added as malate was recovered in organic acids or carbon dioxide.

Bacteriological examination

A bacteriological examination revealed that there were few lactic acid organisms in the fresh grass. This was not unusual since precautions were taken to avoid extraneous contamination during harvesting. Sterile bags, scissors, gloves

and trays were used for handling the grass. Stirling (1953) has found that lactic acid bacteria are scarce in fresh grass. Later work by Stirling and Whittenburg (1963) indicated that harvesting equipment is an important method of propagation for lactic acid bacteria.

VII. DISCUSSION

Fixation of carbon dioxide

Two experiments were conducted to study the possibility of fixation of carbon dioxide in ensilage. In the first experiment, a clostridial type fermentation was observed, characterized by a high pH and the production of butyric acid. A more desirable fermentation was obtained in the main experiment. The fermentation was vigorous and, when the radioactive carbon dioxide was generated after 1 day of incubation, pH was already down to 5.1 units and malic and citric acids had virtually disappeared. Lactic and acetic acids were the main fermentation products.

Under both circumstances, fixation of carbon dioxide was observed. The phenomenon of non-photosynthetic carbon dioxide fixation has been observed in many biological systems (cf Review of Literature), but this is the first direct evidence of carbon dioxide fixation in ensilage.

The quantity of $^{14}CO_2$ fixed was low and this is not surprising since $^{14}CO_2$ was added only once and it became diluted immediately with the CO_2 already in the silo and subsequently with the CO_2 which was formed. As gas was evolved during incubation, the ^{14}C-labelled CO_2 was gradually displaced out of the silo. In the present experiment, a total of 0.77 per cent of the added radioactivity was recovered in organic acids and free amino acids. The quantity of $^{14}CO_2$ which may have been fixed into alcohol, bacterial proteins or lipids was not measured.

Our results, together with the observation by Elsden (1938) that the rate of fixation was directly related to the amount of CO_2 in the medium, suggests that in practical silage making it may be worthwhile to take steps to prevent the escape of carbon dioxide. An added advantage of air-tight structures is the limited amount of oxidation during storage. As noted earlier in this thesis, air-tight storage has usually been associated with low gaseous losses. Further experiments using isotope dilution techniques would be necessary in order to obtain more precise measurements of the importance of the phenomenon of CO_2 fixation in ensilage. Information is also lacking on the time of occurrence of the phenomenon and on conditions conducive to fixation. Some of this information could be obtained with pure strains of silage bacteria.

By consideration of the total activity in the various organic acids of silage, it appears that there are two main products of carbon dioxide fixation in ensilage: lactic acid and succinic acid. The evidence available from experiments with pure strains of bacteria (Clifton, 1957) or with plant enzymes (Jackson and Coleman, 1959) suggests that CO_2 is fixed by pyruvate with the participation of the "malic enzyme" of Ochoa (1951) or by phosphoenolpyruvate with the participation of the enzyme phosphoenolpyruvate carboxylase. In the first case, malate is formed; in the second, oxalacetate results. Oxalacetate or malate can then be converted to succinate by a series of reactions known as the Wood-Werkman (1936) scheme, or they can be fermented to yield lactate and carbon dioxide.

Both succinate and lactate were accumulation products in this experiment. The higher specific activity in succinate suggested that carbon dioxide played a quantitatively more important role in the formation of this salt than in the formation of lactate. However, in view of the relative abundance of lactate in the silage, it accounted for 34.5 per cent of the carbon dioxide fixed, while succinate accounted for only 19.0 per cent of the fixation.

Some activity was detected in propionic and acetic acids, arising probably from the decarboxylation of succinic acid, as demonstrated by Delwiche (1948). That author, working with *Propionibacterium pentosaceum*, found that succinate was decarboxylated and gave rise mostly to acetic acid at a pH near neutrality while propionic acid became preponderant at pH lower than 6.5. In this study, a high specific activity was observed for propionic acid while the specific activity of acetic acid was very low. It was surprising to find propionic acid at a high concentration when butyric acid was very low. Usually the two acids are associated and propionic acid is at a lower concentration than butyric acid. The presence of propionic acid is, possibly, the result of the development of some *Propionibacterium* in this silage, although this genus of bacteria has not been shown to be active in silage fermentations.

A measurement of total activity in the free amino acid fraction indicated that about 3 per cent of the activity fixed was in that fraction. The activity in bacterial proteins was not measured but it was not likely to be high. Stirling (1951)

and others have shown that bacterial numbers reach a peak in the early stage of ensilage. It can be assumed that most of the bacteria present at the time when $^{14}CO_2$ was at peak concentration in ensilage had died long before the end of the 4-month incubation period. At least some of the bacterial proteins were hydrolyzed and recovered as free amino acids by the time the silage was analysed. Even assuming that bacterial proteins resisted hydrolysis (information is lacking on the stability of bacterial proteins in ensilage) the proportion of labelled carbon from that source was not likely to be high. Brady (1966) has calculated that bacterial proteins would constitute less than 2 per cent of the total nitrogen content of mature ryegrass.

Dissimilation of malate, citrate and glucose in ensilage

In the experiment on the dissimilation of malate, citrate and glucose, the fermentation was a typical high pH, butyric acid fermentation. Playne (1964) used the same grass for experiments on the changes in buffering capacity in ensilage and observed a similar fermentative behaviour. He attributed the poor fermentation to the low number of lactic acid bacteria in the grass. This does not seem to be a valid explanation, since it is usual for grass to contain few lactic acid bacteria. In a suitable environment, those bacteria usually develop and eventually dominate. Hirst and Ramstad (1957) also using ryegrass obtained a silage of similar quality and attributed their results to a low initial concentration of fermentable carbohydrates. In this experiment, there was certainly no carbohydrate deficiency since water-soluble carbohydrates accounted

for 16.2 per cent of the dry matter. It appears, therefore, that there was a deficiency in some other substance necessary for the growth of lactic acid bacteria. Brady (1966) has presented data suggesting that protein quality in ryegrass of low nitrogen content may be a limiting factor in the development of lactic acid bacteria. This is the most plausible explanation. Nitrogen content was 2.7 per cent of the dry matter when the grass was cut and chemical analyses of the 31-day silage indicated that extensive proteolysis had taken place during incubation. The breakdown of amino acids, resulting in the formation of volatile bases (19.1 per cent of soluble nitrogen), contributed to the shortage of certain amino acids.

The changes in pH during ensilage were usual for butyric acid silages. There was an initial pH increase in the 24-hour sample, an observation frequently made in studies with silage or pure strains of silage bacteria (McDonald _et al_., 1960; Whittenbury, 1961; Playne, 1964). The increase is the result of the formation of volatile bases by plant enzymes (Mabbitt, 1951) and of the release of cations during decarboxylation of organic acids. Volatile bases and cations are immediately used to neutralize the carbon dioxide evolved and bicarbonate buffers result. There was sufficient production of organic acids to overcome the bicarbonate buffer and the 48-hour sample showed that pH had decreased to the approximate level of the original grass. However, the pH of the 31-day samples stayed high because the main products of the fermentation were weak acids such as succinic acid (pKa = 5.64), acetic acid

(pKa = 4.76) and butyric acid (pKa = 4.82). Lactic acid (pKa = 3.86) was present in relatively low quantities and was mostly in the salt form at the pH of the silages.

Measurements of the evolution of $^{14}CO_2$ from silages to which ^{14}C-labelled malate, citrate or glucose had been added indicated that carbon dioxide was produced from the three substrates at a maximum rate immediately after ensiling and at decreasing rates in the following 15 to 18 hours. This suggests that the period of aerobic respiration by either plant cells or aerobic bacteria is terminated after about 18 hours under conditions similar to those in the laboratory silos. The respiration may take a heavy toll of plant nutrients, since $^{14}CO_2$ production in the period accounted for 14.1, 15.6 and 23.5 per cent of the added glucose, malate and citrate respectively. That those losses were mostly due to plant enzymes was indicated by Playne's results (1964), who found a similar dissimilation of malic acid in silages made from sterile grass. The high evolution of $^{14}CO_2$ from citrate was expected since this salt was labelled in the 1, 5 positions, on the terminal carboxyl groups. The acid production in the period was not measured exactly since the first silos analysed were opened 24 hours after ensiling. From the results obtained with those silos it appears that the decrease in malic, citric and fumaric acids was accompanied by an increase in acetic, lactic and succinic acids. It is not known whether the acid increases occurred during the initial aerobic phase or in the period from 18 to 24 hours after ensiling. From the results of

Playne (1964) using sterile grass, it can be speculated that some of the accumulation of acetate and succinate could have occurred during the phase of aerobiosis, being due to plant enzymes. However, even in that case, it is possible that the accumulation of acids took place after anaerobic conditions became established. Further work would be required using sterile grass and pure strains of silage bacteria to establish the importance of the waste of nutrients in the period when anaerobic conditions become established in the silo. Very short incubation times should be used. This would also show if it is useful to resort to the technique of vacuum silage such as recommended by Doutre (1964) in order to shorten the period of aerobic respiration.

The initial aerobic phase of $^{14}CO_2$ production was followed by a quieter phase lasting from about the 18th hour after ensiling to about the 60th hour. Carbon dioxide production was maintained at a constant low level in the silos labelled with malate and citrate, but reached a new maximum in the silage labelled with glucose. Gas production in this case can be attributed to the action of microorganisms. Losses of ^{14}C-labelled carbon as $^{14}CO_2$ in the period were 2.8 per cent for malate, 4.8 per cent for citrate and 8.4 per cent for glucose silages. The main changes in organic acid content, as judged from the difference between the 24-hour and the 48-hour samples, were a sharp increase in succinic acid content and lower increases of acetic and lactic acids. A large proportion, but not all of the succinic acid formed, arose from

malate as shown by the increase in total activity in the succinic acid fraction and the decrease in specific activity of the same fraction. Contrarily to the previous period, malate was not an important source of lactate. In fact, both the specific activity and the total activity of the fraction decreased, indicating that lactate was utilized. The utilization of lactate does not appear to have been due to the action of *Clostridia* since butyrate is the usual product formed and butyrate was absent in the 48-hour silages.

A third phase was observed in the silages when $^{14}CO_2$ production was low for all treatments. It lasted from the 60th hour to the end of the conservation period. In the period, the largest part of the residual malic and citric acid was utilized. Losses of $^{14}CO_2$ were 1.4 per cent from malate, 6.0 per cent from citrate and 2.4 per cent from glucose. The most significant change in fermentation products was the apparition of butyric and propionic acids, an indication of the development of *Clostridia* or similar organisms.

The detail of the chemical transformations which took place during ensilage is difficult to establish, even with the use of tracers, in view of the variety of agents responsible for the changes. Metabolic changes take place immediately on contact of radioactive compounds with the plant material. The suggestion of Ruschmann (1939) that bacterial multiplication and fermentation take place in the juice exuded from plant material, rather than in plant cell, is disturbing when interpreting changes in silage made with tracers. It means that in

the initial stages of ensilage the tracer will be dissimilated faster than the real compound in the plant, since it is readily available for fermentation, while the compound in the plant will become available only after it has passed into solution. Our results supported Ruschmann's suggestion. After 31 days of ensilage, the specific activity of citric acid was less than half the original specific activity, and the specific activity of malate was only 2.7 per cent of the original. The low specific activity of malate can be explained by the formation of malate during ensilage, for example, during fixation of carbon dioxide as discussed earlier. Citrate formation is not expected, except in the initial aerobic stage. The presence of labelled citrate in the silage to which radioactive malate had been added shows that the oxidation proceeded through the complete Krebs cycle.

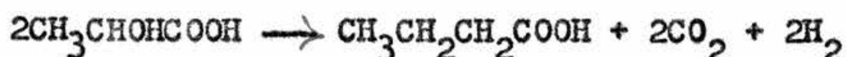
It appears therefore that the sequence of events during incubation was the following: during the initial aerobic stage, the action of plant enzymes and of aerobic bacteria resulted in the dissimilation of malate, citrate, and glucose. Carbon dioxide was the main compound formed but substantial quantities of ^{14}C from malate were found in succinate. The fact that smaller quantities of succinate were formed from citrate is explained by the fact that the label was in the most labile position and was lost as CO_2. Fumarate decreased, but became labelled. Acetic acid was formed by the action of plant enzymes (Playne, 1964) and of coli aerogenes (Allen _et al_., 1937). Lactic acid was probably not formed to any extent in

the aerobic period. The second stage was a period of active fermentation by heterofermentative lactic acid bacteria. Malate was fermented initially with the production of carbon dioxide, succinate and lactate. The reaction then shifted in favour of succinate, and some of the lactate formed was converted to succinate or carbon dioxide. The increase in pH was probably responsible for the shift. It appears that many *Lactobacilli* produce succinate in preference to lactate at high pH. This was observed by Carr (1959) with *Lactobacillus pastorianus* and by Playne (1964) with *Lactobacillus plantarum*. Later on, when the pH decreased, lactate was again formed from malate, and this was visible despite the utilization of some of the lactate by *Clostridia*.

Citrate was fermented to succinate, lactate, acetate and carbon dioxide. According to Phillips *et al*. (1956), lactate and acetate are the main products formed by lactic acid organisms when citrate is metabolized. The high pH of the silages may have been responsible for the shift to a succinate fermentation. In the fermentation of malate, there was a decrease in total activity in lactate and acetate between the 24-hour and the 48-hour silages. In the citrate fermentation increases were observed. It may be that changes of small magnitude which were undetected in the malate fermentation on account of high total activity in the two acids became preponderant in the citrate fermentation. Changes due to carbon dioxide fixation could be of that category.

When the last silos were opened at the end of the 31-day

period of incubation, the fermentation was mostly of the clostridial type. Butyrate and propionate appeared and carbon dioxide was evolved. Carbon dioxide evolution was more evident in the citrate-labelled silage than in the malate or citrate silages. As mentioned earlier, it is generally assumed that butyric acid formation in silage is the result of the fermentation of lactate according to the following equation:



$$2CH_3CHOHCOOH \longrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

Assuming uniform labelling of the lactate molecule - an assumption which is reasonably safe for the fermentation of uniformly-labelled malate to lactate, unless considerable fixation of carbon dioxide has occurred - it can be expected that the specific activity of butyrate will be 1.25 times the specific activity of lactate. In fact, the specific activity of butyrate was only 31 per cent of that in lactate. It appears that some butyrate was formed from compounds other than lactate, possibly from carbohydrates or amino acids. Propionate was a side product of the clostridial fermentation and arose partly from succinate.

Much has been said about the buffering capacity of plants and its effects on silage fermentation (Common, 1941; McDonald and Henderson, 1962; Playne, 1964) and it has been shown that organic acids are the main buffering agents in plant materials and in silages (Playne, 1964). It has even been suggested that selection could be made on the basis of low acid content when considering plants for ensiling (Playne, 1964). It appears,

however, that it is not so much the acid content of plants which is responsible for the difficulties encountered in the conservation of some crops as the fact of the extensive decarboxylations of those acids during the early stages of ensilage. The formation of volatile bases by plant enzymes in the early stages of ensilage is also instrumental in maintaining a high pH.

For a proper understanding of the biochemistry of ensilage, we need to know more about the changes taking place in the early period after ensiling. The action of plant enzymes, especially proteolytic enzymes and decarboxylases, needs to be investigated further. The relationship between growth of lactic acid bacteria and amino acid availability may be a deciding factor in conservation.

VIII. SUMMARY AND CONCLUSION

A method was described for the separation of a wide range of acids in plant material and in silage. The method consists of cold extraction with dilute sulfuric acid, followed by purification of the extract on a silica gel column. The organic acids in the purified extract were separated quantitatively using a continuous gradient-elution system of eluants through a small silica gel column.

The extraction method was found to be more efficient for extracting citric, oxalic and fumaric acids from red clover than a method consisting of repeated ethanol extractions, followed by aqueous extractions and purification on ion-exchange resins.

Most of the acids of the Krebs cycle were detected in red clover. Glyceric acid accounted for more than 37 per cent of the organic acidity. The same acids were found in a second crop of wilted red clover. During ensilage, malic, citric and glyceric acids disappeared almost completely. Large quantities of lactic and acetic acids were produced.

When the method of separation of organic acids was used in conjunction with experiments where ^{14}C-labelled tracers were added to the grass at ensiling, the radioactivity in fractions eluted from the column could be measured directly by liquid scintillation counting on aliquots of the fractions after mixing with a suitable scintillator.

In a grass ensiled with molasses as an additive and ^{14}C-sucrose as a tracer, 85.9 per cent of the activity was

recovered in a silage extract and 5.5 per cent in the silage effluent. Of the activity present in silage, 15.9 per cent was in acetic acid, 12.0 per cent in lactic acid and 5.5 per cent in butyric acid.

It was found that carbon dioxide was fixed during ensilage. Labelled ^{14}C from $^{14}CO_2$ was recovered in lactic, succinic and acetic acids and in amino acids.

In experiments with perennial ryegrass to which ^{14}C-labelled malate, citrate or glucose had been added, it was found that a large proportion of the radioactivity was lost as $^{14}CO_2$ in the initial 15 to 18 hours after ensiling. Subsequent losses of $^{14}CO_2$ were small. The largest part of the residual ^{14}C-activity from malate was recovered in succinic and lactic acids. Small quantities of radioactivity were also found in butyric, acetic and propionic acids and traces in three unidentified acids. It appeared that the radioactive substances added at ensiling were metabolized more readily than the real compound present in the plant cell.

The results presented indicate the need for detailed investigations on the bacteriological changes and the chemical changes in the period immediately following ensiling.

IX. PUBLISHED WORK

Lessard, J. R., and McDonald, P. (1966) A silica gel chromatographic procedure adapted to liquid-scintillation counting of ^{14}C-labelled organic acids from plant material and silage. J. Sci. Fd. Agric. 17: 257.

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APPENDIX I

Tests for particular acids on paper chromatograms

Test 1 - Indicator spray

The reagent due to Hargreaves and modified by Aronoff (Techniques of Radiobiochemistry, The Iowa State College Press, Ames, Iowa, 1957) is used. It is prepared by dissolving 50 mg of methyl yellow and 150 mg of bromphenol blue in 400 ml of 95 per cent ethanol. The solution is adjusted with sodium hydroxide to give optimal sensitivity.

The chromatograms developed in acidic solvents are sprayed without drying the paper completely. Spots varying from yellow to red appear against a blue-green background. Cations register as blue spots.

Test 2 - Ammoniacal silver nitrate spray

The reagent described by Brown (1951) is particularly useful for chromatograms developed in ammoniacal solvents. It is prepared by mixing equal volumes of 0.1 M $AgNO_3$ and 5 N ammonia. The sprayed sheet is dried at room temperature away from direct sunlight. Spots are examined in daylight when the background has become a light tan colour. Yellow spots appear for glyceric, glycolic, lactic, quinic and shikimic acids. In addition, glyceric acid has a purple edge around the spots. Other acids and many other compounds

appear as spots of various shades. Carles, Schneider and Lacoste (Bull. Soc. Chim. biol. 40:221, 1958) have presented the results of a survey of the reaction of over 60 acids to ammoniacal silver nitrate.

Test 3 - Cartwright and Roberts Reagent (1955)

The chromatogram is sprayed with a saturated solution of sodium metaperiodate diluted with 2 volumes of water and, 20 minutes later, with a solution of 0.5 g each of sodium nitroprusside and piperazine in 20 ml of water. The nitroprusside-piperazine solution is mixed with 100 ml of ethanol before spraying. Shikimic and glyceric acids appear in a few minutes without heating, the latter as a white spot and the former as a yellow spot. On heating the sheet for 5 minutes at 100°C, quinic acid appears as a yellow spot and dihydroshikimic as a greenish-yellow spot. Other acids do not react.

Test 4 - Potassium ferrocyanide reagent (Martin, 1955)

The chromatographic sheet is sprayed heavily with aqueous 10 per cent potassium ferrocyanide and allowed to dry. The sheet is then sprayed with 0.5 per cent ferric ammonium sulfate in 70 per cent ethanol and allowed to dry. Spots which develop at this point are marked and the colour noted.

After heating for 5 to 10 minutes at 100°C the chromatogram is discolourized with 10 per cent aqueous ammonia. Spots appear on drying which are often

characteristic. Fumaric and glutaric acids appear as duck-egg green spots. Oxalic acid gives a bright blue spot. The colour reactions of many other acids have been presented by Carles, Schneider and Lacoste (1958).

Test 5 - Diazotized p-nitroaniline reagent (Swain, Biochem. J. 53:200, 1953).

A volume of 25 ml of a 0.3 per cent solution of p-nitroaniline in 80 per cent hydrochloric acid is mixed with 1.5 ml of 5 per cent sodium nitrite solution just before spraying. After drying, the sheet is sprayed with a 20 per cent solution of sodium carbonate.

Malonic acid appears as a green spot. It is the only acid other than the aromatic acids to react with diazotized p-nitroaniline (Wall *et al.*, 1961).

Test 6 - Aroyl-glycine reagent (Smith, I., "Chromatographic and Electrophoretic Techniques", Vol. 1, London. William Heinemann Medical Book Ltd., 1958).

One volume of 5 per cent p-dimethylaminobenzaldehyde in acetic anhydride is mixed with 4 volumes of acetone.

Aconitic acid appears as a violet spot even before heating. Fumaric acid gives a yellowish spot. Citric, α-oxoglutaric and itaconic acids appear as pink spots.

Test 7 - Semicarbazide reagent (Magasanik and Umbarger, 1950)

The developed chromatogram is sprayed with 0.1 per cent aqueous semicarbazide and when dry with 0.15

per cent aqueous sodium acetate. α-oxoacids show as dark areas against the fluorescent background when examined under U.V. light.

Test 8 - Ultraviolet absorption

Acids containing conjugated double bonds such as fumaric and aconitic acids appear as dark areas on chromatograms when examined under U.V. light.

APPENDIX II

Time of sampling and $^{14}CO_2$ production from silages incubated with labelled citrate, malate and glucose (mμc per silo)

Sample No.	Hours after ensiling	Treatment		
		^{14}C-citrate	^{14}C-glucose	^{14}C-malate
1	4	998	672	963
2	7	384	156	190
3	11½	651	225	307
4	14½	253	40.9	85.1
5	17½	59.4	34.9	14.6
6	20½	63.4	73.5	39.8
7	24	35.7	40.0	25.7
8	27	74.2	104.4	43.1
9	30	44.7	78.6	19.8
10	33	44.4	73.2	40.1
11	36	45.4	57.5	33.3
12	39	12.6	36.0	19.5
13	42	16.1	62.0	20.0
14	48	79.1	67.5	27.5
15	60	67.2	83.9	14.9
16	87	140.3	83.7	50.3
17	111	57.4	25.5	22.6
18	136	56.9	43.6	31.5
19	206	25.0	26.5	7.4
20	752	318.7	15.3	70.4