

INVESTIGATIONS ON THE RÔLE OF ORGANIC MATTER IN PLANT NUTRITION.

Part IV. Chemical and Biological Transformations attendant on the application of Cane Molasses to Swamp Soil.

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Received October 6, 1934.

DURING recent years, a number of enquiries have been directed to the study of the decomposition of organic matter in the soil. Considerable amount of valuable information relating to the formation of plant food under different conditions and its bearing on crop production has been obtained. In spite of these useful advances, our knowledge of the mechanism of the related processes is still comparatively meagre. This is particularly so under conditions of dry cultivation in which the changes are so rapid that the intermediate products cannot be easily followed. Thus, the stages through which a cellulosic material has to pass prior to oxidation to carbon dioxide are still obscure. In a like manner, the various steps that lead to the transformation of an organic manure into fertilising ingredients such as potash and phosphate are yet largely unknown. The position with regard to swamp soils is somewhat better defined. Some of the intermediate products are known (Harrison and Iyer, 1913, 1914; Subrahmanyam, 1929). Evidence has also been obtained to show that the products of decomposition play a part in the dissolution of minerals (Ramaswami Sivan, 1925) and in determining the composition of the different mineral fractions of the soil (Sreenivasan and Subrahmanyam, 1934). More detailed information is nevertheless wanting regarding the different products of decomposition and their bearing on the release of plant nutrients from the soil.

The study of the decomposition of cane molasses presents certain unique features of academic as well as practical interest. Being essentially a mixture of readily fermentable sugars with a small percentage of minerals and nitrogen, its application to soil raises various problems of fundamental importance. The nature of the agents that are responsible for the decomposition, the type of products that are formed and their influence on various soil constituents and on general plant growth bring the practice of molasses fertilisation into a class different from most others in common use. On the

practical side, the interest in the research lies in the fact that the abnormally rapid development of the sugar industry in different parts of the World, and more recently in India, has rendered the disposal of the by-product, molasses, an acute problem (Subrahmanyam, 1933). Perhaps the cheapest method of disposal and, at the same time, a convenient way of returning to the soil a large part of the fertilising ingredients removed by the cane would be to utilise the surplus molasses as a fertiliser. The extensive field experiments conducted in Java, Hawaii, Queensland, Natal and elsewhere would, in fact, show that, under favourable conditions, large increase in crop yield may thus be obtained. The researches of the Java workers have also shown that the fertilising value of molasses is largely due to the sugars present in it. It is hardly probable, however, that the sugars could have any direct influence on plant growth. The previous workers (De Sornay, 1919; Tempany and Giraud, 1924; Heck, 1929 and 1930) have drawn attention to the fact that they are mostly decomposed within the first few days after application of molasses to soil. This observation combined with the fact that at least a few weeks have to lapse between manuring and planting would show that the fertilising action is due to the products of decomposition of molasses in the soil. The latter include different organic acids and gases, the precise nature and behaviour of which have not yet been systematically investigated. Some of the micro-organisms associated with the decomposition of molasses in the soil have been described by previous workers (Tempany and Giraud, *loc. cit.*; Kerr, 1932), but further information is needed before the rôle of those and other organisms in the production of plant food can be defined. It is known that the immediate products of decomposition, especially when the molasses is applied in large quantities, are toxic to plant growth (Arrhenius, 1929; Kerr, *loc. cit.*). The nature of the toxic materials and the manner in which they function are still obscure. It need hardly be pointed out that this aspect of the problem is of considerable practical importance and requires thorough investigation. The conditions relating to the application of the molasses should be so standardised that the formation of the toxic materials is either avoided or, at any rate, reduced to such an extent that crop yield is not affected. In addition to these, there is also the need to study the economy of carbon and nitrogen at various stages during decomposition and to devise means to conserve the two constituents, as far as possible, in the soil. The present enquiry was undertaken with the object of throwing some light on the above and related problems.

Experimental.

Materials.—The soil used in the present study was a red, sandy loam of the type commonly met with in many parts of the Deccan plateau. It was

collected from an uncultivated area where there was no vegetation except for a few occasional weeds. The samples were taken to a depth of 9 inches and brought to the laboratory where they were mixed together to make one composite mass and spread out to dry. The air-dried product was freed from adhering roots and other plant residues and then ground to pass the 20-mesh sieve. Since the powder thus obtained represented a wide range of particle sizes, it was re-sieved through the 30-mesh. The particles then remaining on the sieve were used for the biochemical studies described in the present paper.

The soil was analysed for its mechanical composition and the various chemical constituents. The former was determined according to the International method (A.E.A., 1926) and the latter generally according to A.O.A.C. (1930) methods. Total nitrogen was estimated by a method of wet digestion (Sreenivasan and Subrahmanyan, 1933) and total carbon according to the modified method of Narayanayya, Bhagvat and Subrahmanyan (1934). The following were the results :—

Mechanical Composition.

Item	Per cent.	Item	Per cent.
Coarse sand ..	33.4	Silt	7.7
Fine sand ..	26.4	Clay	26.4

Chemical Analysis.

Item	Percentages on air-dry basis	Item	Percentages on air-dry basis
Moisture	3.84	Total Phosphoric acid (P ₂ O ₅)	0.02
Loss on ignition (on oven-dry material) ..	3.19	Total potash (K ₂ O) ..	0.22
Total carbon	0.72	Total lime (CaO) ..	0.10
Total nitrogen	0.04	Total Silica (SiO ₂) ..	77.76
Total carbonate ..	Nil	Iron and alumina (Fe ₂ O ₃ + Al ₂ O ₃) ..	13.75

					Parts per Million
Ammonia (as N)	3.0
Nitrite	0.2
Nitrate	0.2
<i>Numbers of Micro-organisms (in 1 g. of soil)—</i>					Millions
Bacteria (Waksman, 1922)	15.6
,, (Thornton, 1922)	9.0
Actinomyces (Ganesha Rao and Subrahmanyam, 1929)	0.2
Fungi (on wort agar)	1.6
P _H (colorimetric)					6.8

The molasses used in the present research was a solidified product originally imported from Java. Its composition, as determined according to A.O.A.C. methods, was as follows:—

Item	Percentages on moisture-free basis	Item	Percentages on moisture-free basis
Loss on ignition	89.84	Total phosphoric acid (P ₂ O ₅)	0.12
Total carbon	30.70	Total potash (as K ₂ O)	4.20
Total nitrogen	0.35	Total lime (as CaO)	1.72
Sucrose	34.20	Total silica (SiO ₂)	0.44
Reducing sugars (as invert sugar)	24.70	Iron and alumina (Fe ₂ O ₃ + Al ₂ O ₃)	0.44

P_H (colorimetric) 5.3

The molasses was applied to soil as follows:—The dry soil was weighed into a suitable glass container and the molasses (in solution) added in quantity corresponding to 1 per cent. on the weight of the soil (about 10 tons per acre). The soil was then treated with two and a half times its weight of water which proportion was found to be sufficient to keep it well submerged and to yield useful quantities of supernatant for different types of chemical and biological studies. The containers were then plugged with cotton wool and the suspensions kept in wire gauze cages sheltered from rain but otherwise exposed to the prevailing weather conditions.

*Meteorological Observations.**—The researches described in the present paper were carried out from the beginning of March to the end of August 1934. The earlier part of the season which corresponded to that of a severe cold wave which swept through North India showed certain unusual features. The days were fairly warm (Max., 85-88°F.), but the nights were comparatively cold, the temperature dropping by about 30°. The relative humidity also became low falling by about 50 per cent. This condition lasted, however, for only a few days after which warm weather set in, the temperature reaching a maximum of about 98° F. during the months of March, April and May. The days were also bright, the hours of sunshine ranging from 7 to 10. With the outbreak of the South-West Monsoon in the beginning of June, there was marked change in the weather conditions. Occasional showers of rain helped to lower the temperature, the maximum rarely exceeding 85°F. The average relative humidity increased to over 80 per cent. while the frequently clouded sky reduced the hours of sunshine to an average of about 4 per day. The total rainfall was, however, rather poor and did not aggregate to more than 4 inches in any month.

Viewed from the biological point, the earlier part of the season with its wide range of temperature might have been somewhat unfavourable to the progress of decomposition. The warm weather which followed would have been quite helpful while the cooler monsoon period should have slightly retarded the subsequent transformations. It is hardly probable, however, that the effects of the season were so pronounced at any time as to seriously affect the course of the reactions.

Decomposition of Sugars.—The determinations were carried out on separate lots of soil suspensions each made up of 50 g. of soil, 0.5 g. of molasses and 125 c.c. of water. The sugars together with other soluble substances were first separated by filtration. The filtrate was clarified with alumina cream which was found to be the most suitable for the purpose (*vide* Subrahmanyam, 1929). The sugars were estimated by the Bertrand method both before and after hydrolysis. The hydrolysis was carried out according to a modification of the original Clerget method, it being found that less caramelisation occurred on heating with dilute acid (10 c.c., 5 per cent.) for 1 hour than with concentrated acid for 5 minutes. The results have been presented in Fig. 1.

It may be noted that during the first 48 hours there was no appreciable change in the sugar content. After this period, there was

* The data were obtained through the courtesy of the Meteorologist to the Government of Mysore to whom the authors' thanks are due.

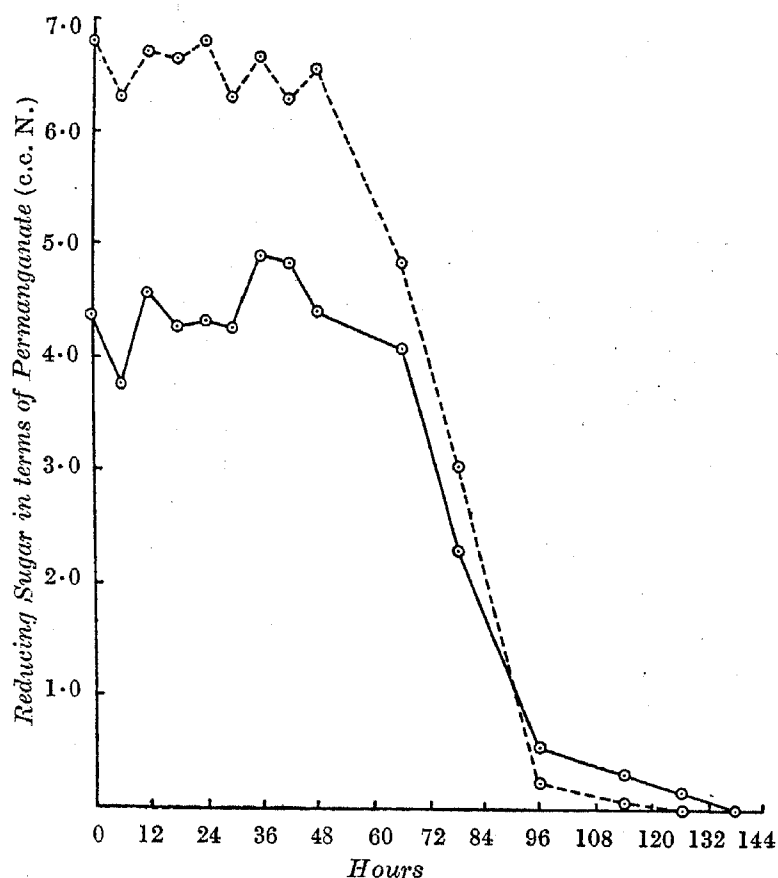


FIG. 1. Decomposition of Sugars

○—○ Before hydrolysis ○---○ After hydrolysis

very rapid fall, the rate being fastest between 72 and 96 hours. Both sucrose and invert sugar were affected to nearly the same extent. This was only to be expected since parallel studies had shown that the associated micro-organisms secrete invertase. It was mostly invert sugar that was fermented after 72 hours. After about 90 hours there was an apparent anomaly, the reducing matter before inversion being greater than that after hydrolysis. This observation suggested that the reduction observed at that stage was not so much due to sugars as to some other substance which reacted with Fehling's solution but was either volatilised or decomposed on heating with dilute mineral acid. Later observations showed that the reducing substance was acetaldehyde which was present in minute quantities during the first few days of decomposition. In view of this finding, it may be concluded that the decomposition of sugars was practically complete at the end of 96 hours.

Hydrogen-ion Concentration.—This was determined colorimetrically, a Hellige comparator being used for the purpose (Fig. 2). The reaction

remained more or less constant (about P_H 7.0) for 42 hours after which there was a rapid fall to P_H 6.0 at the end of 48 hours. Further change which was spread over a number of days was rather slow, but a P_H of 5.0

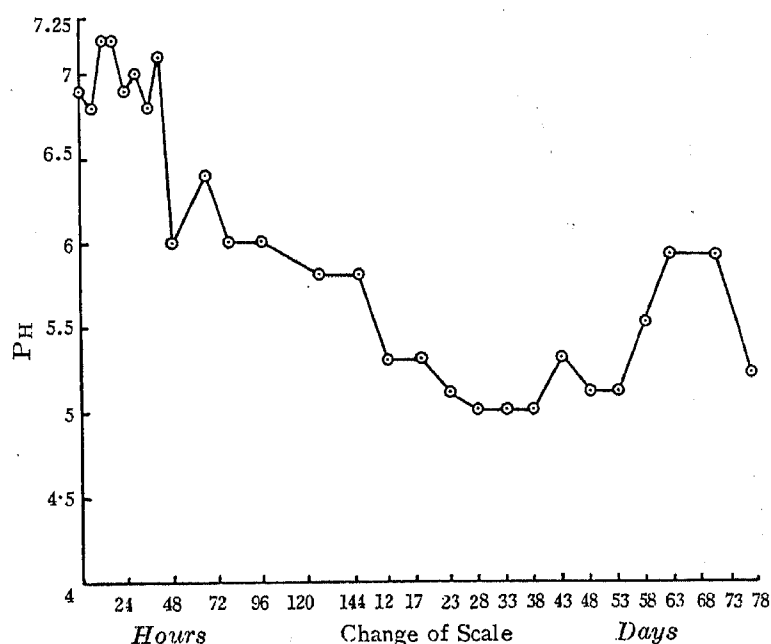


FIG. 2. Hydrogen-ion Concentration

was attained by about the 28th day. After being steady at that level for a few days, the reaction tended to become less acid, the P_H becoming 5.9 on the 71st day. Subsequent change was not pronounced and has therefore not been recorded.

Buffering Capacity.—This was measured by determining the quantity of alkali (c.c. N/10) required to shift the P_H over a constant range, 6.8—8.0 which correspond to the colour changes of bromothymol blue and phenolphthalein respectively. The supernatant (5 c.c.) was first adjusted to P_H 6.8 by addition of the necessary quantity of acid or alkali as the case may be and then titrated in the usual way until P_H 8.0 was attained. The quantities of alkali required at different stages for unit shifts of P_H have been presented in Fig. 3.

In the early stages, the buffering capacity was comparatively low and remained more or less the same for the first 66 hours after which there was rapid increase, attaining a maximum (4.3 c.c.) by the 126th hour. After being again stationary for about a week, it then declined at a fast rate reaching ultimately such a low figure that it was not measurable on the 71st and 77th days.

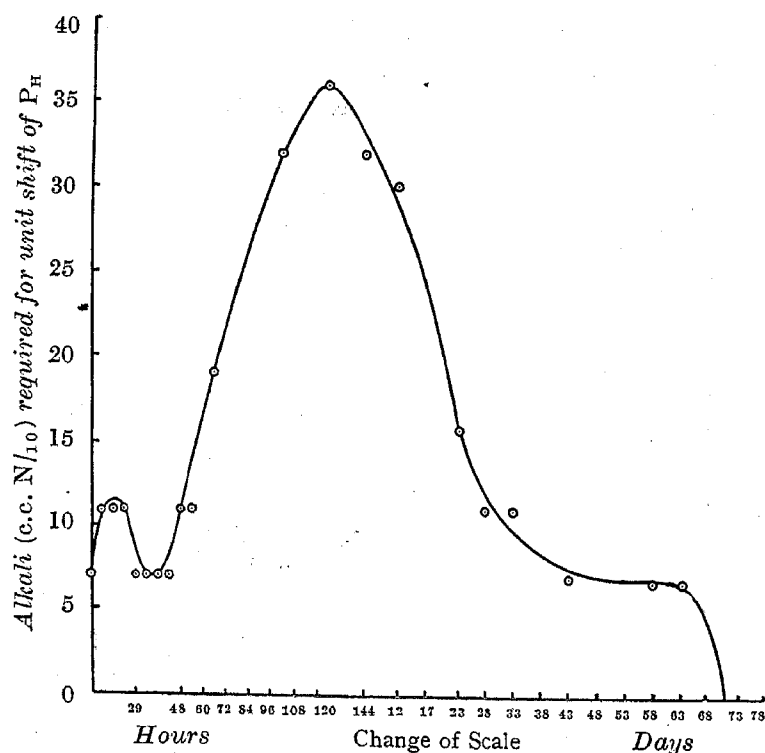


FIG. 3. Buffering Capacity

The estimates for H-ion concentration, together with the corresponding buffering capacities, are of some practical interest. They show that in the early stages, especially between the 5th and the 12th days, the reaction of the soil cannot be easily disturbed, so that the acids, together with the buffering salts, will exercise a definite physiological influence on the crop which may then be growing. Since there is evidence to show that the materials then present in the soil are toxic to plant growth, the present practice of allowing the soil to rest for a few weeks is justified on this account. After the 12th day, although the reaction is still acid, the buffering capacity diminishes so rapidly that even a slight disturbance in the soil system would suffice to restore the neutral condition.

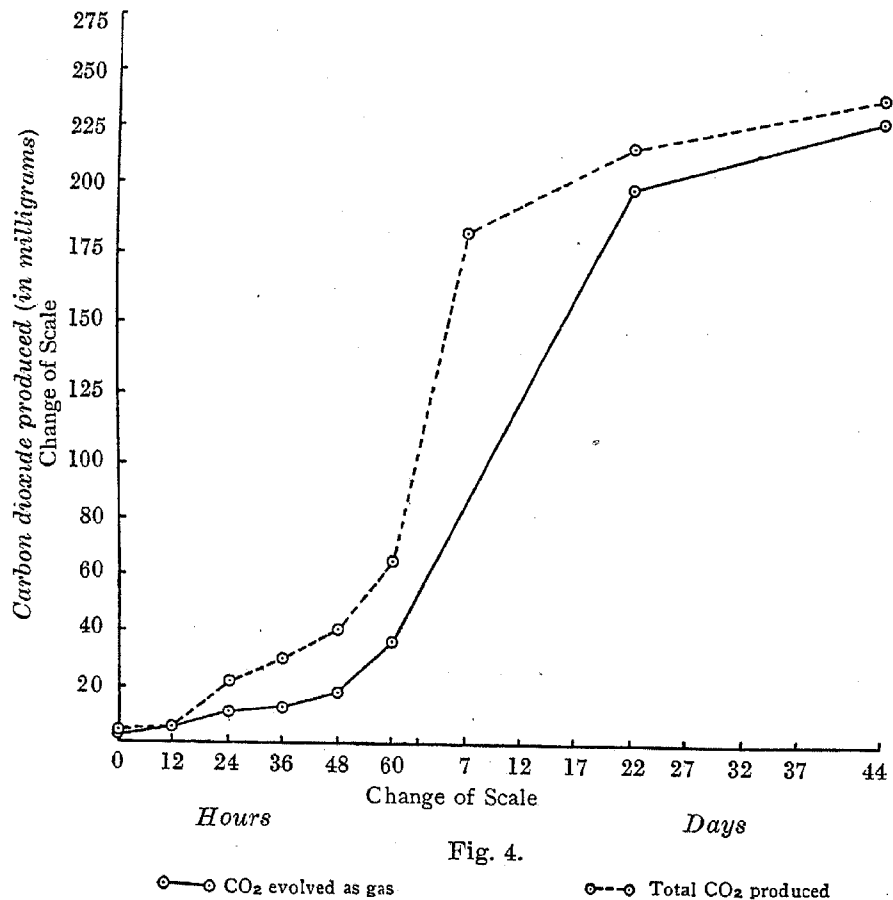
Carbon Dioxide.—The soil (50 g.) was weighed into a number of conical flasks (cap. 200 c.c.) and treated with appropriate quantities of molasses and water. The fermentation was then allowed to proceed in the usual way and the CO₂ produced estimated under three heads: (a) given off as vapour, (b) present in the space above, and (c) dissolved in the supernatant. The first was trapped in alkali in the usual way. The second was displaced by CO₂-free air and estimated separately. There was some difficulty, however, in determining the gas present in solution. The quantities were so small that none of the commoner methods could be applied with

satisfactory results. After a number of trials, the following procedure was adopted.—An aliquot (10 c.c.) of the supernatant was carefully pipetted out into excess of standard alkali without in any way disturbing the liquid layer. The unused alkali was then estimated in the usual way. Another aliquot of the liquid was transferred to a small beaker diluted with excess of water (125 c.c.) and boiled for 1 minute so as to drive off all dissolved carbon dioxide. The same quantity of alkali was then added to it and the unused quantity determined as before. The difference between the back-titre values thus obtained would correspond to the carbon dioxide present in solution. The significance of the above procedure is evident. The first estimate would correspond to carbon dioxide in solution *plus* such substances in solution as could react with the alkali. The second one would provide the correction for the latter. The accuracy of the method was tested by applying it to soil suspensions containing known quantities of carbon dioxide in solution and it was always found to yield accurate results, even in presence of volatile fatty acids. By suitable dilution and restricting the time of boiling, it was possible to reduce the loss of such acids to a negligible figure.

It was noted that the estimates for carbon dioxide passing out of the experimental flask and that was present in the space above were discrepant when determined individually, but yielded more concordant values when added together. This was traced to the fact that although the total quantity of gas given out might be the same in each case, absorption by dilute alkali was not uniform so that the separate estimates were discordant. Since both the forms were only portions of carbon dioxide given off as gas, it was decided to take them together as a single determination.

A further difficulty that complicated the estimation of carbon dioxide was the uneven fermentation in parallel samples. Although much care was taken to ensure proper distribution of soil particles, the associated microflora were still not uniformly distributed—a phenomenon over which there is no control—with the result that while most of the data followed a certain order, there were still some which were quite abnormal and had to be discarded. The quantities for dissolved carbon dioxide also showed a tendency to fluctuate considerably. Taking all these facts into consideration, only the figures for total carbon dioxide (including that in solution) and that which passed out of the soil system as gas have been presented in Fig. 4.

It may be seen from the figure that both the sets of results follow the same order, the quantities steadily increasing with time. For the first 60 hours, the production of gas was comparatively slow, but during the following days considerable quantities were produced. After the 7th day, the rate slackened again though small quantities continued to be produced



throughout the period under observation. As may be naturally expected, the major part of the gas passed out of the flask and was therefore lost to the soil system. The quantities present in solution were invariably small, being under one-tenth of the total amount produced.

Other Gaseous Products of Fermentation.—The gases were collected over water and examined at weekly intervals. It was observed that in addition to carbon dioxide, there were appreciable amounts of hydrogen and small quantities of hydrocarbons among the products of the first week. Small amounts of oxygen were also found but this was presumably derived through displacement of the gas originally present dissolved in the water. During subsequent weeks, the gas evolution tended to diminish. The bulk of it was still carbon dioxide, the total percentage of other gases dwindling to a negligible figure. Carbon monoxide could not be detected at any time during the fermentation. Absence of hydrogen sulphide and ammonia could not be taken to mean that those gases were not formed. Indeed, as observed by Robinson (1930) in the case of submerged soils, increasing quantities of sulphides (especially that of iron) could be detected in the soil sediment thereby

suggesting that hydrogen sulphide was one of the products of the fermentation. In a like manner, ammonia may have been retained as such by the soil complex or combined with organic acids formed during fermentation.

The quantitative studies relating to the gaseous products of fermentation will form the subject of a later communication.

Volatile, non-acid Products of Fermentation.—A number of flasks containing suspensions made up of 100 g. of soil and the corresponding amounts of molasses and water were prepared and allowed to undergo fermentation. At daily intervals for one week and at weekly intervals in the later stages, the flasks were taken out one at a time and the contents examined as follows:—The soil suspension was passed through a coarse filter and the filtrate rendered alkaline. It was then distilled at atmospheric pressure and the distillate tested for the presence of different volatile constituents. It was observed that ethyl alcohol and acetaldehyde were present throughout the first week, but subsequently they could not be detected. Furfural and acetone bodies were absent throughout the period of observation. The distillate contained minute quantities of a volatile substance possessing a rather unpleasant odour. Qualitative tests showed that it was neither an ester nor an alcohol. It was found only in traces so it was not possible to identify it.

In the course of the above study, it was noted that Schiff's reagent is not by itself a sensitive indicator for the presence of aldehydes. Various inorganic substances which can react with sulphurous acid yield a positive reaction even in the absence of aldehydes. Thus, tap water which contains minute quantities of lead and other metals gives the reaction though rather slowly. The error can be eliminated, however, by adding a small quantity of a mineral acid (preferably hydrochloric) to the solution to be tested when only aldehyde gives the reaction.

Volatile Acids.—The alkaline fluid left after the previous distillation was rendered slightly acid and then steam-distilled. The distillate was then examined for its constituents especially volatile fatty acids. In addition to the usual qualitative and orientation tests (Dyer, 1917) the average distilling constants were also determined at each stage to confirm the observations. It was noted that in the early stages there was practically no volatile acid in the medium, but that after 48 hours, minute quantities of propionic acid were first detected. On the third day, both acetic and propionic acids were present. On subsequent days, the two acids were definitely on the increase as may be gathered from the intensity of the colour reactions and the total acidity of the distillate. On the fifth and subsequent days, the presence of butyric acid was also noticed, though only in minute quantities. Other fatty acids seemed to be absent.

Non-volatile Products.—The residue left after steam-distillation was neutralised and concentrated to a small bulk by evaporation on the water-bath. It was examined in the usual way for its various constituents. Lactic acid was noticed even at the end of the first day. On subsequent days, the quantities were apparently on the increase as may be gathered from the intensity of the reactions. The tests for pyruvic acid were throughout negative, but there was evidence for the presence of some other substance which gives the iodoform reaction after standing for some time with iodine and alkali. There are, indeed, indications to show that a part of the sugar is converted into some intermediate product before the volatile acids are formed but further work using large quantities of the fermented product is needed before its identity can be established.

Quantitative studies on the distribution of carbon at various stages are in progress and will be included in a later communication.

Mineral Transformations.

Dissolution of Minerals. Total Quantity.—The soil (300 g.) was weighed out into a number of flasks (cap. 1 litre) and treated with appropriate quantities of molasses and water. The suspensions were then allowed to ferment in the usual way. Another set of flasks containing only soil and water were maintained under similar conditions as controls. At two-day intervals during the first nine days and at weekly intervals during the later stages, the entire supernatant from one flask from each set was decanted out and passed through a coarse filter to remove suspended soil particles. The filtrate was transferred to an evaporating basin and concentrated on the water bath. The concentrate was then transferred to a platinum basin in which it was ignited at about 600°. From the weight of ash thus obtained, the quantities of minerals present in solution at each stage were calculated correction being made for the volume retained in the soil (Fig. 5).

The difference between the two sets of results is too obvious to need any comment. It may be noted, however, that in both the experimental and the control specimens, the quantities increased, upto a point after which they tended to decrease. This would suggest that the dissolved minerals reprecipitated partly on standing.

Iron and Aluminium.—The details were the same as those in the previous experiment. The estimations of iron and aluminium were carried out according to the A.O.A.C. methods.

It may be seen from Figure 6 that large amounts of both the metals were brought into solution as the result of application of molasses. The quantities increased very rapidly reaching a maximum on the 23rd day.

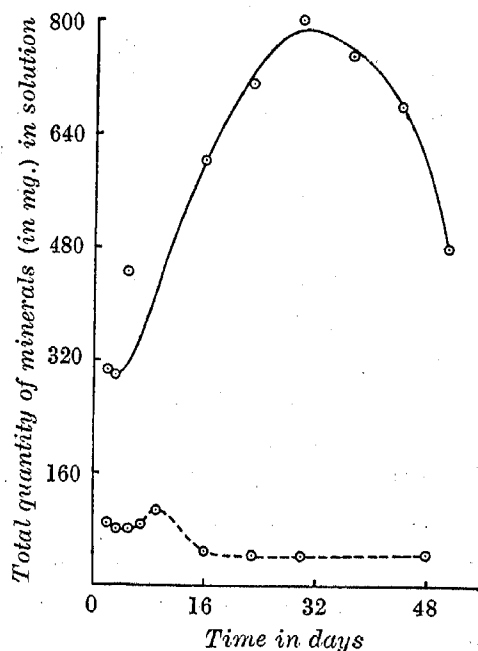


FIG. 5. Total minerals in solution

○—○ Soil treated with molasses
○---○ Untreated (Control)

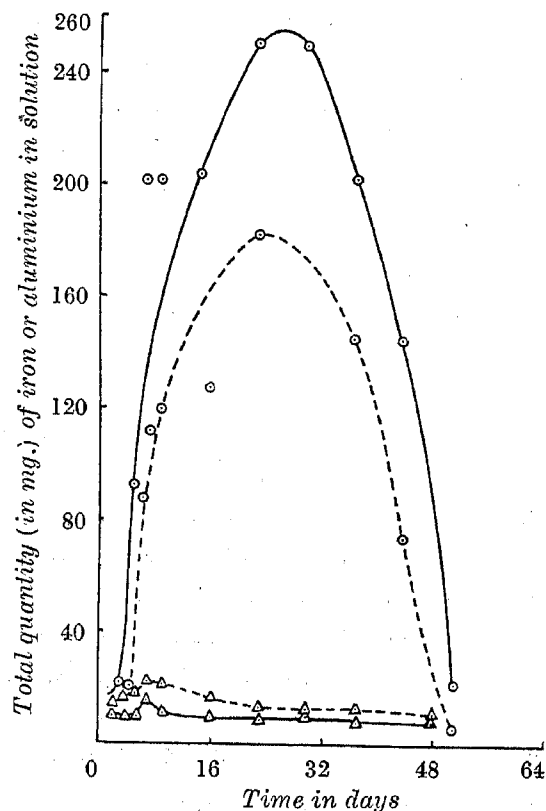


FIG. 6. Dissolution of Iron and Aluminium

○—○ Total iron as Fe₂O₃ (Experimental)
○---○ Total iron as Fe₂O₃ (Control)
△—△ Total aluminium as Al₂O₃ (Experimental)
△---△ Total aluminium as Al₂O₃ (Control)

After that period, they tended to decline at a fast rate attaining nearly the same level as the control on the 51st day.

A remarkable feature about the dissolution of iron was that it was present almost exclusively in the ferrous condition. After some time, however, it began to undergo spontaneous oxidation. Ferric oxide was present in the film formed at the surface and in the ring at the side. It was also found in the light, fluffy, red-brown layer which began depositing from the supernatant when the iron content of the latter was fast diminishing.

On heating the supernatant or blowing in a current of air, the ferrous iron present in it was readily oxidised. There was no separation, however, of any precipitate. This would suggest that the iron was not held in solution by carbon dioxide; for, if it were present, as ferrous bicarbonate, there should have been the immediate precipitation of hydrated ferric oxide, especially on heating. Furthermore, no more than traces of iron could be brought into solution even after prolonged bubbling of carbon dioxide through the soil

suspension. It is not probable therefore that carbon dioxide played any important part in the dissolution of iron or its retention in solution.

The available evidence is hardly adequate to throw light on the mechanism of reduction and dissolution of iron, but it may be of interest to mention that lactic acid which is one of the first products of fermentation reduces ferric iron (whether in the soluble or insoluble condition) and brings small amounts of ferrous iron into solution. When working with soil, laterite or precipitated ferric oxide, the products were found to be acetaldehyde and a soluble ferrous salt which was, presumably, ferrous lactate held in solution by excess of acid.

The concentration of iron and aluminium in solution, especially towards the latter part of the first month (about 300 and 250 parts per million respectively) is far above the tolerance limit for most crops and should therefore be regarded as undesirable. They affect plant growth and are especially toxic to seedlings which turn pale and die out prematurely (Pillai and Subrahmanyam, 1931). Even low concentrations of aluminium are known to injure root hairs and to cause stunted growth (Ligon and Pierre, 1932). In view of these and other observations, the present practice of allowing the soil to rest for some weeks before planting would appear to be justified. Alternatively, the molasses may be applied in small instalments at a time in irrigation water as in Java or suitably combined with lime, nitrogen and minerals that the formation of acid products and the consequent dissolution of iron and aluminium are greatly reduced.

The quantities of iron and aluminium present in solution at various stages may not be identical with those formed under field conditions, but it may be reasonably expected that both the dissolution and the precipitation of the two metals follow about the same order. The nature of the soil, extent of drainage and, at a later stage, the rate of flow of water at the surface would be some of the more important factors determining the rates of various changes. It would be ordinarily difficult to forecast when the toxic materials would be removed under field conditions, but in most cases, the usual period of 3—5 weeks should suffice. A more reliable procedure would be to use a test paper that would indicate the presence of toxic concentrations of iron or aluminium.

Phosphoric Acid.—The quantities of phosphoric acid present in the supernatant at different stages together with those in the corresponding controls have been presented in Fig. 7.

It may be seen from the figure that in neither the experimental nor the control samples was any large quantity of phosphorus brought into solution. The experimental samples contained slightly more than the controls, but

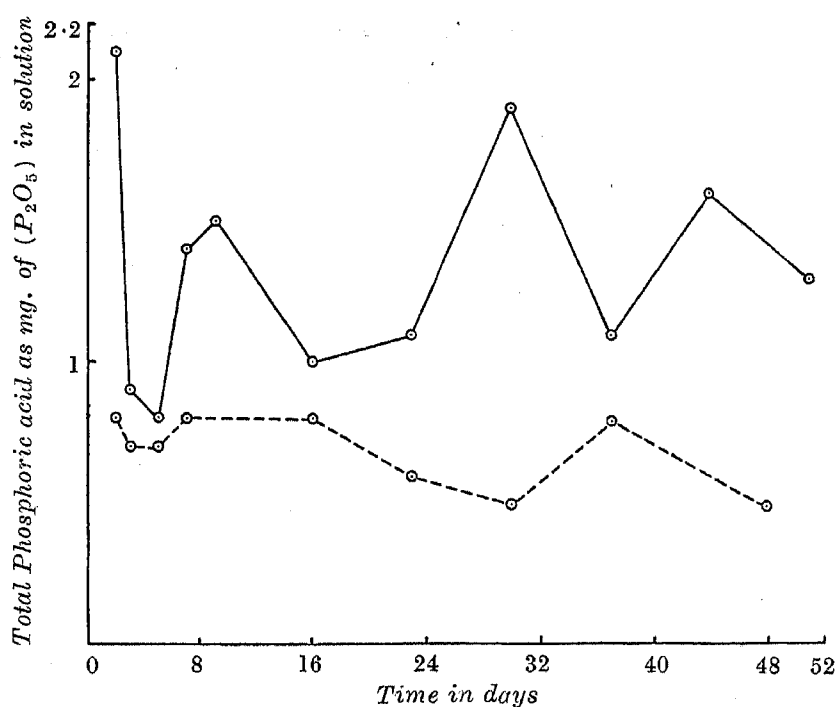


FIG. 7. Dissolution of Phosphorus

○—○ Experimental ○---○ Control

considering the quantities of soil (300 g.) and molasses (3 g.) taken, the difference should be regarded as being insignificant.

Although the medium was fairly acid, the poor dissolution of phosphorus may have been due to one of the following causes:—(a) low phosphorus content of soil and molasses, (b) phosphorus being present as iron or aluminium phosphate which is not easily soluble, or (c) the large quantities of iron and aluminium present in the supernatant combining with any phosphorus that may be brought into solution and thus precipitating it. The total quantity of phosphorus in the soil system was, no doubt, comparatively small (about 60 mg.) but it was nevertheless a substantial amount as compared with that in solution. A large part of the soil phosphorus was presumably present as iron or aluminium phosphate, as may be gathered from their low citric solubilities. The partial dissolution of those two metals should nevertheless have brought some of the associated phosphorus into solution. Since this could not be observed, it has to be inferred that the dissolved phosphorus, if any, was re-precipitated in the soil itself.

The above observations are apparently discordant with those of Sivan (*loc. cit.*) but it may be pointed out that the conditions in the related experiments were different. Sivan added excess of an insoluble phosphate which also contained useful quantities of lime to the green manured soil. In the

present investigation, there was only the small quantity of phosphorus in the soil and in molasses to act on. In Sivan's experiment, the lime must have partly helped to precipitate the excess of iron whereas, in the present experiment, there was none. Furthermore, it is probable that the gravelly soil used in the present study was partly responsible for the non-dissolution of phosphorus. It was particularly rich in iron and aluminium oxides and since the P_H remained throughout the experiment within the range 4.0—7.5, it cannot be reasonably expected that under such conditions any large quantity of phosphorus will pass into solution. It would be necessary therefore to carry out further experiments with other types of soils, with and without addition of lime and other minerals, before the relation between molasses fertilisation and availability of phosphorus can be established.

Calcium and Potassium.—The quantities of these two metals present in the supernatant at different stages have been presented in Fig. 8.

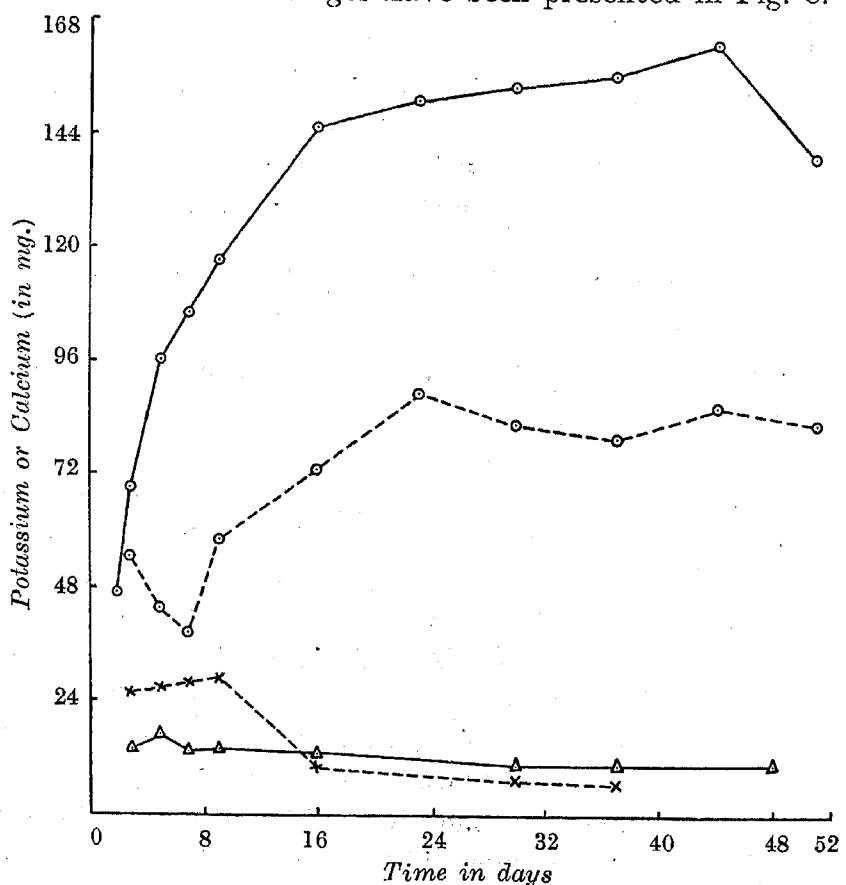


FIG. 8. Dissolution of Calcium and Potassium

○—○ Calcium as (CaO) in solution—(Experimental) ○—○ Potassium as (K₂O) in solution—(Experimental)
 ▲—▲ " " (Control) ×—× " " (Control)

It may be noted that in the experimental samples the quantities of calcium and potassium increased steadily with time, the former to a greater

extent than the latter. Even after the completion of the fermentation, the quantities continued to remain high and there was no indication of re-precipitation such as observed in the case of iron and aluminium. The control samples contained very much smaller quantities of the two metals.

A considerable part of the two metals in solution was presumably derived from the added molasses, but there is evidence to show that a part was also derived from the soil. Their persistence in solution would suggest that although a part may be useful to the crop, the rest would be either carried away by the surface-water or lost in drainage. This would constitute a serious loss and should be remedied by either altering the conditions relating to the application of molasses or making up by addition of lime and potassic fertilisers from time to time.

The mechanism of dissolution of calcium and potassium would appear to be the same as those of other metals, the organic acids and carbon dioxide taking part in the reactions. The formation of the corresponding salts would also account for the metals continuing to remain in solution even after completion of fermentation.

The removal of calcium and potassium, together with the precipitation of iron and aluminium, would point to a change in the mineral structure, at any rate in the surface layers, of the type reported by Sreenivasan and Subrahmanyan (*loc. cit.*).

Other Minerals in Solution.—The quantities of other minerals in solution including manganese, silicon, sodium and magnesium were calculated by adding together the values for known forms and subtracting the sum from the total mineral content. The estimates thus obtained were comparatively small. In the case of the experimental samples, they ranged from 49 to 230 mg. in 750 c.c. of supernatant. The quantities showed a tendency to decrease upto the 23rd day after which they rose steadily reaching the maximum on the 51st day. These observations would suggest that while some of the forms dissolved in the earlier stages were re-precipitated, others were brought into solution in the later period. In this connection, it may be noted that the dissolution of the above-mentioned minerals, taken as a whole, is inversely related to that of iron and aluminium. It would be of interest to determine if there is any chemical relation between the two sets of changes.

Quantity of Molasses and Loss in Solution.—Attention has already been drawn to the fact that the quantities of the different minerals present in the soil are considerably in excess of those brought into solution by the molasses. This process of dissolution may therefore be pictured as the result of

interaction between a large reserve of minerals in insoluble form and a small quantity of an active substance. From this, it would follow that within reasonable limits, the total quantity of minerals brought into solution would be proportional to that of the active substance (molasses) added to the soil. Reckoning on this basis, it should be possible to calculate the quantities of different minerals that would be brought into solution on applying molasses under swamp conditions. The estimates thus obtained would, no doubt, be only approximate, the actual quantities being determined by the nature of the soil, efficiency of drainage and such other factors. They would nevertheless be useful in indicating the probable losses that may be incurred in field practice. Calculations were accordingly made reckoning the quantities that may be dissolved by applying molasses at the rate of 10 tons to the acre.

Assuming that the major part of iron and aluminium would eventually re-precipitate in the soil, the bulk of the loss would relate to calcium (as CaO) and potassium (as K₂O) which may be estimated at 4,500 and 2,200 lbs. respectively which is indeed a considerable figure. The other minerals lost in solution would correspond to about 2,500 lbs. A small part of this would no doubt be derived from the molasses, but the bulk of it will have to come from the soil which will, in consequence, become poorer to the extent of about 4 tons of minerals. This quantity will have to be restored chiefly in the form of lime and potassic fertilisers if the fertility of the soil is to be maintained. Alternately, the conditions relating to the application of molasses should be so modified that loss by solution is either entirely eliminated or, at any rate, greatly reduced.

Exchangeable Bases.—After decanting out the supernatant for the estimation of minerals, the residual soil left at each stage was air-dried and the product used for the determination of exchangeable calcium, iron and aluminium (Hissink, 1922). The results have been presented in Figs. 9 and 10.

There was appreciable decrease in the exchangeable calcium present in the experimental sample while that in the control remained unaffected. A comparison with Fig. 8 would show that with the progress of dissolution, exchangeable calcium in the soil underwent steady decrease.

Decrease in exchangeable calcium, though not very considerable within the period of observation, would, if allowed to continue, have an adverse effect on the physical properties of the soil. The colloidal equilibrium may be disturbed and increasing quantities of unsaturated clay acids formed. The soil would tend to deflocculate and thus become less suitable for plant growth.

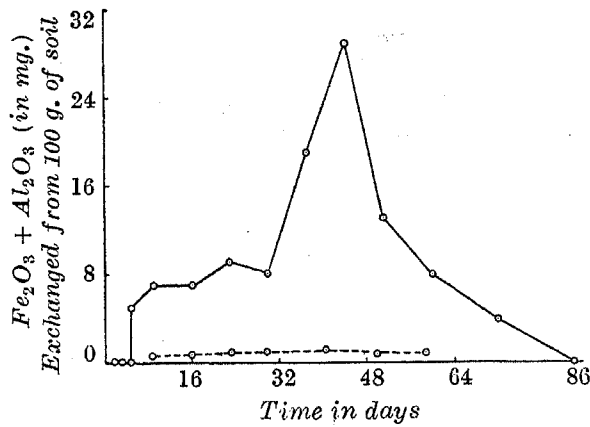


FIG. 9. Exchangeable Iron and Aluminium

○—○ Exchangeable Fe₂O₃ + Al₂O₃ (Experimental)
 ○---○ " " " (Control)

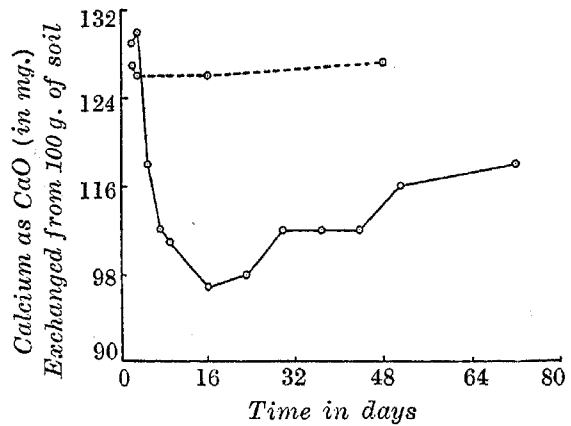


FIG. 10. Exchangeable Calcium

○—○ Exchangeable Calcium (Experimental)
 ○---○ " " " (Control)

There was practically no exchangeable iron and aluminium in the control samples while small quantities were present in the experimental ones. The latter tend to increase for a time and then decrease in the same manner as the dissolved minerals (*vide* Fig. 6). These observations would show that iron and aluminium in the original soil were present in resistant forms and could not be exchanged. The fermentation of molasses had, however, brought a part of them into solution and it may be reasonably expected that some of the dissolved matter still remained in the soil sediment. This would explain why exchangeable iron and aluminium followed nearly the same course as the dissolution of those two metals.

Biological Changes that attend the Application of Cane Molasses to the Swamp Soil.

Preliminary Observations.—Soil suspension containing molasses and water in the same proportions as in the previous experiments was prepared and divided between a number of tubes. The contents of the tubes were examined microscopically at 4-hour intervals for the first 24 hours and at daily intervals in the later stages. The observations were compared with those on controls which were made up with only soil and water.

It was observed that there was no perceptible difference between the two sets of samples in the early stages, the more prominent forms being only certain cocci, a few short, rod-like forms and some amoeba. The position continued to be so for about 20 hours after which there was a striking change, the experimental samples rapidly turning into a dense mass of long, motile rods, which increased at a phenomenal rate almost to the exclusion of other forms. This continued, however, only for a short period, and it was noticed even at the end of two days that the motile forms were definitely on

the decline. There was no further noticeable change in the experimental samples. Neither protozoa nor yeasts were at any time seen prominently in the medium. In the controls, the cocci and the amœba persisted and even seemed to increase for a time, but subsequently they too decreased thereby showing that the conditions were not favourable to microbial activity (*vide* Subrahmanyam, 1927).

Bacterial Numbers.—The soil was weighed out in separate lots (25 g. each) into a number of conical flasks and treated with appropriate quantities of molasses and water. The suspensions were then allowed to ferment in the usual way. At stated intervals, the experimental samples together with the corresponding controls were plated out, the entire contents of one flask being used for each set of dilutions. The platings were carried out at two dilutions and on two count media (Thornton, *loc. cit.*; Waksman, *loc. cit.*).

It may be seen from the results (Figs. 11 and 12) that the plate counts do not even indicate the increase observed by direct examination during the

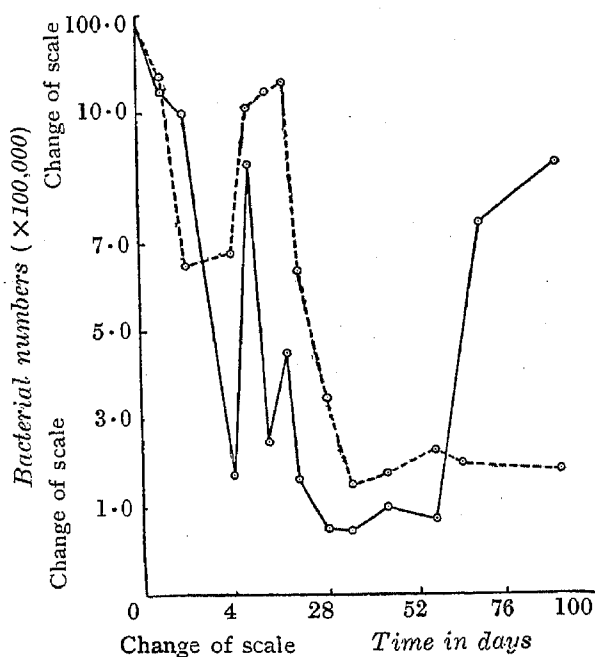


FIG. 11. *Bacterial numbers on Thornton's Medium*

○—○ Soil treated with molasses (Experimental)
 ○---○ .. untreated (Control)

first two days. They showed only continued decrease and at the end of four days the numbers were almost negligible. After that period, they fluctuated between 100,000 and 1,000,000 per gram of soil but this is obviously more due to sampling and plating errors, than to any appreciable change in numbers. The controls also showed a decrease, but the rate of

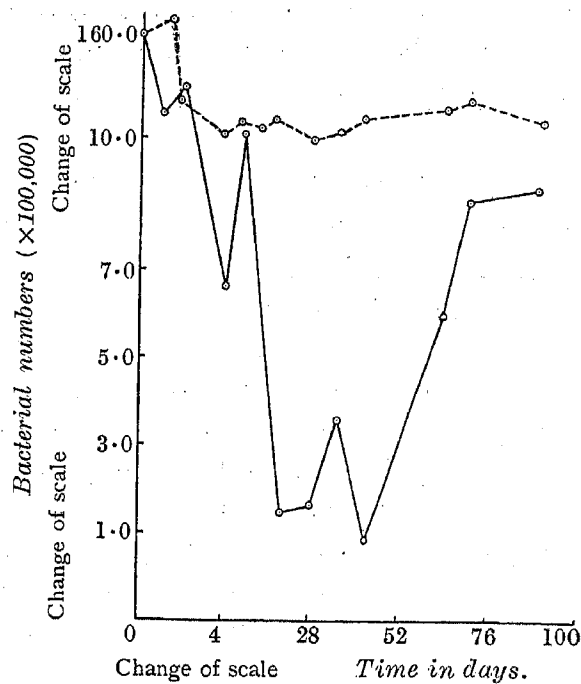


FIG. 12. *Bacterial numbers on Waksman's Medium*

—○— Soil treated with molasses (Experimental)
 - - -○- - - " untreated (Control)

fall was less pronounced than in the experimentals. The general trend of observations was the same on both the media though the counts on Waksman's medium were generally higher than those on Thornton's (*vide* Jensen, 1931).

The foregoing observations are of considerable interest as they provide an instance of the failure of the plate method even for the qualitative study of biological changes in the medium. Both the media were probably unsuited for the growth of the organisms concerned in the fermentation, but that again only emphasises a serious defect in the technique as adopted at present. It would show that the composition of the medium determines the type of flora that shall appear on it and no single medium, however generally useful, would be suitable for all forms of microflora that may be present in a soil. Even under identical conditions, the same medium may not be suitable for all soils and the more recent work of Rajagopalan (private communication) has shown that a medium which is eminently satisfactory in the case of one soil may prove an utter failure in that of another. It would indeed appear to be almost impossible to find any medium, natural or artificial, which would meet the requirements of all types of bacteria present in any soil and under any condition.

Although it was realised that the colonies appearing on the plates were not representative of those actually concerned in the decomposition of

molasses, some of the more prominent forms were nevertheless isolated and their characteristics studied after repeated subculturing on sterile soil-molasses suspensions. The related observations have been described in a later section of the paper.

Some attempts were made to obtain direct counts according to the recent method of Thornton and Gray (1934), but as they included the dead along with the living forms, it was difficult to make out whether there was any perceptible change after the first few days.

Microscopic examination (under low power lens) of apparently empty portions of plates taken out at different stages revealed the presence of numerous minute colonies and quite a number of detached cells which had not germinated. The former represented presumably some of the active forms which were not favoured by the medium while the latter were probably mostly dead cells. Extension of this type of examination promised interesting results, but it was felt that the related conditions should first be standardised before the examination could be regarded as being quantitative. Further work in this direction is in progress and will form the subject of a later communication.

Actinomyces.—Although these organisms might have appeared along with the bacteria, it was yet difficult to count them on the two media that were employed. The colonies were often indistinguishable from those of bacteria, so separate counts were made, the platings being carried out on the starch agar medium of Ganesha Rao and Subrahmanyam (*loc. cit.*) which facilitates easy detection of those forms. The results have been presented in Fig. 13.

It may be noted that there was rapid decrease in both the experimental and the control samples. After about 8 days the counts were almost negligible, while in the still later stages actinomyces were practically absent from the medium.

The foregoing observations, which are also supported by others, would show that actinomyces play no prominent part in the decomposition of cane molasses in the swamp soil. The increasing acidity of the medium and the presence of comparatively high concentrations of mineral salts, especially iron and aluminium, would appear to be unfavourable to those organisms.

Yeasts and Fungi.—The platings were carried out on wort agar, the details being the same as those described in a previous communication (Harihara Iyer, Rajagopalan and Subrahmanyam, 1934).

Yeasts were entirely absent though hundreds of plates representing the various stages of fermentation were examined. This appeared rather strange

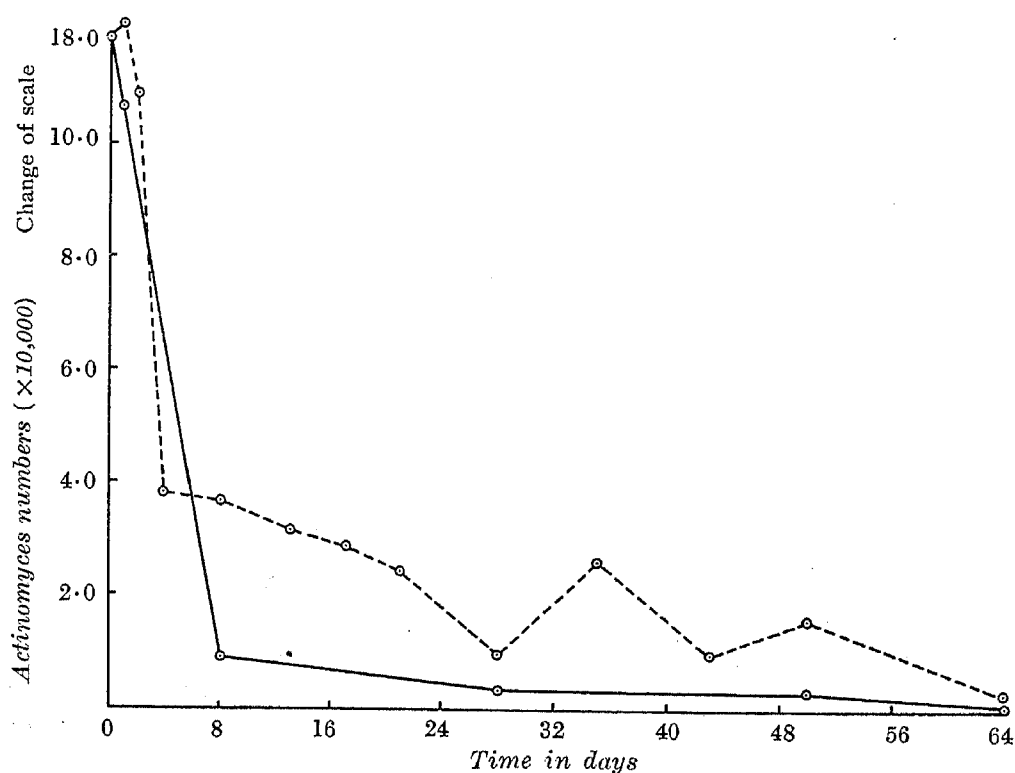


FIG. 13. Actinomycetes on Starch Agar
 ○—○ Soil treated with molasses (Experimental)
 ○-○ " untreated (Control)

since some previous workers have drawn attention to the prominence of certain pseudo-yeasts in soils fertilised with molasses. The platings were therefore repeated on other media. The results have been discussed elsewhere.

It may be seen from the counts for fungi (Fig. 14) that those forms were also adversely affected by the conditions of the experiment. From 1.6 millions, the numbers dropped in the course of 8 days to less than 50,000. Except for a momentary rise to just over 150,000 on the 43rd day, the subsequent change was not appreciable. Both the experimental and the control samples yielded nearly the same type of results, thereby showing that the decrease was really due to swamping and was not influenced by the addition of molasses. Since the commoner forms of soil fungi grow readily on culture media containing molasses, the above observations would suggest that under the conditions of the swamp soil the sugars were not available to the fungi.

A study of the general behaviour of the commoner forms of soil fungi would explain the above phenomenon. In the dry soil, the fungi occur mostly as spores which would require at least 2—3 days to germinate. A further period of at least one day may be required for the vegetative forms to develop, so that it would take the fungi nearly 4 days to establish themselves in the

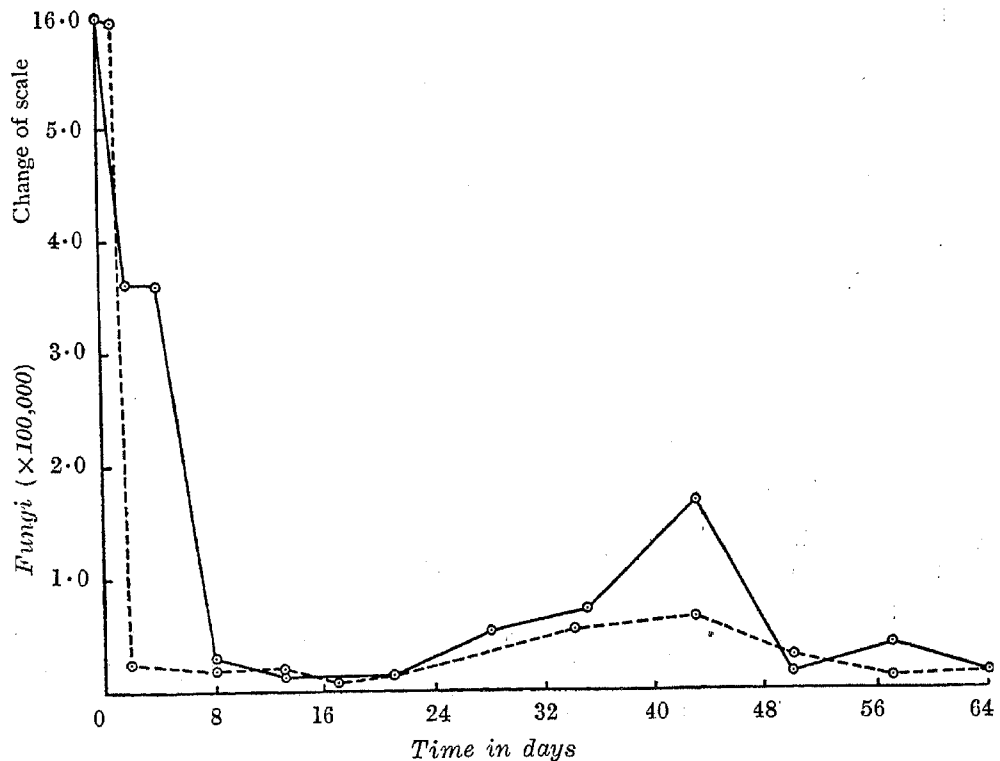


FIG. 14. Fungi on Wort Agar

○—○ Soil treated with molasses (Experimental)
 ○---○ „ untreated (Control)

medium. A comparison with Fig. 1 would show, on the other hand, that the major part of the sugar was decomposed between 48 and 72 hours and that there was practically none left after 90 hours. This would mean that other organisms, especially the bacteria, used up the sugar before the fungi were in a position to do so. The latter were thus left without sufficient nutrition, and in a medium where owing to the preceding fermentation air supply was also inadequate. The fungi could have acted on the organic acids but that too would not appear to have been favoured by the conditions in the soil. It is probable that the dissolved minerals, especially iron, may have something to do with this, but further work is needed before any definite conclusions can be drawn.

Plating on Soil Extract—Molasses Agar.—Since other media had proved unsatisfactory in regard to the isolation of the yeasts and other fermentative organisms, a series of platings were carried out on the above medium, which, it was expected, would provide very nearly the same nutrients as those obtained in soil suspensions. The more prominent forms appearing on the plates were isolated and these, together with those from other media, were studied for their cultural, morphological and physiological characteristics.

About twenty different organisms were thus examined and, of these, the following five would deserve special consideration.—

I. Long rods measuring $2.3-2.8\mu$ by $0.5-0.7\mu$ —Sporulating—Spores measure $1.0-1.2\mu$ by $0.7-1.0\mu$ —Motile—Agar colonies: circular entire with central dot—Agar slant: white, sticky with pellucid dots—Broth: surface growth, white, wavy fragile pellicle—Litmus milk rendered alkaline; peptonisation moderately rapid—Indol production nil—Potato: growth thick, white membranous and wrinkled—Nitrates reduced—No ammonia—No gas—Fermentation of sugars: acid from dextrose and sucrose; no gas: Other products: Ethyl alcohol,+; acetaldehyde,+; Acetone,—; Fusel oil,+; acetic acid,+ (This was verified by determining Duclaux constants); lactic acid,+; Starch not hydrolysed—Aerobic, facultative—Optimum temperature 30°C .—Habitat, soil.

The above description would point to the identity of the organism with *B. graveolens* (Bergey, 1930).

II. Rods, $1.5-3.5\mu$ by $0.7-0.8\mu$, in chains—Motile—Sporulating—Spores central, $0.5-1.0\mu$ —Agar colonies: spreading slightly greyish—Agar slant: white, slightly shiny: thin, membranous—Broth: turbid with sediment—Litmus milk: alkaline, peptonised—Potato: growth abundant—Nitrates reduced but, No ammonia—Acid and gas from sucrose, dextrose and lævulose—Indol,+—Products of fermentation: ethyl alcohol,+; acetaldehyde,+; acetone,—; fusel oil, nil—Aerobic, facultative—gram positive—Optimum temperature, 30°C .—Habitat, soil.

The organism under reference is obviously *B. subtilis*, which is a common soil organism forming nearly 10 per cent. of the total flora.

III. Short rods, $1.2-1.8\mu$ by $0.5-0.7\mu$ —Slightly motile—Sporulating—Spores smaller than those of the other two forms—Agar colonies; circular entire; translucent—Agar slant: white, thick, sticky—Broth: surface white, membranous; broth quite clear—Litmus milk: alkaline; peptonisation, positive—Indol not formed—Potato: abundant growth; thick white, membranous and wrinkled—Nitrates reduced; no NH_3 , no gas—Slight acidity in glucose and sucrose. Products: ethyl alcohol,+; acetaldehyde,+; acetone,—; fusel oil,+; Starch not hydrolysed. Gram positive—Aerobic, facultative—Optimum temperature, 30°C —Habitat, soil.

The above description would not fit in with any of the known forms. Further work on the identification of this bacillus is in progress.

IV. Spheres arranged in packets about 1μ in diameter—Agar colonies: yellow, coarsely granular; circular, raised, moist and glistening—Agar slant: sulphur yellow, smooth—Broth: clear with yellow sediment—Litmus milk: coagulated and becomes alkaline—Indol formation, negative—Potato: growth negligible—Nitrates: reduced to nitrites; no gas, no NH_3 —Sugars: no acid, no gas, Products: ethyl alcohol—aldehyde,—; acetone,—; fusel oil,—; iodoform reaction,+; volatile fatty acids,—; lactic acid, traces— H_2S , traces—Strictly aerobic—Gram positive.

The above description would correspond to that of *Sarcina lutea*.

V. Isolated only from soil-molasses agar—Colonies slightly greyish and glistening—Cells elongated, $1.5-5\mu$ by $1.0-2.0\mu$ —Small fat globules inside the cells—Non-sporulating—Forms only traces of alcohol.

The organism is probably a torula. Further work on its identification is in progress.

Effect of Inoculating True Yeasts into Soil-Molasses Medium.—Since *Saccharomyces* were absent from the media at all stages of fermentation, it was considered probable that all the media that were tried were unsuitable for their isolation. With a view to verifying this, some experiments were carried out inoculating *S. cerevisiæ* into fresh soil suspensions containing 1 per cent. molasses. It was observed, however, that the organism made no growth at all and that after a few days it had almost entirely disappeared from the medium. It would thus be seen that the absence of yeasts from the plates was not fortuitous but was really due to the soil conditions being unfavourable to them.

Protozoa.—These were present only in the early stages of fermentation. Even then the numbers were very small, 20-30 and, sometimes, even 40 slides having to be examined before even a single protozoan could be found. In the later stages they were entirely absent from the medium.

Correlation between Chemical Changes and Microbiological Activity.—Although the foregoing study has brought into relief some of the more prominent organisms concerned in the decomposition of molasses, it is yet difficult to correlate their physiological activity with various chemical transformations reported in the earlier sections of the paper. Some of the known forms, e.g., *B. graveolens* do, no doubt, produce traces of lactic acid but there would also appear to be other forms which can produce even larger quantities and thus account for those observed during the fermentation. The organism responsible for the production of propionic acid has not yet been isolated, but since that acid follows immediately after the production of lactic acid, it would be of interest to determine whether any of the former is derived through reduction of the latter. In a like manner, the possible oxidation of lactic acid to acetic acid through purely chemical or biological agencies will also have to be considered. The various gases would appear to be the result of the direct action of micro-organisms on sugars, but some, especially carbon dioxide, are formed even in the later stages and are presumably derived from the immediate products of decomposition. The dissolution of minerals is probably largely due to purely chemical action between the soil and the acid products of fermentation, but it is nevertheless possible that a part of the related changes, such as the reduction of iron, is indirectly due to biological agencies. Further work on these and related problems is in progress and will form the subjects of later communications.

Summary.

1. The more important products of decomposition of cane molasses in the swamp soil are:—(a) lactic acid, (b) acetic, propionic and butyric acids,

(c) minute quantities of ethyl alcohol, acetaldehyde and fusel oil, and (d) carbon dioxide together with small percentages of hydrogen, methane and other hydrocarbons.

2. The decomposition of sugars, changes in reaction and buffering capacity and production of carbon dioxide have been quantitatively followed. The sugar disappears within four days yielding largely acid products. The buffering capacity of the supernatant increases for a time and then decreases rapidly. Considerable amounts of carbon dioxide are formed but these are mostly lost from the soil system.

3. As the result of the fermentation, increasing quantities of different minerals are brought into solution, but after a time, they are partially re-precipitated in the soil. This is particularly so in the case of iron and aluminium which are first present in highly toxic proportions, but after about a month the concentration in the supernatant decreases rapidly until, finally, it attains nearly the same low level as in the control.

The dissolved iron is present almost exclusively in the ferrous condition. The available evidence would point to its being associated with organic acids present in the medium. The re-precipitated iron is in the ferric condition, mostly as the oxide.

4. Dissolution of phosphorus is not favoured by application of molasses, the presence of iron and aluminium being responsible for its retention in the soil. On the other hand, considerable quantities of calcium and potassium are brought into solution and continue to remain so for nearly two months.

5. The soil residue left after draining the supernatant contains more of soluble iron and aluminium but less of exchangeable calcium than the untreated one.

6. Direct microscopic examination of the molasses-treated soil suspension shows a distinct increase in bacterial numbers for the first two days, followed by rapid fall. The plate counts show, on the other hand, only steady decrease.

7. Actinomyces and fungi show rapid decline in numbers. Protozoa are also adversely affected. True yeasts (*Saccharomyces*) are absent.

8. Some of the more prominent forms associated with the decomposition of molasses have been isolated and their physiological activities studied. They are, (1) *B. subtilis*, (2) *B. graveolens*, (3) an unidentified bacillus, (4) *Sarcina lutea*, and (5) a torula.

9. The relation between micro-biological activity and the various types of chemical changes have been discussed and future lines of work indicated.

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