The Enzymes Concerned in the Decomposition of Glucose and Mannitol by Bacillus coli communis. Part II.—*Experiments of Short Duration with an Emulsion of the Organisms.**

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(Communicated by Dr. A. Harden, F.R.S. Received July 25, 1917.)

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CONTENTS.

The earlier experiments described by the writer upon the decomposition of glucose and mannitol by *B. coli communis*† were open to the objection from the biological side that no attempt was made to distinguish those products which arose by enzyme action from those which might be more particularly associated in their formation with the growth and multiplication of the cells, and from the chemical side that certain substances of unknown composition, such as peptone, were employed, so that it was not possible to be sure that some of the products of fermentation had not been derived from this source.

To overcome these objections the author has adopted a new plan of work which aims at separating the process of growth from that of fermentation.

The method consists in growing the bacteria upon a suitable medium and adding them when sufficiently developed to a solution of the substance to be fermented. The bacteria are washed from the surface of the medium by means of a solution of potassium sulphate, and the emulsion so obtained is added to the substance to be fermented, in the presence of chalk. Under such circumstances an amount of bacteria which would weigh, when dry, 1 grm. will, in the case of *B. coli communis*, bring about the degradation of 40 grm. of glucose in 48 hours.

A period of 48 hours is too long for the fermentation if it be desired to study the separate phases of the fermentation process, nevertheless, in

* Part I of this work appeared in these Proceedings, B, vol. 87, p. 472 (1914).

† Grey, E. C., 'Roy. Soc. Proc.,' B, vol. 87, p. 472 (1914).

this communication a series of experiments will be described wherein the duration of each experiment was of this order, since, though these results represent the average of several fermentation processes which occur together, they have the value of indicating the manner in which the various products of the fermentations vary with changes in the conditions of the experiment and they give information as to the probable origin of succinic acid in this particular fermentation. In Part III an experiment will be described in which the several phases which characterise this bacterial fermentation process have, to a certain extent, been separately studied.

Bacteriological Technique.

For the sake of future reference, and for present uniformity, it has been thought best to employ only standard organisms. In these experiments the *B. coli communis* of the collection of the Institut Pasteur has been used. The treatment of the organism prior to its mixture with the substance to be fermented has also been made to conform to a uniform plan. In each case a loopful taken from a growth on agar has been inoculated into beef bouillon and the fluid incubated for 24 hours at 38° C. This culture has been used to inoculate a series of Roux bottles containing agar prepared after the manner recommended by Dr. Martin. The technique of inoculating the bottles, as well as for the subsequent removal of the growth, is practically that employed by Dr. Salambini, of the Pasteur Institute. Instead of the saline solution employed for the preparation of vaccines a solution of potassium sulphate or mixture of this and magnesium sulphate is employed, as it is not desirable to introduce the salt of any volatile acid such as hydrochloric acid into the fermentation solution. The growth in the Roux bottles has been allowed to take place during 48 hours in each case. At the end of this period about 100 c.c. of sterile sulphate solution is introduced into each bottle. The concentration of the sulphate solution used depends upon the object of the experiment. A very great variation in concentration is permissible. The maximum decomposition of glucose obtained in a series of preliminary experiments was found to occur with a concentration of potassium sulphate equivalent to 3N/40 K_2SO_4. With this concentration of potassium sulphate in the fermentation solution 1 grm. of bacteria decomposed 40 grm. of glucose in 48 hours. The only ion which it was found of value to add to the potassium sulphate solution was magnesium. The addition of sulphate of magnesium will increase the rate of fermentation by about 15 per cent. With the exception of calcium, which the writer has found to be of benefit in the decomposition of sugar by *B. coli communis*, and which is present in excess in these experiments, no other metallic ion nor any negative ion was

found to assist the fermentation when added to the emulsion of bacteria washed off the surface of the agar. Such ions if necessary are required only in the traces in which they exist in the emulsion so obtained.

The metallic ions added were those of manganese, iron, zinc and aluminium; the negative ions, chloride, nitrate, silicate and phosphate.

The writer has found that in order to grow *B. coli communis* on an artificial medium such as a solution of sugar and mineral sulphates together with some source of nitrogen such as ammonium sulphate or an amino-acid (asparagine, alanine, glycine) it is necessary or at least highly beneficial to add a phosphate; nevertheless no beneficial effect was obtained upon the rate of fermentation when phosphates were added to the fully grown organisms.

A solution of 6 grm. of potassium sulphate and 0·5 grm. of magnesium sulphate per litre is suitable for the study of the decomposition of substances allied to glucose under the influence of *B. coli communis.*

If each of the Roux bottles contain about 100 c.c. of agar and the growth be obtained in the manner described, it will be found that 10 bottles will yield about 1 grm. of dried bacilli. The amount of organic matter outside the bodies of the bacteria which is washed off the agar with the potassium sulphate solution has been found to be fairly constantly equal to three times the weight of the yield of dried bacteria. The weights of dried bacteria and soluble organic matter are determined for each experiment by centrifugalising a sample of the emulsion and determining the weight of bacteria directly, and the organic matter in the solution by evaporation in a platinum vessel and weighing after drying at 110° C. and after incinerating, or the bacteria may be determined indirectly by evaporating to dryness two separate samples, from one of which the bacteria have been separated by the centrifuge. The difference in weight represents the weight of bacteria in the sample of emulsion.

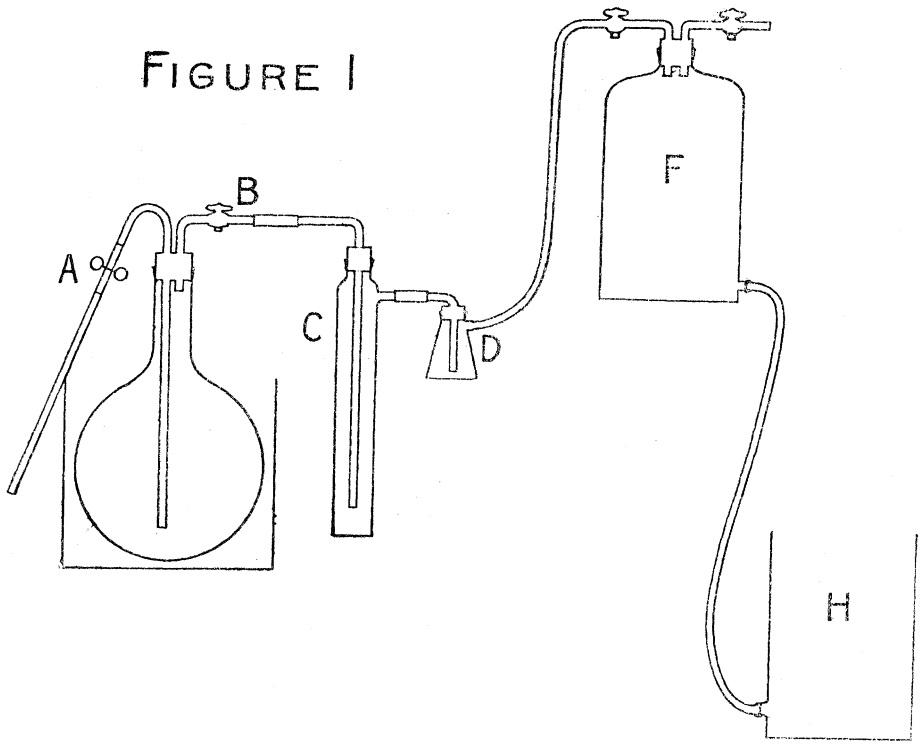
Arrangement of Apparatus for the Study of a Complete Fermentation.

The fermentation flask rests in the water-bath, regulated for the required temperature. The flask has a capacity of about 2 litres. The volume of the fermentation solution at the beginning of the experiment is about 1½ litres. At the end of the experiment water is introduced to displace the gas above the solution in the flask.

In the experiments described below the procedure adopted was as follows: The flask containing chalk and the substance to be fermented dissolved in about 800 c c. of distilled water was sterilised by heat. The solution was cooled to 38° C. and the emulsion of bacteria introduced, the amount added being determined by weighing the flask before and after the introduction of

the emulsion. The flask was sterilised while plugged with cotton wool, and after the introduction of the bacteria the cotton wool plug was replaced by the stopper seen in the figure. The practice has always been adopted of either sterilising the stopper separately by steam or flaming the tubes and rubber previous to its introduction into the flask, but it is almost certain that this precaution is unnecessary, since the size of the population of bacteria introduced at the beginning of the experiment precludes the possibility of the results being appreciably influenced by subsequent contamination even should such occur. This is one of the great advantages of the new technique.

FIGURE 1



At the beginning of the experiment the pinchcock of the tube A is closed and the tube B connected to the water-pump. By this means the air in the flask is exhausted, the solution being brought to the boil under the reduced pressure. The tap B is then closed and, the tube A having been put into communication with a reservoir of nitrogen, the pinchcock is opened and nitrogen allowed to enter to replace the air which has been removed. The operation is repeated a second and third time to ensure complete removal of oxygen from the flask either in the gas space or dissolved in the solution.

The nitrogen which enters through A is filtered through a plug of cotton wool.

The tube B is in connection with a wash-bottle C of 500 c.c. capacity, which contains a solution of sodium hydroxide free from CO_2. If the strength of the solution is about normal, it is found that the one bottle of this capacity suffices to absorb the carbon dioxide from the evolved gases although these may pass through the solution at a considerable rate. A second small bottle D has been introduced for safety; it contains about 100 c.c. of the sodium hydroxide solution. The mixture of nitrogen introduced at the beginning of the experiment and the hydrogen evolved from the fermentation is collected in the reservoir F. By lowering the vessel H the difference of level between F and H may be adjusted to counteract the weight of the column of sodium hydroxide solution which has to be lifted by the gases issuing from the fermentation flask. The gases are thus evolved under atmospheric pressure. The method of collecting the gases here described is much simpler than that in which they are collected in an evacuated flask over mercury. As far as the estimation of CO_2 is concerned the method is just as accurate, but the estimation of hydrogen is not so accurate owing to the difficulty of accurately graduating a vessel of the form F and also because of the solubility of hydrogen in water.

But apart from its simplicity the present method has now been adopted because the rate of evolution of the gases is too great to permit of them being collected through a column of mercury into a vacuum even if the wide form of tube recommended by the writer in an earlier communication be adopted. It may be pointed out, however, that a combination of the sodium hydroxide absorption bottles with the mercury collecting apparatus employed by Harden would be still better than the system here employed if an accurate estimation of small amounts of gases other than carbon dioxide was to be made. The gases in this case would, after passing through the sodium hydroxide solution, pass on into the mercury gas-collecting apparatus, the size of which could be considerably reduced.

A further point to be noted in connection with the form of apparatus now employed is that it is suitable for the study of aërobic as well as anaërobic fermentation, for oxygen can be readily admitted during the course of the experiment by opening the pinchcock A.

In the series of experiments described below, the practice has been adopted of stopping the fermentation at the time desired by raising the temperature of the water-bath to about 70° C. and maintaining it at that temperature for about half-an-hour. At the same time hot distilled water has been introduced to displace the gases above the solution in the flask.

By this operation not only is the reaction of fermentation brought to an end and the solution made practically sterile, but a large part of the dissolved carbon dioxide is evolved, so that it is possible to estimate the carbon dioxide which remains in the solution when cold more accurately than when the solution was saturated as in the older experiments.

Chemical Technique.

Where no particular method is indicated it will be understood that the methods employed by Harden* and already described have been adhered to.

The Estimation of Carbon.—It is sometimes of great help in following the course of the fermentation to be able to estimate in a simple manner the carbon dissolved in the solution. This can be done by the volumetric method described by the writer.†

In cases where the fermentation was interrupted before the whole of the sugar was fermented an estimation of carbon was made in the solution after the removal of all the products known to be formed, but in no case was any appreciable amount of carbon found in excess of that which corresponds to the sugar present. From this it was concluded that if any glycerine had been left over during the fermentation it was in amount too small to be taken into account. The estimation serves as a check that the whole of the products have been accounted for.

Carbon Dioxide.—The carbon dioxide which remains dissolved in the fermentation solution is estimated by removing a sample with a pipette and mixing it with a solution of standard barium hydroxide. This is permissible in the case when the solution is not saturated with gas, as in the experiments described in Part II, but when the concentration of dissolved gas is greater the more accurate method described in Part III is to be recommended.

The method of collecting the evolved carbon dioxide has been described. The sodium hydroxide used for the absorption is prepared as follows: A known volume of a strong solution of sodium hydroxide is placed in a tall cylinder and a sample of the solution used to determine the amount of carbon dioxide which it contains. Sufficient standard barium hydroxide is added to completely precipitate the whole of the CO_2 and the precipitate allowed to settle. The clear fluid is siphoned into a large volume of water which has been previously freed from CO_2 by a rapid current of CO_2-free air.

The estimation of the amount of carbon dioxide in the original sodium hydroxide solution employed may be made in the apparatus described for the

* Harden, A., 'Chem. Soc. Journ.,' 1901, p. 610; Grey, E. C., 'Roy. Soc. Proc.,' B 87, p. 472 (1914).

† Grey, E. C., 'Chem. Soc. Journ.,' 1914.

estimation of carbon. In the absence of such an apparatus an accurate method is to mix the sample with acid and carry off the disengaged gas in a current of CO_2-free air (the CO_2 produced in the experiment being absorbed in standard barium hydroxide). When once a solution free from CO_2 has been obtained all the subsequent estimations of CO_2 are simple titrations.

To determine the amount of carbon dioxide evolved during the fermentation, the sodium hydroxide solution of the bottles C and D is transferred to a graduated flask and diluted to a definite volume. Of this solution a sample is mixed with standard barium hydroxide solution, and after filtration from the precipitate of barium carbonate the diminution of alkalinity is determined by titration with standard acid. An example of an estimation may be given.

250 c.c. of the CO_2-free sodium hydroxide solution was diluted to 1 litre. Of this solution 20 c.c. corresponded to 54·88 c.c. of N/10 H_2SO_4.

Another 250 c.c. of the same solution was diluted with water in the bottles C and D for the absorption of the CO_2 evolved in an experiment, and the solution was ultimately diluted to 1 litre; of this solution 20 c.c. was mixed with 50 c.c. of standard barium hydroxide solution and the mixture filtered; 20 c.c. of the filtrate neutralised 33·50 c.c. of N/10 H_2SO_4.

20 c.c. of the diluted NaOH corresponds to	54·88 N/10 H_2SO_4
50 c.c. of barium hydroxide corresponds to	102·80 N/10 H_2SO_4
	157·68
Alkalinity after absorption $33·5 \times 7/2$	= 117·25
Carbon dioxide in 20 c.c. of NaOH solution	= 40·43 N/10

Total carbon dioxide evolved 202·15 c.c. normal.

The Estimation of Alcohol.—The most accurate and at the same time the simplest method of estimating alcohol is that of Martin.* According to the original method the fermented solution is distilled directly into the mixture of bichromate and sulphuric acid, but this cannot be done in the presence of acids which could themselves reduce the bichromate. A previous distillation from an acid solution must be followed by a distillation from an alkaline solution. It is not necessary to distil the alcohol directly into the bichromate mixture, but the dilute solution of alcohol may be added gradually from a pipette to the oxidising mixture and the solution maintained at the temperature of the water-bath for 10 minutes. The excess of bichromate is titrated with ferrous sulphate solution in the usual way.

* Martin, 'Mon. Sci. Quesn.,' 1904.

Volatile Acids.—Under certain circumstances it has been found that the reduction of mercuric chloride to mercurous chloride in the estimation of formic acid may be accompanied by a blackening indicative of further reduction. It was found that under such circumstances the solution of the sodium salts of the volatile acids obtained from the fermentation gave a precipitate of iodoform when treated in the cold with sodium hydroxide and iodine solution.

The fact that the production of iodoform in the cold occurred after the solution of the sodium salts of the volatile acids had been concentrated from a volume of 2 litres to 200 c.c., and also that the distillate obtained for the estimation of alcohol did not give the reaction, shows that the reducing substance is an acid, and it calls to mind the formation of pyruvic acid observed by Fernbach and Schoen* under the same circumstances from yeast. In this case the amount of acid is very small and no conclusive reaction could be obtained to decide as to its nature.

The Separation of Succinic and Lactic Acids.—The details to which it is necessary to adhere in order that the Pasteur method of separating succinic acid from other acids may yield accurate results have been described by the writer elsewhere.†

An important point to note in connection with the present technique is that the absence of peptone obviates the difficulty which was experienced in the earlier experiments in the examination of the residual solution after the distillation of the volatile acids. Also during the distillation itself there is here none of the objectionable frothing to which solutions of peptone may give rise. Chiefly, however, is it essential that there should be no peptone in the solution which is used for the separation of succinic and lactic acids, as has been pointed out in the special communication referred to.

The Estimation of Residual Carbohydrate or Allied Substance in the Solution after the Fermentation.—Up to the present the number of substances which could be studied as to the decomposition products resulting from their fermentation has been limited by the difficulty of estimating the portion which remained unfermented after the experiment. It was partly for this reason that the writer introduced the volumetric method of estimating carbon. Even in the case of glucose it may not always be safe to rely upon the figure for the residual portion as indicated by the reduction of copper solution, and certainly the reduction should be determined also after the residual solution (in which the glucose is to be determined) has been hydrolysed

* Fernbach and Schoen, 'Comptes Rendus,' vol. 157, p. 1478 (1913); *ibid.*, vol. 158, p. 1719 (1914).

† Grey, E. C., 'Bull. Soc. Chim.,' 1917; 'Biochem. Journ.,' vol. 11, p. 2 (1917).

by acid, as will be seen from the experiments described in Part III. But with the method of studying the fermentation now employed there is a very simple method of getting over the difficulties which might arise from the presence of residual unfermented substance difficult to estimate. It consists in so adjusting the weight of bacteria employed that, in the time during which the experiment is to be continued, the whole of the substance to be examined will be fermented. It is thus possible to control the fermentation in a way which was not possible in the earlier experiments and it should be possible to study the fermentation of carbohydrate and allied substances for which no methods are at hand for their estimation with accuracy, as, for example, the various polyhydric alcohols. The method has been used here for the comparison of the products from mannitol with those from glucose under the same conditions.

The Neutralisation of the Medium by Chalk.—Even if the fermentation flask be repeatedly shaken during the course of the fermentation, there will be periods during which the chalk will settle to the bottom of the flask if, as in the usual experiment, the fermentation be continued overnight without agitation. During such periods the acidity may increase sufficiently to impede or alter the course of a fermentation. The writer has employed a very simple device for preventing the settling of the chalk during the experiment. It consists in introducing the chalk into small sacks of cloth, in each of which is placed also a small piece of cork. The weights of chalk and cork are so balanced that the sacks just sink to the bottom of the flask when they are first introduced; during the course of the fermentation the acid disengaged penetrates the sacks and they become swollen with gas and rise to the top of the solution. During this movement up and down the chalk contained in the sacks becomes gradually liberated, so that the solution is always turbid with chalk without being mechanically agitated from without.

The Fermentation of Glucose in the Presence of Chalk by an Emulsion of B. coli communis.—A series of experiments will be now described in which an emulsion of *B. coli communis* was allowed to act for a period of 48 to 68 hours on a solution of glucose in distilled water, containing varying quantities of potassium sulphate. The fermentations took place in the presence of chalk.

An accidental rise in the temperature during the early stage of one of the first experiments (No. 3) gave rise to a phenomenon which has not since been repeated to the same extent. The bacteria introduced into the fermentation flask contained 0·5 grm. of fatty or waxy material. At the end of the experiment the weight of this material was 3·5 grm. There had been thus a synthesis of 3 grm. of a material which, under normal circumstances, does not accumulate. On the contrary, in most experiments the amount

of fatty material in the bacterial cells appears to be used up during the fermentation.

It is to the attempt to understand the cause of this synthesis of fat or wax (which was accompanied by a high yield of lactic acid) that the series of experiments described in Part II owes its length. Attempts were made to obtain a similar yield of the substance by change of the temperature in various ways, and by alteration in the concentration of the salt. The attempts were never as successful as in Experiment 3, but the variations in the resulting proportions of the products led to the belief that the fermentation by *B. coli communis* really represented the sum of several independent fermentations, which belief proved to be amply justified by the results of the experiment which was devised to settle this question, and which is described in Part III.

In Table I the results are arranged in order of decreasing yield of alcohol. With the method of Martin, the estimation of alcohol is of a high order of accuracy. In fig. 2 these results are recorded graphically. In fig. 3 the same results are arranged from left to right, in order of increasing concentration of the potassium sulphate used in the experiments. It will be seen that, with the exception of the relative positions of No. 3 and No. 5, the results grouped in this way give rise to much the same curves as when arranged according to the yield of alcohol, from which it may be inferred that the cause responsible for the variations in the proportions of the products is the change in the concentration of the salt solution. The manner in which the change in the concentration of the potassium sulphate solution influences the proportion in which the products of the fermentation appear at the end of the experiment was not clear until the experiments described in Part III had been carried out, for, unfortunately, the bacteria were not counted in the earlier experiments. It is now clear, however, that in dilute solution there is a greater diminution in the number of living bacteria, or a greater action of the dying or dead cells, than in the cases where the concentration of potassium sulphate is greater, and, as will be seen later, the death of the cells is accompanied by the production of alcohol and those other products which are formed in conjunction with it, while the production of lactic acid is more closely associated with the rapid multiplication of the cells. Thus, at the one end of the figures the results are to be correlated with rapid death of the bacteria introduced, and at the other with less death and more multiplication.

Note.—In fig. 3 Experiment 3 has been placed at the end of the series. There was uncertainty about the conditions of this experiment. It, however,

Table I.—Collected Results of the Action of *B. coli communis* upon Glucose. The results are expressed as percentages upon the sugar consumed. The numbers refer to the original order in which the experiments were carried out.

	No. 7.	No. 4.	No. 6.	No. 8.	No. 10.	No. 11.	No. 3.	No. 5.
Carbon dioxide	19·38	21·25	16·00	22·46	15·24	14·57	16·50	11·90
Formic acid	5·22 } 24·60	3·88 } 25·13	5·42 } 21·42	3·19 } 25·65	12·29 } 27·53	6·18 } 20·75	7·50 } 24·00	9·02 } 20·92
Acetic acid	18·56	15·64	16·02	17·99	22·02	19·89	9·67	23·23
Lactic acid	16·93	21·25	20·05	19·99	25·46	36·52	49·79	27·09
Succinic acid	17·36	19·59	18·01	15·05	11·23	9·76	3·97	10·69
Alcohol	20·06	16·63	15·02	14·28	13·36	13·17	10·63	8·31
Hydrogen	0·35	—	0·35	0·26	0·21	—	—	0·26
Total	97·86	98·24	92·97	93·22	99·81	100·09	98·06	90·50
Ratio CO_2/H_2	1·25	—	1·05	1·96	1·78	—	—	1·03

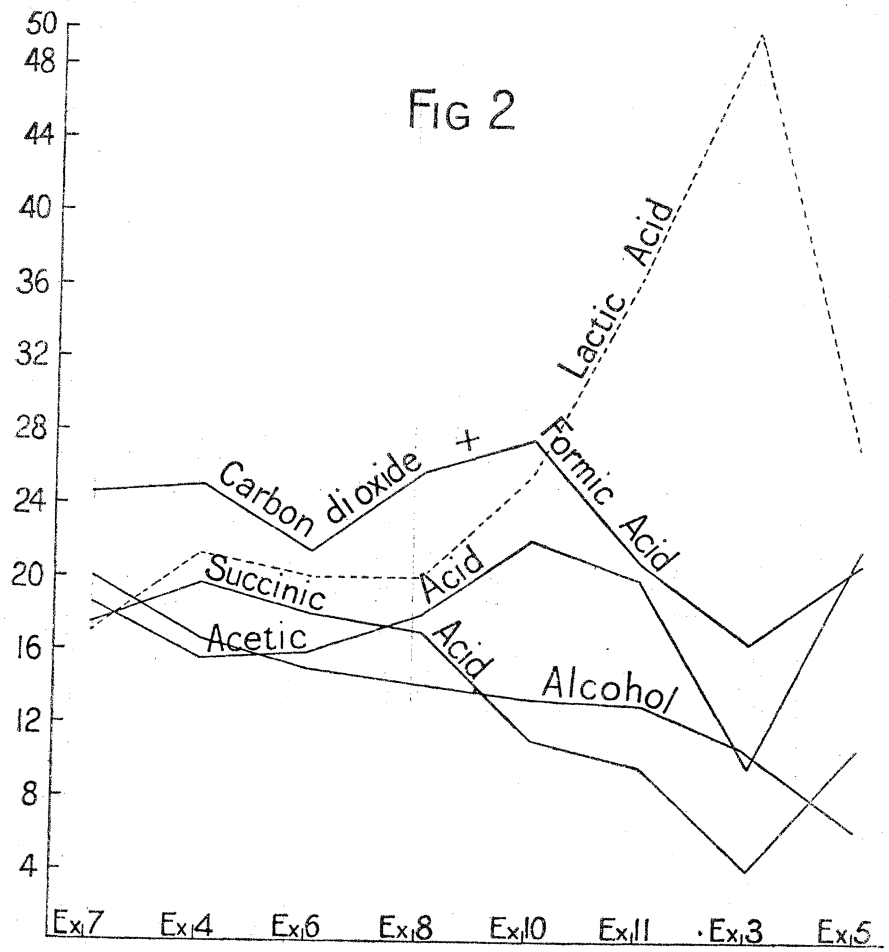
Where a blank has been left hydrogen was not estimated.

Appendix to Table I.—Data as to the Conditions of the Experiments recorded in Table I.

	4.	7.	8.	6.	10.	5.	11.	3.
Concentration of K_2SO_4 in grammes per litre	1·52	2·69	2·90	5·66	6·60	7·40	9·60	7·0 ?
Average temperature	34°	41°	35°	38°	40°	43°	40°	45° ?
Weight of bacteria	0·24	0·49	0·33	0·42	0·81	0·29	0·37	0·66
Sugar employed	18·6	15·5	20·69	15·2	15·0	19·09	15·09	16·46
Sugar fermented	14·6	12·9	10·49	15·2	15·0	13·70	15·09	16·13
Duration in hours of the experiment	47	47	43	48	66	48	67	45
Weight of fat obtained	0·41	Nil	—	—	—	—	0·20	2·53

Note.—Actually the temperature in Experiments 3, 4, 7, 8 and 10 was made to fluctuate in a special manner under the belief that the variation of temperature which occurred accidentally in Experiment 3 was responsible for the synthesis of the fat which was found produced in that experiment. As has been noted, the writer was unable to repeat the phenomenon, but still more because of the results recorded in the next communication it becomes unnecessary to record the exact details as to the fluctuations of temperature to which the fermentations were submitted.

clearly represents a continuation of the process by which the lactic acid-forming enzyme comes to predominate in its action.



The following facts may be deduced from the experimental results recorded in Table I and the accompanying graphical representations :—

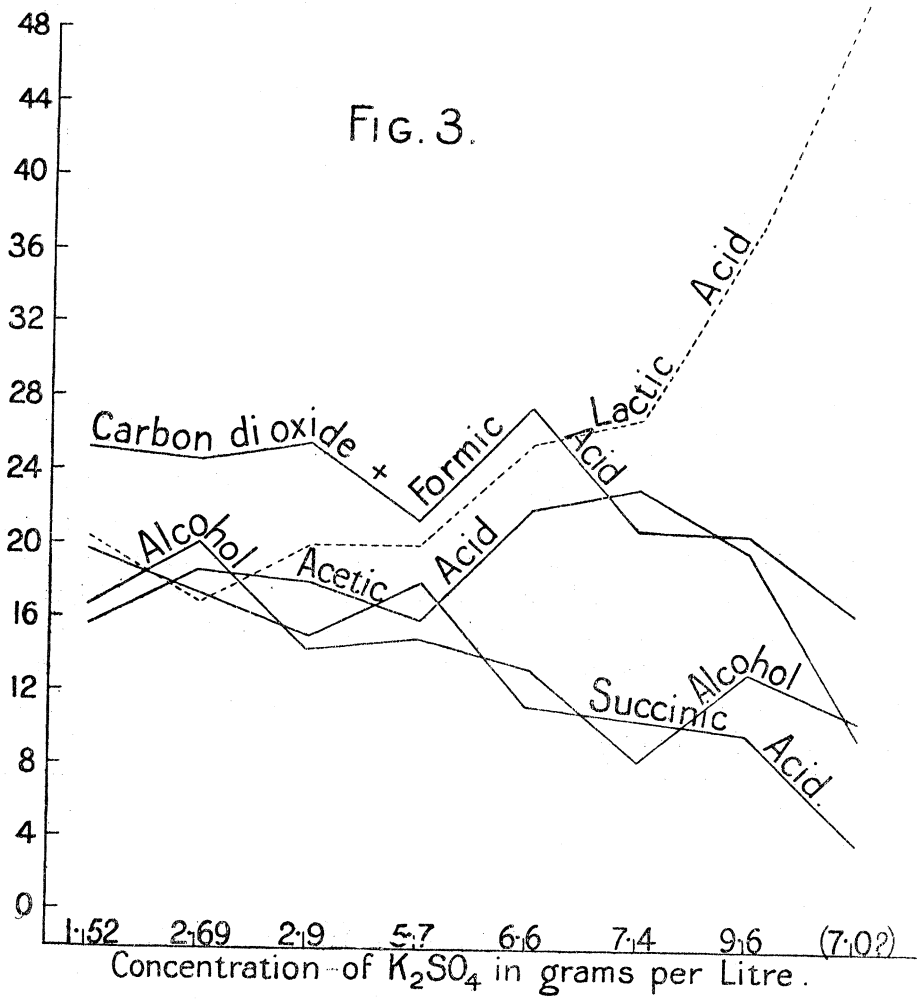
(1) The fermentation takes place in two main directions. On the one hand, there is formation of lactic acid, and, on the other, a group of substances which seem to be related more closely to one another as regards their origin than they are related to lactic acid. This group comprises alcohol, formic acid, and carbon dioxide (which arises from it by further decomposition), acetic acid and succinic acid.

(2) Succinic acid and acetic acid are complementary as regards the extent

of their formation, and are therefore probably produced from a common parent substance.

(3) The average drawn between succinic acid and acetic acid corresponds very closely with the average between acetic acid and alcohol, from which it would appear that the three substances are related to a common intermediate substance, and are produced by the same enzyme action.

(4) The curves for the production of alcohol and succinic acid closely follow one another, indicating that the oxidation of a part of the sugar to succinic acid, and the reduction of a part of the sugar to alcohol, are complementary processes.

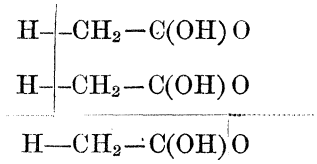


It is of interest to add together the acetic acid and succinic acid produced in each experiment. It will be seen that, in each case, the combined weight does not differ very greatly from 33 per cent. The same applies to the sum of the weight of acetic acid and alcohol. When the fermentation has taken place under certain conditions, as in Experiment 3, the sum of either of these two pairs of products no longer approximates to a constant figure, but the point to which the writer would draw attention is that, throughout conditions which have varied sufficiently to introduce considerable variation in the individual products, the sum of these pairs approaches very closely to a constant. Thus, a variation, as seen between Experiments 4 and 5, represents nearly 100 per cent. on the amount of succinic acid, and yet only about 5 per cent. on the sum of succinic acid and acetic acid. Similarly, the alcohol, as between Experiments 4 and 5, has varied by 100 per cent., but the sum of alcohol and acetic acid has only varied by 4.

	4.	5.	6.	7.	8.	10.	11.	Mean.
Acetic acid.....	15·6	23·2	16·0	18·6	18·0	22·0	19·9	
Succinic acid.....	19·6	10·7	18·0	17·4	15·1	11·2	9·8	
Sum	35·2	33·9	34·0	36·0	33·1	33·2	29·7	33·6
Acetic acid.....	15·6	23·2	16·0	18·6	18·0	22·0	19·9	
Alcohol	16·6	8·3	15·0	20·0	14·3	13·4	13·2	
Sum	32·2	31·5	31·0	38·6	32·3	35·4	33·1	33·4

Doubtless, these regularities are in part due to some constancy in the conditions which is not apparent, but, nevertheless, the results are highly suggestive that succinic acid, which is, in point of fact, di-acetic acid, arises in this fermentation from the same intermediate substance which gives rise to acetic acid. And, again, since acetic acid is shown to be similarly related to alcohol as regards its origin, it seems clear that the three substances arise through the agency of the same enzyme or enzymes. These results suggest, therefore, that succinic acid may arise by the union of two $—CH_2COOH$ groups.

The close relationship of these three substances is pictorially represented below.



Each line is equivalent to a molecule of acetic acid, and the three are thus equivalent to a molecule of glucose.

Above and to the right is succinic acid, below and to the left acetaldehyde. Ignoring the dotted line, to the left is alcohol. On the right below, the removal of oxygen by an acceptor is represented; if this acceptor is acetaldehyde the product becomes acetic acid. Above and to the left hydrogen is represented as being separated from the glucose molecule in proportion corresponding to the oxygen, the two phenomena representing the action of the reductase, the action of which it was suggested was impaired under certain conditions (Grey, 1914). The acetaldehyde is thus represented as having three possibilities as regards transformation. It may, as has been previously suggested to account for the tendency under certain conditions for alcohol and acetic acid to appear in equimolecular proportions (Harden, 1901), undergo the Cannizarro condensation into equimolecular proportions of the acid and the alcohol. It may become oxidised to the acid by the oxygen represented on the right or reduced by the hydrogen represented on the left of the scheme.

The key for the working of such a mechanism is the existence of an enzyme, or a co-operation between enzymes, capable of effecting simultaneously oxidation and reduction. Any interference with such a mechanism would lead to a simultaneous diminution in the production of alcohol, acetic acid and succinic acid. This simultaneous diminution has been established by experiment as actually occurring.

The Comparison between the Fermentation of Glucose and that of Mannitol.

In an earlier communication* the writer expressed the view that the fermentation of mannitol and glucose by *B. coli communis* was brought about by the same set of enzymes and that in general it was highly probable that bacteria dealt with all carbohydrate molecules, and molecules of substances allied to them such as the corresponding alcohols, upon a plan which was characteristic for the bacterium; in other words, that the products obtained by the action of a bacterium depended upon the bacterial content of enzymes rather than on the nature of the sugar. Only in the case of the formation of the first intermediate product might a special enzyme be necessary for a special carbohydrate configuration, but once the first intermediate product is formed the remainder of the fermentation is effected in all cases in the same way, modified only by the secondary reactions which may occur in the direction of further reduction or oxidation, should the conditions give opportunity for such changes.

* Grey, E. C., 'Roy. Soc. Proc.,' B, vol. 87, p. 472 (1914).

It was suggested in the earlier communication that mannitol was thus in a position to give rise to a greater proportion of alcohol than glucose, not simply because it was a compound already more reduced than glucose, but because the hydrogen atoms existing in excess in the case of mannitol rendered less necessary the action of the reductase. If acetaldehyde were the intermediate substance immediately preceding alcohol, it was suggested that an interference with the reducing mechanism or reductase might lead to an accumulation of the aldehyde, which in its turn would react upon the intermediate substance A. If intermediate substance A had no alternative but ultimately to become acetaldehyde and formic acid, the accumulation of the aldehyde resulting from a weakening of the reductase would only lead to a slowing down of the fermentation as a whole, but since the intermediate substance can, according to the hypothesis, become changed by a mere molecular rearrangement into its isomer lactic acid, the result of a weakening of the reductase is a proportional increase in the production of lactic acid.

This hypothesis concords entirely with the results of experiment. Further, it would be expected to follow from this hypothesis that the more completely the phenomenon of bacterial growth and consequent change in the enzymes was excluded from the fermentation, and the less possibility there was for the products of fermentation to accumulate and hinder the reaction, both of which possibilities are minimised by the new technique, the more completely should the fermentation products from mannitol resemble quantitatively those from glucose, so that finally the only difference should be that part of the acetic acid in the case of the glucose should be represented by alcohol in the case of mannitol. How true this is may be seen by the following comparison between one of the analyses of the products from glucose already described and an analysis of the products of a mannitol fermentation carried out under the same conditions.

	Glucose.	Mannitol.
Hydrogen	0·21	0·54
Carbon dioxide and formic acid	27·53	27·61
Lactic acid	25·46	23·11
Succinic acid	11·23	12·03
Acetic acid	22·02 } 35·38	10·51 } 37·73
Ethyl alcohol	13·36 }	27·22 }
Total	99·81	101·02

These results speak very strongly, if not conclusively, in favour of the writer's hypothesis that the two substances are fermented by the same set of enzymes.

With regard to the removal of the hypothetically produced acetaldehyde from the sphere of the fermentation by reduction, it may be noted that one would expect in the case of a mannitol fermentation not to be able to detect the presence of as much acetaldehyde in the solution as in the case of a similar fermentation with glucose. The writer has sought for acetaldehyde in the case of mannitol fermentation and has failed to detect it, a fact in harmony with the above consideration.

Summary and Conclusions.

By allowing *B. coli communis* (suspended in saline solution) to act on glucose it has been found that the proportion between the products of decomposition differs considerably from that obtained in the earlier experiments in which the organism was allowed to grow in a mixture of glucose and peptone, a greater proportion of alcohol, acetic acid, and succinic acid, and smaller proportion of lactic acid being obtained.

The conclusions which may be drawn from the results refer in the first place to *B. coli communis* in particular, and in the second place to bacterial fermentation in general.

In particular it has been shown that:

(1) Succinic acid has an origin in common with acetic acid and alcohol.

(2) The formation of lactic acid is independent of the formation of the above three products.

(3) The enzymes which effect the decomposition of glucose also co-operate in the decomposition of mannitol.

With regard to bacterial fermentation in general the experimental results point to the independence of the intracellular ferments.

The experiments of Harden and Penfold,* and later of the writer,† upon *B. coli communis* grown in the presence of a chloroacetate showed that by artificial selection a strain could be obtained which yielded products from glucose in different proportions from those in which they were formed by the original strain, and since this difference in proportion of products must correspond to a difference in proportion of the enzymes forming them, or of the activity of these enzymes, which for practical purposes is the same thing, it is clear that artificial selection can vary the proportion between certain enzymes, and this is good evidence that they are independent of one another in the original cell. The further results here recorded give additional evidence of this, for they show that with the unselected organism, even when the whole process of fermentation only occupies 48 hours, considerable

* Harden, A., and Penfold, W. J., 'Roy. Soc. Proc.,' B, vol. 85, p. 415 (1912).

† Grey, E. C., 'Roy. Soc. Proc.,' B, vol. 87, p. 472 (1914).

variation in the proportion of the products may be obtained by varying the conditions.

With regard to the results of exactly comparable experiments with mannitol, good evidence has been obtained in confirmation of the view already put forward by the writer that the fermentation of various carbohydrates and allied substances by bacteria is brought about by a single set of enzymes whose actions are common to all such cases of fermentation. This does not exclude the possibility that the first step in the degradation of a particular molecular structure may require a special enzyme in order to produce the first intermediate substance, which according to the writer's hypothesis would be the same for all analogous cases of fermentation.

The Enzymes Concerned in the Decomposition of Glucose and Mannitol by Bacillus coli communis. Part III.—*Various Phases in the Decomposition of Glucose by an Emulsion of the Organisms.*

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(Communicated by Dr. A. Harden, F.R.S. Received July 25, 1917.)

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CONTENTS.

In Part II of this series results have been described which indicate the existence of several independent processes occurring during the one experiment. The experiment which will be now described was undertaken with the object of simplifying results by shortening the time of the fermentation. Arrangements were made also to count the bacteria at various periods, with the object of determining how far the fermentation was due to enzyme action which could be said to be carried on independent of the multiplication of the cells. The plan of the experiment was as follows:—About 50 grm. of glucose was to be fermented in a volume of 5 litres of solution. A sample of about a litre was to be removed every 12 hours and submitted