Studies in the Biochemistry of Micro-organisms.

Part II.—*Quantitative Methods and Technique of Investigation of the Products of Metabolism of Micro-organisms.*

By John Howard Birkinshaw *and* Harold Raistrick.

At the commencement of this work it was decided, as outlined on p. 7 in the introductory paper in this series, to take as the basis of the investigations the quantitative determination of the *types* of compounds formed by micro-organisms. Hence it became necessary to devise methods of separating the products of metabolism arising from glucose into different *types* of chemical compounds, and of isolating them in a form suitable for the subsequent estimation of their carbon content by combustion. The methods adopted will be described.

(A) *The estimation of carbon by wet combustion.*

Several methods have been described from time to time for the estimation of carbon in solution, but the principle involved appears to be the same in almost all, namely, oxidation of the carbon compound with chromic acid, and estimation of the carbon dioxide produced. In the method reported by Grey (1914) the carbon dioxide is estimated by measuring its volume. In other methods it is customary to follow the classical method of estimating carbon by dry combustion, and weighing the carbon dioxide formed. The method finally adopted in this work is a modification of that of Messinger (1890). It consists in the oxidation of the carbon compounds by a mixture of sulphuric and chromic acids. The chief product of oxidation is carbon dioxide, but the combustion gases are passed over heated copper oxide to complete the oxidation of any carbon monoxide produced, and also of any volatile carbon compounds which have escaped oxidation by the combustion mixture. The carbon dioxide is absorbed in standard baryta solution and estimated by titration.

The apparatus used is shown in fig. 1 and consists of three parts.

(1) *Purification of air current.*—The air is purified by the usual arrangement of aspirators (A) for holding air, and of soda-lime towers (B1 and B2) for removing carbon dioxide.

(2) *Combustion.*—The substance is oxidized in the 250 c.c. flask F, which is connected by the ground-glass joint and three-way tap T_1 to the combustion tube CC_1, and to the air by the three-way tap V, thus providing alternative air paths via the funnel H or the tube G. The combustion tube CC_1 is filled for about half its length with granular copper oxide, and for about one-third with lead chromate, a space being left at the end C_1 for the insertion of a copper spiral, which is to be employed if nitrates are present in the solution to be oxidized.

(3) *Absorption of carbon dioxide.*—The carbon dioxide is absorbed in the train of baryta bubblers N, O, P, which contain suitable measured amounts of N/4 baryta. P is of 25 c.c., O of 50 c.c., and N of 50 or 100 c.c. capacity, depending on the amount of carbon dioxide anticipated. The bubblers are connected to the combustion apparatus by the three-way tap T_2. There is thus an alternative air path through the indicator baryta bubbler M.

The combustion is carried out as follows :—10 c.c. of chromic acid solution (100 gm. of chromic anhydride in 100 c.c. solution) are pipetted into flask F, which is then replaced. The combustion tube CC′ is heated and CO_2-free air passed through the whole apparatus

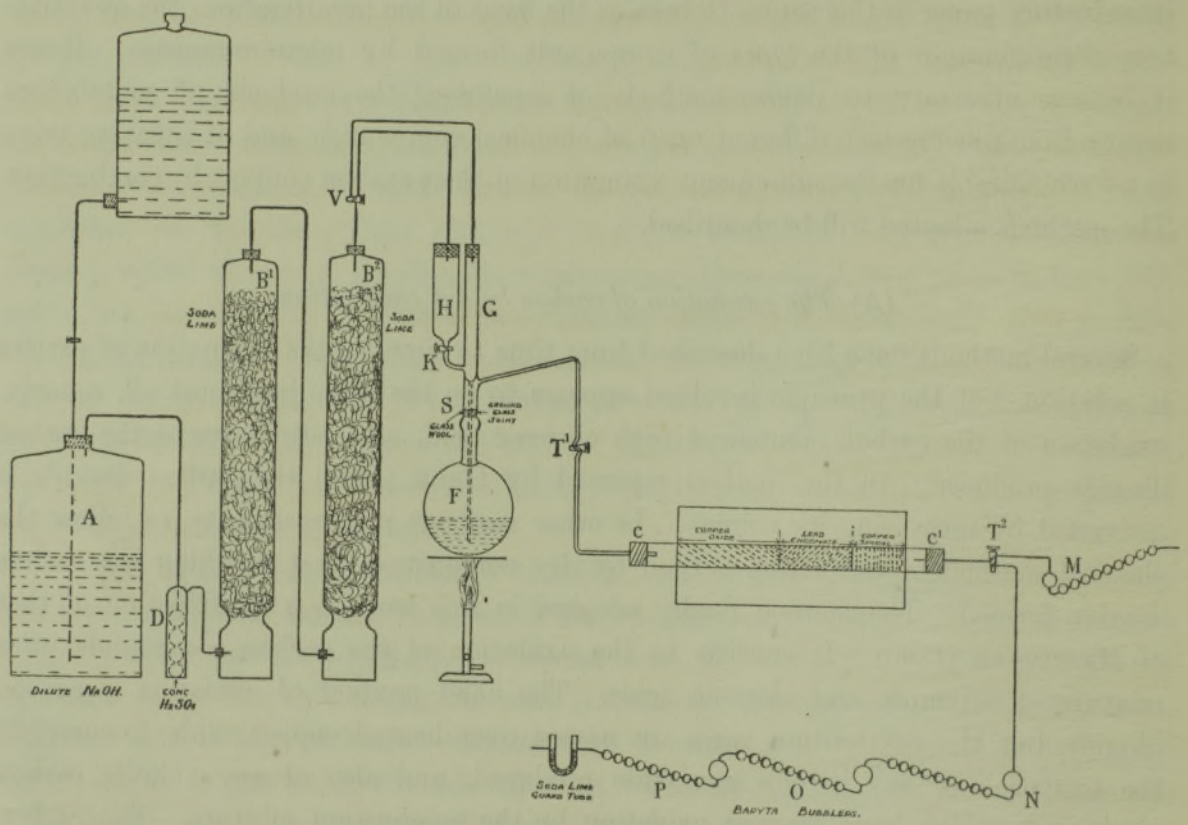

Fig. 1

from A, via tube G, until free from carbon dioxide, as tested by bubbling the air for a short time through M. V is then closed and T_2 connected with the bubblers N, O, P. K is closed, the stopper from H removed, and a suitable amount of the solution to be oxidized is pipetted into H. It is run into F by replacing the stopper in H, connecting V to H and opening K. In a similar way the solution is washed into F, first with a little water, and then with 100 c.c. of concentrated sulphuric acid, divided into three portions and added, by means of a pipette, to wash down the sides of H. A slow stream of air is passed via G through F, which is heated, gently at first and later more strongly, to bring about combustion. When this is complete, heating is stopped and the combustion gases are swept out of the apparatus and through the bubblers N, O, P by a quicker current of air.

If a solid is to be oxidized the procedure is similar, except that the substance is weighed out directly into F at the commencement, and the chromic acid is only run from H after the apparatus has been initially filled with CO_2-free air, and before the sulphuric acid is introduced.

At the end of the combustion the bubblers containing any barium carbonate—usually only N, but occasionally O—are washed out and titrated with N/2 hydrochloric acid to phenolphthalein. The baryta in P should always remain perfectly clear, but if any cloudiness appears another bubbler is immediately added in order to ensure complete absorption.

Blank estimations are, of course, necessary, as both chromic acid and sulphuric acid nearly always contain a little carbon.

To test out the method, a number of carbon estimations were made on a variety of pure substances. The results, which are given in Table 1, indicate that the method is capable of giving perfectly accurate and reliable results. It is also decidedly economical in time.

TABLE 1.—*Estimation of carbon in pure substances by wet combustion.*

Substance analysed.	Percentage Carbon.	
	Found.	Calculated.
Cane sugar	42·43 41·75	42·11
Acetaldehyde ammonium bisulphite	19·11	19·18
Oxalic acid	18·85	19·04
Fumaric acid	41·11	41·37
Succinic acid	40·91	40·68
Mucic acid	34·36	34·27
Acetone, 5 c.c. of stock solution containing 0·2437 gm. acetone	62·21	62·00
Silver acetate	14·47 14·33 14·45	14·38
Acetic acid (10 c.c. of solution)	0·1121 gm.	0·1120 gm. calculated from titration.
Dry mycelium of *Aspergillus niger*	46·86 46·71	46·55 by direct dry combustion.

(B) *Apparatus for metabolism experiments.*

It is evident that in work of this nature, involving the culture of micro-organisms with a view to the subsequent analysis of their metabolic products and the preparation of carbon balance sheets for each organism studied, a suitable type of apparatus must fulfil the following conditions :—

(1) It must permit of the organism being grown in a closed system so that the whole of the metabolic products—gaseous, liquid and solid—are retained in such a manner as to be easily subjected to quantitative examination.

(2) It must allow of the passage of a continuous stream of sterile gas (air, nitrogen, &c.) over the surface of, or through, the liquid where the organism is growing, so that the experiment may be conducted aerobically or anaerobically at will, and so as to enable the gaseous products of metabolism to be collected for analysis.

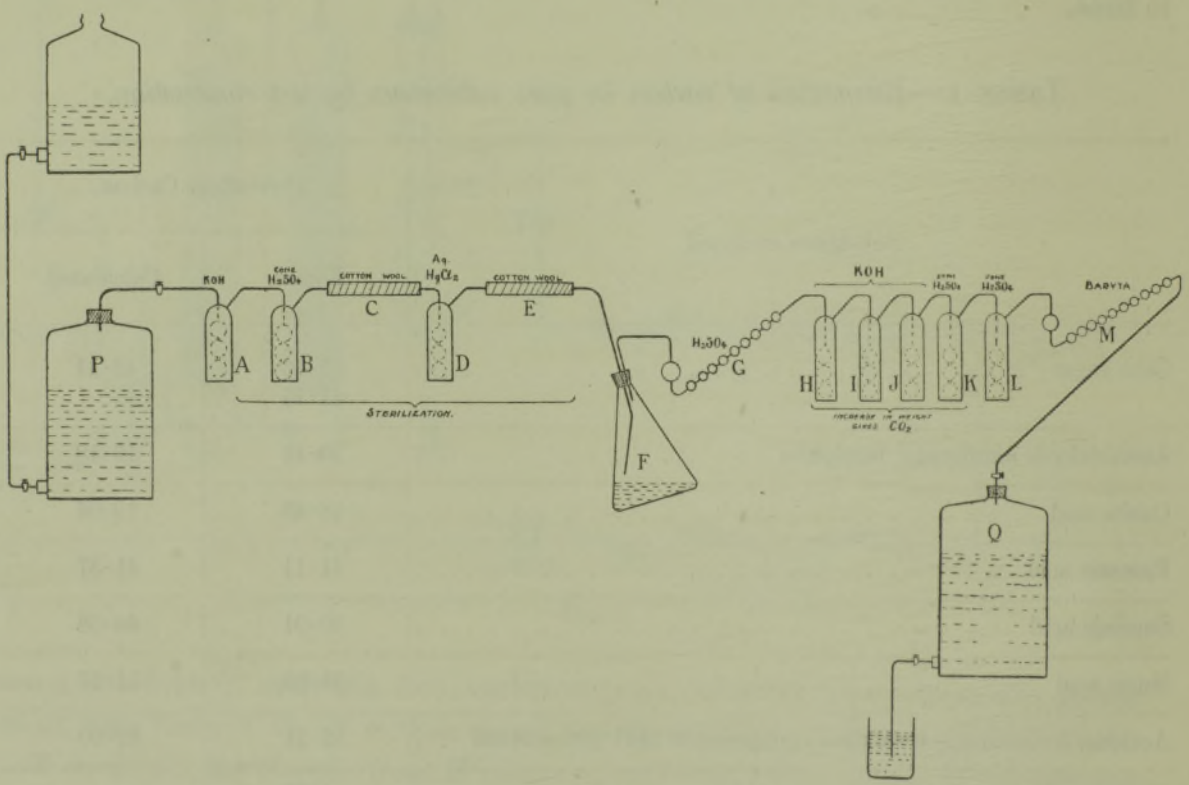

FIG. 2

The form of apparatus finally decided on is shown in fig. 2. It was designed primarily for the cultivation of fungi but, with very slight modification, it is suitable for use with any type of micro-organism.

It consists of three parts designed for :—

(1) *Purification of the gas current.*—Whatever gas is used must be microbiologically sterile and chemically free from carbon dioxide. This is done by passing the gas first

through a potassium hydroxide bubbler A to remove carbon dioxide, then through a concentrated sulphuric acid bubbler B and a sterilized cotton-wool filter C. The gas is further sterilized by bubbling through dilute mercuric chloride solution (1 : 1,000) in D and finally through another sterilized cotton-wool filter E. The bubbler D serves a double purpose. Since it contains mercuric chloride solution, it helps to sterilize the gas in addition to maintaining saturation of the gas with moisture, thus avoiding evaporation of the contents of flask F. The efficiency of the sterilization effected by the above method is shown by the fact that, although relatively large volumes of air are passed through the culture flask F during the course of an experiment, often lasting several weeks, cases of infection have been extremely rare.

(2) *Cultivation of the micro-organism.*—The culture being studied is grown on a suitable culture medium in a 750 c.c. conical flask F, the neck of which is closed by a sterile bung, carrying an inlet glass tube which reaches practically to the surface of the culture medium, and an outlet tube, the end of which only just passes through the bung. Each end of each tube is fitted with a cotton-wool plug, and the whole fitting—bung and glass tubes—is sterilized in the autoclave immediately before use.

(3) *Absorption and collection of the gaseous end-products.*—The gas stream, as it emerges from the flask F, contains carbon in the form of carbon dioxide and traces of volatile organic compounds. The latter are removed by passing the gas through the sulphuric acid bubbler G, which also serves to dry the gas before absorption of the carbon dioxide in the absorption bulbs—three potassium hydroxide bulbs H, I, J and a concentrated sulphuric acid bulb K. These four bulbs are weighed at the beginning and at the end of an experiment, the difference giving the weight of carbon dioxide formed. The bulb L contains concentrated sulphuric acid to protect the bulb K from back diffusion of water vapour. The baryta bubbler M is merely an indicator to show whether absorption of carbon dioxide is complete. A known volume of standard baryta is measured into M at the start of each experiment, so that if any carbon dioxide has escaped absorption in the potassium hydroxide bulbs, as evidenced by the appearance of a turbidity in M, it may be readily estimated by titration. The gaseous products of metabolism, other than carbon dioxide, together with the residual gases passed through F, are collected in the aspirator Q.

(C) *Technique employed for the cultivation of micro-organisms in the metabolism experiments.*

In the metabolism experiments described in Parts III, IV, V and VI it was essential that the medium employed for the cultivation of the fungi should be a purely synthetic one, so as to admit of reproduction at any future time. The medium finally chosen for use was that recommended by Czapek (1902) and later by Dox (1910) and has the composition given in Part I, page 7. It is generally suitable as a standard medium for the cultivation of fungi and is largely used for that purpose. It has only one source

of carbon, has a standard chemical composition, is easily reproducible, and lends itself admirably to analysis.

The medium was made up in large batches of 10–15 litres, and was then accurately pipetted out, in 250 c.c. quantities, into a large number of 750 c.c. conical flasks, all of the same shape. The flasks were then plugged with cotton-wool, and sterilized by steaming for half an hour on each of three consecutive days. Three flasks from each batch were taken for analysis, and the remainder were covered with lead-foil caps to prevent evaporation, and stored for use. Under these conditions the medium kept perfectly, as, even at the end of twelve months' storage at 24° C., analysis failed to reveal any alteration in the carbohydrate content. The flasks were always stored at 24° C. for at least a month before use, in order to detect any which had been imperfectly sterilized.

A number of similar-sized fittings for the necks of the culture flasks—comprising rubber bung and glass delivery tubes, as described on p. 15—were made, so that each would fit any of the culture flasks. These fittings were sterilized before use by wrapping in parchment paper and heating in an autoclave at 120° C. for an hour. Fresh cotton-wool plugs were inserted in the side tubes for each experiment.

At the commencement of a metabolism experiment one of the 750 c.c. culture flasks is sown with spores of the organism under investigation. This is done by emulsifying, in 5 c.c. of sterile distilled water, the spores of a young culture grown at 24° C. on a test-tube slope of Czapek-Dox agar, and adding the emulsion to the medium. This mass sowing, which experiment showed to add only negligible traces of carbon to the medium in the metabolism flask, was chosen in preference to the more usual small sowing, in order to encourage quick growth of a mycelial felt and minimize the risk of infection. In practice it worked very well, especially with the more "delicate" fungi, which, with a small sowing, give only a very feeble growth and are often contaminated. Single-spore cultures of all the fungi investigated were used in every experiment described.

Immediately after sowing, the sterilized rubber bung and side tubes are inserted in the neck of the culture flask in place of the cotton-wool plug, and the flask is inserted in position in the apparatus for these metabolism experiments described on p. 14. A bubbler G, containing 20 c.c. of fresh concentrated sulphuric acid, the carbon dioxide absorption bulbs freshly charged and weighed, and an aspirator Q filled with water are also inserted in their proper places. The whole apparatus is tested for gas leaks and is then incubated for a suitable period, varying from two to eight weeks.

The whole of the metabolism experiments were carried out in a large constant-temperature room maintained at an average temperature of 24° C. (23°–25°), as many as 14 experiments proceeding concurrently.

During the whole of the experiments described in Parts III, IV, V and VI air was the only gas passed through the metabolism apparatus. At first, aspirators were used to supply the air, but later, with the expansion of the work, it became desirable to

use compressed air. This was drawn from taps laid at convenient intervals in a compressed-air supply pipe which circled the incubator room. The method of aeration used at first was to bubble a slow stream of air continuously through the whole apparatus. Later, however, this was abandoned, as it was found that more interesting results were obtained by passing about 400–500 c.c. of sterile air through the apparatus during the space of one hour per day. This method of "controlled aeration" is probably responsible for a number of the interesting results obtained—especially with the *Aspergilli* and *Penicillia*—a fact which is discussed in greater detail in Parts III and IV.

At the end of a metabolism experiment, which is stopped, if possible, before all the glucose has disappeared, in order to minimize destruction of possible metabolism products, a quick current of air is passed through the whole metabolism apparatus for two or three hours. The culture flask F is taken off for examination and analysis, and the various parts—the sulphuric acid bubbler G, the carbon dioxide absorption bulbs H, I, J, K, and the aspirator Q—are disconnected and their contents submitted to analysis. In all cases portions of the mycelium are immediately transferred to Czapek-Dox agar and beer-wort agar and incubated at 24° C. to test the purity of the culture, while another portion is examined microscopically for contaminations.

(D) *Methods of separation and analysis of the metabolic products.*

The carbon originally supplied to the fungus as glucose has been metabolized by it, at the end of a metabolism experiment, into a number of different types of carbon compounds, which, for ease of subsequent description, may be divided first into three different classes :—

(1) Carbon as gaseous compounds.
(2) Carbon as insoluble solid compounds.
(3) Carbon as compounds in solution.

These will be dealt with in turn, and the methods of separation and analysis for each class described as they arise.

(1) *Carbon as gaseous compounds.*—All the gaseous products of metabolism are contained in the sulphuric acid bubbler G, in the potash bulbs H, I, J, in K, and in the aspirator Q. The carbon as gaseous products may thus be subdivided into three sub-classes.

(1*a*) *Carbon as volatile compounds soluble in strong sulphuric acid.*—This sub-class includes small amounts of the vapours of such bodies as the alcohols, aldehydes, ketones, &c. These are carried over by the stream of air passed during the experiment, and are absorbed in the strong sulphuric acid contained in bubbler G. Its carbon content is estimated by wet combustion in the combustion apparatus.

(1*b*) *Carbon as carbon dioxide.*—The four bulbs H, I, J and K are disconnected, wiped clean, allowed to stand in the balance room for an hour and weighed. From the weight of carbon dioxide the carbon as CO_2 is readily calculated.

(1*c*) *Carbon as permanent gases.*—The gas in the aspirator Q, consisting of vitiated air together with any gaseous products, other than carbon dioxide, arising from the decomposition of the sugar by the fungus, is brought to atmospheric pressure in the incubator room by running water into the aspirator until the pressure is adjusted. The temperature and atmospheric pressure are then noted. The volume of the gas is determined, after analysis, by deducting the volume of the residual water from the volume of the aspirator. The gas is then analysed in a BONE-WHEELER gas-analysis apparatus for—

(1) *Oxygen*—which is absorbed in alkaline pyrogallol in the usual way.
(2) *Combustible gases—e.g.*, methane, hydrogen.

These gases are first tested for qualitatively by adding to a known volume of the vitiated air from the aspirator a known volume of explosive gas—made by the electrolysis of acidulated water, and consisting of a mixture of hydrogen and oxygen in the proportion of two volumes of hydrogen to one volume of oxygen. The function of this explosive gas is to initiate the explosion of any methane or hydrogen which may be present. The mixture of vitiated air and explosive gas is then exploded in the BONE-WHEELER apparatus and the volume of residual gas noted. Unless this volume is less than the original volume of vitiated air taken for analysis no methane, hydrogen or other combustible gas can be present. This follows, since an electrolytic explosive gas leaves no residue on explosion, and since, in addition, methane, hydrogen or any other combustible gas likely to arise during fermentation shrinks in volume on exploding with oxygen, as is evident from a consideration of the various equations.

No evidence has so far been obtained of the production of either methane or hydrogen, or of any other combustible gas, by any species of mould tested.

The main use at present of the analysis of the vitiated air is that it makes it possible to estimate the "respiration coefficient" for the fungus under investigation. The respiration coefficient, which is $\frac{\text{Volume of carbon dioxide produced}}{\text{Volume of oxygen absorbed}}$, has proved of considerable value for classification purposes, particularly with the *Aspergilli* and *Penicillia*.

The volume of carbon dioxide produced is readily calculated from the sum of the carbon dioxide absorbed in the potash bulbs (1*b*) and the carbon dioxide dissolved in solution (3*b*).

The volume of oxygen absorbed is also readily calculated by making what appears to be a justifiable assumption, at any rate with any of the fungi used. It is assumed that these fungi do not fix atmospheric nitrogen nor produce nitrogen from the sodium nitrate contained in the medium. Since the final volume of vitiated air and its content of oxygen are known, the final volume of nitrogen can be calculated. Making the above assumption, this volume is also equal to the volume of the nitrogen present in the air originally passed through the apparatus. The volume of this air can be readily

calculated from the volume of nitrogen by using the factor 79·03 per cent. for the percentage of nitrogen in atmospheric air. Then, by deducting from the original volume of air the volume of vitiated air at the finish, the volume of oxygen absorbed is obtained. This figure is probably slightly inaccurate, but not sufficiently so to affect the respiration coefficient appreciably. Since very large differences are observed in the respiration coefficients in different species of fungi this slight error becomes quite negligible in comparing the respective respiration coefficients.

(2) *Carbon as insoluble carbon compounds.*—The only substance included in this class which has so far been encountered is the mycelium of the fungus under observation. A little difficulty was experienced at first in separating satisfactorily the mycelium from the metabolic products in solution. This was due to the fact that it was found in earlier experiments that, when a mould mycelium is filtered through an ordinary filter paper, it is impossible, after drying, to detach the mycelium and the accompanying spores in any quantitative fashion without detaching fibres of filter paper, which vitiate the carbon figures obtained in the subsequent combustion of the mycelium. In order to overcome this difficulty recourse was had to filtration through kieselguhr, since the fact was established by experiment that, on drying a filter paper which has been covered during filtration with a thin layer of kieselguhr, the latter peels off in a layer without detaching any fibres from the paper.

The procedure adopted to separate the mycelium from the soluble metabolic products is as follows :—

The metabolism flask F is detached from the apparatus and, after mycological examination (see p. 17), the contents are neutralised to *p*H 7·0 with standard sodium hydroxide (or hydrochloric acid), using bromthymol blue paper as an external indicator. This titration gives the acid (or alkali) formed by the organism, since the titration value, to the same *p*H, of the original medium is known. The liquid is then filtered through kieselguhr in the following way.

An 11 cm. Buchner funnel with a double, well-fitting filter paper is connected to an Irvine filtering tube (1915). About 0·2 gm. of kieselguhr, which has previously been thoroughly extracted with concentrated hydrochloric acid, then washed, calcined and preserved dry, is accurately weighed into a 500 c.c. flask, and a smooth suspension made by shaking with 300–400 c.c. of distilled water. This is poured on the filter paper so as to give a uniform layer, the flask being finally washed out quantitatively on to the filter.

The liquid contents of flask F are then filtered through the funnel, the mycelium being retained in the flask. It is carefully washed about ten times with separate 10–20 c.c. quantities of hot distilled water, the flask being heated on the boiling water bath for a short time after each successive addition of water. The mycelium is finally washed out into the Buchner funnel, drained dry, and the combined filtrate and washings cooled and made up to 500 c.c. for analysis.

The Buchner funnel and contents are then placed in the oven at 100° C. till the

mycelium is completely dry. The mycelium and most of the kieselguhr are then carefully peeled off the filter paper, weighed in a closed weighing bottle, and ground up in a mortar until a perfectly even sample is obtained. This is again dried and its carbon content determined by wet combustion of a portion. The weight of kieselguhr left adhering to the filter paper is estimated by ashing the latter. From the figures thus obtained the dry weight of mycelium, the total carbon (2), and therefore the percentage of carbon in the mycelium is found.

(3) *Carbon as compounds in solution.*—The total carbon present as compounds in solution (3) has been divided for the purposes of analysis into seven sub-classes :—

(3*a*) Carbon as residual glucose.
(3*b*) Carbon as carbon dioxide in solution.
(3*c*) Carbon as volatile acids.
(3*d*) Carbon as non-volatile acids.
(3*e*) Carbon as volatile neutral compounds.
(3*f*) Carbon as non-volatile neutral compounds (including synthetic compounds).
(3*g*) Carbon unaccounted for.

Hence the sum of (3*a*), (3*b*), (3*c*), (3*d*), (3*e*), (3*f*), (3*g*) is equal to (3), *i.e.*, the total carbon in solution.

The methods used for the separation and analysis of each sub-class will now be described in detail.

(3) *Total carbon as compounds in solution.*—The carbon content of duplicate portions of 10 c.c. of the filtrate from the mycelium (p. 19) is estimated in the usual way by wet combustion.

Here it may be pointed out that a very useful check on the experimental details up to this point is afforded by the fact that the sum of the carbon present in each of the four classes—

(*a*) Carbon in sulphuric bubbler (1*a*),
(*b*) Carbon in carbon dioxide (1*b*),
(*c*) Carbon in mycelium (2),
(*d*) Carbon in compounds in solution (3),

should equal the total carbon present in the culture medium, at the commencement of the metabolism experiment, a figure which is determined for each batch of medium made up. Any appreciable discrepancy points either to a leak of carbon dioxide through faulty joints in the apparatus or to errors in the quantitative estimations. Although almost three hundred complete metabolism experiments on different types of fungi have been carried out in this laboratory, it has been found that the sum of the above four classes accounts for 96–100 per cent. of the carbon initially supplied, in at least 95 per cent. of the experiments.

(3*a*) *Carbon as residual glucose.*—Since the carbon as residual glucose often amounts to the greater part of the carbon in solution, it is essential that the sugar should be determined with as great accuracy as possible. The method adopted for the estimation of glucose, and used in the preparation of carbon balance sheets, is that described by SHAFFER and HARTMANN (1921). This method involves copper reduction and the oxidation of the cuprous oxide by iodine in *acid* solution.

It was feared that the method might not give accurate results, because of the absorption of iodine, in those cases where unsaturated metabolism products are present. This fear seems groundless, however, since in a test on a solution of glucose containing fumaric acid the divergences from the calculated results were all within the limits of experimental error.

A check was, however, kept on the results obtained by the above method by carrying out additional estimations of the glucose by each of three other methods of different types whenever sufficient material was available. Further, these methods occasionally afforded valuable information on the types of metabolism products formed, in those cases where discrepancies were observed between the results obtained by them and by the SHAFFER and HARTMANN method. The three methods used were :—

(*a*) *Polarimeter.*—The glucose was estimated in the usual way by measuring the optical rotation of the solution in the polarimeter. In several cases, which are referred to in more detail in Parts III, IV and VI, there were very marked discrepancies between the figures for glucose as estimated by the SHAFFER and HARTMANN method, and as calculated from the optical rotation. These discrepancies are caused by the production of optically active substances which have no reducing effect on copper solutions, a notable example being gluconic acid.

(*b*) WOOD-OST *method* (1904).—This well-known method, involving copper reduction like the SHAFFER and HARTMANN method, invariably gave results agreeing with those obtained in the latter method, except in cases where traceable mistakes had been made.

(*c*) HINTON *and* MACARA *method* (1924).—The principle involved in this method is the oxidation of the glucose by iodine in *alkaline* solution. It is a very simple method, giving accurate results with pure glucose. However, in the presence of any substance giving rise to iodoform in *alkaline* iodine solution, *e.g.*, alcohol, acetaldehyde, acetone, kojic acid, or any other substance absorbing iodine, values are obtained which are sometimes far in excess of those obtained in the SHAFFER and HARTMANN method. This fact proved of considerable use in investigating the products of metabolism of some of the fungi.

(3*b*) *Carbon as carbon dioxide in solution.*—The carbon present in the metabolism solution as carbon dioxide, whether as the free gas or in combination as carbonates, is estimated by the VAN SLYKE method, originally described for the estimation of carbon dioxide in blood (1917). The method consists in removing the carbon dioxide *in vacuo*, in a special apparatus designed by VAN SLYKE, from the acidified liquid and

measuring its volume. Triplicate estimations are carried out on 1 c.c. quantities, and the weight of carbon calculated from the volume of carbon dioxide measured.

(3*c*) *Carbon as volatile acids.*—Included in this class are all the organic acids which are volatile in steam, including all the lower fatty acids up to, say, valeric acid. In order to isolate and analyse the volatile acids the latter are separated by distillation *in vacuo* of the metabolism liquid, after acidification with phosphoric acid. The acids are absorbed in a measured amount of standard baryta, the excess of baryta is titrated, a correction made for carbon dioxide, and the acidity due to volatile acids is calculated. The solution of the barium salts of the volatile acids is evaporated to dryness, weighed, and the carbon content determined by wet combustion.

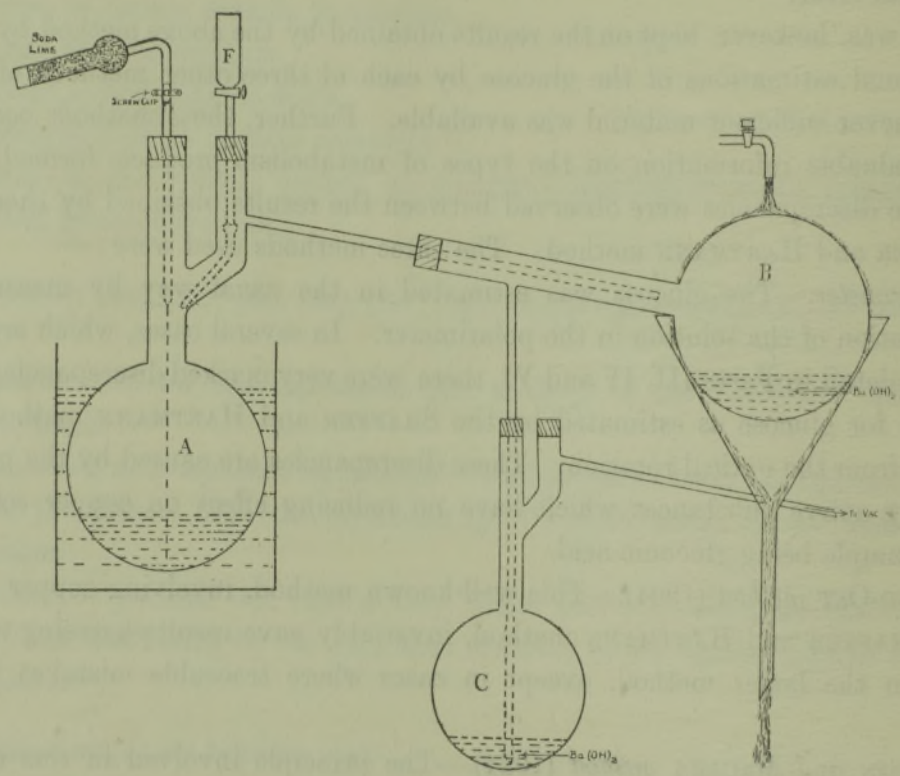

Fig. 3.

The details of the method are as follows: 100 c.c. of the metabolism solution [or, in case of scarcity, the residue from the estimation of volatile neutral matter (3*e*)] are introduced into the litre Claisen distilling flask A, forming part of the vacuum distillation apparatus shown in fig. 3. 20 c.c. of standard N/4 baryta are run from a burette into the flasks B and C—15 c.c. into B and 5 c.c. into C—and washed in with CO_2-free distilled water. 10 c.c. of 10 per cent. phosphoric acid are then added to flask A, the apparatus connected up, evacuated, and the liquid in flask A evaporated to about 20 c.c., the volatile acids being absorbed in the baryta. 150 c.c. of CO_2-free water are run into A through the funnel F, without letting in air, and the distillation is repeated. Three further amounts of 150 c.c. of CO_2-free water are added and distilled, the liquid

being evaporated to about 20 c.c. each time. Under these conditions the volatile acids are completely separated. The baryta from B and C is washed out into a litre conical flask and titrated with N/10 sulphuric acid to neutrality with phenolphthalein. In order to avoid errors in the subsequent combustion of the barium salts, a known amount—25 c.c.—of aqueous phenolphthalein is used as an indicator and a correction is made in the weight of the barium salts, and in their carbon content, for the weight of phenolphthalein in 25 c.c. and for its carbon content. Errors due to carbon dioxide are corrected by rinsing out flasks B and C with 10 c.c. of N/10 H_2SO_4, washing this into the litre conical flask, and boiling under reflux to remove carbon dioxide. After cooling, the excess of sulphuric acid is titrated back with baryta and the carbon dioxide calculated. The solution is then filtered from the precipitate of barium sulphate, and the filtrate evaporated to dryness. The residue of barium salts of the volatile acids is weighed and its carbon content determined by wet combustion.

From the weight of the barium salts of the volatile acids, their titration value, and their carbon content, it is possible to obtain an approximate idea as to their composition.

(3*d*) *Carbon as non-volatile acids.*—The problem of the separation of the non-volatile acids proved to be difficult of solution. A reasonably satisfactory method was obtained, however, by taking advantage of the fact that, while the calcium salts of the volatile acids are relatively soluble in alcohol, those of the non-volatile acids are practically insoluble. The experimental basis for this was obtained by determining the solubilities in 80 per cent. alcohol of the calcium salts of a number of acids likely to be met with in fermentation processes. This was carried out by adding to 20 c.c. of a saturated aqueous solution of the calcium salt of the acid under investigation 80 c.c. of 97 per cent. alcohol, making a final volume of 100 c.c. The mixture, after standing overnight, was filtered, an aliquot part evaporated to dryness, the residue ignited with strong sulphuric acid and the calcium sulphate weighed. From this the weight of organic acid present originally as the calcium salt was calculated.

The results obtained are given in Table II.

It will be seen that the calcium salts of the non-volatile acids have solubilities in 80 per cent. alcohol so low that they may be regarded as insoluble. On the other hand, the calcium salts of the volatile acids are very soluble, so that separation of the two classes by this means is practicable. There still remains the case of lactic acid, a common product of fermentation, which is not readily volatile and thus would not be accurately estimated under section (3*c*). It is unfortunately not included in class (3*d*) either, since its calcium salt is soluble in 80 per cent. alcohol to the extent of 334 mgm. of free lactic acid in 100 c.c. of solution, using the same method of expressing solubilities as in the following table. It is, however, included in class (3*g*) and is referred to later under that heading.

The details of the method used for the separation and analysis of the non-volatile acids are as follows: 50 c.c. of the metabolism solution are pipetted into a 250 c.c. measuring

TABLE II.—Solubilities of the calcium salts of various organic acids in 80 per cent. alcohol at 15° C.

(The solubilities are expressed as milligrams of free acid per 100 c.c. solution.)

Acid.	Solubility.
Non-volatile acids—	
Glycollic	3·6
Oxalic	0·3
Fumaric	2·1
Succinic	3·0
Tartaric	2·5
Citric	6·3
Malic	19·9
Gluconic	3·9
Volatile acids—	
Formic	152·3
Acetic	1,262
Propionic	2,674
Butyric	3,485
Pyruvic	76

flask. To this is added about 150 c.c. of 97 per cent. alcohol, and any precipitate formed is filtered off. To the clear solution is then added a little phenol red and ammonia, drop by drop, until a permanent pink colour is obtained. The calcium salts are then precipitated by the addition of 5 c.c. of 20 per cent. calcium acetate solution, more ammonia if necessary, and sufficient alcohol to make up to 250 c.c. The mixture is well shaken and allowed to stand overnight. It is then filtered on a tared asbestos Gooch filter (the asbestos having been previously extracted with hydrochloric acid and ignited), dried and reweighed. This gives the weight of the calcium salts of the non-volatile acids. Their carbon content is then determined by the usual wet combustion method, the pad of asbestos as a whole being transferred to the combustion flask. From the weight of the calcium salts and their carbon content an approximate idea can be got of the composition of the non-volatile acids.

(3*e*) *Carbon as volatile neutral compounds.*—This class includes such compounds as alcohols, ketones, aldehydes, &c., which are readily separated from volatile acids and other compounds by distillation of the metabolism solution, which, as stated on p. 19, is neutralized to *p*H 7·0 before analysis.

100 c.c. of the metabolism solution are distilled from a Claisen distillation flask, through a condenser, and the distillate is collected in a measuring flask cooled in ice. If only small amounts of volatile compounds are present, only 50 c.c. of distillate are collected. If, on the other hand, relatively large amounts are present, a second 50 c.c. of distillate are collected, after cooling the distillation flask, adding 50 c.c. of distilled water, and redistilling. The carbon content of the distillate is then estimated in an aliquot portion (5 c.c. or 10 c.c.) in the usual apparatus.

(3*f*) *Non-volatile neutral compounds. Synthethic substances.*—No satisfactory method has been evolved for the separation and estimation of all the non-volatile neutral compounds which would be likely to occur in a fermentation solution. This class covers a wide range of compounds of a number of types, including synthetic compounds (proteins, peptones, &c.), polyhydric alcohols and polyhydric neutral compounds generally. It was therefore necessary to estimate the synthetic compounds separately, leaving the rest of the non-volatile neutral compounds to be included in class (3*g*), to be estimated separately later, should occasion arise (*cf.* mannitol in Part X and kojic acid in Part VIII).

The synthetic substances, including proteins, peptones, &c., are separated from the metabolism solution by precipitation with colloidal iron solution, and estimated as follows:—To 100 c.c. of the metabolism solution, 25 c.c. of colloidal iron solution are added drop by drop, the flask being shaken vigorously during the addition. The flask is heated in a boiling water bath to flocculate the iron precipitate which carries down with it any proteins, peptones, &c., present. The precipitate is then filtered on a kieselguhr filter, prepared as described on page 19, washed, dried, peeled from the filter paper and the mixture of ferric hydroxide and kieselguhr analysed in the usual apparatus.

(3*g*) *Carbon unaccounted for.*—This class, which includes the majority of the non-volatile neutral compounds, including particularly the polyhydric alcohols and other neutral compounds, has proved of particular interest. A comparatively large figure in this class is an indication that the fungus in question is worthy of further experimental investigation, since it indicates the production in appreciable quantities of some, possibly new, fermentation compounds, by the micro-organism under investigation. In interpreting the results obtained, however, care must be taken not to overlook the fact that lactic acid is included with the non-volatile neutral compounds (see page 23).

The figure "Carbon unaccounted for" is, of course, not an experimental one, but is obtained by subtracting from the total carbon in solution (3) the sum of the carbon in the sub-classes (3*a*), (3*b*), (3*c*), (3*d*), (3*e*) and (3*f*).

In concluding this section it should be said that it is necessary to keep a strict check on all reagents used during the analysis, and blank estimations were therefore made on all samples of chromic and sulphuric acids used in the wet combustion method, corrections being made later for the carbon found. Particular care is necessary with the chromic acid, some samples of which give large carbon blanks. Complete analyses have also been carried out on different batches of media, treating the unsown Czapek-Dox solution as a metabolism solution and carrying through a complete analysis on each flask so chosen. The averages of these figures for the classes (3*a*) to (3*f*), which were in no case very high, were used to correct the final carbon balance sheets, all of which are thus made up from figures corrected for blanks of reagents used, and for blanks of methods.

Large numbers of these balance sheets were prepared and will be found in Parts III, IV, V and VI of this series.

Summary.

A description is given of the methods elaborated for the quantitative determination of different *types* of metabolic compounds formed by the action of micro-organisms on carbohydrates. From these figures a carbon balance sheet is prepared for the micro-organism under investigation.

The methods adopted are described under the following heads :—

(A) The estimation of carbon by wet combustion (p. 11).

(B) Apparatus for metabolism experiments (p. 14).

(C) Technique employed for the cultivation of micro-organisms in the metabolism experiments (p. 15).

(D) Methods of separation and analysis of the metabolic products (p. 17).