PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Introductory

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PHILOSOPHICAL TRANSACTIONS.

Studies in the Biochemistry of Micro-organisms.

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Part I.—*Introductory.*

By Harold Raistrick *and* William Rintoul.

The application of fermentation processes to the arts, such as brewing, wine-making, the production of vinegar and the tanning process, has been practised from time immemorial, though only during the nineteenth century was it definitely recognized that the processes are accomplished by the aid of living organisms. To Pasteur chiefly is due the establishment of the necessity of the living cell to fermentation and the development of scientific practice to supersede the old rule-of-thumb methods.

In recent years an increasing interest is being taken in the biochemical investigation of micro-organisms and their products.

Of the micro-organisms which are known only the two classes—the Schizomycetes (bacteria) and the Eumycetes (true fungi)—seem to offer much hope of successful chemical investigation because of the relative ease with which they are cultivated in artificial media. Since more attention has hitherto been paid to the chemical activities of the Schizomycetes (bacteria) and of that family of Eumycetes known as the Saccharomycetaceæ (yeasts), it was decided to investigate first the metabolic products of other families of the Eumycetes popularly known as " moulds."

Up to the present these fungi have been investigated chiefly from a morphological point of view. It is neither possible nor desirable here to deal with the mass of taxonomic literature running through two centuries of descriptive botany, but a review of the relatively few investigations dealing with the metabolic products of moulds will make it abundantly clear that certain families would justify chemical investigation with every hope of encountering new fermentation products or new types of fermentation.

Historical.

The formation of acid by moulds is so easily recognized by a simple titration of the medium that it is only natural that acid production early excited attention and most of the pioneer work on mould products deals with carboxylic acids which are formed in considerable amount by many of the commoner species of mould. The first acid to be encountered and recognized was oxalic acid, which is very easy to detect owing to the insolubility of its calcium salt.

Oxalic acid was detected in the tissues of moulds as crystals of calcium oxalate by many observers, but it was first recognized as a definite fermentation product by Wehmer (1891). In a series of classical researches he made an extended study of the conditions of its production from sugars by *Aspergillus niger*. By the addition of calcium carbonate to this medium, consisting of sugar and inorganic salts, he was able to obtain yields of calcium oxalate up to 120 per cent. of the sugar. Other observers have since confirmed and elaborated his findings, amongst whom are Heinze (1903), Emmerling (1903), Currie and Thom (1915) and Elfving (1918).

Citric acid was first shown to be a product of mould fermentation by WEHMER (1893). He obtained it in excellent yield by growing species of *Citromyces* on synthetic media containing sugar as the only source of carbon. He again employed calcium carbonate to fix the acid and by this means claimed to obtain much improved yields. CURRIE (1917) investigated the lag between the total acidity and the oxalic acid produced by strains of black *Aspergillus* and this led him to the discovery that citric acid is produced in large amounts by this species. He showed that the formation of oxalic acid could be entirely inhibited by correct manipulation and by selecting the proper concentration of sugar in the medium, and worked out the conditions of citric acid manufacture from saccharose as a commercial process. Other observers, including MOLLIARD (1919) and BUTKEWITSCH (1922–4), have worked in the field of citric acid production. BLEYER (1926) has patented a process for its production in Germany, and FERNBACH, YUILL and ROWNTREE (1927) have developed a similar patent in this country.

Citric acid is now recognized as one of the commonest products of mould fermentation, as it is formed not only by species of *Citromyces* as was at first thought by WEHMER, but by many species of *Aspergillus*, and also by the majority of species of *Penicillium*, as is shown by CHRZASZCZ and TIUKOV (1929).

d-*Gluconic acid* was first isolated as a mould fermentation product by MOLLIARD (1922) from *Aspergillus niger*, and in a series of subsequent papers (1923, 1924) the conditions for the formation of gluconic, citric and oxalic acids were investigated, particularly with respect to unbalanced media. Partial deprivation of the inorganic constituents was found to lead to an increased yield of gluconic acid. MOLLIARD'S discovery was soon confirmed by other workers, notably BERNHAUER (1924), BUTKEWITSCH (1924) and FALCK and KAPUR (1924). Still more recently MAY, HERRICK, THOM and CHURCH (1927) have shown that *d*-gluconic acid is formed in very high yield by *Penicillium luteum* var. *rubrisclerotium*, and in a later paper of HERRICK and MAY (1928) details are given of the preparation in quantity of gluconic acid from glucose by means of this organism.

Fumaric acid was first observed by EHRLICH (1911) to be a product of *Mucor stolonifer*, and was later shown by WEHMER (1918) to be formed in large amount from sugar by a species of *Aspergillus* which he named *A. fumaricus*. In a culture reputed to be *A. fumaricus* obtained from THOM we were unable to show any production of fumaric acid. It is of special interest, therefore, to note that WEHMER (1928) in a recent paper states that his culture, which originally gave large amounts of fumaric acid, now produces only traces of this substance and that gluconic acid is formed instead.

Malic acid.—Although casual references occur in the literature to the production of malic acid by moulds, there appear to be no well-founded proofs of its formation until the latest paper of WEHMER (1928). Here it is definitely shown that malic acid is produced in small amounts from glucose by *Aspergillus fumaricus*. We have been able to show that malic acid is produced by other moulds. It was isolated as the ester

from the acid products of one of the white species of *Aspergillus* (Ad. 55), (see Part IX), and also from *Aspergillus Wentii* (see Part XVII).

Succinic acid.—Here again there are isolated references to the occurrence of succinic acid as a mould product, without any definite evidence of its identification. We have found that it is actually formed in small amounts from sugar by several species of mould—*Aspergillus* and *Fumago* (see Parts IX and XVII). It was isolated as the ester and identified both as the free acid and by its derivatives. The formation of succinic acid by yeast in alcoholic fermentation has of course long been known, and it has been proved by Ehrlich (1909) to be derived from the de-amination of amino-acids. There is no evidence to show whether the acid formed by moulds is produced in a similar manner or is a direct product of the oxidative breakdown of sugar.

Other Mould Products.

Ethyl alcohol.—The formation of ethyl alcohol by moulds has been recognized in a few cases which have, however, until lately been regarded as exceptional. Wehmer (Lafar : " Handbuch der technischen Mykologie ") says, " Alcoholic fermentation is not found to any marked degree with any of the Aspergillaceæ, with one exception."

Sanguineti (1897) observed alcohol production by *Aspergillus oryzæ* from saccharose, starch and dextrin. Elfving (1890) obtained yields of alcohol up to 4·2 per cent. from " *Penicillium glaucum.*" Mazé (1902) regarded alcohol as a normal intermediate product in the breakdown of sugar by moulds and instanced its formation by *Allescheria gayoni.* We have shown that large groups of *Aspergilli* and *Penicillia* are capable of producing alcohol in quantity and have used this characteristic as one means of division into sub-groups for classification purposes (see Parts III and IV). A recent paper by Yuill (1928) points out that alcohol is formed by *Aspergillus flavus.* This mould belongs actually to the group of *Aspergilli* producing the highest yields of alcohol. Species of *Fusarium* were also recognized as being pre-eminently alcohol-formers ; Anderson and Willaman (1922) showed this to be the case for *Fusarium lini,* and White and Willaman (1928) compared the mechanism of fermentation by *Fusarium lini* with that of yeast. The production of alcohol by *Fusaria* is abundantly confirmed in our studies on this group (see Part V).

Acetaldehyde.—Neuberg and Cohen (1921) found that acetaldehyde was produced from sugar by various moulds, including several species of *Aspergillus* and one species of *Penicillium.* The acetaldehyde was " intercepted " by the addition of a sulphite, as in the production of glycerol by yeast fermentation, and was in some cases obtained in fair yield. The addition of sulphite, however, causes a profound modification of the fermentation process.

We have found in several cases of moulds producing alcohol that, without any interceptor present, acetaldehyde can be isolated as the 2 : 4–dinitrophenylhydrazone from the volatile products (see Part XVII).

Mannitol.—Although the presence of mannitol in the mycelium of fungi has long

been recognized, it has hitherto never been regarded as a definite fermentation product of moulds. We have been able to show that it is produced by many moulds, and by certain of the white species of *Aspergillus* in particular, in yields as high as 50 per cent. of the sugar fermented. An account of the production of mannitol is given in Part IX and it need not, therefore, be considered here.

Polysaccharides.—The recorded polysaccharides from the lower fungi are comparatively few. Cramer (1894) obtained from "*Penicillium glaucum*" spores a carbohydrate hydrolysing completely to glucose and giving a blue colour with iodine. He named it "spore starch." Alsberg and Black (1913) obtained a similar substance from *Penicillium puberulum*. It gave a violet colour with iodine and was considered to be identical with trehalum from manna. Dox and Neidig (1914) isolated from *Penicillium expansum* a substance, mycodextran, giving glucose on hydrolysis and no colour with iodine. Later (1914 (2)) they isolated from *Aspergillus niger* another carbohydrate, mycogalactan, which gave galactose on hydrolysis.

Boas (1917, 1919, 1922) examined in some detail the polysaccharide obtained from *Aspergillus niger* when grown in acid media at a fairly high temperature. It gives a blue colour with iodine and is dextro-rotatory, $[\alpha]_D = +120°$–$160°$. It is formed from various sugars, polyhydric alcohols and carboxylic acids. Lappalainen (1919) examined more closely the conditions of its formation, and further work on this same "mould starch" is described by Schmidt (1925). She finds that it is identical with amylose and therefore with isolichenin.

In the present series of studies on moulds, several polysaccharides have been encountered and they seem to be formed in some cases in considerable amount. A substance resembling, if not identical with, glycogen was isolated from one of the white *Aspergilli*, Ac. 56 (see Part IX). *Fumago vagans* has been shown to synthesize in fair quantity a polysaccharide, hydrolysing to dextrose and giving no colour with iodine (see Part XVII), while *Penicillium digitatum* produces a different polysaccharide having a very high dextro-rotation, and giving no colour with iodine (see Part XVIII). The most interesting product, however, is that formed by *Penicillium luteum* and described in detail in Part XIII. This product, which we have called "Luteic acid," is a complex, built up of units each consisting of two molecules of glucose to one of malonic acid in such a manner that one of the carboxyl groups is still free. It may be described as a malonyl-polyglucose. It is lævo-rotatory and gives rise to viscous solutions, this viscosity of the medium being characteristic of the mould in question. Yields of 12 per cent. of this material can be readily obtained. The product most resembles some of the soluble specific substances isolated by Heidelberger and Goebel (1927) from *Pneumococcus*.

The reason for the elaboration of these complex substances by the mould organism has not been elucidated, the most likely hypothesis being that they are merely reserve foodstuffs stored in a less soluble, non-dialysable form and therefore more easily retained for eventual requirements than the simpler sugars.

Fats.—Although fats derived from yeast have been isolated and investigated, very little work seems to have been devoted to the fats formed by moulds. The earliest observers, NAEGELI and LOEW (1878), and SIEBER (1881), seem to have worked with cultures of doubtful authenticity and purity. There are some recent observations which are of greater interest. In 1921 a German patent was taken out relating to fat production from carbohydrates. The fat in this case is obtained by inoculating turnips, apples, &c., with certain specified fungi. BELIN (1926) refers to the production of fat by *Aspergillus niger*, and BARBER (1927) describes the isolation of fat from a species of *Penicillium* grown on sucrose solution. TERROINE (1927) is concerned with the formation of fat by *Aspergillus niger* from sugar chiefly from an energy standpoint. The function of the fat formed is obscure. It may be merely a reserve foodstuff which is accumulated in the same way as the polysaccharides. Our own observations have not included the fats, although a most interesting and new complex fatty acid, produced by *Penicillium spiculisporum*, has been isolated and investigated. It is described in Part XVI of this series.

Phenolic Bodies.

(*a*) *Kojic acid.*—This curious substance—5-hydroxy-2-hydroxymethyl-γ-pyrone—was first isolated by YABUTA (1912), although its colour reaction with ferric chloride had previously been noted by SAITO (1907). It is formed by various species of the *Aspergillus flavus-oryzæ* group and its production has indeed been used by us as diagnostic of this group. Several observers since YABUTA have described new mould products which from their properties would appear to be identical with kojic acid. Kojic acid forms the subject of two papers in this series—Parts VII and VIII.

(*b*) *Penicillic acid*, $C_8H_{10}O_4.2H_2O$, and *mycophenolic acid*, $C_{17}H_{20}O_6$, were two products of phenolic type obtained by ALSBERG and BLACK (1913) from *Penicillium puberulum* and *Penicillium stoloniferum* respectively. Both these moulds were obtained from spoiled maize. Apart from determining the empirical formulæ and noting their reactions and toxicity towards certain animals, nothing further appears to have been done with these products, their constitution still remaining to be elucidated.

(*c*) Our own observations have led to the discovery of several new phenolic bodies. A yellow benzopyrone derivative to which we have given the name "Citromycetin," and which has the formula $C_{14}H_{10}O_7.2H_2O$, is produced only by certain species of *Citromyces*. The yields of this material are really good and may under certain conditions reach 25–30 per cent. of the glucose fermented. The preparation and constitution of citromycetin are dealt with in Part XI of this series. Another yellow colouring matter, having the empirical formula $C_{13}H_{14}O_5$ to which we have given the name "Citrinin," is a metabolic product of *Penicillium citrinum*. Yields approximating 5 per cent. of the sugar fermented were obtained. This material is discussed in Parts XIV and XV. A purple-coloured quinone having the empirical formula $C_8H_8O_5$ and the chemical

constitution of a methoxy-dihydroxy-toluquinone is produced by certain strains in the *P. spinulosum* series. This material is described in Part XII of this series.

When it was decided in 1922 to commence a comprehensive scheme of work on the general biochemistry of micro-organisms, and the "moulds" were chosen as the first group of micro-organisms for investigation, it at once became evident that with such a wealth of fungi available it would be impossible to investigate in detail, in any reasonable space of time, the chemical compounds formed by even a small proportion of the different known species of fungi. For this reason then, instead of attempting the isolation and identification of the compounds formed by any mould taken at random, it was decided to investigate quantitatively the *types* of compounds formed by each of a large number of fungi, so as to obtain a logical basis for the choice of any particular fungus for later investigation. To this end a method involving the preparation of carbon balance sheets was evolved and is described in detail in Part II of this series. This method is capable of being applied to any organism which will grow in or on synthetic media, and hence is applicable to bacteria as well as to moulds. It gives a clean-cut classification of the various types of products formed and of their quantitative relationships. In order further to reduce the scope of the work and to standardize conditions so as to ensure the possibility of repeating the work, all experiments were carried out using glucose as the sole source of carbon and a CZAPEK-DOX synthetic medium having the following composition was used as the basal metabolism solution :—

Glucose	50 gm.
$NaNO_3$	2 ,,
KH_2PO_4	1 ,,
KCl	0·5 ,,
$MgSO_4.7H_2O$	0·5 ,,
$FeSO_4.7H_2O$	0·01 ,,
Water	1,000 c.c.

By means of the carbon balance sheets it has been possible to eliminate from further investigation all those fungi—constituting by far the greater number—which, under the conditions of our experiments, produce practically nothing else but carbon dioxide from glucose, and thus very greatly to economize time, by only attempting the isolation and identification of end products from a comparatively small number of fungi already proved from their carbon balance sheets to be biochemically interesting, and to give rise to considerable yields of some end product other than carbon dioxide. The quantitative results and carbon balance sheets obtained with 96 species of *Aspergillus* are given in Part III, from 75 species of *Penicillium* and 8 species of *Citromyces* in Part IV, from 23 species of *Fusarium* in Part V, and from 36 miscellaneous species of fungi in Part VI.

Having, by means of the carbon balance sheets, made a choice of species suitable for further intensive investigation, these chosen species were now grown on a larger scale

and their metabolic products investigated. For this purpose an apparatus was designed for the cultivation of fungi in pure culture on a large laboratory scale. This apparatus is described in Part VII.

Cultures.

It was realized at the commencement that in work of this description no pains should be spared to ensure the authenticity and purity of all cultures used. To this end the strictest care was exercised, not only in the choice of cultures for examination but also in sub-culturing them subsequently in the laboratory, in order to avoid errors in nomenclature. Immediately on receipt, all fungi were examined and single-spore cultures prepared from them, so that all the work described was carried out on single-spore cultures.

Most of the cultures used have been obtained from one of the following sources :—

1. Microbiological Laboratory of the United States Department of Agriculture.
2. The American Type Culture Collection at Chicago.
3. Centraalbureau voor Schimmelcultures at Baarn, Holland.
4. Přibřam's Mikrobiologische Sammlung in Vienna.
5. The National Collection of Type Cultures, Lister Institute, London.

The remainder of our cultures were either isolated by ourselves at Ardeer or were received from private donors.

Considerable difficulty was experienced from time to time with cultures purchased from certain of the above sources of supply, since some of the cultures were obviously incorrectly named. In these circumstances it would have been impossible to carry through the work on the *Aspergilli* and *Penicillia* without the whole-hearted co-operation of Dr. Charles Thom of the Microbiological Department of the United States Department of Agriculture. We desire to offer to him and to his colleague, Miss Margaret B. Church, our warmest thanks for their kindly co-operation.

We are also greatly indebted, for expert advice on the cultures of *Citromyces* on which we have worked, to Prof. Carl Wehmer of the Technical High School of Hanover, and to Prof. Philip Biourge of the University of Louvain, to both of whom we offer our best thanks.

Practically all the species of *Fusarium* used were Wollenweber's original cultures obtained from the Centraalbureau at Baarn, and hence should be above suspicion.

We have received several different species of fungi from Mr. F. T. Brooks, F.R.S., of Cambridge, for which, and for the keen interest he has shown in this work since its inception, we desire to thank him.

Sub-cultures of the whole of the cultures worked upon and described in this series of papers have been deposited with the National Collection of Type Cultures at the Lister Institute, London. We wish to express our indebtedness to the Medical Research Council for their kindness in making this possible.

The main results arising from these observations may be briefly summarized as follows :—

1. The carbon balance sheets may be used as a biochemical method for the classification of different species in certain families of fungi. This is particularly true in the case of species of *Aspergillus*. In this genus the different species arranged according to their biochemical characteristics follow closely the classification based on morphological characteristics and used by Dr. THOM (1926) in his recent book ' The *Aspergilli*.' With species of *Fusarium*, on the other hand, there seems little hope of classification on these lines, since all the species tested gave rise principally to alcohol and showed themselves closely allied to the *Saccharomyces*.

2. The experimental data seem to throw some light on the biochemistry of the initial stages of the breakdown of the glucose molecule by fungi. It appears that the first stage is a CANNIZZARO reaction involving the production from two molecules of glucose of one molecule of mannitol and one molecule of gluconic acid. Depending then on whether the mould in question prefers for growth an acid or a neutral medium, either mannitol or gluconic acid (of course, in some cases both) is destroyed. There is ample evidence to support this view. The white *Aspergillus*, Ac. 55, which gives rise to yields of mannitol approximating to but never exceeding 50 per cent. of the glucose metabolized (see Part IX), produces very little if any gluconic acid, refuses to grow on an acid medium, and even when cultivated on a medium with an initial *p*H of 4·6, changes this during the course of its growth to 6·5 to 7·0. On the other hand, species which produce large quantities of gluconic acid, *e.g.*, *Aspergillus Wentii* and *Penicillium chrysogenum* (Catalogue No. Ad. 11), give small amounts of mannitol, on which material they grow quite well, but bring the *p*H of the medium to about 1 to 2 no matter what the original *p*H may be. Other species, again, produce moderate amounts of both mannitol and gluconic acid. The production of these two materials from glucose raises an interesting stereochemical point. Glucose on reduction should give sorbitol, and on oxidation gluconic acid, while, on the other hand, mannitol arises from mannose, which gives mannonic acid on oxidation. The curious fact remains, however, that in spite of frequent search for sorbitol and mannonic acid as metabolic products of fungi, not even a trace of them has been found, although yields of 50 per cent. of mannitol or gluconic acid are easily obtained.

3. Perhaps the most striking fact arising from these investigations is the extraordinary specificity of the different mould products. This has led us to the belief that while such general biochemical reactions as the production from glucose of mannitol and gluconic acid, of ethyl alcohol, of citric and oxalic acids may be regarded as common to many species belonging to many different genera of fungi, there are certain highly specific products which are only produced, in some cases by a single species, and in others by a very small sub-group containing a very few species. Thus, for example, the new product, citrinin, described in Part XIV, is produced only by *Penicillium citrinum* THOM. This is so striking that the cultures of *Penicillium citrinum* in our

collection may be distinguished from all the other five or six hundred species belonging to many different genera, by the purely chemical test of adding ferric chloride to their metabolism solutions, when the colour reaction characteristic of citrinin enables one to pick out cultures of *P. citrinum* from any others. Similarly citromycetin, which is described in Part XI, is produced only by species of *Citromyces*, while the methoxy-dihydroxy-toluquinone, described in Part XII, is produced only by certain strains in the *P. spinulosum* series. Other examples will be found in Parts VII, XIII, XVI and XVIII.

4. The amazing powers of the fungi of bringing about *synthetic* chemical processes is shown from a consideration of the composition and constitution of some of the above specific products. While the general mould products such as citric, fumaric and oxalic acids, ethyl alcohol, &c., are simple breakdown products, the specific products are almost invariably much more complex than the original glucose molecule. The methoxy-dihydroxy-toluquinone ($C_8H_8O_5$) already mentioned is among the simplest of these products. On the other hand, citromycetin ($C_{14}H_{10}O_7.2H_2O$) contains a benzo-pyrone nucleus, while citrinin ($C_{13}H_{14}O_5$), the exact constitution of which is given in Part XV, contains a benzene ring to which is fused another ring containing oxygen. The product ($C_{17}H_{28}O_5$) given by *Penicillium spiculisporum* and described in Part XVI, is of an entirely different type. It is the lactone of a monohydroxy-tricarboxylic fatty acid having a straight chain of 15 carbon atoms. Finally, the colloidal material luteic acid produced by *Penicillium luteum* is a malonyl-polyglucose, somewhat similar in general chemical structure to acetyl cellulose.

5. There is a general resemblance between some of the specific products given by the lower fungi and various members of the so-called lichen acids. Thus the product from *P. spiculisporum* ($C_{17}H_{28}O_5$) is similar both in empirical formula and general chemical properties to oxyroccellic acid ($C_{17}H_{32}O_5$), isolated by Hesse (1898) from different species of the lichen *Roccella* and to proto-α-lichesterinic acid ($C_{18}H_{30}O_5$) which occurs in the lichen *Cetraria islandica*. This resemblance seems to support the view that it is the fungal part of the fungus-alga symbionts constituting the lichen which is responsible for the formation of the lichen acids.

This work was originally undertaken after discussion with Sir Frederick Gowland Hopkins, and we would offer to him our thanks for his continued interest in it during the seven years it has been in progress. We desire also to express our appreciation of the attitude adopted by the Directorate of Messrs. Nobel Industries Limited, in originally sanctioning the undertaking, and our thanks to the Directorate of Imperial Chemical Industries Limited for permission to publish the work. Finally, our best thanks are due to our assistants, Messrs. J. J. Tidd, R. H. Barnett, H. B. Colquhoun, A. Currie, J. Paton and R. Page, without whose loyal help it would have been difficult to carry out what was at times very laborious work.