III. THE CHEMISTRY OF MOULD TISSUE.

VI. FACTORS INFLUENCING THE AMOUNT AND NATURE OF THE FAT PRODUCED BY *ASPERGILLUS FISCHERI*[1].

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THE fat content of mould mycelium, as reported by various authors, ranges from 1 to 40 %, depending upon the species and upon the conditions of growth. Even within a single strain of a species certain characteristics of the fat, as well as its amount, have been reported to vary with the conditions of growth.

From what is known about the chemical composition of mould-fat it appears that glycerides together with free fatty acids constitute the major portion of it. In some cases the mould-fat contains a high percentage of free fatty acids [Pruess *et al.*, 1934]. Barber [1929] found that the nature of the fatty acids, both free and combined, produced by a *Penicillium* sp. remained practically unchanged whether this mould was grown on glucose, sucrose, xylose or glycerol. The more easily extracted fat of *A. sydowi*, exclusive of the phospholipins [Strong and Peterson, 1934] and that of *P. javanicum* [Ward and Jamieson, 1934] have both been shown to yield upon saponification glycerol and oleic, linoleic, palmitic, stearic and small amounts of *n*-tetracosanoic acids. The mould-fat contains variable percentages of phospholipins and sterol, the latter having been definitely shown to be ergosterol in the case of *A. fischeri* [Pruess *et al.*, 1932]. There also occurs a small amount of some other unsaponifiable material which has not yet been studied. A small amount of pigment is presumably also present, since the mould-fat is usually quite deeply coloured.

The mould-fat has been generally regarded as a reserve material [Perrier, 1905], although Kordes [1923], observing that the fat globules in the hyphae persisted even during inanition, believed that this fat should be considered as an "excretory" product. Belin [1926] demonstrated that the fat content of *A. niger* fell to a low value after prolonged inanition. He postulated that the fats of moulds and of other living organisms should be considered as consisting of an "élément constant" and an "élément variable." By the term "élément constant" he designated that minimum percentage of fatty material, assumed by him to be phospholipins, intimately associated with the protoplasm, which is indispensable for the life of the organism; and by the term "élément variable" he designated any additional amount of fat stored as a reserve material.

In the case of animal tissues, three possibilities have been postulated for the function of the phospholipins [Sinclair, 1934]: (1) that they are intermediate

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products in fat metabolism; (2) that they act as oxygen transport agents within the cells; and (3) that they, and possibly also the cholesterol, are concerned in some more strictly physicochemical sense in the structural make-up and activity of the cell. It is possible that in the mould cells the phospholipins and also the sterol (ergosterol) might have functions similar to those postulated above.

The purpose of the present investigation was to determine the effects of the variations of certain factors on the amount and nature of the fat produced by *A. fischeri*, and also to determine any relationships which might exist among the percentages of total fat, phospholipins and sterol in the mycelium.

Experimental.

The mould was grown in 500 ml. pyrex Erlenmeyer flasks, six to eight flasks for each condition of growth, each flask containing 100 ml. of inorganic salt solution to which cerelose (commercial glucose) was added in the amount of 20 g. unless otherwise indicated. Except for certain indicated changes in the amount or nature of the nitrogen source in some of the experiments, the composition of the inorganic salt solution per flask was as follows:

NH_4NO_3	1·00 g.
KH_2PO_4	0·68 g.
$MgSO_4, 7H_2O$	0·50 g.
$FeCl_3, 6H_2O$	0·016 g.
$ZnSO_4, 7H_2O$	0·005 g.
Distilled water	100·0 ml.

To prevent the development of acidity, $CaCO_3$ was added to the media for all experiments except those concerned with the effects of acidity or alkalinity. Those components of the media which would react with each other if heated together, *e.g.* glucose and an alkaline substance, were sterilised separately.

Except for the comparison of the different cultures, in which case soil suspensions of these cultures were used as inocula, an aqueous suspension of the spores from a malt-agar slant of a single spore culture of *A. fischeri* was used as the inoculum in each series of experiments.

At the end of the growth period the mould was killed by autoclaving at 120° for 10 minutes. The mould pads were removed, washed with water, air dried at 37° for 2 days, weighed and ground to pass a 40-mesh sieve. The amounts of unutilised glucose remaining in the media were determined by the Shaffer and Hartmann [1920–21] method.

For the analyses 10 g. samples of the dry mould were weighed out, usually in duplicates if the amounts of material permitted. The samples were extracted with hot absolute alcohol in continuous extractors for at least 12 hours. It was found that this solvent extracted the fat from the mould more completely than did chloroform, ether or light petroleum. Moreover, it was also found that comparatively negligible amounts of additional fatty acids were obtainable upon more drastic treatment of the alcohol-extracted residue with alkali or acid. Since the alcohol also extracted some materials other than fat, the alcohol was distilled from the extracts, and the mixtures of fat and other extracted matter were dried in a vacuum-desiccator over $CaCl_2$ and then treated with hot chloroform, which dissolved only the fat. The chloroform-insoluble material was usually a light yellow water-insoluble powder, which comprised 10–30 % of the total alcoholic extract and 2–4 % of the mould pad. In some cases, which will be mentioned later, the chloroform-insoluble material was of a different nature,

i.e. it was gummy and appeared to contain a large amount of carbohydrate. In these latter cases this fraction comprised the major portions of the alcoholic extracts and in an extreme case it formed 36 % of the mould pad.

The chloroform solution of the fat was in each case made up to a volume of 50 ml. For the determination of the amount of total fat a 20 ml. portion of this solution was evaporated in a tared 50 ml. Erlenmeyer flask, dried in a vacuum-oven at 60° for 2 hours and weighed. An aliquot of this same portion of fat was used for the determination of phosphorus by the Fiske and Subbarow [1925] colorimetric method after ignition with $Mg(NO_3)_2$. In some cases another aliquot of this fat was used for the nitrogen determination [Chiles, 1928].

The $CHCl_3$ was completely removed by distillation from the other 30 ml. portion of the solution and the fat was saponified by boiling for 1½ hours with 50 ml. of 5 % alcoholic KOH. After removal of the greater portion of the alcohol by distillation and addition of 50 ml. of cold water, the unsaponifiable matter was extracted by shaking with three successive 50 ml. portions of ether, the liquids being allowed to stand in the separating funnel until the two layers were distinct and free from emulsions. The combined ether solution of the unsaponifiable matter was washed once with 1 % KOH and twice with water, these washings being added to the aqueous soap solution. The aqueous soap solution was made strongly acid with HCl, and the fatty acids were extracted with ether. After washing this extract with water, distilling off the ether and drying the crude fatty acids in a vacuum-desiccator over $CaCl_2$, light petroleum was used to redissolve the true fatty acids. The small amount of more deeply coloured residue insoluble in light petroleum amounted to 3 % or less of the original fat. After distilling off the solvents and transferring to tared 50 ml. Erlenmeyer flasks, both the unsaponifiable matter and the fatty acids were dried in a vacuum-oven at 60° for 2 hours. This method of drying did not affect the iodine numbers of the fatty acids. The weight obtained for the fatty acids corresponds to that of the non-volatile fatty acids. These were in all cases light yellow in colour and appeared to be solid up to about 30°.

Aliquots of the fatty acids were taken for the determination of the iodine numbers and neutral equivalents. All the iodine numbers were determined by the Rosenmund and Kuhnhenn [1923] method, which, while reported to give somewhat lower values than the Wijs method, is less affected by experimental conditions and gives values which are consistent amongst themselves [Barbour, 1934; Yasuda, 1931]. The neutral equivalents, as determined by titration of an alcoholic solution of the fatty acids with $N/10$ NaOH to the phenolphthalein end-point, were all around 280, with maximum deviations of 5. Because these deviations are not much greater than the experimental error, the individual neutral equivalents are not included in the Tables.

The amount of sterol in the unsaponifiable matter was determined by a colorimetric method based on the Liebermann-Burchard reaction. This method will be described in a later publication.

Comparison of cultures.

Nine different cultures of *A. fischeri* Wehmer, eight of which were recently developed from single spores [Greene, 1933], were grown at 30° on a medium containing 10 % glucose. A series of old soil suspensions (A) and another series of more recently prepared soil suspensions (B) of these cultures were used as inocula. Practically all the glucose was utilised at the end of the 10-day growth period. The yields of mould pad per g. of glucose utilised varied only slightly

from the average value of 0·27 g. In Table I the single spore cultures (S.S.C.) and the parent stock culture are arranged in the approximate order of the decreasing fat contents of the mycelia which they produced.

Table I. *Comparison of cultures.*

S.S.C. No.	Series	Fat %	Percentages of fat: Fatty acids	Unsap. matter	Sterol	P	N	Iodine no. of fatty acids
13	A	20·5	70·3	5·9	3·6	0·51	—	81
	B	16·1	70·7	8·5	4·7	0·52	0·38	79
7	A	15·2	68·5	8·9	4·6	0·53	—	79
	B	14·8	70·0	9·6	4·8	0·47	0·38	79
10	A	14·9	58·0	11·3	6·5	0·59	—	87
	B	15·1	62·2	11·5	6·4	0·58	0·43	84
1	A	14·8	65·3	9·1	5·7	0·47	0·30	77
	B	14·4	66·8	11·1	5·3	0·42	0·32	73
12	A	13·5	63·6	9·5	5·3	0·35	—	83
	B	14·5	67·0	9·9	5·5	0·52	0·43	83
2	A	12·7	64·4	10·8	5·8	0·53	0·22	79
	B	11·0	65·9	11·9	5·1	0·40	0·33	76
9	A	12·5	60·4	12·7	6·3	0·58	—	80
	B	11·5	63·2	13·0	7·6	0·50	0·42	78
20	A	11·2	55·0	10·2	4·2	0·49	0·24	89
	B	10·5	55·2	12·2	5·3	0·51	0·32	89
Stock	A	10·7	54·6	12·0	6·4	0·63	—	86
	B	11·2	58·1	9·2	4·7	0·45	0·28	89

It is seen that the variations in the amount and nature of the fats produced by the different cultures were quite appreciable and were fairly parallel in the two series. The pads produced by S.S.C. No. 13 were the highest in fat content, while those produced by S.S.C. No. 20 and by the stock culture were the lowest. In those cases where the fat content of the mould was low the fatty acids made up comparatively low percentages of the fat (about 55 % in the extreme cases) and the unsaponifiable matter tended to make up increased percentages; but the sum of these two components left considerable percentages of the fat unaccounted for, which might have been due to the presence of some other water-soluble components. The iodine numbers of the fatty acids produced by the different cultures showed fairly regular variations in both series and were highest in the cases of S.S.C. No. 20, S.S.C. No. 10 and the stock culture.

For the subsequent experiments the culture designated as S.S.C. No. 13 was used exclusively. It was believed that by using an inoculum as homogeneous as possible, *i.e.* all its components having the same characteristics, the observed variations in the amount and nature of the fat would be a true picture of the effects due to the variations in the conditions of growth; whereas, by using an inoculum, such as the stock culture, consisting of a mixture of components having different characteristics, the observed variations in the fat would be due partly to the altered proportions in which the different components would co-exist under the changed conditions of growth.

Effect of initial concentration of glucose.

The data for this series are summarised in Table II. The initial concentrations of glucose were varied by adding from 1 to 70 g. of cerelose to 100 ml. of the salt solution. The progressively increasing initial concentrations of glucose in

Table II. *Effect of initial concentration of glucose.*

Glucose added to 100 ml. of sol. g.	Glucose utilised g.	Period of incubation days	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat				Iodine no. of fatty acids
					Fatty acid	Unsap. matter	Sterol	P	
1·0	0·84	4	0·38	10·4	51·0	16·2	5·4	2·30	113
3·0	2·55	5	0·37	11·8	62·4	9·0	4·3	1·21	100
5·0	4·65	7	0·34	10·8	67·5	10·9	6·7	0·76	94
10·0	9·5	8	0·33	13·1	75·0	9·0	6·4	0·66	80
15·0	14·4	10	0·32	15·6	76·3	7·1	4·5	0·66	84
20·0	19·2	12	0·29	18·0	85·0	6·5	4·5	0·40	78
30·0	29·0	14	0·28	23·3	83·3	4·9	3·5	0·30	77
40·0	39·4	16	0·26	28·1	84·5	3·8	2·7	0·23	76
55·0	49·5	22	0·26	33·3	85·7	3·8	2·7	0·13	75
70·0	68·6	28	0·23	36·0	85·8	3·2	2·8	0·15	73

the medium caused certain quite regularly progressing variations in the amount and nature of the fat produced by the mould. The limited amount of nitrogen source compared with the large amount of carbon source in the media high in glucose was doubtless an important factor in producing some of these changes.

The fat content of the mould increased quite distinctly, which is in accord with previous reports [Belin, 1926]. In the case of yeasts also a high fat content is reported to be favoured by a high concentration of fermentable sugar and a low concentration of nitrogen in the medium [Smedley-MacLean, 1922]. Although the yields of mycelium per g. of glucose utilised decreased from 0·38 to 0·23 g., the yields of fat per g. of glucose utilised still showed an increase from 0·0395 to 0·083 g. These changes can be correlated because the conversion of carbohydrate into fat requires energy, hence greater portions of the glucose would have to be more or less completely oxidised in order to furnish the necessary energy for the synthesis of the greater amounts of fat. The "efficiency of the conversion of energy" [Terroine and Bonnet, 1927], which is equivalent to the value of the ratio,

energy content of the mould pad/decrease in energy content of the medium,

would doubtless have shown a much smaller decrease than did the yields in terms of the weight of mould pad per g. of glucose utilised.

The fatty acids obtained after saponification comprised increasing percentages of the original fat, while the unsaponifiable matter comprised decreasing percentages. Also the sum of these two components accounted for the fat more completely when the fat content was high. These facts, together with the decreasing percentages of phosphorus in the fat, indicate that the increment of fat consisted almost entirely of glycerides.

Based upon the mycelium, the percentages of unsaponifiable matter varied slightly around an average value of 1·2 %. The sterol usually comprised from 65 to 75 % of the unsaponifiable matter, except in the first two members of the series where the percentages were lower. Based upon the mycelium, the percentages of lipin-phosphorus were unusually high for the first two members (0·23 and 0·14) and decreased through the series, down to 0·05. Except for the first two members, the ratio of lipin-phosphorus to fat-free mycelium tended to remain fairly constant in this series.

The iodine numbers of the fatty acids decreased. The unusually high iodine numbers for the first three members of the series might be due to the more unsaturated character of the mould-phospholipins, which in these cases appear to make up appreciable percentages of the fat.

Effect of ammonium nitrate concentration.

The data for this series of experiments are summarised in Table III. The mould was allowed to grow at 30° for 10 days.

Table III. *Effect of ammonium nitrate concentration.*

NH_4NO_3 %	Glucose utilised %	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat: Fatty acids	Unsap. matter	Sterol	P	N	Iodine no. of fatty acids
0·2	44	0·45	22·3	81·0	3·6	2·2	0·23	0·18	90
0·5	68	0·37	20·8	78·8	3·9	2·6	0·27	0·26	91
1·0	79	0·33	17·9	79·5	5·6	3·4	0·34	0·39	88
2·0	92	0·35	15·8	81·2	6·7	3·3	0·22	0·37	84
5·0	73	0·25	15·2	78·8	6·7	3·4	0·16	0·33	83
10·0	47	0·20	14·1	80·0	5·0	Traces	0·23	0·45	83

It is seen that the utilisation of the glucose was most complete in the medium containing 2 % NH_4NO_3. It should also be mentioned that the chloroform-insoluble portions of the alcoholic extracts (not shown in Table III) were the greatest in those cases where the mould had been grown on a medium having either an extremely high or extremely low concentration of NH_4NO_3 and where the utilisation of the glucose had been least complete. In these cases this fraction was gummy and appeared to contain a large amount of carbohydrate. This fraction made up 28 % of the mould pad produced on the 10 % NH_4NO_3 medium.

With the increasing initial NH_4NO_3 concentration of the medium the yields of mould pad per g. of glucose utilised decreased, the fat content of the mycelium decreased, and certain characteristics of the fat changed progressively. The unsaponifiable matter formed an increasing percentage of the original fat while the fatty acids made up a fairly constant percentage (about 80). The iodine numbers of the fatty acids decreased.

The percentages of sterol and of lipin-phosphorus were highest in the mycelium produced on the usual 1 % NH_4NO_3 medium, both on the basis of the mycelium and on the basis of the fat.

The mould pads produced on the 10 % NH_4NO_3 medium, and to a lesser extent those produced on the 5 % NH_4NO_3 medium, were slimy and lacked turgidity. The abnormal growth is probably reflected in the very low sterol content produced on the 10 % NH_4NO_3 medium and in the unusually high values of the ratio, lipin-N/lipin-P, for the mould grown on the higher NH_4NO_3 concentrations.

Effect of acidity or alkalinity.

Ammonium salts of mineral acids have a tendency to produce acidity in the medium because of the liberation of mineral acid when the nitrogen of the ammonium radical is utilised. In the case of NH_4NO_3 this does not necessarily apply, because the mould can utilise both ammonium- and nitrate-nitrogen but not necessarily at the same rate. Likewise, the use of the nitrates of the alkali metals would be expected to decrease the acidity or produce alkalinity in the medium.

To determine the effects of the acidity or alkalinity which is produced in the medium by virtue of the nitrogen source, the mould was grown on media in which the NH_4NO_3 was in some cases replaced by equivalent amounts of various other nitrogen sources. Parallel experiments in which either $CaCO_3$ or

potassium acetate was added to some of these media were also performed. The mould was in all cases allowed to grow at 30° for 12 days. The data for these experiments are summarised in Table IV.

Table IV. *Effect of acidity or alkalinity developed in the media by virtue of the nitrogen source.*

N source	Neutralising agent per flask g.	Glucose utilised %	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat: Fatty acids	Unsap. matter	Sterol	P	Iodine no. of fatty acids
NH_4Cl	None	36	0·16	9·0	61·7	11·2	7·7	0·08	100
	$CaCO_3$	51	0·32	23·2	78·5	3·8	3·3	0·26	75
$(NH_4)_2SO_4$	None	47	0·075	10·3	59·0	14·8	7·5	0·14	98
	1 g. KOAc	84	0·20	11·6	67·0	9·5	7·7	0·18	93
	$CaCO_3$	66	0·34	20·3	76·0	5·8	3·9	0·24	82
NH_4NO_3	None	84	0·28	17·3	70·0	6·4	4·9	0·53	75
	$CaCO_3$	84	0·30	23·1	66·6	4·7	4·0	0·39	75
	0·5 g. KOAc	99	0·27	21·2	79·0	7·1	5·9	0·52	79
	1 g. KOAc	99	0·28	22·2	80·0	6·3	5·1	0·46	84
	2 g. KOAc	99	0·25	26·1	82·0	5·2	3·9	0·25	84
$NaNO_3$	None	88	0·24	25·7	78·0	5·2	3·4	0·33	82
$Ca(NO_3)_2$	None	55	0·39	16·8	69·5	6·2	3·1	0·27	58
Urea	None	98	0·26	21·7	69·3	10·4	4·8	0·63	81
	$CaCO_3$	22	0·26	14·3	86·5	5·3	3·4	0·31	68

The growth of the mould on the unneutralised $(NH_4)_2SO_4$ and NH_4Cl media apparently produced such a high degree of acidity that further growth was inhibited. The glucose was very incompletely utilised and the yields of mycelium per g. of glucose utilised were very low. The alcoholic extracts contained large amounts of chloroform-insoluble material similar to that previously described, and in the case of the mycelium produced on the NH_4Cl medium this amounted to 36 % of the mould pad. The mould pads produced on these media had low fat contents. Based on the mycelium, the percentages of sterol and unsaponifiable matter were as high as those observed in other cases; hence, these components amounted to appreciable percentages of the fat. The percentages of lipin-phosphorus were unusually low. The iodine numbers of the fatty acids were comparatively high.

The addition of $CaCO_3$ to these two media caused the production of good yields of mould pad per g. of glucose utilised and high fat contents of the mycelia. The addition of a small amount of potassium acetate partially neutralised the H_2SO_4 which was liberated during growth. The potassium acetate acts as a neutralising agent because of its own slightly alkaline reaction and probably also because of the production of the more alkaline $KHCO_3$ upon utilisation of the acetate radical as an additional carbon source. In this case the utilisation of the glucose was more complete, the yield of mould pad was fair, but the fat content was only slightly increased. The nature of this fat was intermediate between those of the fats produced by the mould on the $(NH_4)_2SO_4$ media with and without $CaCO_3$.

The growth of the mould on the unneutralised NH_4NO_3 medium was fairly normal; however, the addition of $CaCO_3$ or increasing amounts of potassium acetate caused an increase in fat content of the mycelium.

Urea, $NaNO_3$ and $Ca(NO_3)_2$ also served as suitable nitrogen sources and favoured the production of mycelia with high fat contents. The mould pads

produced on the $Ca(NO_3)_2$ medium contained an abnormally high percentage of mineral matter (16 % ash). If corrections were made for the excessive mineral content of these pads (5 % is a more usual ash content), the fat content would appear as a higher percentage than that indicated in the table. The addition of $CaCO_3$ to the urea media proved unfavourable to growth, probably because of the development of excessive alkalinity. The iodine numbers of the fatty acids in the two last-mentioned cases were unusually low.

For the investigation of the effect of the initial p_H of the medium, the usual medium was modified by the substitution of an equivalent amount of urea in place of the NH_4NO_3, and H_2SO_4 or KOH was added to adjust the initial p_H to the values indicated in Table V. The mould was allowed to grow at 37° for 12 days.

Table V. *Effect of initial* p_H.

N reagent added per flask ml.	Initial p_H	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat: Fatty acids	Unsap. matter	Sterol	P	N	Iodine no. of fatty acids
H_2SO_4 2	2	0·200	19·2	64·5	15·7	10·3	0·66	0·24	85
H_2SO_4 0·5	3	0·217	17·0	66·5	14·6	11·3	0·63	0·37	87
0	4·6	0·223	19·5	59·0	12·9	8·6	0·66	0·46	88
KOH 2	6	0·275	23·9	78·5	6·7	4·6	0·46	0·23	89
KOH 6	8	0·270	37·0	76·5	2·9	1·8	0·23	0·11	77

Probably because of the hydrolysis of urea to $(NH_4)_2CO_3$, either by mould enzymes or by ordinary chemical reactions, and because of the low buffer capacities of the media, a final p_H of about 7·5 was observed in all media except the one which had an initial p_H of 8. The growth of the mould on this last medium was unique in that the p_H decreased from 8 to 7, only 67 % of the glucose was utilised (whereas all the glucose was utilised in the other media), and the mycelium had a marked tendency to pile up against the walls of the flasks. This mycelium contained an unusually high percentage (37) of fat. Also, the iodine number of the fatty acids was lower in this case than in the others.

The addition of H_2SO_4 to the urea medium caused a slight decrease in the yields of mycelium, hardly any changes in the percentages of fat or of lipin-phosphorus, but a marked increase in the percentages of sterol and total unsaponifiable matter. The addition of KOH to the urea medium caused an increase in the yields of mycelium and an increase in the percentages of fat, but a decrease in the percentages of sterol, total unsaponifiable matter and lipin-phosphorus, both on the basis of the mycelium and on the basis of the fat.

Pontillon [1930; 1932–33] made a study of the effects of various nitrogen sources and the concomitant changes in the degree of acidity of the media upon the amount and nature of the fatty constituents of *A. niger*. It is difficult to correlate his results and ours; however, it appears that, in regard to the nature of the fat produced, *A. fischeri*, while showing considerable variations, does not tend to exhibit some of the rather extreme variations which, as his data imply, occur in the case of *A. niger*.

Effect of temperature.

The data for these experiments are summarised in Table VI. The mould was allowed to grow at the indicated temperatures until all the glucose was utilised.

Table VI. *Effect of temperature.*

Temp. ° C.	Period of incu-bation days	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat: Fatty acid	Unsap. matter	Sterol	P	N	Iodine no. of fatty acids
20	16	0·331	24·4	79·0	5·9	3·4	0·46	0·20	93
30	12	0·305	25·7	81·5	5·8	4·1	0·39	0·20	82
37	12	0·273	20·0	80·0	8·5	6·8	0·41	0·22	88

It appears that a temperature of 37° is somewhat less favourable for fat production than are the lower temperatures. The percentage of sterol (based on the mycelium) increased greatly while that of the lipin-phosphorus decreased slightly as the temperature of growth was increased.

It has been reported [Pearson and Raper, 1927; Terroine *et al.*, 1927] that in the case of two moulds, *A. niger* and *Rh. nigricans*, and also in the case of the timothy bacillus, the iodine numbers of the fatty acids obtained from these organisms decreased as the temperature at which they were grown increased. Our data do not lend much support to the supposition that this tendency is a general one; for although the iodine number of the fatty acids produced by the mould at 20° was somewhat higher, those of the fatty acids obtained from the mould grown at 30° and 37° were in an order the reverse of that which would have been expected.

Effect of increased aeration.

The data for this experiment are summarised in Table VII. This experiment was performed twice, (A) with the usual 500 ml. Erlenmeyer flasks, and (B) with 3 l. Currie flasks containing 750 ml. of medium. The medium contained 15 %

Table VII. *Effect of increased aeration.*

		Fat %	Percentages of fat: Fatty acid	Unsap. matter	Sterol	P	N	Iodine no. of fatty acids
A	Aerated	23·8	79·0	3·8	2·2	0·24	0·12	83
	Control	24·6	75·5	4·3	2·3	0·26	0·10	83
B	Aerated	27·0	79·0	3·5	2·4	0·21	0·09	82
	Control	29·8	75·5	3·4	2·0	0·23	0·11	79

glucose in both cases. For increased aeration a continuous current of sterile air was introduced just above the surface of the pads. The flasks containing the controls were plugged with cotton wool in the usual manner. The mould was in all cases allowed to grow at 25° for 10 days.

With increased aeration the growth of the mould was more rapid and the glucose was practically all utilised at the end of the 10-day period. In the controls there were considerable amounts of unutilised glucose, but the yields of mycelial pads per unit of glucose utilised did not differ greatly from those produced with increased aeration. No changes in the amount or nature of fat that can be considered significant were found to be produced by increased aeration.

Effect of inanition.

The mould for this series of experiments was grown at 30°. The first sample of six flasks was taken for analysis after 10 days' growth at which time 77 % of the glucose had been utilised. The other pads were allowed to remain on the

medium for longer periods of time. The data for this series are summarised in Table VIII and the changes in the amounts of some of the mycelial constituents per flask are shown graphically in Fig. 1.

Table VIII. *Effect of inanition.*

Period of incubation days	Mycelium per g. glucose utilised g.	Fat %	Percentages of fat: Fatty acids	Unsap. matter	Sterol	P	Iodine no. of fatty acids
10	0·320	23·3	65·0	5·0	2·8	0·45	83
20	0·245	16·2	70·7	9·2	5·6	0·57	82
30	0·212	13·9	69·6	12·4	8·6	0·41	81
40	0·177	11·6	65·0	18·5	12·4	0·11	79
50	0·165	11·5	60·6	19·1	13·5	0·10	78
68	0·074	12·1	65·5	14·7	9·8	Traces	82

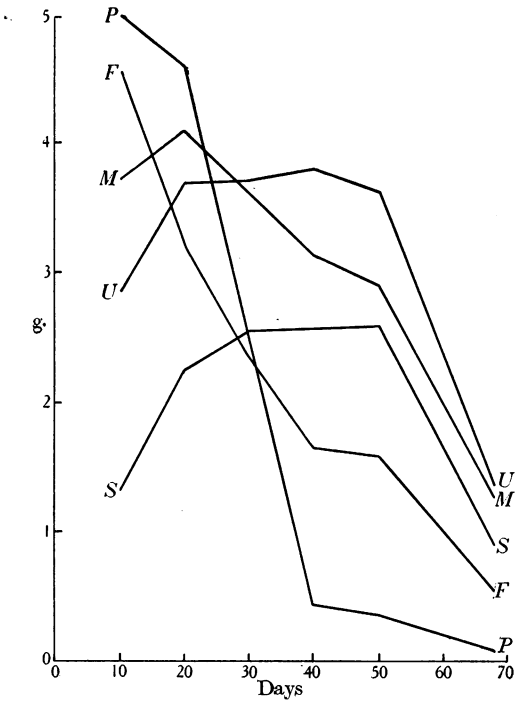

Fig. 1. Effect of inanition on some mycelial constituents, expressed as g. per 100 ml. of medium. M = mycelium *minus* fat; F = fat × 4; U = unsaponifiable matter × 50; S = sterol × 50; P = lipin-P × 1000.

Between the 10th and 20th days the absolute amount of fat began to decrease. The fat decreased more rapidly than did the sum of the other mycelial constituents until about the 40th day, after which both decreased at almost the same rate; consequently, the percentage of fat in the mould pad decreased from 23·3 to 11·6 and then remained fairly constant. Belin [1926] reported that on prolonged inanition the fat content of *A. niger* fell to a low final value corresponding to 1·37 % fatty acids on the basis of the final weight of the mycelium.

While a decrease in the absolute amount of fat was in progress, the amount of unsaponifiable matter, including the sterol, still increased slightly and then

remained fairly constant for a long period before it also finally decreased. Until about the 50th day, the sterol and other unsaponifiable matter amounted to increasing percentages both of the fat and of the mould pad.

The fatty acids remained a fairly constant percentage of the fat. The iodine numbers of the fatty acids showed only a very slight tendency to decrease and the neutral equivalents varied only slightly around 280, just as was observed in the previous series of experiments. If these fatty acids are a mixture consisting of appreciable amounts of both saturated and more or less unsaturated individuals, the almost constant iodine number of this mixture would indicate that the unsaturated fatty acids are not preferentially utilised. It has also been reported [Pearson and Raper, 1927; Terroine and Belin, 1927] that the iodine number of the fatty acids obtained from *A. niger* is not greatly influenced by the period of incubation of the mould.

The amount of lipin-phosphorus decreased very rapidly between the 20th and 40th days. In view of the hypothesis that the phospholipins serve as intermediates in fat metabolism, it might be assumed that the phospholipin would be utilised more readily than the other fatty constituents. However, it is also possible that the observed decrease in lipin-phosphorus might have been due to an enzymic cleavage of the phosphate linkage rather than a complete utilisation of the phospholipin molecules.

Discussion.

In the series of experiments dealing with the initial concentration of glucose, the yields of mycelium and of fat per g. of glucose utilised varied in opposite directions, and this was partly explained by the energy required for the conversion of carbohydrate into fat. In the other series studied the variations in the yields of both mycelium and fat were in the same direction, which indicates that in these cases the effect due to the above-mentioned principle was completely overshadowed by the effects due to other factors.

The percentages of sterol and other unsaponifiable matter, when based on the mycelium, were usually less variable than were the percentages of total fat; consequently, these components generally made up comparatively greater percentages of the fat in those cases where the fat contents of the mycelia were low. Also the undetermined water-soluble components left unaccounted for by the sum of the percentages of fatty acids and unsaponifiable matter amounted to comparatively greater percentages of the fat in these cases.

The amount of lipin-phosphorus was taken as an approximate measure of the amount of phospholipins. In those cases where the lipin-nitrogen was also determined, except for a few apparently abnormal cases, the value of the ratio N/P usually indicated between one and two atoms of nitrogen to one of phosphorus.

At a given temperature of growth and with media that did not become strongly acidic, the percentages of phospholipin (based on the mycelium) and of total fat tended to vary in opposite directions as the initial concentration of glucose, the concentration of NH_4NO_3 (up to 1 %) or the initial p_H was varied.

With a given initial concentration of glucose in the media, the percentages of sterol (based on the mycelium) and of total fat tended to vary in opposite directions as the degree of acidity, the concentration of NH_4NO_3 (up to 1 %) or the temperature of growth was varied.

It was observed that within a certain limited range of growth conditions—a temperature of 25° to 30°, a fairly neutral medium (presence of $CaCO_3$) and

an initial concentration of glucose between 5 and 30 %—the values of the ratio, sterol/lipin-P, were usually within the range of 9 to 11. Under most other conditions of growth the value of this ratio was higher. If the free rather than the total sterol had been determined, an even more direct relationship might have been obtained. It has been suggested [Sinclair, 1934] that in animal tissues the sterol and phospholipin might be concerned in the regulation of some physicochemical property of the cells, and it has also been supposed that these two substances have antagonistic actions. Parallelisms between the percentages of cholesterol and phospholipins in different healthy organs of animals have been reported by numerous authors. In mould grown under a limited range of conditions there appears to be a parallelism between the percentages of sterol and phospholipin. In the mould grown under more extreme conditions different amounts and proportions of these two substances might be necessary for the adjustment of the mould cells to the external conditions.

The neutral equivalents of the fatty acids were in all cases very near to 280 (maximum deviation of 5). Although the neutral equivalent of a mixture of fatty acids is usually considered as the effective average molecular weight of the components, the almost constant value observed for the neutral equivalents of the fatty acids produced by *A. fischeri* under such diverse conditions suggests that there was a very great preponderance of those fatty acids which contain 18 carbon atoms.

Summary.

1. Several single spore cultures of *A. fischeri* were compared in regard to the amount and nature of the fats produced in the mycelia. Appreciable differences were noted.

2. A study was made of the effects of the initial concentration of glucose, concentration of NH_4NO_3, acidity, alkalinity, temperature, increased aeration and period of incubation upon the amount and nature of the fat produced by a single spore culture of *A. fischeri*.

3. The production of mycelia with high fat contents was favoured by neutral or slightly alkaline media, a high initial concentration of glucose and a low concentration of NH_4NO_3.

4. The production of increased percentages of sterol in the mycelium was favoured by a fairly high initial concentration of glucose, 1 % NH_4NO_3 or an initially acid medium containing an equivalent amount of urea, a higher temperature (37°) and a long period of incubation.

5. The production of increased percentages of phospholipin in the mycelium, as judged by the percentages of lipin-phosphorus, was favoured by a low initial concentration of glucose, 1 % NH_4NO_3, and an initially slightly acid medium.

6. During inanition the greater part of the fat (exclusive of unsaponifiable matter) was utilised by the mould.

7. The iodine numbers of the fatty acids obtained from the total fat of the mould were higher when the mould was grown on a low concentration of glucose, on a medium which became strongly acid or at a lower temperature.

8. The neutral equivalents of these fatty acids were in all cases very near to 280.

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