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POSTSCRIPT. Since this paper was in the press we have seen the very valuable account of the chemical and immunological properties of phos-

phorylated crystalline horse serum albumin given by Mayer & Heidelberger (1946). We hope to make full reference to this work later.

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Studies in the Biochemistry of Micro-organisms

77. A SURVEY OF FUNGAL METABOLISM OF INORGANIC SULPHATES

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(Received 9 December 1947)

Clutterbuck, Mukhopadhyay, Oxford & Raistrick (1940), in a communication from this laboratory, described the results of a quantitative survey of the chlorine metabolism of 139 species or strains of moulds grown on Czapek-Dox 5% glucose solution containing 0.5 g. KCl/l. as the sole source of chlorine.

The results are now given of a parallel quantitative survey of the sulphur metabolism of 115 species or strains of moulds grown on the same medium containing 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.01 g. $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ /l. as the sole source of sulphur.

EXPERIMENTAL

Cultural conditions

The medium used throughout this investigation was the well-known Czapek-Dox solution of the following composition: glucose, 50 g.; NaNO_3 , 2.0 g.; KH_2PO_4 , 1.0 g.; KCl, 0.5 g.; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5 g.; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01 g.; distilled water, 1 l. All the constituents of the medium were of analytical reagent purity. Hence the sole source of sulphur was present as the sulphates of magnesium and iron, and amounted to 22.9 mg./flask (6.6 mg./100 ml.) at the time of sowing. This medium (350 ml.) was accurately measured into each of a number of 1 l. conical flasks plugged with cotton wool, and sterilized by steaming for 0.5 hr. on each of 3 consecutive days. The flasks were then inoculated with a pure culture of the chosen species of mould, and incubated at 24° for the requisite period of days.

The sulphate content of duplicate uninoculated flasks from each batch was determined by the method described later. The inoculated flasks were treated as follows. The mycelium from each species was separated quantitatively by filtration through a Buchner funnel, and was thoroughly washed with warm water. It was then dried to constant

weight *in vacuo*, weighed, ground and stored in a closely stoppered bottle prior to examination. The culture filtrate and washings were made up to a total volume of 500 ml., and analyzed by the methods described in the next section.

Analytical methods

I. Sulphur as inorganic sulphate in culture filtrate

The concentration of inorganic sulphate present in the uninoculated medium is quite low (equiv. to 22.9 mg. S/350 ml., or 4.58 mg./100 ml. after dilution to 500 ml.). The medium also contains relatively considerable amounts of nitrate, phosphate and chloride. Further, the culture filtrate, resulting from the growth of different moulds may contain metabolic products of complex and unknown nature and may vary from almost colourless to deeply coloured. After a considerable amount of exploratory work, details of which will be published elsewhere, on a number of published methods the one finally chosen as being the most generally useful for our purpose was that based on Øllgaard's (1934) method. This method involves the precipitation of inorganic sulphate with benzidine in acetone and titration of the precipitate, after liberation of the sulphate with a little NaOH, with a standard BaCl_2 solution using sodium rhodizonate as indicator. Using this method we found that, in solutions containing 0.3 mg. of sulphate S/10 ml. portion, a precision of $\pm 1\%$ in duplicates can in general be assured, and of $\pm 5\%$ with certainty.

Reagents

Trichloroacetic acid. 20% (w/v) solution in water.

Benzidine. 1% solution in pure acetone, freshly prepared each day.

Sodium hydroxide. N-NaOH, in water.

Ethanollic magnesia mixture. NH_4Cl (6 g.) and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (6 g.) in water (100 ml.) and made up to 1 l. with absolute ethanol.

Barium chloride. 0.02 N-BaCl₂, in water.

Indicator. A freshly prepared aqueous solution of sodium rhodizonate of a fairly deep orange colour.

All reagents should be A.R.

Method. The solution (20 ml.) to be examined is acidified with trichloroacetic acid (10 ml.). Any precipitate formed is separated by filtration. Duplicate 10 ml. samples of the clear solution are pipetted into 50 ml. centrifuge tubes. Benzidine solution in acetone (25 ml.) is added to each tube in a fine stream, and the tubes are capped and allowed to stand for at least 0.5 hr. They are then centrifuged at medium speed for 15–20 min., and the clear supernatant fluid is carefully decanted. The tubes are now inverted on filter paper to drain residual acetone. It was found by control experiments that washing of the precipitate with acetone is unnecessary, and that even when the culture filtrate is highly coloured the precipitate left after decantation is almost white, and the amount of residual colouring matter was never sufficient to interfere with subsequent titration. The precipitate is now prepared for titration by dissolving in 0.1 ml. of N-NaOH. Shaking and warming facilitate solution. Ethanolic magnesia mixture (10 ml.) is added to the solution to precipitate phosphates, and the tubes are kept at a temperature of 65–70° in a thermostat during the titration. Sufficient sodium rhodizonate solution is added to give a definite yellow colour to the solution, and care must be taken to add the same amount of indicator to each tube of a series of estimations. 0.02 N-BaCl₂ is added drop by drop from a micro-burette graduated in 0.01 ml. During the titration the contents of the tube are rotated gently. The yellow colour changes through orange and reddish orange to a rather bright red. The BaCl₂ should be added carefully until there is a definite and permanent blue tint in the red, continuing heating in the thermostat and further addition of BaCl₂ if necessary. Illumination needs to be good; a blue-glass electric light placed close to the tube and a white background help considerably.

1 ml. of 0.02 N-BaCl₂ = 0.3207 mg. S.

Of the 119 species and strains of moulds examined only four, which were discarded, gave trouble and then only after a very long period of incubation. In three cases, i.e. *Penicillium frequentans* Westling, P. 28; *P. luteum* Zukal, P. 42; *P. rugulosum* Thom, P. 50, the interference was due to the extreme viscosity of the culture filtrate, and in the fourth, *Aspergillus niger* van Tieghem, no. 33, a heavy precipitate prevented titration.

II. Sulphur as total inorganic sulphate in culture filtrate after heating with 0.3 N-HCl

The method commonly used for the hydrolysis of the ethereal sulphates in urine has been modified and applied to the mould culture filtrates. A portion of the culture filtrate is accurately measured into a conical flask and acidified with $\frac{1}{10}$ vol. pure conc. HCl, so that the final concentration of HCl is approx. 0.3 N. A small funnel and watch glass are inserted in the neck of the flask to lessen evaporation, and its contents are boiled gently for 30 min. After cooling, the solution is transferred to a measuring flask, a little bromocresol green is added and the solution neutralized with NaOH. The total inorganic sulphate now present is determined in portions by the method described in § I. Reagents are checked by means of a blank experiment. Phenyl potassium sulphate, initially free from inorganic sulphates, gave a 96.2% yield of inorganic sulphate when estimated by this method.

III. Sulphur as inorganic sulphate generated in culture filtrate after heating with 0.3 N-HCl

This is obtained by subtracting the value given by the estimation in § I from that obtained as described in § II.

IV. Total sulphur in culture filtrate

To a sample of the culture filtrate (usually 100 ml.) is added fuming HNO₃ (10 ml.) and KNO₃ (0.5 g.). The mixture is evaporated almost to dryness in a Kjeldahl flask, and is then treated exactly as in the estimation of total sulphur in the mycelium described in § VI.

V. Sulphur in culture filtrate not present as inorganic sulphate after heating with 0.3 N-HCl

This is obtained by subtracting the value given by the estimation in § II from that obtained as described in § IV.

VI. Total sulphur in the mycelium

About 1 g. of dried and powdered mycelium is accurately weighed, and is gently digested for 4 hr. in a Kjeldahl flask with fuming HNO₃ (10 ml.) and KNO₃ (0.5 g.). The acid is then removed in a stream of air on a water bath. A further 10 ml. fuming HNO₃ are added, the contents heated for 2 hr. and the acid removed as before. The residue is now digested on a sand bath for 1–2 hr. with H₂O₂ (10 ml. of 100 vol.) added at intervals 2 ml. at a time. The clear solution is evaporated on the water bath, taken up in 0.25 N-HCl (20 ml.) and evaporated to dryness. Evaporation with HCl is repeated, and the final white residue is dissolved in 100 ml. of a mixture of water and trichloroacetic acid (final acid concentration, 6%). The amount of inorganic sulphate is estimated in 10 ml. samples by precipitation with benzidine as described in § I.

VII. Residual glucose in the culture filtrate

This is estimated polarimetrically.

RESULTS

The utilization of sulphur initially present as inorganic sulphate, by 115 mould species or strains

In this part of the investigation only the culture filtrate was examined.

Two flasks, each containing 350 ml. of Czapek-Dox culture medium with a sulphate content equivalent to 22.9 mg. of sulphur/flask, were inoculated at the same time and from the same culture of the species or strain of mould being examined. One flask was, in general, harvested after an incubation period of 17–25 days, after which time the residual glucose was usually between 0.1 and 1.0%. The second flask was harvested after a much longer incubation period, usually 5–10 weeks, by which time the glucose had either been completely metabolized, or its utilization had become extremely slow. The residual inorganic sulphate was estimated in each flask by the method described above.

The results obtained are given in Table 1. The figures given in column 5 show the percentage utilization of sulphate, so that a figure of 97.6% means that only 2.4% of the sulphur originally present in the culture medium as inorganic sulphate remains in that form in the culture filtrate. The moulds examined are listed in descending order of

Table 1. *Utilization of inorganic sulphate by 115 species and strains of fungi*

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO ₄ (%)
Species of <i>Penicillium</i>				
<i>P. puberulum</i> Bainier	P. 47	24	0.2	97.6
		38	0.0	96.0
<i>P. notatum</i> Westling	P. 45	23	0.7	95.6
		38	0.0	96.4
<i>P. citrinum</i> Thom	P. 27	21	0.6	85.3
		39	0.1	70.3
<i>P. olivino-viride</i> Biourge	P. 46	24	0.2	80.6
		37	0.2	97.4
<i>P. brunneo-rubrum</i> Dierckx	P. 8	21	0.4	80.3
		58	0.0	58.0
<i>P. lanosum</i> Westling	P. 86	21	1.0	80.2
		68	0.0	59.6
<i>P. candido-fulvum</i> Dierckx	P. 12	18	3.6	79.6
		65	0.1	72.0
<i>P. chrysogenum</i> Thom	P. 57	25	0.0	79.1
		39	0.0	40.6
<i>P. phaeo-janthinellum</i> Biourge	P. 91	22	0.1	76.6
		56	0.0	60.4
<i>P. griseo-brunneum</i> Biourge	P. 36	22	0.2	76.1
		37	0.0	45.8
<i>P. Hagemi</i> Zaleski	P. 84	21	0.3	74.0
		68	0.0	55.8
<i>P. citreo-nigrum</i> Dierckx	P. 20	21	1.2	73.4
		57	0.0	96.6
<i>P. citrinum</i> Thom	P. 26	24	0.1	73.4
		56	0.0	55.7
<i>P. digitatum</i> Saccardo	P. 33	43	0.0	73.0
		66	0.5	79.7
<i>P. aurantio-brunneum</i> Dierckx	P. 2	20	0.8	68.8
		60	0.0	95.2
<i>P. citreo-viride</i> Biourge	P. 24	24	0.3	68.8
		55	0.0	68.4
<i>P. commune</i> Thom	P. 30	21	0.4	67.8
		38	0.0	50.7
<i>P. patris-mei</i> Zaleski	P. 90	22	0.3	66.6
		68	0.0	50.6
<i>P. solitum</i> Westling	P. 52	24	0.6	66.1
		39	0.1	60.9
<i>P. caseicolum</i> Bainier	P. 11	18	2.0	66.0
		60	0.0	33.9
<i>P. citreo-roseum</i> Dierckx	P. 22	21	0.7	65.5
		57	0.0	98.1
<i>P. chloro-leucon</i> Biourge	P. 14	18	0.4	64.8
		55	0.1	52.8
<i>P. chloro-phaeum</i> Biourge	P. 15	18	1.4	64.8
		57	0.0	76.5
<i>P. ochraceum</i> Bainier	P. 88	22	0.4	63.5
		68	0.0	47.2
<i>P. baculatum</i> Westling	P. 7	21	0.3	63.4
		59	0.0	58.0
<i>P. chrysitis</i> Biourge	P. 16	18	3.1	60.5
		61	0.0	58.4
<i>P. griseo-roseum</i> Dierckx	P. 39	22	0.8	60.4
		39	0.2	38.9
<i>P. flavido-marginatum</i> Biourge	P. 78	20	0.1	59.8
		57	0.1	26.2

* London School of Hygiene and Tropical Medicine (L.S.H.T.M.)

Table 1 (cont.).

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO ₄ (%)
<i>P. Bialowiezense</i> Zaleski	P. 71	19 56	0.2 0.0	59.7 35.9
<i>P. citreo-roseum</i> Dierckx	P. 23	24 55	0.4 0.1	59.2 61.1
<i>P. citrinum</i> Thom	P. 6	20 60	1.1 0.0	58.5 58.4
<i>P. fusco-glaucum</i> Biourge	P. 81	21 56	0.5 0.0	58.1 82.3
<i>P. chrysogenum</i> Thom	P. 19	21 55	0.4 0.0	57.7 60.5
<i>P. frequentans</i> Westling	P. 29	21 39	0.8 0.0	56.3 80.1
<i>P. griseo-fulvum</i> Dierckx	P. 38	22 43	2.0 0.7	54.1 48.9
<i>P. citreo-nigrum</i> Dierckx	P. 21	21 61	2.2 0.2	53.7 56.8
<i>P. Raciborskii</i> Zaleski	P. 92	22 67	0.1 0.0	52.8 42.8
<i>P. stilton</i> Biourge	P. 54	25 64	2.0 0.1	52.8 62.8
<i>P. citreo-sulfuratum</i> Biourge	P. 66	18 71	2.3 0.0	50.7 66.2
<i>P. expansum</i> Link.	P. 41	23 43	1.1 0.1	50.2 95.8
<i>P. biforme</i> Thom	P. 72	19 67	0.1 0.1	49.8 24.5
<i>P. citreo-sulfuratum</i> Biourge	P. 59	26 65	1.7 0.1	49.7 52.0
<i>P. cinerascens</i> Biourge	P. 65	18 71	2.8 1.0	48.5 52.0
<i>P. spinulosum</i> Thom	P. 96	25 67	0.1 0.0	48.0 39.8
<i>P. citrinum</i> Thom	P. 25	24 55	0.3 0.0	47.4 65.6
<i>P. cyaneo-fulvum</i> Biourge	P. 34	22 66	1.2 0.5	45.4 55.0
<i>P. Oledzkii</i> Zaleski	P. 89	22 69	2.4 0.0	44.5 60.9
<i>P. Siemaszki</i> Zaleski	P. 95	25 69	1.0 0.1	44.1 31.0
<i>P. sublateralitium</i> Biourge	P. 55	25 42	0.3 0.0	42.8 60.9
<i>P. purpurogenum</i> Stoll	P. 48	24 39	0.3 0.1	40.6 45.4
<i>P. viridi-dorsum</i> Biourge	P. 56	25 42	1.3 0.1	40.6 43.7
<i>P. fellutanum</i> Biourge	P. 67	19 56	0.5 0.0	40.2 48.5
<i>P. Dierckxii</i> Biourge	P. 32	22 43	2.0 0.1	39.7 61.2
<i>P. roqueforti</i> Thom	P. 93	22 69	3.1 0.0	37.6 40.6
<i>P. cyaneo-fulvum</i> Biourge	P. 31	21 65	1.5 0.5	37.5 50.7
<i>P. aurantio-griseum</i> Dierckx var. <i>poznaniensis</i> Zaleski	P. 69	19 56	0.2 0.0	36.3 55.0
<i>P. Niklewskii</i> Zaleski	P. 61	26 65	0.9 0.1	36.2 37.6

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Table 1 (cont.).

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO ₄ (%)
<i>P. obscurum</i> Biourge	P. 87	21 68	1.6 0.0	35.8 50.7
<i>P. fellutanum</i> Biourge	P. 60	26 65	2.1 0.1	35.4 50.2
<i>P. Biourgei</i> Dierckx	P. 73	20 67	1.8 0.0	35.0 39.8
<i>P. roseo-citreum</i> Biourge	P. 94	25 69	1.4 0.9	34.1 37.2
<i>P. Adametzi</i> Zaleski	P. 58	25 64	2.5 0.1	34.0 58.2
<i>P. granulatum</i> Bainier	P. 35	22 38	0.4 0.2	32.8 45.4
<i>P. flavi-dorsum</i> Biourge	P. 79	20 70	2.2 0.0	32.4 43.2
<i>P. gorgonzola</i> Weidemann	P. 83	21 69	2.6 0.1	32.3 53.3
<i>P. subcinereum</i> Westling	P. 98	25 69	2.8 0.1	32.0 38.4
<i>P. terlikowskii</i> Zaleski	P. 63	18 67	2.5 0.2	30.1 54.2
<i>P. decumbens</i> Thom	P. 76	20 71	2.9 0.0	29.4 40.6
<i>P. chrysogenum</i> Thom	P. 18	21 60	2.4 0.1	29.2 53.3
<i>P. brunneo-rubrum</i> Dierckx	P. 9	18 63	2.9 0.8	28.8 50.1
<i>P. frequentans</i> Westling	P. 80	20 70	3.2 0.0	28.8 44.2
<i>P. chrysogenum</i> Thom	P. 17	21 61	3.3 1.5	27.6 34.4
<i>P. frequentans</i> Westling	P. 82	21 70	3.1 0.1	27.1 65.4
<i>P. carmino-violaceum</i> Dierckx	P. 64	18 71	3.3 0.0	26.7 50.7
<i>P. baiiolum</i> Biourge	P. 70	19 67	1.4 0.0	25.8 43.2
<i>P. Paczoskii</i> Zaleski	P. 62	18 71	2.5 0.0	25.0 45.0
<i>P. aurantio-virens</i> Biourge	P. 4	20 107	3.5 0.0	22.9 32.0
<i>P. meleagrinum</i> Biourge	P. 43	23 78	3.4 2.2	22.7 36.7
<i>P. atramentosum</i> Thom	P. 1	20 64	3.4 0.1	21.7 47.7
<i>P. griseo-fulvum</i> Dierckx	P. 68	19 71	3.5 0.0	20.1 74.8
<i>P. flavo-cinereum</i> Biourge	P. 77	20 70	3.5 0.1	19.6 44.5
<i>P. spiculisporum</i> Lehman	P. 53	25 43	1.6 0.1	17.0 20.0
<i>P. schneegii</i> Boas	P. 51	24 64	4.2 2.4	12.4 32.8
<i>P. minio-luteum</i> Dierckx	P. 44	23 78	3.8 1.8	11.7 28.0
<i>P. jantho-citrinum</i> Biourge	P. 40	23 78	3.8 3.2	10.5 25.8
<i>P. aurantio-candidum</i> Dierckx	P. 3	20 65	4.5 3.6	7.0 42.6
<i>P. brunneo-violaceum</i> Biourge	P. 10	18 61	2.8 0.2	5.8 73.4

* London School of Hygiene and Tropical Medicine (L.S.H.T.M.)

Table 1 (cont.).

Organism	Catalogue no.*	Incubation period (days)	Residual glucose by polarimeter (g./100 ml.)	Utilization of SO ₄ (%)
Species of <i>Aspergillus</i>				
<i>A. fumigatus</i> Fresenius	No. 47	19	2.4	99.3
		47	0.3	85.1
<i>A. terreus</i> Thom	Am. 1	17	0.5	93.0
		59	0.0	82.5
<i>A. terreus</i> Thom	No. 3	17	0.6	90.5
		59	0.0	98.2
<i>A. terreus</i> Thom	Ab. 2	19	0.4	85.5
		57	0.0	97.2
<i>A. terreus</i> Thom	No. 37	17	0.8	75.6
		59	0.0	99.5
<i>A. flavus</i> Link.	No. 51	19	0.5	74.8
		47	0.2	98.7
<i>A. terreus</i> Thom	Ac. 24	17	0.5	71.1
		57	0.6	100.0
<i>A. Sydowi</i> Bainier & Sartory	No. 43	18	0.6	68.5
		46	0.0	88.5
<i>A. terreus</i> Thom	Am. 2	19	0.2	67.1
		57	0.0	97.7
<i>A. ochraceus</i> Wilhelm	No. 69	19	1.5	66.2
		47	0.2	97.9
<i>A. terreus</i> Thom	Ab. 1	19	0.8	61.0
		59	0.0	69.9
<i>A. terreus</i> Thom	Ac. 107	17	0.3	58.5
		57	0.0	63.9
<i>A. oryzae</i> (Ahlburg) Cohn	A. 11	25	0.5	57.0
		48	0.3	50.7
<i>A. Wentii</i> Wehmer	No. 2	19	2.0	42.8
		46	0.6	37.1
<i>A. nidulans</i> Eidam	No. 46	18	1.6	41.4
		46	0.1	69.0
<i>A. versicolor</i> Vuill. (Tiraboschi)	No. 18	19	2.2	41.0
		46	0.1	62.2
<i>A. terreus</i> Thom	Ac. 100	17	2.6	31.6
		60	0.0	57.9
<i>A. tamarii</i> Kita	No. 55	20	3.4	19.7
		47	0.5	42.8
<i>A. candidus</i> Link.	No. 32	25	3.8	17.9
		49	0.1	44.5
Miscellaneous species				
<i>Clasterosporium</i> sp.	Ag. 64	18	0.7	39.7
		46	0.1	42.0
<i>Stysanus stemonites</i> (Pers.) Corda	No. 137	20	2.4	33.2
		48	0.1	41.0
<i>Stemphylium lanuginosum</i> Harz.	No. 56	20	4.2	31.5
		47	2.5	21.8
<i>Syncephalastrum cinereum</i> Bainier	No. 112	20	4.4	22.7
		48	0.4	36.2
<i>Absidia spinosa</i> Lendner	No. 118	20	4.0	19.7
		48	0.6	29.7
<i>Alternaria</i> sp.	Ag. 17	20	4.3	11.8
		49	3.3	24.0
<i>Trichoderma viride</i> Pers. ex Fries	No. 85	19	4.1	11.3
		48	1.8	24.0
<i>Caldariomyces fumago</i> Woronichin	Ag. 92	18	2.9	7.0
		46	1.5	68.4
<i>Cephalosporium</i> sp.	Ag. 30	25	4.3	4.8
		49	3.1	14.4

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the amount of sulphate metabolized after the shorter incubation period. Eighty-seven species or strains of *Penicillium*, 19 of *Aspergillus* and 9 miscellaneous species were examined.

Sulphur balance sheets of fourteen species or strains of moulds

The aims of this part of the investigation which, because of the large number of estimations involved, was of necessity somewhat restricted, were twofold:

(a) To explore the variations in sulphur metabolism of different strains within a single species of mould. For this purpose six authentic strains of *Aspergillus terreus* Thom were chosen, since it was already known, from investigations carried out in this laboratory, that different strains of this species give rise to a large number of different metabolic products. Two of these strains, no. 45 and Am. 2, metabolize considerable amounts of inorganic chloride (Clutterbuck *et al.* 1940).

(b) To explore the variations in sulphur metabolism of different species of moulds within a single genus. Seven different species of *Penicillium* were chosen for this purpose.

Eight flasks, each containing 350 ml. of Czapek-Dox medium, were sterilized, inoculated with the species or strain under investigation, and incubated at 24°. Four flasks of each strain or species were harvested, and the filtered contents made up to 2 l. after (a) substantially all the glucose had been utilized, and four flasks after (b) extended incubation for 9–11 weeks. In the culture filtrate, residual inorganic sulphate, sulphate generated after acid hydrolysis and total sulphur in solution were determined, and total sulphur in the mycelium, by the methods previously described. The results obtained are given in Table 2.

DISCUSSION

An outstanding fact, that emerges from the results presented in Table 1, is the evident importance of sulphur in the metabolism of moulds, as indicated by the large amounts of inorganic sulphate absorbed from the nutrient medium. Thus, of the 115 species or strains examined, seven species of *Penicillium* and nine species or strains of *Aspergillus*, including six strains of *A. terreus* Thom, utilized, after either the shorter or longer incubation period, 90.0% or more of the inorganic sulphate originally present in the nutrient medium. Similarly, 40 species or strains utilized from 60.0 to 89.9% of the inorganic sulphate. With 29 species or strains the percentage utilization of inorganic sulphate was more than 5% greater after the shorter period of incubation than after the longer period. In some of these cases, where the difference is relatively small, it is possible that the difference represents variations between single flasks. In others, it is probably due to the hydrolysis of 'etheral sulphates' formed as metabolic products (see below).

The seven different species of *Penicillium*, six different strains of *Aspergillus terreus* Thom and one of *A. fumigatus* Fresenius, showing a large percentage utilization of inorganic sulphate, were chosen for a more detailed examination of their sulphur metabolism with the results given in Table 2. Consideration of these results leads to the following conclusions:

(1) *Utilization of inorganic sulphate* (column 6, Table 2). All seven species or strains of *Aspergillus* and three species of *Penicillium* utilized the whole of the inorganic sulphate originally present in the medium within the limits of experimental error $\pm 5\%$, and after either the shorter or longer period of incubation. Two of the four remaining species utilized over 90%. The results are thus in substantial agreement with those given in Table 1.

(2) *Inorganic sulphate generated after acid hydrolysis* (column 8, Table 2). With most species or strains examined the amount of inorganic sulphate regenerated after acid hydrolysis under relatively mild conditions (boiling with 0.3N-HCl for 30 min.) is considerable, reaching a maximum of 60.1% with *Penicillium citreo-roseum*, and exceeding 25% with ten other species or strains. The nature of this sulphur has not yet been established, but it is clear that it is present in some relatively labile form possibly as 'etheral sulphates'.

The presumed formation of 'etheral sulphates' does not, however, appear to be a property possessed by all the species examined. Thus the percentage amount of 'etheral sulphates' formed by *P. notatum* (5.3 and 3.9% after 35 and 75 days' incubation respectively), and by *P. aurantio-brunneum* (5.2% after 36 days' incubation) falls within, or very close to, the experimental error of the method of estimation ($\pm 5\%$). A similar observation was made by Rippel & Behr (1936) who cultivated *Aspergillus niger* on a chemically defined medium, somewhat different in nature to Czapek-Dox medium, and containing the sulphates of potassium, magnesium and zinc. They observed the utilization of inorganic sulphate, and showed that boiling the metabolism solution with 0.5N- or N-HCl for 2 hr. gave no appreciable increase in inorganic sulphate. We have confirmed the absence of 'etheral sulphate' formation by our strain of *A. niger* L.S.H.T.M. strain Ac. 1 when grown on Rippel & Behr's medium.

So far as we are aware only one 'etheral sulphate' has been isolated in a state of purity from mould cultures. Woolley & Peterson (1937) extracted cyclic choline sulphate

(β -sulphatoethyltrimethylammonium betaine

Table 2. Balance sheet showing distribution of sulphur originally supplied as inorganic sulphate and metabolized by fourteen species or strains of moulds

Organism	Catalogue no.*	Incubation period (days)	Residual glucose (g./100 ml.)	Weight of mycelium (g./flask)	Residual inorganic SO ₄ I (%)	Total SO ₄ after hydrolysis II (%)	SO ₄ generated after hydrolysis III (%)	Total S in filtrate IV (%)	Non-SO ₄ -S after hydrolysis V (%)	S in mycelium VI (%)	Total S accounted for IV + VI (%)
<i>A. terreus</i>	Ac. 24	29	0.2	3.01	0.0	26.8	26.8	66.9	40.1	32.2	99.1
		71	—	2.18	0.5	16.2	15.7	70.8	54.6	21.8	92.6
<i>A. terreus</i>	No. 45	29	0.2	3.19	0.0	19.8	19.8	52.8	33.0	47.2	100.0
		78	—	1.92	0.0	35.5	35.5	74.7	39.2	29.4	104.1
<i>A. terreus</i>	No. 3	22	0.1	5.78	2.9	18.5	15.6	37.4	18.9	52.6	90.0
		71	—	3.41	0.5	27.1	26.6	48.5	21.4	40.0	88.5
<i>A. terreus</i>	Ab. 2	33	0.1	5.45	12.0	35.5	23.5	49.4	13.9	46.3	95.6
		62	—	4.56	0.0	43.5	43.5	53.9	10.3	47.6	101.4
<i>A. terreus</i>	No. 37	30	0.1	6.04	14.0	27.2	13.2	41.0	13.8	56.8	97.8
		63	—	4.73	0.0	39.0	39.0	51.5	12.5	44.6	96.1
<i>A. terreus</i>	Am. 2	30	0.1	3.40	19.0	35.4	16.4	53.7	18.3	40.3	94.0
		74	—	2.57	1.2	27.8	26.6	49.8	22.0	37.6	87.4
<i>A. fumigatus</i>	No. 47	41	1.5	3.00	0.0	16.5	16.5	48.1	31.6	37.9	86.0
		76	—	3.35	0.7	16.5	15.8	43.2	26.7	50.3	93.5
<i>P. citreo-nigrum</i>	P. 20	41	0.0	2.49	0.0	40.6	40.6	59.9	19.3	32.7	92.6
		74	—	2.15	3.5	45.0	41.5	70.0	25.0	24.0	94.0
<i>P. olivino-viride</i>	P. 46	35	0.0	2.46	6.6	52.1	45.5	83.9	31.8	15.9	99.8
		70	—	1.96	6.5	49.4	42.9	77.8	28.4	22.6	100.4
<i>P. puberulum</i>	P. 47	27	0.0	2.79	4.9	36.1	31.2	64.6	28.5	28.5	93.1
		70	—	1.79	0.0	20.6	20.6	68.1	47.5	27.2	95.3
<i>P. cepanum</i>	P. 41	41	0.4	2.13	34.0	63.1	29.1	75.6	12.5	23.1	98.7
		68	—	1.97	28.2	62.5	34.3	77.4	14.9	26.4	103.8
<i>P. citreo-roseum</i>	P. 22	36	0.2	3.65	21.8	43.6	21.8	59.0	15.4	40.6	99.6
		67	—	3.30	4.5	64.6	60.1	72.1	7.5	31.4	103.5
<i>P. notatum</i>	P. 45	35	0.0	2.26	0.4	5.7	5.3	70.0	64.3	24.8	94.8
		75	—	1.75	8.4	12.3	3.9	96.6	84.3	7.4	104.0
<i>P. aurantiobrunneum</i>	P. 2	36	0.0	3.98	23.4	28.6	5.2	45.9	17.3	43.3	89.2
		68	—	2.80	17.4	26.8	9.4	49.8	23.0	50.6	100.4

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as colourless crystals from the mycelium of *A. Sydowi* Bainier & Sartory grown on a glucose-mineral salts medium. This substance yields choline and sulphuric acid on acid hydrolysis.

(3) *Sulphur in the culture filtrate not present as inorganic sulphate after acid hydrolysis* (column 10, Table 2). All the species and strains examined convert considerable amounts of inorganic sulphate into water-soluble, sulphur-containing compounds, which do not give rise to inorganic sulphates on mild acid hydrolysis. In some cases the conversion is surprisingly large, e.g. *Penicillium notatum*, 75 days' incubation, 84.3 %; *Aspergillus terreus*, strain Ac. 24, 71 days' incubation, 54.6 %; *Penicillium puberulum*, 70 days' incubation, 47.5 %.

Nothing is known at present as to the nature of these sulphur compounds, though it is probable that the sulphur is in organic combination. Hydrogen sulphide and mercaptans were not encountered, although Birkinshaw, Findlay & Webb (1942) established the formation of methyl mercaptan by the wood-rotting fungus *Schizophyllum commune* Fr., when grown on a synthetic medium containing glucose with ammonium and magnesium sulphates as sources of sulphur.

It is improbable that the sulphur is present to any considerable extent as protein or peptone. Thus, although Clutterbuck, Lovell & Raistrick (1932) isolated from culture filtrates of strains of *Penicillium chrysogenum* Thom and *P. notatum* Westling, including the strain P. 45, a protein containing 3.5 % of cystine, the yield, expressed as a percentage of the inorganic sulphate in the medium, is small. Similarly, Rippel & Behr (1936) state that, of the inorganic sulphate utilized by *Aspergillus niger*, less than one seventh is present as protein and none as peptone.

Very few mould metabolic products containing sulphur have been isolated in a state of purity. It may be significant, however, that the different penicillins, metabolic products of *Penicillium notatum*, all give $\beta\beta$ -dimethylcysteine on hydrolysis, and that another antibiotic, gliotoxin, produced by a variety of fungi, including *Aspergillus fumigatus* Fresenius (Menzel, Wintersteiner & Hoogerheide, 1944) and *Penicillium cinerascens* Biourge (Bracken & Raistrick, 1947), has the empirical formula $C_{13}H_{14}O_4N_2S_2$.

Ovcharov (1937) has demonstrated qualitatively the formation of thiourea by cultures of *Verticillium albo-atrum* and *Botrytis cinerea* grown on Richards's (1897) medium, containing magnesium and ferrous sulphates as sole source of sulphur.

(4) *Sulphur in mycelium* (column 11, Table 2). Although the total sulphur in the mycelium amounts in some cases, particularly with strains of *Aspergillus terreus*, to about half that supplied originally as inorganic sulphate, the percentage of sulphur in the dried mycelium is, with three exceptions, fairly

uniform, and is consistently small. The percentage sulphur content in the dried mycelium of the different species and strains examined is as follows (*d.* = incubation period in days): *A. terreus*, Ac. 24. 29d., 0.24 %; 71d., 0.23 %. *A. terreus*, no. 45. 29d., 0.34 %; 78d., 0.35 %. *A. terreus*, no. 3. 22d., 0.21 %; 71d., 0.27 %. *A. terreus*, Ab. 2. 33d., 0.20 %; 62d., 0.24 %. *A. terreus*, no. 37. 30d., 0.22 %; 63d., 0.22 %. *A. terreus*, Am. 2. 30d., 0.27 %; 74d., 0.33 %. *A. fumigatus*, no. 47. 41d., 0.29 %; 76d., 0.34 %. *Penicillium citreo-nigrum*, P. 20. 41d., 0.30 %; 74d., 0.25 %. *P. olivino-viride*, P. 46. 35d., 0.15 %; 70d., 0.26 %. *P. puberulum*, P. 47. 27d., 0.23 %; 70d., 0.35 %. *P. expansum*, P. 41. 41d., 0.25 %; 68d., 0.31 %. *P. citreo-roseum*, P. 22. 36d., 0.25 %; 67d., 0.22 %. *P. notatum*, P. 45. 35d., 0.25 %; 75d., 0.10 %. *P. aurantio-brunneum*, P. 2. 36d., 0.25 %; 68d., 0.41 %.

Thus, although the maximum and minimum weights of dried mycelium/flask for the shorter incubation period are 6.04 g. (*Aspergillus terreus*, no. 37, 30 days) and 2.13 g. (*Penicillium expansum*, P. 41, 41 days), their respective sulphur contents are 0.22 and 0.25 %.

The dry weight of mycelium (Table 2, column 5) is less after the longer period of incubation than after the shorter period, in every case except for *Aspergillus fumigatus*, no. 47, clearly indicating that autolysis has taken place in the interval. Disregarding any results which fall within the limits of experimental error (± 5 %) there has been during this interval:

(a) A loss of sulphur from the mycelium to the metabolism solution in seven cases (limits -8.7 % with *Penicillium citreo-nigrum*, P. 20, to -17.8 % with *Aspergillus terreus*, no. 45) and a gain in three cases (limits $+6.7$ % with *Penicillium olivino-viride*, P. 46, to $+12.4$ % with *Aspergillus fumigatus*, no. 47).

(b) A gain in 'etheral sulphates' in the metabolism solution in seven cases (limits $+5.2$ % with *Penicillium expansum*, P. 41, to $+38.3$ % with *P. citreo-roseum*, P. 22) and a loss in two cases (-10.6 % with *P. puberulum*, P. 47, and -11.1 % with *Aspergillus terreus*, Ac. 24).

(c) A gain in sulphur compounds, other than 'etheral sulphates', in the metabolism solution in six cases (limits $+5.7$ % with *Penicillium citreo-nigrum*, P. 20, and *P. aurantio-brunneum*, P. 2, to $+20.0$ % with *P. notatum*, P. 45) and a loss in one case of -7.9 % with *P. citreo-roseum*, P. 22.

SUMMARY

1. A survey has been made of the utilization of inorganic sulphate by 115 species or strains of moulds, grown on Czapek-Dox glucose solution con-

taining magnesium and ferrous sulphates as the sole source of sulphur.

2. This has demonstrated the universal importance of inorganic sulphates in the metabolism of moulds, since all species or strains tested utilized 10 % or more, 56 utilized 60 % or more, and 16 over 90 % of the inorganic sulphate in the medium.

3. A more detailed examination of the sulphur

metabolism of seven species of *Penicillium*, six strains of *Aspergillus terreus* Thom, and one strain of *A. fumigatus* Fresenius, grown on the same medium, revealed the fact that some of them form considerable amounts of 'ethereal sulphates' while others, particularly *Penicillium notatum* Westling, give large yields of other, probably organic, sulphur compounds, of at present unknown nature.

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Studies on Ruminant Saliva

1. THE COMPOSITION AND OUTPUT OF SHEEP'S SALIVA

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(Received 16 October 1947)

In all domestic animals the principal function of saliva is that of a lubricant to assist mastication and deglutition. In ruminants however, saliva has a still more important role, for the rumen has no secretion of its own and the saliva forms a fluid medium for the transport of ingesta both back to the mouth for remastication and onwards through the stomach to the small intestine. It forms also a buffered medium in which the micro-organisms of the rumen can flourish. Particular interest therefore is attached to the saliva of the ruminant, to its composition and the amounts in which it is formed.

The literature on ruminant saliva is not very extensive: except for a few more recent papers, most of it has been reviewed by Mangold (1929). The sublingual and submaxillary glands, when stimulated

by eating, secrete a saliva of low ash and alkalinity which contains mucin and whose function is limited to lubricating the bolus of food. The parotid gland, on the other hand, secretes during eating and rumination, at a greater rate on the side on which the bolus is being masticated, and its flow continues at a reduced rate during the intervals of rest. The parotid saliva is formed in large amounts, it contains no ptyalin but has a high alkalinity and buffering power. It is consequently the most important secretion for the maintenance of normal rumen function.

The output of saliva by various ruminants as reported in the literature is summarized in Table 1. The quantity produced per day has been estimated at 56 kg. for the ox by Colin (1886) and at 50 l. for the

Table 1. *Published values for outputs of ruminant saliva*

Species	Secretion	Output of saliva		Reference
		ml./hr.	Total/day	
Ox	Parotid	800-2400	56 kg.	Colin (1886)
Cow	Mixed	—	50 l.	Markoff (1913)
Sheep	Parotid	25	—	Scheunert & Trautmann (1921)
	Parotid	3.6-21.6	—	Popov (1921)*
Goat	Parotid	10	—	Do.
	Parotid	60	—	Babichev (1925)*

* Quoted by Babkin (1944).