THE SULPHUR METABOLISM OF MOULD FUNGI: THE USE OF "BIOCHEMICAL MUTANT" STRAINS OF *ASPERGILLUS NIDULANS* IN ELUCIDATING THE BIOSYNTHESIS OF CYSTINE

by

D. J. D. HOCKENHULL

The Faculty of Technology, The University, Manchester (England)

In 1941, STEINBERG[1] showed that many inorganic sulphur sources (sulphate, sulphite, thiosulphate, etc.) were available for the growth of *Aspergillus niger*. He postulated that the mould synthesized organic sulphur compounds *via* "sulphoxylic" acids. Similar results were obtained by HOCKENHULL[2] with *Penicillium notatum*.

In addition, RIPPEL AND BEHR[3] had shown that *Asp. niger*, when supplied with sulphate, liberated considerable quantities of organic sulphur compounds into the medium. Under certain conditions, *P. notatum* could also be induced to convert a large proportion of sulphate in the medium to an unidentified intermediate (HOCKENHULL[2]).

In the present communication, the use of "biochemical mutant" strains of *Asp. nidulans*, in establishing the feasibilities of certain schemes for the biosynthesis of cystine from sulphate, is described. The principle of the method is that many of the steps in such a biosynthesis may be brought about by specific enzymes. The functioning of each of these may, in turn, depend on the intactness of a gene, or set of genes, governing its initial formation. A strain lacking a particular enzyme function for this reason is termed a "biochemical mutant". For example, strains of an organism capable, say, of converting A to intermediate B and intermediate C to final product D, but not of B to C, may be obtained. If D is an essential metabolite, neither A nor B will support growth. However, C (or any substance X easily converted to C) will support growth. By testing possible compounds, the identity of C may be established. This technique has been developed by BR. BEADLES's group at Stanford and is described more fully in a review by BONNER[4].

EXPERIMENTAL

The Organisms Used

The organisms employed were strains of *Asp. nidulans*. The bright green strain, A 69, was kindly given to me by Mr E. YUILL (The American subculture of this is probably N.R.R.L. 195). Y2 was a yellow X-ray mutant of this from Dr G. PONTECORVO. The author's Bα strain was a glaucous mustard-gas mutant of A 69 remarkable for absence of perithecia, dense sporing and rapid growth. "Cystineless" strain, σ, was obtained by Miss J. LASCELLES from strain Y 2.

References p. 335.

MEDIA

1. *C– medium*: glucose 4 %, Na acetate 0.25 %, $NaNO_3$ 0.5 %, $(NH_4)_2SO_4$ 0.5 %, salts PF 1 %, salts HS 1 % in Manchester tapwater, p_H to 5.8–6.0 with KOH.

2. *C+ medium*: glucose 4 %, Na acetate 0.25 %, NH_4NO_3 0.5 %, cystine 0.025 %, salts PF 1 %, salts HC 1 %, in tapwater, p_H to 5.8–6.0 with KOH.

3. *CD medium*: as C– with the addition of 0.5 % casein hydrolysate powder (vitamin free, ASHE).

4. *Basal medium*: as C+ without cystine.

5. *Malt wort*: Sp.Gr. 1.040.

6. *Salts HS*: $MgSO_4.7H_2O$ 5 %, $FeSO_4.7H_2O$ 0.5 %, $MnSO_4.4H_2O$ 0.2 %, $CuSO_4.5H_2O$ 0.2 %, $ZnSO_4.7H_2O$ 0.2 %, $Co_2(SO_4)_3.18H_2O$ 0.2 %, H_2SO_4 0.2 % v/v, in distilled water.

7. *Salts HC*: $MgCl_2.6H_2O$ 5 %, $FeCl_3$ 0.5 %, $MnCl_2.4H_2O$ 0.2 %, $CuCl_2.2H_2O$ 0.2 %, $ZnCl_2$ 0.2 %, $CoCl_2.6H_2O$ 0.2 %, HCl conc. 0.2 % v/v, in distilled water.

8. *Salts PF*: NaH_2PO_4 10 %, NaF 0.1 % in distilled water.

All solid media contained 2.5 % agar.

Sterilization was carried out at a steam pressure of 20 lb per sq. in for 15 min unless otherwise stated.

Production and Isolation of "Cystineless" Mutants

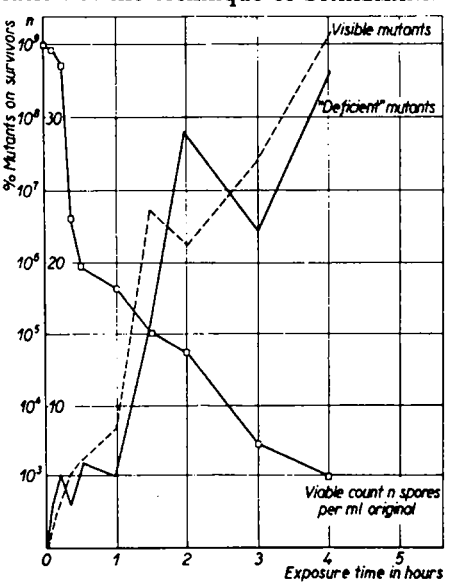
a. *With Mustard Gas in the Vapour Phase.* This technique has already been described (HOCKENHULL[5]).

b. *With Mustard Gas in Aqueous Solution.* Although the vapour exposure method was convenient and clean and gave uniform material which could be stored easily with successful treatments, uniformity between separate experiments was not easily obtained. In addition, the variation introduced by washing off the spores from the discs of felt, the shedding of spores and desiccation during storage and other factors, made difficult the quantitative study of the relation of spore mortality to exposure period. For these reasons, a number of experiments were carried out in which a spore suspension was mixed with mustard gas. This was a modification of the technique of STAHLMANN AND STAUFFER[6] as used with *Neurospora crassa*.

100 ml of a 10^9 conidiospore/per ml suspension, in 0.32 M phosphate buffer p_H 6.8–7.0 containing 0.1 % Calsolene oil H.S. (*ex* I.C:I.Ltd., a wetting-out agent), from a 5–7 day culture of *Asp. nidulans* on malt-wort agar. This suspension was added to 0.25 ml mustard gas ($\beta\beta'$ dichlorodiethylsulphide) in a dry, sterile, 250 ml conical flask containing about 20 7–9 mm glass balls and incubated at 30° with periodic shaking. At intervals, 2.5 ml of the suspension was withdrawn and added to 100 ml of 0.32 M phosphate buffer p_H 6.8–7.0 containing 0.5 % glycine to inactive the unused mustard gas.

After standing for about 30 min, 20 ml of this suspension was mixed with 30 ml C– medium in a sterile 1 l conical flask. The whole was incubated for 16 h to initiate germination.

The graph shows the relation between exposure time, survival rate, and the proportion of mutants on the survivors. The morphological mutants were diagnosed by naked-eye examina-



The Effect of Mustard Gas upon an Aqueous Suspension of *Conidiospores* of *Asp. nidulans*. Strain Bα in phosphate buffer p_H 6.9–7.0 at 30° with 0.25 % mustard gas.

References p. 335.

tion, the "biochemical" ones by isolation and testing upon C– medium and upon malt-wort agar. For isolation of "cystineless" mutants, survival rates of 50–100 organisms per ml (*ca* 10^4 of the original 10^9 spores per ml) were desirable. 90–120 min was the usual exposure period.

c. *Isolation of "Cystineless" Mutants.* The filtration technique of FRIES[7] was abandoned in the later experiments on the grounds that, with a 16 h incubation period at 30°, both normal and "cystineless" strains appeared to germinate to the same degree in C– medium. The pregermination was however continued as it appeared to give more uniform results on plating.

The germinated suspension was plated out at the rate of 1 ml per 10 cm petri dish of C+ agar. After incubation at 30° for 2 ½–3 days, separate colonies showing the green of conidiospores in their centres had grown from each viable spore. On this medium, "cystineless" and normal strains could grow well, but strains with other deficiencies could not.

Plates of C– agar (15 ml per 10 cm petri dish) were marked out with 10 mm circles by means of a sterile corkborer. The centre of each of these marked areas was inoculated from a separate colony of treated material. The technique of transfer with a pointed needle (sabre-shaped tip) was critical. Great care was taken to take up very little material and to make sure that spores were not scattered on the marked plate.

Incubation (at 30°) was again carried out for 2 ½–3 days, or until growth in the fastest isolates had reached the edges of the circles. In some circles, it was noted that no growth, or thin spidery mycelium, only had appeared. Such circles were lifted out bodily on a needle and placed on C+ agar. They were inundated with a few drops of a saturated solution of cystine and incubated a further 3 days or more. From those circles upon which growth appeared, inocula were made onto slopes of C+ and C– media. If growth appeared on the former, but failed on the latter, a retest was made to check that the isolate was genuinely "cystineless". The isolated were stored on CD medium with 0.025% added cystine, at room temperature.

13 "cystineless" mutants were obtained from about 9000 isolates with the vapour method and 3 from about 6000 by the liquid-phase method. The apparent difference between the success of the two techniques is largely based upon one extremely successful set of vapour-exposed discs. Therefore, although the author tends to favour the vapour method, no real argument for this choice can be made. All mutant strains were derived from A 69 except ξ and σ from Y2 and ν from Bα.

Substances used in Testing

Mg ethane sulphinate, from ethyl bromide and SO_2 by the GRIGNARD reaction.
Ba β-sulphopropionate, after KHARASCH AND BROWN[8].
Ca ββ′-dicarboxydipropionyldisulphide, after CHEYNEY AND PIENING[9].
Taurine disulphoxide, after CHRISTIANSEN AND DOLLIVER[10].
β-aminoethylmercaptan and bis-β-aminoethyldisulphidedihydrochloride after MILLS AND BOGERT[11].
Cysteic acid, after FRIEDMANN[12].
Cysteine sulphinic acid and cystine disulphoxide, kindly given by Dr MEDES of the Lankenau Institute, Philadelphia. They were made after LAVINE[13].
Na cysteine S-sulphonate, after CLARKE[14].

Techniques of Growth-Testing upon various Sulphur Sources

25 ml melted basal agar were cooled to 60° and poured into a warm, sterile, 10 cm petri dish. 5 ml of a seitz-filtered solution of the sulphur source at p_H 6–6.5 was added

References p. 335.

to bring the final concentration to about 1 mg per ml. The plate was then cooled and cylinders were transferred each to a separate, sterile, plugged 6" × 5/8" tube. They were then individually needle-inoculated with material from the master-cultures of "cystineless" and normal strains, incubated at 30° for 4 days and examined for growth. (Cystine and homocystine were used as steam-sterilized suspensions).

Two completely different tests were carried out with each substance, and at least two replicates per test. Further replication was made if the results appeared uncertain.

In the case of hydrosulphite which was of great theoretical interest, a further series of tests were carried out. Plates of 10 ml of C– medium were poured and sown each with 2 ml of a spore suspension of each organism under test at about 10^7 spores per ml. They were incubated until microscopic examination indicated that germination had taken place. Crystals of hydrosulphite were then added. The growth response was noted after 4 days. The results of these "auxanographic" tests were the same as those by the method described earlier.

The results of the tests are presented in Tables I and II.

TABLE I

GROWTH OF DEFICIENT STRAINS ON INORGANIC SULPHUR SOURCES

Strain	SO_4^{--}	$SO_3NH_2^-$	$S_2O_6^{--}$	SO_3^{--}	$S_2O_4^{--}$	$S_4O_6^{--}$	$S_2O_3^{--}$	S_2^{--}	S^{--}
α	—	—	—	—	—	—	+	—	—
β	—	—	—	—	—	—	+	±	+
γ	—	—	—	++	++	++	++	+	+
δ	—	—	—	++	++	++	++	+	±
ε	—	—	—	—	—	—	+	—	—
ζ	—	—	—	—	—	—	++	—	—
η	—	——	—	++	+	++	++	±	±
θ	—	—	—	—	no test	—	+	+	+
ι	—	—	—	++	++	++	++	+	++
κ	—	—	—	—	—	—	++	±	±
λ	—	—	—	++	+	++	++	—	—
μ	—	—	—	—	—	—	+	—	—
ν	—	—	—	—	—	—	++	—	—
ξ	—	—	—	—	—	—	++	—	—
ο	—	—	—	—	—	—	+	—	—
σ	—	—	—	++	+	++	++	±	±
υ	—	—	—	++	++	++	++	++	+
A 69	++	++	+	++	+	++	++	±	+
Bα	++	++	+	++	++	++	++	++	++

++ vigorous growth + moderate growth ± doubtful growth — no growth

Strains growing on sulphite but not upon sulphate were termed "sulphiteless", while those growing on thiosulphate but not upon sulphite were termed "thiosulphiteless".

References p. 335.

TA

GROWTH OF DEFICIENT ST

Strain	Na sulphite	L-cystine	Cystine disulphoxide	Cysteine sulphinic acid	Cysteic acid	Ca-bis-β-carboxyethyl disulphide	Ba-β-sulphopropionate	Taurine	β-ethylamine sulphinic acid(?)	Bis β-ethylamine disulphoxide di-HCl	Bis β-ethylamine
α	—	++	—	—	—	—	—	—	—	—	
β	—	++	—	—	—	—	—	—	—	—	
γ	++	++	++	++	++	++	++	++	++	++	++
δ	++	++	++	++	++	++	++	++	++	++	++
ε	—	++	±	—	—	—	—	—	—	—	
ζ	—	++	—	—	—	—	—	—	—	—	
η	++	++	++	++	++	++	++	++	++	++	++
ϑ	—	++	—	—	—	—	—	—	—	—	
ι	++	++	++	++	++	++	++	++	++	++	++
κ	—	++	—	—	—	—	—	—	—	—	
λ	++	++	++	++	++	++	++	++	++	++	++
μ	—	++	±	+	—	—	—	—	—	—	
ν	—	++	±	—	—	—	—	—	—	—	
ξ	—	++	—	—	—	—	—	—	—	—	
ο	—	++	—	—	—	—	—	—	—	—	
σ	++	++	++	++	++	++	++	++	++	++	++
υ	++	++	++	++	++	++	++	++	++	++	++
A69	++	++	++	++	++	++	++	++	++	++	++
Bα	++	++	++	++	++	++	++	++	++	++	++

Genetical Testing of Mutant Strains

Biochemically deficient strains of *Asp. nidulans* have been known for a long time to show the phenomenon of "balanced" heterokaryosis. (Heterokaryosis, the symbiosis of different types of nuclei within the same mould cells, has been described by PONTES CORVO[15] and PONTECORVO AND GEMMELL[16]. It usually occurs after hyphal anastomosi- between two different strains). Two strains, each deficient in a different essential metabolite, may by this means live symbiotically upon a doubly deficient medium. The relative numbers of each type of nuclei in the organism will depend upon the rate at which each can cause the formation of the metabolite essential to the other. This ratio is more or less constant for any particular environment, and the heterokaryon will therefore be "balanced" (in contradistinction to the variable heterokaryosis often observed between non-deficient strains).

Suspensions of about 10^7 spores per ml were made in C– medium from each strain under test. 2 ml of the suspension form each of the two strains under test for symbiosis were mixed in a 6″ × 1″ tube containing a slope of C– medium. After incubation at 30° for 16 h, the suspension was poured away and the residual organisms allowed to

References p. 335.

ORGANIC SULPHUR-SOURCES

β-mercapto ethylamine	Na ethyl sulphate	Na ethane-sulphonate	Mg ethane sulphinate	Diethyl disulphide	"Cyclic" choline sulphate	Acetaldehyde-Na bisulphite	Acetone-Na bisulphite	Formaldehyde-Na-sulphoxylate	Homocystine	Methionine	Cysteine-S-sulphonate	Na sulphite
−	−	−	−	−	−	−	−	−	±	++	+	−
−	−	−	−	−	−	−	−	−	+	++	++	−
++	−	±	±	−	−	++	++	++	++	++	++	++
++	−	−	−	−	−	+	++	−	++	++	++	++
−	−	−	−	−	−	−	−	−	+	++	++	−
−	−	−	−	−	−	−	−	−	+	++	++	−
−+	−	−	−	−	−	++	+	−	++	++	++	++
−	−	−	−	−	−	−	−	−	+	++	++	−
++	−	−	−	−	−	+	+	+	++	++	++	++
−	−	−	−	−	−	−	−	−	±	++	++	−
++	−	−	−	−	−	±	++	−	++	++	++	++
−	−	−	−	−	−	−	−	−	±	++	++	−
−	−	−	−	−	−	−	−	−	±	++	++	−
−	−	−	−	−	−	−	−	−	++	++	++	−
−	−	−	−	−	−	−	−	−	+	++	+	−
++	−	−	−	−	−	++	++	+	++	++	++	++
++	−	−	−	−	−	++	++	++	++	++	++	++
++	++	−	±	±	++	++	++	++	++	++	++	++
++	++	−	−	+	++	++	++	++	++	++	++	++

grow on the slope for another 4 days at 30°. If strong mycelial growth appeared, portions of the growing mycelium were transferred to a second slope of C– medium and again incubated. The presence of continued growth on subculture showed that heterokaryosis had occurred. This finding was checked (to eliminate possibilities of contamination or reversion) by the making of single spore isolations on C+ medium from the non-perithecial portions of the heterokaryons and testing these against C– medium. In all cases of true heterokaryosis, the original strains could be separated out in this way.

The 6 "sulphiteless" strains tested γ, δ, η, i, λ, v) were found to fit perfectly into three exogamous groups: I, γ; II, δ, ι, v and III, η, λ.

The 8 "thiosulphateless" strains tested (α, β, ϑ, κ, μ, ν, ξ, o) were found to fit into three exogamous groups: I, α, β, ν; II, κ. μ, o, and III, ϑ, ξ, with the following discrepancies: $\beta \times \kappa$, ? for −; $\vartheta \times \nu$, ? for +; $\mu \times \nu$ − for +; $\mu \times \xi$, ? + (where + = heterokaryosis, − = no heterokaryosis and ? = unreliable results).

7 Combinations between "sulphiteless" and "thiosulphateless" strains were tested and heterokaryosis was obtained in all cases.

The heterokaryon, $\beta \times \xi$ (green × yellow) was maintained for some months on

References p. 335.

C– agar. On transferring a small piece of mycelium to C+ agar, it readily segregated into the two parent strains in a typical pattern as described by PONTECORVO AND GEMMELL[17].

DISCUSSION

The State of Reduction at which Inorganic Sulphur is combined with Organic Residues

Inorganic sulphur-compounds seem to be reduced by the mould at least as far as thiosulphate before being assimilated into organic molecules. The results with sulphide (of which the inconsistency is probably due to the extreme ease with which sulphide is oxidized by atmospheric oxygen) substantially agree with those of STEINBERG[1], that sulphide is not a precursor in the direct route from sulphate to organic sulphur.

The Reduction of Sulphate

Sulphate appears to be reduced directly to sulphite. Dithionate is not an intermediate in this process as it behaves wholly as does sulphate. The possibility of a common enzyme for the reduction of both sulphate and dithionate must not be ignored.

The Reduction of Sulphite

Two paths have been suggested for the reduction of sulphite:

1. The formation of metabisulphite (which, according to ZACHARIASEN[18], may sometimes exist in the form $H.SO_2.SO_2OH$) with the S–S linkeage), its subsequent reduction to hydrosulphite ($H.SO.SO_2H$), followed by reduction to thiosulphate ($HS.SO_2H$). (*c.f.*, JELLINEK[19] on the electrolytic reduction of sulphite).

2. The direct reduction of sulphite to "sulphoxylate" (H_2SO_2), with either dimerization of this to form thiosulphate, or, alternatively, enzymic or non-enzymic reaction of this with sulphite to form hydrosulphite and spontaneous reduction of this by "sulphoxylate" to form thiosulphate.

The latter hypothesis is favoured on the following grounds:

1. that, although precise evidence on this point is lacking, the concentration of metabisulphite would be very low indeed under the conditions of the experiment (0.1% aqueous sulphite at a p_H value about 6).

2. that hydrosulphite behaved exactly as sulphite in the 9 "thiosulphateless" strains tested. This, while not absolutely excluding its intermediacy, does reduce the probability of it very considerably.

The Intermediacy of Thiosulphate

In the preceding paragraph, it has been assumed that thiosulphate and not sulphoxylate, is the form in which sulphate combines with organic sulphur. Although not completely cogent (for the lack of trials with substances of general formula, $-S.CH_2.CO.COOH$, presents a loophole), the fact that none of the possible intermediates suggested by various possible breakdown routes of cystine and cysteine, could be utilized by any of the thiosulphateless mutants, strongly indicates the irreversibility of such processes. Oxidation of the sulphur of cystine to the sulphinic acid group appears to render it unavailable to "thiosulphateless" strains. Decarboxylation or reductive deamination, with or without oxidation of the sulphur atom, appear also to render the sulphur unavaible to the latter strains.

The observation that the thiosulphate of serine- cysteine S-sulphonate-supports

References p. 335.

good growth of the "thiosulphateless" strains, adds a little weight to the above hypothesis. The luxuriant growth of the "thiosulphateless" strains when serine was added together with thiosulphate, is also noteworthy. Chemically, the breakdown of cysteine S-sulphonate in presence of mineral acid to give cysteine and sulphate, or in presence of alkali to give sulphenic acid and sulphite, have been described by CLARKE[14]. In addition, CHALLENGER AND BRISCOE[20] report that, with the mould fungi, *P. brevicaule* and *Schizophyllum commune*, they have obtained methyl and ethyl mercaptans from the corresponding alkylthiosulphates (Bunte salts). This implies a biologically catalysed fission of the type postulated for cysteine S-sulphonate.

A further point is that no mutants have been isolated which lacked the power to utilize thiosulphate. This makes it extremely unlikely (save if there is more than one route for the synthesis) that more than one enzymic step is involved. It is obvious that the S-sulphonate route entails the least number of possible enzyme-catalysed steps.

Again, the presence of thiosulphate has been noted in many higher organisms, notably by FROMAGEOT AND ROYER[21] in the urine of mammals. The possibility that the non-sulphate fraction in *P. notatum*, noted by HOCKENHULL[2], may be thiosulphate is now under investigation.

The Utilization of Organic Sulphur Compounds

The organic compounds tested, with the exception of cysteine S-sulphonate, homocystine and methionine, were not utilized by the "thiosulphateless" strains of the mould.

The ethyl-S compounds (diethyl disulphide, Na ethanesulphonate, etc.) were either not utilized, or only utilized with extreme difficulty, by all strains, including the normal ones. This implies that substitution is necessary before fission of the S-C linkage can occur.

The substituted S-ethyl compounds (with β-carboxy, β-amino or β-carboxy β-amino) were all utilizable by the "sulphiteless" strains while being non-utilizable by the "thiosulphateless" ones. This pointed to a breakdown to yield sulphite.

Recent work by FROMAGEOT, CHATAGNER AND BERGERET[22] indicates that, in mammals, cysteine sulphinic acid is broken down by a sulphinicase to yield alanine and sulphite, is pertinent, especially in view of their claim that the S-C linkage in the compound is very easily broken. It is, however, not easy to see how the action of such an enzyme could explain the breakdown of the *sulphonic* acids tested to yield sulphite. At this stage, therefore, it is unwise to speculate upon possible breakdown mechanisms.

In connexion with this phenomenon, however, it has been noted by HOCKENHULL[2] that *P. notatum* can produce sulphate from cystine. Similar results have been obtained by MÖTHES[23] with *Asp. niger*.

All strains tested grew with homocystine and methionine, especially well with the latter. HOROWITZ[24], with mutant strains of *Neurospora*, has shown that methionine is synthesized from cysteine *via* L(+)cystathionine and homocysteine. LAMPEN, ROEPKE, AND JONES[25] found, with *Esch. coli* that, while homocystine or methionine could support growth in *all* "cystineless" mutants, cystine or cysteine both failed to support growth in some "methionineless" strains. That is to say, in the latter cysteine was primary and that further steps in methionine synthesis were blocked. SIMMONDS[26], however, has reported a cystineless strain in which none of homocysteine, cystathionine and methionine, supported growth. In this instance the back-reaction to give cysteine from homocysteine appears to have been lost also.

References p. 335.

The other miscellaneous compounds tested gave no unexpected results. "Cyclic" choline sulphate behaved exactly as sulphate, the two bisulphite compounds as sulphite, but formosol was not a very good sulphur-source with any of the mutant strains. This last result does not, however, jeopardize the standing of "sulphoxylate" as an intermediate.

The Genic Control of Enzymic Reactions

The heterokaryotic phenomena indicate that at least two, and probably three, genes are implicated in each of the changes, sulphate-sulphite and sulphite-thiosulphate.

The author wishes to thank Professor CHALLENGER and Dr BRISCOE of Leeds for helpful discussion and criticism.

SUMMARY

1. Methods for the isolation of mutant strains with particular biochemical deficiencies are described.

2. "Cystineless" mutants obtained by these techniques have been used in the elucidation of biosynthetic mechanisms. Sulphate appears to be reduced directly to sulphite. It is most probable that sulphite is reduced directly to "sulphoxylate" and does not pass through the metabisulphite-hydrosulphite route. However, it seems that "sulphoxylate" does not combine directly with an organic residue but first dimerized to thiosulphate. The possibility is discussed that cysteine S-sulphonate (serine thiosulphate) may be the next step in the synthesis of cysteine, the most simple hypothesis for cystine synthesis to fit the experimental facts is:

$$H_2SO_4 \xrightarrow{\text{genes 1, 2, 3}} H_2SO_3 \xrightarrow{\text{genes 4, 5, 6}} \underset{\text{hypothetical}}{(H_2SO_2)} \rightarrow H_2S_2O_3 \rightarrow HSO_3.S.CH_2.CH(NH_2)COOH \rightarrow$$
$$\rightarrow \underset{\text{cysteine}}{HS.CH'.CH(NH_2)COOH}$$

3. None of the possible breakdown routes of cystine tested appear to be reversible. The possible products of such breakdowns by: a) oxidation of the sulphur atom stepwise to the sulphonic acid; b) reductive deamination followed by a); c) decarboxylation followed by a); appear to yield sulphite in presence of the mould.

4. Cystine can be formed from methionine or homocystine without prior breakdown to sulphite.

5. The conversion of sulphate to sulphite and that of sulphite to thiosulphate appear in each case to be governed by at least two and probably three genes.

RÉSUMÉ

1. On décrit deux moyens pour isoler des souches mutantes à déficiences biochimiques spécifiques.

2. On s'est servi de telles souches mutantes "sans-cystine" obtenues par ces moyens-ci pour éclaircir des mécanismes biosynthétiques. Il semble que le sulphate est réduit au sulphite sans intermédiare. Il est très probable que le sulphite est réduit directement au "sulphoxylate", et ne passe pas par le métabisulphite et l'hydrosulphite au thiosulphate. Néanmoins, il semble que le "sulphoxylate ne se joint pas à un résidu organique directement, mais, au contraire, il se dimérise pour former le thiosulphate. Il se peut que le cystéine S-sulphonate (thiosulphate de sérine) soit la phase prochaine de la synthèse de la cystine.

L'hypothèse la plus simple qui se rapporte aux faits expérimentaux est:

$$H_2SO_4 \xrightarrow{\text{gènes 1, 2, 3}} H_2SO_3 \xrightarrow{\text{gènes 4, 5, 6}} \underset{\text{hypothétique}}{(H_2SO')} \rightarrow H_2S_2O_3 \rightarrow HSO_3.S.CH_2.CH(NH_2)COOH \rightarrow$$
$$\rightarrow \underset{\text{cystéine} \rightarrow \text{cystine}}{HS.CH_2.CH(NH_2)COOH}$$

3. Aucune voie de décomposition de la cystine qui a été étudié ne semble être réversible. Les produits possibles d'une telle décomposition par: a) l'oxydation graduelle de l'atome de soufre jusqu'à l'acide sulphonique; b) la déamination réductive qui est suivie d'(a); c) la décarboxylation qui est suivie d'(a), donnent tous l'acide sulphureux en présence du champignon.

References p. 335.

4. La cystine peut se former à partir de la méthionine ou de l'homocystine sans décomposition préalable au sulphite.

5. Les symbioses hétérokaryotiques indiquent que deux déterminants (gènes) au moins (plus probablement trois) prennent part dans chaque transformation, sulphate-sulphite et sulphite-thiosulphate.

ZUSAMMENFASSUNG

1. Es werden zwei Methoden beschrieben, um mutierende Stämme mit besonderen biochemischen Mängeln zu trennen.

2. Auf diese Weise isolierte "cystinlose" Stämme wurden zur Aufklärung von biosynthetischen Mechanismen herangezogen. Es scheint, dass Sulfat direkt zu Sulfit reduziert wird. Sulfit wird sehr wahrscheinlich direkt zum "Sulfoxylat" reduziert, und nicht über Metabisulfit und Hydrosulfit zum Thiosulfat. Es scheint aber, dass sich das "Sulfoxylat" nicht direkt mit organischen Resten verbindet, sondern sich zuerst zum Thiosulfat dimerisiert. Möglicherweise ist das Cystein-S-sulfonat (Serinthiosulfat) die nächste Stufe in der Synthese des Cysteins.

Die einfachste Hypothese für die Cystinsynthese die mit den Versuchsergebnissen übereinstimmt, ist die Folgende:

$$H_2SO_4 \xrightarrow{\text{Gene 1, 2, 3}} H_2SO_3 \xrightarrow{\text{Gene 4, 5, 6}} \underset{\text{hypotetisch}}{(H_2SO_2)} \rightarrow H_2S_2O_2 \rightarrow HSO_3.S.CH_2.CH(NH_2)COOH \rightarrow$$

$$\rightarrow \underset{\text{Cystein}}{HS.CH_2.CH(NH_2)COOH}$$

3. Es scheint, dass keiner der möglichen Abbaumechanismen, die der Verfasser untersuchte, reversibel sei. Es zeigte sich, dass ein solcher Abbau a) durch stufenweise Oxydation des Schwefelatoms zur Sulfonsäure b) durch reduzierende Desaminierung gefolgt von a) oder c) durch Decarboxylierung gefolgt von a) in Gegenwart des Schimmelpilzes Sulfit gibt.

4. Cystin kann aus Methionin oder Homocystin ohne vorhergehenden Abbau zu Sulfit entstehen.

5. Die Umwandlungen von Sulfat zu Sulfit und von Sulfit zu Thiosulfat scheinen in jedem Falle von mindenstens zwei, wahrscheinlich aber von drei Erbfaktoren bestimmt zu werden.

REFERENCES

1 R. A. STEINBERG, *J. Agr. Research*, 63 (1941) 109.
2 D. J. D. HOCKENHULL, *Biochem. J.*, in press.
3 A. RIPPEL AND G. BEHR, *Arch. Mikrobiol.*, 7 (1936) 584.
4 D. BONNER, *Cold Spring Harbor Symposia Quant. Biol.*, XI (1946) 14.
5 D. J. D. HOCKENHULL, *Nature*, 161 (1948) 100.
6 M. A. STAHLMANN AND J. F. STAUFFER, *Science*, 106 (1947) 32.
7 N. FRIES, *Nature*, 159 (1947) 199.
8 M. S. KHARASCH AND H. C. BROWN, *J. Am Chem. Soc.*, 62 (1940) 925.
9 L. C. CHEYNEY AND R. J. PIENING, *J. Am. Chem. Soc.*, 67 (1945) 731.
10 W. G. CHRISTIANSEN AND M. A. DOLLIVER, *U. S. Patent*, 2, 242, 236.
11 E. J. MILLS AND M. T. BOGERT, *J. Am. Chem. Soc.*, 62 (1940) 1173 and 63 (1941) 2363.
12 E. FRIEDMANN, *J. Biol. Chem.*, 94 (1931) 550.
13 T. F. LAVINE, *J. Biol. Chem.*, 113 (1936) 580 and 589.
14 H. T. CLARKE *J. Biol. Chem.*, 97 (1932) 235.
15 G. PONTECORVO, *Cold Spring Harbor Symposia Quant. Biol.*, XI (1946) 193.
16 G. PONTECORVO AND A. R. GEMMELL, *Nature*, 154 (1944) 514.
17 G. PONTECORVO AND A. R. GEMMELL, *Nature*, 154 (1944) 532.
18 W. H. ZACHARIASEN, *Physiol. Rev.*, 40 (1932) 923.
19 E. JELLINEK, *Z. physik. Chem.*, 93 (1919) 325.
20 F. CHALLENGER AND P. A. BRISCOE, private communication.
21 C. FROMAGEOT AND A. ROYER, *Enzymologia*, 11 (1945) 361.
22 C. FROMAGEOT, F. CHATAGNER, AND B. BERGERET, *Biochim. Biophys. Acta*, 2 (1948) 294.
23 K. MÖTHES, *Planta*, 29 (1936) 67.
24 N. H. HOROWITZ, *J. Biol. Chem.*, 171 (1947) 255.
25 J. O. LAMPEN, R. R. ROEPKE, AND M. J. JONES, *Arch. Biochem.*, 3 (1947) 55.
26 S. SIMMONDS, *J. Biol. Chem.*, 174 (1948) 717.

Received October 23rd, 1948