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Formation of Sulphate, Sulphite and *S*-sulphocysteine by the Fungus *Microsporum gypseum* During Growth on Cystine

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ABSTRACT. The dermatophyte *Microsporum gypseum* was cultivated in media containing 0.5% cystine in suspension, and 0.05% peptone or 1% glucose and 0.05% peptone. During growth on cystine the excess sulphur was oxidized and excreted into the medium not only in the form of sulphate but also in the form of sulphite. Sulphite was produced especially during first phases of growth, where its quantity was higher than that of sulphate and its maximum concentration exceeded 1 mg/ml. *S*-sulphocysteine, detected chromatographically and determined quantitatively, originated in large quantities by the reaction of sulphite with cystine in the medium. Both sulphite and *S*-sulphocysteine were further oxidized to sulphate. After exhaustion of cystine 90–93% of the sulphur present was converted to sulphate in the cultivation medium.

Dermatophytes, *i.e.* fungal parasites of the skin of higher vertebrates are able to utilize keratin as the only source of nutrition. Scleroprotein keratin is characterized by a high sulphur content, namely cystine, its quantity reaching up to 18%. Studies of metabolism of cystine in dermatophytes can thus contribute substantially to understanding of their physiology.

Up to now, most studies have been concerned primarily with the suitability of sulphur amino acids as nitrogen sources (Robbins and Ma, 1945; Archibald and Reiss, 1950; Giblett and Henry, 1950; Stockdale, 1953; Fujii, 1957; Bereston *et al.* 1958; Raubitschek, 1962; Horváth, 1966; Ragot, 1966*a*). It was found that free cysteine is very bad and sometimes the worst nitrogen source for dermatophytes. Higher concentrations of cysteine are inhibitory even after addition to media that are otherwise suitable (Robbins and Ma, 1945; Raubitschek, 1962). This effect is attributed to reactivity of the sulphydryl group. Also cystine is considered by most authors as a poor or at best an average source of nutrition. Only Bereston and co-workers (1958) found a good growth of the genus *Microsporum* in a medium containing cystine. A long lag phase is typical for growth on cystine media. The addition of cystine at a concentration higher than 0.2 mg/ml to media with other carbon and nitrogen sources prolongs development of cultures and decreases production of dry weight (Stahl *et al.*, 1949; Ziegler and Reichmann, 1968; Ziegler *et al.*, 1969; Schaper and Ziegler, 1970, 1972).

A relatively poor growth on media containing cystine is not caused by the inability of dermatophytes to metabolize this amino acid. On the contrary, cystine is rapidly utilized even in the presence of more suitable nutrients, and the sulphur contained in it is excreted into the medium in the form of sulphate. The ability of dermato-

phytes to oxidize cystine intensively to sulphate was discovered by Stahl *et al.* (1949). Production of sulphate is intensive even during growth on keratin containing protein-bonded cystine (Ragot, 1966*b*, 1968, 1969; Ziegler and Reichmann, 1968; Ziegler *et al.*, 1969; Clerivet, 1971). The final conversion of the excess sulphur to sulphate is complete, other products (*e.g.* hydrogen sulphide) were not detected. The oxidation of cystine to sulphate in dermatophytes is not well understood at present. Suggested biochemical mechanisms (Schaper and Ziegler, 1970, 1972) are based mainly on data concerning the oxidation of sulphur compounds in other organisms.

Our preliminary results showed that the dermatophyte *Microsporum gypseum* can grow on media containing cystine as the only source of nitrogen, or even as the only source of nutrition. In addition to sulphate, considerable quantities of sulphite were found in filtrates of such cultures. Chromatographic studies then showed that the cultivation liquid contains in addition to cystine and cysteine another ninhydrin positive compound. Therefore, we considered it useful to extend these experiments, aiming to identify products of the oxidation of cystine excreted during growth into the medium.

MATERIALS AND METHODS

Fungal strain and inoculum. The strain MG 155 of the dermatophyte *Microsporum gypseum* (Bodin) Guiart et Grigoraki 1928 from the Czechoslovak Collection of Microorganisms, dermatophytes section, at the Department of Biology, Medical School, Palacký University, was used throughout. Spores from the surface of 10-day cultures on the Sabouraud glucose-peptone agar cultivated at 26°C served for preparation of inoculum. The spores were suspended in physiological saline by shaking with glass beads and filtered through a sterile gauze. The density of the suspension was measured in a haemocytometer and set by dilution to 10^8 spores per ml.

Preparation of media and cultivation. In order to prepare a sterile suspension of cystine a 10% solution of cystine in 1N HCl was prepared on a boiling water bath and the warm solution was pipetted as 2.5 ml portions to sterile Erlenmeyer flasks (250 ml) containing 10 ml of distilled water. About 2.5 ml of 1N NaOH were then added, so that the resulting pH of the solution was roughly 6. The flasks were then left overnight at 4°C. Cystine precipitated in the form of a fine sediment was then washed by decantation under sterile conditions with 50 ml distilled water, 50 ml basic medium and finally suspended in a further 50 ml of this medium.

As a basic medium in the 1st and 2nd series a 0.1N Sörensen phosphate buffer pH 7.5 containing 50 mg $MgCl_2 . 6 H_2O$, 10 mg of $FeCl_3$ and 500 mg of peptone Spofa per 1000 ml was used. In the 3rd series this solution contained also 10 g glucose. The concentrations of peptone and glucose were thus 0.05% and 1% respectively. As shown by additional measurements the final concentration of cystine was on average 0.46% instead of 0.5%, due to incomplete precipitation and losses during decantation. Flasks with 50 ml of the medium were inoculated by adding 1 ml of the spore suspension and incubated at 28°C in the dark. The first and third series were incubated under stationary conditions without additional treatments. In the second series the concentration of peptone was adjusted after 7, 14 and 22 days to the original 0.05% by adding 2 ml of a 1.25% peptone solution. Always 4 flasks of each series were taken at regular intervals, mycelium and cystine particles were separated by filtration and the cultivation liquid was used for analytical measurements.

Analytical methods. Sulphate in the filtrates was determined by a modified turbidimetric method according to Chopra (1964). pH of the resulting solution was decreased to 1.5 to avoid errors introduced by precipitation of barium sulphite. Sulphite was determined colorimetrically by means of a fuchsin-formaldehyde method according to West and Gaeke (1956). The quantity of sulphydryl compounds was measured according to Saville (1958).

A colorimetric method developed in this laboratory was used to determine *S*-sulphocysteine. The method is based on decomposition of this compound with cyanide and the determination of the sulphite released. A more detailed description of this method has already been published (Kunert, 1973). A very similar method was published independently by Gunnison and Palmes (1973).

Chromatographic demonstration of S-sulphocysteine. Filtrates of cultures of the first series, 15, 22 and 30 days old were used. The filtrate was set at pH 4 with acetic acid and both the sulphate and sulphite were removed by adding a solution of barium acetate to a slight excess of Ba^{2+} ions. The precipitate was filtered off and the solution (50 ml) was passed through a Dowex W50 × 8 column (0.9 × 7 cm) in the H^+ form. *S*-sulphocysteine is not retarded by this strongly acidic ion-exchanger whereas practically all other amino acids are adsorbed. The resulting solution was reduced in a rotary vacuum evaporator to about 3 mg/ml of *S*-sulphocysteine. Partition chromatography in thin layers of non-activated silica gel (Silufol^R, 15 × 15 cm; Kavalier Glass Works, Sázava, ČSSR) was used for demonstration. Two µl of the solution containing 5—10 µg of the studied compounds were applied and subjected to ascendent chromatography in chambers saturated with vapours of solvents. The seven following elution systems were used: I. phenol-water (75 : 25, w/w); II. 96% ethanol-concentrated ammonia (7 : 3, v/v); III. 96% ethanol-water (7 : 3, v/v) IV. methanol-pyridine-water (20 : 1 : 5, v/v/v); V. chloroform-methanol-concentrated ammonia-water (10 : 10 : 2 : 1, v/v/v/v); VI. *n*-propanol-concentrated ammonia (7 : 3, v/v); VII. *n*-propanol-water (7 : 3, v/v).

Systems I, II, III, VI and VII were presented by Brenner and Niederwieser (1960), the system IV was used by Luchi and de Marco (1969). *S*-sulphocysteine synthesized according to Milligan and Swan (1962*a*) and cysteic acid (Reanal, Budapest) were always co-chromatographed with the sample. Detection was performed by spraying with 0.2% ethanolic solution of ninhydrin or with a freshly prepared mixture consisting of 50 ml 0.2% ethanolic ninhydrin, 10 ml glacial acetic acid and 2 ml 2,4,6-collidine. The colour developed during 10 min at 90°C. Compounds containing divalent sulphur were detected with aniline and bromine vapours according to Bayfield and co-workers (1965).

RESULTS

The fungus *Microsporum gypseum* grew on all the media used. As it was not possible to separate the mycelium and cystine particles either by filtration or by a flotation method the growth could be evaluated only visually. The growth was poor in the medium containing in addition to cystine only 0.05% peptone (1st series). Only a discontinuous, fluffy aerial mycelium formed at the surface, sporulation was minimal, as well as pigmentation into the medium. A complete dissolution of the suspended cystine was observed after about 28 days of growth. In the 2nd series the growth was slightly more intensive. The amount of the surface mycelium always increased after the addition of peptone. Also the pigmentation was more pronounced (colour of the cultivation liquid yellowish). Cystine particles in cultures dissolved

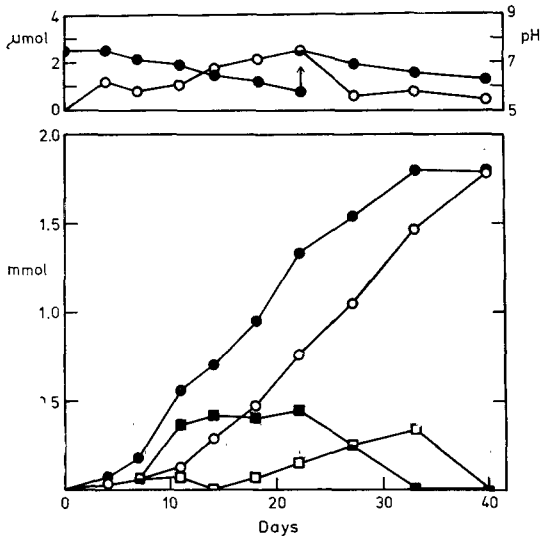

FIG. 1. Growth of *Microsporum gypseum* on a cystine suspension in the presence of 0.05% peptone. Analysis of cultivation liquid. Upper panel: ●, pH; ○, sulphydryl compounds (cysteine). Lower panel, ○ sulphate; □, free sulphite; ■, *S*-sulphocysteine (bound sulphite); ●, sum of all determined sulphur compounds. Concentration of all compounds is expressed in millimoles or micromoles per 50 ml of the medium (1 culture). Initial concentration of cystine was 1.91 mmol/50 ml.

after 22—25 days. The growth was very good in the medium with cystine and glucose (3rd series). Abundant aerial mycelium occurred at the surface after 10 days. The mycelium was ochre and was characterized by an abundant sporulation. Cystine particles at the bottom of the flasks dissolved after about 15 days. After 18 days, indications of autolysis began to appear; the mycelial film became settled and crumbled and the medium turned gradually dirty yellow. In the 1st and 2nd series the autolysis occurred only after 4 weeks and was less intensive.

Main attention was devoted to analysis of the cultivation liquid during development of the cultures. The results are presented in Figs. 1—3. Cystine was metabolized in all series from the very beginning of growth. This is indicated by well demonstrated quantities of sulphate in the medium occurring already after 4 days. The amount of sulphate as the final product of oxidation of the cystine sulphur then further increased during growth. At the end of the experiment sulphate was practically the only compound of the studied sulphur components in the medium. This held true for all series. Its concentration reached almost 5 mg/ml of the medium and the total amount corresponded to a 93.0, 92.8 and 90.4% conversion of the cystine sulphur to sulphate in the 1st, 2nd and 3rd series, respectively. The increase of concentration was relatively continuous thus indicating that an inhibitory effect of the accumulated sulphate on a further oxidation of cystine can be excluded. In the 2nd and 3rd series a slight decrease of a determinable amount of sulphate in the medium could be detected during the autolytic phase.

Also the lower oxidation product — sulphite — was excreted into the medium during growth of all cultures. In the cultures without glucose (series 1 and 2), the free sulphite occurred at a concentration of 0.1—0.2 mg/ml already during the initial phase of growth. Furthermore, it accumulated in all series mainly in the period of exhaustion of cystine when the oxidation terminated. The highest concentration (over 1 mg/ml) was found in the series 3 at the beginning of the autolytic phase. During the final phases of development of the cultures the free sulphite disappeared again relatively rapidly. This process was associated with a corresponding increase of concentration of the sulphate.

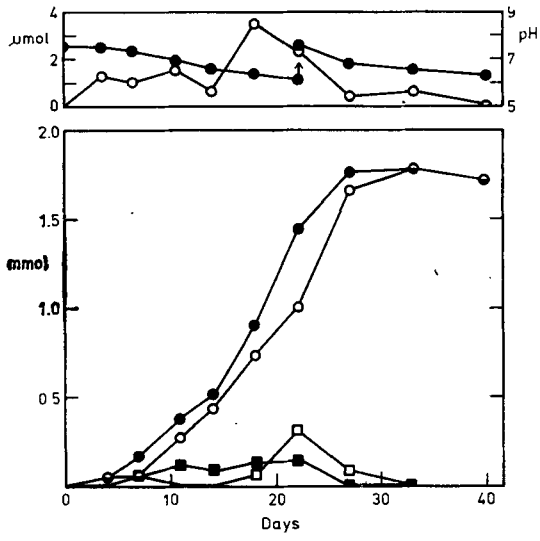

FIG. 2. Growth of *Microsporum gypseum* on a cystine suspension during repeated addition of peptone. Analysis of cultivation liquid. See Fig. 1 for explanation.

Apart from cystine, *S*-sulphocysteine was the main sulphur-containing organic compound detected in the medium. It was determined as sulphite released by cyanide. A chromatographic demonstration of *S*-sulphocysteine performed simultaneously confirmed the specificity of the reaction used. In all series *S*-sulphocysteine was produced during the main growth phase and gradually disappeared when the solid cystine had been exhausted. Large quantities of this compound were detected in the series 1 and especially in the series 3, where the maximum concentration exceeded 1.3 mg/ml. As *S*-sulphocysteine originated most probably by reaction of the excreted sulphite with cystine in the medium (see details in the Discussion) it represents a certain form of a "bound sulphite". When molar concentrations of the free and bound sulphite in culture filtrates are summed up, an assumption can be made that sulphite was excreted in higher quantities than sulphate, primarily during first phases of oxidation of cystine.

The reaction according to Saville (1958) revealed in all series the presence of small amounts of thiols. This reaction was most probably caused by cysteine originating by reaction of sulphite with cystine.

Irrespective of a buffering capacity of the medium, the intensive production of sulphate, sulphite and *S*-sulphocysteine led in all media to a gradual decrease of pH below 6.0. It was hence necessary to increase pH artificially by adding NaOH to older cultures.

Comparison of the development of cultures in individual series (Figs. 1—3) shows that the oxidation of cystine was slowest in the series 1 characterized by the poorest growth. In this very series cystine served as the only source of nutrition (after exhaustion of the small amount of peptone). The production of sulphite was relatively high; during the first phase of growth its molar concentration exceeded that of sulphate by about three times.

In the series 2 the addition of peptone brought about intensification of growth and acceleration of the oxidation of cystine to sulphate. However, the production of the free sulphite and particularly of *S*-sulphocysteine decreased considerably. The molar concentration of sulphite exceeded that of sulphate only at the very beginning of the oxidation.

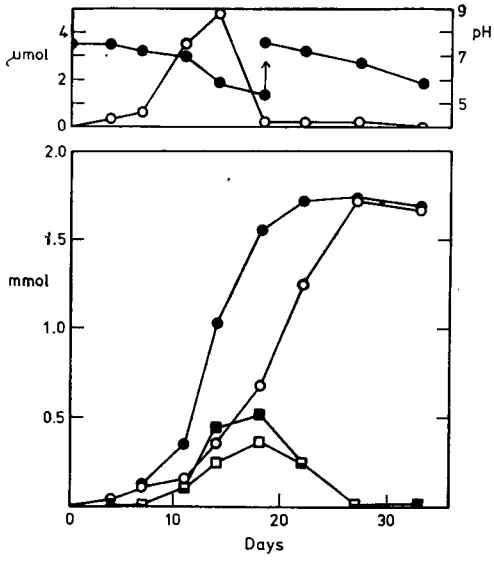

FIG. 3. Growth of *Microsporum gypseum* on a cystine suspension in the presence of 1% glucose and 0.05% peptone. Analysis of cultivation liquid. See Fig. 1 for explanation.

The fastest growth as well as the fastest oxidation of cystine was observed in the medium with glucose (series 3). After a relatively slow beginning, intensive production of both sulphate and sulphite occurred. During the first phase of oxidation, the concentration of the free and "bound" sulphite exceeded concentration of sulphate up to two times. In the series 3 acidification of the medium was most pronounced and the detected concentration of the sulphydryl compounds was the highest.

Filtrates of cultures of the 1st series were analyzed also chromatographically. It was the main aim to identify a strongly acidic ninhydrin-positive compound, the presence of which was demonstrated in preliminary experiments. Only one ninhydrin-positive compound was detected on chromatograms of purified preparations from cultures of different age. The compound had R_F identical with *S*-sulphocysteine in all used elution systems and cochromatographed with it. Both compounds were also similar with respect to detection with ninhydrin yielding a brick red colour. Both compounds also formed a purple spot with ninhydrin and collidine and gave a weakly positive reaction for divalent sulphur. The above-mentioned findings clearly indicate the presence of *S*-sulphocysteine in the studied culture filtrates.

For comparison, cysteic acid, an amino acid closely related with sulphocysteine by its composition, was developed in all systems. Most elution systems were not useful for separation of the two compounds as their R_F values were only slightly different. In systems V (R_F of *S*-sulphocysteine and cysteic acid — 0.13 and 0.08, respectively) and VI (R_F values of 0.15 and 0.08, respectively) the resolution was clear and could be improved even further on repeated development. In addition, cysteic acid differed by a blue-violet colour with ninhydrin and collidine and by a negative reaction for divalent sulphur.

When using culture filtrates that were not purified on ion exchangers, two weaker ninhydrin-positive spots could be observed in cultures 15 and 22 days old, in addition to *S*-sulphocysteine. By co-chromatography with the authentic sample in five elution systems it was possible to identify the more marked substantial spot as

cystine. In filtrates of 30 days old cultures the above-mentioned additional spots could not be detected even when applying 15 μl of the sample.

DISCUSSION

As already mentioned above, free cystine is a poor source of nutrition for dermatophytes. This was confirmed also in this work. The used strain could grow on a plain suspension of cystine in a mineral solution in preliminary experiments; however, the growth was very slow and weak. The presence of small amounts of peptone accelerated substantially the overall development of cultures and improved production of the mycelium. In the presence of glucose good growth was obtained and all cystine was rapidly metabolized. Hence, it can be assumed that the presence of further nutrients in the medium was not a precondition for the utilization of cystine but accelerated it. At the same time, cystine was utilized from the very beginning of growth of the culture, even in the presence of readily accessible sources of nutrition. Also the results of Ziegler and Reichmann (1968) and Ziegler and co-workers (1969) indicate that cystine is metabolized rapidly even in the presence of additional nutrients.

The good growth in the medium with cystine and glucose and the absence of pronounced lag phase in such cultures is in a way contradictory to data of a number of authors, *viz.* that cystine is a poor source of nitrogen (see the Introduction). It is possible that the results presented by them were influenced by a non-controlled acidification of media by products of oxidation of cystine or by accumulation of sulphite at an inhibitory concentration.

The poor growth in media, in which cystine served as almost the only source of nutrition, was not associated with its slow utilization. This was clearly manifested by the rate of disappearance of the cystine suspension as well as by the accumulation of products of its oxidation in the medium. The poor growth of the mycelium is thus caused rather by an uneconomical metabolism of cystine (low economic coefficient of growth). The unsuitability of cystine as a nutritional source is due mainly to its high sulphur content or rather to an unsuitable C : N : S ratio (Stahl *et al.*, 1949; Ziegler and Reichmann, 1968). Similarly to most fungi, dermatophytes get rid of the excess sulphur by its oxidation to sulphate. In agreement with the experiments of Ziegler and co-workers (Ziegler and Reichmann, 1968; Ziegler *et al.*, 1969; Schaper and Ziegler, 1970, 1972) it was found that the used cultures converted more than 90% of the cystine sulphur to sulphate. As the chromatography revealed a complete disappearance of cystine from the medium, the remaining portion of cystine must have apparently been utilized for production of biomass of the mycelium. However, the possibility cannot be excluded that small amounts of unidentified compounds of sulphur remain in the medium. In all series the oxidation of cystine terminated at the beginning of the autolytic phase. After reaching its maximum the concentration of sulphate in the autolytic phase again gradually decreased. This phenomenon was also described in the above-mentioned papers of Ziegler and co-workers, however, a satisfactory explanation of this observation is not available so far.

In all experimental series, sulphite, the lower oxidation product, occurred at least during some phases in amounts comparable roughly with those of sulphate. It is known that in a neutral and alkaline environment sulphite reacts with disulphides

giving origin to a thiol and an *S*-thiosulphate ester (sulphenyl-sulphite, *S*-sulpho compound):

$$R_1{-}SS{-}R_2 + SO_3'' \leftrightarrows R_1{-}S' + R_2{-}SSO_3'$$

The reaction has been studied by a number of authors. The reaction itself, as well as properties of *S*-thiosulphate esters were reviewed by Milligan and Swan (1962*b*). In the cultures used by us, cysteine and a compound most frequently called *S*-sulphocysteine (SSC) should hence originate by reaction of sulphite with cystine. *S*-sulphocysteine was in fact detected chromatographically. The colorimetric determination showed that SSC was present in the medium in all series and almost for the whole time of oxidation of cystine. Sulphite was thus apparently produced for the whole time of growth of the cultures. A relatively highest production of sulphite (as compared with sulphate) was found in young cultures and in cultures with the poorest growth (series 1). In older cultures SSC rapidly disappeared and excretion of sulphate continued at the same time. In addition, sulphite was produced in the series 1. Hence, SSC was also utilized as a source of nutrition and the present sulphur was excreted in the form of sulphate and perhaps also sulphite.

S-sulphocysteine is known as an intracellular intermediate of cysteine biosynthesis in fungi (Roy and Trudinger, 1970). The extracellular production of SSC in fungi was first demonstrated by Matsunaga (1970) in cultures of representatives of *Aspergillaceae* and *Mucorales* growing in a cystine suspension. The author did not study excretion of sulphite; however it appears that even in this case SSC originated by "sulphitolysis" of cystine in the medium.

Cysteine is the other product of reaction of sulphite with cystine. However, only negligible concentrations of cysteine were detected. It is likely that at a given pH and supply of oxygen this compound was rapidly reoxidized to cystine. A rapid oxidation of cysteine originating by sulphitolysis of cystine in the presence of air was described also by Bailey and Cole (1959). However, it is also possible that just this very compound is most rapidly taken up by the mycelium.

As the reaction of cystine and sulphite is very rapid, the question arises, why free sulphite accumulated in the cultures before the available cystine had been exhausted. It is likely that this phenomenon is due to a slow diffusion of compounds in the stationary culture and a slow dissolution of the suspension of cystine, the particles of which are directly covered and isolated by the mycelium. Under these conditions a local exhaustion of cystine can occur. In older cultures the consumption of cystine as well as a decrease of pH of the medium could lead to the accumulation of sulphite. The reaction proceeds at a much slower rate with HSO_3' ions as compared with SO_3''. It thus decelerates already at pH about 6 (Stricks and Kolthoff, 1951). A gradual decrease of the free sulphite is certainly caused also by its aerial oxidation to sulphate. This oxidation may be the predominating factor in old cultures after the exhaustion of cystine.

Almost all products of metabolism of cystine are of strongly acidic nature (also SSC may be compared with mineral acids with respect to acidity — Wolfram and Bruggemans, 1970). The substantial acidification tendency in all cultures is thus not surprising. This tendency was most considerable in the medium with glucose. This fact can be explained by production of organic acids from the excess glucose and especially by a complete exhaustion of the cystine nitrogen. In the series 1 and 2 the excess of amino nitrogen was apparently released as ammonia contributing to the neutralization of acidic products.

The demonstrated extracellular production of sulphite from cystine in the fungus *Microsporum gypseum* is of interest from the point of view of physiology of fungi

To the best of our knowledge this phenomenon has not yet been described in fungi. Several yeasts secrete a small amount of sulphite into the medium (Dittrich and Staudenmayer, 1970); however in this case sulphite is the product of reduction of sulphate. On the other hand, the results of Matsunaga (1970) indicate that oxidation of cystine to sulphite can represent a phenomenon that has not been thoroughly studied so far but can be rather frequent and present not only in dermatophytes or keratinophilic fungi.

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