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Uptake and metabolism of ^{35}S-sulphate by wine yeast

by

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Aufnahme und Umsetzung von ^{35}S-Sulfat durch Weinhefen

Zusammenfassung. — Nach Vergärung eines Substrates mit ^{35}S-Sulfat als einziger Schwefelquelle durch drei Weinhefenstämme — viel H_2S, viel SO_2 und sowohl wenig H_2S als auch SO_2 bildend — waren folgende Stoffwechselprodukte radioaktiv markiert: Methionin, Cystin, Cystathionin, Glutathion und S-Adenosylmethionin (SAM). Zwei weitere nicht identifizierte Komponenten könnten Anlagerungsprodukte von Sulfit mit Zellwandkomponenten darstellen.

Aus der relativen ^{35}S-Aktivität der Stoffwechselprodukte bei den verschiedenen Hefestämmen kann auf eine verminderte Synthese von Methionin und seinen Metaboliten geschlossen werden. Die möglichen Auswirkungen auf die H_2S- und SO_2-Bildung werden diskutiert.

Introduction

There exist a variety of reasons for the production of sulphite and sulphide by yeast during fermentation (see review Eschenbruch 1974, also Eschenbruch *et al.* 1978). Often these compounds are produced as side products of the reduction of sulphate to cysteine and methionine. The production of sulphite or sulphide frequently varies with yeast strain suggesting a genetic basis to this variation in metabolism (Romano *et al.* 1976, Dott *et al.* 1977).

In a series of recent papers, Trüper and co-workers have sought a metabolic explanation for the production of sulphite by some yeast strains. They have found that ATP-sulphurylase is not inhibited by the end product of the assimilatory sulphate reduction pathway (sulphide) in sulphite producing yeasts, while the reverse was found to be true for a low sulphite forming strain (Heinzel and Trüper 1976). They later showed that high sulphite producing strains differed in the regulation and biosynthesis of ATP-sulphurylase and this, taken together with a decreased activity of sulphite reductase in these strains (Dott and Trüper 1976), was taken as a possible explanation for sulphite production (Heinzel and Trüper 1978). The uptake of sulphate by yeast cells was shown to be an active process mediated by sulphate permease (Dott *et al.* 1977). Differences between the modes of repression of this enzyme, particularly by methionine, were suggested to provide a partial explanation for sulphite and sulphide formation by different yeast strains. In their most recent paper (Dott and Trüper 1978) Trüper and his co-workers have shown an increase of NADPH-dependent sulphite reductase during the exponential growth phase of low sulphite producing strains that was not observed in high sulphite producing strains. Various sulphur sources, especially methionine and cysteine, prevented this derepression. The authors believed that this behaviour by the sulphite producing strains must be the reason for the release of large quantities of sulphite during fermentation.

WAINWRIGHT (1970) has shown that the addition of exogenous methionine to a fermentation medium may reduce the ability of a yeast to produce sulphide. On the other hand, the addition of exogenous methionine alters the internal pool of some sulphur metabolites (CHEREST *et al.* 1973), which may cause sulphide production to cease. Some attempts have been made to determine the extent of the sulphur pool of yeast, but often the determinations have been carried out on total (including protein) sulphur (MAW 1965) or individual metabolites (SCHLENK and DE PALMA 1957, MAW 1965, SCHLENK *et al.* 1970, CHEREST *et al.* 1973, WARNER *et al.* 1976).

In order to extend the knowledge already gained in the field of sulphur gas production by wine yeasts, the incorporation of ^{35}S from ^{35}S-sulphate into a variety of sulphur metabolites from three strains of wine yeast was attempted. In this way, it was hoped that possible disturbances to the assimilatory metabolism of sulphur by wine yeasts could be observed.

Materials and methods

Three strains of pure cultured wine yeasts described by ESCHENBRUCH *et al.* (1978) were used: R 93 *(Saccharomyces chevalieri)* was a high sulphide producer, R 104 *(S. cerevisiae)* a high sulphite producer and R 92 *(S. cerevisiae)* formed low levels of sulphide and sulphite. The synthetic fermentation medium was prepared according to TOKUYAMA *et al.* (1973) except that the amount of anhydrous Na_2SO_4 was reduced to 10 mg/l.

Flasks containing 150 ml of medium were inoculated with yeast and their growth at 25 °C was followed by measuring the increase in absorbance at 620 nm. When the upper quartile of the logarithmic phase of growth was reached, 1—3 mCi of ^{35}S-sulphate was added to the medium. At 5 min intervals (up to 45 min) 10 ml aliquots were withdrawn and the yeast suspension centrifuged at 3000 rev/min to form a pellet. The supernatant was discarded and the pellet frozen in dry ice prior to extraction.

The yeast pellet was extracted with 250 µl of 80% aqueous ethanol for 3 min at 80 °C. The resulting suspension was centrifuged at 3000 rev/min for 3 min before 2.5—5 µl of the supernatant was subjected to two-dimensional paper chromatography on Whatman No. 4 paper in phenol/water (80/20 w/v) and then n-butanol/propanoic acid/water (BENSON *et al.* 1950). Radioactive areas on the chromatograms were located by autoradiography. The ^{35}S activity of these areas when cut from the chromatograms was determined by liquid scintillation spectrometry. Identification of ^{35}S-labelled metabolites was by co-chromatography with authentic samples. All radiochemicals were supplied by the Radiochemical Centre, Amersham, England.

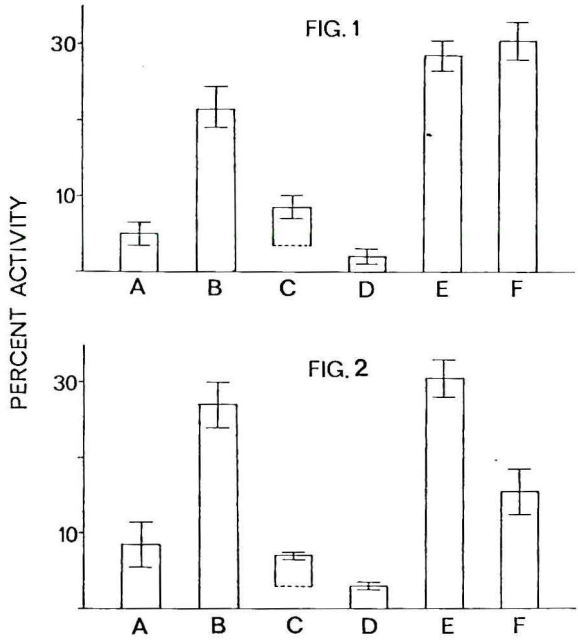
Results and discussion

Qualitatively, there was no difference between the chromatograms of the extracts of each three strains of yeast. The ^{35}S-labelled compounds found labelled were: sulphate, cystathionine, oxidised glutathione, S-adenosylmethionine (SAM), methionine sulphoxide, 5'-methylthioribose sulphoxide, 5'-methylthioadenosine sulphoxide, thiamine and four unidentified compounds. 5'-methylthioribose sulphoxide, 5'-methylthioadenosine sulphoxide and one unknown compound were shown to be degradation products of SAM formed during the extraction and separation processes

(SCHLENK and DE PALMA 1957, WARNER *et al.* 1976). The ^{35}S activity of these compounds was summed and added to that of SAM.

Two of the unknown ^{35}S-labelled compounds (f, g) were thought to have been formed by the addition of $^{35}SO_2$ (obtained from the reduction of the ^{35}S-sulphate by the yeast during the experiment) to yeast cell wall components or to other metabolites. In this laboratory, compounds of similar chromatographic behaviour have been found in extracts of *Sinapis alba* seeds and young *Hordeum vulgare* leaves that had been exposed to $^{35}SO_2$. In the case of *Sinapis* seeds, it was demonstrated that the compounds were formed non-enzymically and were very likely carbohydrate sulphates.

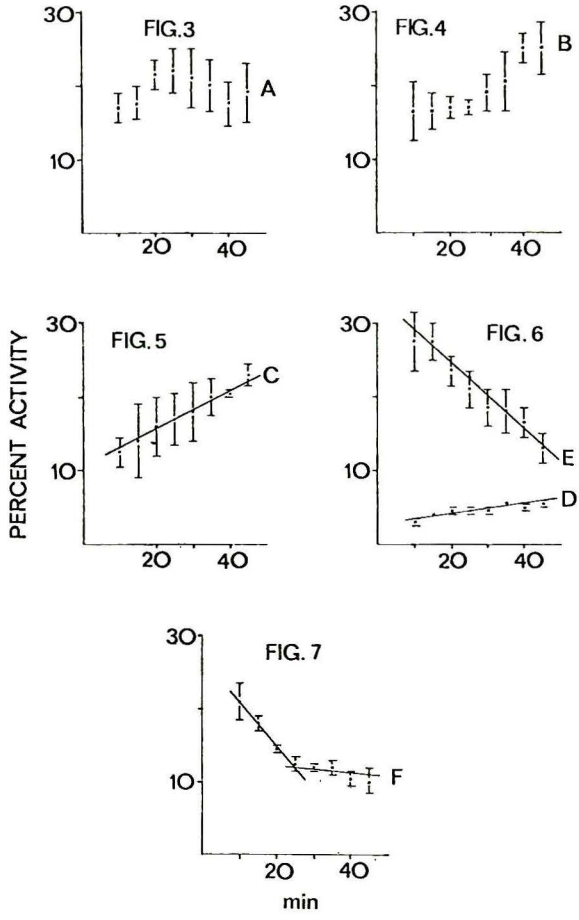
Cystine and glutathione were not always completely resolved on the chromatograms, hence their ^{35}S activities have been summed. ^{35}S-thiamine was found in extracts of all strains but the ^{35}S activity was always less than 1% of the total, hence it was ignored. Homocysteine was not detected on autoradiographs of any of the chromatograms. The percentage distribution of ^{35}S activity of the various sulphur metabolites of yeast R 92 is shown in Fig. 1. All but one of the compounds have a



Figs. 1 and 2: Distribution of ^{35}S in strains R 92 (1) and R 93 (2). As the % distribution of all compounds except „f" remained constant with time, the data are represented as averages of samples from all time intervals investigated. The vertical bars represent standard deviation. — A = cystathionine, B = cystine + glutathione, C = compound f, D = compound g, E = S-adenosylmethionine, F = methionine.

Verteilung von ^{35}S in den Hefenstämmen R 92 (1) und R 93 (2). Da die prozentuale Verteilung aller Komponenten außer „f" über die Versuchszeit konstant blieb, wurden die Werte der einzelnen Zeitintervalle gemittelt. Die senkrechten Linien zeigen die jeweilige Standardabweichung an. — A = Cystathionin, B = Cystin + Glutathion, C = Komponente f, D = Komponente g, E = S-Adenosylmethionin, F = Methionin.

constant percentage activity over the time course of the experiment. Compound f showed a small steady statistically significant increase in ^{35}S activity with time. This rise was not offset by a fall in ^{35}S activity by any other single compound. This suggests that there was an approximate decrease of 1—2% in the ^{35}S activity of two or more of the other compounds, this drop being too small to be detected as statistically significant. In Figs. 1 and 2, this rise in the activity of compound f is represented by a horizontal dotted line as the average % ^{35}S distribution at 5 min after ^{35}S-sulphate addition, and by a solid horizontal line as the average % ^{35}S distribution 45 min after the addition. The results for yeast R 93 are similar to those of R 92 and are illustrated in Fig. 2.



Figs. 3—7: Plots of % ^{35}S activity versus time for ^{35}S-labelled metabolites in yeast R 104. Vertical bars represent standard deviation. — A = cystathionine, B = cystine + glutathione, C = compound f, D = compound g, E = S-adenosylmethionine, F = methionine.

Prozentuale Aktivität ^{35}S-markierter Metabolite des Hefenstammes R 104 in Beziehung zur Versuchsdauer. Senkrechte Linien: Standardabweichung. — A = Cystathionin, B = Cystin + Glutathion, C = Komponente f, D = Komponente g, E =S-Adenosylmethionin, F = Methionin.

With the yeast R 104 a different behaviour of the relative distribution of ^{35}S amongst the metabolites with time was found. These data are illustrated in Figs. 3—7. As with yeasts R 92 and R 93 a significant increase in ^{35}S activity in compound f with time was found (Fig. 5). Compound g also showed a small but significant increase in ^{35}S activity (Fig. 6). The % ^{35}S activity in SAM (Fig. 6) and methionine (Fig. 7) both decreased with time to a greater extent than the increase associated with compounds f and g. It might thus be expected that the % ^{35}S activity of cystathionine and/or cystine + glutathione (Figs. 3 and 4, respectively) would increase. The standard deviations of the points on Figs. 3 and 4 are too large to allow a statistical test of this hypothesis.

That the % ^{35}S activities of SAM and methionine should change with time is surprising. There exists no overt reason for the change. The mass of ^{35}S-sulphate added was negligible, as was the radiation dose associated with the ^{35}S. So the concentration of sulphate in the medium was not changed markedly by the addition of ^{35}S-sulphate and did not change significantly during experimental period of 45 min. The experiments with strain R 104 were identical to those with strains R 92 and R 93 and such changes were not seen in the latter cases. It should be noted that both strains R 104 and R 92 are of the species S. cerevisiae while strain R 93 is *S. chevalieri,* so that it is unlikely that this is a species difference as observed by Dott *et al.* (1977) for active sulphate uptake by different strains of wine yeast species. Any explanation of this phenomenon is clouded by the lack of a statistically significant change in the % ^{35}S activity in cystathionine and cystine/glutathione. An answer must await future experiments on this aspect of the sulphur metabolism of yeast strain R 104.

As a basis for the comparison of the percentage distribution of ^{35}S-activity in the compounds of the three yeast strains, yeast R 92 will be considered to have a "normal" distribution of ^{35}S. This latter strain forms relatively small amounts of sulphite and sulphide under normal conditions so that any significant deviation in ^{35}S distribution in the other two strains may either contribute to their formation of sulphide or sulphite, or be caused by it.

The % ^{35}S in cystathionine shows a small but statistically significant difference between R 92 and R 93. On the other hand, strain R 104 has a much larger % ^{35}S content in cystathionine than either strain R 92 or R 93. The % ^{35}S of SAM in strain R 92 and R 93 are not greatly different but once again strain R 104 shows a different behaviour. In this case, the % ^{35}S activity in SAM in strain R 104 started at a value similiar to those in R 92 and R 93 but dropped by about half during the time of the experiment. With methionine it was found that R 92 maintained a constant % ^{35}S of 31% while the constant value for strain R 93 was 16%. Strain R 104 commenced at 21 % ^{35}S in methionine and dropped to 12 % in 25 min, after which it remained constant too.

As cystine and oxidised glutathione were not always separated on the chromatograms their % ^{35}S activities were summed. Since the sulphur containing moiety of glutathione is a cysteinyl unit, this summing is probably not unreasonable. There were no great differences in the ^{35}S activities of cystine/glutathione between any of the three strains. This result suggests an overall tighter control of the biosynthesis of cysteine compared with methionine in the three yeast strains investigated.

The % ^{35}S distribution of compounds f and g were of interest as it was suspected that these compounds may have been formed from sulphite reacting non-enzymically with yeast metabolites or cell wall fragments. In both R 92 and R 93 the % ^{35}S activities of compound f were low and similar, whereas in strain R 104, the high

sulphite former, it was considerably greater. If higher ^{35}S activities were to be found in compound f in any yeast strain it would be expected to be the strain forming larger quantities of sulphite. Compound g showed a similar behaviour.

In summary, it may be stated that the only major difference in the % ^{35}S distribution between strains R 92 and R 93 was the considerably lower proportion of ^{35}S in methionine in strain R 93. It is of importance to again point out that the strains showing the most similar ^{35}S distributions are two derived from different species and that more differences were seen between strains of the same species i. e. *Saccharomyces cerevisiae* strains R 92 and R 104. These differences were:

a) A higher average % ^{35}S in cystathionine in R 104;
b) a higher % ^{35}S in compound f in R 104;
c) a decreasing % ^{35}S activity in SAM in R 104 while it remained constant with time in R 92; and
d) a decreasing % ^{35}S activity in methionine in R 104 compared to a constant value in R 92.

A frequently cited cause for sulphide production in yeast is methionine deficiency (WAINWRIGHT 1971). It is known that the addition of methionine to a fermentation medium inhibits high sulphide formation by yeast (WAINWRIGHT 1970). Also, deficiencies of the vitamins pantothenate and pyridoxine are said to cause methionine deficiencies with subsequent sulphide formation (MAW 1965, WAINWRIGHT 1970). It is possible that the reason for sulphide production by strain R 93 compared to R 92 may have been due to a lower concentration of methionine associated with a lower rate of biosynthesis of that amino acid. Certainly proportionately less ^{35}S from ^{35}S-sulphate reaches methionine in strain R 93 compared to strain R 92. It should however be noted that both yeast strains were growing in adequate supplies of pantothenate and pyridoxine so that, unless strain R 93 was unable to utilise these vitamins, the lower proportion of ^{35}S in methionine must have been due to other causes.

CHEREST *et al.* (1971) and DOTT and TRÜPER (1978) have shown that yeast sulphite reductase is sensitive to methionine repression. The latter workers found that sulphite reductases of normal strains were derepressed only during the logarithmic phase of growth and repressed again on reaching the stationary phase, possibly by methionine. It is possible that in the sulphide producing strain R 93, the concentration of methionine is sufficiently low to cause little or no repression in the stationary phase and could thus lead to sulphide formation.

The relationship of the present results to the production of sulphite by strain R 104 appears to be more complex. When a yeast is grown on a medium containing excess methionine, some enzymes in the sulphate reduction and amino acid production pathways are repressed. ATP sulphurylase is notable amongst these (CHEREST *et al.* 1971, HEINZEL and TRÜPER 1978). In the sulphate reduction pathway this enzyme is involved in the first step of the activation of sulphate by adenylation, hence any repression of ATP sulphurylase might also affect the production of sulphite. SAM and, via it, methionine show a definite repression of ATP sulphurylase (CHEREST *et al.* 1973, BRETON and SURDIN-KERJAN 1977, HEINZEL and TRÜPER 1978). It should be noted that strain R 104 showed a decrease in % ^{35}S in SAM and methionine with time. This might reasonably suggest that the concentration of these two metabolites within the yeast cells are decreasing with time in strain R 104.

Such a decrease could lead to derepression of ATP sulphurylase and, hence, increased production of sulphite via the activated intermediates of the sulphate assimilatory pathway. It is of interest to note that ESCHENBRUCH and BONISH (1976)

have found an increase in the specific activity of ATP sulphurylase in strain R 104 during fermentation of an artificial must while strain R 92 showed constant specific activity of ATP sulphurylase under the same conditions. It has been postulated (CHEREST *et al.* 1973, BRETON and SURDIN-KERJAN 1977) that methionine, through activated intermediates such as SAM and $tRNA^{met}$, has a regulatory function in the synthesis of four enzymes (group 1 enzymes) involved in methionine biosynthesis, namely, homoserine-o-transacetylase, homocysteine synthetase, ATP sulphurylase and sulphite reductase. These authors further suggested that the synthesis of these enzymes is co-ordinated. This would imply that any derepression of ATP sulphurylase by a decrease in methionine and, especially in SAM concentration, would also result in a derepression of sulphite reductase. This latter effect would then enable the extra sulphite produced due to increasing ATP sulphurylase activity to be reduced to sulphide and probably incorporated into cysteine and methionine. HEINZEL and TRÜPER (1978) have recently shown that in a high sulphite producing strain of wine yeast the group 1 enzymes are not under co-ordinated control by methionine. DOTT and TRÜPER (1978) have also shown that in high sulphite producing strains sulphite reductase has a low activity. The data presented in the present paper suggest that a decreasing incorporation of ^{35}S in SAM could be an indicator of decreasing SAM concentration in the yeast, leading to derepression of ATP sulphurylase and the ultimate production of more sulphite than the sulphite reductase, with its resulting low activity, is able to further metabolise. The final result is an accumulation of sulphite.

There exist many strains of yeast that produce sulphite or sulphide. Their behaviour is usually attributed to a genetic mutation of some kind. The purpose of this investigation was to gain some insight into possible metabolic factors leading to sulphide and sulphite formation by yeast. An increased understanding could possibly lead to methods for controlling the excretion of these undesirable metabolites into wine. The data presented here suggest that strain R 93 produces sulphide due to a low efficiency in reducing sulphate to be incorporated into methionine, while strain R 104 produces sulphite due to a low efficiency in the incorporation of sulphur into SAM. Presumably, these defects could be corrected by adding methionine to the fermenting must. CHEREST *et al.* (1973) have shown that addition of exogenous methionine increases the internal pool of SAM. Further, ESCHENBRUCH (1978) has demonstrated that the addition of methionine to media being fermented by strains R 93 and R 104 causes a decrease in the production sulphide and sulphite respectively. Such an addition however may cause yeast to form other organoleptically undesirable sulphur compounds and, specifically in the case of strain R 104, also lead to the production of significant amounts of sulphide.

Summary

The ^{35}S-labelled metabolites obtained by growing three strains of wine yeast in a medium with ^{35}S-sulphate as the sole source of sulphur were methionine, cystine, cystathionine, glutathione and S-adenosylmethionine. Two further compounds were separated and thought to be adducts of sulphite associated non-metabolically with cell wall components.

The yeast strains were chosen to represent high sulphide formation, high sulphite formation and a combination of low sulphite and sulphide formation. Comparison of the relative ^{35}S activities of the compounds formed by each strain suggested that

sulphide production could be explained in terms of a lowered rate of synthesis of methionine and its activated metabolites leading to lowered control over the production of sulphite reductase. Lowered SAM and methionine production allowing derepression of ATP sulphurylase, together with the reported low activity of sulphite reductase in sulphite producing yeast, could be an explanation for sulphite production.

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