JOURNAL OF

THE

INDIAN INSTITUTE OF SCIENCE

VOLUME 44 OCTOBER 1962 NUMBER 4

STUDIES ON SOIL BACTERIA DECOMPOSING GLYCEROL

Part II: Metabolism of *Streptomyces* and *Azotobacter* species

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Received on July 26, 1962

ABSTRACT

Streptomyces produce acetylmethylcarbinol under certain conditions. Glycerol is metabolised by *Streptomyces* via the Embden-Meyerhoff-Parnas scheme as well as some other route. *Azotobacter* species fix nitrogen with glycerol as the sole source of carbon. 0.001 *M* sodium arsenite and 0.1 *M* sodium fluoride completely inhibit both glycerol decomposition and nitrogen fixation in both the strains studied whereas 0.025 *M* fluoride has a variable effect on the two processes in two different strains. Similarly 0.0001 *M* sodium azide and 0.0005 *M* 2-4 dinitrophenol do not inhibit glycerol decomposition while their effect on nitrogen fixation was different on two strains. Cell-free extracts of *Azotobacter* require Mg^{++} and *ATP* for activity and these oxidised glycerol but no oxygen uptake was obtained with α-glycerophosphate and glycolate. 0.001 *M* and 0.0001 *M* sodium azide and 0.001 *M* arsenite had no effect on oxygen uptake by cell-free extracts. Results with sodium fluoride were variable.

INTRODUCTION

That glycerol could serve as a suitable carbon source for actinomyces has been known since decades. Nevertheless nothing noteworthy seems to have been reported on the glycerol metabolism of actinomyces in general and/or *Streptomyces* in particular. In the work under report some experiments on the glycerol utilisation by *Streptomyces* and the effect of sodium fluoride thereon have been detailed.

Surprisingly only scanty information on the glycerol metabolism of *Azotobacter* was witnessed in the literature notwithstanding the fact that this

genus has in other respects been a subject of extensive investigations. Attempts were therefore made to isolate *Azotobacter* strains from nitrogen-free enrichments set with glycerol, instead of mannitol, as sole source of carbon and energy[1], and the isolated strains were studied with respect to their power to utilise glycerol to fix nitrogen. In order to gain an insight into the mechanism of glycerol utilisation in relation to nitrogen fixation, the effect of inhibitors on these processes was studied. The effect of inhibitors on oxygen uptake by cell-free extracts was also undertaken and the results of all these investigations are presented in this paper.

MATERIALS AND METHODS

Streptomycete Culture:—The medium employed for growth studies consisted of the following: mineral salt solution 100 ml, a nitrogen source, either ammonium sulphate, glutamic acid or asparagine (the concentrations used are mentioned in each experiment), Difco yeast extract—0.01 *g*, glycerol—1 *g*, and bromocresol purple solution—1 ml (wherever necessary).

The circular paper chromatographic technique of Giri *et al.*[2] was used for detecting organic acids in *Streptomyces* culture filtrates.

Azotobacter culture:—Autoclaved solutions of sodium fluoride, 2-4 dinitrophenol and sodium azide were prepared in hundred times the required strength in distilled water and were added aseptically to media previously sterilised. Sodium arsenite solution was used after Sietz filtration. The preparation of inhibitor solutions for manometric experiments as well as the techniques followed for inoculation, incubation and glycerol estimation have been detailed elsewhere[3].

Nitrogen was estimated by the micro-Kjeldahl method. The cell-free extracts of *Azotobacter* were prepared by alumina grinding according to the technique of McIllwain.[4] The protein content of cell-free extract was determined by the biuret method and extract containing 6-8 mg protein was used in each Warburg flask. The following cofactors were added to the cell-free extract in each Warburg flask: Coenzyme A—20 μg, Adenosine triphosphate (ATP)—5 μ moles, Diphosphopyridine nucleotide (DPN)—100 μg, Triphosphopyridine nucleotide (TPN)—20 μg. One mg of magnesium chloride was also added to each reaction mixture. The manometric studies were carried out according to conventional methods[5] for studying oxygen uptake.

RESULTS AND DISCUSSION

Five species of *Streptomyces* were examined for their power to decompose glycerol and for the products they produced when ammonium sulphate (0.05%) was the nitrogen source in the medium of initial pH 7.0, and incubated at

room temperature (20-25 C) for two weeks. All the 5 species, *viz.*, *S. pluricolor*, *S. cacaoi*, *S. longissimus*, *S. bikiniensis* and *S. noursei* decomposed glycerol only to an extent of about 10 per cent but the final pH reached was 3.8 in all cases except in *S. noursei* in which it was 5.7. It was observed that further decomposition of glycerol was prevented by the low pH reached. It was further observed that all species of *Streptomyces* tested produced small amounts of acetylmethyl carbinol (amc). It should be mentioned here that amc production was repeatedly tested and found to give variable results. It is of interest to mention that its formation from glucose in the presence of arsenite has recently been reported by Musilek *et al.*[6,7] However, the full significance of its formation has not been elucidated. It is likely that in the present instance glycerol is broken down to pyruvate and therefrom amc results.

Paper chromatography of the culture filtrates of the above experiment showed 3 bands, one of them having the same R_f value as that of oxalic acid. Six other species similarly examined *viz.*, *S. faciculus*, *S. gadenensis*, *S. aureofaciens*, *S. californicus*, *S. longisporus* and *S. albus*, gave bands in identical position suggesting thereby that most species of *Streptomyces* tested could metabolise glycerol to similar acid products which tend to arrest further utilisation of the substrate.

The results of the effect of sodium fluoride on glycerol decomposition and the resultant fluctuations in pH are recorded in Table I. Asparagine (0.05 %)

TABLE I

Effect of sodium fluoride on glycerol decomposition

Species with lab No.	*pH* changes		% glycerol decomposed
	Initial	Final	
S. longissimus C. F.-6	6.8	4.6	34
,, +0.025 M NaF	6.7	6.1	32
S. longisporus C. F.-21	6.8	5.2	38
,, +0.025 M NaF	6.7	6.3	22
S. aureofaciens C. F.-38	6.9	5.2	16
,, +0.025 M NaF	6 8	6.3	17

was the nitrogen source used since organic nitrogen was found to be a more suitable nitrogen source than an inorganic source such as ammonium sulphate. The incubation was for two weeks at room temperature, unshaken.

It was interesting to observe that in strain CF-6 (Table I) addition of sodium fluoride enhanced growth and pigmentation as observed visually. However, it had the effect of preventing acid formation in all cases since the pH of the media had not fallen to the level it did in cultures without the inhibitor. The production of amc was clearly observed in the *S. longissimus* cultures without NaF while only a trace was detected in its presence.

Since 0.025 M NaF had no inhibitory effect on glycerol decomposition (except in case of *S. longisporus*) it was considered possible that in addition to the Embden-Meyerhoff-Parnas pathway some other mechanism of glycerol breakdown was also functioning in these species of *Streptomyces*. The latter explanation appears to be plausible in that a trace of amc was detected in the presence of NaF. The suppression of amc formation in *S. longissimus* by fluoride would indicate the normal working of the phosphorylative Embden-Meyer-Parnas scheme. Mitra and Ray[8] have shown the enzymes of glycolytic and hexose monophosphate shunt in *S. olivaceus*.

METABOLISM OF GLYCEROL BY AZOTOBACTER

Generally speaking nitrogen fixation was observed to be more or less commensurate with glycerol utilization though it was not so in all the cases studied (Table II).

TABLE II

No.	% glycerol decomposed	*mg* nitrogen fixed 100 ml. medium
C6a	93	3.997
C_{11}	83	3.917
C_4	59	1.908
C_5	30	1.654
11a	27	1.47
C_3	21	1.137
C9 b	73	1.087
C9 a	46	0.94
C6	60	0.91

It would appear that different strains vary in their relative efficiency of glycerol utilisation and nitrogen fixation and this a finding consistent with the

available reports on the organism. A word of caution has however to be sounded on the whole question of nitrogen fixation and this pertains, more to the methods of estimating nitrogen than doubts on the ability or otherwise of the organism concerned. The limitations of estimating nitrogen by the Kjeldahl method have been pertinently expressed by Wilson[9]. However, only the first two clauses of the above limitations are applicable in our tests and we had no other means of estimating the nitrogen fixed by these organism. The majority of the strains, it may be noted, could fix nitrogen in excess of 10 μg/ml medium. Hence there is *a priori* reason to conclude that our strains possess the power to fix nitrogen. Nitrogen fixation has also been recorded in the presence and absence of 2-4 dinitrophenol and sodium azide both of which contain nitrogen (*vide*, supra). The values of nitrogen fixed were estimated as between 30-50 μg/ml. which are far above the minimum values set by Wilson[9] and which at the same time satisfy the objection in clause 2.

A minor modification was made in the digestion procedure for cultures. The wet digestion method was observed to cause excessive frothing and to avoid the possible loss of material from Kjeldahl flasks, large aliquots of culture were evaporated to dryness prior to digestion. After evaporation the material was transferred to Kjeldahl flasks. This method avoided frothing and has been reported to be satisfactory by Gadgil and Bhide[10].

Taking into consideration the physiological nature of *Azotobacter* it may be surmised that glycerol can serve as an important carbon source in its isolation from soil and as such deserves further attention from the point of view of studies relating to their population in soils and other environs.

TABLE III

The effect of inhibitors on glycerol decomposition and nitrogen fixation

Inhibitor	% glycerol utilised		*mg* nitrogen fixed 100 ml. medium	
	Strain C6a	Strain C4	Strain C6a	Strain C4
Nil	100	100	4.446	3.917
0.025 M NaF	64	96	3.45	5.76
0.1 M NaF	NG	NG	Nil	Nil
0.0001 M NaN_3	100	100	2.752	3.244
0.0005 M 2-4 DNP	100	100	2.156	5.526
0.001 M arsenite	NG	NG	Nil	Nil

Initial glycerol added—1%. NG—no growth.

Since arsenite could inhibit growth completely it would suggest that all pathways of entry into the tricarboxylic cycle pass through pyruvate.

In strain C_4, 0.025 M NaF could neither inhibit glycerol decomposition nor nitrogen fixation. The latter activity was in fact enhanced. In C6a both glycerol utilisation and nitrogen fixation were suppressed to a slight extent by NaF. Both sodium azide and 2-4 DNP had the effect of suppressing fixation in strain C6a, however these had no effect on glycerol decomposition.

In strain C_4 it is interesting to note that sodium azide could suppress nitrogen fixation only to a small extent whereas 2-4 DNP actually had a stimulatory effect for which no explanation can be offered at the moment, the nitrogen in the inhibitor being too little to account for the increase. None of these inhibitors had any effect on glycerol decomposition in strain C_4.

That sodium fluoride, regarded as a strong inhibitor of glycolysis (enolase) and azide and 2-4 DNP both established uncouplers of oxidative phosphorylation should in the concentrations tried, allow metabolism of glycerol in these strains, suggests operation of some other route than the glycolytic pathway and that triphosphate synthesis may not be essential to drive the mechanism of nitrogen fixation. Arsenate, on the other hand proved to be a powerful uncoupler of phosphorylation, could strongly inhibit nitrogen fixation by whole cells of *B. polymyxa* when pyruvate was the carbon source and this led Grau and Wilson[11] to conclude that high energy phosphate may be involved in the process of fixation or alternatively in the absence of phosphorylation, uncontrolled pyruvate oxidation saturates the nitrogen acceptor site with H so that nitrogen cannot be absorbed.

Rose and Ochoa,[12] while studying oxidative phosphorylation with cell free extracts of *Azotobacter vinelandii* in the presence of 2-4 DNP, also observed 80 per cent inhibition of inorganic phosphorus uptake at pH 7.0. They had suggested that in *Azotobacter*, unlike in animal mitochondria, oxidation of substrate is independent of the presence of the phosphate acceptor; in other words, phosphorylation was largely uncoupled with oxidative processes. Hartman *et al.*[13] also found oxidative phosphorylation in cell free extracts of *Azotobacter* to be resistant to 2-4 DNP.

The few manometric experiments carried out with cell-free extracts of strain C6a isolated from glycerol also showed that glycerol was oxidisable by cell-free extracts but not glycolate and α-glycerophosphate. Sodium azide in a concentration of 10^{-4} and 10^{-3}M did not effect oxygen uptake with glycerol as the substrate. Likewise 10^{-3}M arsenite had very little effect on oxidation of glycerol but 10^{-2}M arsenite suppressed oxygen uptake significantly. The results recorded with sodium fluoride as the inhibitor were somewhat variable inasmuch as the extracts were extremely labile and inactive in the absence of ATP and Mg^{++}.

The mechanism of nitrogen fixation has by and large remained an unsolved mystery in spite of considerable efforts made by different groups of workers. The demonstration of nitrogen fixation by cell free extracts of *Clostridium pasteurianum* and other organisms[14] marks the beginning of the solution to the problem. The significance of the very high oxygen uptake and the consequentially high respiratory quotient observed in *Azotobacter*, though not understood, has been a subject of such discussion. Though the present investigation was not aimed at elucidating the mechanism of nitrogen fixation, from the little that has been attempted on the cell-free extracts of *Azotobactor*, one feels that what is probably happening is that the vigorous oxidation of the carbon source results in the simultaneous reduction of atmospheric nitrogen, the two reactions being coupled.

ACKNOWLEDGEMENT

The authors thank Dr. S. Bhagavantam, Director, Indian Institute of Science for his keen interest.

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