ARTMAN, M. (1956). *J. gen. Microbiol.* **14**, 315–322

The Production of Hydrogen Sulphide from Thiosulphate by *Escherichia coli*

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SUMMARY: Suspensions of non-proliferating *Escherichia coli* produced H_2S from thiosulphate in the presence of pyruvate or acetaldehyde. Production of H_2S was slight in the presence of α-ketoglutarate and α-ketobutyrate. Organic acids such as malate, fumarate, succinate, lactate, formate and acetate, aldehydes other than acetaldehyde and monohydric alcohols either had no effect or inhibited H_2S production from thiosulphate. H_2S was not produced from sulphite, bisulphite or sulphate, either in the presence or in the absence of the above-named compounds. Crude cell-free extracts of *Escherichia coli* produced H_2S from thiosulphate in the presence of pyruvate. Experiments with dialysed extracts showed that inorganic phosphate, Mg ions and cocarboxylase were essential for H_2S production. Treatment of the extracts with anion exchange resin revealed that in addition, coenzyme A was indispensable for H_2S production from thiosulphate. The addition of DPN to extracts dialysed or treated with anion exchange resin did not influence H_2S production to a marked degree.

Suspensions of non-proliferating cells of various species of Enterobacteriaceae produce H_2S in the presence of such sulphur-containing compounds as cysteine and thiosulphate. The work carried out in this field by many authors has been recently reviewed by Clarke (1953) and by Olitzki (1954). While quantitative differences in the intensity of H_2S production by different micro-organisms are well known, no data exist which explain the mechanism of their H_2S production from inorganic sulphur compounds. The purpose of the present study was to examine the factors governing H_2S production from thiosulphate by non-proliferating organisms and cell-free extracts of *Escherichia coli*.

METHODS

Preparation of suspensions of non-proliferating organisms

Escherichia coli strain B/r was used throughout. The organisms were grown on Difco nutrient agar in Roux bottles. The 18 hr. growth was washed off with distilled water, washed twice in phosphate buffer (pH 6·8) and the suspension brought to the required density as measured in the Coleman Junior spectrophotometer at a wavelength of 550 mμ.

Preparation of cell-free extracts. The organisms were grown as described above. After harvesting and centrifugation they were washed in distilled water and subjected to sonic vibration for 15–20 min. in a Raytheon Magnetostriction Oscillator and then centrifuged at 10,000 r.p.m. for 10 min. The cell-free supernatant solution was used in all experiments. Protein was determined by the

biuret method of Mehl (1945), using a Coleman Junior spectrophotometer at 540 mμ.

Treatment with anion exchange resin. The anion exchange resin 'Amberlite' Ira 410 was charged with 4 % (w/v) sodium chloride until the pH of the effluent was neutral. The resin was then washed with distilled water until no chloride could be detected in the effluent. The resin was added directly to the extracts in equal volume, the suspension stirred for 15 min., centrifuged and the supernatant finally filtered to remove the last traces of resin. This treatment removed coenzyme A as judged by arsenolysis (Stadtman, Novelli & Lipmann, 1951). The cocarboxylase was removed by the same procedure.

Experimental procedure. Sodium thiosulphate was brought in contact with a cell suspension or cell-free extracts in the presence of sodium phosphate buffer (pH 6·8) and incubated at 37° in small test tubes closed with a rubber stopper to prevent any losses of H_2S. After the required incubation, the reaction was stopped by the addition of two drops of 20 % sodium hydroxide and sulphide determined by the method of Delwiche (1951).

RESULTS

Optimal conditions for H_2S *production from thiosulphate.* Suspensions of non-proliferating organisms were incubated with different amounts of sodium thiosulphate and the quantities of H_2S produced were determined after 2 and 24 hr. of incubation. Under the specified conditions the optimum amount of thiosulphate was about 800 μmole (Table 1). The optimum pH was of the order of 6·8 (Fig. 1).

Enhancement of H_2S *production by glucose.* Since Braun & Silberstein (1942) reported that fermentable carbohydrates enhance the production of H_2S from inorganic sulphur compounds by growing organisms, this effect was tested by using non-proliferating cells. Glucose markedly enhanced H_2S production from thiosulphate (Fig. 2), but of several fermentable carbohydrates tested only glucose increased H_2S production, and this to a marked degree (Table 2).

The influence of inhibitors of glycolysis on H_2S *production from thiosulphate.* To ascertain whether glucose itself or some intermediary product arising from glucose by glycolysis was responsible for the enhancing effect in H_2S production, various known inhibitors of glycolysis were tested. These experiments showed that the various inhibitors reduced or completely arrested H_2S production (Table 3). The almost complete inhibition of H_2S production from thiosulphate by bisulphite, both in the presence and absence of glucose, suggested that the enhancing effect of glucose on H_2S production was due to pyruvic acid or some lower intermediary compound produced in the course of glycolysis, since bisulphite is known to give addition compounds with aldehydes and some ketones. The production of H_2S from thiosulphate by non-proliferating cells in the absence of glucose may thus be explained by the presence of endogenic substrate. Further evidence that without pyruvate there was no H_2S production was obtained in studies with cell-free extracts. Experiments carried out with pyruvic acid showed that this compound strongly stimulated

Table 1. H_2S *production from thiosulphate by non-proliferating* Escherichia coli

Bacterial suspension (organisms/ml.)	Thiosulphate (μmole.)	Incubation (hr.)	Total H_2S produced (μmole)
$2{\cdot}5 \times 10^8$	800	2	0
$2{\cdot}5 \times 10^8$	800	24	0
$2{\cdot}5 \times 10^8$	500	2	0
$2{\cdot}5 \times 10^8$	500	24	0
$2{\cdot}5 \times 10^8$	200	2	0
$2{\cdot}5 \times 10^8$	200	24	0
$2{\cdot}5 \times 10^8$	100	2	0
$2{\cdot}5 \times 10^8$	100	24	0
$2{\cdot}5 \times 10^9$	800	2	0·20
$2{\cdot}5 \times 10^9$	800	24	0·60
$2{\cdot}5 \times 10^9$	500	2	0·15
$2{\cdot}5 \times 10^9$	500	24	0·45
$2{\cdot}5 \times 10^9$	200	2	0·10
$2{\cdot}5 \times 10^9$	200	24	0·40
$2{\cdot}5 \times 10^9$	100	2	0·10
$2{\cdot}5 \times 10^9$	100	24	0·40
$1{\cdot}25 \times 10^{10}$	800	2	0·30
$1{\cdot}25 \times 10^{10}$	800	24	2·00
$1{\cdot}25 \times 10^{10}$	500	2	0·20
$1{\cdot}25 \times 10^{10}$	500	24	1·20
$1{\cdot}25 \times 10^{10}$	200	2	0·11
$1{\cdot}25 \times 10^{10}$	200	24	1·00
$1{\cdot}25 \times 10^{10}$	100	2	0·11
$1{\cdot}25 \times 10^{10}$	100	24	0·70

Each tube contained: sodium phosphate buffer (pH 6·8), 100 μmole; cells and thiosulphate as indicated; total vol., 4 ml.

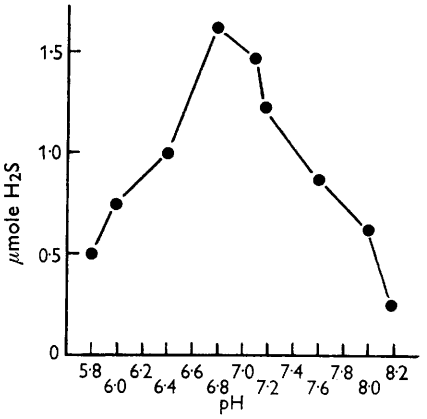


Fig. 1

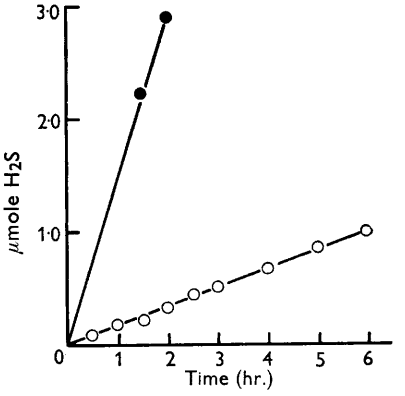


Fig. 2

Fig. 1. Effect of pH value on H_2S production from thiosulphate by non-proliferating *Escherichia coli*. Organism concentration $1{\cdot}25 \times 10^{10}$/ml.; thiosulphate, 800 μmole; sodium phosphate buffer of various pH values, 100 μmole; final vol., 4 ml.; time of incubation, 4 hr.

Fig. 2. The effect of glucose on H_2S production from thiosulphate by non-proliferating *Escherichia coli*. The reaction mixture contained: glucose, when added, 100 μmole; thiosulphate, 800 μmole; sodium phosphate buffer (pH 6·8), 100 μmole; organism concentration $1{\cdot}25 \times 10^{10}$; final vol., 4 ml. –○–○–, without glucose; –●–●–, with glucose.

H_2S production and the magnitude of this effect was equal to that obtained with equimolar glucose. As expected, the production of H_2S in the presence of pyruvate was not affected by fluoride, in contrast to the results obtained with glucose.

Table 2. *Effect of various fermentable carbohydrates on* H_2S *production by non-proliferating* Escherichia coli

Carbohydrates (100 μmole)	H_2S produced (μmole)
Glucose	2·70
Maltose	0·27
Lactose	0·30
Sucrose	0·33
Galactose	0·33
Xylose	0·27
None	0·33

Each vessel contained: carbohydrates as indicated; thiosulphate, 800 μmole; sodium phosphate buffer (pH 6·8), 100 μmole; organism concentration, $1{\cdot}25 \times 10^{10}$/ml.; final vol., 4 ml. H_2S determined after 2 hr. at 37°.

Table 3. *The effect of glycolysis inhibitors on* H_2S *production from thiosulphate in the presence and absence of glucose*

		Glucose present		Glucose absent	
Inhibitors	Concentrations (M)	H_2S (μmole)	Inhibition (%)	H_2S (μmole)	Inhibition (%)
Na bisulphite	0·01	0·10	96	0	100
Na bisulphite	0·001	0·36	89	0	100
Na fluoride	0·01	0·33	90	0·40	—
Na fluoride	0·001	0·73	78	0·40	—
Na iodoacetate	0·01	0·20	94	0·20	50
Na iodoacetate	0·001	0·30	91	0·20	50
None		3·20	—	0·40	—

Each reaction mixture contained: thiosulphate, 800 μmole; Na phosphate buffer (pH 6·8), 100 μmole; glucose, when present, 100 μmole; inhibitors as indicated. Concentration of organisms, 25×10^{10}/ml.; total vol. 44 ml. Sulphide determined after 3 hr.

The effect of various organic acids and alcohols on H_2S *production from thiosulphate.* Results of the above experiments suggested that H_2S production from thiosulphate is conditioned by the presence of hydrogen donors and thus the effect of adding various hydrogen donors was examined. The results of these experiments (Table 4) showed, surprisingly, that none of the compounds tested enhanced the production of H_2S from thiosulphate. The following organic acids and alcohols were without effect, whether in the presence or absence of glucose: succinate, acetate, formate, lactate, citrate, methanol and propanol. Butanol had a marked inhibitory effect. Malate and fumarate inhibited the reaction completely in the absence of glucose. This result may be tentatively explained by the prevention of endogenic pyruvate formation due to the inhibition of oxaloacetate decarboxylation to pyruvate by malate (Ochoa & Weisz-Tabori, 1948). The same explanation is valid in the case of fumarate

since these acids are readily interconvertible. Another interesting observation that arose from the above experiments was that ethanol in the presence of glucose almost doubled H_2S production. Further experiments showed that only ethanol (among other alcohols examined) enhanced H_2S production from thiosulphate, and then only in the presence of glucose or pyruvate. Ethanol alone or in the presence of all the above-mentioned organic acids had no effect.

Table 4. *The effect of various organic acids and alcohols on* H_2S *production from thiosulphate in the presence and absence of glucose*

	H_2S (μmole)	
Organic acids and alcohols	Glucose present	Glucose absent
None	3·00	0·20
Pyruvate	3·70	3·20
Fumarate	3·00	0·00
Malate	2·90	0·00
Acetate	2·90	0·20
Formate	3·00	0·19
Lactate	3·00	0·20
Succinate	2·90	0·20
Citrate	3·00	0·20
α-Ketoglutarate	3·70	0·43
α-Ketobutyrate	3·30	0·53
Methanol	3·00	0·20
Ethanol	6·00	0·30
Propanol	2·30	0·20
Butanol	2·00	0·10

Experimental conditions as given in Table 3. The organic acids were neutralized with sodium hydroxide to pH 6·8 prior to addition. Organic acids and alcohols, when added, 100 μmole. H_2S determined after 3 hr.

If an intermediary compound responsible for H_2S production arises it might give rise to ethanol, and addition of ethanol would then cause accumulation of the intermediary compound and thus enhance H_2S production. Although the fermentation processes in *Escherichia coli* leading from pyruvate to acetaldehyde are not known, these experiments suggest that acetaldehyde may be the intermediary compound responsible for H_2S production from thiosulphate, since acetaldehyde enhanced H_2S production to the same degree as glucose or pyruvate (Table 5). Among the inhibitors of glycolysis only bisulphite inhibited H_2S production. No other aldehydes had any stimulatory effect on H_2S production; on the contrary, higher aldehydes from 4-C exerted an inhibitory effect.

H_2S *production from various inorganic compounds.* Experiments carried out with bisulphite, sulphite and sulphate in the presence and absence of various carbohydrates, organic acids, alcohols and aldehydes, showed no production of H_2S.

H_2S *production from thiosulphate by crude cell-free extracts.* Production of H_2S from thiosulphate by crude extracts took place only in the presence of pyruvate. In the presence of glucose little if any H_2S was produced. There was

no H_2S production from thiosulphate in the presence of various organic acids and alcohols.

H_2S *production by dialysed extracts.* Cell-free extracts after dialysis against distilled water for 18 hr. in a cold room at 5° failed to produce H_2S from thiosulphate in the presence of pyruvate unless Mg ions, cocarboxylase and inorganic phosphate were added. The addition of DPN with the above-mentioned cofactors had no appreciable effect on H_2S production (Table 6).

Table 5. (*a*) *The effect of various aldehydes on* H_2S *production from thiosulphate.* (*b*) *The effect of inhibitors of glycolysis on* H_2S *production from thiosulphate in the presence of acetaldehyde*

Aldehydes	Inhibitors of glycolysis	H_2S (μmole)
(*a*) Formaldehyde	—	0·20
Acetaldehyde	—	2·00
Propylaldehyde	—	0·10
Butylaldehyde	—	0·00
(*b*) Acetaldehyde	Na fluoride 0·01 M	2·30
Acetaldehyde	Na fluoride 0·001 M	2·30
Acetaldehyde	Na bisulphite 0·01 M	0·00
Acetaldehyde	Na bisulphite 0·001 M	0·00

Each test tube contained: thiosulphate, 800μmole; aldehydes, 80μmole; sodium phosphate buffer (pH 6·8), 100μmole; organism concentration $1{\cdot}25 \times 10^{10}$/ml.; inhibitors as indicated; final vol., 4 ml. H_2S determined after 2 hr. at 37°.

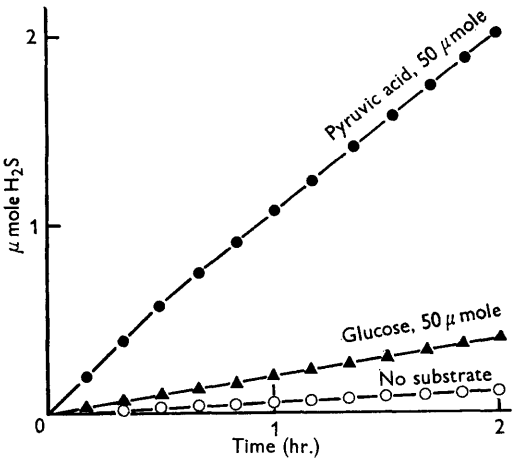


Fig. 3. H_2S production from thiosulphate by crude extracts of *Escherichia coli*. Each test tube contained: extract containing 20 mg. protein; thiosulphate, 300μmole; sodium phosphate buffer (pH 6·8), 100μmole; total vol., 3 ml.

H_2S *production by extracts treated with anion exchange resin.* These extracts, after treatment with anion exchange resin, did not produce H_2S from thiosulphate in the presence of pyruvate unless Mg ions, cocarboxylase, inorganic phosphate and coenzyme A were added. The addition of DPN with the above-mentioned activators did not affect H_2S production appreciably. A typical

experiment is shown in Table 7. In the presence of α-ketoglutarate the results were similar to those obtained with pyruvate, but the amount of H_2S produced was considerably smaller.

Table 6. H_2S *production from thiosulphate by dialysed extracts*

Cofactors	Inorganic phosphate	H_2S (μmole)
Control before dialysis	Added	2·10
None	None	0·00
None	Added	0·10
$MgCl_2$, cocarboxylase	Added	1·80
$MgCl_2$, cocarboxylase, DPN	Added	1·60

Each vessel contained: extracts containing 20 mg. protein; thiosulphate, 300 μmole; pyruvate, 50 μmole; cocarboxylase, when added, 200 μg.; $MgCl_2$, if added, 32 μmole; veronal buffer (pH 6·8), 100 μmole; sodium phosphate (pH 6·8), if added, 100 μmole; DPN, when added, 200 μg.; total vol., 3 ml. H_2S determined after 2 hr. at 37°.

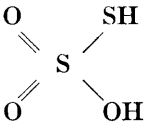
Table 7. H_2S *production from thiosulphate in the presence of pyruvate by extracts treated with anion exchange resin*

Cofactors	Phosphate	H_2S produced (μmole)
None	Added	0·23
$MgCl_2$, cocarboxylase	None	0·29
$MgCl_2$, cocarboxylase, coenzyme A, DPN	None	0·31
$MgCl_2$, cocarboxylase, coenzyme A, DPN	Added	1·61
$MgCl_2$, coenzyme A, cocarboxylase	Added	1·91
$MgCl_2$, coenzyme A	Added	0·69
Untreated control	Added	2·30

Experimental conditions as given in Table 6. Coenzyme A, when added, 30 units.

DISCUSSION

The exact mechanism involved in H_2S production from thiosulphate still remains to be elucidated. The requirement for pyruvate (pyruvate could be replaced to some extent by α-ketoglutarate and α-ketobutyrate, and in non-proliferating cell suspensions by acetaldehyde) as well as for some of the cofactors of the pyruvic dehydrogenase system seems to indicate that H_2S formation from thiosulphate is linked to a reductive step, in which pyruvate serves as an obligate hydrogen donor. The specificity of the hydrogen donor appears remarkable. It will be recalled in this connexion that the oxidation of pyruvate in bacteria requires thioctic acid as a primary hydrogen acceptor (reviewed by Gunsalus, 1953). If the formula of thiosulphate is written as follows:



(cf. Partington, 1953)

the presence of an S—S bond becomes apparent. It is tempting to speculate that the S—S bond confers on the thiosulphate the capacity to function as a primary hydrogen acceptor, alternative to thioctic acid. This interpretation would readily account for the specificity of hydrogen donor, the failure of other inorganic sulphur compounds to produce H_2S, and the finding that the system mediating H_2S production from thiosulphate, in contrast to pyruvic dehydrogenase, does not depend on DPN as an essential cofactor. It will be pointed out that, according to the scheme of Gunsalus (1954), DPN serves to regenerate the S—S linkage of the reduced thioctic acid. In the reaction described by me the acceptor (thiosulphate) is supplied in excess and therefore the regeneration mechanism is obviously not required.

I wish to express my gratitude to Prof. A. L. Olitzki for having suggested to me the subject of the study, and for constant guidance and encouragement throughout this work, which forms part of a study submitted for the degree of Ph.D. to the Senate of the Hebrew University.

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(*Received* 7 *September* 1955)