

## Studies on the Mechanism of Inhibition of Growth of *Vibrio cholerae* by Erythrose

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### SUMMARY

Erythrose strongly inhibits the growth of *Vibrio cholerae* and *Vibrio eltor*. The inhibition can be reversed by washing the cells free from erythrose with sterile normal saline. The respiration of *V. cholerae* cells in the presence of glucose is markedly inhibited by erythrose but the oxygen uptake of cell-free extract under the same conditions is not affected. From the results of experiments on the uptake of [<sup>14</sup>C]glucose and [ $\alpha$ -<sup>14</sup>C]methylglucoside, it may be concluded that erythrose inhibits the transport of glucose across the cell membrane and thereby inhibits the growth of the organism.

### INTRODUCTION

It has been reported (Roy Chowdhury & Datta, 1965) that erythrose has a marked inhibitory effect on the growth of *Vibrio cholerae*, when grown on synthetic medium containing glucose. This growth inhibitory effect of erythrose could be demonstrated even when glucose was replaced by different hexoses or pentoses. The growth inhibitory effect of erythrose was found to be proportional to the concentration of erythrose in the medium; it could also be demonstrated in experiments *in vivo* (Bhattacharya, Roy Chowdhury & Datta, 1965). This paper presents a more detailed biochemical study on the mechanism of this growth-inhibitory effect of erythrose.

### METHODS

*Organisms and media.* *Vibrio cholerae* (Inaba type) or *Vibrio eltor* were grown for 18 h. at 37° on a synthetic medium of Finkelstein & Lankford (1955) containing glucose instead of sucrose, as the sole carbon source. Glucose was added aseptically, according to the concentration needed, as a 50% (w/v) solution sterilized by steaming for three periods of half an hour each. Growth was assayed by measuring the turbidity increase in 18 h. using a Klett Summerson photoelectric colorimeter at 540 nm. For this purpose 50 ml. conical flasks, with optically matched sidearms to fit in the colorimeter, were used.

*Respiration studies.* The vibrios were grown on the synthetic medium and washed by centrifuging three times in cold normal saline (pH 7.8). Oxygen consumption was measured in a Warburg apparatus by the conventional method (Umbreit, Burris & Stauffer, 1959) at 37° using air as the gas phase. The results have been corrected for endogenous respiration.

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*Preparation of cell-free extract.* The vibrios were grown in 500 ml. conical flasks containing 100 ml. of synthetic medium with the requisite amount of glucose, on a rotary shaker at 35°. Organisms from two to three flasks were pooled and washed three times in cold normal saline by centrifugation at 3500g in a Sorvall refrigerated centrifuge. The packed cells were transferred to a chilled mortar with a minimal volume of cold 0.05M-K phosphate buffer (pH 7.4) and homogenized with an equal weight of chilled glass-powder for 1 to 2 min. The mixture was then extracted with a minimal volume of the same buffer, quickly transferred to a chilled centrifuge tube, and the glass powder and cell debris removed by centrifugation at 10,000g for 10 min. The resulting supernatant (cell-free extract) was dialysed for 2 h. against deionized water and used for the respirometric experiments. The whole operation was carried out at 0 to 4°.

*Assay of different enzymes.* Undialysed cell-free extract of *Vibrio cholerae*, prepared as stated above, was used. Hexokinase (Racker, 1947), glucose-6-phosphate dehydrogenase (Kornberg & Horecker, 1955) and phosphohexoseisomerase (Slein, 1955) were assayed by measuring the increase in absorbance at 340 nm. in a Zeiss spectrophotometer, using 1 cm. light-path cuvettes. Protein concentrations were estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) with a bovine serum albumin (Sigma Chemicals) as standard.

*[<sup>14</sup>C]Glucose uptake.* *Vibrio cholerae* was grown overnight in synthetic medium, harvested in a refrigerated centrifuge, washed twice with five volumes of cold normal saline and finally suspended in two volumes of cold normal saline. Usually 0.5 ml. of this cell suspension (0.25 g. organisms, wet wt) was incubated with 550  $\mu$ moles of [<sup>14</sup>C] glucose (36,000 counts/min.) at 37° for 30 min. either in the presence or absence of 50  $\mu$ moles of erythrose. After incubation, the bacteria were washed three times with five volumes of cold normal saline and finally bacteria suspended in 1 ml. of distilled water: 0.5 ml. of this suspension was dried on an aluminium planchet by means of an infrared lamp for measurement of radioactive counts. In addition a suitable sample of the suspension was used for determination of the protein content.

*[ $\alpha$ -<sup>14</sup>C]Methylglucoside.* This radiochemical was prepared according to the method of Bollenback (1963) using uniformly labelled [<sup>14</sup>C]glucose and methanol in the presence of a cation exchange resin.

<sup>14</sup>Carbon counts were measured in a Tracer Lab Super scaler Gas-flow counter, Model SC 18 A.

*Chemicals.* The following were obtained from Sigma: glycolaldehyde, erythrose, glyceraldehyde, sodium pyruvate, DL-glyceraldehyde-3-phosphate (diethyl acetal barium salt), glucose-6-phosphate, 6-phosphogluconate, ATP, NAD, NADP and tris (hydroxymethylaminomethane). Analar (British Drug Houses, Poole, England) glucose, 2,4-dinitrophenol (DNP) and erythritol were used in these experiments. Erythrose was obtained from L. Light & Co., Colnbrook, England. D-Threose was kindly supplied by Dr Perlin, and erythrose-4-phosphate was a gift from Professor B. L. Horecker. [<sup>14</sup>C]Glucose (uniformly labelled) was supplied by the Bhabha Atomic Research Centre, India.

## RESULTS

*Inhibition of growth*

When it was observed that five- and six-carbon sugars did not inhibit the growth of *Vibrio cholerae* (Roy Chowdhury & Datta, 1965), sugars of shorter carbon-chain length and their derivatives were tested using the same procedure as described earlier. They were particularly chosen to provide either terminal groups identical with those of erythrose, or only one terminal group similar to erythrose. The compounds tested were threose, erythritol, glyceraldehyde, glyceraldehyde-3-phosphate, erythrose-4-phosphate and glycolaldehyde: all except erythritol inhibited growth (Table 1). The inhibitory compounds have the common characteristic of a free aldehyde group on the first carbon. Erythritol, in contrast to the rest of the compounds, does not have this reducing group on the first carbon and it may be noted that it did not produce any inhibitory effect on the growth of *V. cholerae*. Erythrose similarly inhibited the growth of *V. eltor*, the causative organism of a closely related disease to cholera. Under the same experimental conditions as described in Table 1, the turbidity change due to growth of *V. eltor* with glucose as sole carbon source was 300 whereas in presence of glucose and erythrose the turbidity change was only 20.

Since the experiments *in vivo* (Bhattacharya *et al.* 1965) indicated that erythrose is only vibriostatic and not vibriocidal, an attempt was made to study the same phenomenon *in vitro*. The inhibitory effect of erythrose was found to be a reversible process and could be removed by merely washing the vibrios. When the organisms were incubated in synthetic medium containing erythrose, no turbidity increase occurred in 18 h. If these inhibited vibrios were centrifuged, washed aseptically three times in sterile normal saline and then transferred to fresh medium containing glucose, growth occurred during the next 18 h. The increase in turbidity was similar to that of organisms which had not been exposed to erythrose. This alternate inhibition and restoration of growth after washing could be repeated at least three successive times with the same culture.

Growth of *Vibrio cholerae* on peptone broth medium instead of the synthetic medium was not inhibited by erythrose, and the addition of a mixture of 20 amino acids to the synthetic medium completely prevented inhibition by erythrose. Cysteine alone could reverse the inhibitory effect of erythrose but could not support the growth of the organism when added as sole carbon source (Table 2). Cysteine, by itself, has very little effect on the growth of *V. cholerae*.

The growth-inhibitory effect was thus specific for the aldehyde group at the carbon-one position of the sugar molecule; it was not bactericidal and could be removed by washing the organisms.

*Inhibition of glucose metabolism*

The glucose respiration of non-dividing *Vibrio cholerae* was almost completely inhibited in the presence of erythrose (Fig. 1). Although there was a small oxygen consumption with erythrose alone as substrate, the oxygen consumption was almost nil when both glucose and erythrose were added together. With cell-free extracts of *V. cholerae* using glucose as substrate, however, there was no appreciable inhibition in the presence of erythrose (Fig. 2). Attempts to abolish the initial lag in the uptake of oxygen by the addition of ATP and almost all the known cofactors were unsuccessful. When

Table 1. *Effect of sugars and sugar derivatives on the growth of Vibrio cholerae*

The increase in turbidity (in Klett units) was determined after 18 h. incubation at 37° in 10 ml. synthetic medium + 550  $\mu$ moles glucose + 50  $\mu$ moles of additional sugar or derivative.

System	Turbidity increase
Glucose	200
Glucose + erythrose	0
Glucose + threose	0
Glucose + glyceraldehyde	0
Glucose + erythrose-4-phosphate	0
Glucose + glyceraldehyde-3-phosphate	0
Glucose + erythritol	195

Table 2. *Effect of cysteine on the growth inhibitory effect of erythrose on Vibrio cholerae*

Turbidity change (in Klett units) determined after 18 h. at 37° in 10 ml. synthetic medium + 550  $\mu$ moles glucose; cysteine and erythrose (50  $\mu$ moles of each) added as indicated.

System	Turbidity increase
Glucose	198
Glucose + cysteine	210
Glucose + erythrose	0
Glucose + erythrose + cysteine	195
Cysteine	45

6-phosphogluconate and pyruvate, the primary reactants of the hexose monophosphate shunt pathway and the tricarboxylic acid cycle, were used as substrates, oxygen uptake by cell-free extract was also not inhibited by erythrose.

It has been reported by Racker, Klybas & Schramm (1959) and Fluharty & Ballou (1959) that D-threose 2,4-diphosphate strongly inhibits glyceraldehyde-3-phosphate dehydrogenase. Furthermore, Grazi, De Flora & Pontremoli (1960) and subsequently Venkataraman & Racker (1961) have shown that erythrose-4-phosphate strongly inhibits phosphohexose-isomerase. Hence studies were made with cell-free extract of *Vibrio cholerae*, in which the activities of certain enzymes were assayed spectrophotometrically in the presence and absence of erythrose. The activities of hexokinase, glucose-6-phosphate dehydrogenase, phosphohexoseisomerase and glyceraldehyde-3-phosphate dehydrogenase were not inhibited significantly by erythrose.

Inhibition of oxygen consumption of whole cells by erythrose and failure to produce such inhibition in experiments with cell-free extracts led us to investigate the effect of erythrose on the uptake of glucose by whole cells.

#### *Uptake of glucose*

When *Vibrio cholerae* was grown overnight (18 h.) in a synthetic medium in the presence and absence of erythrose together with [<sup>14</sup>C]glucose, parallel effects by erythrose on inhibition of growth and inhibition of [<sup>14</sup>C]glucose uptake were found (Table 3).

Since in this experiment glucose uptake by *Vibrio cholerae* cells was measured during an 18 h. growth period, with the possibility of glucose being metabolized during that long period of incubation, short-term incorporation studies were made using resting

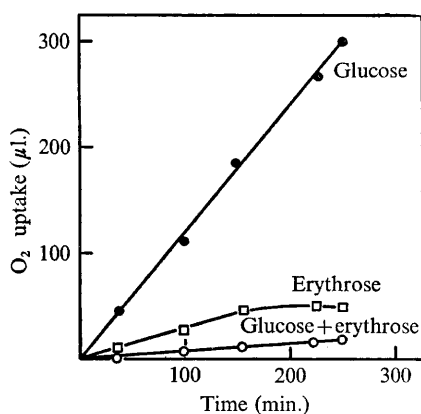


Fig. 1

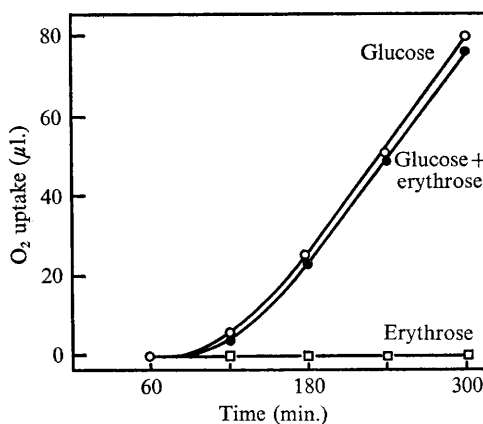


Fig. 2

Fig. 1. Effect of erythrose on the oxygen uptake of non-dividing *Vibrio cholerae*. Final volume 3 ml., containing 150  $\mu$ moles K phosphate buffer, pH 7.4; 10  $\mu$ moles glucose; 1  $\mu$ mole erythrose (where indicated) and 1.0 ml. bacterial suspension (6 mg. protein). The centre well contained 0.15 ml. 30% KOH. Gas phase, air; temp. 37°.

Fig. 2. Effect of erythrose on the oxygen uptake of cell-free extracts of *Vibrio cholerae*. Final volume 3 ml. containing 150  $\mu$ moles K phosphate buffer, pH 7.4; 10  $\mu$ moles glucose; 3  $\mu$ moles ATP; 1  $\mu$ mole erythrose and 1.0 ml. of cell-free extract (4.5 mg. protein). The centre well contained 0.15 ml. of 30% KOH. Gas phase, air; temp. 37°.

Table 3. *Effect of erythrose on [<sup>14</sup>C]glucose uptake during growth of Vibrio cholerae*

Flasks containing 560  $\mu$ moles of unlabelled glucose, 1  $\mu$ Ci of [<sup>14</sup>C]glucose and 50  $\mu$ moles of erythrose (where required) in a final volume of 10 ml. synthetic medium were inoculated with *V. cholerae* and incubated at 37° for 18 h. Growth was measured in terms of increase in turbidity (Klett units). After 18 h. the organisms were washed twice with normal saline and once with distilled water, suspended in 1 ml. of distilled water and 0.5 ml. of this suspension used for counting the radioactivity. Protein was measured on the remaining suspension and the results are expressed as counts/min. mg. protein.

System	Uptake of <sup>14</sup> C		Growth	
	counts/min./ mg. protein	Inhibition (%)	Turbidity increase in 18 h.	Inhibition (%)
Glucose	1012	—	145	—
Glucose + erythrose	150	85	20	86

suspensions of *V. cholerae*. The results in Table 4 confirm that erythrose effectively inhibited the uptake of [<sup>14</sup>C]glucose by *V. cholerae*. It may be recalled (see Table 2) that the inhibitory effect of erythrose on the growth of the organism was reversed by the addition of cysteine. From Table 4 it may be seen that the inhibition of [<sup>14</sup>C]glucose uptake by the *V. cholerae* in the presence of erythrose was also reversed by cysteine which was without effect in the absence of erythrose. Table 4 also shows that the inhibition of glucose uptake by 2,4-dinitrophenol was accentuated by erythrose.

Cohen & Monod (1957) have shown that  $\alpha$ -methylglucoside, normally a non-

metabolizable glucose derivative, is useful in the study of the glucose permeation mechanism in bacteria. When  $\alpha$ -methylglucoside was added to the synthetic medium in place of glucose, it did not support growth of *Vibrio cholerae* indicating that it is not measurably metabolized by the organism. The compound accumulated inside the cell, however, at a linear rate for 2 h. (Fig. 3). Glucose at a concentration of  $10^{-4}$  M considerably inhibited the uptake of [ $\alpha$ - $^{14}$ C]methylglucoside (Fig. 3).

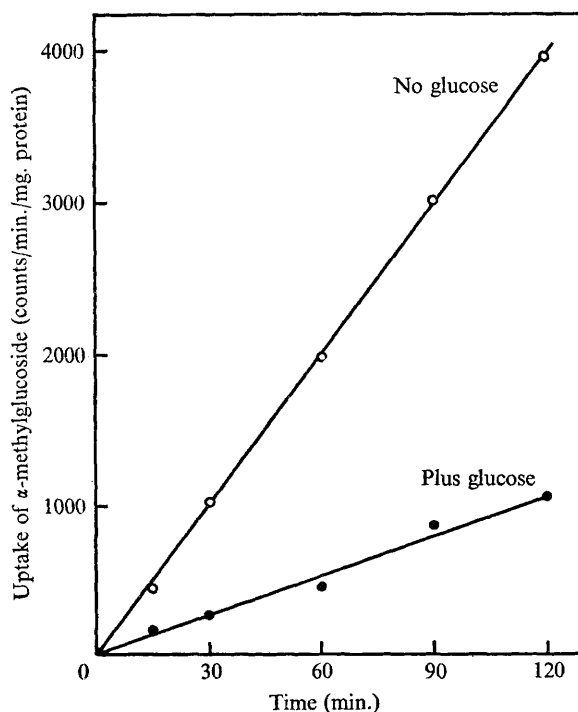


Fig. 3. Effect of glucose on the uptake of [ $\alpha$ - $^{14}$ C]methylglucoside by *Vibrio cholerae*. Incubation mixture contained 0.5 ml. bacterial suspension, 10  $\mu$ moles [ $\alpha$ - $^{14}$ C]methylglucoside (1000 counts/min./ $\mu$ mole) and 0.1  $\mu$ mole glucose in a final volume of 1.0 ml. The reaction mixtures were incubated for different periods of time and uptake measured as Table 3.

Table 4. Effect of erythrose on the uptake of [ $^{14}$ C]glucose by non-growing *Vibrio cholerae*

The incubation mixture contained 550  $\mu$ moles of [ $^{14}$ C]glucose (36,000 total counts/min.) and 0.5 ml. bacterial suspension in a final volume of 10 ml. normal saline with, where indicated, erythrose, 50  $\mu$ moles; 2,4-dinitrophenol, 25  $\mu$ moles; cysteine, 10  $\mu$ moles. After 30 min. incubation at 37° the organisms were centrifuged, washed and treated as described in Table 3.

System	Uptake of $^{14}$ C (counts/min./ mg. protein)
Glucose	3030
Glucose+erythrose	218
Glucose+2,4-dinitrophenol	607
Glucose+2,4-dinitrophenol+erythrose	101
Glucose+cysteine	3010
Glucose+cysteine+erythrose	2800

To check whether [ $\alpha$ - $^{14}\text{C}$ ]methylglucoside was transported intact into the bacteria, *Vibrio cholerae* was exposed to the glucoside and then collected by centrifugation. After washing twice with 5 ml. normal saline the packed organisms were extracted with  $3 \times 5$  ml. portions of hot water. The combined washings were concentrated to about 1.0 ml. and 0.05 ml. samples were chromatographed on Whatman no. 1 filter paper using butanol:acetic acid:water (60:30:10). Radioautograms of these extracts showed a single radioactive spot with an  $R_f$  value identical to that of authentic  $\alpha$ -methylglucoside. Furthermore, 80% of the total radioactivity applied to the paper could be eluted from the  $\alpha$ -methylglucoside region and when co-chromatographed with authentic [ $\alpha$ - $^{14}\text{C}$ ]methylglucoside gave one single radioactive spot. The above observations suggest that  $\alpha$ -methylglucoside was transported into *V. cholerae* as free  $\alpha$ -methylglucoside through the mediation of the permease responsible for the entry of glucose.

Table 5 shows that erythrose inhibited the uptake of [ $\alpha$ - $^{14}\text{C}$ ]methylglucoside by *Vibrio cholerae*. Attempts were made to check whether the inhibition of  $\alpha$ -methylglucoside uptake by erythrose was of competitive nature by plotting the reciprocals

Table 5. *Effect of erythrose on the uptake of [ $\alpha$ - $^{14}\text{C}$ ]methylglucoside by non-growing *Vibrio cholerae**

The incubation mixture contained 0.5 ml. bacterial suspension, 10  $\mu$ moles of [ $\alpha$ - $^{14}\text{C}$ ]methylglucoside (1000 counts/min./ $\mu$ mole) in a final volume of 1.0 ml. normal saline. Erythrose, 1  $\mu$ mole. Incubation for 30 min. at 37°, and then the uptake of  $^{14}\text{C}$  measured as in Table 3.

System	Uptake of $^{14}\text{C}$ (counts/min./ mg. protein)
$\alpha$ -Methylglucoside	1058
$\alpha$ -Methylglucoside + erythrose	328

of the observed uptakes of  $\alpha$ -methylglucoside at different concentrations of the glucoside in the presence and absence of erythrose. Results of these experiments were erratic and no definite conclusion could be made.

#### DISCUSSION

The growth inhibitory effect described was dependent on the presence of a free terminal aldehyde group in a sugar molecule with a chain length of not more than four carbons; the lack of inhibition by pentoses or hexoses may be due to the fact that when in a ring form their aldehyde groups are masked.

The results indicate that erythrose does not inhibit any one of the major pathways of glucose catabolism (e.g. glycolytic, shunt pathway and tricarboxylic acid cycle) in cell-free extracts of *Vibrio cholerae*, whereas it does inhibit very strongly the oxygen uptake of whole organisms with glucose as substrate. This effect is explained by the marked inhibitory effect of erythrose on the uptake of [ $^{14}\text{C}$ ]glucose and [ $\alpha$ - $^{14}\text{C}$ ]methylglucoside by *V. cholerae*, an effect demonstrable even with bacteria in the growth phase. Since transport of substrates across bacterial membranes is believed to be mediated by stereospecific proteins, or 'permeases' (Cohen & Monod, 1957), the growth inhibitory effect of erythrose on *V. cholerae* might be on the 'gluco-permease' system. Such systems are likely to be depressed in the presence of a typical uncoupling agent

like 2,4-dinitrophenol which markedly reduced the glucose uptake by non-growing organisms, and this inhibitory effect was increased by erythrose.

Results presented in Table 3 to 5 strongly suggest the inhibitory effect of erythrose to be on the transport of glucose across the bacterial cell membrane. The concluding experiment, which was done to establish this 'glucose-permease' inhibition by erythrose, was to study the uptake of [ $\alpha$ - $^{14}$ C]methylglucoside in non-growing *Vibrio cholerae*. This glucoside appears to be transported into bacteria by the same 'permease mechanism as is needed for glucose (Cohen & Monod, 1957) and so the rate of uptake of  $\alpha$ -methylglucoside by bacteria can be used as an indirect measure of the 'glucose-permease' activity of an organism. It was very interesting to note that erythrose caused 70% inhibition of the uptake of  $\alpha$ -methylglucoside (Table 5). This inhibition of  $\alpha$ -methylglucoside uptake confirms the mode of inhibition by this four-carbon sugar on *V. cholerae* to be on the permeation of glucose into the organism.

Recently Együd & Szent-Györgyi (1966a) reported that methylglyoxal, a ketoaldehyde, inhibits strongly the growth of *Escherichia coli* as well as the synthesis of protein in the same organism (Együd & Szent-Györgyi, 1966b). The inhibition of protein synthesis by methylglyoxal can be reversed by the addition of cysteine (Együd & Szent-Györgyi, 1966b). Szent-Györgyi, Együd & McLaughlin (1967) suggested that the methylglyoxal inhibition could be due to a stoichiometric reaction between the ketone aldehyde and certain sulphhydryl groups and the reversal by cysteine could be due to regeneration of these sulphhydryl groups. In our system, growth inhibition by erythrose could be due to an interaction between the four-carbon sugar and sulphhydryl groups, of the gluco-permease of *Vibrio cholerae* and the reversal by cysteine could be due to the regeneration of these groups.

In conclusion, it may be said that erythrose primarily inhibits the glucose uptake by *Vibrio cholerae*, which, secondarily, leads to the inhibition of growth of this organism.

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