THE SYNTHESIS OF THE INDUCED ENZYME, "CYANASE", IN *E. COLI*

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SUMMARY

An enzyme, cyanase, which breaks down cyanate to CO_2 and ammonia has been demonstrated in *E. coli*. The enzyme is adaptive and is synthesised in the lag as well as the logarithmic growth phase of the cells. Concentrations that inhibit the growth of *E. coli* permit enzyme synthesis in the lag phase but not in the logarithmic growth phase unless the preliminary growth of the cells occurs in low concentrations of cyanate. The enzyme has been partially purified, and its K_m value, pH sensitivity and stability have been described. The synthesis of the enzyme may be inhibited by various nucleic acid antagonists as well as by the inhibition of protein synthesis. Cyanase activity itself cannot be inhibited by KCN, NaF, and only the activity is depressed by KCNS while the yield remains unaffected. The effect of bacteriophage infection on the synthesis of the enzyme has been described, and it has also been shown that simultaneous synthesis of cyanase and β-galactosidase can take place.

INTRODUCTION

In a preliminary report it was stated[1] that addition of KCNO to the growth medium of *E. coli* prevents growth only for a period. It was also found that when *E. coli* was grown in the presence of low concentrations of cyanate, further addition of higher concentrations of KCNO did not inhibit the growth of the cells. In contrast, bacteria grown without addition of low concentrations of KCNO were inhibited by 0.02 *M* KCNO when it was added during the logarithmic growth phase. These findings suggested that an induced enzyme may be formed in the cells in the presence of KCNO and that this enzyme breaks down KCNO to a harmless product permitting cell growth. After an unusually long lag phase, the length of which depends on the concentrations of cyanate, the bacteria begin to grow.

The purpose of the experiments reported here was to confirm the presence of such an inducible enzyme, to characterize it and to determine the conditions permitting its synthesis.

Abbreviations: DEAE, diethyl aminoethyl; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

METHODS AND MATERIALS

Organisms and growth conditions

E. coli B and B/1,5 and *B. megaterium* were maintained on nutrient agar slopes and were subcultured daily. The cultures were grown with aeration in Difco nutrient broth and the concentration of cells was determined turbidimetrically. The bacteriophage T_1 was prepared by the method of CREASER AND TAUSSIG[2] and assayed as described by ADAMS[3]. Cell free extracts of bacteria were prepared by shaking with glass beads. Protoplasts of *E. coli* were prepared using lysozyme and Versene as described earlier[4], and protoplasts from *B. megaterium* were prepared according to the method of WEIBULL[5]. 5 m*M* phosphate buffer, pH 7.0, containing 3 m*M* $CaCl_2$ was used to wash and suspend the cells.

Chemicals and determinations

All chemicals used were reagent grade. Carbamyl phosphate was prepared according to the method of JONES *et al.*[6], and assayed by its ability to convert ornithine to citrulline in the presence of ornithine transcarbamylase[6]. DEAE was prepared as described by PETERSON AND SOBER[7]. Ammonia was determined according to the method of BRAGANCA *et al.*[8]. β-galactosidase was measured by the method of LEDERBERG[9].

RESULTS

Cyanase determination

The induced enzyme, which will be referred to as cyanase, was determined manometrically. The principle of the determination was as follows: It was observed that when KCNO was incubated in the presence of the enzyme the medium became alkaline. When the incubation was carried out in the presence of a bicarbonate buffer

TABLE I

RELATION BETWEEN CYANATE CONCENTRATION AND CO_2 UPTAKE

Adapted bacterial extracts were the source of enzyme and the results were expressed as μmoles CO_2 uptake when the experiment was allowed to go to completion.

KCNO concentration	*μmoles CO_2 uptake*	*% theoretical*
20 μmoles	15.4	77
15 μmoles	11.5	77
10 μmoles	7.5	75

anaerobically in conventional Warburg vessels using N_2–O_2 (95:5) as the gas phase, CO_2 was taken up. The CO_2 uptake was proportional to the substrate concentration. It can be seen in Table I that when the reaction was allowed to proceed to equilibrium, for each mole of KCNO about 0.75 mole of CO_2 was taken up. The results conform with the following reactions:

$$3H_2O + CNO^- \rightleftharpoons NH_4^+ + OH^- + HCO_3^- \quad (1)$$

$$OH^- + CO_2 \rightleftharpoons HCO_3^- \quad (2)$$

i.e. Total reaction =

$$3H_2O + KCNO + CO_2 \longrightarrow NH_4HCO_3 + KHCO_3 \quad (3)$$

i.e. in an atmosphere of CO_2 there is one mole of CO_2 taken up for each mole of KCNO decomposed.

Consequently, enzyme activity may be measured manometrically by measuring the rate of CO_2 uptake by the alkali produced by KCNO breakdown. There was no spontaneous breakdown of KCNO under the present incubation conditions.

The fact that there is no equivalence of KCNO decomposition and CO_2 uptake may be explained as follows: The alkalinity due to KCNO breakdown appears to be neutralized to about 75% by the CO_2 in the atmosphere, while the rest is not neutralized but is reflected by a change in pH. When more bicarbonate buffer and more CO_2 in the atmosphere was used, the reaction went to a higher percentage of the theoretical and the shift in pH was also less. It is not due to the equilibrium of the reaction ($3H_2O + KCNO \rightarrow NH_4HCO_3 + KOH$) since estimation of the ammonia produced indicated that when extracts of adapted cells were incubated with KCNO, the nitrogen was quantitatively converted to ammonia.

Production of cyanase

E. coli B/1 was grown in the presence and absence of 0.01 *M* KCNO in nutrient broth containing 1 mg/ml glucose to a concentration of about 10^9 cells/ml. After the cells were harvested they were resuspended in phosphate–calcium buffer and the cyanase activity was measured manometrically. It can be seen from Fig. 1 that cells which were grown in the presence of KCNO decomposed cyanate but not those grown in its absence.

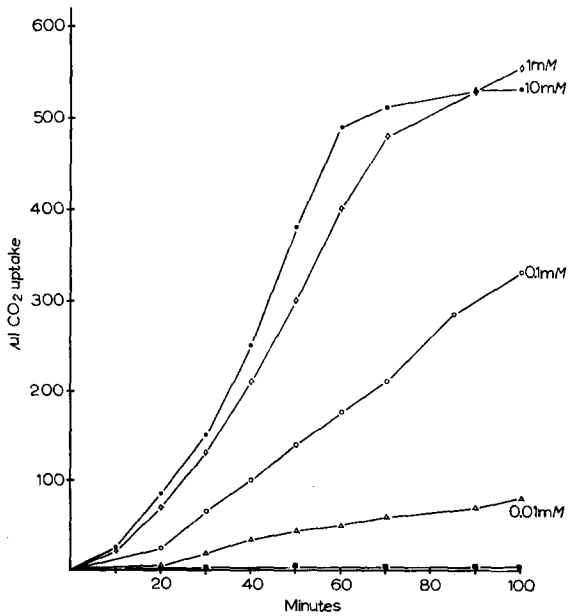

Fig. 1. Breakdown of KCNO by *E. coli* B/1,5 grown in the presence and absence of cyanate. Results expressed as μl CO_2 uptake from 30 μmoles KCNO by 10^{10} cells. Cells grown to 10^9/ml in nutrient broth plus 1 mg/ml glucose and ■—■, Nil; △—△, 0.01 m*M* KCNO; ○—○, 0.1 m*M* KCNO; ◇—◇, 1.0 m*M* KCNO; ●—●, 10.0 m*M* KCNO.

Purification and some properties of cyanase

Attempts to purify and concentrate the enzyme have been made by means of high speed centrifugation of cellular extracts and fractionation of the high speed supernatant on DEAE cellulose. The fractionation was carried out as described earlier[2].

Results of this purification may be seen in Table II.

TABLE II

PURIFICATION OF CYANASE

Fraction	*Enzyme activity/mg protein μmoles CO_2 uptake in 30 min/mg protein*
Crude extract	11
144,000 × *g* supernatant	21
Sediment	0
DEAE: not absorbed	0
0.1 *M* NaCl	0
0.2 *M* NaCl	38
0.3 *M* NaCl	31
0.4 *M* NaCl	2.4
0.5 *M* NaCl	0

The 0.2 *M* NaCl eluted fraction was considered sufficiently pure to determine the K_m value of the enzyme, and Fig. 2 shows that the K_m value for cyanase is $2.9 \cdot 10^{-2}$ *M*. The pH sensitivity of the enzyme is not very great. Although the optimal pH seems to be around 7, activity is quite high at pH 6 as well as at pH 8. The extract keeps well in the frozen state at —20° for weeks, but 1 min boiling destroys the activity completely. Dialysis against distilled water has no effect on the activity of cyanase.

Induction of enzyme synthesis related to growth cycle of bacteria

Experiments were performed to establish the phase of the growth cycle of *E. coli* when enzyme synthesis occurs.

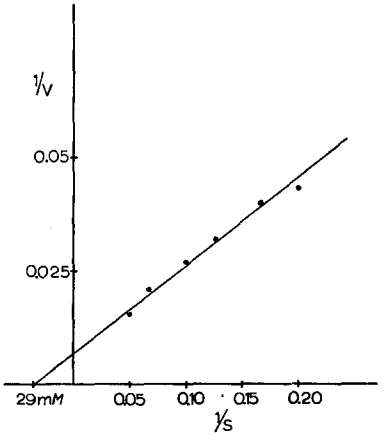

Fig. 2. The effect of substrate concentration on the velocity of cyanate decomposition. 0.2 *M* NaCl eluate from DEAE was used as source of enzyme. The rates are measured as described in text using μl CO_2 uptake in 10 min.

1.5 l of nutrient broth were inoculated with 2-day old *E. coli* B/1,5 slant so that the final concentration of cells was $2.5 \cdot 10^8$ cells/ml. Samples were taken at 30-, 60-, and 90-min intervals. The bacteria were centrifuged, washed, resuspended in phosphate–calcium buffer, and cyanase activity was determined.

The cyanase activity expressed as μmoles CO_2 uptake in 30 min brought about by 10^{11} cells exposed to KCNO for varying periods is shown in Table III.

TABLE III

CYANASE SYNTHESIS IN THE LAG PHASE OF GROWTH BY *E. coli* B/1,5

Bacteria were incubated in presence of 10 m*M* KCNO for the stated time. Activity is expressed as μl CO_2 uptake in 10 min by 30 μmoles KCNO in presence of 10^{11} cells.

Time of exposure to KCNO minutes	*0*	*30*	*60*	*90*
Cell count/ml	$3 \cdot 10^8$	$3.1 \cdot 10^8$	$3.3 \cdot 10^8$	$5 \cdot 10^8$
Cyanase activity	0	102	152	360

It can be seen from Table III that synthesis of cyanase takes place before the bacteria reach the logarithmic growth phase, although it cannot be unequivocally stated that no growth took place during this time.

The rate of cyanase synthesis was determined by allowing 1 m*M* KCNO to be present in cultures of bacteria growing logarithmically for various times. The results of this experiment are shown in Fig. 3. It can be seen that synthesis of cyanase begins immediately and the maximum amount is reached somewhere between 15 and 30 min.

Concentration of substrate necessary for induction

It was also of interest to find out the minimum amount of KCNO that will induce enzyme formation and the maximum amount that will permit cyanase synthesis.

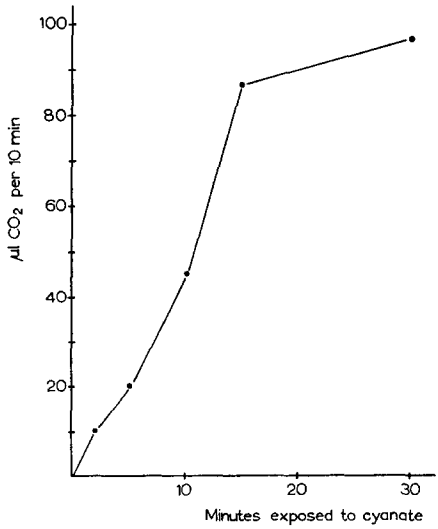

Fig. 3. Rate of cyanase synthesis by *E. coli*. Cells were grown in nutrient broth-glucose to about $5 \cdot 10^8$ cells/ml. KCNO was then added at a concentration of 1 m*M* for increasing periods. Samples were taken at these times, washed and resuspended to a concentration of $1.5 \cdot 10^{10}$ cells/3 ml containing 15 μmoles KCNO.

The results of this experiment are shown in Table IV. It can be seen that 0.01 m*M* KCNO is sufficient to induce synthesis of enzyme and that addition of 20 m*M* KCNO does not permit enzyme synthesis. In all these cases KCNO was added to logarithmically growing *E. coli* B/1,5 and was allowed to act for 15 min. However, it is also shown in Table IV that if the substrate is allowed to be present for not a predetermined period but for as long as the bacteria reach a given cell concentration, the results are strikingly different, suggesting that exhaustion of the substrate results in cessation of further synthesis and consequent dilution of the enzyme occurs. This was further confirmed in an experiment where exposure of *E. coli* to KCNO for various times was followed by removal of KCNO by centrifugation of the cells and resuspension and further growth of the cells in fresh nutrient broth. The results shown in Table V, expressed as enzyme activity per cell, indicate that enzyme activity drops if cells are reincubated in the absence of substrate.

TABLE IV

CYANASE SYNTHESIS IN THE PRESENCE OF VARIOUS AMOUNTS OF KCNO

Bacteria (a) exposed to KCNO for 15 min, or (b) grown in the presence of the stated amounts of KCNO to 10^9 cells/ml. Activity: μl CO_2 uptake from 30 μmoles KCNO in 30 min per 10^{10} cells.

KCNO mM	*0*	*0.01*	*0.1*	*1.0*	*10*	*20*
(a)	0	75	375	258	18	0
(b)	0	22	63	130	155	160

TABLE V

EFFECT OF LENGTH OF EXPOSURE TO CYANATE ON ENZYME SYNTHESIS

Expressed as μl CO_2 uptake in 30 min per 10^{10} cells from 30 μmoles KCNO

Time of exposure to KCNO minutes	*0*	*5*	*10*	*15*
Tested immediately	0	145	276	420
Reincubated 4 hours in absence of substrate	0	56	90	76

Effect of low concentrations of cyanate in the growth medium on enzyme synthesis in the logarithmic growth phase

E. coli B/1,5 was grown in the presence and absence of 1 m*M* KCNO to a concentration of $5 \cdot 10^8$ cells/ml. At this time cyanate was added to a final concentration of 20 m*M* and the bacteria were incubated for a further 20 min. Table VI demonstrates the level of enzyme in the cells at the beginning and end of the experiment as well as the growth of the cells under these conditions, and it can be seen that the cells which were grown in the presence of KCNO were able to grow and synthesize further amounts of the enzyme, while those grown in the absence of cyanate could neither grow nor could they be induced.

TABLE VI

EFFECT OF CYANATE IN THE GROWTH MEDIUM ON SUBSEQUENT CYANASE SYNTHESIS

Cyanate concentration in growth medium	*Amount of enzyme**		E. coli/*ml*		*Factor of increase in growth*
	at 0 time	*at 20 min*	*0 min*	*20 min*	
1 m*M*	6	12	5.9×10^8	7.8×10^8	1.32
Nil	0	0	6.8×10^8	7.1×10^8	1.04

* Amount of enzyme expressed as μmoles CO_2 uptake per 10^{10} cells from 30 μmoles KCNO in 30 min.

Inhibition of enzyme synthesis

In order to gain an insight into the cellular mechanisms necessary for the synthesis of cyanase, various substances that are known to have an effect on protein or nucleic acid synthesis or on the metabolism of the cells were tested for their effect on cyanase production. Table VII illustrates the effect of these substances. Cyanase activity is expressed as μl CO_2 uptake by 10^{10} cells from 30 μmoles KCNO in 30 min. It can be seen from Table VII that chloramphenicol and KCN inhibit the synthesis of the enzyme but the other substances produce complete inhibition only if added prior to the substrate. When the bacteria were grown aerobically and induction took place under anaerobic conditions, or when growth as well as induction took place anaerobically, there was no noticeable diminution in the level of enzyme activity.

TABLE VII

EFFECT OF VARIOUS SUBSTANCES ON CYANASE SYNTHESIS

Substance	*Time of KCNO addition*	*μl CO_2/30 min per 10^{10} cells*	*% inhibition*
Nil	0	485	0
100 μg streptomycin	0	286	41
100 μg streptomycin	30	15	97
20 μg chloramphenicol	0	0	100
20 μg chloramphenicol	30	0	100
10 μg vancomycin	0	384	21
10 μg vancomycin	30	100	80
2 μg mitomycin	0	420	13
2 μg mitomycin	30	160	67
20 μg mitomycin	0	300	38
20 μg mitomycin	30	0	100
100 μg 8-azaguanine	0	480	2
100 μg 8-azaguanine	30	80	84
100 μg 5-bromouracil	0	490	0
100 μg 5-bromouracil	30	68	86
100 μg 5-nitroorotic acid	0	495	0
100 μg 5-nitroorotic acid	30	130	73
100 μg 2-4-dichloropyrimidine	0	400	18
100 μg 2-4-dichloropyrimidine	30	120	75
0.01 *M* KCN	0	25	95
0.01 *M* KCN	30	25	95

1 m*M* KCNO was added to each sample at indicated time and was allowed to be present for 30 min.

The effect of phage on enzyme synthesis

The effect of phage infection on cyanase synthesis is demonstrated in Table VIII. It can be seen in Table VIII that infection with T_1 prevents adaptive enzyme synthesis to a large extent even if added together with the inducer and that there is complete inhibition of synthesis if infection precedes induction. However, Table VIII also shows that while the infection may reduce the amount of enzyme in the cells, if virus is allowed to be present for longer periods it does not abolish the already existing enzyme.

TABLE VIII

EFFECT OF BACTERIOPHAGE T_1 ON CYANASE SYNTHESIS

E. coli B was grown in nutrient broth-glucose to $5 \cdot 10^8$ cells/ml. 0.1 μmoles/ml KCNO was added for 15 min, except when stated differently, then T_1 was added for the stated times in a 10-fold excess. Results expressed as μl CO_2 per $2 \cdot 10^{10}$ cells per 30 min released from 30 μmoles KCNO.

Conditions	*μl CO_2*
No virus, 1 m*M* KCNO added at 0 time (for 10 min)	210
T_1 and 1 m*M* KCNO added at 0 time (for 10 min)	45
T_1 at 0 time, 1 m*M* KCNO 5 min later (KCNO for 10 min)	0
Time of exposure to particles	
0	580
5	570
8	540
10	420
12	309
Lysate	360

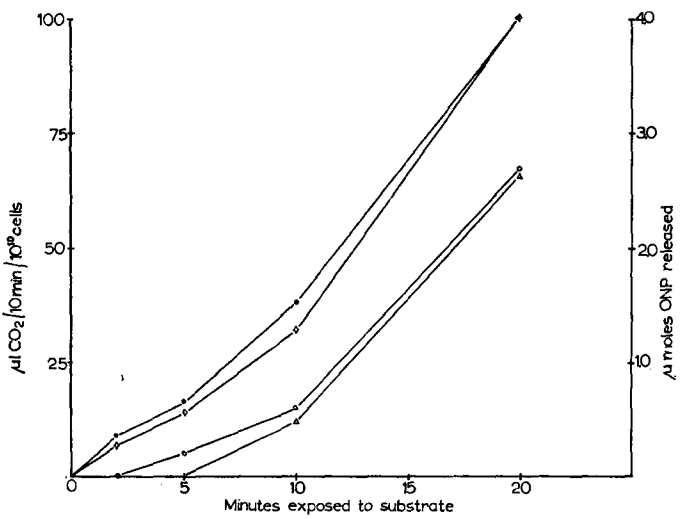

Fig. 4. Simultaneous induction of cyanase and β-galactosidase synthesis in *E. coli*. Cells were grown in nutrient broth and 1 mg/ml glucose to a concentration of $5 \cdot 10^8$ cells/ml. Then flasks were set up to contain 5 mg/ml lactose and/or 1 μmole/ml KCNO for varying periods. Samples were centrifuged washed and resuspended in the appropriate buffer for determinations. Results expressed as μl CO_2 uptake from 30 μmoles KCNO per 10^{10} cells and as μmoles *o*-nitrophenol released from ONPG by 10^9 cells. The cells were exposed to ●—●, 1 m*M* KCNO, and ◇—◇, 1 m*M* KCNO + 5 mg/ml lactose: cyanase activity; ○—○, 5 mg/ml lactose and △—△, 5 mg/ml lactose + 1 m*M* KCNO: β-galactosidase activity.

Simultaneous induction to two substrates

It was of interest to investigate the effect of the simultaneous induction by another substrate, such as lactose, to induce β-galactosidase, on the synthesis of cyanase. Fig. 4 demonstrates that simultaneous exposure to lactose and KCNO has no adverse effect on either of the two enzyme systems and it can be seen that both enzymes are synthesised at a rapid rate.

Specificity

Attempts were made to induce cyanase synthesis by KCNS, but it was not successful. Carbamyl phosphate acted as an inducer; however, this may be due to the fact that low concentrations of KCNO are always present when carbamyl phosphate is added to the growth medium and these low concentrations may be sufficient to induce cyanase synthesis. Cyanase was found to break down carbamyl phosphate although somewhat slower than it broke down cyanate, but had no effect on KCNS.

However, KCNS did reduce cyanase activity and the reduction was proportionate to the KCNS concentration, but the final amounts of both CO_2 uptake and ammonia released from KCNO breakdown were the same. KCN or NaF inhibited neither the rate of cyanate breakdown nor the final amount of KCNO broken down.

Attempts were also made to induce cyanase synthesis in *B. megaterium*, the protoplasts prepared from *B. megaterium* and protoplasts prepared from *E. coli*. In none of these systems was there any evidence of cyanase production.

DISCUSSION

An adaptive enzyme that breaks down the growth inhibitory substance, cyanate, has been demonstrated in *E. coli*. This enzyme behaves similarly to other induced enzymes in that enzyme synthesis stops if the substrate is removed. However, it appears that the bacteria can synthesize the enzyme in the lag phase suggesting that growth is not essential for its synthesis. Further experiments are needed to confirm this point, but it is certain that the synthesis of the enzyme takes place in the lag phase at such concentrations of KCNO that do not permit bacterial growth for as long as 4 h. It is interesting to note that 20 m*M* KCNO did not induce cyanase when added in the logarithmic growth phase while it did when added during the lag phase of *E. coli*. Also, addition of 20 m*M* KCNO to logarithmically growing cells *will* produce cyanase synthesis if the bacteria were grown in the presence of low (1 m*M*) concentrations of cyanate. The reason for this is difficult to explain especially since the organisms grown in the presence of KCNO do not have a preformed enzyme in sufficient quantity to rapidly break down KCNO as it can be seen from b of Table IV or from Table VI. It may be calculated that if the adapted cells were to begin breaking down cyanate at the rate prevailing at the end of 30 min, they would dispose of only about 0.6 μmole of KCNO/30 min from the growth medium still leaving 19.4 μmoles KCNO/ml. These cells can also grow in the presence of 20 m*M* KCNO in contrast to organisms grown in the absence of low concentrations of cyanate.

The rate of synthesis has been found comparable with the rate of synthesis of β-galactosidase, used as an example of another induced enzyme. The concentration of KCNO necessary to bring about induction is quite low, 0.01 m*M* KCNO being

sufficient. Optimal enzyme synthesis occurs in the logarithmic growth phase at concentrations which do not inhibit growth.

The results obtained with the few inhibitors are not unusual. The various structural analogues of purines and pyrimidines were examined in the hope that it would be possible to show the need for either RNA or DNA only for enzyme synthesis. However, it can be seen in Table VI that all the substances tested inhibited to some degree the synthesis of cyanase. It is not surprising that the substances which influence nucleic acid caused complete inhibition of enzyme only if added prior to the inducer, since the synthesis of cyanase begins immediately and it would go on until normal nucleic acid was available. Since it would take some 10 min for, *e.g.*, 8-azaguanine to exert its effect on nucleic acids[10], it is natural that for 10 min or so cyanase synthesis should go on unhindered. In that period, a near maximum amount of cyanase would be synthesized. In the case of the effect of T_1 infection, it is clear that infection before induction will prevent induction, while the results shown in Table VIII suggest that there is no effect on the already formed enzyme until 10–12 min after infection, at which time the enzyme begins to decrease because it is being used for virus protein synthesis. Unfortunately, at present the objection that perhaps at later stages of the infection some lysis of the bacteria results in dilution of the enzyme cannot be answered, although every precaution has been taken in the experiments to prevent this and the results shown were typical.

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