DEGRADATION OF DEOXYRIBOSE BY *E. COLI*. STUDIES WITH CELL-FREE EXTRACT AND ISOLATION OF 2-DEOXY-D-RIBOSE 5-PHOSPHATE

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SUMMARY

The preparation of an extract stable at —10° from deoxyribose-adapted *E. coli* which degrade deoxyribose to diphenylamine negative material is described. The presence in the extract of a deoxyribokinase requiring ATP and Mg^{++} is suggested. Accumulation of a sugar phosphate in the presence of $(NH_4)_2SO_4$ is demonstrated. The evidence indicates it to be deoxyribose 5-phosphate.

INTRODUCTION

Previous papers[1,2] described experiments on the adaptation of *E. coli* to deoxyribose. These showed that resting cells from organisms previously grown in the presence of deoxyribose rapidly degraded it to products which were no longer detectable by the Dische diphenylamine test.

The present paper describes experiments with cell-free extracts prepared from deoxyribose adapted cells. Such extracts degrade the pentose with the intermediate formation of a sugar phosphate identified as deoxyribose 5-phosphate.

MATERIALS AND METHODS

Deoxyribose was prepared by the method of SOWDEN[3]. Adenosine triphosphate was obtained as the barium salt from Light and Co. Ltd., converted into the sodium salt and stored as a frozen 0.1 *M* solution at —10°. Deoxyribose 5-phosphate for reference purposes was prepared by hydrolysis of deoxyadenosine 5'-phosphate. 5 mg was dissolved in 10 ml of water and shaken at room temperature with 1 g Amberlite IR 120 (H) (dry form) for 30 min. The resin was filtered off and washed with 5 ml water. The combined filtrates were neutralized to pH 7.5 with NaOH and finally freeze-dried. Deoxyribose 1-phosphate was prepared* from thymidine by the method of FRIEDKIN AND ROBERTS[4].

E. coli 207/56[2] was used throughout all experiments. The strain was maintained on slopes of the following composition: agar (Difco) 2.67 %, yeast extract (Difco)

The following abbreviations are used: deoxyribose = 2-deoxy-D-ribose; Tris = tris(hydroxymethyl)aminomethane.

* We are grateful to Mr. E. CHRISTIANSEN for preparing deoxyribose 1-phosphate.

References p. 123.

0.1 %, peptone (Difco) 0.1 %, $NH_4H_2PO_4$ 0.07 %, NaCl 0.17 %, glucose 0.13 %. Cells capable of rapid fermentation of deoxyribose were prepared as described earlier[1,2].

Estimations

Deoxyribose and deoxyribose 5-phosphate were estimated by the Dische diphenylamine method[5]. Deoxyribose 5-phosphate in the presence of deoxyribose was determined by a method similar to that described for ribose and ribose 5-phosphate by HEALD AND LONG[6]. A volume of the mixture to be analyzed was added equal volumes of 5 % $ZnSO_4$ and 0.3 *N* $Ba(OH)_2$ respectively. Deoxyribose 5-phosphate was calculated from the total deoxyribose content before and after precipitation with the $ZnSO_4$–$Ba(OH)_2$ reagents.

Paper chromatography

Deoxyribose, deoxyribose 5-phosphate and deoxyribose 1-phosphate were chromatographed on Whatman No. 1 paper by the descending method and the chromatograms developed by the diphenylamine reagent of BUCHANAN *et al.*[7].

Paper electrophoresis

The sugar phosphates were run in a 0.05 *M* phosphate buffer pH 7.4 and a borate buffer (7.44 g boric acid and 4 g NaOH per liter) pH 10 in the apparatus described by FOSTER[8].

Preparation of cell-free extracts

Washed adapted cells in the range of 1–8 g were placed in the steel cylinder of Hughes bacterial press[9] without abrasives added. The press had previously been cooled on solid CO_2. The cells froze rapidly and the press was again placed on solid ice for 15 min. The bacteria were then subjected to sudden blows by the steel piston. Usually the number of blows necessary to achieve complete disintegration of the cells was greater than that reported[9]. Occasionally about 20 % was not forced into the reservoir of the press as easily as the rest of the cells. When the press was left for a few minutes at room temperature, the remaining cells were easily disintegrated after further blows. The extract was removed in a frozen state from the press, ice-cold 0.05 *M* tris buffer pH 7.6 added. It was left in an ice bath with occasional stirring and then subjected to centrifugation at 0° at 1000 *g* for 15 min (crude extract).

Experiments with cell-free extract

Incubation was carried out at 37°. The incubation mixture contained cell-free extract, deoxyribose, ATP, $MgCl_2$ and tris buffer pH 7.6. The degradation of deoxyribose was followed by the Dische diphenylamine test on aliquots of the incubation mixtures. In several experiments the incubation mixtures contained $(NH_4)_2SO_4$. To precipitate sulphate ions, 2 *M* Ba acetate, either alone or in combination with the $ZnSO_4$–$Ba(OH)_2$ reagents was used.

Preparation of biosynthetic deoxyribose 5-phosphate

1.85 g of *E. coli* (wet weight) adapted to deoxyribose was treated with the Hughes bacterial press. 5 ml 0.05 *M* tris buffer of pH 7.6 was added. After 15 min at 0° the suspension was centrifuged at +4° for 15 min at 1000 *g*. The sediment was again

References p. 123.

extracted with 5 ml buffer and centrifuged as above. The combined supernatants were centrifuged at 12,000 *g* for 30 min. In a final volume of 10 ml the following mixture was prepared:

$(NH_4)_2SO_4$	0.28 *M*
ATP	0.005 *M*
$MgCl_2$	0.005 *M*
Deoxyribose	0.004 *M*
Extract	0.5 ml
Tris buffer	0.05 *M*, pH 7.6

The mixture was incubated at 37° for 20 min. 1.5 ml of 2 *M* Ba acetate was then added and the mixture kept at +4° for 2 h. After centrifugation, the sediment was washed three times with slightly alkaline 0.1 *M* Ba acetate. The washed precipitate was extracted four times with 1 ml portions of 0.1 *N* HCl and the combined extracts were adjusted to pH 6.0 and left in the refrigerator for 2 h. A precipitate was obtained which gave a negative diphenylamine test. The supernatant was again adjusted to pH 8.0 and left in the refrigerator for 2 h. Further precipitate was removed by centrifugation and the supernatant was acidified to pH 6.0 and 4 volumes of ethanol were added. The mixture was left in the refrigerator overnight and then centrifuged. The precipitate was dissolved in 1 ml 0.1 *N* HCl, pH was adjusted to 6.0 and a slight excess of Ba acetate was added. After centrifugation pH was adjusted to 8.0, insoluble material removed as above and the supernatant precipitated with ethanol after acidification to pH 6.0. The procedure was repeated once. The final precipitate was washed with ethanol and ether. It was then shaken with Dowex-50 in the hydrogen form in the presence of 1 ml of water. The resin was filtered off after 15 min and the solution neutralized and freeze-dried.

RESULTS

(1) *Tests on the separation of deoxyribose and deoxyribose 5-phosphate*

Experiments to test the efficiency of the $ZnSO_4$–$Ba(OH)_2$ procedure in the separation of deoxyribose and deoxyribose-5-phosphate were carried out.

Table I shows the results with solutions containing either the free sugar, the sugar phosphate, or mixtures of both. The data show a nearly complete precipitation of deoxyribose 5-phosphate from the original solution and 97 % of this could be extracted from the precipitate. The free pentose was not precipitated and the presence of deoxyribose had no effect on the precipitation or recovery of deoxyribose 5-phosphate.

(2) *Recovery of deoxyribose 5-phosphate from incubation mixture*

Experiments to determine the yield of recovery of deoxyribose 5-phosphate when present in a complete incubation mixture were carried out. In these experiments the yield obtained by elution with 0.1 *N* HCl was also compared with that obtained by treatment of precipitates with Dowex-50 (H). The experiments were made as indicated in Table II. They showed that the resin treatment was an efficient elution procedure. In the presence of $(NH_4)_2SO_4$ the addition of $ZnSO_4$–$Ba(OH)_2$ did not precipitate the sugar phosphate quantitatively. Complete precipitation was achieved with Ba acetate alone or in mixture with $ZnSO_4$–$Ba(OH)_2$.

References p. 123.

TABLE I

THE EFFECT OF ADDITION OF $ZnSO_4$-$Ba(OH)_2$ TO SOLUTIONS CONTAINING DEOXYRIBOSE AND DEOXYRIBOSE 5-PHOSPHATE

Sugar	*μg Substance in solution*					
	before treatment	*recovered in supernatant*	*before treatment*	*recovered in supernatant*	*before treatment*	*recovered in supernatant*
Deoxyribose	500	500			500	
Deoxyribose 5-phosphate			320	10	320	515
Extraction No.	*Sugars extracted from precipitate*					
1	0		60		50	
2	0		225		230	
3			20		15	
4			5		10	
5			0		0	
Total recovery	0		310		305	

The substances were dissolved in 1 ml distilled water. 1 ml 5% $Zn(SO)_4$ was added, followed by 1 ml 0.3 *N* $Ba(OH)_2$. Mixture left in ice-bath for 15 min, centrifuged and sediment extracted several times at room temperature with 1 ml portions of 0.1 *M* HCl, 5 min each time. The amount of material in supernatant and extracts determined by the diphenylamine method.

TABLE II

CONTROL EXPERIMENTS FOR THE PRECIPITATION OF DEOXYRIBOSE 5-PHOSPHATE FROM PREPARED MIXTURES

Precipitating reagents	*Precipitation of deoxyribose 5-phosphate*	*Recovery of deoxyribose 5-phosphate from precipitate by*	
		HCl extr.	*Dowex-50(H) treatment*
1 ml $ZnSO_4$ 1 ml $Ba(OH)_2$ 100 μl 2 *M* Ba acetate	Complete	100%	97%
150 μl 2 *M* Ba acetate	Complete		104%
1 ml $ZnSO_4$ 1 ml $Ba(OH)_2$	Incomplete	105%	100%

The final mixtures contained: ATP (0.005 *M*), tris buffer pH 7.6 (0.05 *M*), $MgCl_2$ (0.005 *M*), $(NH_4)_2SO_4$ (0.28 *M*) and deoxyribose 5-phosphate (1.4 μmole) in a total volume of 1 ml. Each precipitate was washed with 2 ml of ethanol followed by 4 ml ether. Half of the precipitate was eluted with 0.1 *N* HCl, the other half was shaken with resin in the presence of a small amount of distilled water. The deoxyribose 5-phosphate dissolved was estimated on aliquots.

(3) *The utilization of deoxyribose*

The degradation of the pentose was followed by using several lots of the crude extract:

(a) freshly made extract; (b) extract stored at +4° for 24 h; (c) extract stored at −10° for 24 h; (d) extract stored at −10° for 30 days.

The results of these experiments are shown in Table III. It will be seen that

References p. 123.

the degradation is not appreciably affected by storage at —10° for 30 days, whereas storage for 24 h at 4° caused a marked drop in the activity. It is also apparent that $MgCl_2$ and ATP are required for the degradation. When crude extracts were centrifuged at 0° at 12,000 *g* for 30 min a heavy sediment was obtained. The degradation of deoxyribose by the supernatant showed that the whole enzymic activity was present in the supernatant (Fig. 1). None of these extracts catalysed the phosphorylation of ribose.

TABLE III

DEGRADATION OF DEOXYRIBOSE BY CELL-FREE EXTRACTS AND STABILITY OF THE EXTRACTS

		μg Deoxyribose in mixture		*μg Deoxyribose degraded*
		before incubation	*after incubation*	
Expt. 1.	Fresh extract with ATP and $MgCl_2$	345	166	179
	with ATP	345	345	—
	with $MgCl_2$	345	340	5
	without ATP, $MgCl_2$	345	350	—
Expt. 2.	Extr. stored 24 h 5°*	345	259	86
	Extr. frozen 24 h —10°*	345	150	195
	Extr. frozen 24 h —10° (control)**	345	335	10
Expt. 3.	Extr. stored 30 days 5°*	350	355	—
	Extr. frozen 30 days —10°*	350	165	185
	Extr. frozen 30 days —10° (control)**	350	355	—

The complete mixtures contained deoxyribose (1.7 μmole), ATP (0.005 *M*), $MgCl_2$ (0.005 *M*), tris (0.05 *M*, pH 7.6) and extract (0.2 ml) in a total volume of 0.85 ml. The mixture was incubated at 37° for 1 h. Aliquots of 0.1 ml were used to follow the decrease in deoxyribose.

* Complete system (*i.e.* ATP and $MgCl_2$ added).

** ATP and $MgCl_2$ omitted.

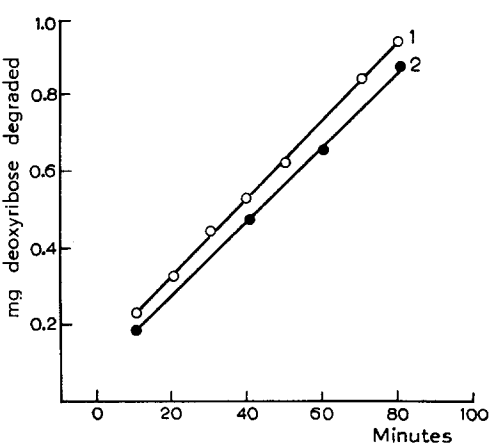

Fig. 1. Degradation of deoxyribose by cell-free extract of *E. coli* as measured by the disappearance of diphenylamine-positive material. Curve 1 was obtained with crude extract and curve 2 with extract centrifuged at 12,000 *g*.

(4) *The effect of* $(NH_4)_2SO_4$ *on the degradation of deoxyribose*

HEALD AND LONG[6], using a ribokinase-containing extract of *E. coli*, showed that the presence of 0.28 *M* $(NH_4)_2SO_4$ caused the accumulation of ribose 5-phosphate. These results were readily confirmed in this laboratory. Similar experiments to investigate the effect of different concentrations of $(NH_4)_2SO_4$ showed no effect on the rate of degradation of deoxyribose (Table IV). In some cases an accelerating effect was noted but this was not generally the rule. The reason for this cannot be given at the moment. However, it is seen from Table IV that even in cases with an accelerating effect, deoxycompounds, which could be precipitated with $ZnSO_4$–$Ba(OH)_2$, accumulated.

References p. 123.

(5) *Isolation and identification of biosynthetic deoxyribose 5-phosphate*

Deoxyribose phosphate was isolated from an incubation mixture consisting of $(NH_4)_2SO_4$, ATP, $MgCl_2$, tris buffer, deoxyribose and bacterial extract. Deoxyribose phosphate coprecipitated with $BaSO_4$ upon addition of $BaAc_2$ to the incubation mixture and behaved therefore as ribose 5-phosphate, as described by HEALD AND LONG[6]. The sugar phosphate was extracted with 0.1 *N* HCl and purified further by repeated precipitation of the barium salts with ethanol.

TABLE IV

THE EFFECT OF $(NH_4)_2SO_4$ ON THE DEGRADATION OF DEOXYRIBOSE BY *E. coli* EXTRACTS

Incubation time (min)	*μg Pentose* in mixtures*							
	no $(NH_4)_2SO_4$		*0.28 M $(NH_4)_2SO_4$*		*0.56 M $(NH_4)_2SO_4$*		*0.84 M $(NH_4)_2SO_4$*	
	before ppn.	*after ppn.*	*before ppn.*	*after ppn.*	*before ppn.*	*after ppn.*	*before ppn.*	*after ppn.*
0	560	560	550	560	555	560	555	555
20	500		465		470		475	
40	475	480	420	310	415	310	430	320
Deoxyribose 5′-phosphate**	—		110		105		110	

Incubation mixture contained deoxyribose (2.6 μmole) and ATP (0.005 *M*), $MgCl_2$ (0.005 *M*), tris (0.05 *M*, pH 7.6), ammonium sulphate and extract (0.1 ml) in a total volume of 1 ml.

* The amount of pentose is expressed as μg deoxyribose.

** Expressed as μg deoxyribose in the precipitate obtained with the $ZnSO_4$–$Ba(OH)_2$ reagent.

The biosynthetic sugar phosphate was examined by paper chromatography in conc. NH_3-*n*-propanol (40:60 v/v) and when sprayed with the diphenylamine reagent gave a spot with the same mobility as deoxyribose 5-phosphate but different from deoxyribose and its 1-phosphate. Some diphenylamine-positive material remained at the site of application on the chromatogram. When the purification procedure (repeated precipitation of barium salt) was repeated a smaller proportion of material remained at the site of application. The purified deoxyribose 5-phosphate behaved identically to authentic material in paper electrophoresis in borate buffer pH 10.

Also, when the freeze-dried incubation mixture was subjected to chromatography and electrophoresis, there was no indication of other diphenylamine-positive spots than those corresponding to deoxyribose and its 5-phosphate. In experiments with extracts prepared from *E. coli* grown in the absence of deoxyribose no formation of deoxyribose phosphate could be demonstrated.

DISCUSSION

Previous experiments[1,2] with *E. coli* 207/56 had shown that this strain could utilize deoxyribose as the sole carbon source. The kinetics of the degradation by growing cells indicated that the formation of the primary reaction product was catalysed by adaptive enzymes[1]. In a previous short communication[1] we reported the failure to prepare bacterial extracts which would degrade deoxyribose in the presence of ATP and Mg^{++}. By using the bacterial press of HUGHES we have now obtained bacterial

References p. 123.

extracts which will degrade deoxyribose, provided ATP and Mg^{++} are present. This, together with the fact that phosphorylated deoxyribose[10,11] is readily degraded by bacterial extracts from *E. coli*, suggests that the adapted enzyme is a deoxyribokinase. An enzyme which catalyses the phosphorylation of D-ribose[12] present in liver will also catalyze the phosphorylation of deoxyribose. Apart from this report, no deoxyribosekinase has been described. In this connection it is of interest to note that our extract containing deoxyribokinase was specific for deoxyribose and could not catalyse the phosphorylation of ribose.

Degradation of deoxyribose phosphate by the extracts is rapid and therefore the detection of such a phosphate is difficult. However, in the presence of ammonium sulphate an accumulation of a sugar phosphate identical to deoxyribose 5-phosphate occurred. In this respect there is close resemblance to ribose 5-phosphate which also accumulates in a system consisting of ribose, ATP, Mg^{++} and an extract of ribose adapted *E. coli*[6]. Evidence for the sugar phosphate being deoxyribose 5-phosphate is a positive diphenylamine test, identity with authentic material in paper chromatography and paper electrophoresis in borate buffer. The results of paper chromatography rules out any possibility of the substance being deoxyribose 1-phosphate. The possibility of the pentose phosphate being a 3-phosphate can not be ruled out, although paper electrophoresis in borate buffer would be expected to distinguish between the 3- and 5-phosphate. The formation of a deoxyxylose phosphate which would also give a positive diphenylamine test[13] seems highly unlikely and electrophoresis in borate buffer would be expected to distinguish it from deoxyribose 5-phosphate.

The transfer of a phosphate group from ATP to deoxyribose catalysed by extract of deoxyribose-adapted *E. coli* cells offers possibilities for the preparation of deoxyribose-5-phosphate and for the ^{32}P-labelled phosphate. Large scale enzymic preparation of deoxyribose 5-phosphate together with a chemical analysis of the product is in progress.

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