STUDIES ON PHOSPHODEOXYRIBOMUTASE

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SUMMARY

A phosphodeoxyribomutase has been demonstrated in cell-free extracts of *Sarcina lutea*. The enzyme could be heated to 55° for at least 2 h and still retain activity. It had a pH optimum around 7.2, and the most highly purified preparation showed a specific activity of approx. 60 μmoles of deoxyribose 1-phosphate converted to deoxyribose 5-phosphate/mg protein/h. The enzyme was constitutive in the above organism, but could not be demonstrated conclusively in extracts of 14 other microorganisms tested.

The enzymic conversion of deoxyribose 1-phosphate to deoxyribose 5-phosphate was established by isolation and enzymic characterization of the product. The enzyme was also able to convert deoxyribose 5-phosphate to pyrimidine-bound deoxyribose, presumably through deoxyribose 1-phosphate. Chromatographic and radiographic studies indicated that, at equilibrium, the reaction contains approx. 95% deoxyribose 5-phosphate and 5% deoxyribose 1-phosphate.

The mutase was shown to be inhibited by inorganic phosphate at low concentrations, and by deoxyribose 1-phosphate, deoxyribose 5-phosphate, potassium sulfate and fructose 6-phosphate. The enzyme appeared to be stimulated by fructose 1,6-diphosphate. The inhibitions by inorganic phosphate and sulfate confirm previous reports in the literature.

Acetaldehyde and an aqueous extract of Whatman No. 1 filter paper were shown to interfere with the cysteine–sulfuric acid reaction with deoxyribose.

INTRODUCTION

The metabolic degradation of nucleoside-DR in animal and microbial cells has been shown to proceed via DR 1-P[1,2] to DR 5-P[3–5] which is finally split to glyceraldehyde phosphate and acetaldehyde[3,4,6]. The conversion of DR 5-P to nucleoside-bound DR, presumably *via* DR 1-P, has also been reported[7]. The isolation of DR 1,5-diP from ling-cod muscle[8] further suggests the presence of a phosphodeoxyribomutase, requiring the diphosphate as co-enzyme. Although it appears that the deoxyribosyl moiety may arise by reduction of the ribosyl moiety[9–14], probably at the nucleotide level,

Abbreviations: deoxyribose, DR; deoxyribose 1-phosphate, DR 1-P; deoxyribose 5-phosphate, DR 5-P; deoxyribose 1,5-diphosphate, DR 1,5-diP; thymine, T.

the degradation of nucleoside-bound deoxyribose would seem to occur *via* the intermediates noted above[6,9].

The work reported herein was undertaken in an effort to produce deoxyribosides in quantity from the inexpensive starting materials acetaldehyde and hexosediphosphate. The interconversion of DR 1-P and DR 5-P, which could be demonstrated in the presence of a cell free extract of *Sarcina lutea* PCI-1001 (UC-128) is reported in this publication.

MATERIALS AND METHODS

The initial test for the detection of mutase activity was carried out in a reaction mixture containing 10 μmoles/ml each of deoxyadenosine and Pi in 0.05 *M* maleate buffer at pH 6.5, plus approx. 3 mg/ml of protein extracted from *Escherichia coli* Wc–. The *E. coli* preparation was obtained from cells which had previously been grown aerobically at 32° in a medium containing glucose, 10 g/l of distilled water; acetic acid, 5 g/l; yeast extract, 3 g/l; beef extract, 3 g/l; potassium phosphate, 2 g/l; magnesium sulfate, 0.2 g/l; manganese, iron and zinc, 0.001 g/l each; and cytosine, 0.5 g/l. The presterilization pH was adjusted to 8 with NH_3. The cells, obtained in a concentration of 8 to 10 g/l (dry wt.), were harvested after approx. 16 h growth, washed with one volume of distilled water and ground for several minutes in a mortar with an equal weight of powdered Pyrex glass (300 mesh). The ground residue was extracted with 5–10 volumes of distilled water and this suspension was centrifuged at 0° for 30 min at 30 000 × *g*. The supernatant fraction, containing approx. 10 mg/ml of protein, was diluted four fold for the mutase assay. In the above reaction mixture, deoxyadenosine was added as a source of DR 1-P which was not metabolized further unless *S. lutea* extract was also added. Total DR (0.1 to 0.8 μmole/6 ml reaction) was determined by the method of DISCHE[15] using deoxyadenosine as standard and measuring A_{595}—A_{650}. Mutase activity was indicated by a disappearance of the DR moiety. Excess deoxyribose phosphate aldolase[7] for the degradation of DR 5-P was provided in the *E. coli* extract. Aqueous extracts of the following organisms, prepared by the procedure described above, showed no mutase activity in the deoxyadenosine–*E. coli* system: *Salmonella typhi*, *Salmonella pullorum*,

TABLE I

EFFECT OF ACETALDEHYDE ON CYSTEINE–SULFURIC ACID REACTION

Thymidine concentration (μg/ml)	*Acetaldehyde concentration (μg/ml)*	A_{490}*	
		Unheated	*Heated***
14.5	0	0.54	
14.5	0.18	0.44	
14.5	1.8	0.17	
14.5	18	0.11	
0	18	0.0	
14.5	0	0.54	0.62
14.5	3.6	0.15	0.61
7.2	0	0.20	0.23
7.2	3.6	0.06	0.23

* Determined with a Beckman Spectrophotometer, Model B. Dose-response curves gave straight line functions which did not pass through the origin.

** Steam bath for 10 min before the addition of cysteine or sulfuric acid.

Proteus vulgaris, Mycobacterium avium, Candida albicans, Saccharomyces pastorianus, Saccharomyces cerevisiae, Klebsiella pneumoniae, Pseudomonas aeruginosa, Salmonella paratyphi, Serratia marcescens, Staphylococcus albus and *Staphylococcus aureus*. Protein content of the above extracts varied from 4 to 19 mg/ml.

When an extract of *S. lutea*, to give 1.5 mg of protein/ml, was added to the mixture of deoxyadenosine (or thymidine), *E. coli* extract and buffer, DR disappeared after 30 min incubation at 37°.

When thymidine was used as substrate for the mutase studies, DR was determined by the cysteine–sulfuric acid method of STUMPF[16]. During the course of these investigations, it was observed that acetaldehyde, formed from DR 5-P by deoxyribose aldolase[5,7], interfered with the cysteine–sulfuric acid reaction for DR as illustrated in Table I. These data show that acetaldehyde could be removed from the reaction mixture before the cysteine and sulfuric acid were added by steaming for 10 min, and this procedure was adopted for all subsequent determinations. Acetaldehyde *per se* did not react with the cysteine–sulfuric acid reagent.

An aqueous extract of Whatman No. 1 filter paper was also found to interfere with the cysteine–sulfuric acid reaction with DR. When the paper was extracted with hot water and the concentrated extract added to deoxyadenosine standards, development of color with cysteine–sulfuric acid resulted in (a) an approximate five fold decrease in absorbancy/unit weight of DR and (b) a change in the spectral curve which was found to contain a shoulder at 475 mμ in addition to the expected peak at 490–495 mμ. These changes were also evident with DR samples which were chromatogrammed on Whatman No. 1 paper and isolated by extraction with hot water.

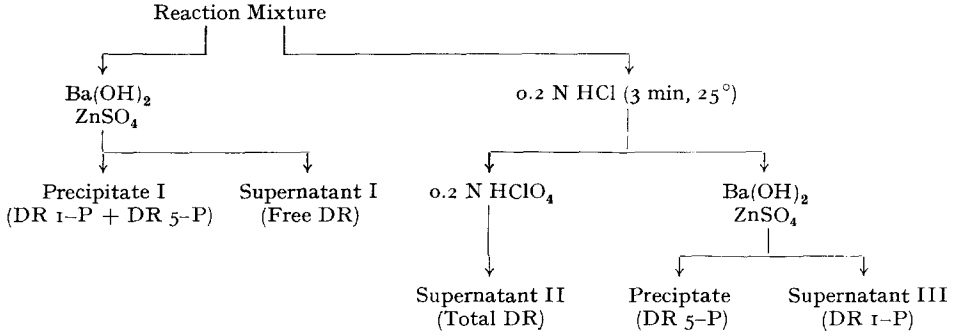

Fig. 1. Precipitation scheme for analysis of DR 1-P conversion.

Crystalline DR 1-P was prepared as the dicyclohexylamine salt according to the method of FRIEDKIN[17] as modified by MCDONALD AND FLETCHER[18]. DR 5-P was prepared by the method of PRICER[21] and was characterized as described by RACKER[22]. The conversion of DR 1-P to DR 5-P was measured quantitatively using the precipitation scheme shown in Fig. 1. The salient features of this scheme are the following. (a) The initial precipitation with barium hydroxide (I) measures any reaction which might liberate free DR (supernatant) from DR 1-P or DR 5-P (precipitate). (b) Following incubation for 3 min at 25° in 0.2 *N* HCl, DR 1-P is cleaved rapidly to free DR whereas DR 5-P is not. In fraction II, total DR in the reaction mixture is measured and any disappearance of the sugar moiety would be observed here (*e.g.*, pyrimidine

binding or aldolase activity). In fraction III, free DR (from DR 1-P) is present in the supernatant fluid whereas DR 5-P is precipitated as the barium salt.

When DR 1-P is converted to DR 5-P, there is no change in the barium soluble fraction (I) or the total DR moiety (II), but there is a decrease in the free DR after acid treatment (III), as shown in Fig. 2.

S. lutea extracts were prepared as described above for *E. coli* or in an Eppenbach colloid mill through the courtesy of Dr. J. GARVER[19]. *S. lutea* was grown in a medium containing glucose, 10 g/l; acetic acid, 5 g/l; yeast extract, 3 g/l; beef extract, 3 g/l; KH_2PO_4, 2 g/l; $MgSO_4$, 0.2 g/l; Mn, Fe and Zn, 0.001 g/l; NH_3 to pH 8, on a Gump rotary shaker at 250 rev./min and 32° in an indented shake flask[20]. Under these conditions of growth, approx. 8 g/l of dry cell weight were obtained after 18–20 h incubation. Radioactivity was measured with a Geiger-Müller counter using a Micromil gas flow window.

EXPERIMENTAL

Purification of mutase

The original *S. lutea* extract, prepared as described above, contained pyrimidine phosphorylase and deoxyribose–aldolase activities which interfered with the determinations of DR 1-P and DR 5-P. When the crude enzyme preparation was heated at 55° for 15, 30, 60 and 120 min, and mutase activity determined in the presence of thymidine, P_i and *E. coli* extract, the specific mutase activity was increased approximately three fold (Table II) while phosphorylase and aldolase activities were completely destroyed. Following the heat treatment, the crude preparation was adjusted to pH 4.5 with acetic acid at 0°, the precipitate removed by centrifugation and the supernatant fluid readjusted to pH 6.5 to 7. This procedure also increased the specific activity as shown in Table II. Approx. 50 ml of enzyme preparation was next dialyzed for 48 h against 5 l of deionized water which resulted in an over-all purification (units/mg protein) of approximately ten fold. More important perhaps than the absolute increase in specific activity is the fact that the heat treatment rendered this preparation essentially free of contaminating enzyme activities such as aldolase and nucleoside phosphorylase and allowed the direct measurement of the conversion of DR 1-P to DR 5-P.

TABLE II

PREPARATION OF MUTASE FROM *S. lutea*

*Treatment**	*Specific activity μmoles converted/mg protein/h***
1. None	0.8
2. 55° for 15–120 min***	3.5
3. Precipitate with acetic acid at pH 4.5	
(a) Supernatant fluid	4–6
(b) Precipitate	0.5
4. Supernatant fluid dialyzed 48 h against water	8

* Details of purification treatments given in text.

** Determined as disappearance of DR from thymidine in the presence of phosphate and extract of *E. coli*, as described in text.

*** 100° for 3 min completely inactivated the preparation.

Conversion of DR 1-P to DR 5-P

The conversion of DR 1-P (12 μmoles/ml) to DR 5-P, using the heat treated dialyzed enzyme preparation (at 3 mg/ml total solids; 1 mg protein/ml) shown in Table II, was carried out in 0.05 *M* maleate buffer at pH 7.5 and 37°. Total DR and DR 1-P were determined by the DISCHE reaction (see Fig. 1 and METHODS) and the results are presented in Fig. 2. Subsequent investigations showed that approximately 90% of the DR 1-P was converted to acid stable, barium-insoluble DR (DR-5-P) after 2 h incubation.

Aliquots removed at 0 and 100 min were chromatogrammed in a solvent system consisting of 1-butanol–glacial acetic acid–water (2:1:1, v/v). After development overnight, the papers were sprayed with cysteine–sulfuric acid[23] and thiobarbituric acid reagents[24] to visualize DR. The 0-min sample contained only DR 1-P, which migrated as the free sugar in the acid system employed. After 60 and 100 min of enzyme treatment, however, the DR spot (R_F 0.65) decreased sharply in intensity and a spot which migrated with DR 5-P (R_F 0.35) appeared and increased in intensity. When these spots were eluted from the papergram and reacted with cysteine–sulfuric acid[16] and the DISCHE reagent[15], both showed spectra which could not be differentiated from known DR.

Isolation of DR 5-P

A large scale experiment was carried out to isolate and identify the product formed from DR 1-P by the *S. lutea* preparation. Twenty millilitres of reaction mixture (as described above), containing 440 μmoles of DR 5-P by assay, were steamed for 3–4 min, and freeze-dried. The dried residue was taken up in 10 ml of cold H_2O and the protein precipitated by the addition of 0.5 ml of cold 70% $HClO_4$. The precipitated protein was removed by centrifugation. The supernatant fluid was adjusted to pH 7.2 with KOH and the $KClO_4$ which precipitated was removed by filtration. A portion of this filtrate, containing 50 μmoles of deoxyribose, was chromatographed at 0° on a column of Dowex-1 acetate (1.2 cm in diameter by 15 cm in length) using the gradient solvent system (ammonium acetate) of PRICER[21]. The material eluted between 100 and 210 ml gave a positive reaction with diphenylamine as did an authentic sample of DR 5-P chromatographed in the same way. 70% of the starting material was recovered in these fractions in the case of the experimental and 88% with the authentic sample. Precipitation as the barium salt[21] by the addition of $BaBr_2$ and four volumes of acetone at —10° yielded a water soluble product in 63% yield which assayed 92% pure by diphenylamine (the authentic sample was 95% pure by the same criterion). An aliquot was converted to a mixture of acetaldehyde and 3-phosphoglyceraldehyde with purified deoxyribose phosphate aldolase[21], and the products assayed enzymically[22] using alcohol dehydrogenase and diphosphopyridine nucleotide for acetaldehyde and triose phosphate isomerase, α-glycerophosphate dehydrogenase and diphosphopyridine nucleotide for glyceraldehyde phosphate. 96 and 94%, respectively, of the theoretical amounts of the products were obtained.

Effect of pH on mutase activity

The pH optimum of the mutase reaction was determined in maleate buffer using DR 1-P as described above and the results are shown in Table III. A pH optimum of approx. 7.2 was confirmed using buffers other than maleate.

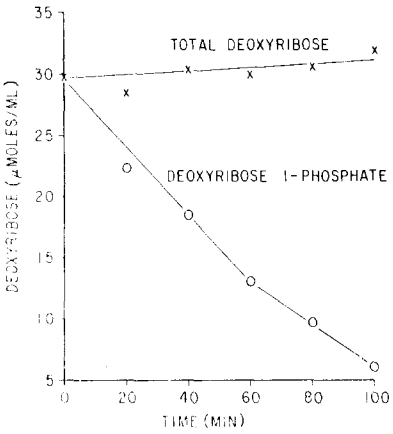

Fig. 2. Conversion of DR 1-P to DR 5-P.

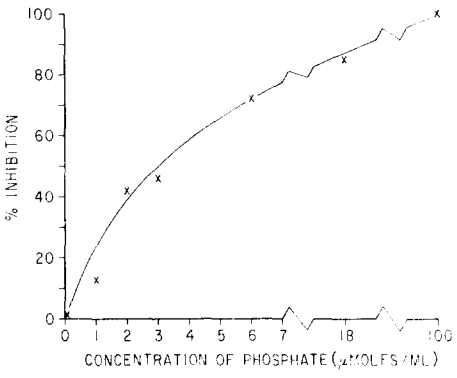

Fig. 3. Effect of phosphate on mutase reaction.

TABLE III

EFFECT ON pH ON MUTASE ACTIVITY

Reaction mixture contained 12 μmoles DR 1-P + 1 mg *S. lutea* protein in 1 ml 0.05 *M* maleate buffer. Temperature of reaction was 37°. Conversion was determined as shown in Fig. 1.

pH	*DR 1-P converted (μmoles/ml)*
5.5	3.2
5.7	4.0
6.4	6.0
7.2	6.8
7.3	6.2
7.6	5.5
8.1	0.4
9.3	0

TABLE IV

EFFECT OF DEOXYRIBOSE PHOSPHATES ON MUTASE ACTIVITY

DR 1-P concentration (μmoles/ml)*	*DR 5-P concentration (μmoles/ml)*	*Relative rate of reaction***
6	0	1.0
8.6	0	0.67
12.2	0	0.5
27	0	0.35
55	0	0
12.5	0	1.0
12.5	10	0.55

* At beginning of reaction, added as dicyclohexylamine salt.

** Measured at 15-20 min with 12 μmoles/ml of DR 1-P as substrate in 1 ml of 0.05 *M* maleate buffer pH 7.5, as shown in Fig. 1. *S. lutea* mutase preparation added at a final concentration of 1 mg of protein/ml. Temperature of incubation 37°. Maximum specific activity observed was 60 μmoles DR 1-P converted/mg protein/h. Cyclohexylamine·HCl was not inhibitory at a concentration of 50 μmoles/ml.

Effect of substrate and product concentration on reaction rate

Both DR 1-P and DR 5-P were found to inhibit the rate of mutase reaction as shown in Table IV. The inhibition by both substrate and product made the determination of the Michaelis constant essentially meaningless, since the rate was changing throughout the course of the reaction as a function of the relative concentrations of DR 1-P and DR 5-P.

Effect of phosphate and sulfate on the rate of reaction

HOFFMAN AND LAMPEN[3] described the inhibition of the conversion of DR 1-P to DR 5-P in *E. coli* by P_i and sulfate. The effect of P_i on the conversion of DR 1-P by the *S. lutea* mutase was determined by incubating 1 ml of reaction mixture containing 12 μmoles/ml of DR 1-P, varying concentrations of P_i, and 3 mg/ml of enzyme preparation in 0.05 *M* maleate buffer at pH 7.5 at 37° and measuring residual DR 1-P as shown in Fig. 1. The results of this study are shown in Fig. 3. The mutase reaction was inhibited 50% in the presence of 3 μmoles/ml of P_i.

The inhibition by phosphate did not appear to be competitive since it varied only from 48% to 55% when the DR 1-P concentration was varied from 12 to 6 μmoles/ml in the presence and absence of 4 μmoles/ml of P_i. The mutase reaction did not appear to be inhibited by P_i when the substrate was a purine deoxyriboside or thymidine, but this observation was complicated by the fact that P_i was a required intermediate in this reaction.

Sulfate was also inhibitory to the mutase as previously described[3]. When potassium sulfate was added at a concentration of 50 μmoles/ml, the reaction was inhibited approx. 30% (DR 1-P at 12 μmoles/ml).

Effect of other sugar phosphates on the mutase reaction

Since DR 1-P, DR 5-P and P_i all inhibited the conversion of DR 1-P to DR 5-P, the effects of other sugar phosphates were also investigated. Barium ion was removed where required by precipitation with oxalate, which itself was shown not to interfere with the mutase reaction. Results of these studies are presented in Table V. These data show marked (albeit opposite) effects on the mutase reaction only by fructose 6-phosphate and fructose 1,6-diphosphate.

TABLE V

EFFECT OF VARIOUS SUGAR PHOSPHATES ON MUTASE REACTION

Reaction mixture contained DR 1-P, 12.5 μmoles/ml in maleate buffer, as described in Table IV. Sugars, obtained from Nutritional Biochemical Corporation were added at zero time.

Sugar added (at 10 μmoles/ml)	*Per cent inhibition*	*Per cent stimulation*
Ribose 5-phosphate	9	
Galactose 6-phosphate	15	
Glucose 6-phosphate	9	
3-Phosphoglyceric acid		4
Fructose 6-phosphate	44, 40	
Fructose 1,6-diphosphate		48, 25
Glucose 1-phosphate	0	

Effect of magnesium and cysteine

Magnesium and cysteine are required for optimum activity of phosphoglucomutase[25,26]. Cysteine, at 5 μmoles/ml, was found to accelerate the conversion of DR 1 to DR 5-P only slightly, whereas magnesium ion had no effect on the reaction.

Reversibility of the mutase reaction

RACKER described the synthesis of hypoxanthine deoxyriboside from DR 5-P and hypoxanthine in the presence of *E. coli* extract[7]. When DR 5-P (8 μmoles/ml) and thymine (5 μmoles/ml) were incubated at 37° with the *S. lutea* mutase (3 mg/ml) in 0.05 *M* maleate buffer at pH 7.5 in the presence of *E. coli* thymidine phosphorylase (specific activity of 430 μmoles thymine liberated/mg protein/h), 1–1.75 μmoles/ml of thymidine were formed. This conversion is shown chromatographically in Fig. 4, which establishes the reversibility of the mutase.

When DR 5-P was incubated with the mutase in the absence of an acceptor for DR 1-P, approx. 0.5 μmole/ml of DR 1-P were formed in 20 min from 8 μmoles/ml of DR 5-P. The conversion was established by (a) the production of acid labile DR-P and (b) paper chromatographic analysis using the thiobarbituric acid spray reagent[21]. Since approx. 0.5 μmole of DR 1-P were present per millilitre when the conversion of DR 1-P (12 μmoles/ml) to DR 5-P was measured, these data indicate that, at equilibrium, approx. 95% of the sugar is present as the 5-phosphate.

[^{14}C]DR 5-P, prepared from fructose [1,6-^{14}C]diphosphate by the method of PRICER[21] was submitted to the *S. lutea* preparation at 3.3 μmole of DR 5-P/ml in 0.5 *M* Tris buffer at pH 7.5. After 90 min, 0.5 ml of the incubation mixture was exposed to 0.2 *N* HCl at room temperature for 20 min to hydrolyze any DR 1-P

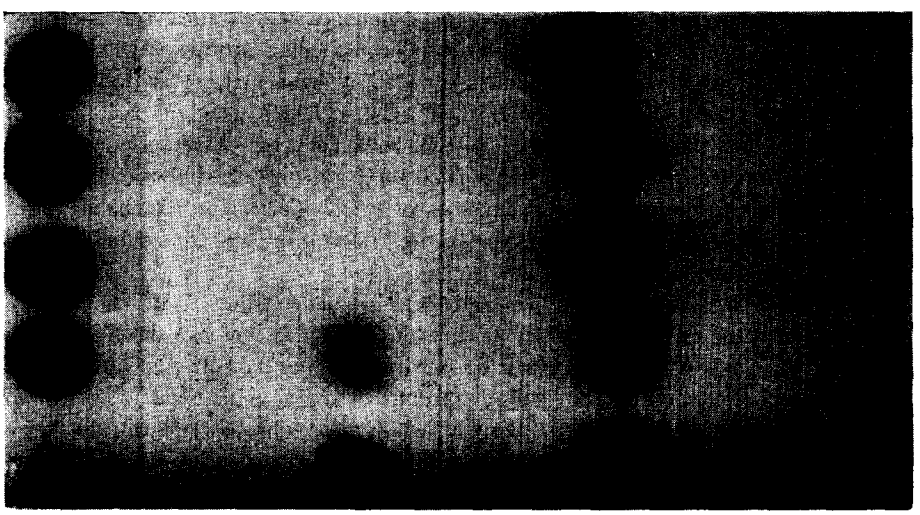

Fig. 4. Papergram demonstration of thymine synthesis. 1, Zero-min sample, DR 5-P omitted*; 2, 40-min sample, DR 5-P omitted; 3, Zero-min sample, DR 5-P present; 4, 40-min sample, DR 5-P present; 5, thymidine (50 μg) + thymine (25 μg) standard.

* Reaction mixture contained thymine (5 μ*M*/ml), *S. lutea* preparation (3 mg/ml) and thymidine phosphorylase (1 mg/ml) in maleate buffer. DR 5-P added as noted above. Papergram was developed for 16 h by the descending technique in ethyl acetate saturated with 0.05 *M* phosphate buffer at pH 6 after equilibration for 3 h.

formed and chromatogrammed on a column of Dowex-1 acetate as previously described. Any free deoxyribose formed by hydrolysis of DR 1-P in 0.2 *N* HCl would not adsorb on the resin and would be seen as ^{14}C in the effluent. The starting [^{14}C]DR 5-P had a specific activity of 12 300 counts/min/μmole. The ^{14}C which did not adsorb on the column after 90 min of incubation corresponded to the conversion of a minimum of 0.1 μmole/ml of DR 5-P to DR 1-P respectively. Further purification of this enzyme will be required before an accurate determination of the equilibrium point is possible.

DISCUSSION

The above data confirm the existence of a phosphodeoxyribomutase which catalyzes the interconversion of DR 1-P and DR 5-P. Deoxyribosides can be converted to DR 1-P *via* the action of purine and pyrimidine phosphorylases[1,2]. The DR 1-P can then be converted to DR 5-P in the presence of the mutase[3,4] and DR 5-P finally cleaved by DR-aldolase to triosephosphate and acetaldehyde[7].

The synthesis of deoxyribonucleotides appears to proceed in bacteria and animal cells by the direct reduction of ribonucleotides[13,14]. The existence of a mutase which can convert DR 5-P to DR 1-P points to this pathway as a possible alternate for deoxynucleoside synthesis. RACKER[7] was able to demonstrate the synthesis of deoxyribosides from DR 5-P and hypoxanthine and the data presented above illustrate the synthesis of thymidine from thymine and DR 5-P. In the presence of small amounts of phosphate (5–10 μmoles/ml), however, no conversion of DR 5-P to DR 1-P could be demonstrated in the *S. lutea* system. It was further noted in the above studies that DR 1-P, DR 5-P and fructose 6-phosphate all inhibited the mutase reaction. With these possibilities for physiologic control of phosphodeoxyribomutase, one cannot predict or estimate the extent to which this enzyme may be employed in the synthesis of deoxyribosides in intact cells. Studies with radioactive compounds have indicated that the mutase pathway from DR 5-P is not important in the synthesis of deoxyribosides in *E. coli*[9]. Using *E. coli*$_{15}$, whole cells of which degrade thymidine to thymine, ethanol, CO_2 and acetate, extremely low mutase activity was observed in a cell-free preparation tested as described above. The mutase was very difficult to demonstrate in extracts of *E. coli* even after extensive dialysis to remove P_i, and further work is needed to explain the difference between this organism and *S. lutea*.

The interference of acetaldehyde and an aqueous extract of Whatman No. 1 filter paper with the cysteine–sulfuric acid reaction for deoxyribose should be noted. When this reagent is used to measure deoxyribose under conditions where acetaldehyde is being produced, the apparent amount of sugar present will decrease with increasing aldehyde concentration. Likewise, when paper chromatograms are sprayed with the cysteine–sulfuric acid reagent, the intensity of the reaction is much reduced with Whatman No. 1 paper. Extracts of this paper added to known deoxyribose compounds also show a much reduced intensity of reaction with the cysteine–sulfuric acid reagent and a change in the spectral curve for DR. The effects of other papers were not investigated.

No absolute requirement could be shown with the *S. lutea* mutase for DR 1,5-diphosphate, magnesium or cysteine, although fructose 1,6-diphosphate did stimulate the reaction and DR 1,5-diphosphate was not tested *per se*. The only attempt to

remove such co-factors was a 48-h dialysis against de-ionized water and it is entirely possible that the necessary co-factors were bound tightly to the enzyme and were not removed under these conditions.

NOTE ADDED IN PROOF

Most recent investigations indicate that, when all of the fructose-diphosphate is degraded enzymically or chemically, stimulatory activity is still present which appears to be due to glucose-1,6-phosphate.

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