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The Enzymically Catalysed Transfer of the Deoxyribosyl Group from One Purine or Pyrimidine to Another

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Since several of the natural deoxyribosides serve equally well as growth-promoting substances for certain bacteria (Kitay, MacNutt & Snell, 1949, 1950; Hoff-Jørgensen, 1949; Kocher & Schindler, 1949) it seems probable that in such organisms the administered deoxyriboside is converted into the other deoxyribosides of which the bacterial deoxyribonucleic acid is constituted. The only mechanism which has been established for the biological synthesis of nucleosides (Kalckar, 1947; Friedkin & Kalckar, 1950) would depict the interconversion as follows:

where R, R¹, etc. represent the purines and pyrimidines (or their precursors) which are characteristic of the deoxyribonucleic acid of the bacterium.

The experiments reported below were designed to test such a mechanism in representatives of the group of bacteria which require deoxyribosides for growth. Dialysed enzyme preparations from *Lactobacillus helveticus* S have been shown, by a series of indirect methods, to be capable of bringing about a wide variety of interconversions when incubated with a deoxyriboside and a free purine or pyrimidine. Contrary to expectation, deoxyribose-1-phosphate did not react, in this system, with purines or pyrimidines to yield deoxyribosides. These findings have been reported in brief (MacNutt,

1950). Enzyme solutions have been prepared from two additional bacteria, both of which require deoxyribosides for growth, and these, too, have been found to catalyse an exchange. The exchange of the deoxyribosyl group from one acceptor to another can hardly proceed through hydrolysis followed by resynthesis, since hydrolysed deoxyribose-1-phosphate was also inactive in this system. The enzyme preparation is therefore considered to be a trans-*N*-glycosidase, analogous to the trans-*O*-glycosidase from *Pseudomonas saccharophila* (Doudoroff, Barker & Hassid, 1947).

EXPERIMENTAL

Materials

All melting points reported are uncorrected. Optical rotations were measured in a 2 dm. tube.

Purines and pyrimidines. Commercial preparations (Schwartz Laboratories; Hoffmann La Roche and Co.) were used. 5-Methyleytosine was generously supplied by Dr H. K. Mitchell. It melted at 276–278° with gas evolution after softening at 268°. (Found in material dried at 100° over P₂O₅ for 4 hr. (1 mm.): C, 48.1; H, 5.8; N, 33.7. Calc. for C₈H₇ON₃: C, 48.0; H, 5.6; N, 33.6%). The picrate melted at 297–299° (decomp.).

4-Aminoglyoxaline-5-carbonamide hydrochloride was prepared from formamidomalonanamide hydrochloride (a gift from Dr D. W. Woolley) by ring closure (Shaw & Woolley, 1949). Buff-coloured material was obtained which decomposed at 253° without melting completely. The picrate decomposed at 237°.

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Deoxyribosides. Adenine deoxyriboside was kindly supplied by Dr T. G. Brady. The other deoxyribosides were prepared by a modification of Klein's (1938) procedure. Sodium deoxyribonucleate (25 g.) (7.55% P), prepared by Hammarsten's (1924) method, was dissolved in 500 ml. of water, adjusted to pH 8.0 with 15N-NH₄OH, and 500 mg. of MgSO₄·7H₂O in 5 ml. of water were added followed by 1 mg. of crystalline deoxyribonuclease (Nutritional Biochemicals Corporation) in 3 ml. of water. After a few minutes at 23° the viscosity of the solution had dropped. After 2 hr. 100 ml. of saturated (NH₄)₂SO₄ solution, 50 g. of MgSO₄·7H₂O in 100 ml. of water, sufficient 15N-NH₄OH to bring the pH to 8.7, and water to make a total volume of 800 ml. were added, followed by 200 ml. of calf intestinal phosphatase purified to the stage prior to reprecipitation with 0.8 saturated (NH₄)₂SO₄ (Schmidt & Thannhauser, 1943). The solution was covered with toluene and incubated at 37° for 2.5 days, NH₄OH being added from time to time to maintain the pH at 8.7-9.0.

Klein's (1938) procedure was followed for the separation of guanine deoxyriboside as a gel. The two portions of the gel were dissolved in 300 ml. of water and extracted with 250 ml. portions of *n*-butanol saturated with water until 500 ml. of washed butanol solution gave no appreciable amount of deoxyriboside upon evaporation. The total volume of *n*-butanol used was 4.5 l. The combined washed butanol layers were evaporated *in vacuo* to yield a white crystalline residue consisting of guanine deoxyriboside and hypoxanthine deoxyriboside from which 1.23 g. of guanine deoxyriboside were obtained by repeated crystallization from hot water. The compound began darkening around 208° (moderate rate of heating), continued to darken slowly as the temperature rose, and at 305° was dark brown. It did not shrink or decompose suddenly. $[\alpha]_D^{25} = -42.7^\circ$ in 1N-NaOH (*c*, 2). The ultraviolet absorption is shown in Fig. 1. (Found in material dried in air at 25°: C, 41.3; H, 5.5; N, 24.6. Calc. for C₁₀H₁₃O₄N₅·1½H₂O: C, 41.3; H, 5.4; N, 24.2%.) (Found in material dried to constant weight over P₂O₅ at 100° (4 hr.; 1 mm.): C, 42.3; H, 5.0; N, 25.0%. Calc. for C₁₀H₁₃O₄N₅·H₂O: C, 42.1; H, 5.3; N, 24.6%.) (Found in material dried to constant weight over P₂O₅ at 140° (4 hr.; 1 mm.): C, 44.6; H, 5.2; N, 26.4, 26.3%. Calc. for C₁₀H₁₃O₄N₅: C, 44.9; H, 4.9; N, 26.2%.)

Crude hypoxanthine deoxyriboside was separated by Klein's (1938) procedure. To this was added the crude hypoxanthine deoxyriboside from the butanol extract. The yield of substance crystallized three times from hot water was 1.55 g. Further crystallization was required to obtain the pure compound. (Found in material dried in air at 25°: C, 47.2; H, 4.7; N, 22.4. Calc. for C₁₀H₁₂O₄N₄: C, 47.6; H, 4.8; N, 22.2%.) At about 206° the compound shrank somewhat in volume and began darkening slowly until at 310° it was brown. It did not melt or decompose suddenly. The ultraviolet absorption spectrum is shown in Fig. 2. $[\alpha]_D^{26} = -21.5^\circ$ in water (*c*, 1).

Thymine deoxyriboside (Klein's (1938) procedure). Yield of material melting at 182-184° = 0.53 g. The compound was crystallized once more from hot water: m.p. = 186-187°. (Found in material dried in air at 25°: C, 49.7; H, 5.7; N, 11.9. Calc. for C₁₀H₁₄O₆N₂: C, 49.6; H, 5.8; N, 11.6%.) $[\alpha]_D^{23} = +18.5^\circ$ in water (*c*, 2). The ultraviolet absorption spectrum is shown in Fig. 3.

Cytosine deoxyriboside was separated as the picrate by Klein's procedure (1938). The yield of the recrystallized salt was 2.37 g. It decomposed (black) without melting at

188-192°. (Found in material dried in air at 25°: C, 39.3; H, 3.2; N, 18.3. Calc. for C₁₁H₁₀O₁₁N₆: C, 39.5; H, 3.5; N, 18.4%.) Cytosine deoxyriboside was obtained in 80% yield by decomposition of the picrate. It crystallizes easily from hot absolute ethanol: m.p. 207-209°. (Found in material dried in air at 25°: C, 48.1; H, 5.8; N, 18.8. Calc. for C₉H₁₃O₄N₃: C, 47.6; H, 5.8; N, 18.5%.) $[\alpha]_D^{23} = +57.6^\circ$ in water (*c*, 2). The ultraviolet absorption spectrum is shown in Fig. 4.

Thymine deoxyriboside and cytosine deoxyriboside were more rapidly and conveniently prepared by the action of intestinal phosphatase upon the pyrimidine nucleotides. The dibarium salts (14.6 g.) of the pyrimidine diphosphonucleotides (Levene, 1921) were dissolved in cold water. (NH₄)₂SO₄ was added to precipitate the barium. The filtrate and washings were diluted to 500 ml.; 8 g. of MgSO₄·7H₂O and 50 ml. of saturated (NH₄)₂SO₄ solution were added. The pH was adjusted to 9.0 with 15N-NH₄OH; 50 ml. of calf intestinal phosphatase (Schmidt & Thannhauser, 1943) were added, and the solution was incubated as described previously. The filtrate was worked up for thymine deoxyriboside and cytosine deoxyriboside as before. Yield of thymine deoxyriboside of m.p. 184-185° = 0.44 g. Yield of cytosine deoxyriboside picrate (decomp. without melting at 184-189°) = 1.03 g.

Uracil deoxyriboside was prepared by the chemical deamination of cytosine deoxyriboside: 0.5 g. of cytosine deoxyriboside (m.p. 207-209°) was dissolved in 3.5 ml. of water. NaNO₂ (1.5 g.) and 1.5 ml. of glacial acetic acid were added. After 8.2 hr. at 24°, 0.4 g. of NaNO₂ and 0.8 ml. of glacial acetic acid were added. After standing at 24° for an additional 12 hr. the acetic acid was distilled off at 40° *in vacuo*. Water (30 ml.) was added, and the solution was again taken to dryness. The residue was dissolved in 180 ml. of water and 50 g. of moist H⁺-charged amberlite IR-100 were added to the solution. To remove the acetic acid released, the filtrate and washings were reduced to 10 ml. (inside temperature 15°). Water (20 ml.) was added, and the solution was reduced as before to 4 ml. The pH was adjusted to 7 with Ba(OH)₂. Absolute ethanol (100 ml.) was added, whereupon a yellow precipitate formed and was discarded. The filtrate was reduced to dryness. The white crystalline mass was taken up in 6 ml. of hot absolute ethanol, and a small amount of insoluble material was filtered off. Upon reducing the filtrate to 4 ml. on a hot-water bath and placing the beaker at room temperature crystallization began (10 min.). The material was filtered off, sucked dry, and washed with a small portion of absolute ethanol. Yield: 0.285 g., m.p. 162.5-163°. Second crop: 0.038 g., m.p. 162-163°. 65% of the theoretical was recovered in the combined portions. The compound was crystallized twice more from absolute ethanol. It separated in clusters of platelets about 1 mm. long; m.p. 163-163.5°. It solidifies upon cooling and melts the second time at 161-162.5°. $[\alpha]_D^{26} = +30.0^\circ$ in water (*c*, 2). (Found in material dried in air at 25°: C, 47.7; H, 5.3; N, 12.2. Calc. for C₉H₁₂O₄N₂: C, 47.6; H, 5.3; N, 12.3%.) The spectral data (Fig. 5) were identical with those reported for uridine (Ploeser & Loring, 1949).

A crude barium salt of deoxyribose-1-phosphate was prepared by a method similar to that reported (Friedkin, 1950). Hypoxanthine deoxyriboside (400 mg.) yielded 200 mg. of crude barium deoxyribose phosphate. Found: *ortho* P, precipitated as MgNH₄PO₄, nil; *ortho* P, precipitated as MgNH₄PO₄, after hydrolysis for 10 hr. at pH 4.0 and 22° (determined by the method of Berenblum & Chain, 1938),

Fig. 1. Guanine deoxyriboside. E calc. on the air-dried sample as $C_{10}H_{18}O_4N_5 \cdot 1\frac{1}{2}H_2O$.

Fig. 2. Hypoxanthine deoxyriboside.

Fig. 3. Thymine deoxyriboside.

Fig. 4. Cytosine deoxyriboside.

Figs. 1-5. Ultraviolet absorption spectra of deoxyribosides. The solutions at concentrations of 10-12 $\mu\text{g./ml.}$ were examined in a Beckman spectrophotometer. +—+, pH at 7.0 (weakly buffered with phosphate); o—o, pH at about 12 (30 $\mu\text{l.}$ of 5N-NaOH added per 3 ml. of weakly buffered solution); Δ — Δ , pH at about 1 (50 $\mu\text{l.}$ of 15N-HCl added per 3.03 ml. of alkaline solution).

3.75%. Barium deoxyribose-1-phosphate content, calculated from the labile P, = 43%. Yield = 16% of the theoretical. 10 μ l. (0.125 μ mol.) of deoxyribose-1-phosphate (calc. upon the basis of the labile P), incubated with 125 μ g. of hypoxanthine in 125 μ l. of 0.1 M-glycine buffer (pH, 8.8) and 125 μ l. (1.9 mg. dry wt.) of calf liver nucleoside phosphorylase (Friedkin & Kalckar, 1950) for 11 hr. at 24°, showed a net synthesis of 0.115 μ mol. of deoxyriboside as judged by the microbiological test.

Fig. 5. Uracil deoxyriboside.

Bacterial enzymes. *Lactobacillus helveticus* S and *Lb. delbrueckii* ATCC 9649 were transferred to the inoculum medium and grown in 250 ml. lots of the complete basal medium as previously described (MacNutt & Snell, 1950). Thymidine was used as the growth-promoting substance at

a level of 1 mg./250 ml. of final medium. After 24 hr. incubation at 37° the cells from 500 ml. of medium were collected by centrifugation and washed twice with 75 ml. portions of 0.05 M-citrate buffer at pH 6.0. The washed cells were suspended in 15 ml. of the same buffer and were broken up by agitation with glass beads for 30 min. in the electric tuning fork shaker (Mickle apparatus). During this process the cell suspension was cooled intermittently. Occasionally this suspension of broken bacteria was used, in which case it is referred to as 'undialysed enzyme'. 'Dialysed enzyme' was prepared by dialysing the above suspension against distilled water at 3°. The insoluble material was removed by centrifugation at low speed after the period of dialysis. The solution, preserved by freezing, retained its activity relatively well. The enzyme from *Thermobacterium acidophilus* R 26 was prepared as above from cells grown in a basal medium (Hoff-Jørgensen, 1951), supplemented with thymidine as described above.

Methods

The microbiological assay of deoxyribosides with *Thermobacterium acidophilus* R 26 was employed according to the described method (Hoff-Jørgensen, 1952). Thymine deoxyriboside was used as the standard.

Chromatography. One-dimensional ascending chromatography on paper was carried out in glass jars 11 × 48 cm. The compounds were placed along Whatman no. 4 filter paper in quantities of 10–20 μ g. of purine or pyrimidine per cm. The solvent (25–30 ml.) was added to the dry vessel; the paper was suspended above the solvent, and the system equilibrated for the periods indicated. The distance traversed by the solvent was 45 cm. No special precautions were taken to ensure a constant temperature (usually about 23°), and R_F values varied somewhat on independent runs. The following systems were employed: *n*-butanol-water-ammonia: 1 ml. of 15N-NH₄OH was added to 100 ml. of *n*-butanol saturated with water. After shaking thoroughly the clear supernatant was used. Equilibrated for 11 hr. Time to traverse 45 cm. = 1.5–2 days. *n*-Butanol-water-acetic acid: 25 ml. of *n*-butanol saturated with water + 5 ml. of glacial acetic acid.

Table 1. R_F values of deoxyribosides and related compounds

Compound	R_F values in system			
	<i>n</i> -Butanol-water-ammonia	<i>n</i> -Butanol-water-acetic acid	<i>n</i> -Butanol-water-ethyl acetate-morpholine	<i>n</i> -Butanol-water-morpholine-methyl glycol
Adenine	0.40	—	0.48	0.45
Guanine	0.15*	—	—	—
Hypoxanthine	0.19	—	0.36	—
Cytosine	0.28	—	—	—
Uracil	0.33	—	—	—
Thymine	0.50	—	—	—
4-Aminoglyoxaline-5-carbonamide	0.32	—	0.44	—
Adenine deoxyriboside	0.41	—	0.70	0.66
Guanine deoxyriboside	0.18	—	0.57	0.58
Hypoxanthine deoxyriboside	0.17	—	0.54	0.55
Cytosine deoxyriboside	0.26	0.35	0.70	0.68
Uracil deoxyriboside	0.34	0.44	0.88	0.77
Thymine deoxyriboside	0.48	0.56	0.94	0.85
Cytidine	0.15	—	—	—
5-Methylcytosine	0.36	—	—	—
Inosine	0.08	—	—	—
Uric acid	0.02	—	—	—

* It has been pointed out by Markham & Smith (1949) that the capacity of the system for guanine is very limited.

Equilibrated for 8 hr. Time to traverse 45 cm. = 1.5–2 days. *n*-Butanol-water-ethyl acetate-morpholine: 5 ml. of *n*-butanol saturated with water + 5 ml. of ethyl acetate saturated with water + 10 ml. of morpholine equilibrated for 24 hr. Time to traverse 45 cm. = 2–2.5 days. *n*-Butanol-water-morpholine-methylglycol: 10 ml. of *n*-butanol saturated with water + 10 ml. of morpholine + 5 ml. of methylglycol + 2 ml. of water. Equilibrated for 24 hr. Time to traverse 45 cm. = 3–3.5 days. The R_F values of the deoxyribosides and certain related compounds in the several systems are shown in Table 1.

RESULTS

The formation of thymine deoxyriboside by interaction of hypoxanthine deoxyriboside and thymine

The ease with which purine deoxyribosides are hydrolysed by acid (Levene & London, 1929) affords a convenient way of selectively destroying all the purine deoxyribosides in a mixture. This has also been demonstrated microbiologically (Hoff-Jørgensen, 1952). Thus, if a pyrimidine deoxyriboside were formed by the interaction of a purine deoxyriboside and a pyrimidine, it could easily be detected by the growth-promoting effect upon *Thermobact. acidophilus* R 26 after the purine deoxyriboside had been hydrolysed by dilute acid.

It was assumed at the outset that the exchange of the aglycone would proceed through the phosphorolytic cleavage of the nucleoside, followed by the exchange of the phosphate group of deoxyribose-1-phosphate for another purine or pyrimidine. For this reason xanthine oxidase was included in the incubation mixture in the following experiment.

Hypoxanthine deoxyriboside (0.78 μ mol., 40 μ l.), 1 ml. of 0.2M-phosphate buffer at pH 8.04, 1 mg. of thymine, 1 ml. of undialysed *Lb. helveticus* enzyme (20 mg. dry wt.), and xanthine oxidase (1 mg. of protein, 50 μ l.) from cream (Kalckar, Kjeldgaard & Klenow, 1950) were incubated at 37° (toluene added) for 10 hr. The control tube differed from the experimental in that hypoxanthine deoxyriboside was added after acidification. After adding 70 μ l. of 5N-HCl the tubes were heated in a boiling-water bath for 5 min.; water was added and the precipitate filtered off. The filtrate was neutralized with NaOH and assayed microbiologically. From the difference in the activity between control and experimental samples the total amount of pyrimidine deoxyriboside formed was computed.

To identify the pyrimidine deoxyriboside which had been formed, the solutions were freed from protein and excess inorganic salts and chromatographed on paper: eight-tenths of each sample (8 ml.) was reduced to 0.7 ml. at 70°. Absolute ethanol (5 ml.) was added. The supernatant and washings were evaporated to dryness at 65°, and the residue was extracted with 2 ml. of warm absolute ethanol. The filtrate and washings were again reduced to dryness. Water (1 ml.) was added to the residue. A sample (100 μ l.) was placed along 4 cm. of paper and chromatographed in the *n*-butanol-water-ammonia system. Indicator spots: thymine, thymine deoxyriboside and uracil. Beneath ultra-violet light the compounds appeared as dark spots on the paper.

The thymine-thymine deoxyriboside spots (R_F = 0.51) were cut out and extracted with water. Upon the basis of the difference in microbiological activity between the filtrates of the control and experimental spots, the total amount of pyrimidine deoxyriboside recovered in the spot at R_F = 0.51 was calculated. The spots of hypoxanthine which appeared on the chromatogram were also extracted with water, the amounts present being estimated enzymically (ΔE_{290}) (Kalckar, 1947). Any hypoxanthine set free by the bacterial enzyme should be immediately oxidized to uric acid by the xanthine oxidase present. Hence, the difference in hypoxanthine content between control and experimental areas should serve as a measure of the hypoxanthine released from the nucleoside during the course of the reaction.

In this experiment 0.55 μ mol. of pyrimidine deoxyriboside (71 % conversion) was formed in the reaction mixture and 0.36 μ mol. (65 % of the pyrimidine deoxyriboside found) was recovered from the spot on the chromatogram corresponding to thymine deoxyriboside. The poor recovery by

Fig. 6. The rate of formation of thymine deoxyriboside from hypoxanthine deoxyriboside plus thymine. 0.099 μ mol. of hypoxanthine deoxyriboside, 125 μ g. of thymine in 125 μ l. of 0.2M-phosphate buffer (pH 8.0), and *Lb. helveticus* enzyme (dialysed 7 hr.) 125 μ l. (2.4 mg. dry wt.). The reaction (at 37°) was initiated by the addition of enzyme and was stopped by the addition of 7 μ l. of 5N-HCl. The samples were hydrolysed and assayed as usual.

chromatography was most likely due to losses in manipulation, since only 0.49 μ mol. hypoxanthine (63 % of the original) was accounted for on the control chromatogram. These results suggest strongly that thymine exchanged enzymically with the hypoxanthine of hypoxanthine deoxyriboside to give thymine deoxyriboside. The amount of hypoxanthine released from the nucleosidic linkage (0.47 μ mol.) agreed fairly well with the amount of thymine deoxyriboside formed (0.36 μ mol.).

It was subsequently found that the exchange occurred equally well in the absence of phosphate buffer and xanthine oxidase. This led to a testing of deoxyribose-1-phosphate and a source of 2-deoxy-D-ribose (hydrolysed deoxyribose-1-phosphate) in the exchange system. Both proved to be ineffective. These results are reported below.

The rate of the reaction. In Fig. 6 the rate of formation of thymine deoxyriboside from hypoxanthine deoxyriboside plus thymine is presented. Under the conditions specified, equilibrium had been established within 6 hr. An incubation period of 10–11 hr. was adopted for all the subsequent experiments. It will be noted that in all the experiments a considerable excess of the purine or pyrimidine was employed.

The formation of various pyrimidine deoxyribosides.

Various pyrimidines were tested for their ability to react with hypoxanthine deoxyriboside to give pyrimidine deoxyribosides active as growth factors for *Thermobact. acidophilus* R 26. Thymine, uracil, and 5-methylcytosine were active (Table 2). Since

Table 2. *The ability of various pyrimidines to form microbiologically active pyrimidine deoxyribosides by the exchange reaction*

(0.39 μ mol. of hypoxanthine deoxyriboside, 500 μ g. of pyrimidine in 500 μ l. of 0.05M-alaninylglycine buffer (pH 8.5), and 500 μ l. (1.7 mg. dry wt.) of *Lb. helveticus* enzyme (dialysed 4 days). Hydrolysed and assayed as usual.)

Pyrimidine tested	Pyrimidine deoxyriboside formed (μ mol.)
Thymine	0.18
Uracil	0.17
5-Methylcytosine	0.20*
Methyl thiouracil	<0.005
5:5-Diethylbarbituric acid	<0.005
4:5:6-Triaminopyrimidine	<0.005

* Carried out in phosphate buffer (pH 8.04).

the exchange proceeded in the absence of phosphate there was no evidence that nucleoside phosphorylase played any important function in the transfer of the deoxyribosyl group from hypoxanthine to the pyrimidine in question.

Uracil deoxyriboside from hypoxanthine deoxyriboside. Uracil was incubated with hypoxanthine deoxyriboside as in the experiment with thymine except that dialysed (3 days) *Lb. helveticus* enzyme (4 mg. dry wt.) was used. The preparation of the solution for chromatography also differed in that the residue was extracted four times with boiling absolute ethanol (1.5 ml. each time). Chromatographic separation was carried out in the *n*-butanol-water-acetic acid system. Indicator spots: thymine deoxyriboside, uracil deoxyriboside, and cytosine deoxyriboside.

Hydrolysis and assay showed that 0.76 μ mol. of pyrimidine deoxyriboside was formed from 0.78 μ mol. of hypoxanthine deoxyriboside (97% conversion), while chromatography (Table 3) showed that 90% of the pyrimidine deoxyriboside formed was recovered from the spot with $R_F=0.40$ in the *n*-butanol-water-acetic acid system—the same value as that of uracil deoxyriboside. Uracil deoxyriboside, showed an activity of 1.03 per μ mol. in the microbiological assay, where 1 μ mol. of thymine deoxyriboside = 1.00. Uracil deoxyriboside is thus as active as the other natural deoxyribosides in supporting the growth of *Thermobact. acidophilus* R 26.

Thymine deoxyriboside and uracil deoxyriboside from guanine deoxyriboside. Guanine deoxyriboside (0.75 μ mol.) was incubated with thymine as described in Table 3, the only difference being that xanthine oxidase was omitted. Hydrolysis and microbiological assay revealed that 0.56 μ mol. of pyrimidine deoxyriboside had been formed (75% conversion). Chromatography of the ethanolic extract of the hydrolysed solutions in the *n*-butanol-water-ammonia system and assay of the appropriate areas showed that 0.48 μ mol. (86%) of the pyrimidine deoxyriboside formed was accounted for in the thymine deoxyriboside spot ($R_F=0.49$). The uracil deoxyriboside spot ($R_F=0.32$) and the cytosine deoxyriboside spot ($R_F=0.25$) each showed less than 0.01 μ mol. of pyrimidine deoxyriboside.

Similar figures were obtained when uracil was used. 0.54 μ mol. (72% conversion) of pyrimidine deoxyriboside was formed, of which, by chromatography in the *n*-butanol-water-acetic acid system, 0.47 μ mol. (87%) was accounted for in the uracil deoxyriboside spot ($R_F=0.39$). The thymine deoxyriboside ($R_F=0.49$) and cytosine deoxyriboside ($R_F=0.30$) spots each showed less than 0.01 μ mol. of pyrimidine deoxyriboside.

Cytosine deoxyriboside from hypoxanthine deoxyriboside and cytidine. 0.20 μ mol. of hypoxanthine deoxyriboside, 400 μ g. of cytidine (Hoffman-La Roche), 200 μ l. of 0.2M-phosphate buffer at pH 8.04, and 200 μ l. of *Lb. helveticus* enzyme (dialysed 7 hr.; 3.9 mg. dry wt.) were incubated as usual. The reaction was stopped by the addition of 7 ml. of ethanol. In the control tube the hypoxanthine deoxyriboside was added after ethanol had been added. The ethanolic extract was prepared and chromatographed in the *n*-

Table 3. *Formation of uracil deoxyriboside from hypoxanthine deoxyriboside*
(0.76 μ mol. total pyrimidine deoxyriboside was found by direct assay of the hydrolysed mixture.)

Sample	Deoxyriboside found (μ mol.)		
	Thymine deoxyriboside spot ($R_F=0.50$)	Uracil deoxyriboside spot ($R_F=0.40$)	Cytosine deoxyriboside spot ($R_F=0.32$)
Control	<0.01	<0.01	<0.01
Experimental	0.02	0.70	0.01

butanol-water-ammonia system. Found: 0.13 μ mol. of cytosine deoxyriboside ($R_F=0.26$) formed, and 0.12 μ mol. of hypoxanthine deoxyriboside ($R_F=0.14$) disappeared.

Since large amounts of cytosine appeared on the chromatogram it seems likely that the reaction involved the prior splitting of cytidine to give cytosine which then entered into the exchange reaction. The difference in the rate at which these enzyme preparations split the ribosides and the deoxyribosides is remarkable (see Kalckar, MacNutt & Hoff-Jørgensen, 1952). It should be noted that cytosine deoxyriboside was not deaminated by the enzyme preparation.

Thymine deoxyriboside and uracil deoxyriboside from cytosine deoxyriboside. 0.44 μ mol. of cytosine deoxyriboside (40 μ l.), 500 μ l. of pyrimidine, 500 μ l. of *Lb. helveticus* enzyme (dialysed 3 days, 4.0 mg.), 500 μ l. of 0.2M-phosphate buffer at pH 8.04 were incubated and hydrolysed as usual. The ethanolic extract was chromatographed in the *n*-butanol-water-acetic acid system. The results (Table 4) show that thymine and uracil exchange enzymically with the cytosine of cytosine deoxyriboside. Since the residual cytosine deoxyriboside from the exchange reaction with thymine (Exp. A) was not deaminated to uracil deoxyriboside the origin of uracil deoxyriboside in Exp. B must proceed by the exchange of the entire pyrimidine or by a transamination involving positions six of cytosine deoxyriboside and uracil. This latter possibility was not excluded, but an analogous case in the purine series (adenine and hypoxanthine deoxyriboside) has been shown to involve the transfer of the purine skeleton (Kalckar *et al.* 1952).

5-Methylcytosine deoxyriboside and its chemical deamination to give thymine deoxyriboside. 0.37 μ mol. of guanine deoxyriboside, 500 μ l. of 5-methylcytosine, 500 μ l. of 0.2M-phosphate buffer at pH 8.04, and 500 μ l. of *Lb. helveticus* enzyme (dialysed 1 day, 1.7 mg.) were incubated. Ethanol was added and the ethanolic extract was chromatographed in the *n*-butanol-water-ammonia system. The spots were assayed as usual. The results (Table 5) indicated that 5-methylcytosine deoxyriboside had an R_F value of 0.32, which would distinguish it from thymine deoxyriboside and cytosine deoxyriboside but not from uracil deoxyriboside ($R_F=0.30$). The presumed 5-methylcytosine deoxyriboside was next separated from uracil deoxyriboside in the *n*-butanol-water-morpholine-methyl glycol system. The

results were as follows: thymine deoxyriboside formed ($R_F=0.83$), <0.01 μ mol.; uracil deoxyriboside formed ($R_F=0.75$), <0.01 μ mol.; deoxyriboside formed ($R_F=0.66$), 0.25 μ mol.; guanine deoxyriboside lost ($R_F=0.57$), 0.22 μ mol. The presumed 5-methylcytosine deoxyriboside has an R_F value in the *n*-butanol-water-morpholine-methyl glycol system which lies close to that of cytosine deoxyriboside.

Thus, by means of chromatography in two systems, it was shown that 5-methylcytosine reacted enzymically with purine deoxyribosides to yield a pyrimidine deoxyriboside which differed from thymine, uracil and cytosine deoxyribosides. To identify it as 5-methylcytosine deoxyriboside the substance was converted into thymine deoxyriboside by treatment with HNO_3 .

Portions (50 μ l.) of the ethanolic extracts were placed on two sheets and chromatographed in the *n*-butanol-water-ammonia system. From one of them the desired areas were removed and saved for assay. From the other, the control and experimental areas corresponding to the location of the new deoxyriboside ($R_F=0.35$) were excised and extracted with boiling ethanol. The supernatant and washings were evaporated to dryness on the water bath. The residues were treated with 65 μ l. of water, 65 μ l. of glacial acetic acid, and 70 mg. of NaNO_2 added in portions over a period of 2 days. The acetic acid was distilled off, the residues were dissolved in water and treated with 2 g. of H^+ -charged amberlite IR-100-H. The filtrate and washings were reduced to 2 ml. at 40°. $\text{Ba}(\text{OH})_2$ was added to adjust the pH to 8.0. CO_2 was passed in to neutralize the excess of $\text{Ba}(\text{OH})_2$. The residues were extracted with ethanol, but since considerable amounts of inorganic material dissolved, the final extraction was carried out with ethyl acetate. The residues were transferred to the paper and chromatographed in the *n*-butanol-water-ammonia system. The appropriate areas were cut out and assayed microbiologically. Since, of the five deoxyribosides tested (Kitay *et al.* 1949) *Leuconostoc citrovorum* 8081 responds only to thymine deoxyriboside, the untreated and the deaminated substances were tested as possible growth factors for this organism. The results (Table 6) show that the substance presumed to be 5-methylcytosine deoxyriboside was inactive as a growth factor for *Leuc. citrovorum* and was converted into thymine deoxyriboside by treatment with HNO_3 . The recovery was poor (15–16 %).

Table 4. Conversion of cytosine deoxyriboside into thymine and uracil deoxyriboside

Exp.	Pyrimidine added	Sample	Deoxyriboside found (μ mol.)		
			Thymine deoxyriboside spot ($R_F=0.54$)	Uracil deoxyriboside spot ($R_F=0.44$)	Cytosine deoxyriboside spot ($R_F=0.35$)
A	Thymine	Control	<0.01	<0.01	0.33
		Experimental	0.32	<0.01	0.04
B	Uracil	Control	<0.01	0.01	0.36
		Experimental	<0.01	0.41	0.05

Table 5. Formation of 5-methylcytosine deoxyriboside from guanine deoxyriboside

Sample	Deoxyriboside found (μ mol.)			
	Thymine deoxyriboside spot ($R_F=0.46$)	5-Methylcytosine deoxyriboside spot ($R_F=0.32$)	Cytosine deoxyriboside spot ($R_F=0.25$)	Guanine deoxyriboside spot ($R_F=0.16$)
Control	<0.01	0.01	<0.01	0.30
Experimental	<0.01	0.23	<0.02	0.11

Table 6. *Deamination of 5-methylcytosine deoxyriboside to thymine deoxyriboside*

Treatment	Net amount* of deoxyriboside found ($\mu\text{mol.} \times 10^{-6}$)			
	Assayed with <i>Thermobact. acidophilus</i> R 26		Assayed with <i>Leuc. citrovorum</i> 8081†	
	Thymine deoxyriboside spot ($R_F=0.47$)	5-Methylcytosine deoxyriboside spot ($R_F=0.34$)	Thymine deoxyriboside spot ($R_F=0.48$)	5-Methylcytosine deoxyriboside spot ($R_F=0.34$)
None	8	850	1	2
Deaminated with nitrous acid	140	9	130	-1

* The amount in the experimental sample minus the amount in the control sample (thymine deoxyriboside as the standard).

† 48 hr. growth in a described medium (MacNutt & Snell, 1950).

The formation of purine deoxyribosides

When purines are tested in the exchange system by the reverse process to that shown in Table 2, the exchange of any purine with the pyrimidine of a pyrimidine deoxyriboside will cause a loss in the growth-promoting activity of the mixture after hydrolysis, whether the purine deoxyriboside formed be itself active or inactive as a growth factor for *Thermobact. acidophilus*. Also, were the pyrimidine deoxyriboside merely split by the enzyme every purine tested would seem to enter into

Table 7. *Ability of various purines and related compounds to enter into the exchange reaction*

(0.368 $\mu\text{mol.}$ of thymine deoxyriboside, 500 $\mu\text{g.}$ of the given compound in 500 $\mu\text{l.}$ of alanyl-glycine buffer, etc. as in Table 2.)

Purine or related compound	Purine deoxyribose formed ($\mu\text{mol.}$)
Adenine	0.260
Guanine	0.155*
Hypoxanthine	0.295
Xanthine	0.300
4-Aminoglyoxaline-5-carbonamide.HCl	0.295
	0.303†
Formamido malonamide.HCl	0.02
Uric acid	0.02
2:6-Diaminopurine. $\frac{1}{2}\text{H}_2\text{O} \cdot \frac{1}{2}\text{H}_2\text{SO}_4$	0.03
4:5:6-Triaminopyrimidine	0.03
Formyl-4:5:6-triaminopyrimidine	-0.01
Dihydrothymine†	0.02†

* Since guanine is so insoluble its concentration could not be brought to that of the other compounds tested.

† Phosphate buffer at pH 8.04.

† 0.44 $\mu\text{mol.}$ of cytosine deoxyriboside incubated with 500 $\mu\text{g.}$ of dihydrothymine (a gift from Dr H. K. Mitchell).

the exchange reaction, unless the purine were by chance an inhibitor of the hydrolysis. Since previous experiments had shown that relatively good yields of pyrimidine deoxyribosides could be obtained it did not seem likely that the splitting of the pyrimidine deoxyriboside would offer serious difficulty. Results showing the amount of deoxyriboside split by this enzyme preparation in the absence of an acceptor (about 20 %) are given later.

Four purines and 4-aminoglyoxaline-5-carbonamide (Table 7) apparently reacted enzymically with thymine deoxyriboside to give purine deoxyribosides, in that there was a loss of growth-promoting activity after mild treatment of the mixture with acid. Dihydrothymine was tested as though it were a purine, since it has been shown (Levene & Bass, 1931) that dihydrouridine is as easily hydrolysed by acid as purine ribosides. The negative result with uric acid was further investigated to determine whether it was due to failure to enter into the exchange reaction or to the formation of a microbiologically active deoxyriboside which resists mild acid hydrolysis, since uric acid riboside shows abnormal resistance to acid hydrolysis (Davis, Newton & Benedict, 1922).

Thymine deoxyriboside, uric acid, phosphate buffer, and enzyme were incubated as usual and treated with ethanol; the ethanolic extract was chromatographed in the *n*-butanol-water-ammonia system. In this system one would expect uric acid deoxyriboside to have an R_F value close to that of uric acid ($R_F=0.02$) and certainly much lower than that of thymine deoxyriboside. The amount of thymine deoxyriboside ($R_F=0.48$) recovered from the experimental sample (0.45 $\mu\text{mol.}$) was in very good agreement with that of the control (0.46 $\mu\text{mol.}$). It is inferred, therefore, that uric acid did not enter into the exchange reaction.

The formation of adenine deoxyriboside from uracil deoxyriboside. A further investigation of the purine deoxyriboside formed by the interaction of adenine and pyrimidine deoxyribosides showed that adenine deoxyriboside was formed and that it was not deaminated to hypoxanthine deoxyriboside.

Uracil deoxyriboside (0.44 $\mu\text{mol.}$), adenine (500 $\mu\text{g.}$), etc. were incubated in the usual way. The ethanolic extract of the unhydrolysed incubation mixture was chromatographed in the *n*-butanol-water-ammonia system, and the chromatogram assayed. 0.37 $\mu\text{mol.}$ of adenine deoxyriboside ($R_F=0.40$) was formed, 0.45 $\mu\text{mol.}$ of uracil deoxyriboside ($R_F=0.34$) disappeared, and less than 0.01 $\mu\text{mol.}$ of hypoxanthine deoxyriboside ($R_F=0.15$) was formed.

The exchange of one purine for another. Since the enzyme preparation was without adenine deoxyriboside deaminase activity, the exchange of adenine for the hypoxanthine of hypoxanthine deoxyriboside and the guanine of guanine deoxyriboside could easily be demonstrated (Table 8). The possibility that transamination between positions six of adenine and hypoxanthine deoxyriboside occurs has been ruled out by use of radioactive adenine (Kalekar *et al.* 1951).

The unknown deoxyriboside formed from the reaction of xanthine with uracil deoxyriboside. Uracil deoxyriboside (0.44 $\mu\text{mol.}$), xanthine (500 $\mu\text{g.}$), etc., were incubated, the

Table 8. *Exchange of adenine for the hypoxanthine of hypoxanthine deoxyriboside and the guanine of guanine deoxyriboside*(Purine deoxyriboside, 500 μ g. of adenine, and conditions as in Table 5.)

Deoxyriboside added	Sample	Deoxyriboside found (μ mol.)		
		Adenine deoxyriboside spot ($R_F=0.40-0.42$)	Hypoxanthine deoxyriboside and guanine deoxyriboside spots ($R_F=0.16-0.15$)	All other areas
Hypoxanthine deoxyriboside (0.41 μ mol.)	Control	<0.01	0.32	<0.01
	Experimental	0.12	0.14	<0.01
Guanine deoxyriboside (0.40 μ mol.)	Control	<0.01	0.38	<0.01
	Experimental	0.28	0.05	<0.01

Table 9. *The deoxyriboside formed when 4-aminoglyoxaline-5-carbonamide is incubated with thymine deoxyriboside*

(Thymine deoxyriboside (0.44 μ mol.), 4-aminoglyoxaline-5-carbonamide.HCl (500 μ g.) in 500 μ l. of 0.2M-phosphate buffer (pH 8.04), 500 μ l. (1.7 mg.) of *Lb. helveticus* enzyme, dialysed 1 day, were incubated as usual. The total 'purine deoxyriboside' formed, estimated by hydrolysis and assay was 0.37 μ mol.)

Sample	Deoxyriboside found by chromatography of the unhydrolysed samples in the <i>n</i> -butanol-water-ammonia system and assay of the appropriate areas (μ mol.)			
	Thymine deoxyriboside spot. $R_F=0.49$	Adenine deoxyriboside. $R_F=0.39$	Hypoxanthine and guanine deoxyribosides. $R_F=0.16$	Unknown deoxyriboside spot. $R_F=0.31$
Control	0.47	0.01	0.01	0.03
Experimental	0.10	0.01	0.02	0.10

ethanolic extract prepared and chromatographed as in Table 5. Beneath ultraviolet light the control sample showed two spots: $R_F=0.05$ (xanthine) and $R_F=3.04$ (uracil deoxyriboside). In the experimental sample three spots appeared: $R_F=0.34$ (uracil and residual uracil deoxyriboside), $R_F=0.05$ (xanthine), and a third dark area, partially overlapping the xanthine spot, at $R_F=0.06$. The areas at $R_F=0.34$, $R_F=0.05-0.06$, and $R_F=0.18-0.20$ (where hypoxanthine deoxyriboside and guanine deoxyriboside would appear) were assayed and gave the following results: Uracil deoxyriboside lost, 0.37 μ mol.; unknown deoxyriboside ($R_F=0.05-0.06$) formed, 0.16 μ mol.; hypoxanthine or guanine deoxyriboside formed, 0.02 μ mol. Since in the *n*-butanol-water-ammonia system the known deoxyribosides and their respective purines and pyrimidines have R_F values which are close together one would expect xanthine deoxyriboside to appear near the xanthine spot. The R_F value of the unknown deoxyriboside was less than that of any of the known deoxyribosides; whether it was actually xanthine deoxyriboside was not further investigated.

The quantitative aspects of this experiment were very unsatisfactory, only 43% of the uracil deoxyriboside which disappeared being recovered in the form of the unknown deoxyriboside. There was a pronounced drift in the microbiological assay of this substance, which possibly means that the shape of the growth curve with the unknown deoxyriboside differed from that of the standard curve with thymine deoxyriboside. The substance may not have been as active in supporting growth as an equimolar quantity of thymine deoxyriboside; greater losses than those encountered in the case of other deoxyribosides may have

occurred in the preparation of solutions of this substance for chromatography; or actual loss may have occurred when this substance was autoclaved with the medium (pH 6.8) at 120°.

The reaction product of 4-aminoglyoxaline-5-carbonamide and thymine deoxyriboside. It may be seen from Table 8 that a 'purine deoxyriboside' was apparently formed when 4-aminoglyoxaline-5-carbonamide was incubated with thymidine in the presence of the enzyme from *Lb. helveticus*. Since it has been claimed that 4-amino-5-imidazole carboxamide is a precursor of purines in bacteria (Shive, Ackermann, Gordon, Getzendaner & Eakin, 1947) the possibility that the deoxyriboside of this compound had been synthesized enzymically was further examined.

A typical balance experiment (Table 9) shows that the amount of 'purine deoxyriboside' formed (0.37 μ mol.), as judged by the loss of microbiological activity following hydrolysis, agreed well with the amount of thymine deoxyriboside which disappeared (0.37 μ mol.), as judged by chromatography of the unhydrolysed mixtures and assay. But the amount of unknown deoxyriboside formed (0.07 μ mol.), as measured by direct assay of the chromatogram, was only 19% of the thymine deoxyriboside which disappeared, an even lower yield of purine deoxyriboside than that obtained from the reaction of xanthine and uracil deoxyriboside (43%). In the microbiological assay of this substance, too, the drift was serious. The data merely suggested that a purine deoxyriboside, which differed from all of the known purine deoxyribosides in having an R_F value of 0.31 in the *n*-butanol-water-ammonia system and which showed some microbiological activity toward *Thermobact. acidophilus* R 26, was formed in the reaction between

4-aminoglyoxaline-5-carbonamide and thymine deoxyriboside.

Another experiment was therefore carried out to identify the product more certainly. Thymine deoxyriboside (0.740 $\mu\text{mol.}$), 500 $\mu\text{g.}$ of 4-aminoglyoxaline-5-carbonamide. HCl, 500 $\mu\text{l.}$ of 0.2 M-phosphate buffer (pH 8.04), and 500 $\mu\text{l.}$ (3.2 mg.) of *Lb. delbrueckii* enzyme (dialysed for 3 days) were incubated at 37° for 10 hr. (toluene used). Ethanol was added, and a sample of the ethanolic extract was chromatographed in the *n*-butanol-ethyl acetate-water-morpholine system. The control sample showed two spots, one at $R_F = 0.94$ (thymine deoxyriboside) and the other at $R_F = 0.44$ (4-aminoglyoxaline-5-carbonamide). The experimental sample showed three spots, one at $R_F = 0.83$ (presumably thymine), another at $R_F = 0.44$ (4-aminoglyoxaline-5-carbonamide), and a faint spot at $R_F = 0.59$. Microbiological assay of the sheet showed that 0.71 $\mu\text{mol.}$ of thymine deoxyriboside had disappeared. The areas at $R_F = 0.59$ assayed as follows: control, 0.007 $\mu\text{mol.}$, experimental, 0.150 $\mu\text{mol.}$ of deoxyriboside present. The activity thus found by direct assay (0.143 $\mu\text{mol.}$) accounted for only 20% of the thymine deoxyriboside which disappeared, a result similar to that obtained with the *Lb. helveticus* enzyme (Table 9). There was also considerable drift in the assay of the substance.

Several chromatograms were run, and the control and experimental areas corresponding to $R_F = 0.59$ were collected, cut into small pieces and extracted with water. The solutions were reduced to dryness *in vacuo*. Microbiological assay showed that during the course of this operation the activity had been destroyed, due presumably to hydrolysis in the unbuffered solution. The material thus obtained was re-chromatographed in the *n*-butanol-water-ammonia system.

In the control sample nothing was visible, while the experimental sample showed a dark spot at $R_F = 0.30$. (Found for 4-aminoglyoxaline-5-carbonamide, $R_F = 0.32$.) The two areas were cut out, extracted with water, and the amounts of 4-aminoglyoxaline-5-carbonamide present in the filtrates determined by the Bratton & Marshall (1939) test at $\lambda = 530$; found in the control, 0.002 $\mu\text{mol.}$, and in the experimental, 0.105 $\mu\text{mol.}$

The filtrate from the control area was placed in the reference cuvette, and the ultraviolet absorption spectrum of the substance was determined. Found, $E_{\text{max.}} = 264 \text{ m}\mu$. (Found by Stetten & Fox (1945) for 4-aminoglyoxaline-5-carbonamide, $E_{\text{max.}} = 264 \text{ m}\mu$.) Upon the basis of the extinction at 264 $\text{m}\mu$. the amount of 4-aminoglyoxaline-5-carbonamide was 0.111 $\mu\text{mol.}$, a value in fair agreement with that found by the Bratton & Marshall test (0.103 $\mu\text{mol.}$).

These findings are consistent with the idea that 4-aminoglyoxaline-5-carbonamide deoxyriboside was formed by the exchange reaction.

The inactivity of deoxyribose-1-phosphate in the exchange system

Deoxyribose-1-phosphate was tested in this system with the various purines and pyrimidines which entered into the exchange reaction. In no case was any appreciable deoxyriboside formed.

Barium deoxyribose-1-phosphate preparation (8.5 mg.) was dissolved in 1.3 ml. of water. Na_2SO_4 (7 mg.) was added, and the BaSO_4 was removed by centrifugation. A sample (600 $\mu\text{l.}$) of the supernatant and 500 $\mu\text{l.}$ of M-citrate buffer (pH = 3.7) were left at 26° for 10 hr.; 150 $\mu\text{l.}$ of 5 N-NaOH were then added bringing the pH to 7. This hydrolysed solution was used in the control mixture. Another sample of 600 $\mu\text{l.}$ of the supernatant was kept in the frozen state while the first portion was hydrolysing. It was then melted and added last to 650 $\mu\text{l.}$ of the neutralized citrate solution. Samples (125 $\mu\text{l.} = 0.474 \mu\text{mol.}$) of the deoxyribose-1-phosphate solution or the hydrolysed solution were incubated with 200 $\mu\text{g.}$ of purine or pyrimidine in 200 $\mu\text{l.}$ of 0.1 M-sodium citrate (pH = 7.0), and 200 $\mu\text{l.}$ of *Lb. helveticus* enzyme (dialysed 3 days). The tubes were then heated at 100° for 5 min. The solutions were diluted, filtered, if necessary, and assayed microbiologically. The difference between control and experimental samples was used to calculate the amount of deoxyriboside formed. The authenticity of the deoxyribose-1-phosphate preparation was checked with the calf liver nucleoside phosphorylase system, in which 0.474 $\mu\text{mol.}$ formed 0.36 $\mu\text{mol.}$ of deoxyriboside on incubation with hypoxanthine.

The results (Table 10) show that the *Lb. helveticus* enzyme did not catalyse the synthesis of deoxyribosides from deoxyribose-1-phosphate and the various purines and pyrimidines which enter into the exchange reaction. Moreover, the deoxyribose-1-phosphate preparation did not contain any inhibitor of the *Lb. helveticus* enzyme, since the same amount of exchange took place in the presence of the deoxyribose-1-phosphate as in its absence, and large amounts of phosphate did not inhibit the exchange reaction, although an inhibition might reasonably be expected if deoxyribose-1-phosphate were the intermediate in the exchange reaction.

Table 10. *Inactivity of deoxyribose-1-phosphate in the exchange system with Lb. helveticus extract*

(Description in text.)			
Purine or pyrimidine added	Deoxyribose-1-phosphate	Hypoxanthine deoxyriboside	Deoxyriboside formed ($\mu\text{mol.}$)
Hypoxanthine*	+	—	<0.005
Thymine	+	—	<0.005
	+	+	0.11†
	—	+	0.12†
	—	+	0.12†‡

* Also, adenine, guanine, xanthine, 4-amino-5-imidazole carboxamide, and uracil.

† Determined in the usual way following mild acid hydrolysis.

‡ 0.2 M-Phosphate buffer (pH 7.0) used instead of citrate buffer.

There is nothing here to suggest that deoxyribose-1-phosphate plays any role in the exchange reaction.

The inactivity of deoxyribose in the exchange system

If deoxyribose-1-phosphate is dismissed as the intermediate in the interconversion of deoxyribosides, the only reasonable question remaining is whether the reaction involves (1) hydrolysis followed by resynthesis, or (2) trans-*N*-glycosidation. An investigation was made of the comparative amounts of deoxyriboside hydrolysed and of deoxyribose transferred by these enzyme preparations in the same period of time.

1.9 mg.). (2) Same as (1) plus 0.24 μ mol. of deoxyribose-1-phosphate, hydrolysed at pH 4.0 for 11 hr. at 28° (60 μ l. of solution). (3) Same as (2) plus 0.099 μ mol. of hypoxanthine deoxyriboside (10 μ l. of solution). The tubes were incubated and adjusted to pH 1. To (1) and (2) 10 μ l. of hypoxanthine deoxyriboside solution was added, and all tubes were heated at 100° for 5 min., filtered, the filtrates neutralized and assayed microbiologically for pyrimidine deoxyriboside. Found (μ mol.): (1) 0.005; (2) 0.005; (3) 0.063. It was hence concluded that the mechanism of the transfer does not involve mere hydrolysis followed by resynthesis.

Table 11. *The comparative amounts of deoxyriboside hydrolysed and deoxyribose transferred*

Deoxyriboside tested	μ mol. added	Hydrolysis experiment		Transfer experiment	
		Deoxyriboside hydrolysed		Purine or pyrimidine added	Deoxyribose transferred
		(μ mol.)	(%)		(μ mol.) (%)
Cytosine deoxyriboside	0.110	—	—	Adenine	-0.004* -4
Cytosine deoxyriboside	0.110	0.019	17	Adenine	0.097 88
Uracil deoxyriboside	0.088	0.019	23	Adenine	0.083 94
Thymine deoxyriboside	0.103	0.023	22	Adenine	0.085 82
Hypoxanthine deoxyriboside	0.099	0.020	20	Thymine	0.076 76
Guanine deoxyriboside	0.094	0.017	18	Thymine	0.068 72

* Enzyme boiled prior to incubation.

Transfer reaction. 125 μ g. of purine or pyrimidine, 125 μ l. of 0.1 M-glycine buffer (pH 8.5), 10–15 μ l. of deoxyriboside solution and 125 μ l. (0.42 mg.) of *Lb. helveticus* enzyme, (dialysed 1 day) were incubated at 37° for 10 hr. The incubation was stopped with 8 μ l. of 5N-HCl. In the control tubes the deoxyribosides were added after the acid had been added. The solutions were hydrolysed, diluted and centrifuged, the supernatant solutions, after neutralization, being assayed as usual.

Hydrolysis. As above, with the purine or pyrimidine omitted. In the case of the purine deoxyribosides, however, the reaction was stopped by heating the incubation mixture at 100° for 5 min., the control tube having the purine deoxyriboside added after the period of heating.

The results (Table 11) show that during a period in which about 80 % of deoxyribose was transferred, about 20 % of the deoxyriboside was split. It might be argued that in the presence of an excess of acceptor, which would combine with one of the products of hydrolysis, an exchange in excess of the hydrolysis in the absence of an acceptor would take place. Thus, 80 % of deoxyribose transferred might not be considered as unreasonable if resynthesis occurred.

The question of resynthesis was next examined. Since crystalline 2-deoxy-D-ribose was not available for testing, a solution of deoxyribose was prepared by the very mild hydrolysis of deoxyribose-1-phosphate. The mixtures were as follows: (1) Thymine (100 μ g.) in 100 μ l. of 0.1 M-sodium citrate at pH 7.0, 100 μ l. of *Lb. helveticus* enzyme (dialysed for 7 hr.;

The presence of the enzyme in other bacteria

Enzyme preparations from two other bacteria, *Lb. delbrueckii* and *Thermobact. acidophilus* R 26, both of which require a deoxyriboside for growth, also catalyse the transfer (Table 12). The enzyme

Table 12. *Exchange reaction using enzymes from Lb. delbrueckii and Therm. acidophilus R 26*

(Hypoxanthine deoxyriboside (0.099 μ mol.) added as donor.)

Bacterial enzyme	Pyrimidine added	Pyrimidine deoxyriboside formed (μ mol.)
<i>Thermobact. acidophilus</i> R 26 (1.1 mg.)	Thymine	0.069
	Uracil	0.057
<i>Lb. delbrueckii</i> (0.81 mg.)	Thymine	0.064
	Uracil	0.061

preparation from *Thermobact. acidophilus* R 26 was tested for its ability to catalyse the synthesis of hypoxanthine deoxyriboside from deoxyribose-1-phosphate plus hypoxanthine. The result was negative: Found, 0.004 μ mol. of deoxyriboside formed from 0.474 μ mol. of deoxyribose phosphate. The possibility that enzymes of the type described may in some way be related to the requirement of bacteria for deoxyribosides should be further investigated.

An attempt to demonstrate the trans-reaction in a growth experiment with cells of Leuc. citrovorum

This organism responds specifically to thymine deoxyriboside. If, thymine and any of the deoxyribosides to which the organism does not respond in growth are added to the basal medium; if the substances enter the cell, and if a trans-reaction of the above type occurs, thymine deoxyriboside would be formed, and growth would result. A positive result would be consistent with the other experiments. A negative result, of itself, would tell very little: Three deoxyribosides (uracil deoxyriboside, cytosine deoxyriboside and hypoxanthine deoxyriboside) were each tested in combination with thymine. In no case was any growth detected after 28 hr. incubation.

DISCUSSION

Through the use of bacterial enzymes, which catalyse the transfer of the deoxyribosyl group from one purine or pyrimidine to another, it was possible to synthesize certain deoxyribosides, identified by chromatographic methods and microbiological assay, which have not yet been detected as constituents of cells. The questions arise as to whether these deoxyribosides may be components of the bacterial nucleic acid, whether they serve as intermediates in the synthesis of bacterial nucleic acid, or whether they are mere biochemical curiosities.

A considerable number of purines and pyrimidines act as acceptors in the transfer of the deoxyribosyl group, but uric acid, 2:6-diaminopurine, dihydrothymine, and other compounds are inactive. Of the new deoxyribosides which have been detected in this way uracil deoxyriboside is of interest, since it may be envisaged as a precursor of both cytosine deoxyriboside and thymine deoxyriboside. The existence of an enzymic mechanism for the synthesis of 5-methylcytosine deoxyriboside seems reasonable in view of recent work in which it has been established that 5-methylcytosine is a component of the deoxyribonucleic acid of several animal and plant species (Wyatt, 1950). Ever since the original claims made for 4-aminoglyoxaline-5-carbonamide (Shive *et al.* 1947) this compound has been mooted as an intermediate in purine metabolism (Fries, Bergström & Rottenberg, 1949; Greenberg, 1950; Miller, Gurin & Wilson, 1950; Schulman, Buchanan & Miller, 1950; Gotz, 1950). The possibility that 4-aminoglyoxaline-5-carbonamide deoxyriboside may be formed by these bacterial enzymes does not clarify the part it might play in the formation of the bacterial deoxyribonucleic acid. The organism may simply replace the aglycone with appropriate and completely formed purines or pyrimidines which are present in the

medium or which the bacterium may synthesize. Such an exchange between thymine and the aglycones of several deoxyribosides was investigated in living cells of *Leuc. citrovorum*. In no case was thymine deoxyriboside formed, but the negative finding is of little value without knowledge of the permeability of the cell to thymine and the deoxyribosides.

Whether enzymes of this type function in the biosynthesis of nucleic acids in higher animals is difficult to assess. The findings with these bacterial enzymes are in contrast with the data showing that pyrimidines are not incorporated into the nucleic acids of rats, while the cytosine of cytidine (Hammarsten, Reichard & Saluste, 1950) and the cytosine and thymine of the corresponding deoxyribosides are incorporated. It would be of interest to know, whether the ribose or deoxyribose of the administered nucleoside is distributed among all of the purines and pyrimidines of the nucleic acids, for which purpose nucleosides labelled not in the pyrimidine- but in the sugar-portion of the molecules would be required, and whether the bacterial enzymes will catalyse the transfer of purines and pyrimidines in polynucleotides.

The remarkable rapidity with which the enzyme preparations split the ribosides is a technical difficulty which must be first solved before any trans-type reaction can be demonstrated in this series by use of these enzymes—if, indeed, such occurs (Kalckar *et al.* 1951).

Since both deoxyribose-1-phosphate and deoxyribose (the only two compounds which might reasonably be expected to act as intermediates) are inactive in the exchange system the enzyme is considered to be a trans-*N*-glycosidase. The similarity of these reactions to those catalysed by the trans-glucosidase from *Pseudomonas saccharophila* (Doudoroff *et al.* 1947) is most remarkable, although alcoholysis (and phosphorolysis) of glucose-1-phosphate is catalysed by that enzyme preparation, whereas, in the system here described, aminolysis of deoxyribose-1-phosphate does not occur. A β -glucosidase from *Salix purpurea* L. has been described which catalyses the transfer of the glucosyl group from phenols to primary alcohols (Rabaté, 1935). 'Trans-pentosidation' from guanine to hypoxanthine has been claimed (Kritskii, 1950). A trans-phosphorylase catalyses the transfer of the phosphoryl group from phenols to primary alcohols (Axelrod, 1948), and the same type of reaction has been extended to include the phosphorylation of glycerol by creatine phosphate (Meyerhof & Green, 1950). A trans-peptidase has been reported (Hanes, Hird & Isherwood, 1950) which catalyses the aminolysis of γ -glutamyl peptides by amino-acids. In analogy with this trans-peptidase one may suppose that esterases which catalyse the transfer of acyl

groups from one alcohol to another will be discovered when a serious search is made for them.

Since lactose and other β -galactosides but not galactose serve equally well as the substrate for *Lactobacillus bulgaricus* in a medium containing glucose (Snell, Kitay & Hoff-Jørgensen, 1948) the interesting problem is presented as to whether a trans- β -galactosidase may function in the nutrition of certain bacteria. No completely satisfying explanation of how such trans-reactions are brought about has yet been devised. It is becoming apparent that trans-reactions play a more general role in biological processes than has been previously supposed.

SUMMARY

1. Dialysed enzyme preparations from *Lactobacillus helveticus* have been shown, by indirect methods, to catalyse the transfer of the deoxy-ribosyl group from one purine or pyrimidine to another.

2. By these methods, uracil deoxyriboside, thymine deoxyriboside, 5-methylcytosine deoxyriboside, and cytosine deoxyriboside were shown to be formed by the interaction of the corresponding pyrimidine and purine deoxyribosides. Orotic acid was not tested.

3. Conversely, substances which showed the lability to mild acid hydrolysis characteristic of purine deoxyribosides were formed when pyrimidine deoxyribosides reacted enzymically with adenine, guanine, hypoxanthine, xanthine, and 4-amino-

glyoxaline-5-carbonamide. Uric acid, 2:6-diaminopurine, and certain other compounds were inactive.

4. The transfer was catalysed in phosphate-free buffers by enzyme preparations which had been dialysed for several days.

5. Neither deoxyribose nor deoxyribose-1-phosphate was active in the transfer reaction.

6. The enzyme involved is considered to be a trans-*N*-glycosidase, analogous to the trans-*O*-glycosidase from *Pseudomonas saccharophila* (Doudoroff *et al.* 1947).

7. Enzyme preparations from two other deoxyriboside-requiring bacteria (*Lactobacillus delbrueckii* and *Thermobacterium acidophilus* R 26) also catalysed the transfer.

8. An attempt to demonstrate the trans-reaction in living cells of *Leuconostoc citrovorum* was unsuccessful.

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Trans-*N*-Glycosidase Studied with Radioactive Adenine

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It has been shown (MacNutt, 1952) that extracts from *Lactobacillus helveticus* contain an enzyme—in effect a trans-*N*-glycosidase—which catalyses an exchange between purines incorporated in the deoxyribosidic linkage and free pyrimidines, and the converse exchange between a pyrimidine deoxyriboside and a free purine. The same type of exchange was also shown to take place between one purine of a purine deoxyriboside and another purine base; e.g. adenine + guanine deoxyriboside $\rightleftharpoons$ guanine + adenine deoxyriboside.

In the closely related reaction demonstrated by MacNutt (1952): adenine + hypoxanthine deoxyriboside (HDR) $\rightleftharpoons$ hypoxanthine + adenine deoxyriboside (ADR), the formation of adenine nucleoside could be the result of a transamination, involving only the 6-amino group of the free adenine. Alternatively, the reaction could be due to a transglycosidase involving transfer of the whole adenine molecule. The mechanism of the reaction has now been investigated using adenine labelled at carbon atom number 8 with ^{14}C , and determining the isotope concentration in the reaction products. The two types of reactions can be illustrated as follows:

(1) Transamination

$[^{14}\text{C}]\text{-adenine} + \text{HDR} \rightleftharpoons [^{14}\text{C}]\text{-hypoxanthine} + \text{ADR}$.

In this case the free hypoxanthine contains undiluted isotopic carbon, and the adenine nucleoside does not contain any excess isotopic carbon.

(2) Trans-*N*-glycosidase

$[^{14}\text{C}]\text{-adenine} + \text{HDR} \rightleftharpoons \text{hypoxanthine} + [^{14}\text{C}]\text{-ADR}$.

In this case the radioactivity of the reaction products will be found exclusively in the adenine nucleoside, in which the isotopic concentration will be as high as that of the radioactive adenine added, while the free hypoxanthine will be devoid of radioactivity.

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EXPERIMENTAL

Materials

Adenine labelled in position 8 with ^{14}C , was made from $[^{14}\text{C}]\text{-formic acid}$ and 4:5:6-triaminopyrimidine (Clark & Kalckar, 1950). The preparation used contained about 0.8 mc./ μmol .

Commercial preparations of hypoxanthine were used.

Hypoxanthine deoxyriboside was prepared from enzymically degraded thymus nucleic acid as described by MacNutt (1952).

Adenosine deoxyriboside was supplied as the monohydrate through the kindness of Dr T. G. Brady, University of Dublin, Ireland (Brady, 1941).

Adenosine was obtained either from Fordomes Trading Company, Arlington, N.J., U.S.A., or from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

Inosine was prepared from adenosine by enzymic deamination (Kalckar, 1947*a*).

The enzyme preparation from *Lb. helveticus* was that described by MacNutt (1952).

Methods

Chromatography with *n*-butanol-water-ammonia or *n*-butanol-water-ethyl acetate-morpholine was performed as described by MacNutt (1952). Hypoxanthine was determined by addition of xanthine oxidase and recording the increase in density at 293 m μ . (Kalckar, 1947*a*).

Adenine was characterized by its R_f value in paper chromatography and determined by microbiological assay with *Lb. casei* (Roberts & Snell, 1946). Since this assay, although quite accurate from a quantitative point of view, is neither specific nor sensitive, the optical method for the determination of adenine described by Klenow (1952) was used.

Deoxynucleosides were measured by a microbiological assay using *Thermobacterium acidophilus* (Hoff-Jørgensen, 1952) and differentiated by chromatography on paper (MacNutt, 1952) and by enzymic ultraviolet spectrophotometry (Kalckar, 1947*a, b*). The procedure for adenosine deoxyriboside (ADR) was as follows:

The total amount of deoxyriboside present (e.g. in an eluate from a chromatogram spot) was determined by microbiological assay. The ADR content was determined spectro-