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Trans-*N*-Glycosidase Studied with Radioactive Adenine

BY H. M. KALCKAR, W. S. MACNUTT* AND E. HOFF-JØRGENSEN

Institutes of Cell Physiology and Biochemistry, University of Copenhagen

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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.

* Present address: University Chemical Laboratory, Cambridge, England.

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-

photometrically by addition of intestinal deaminase and measurement of the decrease in density at 265 m μ . (Kalckar, 1947*b*); this estimation was checked by determination of the hypoxanthine deoxyriboside (HDR) formed, by means of the spectrophotometric assay using liver nucleoside phosphorylase and xanthine oxidase (Kalckar, 1947*a*; Friedkin & Kalckar, 1950). The second method frequently gave lower values than the first. Both spectrophotometric determinations, especially that of HDR, were greatly hampered by the presence of some strongly absorbing materials in the eluate, presumably a breakdown product of morpholine. Upon addition of adenosine deaminase or xanthine oxidase to eluates of filter paper treated with this solvent alone, the density at 265 m μ . did not change as a result of enzymic conversion of the unknown absorbing material. It would, nevertheless, be advisable in future to attempt to remove these disturbing substances.

RESULTS

Enzymic interaction of [¹⁴C]-adenine and hypoxanthine deoxyriboside

The experimental mixture contained 500 μ g. of [¹⁴C]-adenine. HCl. $\frac{1}{2}$ H₂O in 500 μ l. of 0.2M-phosphate buffer at pH 7.0, 50 μ l. of HDR (125 μ g., 0.496 μ mol.) and 500 μ l. of *Lb. helveticus* extract (dialysed 3 days; 1.7 mg. dry weight. The control mixture was the same except that the HDR was added after the period of incubation had been terminated by the addition of ethanol. The mixtures were incubated for 12 hr. at 37° (1 drop of toluene added) and deproteinized by addition of 7 ml. of ethanol. The ethanolic extracts were prepared as described previously (MacNutt, 1952). Final volume = 500 μ l.

First chromatographic separation. The entire volumes were chromatographed in the *n*-butanol-water-ammonia system. For each paper chromatogram 100 μ l. of ethanolic extract were placed on paper along 4 cm., using adenine and HDR as indicators. Adenine and ADR appeared at one spot (R_F = 0.40), hypoxanthine and HDR at the other spot (R_F = 0.18). The appropriate spots from several chromatograms were pooled, cut into small pieces and extracted with water.

The filtered solutions from the ADR-adenine spots were assayed microbiologically. The experimental sample contained ADR equivalent to 0.365 μ mol. in the total incubation mixture, while the control sample contained none. This yield corresponds to a conversion of 74 % of the initial HDR. HDR and hypoxanthine were not determined until after these filtrates had been rechromatographed.

Second chromatographic separation. ADR was then separated from adenine, and HDR from hypoxanthine, by chromatography of these separate filtrates in the *n*-butanol-water-morpholine-ethyl acetate system (MacNutt, 1952). The individual spots were cut out and extracted with water as before. The volume of filtrate in each case was 4.0 ml. Hypoxanthine was estimated by the increase in density at 293 m μ . upon addition of xanthine oxidase and HDR and ADR by microbiological assay. The results were as follows:

Sample	μ mol./4 ml. eluate		
	ADR	HDR	Hypoxanthine
Control	0.005	0.132	0.063
Experimental	0.241	0.004	0.140

Radioactivity of the filtrates from the second chromatogram. Samples of 50, 100 and 150 μ l. were dispensed from constriction pipettes on to clean aluminium dishes and dried beneath an infrared lamp to form areas of about 4 mm. in diameter. Radioactivity counts were taken. The results were averaged and calculations made upon the basis of the counts above background per μ g. of purine. ADR and adenine were expressed as C₅H₅N₅. HCl. $\frac{1}{2}$ H₂O. The results set out in Table 1 show unambiguously (1) that free adenine can be exchanged with the hypoxanthine in HDR, and (2) that in this enzyme system no transamination between the 6-amino group of adenine and HDR took place; otherwise the adenine in the ADR which was formed should have shown a lower ¹⁴C concentration than that of the free [¹⁴C]-adenine.

Table 1. *Enzymic formation of [¹⁴C]-adenine deoxyriboside from hypoxanthine deoxyriboside and [¹⁴C]-adenine*

Sample analysed		Purine for counting (μ g.)	Counts/min.	Counts/min./ μ mol. purine ($\times 10^4$)
Eluate from ADR spot	Control	0.010	5.0	—
	Experimental	0.273	100.8	6.62
		0.546	208.0	6.88
Eluate from HDR spot	Control	0.200	1	—
	Experimental	0.006	1	—
Eluate from hypoxanthine spot	Control	0.100	1.0	—
		0.200	1.2	—
Standard [¹⁴ C]-adenine	—	0.250	91.2	6.60
		0.500	183.6	6.63

Enzymic exchange of free [¹⁴C]-adenine with the adenine of adenine deoxyriboside

It was considered of interest to investigate whether the adenine of ADR would be exchanged with free [¹⁴C]-adenine in the presence of the bacterial enzyme, for a reaction of this type would raise problems related to those encountered in the enzymic exchange of ³²P with the phosphorus of glucose-1-phosphate in the absence of added sugars (Doudoroff, Barker & Hassid, 1947). It was found indeed that on incubation of ADR with the bacterial enzyme and a fivefold excess of [¹⁴C]-adenine the exchange took place and was practically complete after 10.5 hr. incubation. The enzyme used, however, contained traces of hypoxanthine, which might have acted as a 'carrier' of the deoxyribosyl group through the reactions previously established:

It is, therefore, not yet clear if a direct exchange takes place; further work with an enzyme preparation free from potential 'carriers' is necessary to clarify this point.

Incubation of [¹⁴C]-adenine with adenine riboside and Lb. helveticus

Stephenson & Trim (1938) showed that *Escherichia coli* cells catalysed the deamination of adenine to hypoxanthine provided that either adenosine (or inosine) was present in appreciable amounts. Since the amount of the purine nucleoside required was only 10–20 % of the amount of adenine metabolized and the adenosine was rapidly deaminated, they concluded that the nucleoside acts as a transfer system. They formulated the reaction as a transamination:

- (1) adenine + inosine = hypoxanthine + adenosine,
- (2) adenosine = inosine + ammonia.

We were interested to study this reaction of riboside compounds which could be either a transamination or a trans-*N*-glycosidase type of reaction as it is analogous to the reaction with deoxyribosides.

Attempts were made to study the reaction using *Lb. helveticus* as a source of the enzyme, since *Esch. coli* extracts are rich in purine riboside phosphorylase (Kalckar, unpublished) and were, therefore, considered unsuitable for this purpose.

Mixtures were made as follows: *Experimental sample*: 100 µl. (212 µg.) [¹⁴C]-adenine.HCl. $\frac{1}{2}$ H₂O, 250 µl. 0.067 M-phosphate buffer at pH 8.3, 250 µl. of dialysed *Lb. helveticus* enzyme (0.8 mg. dry weight) and 50 µl. (57 µg.) adenosine. *Control* (1): same as the experimental sample except that the adenosine was added after the incubation period had been terminated by the addition of ethanol. *Control* (2): same as

the experimental sample except that the enzyme was added after incubation. The samples were incubated at 35° for 2 hr. at which time 7 ml. of ethanol were added, and the ethanolic extracts prepared in the usual way. The extracts were chromatographed in the *n*-butanol-water-ammonia system.

The chromatogram showed the following phenomena: (a) there was neither an adenosine nor an inosine spot in the experimental sample; (b) in the experimental sample there was a strong hypoxanthine spot which was devoid of radioactivity; (c) all the adenine spots were strongly radioactive; (d) the two controls showed highly radioactive adenine spots (*R_F*, 0.37) and non-radioactive adenosine spots (*R_F*, 0.30).

It was concluded, therefore, that adenosine had been deaminated and that the inosine (and perhaps the adenosine) had been completely split within 2 hr. by a ribosidase. No exchange occurred during this time, since the hypoxanthine was devoid of radioactivity.

It appears that extracts of *Lb. helveticus* contain a ribosidase and also a deaminase which catalyses the deamination of adenosine but not (according to the earlier experiments) adenine deoxyriboside. This is in contrast to the action of the mammalian adenosine deaminase as well as the deaminases of *Esch. coli* and *Lb. casei*, which bring about a deamination of both adenine deoxyriboside and adenosine.

SUMMARY

1. The exchange between adenine and the hypoxanthine of hypoxanthine deoxyriboside catalysed by an enzyme preparation from *Lactobacillus helveticus* has been studied. Using [¹⁴C]-adenine, the reaction gave rise to adenine deoxyriboside with a molar ¹⁴C concentration as high as that of the initial adenine.

2. The reaction is therefore not a transamination involving the 6-positions of adenine and hypoxanthine deoxyriboside, but is probably an exchange of the adenine skeleton with the bound hypoxanthine, catalysed by a trans-glycosidase.

3. The enzyme preparation catalysed an exchange reaction between the adenine of adenine deoxyriboside and [¹⁴C]-adenine. It also rapidly degraded adenine riboside (adenosine) by hydrolysis and deamination, but did not deaminate adenine deoxyriboside.

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A Microbiological Assay of Deoxyribonucleosides and Deoxyribonucleic Acid

By E. HOFF-JØRGENSEN

Institute of Biochemistry, University of Copenhagen, Denmark

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In the course of a survey of the nutritional requirements of certain lactic acid bacteria a strain, *Thermobacterium acidophilus* R 26, was encountered whose requirement for a deoxyriboside could not be replaced either by vitamin B₁₂ or by commercial antipernicious anaemia preparations from liver. This organism, therefore, seemed well suited as a test organism for a microbiological assay of deoxyribonucleic acid (DNA) more specific and more sensitive than the methods of Schmidt & Thannhauser (1945) and Schneider (1945). The method described below allows the determination of a few µg. of DNA with an accuracy of about 10 %, even in tissues, such as frogs eggs, where the concentration of DNA is less than 0.01 %.

EXPERIMENTAL

Methods

Stock cultures are maintained by weekly transfer in skimmed milk to which is added 0.1 % cysteine and 0.5 % 'Difco' yeast extract. The tubes are autoclaved for 10 min. at 120° and stored at about 10°.

Inoculum medium. Double-strength basal medium (100 ml.) and 100 ml. of 0.02 M-deoxyriboside are mixed and autoclaved in 5 ml. portions for 10 min. at 120°.

Inoculum. A small loopful of the stock milk culture is transferred to a tube containing 5 ml. of the inoculum medium. After incubation at 37° for 20–24 hr., the cells are centrifuged, washed once with 10 ml. of sterile saline, and resuspended in 10 ml. of sterile saline. One small drop of this suspension is used to inoculate each assay tube.

Standard. Stock solution: 10⁻⁴ g.mol. of deoxyriboside, e.g. 24.2 mg. of thymidine, is dissolved in 100 ml.: 25 % ethanol. This solution is stable for at least 1 year. To make a working standard containing 5 × 10⁻⁹ g.mol. of deoxyriboside/ml., 0.050 ml. of the stock solution is diluted to 10 ml. with water.

Table 1. *Double-strength basal medium*

Component	Amount (g./100 ml.)
HCl-hydrolysed casein*	2.5
Enzyme-hydrolysed casein†	0.5
Sodium acetate	3.0
Glucose	3.0
'Tween 80'	0.1
DL-Cysteine	0.05
DL-Tryptophan	0.02
KH ₂ PO ₄	0.2
K ₂ HPO ₄	0.2
MgSO ₄ ·7H ₂ O	0.02
MnSO ₄ ·4H ₂ O	0.004
FeSO ₄ ·7H ₂ O	0.002
	(mg./100 ml.)
Adenine	2
Guanine	2
Thymine	2
Cytidylic acid	5
Riboflavin	0.1
Nicotinic acid	0.1
Calcium pantothenate	0.1
p-Aminobenzoic acid	0.1
Pteroylglutamic acid adjusted to pH 6.8 with NaOH	0.005

* Prepared according to Snell (1950), neutralized with NH₃ instead of NaOH.

† Prepared according to Roberts & Snell (1946).

Basal medium. The composition of the basal medium is shown in Table 1. The addition of the following substances to the basal medium had no effect on the growth curve: ribonucleic acid, depolymerized ribonucleic acid, ribose, asparagine, ascorbic acid (20 mg./100 ml.); adenosine, adenylic acid, uracil, uridine, guanine, guanylic acid, cytosine, cytidine, oleic acid, deoxyribose, deoxyribose-1-phosphate, choline, inositol, cadaverine, putrescine (2 mg./100 ml.); aneurin, biotin (0.1 mg./100 ml.); vitamin B₁₂ (1 µg./100 ml.); *Citrovorum* factor (approx. 0.1 mg./100 ml.) and cozymase (1 mg./100 ml.).