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A Microbiological Assay of Deoxyribonucleosides and Deoxyribonucleic Acid

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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 B_{12} 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_2PO_4	0·2
K_2HPO_4	0·2
$MgSO_4.7H_2O$	0·02
$MnSO_4.4H_2O$	0·004
$FeSO_4.7H_2O$	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 NH_3 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 B_{12} (1 μg./100 ml.); *Citrovorum* factor (approx. 0·1 mg./100 ml.) and cozymase (1 mg./100 ml.).

Assay procedure. The tubes used for the assay are ordinary rimless Pyrex tubes 100 × 12 mm. The internal diameter of the tubes should not differ by more than 1 mm. at most. The standard working solution is added in amounts of 0·0, 0·1, 0·2, 0·4, 0·6, 0·8 and 1·0 ml. in duplicate. The sample is usually added in amounts of 0·2, 0·4, 0·6 and 0·8 ml. The contents of the tubes are diluted to 1 ml. with water and 1 ml. of the double-strength basal medium is added.

The solutions of the samples and the standard should be measured with an accuracy of 2% or better. The tubes are covered with caps and shaken to ensure thorough mixing. After autoclaving for 5 min. at 120° the tubes are cooled to room temperature, inoculated and incubated at 37° for 24 hr.

Determination of response. The tubes are shaken vigorously, and the contents are transferred to a round Coleman cuvette, type 6-310 B (10 × 75 mm.). The turbidity is read in a photoelectric colorimeter. We have used the apparatus described by Rehberg (1943). 100 = reading of the instrument with uninoculated medium, 0 = complete darkness.

The results are calculated from the standard dose-response curve (Fig. 1) according to Snell (1950) and expressed in g.mol. of deoxyriboside. 1 g.mol. deoxyriboside is equivalent to 327 g. DNA.

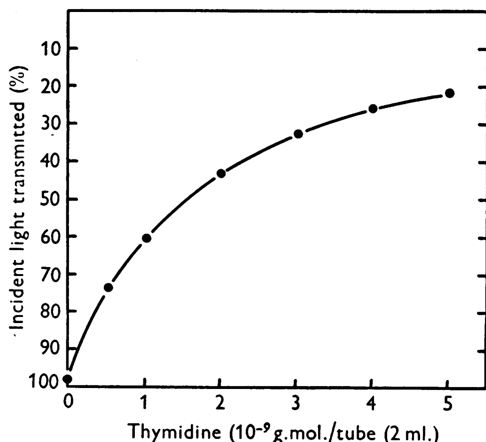

Fig. 1. The growth curve with thymidine (*Thermobacterium acidophilus* R 26). Total volume = 2 ml./tube.

The growth effect of different deoxyribosides

The growth effect of several deoxyribosides was compared, using a sample of thymidine, recrystallized three times, as a standard. The figures in Table 2 indicate that within the experimental error all the deoxyribosides tested have the same growth effect. After boiling at pH 2 for 5 min. the growth effect of the purine deoxyribosides completely disappeared, while the effect of the pyrimidine deoxyribosides was unimpaired.

Table 2. *The growth effect of some deoxyribosides*

Deoxyriboside (equimolar concentrations)	Growth effect	Growth effect after boiling at pH 2 for 5 min.
Thymidine*	100	100
Thymidine†	88, 90, 94	90
Thymidine‡	101, 98, 87	96
Guanine deoxyriboside§	104, 96, 96	0
Guanine deoxyriboside*	95, 97, 100	0
Adenine deoxyriboside‖	106, 108, 112	0
Cytosine deoxyriboside*	98, 106, 105	104
Hypoxanthine deoxyriboside*	92, 96, 90	0

* Prepared by Dr W. S. MacNutt in this Institute.

† Gift from Dr R. Hotchkiss, Rockefeller Institute for Medical Research.

‡ Gift from Dr W. Shive, University of Texas.

§ Prepared by Dr M. Friedkin in the Institute of Cytophysiology, Copenhagen.

‖ Gift from Dr G. O. Brady, University of Dublin.

Comparison of the growth effect of DNA, deoxyribonucleotides and deoxyribonucleosides

A solution (*a*) of DNA was prepared by dissolving 1 mg. of sodium thymonucleate (Hammarsten, 1924) (P = 9·15%, N = 15·97%) in 5 ml. of phosphate buffer pH 6·8.

A solution of deoxyribonucleotides (*b*) was prepared by incubating 1 ml. of the DNA solution (*a*) and 0·1 ml. 0·01 M-$MgSO_4$ with 100 μg. crystalline deoxyribonuclease (obtained from Worthington Biochemical Laboratory Freehold, New Jersey, U.S.A.) for 10 hr. at 37°. A solution of deoxyribonucleosides was prepared by incubating 0·5 ml. of solution (*b*) with 0·1 ml. intestinal phosphatase (Schmidt & Thannhauser, 1943) for 10 hr. at 37°.

From the results shown in Table 3 it appears that DNA prepared according to Hammarsten (1924) has a growth effect for *Thermobact. acidophilus* R 26 which is less than 1% of the effect of the amount of deoxyribonucleosides present in the DNA.

If, however, the DNA is depolymerized by deoxyribonuclease without liberation of inorganic phosphate (Kunitz, 1950), the growth response is maximum, i.e. the effect is equivalent to the effect of the calculated content of deoxyribosides in the DNA (70·6%). The release of inorganic phosphate by

Table 3. *The growth effect for* Thermobact. acidophilus R 26 *of DNA, deoxynucleotides and deoxynucleosides*

Growth factor	Content of deoxynucleoside (10^{-6} g.mol./mg. DNA): Found by growth effect	Calculated*
DNA	0·021, 0·020, 0·022, 0·022	3·06
DNA, depolymerized	2·95, 2·85, 2·85, 3·15	3·06
DNA, depolymerized and dephosphorylated	3·20, 3·00, 3·30, 2·85	3·06

* Assuming 1 g.mol. deoxynucleoside ~ 327 g. DNA.

intestinal phosphatase is not associated with any additional increase or any decrease in the growth effect. Deoxyribonucleotides therefore have the same effect as deoxyribonucleosides in equimolar concentrations.

The determination of DNA

A piece of tissue containing at least 0·3 μg. P as DNA is placed in a small test tube. After addition of an exactly measured amount of 0·5N-sodium hydroxide (at least 5 μl./mg. of fresh tissue) the tube is placed in a boiling-water bath for 15 min. During this time the tissue is disintegrated with a glass rod. After the incubation at 100° 5 vol. of a solution containing 0·06 g.mol. of maleic acid and 0·01 g.mol. of magnesium sulphate per l. are added for each vol. of 0·5N-sodium hydroxide used above. After addition of 5 μg. of crystalline deoxyribonuclease/mg. fresh tissue the tube is incubated at 37° for at least 16 hr. The mixture is then diluted to contain about 3×10^{-9} g.mol. deoxyriboside/ml., centrifuged if necessary, and assayed.

Samples of bacteria, yeast or tissue may either be analysed in the wet state or after drying with acetone. For the analysis of bacteria and yeast, the cells should be disintegrated, preferably in 'the tuning fork disintegrator' (obtained from H. Mickle, Middlesex, England).

Accuracy of the method. An assay of DNA in 0·5 mg. dry *Lactobacillus casei* cells was repeated ten times within 2 months. The mean value found was $1{\cdot}25 \times 10^{-6}$ g.mol. deoxyriboside/100 mg. dry cell. The standard deviation was $0{\cdot}08 \times 10^{-6}$ g.mol.

Comparison of the results obtained with two different methods for the content of DNA in some tissues. A male rat, weight 230 g., was killed by decapitation. The tissues were analysed immediately by the microbiological method described in this paper and by the method of Schmidt & Thannhauser (1945). Table 4 shows that the results of the two methods agree reasonably well.

Determination of DNA in tissues, etc., which contain very little DNA. The results of the microbiological assay of the DNA in tissues which contain less than about 0·1 % DNA cannot be verified by comparison with the results obtained by other methods, as no other method allows the determination of DNA in such tissues. The validity of the results given in Table 5 is, however, supported by the facts that added amounts of DNA are recovered, and that about half the growth effect is destroyed by mild acid hydrolysis of samples in which the DNA has been depolymerized.

The content of DNA in certain bacteria. Mixtures of 50 ml. of the basal medium described on p. 400 and 50 ml. of water containing varying amounts of thymidine were inoculated and incubated for 24 hr. at 37°. The cells were spun down, washed with distilled water, treated twice with 10 ml. of acetone and dried in a vacuum over sulphuric acid. The content of DNA in the cells and the residual deoxyriboside in the medium was then determined. It is seen from Table 6 that with both *Thermobact. acidophilus* R 26 and *Thermobact. lactis* I the amount of thymidine taken up from the medium is quantitatively recovered from the cells as DNA.

Table 4. *Estimation of DNA in rat tissues by two methods*

	Microbiological assay		Method of Schmidt & Thannhauser
Tissue	10^{-3} g.mol. deoxyriboside/100 g. fresh wt.	DNA-P* mg./100 g. fresh wt.	(mg. DNA-P/100 g. fresh wt.)
Liver	1·13	35	31
Kidney	1·20	37	33
Testes	0·95	30	24
Spleen	3·9	121	114
Brain	0·33	10·2	13

* DNA-P calculated from the microbiological assay assuming 1 atom P/mol. deoxyriboside.

Table 5. *Content of DNA in tissue etc. very low in DNA*

Tissue, etc.	Added DNA (mg./100 g.)	Deoxyriboside found as DNA (mg./100 g.)	Deoxyriboside found as DNA after acid hydrolysis* (mg./100 g.)
Rat muscle	0	43	20
	50	91	42
Frog eggs	0	6·5	3·4
	5	11·3	5·4
Tobacco mosaic virus	0	62	24
	60	130	60

* pH 1, 100°, 5 min. after depolymerization.

Table 6. *The content of DNA in two lactic acid bacteria*

(100 ml. of medium.* 24 hr. at 37°.)

Organism	Thymidine added (10^{-6} g.mol.)	Yield of dry cells (mg.)	Deoxyriboside (10^{-6} g.mol.) As DNA in the cells	Residual in medium	Total recovered
Thermobact. acidophilus R 26	0·2	25·9	0·16	0·03	0·19
Thermobact. acidophilus R 26	0·4	31·9	0·35	0·03	0·38
Thermobact. acidophilus R 26	0·8	38·5	0·72	0·04	0·76
Thermobact. lactis I†	0·5	49·6	0·45	0·03	0·48
Thermobact. lactis I	1·0	95·2	0·86	0·07	0·93

* Basal medium (50 ml.) as described in Table 1 and 50 ml. of water.
† This organism will grow either on vitamin B_{12} or on a deoxyriboside.

DISCUSSION

The method presented in this paper is based on the finding that deoxyribonucleosides are essential growth factors for *Thermobact. acidophilus* R 26.

The sensitivity of the method is limited only by the requirement of the organism for deoxyribonucleosides. In a medium containing all other essential and stimulatory growth factors half maximum growth is produced by about 10^{-9} mole deoxyriboside/ml., corresponding to about 0·3 μg. DNA/ml. As no other substances seem to interfere with the assay, analysis can be made of tissues etc. which are either very poor in DNA or of which only small amounts can be obtained.

The essential requirement of *Thermobact. acidophilus* R 26 for a deoxyribonucleoside is elucidated by the finding that the amount of DNA found in the cells is equal to the amount of deoxyribonucleoside taken up from the medium. As the organism will grow equally well on each one of at least five different deoxyribonucleosides, some process in the cell must take place by which the bound deoxyribose taken up from the medium is distributed among the four different purine- and pyrimidine-deoxyribosides found in the DNA. Such a mechanism has been demonstrated in some deoxyriboside-requiring organism by Kalckar, MacNutt & Hoff-Jørgensen (1952).

SUMMARY

1. A microbiological method for the determination of deoxyribosides and deoxyribonucleic acid has been devised, using *Thermobacterium acidophilus* R 26.

2. The method is very specific and allows the determination of a few μg. of deoxyribonucleic acid with a standard deviation of about 10% even at a low concentration.

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