The Deoxyribonucleic Acid of *Micrococcus radiodurans*

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The DNA of *Micrococcus radiodurans* was prepared by three methods. Although the recovery of DNA varied considerably, the percentage molar base ratios of the DNA from the three preparations were essentially the same: guanine, 33 ± 2 ; adenine, 18 ± 1 ; cytosine, 33 ± 2 ; thymine, 17 ± 1 . Base compositions calculated from T_m values and from density in caesium chloride gradients also yielded guanine + cytosine contents of 66 and 68% of total bases respectively. No unusual bases were observed. The $S_{20,w}$ values were characteristic of high-molecular-weight DNA. Electron microscopy showed the purified DNA in long strands; occasionally these were coiled.

Micrococcus radiodurans is a red-pigmented non-sporing bacterium that is extremely resistant to ionizing and ultraviolet radiation (Anderson, Nordan, Cain, Parrish & Duggan, 1956; Duggan, Anderson, Elliker & Cain, 1959). Some alternative hypotheses for its resistance to radiation have been advanced (Setlow & Duggan, 1964; Moseley & Schein, 1964; Moseley & Laser, 1965). Among the possibilities was that the DNA of the micro-organism has unique quantitative or qualitative base composition. Details of the preparation, chemical and physical properties of the DNA of *M. radiodurans* are the subject of the present study. Neither the cellular concentrations nor the base ratios of *M. radiodurans* DNA were unique, the latter falling within the ranges observed for other micro-organisms (Schildkraut, Marmur & Doty, 1962).

A previous note summarizing some radiobiological and analytical work with *M. radiodurans* and *M. radiodurans* DNA has been published (Moseley & Schein, 1964).

METHODS

Chemicals. Deoxyribose was purchased from Calbiochem (Los Angeles, Calif., U.S.A.). Caesium chloride (80%, w/w) was obtained from Gallard-Schlesinger (New York, N.Y., U.S.A.). Crystalline muramidase (EC3.2.1.17) was supplied by the Armour Co. (Chicago, Ill., U.S.A.) and Worthington Biochemical Corp. (Freehold, N.J., U.S.A.). Deoxyribonuclease was a gift from Dr D. B. Dunn. All other chemicals including EDTA were of analytical grade except Duponol (sodium dodecyl sulphate), which was recrystallized from ethanol.

Organisms. Organisms employed as a source of DNA were wild-type red-pigmented *M. radiodurans* and a non-pigmented white mutant strain W1 (Moseley, 1963).

In the early stages of this work *M. radiodurans* was grown as described by Moseley & Schein (1964). Subsequently the micro-organisms were grown aseptically in 16–18l. batches at 37° with vigorous aeration in a medium containing (g./l.): tryptone (Difco), 5.0; yeast extract (Difco), 5.0; glucose, 1.0; K_2HPO_4 , 1.0; adjusted to pH 7.0. The generation time was about 90 min. Cocci were harvested during the late exponential phase and washed with 0.15M-NaCl and water in a continuous-flow Servall KSB rotor.

Extraction of lipids and pigments. The cocci were either processed immediately after growth or stored in the frozen state until lipid extraction. Washed cocci were stirred with 10 vol. of 90% (v/v) ethanol for 5–10 min. and centrifuged. Most of the colour was extracted by two further treatments with ethanol. The cocci were treated twice with ethanol, twice with ethanol-ether (1:1, v/v) and twice with ether and dried overnight with silica gel in a vacuum desiccator. The yield of dry lipid-extracted micro-organisms was about $13 \pm 1\%$ of the centrifuged packed wet weight.

Preparation of DNA from *M. radiodurans*. Three methods for preparation of DNA were used for the study.

Method 1. Dried cocci (350 mg.) were suspended in 70 ml. of saline-EDTA (0.15M-NaCl-0.1M-EDTA) containing 35 mg. of muramidase. The suspension was intermittently swirled in a water bath for 1 hr. at 37°. Clumps of organisms were dispersed with a glass rod. The cooled coccal suspension cleared immediately and became very viscous when 0.7 ml. of 25% (v/v) Duponol was added. The solution was made 1N with respect to NaOH and kept at 22° for 20 hr. The addition of 2 vol. of ethanol plus a few crystals of $MgCl_2$ resulted in the formation of a white flocculent precipitate. The suspension was refrigerated for 2 hr. and centrifuged. The precipitate was washed five times with 20 ml. portions of acidified ethanol [N-acetic acid in 70% (v/v) ethanol], dissolved in 3 ml. of saline-citrate (0.15M-NaCl-15 mM-trisodium citrate) and reprecipitated by adding 2 vol. of ethanol. The precipitate was washed with acidified ethanol until the $E_{280m\mu}^{1cm}$ value of the washes was less than 0.05. The precipitate was then washed successively with 70% ethanol, 95% ethanol, 100% ethanol and ether and dried at room temperature in a vacuum desiccator with silica gel.

Method 2. Dried cocci (5g.) were added to 250ml. of saline-EDTA containing 200mg. of muramidase and the suspension was stirred magnetically at 37° for 1 hr. The addition of 11 ml. of 25% (v/v) Duponol to the homogeneous suspension resulted in a completely clear and very viscous solution that was then heated to 65° and maintained at this temperature for 15 min. Cooling and making the solution 1M with respect to perchlorate by adding 5M-NaClO₄ gave a white turbidity. The suspension was divided among several glass-stoppered Erlenmeyer flasks and was shaken with equal volumes of chloroform-3-methylbutan-1-ol (24:1, v/v) for 30 min. (Sevag, Lackman & Smolens, 1938). The emulsion was separated at $1.73 \times 10^4 g$ (1.2×10^4 rev./min.) at room temperature for 30 min. in the SS34 rotor of the Servall RC2 centrifuge. The clear top aqueous layers were combined. The turbid white middle zones were combined and re-centrifuged, and the aqueous layers were drawn off. The bottom chloroform layers were discarded. The combined aqueous layers were deproteinized twice more with chloroform-3-methylbutan-1-ol, precipitated with 2 vol. of ethanol and treated with ribonuclease [0.2% (w/v) in 0.15M-NaCl, pH 5; heated at 80° for 10 min. to inactive deoxyribonuclease (Marmur, 1961)] for 30 min. at 37°. The ribonuclease-treated solution was further deproteinized five times with redistilled phenol (Saito & Miura, 1963) before precipitating the DNA with 0.54 vol. of propan-2-ol. Subsequent purification was by the procedure of Marmur (1961).

Method 3. This was the same as method 2 up to and including the ribonuclease treatment, but was thereafter followed by a series of chloroform-3-methylbutan-1-ol deproteinizations and subsequent purification with propan-2-ol (Marmur, 1961).

Chemical and physical characterization of DNA of M. radiodurans. Spectra were measured in a Cary model 15 recording spectrophotometer. DNA was determined by the diphenylamine reactions (Dische, 1955; Burton, 1956), with herring-sperm DNA (ϵ_{260}^{1cm} 6700) and deoxyribose as standards. Phosphorus was determined by the method of Allen (1940).

Sedimentation coefficients at various concentrations of DNA in 0.1M-NaCl were measured in a Spinco model E ultracentrifuge equipped with schlieren and ultraviolet optics. The plates from the ultraviolet runs were scanned in a Joyce-Loebl microdensitometer.

DNA specimens were treated with ammonium acetate-cytochrome c-propan-2-ol by the technique of Kleinschmidt Lany, Jackerts & Zahn (1962), shadowed with platinum-gold and photographed in a Siemens Elmiskop I electron microscope.

Density determinations of DNA were made in a CsCl gradient (Schildkraut *et al.* 1962) at 3.5×10^4 rev./min. for 72 hr. at 20° in the SW39L rotor of the Spinco model L ultracentrifuge. The refractive index of a series of CsCl solutions of known density was measured in an Abbe-Zeiss refractometer.

T_m values were measured by following $E_{260m\mu}$ as a function of temperature of the DNA solution and establishing the mid-point of the hyperchromic rise (Marmur & Doty, 1962). The determinations were done in glass-stoppered 1 cm. quartz cuvettes in a Beckman DU spectrophotometer with an immersed thermometer protruding through a drilled-out top-plate. The cuvettes were heated by an external temperature-controlled bath and pump that circulated a

mixture of water and ethylene glycol through surrounding heating coils. Under these conditions calf-thymus DNA had a T_m of 87° (Marmur & Doty, 1962).

Hydrolysis of DNA with formic acid, chromatography of the hydrolysates in propan-2-ol-2N-HCl and estimation of bases were carried out as described by Markham (1955).

Search for unusual bases. A 35 mg. sample of DNA from *M. radiodurans* was hydrolysed with formic acid. The formic acid was evaporated off, the hydrolysate was taken up in N-HCl, and volumes containing approx. 4 mg. of mixed nucleotides were streaked on Whatman no. 1 filter paper and chromatographed with propan-2-ol-2N-HCl. Five distinct ultraviolet-absorbing bands were present: the band nearest the origin was guanine, followed by adenine, cytosine, 'X' and thymine. The major component of 'X' was identified as uracil, an artifact of formic acid hydrolysis (Schein, 1966).

To detect minor amounts of unusual bases that might have been obscured by the major ultraviolet-absorbing regions, each of the bands was eluted with water, or for guanine with 0.01N-HCl, the eluate evaporated to dryness *in vacuo*, and the solid residue dissolved in 100 μ l. of water and streaked on Whatman 3MM paper. Chromatography was carried out in propan-2-ol-aq. NH₃ (Markham, 1955). Marker bases (guanine, adenine, cytosine, uracil, thymine, 6-methylaminopurine, 6-dimethylaminopurine and 5-methylcytosine) were chromatographed in adjacent lanes in the second solvent.

RESULTS

The dried lipid-extracted wild-type *M. radiodurans* contained 1.50% (± 0.24 S.D., $n = 13$) of DNA. Duplicate determinations each yielded 1.5% of DNA for the white mutant W1.

Recovery of DNA (Burton, 1956) by method 1 was approx. 75% (Table 1). For methods 2 and 3 the recoveries were less than 10% and 7-36% respectively. The losses of DNA associated with the steps of method 3 were as follows: (1) 10-20%

Table 1. *Recovery of DNA from M. radiodurans*

Strain	Preparative method	DNA recovery (%)
Wild-type	1	76
Wild-type	1	75
Wild-type	1	73
Wild-type	2*	9
W1	2	1.4
Wild-type	2	9
W1	2	6
Wild-type	3†	7
Wild-type	3	35
Wild-type	3	36
Wild-type	3	17

* Chloroform-3-methylbutan-1-ol and phenol deproteinization.

† Chloroform-3-methylbutan-1-ol deproteinization.

EXPLANATION OF PLATE 1

Electron micrographs of *M. radiodurans* DNA. The technique of Kleinschmidt *et al.* (1962) was used. Pictures are in order of increasing number of purification steps: (a) after lysis with muramidase and Duponol; (b) after three treatments with chloroform-3-methylbutan-1-ol, digestion with ribonuclease and four treatments with phenol; (c) subsequent precipitation first with ethanol and then with 0.54 vol. of propan-2-ol; (d) reprecipitation with propan-2-ol; (e) solution of (c) treated with deoxyribonuclease. Magnification 23200 $\times$.

Table 2. *Base ratios of M. radiodurans DNA*

Base ratios were determined after formic acid hydrolysis. Each set of values except those for the last line is the average of duplicate determinations. The reproducibility of each set of duplicate determinations varied from 1 to 8%. The molar base ratios add up to 100±2%.

Preparative method	Strain	Composition (moles/100 moles of total bases)			
		Guanine	Adenine	Cytosine	Thymine
1	Wild-type	32	17	35	16
1	Wild-type	33	17	35	17
2	Wild-type	34	17	33	16
2	W1	32	17	34	17
3	Wild-type	32	18	33	17
3	Wild-type	35	19	31	16

Fig. 1. Sedimentation of DNA from *M. radiodurans* in 0.1M-NaCl in the Spinco model E ultracentrifuge at 4.47×10⁴ rev./min. for approx. 96 min. Schlieren photographs are shown, sedimentation being from left to right. (a) Wild-type (260 µg./ml.), *S*_{20,w} 20 s; (b) white mutant W1 (460 µg./ml.), *S*_{20,w} 9.9 s.

chloride the *S*_{20,w} of DNA from the wild-type and from the mutant W1 was 22 s. At a concentration of 260–460 µg./ml. the *S*_{20,w} was 10 s. The schlieren boundary was quite sharp (Fig. 1).

Electron microscopy of the shadowed DNA prepared by method 2 showed long strands; occasionally they were coiled. The DNA gradually unfolded or unwound with purification (Plate 1a–d). Treatment of the DNA with pancreatic deoxyribonuclease resulted in complete disappearance of the strands; only the cytochrome *c* background could be discerned (Plate 1e).

The *T*_m value of *M. radiodurans* DNA was 96.6° and its density was 1.728. No unusual bases in measurable quantities were observed in the formic acid hydrolysates of *M. radiodurans* DNA when chromatographed initially with propan-2-ol–2N-hydrochloric acid and then with propan-2-ol–aq. ammonia.

DISCUSSION

for initial treatment of the bacterial lysate with chloroform–3-methylbutan-1-ol [Saito & Muira (1963) also experienced large losses at this stage]; (2) 3–6% for each of the deproteinizations with chloroform–3-methylbutan-1-ol; (3) approx. 20% for the steps involving precipitation of DNA with ethanol followed by solution in saline–citrate, ribonuclease treatment and deproteinization with chloroform–3-methylbutan-1-ol; (4) 15–20% for precipitation of DNA with ethanol followed by solution in acetate–EDTA and reprecipitation with propan-2-ol. Thus the low (7%) recovery of method 3 was associated with the number of chloroform–3-methylbutan-1-ol deproteinizations (15 in this case). When the number of deproteinizations was less the recoveries were much higher, although presumably the DNA was less pure. Base ratios of DNA from *M. radiodurans* are listed in Table 2.

Various modifications of the three methods described for the preparation of DNA from *M. radiodurans* were tested and abandoned. Thus initial treatment of the lysed bacteria with aqueous phenol instead of with chloroform–3-methylbutan-1-ol caused very large losses of DNA. The losses of DNA with phenol deproteinization (method 2; Table 1) were greater than those with chloroform–3-methylbutan-1-ol (method 3). DNA recoveries were estimated at each purification step except that the aqueous solution overlaying phenol could not be analysed by the diphenylamine reaction until phenol had been completely removed. This was done too infrequently to include stepwise estimation of losses with phenol in Table 1.

There was no essential difference in the base ratios of DNA from the two strains of *M. radiodurans*. Also, the base ratios of the DNA specimens isolated by methods 1, 2 and 3 were similar even though the DNA recovery by the various methods

varied considerably (Table 2). Setlow & Duggan (1964) have reported the base ratios of *M. radiodurans* as (average values): guanine, 30.0; adenine, 19.3; cytosine, 30.9; thymine, 19.6.

The observed T_m value of DNA from *M. radiodurans*, 96.6°, corresponds to a guanine + cytosine content of 66 moles/100 moles of total bases (Marmur & Doty, 1962). Schildkraut *et al.* (1962) related the guanine + cytosine content (in moles/100 moles) to DNA buoyant density; plotted on their curve the value d 1.728 corresponds to 68 moles of guanine + cytosine/100 moles. Thus base estimation by physical means compared well with analytical results obtained by chemical analysis (Table 2).

The $S_{20,w}$ values are characteristic of high-molecular-weight DNA (Chargaff, 1955).

The electron micrographs of DNA prepared from *M. radiodurans* by the technique of Kleinschmidt *et al.* (1962) are similar to those published for DNA by Young & Sinsheimer (1965). A unique feature was the gradual appearance of strands as purification proceeded. It appeared as if DNA gradually unwound from tightly coiled spheres; the strands increased in number whereas dense spheres decreased as purification proceeded (Plate 1a-d). In all of the preparations, treatment with deoxyribonuclease caused a complete disappearance of all of the strands and most of the spheres.

The chromatographic method used to detect unusual bases should have detected minimum contents of approx. 1 mole of unusual base/100 moles of total bases, unless an unusual base has the same R_f as one of the major bases in the two solvents employed. The obvious limits of sensitivity of chromatography necessitates a different approach, and the possible occurrence of methylated bases in *M. radiodurans* DNA is being investigated by employing [$Me-^{14}C$]methionine in the growth medium (A. Schein, unpublished work).

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