acid. Alanine, when present in the immunogenic molecule, also contributes to specificity.

3. Substances which are not immunogenic, because the area important for immunogenicity is not accessible to the biosynthetic site, may nevertheless cross-react with antibodies formed against substances closely related chemically. Apparently the capacity of a molecule to react with the combining site of an antibody is not parallel with its ability to elicit the formation of antibodies.

4. The antigenic specificity is governed by the area of the molecule most exposed to the surroundings. Thus tyrosine and glutamic acid contribute more to the specificity of p(Tyr,Glu)-pAla-pLys, whereas alanine contributes more to that of pAla-p(Tyr,Glu)-p(Lys,Ala).

5. Antibodies to linear polypeptide antigens gave much better precipitation with linear than with multichain polymers.

6. The efficiency of inhibition of some systems with specificity directed to peptides of tyrosine and glutamic acid by means of copolymers of tyrosine and glutamic acid was strongly dependent on the molar ratio of two amino acids in the copolymer. Some copolymers caused complete inhibition of the homologous reaction of the system p(Tyr,Glu)-pLys-anti-p(Tyr,Glu)-pLys. The molar ratios of two such inhibitors to the immunogen at 50 % of the total inhibition were 70 and 94.

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Biochem. J. (1963) **87**, 79

Studies on Incorporation of Deuterium into Bacteria

BY ROSALIE DE GIOVANNI AND S. ZAMENHOF

Department of Biochemistry and Department of Zoology, Columbia University, New York, N.Y., U.S.A.

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The mutagenic effect of deuterium oxide has been reported for several strains of bacteria (De Giovanni & Zamenhof, 1959; De Giovanni, 1960, 1961) and for the bacteriophage T4 (Konrad, 1960). With bacteria the growth of several strains was inhibited by media containing deuterium oxide and the degree of inhibition was strain-specific. No adaptation to better growth in media containing deuterium oxide was obtained in any of the strains tested; however, a deuterium-resistant mutant was obtained from one strain. Two strains tested formed enlarged distorted cells when grown for more than eight generations in media containing deuterium oxide. Deuterium induced the occurrence of forward mutants in some strains. Many loci, but not all, showed an up to 54-fold increase in the back-mutation rate over the spontaneous level, indicat-

ing some specificity of action by deuterium. Deuterated bases, obtained from a strain grown in a medium containing deuterium oxide, did not induce any phenotypic or genotypic effects when supplied to specific base-requiring strains.

The mechanism of such mutagenic action is not understood; however, it may be related to the extent of deuterium incorporation by bacteria or phage. Differences in the nuclear mass of hydrogen and deuterium may possibly cause disturbances in the synthesis of DNA, leading to permanent changes in its structure and consequently in the genotype it controls.

The purpose of the present study was to investigate the extent of deuterium incorporation in cells of *Escherichia coli* and in nitrogenous bases of their DNA, in an attempt to correlate such incorporation

with the phenotypic and genotypic effects of deuterium on these cells (De Giovanni & Zamenhof, 1959; De Giovanni, 1960, 1961). Hence deuterium analyses were performed on whole cells grown in a medium containing deuterium oxide under mutagenic conditions, and on purines and pyrimidines from the DNA of such cells.

EXPERIMENTAL

Materials. Deuterium oxide (supplied as 99.5% D_2O by General Dynamics Corp., San Carlos, Calif., U.S.A.) was purified by distillation from alkaline permanganate to eliminate any inhibition of bacterial growth by contaminating metal ions and other impurities. The Penassay (Difco) medium containing deuterium oxide used for the growth of bacteria was as described by De Giovanni (1961).

As calculated from the deuterium oxide content of heavy water and the amounts of non-deuterated solids used, the deuterium content of freshly prepared growth medium was 97.6%. The analysis (mass spectrometer) of such a medium after autoclaving gave a value of 92%, indicating a dilution by normal water in the process of autoclaving. A similar dilution was observed by Marmur & Schildkraut (1961), who devised a synthetic medium containing a higher deuterium content. No such attempt was made in the present work since the deuterium content used produced pronounced phenotypic and genotypic effects (De Giovanni, 1961) without excessive inhibition of growth.

Bacterial strains. The strains of *Escherichia coli* used in the present work were as described by De Giovanni (1961). They included B/Sd, a streptomycin-dependent mutant of strain B, and B/S, a streptomycin-sensitive mutant derived from B/Sd. In addition, strain 15 was used.

Bacteria were grown in synchronized and non-synchronized cultures as described by De Giovanni (1961).

Isolation of highly polymerized deuterated deoxyribonucleic acid and its bases. The cells (strain 15), grown for 24 hr. in the medium containing deuterium oxide, were harvested by centrifuging for 30 min. at 19 000g and washed five times, each time with 1 vol. of 0.85% NaCl. The highly polymerized DNA was isolated from the cells by the method of Zamenhof, Reiner, De Giovanni & Rich (1956). A micromodification of the Dische diphenylamine test was used to estimate the amount of DNA obtained (Zamenhof, 1957).

For the isolation of bases, the DNA preparation was dried overnight over P_2O_5 in a vacuum desiccator. A sample (10 mg.) of DNA was hydrolysed in 1 ml. of 7.5N-HClO₄ for 1 hr. at 100°. The solution was centrifuged and the brown supernatant fluid was subjected to chromatography on Whatman no. 1 filter paper. A row of sixty 1 μ l. spots was placed on the starting line. With propan-2-ol-HCl as the developing solvent (Wyatt, 1951), a separation of thymine, guanine, cytosine and adenine was achieved after 30 hr. (Marshak & Vogel, 1951).

All the spots were cut out and eluted for 24 hr. at 23°; the pyrimidines were extracted in 10 ml. of water and the purines in 10 ml. of 0.1N-HCl. The concentration of the base in solution was determined from the ultraviolet-light absorption (Vischer & Chargaff, 1948).

Deuterium analyses of bases, bacteria and medium. A solution of a known amount of a deuterated base was diluted with a known amount of the normal base in solution (total,

2-3 mg. of base), placed dropwise into a dry platinum boat, allowed to dry under an infrared light, and further dried over P_2O_5 in a vacuum desiccator overnight. The samples were subject to combustion in a micro-furnace and the water was collected in a U-tube immersed in solid carbon dioxide, as described by Graff & Rittenberg (1952). The U-tube was attached to the mass spectrometer, the water vapour was passed over hot uranium, and the hydrogen formed analysed for its deuterium concentration (Botter & Nieff, 1959).

For the total deuterium content of cells grown in the medium containing deuterium oxide, the cultures were washed twice with 0.85% NaCl in 92% deuterium oxide and dried in a vacuum oven at 103-105° for 24 hr. The samples were diluted with a known amount of control cells (total, 2-3 mg.), placed in dry platinum boats and handled as described above.

The medium containing deuterium oxide used for the growth of bacteria was diluted 100-fold for analysis. Approx. 0.1 ml. of solution was placed in a dry platinum boat. The sample was subjected to combustion and analysed as described above.

RESULTS AND DISCUSSION

Deuterium content of bacterial cells. Cells of B/S strain, grown for four generations (cell divisions) in synchronized cultures in medium containing 92% of deuterium oxide, did not exhibit distortions or an increased yield of mutants; however, after eight generations, distorted cells and a 37-fold increase in the mutation rate were detected (De Giovanni, 1961). Analysis of the water obtained from the combustion of dried cells showed that the former cells contained 15.1% of deuterium and the latter 34.3%. These results indicate that there was a preferential uptake of hydrogen especially in the earlier generations. Marmur & Schildkraut (1961) also noted a preferential uptake of hydrogen in the initial stages of bacterial growth. Weinberger & Porter (1954) reported that the cells of *Chlorella pyrenoidosa* grown in a medium containing deuterium oxide incorporated deuterium more slowly than hydrogen; the deuterium:hydrogen ratio of incorporation after five divisions was 0.4:1. Moses, Holm-Hansen & Calvin (1958) reported that 'unadapted' cells of *C. pyrenoidosa* took up hydrogen from the growth medium in preference to deuterium; however, 'adapted' cells preferentially took up deuterium. No preferential uptake of hydrogen was reported by Chorney, Scully, Crespi & Katz (1960) in their study with *Scenedesmus obliquus*; however, such cells were tested after prolonged cultivation and perhaps were also 'adapted' cells. It therefore appears that, during initial divisions, cells preferentially take up hydrogen, and that, during subsequent divisions, the concentration of deuterium in the cell increases.

The incorporation of deuterium has a pronounced effect on the phenotype and genotype of bacteria

Table 1. *Deuterium content of nitrogenous bases from the deoxyribonucleic acids of Escherichia coli strain 15 grown in a medium containing deuterium oxide*

Nitrogenous base	Theoretical D expected for 1 non-labile H atom replaced by D (atom % excess)	Actual D (atom % excess)	Non-labile H replaced by D (atoms/molecule)	Total non-labile H (atoms/molecule)
Adenine	0.082	0.064 0.061	0.8 0.7	} 2
Guanine	0.098	0.028 0.018	0.3 0.2	
Cytosine	0.050	0.107 0.119	2.1 2.4	} 2
Thymine	(a)* 0.077 (b)* 0.114 (c)* 0.140	0.132 0.18 0.25	1.7 1.6 1.8	} 4

* (a), (b) and (c) refer to three separate preparations of thymine analysed.

(De Giovanni & Zamenhof, 1959; De Giovanni, 1960, 1961); the extent of the effect appears to be related to the extent of deuterium incorporation. In a study of the effects of isotopes on bacterial growth Borek & Rittenberg (1960) suggested that the presence of the mixture of isotopes rather than the presence of the rare isotope may be responsible for the phenotypic effects. A change in enzymic reaction rates due to the incorporation of deuterium into substrate(s) or enzyme(s) of *E. coli* B has also been reported (Rittenberg & Borek, 1961). In a study of the effects of deuterium on genotype, Konrad (1960), who estimated a 21 % replacement of hydrogen in bacteriophage T4 prepared in a medium containing 50 % of deuterium oxide, found that some r_{II} bacteriophage mutants exhibited a 18-fold increase in their reversion rate to wild-type. Fewer revertants were obtained from bacteriophages prepared in media containing lower concentrations of deuterium oxide.

Deuterium content of nitrogenous bases. Nitrogenous bases were isolated from the DNA of cells grown for more than eight divisions in the medium containing deuterium oxide, and the deuterium content of these bases was determined as described above. The method of isolation precludes the detection of labile (O- and N-bound) deuterium atoms initially present, since they are replaced by hydrogen of the water used during isolation. The non-labile (C-bound) deuterium content of isolated bases is represented in Table 1; this Table also lists the theoretical values expected for the replacement of one non-labile hydrogen atom/molecule by deuterium, with correction for the dilution factor.

The incorporation of deuterium into adenine and guanine was low; however, only the water of the growth medium was deuterated. Two non-labile hydrogen atoms in the thymine molecule and two in the cytosine molecule were replaced by deuterium; such results are to be expected from consideration of the biosynthetic pathway of these pyrimidines.

As reported by De Giovanni (1961), none of the bases so deuterated was able to alter the phenotype or genotype of cells that require these bases for growth and presumably incorporate them into their DNA. Thus the increase in mutation rate after growth in deuterated media is likely to be the result of a disturbance other than the deuteration of bases to the extent reported.

The deuterated bases that are incorporated into DNA are supplied with deuterium only in the non-labile positions. Thus it is possible that a similar 'extent of deuteration' with the deuterium in the labile positions in the bases could give mutational changes when the bases were incorporated into DNA. In view of the importance of hydrogen-bonding in functional DNA such an explanation is not unlikely.

SUMMARY

1. Cells of *Escherichia coli* were grown in synchronized and non-synchronized cultures in a medium containing 92 % of deuterium oxide.

2. The incorporation of deuterium into cells was 15.1 % after four generations and 34.3 % after eight generations, indicating selectivity of incorporation dependent on length of exposure to deuterium. Cell distortion and increase in mutation rate could be correlated with the amount of incorporation.

3. The incorporation of deuterium into the nitrogenous bases of the DNA of these cells has been determined.

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Biochem. J. (1963) **87**, 82

Metabolism of D-Glucuronolactone in Mammalian Systems

2. CONVERSION OF D-GLUCURONOLACTONE INTO D-GLUCARIC ACID BY TISSUE PREPARATIONS*

By C. A. MARSH

Rowett Research Institute, Bucksburn, Aberdeen

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Normal mammalian urine contains D-glucaric acid, which is partly responsible for the inhibitory effect of boiled urine on β -glucuronidase (Marsh, 1962, 1963). Excretion of D-glucaric acid by normal human adults was about 10 mg./day, but it was increased approximately 100-fold within 24 hr. of the oral administration of a 5 g. dose of D-glucurono-(6 $\rightarrow$ 3)-lactone, whereas the administration of D-glucuronic acid had little effect. From their close structural similarity it seemed probable that D-glucuronolactone was a direct precursor of biosynthetic D-glucaric acid, and mammalian tissue preparations were examined with the purpose of identifying the enzyme system that would accomplish such a conversion. The probable source of D-glucuronolactone *in vivo* is free D-glucuronic acid, which appears to be formed in tissues solely from UDP-glucuronic acid, either by direct action of phosphatases (Ginsburg, Weissbach & Maxwell, 1958) or by the hydrolysis of glucuronides formed by transfer of the D-glucuronic acid moiety to unknown acceptors (Hollmann & Touster, 1962). The additional possibility of dietary sources of D-glucuronic acid, e.g. from breakdown of acidic mucopolysaccharides, cannot be excluded.

A preliminary account of some of this work has been published (Marsh, 1961).

METHODS AND MATERIALS

Substrates. D-Glucurono-(6 $\rightarrow$ 3)-lactone (Corn Products Refining Co., New York, U.S.A.), recrystallized once from water, had m.p. 175–177°. It was converted in solution into sodium D-glucuronate as described by Marsh (1963). NAD and NADH₂ (sodium salt) were 'Sigma Grade' (Sigma Chemical Co., St Louis, Mo., U.S.A.), for which 97.5–100% purity was claimed. Phenolphthalein β -glucuronide was prepared biosynthetically (Levy & Marsh, 1959).

Tissue preparations. Adult human tissues, supplied by Dr A. J. Carr, Pathology Department, Aberdeen University, were apparently non-diseased autopsy specimens obtained within 6 hr. of death and stored at -20° . Fresh pig liver was provided by Robert Lawson and Sons (Dyce) Ltd. The rats used were hooded Lister strain, and the white mice were from the Institute stock (Tyler's original, Mill Hill colony). The C3H mice were originally from the Institute of Physiology, Glasgow University.

For the assay of 'glucurone dehydrogenase' the tissues were treated for 1 min. (average speed 5000 rev/min.) in a chilled glass homogenizer (Jencon's Ltd.) with sufficient ice-cold 0.17M-KCl (to preserve intracellular particles) to give a moist-tissue concentration of 10%. All further manipulations were carried out at 0° . The homogenate was centrifuged at 75 000g for 40 min., and the clear non-

* Part 1: Marsh (1963).