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18, 440.  
Wash. 63, 556.

*J. Mol. Biol.* (1972) 63, 9-19

Host Specificity of DNA produced by *Escherichia coli*  
X.V. The Role of Nucleotide Methylation in *in vitro*  
B-specific Modification

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(Received 29 March 1971, and in revised form 11 August 1971)

It is shown that *in vitro* *Escherichia coli* strain B-specific modification of the replicative form of bacteriophage fd DNA is accompanied by the methylation of certain adenine moieties to form *N*-6-methyladenine. The reaction follows first order kinetics and saturation is reached when about four adenines are methylated per replicative form. No methyl groups are transferred to B-modified DNA.

The replicative form of a one step mutant of fd, which has a reduced sensitivity towards B-specific restriction, has lost two of the four methyl acceptor sites. The replicative form of a second step mutant, which is not subject to B-specific restriction, is completely refractory to methylation by the modification enzyme. It is therefore concluded that the B-modification and the B-restriction enzyme react with the same sites on the substrate DNA and that the replicative form of wild type fd has two such sites. The number of *N*-6-methyladenines per B-specificity site of fully modified double-stranded DNA is two.

### 1. Introduction

Kühnlein, Linn & Arber (1969) partially purified the DNA-modification enzyme from extracts of *Escherichia coli* strain B. The enzyme was identified by its ability to render the unmodified replicative form of phage fd DNA resistant to B-specific restriction. This *in vitro* modification requires *S*-adenosyl methionine. The role of this factor has now been explored: it serves as methyl donor in a reaction producing *N*-6-methyladenine. *In vitro* B-specific modification is thus obtained by nucleotide methylation in confirmation of an old hypothesis (Arber, 1965 *a, b*). Data presented in an accompanying paper lead to the same conclusion for *in vivo* B-specific modification (Smith, Arber & Kühnlein, 1972).

### 2. Materials and Methods

#### (a) Chemicals

Tritiated *S*-adenosylmethionine and [<sup>32</sup>P]orthophosphate were products of the Radiochemical Centre (Amersham). Sophadex G100 was purchased from Pharmacia (Uppsala), and benzoylated-naphthoylated DEAE-cellulose, prepared as described by Gillam *et al.* (1967), was a gift of Dr T. Young. Toluene scintillation liquid was 5 g PPO and 0.3 g POPOP dissolved in 1 l. of toluene. All other reagents were the same as described previously (Kühnlein *et al.*, 1969).

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(b) *Bacteriophage and bacterial strains*

Phage fd is the strain isolated by Hoffman-Berling, Marvin & Dürwald (1963).

The following strains of *E. coli* were used: 993 (Arber, 1966), an  $F^+r_K^-m_K^-$  strain; 991 (Arber, 1966), an  $F^+r_K^+m_K^+$  strain; 2027 (Arber, 1966), an  $F^+$  derivative of a K-B transduction hybrid  $r_B^+m_B^+thr_B^+leu_K^-met_K^-$ ; 2539, a partial diploid ( $r_B^+m_B^+thr^-leu^-/F' r_B^+m_B^+thr^+leu^-$ ) derivative of Bc 251, prepared by Drs S. Linn and C. Berg with the F-prime derived from KLF1 (Low, 1968).

According to the host specificity phenotype, 993 is an O-strain, 991 a K-strain and 2027 a B-strain. Since the DNA of phage fd is insensitive to K-specific restriction (Arber, 1966) and modification (Smith *et al.*, 1972), the K-strain will also be used as O-strain in this paper.

(c) *Selection of restriction-deficient mutants of phage fd*

Mutants of phage fd with a reduced sensitivity towards B-specific restriction were selected by growing phage alternatively on the B-strain 2027 and on the O-strain 993. Bacteria were grown in Tryptone broth to a concentration of  $2 \times 10^8$  cells/ml. and mixed with 1 to 5 phage particles/cell. After incubation for 15 min at 30°C, the unabsorbed phage was removed by washing the cells twice in Tryptone broth. The cells were resuspended in the original volume of Tryptone broth and incubated for an additional 2 hr at 30°C, after which time the bacteria were removed by centrifugation. The phage contained in the supernatant was used to initiate the next growth cycle.

(d) *Purification of the B-specific modification enzyme*

The B-specific modification enzyme was isolated from an extract of the partial diploid strain 2539 as described previously (Kühnlein *et al.*, 1969). The purification was approximately 200-fold. Incubation of unmodified fd RF† with the enzyme preparation under modifying conditions for 5 hr or restricting conditions (Linn & Arber, 1968) for 30 min did not lead to detectable loss of infectivity on strain O. This was taken as evidence that the enzyme preparation was relatively free of endonuclease activity.

(e) *Isolation of the replicative form of fd DNA*

Bacteria were grown in Tryptone broth at 37°C to a density of  $3 \times 10^8$  cells/ml. and infected with a multiplicity of 8 phage particles/bacterium. The aeration was reduced for 15 min in order to facilitate infection. Then the aeration was restored and the incubation at 37°C continued for another 75 min. The infected cells were chilled, harvested by centrifugation and the RF extracted according to the method described by Komano & Sinsheimer (1968) with the following modifications: the denaturation step before chromatography on BN-cellulose was omitted and the BN-cellulose was eluted with a linear NaCl gradient between 0.4 and 1 M-salt concentration. The purest RF fractions obtained from this column contained less than 10% bacterial DNA as determined by electron microscopy. The DNA concentration of the RF preparation was determined by the method of Burton (1956).

For the preparation of  $^{32}P$ -labelled fd RF, cells were grown in TPG2A medium (Lindquist & Sinsheimer, 1967) supplemented with  $1.7 \times 10^{-3}$  M- $KH_2PO_4$ . At a density of  $2 \times 10^8$  cells/ml., the bacteria were concentrated fivefold in the same medium containing no phosphate, and infected with phage at a multiplicity of 10. After 10 min at room temperature, the infected cells were diluted fivefold into TPG2A medium containing 3 mCi of [ $^{32}P$ ]orthophosphate/l. and a total phosphate concentration of  $1.7 \times 10^{-4}$  M (adjusted with  $KH_2PO_4$ ). The RF was isolated after 75 min of incubation at 37°C following the procedure described above. The relatively low DNA concentrations did not allow accurate measurements with the method of Burton (1956). They were therefore determined by comparing the infectivity of the preparations with the infectivity of an RF

†Abbreviations used: RF, replicative form; BN-cellulose, benzoylated-naphthoylated DEAE cellulose; S-AM, S-adenosylmethionine.

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preparation of known DNA concentration. The purified RF had a specific activity of approximately 50  $\mu\text{Ci}/\text{mg}$ .

The host strain 2027 was used to isolate B-modified RF (fd RF·B) and the host strain 991 to isolate unmodified RF (fd RF·0).

(f) Assay conditions for in vitro modification

Reaction mixtures contained 0.07 M-potassium phosphate (pH 6.7), 7 mM-EDTA and 1 mM-2-mercaptoethanol. The amounts of enzyme, fd RF and [*methyl*- $^3\text{H}$ ]S-AM are indicated in each assay. [*methyl*- $^3\text{H}$ ]S-AM, stored in dilute sulphuric acid (pH 2.5 to 3.5), was added without being neutralized. This did not markedly change the pH of the reaction mixture. Incubations were carried out at 37°C.

(g) Measurement of the extent of methylation

The RF sample to be tested was mixed in known proportions with  $^{32}\text{P}$ -labelled fd sB-1°sB-2°RF·B. As shown in the Results section, this mutant does not accept methyl groups in the modification reaction, even if isolated from strain 0. The modification reaction was carried out as described above and was stopped by the addition of 0.075 ml. of 0.5 M-sucrose, 0.7 M-NaCl, 0.01 M-glycine-NaOH (pH 9.5) to the reaction mixture (0.15 ml. or less) and subsequent incubation at 65°C for 10 min. The sample was layered between the gel surface and the eluent layer of a 15 cm  $\times$  1 cm Sephadex G100 column and eluted with a buffer containing 0.3 M-NaCl,  $10^{-2}$  M-EDTA and  $10^{-2}$  M-Tris-HCl (pH 8.0). The flow rate was approximately 0.2 ml./min, and fractions of 1 ml. were collected. After adding 0.25 mg of carrier DNA to each column fraction, the DNA was precipitated by adding 4 ml. of 2 N-HCl and incubating for 30 min at 0°C. The precipitate was collected by filtration through Whatman glass fiber filters (type GF/C). The filters were successively washed with 2  $\times$  20 ml. 2 N-HCl and 4 ml. of ethanol. They were dried and counted with toluene scintillation fluid in a Beckman scintillation counter in the  $^3\text{H}$ -channel and the  $^{32}\text{P}$ - $^3\text{H}$ -channel. Most of the RF ( $^{32}\text{P}$  counts) was found in 3 adjacent fractions, separated from the bulk of [*methyl*- $^3\text{H}$ ]S-AM. The recovery of RF was between 70% and 80%.

The  $^3\text{H}$  counts which eluted with the RF were first corrected for the counting background, second for the  $^{32}\text{P}$ -overlap, and third for an additional background due to incomplete removal of [*methyl*- $^3\text{H}$ ]S-AM. This background was determined graphically by plotting the acid-precipitable  $^3\text{H}$ -counts versus the  $^{32}\text{P}$ -counts for each column fraction. A straight line was obtained, and the intercept with the ordinate was taken as background value. The values obtained varied between 10 and 75 cts/min, depending on the concentration of [*methyl*- $^3\text{H}$ ]S-AM of the reaction mixture.

The ratio of  $^3\text{H}$  counts to  $^{32}\text{P}$  counts, corrected for the  $^3\text{H}$ -background as described above, gives the relative incorporation of methyl groups into the substrate DNA. From this value and the proportion of substrate fd RF to  $^{32}\text{P}$ -labelled fd sB-1°sB-2° RF·B, the number of methyl groups incorporated per RF molecule was calculated. Two parameters enter this calculation, the counting efficiency of  $^3\text{H}$  and the specific activity of [*methyl*- $^3\text{H}$ ]S-AM. For the determination of the counting efficiency, samples of [*methyl*- $^3\text{H}$ ]S-AM were applied to a filter, dried and counted with toluene scintillation liquid. For the specific activity, the value given by the commercial source was used.

(h) DNA hydrolysis and chromatography of the bases

After the modification reaction, the RF was separated from [*methyl*- $^3\text{H}$ ]S-AM by filtration on a Sephadex column as described above. The fractions which contained the RF were pooled, and 0.2 mg of carrier DNA were added. The total DNA was precipitated with 5% trichloroacetic acid for 30 min at 0°C, collected by centrifugation and redissolved in 0.5 ml. of 0.2 N-NaOH. After another precipitation with 10% trichloroacetic acid, the DNA was re-dissolved in 0.5 ml. formic acid. The formic acid hydrolysis of the DNA was then carried out as described by Wyatt & Cohen (1953). The resulting bases, together with markers of 5-methyl-cytosine and N-6-methyladenine (4 O.D.<sub>260</sub> units each), were chromatographed on Whatman no. 1 chromatography paper in an ammonia-vapour phase with n-butanol-water (86 : 14, v/v) as the solvent (Hotchkiss, 1948). After approximately 30 hr the bases were located by u.v. light. The chromatogram was cut into strips, 2 cm

wide and 1 cm long (i.e. in the direction of solvent migration). Since enough counts were available, the strips were directly placed into vials, toluene scintillation fluid was added, and the radioactivity was determined.

#### (i) Other methods

The techniques used to assay bacteriophage fd and to prepare phage stocks were those described by Arber (1966), except that the host cells were grown in Tryptone broth without maltose. Assays for infectious RF were carried out according to Benzinger (1968). Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), and phosphate concentration by the method of Chen, Toribara & Warner (1956).

### 3. Results

#### (a) Correlation of B-specific modification with methylation of DNA

*S*-adenosylmethionine is required for *in vitro* B-specific modification (Kühnlein *et al.*, 1969). The role played by *S*-AM in this reaction was explored in the experiment presented in Figure 1. Two reaction mixtures for *in vitro* modification were prepared with [*methyl*-<sup>3</sup>H]*S*-AM. Unmodified replicative form of fd DNA (fd RF·0) was added as substrate to one reaction mixture and B-modified fd RF (fd RF·B) to the other. Both were incubated at 37°C for five hours, allowing extensive B-specific modification, and then dialysed in order to remove most of the unincorporated radioactivity. The DNA was reisolated and analysed by sucrose-gradient sedimentation. Assays of the gradient fractions for biological activity revealed two peaks. Their relative positions correspond to those expected for the two forms of fd RF, i.e. RF I (24 s, supercoiled) and RF II (19 s, nicked) (see Marvin & Hohn, 1969). Both peaks obtained with the unmodified substrate, fd RF·0, contained radioactivity, indicating incorporation of <sup>3</sup>H-label into the DNA. No such incorporation was obtained with the B-modified substrate, fd RF·B. These observations demonstrate a correlation between B-specific modification and transfer of the methyl group of the *S*-AM to the DNA substrate.

The chemical nature of the reaction product was further analysed (Fig. 2). A sample of fd RF·0 was modified *in vitro*, purified from non-integrated radioactivity and hydrolysed with formic acid which degrades DNA into bases. These were analysed by paper chromatography together with 5-methylcytosine and *N*-6-methyladenine markers. The radioactivity of the hydrolysate migrated with *N*-6-methyladenine. Hence, the product of B-specific modification is *N*-6-methyladenine, and the modification enzyme is a methylase, to be called DNA methylase M·B as suggested by Arber & Linn (1969).

#### (b) Characterization of specificity site mutants of fd

If phage fd is grown alternatively on host strains of type 0 and of type B, each infection of B selectively favours the growth of mutants of fd, which are less efficiently restricted by B than wild type phage. Such mutants are eventually highly enriched and may form the majority of the phage population. The following typical experiment made with non-mutagenized phage fd confirms preliminary data published by Arber & Kühnlein (1967). After three selective cycles of fd on host B and readaptation of the phage to strain 0, phage lysates were tested for mutants. Among 60 isolates from two independently cycled lysates, approximately 70% had a relative infectivity on B

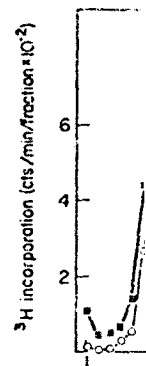


FIG. 1. Incorporation of <sup>3</sup>H into DNA fractions during B-specific modification.

Samples of fd RF·0 and [*methyl*-<sup>3</sup>H]*S*-AM and analysis of the reaction product as described in the text. The radioactivity of the fractions (4.2 Ci/m-mole), 5 ml of RF. After incubation for 5 hours at 37°C, the reaction mixture was dialysed and centrifuged in a Spinco 500 ml. of 10<sup>-3</sup>M-EDTA, 5 ml. sucrose gradients (50% sucrose) at 100,000 rev./min. for 2 hours. The fractions were collected through a tube. The radioactivity of the fractions (■) were determined by scintillation counting. The infectivity (○) was determined by plaque assay as described by Benzinger (1968).

(plaque forming units) of 3.2 × 10<sup>-2</sup> (Table 1). The wild type phage, i.e. a mutant of type 0.

To four of the mutants the phage was applied again. Thirty per cent of the isolates still had a relative infectivity on B. The mutants which had no relative infectivity on B are believed to be mutants of type 0.

Together with other results it is concluded that the DNA of phage fd is modified by B, of which can be lost by B. According to Benzinger (1969), the intact specificity site is indicated by the symbol sB-1. The mutants either sB-1°sB-2 or sB-1°sB-1 do not reveal whether a mutant of type B has lost the specificity site for further experiments.

Besides their changed plaque morphology, the mutants towards B

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### Modification of DNA

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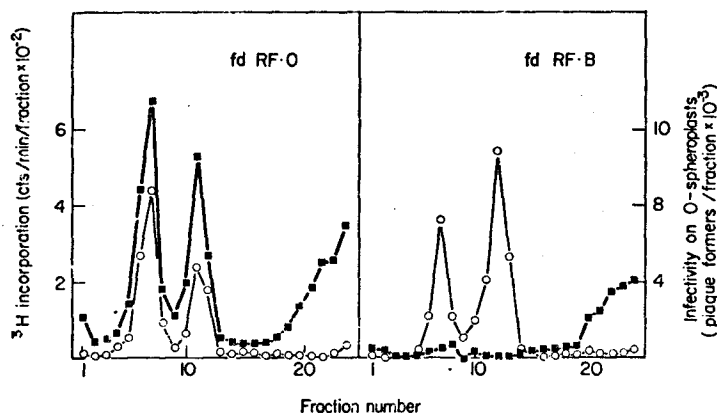


FIG. 1. Incorporation of radioactivity from [methyl- $^3\text{H}$ ]S-AM into fd RF during *in vitro* B-specific modification.

Samples of fd RF-0 and fd RF-B were exposed to the modification enzyme in the presence of [methyl- $^3\text{H}$ ]S-AM and analysed on a sucrose gradient. The reaction mixture (130  $\mu\text{l}$ ), with the composition described in Materials and Methods, contained 50  $\mu\text{M}$  [methyl- $^3\text{H}$ ]S-AM (specific activity 4.2 Ci/m-mole),  $5 \times 10^{-2}$  mg/ml. of the purified enzyme fraction and  $2.5 \times 10^{-2}$  mg/ml. of RF. After incubation for 5 hr at 37°C the reaction mixtures were dialysed for 30 hr against  $4 \times 500$  ml. of  $10^{-3}$  M-EDTA, 0.02 M-glycine-NaOH (pH 9.5). The 2 samples were layered on 5-ml. sucrose gradients (5% to 20% sucrose in  $10^{-3}$  M-EDTA, 0.02 M-glycine-NaOH (pH 9.5)) and centrifuged in a Spinco SW39 rotor for 6 hr at 35,000 rev./min at 15°C. Fractions of 0.2 ml. were collected through a hole punched in the bottom of the tubes. The  $^3\text{H}$  counts of the fractions (—■—■—) were determined by counting 0.1-ml. samples in 10 ml. Bray scintillation liquid. The infectivity (—○—○—) was determined by plating on spheroplasts prepared from strain 991 as described by Benzinger (1968).

(plaque forming units on strain B divided by plaque forming units on strain 0) of  $3.2 \times 10^{-2}$  (Table 1). The remaining 30% had retained the restriction response of the wild type phage, i.e. a relative infectivity on B of  $7 \times 10^{-4}$ .

To four of the mutants (two of each lysate) the same enrichment procedure was applied again. Thirty phage lines were tested of each of the four lysates. On the average 20% of the isolates still grew with an efficiency of  $3 \times 10^{-2}$  on B, while 80% were new mutants which had entirely lost their sensitivity to B-specific restriction. These phages are believed to be double mutants.

Together with other results to be discussed below, these data are taken as evidence that the DNA of phage fd carries two sites with affinity for B-specific restriction, each of which can be lost by mutation. According to the nomenclature of Arber & Liun (1969), the intact specificity sites will be called *sB-1* and *sB-2* and their loss will be indicated by the symbols *sB-1*<sup>o</sup> and *sB-2*<sup>o</sup>, respectively. Thus the one step mutants are either *sB-1*<sup>o</sup>*sB-2* or *sB-1sB-2*<sup>o</sup> and the double mutants are *sB-1*<sup>o</sup>*sB-2*<sup>o</sup>. Our experiments do not reveal whether a particular mutant with restriction response at the intermediate level has lost the specificity site 1 or the specificity site 2. The mutant strain 101 used for further experiments is arbitrarily called *sB-1*<sup>o</sup>*sB-2*.

Besides their changed properties towards B-specific restriction, no difference between any of the mutants tested and the wild type phage could be detected. The plaque morphology, the yield of phage in lysates grown on host 0 and the sensitivity of the mutants towards P1-specific restriction were not altered.

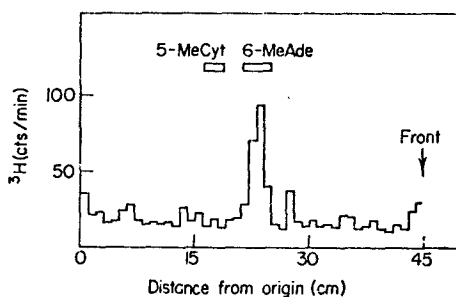


Fig. 2. Chromatogram of the methylated bases of *in vitro* modified fd RF.

The reaction mixture for modification was as indicated in Materials and Methods, with  $5.3 \times 10^{-3}$  mg/ml. of the enzyme fraction,  $5 \times 10^{-2}$  mg of fd RF-0/ml. and  $14 \mu\text{m}$  [methyl- $^3\text{H}$ ]S-AM (specific activity 4.7 Ci/m-mole). Incubation was overnight at  $37^\circ\text{C}$ . The RF was purified from [methyl- $^3\text{H}$ ]S-AM, hydrolysed and the bases separated as indicated in Materials and Methods. The squares indicate regions of authentic 5-methylcytosine (5-MeCyt) and *N*-6-methyladenine (6-MeAde) localized by their absorption of ultraviolet light.

TABLE I

Relative infectivity on B of bacteriophage fd and of its specificity site mutants

Strain	Genetic symbol	Grown on	Relative infectivity on B
Wild-type fd	sB-1 sB-2	0	0.0007
		B	1
One-step mutants	sB-1°sB-2 or sB-1 sB-2°	0	0.032
		B	1
Two-step mutants	sB-1°sB-2°	0	1
		B	1

The relative infectivity on B was determined by dividing the number of plaques formed on the B-strain 2027 by the number of plaques formed on the 0-strain 991. Strain 991 was used to grow stocks of unmodified phage, and strain 2027 was used to produce B-modified phage. The designation of the one-step mutants is arbitrary. As discussed in the text, they might include the two classes sB-1°sB-2 and sB-1 sB-2°.

(c) *The sensitivity of the replicative form of specificity site mutants of fd to DNA methylase M·B and the quantitation of methylation*

Having defined specificity site mutants as refractory to B-specific restriction, we examined whether the RF of such mutants is still a substrate for methylase M·B, i.e. whether mutated sites undergo modification.

The unmodified replicative forms of fd sB-1°sB-2 (strain 101) and of fd sB-1°sB-2° (strain 601, derived as a second-step mutant from strain 101) were isolated, and their capacity to accept methyl groups in the modification reaction was compared with that of the wild type RF. In these experiments, the RF samples to be tested were mixed in known proportions with  $^{32}\text{P}$ -labelled fd sB-1°sB-2° RF·B which is refractory to  $^3\text{H}$ -

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fd sB-1°sB-2° RF-B

The methylated in re of the reaction (ratio of 0.2 (0.0007), 6 (0.032) and 7 (0.032) RF B) (upper) was carried out. The  $^3\text{H}$  was determined

The incorporation represented in Figure methyl groups, measured 1  $\mu\text{m}$  further indicating that the enzyme during the

incorporation (see below). After overnight incubation to allow the completion of the reaction (see section d), the [*methyl*-<sup>3</sup>H]S-AM was separated from the RF by filtration on Sephadex G100. The RF contained in the column fractions was precipitated, collected on filters and the ratio of <sup>3</sup>H counts : <sup>32</sup>P counts was measured.

The results are summarized in Table 2. The amount of <sup>3</sup>H counts incorporated per RF molecule of the mutant fd sB-1°sB-2 was half of that found with the RF of the wild type phage. Essentially no incorporation was obtained with RF of the double mutant fd sB-1°sB-2° as substrate. Again, the *in vivo* modified wild type RF, fd RF·B, run as a control, gave very little incorporation.

The calculation of the number of methyl groups incorporated per RF molecule was based on the specific activity of the [*methyl*-<sup>3</sup>H]S-AM as given by the producing firm and on a molecular weight of fd RF of  $4 \times 10^6$  daltons (see Marvin & Hohn, 1969). The values obtained were 4.5 methyl groups incorporated per wild type RF molecule, and 2.2 methyl groups per RF for the mutant sB-1°sB-2. One specificity site would thus receive two methyl groups in B-specific modification.

TABLE 2

Relative incorporation of methyl groups into RF prepared from phage fd and from two of its specificity site mutants

Substrate	RF concentration of reaction mixture (mg/ml. $\times 10^2$ )	Incorporation of methyl groups		
		Ratio <sup>3</sup> H cts : <sup>32</sup> P cts	<sup>3</sup> H cts/mg of RF (cts/min $\times 10^{-3}$ )	Relative incorporation of methyl groups/RF molecule
fd sB-1 sB-2 RF-0	0.73	0.72	4320	=100%
	1.45	1.35	4060	
fd sB-1°sB-2 RF-0	0.75	0.36	2100	49%
	1.39	0.65	2100	
	1.50	0.67	1950	
fd sB-1°sB-2°RF-0	1.45	0.03	90	2%
fd sB-1 sB-2 RF·B	1.58	0.10	280	7%

The methylation reactions were carried out as indicated in Materials and Methods. The volume of the reaction mixtures was 50  $\mu$ l. and contained 80  $\mu$ M of [*methyl*-<sup>3</sup>H]S-AM (specific activity 4.2 Ci/m-mole),  $6 \times 10^{-2}$  mg/ml. of the enzyme fraction, 43,600 cts/min./ml. of <sup>32</sup>P-labelled fd sB-1°sB-2° RF·B (approx.  $4 \times 10^{-4}$  mg/ml.), and the amounts of unlabelled RF indicated. Incubation was carried out for 12 hr at 37°C. The [*methyl*-<sup>3</sup>H]S-AM was removed and the incorporation of <sup>3</sup>H was determined as indicated in Materials and Methods.

#### (d) Kinetics of the incorporation of methyl groups

The incorporation of methyl groups into fd sB-1°sB-2 RF as a function of time is represented in Figure 3. In the course of about 4 hr of incubation the incorporation of methyl groups, measured as the ratio of <sup>3</sup>H counts : <sup>32</sup>P counts, reaches a saturation level. Upon further addition of RF, the incorporation of methyl groups resumes, indicating that the plateau is not due to exhaustion of S-AM or inactivation of the enzyme during the reaction.

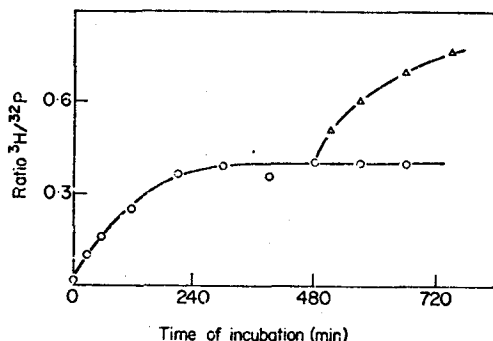


FIG. 3. Incorporation of methyl groups as a function of the time of incubation.

The reaction mixtures (0.4 ml.) contained  $90 \mu\text{M}$  [methyl- $^3\text{H}$ ]S-AM (spec. act. 4.2 Ci/m-mole),  $5.2 \times 10^{-2}$  mg of the purified enzyme fraction/ml., 54,600 cts/min/ml. of  $^{32}\text{P}$ -labelled fd sB-1 $^{\circ}$ sB-2 $^{\circ}$  RF-B (approx.  $3.3 \times 10^{-5}$  mg/ml.) and  $1.1 \times 10^{-2}$  mg/ml. of fd sB-1 $^{\circ}$ sB-2 RF-0. At the times indicated, samples of 20  $\mu\text{l}$ . were removed and the ratio of  $^3\text{H}$  counts :  $^{32}\text{P}$  counts of the RF was determined as described in Materials and Methods (O). After 480 min. of incubation, additional fd sB-1 $^{\circ}$ sB-2 RF-0 was added to a portion of the reaction mixture to give a total RF concentration of  $2.0 \times 10^{-2}$  mg/ml. ( $\Delta$ ).

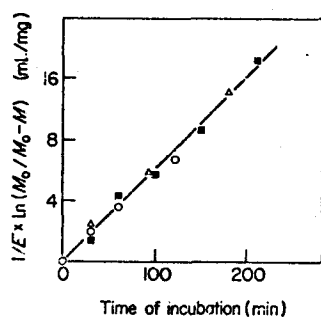


FIG. 4. Semilogarithmic plot of the time-dependence of the methylation reaction.

From the data represented in Fig. 3, the values  $1/E \times \ln(M_0/M_0 - M)$  were calculated and plotted *versus* the time of incubation.  $E$  = enzyme concentration,  $M_0$  = saturation value of the ratio of  $^3\text{H}$  counts :  $^{32}\text{P}$  counts,  $M$  = ratio of  $^3\text{H}$  counts :  $^{32}\text{P}$  counts at time  $t$ . The points were calculated from the beginning of the reaction ( $M_0 = 0.4$ ; O) and from the reaction where additional RF had been added ( $M_0 = 0.8$ ;  $\Delta$ ). A similar experiment was carried out with wild type fd RF as substrate ( $\blacksquare$ ). In this reaction the [methyl- $^3\text{H}$ ]S-AM was  $54 \mu\text{M}$  and the enzyme concentration  $4.7 \times 10^{-2}$  mg/ml.

The semilogarithmic plot of the time dependence of the methylation reaction gives a straight line as expected of a first-order reaction. The same result was obtained with the wild type RF which carries twice the number of methyl acceptor sites per molecule. Again the reaction follows first-order kinetics and the velocity constant is the same as for the mutant RF (Fig. 4).

In agreement with first-order kinetics, the velocity of methylation is proportional to the enzyme concentration (Fig. 5) and the S-AM dependence of the reaction follows

Materials-Method  
to those of Arber et al.  
(1964).

FIG. 5. De

The reaction mix  
0.4 ml.,  $2.6 \times 10^{-2}$   
volume of the react  
at 270, 420 min  
 $^{32}\text{P}$  counts were dete

FIG. 6. Linewe

The reaction mix  
 $3.1 \times 10^{-2}$  mg of fd  
mutant substituted A  
RF-B were added to  
as indicated in Mate  
 $0.4 \text{ ml}$  of  $^3\text{H}$  cts  
The substrate with t

Good experiment  
the observed base  
Smith et al. (1973)  
level of N 6 meth  
this paper demon  
methyl group of  
double-stranded t  
substrate. No meth

Michaelis-Menten kinetics (Fig. 6). The Michaelis constant is  $4 \times 10^{-6}$  M. This value is 10 times lower than the value for the DNA methylases isolated by Gold & Hurwitz (1964).

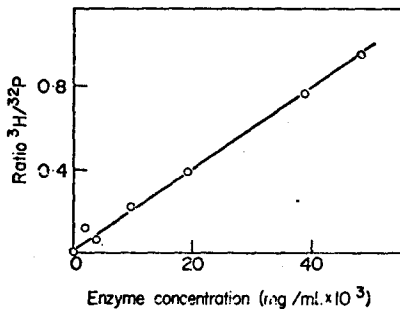


Fig. 5. Dependence of the reaction velocity upon the enzyme concentration.

The reaction mixtures (0.105 ml.) contained  $35 \mu\text{M}$  [*methyl- $^3\text{H}$* ]S-AM (spec. act. 4.7 Ci/m-mole),  $3.6 \times 10^{-2}$  mg of fd sB-1'sB-2 RF-0/ml. and the amounts of enzyme indicated. The volumes of the reaction mixtures were adjusted with 50% glycerol. After incubation for 40 min at  $37^\circ\text{C}$ , 820 cts/min of  $^{32}\text{P}$ -labelled fd sB-1'sB-2 RF-B were added and the ratios of  $^3\text{H}$  counts to  $^{32}\text{P}$  counts were determined as described in Materials and Methods.

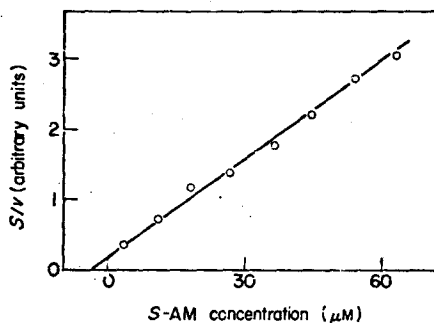


Fig. 6. Lineweaver-Burk plot of the S-AM dependence of the methylation reaction.

The reaction mixtures (0.1 ml.) contained  $1.4 \times 10^{-2}$  mg of the enzyme preparation/ml.,  $3.1 \times 10^{-2}$  mg of fd sB-1'sB-2 RF-0/ml. and the amount of [*methyl- $^3\text{H}$* ]S-AM (spec. act. 4.7 Ci/m-mole) indicated. After incubation for 40 min at  $37^\circ\text{C}$ , 1290 cts/min of  $^{32}\text{P}$ -labelled fd sB-1'sB-2 RF-B were added to each reaction mixture and the ratio of  $^3\text{H}$  counts :  $^{32}\text{P}$  counts was determined as indicated in Materials and Methods. In the Figure, the values S/v (S = concentration of S-AM, v = ratio of  $^3\text{H}$  cts :  $^{32}\text{P}$  cts after 40 min of incubation) are plotted versus the S-AM concentration. The intercept with the abscissa is the negative value of the Michaelis constant.

#### 4. Discussion

Good experimental evidence has now been obtained that nucleotide methylation is the chemical basis for B-specific modification of DNA. The *in vivo* data presented by Smith *et al.* (1972) show that B-modified phage fd DNA contains a twofold higher level of N-6-methyladenine than unmodified fd DNA. The *in vitro* data presented in this paper demonstrate that the B-modification enzyme is a methylase: it transfers the methyl group of the coenzyme S-adenosylmethionine to the substrate DNA, the double-stranded replicative form of fd DNA. The reaction product is N-6-methyladenine. No methylation is obtained with B-modified DNA as substrate.

The correlation between modification and nucleotide methylation is also reflected in the response of specificity site mutants to modification. These mutants were isolated as refractory to B-specific restriction. They are now found to have no affinity for B-specific methylation, both *in vivo* and *in vitro*. The simultaneous loss of sensitivity for restriction and modification indicates that the two responsible enzymes recognize the same base sequences on the substrate DNA. However, only the mutated specificity sites of strain 601 have been tested, and we do not know whether restriction refractory sites can be obtained which still accept strain-specific methylation.

The strains of fd which are completely refractory to B-specific restriction could only be obtained in two discrete steps of mutation. This is taken as evidence that the DNA of wild-type fd carries two B-specificity sites. Support for this thesis comes (1) from Linn's (personal communication) electron microscopical observation that *in vitro* B-specific restriction of the circular replicative form of fd produces two DNA fragments of unequal length, and (2) from the quantitative measurement of B-specific methylation of fd and its mutants (see below). Two B-specificity sites are also carried on the DNA of phage f1, a close relative of fd. The sites of f1 have been genetically mapped (Boon & Zinder, 1971).

Wild-type fd with two B-specificity sites bypasses restriction with a probability amounting to roughly the square of the value found for the mutants which have lost one of the sites. This observation indicates that successful infection of restrictive bacteria with DNA molecules carrying a small number of specificity sites is primarily a function of the number of sites recognized by the host-specificity enzymes, and only secondarily a function of "exceptional" host cells, which are believed to determine whether a DNA molecule with a higher number of specificity sites escapes restriction (see Arber & Linn, 1969).

The number of methyl groups incorporated per fully modified RF molecule is close to 4 for the wild type fd and 2 for the mutant fd *sB-1<sup>s</sup>B-2*. Thus, each B-specificity site of the double-stranded RF receives two methyl groups in complete modification. Experiments of Kellenberger, Symonds & Arber (1966) and of Meselson & Yuan (1968) indicate that modification alters both strands at each specificity site. One might consequently expect that the two methyl groups of a modified site are located on opposite strands of the DNA. This conclusion is supported by the result of Smith *et al.* (1972) that *in vivo* modified single-stranded fd DNA carries one *N*-6-methyladenine per B-specificity site.

The methylation reaction is of first order and the *S*-AM dependence of the reaction velocity follows Michaelis-Menten kinetics. These data indicate that the four methyl groups of modified fd RF (two per specificity site) are transferred to the DNA in independent reactions with almost identical rate constants. The equal reactivity of the methyl acceptor sites suggests that (1) the specificity sites have similar structures (i.e. base sequences) and (2) the sites themselves possess an internal symmetry which allows the enzyme to react with equal probability with either of the strands. Kelly & Smith (1970) have determined part of the base sequence of the sites which are recognized by a restriction enzyme from *Haemophilus influenzae*. They found that all these sites contain the sequence pGpTpPy'pPupApC (the apostrophe indicates where restriction scission occurs). This sequence is symmetric: the complementary sequence, also read in the 5' to 3' direction, is again pGpTpPy'pPupApC. It is not yet known if symmetry is also a property of the B-specificity sites. However, the kinetic of B-specific methylation is suggestive.

We thank Dr  
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We thank Dr. Stuart Linn for his valuable advice and stimulating discussions. This research was supported by grant 3.148.69 from the Swiss National Foundation for Scientific Research.

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