On the Nature of the Deoxyribonucleic Acid Methylases. Biological Evidence for the Multiple Nature of the Enzymes*

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ABSTRACT: The minor components of DNA of several bacterial species were determined by growing the organisms in the presence of [*methyl*-^{14}C]methionine and analyzing the labeled bases by isotope dilution. The DNA's were found to contain either 6-methylaminopurine or both 6-methylaminopurine and 5-methylcytosine. The interaction of extracts derived from some of these organisms with a variety of DNA's was also examined. The results of these studies suggest that the DNA methylases as well as the substrate DNA's possess directive specificity regarding the pattern of methylation. The findings confirm that the DNA methylases are species specific and also demonstrate the existence of two types of DNA methylases: one capable of methylating adenine and the other cytosine. Infection by T2 bacteriophage elevates the DNA methylase activity of both *E. coli* B and *E. coli* K_{12}. Extracts of *E. coli* B can methylate only adenine of DNA, whereas in *E. coli* K_{12} two methylating capacities are normally present, producing 5-methylcytosine and 6-methylaminopurine. However, on phage infection only the level of the adenine methylase was elevated.

Several years ago, Kornberg *et al.* (1959) suggested the possibility that the methylated bases of DNA may be acquired by the methylation of the primary bases of the preformed polymer. The discovery of the species specificity of the enzymes which methylate transfer ribonucleic acid (t-RNA), the t-RNA methylases, offered a means of testing this hypothesis, for it appeared highly likely that the DNA methylases may also turn out to be species specific. Evidence for the methylation of DNA at the polymer level and for the species specificity of the DNA methylases has recently been published by Gold and Hurwitz (1963, 1964a), but they have not as yet been able to resolve the question as to whether the methylation of DNA is achieved by a multienzyme system, as is the case with the t-RNA methylases.

We wish to report here our experience with the species variation of the DNA methylases. Our studies of DNA methylation *in vivo* and *in vitro* with a variety of microorganisms under varied conditions lead to the conclusion that two separate enzyme potencies exist, one of which methylates cytosine and the other adenine.

Experimental Section

The bacterial strains used in this investigation are from our collection. We are grateful to Mrs. Charlotte Shemin for the strain of *Shigella dysenteriae* and to Dr. Stephen Zamenhof for the strain of *Bacillus subtilis*. [*methyl*-^{14}C]*S*-Adenosyl-L-methionine was purchased from New England Nuclear Corp., Boston, Mass., and [*methyl*-^{14}C]methionine from California Corp. for Biochemical Research. Calf thymus DNA was obtained from Worthington Biochemical Corp., Freehold, N. J.

Preparation of Enzyme Extract. The organisms were grown on nutrient broth (Difco) supplemented with glucose (0.5%) and were harvested in the logarithmic phase of growth. *Bacillus subtilis* was grown on Penn assay broth (Difco) supplemented with 1% glucose. The following complex medium was used for growing *Proteus vulgaris:* NaH_2PO_4, 7.9 g; K_2HPO_4, 1.5 g; nutrient broth (Difco), 10 g; NaCl, 5 g; Casamino acid (Difco), 5.0 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; glucose, 5.0 g, and distilled water to 1 l. *Rhodopseudomonas spheroides* was grown anaerobically in the presence of light in medium S of Lascelles (1956) in 2-l. bottles. *Pseudomonas fluorescens* was grown at 30°.

For the extraction of the enzymes 2 g wet weight of cells was suspended in 10 ml of 0.02 M Tris buffer, pH 8.0, containing 10 μmoles of $MgCl_2$, 3 μmoles of disodium ethylenediaminetetraacetate, and 10 μmoles of mercaptoethanol and were disrupted by means of sonic oscillation for 15 min in a 9-kc Raytheon oscillator. The cell debris and unbroken cells were eliminated by centrifugation for 1 hr at 105,000 × *g* in the Spinco preparative centrifuge, and the clear extracts thus obtained were utilized as the source of the enzymes.

Assay of DNA Methylase Activity. The activity of the enzyme was determined by measuring the incorporation of radioactivity into an acid-insoluble product which on hydrolysis yielded specific methylated bases.

The incubation mixture contained 25 μmoles of Tris buffer, pH 8.0, 1 μmole of $MgCl_2$, 5 μmoles of mer-

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captoethanol, 100 mμmoles of the indicated DNA (expressed on a nucleotide basis), 5 mμmoles of [*methyl*-^{14}C]adenosylmethionine (0.2 μcurie), and 0.05 ml of the enzyme extract (1 mg of protein) in a total volume of 0.25 ml. The incubations were carried out at 37° for 30 min and the reaction was terminated by the addition of cold trichloroacetic acid (5%). The contents were then filtered through millipore filters and were washed extensively with cold trichloroacetic acid (5%). The membrane filter containing the precipitate was then mounted on a metal planchet, dried at 60° for 30 min, and counted to assess the incorporation of radioactivity. With each extract a control experiment was performed to determine the incorporation in the absence of any added DNA, and such background values were subtracted from the results presented in the tables. The protein content of bacterial extracts was measured by the method described by Lowry *et al.* (1951) using bovine serum albumin as the standard.

Pattern of Methylation. In these experiments incubations were carried out on a scale ten times larger than that employed for evaluating the DNA methylase activity. At the end of the incubation an equal volume of phenol saturated with H_2O was added, and the mixture was shaken for 10 min and centrifuged. To the aqueous layer containing the DNA, 0.33 volume of cold 20% trichloroacetic acid was added and the precipitate was centrifuged. The precipitate was repeatedly washed with cold 5% trichloroacetic acid, 80% ethanol, and finally ethanol–ether (3:1). In some cases the reaction was stopped by the addition of 20% trichloroacetic acid to a final concentration of 5%. The precipitate thus obtained was washed three times with cold trichloroacetic acid (5%), once with 80% ethanol, and finally with ethanol–ether (3:1). It was then hydrolyzed with 98% formic acid in sealed tubes and processed by the method outlined for the determination of methylated bases of DNA.

Isolation of DNA for Enzymatic Methylation. DNA was isolated essentially by the method of Marmur (1961) except for a minor modification. After ribonuclease treatment, deproteinization was carried out by shaking with phenol instead of chloroform–isoamyl alcohol mixture. DNA from *Micrococcus lysodeikticus* was prepared from spray-dried cells purchased from Miles Chemical Laboratories, Elkhart, Ind. The estimation of DNA was performed by the diphenylamine reaction. DNA's from *E. coli* $K_{12}W_6$ and *E. coli* W122–33 after starvation of methionine were prepared from cells of these two strains starved by the previously described procedure (Borek *et al.*, 1955) as modified by Peterkofsky *et al.* (1964).

Isolation of DNA from Cells Grown on [methyl-^{14}C]-Methionine and Determination of Radioactivity in 6-Methylaminopurine and 5-Methylcytosine. For *in vivo* labeling of the methylated bases the bacterial strains were grown on appropriate media with either 50 or 100 μcuries of [*methyl*-^{14}C]methionine per liter of medium and harvested before the end of the exponential growth phase. The method employed for the isolation of DNA from approximately 1 g of wet cells was essentially similar to the procedure outlined by Marmur (1961) except for the deproteinization by phenol. The aqueous layer from this step was treated with three volumes of ethanol and centrifuged, and the precipitated DNA was treated with 0.5–1 ml of 0.1 N NaOH and incubated at 30° for 24 hr. Acetic acid (1 N) was then added to neutralize the base and two volumes of ethanol was added to precipitate the DNA. The DNA precipitate was washed successively with 66% ethanol, 80% ethanol, 95% ethanol, and ethanol–ether (3:1) and finally dried *in vacuo* in a desiccator over P_2O_5 and KOH. The dry residue was then hydrolyzed in a sealed tube with 0.1–0.2 ml of 90% HCOOH at 175° for 30 min. The hydrolysate (50–100 μl) was applied on Whatman No. 1 paper with 1 μmole each of 6-methylaminopurine and 5-methylcytosine as carriers. The paper was then subjected to two-dimensional chromatography. Butanol–0.1 N NH_4OH (6:1) was used in the first dimension for 18–24 hr followed by 2-propanol–concentrated HCl–H_2O (65:17.2:17.8) in the second dimension. The 6-methylaminopurine and 5-methylcytosine spots were detected by ultraviolet light. The spots were cut into thin strips and extracted with 2 ml of 0.1 N HCl by placing the tubes at 37° for 18 hr. Aliquots of these solutions were transferred to stainless steel planchets, dried, and counted. The adenine spot was also eluted by a similar procedure and the amount was estimated by customary spectrophotometric methods. The results are expressed as counts per minute incorporated into 6-methylaminopurine and 5-methylcytosine per μmole of adenine. The amount of adenine in most experiments ranged from 0.5 to 1.2 μmoles. In some instances the specific radioactivities of 6-methylaminopurine and 5-methylcytosine present in the eluate were determined. Aliquots were dried and subjected to further chromatography in two different solvent systems: butanol–acetic acid–H_2O (4:1:2) and 2-propanol–H_2O (6:4). The ultraviolet-absorbing spots were identified, cut, and eluted in 0.1 N HCl. The specific radioactivities were again determined and found to be essentially identical with the value determined earlier.

T2 Infection of E. coli B and E. coli K_{12}. *E. coli* B or *E. coli* $K_{12}W_6$ was grown to logarithmic phase to a population of about 5×10^8 cells/ml in Difco nutrient broth (0.8%) containing 0.5% NaCl. For effective infection of *E. coli* $K_{12}W_6$ the medium had to be supplemented with $MgCl_2$ (10^{-3} M) and L-tryptophan (30 mg/l.). T2 bacteriophage was added to 1-l. lots of the bacterial cultures to a multiplicity of 8 to 10. The cultures were vigorously shaken and well aerated for 10 min. The infection was aborted by pouring the cultures onto cracked ice. The infected cells were harvested by centrifugation at 10,000 × *g* in the cold. The bacterial pellet was washed once with 0.9% NaCl. For the preparation of enzyme extracts 0.6 g of normal and infected cells was suspended in 5.5 ml of buffer and disrupted as described earlier. The rate of methylation was determined by adding varying amounts of enzyme extract of normal and T2 infected cells to reaction mixtures. The apparent extent of methylation presented in

TABLE I: Interaction of Bacterial DNA's and DNA Methylases.[a]

Source of DNA	A + T / G + C	Source of Enzyme Extract: *E. coli* B	*E. coli* $K_{12}W_6$	*E. coli* W122–33	*Shigella dysenteriae*
		CH_3 Incorporation ($\mu\mu$moles)			
M. lysodeikticus	0.39	127	127	133	180
Rh. spheroides	0.47	178	129	248	257
Ps. aeruginosa	0.55	116	98	158	202
Ps. fluorescens	0.58	78	94	131	144
S. dysenteriae	0.87	6	7	0	0
S. typhimurium	0.92	7	0	7	0
E. coli $K_{12}W_6$	0.96	4	5	0	0
B. subtilis	1.36	137	162	121	171
Proteus vulgaris	1.60	9	26	16	1
B. cereus	2.00	62	70	72	63

[a] Activity is expressed as $\mu\mu$moles of methyl groups incorporated into 100 mμmoles of DNA (on a nucleotide basis) with [$^{14}CH_3$]S-adenosylmethionine as methyl donor.

Table V was obtained by using 0.19 mg of protein of T2 infected cell extract and 0.58 mg of *E. coli* B extract. The extent of methylation reported in Table VI was obtained by using 0.25 mg of T2 infected cell extract and 0.4 mg of *E. coli* $K_{12}W_6$ extract. The patterns of methylation were determined by carrying out incubations on a scale five times larger than that employed for evaluating the extent of methylation.

All radioactivity determinations were carried out in a Nuclear Chicago low background end-window gas flow counter whose average background was 3 cpm (efficiency *ca.* 20%).

Results and Discussion

Studies of the interaction of enzyme extracts from four different organisms with DNA from a variety of sources are presented in Table I. It is quite apparent that the introduction *in vitro* of supernumerary methyl groups into DNA is independent of the over-all primary base composition of the substrate DNA. The species specificity of the DNA methylating enzymes is also equally evident. The substrate DNA's prepared from *S. dysenteriae*, *S. typhimurium*, and *E. coli* $K_{12}W_6$ were unable to serve as recipients of methyl groups with extracts derived from *E. coli* B, *E. coli* K_{12}, and *S. dysenteriae*, indicating that DNA from a given source is fully saturated not only with respect to its own enzymes but also with respect to extracts derived from closely related organisms, a phenomenon already amply documented in the species variation of t-RNA methylases.

The normal distribution of the methylated bases of DNA, 6-methylaminopurine and 5-methylcytosine, is presented in Table II. Of the nine organisms investigated, only three contain predominantly 6-methylaminopurine, *i.e.*, *E. coli* B, *Proteus vulgaris*, and *Pseudomonas aeruginosa*. Early reports based on chromatographic analyses minimized the presence of 5-methylcytosine in the DNA of bacterial species. The

TABLE II: Incorporation of $^{14}CH_3$ into DNA of Organisms Grown on [*methyl*-$^{14}CH_3$]Methionine.

Organism	cpm/μmole of Adenine[a]: 6-Methylaminopurine	5-Methylcytosine	Ratio 6-MAP/5-MC[1]
E. coli B	5760	25	230
E. coli $K_{12}W_6$	4680	2260	2.1
E. coli W122–33	3440	2280	1.5
S. dysenteriae	2430	1130	2.2
B. cereus	120	30	4.0
B. subtilis	190	110	1.7
Proteus vulgaris	280	(4)[b]	Predominantly 6-MAP
Ps. aeruginosa	290	(2)[b]	Predominantly 6-MAP
Ps. fluorescens	140	30	4.7

[a] The values given in the table are obtained from two independent determinations of the formic acid hydrolysate. [b] Since the average background count is 3 cpm, these values have but marginal significance.

[1] The following abbreviations have been used: 6-MAP, 6-methylaminopurine; 5-MC, 5-methylcytosine.

TABLE III: Interaction of Heterologous DNA's with DNA Methylases. Pattern of Methylation (Ratio of 6-Methylaminopurine to 5-Methylcytosine).[a]

Source of DNA	$\frac{A+T}{G+C}$	Source of Enzyme Extract			
		E. coli B	*E. coli* $K_{12}W_6$	*E. coli* W122–33	*Shigella dysenteriae*
		(Ratio of 6-MAP to 5 MC)			
M. lysodeikticus	0.39	>200[b]	3.6	6.3	2.6
Rh. spheroides	0.47	>200[b]	12	33	25
Ps. aeruginosa	0.55	>200[b]	3.3	7.3	4.3
Ps. fluorescens	0.58	>200[b]	3.1	5.0	5.3
B. subtilis	1.36	>200[b]	23	54	32
B. cereus	2.00	>200[b]	6.8	8.1	13

[a] The values given in the table are derived from two independent determinations with each bacterial extract. [b] Predominantly 6-methylaminopurine; incorporation into 5-MC was negligible. In all the other experiments the ratios were calculated from incorporations where the radioactivity of 5-MC was at least 20 times background count.

possibility of the specific labeling of the methylated bases by the incorporation of labeled methyl groups permits more dependable assessment of their presence. The variations of the levels of the two bases can be striking, as exemplified by the hundredfold difference in the ratios of the two bases in two different strains of *E. coli:* B and K_{12}. These findings are in complete agreement with a recent report demonstrating the occurrence of 5-methylcytosine in bacteria (Doskocil and Soronova, 1965). In view of these results one can conclude that bacterial DNA may contain either 6-methylaminopurine or 5-methylcytosine or both, depending on the microorganism.

The occurrence of either 6-methylaminopurine or 6-methylaminopurine with 5-methylcytosine in the DNA of some bacterial strains permitted the probing of an interesting question on the interrelationship between the nature of the methylated bases present in the DNA of a particular strain and the pattern of methylation achieved by its enzyme extracts against heterologous DNA's.

The ratios of the two methylated bases introduced by enzyme extracts into heterologous DNA's are presented in Table III. The original ratio of the two bases in the DNA of the organism which is the source of the enzyme is not necessarily imposed upon the heterologous substrates. Enzymes from *E. coli* $K_{12}W_6$, *E. coli* W122–33, and *Shigella dysenteriae* can introduce methyl groups into heterologous DNA's, yielding ratios of 6-methylaminopurine to 5-methylcytosine in over tenfold excess of that of the DNA of the enzyme's donor. This finding seems to indicate that the recipient DNA has a directive influence over the sites to be methylated. On the other hand, *E. coli* B, which has only 6-methylaminopurine in its DNA, yields enzyme extracts which are incapable of methylating cytosine in any heterologous DNA. This finding indicates that such an enzymatic capacity may be lacking in *E. coli* B and it may be inferred, therefore, that a separate enzyme must exist for the methylation of cytosine and of adenine. The changes in the DNA methylases which occur as a result of bacteriophage infection, discussed later in this communication, also support such an inference.

Gold and Hurwitz (1963) have reported that interspecies methylation among various strains of *E. coli* is possible. Our observations have confirmed such a conclusion. From the data in Table IV it is evident that enzymes obtained from *E. coli* B cannot methylate either homologous DNA or the DNA originating from *E. coli* K_{12}. On the other hand, DNA from *E. coli* B can act as a recipient substrate of methyl groups for enzymes extracted either from *E. coli* W or *E. coli* K_{12}. Both of these extracts methylate cytosine in the DNA of *E. coli* B even though the indigenous enzymes of that strain of *E. coli* lack such a capacity.

The enzymes from all three strains of *E. coli* can methylate DNA extracted from cultures of *E. coli* K_{12}-W_6 RC^{rel} which had been starved of methionine for 3 hr. On the other hand, the DNA extracted from *E. coli* W122–33 RC^{Str} after methionine starvation of that organism does not serve as a substrate for methylation by any of the three enzymes. *E. coli* $K_{12}W_6$ and *E. coli* W122–33 are both auxotrophs for methionine with blocks before cystathionine, but differ in that the former has lost its genetic control over RNA synthesis, and consequently RNA accumulates during deprivation of the amino acid. The accumulation of DNA during methionine starvation had been found to be the same (about 25%) in the organisms with the relaxed and stringent controls. On the other hand, Gold and Hurwitz (1964a,b) have reported increases of as much as 100% in the DNA content of cells of *E. coli* $K_{12}W_6$ after methionine starvation and suggested that DNA as well as RNA synthesis is under relaxed control. However, two other groups of investigators (Dagley *et al.*, 1963; Friesen and Maaløe, 1965), as well as the original investigators (E. Borek and A. Ryan, unpublished observations), on a re-examination of the problem could

TABLE IV: Intermethylations among Strains of *E. coli*.

Source of DNA	Source of Enzyme Extract: *E. coli* B Activity[a]	*E. coli* B Ratio[b] 6-MAP/5-MC	*E. coli* $K_{12}W_6$ Activity	*E. coli* $K_{12}W_6$ Ratio[b] 6-MAP/5-MC	*E. coli* W122–33 Activity	*E. coli* W122–33 Ratio[b] 6-MAP/5-MC
E. coli B	4		22	0.13	27	0.12
E. coli $K_{12}W_6$ RC^{rel} "log"	4		5		0	
E. coli $K_{12}W_6$ RC^{rel} "starved"	58	>200	31	2.5	59	2.1
E. coli W122-33 RC^{Str} "starved"			0		0	

[a] $\mu\mu$moles of methyl groups incorporated into 100 mμmoles of DNA (expressed on a nucleotide basis). [b] The pattern of methylation was evaluated from two independent experiments with each bacterial extract.

find no appreciable difference in DNA synthesis during methionine deprivation of organisms with stringent and relaxed controls. The preponderant evidence, originating in particular from Maaløe's laboratory, refutes the coincidence of simultaneous relaxed control over RNA and DNA synthesis. However, as can be seen from the data in Table IV, the DNA's isolated from the methionine-starved organisms with the relaxed control over RNA synthesis do have the unique capacity to accept methyl groups from homologous enzymes. The origin of this anomaly may be rooted in a consequence of the relaxed control over RNA synthesis. Even in a normal cell RNA is present in preponderately larger amounts than DNA, and in the organisms with the relaxed control extensive synthesis of all RNA's continues unabated even though the pool of methionine is diminishing. The nascent RNA may compete successfully with the DNA for the last traces of methyl groups and therefore the last round of DNA synthesis may produce a product devoid of methyl groups.[2]

It is noteworthy that the pattern of methylation of *E. coli* $K_{12}W_6$ "starved DNA" by heterologous enzymes derived from *E. coli* B, *E. coli* K_{12}, and *E. coli* W mirrors very closely the ratio of 6-methylaminopurine to 5-methylcytosine found in the strain which yields the enzymes. Thus *E. coli* B contains predominantly 6-methylaminopurine in its DNA, and its extracts introduce *only* 6-methylaminopurine into $K_{12}W_6$ starved DNA. On the other hand, *E. coli* K_{12} and *E. coli* W, which contain both 6-methylaminopurine and 5-methylcytosine in their DNA's, are capable of introducing methyl groups into both adenine and cytosine and yield a ratio of 6-methylaminopurine to 5-methylcytosine similar to that obtained *in vivo*. These studies again reflect the occurrence of two types of DNA methylases: adenine methylase and a cytosine methylase.

An increase in DNA methylase activity in the host bacteria after infection by T2 bacteriophage has been

TABLE V: Effect of T2 Infection on *E. coli* B.

	Infected	Normal
A. Rate of methylation: substrate *M. lysodeikticus* DNA: mμmoles/mg of protein/30 min	36.4	0.76
B. Apparent extent of methylation: $\mu\mu$moles of methyl groups incorporated per 100 mμmoles of DNA (expressed on a nucleotide basis)		
M. lysodeikticus DNA	1000	182
Rh. spheroides DNA	1530	229
Ps. aeruginosa DNA	610	116
Ps. fluorescens DNA	652	132
S. dysenteriae DNA	2	3
S. typhimurium DNA	6	0
E. coli B DNA	0	0
E. coli $K_{12}W_6$ DNA	0	0
E. coli $K_{12}W_6$ starved DNA	48	41
B. subtilis DNA	771	197
Proteus vulgaris DNA	8	2
B. cereus DNA	318	—
Calf thymus DNA	435	75
Rat spleen DNA	108	33
C. Pattern of methylation (ratio 6-MAP/5-MC)		
M. lysodeikticus DNA	>500	>500
B. subtilis DNA	>500	>500

[2] The difference in the DNA's produced during the starvation of the RC^{rel} and RC^{Str} strains has also emerged from a different biological phenomenon. Arber (1965) has shown that λ bacteriophage produced during partial deprivation of methionine in the RC^{rel} mutant has a highly altered host range specificity. In an RC^{Str} auxotroph methionine deprivation yields λ less altered with respect to host range specificity. The difference must stem from the greater ease of production of methyl-deficient DNA in the RC^{rel} strain.

reported (Gold and Hurwitz, 1964b). We have confirmed this basic observation and we also studied rates and patterns of methylation by enzymes from normal *E. coli* B and *E. coli* K_{12} and infected cells of these two strains. These studies are presented in Tables V and VI.

TABLE VI: Effect of T2 Infection on *E. coli* $K_{12}W_6$.

	Infected	Normal
A. Rate of methylation: substrate *M. lysodeikticus* DNA: mμmoles/mg of protein/30 min	16.0	0.62
B. Apparent extent of methylation: μμmoles of methyl groups incorporated per 100 mμmoles of DNA (expressed on a nucleotide basis)		
M. lysodeikticus DNA	841	121
E. coli $K_{12}W_6$ DNA	0	0
E. coli $K_{12}W_6$ "starved" DNA	58	56
B. subtilis DNA	657	103
C. Pattern of methylation (ratio of 6-MAP/5-MC)		
M. lysodeikticus DNA	45	3.6
Ps. fluorescens DNA	51	3.2
B. subtilis DNA	190	23

Both the rates of methylation and the apparent extent of methylation are elevated by enzymes extracted from infected cells of the two strains of *E. coli*. Studies of the patterns of methylation by enzymes from the two organisms promised to be fruitful, because in *E. coli* $K_{12}W_6$ the potencies to methylate adenine and cytosine are both present, whereas in *E. coli* B the DNA cytosine methylase is essentially absent. T2 infection did not change the patterns of methylation inherent in *E. coli* B either with homologous or heterologous DNA's. Both before and after the infection the DNA adenine methylase capacity preponderates. On the other hand, the infection of *E. coli* $K_{12}W_6$ by T2 bacteriophage produced a profound change in the relative methylating capacities for the two bases. There was a tenfold increase in the adenine methylase activity, with three different DNA substrates, indicating that only the adenine methylase activity was enhanced by T2 infection. The preferential elevation of the adenine methylase capacity as a concomitance of phage infection is especially significant if we recall that T2 phage contains 6-methylaminopurine (Dunn and Smith, 1958). Whether the enhanced methylase activity is the result of a new enzyme induced by the phage remains to be explored.

The species specificity of enzymes which alter macromolecules at the t-RNA and DNA level may have profound biological significance. They may determine *inter alia* the species individuality of nucleic acids. That such individuality can be achieved by methylation of DNA has been recently demonstrated by the meticulous studies of Arber (1965), who has provided evidence that the host range specificity of lambda bacteriophage can be altered by a deficiency of methylation of the phage DNA in an RC^{rel} mutant.

The species specificity of the DNA methylases enables *in vitro* heterologous methylations and opens a new approach to the study of the biosynthesis of DNA. It is apparent that both the DNA and its methylating enzymes have directive specificity in the levels and patterns of methylation to be achieved. However, the geographic distribution of the methyl groups in DNA is not yet accessible to study and therefore the base sequences which may direct methylation of specific sites must remain obscure for the present.

The enzymes which catalyze methylation of the two bases in DNA have not as yet been physically separated, but the biological evidence presented here points to the existence of two separate potencies. This was to be expected both from theoretical considerations and from experience with the t-RNA methylations. In cytosine a carbon–carbon bond is achieved whereas in adenine an amino group is methylated. No single enzyme could be expected to have such wide-ranging versatility. It may be argued that two types of DNA methylases exist, one catalyzing the methylation of adenine and the other both adenine and cytosine. This is unlikely in view of the demonstration of Doskocil and Sornova (1965) that a strain of *B. subtilis var atterimus* contains predominantly 5-methylcytosine.

The alteration of the relative levels of DNA methylating enzymes in *E. coli* K_{12} by T2 infection may have a significance not restricted to that host and virus pair. It has recently been shown in our laboratories (Wainfan *et al.*, 1965) that the total t-RNA methylating capacity as well as the pattern of t-RNA methylation is altered by T2 infection of *E. coli* B. After the discovery of the species specificity of the nucleic acid methylating enzymes we postulated the possibility of oncogenesis by aberrant or excessive methylations by enzymes induced in the host cell by an oncogenic virus (Srinivasan and Borek, 1964). The findings of changes in the RNA and DNA methylases produced by T2 bacteriophage provide a tool for the study of this hypothesis in microbial systems. Studies of the possibility of parallel phenomena in mammalian cells are under way.

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The Biosynthesis of Tyrosine from Labeled Glucose in *Escherichia coli**

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ABSTRACT: The pathway of biosynthesis of tyrosine in *Escherichia coli* was investigated with labeled glucose. C-6 of glucose was incorporated into the ring of tyrosine only in C-2,6 and to the extent of 1.1 atoms. This result is identical with that observed earlier in the ring of shikimic acid, a precursor of tyrosine and several other aromatic metabolites. Similar correspondence between the rings of tyrosine and shikimic acid had already been observed with [1-^{14}C]glucose in yeast. Confirmation is therefore provided for the earlier conclusion, based on the enzymic reactions involved in the formation and function of prephenic acid, that in the conversion of shikimate to tyrosine the ring remains intact, C-1 to C-6 of shikimate corresponding to C-1 to C-6, respectively, of tyrosine. In view of the known origin of the rest of the ring of shikimic acid, these results are in conflict with the observation of other workers that in *Aerobacter aerogenes* [3,4-^{14}C]glucose gives rise to extensive labeling in three successive atoms, either C-2,3,4 or C-4,5,6, of tyrosine. Certain limitations of the degradation of labeled tyrosine are discussed, and a modification of the procedure is introduced.

Several lines of evidence have shown that shikimic acid is an intermediate in the biosynthesis of tyrosine, phenylalanine, and tryptophan as well as certain aromatic vitamins (Davis, 1951, 1955; Tatum *et al.*, 1954). On structural grounds it seemed reasonable to assume that in the formation of the benzene ring of these compounds the carbon ring of shikimate would remain intact.

This assumption was supported by the results of enzymic and other studies on the conversion of shikimate (I) to phenylalanine, tyrosine, and tryptophan (Scheme I). Shikimate 5-phosphate (II), formed from shikimate and adenosine triphosphate (Fewster, 1962), was shown to react with enolpyruvate phosphate to yield 3-enolpyruvylshikimate 5-phosphate (III) (Levin and Sprinson, 1964). Elimination of orthophosphate from III gave chorismic acid (IV) (Clark *et al.*, 1964), which was originally isolated from culture filtrates of a multiple mutant of *Aerobacter aerogenes* and shown to be the branch point intermediate for the formation of prephenate (V) and anthranilate (VI) (Rivera and Srinivasan, 1963; Gibson and Gibson, 1964). Hence, the carboxyl group on C-1 of prephenate is the original carboxyl of shikimic acid. This carboxyl group is lost during aromatization of prephenate (Katagiri and Sato, 1953; Davis, 1953), both to phenylpyruvate (VII) by prephenate dehydratase (Weiss *et al.*, 1954; Gilvarg, 1955) and to *p*-hydroxyphenylpyruvate (VIII) by prephenate dehydrogenase (Schwinck and Adams, 1959). The detailed knowledge now available of the reactions leading to the formation of the benzene rings of phenylalanine, tyrosine, and tryptophan from shikimate (I) indicates, on structural and mechanistic grounds, that the C–C bonds of the ring of shikimate remain unbroken throughout these conversions.

Since the pyruvic acid side chain and the carboxyl of prephenate (V) are attached to C-1 of shikimate these findings further suggested that carbon atoms 1–6 of the ring of shikimate become atoms 1–6, respectively,

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