A New Assay for Phage Hydroxymethylases and Its Use in *Bacillus subtilis* Transfection*

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ABSTRACT: The enzymatic synthesis of 5-hydroxymethyl-5′-deoxyuridylic acid, which replaces thymidylic acid in the deoxyribonucleic acid of certain bacteriophages infecting *Bacillus subtilis*, is reversible. This property can be utilized in measuring the enzyme that catalyzes the reaction. A mononucleotide labeled with radioactive formaldehyde is first prepared enzymatically. To assay the enzyme, the release of formaldehyde from this radioactive substrate is then determined. The method described is highly sensitive, simple, and reproducible and allows the easy detection of the low enzyme levels present in competent cells infected with purified phage deoxyribonucleic acid. The 5′-deoxycytidylic acid hydroxymethylase induced in *Escherichia coli* by T2 bacteriophage also catalyzes a reversible reaction and can be assayed in a similar way. However, the deoxyuridylic acid and the deoxycytidylic acid phage-induced hydroxymethylases act only on their respective substrate. Some preliminary data on the induction of deoxycytidylic acid hydroxymethylase by phage-SP82 deoxyribonucleic acid in B. *subtilis* competent cells are presented.

The infection of competent *Bacillus subtilis* cells by phage DNA (Romig, 1962; Földes and Trautner, 1964; Okubo *et al.*, 1964; Green, 1964; Reilly and Spizizen, 1965) offers the opportunity to study any effects of *in vivo* or *in vitro* modifications of the DNA molecule on the expression and reproduction of the viral genome. The detection of changes in the levels of specific phage-induced proteins is, however, difficult due to the low frequency of the cell population susceptible to transfection (see Spizizen *et al.*, 1966). Assays sensitive enough to detect the low protein levels produced under such circumstances are then required.

A virulent group of *B. subtilis* bacteriophages has been described whose DNA contains hydroxymethyluracil in place of thymine (Kallen *et al.*, 1963; Okubo *et al.*, 1964; Green, 1964; Pène and Marmur, 1964; Roscoe and Tucker, 1964). The enzyme responsible for the synthesis of the new pyrimidine mononucleotide, dUMP hydroxymethylase (Kahan *et al.*, 1964; Roscoe and Tucker, 1966), catalyzes the reaction dUMP + HCHO $\xrightarrow{\text{THF}^1}$ HMdUMP, which is analogous to the synthesis of HMdCMP, the nucleotide replacing dCMP in the DNA of the *Escherichia coli* T-even bacteriophages (Flaks and Cohen, 1959).

The present report describes a very sensitive assay for the dUMP hydroxymethylase induced in cells of *B. subtilis* infected with the virulent phage SP82 (Green, 1964). The assay also allows the easy detection of such an enzyme in competent cells infected with purified SP82 DNA (Green, 1964) and can be used with other phages of the same group such as phage 2C (Pène and Marmur, 1964). Extension of the procedure to the dCMP hydroxymethylase induced by *E. coli* phage T2 has shown that this enzyme can also be assayed in a similar manner.

Materials and Methods

Strains. B. subtilis thyA thyB trp-2 (Farmer and Rothman, 1965) was obtained as SB566 from Dr. A. T. Ganesan. *B. subtilis* A26U⁻ is a uracil-requiring derivative of *B. subtilis* 168 (Dubnau *et al.*, 1965). *B. subtilis* SB19 *str-1 ery-1 mic-1* (Dubnau *et al.*, 1965) is a prototroph resistant to streptomycin, erythromycin, and micrococcin. Phage SP82 was obtained from Dr. D. Green and phage T2 and *E. coli* B from Dr. M. Gold.

Materials. dUMP, dCMP, ascorbic acid, and streptomycin were obtained from Calbiochem; THFA from Sigma Chemical Co.; formaldehyde solution and α,α′-dipyridyl from Fisher Scientific Co.; and dimedone (5,5-dimethyl-1,3-cyclohexanedione) from Eastman Organic Chemicals. [³H]Formaldehyde (120 mCi/mmole) was purchased from New England Nuclear Corp. DEAE-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: THFA, dl,L-tetrahydrofolic acid; THF, tetrahydrofolate; pfu, plaque-forming units; SSC, 0.15 M sodium chloride plus 0.015 M sodium citrate (pH 7.0); BHI, brain heart infusion; BAL, dimercaptopropanol. Genetic markers *thy*, *trp*, and *his* denote mutations affecting the synthesis of thymine, tryptophan, and histidine, respectively.

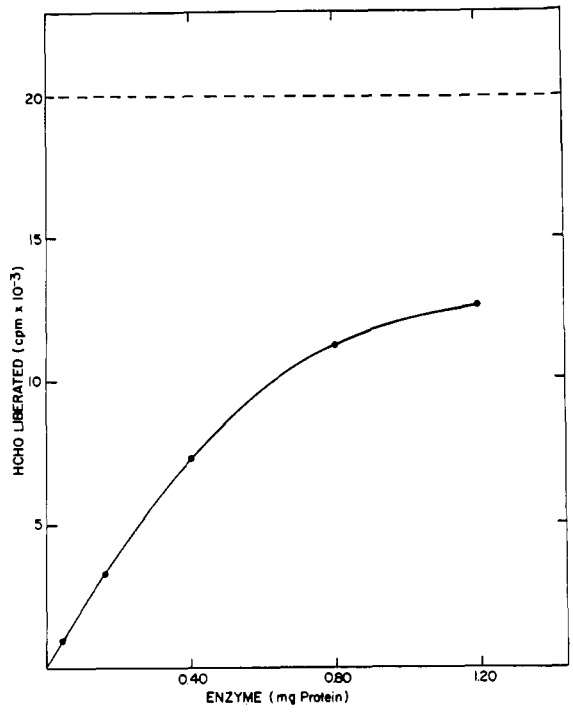

FIGURE 1: Dependence of dUMP hydroxymethylase assay on enzyme concentration. A crude extract of *B. subtilis* 168 *thyA thyB trp-2* infected with intact SP82 was used. A total of 2.0×10^4 cpm of [^{3}H]HMdUMP (12 mμmoles) was present in the reaction mixture. Incubation was for 2 hr at 37°. The dashed line indicates the theoretical amount of formaldehyde that could be liberated.

cellulose (Cellex-D) was obtained from Bio-Rad Laboratories.

Nonradioactive HMdUMP was obtained by enzymatic hydrolysis of SP82 DNA or by chemical synthesis (Alegria, 1967). Nonradioactive HMdCMP was obtained by chemical coupling of formaldehyde to dCMP (Alegria, 1967). The VY medium used for growing *B. subtilis* contained 25 g of veal infusion and 5 g of yeast extract per l. (Difco).

Enzyme Preparations. The partially purified dUMP hydroxymethylase utilized in the synthesis of [^{3}H]HMdUMP was obtained from *B. subtilis thyA thyB trp-2* infected with phage SP82. This strain lacks thymidylate synthetase activity (Wilson *et al.*, 1966) and competition for the radioactive formaldehyde utilized in the reaction is thus prevented. Cells were grown in BHI (Difco) broth and infected in the exponential phase of growth with about 5 pfu/cell. After 25 min the cells were chilled rapidly in an ice bath and harvested in a Sharples supercentrifuge. They were washed once with cold 0.02 M potassium phosphate (pH 7.0) and the pellet was stored at −20°. No loss of enzyme content was observed after 6 months.

Crude enzyme extracts were prepared by disrupting the cells (25 g) in the cell press described by Eaton (1962) (W. A. Schaerr, Brooklyn, N. Y.) after resuspension in 0.02 M potassium phosphate (pH 7.0) plus 0.01 M β-mercaptoethanol. After centrifugation at 10,000*g* for 10 min to eliminate cell debris, one-third volume of 5% streptomycin was added and the precipitate was discarded. The supernatant (100 ml, about 15 mg of protein/ml) was diluted with an equal volume of water and applied directly to a DEAE-cellulose column (15 × 1.9 cm) equilibrated with the same buffer used for the resuspension of the cells. After washing with two column volumes of this buffer, the enzyme was eluted with 800 ml of a linear NaCl gradient (0.04–0.80 M). The fractions containing the enzyme, as determined by the assay procedure described later,[2] were pooled and the enzyme was precipitated by adding ammonium sulfate to 70% saturation. The precipitate was resuspended in 5 ml of 0.05 M Tris-HCl (pH 7.4) plus 0.01 M β-mercaptoethanol. The partially purified enzyme preparation (about 20-fold, 10 mg of protein/ml) can be stored at −20° for at least 1 year with little loss in activity. Crude enzymatic extracts obtained after streptomycin treatment and ammonium sulfate precipitation were used in some of the experiments.

dCMP hydroxymethylase (Flaks and Cohen, 1959) was obtained from T2-infected *E. coli* B cells as described by Matthews *et al.* (1964); however, purification was carried out only as far as the ammonium sulfate fractionation step.

[^{3}H]HMdUMP was prepared by incubating 82 μmoles of dUMP with 8 μmoles of [^{3}H]formaldehyde (120 mCi/mmole), 2 μmoles of THFA, 400 μmoles of KF, 400 μmoles of Tris-HCl (pH 7.4), 20 μmoles of $MgCl_2$, 280 μmoles of β-mercaptoethanol, and 0.4 ml of partially purified hydroxymethylase in a volume of 4.0 ml for 4 hr at 37°. The reaction was terminated by the addition of 3.5 ml of 1 M sodium acetate plus 2 M acetic acid and 1 ml of 0.4 M dimedone in 50% ethanol and the mixture was heated in a boiling water bath for 5 min. The ice-cooled mixture was extracted four times with 10 ml of toluene to eliminate the formaldehyde–dimedone complex. The solution was then passed through a Dowex 50-X8 (H^+, 200–400 mesh, 0.8 × 25 cm) column. The effluent was diluted to 1 l. with water and its pH was adjusted to 11 with KOH. It was then absorbed on a Dowex 1-X8 (Cl^-, 200–400 mesh, 1.0 × 12 cm) column. After washing with water, elution was carried out with 0.08 M ammonium carbonate. The fractions containing the radioactive peak were pooled and lyophilized and the mixture of dUMP and [^{3}H]HMdUMP was separated by paper chromatography in the isobutyric acid–ammonia–water (66:1:33, v/v) solvent (see Alegria, 1967). The final yield in terms of the formaldehyde used was about 70%.

[^{3}H]HMdCMP was prepared according to Flaks and Cohen (1959).

dUMP Hydroxymethylase Assay. The principle of the assay is similar to the one described by Taylor and Weissbach (1965) for serine transhydroxymethylase. In a reaction which is the reverse of the synthesis, the enzyme is used to strip the radioactive formaldehyde from the [^{3}H]HMdUMP. The assay mixture contained 12 mμmoles of [^{3}H]HMdUMP, 1 μmole of THFA, 25

[2] Since the radioactive HMdUMP used in this assay would be initially unavailable, a method such as the one described by Roscoe and Tucker (1966) can be used for the detection of the enzyme in the effluent from the column.

μmoles of KF, 2 μmoles of $MgCl_2$, 25 μmoles Tris-HCl (pH 7.4), and 5 μmoles of β-mercaptoethanol plus enzyme in a volume of 0.5 ml. Incubation was carried out at 37° for 2 hr in 12-ml conical centrifuge tubes with rubber stoppers and covered with aluminum foil to reduce any light effects on the THFA. The reaction was stopped by the addition of 0.4 ml of 1 M sodium acetate plus 2 M acetic acid and afterwards 0.3 ml of 0.1 M formaldehyde solution and 0.4 ml of 0.4 M dimedone in 50% ethanol were successively added and the mixture was heated in a boiling water bath for 5 min. After cooling the tubes in ice, the formaldehyde–dimedone complex was extracted with 5 ml of toluene. The two phases were separated by centrifugation at 1000*g* for 5 min and then the toluene phase was added to 18 ml of toluene containing 0.1 g of *p*-bis[2-(5-phenyloxazolyl)]benzene (Pilot Chemicals) and 4 g of 2,5-diphenyloxazole (Pilot) per l. Radioactivity was determined in a Packard Tri-Carb or a Nuclear-Chicago liquid scintillation counter.

The determination of the dCMP hydroxymethylase was carried out in the same way, replacing HMdUMP by HMdCMP at the same concentration.

When a very high sensitivity was unnecessary, the radioactive substrate synthesized enzymatically (120 mCi/mmole) was suitably diluted with nonradioactive mononucleotide.

Cells of *B. subtilis thyA thyB trp-2* were grown to competence essentially as described by Farmer and Rothman (1965). However, thymine was replaced by thymidine and 20 μg/ml of 2,2′-dipyridyl was added to the second transformation medium (Anagnostopoulos and Spizizen, 1961). Incubation in this second medium was carried out at 30° for 150 min as suggested by Dr. A. T. Ganesan (personal communication). Transformation frequencies to thymine independence, assayed with *B. subtilis* SB 19 *str-1 ery-1 mic-1* DNA at a concentration of 2 μg/ml, were generally 1–2%. The assay for dUMP hydroxymethylase need not be restricted to thymine-requiring cells as shown by the experiments reported in Figure 4 with *B. subtilis* A26U⁻. Other *B. subtilis* strains such as SB1 (*hisA1, trp-2*) (Nester *et al.*, 1963) have also been used successfully for enzyme determinations in transfection (A. Alegria, unpublished observations). Interference by any thymidylate synthetase present in cells which do not require thymine is probably small, particularly in the transfection experiments where the total amount of dUMP liberated during the assay is minor.

SP82 DNA was isolated as described by Green (1964) and bacterial DNA was isolated by the method of Marmur (1961). The sedimentation coefficient, $s_{20,w}$, of SP82 DNA, determined by boundary sedimentation, was found to be 53 S at a DNA concentration of 20 μg/ml in SSC (35,600 rpm), indicating that the molecules were largely intact (see Green, 1964).

Protein determinations were carried out by the method of Lowry *et al.* (1951).

Results

Figure 1 shows the release of [³H]formaldehyde from [³H]HMdUMP, catalyzed by increasing amounts of a dUMP hydroxymethylase preparation derived from SP82-infected *B. subtilis*. The amount of formaldehyde liberated is approximately proportional to enzyme concentration until about 30–35% of the substrate has been consumed and decreases progressively thereafter. The reaction is not complete at high enzyme concentrations and the conversion essentially ceases under these conditions when about 60% of the HMdUMP has been split. That the reversibility of the reaction, rather than destruction of the substrate during the incubation, is probably responsible for this lack of completion was demonstrated by isolating the products after the reaction had stopped and showing by paper chromatography in the isobutyric acid–ammonia–water system that most of the counts remaining in the water phase still migrate as HMdUMP. Possible consumption of the released formaldehyde through other enzymatic pathways, such as oxidation or reduction of the methylene tetrahydrofolate intermediate, was minimized in these experiments by precipitating the enzyme preparation with ammonium sulfate as described earlier. In this way the concentration of cofactors necessary for the mentioned side reactions was drastically reduced.

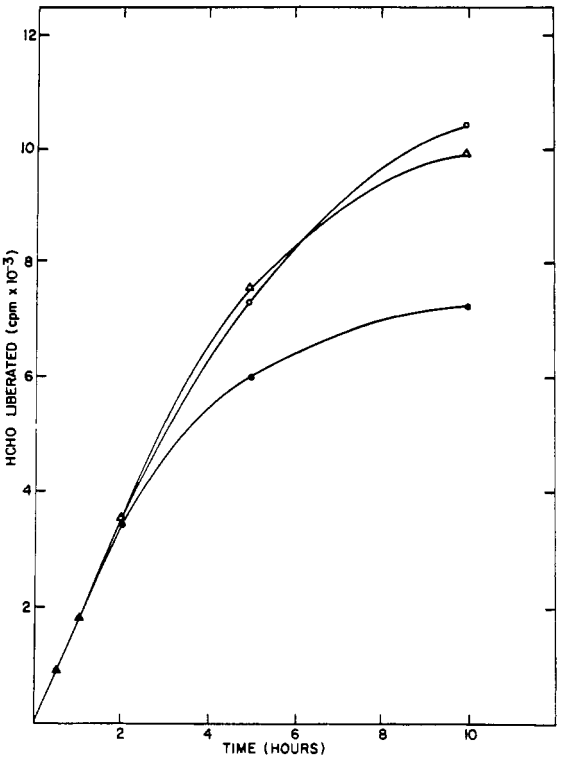

FIGURE 2: Time course of dUMP hydroxymethylase assay A crude extract of *B. subtilis* 168 *thyA thyB trp-2* infected with intact SP82 equivalent to 0.2 mg of protein was used for each time point. [³H]HMdUMP: 2.0 × 10⁴ cpm (12 mμmoles). (●——●) No addition; (○——○) potassium ascorbate (10% solution in water, pH 7) added to 0.15 M final concentration; (△——△) BAL (4 M solution in ethanol) added to 10 mM final concentration.

Figure 2 shows the time course of the reaction. It is approximately linear for about 2 hr and then diminishes. This decrease is largely due to the oxidation of the THFA. Addition to the reaction mixture of agents that protect THFA from being rapidly oxidized, such as

TABLE I: Substrate Specificity of Hydroxymethylases.

Additions	Formaldehyde Released (cpm)
AM[a] + [^{3}H]HMdUMP (10^5 cpm = 12 mμmoles)	15
AM[a] + [^{3}H]HMdUMP + dUMP hydroxymethylase[b] (2 μl)	4530
AM[a] + [^{3}H]HMdUMP + dCMP hydroxymethylase[c] (100 μl)	87
AM[a] + [^{3}H]HMdUMP + HMdCMP (180 mμmoles) + dUMP hydroxymethylase[b] (2 μl)	3512
AM[a] + [^{3}H]HMdCMP (2.0×10^4 cpm = 12 mμmoles)	5
AM[a] + [^{3}H]HMdCMP + dCMP hydroxymethylase[c] (10 μl)	4329
AM[a] + [^{3}H]HMdCMP + dUMP hydroxymethylase[b] (200 μl)	17
AM[a] + [^{3}H]HMdCMP + HMdUMP (120 mμmoles) + dCMP hydroxymethylase[c] (10 μl)	4659

[a] AM: assay mixture as described under Materials and Methods except for the nucleotide. [b] Partially purified dUMP hydroxymethylase preparation from *B. subtilis* infected with phage SP82 (see Materials and Methods) (about 10 mg of protein/ml). [c] dCMP hydroxymethylase preparation from *E. coli* infected with phage T2 (see Materials and Methods) (about 10 mg of protein/ml).

BAL (Blakley, 1960) or ascorbic acid (Greenberg and Jaenicke, 1957), help to maintain the release of formaldehyde over a longer period of time (Figure 2).

That the dCMP hydroxymethylase present in *E. coli* B cells infected with phage T2 can be assayed similarly is shown in Figure 3. Noteworthy is the fact that this enzyme preparation seemed to be able, under the same conditions, to carry the reverse reaction much farther than the dUMP hydroxymethylase preparation. Almost complete release of the [^{3}H]formaldehyde was obtained at high enzyme concentrations and it was proportional to the enzyme concentration until about 50% of the substrate had been consumed. It should be recalled that infection by phage T2 also induces the formation of a potent dCMP deaminase in *E. coli* cells (Keck *et al.*, 1960; Maley and Maley, 1966, 1967). The presence of this enzyme in the preparation used would explain the practically complete release of formaldehyde observed since dCMP, the substrate for the synthetic reaction, would be transformed to dUMP by the deaminase. To establish whether the kinetic characteristics of dUMP and dCMP hydroxymethylases are really different, the use of purified preparations free of any contaminating enzymes would be required.

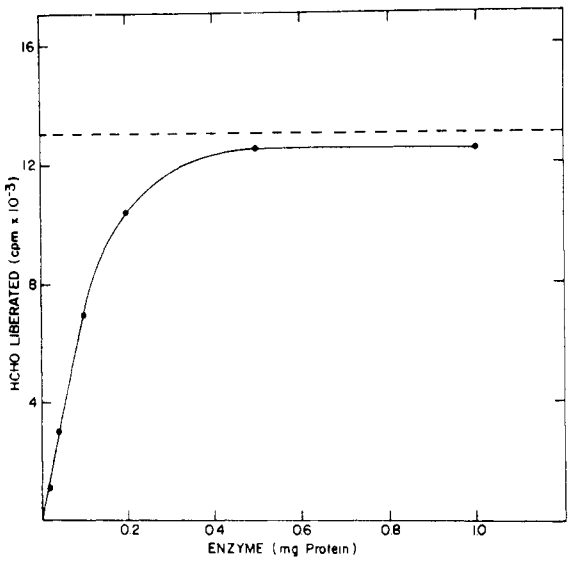

FIGURE 3: Dependence of dCMP hydroxymethylase assay upon enzyme concentration. A T2-infected *E. coli* B extract prepared as described under Materials and Methods (about 10 mg of protein/ml) was used. [^{3}H]HMdCMP: 1.3×10^4 cpm (12 mμmoles). Incubation was for 2 hr at 37°. The dashed line indicates the theoretical amount of formaldehyde that could be liberated.

The clear-cut specificity of both the dUMP hydroxymethylase (SP82) and the dCMP hydroxymethylase (T2) for their corresponding substrate is shown in Table I. Neither of the two enzymes is able to catalyze the liberation of formaldehyde from the other's substrate. Furthermore, no significant inhibition of either reaction could be observed when the alternate substrate was added to the assay mixture.

The dUMP hydroxymethylase assay can be used for the determination of enzyme levels during normal bacteriophage infection. Figure 4 shows the time course for the induction of this enzyme in *B. subtilis* A26U infected by the SP82-related phage 2C (Pène and Marmur, 1964). Comparison with the data obtained with the same extracts by Pène and Marmur (1967; Figure 1) for the induction of the dCMP deaminase, an early enzyme, and the phage endolysin, a late one, indicate that the dUMP hydroxymethylase appears at an intermediate stage, about 2 min after the deaminase and 7 min before the onset of endolysin synthesis.

However, the dUMP hydroxymethylase assay was developed mainly for the purpose of detecting the low levels of enzyme produced during transfection of competent *B. subtilis* cells with bacteriophage SP82 DNA. A substrate with a higher specific radioactivity (120 mCi/mmole), at the same concentration, was used in this case. Table II presents a comparison between the levels of enzyme (as radioactive counts) induced in competent cells exposed to phage DNA for different periods of time and the number of infective centers determined simultaneously.

TABLE II: dUMP Hydroxymethylase in Transfected *B. subtilis*.[a]

Time of DNase Addn (min)	Formaldehyde Released (cpm)	Infective Centers (pfu/ml)
0	5	0
6	1,827	0
20	53,108	9.6×10^3
45	69,195	1.9×10^5

[a] Aliquots (18 ml) of a *B. subtilis thyA thyB trp-2* competent culture (about 9×10^8 cells/ml) incubated at 37° were exposed to 2 ml of SP82 phage DNA solution containing 100 μg/ml in SSC. Pancreatic DNase II (100 μg/ml) was then added to different samples at the times indicated and incubation was continued at the same temperature. Forty-five minutes after the DNA addition sodium azide was added to a final concentration of 0.01 M and the cells were rapidly chilled in ice water. After sedimentation in the centrifuge they were resuspended in 5 ml of cold 0.05 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, and 0.01 M β-mercaptoethanol, frozen, and disrupted in the Eaton press (Eaton, 1962). Cell debris was eliminated by centrifugation and ammonium sulfate to 70% saturation was added to the supernatant. dUMP hydroxymethylase activity was determined in the redissolved precipitate by the methods described under Materials and Methods. Infective centers were measured as described by Green (1964) before adding the azide. [^{3}H]HMdUMP: 8×10^5 cpm (12 mμmoles) were used for each assay. A background of 200 cpm was subtracted in every case.

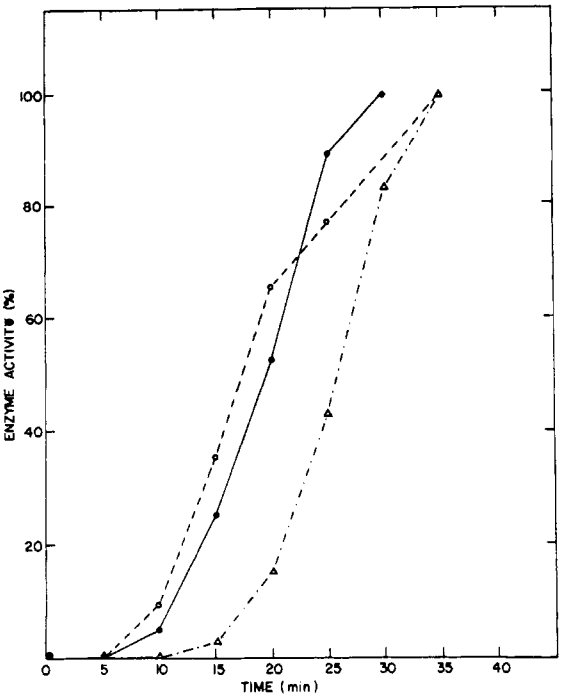

FIGURE 4: Time course of dUMP hydroxymethylase development after phage infection. *B. subtilis* A26U⁻ was infected with intact phage 2C. The extracts used are the same as those prepared by Péne and Marmur (1967). The extract (0.1 ml) (1 mg of protein/ml) was utilized for each point [^{3}H]HMdUMP: 2.0×10^5 cpm (12 mμmoles). The data of Péne and Marmur (1967) for the dCMP deaminase and the endolysin are presented for comparison, after normalizing and subtracting the backgrounds: (●——●) dUMP hydroxymethylase, 100% = 8.4×10^4 cpm. (○——○) dCMP deaminase, 100% = 0.36 μmole of dUMP/hr per 5×10^8 cells; (△——△) endolysin 100% = 20 units/5×10^8 cells.

When the assay is used for detecting the enzyme in transfected cells, a blank value of about 200 cpm (including the natural background) is obtained, corresponding to about 0.03% of the number of counts used. This background is highly reproducible from one assay to another. No increase in it is produced by the presence of extracts obtained from uninfected cells, indicating that it is largely due to decomposition of the substrate during storage or during the assay manipulations.

When the enzyme content of cells infected with phage DNA is measured, the assay is carried out on the ammonium sulfate precipitate of the extract obtained from mechanically disrupted transfected cells. Complete solution of this precipitate in the assay mixture is not generally achieved and, furthermore, it has been found that the ammonium sulfate present under these conditions inhibits the reaction about 30%. To test whether the release of radioactive formaldehyde thus measured gives an accurate estimation of the amount of enzyme present, cells from a competent culture were infected with small and variable amounts of *intact* SP82 phage, at levels similar to the phage titers obtained in the transfection experiments. These cells were then processed for the assay of dUMP hydroxymethylase in the same way as cells infected with phage DNA (see legend to Table II). At these low phage multiplicities (about 0.005 or less) a direct proportionality is to be expected between the titers of infecting phage and the amounts of induced enzyme if the assay procedure is still accurate under such circumstances, and it is found to be so (Figure 5). In the same figure a comparison with the enzyme levels obtained in exponentially growing cells exposed to the same phage concentrations is also presented. Again a linear relationship is observed, but the enzyme levels are much higher. Possible reasons for the different enzyme levels in phage-infected transformable and exponentially growing cells are presented in the Discussion.

Figure 6 presents the time course of enzyme development in transfected cells following the addition of DNase at 15 min. A substantial lag in the development of the enzyme is observed when compared with the enzyme induced in exponentially growing cells by intact phage (Figure 6). The figure also shows that a shorter lag is observed in the induction of the enzyme when a competent culture is infected with whole SP82 phage.

Discussion

Although the mechanism of the reactions catalyzed by dUMP hydroxymethylase and serine transhydroxy-

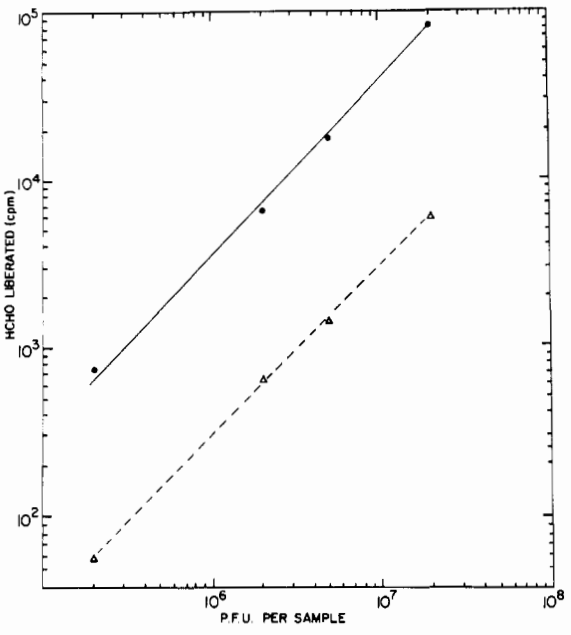

FIGURE 5: Correlation between amount of infecting SP82 phage and enzyme activity. Aliquots (20 ml) of cells from both competent and exponentially growing *B. subtilis thyA thyB trp-2* cultures were infected with the indicated amounts of SP82 phage. Twenty minutes later sodium azide was added to a final concentration of 0.01 M and the cells were rapidly chilled in an ice bath. They were then processed for dUMP hydroxymethylase determination in the same way as indicated in the legend to Table II. (●——●) Cells growing exponentially in VY medium (about 5 × 10^8 cells/ml); (△——△) competent cells (about 9 × 10^8 cells/ml).

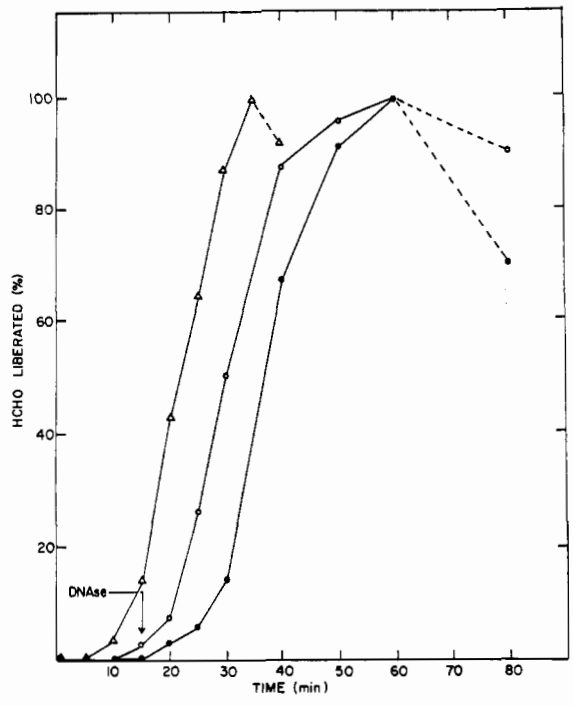

FIGURE 6: Time course of dUMP hydroxymethylase formation in transfected cells. Aliquots (18 ml) of competent *B. subtilis thyA thyB trp-2* cells (about 7 × 10^8 cells/ml) incubated at 37° were exposed to 2 ml of SP82 DNA containing 100 μg/ml in SSC. Pancreatic DNase (100 μg/ml) was added 15 min later to the samples, except those at 0 and 10 min. At the times indicated sodium azide was added to a final concentration of 0.01 M and the cells were rapidly cooled in ice water. They were then treated in the same way described in the legend to Table II. Infective centers, determined 40 min after DNA addition, were about 0.9 × 10^4 pfu/ml. Simultaneously 20-ml aliquots of the same competent culture were each infected with about 2.5 × 10^6 SP82 phages particles and treated at the indicated times in the same way as the transfected samples. From an exponentially growing culture of the same *B. subtilis* strain in VY medium ((100 ml, about 5 × 10^8 cells/ml), infected with about 2.5 × 10^6 SP82 phages/ml), 10-ml aliquots were taken at the times indicated and processed identically. The results have been normalized for the maximum value of each curve, given below. (●——●) Competent cells infected with SP82 DNA; 100%, 5.23 × 10^4 cpm. (○——○) Competent cells infected with intact SP82 phage; 100%, 1.12 × 10^4 cpm. (△——△) Exponentially growing cells infected with intact SP82 phage 100%, 1.07 × 10^5 cpm.

methylase may be somewhat different, since no requirement for pyridoxal 5-phosphate has been observed for the former, the over-all similarities led us to investigate the possibility that the reaction catalyzed by the phage enzyme was also reversible. That such is indeed the case is demonstrated by the fact that the dUMP hydroxymethylase assay can be carried out as described. The dCMP hydroxymethylase reaction is also reversible and this might explain the competitive inhibition that HMdCMP produced on its own synthesis, first observed by Pizer and Cohen (1962), although detailed kinetic studies have not been carried out.

Yeh and Greenberg (1967) have shown that the first step in the enzymatic synthesis of HMdCMP is the reversible removal of the hydrogen atom in the 5th position of the pyrimidine ring, the second step being the coupling of formaldehyde to the vacant site. That this step is also reversible has been proven in the present report. Although no detailed investigation of the mechanism of action of the dUMP hydroxymethylase has been carried out so far, it will, in all likelihood, be very similar to the one revealed for the dCMP hydroxymethylase. Whether the reversibility of the reaction plays any role in the control of the phage DNA synthesis remains also to be established.

The easy detection of dUMP hydroxymethylase in *B. subtilis* competent cells infected with SP82 DNA provides a new way to gain insight into the events that occur during transfection, with the hope that these results can be useful in understanding the stages of phage development. For instance, purified phage DNA, isolated from mature phage particles or from the infected cell, can be manipulated before transfection to see what role the molecular configuration of the DNA plays in the induction and control of this early phage-specific enzyme.

The preliminary data obtained seem to indicate that the formation of a phage DNA-infective center is not a prerequisite for the induction of the hydroxymethylase during transfection (Table II, 6-min point). A rough estimate of the amount of enzyme activity per infective center (as measured by formaldehyde release) gives maximal values of about 4 × 10^{-3} cpm/pfu for both competent and exponentially growing cells infected with whole phage (legend to Figure 6) and a much higher value of about 2 × 10^{-2} cpm/pfu for transfected cells

after 45-min contact with DNA (Table II). An even higher value (0.2–0.3 cpm/pfu) is obtained if DNase is added 15–20 min after DNA (Table II, Figure 6). These results indicate again that infecting DNA is able in many instances to induce enzyme formation but unable to produce an infective center. Green (1964) has presented evidence from dosage–response curves suggesting that four whole-phage DNA molecules are required to initiate an infective center and this has been confirmed in transfection experiments using *B. subtilis thyA thyB trp-2* as the recipient (A. Alegria, unpublished results). The large amount of enzyme that cannot be accounted for by the number of infective centers suggests that either a smaller number of whole DNA molecules or fragmented ones might be sufficient for enzyme induction.

Several factors are probably implicated in the delayed appearance of the dUMP hydroxymethylase during transfection (Figure 6) as compared with the enzyme induced by intact phage in exponentially growing cells. It is possible that injection of DNA into the cells by intact phage particles is faster than the uptake of DNA during transfection. Green (1964) has calculated that about 13 min are necessary for phage DNA-infective centers to become completely DNase resistant and this finding suggests that the uptake process is relatively slow, although the requirement of four whole DNA molecules per infective center makes the argument less compelling. That the uptake of DNA during transfection is also an asynchronous process is shown by the increasing number of infective centers produced when the contact between cells and DNA is prolonged even as much as 45 min (Table II; see also Green, 1964). Since a certain number of hydroxymethylase genes must accumulate before the amount of enzyme they induce can be measured, an uptake process which is both slow and asynchronous would delay the appearance of the enzyme. It has also been shown that *B. subtilis* competent cells have a decreased metabolism as compared with the cell population as a whole (Nester and Stocker, 1963; Nester, 1964; McCarthy and Nester, 1967). This may influence the ability of these cells to synthesize a phage-induced enzyme. In this respect it must be noted, however, that the rate of enzyme increase does not seem to be very different in transfected cells (Figure 6) and this may be related to the recent finding of McCarthy and Nester (1967) that newly transformed cells are still able to carry out extensive protein synthesis.

The reasons for the lag observed in the induction of the dUMP hydroxymethylase in a competent culture infected with intact phage (Figure 6) are not clear. It is known, however, that chemical modifications occur in the cell wall of *B. subtilis* cells during competence (Young *et al.*, 1963; Young and Spizizen, 1963) and these changes might influence both the attachment and injection of DNA from phage particles. In any case, this lag largely explains the discrepancy in the amount of enzyme observed between the competent and the exponentially growing cultures infected with equal amounts of virus, shown in Figure 5.

Recently Pène (1967) has reported some data on the induction of phage 2C endolysin in competent cells infected with phage DNA. With this late enzyme an even more prolonged lag (about 35 min) was observed with respect to enzyme induction in phage-infected, exponentially growing cells (Pène and Marmur, 1967).

Work is currently in progress to define other characteristics of the system such as dependence of enzyme induction upon DNA dosage and degree of integrity of the phage genome necessary for phage-specific enzyme induction.

The possibility of studying enzyme induction in *E. coli* infected with T-even phage DNA is also being explored.

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Subfragment 1 of Myosin: Adenosine Triphosphatase Activation by Actin*

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ABSTRACT: Adenosine triphosphatase measurements were used to study the interaction of actin with subfragment 1, a tryptic digestion product of heavy meromyosin. As is true for myosin, the adenosine triphosphatase activity of subfragment 1 is significantly activated by actin at low ionic strengths and this activation requires the presence of magnesium. Furthermore, as we previously found for heavy meromyosin, actin activation of the subfragment 1 adenosine triphosphatase increases markedly as the actin concentration is increased above the stoichiometric binding ratio of actin to subfragment 1. This shows that adenosine triphosphate causes marked dissociation of the acto-subfragment 1 complex even at low ionic strength; and if, as appears quite likely subfragment 1 has only a single binding site for nucleotide, then we can conclude that dissociation of the acto-subfragment 1 complex must be caused by the binding of adenosine triphosphate to the hydrolytic site of the subfragment 1 rather than to some separate dissociating site. This conclusion in turn leads to the possibility that motion is generated in muscle by repeated cycles consisting of formation of the rigor actin–myosin link in conjunction with adenosine triphosphate hydrolysis followed by dissociation of this actin–myosin link in conjunction with the rebinding of adenosine triphosphate at the hydrolytic site.

It has long been known that heavy meromyosin, a tryptic digestion product of myosin, retains both the ATPase activity and the actin-binding properties of myosin (Mihalyi and Szent-Györgyi, 1953). Recently a further tryptic digestion product of myosin, subfragment 1, was also found to retain the ATPase activity and actin-binding properties of myosin (Mueller and Perry, 1962); and since it has a molecular weight only one-third that of heavy meromyosin, several workers have suggested that a single molecule of heavy meromyosin might contain two or three identical subfragment 1 subunits (Mueller, 1965; Young *et al.*, 1965; Slayter and Lowey, 1967).

In relation to the role of myosin in muscle contraction, one of the most important questions concerning subfragment 1 is whether its ATPase activity, like that of myosin and heavy meromyosin, is activated by actin at low ionic strength in the presence of magnesium. There have been several reports that the subfragment 1 ATPase is activated very little, if at all, by actin and magnesium (Jones and Perry, 1966; Yagi and Yazawa, 1966); and the possibility therefore arises that the prop-

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