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Influence of Deoxyribonucleic Acid Degradation Products and Orthophosphate on Deoxynucleotide Kinase Activity and Deoxyribonucleic Acid Synthesis in Pneumococcus Type III

WILLIAM FIRSHEIN

Department of Biology, Wesleyan University, Middletown, Connecticut

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ABSTRACT

FIRSHEIN, WILLIAM (Wesleyan University, Middletown, Conn.). Influence of deoxyribonucleic acid degradation products and orthophosphate on deoxynucleotide kinase activity and deoxyribonucleic acid synthesis in pneumococcus type III. J. Bacteriol. 90:327-336. 1965.—An oligodeoxynucleotide fraction derived from a deoxyribonuclease-treated calf-thymus deoxyribonucleic acid (DNA) can enhance the activity of deoxycytidylic acid (dCMP) and deoxyguanylic acid (dGMP) kinases in cell suspensions of type III pneumococci. High levels of orthophosphate can produce similar effects. For part of the incubation period, the activity of dCMP and dGMP kinases is very low or undetectable in unsupplemented-cell suspensions of pneumococci. In contrast, the remaining kinases, deoxyadenylic acid and thymidylic acid, which are present in ample amounts in control and supplemented cells throughout the incubation period, are unaffected by the addition of oligodeoxynucleotides and orthophosphate. The stimulation of kinase activity is amino acid-dependent and can be abolished by adding chloramphenicol. When the oligodeoxynucleotide fraction and orthophosphate are further supplemented with all eight of the naturally occurring deoxynucleosides and deoxynucleotides (which do not affect kinase activity), a preferential enhancement of DNA synthesis occurs in comparison with cell growth or protein synthesis. Addition of deoxynucleosides and deoxynucleotides to unsupplemented cells produces only a slight increase in DNA synthesis. The preferential enhancement of DNA synthesis can be prevented by adding chloramphenicol at a certain time during incubation.

Although the metabolic pathways leading to the biosynthesis of deoxyribonucleic acid (DNA) have been studied intensively during the past decade (Magasanik, 1962; Lehman et al., 1958), little has been learned about the factors controlling rates of synthesis of this important macromolecule. Most investigations on control mechanisms have been concerned with purine and pyrimidine deoxynucleotide synthesis (Magasanik, 1962). Recently, Jacob, Brenner, and Cuzin (1963) proposed a model for the control of bacterial DNA synthesis involving two elements, an "initiator" which controls the starting point of replication, and a "replicator" which is activated by the initiator and which controls the actual duplication of the DNA strands. Some support for this model has come from findings of Lark and Lark (1964), who detected the presence of two substances which play a role in the regulation of DNA replication in a thymineless auxotroph of *Escherichia coli*. However, a role for deoxynucleotide kinases in the control of DNA synthesis can be suspected on the basis of a number of investigations. Thus, several recent studies have shown that increased levels of deoxynucleosides or other DNA degradation products in vivo can raise deoxynucleotide kinase activity (Hotta and Stern, 1961; Takats, 1962). Such elevation is invariably followed by a burst of DNA synthesis. Hotta and Stern (1961) described the capacity of microspores of *Lilium* to phosphorylate deoxynucleosides for brief periods just prior to DNA synthesis, i.e., when high levels of deoxynucleosides had accumulated. It was suggested by Takats (1962) that such deoxynucleosides were derived from degraded tapetal DNA and that they induced the synthesis of certain deoxynucleotide kinases in *Lilium*. In experiments with rat-liver extracts, the absence of one deoxynucleotide kinase, thymidylic acid (dTMP) kinase, was found to be correlated with the inability of the extracts to synthesize DNA (Mantsavinos and Canellakis,

1959; Bollum and Potter, 1957). Although it seems that the absence of the kinase is due to its extreme lability (Grav and Smellie, 1965) and not to any specific repression of synthesis of the enzyme, the important point in these experiments is the requirement for all four deoxynucleotide kinases in order for DNA synthesis to occur. Finally, Mantsavinos and Canellakis (1959) observed that dTMP incorporation into DNA of regenerating rat-liver extracts could be stimulated by adding other deoxynucleotides. Although this stimulation may reflect the requirements for DNA synthesis, it may also reflect the stimulation of dTMP kinase activity by these deoxynucleotides.

In previously reported studies with virulent strains of pneumococci (Firshein, 1960, 1961), it was observed that DNA degradation products can selectively enhance DNA synthesis in comparison with the rate of ribonucleic acid (RNA) or protein synthesis in resting-cell suspensions. Avirulent strains were unaffected by such DNA degradation products. The usually employed and most effective supplement consisted of a deoxyribonuclease-treated calf-thymus DNA further supplemented with all eight of the naturally occurring deoxynucleosides and deoxynucleotides. DNA or deoxyribonuclease alone, or RNA derivatives, were inactive in stimulating DNA synthesis. It was subsequently found that (i) the components of the DNA digest are not incorporated significantly into intracellular DNA, but are taken up by the cells, and (ii) the active component(s) in the digest is an oligodeoxynucleotide of low molecular weight. Finally, it has been observed that orthophosphate can enhance the effects of the DNA degradation products on DNA synthesis, but is inactive by itself. The manner in which DNA degradation products and phosphate can stimulate DNA synthesis of virulent pneumococci has remained obscure, but, in view of the results of others which implicated deoxynucleotide kinases in control of DNA synthesis, an investigation of the effects of DNA degradation products and phosphate on the synthesis and activity of these enzymes was initiated.

MATERIALS AND METHODS

Organism. A virulent strain of type III *Diplococcus pneumoniae*, A66, which responded maximally to the DNA degradation products and phosphate with respect to enhanced DNA synthesis was used. Stock cultures of this strain were maintained on Brain Heart Infusion Agar (Difco) plates containing 5% defibrinated rabbit blood.

Preparation of resting cells and growth medium, and methods for chemical analysis. A 30-ml (6 hr, 37 C) starter culture of A66 was grown in a medium containing Casitone, Tryptone (both Difco), albumin (fraction V from bovine serum albumin; Armour Pharmaceutical Co., Kankakee, Ill.), glucose, and K_2HPO_4 (Marmur and Hotchkiss, 1955). The culture was inoculated into 9 liters of medium and incubated for 16 hr at 37 C. More glucose and phosphate were added, and the culture was incubated for 3 hr. The cells were harvested by centrifugation at 30,000 $\times$ *g*, washed once with 0.02 M sodium-potassium phosphate buffer (*p*H 7.5), and resuspended to a density of 1.5×10^9 viable cells per milliliter in 3 ml of the same buffer augmented with glucose, Casitone (vitamin-free; both 1.0%), and catalase (0.005%; Nutritional Biochemicals Corp., Cleveland, Ohio). The suspending medium was prepared double strength and supplemented with DNA degradation products and excess phosphate. The cells were shaken at 200 rev/min in a New Brunswick gyrotory water-bath shaker for various periods of time. DNA and protein were extracted and measured as described previously (Firshein, 1961). The sensitivity of the DNA and protein determinations was good to within 5 and 3%, respectively. Since no multiplication of the population occurred during the incubation period (Firshein, 1961), protein synthesis was a measure of cell growth only.

Enzyme extraction. Cells were centrifuged for 20 min at 30,000 $\times$ *g*, washed twice with 0.01 M potassium-phosphate buffer (*p*H 7.4), and disrupted in a Servall Omni-mixer by the method of Canellakis, Gottesman, and Kammen (1960). The cell debris was centrifuged for 30 min at 30,000 $\times$ *g*, leaving a turbid supernatant fluid. Deoxynucleotide kinases were partially purified (20-fold) from the supernatant fluid by the method of Lehman et al. (1958). At any given time, one preparation served as the source for all the kinases. No attempt was made to separate deoxynucleotide kinases from deoxynucleoside diphosphokinases, since it was important only to ascertain whether phosphorylating activity was present in the preparations. The presence of both types of kinases was evident from the assay (see below). Activities of partially purified kinases for different substrates were checked against those of fresh extracts to make sure that the purification procedure was not responsible for differences of activity for different substrates. All extraction procedures were carried out in the cold (4 C).

Assay of deoxynucleotide kinase activity. This assay was a modification of the method employed by Lehman et al. (1958). Various C^{14}-deoxynucleotides (Schwarz Laboratories, Inc., Mt. Vernon, N.Y.) were used as substrates. After incubation of the assay mixture, an equal volume of 70% methanol was added, and a sample was chromatographed on Whatman no. 1 paper for 48 hr at 15 C in a solvent consisting of isobuytric acid, 1 N NH_4OH, and 0.2 M sodium ethylenediaminetetraacetic acid (50:30:0.5, v/v). The spots were detected by ultraviolet light (2,538 A), and paper strips were cut out. The radioactivity of the spots

was assayed in a Vanguard chromatogram scanner, model 880. Planimeter measurements were used to calculate the area under the radioactive peaks. These areas were proportional to the amounts of substrates and products present on the paper strip. Specific activity was defined as the product-substrate ratio per milligram of protein in the partially purified preparations.

Preparation of DNA degradation products and fractionation of DNA digest. Deoxynucleosides, deoxynucleotides, nucleosides, and nucleotides were purchased from Sigma Chemical Co., St. Louis, Mo. Highly polymerized calf-thymus Na-DNA was prepared by the method of Kay, Simmons, and Dounce (1952). Once recrystallized pancreatic deoxyribonuclease was obtained from Worthington Biochemical Corp., Freehold, N.J. The deoxyribonuclease-treated DNA was prepared as described previously (Firshein, 1961). An oligodeoxynucleotide fraction was obtained by ion-exchange column chromatography of the DNA digest on ECTEOLA cellulose (Bendich et al., 1955). After elution of the DNA degradation products from the column by 0.01 M Na-K phosphate buffer (*p*H 7.0), a fraction containing oligodeoxynucleotides of small molecular weight was concentrated by evaporation in a Rinco rotary evaporator. A concentrate was obtained which gave an absorbancy of 0.450 at 260 mμ in a Beckman spectrophotometer for a sample diluted 10-fold. This reduction in volume increased the concentration of the phosphate buffer to 0.25 M. (Unless otherwise indicated, this Na-K phosphate buffer will be referred to as "phosphate.") Separation of the phosphate from the oligodeoxynucleotide fraction was achieved by adsorption of the oligodeoxynucleotides on Norit and elution as described by Lehman et al. (1958). Usually, 0.5 ml of the oligodeoxynucleotides with or without phosphate, or 0.5 ml of 0.25 M phosphate was diluted sixfold in the suspending medium.

Transformation experiments. Transforming DNA was prepared as described by Hotchkiss (1957) from a streptomycin-resistant mutant of A66 before and after exposure of the cells to the DNA degradation products and phosphate. The recipient strain (streptomycin-sensitive) was an avirulent mutant of A66 (strain 471). The method of assay and medium were those described by Abe and Mizuno (1958).

RESULTS

Effects of supplement on deoxynucleotide kinase activity. The effects of the supplement (oligodeoxynucleotides and phosphate, further supplemented with all eight of the naturally occurring deoxynucleosides and deoxynucleotides) on deoxynucleotide kinase activity in suspensions of virulent cells were tested as follows. Pneumococci were exposed to the supplement for various periods of time, and kinase activity was determined for all four deoxynucleotides (Fig. 1). Specific

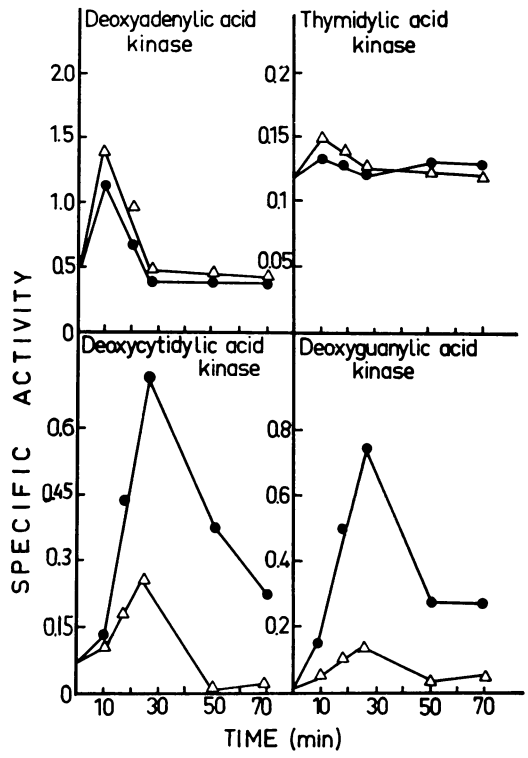

FIG. 1. *Deoxynucleotide kinase activity in partially purified preparations from supplemented and control cells. Concentration of supplement in suspending medium (μg/ml): oligodeoxyribonucleotides, 75; phosphate, 3,800; deoxynucleosides, 800 (200 each of deoxyadenosine, deoxyguanosine, thymidine, and deoxycytidine); deoxynucleotides, 800 (200 each of the phosphorylated deoxynucleosides). Incubation mixture for kinase assay (total volume, 0.6 ml): tris(hydroxymethyl)aminomethane buffer (pH 7.5), 20 μmoles; adenosine triphosphate, 4 μmoles; $MgCl_2 \cdot 6H_2O$, 8 μmoles; (C^{14}) deoxynucleotide, 1.6 μmoles total, including either (C^{14}) dCMP (0.5 μc/0.05 μmole), (C^{14}) dTMP (0.5 μc/0.03 μmole), (C^{14}) dAMP (0.5 μc/0.03 μmole), or (C^{14}) dGMP (0.5 μc/0.03 μmole). To make up the total concentration of 1.6 μmoles, each isotope was diluted with its nonlabeled counterpart deoxynucleotide. Concentration of kinases, 60 to 120 μg of protein per ml (0.2 to 0.4 ml). Incubation time and temperature for all kinases except dTMP was 40 min at 37 C. For dTMP kinase, the time was 70 min. Preliminary results (Firshein, 1963) had suggested that only dCMP kinase activity was affected by the supplement, and not dGMP kinase activity. However, these preliminary results were made with only one time period, and nonspecific variability occurred with respect to the time of optimal enhancement of dGMP kinase activity. More stringent control of the extraction process has eliminated this variability. Symbols: ●, supplemented; △, control.*

activity of all the kinases increased during the initial 10 or 25 min in control or supplemented cells, and then declined. As far as deoxyadenylic acid (dAMP) and dTMP kinases were concerned, there was little difference between control and supplemented cells. In regard to deoxyguanylic acid (dGMP) and deoxycytidylic acid (dCMP) kinases, however, there was a marked difference. A much greater increase in activity of dCMP and dGMP kinases was observed in supplemented cells than in control cells during this initial period. Subsequent declines in specific activity of these latter two kinases did not alter this difference. The declines were such that dCMP kinase activity was almost negligible in control cells, whereas significant activity remained in supplemented cells. dGMP kinase activity also decreased to low levels in control cells, but its activity was still detectable. An important observation was that the major increases in activity of dCMP and dGMP kinases occurred between 10 and 25 min in control and supplemented cells, whereas the increases for dAMP and dTMP kinases occurred during the first 10 min.

Another way of viewing these results is to plot the change in specific activity for each kinase of supplemented cells relative to that of control cells, as a function of time. Figure 2 shows that a progressive increase in dGMP and dCMP kinase activity occurred in supplemented cells compared with control cells until 25 and 50 min, respectively. After these periods, activity decreased, but the relative difference between supplemented and control cells was still marked. With dAMP and dTMP kinases, controls had slightly higher specific activities than supplements during the first 25 min. This gap narrowed with incubation time until, after 70 min, there was either no difference in specific activity (dAMP kinase) or an increase in favor of supplemented cells (dTMP kinase).

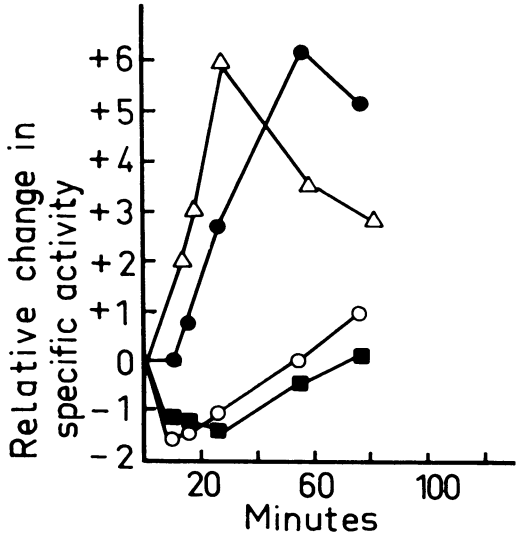

FIG. 2. *Relative change in specific activity of deoxynucleotide kinases between supplemented cells and control cells. The ordinate represents the number of times greater (+) or less (−) that the specific activity of kinases from supplemented cells differ from those of controls. Symbols: ●, dCMP kinase; △, dGMP kinase; ○, dTMP kinase; ■, dAMP kinase.*

Effects of supplement components on kinase specific activity. Next, an attempt was made to determine which part of the supplement increased dCMP and dGMP kinase activity. Kinases were prepared from cells exposed to various moieties of the supplement and then assayed for activity (Table 1). The most striking observation was that only the oligodeoxynucleotides and phosphate, alone or in combination, showed any marked stimulatory activity. Deoxynucleosides and deoxynucleotides actually depressed the activity of the oligodeoxynucleotides and phosphate somewhat, but, nevertheless, the levels were still much higher than those of the controls. By themselves, deoxynucleosides and deoxynucleotides were inactive in affecting dCMP and dGMP kinase activity. Phosphate was more active in stimulating dCMP and dGMP kinase activity than the oligodeoxynucleotides, and the effects of both components together were approximately equal to the sum of their effects separately (with respect to specific activity levels). It is important to note that the effects of the oligodeoxynucleotides cannot be ascribed to their phosphate content, because the amount of phosphate present in the oligodeoxynucleotide fraction as part of the deoxynucleotide structure (see Fig. 1 for concentrations of each) is only a small fraction of the amount of phosphate required to affect kinase activity.

Kinetics of stimulation, requirements for amino acids, inhibition by chloramphenicol. To determine whether protein synthesis was involved in the increase of dCMP and dGMP kinase activity, the following experiments were performed. First, the requirement for an external source of amino acids was examined. Second, the sensitivity of the stimulation to chloramphenicol was determined. Table 2 shows that the removal of Casitone (vitamin-free enzymatic digest of casein used as the source of amino acids in the suspending medium) from the suspending medium resulted in an almost complete inhibition of the stimulatory effect, whereas little inhibition was observed in control cells when Casitone was deleted. Table 2 also shows that the increases in kinase activity

TABLE 1. *Deoxynucleotide kinase activity in extracts taken from cells exposed to supplement moieties**

Additions				Kinase assayed	Specific activity (product-substrate ratio/mg of protein)
Deoxynucleosides and deoxynucleotides	Oligodeoxyribonucleotides	Phosphate	Specific substrate		
+	+	+	−	dCMP	0.53
+	+	−	−		0.18
+	−	+	−		0.42
+	−	−	−		0.07
−	+	+	−		0.63
−	+	−	−		0.23
−	−	+	−		0.45
−	−	−	+		0.05
−	−	−	−		0.05
+	+	+	−	dGMP	0.26
+	+	−	−		0.18
+	−	+	−		0.35
+	−	−	−		0.05
−	+	+	−		0.44
−	+	−	−		0.20
−	−	+	−		0.40
−	−	−	+		0.06
−	−	−	−		0.05
+	+	+	−	dAMP	1.10
+	+	−	−		1.33
+	−	+	−		1.09
+	−	−	−		1.55
−	+	+	−		1.22
−	+	−	−		1.45
−	−	+	−		1.37
−	−	−	+		1.13
−	−	−	−		1.49
+	+	+	−	dTMP	0.06
+	+	−	−		0.045
+	−	+	−		0.04
+	−	−	−		0.06
−	+	+	−		0.05
−	+	−	−		0.05
−	−	+	−		0.07
−	−	−	+		0.065
−	−	−	−		0.07

* Reaction mixtures were as described in the standard assay system (see Fig. 1). For concentrations of moieties from the supplement, see Fig. 1. Incubation time of the cells before extraction of kinases was 25 min.

were abolished when chloramphenicol was added at zero-time. Hardly any effects were noted in control cells when the antibiotic was added.

With total kinase activity as a parameter of kinase-protein synthesis, Fig. 3 shows that the rate of dCMP and dGMP kinase synthesis increases when oligodeoxynucleotides and phosphate are added to the cells without a concomitant increase in the *rate* of total protein synthesis of the cells.

TABLE 2. *Inhibition of effects of oligodeoxyribonucleotides and phosphate by chloramphenicol and lack of an external source of amino acids**

Additions			Kinase assayed	Specific activity (product-substrate ratio/mg of protein)
Oligodeoxyribonucleotides and phosphate	Chloramphenicol	Casitone		
+	−	+	dCMP	0.71
+	+	+		0.09
+	−	−		0.12
−	−	+		0.08
−	+	+		0.07
−	−	−		0.055
+	−	+	dGMP	0.52
+	+	+		0.10
+	−	−		0.14
−	−	+		0.08
−	+	+		0.09
−	−	−		0.06

* Reaction mixtures were as described in the standard assay system (see Fig. 1). For concentrations of oligodeoxyribonucleotides and phosphate, see Fig. 1. Cells were washed four times with 0.85% saline adjusted to *p*H 7.2 with dilute NaOH, and were allowed to "starve" in this solution for 20 min at 25 C before inoculation into the suspending medium. This was done to remove as much of the intracellular amino acid pool as possible. Casitone (enzymatic digest of casein used as the external amino acid source) is a normal constituent of the suspending medium. In this experiment, it was omitted and added as shown in the table at a final concentration of 1%. Concentration of chloramphenicol was 100 μg/ml in the suspending medium. Cells were extracted for kinase activity after 25 min of incubation.

Test for possible direct effects of supplement components on activity of isolated kinases. Table 3 shows that the components from the supplement had no stimulatory effects on dCMP and dGMP kinase activity when the enzymes were first extracted from the cells. In fact, the supplement components had a depressing effect on kinase activity.

Detection of monophosphatases for dAMP and dTMP in kinase preparations. The decline in specific activity of all the kinases after 10 or 25 min in the partially purified kinase preparations of controls and supplements could be due to a

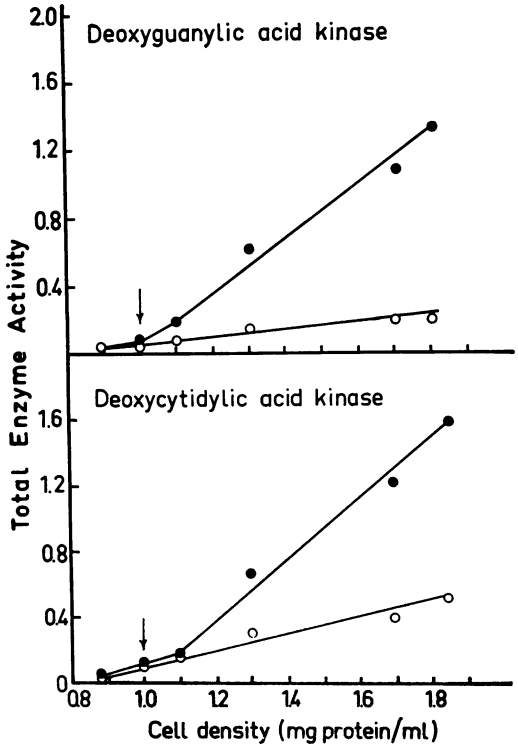

FIG. 3. *dCMP and dGMP kinase activity as a function of cell density (mg of protein per ml) before and after exposure of the cells to oligodeoxyribonucleotides and phosphate. Reaction mixtures were as described in the standard assay system (see Fig. 1). The arrows signify the time of addition of the components to the cells. The entire experiment was carried out over a period of approximately 30 min after inoculation of the cells into the suspending medium. This was the time of active protein synthesis by the cells. Symbols: ●, supplemented; ○, control.*

TABLE 3. *Effects of various supplement moieties on isolated dCMP and dGMP kinase activities**

Additions to assay mixture		Enzyme source	Kinase assayed	Specific activity (product-substrate ratio/mg of protein)
Oligodeoxyribonucleotides and phosphate	Deoxynucleosides and deoxynucleotides			
+	−	Supplemented cells	dCMP	0.20
−	+			0.60
+	+			0.51
−	−			0.63
+	−	Control cells		0.12
−	+			0.09
+	+			0.15
−	−			0.12
+	−	Supplemented cells	dGMP	0.40
−	+			0.50
+	+			0.44
−	−			0.70
+	−	Control cells		0.11
−	+			0.10
+	+			0.11
−	−			0.13

* Reaction mixtures were as described in the standard assay system (see Fig. 1). For concentrations of supplement moieties, see Fig. 1.

number of factors. These include (i) the presence of proteases or phosphatases (which could degrade either the kinases or substrates, respectively), (ii) the accumulation of inhibitors or end products during incubation, and (iii) the loss of specific activators. Thus far, monophosphatases have been demonstrated, but only for the substrates dAMP and dTMP (Table 4).

Relationship between stimulated kinase activity and preferential DNA synthesis. The ability of the supplement to enhance DNA synthesis by stimulating dCMP and dGMP kinase activity was supported by several observations. First, the selective rise in DNA of supplemented cells was dependent on new protein synthesis which occurred between 10 and 30 min (Fig. 4). This was shown by the fact that chloramphenicol prevented any selective rise in DNA over that which occurred in controls when added at 10 min of incubation. When added at 30 min, however, the antibiotic did not prevent the increase in DNA. In contrast, chloramphenicol had no effect on DNA synthesis of control cells when added at either time; yet, protein synthesis in controls and supplements was inhibited immediately upon addition of the drug at either time. It will be recalled that (i) chloramphenicol abolished the stimulation of dCMP and dGMP kinase activity (see Table 2), and (ii) the greatest increase in dCMP and dGMP kinase activity occurred between 10 and 30 min, namely, at 25 min (see Fig. 1), or just prior to the onset of excess DNA synthesis. It was possible to detect the onset of enhanced DNA synthesis, because DNA and protein syntheses increased at approximately the same rates in controls and supplements for the first 25 min, whereas, after this time (as shown in Fig. 4), DNA synthesis continued to increase for 45 min in supplemented cells without a parallel increase of protein synthesis. No further DNA and protein syntheses occurred in control cells after 25 min. Second, it was possible to correlate the ability of oligodeoxynucleotides and phosphate to stimulate dCMP and dGMP kinase activity with their ability to enhance DNA synthesis. As shown in Fig.

TABLE 4. *Phosphatases for dAMP and dTMP in extracts of supplemented and control cells**

Source of enzyme		Deoxynucleotide	Time (min)	Specific activity (product-substrate ratio/mg of protein)
Supplemented cells	Control cells			
+	−	dTMP	10	0.098
			25	0.18
			50	0.11
			75	0.098
−	+		10	0.15
			25	0.24
			50	0.16
			75	0.10
+	−	dAMP	10	0.78
			25	0.60
			50	0.70
			75	0.66
−	+		10	1.00
			25	0.70
			50	0.70
			75	0.67

* Reaction mixtures were as described in the standard assay system (see Fig. 1). The degradation of deoxynucleotides to deoxynucleosides was detected and measured on the chromatogram in the same manner as the synthesis of deoxynucleoside di- and triphosphates.

5, the greater the stimulation of the activity of dCMP and dGMP kinases by various concentrations of oligodeoxynucleotides and phosphate, the greater was the enhancement of DNA synthesis. However, this correlation was obtained only in the presence of the deoxynucleosides and deoxynucleotides. In their absence, enhanced DNA synthesis did not occur, despite the stimulation of kinase activity by oligodeoxynucleotides and phosphate.

Specificity of observed effects for DNA degradation products and phosphate. It could be argued that the supplement contains agents which affect the overall metabolic rate, and, therefore, the observed effects may not be truly specific for enzymes involved in DNA synthesis. Table 5 summarizes the effects of several other metabolites, including RNA degradation products, on enhanced DNA synthesis and kinase activity. None of these metabolites produced effects similar to those elicited by the regular supplement. Furthermore, when such metabolites were mixed with parts of the usual supplement, the only effects observed were those contributed by moieties of the supplement.

Genetic competence of excess DNA. Consideration was given as to whether the excess DNA synthesized in supplemented cells was genetically competent. If not, then control of such DNA synthesis might not reflect normal processes involved in DNA metabolism. Thus, transformation experiments were performed with DNA extracted from streptomycin-resistant pneumococci which had been exposed to the supplement. If a greater amount of genetically competent DNA were synthesized in supplemented cells than in control cells, the specific activity (transformants per milligram of cell protein) of the extracted DNA would be proportionately greater from supplemented than from control cells. As Table 6 shows for the streptomycin locus, the excess DNA was genetically competent.

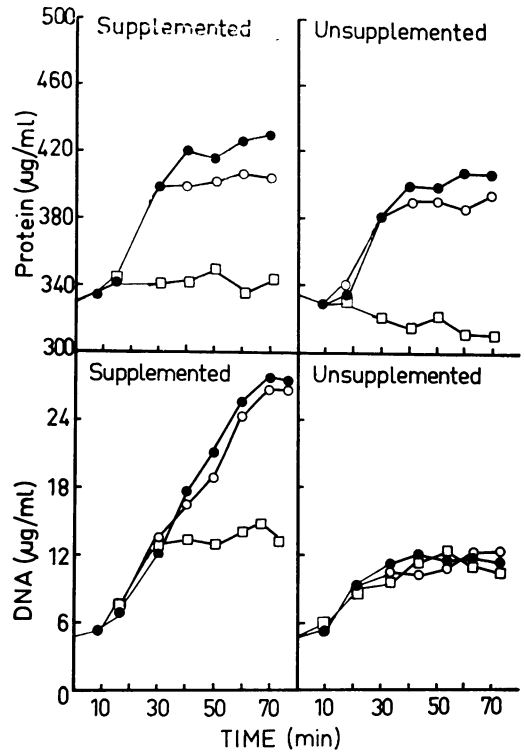

FIG. 4. *Effects of chloramphenicol on DNA and protein syntheses of supplemented- and control-cell suspensions. For concentration of supplement, see Fig. 1. Concentration of chloramphenicol was 100 µg/ml in the suspending medium. Symbols:* □, *chloramphenicol added at 10 min;* ○, *chloramphenicol added at 30 min;* ●, *no chloramphenicol.*

DISCUSSION

The foregoing data indicate a correlation between the selective stimulation of DNA synthesis elicited by DNA degradation products and phosphate, and the increased activities of

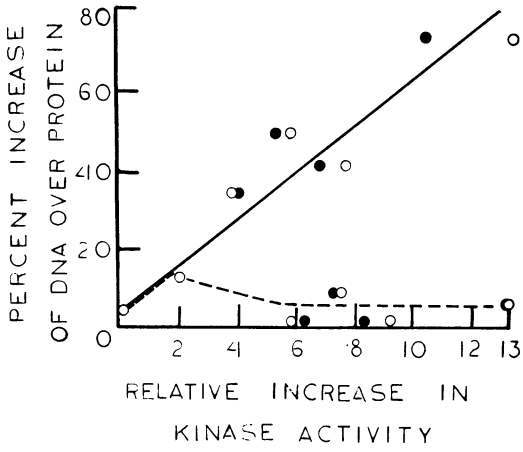

FIG. 5. *Per cent increase of DNA synthesis over protein synthesis as a function of relative increase in dCMP and dGMP kinase activity. Each point (except the first four nearest the origin—the unshaded circles are superimposed over the shaded circles) depicts the ability of increasing concentrations of oligodeoxyribonucleotides and phosphate to enhance dCMP and dGMP kinase activity and DNA synthesis in the presence and absence of a constant amount of deoxynucleosides and deoxynucleotides. The first four points depict the effects of deoxynucleosides and deoxynucleotides alone, and the control (nearest to the origin). Incubation time for determination of DNA and protein syntheses and kinase activity was 70 min. For concentration of supplement components, see Fig. 1. The term "relative increase" connotes the increase in dCMP and dGMP kinase activity from control levels. Per cent increase of DNA over protein was calculated as described previously (Firshein and Zimmerman, 1964). Solid lines, with deoxynucleosides and deoxynucleotides; dashed lines, without deoxynucleosides and dexoynucleotides;* ○, *dCMP kinase activity;* ●, *dGMP kinase activity.*

TABLE 5. *Effects of RNA degradation products and yeast extract on kinase activity and excess DNA synthesis in the presence and absence of moieties of the supplement**

Additions	Relative increase over control of specific activity		Per cent increase of DNA over protein
	dCMP	dGMP	
a. Oligo(deoxy)nucleotides and phosphate	10.6	5.2	6
b. Deoxynucleosides and deoxynucleotides	1.4	—	12
c. a + b	8.5	6.8	55
d. RNA oligonucleotides and phosphate	9.2	7.7	—
e. Nucleosides and nucleotides	1.1	1.0	—
f. d + e	11.2	8.3	1
g. Oligo(deoxy)nucleotides and deoxynucleosides and deoxynucleotides	7.7	4.4	40
h. Oligonucleotides (RNA) and nucleosides and nucleotides	—	1.1	1
i. Oligo(deoxy)nucleotides and nucleosides and nucleotides	4.1	3.9	5
j. RNA oligonucleotides and deoxynucleosides and deoxynucleotides	1.8	1.5	10
k. Phosphate and nucleosides and nucleotides	9.8	7.1	—
l. Phosphate	10.9	8.8	1
m. Yeast extract	1.3	1.3	—
n. Phosphate and yeast extract	12.0	8.8	—
o. Yeast extract and deoxynucleosides and deoxynucleotides	1.4	1.0	11

* Reaction mixtures for determining kinase activity were as described in Fig. 1. Cells were incubated for 25 min before extraction. DNA and protein were determined after 70 min of incubation. Per cent increase of DNA over protein was calculated as described previously (Firshein, and Zimmerman, 1964). The RNA oligonucleotides were prepared as described by Staehelin (1963). For concentration of moieties of supplement, see Fig. 1. Concentrations of RNA degradation products were identical with their DNA degradation product counterparts in the supplement. Concentration of yeast extract in the suspending medium was 0.1%.

dCMP and dGMP kinases. This correlation is supported by several lines of evidence. First, there is a direct relationship between the levels of dCMP and dGMP kinase activity, and the extent of DNA synthesis. The greater the activity of dCMP and dGMP kinases, the greater was the enhancement of DNA synthesis. Second, chloramphenicol prevents the selective rise in DNA if added at the appropriate time (10 min), and the antibiotic also inhibits stimulated dCMP and dGMP kinase activity. These observations indicate that new protein synthesis is required both for enhanced DNA synthesis and stimulated dCMP and dGMP kinase activity. Third, the greatest enhancement of dCMP and dGMP kinase activity occurs between 10 and 30 min, namely, at 25 min, or just prior to the onset of enhanced DNA synthesis. In contrast, the increases in activity of the remaining two kinases occurs during the first 10 min. The fact that chloramphenicol is inactive in preventing enhanced DNA synthesis when added at 30 min, but active in this respect at 10 min, suggests

TABLE 6. *Genetic competency of excess DNA synthesized in the presence of the supplement*

Condition	DNA after 70 min	Protein after 70 min	Specific activity* ($\times 10^2$)
	μg/ml	*μg/ml*	
Supplemented cells	24 ± 2.4	406 ± 8.1	130
Unsupplemented cells	15 ± 1.7	399 ± 6.0	90
Per cent increase of supplemented cells over control cells	52	1.7	44

* Specific activity is defined as transformants obtained from DNA per milligram of cell protein. For concentration of supplement, see Fig. 1. Each DNA preparation was diluted 1:75 before assaying for transforming activity. This dilution was made to ensure that the final concentration of transforming DNA in the assay medium per milliliter would be well below the saturating levels. The results represent an average of eight replicate experiments.

that the proteins involved in stimulating DNA synthesis are themselves synthesized between 10 and 30 min.

Presumably, the increase in the activity of dCMP and dGMP kinases results in an elevation of DNA synthesis in supplemented cells because such enhancement provides sufficient levels of all four deoxynucleoside triphosphates required for DNA synthesis throughout the incubation period. In contrast, in control cells, the activity of dCMP and dGMP kinases decreases to low or almost undetectable levels between 25 and 50 min. In addition to appropriate enzyme levels, the results suggest that a sufficient supply of substrates for kinase reactions (deoxynucleosides and deoxynucleotides) is required for enhanced DNA synthesis to occur; oligodeoxynucleotides and phosphate are incapable of stimulating DNA synthesis in the absence of added deoxynucleosides and deoxynucleotides.

Oligodeoxynucleotides and phosphate, alone or in combination, may act as inducers of higher levels of dCMP and dGMP kinases. The evidence for induction is as follows. (i) An inincrease in the rate of kinase synthesis occurs when oligodeoxynucleotides and phosphate are added to cell suspensions without a concomitant increase in the *rate* of cell protein synthesis. (ii) The components of the supplement are incapable of stimulating the activity of isolated kinases. (iii) The enhancement of dCMP and dGMP kinase activity requires an external source of amino acids. (iv) The effects of oligodeoxynucleotides and phosphate are inhibited by adding chloramphenicol. It is not known whether the oligodeoxynucleotides and phosphate act in the same manner to induce dCMP and dGMP kinases. That is, it is possible for one component to act directly on a kinase repressor, while the other acts indirectly by activating an intracellular inducer. Since phosphate can participate in a number of cellular processes, particularly those related to energy-generating systems, it may be that this anion activates an intracellular inducer related to, but more effective than, the oligodeoxynucleotides. This hypothesis is under investigation at present, as well as attempts to isolate the active component(s) from the oligodeoxynucleotide fraction.

The phenomenon of enhanced DNA synthesis in pneumococci is a result of "forced-feeding" of the cells with DNA degradation products and excess phosphate. It is not certain whether such DNA degradation products naturally occur intracellularly in pneumococci, or whether high concentrations of phosphate are normally available to the cells. However, since the cells contain enzyme systems that respond to added DNA degradation products and excess phosphate in the manner described, it is suggestive that such systems may also operate under conditions where no external supplement is added. Also, at least one explanation for the observation that the activity of dCMP and dGMP kinases increases in unsupplemented-cell suspensions during the first 25 min (see Fig. 1) is that inducers and substrates for these two kinases have been produced in limited amounts de novo. Some support for the natural occurrence of oligodeoxynucleotides in other microorganisms, and their possible role in deoxynucleotide kinase metabolism, has come from recent studies by Stone and Burton (1962) and Weissbach and Korn (1963). Both groups of investigators have shown that several kinds of deoxyribonucleases are produced in phage-infected cells or in induced lysogenic cells after bacterial DNA has been degraded. Most important is the finding that the deoxyribonucleases are produced at the same time or just prior to the synthesis (by the phage) of dCMP or dTMP kinases. Since the extent of degradation of the bacterial DNA is not known completely, it is possible that at least one function for the deoxyribonucleases is to provide more suitable oligodeoxynucleotides from the degraded DNA to act as inducers for phage deoxynucleotide kinases. However, it should be stated that other mechanisms for the deoxyribonucleases have been proposed (Lehman, 1963).

Finally, a point may be made concerning the possible relationship between the role of DNA degradation products and phosphate in control of enhanced DNA synthesis in pneumococci, and the "initiator-replicator" model for control of bacterial DNA synthesis proposed by Jacob et al. (1963). Presumably, the "replicator" would require the presence of an adequate substrate pool (deoxynucleoside triphosphates) for polymerization purposes. Such substrates could be made available by reactions involving DNA degradation products and phosphate. In fact, one might visualize the "replicator" as a gene site regulating reactions that produce substrates and inducers for deoxynucleotide kinases. It is probable that this hypothesis will have to be revised as new experiments are forthcoming. However, it serves the purpose of focusing attention on an in vitro system for further analysis of the "initiator-replicator" model.

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