EFFECTS OF DEOXYRIBONUCLEIC ACID PRODUCTS ON DEOXYRIBONUCLEIC ACID SYNTHESIS OF VIRULENT AND AVIRULENT PNEUMOCOCCI

W. FIRSHEIN

Department of Biology, Wesleyan University, Middletown, Connecticut

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ABSTRACT

FIRSHEIN, W. (Wesleyan University, Middletown, Conn.). Effects of deoxyribonucleic acid products on deoxyribonucleic acid synthesis of virulent and avirulent pneumococci. J. Bacteriol. **82**:169–180. 1961.—Cell suspensions of virulent pneumococci synthesize a greater amount of deoxyribonucleic acid (DNA) per cell in the presence of DNA + deoxyribonuclease + mixtures of deoxynucleosides and deoxynucleotides (supplement-1) than unsupplemented suspensions. Under identical conditions, avirulent and weakly virulent pneumococci do not respond to these DNA breakdown products. Glucose and casitone must be present for maximal effects to occur.

A DNA turnover occurs in virulent cells. This has been demonstrated by a decrease in specific activity of H^3-DNA extracted from virulent (S) cells exposed to nonradioactive supplement-1.

Under laboratory conditions, most pathogenic bacteria undergo population changes resulting in the establishment of spontaneously arising avirulent mutants. But in susceptible hosts, the virulent type predominates and the selection of avirulent mutants is suppressed. Environmental factors responsible for these population changes are poorly understood. Their elucidation would contribute greatly to an understanding of the metabolic basis of pathogenicity and the metabolic processes associated with dissimilar cell types. Several investigators have attempted recently to duplicate the events occurring in susceptible hosts in vitro by establishing conditions that stimulate the selection and proliferation of virulent mutant cells in initially predominantly avirulent populations. Braun and Whallon (1954) and Firshein and Braun (1960) observed that deoxyribonucleic acid (DNA) + deoxyribonuclease selectively enhanced population changes from avirulent (R) → virulent (S) in cultures of *Brucella abortus* and *Diplococcus pneumoniae*. Although the same end results were obtained, the mechanisms responsible for these effects were completely different in both microorganisms. Whereas virulent brucella mutants were selected because of an inhibition of R cells without any effect against S cells, selection in pneumococci was due to a stimulation of the multiplication of S cells without any effect against R cells. In addition to the presence of the DNA digest, mixtures of deoxynucleosides, deoxynucleotides, and nucleoside diphosphates were required for maximal effects in pneumococci. Preliminary experiments with resting cells demonstrated that the DNA products enhanced DNA synthesis in S cell suspensions, but had no effect when R cells were employed.

This report will be concerned with the metabolic basis for the differential response by S and R pneumococci to the DNA products, as well as the role of these components in the metabolism of S pneumococci.

MATERIALS AND METHODS

Organisms. Virulent and avirulent strains of types I, II, and III *D. pneumoniae* were obtained from R. Austrian. The intermediate (I) strains were freshly isolated from their corresponding S parents by prolonged culture in brain heart infusion (Difco) liquid medium supplemented with 0.2% sterile defibrinated rabbit blood. All strains were maintained on brain heart infusion agar medium supplemented with 5% rabbit blood. Many experiments were carried out with type I pneumococci although the R strain was not closely related to, or derived from, the S strain employed. But it was felt that the results obtained with these strains reflected naturally occurring events for several reasons: (i) The DNA products acted primarily on S cells without any effects against R cells,

TABLE 1. *Effects of type III capsular hydrolyzing enzyme on ability of type III virulent pneumococci to incorporate H^3-thymidine and synthesize DNA in the presence of supplement-1*

Additions	Capsular-hydrolyzing enzyme*	DNA†	Protein†	Specific activity
	units/ml	*μg/ml*		*counts: min: mg DNA*
Supplement-1 containing H^3-thymidine‡	1.5	9.0	350	19,908
		14.0	366	32,293
None	1.5	8.8	340	
		8.7	342	
At zero time.....		7.3	335	

Inoculum: A66 (Type III, virulent). Supplement-1 (per ml): DNA, 130 μg; deoxyribonuclease, 25 μg; deoxynucleosides, 800 μg (200 each of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine); deoxynucleotides, 800 μg (200 each of the phosphorylated deoxynucleosides mentioned above); glucose and casitone, 10 and 20 mg, respectively.

* One unit of enzyme is that amount hydrolyzing 500 μg of polysaccharide in 1 ml solution in 18 hr at 37 C.

† After 70 min.

‡ The final concentration of H^3-thymidine in the suspensions was 2 μc. The ratio of radioactive isotope to nonradioactive carrier was 1:1,000.

regardless of the origin of the strains under study. (ii) The DNA products always stimulated S cells. (iii) Experiments with more closely related R or I mutants produced essentially identical results as the type I model system.

Resting cell experiments and growth medium. To obtain large numbers of viable cells for resting cell experiments, R and S pneumococci were grown in a casein-tryptone-yeast extract medium (Marmur and Hotchkiss, 1955), washed once with 0.02 M phosphate buffer (pH 7.5), and suspended in 1.5 ml of the same buffer supplemented with 1% glucose, 2% casitone (enzymatic digest of casein, Difco), and catalase (50 μg per ml). The suspending medium was prepared double strength, and before final dilution supplemented with the various DNA products to be tested. The cell suspensions were shaken vigorously in a gyrotory water bath shaker for various periods of time at 37 C, and then sampled for their nucleic acids and protein. Each assay was performed in duplicate.

Preparation of DNA, depolymerized DNA, and other substances. Highly polyermized calf-thymus DNA (sodium salt) was prepared by the method of Kay, Simmons, and Dounce (1952). The DNA was stored as a precipitate in 95% ethanol at 4 C. Depolymerized DNA was prepared by treating a solution of DNA (1,100 μg per ml) with pancreatic deoxyribonuclease (500 μg per ml, Worthington, 1 × crystallized) in the presence of 0.03 M $MgSO_4 \cdot 7H_2O$ at 25 C for 2 hr. The digestion was followed by the increase in ultraviolet absorption (27 to 30%) at 260 mμ in a spectrophotometer (Kunitz, 1950). The proportion of DNA to deoxyribonuclease by volume was always 2:1. In routine use, 0.25 ml of the enzymatic DNA digest was added to 1.25 ml of the resting cell suspension. All of the remaining compounds that included the naturally occurring deoxynucleosides and deoxynucleotides (California Corporation for Biochemical Research) were dissolved in distilled water for use in the experiments. Their specific concentrations are described in Table 1. Nucleoside diphosphate mixtures which had been effective in stimulating R → S population changes were ineffective in the resting cell suspensions and were excluded from further study.

Fractionation of enzymatic DNA digests. The method of Bendich et al. (1955) was employed to separate small and large molecular weight components in the enzymatic DNA digest. When all of the fractions detectable by ultraviolet at 240, 260, and 280 mμ had been eluted, each of them was lyophilized, reconstituted to a smaller volume, and tested for activity in the resting cell suspension. Excess salt was removed by addition of 10 volumes of 95% ethanol to each fraction. A considerable amount of salt was precipitated leaving the DNA products in solution. The alcohol was evaporated under reduced pressure.

Protein determination in resting cells as a parameter of cell growth and multiplication. Because avirulent pneumococci (and to a lesser extent virulent pneumococci) form chains, errors in viable counts may be as high as 70 to 80%. To obviate this difficulty, protein increase was employed as a more reliable parameter of growth (cellular and reproductive) in the resting cell

suspension (Lamanna and Mallette, 1954). Protein was estimated in the precipitate remaining after hot trichloroacetic acid extraction of the nucleic acids with the Folin-Ciocalteau reagent according to Lowry et al. (1951). The sensitivity of this determination was good to within 3 to 5%.

Nucleic acid extraction and estimation. Nucleic acids were extracted by a modification of two methods (Schneider, 1945; Morse and Carter, 1949). Both procedures individually were found unsuitable for pneumococci either because of inadequate washing, or because of the inability to extract all the DNA present.

The cells were precipitated with cold trichloroacetic acid at a final concentration of 10%. The nucleic acids were extracted from the precipitate with 2 ml of 5% trichloroacetic acid at 95 C for 30 min after washing once with 10% trichloroacetic acid, twice with 95% ethanol, and once with ethanol-ether (3:1) at 60 C for 15 min. Ribonucleic acid (RNA) was estimated by the orcinol reagent of Drury (1948) and DNA by a modification of the cysteine-sulfuric acid reagent of Stumpf (Brody, 1953). The sensitivity of both of these analyses was good to within 5 to 7%.

Preparation of enzyme hydrolyzing type III pneumococcal capsular polysaccharide. This enzyme, derived from *Bacillus palustris*, was prepared by the method of Dubos (1935) as modified by Smith et al. (1960). One unit of enzyme was that amount hydrolyzing 500 μg of polysaccharide in 1 ml solution in 18 hr at 37 C. Type III capsular polysaccharide was estimated photometrically by measurement of the turbidity of the specific antigen-antibody precipitate formed in the presence of excess antibody (Bernheimer, 1953).

Isotope procedures. H^3-Thymidine, H^3-deoxycytidine, and H^3-deoxycytidylic acid (Schwarz Laboratories) were diluted with their nonradioactive carrier compounds such that the specific activity of each isotope in the resting cell suspension was 2 μc per 200 μg. Radioactive measurements were performed on soluble nucleic acid extracts obtained as described previously. That all the radioactivity measured in these extracts was derived from DNA was established by the following experiments: (i) The incorporated radioactivity was acid-insoluble, but could be solubilized by treatment with deoxyribonuclease. (ii) The distribution of H^3-thymidine in DNA separated from RNA by the method of Davidson and Smellie (1952) was examined by hydrolysis with 98% formic acid for 2 hr in a sealed tube at 165 C (Chargaff and Davidson, 1955) and by paper chromatography of the bases (Wyatt, 1951). The chromatogram showed only one radioactive spot, coincident with an ultraviolet quenching spot, with the R_F and the spectrum corresponding to thymine. (iii) None of the mononucleotides obtained by alkaline hydrolysis of RNA separated from DNA by the above procedure was radioactive.

Measurements were made at infinite thinness in a windowless flow counter (Technical Measurement Corporation).

Preparation of S cells containing DNA labeled in the thymidine moiety with H^3. Virulent type I cells were grown overnight in casein-tryptone-yeast extract medium (1.5 liters) containing 0.5 μc per ml of H^3-thymidine. When growth had ceased, approximately 80 to 90% of the isotope had been removed from the medium by the cells. The harvested bacteria were then treated in a manner similar to that described for nonlabeled cells. Representative DNA extracts had a specific activity of 0.2 μc per mg, corresponding to 1 mμmole thymidine per mg DNA.

RESULTS

It was suspected that the selective stimulation of the multiplication of S cells in complete media containing DNA products involved an effect on DNA metabolism. To investigate this possibility, it was of particular value to measure DNA and protein syntheses in the presence and absence of these components in resting S and R suspensions. A survey of three pneumococcal types (I, II, and III) revealed that when S cells of all three types were augmented with DNA + deoxyribonuclease + mixtures of deoxynucleosides and deoxynucleotides (supplement-1), a significant selective stimulation of DNA synthesis occurred over protein synthesis. In contrast, this selective increase was not observed in unsupplemented S, I (weakly virulent), or R suspensions of these types, or in R and I suspensions containing supplement-1 (Fig. 1, 2, and 3). Parallel increases in DNA and protein were observed in these latter cases, however, suggesting that some cell growth and multiplication occurred (Morse and Carter, 1949). These parallel increases were due primarily to the constituents of the suspending medium (glucose and casitone)

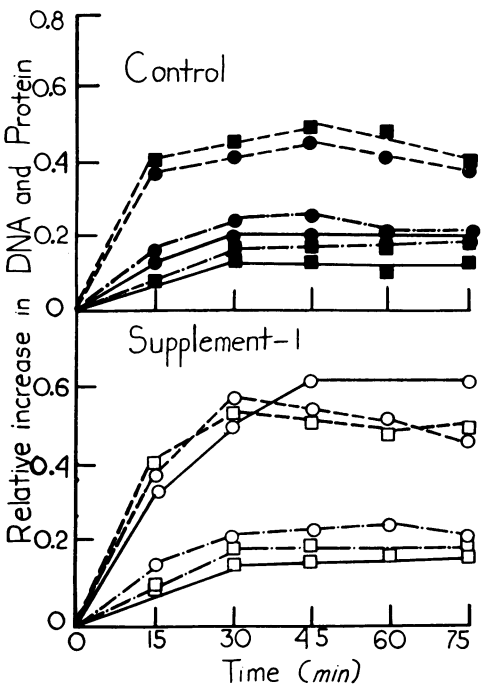

FIG. 1. *Effects of supplement-1 on DNA and protein syntheses of S, I, and R type I pneumococci. S* (——), *I* (-·-·), *R*(– – –); *supplement-1, DNA* (○), *protein* (□); *control, DNA* (●), *protein* (■). *Inoculum: SVI* (*S*), *ISVI* (*I*), *RI192* (*R*) (letters and roman numerals represent terminology of strains used; *S* = virulent, *R* = avirulent, *I* = intermediate). For *concentration of supplement-1, glucose, and casitone, see Table 1. The term "relative increase" on the graph connotes the increase of DNA and protein from zero time.*

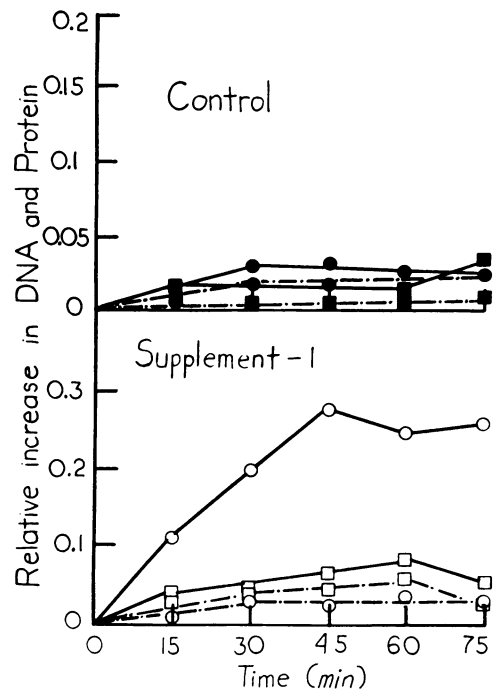

FIG. 2. *Effects of supplement-1 on DNA and protein syntheses of S and I type II pneumococci. S* (——), *I* (-·-·-); *supplement-1, DNA* (○), *protein* (□); *control, DNA* (●), *protein* (■). *Inoculum: D39S* (*S*), *D39I* (*I*). *For concentration of supplement-1, glucose, and casitone, see Table 1. The term "relative increase" on the graph connotes the increase of DNA and protein from zero time.*

rather than to supplement-1. This is seen by a comparison of the results obtained with controls and augmented suspensions of each R and I strain. No major differences in over-all rates of DNA and protein syntheses were observed, except for a slight enhancement in type I R suspensions. In some cases, the differences between supplement-1 augmented S suspensions and controls were not great, but they were considered significant as judged by repeated trials. Since the increases in protein were minimal in comparison to the increases in DNA, it was concluded that S cells of types I, II, and III pneumococci synthesized a greater amount of DNA per cell in the presence of supplement-1 than unexposed S cells.

It was conceivable that the differential response of S and R cells to supplement-1 was due to the inability of R cells to incorporate appropriate DNA products as efficiently as S cells. This possibility was examined by isotope studies using H^3-thymidine. The isotope was added to S and R cell suspensions as part of supplement-1 and after specified intervals, DNA was extracted from duplicate samples, assayed, and a portion taken from this extract for radioactive determination. As illustrated in Fig. 4, S cells incorporated a greater amount of H^3-thymidine per mg DNA than R cells. That this accelerated incorporation was at least partially dependent on cell surface characteristics was shown by the results obtained in Table 1. Virulent cells, whose capsule was removed by an enzyme derived from *B. palustris*, failed to respond to supplement-1 as did fully capsulated pneumococci. No selectively increased DNA levels were produced by such decapsulated cells and they were incapable of incorporating H^3-

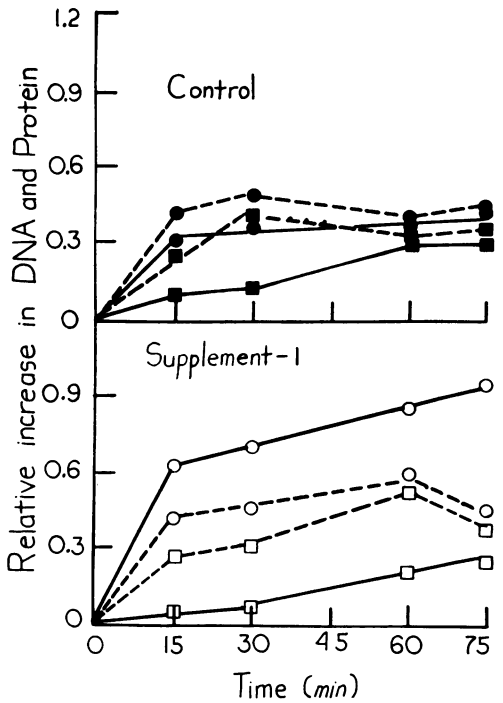

FIG. 3. *Effects of supplement-1 on DNA and protein syntheses of S and R type III pneumococci. S* (——), *R* (---); *supplement-1, DNA* (○), *protein* (□); *control, DNA* (●), *protein* (■). *Inoculum: A66 (S), A66R (R). For concentration of supplement-1, glucose, and casitone, see Table 1. The term "relative increase" on the graph connotes the increase of DNA and protein from zero time.*

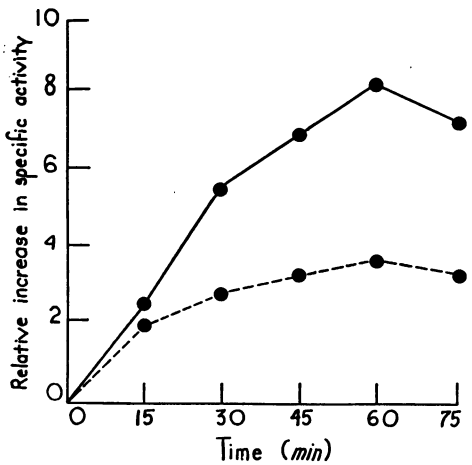

FIG. 4. *Comparison of the ability of S and R type I pneumococci to incorporate H³-thymidine into DNA. S* (——), *R*(---). *Inoculum: SVI (S), RI192 (R). For concentration of supplement-1, glucose, and casitone, see Table 1. The final concentration of H³-thymidine in the suspensions was 2 μc. The ratio of radioactive isotope to nonradioactive carrier was 1:1,000. The term "relative increase" on the graph connotes the increase in specific activity of the extracted S or R DNA from 30 sec (since specific activity at zero time was "zero," it was necessary to have some incorporation figure on which to base the increase and 30 sec was arbitrarily chosen for this purpose. At this time it was possible to extract labeled DNA from S and R cells).*

thymidine as efficiently as unaltered cells. According to Dubos (1935), the hydrolytic enzyme renders a genotypically virulent population phenotypically avirulent by removing the capsule. For many years, the capsule has been regarded as a protective covering around the surface of the cell that prevents phagocytosis (White, 1938; Dubos, 1954). However, the present results suggest that the capsule may be of some importance in regulating the metabolic activities of the cell. This suggestion will be discussed in greater detail later.

The increased metabolism of DNA in S cells in the presence of supplement-1 may have resulted in a significant DNA turnover. To investigate this possibility, the specific activity of thymidine-labeled H³-DNA extracted from S cells was measured over a 10-min incubation period in unsupplemented suspensions, and in suspensions containing either supplement-1, thymidine, deoxyguanosine, deoxyadenosine, or deoxycytidine. The concentrations of the individual deoxynucleosides were identical to those of their counterparts in supplement-1, and the 10-min incubation period was employed to avoid any complications arising from cell lysis and reincorporation of the label. A DNA turnover would be indicated by a decrease in specific activity in the supplemented suspension. As summarized in Fig. 5, the specific activity remained constant in control suspensions and in suspensions containing each of the individual deoxynucleosides except thymidine. A 14% decrease in specific activity occurred when thymidine was present and a 45% decrease when supplement-1 was employed. DNA and protein remained constant in each suspension during the limited incubation period except in suspensions containing supplement-1 where a slight enhancement of DNA synthesis occurred (8%).

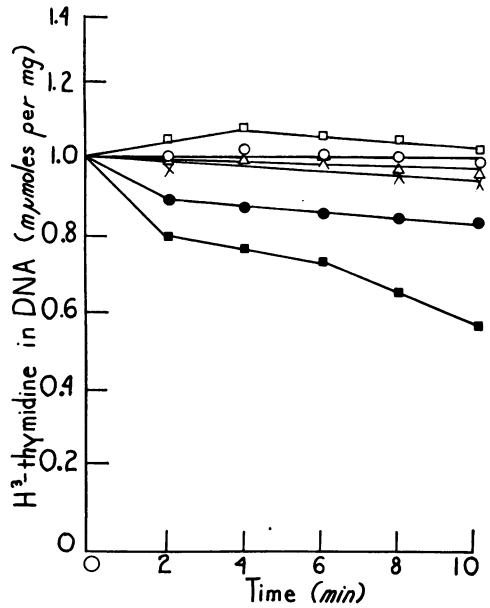

FIG. 5. *Effect of supplement-1 and single deoxynucleosides on metabolic stability of intracellular DNA of type I S cells, labeled with H^3 in the thymidine moiety. Control (○), deoxyadenosine (□), deoxyguanosine (△), deoxycytidine (×), thymidine (●), supplement-1 (■). Inoculum: SVI. For concentration of supplement-1, glucose, and casitone, see Table 1. Single deoxynucleosides = 200 μg per ml. Cells containing H^3-DNA were prepared as described in Materials and Methods.*

The fact that none of the deoxynucleosides except thymidine decreased specific activity did not mean that they were inactive in the turnover reaction, but rather it suggested that they were exchanging specifically with their related deoxynucleosides in DNA. Thus, if the cells had been labeled with deoxyadenosine instead of with thymidine, only the addition of deoxyadenosine would have decreased specific activity. Furthermore, it would appear that supplement-1 accelerated these specific exchanges.

A number of investigators working with animal tissues and with microorganisms have reported that DNA turnover is relatively insignificant in comparison to other cell components, such as RNA (Hershey, 1954; Siminovitch and Graham, 1956; Fresco and Bendich, 1960). According to these investigators, this stability of DNA would be in keeping with its function as genetic material. However, none of these experiments were carried out in an environment where DNA synthesis was enhanced to the extent occurring in virulent pneumococcal suspensions augmented with supplement-1. It is possible that a significant turnover could have been demonstrated if DNA metabolism were accelerated by the presence of a proper supplement.

To investigate the mechanism of action of supplement-1 in the metabolism of S pneumococci, it was decided first to assay various moieties of supplement-1 for their ability to enhance DNA synthesis selectively in S suspensions. The results (Table 2) showed (i) that the addition of DNA + deoxyribonuclease to mixtures of deoxynucleosides and deoxynucleotides, present in combination or by themselves, stimulated DNA synthesis to a greater extent than such mixtures lacking the DNA digest; (ii) that mixtures of deoxynucleosides and deoxynucleotides (with or without the DNA digest) enhanced DNA synthesis more than either mixture alone; (iii) that deoxynucleoside mixtures exerted greater effects on DNA synthesis than mixtures of deoxynucleotides (with or without the DNA digest); and (iv) that all of the partial supplements enhanced DNA synthesis to a greater extent than that observed in unsupplemented

TABLE 2. *Effects of various parts of supplement-1 on DNA synthesis by virulent type I pneumococci*

Additions			Percentage increase over zero time after 70 min		Net increase of DNA over protein
DNA + deoxyribonuclease	Deoxynucleosides	Deoxynucleotides	DNA	Protein	
			%	%	%
+	+	+	65	19	46
+	+	−	48	17	31
+	−	+	39	15	24
+	−	−	23	15	8
−	+	+	45	16	29
−	+	−	34	15	19
−	−	+	23	14	9
−	−	−	21	15	6

Inoculum: SVI (type I, virulent). For concentrations of parts of supplement-1, glucose, and casitone, *see* Table 1. When either the deoxynucleosides or deoxynucleotides were used separately, their concentrations were doubled to account for the loss in concentration resulting from the omission of the other mixture. The DNA digest was not altered because its concentration represented only 8% of the entire supplement.

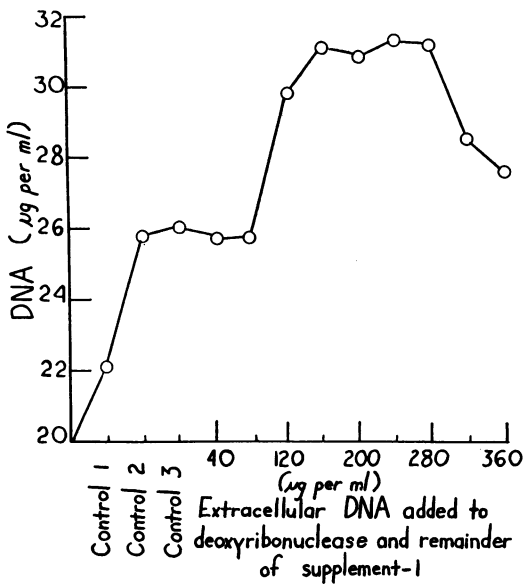

FIG. 6. *Effect of varying the concentration of extracellular DNA on levels of intracellular DNA in type I S cells in the presence of deoxyribonuclease and the remainder of supplement-1 (mixtures of deoxynucleosides and deoxynucleotides). Inoculum: SVI (S). For concentration of supplement-1 constituents, glucose, and casitone, see Table 1. Control 1 is unsupplemented, control 2 contains everything except for DNA, control 3 contains everything except deoxyribonuclease (the DNA concentration in control 3 was 160 μg per ml). Incubation time was 70 min. Concentration of intracellular DNA at zero time in the suspension was 20 μg per ml.*

suspensions. A comparison of these results with those obtained previously in growing cultures (Firshein and Braun, 1960) showed that the intracellular increases in DNA brought about by the partial supplements closely paralleled their effects in stimulating the multiplication of S cells.

The activity of the DNA digest was of particular interest for the following reasons. First, when the digest was added to mixtures of deoxynucleosides and deoxynucleotides, a 40% increase in DNA synthesis occurred over that elicited by the mixtures. However, in the absence of these deoxynucleosides and deoxynucleotides, the digest stimulated DNA synthesis only slightly above control levels without any DNA products (9%). Second, although the addition of digested DNA to mixtures of deoxynucleosides and deoxynucleotides raised the total DNA-product concentration only 8% (from 1,600 to 1,730 μg per ml), the enhancement of DNA synthesis was greater than could be accounted for by the addition of an equivalent amount of these aforementioned mixtures. An 8% increase in the total amount of deoxynucleosides and deoxynucleotides present should have elicited a negligible stimulation of DNA synthesis (3%) in lieu of the 40% actually observed when the digested DNA was added. One possible interpretation of these results was that the digest was controlling the extent to which these mixtures stimulated DNA synthesis, instead of acting merely as an additional source of DNA precursors. If this hypothe-

TABLE 3. *Effects of 2 fractions derived from DNA + deoxyribonuclease by column chromatography on DNA synthesis by virulent type I pneumococci in the presence and absence of deoxynucleosides and deoxynucleotides*

Concn of fraction (per ml of deoxypentose)		Percentage increase over zero time after 70 min		Net increase of DNA over protein	DNA breakdown supplement
I	II	DNA	Protein		
μg	μg	%	%	%	
186		68	18	50	Mixtures of deoxynucleosides and deoxynucleotides
93		65	17	48	
93*	33*	51	14	37	
	66	18	11	7	
	33	35	13	22	
		40	14	26	
186		33	13	20	None
	66	16	12	4	
		19	13	6	
		57	15	42	Supplement-1

Inoculum: SVI. For concentrations of supplement-1, parts thereof, glucose, and casitone, *see* Table 1. By comparing ultraviolet absorption spectra from Fig. 7 of the 2 fractions with that of the entire enzymatic DNA digest at 260 mμ, it was possible to calculate the proportion of the fractions in the digest on a percentage basis. Deoxypentose analyses (Brody, 1953) were then performed on both fractions and on the enzymatic DNA digest. After lyophilization of the fractions, they were reconstituted separately with distilled water to volumes which would produce the desired proportion on a μg per ml basis.

* Actual proportions of 2 fractions in DNA + deoxyribonuclease.

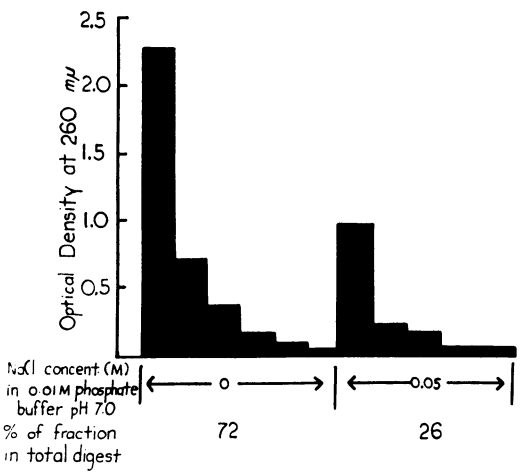

FIG. 7. *Fractionation pattern of DNA + deoxyribonuclease on "ECTEOLA" cellulose. Ten milligrams of calf thymus DNA (Na-salt) in 5 ml of distilled water were depolymerized with 2.5 ml of a solution containing 500 μg per ml of deoxyribonuclease in the presence of 0.03 M $MgSO_4 \cdot 7H_2O$ for 2 hr at room temperature. This digest was placed on a column of "ECTEOLA" cellulose (Bendich et al., 1955). The flow rate was about 12 ml per hr. The percentage of each fraction in the total digest was determined by comparing the ultraviolet absorption of the entire DNA digest at 260 mμ before fractionation with the absorptions of the fractions.*

sis were correct, a critical amount of digested DNA might suffice to stimulate the reaction, and the amount of intracellular DNA synthesized might be independent of the amount of digested DNA added, at least over a certain range. Accordingly, a number of modified supplement-1 solutions were prepared containing varying concentrations of digested DNA. They were added to S suspensions together with the controls lacking either DNA, deoxyribonuclease, or both. The results, averaged from three independent experiments, are summarized in Fig. 6. It was obvious that the levels of intracellular DNA synthesized were not proportional to the amounts of digested DNA added. The type of enhancement obtained suggested a threshold effect in that concentrations of digested DNA below 80 μg per ml produced no enhancing effects above those elicited by mixtures of deoxynucleosides and deoxynucleotides, whereas concentrations of digested DNA from 120 to 280 μg per ml yielded approximately equivalent responses. The inability of high concentrations of digested DNA to enhance the effects of deoxynucleosides and deoxynucleotides can be explained either by the fact that (i) the active component in the digest was toxic at high levels, or (ii) another factor was present which antagonized the selective stimulation of DNA synthesis, but only exerted its effects at high levels. Subsequent results demonstrated that the latter possibility was correct (Table 3). Using the method of Bendich et al. (1955), the digest was fractionated by ion exchange chromatography (Fig. 7) and the two major fractions obtained tested for DNA synthesis-enhancing activity in S suspensions augmented with deoxynucleosides and deoxynucleotides. It was evident that the first fraction elicited the maximal response in stimulating DNA synthesis. This enhancement was greater than that elicited by the entire DNA digest, even though the products in this fraction did not represent all of the products eluted from the column (only 70%). In contrast to the effects of the first fraction, the second fraction depressed DNA synthesis from those levels obtained with the first fraction, as well as those levels obtained with the deoxynucleosides and deoxynucleotides. It was interesting to note that whereas the antagonist exerted its effects in proportion to its concentration, the active fraction behaved in an

TABLE 4. *Comparison of the ability of virulent type I pneumococci to incorporate H^3-deoxycytidine and H^3-deoxycytidylic acid when added as part of supplement-1*

Isotope added to supplement-1*		DNA†	Protein†	Specific activity
Deoxycytidine	Deoxycytidylic acid	μg/ml	μg/ml	*counts:min:mg DNA*
+	−	39	450	35,353
−	+	37	445	20,001
−	−	37	448	
At zero time..		21	390	

Inoculum: SVI. For concentration of supplement-1, glucose, and casitone, *see* Table 1.

* The final concentration of each isotope in the suspension was 2 μc. The ratio of the radioactive isotope to the nonradioactive carrier was 1:1,000.

† After 70 min.

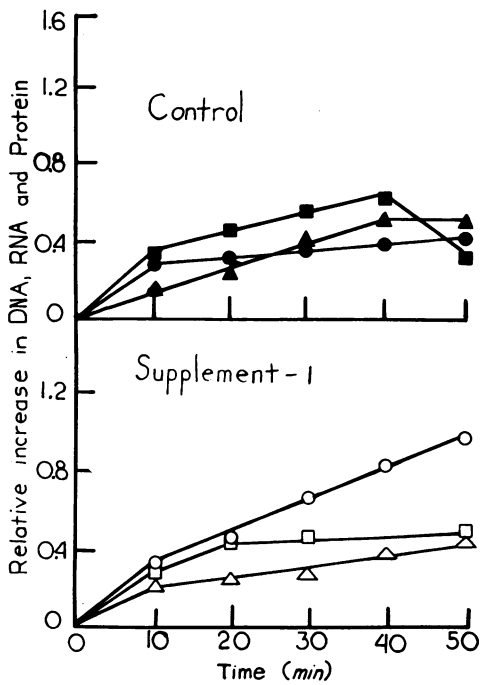

FIG. 8. *Effect of supplement-1 on DNA, RNA, and protein syntheses of type I S cells. Supplement-1, DNA* (○), *RNA* (□), *protein* (△); *control, DNA* (●), *RNA* (■), *protein* (▲) *Inoculum: SVI* (*S*). *For concentration of supplement-1, glucose, and casitone, see Table 1. The term "relative increase" on the graph connotes the increase of DNA, RNA, and protein from zero time.*

opposite manner, similar to that of the entire DNA digest (*see* Fig. 6). From this experiment, it was not possible to ascertain whether the enhancement of DNA synthesis brought about by the active fraction, or the inhibition of the second fraction, was due to one or more components, since both fractions are composed of complex mixtures of nucleotides (Sinsheimer, 1954).

The ability of S cells to incorporate H^3-deoxycytidine into DNA more efficiently than H^3-deoxycytidylic acid apparently accounts for the fact that deoxynucleoside mixtures are more effective in stimulating DNA synthesis than mixtures of deoxynucleotides (Table 4). However, it does not explain why both mixtures in combination elicit greater effects than either by themselves even though the total concentrations are identical. Results to be described in a subsequent report suggest that the answer to this problem lies in the ability of deoxynucleotides to enhance glucose oxidation in S cells (Firshein, 1961).

TABLE 5. *Effects of supplement-1 on DNA, RNA, and protein syntheses of virulent type I pneumococci in the presence and absence of glucose and casitone*

Addition	Glucose	Casitone	Percentage increase over zero time after 70 min			Net increase of DNA over protein
			DNA	RNA	Protein	
			%	%	%	%
Supplement-1	+	+	61	10	14	48
	+	−	25	6	5	20
	−	+	12	5	3	9
	−	−	0	3	2	0
None	+	+	8	3	10	0
	+	−	3	0	2	1
	−	+	0	0	0	0
	−	−	0	0	0	0

Inoculum: SVI. For concentrations of supplement-1, glucose, and casitone, *see* Table 1.

That supplement-1 was specific for DNA synthesis and had no effects on RNA synthesis is shown by the results of Fig. 8.

Efforts to determine whether glucose and casitone (the major components of the suspending medium) affected the activity of supplement-1 showed that both components were necessary for maximal effects. The level of DNA synthesis was reduced considerably in the absence of either one, but more so if glucose were omitted (Table 5). Also, reductions in RNA and protein syntheses were noted in the deficient suspensions.

DISCUSSION

It is important to emphasize at the outset that although some of the differences between controls and augmented suspensions were not great, they were considered significant for the reasons mentioned previously, i.e. repeated trials, and characteristic responses by the various S types examined. In 2 years of experimentation, there have been relatively few instances where significant deviations from the expected results with supplement-1 or parts thereof, were obtained, and these were due to faulty techniques. Secondly, although environmental conditions in the suspending medium and in growing cultures were dissimilar, the results obtained closely paralleled

each other. Most important was the observation that the growth enhancing effects of parts of supplement-1 were proportional to the effects of these same parts on DNA synthesis in S suspensions. Thus, it was plausible to ascribe this growth stimulation (and R → S population changes) to primary effects upon DNA synthesis in S pneumococci.

The metabolic basis for the differential response appears to reside in the ability of S cells to incorporate DNA precursors and factors enhancing their utilization (the active fraction from DNA + deoxyribonuclease) more efficiently than R cells. In addition, the incorporation of these factors by S cells is enhanced considerably by the presence of the specific polysaccharide capsule surrounding the bacterial cells. This connection between incorporation of DNA products and the presence of a capsule may provide an important clue for an understanding of the relationship between virulence and the capsule. It is well known that encapsulated pneumococci resist phagocytosis in vivo (White, 1938; Dubos, 1954) and further, that it is possible to correlate the amount of polysaccharide produced in vitro by various pneumococcal types with the virulence of such types in vivo (Macleod and Krauss, 1950). However, the isolated capsule is nontoxic when injected into susceptible hosts. Thus, it has been postulated that the protection afforded to the cells by the capsule permits extensive proliferation and elaboration of toxins. The greater the amount of capsule, the greater the protection, and the greater the ultimate proliferation. Nevertheless, active metabolism for growth and production of the capsule undoubtedly requires proper environmental conditions in vivo that are found in susceptible hosts, but not in resistant ones. This conclusion is supported by the findings of Hitchings and Falco (1946) and Firshein and Braun (1958). Both studies revealed that virulence of pneumococci in susceptible hosts could be increased by adding substances known to stimulate the multiplication of pneumococci in vitro. In the latter studies, it was demonstrated that mice treated with DNA + deoxyribonuclease succumbed much faster to intraperitoneal infection with virulent pneumococci. Viable counts of peritoneal washings revealed a remarkable 4 log enhancement of multiplication in the treated animals as compared to the untreated ones. It is entirely possible that DNA products occur in significant amounts in vivo during the spreading phase of the disease as a result of cellular disintegrations. The ability of the capsule to enhance incorporation of these compounds, and the resultant effects of this incorporation on the proliferation of S pneumococci, could contribute greatly to the pathological condition.

The possibility that other factors contribute to the differential response by S and R pneumococci to supplement-1 besides cell surface or permeability phenomena is suggested by results obtained with R cells. First, these cells can incorporate H^3-thymidine into intracellular DNA despite the lack of a capsule. Second, they are completely inert to supplement-1 as far as selectively enhanced DNA synthesis is concerned, or as far as significant growth stimulation is concerned (Firshein and Braun, 1960). This latter observation is unusual in view of the fact that R cells are usually more active metabolically than S cells in vitro (Dubos, 1954). A plausible explanation is that R cells do not possess the necessary enzyme systems to act on supplement-1, or they may possess alternate pathways to DNA which do not depend extensively upon the presence of the DNA products used by S cells. These alternative hypotheses are under investigation at present.

The excess DNA synthesized in S pneumococci in the presence of supplement-1 raises the question of its genetic competency and organization. Nuclear segregation experiments patterned after those of Ryan and Wainwright (1954) can indicate the organization, whereas transformation experiments could ascertain whether the excess DNA is genetically competent.

The significance of the DNA turnover in S cells is unknown, but the fact that it occurs to the greatest extent under conditions of maximal DNA synthesis (in supplement-1 suspensions) suggests that this newly formed DNA comprises a substantial part of the total DNA of the cell. In addition, it means that the original DNA is unstable as long as supplement-1 is present.

Certain conclusions can be made regarding the mechanism of action of supplement-1 in S cells. The enzymatic DNA digest contains at least two factors; one that enhances the response of the deoxynucleosides and deoxynucleotides and another which antagonizes these effects. The active factor is of relatively small molecular size and acts only over a finite critical range of

concentration. In contrast, the antagonist is of larger molecular weight and appears to act in proportion to its concentration. Their mechanisms of action and chemical structures are at present speculative. However, the importance of a DNA digest in relation to systems involved in DNA synthesis has been cited in several reports (Butros, 1959; Taliaferro and Jaroslow, 1960). As far as the antagonist is concerned, a literature survey has not revealed the existence of such a component.

Further work will have to be undertaken to assess the role of the mixtures of deoxynucleosides and deoxynucleotides. The most important problem is why both mixtures in combination elicit greater effects than either by themselves. A subsequent report (Firshein, 1961) has demonstrated that deoxynucleotides stimulate glucose oxidation in S cells, whereas deoxynucleosides have no effects. It has been shown in the present studies that the effectiveness of deoxynucleosides in stimulating DNA synthesis is related to the ability of S cells to incorporate these compounds more efficiently than deoxynucleotides. It is possible that when both mixtures are present simultaneously, the excess energy available from the enhancement of glucose oxidation by the deoxynucleotides enhances the incorporation of the deoxynucleosides to a greater extent than if such compounds were absent. The validity of this interpretation can be ascertained by measuring incorporation rates of deoxynucleosides into DNA in the presence and absence of deoxynucleotides.

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