EFFECTS OF ENZYMATIC DEOXYRIBONUCLEIC ACID DIGESTS ON POPULATION CHANGES AND DEOXYRIBONUCLEIC ACID SYNTHESIS OF PNEUMOCOCCI[1]

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Received for publication July 20, 1959

Under most conditions *in vitro*, bacteria belonging to pathogenic species tend to undergo population changes that involve the progressive establishment of avirulent (e. g., R) variants in initially virulent populations (Braun, 1953). In contrast, virulent (e. g., S) cell types attain high selection values while in competition with R type cells *in vivo*, i. e., within susceptible hosts. In the course of recent studies with *Brucella* species, it had been found that this typical direction of population changes, i. e., from R → S, *in vivo*, could be duplicated *in vitro* provided deoxyribonucleic acid (DNA) + deoxyribonuclease were added to the culture medium (Braun and Whallon, 1954; Braun, 1956). It subsequently was demonstrated that this selective effect was independent of the source of the DNA, could not be reproduced by any known DNA breakdown product, and involved a capability of the virulent S cells to convert a DNA product of relatively large molecular size into a toxic factor for avirulent non-S cells (Braun *et al.*, 1957*b*; Braun, 1958). A cursory survey among a few bacterial species, including *Diplococcus pneumoniae*, *Salmonella typhosa*, *Staphylococcus aureus*, and *Vibrio comma*, indicated that the selective effects *in vitro* of DNA + deoxyribonuclease were not restricted to brucellae. Further investigations with pneumococci, however, revealed that the mechanism responsible for the altered selective value of virulent cells in cultures containing DNA + deoxyribonuclease can differ significantly from that previously elucidated in studies with brucella populations. In the case of pneumococcal populations subjected to the effects of DNA + deoxyribonuclease, a selective stimulation of the multiplication of virulent cells, rather than a selective inhibition of the avirulent cells, proved to play a major role (Firshein and Braun, 1958). Details of this selective mechanism and its role in certain phenomena *in vitro* and *in vivo* will be described in the present report.

[1] This work was supported by U. S. Public Health Service grant E-1137 and National Science Foundation grant G-2184.

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MATERIALS AND METHODS

S and R strains of *D. pneumoniae*, belonging to or derived from types I, II, III, V, VII, and XIV were kindly supplied by Dr. R. Austrian. These strains were maintained on, and for the preparation of inocula grown on, brain heart infusion agar medium (Difco) supplemented with 5 per cent sterile defibrinated rabbit blood. The blood supplement was reduced to 1 to 2 per cent in plates used for the detection of smooth and rough colonial characteristics. Liquid brain heart infusion medium, supplemented with 0.25 per cent rabbit blood, was employed in all growth and population studies. Cultures were routinely inoculated with 1×10^7 cells per ml, obtained from 18- to 20-hr-old agar slant cultures, and incubated at 37 C, usually for 48 hr. Inocula were adjusted turbimetrically, and in the case of mixed inocula (99.9 per cent R + 0.1 per cent S), appropriate dilutions of R and S cell suspensions were mixed before the addition of 0.2 ml of the cell suspension in saline to 3.0 ml of medium per test tube. Stock media were prepared in double strength, and for use were diluted 1:1 with distilled H_2O or with supplements dissolved in H_2O. The extent and rate of growth were determined by conventional viable counts (serial dilutions of samples and plating on brain heart infusion-blood agar). In the case of rough cells, where clumping and chain formation tend to complicate counting of minimal reproductive units (Lerman and Tolmach, 1957), control comparisons were made microscopically to assure that changes in viable counts following certain al-

terations in environmental conditions were not due to an altered degree of clumping or chain formation. Population changes were determined as described for studies with brucellae (Braun, 1946). This involved principally the streaking on agar media of aliquots from at least duplicate liquid cultures, and subsequent determination, by checking at least 100 colonies per plate, of the percentage of different colonial types. Appropriate preliminary tests on replicate cultures established the statistical significance of this procedure as applied to the estimation of pneumococcal population changes. Since repeated sampling of one culture tended to alter the rate and extent of population changes, presumably due to the resulting alterations in oxygen tension, separate sets of duplicate cultures were prepared for each sampling period in cases where readings were made repeatedly over a period of time.

DNA was extracted and purified from *Brucella abortus* strain 19-M, by the procedure of Braun *et al.* (1957*a*), from pneumococci by a method described by Hotchkiss (1957), and from calf thymus by the procedure of Kay *et al.* (1952). All DNA preparations were stored as a precipitate in 95 per cent ethanol at 5 C and dissolved in saline for use. Pancreatic deoxyribonuclease (DNAase, Worthington) was prepared by dissolving the 1× crystallized enzyme in 0.03 M $MgSO_4 \cdot 7H_2O$. In routine use, 450 μg DNA, as determined by the Stumpf (1947) modification of the Dische reaction, and 100 μg deoxyribonuclease were added to each culture (final culture volume, 3 ml). Before addition to the concentrated culture medium, DNA and deoxyribonuclease usually were mixed, allowed to stand for 1 hr at room temperature, and sterilized by filtration through sintered glass. All of the commercially prepared nucleic acid breakdown products employed in this study were similarly sterilized by filtration through sintered glass.

For studies with resting cells, the bacteria were grown in a casein-tryptone-yeast extract medium (CAT medium, Marmur and Hotchkiss, 1955), washed twice and suspended in 0.02 M phosphate buffer, pH 7.5, supplemented with 0.1 per cent glucose, 1 per cent enzymatic digest of casein (N.Z.case), and catalase (25 μg per ml). As a rule, the suspending medium was prepared in double strength, and before final dilution supplemented with the various DNA breakdown products to be tested. Deoxyribonuclease was allowed to act on DNA for 2 hr before addition to the suspensions. DNA determinations on these resting cell suspensions, shaken at 37 C for various periods of time, were made by hot trichloroacetic acid extractions of acid precipitates (Morse and Carter, 1949) which had been defatted by alcohol-ether mixtures. The level of acid-insoluble deoxypentose in these extracts was then determined colorimetrically (Stumpf, 1947).

RESULTS

Initial tests with inocula of R cells derived from types I, II, III, V, VII, and XIV confirmed that even after prolonged incubation (up to 5 days) in either brain heart infusion, casein-tryptone-yeast extract, or neopeptone broth (Austrian, 1953), few (in the case of type V-derived R cultures) or no population changes, involving the establishment of S mutants, took place. This population stability may be regarded as a reflection of extremely low spontaneous mutation rates from R → S in most of these strains, a fact that has made such populations particularly good material for the study of transformation reactions. Even when a few S cells (<0.1 per cent), of the type specificity corresponding to the progenitor of the R population, were added with the inoculum, either no population changes to S (types I, II, and VII) or only a limited selective establishment of S cells occurred (types III and XIV); examples demonstrating the fate of such populations in brain heart infusion broth are shown in table 1. However, after the addition of DNA + deoxyribonuclease to such cultures, rapid population changes involving the establishment of S type cells were observed with all of the R cultures to which a few S cells had been added initially (see examples in table 2). The requirement for the addition of a few S cells to initiate R → S population changes in the initially predominantly R cultures is believed to be due to the already mentioned low spontaneous rates of mutation from R → S, which may cause a lack of "raw material" for selection in the course of the relatively limited period of growth in broth cultures of pneumococci. However, despite the need for mixed inocula to initiate R → S population changes in the presence of

TABLE 1

Population changes in aging broth cultures initiated with either homogeneous or mixed inocula of several pneumococcal strains

Pneumococcal Strains	Inoculum	Percentage of Smooth in Brain Heart Infusion-Blood Broth*			
		0 hr	24 hr	48 hr	5 days
S-VI	100% S	100	100	100	NG
R-I192	100% R	0	0	0	0
S-VI + R-I192	0.1% S + 99.9% R	0.1	0	0	0
	99% S + 1% R	99	40	0.1	0.1
S-D39	100% S	100	98	85	NG
R-36A	100% R	0	0	0	0
S-D39 + R-36A	0.1% S + 99.9% R	0.1	0.1	0	0
	99% S + 1% R	99	10	0	0
S-A66	100% S	100	100	100	100
R-A66	100% R	0	0	0	0
S-A66 + R-A66	0.1% S + 99.9% R	0.1	8	15	20
	99% S + 1% R	99	85	89	70
S-VII	100% S	100	50	0	0
R-VII	100% R	0	0	0	0
S-VII + R-VII	1% S + 99% R	1	0	0	0
	99% S + 1% R	99	0	0	0

* Averages from duplicate samples. NG = no growth.

DNA + deoxyribonuclease, several reasons suggest that the results may be regarded as reflecting natural trends. First, a rough culture (R-V 497) was found that had sufficiently high mutation rates to permit population changes to S to occur in initially 100 per cent R, DNA + deoxyribonuclease-supplemented cultures (table 2); also, in subsequent tests with other experimental culture modifications this R culture displayed responses similar to those occurring in initially R + S cultures. Moreover, subsequent findings have revealed that the effects of DNA + deoxyribonuclease involve primarily modified growth responses of S cells, thus minimizing the importance of the origin of R cells, and with it any possible criticism of a misleading artificial situation that might result from the use of mixed inocula containing, in some cases, only distantly related S and R cells.

Using cultures with mixed inocula as convenient model systems, it was established that supplementation of cultures with either DNA or deoxyribonuclease alone failed to promote R → S population changes whereas DNA + deoxyribonuclease did support such changes. The rate of changes occurring in the presence of DNA + deoxyribonuclease, as well as the amount of supplementation required, varied with the strains used (table 2).

As illustrated by the example in table 3, the DNA + deoxyribonuclease effect was essentially unaffected by the source of the DNA and the medium employed. Subsequent tests showed that DNA freshly isolated from calf thymus by either the 0.5 per cent phenol (Braun *et al.*, 1957*a*) or the duponol (Kay *et al.*, 1952) extraction procedures yielded equally effective starting materials. Within the range tested, the effects proved independent of the initial percentage of S cells (ranging from 0.1 to 16), the inoculum size (1×10^6 to 1×10^9), and the concentration of blood (0.5 to 2.0 per cent) in the medium.

These data demonstrated that similarly to the previous observations (Braun and Whallon, 1954; Braun, 1956) with *B. abortus*, supplementation of growth media *in vitro* with enzymatic digests of DNA produced a significant selective advantage for virulent pneumococci in initially predominantly avirulent populations. That this

TABLE 2

Effect of deoxyribonucleic acid (DNA) + deoxyribonuclease upon population changes of various pneumococcal strains in aging broth cultures

Conc of Supplement in Brain Heart Infusion-Blood Broth (μg/ml)		Inoculum	Percentage of Smooth in Brain Heart Infusion-Blood Broth*	
DNA†	Deoxyribonuclease		24 hr	48 hr
130	25	S-VI (1%) + R-I192 (99%) (both of type I origin)	100	100
65	13		100	100
130	—		6	8
—	25		1	0
—	—		0	0
130	25	S-VI (1%) + R-36A (99%) (S = type I; R originated from type II)	2	75
65	13		1	26
130	—		1	2
—	25		0	1
—	—		1	1
130	25	S-A66 (1%) + R-A66 (99%) (both of type III origin)	4	38
65	13		10	95
32	6		6	49
65	—		8	10
—	13		5	7
—	—		4	20
130	25	R-V497 (100%) (originated from type V)	16	80
65	13		4	20
130	—		1	3
—	25		1	4
—	—		1	4

* Averages from duplicate samples.

† From *Brucella abortus*.

result, as in the case of *B. abortus*, was due to an alteration of selective effects rather than any process involving transformation reactions, was supported by the data shown in table 4. Using DNA derived from streptomycin-sensitive, type II, smooth pneumococci, added in conjunction with deoxyribonuclease to cultures initiated with 99 per cent streptomycin-resistant R cells + 1 per cent streptomycin-sensitive S cells, population changes were obtained that involved the gradual establishment of the initially present streptomycin-sensitive S cell type (rather than the resistant S type derivable by single-step transformation from the resistant R cells).

At this point, analysis of the growth dynamics of the R and S cells in the initially

TABLE 3

Effects of the source of deoxyribonucleic acid (DNA) upon population changes of pneumococci in two different media. Inoculum: S-VI (1 per cent) + R-36A (99 per cent)

Medium	DNA Source*			Percentage of Smooth after 48 hr†
	Brucella	Pneumococcus	Thymus	
Brain heart infusion-blood broth	+	−	−	75
	−	+	−	82
	−	−	+	85
	−	−	−	1
Casein-tryptone-yeast extract medium	+	−	−	98
	−	+	−	100
	−	−	+	100
	−	−	−	15

* DNA: Brucella = 0.5 per cent phenol-extracted; thymus = duponol-extracted; pneumococcus = deoxycholate-extracted.

† Averages from duplicate samples.

TABLE 4

Population changes in the presence and absence of deoxyribonucleic acid (DNA) + deoxyribonuclease as detected with the aid of streptomycin-containing agar and "labeled" (streptomycin-resistant) rough cells. Inoculum: S-VI (1 per cent) + R-I192/sr (99 per cent)

Additions to Brain Heart Infusion-Blood Broth	Percentage of Smooth after 48 hr*	
	Brain heart infusion-blood agar plates + 150 μg/ml streptomycin	Plain brain heart infusion-blood agar plates
None	0	0
Brucella DNA (130 μg/ml) + deoxyribonuclease (25 μg/ml)	0	98
Pneumococcus DNA (130 μg/ml) + deoxyribonuclease (25 μg/ml)	0	98

* Averages from duplicate samples.

99.9 per cent R cultures revealed that, in contrast to the brucella studies, where supplementation with DNA + deoxyribonuclease had favored S cells through selective inhibition of non-S cells, R → S population changes in similarly supplemented cultures of pneumococci in-

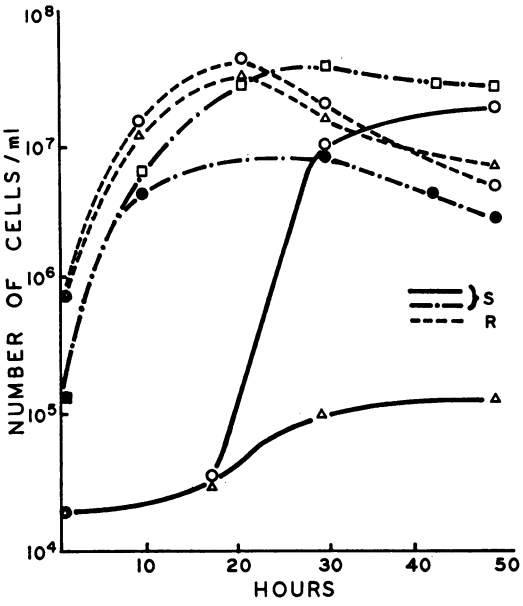

Figure 1. Viable counts on initially 99.999 per cent R + 0.001 per cent S cultures, and on 100 per cent S (-•-) cultures, in the presence (□——□, ○——○) and absence (●——●, △——△) of DNA + deoxyribonuclease. Inoculum: R-36A + S-VI, or S-VI alone.

TABLE 5

Effects of kinetin upon population changes of initially predominantly rough pneumococcal populations in the presence and absence of deoxyribonucleic acid (DNA) + deoxyribonuclease. Inoculum: S-A66 (1 per cent) + R-36NC (99 per cent)

DNA (130 μg/ml) Deoxyribonuclease (25 μg/ml)	Kinetin (μg/ml)	Percentage Smooth after 48 hr in Brain Heart Infusion-Blood Broth*
−	10	2
	3	7
	1	20
	0.3	27
	0.1	40
+	10	45
	3	50
	1	48
	0.3	48
	0.1	49
	—	51
−	—	4

* Averages from duplicate samples.

TABLE 6

Effects of various nucleic acid analogues on population changes of pneumococci in the presence and absence of deoxyribonucleic acid (DNA) + deoxyribonuclease. Inoculum: S-VI (1 per cent) + R-36A (99 per cent)

Analogue	Concentrations Tested (μg/ml)	DNA (130 μg/ml) + Deoxyribonuclease (25 μg/ml)	Percentage of Smooth after 48 hr in Brain Heart Infusion-Blood Broth*
5-Bromouracil	50 or 100	+	90
		−	2
6-Thiouracil	30 or 60	+	83
		−	1
Uracil disulfide	25 or 50	+	85
		−	5
8-Azaguanine	50 or 100	+	96
		−	0
6-Mercaptopurine	50 or 100	+	81
		−	0
5-Bromodeoxyuridine	25 or 50	*	90
		−	0
Puromycin	10 or 25	+	87
		−	2
Caffeine	100 or 200	+	85
		−	1
−	−	+	81
−	−	−	1

* Averages from duplicate samples.

volved a selective stimulation of the growth of S cells without any apparent effects on R cells (figure 1). We shall return to a more detailed consideration of these growth dynamics subsequently.

As in the case of brucellae, the active factor derived from enzymatic digests of DNA was not replaceable either by single or combined supplementations of media with any of the deoxynucleosides, deoxynucleotides, purines, or pyrimidines, or by adenosine tri- and di-phosphate, diphosphopyridine nucleotide, or acid-hydrolyzed DNA, when employed in concentrations ranging from 0.1 to 10 μg per ml. However, high concentrations (1600 μg per ml) of adenine and deoxyadenosine yielded significant selective effects in favor of S cells in initially predominantly R populations of pneumococci

derived from type I. As in brucella (Braun, 1956), the only known nucleic acid derivative that in low concentrations produced any significant selective activity resembling that of the DNA digest factor proved to be 6-furfurylamino purine (kinetin). Table 5 illustrates that kinetin, as in other systems where it has displayed biological activity (Strong, 1958; Obrecht, 1958), was increasingly active with decreasing concentration. However, it is unlikely that the active factor in enzymatic DNA digests is kinetin, since the activity of this compound showed great variability depending upon the strain and environmental conditions employed, i. e., under conditions where DNA + deoxyribonuclease effects remained constant.

A series of nucleic acid analogues tested (table 6) neither produced DNA + deoxyribonuclease-type effects nor antagonized such effects.

Fractionation of enzymatic DNA digests by Ecteola cellulose (Bendich *et al.*, 1955) yielded four fractions (figure 2), of which only the fraction coming off the column first, i. e., the small molecule fraction, displayed selective activity. A similarly active fraction was obtained when the period of DNA digestion with deoxyribonuclease prior to fractionation was prolonged from 5 min to 30 min, whereupon the active first fraction eluted from the column represented 99 per cent of the total digest.

The activity just described of the DNA digest fractions was tested in brain heart infusion cultures supplemented with 54 μg Mn^{++} per ml. This Mn^{++} supplementation became a standard procedure when it was recognized that the presence of this ion, but not of Mg^{++}, Zn^{++}, Cu^{++}, Fe^{++}, Co^{++}, or Pb^{++}, significantly enhanced the selective effects of DNA + deoxyribonuclease. Media employed prior to these tests had been supplemented unwittingly with an excess of Mn^{++} required for optimal DNA + deoxyribonucleic acid effects. Following the recognition of the supplemental role of Mn^{++} (figure 3), it became possible to adjust environmental conditions in a manner permitting control over the extent of these effects, which in turn permitted the recognition of other fractions enhancing DNA + deoxyribonuclease activity.

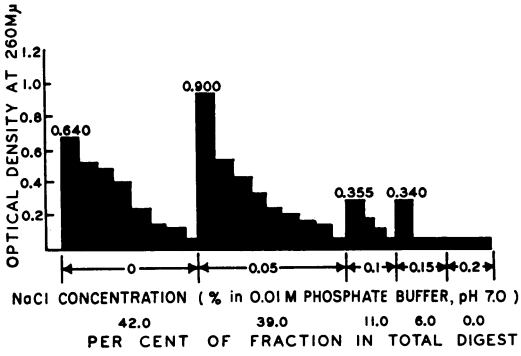

Figure 2. Fractionation pattern of enzymatic DNA digests on Ecteola cellulose: 6.7 mg of thymus DNA + 1.5 mg doxyribonuclease hydrolyzed for 1 min at room temperature. Solution placed on column of 1.0 g Ecteola cellulose. Flow rate, 5 ml per hr. Discontinuous concentration change.

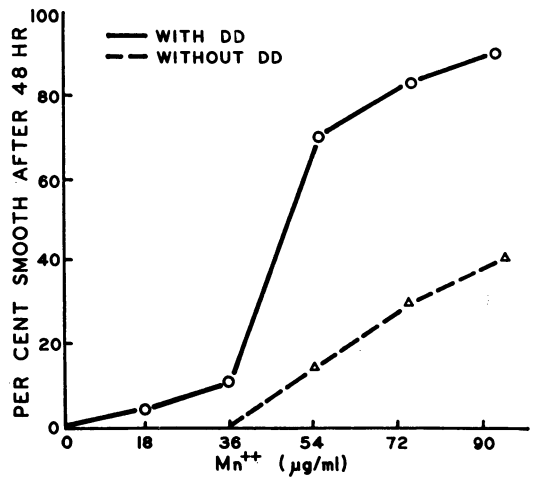

Figure 3. Effects of various concentrations of manganese in brain heart infusion broth on population changes of Type I pneumococci in the presence and absence of DNA + deoxyribonuclease (*DD*). Inoculum: 99 per cent R-I192 + 1 per cent S-VI.

In a search for agents, other than Mn^{++}, capable of enhancing the selective effects of enzymatic DNA digests, mixtures of deoxynucleosides, mixtures of deoxynucleotides, and mixtures of nucleoside diphosphates were added to predominantly R cultures of pneumococci in Mn^{++}-deficient brain heart infusion media. As shown in figure 4, DNA + deoxyribonuclease in combination with deoxynucleosides or deoxynucleotides enhanced R $\longrightarrow$ S population changes

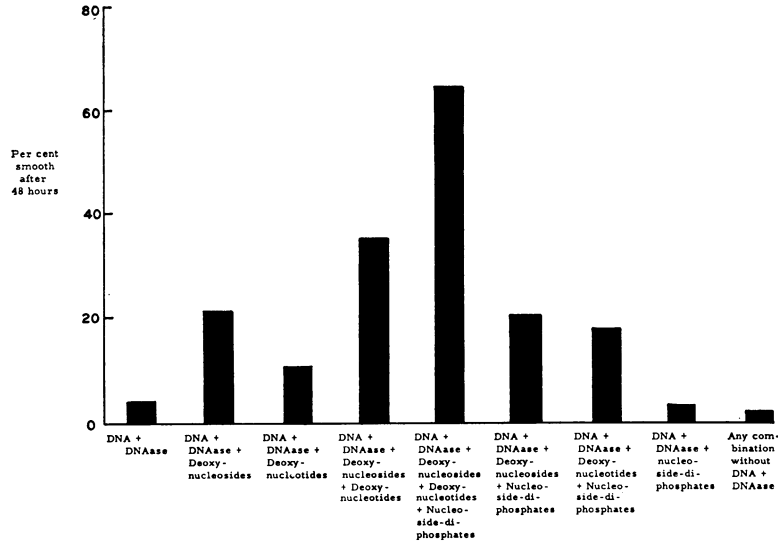

Figure 4. Effects of mixtures of deoxynucleosides, deoxynucleotides, and nucleoside diphosphates on R → S population changes of type I-derived pneumococci. Amount of supplementations (μg per ml): DNA = 130; deoxyribonuclease (*DNAase*) = 25; deoxynucleoside mixture = 800 (200 each of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine); deoxynucleotide mixture = 800 (200 each of the phosphorylated deoxynucleosides named above); nucleoside diphosphate mixture = 100 (25 each of the diphosphates of adenosine, guanosine, cytidine, and uridine). When the "deoxynucleotide mixture" or "deoxynucleoside mixture" were used separately with DNA + deoxyribonuclease, their concentration was raised to 1600 μg per ml.

in comparison to those occurring in cultures containing only DNA + deoxyribonuclease. When both nucleosides and nucleotides were added in conjunction with DNA + deoxyribonuclease at a total concentration equaling those employed for either nucleosides or nucleotides alone, an even stronger selective advantage in favor of S cells occurred. So far, optimal selective effects have been obtained when cultures were supplemented with DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates, the total supplementation employed again approximating those used for supplementation with either deoxynucleosides or deoxynucleotides alone. It should be noted that nucleoside diphosphates produced an effect only when added in conjunction with deoxynucleosides and deoxynucleotides; under these conditions supplementation with nucleotide mixtures or mixtures of nucleoside triphosphates failed to duplicate the nucleoside diphosphate effects. In addition, the effects of enzymatic ribonucleic acid (RNA) digests, mixtures of nucleosides, and nucleotides in cultures containing DNA + deoxyribonuclease were tested and found to be nil. Also, the omission of any one of the deoxynucleosides from the nucleoside mixture in DNA + deoxyribonuclease-supplemented cultures significantly reduced or abolished the deoxynucleoside effect, suggesting a requirement for all four bases for optimal enhancement. This suggestion is further supported by the observation that elevation of single deoxynucleosides or deoxynucleotides to concentrations equaling those of the deoxynucleoside or the deoxynucleotide mixtures failed to produce enhancing effects or produced effects that were significantly below those produced by the deoxynucleoside or the deoxynucleotide mixtures (the only relatively effective compounds being thymidylic acid, deoxyadenosine, and deoxyguanosine). Omission of DNA + deoxyribonuclease, regardless of the presence of the other supplements (except for high concentrations of deoxyadenosine), resulted in a virtual abolition of all effects. Since the breakdown products resulting from deoxyribonuclease treatment of DNA represent only 6 per cent of the total supplementation in the case of deoxynucleoside- or deoxynucleotide-enriched cultures,

it can be assumed that the active digest factor represents an as yet unrecognized component or that it is not identical with compounds present in the nucleoside or nucleotide mixtures. Finally, it is noteworthy that in the presence of the "optimal supplementation system" (DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates) the requirement for deoxyribonuclease disappears and optimal selective effects can be obtained by adding DNA alone to deoxynucleosides + deoxynucleotides + nucleoside diphosphates.

Having observed that the selective effects of DNA + deoxyribonuclease can be enhanced by Mn^{++} and by the addition of deoxynucleosides, deoxynucleotides, and nucleoside diphosphates, a survey was made regarding the effects of these supplementations on cultures initiated with predominantly R populations of different strains of pneumococci. Both population changes and the associated growth dynamics of S and R cells in these cultures were checked. With one exception, all initially predominantly R cultures tested (R derived from types I, II, VII, and XIV), containing a few S cells of the corresponding progenitor type, underwent far more extensive R to S population changes in the presence of DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates than in cultures containing DNA + deoxyribonuclease alone. The exception was in the case of cultures initiated with type III "derivatives," i.e., 99 per cent R-A66 + 1 per cent S-A66, where the deoxynucleosides + deoxynucleotides + nucleoside diphosphate supplementation actually inhibited R → S population changes in comparison to those occurring in cultures containing DNA + deoxyribonuclease only; this effect might involve an unusual susceptibility of type III cells to high concentrations of the DNA breakdown products. In the presence of DNA + deoxyribonuclease, the addition of Mn^{++} (54 or 72 μg per ml) further enhanced R → S population changes in cultures initiated with types I, II, III, and VII "derivatives," but not with type XIV. In the case of cultures started with types III, VII, and XIV "derivatives," Mn^{++} addition alone, in the absence of DNA + deoxyribonuclease, yielded significant R → S population changes, an effect that did not occur with the other types tested. It should be mentioned at this point that subsequent metabolic studies on Mn^{++} effects, such as those here observed with types III and XIV, have shown that these involve stimulatory effects of a different nature than those mediated by the nucleic acid breakdown products (see Discussion). But despite such differences in the mode of action of Mn^{++} and DNA + deoxyribonuclease, the differences in response of different strains of pneumococci to the various supplementations demonstrate that it would be dangerous to draw any generalized conclusions regarding modifications of populations shifts on

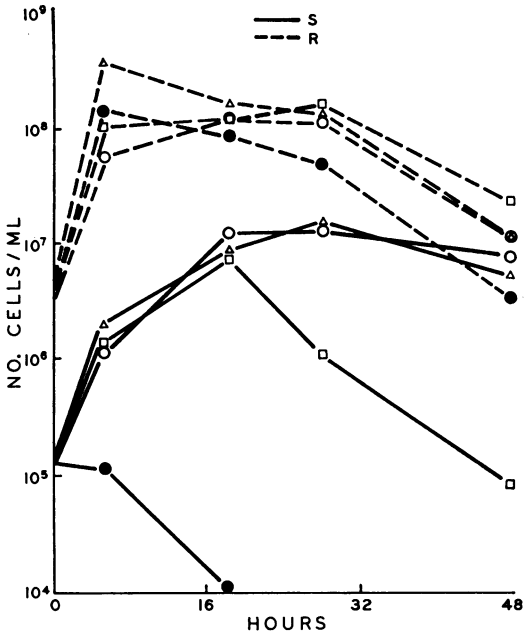

Figure 5. Viable counts of S and R cells in cultures of pneumococci undergoing population changes in the presence and absence of various combinations of DNA + deoxyribonuclease, manganese, nucleotides, and nucleosides in brain heart infusion broth. Inoculum: 1 per cent S-VI + 99 per cent R-I192. ○ = DNA + deoxyribonuclease + Mn; △ = DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates; □ = Mn; ● = control. Amount of supplementations (μg per ml): DNA = 130; deoxyribonuclease = 25; deoxynucleoside mixture = 800 (200 each of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine); deoxynucleotide mixture = 800 (200 each of the phosphorylated deoxynucleosides named above); nucleoside diphosphate mixture - 100 (25 each of the diphosphates of adenosine, guanosine, cytidine, and uridine); Mn^{++} = 55.

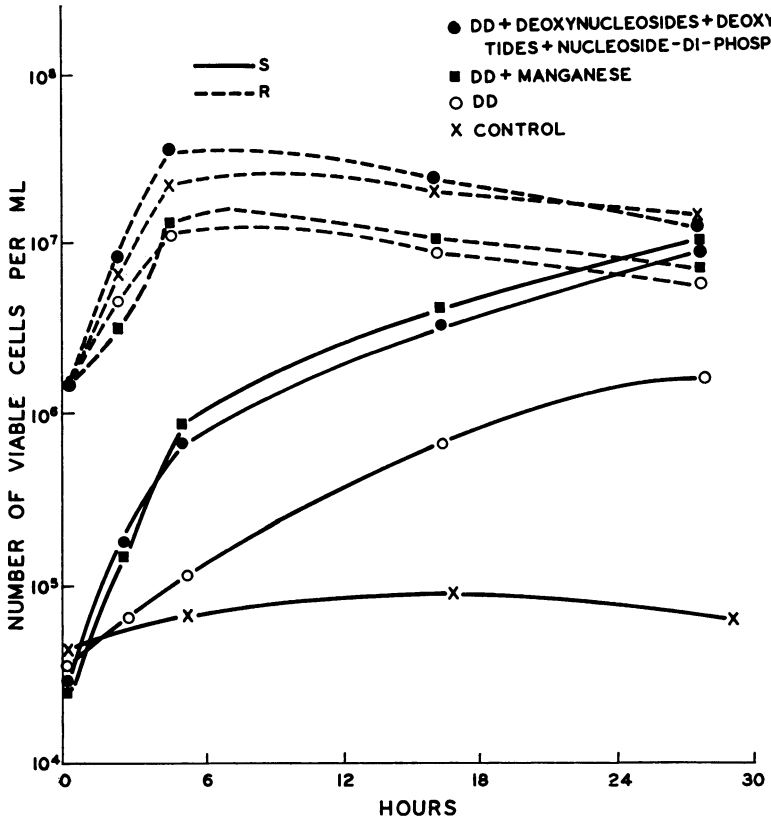

Figure 6. Viable counts of S and R cells in cultures of pneumococci undergoing population changes in the presence and absence of various combinations of DNA + deoxyribonuclease (*DD*), manganese, deoxynucleotides, and deoxynucleosides in casein-tryptone-yeast extract medium. Inoculum: 1 per cent S-VI + 99 per cent R-I192. For amount of supplementations, see figure 5.

the basis of data obtained with derivatives from only one type-specific strain.

There was, however, one common phenomenon in all of these population tests with DNA breakdown products and Mn^{++}, which was revealed when actual viable counts of R and S cells in these cultures were performed: a stimulation of the rate and extent of multiplication of S cells, without any growth-enhancing effects on R cells, occurred in all cases. Typcial examples of such effects upon growth of S cells, either when they were in mixtures with R cells or by themselves are shown in figures 5 to 9. As in the previous observations on DNA + deoxyribonuclease-stimulated R → S population changes in cultures initiated with different R and S strains, strain differences in the growth response of S cells to this stimulation became evident, and in many instances the greatest stimulation of growth occurred with those S strains that also had exhibited greatest DNA + deoxyribonuclease-promoted selection values in the tests on population changes. However, this relationship did not hold true in all instances because population changes did not only depend on the growth rates of S cells but also on the comparative growth rate of R cells and the rate of lysis of R and S cells. In addition to the effects of DNA + deoxyribonuclease on rate and extent of multiplication of S cells, this supplementation also produced a delay in autolysis of S cells in certain strains (figures 5 and 7); similar delays in autolysis did not occur in Mn^{++}-stimulated cultures. Figures 5 and 6 demonstrate that the DNA + deoxyribonuclease effects on growth of S cells were essentially similar in two different media.

In view of the striking stimulation of the growth of S cells in the presence of DNA + deoxyribonuclease, particularly in the presence

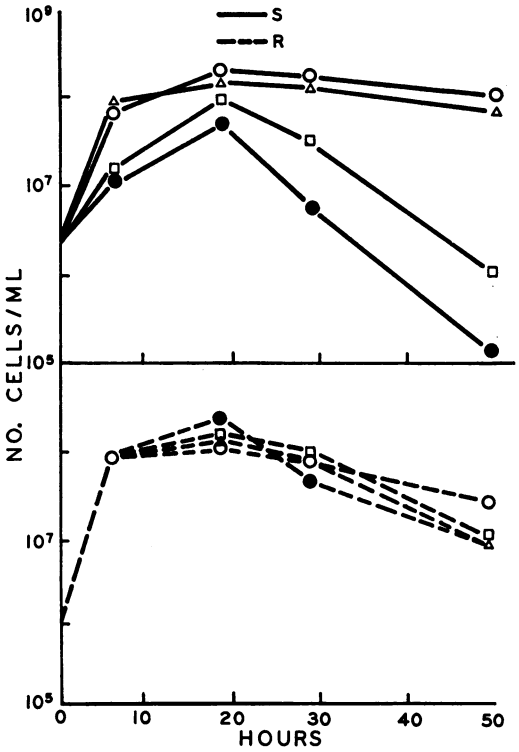

Figure 7. Viable counts of initially homogeneous S or R cultures in the presence and absence of various combinations of DNA + deoxyribonuclease, manganese, deoxynucleosides, and deoxynucleotides in brain heart infusion broth. Inoculum: 100 per cent S-VI or 100 per cent R-I192 (derived from type I). ○ = DNA + deoxyribonuclease + Mn; △ = DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates; □ = Mn; ● = control. For amount of supplementations, see figure 5.

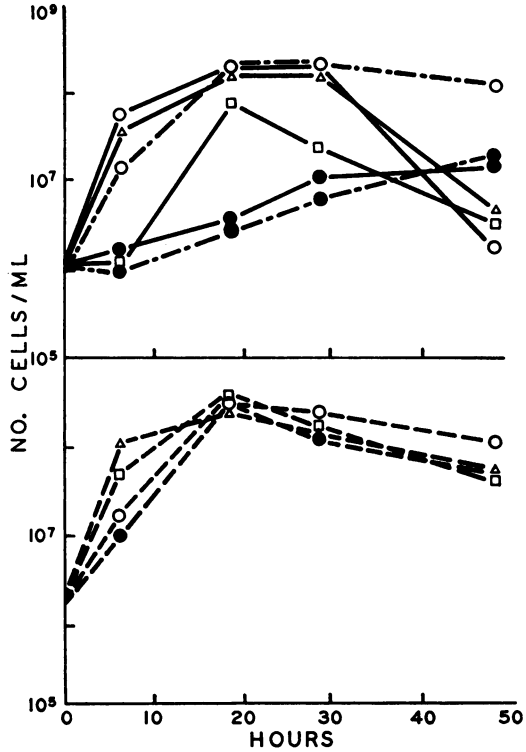

Figure 8. Viable counts of initially homogeneous S cultures (*upper graph*), derived from type II (——) and VII (-·-), and of type II-derived R cultures (- - -, *lower graph*) in the presence and absence of various combinations of DNA + deoxyribonuclease, manganese, deoxynucleosides and deoxynucleotides in brain heart infusion broth. Inoculum: 100 per cent S-D39 (——), 100 per cent S-VII (-·-), or 100 per cent R-36A (- - -). ○ = DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates; △ = DNA + deoxyribonuclease + Mn; □ = Mn; ● = control. For amount of supplementations, see figure 5, except that manganese concentration was 18 μg per ml.

of the "optimal supplementation system," and in view of the similarity between the requirement for all four bases in the here described optimal supplementation system and in systems known to affect DNA synthesis (Kornberg, 1957), it was decided to measure the effects of our effective supplementation upon DNA synthesis. Resting smooth (type I), as well as related rough cells, were exposed, in the presence of 1 per cent N.Z.case and 0.1 per cent glucose in phosphate buffer (see Materials and Methods), to DNA + deoxyribonuclease alone or with deoxynucleosides + deoxynucleotides + nucleoside diphosphate. As illustrated in figure 10, the acid-insoluble deoxypentose content of resting S cell suspensions increased significantly and rapidly under the influence of DNA + deoxyribonuclease, and even more strikingly under the influence of DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates. The intracellular increases in acid-insoluble deoxypentoses (presumably DNA) were directly proportional to the effects of the tested supplements on growth of S cells (figure 6) and on R → S population changes. Therefore, it appeared reasonable to ascribe the observed effects of DNA breakdown products on population changes and growth of

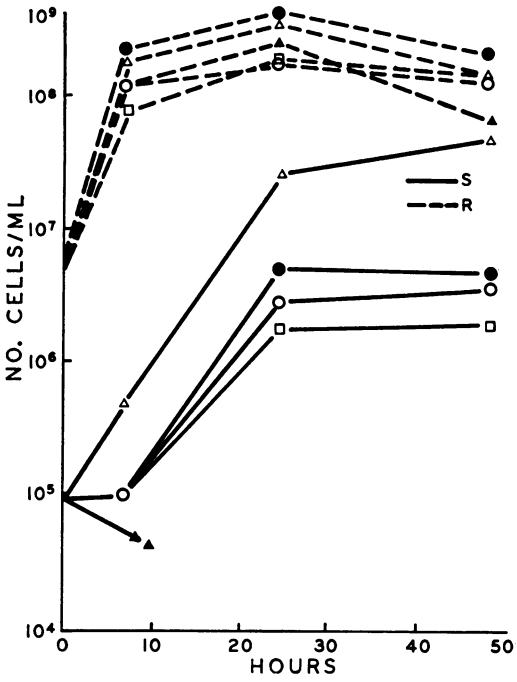

Figure 9. Viable counts of S and R cells in cultures of pneumococci undergoing population changes in the presence and absence of various combinations of DNA + deoxyribonuclease, manganese, deoxynucleotides, and deoxynucleosides in brain heart infusion medium. Inoculum: 1 per cent S-VII + 99 per cent R-VII. ○ = DNA + deoxyribonuclease + Mn; △ = DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates; □ = Mn; ● = deoxynucleosides + deoxynucleotides + nucleoside diphosphates; ▲ = control. For amount of supplementation, see figure 5, except that manganese concentration was 18 μg per ml.

S cells to a primary effect upon DNA synthesis of S cells. There were no comparable effects upon R cells, either in the growth or in the resting cell studies. The possibility that the addition of DNA + deoxyribonuclease may serve merely as a supplemental source of breakdown products for intracellular incorporation was made unlikely by the observation that the described effects on intracellular DNA levels of resting S cells were not directly proportional to the quantity of DNA present in the extracellular environment. The addition of 28 μg DNA per ml failed to produce any stimulation despite the presence of deoxyribonuclease, deoxynucleosides, deoxynucleotides, and nucleoside diphosphates, whereas 56 μg and 112 μg of DNA per ml yielded equal enhancements.

The readings for acid-insoluble deoxypentoses obtained in these resting cell studies were higher than those reported by others (Lerman and Tolmach, 1957; Fox, 1957). Subsequent tests, to be reported separately, utilized more rigorous washing procedures which resulted in an appreciable, relative decrease of deoxypentose values per ml, but these decreases did not alter the extent of increase in DNA levels between control and supplemented S cell suspensions. Also, in contrast to the early data as shown in figure 10, the more recent resting cell experiments failed to evoke any enhancement of synthesis in R cell suspensions containing the full supplementation, thus placing R cells into a truly nonresponsive class. Finally, figure 10 shows that in DNA + deoxyribonuclease-supplemented S cell suspensions, a decrease from the point of optimal levels of acid-insoluble deoxypentoses per ml occurred after the initial 30-min period. This decrease was also noted in all of the subsequent tests and appears to be a real effect; its cause is unknown but it may involve the initial availability of a critical amount of intracellular DNA precursors.

The effects of enzymatic DNA digests on growth of S pneumococci also were demonstrable *in vivo* (Braun *et al.*, 1957*b*; Firshein and Braun, 1958). Intraperitoneal inoculation of white Swiss mice with 1 LD_{50} of S (type I) pneumococci resulted in 100 per cent mortality within 48 to 60 hr when DNA digests (450 μg DNA + 100 μg deoxyribonuclease per mouse) were administered subcutaneously (dorsally) at time of infection or 24 hr prior thereto. Sampling of peritoneal washings obtained by the intraperitoneal injection of 2 ml of saline per mouse, initially infected with 1200 organisms, revealed that these DNA digest injections resulted in a 10,000-fold increase in pneumococcal counts within 25 hr. Thus, the average count per ml of peritoneal washings from 8 untreated mice, 25 hr after infection, was $2.7 \pm 0.9 \times 10^5$, whereas the average count from 8 treated animals was $2.9 \pm 0.7 \times 10^9$.

Limited efforts to detect antagonists capable of interfering with the DNA + deoxyribonuclease-promoted enhancement of growth of virulent pneumococci showed that elevated Ca^{++} levels (20 μg per ml or more), kinetin riboside,

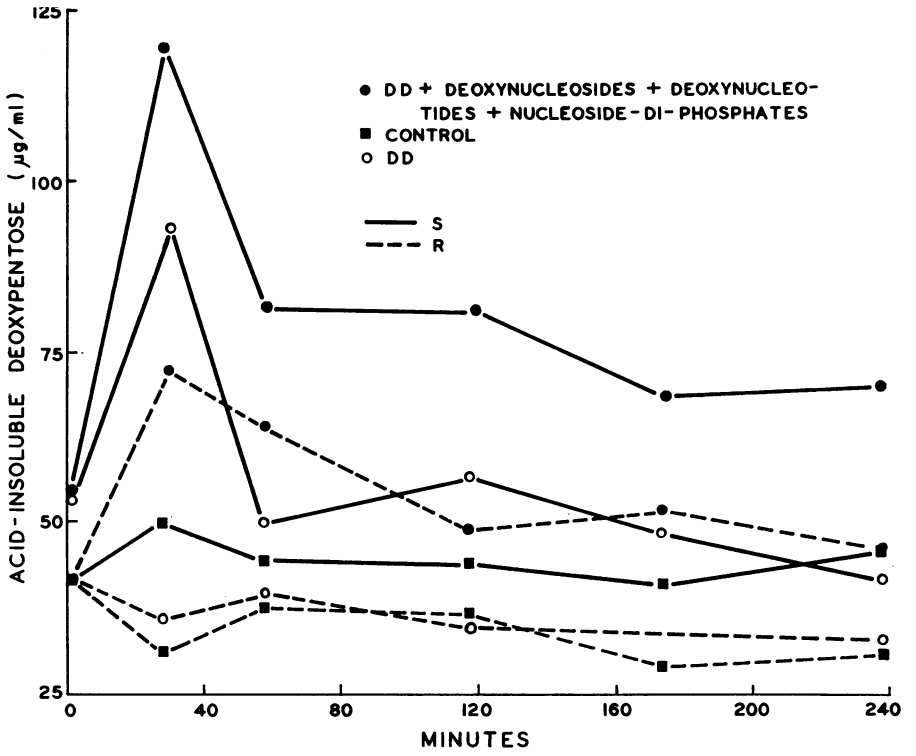

Figure 10. Acid-insoluble deoxypentose content of resting S (S-VI) and R (R-I192) pneumococci in the presence of various nucleic acid breakdown products. *DD* = DNA + deoxyribonuclease.

and salmine are capable of producing such effects *in vitro* and *in vivo* (Braun, 1958).

DISCUSSION

The foregoing data have demonstrated that the effects of enzymatic digests of DNA upon population changes from R → S in cultures of pneumococci, though similar in end results, differ significantly with regard to the mechanism involved from phenomena previously elucidated in studies with brucellae (Braun, 1958). In contrast to the events in brucella populations, where DNA digests led to a selective inhibition of the growth of non-S types, the essential mechanism of the selective effect upon pneumococcal populations now has been shown to involve a selective stimulation of the growth of virulent smooth cells in the presence of extracellular DNA digests. The most remarkable finding appears to be the fact that such stimulation is directly associated with, and presumably based upon, a selective stimulation of DNA synthesis of S cells, and that R cells do not respond to such influences.

It is interesting that in the presence of enzymatic DNA digests, mixtures of nucleoside derivatives can attain a significant capability to support such selective population changes, as well as the associated enhancement of growth and DNA synthesis of S pneumococci. In fact, the effectiveness of DNA + deoxyribonuclease supplementations has been shown to depend, in the case of some pneumococcal strains, upon the presence of such added DNA breakdown products. The primary requirement, however, appears to be the so far unidentified factor present in enzymatic DNA digests, which although of relatively small molecular size, is not identical with known purines, pyrimidines, nucleosides, or nucleotides. In many respects this factor bears an amazing resemblance to Gale's (1957) incorporation factor, which appears to stimulate RNA synthesis but, on the basis of all available evidence, it is not identical with it. The requirement for mixtures of the four deoxynucleosides and deoxynucleotides for optimal enhancement of DNA + deoxyribonuclease effects further places this phenomenon into the

realm of known systems supporting nucleic acid synthesis (Kornberg, 1957).

Okazaki and Okazaki (1958) reported recently that the growth of *Lactobacillus acidophilus* ordinarily cannot be stimulated by acid-soluble cell fractions containing deoxyriboside unless these have been digested enzymatically with crude snake venom; however, they will be utilized for DNA synthesis if a known deoxyriboside (e. g., thymidine) is added simultaneously. In view of such observations, it can be suggested that the ability of polymerized DNA to effect the stimulation of events in S pneumococci in the presence of deoxynucleosides + deoxynucleotides + nucleoside diphosphates possibly may reflect an ability of DNA to get into the cells in the presence of the "optimal supplementation." The digest factor may then be released by intracellular breakdown of DNA.

The significant role played by Mn^{++} in support of, or in lieu of, the factor(s) present in enzymatic DNA digests appears to involve additive effects of a different nature rather than identical effects. Studies with resting cells, to be reported subsequently, have indicated that the Mn^{++} effect is associated with a general enhancement of DNA, RNA, and protein syntheses, whereas DNA + deoxyribonuclease effects upon resting cells result in a selective enhancement of DNA synthesis with only minor effects upon RNA and protein syntheses. It should be noted that since Mn^{++}, through its apparent general stimulation of metabolic processes of S cells, can also enhance the rate of selective establishment of S cells in initially predominantly R populations, it often becomes difficult, particularly with certain batches of Mn^{++}-supplemented complex media and with certain strains, to detect DNA + deoxyribonuclease effects. When such complications arose, the actual contribution of DNA + deoxyribonuclease to the control of R $\rightarrow$ S population changes (and to DNA synthesis) became pronounced only when the Mn^{++} concentration was reduced to levels that yielded a lesser selective effect of Mn^{++}, e. g., to concentrations between 36 and 54 μg per ml, as shown in figure 3.

The capability of the smooth types to respond to extracellular metabolic factors by increased DNA synthesis and associated stimulation of multiplication would appear to be an entirely appropriate property for a virulent, parasitic form. The inability of the avirulent rough type to react similarly could be regarded as an appropriate indicator of self-sufficiency. Such teleological interpretations, however, fail to reveal the basic difference between virulent and avirulent pneumococci that could be held responsible for the observed differences. Future studies must attempt to elucidate whether the responsiveness of the virulent pneumococci to extracellular stimulation of DNA synthesis and the nonresponsiveness of avirulent cells involves differences in permeability, enzymatic capabilities, or energy availability. But even in the absence of such information, the demonstrated ability of virulent pneumococci to display striking growth responses in the presence of the type of compounds that may occur in appreciable amounts under conditions *in vivo* suggests the possibility of a significant natural role of such enhancement systems. This, in turn, suggests the possible future control of the fate of virulent populations *in vivo* through appropriate interference with the activity or presence of factors that promote the growth of virulent cells in the manner elucidated in the present studies.

Preliminary tests have indicated that supplementation of media with DNA + deoxyribonuclease + deoxynucleosides + deoxynucleotides + nucleoside diphosphates also can result in enhanced growth of virulent strains of streptococci and staphylococci. However, it has not been established so far whether or not such effects are also associated with increased DNA synthesis and whether they are also specific for virulent types. It further has been observed that enzymatic DNA digests or kinetin promote R $\rightarrow$ S population changes in cultures of *Shigella boydii* (Ikari *et al.*, 1958), *Clostridium botulinum* (N. Grossevicz, *unpublished data*), *Vibrio cholera* (W. Gardner, *unpublished data*), and apparently also in *Pasteurella pestis* (Eisler *et al.*, 1959). It will be interesting to determine whether enzymatic DNA digests play a rather general role in influencing bacterial population changes involving the establishment of virulent variants. It will also be interesting to learn whether gram-negative species tend to respond to DNA + deoxyribonuclease effects by mechanisms of

selective inhibition of avirulent types, previously elucidated for brucellae, and gram-positive species by selective stimulation of virulent types as now elucidated for pneumococci.

SUMMARY

Enzymatic digests of deoxyribonucleic acid (DNA), regardless of the source of DNA, were found to promote R → S population changes in initially R, or predominantly R, cultures of *Diplococcus pneumoniae*. Such population changes do not occur under ordinary laboratory conditions, and, with one major exception, known breakdown products of DNA do not have similar effects. The one exception is kinetin, but this compound does not appear to be identical with the factor present in the DNA digest, which, according to Ecteola fractionation is of relatively small molecular size. The effects of the DNA digest factor were enhanced by Mn^{++} and by the addition of mixtures of deoxynucleosides, deoxynucleotides, and, in the presence of deoxynucleosides + deoxynucleotides, by nucleoside diphosphates. With the help of R cells derived from a variety of type-specific S strains, it was shown that the extent and rate of DNA digest-promoted and Mn^{++}-, deoxynucleoside-, or deoxynucleotide-enhanced population changes were strain-dependent. Also, in deoxynucleoside + deoxynucleotide-supplemented cultures, the effects were significantly reduced in the absence of any one of the components of the deoxynucleoside or deoxynucleotide mixture. Contrary to previous findings with brucellae, where DNA digests were found to exhibit their selective effects via a selective inhibition of non-S types, R → S population changes of pneumococci in the presence of DNA + deoxyribonuclease have proved to involve a selective stimulation of the rate and extent of multiplication of S cells, without an effect on R cells. Experiments with resting cells demonstrated that the selective effects of DNA digests observed in growing cultures are directly correlated with a significant selective stimulation of DNA synthesis in S cells; R cells did not respond. Enzymatic DNA digests also influenced rates of multiplication of S pneumococci *in vivo*, and thus strikingly modified the outcome of the disease in mice. Some antagonists capable of interfering with these effects have been detected; their potential role in the control of infections, as well as the possibility of a generalized role of selective DNA digest effects among bacteria, have been discussed.

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