STUDIES ON IMMUNITY IN ANTHRAX

IX. Effect of Variations in Cultural Conditions on Elaboration of Protective Antigen by Strains of *Bacillus anthracis*

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ABSTRACT

Wright, George G. (Fort Detrick, Frederick, Md.), Milton Puziss, and W. Brook Neely. Studies on immunity in anthrax. IX. Effect of variation in cultural conditions on elaboration of protective antigen by strains of *Bacillus anthracis*. J. Bacteriol. **83**:515-522. 1962.—Nonproteolytic and nonencapsulated avirulent mutants were isolated from six virulent strains of *Bacillus anthracis* and tested for elaboration of protective antigen in the chemically defined medium developed previously (599 medium). Initially the strains grew rather slowly in 599 medium; serial transfer in the medium increased the rate of growth but reduced the elaboration of antigen. Two of the strains tended to revert to the encapsulated form during serial passage in 599 medium. Modifications in the medium and cultural conditions were studied in an attempt to obtain improved growth; the most significant alterations were the addition of adenosine and L-alanine, reduction in the concentration of ferrous sulfate, and growth under anaerobic conditions with agitation. Study of amino acid utilization in the medium revealed that the concentrations of certain amino acids could be reduced. These modifications, together with substitution of more productive strains, resulted in an approximately fivefold increase in elaboration of protective antigen, and greatly simplified production of the antigen on a practical scale. Omission of bicarbonate from the medium had no significant influence on utilization of amino acids.

Elaboration of the protective antigen of *Bacillus anthracis* in chemically defined media was initially reported in a previous paper (Wright, Hedberg, and Slein, 1954). Subsequently, the culture media were improved and simplified, and certain of the conditions influencing accumulation of the antigen in culture filtrates were recognized (Belton and Strange, 1954; Puziss and Wright, 1954; Thorne and Belton, 1957; Puziss and Wright, 1959). Gladstone (1946), in his initial report of elaboration of antigen in vitro, demonstrated antigen production by virulent and certain avirulent strains of *B. anthracis*. Subsequent studies, however, have been limited to the Sterne or Weybridge strain and mutants of the Vollum strain. The present investigation was undertaken to obtain information on antigen production in chemically defined media by additional strains of *B. anthracis*, in an effort to obtain filtrates of greater antigenic activity, and to provide a broader basis for conclusions regarding elaboration of the antigen.

MATERIALS AND METHODS

Cultures. The strains of *B. anthracis* from which mutants were derived for study of antigen elaboration had been isolated from man and animals primarily in widely separated outbreaks (Auerbach and Wright, 1955). All had been maintained as spore suspensions since they were received. Strain R1-NP, a nonencapsulated (R), nonproteolytic (NP) mutant of the Vollum strain, has been described previously (Puziss and Wright, 1954). A standard spore suspension of the Vollum strain was used for challenge of animals in the assay of protective antigen.

Media and methods for elaboration of protective antigen. The 599 medium of Puziss and Wright (1954) was used in initial studies; subsequent modifications are described in the Results. For routine study of antigen elaboration under aerobic conditions, 500-ml volumes of medium in Fernbach flasks were employed. For anaerobic cultures, 400-ml volumes in 600-ml centrifuge bottles were incubated under nitrogen in desicca-

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tor jars, except as otherwise noted. It was found necessary to avoid excessive heat in preparing the concentrated stock solution containing the amino acids; otherwise growth in the medium was delayed. The constituents were dissolved with the aid of vigorous shaking between 40 and 50 C. In most of the studies to be reported, water redistilled in glass was used for preparation of media. In the final experiments, however, passage through a deionizing column was substituted for the second distillation. This alteration proved satisfactory and simplified large-scale preparation of medium. After incubation the cultures were adjusted to pH 8, filtered through ultrafine sintered glass filters, and lyophilized.

Estimation of protective antigen. Complement-fixation titrations and active immunization assays in guinea pigs were employed as described previously (McGann, Stearman, and Wright, 1961). Complement-fixation titers are expressed in terms of 50% units per ml of filtrate; the results of the in vivo assays are expressed in relative potency units per ml of filtrate.

RESULTS

Isolation and properties of NP and R mutant strains. Mutants were isolated from six typical virulent strains of *B. anthracis*. NP colonies were selected on milk agar plates as described previously (Wright, Hedberg, and Feinberg, 1951), except that ultraviolet irradiation of the parent spore suspension was found to be unnecessary and was omitted. NP clones evidently arise spontaneously in laboratory strains of *B. anthracis*. R strains were isolated from the respective NP mutants by the method of Sterne (1937), so that NP and NP-R substrains were obtained from each parent strain. Spore suspensions were prepared for each mutant strain and retested to determine whether the mutant characteristics had been maintained. In mice the R strains were not lethal when injected subcutaneously in doses of 5000 spores.

Preliminary experiments had indicated that the virulence of NP mutants was similar to that of the parent Vollum strain from which they had been isolated (Wright et al., 1951). Additional virulence titrations were carried out in guinea pigs with five of the NP strains described above. Spore suspensions of the parent and NP mutant strains were diluted in water to contain 45 15, and 5 spores per 0.25-ml dose; the dilutions were injected intracutaneously into guinea pigs, five animals per group. Plate counts confirmed that the concentration of spores was close to the desired number in each case. Over 99% of the deaths occurred within 6 days; surviving animals were held 13 days. The LD_{50} of each strain was of the order of five spores. The results suggested that the LD_{50} of the mutant might be lower than that of the parent strain, although the numbers of animals were insufficient to allow calculation of individual LD_{50} values. Table 1 presents the combined results obtained with the five NP and the five parent strains. The data give no indication that loss of the proteolytic character is accompanied by a reduction in virulence for guinea pigs by the intracutaneous route of challenge.

TABLE 1. *Virulence of parent strains and their nonproteolytic mutants for guinea pigs*

Strains injected	Number dead/total injected			
	45 Spores	15 Spores	5 Spores	Total
Parent strains (5) . .	22/25	16/25	12/25	50/75
Nonproteolytic mutants (5)	25/25	19/25	18/25	62/75

Antigen production in 599 medium. The newly isolated NP and R mutants of the six virulent strains were compared with strain R1-NP with respect to growth and elaboration of antigen in 599 medium (Puziss and Wright, 1954). All of the newly isolated strains grew much more slowly than did R1-NP; even after incubation for 48 hr, growth of several of the strains was not as heavy as that of R1-NP within 24 hr. Determinations of antigen activity in the filtrates are summarized in Table 2. Although antigen production by the newly isolated strains was not consistently high, there were indications that two of the strains were superior to R1-NP, despite their relatively slow growth.

The parent strains, from which the NP and R mutants had been isolated, and the NP single mutants were also tested for growth in 599 medium. The rates of growth of these strains were similar to those of the corresponding NP-R mutants, suggesting that the delayed growth relative to R1-NP is a characteristic derived from the parent strains, and not a result of the mutations.

TABLE 2. *Antigen elaboration by various strains under different cultural conditions*

Strain	Aerobic growth in 599 medium			Aerobic growth in 968 medium			Anaerobic growth in 968 medium[a]		
	Growth time[b]	Complement-fixation titer	Relative potency units	Growth time[b]	Complement fixation titer	Relative potency units	Growth time[b]	Complement-fixation titer	Relative potency units
	hr	*units/ml*	*units/ml*	*hr*	*units/ml*	*units/ml*	*hr*	*units/ml*	*units/ml*
R1-NP	29 (4)	38	1.08	32 (4)	50	0.82	48 (4)	42	0.98
V770-NP1-R	40	15	0.09	42 (3)	57	1.4	48 (7)	63	1.4
1062-NP1-R3	40	5	0	47	15	0.25	50[c]		
107-NP2-R1	48	60	3.62	41(3)	90	2.1	48 (7)	80	2.1
108-NP1-R2	48	7.5	0.43	47	20	1.93	50	15	0.43
116-NP1-R2	48	60	—[d]	29	120	5.9	49 (3)	180	8.5
1133-NP1-R3	48	10	0.61	47	30	2.32	50[c]		

[a] Slightly modified; see text.

[b] Figures in parentheses refer to the number of replicate preparations when greater than 1. Mean values are reported in these cases.

[c] Only slight growth was obtained; antigen production was not tested.

[d] Value was above the applicable range of the method at the dose used.

The foregoing observations indicated that 599 medium was not well suited to growth and antigen elaboration by most of the strains and mutants. Accordingly, serial transfers were carried out with three of the NP-R mutants mentioned above in an attempt to adapt them to growth in the medium. After five passages, the growth was considerably more rapid, so that heavy growth was obtained within 24 hr. The fourth-passage cultures were tested for alteration of the R and NP characters, and then introduced into fresh medium for estimation of protective antigen production. Although all of the cultures remained completely NP, definite evidence was obtained of the emergence of encapsulated forms in two of the three cultures. Strain R1-NP reverted almost completely to the encapsulated form, and the fourth-passage culture was virulent for mice at a dose of 30 spores. Strain 116-NP1-R2 reverted slightly, as shown by development of mucoid areas on heavily seeded plates; strain 1133-NP1-R3 was unchanged. Elaboration of antigen by the passage strains was markedly reduced, as compared with the original culture, in the case of strains R1-NP and 116-NP1-R2; it remained at essentially the same low level with strain 1133-NP1-R3. It was evident that serial passage in 599 medium did not increase elaboration of antigen by these strains.

Modification of 599 medium. Alterations in the medium were investigated in an effort to obtain more rapid growth and increased elaboration of antigen by these strains. The following modifications produced no improvement: sterilization of the medium by filtration rather than by autoclaving, addition of 0.001 M glycine or 0.0005 M glutamine, substitution of cystine for cysteine and alteration in the serine-threonine ratio. Slightly improved growth was obtained by reduction in the concentration of ferrous sulfate to 0.00005 M. Marked stimulation of growth was obtained by addition of yeast extract; concentrations as low as 3 μg per ml of medium produced significant stimulation.

The effect of yeast extract could not be duplicated by a number of vitamins and growth factors known to be present in yeast. The results were suggestive of the stimulation of spore germination by yeast extract; this effect was duplicated by a combination of adenosine, L-alanine, and tyrosine (Hills, 1949). Accordingly, the influence of adenosine and L-alanine was tested; concentrations of 1 μg per ml of adenosine and 0.0001 M L-alanine gave optimal stimulation of growth, and their combined effect was essentially equivalent to that of yeast extract. Adenine, adenylic acid, guanylic acid, and guanosine were inactive or only slightly active as replacements for adenosine. Addition of tyrosine produced no further stimulation, presumably because of the presence of phenylalanine.

Further modifications were investigated in an effort to improve and simplify the medium. Omission of guanine had no visible effect on growth when adenosine was present, but the omission reduced the elaboration of antigen considerably.

Indications were obtained that a threefold increase in the concentration of guanine increased the elaboration of antigen. Omission of cysteine had no effect, and under these conditions glucose could be autoclaved in the final medium. Reduction in the concentration of ferrous sulfate to 0.00005 M was satisfactory, and this concentration of ferrous sulfate could be added to the medium prior to autoclaving. The effect of addition of glycine was further investigated, and it was determined that this amino acid at 0.0003 to 0.00001 M concentrations produced significant stimulation of growth of the five strains tested. Addition of 0.0003 M glycine appeared to increase the elaboration of antigen detectably; higher concentrations appeared to be somewhat inhibitory.

The combination of these modifications was referred to as 968 medium; it differed from 599 medium in addition of 1 μg per ml of adenosine, 0.0001 M L-alanine, and 0.0003 M glycine, increase in guanine to 0.000045 M, elimination of cysteine, reduction of ferrous sulfate to 0.00005 M, and autoclaving of glucose and ferrous sulfate in the medium. Seven strains were tested for antigen production in this medium (Table 2). The incubation times for the several strains were adjusted as required for development of heavy growth. Results of both complement-fixation titrations and active immunization assays indicated that in almost all cases higher titers of antigen were produced in 968 medium than in 599 medium. Growth was heavier and more rapid, and it was evident that considerable adaptation to the new strains had been achieved, particularly in the case of V770, 107, and 116 mutants.

Antigen elaboration under anaerobic conditions. It was observed that protective antigen was elaborated not only under aerobic conditions, but also during growth under strict anaerobic conditions (Wright and Puziss, 1957; Puziss and Wright, 1959). Exploratory investigations with 968 medium suggested that slightly greater elaboration of antigen was obtained when the concentration of ferrous sulfate was reduced to 0.00001 M and added aseptically to the autoclaved

TABLE 3. *Utilization of amino acids during growth of strain V770-NP1-R in 968 medium*

Amino acid[a]	Amino acid remaining, mg per liter					
	Uninoculated medium		Aerobic culture filtrate		Anaerobic culture filtrate	
	Theoretical	Found	Complete medium	Bicarbonate omitted	Complete medium	Bicarbonate omitted
Glycine	22		15	16	18	17
L-Alanine	9	9	19	20	14	15
DL-Valine	60	69	51	56	46	50
DL-Leucine	128	119	33	43	40	45
DL-Isoleucine	128	87	38	46	63	64
DL-Serine	21	22	—[b]	—	11	12
DL-Threonine	60	63	—	—	6	6
DL-Methionine	30	32	U[c]	U	U	U
DL-Aspartic acid	64	69	Trace	Trace	Trace	Trace
L-Glutamic acid	168	135	A[d]	A	A	A
L-Proline	30		—	—	—	—
DL-Phenylalanine	68	66	21	32	32	33
L-Tryptophan	52	53	52	51	52	51
L-Arginine·HCl	104		114		107	108
L-Histidine·HCl	96	81	12	27	30	30
Asparagine			30[e]	28[e]		

[a] Optical activities are those of the original medium constituents. Optical activities of the amino acids in culture filtrates were not determined.

[b] — = Not detectable.

[c] Unidentified component interfered with analysis for methionine.

[d] Presence of asparagine interfered with analysis for glutamic acid.

[e] Determined by paper chromatography.

medium. This modification was adopted in further investigations of anaerobic growth and antigen production by the various strains (Table 2). Strains R1-NP, V770-NP1-R, 107-NP2-R1, and 116-NP1-R2 were well adapted to anaerobic growth, and the titers of antigen were not significantly different from those obtained under aerobic conditions. Strains 1062-NP1-R3, 108-NP1-R2, and 1133-NP1-R3 were unsatisfactory and were not investigated further.

Utilization of amino acids in 968 medium. Filtrates of cultures grown under aerobic and anaerobic conditions were analyzed for residual amino acids to determine whether the medium was deficient or contained significant excesses of these compounds. Filtrates of cultures in which bicarbonate was omitted from the medium were also analyzed to determine whether bicarbonate influenced the utilization of amino acids. Strain V770-NP1-R was grown in 968 medium under aerobic and anaerobic conditions; similar cultures were prepared in which sodium bicarbonate was omitted from the medium. Filtrates from the complete medium were of typical antigenic activity by in vivo assay; the bicarbonate-free filtrate was inactive after aerobic growth and very slightly active after anaerobic growth, as reported previously (Puziss and Wright, 1959). Amino acids were determined in the uninoculated medium and in the culture filtrates by column chromatography (Moore and Stein, 1954). These results are presented in Table 3.

Analyses of the uninoculated medium gave results that were close to theoretical in most cases, thus confirming the adequacy of the method. Recoveries of isoleucine and glutamic acid were somewhat low. Analysis of the culture filtrates revealed little or no utilization of arginine, tryptophan, or valine. Net synthesis of alanine occurred, presumably as a result of the transaminase activity of the organism (Thorne and Molnar, 1955). Methionine had evidently disappeared, but this could not be determined with certainty because of the presence of an unidentified component in the same elution region. If a ninhydrin color density similar to that of an amino acid is assumed, the concentration of the unknown was relatively high; this unknown substance was present in considerably greater concentrations in the aerobic than in the anaerobic cultures. An additional unknown component was detected between the valine and methionine peaks. Determination of glutamic acid was prevented by the appearance of a component which was subsequently identified as asparagine. Serine, proline, threonine, and aspartic acid were almost completely utilized, particularly under aerobic conditions. Evidently

TABLE 4. *Composition of 1095 medium*

Constituent[a]	Concn	Constituent[a]	Concn
	g/liter		*g/liter*
Glycine	0.022	L-Histidine·HCl	0.096
L-Alanine	0.009	Guanine·HCl[b]	0.009
DL-Valine	0.060	Adenosine	0.001
DL-Leucine	0.128	Thiamine·HCl	0.0004
DL-Isoleucine	0.064	Pyridoxal·HCl[c]	0.001
DL-Serine	0.021	Biotin	0.0004
DL-Threonine	0.060	Glucose	1.0
DL-Methionine	0.030	Calcium chloride·$2H_2O$	0.015
DL-Aspartic acid	0.064	Magnesium sulfate·$7H_2O$	0.010
L-Glutamic acid	0.168	Manganous sulfate·H_2O	0.004
L-Proline	0.030	Potassium dihydrogen phosphate	0.680
DL-Phenylalanine	0.068	Dipotassium hydrogen phosphate	0.870
L-Tryptophan	0.010	Sodium bicarbonate[c]	2.500
L-Arginine·HCl	0.021	Ferrous sulfate[c]	0.0015

[a] All constituents except pyridoxal, phosphate salts, sodium bicarbonate, and ferrous sulfate were dissolved as a 25-fold concentrated stock solution, which was diluted as required.

[b] Dissolved separately in a small amount of concentrated HCl.

[c] Dissolved and sterilized separately as stock solutions; added aseptically to autoclaved medium.

the D as well as the L forms of several of the amino acids are utilized.

Asparagine was detected in culture filtrates as a new component with an elution peak close to that of glutamic acid; it was further identified by paper chromatography. An orange spot, with an R_F value of 0.38 in a phenol-water solvent, appeared after ninhydrin treatment; authentic asparagine gave an identical spot. Among the original constituents of the medium, only serine gave a similar R_F value; however, serine was completely utilized during aerobic growth. Asparagine was identified in filtrates of four additional encapsulated and R strains. Omission from the medium of aspartic acid, arginine, or threonine did not interfere with appearance of asparagine, suggesting that none of these amino acids was a precursor. Omission of bicarbonate from the medium produced no significant difference in utilization of amino acids or the appearance of new components.

Modifications of 968 medium. The foregoing observations indicated that further adjustments in the amino acid composition of the medium would be desirable. Attention was directed primarily to adaptation of the medium to anaerobic growth, because anaerobic culture was more readily applicable to large-scale production of antigen. The 968 medium evidently contained substantial excesses of tryptophan, arginine, valine, and isoleucine. Although no utilization of tryptophan was detected, omission of this amino acid delayed growth of most strains. Reduction of tryptophan and arginine to one-fifth and reduction of isoleucine to one-half that present in 968 medium had no detectable effect on growth or elaboration of antigen. Indications have been obtained that the medium was deficient in serine, threonine, proline, methionine, and aspartic acid. Increased concentrations of these amino acids, however, did not increase, and in several instances appeared to inhibit, the elaboration of antigen. Addition of 0.4 μg/ml of biotin and 1 μg/ml of pyridoxal hydrochloride produced a small but consistent stimulation of the growth of most strains; there were indications that elaboration of antigen was stimulated slightly. Increase in the concentration of thiamine to 0.4 μg/ml was satisfactory and provided a moderate excess. The final composition, referred to as 1095 medium, is presented in Table 4.

When large volumes of culture were grown statically under anaerobic conditions, the organisms tended to settle out on the bottom of the vessel. The effect of agitation of the culture was investigated, in an effort to increase the growth rate and shorten the incubation time. After 14 to 18 hr incubation, when visible growth had appeared, a Teflon-covered stirring bar previously autoclaved in the culture vesse

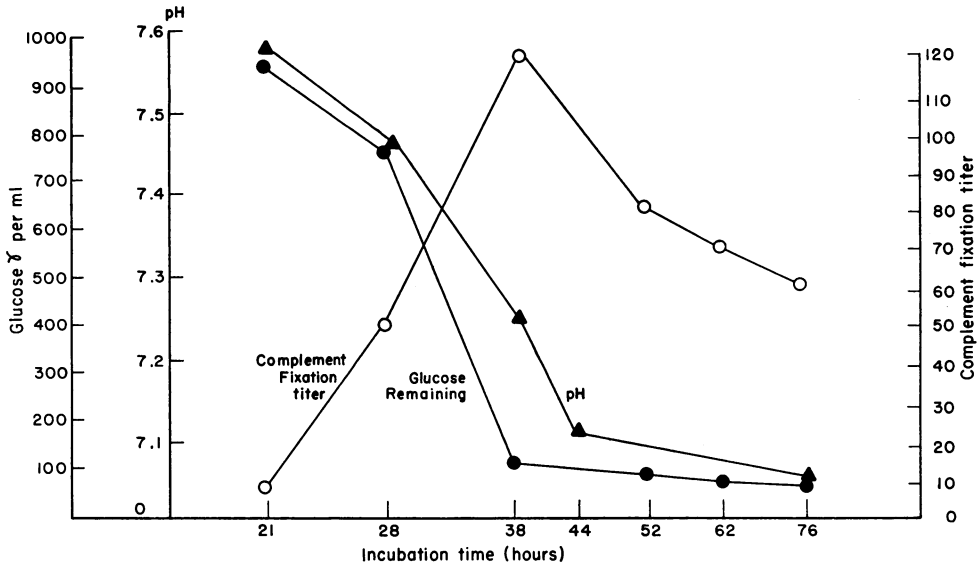

FIG. 1. *Elaboration of antigen, utilization of glucose, and pH change during anaerobic growth with stirring.*

TABLE 5. *Protective antigen production in stirred cultures in 1095 medium*

Preparation	Strain	Volume of culture	Incubation time	Complement-fixation titer	Relative potency units
		ml	*hr*	*units/ml*	*units/ml*
1	116-NP1-R2	600	43	140	1.1
2	V770-NP1-R	600	43	100	4.3
3	V770-NP1-R	3,000	45.5	240	6.7
4	V770-NP1-R	4,000	43	70	1.7
5	V770-NP1-R	10,000	41	>160	17
6	107-NP2-R1	4,000	42	>160	17
7	107-NP2-R1	600	43	100	6.7
8	107-NP2-R1	4,000	43	160	1.0
9	107-NP2-R1	2,000	44	>160	30
Median			43	160	6.7

was rotated slowly by a magnetic stirring motor for the remainder of the growth period. The rate of rotation was just sufficient to maintain a turbid suspension without foaming and with minimal vortex formation. Determinations at intervals during growth revealed that essentially complete utilization of glucose now occurred after 38 hr instead of after 46 hr (Fig. 1). Elaboration of antigen as estimated by complement-fixation titer, and the drop in the pH of the culture, also occurred more rapidly than in unstirred cultures (Puziss and Wright, 1959).

A series of anaerobic cultures was grown in 1095 medium with stirring, using the three strains that had appeared most promising in previous tests. The volumes of the cultures ranged from 600 to 10,000 ml, and the incubation times from 41 to 45.5 hr. Filtrates of high antigenic activity were obtained under these conditions (Table 5).

DISCUSSION

The virulence of NP mutants for the guinea pig did not appear to be significantly lower than the virulence of the wild-type strains. This observation would suggest that proteolytic activity plays no role in infection, although it is possible that differences in virulence would be detected in other hosts or by other routes of challenge. If, however, protease is of no significance in infection, it is not clear how protease production is maintained in all of the virulent strains thus far examined, despite the spontaneous occurrence of NP mutants. It seems possible that the protease represents an adaptation to the saprophytic existence of the organism.

Indications were obtained that virulent organisms emerge during serial transfer of R mutants in chemically defined medium. Should further investigation confirm the reversion of the R mutant to the virulent form, R mutants must not be assumed to remain avirulent after laboratory or other manipulations.

Although bicarbonate has been demonstrated to be a strict requirement for elaboration of antigen in both aerobic and anaerobic cultures, the role of this compound has not been clarified. Bicarbonate could not be replaced by any of a number of metabolically related substances, and had no effect on the metabolism of glucose that could account for its influence on elaboration of antigen. Retention of metabolic carbon dioxide was evidently responsible for elaboration of traces of antigen in bicarbonate-free media (Puziss and Wright, 1959). The study of amino acid utilization was undertaken to explore the possibility that the effect of bicarbonate on elaboration of antigen is associated with and perhaps caused by an alteration in the metabolism of amino acids. No significant effect of omission of bicarbonate on utilization of amino acids was detected, however, and the role of this substance in elaboration of antigen remains unknown.

Comparison of the titer of protective antigen obtained during aerobic growth of strain R1-NP in 599 medium (Table 2) with that produced during anaerobic growth of V770 or 107 mutants in 1095 medium (Table 5) indicates that considerably greater elaboration of antigen was obtained under the latter conditions. On the basis of complement-fixation titrations, it appears that the increase is at least fourfold. From the results of assay of immunizing activity in guinea pigs, the difference appears to be of the order of

sixfold. The new procedures not only increased the elaboration of antigen, but also appear to be more readily applied on a large scale. Use of anaerobic conditions of growth avoided the problems associated with aeration of bicarbonate-containing cultures, and facilitated increase in the size of the batch. Culture volumes of 10 liters proved satisfactory, and there is no apparent reason why larger bottles or tanks should not be used. Methods for combining the antigen with adjuvant in a form suitable for human immunization will be presented in a subsequent paper.

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