TERMINAL PROGRESS REPORT

Period Covered: 1 September 1965 to 31 December 1966

Responsible Investigator:

Robert A. Mah, Assistant Professor

Co-responsible Investigator:

Lloyd W. Regier, Associate Professor

Department of Environmental Sciences and Engineering
School of Public Health
University of North Carolina
Chapel Hill, North Carolina

Subject of Report:

NUTRITIONAL REQUIREMENTS OF ENTEROTOXIGENIC STRAINS OF STAPHYLOCOCCUS AUREUS

Contract Number: DA-49-193-MD-2374

INTRODUCTION

Previous investigations of the nutritional requirements in Staphylococcus aureus were reported in the studies of Sulzer and Peters (15,16) and Mah and Regier (7). A synthetic medium containing 8 amino acids, inorganic salts, and vitamins was devised (16). Such a combination supported growth comparable to that obtained in an equivalent amount of complex medium. It was also found that when glutamic acid served as carbon source, enterotoxin B was produced at a level greater than with glucose as carbon source. Ribose, glycerol, and pyruvate supported growth but not toxin production; Kreb's cycle intermediates did not support growth.

Subsequently, experiments were continued on the composition of the synthetic medium under more stringent conditions; the influence of pH, gas atmosphere, and temperature were also investigated. The following terminal report concerns the nutritional and physical factors required for growth and toxigenesis of S. aureus S-6. It is divided into 3 parts: I. Development of a Defined Medium. II. Studies of pH Effects on Growth and Toxigenesis. III. Effect of Gas Atmosphere and Temperature on Growth and Toxigenesis.

I. Development of a Defined Medium

In all previous experiments, unwashed cells harvested from complex media were used as inocula. Although such experiments were performed with appropriate controls, it was desirable to determine the effect of the complex organic compounds carried over with the inoculum. Experiments were therefore performed with cells washed 3 times in sterile salts solution before use as an inoculum. Introduction of such inocula into the 7 amino acid medium resulted in little or no growth, indicating the necessity for some additional factors present in the complex medium.

A review of the literature pertaining to the development and use of several types of defined media for growth of S. aureus has been given in our previous reports (7,15,16). The nutritional requirements have been known since the investigations of Gladstone (4), who showed that nicotinic acid and thiamine were necessary growth factors in S. aureus. Gretler et al. demonstrated that biotin was an additional requirement in some strains of S. aureus, primarily the non-pathogenic ones. It seemed likely that growth factors such as vitamins were not the deficient ingredients in the synthetic medium so attention was turned toward the composition of the amino acids.

Materials and Methods

The vitamins and inorganic salts solution were the same as previously reported but in further experiments, the latter was modified as described in the text. Enterotoxin was assayed according to the method of Morse and Mah (10) and Weirether et al. (19).

The Inoculum. Small samples (3 ml) of a stationary phase culture of S. aureus S-6 grown aerobically on 2% protein hydrolysate (Mead Johnson Int.,

Evansville, Ind.) broth plus vitamins at 37 C were aseptically transferred to sterile culture tubes, quick-frozen in a dry ice-acetone bath and kept at -15 C. The samples were thawed as needed, a 1% inoculum introduced into protein hydrolysate (PHP) broth and the culture incubated on a shaker at 37 C for 12 hr. It was then centrifuged and washed aseptically in salts solution to remove soluble organic compounds, resuspended to the initial volume and used as a 1% inoculum in the experimental vessels. The vessels of choice were 300-ml Nephloflasks (Bellco Glass Inc., Vineland, N. J.) containing a total of 50 ml medium. The carbon source was 1% glucose in all cases unless otherwise stated; it was autoclaved and added separately.

Growth in defined media reported by previous workers for S. aureus was not evaluated in quantitative terms; relative degrees of turbidity were noted after daily intervals often up to 6 days (17). In the present investigation, turbidity was measured by Klett-Summerson colorimetry (green filter at 540 mμ) after 24 hr incubation at 37 C under aerobic conditions (shaken at 180 rpm, Psycrotherm incubator-shaker, New Brunswick Sci. Inc., New Brunswick, N. J.). On complex media, cells were in the maximum stationary phase after this length of incubation.

RESULTS

The Amino Acid Requirements. Since PHP medium supported optimal growth of S-6 in the presence of nicotinic acid and thiamine, the initial composition of the defined medium was based on the constituent 18 amino acids present in PHP. These consisted of glycine, alanine, aspartic acid, valine, leucine, isoleucine, serine, threonine, phenylalanine, tyrosine, tryptophan, lysine, arginine, histidine, glutamic acid, cystine, methionine, and proline.

All amino acids were obtained from commercial sources and were chromatographically pure. Each was added at a final concentration of 25 µg/ml. From this mixture of amino acids, it was calculated that an equivalent amount of amino nitrogen was present in 0.08% PHP, and this concentration was used as an index of the maximal growth obtainable under the previously stated conditions. In the presence of glucose as a carbon source, growth in 0.08% PHP [270 Klett Units, (KU)] was similar to that in the 18 amino acid defined medium (250 KU). To determine the essential amino acids for optimal growth of S-6, an arbitrary minimum yield of 200 KU in 24 hr was chosen. Media resulting in an extended lag phase might give rise to less than this arbitrary value even though the final cell yield may reach 200 KU. Such media would not be selected under these conditions.

To determine the amino acid requirement, the following protocol was adopted. Starting with the 18 amino acids in inorganic salts solution plus vitamins, each amino acid was singly deleted and the growth recorded. Omission of some amino acids satisfied the specified growth criteria of 200 KU in 24 hr under aerobic conditions at 37 C. Addition of such amino acids was presumed unnecessary for optimal growth of S-6. Furthermore, by deleting such an amino acid in combination with the deletion of each of the remaining 17 amino acids, pairs of amino acids may be found unnecessary. Deleted pairs resulting in equivalent or better growth may be tested in the same fashion with the remaining 16 amino acids. Such a protocol would avoid a factorial series of combinations while providing some practical basis for selection of the appropriate amino acids.

In the initial deletion experiments, any one of several amino acids could be omitted. However, only one of the possibilities, usually the deletion

yielding the highest growth, was tested. In the final experiments, deletion of an additional amino acid resulted in equivalent or better growth in only one or two cases. In such instances, both deletions were tested further.

In the first series, each individual amino acid was omitted in succession. A control flask containing all 18 amino acids, vitamins and glucose as carbon source, was prepared in the basal salt solution previously described (7). Experimental flasks were identical to the control except for the amino acid mixture. A different amino acid was omitted from each of the 18 flasks, leaving 17 amino acids in each flask. All 19 flasks were then autoclaved, inoculated, and incubated for 24 hr under the conditions previously described.

Of the 18 amino acids, 14 were not absolutely essential for growth. In fact, omission of some (aspartic acid, isoleucine, alanine, tryptophan, methionine and tyrosine) led to growth yields which satisfied the criterion of 200 KU in 24 hr. These results are depicted in Fig 1.

In the second experiment, aspartic acid was completely omitted; it was arbitrarily chosen for this experiment since it was non-essential. In addition to aspartic acid, a different amino acid was omitted from each of the 17 flasks leaving 16 amino acids in each flask. A control containing 17 amino acids (no aspartic acid) was maintained. All 18 flasks were autoclaved, inoculated, and incubated for 24 hr as previously described. The growth yields are shown in Fig 2.

The results showed that the double omission of aspartic acid and any one of the following: isoleucine (218 KU), lysine (198 KU), tryptophan (208 KU), glutamic acid (198 KU), tyrosine (204 KU), and serine (198 KU), again resulted in equivalent or improved growth. The 17 amino acid control gave a reading

Figure 1

1-Amino Acid Deletion plus Glucose

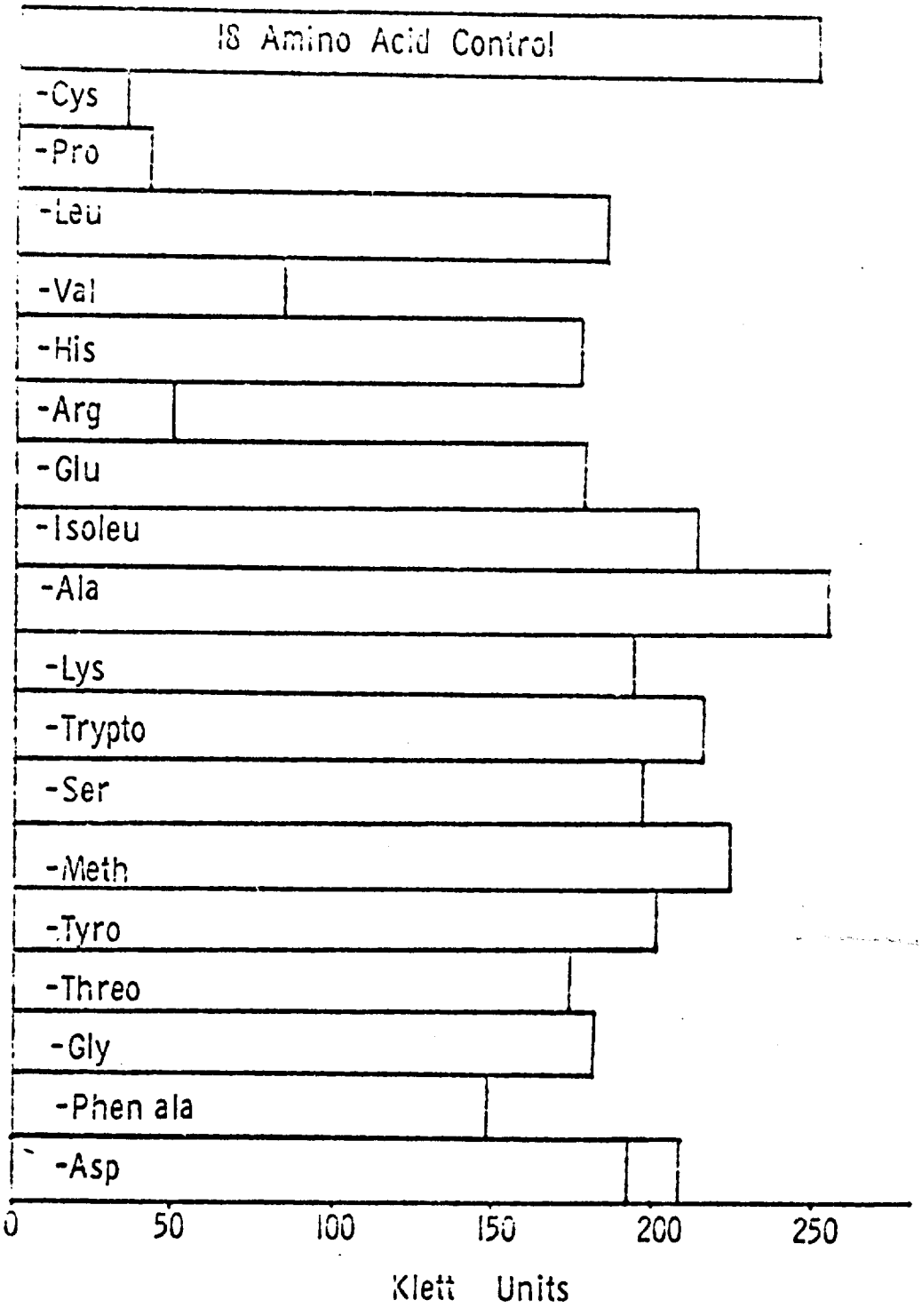
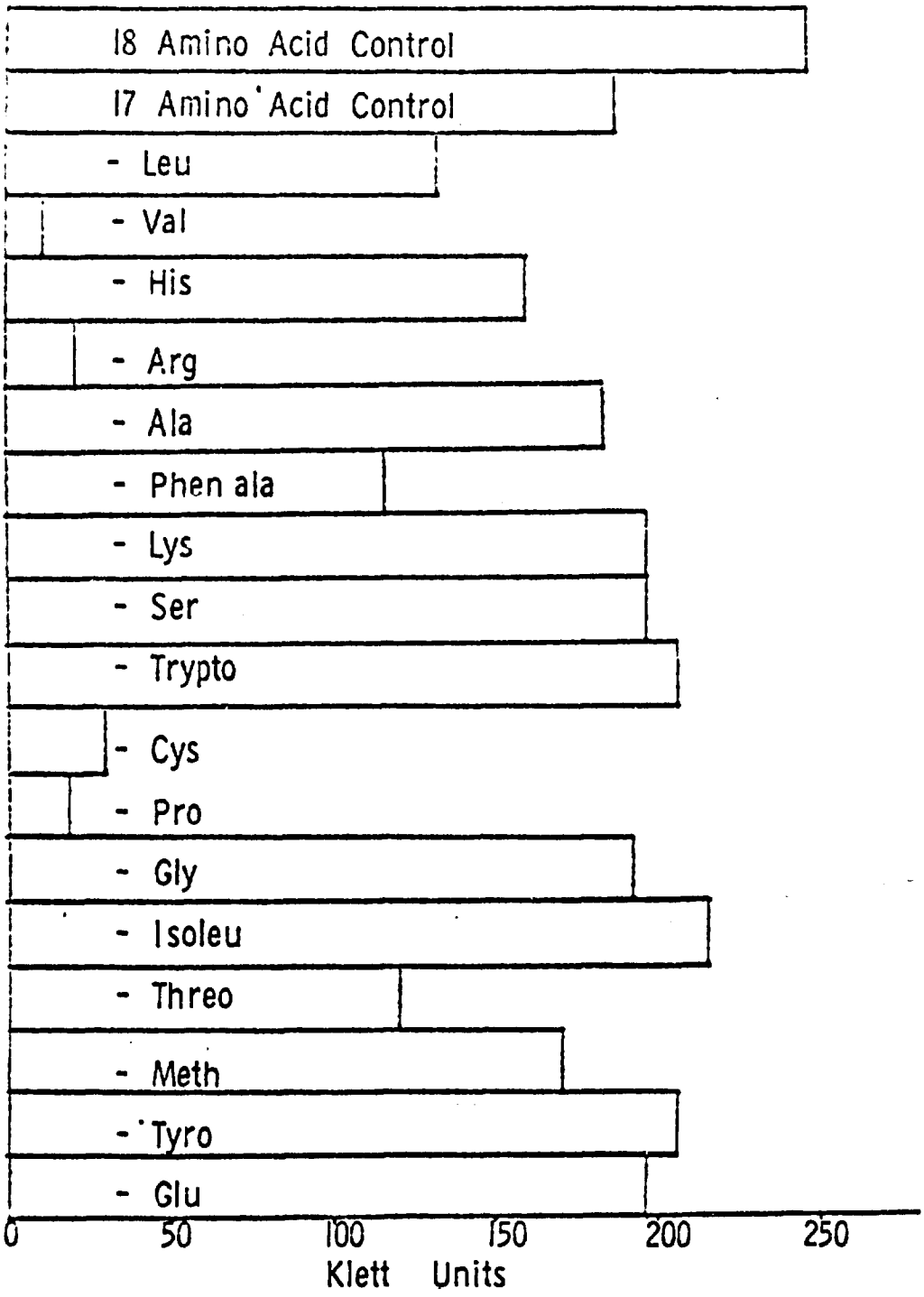


Fig 2 2-Amino Acid Deletion plus Glucose



of 191 KU. The deletion of the aspartic acid-isoleucine pair yielded the highest growth in this series.

Omission of aspartic acid and isoleucine in a flask containing all the other components of the defined medium was selected as the test pair for the next set of experiments. Using this pair as a control, a total of 3 amino acids was deleted from the defined medium. In this case, 16 flasks were prepared. An additional amino acid besides the aspartic acid-isoleucine pair was omitted from each of the 16 flasks, leaving 15 amino acids in each flask. All 17 flasks were again autoclaved and incubated after inoculating as previously described. The results of this experiment are tabulated in Fig 3.

The 16 amino acid control showed a yield of 245 KU. The triple omission of aspartic acid, isoleucine, and any one of the following: alanine (246 KU), lysine (215 KU), tryptophan (197 KU), serine (214 KU), and glycine (219 KU), again resulted in equivalent or better growth.

Since omission of aspartic acid, isoleucine and alanine gave the highest growth, they were deleted as the control of the next experiment in which a total of 4 amino acids was omitted. Again the same protocol was followed and a different amino acid was omitted from each of 15 flasks, leaving 14 amino acids in each vessel. The flasks were treated as previously described and the results are tabulated in Fig 4.

The 15 amino acid control flask yielded 240 KU. The combined omission of aspartic acid, isoleucine, alanine, and any one of the following: lysine (230 KU), tryptophan (238 KU), glutamic acid (226 KU), serine (211 KU), histidine (212 KU), tyrosine (206 KU), threonine (194 KU), and leucine (212 KU) again resulted in equivalent or better growth.

Fig 3

3-Amino Acid Deletion plus Glucose

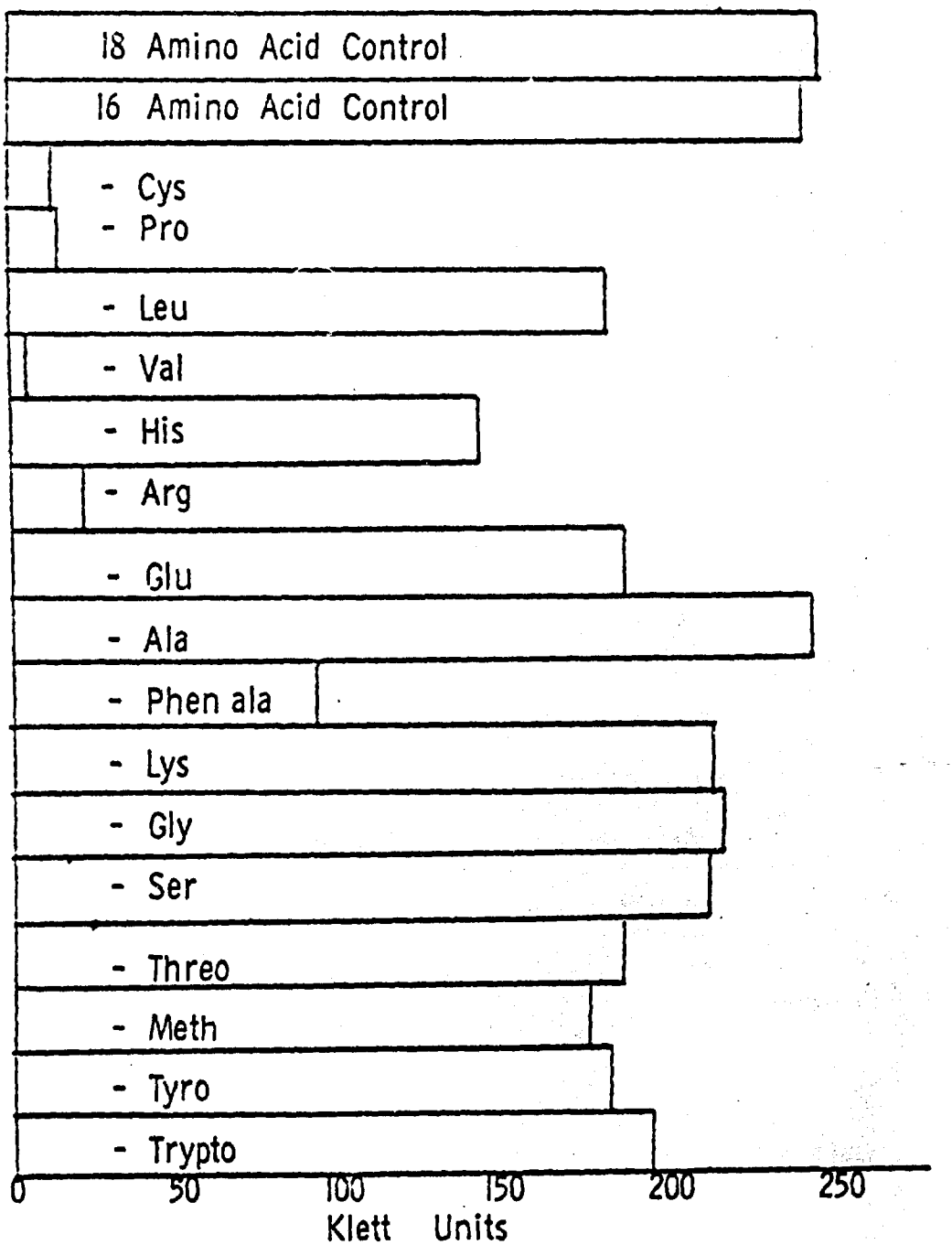
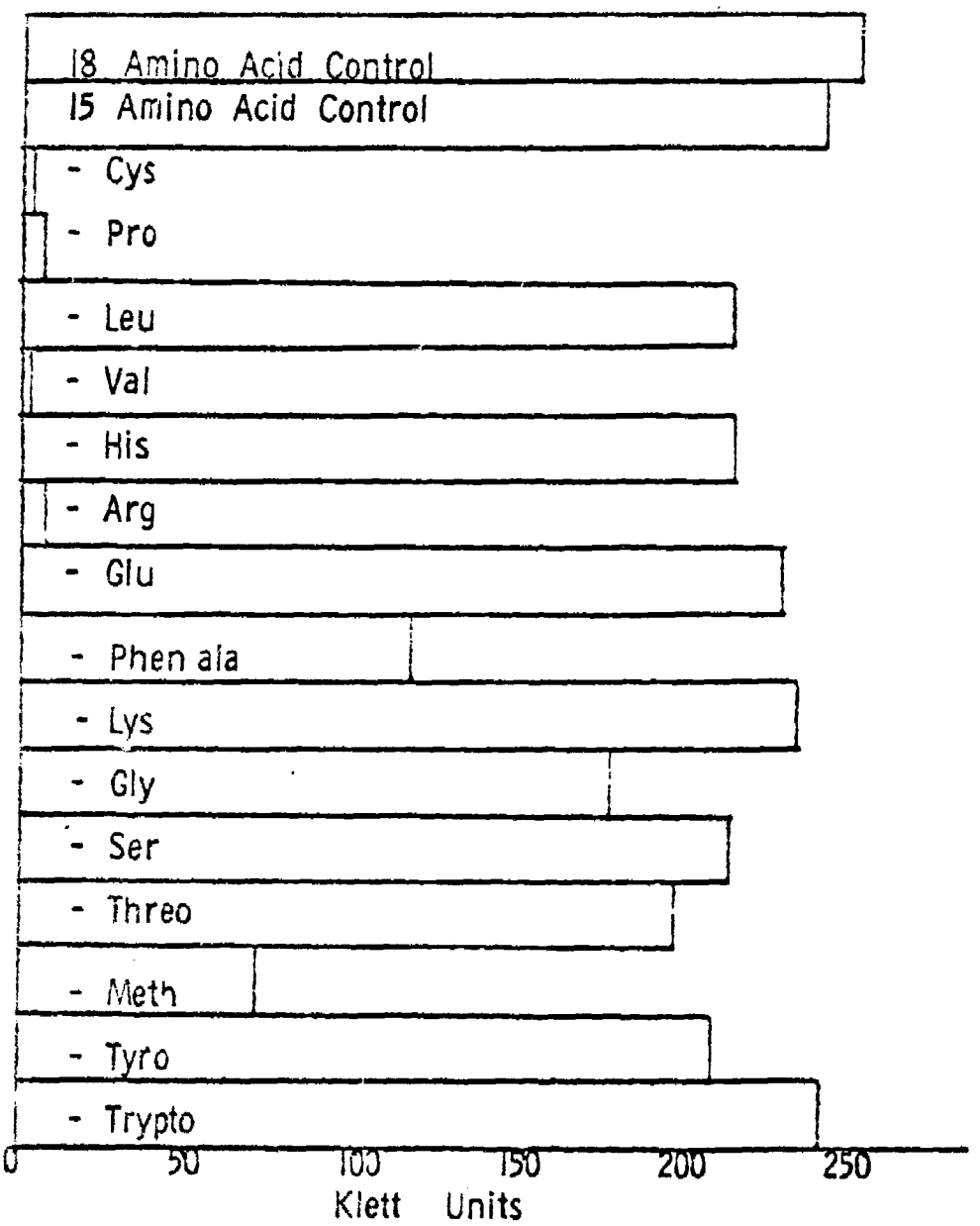


Fig 4.

4-Amino Acid Deletion plus Glucose



The omission of lysine, aspartic acid, isoleucine, and alanine from the defined medium was selected for the next series in which 5 amino acids were omitted. The protocol was again the same as previously described, this time with 14 vessels and 13 amino acids in each. The results are tabulated in Fig 5.

The combined omission of aspartic acid, isoleucine, alanine, lysine, and any one of the following: tryptophan (256 KU), tyrosine (244 KU), glutamic acid (244 KU), glycine (214 KU), serine (216 KU), threonine (214 KU), and methionine (216 KU) again resulted in equivalent or better growth. The 14 amino acid control in this case was 258 KU. The combined omission of tryptophan and the previous 4 non-essential amino acids was chosen as the control for the next series in which 6 amino acids were deleted. Again, the previous protocol was followed, this time with 13 flasks and 12 amino acids in each. The data are tabulated in Fig 6.

The 13 amino acid control was 225 KU. The combined omission of aspartic acid, isoleucine, alanine, lysine, tryptophan, and any one of the following: glutamic acid (226 KU), serine (220 KU), and tyrosine (200 KU) again resulted in equivalent growth.

Both the glutamic acid-less and serine-less mixtures were tested further. The standard protocol was used, this time with 12 flasks and 11 amino acids in each. The results are shown in Fig 7. Omission of aspartic acid, isoleucine, alanine, glycine, tryptophan, glutamic acid and any one of the following: serine (209 KU) and methionine (218 KU) resulted in equivalent growth. The 12 amino acid control was 224 KU.

In a similar experiment, serine, instead of glutamic acid, was deleted; otherwise, the conditions were the same. The results are depicted in Fig 8.

Fig.5

5-Amino Acid Deletion plus Glucose

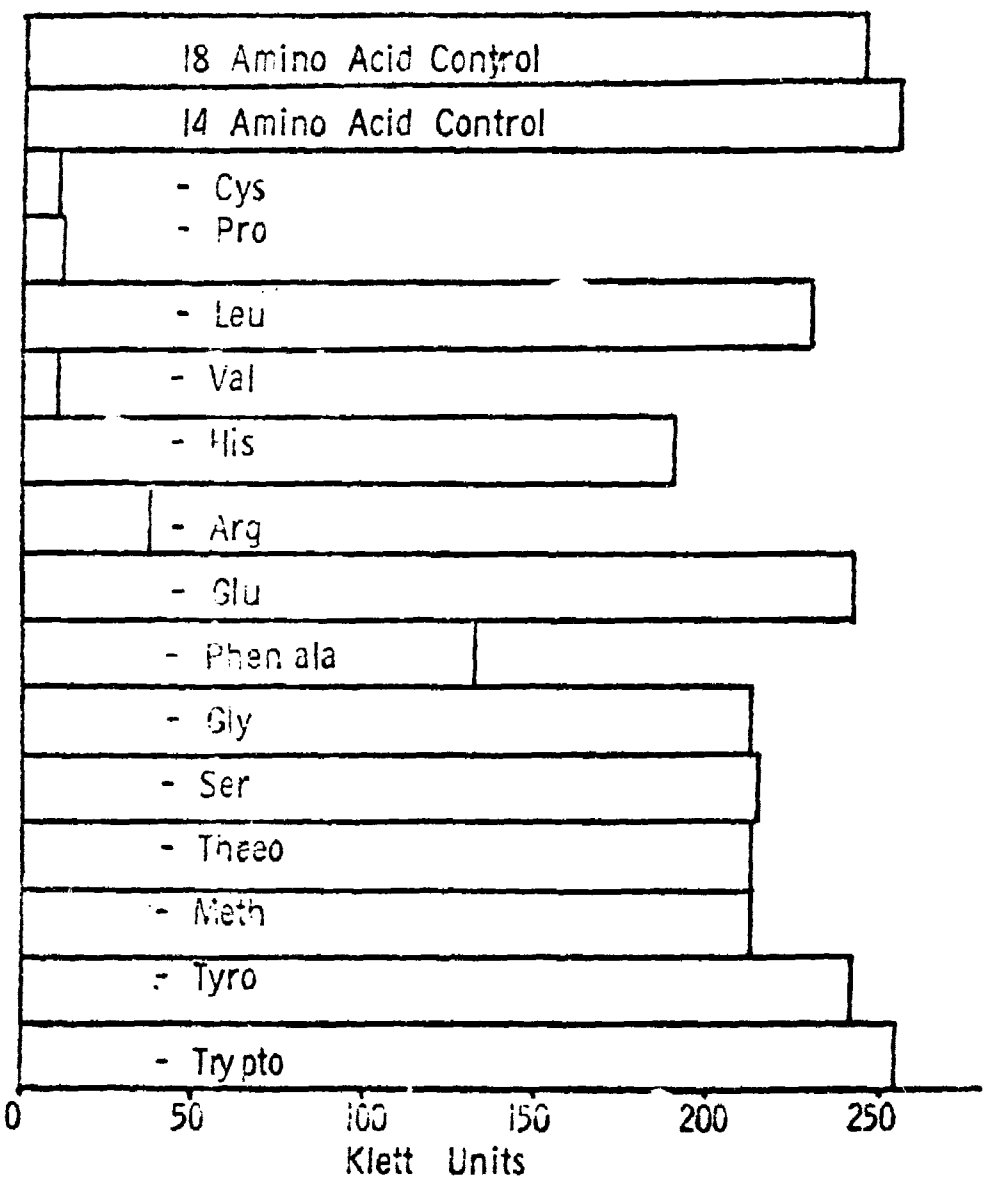


Fig 6

6-Amino Acid Deletion plus Glucose

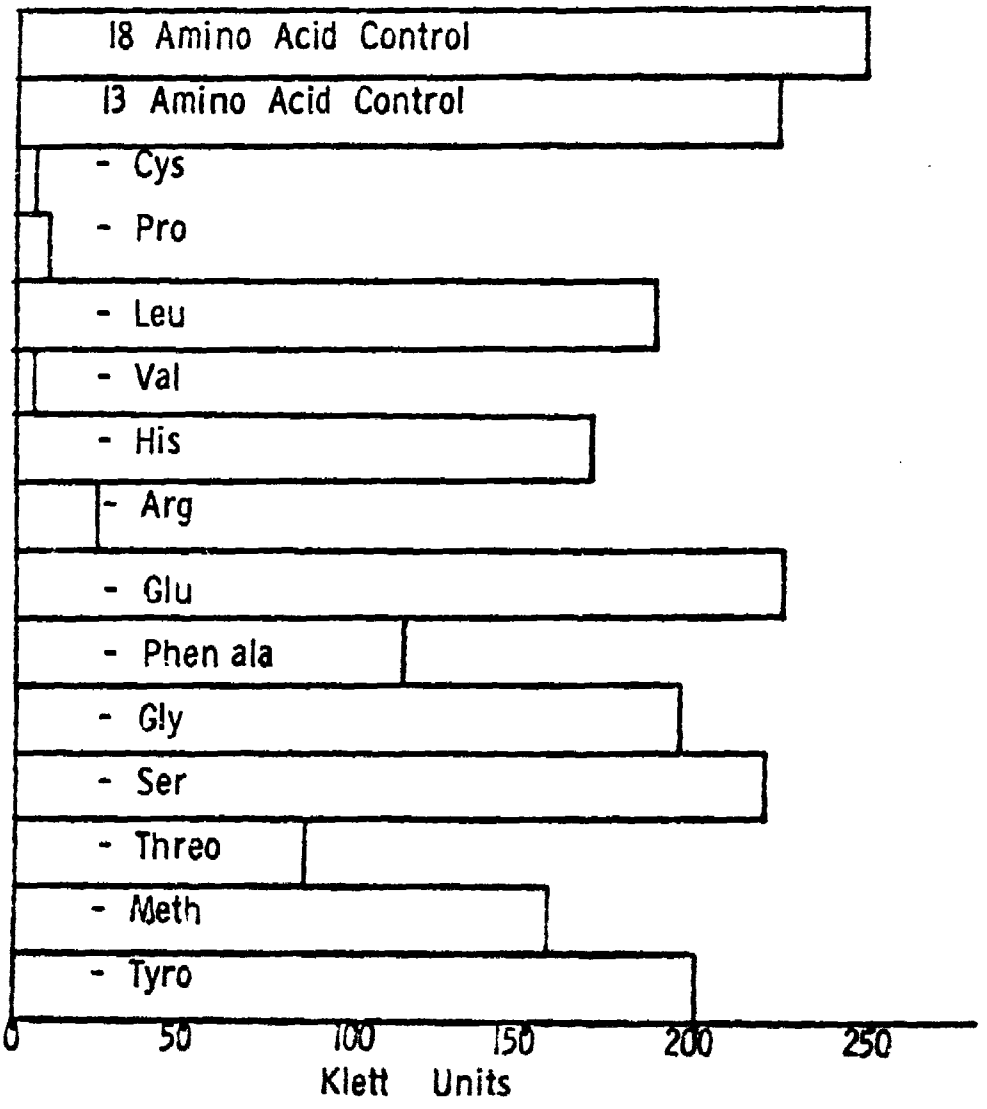


Fig 7

7-Amino Acid Deletion plus Glucose

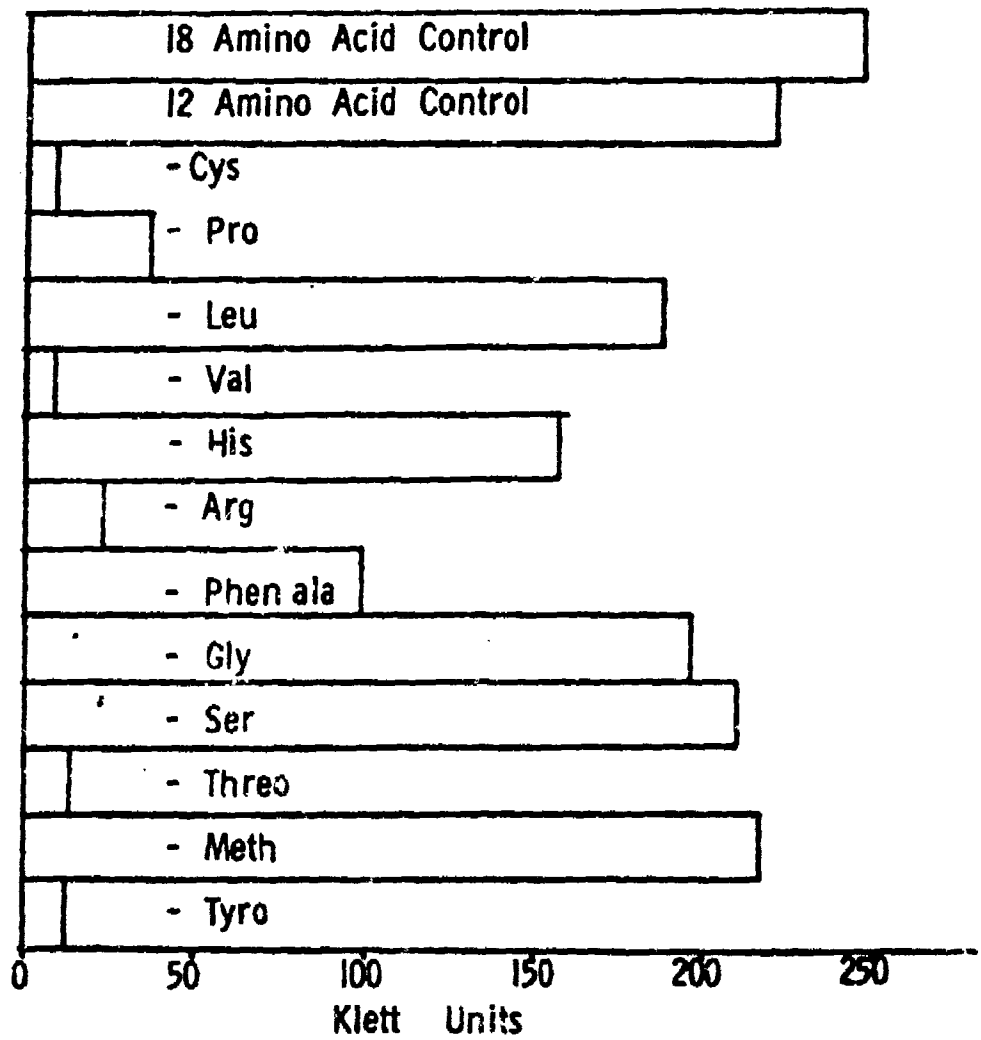
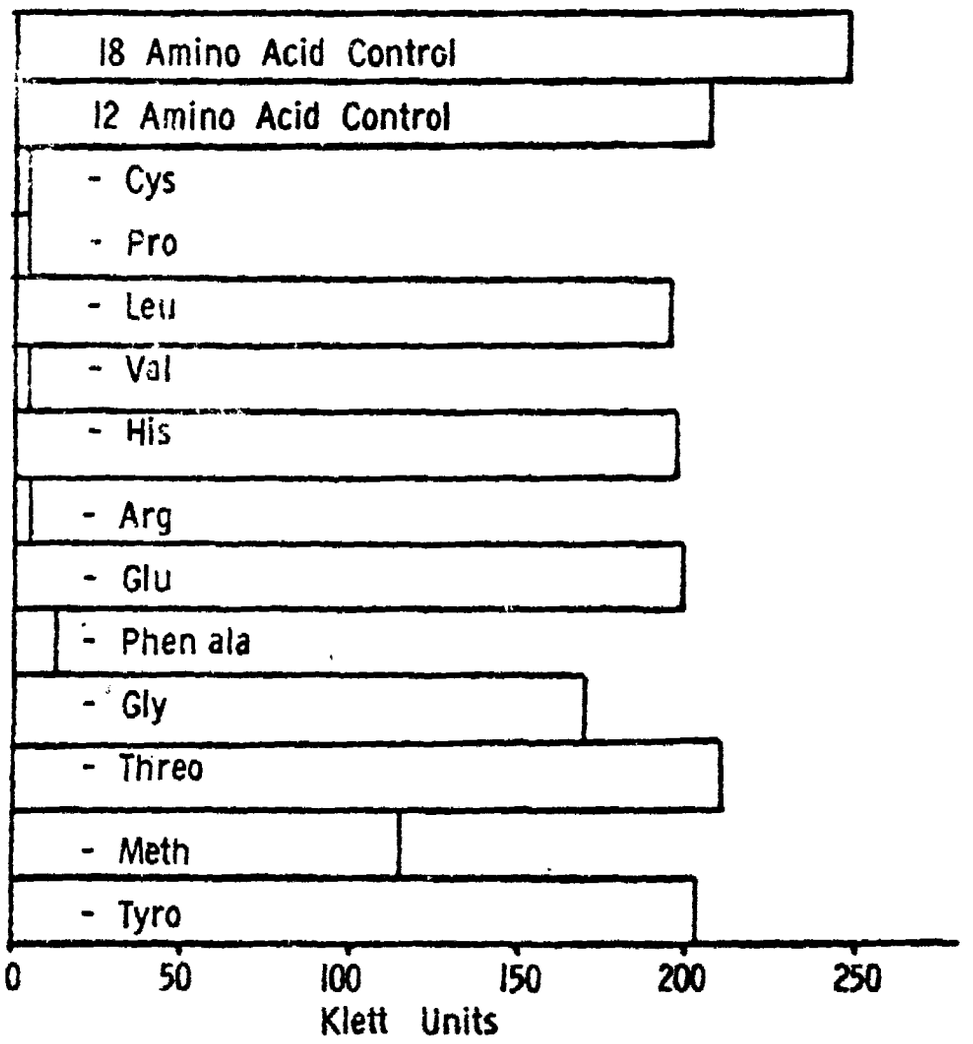


Fig 8

7-Amino Acid Deletion p_us Glucose



The combined deletion of aspartic acid, isoleucine, alanine, lysine, tryptophan, serine and any one of the following: glutamic acid (200 KU), threonine (212 KU), and tyrosine (204 KU) again resulted in equivalent growth. The 12 amino acid control was 208 KU. It should be noted that in the glutamic acid-less series, omission of serine gave equivalent growth, while in the serine-less series, omission of glutamic acid gave satisfactory growth. In both of these cases, the combination of amino acids actually deleted was the same.

The threonine-less and glutamic acid-less combinations were both investigated further. In the threonine-less series, the same protocol was again used; a control omitting the previous 6 non-essential amino acids in addition to threonine served as a reference medium. The omission of 8 amino acids was tested with respect to this culture; the results are shown in Fig 9. The control yielded 172 KU but none of the experimental vessels gave permissible growth yields. Thus, this series did not result in satisfactory growth according to the predefined criteria.

An experiment identical to the above in which glutamic acid instead of threonine was omitted, gave the results shown in Fig 10. The glutamic acid-less control gave a value of 195 KU. In this series, only the methionine-less flask showed equivalent growth; the absence of methionine was tested in an attempt to eliminate 9 amino acids. The results of this experiment are given in Fig 11. The control culture produced 197 KU, and only the histidine-less flask (209 KU) showed satisfactory growth.

Elimination of 10 amino acids from the defined medium was tested on the basis of the histidine-less findings. Again, the procedures were the same as previously described, this time with 8 amino acids. The results are given in Fig 12. The control yielded an unacceptable value of 95 KU, and none of the

Fig 9

8-Amino Acid Deletion plus Glucose

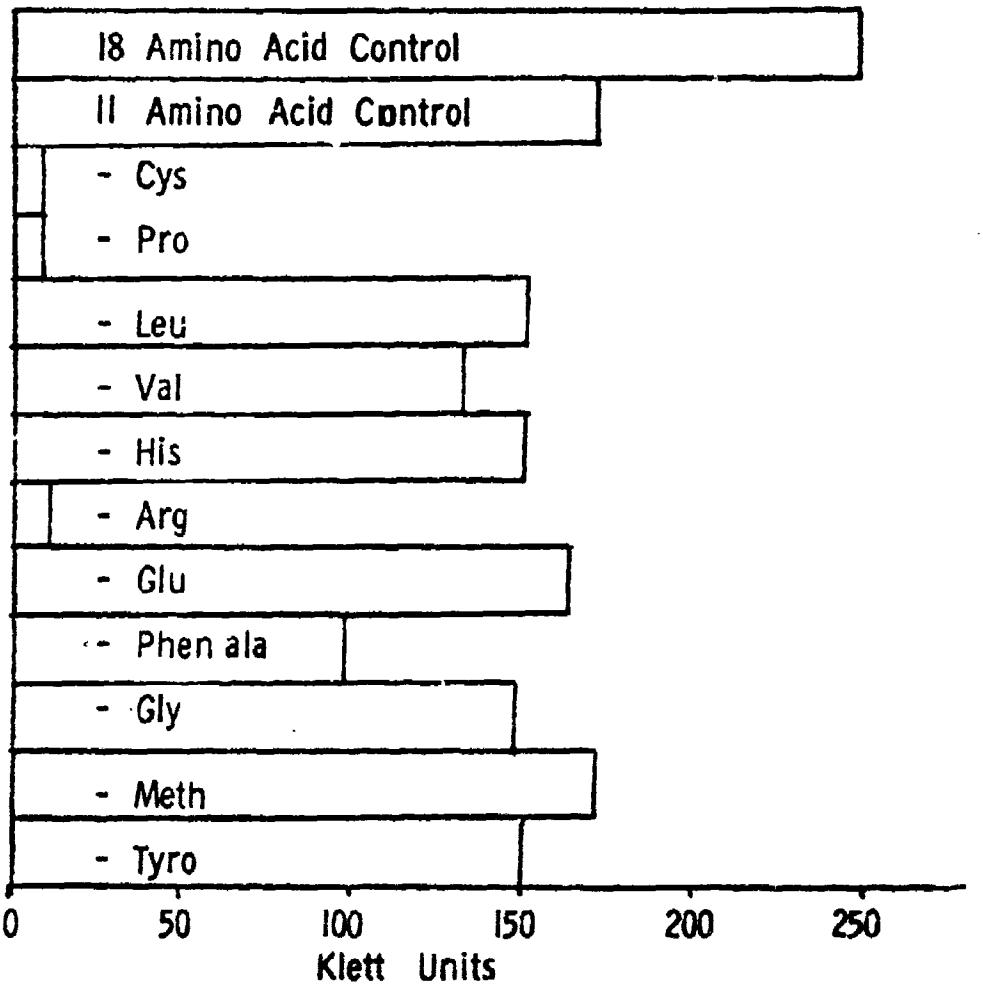


Fig. 10
8-Amino Acid Deletion plus Glucose

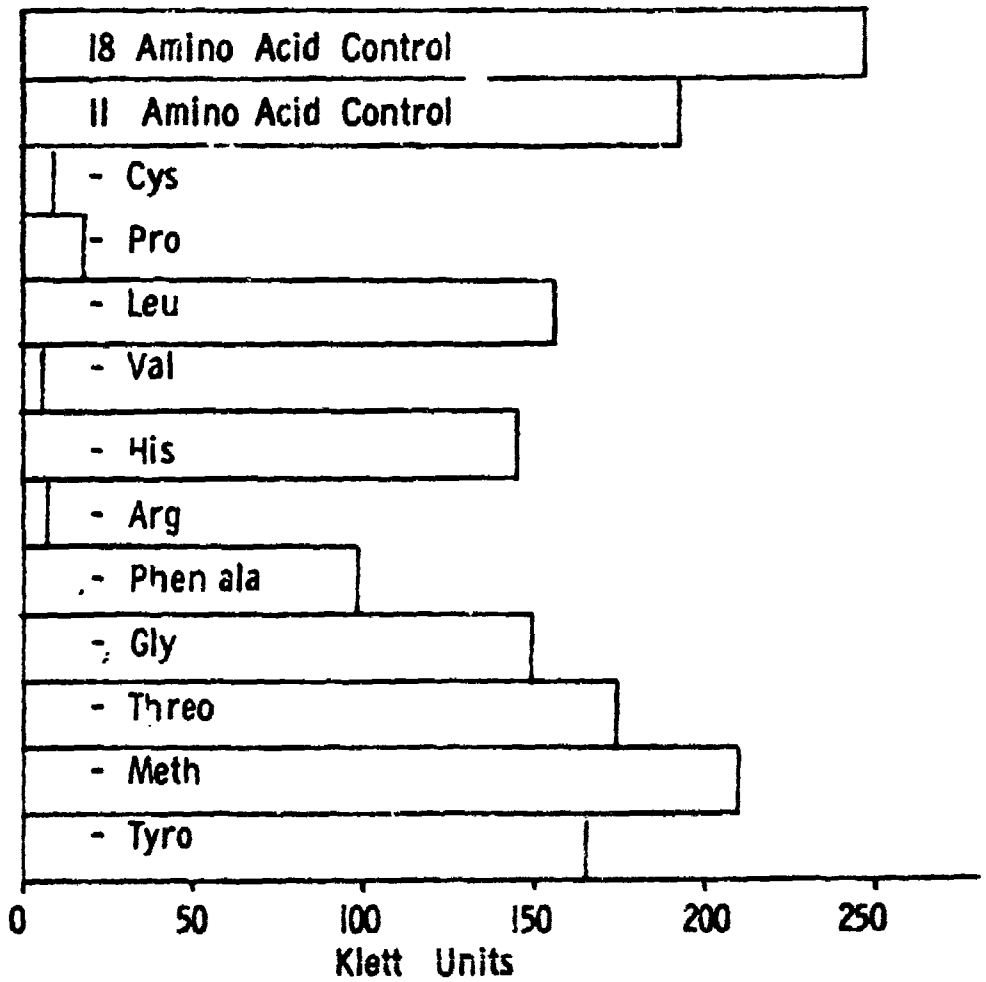
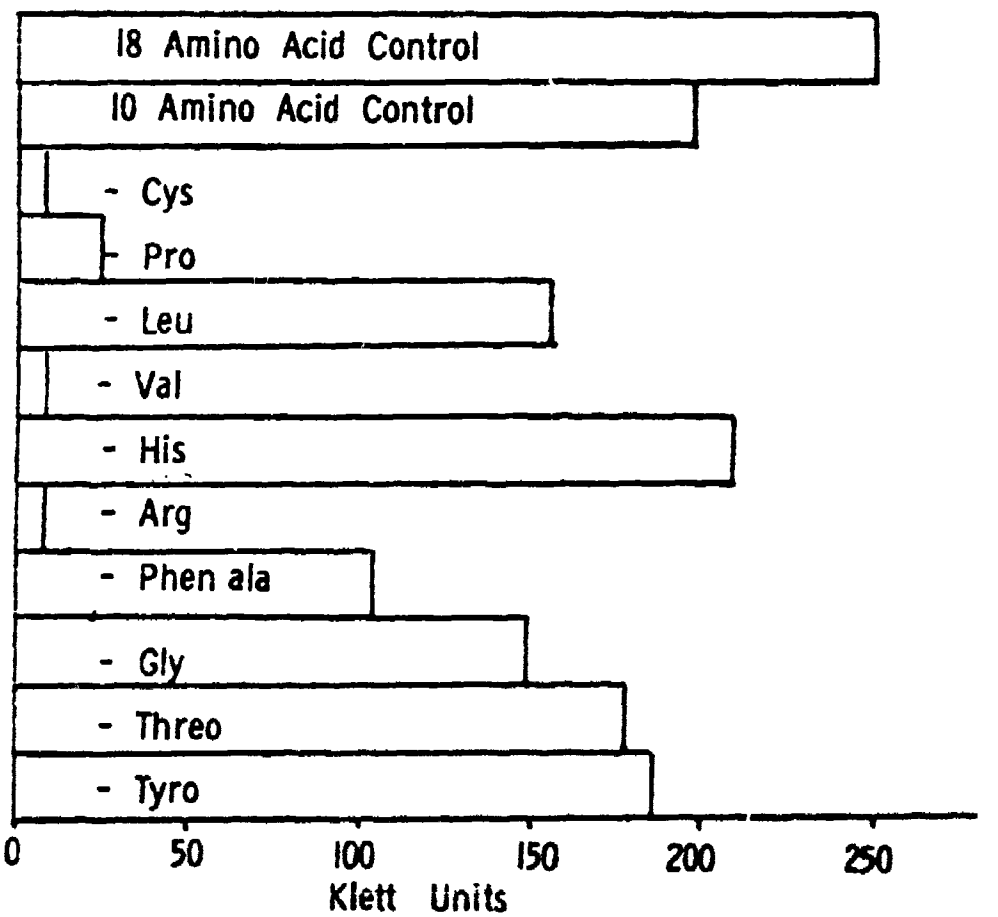


Fig 11

9-Amino Acid Deletion plus Glucose



experimental flasks showed satisfactory growth. With these results the amino acid deletion experiments were terminated.

The final amino acid mixture consisted of glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteine, methionine, proline, arginine, and histidine. The results of testing all the previous controls used in the deletion experiments are depicted in Fig 13. The results are in accordance with the findings of the previous experiments; the first 8 vessels yielded acceptable growth while the last two, in which methionine and histidine were deleted, were unacceptable.

Omission of any one of the 4 amino acids, proline, arginine, valine, or cysteine, resulted in little or no growth. However, a mixture composed solely of these apparently essential amino acids did not support growth.

Glutamic Acid. Since S-6 grew well in PHP alone, it must use amino acids as a carbon source because other organic compounds are not significant in this medium. Endogenous respiration of S. aureus can be attributed in part to the disappearance of glutamic acid from the free amino acid pool (5,13). Furthermore, suspensions of S. aureus metabolize glutamic acid in the presence of either an endogenous (5) or exogenous (3,5) energy source. This energy requirement is apparently necessary for transport of the amino acid across the cell membrane.

Growth of S-6 was tested on glutamic acid (as monosodium glutamate, MSG) as the main carbon source in the defined medium. The yield was in the same range when grown on MSG (196 KU) or glucose (208 KU).

Other amino acids were also tested as carbon sources in the defined medium. None of the following amino acids known to exhibit O_2 uptake (5) were utilized: threonine, proline, glycine, alanine, arginine, cysteine, histidine

Fig. 12
10-Amino Acid Deletion plus Glucose

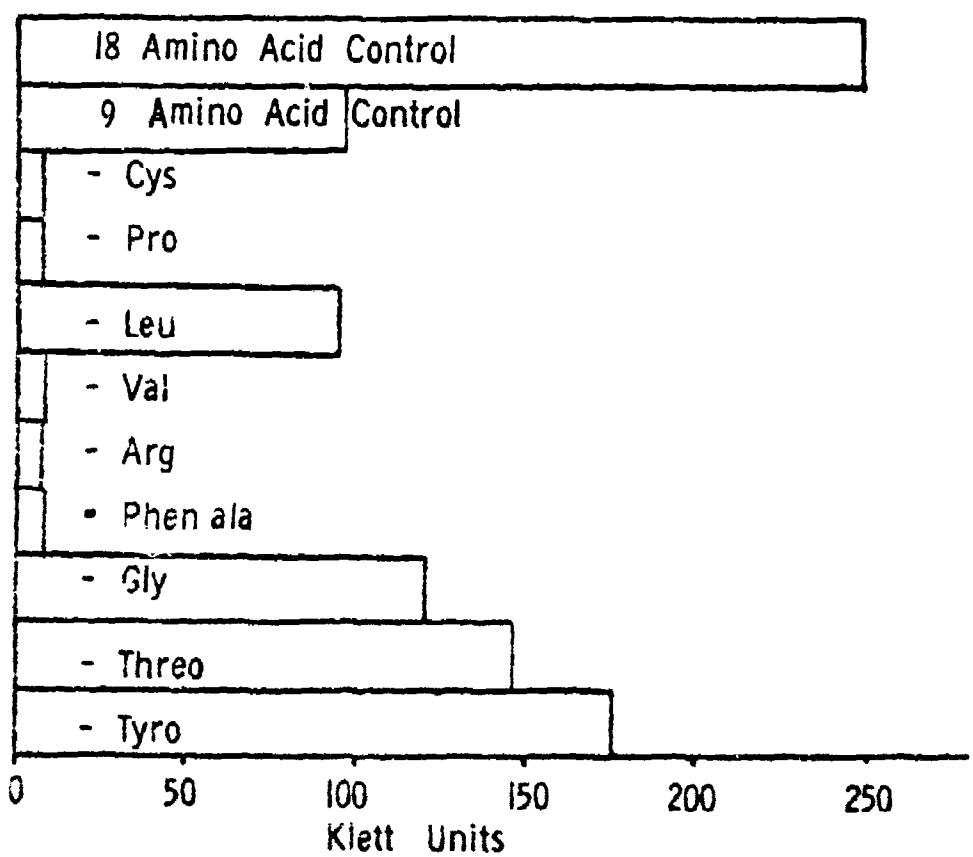
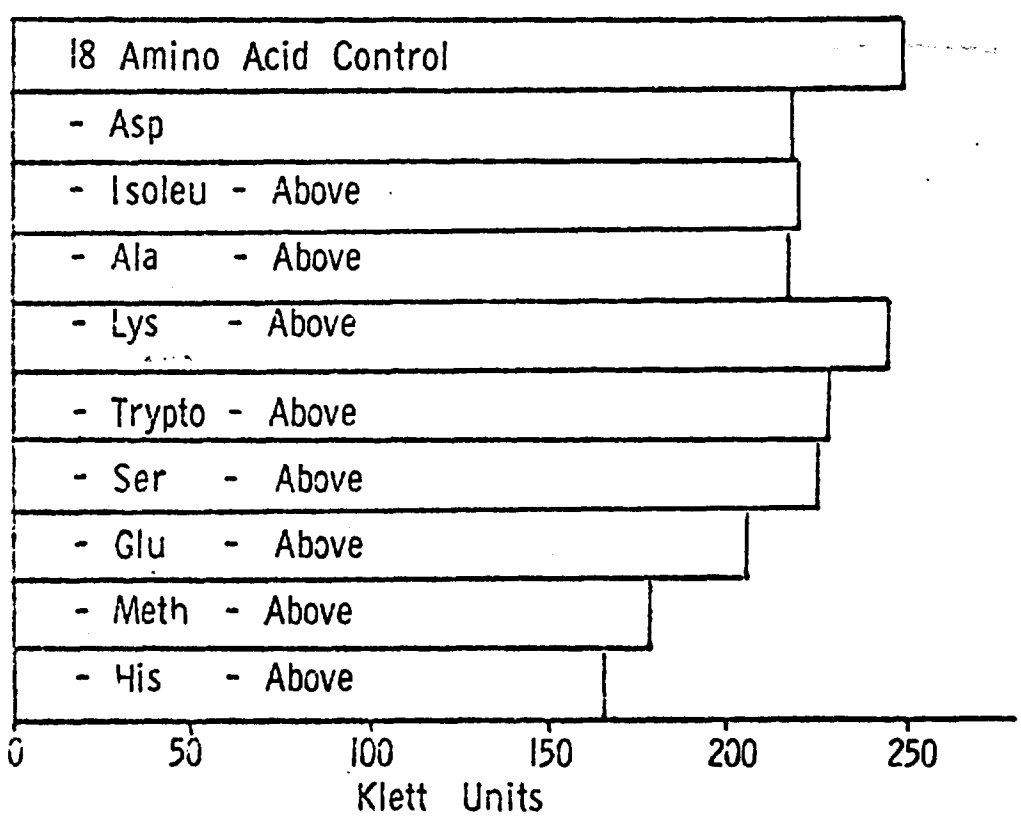


Fig 13

Growth in 10 Controls plus Glucose



and serine. Each was tested by addition at a concentration of 0.2% and compared with the growth yield on 0.2% MSG. Proline gave the highest value (46 KU) compared to MSG (116 KU).

Biotin. In early experiments only thiamine and nicotinic acid were incorporated into the medium to satisfy the known vitamin requirements of S. aureus. Growth of S-6 in defined medium containing glucose as carbon source was virtually unaffected by the addition of yeast extract.

However, MSG was not metabolized in defined medium in the presence of thiamine and nicotinic acid as the only added vitamins; with the addition of yeast extract, MSG was used as a carbon source. Biotin substituted for this growth factor requirement; cells incubated without biotin yielded ca. 47 KU. Addition of biotin to glucose-grown cells did not stimulate growth.

The Salts. The inorganic salts are reported as wt/vol, final % concentration. The ammonium ion concentration was tested by deletion of the 0.4% $(NH_4)_2HPO_4$ in Gale's solution (2), replacement of the phosphate by 0.33% Na_2HPO_4, and addition of 0.05% NH_4Cl. The final % concentrations of the other salts were: KH_2PO_4, 0.1; $MgSO_4 \cdot 7H_2O$, 0.07; NaCl, 0.1; and $FeSO_4 \cdot 7H_2O$, 0.001; the final pH was 7.1. These modifications led to an increase in the growth yield when either glucose or glutamic acid served as carbon source. The results are shown in Table 1. This mixture of inorganic salts was adopted

TABLE 1

GROWTH IN BASAL MEDIUM PLUS MSG OR GLUCOSE

Gale's basal media		Modified basal media	
MSG	Glucose	MSG	Glucose
196 KU	256 KU	216 KU	282 KU

as the final combination.

Enterotoxin Formation. Growth in 0.5% PHP without added glucose or MSG resulted in about 7X more toxin than that produced in defined medium with glucose or MSG. This concentration of PHP supported a cell yield equivalent to the defined medium with added carbon source. Upon addition of glucose or MSG to the minimum PHP concentration (0.06%) necessary to support growth equivalent to the defined medium, toxigenesis was again very low. These results indicated that the quantity of toxin produced in the defined medium must be limited by available precursors and that the low values are most likely a response to their presence or absence rather than to the ability to produce toxin.

II. Effect of pH on Growth and Toxigenesis

It was previously shown by Sulzer and Peters (16) and Mah and Regier (7) that changes in pH which accompany the growth of enterotoxigenic strains of Staphylococcus aureus in PHP medium underwent a sequence of changes starting with an acid pH and ending with an alkaline pH. Such results were typical of cells grown on PHP and could be repeated from one experiment to another. Peters (12) showed that little or no toxin was produced in the presence of 1% glucose in PHP. Examination of the pH of cultures grown under these conditions revealed a terminal pH of 5 or lower.

Because of these observations, further work was performed in our laboratory by R. E. Marland (9) and W. Biggs to determine whether there is, in fact, an effect of pH on enterotoxin production. The effect of pH was investigated under the following conditions: 1) the initial pH was adjusted to various predetermined levels by the addition of standard HCl or NaOH. The medium was not buffered, and the pH was permitted to change as a result of cell growth; 2) the pH of the medium was adjusted to a selected level by the addition of phosphate buffer at a molarity which would buffer effectively; 3) the pH was held constant at a selected concentration during the course of growth of cells by continuous addition of sterile acid or base.

The results of growth and toxigenesis were compared to a culture in which no attempt was made to adjust or control the pH of the PHP medium. In this case the changes in pH were simply recorded.

Methods

Fernbach flasks were modified by making three indentations (baffles) at the base of the vessels to permit greater agitation of the culture medium

during shaking. In addition, the flasks had two ports to permit sampling and adjustment of pH. Each vessel received 400 ml of PHP medium which was adjusted to the desired pH prior to sterilization by the addition of standard acid or base. The pH was not altered after sterilization; the values selected for the experiment were the following: 5.0, 6.0, 7.0, 8.0, and 6.2; the latter value represented the pH of the normal unadjusted PHP medium. The flasks were inoculated with the standard inoculum and shaken on a rotary shaker at 37 C. Using a sterile syringe and hypodermic needle, samples were removed at 0, 6, 12, 24, and 36 hr. Again growth was measured by the Klett-Summerson colorimetry. The pH was measured with a Beckman Zeromatic pH Meter, and the enterotoxin was estimated by the method of Wadsworth et al. (18).

RESULTS

The results of these experiments are shown in Table 2. Examination of this table shows that the pH of these cultures did not remain at the initial pH for long. A comparison of the values obtained at the 36 hr sampling showed that none of the adjusted samples varied from pH 7.6 by more than 0.1 units. In addition, the samples showed little difference in growth as determined by turbidity measurements. On the other hand, both the pH 7 and pH 8 samples yielded 100 µg/ml of enterotoxin compared with 50 µg/ml in the pH 5 and 6 samples. The control cultures with unadjusted pH permitted a higher growth and toxin yield. These values were virtually the same as those obtained when the same medium was introduced in 50 ml aliquots into the smaller Nephloflasks.

In the next series of experiments the pH was buffered at 7.0 by the addition of phosphate buffers containing equimolar amounts of both mono- and dibasic sodium salts. A renge of 6 molarities between 0.1 M and 1.0 M was

prepared. After addition of the phosphate, the medium was adjusted to pH 7.0 by the addition of standard NaOH. Again the medium was sterilized in the modified Fernbach flasks in 400 ml quantities. The protocol was, otherwise, the same as previously described.

The results of these experiments are depicted in Table 3. Throughout the range of molarities chosen, the final pH remained close to the initial reading of 7.0; however, the effect is one of inhibition of both growth and

Table 2. Comparison of Growth, pH, and Enterotoxin Production in Cultures of Protein Hydrolysate at Several Initial pH Values

Condition	Time (Hours)	pH	Turbidity (Klett Units)	Enterotoxin (μg/ml)
Initial pH adjusted to 5.0	0	5.0	0	0
	6	5.0	125	1.5
	12	5.8	252	10
	24	6.9	375	--
	36	7.5	465	50
Initial pH adjusted to 6.0	0	6.0	0	0
	6	5.4	165	4
	12	6.4	253	8
	24	7.2	370	20
	36	7.5	445	50
Initial pH adjusted to 7.0	0	7.0	0	0
	6	5.6	175	3
	12	6.7	261	10
	24	7.4	405	20
	36	7.6	475	100
Initial pH adjusted to 8.0	0	8.0	0	0
	6	6.5	171	1
	12	6.9	247	8
	24	7.5	375	20
	36	7.7	445	100
pH not adjusted	0	5.8	0	0
	6	5.4	222	0
	12	7.2	465	100
	24	7.9	520	260
	36	8.1	510	260

toxin production. The inhibition is proportional to the concentration of the phosphate buffer, with less growth and toxin produced at higher values of phosphate. In earlier experiments, it was found that in cultures containing 0.03 M phosphate at pH 7.0 maximum growth and normal amounts of toxin were produced. In these cultures, however, the terminal pH values were approximately 8.0. These findings indicated that the presence of phosphate molerities sufficient to buffer against pH changes inhibited both growth and toxin production The increased concentration of the phosphate ion and not its effect on the pH alone appeared to be the primary inhibitory factor.

Teble 3. The Effect of Phosphate on pH, Growth, and Enterotoxigenesis

Molarity of PO_4	24 hour pH	Turbidity (Klett Units)	Enterotoxin (µg/ml)
0.1	7.22	490	130
0.2	7.20	482	65
0.3	6.95	425	32
0.4	6.95	396	16
0.5	7.0	128	0
1.0	7.0	0	0

Consequently, the pH of the medium was held constant by the addition of sterile acid or base during growth of the cultures; the pH was maintained at the following values: 5.0, 6.0, 7.0, 8.0, and 9.0. This was accomplished by using a modified Coleman Titrimeter. Glass tubing of 4 mm internal diameter was drawn to a small tip and inserted through a rubber stopper into the culture medium. The glass tubing was connected to 1/8" rubber tubing which was passed through an actuating mechanism on the titrimeter and attached to a

sterile burette containing either sterile 1.0 N NaOH or sterile N HCl. The cultures were inoculated and incubated as previously described; they were checked at close intervals, particularly between 6 and 8 hr, at which time it was necessary to convert from base to acid additions. The titrimeter was actuated by changes in pH registered by a Beckman pH Meter from a combination electrode inserted into the flask.

The data obtained from these experiments are shown in Table 4. Maintenance of pH at a constant level resulted in toxin yields which were much lower than those obtained when pH was uncontrolled. There was no apparent trend in correlation between pH and toxin production.

Table 4. Growth and Toxigenesis at Constant pH Maintained by Titration

pH (± 0.2)	Turbidity (Klett Units)	Enterotoxin (µg/ml)
5.0	315	0
6.0	365	32
7.0	378	100
8.0	370	50
9.0	328	0

The control flasks in which the pH was normally unadjusted is depicted in Fig 14. The initial pH of unadjusted PHP medium is normally 5.8. The culture remains on the acid side for a period of approximately 3 hr. At the end of this time, there is a rapid rise in pH, increasing to a value of ca 8.2 at the end of 9 hr; the pH remains virtually unchanged from this point until 24 hr when the cultures were normally terminated. The final turbidity at the end of 24 hr was approximately 530 KU, and the final toxin value ca 260 µg/ml.

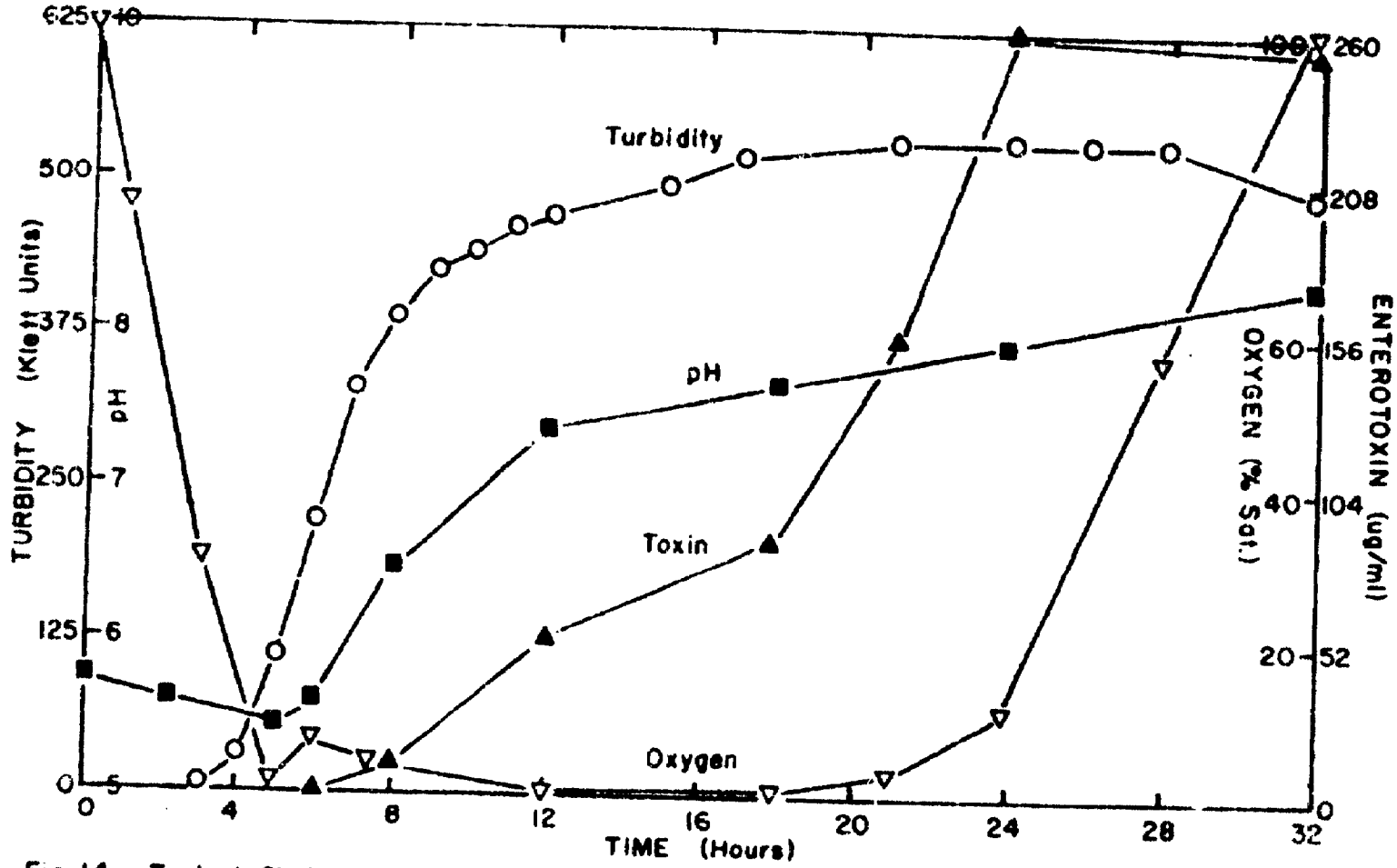


Fig 14 Typical Shake Culture Growth Curve for Strain S-6 Staphylococcus aureus in PHP Medium; Showing Enterotoxin Production, Changes in pH, and Dissolved Oxygen

All of these findings indicated that the pH had an effect on the quantity of toxin produced, but the formation of toxin is not completely eliminated by alteration of pH. Further investigations are underway to clarify these findings.

III. Effect of Gas Atmosphere and Temperature on Growth and Toxigenesis

Methods

Description of Equipment. The apparatus used for these experiments was designed and constructed especially for this purpose. Figure 15 shows the schematic diagram for the fermenter; it permitted the measurement of turbidity, pH, dissolved oxygen, and temperature without necessity for sample removal. In addition, it permitted a wide range of temperatures to be used, and it permitted the testing of various gas atmospheres.

The operation of the fermenter is as follows. The medium was introduced into a 1000 ml pyrex jar which contained a ground glass flange top. This cover contained 4 openings which could accommodate #4 rubber stoppers. A Model 0 Brosites Pump (Brosites Machine Co., 60 Church St., N.Y., N.Y.) was connected to the culture jar by rubber tubing. The medium was circulated by pumping from the culture jar to a coiled glass tube immersed in a water bath. Following heating, the medium flowed through a cuvette inserted into a Klett-Summerson Colorimeter equipped with a 540 mμ filter. The medium was then returned to the jar and the cycle repeated. The oxygen and pH electrodes and capillary outlets for sampling and pH adjustments were present at both the inlet and outlet of the culture jar. The gas mixture which served as the atmosphere for this system was passed through a sterile Gelman-type GA filter with a pore size of 0.2 μ, held in a sterile Swinny adaptor. The gas entered the system through a glass frit diffuser situated at the bottom of the culture jar. Gas flow was regulated by a needle valve at the cylinder outlet in addition to a water manometer in the line immediately in front of the culture jar. Spent gas was removed from the top of the culture jar and passed

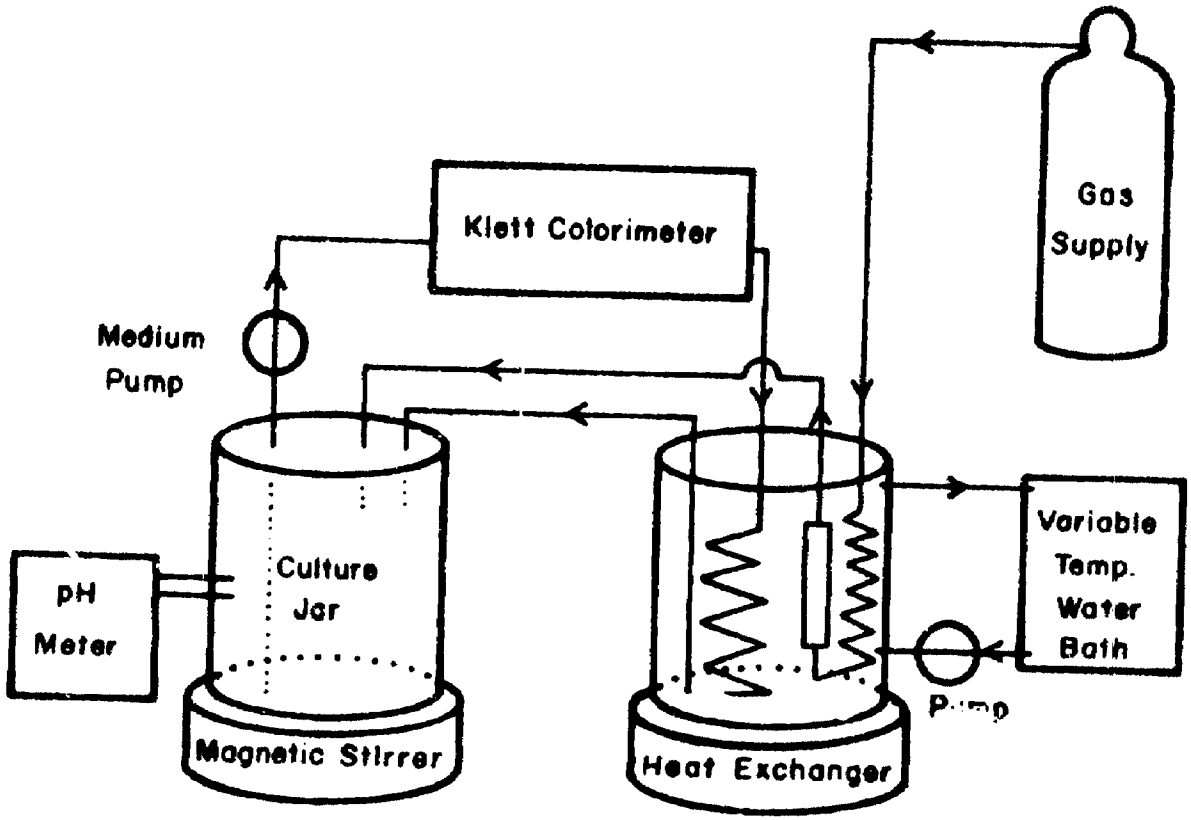


Fig. 15 DIAGRAM OF EXPERIMENTAL FERMENTER APPARATUS

through a moisture trap. The flow rate of the gas was calibrated by measuring the water displaced from an inverted graduated cylinder at the end of the exhaust line; flow rates ranged from 10 cc/min to 1200 cc/min.

Constant agitation was maintained by setting the flow of the medium through the Brosites pump at such a rate that an equivalent of the entire volume (500 ml) of medium circulated through the system in 5 min. In addition to the recirculation, agitation was insured by use of a magnetic stirrer set at a fixed speed which resulted in a vortex within the culture and immediately adjacent to the gas diffusing frit.

The entire apparatus was sterilized by autocleving at 15 lbs steam pressure for 15 min at 120 C. The Gelman filter, through which the gas passed, was sterilized in the line at the same time. When either the oxygen of pH electrodes were used, they were sterilized separately in chlorine, rinsed in sterile distilled water, and inserted into the sterilized culture jar.

The Oxygen Electrode. The dissolved O_2 content of the cultures was measured by means of a Galvanic Cell Oxygen Analyzer developed by Mancy and Westgarth (8). When used with the fermenter, the electrode was attached to a Bausch and Lomb Model VOM 6 microamp meter equipped with a recorder. A new electrode was prepared for each experiment and calibrated in O_2 saturated medium before use. The recorded value was expressed as per cent saturation with O_2 and was read from the calibration curve established for that electrode. The zero per cent saturation is that reading established in a saturated solution of sodium sulfite.

Other Methods. Protein was determined by the procedure of Lowry, et al. (6). Relative lipase was measured by the method of Nelson (11). In this

method, a reagent blank containing water instead of culture filtrate served as a control; both 30 and 60 min readings were recorded.

RESULTS

The effect of air as a gas atmosphere was tested by varying the flow rate as it passed through the cultures in the fermenter. Flow rates from 0 cc/min/900 ml culture liquid to 1200 cc/min/500 ml culture medium were tested. The liquid volume of the fermenter was purposely increased to 900 ml to reduce the volume of the head space in the fermenter. However, 500 ml was the standard liquid volume used in the fermenter when other flow rates were tested. The flow rates could be maintained throughout the entire experiment with fluctuations in rate not exceeding 10%. Measurement of the per cent saturation of the cultures with dissolved oxygen was made on both reference and fermenter samples.

When the flow rate of air was 1200 cc/min/500 ml of culture, there was a reduction in growth and enterotoxin formation. The data for this rate of flow are given in Fig 16. The yield of enterotoxin varied between 16 and 32 μg/ml at the 24 hr sampling time. The turbidity of the cultures ranged from 370 to 570 KU; the latter sample yielded only 20 μg/ml of enterotoxin.

At zero flow rates of air, 3 liquid volumes, 900 ml, 700 ml, and 500 ml were tested. The resulting head spaces above the media were 300 ml, 500 ml, and 700 ml respectively. The only oxygen available to these cultures was that concentration already present in the head space. The cultures were agitated by recirculation and magnetic stirring as previously described. The results of these findings are shown in Fig 17 and Fig 18. The enterotoxin and growth yields were directly related to the concentration of oxygen ori-

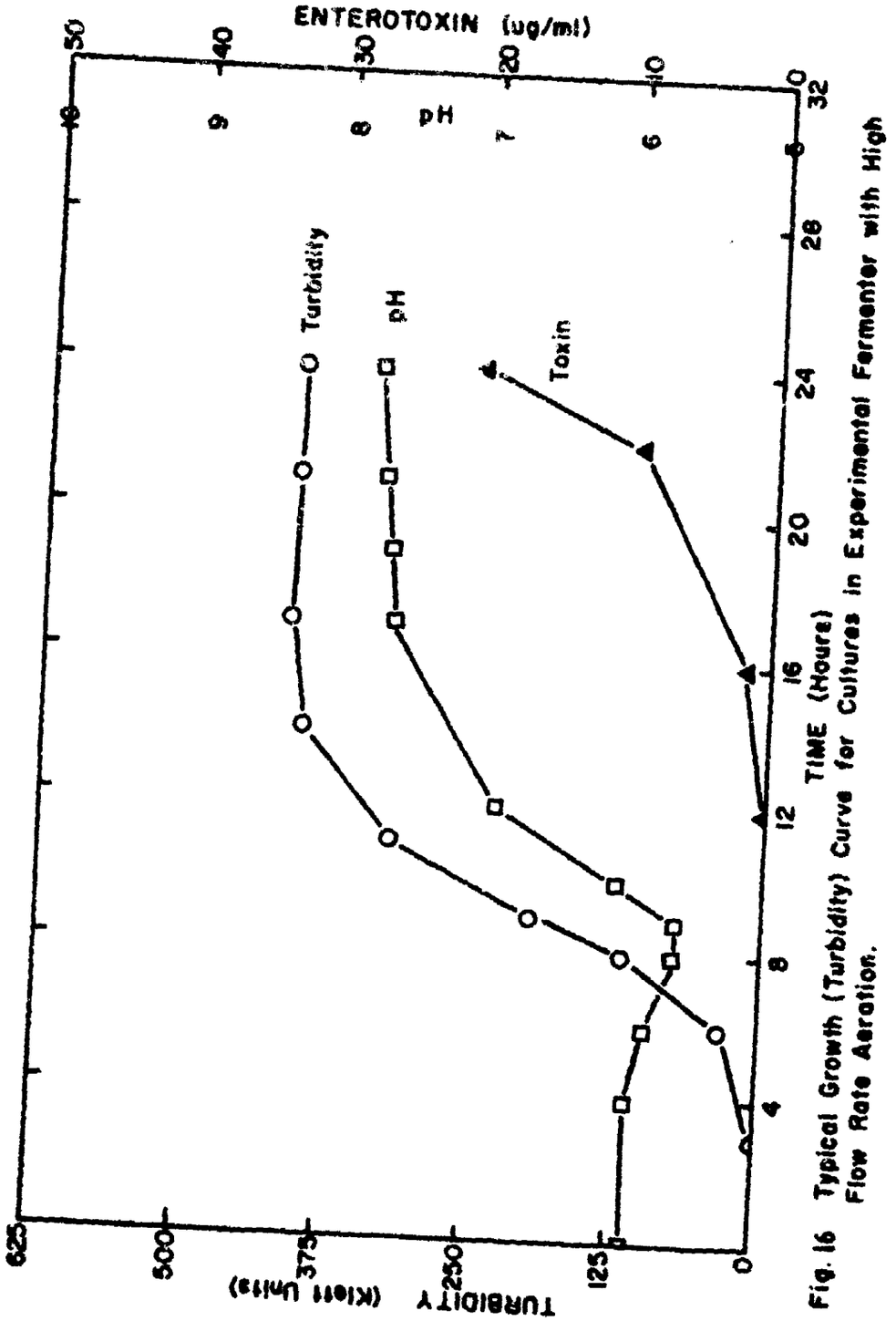


Fig. 16 Typical Growth (Turbidity) Curve for Cultures in Experimental Fermenter with High Flow Rate Aeration.

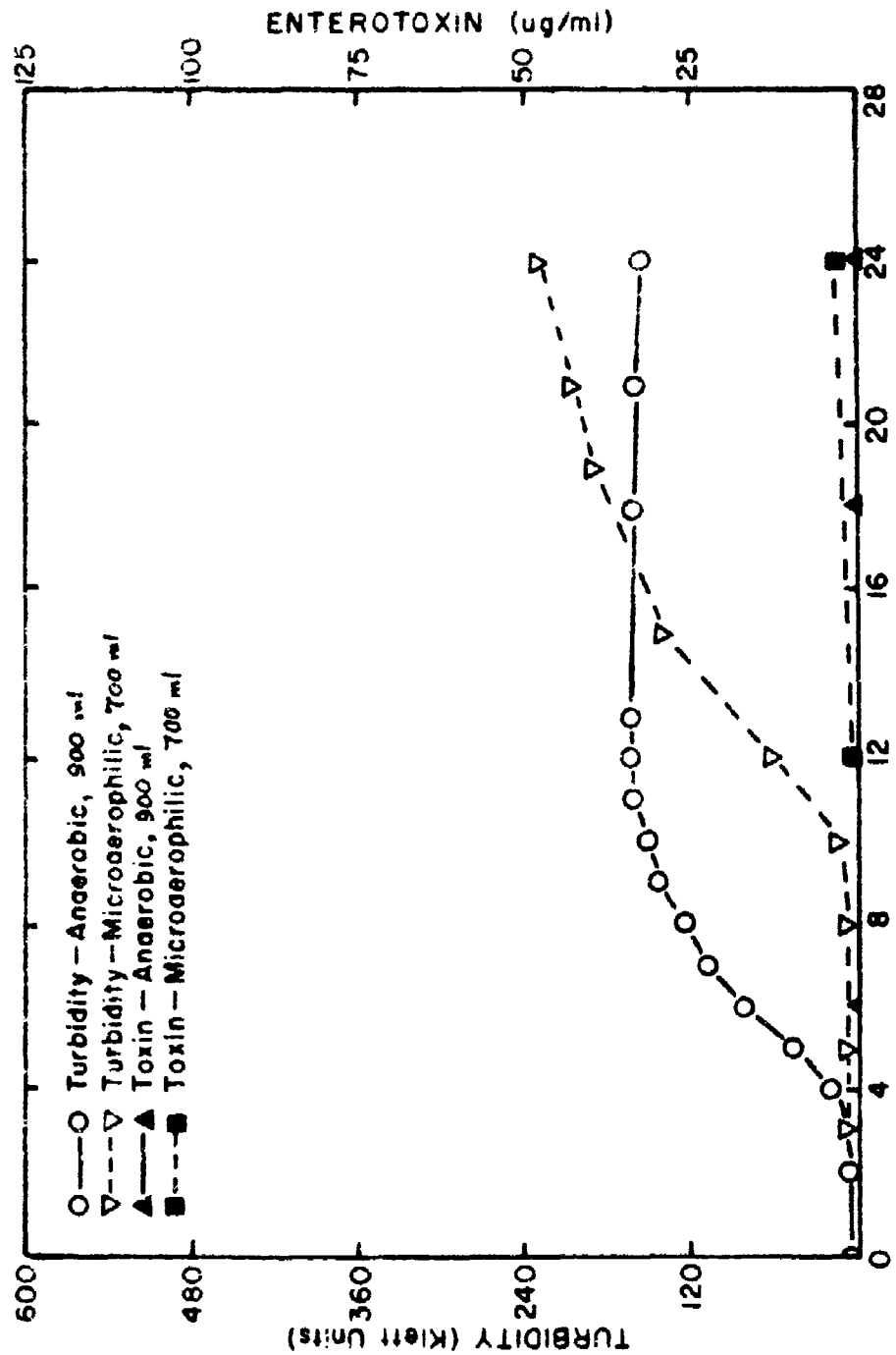


Fig. 17 Effects of Anaerobic and Microaerophilic Conditions on the Growth and Enterotoxin Production by Strain S-6 *Staphylococcus aureus*.

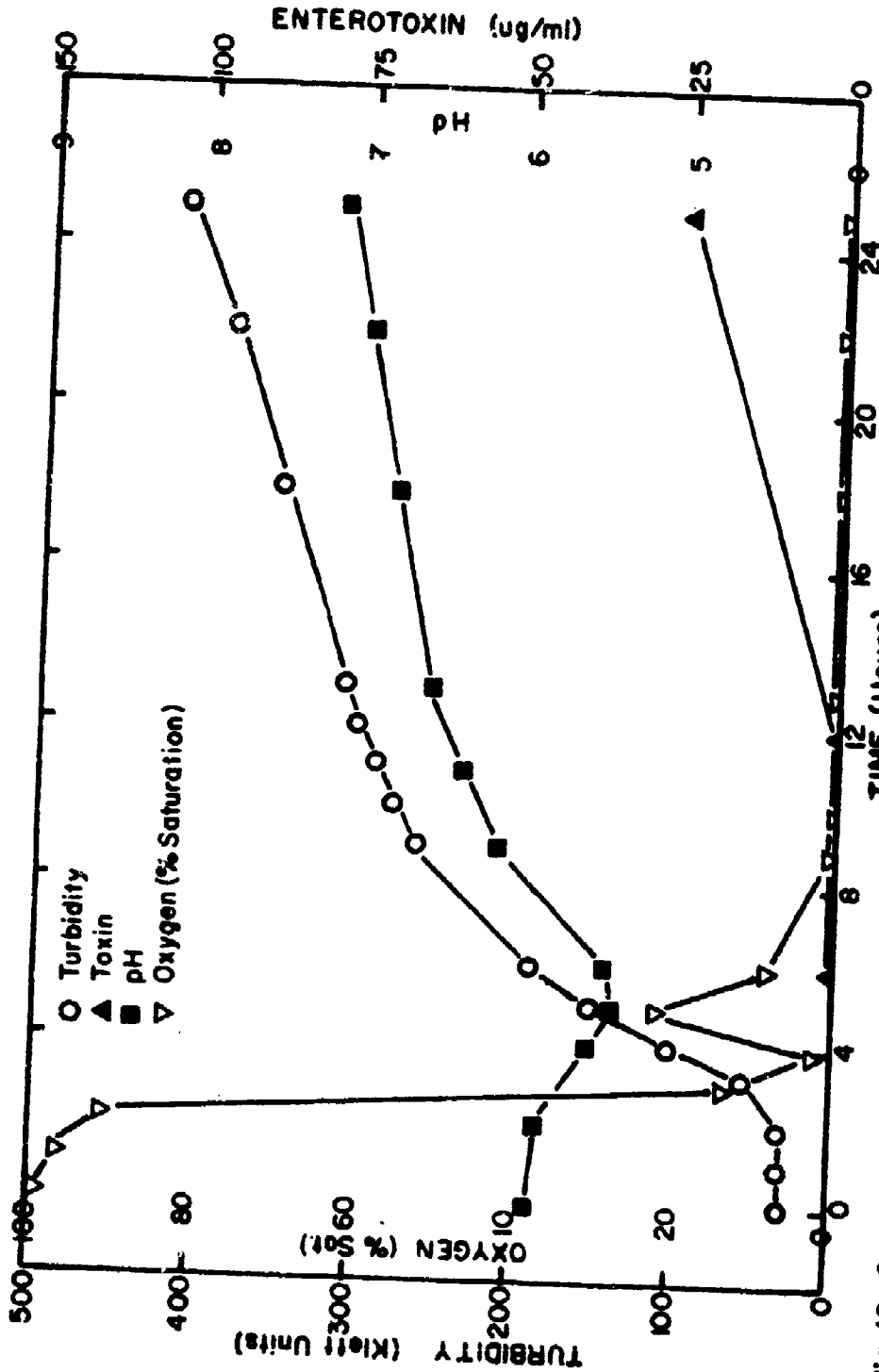


Fig. 18 Growth of and Production of Enterotoxin by Strain S-6 Staphylococcus aureus Experimental Fermenter Apparatus, 500 ml

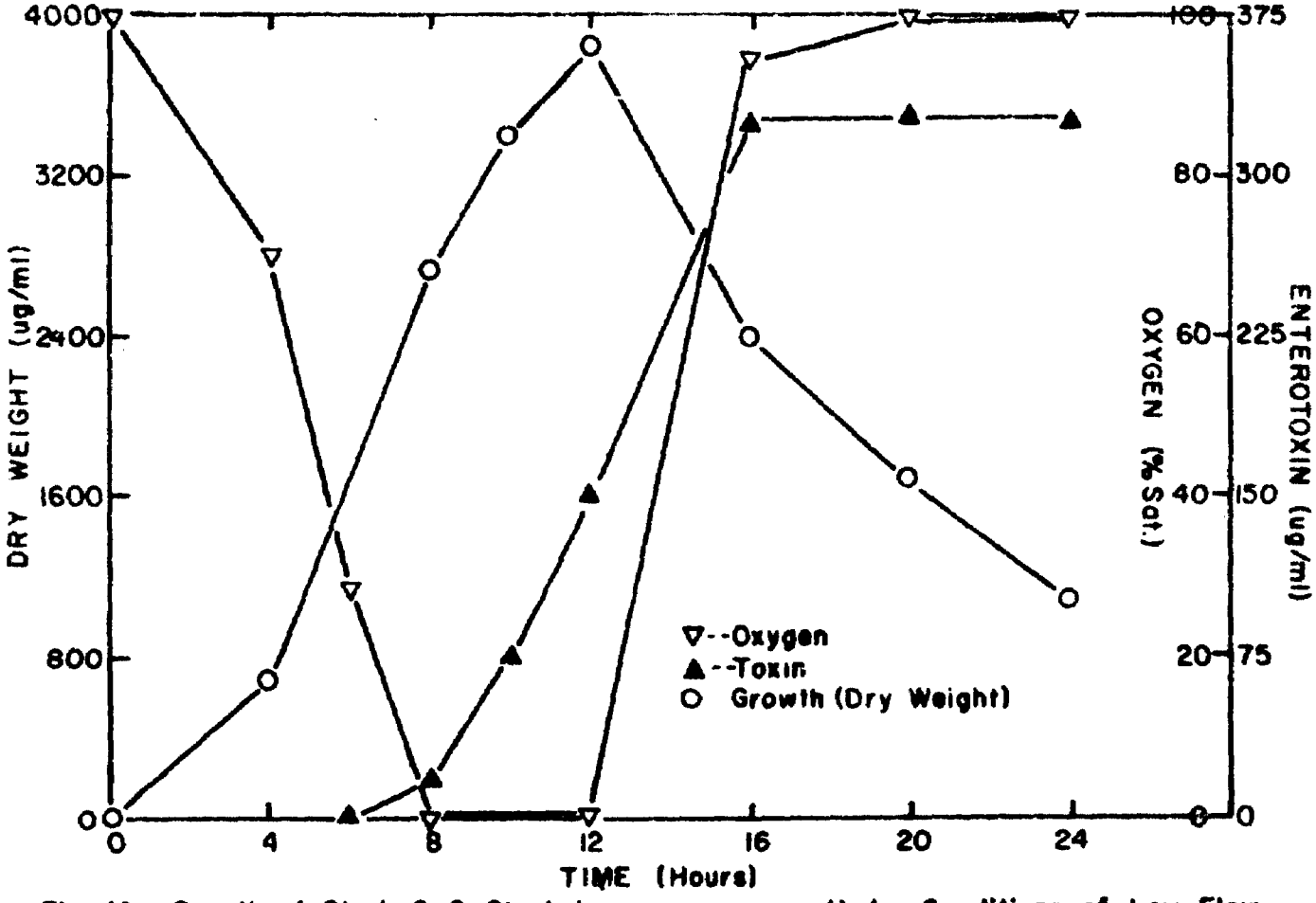


Fig. 19 Growth of Strain S-6 Staphylococcus aureus Under Conditions of Low Flow Rate Oxygen, Showing Enterotoxin and Dissolved Oxygen.

ginally present in the air space. The data showed that both growth and enterotoxin are higher when greater amounts of air are available. The interesting finding is that little or no toxin is produced when the head space, and the available amount of oxygen, is greatly reduced.

Further investigations revealed that a flow rate of 20 cc/min/500 ml culture medium was optimum for growth and toxin production in the fermenter. The data obtained from such an experiment are plotted in Fig 19. The maximum growth was obtained in 16 hr with a turbidity of 580 KU and a maximum amount of enterotoxin of 320 µg/ml. No other flow rate of air tested in these experiments resulted in greater growth or toxin production. These figures corresponded to those obtained on shake cultures using the standard Nephloflasks. This figure also shows the results obtained when pure oxygen is diffused through the fermenter at a rate of 20 cc/min/500 ml culture medium. Although the oxygen resulted in greater rate of growth and a higher total yield, the amount of enterotoxin present at the end of 24 hr was approximately the same (320 µg/ml) as found in cultures receiving air at the same flow rate.

The effect of higher flow rates of oxygen was measured by increasing the rate of flow of pure oxygen to 200 cc/min/500 ml culture. Maximum growth was then reached at the end of 8 hr, and the dry weight at this time was 2680 µg/ml while the turbidity corresponded to 540 KU. The 24 hr sample showed that the maximum toxin produced was 130 µg/ml, approximately 3/8 as much as with the lower flow rate.

Choosing the highest flow rate of 200 cc/min/500 ml of medium, this rate was used to measure the effect of various mixtures of O_2 and CO_2 and air and CO_2. Dack (1) reported that the presence of 10 to 20% CO_2 resulted in greater

enterotoxin production although there was considerable variation in the same strain under similar conditions. These observations supported the hypothesis that CO_2 may enhance enterotoxin production since it might be expected that high flow rates of either air or O_2 resulted in a rapid removal of the CO_2 which was formed by cell metabolism; consequently, a decrease in the enterotoxin yield would be observed. Similarly, lower flow rates resulted in less CO_2 loss and higher amounts of enterotoxin would be produced.

Because of these findings, various concentrations, 5, 10, and 20% CO_2 in oxygen were tested; a 5% CO_2 concentration in air was also examined. The results obtained were variable and did not correspond to increasing or decreasing concentrations of CO_2 insofar as enterotoxin or cell yield was concerned. When the level of CO_2 was 20%, the growth of cells was the highest observed, but the amount of enterotoxin produced was half as much as normal. It can be concluded from these findings that CO_2 does not cause a marked stimulation of toxin production.

Measurement of dissolved oxygen by means of Galvanic Cell Oxygen Analyzer showed that even at high flow rates of air or oxygen, there was no detectable dissolved oxygen at the early stationary phase even if pure oxygen were used Dissolved O_2 measurements for the shake cultures showed a longer time period during which the culture contained no free dissolved O_2.

DISCUSSION

Although both growth and enterotoxin production were dependent on the concentration of O_2 present in the culture medium, the range of flow rates and the concentration of O_2 used in these experiments showed that the levels were not absolutely critical. At low O_2 concentrations, S. aureus grew poorly

and little or no toxin could be demonstrated. At higher concentrations of O_2, both growth and toxin formation were stimulated. However, at the highest oxygen concentrations, growth but not toxin formation was increased.

O_2 was utilized so rapidly that there was no dissolved O_2 measureable in cultures during either logarithmic or early stationary phase when it was supplied at a rate of 200 cc/min/500 ml culture. The period of growth during which this rapid uptake of O_2 occurs was quite brief. During this short period, the rate of metabolism of the cells is maximum, and the population of organisms present is near maximum. Most of the nutrients should be utilized during the latter part of this logarithmic growth phase. When the cells enter the stationary phase, they do so because some available nutrient has become limiting. It is expected that O_2 uptake of cells in the stationary phase would be diminished; the appearance of dissolved O_2 at this point can be reasonably explained on this basis.

The pertinent findings from these experiments are as follows. At high flow rates of O_2 or air, the growth rate of the cultures is increased to maximum level. Under these conditions, cells are capable of using dissolved O_2 as well as O_2 present at a gas-to-cell interphase. The rate of utilization of O_2 under these conditions cannot be estimated from dissolved O_2 data since its concentration in solution should be zero when the cells are actively metabolizing (This also means that O_2 would be available for the complete combustion of intermediates which would accumulate as end products under conditions of limiting O_2.) It is conceivable that such end products could serve as intermediates for the synthesis of such extracellular proteins as enterotoxin. The present findings support this hypothesis; little or no enterotoxin is produced at high flow rates of air or O_2. At low flow rates,

where less O_2 is available, the efficiency of utilization of substrates is decreased; the growth rate is lowered; but, enterotoxin production is much higher. Greater O_2 concentrations permit a more efficient combustion of substrates and, because of this efficiency, fewer end products are available to serve as intermediates for the synthesis of enterotoxin.

TEMPERATURE AND OTHER STUDIES

The temperature of incubation for all the present experiments was 37 C, the optimum temperature of growth for S. aureus. Because of the etiology of natural staphylococcal food poisoning outbreaks, however, growth of this organism at less than the optimum temperature must result in the elaboration of enterotoxin. Quantitative measurements for enterotoxin have not been reported at temperatures other than the optimum 37 C; therefore, the temperature range for enterotoxigenesis was examined.

Qualitative analyses for enterotoxin previously reported by Segalove and Dack (14) indicated that enterotoxin might be produced between 20 and 23 C if the cultures were incubated from 3 to 7 days. No detectable enterotoxin was produced on laboratory medium after 3 days incubation at 15 C. Results reported by these investigators were based on animal assays.

The effect of various temperatures was consequently reinvestigated and enterotoxigenesis evaluated using quantitative serological methods. The temperatures chosen for these experiments were 43 C, 45 C, 46 C, 23 C, and 15 C. The results of growth and toxin produced at these temperatures are shown in Table 5. Because of the slower growth rate at lower temperatures it was necessary to permit all cultures to grow until they reached the maximum stationary phase in order to have a basis for comparison. At temperatures

Table 5. Growth and Toxigenesis During Various Times and Temperatures of Incubation

Incubation		Maximum Growth		Enterotoxin	Ratio
Temp. °C	Time	Dry Weight (µg/ml)	Plate Count/ml	(µg/ml)	Toxin/Dry Weight
15	168 hr	1575	1.1×10^{10}	2	.001
23	72 hr	1510	1.4×10^{10}	16	.01
37	24 hr	3500	2.0×10^{10}	320	.1
43	24 hr	2630	1.2×10^{10}	50	.02
45	31 hr	473	1.1×10^{10}	0	-
46	18 hr	0	-	0	-

above 43 C, growth and enterotoxigenesis were sharply reduced. Some measureable growth was produced at 45 C, but no toxin was detectable; cultures failed to grow or produce toxin at 46 C. At the lower temperature range, both growth and toxigenesis were again diminished. Toxin was greatly reduced at 23 C, and only barely detectable at 15 C. Although the concentration of toxin in the latter experiment was only 2 µg/ml, nonetheless, this showed that enterotoxin can be formed at low temperatures

The data obtained from cultures grown under certain specified conditions are depicted and described in Table 6; dry weight, toxin, relative lipase activity, and extracellular protein were measured and compared. Lipase activity varied directly with toxin production. In addition, the quantity of toxin produced was directly related to the amount of extracellular protein present in the medium; the most interesting finding here is the fact that the amount of toxin present accounts in most cases for approximately 50% of

the total extracellular protein produced. It appears from these data that the elaboration of extracellular protein (such as lipase) other than toxin is under a similar type of control mechanism. Culture conditions which affect total extracellular protein apparently affect lipase and enterotoxin formation as well.

Table 6. Relationship of Dry Weight and Enterotoxin to Lipase Activity and Extracellular Protein

Conditions of Experiment	Dry Weight (µg/ml)	Toxin (µg/ml)	Relative Lipase Activity*	Extra-Cellular Protein** (µg/ml)
Shake Culture	3205	260	64	460
Low Flow Rate Air	2750	190	64	268
Low Flow Rate Oxygen	3850	320	128	683
Constant pH 7.0	1360	65	16	155
Constant pH 7.0 with 1% Glucose	2520	75	64	230
Anaerobic	464	0	0	30
Temperature 15.2°	1575	2	4	52
Temperature 45.2°	444	0	2	less than 20

*The value stated for the relative lipase activity is the denominator of smallest fractional portion of the diluted filtrate which gave a color change in one hour's incubation.

**Extra-cellular protein is reported in equivalent µg/ml of bovine serum albumin. When measured by this method, the B enterotoxin of 95% purity gave a value of 240 equivalent µg/ml The actual concentration of the enterotoxin was 200 µg/ml.

References

1. Dack, G. M. Food Poisoning, 109-158, University of Chicago Press, Chicago, Ill., 1956.

2. Gale, E. F. The Assimilation of Amino Acids by Bacteria. 1. Passage of Certain Amino Acids Across the Cell Wall and their Concentration in the Internal Environment of Streptococcus faecalis. J. Gen. Microbiol., 1, 53-76, 1947.

3. Gale, E. F. The Assimilation of Amino Acids by Gram-positive Bacteria and some Actions of Antibiotics Thereon. Adv. Protein Chem., 8, 285-391, 1953.

4. Gladstone, G. P. Nutrition of Staphylococcus aureus: Nitrogen Requirements. Brit. J. Expt. Path., 18, 322-333, 1937.

5. Ivler, D. Comparative Metabolism of Virulent and Avirulent Staphylococci. Ann. N.Y. Acad. Sci., 128, 62-80, 1965.

6. Lowry, O. H., Rosebrough, N. J., Farr, A. L and Randall, R. J. Protein Measurement with the Folin Phenol Reagent. J. Biol. Chem., 193, 265, 1951

7. Mah, R. A. and Regier, L. W. Nutritional Requirements of Enterotoxigenic Strains of Staphylococcus aureus. Annual Progress Report to the U. S. Army Med. Res. and Dev. Command, Contract No. DA-49-193-MD-2374, 1965.

8. Mancy, K. H. and Westgarth, W. C. A Galvanic Cell Oxygen Analyzer. J. Water Poll. Cont. Fed., 34, 1037, 1963.

9. Marland, R. E., Ph.D. Thesis. The Effects of Several Environmental Factors on the Growth and Enterotoxigenicity of Strain F-6 Staphylococcus aureus. The University of North Carolina, Chapel Hill, 1965.

10. Morse, S. A. and Mah, R. A. Microtiter Hemagglutination-Inhibition Assay for Staphylococcal Enterotoxin B. Appl. Microbiol., 15, 58-61, 1967.

11. Natelson, S. Microtechniques of Clinical Chemistry. 2nd Ed., Charles C. Thomas, Springfield, Ill., 1963.

12. Peters, H., Ph.D. Thesis. The University of North Carolina, Chapel Hill, 1964.

13. Ramsey, H. H. Endogenous Respiration of Staphylococcus aureus. J. Bacteriol., 83, 507-514, 1962.

14. Segalove, M., and Dack, G. M. Relation of Time and Temperature to Growth and Enterotoxin Production of Staphylococci. Food Res., 6, 127, 1941.

15. Sulzer, F. P. and Peters, H. Nutritional Requirements of Enterotoxigenic Strains of Staphylococcus aureus. Annual Progress Report to U. S. Army Med. Res. and Dev. Command, Contract No. DA-49-193-MD-2374, 1963.

16. Sulzer, F. P. and Peters, H. Nutritional Requirements of Enterotoxigenic Strains of Staphylococcus aureus. 1964.

17. Surgalla, J. M. A Study of the Production of Staphylococcal Enterotoxin in Chemically Defined Medium. J. Inf. Dis., 81, No. 2, 97-111, 1947.

18. Wadsworth, C. A Slide Microtechnique for the Analysis of the New Precipitates in Gel. Int. Arch. Allergy, 10, 355, 1957.

19. Weirether, F. J., Lewis, P. E., Rosenwald, A. J., and Lincoln, R. E. Rapid Quantitative Serological Assay of Staphylococcal Enterotoxin B. Appl. Microbiol., 14, 284-291, 1966.

Security Classification

DOCUMENT CONTROL DATA - R&D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified.)

1 ORIGINATING ACTIVITY (Corporate author)
Department of Environmental Sciences & Engineering,
School of Public Health, University of North Carolina,
Chapel Hill, N. C. 27514

2a REPORT SECURITY CLASSIFICATION

2b GROUP

3 REPORT TITLE

NUTRITIONAL REQUIREMENTS OF ENTEROTOXIGENIC STRAINS OF STAPHYLOCOCCUS AUREUS

4 DESCRIPTIVE NOTES (Type of report and inclusive dates)

Terminal Progress Report (1 September 1965 - 31 December 1966)

5 AUTHOR(S) (Last name, first name, initial)

Responsible Investigator, Mah, Robert A., Assistant Professor
Co-responsible Investigator, Regier, Lloyd W., Associate Professor

6 REPORT DATE	7a TOTAL NO OF PAGES	7b NO OF REFS
12 June 1967	47	19

8a CONTRACT OR GRANT NO	9a ORIGINATOR'S REPORT NUMBER(S)
DA-49-193-MD-2347	
b PROJECT NO	4 (Progress Report)
c.	9b OTHER REPORT NO(S) (Any other numbers that may be assigned this report)
d.	

10 AVAILABILITY/LIMITATION NOTICES

Qualified requesters may obtain copies of this reprint from DDC

11 SUPPLEMENTARY NOTES	12 SPONSORING MILITARY ACTIVITY
	U.S. Army Medical Research and Development Command, Department of the Army, Washington, D. C. 20315

13 ABSTRACT In developing a synthetic medium for Staphylococcus aureus S-6, the following criteria were chosen: growth for 24 hr at 37 C with continuous shaking, minimum acceptable yield of 200 Klett Units. Growth was defined on the basis of these stringent, arbitrary criteria; positive growth was reported only if they were met. The defined medium developed was composed of inorganic salts, 11 amino acids (glycine, valine, leucine, threonine, phenylalanine, tyrosine, cysteine, methionine, proline, arginine, and histidine), and 3 vitamins, thiamine, nicotinic acid, and biotin. Biotin was a growth factor requirement in S-6 when glutamic acid but not glucose was used as a carbon source. The quantity of enterotoxin B was ca 7 times less in the defined than in complex medium, even though growth yields were similar.

Any modification of the initial pH of the medium resulted in some loss of enterotoxin production, regardless of whether it was initially altered without further change, buffered with phosphate at constant level, or adjusted throughout the growth cycle by addition of acid or base. Both growth yield and toxigenesis were diminished at the two extremes of temperature, 15 and 43 C, tested; it is of practical importance that enterotoxin can be produced at these temperatures.

Varying concentrations of CO_2 in pure O_2 were not beneficial for toxigenesis. With increased sparging of air or pure O_2 into growing cultures, growth but not toxigenesis was stimulated; however, at high sparge rates, both were drastically reduced. An optimum air or O_2 sparge-rate exists for growth or toxigenesis.

DD FORM 1 JAN 64 1473

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Staphylococcus aureus Enterotoxin Food Poisoning						

INSTRUCTIONS

1. ORIGINATING ACTIVITY: Enter the name and address of the contractor, subcontractor, grantee, Department of Defense activity or other organization *(corporate author)* issuing the report.

2a. REPORT SECURITY CLASSIFICATION: Enter the overall security classification of the report. Indicate whether "Restricted Data" is included. Marking is to be in accordance with appropriate security regulations.

2b. GROUP: Automatic downgrading is specified in DoD Directive 5200.10 and Armed Forces Industrial Manual. Enter the group number. Also, when applicable, show that optional markings have been used for Group 3 and Group 4 as authorized.

3. REPORT TITLE: Enter the complete report title in all capital letters. Titles in all cases should be unclassified. If a meaningful title cannot be selected without classification, show title classification in all capitals in parenthesis immediately following the title.

4. DESCRIPTIVE NOTES: If appropriate, enter the type of report, e.g., interim, progress, summary, annual, or final. Give the inclusive dates when a specific reporting period is covered.

5. AUTHOR(S): Enter the name(s) of author(s) as shown on or in the report. Enter last name, first name, middle initial. If military, show rank and branch of service. The name of the principal author is an absolute minimum requirement.

6. REPORT DATE: Enter the date of the report as day, month, year, or month, year. If more than one date appears on the report, use date of publication.

7a. TOTAL NUMBER OF PAGES: The total page count should follow normal pagination procedures, i.e., enter the number of pages containing information.

7b. NUMBER OF REFERENCES: Enter the total number of references cited in the report.

8a. CONTRACT OR GRANT NUMBER: If appropriate, enter the applicable number of the contract or grant under which the report was written.

8b, 8c, & 8d. PROJECT NUMBER: Enter the appropriate military department identification, such as project number, subproject number, system numbers, task number, etc.

9a. ORIGINATOR'S REPORT NUMBER(S): Enter the official report number by which the document will be identified and controlled by the originating activity. This number must be unique to this report.

9b. OTHER REPORT NUMBER(S): If the report has been assigned any other report numbers *(either by the originator or by the sponsor)*, also enter this number(s).

10. AVAILABILITY/LIMITATION NOTICES: Enter any limitations on further dissemination of the report, other than those imposed by security classification, using standard statements such as:

(1) "Qualified requesters may obtain copies of this report from DDC."

(2) "Foreign announcement and dissemination of this report by DDC is not authorized."

(3) "U. S. Government agencies may obtain copies of this report directly from DDC. Other qualified DDC users shall request through _____."

(4) "U. S. military agencies may obtain copies of this report directly from DDC. Other qualified users shall request through _____."

(5) "All distribution of this report is controlled. Qualified DDC users shall request through _____."

If the report has been furnished to the Office of Technical Services, Department of Commerce, for sale to the public, indicate this fact and enter the price, if known.

11. SUPPLEMENTARY NOTES: Use for additional explanatory notes.

12. SPONSORING MILITARY ACTIVITY: Enter the name of the departmental project office or laboratory sponsoring *(paying for)* the research and development. Include address.

13. ABSTRACT: Enter an abstract giving a brief and factual summary of the document indicative of the report, even though it may also appear elsewhere in the body of the technical report. If additional space is required, a continuation sheet shall be attached.

It is highly desirable that the abstract of classified reports be unclassified. Each paragraph of the abstract shall end with an indication of the military security classification of the information in the paragraph, represented as *(TS)*, *(S)*, *(C)*, or *(U)*.

There is no limitation on the length of the abstract. However, the suggested length is from 150 to 225 words.

14. KEY WORDS: Key words are technically meaningful terms or short phrases that characterize a report and may be used as index entries for cataloging the report. Key words must be selected so that no security classification is required. Identifiers, such as equipment model designation, trade name, military project code name, geographic location, may be used as key words but will be followed by an indication of technical context. The assignment of links, rules, and weights is optional.