

AD 624249

ANNUAL PROGRESS REPORT

Period Covered: 1 September 1964 to 1 September 1965

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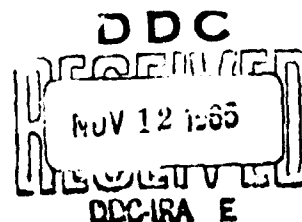
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Subject of Report:

NUTRITIONAL REQUIREMENTS OF ENTEROTOXIGENIC

STRAINS OF STAPHYLOCOCCUS AUREUS

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



ABSTRACT

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School of Public Health, University of North Carolina,
Chapel Hill, North Carolina.

Title of Report: Nutritional Requirements of Enterotoxigenic Strains
of Staphylococcus aureus.

Annual Progress Report: 1 November 1965

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

Supported by: U.S. Army Medical Research and Development Command,
Department of the Army, Washington 25, D.C.

A 7-amino acid medium has been devised which permits good growth of S. aureus S-6 comparable to that obtained on an equivalent amount of complex medium. Further work is in progress to simplify this medium. Current findings show that when the amino acid, glutamic acid, serves as a carbon source in defined media, enterotoxin B is produced. The amount of toxin formed under these conditions is approximately 25 times greater than that when glucose serves as a carbon source. Substrates which supported growth but not toxin formation were ribose, glycerol, and pyruvate. Of the five-carbon sugars tested, only ribose supported growth whereas xylose and arabinose did not. The Krebs's cycle intermediates, succinate, citrate, acetate, oxaloacetate, alpha-ketoglutarate, and malate did not support growth.

A new method for the assay of enterotoxin has been developed which permits the detection of 0.06 μ g of toxin. This is the microtiter hemagglutination inhibition method which is fast, accurate, repeatable, easy to read, and requires only micro-amounts of reactants. It has been adopted for routine use in our laboratory.

Ether-extracted protein hydrolysate powder (PHP) still supported growth and toxin production when vitamins were added to the medium. In the absence of added vitamins, growth occurs but no toxin is produced. Further investigations are in progress to examine this finding.

KEY WORDS:

Staphylococcus aureus: Enterotoxin production in synthetic media; effect of amino and organic acids and carbohydrates on growth and enterotoxin production.

Toxin: Nutritional requirements for enterotoxin production in Staphylococcus aureus.

Food Poisoning: Nutritional requirements for enterotoxin production of Staphylococcus aureus.

INTRODUCTION

Progress on the nutritional requirements for the growth of enterotoxigenic strains of Staphylococcus aureus has been reported in the previous studies of Sulzer and Peters (1963, 1964). Synthetic medium containing 8 amino acids, inorganic salts, and vitamins, with glucose as a carbon source was devised. The maximum growth of S. aureus supported by this medium was approximately 60 Klett units after 24 hours. Little or no toxin was produced under these conditions.

Growth and enterotoxin production in complex media were also studied with respect to glucose concentration, pH, and high concentrations of inorganic salts. Addition of glucose to such media resulted in increased growth but lower toxin concentrations. High concentrations of glucose (25%) suppressed both growth and toxin production. Addition of 10% NaCl did not result in significant differences in cell yield, although the toxin concentration was markedly reduced (to approximately 10% of the normal medium). Concentrations of NaCl above 15% were found to be inhibitory to growth as well as enterotoxin production. Similar findings occurred with inorganic salts. Adjustment of pH with acid or base during growth of the organisms showed that values above or below pH 7 reduced toxin formation but not growth. Adjustment of the initial pH without further manipulation during growth had little or no effect on toxin or growth.

Because of the limited growth and low yield of toxin (less than 1 µg/ml) on the above synthetic medium, further investigations were continued to devise an appropriate medium. Studies on pH were also continued. The role of temperature and gas atmospheres has been examined, and work has been initiated on the role of vitamins and possible substrates or cofactors necessary for growth and/or synthesis of the enterotoxin.

Although it was not the original purpose of this investigation to examine the assay method for toxin analysis, it became apparent that the existing methods were time-consuming as well as difficult to read. Evaluation of toxin production could not be made until 3-7 days after an experiment, owing to the nature of the assay. Robinson (1965) reported the development of a new microtiter technique based on hemagglutination-inhibition which allowed assay of toxin within hours. This method was presumably* worked out for enterotoxin A but not B, and the latter is the toxin of primary concern here. Robinson's method has been drastically modified in our investigations and has resulted in a new assay technique for enterotoxin B.

The results of these investigations are detailed in the following report.

* Bergdoll, personal communication: The hemagglutination-inhibition method of Robinson was not specific for enterotoxin A; some other cross-reacting material was present and enterotoxin was actually not detected in these assays.

DEFINED MEDIA

A review of the literature showed that there have been many types of media devised for growth of S. aureus. The first of these employed complex compounds such as gelatine (Knight, 1935). Later, defined media were developed (Fildes, et al., 1936) which required unidentified growth factors. Identification of these factors as nicotinic acid and vitamin B₁ led to the first completely defined medium reported by Gladstone (1937). This medium was composed of 16 amino acids, vitamins, and inorganic salts. It was found that the amino acid requirement was due to strain differences, although with "training," these differences disappeared and, indeed, strains were produced which grew on ammonia as the only nitrogen source. (Such training procedures undoubtedly resulted in selection of mutants or recombinants.)

More recently, Ramsey and Padron (1954) developed a synthetic basal medium in determining the essential amino acids of a strain of S. aureus. A comparison of the inorganic salts used by these and previous workers is summarized in Table 1.

It can be seen from the table that the inorganic salt composition has varied considerably from one investigation to another. The mixtures reported by Gale (1945, 1947) and by Ramsey and Padron appear to be the most complete and were tested in experiments using glucose as a carbon source and supplemented with appropriate vitamins. The seven amino acids shown by the latter workers to be essential for maximum growth of their strain of S. aureus were incorporated into this medium as nitrogen source.

Materials and Methods

The following amino acids were tested at a final concentration of 25 µg/ml: L arginine monochloride, L cysteine hydrochloride, L glutamic acid, L leucine, L proline, DL valine, and L histidine. All media contained final concentrations

Table 1

Percent Concentration

	Knight 1935	Fildes et al., 1936	Gladstone 1937	Kligler et al., 1943	Gale 1945	Gale 1947	Surgalla 1947	Ramsay & Padron 1954	Peters 1964
Inorganic salts									
$(\text{NH}_4)_2\text{HPO}_4$	—	—	—	—	0.4	—	—	—	—
KH_2PO_4	0.45	0.45	0.45	0.35	0.1	0.1	0.45	—	0.2
K_2HPO_4	—	—	—	—	—	—	—	0.4	—
Na_2HPO_4	—	—	—	0.25	—	0.33	—	—	—
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.004	—	0.07	—	0.07	0.07	0.002	0.04	0.01
$\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$	—	—	—	—	—	—	—	0.002	—
NaCl	—	—	—	0.5	0.1	0.1	—	0.01	—
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	—	—	—	—	0.001	—	—	0.002	0.001
$\text{FeSO}_4(\text{NH}_4)_2$	—	—	—	—	—	—	—	—	—
$\text{SO}_4 \cdot 6\text{H}_2\text{O}$	—	0.05	0.05	—	—	—	0.0145	—	—
$(\text{NH}_4)_2\text{SO}_4$	—	—	—	—	—	—	—	0.002	—
pH	7.4	7.4	7.4	7.2	—	—	7.6	—	7.0

of 20 mg/ml glucose and 1.2 μ g/ml nicotinic acid, 0.033 μ g/ml thiamine, and 0.001 μ g/ml calcium pantothenate.

Three salt solutions were tested:

1. Gale's (1947) salt solution for diffusion experiments with resting cells was modified by the addition of CaCl_2 , NH_4Cl , and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and tested as a basal salts solution in growth experiments. The inorganic salts solution had the following composition in final percent concentrations:

- A) KH_2PO_4 , 0.1; Na_2HPO_4 , 0.33; NaCl , 0.1, NH_4Cl , 0.05.
- B) $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07.
- C) CaCl_2 , 0.005.

The amino acids and vitamins were made up in salt solution A and autoclaved for 15 min at 15 pounds pressure, 120°C. Solution B was sterilized separately in a Morton filter apparatus and added to the autoclaved amino acid salt solution. Solution C and glucose were autoclaved separately and also added to the above medium. The individual treatment of these solutions was necessary to avoid precipitation.

2. The following salt solution was used by Gale (1945) as a culture medium. The composition is given in final percent concentrations: KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.07; NaCl , 0.1; $(\text{NH}_4)_2\text{HPO}_4$, 0.4; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001. The ferrous and magnesium sulfates were sterilized by filtration and added separately to the other salts which were sterilized by autoclaving. Amino acids and vitamins were autoclaved in the salts solution. Glucose was autoclaved and added separately.

3. Ramsey and Padron (1954) employed a salt solution having the final percent concentration and composition as follows: KH_2PO_4 , 0.4; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04; NaCl , 0.01; $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002; $(\text{NH}_4)_2\text{SO}_4$, 0.002; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.002. Again, the ferrous and magnesium sulfates, the other salts, amino acids, vitamins, and glucose were treated as previously described.

A suspension of S. aureus S-6 dried on a porcelain bead was inoculated into 2% PHP broth medium and incubated on a shaker for 24 hours at 37°C. Five-milliliter aliquots of this culture were transferred aseptically into screw cap tubes and quickly frozen in an acetone-dry ice bath. The frozen cultures were stored at -15°C. When used in experiments, the cultures were thawed in warm water and a 1% inoculum introduced into 2% PHP broth. The culture was allowed to incubate on a shaker at 37°C for 17 to 17.5 hours. The turbidity was between 460-490 Klett units. All cultures were inoculated with a 1% inoculum of cells grown for this length of time under the conditions described.

Growth was measured by increase in turbidity as determined by Klett colorimetry, using a green filter, 540 mμ.

The results of this experiment are given in Table 2. It can be seen from the data that Gale's 1945 medium allowed the best growth. The modified Gale's salt solution also permitted good growth. Ramsey and Padron's medium was not suitable for S-6; this may be a result of strain differences in sensitivity to manganese ion since this was the only different ion present. The lower growth in the modified Gale's salt solution may be due to inhibition by calcium ions. These discrepancies are being further investigated.

Growth in synthetic medium with glucose was evaluated by comparison with that obtained upon substitution of protein hydrolysate powder (PHP) for the amino acid mixture. This was tested only in the modified salt solution of Gale. The two concentrations of PHP used were calculated on the basis of the total amino nitrogen or amino acid concentration present in the synthetic medium. PHP contains 10% nitrogen as amino nitrogen.

1. Amino nitrogen present in PHP calculated as amino nitrogen present in the synthetic medium.

2. Weight of PHP calculated as the total weight of amino acids present in the synthetic medium.

Each concentration of PHP was tested with and without glucose as a carbon source and compared to the growth obtained in synthetic medium with and without glucose. The final turbidity after 24 hours is given in Table 3. A graph of the growth response is given in Figures 1 and 2.

These results show that growth in synthetic medium was virtually the same as that in complex medium. The lower value obtained for PHP #2 can be explained by the fact that it contained less PHP than PHP #1. (The amount of PHP calculated as weight of amino acids in the synthetic medium is less than the figure calculated as amino nitrogen.)

Figures 1 and 2 are log plots of the Klett readings versus time. In the presence of glucose (Figure 1) as carbon and energy source, a typical growth curve is seen. In PHP #1 there is no lag because the young culture used as inoculum was grown up on the same complex medium. PHP #2 showed similar kinetics but had a slightly lower cell yield. The defined medium does show a lag but the same cell yield is obtained at the end of 24 hours.

In the absence of glucose (Figure 2) growth was erratic. Since no other carbon source was present in substrate amounts, this was not a surprising finding. These results indicate that little growth occurred in the absence of added carbon source. The amount of growth which does occur is likely due to utilization of the added amino acids or PHP rather than to carry-over of nutrients with the inoculum.

Since growth in the synthetic amino acids medium was equivalent to that found in the PHP medium all essential amino acids must have been present. However, the possibility exists that the essential amino acid requirement for S. aureus 6-6 is not as stringent as that reported for the strain of Ramsey and Padron. Consequently, each of the seven amino acids will be individually

Table 2

Medium	Klett Units	
	0 hr	24 hr
Gale 1945		
Amino acids glucose	15	211
Equivalent PHP glucose	13	210
Amino acids no added glucose	14	40
Gale 1947, modified		
Amino acids glucose	15	169
Equivalent PHP glucose	14	166
Amino acids no added glucose	14	14
Ramsey and Padron		
Amino acids glucose	17	11
Equivalent PHP glucose	16	106
Amino acids no added glucose	17	10

Table 3

Medium	Klett Units	
	No Glucose	Glucose
Defined	51	208
PHP #1	60	181
PHP #2	35	145

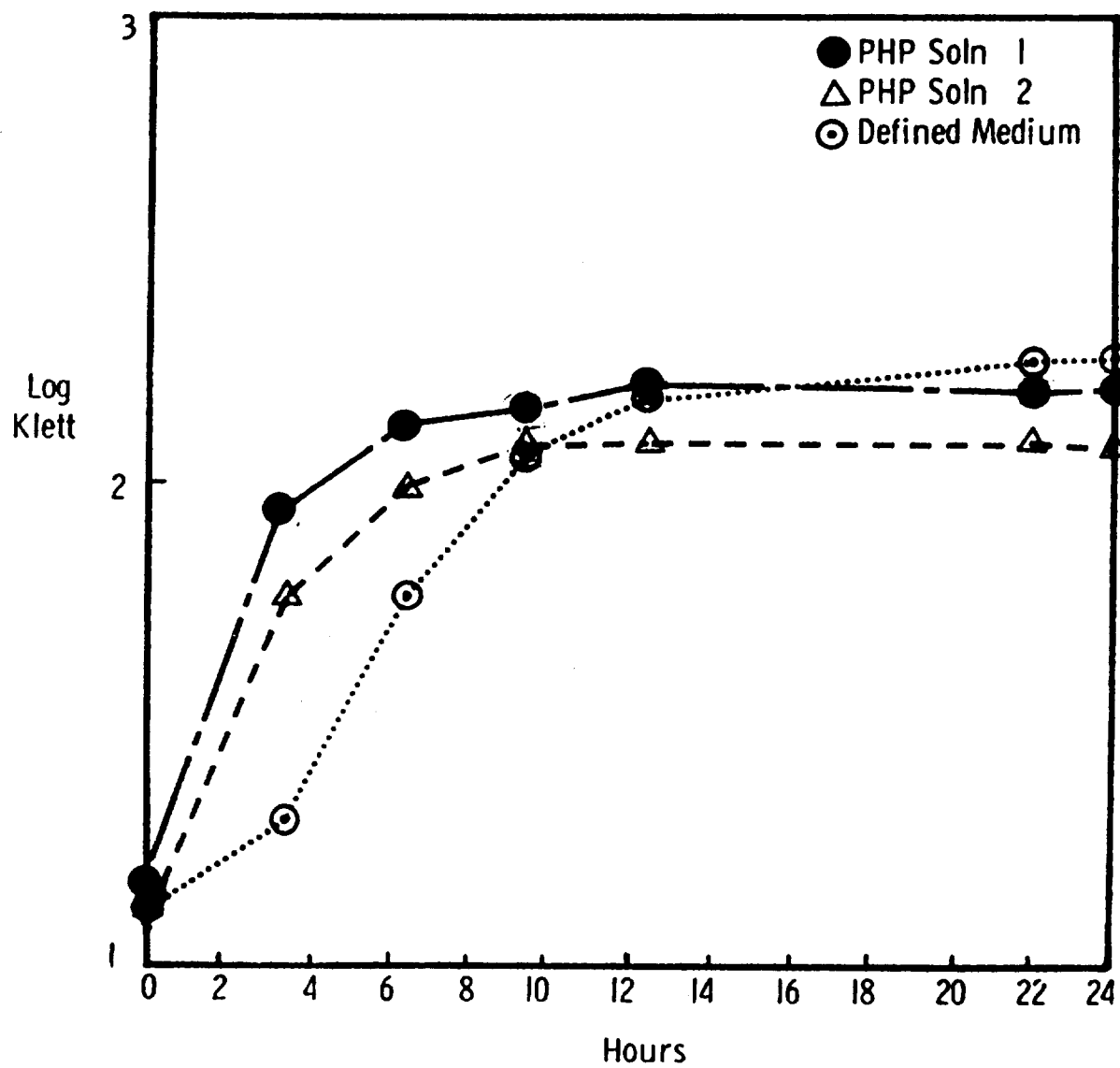


Figure 1

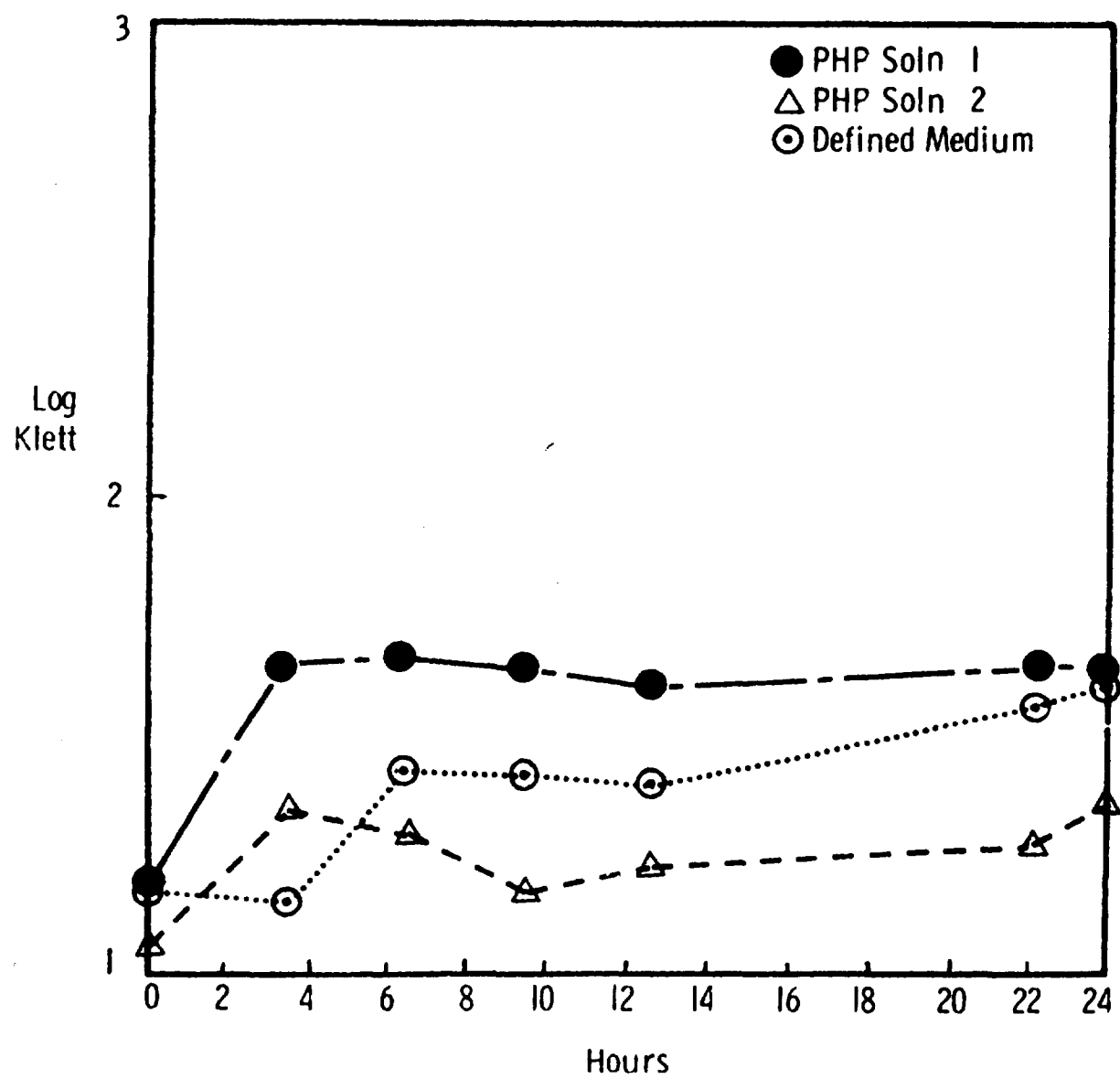


Figure 2

eliminated from the medium to test this hypothesis in a future investigation.

The above experiments are based on the premise that growth of S-6 will occur immediately if the proper compounds are present. For this reason, a complex medium (PHP) was used as a positive control. Growth is easily measured by inoculation into liquid medium and determining increase in turbidity. Inoculation on solid medium is not as satisfactory because of the inability to measure quantitatively the resulting growth.

Carbon Source Experiments

Strasters and Winkler (1963) showed that glucose is metabolized fermentatively by S. aureus even in the presence of oxygen. Manometric experiments on resting cell suspensions and cell-free extracts showed that there was a widespread glucose repression of the oxidative enzymes. It was shown by Peters (1964) that glucose also suppressed formation of enterotoxin. Because of these observations, it was hypothesized that synthesis of enterotoxin may very well be related to the general repression of the oxidative enzymes. If a suitable carbon source were found which could be used oxidatively, toxin might then be produced. Glucose was used in the initial investigations for establishment of the proper basal medium since it was known that it can serve as a carbon source. Thereafter, glucose was used as a positive control when other carbon sources were tested in basal medium containing amino acids or an equivalent amount of PHP. Defined medium without added carbon source was kept as another control.

Three main groups of compounds were examined: the tricarboxylic acid cycle (TCA) intermediates, five-carbon sugars, and amino acids. All possible carbon sources were tested in concentrations of 2%; treatment of inoculum and other growth conditions was the same as previously described.

1. The TCA intermediates were sodium succinate, sodium citrate, sodium acetate, oxaloacetic acid, alpha-ketoglutaric acid, and malic acid. The pH of the defined medium was adjusted between 7 and 7.3 in one experiment and unadjusted in another. The results showed that none of the compounds tested were used to an appreciable extent compared to that of glucose. A summary of these data is given in Table 4.

In a separate experiment, glycerol and pyruvate were used as carbon sources. These substrates were tested both in synthetic amino acid medium and

Table 4

Carbon Source	pH Unadj.	Klett Units		pH Adj.	Klett Units	
		0 hr	24 hr		0 hr	24 hr
Glucose	7.30	13	199	7.30	18	73
Alphaketoglutarate	1.95	13	7	7.00	16	16
Oxaloacetate	2.10	14	16	7.00	15	14
Acetate	7.15	12	21	7.15	17	11
Citrate	7.30	10	13	7.30	16	9
Malate	5.40	13	5	7.00	13	13
Succinate	7.21	11	32	7.21	18	14

in medium containing an equivalent amount of PHP. The results, figures 3 and 4, show that both glycerol and pyruvate were used as substrates. Growth on glycerol was equal to or more than the positive glucose control. Since glycerol can be converted to dihydroxyacetonephosphate, its utilization may also be via a fermentative pathway. Furthermore, enterotoxin was assayed in these cultures and the yield (μg enterotoxin/mg cell) on glucose and glycerol was lower than pyruvate. Pyruvate, however, did not serve as a good carbon source; the cell yield after 24 hours was less than one-half that of the glucose and glycerol cultures.

2. Five-carbon sugars were examined since it was known that ribose is used oxidatively by S. aureus and that the pentose cycle was present (Strasters and Winkler, 1963). Growth on ribose, xylose, and arabinose was examined. The results, given in figure 5, show conclusively that ribose is metabolized. However, little or no toxin was produced on any of these substrates.

3. The amino acid, glutamic acid (as monosodium glutamate), was the only one tested as a carbon source since it is readily metabolized by some strains of S. aureus. The results are shown below in Table 5. Glutamate was used as a carbon source, and growth was twice that on glucose. The absolute quantity of toxin produced was not high ($25 \mu\text{g/ml}$), but it was approximately 25 times that produced in glucose, which was expected to be low. Further investigations are in progress to test other amino acids and combinations of amino acids for growth and toxin production.

Table 5

Carbon Source	24 hr Klett	Cell yield mg/ml	Enterotoxin $\mu\text{g/ml}$	μg toxin/ mg cell
Glutamate	254	21.5	24.96	1.36
Glucose	124	9.4	1.56	0.17

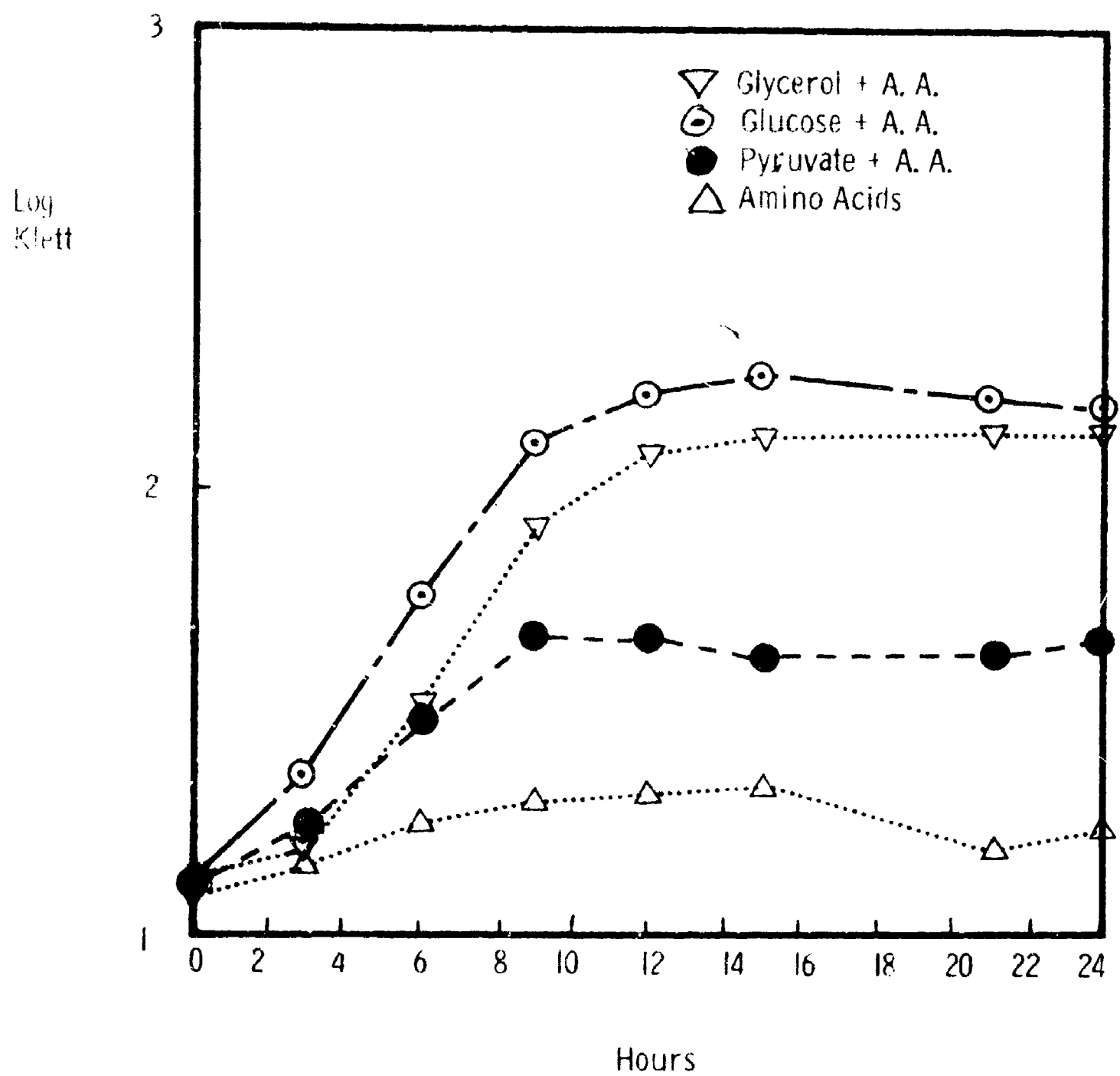


Figure 3

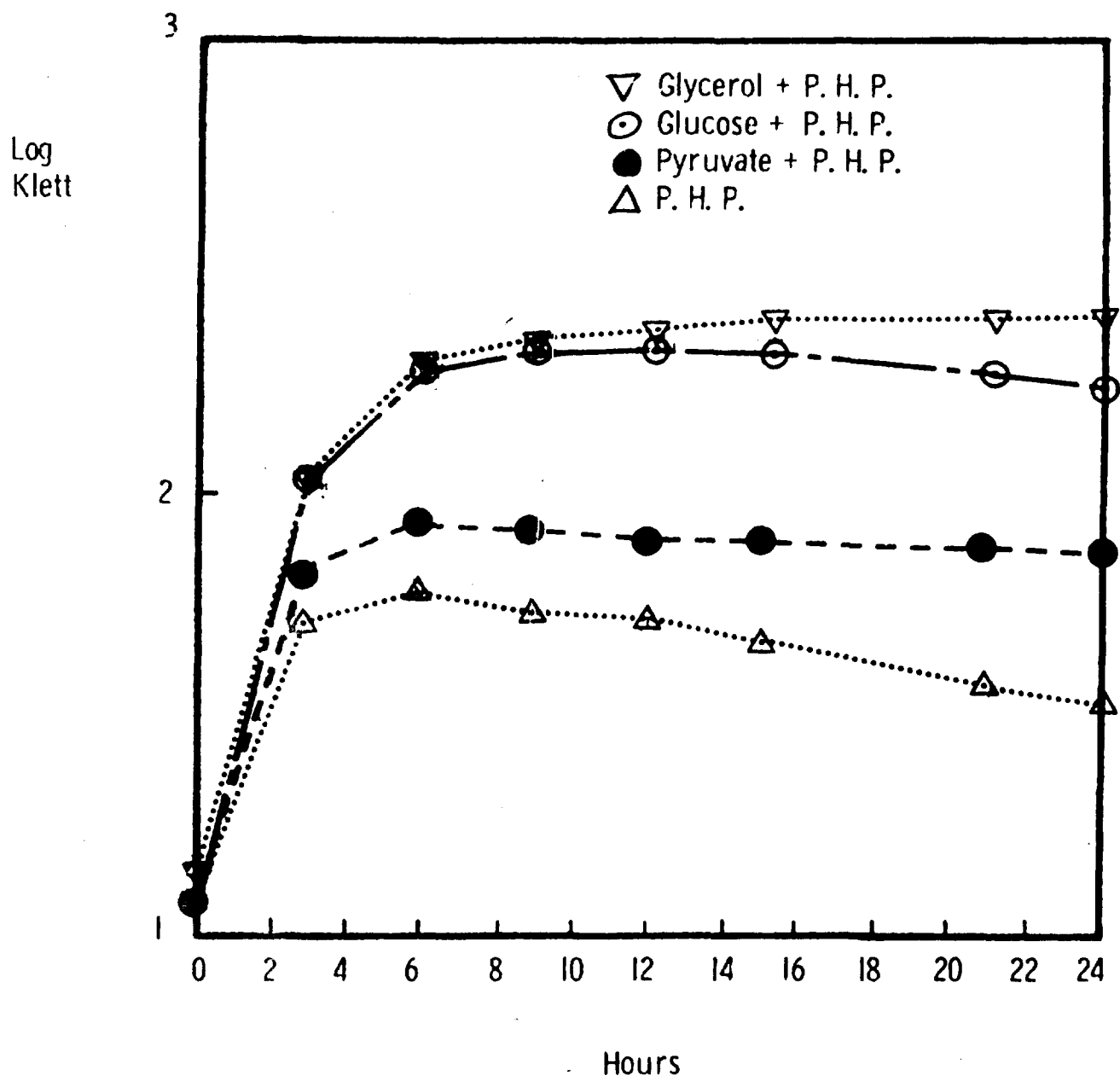


Figure 4

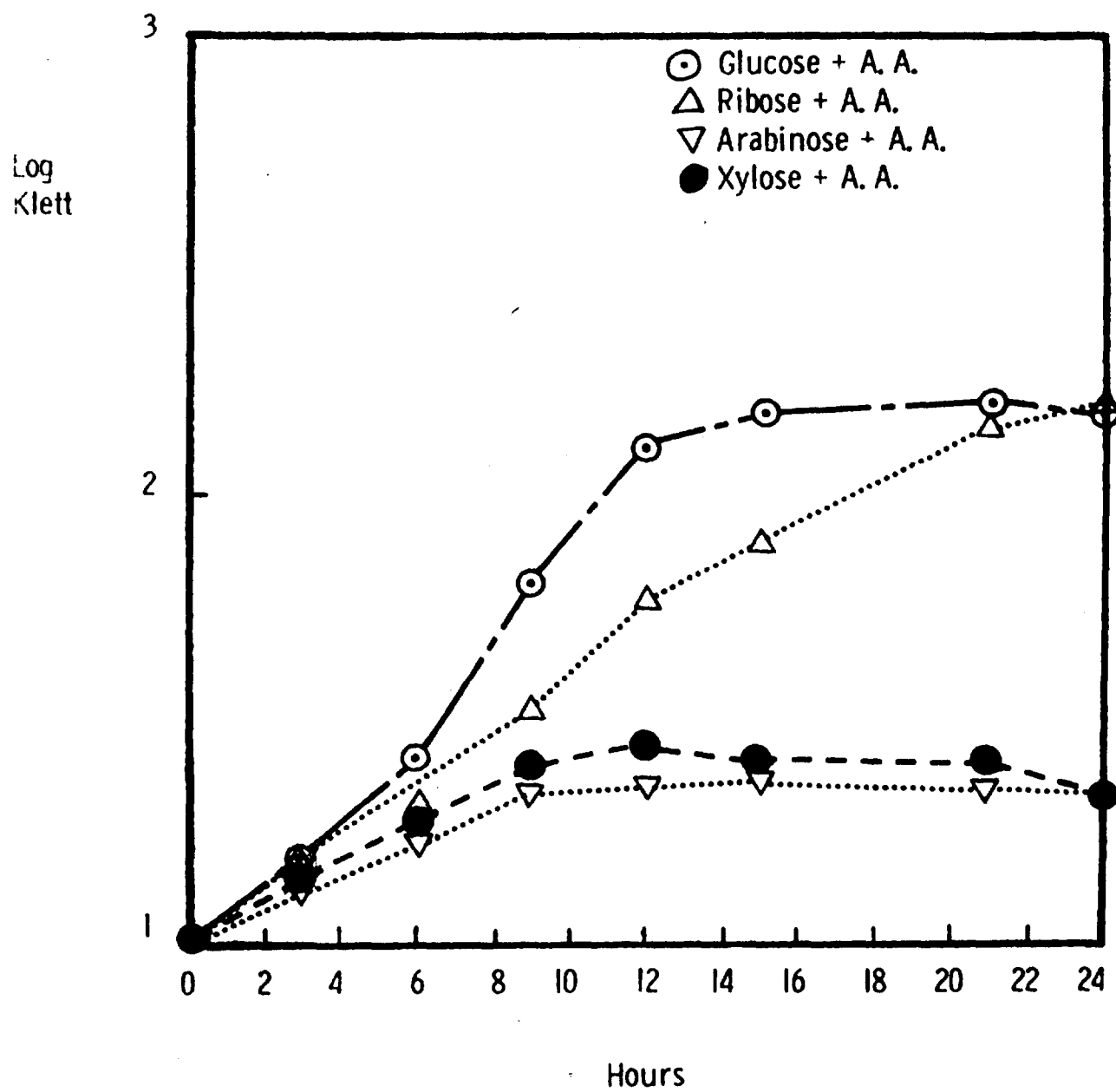


Figure 5

Hemagglutination Inhibition Assay for Enterotoxin B

The methods of enterotoxin assay previously used in our laboratory by Peters (1964) and Marland (1965) suffered certain disadvantages: 1) they were time-consuming, requiring from a few days to a week to obtain readable results; 2) the endpoint was not always clear-cut; 3) a considerable amount of skill was involved in the preparation of the tests. Nevertheless, these immunodiffusion tests were far superior to any in vivo methods available (monkey and kitten tests, etc.) and had the further advantage of requiring micro-amounts of toxin and antiserum for quantitative analyses.

Robinson (1965) reported the development of a hemagglutination inhibition test which circumvented the disadvantages of the immunodiffusion method and at the same time retained the advantage of using micro-amounts of reactants. However, the test was developed for enterotoxin A and not B. Since these enterotoxins cause similar reactions in animals, it was assumed that they could also be similar in immunological response and that Robinson's method would also apply to enterotoxin B. Robinson's procedures, however, were never published, but we were able to piece together enough information from his paper delivered at the 1965 ASM meetings to attempt to duplicate his results with B.

Robinson's methods in which the microtiter technique is used is outlined as follows:

1. The cells are sensitized by incubating 8 ml 2% formalin-treated sheep erythrocytes with 0.5 ml toxin (150 μ g/ml) at 35°C for 15 min.
2. The sensitized cells are washed three times in tris buffer (0.3%) containing 0.85% NaCl and 0.36% KCl adjusted to pH 7.5 with concentrated HCl. Centrifuge at room temperature.

3. An appropriate serum dilution is made in the above buffer.

Assay

4. 0.025 ml toxin is added to the antiserum and incubated for one hour at room temperature.
5. 0.025 ml sensitized cells from step 1 is added to each well, the plate is agitated and then incubated with cover for 3/4 hr. before reading.

Repeated trials with this technique were unsuccessful and results were uniformly negative, with agglutination showing in all wells of the micro-titer plate. Parallel tests using the accepted micro-immunodiffusion method of Casman and Bennett (1963) with the same standard enterotoxin and antiserum gave the expected results. It was not likely that these reactants were at fault. The order of addition of reactants and the length of incubation time were tested. Likewise, the sensitization of the cells was re-examined. It was found that the cells sensitized by the method described by Robinson was not satisfactory. It was necessary to treat the formalinized cells first with tannic acid prior to sensitization (Boyden, 1950).

The final protocol for the method adopted is as follows:

Formalinization. Sheep erythrocytes collected in Alsever's solution were formalinized according to the method of Csizmas (1960). The formalinized sheep erythrocytes (FSE) are stored frozen in small aliquots until needed.

Sensitization. Frozen FSE is defrosted and sensitized with specific antigen (enterotoxin B) according to these procedures:

1. FSE is washed 4 times in 0.85% saline with 1:10,000 merthiolate.
2. The packed-cell volume (PCV) is determined and the suspension diluted to 2.5% with the above saline-merthiolate solution.

3. An equal volume of 1:2000 tannic acid preheated to 37°C is added and the cells incubated for 10 min. at 37°C.
4. The cells are washed in tris buffer, the PCV again determined, and the suspension diluted to 5% in tris buffer.
5. An equal volume of enterotoxin B at a concentration of 20 µg/ml is added and the cells incubated at room temperature for 30 min. with frequent mixing.
6. The cells are reconcentrated by centrifugation and washed once in 0.1% bovine serum albumin (BSA) in tris buffer.
7. 0.1% BSA in tris buffer is used to resuspend the cells to a final concentration of 0.5%.

Sensitized cells prepared according to this method are not stable and must be made fresh daily.

Antiserum. Antiserum B was diluted to a final concentration of 1:200 with tris buffer containing 0.1% BSA.

Standard Enterotoxin. Known pure enterotoxin B was dissolved in tris buffer to give a final concentration of 25 µg/ml.

Assay Procedure. The microtitration procedure of Takatsy as outlined by Sever (1962) was used. All supplies were purchased from the Cooke Engineering Company.

1. Each well except the first contains 0.025 ml tris buffer. The first well is left empty and 0.025 ml of unknown sample or standard is added to it.
2. Serial dilutions are made with pretested, calibrated loops.
3. 0.025 ml of 1:200 antiserum B is added to each well and the plate is incubated for 1 hour at room temperature. The plate is covered to prevent evaporation.

4. 0.05 ml of sensitized cells is added to each well; the plate is then shaken on a Thomas shaking apparatus for 30-45 sec. until the cells are thoroughly suspended.
5. The plates are incubated at room temperature, undisturbed by further agitation and read after one hour. Better results are obtained after incubation overnight at refrigerator temperature.

Hemagglutination inhibition is indicated by a uniform dark button with a clear supernate. The endpoint is the highest dilution which shows a small central button. To make the test quantitative, the dilution factor at the endpoint is multiplied by a sensitivity factor, determined from the highest dilution of standard toxin which can be detected.

Results. The optimal concentration of antiserum was determined by testing various dilutions with standard toxin diluted serially. The results of such an experiment are given in figure 6. These data show that the highest possible dilution giving a positive inhibition is between 1:160 and 1:240. A 1:200 antiserum B dilution was chosen for all subsequent tests. This dilution gave a sensitivity of 0.06 μ g absolute toxin concentration per well; this figure corresponds to a concentration of 2.4 μ g/ml which must be present in the sample to be measureable.

The accuracy of the test was determined by adding known amounts of standard toxin to various solutions and calculating the percentage recovery. Three diluents, tris buffer, 2% PHP, and defined medium, were used; the results are depicted in Table 6. These data indicate that 95-100% of the enterotoxin was detected in all three cases. Whether or not there are cross-reacting materials present in culture media as a result of cell growth is not known, but experiments are in progress to elucidate this aspect.

The hemagglutination inhibition method was tested against the established micro-immunodiffusion method, and the results were the same,

if not comparable, in every sample used. Table 7 gives the data from such an experiment.

Dr. Bergdoll has kindly agreed to test samples of culture media by animal assay in monkeys and by single-gel diffusion techniques. Samples of culture media assayed in our laboratory by hemagglutination inhibition and micro-immunodiffusion have already been sent to him for this purpose.

Table 6
Percent Recovery of Enterotoxin B

Diluent	Amount Added $\mu\text{g/ml}$	Amount Recovered $\mu\text{g/ml}$	Percent Recovery
Tris buffer	25.0	25.3	101
2% PHP	20.0	19.2	96
Defined Medium	20.0	19.2	96

Table 7
Microimmunidiffusion (MID) vs.
Hemagglutination Inhibition (HAI)

Sample	MID $\mu\text{g/ml}$	HAI $\mu\text{g/ml}$
1	48	50
2	384	399
3	768	799

Experiments in Progress

Growth of S-6 on 2% PHP was always abundant and accompanied by high concentrations of toxin. No significant amounts of carbohydrates or carbon sources other than amino acids are present in PHP, and it must be assumed that growth is due to the utilization of amino acids. Toxin formation, on the other hand, may be a result of the presence of suitable energy sources and/or the presence of precursors or cofactors needed for its enzymatic synthesis. Fractionation of the PHP would permit an examination of the effect of its various components on growth and toxin formation.

These studies are still in the preliminary stages since the actual evaluation of the PHP fractions must await the resolution of a suitable defined medium for testing. The initial experiments fall into two categories: 1) extraction of the PHP with ether to remove any fatty acids or ether-soluble compounds which may be present. 2) fractionation of PHP by use of gel-filtration on sephadex columns.

1. Demain et al. (1959) reported that fatty acids were present in vitamin-free casein hydrolysates and that these fatty acids served as growth stimulants in Sarcina. Ether extraction of PHP to separate these and other ether-soluble compounds would yield a purified substrate which could be tested in S-6.

Materials and Methods. 20% PHP in distilled water was adjusted to pH 2.0 with 5N HCl and extracted 5 times with 40 ml absolute ethyl ether in a 1 L separatory funnel. The extracted PHP was refrigerated at 4°C. The ether fraction was collected and the ether removed by drying in a vacuum evaporator. The resulting residue was resuspended in 10 ml 95% ethanol; it had a strong odor of fatty acids.

The pH of the extracted PHP was readjusted to 6 with concentrated NaOH. Medium containing a concentration of extracted PHP equivalent to that of unextracted 2% PHP was prepared. One flask received an amount of ether soluble extract equivalent to the amount normally present in 2% PHP. A second flask received three times the normal amount, and a third flask received none. Unextracted 2% PHP was similarly treated. All media contained the usual concentrations of added vitamins. The flasks were inoculated and treated as previously described; results are found in Table 8.

These data indicate that ether-soluble compounds are not essential for growth and/or enterotoxin production. The amount of toxin produced in the absence of ether-soluble compounds is approximately 60% that of the normal substrate. Addition of these compounds to normal PHP did not stimulate enterotoxin formation.

However, in one preliminary experiment, the observation was made that in the absence of ether-soluble compounds and added vitamins, growth (178 Klett units after 24 hours) occurred but no enterotoxin was produced. This suggests that vitamins may play an important role in the synthesis of enterotoxin and that the ether-soluble fraction may contain vitamin precursors or the actual vitamins necessary for growth and toxin production. Experiments are now in progress to examine this interesting finding.

2. Phillips and Gibbs (1961) reported a technique for the fractionation of peptides in casein using a gel-filtration method with sephadex. Sephadex G-25 powder was soaked for four hours in M/15 sodium phosphate buffer at pH 6.8. The column was packed at the same temperature of soaking to avoid volume changes. Column dimensions were approximately 250 mm by 39 mm. Phosphate buffer was eluted with 0.2M NH_4OH before addition of casein (solubilized in water). The sample was then brought on to the column before

Table 8

Medium	Ether Extract	0 hr	24 hr	Toxin/ml
Extracted PHP				
1	0	11	468	450
2	1X	11	488	600
3	3X	14	389	600
Normal PHP				
1	0	11	463	750
2	1X	13	529	750
3	3X	13	361	600

further addition of eluant. The flow rate was 70-80 ml/hr; the fractions were collected in 10 ml aliquots and assayed for aromatic amino acids by determining the extinction at 280 m μ . The results were in good agreement with those reported by Phillips and Gibbs. Further analysis of the fractions by ninhydrin reaction are planned if any positive growth or toxin responses are shown. PEP fractions obtained by this method have not yet been collected in sufficient quantities to test. Preparative fractionation of workable amounts is planned in the near future. These fractions will most likely be tested in defined media.

Summary

Defined media containing inorganic salts (Gale, 1945) supplemented with vitamins and 7 amino acids has been devised which supports growth of S. aureus S-6 equivalent to that produced in an equal amount of PHP. The effect of inorganic ions in these defined media is being investigated further. It appears that both calcium and manganese ions have some inhibitory effects.

The role of carbon source is of primary importance in growth and toxinogenesis. Glucose supports good growth in the synthetic media (200 Klett units in 24 hr) but little or no toxin is formed. Similar results are found for glycerol, pyruvate, and ribose. However, utilization of glutamic acid yields both good growth (254 Klett units in 24 hr) and toxin production (25 µg/ml). This effect of the carbon source on toxin formation may be related to the pathway of metabolism of the carbon source: the carbohydrates are used fermentatively even in the presence of oxygen whereas glutamic acid is used oxidatively. Repression of oxidative metabolism during utilization of glucose is a phenomenon known to exist in S. aureus and other bacteria. The failure to form toxin under these conditions may be due to the unavailability of necessary intermediates or substrates produced during oxidative metabolism or to the direct repression of enzyme systems for toxin synthesis.

Other substrates tested were the Kreb's cycle intermediates, succinate, citrate, acetate, oxaloacetate, alpha-ketoglutarate, and malate. Glutamic acid was the only amino acid examined at this time, but experiments are presently under way to test the effects of others both as nitrogen and carbon source.

A microtiter hemagglutination inhibition assay has been developed for the detection of enterotoxin B. The method relies on the use of formalin treated, tanned, sheep erythrocytes which are sensitized with enterotoxin B. Antiserum

and toxin samples are incubated together in 0.025 ml quantities before addition of sensitized cells. If toxin were present in the sample, the antiserum would be used up and an inhibition of agglutination of the erythrocytes occurred. If toxin is not present, the antiserum is free and will cause an agglutination of the sensitized erythrocytes. The method is sensitive enough to permit detection of 0.06 μ g of toxin; it is fast, accurate, and easy to read. The development of this assay has resulted in a significant saving of time in laboratory analyses and has proved to be an important tool in our investigations.

Ether-extracted PHP supported growth and toxin formation when supplemented with the usual vitamins. However, in the absence of vitamins some growth, but no toxin was produced. It is known that cofactors are important for growth of S. aureus and the amount of growth here may have been due to carry-over of cofactors in the inoculum or the presence of trace amounts in the extracted PHP. Whether toxin formation is also dependent upon the presence of cofactors is still unresolved, but these data do lend some support to this view at present.

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