phosphate by chemical methods, 62%, by microbiological assay, 57%; pyridoxamine phosphate, chemical, 69%, microbiological, 57%. The sample of pyridoxal phosphate used contained 1.5 times as much phosphate as pyridoxal, the excess phosphate being released by alkaline hydrolysis under conditions where pyridoxal phosphate is stable.

Absorption spectra of the vitamin B_6 phosphates have been reported.[13,15] Pyridoxal phosphate can be specifically estimated at *p*H 6.7 by its absorption at 385 mμ. Measurements at 326 mμ were made as a check.

Ca. 0.001 *M* solutions of the vitamin B_6 phosphates were heated at various *p*H values at 100° and the amount of hydrolysis measured by inorganic phosphate release and spectrum changes. In the *p*H range 2–12 hydrolysis rates decreased as the *p*H was increased; at *p*H 7 pyridoxal phosphate was hydrolyzed to the extent of 20% in 1 hr.; pyridoxamine phosphate 5%.

Transamination was carried out at *p*H 7 to avoid excessive hydrolysis. By contrast to the reactions with free pyridoxamine and pyridoxal, a reproducible catalytic effect could not be demonstrated for salts of metals other than copper in transamination reactions with the vitamin B_6 phosphates, apparently due to the formation of insoluble metal salts with these compounds. However, from the data obtained, it appears reasonably certain that aluminum and iron(II) and -(III) salts have some catalytic effect; copper sulfate is highly active as a catalyst.

The transamination equilibrium lies decidedly in favor of pyridoxamine phosphate as shown in Fig. 6 which gives data for the copper-catalyzed transamination in this system with 0.004 *M* reactants in the presence of 0.02 *M* excess ketoglutarate. The reactions were carried out under nitrogen to avoid excessive air oxidation of pyridoxamine phosphate to pyridoxal phosphate. When the reactions were carried out with a 2.5-fold higher copper sulfate concentration, anomalous results were obtained. The initial reaction rates were increased, but decreased rapidly so that at 60 min. the system appeared to be farther from equilibrium than with the lower catalyst concentration, apparently due to the precipitation of certain reactants by the copper.

The fact that the equilibrium favors pyridoxamine phosphate probably explains the reported failure to obtain complete transamination of pyridoxamine phosphate by ketoglutarate.[13] It is also consistent with several observations[cf. 15] that pyridoxal can exist as a cyclic hemi-acetal whereas pyridoxal phosphate cannot. If at *p*H 7 pyridoxal exists largely as the hemiacetal, the concentration of the free aldehyde form in a pyridoxal phosphate solution will be much larger than that in a pyridoxal solution. Thus, if the equilibrium constants for transamination with the aldehyde forms are similar for pyridoxal phosphate and for pyridoxal, the over-all transamination equilibrium with pyridoxal phosphate will lie in favor of the amine form as compared to the equilibrium with pyridoxal.

Rate *vs.* *p*H curves for transamination of pyridoxal phosphate with glutamate using copper sulfate as catalyst were found to be very similar to those in Fig. 4 for the reaction of pyridoxamine and ketoglutarate.

(15) D. Heyl, E. Luz, S. A. Harris and K. Folkers, THIS JOURNAL, **73**, 3430 (1951).

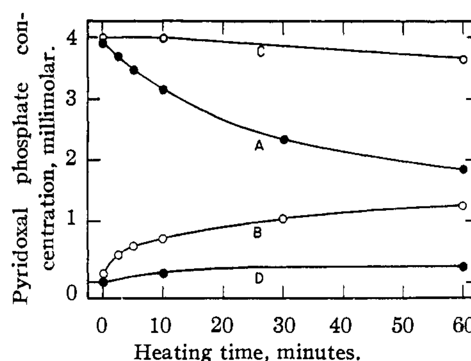

Fig. 6.—Pyridoxal phosphate concentration *vs.* time during transamination at 100°: A, between 0.004 *M* pyridoxal phosphate and 0.004 *M* glutamate in the presence of 0.02 *M* α-ketoglutarate; B, between 0.004 *M* pyridoxamine phosphate and 0.024 *M* α-ketoglutarate; C, 0.004 *M* pyridoxal phosphate heated alone; D, 0.004 *M* pyridoxamine phosphate heated alone. All solutions were 0.0002 *M* in copper sulfate and 0.1 *M* in phosphate buffer, *p*H 7.0.

AUSTIN 12, TEXAS RECEIVED SEPTEMBER 10, 1951

[CONTRIBUTION FROM DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

An Assay Procedure for the Determination of a Growth Stimulatory Factor for *Staphylococcus Albus*

BY NATHAN H. SLOANE[1] AND RALPH W. MCKEE[2]

A satisfactory nutrient medium was devised and appropriate conditions determined for the assay of a growth stimulatory factor for the bacterium, *Staphylococcus albus*. A standard growth stimulatory preparation, 4AB148, was prepared from beef plasma. This stable material provided a reference for the assay of unknown source materials. Employing the assay procedure the relative distribution of growth stimulatory activity was determined in a large number of natural products.

Introduction

The growth requirements and optimal conditions for multiplication of the bacterium, *Staphyloccocus albus*, have been the subjects of numerous and extensive studies.[3,4,5,6] In spite of the various investigations on this organism its growth requirements are still incompletely defined.

It was shown recently[7,8] that a specific growth stimulatory material reduces the lag phase of *Staphylococcus albus*. As will be described in the succeeding paper this growth stimulatory factor, which is present in a wide variety of natural materials, has been obtained in a relatively high state of purity from the blood plasma of cattle.

It is the purpose of this paper to describe a technique which has been developed for the assay of this growth stimulatory factor required by *Staphylococcus albus*. This study resulted from work initiated in the Department of Bacteriology by Professor J. Howard Mueller.

(1) United States Public Health Service, National Institutes of Health Predoctoral Research Fellow, 1948–1949.

(2) Supported in part by the Higgins Fund.

(3) T. P. Hughes, *J. Bact.*, **23**, 437 (1932).

(4) B. C. J. G. Knight, *Brit. J. Exp. Path.*, **16**, 315 (1935).

(5) P. Fildes, G. M. Richardson, B. C. J. G. Knight and G. P. Gladstone, *ibid.*, **17**, 481 (1936).

(6) B. C. J. G. Knight, *Biochem. J.*, **31**, 371 (1937).

(7) N. H. Sloane and R. W. McKee, *Federation Proc.*, **8**, 252 (1949).

(8) N. H. Sloane, Thesis, Harvard University, 1950.

Experimental

Organism.—The *Staphylococcus albus* organism used in this study was a non-hemolytic strain, maintained for a number of years in the Department of Bacteriology.

Twenty-hour cultures grown on nutrient agar slants were stored in the cold room at +5° and transplanted every 2–3 weeks. Eighteen-hour cultures grown at 37° in tryptic digest of beef heart infusion[9] were prepared from the cold room slant and used as the inoculum for the assay.

Lag Phase.—Maximum growth of the *Staphylococcus albus* was obtained in 12 hours at 37° using a large inoculum of organisms (approximately ten million) in 10 ml. of the tryptic digest broth or in the synthetic medium, containing either pure amino acids or an acid hydrolysate of casein (Table I).

TABLE I

COMPOSITION OF BASAL MEDIUM, 3A30-1

Component	Amount per culture tube Mg.	Amount per culture tube μg	Blocks of nutrients[a] No.	Blocks of nutrients[a] Ml.
Acid hydrolysate of vitamin-free casein	100		1	1.0
Cystine	2		2	0.25
Tryptophan	2		3	.25
Tyrosine	4		4	.25
Na_2HPO_4	3		5	
KCl	2		5	.25
Adenine		100	6	.25
Guanine		50	7	.25
Xanthine		50	8	.25
Uracil		20	9	.25
Biotin		0.04	10	
d-Calcium pantothenate		20	10	
Choline	1		10	
Folic acid		0.4	10	
Inositol		100	10	
Nicotinic acid		10	10	
p-Aminobenzoic acid		5	10	
Pyridoxal		9	10	
Pyridoxamine		9	10	
Pyridoxine		5	10	
Riboflavin		5.2	10	
Thiamin HCl		5.2	10	
Asparagine	1		10	
Oleic acid		5	10	1.5
$FeCl_2 \cdot 6H_2O$		50	11	
$CuSO_4 \cdot 5H_2O$		10	11	
$ZnSO_4 \cdot 7H_2O$		3	11	
$MnCl_2 \cdot 4H_2O$		3	11	
$MgSO_4 \cdot 7H_2O$	65		11	0.25[b]
Glucose	25		12	0.25[c]

[a] The basal medium was prepared fresh for each assay from the twelve different component blocks previously prepared and stored at +5° with chloroform as a preservative. [b] The mineral solution was adjusted to *p*H 2 before autoclaving and added aseptically to the sterilized medium. [c] The sterile glucose solution was added aseptically to the sterilized medium.

However, when the basal medium, 3A30-1 (Table I) was inoculated with only 10 to 100 organisms a longer lag phase was observed. This lag period could be reduced by adding a standard preparation of growth stimulatory material, 4AB148 (Fig. 1), obtained from deproteinated beef plasma or other natural materials containing the growth stimulatory factor.

(9) *Tryptic Digest of Beef Heart Infusion:* The tryptic digest broth was prepared according to the method of Mr. V. Fields of the Department of Bacteriology. Three hundred grams of fat-free ground beef heart was infused overnight at 4° in 1800 ml. of tap water. The material was then heated at 75–80° for 5 minutes, cooled to 30° and 0.6 g. of powdered trypsin (Pfanstiehl 1:110) was added. After incubating at 37° for six hours, 2.1 ml. of glacial acetic acid was added and the mixture boiled for ten minutes, cooled and refrigerated overnight. The material was then filtered, adjusted to *p*H 7.6 and diluted with an equal volume of tap water containing 1% NaCl. The broth was then sterilized by autoclaving at 15 pounds pressure (121°) for 15 minutes. A sterile 10% glucose solution was added to make 0.1%.

The longer lag phase that occurs with the basal medium could not be attributed to suboptimal or toxic concentrations of any of the ingredients of the medium, or to a deficiency of any known biologically active compound tested (Table II).

TABLE II

SUBSTANCES NOT AFFECTING THE LAG PHASE OF *Staphylococcus albus*

Substance	Amount of material per culture tube, μg. unless specified
N-Acetylglucosamine	0–1000
Adenine	50–250
Adenosine triphosphate	0–1000
Adenylic acid	0–200
β-Alanine	0–50
p-Aminobenzoic acid	0–10
Biotin	0.02–1
Carbon dioxide	0–5% in air
Choline	0–2000
Citrovorum factor	0–10
Cocarboxylase	0–40
Cytosine	0–1000
Ergothionine	0–100
Folic acid	0.2–2
Glucosamine	0–1000
Glucose	25–50
Glutamine	0–1000
Glutathione	0–4000
Guanine	0–100
Guanylic acid	0–1000
Inositol	0–200
Lactobacillus bulgaricus factor (LBF)	0– ≅ 100 g. yeast
Liver extract, Lederle, antipernicious anemia	0–1.5 units
Nicotinamide	0–100
Nicotinic acid	5–100
Nucleic acid, thymus	0–1000
Nucleic acid, yeast	0–1000
Oleic acid	0–10
Orotic acid	0–10
d-Calcium pantothenate	10–50
Protogen	0–50 units
Pyridoxamine	0–18
Pyridoxal	0–18
Pyridoxine	0–50
Riboflavin	0.1–50
Ribose	0–50
Sodium bicarbonate	0–1000
Thiamin·HCl	0.1–100
Thymine	0–50
Uracil	10–50
Vitamin B_{12}, crystalline	0–0.1
Xanthine	0–100
Xanthopterin	0–100

Preparation of Basal Medium, 3A30-1.—The medium designated 3A30-1 (Table I) is the basal nutrient medium devised and used for the assay of the growth stimulatory factor for *Staphylococcus albus*. The components of the nutrient medium were prepared in twelve different blocks and stored at +5°, using chloroform as a preservative. These blocks of nutrients are freshly prepared every two weeks.

The basal medium was prepared fresh for each assay. All the blocks except 10, 11 and 12 were combined for the desired number of culture tubes in the proportions indicated in Table I. The solution was adjusted to about *p*H 7 and then the required amount of the vitamin block (No. 10) was added. The resulting medium was adjusted to *p*H 7.4–

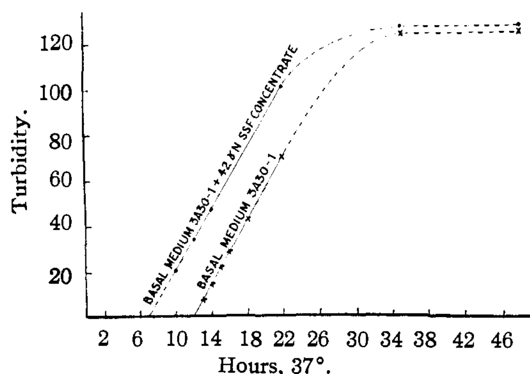

Fig. 1.—Growth rate curves of *S. albus* on the basal medium and the basal medium plus the SSF concentrate, (4AB148).

7.6. This constitutes the double-strength medium which is added to the standard and test materials after they have been prepared (see Preparation of Culture Tubes for Assay).

Preparation of Standard and Test Materials.—It was determined that the growth stimulatory factor present in bovine plasma and human urine was susceptible to spontaneous oxidation in air. It was thus necessary to reduce the material and to maintain it in the reduced state during the incubation period of the assay. This could be accomplished by treating the growth stimulatory material with either cysteine or glutathione. Due to the slower rate of oxidation by air of glutathione this compound was employed throughout most of the studies. The glutathione (2 mg. per 0.1 ml.) was dissolved and neutralized (*p*H 7) immediately prior to its addition to the culture tubes.

Aqueous solutions of the growth stimulatory material to be assayed were adjusted to *p*H 7.4–7.6. These solutions were measured into culture tubes (16 × 150 mm.) and water added to make a total of 4.4 ml. The glutathione solution (0.1 ml.) was added next and the tubes allowed to stand at room temperature for 15 minutes.

Preparation of Organisms.—The day before the assay was to be performed a loopful of organisms from the nutrient agar slant stored at +5° was transferred aseptically to 5 ml. of tryptic digest broth and grown at 37° for 18 hours. The culture was then centrifuged for 10 minutes at 1500 r.p.m. and the supernatant broth discarded. The cells were washed by suspending in 10 ml. of sterile saline and recentrifuging. The bacteria were resuspended in 5 ml. of sterile saline and six tenfold serial dilutions of the washed organisms were made with sterile saline. One drop of the sixth dilution of *Staphylococcus albus* (10–100 organisms), measured with a Pasteur pipet (0.02 ml.), was added to each culture tube as indicated in the next section.

Preparation of Culture Tubes for Assay.—Culture tubes (a) without growth stimulatory material (blanks), (b) with several different amounts of the standard, and (c) with various levels of the substances, were prepared in triplicate. As indicated previously aqueous solutions of the materials were treated for 15 minutes at room temperature with glutathione.

To these tubes was then added 5.0 ml. of the double strength medium. The tubes were plugged with cotton and autoclaved at 15 pounds pressure for 10 minutes. After cooling the tubes, 0.25 ml. of the sterile solution of inorganic components (No. 11) and 0.25 ml. of the 10% glucose solution (No. 12) was added to each tube. Finally one drop of the diluted organism was added to each of two of the three tubes prepared. The third tube was the non-inoculated control. The contents of the tubes were mixed by agitation and placed at 37° for the assay period of 12 to 15 hours.

Measurement of Growth.—Growth of the bacteria was measured turbidimetrically by means of a Klett–Summerson photoelectric colorimeter fitted with a No. 54 filter. Over the range of turbidities studied the instrument readings are proportional to the numbers of organisms. Thus the measurements are expressed simply as "turbidity" using the numerical values obtained with the photoelectric colorimeter.

Each culture was thoroughly mixed and transferred to a colorimeter tube. The instrument was adjusted to zero with a water tube and then each of the cultures read against that tube. The reading of each non-inoculated tube was subtracted from the readings of the two inoculated cultures.

Preparation of Stable Standard and the Evaluation of Test Materials.—Although the growth stimulatory response is proportional to the concentrations of growth stimulatory factor (Fig. 2), a constant half-maximal growth response unit could not be obtained. Neither is there, from one assay to another, the same amount of growth with the same concentration of the growth stimulatory factor. For these reasons at least three different levels of the standard preparation are run with each assay so that a more direct comparison of the unknowns could be made with the standard. When such comparisons are made from one assay to another the error of assay is approximately ± 10%.

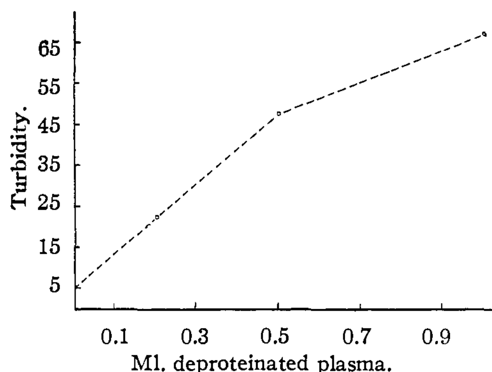

Fig. 2.—Growth response curve of *Staphylococcus albus* to deproteinated bovine oxalated plasma, 13 hours, 37°.

The standards of reference initially used were tryptic digest broth and deproteinated plasma. However, a dried preparation, 4AB148, was obtained from plasma and this was employed in all the subsequent work as the reference standard. This material, as will be more completely described in the next paper, was obtained by diluting oxalated beef plasma with an equal volume of water, adjusting to *p*H 5–6 with acetic acid, heating in a boiling water-bath for 5 minutes to remove the heat coagulable protein, precipitating the active material with silver, decomposing the silver proteinate with sodium chloride and lyophilizing the supernatant material. With this material it was determined that a quantity containing 21 µg. of nitrogen yielded in 12–15 hours growth adequate for assay. Therefore amounts of 4AB148 containing from 4.2 to 21 µg of nitrogen were used as reference quantities for the assays.

A unit of growth stimulatory response was defined as the SSF activity present in 0.20 ml. of oxalated plasma (0.4 ml. of deproteinated plasma) or 8.4 µg. of nitrogen of the stable silver salt, 4AB148, prepared from bovine plasma (equivalent in SSF activity to 0.4 ml. of deproteinated plasma) (Fig. 3).

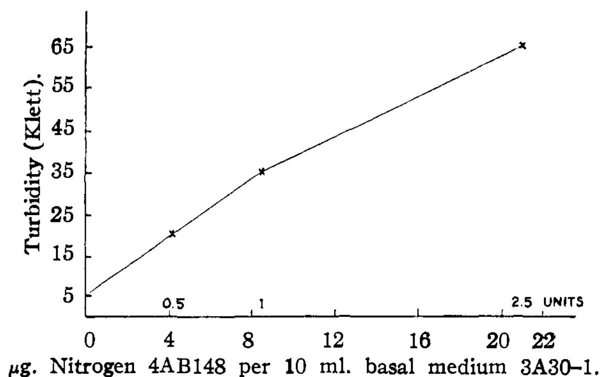

Fig. 3.—Growth response curve of *S. albus* to decomposed silver salt (4AB148) of SSF prepared from oxalated bovine plasma, 12 hours, 37°.

Distribution of Growth Stimulatory Factor in Naturally Occurring Materials.—Utilizing the above-described assay

procedure it was possible to survey a rather wide variety of natural products and to determine the relative concentrations of the growth stimulatory factor in these materials. Table III indicates the wide-spread distribution of the factor in both plant and animal sources.

It is of interest to note its high content in human plasma protein fraction IV-4, and its absence from ultrafiltrates of bovine serum. The isolation studies presented in the following paper indicate the protein nature of the stimulatory factor as it is present in bovine plasma and human urine.

TABLE III

RELATIVE CONCENTRATIONS OF SSF IN NATURAL MATERIALS

Test material	Relative concentration of SSF (based on dry weight)
Beef heart infusion	100
Liver extract (aqueous extract of bacto-liver)	7
Difco yeast extract	50
Milk whey	2
Pseudomonas aeruginosa culture filtrate	400
Staphylococcus albus culture filtrate (grown in basal medium 3A30-1)	175
Human male urine	50
Human plasma	100
Bovine plasma	100
Bovine serum ultrafiltrate[a]	0
Human plasma fractions Cohn, *et al.*[b]	
Fraction I	0
Fraction II plus III	50
Fraction IV-1	400
Fraction IV-4	900
Fraction IV-6	400
Fraction V	100
Fraction VI	0

[a] We wish to thank Dr. John Hanks, Department of Bacteriology, for supplying this material. [b] We wish to thank Dr. Douglas Surgenor, Department of Physical Chemistry, for these fractions.

Discussion

Any microörganism shows a delay (lag phase) in its growth when transplanted to a fresh culture. The length of the lag phase will depend on several factors, principally, however: (1) the proportion of organisms to nutrient medium in the new culture, a fact that was used to advantage in this study, and (2) whether the culture medium contains the essential growth factors performed. If the microörganism has the ability to synthesize its growth factors then maximal growth will ensue even after a prolonged lag period in the absence of these factors.

The basal medium when inoculated with a small number of *S. albus* organisms allows maximal growth to occur, following a 12-hour lag period (Fig. 1). The addition to the culture of the growth stimulatory material, hastens the onset of growth but without increasing the maximal growth which is obtained in 36 hours (Fig. 1). This means that these bacteria produce the growth factor (Table III) and when the number of organisms becomes large enough to produce an adequate concentration of the essential factor there is rapid growth. This is in agreement with the concept of Hinshelwood[10] that when the optimal concentrations of the essential metabolites have been supplied to or produced by the cells a logarithmic growth rate will occur.

Under the conditions employed in this study, namely a culture containing only a few organisms and therefore a limited ability to synthesize the SSF material, the lag phase is proportional to the amount of added SSF protein. By choosing a particular time (12–15 hours) at which little or no growth is manifested in the tubes without added SSF then this proportionality between the amount of added SSF and growth can be measured. This graded growth response of a microörganism to an essential growth factor, and its correlation with the synthesizing ability of the bacteria, has been demonstrated by the studies of Bohonos, Hutchings and Peterson.[11] Employing the basal medium, 3A30-1, it was found that the growth stimulatory response to a primary standard was proportional to the amount of extract added, but the results of numerous assays showed a wide variation in the half maximal growth response for a unit of SSF material. Bohonos, Hutchings and Peterson[11] observed that *Lactobacillus casei* did not give a constant amount of growth response to a given level of pyridoxine. Similarly, Woolley[12] in studies on the nutrition of Group A hemolytic streptococci found that an absolute value for half maximal growth response could not be achieved. Thus in these studies several different amounts of the 4AB148 standard were run with each assay and a unit of activity arbitrarily defined as the amount of growth obtained with an amount of the standard preparation, 4AB148, containing 8.4 μg. of nitrogen.

The important problem of the interrelationships of essential metabolites in the growth of the microorganism was considered in this study by testing a rather wide range of concentrations of the nutrient components and of a number of compounds not used in the medium (Table II). There appeared to be little influence on growth due to these variations in nutrient concentrations. It thus appears that a satisfactory system was obtained with only one principal variant to consider, namely, the growth stimulatory factor.

BOSTON, MASSACHUSETTS RECEIVED AUGUST 6, 1951

(10) C. N. Hinshelwood, "Chemical Kinetics of the Bacterial Cell," Oxford University Press, London, 1946, p. 49.

(11) N. Bohonos, B. L. Hutchings and W. H. Peterson, *J. Bact.*, **44**, 479 (1942).

(12) D. W. Woolley, *J. Exp. Med.*, **73**, 487 (1941).