Group A Hemolytic Streptococci. I. A Chemically Defined Medium for Growth from Small Inocula. (23406)

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Several attempts have been made to cultivate group A streptococci in chemically defined media(1-8). Slade, Knox and Slamp, and Slade and Slamp(7,8) obtained luxuriant growth of various strains of hemolytic streptococci by using media of 15 or 16 amino acids to which either a peptide preparation (CR14A) or heated crystalline ovalbumin was added; when, however, ovalbumin or the peptide preparation were omitted, no growth was obtained under defined conditions.

A defined medium allowing good growth and streptolysin 'O' production by various strains of group A streptococci has been described previously(9). The present study was performed to determine the nutritional factors necessary for optimal growth of group A streptococci. Details on streptolysin 'O' production in this medium will be reported elsewhere.

Materials and methods. Table I lists all components of the medium. Complete synthetic medium—COM-SYN-6—is composed of: basal medium, 22 amino acids and 6 vitamins. To study the nutritional demands of the various strains used, additional media were employed listed in Table I. All media were dispensed in 10 ml quantities into pyrex tubes 22 x 160 mm and were incubated at 37°C. Uninoculated tubes served as controls. Growth was determined as optical density (O.D.) and was measured with a 6A model Coleman Spectrophotometer at 550 mμ. Three strains of group A streptococci were used.† 1) Strain S84 (type 3) was obtained from the State Serum Institute, Copenhagen. 2) Richard's strain R 8191 (type 6) was obtained from the NCTC, London, and 3) Strain M, a local strain. *Inoculum.* Cultures maintained on blood agar were subcultured monthly. Single hemolytic colonies were inoculated into BHI medium and grown for 18 hours at 37°C. The bacterial growth was washed twice with an equal volume of phosphate buffer M/15, pH 7.2 made up in saline. Two consecutive passages in COM-SYN-6 medium were then performed using an inoculum of approximately 5 x 10^7/ml organisms (barely visible suspension, O.D. = 0.01). After 6-8 hours of incubation growth was diluted to give a barely visible suspension which was either further diluted or used as such; 0.05 ml aliquots were used to inoculate each assay tube. The medium was not considered adequate unless 2 consecutive passages in it without diminution of growth were obtained.

Results. Growth of different strains of streptococci in various media. Using a large inoculum (5 x 10^6 cells/ml) heavy growth was obtained in the following media: COM-SYN-6, CAS-AMINO-6, ENZ-AMINO-6, BHI, and Slade and Slamp medium(8). Cysteine HCl (100 μg/ml) was added prior to inoculation to all media except for BHI medium. Growth in BHI medium appeared earlier than in others and could be initiated from smaller inocula. No reduction in growth was observed even after 12 consecutive passages on all media used. *Influence of inoculum size on growth of Strain S84 on various media.* As seen in Table II, growth in BHI medium was initiated from as few as 500 cells. On the other hand, with COM-SYN-6 medium with added cysteine (100 μg/ml) or semi-defined media containing the same amount of cysteine, no growth from inocula smaller than 5 x 10^6/ml could be obtained. It was considered that the excellent growth obtained in BHI medium might have been due to its reducing and buffer properties as

* This paper forms part of a Ph.D. thesis to be submitted.

† While this paper was submitted for publication 2 additional strains (c-203S and Blackmore) were grown successfully in the COM-SYN-6 medium. These strains were kindly supplied by Dr. A. W. Bernheimer, N. Y. University Medical College.

well as to its nutritional adequacy. Therefore, the effect of increasing concentrations of various reducing agents on growth from small inocula was tried. *Effect of increasing concentration of reducing agents on growth of streptococci.* Slade & Knox(6) stressed the importance of reducing agents for streptolysin 'O' formation on a semi-defined medium. Under their experimental conditions cysteine at a concentration of 75-100 μg/ml allowed optimal streptolysin 'O' production by the Richard's strain from an inoculum of approximately 1 x 10^7 organisms/tube (about 0.015 ml of O.D. of 0.25); concentrations of cysteine higher than 200 μg/ml were slightly inhibitory. However, we failed to obtain visible

TABLE I. Composition of the Complete Synthetic Medium (COM-SYN-6).*

Basal medium	mg/ml	"3 vitamins"	μg/ml	"22 amino acids" 13 essential	μg/ml	9 non-essential	μg/ml
$Na_2HPO_4 \cdot 12H_2O$	3.4	Nicotinic acid	10	L-Arginine	75	L-Alanine	35
KH_2PO_4	.3	Pantothenate-Ca	"	L-Cysteine HCl†		L-Aspartic acid	120
$FeSO_4 \cdot 7H_2O$	.05	Riboflavin	"	Glycine	10	L-Asparagine	100
$MnCl_2 \cdot 4H_2O$	.025	"4 vitamins"		L-Glutamine	100	L-Cystine	70
$ZnSO_4 \cdot 7H_2O$	.01			L-Histidine	50	L-Glutamic acid	400
Glucose	4.0	Nicotinic acid	10	L-Leucine	50	DL-Isoleucine	200
$NaHCO_3$	.5	Pantothenate-Ca	"	L-Lysine	65	DL-Methionine	120
$MgSO_4$	.04	Pyridoxal • HCl	"	L-Phenylalanine	80	L-Proline	350
NaCl	.3	Riboflavin	"	DL-Serine	100	L-OH Proline	5
	μg/ml	"6 vitamins"		L-Tryptophane	200		
Guanine	10	(Essential and		L-Threonine	70		
Xanthine	10	non-essential)		L-Tyrosine	110		
Uracil	10	Biotin	.2	L-Valine	160		
		Nicotinic acid	10				
		Pantothenate-Ca	"				
		Pyridoxal • HCl	"				
		Riboflavin	"				
		Thiamine • HCl	.6				

* Other media employed:

1) COM-SYN-3 medium—basal medium, 22 amino acids, and 3 vitamins.
2) 13-SYN-6 " " " 13 " " and 6 " .
3) 13-SYN-4 " " " 13 " " and 4 " .
4) 13-SYN-3 " " " 13 " " and 3 " .
5) 12-SYN-6 " " " 12 " " (Cysteine omitted from 13 amino acids), and 6 vit.
6) 12-SYN-3 " " " 12 " " (as in 5), and 3 vit.
7) CAS-AMINO-6 " " " supplemented with 150 mg/10 ml casamino acid (Difco), and 6 vit.
8) ENZ-AMINO-6 " " " supplemented with 150 mg/10 ml enzymatic hydrolysate of casein (N.B.C.), and 6 vit.
9) BHI medium—Brain heart infusion medium (Difco).
10) Slade & Slamp medium—basal medium supplemented with 16 amino acids according to Slade & Slamp(8), and 6 vit.

† The amt of cysteine varied in different experiments; its concentration, therefore, is given separately. Cysteine was freshly prepared before inoculation. Glucose, vitamins, glutamine, bicarbonate, magnesium sulfate and cysteine were Seitz sterilized; all other materials were sterilized by steam pressure at 15 lb for 15 min. Solutions were made up with bidistilled water. The pH of the medium was adjusted to 7.6. All chemicals were purchased from NBC, Cleveland, Ohio.

TABLE II. Influence of Inoculum Size on Growth of Strain S84.

Medium	Cells per ml 5×10^6	5×10^5	5×10^4	5×10^3	5×10^2
COM-SYN-6	.19	.07	.02	.02	.02
CAS-AMINO-6	.28	.11	.04	.02	.02
BHI	.32	.32	.32	.32	.28

Cysteine (100 μg/ml) was added prior to inoculation except for BHI medium. The inoculum consisted of cells grown for 6 hr in COM-SYN-6 medium; O.D. readings were performed after 18 hr of incubation at 37°C.

TABLE III. Effect of Increasing Concentrations of Cysteine HCl and Ascorbic Acid on Growth of Streptococci from Small Inocula.

Cysteine HCl, mg/ml	Cells per ml				
	2×10^5	1×10^5	1×10^4	1×10^3	5×10^2
.1	.02	.02	.02	.02	.02
.25	.18	.09	.02	.02	.02
.5	.26	.26	.24	.18	.15
.8	.32	.32	.32	.29	.23
1.2	.38	.35	.35	.29	.25
Ascorbic acid, mg/ml					
1.5	.12	.06	.02	.02	.02
2.0	.17	.12	.12	.02	.02
3.0	.22	.18	.15	.10	.02

The medium employed was COM-SYN-6. Cysteine and ascorbic acid were freshly prepared before inoculation; O.D. readings were taken after 18 hr of incubation at 37°C. The strain employed was S84.

growth from inocula smaller than 5 x 10^6 cells/ml in the presence of 100 μg/ml of cysteine. On the other hand, increasing the concentration of cysteine from 100 to 500 μg/ml allowed optimal growth from as few as 10^3 cells/ml. Even concentrations as high as 1200 μg/ml were effective in promoting growth from small inocula (5 x 10^2 cells/ml, Table III). Similar results were obtained with the 2 other strains employed. Ascorbic, thiomalic and thioglycollic acids were less effective than cysteine. *Specificity of reducing agents for growth of streptococci.* Koser & Thomas(10) using *Lactobacillus fermenti* found that the requirements for cysteine could be satisfied by ascorbic acid, while strains of *L. casei* and the majority of *L. plantarum* strains required cysteine for growth. Table IV shows that with the streptococcus employed ascorbic acid could replace cysteine only when a small amount (10 μg/ml) of cystine was present. Methionine, ethionine and thiosulfate were ineffective in the presence of ascorbic acid. Similar results were obtained with the 2 other strains used. *Nutritional requirements of streptococci in complete synthetic medium.* Omission from the complete medium (22 amino acids and 6 vitamins) of 2 vitamins (thiamine and biotin) and 9 non-essential amino acids, did not influence the growth of all strains employed. However, when both amino acids and vitamins were reduced to 13 and 3 respectively, the onset of growth was delayed to 36 hours (instead of 13-15 hr) and the final yield was about 25% of that obtained in COM-SYN-6 medium. Addition of pyridoxal to the 13-SYN-3 medium resulted in rapid and optimal growth after 15 hours (Table V). The need for pyridoxal in the 13 amino acids medium (13-SYN-3) in spite of its dispensability in the 22 amino acids medium (COM-SYN-3) suggested that non-essential amino acids may replace pyridoxal. The effect of different non-essential amino acids in a medium containing no pyridoxal was thus tried. It was

TABLE V. Growth of Strain S84 on Various Combinations of Amino Acids and Vitamins.

Medium	Hr of incubation			
	15	19	25	36
13-SYN-6	.23	.23	.23	.23
13-SYN-3	.05	.06	.06	.06
13- " + pyridoxal	.19	.23	.23	.23
13- " + DL-alanine	.19	.22	.22	.22
13- " + L-alanine	.18	.20	.23	.23

Concentration of cysteine was 500 μg/ml, of pyridoxal 10 μg/ml, of DL- and L-alanine: 70 and 35 μg/ml respectively. The inoculum consisted of 10^4/ml cells of a 5 hr culture grown on 13-SYN-4 medium. Growth was determined as O.D. at 550 mμ.

TABLE IV. Effect of Cysteine and Ascorbic Acid on Growth of Strain S84.

Medium	Cysteine·HCl, .3 mg/ml	Ascorbic acid, 3.0 mg/ml	Hr of incubation 15	19	24
COM-SYN-6*	+	—	.21	.23	.24
COM-SYN-3*	—	+	.22	.23	.23
12-SYN-6	—	+	.02	.02	.02
COM-SYN-6 + cystine, 10 μg/ml	—	+	.21	.23	.23
12-SYN-3 + " 10 "	—	+	.10	.10	.10
Idem + pyridoxal, 10 μg/ml	—	+	.18	.23	.24

* COM-SYN-6 and COM-SYN-3 contain cystine 5 μg/ml, but no cysteine. Growth was determined as O.D. at 550 mμ.

TABLE VI. Effect of Glutamine and Glutamic Acid on Growth of Strain S84.

Medium	Glutamine	Glutamic acid	Hr of incubation 13	15	19	36
13-SYN-6	—	—	.23	.23	.23	.23
" *	+	+	.22	.22	.22	.22
" *	—	+	.02	.05	.15	.23
" *	+	—	.20	.23	.24	.24

* 13-SYN-6 medium less glutamine. Concentration of glutamine and glutamic acid were 100 μg/ml. All tubes contained 400 μg/ml of cysteine. The inoculum consisted of 10^6/ml cells obtained from a 6 hr culture grown on 13-SYN-6 medium. Growth was determined as O.D. at 550 mμ.

found that both L- and DL-alanine effectively replaced pyridoxal when strain S84 and R8191 were used. With the M strain little or no growth was obtained in the presence of alanine.

Requirements for purines and pyrimidines. The complete medium contains guanine, xanthine and uracil. However, optimal growth was obtained when either one of the bases was used in a medium which consisted of 13 amino acids and 4 vitamins. Thus the findings of Slade & Knox(6) regarding interchangeability of purine and pyrimidine bases for growth of the Richard's strain was confirmed in a complete synthetic medium. Similar results were obtained with the other 2 strains employed. *Requirements for glutamine and glutamic acid.* Glutamine was necessary for growth of hemolytic streptococci and was non-replaceable by a series of compounds related to it(11,12). As can be seen from Table VI, optimal growth was obtained when the mixture of 12 amino acids and 6 vitamins was supplemented with glutamine and glutamic acid or with glutamine alone. In the presence of glutamic acid alone growth was considerably delayed and an optimum reached after 26 hours (in comparison to 13-15 hours in the presence of glutamine). Thus the importance of glutamine for growth of streptococci as reported by McIllwain *et al.* (11,12) is confirmed.

Discussion. Cultivation of group A streptococci in defined media is very much dependent on size of inoculum used, while no such limitations are encountered when media like brain heart infusions are employed. With small inocula (less than $5x10^6$/ml) no growth in defined media was obtained unless cysteine or other reducing agents were added. However, the amounts of cysteine needed were surprisingly high. An initial concentration of 600-1200 μg/ml produced growth from as few as 10^3/ml organisms, while 100 μg/ml cysteine allowed growth from not less than 5 $x10^6$/ml cells. Thus growth promoting conditions similar to those of BHI medium were obtained. The failure to cultivate streptococci in synthetic media from small inocula by other investigators was apparently due to an inadequacy of reducing conditions rather than lack of unidentified growth factors. It is conceivable that the heated crystalline ovalbumin used by Slade & Slamp(8) partly served to improve reducing conditions. It is difficult to explain the inability of cysteine to replace heated albumin unless this amino acid was used in insufficient amounts (concentrations of cysteine were not stated). However, the possibility is not ruled out that different strains possess different nutritional and reducing demands.

Cysteine was not only a superb reducing agent but also an essential amino acid for all the strains used. Ten μg/ml of either cysteine or cystine were adequate, provided a suitable reducing agent (ascorbic acid) was present. Another point of interest is the need for pyridoxal in a medium containing essential amino acids only (13-SYN-3). In fact, it was possible to replace pyridoxal by DL- or L-alanine. These results are in accord with the findings describing the replaceability of pyridoxal by DL-alanine(13,14,15). As L-alanine was also effective in replacing pyridoxal it seems plausible that the strains used (S84, R8191) possess an alanine racemase allowing utilization of the L-enanthiomorph (16).

Summary. 1) Defined media which allow

heavy growth of 3 strains of group A streptococci have been developed. The medium consists of either a) 21 amino acids, glutamine, 6 vitamins, salts, purines, pyrimidines and glucose or b) 13 amino acids and 4 vitamins. 2) Cysteine is important both as an essential amino acid and as a reducing agent. As an amino acid only a small amount (10 μg ml) is needed and this can be substituted by an equivalent amount of cystine. As a reducing agent it can be replaced by ascorbic acid and less effectively by thiomalic or thioglycollic acids. Concentration of cysteine was critical for initiation of growth from small inocula. With less than 5 x 10^6 ml cells at least 350 μg ml cysteine HCl are needed for obtaining visible growth. 3) Pyridoxal was necessary in a medium of 13 amino acids and 3 vitamins (nicotinic acid, pantothenate, riboflavin) whereas in a complete medium (22 amino acids) no need for pyridoxal was found. Pyridoxal could be replaced by L- or DL-alanine.

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Received May 15, 1957. P.S.E.B.M., 1957, v96.

Survival of Labeled Platelets in X-Irradiated Rabbits.* (23407)

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Although several excellent studies of radiation injury to animals have shown that the cause of the thrombocytopenia appears to be depression of platelet formation in the marrow(1,2,3), none of these studies showed the effect of radiation on a specific population of platelets. The development of a satisfactory method of labeling platelets made this type of study possible(4). This paper reports observations on the survival of Cr^{51} labeled platelets reinjected into rabbits irradiated with X-ray.

Methods and materials. 13 rabbits were exposed to 750 r of whole body X-ray, 24 hours after injection of autologous platelets labeled with sodium chromate^{51}. Twelve rabbits were subjected to the same procedure but with "sham" irradiation.

A Kellikoet X-ray machine was used set at 20 ma, 200 KV with HVL 1.1 mm of Cu using a 0.5 mm Cu filter. The unit was calibrated by the hospital physicist with a Victoreen 250 r chamber, and had an output of 12 r/min with back scatter. The tsd was 1 meter to the dorsal skin of the rabbit, and the animal was entirely within the 90% isodose line.

The platelets from 40 ml of rabbits heart blood were separated and labeled with 450-500 μc of sodium chromate^{51} (sp. activity 20-25 μc/μg) by the technic previously described(4). The labeled platelets

* Supported by U. S. Atomic Energy Com.