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Effect of Certain Purines, and CO_2 on Growth of Strain of Group A Hemolytic Streptococcus.*

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Rapid luxuriant growth of the C203S strain of Group A hemolytic streptococcus, equal to that occurring in broth under comparable conditions, has been obtained on a medium of essentially known composition. The complete medium is made up as follows:

I. 40 cc of stock solution of acid hydrolyzed gelatin equivalent to 25% gelatin[1] are diluted with distilled water to 500 cc and 500 mg cystine dissolved in a few cc of dilute HCl, 3 g KH_2PO_4, 1 g Na_2HPO_4 (anhydrous), and enough 5N NaOH to bring the pH to 7.4-7.6 are added. The solution is boiled gently for 5 minutes and filtered.

II. To the filtrate are added 50 mg tryptophane, 100 mg tyrosine, 15 mg adenine sulfate, 10 mg uracil, 0.2 mg nicotinic acid, 2 mg synthetic vitamin B_6,† 0.1 mg of biotin concentrate‡ and 2 cc of salt mixture (25 g $MgSO_4 \cdot 7\ H_2O$, 20 mg $MnCl_2 \cdot 4\ H_2O$, 5 mg $CuSO_4$, 2 mg $FeSO_4 \cdot 7\ H_2O$, and 2 mg $ZnSO_4 \cdot 7\ H_2O$ made up to 100 cc with water containing a few drops of concentrated HCl). The volume is made up to 900 cc, the solution readjusted to pH 7.4-7.6, tubed in 9 cc amounts and autoclaved at 10 lb for 10 minutes.

III. To each tube is added 0.1 cc of the following solutions which have been sterilized separately: 0.1 mg thiamin (vitamin B_1) per cc, 0.05 mg riboflavin per cc, 0.1 mg synthetic d-calcium pantothenate per cc, 1% neutralized thioglycollic acid containing 0.2 mg glutathione per cc and 5 mg glutamine per cc. The last three solutions

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1 Pappenheimer, A. M., Jr., and Johnson, S. J., *Brit. J. Exp. Path.*, 1937, **18**, 239.

† We are greatly indebted to Merck & Co. for generous samples of synthetic vitamin B_6 and calcium pantothenate.

‡ We are indebted to Dr. D. W. Woolley of the Rockefeller Institute, New York, for this concentrate which was prepared according to the procedure of Woolley, D. W., McDaniel, L. E., and Peterson, W. H., *J. Biol. Chem.*, 1939, **131**, 381. From its activity in promoting growth of *Clostridium butylicum* Dr. Woolley estimates that approximately 4% of the material is actual biotin. (See also Peterson, W. H., McDaniel, L. E., and McCoy, E., *J. Biol. Chem.*, 1940, **133**, lxxv.)

are sterilized by filtration. Finally, 0.5 cc of 5% glucose containing 0.04% $CaCl_2 \cdot 2\ H_2O$ is added to each tube.

IV. The inoculum consists of one drop of 6-8-hour broth culture of C203S which has been twice washed with saline and then made up to slight turbidity with saline. Tubes are incubated under 8 mm CO_2 tension in air for 40 hours. The amount of growth is determined with a photoelectric colorimeter and the readings correlated with standard suspensions of known bacterial nitrogen content.

The effect of omitting each of the above factors in turn from the complete medium is listed in Table I. Figures in the second column indicate the minimum amount of substance necessary for optimum growth in 10 cc of medium.

All the substances listed in Table I are essential for rapid growth with the exception of uracil. Uracil, while not essential, appears to increase growth slightly.

In the complete medium the limiting factor appears to be glucose. Addition of more glucose will increase growth proportionately until sufficient acid has been produced to kill the organisms.

The need for pantothenic acid and riboflavin agrees with the results of Rane and Subbarow,[2-3], McIlwain,[4], and Woolley and Hutchings[5] using other strains. The necessity of glutamine for rapid growth confirms the work of McIlwain *et al.*[6] In agreement with McIlwain we have found that glutathione is not essential provided the thioglycollic acid concentration is increased to 10^{-3} molar in the final medium. Our findings with respect to vitamin B_6 would also seem to confirm those of McIlwain and of Woolley and Hutchings[5] on Group D strains. Thiamin has not previously been reported essential for growth of hemolytic streptococcus. While it does not seem improbable that biotin is necessary for growth of the C203S strain, this cannot be regarded as certain until pure biotin becomes available for test. It is also possible that some other essential factor may be present as impurity in the gelatin or in the glutamine, both of which were prepared from natural sources. This seems unlikely in view of the good growth obtained.

We have been particularly interested in the requirement of strain

2 Rane, L., and Subbarow, Y., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 837.

3 Subbarow, Y., and Rane, L., *J. Am. Chem. Soc.*, 1939, **61**, 1616.

4 McIlwain, H., *Brit. J. Exp. Path.*, 1939, **20**, 330; *Brit. J. Exp. Path.*, 1940, **21**, 25.

5 Woolley, D. W., and Hutchings, B. L., *J. Bact.*, 1939, **38**, 285.

6 McIlwain, H., Fildes, P., Gladstone, G. P., and Knight, B. C. J. G., *Biochem. J.*, 1939, **33**, 223.

TABLE I.
Effect of Omitting Certain Factors from Complete Medium on Growth of C203S Strain.

Substance	Amt necessary for optimal growth	Mg bacterial N after 40 hr incubation from 10 cc × 50
Complete medium	—	10-13
No glucose	25 mg	0.1
" glutamine	500 μg	variable*
" tyrosine	1000 "	3.9
" tryptophane	500 "	0.8
" uracil	100 "	9.9
" adenylic acid (0.4 mm CO_2 tension)	100 "	0.7
" CO_2 (CO_2-free air)	8 mm	2.2
" thioglycollic acid	1 mg	}
" glutathione	4 μg	} 0.1
" thiamin	0.01 "	3.1
" nicotinic acid	1.0 "	1.9
" pantothenic acid	10 "	0.2
" riboflavin	0.04 "	0.8
" vitamin B_6	20 "	5.9-7.1†
" "biotin" concentrate	1.0 "	1.8
Broth (25 mg glucose in 10 cc)	—	10-17

*No significant growth at 20 hours without glutamine. Growth at 40 hours at 8 mm CO_2 tension is variable.

†No significant growth at atmospheric CO_2 tension without vitamin B_6.

C203S for adenine or related compounds. If purine is omitted from the complete medium no growth occurs within 40 hours. Addition of adenine permits growth to occur. Adenine may be replaced by adenosine or adenylic acid, by guanine, guanosine or guanylic acid and by xanthine or hypoxanthine. It cannot be replaced by uric acid, caffein or theophylline or by the pyrimidines uracil and cytosine. Subbarow and Rane[3] have reported that certain of the above purines "may be of significance" in the growth of the N.Y. No. 5 strain of hemolytic streptococcus. McIlwain[4] has included a number of purines in his medium on general grounds and Möller[7] has shown that adenine or guanine but not xanthine or hypoxanthine increase the growth of *Lactobacillus plantarum.*

The purine requirement of strain C203S was discovered before all of the other growth factors had been identified. Upon reexamining the purine requirements using the more defined medium given in detail above, it was noted that the presence of 5% carbon dioxide in the atmosphere above the culture greatly accelerated growth and the surprising observation was made that even when purine was absent growth occurred. We therefore examined the effect of carbon dioxide in some detail and in Table II are shown some of the

7 Möller, E. F., *Z. Physiol. Chem.*, 1939, **260**, 246.

TABLE II.
Effect of Adenylic Acid on Growth of Strain C203S at Different Carbon Dioxide Tensions.

Carbon dioxide tension (mm Hg)	No adenylic acid		Adenylic acid, 10 μg/cc	
	20 hr	40 hr	20 hr	40 hr
CO_2-free air	—	0.3	—	2.2
0.4	0.0	0.7	1.9	6.8-8.2*
1.4	0.1	0.7	3.1	8.4
2.4	0.15	3.8	3.0	9.1
4.3	0.15	9.0	10.9	10.1
8	1.4	9.1	11.3	10.4
20	1.0	9.2	9.6	10.8
40	10.1	12.2	12.2	12.4
8 mm CO_2, 730 mm nitrogen, no oxygen	—	12.5	—	12.5

*Growth is quite variable at atmospheric CO_2 tension which has been assumed to be 0.4 mm Hg.

Note that readings at 20 and 40 hours were from different experiments in different tubes. Growth is given as milligrams bacterial nitrogen per 10 cc × 50. Figures represent averages of at least 2 tubes.

The oxygen tension was kept constant at 120 mm throughout except in the anærobic experiments and those done at atmospheric CO_2 tensions. The gas pressure was made up to 740 mm with nitrogen in each case.

results at different CO_2 tensions with and without adenylic acid. It will be noted that maximal growth when adenylic acid is present occurs within 20 hours provided the CO_2 tension is 4 mm or greater, that no significant growth occurs even after 40 hours' incubation in the absence of adenylic acid when the CO_2 tension is below 2 mm and that even when the CO_2 tension is high, a small but consistent increase in growth is apparent at 40 hours when adenylic acid is added to the medium. It has been observed that the bicarbonate ion cannot replace carbon dioxide when no purine is present. However, in the presence of both adenylic acid and bicarbonate, any slight growth of the organisms may liberate sufficient carbon dioxide through action of the acid produced to accelerate growth. These observations on the accelerating effect of carbon dioxide are in harmony with those of other workers[8] and indeed McIlwain[4] grew his cultures in 5% carbon dioxide.

At present we have no clue as to the significance of these findings. Whether carbon dioxide is necessary for purine synthesis or whether purine plays a rôle in the production of carbon dioxide by the organisms cannot be decided at this time. We may point out, however, that a somewhat analogous situation has been reported in the case of *Staphylococcus aureus* by Richardson.[9] With this organism no

[8] Gladstone, G. P., Fildes, P., and Richardson, G. H., *Brit. J. Exp. Path.*, 1935, **16**, 335.

[9] Richardson, G. H., *Biochem. J.*, 1936, **30**, 2184.

growth occurs under anaerobic conditions unless uracil is added. In the presence of oxygen uracil is non-essential.

The C203S strain of streptococcus is a powerful hemolytic strain. In our experience hemolytic titers on this medium are equivalent to those obtained in broth, provided care is taken to avoid accumulation of acid during growth. The growth and hemolysin titer may be increased by addition of more glucose to the medium and periodic neutralization of the acid formed. However, preliminary work in this direction indicates that some factor, as yet unidentified, becomes the limiting one under these conditions.

Summary. Rapid, heavy growth and hemolysin production of the C203S strain of Group A hemolytic streptococcus have been obtained on a medium of essentially known composition. In addition to factors reported by previous workers, we have found that thiamin, nicotinic acid, adenine or related purines, and an unknown factor which may possibly be biotin are necessary for growth of this strain. The relation of carbon dioxide tension to the purine requirement has been studied.

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Modifying Influence of Light on Chick's Comb Response to Androsterone.

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The assay of androgenic material by biologic methods offers definite advantages over colorimetric determinations when we consider that in measuring the 17 ketosteroids by chromogenic effect we are determining both biologically active and inactive material.[1] In many cases the important consideration is, to what degree is biological activity present in a given specimen. In consequence of this, we made comparisons of colorimetric[2] and biologic determinations in some of our studies. The comb response of one-day-old

[1] Callow, N. H., Callow, R. K., Emmens, C. W., and Stroud, S. W., *J. Endo.*, 1939, **1**, 76.

[2] Neustadt, Rudolph, *Endo.*, 1938, **23**, 711.