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Studies on the Nutritional Requirements of Hemolytic Streptococci. I. Effect of Various Substances Isolated from Liver Extract on Hemolytic Streptococci.

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Attempts to substitute a relatively simple, chemically defined medium for the usual complex media in the cultivation of hemolytic streptococci have indicated the need for essential accessory growth factors. Considered in themselves multiple or of complex nature, they have hitherto been unidentified. Our preliminary experiments have demonstrated the presence of these indispensable factors in liver extract. A medium of gelatin hydrolysate, amino acids, inorganic salts, and glucose is incapable of inducing the growth of hemolytic streptococci, but the addition of liver extract yields a growth equivalent to that obtained in meat infusion broth. By fractionation glutathione, thiochrome, nicotinic acid, betaine, flavin and glucosamine were isolated from liver extract.† Two to 10 μg of glutathione per cc of medium, 0.00001-0.0001 μg of thiochrome per cc of medium, 40-200 μg of nicotinic acid per cc of medium, 4-20 μg of betaine per cc of medium, 0.01-0.1 μg of flavin per cc of medium definitely contributed to the growth of the Dochez NY5 strain of hemolytic streptococcus while 200 μg of glucosamine per cc of medium noticeably shortened the lag period.‡ These substances are, however, active only with an additional fraction of liver extract of which the complete identity is at this time still undetermined.

Basal Medium:

Gelatin hydrolysate[1]	25	cc
l-Tyrosine	0.1	g
l-Cystine	0.025	"
Distilled water	1000	cc

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† The details of the isolation and the identification of these substances will be reported in a subsequent paper (Y.S.).

‡ To eliminate the question of adsorption, synthetic products were substituted wherever possible. Synthetic glutathione was kindly supplied by Dr. A. Baird Hastings of the Harvard Medical School. Pfanstiehl's glucosamine was used.

[1] For preparation see Pappenheimer, A. M., Jr., and Johnson, S. J., *Brit. J. Exp. Path.*, 1937, **28**, 239.

Combined, pH adjusted with 5 N NaOH to 7.6, added:		
Calcium alcoholic precipitate of liver extract[2]	0.5	g
Sodium citrate	1.0	"
$NaH_2PO_4 \cdot H_2O$	2.0	"
Mixture heated gently for 10 minutes, filtered. Added:		
d-Glutamic acid	0.2	"
l-Tryptophane	0.1	"
dl-Methionine	0.05	"
dl-Valine	0.05	"
NaCl	1.0	"
$Na_2HPO_4 \cdot 12H_2O$	1.0	"
KH_2PO_4	1.0	"
K_2HPO_4	1.0	"
$MgCl_2 \cdot 6H_2O$	0.3	"

Tubed in 9.0 cc amounts. Volume brought to 9.7 cc with the addition of test substances and distilled water. Autoclaved 10 min. at 112°C. 0.1 cc flavin previously autoclaved in acid solution (pH 4.5) for 10 minutes at 112°C, 0.1 cc 50% glucose solution also autoclaved separately, and 0.1 cc culture added to test media. Final volume 10 cc.

Note: The amino acids and inorganic salts were included on general grounds. Detailed studies on the amino acid and inorganic salt requirements for growth have been deferred until all accessory growth factors have been determined.

Inoculum: A culture of the Dochez NY5 strain of hemolytic streptococcus grown for 6 hours in meat infusion broth at 37°C was centrifuged, washed thoroughly in sterile distilled water, and resuspended in sterile distilled water. It was then inoculated in 0.1 cc amounts.

Incubation: 40 hours at 37°C.

Readings: For greater accuracy direct readings based on turbidity were made by means of a modified Gates nephelometer,[3] rather than by visual approximation. A 2.3 reading was obtained for the growth of the Dochez NY5 strain in meat infusion broth§ incubated for 40 hours at 37°C. Readings of the growth in different test media varied from 2.9 to more than 5.0 (limit of direct reading).

Results: A summary of typical results is presented in Table I.

Conclusion. Glutathione, thiochrome, flavin, nicotinic acid, betaine and glucosamine, in the presence of a calcium-alcoholic precipitate of a highly purified liver extract,** are clearly significant for the growth of the Dochez NY5 strain of hemolytic streptococcus in a deficient medium. The presence of all these factors provides almost optimum conditions: omission of one or more decreases the amount of growth.

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[2] Subbarow, Y., Jacobson, B. M., and Fiske, C. H., *New Eng. J. Med.*, 1936, **214**, 194.

[3] Feemster, R. F., Wetterlow, L. H., and Cianciarulo, J., *Am. J. Public Health*, 1936, **26**, 1176.

§ A beef-heart infusion containing 2% peptone, 0.5% NaCl, and 0.1% dextrose.

** For the supply of liver extract we are indebted to Dr. Guy W. Clark of the Lederle Laboratories, Pearl River, N. Y.

TABLE I.

Influence of Accessory Substances on Growth of Dochez NY5 Strain of Hemolytic Streptococcus. Control: Growth in Meat Infusion Broth. Nephelometer Reading = 2.3.

Basal Medium containing Ca-alc. ppt. of liver extract + 200 μg of glucosamine per cc of medium		Micrograms per cc. medium	Nephelometer Reading 40 hr	Micrograms per cc Medium	Nephelometer Reading 40 hr	Micrograms per cc Medium	Nephelometer Reading 40 hr
Basal Medium		—	>5.0*	—	>5.0	—	>5.0
Added individually to Basal Medium:							
Glutathione	(G)‡	10	4.9	2	> "	0.4	> "
Flavin	(F)	0.1	>5.0	0.1	> "	0.1	> "
Nicotinic Acid	(N.A.)§	200	> "	200	> "	200	> "
Thiochrome	(T)	0.00001	> "	0.00001	> "	0.00001	> "
Betaine	(B)§	4	> "	4	> "	4	> "
Added in combination to Basal Medium:							
(G) + (N.A.)		as above	4.9	as above	> "	as above	> "
(G) + (T)		"	"	"	> "	"	> "
(G) + (B)		"	"	"	> "	"	> "
(G) + (F)		"	4.5	"	> "	"	> "
(G) + (T) + (B)		"	4.9	"	> "	"	> "
(G) + (N.A.) + (T)		"	"	"	> "	"	> "
(G) + (N.A.) + (B)		"	"	"	> "	"	> "
(G) + (F) + (B)		"	4.5	"	> "	"	> "
(G) + (F) + (T)		"	4.3	"	> "	"	> "
(G) + (F) + (N.A.)		"	3.9	"	4.7	"	> "
(G) + (N.A.) + (T) + (B)		"	4.9	"	>5.0	"	> "
(G) + (F) + (T) + (B)		"	4.3	"	> "	"	> "
(G) + (F) + (N.A.) + (T)		"	3.5	"	4.5	"	> "
(G) + (F) + (N.A.) + (B)		"	3.3	"	4.0	"	4.8
(G) + (F) + (N.A.) + (T) + (B)		"	2.9†	"	3.6	"	4.7

*Nephelometer Readings of 5.0 or more indicate essentially no growth.

†Nephelometer Readings of 3.0 or less indicate good growth.

‡No growth without glutathione.

§Although these substances as such were isolated, we have reasons to believe that there are still others in liver extract closely allied but more active.