NUTRITION OF SHIGELLA: GROWTH OF *SHIGELLA FLEXNERI* IN A SIMPLE CHEMICALLY DEFINED MEDIUM[1]

ARVID L. ERLANDSON, JR.,[2] AND WILLIAM H. MACKEY

Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland

Received for publication August 26, 1957

The nutritional requirements of Shigella grown in chemically defined media have been investigated by Koser *et al.* (1938*a*, *b*), Kligler and Grosowitz (1939), and Weil and Black (1944), among others. These investigators have reported the successful cultivation of various strains of Shigella in chemically defined, synthetic media of variable composition. All of these media consisted of inorganic salts, glucose, nicotinic acid, and various combinations of several amino acids. Recent metabolic studies with *Shigella flexneri* 3 strain 1013, (Erlandson and Ruhl, 1956) have shown that this organism possesses a very active metabolic mechanism for dissimilating amino acids and related nitrogen compounds. Thus, the previously described synthetic media seemed unduly complex for the growth of *S. flexneri* 3 by reason of the number of amino acids required in their preparation. Consequently, the present study was undertaken to determine whether a single amino acid or other nitrogen compound could be utilized as the nitrogen source for growth and subsequently to develop a simple, chemically defined medium for the cultivation of Shigella.

Interest in this laboratory has centered on *S. flexneri* 3 strain 1013, which was used in previous studies. The nutritional requirements of this culture were studied extensively and the findings applied subsequently to other serotypes and species of *Shigella*.

EXPERIMENTAL METHODS

Strain 1013 of *Shigella flexneri* 3 was employed routinely throughout the experimental study and all data refer to this organism, unless specifically stated to the contrary. The strain was biochemically and serologically characteristic of the species and was maintained in a lyophilized state. Other serotypes and species of *Shigella* used in later studies were also biochemically and serologically characteristic.

Pyrex glassware was used in all experiments. Prior to use, it was rigorously cleaned in aqua regia and thoroughly rinsed in deionized, distilled water. The highest grade chemicals commercially available were used throughout the study. The accessory growth factors employed were crystalline preparations from reputable sources.

Inocula preparation was rigidly standardized, both to minimize variation in numbers and activity, and to reduce the possibility of nutrient carry-over. Cells were carefully washed off the surface of an 18-hr brain-heart infusion agar (Difco) Kolle flask to 0.067 M phosphate buffered saline, washed three times, and resuspended in buffered saline to a given turbidity. A 0.1 ml aliquot of appropriate dilution was used for the inoculation of experimental cultures. In order that this standard inoculum might be interpreted in terms of numbers of viable cells, a curve was prepared to show the relationship between viable cell count and percentage light transmittance for suspensions prepared in this manner. Plate counts of the inocula in each experiment were made also.

Cultures for static incubation were grown in 10 ml aliquots of media in test tubes. Aerated cultures were grown in 10 ml aliquots in 25-ml Erlenmeyer flasks mounted on a shaker and in 100-ml aliquots in "Gas-Washing" flasks. All samples were incubated at 37 C.

Growth was evaluated turbidimetrically using a Coleman Universal spectrophotometer with a 600 mμ filter; the uninoculated medium and the inoculated basal medium served as controls. Readings were made on duplicate or triplicate samples. Plate counts were made by the usual pour plate method. The pH was determined electrometrically. Postincubation characteriza-

[1] The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large. A preliminary report of this work appeared previously (Erlandson and Mackey, 1957).

[2] Present address: Research Division, Parke, Davis and Company, Detroit, Michigan.

TABLE 1

Comparison of inorganic ammonium salts, amino acids, and related compounds used singly as a nitrogen source for the growth of Shigella flexneri 3 strain 1013

Nitrogen Source Added to Basal Medium	Percentage Light Transmittance	
Compound*	Cells per ml inoculum, 48 hr incubation	
	1×10^8	1×10
Basal medium control	100	100
Ammonium carbonate†	100	100
Ammonium citrate†	71	77
Ammonium chloride†	100	100
Ammonium phosphate (monobasic)†	89	88
Ammonium sulphate†	100	99
L-Alanine	82	77
L-Arginine	99	96
L-Asparagine†	84	82
L-Aspartic Acid	20	21
L-Cystine‡	99	99
L-Cysteine	97	95
L-Glutamic Acid	70	76
Glutamine†	83	85
Glutathione	85	88
DL-Glycine	65	85
Glycyl-glycine	65	80
Glycyl-glycyl-glycine	68	77
Histamine†	98	96
L-Histidine	97	92
DL-Homocystine	100	99
Hydroxy-L-Proline	100	96
DL-Isoleucine	96	94
L-Leucine	99	100
L-Lysine	96	96
L-Methionine	100	100
DL-Norleucine	100	100
DL-Ornithine	88	91
L-Phenylalanine	99	100
L-Proline	100	98
L-Serine	48	97
L-Threonine	89	90
L-Tryptophan	99	100
L-Tyrosine‡	98	99
DL-Valine	96	94

* Concentration of 0.25 per cent.

† Sterilized separately by filtration and added aseptically to the medium.

‡ Present in saturated solution.

tion of cells was accomplished by streaking on brain-heart infusion agar plates to check purity and colonial variation. Serology and cellular morphology were also checked.

The basal medium, to which were added singly the various nitrogen sources, had the following composition: 0.067 M dipotassium phosphate, 0.067 M monopotassium phosphate, 1 per cent glucose, 0.05 per cent magnesium sulphate, 0.03 per cent thiamin hydrochloride, and 0.001 per cent nicotinic acid. The pH was adjusted to 7.0 with concentrated sodium hydroxide or phosphoric acid and this basal medium sterilized by autoclaving. Glucose solutions were sterilized by Seitz filtration and aseptically added after autoclaving. This basal medium was selected on the basis of growth results obtained in preliminary experiments.

RESULTS

A number of inorganic ammonium salts, amino acids, and related nitrogen compounds were surveyed to determine which might serve as the nitrogen source for the growth of *S. flexneri* 3 when supplied singly to the basal medium. The compounds were added to the basal medium at a concentration of 0.25 per cent. The results are presented in table 1. Following the initial 48 hr, no significant change in turbidity occurred upon continued incubation up to 10 days.

A selective utilization of certain nitrogen compounds is apparent immediately. Aspartic acid supported excellent growth at both inocula levels. Serine supported good growth at the higher inoculum but no growth at the lower inoculum. Glutamic acid, glycine, and the glycine di- and tripeptides permitted moderate growth at both inocula levels; alanine, asparagine, glutamine, and threonine permitted light growth, and the other compounds allowed negligible or no growth of the test organism. Of the inorganic ammonium salts, only ammonium citrate and ammonium phosphate were capable of supporting fair growth. The sufficiency of L-aspartic acid as a nitrogen source was further demonstrated by maintaining serial transfers of the organism in the medium with no appreciable change in the growth rate, maximum turbidity being attained in 24 to 48 hr. Further, the degree of variation usually associated with synthetic media was found to be comparable to that obtained with the usual liquid media.

The utilization of stereo-isomers of aspartic acid for the growth was investigated on a limited basis, since previous metabolic studies (Erlandson and Ruhl, 1956) had indicated that the D-isomer of aspartic acid inhibited the oxidation

of the L-isomer, the extent of inhibition being dependent on the concentration of the D-isomer present. No such inhibition was found nutritionally. While no growth was obtained with the D-isomer, no inhibition of growth was noted with either the racemic mixture or when the proportion of the D- to the L-isomer was increased as high as 5 to 1. Comparable results were obtained with both large and small inocula.

The effect of pH upon the growth of *S. flexneri* 3 in the aspartic acid medium is shown in figure 1. It should be noted that, with a large inoculum, the pH optimum occurred over a wide range, whereas with a small inoculum, the pH optimum occurred approximately at neutrality.

The effect of substrate concentration on growth was negligible. Very little, if any, change in growth rate was found to occur within the concentration range of 0.25 per cent to 2 per cent aspartic acid.

Since previous evidence (Erlandson and Ruhl, 1956) indicated that aspartic acid, glutamic acid, and asparagine are oxidized at significant rates by nonproliferating cells of *Shigella*, the question arose as to whether the organism could utilize one of these compounds not only as a nitrogen source, but as a carbon and energy source as well. No growth was obtained with either glutamic acid or asparagine as the carbon, nitrogen and energy source and only sparse growth was obtained using aspartic acid. These data indicated the necessity of a carbohydrate energy source for good growth. Subsequent investigations indicated that mannose, fructose, or galactose could be substituted for glucose with no deleterious effects. Attempts to substitute other carbohydrates, either monosaccharides, disaccharides or polysaccharides, were unsuccessful. Optimal carbohydrate concentration was between 0.5 per cent and 1 per cent. Concentrations higher than 1 per cent were slightly inhibitory.

Since earlier studies showed that nonproliferating cells had increased metabolic activity under aerobic conditions, it was anticipated that aeration would provide stimulated growth rates. This was found to be true. Aeration increased the rate of growth to about one and one half times the static rate but total growth was not altered.

Size of inoculum being of such importance with synthetic media, the ability of the aspartic acid medium to sustain growth at low levels of inoculation was tested. These data, presented in table 2, indicate the ability of the medium to support growth of *S. flexneri* 3 at very low inoculum levels (10^2 cells per ml). Though the rate of growth from these small inocula was initially slow, the same total growth was eventually reached with all inocula.

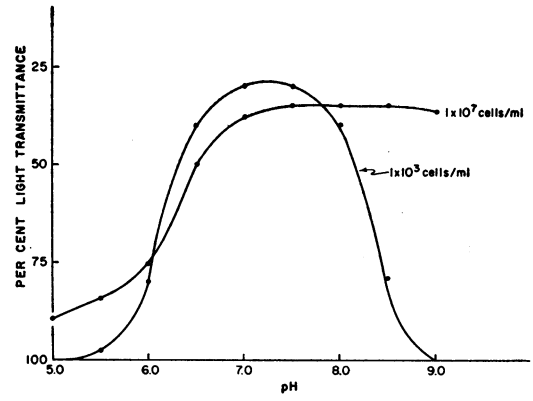

Figure 1. The relationship between pH and growth of *Shigella flexneri* 3 strain 1013, in a synthetic medium, using large and small inocula.

The sufficiency of the aspartic acid medium was further demonstrated by maintaining serial transfers of the organism for 15 days with no appreciable change in either the rate or extent of growth. Further, the extent of colonial variation encountered therein was found to be comparable to that obtained with the usual undefined broths.

In order to determine the efficacy of this synthetic medium in terms of its comparison with a maximal medium (brain-heart infusion broth,

TABLE 2

The effect of inocula size on the growth of Shigella flexneri 3 strain 1013

Cells per ml Inoculum	Percentage Light Transmittance			
	24 hr	48 hr	72 hr	96 hr
1×10^8	20	20	20	20
1×10^7	21	21	21	21
1×10^6	33	22	22	22
1×10^5	92	20	20	20
1×10^4	94	18	18	18
1×10^3	97	18	17	17
1×10^2	99	26	19	19
1×10	100	100	100	100
0	100	100	100	100

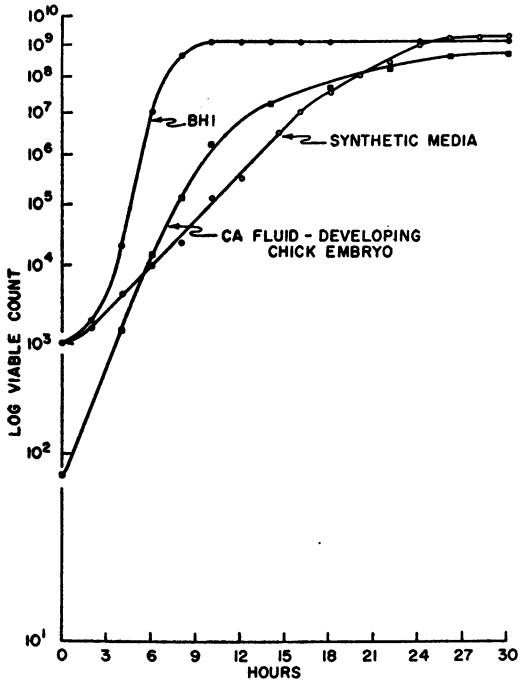

Figure 2. Growth curves for *Shigella flexneri* 3 strain 1013, in a synthetic medium, brain-heart infusion broth, and the chorioallantoic fluid of the developing chick embryo.

Difco), growth curves for the test organism were determined. As can be seen in figure 2, growth in the maximal medium was initially much more rapid than in the synthetic medium. However, total viable cell counts indicate that at 24 hr, multiplication rates in both media are comparable. Since studies on growth of *Shigella* in 8-day-old, developing chick embryos were being conducted at the same time, the rate of growth of the test organism in this environment was further compared with growth rates in both the maximal and synthetic media. As will be noted in figure 2, the rate of growth in the chorio-allantoic fluid of the chick embryo was intermediate between the other two media. It may be noted, however, that at 24 hr, growth rates were comparable in all three environments. With these observations the present formulation of our definitive medium was considered sufficient. The final concentrations of the constituents are shown in table 3.

In order to ascertain the scope of the ability of the aspartic acid medium to support growth, the medium was tested for its ability to support the growth of a number of other strains, serotypes, and species of *Shigella*. Since *S. flexneri* 3, strain 1013, was used as the test organism, three strains of each of the six serotypes of *S. flexneri* were tested first. These strains were selected at random from the Naval Medical Reference Collection of Enterobacteriaceae. All of these strains grew readily in the synthetic medium at the high inocula level and all grew consistently at the small inocula level. Average yields of from 1 to 5 billion viable cells per ml were obtained in 24 hr. Further, all were easily maintained for a minimum of 10 successive serial transfers without significant change in growth rate. The medium also was capable of supporting the growth of *S. sonnei*, with results comparable to that found with *S. flexneri*. However, the medium was unable to support the growth of seven serotypes of *S. boydii* or seven serotypes of *S. dysenteriae*.

TABLE 3

Composition of chemically defined synthetic medium

L-Aspartic Acid	0.25%
Glucose	1.00%
NaCl	0.85%
Mg^{++} (as $MgSO_4 \cdot 7H_2O$)	0.05%
K_2HPO_4	0.067 M
KH_2PO_4	0.067 M
Thiamin hydrochloride	0.03%
Niacin	0.001%

DISCUSSION

This investigation has attempted to demonstrate that many strains of Shigella may be cultivated in a relatively simple medium, in which all constituents are chemically defined. Our interest in these studies has centered about the 6 serotypes of *Shigella flexneri*. The essential nutritive requirements of these organisms may be met by a combination of mineral salts, two accessory growth factors, a single carbohydrate energy source, and a single amino acid nitrogen source. Although certain inorganic ammonium compounds and amino acids were capable of serving as the sole nitrogen source, maximal growth was obtained using aspartic acid. The degree of selectivity for nutrients was indicated by the fact that, although closely related in structure, neither asparagine nor glutamic acid were capable of successfully substituting for aspartic acid as the nitrogen source for growth.

The utilization of only the natural isomer by the organism was suggested by its failure to grow in the presence of D-aspartic acid. However, no inhibition of the natural isomer by the D-isomer was found. It was of interest to note that of all the nitrogen compounds tested, those compounds which supported the growth of the test organism, when singly supplied as the nitrogen source, were qualitatively correlated with those compounds which were actively oxidized and deaminated by nonproliferating cells (Erlandson and Ruhl, 1956). This correlation, however, appeared strictly qualitative and no quantitative correlation was evident. This observation and the fact that the ammonium ion may serve as nitrogen source for growth suggests the hypothesis that amino acids may serve as an organic source of ammonia, this in turn serving as the primary nitrogen source for synthetic reactions. This does not exclude the possibility of direct assimilation of the preformed amino acids as a pathway for nitrogen anabolism. Further, these observations suggest that the combinations of amino acids, which other investigators have indicated as necessary for growth, may function in a stimulatory rather than an essential role in the nutritional requirements of these organisms.

It seems probable that the maximal level of growth attained with any one strain might be considerably improved by minor modifications in the basic formulation of the medium described. However, the growth curve comparisons between the synthetic and maximal media attest to the sufficiency of the synthetic medium. Although the medium presented does not support the growth of all species of *Shigella* and may not furnish the optimal nutritional requirements of any single strain, it affords an extremely useful tool in metabolic and nutritional studies with these organisms. The inability of the medium to support the growth of any of the serotypes of *S. boydii* and *S. dysenteriae* is a problem of interest. Whether this is the result of significant nutritional differences or merely the absence of an essential nutrient merits consideration in future studies.

SUMMARY

An investigation of the nutritional requirements of *Shigella flexneri* 3 strain 1013, has resulted in the formulation of a medium consisting of mineral salts, two accessory growth factors, a single carbohydrate energy source, and a single amino acid nitrogen source. Although the inorganic ammonium ion and certain amino acids could serve as the sole nitrogen source, aspartic acid yielded maximal growth.

The effects of pH, concentration, aeration, inocula size, and other factors were determined. The medium was capable of supporting the growth of all 6 serotypes of *Shigella flexneri*.

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