

THE NUTRITION OF BRUCELLAE: GROWTH IN SIMPLE CHEMICALLY DEFINED MEDIA¹

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The nutritional requirements of brucellae grown in chemically defined media have been investigated by ZoBell and Meyer (1932*a,b*), Koser, Breslove, and Dorfman (1941), N. B. McCullough and Dick (1942*a,b*, 1943), W. G. McCullough *et al.* (1947), and others. With one exception (McCullough and Dick, 1943), however, the media employed seemed unduly complex by reason of the number of amino acids required in their preparation. Utilization of inorganic ammonium salts as the sole nitrogen source for these organisms has been demonstrated by ZoBell and Meyer (1932*a*), McCullough and Dick (1943), and Gerhardt and Wilson (1947). Consequently, the present study was undertaken in an effort to obtain a simple, chemically defined medium for cultivation of brucellae.

Interest in this laboratory has centered on the "avirulent" *Brucella abortus* strain 19, widely used for animal immunization. The nutritional requirements of this culture were studied intensively and the findings applied subsequently to a number of other strains.

METHODS

Pyrex glassware was employed for all experimental work; before use it was rigorously cleaned with detergent or acid cleaning solution and rinsed with glass-distilled water. When it was necessary to use chromic acid cleaning solution, extra precautions were taken in rinsing. The highest grade chemicals commercially available were used throughout the study. The accessory growth factors employed were crystalline preparations from reputable sources.

Cultures were grown in 10-ml aliquots of the medium in pyrex milk dilution bottles, which were plugged with cotton enclosed in gauze wrapping. When extended incubation of cultures was necessary, an aluminum foil covering was used to minimize evaporation. The constituents of the medium were made to volume in the culture vessels and sterilized by autoclaving for 20 minutes at 120 C. Duplicate or triplicate cultures were incubated at 35 to 37 C in a humidified atmosphere. Aeration was accomplished by means of a shaker apparatus. Growth usually was evaluated turbidimetrically; light transmittance was determined with an Evelyn photometer, using matched 18-mm test tubes as cuvettes, a 660-m μ filter, and the original, uninoculated medium as the reference. Readings were made on aggregate samples of two or more replicate cultures.

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Plate counts were made by the usual poured plate methods. The pH was determined electrometrically.

A majority of the experiments were conducted with *B. abortus* strain 19 (Cotton, Buck, and Smith, 1933), sent to us from the U.S. Department of Agriculture through the courtesy of Dr. A. B. Crawford (U.S.B.A. I., *B. abortus* no. 19-18B, March 8, 1946). This strain is distinguished by its relatively low virulence for experimental animals and by its reaction to dye bacteriostasis (Levine and Wilson, 1947).

Preparation of the inocula for experimental cultures was rigorously standardized, both to minimize variation in numbers and activity as well as to reduce the possibility of nutrient carry-over. Cells were removed carefully from a 24-hour, tryptose agar culture to physiological saline; this suspension was adjusted to a given turbidity and 0.1-ml aliquots of appropriate dilutions were 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 of light transmittance for suspensions prepared in this manner. Of necessity, early experiments having ammonium sulfate as the nitrogen source were with relatively large inocula; this requirement was subsequently obviated.

RESULTS

The study was begun with attempts to cultivate *B. abortus* strain 19 on the simplified medium of N. B. McCullough and Dick (1943); negative results were obtained in each instance. Similar findings have been reported by Stimmell (1946), Polding (1946), and W. G. McCullough *et al.* (1947). Experiments were then conducted to demonstrate utilization of ammonium salts as the sole nitrogen source by the organism. This accomplished, the study was extended to investigate the nutrition of the organism in relatively simple substrates and to define conditions under which maximum growth might be obtained.

Initial experiments were with a medium arbitrarily devised from the results of N. B. McCullough and Dick (1943) and other investigators. As the study progressed, the medium was changed to incorporate the experimental findings; thus, the various data may not be directly comparable. The basal medium used in most of the work had the following composition: 0.75 per cent sodium chloride, 1.00 per cent dipotassium phosphate, 0.01 per cent sodium thiosulfate, 10.0 μg per ml Mg^{++} (as magnesium sulfate), 0.10 μg per ml Fe^{++} (as ferrous sulfate), 0.10 μg per ml Mn^{++} (as manganous sulfate), 0.20 μg per ml thiamine hydrochloride, 0.20 μg per ml nicotinic acid, 0.04 μg per ml calcium pantothenate, 0.001 μg per ml biotin, and pH adjustment to 6.8 to 7.0.

Limited studies on the respiratory activity of *B. abortus* strain 19 on various energy sources indicated that the rate of oxygen uptake of washed, resting cells in the presence of sodium lactate compared favorably with that for glucose or glycerol.² The latter often are employed as energy sources for brucellae. These

² Studies on the respiration of brucellae are being extended and will be reported at a future date.

results suggested the incorporation of lactate into the basal medium, which previously had failed to support growth with ammonium sulfate as the nitrogen source and glucose as the carbon source. When the medium was modified by the addition of lactate, moderate growth was obtained. Subsequently, a marked stimulatory effect of glycerol was observed.

The relationships between varying concentrations of lactate and glucose, and of lactate and glycerol, are given in figures 1 and 2, respectively. The results were plotted as three-dimensional graphs to illustrate the relationships between the two independent variables (lactate versus glucose or glycerol concentrations) in terms of the dependent variable (percentage of light transmittance). The

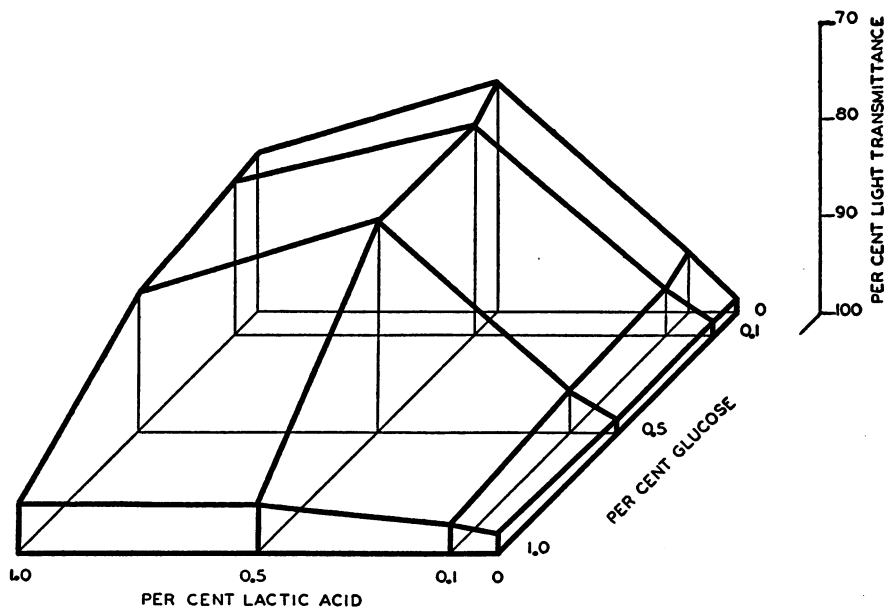


Figure 1. Relationship between varying concentrations of lactic acid and glucose on the growth of *Brucella abortus* strain 19. Medium: Basal medium constituents plus 0.05 per cent $(\text{NH}_4)_2\text{SO}_4$. Inoculum: 1×10^8 viable cells per ml. Incubation: 96 hours, not on shaker.

following working conclusions were drawn from the data of figure 1: (1) a concentration of 0.5 per cent lactate was optimal for all concentrations of glucose used; (2) lactate may serve as the sole carbon and energy source; and (3) glucose alone failed to support growth and apparently inhibited growth in the presence of lactate. The following working conclusions were drawn from the data of figure 2: (1) a concentration of 0.5 per cent lactate was optimal for all concentrations of glycerol used; (2) the combined effect of lactate and glycerol together was greater than that of either alone; and (3) although either may serve as the sole carbon and energy source, the effect of lactate was more pronounced than that of glycerol. These results were subsequently extended to determine more accurately the optimal levels of lactate and glycerol, each in the presence of optimal amounts of the other. In this respect, a level of 0.5 per cent lactic acid

was confirmed as optimal, whereas increasing the concentration of glycerol to 3.0 per cent resulted in further increases in the growth response. The range of the optimal concentration of glycerol was found to be very broad.

In this preliminary work, the basal medium included 0.05 per cent ammonium sulfate as the nitrogen source, the culture vessels being incubated statically. That oxygen supply had become a limiting factor soon became evident; consequently, increased aeration was employed in subsequent experiments. Moreover, the use of ammonium sulfate offered serious disadvantages as a nitrogen source: (1) ammonia determinations indicated that appreciable and variable amounts of ammonia (e.g., 12.5 to 36.0 per cent) were lost from the medium on

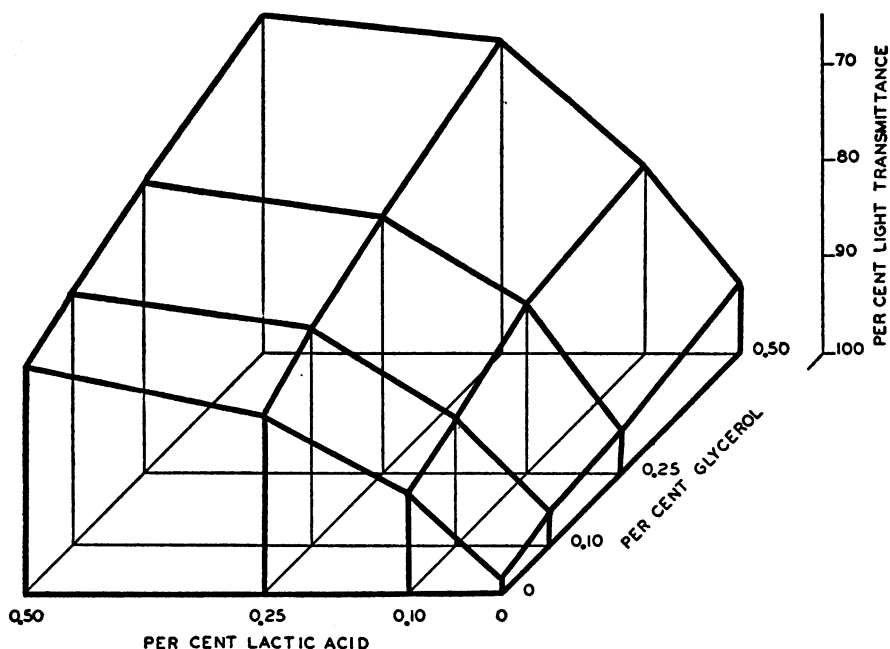


Figure 2. Relationship between varying concentrations of lactic acid and glycerol on the growth of *Brucella abortus* strain 19. Medium: Basal medium constituents plus 0.05 per cent $(\text{NH}_4)_2\text{SO}_4$. Inoculum: 1×10^8 viable cells per ml. Incubation: 96 hours, not on shaker

autoclaving; and (2) although growth of the organism could be maintained in the medium with prolonged serial transfer, the initiation of growth by small inocula was delayed and sporadic unless the physical character of the medium was altered with small amounts of agar or methyl cellulose. Consequently, a series of experiments was conducted in an effort to obviate these difficulties and yet avoid the use of a multiple nitrogen source. The effect of various nitrogen sources employed singly in the basal medium was investigated. The results indicated that various sources of the inorganic ammonium ion (e.g., ammonium carbonate, hydroxide, phosphate, or citrate) may satisfactorily substitute for ammonium sulfate but with no apparent advantage, either in the growth response or in the chemical properties of the compounds. However, the response of the organism to individual amino acids and related compounds could be demon-

strated. The favorable results with asparagine, in particular, suggested further investigation. Moreover, the stimulatory effects of asparagine on the growth of brucellae had been observed previously by ZoBell and Meyer (1932a) and others.

Representative results for the growth response of the organism to asparagine as the sole nitrogen source are given in table 1. Not only were the levels of growth attained comparable to those with ammonium sulfate, but the organism grew readily from smaller inocula. From these and subsequent data, the optimal concentration of DL-asparagine was determined as 0.3 per cent. Under these conditions visible growth of *B. abortus* strain 19 became evident after incubation periods of approximately 1, 3, 5, and 7 days with inocula of 1×10^8 , 1×10^6 , 1×10^4 , and 1×10^2 viable cells per ml, respectively. The maximum levels of

TABLE 1

The effect of varying concentrations of asparagine on the growth of Brucella abortus strain 19

NITROGEN SOURCE ADDED TO MEDIUM			PERCENTAGE OF LIGHT TRANSMITTANCE	
Percentage of compound	Compound	Nitrogen percentage	1×10^8 cells/ml inoculum, 96 hr	1×10^4 cells/ml inoculum, 232 hr
0	None	0	98.5	100.0
0.05	(NH ₄) ₂ SO ₄	0.0106	56.0	100.0
0.01	DL-Asparagine	0.0021	83.5	93.0
0.05	DL-Asparagine	0.0106	47.0	52.5
0.10	DL-Asparagine	0.0212	50.5	45.5
0.30	DL-Asparagine	0.0636	49.0	47.0
0.50	DL-Asparagine	0.1060	55.0	100.0
1.00	DL-Asparagine	0.2120	98.5	100.0

Medium: Basal medium constituents plus 0.5 per cent lactic acid and 3.0 per cent glycerol. Incubation: On shaker.

growth attained in each instance were approximately the same: 45 to 50 per cent light transmittance and a viable cell count of 1.0 to 2.0×10^9 viable cells per ml. An interesting adjunct to the effect of asparagine is the fact that, at certain levels of the compound, the culture became distinctly colored, usually brown ochre but at times a distinct pink. Centrifugation of such cultures revealed the pigmentation to be concentrated in the supernatant.

A partial analysis of the utilization of asparagine by the organism is given in table 2. Aspartic acid, the amino acid analogue of asparagine, could not be substituted. Contrary to expectations, L-glutamine, differing from asparagine only in the length of its carbon chain, gave no growth response, whereas L-glutamic acid may serve as the sole nitrogen source. L-Histidine also may replace asparagine. Of the other amino acids examined, none supported significant growth.

As a result of the preceding studies, a simple, chemically defined medium was formulated, having DL-asparagine as the sole nitrogen source. Its complete composition is given in table 3. *B. abortus* strain 19 was carried through 10 serial transfers in this medium and then examined for possible changes in its characteristics. Thus far, no changes have been detected in its cellular mor-

phology, susceptibility to the bacteriostatic effect of thionin blue, or pathogenicity for guinea pigs. Limited investigation of the dissociation pattern showed characteristics of 65 per cent rough, 10 per cent intermediate rough, 10 per cent intermediate, and 15 per cent smooth colonies.

TABLE 2

The effect of various amino acids as nitrogen source on the growth of Brucella abortus strain 19

NITROGEN SOURCE ADDED TO MEDIUM			PERCENTAGE OF LIGHT TRANSMITTANCE
Percentage of compound	Compound*	Nitrogen percentage	
0.10	DL-Asparagine	0.0212	51.0
0.201	DL-Aspartic acid	0.0212	97.0
0.111	L-Glutamine†	0.0212	100.0
0.222	L-Glutamic acid	0.0212	60.5
0.114	Glycine	0.0212	98.0
0.159	DL-Serine	0.0212	97.0
0.182	L-Cystine‡	0.0212	100.0
0.080	L-Arginine	0.0212	94.5
0.106	L-Histidine	0.0212	74.5
0.274	L-Tyrosine‡	0.0212	100.0
0.250	DL-Phenylalanine	0.0212	99.5
0.155	L-Tryptophan	0.0212	97.5

Medium: Basal medium constituents plus 0.50 per cent lactic acid and 3.0 per cent glycerol. Inoculum: 1×10^4 viable cells per ml. Incubation: 240 hours on shaker.

* With the exception of asparagine and glutamine, these amino acids have been reported (W. G. McCullough *et al.*, 1947) as essential or stimulatory for growth of a strain of *B. suis*.

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

‡ Present in saturated solution.

TABLE 3

Composition of chemically defined medium

DL-Asparagine	0.30 %
Lactic acid	0.50 %
Glycerol	3.00 %
NaCl	0.75 %
K ₂ HPO ₄	1.00 %
Na ₂ S ₂ O ₃ ·5H ₂ O	0.01 %
Mg ⁺⁺ (as MgSO ₄ ·7H ₂ O)	10.0 µg/ml
Fe ⁺⁺ (as FeSO ₄ ·7H ₂ O)	0.10 µg/ml
Mn ⁺⁺ (as MnSO ₄ ·4H ₂ O)	0.10 µg/ml
Thiamine hydrochloride	0.20 µg/ml
Nicotinic acid	0.20 µg/ml
Calcium pantothenate	0.04 µg/ml
Biotin	0.001 µg/ml

Adjustment to pH 6.8 to 7.0 with NaOH.

The asparagine medium (table 3) was tested for its ability to support growth of 28 strains of *B. abortus*, *Brucella suis*, and *Brucella melitensis* contained in our culture collection. All strains grew readily in an air atmosphere on tryptose (Difco) agar. Of these 28 strains, all but 4 grew from an inoculum of approxi-

mately 1×10^4 viable cells per ml within 8 days in the asparagine medium. The yields varied from barely visible growth (97.0 per cent light transmittance) to heavy growth (6.5 per cent light transmittance). Heaviest growth was obtained with *B. abortus* strain ORF; a maximum level of 19.0×10^8 viable cells per ml was reached after 168 hours from an inoculum of approximately 1×10^4 viable cells per ml. With this medium, attempts failed in every instance to cultivate strains of *B. abortus* that required an increased carbon dioxide tension.

DISCUSSION

This investigation has attempted to demonstrate that many strains of *Brucella* may be cultivated on a relatively simple medium, in which all constituents are chemically defined and readily available to most laboratories. The essential nutritive requirements of these organisms may be met by a combination of mineral salts, four accessory growth factors, lactate, glycerol, and a single nitrogen source. Although the inorganic ammonium ion may serve as the sole nitrogen source, best results were attained using DL-asparagine. Glutamic acid or histidine, employed singly, may substitute for asparagine. Whereas McCullough *et al.* (1947) have reported that cystine, tyrosine, phenylalanine, tryptophan, and histidine were essential for growth of a strain of *B. suis* (originally received from Dr. I. F. Huddleson, his strain no. 1772-A), in our hands this strain was among those that grew in the asparagine medium. These results collectively would seem to indicate that combinations of amino acids have a stimulatory rather than an essential role in the nutritional requirements of these organisms.

Although *B. abortus* strain 19 is known to utilize glucose, no response could be obtained with this compound when ammonium sulfate was used as the sole nitrogen source in the basal medium. However, when lactic acid was substituted as the carbon and energy source in an otherwise similar medium, growth was obtained and glucose actually appeared inhibitory. The stimulatory effect of lactate for these organisms has been reported previously, but its preferential use by a strain of *B. abortus* appeared noteworthy. Our data confirm previous observations on the effect of glycerol with the modification that a relatively high level of glycerol was found to be of value, supporting the view that glycerol may function in ways other than as a primary nutrient.

For possible applications to other studies, the medium that supported the best growth of the test organism was investigated for its ability to support the growth of a number of other strains. These included at least five strains of each species of *Brucella*. All of these strains grew in an air atmosphere. Approximately 85 per cent of the cultures initiated growth in the chemically defined medium from relatively small inocula. It seemed probable that those strains which failed to grow might be induced to do so by minor modifications in the medium, as variation in the nutritional requirements of different strains of *Brucella* has been observed previously (e.g., Koser, Bresolve, and Dorfman, 1941). Similarly, it seemed probable that the maximum level of growth attained by any one strain might be considerably improved by minor modifications in the medium. However, cultivation of those strains of *B. abortus* that require an

increased carbon dioxide tension seemingly must await further study of their requirements. It may be noted, in this respect, that N. B. McCullough and Dick (1942b) reported that 41 recently isolated strains of *B. abortus* (requiring an increased carbon dioxide tension) were not successfully grown in an amino acid medium that they employed.

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SUMMARY

An investigation of the nutritional requirements of *Brucella abortus* strain 19 resulted in the formulation of a medium consisting of mineral salts, four accessory growth factors, lactate, glycerol, and a single nitrogen source. Although the inorganic ammonium ion may serve as the sole nitrogen source, best results were attained with DL-asparagine. L-Glutamic acid or L-histidine, employed singly, may substitute for asparagine.

Of 28 "aerobic" strains of *Brucella abortus*, *Brucella suis*, and *Brucella melitensis* tested, all but 4 grew in the asparagine medium from relatively small inocula. Yields varied up to 19 billion viable cells per ml.

REFERENCES

- COTTON, W. E., BUCK, J. M., and SMITH, H. E. 1933 Efficacy of an avirulent strain of *Brucella abortus* for vaccinating pregnant cattle. *J. Agr. Research*, **46**, 291-296.
- GERHARDT, P., and WILSON, J. B. 1947 Ammonium as the primary nitrogen source in the nutrition of *Brucella abortus* strain 19. *J. Bact.*, **54**, 15-16.
- KOSER, S. A., BRESLOVE, B. B., and DORFMAN, A. 1941 Accessory growth factor requirements of some representatives of the *Brucella* group. *J. Infectious Diseases*, **69**, 114-124.
- LEVINE, H. B., and WILSON, J. B. 1947 Differentiation of *Brucella abortus* strain 19 by dye bacteriostasis. *J. Bact.*, **54**, 12.
- MCCULLOUGH, N. B., and DICK, L. A. 1942a Physiological studies of *Brucella*. I. Quantitative accessory growth factor requirement of certain strains of *Brucella*. *J. Infectious Diseases*, **71**, 193-197.
- MCCULLOUGH, N. B., and DICK, L. A. 1942b Physiological studies of *Brucella*. II. Accessory growth factor requirement of recently isolated strains of *Brucella abortus*. *J. Infectious Diseases*, **71**, 198-200.
- MCCULLOUGH, N. B., and DICK, L. A. 1943 Growth of *Brucella* in a simple chemically defined medium. *Proc. Soc. Exptl. Biol. Med.*, **52**, 310-311.
- MCCULLOUGH, W. G., MILLS, R. C., HERBST, E. J., ROESSLER, W. G., and BREWER, C. R. 1947 Studies on the nutritional requirements of *Brucella suis*. *J. Bact.*, **53**, 5-15.
- FOLDING, J. B. 1946 Some peculiarities in the germination of *Brucella*. *J. Comp. Path.*, **56**, 215-236.
- STIMMEL, M. P. 1946 *Unpublished*.
- ZOBELL, C. E., and MEYER, K. F. 1932a Metabolism studies of the *Brucella* group. VIII. Nutrient requirements in synthetic media. *J. Infectious Diseases*, **51**, 344-360.
- ZOBELL, C. E., and MEYER, K. F. 1932b Metabolism studies of the *Brucella* group. IX. Physicochemical requirements in synthetic media. *J. Infectious Diseases*, **51**, 361-381.