STUDIES ON THE NUTRITION OF BRUCELLA MELITENSIS

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Koser, Breslove, and Dorfman (1941) obtained moderate growth (one billion cells per ml) of the three species, *Brucella melitensis, Brucella abortus,* and *Brucella suis,* in a chemically defined medium consisting of 17 amino acids, glucose, inorganic salts, thiamin, nicotinamide, biotin, and pantothenic acid. All of the strains studied required thiamin and nicotinamide, and all were stimulated by pantothenic acid. Growth of *B. abortus* strains was stimulated by a biotin concentrate. McCullough *et al.* (1947) developed a chemically defined medium for *B. suis* consisting of 14 amino acids (5 of which were considered essential and 9 stimulatory), glucose, inorganic salts, thiamin, nicotinic acid, biotin, and pantothenate. With this medium they obtained yields of approximately 35 billion cells per ml. With 12 amino acids and added purines they obtained more than 50 billion cells per ml.

The nutritional requirements of brucellae also have been studied by many other investigators. The media employed, however, were either unduly complex because of the number of amino acids required in their preparation or failed to produce a high yield of viable cells. The present investigation of the nutritional requirements of strain 4247 of *B. melitensis* was undertaken to devise a simple synthetic medium that would give abundant growth. A preliminary report of this work was presented by Sanders, Higuchi, and Brewer (1951).

EXPERIMENTAL METHODS

The components of the medium excepting glucose were made to volume (10 ml) in 150 ml pyrex milk dilution bottles, adjusted to pH 7.5 with NaOH, and sterilized by autoclaving for 20 minutes at 121 C. Glucose was sterilized separately and added aseptically to the medium. The bottles were plugged with cotton enclosed in gauze. Inoculations were made with suitable dilutions of a culture grown in partially hydrolyzed casein medium to provide about 100 million cells per ml. However, in determining the vitamin requirements much smaller inocula were used. Triplicate cultures were incubated on a reciprocating shaker in a humidified atmosphere at 37 C. Pyrex glassware, cleaned with chromic acid and rinsed with glass-distilled water, was used in preparing media and in growing cultures.

In preliminary experiments, bacterial growth was measured by plate count methods; however, turbidimetric procedures were found to be quite reliable and to require much less time and effort. The turbidities of triplicate samples were measured with a Coleman photonephelometer using matched 18 mm test tubes. A turbidity value of 160 (obtained with a culture diluted 1:10) was equivalent to approximately 100 billion cells per ml in the original culture. The numbers of viable cells were estimated by plating tryptose saline dilutions in triplicate on tryptose agar and counting after 4 to 5 days' incubation at 37 C. The bacterial counts reported are averages of triplicate plates. Stock cultures were carried on tryptose agar slants with incubation for 3 to 5 days.

RESULTS

The study was begun with attempts to grow *B. melitensis* in tryptose broth and in a casein hydrolyzate medium. Although the tryptose medium produced moderate growth, the casein partial hydrolyzate medium gave very poor growth because of the high salt content which resulted from the neutralization of the sulfuric acid used for the protein hydrolysis. A medium containing casein partial hydrolyzate that had been deacidified with an anion exchange resin ("amberlite IR-4B") supported excellent growth. Therefore, it was thought that a mixture of amino acids combined in the relative concentrations in which they occur in casein likewise should be satisfactory. Initial experiments were conducted with a medium composed of 18 amino acids at concentrations equivalent to those in 1.7 per cent

of casein (Block and Bolling, 1940) together with glucose, inorganic salts, and vitamins. This mixture gave yields of approximately 105 billion cells per ml. Having obtained excellent growth, attempts were made to eliminate all amino acids, vitamins, and inorganic salts that did not contribute to the high yield of viable cells. After elimination of the unnecessary components, a medium was formulated in which only 6 of the 18 amino acids in the simulated casein mixture were found to be essential for optimal yields (table 1).

In the synthetic medium containing potassium and sodium ions (added as 0.01 M K_2HPO_4 and the approximately 0.025 M NaOH necessary for pH adjustment) the only demonstrable metallic cation requirements were for iron, magnesium, and manganese. The effects of these inorganic ions on the growth of *B. melitensis* are shown in table 2. No growth occurred in the absence of all three of the added metals. When iron or manganese (either singly or together) was the only added metal, there was very little growth. The addition of magnesium alone (0.0025 M) gave

TABLE 1

Chemically defined medium for Brucella melitensis

	mg/ml		μg/ml
DL-Alanine	1.20	$FeSO_4 \cdot 7H_2O$	13.9
L-Arginine·HCl	0.77	$MnSO_4 \cdot H_2O$	16.9
L-Cystine	0.10	Nicotinic acid	2.0
L-Glutamic acid	3.90	Thiamin·HCl	0.15
L-Lysine·HCl	0.77		
DL-Methionine	0.94		
Glucose	25.00		
K_2HPO_4	1.74		
$MgSO_4 \cdot 7H_2O$	0.62	pH adjusted to 7.5 with NaOH	

TABLE 2

Effect of metallic ions on the growth of Brucella melitensis in a chemically defined medium

METAL ADDED TO BASAL MEDIUM*					TURBIDITY OF 1:10 DILUTION OF CULTURE		VIABLE CELLS
Fe^{++}		Mn^{++}		Mg^{++}			
(5×10^{-5} M)	(5×10^{-6} M)	(1×10^{-3} M)	(1×10^{-4} M)	(2.5×10^{-3} M)	72 hours	96 hours	96 hours
							× 10^9/ml
−	−	−	−	−	3	3	—
+	−	−	−	−	11	14	5
−	−	−	+	−	11	13	5
−	−	+	−	−	10	13	—
−	−	−	−	+	43	95	63
+	−	−	+	−	10	14	5
+	−	+	−	−	10	13	—
+	−	−	−	+	73	168	130
−	−	+	−	+	27	51	27
−	−	−	+	+	47	87	47
+	−	+	−	+	75	176	141
+	−	−	+	+	101	185	151
−	+	−	−	−	10	15	—
−	+	−	−	+	66	155	105
−	+	+	−	−	10	13	—
−	+	+	−	+	69	161	103
−	+	−	+	+	80	144	89

* The basal medium is given in table 1, with the omission of metallic ions concerned.

approximately half the maximal growth. The optimal concentration of K_2HPO_4 was found to be 0.01 M, but yields in excess of 10^{11} cells per ml were obtained with levels as high as 0.075 M.

Thiamin and nicotinic acid were the only vitamins required in the chemically defined medium. Biotin and calcium pantothenate had little effect on the final growth. Figure 1 shows the growth response curves obtained with thiamin and nicotinic acid. About 0.15 μg per ml of thiamin·HCl and 2.0 μg per ml of nicotinic acid

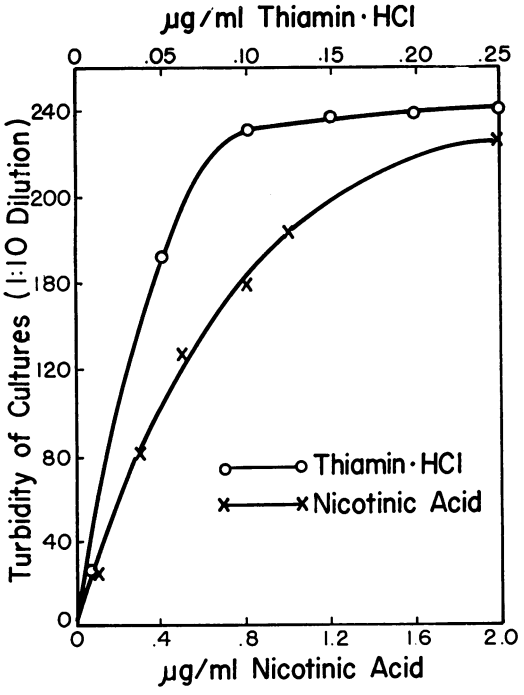

Figure 1. Effects of varying concentration of thiamin and nicotinic acid on the growth of *Brucella melitensis*.

allowed maximal growth of the organism under the conditions of the experiment.

The optimal glucose concentration of 2.5 per cent was found to be unusually high compared to that used for growing other species of *Brucella*. Reducing values determined after growth showed that the 2.5 per cent of added glucose was almost completely utilized. Galactose and fructose were found to replace glucose in the medium with little change in cell yield. This was in contrast to cultures in complex medium (casein digest-yeast autolysate) where *B. melitensis* grew only one-fifth as well in galactose medium as in glucose medium. A keto acid, identified as pyruvate, accumulated in large amounts (1 mg/ml) in the galactose cultures in the complex medium, and the resulting acidity was believed at least partially responsible for the poor growth (Higuchi *et al.*, 1951). When glucose was replaced in the synthetic medium with L-arabinose or D-xylose, the best yields were obtained at a concentration of 1 per cent carbohydrate, and the yields never equalled those obtained with glucose. A distinct acetamide-like odor was detected during bacterial growth in media containing L-arabinose. Little or no growth occurred when glucose was replaced with D-ribose, D-lyxose, or various disaccharides or polysaccharides.

TABLE 3

*Effects of varying the concentrations of single amino acids on the growth of Brucella melitensis in a chemically defined medium**

AMINO ACID	MILLIGRAMS PER ML	TURBIDITY		VIABLE CELLS
		71 hours	95 hours	96 hours
				× 10^9/ml
DL-Methionine	0	78	92	31
DL-Methionine	0.47	149	223	124
DL-Methionine	0.94	97	217	124
DL-Methionine	1.41	72	201	—
L-Lysine	0	76	206	114
L-Lysine	0.31	82	219	136
L-Lysine	0.62	95	211	123
L-Lysine	0.93	101	214	—
L-Arginine	0	73	186	97
L-Arginine	0.32	95	204	114
L-Arginine	0.64	94	215	126
L-Arginine	0.96	106	223	116
DL-Alanine	0	18	81	49
DL-Alanine	0.6	72	205	130
DL-Alanine	1.2	101	232	136
DL-Alanine	1.8	108	233	140
L-Glutamic acid	0	9	44	35
L-Glutamic acid	1.95	62	138	78
L-Glutamic acid	3.90	94	220	126
L-Glutamic acid	5.85	100	244	144
L-Cystine	0	0	1	—
L-Cystine	0.05	93	208	125
L-Cystine	0.10	105	221	128
L-Cystine	0.15	42	214	—

* The composition of the medium is that given in table 1, except for the designated amino acid.

Table 3 shows the effects of varying the concentrations of the amino acids one at a time in the

medium consisting of six amino acids. For maximal growth methionine, alanine, glutamic acid, and cystine were essential. The single omissions of either lysine or arginine affected the final growth in lesser degrees. In general, the levels of the six amino acids in the medium were not critical and the recommended quantities for each amino acid are based on a number of experiments where both early and final growth were considered.

In some of the early experiments the requirement for cystine was less definite than in subsequent work. In trying to reduce further the number of amino acids in the synthetic medium, it was thought that cystine might be replaceable by inorganic sulfur compounds. In the early experiments, cystine was replaceable by sodium sulfide, sodium hydrosulfite, or sodium thioglycolate. However, in later work (tables 3 and 4) the growth obtained with the inorganic sulfur compounds was approximately half or less of that obtained with cystine.

The supplementation of the complete medium (table 1) with 0.05 and 0.10 per cent of certain amino acids (aspartic acid, glycine, histidine, phenylalanine, serine, proline, threonine, tryptophan, tyrosine, and hydroxyproline) did not affect the final yield. Partial inhibition resulted from supplementation with 0.01 per cent of norleucine, leucine, isoleucine, or valine.

Cultures of *B. melitensis* were carried through 10 serial transfers in the synthetic medium without any evidence of diminished growth. Examination of the bacterial dissociation pattern showed characteristics of 100 per cent smooth colonies.

Evidence was obtained that D-methionine, D-alanine, D-cystine, and D-glutamic acid were utilized by *B. melitensis*. As seen in table 4, the growth at 70 hours at each level of DL-alanine was at least equal to that with the same molarity of L-alanine. Similar evidence for the utilization of the D-isomers of glutamic acid and methionine also was obtained.

Because the medium was developed for a specific strain of *B. melitensis*, its suitability for other strains and species of *Brucella* was tested with a number of representative strains of *B. melitensis*, *B. suis*, and *B. abortus* (not CO_2 requiring types). Of the 14 strains of *B. melitensis* examined, 7 grew to produce turbidities of 150 or higher, 4 were in the range of 50 to 150, and only 3 strains yielded turbidities below 50. On the other hand, none of the 7 strains of *B. suis* tested grew to a turbidity of above 150, 4 were in the 50 to 150 range, and 3 were below 50. Of the 7 *B. abortus* strains tested, 6 failed to grow at all and only 1 grew in the turbidity range of 0 to 50. These results indicated that the synthetic medium was especially suitable for several strains of *B. melitensis*.

A comparison of the growth obtained with strain 4247 of *B. melitensis* in three published chemically defined media was made. As stated previously, the medium of McCullough *et al.* (1947) is composed of 14 amino acids, purines, glucose, inorganic salts, and vitamins; it was developed for *B. suis*. Rode, Oglesby, and Schuhardt (1950) failed to obtain consistent growth of *B. melitensis*, strain 2459, in the medium of McCullough *et al.* (1947) and correlated this with the high histidine content of the medium. However, in the present work, strain 4247 of *B. melitensis* failed to grow even when the histidine content was reduced to 0.06 per cent. The medium of Gerhardt and Wilson (1948) supported

TABLE 4

*Effect of the configuration of the amino acids on the growth of Brucella melitensis in a chemically defined medium**

AMINO ACID	MILLIGRAMS PER ML	TURBIDITY	
		70 hours	96 hours
No alanine		50	126
L-Alanine	0.3	74	178
DL-Alanine	0.3	93	200
L-Alanine	0.6	119	217
DL-Alanine	0.6	126	215
L-Alanine	1.2	158	244
DL-Alanine	1.2	172	237
No methionine		75	80
L-Methionine	0.47	177	218
DL-Methionine	0.94	155	206
D-Methionine	0.94	145	206
No glutamic acid		44	58
L-Glutamic acid	2.0	135	164
DL-Glutamic acid	2.0	111	160
D-Glutamic acid	2.0	70	156
No cystine		38†	120†
L-Cystine	0.1	152	260
DL-Cystine	0.1	210	235

* The composition of the basal medium is that given in table 1, except that the designated amino acid was omitted.

† Subsequent results showed much less growth in media without cystine.

only scanty growth (turbidity of 5) of strain 4247 of *B. melitensis* with either asparagine or glutamic acid as the nitrogen source. The medium of Rode, Oglesby, and Schuhardt (1950) (composed of 18 amino acids, glucose, inorganic salts, and vitamins) yielded a turbidity of only 24 with *B. melitensis*, strain 4247. By supplementing this medium with larger amounts of amino acids, vitamins, and glucose, the growth was doubled but turbidities greater than 55 were not obtained.

Concurrent with studies on the formulation of a synthetic medium it was observed that the addition of autolyzed yeast (0.25 mg/ml) to the synthetic medium decreased the lag phase of growth of *B. melitensis* by approximately 50 per cent. However, the final total growth was unchanged. In an effort to duplicate the marked stimulation shown by yeast, various amino acids (either singly or combined) were added to the basal medium at varying concentrations. Serine (0.77 mg/ml) was the only amino acid which showed significant stimulation (approximately 30 per cent) of early growth, but it did not approach that shown with the autolyzed yeast. Incidental to the stimulation of early growth it was found that excellent growth was obtained when serine or proline was substituted for lysine (or for lysine and arginine) in the complete medium. The known vitamins and other likely compounds also were tested in the basal medium, but none gave the activity of autolyzed yeast. A strepogenin concentrate gave stimulation of early growth equivalent to approximately 46 per cent of autolyzed yeast on a dry weight basis. Very little loss of the activity of yeast autolysate resulted from acid or alkaline hydrolysis (5.4 N H_2SO_4 or 5.4 N NaOH at 126 C for 6 hours). Nitrous acid destroyed the activity but hydrogen peroxide did not. Ethyl ether extraction of a yeast autolysate solution at pH 1.8 for 1 to 7 days removed no more than 10 per cent of the activity; ether extracts made at pH 7 and pH 9 contained no appreciable activity.

Treatment of the yeast autolysate with twice its weight of charcoal removed approximately half of the solids, which included most of the stimulative activity. The activity was not eluted significantly with either dilute HCl or NH_4OH as judged on a dry weight basis. When the charcoal was eluted with ethanol, the eluate was approximately 4 times as active as the yeast autolysate on a dry weight basis; however, only about 2.5 per cent of the original solids were recovered in this fraction. Further work is necessary to clarify the nature of the stimulatory factor(s) in yeast. However, because neither amino acids, yeast ash, nor known growth factors replaced the activity shown with autolyzed yeast, an unknown factor may be involved.

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SUMMARY

The nutritional requirements of strain 4247 of *Brucella melitensis* were studied intensively, and a synthetic medium was formulated that consistently gave yields of over 125 billion cells per ml. The chemically defined medium consists of 6 amino acids, glucose, inorganic salts, and vitamins.

Magnesium, iron, and manganese salts are necessary for good growth. In agreement with Koser and co-workers nicotinic acid and thiamin are essential.

A relatively high level of glucose, 2.5 per cent, is required in the medium for maximal growth.

Evidence was obtained that D-methionine, D-alanine, D-cystine, and D-glutamic acid are utilized by *B. melitensis*.

Based on tests with representative strains of the three species of brucellae, the synthetic medium was found to be especially suitable for the growth of several strains of *B. melitensis*. Moderate to poor growth was obtained with strains of *B. suis* and *B. abortus*.

A substance contained in yeast autolysate which stimulates early growth could not be duplicated by any other compound tested. The nature of the yeast stimulation is only partially understood.

Combinations of amino acids play an important role in the nutritional requirements of *B. melitensis*. No single amino acid was found to be essential.

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