THE NUTRITION OF BRUCELLAE

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I. INTRODUCTION

Sir David Bruce in 1887 isolated on peptone-beef extract agar the causative "micrococci" from fatal cases of human brucellosis (18). During the next forty-five years of investigation of the brucellae, the major basic finding in their nutrition was the observation that freshly isolated *Brucella abortus* requires an added amount of carbon dioxide for growth (6, 41, 100). About 1932, the comprehensive work of ZoBell and Meyer (103–106) established a solid foundation for the study of growth requirements in defined environments and since then significant advances in knowledge of the nutrition of brucellae have been accomplished. The limited objectives of early investigation, better methods of isolation and classification, have since been enlarged to include other practical applications, a directed approach to chemotherapy in brucellosis, and perhaps most importantly, basic information on the physiology of these pathogens and of bacteria in general.

II. COMPOSITION OF MEDIA

A. Undefined Media

A variety of undefined media, usually meat infusion comparable to that originally employed by Bruce (18), have been used in the cultivation of brucellae. The beef liver infusion medium of Stafseth (94) is typical and earlier found considerable use. Potato infusion agar (93) was recommended by the Bureau of Animal Industry, U. S. Department of Agriculture, and is still in use for the preparation of standardized antigen and of vaccine for cattle.

The variability in composition and nuisance of preparation of such infusion media were obviated about 1938 by the commercial development of a peptone in dehydrated form, which was prepared from a pancreatic digest of casein and which supported superior growth of brucellae (42). In its present form, this medium contains 2 per cent peptone, 0.1 per cent glucose, 0.5 per cent sodium chloride, and 0.5 mg per cent thiamin. A number of similar casein-digest media are available commercially; the choice between them is largely a matter of individual preference. Media prepared with acid-hydrolyzed casein, preferably if the hydrolyzate is subsequently deionized, also support excellent growth of brucellae (82, 87).

Modifications of these complex media have appeared from time to time in the literature and in the manufacturers' formulas. Yeast (67, 74) and liver (40, 88) extracts and hemin (59) have

been reported to enhance growth in some cases, and the growth-stimulating effect of thiamin when this vitamin is added to some undefined media suggests that it may sometimes be a limiting factor (53, 67, 85). The addition of iron and higher concentrations of glucose increased the growth of *Brucella suis* in one medium (67). When the supply of oxygen is greatly increased, the usual concentrations of peptone and glucose may become limiting (85). Yeast extract, thiamin, sodium bisulfite, and phosphate have been included in various commercial preparations along with the usual peptones, salt, and glucose.

Some of the highest yields reported for growth of brucellae, up to 1×10^{12} viable cells per ml, have been attained in a medium consisting of 3 per cent peptone, 3 per cent glucose, 1 per cent yeast extract, and 0.15 per cent Na_2HPO_4 at pH 6.4, sterilized by filtration (94*a*, 98*a*). In attempts to confirm these results. Tyrrell and Gerhardt (*unpublished*) found that nearly a decimal increase in the yield could be attributed to the use of filtration rather than heat for sterilization of the medium.

A comprehensive investigation of the toxicity of some lots of a peptone for brucellae was reported in a series of papers by Schuhardt and his associates at the University of Texas (89–92); their observation has been confirmed, and the peptone also has been reported to affect agglutinability of brucellae (19). The Texas group initially detected a brucellacidal factor which could be extracted and concentrated 60-fold. It was neutralized by agar, blood, and other substances, and seemed to consist of oxidized amino acids (89). Subsequently it was also found that the toxicity developed upon aging and was not the result of gross exposure to laboratory environments (91). When amino acids were systematically added to a synthetic medium originally lacking amino acids, cystine was found to be toxic (90). However, the apparent cystine toxicity in synthetic medium could be neutralized by adding nontoxic peptone, and cystine reversed the inhibition of growth caused by adding toxic peptone. When cystine was heat-sterilized at pH 7 and was then added to nontoxic peptone, the resulting medium became toxic for brucellae (91). These findings implicated a degradation product of cystine as the antibrucella factor and this interpretation subsequently was confirmed. Elemental sulfur was isolated from autoclaved cystine solutions, and the previously observed toxicity was duplicated by the addition of colloidal dispersions of sulfur from this or other sources in concentrations as low as 0.06 μg per ml of medium (92). Sulfur toxicity, which develops with aging of the peptone studied and possibly of other peptones as well, is neutralized in culture media containing either blood or agar but may require consideration if such broth media are not supplemented.

The growth of brucellae on undefined media may be inhibited by certain fatty acids. Boyd and Casman (7) found that such components probably account for the toxicity of media filtered through cotton or for the original toxicity of a commercial peptone. These toxic materials, to which strains of the organism apparently vary in sensitivity, may be extracted by fat solvent or neutralized by starch. Huddleson similarly reported (46, 48) the enhancement of growth, especially of Wilson strains of *B. abortus*, after treatment of peptones with charcoal, serum, or Tween; the original toxicity was duplicated in nontoxic peptones by the addition of oleic acid.

Living animals, cell suspensions, and body fluids constitute exceedingly important "media" for growth of brucellae. Embryonated chickens' eggs (93), leucocytes (80*a*), and tissue cultures (39*a*) provide especially useful *in vivo* systems.

B. Chemically Defined Media

Critical study of the nutritional requirements of brucellae inevitably led to development of media in which all components were known. Such an approach not only resulted in a rational improvement of undefined media (67) but has provided controlled environments for allied studies of intermediary metabolism, regulatory mechanisms, genetics, and virulence.

Koser and Rettger in 1919 (55) were among the first of several workers who unsuccessfully attempted to grow brucellae in a synthetic medium. The comprehensive investigation of ZoBell and Meyer (103–106) and ZoBell and ZoBell (107), beginning in 1930, resulted in successful cultivation of brucellae in chemically defined media; however, relatively large inocula were required, the growth period was prolonged, the actual cell yields were low, and serial transfer was not possible. In this connection ZoBell and Meyer correctly noted that the failure of small inocula to grow "may be interpreted as a mani-

TABLE 1

*Composition of some chemically defined media formulated for the growth of brucellae**

Composition	Koser *et al.* (54)	McCullough *et al.* (67)	Rode *et al.* (84)	Sanders *et al.* (87)	McCullough and Dick (66)	Gerhardt and Wilson (31)
mg/ml						
Sodium chloride	8.0	8.0	7.5		7.5	7.5
Dipotassium phosphate	1.0	1.0	1.0	1.74	1.0	10.0
Sodium thiosulfate			0.1		0.1	0.1
Glucose	3.0	10.0	4.0	25.0	1.0	
Glycerol		1.0				30.0
Lactic acid						5.0
μg/ml						
Mg^{++}	10.0	10.0	10.0	62.0	10.0	10.0
Fe^{++}		4.00	0.10	2.8		0.10
Mn^{++}		0.10	0.10	5.5		0.10
Thiamine·HCl	0.20	0.10	0.20	0.15	0.20	0.20
Nicotinic acid	0.20	0.80	0.20	2.0	0.20	0.20
Ca pantothenate	0.20	0.30	0.04		0.04	0.04
Biotin	0.0001	0.0001	0.0001		0.0001	0.0001
Ammonium sulfate	500				500	
DL-Asparagine						3000
Glycine	200	337	100			
DL-α-Alanine	500		100	1200		
DL-Valine	100		100			
DL-Leucine	100		50			
DL-Isoleucine		59	50			
DL-Aspartic acid		665	200			
L-Glutamic acid	500	411	(DL) 3000	3900		(1500)†
DL-Serine	15	16	25			
DL-Threonine	15	47	25			
L-Proline	100		100			
L-Hydroxyproline	100					
L-Cystine	150	192	5	100		
DL-Methionine	100	104	50	940		
DL-Phenylalanine	100	99	25			
L-Tyrosine	50	49	25			
L-Tryptophan	200	20	25			
L-Arginine	100	82	100	770		
L-Lysine	200	146	(DL) 100	770		
L-Histidine	200	4464	100			
Initial pH	6.8–7	7	6.8–7	7.5	6.8–7.2	6.8–7

* Modified from a table by Rode *et al.* (84).

† Recommended now in place of asparagine.

festation of the requirement of a vitamin, hormone, or accessory growth factor." They also observed the enhancement of growth from small inocula when preparations of killed brucella cells or products of the growth of other bacteria were added to the medium. These results now may be attributed to a lack of accessory growth factors in the media and a probable carry-over of traces of such substances in the inoculum. Concurrently, McNutt and Purwin (68), Olitzki and Bromberg (78), and Huddleson (see 106) also reported partial success with synthetic media. Although limited by the absence of knowledge of vitamins, these early studies provided a wealth of valid information on nitrogen and carbon sources, inorganic salts, minerals, and physical-chemical balances required for the growth of brucellae.

Probably the first fully defined and nutritionally adequate medium was published by Koser *et al.* in 1941 (54), at which time the specific preformed vitamin requirements of the

brucellae were discovered. The constituents of this and several subsequently described media are summarized in table 1.

The medium of Koser *et al.* supported moderately high cell yields (0.5 to 1 $\times$ 10^9 viable cells per ml) in 3 days from small inocula (1 $\times$ 10^4) of 7 of the 8 strains tested and permitted serial transfers, which are reasonable standards of nutritional adequacy. Their results have been confirmed by others (64, 84) and recently their medium has been modified to contain fewer amino acids (56*a*, 102*a*).

The Fort Detrick group published reports on two intensive studies of the nutrition of single strains (67, 87). In both cases the cell yields obtained (*ca.* 1 $\times$ 10^{10} viable cells per ml) were as high as or higher than those attainable at that time with undefined media and the results were applicable to most other strains. McCullough *et al.* (67) and Sanders *et al.* (87) used strains of *B. suis* and *Brucella melitensis*, respectively. These investigations demonstrate what may be accomplished in nutritional studies involving specific objectives and specific organisms.

In contrast, the medium of Rode *et al.* (84) was designed to promote growth from small inocula of a large number of strains of the 3 species of *Brucella*. Of the 61 strains tested, including 47 that required additional CO_2, all grew in this medium, although wide differences were observed in the nutritional requirements of the various strains.

The early demonstration of the adequacy of the ammonium ion as a nitrogen source (68, 105) and the subsequent definition of the added vitamin requirements for growth of brucellae (54, 64) led to the formulation by McCullough and Dick (66) of a different type of chemically defined medium, which contains a minimum number of constituents and an ammonium salt as the nitrogen source. This medium and others like it offer the considerable merit of simplicity, although this usually is obtained at the expense of high yields of cells.

The disadvantages in using ammonium salts were overcome in the medium of Gerhardt and Wilson (31), who employed instead a single amino acid. This medium was further studied and modified (27, 30, 35, 72), and L-glutamate rather than the DL-asparagine used originally is now recommended as the nitrogen source. For growth of at least one strain, maximum simplicity may be obtained by dispensing with the glycerol and lactate and using 0.5 per cent L-glutamate as the sole source carbon, energy, and nitrogen (30).

In retrospect, the development and application of synthetic media have tended toward either of two types: media that contain multiple sources of nitrogen and give maximum growth (54, 67, 84, 87, 102*a*) or media that contain a single source of nitrogen and have maximum simplicity compatible with moderate growth (31, 66). Very frequently these media have been developed for a selected strain and then tested with a representative group (31, 67, 87). Even if the goal has been general (84), differences in species, strains, level of inoculum, and aeration have tended to make comparisons of the value of the several formulations relative. All of the more recent media that contain multiple amino acids support growth of most strains from a small inoculum at rates and to levels at least comparable to those obtainable with undefined media. Even the simpler synthetic media often produce yields within a logarithmic unit of those obtainable with undefined media and support growth from small inocula, particularly when thickening agents are added. The choice of a medium, then, depends on the purpose of the study in which it is to be used.

III. BASIC REQUIREMENTS

A. Inorganic Materials

The need for a high buffering capacity near neutrality, for inorganic phosphate, and probably for potassium ions has made the incorporation of K_2HPO_4 common to all defined media for brucellae. Sodium ions usually have been added at the same concentration as in the traditional "physiological saline" (0.85 per cent NaCl), although the addition of NaCl probably has little effect (87). Sodium and potassium ions appear interchangeable (54, 105).

The requirement for sulfur may be met by any of a number of inorganic or organic sources; thiosulfate and cystine have been used most commonly. ZoBell and Meyer (105) recognized the relatively low levels of sulfur required by the organisms and also present in many chemicals. They found that cystine or cysteine, thiogylcolate, sulfate, sulfite, and thiosulfate were utilized less well in the order given; sulfide and elemental sulfur did not support growth. In a

more recent and detailed investigation of the sulfur nutrition of *B. suis* in sulfur-free media, the Texas group (57, 58, 83) found that none of 10 strains assimilated inorganic sulfur at an oxidation level above that of hydrosulfite. All cultures utilized thiosulfate, sulfide, and hydrosulfite, but not elemental sulfur. L-Cystine or L-cysteine seemed to be the best organic source of sulfur. The toxic effect of elemental sulfur and the production of it on autoclaving cystine have been discussed previously in this review.

Relatively large amounts of magnesium are obligatory for growth of brucellae. Since the components of synthetic media usually are contaminated with traces of various minerals, the essentiality of such an element can best be demonstrated by the absence of growth in media extracted with chelating agents or, if sparse growth occurs, by the failure of subcultures to grow in such a depleted medium. An absolute requirement of brucellae for magnesium was demonstrated by these methods (27) and was strongly implied by less rigorous methods (67, 105). Magnesium apparently cannot be replaced by manganese (27).

Treatment of the medium with chelating agents revealed an absolute requirement for iron by strain 19 of *B. abortus* (27), although in similar experiments iron appeared only stimulatory for strain PS-1 of *B. suis* (99), as well as for other strains in studies in which special precautions were not taken to remove traces of iron (67, 105). Such apparent differences in the need for iron and other trace elements and the rather wide variation in the amounts reported to support maximum growth may reflect the degree of depletion in the basal medium, subsequent prevention of contamination, carry-over in the inoculum, presence of sequestering compounds in the medium, strains used, population levels reached, or other variables.

The beneficial effects of the addition of other inorganic elements in trace amounts are less pronounced. Although manganese often is included in defined media for brucellae and appears moderately stimulatory, the one attempt to demonstrate a requirement for the element gave equivocal results (27). Calcium has been reported to be stimulatory (67, 105) and traces often are added in the form of calcium pantothenate. Low levels of cadmium, cobalt, zinc, or nickel are either ineffectual or toxic (67).

B. Nitrogen Sources

The success of early workers (68, 78, 103, 105) in obtaining growth of brucellae by using an ammonium salt, or a readily deaminated organic compound, strongly indicated the sufficiency of the ammonium ion as a nitrogen source. This since has been amply confirmed by experiments using adequately supplemented media, small inocula, and serial transfers (31, 66). Any of the ammonium salts serve equally well, although they are unstable on autoclaving, and growth from small inocula often is obtained only if the medium is semisolid (31, 106). Nitrates and nitrites usually are reduced to ammonia by the brucellae (79), yet alone they fail to support growth, possibly because the necessarily large initial concentration of these oxidized nitrogen sources poises the medium at an unfavorably high Eh. Readily deaminated compounds such as urea, amino acids, or amides circumvent the disadvantages of ammonium salts and singly replace them (30, 31, 105). Of these substances, L-glutamate appears to be the most satisfactory.

Consideration of the amino acid composition of casein, hydrolyzates of which provide the base of most undefined media, has led to several very satisfactory combinations of amino acids, depending on the cultural conditions, strains, and criteria employed. The simplest combination consistent with maximum growth of *B. melitensis* indicated a requirement for glutamate, alanine, lysine, histidine, methionine, and cystine in one instance (87) and for aspartate, cystine, arginine, histidine, phenylalanine, and serine in another (56*a*, 102*a*). In an investigation of a *B. suis* strain (67), cystine, histidine, tyrosine, phenylalanine, and tryptophan were reported to be essential and 9 other amino acids were stimulatory. In a larger survey of 61 strains, the full component of 18 amino acids appeared necessary (84). Upon assessing the amino acids apparently essential or even common to such formulations, one gains the impression that a balance rather than any specific amino acids are essential for maximum growth of brucellae. However, differences in synthesizing capacity among strains also may account for the apparent inconsistencies in such an analysis.

The exact function of exogenous amino acids in the nutrition of brucellae is debatable. Preformed amino acids may be assimilated *per se*, relieving the necessity for their synthesis and

thus enhancing the rate or maximum extent of growth. However, it seems more probable that mixtures of amino acids may simply supply a balanced reservoir of ammonium or amino nitrogen, which serves as the primary source for growth. This concept is supported by observations that the optimum mixtures of amino acids vary considerably (table 1), that single amino acids are adequate for growth (30), that there is a positive correlation between the nutritional and metabolic specificity for single amino acids (29*a*, 30), that amino acids are aerobically deaminated (29), and that the ammonium ion supports growth of the brucellae (31, 66, 103, 105).

Utilization by brucellae of organic nitrogen sources other than amino acids has received little attention. It is not known whether simple peptides will suffice or offer advantages for growth, although alanyl dipeptides recently have been investigated for their effect on population changes (3). Catalytic amounts of ribose nucleic acid or any of its component purines or pyrimidines stimulate the rate of growth of *B. suis* in a multiple amino acid medium (67).

One of the very intriguing aspects of the nutrition of this group of bacteria concerns their utilization of D-amino acids. The extensive work on the apparent relationship between D-alanine and the selective establishment of nonsmooth mutants will be considered subsequently in this review. Population changes are observed when most smooth strains of *Brucella* are grown under nonaerated conditions in Gerhardt-Wilson medium where DL-asparagine is employed as the sole nitrogen source. D-Asparagine, alone or as the racemate, supports growth of several smooth strains to varying degrees, but it greatly affects the accumulation of intermediates and the selection of the nonsmooth mutants (35), and even is associated with formation of a red pigment (30). The utilization of D-alanine in a synthetic medium (33) or of other D-amino acids in undefined media, similarly is associated with the selection of nonsmooth mutants (86, 101). Some of these variants develop a requirement for alanine, presumably in the D-form (33). *B. melitensis* can be grown on D-glutamate, D-methionine, D-cystine, and D-alanine, although the last two were tested as the racemate (87). D-Cystine and D-methionine were reported to support growth of *B. suis* (83). It is apparent that at least several D-amino acids are metabolized by and support growth of smooth strains of brucellae, but that better utilization of, or lesser sensitivity to, the D-forms may be one of the distinguishing properties of nonsmooth mutants. These apparent differences between mutant types emphasize the importance of recognizing and controlling population changes in nutritional studies on brucellae (12, 33).

Using a casein-digest medium to which were added the D- or DL-amino acids essential in human nutrition, Yaw and Kakavas (101) found the racemic mixtures to be toxic for three strains of *B. abortus*. A slowly bactericidal as well as a bacteriostatic effect was indicated for the separate D-isomers of five of the amino acids. Continued subculture in the presence of any of several such amino acids selected the less virulent mucoid mutants (25, 51, 102).

The metabolism of sulfur-containing amino acids and the utilization of the stereoisomers by brucellae have been studied in detail by Lankford *et al.* (57, 58) and Rode *et al.* (83).

Glycine added in high concentrations to a growth medium uniquely causes the formation of protoplast-like bodies from *brucella* cells. Unpublished results by the author have shown that several strains incubated for 16 to 24 hr in Albimi broth containing 1.25 per cent glycine are converted almost entirely to these bodies. Gentle agitation during the incubation, a heavy inoculum of actively growing cells, the strain of the organism, and the concentration of glycine were found critical, whereas moderate variation in pH near neutrality or the addition of sucrose made little difference. The brucella "protoplasts" appeared in the phase microscope as round objects having a delicate membrane, which remained after plasmoptysis; unfortunately, the cytoplasm often appeared to be retracted to a small portion of the "protoplast" and the original cell (or cell wall) appeared to adhere to it, thus limiting their potential application to physiological and immunological problems.

C. Carbon and Energy Sources

Glucose traditionally is included in most bacteriological media as a source of carbon and energy. The concentration generally employed for brucellae has been 0.1 to 0.4 per cent; however, higher levels (1 to 3 per cent) were found optimum and were completely utilized in several

instances characterized by unusually high aeration and yields (67, 85, 87, 94*a*, 98*a*, 98*b*). Other sugars may be employed less satisfactorily: 2.5 per cent glucose can be replaced completely by 2.5 per cent galactose or fructose (but not in a casein-digest medium), partially by 1 per cent arabinose or xylose, but not at all by ribose, lyxose, or various disaccharides or polysaccharides for growth of a strain of *B. melitensis* in a synthetic medium (87). Using 12 strains of the three species in McCullough-Dick medium, McCullough and Beal (63) found that the lag period was shortest and all strains could be carried through serial transfers when erythritol was used as the sole carbon and energy source. Glucose appeared next best of 9 carbohydrates tested. Since strains vary in their carbohydrate requirements, classification within the genus on the basis of glucose utilization during growth was advocated (60, 61) but later refuted (104). Fermentation of carbohydrates by resting cells similarly has been suggested (80) and questioned (1) as a convenient criterion of speciation.

Several compounds support a greater rate of respiration than does glucose and consequently have been used as a carbon and energy source in defined media. L-Glutamate appears the most likely replacement and its value when used singly or in combination (table 1) as a nitrogen source possibly may be attributed in part to the concomitant oxidation of its carbon skeleton. The same may be said for asparagine. Used alone, either was found to support growth as the sole nitrogen, carbon, and energy source (30). Of the non-nitrogenous organic acids, L-malate is rapidly metabolized and was used by Marr and Wilson (72) to replace lactate and glycerol in Gerhardt-Wilson medium. In this medium lactate and the ammonium ion support the growth of *B. abortus* strain 19 (31) but in a peptone medium lactate offers no advantages as a replacement for glucose (85). A unique effect of lactate, permitting the utilization of methionine and cystathionine, has been reported (83).

Glycerol was recommended as a medium supplement in several early investigations (78, 105). Although the effect of glycerol in these experiments may be partially attributed to a probable contamination with vitamins, the high concentration optima observed both in these vitamin-deficient and in a subsequent vitamin-sufficient medium (31) "are very suggestive that physical and not chemical factors are responsible for the growth-promoting properties of glycerol" (105). Non-nutritive properties may also apply in part to the effect of glucose, which has reducing properties and, when heat-sterilized in the medium, was shown to neutralize the toxicity associated with some peptones (89).

D. Accessory Growth Factors

The beneficial effect of supplements such as liver extract in natural media upon growth of brucellae was recognized early (40). Kerby (53) noted that growth of *B. abortus* on a peptone medium was enhanced by the addition of nicotinic acid and thiamin. In 1941, Koser *et al.* (54) first demonstrated that seven representative strains of the three species require thiamin, calcium pantothenate, nicotinic acid (or nicotinamide), and probably biotin for growth in a chemically defined medium. The need for biotin was subsequently confirmed and the pyrimidine, but not thiazole, component of thiamin was found to be required (56). McCullough and Dick (65) were unable to correlate a vitamin requirement with their earlier failure to obtain growth of CO_2-sensitized strains of *B. abortus* in a defined medium (54); after acclimatization to normal atmospheric conditions, however, 30 of 41 such strains grew in the medium, 23 of these with only thiamin and biotin as added vitamins. Quantitative requirements for the four vitamins subsequently were determined for various strains and media (64, 67, 87). Recent investigations have centered on the role of pantothenate in population changes (3, 73) and they will be discussed later in this review. Mika *et al.* (73) concluded that strains vary in their utilization of preformed pantothenate and that it is a stimulatory rather than an absolute nutritional requirement for brucellae. In reviewing these data on vitamins, it appears that thiamin is indispensable for growth of all strains, niacin is usually so for *B. suis* and *B. melitensis*, and biotin is essential for *B. abortus*; niacin, biotin, and pantothenate often are stimulatory, if not absolutely required.

Vitamin B_{12} has been studied in regard to its effect on utilization of sulfur-containing amino acids (58). Vitamin A has been reported to be an essential growth factor for some strains of *Brucella* (95).

Several other supplements have been reported to accelerate the initiation and rate of multi-

plication of brucellae. As noted before, ribose nucleic acid, a mixture of its constituents, or adenine, guanine, or cytosine alone stimulated the rate of growth but not the yield of cells from small inocula of *B. suis* in a multiple amino acid medium (67). Two attempts have been made to demonstrate a new growth factor in autolyzed yeast, which frequently is observed to stimulate growth of brucellae even in relatively complete media. In both cases (23, 87), the factors were soluble in water and ethanol, insoluble in ethyl ether, adsorbed by charcoal, and stable to acid and alkaline hydrolysis. In neither case was the material singly replaced by a mineral, known vitamin, amino acid, purine, or pyrimidine.

E. Oxygen

The popular emphasis on chemical factors in bacterial nutrition is evident also in studies with brucellae, although the obvious effects of aeration and the requirements of some strains of *B. abortus* for an increased pCO_2 have focused attention on gaseous requirements, in particular where small inocula are employed or large cell crops are desired.

The brucellae are aerobic and do not grow under anaerobic conditions (106). In exception to this statement, Zottner (108) reported the isolation of *B. abortus* in "pure" CO_2; however, it seems likely that the commercial CO_2 he used contained some oxygen or that he inadequately removed air from the containers before replacing with CO_2. Earlier, Wilson (100) observed growth in CO_2 if as little as 0.5 per cent oxygen was present.

As with most aerobes, growth initiation of brucellae is favored by a reduced air supply but considerable increases of both the rate and extent of growth result when cultures are adequately aerated by either shaking or sparging (28, 29, 31, 67, 82, 85, 87, 98*a*). Agitation alone is ineffectual (85) but turbulence is necessary for a high rate of oxygen transfer. The type of sparger and the antifoam agent used are important factors in sparger aeration systems for mass production of brucellae; air economy and better foam control result from the use of a gradually increasing rate of aeration (28). It is important to recognize that high aeration rates from shaking or sparging of cultures often result in a considerable and variable amount of evaporation during the usual incubation period unless the atmosphere is humidified. Sanders and Huddleson (85) reported that a flow of pure oxygen over shaken cultures dramatically increased the rates of growth and the total yields of three strains. This effect was especially pronounced when the concentrations of peptone and glucose were increased to 3 and 2 per cent, respectively. Representative strains of the three species differed in their response to various O_2 and CO_2 atmospheres (85). However, a correlation between growth of brucellae and direct determinations of dissolved oxygen in the medium has not been made and there is reason to believe that the maximum oxygen demand of brucellae has not yet been realized.

Aerobiosis also is a major selective factor in population changes of brucella cultures, anoxia generally favoring the growth of nonsmooth mutants (5, 8, 13, 86). This important factor will be considered in detail subsequently in this review.

Oxygen is only slightly soluble and diffuses slowly into static media. However, tradition and convenience continue to favor the use of test tubes for cultivating aerobes such as brucellae, even in nutritional work where the limitation of growth by a vital requirement casts doubt on conclusions. This limitation seems more readily accepted with regard to vitamin deficiencies than with oxygen starvation. Shaken flasks or bottles have been employed with considerable success to insure adequate aeration. Cuvette tubes that are attached as a sidearm to shaken flasks (23), directly shaken longitudinally (27), swirled rapidly on a rotary shaker, or agitated internally with magnetic impellers provide reasonable aeration, can be read directly in a colorimeter, and still retain the other advantages of test tubes.

A modification in this recommendation for aeration is warranted when the sole criterion of "growth" is its initiation, especially from a small inoculum. Then, an increase in oxygen supply becomes deleterious, possibly because of a higher Eh, and measures to prevent or neutralize the absorption of air actually become desirable. Addition of a thickening agent such as 0.1 per cent agar to provide a semisolid medium appears to be the most effective measure, although stratification of growth results. It was demonstrated that the action of the agar is physical and that this effect of agar may be duplicated with gelatin or synthetic methyl cellulose (31, 106) but not

with various concentrations of reducing agents such as thiosulfate or cysteine. An attractive alternative to the above idea is supplied by the experimental observations and literature review by Schuhardt *et al.* (89) concerning the possible neutralization of toxic materials by small amounts of agar. In any case, the use of semisolid media is recommended for growth from a very small inoculum of brucellae, especially in isolation procedures from clinical specimens (26).

Chemical reducing agents presumably favor initiation of growth from small inocula, although it is difficult to separate this Eh effect from the primary purpose for which such components are usually added, and no adequately controlled experiments have been reported. Thiosulfate as a sulfur source and glucose as a carbon and energy source probably are also effective in lowering the Eh, and the high concentration optimum for glycerol suggests that its growth-promoting properties are due to physical factors, possibly including Eh.

Several workers have described the negative drift of Eh that occurs with growth of brucellae (29, 96, 106), although the observation in itself has dubious significance.

F. Carbon Dioxide

Many freshly isolated strains of *B. abortus* differ from others of the group in requiring a quantity of CO_2 in excess of that needed by most heterotrophs. Five per cent CO_2 in an air atmosphere seems adequate for such dependent strains (44, 45); 10 per cent frequently is used but may result in unfavorable pH changes (44, 45), although either these increased levels or complete exclusion inhibits the growth of nondependent strains (62). Zottner reported that *B. abortus* could be isolated equally well in "pure" CO_2 (108); if confirmed, the observation would permit a simpler apparatus for isolation, as he suggested, but it seems probable that such strains are obligately aerobic, as discussed previously.

The CO_2 requirement of *B. abortus* apparently was first studied by Bang in 1897 (6); observing a subsurface layer of growth in semisolid medium incubated in air and using different atmospheres of O_2 and CO_2, he thought that growth of the organism was influenced by lowered pO_2. This idea of a reduced oxygen tension also was used to explain why primary isolates grow well in closed containers with cultures of *Bacillus subtilis* (77) and in sealed tubes (81). Later, following others' clarification of the similar requirement by gonococci and meningococci, Huddleson (41, 49) clearly showed that the increased pCO_2 rather than reduced pO_2 was responsible for stimulating growth of *B. abortus*, but he concluded that this CO_2 in part "was necessary to produce the pH change in the atmosphere." In 1931, Wilson (100) demonstrated unequivocally that CO_2 is required *per se*.

On continued subculture, isolates of *B. abortus* frequency lose the requirement for added CO_2 and may then be grown in air alone. Marr and Wilson (71) found that this "acclimatization" consists in the selective establishment of spontaneously occurring mutants rather than the gradual adaptation of the culture as a whole. The mutation rate for nine strains was shown to be fairly constant and relatively low, varying from 1.5 to 6.4×10^{-10} mutations per cell division, which indicated a high degree of stability for the CO_2 requirement in these cultures.

The biochemical basis of the CO_2 requirement is unknown. Unlike comparable requirements in some other bacteria, the added CO_2 needed by *B. abortus* apparently cannot be replaced by cell extracts, complex nutrients, Krebs cycle intermediates, or purines or pyrimidines (32, 72). A basic similarity in their other growth requirements and in their oxidative metabolism apparently exists for both parent and mutant CO_2 types (70). With isotopic techniques, the major products of CO_2 fixation in the protein of a dependent strain were identified as glycine and alanine (70), and in nucleic acids as pyrimidines (75, 76); however, in neither case did a CO_2-independent mutant differ from its parent in the distribution of the fixed CO_2 (70, 75, 76).

G. Other Physicochemical Factors

"These organisms thrive in rather wide ranges of hydrogen ion concentration. The maximum multiplication was observed between pH 6.6 and 7.4 with some growth at from pH 6.1 to 8.4. Hydrogen ion concentration has a more specific effect on the viability of the organisms suspended in salt solutions." These statements of Zobell and Meyer (106) still prevail, although confirmatory data of comparable detail are wanting. A significant qualification, especially in primary isolation of *B. abortus*, was emphasized by the experi-

ments of Huddleson (44). These indicated a greater pH sensitivity of some CO_2-dependent strains and the necessity for increased buffering capacity and/or a higher initial pH to counter the acidity resulting from added CO_2 and blood. The several defined media (table 1) are adjusted initially to pH 6.8 to 7.5 and contain 0.1 to 1.0 per cent phosphate buffer; in these media, the pH does not appreciably change during growth of brucellae.

Tradition and convenience often have dictated the use of an incubation temperature of 37.5 C for brucellae. A scant literature (23) and unpublished information indicate that both the rate and extent of growth are greater at 34 C. Moreover, a precipitous drop beyond the optimum usually is seen in a temperature curve for bacterial growth, suggesting the practicality of underestimating the incubation temperature. Fortunately, adiabatic cooling probably occurs in aerated cultures, so that actual temperatures are somewhat below incubation temperatures.

In apparently the only pertinent investigation (106), the optimum osmotic pressure for growth of brucellae, as determined by cryoscopic methods, seemed to be between 2 and 6 atm, which is somewhat lower than that of usual culture media (about 7 to 8 atm). Solutions as hypertonic as 10 atm are inimical both to multiplication and to viability. The organisms apparently are more sensitive to hypertonic than to hypotonic salt solutions.

Surface tension influences the growth and, even more so, the morphology of brucellae, although only qualitative data have been obtained (106).

IV. SELECTIVE FACTORS IN POPULATION CHANGES

Mutation in brucellae is readily observed as smooth-rough colony changes on solid media. Since such "dissociation" also is correlated with virulence, considerable research has been directed to this aspect of genetics and, pertinent to this review, to the nutritional basis for population changes involving such genetically altered types. There is ample evidence that such changes represent spontaneous mutations and that the emergence of a particular type in a population results from environmental selection (9). Braun has periodically reviewed the subject (9, 11, 12) and papers by Henry (38) and Huddleson (43) are basic to the study of dissociation in brucellae.

An important segment of this work stems from an observation of Goodlow *et al.* (34) that smooth *B. abortus* cells growing in unaerated Gerhardt-Wilson medium (31) containing DL-asparagine as the sole source of nitrogen are supplanted by a predominantly rough population late in the stationary phase of growth, at about which time alanine accumulates extracellularly. Further investigation revealed that the addition of old culture filtrate or of DL- or D-alanine to smooth cultures early in growth accelerated the outgrowth of alanine-resistant, nonsmooth types in the population; L-alanine had no effect except in very high concentrations (33). When smooth *B. abortus* cells were inoculated into Gerhardt-Wilson medium containing the L-isomer of asparagine, the change to a predominantly rough population did not occur, nor did alanine accumulate; with D-asparagine, the result was similar to that in the racemic mixture (35). However, these effects associated with alanine are usually delayed until late in the growth cycle, suggesting the possibility of an indirect action of alanine on the synthesis or utilization of a substance vital for the multiplication of alanine-susceptible cells.

The known association of alanine with pantothenic acid synthesis led to the investigation of this relationship with respect to population changes of brucellae. Mika *et al.* (73) observed that increased levels of pantothenate depressed the effect of alanine, that pantothenate-starved cells displayed more pronounced population changes than pantothenate-rich cells (although the latter comparison was unfortunately made with two different basal media), and that some inhibitors of pantothenate synthesis duplicated the effect of alanine. It is tempting to conclude that D-alanine selects nonsmooth cells by interfering with pantothenate synthesis, possibly competing with β-alanine as a substrate analogue (12, 73). This interpretation was furthered by the concurrent observation of Altenbern and Ginoza (2) that smooth cells can synthesize pantothenate more rapidly than rough cells. However, a rigorous analysis of the competition between D-alanine and β-alanine has not been published. Interference of D-alanine with pantothenate synthesis could not be demonstrated in resting cells (2); D-asparagine, however, was shown to interfere with pantothenate synthesis in resting cells at pH 5.4 but not at pH 7.4 (2). Recently, Altenbern *et al.* (3) have found that the selective action of D-alanine in promoting population changes was

antagonized competitively by L-alanine and apparently noncompetitively by L-alanyl-L-valine and L-alanyl-L-leucine, and that there was no effect of L-alanine in suppressing population changes in cultures not supplemented with D-alanine. Resting smooth cells synthesized and excreted panthothenate, whereas rough cells utilized the pantothenate present in the medium; this difference was reported to be reflected in a greater rate of pantothenate uptake by rough cells, although small cell masses were used and the rate differences were not great.

The evidence to date on alanine and pantothenate may be summarized as follows: (1) alanine, presumably the D-isomer, accumulates in nonaerated cultures of originally smooth *B. abortus* growing in a D-asparagine medium, and there occurs at about the same time a selective establishment of nonsmooth, alanine-resistant mutants in the population; (2) it cannot be concluded unequivocally that pantothenate synthesis is the primary site nor even that D-alanine is the primary cause of the selective establishment of nonsmooth mutants.

This degree of skepticism is also supported by the diversity of other environmental changes that effect population changes of smooth to rough types, suggesting some underlying common mechanism. Thus, population changes in originally smooth brucella cultures were found to be suppressed by normal serum and globulin fractions of susceptible animals (10), pyrophosphate (22), or chelating agents (21), and to be enhanced by homologous antiserum (8, 9), pH, Eh, and temperature (8), penicillin (16), metallic ions (22, 99), valine (35), sugars (86), and oxygen deficiency (5, 8, 13, 35, 86). Kinetin (14) and breakdown products of deoxyribonucleic acid (14, 17) uniquely favor change from rough to smooth colonial types and may have to be considered separately. Of the factors listed, oxygen deficiency offers the most likely alternative to the D-alanine-pantothenate hypothesis.

Recognition of the effects of aeration on population changes has developed over a period of time. In his initial work on dissociation of brucellae, Braun (8) observed that lowered growth rates generally were associated with reduced population changes; this effect occurred with "daily disturbance" of the culture tubes, and "the most striking demonstration of the effect of growth rates upon the establishment of dissociated types within a population was obtained when broth with a lowered oxidation-reduction potential was used." However, considerable subsequent work has indicated the opposite: rapid growth specifically associated with aerobic conditions brings about markedly *reduced* outgrowth of nonsmooth types. Although interpreted in terms of "population pressure," Braun in the same paper (8) observed that little dissociation occurred when growth took place on the surface of a solid medium. Goodlow *et al.* (12, 35) noted briefly that less dissociation in Gerhardt-Wilson medium occurred when cultures were shaken or held statically with a thin layer of medium in the flask. The critical role of aeration in dissociation was then clearly recognized by Braun *et al.* (13), who found that the enhancement of dissociation by homologous antiserum did not occur in aerated cultures grown in either complex or defined media. Sanders and Huddleson (86) definitely established that the increased population changes of originally smooth brucellae, brought about by the addition of sugars or DL-alanine to a peptone medium, could be reversed by an adequate supply of air or oxygen. In a mixed inoculum of smooth and nonsmooth cells in a glucose medium, the growth of nonsmooth types was suppressed by aeration and enhanced by the absence of aeration. These findings were substantiated by the observations of Altenbern *et al.* (5) that the population change to nonsmooth types was greatly accelerated by incubation under low air pressure and that a rough variant grew more rapidly than its smooth parent at reduced pressure. They made the further observation that other hydrogen acceptors (nitrate, methylene blue, or resazurin) in Gerhardt-Wilson synthetic medium or in several undefined media could replace oxygen in suppressing population changes to nonsmooth types. It thus appears clear that the supply of oxygen, acting either as a hydrogen acceptor or as an oxidizing agent, is a critical factor in population changes of brucellae.

How may this fact be reconciled with the effects of alanine and of the other factors observed to be influential? At present, only partial ties are apparent. The brucellae are known to excrete pyruvate and alanine and to possess the enzymes necessary for interconversion and racemization of these products (4, 69); greater amounts of such products commonly occur in bacterial metabolism as a consequence of anaerobiosis or

of excess carbohydrate supply. Thus the natural accumulation of D-alanine, or even valine, in brucellae may be the result of anoxia; this possibility is borne out by the fact that alanine usually appears in detectable concentrations only after conditions selectively favoring nonsmooth types have been established, at least for strain 19 (3). Although added D-alanine is demonstrably selective, the amount required is considerably in excess of that produced naturally by cultures (3). Moreover, L-alanine at still higher levels (33), or for that matter several sugars (86), will produce the same effect in unaerated media. A possible relationship between aeration and pantothenate is not immediately apparent. In the case of enhancement of dissociation by metals, and of the opposing action of pyrophosphate or specific chelating agents or possibly other factors that may act by sequestering metals, the effects may be related to electron transport systems containing metals, such as the cytochromes. That is, rough and smooth types conceivably may differ in their dependence on these pathways to oxygen. Possible relationships between aeration and other selective factors are obscure and generally have not been examined, although anoxia was found to explain the seemingly selective effect of homologous antiserum (13) and aeration was reported (Braun, *personal communication*) to antagonize rough-to-smooth changes in the presence of breakdown products of deoxyribonucleic acid. It may be that there is no simple relationship between oxygen availability and the survival ability of a mutant. However, it seems doubtful that an understanding of the physiological basis of population changes in brucellae can be furthered by enumeration of separately effective factors, which must be endless, or by the detailed study of any one of them without adequate consideration of the others.

V. PRACTICAL APPLICATIONS

A. Primary Isolation

Laboratory procedures for the primary isolation of brucellae from clinical samples have frequently and adequately been reviewed elsewhere (93). Although a complete appraisal of such methods is beyond the scope of this review, basic nutritional investigations have disclosed several principles that would seem applicable.

The problem of primary isolation of brucellae essentially is one of obtaining growth of a strain-variable heterotroph from an often small inoculum under an environment different from the host. The essential prerequisite is a clinical sample that actually contains the organism; inattention to this requirement undoubtedly accounts for many failures. However, even ideal sampling of blood at the crest of pyrexia, or biopsy of bone marrow or lymph nodes, often will yield specimens containing relatively few brucellae. Consequently, preliminary concentration of the few cells by centrifugation or filtration (15) makes good sense.

Frequently described as fastidious in their growth requirements, the brucellae actually are fairly typical gram-negative organisms with basically simple nutritional requirements and relatively complete synthetic capacities. Although strains differ in specific requirements, the inclusion of complex nutrients with the clinical sample makes the selection of a basal medium relatively unimportant. The sulfur toxicity occurring in some lots of peptone undoubtedly is neutralized under the conditions of primary isolation.

A semisolid medium with 0.1 to 0.3 per cent agar (26, 31, 105) has much to recommend it over liquid or solid media alone or even Castaneda's widely used combination (20). Heating and cooling the tube of semisolid medium just before use, a routine procedure with pneumococci, would be a simple precaution against excessive oxidation. A solid sample (*i.e.*, one collected on a membrane filter) should be arranged longitudinally in the tube as growth stratification may be expected to occur.

The crude but rational procedure of enriching a culture by adding killed cells or extracts of brucellae to the medium (45, 47, 48, 103) also seems worthy of a wider test for primary isolation. This procedure has been found especially useful in inducing growth of the so-called Wilson strains of *B. abortus*, which frequently are missed in the usual procedures (45, 47, 48).

Huddleson (44) recently has re-emphasized the necessity of pH control in the isolation of CO_2-dependent strains from blood. Any of several procedural faults may allow the pH to fall critically below the optimum (pH 7.2 to 7.6) for initiation of growth from small numbers of such strains: inadequate levels of buffer, or inhibitory types, *e.g.*, K_2HPO_4; an atmospheric concentration of CO_2 above the adequate level of 5 per

cent; or failure to compensate for the acidifying effects of erythrocytes. A practical means of accomplishing adequate control and of dispensing with the usual CO_2 jar is to add to a diaphragm-sealed container sufficient carbonate to bring the pH of the medium initially to 7.5 (44).

Methods for isolating brucellae from sources other than the usual clinical specimens have been summarized by Spink (93). A highly selective medium was described recently by Morris (74*a*).

B. Mass Propagation

The need for large numbers of cells requires efficient and safe laboratory methods for growth and concentration of brucellae. The usual procedure, and possibly the best for good yield and minimum dissociation, is simply to inoculate evenly a large, previously dried surface of agar medium in a Roux, Povitsky, or penicillin-culture bottle; the resultant growth is harvested by dislodging the growth with a small amount of diluent. The suspension then is removed by aspiration and the cells are washed by centrifugation in plugged cups.

When larger numbers of cells are needed, it becomes necessary to turn to aerated liquid cultures in large containers. Although limited by volume requirements, large shaker flasks have the obvious advantages of simplicity and relative safety. In using carboys or similar vessels, the main consideration is to provide adequate agitation and aeration; in all probability, it will be physically impossible to over-aerate, and one may in practice be guided by the limit of foam control. Vortex aeration may offer some advantages (98*b*). Several procedures are worth special attention: use of a gradually increasing rate of aeration (28); oxygen-enriched air, while maintaining adequate CO_2 levels, together with a more concentrated medium (85); a means for obtaining turbulence as well as air passage; and an effective but nontoxic antifoam agent. A stable silicone emulsion such as General Electric antifoam no. 60 is excellent; it is effective and nontoxic in concentrations of 50 parts per million. Considerable increases in yields can be effected by sterilizing the medium by filtration rather than by heat (94*a*, 98*a*). Precaution should be taken against the rapid decline of viability and the selective establishment of nonsmooth mutants after maximum populations are obtained.

Continuous culture of brucellae is possible and offers considerably increased efficiency as well as a physiologically uniform product (28*a*); however, these advantages are largely lost unless subsequent operations also are done continuously.

Since concentration of the cells imposes the greatest safety problem, the possibility of growing brucellae in concentrated culture has obvious merit. One approach to this objective was to cultivate the cells in a charcoal-cellophane system contained in a shaken flask (36). A thin-walled cellophane sac containing the nutrient medium was immersed in saline containing charcoal and the growing organisms. As an example of the results, populations of strain 19 of *B. abortus* rose to 1.4×10^{11} viable cells per ml, compared to an equal-volume control of 5.4×10^{10}, a concentration ratio of 2.6. Unfortunately, this comparison did not fully take into account aeration and toxicity differences, and the presence of charcoal with the cells may be impractical for some applications. Recently Sterne (94*a*) successfully used a cellophane tube apparatus for production of strain 19 vaccine, with yields up to 1×10^{12} viable cells per ml reported.

This principle of physically confining growth so as to obtain concentrated cell populations was further exploited by using a biphasic growth system, which consists simply of a layer of solid nutrient medium overlaid with a small volume of nutrient broth in a flask aerated by shaking (97). Such cultures of three *Brucella* species in a heat-sterilized casein-digest medium yielded 0.3 to 2.6×10^{11} viable cells per ml as compared to populations in control flasks of 0.3 to 1.6×10^{10}, with concentration ratios of 10 to 20. When a filter-sterilized medium was used, yields of strain 19, for example, rose to 1.2×10^{11} viable cells per ml in the biphasic system as compared to 2.2×10^{10} in the control flask, with a concentration ratio of 5.5 (Tyrrell and Gerhardt, *unpublished*). Adequate aeration of the broth and the depth rather than quantity of the nutrient agar were found to be important in determining the optimum ratio of liquid to solid phase. The relative efficiency of the system was found to vary with different media and organisms.

A remarkable concentration of *B. abortus* in the placental cotyledon of the bovine foetus has been reported by H. Smith, J. Keppie, and R. Fuller, in a personal communication; these workers have separated masses of *in vivo* grown

cells from this tissue for the purpose of chemical and antigenic studies.

In all the above cases, the eventual problem of separating the cells from the menstruum, a normally simple procedure, is here complicated by the infectious hazard associated with the most effective methods, *e.g.*, continuous centrifugation. Enclosure of centrifugal equipment in an autoclave, use of settling columns with a hydrophilic flocculating agent such as methyl cellulose or gelatin (39), or various types of filtration (52) may prove practical in special situations. Replicated batch sedimentation in some kind of a bucket centrifuge usually proves to be the most practical method in a laboratory and high speed, large-capacity centrifuges are available.

C. *Preservation of Viability*

Maintaining the viability of brucellae over periods of time becomes of concern in the use of living vaccines, stock cultures, dilute suspensions for counting, and concentrated suspensions for metabolic work. Several observations concerning these problems may be mentioned.

Freeze-drying has obvious merit and is widely used for preserving permanent stock cultures and vaccines. The latter purpose requires a higher viability retention during the freeze-drying process, when most of the killing occurs. A preliminary study of some physical factors that influence the survival of *B. abortus* during freeze-drying was reported by Hutton *et al.* (50). Van der Scheer (98) recently reviewed the pertinent literature. He also reported the experimental development of a suspension medium containing 2 per cent peptone, 0.75 per cent thiourea, and 0.5 per cent freshly prepared and neutralized ascorbic acid, which gave over 82 per cent survival of *B. abortus* strain 19. It has been pointed out (11) that lyophilization does not necessarily preclude the appearance of mutant types.

Brucellae also may be stored effectively and conveniently on slants under paraffin oil (37) or as suspensions in an equal mixture of horse serum and peptone broth (93), in either case frozen hard to minimize mutation. This serious and frequently incurred difficulty also may be lessened by growing the cultures before storage as briefly as feasible, certainly not beyond the logarithmic phase, and by transferring the cultures from selected and tested small single colonies (12).

It is now commonly recognized that distilled water, saline, buffered saline, or other similar diluents are toxic to dilute suspensions of brucellae, and some quantitative data on toxicity have been published (24, 107). Dilute peptone (24) or peptone-saline are generally preferred as diluents for counting. Among seven diluents tested in unpublished work by the writer, phosphate-saline (pH 6.8, M/15 phosphate, 0.85 per cent NaCl) supported a relatively constant endogenous and exogenous (glucose, glutamate, or asparagine) respiration of heavy suspensions of strain 19 of *B. abortus* for the period of at least 1 week.

D. *Identification and Classification*

Nutritional studies have found considerable application in the identification and classification of brucellae. These methods have been appraised in Spink's recent monograph (93).

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