SOME BIOCHEMICAL CHARACTERISTICS OF *VIBRIO FETUS* AND OTHER RELATED VIBRIOS ISOLATED FROM ANIMALS[1]

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Microaerophilic, motile, gram-negative, comma-like bacteria have been found associated with the genital organs of the ovine and bovine species. These curved rods are believed to be an important cause of sterility in cows and sheep. Smith and Taylor (1919) proposed the name *Vibrio fetus* for these microorganisms. Recently, Bryner and Frank (1955) and Reich *et al.* (1956) found, using catalase and H_2S activity as criteria, that organisms of the species *V. fetus* could be separated into two groups. The first group was catalase-positive and H_2S-negative. This group was believed to be the true *V. fetus* and a pathogen. The second group contained the organisms that were catalase-negative and H_2S-positive. They were thought of as saprophytes or nonpathogens belonging to a yet unnamed species.

Similar vibrios have been isolated from cattle and calves with a diarrhea called winter scours and from pigs (Doyle, 1944) with a diarrhea called bloody dysentery. The vibrio of winter scours was named *Vibrio jejuni* by Jones *et al.* (1931).

The organisms enumerated above are not only morphologically similar, but in addition, they are similar in that they lack fermentative powers (as measured by acid and gas production) (Plastridge, 1955). Since characteristics that are contingent on the ability of growing cultures to ferment substrates to acid and gas are lacking, other biochemical characteristics that might aid in classifying these vibrios were investigated. The approach taken was to study under standard conditions the oxidative capacity of resting vibrio cells (originating from different hosts and anatomical locations) towards a variety of substrates. Standardization of a metabolic testing system and results obtained from the use of this system is the subject of this communication.

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MATERIALS AND METHODS

Stock cultures and inocula. Stock cultures were maintained at room temperature in thiol broth with 0.3 per cent added agar. They were transferred at four-week intervals. Inocula were prepared by seeding heart infusion broth (pH 7.0) with organisms from the stock cultures and incubating for 24 to 48 hr. These heart infusion cultures served as a source for either inoculating other tubes of heart infusion, or inoculating media for the growth of cell crops. Care was taken to use as inocula cultures no older than 48 hr.

A total of 27 strains of vibrios from a variety of sources were employed in this study. Fourteen of these strains, from Maryland, Florida, Wisconsin, and North Carolina, were considered *V. fetus* since they were catalase-positive and were isolated from either aborted fetuses, or from the genital tract of cows or bulls that were associated with infertility problems. Six similar catalase-positive strains of ovine origin, also designated *V. fetus*, originated from aborted ovine fetuses in Montana, Colorado, and Illinois. The remainder of the strains studied for oxidative action were 3 catalase-positive strains isolated from Maryland and Indiana pigs and 4 catalase-negative strains (thought to be nonpathogens) isolated from bovine genital tracts in Maryland and Wisconsin. The isolation dates for these 27 strains ranged from 1939 to 1956.

Effect of adding formate and glycine on growth. Sodium formate was added to heart infusion broth in order to determine whether the growth of vibrios could be stimulated by this substrate. Five catalase-positive strains served as testing organisms. The effect of 0.8 per cent glycine in heart infusion broth, pH 7.0, on the growth of vibrios was determined for 17 catalase-positive strains, 5 of which were of ovine origin and 12 of

bovine origin. Growth was roughly quantitated by the relative time required for the culture to yield detectable visible turbidity and the amount of turbidity observed at 40 hr, 2 days, 3 days, 4 days, and 5 days of incubation.

Preparation of resting cells. Bottles containing 160 ml of heart infusion broth (pH 7.0) were steamed immediately before use and when cool inoculated with 0.2 ml of 24 to 48-hr inoculum grown in heart infusion broth. Cultures were incubated at 37 C in a candle jar. Approximately 24 hr after the appearance of growth (usually about 48 hr after inoculation) the cells were harvested by centrifuging at 7000 rpm for 10 min. They were then resuspended and washed in M/15, pH 6.8, phosphate buffer at $\frac{1}{10}$ their original volume and centrifuged again at 7000 rpm for 10 min. The sedimented cells were then diluted in M/15, pH 6.8, phosphate buffer to equal an optical density of between 0.40 and 0.50 at a wave length of 450 mμ in a Bausch and Lomb spectronic 20 colorimeter (a dry weight of 480 to 622 μg/ml). These cells were free of detectable endogenous reductive activity and had the desired exogenous activity. An attempt was made to use cells harvested from solid media as a source of metabolically active resting cells. Such cells were harvested, after 40 hr of incubation in a candle jar at 37 C, from blood agar plates (trypticase soy agar + 5 per cent bovine blood), blood agar plates plus 0.5 per cent lactate, and heart infusion agar plates (pH 7.0). Cells from these 3 different solid media had only slight reductive activity in the presence of lactate and formate. Boiled cells from metabolically active, broth-grown resting cells added to resting cells harvested from solid media were not able to stimulate lactate and formate metabolic activity, and also boiled cells from blood plates added to active, broth-grown resting cells did not inhibit metabolic activity.

Substrates tested. The following 30 substrates were tested for their potential to serve as electron donors in the presence of resting vibrio cells: lactate, pyruvate, acetate, glutamate, malate, citrate, α-ketoglutarate, succinate, fumarate, formate, aspartate, glycerol, rhamnose, arabinose, fructose, lactose, sucrose, mannose, galactose, glucose, gluconate, xylose, ribose, maltose, ethyl alcohol, trehalose, sorbitol, mannitol, alanine, and glycine. The substrates were obtained from commercial sources, and acids (with the exception of potassium gluconate) were used as their sodium salts.

Reduction testing system. Unless the conditions of the experiment dictated otherwise, the metabolic reaction system consisted of 100 to 150 μmoles of substrate, 0.2 or 0.3 mg of triphenyl tetrazolium chloride, and 2 ml of washed resting cells prepared as above. The total volume of the reaction was 2.5 ml and was carried out in Thunberg tubes at 37 C. Nitrogen was substituted for atmospheric air in the reaction vessel by evacuating and flushing four times with commerical nitrogen. The reaction time for lactate and formate was 20 min; pyruvate, 1 hr; and 2 hr for the remaining substrates. At the conclusion of the reaction the degree of reduction was quantitated by extracting the reduced triphenyl tetrazolium chloride (formazan) with acetone. The contents of the Thunberg tubes were poured into a tube calibrated to 6 ml, and acetone was added to bring the volume up to this calibration. These tubes were then spun at 2000 rpm for 10 min to sediment the cellular debris. The optical density of the supernatant containing the clear, red, formazan solution was measured at 480 mμ (if necessary, the 6 ml acetone-formazan was further diluted with 66 per cent acetone). The optical density was related to μg formazan by referring to a standard curve prepared by reducing known quantities of triphenyl tetrazolium chloride with alkaline ascorbic acid.

EXPERIMENTAL PROCEDURES AND RESULTS

Standardization of a metabolic testing system. The first step in determining some of the biochemical properties of *V. fetus* and related vibrios involved rigidly standardizing one metabolic system at optimal conditions for one strain of

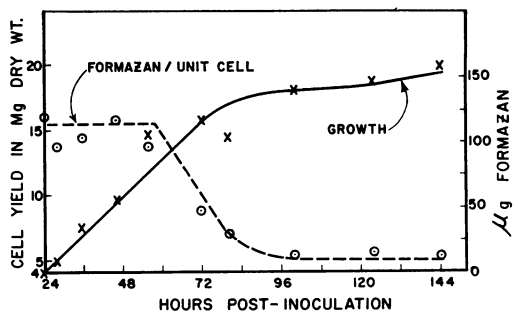

Figure 1. Optimal harvest time for yield of metabolically active, resting cells of *Vibrio fetus* strain D. Thunberg tubes contained: 1.24 mg of cells in buffer, pH 7.6, 0.2 mg of triphenyl tetrazolium chloride, and 100 μmoles of lactate. Total volume, 2.5 ml; reaction time, 1 hr.

vibrio. The reduction of triphenyl tetrazolium chloride by resting cells of a typical strain of *V. fetus* (strain D) in the presence of sodium lactate was chosen as the model system for standardization. The reaction was standardized with respect to the following optima: (1) harvest time, (2) pH of reductive reaction, (3) triphenyl tetrazolium chloride concentration, (4) cellular concentration, and (5) substrate concentration. Results leading to the adoption of standard conditions are summarized in figures 1 and 2. As can be seen in figure 1, good cellular yield and high lactate activity were obtained with cells harvested about 48 hr after inoculation (approximately 24 hr after first visible turbidity). From the data expressed in figure 2, it seemed that conditions were optimal for the reduction of triphenyl tetrazolium chloride by strain D when 100 to 150 μmoles of lactate (*B*), and 0.3 mg of triphenyl tetrazolium chloride (*D*) were added to 0.96 to 1.24 mg of resting cells (*C*) that had been washed and resuspended in phosphate buffer of pH 6.8 (*A*). These optima were then adopted as standard conditions for determining the activity of this strain with 29 other substrates. Twenty-six other strains of diverse origin were tested in a like manner.

Oxidative activity of V. fetus and other related vibrios towards 30 substrates. It was observed that, under the standard conditions described, only a few substrates out of some 30 were able to serve as a source of electrons for the reduction of triphenyl tetrazolium chloride by *V. fetus* and other vibrios isolated from animals (table 1). With the exception of lactate, formate, pyruvate, α-ketoglutarate, and succinate (glutamate was slightly active with 4 strains) the other substrates enumerated under Materials and Methods were negative after a reaction time of 2 hr.

It should be emphasized that the arithmetic means of the reductive activity as tabulated in table 1 reflect the magnitude and order of ind

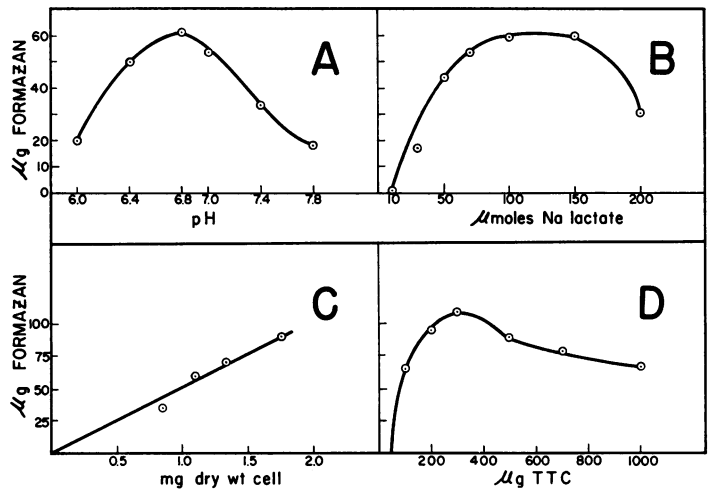

Figure 2. Optimal pH and concentration of triphenyl tetrazolium chloride, substrate, and cells for the reduction of triphenyl tetrazolium chloride by resting cells of *Vibrio fetus* strain D in the presence of lactate.

A. pH Optimum: Thunberg tubes contained 100 μmoles of lactate, 0.2 mg of triphenyl tetrazolium chloride, and 1.12 mg of cells washed and resuspended in M/15 phosphate buffer at various pH's. Total volume, 2.5 ml; reaction time 30 min.

B. Optimal substrate concentration: Thunberg tubes contained 10 to 200 μmoles of lactate, 0.3 mg of triphenyl tetrazolium chloride, and 1.24 mg of cells in buffer, pH 6.8. Total volume, 2.5 ml; reaction time, 20 min.

C. Optimal cell concentration: Thunberg tubes contained 100 μmoles of lactate, 0.3 mg of triphenyl tetrazolium chloride, and 0.86 to 1.74 mg of cells in buffer, pH 6.8. Total volume, 2.5 ml; reaction time, 20 min.

D. Optimal triphenyl tetrazolium chloride concentration: Thunberg tubes contained 100 μmoles of lactate, 0.1 to 1.0 mg of triphenyl tetrazolium chloride, and 1.25 mg of cells in buffer, pH 6.8. Total volume, 2.5 ml; reaction time, 45 min.

TABLE 1

Oxidative capacity of 27 strains of vibrios isolated from bovine, ovine, and porcine sources

Substrate*	Reaction Time	Total μg Formazan from 2.5 ml Reaction							
		14 Catalase + bovine strains		6 Catalase + ovine strains		3 Catalase + porcine strains		4 Catalase − bovine strains	
	min	*range*	*mean*	*range*	*mean*	*range*	*mean*	*range*	*mean*
Lactate	20	37–72	50	54–96	72	0–76	42	60–108	83
Formate	20	75–275	181	180–250	215	198–274	242	225–278	254
Pyruvate	60	22–104	60	39–96	79	18–132	80	86–145	110
α-Ketoglutarate	120	40–98	66	48–142	84	36–88	61	24–66	36
Succinate	120	8–36	15	8–26	16	12–22	15	6–12	9

* Acetate, glutamate, malate, citrate, fumarate, asparate, glycerol, rhamnose, arabinose, fructose, lactose, sucrose, mannose, galactose, glucose, gluconate, xylose, ribose, maltose, ethyl alcohol, trehalose, sorbitol, mannitol, alanine, and glycine were negative after a reaction time of 2 hr.

vidual results that comprise the mean; that is, with each strain at the end of a 20-min reaction, there was nearly always three times more formazan produced in the tubes containing formate than tubes with lactate. In addition, in order to reach a degree of reduction comparable to lactate, pyruvate required a reaction time of 60 min and α-ketoglutarate required 2 hr. The degree of reduction with succinate was markedly lower than the other four substrates even after a reaction time of 2 hr. These results indicated a remarkable similarity not only in the substrates that were active, but also in the degree of their activity.

It was further observed that the bovine catalase-positive strains did not appear to be as vigorous in their growth and their reductive capacity as the ovine catalase-positive and the bovine catalase-negative strains.

Effect of adding formate and glycine on growth. Since formate proved to be the most active electron donor, it was decided to determine if this substrate, when added to heart infusion broth (pH 7.0) could stimulate the growth of vibrios. Actually formate proved to inhibit completely the growth of vibrios at a concentration of 0.5 per cent and to delay their growth at a concentration of 0.2 per cent. Inhibition of growth of *Escherichia coli* by formate was noticed by Gale and Epps (1942).

In an unsuccessful attempt to antagonize formate inhibition with glycine, it was noticed in the controls containing glycine alone that, although the glycine was also inhibitory, it seemed to indicate a possible selective inhibition based on the animal from which the vibrio was isolated. To pursue this lead, the effect of 0.8 per cent glycine in heart infusion broth (pH 7.0) was determined for all the catalase positive vibrios in our collection at that time. It was determined that 5 out of the 5 sheep strains were not inhibited by glycine while 11 out of 12 bovine vibrios were (table 2).

TABLE 2

Effect of 0.8 per cent glycine in heart infusion broth (pH 7.0) on the growth of catalase positive vibrios of ovine and bovine origin

Strain Origin	No. Tested	No. Inhibited by 0.8 per cent Glycine	Per cent Inhibited
Ovine	5	0	0
Bovine	12	11	92

DISCUSSION

Motivation for this study stemmed from a desire to learn positive biochemical characteristics that might aid in further defining the species *V. fetus*. In that context, it should be noted that, under our standard conditions, 27 strains of vibrios of diverse origin and catalase activity, ranging in isolation dates from 1939 to 1956, were remarkably similar in their oxidative capacity towards a variety of substrates. Results compatible with these are to be found in the work of Alexander (1957) with 2 ovine strains of *V. fetus* and Kiggins and Plastridge (1958) with 2 bovine strains of *V. fetus*.

Up until 1955, motile, microaerophilic vibrios isolated from either aborted bovine and ovine fetuses, or bovine and ovine genital tracts were regarded as *V. fetus*. Since the report of Bryner and Frank (1955) and Reich *et al.* (1956), these vibrios have been divided into two categories:

(1) catalase-positive, H_2S-negative vibrios were considered pathogenic, true *V. fetus*, (2) catalase-negative, H_2S-positive vibrios were considered saprophytes belonging to a yet unnamed vibrio species. Recent evidence indicates that this is not a meaningful separation: (1) Akkermans *et al.* (1956) isolated what they considered pathogenic vibrios in aborted bovine fetuses. These bacteria were catalase-positive and H_2S-positive, (2) Bond's (1957) discovery that catalase activity correlated with colonial type indicates catalase-positive, H_2S-negative vibrios and catalase-negative, H_2S-positive vibrios are progeny derived from the same parent organism, and (3) data in this report point up similarities between the ovine and bovine catalase-positive, H_2S-negative genital vibrios, bovine catalase-negative, H_2S-positive genital vibrios, and porcine dysentery vibrios. It is proposed in the light of data cited above that catalase and H_2S activity in genital vibrios is too tenuous a characteristic to differentiate the species.

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SUMMARY

The reduction of triphenyl tetrazolium chloride in a Thunberg tube by resting cells of a typical strain of *Vibrio fetus* in the presence of sodium lactate was chosen as a model system that was standardized with respect to the following optima: harvest time, pH of reduction reaction, concentration of triphenyl tetrazolium chloride, cellular concentration and substrate concentration. Under these standard conditions other substrates were tested for their electron donating capacity. This yielded a pattern of oxidative activity for one strain. The pattern for this strain was compared to the pattern of 26 other strains examined in a like manner. It was determined that the oxidative capacity of vibrios isolated from farm animals was quite similar regardless of whether they were isolated from different hosts, different anatomical locations, or different H_2S-catalase types from the same host and same anatomical location. Only lactate, formate, pyruvate, α-ketoglutarate, and succinate out of 30 substrates tested served as electron donors.

The growth in heart infusion broth of 11 out of 12 bovine catalase positive strains of *V. fetus* was inhibited by the addition of 0.8 per cent glycine while none of 5 similar ovine strains was inhibited.

REFERENCES

ALEXANDER, J. K. 1957 Energy sources utilized by *Vibrio fetus*. J. Bacteriol., **74,** 168–170.

AKKERMANS, J. P., TERPSTRA, J. I., AND VAN WAVEREN, H. G. 1956 Over de betekenis van verschillende vibrionen voor de steriliteit van het rund. Tijdschr. Diergeneesk, **81,** 430–435. Eng. summary seen in: Vet. Bull., **26,** 421, 1956.

BOND, J. M. 1957 Characteristics of colonial forms of *Vibrio fetus*. Am. J. Vet. Research, **18,** 449–455.

BRYNER, J. H. AND FRANK, A. H. 1955 A preliminary report on the identification of *Vibrio fetus*. Am. J. Vet. Research, **16,** 76–78.

DOYLE, L. P. 1944 A vibrio associated with swine dysentery. Am. J. Vet. Research, **5,** 3–5.

GALE, E. F. AND EPPS, H. M. 1942 The effect of the pH of the medium during growth on the enzymatic activities of bacteria (*Escherichia coli* and *Micrococcus lysodeikticus*) and the biological significance of the changes produced. Biochem. J. (London), **36,** 600–618.

JONES, R. S., ORCUTT, M., AND LITTLE, R. B. 1931 Vibriosis (*Vibrio jejuni* n. sp.) associated with intestinal disorders of cows and calves. J. Exptl. Med., **53,** 853–863.

KIGGINS, E. M. AND PLASTRIDGE, W. N. 1958 Some metabolic activities of *Vibrio fetus* of bovine origin. J. Bacteriol., **75,** 205–208.

PLASTRIDGE, W. N. 1955 Vibriosis. Advances in Vet. Sci., **2,** 327–374.

REICH, C. V., MORSE, E. V., AND WILSON, J. B. 1956 Gaseous requirements for growth of *Vibrio fetus*. Am. J. Vet. Research, **17,** 140–143.

SMITH, T. AND TAYLOR, M. S. 1919 Some morphological and biological characteristics of the spirilla (*Vibrio fetus* n. sp.) associated with disease of the fetal membranes in cattle. J. Exptl. Med., **30,** 299–312.