FACTORS AFFECTING THE GROWTH OF PROPIONIBACTERIA[1]

MARVIN F. FIELD AND HERMAN C. LICHSTEIN

Department of Bacteriology and Immunology, University of Minnesota, Minneapolis, Minnesota

Received for publication July 26, 1956

Many species of the genera *Lactobacillus* and *Streptococcus* exhibit a prolonged delay in initiation of growth when inoculated into synthetic media which have been sterilized by filtration or autoclaved without fermentable carbohydrate. In contrast, growth is initiated with little or no lag when the complete medium is sterilized in the autoclave. The nature of this effect has been studied in species of lactobacilli and streptococci by Smiley *et al.* (1943), Snell *et al.* (1948), Orla-Jensen (1933), Neilsen and Eistrup (1940), Rabinowitz and Snell (1947), and Rogers *et al.* (1953).

The present paper reports studies on similar heat activation effects observed with species of propionibacteria.

MATERIALS AND METHODS

The following strains of propionibacteria were employed: *Propionibacterium pentosaceum* strain E 214, from the collection of Professor C. B. van Niel; *Propionibacterium freudenreichii* strain ATCC 6207; *Propionibacterium pentosaceum* strain ATCC 4875; and *Propionibacterium petersonii* strain ATCC 4870. Stock cultures were maintained in a complex medium consisting of 1 per cent each of yeast extract (Difco), casitone (Difco), and glucose, and 0.5 per cent K_2HPO_4 adjusted to pH 6.7–6.9. The growth experiments were carried out employing the synthetic medium of Delwiche (1950) at a pH of 6.7. The concentration of glucose or other carbohydrate was 1 per cent. The medium (7.5 ml) was introduced into Klett tubes and after sterilization and addition of inoculum, the tubes were fitted with sterile rubber stoppers to permit mixing of the contents prior to turbidity readings. The inoculum was prepared from a 48-hr culture grown at 30 C in the complex medium. The bacterial cells were harvested by centrifugation, washed three times in sterile distilled water, diluted 1:100 in the same menstruum, and added in 0.5-ml amounts to each tube. The experimental tubes were incubated at 30 C and growth determined at desired intervals in a Klett-Summerson photoelectric colorimeter at 640–670 mμ (red filter). The N-D-glucosylglycine employed in these studies was kindly supplied by Dr. V. H. Cheldelin.

[1] This work was supported in part by a grant from Eli Lilly and Company.

RESULTS AND DISCUSSION

Representative data demonstrating the marked enhancement of growth of each of the four strains of propionibacteria studied by autoclaving the glucose with the other constituents of the medium are given in table 1. It is to be noted that in some experiments the organism failed to proliferate at all for as long as 4 to 5 days when inoculated into the synthetic medium to which glucose was added aseptically after autoclaving. It should be emphasized that although the extent of growth enhancement, due to autoclaving the glucose with the medium, varied in different experiments with the same strain, the effect was always measurable and in most cases very extensive. The effect of inoculum size is shown by the results given for strain 6207. A 10-fold increase in number of cells inoculated resulted in growth in the aseptically added glucose medium, although the lag period was considerably longer than that found in the same medium when the sugar was added prior to autoclaving. It is also pertinent to note that if growth was initiated in the medium to which glucose was added after autoclaving, it followed the normal growth pattern. It appears, therefore, that this "glucose effect" can be simulated to some extent by increasing the size of inoculum and in some cases by prolonged incubation of small inocula, thus suggesting that some metabolic product of the organism is required in sufficient concentration before optimal growth and reproduction can proceed.

P. freudenreichii strain ATCC 6207 was chosen for further investigation of this growth enhancement effect. To determine the specificity of glucose other carbohydrates were tested. None of

TABLE 1

*Enhancement of growth of propionibacteria by autoclaving glucose with the other constituents of the growth medium**

Strain	Glucose Addition	Inoculum Dilution	Time of Incubation (Hr)					
			21	33	45	51	70	100
E-214	Aseptic	1:100	0†	0	2	6	30	290
	Autoclaved		41	224	420	455	480	480
4875	Aseptic	1:100	0	0	0	0	0	0
	Autoclaved		11	64	260	330	420	435
4870	Aseptic	1:100	0	0	0	2	10	320
	Autoclaved		9	65	274	305	345	350
6207	Aseptic	1:100	0	0	0	0	0	6
	Autoclaved		3	13	55	110	360	455
6207	Aseptic	1:10	7	18	140	320	410	—
	Autoclaved		20	56	198	335	415	—

* Autoclave time = 10 min.

† Klett-Summerson readings (640–670 mμ).

TABLE 2

*Replacement of glucose by other carbohydrates**

Carbohydrate Tested	Added Aseptically	Autoclaved† with Medium	Aseptic CHO + Aseptic Glucose	Autoclaved† CHO + Aseptic Glucose
Maltose	0‡	40	0	275
Lactose	0	0	0	260
Ribose	2	15	0	340
Sucrose	0	0	0	0
Mannitol	0	0	0	0
Glycerol	0	0	0	0
Fructose	0	305	0	315
Mannose	5	236	0	255
Glucose	0	310	—	—

* *Propionibacterium freudenreichii* strain ATCC 6207.

† Autoclave time = 10 min.

‡ Klett-Summerson readings (640–670 mμ) after 72 hr incubation.

the sugars examined supported growth of the organism when added aseptically to the medium, while fructose and mannose were as active as glucose when incorporated into the medium prior to heat sterilization (table 2). Combinations of each sugar with glucose, when added aseptically after autoclaving of the medium, were also inactive. Of considerable interest was the finding that maltose, lactose, and ribose, which were without effect on the growth of *P. freudenreichii* when added either prior to or after autoclaving of the medium, were highly active when autoclaved with the medium in the absence of glucose but with the addition of glucose after heat sterilization. The nature of this effect is obscure. These results demonstrate that glucose can be replaced by other reducing sugars such as fructose and mannose. There are numerous possibilities for reaction of the reducing portion of the carbohydrate with other constituents of the medium. For example, Maillard (1916) type reactions in which carbonyl groups react with amino groups must be considered as well as possible reactions with purines, pyrimidines, or vitamins.

Subsequent studies revealed that the growth enhancement effect obtained by autoclaving the medium with glucose or other reducing sugar could be duplicated by extended time of autoclaving in the absence of added carbohydrate (figure 1) or by autoclaving for 10 min at a more acid pH in the absence of added sugar (figure 2). In the latter case, the medium autoclaved at pH

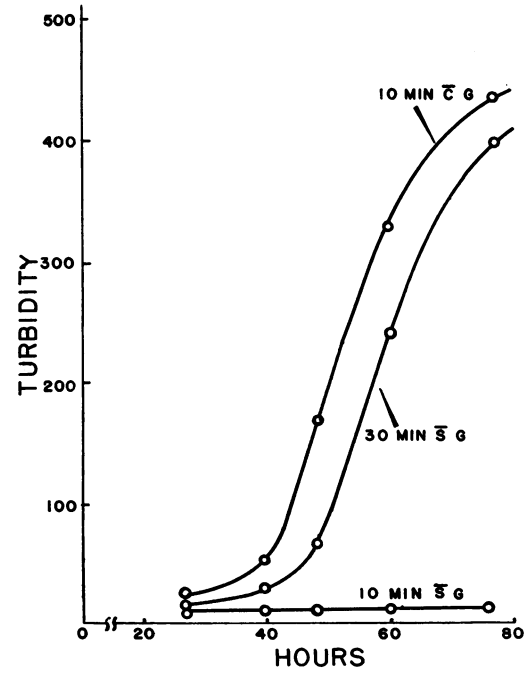

Figure 1. Effect of autoclaving time on growth response of *Propionibacterium freudenreichii*. c̄G = with glucose; s̄G = without glucose.

5 was adjusted to pH 6.7 after sterilization by the aseptic addition of sterile NaOH. With regard to time of autoclaving at pH 6.7 it should be noted that some inhibition of growth occurred if the glucose-containing medium was held at 121 C in excess of 15 min. It seems apparent, therefore, that the reaction(s) occurring under the usual conditions of sterilization in the presence of reducing sugar (i. e., pH 6.7, 121 C, 10 min) can be duplicated in the absence of added sugar, presumably by some other component of the medium, by holding the medium at 121 C for 30 min at pH 6.7 or for 10 min at pH 5.

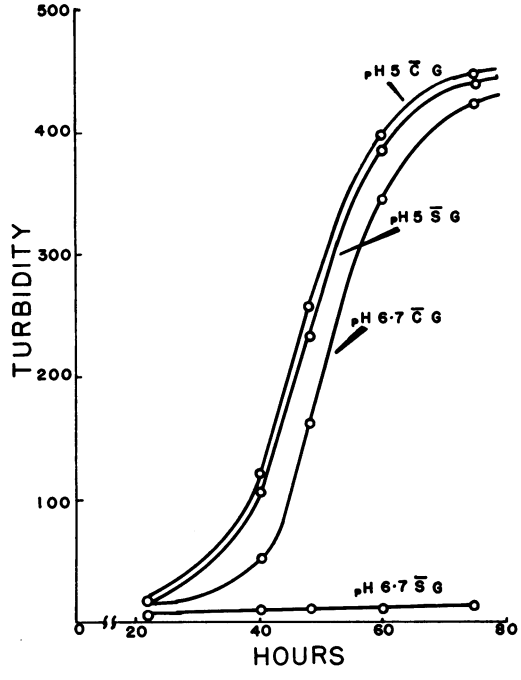

Figure 2. Effect of pH during autoclaving on growth response of *Propionibacterium freudenreichii*. Autoclave time = 10 min.

Attempts to replace the growth stimulating effect of media autoclaved with glucose at pH 6.7 for 10 min by the addition of known compounds to media autoclaved under the same conditions in the absence of added sugar gave the following results. Glyoxal, glyoxylic acid, and sodium pyruvate were inactive at a concentration of 1 mg per ml of medium. Smiley *et al.* (1943) and Snell *et al.* (1948) reported that pyruvate replaced the effect of autoclaved carbohydrate for *Streptococcus salivarius* and *Lactobacillus bulgaricus* respectively. Cysteine (50 μg per ml of medium) was inactive in contrast to the work of Rabinowitz and Snell (1947) with *Streptococcus faecalis*. Negative results with *P. freudenreichii* were also obtained by the addition of 0.5 μg per ml of medium of nicotinic acid, pyridoxal, folic acid, riboflavin, or vitamin B_{12}, 5 μg of inositol, or 50 μg of glutathione per ml of medium. The addition of a complex natural material such as yeast extract was found to be active but only at a concentration in excess of 1 mg per ml of medium.

Inasmuch as Rogers *et al.* (1953) have reported that N-D-glucosylglycine was able to replace the growth stimulating effect of autoclaved glucose

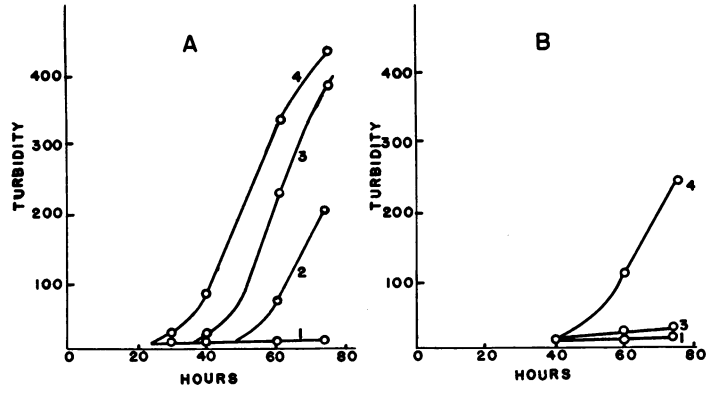

Figure 3. Effect of N-D-glucosylglycine on the growth of *Propionibacterium freudenreichii*. *A* = Delwiche medium; *B* = Delwiche medium modified by replacement of casein hydrolyzate with amino acids; curve 1 = glucose added aseptically after autoclaving; curve 2 = glucose added aseptically after autoclaving + 1 mg glucosylglycine per 10 ml medium; curve 3 = glucose added aseptically after autoclaving + 8 mg glucosylglycine per 10 ml medium; curve 4 = glucose autoclaved with medium.

on *Lactobacillus gayoni*, it was advisable to test this compound on *P. freudenreichii*. The results of such studies are presented in figure 3. It is apparent that glucosylglycine in concentrations of 1–8 mg per 10 ml of medium was capable of replacing to some extent the growth stimulating effect of glucose autoclaved with the medium. Maximal effect was noted with 8 mg of glucosylglycine, and even in the presence of this amount the lag period was decidedly longer than that obtained in the autoclaved glucose medium. However, glucosylglycine was inactive when added to media in which the hydrolyzed casein had been replaced by a mixture of 19 synthetic amino acids. Moreover, it is important to note that the rate of growth in the latter media even in the presence of autoclaved glucose was considerably slower than in the same medium containing hydrolyzed casein. It is probable that some factor(s) present in commercial preparations of vitamin-free hydrolyzed casein is required for optimal growth and for utilization of N-D-glucosylglycine.

Although the data presented do not reveal the nature of the growth enhancement caused by autoclaving glucose with the other medium constituents, they do make known some of the factors of importance in this phenomenon. Investigations are in progress in this laboratory attempting to ascertain the factor(s) required for initiation of growth of propionibacteria from small inocula and to correlate such studies with the growth stimulating effect obtained by autoclaving reducing sugars with the medium.

SUMMARY

Growth of four strains of propionibacteria has been shown to be delayed or not initiated at all when small inocula are introduced into media where the glucose is added aseptically after autoclaving of the other medium constituents. Prompt growth results when the carbohydrate is autoclaved with the medium. Other reducing sugars such as fructose and mannose are as effective as glucose.

The growth stimulating effect obtained by autoclaving reducing sugars with the medium can be duplicated by autoclaving sugar free media at a more acid pH (5) for short periods of time (10 min) or at the usual pH (6.7) for longer periods (30 min).

N-D-Glucosylglycine replaces partially, but only under certain conditions, the growth enhancement produced by autoclaving glucose with the medium.

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