Comparison of Physiological and Biochemical Characters of *Actinomyces* spp. with those of *Lactobacillus bifidus*

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SUMMARY: Evidence is presented that under the conditions described 11 strains of *Actinomyces* spp., representing strains described as *A. israelii*, *A. israelii*-like or *A. naeslundii*, require carbon dioxide for anaerobic growth. Some of these strains, under these conditions, are obligate anaerobes to microaerophils, while others appear to be facultative anaerobes. Cultures which are capable of aerobic growth may or may not require carbon dioxide for such growth. Of three strains of *Lactobacillus bifidus* tested, all required carbon dioxide for anaerobic growth. One avian strain required carbon dioxide to give limited aerobic growth; the remaining strains did not grow significantly under aerobic conditions. Comparisons of several strains of *Actinomyces* spp. with *L. bifidus* indicated that of eleven sugars tested, the sugar of choice for growth of *Actinomyces* spp. was glucose or maltose, whereas lactose or maltose was preferred by strains of *L. bifidus*. All strains of each group of organisms were found to be catalase-negative; none liquefied gelatin; all eleven strains of *Actinomyces* spp. reduced nitrate to nitrite, but none of the bifid strains possessed this ability; production of acetylmethylcarbinol was variable in both groups. All strains of *Actinomyces* spp. tested formed L (+) lactic acid, although the results suggested that small amounts of D (-) lactic acid were also formed. Fermentation analyses indicated that strains of *L. bifidus* and *Actinomyces* spp. form the same products from glucose and carbon dioxide (lactic, acetic, formic and succinic acids). However, strains of *Actinomyces* spp. form predominantly lactic acid with small amounts of acetic, formic and succinic acids; whereas the strains of *L. bifidus* form approximately equal amounts of lactic and acetic acids (based on glucose fermented) with trace amounts of succinic and formic acids. *Actinomyces* strains fermented but 34-59 % of the glucose supplied as compared to the strains of *L. bifidus* which used from 59 to 89 % of the glucose (1 % glucose medium).

The relationship of *Lactobacillus bifidus* to the genus *Actinomyces* was apparently first noted by Vuillemin (1931). Puntoni (1937) noted certain morphological, biochemical and immunological resemblances between strains of *Actinomyces* spp. and *L. bifidus*. He concluded that the organisms were sufficiently similar to be placed in the same genus and species, although he felt a species differentiation might be made. Negroni & Fischer (1944), from a study of *L. bifidus*, concluded that this organism should be placed in the genus *Cohnistreptothrix* (*Actinomyces*). Frank & Skinner (1954), on primarily morphological grounds, suggested that *L. bifidus* be placed in the genus *Actinomyces* as *A. bifidus*. However, each of these studies was somewhat limited in extent. In addition,

any conclusions regarding the relationship of these organisms to one another have been limited by the lack of information on certain biochemical properties of organisms of the genus *Actinomyces*. Consequently, it seemed worth while to make comparisons of these two groups of organisms on the basis of: (1) their ability to grow aerobically or anaerobically, with and without added carbon dioxide; (2) their ability to utilize various sugars; (3) their fermentation products from glucose; (4) certain physiological properties. The results of these experiments are presented here.

METHODS

Strains of organisms

Twelve strains of *Actinomyces* spp. and four of *Lactobacillus bifidus* were studied. The strains of *Actinomyces* spp. were those described previously (Howell & Pine, 1956), except that the bovine isolates, strains 278 and 284, were not included in the present study. Cultures 306 and 307 were avian strains of *L. bifidus* obtained through the courtesy of Mr Morrison Rogosa, National Institute of Dental Research. Strains 308 and 309 were human isolates of *L. bifidus* described as 'Timberlaine' and 'Jackson' strains, respectively, by Norris, Flanders, Tomarelli & György (1950), and were obtained from Dr Paul György, Department of Pediatrics, University of Pennsylvania Hospital. Cultures of *Actinomyces* spp. were maintained as described previously (Howell & Pine, 1956); those of *L. bifidus* were maintained on the acetate medium of Norris *et al.* (1950) modified by the omission of pancreatin and sorbitan mono-oleate as recommended by Norris (personal communication).

General methods

The procedures for the growth and preparation of inoculum for all cultures and measurements of growth were those described earlier (Howell & Pine, 1956). To study growth anaerobically in the absence of carbon dioxide, a pyrogallol + KOH (10 %, w/v) seal was used; to measure growth aerobically in the presence of increased carbon dioxide, a 10 % (w/v) $\text{Na}_2\text{CO}_3 + \text{m-KH}_2\text{PO}_4$ seal was used. In either case five drops of each reagent were used per growth tube. For studies on growth in air, the cotton plug and rubber stopper were replaced by an aluminium cap. In the experiments in which oxygen tolerance or carbon dioxide requirements were studied, all cultures were incubated at 37° on a rotary shaker as described previously (Howell & Pine, 1956). With the conditions of shaking under which such cultures were incubated in the presence or absence of added carbon dioxide, methylene blue (2 drops of a 10^{-4} dilution/5 ml. medium) was not reduced. In those cultures in which growth was obtained in the presence of oxygen, serial transfers were made when the optical density reached 0.500 or greater. For other studies anaerobic cultures were incubated stagnant inasmuch as shaking did not appear significantly to affect growth of actinomycetes (Howell & Pine, 1956).

Unless stated otherwise, the medium used for all comparative growth experiments was the liquid defined medium with starch and Casitone described

by Howell & Pine (1956) containing 0.5 % (w/v) sugar. For fermentation analyses the same medium with 0.5 or 1.0 % (w/v) glucose and either casein hydrolysate, Casitone, or Casitone supplemented with 0.5 % (w/v) yeast extract was used. The inoculum was grown anaerobically in the 0.5 % (w/v) glucose Casitone medium.

Cultural methods for fermentation analyses

As reported previously, several of the *Actinomyces* strains show poor growth at pH 7.0 in the casein hydrolysate medium, although excellent growth is obtained at pH 6.5. Consequently, in cases where accurate carbon dioxide

Fig. 1. Diagram of fermentation tube used for carbon dioxide analyses.

determinations were to be made a system was necessary which would allow the addition of gaseous carbon dioxide at pH 6.5 and analyses of residual carbon dioxide without loss to, or contamination from, the air. Initially, 100 ml. volumetric flasks filled to the mark were sealed with sterile vaccine bottle stoppers. By the use of hypodermic needles pushed through the stoppers, the air phase was replaced with a 95 % N_2 + 5 % CO_2 (v/v) mixture. When growth had apparently ceased, carbon dioxide-free NaOH was injected through the stopper until the medium was alkaline, and a sample was then withdrawn for analysis. As will be described later, such flasks, although usable, were not entirely satisfactory. Good growth was more consistently obtained with the type of tube shown in Fig. 1, containing 25–30 ml. of medium. In this case, after the medium was inoculated, the cotton stopper, used in autoclaving before

the introduction of the medium, was pushed into the tube to block the side arm orifice and the tube was sealed with a no. 4 rubber stopper, *A*. A no. 20 hypodermic needle attached by a rubber hose to a vacuum pump was then pushed through the vaccine stopper, *B*, and the system evacuated for 1–2 min. The needle was withdrawn and 3 ml. of a pyrogallol + H_2SO_4 solution (30 g. pyrogallol dissolved in and made to 100 ml. with $\text{N-H}_2\text{SO}_4$) were introduced with a syringe into the side bulb through *B*. Next 4 ml. of 10 % Na_2CO_3 (w/v) solution were injected slowly into the bulb. The cotton stopper prevents contamination by vapour formed by the release of carbon dioxide. At the end of the growth period sufficient carbon dioxide-free NaOH to make the medium pH 9 or greater was injected directly into the medium through the stopper at *C*. The tube was incubated overnight or longer at 5° to absorb the carbon dioxide into the medium. One ml. samples were then withdrawn with an Ostwald-Van Slyke pipette with stopcock and rubber tip for determination of the final carbon dioxide content of the medium. In this type of tube the granules of *Actinomyces* strains rapidly settled to the bottom and growth was localized there. Therefore a magnet, *D*, was used in conjunction with a magnetic stirrer to mix the culture sediment throughout the medium several times each day. Uninoculated controls were used to determine the initial carbon dioxide concentration. In one experiment four replicate determinations of the initial carbon dioxide present in uninoculated controls gave values of 25.62, 24.25, 26.71, and 24.73 $\mu\text{mole/ml}$.

In some cases, where carbon dioxide was not determined, cultures were grown in 25 × 200 mm. test tubes containing 25 or 30 ml. of medium with a pyrogallol + Na_2CO_3 seal.

Chemical methods

Nitrate reduction was tested on horse meat infusion broth containing 1 % trypticase, 0.5 % NaCl and 0.1 % KNO_3 . Qualitative nitrite determinations were made daily for 7 days (*Manual of Methods for Pure Culture Study of Bacteria*, 1946); residual nitrate was determined after 7 days incubation. Gelatin liquefaction was tested after 10 days incubation on the horse meat infusion broth containing 10 % (w/v) gelatin. Cultures on litmus milk were incubated for 10 days. Catalase was determined on colonies of actinomycetes grown for 7 days on brain heart infusion agar plates by adding 1–2 drops of a freshly prepared 10 % (v/v) solution of Superoxol (Merck and Co., Reagent, hydrogen peroxide 30 %) and observing gas formation. Cells of *Candida albicans* served as a positive control. All cultures for determination of nitrate reduction, gelatin liquefaction, action on litmus milk and catalase reaction were incubated anaerobically in Brewer jars with 95 % N_2 + 5 % CO_2 at 37°.

Carbon dioxide was measured by the method of Peters & Van Slyke (1932) or with the Warburg apparatus. Initial and residual glucose were determined by the method given by Umbreit, Burris & Stauffer (1949). Ethanol was identified qualitatively by diffusion into dichromate (Winnick, 1942) and the acid formed by its Duclaux value. Quantitative determinations of acetoin were made by the procedure of Westerfeld (1945). Quantitative estimation of

glycerol in several fermentations by the procedure of Smith (1950) showed no significant formation of this compound. Tests for organic compounds not mentioned above were made by the dichromate oxidation procedure of Johnson (1949). Formic and acetic acids were identified qualitatively as a mixture by their Duclaux values and were then identified by paper chromatography and R_f values by the procedure of Kennedy & Barker (1951). Formate was also shown to be present at the correct R_f by spraying with 5 % (w/v) ammoniacal AgNO_3 . Formic and acetic acids formed by strains of *Actinomyces* spp. were then determined quantitatively by the procedure of Friedemann (1938) as modified by Rabinowitz & Barker (1956). Formic acid formed by strains of *Lactobacillus bifidus* in the acetate medium (Norris *et al.* 1950) containing glucose or lactose, was identified and estimated in acid steam distillate by the formic acid hydrogenlyase method of Gest (1952). Four ml. Warburg vessels were used. It was estimated in other media by the procedure of Rabinowitz & Barker (1956).

Succinic acid was identified as a product of glucose fermentation by actinomycetes by fermenting glucose- ^{14}C with strains 279 and 295. Isolation of an unknown radioactive non-volatile acid was accomplished by paper chromatography (Block, Durrum & Zweig, 1955) and subsequent radioautographs. Its identity was indicated by a dilution and spreading of the radioactive spot upon the addition of unlabelled succinic acid to paper chromatograms. Ethanol + ammonia (Kennedy & Barker, 1951) and methanol + pyridine (Redfield, 1953) were used as solvents. The unknown compound was separated from lactic acid by the procedure of Phares, Mosbach, Denison & Carson (1952). Titration values and radioactivity determinations on the effluents of a Celite column showed a direct correspondence of the peak with known succinic acid. Separation from lactic acid was also accomplished by eluting the radioactive strip from paper chromatograms. The separated succinic acid fractions were assayed by the succinoxidase procedure of Umbreit *et al.* (1949) using 5 ml. Warburg vessels. Subsequently, fermentation analyses for succinic acid were made directly on the non-volatile ether-extractible acid fraction. Lactic acid did not interfere with the determination.

Lactic acid was isolated and identified as the zinc salt according to the procedure of Pederson, Peterson & Fred (1926). It was determined quantitatively by the method of Barker & Summerson (1941). The oxidation-reduction indices (redox values) were calculated according to the method of Neish (1950).

RESULTS

Comparison of media for growth of Lactobacillus bifidus

Preliminary experiments indicated that the rates of growth of each of the four strains of *Lactobacillus bifidus* in the liquid medium with starch (Casitone and 0.5 % lactose; Howell & Pine, 1956) were essentially comparable to those obtained in the acetate lactose medium of Norris *et al.* (1950) (Fig. 2). Furthermore, although the amount of growth obtained with the human bifid strains was less than that obtained on the lactose medium of Norris *et al.*

(1950), the amount of growth of the avian strains was significantly greater on the lactose Casitone starch medium than on the lactose medium. Gram stains of bifid cultures 308 and 309 on the former medium showed more 'straight rod' forms than were usually obtained on the medium. However, subcultures on to the lactose agar of Norris *et al.* (1950) from cultures of strains 308 and 309 on the lactose Casitone starch medium, made after maximum growth was obtained, yielded only the typical 'bifid type' colonies described by Norris *et al.* (1950). Gram stained films of these colonies showed consistently and almost exclusively bifid morphology. Similar results were obtained when glucose was substituted for lactose in the Casitone medium, with the exception that strain 306 failed to grow on Casitone glucose. Since the casitone starch medium appeared to be a satisfactory medium for the growth of all four strains of *L. bifidus*, did not induce 'mutations' in these cultures, and had previously been shown to support excellent growth of all strains of *Actinomyces* spp., it was chosen as the basic medium for the comparative study of cultures of *L. bifidus* and *Actinomyces*.

Oxygen tolerance and carbon dioxide requirements of strains of Actinomyces spp. and Lactobacillus bifidus

From Table 1, it is seen that all cultures of *Actinomyces* spp. and *Lactobacillus bifidus* required carbon dioxide for anaerobic growth in the liquid glucose Casitone starch medium. Four of the six *Actinomyces* capable of aerobic growth under these conditions and the single strain of *L. bifidus* capable of limited aerobic growth required carbon dioxide for such growth. Subsequent experiments, however, indicated that growth of strain 296 in air without carbon dioxide, and of strain 282 in air+carbon dioxide was not a constant finding.

Of the *Actinomyces* capable of aerobic growth on the first transfer, five appeared capable of continuous transfer in air of increased carbon dioxide tension. These cultures gave as much or more (in several instances twice as much) growth aerobically as they did anaerobically with carbon dioxide (Table 1). In those cultures grown aerobically or in air plus carbon dioxide on which a final pH value was determined, the terminal pH value was found to be approximately 5.0–6.1 as compared to about 4.5 usually obtained in cultures grown anaerobically. Strains 279, 282 and 296 showed no

Fig. 2. Rate of growth of human (308) and avian (307) strains of *Lactobacillus bifidus* in the lactose medium of Norris *et al.* (1950) and on the lactose-Casitone medium. Strain 307: ▲, △; strain 308: ●, ○; ▲, ● = 1.75% lactose, medium; △, ○ = 0.5% lactose, Casitone medium.

decrease in rate or amount of anaerobic growth after four to six consecutive aerobic transfers (with added carbon dioxide). These cultures, particularly those which grew aerobically, were examined by Gram reaction and by wet mounts made at the period of maximum growth. In no instance in the reported experiments was there observed any indication of contamination or gross change in morphology. In other experiments (Howell, unpublished), colonies of strains 279 and 296 developed on plates of this medium containing 1.5 % (w/v) agar in air + 5 % (v/v) carbon dioxide. These colonies, though

Table 1. *Amount of growth of strains of Actinomyces spp. and Lactobacillus bifidus obtained anaerobically and aerobically in the presence and absence of added carbon dioxide*

	Anaerobic		Aerobic*	
	- CO ₂	+ CO ₂	- CO ₂	+ CO ₂
	Maximal growth units obtained			
<i>Actinomyces</i> spp.				
261	None	2.90	0.10 (1)	2.68 (1)
262	None	1.83	0.14 (1)	0.12 (1)
263	None	2.25	0.10 (1)	3.23 (5)
279	None	3.00	3.60 (5)	5.60 (5)
281	None	2.32	0.11 (1)	0.47 (1)
282	None	2.75	0.07 (1)	4.40 (4)
283	None	1.60	0.02 (1)	0.06 (1)
286	None	3.50	0.13 (1)	6.20 (6)
287	None	0.80	0.13 (1)	0.13 (1)
295	None	2.63	0.20 (1)	0.52 (1)
296	None	2.35	0.71 (2)	4.48 (5)
<i>L. bifidus</i>				
307	None	1.40	0.00	0.70 (1)
308	None	3.11	0.00	0.12 (1)
309	None	3.17	0.00	0.00

* The numbers in parentheses indicate the number of consecutive transfers in which growth was obtained and the maximum growth (expressed as growth units) obtained on the first transfer.

fewer in number than on similar plates incubated in an atmosphere of 5 % CO₂ + 95 % N₂ (v/v) were identical in colony morphology with those developed on the plates incubated anaerobically. Similar studies (Pine, unpublished) with strain 296 using large agar slants and both the potassium phosphate + sodium carbonate, or pyrogallol + sodium carbonate seals described above, gave identical colonies and cell morphology. From these results, and those presented in Table 1, it would appear that under these conditions some strains of the genus *Actinomyces* are facultative anaerobes, whereas others are obligate anaerobes or microaerophils.

A single strain of *Lactobacillus bifidus* (307) showed some aerobic growth in the presence of added carbon dioxide, but the amount was but half that obtained anaerobically with carbon dioxide and did not continue on the second transfer (Table 1). The other two lactobacillus cultures showed no aerobic growth. These cultures would appear to be anaerobes or microaerophils.

Cultures of *Lactobacillus bifidus* were also tested for an anaerobic carbon dioxide requirement on the same medium with 0.5 % (w/v) lactose. Of the four strains tested, only the avian strains (306 and 307) grew anaerobically on lactose without added carbon dioxide. However, only half the amount of growth was obtained under these conditions as was obtained on this medium with added carbon dioxide. It would appear, therefore, that with *L. bifidus* the medium used may affect the requirement for carbon dioxide.

Growth of Lactobacillus bifidus and Actinomyces spp. on sugars

Rose, Luchi, Avery, Norris & György (1953) reported that human strains of *Lactobacillus bifidus* utilized the disaccharides lactose and maltose more readily than glucose and implied that this finding might be of value in differentiating *L. bifidus* from its straight rod variants and *L. acidophilus*. Comparisons were

Fig. 3. Rate of growth of strains of *Lactobacillus bifidus* and *Actinomyces* spp. on glucose and lactose. A: *L. bifidus*; B, C: *Actinomyces* spp.; ●, ▲ = glucose; ○, △ = lactose.

made, therefore, of the relative utilization of these three sugars by strains of *L. bifidus* and the actinomycetes to determine whether or not these two groups of organisms behaved similarly under these conditions.

Under the conditions of this experiment, it was found that the rates and amounts of growth of all lactobacilli tested were, in general, similar on lactose and glucose except for strain 306 which did not grow on glucose (Fig. 3A). In some instances the rates of growth were slightly higher on lactose but these results were not consistent. However, growth on glucose was never significantly greater than on lactose. In a single experiment, rates

of growth of strains 307, 308 and 309 were slightly higher on maltose than on glucose but the difference was not regarded as significant. Strain 306 was not tested on maltose.

On the other hand, most cultures of the *Actinomyces* were more 'active' on glucose than on lactose. One group of strains (277, 279, 286 and 287) grew more slowly and erratically with lactose as a substrate than with glucose and the final amounts of growth were much greater with glucose (Fig. 3B). A second group (strains 295 and 296) showed equal rates of growth on the two sugars but the amount of growth on glucose was three to four times that obtained on lactose (Fig. 3C). Other strains (261, 263, 282 and 283) showed equal rates and amounts of growth on the two sugars (Fig. 3C). In no instance, however, was growth on lactose considered to be superior to that on glucose. Results with maltose were essentially identical with those on glucose with each of the ten strains tested.

The relative ability of these two groups of organisms (except strain 306) to utilize other carbohydrates and pyruvate and gluconate as carbon sources was then tested. Carbohydrates employed were arabinose, mannitol, mannose, raffinose, rhamnose, ribose, salicin and sucrose. In general, no single one of these substrates showed a clear differentiation of lactobacilli as a group from the actinomycetes as a group (Table 2). Little or no growth of either group was obtained on the basal medium alone.

In addition to the results reported above, the actinomycetes and *Lactobacillus bifidus* were tested for the presence or absence of certain other biological properties. All strains of each group of organisms were found to be catalase-negative; none liquefied gelatin; all eleven strains of *Actinomyces* spp. were found to reduce nitrates to nitrites, but none of the lactobacilli strains possessed this ability; production of acetylmethylcarbinol was variable in both groups, apparently being dependent on the medium; and finally, both groups showed similar activity on litmus milk in that, in general, they produced acid and showed some reduction, both properties varying in extent, dependent on the strain.

Studies on the fermentation of glucose by Actinomyces and Lactobacillus bifidus

The results of analyses of fermentations of seven *Actinomyces* strains on 1% (w/v) glucose in the basal medium prepared with casein hydrolysate are given in Table 3. In this medium and under the conditions employed, c. 90–100% of the carbon of the glucose fermented by these strains was determined as lactic acid. All cultures, however, produced small amounts of formic and acetic acids (3.5–11.8% of the glucose fermented). A compound tentatively identified as ethanol accounted for a maximum of 2% of the glucose fermented. Although acetylmethylcarbinol was found in trace amounts in other media, none was formed in the fermentations presented in Table 3. Starch, which is present in the medium at a concentration of 0.05% (w/v), is known to support a small amount of growth of at least strains 277, 279, 286 and 287; its presence is accounted for in the calculations of percentage

Table 2. Maximum amount of growth* obtained on specified substrates with strains of *Lactobacillus bifidus* incubated 10 days and *Actinomyces* spp. incubated for 7 days

Strain	Substrate													None
	Glucose	Lactose	Maltose	Sucrose	Mannitol	Rhamnose	Arabinose	Raffinose	Salicin	Ribose	Mannose	Gluconate	Pyruvate	
<i>Actinomyces</i> spp.														
261	2.44	2.70	1.82	1.06	2.25	0.24	0.99	2.92	1.18	2.40	1.95	0.79	0.70	0.20
263	3.20	2.12	1.85	—	0.14	0.12	0.19	—	—	—	—	0.20	0.11	0.21
277	2.41	0.59	2.37	0.82	1.90	0.32	0.62	—	—	—	—	0.36	0.56	0.33
279	3.60	0.42	3.28	—	0.14	0.26	0.23	2.22	1.62	—	0.95	0.25	0.93	0.26
281	—	—	—	—	—	0.12	0.40	0.53	0.66	0.60	0.97	0.20	—	—
282	3.00	2.80	4.10	—	1.34	0.50	0.40	1.35	1.29	1.88	1.09	0.15	1.13	0.38
283	2.64	2.32	3.77	3.33	2.23	0.52	0.19	2.72	2.10	1.51	1.09	0.12	0.70	0.17
286	2.95	0.28	3.04	3.78	0.20	0.19	0.14	2.02	0.80	0.08	0.88	0.09	0.23	0.07
287	2.32	1.47	2.17	0.78	0.68	0.18	0.29	0.60	0.73	0.78	0.53	0.21	0.50	0.13
295	3.78	0.90	2.57	2.88	2.30	0.36	0.61	0.76	0.97	0.76	0.43	0.13	0.48	0.21
296	3.00	0.98	1.14	—	1.19	0.43	1.75	0.66	0.99	2.12	0.76	0.16	0.67	0.29
<i>L. bifidus</i>														
307	1.04	0.64	0.50	0.46	0.03	0.06	0.84	1.75	2.08	0.59	0.62	0.04	—	0.02
308	3.03	3.15	2.55	2.95	0.08	0.09	0.16	3.20	0.05	0.69	0.56	0.12	0.14	0.13
309	3.72	3.00	2.85	2.77	0.04	0.06	0.07	2.52	0.06	1.19	0.45	0.05	0.07	0.03

* Readings taken daily and expressed as growth units (optical density $\times 1$ /dil.).

carbon recovered. In general carbon recoveries in this medium were high. The values obtained for carbon dioxide are questionable since the determinations employed the Warburg apparatus and were susceptible to contamination with carbon dioxide from the air. No fermentation analysis gave a satisfactory redox balance. Because of the small concentration of reduced or oxidized products, this was not considered important.

Table 3. *Analysis of glucose (1.0 %, w/v) fermentations by strains of Actinomyces spp. in basal medium with casein hydrolysate in 100 ml. volumetric flasks filled to the mark, and flushed 1-2 min. with 95 % N₂-5 % CO₂ to remove air*

	Strain number						
	277	279	281	282	286	287	295
	$\mu\text{mole/ml.}$						
Glucose fermented	32.4	27.3	20.6	29.1	30.1	23.5	19.0
Products							
Carbon dioxide	0.2	0.6	0.0	0.3	0.5	0.0	0.4
Formic acid	3.2	3.6	4.2	3.9	2.4	4.7	4.5
Acetic acid	1.7	1.7	2.1	1.7	0.7	2.7	3.8
Lactic acid	64.4	53.2	41.0	57.1	58.5	42.3	38.1
Ethanol	—	1.2	—	—	1.5	1.8	0.7
Percentage carbon recovered*	101.5	98.8	103.6	99.7	98.6	96.4	108.3
Redox value	—	2.00	—	—	1.13	1.30	3.78
Percentage glucose fermented	57.9	49.1	37.0	52.4	54.2	42.4	34.2

* Values of percentage carbon recovered have been corrected for the presence of 0.05 % (w/v) starch assuming utilization of the starch and weight to weight equivalence of starch to glucose.

In an attempt to determine whether or not the composition of the medium would significantly affect the products of glucose fermentations, three strains of *Actinomyces* were grown in the casein hydrolysate medium (1 %, w/v, glucose) and in the Casitone medium supplemented with 0.5 % (w/v) yeast extract, with and without 0.5 % (w/v) glucose. The results obtained are given in Table 4. A small amount of growth and fermentation occurred in the Casitone yeast-extract medium without glucose, but this growth was accounted for by the disappearance of reducing substances from the medium. Growth of these strains in the two sugar-containing media showed a wide diversity in the amount of lactic acid formed, the ratio of lactic acid to volatile acid formed, and in the ratio of formic to acetic acid formed. The amount of lactic acid formed in the casein hydrolysate medium was from 80 to 98 % of the glucose fermented. In the Casitone yeast-extract medium, lactic acid accounted for 63-82 % of the glucose fermented. Furthermore, carbon recoveries in the latter medium were significantly lower for two strains than that obtained in the casein hydrolysate medium.

In other experiments, carbon recoveries in the Casitone medium were generally lower than those obtained using casein hydrolysate. For example, analysis of fermentations of strains 277 and 282, in the Casitone medium with

Table 4. *Effect of the medium on the products of glucose fermentation by strains of Actinomyces spp. incubated in 25 × 200 mm. test tubes with pyrogallol-Na₂CO₃ seal*

	Strain					
	279			295		
	Medium			Medium		
	279			295		
	Casitone yeast extract + 0.5 % glucose†	Casein hydrolysate + 1.0 % glucose	Casitone yeast extract + 0.5 % glucose†	Casitone yeast extract + 0.5 % glucose†	Casein hydrolysate + 1.0 % glucose	Casitone yeast extract + 0.5 % glucose†
Glucose fermented	0.8	26.0	27.1	1.7	23.7	32.0
Products						
Formic acid	3.1	8.0	6.9	3.0	11.6	10.8
Acetic acid	4.6	2.2	1.5	3.1	8.5	4.9
Lactic acid	-2.4	31.9	44.1	0.5	29.9	57.0
Ethanol	0.2	0.0	0.2	0.8	0.0	1.0
Percentage carbon recovered	—	69.2	87.5	—	82.7	99.7
				0.8	24.0	32.3
				4.3	11.1	10.5
				3.1	9.4	5.3
				0.8	39.8	59.9
				0.7	0.0	0.6
				—	103.5	104.0

* Net disappearance of initial reducing substances was calculated as glucose fermented.
 Values given are corrected for the values obtained in the absence of added sugar.

and without glucose, gave essentially the same results obtained with strain 295. Carbon recoveries in the presence of added sugar were 86 % in all cases. These results suggested that an unknown product was being formed. Subsequently, analyses of fermentations of glucose- ^{14}C with strains 279 and 295 in the Casitone yeast-extract medium revealed the formation of significant amounts of succinic acid. No additional products were found.

Table 5. *Analysis of glucose (1 %) fermentations by specified strains of Actinomyces spp. and Lactobacillus bifidus in basal medium with Casitone and 1.0 % (w/v) glucose*

	Actinomyces spp. Strain					L. bifidus Strain		
	277	279	282	295	296	307	308	309
	$\mu\text{mole used or formed per ml.}$							
Glucose fermented	25.2	24.9	24.3	26.6	25.4	32.9	49.2	47.6
Carbon dioxide utilized	4.5	8.9	4.7	8.7	10.8	11.2	4.8	0.8
Products								
Formic acid	6.0	7.9	5.8	7.8	11.5	10.4	5.8	7.5
Acetic acid	3.4	3.2	5.4	6.3	12.0	54.8	66.7	70.4
Lactic acid	44.3	42.1	38.2	37.1	24.5	22.1	46.3	38.9
Succinic acid	3.0	4.7	6.1	8.6	12.8	Trace	3.6	1.2
Percentage carbon recovered	101.3	100.7	103.4	98.7	98.1	89.4	97.3	97.4
Redox value	1.00	0.77	1.26	0.94	1.12	0.46	0.98	5.40
Percentage glucose fermented	45.4	44.8	43.8	47.9	45.8	59.2	88.6	85.7

With the change in fermentation noted in the Casitone yeast-extract medium, the necessity for accurate redox balances was emphasized. It had been observed that cultures grown in volumetric flasks from which oxygen was removed by gassing with 95 % N_2 + 5 % CO_2 had long lag periods, gave small amounts of growth and often required re-inoculation to obtain growth. Occasionally similar results were obtained with these flasks using a pyrogallol + Na_2CO_3 seal. However, cultures in the 25 × 200 mm. test-tubes sealed with pyrogallol + Na_2CO_3 seals (12 to 15 drops each) invariably gave good growth. These results, together with the fact that carbon dioxide was known to be required by all strains for anaerobic growth, suggested that carbon dioxide was a limiting factor, and that net fixation of carbon dioxide was occurring. Consequently, the fermentation flasks illustrated in Fig. 1 were used with the Van Slyke blood gas apparatus to determine the initial and final carbon dioxide content of the medium. The results presented in Table 5 show that there is a significant net carbon dioxide fixation by Actinomyces strains 279, 295 and 296, and probably lesser carbon dioxide fixation by strains 277 and 282, and suggest that carbon dioxide may not be a product of glucose fermentation as indicated by the results in Table 3.

The results in Table 5 show that, in general, the products formed by strains of *Actinomyces* and *Lactobacillus bifidus* on the glucose Casitone medium are identical but differ quite markedly in the relative amounts of acetic acid formed. In these fermentations, the acetic acid formed by actinomycetes accounted for 4.3–15.8 % of the glucose fermented, whereas that formed by the lactobacilli was from 45.2 to 54.5 % of the glucose utilized. Furthermore, of the total glucose added to the medium, the actinomycetes fermented from 43.8 to 47.9 %, whereas the lactobacilli utilized from 59.2 to 88.6 %. All cultures showed an apparent fixation of gaseous carbon dioxide, although in all probability the value given for strain 309 is in error. Strain 307 of *L. bifidus* formed but a trace of succinic acid. Similarly, in other fermentations done with *L. bifidus*, strain 308, succinate was not found.

Negroni & Fischer (1944) and Norris *et al.* (1950) have shown that lactobacilli cultures isolated from human sources form L (+) lactic acid. The type of lactic acid formed by actinomycetes has not been reported. Cultures of strains 277, 279, 282 and 295 were harvested and the lactic acid formed from glucose was isolated as the zinc salt. 4 % solutions (w/v) of the anhydrous salts gave specific rotations of -5.35 to -8.10 (specific rotation of a 4 % solution of active zinc lactate was reported by Pederson *et al.* (1926) to be -8.25). The 4 % (w/v) solutions of zinc lactate tested were found to be contaminated with a maximum of 0.7 % (w/v) succinic acid. The rotations reported are based on lactic acid concentrations determined by the method of Barker & Summerson (1941). It would therefore appear that L (+) lactic acid is formed by these strains of *Actinomyces* spp. but small amounts of D (–) lactic acid may also be formed.

DISCUSSION

It was indicated above that strains representative of *Actinomyces bovis* as described by Erikson (1940) and Thompson (1950) were not included in the present study. Initially two cultures typical of the description of *A. bovis* (Erikson, 1940; Thompson, 1950) were tested for growth on the basal medium used in these experiments (Howell & Pine, 1956). Of these, one strain (284) gave satisfactory growth on this medium but unfortunately this strain was lost during the present investigation. The second strain (278) gave only a limited amount of growth on this medium and therefore was not used in the present study. Attempts were made to obtain other cultures similar to the above two bovine isolates. However, all such cultures available for study were found to be identical, in certain major characteristics (indole, H_2S , and catalase production; action on milk and gelatin) with *Corynebacterium acnes* as described by Beerens (1953). It was therefore considered unwise to include these cultures in the present study.

Certain strains of *Actinomyces* spp. and *Lactobacillus bifidus* have been described previously as anaerobes or microaerophiles (Erikson, 1940; Gyllenberg, 1955); as requiring or being stimulated by carbon dioxide (Erikson, 1940; Rosebury, Epps & Clark, 1944; Norris *et al.* 1950); of being capable of aerobic growth (Thompson & Lovestedt, 1951; Veltre, Shorb & Pelczar, 1953);

of being able to grow in the presence of small amounts of oxygen (Rosebury *et al.* 1944; Norris *et al.* 1950); of being able to adapt to aerobic conditions (Ludwig & Sullivan, 1952); or, in the case of *L. bifidus*, of being able to adapt to growth in air with the formation of 'straight' rod forms (Gyllenberg, 1955; Hayward, Hale & Bisset, 1955). Erikson & Porteous (1955) have also reported the conversion of a single 'rough' strain of *A. israelii* to a 'smooth' type more tolerant of oxygen. They found, however, that this apparent conversion was due to the presence in cultures of this strain of a facultatively anaerobic staphylococcus which affected both colony morphology and oxygen tolerance of the host strain.

The results reported here show quite clearly that some strains of actinomycetes may be facultative anaerobes, whereas others are microaerophils to obligate anaerobes. Carbon dioxide may or may not be required for growth in air, but is apparently required by all strains of actinomycetes for anaerobic growth under the conditions described above. Of the strains of *Lactobacillus bifidus* tested all required carbon dioxide for anaerobic growth. Only one of the three strains tested grew in air; the growth which was obtained was very limited in amount and carbon dioxide was required. Therefore, it would appear that these strains of *L. bifidus* are essentially microaerophils or anaerobes.

The value of the comparative rates of growth of *Actinomyces* spp. and *Lactobacillus bifidus* on glucose, maltose and lactose is questionable. It would appear that some *Actinomyces* strains grow slowly on lactose, must adapt to its utilization, or lack the ability of utilizing the sugar as efficiently as they do glucose. However, other strains appear to utilize lactose as efficiently as they do glucose or maltose (maltose was utilized as well as glucose by all *Actinomyces* strains tested). Therefore, the use of these sugars for the differentiation of *L. bifidus* from the genus *Actinomyces* would seem to have but limited value, would require exact determination of rates of growth, total cell yields, etc. Nevertheless, considering the results of Rose *et al.* (1953), György, Norris & Rose (1954), Huhtanen (1955), and the results reported above, it would appear that lactose is the preferred sugar for growth of *L. bifidus*, whereas glucose or maltose is the preferred sugar for *Actinomyces* spp.

In most instances, results obtained with *Actinomyces* show that lactic acid was the primary product of glucose fermentation regardless of the medium or conditions employed. In one case reported above (strain 296, Table 5) lactic acid accounted for only 43 % of the glucose fermented; succinic, acetic, and formic acids accounted for the remaining carbon. Depending upon the strain and the medium used, other products were formed but in an amount seldom accounting for more than 20 % of the glucose fermented.

Results obtained with the three strains of *Lactobacillus bifidus* show that lactic and acetic acids in approximately equal amounts (based on the glucose fermented) are the primary products of glucose fermentation, although trace amounts (4–7 % of the glucose fermented) of formic and succinic acids may also be formed. The formation of lactic and acetic acids as the major products of glucose fermentation by human lactobacilli has been reported previously by Orla-Jensen (1943), Negroni & Fischer (1944), and by Norris *et al.* (1950).

The identity of the compound identified as ethanol in the *Actinomyces* fermentations is questionable since the basal medium contained a small amount of ethanol as a solvent for thioctic acid. The values reported were obtained as small differences between the zero time and final determinations. Acetoin production varied with the medium used, and in general, the greater the concentration of yeast extract or Casitone, the greater the acetoin formation; no acetoin was found to be produced in the glucose casein hydrolysate medium. Acetoin was never formed in amounts sufficiently great to affect the quantitative aspect of the fermentation balances. No other products besides formic, acetic and succinic acids were found in any of the fermentations.

The relative proportion and amounts of the products formed by actinomycetes apparently may be influenced somewhat by the presence of crude organic substances in the medium. Erikson & Porteous (1953) found that on a heart broth casein digest medium, *Actinomyces* strains produced lactic acid equivalent to only 30–60 % of the glucose utilized; other products were not reported.

Changes in the character of fermentations due to changes in the medium or methods of handling the cultures have been described by Pappenheimer & Shaskan (1944), Waring & Werkman (1944), Bard & Gunsalus (1950), and Hodges, Coolidge & Harrison (1951).

Increased carbon dioxide concentrations have previously been shown to be beneficial to the growth of *Actinomyces* strains (Erikson, 1940; Rosebury *et al.* 1944). That strains of *Lactobacillus bifidus* require carbon dioxide for anaerobic growth on solid media has been reported by Norris *et al.* (1950). The fermentative data presented in Table 5 show a net carbon dioxide fixation by both groups of organisms. However, in some cases the values for carbon dioxide fixed as shown in Table 5 are within or closely approaching experimental error. A net fixation in these instances is only suggestive.

If one uses the fermentation data of *Actinomyces* strains 282 or 296, and *Lactobacillus bifidus*, strain 308, as given in Table 5, recognizing the limitations of the data, over-all reactions based on the stoichiometry for glucose utilization for these organisms would approximate the following equations:

The apparent absence of reaction 3 in *Actinomyces* spp. suggests a major metabolic difference between *Actinomyces* spp. and *Lactobacillus bifidus*.

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