seem to rule out ergotamine inhibiting milk let-down in lactating rats by vasoconstriction of mammary vessels. There remain at least two other possibilities to explain this inhibition. It is generally accepted(9,10) that mammary myoepithelial cells are the contractile elements responsible for squeezing milk from alveoli and ducts. There may then be a competition between oxytocin and ergotamine at the myoepithelium level for results from injecting ergotamine 2 minutes prior to nursing, suggests a certain level of the drug must be present in general circulation before full inhibition of milk let-down is assured. The other possibility is ergotamine through sympatholytic action and central nervous system depression blocks release of oxytocin from posterior pituitary gland. As neural pathways involved in the milk let-down reflex are obscure, it is perhaps premature to speculate on the site of neural blockage by ergotamine. It has been shown, however, that electrical stimulation of anterior hypothalamus(11) and vagi(12) in lactating sheep and goats, and supraoptico-hypophyseal tract(2,13,14) in lactating rabbits resulted in let-down in milk. It is possible that ergotamine inhibits milk let-down in lactating rats through blockage of one or more of these areas.

Summary. 1. The quantity of milk secured by litters of lactating rats treated with ergotamine 10 minutes before nursing was significantly less than that obtained by control offspring. Oxytocin administered before or after ergotamine restored normal milk let-down. 2. Topical application of ergotamine to living rat mammary tissue resulted in arteriole constriction but did not interfere with normal myoepithelial contraction induced by oxytocin. 3. Results indicate ergotamine does not inhibit milk let-down through vasoconstriction of mammary blood vessels thus prohibiting access of oxytocin to effector myoepithelium. Rather, a competition between ergotamine and oxytocin for the effector tissue or a neural block inhibiting release of oxytocin by posterior pituitary gland seem more probable.

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Fixation of Carbon Dioxide by *Actinomyces* and *Lactobacillus bifidus*. (22790)

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In recent studies on fermentation of glucose by *Actinomyces bovis* and *Lactobacillus bifidus*(1) a net fixation of CO_2 was definitely observed in some fermentations and suggested in others. The overall stoichiometry of 2 fermentations by *Actinomyces* indicated that CO_2 was being fixed in a manner consistent with the operation of the Wood-Werkman reaction(2) with the formation of succinic acid. The data suggested that this same reaction was functioning to a minor extent in one strain of *L. bifidus;* but that the major path-

way in this organism was one which satisfied the equation: glucose→3 acetic acid. In view of the facts that the same stoichiometry of glucose fermentation occurs in fermentations by *Clostridium thermoaceticum*(3), that carbon dioxide is reduced to acetic acid in the clostridial fermentation(4,5), and that both *Actinomyces* and *Lactobacillus bifidus* have been shown to require carbon dioxide for growth under certain conditions(1), it was decided to conduct fermentations in the presence of carbon dioxide-C^{14} and to determine the extent of incorporation of radioactivity into the carbon atoms of the products.

Materials and methods. The organisms used were *Actinomyces bovis,* strains ATCC #10048 and #10049, and *Lactobacillus bifidus,* strain #308, obtained from Dr. Paul Gyorgi, University of Pennsylvania Hospital. They were grown on casitone-starch medium with 1% glucose in fermentation tubes as described earlier(1). However, sodium carbonate-C^{14} instead of sodium carbonate-C^{12} was used in the pyrogallol seal to induce anaerobiosis. In aerobic fermentation described in Table II, the culture grew anaerobically 24 hours. The culture was then attached through the top stopper of fermentation tube to a one liter flask containing pure oxygen, and the culture mixed for additional 24 hours with magnetic stirrer. Quantitative determinations of the products were done as described previously(1). Pyruvic acid was identified and estimated by the method of Koepsell and Sharpe(6). Formate was degraded in the presence of acetic acid by oxidation with mercuric sulfate and recovered and counted as barium carbonate. The residual acetic acid was steam distilled and its purity verified by its Duclaux constant. In Table I, acetate was degraded by wet oxidation to determine total specific activity; radioactivity in the carboxyl carbon was determined using the Schmidt degradation(7); and radioactivity of methyl carbon was calculated as the difference. In all other fermentations acetate was recovered as barium acetate and counted as such, applying self absorption corrections determined for barium carbonate. In the fermentation in Table I, succinic acid was separated from lactic acid using a celite column according to procedure of Phares *et al.* (8). Only those fractions were used which gave a negative spot test for lactic acid(9). The succinate was degraded as described below. In all other fermentations, non-volatile acids were chromatogramed on paper (S and S #507) using ammonia-ethanol-water solvent of Cheftel *et al.*(10). Acid bands were demonstrated using brom cresol green (0.05%) adjusted to blue with 0.1 N NaOH. A much more sensitive and satisfactory spray was 0.3% solution of ninhydrin used after the papers were air dried and no longer smelled of ammonia. The color was developed in a 105°C oven for 5 to 10 minutes. This procedure was used in those cases where the acids were not recovered from the paper. The radioactive bands obtained from the *Actinomyces* fermentations were located by radioautograms and were checked for coincidence with the acid bands. The acids were identified by their Rf values and were cut from the paper using the radioautogram as a guide. The single radioactive band obtained from the *L. bifidus* fermentation was located using a hand probe. In all cases there was a wide separation of the lactic acid band from that of the succinic acid. In all cases radioactivity and acid bands were exactly coincident

TABLE I. Anaerobic Fermentation of Glucose plus Carbon Dioxide-C^{14} by *Actinomyces* Strain #10049.*

Substrate or product	μmoles/ml	Cts/min. /μmole
Glucose utilized	34.4	
Carbon dioxide (initial)	33.4	523.4
" " (final)	14.1	492.8
Formic acid	12.6	50.6
Acetic acid	10.3	.36
–COOH		.23
$-CH_3$		.13
Lactic acid	43.2	n.d.†
Succinic acid	11.0	527.2
–COOH		143.3
$-CH_2-$		120.3
Beta alanine (predicted)		383.9
(found)		377.0

* Fermentation of glucose was done by the fermentation procedure described by Pine and Howell (1) using a 1% glucose casitone medium with starch.

† n.d. = not determined.

TABLE II. Fermentations of Glucose plus Carbon Dioxide-C^{14} by *Actinomyces* Strains #10048 and #10049 and by *Lactobacillus bifidus.**

Substrate or product	*Actinomyces* 10048 Anaerobic		*Actinomyces* 10049 Anaerobic		*Actinomyces* 10049 Aerobic		*L. bifidus* Anaerobic	
	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM	μM/ml	Cts/min. /μM
Glucose utilized	22.8		31.8		31.5		49.5	
CO_2 (initial)		1995.0		1995.0		1730.0		1995.0
(final)		1696.0		1867.0		857.5		1505.0
Formic acid	8.62	119.7	11.7	98.4	15.0	61.8	2.0	1.0
Acetic "	6.52	2.4	8.7	.8	13.3	.6	69.8	.0
Lactic "	24.6	120.5	43.0	72.6	29.9	34.7	49.2	.3
-COOH		118.8		72.2		34.7		—
CH_3CHOH-		1.7		.4		.0		—
Succinic acid	8.18	1169.2	11.2	1301.2	7.04	368.0	.6	529.6
-COOH		159.5		248.4		19.1		20.4
$-CH_2-$		425.1		402.2		164.9		244.6
Beta alanine (predicted)		1009.7		1052.8		348.9		—
" " (found)		1060.0		1020.0		358.0		—

* Fermentation of glucose was done by fermentation procedure described by Pine and Howell(1) using 1% glucose casitone medium with starch. The procedure for aerobic fermentation is given in text.

with one exception. An apparently non acidic single band of high radioactivity was found running behind and not quite touching the succinate band from the aerobic #10049 fermentation of Table II. The identity of this compound has not as yet been determined. The radioactive compounds were eluted from the paper with water. The samples of succinate so obtained from the *Actinomyces* fermentation were acidified with one or 2 drops of 25% HCl, evaporated to dryness on steam bath, and sublimed *in vacuo*. Melting points were taken and all melted within 2 degrees of an authentic sample of succinic acid and with no depression observed with mixtures of the two. The succinic acid of the *L. bifidus* fermentation was not sublimed but was degraded directly. Lactic acid samples were passed through Dowex 50 resin to convert them to free acid, and were neutralized with $Ba(OH)_2$. Succinic acid was wet ashed using the reagents of Van Slyke *et al.*(11) to determine the average specific activity of its carbon atoms. A sample was then decarboxylated and the specific activity of the carbon dioxide determined according to the procedure of Phares(7) maintaining the temperature at 70°C for one half hour or for one hour at 45°C. Under our conditions, approximately 5 to 7% of the methylene carbons of known succinate-2, 3-C^{14} was obtained as the carboxyl carbon. Attempts to isolate ethylene diamine according to the procedure of Phares and Long(12) failed. Upon the suggestion of Dr. Richard Hendler, National Heart Institute, National Institutes of Health, analyses were made for amino acid in the reaction mixture. An amino acid, presumably beta alanine (13) was found. The reaction mixture was neutralized with 50% NaOH, a few ml of water added, and the Na_2SO_4 was allowed to crystallize out over night at 5°C. Ten ml of ethanol were added, the suspension was centrifuged and the supernatant was removed. The precipitate was washed twice with alcohol, and the alcohol fractions combined. The presence of beta alanine was shown using paper chromatograms run with the acetic acid-butanol-water solvent of Calvin *et al.*(14) and the ethanol-ammonia-water solvent. The former solvent was used to separate the beta alanine from residual succinic acid and trace amounts of ethylene-diamine which were also formed. The beta alanine was eluted with water from such chromatograms; its concentration determined using the ninhydrin procedure of Troll and Cannan(15); and its specific activity was determined by plating and counting directly known amounts of the amino acid. Barium lactate was decarboxy-

lated using chromic acid(16). The residual acetic acid was steam distilled, neutralized with $Ba(OH)_2$ and counted as barium acetate, applying the same correction factors used for barium carbonate.

Results. In initial experiments using *Actinomyces* strain #10049 both the volatile and non-volatile acids were radioactive. A small decrease in the specific activity of the carbon dioxide occurred during the fermentation. Later fermentations using strains #10048 and #10049 and *L. bifidus* showed that each of the 3 fixed from one to 3% of the added counts into the cells and that the volatile acids formed by *Actinomyces* were radioactive while those formed by *L. bifidus* were not. All organisms had incorporated radioactive carbon dioxide into the non-volatile acids but the total counts in this fraction from *L. bifidus* were low. An analysis was then made of a fermentation by *Actinomyces* strain #10049, the results of which are presented in Table I. Again there was observed a small decrease in the specific activity of the carbon dioxide although a net fixation of carbon dioxide occurred. The proportion of formate to acetate to succinate formed was approximately 1/1/1 but the ratio of carbon dioxide fixed to any of these products was approximately 1.54. Relatively low carbon recovery (91%) and a high redox index (1.6) indicated the formation of other products having a high redox value. Attempts to find such products failed and revealed only an additional 0.5 micromole of pyruvate formed per ml. Apparently more carbon dioxide was fixed than was required for the operation of the Wood-Werkman reaction. The specific activity of the succinate was slightly higher than that of the initial carbon dioxide. Upon degradation of the succinic acid, approximately 46% of the total activity was found in the methylene carbons. Isolation of the beta alanine verified these results.

The fermentations were repeated using *Actinomyces* strains #10048 and #10049 and *L. bifidus*. An aerobic fermentation of #10049 was also included as described above. The results are given in Table II. In the 2 anaerobic *Actinomyces* fermentations, the ratio of activity of methylene carbons to carboxyl carbons of the succinic acid was 2.67 and 1.62 for strains #10048 and #10049, respectively. For the aerobic fermentation by *Actinomyces* #10049 and the anaerobic *L. bifidus* fermentation the ratio increased to 8.6 and 12.0, respectively. No significant activity was found in any other products of *L. bifidus*.

In the anaerobic *Actinomyces* fermentations, formate and the lactate carboxyl were of approximately the same specific activity but much lower in specific activity than the final carbon dioxide. The specific activity of the final carbon dioxide in the aerobic fermentations was one half its original activity. Similarly, formate and lactate carboxyl carbon of this fermentation had a specific activity one half that found in the sister anaerobic fermentation. The lactic acid of these fermentations was labeled essentially only in the carboxyl carbon.

In aerobic *Actinomyces* fermentation it appeared that lactate was oxidized to acetate and carbon dioxide with a subsequent decrease in the amount of succinic acid formed. Little fixation of carbon dioxide into the carboxyl carbons of succinic acid occurred in this case. However, the reduction of specific activity in the methylene carbons remained proportional to the decrease in specific activity of the carbon dioxide and again was approximately one half that found in the anaerobic fermentation. In general, the calculated values of specific activity for the beta alanine were approximated by the observed values with the exception of the beta alanine isolated from the succinate of *L. bifidus*. In this case, however, the sample of beta alanine was heavily contaminated with salt and no attempt was made to purify it further.

The results of the fermentation of glucose by *L. bifidus* as shown in Table II may be expressed as being essentially: glucose→lactic acid + 1.41 acetic acid + unknown products. The results obtained with the carbon dioxide-C^{14} showed no incorporation of radioactivity into these products. That the added carbon dioxide was available to and was metabolized by this organism was shown by the high specific activity of the succinic acid which it formed. These results are in

agreement with those of Kuhn and Tiedemann(17) who found no significant incorporation of carbon dioxide into lactic and acetic acids. These workers fermented glucose-1-C^{14} with *L. bifidus* and obtained lactic and acetic acids having the same specific activity. They did not degrade these products, however. It would appear therefore that the formation of acetic acid from glucose by this organism involves a molecular rearrangement without the concomitant fixation of carbon dioxide as occurs in *Clostridium thermoaceticum*(4,5) or in *Butyribacterium rettgeri*(18).

It is of interest to note the similarity of labeling of succinic acid found in the *L. bifidus* fermentation and the aerobic fermentation by *Actinomyces* #10049. Similarities of the glucose fermentation by these organisms have been reported previously(1). It is also noted that not only do these fermentations of glucose by *Actinomyces* differ qualitatively from the fermentations of the genus *Lactobacillus* (which do not generally form succinic acid) and from members of the genera *Corynebacterium* and *Propionibacterium* (which form propionic acid) but they also differ from *Propionibacterium* in the mechanism by which they utilize carbon dioxide for succinate synthesis.

It is believed that this is the first report of the net fixation of carbon dioxide with its major incorporation into the methylene carbons of succinic acid. However, the results are similar to those reported by Jefferson *et al.* (19) who found that washed suspensions of *Rhizopus nigricans* fermented glucose in the presence of formate-C^{14} with the formation of succinic and fumaric acids having the greatest radioactivity in the central carbons. However, in this fermentation, lactic acid and ethanol formed had their greatest radioactivity in the methyl carbons while in the presence of carbon dioxide-C^{14} only carboxyl labeled acids were formed. Results similar to those of Jefferson *et al.* were obtained with *Escherichia coli* by Nutting and Carson(20).

Summary. Fermentations of glucose in the presence of carbon dioxide-C^{14} were carried out with strains of *Actinomyces* under aerobic and anaerobic conditions and with *Lactobacillus bifidus*. Fermentation products were isolated and degraded. It was found that succinic acid formed by these organisms had a high percentage of its radioactivity in the methylene carbons. When one strain of *Actinomyces* was grown in the presence of oxygen, 90% of the total activity of the succinic acid was found in the methylene carbons as compared to 62% when it was grown anaerobically.

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