

# STUDIES ON THE SULFUR NUTRITION OF *LACTOBACILLUS ARABINOSUS*

TETSUO SHIOTA<sup>1</sup> AND FRANCIS M. CLARK

*Department of Bacteriology, University of Illinois, Urbana, Illinois*

Received for publication March 28, 1955

The sulfur nutrition of microorganisms has been studied by many investigators. Early workers were interested essentially in sulfur compounds used by organisms as sulfur sources. Recent efforts have been directed toward the determination of mechanisms involved in the utilization of sulfur compounds. Results of investigations by Horowitz (1947), Lampen *et al.* (1947), Teas *et al.* (1948), Binkley and Hudgins (1953), and Wijesundera and Woods (1953) have led to the following proposed mechanism. Sulfate sulfur is reduced to sulfide, which is converted to organic sulfur, probably in the form of cysteine. At this stage the cysteine can condense with a 4-carbon compound, homoserine, to form the asymmetric thio ether, cystathionine. The cleavage of this compound forms homocysteine, which can then be methylated to form methionine.

In this investigation, conditions for the growth of *Lactobacillus arabinosus* were studied when various sulfur compounds were supplied as sulfur sources. Further, efforts were made to demonstrate the mechanism of sulfur utilization.

## EXPERIMENTAL METHODS

The stock cultures of *L. arabinosus* strain 17-5, used in this work, were maintained on L-agar (Baltimore Biological Laboratory) as stabs. New stabs were prepared once a month, and stored in a refrigerator. The inoculum was prepared by transferring the organisms from a stab to synthetic medium or to Difco's micro inoculum broth, incubating at 37 C for about 18 hours, centrifuging and washing the cells. One drop, representing approximately  $1 \times 10^6$  cells per ml final concentration, was used to inoculate all tubes. Growth was measured with a "lumetron" colorimeter, model 400-A at 650  $m\mu$ .

Synthetic medium C (Dunn *et al.*, 1945) was used to study the sulfur nutrition of *L. arabinosus*. A stock medium was prepared double strength, omitting glucose, phosphate and organic sulfur

compounds. Glucose and phosphate solutions were sterilized separately by autoclaving, and were pooled. Sodium pyruvate and the various sulfur compounds were prepared fresh and filter sterilized with UF sintered glass filters. Vitamins and amino acids were omitted from the basal medium when the effect of these substances was studied.

Three media, synthetic medium C, H<sub>2</sub>O<sub>2</sub> treated peptone medium (Lyman *et al.*, 1946) and AC broth (tryptone, 10 g; yeast extract, 10 g; K<sub>2</sub>HPO<sub>4</sub>, 5 g; glucose, 1 g; 5 ml of salts B, in 1000 ml of H<sub>2</sub>O) were used to grow cells for resting and dried cell experiments.

Determination of methionine and serine were made microbiologically using *Leuconostoc mesenteroides* strain P-60 according to the procedures outlined by Barton-Wright (1952). Paper chromatograms were used for the detection of amino acids according to the method of Consden and coworkers (1944). The detection of alpha-keto acids was in accordance with the method of Cavallini *et al.* (1949). Chemical determinations for pyruvate (Freidemann and Haugen, 1943), cysteine (Sullivan and Hess, 1936), and lactic acid (Barker and Summerson, 1941) were also run. H<sub>2</sub>S was detected qualitatively with filter paper soaked with lead acetate solution, and ammonia by Nessler's reagent.

## RESULTS

*Nutritional studies.* A series of sulfur compounds were used to determine their ability to serve as sole sulfur sources for the growth of *L. arabinosus*. The compounds tested were L-cysteine, L-cystathionine, DL-homocysteine, DL-homocystine, sodium sulfide and sodium thioglycolate. The  $\alpha$ -hydroxy analogue of methionine ( $\gamma$ -methio- $\alpha$ -hydroxy butyric acid) was also tested. DL-Methionine was used as a check. The basal medium contained sulfur compounds in the form of the sulfates of magnesium, manganese, iron, and adenine, but this sulfur did not support growth.

<sup>1</sup> Present address: National Institutes of Health, Bethesda 14, Maryland.

TABLE 1  
Growth response of *Lactobacillus arabinosus* strain 17-5 to various sulfur compounds

Sulfur Source	$\mu\text{M}$ Added	Optical Density $\times 10^3$	Sulfur Source	$\mu\text{M}$ Added	Optical Density $\times 10^3$
DL-Methionine	0	26	DL-Homocysteine	0.5	25
	0.1	52		1.0	32
	0.4	87		2.0	41
	0.8	95		5.0	76
	1.0	100		10.0	96
$\gamma$ -Methio- $\alpha$ -hydroxybutyric acid	0.1	51	DL-Homocysteine	0.5	23
	0.4	86		1.0	26
	0.8	97		2.0	32
	1.0	97		5.0	52
L-Cysteine	0.1	37	L-Cystathionine	10.0	71
	0.4	63		0.2	42
	0.8	72		0.6	64
	1.0	74		1.0	61
	5.0	74		5.0	80
	10.0	73		8.0	87
			Sodium sulfide	0-5	30-34
			Na thioglycolate	0-5	30-36

Growth experiments using *L. arabinosus* and the compounds tested, when added on an equal molar basis, gave the results shown in table 1.

The hydroxy analogue of methionine gave growth essentially equal to that of methionine. Considering growth of this organism when supplied 1.0  $\mu\text{M}$  of methionine as 100 per cent, L-cysteine produced 74 per cent; L-cystathionine, 61 per cent; DL-homocysteine, 32 per cent; and DL-homocysteine, 26 per cent of the growth compared to methionine. At higher concentrations all compounds mentioned gave increased growth with the exception of L-cysteine. Sodium sulfide and sodium thioglycolate supported essentially no growth in this sulfur-free medium.

In order to determine whether cysteine or homocysteine was converted to methionine, *L. arabinosus* was grown in a synthetic medium with cysteine or homocysteine as sole sulfur source. Cells grown in the same synthetic medium with methionine were used as a check. These cells were washed, hydrolyzed and their methionine content determined microbiologically. Results are shown in table 2.

From these results it seems logical to conclude that both L-cysteine and DL-homocysteine were converted to methionine by the cells of *L. arabinosus* strain 17-5. As indicated above, more homocysteine is required to produce growth comparable to that of cysteine and methionine. Also

TABLE 2  
Comparative growth and methionine content of cells of *Lactobacillus arabinosus* using L-cysteine and DL-homocysteine as sulfur sources

Sulfur Source	$\mu\text{M/L}$ Supplied	Cells, g Dry Wt/L	Methionine, Per Cent Dry Weight
DL-Methionine.....	100	0.698	1.75
L-Cysteine.....	100	0.572	1.47
DL-Homocysteine....	500	0.551	1.27

the yield of methionine in the cells is lower when homocysteine is used as the sole sulfur source.

The effect of vitamins on the utilization of sulfur compounds by *L. arabinosus* was studied by omitting various vitamins from a mixture of vitamins. Regardless of the sulfur compound used, this culture of *L. arabinosus* required biotin, nicotinic acid, Ca pantothenate and pyridoxal. With methionine or its  $\alpha$ -hydroxy analogue as sole sulfur source, *p*-aminobenzoic acid was not required for growth. When cysteine, homocysteine or cystathionine was supplied, maximum growth of *L. arabinosus* depended upon the addition of *p*-aminobenzoic acid to the medium. The omission of folic acid had no restricting effect on growth of this organism on any sulfur compounds tested.

Inhibition studies using sulfanilamide were used to check the *p*-aminobenzoic acid require-

ment. With cysteine or homocysteine, sulfanilamide (300  $\mu\text{g/ml}$  and above) inhibited the growth of *L. arabinosus*. When methionine was supplied as a sulfur source, a concentration of 3000  $\mu\text{g/ml}$  of sulfanilamide did not inhibit growth.

Efforts to show an increased growth response of *L. arabinosus* to homocysteine in the absence of *p*-aminobenzoic acid or with suboptimal amounts of homocysteine by supplying various methyl sources were of no avail. The substances tested were sodium formate, sodium bicarbonate, choline, betaine, and sarcosine.

The effect of amino acids on the utilization of sulfur compounds when single amino acids were omitted from a mixture of amino acids indicated a requirement for serine when methionine, its  $\alpha$ -hydroxy analogue or homocysteine was supplied as a sulfur source. The use of cysteine or cystathione, however, eliminated the serine requirement. Hift and Wallace (1949) also demonstrated with two microorganisms, *L. arabinosus* strain 17-5 and *L. mesenteroides* strain P-60, that cysteine replaced serine.

There seemed a possibility that pyruvate might replace serine when methionine was used as a sulfur source. Using this combination the maximum growth was obtained with the addition of 2  $\mu\text{M}$  of sodium pyruvate. However, this growth corresponded to slightly more than half-maximum as compared to the growth when serine and methionine were supplied. Pyruvate and sulfide in the absence of any organic sulfur source did not support growth.

To test the conversion of cysteine to serine by growing cells of *L. arabinosus*, this organism was grown in a synthetic medium containing cysteine but without serine. As a check, cultures were grown in a medium containing methionine and serine as well as cysteine and serine. The cells were hydrolyzed and treated as before for methionine determination. The results are given in table 3. The serine content of cells grown in a medium containing cysteine without serine was as high as that grown in a similar medium with serine. This indicated a possibility that cysteine could be converted to serine under these conditions.

*Resting and dried cell studies.* Since growth experiments involving the use of various methyl sources failed to increase the growth response of *L. arabinosus* to homocysteine, a similar attempt

TABLE 3  
*Serine content of cells of Lactobacillus arabinosus*

<i>L. arabinosus</i> Grown On	$\mu\text{M/L}$ Supplied	Cells g Dry Wt/L	Serine, Per Cent Dry Weight
DL-Methionine with serine.....	100	0.698	2.0
L-Cysteine with serine.....	100	0.572	2.9
L-Cysteine without serine.....	100	0.620	2.8

was made to demonstrate the role of these compounds with live and dried cell preparations.

Cells from an 18 hour culture of *L. arabinosus* were centrifuged, washed once and suspended in phosphate buffer  $\text{M}/10$ , pH 7, to a cell concentration of about  $5 \times 10^{10}$  cells/ml. The cells were grown in AC broth or a medium containing  $\text{H}_2\text{O}_2$  treated peptone to which was added homocysteine. The total volume of the reaction mixture was 4.5 ml. These tubes were incubated at 37°C. The reaction was stopped by placing the tube in a boiling water bath for 10 minutes.

The compounds used were homocyst(e)ine (10  $\mu\text{M/ml}$ ) and glucose (5–10  $\mu\text{M/ml}$ ) with either choline (10–20  $\mu\text{M/ml}$ ), betaine (5–10  $\mu\text{M/ml}$ ) or sarcosine (5–10  $\mu\text{M/ml}$ ). Vitamin B<sub>12</sub> (15  $\text{m}\mu\text{/ml}$ ), *p*-aminobenzoic acid (5–10  $\mu\text{g/ml}$ ) and 0.2 ml of 100  $\times$  vitamin mixture as described for medium C were also used in conjunction with methyl donors.

No methionine or other amino acids except the homocyst(e)ine was detected on paper chromatograms in samples removed from the reaction mixture at various time intervals over a period of 24 hours.

The utilization of cysteine and homocysteine was tested in a similar system as described above, by supplying cysteine (10  $\mu\text{M/ml}$ ) and homoserine (10  $\mu\text{M/ml}$ ) or homocysteine and serine (10  $\mu\text{M/ml}$ ). Such cell suspension did not synthesize any amino acids even with the additions of glucose, vitamin B<sub>12</sub> and *p*-aminobenzoic acid.

Suspensions of cysteine grown dried cells also failed to form any amino acids when incubated at 37 C over a period of 24 hours with, (1) DL-homocysteine alone or with DL-serine, (2) L-cysteine alone or with DL-homoserine. These were tested also in the presence of pyridoxal phosphate (45  $\mu\text{g/ml}$ ), *p*-aminobenzoic acid, and adenosine triphosphate (50  $\mu\text{M}$ ). The total volume of the reaction mixture was 2.5 ml.

TABLE 4

Products from the breakdown of *L*-cystathionine using a dried cell preparation of *Lactobacillus arabinosus*

	Hours	Products				
		H <sub>2</sub> S	NH <sub>3</sub>	Cysteine	Pyruvate	Methionine
				μg	μg	μg
Cells	0			0	0	0
	8	—	—	0	0	0
	14			0	0	0
	24	—	+	0	0	9.0
Cells + cystathionine	0				0	0
	8	—	—			
	14	+	+		40.0	8.0
	24	+	++		0	26.0
Cells + cystathionine + <i>p</i> -amino- benzoic acid	0				0	0
	8	—	—			
	14	+	++		46.0	9.0
	24	+	++		0	22.5
Cells + cystathionine + pyridoxal phosphate	0	—	—		0	0
	8	+	++			
	14				86.0	9.0
	24	+	++		0	30.0
Cells + cystathionine + <i>p</i> -amino- benzoic acid + pyridoxal phosphate	0			0	0	0
	8	+	++	0		
	14	+	++	0	86.0	9.0
	24			0	0	30.0

Live cell suspensions of *L. arabinosus* grown on cysteine did not attack cystathionine (10 μM/ml) alone or with the additions of *p*-aminobenzoic acid and pyridoxal (40 μg/ml). Dried cell suspensions, however, appeared to attack cystathionine. The results are tabulated in table 4, which demonstrates the formation of H<sub>2</sub>S, NH<sub>3</sub>, pyruvate and some methionine, as a result of cystathionine cleavage. Paper chromatograms were run on these samples and the results showed the formation of homocysteine regardless of whether pyridoxal phosphate or *p*-aminobenzoic acid was present. Homocysteine appears to have been formed in 8 hours and by 24 hours there was a very faint spot which corresponded to methionine. At this time there was also a streak appearing just below the cystathionine spot. This was not identified.

Since the formation of pyruvate indicated that these dried cells contained either serine dehydrase or cysteine desulfhydrase, the dried cells were tested for the presence of these enzymes by following the reaction for the formation of pyruvate, ammonia and hydrogen sulfide. Pyridoxal phosphate appeared to be required for the greater activity of cysteine desulfhydrase and serine

dehydrase. The amount of pyruvate found at 24 hours was considerably less than that present at 15 hours. Tests indicated that the pyruvate was converted to lactate.

A similar experiment using homocysteine and homoserine was conducted. When homocysteine was supplied to dried cells, a small amount of H<sub>2</sub>S and NH<sub>3</sub> was detected. Paper chromatograms of the reaction mixture showed the presence of a material corresponding to known 2,4-dinitrophenyl hydrazone of  $\alpha$ -keto butyric acid. Homoserine, however, was not attacked.

The ability of cell suspensions of *L. arabinosus* to synthesize methionine from the  $\alpha$ -hydroxy analogue was investigated by growing these cells in the presence of  $\gamma$ -methio- $\alpha$ -hydroxy butyric acid. Live and dried cell experiments were carried out. The results from live cell preparation are presented in table 5.

Live cells formed methionine from the  $\alpha$ -hydroxy analogue of methionine either with or without the addition of pyridoxal, glutamic acid or aspartic acid. Dried cells gave similar results with considerable less activity. Live cells were investigated for the presence of a dehydrogenase by the reduction of methylene blue in Thunberg

TABLE 5

Methionine formation from its  $\alpha$ -hydroxy analogue by live cell preparation of *Lactobacillus arabinosus*

	$\mu$ g Methionine at Hours			
	0	4	8	24
Cells.....	0	0	4.5	10.5
Cells + $\gamma$ -CH <sub>2</sub> - $\alpha$ -OH butyric acid + B <sub>6a1</sub> .....	0	60	75	180
Cells + $\gamma$ -CH <sub>2</sub> - $\alpha$ -OH butyric acid + glutamic acid.....	0	60	71	180
Cells + $\gamma$ -CH <sub>2</sub> - $\alpha$ -OH butyric acid + glutamic acid + B <sub>6a1</sub> .....	0	60	75	135
Cells + $\gamma$ -CH <sub>2</sub> - $\alpha$ -OH butyric acid + aspartic acid.....	0	66	90	180
Cells + $\gamma$ -CH <sub>2</sub> - $\alpha$ -OH butyric acid + aspartic acid + B <sub>6a1</sub> .....	0	60	72	114

tubes. Cell suspensions with  $\gamma$ -methio- $\alpha$ -hydroxybutyric acid reduced methylene blue in a half hour, while the control tube with cells only showed that methylene blue was not reduced for one hour.

## DISCUSSION

The lack of evidence to show the methylation of homocysteine by the various sources of methyl groups indicates either (1) this is not the mechanism for the formation of methionine from homocysteine, or (2) the proper methyl source and conditions were not selected. If methylation of homocysteine is the mechanism, the growth obtained indicates the cell is capable of synthesizing and transferring the methyl group to homocysteine. When methionine or its  $\alpha$ -hydroxy analogue was supplied, *L. arabinosus* grew in the absence of *p*-aminobenzoic acid. It appears that this vitamin is concerned in the synthesis of methionine, but one cannot say *a priori* that methylation is the mechanism. More recently, Schwartz and Shapiro (1954), using a mutant of *Aerobacter aerogenes*, requiring methionine or thiomethyladenosine, demonstrated a thio-methylation of a 4 carbon compound to form methionine.

The requirement for *p*-aminobenzoic acid by *L. arabinosus* strain 17-5 has been discussed by several investigators (Isbell, 1942; Lewis, 1942;

Snell and Mitchell, 1942; Shankman, 1943; Shankman *et al.*, 1947; and Pennington, 1946).

The difference between the results of the present investigation and those of others regarding the *p*-aminobenzoic acid requirement might be attributed to the use of the *p*-aminobenzoic acid independent strain of *L. arabinosus* with respect to methionine.

The ability of the organism to use cysteine or cystathionine as a sole sulfur source and a probable precursor of serine indicates that *L. arabinosus* may possess a system which is capable of converting cysteine according to the proposed mechanism by Teas *et al.* (1948). Such a mechanism would satisfy both the serine and sulfur requirements of the organism when cysteine or cystathionine is supplied to the growth medium in the absence of serine. This is a most attractive mechanism to explain the replacement of serine with cysteine and cystathionine.

When dried cell suspensions of *L. arabinosus* were allowed to act on cystathionine, the formation of serine could not be demonstrated on paper chromatograms. Pyruvate was found as one of the products of the above reaction. One cannot decide whether pyruvate is formed directly as a result of the cystathionine cleavage, or formed with the dehydration and deamination of serine. The presence of serine dehydrase in these cells would indicate that this is a possibility. On the basis of growth experiments, it would seem that pyruvate could substitute for serine to a limited extent.

The addition of cysteine and homoserine or homocysteine and serine to live and dried cell suspensions did not yield any amino acids (cystathionine, homocysteine and methionine) as detectable on paper chromatograms. If the transformations of the above amino acids occur with the conditions employed, the products of these reactions should be detected. At the present, however, no explanation for the failure to show such reactions can be offered.

The formation of methionine from cystathionine, though in small amount, is difficult to explain in view of the negative results obtained from the additions of various materials to the dried cell suspensions as was mentioned elsewhere. The formation of H<sub>2</sub>S as one of the products of the above reaction is probably due to the desulfhydration of homocysteine.

## SUMMARY

DL-Methionine, the  $\alpha$ -hydroxy analogue of methionine, L-cysteine, L-cystathionine, and DL-homocysteine can serve as sole sulfur source for the growth of *Lactobacillus arabinosus* strain 17-5. When cysteine, cystathionine or homocysteine is supplied, *p*-aminobenzoic acid is required for growth. When methionine or its  $\alpha$ -hydroxy analogue is supplied, however, the *p*-aminobenzoic acid requirement is dispensable.

Serine was required for the growth of *L. arabinosus* when methionine, its  $\alpha$ -hydroxy analogue, or homocysteine was supplied as sulfur source. With cysteine or cystathionine, serine could be omitted from the medium.

The methylation of homocysteine could not be demonstrated by growth, resting, or dried cell experiments. Furthermore, cysteine, with or without homoserine and homocysteine, with or without serine, supplied to resting or dried cell suspensions under various conditions, did not yield any new amino acids. However, these cell preparations were capable of forming methionine from  $\gamma$ -methio- $\alpha$ -hydroxybutyric acid, desulfhydrating cysteine, and to some extent homocysteine, and dehydrating serine.

Dried cell preparations attacked cystathionine with the formation of homocysteine, ammonia, hydrogen sulfide, and methionine.

## REFERENCES

- BARKER, S. B., AND SUMMERSON, W. H. 1941 The colorimetric determination of lactic acid in biological material. *J. Biol. Chem.*, **138**, 535-554.
- BARTON-WRIGHT, E. C. 1952 *The microbiological assay of vitamin B-complex and amino acids*. Pitman Publishing Corp., New York, N. Y.
- BINKLEY, F., AND HUDGINS, A. 1953 Comparative studies of sulfur metabolism. *Federation Proc.*, **12**, 178-179.
- CAVALLINI, D., FRONTALLI, N., AND TOSCHI, G. 1949 Determination of keto-acids by partition chromatography on filter paper. *Nature*, **163**, 568-569.
- CONSDEN, R., GORDEN, A. H., AND MARTIN, A. J. P. 1944 Qualitative analysis of proteins: a partition chromatographic method using paper. *Biochem. J. (London)*, **38**, 224-232.
- DUNN, M. S., CAMIEN, M. N., AND SHANKMAN, S. 1945 Investigations of amino acids, peptides and proteins. XXIV. The amino acid requirements of *Lactobacillus fermenti* 36. *J. Biol. Chem.*, **161**, 657-667.
- FRIEDEMANN, T. E., AND HAUGEN, G. E. 1943 Pyruvic acid. II. The determination of keto acids in blood and urine. *J. Biol. Chem.*, **147**, 415-442.
- HIFT, H., AND WALLACE, G. I. 1949 A study on the synthesis of cystine by some lactic acid bacteria. *J. Biol. Chem.*, **177**, 929-931.
- HOROWITZ, N. H. 1947 Methionine synthesis in *Neurospora*. The isolation of cystathionine. *J. Biol. Chem.*, **171**, 255-264.
- ISELL, H. 1942 Effect of *p*-aminobenzoic acid on the microbiological assay for nicotinic acid. *J. Biol. Chem.*, **144**, 567-568.
- LAMPEN, J. O., ROEPKE, R. R., AND JONES, M. J. 1947 Studies on the sulfur metabolism of *Escherichia coli*. III. Mutant strains of *Escherichia coli* unable to utilize sulfate for their complete sulfur requirements. *Arch. Biochem. and Biophys.* **13**, 55-66.
- LEWIS, J. C. 1942 A lactobacillus assay method for *p*-aminobenzoic acid. *J. Biol. Chem.*, **146**, 441-450.
- LYMAN, C. M., MOSELEY, O., WOOD, S., AND HALE, F. 1946 Note on the use of hydrogen peroxide-treated peptone in media for the microbiological determination of amino acids. *Arch. Biochem. and Biophys.*, **10**, 427-431.
- PENNINGTON, D. 1946 Assay of *p*-aminobenzoic acid. *Science*, **103**, 397.
- SCHWARTZ, M., AND SHAPIRO, S. K. 1954 The mechanism of utilization of thiomethyladenosine in the biosynthesis of methionine. *J. Bacteriol.*, **67**, 98-102.
- SHANKMAN, S. 1943 Amino acid nutrition of *Lactobacillus arabinosus*. *J. Biol. Chem.*, **150**, 305-310.
- SHANKMAN, S., CAMIEN, M. N., BLOCK, H., MERRIFIELD, B. R., AND DUNN, M. S. 1947 Vitamin requirements of twenty-three lactic acid bacteria. *J. Biol. Chem.*, **168**, 23-31.
- SNELL, E. E., AND MITCHELL, H. K. 1942 Some sulfanilamide antagonists as growth factors for lactic acid bacteria. *Arch. Biochem. and Biophys.* **1**, 93-101.
- SULLIVAN, M. X., AND HESS, W. C. 1936 The determination of cystine in urine. *J. Biol. Chem.*, **116**, 221-232.
- TEAS, H. J., HOROWITZ, N. H., AND FLING, M. 1948 Homoserine as a precursor of threonine and methionine in *Neurospora*. *J. Biol. Chem.*, **172**, 651-658.
- WIJESUNDERA, S., AND WOODS, D. D. 1953 Cystathionine in relation to methionine synthesis by *Bacterium coli*. *J. Gen. Microbiol.*, **9**, III.