Serine Synthesis by *Leuconostoc mesenteroides*. (27675)

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The widespread employment of *Leuconostoc mesenteroides* (ATCC 8042) in microbiological assay of essential nutrients has made it desirable to obtain as much specific information as possible concerning the nutrition of this micoorganism. Continuing work in this area has led to a consideration of the growth of *L. mesenteroides* in the absence of serine. Earlier investigators have established a role for Vit. B_6, p-aminobenzoic acid, and folinic acid in the serine nutrition of *L. mesenteroides*(1,2). In a recent report describing the synthesis of serine by cell suspensions of *L. mesenteroides* (ATCC 7881), p-aminobenzoic acid and leucovorin were found to have equal activity as cofactors(3). The present study consists of an attempt to evaluate the relative effectiveness of certain derivatives of folic acid in mediating the synthesis of serine by *L. mesenteroides* (ATCC 8042).

Materials and methods. Growth of the test organism. The medium of Williams(4) was employed for the growth of *Leuconostoc mesenteroides* strain P-60 (ATCC 8042). Alanine, hydroxyproline, norleucine, and folic acid were omitted. Inocula were obtained from tube cultures of *L. mesenteroides* which had been incubated for 16 hr at 37°C in a yeast extract-peptone media(5). The cells were washed twice with saline and adjusted to 65-70% transmission at 550 mμ. For the growth experiments reported in Table I, tubes were inoculated with one drop of this suspension. Cells used in studies with C^{14}-labeled formate were grown in 100-ml aliquots of the basal medium containing serine, 0.01 mg/ml of aseptically added thymidine, but no p-aminobenzoic acid. Flasks were inoculated with 0.8 ml of inoculum and held for 16 hr at 37°C. The turbid cultures were centrifuged, washed with an equal volume of cold saline, and resuspended in distilled water so that aliquots equivalent to 3 to 5 mg of dry cells were added per tube.

Estimation of radioactive serine. At the conclusion of the incubation period all tubes were boiled for 5 min, centrifuged, and the supernate transferred to plastic tubes. A weighed amount of Dowex-50 resin (hydrogen form) was added to the tubes and stirred for 2 min. The resin was recovered by centrifugation, washed with 30 ml of distilled water, and finally stirred with 20 ml of ammonium hydroxide solution (concentrated diluted 1:3). The tube was capped, centrifuged, and aliquots of the supernate dried on planchets. The amount of radioactivity present was determined to a counting error of 0.95. Each time the resin was regenerated, the amount necessary to adsorb the amino acids from simulated incubation mixtures was established. With known amounts of amino acids recovery values of 95% or better were routinely obtained.

To verify that the radioactivity recovered by the above procedure was in reality a measure of the serine present, aliquots of incubation mixtures plus carrier serine were placed on a Dowex-50 resin column (hydrogen form), washed with water, and eluted with increasing normalities of HCl(6). All of the radioactivity was found to elute with the microbiologically determined serine. Paper chromatograms of the eluate also indicated that the radioactivity migrated with serine.

Folic acid compounds. Tetrahydrofolic acid was purchased from Nutritional Biochemicals Corp. and also synthesized according to Hatefi *et al.*(7). Solutions of this compound were stabilized with 0.2% 2-mercaptoethanol and stored at −20°C. At the time of use, samples were formylated according to the method of Futterman and Silverman(8) and assayed with *L. citrovorum* (ATCC 8081). Leucovorin (5-formyltetrahydrofolic acid) was used for the standard curve. Molarities are given in terms of the total (dl-isomer) reduced folic acid compound.

Results. The data recorded in Table I show the response of *L. mesenteroides* to p-aminobenzoic acid, tetrahydrofolic acid, and 5-formyltetrahydrofolic acid in serine-free media. No growth occurred unless one of these 3 compounds was present. When the time of incubation was extended to 40 hr, p-aminobenzoic acid was as effective as the reduced forms of folic acid in promoting growth. Under these conditions there was no similar delay in growth with tetrahydrofolic acid or 5-formyltetrahydrofolic acid.

TABLE I. Effect of p-Aminobenzoic Acid, Tetrahydrofolic Acid, and 5-Formyltetrahydrofolic Acid upon Growth of *Leuconostoc mesenteroides* without Serine.*

	Additions			Optical density	
Tube	PABA	fFH_4	FH_4	20 hr	40 hr
1	—	—	—	.01	.03
2	—	—	+	1.13	1.49
3	—	+	—	1.18	1.49
4	—	+	+	1.19	1.50
5	+	—	—	.30	1.50
6	+	—	+	1.12	1.50
7	+	+	—	1.26	1.52
8	+	+	+	1.30	1.53

* Each 10 ml stoppered tube contained 0.2% 2-mercaptoethanol. Thymidine was added aseptically at 0.01 mg/ml to the basal medium, which was added aseptically to the tubes. Glycine present at 1.33×10^{-2} M; p-aminobenzoic acid at 7.32×10^{-7} M; tetrahydrofolic acid (FH_4) at 3.22×10^{-7} M; and 5-formyltetrahydrofolic acid (fFH_4) at 5.27×10^{-7} M. Incubation at 37°C.

In order to show a more direct effect of the above compounds, the synthesis of serine by washed suspensions of *L. mesenteroides* was examined. Cells grown in the presence of serine, but without p-aminobenzoic acid or any other derivative of folic acid, appeared to have no discernible preformed folic acid cofactor. To cells of this type it was necessary to add glucose, glycine, formate, and a suitable derivative of folic acid for significant serine synthesis. By including C^{14}-labeled formate in the incubation mixture, the amount of radioactivity fixed into serine could be used to estimate the extent of reaction. Incorporation of C^{14}-labeled formate into serine as a function of time is shown in Fig. 1.

Further consideration of the incorporation of C^{14}-labeled formate into serine indicated a relationship between p-aminobenzoic acid, 5-formyltetrahydrofolic acid, and tetrahydrofolic acid, which was quite similar to that observed with cells growing without serine. Although tetrahydrofolic acid apparently was more effective than 5-formyltetrahydrofolic acid in stimulating the synthesis of C^{14}-labeled serine, both were significantly superior to p-aminobenzoic acid in this respect (Table II).

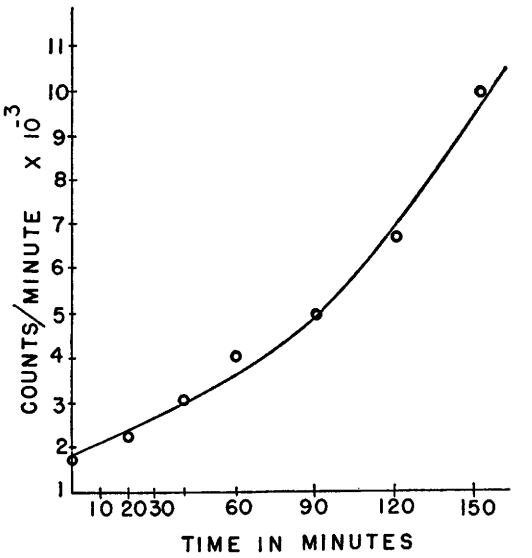

FIG. 1. Synthesis of C^{14}-labeled serine by washed suspensions of *L. mesenteroides*. Each 10-ml tube contained 2.67×10^{-3} M glycine, 5.56×10^{-3} M glucose, 3.12×10^{-4} M C^{14}-labeled formate (potassium and sodium salt) containing 3.26×10^{5} counts/min, 0.1 M phosphate buffer, pH 6.9, pyridoxal hydrochloride 3.22×10^{-7} M. Incubation at 37°C.

Discussion. The data presented in Table I show that both leucovorin and tetrahydrofolic

TABLE II. Effect of Various Compounds upon Synthesis of C^{14}-Labeled Serine by Washed Suspensions of *Leuconostoc mesenteroides*.*

Addition	Radioactivity found in serine (counts/min)
Tetrahydrofolic acid	6250
5-Formyltetrahydrofolic acid	3600
p-Aminobenzoic acid	480
Folic acid	67
None	54

* All additions at 3.22×10^{-7} M. Each 10-ml tube contained 2.67×10^{-3} M glycine, 5.56×10^{-3} M glucose, 3.23×10^{-4} M C^{14}-labeled formate (potassium and sodium salt) containing 5.26×10^{5} cpm, 0.1 M phosphate buffer, pH 6.9, 3.22×10^{-7} M pyridoxal hydrochloride and 0.2% 2-mercaptoethanol. Incubation at 37°C for 2½ hr.

acid were initially more effective than p-aminobenzoic acid in allowing *L. mesenteroides* to proliferate in the absence of serine. However, it is clear that with prolonged incubation a maximal level of growth was attained with p-aminobenzoic acid. The simplest explanation is that *L. mesenteroides* possesses the necessary pathways for converting p-aminobenzoic acid to an active form of folic acid.

The studies with washed suspensions indicated more directly that the folic acid compounds actually affected the synthesis of serine by *L. mesenteroides*. In addition, the results shown in Table II substantiated the growth studies in that leucovorin and tetrahydrofolic were found to be more effective than p-aminobenzoic acid. This discrepancy with respect to the finding of Cross(3) that leucovorin and p-aminobenzoic are of equal activity for *L. mesenteroides* (ATCC 7881) may be the result of his having used a longer incubation period (24 hr). During this period, p-aminobenzoic acid could be converted to an actvie form of folic acid. In the present study, the use of a short incubation period in conjunction with C^{14}-labeled substrate allowed for a minimum exposure of compounds to the action of enzymes present in the cells.

Summary. *Leuconostoc mesenteroides* (ATCC 8042) required a member of the folic acid group of compounds for growth in the absence of serine. p-Aminobenzoic acid, or more efficiently, 5-formyltetrahydrofolic acid and tetrahydrofolic acid, met this requirement. With washed cell suspensions, serine synthesis was followed by incorporation of C^{14}-labeled formate into serine. Over a relatively short incubation period p-aminobenzoic acid had little effect, while the reduced derivatives of folic acid effectively catalyzed the synthesis of serine.

1. Wold, F., Sirny, R. J., *Fed. Proc.*, 1953, v12, 292.

2. Lascelles, J., Cross, M. J., Woods, D. D., *J. Gen. Microbiol.*, 1954, v10, 267.

3. Cross, M. J., *J. Gen. Microbiol.*, 1960, v23, 105.

4. Williams, H. H., Memoir 337, Cornell Univ. Agri. Exp. Station, Ithaca, N. Y., 1955.

5. O'Barr, T. P., Levin, H., Reynolds, H., *J. Bact.*, 1958, v75, 429.

6. Stein, W. H., Moore, S., *Cold Spring Harbor Symposia Quant. Biol.*, 1949, v14, 179.

7. Hatefi, Y., Talbert, P. T., Osborn, M. J., Huennekens, F. M., *Biochem. Preparations*, 1959, v7, 89.

8. Futterman, S., Silverman, M., *J. Biol. Chem.*, 1957, v224, 31.

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Potency Relationships of Various C-21 Steroids as Measured by Two Methods of Liver Glycogen Deposition. (27676)

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In the area of glucocorticoid research numerous observations have been made showing the disparity between anti-inflammatory activity in humans and laboratory animals(1, 2,3). Even more prevalent in the bioassay literature is disagreement between the estimates of adrenocortical steroid efficacy assessed by different criteria of response. One response which has shown wide divergence in steroid potency estimates from different laboratories is liver glycogen deposition(2,3,4). To determine if a relationship exists between liver glycogen deposition potencies assessed by 2 different bioassay methods, a series of 15 substituted adrenocortical steroids was evaluated at Lederle and Upjohn Laboratories.

Materials and methods. Steroids* were ad-

* Steroids kindly supplied by the Organic and Biochemical Research Sections, Lederle Laboratories, and by the Chemistry Dept., Upjohn Co.