sate (subject C) and crystalline egg-white lysozyme‖ to decreasing concentrations of saline. It will be noted that while no consistent change occurs with lysate or crystalline lysozyme, there is a steady increase in lysis of substrate in the presence of leukocytes as the leukocytes are exposed to decreasing concentrations of saline.

Table II compares the percent lysis of substrate in the presence of normal human leukocytes as compared to that observed in the presence of leukocytes from the same suspension after exposure to leukotoxin for one hour. The marked increase in lysis of substrate after exposure of the leukocytes to leukotoxin is evident.

Summary. A method is described for the detection of cell injury by the measurement of intracellular substances released upon injury. The cells studied have been human leukocytes, and the intracellular substance selected for measurement has been an enzyme with the properties of lysozyme. Application of the method is illustrated by data concerning the adverse effect of hypotonicity and of an antiserum to human leukocytes on the cells. In further studies beyond the scope of the present report, the method has been found to be useful in screening a variety of physical, chemical and biologic agents for adverse effect on the metabolism of human leukocytes, as well as for detecting reversal of such injurious effects by protective agents.

‖ Obtained through the courtesy of Armour and Co.

1. Rossiter, R. J., *J. Physiol.*, 1949, v110, 136.
2. Walker, P. G., *Biochem. J.*, 1952, v51, 223.
3. *Separation of Formed Elements, The Protein, Carbohydrate, Steroid, Peptide and Other Components of Plasma.* University Laboratory of Physical Chemistry Related to Medicine and Public Health, Harvard University, July, 1950.
4. Pringle, B. H., DePaulis, D. C., and Pemrick, T. D., *Am. J. Clin. Path.*, 1951, v21, 1039.

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Uracil in Growth and Pyrimidine Nucleotide Synthesis of *Lactobacillus bulgaricus* 09. (19801)

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Orotic acid or a potential source of the compound is required for rapid growth of *Lactobacillus bulgaricus* 09(1-4). Ureidosuccinic acid has small but definite growth-promoting activity(2). Ureides of a number of other mono- and di-carboxylic acids which could not be visualized to yield orotic acid were synthesized and found to be essentially inactive (5). In experiments with *Lactobacillus bulgaricus* orotic acid or ureidosuccinic acid labelled with C^{14} in the ureido carbon gives rise to C^{14}-labelled uridylic and cytidylic acids of the bacterial cell(6). A considerable amount of non-radioactive dilution was observed in the nucleotides isolated despite the essential nature of a utilizable pyrimidine source for growth. Somewhat analogous findings were reported simultaneously by Abrams (7) who observed that a purine-requiring mutant of *Saccharomyces cerevisiae* synthesizes adenine and guanine from 1-C^{14}-labelled glycine as readily as does the wild type parent strain with no purine requirement. It has been reported(1,2) that uracil, uridine, uridylic acid, cytosine, cytidine or cytidylic acid are inactive in satisfying the requirement of *Lactobacillus bulgaricus* 09 for a pyrimidine. These findings led to the hypothesis that nucleic acid synthesis in *Lactobacillus bulgaricus* 09, and possibly in other species as well, normally does not involve the pyrimidine nucleosides and nucleotides as intermediates but instead involves the participation of an orotic acid nucleoside and an orotic acid nucleotide. According to this concept decarboxylation of orotic acid would not occur prior to incorpora-

tion into a "modified" nucleic acid. More extended studies reported here have now shown that while uracil is inactive in satisfying the pyrimidine requirement of *Lactobacillus bulgaricus* 09 under the usual conditions of assay (1-3 days of incubation), by extending the period of incubation (3-7 days) uracil promotes nearly maximal growth. Response curves to orotic acid determined with uracil as a constituent of the basal medium are "catenary" in type. Similar response curves have been reported previously showing the behavior of a tryptophane-synthesizing strain of *Lactobacillus arabinosus* to tryptophane(8) or of a histidine-synthesizing mutant of *Escherichia coli* histidine-less to histidine(9-12). Ryan and Schneider(9-12) have shown that these "catenary" type curves are to be expected if it is assumed that: (a) the bacterial population under study consists of a mixture of metabolite-requiring and metabolite-synthesizing strains, (b) growth in the "blanks" is due to unrestrained growth of the synthesizing strain, and (c) conformity to the expected response curve at intermediate and high levels of the metabolite is due to the existence of a certain "selection pressure" favoring the metabolite-dependent strains.

In addition to demonstrating the type of curves obtained with *Lactobacillus bulgaricus* 09 in response to orotic acid determined with and without uracil as a component of the basal medium, data are presented in this paper on the utilization of C^{14}-labelled uracil for the synthesis of bacterial uridylic and cytidylic acids when *Lactobacillus bulgaricus* 09 is grown under the following conditions: (a) in the presence of C^{14}-uracil as the only known source of pyrimidine (requires prolonged incubation) and, (b) in the presence of equal amounts of unlabelled orotic acid and C^{14}-uracil (rapid growth of the organism).

Experimental. Lactobacillus bulgaricus 09 was maintained as described in previous papers(1,2). Other procedures were according to customary technics with lactic acid bacteria (13). The basal medium had the composition given in Table I. Where uracil was used as a component of the basal medium it was present to the extent of 10 mg per liter of double strength medium. Titration of acid production using bromthymol blue as an indicator was used as a measure of the extent of bacterial growth. In the radioisotope experiments, *Lactobacillus bulgaricus* 09 was grown in 500 ml amounts of medium supplemented with 5 mg of C^{14}-uracil where this compound was used alone to promote growth and with 5 mg of orotic acid and 5 mg of C^{14}-uracil where the 2 compounds were used together. Thus these experiments were comparable in their design to those reported earlier in a preliminary note(6) where *Lactobacillus bulgaricus* 09 was grown with 5 mg of C^{14}-labelled orotic acid or with 15 mg of C^{14}-labelled DL-ureidosuccinic acid. The 2-C^{14}-labelled uracil was synthesized by the condensation of C^{14}-urea with malic acid according to a modification of the method of Johnson and Flint(14). Following the growth of *Lactobacillus bulgaricus* 09 at 37°C for 3 days with the combined supplement of orotic acid and C^{14}-uracil and for 7 days with the C^{14}-uracil alone the cells were centrifuged and washed once with water. The washed cells then were dried in a desiccator over Drierite. The dry cells (about 150 mg) were finely ground in the presence of 0.2 ml of 5% tri-

TABLE I. Basal Medium.*

Acid-hydrolyzed, norit-treated, vit.-free casein	10	g
Tryptic-digested, norit-treated, vit.-free casein	10	
DL-tryptophane	400	mg
L-cystine	200	
Glucose	40	g
Sodium acetate · 3 H_2O	20	
Adenine	10	mg
Guanine	10	
Xanthine	10	
Salts A	10	ml
Salts B	10	
Thiamine	2	mg
Riboflavin	2	
Nicotinic acid	2	
Ca pantothenate	2	
Pyridoxine	4	
PABA	1	
Biotin	10	γ
Folic acid	500	
Pyridoxal	500	
Vit. B_{12}	.4	
Tween 80	2	ml
pH to 5.8		

* Quantities given are for the preparation of one liter of double strength medium.

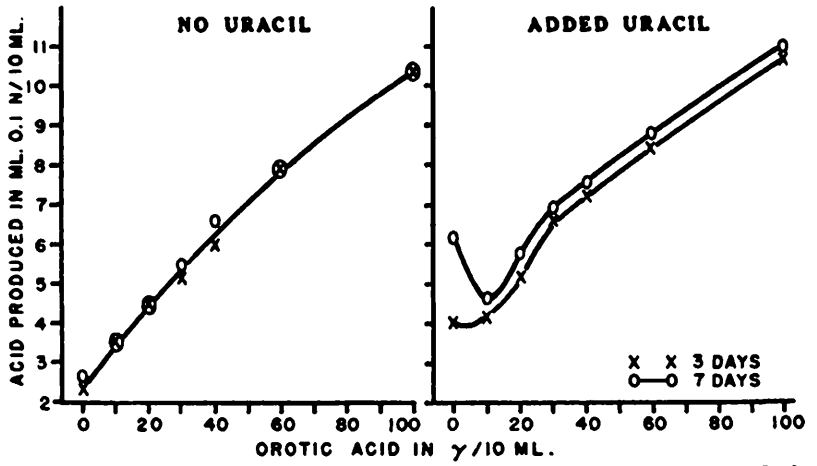

FIG. 1. Response of *Lactobacillus bulgaricus* 09 to orotic acid in the presence and absence of uracil as a component of the basal medium.

chloroacetic acid and the nucleoproteins then were hydrolyzed with 2 ml of 5% trichloroacetic acid at 100°C for 30 minutes. The precipitated proteins were centrifuged and the supernatant solutions containing the liberated nucleic acids hydrolyzed with equal volumes of 2 N HCl at 100°C for one hour. The hydrolysates containing free purines bases and pyrimidine nucleotides were chromatographed on paper using the tertiary butanol-0.8 N HCl mixture of Smith and Markham(15). The pyrimidine nucleotides were located with the aid of a Mineralight lamp and were eluted with water. The water eluates (5 to 10 ml) of each nucleotide were chromatographed on Dowex-1 (Cl^-) columns (about 0.7 cm x 15 cm) according to Cohn(16). Cytidylic acid was eluted with 0.002 N and uridylic acid with 0.01-0.02 N HCl. Those fractions with satisfactory absorption ratios at 278/262 mμ were quantitated in the Beckman spectrophotometer and evaporated to dryness. The molecular extinction coefficients of Ploeser and Loring(17) were used in these calculations. The published molecular extinction coefficients were found to be in good agreement with those found in this laboratory for high quality commercial samples of uridylic and cytidylic acids. The dried purified pyrimidine nucleotides were taken up in small volumes of water and plated. Counts on the labelled compounds and the isolated pyrimidine nucleotides were obtained with a gas flow counter.

Results and discussion. Results of a growth experiment are summarized in Fig. 1. In the absence of uracil from the basal medium growth with or without orotic acid is not influenced by increasing the period of incubation from 3 to 7 days. In the presence of uracil as a component of the basal medium, the growth response to orotic acid is not influenced by prolonging the period of incubation from 3 to 7 days. On the other hand, with uracil growth occurs in the blanks (no added orotic acid) to yield the catenary type curves previously referred to. The mechanism of the selection pressure in favor of those organisms dependent upon orotic acid for growth has not been elucidated.

Results of the isotopically-labelled incorporation experiments are summarized in Table II. It is apparent that if incubation be prolonged (7 days) (Exp. 3) bacterial uridylic and cytidylic acids may be derived from uracil as well as orotic and ureidosuccinic acids (Exp. 1 and 2). These data, together with the results obtained from the growth studies, would appear to indicate the existence in *Lactobacillus bulgaricus* 09 cultures of spontaneously occurring mutants that utilize uracil for purposes of pyrimidine nucleotide synthesis. In the presence of equal amounts of orotic acid and C^{14}-labelled uracil about 5% of the uricylic and cytidylic acids synthesized appear to be derived from uracil. Of special interest is the fact that despite the variety of

TABLE II. Utilization of C^{14}-Uracil for the Synthesis of Uridylic and Cytidylic Acids by *Lactobacillus bulgaricus* 09.

Exp. No.	Pyrimidine source	Incubation, days	Activities: Compound counted	Activities: cpm/μM	Activities: % of pyrimidine source
1	C^{14}-orotic acid*	3	Orotic acid	11650	
			Uridylic "	7500	64
			Cytidylic "	7950	68
2	C^{14}-ureidosuccinic acid*	3	Ureidosuccinic acid	12060	
			Uridylic "	7600	63
			Cytidylic "	7000	58
3	C^{14}-uracil	7	Uracil	14936	
			Uridylic acid	13019	87
			Cytidylic "	11338	76
4	Unlabelled orotic acid C^{14}-uracil	3	Uracil	14936	
			Uridylic acid	1013	6.8
			Cytidylic "	693	4.6

* Data from these experiments have been presented in a preliminary note(6).

pyrimidine sources employed to promote growth and the differences in time required to attain maximal growth (1-2 days with orotic acid (Exp. 1), 2-3 days with ureidosuccinic acid (Exp. 2), and 7 days with uracil alone (Exp. 3)) all of the pyrimidine nucleotides isolated were less active than were the labelled compounds used to promote growth. Some possible explanations for this phenomenon are the following: (a) orotic acid and/or uracil is required in nucleic acid synthesis only as a "primer" to initiate growth; (b) alternative pathways for pyrimidine nucleotide synthesis may exist that do not involve orotic acid, uracil, or ureidosuccinic acid as intermediates; (c) bacterial uridylic and cytidylic acids may be of two "types," one type derivable from known precursors on which *Lactobacillus bulgaricus* 09 is dependent for growth and the other type(s) derivable from other unrecognized components of the basal medium; and (d) orotic acid and/or uracil is required for some essential function quite unrelated to pyrimidine nucleotide synthesis and its incorporation into nucleic acid components is only incidental.

Summary. Growth and isotopically-labelled incorporation studies have shown that *Lactobacillus bulgaricus* 09 utilizes uracil in lieu of orotic acid provided the incubation time is greatly prolonged. Some implications of this finding are discussed.

1. Wright, L. D., Huff, J. W., Skeggs, H. R., Valentik, K. A., and Bosshardt, D. K., *J. Am. Chem. Soc.*, 1950, v72, 2312.

2. Wright, L. D., Valentik, K. A., Spicer, D. S., Huff, J. W., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 293.

3. Huff, J. W., Bosshardt, D. K., Wright, L. D., Spicer, D. S., Valentik, K. A., and Skeggs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1950, v75, 297.

4. Wieland, O. P., Avener, J., Boggiano, E. M., Bohonos, N., Hutchings, B. L., and Williams, J. H., *J. Biol. Chem.*, 1950, v186, 737.

5. Spicer, D. S., Liebert, K. V., Wright, L. D., and Huff, J. W., PROC. SOC. EXP. BIOL. AND MED., 1952, v79, 587.

6. Wright, L. D., Miller, C. S., Skeggs, H. R., Huff, J. W., Weed, L. L., and Wilson, D. W., *J. Am. Chem. Soc.*, 1951, v73, 1898.

7. Abrams, R., *J. Am. Chem. Soc.*, 1951, v73, 1888.

8. Wright, L. D., and Skeggs, H. R., *J. Biol. Chem.*, 1945, v159, 611.

9. Ryan, F. J., and Schneider, L. K., *J. Bact.*, 1948, v56, 699.

10. ———, *J. Bact.*, 1949, v58, 181.

11. ———, *J. Bact.*, 1949, v58, 191.

12. ———, *J. Bact.*, 1949, v58, 201.

13. Snell, E. E., *Vitamin Methods*, vI, 1950, Academic Press, New York, p327.

14. Johnson, T. B., and Flint, R. B., *J. Am. Chem. Soc.*, 1931, v53, 1079.

15. Smith, J. D., and Markham, R., *Biochem. J.*, 1950, v46, 509.

16. Cohn, W. E., *J. Am. Chem. Soc.*, 1950, v72, 1471.

17. Ploeser, J. M., and Loring, H. S., *J. Biol. Chem.*, 1949, v178, 431.

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