Biosynthesis of Purine and Pyrimidine Nucleotides in *Pasteurella multocida.** (28547)

A. S. Issaly† and A. O. M. Stoppani

Institute of Biochemistry, School of Medicine of Buenos Aires, and Laboratory for Cell Metabolism, National Atomic Energy Commission

The ability of bacteria to synthesize purine and pyrimidine nucleotides varies greatly with the species. For example, in *Escherichia coli* (1,2) and yeasts(3), synthesis *de novo* takes place while in *Tetrahymena gelei*(4) and *Corynebacterium diphtheriae*(5) bases or nucleosides must be supplied exogenously. The observations summarized below support the inclusion of *Pasteurella multocida* in the first group of microorganisms.

Materials and methods. *Past. multocida* (strain Beaufort No. 28 from Inst. Pasteur, Paris) was grown in a liquid medium consisting of meat extract (Bovril), 5 g; peptone (Biochem) 30 g; yeast extract (Difco), 1 g; glucose, 20 g; sodium chloride, 5 g and distilled water to 1 l; pH 7.2. After incubation for 16 hours at 37° the cells were transferred to fresh medium containing the radioactive substrates (experiment with proliferating cells). For experiments with resting cells, portions of the liquid culture were layered on 150 ml of medium solidified with agar (25 g/l) in Roux bottles and further incubated at 37° for 24 hours. The colonies were suspended in 0.85% NaCl and collected by centrifugation at 13,000 *g* three times. The concentration of the cell suspensions was determined by comparison of the optical density with a standard curve made with a cell suspension the concentration of which was known by measurement of dry weight (at 100°). The radioactive substances were obtained from The Radiochemical Centre, Amersham, England, with the following specific activities (mC/mmole); formate-C^{14}, 5.0 and 11.0; pyruvate-2-C^{14}, 3.22; glycine-2-C^{14}, 3.70; glucose-6-C^{14}, 2.18 and adenine-8-C^{14}, 4.46. Phosphate-P^{32} was carrier-free. Under conditions of radioassay used 1 mC was equivalent to 1.18×10^8 cts/min. Chloramphenicol was from Parke Davis & Co.

Past. multocida cells were incubated with the radioactive substrates in Erlenmeyer flasks as described in Table I. Portions of the cell suspensions were thoroughly mixed with 9 vol. of methanol and centrifuged. The residue was suspended in 3 ml 5% (w/v) trichloracetic acid (TCA), heated at 90° for 30 minutes, cooled and centrifuged(1,6); the residue was washed in the centrifuge with 1 ml cold TCA solution and the supernatants combined (nucleic acid extract). When necessary, the precipitates remaining after the TCA extraction were washed once with methanol, plated and counted for ^{14}C (protein fraction). The nucleic acid extracts were hydrolysed by treatment with 0.05 *N* HCl at 100° for 1 hour with frequent stirring(7). After cooling, the digests were freed of TCA by extraction with ethyl ether and brought to a small volume *in vacuo* at 4°. Nucleotides and bases were chromatographed according to Wyatt(8) and radioautographed. The radioactive substances were counted directly on the chromatograms with Scott tubes and identified by cochromatography with pure specimens. In the experiment with glucose-6-C^{14}, cytidylic acid was hydrolysed with perchloric acid. The resulting cytosine was chromatographed on paper(9) and counted for C^{14}. Total radioactivity in each substance, calculated according to Stoppani *et al.*(10), is expressed as cts./min./mg of cells. Proteins were determined according to Lowry *et al.*(11) and the nucleic acid content of cells according to Mitchell(12).

Results and discussion. After incubation of *Past. multocida* with the substrates listed in Table I, radioactive adenine, guanine, cytosine and the respective nucleotides (AMP, GMP and CMP) as well as uridylic acid (UMP) and deoxythymidylic acid (d-TMP) were identified in the nucleic acid digest. With formate-C^{14} as substrate (Exp. i), adenine,

* Supported by grants from Rockefeller Foundation and Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina).

† On leave from Inst. Nacional de Microbiol.

TABLE I. Incorporation of Labeled Precursors into Components of Nucleic Acids from *Past. multocida*.

Reaction medium: 1.0 mM phosphate buffer, pH 7.2 (expts. with resting (*R*) cells) or culture medium (expts. with proliferating (*P*) cells). 1.7 (expt. *R*) and 1.5 mM (expt. *P*) formate-C^{14}; 0.17 mM glycine-2-C^{14}; 0.05 mM adenine-8-C^{14}; 1.3 mM pyruvate-2-C^{14}; 0.1 mM glucose-6-C^{14} and 5.0 mM glucose. Total vol. of reaction mixture, 10 ml except expt. i (*R*)(12 ml) and i(*P*)(14 ml). C^{14}(cts./min. $\times 10^7$): 22.9 (formate-C^{14}); 1.84 (glycine-2-C^{14}); 1.87 (adenine-8-C^{14}); 8.85 (pyruvate-2-C^{14}) and 0.66 (glucose-6-C^{14}). A, adenine; G, guanine; AMP, adenylic; GMP, guanylic; CMP, cytidylic and d-TMP, deoxythymidylic acid.

Exp	*Past. multocida* (mg/ml)	Substrate	Incubation time (min.)	C^{14} (cts./min./mg of cells) A	AMP	G	GMP	CMP	UMP	d-TMP
i(*R*)	26	Formate-C^{14}	.5	120	78	68	—	175	60	198
i(*R*)	26	"	1.0	98	55	70	—	140	43	220
i(*R*)	26	"	5.0	68	78	50	—	92	42	107
i(*P*)	2.7	"	.5	157	357	460	220	440	—	240
i(*P*)	2.7	"	1.0	128	160	213	100	335	—	290
i(*P*)	2.7	"	5.0	200	220	175	—	490	—	440
ii(*R*)	23	Glycine-2-C^{14}	.5	65	100	—	100	—	40	35
ii(*R*)	23	"	1.0	75	120	—	91	—	40	44
ii(*R*)	23	"	5.0	93	184	—	144	—	67	74
ii(*R*)	23	Glycine-2-C^{14} + glucose	.5	50	230	—	150	—	50	60
ii(*R*)	23	*Idem*	5.0	154	297	—	320	—	102	114
ii(*P*)	1.8	Glycine-2-C^{14}	.5	44	132	—	92	—	—	—
ii(*P*)	1.8	"	5.0	80	262	—	138	—	—	—
iii(*R*)	23	Adenine-8-C^{14}	5.0	28	71	—	104	—	—	—
iii(*P*)	1.8	"	5.0	73	43	34	32	—	—	—
iv(*R*)	31	Pyruvate-2-C^{14}	.5	146	160	—	90	190	—	290
iv(*R*)	31	"	1.0	300	390	—	300	460	—	960
iv(*R*)	31	"	3.0	125	108	—	100	170	—	300
iv(*R*)	31	"	5.0	90	150	—	100	140	—	330
v(*R*)	26	Glucose-6-C^{14}	3.0	52	40	—	—	131	—	917
v(*R*)	26	"	5.0	11	15	—	—	29	—	100
v(*P*)	3.1	"	3.0	39	73	33	—	208*	—	78
v(*P*)	3.1	"	5.0	46	42	68	—	265†	—	45

C^{14} in cytosine. (%): * 65. Same, † 54.

AMP, GMP, CMP, UMP and d-TMP appear radioactive with a relatively higher proportion of C^{14} in the pyrimidine compounds. Since in *Past. multocida* formate is not a precursor of glucose, pentose or the related phosphates(13), the radioactivity of nucleotides is due to the bases. With glycine-2-C^{14} as substrate (Exp. ii), adenine, AMP, GMP and UMP become radioactive and significant labeling of the purine compounds is seen. The introduction of glycine-2-C^{14} and the introduction of formate-C^{14} into the purine molecule, are two essential steps of the standard metabolic pathway for adenine biosynthesis in animal tissues and bacteria(14) and according to Exp. ii in *Past. multocida* guanine can be formed from adenine or a common precursor. The incorporation of carbon atoms from formate-C^{14} (Exp. i), pyruvate-2-C^{14} (Exp. iv) and glycine-8-C^{14} into purines is relatively fast with the first two and slow with the latter. This is consistent with the earlier introduction of glycine into the purine molecule. Active synthesis of nucleic acids in proliferating cells determines a higher rate of formate-C^{14} fixation in the total purine bases (nucleotide plus free base values) notwithstanding the eventual dilution of formate-C^{14} with related "1-C" compounds formed from the substrates present in the culture medium. Furthermore, glucose increases the incorporation of glycine-2-C^{14} into the purines, which agrees with the role of glucose as a source of energy and pentose-5-phosphate for the synthesis of purine nucleotides. In this connection it is noteworthy that the pentose pathway is a major route of glucose oxidation in *Past. multocida*(15).

With pyruvate-2-C^{14} (Exp. iv) or glucose-6-C^{14} (Exp. v) as substrates, adenine, guanine, cytosine, AMP, GMP, CMP, and d-TMP incorporate radioactivity. Glucose yields pyru-

TABLE II. Effect of Chloramphenicol on Protein and Nucleic Acids Biosynthesis in *Past. multocida*.

Past. multocida (6 hr proliferating suspension; 0.5 mg/ml) incubated for 2 hr in 40 ml culture medium at 37°. Additional substrates: glycine-2-C^{14}, 8.60 μmole (1.84×10^7 cpm) and adenine-8-C^{14}, 4.57 μmole (1.87×10^7 cpm).

Chloramphenicol (μg/ml)	Final concentration: Cells dry wt (μg/ml)	Protein (μg/ml)	Nucleic acids (μg/ml)	Nucleic acids (μg/mg cells)	Incorporation of C^{14} (cpm/mg of cells): Glycine-2-C^{14} Nucleic acids	Glycine-2-C^{14} Protein fraction	Adenine-8-C^{14} Nucleic acids
0	2800	278	320	114	1800	1400	12300
5	1500	53	280	185	2000	620	16800
15	820	44	270	330	2250	200	23200
25	500	29	235	470	2470	220	25600

vate either through glycolysis or the pentose pathway(15) and therefore, incorporation of glucose and pyruvate carbon atoms into the bases must follow a similar pattern. Pyruvate enters the Krebs cycle and subsequently, formation of aspartic acid would precede the incorporation of pyruvate carbon atoms into the pyrimidines. Similarly, conversion of pyruvate into serine, glycine and formate should be prior steps to purine labeling. On the other hand, the methyl carbon of thymine could be labeled directly by formate or related "1-C" compounds. These reaction mechanisms are borne out by the distribution of formate-C^{14} and pyruvate-2-C^{14} in the methanol-water soluble fraction of *Past. multocida*(13). Thus, after 60 minutes incubation of formate-C^{14} with resting cells, glycine, serine and aspartic acid fixed 1.4, 2.8 and 3.3% of total C^{14} in the soluble fraction; with pyruvate-2-C^{14} as substrate the corresponding values for glycine and aspartic acid were 0.7, respectively, and with proliferating cells, 2.1 (serine) and 1.6 (glycine).

The synthesis of nucleotides requires phosphate incorporation which is much faster with proliferating than with resting cells. In fact, with resting *Past. multocida* (57 mg) incubated at 37°, for 5 minutes with 15 ml pH 7.2 m*M* phosphate buffer-P^{32} (3.68×10^8 cts/min), the activity in the nucleic fraction was 4700 cts/min/mg of cells. P^{32} incorporation was not increased by 5 m*M* glucose or 10 m*M* pyruvate. Under the same experimental conditions, with 41 mg of cells proliferating in culture medium with phosphate-P^{32} (carrier-free, same activity as above) the activity incorporated in the nucleic acid fraction was 25000.

In *Past. multocida* as in other bacteria(16) nucleic acid synthesis and protein synthesis can be dissociated with chloramphenicol (Table II). With glycine-2-C^{14} as substrate, C^{14} incorporation into the "protein fraction" is proportional to cell growth. The antibiotic prevents to nearly the same extent both cell growth (measured by dry weight and protein concentration of the cell suspension) and incorporation of glycine-2-C^{14} into the protein residue after fractionation with TCA. On the other hand, there is a lesser diminution in nucleic acid synthesis, as indicated by the concentration of the latter in the suspension medium. Nevertheless, in the presence of chloramphenicol the relative proportion of nucleic acid *vs* cell dry weight increases significantly, like the incorporation of adenine-8-C^{14} into the nucleic acid fraction. The incorporation of glycine-2-C^{14} into the latter is similarly affected by the antibiotic although calculation of specific activities on the basis of nucleic acid concentration shows an increase with adenine-8-C^{14} and a diminution with glycine-2-C^{14}.

Summary. *Past. multocida* synthesizes *de novo* nucleic acid components. Purine and pyrimidine bases incorporate the radioactive carbon from formate-C^{14}, glycine-2-C^{14}, pyruvate-2-C^{14} and glucose-6-C^{14} as substrates. Adenine and guanine incorporate adenine-8-C^{14}. Nucleic acids take up phosphate-P^{32} at a higher rate during cell proliferation. Chloramphenicol produces an apparent stimulation of nucleic acids synthesis and simultaneously,

an effective inhibition of protein synthesis.

1. Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T., Britten, R. J., *Studies of Biosynthesis in* Escherichia coli, Carnegie Inst. of Washington Publ. 607, Washington, D. C., 1955.

2. Magasanik, B., *Ann. Rev. Microbiol.,* 1957, v11, 221.

3. Harris, G., in *Chemistry and Biology of Yeasts,* Cook, A. H., ed., Academic Press Inc. New York, 1958, p. 437.

4. Kidder, G. W., Dewey, V. C., *Proc. Nat. Acad. Sci.,* 1948, v34, 566.

5. Dalby, A., Holdsworth, E., *J. Gen. Microbiol.,* 1956, v15, 335.

6. Schneider, W. C., *J. Biol. Chem.,* 1945, v161, 293.

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

8. Wyatt, G. R., *ibid.,* 1951, v48, 584.

9. Marshak, A., Vogel, H. J., *ibid.,* 1951, v189, 597.

10. Stoppani, A. O. M., Conches, L., de Favelukes, S. L. S., Sacerdote, F. L., *Biochem. J.,* 1958, v70, 438.

11. Lowry, O. H., Rosebrough, N. I., Lewis Farr, A., Randall, R. J., *J. Biol. Chem.,* 1951, v193, 265.

12. Mitchell, P., *J. Gen. Microbiol.,* 1950, v4, 389.

13. Issaly, I. S. M., de, *Ph.D. Thesis,* Univ. of Cordoba, Argentina, 1962.

14. Shulman, M. P., *Metabolic Pathways,* Greenberg, D. M., ed. Academic Press, Inc., New York, 1961, v2, 389.

15. Issaly, I. S. M., de, Issaly, A. S., Stoppani, A. O. M., *Nature,* 1961, v191 727.

16. Gale E. F., *The Bacteria,* Gunsalus, I. C. and Stanier, R. Y., eds. Academic Press Inc., New York, 1962, v3, 471.

Received May 16, 1963. P.S.E.B.M., 1963, v113.

A Simplified Method for Sampling Small Animal Carcasses for Analyses.* (28548)

E. W. HARTSOOK AND T. V. HERSHBERGER (Introduced by N. B. Guerrant)
Department of Animal Industry and Nutrition, Pennsylvania State University, University Park

One of the greatest difficulties encountered in metabolic balance studies, using small laboratory animals as experimental subjects, is that of obtaining representative samples of the carcass of the animal for analyses.

Various methods have been employed to effect representative sampling. Such methods have recently been discussed by Mickelson and Anderson(1), who also presented a method of preparing carcasses for analysis. The carcass is autoclaved, slurried in a Waring blendor, and homogenized in a colloid mill. This method has the advantages of allowing the animal skin to be readily disintegrated (due to autoclaving), allows the analysis of multiple aliquots of the carcass for any and all components, produces a homogenate of very small particle size, and results in analytical values with very small standard deviations. However, quantitative transfer of the slurry from the blendor container to the colloid mill is necessary and the colloid mill is expensive. This led us to investigate a technique which would obviate a colloid mill.

The method described consists of autoclaving the carcass, blending it in a Waring blendor, and transferring aliquots of the slurry directly to tared weighing bottles. A large-bore pipette is used to sample the slurry while the blendor is in operation. This technique was used to prepare the carcasses of 5 weanling and 28 young-adult male albino rats for moisture, total nitrogen and energy determinations. Analytical values obtained are reported and discussed.

Materials and methods. The animals were 33 weanling male albino rats averaging 78 g in body weight. Twenty-eight of the animals were the subjects in a 53-day metabolic study involving 7 quadruplicates of animals and equalized daily feeding within each quadruplicate of 4 experimental isocaloric diets containing 8-57% protein (casein), 18.6-2% fat (lard and corn oil), and 65-21% carbohydrate (cerelose). At completion of the metabolic study the experimental animals

* Paper No. 2745 in the Journal Series, Pennsylvania Agri. Exp. Station.