CHANGE IN PYRIMIDINE DEOXYRIBONUCLEOSIDE METABOLISM IN CELL CULTURE CAUSED BY MYCOPLASMA (PPLO) CONTAMINATION*

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Several lines of mammalian cells including Sarcoma 180 (S-180), human carcinoma (HeLa) and human monocytic leukemia (J-111) cells when grown in a modified medium containing amethopterin can utilize thymidine, thymidylic acid and 5-methylcytosine deoxyribonucleoside as sources of DNA-thymine (Hakala, M. T. and Taylor, E., 1959). When this medium contained 5-bromodeoxyuridine in place of thymidine, growth also occurred (Hakala, M. T., 1959 and 1962). All of the cell lines studied in this laboratory were capable of growth in such media. Recently, however, an exception was noted. The growth of a HeLa cell line purchased from a commercial supplier was not supported by these deoxyribonucleosides and also had lost its sensitivity to 5-fluorodeoxyuridine. Upon finding that these observations were related to contamination of the culture by Mycoplasma, this line was designated as HeLa/PPLO.

Materials and Methods.--The mammalian cell lines used in this study, mouse Sarcoma-180 (S-180) cells and human carcinoma (HeLa/PPLO) cells were purchased from Microbiological Associates, Inc., Bethesda, Maryland. The S-180 cell line has been carried in this laboratory for six years while HeLa/PPLO was purchased in the summer of 1962. The strain of Mycoplasma, Laidlaw, B., was kindly supplied to us by Dr. H. E. Morton, University of Pennsylvania, Philadelphia, Pennsylvania.

The cell cultures are ordinarily maintained in Eagle's medium (Eagle, H., 1959) containing 10^{-6}M folic acid and 10% horse serum. The amethopterin medium used in this study consists of Eagle's medium supplemented with amethopterin (1 μM), hypoxanthine (100 μM), thymidine (30 μM) and glycine (100 μM) (Hakala, M. T. and

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Taylor, E., 1959). The liquid medium used for growing the different strains of Mycoplasma was beef heart infusion broth (Difco), pH 7.8, to which 15% of horse serum had been added.

5-Fluorodeoxyuridine-2-C^{14} with an activity of 10.9 mc/m mole was kindly supplied to us by Dr. Robert Duschinsky, Hoffmann-LaRoche, Inc., Nutley, New Jersey.

Results and Discussion.--When maintained on the amethopterin medium a previous HeLa cell line also from Microbiological Associates, Inc., had grown to 1/2 maximum in the presence of 5 to 6 μM thymidine or its derivatives while 30 to 100 μM supported maximal growth (Hakala, M. T. and Taylor, E., 1959). However, HeLa/PPLO completely failed to grow (0.3 x inoculum) at concentrations of thymidine, thymidylic acid or 5-methylcytosine deoxyribonucleoside ranging from 10 to 100 μM while the control in folic acid medium grew 17-fold (Table I). 5-Bromodeoxyuridine and 5-iododeoxyuridine also failed to support growth under these conditions.

TABLE I

EFFECT OF DEOXYRIBONUCLEOSIDES ON THE GROWTH OF CULTURED CELLS

Cell Line	50% Inhibitory [1] Concentration		Thymidine [2] Required for 1/2 Maximal Growth
	5-Fluoro-uracil μM	5-Fluoro-deoxyuridine μM	μM
S-180	1.5	0.035	2.5
HeLa/PPLO	2.0	2.0	10-100 no growth

1 The basic medium used was that of Eagle (1959); the experiments were carried for 7 days with four changes of the medium.

2 Determined in amethopterin (1.0 μM) medium supplemented with hypoxanthine (100 μM) and glycine (100 μM), (Hakala, M. T. and Taylor, E., 1959).

Incubation of thymidine (0.96 mM) with HeLa/PPLO (4 x 10^6 cells/ml) caused the cleavage of 77 mμmoles/ml/hr (Table II) while the S-180 cell line completely

failed to cleave thymidine under identical conditions. The observation that 5-fluorouracil and 5-fluorodeoxyuridine were equally effective inhibitors of HeLa/PPLO while for S-180 the nucleoside was 43 times more inhibitory than the free base (Table I) suggested that 5-fluorodeoxyuridine was also being cleaved by HeLa/PPLO. Rapid cleavage of 5-fluorodeoxyuridine (60 μM) was indeed demonstrated (Table II).

TABLE II

CLEAVAGE OF DEOXYRIBONUCLEOSIDES BY CULTURED CELLS

Cell Line	μmoles Recovered [1] After Incubation		mμmoles Recovered After [2] Incubation		
	Thymine	Thymidine	Time min.	5-Fluoro-uracil 2-C^{14}	5-Fluoro-deoxyuridine 2-C^{14}
None	0	4.8		-	-
S-180	0	4.4		-	-
None	0	4.7	30	3	53
HeLa/PPLO	2.3	2.2	15	29	27
			30	49	10
			60	57	0

[1] 2 x 10^7 cells incubated for 6 hours at 36°C with frequent stirring in 5 ml of amethopterin medium containing 0.96 mM thymidine; horse serum, phenol red and antibiotics were omitted. Supernatant (0.2 ml) was chromatographed on Whatman #1 filter paper strip (1 inch) using butanol-ammonia (3:1) as the solvent. Ultraviolet absorbing spots were eluted into 4 cc of 0.1 N HCl. Spectra were identified and quantitated using a Zeiss Spectrophotometer.

[2] 5.8 x 10^6 cells incubated in a shaking waterbath at 37°C in 1.1 ml of Eagle's medium containing 60 μM 5-fluorodeoxyuridine 2-C^{14}; horse serum, phenol red and antibiotics were omitted. Supernatant (0.05 ml) was chromatographed on Whatman #1 filter paper strip (1.5 inch) using isopropanol-hydrochloric acid-water (4:1:1) as the solvent. Strips were air dried, divided in 0.5 cm transverse strips, immersed in toluene containing 2,5-diphenyloxazole and 1,4-bis-2 (4-methyl-5-phenyloxazolyl)-benzene and counted in a Tri Carb liquid scintillation counter.

Since HeLa/PPLO was found to be more sensitive to 6-mercaptopurine (50% inhibition at 0.2 μM) in folic acid medium than the HeLa cell line studied previously

(Hakala, M. T. and Nichol, C. A., 1959) it is concluded that the enzyme system for metabolizing hypoxanthine was not impaired (Brockman, R. W. et al., 1961).

HeLa/PPLO cultures were found to contain Mycoplasma (PPLO), and during this period contamination of two other cell lines by this organism occurred in our laboratory. After this contamination these two cell lines also became unable to grow in the amethopterin medium even though their growth in Eagle's medium continued in satisfactory manner. One PPLO strain (#152) was isolated from HeLa/PPLO. This organism and also four other strains from various sources were incubated with 0.88 mM thymidine. Two strains of PPLO (#152 and #880) were also incubated with 5-fluorodeoxyuridine (60 μM). The results are shown in Table III. It is interesting to note that nucleosides are cleaved much more rapidly by PPLO (#152) together with host cells than by the isolated organisms themselves. There seems to be some conflict between these results and those of Razin, S. (1960 and 1962) who demonstrated that in the absence of folinic acid both thymine and thymidine could support the growth of the saprophytic strains of PPLO (Mycoplasma laidlawii A and B), but that thymidine was more effective. If rapid cleavage had occurred in Razin's experiments the two compounds should have been equal in promoting growth.

Although the PPLO associated enzyme which performs the cleavage of the deoxyribonucleosides as described here was not identified, it seems likely that this enzyme is a pyrimidine nucleoside phosphorylase. In addition to thymidine and 5-fluorodeoxyuridine, the evidence from growth studies suggests that 5-methylcytosine deoxyribonucleoside, 5-bromodeoxyuridine and 5-iododeoxyuridine are also among the substrates being cleaved. Thus, the substrate specificity indicates similarity with the pyrimidine nucleoside phosphorylase isolated from horse liver (Friedkin, M. and Roberts, D., 1954).

That PPLO can modify or interfere with the metabolism of host cells has previously been suggested but not with respect to pyrimidine nucleosides. The amino

TABLE III

CLEAVAGE OF DEOXYRIBONUCLEOSIDES BY MYCOPLASMA

Mycoplasma Strain [1]	μmoles Recovered [2] After Incubation		mμmoles Recovered After [3] Incubation		
	Thymine	Thymidine	Time	5-Fluoro-uracil-2-C ^{14}	5-Fluoro-deoxyuridine 2-C ^{14}
None	0	1.40			
#880	1.00	0.37	15 min.	46	14
			30 min.	57	0
#152	0.34	0.96	1 hr.	5	52
			3 hrs.	11	45
			6 hrs.	18	38
#511	0.48	1.00			
SW 1	0.90	0.48			
Laidlaw B	0.68	0.44			

[1] Strain #152 isolated in 1962 by J. S. H. from HeLa/PPLO; #511 in 1957 from human vagina (Horoszewicz, J. S., 1961); SW 1 in 1961 by J. S. H. from the snout of a swine; Laidlaw B from raw sewage (Laidlaw, P. and Elford, W. J., 1936); #880 in 1961 from the spleen of a patient with chronic lymphocytic leukemia (Grace, J. T., et al.).

[2] The washed PPLO pellet originating from 200 ml of 6 day old liquid culture was incubated for 6 hours at 37°C with 2 cc of amethopterin medium containing 0.88 mM thymidine; horse serum, antibiotics and phenol red omitted. The supernatants were analysed as described in footnote 1, Table II. The quantities of PPLO used for each incubation were not standardized. Thus, e.g., #880 which grows fast and #152 which grows very poorly might be expected to give quantitatively different results in this experiment.

[3] The PPLO pellet originating from 30 ml of a 5 day old liquid culture was incubated in a shaking water bath at 37°C in 1.1 ml Eagle's medium containing 60 μM 5-fluoro-deoxyuridine-2-C^{14}; horse serum, phenol red and antibiotics were omitted. Aliquots of the suspension (0.05 ml) were chromatographed and counted as in footnote 2, Table II.

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acid metabolism of cultured cells was found to be altered by a contamination with PPLO strains of avian or sheep origin (Powelson, D. M., 1961). The importance of

the present observations cannot be overemphasized since PPLO is a common contaminant not only in mammalian cell cultures but also in animals and man. In a recent publication, Horoszewicz, J. S. (1961) reported that 600 out of 1221 persons carried PPLO in the genital tract or in the oral cavity, and cytopathogenic PPLO strains have also been isolated from human leukemic and neoplastic tissues (Grace, J. T., et al.). Barile, M. F., et al. (1962) found PPLO in 10 out of 11 tested cell lines obtained from commercial tissue culture laboratories. Chemotherapy using pyrimidine nucleosides such as 5-fluorodeoxyuridine or 5-iododeoxyuridine is seriously hampered by their extensive cleavage in vivo (Harbers, E., et al., 1959; Prusoff, W. H., et al., 1960). In any studies involving deoxyribonucleosides in mammalian systems it seems advisable to take into consideration the possible presence of Mycoplasma which could drastically affect the experimental results with these compounds.

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