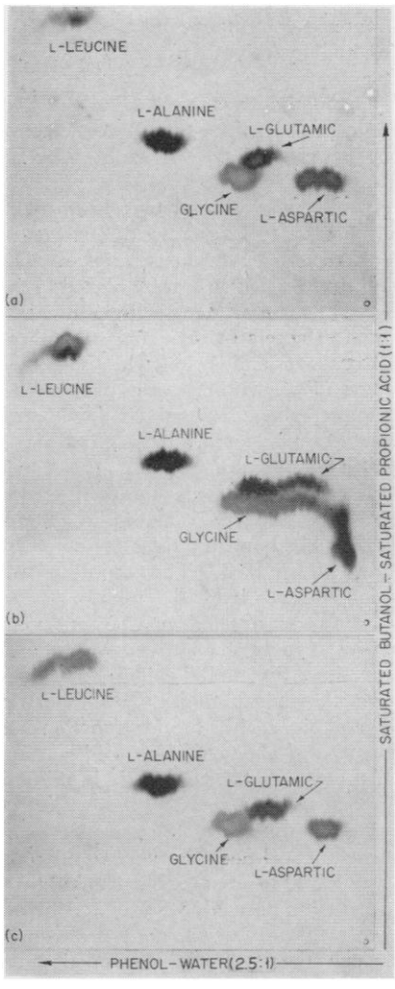

Fig. 1. Chromatograms of an amino acid mixture: (a) water solution, (b) 1*N* NaCl solution, (c) 1*N* NaCl solution followed by desalting.

1.5 mg/ml of each amino acid present. These are typical of results obtained when a variety of amino acids as well as other salts were used, including $CaCl_2$, $MgCl_2$, and KCl. The tailing and overlapping in the presence of NaCl and the improvement effected by the desalting procedure are evident.

To determine the extent of migration on the filter paper and to check the recovery of the amino acids, strips were sprayed with Ninhydrin after electrophoresis and both before and after elution with 20 ml of water. Sprayings before elution (*6*) revealed that the amino acids concentrated rapidly in a band 1 to 2 cm wide and 0 to 3 cm from the point of application, depending on their isoelectric points and the *p*H gradient in the strip. Sprayings after elution did not result in any color development, even on prolonged heating at 105°C, indicating fairly complete removal of both the amino acids and the ammonium formate. In the foregoing, solutions of glycine, glycylglycine, L-alanine, L-glutamic acid, L-cystine, L-methionine, DL-phenylalanine, L-arginine, L-lysine, L-tyrosine, L-tryptophan, and L-valine, were used.

Recovery of DL-phenylalanine and the extent of desalting were also investigated by light absorption and electrical conductance measurements. A typical set of results is shown in Table 1 for experiments in which Whatman No. 1 paper treated with LiOH was used. Because of soluble light-absorbing and conducting material in the paper, corrections with a blank (*7*) were necessary. All absorbances and conductances are for samples diluted to 5 ml with water. The conductances (corrected for solvent) are expressed as milligrams of NaCl. The data show that both the recovery of the amino acid and the removal of salt are essentially complete.

Preliminary experiments with 2′- and 3′-uridylic and 2′- and 3′-cytidylic acids indicate that the procedure can be applied to nucleotides. A strongly acid buffer (for example, 0.1*M* ammonium formate in 50 percent formic acid for uridylic acid) was necessary on the low *p*H side because of the low isoelectric points of these substances (*8*).

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4. A few runs, which proved quite satisfactory, were made with the Spinco model R eight-strip Durrum-type apparatus with Whatman No. 1 paper. With heavier papers, such as 3MM, desalting was not so effective.
5. A laboratory Glass and Instruments Corporation Evapo-Mix evaporator accommodating ten sample tubes was used. The small quantity of ammonium formate present either was completely removed to the cold trap or was condensed as a few hard crystals in the lead-off line between the sample tubes and the trap.
6. If the strip is heated after spraying, color caused by the presence of ammonium formate develops rapidly and obscures the amino acid color. Therefore, the strips were allowed to develop at room temperature.
7. The paper could be temporarily freed of absorbing and conducting material by thorough washing with water or with dilute acid or alkali followed by water. However, the offending material would again appear in reduced amounts in eluates obtained a few hours later. Finally, this effect was eliminated by heating the strips in 1 percent LiOH, at just below boiling temperature overnight, and washing thoroughly with water, although this treatment did not reduce the blank correction to zero (Table 1). Since it was found that the chromatograms (Fig. 1) were not significantly improved by the LiOH treatment, the matter was not pursued further. Evidently a much more thorough treatment, possibly one such as that developed by G. E. Connell, G. H. Dixon, and C. S. Hanes [*Can. J. Biochem. and Physiol.* 33, 416 (1955)], which included a 22-day elution with LiOH, would be necessary to remove all soluble conducting and light absorbing matter.
8. The work described in this report originated in discussions with Dr. N. G. Anderson of the Biology Division, Oak Ridge National Laboratory. I wish to thank Mr. R. E. Canning for running the chromatograms.
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26 January 1959

Incorporation of Unnatural Pyrimidine Bases into Deoxyribonucleic Acid of Mammalian Cells

Abstract. When a mammalian cell strain was incubated with 5-iododeoxyuridine and 5-bromodeoxyuridine, DNA thymine was partially replaced by the halogen-containing pyrimidines. The extent of incorporation of the unnatural bases increased when amethopterin and hypoxanthine were added to the medium. It is thus evident that the replacement of DNA thymine by selected structural analogs, a phenomenon previously reported for bacterial systems, is applicable to cells of higher organisms.

Following preliminary observations of Weygand *et al.* (*1*), Zamenhof *et al.* and Dunn and Smith (*2–5*) have demonstrated the introduction of 5-chloro, 5-bromo, and 5-iodouracil into the deoxyribonucleic acid (DNA) of several bacterial strains. These pyrimidines are considered structural analogs of thymine for which the Van der Waals' radii of the halogen atoms are approximately equal to the radius of the methyl group (*5, 6*). The extensive incorporation (reported in this paper) of 5-bromouracil and 5-iodouracil into the DNA of a mammalian cell strain demonstrates that this phenomenon can be extended to cells of higher organisms.

In vitro experiments in our laboratory had shown that 5-bromodeoxyuridine severely depressed the incorporation of labeled thymidine into the DNA of H.S. No. 1 human tumor transplant slices (*7*). However, a search for incorporation of bromouracil into the DNA of this tissue slice system had yielded negative results.

Cells (H.Ep. No. 1) derived from a human cervical carcinoma (*8*) were grown in large Blake bottles with Eagle's medium (*9*) plus 20 percent horse serum. After cell growth on glass had been established, 5-iododeoxyuridine (*10*), 5-bromodeoxyuridine, hypoxanthine, and amethopterin were added to the culture medium as indicated in Table 1. After approximately 3 days the cells were washed with saline and harvested with 0.05 percent trypsin (*11*). The DNA bases were obtained by a procedure previously described (*12*) and separated from each other by two-dimensional paper chromatography. In agreement with the observations of Dunn and Smith, the Marshak-Vogel hydrolytic procedure for DNA containing iodouracil yielded a compound identified by chromatography and ultraviolet spectrum as uracil (*3*). In the case of DNA containing bromouracil, the hydrolytic procedure yielded a mixture of bromouracil and uracil. Consequently, the data for iodouracil and bromouracil shown in Table 1 are based on the yield of these hydrolysis products.

Table 1. Ratio of halogen-containing bases to thymine in the DNA of H. Ep. No. 1 cells.

Compound	Molar concentration in culture medium	
	A	B
Experiment No. 1		
5-Iododeoxyuridine	1.4×10^{-4}	1.4×10^{-4}
Hypoxanthine		3.6×10^{-5}
Amethopterin		1.0×10^{-7}
Ratio in DNA: I-containing base/thymine	0.30	0.61
Experiment No. 2		
5-Bromodeoxyuridine	1.7×10^{-4}	1.7×10^{-4}
Hypoxanthine		3.6×10^{-5}
Amethopterin	1.0×10^{-7}	1.0×10^{-7}
Ratio in DNA: Br-containing base/thymine	0.76	0.84

In order to demonstrate directly the incorporation of the iodouracil moiety, cells were harvested from a medium containing 5-iododeoxyuridine labeled with I^{131}. The residual solution following extraction of the defatted cells with 10 percent sodium chloride, protein removal by chloroform gel formation (*3, 13*), and subsequent dialysis was analyzed by paper chromatographic methods and scintillation counting. The radioactivity in a compound chromatographically identical with 5-iododeoxyuridine could be recovered only after treatment of the above solution with deoxyribonuclease followed by treatment with snake-venom phosphoesterases (*3*).

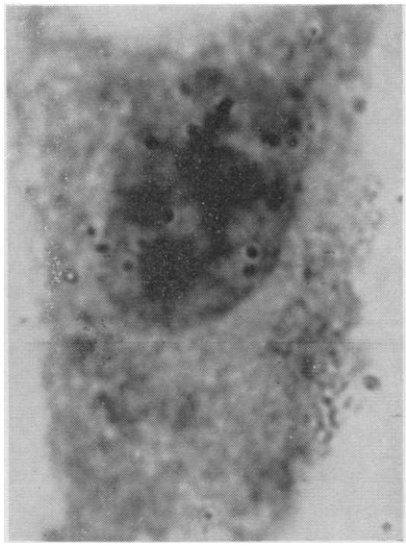

Fig. 1. Autoradiograph of H. Ep. No. 1 cell after incubation in tritium-labeled 5-bromodeoxyuridine for 3 days (in the presence of amethopterin and hypoxanthine) followed by incubation in unlabeled medium for 2 days. Localization of reduced silver grains over the nucleus can be observed. Kodak AR 10 stripping film and MacNeal Tetrachrome stain were employed.

The iodouracil moiety can be incorporated into the DNA of these cells even in the absence of amethopterin, which acts to depress *de novo* thymine synthesis (Table 1, expt. 1A). In experiments 1B and 2B, amethopterin was added to enhance the utilization of the exogenous thymine analogs, while hypoxanthine provided a source of preformed purine moiety. The results of experiment 2 indicate that the addition of hypoxanthine in the presence of amethopterin did not appreciably change the incorporation of the bromouracil moiety. These results may be related to Hakala's observation that 5-bromodeoxyuridine can support HeLa cell growth in a culture medium containing amethopterin, glycine, and hypoxanthine or adenine (*14*).

The molar ratio of DNA thymine to adenine is close to 1.0 for cells grown in normal medium, while in the experiments listed in Table 1 the sum of thymine plus halogen-containing pyrimidine more closely fits this relationship. These results suggest that the halogen-containing pyrimidines partially replace thymine, as has been observed in the bacterial systems studied (*2–5*).

The nuclear localization of the halogen-containing pyrimidines was confirmed by autoradiographic studies in which 5-bromodeoxyuridine labeled with tritium (*15*) and 5-iododeoxyuridine labeled with I^{131} were used. The cell culture conditions were equivalent to experiments 1B and 2B of Table 1. An autoradiograph made with tritium-labeled 5-bromodeoxyuridine is shown in Fig. 1 (*16*).

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16. These studies were aided by research grants from the National Institutes of Health (CY 3328 and C 3811) and the U.S. Atomic Energy Commission (AT-(30-1)-910). We are indebted to E. Simmel and M. Black for the preparation of autoradiographs and to A. Perez and S. Wolfe for assistance in cell cultivation studies. The stock culture of H. Ep. No. 1 cells was kindly given to our laboratory by Miss L. Diamond and Dr. A. E. Moore of the Virus Study Section, Sloan-Kettering Institute.

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Low-Level X-ray Damage to Amphibian Erythrocytes

Abstract. In vitro x-irradiation of frog and *Amphiuma* erythrocytes caused cytophysiological damage to part of the cell population. There was a significant decrease in the percentage of normal cells and some hemolysis. Changes were also observed in the electrical capacitance and potassium-42 uptake of irradiated erythrocytes.

There is considerable evidence that low-level ionizing radiation causes significant changes in populations of dividing cells (*1*). Until recently there has been little information available on the effect of small doses of x-irradiation on the cytophysiology of nondividing (postmitotic) cells (*2*). I chose nucleated amphibian erythrocytes as a biological representative of the postmitotic cell type because of their large size and relatively high metabolic rate and because large numbers of intact cells were easily obtained. Although many millions of cells were observed in these studies, I did not find dividing erythrocytes in the blood of the species studied (*3*).

Curarized animals were bled from the heart (bullfrog) or tail vein (*Amphiuma*) (*4*), and the heparinized blood was washed in physiological saline (*5*). Pooled blood samples were kept at approximately 5°C during all procedures of preparation and were exposed to room temperature (22° to 25°C) only during the experiment. Cell suspensions (hematocrit 33) were divided into control (unirradiated) and irradiated aliquots and poured into Lucite dishes 3½ in. in diameter to a depth of 2 to 3 mm. Irradiations were carried out with a 100-kv (peak) x-ray tube operated at 15 ma (*6*). Because of variation in the degree of response produced by x-irradiation, all experimental procedures were either carried out on the same blood samples or repeated on a sufficient number of samples to obtain averages representative of the cell population. Except for the ir-