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*).

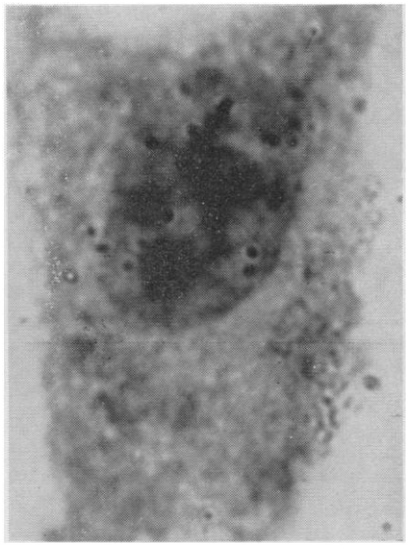

Fig. 1. Autoradiograph of H. Ep. No. 1 cell after incubation in tritium-labeled 5-bromodeoxyuridine for 3 days (in the presence of amethopterin and hypoxanthine) followed by incubation in unlabeled medium for 2 days. Localization of reduced silver grains over the nucleus can be observed. Kodak AR 10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

radiation, exactly the same procedures were applied to both the control and experimental samples.

Phase-microscope studies of unirradiated and irradiated erythrocytes (Fig. 1) indicated that x-irradiation caused an increase in the number of irregularly shaped cells (cells with folded and ruffled membranes) and of irregular nuclei. Immediately following 50- and 100-r x-irradiation, some hemolysis was observed, but none was observed following 10-r x-irradiation. Irradiated erythrocyte suspensions showed further hemolysis after they were transferred to conical tubes and centrifuged. The supernatant was carefully replaced with cold isotonic saline, and the cells were stored at 5°C for 24 and 48 hours, respectively. Further hemolysis was observed only in the erythrocyte suspensions irradiated at 100 r with little or none in the aliquots irradiated at 10 and 50 r. There was apparently a rapid hemolysis of damaged, fragile, and aged erythrocytes; this left a relatively hemolysis-resistant population of cells after 24 hours. An analogous result was reported by Alpen *et al.* (7), who, after 500-r whole-body x-irradiation of rats, found an increased resistance to osmotic hemolysis after 24 hours.

In an effort to follow the course of hemolysis, cell counts of relatively undisturbed erythrocyte suspensions were carried out. There was an increase in the number (per 1000 cells counted) of irregularly shaped and cytologically abnormal cells following 50-r x-irradiation of frog and *Amphiuma* red cells. Phase-microscope cell counts after 100-r irradiation indicated a 10-percent increase in the percentage of damaged cells in the irradiated suspensions as compared to the controls. No significant change in the percentage of damaged cells was found after 24 and 48 hours of storage (at 5°C) of irradiated erythrocytes in fresh (unirradiated) physiological saline. The decrease in the percentage of cytologically normal cells is apparently an indication of irreversible cellular damage due to radiation.

The effect of x-rays on the red-cell envelope was investigated by means of radioisotope and electrical techniques. The capacitance of paired aliquots of unirradiated and 100-r x-irradiated frog erythrocytes at 28°C was determined. Measurements of washed packed cells were carried out in a Lucite cell with the two end walls made of 1-cm² platinum plates placed 0.3 cm apart. Observations were made in accordance with Fricke's theoretical and experimental method (8), over a frequency range of 10 to 100 kcy/sec, by means of an alternating-current Wheatstone bridge, with an oscilloscope as a detector. The irradiated cells showed a consistent elevation in capacitance, as compared to the unirradiated cells, over the entire frequency range studied (Fig. 2). This may be interpreted as physical evidence of changes in cell geometry or in membrane ultrastructure, or in both.

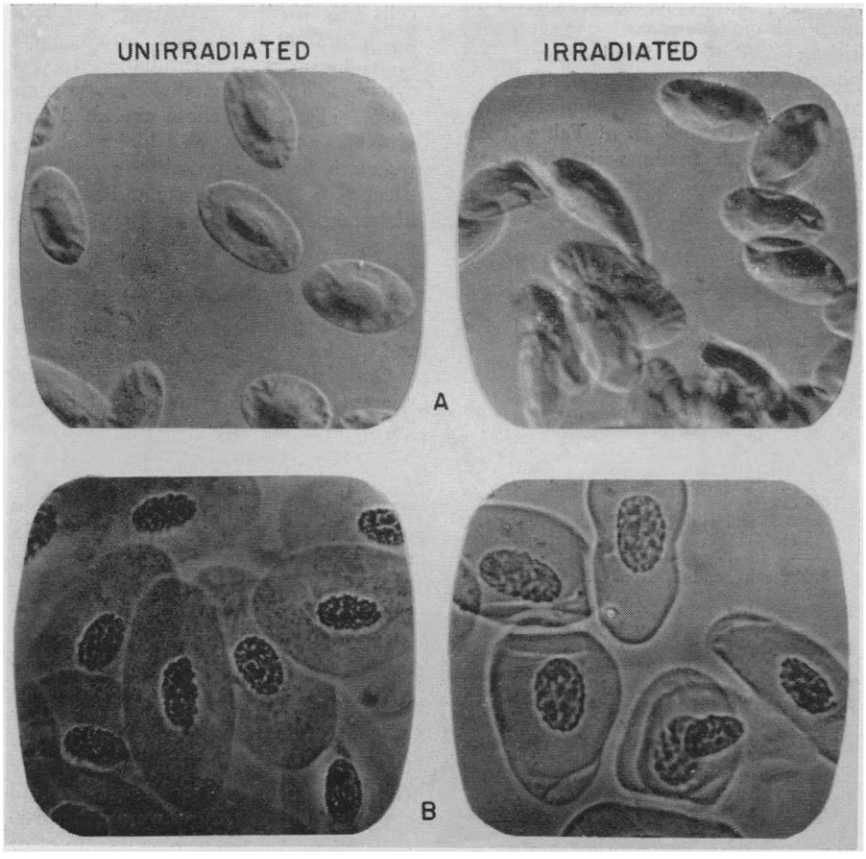

Fig. 1. Phase-microscope pictures of *Amphiuma* erythrocytes. (A) Living cells; (B) fixed and Feulgen-stained cells. Note ruffled and folded cell membranes and irregular cell and nuclear outlines.

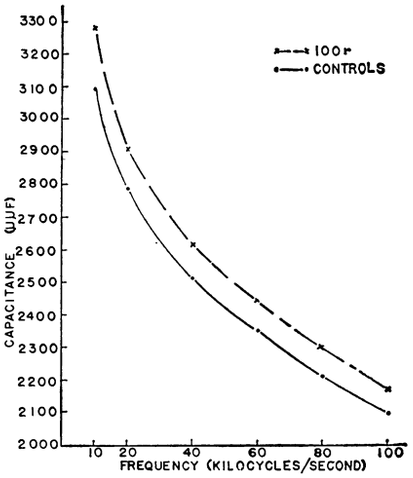

Fig. 2. Capacitance (in micromicrofarads) of paired aliquots of unirradiated and 100-r x-irradiated frog erythrocytes at 28°C.

Additional evidence for cell membrane changes was shown by increased $K_2{}^{42}CO_3$ uptake immediately after irradiation at 100 r. This was followed by a loss in K^{42} after 90 minutes in both frog and *Amphiuma* cells. There were three capacitance experiments which indicated greater differences between control and irradiated cells ½ hour after radiation treatment than were apparent 1½ hours after irradiation. This suggests that some of the changes observed are reversible in nature and follow trends similar to those found in respiration studies of the same cells (2).

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Phenylpyruvic Acid as a Possible Precursor of *o*-Hydroxyphenylacetic Acid in Man

Abstract. The oral administration of phenylpyruvic acid to human subjects results in increased urinary excretion of *o*-hydroxyphenylacetic acid. This demonstrates that phenylpyruvic acid may act as a precursor for *o*-hydroxy derivatives of phenylalanine and suggests that the formation of *o*-tyrosine is not necessary to account for the excretion of *o*-hydroxyphenylacetic acid in phenylketonuria.

o-Hydroxyphenylacetic acid (*o*-HPAA) has been found to be the major hydroxy metabolite of phenylalanine excreted in the urine of patients with the hereditary metabolic disorder known as phenylketonuria (*1–3*). Such a conversion could theoretically occur by the *o*-hydroxylation of phenylalanine, phenylpyruvic acid, or phenylacetic acid. Armstrong and Shaw (*4*) found that the excretion of *o*-HPAA followed the oral administration of *o*-tyrosine in man, and postulated that *o*-tyrosine was a probable intermediate in the formation of *o*-HPAA in phenylketonurics. Mitoma *et al.* (*5*) suggested that the overproduction of *o*-tyramine, the decarboxylation product of *o*-tyrosine, was perhaps responsible for the mental defect in phenylketonuria. The presence of *o*-tyrosine or *o*-tyramine has not as yet been demonstrated in the tissues of normal or phenylketonuric individuals. It has been reported, however, that beef adrenals normally contain free *o*-tyrosine (*6*), and it is possible that the normal excretion of *o*-HPAA in man could originate from such a source.

When Berry *et al.* (*7*) applied phenylalanine tolerance tests (*8*) to individuals heterozygous for phenylketonuria (0.1 g of L-phenylalanine per kilogram of body weight), they found that *o*-HPAA but not phenylpyruvic acid was excreted in the urine in increased amounts. Cullen and Knox (*9*) recently confirmed these findings and also showed that a dose of at least 0.13 g of L-phenylalanine per kilogram is usually required before any increased *o*-HPAA can be detected in the urine of normal subjects.

In the course of experiments in this laboratory (*10*), it was found that ingestion of the D-isomer of phenylalanine in man in amounts as low as 0.015 g/kg regularly results in the urinary excretion of phenylpyruvic acid and of increased amounts of *o*-HPAA. This raised the question whether D-phenylalanine or one of its metabolites might be the substrate for *o*-hydroxylation.

In order to test for the possible *o*-hydroxylation of phenylpyruvic acid or its metabolites, the sodium salt of phenylpyruvic acid (Nutritional Biochemicals Corp.) was orally administered to three normal, adult male subjects. Each urine sample following the ingestion of phenylpyruvic acid was immediately assayed for phenylpyruvic acid by a modification of the procedure of Berry and Woolf (*11*), and the collection of urine was continued until no further phenylpyruvic acid could be detected. The individual urine samples in which phenylpyruvic acid was present were then pooled and assayed for *o*-HPAA by a modification of the paper-chromatography technique of Armstrong *et al.* (*2, 12*) in which the chromatogram was developed with 2,6-dichloroquinone chlorimide.

The results (Table 1) show that the ingestion of phenylpyruvic acid causes an increased excretion of *o*-HPAA and indicates that phenylpyruvic acid may be a precursor for *o*-HPAA. However, since no isotope study was carried out with labeled phenylpyruvic acid, the possibility remains that the administration of phenylpyruvic acid might be indirectly increasing the excretion of *o*-HPAA.

Because the evidence presented here shows that the ingestion of phenylpyruvic acid can cause an increased urinary excretion of *o*-HPAA, it is not necessary to assume an increased production of *o*-tyrosine in phenylketonuria in order to account for the increase of *o*-hydroxy derivatives of phenylalanine (*13*).

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Table 1. Urinary excretion of phenylpyruvic acid (PPA) and *o*-hydroxyphenylacetic acid (*o*-HPAA) following the oral administration of sodium phenylpyruvic acid.

PPA ingested (mmole)	Total PPA excreted (μmole)	Collection time (hr) (*X*)	*o*-HPAA excreted (μmole/24 hr) (control)* (*Y*)	Total *o*-HPAA excreted during *X* (μmole) (*Z*)	*Z* less estimated normal excretion of *o*-HPAA during *X* (μmole) [(*Z* − *XY*)/24]
			Subject A		
0			2.6		
2.5	185	3.0		8.5	8.2
5.0	711	6.5		42	41
			Subject B		
0			5.0		
5.0	598	4.5		19	18
			Subject C		
0			10.6		
5.0	581	7.0		51	48

* The normal daily excretion of *o*-HPAA for seven subjects, including subjects A, B, and C, ranged from 2.0 to 10.6 μmole (mean, 5.6 μmole).

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