120 minutes the protein of the brain was labeled to the extent of 10 percent of the concentration of $C^{14}O_2$ in the atmosphere. The lipid and nucleic acid fractions, however, were not significantly labeled during this time. It appears, therefore, that, in agreement with many other studies, the brain proteins incorporate C^{14} very rapidly (*11*). The turnover of lipids in the white matter of the brain is not so readily explained, however, and this observation must be clarified by further experimentation.

Some correlation appears to exist between the concentration of C^{14} in the atmosphere and that observed in human blood. The erythrocyte protein of the blood donated in January 1964 reflects the amount of C^{14} in the atmosphere during 1962, a difference of about 1½ years, which is in agreement with the conclusions of Broecker *et al.* (*1*). Since plants, during their periods of growth, would be expected to possess essentially the same amount of C^{14} as that in the surrounding atmosphere, radiocarbon would be incorporated into primary foodstuffs such as grain and hay mainly during the spring. Therefore, the erythrocyte and plasma samples appear to reflect the atmospheric radioactivity at the time of maximum photosynthesis in the spring preceeding the corresponding harvest. Presumably, the grain of the 1962 harvest, and the domestic animals fed with it, contained the same amount of radioactivity as was present in the atmosphere during spring 1962. At the time the new harvest of 1963 was being used as human food, its radioactivity had risen to the values found in the blood plasma obtained during January 1964. As more and more 1963 foodstuffs replaced those of 1962, the radioactivity of the erythrocyte protein rose to the same value as that in the plasma obtained from subject HU-21. The radioactivity of the erythrocyte protein has not increased further because that of the bulk of foodstuffs still remains at the spring-1963 value. Similarly, the radioactivity of the plasma sample of September 1963 was at a level comparable to that of the erythrocytes of January 1964. Presumably, erythrocytes during the fall of 1963 may have shown about the same radioactivity as the respective plasma.

Since the radiocarbon activity of erythrocytes and several other body tissues are grossly similar, it may be possible to make reasonable deductions from these data about other body components when only blood data are known. It will be necessary to amass additional data for greater understanding of this problem.

Several other questions arise as a result of this work. For example, what is the significance of the incorporation of appreciable amounts of C^{14} into the white matter (and presumably myelin), proteins, and lipids of the brain? Could analyses of blood samples obtained serially during changes in the C^{14} content of the atmosphere yield information concerning equilibration rates of various tissues? Or could such information be obtained by examining blood samples from individuals arriving in the Northern Hemisphere after they have resided for long periods in the Southern Hemisphere, where the concentration of atmospheric C^{14} lags behind the concentration in the Northern Hemisphere by at least 1 year?

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3. Solution B of the National Institutes of Health.
4. The samples of plasma collected in 1962 and 1963 were kindly provided by Dr. E. P. Adashek and James E. Casey of the American Red Cross Regional Blood Service of Los Angeles and Orange Counties, whose assistance is gratefully acknowledged. We also gratefully acknowledge the assistance of Miss Patricia Taylor of the UCLA Blood Bank in the collection of the blood samples.
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Hydroxyurea: Inhibitory Effect on DNA Metabolism

Abstract. *Hydroxyurea, hydroxyurethane, and dihydroxyurea inhibit incorporation of thymidine into the DNA of monolayers of HeLa cells. They do not affect incorporation of uridine into RNA or of leucine into protein. In contrast, hydroxylamine inhibits cellular incorporation of all three precursors: thymidine, uridine, and leucine. Hydroxyurea does not affect thymidine kinase, thymidylate kinase, or DNA polymerase reactions, but it does inhibit incorporation of cytidylic and guanylic acids into DNA in cell-free supernatants.*

Hydroxyurea is under study as a cancer chemotherapeutic agent. It has antileukemic activity in mouse (*1*) and man (*2*). The mechanism by which it produces biological effects is unknown, but two proposals have been advanced. (i) Hydroxyurea is hydrolyzed in vivo and yields free hydroxylamine. This then cleaves thioesters, in particular acetyl-coenzyme A, disrupting oxidative phosphorylation and reducing concentrations of cellular and mitochondrial adenosine triphosphate (ATP) (*3*). (ii) Hydroxyurea and a number of related derivatives of hydroxylamine cause fragmentation of isolated DNA and induce chromosomal abnormalities in mammalian cells, cultured in vitro (*4*). The postulated common mechanism is that oxidative transformation of these compounds occurs, forming the nitroxyl radical (HON=) which becomes dimerized as hyponitrous acid (HON=NOH). This induces cleavage of the main chain of cellular DNA (*4*). As already reported, hydroxyurea inhibits incorporation of radioactive thymidine into the DNA of monolayers of HeLa cells (*5*). We now examine effects of hydroxyurea and a number of related compounds on the incorporation of thymidine into DNA, of uridine into RNA, and of leucine into protein of HeLa cells. We also report our efforts to identify a site of hydroxyurea-induced inhibition in DNA metabolism.

A sequential isotope technique was used to study acute effects of drugs on protein and nucleic acid metabolism of monolayers of HeLa cells grown on glass coverslips (*6*). The technique involved 15- to 30-minute prior incubation of the cells, without drugs, in

the presence of a precursor labeled with C^{14}, followed by an experimental incubation, with drugs, in the presence of the same precursor labeled with tritium. The precursors used were C^{14}- and H^3-thymidine for DNA, C^{14}- and H^3-uridine for RNA, and C^{14}- and H^3-leucine for protein. The C^{14} and tritium contents of the monolayers were determined by liquid scintillation techniques (*7*). The tritium : carbon-14 ratios of replicate monolayers did not vary more than 11 percent about their mean. Deviation from the control tritium : carbon-14 ratio seen in samples which had been exposed to a drug served as a measure of drug effect.

Hydroxyurea and five related compounds inhibited incorporation of thymidine into DNA (Fig. 1). When compared at the concentrations which inhibited incorporation of thymidine by 50 percent, hydroxyurea and hydroxyurethane were equipotent. Dihydroxyurea was about twice as potent as hydroxyurea, while the alkyl hydroxamate and the hydroxylamine derivatives were less potent by an order of magnitude. Sodium hyponitrite and hydroxyaminoacetic acid ($10^{-2}M$) had no effect. The hydroxamic acids tested (hydroxyurea, hydroxyurethane, dihydroxyurea, and acetohydroxamic acid) did not alter cellular incorporation of uridine or leucine at drug concentrations which decreased uptake of thymidine by 90 percent. However, hydroxylamine inhibited incorporation of thymidine, uridine, and leucine to an equal degree, and *N*-methylhydroxylamine had a modest inhibitory effect on incorporation of leucine. Since hydroxyurea and hydroxylamine differ markedly in their inhibitory effects in this test system, it is unlikely that the observed action of hydroxyurea is dependent upon its hydrolysis to hydroxylamine.

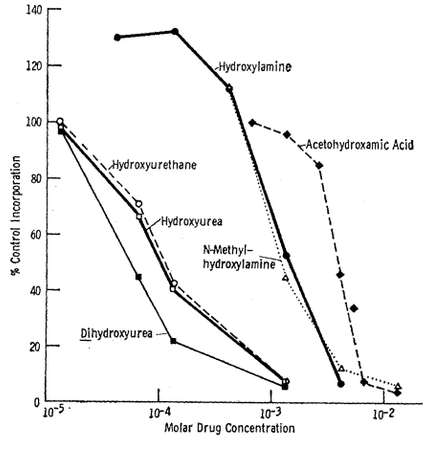

Fig. 1. Acute effects of hydroxyurea and related compounds on incorporation of H^3-thymidine into HeLa monolayers. Percent control incorporation = $[(H^3/C^{14})_{expt}/(H^3/C^{14})_{control}] \times 100$. Time, 30 minutes; drugs were present at zero time.

Table 1. Inhibitory effect of hydroxyurea ($1.3 \times 10^{-3}M$) on incorporation of ribonucleotides into DNA by cell-free HeLa supernatant. The concentrations in the incubation mixture were: (m*M*) ATP 5, $MgCl_2$ 5, tris-HCl (*p*H 8) 40, L-cysteine 0.5, deoxyadenylate triphosphate, deoxythymidylate triphosphate, deoxyguanylate triphosphate, deoxycytidylate triphosphate 0.05 (each), 250 μg DNA primer, cell-free supernatant (approximately 2 mg protein) and hydroxyurea in a total volume of 0.5 ml. Appropriate deoxynucleotide triphosphates and DNA primer were added 15 minutes (Expt. 1) or 30 minutes (Expt. 2) after the addition of the labeled ribonucleotides (4 nmole CMP or 3 nmole GMP). Incubation was continued for 60 minutes; RNA was extracted, and the radioactivity of the DNA-protein residue was measured.

Addition	Ribotide incorporated into DNA (pmole)	
	Expt. 1	Expt. 2
	H^3-CMP	
None	11.0	23
Hydroxyurea	0.5	0.6
	C^{14}-GMP	
None	6.9	7.8
Hydroxyurea	0.3	0.6

Hydroxyurea-induced inhibition of incorporation of thymidine was almost instantaneous in its onset; recovery from inhibition after exposure for 30 minutes was equally rapid when cells were rinsed and placed into drug-free medium (Fig. 2). It is unlikely that synthesis of DNA would recover so rapidly from inhibition caused by cleavage of the main chain of cellular DNA. Further experimental evidence suggests that fragmentation of isolated DNA and inhibition of incorporation of thymidine into DNA of intact cells are separate effects of the drugs under discussion. Hydroxyaminoacetic acid and sodium hyponitrite are active compounds in the former (*4*) and inert in the latter test system.

The inhibitory effect on incorporation of thymidine into DNA of HeLa cells seems pertinent to the action of hydroxyurea in man. The following evidence supports this suggestion. (i) Plasma concentrations in the inhibitory range occur routinely in patients under therapy with hydroxyurea (*8*). (ii) Hydroxyurea (5 μg/ml) inhibits growth in cultures of HeLa cells by 50 percent (*9*). This concentration inhibited incorporation of thymidine by 30 percent in our test system. (iii) Hydroxyurea acutely inhibits incorporation of thymidine into DNA of small intestine, thymus, and regenerating liver in vivo after administration of the drug to rats (*10*).

We have utilized cell-free enzyme systems extracted from HeLa monolayers in an attempt to isolate a site of enzymatic inhibition induced by hydroxyurea in cellular synthesis of DNA. Cells were frozen, thawed, and homogenized; the homogenate was centrifuged at 105,000*g*, and the DNA polymerase activity in the supernatant was investigated by the method of Bollum (*11*). Hydroxyurea ($10^{-2}M$) had no effect on the incorporation of labeled thymidylate into DNA. Thus, high concentrations of hydroxyurea did not inhibit phosphorylation of thymidylate or the DNA polymerase reaction. Thymidine kinase activity in the cell-free supernatant was analyzed by measuring the incorporation of H^3-thymidine into the mixed nucleotide fraction quantitatively absorbed onto diethylaminoethyl-cellulose (DEAE) discs (*12*). This enzymatic reaction was also insensitive to hydroxyurea ($10^{-2}M$), although appropriately sensitive to feedback inhibition by deoxy-

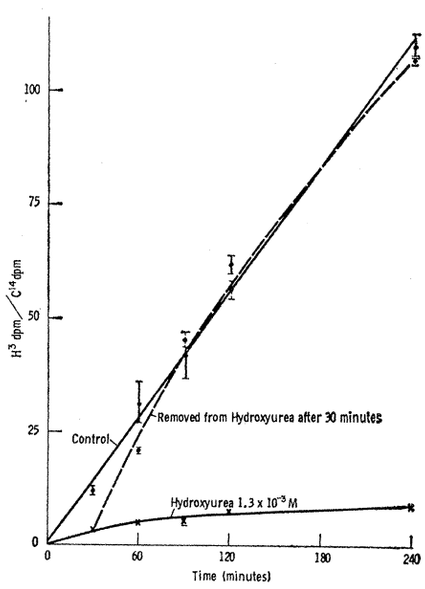

Fig. 2. Inhibition of incorporation of H^3-thymidine into HeLa monolayers by hydroxyurea. Recovery after cell removal to drug-free medium. *dpm*, Disintegrations per minute.

thymidylate triphosphate (dTTP). Results of experiments in which reduction of labeled cytidylic acid (CMP) and guanylic acid (GMP) was coupled with the DNA polymerase system (*13*) are shown in Table 1. Hydroxyurea ($1.3 \times 10^{-3}M$) inhibited incorporation of CMP and GMP into DNA in this subcellular system by more than 90 percent. This inhibition is comparable in degree to the inhibitory effect of hydroxyurea ($1.3 \times 10^{-3}M$) on incorporation of thymidine into intact cells.

Two observations offer strong evidence against the concept that a disruption of oxidative phosphorylation is pertinent to the inhibitory effects of hydroxyurea on incorporation of ribonucleotides or thymidine into DNA. (i) An ATP-generating source was not used in the subcellular studies. (ii) 2,4-dinitrophenol, a compound known to induce a decrease in cellular ATP concentrations, inhibited incorporation of leucine but not thymidine in our cell system (*6*).

Deoxyadenylate triphosphate, deoxyguanylate triphosphate, and a metabolite of cytosine arabinoside are reported to inhibit the reduction of purine or pyrimidine ribonucleotide diphosphates, (*13, 14*). Deoxyadenosine ($10^{-4}M$) and cytosine arabinoside ($10^{-6}M$) inhibited incorporation of H^3-thymidine in our test system (*6*). Deoxyguanosine ($10^{-3}M$), however, was apparently inert; this nucleoside may be poorly phosphorylated by our strain of HeLa cells.

The cellular and subcellular data presented suggest that hydroxyurea alters cellular synthesis of DNA by interfering with ribonucleotide (diphosphate) reduction. The inhibitory effects of hydroxyurea on incorporation of thymidine into intestine, thymus, and regenerating liver in the rat (*10*) suggest that these tissues may provide a satisfactory source of enzymes for further studies.

After this report was submitted, results of the studies of Frenkel, Skinner, and Smiley became available (*15*). They observed decreased conversion of cytidylic acid to deoxycytidylic acid by subcellular extracts of bone marrow taken from rats and patients after treatment with hydroxyurea. Their data suggest that hydroxyurea inhibits the conversion of ribonucleotides to deoxyribonucleotides in vivo as well as in vitro. However, since they did not observe inhibition of incorporation of thymidine into DNA of treated rats, the relation between the effects of hydroxyurea in isolated systems and in intact animals requires further clarification.

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Catalase Hybrid Enzymes in Maize

Abstract. *In maize endosperm there are two electrophoretic variants of catalase. The variations are under genetic control, and the heterozygote shows three hybrid enzymes with mobilities intermediate between the parental enzymes. Thus, maize catalase may exist as a tetramer, and the hybrid enzymes may be formed by random association of two different catalase monomers.*

A large number of genetic enzyme variations in various organisms have been described (*1*). Isozymes (*2*) have not been studied with the same intensity in plants as in animals, but some important findings have been made concerning electrophoretic enzyme variations in maize endosperm (*3*). Of special interest are the formation of hybrid esterase enzymes and the gene dosage variations of those enzymes in the triploid endosperm demonstrable in reciprocal crosses. We have been investigating the formation of catalase hybrid enzymes in maize.

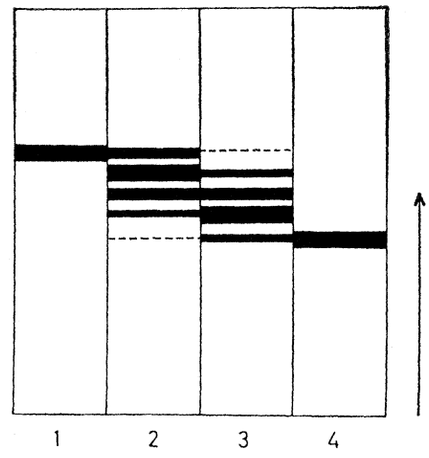

Fig. 1. Scheme of electrophoretic variations of catalase from maize endosperm; *1*, F; *2*, FH; *3*, SH; and *4*, S. The arrow shows the direction of migration toward the anode. The broken line indicates a hypothetical catalase zone that has not been visible in the zymograms. In sample *2*, the band above the dotted line, and in sample *3*, the band below the dotted line, are only occasionally visible in the zymograms.

Individual maize kernels were removed from freshly harvested ears and punctured to release the liquid endosperm, which was collected on a piece of filter paper (5×5 cm). The ears were collected on the 16th day after pollination, the time at which the liquid content of the individual kernels is almost entirely composed of endosperm. This simple method of extracting endosperm is entirely satisfactory and gives the same result as that obtained after a more complicated treatment involving homogenization and centrifugation (*3*). The filter papers were inserted into a starch gel and subjected to electrophoresis in a discontinuous buffer system (*p*H 8.6) (*4*) until the visible borate front zone had migrated about 7 cm from the sample slot. After the electrophoresis the gels were sliced horizontally and stained for catalase activity by a modification of Hale's technique (*5*). The gel was soaked first for 1 minute in 0.5 percent hydrogen