In some of the experiments with H_2O_2 the oxidant was added to the reference cell in the cytochrome b_5 assay also, but no evidence was found for spontaneous reduction of cytochrome b_5 under these conditions. Since the redox potential of cytochrome b_5[9] is higher than that of cytochrome P-450[10] and cytochrome P-450 is rapidly oxidized in the presence of O_2[1], the "spontaneous" reduction found in the protohaem assay is probably related to the specific conditions of that assay. However, the degree of reduction tended to vary between microsome preparations while the reagents used remained unchanged. It may be that microsomes can contain varying amounts of a reductant which only acts on the haem of the microsomal cytochromes at alkaline pH and/or in the presence of pyridine.

Acknowledgements—The author is greatful to Mrs. B. Summers for her excellent technical assistance and to Dr. F. De Matteis of valuable discussions.

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Inhibition of 1-β-D-arabinofuranosyl cytosine phosphorylation in human livers by tetrahydrouridine*

(*Received* 8 *September* 1971; *accepted* 2 *June* 1972)

1-β-D-ARABINOFURANOSYL cytosine or ara-C (NSC-63878),† one of the most active antitumor agents, not only inhibits L1210 mouse leukemia and other transplantable rodent tumors,[1] but also induces remission in adult acute leukemia.[2] Ara-C is rapidly deaminated to the inactive product, 1-β-D-arabinofuranosyl uracil, ara-U.[3,4] A reduced pyrimidine nucleoside, tetrahydrouridine (THU), was found to be a potent inhibitor of the deamination of ara-C by enzyme preparations made from human livers or mouse kidneys.[5] Response to ara-C was related to the rate of its phosphorylation.[6,7] This report describes the phosphorylation of ara-C and the effect of THU on the phosphorylation with various tissues from man, hamster and mouse.

Liver samples were obtained at autopsy from persons who had died of cancer or after accidents. All other tissues were freshly collected from patients after surgery or from mouse or hamster. Chronic lymphocytic leukemia (CLL) cells were isolated from 60–100 ml of blood from patients with CLL.

* This work was supported by Contract PH 43-66-1156 and Grant CA 05831, Chemotherapy, National Cancer Institute, National Institutes of Health, United States Public Health Service.

† 1-β-D-arabinofuranosyl cytosine and tetrahydrouridine were supplied by the Drug Development Branch, Cancer Chemotherapy National Service Center, National Cancer Institute, National Institutes of Health, Public Health Service.

The red blood cells were removed by hypotonic shock as previously reported.[8] The tissues were homogenated with 3 vol. of 0·25 M sucrose. The homogenate was centrifuged at 100,000 *g* for 1 hr at 4°, and the supernatant was used for the enzyme assay. The assay mixture contained Tris buffer, pH 8·0, 40 μmoles; ATP, 4 μmoles; 3-phosphoglyceric acid (sodium salt), 2 μmoles; $MgCl_2$, 26 μmoles; phosphoenolpyruvate kinase, 6 units; ara-C, 8 nmoles (1·0 μCi of the generally labeled tritium compound); and an appropriate amount of the enzyme preparation in a total volume of 0·4 ml, with or without 0·04 μmole of THU. The reaction mixture was incubated at 37° for 30–60 min. The assay was terminated by adding perchloric acid to a final concentration of 4 per cent. After a brief centrifugation, the supernatant was recovered, neutralized, and 40 μl was applied onto Whatman No. 1 filter paper. The paper chromatogram was developed 7 hr with descending flow in a solvent system of isopropanol–H_2O–ethylacetate (13·7:7·3:39) and the radioactive peaks were located by radioscanning. The R_f values of nucleotide, ara-C and ara-U are 0·03, 0·1 and 0·32 respectively. The addition of THU did not change the R_f of the compounds. The spots were cut out, eluted with 1 ml of 4% perchloric acid and counted with a Packard liquid scintillation spectrometer in 10 ml of 10% BBS counting solution (100 ml of Beckman Bio-Solv, 4 g of 2,5-diphenyloxazole, 0·2 g of 1,4-bis-2-(5-phenyloxazolyl) benzene and toluene to make 1 l). The counting efficiency for tritium was approximately 35 per cent. The phosphorylated compound(s) was further identified by incubating it with alkaline phosphatase of calf intestinal mucosa; the only product formed was shown to be ara-C.

Two products, ara-C nucleotide and ara-U, were found in the assay of ara-C kinase by paper chromatography and u.v. spectrophotometric techniques. Centrifugation of the homogenates at 100,000 *g* for 5 hr could not remove either of the enzyme activities. The deaminase reaction did not require any cofactors, and the presence of the deaminase enzyme interfered with the kinase assay by inactivating the ara-C to ara-U. Thus the substrate concentration in the reaction mixture could become a limiting factor for the kinase assay. An ara-C deaminase inhibitor, THU, at 1×10^{-4} M, was therefore routinely employed in the kinase assay. As shown in Table 1, THU has no effect on the phosphorylation of ara-C in mouse or hamster liver preparations, but it produced an average of approx. 50 per cent inhibition of human liver kinases. For comparison, the phosphorylation of

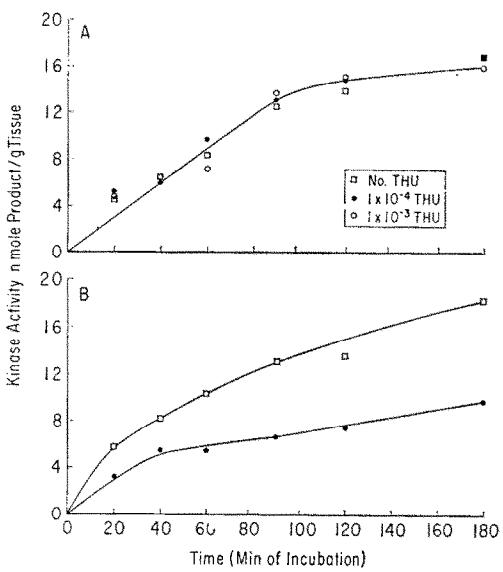

FIG. 1. Phosphorylation of ara-C of mouse liver (A) and human liver (B) was studied with and without THU at various times of incubation. The assay mixture contained Tris buffer, pH 8·0, 40 μmoles; ATP, 4 μmoles; 3-phosphoglyceric acid (sodium salt), 2 μmoles; $MgCl_2$, 26 μmoles; phosphoenolpyruvate kinase, 6 units; ara-C, 8 nmoles (1·0 μCi of tritium-labeled compound); and an enzyme preparation in a total volume of 0·4 ml.

ara-C of both mouse and human liver was studied, with and without THU at various times of incubation (Fig. 1A and 1B). A 10-fold increase in concentration of THU, 1×10^{-3} M, showed no effect on the mouse liver kinase activity after up to 3 hr of incubation, and the enzyme activity was linear for up to 90 min (Fig. 1A). On the other hand, 1×10^{-4} M of THU inhibited the human liver kinase

activity by about 50 per cent at all times during a 3-hr interval (Fig. 1B). The results were not altered when a 10-fold higher ara-C concentration (2×10^{-4} M) was employed.

TABLE 1. EFFECT OF TETRAHYDROURIDINE (THU) ON ARA-C KINASE ACTIVITIES OF HAMSTER, MOUSE AND HUMAN LIVERS *in vitro*

Liver	Ara-C kinase activity (nmoles/g tissue/hr)		
	No THU	THU (1×10^{-4} M)	Inhibition by THU (%)
Hamster	3·2	3·3	0
DBA_2 mouse*	10·4	10·4	0
DBA_2 mouse	8·8	9·2	0
DBA_2 mouse	11·2	11·6	0
DBA_2 mouse	9·6	9·4	0
DBA_2 mouse	12·8	12·8	0
Human autopsy 1	14·4	6·8	53
Human autopsy 2	21·2	8·8	58
Human autopsy 3	24·8	14·0	44
Human autopsy 4	13·2	5·6	58
Human autopsy 5	15·6	8·8	44
Human autopsy 6	13·2	5·2	60

* Livers from at least three mice were combined for each determination. Each determination was done in triplicate. The assay mixture contained Tris buffer, pH 8·0, 40 μmoles; ATP, 4 μmoles; 3-phosphoglyceric acid (sodium salt), 2 μmoles; $MgCl_2$, 26 μmoles; phosphoenolpyruvate kinase, 6 units; ara-C, 8 nmoles (1·0 μCi generally labeled with tritium) and the enzyme preparation in a total volume of 0·4 ml.

The following mechanisms of inhibitory action of THU on the human liver kinase activity were postulated and several experiments were conducted to test the postulates.

(1) The time from death of the patients to obtaining the samples varied from 5–16 hr. This suggested that it was possible for post-mortem changes to have occurred and altered the enzyme activity. Therefore four mice were killed and kept in the refrigerator for 24 hr. The kinase activity from these mouse tissues, with and without THU, was no different from that of freshly collected mouse tissues. These results suggested that the inhibitory activity of THU on the human liver kinase was probably not due to post-mortem changes.

(2) It was asked whether or not THU inhibited only the kinase activity of normal human tissues, but not neoplastic tissues. If so, this would represent another mechanism, in addition to the inhibition of the deaminase, by which THU might potentiate the action of ara-C in the treatment of human neoplasms. The ara-C kinase activity of various normal and neoplastic human tissues was measured with and without THU. Depending on the amount of deaminase activity present in the ara-C kinase assay, THU had no effect or "activated" the human kinase activity with the exception of human liver kinase. Two typical examples (Fig. 2A and B) show the kinase activity measured with and without THU in a human spleen and lung tumor (neurofibrosarcoma) respectively. The amount of deamination occurring in the kinase assay is also illustrated. The lung tumor had only a small amount of deaminase activity; even at 60 min of incubation without the inhibition of deaminase by THU, the substrate, ara-C did not become a limiting factor for assaying the kinase activity. Thus, the amount of ara-C nucleotide formed was not influenced by the presence or absence of THU (Fig. 2A). On the other hand, the human spleen contained more deaminase than did the lung tumor. In the absence of THU, ara-C was mostly converted to ara-U by spleen preparations even at 10 min of incubation, and a very small amount of substrate was left for the kinase reaction (Fig. 2B). However, when THU was added, the phosphorylation of ara-C could proceed in linear fashion, and more ara-C nucleotide was formed than without THU (Fig. 2B). In contrast, the human liver, like the spleen, contained a large amount of deaminase activity in the kinase assay. However, in the presence of THU, the deamination was inhibited and all of the substrate was available for the kinase reaction and yet the ara-C nucleotide formed was less than without THU (Table 2). Thus, with the exception of human liver, it appears that THU does not affect the kinase activity of normal or neoplastic human tissues.

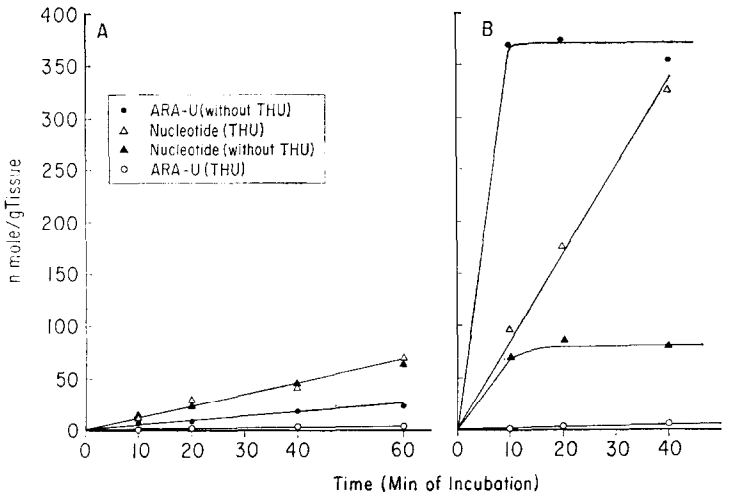

FIG. 2. Ara-C kinase activity measured with and without THU in a human spleen (A) and lung tumor (neurofibrosarcoma) (B). The amount of deamination occurring in the kinase assay is also illustrated. The assay condition is the same as Fig. 1.

TABLE 2. EFFECT OF INCUBATED HUMAN LIVER WITH TETRAHYDROURIDINE (THU) ON THE KINASE ACTIVITY OF CHRONIC LYMPHOCYTIC LEUKEMIA (CLL) CELLS*

Assay mixture	Enzyme activity (nmoles/g tissue/hr)	
	Kinase	Deaminase
Human liver	27	240
Human liver and THU†	14	2
Human liver and THU preincubated with human liver	14	2
CLL	316	144
CLL and THU	316	0
CLL and THU preincubated with human liver	320	0

* Human liver homogenate was preincubated with THU for 2 hr at 37°. The reaction was terminated by heating the tubes in a boiling water bath. After a brief centrifugation the supernatant was used to test its effect on the kinase activity of both human liver and CLL cells.

† Final THU concentration was 2×10^{-4} M.

(3) Does the human liver metabolize THU to a compound(s) which is inhibitory to the kinase activity? An experiment was conducted as follows: human liver homogenate was preincubated with THU for 2 hr at 37°. The reaction was terminated by heating the tubes in a boiling water bath. After a brief centrifugation, the supernatant was used to test its effect on the kinase activity of both human liver and CLL cells. The CLL cells were chosen because of their high kinase activity and availability. The results (Table 2) showed that THU preincubated with the human liver homogenates had no effect on the CLL kinase activity, but still inhibited human liver kinase. In addition, the inhibitory effect of THU (preincubated with or without human liver homogenate) on ara-U formation (deaminase) in the kinase reaction of both human liver and CLL cells would seem to further indicate that the incubated THU is probably still unchanged THU.

(4) In the presence of THU and human liver homogenate, ara-C might be metabolized to a product which is no longer a substrate for ara-C kinase and deaminase reactions. This hypothesis was tested by the following experiment: ara-C, ara-C with THU, or ara-C with THU and human liver homogenate were preincubated at 37° for 90 min, and the incubation was terminated by boiling for 10 min in a water bath. A CLL enzyme preparation and cofactors for the kinase reaction were added and reincubated. The amount of nucleotide and ara-U formed in each sample was measured. Regardless of preincubation with THU and/or human liver homogenate, ara-C served equally well as a substrate for the ara-C kinase or deaminase reaction (Table 3).

In view of the above results, it appears that the ara-C kinase of human liver differs from the ara-C kinase of all the other tissues studied by being sensitive to inhibition by THU. Further work is necessary to verify this point.

TABLE 3. EFFECT OF TETRAHYDROURIDINE (THU) and HUMAN LIVER ON ARA-C*

Preincubation mixture	CLL enzyme activity (nmoles/g/hr)	
	Kinase	Deaminase
Ara-C	154	188
Ara-C and THU	160	0
Ara-C and THU and human liver	154	2

* In the presence of tetrahydrouridine (THU) and human liver, is ara-C unchanged ara-C? Ara-C, ara-C with THU, or ara-C with THU and human liver homogenate were preincubated at 37° for 90 min, and the incubation was terminated by boiling for 10 min in a water bath. An enzyme preparation of chronic lymphocytic leukemic (CLL) cells and cofactors were added for the kinase reaction. Final THU concentration was 2×10^{-4} M.

Acknowledgements—The author would like to thank Dr. William C. Werkheiser, Dr. G. A. LePage and Dr. Emil Frei, III, for the stimulating discussion, and Mrs. Carol Jean Carter for her excellent and capable technical assistance.

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