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MECHANISMS RESPONSIBLE FOR THE LOW INCORPORATION INTO DNA OF THE THYMIDINE ANALOGUE, 5-IODO-2′-DEOXYURIDINE

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Abstract—The comparison of the specific radioactivities of DNA in various tissues of the rat, 1 or 2 hr after injection of either [2-^{14}C] thymidine or [2-^{14}C] iododeoxyuridine confirms that IdU is less readily incorporated into DNA than dT and that the ratio between the uptake of IdU and dT varies from tissue to tissue.

By incubating identical amounts of either [2-^{14}C]-IdU or [2-^{14}C]-dT with extracts of the same tissues, a great difference exists between the phosphorylation of the two substrates, phosphorylation of IdU being almost stopped at the monophosphate level, whereas that of dT proceeds to the triphosphate. The low level of IdUTP formation by the kinase system might account for the poor incorporation of IdU into DNA. The comparison of the results obtained with normal and regenerating liver supports the hypothesis that phosphorylation of dT and IdU are catalyzed by the same sequence of enzymes.

A NUMBER of clinical and biochemical studies have been devoted to the possible applications of 5-iodo-2′-deoxyuridine as cytotoxic agent,[1] and radiosensitizer.[6–9] It has been shown that, at least in most cases, this halogenated analogue of thymidine is less readily incorporated into DNA and more rapidly catabolized than dT.[10–12]

The possibility of using DNA labelled with iododeoxyuridine as a suitable marker for studies on reutilization has also been considered.[11, 13–15] For these reasons, it is interesting to know at which stages of the DNA biosynthesis, dT and IdU behave differently.

The present work shows that a marked difference exists in the phosphorylation rates of thymidine and iododeoxyuridine by various tissue extracts.

METHODS

Animals used

Male Wistar rats, weighing approximately 180–200 g, were used throughout. Partial hepatectomies were performed according to the technique of Higgins and Anderson.[16] The rats were fasted for 24 hr before sacrifice and killed by decapitation.

Preparation of the tissue extracts

After sacrifice, the organs to be studied were quickly excised. The marrow was removed from femur, tibia and humerus by injecting icecold 0·9% NaCl in the lumen

Abbreviations used: 5-IdU: 5-iodo-2′-deoxyuridine; dT: thymidine; TMP: thymidine monophosphate; TDP: thymidine diphosphate; TTP: thymidine triphosphate; IdUMP: iodo-deoxyuridine monophosphate; IdUTP: iodo-deoxyuridine triphosphate.

of the bones. The suspension of bone marrow was then transferred in plastic centrifuge tubes and spun for 15 min at 1,500 *g*. The supernatant was discarded and the pooled bone marrow of 6–8 rats (about 250 mg/rat) was homogenized in 0·01 M Tris-HCl buffer pH 7·0 (20% w/v) by means of the tissue grinder Ultraturrax at 12,000 rpm for 45 sec.

Normal and regenerating livers and spleen were perfused with ice-cold 0·9% NaCl. Normal or regenerating liver, thymus and spleen of 8–10 rats were homogenized in 0·01 M Tris HCl buffer pH 7·0 (30% w/v for livers and 20% w/v for spleen and thymus) by means of Potter tubes fitted with teflon pestles.

The homogenates were spun at 300,000 *g* for 60 min and the supernatants were used as enzyme sources. The protein concentration of the enzymatic extracts was determined with the Folin–Ciocalteu reagent.

Enzymatic assays

The conditions of the enzymatic assays have been discussed in details elsewhere.[17] The composition of the incubation medium was the following, in a final volume of 0·8 ml:

Tris-HCl buffer pH 7·9	55 μmole
ATP	5 μmole
$MgCl_2$	2·5 μmole
[2-^{14}C]-dT or [2-^{14}C]-IdU	1 μCi $\pm$ 40 nmole
enzyme extract	0·5–10 mg protein

At least 4 different concentrations were used for each tissue extract and all the enzymatic assays were made in duplicate. The incubation times are indicated in the legends of the figures. After incubation at 37°C, the reaction was stopped by cooling the mixture and adding 0·1 ml 10 N $HClO_4$. The acid-soluble fraction was neutralized with 5 N KOH and 50 μl were submitted for 18 hr to ascending chromatography on strips of diethylaminoethylcellulose paper (Whatman DE81). The solvent was made of 4 N formic acid, 0·1 M ammonium formate. The radioactivity of the nucleotides peaks was automatically recorded on a 4π counting Scanner Packard. The amount of mono-, di- and triphosphates formed from dT or IdU are plotted as a function of the protein concentration of the samples and the enzymatic activities determined from the slope of the curves, are expressed in n-moles phosphorylated products per mg protein.

DNA synthesis in vivo

In these experiments, the rats were injected with 4 μ Ci (130 n-mole) of either [2-^{14}C]-dT or [2-^{14}C]-IdU, administered in the penis vein under slight ether anesthesia. They were sacrificed 1 or 2 hr after the administration of the labelled precursor (Fig. 1).

The liver, spleen and thymus were quickly excised and dropped into flasks immersed in aceton-dry ice; the bone marrow was removed as described above, the suspension was spun and the sediment, frozen and stored at — 30° until the extraction of DNA. The organs were then separately homogenized in 7–10 vol. of 0·01 M Tris-HCl buffer pH 7·0. 5 ml samples of each homogenate were added with 5 ml ice-cold 1 N $HClO_4$ and allowed to stand at 4° for 15 min. The supernatant was then removed by centrifugation at 3000 *g* for 15 min and the sediment washed three times with ice-cold

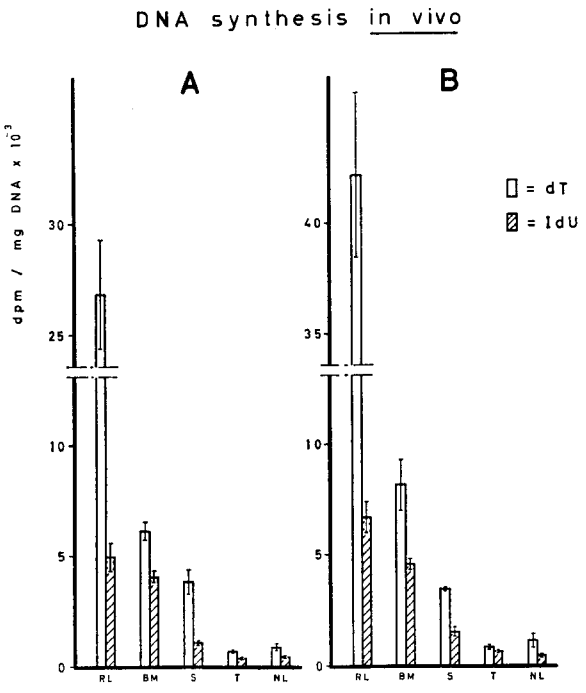

FIG. 1. *In vivo* incorporation of thymidine (dT-white bars) and 5-iodo-2′-deoxyuridine (IdU-hatched bars) into DNA of various rat tissues. *Left* (A): animals sacrificed 1 hr after i.v. injection of 4 μCi of the labelled precursor. *Right* (B): animals sacrificed 2 hr after injection. Ordinates: specific radioactivity of DNA (in dpm per mg DNA). Abbreviations: RL: regenerating rat liver, 24 hr after partial hepatectomy. BM: bone marrow; S: spleen; T: thymus; NL: normal liver. S.D.'s are calculated for 8–10 rats, on the top of each column.

0·5 N $HClO_4$. The DNA was extracted by heating with two portions of 5 ml of 0·5 N $HClO_4$ at 80° for 20 min.

The amount of DNA was determined with the diphenylamine reagent[18] and the radioactivity was measured by liquid scintillation in a Tri-Carb Packard counter.

RESULTS

We have first compared the incorporation *in vivo* of thymidine and 5-iododeoxyuridine into the DNA of various rat tissues. Fig. 1 shows the specific radioactivities of DNA, 1 (A) or 2 (B) hr after i.v. injection of the precursors. In bone marrow, thymus and normal liver, the uptake of thymidine is about two times as great as that of iododeoxyuridine. In the spleen, the ratio of the specific activities reaches 3·8, 1 hr after injection. In order to compare the incorporation of dT and its analogue in a rapidly proliferating tissue, we have chosen regenerating rat liver; the animals were sacrificed 24 hr after partial hepatectomy at the optimum of DNA biosynthesis. It can be seen from Fig. 1 that in this case, dT is incorporated to a 5- to 6-fold greater extent than IdU.

In vitro assays have also been made in order to test the ability of 5-iododeoxyuridine to be used as substrate in place of thymidine for enzymatic phosphorylations by phosphotransferases (kinases) contained in high speed supernatants of tissue

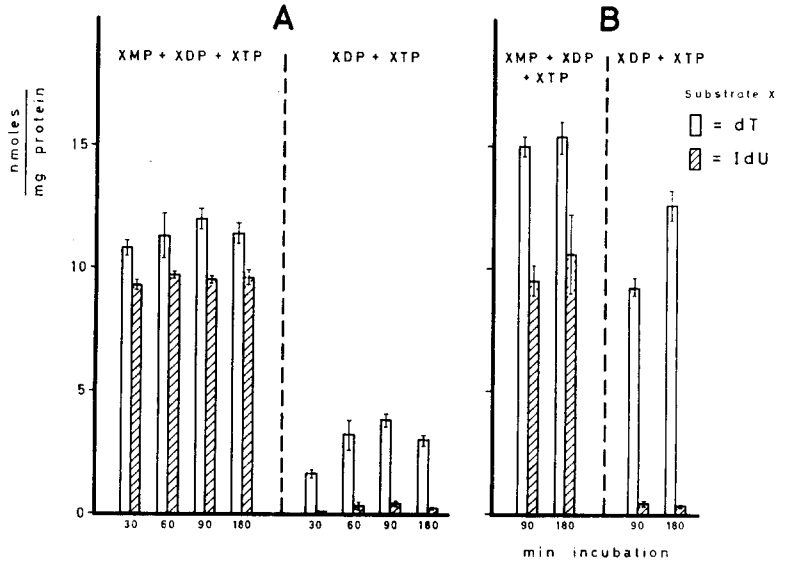

FIG. 2. *In vitro* phosphorylation of either thymidine (dT = white bars) or 5-iodo-2′-deoxyuridine (IdU = hatched bars) in presence of regenerating liver extracts. *Left* (A): rats sacrificed 24 hr after partial hepatectomy; *right* (B): rats sacrificed 39 hr after partial hepatectomy; ordinates: specific enzymatic activities (in n-moles/mg protein); abscissae: incubation times (min). On top of each column S.D.'s are calculated from 3 experiments where 6–8 rat livers were homogenized together.

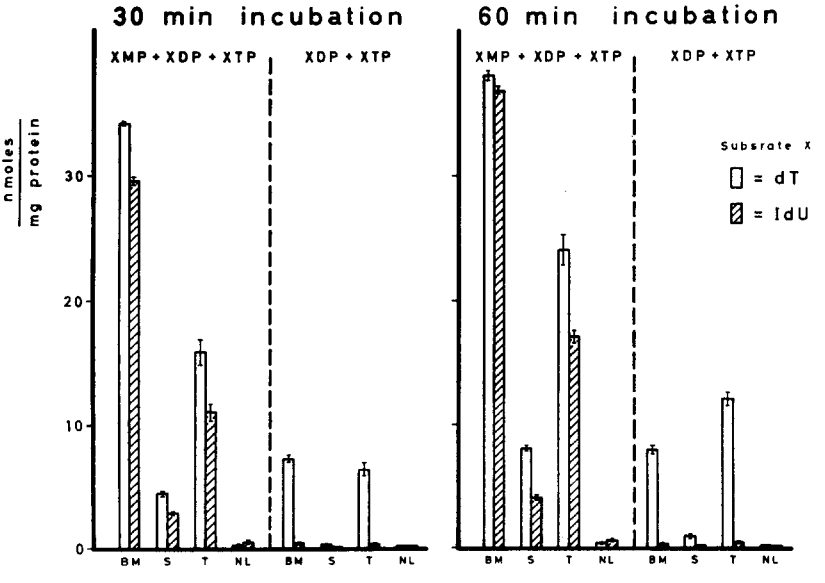

FIG. 3. *In vitro* phosphorylation of either thymidine (dT = white bars) or 5-iodo-deoxyuridine (IdU = hatched bars) in presence of various tissue extracts. Ordinates and S.D.'s: see Fig. 2. Abbreviations: see Fig. 1.

homogenates. The activity of these enzymes has been measured by incubating [2-^{14}C]-dT or [2-^{14}C]-IdU in presence of increasing amounts of tissue extracts. Fig. 2 presents the amounts of mono-, di- and triphosphates per mg protein, formed from identical amounts of either thymidine or iododeoxyuridine incubated with 24 or 39 hr regenerating liver extracts. In the same way, Fig. 3 gives the results obtained with bone marrow, spleen, thymus and normal liver extracts.

The values reported in Figs. 2 and 3 as the sum (XMP + XDP + XTP) represent the activity of thymidine kinase (ATP; thymidine-5′-phosphotransferase E.C. 2.7.1.21) in presence of either thymidine or 5-iododeoxyuridine as substrate. Thymidine and IdU seem indeed to be phosphorylated by the same enzymes.[19]

By increasing the incubation time, no appreciable changes in the ratio of the reaction products have been observed.

For all the tissues considered, except for normal rat liver, a higher amount of dT than IdU is phosphorylated per mg protein. The results presented in Figs. 2 and 3 indicate that the difference becomes much more important at the level of di- and triphosphate formation. The values found for normal liver are extremely low but for 24 hr regenerating liver, the ratio TDP + TTP/IdUDP + IdUTP is about 10·0 and reaches 20·0 with liver extracts of rats sacrificed 39 hr after partial hepatectomy. With extracts of bone marrow and thymus, we have found up to 40 times more TDP + TTP than IdUDP + IdUTP after 60 min incubation. The spleen extracts show a significant phosphorylation of dT to TDP + TTP but no formation of IdUDP + IdUTP from IdU.

From Figs. 2 and 3, it appears that the values of XMP alone are slightly higher for IdUMP than for TMP. This obviously, is the result of a block of further phosphorylation of IdUMP.

The values obtained for di- and triphosphates have not been dissociated; indeed, the nucleoside diphosphokinase has been shown to be nonspecific[20] and its activity being one thousand times as great as dT- and TMP-kinases activities[21, 22] it could not interfere with the present results.

DISCUSSION

That IdU is less readily incorporated into DNA than dT has already been known for some time, as well as the fact that the ratio between uptake of IdU and uptake of dT into DNA varies from tissue to tissue.[10–12] We wanted to compare the rate of incorporation of the two nucleosides *in vivo* in various tissues to their enzymatic phosphorylation by extracts of the same tissues.

Before being incorporated into DNA, nucleosides first undergo phosphorylation to the triphosphate stage. Our results have clearly demonstrated that a great difference in phosphorylation level existed between dT and IdU. Phosphorylation of IdU is almost stopped at the monophosphate level, whereas that of dT readily proceeds to TTP.

The very small amount of IdUTP formed did not allow any *in vitro* measurement of DNA-polymerase activity. But obviously, the large difference between the formation of IdUTP and that of TTP *in vitro* could already account for the difference in the incorporation of the corresponding deoxynucleosides into DNA *in vivo*.

Delamore and Prusoff[19] concluded that phosphorylation of dT and of IdU are catalyzed by the same enzymes. This conclusion finds some support in our observation

that after partial hepatectomy, incorporation of IdU into DNA is much increased, as is the case for dT[23–25] but not for the other deoxynucleosides.[26]

Prusoff[27] proved that IdU acted as a competitive inhibitor for incorporation of dT into DNA and suggested that competition might occur at the level of mono- or triphosphate. Our results show that competition can mainly exist at the monophosphate level, since IdUTP is produced in too small amounts *in vitro* to compete with TTP.

On the other hand, the reverse situation must obtain, and dT may competitively inhibit incorporation of IdU into DNA.[28] This may very well explain the very large difference observed in the labelling of regenerating liver DNA by the two precursors: IdU is catabolyzed more rapidly than dT,[11, 12] most certainly in the liver, and this catabolism will be in favor of dT and therefore enhance the efficiency of the competition by dT. This conception has been proposed by Prusoff *et al.*[28] and is supported by our results.

In other organs, pool dilution may affect the incorporation level of dT, as already suggested by Nygaard and Potter[29] to account for the relatively poor incorporation of dT into thymus DNA, compared to spleen and intestine DNA. The same explanation might be put forward when comparing normal liver DNA to thymus DNA: there is hardly any dT in liver[30] and the specific radioactivity of liver DNA is not much lower than that of thymus DNA (Fig. 1), although DNA synthesis is much more active in thymus than in liver. Indeed, when extracts of these two organs are assayed *in vitro*, in conditions where pool effects are suppressed and catabolic action diminished, the kinase activity is very low in normal liver and of the same order of magnitude in thymus as in regenerating liver (Figs. 2 and 3). Lack of catabolism of IdU in the thymus may also contribute to the fact that IdU incorporation is closer to dT incorporation in thymus than it is in liver.

The chemical mechanism responsible for the low level of IdUTP formation is not clear. Differences in Van der Waals forces would not seem to be involved, since Van der Waals' radius of iodine (2·15 Å) is of the same order of magnitude as that of the methyl group (2·0 Å).[31] The increase in the size of the molecule, however, due to the presence of the I atom, might theoretically interfere with the formation of the enzyme substrate complex. If this factor is involved in the enzymatic phosphorylation, our results suggest that it would act mainly on the TMP kinase activity and not so much on dT kinase activity.

The problem of the use of labelled IdU incorporated into DNA for studies of cell renewal has been discussed by various authors[11, 13–15]. The interpretation of such studies is complicated by eventual reutilization of DNA breakdown products. The fact that IdU is much less phosphorylated to the triphosphate stage than dT decreases very much the probability of its reutilization for DNA synthesis and justifies the use of IdU as a suitable marker for DNA in cell renewal studies.

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