the presence of ethanol, synaptosomal membranes from ethanol-treated mice were less responsive to ethanol *in vitro* than membranes from the control animals. Recent work by Curran and Seeman [17] also supports this finding. These investigators showed that a significant difference between control and ethanol-tolerant groups in miniature end plate potential frequency in phrenic nerve terminals could be brought about only by readdition of ethanol. Recent work in our laboratories has provided a similar finding in that membrane tolerance to chronic barbiturate administration was only observable when synaptosomes were challenged with *in vitro* pentobarbital [6].

These studies point to a cellular mechanism involving calcium-mediated stimulus–secretion coupling events for the production of sedation. Previous work in our laboratories [6] and work done recently by other [4, 5] have shown that barbiturates inhibit synaptosomal calcium influx. We have shown that tolerance develops to this inhibitory effect on $^{45}Ca^{2+}$ influx at the same time as the appearance of behavioral signs of tolerance. The data we have obtained with chlorpromazine are particularly interesting since this drug produces sedation to which tolerance develops, but chronic administration does not result in the production of physical dependence, at least not to the extent of the dependence which is produced by ethanol or barbiturates. Our work shows that, as occurs with barbiturates, tolerance at the membrane level (to the inhibitory effects of chlorpromazine on synaptosomal $^{45}Ca^{2+}$ influx) occurs during the same time frame as the development of behavioral signs of tolerance.

Also of interest is our finding that phenobarbital and chlorpromazine-induced sedation are cross-tolerant. DBA mice rendered tolerant to phenobarbital sedation were also tolerant to chlorpromazine-induced sedation and vice versa. On the other hand, previous work has shown that if chlorpromazine is administered to animals physically dependent upon barbiturates [11] or ethanol [12], withdrawal symptomatology is made worse. This information has significant implications in that it suggests that cellular mechanisms involved in tolerance to sedation may be different from mechanisms involved in physical dependence production. It appears plausible to suggest that "stimulus–secretion coupling" events may be involved in drug-induced sedation and in the development of tolerance to sedation but that other factors, which as yet remain undefined, may be more intimately involved in physical dependence production.

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Incorporation of ψ-isocytidine, a new antitumour C-nucleoside, into mammalian RNA and DNA

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ψ-Isocytidine [5-(β-D-ribofuranosyl)-isocytosine], synthesized by Chu *et al.* [1], is effective against several transplantable murine leukemias including the leukemic cell line P815/ara-C that is resistant to treatment by ara-C (1-β-D-arabinofuranosylcytosine) [2]. This is probably due to the fact that, whereas ara-C is phosphorylated by deoxycytidine kinase, the activity of which is reduced markedly in P815/ara-C [3], ψ-isocytidine is apparently phosphorylated by cytidine kinase [4]. ψ-isocytidine differs from other cytosine nucleosides, such as ara-C, in that it is a C-nucleoside in which the ribose moiety is attached to the base via a carbon–carbon linkage (Fig. 1). This structural feature renders ψ-isocytidine significantly more resistant than cytidine or ara-C to deamination by pyrimidine nucleoside deaminase [2]. The mechanism of action of ψ-isocytidine is different from that of ara-C. Whereas the triphosphate nucleotide form of ara-C inhibits DNA polymerase and thereby DNA synthesis [5], DNA synthesis in mouse small intestine appears not to be directly inhibited by ψ-isocytidine (unpublished observations). Another analog of cytidine which is also isosteric with ψ-isocytidine, 5-azacytidine (Fig. 1), in the monophosphate form inhibits orotidylate decarboxylase [6]. Preliminary studies indicate that, unlike that found using 5-azacytidine, orotidylate decarboxylase activity in homogenates of livers from rats treated with ψ-isocytidine is not inhibited. Since both ara-C [7] and 5-azacytidine [8] are incorporated into nucleic acids, it was of interest, as a possible explanation for the biological activity of ψ-isocytidine, to determine whether this substance could also be utilized as a precursor for RNA and DNA syntheses.

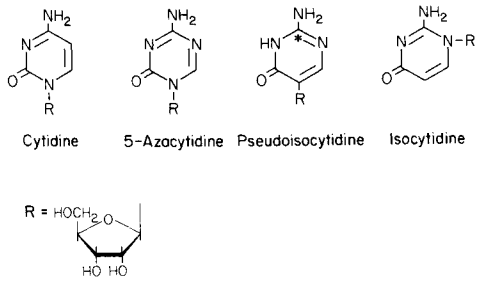

Fig. 1. Structural formulae of cytidine, 5-azacytidine, ψ-isocytidine and isocytidine. The asterisk in ψ-isocytidine denotes the position of the ^{14}C-atom.

The incorporation of [2-^{14}C]ψ-isocytidine into total liver RNA is shown in Fig. 2. The radioactivity and the corresponding u.v.-absorbing material band at a density of 1.6600 g/ml; this value for RNA is similar to that observed by others [12]. The amount of ψ-isocytidine incorporated is 200 pmoles/A_{260} RNA. The non-radioactive u.v.-absorbing material in fractions 4–7 is probably poly ADP-ribose extracted along with the RNA in these procedures using total liver. This material has a density of 1.4900 g/ml; the density of poly ADP-ribose in $CsSO_4$ gradients without formaldehyde is 1.5700 [13]. Other experiments indicated that, unlike the radioactive peak, this material is not alkali-digestible. These properties correspond to those of poly-ADP ribose [13]. The results in Fig. 3 show that [2-^{14}C]ψ-isocytidine can be incorporated into presumably 4s, 18s and 28s RNA.

The incorporation of radioactivity from [2-^{14}C]ψ-isocytidine into DNA was also studied using small intestine and spleen for analyses because cells in these organs continually proliferate and would allow, therefore, maximal incorporation

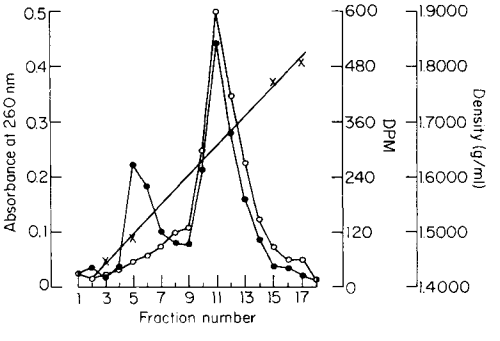

Fig. 2. Incorporation of [2-^{14}C]ψ-isocytidine into total liver RNA. Male weanling (40–60 g) Sprague–Dawley rats (CD line, Charles River Breeding Laboratories, Brookline, MA) were given i.v. [2-^{14}C]ψ-isocytidine (11.3 μCi/mg), 100 mg/kg in 0.9% NaCl solution. At 24 hr the livers, small intestines and spleens were removed and frozen. Total liver RNA was isolated [9]; 50 μl of RNA solution (2 A_{260} units, 1000 dis./min/A_{260}) was added to 4.8 ml of 0.01 M sodium phosphate buffer, pH 7.0, containing $CsSO_4$ (Schwarz/Mann, Orangeburg, NY) to provide an initial density of 1.600 g/ml, and 1% HCHO [10]. Following centrifugation in a Beckman SW 50.1 rotor at 35,000 rev/min at 20° for 66 hr, the fractions (0.27 ml) were collected with a Buchler Densi-Flow. Densities of the fractions were determined with a Bausch and Lomb Abbe-3L Refractometer. To each fraction was added 1 ml of glass-distilled water, its absorbance at 260 nm was determined (Beckman DU-1 cm width cuvette) and its radioactivity content was analyzed with a Packard Tri-Carb liquid scintillation spectrometer using Triton-X based scintillator [11]. Key (●) absorbance at 260 nm; (○) dis./min; and (×) density.

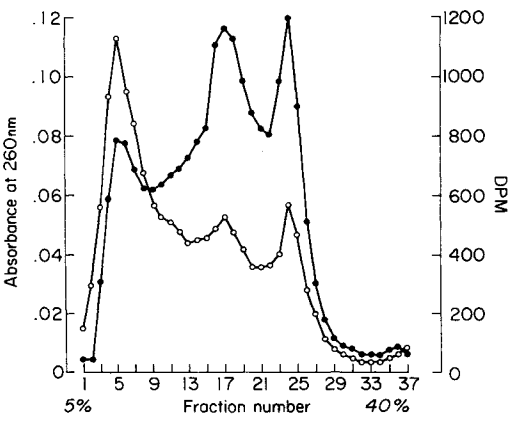

Fig. 3. Incorporation of [2-^{14}C]ψ-isocytidine into the various classes of RNA. Two hundred and fifty μl of the RNA solution (described in Fig. 2) was layered on top of a 5%–40% ribonuclease-free sucrose (Schwarz/Mann) gradient prepared in 0.1 M sodium acetate buffer, pH 5.1, containing 0.1 M NaCl and 0.001 M sodium EDTA and centrifuged in a Beckman SW 27 rotor at 23,000 rev/min at 4° for 20 hr. The 1-ml fractions were collected and the absorbance at 260 nm was determined with a Buchler Fracto-Scan (cuvette light path length of 0.25 cm); the radioactivity of each fraction was measured. Key: (●) absorbance at 260 nm; and (○) dis./min.

of DNA precursors. Incorporation of radioactive product into small intestine is shown in Fig. 4. The u.v.-absorbing material at the density of 1.715 g/ml corresponds to that of single-stranded DNA [16]. The amount of material incorporated is equivalent to 40 pmoles ψ-isocytidine/A_{260} DNA. The u.v.-absorbing material at the densest portion of the gradient with the high specific activity is probably RNA released during the denaturation procedures (see Legend of Fig. 4).

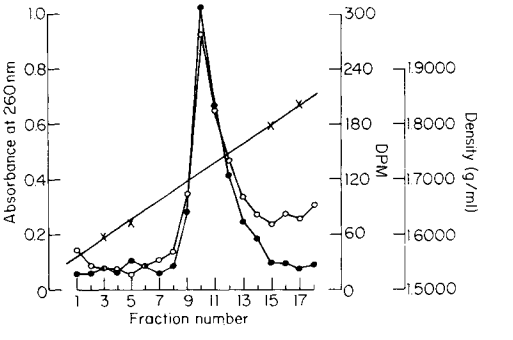

Fig. 4. Incorporation of radioactive material into small intestine DNA from rats treated with [2-^{14}C]ψ-isocytidine. Total DNA from small intestine was isolated by a modified procedure of Marmur [14], as described previously [15]. The isolated DNA was purified using $CsCl_2$ gradients and collected as described [15]. To further remove any [2-^{14}C]ψ-isocytidine-containing RNA from this DNA, the DNA was dialyzed against a 0.01 M NaCl solution and denatured by heating in a boiling water bath for 10 min followed by quick immersion in an ice-bath. To the denatured solution, Tris buffer and $CsCl_2$ were again added. The solution was centrifuged and the DNA again collected as described above. The fractions were collected and their absorbance at 260 nm and radioactivity content determined as described in the legend to Fig. 2. Calf thymus DNA, denatured in a similar manner banded at a density of 1.722 g/ml. Key: (●) absorbance at 260 nm; (○) dis./min; and (×) density.

The radioactive products incorporated into spleen DNA and hepatic RNA were identified (Fig. 5). The radioactive peaks in DNA and RNA correspond to 2′-deoxy-ψ-isocytidine and ψ-isocytidine respectively. Though deoxyguanosine (GdR) has an R_f value similar to that of 2′-deoxy-ψ-isocytidine, it is highly unlikely that the radioactivity from [2-^{14}C]ψ-isocytidine is released and reutilized for synthesis of purines; none of the other deoxy- or ribonucleosides are radioactive. The data indicate that the nucleotide form of ψ-isocytidine is incorporated into RNA and that 2′-deoxy-ψ-isocytidylic acid is incorporated into DNA. It may be presumed that ψ-isocytidine diphosphate is metabolized to the corresponding deoxynucleotide form which is then utilized for DNA synthesis. Alternatively, though unlikely, should the ribose moiety be cleaved from the nucleoside *in vivo* and the resulting free base be metabolized to an N-nucleoside, the radioactive product would not contain cytosine but rather an isocytosine moiety (e.g. isocytidine, Fig. 1). Either way, the incorporation of the ribo- and deoxyribonucleotide forms of ψ-isocytidine or isocytidine into RNA and DNA, respectively, would result in the formation of abnormal nucleic acids, a phenomenon which might account for the biologic activity of ψ-isocytidine. In fact, ψ-isocytidine has been shown to be weakly mutagenic [17].

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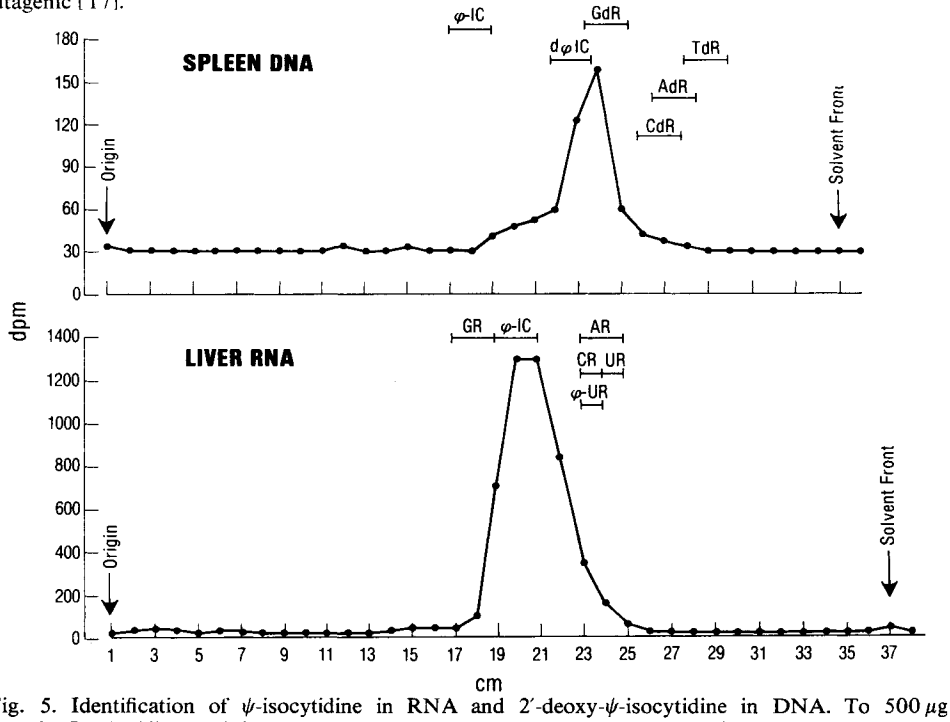

Fig. 5. Identification of ψ-isocytidine in RNA and 2′-deoxy-ψ-isocytidine in DNA. To 500 μg of hepatic RNA (dissolved in 0.1 M sodium acetate–EDTA buffer, pH 5.1) was added 0.5 units T_2 RNAse, 385 units T_1 RNAse and 150 units pancreatic RNAse. The mixture was incubated at 37° for 6 hr after which the pH was raised to 8.5, 500 μg (19 units) of bacterial alkaline phosphatase was added and the mixture incubated for an additional 16 hr. DNA isolated from spleen was treated as follows to remove any traces of RNA. The DNA was purified using $CsCl_2$ density gradient centrifugation [15]. The DNA was collected and incubated in 0.3 M KOH at 37° for 16 hr. The solution was dialyzed against water to remove digested RNA and salts. The DNA was then denatured by heating the solution in a boiling water bath for 10 min followed by quick immersion in an ice-bath. The denatured DNA was again purified by $CsCl_2$ gradient centrifugation and the DNA fractions were collected and dialyzed against water to remove $CsCl_2$. The DNA solution (14 ml, 500 μg) was then adjusted to pH 7.0 and incubated with Mg acetate, 15 mM, and 300 units DNAse I for 3 hr at 37°. Afterward, the pH was raised to 8.5, 500 μg of bacterial alkaline phosphatase and 0.02 units of snake venom phosphodiesterase were added, and the mixture was incubated for an additional 16 hr. Following the incubation periods, an equal volume of chloroform was added to both the RNA and DNA solutions, and the mixtures were shaken and then centrifuged. The deproteinized supernatant fractions were concentrated and the ribonucleosides or deoxyribonucleosides were separated on Whatman No. 1 chromatography paper, using isopropanol–concentrated NH_4OH–0.1 M H_3BO_3 (70:10:20). The paper was cut into 1 cm strips which were placed in counting vials containing water to elute the substances. After 2 hr, liquid scintillator was added and the radioactivity determined. The R_f of each of the standard deoxy- and ribonucleosides is indicated. Abbreviations: ψIC, ψ-isocytidine; dψ-IC, 2′-deoxy-ψ-isocytidine; and ψ-UR, ψ-uridine.

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Kinetics of phosphorylation of 5-aza-2′-deoxycytidine by deoxycytidine kinase*

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5-Aza-2′-deoxycytidine (5-AZA-CdR) is a potent cytotoxic agent to tumor cells *in vitro* [1] and a very effective antileukemic agent in mice [2, 3]. In general, in order for nucleoside analogs to be active inhibitors in the cell, they must first be phosphorylated. Apparently, deoxycytidine (CdR) kinase catalyzes the phosphorylation of 5-AZA-CdR since leukemic cells resistant to this analog are deficient in this enzyme [4, 5]. In this report, we have investigated the interaction of 5-AZA-CdR and its triphosphate form with CdR kinase.

[5-^{3}H]CdR and [2-^{14}C]CdR were obtained from New England Nuclear (Boston, MA). [6-^{14}C]5-AZA-CdR was synthesized by chemical methods [6] and purified by paper chromatography in *n*-butanol–H_2O (86:14). dCTP was obtained from Schwartz–Mann (Orangeburg, NY) and purified by thin-layer chromatography on DEAE-cellulose in 0.05 N HCl at 5°. 5-Aza-2′-deoxycytidine 5′-triphosphate (5-AZA-dCTP) was prepared enzymatically as described previously [7] from AZA-CdR (Chemapol, Prague). CdR kinase was purified from calf thymus as described previously [8] and had a specific activity of 100 units/mg. One unit of enzyme activity is defined as the amount of enzyme that converts 1.0 nmole of CdR to dCMP in 10 min at 37°.

The phosphorylation of CdR and AZA-CdR was assayed as described previously [8], separating the product from the substrate by adherence of the nucleotide to DEAE-cellulose discs. The reaction mixture contained in 0.1 ml: 100 mM Tris–HCl, pH 8.0, or 100 mM imidazole–HCl, pH 6.8; 5 mM ATP; 5 mM $MgCl_2$; 10 mM 2-mercaptoethanol, 0.05 μCi [^{14}C] nucleoside or 1.0 μCi [^{3}H]nucleoside and 0.3 unit CdR kinase.

In Fig. 1 is shown the Lineweaver–Burk plot of different concentrations of radioactive 5-AZA-CdR in the absence and presence of non-radioactive CdR. The apparent K_m of 5-AZA-CdR was estimated to be about 63 μM. CdR was a potent competitive inhibitor of the phosphorylation of AZA-CdR. The apparent K_i for CdR in this reaction was estimated to be 9 μM, a value which is close to its K_m value (14 μM) published previously [8]. The CdR analog, cytosine arabinoside, also has a higher K_m than the natural substrate CdR [8]. In both these cases, a structural change in the base or sugar portion of CdR produced analogs which have a lower binding affinity for the catalytic site of CdR kinase than the natural substrate.

The lower K_i of CdR as compared to the K_m of AZA-CdR is probably one of the key factors for the prevention of the antineoplastic action of this analog by CdR both *in vitro* and *in vivo* [1, 2].

Certain neoplastic cell lines resistant to the CdR analog, cytosine arabinoside, have been observed to have an increased intracellular pool of dCTP [9, 10]. Since the feedback inhibition of CdR kinase by dCTP [8] may be the biochemical mechanism of this resistance, we have investigated the effect of dCTP on the phosphorylation of AZA-CdR by CdR kinase (Table 1). In addition, we studied the effect of 5-AZA-dCTP on this reaction which could be another mechanism by which the cell modulates the intracellular pool size of 5-AZA-CdR nucleotides. At a concentration of dCTP that is within the range found in the cell [11], this nucleotide produced a significant inhibition of the phosphorylation of 5-AZA-CdR. For example, dCTP at a concentration of 10 μM produced a

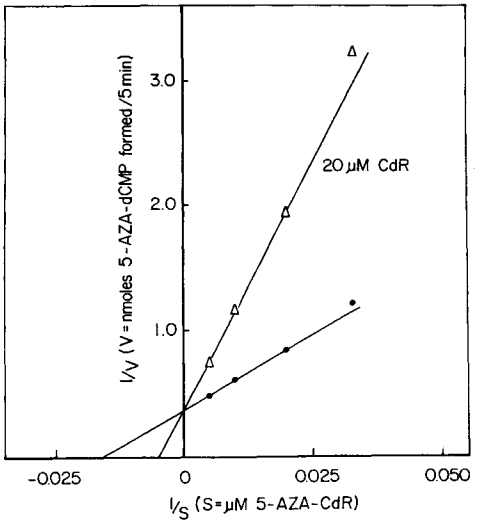

Fig. 1. Lineweaver–Burk plot of the effect of CdR on the phosphorylation of [6-^{14}C]5-AZA-CdR by CdR kinase. The standard reaction mixture with 100 mM Tris–HCl, pH 8.0, contained variable concentrations of [6-^{14}C]5-AZA-CdR. The mixture was incubated for 5 min at 37° in the presence of 0.3 unit CdR kinase and no CdR (●——●), or 20 μM CdR (△——△).

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