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MEASUREMENT OF CARBAMOYLATING ACTIVITY OF NITROSOUREAS AND ISOCYANATES BY A NOVEL HIGH-PRESSURE LIQUID CHROMATOGRAPHY ASSAY

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Abstract—A new assay for carbamoylating activity using reversed phase liquid chromatography (RPLC) is described which offers several advantages over existing assays. A comparison was made of the extent of carbamoylation of the amino group of 5′-amino-5′-deoxythymidine (5′-AdThd) by chloroethyl isocyanate and a series of six nitrosoureas upon incubation (37°) at pH 7.4 in phosphate-buffered saline. Determinations can be made in less than 30 min, whereas more than 24 hr are required in the paper chromatography method of Wheeler *et al.* [*Cancer Res.* **34**, 194 (1974)]. There is a very high coefficient of correlation ($r = 0.986$, $N = 7$, $P < 0.001$) between these two methods of analysis which allows compounds assayed by the new procedure to be compared to the data compiled through the earlier assay. The application of the new assay to the analysis of the rate of carbamoylating activity for these agents is presented, and a mechanistic basis for the marked differences among these structurally similar compounds is discussed.

The anticancer activity both *in vitro* and *in vivo* of certain 2-haloethyl nitrosoureas is well established [1, 2]. Many are in clinical trials; BCNU‡, CCNU and MeCCNU are in clinical use. Under physiological conditions, these highly reactive compounds decompose (Fig. 1) generating alkylating (II) and carbamoylating (III) moieties, which are responsible for the alkylation of nucleic acids and proteins [3, 4] and the carbamoylation of proteins [3, 5]. A number of studies [6–8] have been directed toward deriving correlations between alkylation, carbamoylation, and therapeutic efficacy, using the radiolabeled lysine assay of Wheeler *et al.* [6]. Recently, there has been increased interest in determining the carbamoylating activity of isocyanates, latent-isocyanate prodrugs, and the carbamoylating moieties derived from nitrosourea decomposition. Carbamoylation has been linked to active site specific inactivation of chymotrypsin [9], DNA repair inhibition [10], toxicity of 2-haloethyl nitrosoureas [11], and inactivation of glutathione reductase [12].

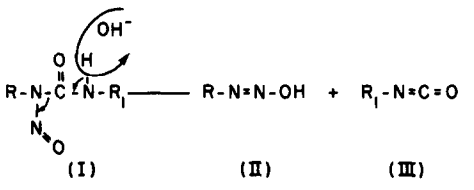

Fig. 1. Decomposition of alkylnitrosoureas (I) to produce an alkyldiazohydroxide (II) and isocyanate (III). 3′-CTNU [$R = CH_2CH_2Cl$, R_1 = 3′-(3′-deoxythymidyl)], 3′-CFdUNU [$R = CH_2CH_2Cl$, R_1 = 3′-(2′,3′-dideoxy-5-fluorouridyl)], 3′-CdUNU [$R = CH_2CH_2Cl$, R_1 = 3′-(2′-3′-dideoxyuridyl], BCNU [$R = R_1 = CH_2CH_2Cl$], chlorozotocin [$R = CH_2CH_2Cl$, R_1 = 2-D-glucopyranosyl], streptozotocin [$R = CH_3$, R_1 = 2-D-glucopyranosyl], and CEI [III, $R_1 = CH_2CH_2Cl$].

The assay of Wheeler *et al.* [6] using the extent of carbamoylation of radiolabeled lysine (Fig. 2) has a number of disadvantages. The use of the radioisotope incurs the expense of the isotope and scintillant, liquid scintillation counter time, and the usual health safety precautions, but more importantly the assay is tedious and not amenable to the rapid analysis of multiple samples. The assay of one compound by this method with appropriate controls in duplicate at a single time point (typically 6 hr) can involve the cutting and preparation of 100 paper chromatography fractions to generate a radiochromatogram by liquid scintillation counting.

We describe here a rapid, convenient HPLC assay based upon the carbamoylation of the amino group of 5′-amino-5′-deoxythymidine (5′-AdThd) to yield the corresponding urea (Fig. 2). No radioisotope is required, since the strong ultraviolet absorption of the nucleoside allows quantitation of the extent of carbamoylation by measurement of the decrease in the height of the 5′-AdThd peak. This new assay

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‡ Abbreviations: BCNU, 1,3-bis(2-chloroethyl)-1-nitrosourea; CCNU, 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea; MeCCNU, 1-(2-chloroethyl)-3-(*trans*-4-methylcyclohexyl)-1-nitrosourea; 5′-AdThd, 5′-amino-5′-deoxythymidine; HPLC, high-pressure liquid chromatography; CEI, chloroethyl isocyanate; Sn_2, substitution nucleophilic bimolecular; 3′-CTNU, 3′-[3-(2-chloroethyl)-3-nitrosoureido]-3′-deoxythymidine; 3′-CFdUNU, 3′-[3-(2-chloroethyl)-3-nitrosoureido]-2′, 3′- dideoxy - 5 - fluorouridine; 3′-CdUNU, 3′-(3-(2-chloroethyl)-3-nitrosoureido]-2′,3′-dideoxyuridine; GANU, 1-(2-chloroethyl)-3-(β-D-glucopyranosyl)-1-nitrosourea; CHLZ, 2-[3-(2-chloroethyl)-3-nitrosoureido]-D-glucopyranose; STRPZ, 2-(3-methyl-3-nitrosoureido)-D-glucopyranose; and PBS, phosphate-buffered saline (pH 7.4).

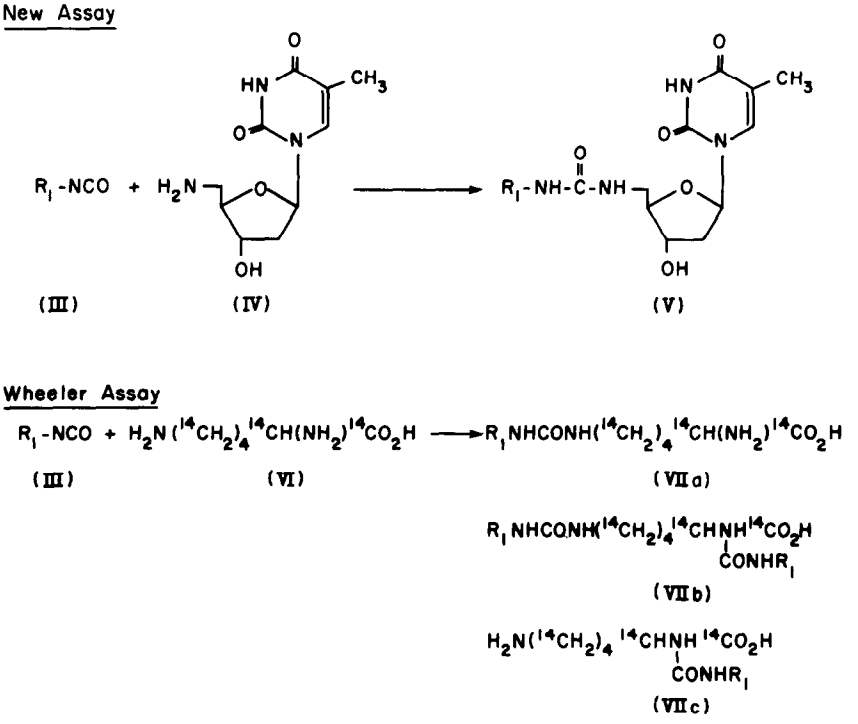

Fig. 2. Assays for carbamoylating activity of nitrosoureas and isocyanates. R_1 as defined in Fig. 1.

simplifies the screening of multiple samples and makes feasible the detailed examination of the time course of the production of the carbamoylating moiety.

MATERIALS AND METHODS

Chlorozotocin, streptozotocin, and BCNU were obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute. 3′-CTNU, 3′-CFdUNU, and 3′-CdUNU were provided by Dr. T.-S. Lin. 5′-AdThd was synthesized by the procedure of Lin and Prusoff [13] and can be purchased from the Sigma Chemical Co. Chloroethyl isocyanate and L-lysine monohydrochloride were obtained from the Aldrich Chemical Co. L-[U-^{14}C]Lysine monohydrochloride was obtained from Amersham. 3′-CTNU radiolabeled with ^{14}C in the alkylating moiety was synthesized by the procedure of Brubaker and Prusoff [14].

Chromatographic methods. A Gilson chromatographic system as described previously [15] was used for reversed phase gradient elution analyses. Analyses were performed using a Whatman Partisil ODS-2 (25 × 0.46 cm, 10 μm) column. Mobile phase A was 95% 0.01 M KH_2PO_4 (pH 3.0)/5% methanol and mobile phase B was 55% 0.01 M KH_2PO_4 (pH 3.0)/45% methanol. A linear gradient was used, 0–100% B in 30 min at a flow rate of 1.75 ml/min at ambient temperature.

Assay of carbamoylating activity. A stock assay solution was prepared and adjusted by u.v. scan to a concentration of 5 μmoles of 5′-AdThd per 450 μl PBS (pH 7.4). Periodic analysis of an aliquot by HPLC indicated no degradation or concentration change during storage at 2° throughout the course of these experiments. Streptozotocin and chlorozotocin were dissolved in 50% dioxane/50% PBS, chloroethyl isocyanate in dioxane, and the remaining nitrosoureas in ethanol at a concentration of 5 μmoles per 50 μl vehicle.

The assay procedure was performed as follows. A 1-ml glass vial was charged with 450 μl of the stock assay solution and warmed to 37°. To this was added 5 μmoles of the compound under study in 50 μl of vehicle. The mixture was vortexed for 10 sec and the t_0 aliquot removed and injected for analysis. The reaction mixture was incubated at 37° in a WCLID model 2156 water bath shaker, and aliquots were withdrawn for analysis each hour for 6 hr.

Standard curves of 5′-AdThd were constructed by triplicate injections at three concentrations in the range of interest. All plots of peak height (254 nm u.v. detector) versus nmoles of 5′-AdThd were linear and passed through the origin. Concentration of 5′-AdThd at each time point was measured by comparing peak height to the standard curve. Carbamoylating activity was determined by the following equation:

$$100 \times \frac{[5'\text{-AdThd}]_{t_0} - [5'\text{-AdThd}]_{t_x}}{[Y]_{t_0}}$$

= % carbamoylation of 5′-AdThd by compound Y at time x.

For the purpose of comparison, carbamoylating activity was also measured by incubation of the nitrosourea with radiolabeled lysine via the method of Wheeler *et al.* [6]. A stock solution of L-[U-^{14}C]-lysine was prepared as follows. Ten microcuries of

L-[U-^{14}C]lysine (sp. act. 336 mCi/nmole) was diluted with 25 μmoles of nonradioactive lysine in 2250 μl of PBS (pH 7.4). To 225 μl of this stock solution was added 2.5 μmoles of the compound to be assayed dissolved in 25 μl of the appropriate vehicle, giving a final solution 10 mM in both lysine and nitrosourea. The vehicle used for each compound was the same as that used in the 5′-AdThd assay. The reaction mixtures with the appropriate vehicle but without nitrosourea were used as controls. After incubation of the mixture at 37° for 6 hr, 5 μl was spotted on Whatman No. 1 paper, and ascending chromatography was performed using 65% isopropanol/17% concentrated hydrochloric acid/18% water as the eluent. The chromatograms were dried, cut into 1-cm strips, and immersed in Econofluor (New England Nuclear). Radioactivity was measured with a Beckman LS 7500 liquid scintillation counter. Radioactivity present as positions other than that corresponding to the control R_f of lysine was attributed to products of carbamoylation. Assays were corrected for small quantities of extraneous radioactivity detected in the control chromatograms. The percentage of total radioactivity associated with carbamoylated products was used as the measure of carbamoylating activity.

RESULTS AND DISCUSSION

Figures 3 and 4 compare the resolution of the HPLC assay versus the Wheeler assay. The chromatograms represent the 6-hr time point from each assay. Three unresolved peaks corresponding to the two mono-carbamoylation and one di-carbamoylation product can be seen in Fig. 3 in addition to the lysine peak; these four peaks obscure 75% of the chromatographic field. In contrast, 5′-AdThd in the HPLC (Fig. 4) assay is present as a sharp, easily quantified peak, well-resolved from eleven other peaks corresponding to carbamoylation and decomposition products. Clearly, the superior resolution obtained by HPLC minimizes the possibility of any carbamoylation products coeluting with the peak of interest and thus compromising the data obtained. In addition, any interfering overlap of peaks could be easily remedied by adjustments in the HPLC gradient profile, whereas the isocratic nature of paper chromatography limits its flexibility in such a situation.

A control experiment was performed to determine if alkylation of 5′-AdThd would interfere with the measurement of carbamoylation. The nitrosourea nucleoside 3′-CTNU radiolabeled with ^{14}C in the alkylating moiety was incubated with 5′-AdThd under the assay conditions. If alkylation of the 5′-AdThd occurred, then superimposing the u.v. chromatogram and the radiochromatogram should show some peaks exhibiting both u.v.-absorbance and radioactivity, i.e. a covalent reaction product of the labeled alkylating moiety with the u.v. detectable nucleoside. This situation could also result from the coincidental co-elution of nucleoside products and volatile decomposition products of the alkylating moiety such as chloroethanol or acetaldehyde.

Following injections of an aliquot of the reaction mixture at 2 and 6 hr, the HPLC column effluent was collected in 1-min fractions and the corresponding radiochromatogram was generated. The fractions containing counts above background were combined and evaporated to dryness using a rotary evaporator. Analysis of the residue by HPLC and liquid scintillation counting indicated that, while there was no loss in u.v.-absorbance, the radioactivity was now at

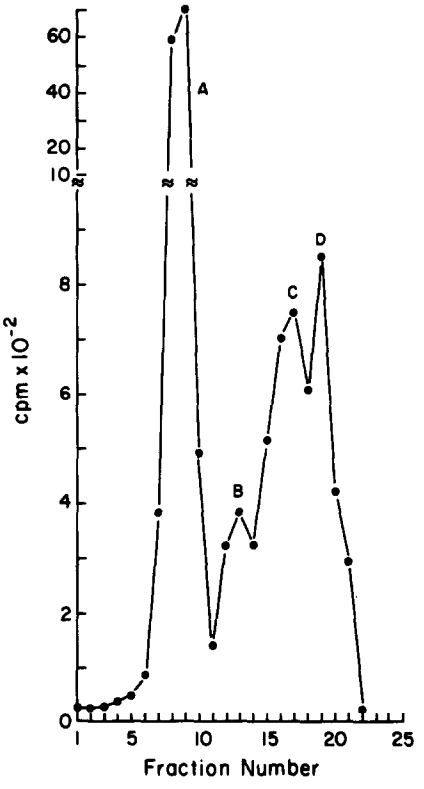

Fig. 3. Radiochromatogram generated from the carbamoylation of [^{14}C]lysine (Wheeler assay) by 3′-CTNU. Five microliters of the reaction mixture was removed following incubation at 37° for 6 hr, spotted on Whatman No. 1 paper, and developed by ascending chromatography using 65% isopropanol/17% concentrated hydrochloric acid/18% water as the eluent. After drying, the chromatograms were cut into 1-cm strips and immersed in Econofluor, and the radioactivity was measured. A = unreacted [^{14}C]lysine; B, C and D = mono- and di-carbamoylation products.

Table 1. Results of measurement of carbamoylating activity of nitrosoureas and chloroethyl isocyanate

Compound	% Carbamoylation	
	New assay*	Wheeler assay†
STRPZ	7.00 ± 0.89	0.764 ± 0.10
CHLZ	9.95 ± 0.90	1.07 ± 0.11
CFdUNU	60.0 ± 0.61	19.9 ± 0.30
CdUNU	66.0 ± 1.72	23.6 ± 1.75
CTNU	66.4 ± 0.25	23.6 ± 3.00
BCNU	78.1 ± 1.92	32.2 ± 2.12
CEI	86.4 ± 0.03	38.0 ± 2.72

Values are the mean ± S.E., N = 3.
* Expressed as percent reaction with 5′-AdThd.
† Expressed as percent reaction with [^{14}C]lysine.

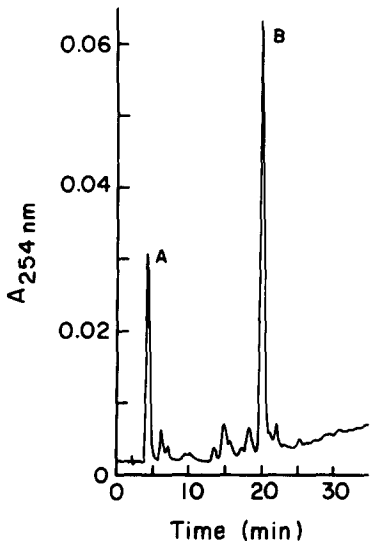

Fig. 4. HPLC assay for carbamoylating activity. Following incubation of 3′-CTNU with the assay reagent (A, 5′-AdThd) at 37° for 6 hr, an aliquot was injected and the extent of carbamoylation was measured by the decrease in the 5′-AdThd peak from its initial concentration. B is the major carbamoylation product (Structure V, Fig. 2). Remaining peaks are minor carbamoylation and decomposition products. Column and conditions are as described in Materials and Methods.

background level. Therefore, there was no measurable alkylation of the u.v. detectable 5′-AdThd by the labeled alkylating moiety of the nitrosourea.

Comparison of carbamoylation data. Both our assay and the Wheeler assay were performed under identical conditions on the seven compounds listed in Table 1. Chloroethyl isocyanate was included as an indicator of the maximum value for carbamoylation obtainable in each assay. Chlorozotocin and streptozotocin, two nitrosoureas known for their poor carbamoylating activity, were included to define the lower end of the spectrum of carbamoylating activity. The data in Table 1 represent the extent of carbamoylation after 6 hr. Clearly, the order of the compounds as defined by carbamoylating activity is identical in both assays. Values obtained by either assay can be correlated by the linear regression equation $Y = 9.64 + 2.20X$ (X = Wheeler %, Y = new assay %; $r = 0.986$, $N = 7$, $P < 0.001$), or by running a known compound such as BCNU to obtain a benchmark. While the relative order of compounds remains the same, the large difference in the extent of carbamoylation, as measured by each assay for a given compound, illustrates another advantage of the new assay. The Wheeler assay, as ordinarily used, yields a low value for total absolute carbamoylation, because the di-carbamoylated product would contain no more radioactivity than a mono-carbamoylated product. Therefore, it would be necessary to multiply the amount of radioactivity associated with the di-carbamoylated product by 2 in order to measure total carbamoylation. The new method does not require a similar correction. The Wheeler assay was designed for comparing the relative, rather than the absolute, extents of carbamoylation by nitrosoureas, and it has been useful for that purpose. However, the new method described here has definite advantages.

CEI, which reacts directly with the amines without prior chemical transformation, demonstrated the highest carbamoylating activity of the seven compounds studied. The carbamoylating activity of BCNU approached that of CEI, which is not surprising since the isocyanate produced during the decomposition of BCNU is, in fact, CEI. The chemical structures of the isocyanates produced in the decomposition of the other nitrosoureas differs greatly from CEI, and that is evident in the differences in carbamoylating activity. The nitrosourea sugars streptozotocin and chlorozotocin are known for their relative lack of carbamoylating activity,

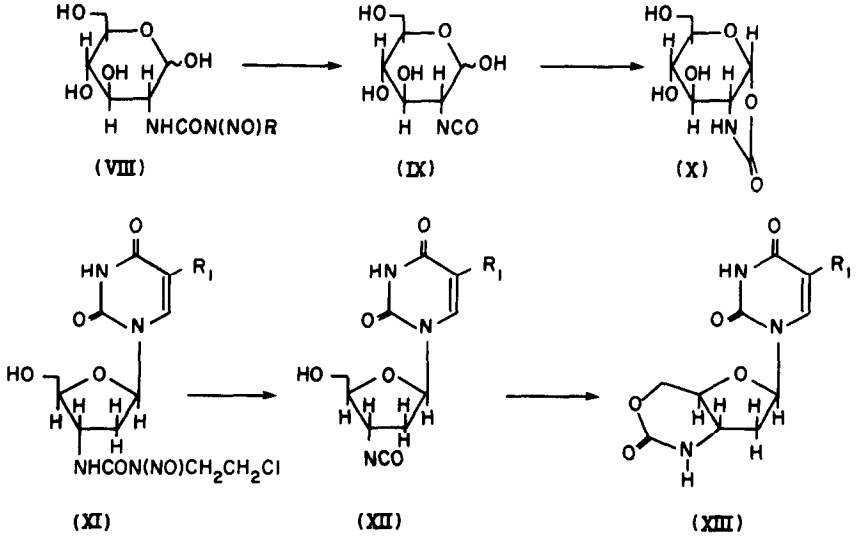

Fig. 5. Formation of bicyclic carbamates by the decomposition of sugar and nucleoside nitrosoureas. R = CH_3 or CH_2CH_2Cl, R_1 = F, H or CH_3.

presumably due to the competing internal cyclization to form the cyclic carbamate [16] (X), which in these cases is apparently inert to further reaction with the amino compounds used in both assays (Fig. 5). The carbamoylating activity of the three nucleoside nitrosoureas was considerably greater than that of the sugar nitrosoureas, in spite of the fact that it has been demonstrated [17] that these compounds undergo an analogous internal cyclization to form the 3′,5′-cyclic carbamates (XIII, Fig. 5). While the carbamates formed by streptozotocin and chlorozotocin were stable, those formed by the nucleoside analogs exhibited greater lability, evidenced both by their greater carbamoylating activity as measured in these assays and by reaction of the isolated 3′,5′-cyclic carbamates with 5′-AdThd [17].

Time course of carbamoylating activity. Using the new assay, we were able to follow the time course of carbamoylation for each of the seven compounds studied (Fig. 6). These data provide a clearer mechanistic picture than that derived from the extent of carbamoylation alone. With CEI, an isocyanate, reaction was complete in minutes. This is not surprising, since the isocyanate and amine can react directly with each other in a facile Sn_2 reaction. At the apparent t_0 time point, the carbamoylation reaction was already complete. In contrast, the extent of carbamoylation by BCNU increased with time, reaching a plateau at 5–6 hr. Carbamoylation by nitrosoureas first requires decomposition of the intact drug to yield an isocyanate. Of the compounds studied, BCNU with its reported [18] half-life of approximately 50 min (PBS, pH 7.4, 37°) was the most stable. It should be noted that, while the other nitrosoureas and CEI had decomposed completely in 1–2 hr (determined by disappearance of parent drug peak), intact BCNU was present through the 5-hr time point, and the rate of carbamoylation by BCNU closely followed the rate of decomposition of parent drug. Since the rate-limiting step in the production of the carbamoylating moiety (CEI) from BCNU is the abstraction of the N-3 proton by base (see Fig. 1), and the subsequent reaction of the CEI with the amine is extremely rapid, one would expect the rate of carbamoylation to parallel the rate of decomposition of BCNU.

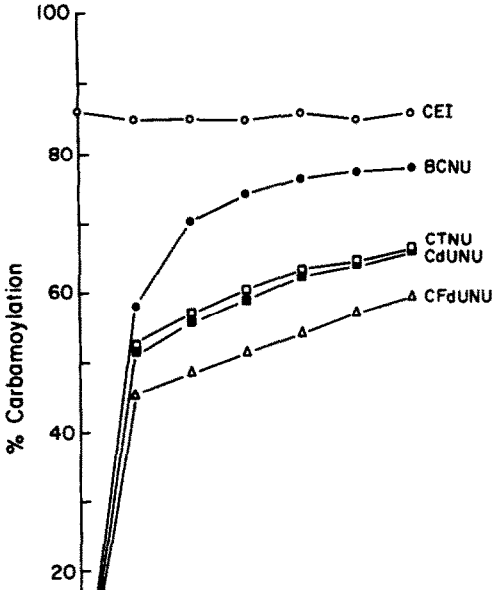

Fig. 6. Plot of extent of carbamoylation of 5′-AdThd by chloroethyl isocyanate and various nitrosoureas versus time. The compounds assayed were incubated at 37° with 5′-AdThd in PBS (pH 7.4). Aliquots were taken every hour, analyzed by the HPLC method described and the extent of carbamoylation determined by the decrease in the 5′-AdThd peak. Values represent the mean of three experiments.

The time course profiles of carbamoylation by BCNU and CEI differ both from each other and from the nitrosourea sugars and nucleosides, whereas the compounds within a class, i.e. the nitrosourea sugars or nucleosides, demonstrate qualitative and quantitative similarities. As illustrated in Fig. 5, the isocyanate formed in the decomposition of the nitrosourea sugars and nucleosides has the option of reaction with intramolecular nucleophilic sites. Streptozotocin and chlorozotocin produced identical isocyanates upon decomposition and exhibited essentially the same carbamoylating activity. The isocyanate formed from streptozotocin and chlorozotocin preferentially undergoes an internal cyclization, and the resultant bicyclic carbamate was relatively inert to further reaction with nucleophiles such as the amino compound used in this assay. This is supported by both the minimal extent of carbamoylation observed, and the fact that this low level did not increase following disappearance (approximately 1 hr) of the parent drug. In contrast, carbamoylation continued to increase even after disappearance of intact drug in the case of the nitrosourea nucleosides. This is consistent with initial conversion of the nitrosourea nucleoside into an intermediate 3′,5′-cyclic carbamate (Fig. 5), which is not inert and acts to potentiate the rate of carbamoylation. The bicyclic carbamates from the nitrosourea nucleosides may be converted to the isocyanates by base-catalyzed abstraction of the RNHCOOR proton followed by elimination of $^-$OR; alternatively, the possibility exists that the amine reacts directly with the bicyclic carbamates. If the isolated nucleoside bicyclic carbamate is incubated in PBS with 3′-AdThd, a 3′,3″-ureido dimer is formed, indicating the ability of this compound or its open isocyanate form to carbamoylate amines [17]. The limits of carbamoylation (>6 hr) for the nitrosourea nucleosides were reached concurrently with the disappearance of the peak corresponding to their 3′,5′-cyclic carbamates.

The question arises, why should there be such a pronounced difference in the carbamoylating ability of the two types of bicyclic carbamate? A plausible explanation can be derived from an examination of the stereochemistry of the molecules (Fig. 7).

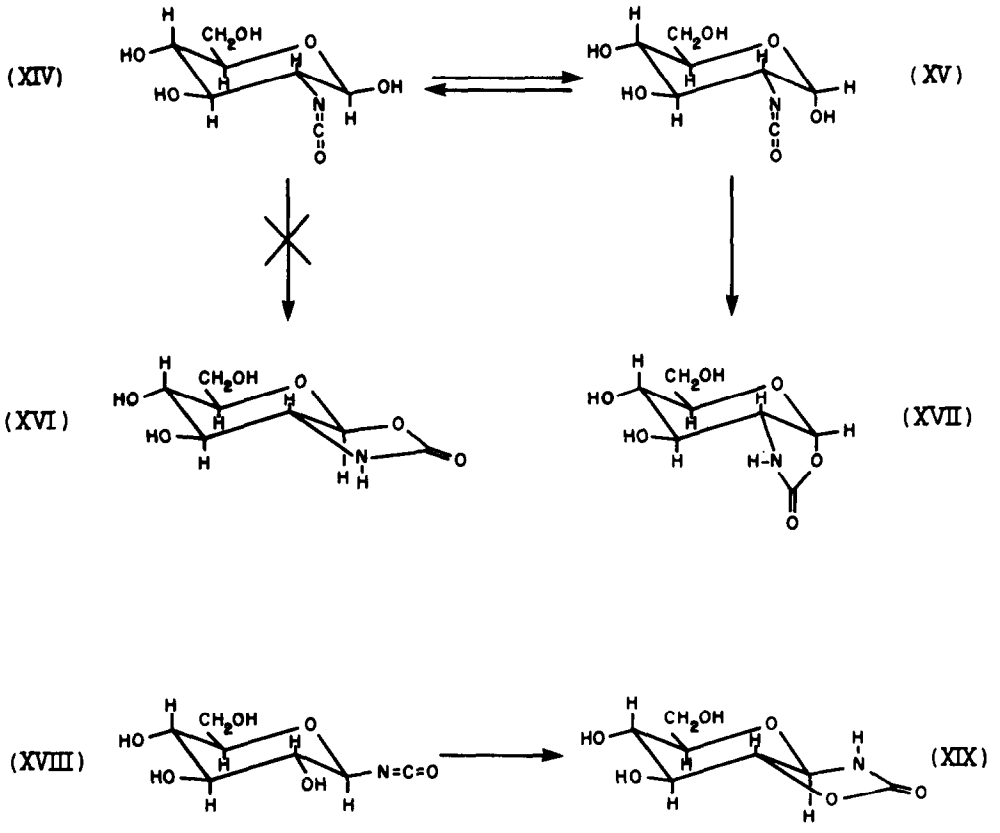

Fig. 7. Stereochemistry of the formation of bicyclic carbamates from sugar nitrosoureas.

A *trans* relationship exists between the C-4′ -CH_2OH group and the C-3′ isocyanate group derived from the nitrosourea nucleosides (XII and XIII, Fig. 5); conversion to the *cis* form is impossible. Therefore, cyclization results in a 6-membered cyclic carbamate fused in a *trans* relationship with the furanose ring. The stereochemistry of the cyclization product is not as easily defined for the sugar nitrosoureas. Epimerization occurs at C-1 of the sugar ring, and for glucose the α and β forms (XIV and XV, Fig. 7) exist in equilibrium in the conformations shown. Therefore, ring closure of the isocyanate could produce a mixture of both the *cis* and *trans* fused ring systems. However, this ring closure reaction is analogous to the protection of diols by reaction with acetone/H^+ to yield a cyclic acetal, and when the diol is cyclic as in the case of glucose, it is well known that for geometric reasons ring closure occurs predominantly when the two -OH groups are *cis*. This essentially acts as a trap for the *cis* form (XVII) of the equilibrium pair. These same factors in the intramolecular closure of the glucose isocyanate may select for the *cis* fused bicyclic carbamate, which apparently is relatively stable. The nitrosourea nucleosides can only form the *trans* isomer, which is labile, as evidenced by its ability to carbamoylate amines. The possibility that it is the stereochemistry of the fused ring carbamates that determines their reactivity is corroborated by the fact that the isocyanate (XVIII) derived from the nitrosourea sugar GANU (Fig. 7), in which the isocyanate moiety is located on C-1, can only produce a *trans* fused ring (XIX) and exhibits carbamoylating activity only slightly less than that of BCNU [8].

The carbamoylation assay described here has been shown to have significant advantages in speed and simplicity over the literature method. In addition, the necessity of working with radioisotopes is eliminated. Its utility for detailed investigations of the rates as well as the extent of carbamoylation by various agents has been demonstrated. It is hoped that the availability of this new assay will promote other detailed investigations of the rate of carbamoylation by nitrosoureas and isocyanates, since the rate of production of carbamoylating moiety may prove to be as critical biologically as the actual extent of reaction.

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