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Reaction of Formaldehyde with Heterocyclic Imino Nitrogen of Purine and Pyrimidine Nucleosides*

Edward J. Eyring† and James Ofengand

ABSTRACT: Hydroxymethylation of pseudouridines B and C, uridine, thymidine, inosinic acid, and polyuridylic acid by reaction with formaldehyde has been shown to take place by the use of spectrophotometric techniques. Reaction is almost instantaneous at room temperature and neutral or alkaline pH, resulting in addition to the N_1 and N_3 atoms of the pyrimidine ring, where available, and to the N_1 atom of the purine ring. Reactants and products are in rapid equilibrium such that after excess formaldehyde is removed by chromatography, no hydroxymethyl adduct remains. The equilibrium constant for reaction with uridine and inosinic acid was determined to be 2.5 and 1.7 l./mole, respectively. This reaction becomes significant when nucleic acids are studied in the presence of formaldehyde, since under these conditions an appreciable fraction of the uridylic or thymidylic acid will be derivatized.

Hydroxymethylation with formaldehyde has been widely used as a mild means of alteration of the secondary structure of nucleic acids as a result of the initial observations of Fraenkel-Conrat (1954) and Staehelin (1958), confirmed by others (Grossman *et al.*, 1961; Haselkorn and Doty, 1961), that reaction occurs wi h amino group containing nucleotides in both RNA and DNA. Several studies have shown that nucleic acids possessing large amounts of secondary structure such as native DNA, synthetic copolymers, or RNA in the presence of magnesium do not react with formaldehyde, while nucleic acids in which the secondary structure has been disrupted do so readily (Staehelin, 1958; Grossman *et al.*, 1961; Haselkorn and Doty, 1961; Staehelin, 1959; Sinsheimer, 1959; Sarkar and Dounce, 1961; Penniston and Doty, 1963; Marciello and Zubay, 1964; Fasman *et al.*, 1965). Attempts have been made to use this reaction as a means of distinguishing structured from nonstructured regions and to quantitate the degree of structure (Haselkorn and Doty, 1961; Penniston and Doty, 1963). In all of these studies, it has been assumed that formaldehyde does not react with the heterocyclic imino nitrogen atoms of purine or pyrimidine nucleotides such as uridylic acid because of the spectral study reported by Fraenkel-Conrat (1954). He observed that while the reaction with adenylic, cytidylic, and guanylic acids was relatively slow and led to marked spectral changes, only minor changes were noted in the absorption of uridine upon addition of formaldehyde, and there was no progressive alteration of the spectrum with time. These results were supported by the failure of [^{14}C]formaldehyde to become bound in a stable form to poly U or to poly I (Staehelin, 1958).

In this paper we report experiments showing that formaldehyde does react with the imino nitrogen atoms of pseudouridine, uridine, thymidine, and inosinic acid as well as with polyuridylic acid. The reaction is very rapid. However, the hydroxymethyl adducts are unstable and decompose when formaldehyde is removed. The reaction is of significance, therefore, only in those studies in which the nucleic acid is studied in the presence of formaldehyde.

Experimental Section

Materials

Uridine (pseudouridine free),[1] thymidine, and inosinic acid, all A grade, were obtained from CalBiochem and used without further purification. Pseudouridine, purchased from CalBiochem, was separated into its B and C isomers (α and β anomers, respectively) by thin layer chromatography (Ofengand and Schaefer, 1965). [^{14}C]Formaldehyde was obtained from New England Nuclear Corp. Polyuridylic acid was synthesized

* From the Department of Biochemistry, University of California Medical Center, San Francisco, California. *Received March 27, 1967.* Supported by Grant GM 11506 from the National Institutes of Health.

† Special Fellow of the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health. Present address: Children's Hospital, Department of Orthopaedics, Columbus, Ohio.

[1] Actual chromatographic analysis showed the presence of 1.0% pseudouridine in this sample (lot 40046).

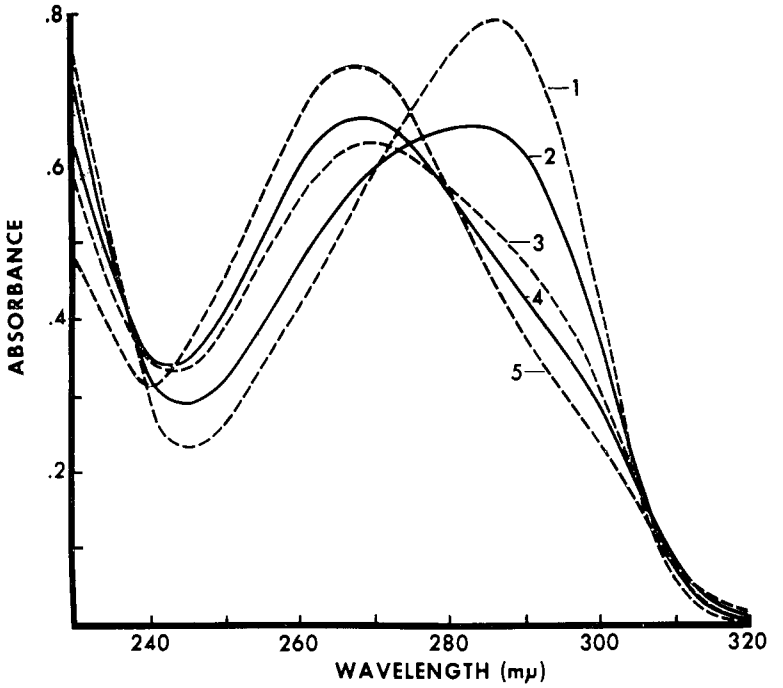

FIGURE 1: Spectrum of pseudouridine C as a function of formaldehyde concentration. Initial pH 10.01 (curve 1); final pH 9.80 (curve 5). Curve 1: pseudouridine C; curve 2: same as curve 1 plus 0.22 M HCHO; curve 3: same as curve 1 plus 1.03 M HCHO; curve 4: same as curve 1 plus 1.90 M HCHO; and curve 5: same as curve 1 plus 3.33 M HCHO.

from UDP[2] using *Micrococcus lysodeikticus* polynucleotide phosphorylase.

Methods

Concentrations were determined by ultraviolet absorption using the following extinction coefficients. For uridine, thymidine, and inosinic acid, the values given by Pabst Laboratories were used.[3] For the pseudouridine isomers, the values determined by Chambers (Shapiro and Chambers, 1961; Chambers, 1966) were used in conjunction with the spectrophotometric titration data previously obtained by us (Ofengand and Schaefer, 1965). The concentration of polyuridylic acid was determined using the extinction coefficient for uridylic acid.

Formaldehyde (J. T. Baker reagent grade 36% containing 12% methanol) was used without further purification since it had been previously shown to be satisfactory for hydroxymethylation experiments (Grossman *et al.*, 1961; Haselkorn and Doty, 1961).

pH was measured at 30° before and after all spectral readings with a Radiometer pHM25SE pH meter standardized against pH 7.00 and 10.00 Beckman standard buffers at 30°. Reactions at pH 10 were buffered with 0.1 M $NaHCO_3$–Na_2CO_3 buffer; those at pH 7 with 0.03 M NaH_2PO_4–Na_2HPO_4 buffer except where indicated otherwise.

Spectra were determined in a Cary Model 14 spectrophotometer in cuvets thermostated at 30°. The reference cell contained the same solution as the sample cell with the exception of the nucleoside or nucleic acid. Formaldehyde solution was added in equal amounts to sample and reference cells. All curves were replotted manually taking into account the percentage dilution of nucleoside by addition of formaldehyde solution. This was never more than 25%. The addition of formaldehyde produced a small drop in pH but this never exceeded 0.2 pH unit, and all measurements were made well away from the pK of the nucleosides in question.

The tandem cell measurements were done as follows. Solutions of nucleoside buffer and formaldehyde buffer were prepared using a fixed nucleoside concentration and several formaldehyde concentrations. The reference pair of 1-cm path cells contained in one cell the nucleoside buffer and in the second cell the appropriate formaldehyde buffer. The sample pair of cells were both filled with a 1:1 mixture of the nucleoside buffer plus formaldehyde buffer, and the difference spectra at 23° were recorded. Thin layer chromatography was performed as described previously (Ofengand and Schaefer, 1965).

[2] Abbreviations used: UDP, uridine diphosphate; IMP, inosine monophosphate; UMP, uridine monophosphate; AMP, adenosine monophosphate; CMP, cytidine monophosphate; GMP, guanosine monophosphate.

[3] P-L Biochemicals, Inc., pp 44–46 (1965).

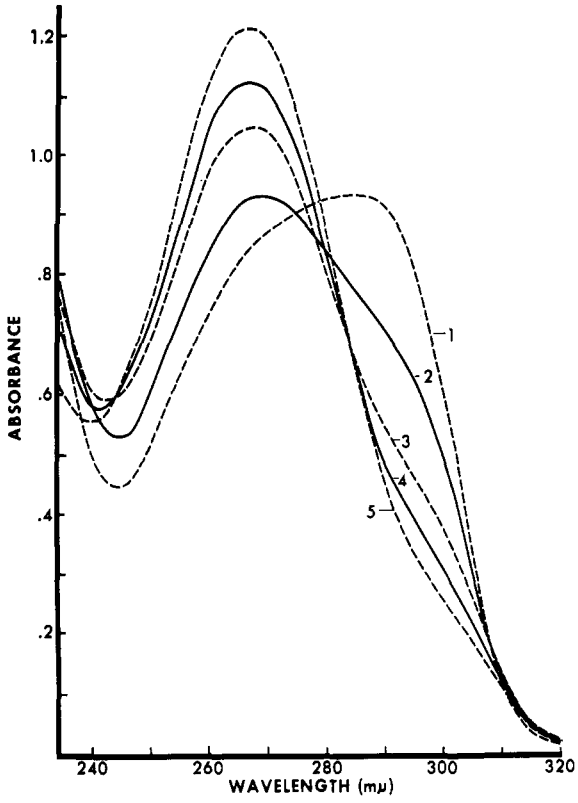

FIGURE 2: Spectrum of pseudouridine B as a function of formaldehyde concentration. Initial pH 9.97 (curve 1); final pH 9.78 (curve 5). Curve 1: pseudouridine B; curves 2–5: same as Figure 1.

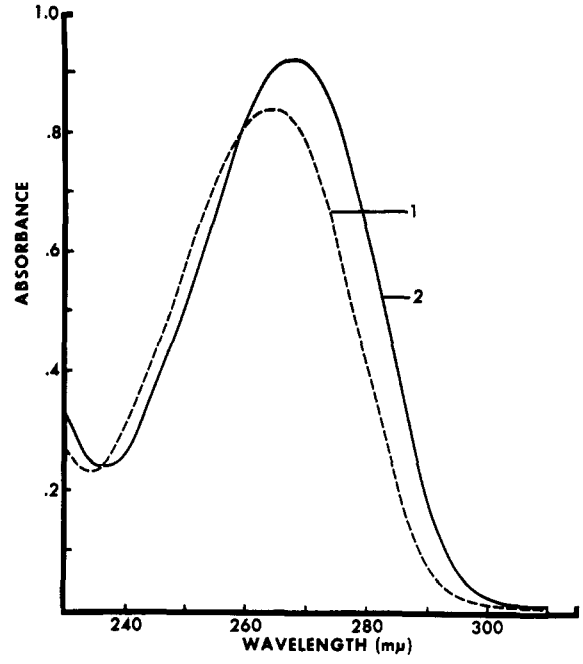

FIGURE 3: Spectrum of pseudouridine B as a function of formaldehyde concentration. Initial pH 7.11; final pH 6.89. Curve 1: pseudouridine B; curve 2; same as curve 1 plus 3.3 M HCHO.

Results

Reaction of Pyrimidine Nucleosides and Nucleic Acid with Formaldehyde. Because of previous observations which demonstrated a much greater reactivity to acrylonitrile of the N_1 position of pseudouridine over that of the N_3 position of pseudouridine or of uridine (Ofengand, 1965), it was expected that formaldehyde would react readily with pseudouridine. Spectral evidence for such a reaction was readily obtained and is given in Figures 1 and 2. The reaction was very rapid. Thus curves 2–5 were observed immediately after mixing and did not change during periods of observation as long as 20 hr. However, the reaction did not go to completion even in the presence of more than a 10^4-fold excess of formaldehyde as judged from the appearance of small increments in absorption with each subsequent addition of HCHO and the failure to reach the expected absorption of an N_1-substituted pseudouridine.

The marked hypsochromic shift of the absorption maximum of both pseudouridine isomers at pH 10 was similar to that obtained by the addition of hydrogen ion (Ofengand and Schaefer, 1965) or a cyanoethyl group (Ofengand, 1965) to the N_1 position suggesting that hydroxymethylation had occurred on that imino nitrogen atom. There was, however, no true isosbestic point. The intersections of the absorption curves shifted with each subsequent addition of formaldehyde, pointing to the presence of more than two species. Since only one molecule of formaldehyde can add to the N_1 position, additional species are probably the N_3 and the N_1,N_3 hydroxymethyl adducts.

Figure 3 illustrates the reaction of pseudouridine B with formaldehyde at neutral pH. At this pH, a hyperchromic and bathochromic shift of the absorption maximum is seen. Hyperchromicity was approximately 10% and bathochromicity approximately 4 mμ. A virtually identical result was obtained with pseudouridine C (not shown). Thus, reaction of both pseudouridine isomers occurred readily at both neutral and alkaline pH.

These findings, especially the apparent reaction with the N_3 position of pseudouridine, suggested that uridine might also form an adduct with formaldehyde. Evidence for this reaction is presented in Figure 4A. A bathochromic shift of approximately 3 mμ and a hypochromic shift of 2% in the absorption peak is present. The magnitude of these changes is similar to that previously observed by Fraenkel-Conrat (1954), and might be overlooked in the absence of the data for pseudouridine. As was the case with pseudouridine, reaction with uridine occurred within 30 sec and did not change during a 24-hr observation period.

In order to accentuate the spectral shift a series of difference curves were obtained by the tandem cell technique. The results (Figure 4B) show clearly that the extent of the reaction is dependent on the amount of

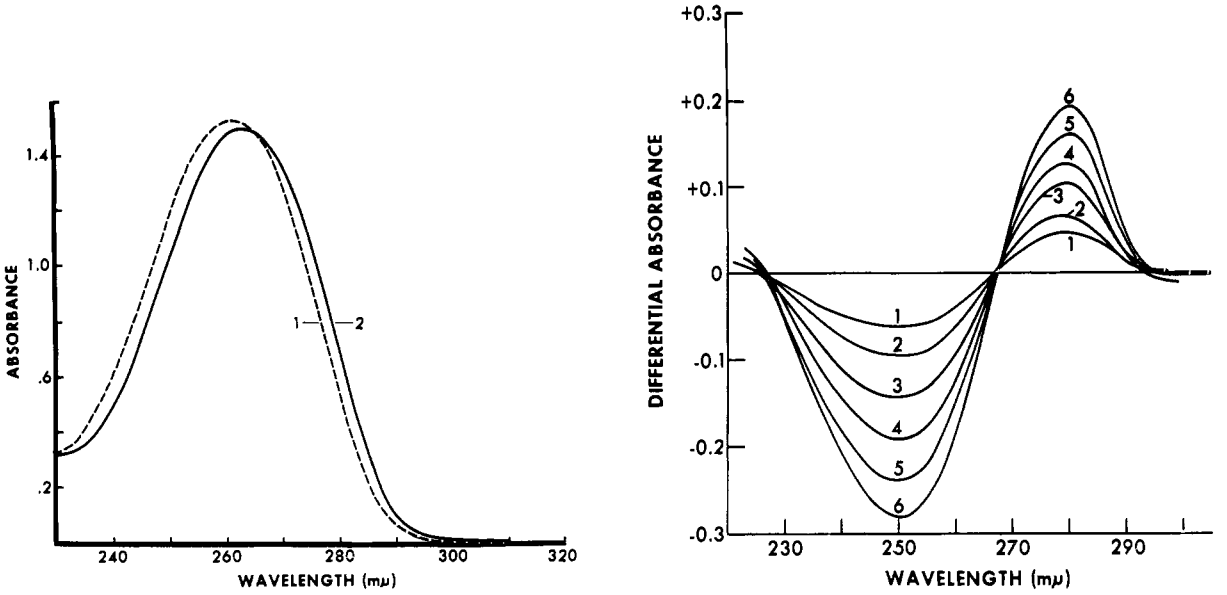

FIGURE 4: Spectrum of uridine as a function of formaldehyde concentration and difference spectrum of uridine–formaldehyde adduct minus uridine. (A) (left) Initial pH 6.97; final pH 6.80. Curve 1: uridine; curve 2: uridine plus 3.3 M HCHO. (B) (right) K_2HPO_4–KH_2PO_4 buffer (0.25 M), initial pH 6.78 (curve 1); final pH 6.61 (curve 6). Curve 1: 0.11 M HCHO; curve 2: 0.20 M HCHO; curve 3; 0.40 M HCHO; curve 4: 0.80 M HCHO; curve 5: 1.99 M HCHO; and curve 6: 3.34 M HCHO.

formaldehyde present. In addition, a true isosbestic point at $\Delta A = 0$ is obtained at approximately 267 mμ, signifying the presence of a single adduct in this case, in sharp contrast to the previous result with pseudouridine. Addition at C_5 seemed unlikely in view of the results of Cline *et al.* (1959) which showed that C_5 addition required either incubation at 50° for 73 hr in 0.42 N KOH or refluxing for 25 hr in 0.5 N HCl. This suggested that reaction had occurred instead at the N_3 position. In order to test this, reaction with thymidine was examined. Thymidine, with both the C_5 and N_1 positions blocked, could add formaldehyde only at the N_3 position. The spectral changes observed on reaction of formaldehyde with thymidine (Figure 5) were very similar to those obtained above for uridine, and provide further support for the assignment of N_3 as the site of hydroxymethylation.

Reaction with formaldehyde is not limited to nucleosides. Polyuridylic acid in 0.1 M sodium phosphate buffer, pH 7.0, and 3.3 M formaldehyde showed spectral shifts virtually identical with those seen with uridine in Figure 4A at the same formaldehyde concentration (data not shown). Thus, these spectral studies showed that a rapid reaction with the available N_1 and N_3 atoms of pseudouridine, uridine, and thymidine occurs with formaldehyde under neutral and alkaline conditions.

Reaction of Inosinic Acid with Formaldehyde. The above findings suggested that other nucleotides previously thought to be unreactive with formaldehyde might also form adducts that could be detected spectrally. Attention was directed specifically toward inosinic acid because of the presence of the same HNCO grouping as is present in the uridine nucleoside series and the previous observation of the high reactivity of this site with acrylonitrile (Ofengand, 1966; Yoshida and Ukita, 1965).

Because major spectral shifts were not expected at pH 7 where the presumed substitution is a hydroxymethyl group for a proton on N_1, the tandem cell technique was used in order to increase sensitivity and accuracy. The results (Figure 6) show clearly that a reaction does take place in the same range of formaldehyde concentrations as used in the pyrimidine nucleoside series above.

Stability of Hydroxymethyl Nucleoside Adducts. In view of the previous claims for unreactivity of these nucleosides (Fraenkel-Conrat, 1954; Staehelin, 1958; Grossman *et al.*, 1961), it was important to ask whether these derivatives were stable in the absence of excess formaldehyde. Initially, it was supposed that evaporation *in vacuo* would be sufficient to remove unreacted formaldehyde and thus decompose the adduct if it were unstable. Indeed, preliminary experiments suggested that the adducts were stable since evaporation of buffered nucleoside–formaldehyde mixtures followed by reconstitution with water reproduced the original adduct spectra. However, such a procedure is apparently inadequate to remove formaldehyde, as shown in the following experiment. Two solutions, both buffered at pH 10 and 4 M in formaldehyde, were prepared, one containing in addition 10^{-4} M pseudouridine C. Both solutions were evaporated to apparent dryness on a vacuum pump, and the residue

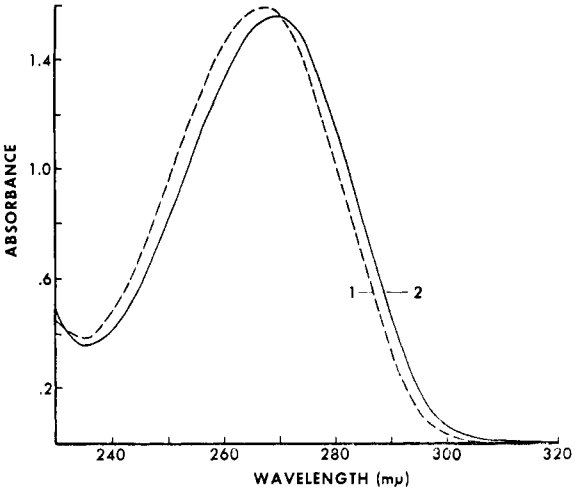

FIGURE 5: Spectrum of thymidine as a function of formaldehyde concentration. Initial pH 7.07; final pH 6.92. Curve 1: thymidine; curve 2: same as curve 1 plus 3.3 M HCHO.

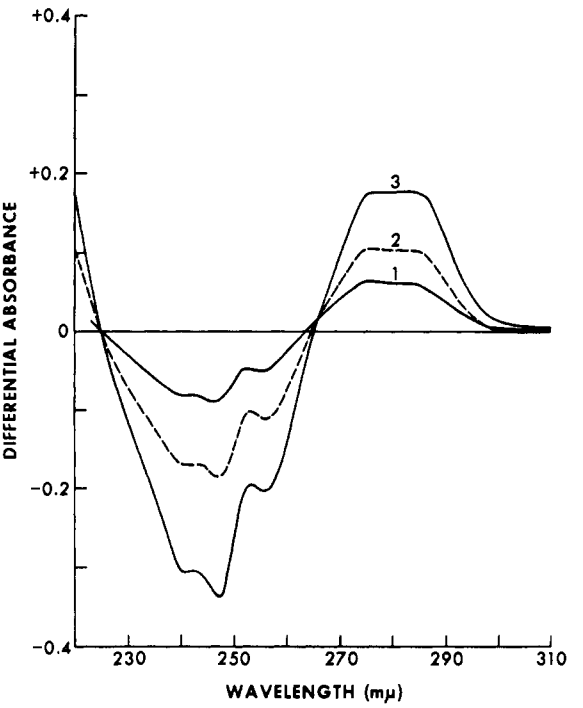

FIGURE 6: Difference spectrum of inosinic acid–formaldehyde adduct minus inosinic acid. K_2HPO_4–KH_2PO_4 buffer (0.25 M); initial pH 6.81 (curve 1); final pH 6.65 (curve 3). Curve 1: 0.17 M HCHO; curve 2: 0.50 M HCHO; curve 3: 3.34 M HCHO.

was reconstituted with water. Pseudouridine was then added to the solution lacking nucleoside, and the spectra of both solutions were measured. In both cases, spectra similar to curve 3 of Figure 1 were obtained, indicating the presence of a formaldehyde species, even after evaporation to dryness, which was capable of reaction with pseudouridine. The result was confirmed by the use of [^{14}C]formaldehyde in a repeat of the above experiment. In this case, more than 75% of the radioactivity was resistant to removal by evaporation.

Thin layer chromatography of the nucleoside–formaldehyde reaction mixtures eventually proved to be the method of choice for the separation of excess formaldehyde from nucleoside. Thus, when a solution of pseudouridine and formaldehyde buffered at pH 10 was concentrated and chromatographed in isobutyl alcohol–water (88:12) only a single band of nucleoside was obtained which on elution proved to be unreacted pseudouridine as judged by its absorption spectrum at pH 10. Similar findings were obtained when reaction was allowed to occur in a pH 7 buffer. In this case, formaldehyde was removed by chromatography in isopropyl alcohol–1% ammonium sulfate (2:1), and the single nucleoside band (moving identically with a pseudouridine marker) was eluted, and characterized spectrally as unreacted pseudouridine (Figure 7). The typical bathochromic shift in alkali, characteristic of pseudouridine (Ofengand and Schaefer, 1965) but not of the formaldehyde adduct, is seen. It is apparent, then, that the adduct formed with pseudouridine under neutral or alkaline conditions is unstable and decomposes in the absence of formaldehyde.

Although not directly tested as above, it is probable that the other nucleoside adducts are similarly unstable. Thus, when mixtures of formaldehyde plus uridine or thymidine were chromatographed in either the isobutyl alcohol–water or isopropyl alcohol–ammonium sulfate solvents, only a single nucleoside spot could be detected which ran identically with control nucleosides. By analogy with the known R_F of the cyanoethyl derivatives of these nucleosides (Ofengand, 1965), a stable nucleoside–formaldehyde adduct would have been detectable in these two solvents. It appears, therefore, that all the nucleotide derivatives described above are in rapid equilibrium with their hydroxymethylated form in the presence of formaldehyde, and revert to the parent compound in its absence.

Extent of Reaction of Inosinic Acid and Uridine with Formaldehyde. The equilibrium position for the reaction

$$\text{IMP} + \text{formaldehyde} \underset{K}{\rightleftharpoons} \text{N}_1\text{-hydroxymethyl-IMP}$$

can be evaluated from the data of Figure 6 by a modification of the method described by Lewin (1964a) which does not require knowledge of either the rates of reaction or the extinction coefficient of the hydroxymethylated species. This method, which has general applicability, makes use of the readily derived relationship $\Delta A/[F] = (K[C](\epsilon_{NF} - \epsilon_N) - K\Delta A)$, where ΔA is the difference in absorption at any selected wavelength, [F] is the formaldehyde concentration, K is the equilibrium constant, [C] is the concentration

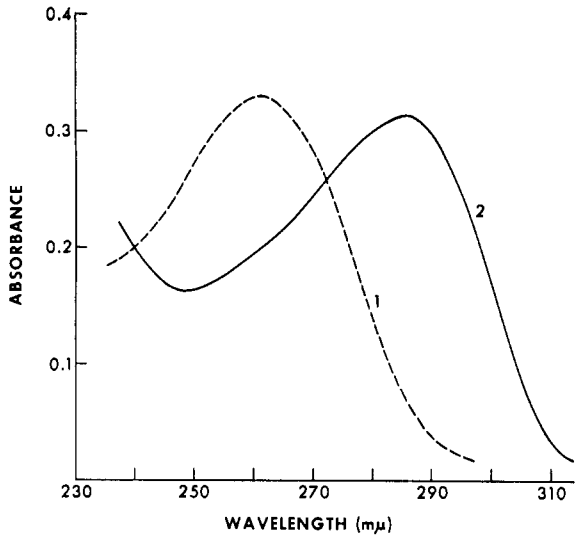

FIGURE 7: Spectrum of pseudouridine C plus formaldehyde after thin layer chromatography for separation of excess formaldehyde from nucleoside. Preparation of adduct and chromatographic separation were performed as described in the text. Curve 1: pH 7.2; and curve 2: pH 10.4.

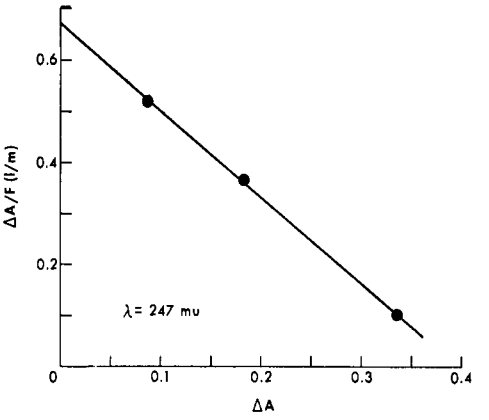

FIGURE 8: Determination of the equilibrium constant for the reaction of inosinic acid with formaldehyde from a plot of $\Delta A/[F]$ *vs.* ΔA. Definition of terms and equation as in the text. Data were taken from the experiment of Figure 6.

of nucleoside plus hydroxymethyl nucleoside, and ϵ_{NF} and ϵ_N are the extinction coefficients of the hydroxymethylated nucleoside and nucleoside, respectively. A plot of $\Delta A/[F]$ *vs.* ΔA should give a straight line whose slope is $-K$. The result of such an analysis applied to the data given in Figure 6 for IMP is shown in Figure 8. The calculated value for the equilibrium constant is 1.7 l./mole. A similar analysis (not shown) applied to the uridine data of Figure 4B gives an equilibrium constant of 2.5 l./mole. It is not possible to make such a calculation for the pseudouridine reaction since more than one hydroxymethylated species is produced in this case.

Discussion

The impetus to these experiments arose from considerations of the reactivity of the N_1 of pseudouridine with acrylonitrile (Ofengand, 1965), and the expectation of a similar reactivity with formaldehyde. The positive findings obtained (Figures 1 and 2) led to the view that not only the N_1 atom but also the N_3 might be involved in reaction with formaldehyde despite previous reports to the contrary for the analogous cases of uridine and thymidine.

The experiments described here show clearly that such a reaction does occur, that the spectral shifts obtained almost certainly reflect addition to N_3 in the cases of uridine and thymidine, and that both the forward and backward rates of reaction are rapid, *i.e.*, it was not possible to readily isolate the hydroxymethylated compound. Although less well studied, it is probable that these comments can be extended to the reaction with inosinic acid as well, in which case reaction probably takes place on N_1 by analogy with ionization and cyanoethylation studies (Ofengand, 1966; Yoshida and Ukita, 1965).

These results are in agreement with studies reported by Lewin on the reaction of formaldehyde with uracil and thymine (Lewin, 1964a; Lewin and Barnes, 1966) bearing in mind the close analogy between uracil and thymine on the one hand and pseudouridine on the other hand. Thus the spectra obtained for uracil plus formaldehyde showed a hyperchromic and bathochromic shift which was very similar to that seen with pseudouridine (Figure 3), and the failure to demonstrate a uracil–formaldehyde adduct by chromatography is also consistent with our findings. It should be pointed out, however, that the methods used by these workers could not detect reaction of the second available nitrogen atom in these compounds while from our data it seems clear that addition to both nitrogen atoms can occur.

Our finding of a hydroxymethylation reaction with the N_3 atom of uridine and thymidine is consistent with the recent report of Aylward (1966) on the hydroxymethylation of UMP. For example, Aylward reported an equilibrium constant for hydroxymethyl-UMP formation of 2.6 at 20° and 2.1 at 30° which should be compared to our value of 2.5 at 23° for uridine. In addition, he measured the rate of approach to equilibrium for UMP and poly U and in essence found the reaction to be almost instantaneous at neutral pH and room temperature. In our experiments we found the reaction with all nucleosides as well as poly U to be over in less than 30 sec. He also concluded that hydroxymethylation occurred on the N_3 atom of UMP and poly U because of the increased pH observed on hydroxymethylation of this acidic imino nitrogen atom, while our similar conclusion was based on the spectral changes observed with

thymidine. Our data complement his findings in that by examining the complete spectral curves for the reaction with uridine we have been able to show that well-defined spectral changes occur and that only a single hydroxymethyl product is formed. This conclusion is based on the fact that even over a wide range of formaldehyde concentrations, a clean isosbestic point was found at $\Delta A = 0$, in complete agreement with expectations.

Reaction with inosine had previously been reported by Lewin (1964b) based on an observed spectral change on addition of formaldehyde and on a differential titration curve. Our results obtained with IMP confirm and extend this report by showing that well-defined spectral changes occur as a function of formaldehyde concentration and that an equilibrium constant for the hydroxymethylation of IMP can be calculated which is not very different from that for the uridine series of compounds.

Although reaction with the imino nitrogen atom is so much faster than the reaction with what are presumed to be the amino groups of adenylic, guanylic, and cytidylic acids, it is of interest that the equilibrium position for the reaction does not differ too widely. Thus, the values for AMP, CMP, and GMP given by Penniston and Doty (1963) are 11.4, 16.6, and 5.3 l./mole, respectively, while the corresponding value for uridine is 2.5 and for inosinic acid, 1.7.

The significance of this lies in the interpretation of studies in which RNA or DNA is examined in the presence of amounts of formaldehyde that lead to appreciable reaction of AMP, GMP, and CMP (*e.g.*, Haselkorn and Doty, 1961). From the above comparison of equilibrium constants, it can be seen that appreciable amounts of uridylic or thymidylic acid will also be converted to hydroxymethyl derivatives, and this fact needs to be taken into consideration. For example, at a 1% formaldehyde concentration, the ratio of hydroxymethylated UMP to free UMP would be 0.9.

A potentially more serious situation arises in cases where the secondary structure of tRNA in the presence of formaldehyde has been investigated (*e.g.*, Fasman *et al.*, 1965), since the pseudouridylate residues, known to be important in maintaining the proper secondary structure (Rake and Tener, 1966; J. Ofengand, in preparation), would be derivatized at even moderate formaldehyde concentrations and may account for a considerable part of the structural deformation observed.

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