mit the drawing of any general conclusions concerning the origin of the tellurium in the nodules. However, Wedepohl (*3*), and Hewett, Fleischer, and Conklin (*2*) have suggested that volcanic emanations may have contributed some of the elements that are concentrated in manganese oxide nodules.

The first two samples in Table 1 were both taken down the prevailing wind and current from Hawaiian volcanos whose sulfur fraction is known to contain tellurium (*4*).

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7 October 1963

Tautomerism and Site of Protonation of 1-Methylcytosine: Proof by Nuclear Magnetic Resonance Spin-Spin Coupling

Abstract. *New nuclear magnetic resonance data lead to a completely unambiguous proof that the predominant tautomeric form of 1-methylcytosine is the amino form and that in acid solution the molecule protonates at 3-N. The close similarity of the nuclear magnetic resonance spectra of this compound to the spectra of the cytosine nucleosides indicates that these structures also exist in the nucleosides. These conclusions are reached on the basis of the spectra of analogs labeled with N^{15} and of the proton spin decoupling measurements made at low temperature.*

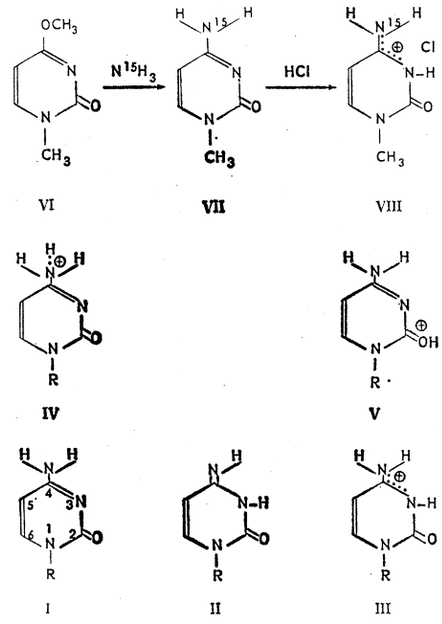
Cytosine and its nucleosides have been the subject of several recent structural studies (*1–7*), stimulated in part by the relevance of these substances to nucleic acid structure and function. Infrared studies (*1*, *2*) have shown that the predominant tautomeric form of cytidine is amino (I) and that in acid solution protonation occurs at 3-N (III). Most nuclear magnetic resonance (NMR) investigations (*2*, *5*, *7*) support these conclusions, but the interpretation of the NMR results is not completely unequivocal since it rests on arguments of relative chemical shifts expected for different tautomers (*5*) and upon the observation of a single peak with an area corresponding to two protons rather than two single proton peaks that would be expected from the imino structure, II (*2*). The foregoing observation provides strong presumptive evidence for the amino form I, but the remote possibility of accidental coincidence of two imino peaks could vitiate this assignment. In the protonated forms the location of the NMR peak resulting from the additional proton is in doubt.

We have made a detailed NMR study of some pertinent cytosine derivatives and wish to report from this work several new experiments and certain conclusions concerning structure which may be unambiguously derived from them. In making peak assignments we have relied primarily upon synthesis of labeled N^{15} analogs, proton-spin decoupling, and temperature variation (*8*).

The spectra were obtained with a Varian A-60 spectrometer and 30-cm-magnet system. The spin decoupling measurements were made with a Varian HR-60 spectrometer and a single sideband proton-proton decoupler (*9*). Chemical shifts are reported in parts per million relative to tetramethylsilane which was used as an internal reference.

1-Methylcytosine (*10*) was prepared by heating 1-methyl-4-methoxypyrimidone-2 (VI) in methanolic ammonia in a sealed tube at 155°C for 10 hours and the product was recrystallized from methanol. The corresponding N^{15} analog (VII) was prepared by distilling 250 mg $N^{15}H_3$ (*11*) into a frozen mixture of 3 ml of methanol and 700 mg of VI in a high vacuum (about 10^{-5} mm-Hg). The chilled tube was sealed at atmospheric pressure and the reaction was heated at 155°C for 10 hours. In a second preparation $N^{15}H_3$ in an amount about 10 percent in excess of VI was used, and the conversion was as good as when the excess of $N^{15}H_3$ was larger. The crude yield of VII was 600 mg. After recrystallization the melting point was 297° to 301°C, and it was not depressed by admixture with an authentic sample of the N^{14} compound.

Figures 1*C* and *D* show that the hydrochloride of 1-methylcytosine has



two broad peaks on the low field side of the 6-H doublet, at 8.85 and 10.00 parts per million (ppm), while its N^{15} analog shows each of these split by N^{15} (peaks at 484, 576, 551, and 643 cy/sec; mean values of the appropriate pairs at 530 and 597 cy/sec, or 8.83 and 9.95 ppm). The only other difference in the spectra of the two molecules is the presence of a small splitting of the 5-H doublet (the spin-spin coupling constant, $J = 0.7$ cy/sec) in the case of the N^{15} compound, presumably as a result of long range coupling with the N^{15}.

When 1-methylcytosine hydrochloride was heated, the two peaks at 8.85 and 10.00 ppm first broadened (~40°C), then disappeared (by 60°C), and finally reemerged as a single peak at 7.13 ppm (~160°C).

Since dimethyl sulfoxide freezes at 18°C, liquid sulfur dioxide was used as a solvent in order to make measurements at low temperatures. At −60°C, 1-methylcytosine hydrochloride has the spectrum shown in Fig. 1*E*. Irradiation with a strong proton-spin decoupling field at 11.43 ppm caused collapse of the 2.5 cy/sec splitting of the 5-H multiplet, leaving a 7 cy/sec doublet. Irradiation at 7.35 ppm had no effect on the 5-H absorption, while irradiation at 7.82 ppm (where the third NH peak virtually coincides with the 6-H peak) caused collapse of the 7 cy/sec splitting, but did not affect the 2.5 cy/sec splitting. On warming, the 11.43 ppm peak broadens, presumably because of proton exchange at an inter-

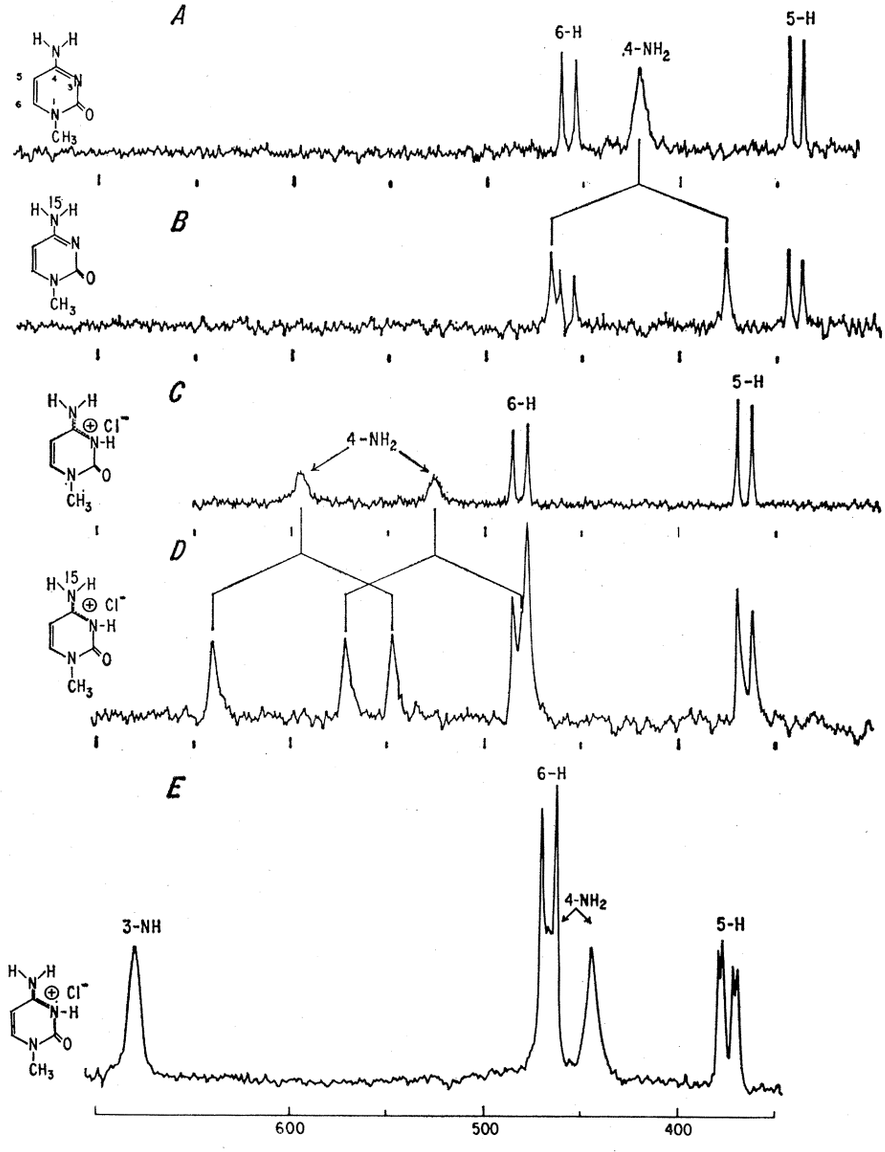


Fig. 1. Nuclear magnetic resonance spectra in dimethyl sulfoxide at 23°C (*A–D*) and in liquid SO_2 at −60°C (*E*). Abscissa is given in cycles per second with respect to tetramethylsilane as an internal reference; magnetic field increases from left to right.

mediate rate, and by room temperature this peak (at 11.70 ppm) has broadened almost beyond recognition. With temperature increase the 2.5 cy/sec splitting of the 5-H doublet becomes less pronounced and completely disappears by 20°C.

1-Methyl-4-N^{15}-cytosine hydrochloride shows at −60°C a 94 cy/sec splitting of the peaks at 7.35 and 7.82 and no splitting of the 11.43 ppm peak.

The spectra of cytidine and deoxycytidine and of their hydrochlorides (in dimethyl sulfoxide at room temperature) have been reported previously (*2–4*) and in the region of the pyrimidine protons have essentially the same spectra as 1-methylcytosine.

The NMR assignment of the amino structure (I) to the cytosine nucleosides has rested upon the observation of a single peak with an area corresponding to two protons rather than the two single-proton peaks that would be expected of an imino structure (II). The splitting of this two proton peak at 7.00 ppm by N^{15} (Fig. 1*B*) provides a completely unambiguous proof of the amino structure.

The site of protonation when cytosine or 1-substituted cytosines are treated with acid has been subject to some uncertainty. Early NMR data were interpreted in terms of protonation of the amino group of cytosine, (IV), (*3*) but this interpretation was later demonstrated to be incorrect (*6, 7*).

A choice between the remaining sites, 3-N and 0, could not previously be made rigorously on the basis of NMR data alone, though the NMR results have been shown to be consistent with the 3-N site derived from IR (*1, 2*) and UV (*12*) studies.

Only two of the three exchangable protons are observed in the NMR spectrum of 1-methylcytosine hydrochloride in dimethyl sulfoxide (Fig. 1*C*), and the spectrum of the N^{15} analog (Fig. 1*D*) shows that these are the amino protons. The difference in their chemical shifts results from hindered rotation of the amino group, as suggested earlier (*7*). That there is a barrier to rotation is supported by the results of the heating experiments in which the separate peaks coalesced at high temperature as a result of rapid rotation. The third exchangeable proton 3-NH (or conceivably 2-OH) is unobserved in the spectra of dimethyl sulfoxide solutions (probably broadened by exchange), but in SO_2 solution at −60°C separate peaks due to each of the three protons are observed (Fig. 1*E*).

The N^{15} results show that the peaks at 7.35 and 7.82 ppm are due to the amino group. The spin decoupling results show that the proton giving rise to the peak from 11.43 ppm is coupled to 5-H with $J = 2.5$ cy/sec. This is a reasonable value for $J_{3,5}$, which involves coupling through four chemical bonds in a partially aromatic ring (see typical *meta* coupling constants of 2-3 cy/sec) (*13*). On the other hand, if the molecule were protonated at the 2-oxygen (V), the OH-proton could scarcely couple to 5-H through six bonds with a coupling constant of 2.5 cy/sec, since even 5-bond aromatic *para* coupling constants are less than 1 cy/sec (*13*). Thus the NMR data prove that 3-N is the site of protonation.

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30 August 1963

Preparation of Retine from Human Urine

Abstract. *Human urine contains "retine." Its partial isolation is described.*

We have reported (*1*) that certain fractions of the urine of children have an inhibitory action on the growth of transplanted malignant tumors in mice. This action was similar to the inhibitory action of analogous extracts of different tissues. The substance responsible for this action was called "retine". We have since found a similar activity in the urine of adults of the age group of about 20 to 25 years. The availability of this material opens the way to large-scale preparation. Since there is less extractable material in urine than in tissues, urine offers the most propitious material for attempts at isolating retine.

Figure 1 shows the effect of retine, prepared from urine, on the transplanted Krebs-2 ascites tumor of Swiss albino mice. The top row, left, shows the size of the tumors on the 4th day after inoculation with 30 million cancer cells subcutaneously into the shoulder region, without treatment. The bottom row shows the tumors of untreated animals a week later. The third row shows the size of the tumors after the animals had been treated from the 4th to the 11th day with one unit of retine daily (a unit was called the quantity which inhibits growth by 50 percent). The second row shows the tumors after a similar treatment with three units of retine. The top row, right, shows the tumors under similar condition after treatment with six units daily. These tumors contained very little live cancer tissue and consisted chiefly of necrotic cancer cells. Injected daily in this quantity, retine produced no observable toxic effect.

As Fig. 1 shows, while smaller doses inhibit growth, bigger ones make the tumors regress. However, our present studies are not aimed at the detailed study of the action of retine but at its final isolation.

The methods used in our test have been described earlier (*1*). The method of purification used in preparing the substance, the action of which is shown in Fig. 1, is as follows.

One thousand liters of urine were concentrated to 80 liters in a Turba Film Evaporator at moderate temperature; the concentrate was chilled to 2° to 4°C, the *p*H was adjusted to 1, and the fluid was extracted four times with 10 liters of chloroform and filtered through Whatman No. 3 paper. The filtrate was cooled to −20°C and filtered again. At this stage the total dry weight was 61.2 g. The chloroform extract was concentrated in a Turba Film Evaporator to 3.5 liters, cooled overnight at −20°C and filtered in the cold to eliminate the hydrochloric acid. Then the chloroform solution was concentrated at reduced pressure to 100 ml and extracted five times with an equal volume of 0.1*N* NaOH. The final *p*H was 11 to 12. The watery extracts were collected, cooled in a bath of 0°C and centrifuged after the *p*H was adjusted to 7.5 to 7.6. The watery extract was filtered through 200 g of Sephadex G-100 gel at 2° to 3°C. The column was buffered with 0.1*M* phosphate buffer of *p*H 7.38.

The void volume (V_0) of the column was 930 ml. The first five volumes contained 30.5 g of material (dry wt.) with about 10 percent of the total retine activity. The effluent was collected from the sixth V_0 up to the tenth (Fig. 2), cooled to 2°C, acidified to *p*H 1, and extracted three times with 1 liter of chloroform. The chloroform extract contained 0.52 g of material (dry wt.) It was cooled to −20°C and filtered. After the filtration it was concentrated to 50 ml and extracted three times with 50 ml of 0.01*N* NaOH. The watery extract was chilled to 2°C and extracted three times with 30 ml of benzene after the *p*H was adjusted to 1. The benzene extract was filtered through Whatman No. 3 paper. This "benzene filter" was washed with benzene and saved. The benzene extract contained 152 mg of inactive material and it was discarded. The cloudy, watery extract was shaken three times with 100 ml of chloroform. Then the chloroform extract was filtered through the "benzene filter," shaken once again with one-third its volume of 1*N* HCl, cooled and filtered at −20°C. It contained 34.2 mg of material with the total activity of 8000 retine units. This corresponds to about 4 μg per

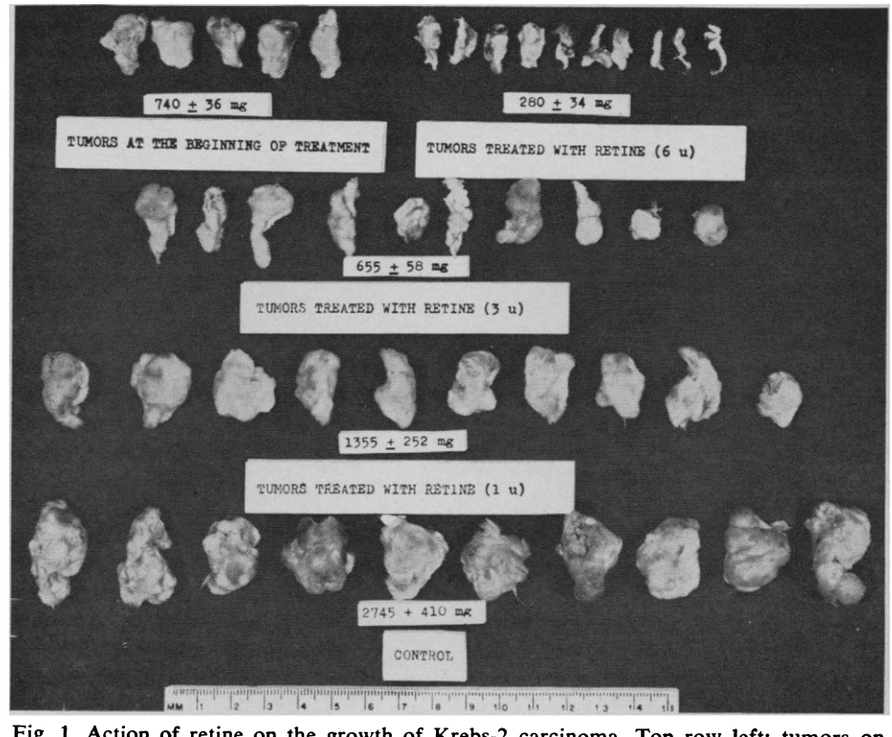

Fig. 1. Action of retine on the growth of Krebs-2 carcinoma. Top row left: tumors on the 4th day after inoculation, untreated. Top row right: treated daily from the 4th to 11th day with six units of retine. Second row: similar treatment with three units. Third row: treatment with one unit. Bottom row: untreated.