A Model for the Molecular Conformation of α-Pseudouridine from Nuclear Magnetic Resonance Data[1]

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Abstract: A computer analysis of the 100-MHz nmr spectrum of α-pseudouridine in aqueous solution is reported. The spectral data were used to determine a model for the conformation of this nucleoside. A comparison is made with the model previously proposed for the naturally occurring β anomer. The conformation of the furanose rings is best described by an equilibrium between several puckered conformations. Both anomers exist predominantly in their anti forms. The molecule rotates freely about the exocyclic $C_{4'}$–$C_{5'}$ bond, but a slight preference is indicated for the gauche–gauche rotamer.

Recently we reported[3,4] a complete analysis of the 100-MHz proton magnetic resonance spectrum of β-pseudouridine, β-ψ (Figure 1), in aqueous solution. β-ψ, a so-called modified nucleoside, is present in tRNA molecules in the sequence TψC,[5,6] and occurs in the anticodon region of some species, such as tRNA (Tyr).[7] Consideration of the proton chemical shifts and coupling constants led to a model for its molecular conformation. In particular, the data precluded the existence of the ribose ring in a single puckered form but were consistent with the presence of a rapid equilibrium between several conformations. Comparison of the data with those of uridine (U)[3,4,8] suggested that the two isomers have similar conformations in aqueous solution and that β-ψ exists predominantly in its anti conformation. We report here the analysis of the 100-MHz spectrum of α-pseudouridine, α-ψ (Figure 1), in aqueous solution. Complete data for the two anomers allow a comparison of their molecular conformations to be made and provides a test of the proposals made regarding the structure of the biologically important β anomer.

Experimental Section

α-ψ (100% anomeric purity) was obtained from Calbiochem and used without further purification. The internal reference, 3-trimethylsilylpropanesulfonic acid, sodium salt (DSS), was a product of E. Merck, Germany. Spectra were obtained in D_2O solution, 0.12 *M* in nucleoside and 0.15 *M* in DSS. The pD of the solution was adjusted to 6.7 by addition of dilute DCl or NaOD (pD = pH + 0.4).[9] Samples were lyophilized three times with D_2O to minimize the concentration of HDO whose resonance might obscure those of interest. Nmr spectra were obtained on a Varian HA-100 spectrometer.

Results and Discussion

A. Spectral Assignment. Figure 2a shows the observed spectrum of α-ψ at 30°. The ribose ring hydrogens were assigned originally by comparison of the spectra with that of β-ψ; the assignments were confirmed by double resonance techniques.[10] The low-field doublet at 7.561 ppm was readily attributed to the proton on the uracil base since C-6 is bonded to an electronegative nitrogen atom. The small splitting (1.2 Hz) is due to the four-bond allylic coupling interaction with the anomeric proton, $H_{1'}$. The spectrum was analyzed using a version of LAOCOON II modified to give spin tickling information.[11] The calculated spectrum in Figure 2b simulates the strongly coupled 2′–5′ region. Good agreement exists between the calculated and observed line positions and intensities.

Chemical shifts and coupling constant data for α-ψ are given in Tables I and II. Also included for comparison are data for β-ψ obtained under similar conditions.[3,4]

Table I. Proton Chemical Shifts[a] of α-ψ and β-ψ at 30°

Proton	α-ψ	β-ψ
H_6	7.561	7.660
$H_{1'}$	4.991	4.674
$H_{2'}$	4.358	4.279
$H_{3'}$	4.328	4.141
$H_{4'}$	3.998	4.009
$H_{5'B}$	3.881	3.840
$H_{5'C}$	3.708	3.726

[a] All shifts are in parts per million and positive values indicate resonance at low field relative to internal DSS.

B. Conformation of the Furanose Ring. In our previous reports we showed how consideration of the ribose coupling constants—$J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$—could lead (*via* the Karplus relation[12,13] between vicinal

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(2) (a) NRCC Postdoctoral Fellow 1967–1969; (b) National Research Council of Canada; (c) The University of Winnipeg.

(3) F. E. Hruska, A. A. Grey, and I. C. P. Smith, *J. Amer. Chem. Soc.*, **92**, 214 (1970).

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(10) Configurational assignment of α- and β-ψ was based on the chemical-shift differential between their respective $H_{1'}$ chemical shifts and was discussed in greater detail in ref 4.

(11) The program was modified to give the origin of all the calculated transitions including their regressively and progressively connected levels. Address correspondence regarding these modifications to A. A. Grey.

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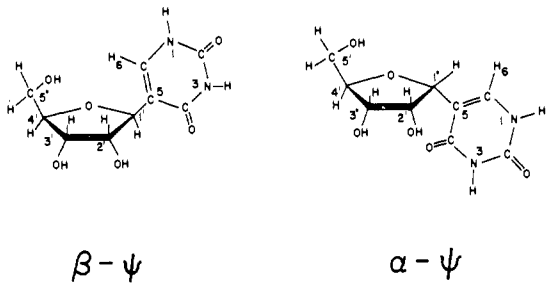

Figure 1. Structural formulas, α- (syn) and β-pseudouridine (anti).

and the corresponding vicinal coupling constants for twenty possible conformations of D-ribose and D-deoxyribose rings.[15] All conformations in which one of the members lies out of the plane were treated, as were conformations in which a pair of adjacent members are puckered out of the plane but in an opposite sense. We thus can make a more complete comparison between our observed coupling constant data and the theoretical values. We find however that the observed values of $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$ are not predicted by a single conformation, and we are led as before to propose the existence of a rapid equilibrium between two or more

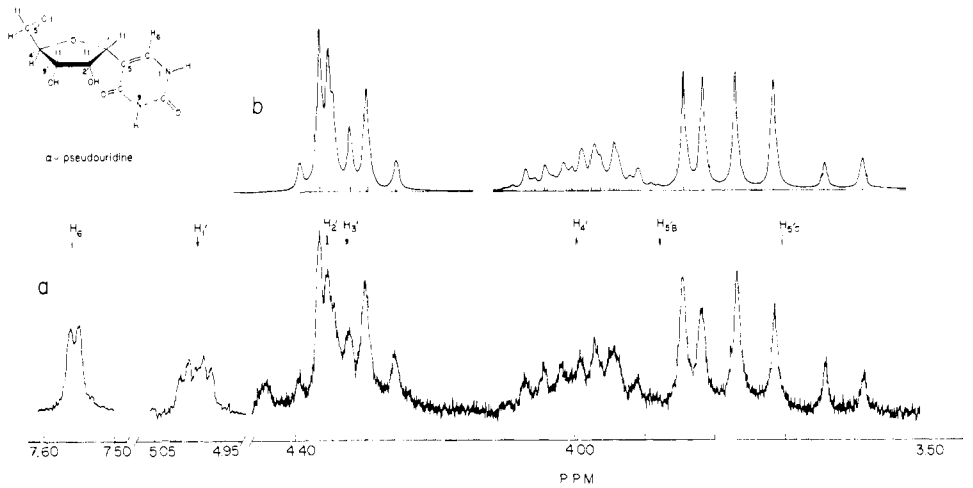

Figure 2. (a) 100-MHz nmr spectrum of α-pseudouridine in D_2O; pD 6.3, 0.12 *M*, 30°. Chemical shifts are expressed relative to internal DSS. (b) Computer-simulated nmr spectrum of the region due to 2′ to 5′ hydrogens.

couplings and dihedral angles) to a model of the furanose ring of β-ψ. We suggested that the data were reasonable only if a rapid equilibrium between several puckered forms was assumed. At that time, however, we considered only conformations in which

Table II. Coupling Constants (Hertz) of α-ψ and β-ψ at 30°

Coupling constants	α-ψ	β-ψ
$J_{61'}$	1.3	0.8
$J_{1'2'}$	3.3	5.0
$J_{2'3'}$	4.2	5.0
$J_{3'4'}$	7.9	5.2
$J_{4'5'B}$	2.4	3.2
$J_{4'5'C}$	5.7	4.6
$J_{5'B5'C}$	−12.7	−12.7

C-2′ and/or C-3′ lay out of the plane defined by the remaining members of the ring. There is, however, no justification in assuming *a priori* the absence of conformations in which O-1′, C-1′, or C-4′ are found out of the plane. Indeed, that C-1′, C-4′, and O-1′ puckered conformations may be favored in some instances is brought out by the studies by Sarma and Kaplan on pyridine nucleotides and by Hall and associates on pentofuranosyl fluorides.[14]

Smith and Jardetzky have published a correlation between the dihedral angles $\phi_{1'2'}$, $\phi_{2'3'}$, and $\phi_{3'4'}$, conventionally puckered forms. The observed data are adequately discussed in terms of several equilibria: (1) endo C-2′ ↔ endo C-3′, (2) exo C-2′ ↔ exo C-3′, or (3) endo C-2′, exo C-3′ ↔ exo C-2′, endo C-3′, which may be considered an intermediate between 1 and 2. These equilibria have, however, one important feature in common—during the interconversion of the forms the hydroxyl groups at the 2′ and 3′ positions pass through the eclipsed conformation. Thus, any barrier to inversion of the ribose ring resulting from steric repulsion of the bulky hydroxyl groups must be small. Recent theoretical calculations by Flory and associates[16] suggest no difficulty in treating the ring as a mixture of forms.

In a subsequent section we shall show that α-ψ, like β-ψ, is present in aqueous solution predominantly as the anti conformer. Examination of molecular models of the two anomers in this conformation reveals no close contact interactions in either which would drastically reduce the flexibility of the ribose ring, and we expect therefore that the equilibrium model should be applicable in both molecules. Our expectations are borne out by comparison of the coupling constant data for the α-ψ anomer with the theoretical values of Smith and Jardetzky; no single puckered conformation, nor the planar form, adequately accounts for the values of $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$. A choice of possible equilibria is, however, less obvious in this instance than for β-ψ. Equilibria involving only C-2′ and C-3′ puckering do

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not describe the observed trend. Presumably then the best description involves puckering at other positions of the ring. Some differences in the ring puckering of the two anomers are indicated by the larger value of $J_{3'4'} = 7.9$ Hz in α-ψ *vs.* 5.2 Hz in β-ψ. This suggests that endo C-3′ and/or exo C-2′ conformations are more favored in the α anomer. Examination of space-filling molecular models reveals a close-contact interaction between H-6 of the uracil base and the hydroxyl group at the 3′ position when this carbon is puckered exo. This contact between the ribose and base substituents would reduce the torsional motion of the molecule about the glycosidic linkage; when the 3′ carbon is puckered endo, however, no hindrance of the torsional motion is present from this source.

C. The Sugar–Base Torsion Angle. To facilitate discussion of the molecular conformations of nucleosides and nucleotides about their glycosidic bonds, Donohue and Trueblood[17] have defined the sugar–base torsion angle and suggested for it the existence of two possible ranges, the so-called syn and anti conformations. In the syn range of the pseudouridines the C-4 position of the base lies above the furanose ring; in the anti range C-6 is found above the ring. Considerable evidence has been accumulated to indicate that the anti conformation of normal nucleosides and nucleotides is favored in the crystalline state[18,19] as well as in aqueous solution.[8,20–23] As yet no X-ray analysis of either anomer of ψ has appeared. From the studies of the chemical shifts of the uracil protons in U and 5′-UMP Schweizer, *et al.*, concluded that base has the anti orientation in these compounds.[21] The similarity between the ribose proton chemical shifts in U and β-ψ suggested that the base in β-ψ also has this orientation.[3,4] More recently, Dugas, *et al.*, have demonstrated significant changes in the ribose proton chemical shifts of β-cyanuric acid riboside due to the presence of a keto group over the ribose ring,[24] confirming the previous conclusions.

Comparison of the data in Table I reveals that the H-1′, H-2′, and H-3 resonances occur at slightly lower fields in α-ψ than in β-ψ. No measurable difference is noted, however, for H-4′; the difference in the average chemical shift of the 5′-methylene hydrogens is small, *ca.* 1 Hz. We note from models of α-ψ that close contact can be made between the 4-keto group on the uracil base and the 4′ proton, if the torsion angle is rotated into its syn range, Figure 3. The magnetic environment is then expected to be influenced by the electric and magnetic fields associated with the anisotropic carbonyl bond.[24] In β-ψ, however, the base moiety lies on the opposite side of the furanose ring and is not expected to influence the shielding of the 4′ hydrogen. Thus the absence of any measurable shift difference in the 4′ resonances of the two anomers is reasonable only if the α anomer exists in the anti

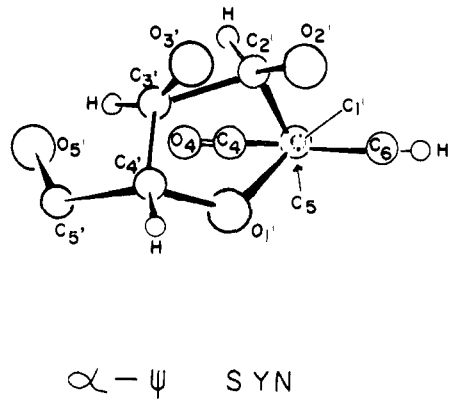

Figure 3. Syn conformer of α-pseudouridine.

conformation. This is not surprising since multiple close contacts are made in the syn conformation by the 4-keto and the hydroxyl groups at the 2′ and 3′ position and severely restrict the torsional motions of the ribose ring and glycosyl bond. Tougard has observed that crystalline 5-bromouracil arabinofuranoside (in which the 2′-hydroxyl and uracil base lie on the same side of the sugar ring) is found in the anti conformation.[25] In the anti conformation the furanose and base ring substituents experience less crowding and consequently this conformation is expected to be favored.

The small difference in the average chemical shift of the 5′ hydrogens is also consistent with the proposed existence of the anomers in their anti conformations.[26] In the syn conformation of β-ψ the 4-keto group of the uracil base can approach the methylene hydrogens closely when the $C_{4'}$–$C_{5'}$ bond is rotated into either its trans–gauche or gauche–trans rotamer (*vide infra*) and should influence the shielding at this position. In the α anomer the methylene and base groups lie on opposite sides of the ribose moiety and any mutual magnetic interactions should be minimal. Thus the similarity of the average 5′ shifts of α-ψ and β-ψ is further confirmation that the β anomer exists predominantly in the anti conformation.

Larger differential shifts are found for the remaining ribose hydrogens. The largest, 0.32 ppm observed for the H-1′ position, is adequately discussed on the basis of the shielding effect of the cis hydroxyl group at the 2′ position of β-ψ. Smaller differential shifts of 0.08 and 0.19 ppm are found for the 2′ and 3′ hydrogens, respectively. A number of factors including the diamagnetic anisotropy of the uracil base and the magnetic and electric fields associated with the 4-keto group could contribute significantly to the shielding at these positions. Differential solvation of the ribose may also be important. In view of the uncertainties in the magnitudes of these effects, and the limitations in their theoretical calculation, we feel that a more precise evaluation of the torsion angle on the basis of these differential shifts should not be attempted.

The upfield shift (0.10 ppm) of H-6 (α-ψ) relative to its resonant field in β-ψ is interesting in the light of recent studies of uridine and its mononucleotides by

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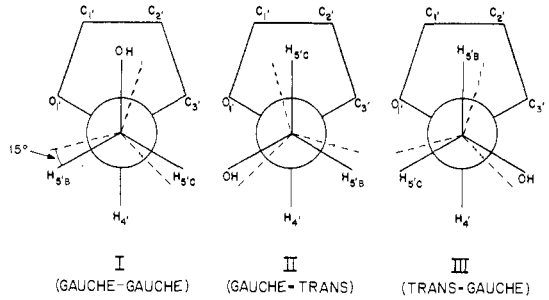

Figure 4. Rotational isomers around the $C_{4'}$–$C_{5'}$ exocyclic bond (---); classical, all dihedral angles 60° (—); nonclassical, 15° distortion introduced to account for O–O repulsions.

Prestegard and Chan.[27] They argued that variations in the torsional angle, within the anti range, should be manifest in the resonant field of H-6. Comparison of the H-6 resonance of pyrimidine bases and their nucleosides reveals an appreciable deshielding induced by the electric and magnetic fields associated with the furanose ring. In view of its proximity to this position in the anti conformation the ethereal oxygen is presumably largely responsible for this deshielding. Prestegard and Chan pointed out that perturbations of the torsion angle to more negative values, resulting in increased separation between the H-6 and ether oxygen, should result in a reduction of this effect and a subsequent upfield shift of this resonance. It is tempting to conclude on this basis that the average torsional angle is somewhat more negative in the α anomer. However the shielding constant of H-6 (α-ψ), but not H-6 (β-ψ), should experience sizeable contributions from the 2′ and 3′ hydroxyl groups, particularly at large negative values of the torsion angle. The interpretation of the differential in the H-6 resonances is therefore less straightforward.

D. Conformation of the Exocyclic CH_2OH Group. A third conformational parameter of interest in nucleoside structure is the orientation of the 5′ hydroxyl group relative to the furanose ring. In Figure 4 we have shown the three possible conformations determined by rotation about the exocyclic $C_{4'}$–$C_{5'}$ bond. In rotamer I the $C_{5'}$–$O_{5'}$ bond is gauche to both the $O_{1'}$–$C_{4'}$ and $C_{3'}$–$C_{4'}$ bonds; this conformation is referred to as gauche–gauche, the most common form found in crystalline nucleosides and nucleotides.[28] In rotamers II and III and $C_{5'}$–$O_{5'}$ bond is trans to either the $C_{3'}$–$C_{4'}$ or $C_{1'}$–$C_{4'}$ bonds; they are referred to as the gauche–trans or trans–gauche conformations, respectively. In our earlier report we showed that a Karplus treatment of the vicinal $J_{4'-5'}$ coupling constants could yield information about the relative rotamer populations in aqueous solution. In particular a slight preference for the gauche–gauche form was indicated but significant populations (>10%) were indicated for the other isomers. It would be useful to calculate the populations of the three rotamers for aqueous α-ψ for comparison with the data of β-ψ. Useful information regarding possible interaction between the hydroxy methyl group and the base at the anomeric carbon may be revealed.

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Inherent in the application of the Karplus equation to this structural problem are several drawbacks; these have been discussed in some detail by Blackburn, *et al.*, in considering uridine.[8] First, the value of J_0, the constant term in the equation, is known to depend on substituent electronegativity and orientation, and ranges from 8 to 16 Hz.[29] The derived populations are dependent on the choice of J_0 and, unfortunately, the best value is not chosen with any degree of certainty. Secondly, the treatment requires prior knowledge of molecular energy as a function of angle of rotation about the C–C bond, in particular the angles at which the relative energy minima occur. There is no justification for assuming that the classically staggered conformations, *i.e.*, all dihedral angles equal to 60°, are energy minima. Certainly when bulky substituents are oriented gauche on the C–C fragment the minimum is expected at an angle somewhat larger than 60°. Distortions as large as 15° from the classical conformations are observed in crystalline nucleosides.[28,30,31] In view of these uncertainties it was deemed necessary to estimate the rotamer populations for a series of J_0 values. Furthermore the calculations were carried out using both the classical and a nonclassical basis set of staggered rotamers shown in Figure 4. In the nonclassical set we have introduced a distortion of 15° to account for repulsion between the oxygen atoms at the 4′ and 5′ positions. Our calculations yield therefore a range of relative populations for each of the three possible rotamers about the $C_{4'}$–$C_{5'}$ bond; the data are presented in Table III.

Table III. Calculated Range of Rotamer Populations of the Exocyclic $C_{4'}$–$C_{5'}$ Bond of α-ψ and β-ψ at 30°

	I (gauche–gauche)	II (gauche–trans)	III (trans–gauche)
α-ψ	0.3–0.7	0–0.5	0–0.5
β-ψ	0.4–0.8	0–0.3	0–0.5

In view of the uncertainties in this treatment it is most reasonable to conclude that each of the rotamers is present to a significant extent in both α-ψ and β-ψ. Therefore energy differences between them must be small (<1 kcal). The data do indicate a slight preference for the gauche–gauche rotamer in each case, and this preference is slightly more pronounced for β-ψ. Thus we must conclude that the conformational properties of the exocyclic bond are not measurably dependent upon the configuration at the anomeric carbon. Temperature independent spectra were obtained for both anomers in the range 30°–70°. Thus the preference for the gauche–gauche rotamer exists at elevated temperature.

Conclusion

The furanose ring of the pseudouridines is a flexible unit and is best described as an equilibrium between several puckered forms. The molecules are restricted to the anti conformations, presumably a consequence of unfavorable steric interactions in the syn conformations. The barriers to rotation about the $C_{4'}$–$C_{5'}$ bond are

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small in each case, but a slight preference for the gauche–gauche rotamer is indicated.[32]

(32) NOTE ADDED IN PROOF. After submission of this manuscript an X-ray study of crystalline α-ψ appeared (D. C. Rohrer and M. Sundaralingam, *J. Amer. Chem. Soc.*, **92**, 4950 (1970)). It demonstrated that in the solid state the ribose ring of α-ψ is puckered exo C-2′, the base has the anti conformation, and the exocyclic hydroxymethyl group is gauche–trans (II in Figure 3). Since the properties of the nucleoside in a crystal are strongly dependent on interactions with neighboring molecules, we believe the solution data to be more relevant to the conformation of a nucleoside in a biological system.

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Conformational Properties of Poly-L-proline Form II in Dilute Solution[1]

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Abstract: The intrinsic viscosity of poly-L-proline has been studied as a function of molecular weight and temperature in five commonly used solvents: water, trifluoroethanol, acetic acid, propionic acid, and benzyl alcohol. The molecular weight range covered was 4400–99,000. The second virial coefficient of the high molecular weight sample has been determined as a function of temperature in four solvents. The $[\eta]$ are higher in the organic solvents than in water, but the log $[\eta]$ *vs.* log M_W plots are of the same shape in all solvents. The characteristic ratio is 14 in water and 18–20 in the organic solvents at 30°, and d ln $\langle r^2\rangle_0/dT$ is negative. The theoretical rotational potential function obtained by Hopfinger and Walton for L-prolyl-L-prolyl-L-prolyl-L-proline correctly predicts the characteristic ratio at 30° but predicts the wrong sign for d ln $\langle r^2\rangle_0/dT$. The conformational and hydrodynamic properties of poly-L-proline and cellulose derivatives have many features in common. The occurrence of cis–trans isomerization of the peptide bond is suggested in concentrated aqueous calcium chloride solutions.

In the solid state poly-L-proline has been observed to form an ordered structure in which the chain conformation is a right-handed helix with *cis*-peptide bonds (form I)[3] or a left-handed helix with *trans*-peptide bonds (form II).[4,5] In dilute solution poly-L-proline can also exist with all peptide bonds in either the cis or trans conformation.[6–14] These two forms can be reversibly interconverted by appropriate changes in solvent composition.[9,10,14] A less extended form of poly-L-proline exists in concentrated solutions of several salts.[6–8,15–17]

Theoretical conformational studies agree that there are severe steric restraints to rotation about the carbonyl carbon–α carbon bond, ψ, in both forms.[18–23] From their theoretical rotational potential function, Schimmel and Flory[19] predicted a limiting characteristic ratio of 116 for high molecular weight poly-L-proline with *trans*-peptide bonds. The other authors did not calculate the characteristic ratios predicted from their theoretical rotational potential functions. Upon carrying out the requisite calculations we have found that the predicted limiting characteristic ratios vary by more than an order of magnitude among the different investigators. All of these studies predict energy minima which yield reasonable agreement with the observed ordered structure of poly-L-proline form II.[4,5] Prediction of ordered structures only tests the minimum in the theoretical rotational potential function. The characteristic ratio is dependent upon both the location of the minimum and upon the shape of the rotational potential function and is a more exacting experimental test of the validity of the theoretical work. The measurement of the characteristic ratio of polypeptides with $-CH_2R$ side chains,[24] which was

(1) This work was supported by a Grant from the Division of Biology and Medicine, Atomic Energy Commission.
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