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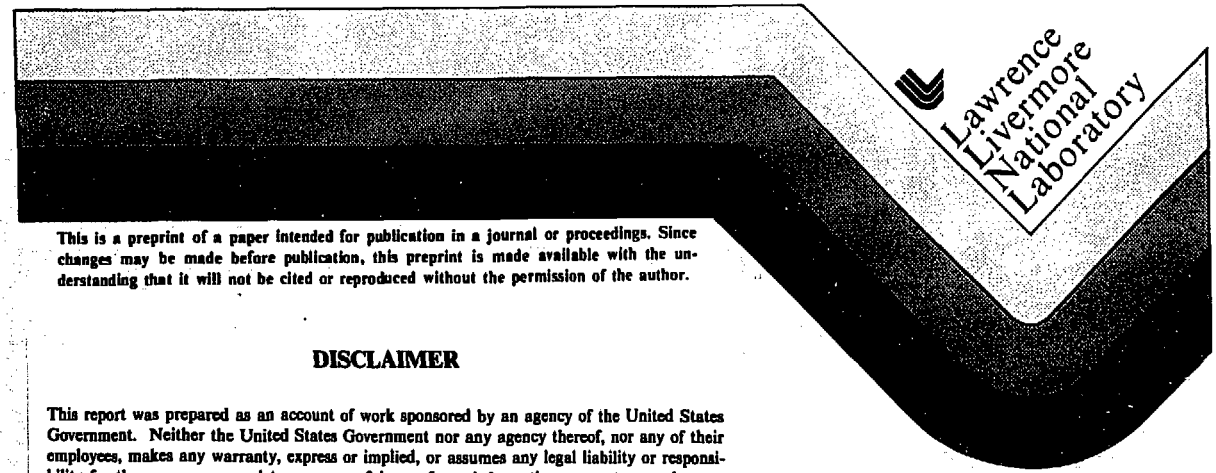

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ABSTRACT

Amino acids and peptides were labeled with liquid and solid tritium at 21 K and 9 K. At these low temperatures radiation degradation is minimal, and tritium incorporation increases with tritium concentration and exposure time. Ring saturation in L-phenylalanine does not occur. Peptide linkage in oligopeptides is stable toward tritium. Deiodination in 3-iodotyrosine and 3,5-diiodotyrosine occurs readily and proceeds in steps by losing one iodine atom at a time. Nickel and noble metal supported catalysts when used as supports for dispersion of the substrate promote tritium labeling at 21 K. Our study shows that both liquid and solid tritiums are potentially useful agents for labeling peptides and proteins.

INTRODUCTION

Tritium labeling by T-for-H exchange induced by radiation from tritium β decay at ambient temperature has been used for labeling polypeptides and proteins.[1] Labeling by radiation induced methods can cause excessive degradation of the products. Radiation decomposition manifests itself only when molecular fragments formed originally by the passage of ionizing radiation move away by diffusion from the site of formation (the spur) and enter into reaction with other fragments. These reactions are temperature dependent. At temperatures near absolute zero, molecular diffusion and migration are greatly reduced and chemical reactions requiring energy of activation will cease; these effects combine to minimize the radiation damage. Cholecalciferol (vitamin D_3)[2] and oleate[3] when labeled at 77 K with tritium gas showed considerably less degradation than at 298 K.

At 21 K, the triple point, tritium gas condenses into liquid and at about 9 K into solid.[4] One gram of tritium has 9,665 Ci of radioactivity and will release 7.35×10^{21} eV of energy per hour. In the condensed form tritium can be used for labeling, but the cryogenic technology required for maintaining tritium in the liquid or solid form and the radiation safety procedures for handling high levels of tritium will be formidable. From both theoretical and practical points of view, labeling with liquid and solid tritium represents a new form of radiation induced labeling. Whether tritium incorporation will dominate over radiation degradation at these low temperatures or not is a matter of profound interest and speculation.

In the study reported here, we have selected some amino acids and peptides for labeling with gaseous, liquid and solid tritium in order to determine the characteristics of tritium labeling at near absolute zero temperatures, especially with respect to the extent of dehalogenation, ring saturation, tritium incorporation and the stability of peptide bond.

EXPERIMENTAL METHODS

Samples of L-phenylalanine, L-tyrosine, 3-iodo-tyrosine, 3,5-diiodotyrosine, glycyl-glycyl-L-leucine, glycylglycyl-L-phenylalanine and leucine-enkephalin were dispersed, as previously described[5] on pellets (0.32 cm or 1/8 inch in diameter) of silica-alumina catalyst support or of supported Ni catalyst. Each pellet contained approximately 0.5 mg of the sample. The pellets were placed in a special liquid helium-cooled cryogenic sample cell (vide infra) operated at the Lawrence Livermore National Laboratory and were exposed (a) to gaseous tritium at 1.33×10^4 Pa at room temperature, at liquid nitrogen temperature or at 22 K, (b) to liquid tritium at about 21.0 K and (c) to solid tritium at about 9 K. After exposure and exhaustive evacuation to remove residual tritium gas, the labeled product was eluted off the treated pellet with water or

dilute acid, freeze-dried to remove labile tritium, radioassayed by liquid scintillation counting and then analyzed.

Analysis of the labeled products was by gradient-elution reversed-phase HPLC on Radial-Pak μBondapak or Nova-pak C18 column (Waters), after precolumn derivatization with o-phthalaldehyde (OPA)[5] in a Waters gradient elution system. This system consisted of two Waters Model 510 pumps, Model 680 gradient elution controller, a Kratos 757 Spectroflow absorbance detector, a Houston Miniscribe recorder, and a HP Model 3393A integrator. The mobile phase was a mixture of acetonitrile and 0.02 M phosphate buffer (pH 6.8). The linear gradient elution began at a flowrate of 1.4 ml/min with a composition of 5% acetonitrile and 95% buffer, changing to 50:50% (v/v) in 45 minutes. The mass peak was monitored at 340 nm. The eluate was collected in 0.35 ml fractions in plastic pico vials which were counted directly in a Beckman LS9000 liquid scintillation counter after addition of 4 ml of an emulsion-type scintillator. The specific activity of the labeled product was obtained from the mass peak and the radioactivity peak. The response of the uv detector was calibrated with a known standard of the sample daily or before each run. A few samples of [^{3}H]L-phenylalanine were further purified by thin-layer chromatography on silica-gel plates, with ethanol and water (70:30%, v/v) as the developing solvent. The edge of the thin-layer plate was developed with ninhydrin to mark the position of the amino acid, and the band of the silica gel containing the labeled amino acid was then collected and eluted with water. The eluate was freeze-dried and re-analyzed by HPLC to determine the constancy of the specific activity values.

Many of the HPLC analyses were performed in replicate. Samples were analyzed promptly after labeling, but a few samples were partially delayed as indicated. Their observed peak percent values appeared to be lower than expected.

Cryogenic Experiment

The cryostat is a 1-W helium flow cryostat. Cold helium gas is blown through an orifice and partially liquifies because of Joule-Thomson expansion. This chills a copper block to which the sample cell is attached. By changing the helium flow rate and by turning on heaters, the temperature is easily stabilized between 4.2 and 23 K. The cryostat is made by Air Products, Allentown, PA.

A special sample cell is screwed onto the copper block. This cell is a copper post with a cylindrical cavity hollowed through the bottom. Two end plates seal this cavity. The front plate is a stainless steel ring with a sapphire window. The end plates are sealed with indium O-rings. Bolts run through the copper cylinder to tighten the seals. Figure 1 shows the front and side view of the sample cell.

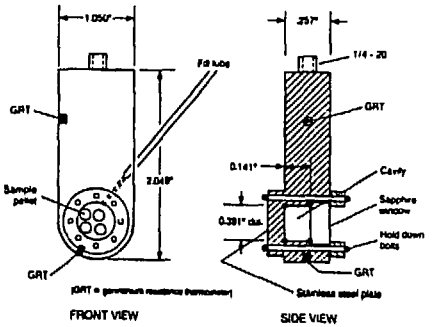

Figure 1. Sketch of Cryogenic Sample Cell

A fill tube runs into the cell in order to provide the tritium. It is stainless steel of 2.7 mm diameter and causes only a small heat leak. The temperature is measured by germanium resistance thermometers mounted on the copper cell away from the radiation of the tritium. The pressure is measured with a 1000 mm baratron (capacitance device), which has been calibrated with a dead weight tester.

The sample pellets were cylindrical with dimensions of 3.0 mm diameter by 3.0 mm length. Up to four could be placed at once in the sample cell.

The cell is cooled to about 21 K and valve opened to a bottle of tritium gas at room temperature. The tritium cryopumps itself into the cell and appears there as the liquid, with a vapor pressure of about 2.93×10^4 Pa (220 torr). The tritium freezes at 20.6 K and may be cooled to about 6 K. Self-heating, caused by the 1.95 W/mol of radioactive decay heat, prevents the samples from reaching the 4.2 K liquid helium temperature. A 500-liter dewar of liquid helium allows continuous operation for ten days. At the end of the experiment, the cryostat is heated and the tritium is gettered onto a palladium bed. The sample cell is unscrewed and opened in a hood. Approximately 18.5 TBq (500 Ci) of liquid or solid tritium are used for one run.

RESULTS AND DISCUSSION

Table 1 shows the results of labeling L-phenylalanine with gaseous tritium at a pres-

Table 1. Labeling of L-Phenylalanine and L-Tyrosine with Gaseous, Liquid and Solid Tritium

Code	Support[a]	State[b]	Condition[c]	Activity[d] (MBq)	Sp. Act. (Gbq/mmol)	Peak %[e]
a. Blank Pellet						
6066-18a	A	G	77K/21h	5.18	---	---
6066-18b	B	G	77K/21h	4.44	---	---
b. L-Phenylalanine						
5016-4a	B	G	300K/21h	7.77	0.052	7.14
5016-4b	A	G	300K/21h	12.58	0.097	10.77
6066-17a	B	G	77K/21h	10.0	0.44	18.8
6066-17b	A	G	77K/21h	25.9	0.63	8.1
3256-5	A	G	21.6K/3h	49.2	6.92	49.51
4036-4	B	G	21.5K/17h	115.2	22.64	30.00
4106-3	B	G	21.0K/47h	518.0	55.50	21.26
3056	A	L	1m	49.95	3.54	60.66
3136-1	A	L	20m	31.45	6.33	48.31
3136-2	A	L	40m	21.83	15.91	47.22
4036-10	B	L	3h	79.55	15.93	24.41
4036-6	B	L	17h	334.85	50.9	18.49
3256-8	A	L	21h	538.35	22.20	8.64
7306-2[f]	B	L	43h	418.47	187.96	9.30
6066-1[f]	B	S	12h	440.30	66.23	13.03
7306-5[f]	B	S	21h	392.94	156.14	5.38
c. Tyrosine						
5016-7a[f]	B	G	77K/21h	(Activity too low for analysis)		
5016-7b[f]	A	G	77K/21h	(Activity too low for analysis)		
3256-6	A	G	3h	38.85	4.22	32.11
4036-2	B	G	47h	93.24	31.31	23.40
3136-1	A	L	20m	15.91	7.09	29.11
3136-2	A	L	40m	14.80	4.79	30.55
4036-8	B	L	3h	55.50	7.05	17.24
4036-5	B	L	17h	131.72	15.77	11.41
3256-7	A	L	21h	364.82	4.32	2.12
7306-3	B	L	43h	65.86	2.55	1.88
6066-3[f]	B	S	12h	215.34	---[g]	---
7306-6	B	S	21h	32.93	0.26	0.94

[a] The substrate was supported on pellets of (A) silica-gel (#980-25, Davison Chemical, Baltimore, MD) and (B) 1% Ni catalyst on A (Cf. Ref. 5).

[b] G stands for gaseous tritium at a temperature to be specified, L for liquid tritium at 21 K, and S for solid tritium at 9 K.

[c] Condition includes temperature and length of contact with tritium. m = minutes, h = hour. The temperature of gaseous tritium (G) if not specified is taken to be 21 K.

[d] Activity represents the gross radiochemical yield of the labeled sample after removal of labile tritium.

[e] Peak % represents the fraction of injected radioactivity that appears as labeled product.

[f] Sample analysis was partially delayed.

[g] The purified sample had activity which was insufficient for HPLC analysis.

sure of 1.33×10^4 Pa (100 torr) at room temperature (300 K), at liquid nitrogen temperature (77 K) and at the triple point of tritium (21 K). The radiochemical yields after removal of labile tritium by equilibration with water and freeze-drying varied from a few to hundreds of megabacquerels per sample. The labeled amino acids had specific activities ranging from a few to a hundred GBq/mmol, increasing with decreasing temperature of the tritium gas. The specific activity of labeled phenylalanine at 21 K is 10 times greater than that at 77 K which in turn is 6 times more than that at 300 K. The density of tritium gas is 5.4 mol/m^3 at 300 K, 20.6 mol/m^3 at 77 K and 246/mol/m^3 at 21.7 K.[6] Apparently, the specific activity of the [^{3}H]L-phenylalanine is a function of tritium gas in the cryogenic sample cell. Other factors may likely contribute to the low temperature enhancement of the specific activity.

In this study, no ring saturated product of phenylalanine was detected and only traces of saturated products of tyrosine were found, indicating that ring saturation is minimal in the labeling with gaseous, liquid or solid tritium at temperatures near absolute zero. In contrast, labeling of phenylalanine and tyrosine by microwave discharge activation of tritium gas has always resulted in the formation of ring saturated by-product.[7]

In general, the gross radiochemical yield and specific activity of [^{3}H]phenylalanine exhibit an approximately exponential relationship with increase in exposure time. The specific activity of labeled phenylalanine increased from 6.42 GBq/mmol for a 3 hour exposure to 55.50 GBq/mmol for a 47 hour exposure to tritium gas at 21 K, and the corresponding radiochemical yield increased from 49.2 MBq to 518 MBq. This clearly demonstrates that tritium incorporation is dependent on exposure time. We will use specific activity rather than gross radiochemcial yield as a measure for tritium incorporation in the labeled substrate because the latter may include the yield of labeled by-products.

For equal exposure time, labeling with liquid and solid tritium is more efficient than labeling with gaseous tritium at 21 K. Liquid tritium has a density of 4,500 mol/m^3 and solid tritium 5,300 mol/m^3.[6] In the condensed phase, more tritium will come in contact with the substrate than in the gas phase at 21 K. Radiation damage from the β decay of tritium at this low temperature is minimal. Even after exposure to solid tritium for 47 hours with accumulation of 1.79×10^{22} eV of energy, the substrate phenylalanine showed no appreciable degradation.

In comparison with L-phenylalanine, L-tyrosine showed less tritium incorporation, as indicated by the gross radiochemical yield. The p-hydroxyl hydrogen in the phenyl ring of tyrosine is labile. Tritium incorporated into that position is readily back-exchanged, lowering the specific activity accordingly. In addition, orientation of the -OH group in the tyrosine molecule adsorbed on silica-alumina support may also exert an effect on tritium incorporation.

Table 2 shows the labeling of tripeptides glycylglycyl-L-leucine (GGL) and glycylglycyl-L-phenylalanine (GGF) and the penta-peptide leucine-enkaphalin with liquid and gaseous tritium at 21 K. GGL on exposure to liquid tritium for 20 minutes yielded labeled product with a specific activity of 3.06 GBq/mmol, which is higher than that of 2.63 GBq/mmol obtained by exposure to tritium gas at 21 K for 3 hours. GGF when exposed to liquid tritium for 17 hours attained a specific activity of 54.54 GBq/mmol, as compared to 26.54 GBq/mmol from equal exposure to tritium gas at 21 k. The advantage of using liquid tritium over tritium gas at 21 K in peptide labeling is that high specific activity can be achieved with shorter exposure. Tritium either as gas or liquid at this low temperature causes no disruption of the peptide bond.

The use of noble metal catalysts as supports favors tritium incorporation; Ru catalysts appear to be more effective in this respect than Pt and Rh catalysts. The silica-alumina support has a strongly acidic surface and when dehydrated is a Lewis acid; excited tritium from microwave discharge can adsorb on its surface and act as an electrophile. The effect of the support acidity on the stability of biomacromolecules at near absolute zero temperatures has to be investigated.

Table 3 shows both the labeling and deiodination of 3-iodotyrosine and 3,5-diiodotyrosine with tritium gas at 21 K. 3-Iodotyrosine yielded [^{3}H]3-iodotyrosine and [^{3}H]tyrosine, the deiodinated product, in a ratio from 1:7 to 1:9, irrespective of whether Ni catalyst or silica-alumina was used as support. The radiochemical yield of [^{3}H]3-iodotyrosine decreased from 8.66% to 3.12% while its specific activity increased from 1.28 to 19.71 GBq/mmol for an exposure from 3 to 47 hour exposure. [^{3}H]Tyrosine, the deiodinated product, was formed in the carrier-free state with a specific activity of 1.073 TBq/mmol and radiochemical yield of about 59%, for a 3 hour exposure. The yield decreased to 32% at 47 hour exposure. The value of its specific activity is from theory, because the extremely small mass of [^{3}H]tyrosine prevents direct measurement. The specific activity may be decreased by the presence of extraneous tyrosine in the sample or enhanced by additional tritium incorporation besides the iodine substitution.

Table 2. Labeling of Peptides with Gaseous and Liquid Tritium

Code	Support[a]	State[b]	Condition[c]	Activity[d] (MBq)	Sp. Act. (GBq/mmol)	Peak %[e]
a. Glycylglycyl-L-leucine						
3256-4	A	G	3h	35.15	2.63	16.44
3136	A	L	20m	48.84	3.06	15.82
3136	A	L	40m	21.09	3.05	13.78
3256-9	A	L	21h	313.02	4.31	3.85
b. Glycylglycyl-L-phenylalanine						
4036-1	B	G	17h	75.85	26.54	23.40
4106-4	B	G	47h	347.06	47.87	12.09
4036-11	B	L	3h	48.84	18.07	19.27
4036-7	B	L	17h	323.38	54.54	10.90
6066-13[f]	Rh[g]	L	17h	73.26	35.89	3.20
6066-14[f]	Ru[g]	L	17h	29.60	57.35	4.42
6066-15[f]	Pt[g]	L	17h	21.09	33.30	4.07
c. Leucine-enkephalin						
3256-1	A	G	3h	15.54	12.93	33.96

[a-f] See footnotes in Table 1.
[g] The noble metal catalyst were prepared similarly to Ni catalyst according to Ref. (8).

Table 3. Deiodination of Iodinated Tyrosines by Gaseous and Liquid Tritium

Code	Support[a]	State[b]	Condition[c]	Activity[d] (MBq)	Sp. Act. (GBq/mmol)	Peak %[e]
a. 3-Iodotyrosine						
3256-2	A	G	3h	29.97	1.28	8.66
		[^{3}H]Tyrosine[f]			C.F.	59.06
4036-3	B	G	17h	92.50	10.09	6.82
		[^{3}H]Tyrosine[f]			C.F.	50.00
4106-2	B	G	47h	137.64	19.71	3.12
		[^{3}H]Tyrosine[f]			C.F.	32.66
4036-9	B	L	3h	66.60	6.94	5.00
		[^{3}H]Tyrosine[f]			C.F.	41.45
b. 3,5-Diiodotyrosine						
3256-3	A	G	3h	37.37	0.93	2.43
		[^{3}H]3-Iodotyrosine[g]			C.F.	52.60
		[^{3}H]Tyrosine[f]			C.F.	10.43

[a-e] See footnotes in Table 1.
[f] [^{3}H]Tyrosine is the deiodinated product of the iodotyrosines and formed in the carrier-free (C.F.) state with a specific activity of 1.073 TBq/mmol (29 Ci/mmol) from 3-iodotyrosine or 2.146 TBq/mmol (58 Ci/mmol) from 3,5-diiodotyrosine.
[g] [^{3}H]3-Iodotyrosine was formed in the C.F. state from 3,5-diiodotyrosine by deiodination.

Exposure of 3,5-diiodotyrosine to tritium gas at 21 K yielded [^{3}H]3,5-diiodotyrosine, [^{3}H]3-iodotyrosine and [^{3}H]tyrosine in the ratio of 1:21:4, showing that the deiodination of the diiodinated tyrosine proceeded in steps, losing one iodine atom at a time. The tritiated 3-iodotyrosine and tyrosine were formed, respectively, in the carrier-free state with a specific activity of 1.07 and 2.14 TBq/mmol. It is likely that their specific activity could vary with the presence of extraneous carriers and additional labeling as discussed above. The deiodination products were not accompanied by labeled by-products from ring saturation and were found cleaner than those from deiodination by microwave discharge activation of tritium gas.

CONCLUSIONS

Labeling with liquid or solid tritium involves the handling of large amounts of tritium and advanced cryogenic technology and has not been previously attempted. The high concentrations of tritium required for labeling would have caused extensive radiation damage to the substrate and the labeled product, had the labeling been allowed to proceed at room temperature. At 21 K or lower, the reaction rate is greatly reduced and the radiation damage becomes minimal. Tritium incorporation into the labeled amino acids and peptides increases with exposure time and can attain specific activities in the range of tens and hundreds of GBq/mmol with the labeled products relatively free of by-products and radioimpurities. Ring saturation, which occurs readily in the labeling of L-phenylalanine and L-tyrosine with activated species of tritium generated by microwave discharge activation of tritium gas, did not occur in the present study. Deiodination of 3-iodotyrosine and 3,5-diiodotyrosine was found to occur in a stepwise manner, and the labeled products were unadulterated with tritiated by-products. The ability of Ni and other noble metal catalysts to enhance tritium incorporation in amino acids and peptides as compared with silica-alumina indicated that their catalytic action persisted down to liquid hydrogen temperature.

Reaction mechanisms for tritium labeling at the low temperatures remains speculative. That the specific activity of the labeled product approximately parallels the tritium concentration indicates that the rate may be first order in tritium concentration. The low temperature precludes the dominance of Arrhenius type reactions. Reactions requiring the energy of activation will be greatly reduced in favor of reactions that are temperature independent.[9,10] The low temperature also favors exothermic reactions. Electron tunneling, proton tunneling, activationless ion-molecule reactions[9,10] and "hot" molecule reactions[11] are all probable. For example, deiodination at temperatures near absolute zero requires heavy atom migration. It may involve electron tunneling to facilitate the dissociation of the C-I bond to form I^- ion followed by recombination with tritium, or the heavy atom facilitates intersystem crossing to form a triplet and then to undergo deiodination. The tritium β decay energy dissipates in the medium and may create ions, atoms, radicals and "hot" molecules that can directly participate in reactions to achieve labeling. Vibrational assisted tunneling (VAT)[11] is also possible. The results of our study suggest that liquid and solid tritiums are potentially useful agents for labeling peptides and proteins. Other aspects of tritium labeling at temperatures near absolute zero, related to the use of metal catalyst to promote labeling, the behavior of substrate support for dispersion and the stability of sensitive biomolecules on support need to be further explored.

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REFERENCES

1. C. T. PENG and R. L. HUA, "Tritium Labeling of Peptides and Proteins. A Review," Fusion Technol., 8, 2265-2272 (1985).

2. C. T. PENG, "Preparation of Tritio-Cholecalciferol (Tritiated Vitamin D_3)," J. Pharm. Sci., 52, 861-864 (1963).

3. C. T. PENG, "Mechanism of Addition of Tritium to Oleate by Exposure to Tritium Gas," J. Phys. Chem., 70, 1297-1304 (1966).

4. P. C. SOUERS, Hydrogen Properties for Fusion Energy, University of California Press, Berkeley (1986).

5. R. L. HUA and C. T. PENG, "Relative Efficiencies of Tritium Atoms and Ionic Species in Peptide Labeling," J. Labelled Compd. Radiopharm., 24, 1095-1106 (1987).

6. P. C. SOUERS, unpublished data.

7. C. T. PENG, B. E. GORDON, W. R. ERWIN and R. M. LEMMON, "Dehalogenation and Ring Saturation by Tritium Atoms," Int. J. Appl. Radiat. Isot., 33, 419-427 (1982).

8. C. T. PENG and O. BUCHMAN, "Tritium Exchange Labeling of Compounds Containing $-NO_2$, -I, -C=C- and -C=O groups," Tetrahedr. Lett., 26, 1375-1378 (1985).

9. V. I. GOLDANSKII, "Chemical Reactions At Very Low Temperatures," Ann. Rev. Phys. Chem., 27, 85-126 (1976).

10. V. I. GOLDANSKII (1979). "Quantum Chemical Reactivity Near Absolute Zero: Biological, Chemical and Astrophysical Aspects," in Tunneling in Biological Systems, (B. Chance, R. A. Marcus, D. C. DeVault, J. R. Schrieffer, H. Frauenfelder, and N. Sutin, eds.), Academic Press, New York, pp. 661-711.

11. R. JAIN, L. MCELWEE-WHITE, and D. A. DOUGHERTY, "Rapid, Multistep Rearrangements of Hydrocarbon Triplet Biradicals at 4 K. A Possible Example of Hot Molecule Effects in Frozen Organic Solvents," J. Am. Chem. Soc., 110, 552-560 (1988).