where ΔS is the entropy of formation of the form stable at high temperature. From the experimental data we can, therefore, compute the following values, assuming a degree of polymerization $n = 700$: $\Delta S/n = -0.30$ e.u. and $\Delta H/n = -94$ cal./mole for the hydrogen-containing molecule, and $\delta S/n = -0.09$ e.u., $\delta H/n = -24$ cal./mole, $\delta F/n = 4$ cal./mole for the changes accompanying deuterium–hydrogen substitution. The values of $\Delta H/n$ and $\Delta S/n$ are extremely small compared with those usually encountered,[16] and this is, of course, in agreement with the conclusion drawn in the preceding paragraph, *i.e.*, the values $\Delta H/n$ and $\Delta S/n$ obtained here are values for the over-all reaction (Fig. 2) involving ΔH and ΔS of several different species of hydrogen-bonds, and are necessarily small.[17]

In order to provide a quantitative argument to account for the magnitude and direction of the change in T_{tr}, the enthalpies and entropies of formation of each of these kinds of hydrogen bonds would have to be estimated. However, in the present state of our knowledge, it is not possible to make these estimates with sufficient accuracy, so that the observed decrease of 11° in T_{tr} must be reconciled qualitatively in terms of the explanation provided above, *viz.*, deuterium substitution affects all the hydrogen bonds indicated in Fig. 2 and the net effect on T_{tr} can be either an increase or a decrease depending on the relative stability of the various kinds of hydrogen bonds involved.

From an experimental point of view, a measurable change in T_{tr} upon deuterium-hydrogen substitution has been demonstrated. The reconciliation with theory must await a more accurate estimate of the thermodynamic parameters for the formation of various kinds of hydrogen bonds.

It is of interest to note from Fig. 1 that, at a temperature of *ca.* 35°, the fraction of molecules in the helical form is very small for the hydrogen-containing sample and very large for the deuterated sample, showing the very large effect of deuterium substitution in a small temperature range. It is thus very interesting to speculate that the ratio of the concentrations of helices to random coils in a biological system (*e.g.*, in genes) can be markedly affected by deuteration. The effect of deuteration on the helix/random coil ratio also has implications for the interpretation of data from experiments on the kinetics of deuterium–hydrogen exchange.[18] In the latter technique, it is assumed that the substitution of deuterium for hydrogen does not change the helix/random coil ratio. However, if such a study were carried out at a temperature near the transition region (*e.g.*, 35° in the case considered here), deuteration would affect this ratio. Of course, if the kinetics experiment is carried out at a temperature sufficiently removed from the transition region, then this ratio will presumably not be affected. It seems probable that most of the experiments heretofore carried out on the kinetics of deuterium–hydrogen exchange have not been performed near the transition region. The effects of D–H substitution on the stability of helical structures in proteins is at present under investigation and will be reported shortly.

Acknowledgment.—We are indebted to Prof. S. H. Bauer for helpful discussions of this problem.

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(17) It may be noticed that the ratio of the *experimental* quantities $\delta H/\Delta H$ is ~1/4, which is rather large. This large ratio probably arises because small changes in the degree of hydrogen bonding in each of the species of Fig. 2 (produced here by D–H substitution) can produce relatively large changes in the small over-all ΔH.

(18) See, for example, K. Linderstrøm-Lang, "Symposium on Peptide Chemistry," The Chemical Society, London, 1955, Special Publication No. 2, p. 1.

[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY, UNIVERSITY OF KANSAS AND PRINCETON UNIVERSITY]

Recoil Tritium Reactions at an Asymmetric Carbon: L(+)-Alanine[1]

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Recoil tritium reactions with L(+)-alanine have been investigated in both crystalline form and in aqueous solution. The T for H substitution at the asymmetric carbon atom occurs with complete retention of configuration in the crystal and occurs for only 0.01% of the total tritium in the liquid solution. More than 85% of the T for H substitutions in the methyl group in both cases proceed with retention of optical configuration.

Introduction

Recoil tritium atoms from the $Li^6(n,\alpha)H^3$ reaction have been shown in previous studies to replace protium in C–H bonds in a variety of molecules without affecting the molecule involved in any other permanent manner.[2] Isolation and intramolecular degradation of tritium labeled glucose and galactose molecules have demonstrated that substantial percentages of this radioactivity are bound in non-labile positions at asymmetric carbon atoms.[3,4] Experiments in both gas phase,[5] and condensed phases[6] indicate that the substitution of T for H in other molecules takes place through an intermediate complex rather than through a free radical mechanism. Considerable information can be obtained about the nature of such a complex by study of the reactions of recoil

(1) Research supported in part by A. E. C. Contract No. AT-(11-1)-407. Presented in part at the 130th Meeting of the American Chemical Society, Atlantic City, September, 1956.

(2) See, for example, F. S. Rowland and R. Wolfgang, *Nucleonics*, **14**, No. 8, 58 (1956).

(3) F. S. Rowland, C. N. Turton and R. Wolfgang, THIS JOURNAL, **78**, 2354 (1956).

(4) H. Keller and F. S. Rowland, *J. Phys. Chem.*, **62**, 1373 (1958).

(5) M. El-Sayed, P. Estrup and R. Wolfgang, *ibid.*, **62**, 1356 (1958).

(6) W. J. Hoff, Jr., and F. S. Rowland, THIS JOURNAL, **79**, 4867 (1957).

tritium at an asymmetric carbon atom, noting in particular whether the substitution is accompanied by retention, racemization or inversion of the stereochemical structure around the asymmetric carbon atom. Labeled galactose from irradiated glucose had not been found in earlier work.[4] A less complex, readily resolvable, optically-active material was sought and L(+)-alanine was chosen for these experiments.

Experimental

Chemicals.—All chemicals used were analytical reagent grade and were not submitted to further purification. The L(+)-alanine used in the irradiations was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Irradiations.—The crystalline sample consisted of an intimate mixture of 0.99 g. of L(+)-alanine and 30 mg. of Li_2CO_3, and the aqueous solution contained 1.01 g. of L(+)-alanine and 190 mg. of LiCl in 7 ml. of solution. Each was irradiated for 24 hr. in a neutron flux of 1.8×10^{12} n/cm.^{2}/sec. at the Brookhaven reactor and showed a slight brownish discoloration from radiation decomposition at the end of the irradiation.

Purification.—After irradiation, each sample was added to an aqueous solution containing 10 g. of *dl*-alanine carrier, and a series of alanine recrystallizations were carried out. Radiochemical purity was checked both on the recrystallized alanine and on the *p*-toluenesulfonyl derivative. When radiochemical purity of the bulk "*dl*"-alanine[7] appeared to have been obtained, the optical isomers were resolved following the method of Pacsu and Mullen,[8] with only slight modification. The successive steps in the resolution were

(1) Formation of benzoyl-*dl*-alanine
(2) Formation of strychnine salt of benzoyl-*dl*-alanine
(3) Crystallization of strychnine salt of benzoyl-L(+)-alanine, leaving the D(−) form in the mother liquor
(4) Removal of strychnine and addition of brucine to the benzoylalanine of the mother liquor
(5) Crystallization of brucine salt of benzoyl-D(−)-alanine, leaving any L(+)-salt in the mother liquor
(6) Hydrolysis of both crystalline alkaloid salts back to benzoylalanine
(7) Removal of the benzoyl group, giving both pure D(−)- and L(+)-alanine

The intramolecular distribution of the tritium activity in each alanine sample was determined by oxidation to acetic acid, and recovery as thallous acetate, following the method of Wolf and Redvanly.[9]

Chemical Procedures.—**Benzoyl-"dl"-alanine.**—4.7 g. of "*dl*"-alanine and 31 g. of $NaHCO_3$ were dissolved in 40 ml. of hot water. After cooling, 17 ml. of benzoyl chloride was added dropwise and stirred for 4 hr. After filtering off excess bicarbonate, 50 ml. of 6 *N* HCl precipitated the benzoyl-"*dl*"-alanine and benzoic acid. The benzoic acid was extracted with hot ligroin, and then the benzoyl-"*dl*"-alanine was recrystallized from water.

Benzoyl-L(+)-alanine.—11.3 g. of strychnine was added to 6.5 g. of benzoyl-"*dl*"-alanine in 150 ml. of water. Excess strychnine was filtered off. The strychnine salt of benzoyl-L(+)-alanine crystallized out on standing for 24 hours at 0°. It then was filtered and recrystallized from water.

The strychnine from 6 g. of salt was hydrolyzed with 10 ml. of 1.25 *N* KOH in 50 ml. of hot water and filtered off. After acidifying with 9 ml. of 1.4 *N* HCl, the solution was concentrated *in vacuo* until benzoyl-L(+)-alanine crystallized.

Benzoyl-D(−)-alanine.—The solution remaining after filtering off the strychnine-benzoyl-L(+)-alanine was concentrated *in vacuo* to a sirup and hydrolyzed with 20 ml. of 1 *N* KOH. After filtration, 20 ml. of 1 *N* HCl and 8.5 g. of brucine tetrahydrate were added to the solution, and the brucine salt of benzoyl-D(−)-alanine crystallized out.

The brucine salt was hydrolyzed by addition of 1.25 *N* KOH to the aqueous salt solution. After filtration and acidification with 1.4 *N* HCl, the solution was cooled and concentrated until benzoyl-D(−)-alanine crystallized.

Recovery of Alanine from Benzoyl-alanine.—1.3 g. of benzoyl derivative was dissolved in 10 ml. of 6 *N* HCl and hydrolyzed by heating under reflux for 3 hr. After cooling, benzoic acid was removed by four ether extractions. The aqueous layer was concentrated to a solid, dissolved in ethanol and evaporated to dryness 4 times. Alanine then was precipitated from a solution of alanine hydrochloride in 95% ethanol by the addition of aniline. The alanine was filtered at 0° and washed with ethanol and then ether.

Thallous Acetate.—0.3 g. of alanine was oxidized to acetaldehyde with 1.5 g. of ninhydrin in citrate buffer. The acetaldehyde was swept in a nitrogen stream into a CrO_3 solution in 1 *M* H_2SO_4. After standing overnight, the acetic acid was steam distilled and titrated with TlOH. After evaporation to dryness, the thallous acetate was extracted with methanol, decolorized with charcoal and recrystallized from methanol–acetone solution.

Tritium Counting.—After each separation of a pure material, a 10-mg. sample was removed for tritium assay. All samples were combusted by the method of Wilzbach, Kaplan and Brown[10] and counted in a silver-walled glass proportional counter with ethane or propane as the counter gas. The reproducibility of samples of the same material is about ±2%; the absolute accuracy is about ±5%.

Optical Rotations.—No optical rotations were measured on the radioactive samples, since radiochemical purity implies optical purity. Measurements on practice runs had agreed with the observations of Pacsu and Mullen.

Results

A summary of the derivatives isolated, and the specific radioactivity of each, expressed in activity per alanine residue, is shown in the form of flow diagrams in Figs. 1 and 2. These diagrams show the gradual purification of each sample by crystallization and then by formation of suitable derivatives. The data indicate that each sample was essentially radiochemically pure at the benzoyl-"*dl*"-alanine stage. The subsequent resolution and purification of D(−) and L(+) fractions from the crystalline irradiation shows no further loss of activity: both fractions are constant through 3 chemical steps, and the average specific activity is approximately equal to the specific activity of the original "*dl*"-mixture.[11]

Discussion

The primary object of this research was to investigate the mechanism of replacement of H by T at an asymmetric carbon atom through observation of the radioactivity in that position for each optical form of the molecule. In the course of the

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[7] The original irradiated material contained pure L(+)-alanine, and we assume no appreciable racemization during irradiation. After the addition of carrier *dl*-alanine to the irradiated sample, the resulting mixture is not an exact *dl*-mixture, but rather contains 5.0 g. of D(−)-alanine and (5.0 × *x*) g. of L(+)-alanine. This mixture we have designated throughout as "*dl*"-alanine. The value of *x* would have been 1.0 for each irradiation, if none of the L(+)-alanine had been destroyed by radiation damage; the actual value is estimated to be ~0.9 from *G*-value calculations.

The subsequent crystallizations, prior to the resolution, will distort this 5.9/5.0 ratio of L(+)/D(−) in accord with the individual phase diagrams involved and the nearness to equilibrium in the crystallization processes. Hence, the "*dl*"-alanine and benzoyl-"*dl*"-alanine have a small uncertainty in the actual L(+)/D(−) ratio of the indidual samples.

[8] E. Pacsu and J. Mullen, *J. Biol. Chem.*, **136**, 335 (1940).

[9] A. P. Wolf and C. S. Redvanly, private communication.

[10] K. Wilzbach, L. Kaplan and W. G. Brown, *Science*, **118**, 522 (1953).

[11] The small uncertainty in the L(+)/D(−) ratio in the "*dl*"-mixture introduces an uncertainty into the calculation of the average specific activity. An average of 5.9 L(+) and 5.0 D(−) gives 58×10^7 DPM/mole, while 5.0 L(+) and 5.0 D(−) gives 55×10^7 DPM/mole. Either limiting figure is in reasonable agreement with the measured 57×10^7 DPM/mole of the "*dl*" compound.

TABLE I

SPECIFIC TRITIUM ACTIVITIES ON α- AND β-CARBON ATOMS OF L(+) AND D(−)-ALANINE FROM IRRADIATED L(+)-ALANINE[7] (10^7 DPM/MOLE)

Irradiated material	Isolated fraction	Total activity	β (TlAc)	α (by difference)	Av. activity "dl"-alanine	% Total tritium as alanine
Crystalline	L(+)-alanine	94 ± 1	75 ± 1	19 ± 2	58 ± 2	≥12
	D(−)-alanine	15 ± 1	15 ± 1	0 ± 1		
Aqueous soln.	L(+)-alanine	22 ± 2	22 ± 1	0 ± 2	14 ± 1	≥ 0.5
	D(−)-alanine	4.7 ± 0.2	4.1 ± 0.1	0.6 ± 0.2		

degradations, the experiments would also indicate whether substitution of T for H at the β-carbon has any effect on the configuration at the α-carbon position.

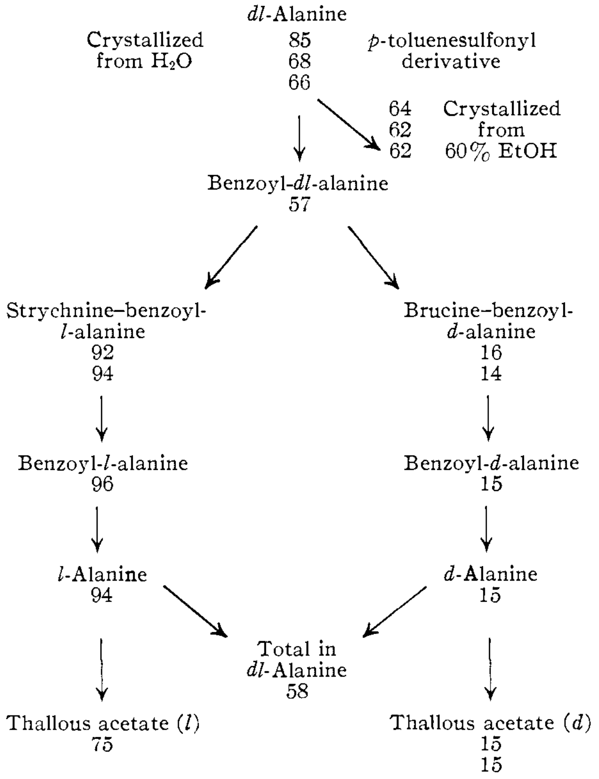

Fig. 1.—Specific activities (10^7 DPM/mole) of crystalline *l*-alanine irradiation.

Racemization and Exchange Reactions.—The original experiments of Pacsu and Mullen indicated that racemization occurred in the benzoylation process prior to the resolution of the optical isomers to an extent that varied somewhat with conditions.[8] Such racemization reactions would alter the original distribution of radioactivity in an irradiated sample as follows. The tritium activity attached to the α-carbon of either an L(+) or D(−) molecule would be lost in the racemization process by exchange with the solvent, resulting in a loss of radioactivity from the alanine molecule. The tritium activity of the β-carbon would be interchanged by racemization between the L(+) and D(−) forms—if racemization were complete, the specific activities of the two forms would be the same. Such racemization is practically undetectable in these experiments by optical activity measurements, since the benzoyl-"*dl*"-alanine was already very nearly optically inactive. The observable effects of racemization would be a diminution in total activity in the alanine molecule and/or a mixing of radioactivity between the L(+) and D(−) forms.

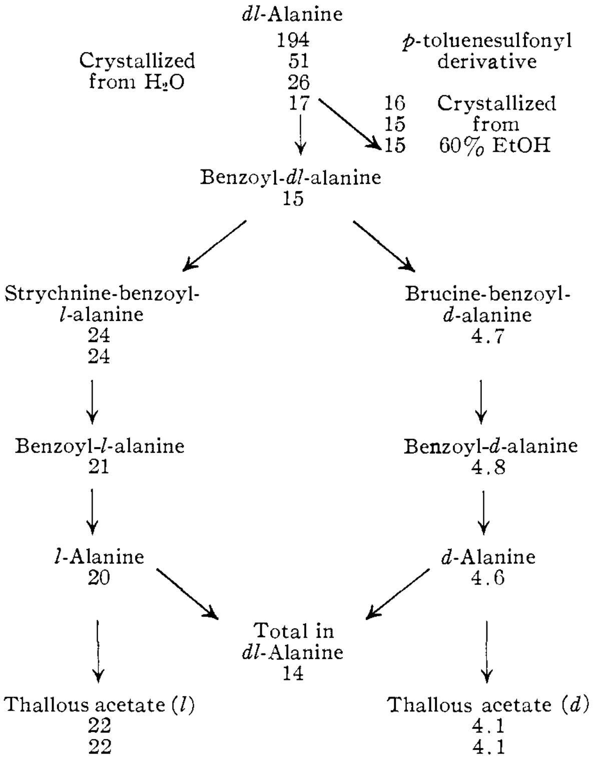

Fig. 2.—Specific activities (10^7 DPM/mole) of *l*-alanine in aqueous solution.

Exchange reactions are of course a possibility at every step. However, the constant specific activity of the separated alanines and derivatives indicates that such reactions were negligible from the crystallization of benzoyl-"*dl*"-alanine to the

TABLE II

PERCENTAGE DISTRIBUTION OF RADIOACTIVITY IN ALANINE ISOLATED FROM L(+)-ALANINE IRRADIATIONS[7]

Crystalline L(+)-alanine

Position Molecule	β	α	% L(+) or D(−)
L(+)	70 ± 1	18 ± 2	88 ± 1
D(−)	12 ± 1	0 ± 1	12 ± 1
% α or β	82 ± 2	18 ± 2	

Aqueous L(+)-alanine

Position Molecule	β	α	% L(+) or D(−)
L(+)	85 ± 3	0 ± 3	85 ± 1
D(−)	13 ± 1	2 ± 1	15 ± 1
% α or β	98 ± 2	2 ± 3	

isolation of pure L(+) and D(−)-alanine. Control experiments also have been run for the oxidation of alanine and showed negligible exchange of methyl hydrogen in the formation of thallous acetate.

α-Carbon Substitution.—Conclusions about the tritium activity on the asymmetric carbon atoms are straightforward for both irradiations:

(a) In irradiated crystalline L(+)-alanine, the tritium activity which is found on the α-carbon is, within error, all in the L(+) form—the substitution reaction proceeds with retention of configuration, and *not* racemization or inversion. This result is in accord with the essential absence of labeled galactose in irradiated crystalline glucose.[4]

(b) In an aqueous solution of L(+)-alanine, substitution of tritium on the α-carbon is a very unlikely process, accounting for only 0.01% of the total tritium activity. Substitution onto the β-carbon position, and, especially, reaction with water are very much more likely processes for recoil tritium atoms. This conclusion is consistent with data for aqueous glucose solutions, in which the glucose does not compete very well with water for recoil tritium.[4] The α-carbon activity which is found is apparently in the D(−) form, as by inversion, but, since the fraction of the total tritium is negligible, many minor processes could be responsible.

It is not possible from the present experimental results to determine whether the retention of configuration during α-carbon substitution in crystals is caused by directional effects of the surrounding crystal, by substitution through some intermediate which maintains asymmetry, or by some other mechanism. Such distinction will require experiments in a medium in which crystal structure effects have been eliminated—ideally, in reactions with a suitable gaseous molecule, or else in reactions in a solvent in which the asymmetric carbon is able to compete more successfully than in water.

The very low α-carbon activity in the alanine from aqueous solution suggests that many reactions at that position are leading to products other than the parent molecule, such as ethylamine and propionic acid by substitution of T for $-COOH$ and $-NH_2$, respectively. These latter substitutions have been observed in good yield with aminobenzoic acids, leading to labeled aniline and benzoic acid, respectively.[12]

Correction for racemization and exchange during benzoylation, as discussed below, would increase the absolute amounts of activity found in the α-position in either irradiation but would not change the lack of α-carbon radioactivity in the D(−) form for the crystalline irradiation or in either form for the aqueous solution. Hence, these conclusions would be unchanged by such corrections.

β-Carbon Substitution.—One would *a priori* expect that tritium would substitute on a β-carbon atom in most cases without affecting the configuration about the adjacent α-carbon atom This is in agreement with the heavy preference found for the L(+) configuration in Tables I and II. It might also be possible for an occasional substitution to produce a molecule sufficiently excited to cause an inversion elsewhere in the molecule. The β-carbon activity observed in the D(−)-alanines in Tables I and II could then have its origin in such an energetic reaction or in racemization from the L(+) form occurring during benzoylation.

The extent of racemization in benzoylation observed by Pacsu aud Mullen is approximately that necessary to produce the observed β-D(−)activity and could have produced all of it. The lower activity of the benzoyl-"*dl*"-alanine, compared to the "*dl*"-alanine and the *p*-toluenesulfonyl derivative, also is consistent with racemization (and loss of α-carbon activity) of the right magnitude. Hence, 85%, and possibly 95 to 100%, of recoil tritium substitutions for hydrogen on a β-carbon occurs with retention of configuration in both liquid and solid; however, a small per cent. of substitution accompanied by sufficient energy to cause inversion at the α-carbon cannot be ruled out by the present experiments.

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[12] R. M. White and F. S. Rowland, 135th Meeting of the American Chemical Society, Boston, April, 1959.