Tritiated Peptides. Part 14.[1] Catalytic exchange labelling of various synthetic peptides

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Several synthetic protected peptides have been catalytically exchange-labelled using tritium gas to a level between 0.1 and 12.5 Ci mmol $^{-1}$. High specific activities could be achieved for histidine-containing peptides, and the factors influencing this have been investigated as have the location and stability of the introduced label. L-Cysteine-containing peptides gave rise to the corresponding peptides containing L-alanine heavily labelled with tritium. The substitution pattern within the alanine residue has been established.

Key words: exchange labelling, tritiation, ^{3}H-histidine peptides, ^{3}H-alanine peptides.

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We reported earlier[2] the labelling of protected β-corticotrophin-(1-24)-tetracosapeptide by catalytic exchange using tritium gas. Over 80% of the incorporated tritium was in the histidine residue, with smaller amounts in tyrosine, phenylalanine, tryptophan and methionine, and in aminobutyric acid which was formed by degradation of methionine.

This exchange method has the potential of being a simple and general method to label peptides containing aromatic amino acid residues. All laboratories practising classical synthetic peptide chemistry have stores of protected intermediates from which labelled peptides might be available without the need to synthesise specific halogenated or unsaturated precursors. We wished to test this possibility by studying the catalytic exchange labelling of various representative protected peptides which presented varying possible constraints on the method.

In the following discussion, the free peptides derived from the compounds A to F are denoted A* to F*. The labelling method and the general methods of analysis were as previously described.[2] In the present study we used substantial purification procedures to eliminate side-products where these arose (e.g. samples B* and D3*, Table 1).

In agreement with our previous study,[2] we observed that peptides containing histidine were more heavily labelled than others (Table 1) and that in these compounds the majority of the label was associated with histidine (Table 2). We again observed (for compound B* only) that some (25%) of the label introduced into tyrosine was labile to acidic hydrolysis, whereas all the label associated with tyrosine in compound A* was stable to acid. Compound A differs from B and from that in the previous study[2] in that the ring hydroxyl is protected as a tertiary butyl ether function. Label at the positions ortho to the hydroxyl group is known to be acid labile.[3] This suggests that the protecting group prevents exchange of tritium into the ortho positions.

As previously observed,[2] histidine was labelled under our experimental conditions far more heavily than the other aromatic residues (Table 2). Inspection of the results in Table 1 shows that the degree of labelling fell off sharply with increasing molecular weight of the compound, presumably for steric reasons. Small compounds such as the protected hexa- and penta-peptides E and F and the tripeptide G were labelled very efficiently. An

Table 1

Amino acid ratios, recoveries and specific

Amino Acid	Compound			
	A*	B*	C*	1
Ala	1.98(2)	1.99(2)	1.01(1)	0.32
Arg	-	-	-	-
Asp	1.97(2)	2.01(2)	1.01(1)	1.12
Cys**	-	-	1.10(2)	1.86
Glu	1.99(2)	1.98(2)	-	-
Gly	2.01(2)	1.98(2)	1.02(1)	1.31
His	1.00(1)	0.99(1)	-	-
Ile	1.02(1)	0.99(1)	-	-
Leu	-	-	-	-
Lys	1.03(1)	1.00(1)	2.08(2)	2.25
Phe	3.00(3)	3.00(3)	3.00(3)	3.00
Pro	2.05(2)	1.99(2)	-	-
Ser	-	-	2.67(3)	2.96
Thr	3.67(4)	3.76(4)		
Trp	-	-	0.42(1)	0
Tyr	0.85(1)	0.97(1)	-	-
Val	1.01(1)	1.00(1)	-	-
% Recovery	48	6	24	3×10^{-4}
Specific Activity (Ci mmol^{-1})	0.55	0.75	0.11	1.48
No. of Residues	22	22	14	13

** Sum of all Cys species

Theoretical values are given in parentheses

Table 1 (contd.)

activities of exchange-labelled peptides A*-G

Compound					
D* Fractions			E*	F*	G
2	3	Theory			
0.36	0.02	(0)	1.00(1)	1.00(1)	-
-	-	-	0.99(1)	-	-
0.99	1.01	(1)	-	-	-
1.17	0.29	(2)	-	-	-
-	-	-	-	-	1.00(1)
1.04	1.03	(1)	1.02(1)	1.00(1)	-
-	-	-	1.00(1)	1.00(1)	1.00(1)
-	-	-	-	-	-
-	-	-	1.98(2)	1.99(2)	-
2.08	2.10	(2)	-	-	-
3.00	3.00	(3)	-	-	-
-	-	-	-	-	1.05(1)
2.71	2.73	(3)	-	-	-
			-	-	-
0.14	0.33	(1)	-	-	-
-	-	-	-	-	-
-	-	-	-	-	-
3	14	-	50	62	-
1.19	0.09	-	3.12	12.5	8.8
13	13	-	6	5	3

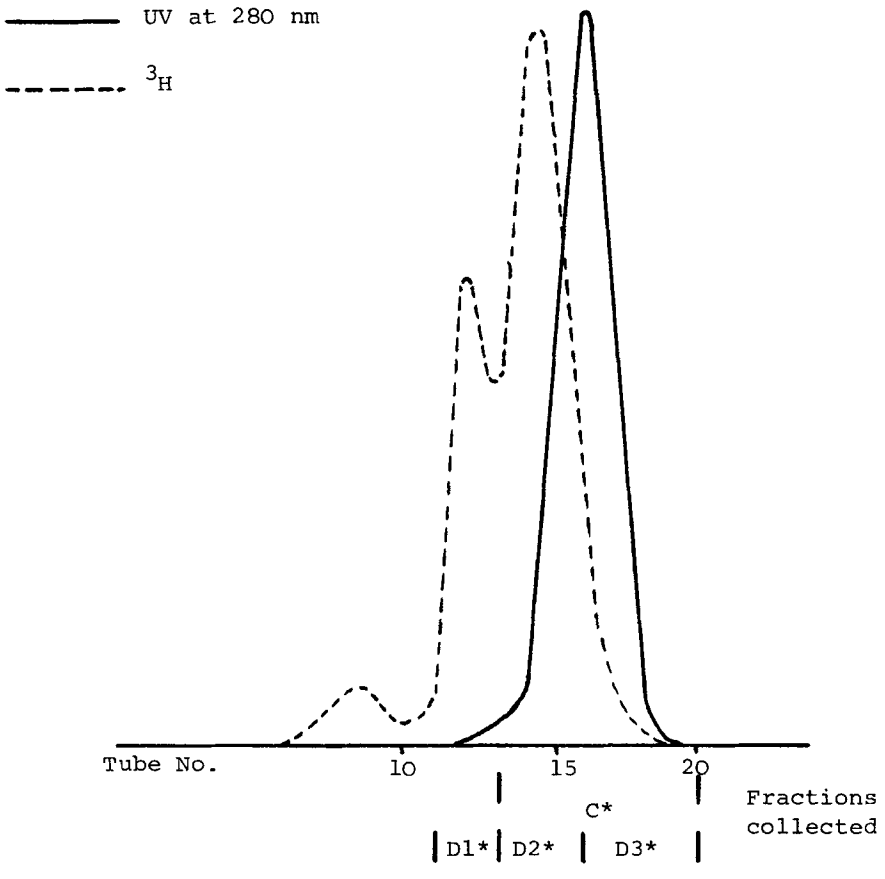

Figure 1.
Ion-exchange chromatography of the exchange-labelled mixtures derived from compounds C and D after cyclisation and deprotection.

additional attraction for these three compounds is that, as they contain only the one aromatic residue, labelling was also exclusive to that residue (Table 2).

Our results demonstrate that small histidine-containing compounds can be efficiently labelled without resort to the greater labour of iodination followed by purification and then catalytic tritiation. The iodination approach has been applied, for instance, to the tripeptide thyrotropin releasing factor[4] (TRF, Glp-His-Pro-NH_2). In that case, the high specific activity, 60 Ci $mmol^{-1}$, (compared to our value of 8.8 Ci $mmol^{-1}$ by exchange), was achieved at the expense of a comparatively elaborate preparative procedure. Workers who are limited by a relatively small isotope handling licence may find our simpler approach useful, especially as pre-equilibration of the catalyst with tritium gas is not used, always provided that the lower specific activity of the product is acceptable. Additionally, exchange labelling is potentially more attractive for use with larger, more complex peptides, as side-reactions associated with iodination[5] (i.e. damage to tryptophan, methionine etc.) are avoided.

Large molecular weight peptides containing histidine (compounds A and B) labelled to approximately 0.5 Ci $mmol^{-1}$. These results are in accordance with that obtained in our previous study with corticotrophin-(1-24)-tetracosapeptide[2] and suggest that the presence of a thioether function in a substrate may not lead to inhibition of exchange labelling. Compounds A and B are sulphur-free and yet did not label any more heavily than the methionine-containing tetracosapeptide.

Compound C contains two sulphur-containing S-trityl cysteine residues and no histidine. The results in Table 2 show an apparently high degree of labelling of alanine. However, it is known that S-trityl cysteine gives alanine slowly on catalytic hydrogenation.[6] The ion-exchange elution profile of the final product from this compound (Figure 1) clearly demonstrates the formation of small amounts of highly-labelled side-products which elute earlier than the main product, somatostatin. A similar degradation of cystine to alanine has been observed in the oxytocin series.[7] In the experiment with compound C, no attempt was made to purify the product of these alanine-containing impurities. Although the amino acid analysis of the product would be accep-

Table 2

The percentage of incorporated tritium associated with amino acid residues after acidic hydrolysis.

Amino Acid	Compound						
	A*	B*	C*	D2*	E*	F*	G
Ala	0	0	76	82	0	0	(NP)
His	94.7	87.5	(NP)	(NP)	100	100	98.5
Phe	3.4	6.5	22	17.5	(NP)	(NP)	(NP)
Trp	(NP)	(NP)	2	0.5	(NP)	(NP)	(NP)
Tyr	1.9	6.0	(NP)	(NP)	(NP)	(NP)	(NP)

(NP) - Amino acid residue not present in compound

table for pure somatostatin (compound C*, Table 1), the presence of tritiated alanine demonstrates its impurity (Table 2). To simplify the analysis, we repeated the exchange-labelling with the corresponding des-alanine somatostatin precursor (compound D). From this experiment it is evident that des-Ala[1] somatostatin (compound D3*) containing no ^{3}H-alanine can be isolated after chromatography as demonstrated by the comparatively low specific activity of the product (90 mCi mmol $^{-1}$) compared to those of the heavily-labelled side-fractions (compounds D1* and D2*).

To conclude this present work, we decided to investigate the position and stability of the histidine label and to characterise the labelled alanine formed from S-trityl cysteine.

^{3}H-NMR analysis of compound F* showed that the tritium was present entirely at the 2-position of the histidine imidazole nucleus. When compared to the report that aromatic amino acids are labelled at the benzylic position by exchange in buffered aqueous solution or in acetic acid,[8] it can be seen that dramatic effects of selectivity in labelling position are possible by changing experimental variables, in this case solvent and/or catalyst. Labelling of the imidazole nucleus of histidine in aqueous buffer has been shown[8] to be time dependent with extended reaction leading to removal of incorporated label by transfer to solvent. We have not investigated whether a similar effect is present under our experimental conditions using non-aqueous solvent. The failure of the tripeptide G to be labelled to a higher degree than the pentapeptide F may either be indicative of that level being the maximum attainable by our simple experimental procedure or be due to such time-course effects. If exchange labelling is time dependent, the time course would be expected to be affected by the molecular weight of the peptide, smaller compounds showing a more rapid time course than larger ones.

We have previously failed to notice any exchangeability of histidine C-2 label in experiments[9] where enzymic digestion was carried out at pH 7.4 at 25^{o} for up to 48 h.[10] Results reported here (Figure 2) show that exchange was overlooked previously[9] as the effect would not have been large under the conditions used. We found that isotope at the imidazole C-2 position in compound F* was completely exchangeable on more extended storage at pH 7.5,

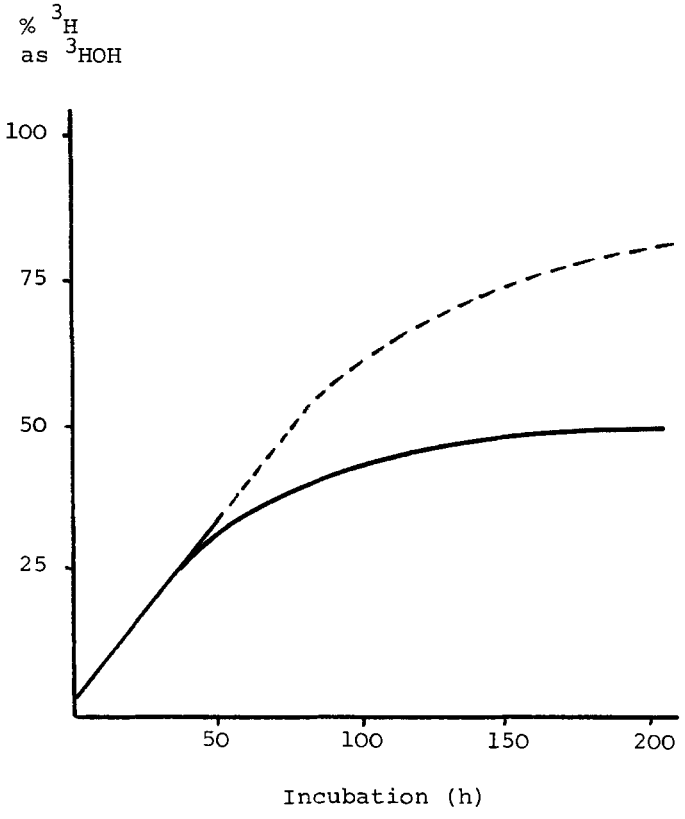

Figure 2.
Release of labile ^{3}H as ^{3}HOH from [2,5-$^3H_2$-His^6]-β-corticotrophin-(1-24)-tetracosapeptide^9 (——) and from ^{3}H-compound F* during incubation at pH 7.4 and 37°C (----).

in agreement with other reports.[11-14] A similar experiment using [2,5-^{3}H-His^{6}]-β-corticotrophin-(1-24)-tetracosapeptide[9] revealed that 50% of the tritium was exchangeable (Figure 2) implying that label at the C-5 position is resistant to aqueous exchange at pH 7.5. This contrasts with a report which implies that the label at the C-5 position is labilised at pH 7.5.[15] The rate of exchange we observed for the C-2 triton is sufficiently slow for the effect to be of no concern in the short-term studies of labelled peptides such as distribution and metabolism *in vivo*, where times of several hours are not exceeded. Nevertheless our results indicate that labelling with tritium by reductive dehalogenation at the C-5 position would give products of absolute stability to exchange at physiological pH.

The alanine produced from *S*-trityl cysteine in compounds C and D was shown to be largely unracemised (Table 3). No values are given as controls for racemisation due to acidic hydrolysis in Table 3 because meaningful results would only come from the hydrolysis of synthetic compounds bearing alanine in place of *S*-trityl cysteine in compounds C and D. The labour involved in synthesising and degrading these compounds does not seem worthwhile when the values for racemisation observed are of the same order as those seen for other amino acids and where racemisation in the labelling step is not suspected (Table 3). By racemisation of the isolated alanine and inverse isotope dilution analysis we showed that 50% of the tritium was located at each of the carbon atoms of the side chain. This result allows only one mechanism of formation. *S*-Trityl cysteine undergoes β-elimination on the catalyst surface to give dehydroalanine. This remains tightly bound and is reduced there to give stereochemically-pure [α,β-$^{3}H_{2}$]-L-alanine with complete retention of configuration at the asymmetric centre. The resultant amino acid was labelled to the extent of 4-5 Ci mmol $^{-1}$. It is possible that highly-labelled alanine-containing peptides, which are not otherwise easily accessible, could be synthesised in favourable cases by reduction of starting materials containing *S*-trityl cysteine in place of alanine.

We tested the possibility that labelled water arising from traces of moisture present rather than tritium gas might be the intermediate responsible for exchange labelling by using a constructed mixture of tritiated water in the DMF solvent and

Table 3

Racemisation of amino acids recovered after acidic hydrolysis of labelled (^{3}H) and unlabelled (^{1}H) compounds.

Compound		Residue			
		Ala	His	Phe	Tyr
A*	^{3}H	(NL)	7.2 ± 0.5	5.4 ± 0.4	5.0 ± 1.2
	^{1}H	-	6.9 ± 0.7	5.0 ± 0.5	4.4 ± 1.5
B*	^{3}H	(NL)	7.8 ± 0.3	4.3 ± 0.7	3.4 ± 0.9
	^{1}H	-	4.9 ± 0.6	5.8 ± 0.6	3.4 ± 1.8
C*	^{3}H	5.5 ± 0.3	(NP)	4.6 ± 1.1	(NP)
	^{1}H	(ND)	-	3.0 ± 0.5	-
D2*	^{3}H	3.5 ± 0.1	(NP)	(ND)	(NP)
	^{1}H	(NP)	-	(ND)	-
E*	^{3}H	(NL)	8.0 ± 0.4	(NP)	(NP)
	^{1}H	-	6.6 ± 0.1	-	-
F*	^{3}H	(NL)	6.4 ± 0.1	(NP)	(NP)
	^{1}H	-	6.5 ± 0.1	-	-
G	^{3}H	(NP)	6.2 ± 0.2	(NP)	(NP)
	^{1}H	-	4.5 ± 0.2	-	-

(ND) = not determined
(NL) = Amino acid present in compound but not labelled by exchange
(NP) = Amino acid not present in compound

protium gas for the exchange labelling of compound F. The achieved specific activity (0.13 Ci mmol $^{-1}$) compared to that theoretically possible (9.1 Ci mmol $^{-1}$) if rapid ^{3}H-^{1}H exchange had occurred and labelling had proceeded to the same degree as in the experiment using tritium gas allows us to conclude that water does not play a significant role in catalytic gas exchange labelling which is in agreement with the findings of other workers.[8]

The foregoing along with previous results[2] establishes that acid-stable tritium can be introduced without racemisation (Table 3) into histidine, phenylalanine, tryptophan and tyrosine provided this residue has the ring hydroxyl function blocked. When sulphur-containing residues are present, reduction gives small amounts of highly-labelled alanine and amino-butyric acid from cysteine derivatives and methionine respectively. Whether these impurities are removable will be a matter for investigation in each individual case studied. No other amino acid residues, that is those with aliphatic side chains, were labelled by our procedure. Small peptides containing histidine can be heavily labelled. Large histidine-containing peptides and peptides devoid of histidine but containing other aromatic residues are less heavily labelled. An investigation into factors we have not examined, such as the temperature of reduction, the duration of reduction, the nature of catalyst and the nature of solvent which might lead to more efficient labelling could be rewarding. For instance, we have observed[16] that isotope can be incorporated into histidine in a protected octadecapeptide to a level of 11 Ci mmol^{-1} using more forcing conditions (24 h, 50^{o}) than those used in this present study.

EXPERIMENTAL

Materials

Compound A[17] - H-Thr(Bu^t)-Tyr(Bu^t)-Thr(Bu^t)-Gln-Asp(OBu^t)-Phe-Asn-Lys(Boc)-Phe-His-Thr(Bu^t)-Phe-Pro-Gln-Thr(Bu^t)-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2: protected (11-32) human calcitonin.

Compound B[17] - H-Thr(Bu^t)-Tyr-Thr(Bu^t)-Gln-Asp(OBu^t)-Phe-Asn-Lys(Boc)-Phe-His-Thr(Bu^t)-Phe-Pro-Gln-Thr(Bu^t)-Ala-Ile-Gly-Val-Gly-Ala-Pro-NH_2: partially protected (11-32) human calcitonin.

Compound C - Boc-Ala-Gly-Cys(Trt)-Lys(Boc)-Asn-Phe-Phe-Trp-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(Trt)-OBu^t: protected somatostatin precursor.

Compound D[6] Boc-Gly-Cys(Trt)-Lys(Boc)-Asn-Phe-Phe-Trp-Lys(Boc)-Thr(Bu^t)-Phe-Thr(Bu^t)-Ser(Bu^t)-Cys(Trt)-OBu^t: protected (2-14) somatostatin precursor.

Compound E - Z-His-Leu-Gly-Leu-Ala-Arg-OBzl: protected partial sequence of complement third component.

Compound F - Boc-His-Leu-Gly-Leu-Ala-OBu^t: protected partial sequence of complement third component.

Compound G - Glp-His-Pro-NH_2: thyrotropin releasing factor, TRF (from Cambridge Research Biochemicals).

Compound C was synthesised from described intermediates as detailed for compound D[6]. After recrystallisation from methanol: dimethylformamide C had mp 231^o (dec.). Found, %: C 65.78; H 7.29; N 9.43; S 2.49. $C_{145}H_{190}N_{18}O_{25}S_2$ required C 65.73; H 7.22; N 9.51; S 2.42.

Compounds E and F were synthesised by Dr. R. Andreatta, CIBA-GEIGY Ltd., Basle, Switzerland.

The ^{3}H-FTNMR spectrum was recorded by Dr. J.R. Jones of the University of Surrey.

Free peptides derived from compounds A to F are denoted A* to F*.

Catalytic exchange labelling - Compounds were labelled using $^3H_2$ gas in the presence of mixed catalysts (Pd/C and Rh/$CaCO_3$) in DMF for 45 min at room temperature as described previously.[2]

Compounds A and B were deprotected and the products were purified by ion-exchange chromatography followed by reverse-phase high-pressure liquid chromatography using the exact conditions described for the purification of [^{3}H]-calcitonins.[17]

Compounds C and D were cyclised, deprotected and chromatographed on carboxymethylcellulose as described for the synthesis of [^{3}H]-somatostatins.[6] Fractions collected from the column were examined at 280 nm (for tryptophan-containing material) and for ^{3}H. Representative elution profiles for C* and D* are shown in Figure 1. In the case of compound C, fractions corresponding to the product as shown by the UV trace were worked up. In the case of compound D fractions were combined as indicated and recovered materials were purified further by h.p.l.c.[6] as appropriate.

Compound E was deprotected during the reduction employed for exchange labelling. Compound F required acidolytic deprotection (90% trifluoroacetic acid, 30 min, 20°). Both compounds were then purified by semipreparative h.p.l.c. as described previously[17] but using a constant volume gradient of MeOH:H_2O:H_3PO_4 (from 100:900:1 to 500:500:1).

Compound G is a natural product but can nevertheless be regarded as a protected peptide since it possesses no amino or carboxyl groups. [^{3}H]-TRF was purified by semi-preparative h.p.l.c. on a column of Nucleosil 10C_{18} by elution with MeOH:H_2O:H_3PO_4 (20:980:1) and was worked up as described previously.[6]

The analyses after acidic hydrolysis of the recovered exchange-labelled compounds A* to G are given in Table 1. The compounds were also characterised satisfactorily by enzymic hydrolysis as reported previously[6,17] and these results are not presented here. Fraction 2 from the ion-exchange chromatography of compound D* (Figure 1) is obviously a mixed fraction.

Analysis of the distribution of tritium - This was done as described previously.[2] The quantities of tritium associated with amino acids after acidic hydrolysis are given in Table 2.

Analysis of the optical purity of the tritiated amino acids - Labelled amino acids were isolated from acidic hydrolysates of the compounds A* to G derivatised with either leucine- or glutamic acid-NCA[18] and the diastereomeric mixtures were analysed as previously detailed.[2] Racemisation of amino acids in the compounds that was due to acidic hydrolysis alone was estimated

using hydrolysates of unlabelled compounds.[2] Results are given in Table 3.

Position of exchange label in histidine - A sample (38.8 mCi) of compound F* was evaporated to dryness three times from D_2O (0.5 ml) and the residue was dissolved in D_2O (75 µl). The solution was sealed in a cylindrical microcell which was inserted into a standard (5 mm) NMR tube which was capped. The triton spectrum (with ^{1}H decoupling) was recorded at 96 MHz and 25° using a Bruker WH 90 pulse spectrometer.[19]

Stability of exchange label in histidine - A solution (2.0 ml) of compound F* (7.8 mCi) in 0.05 M Tris buffer pH 7.4 containing 250 I.U. penicillin G and 250 µg streptomycin was divided into portions (0.2 ml) which were sealed and kept at 37°. Samples (20 µl) were chromatographed at intervals on a column (30 x 0.46 cm) of Nucleosil $10C_{18}$ which was eluted at a flow rate of 2.4 ml min^{-1} with MeCN:H_2O:H_3PO_4 (100:900:1, by vol.). Fractions (10 x 0.6 min) were collected automatically and samples (10 µl) were assayed for ^{3}H. Under these conditions, labilised ^{3}H eluted as ^{3}HOH at the column void volume. The results are shown in Figure 2. The preparation contained 2.2 ± 0.3% ^{3}HOH at zero time. This had accumulated on storage at -196° during one month.

For the purposes of comparison, a sample of [2,5-$^3H_2$-His^6]-β-corticotrophin-(1-24)-tetracosapeptide[9] was similarly investigated. Elution was with a constant volume (20 ml) gradient of MeCN:H_2O:H_3PO_4 (from 100:900:1 to 250:750:1 by vol.). The results are shown in Figure 2. The preparation contained 3.5 ± 0.3% ^{3}HOH at zero time. This had accumulated on storage at -196° during six months.

Stereochemical and substitution pattern analysis of labelled alanine formed from compounds C and D - L-Alanine (2 mg) was added as carrier to the acidic hydrolysates of fractions C* and D2* and alanine was isolated by descending preparative paper chromatography[2] (16 h) using the solvent system butan-2-ol:butan-2-one:dicyclohexylamine:water (10:10:2:5, by vol.). After derivatisation of portions (10 µCi) of the materials with L-leucine NCA, the mixtures were separated using the amino acid analyser and diastereomers were quantified in the eluates by counting for ^{3}H. D-Alanine was present as 5.5 ± 0.3% and 3.5 ± 0.1% of total ^{3}H-alanine in C* and D2* respectively.

(i) Acetylation without racemisation - Alanine (~3 μCi) from compound C* was dispensed into water (100 μl). N-NaOH (0.12 ml) was added, followed by Ac_2O (5 μl). After 30 min at 20° N-H_2SO_4 (0.12 ml) and acetyl-L-alanine (244.3 mg) were added and the mixture was evaporated to dryness. The residue was extracted with hot ethyl acetate, Na_2SO_4 was removed (filtration) and the recovered product was crystallised to constant specific activity.

(ii) Acetylation with racemisation - To an identical alanine sample was added N-NaOH (0.05 ml) and Ac_2O (20 μl in two portions 1 min apart). The mixture was worked up as sample I but with addition of acetyl-DL-alanine (218.5 mg) and the product was crystallised to constant specific activity.

Calculation showed that the acetyl-DL-alanine retained 50.7 ± 4.3% of the isotope present in the acetyl-L-alanine.

Possible involvement of water as a labelling intermediate in exchange reactions - Compound F was catalytically hydrogenated as described but using $^1H_2$ (4 ml) and ^3H_2O (5 Ci, 98% isotopic abundance) in DMF (0.5 ml) as solvent. After work-up, the recovered compound F* (35% yield) had a specific activity of 130 mCi $mmol^{-1}$.

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