Tritiated Peptides. Part 10[1]. Catalytic Exchange Labelling of β-Corticotrophin-(1-24)-tetracosapeptide.

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β-Corticotrophin-(1-24)-tetracosapeptide‡ was labelled to a level of 0.48 Ci mmol^{-1} by catalytic exchange of a protected synthetic precursor with tritium gas. Over 80% of the isotope was incorporated into acid-stable sites in the histidine residue. Smaller amounts were incorporated into methionine (5%), tyrosine (3%), phenylalanine (5%) and tryptophan (2%). Aminobutyric acid was formed (1%) by degradation of methionine and had incorporated tritium (5%). The labelled amino acid residues were stereochemically pure within the limits of experimental error.

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

‡ β-Corticotrophin-(1-24)-tetracosapeptide: tetracosactrin, Synacthen® (trade name of CIBA-GEIGY Basle), Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Gly-Lys-Lys-Arg-Arg-Pro-Val-Lys-Val-Tyr-Pro.

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In our studies on the preparation of tritiated peptides, which we have obtained by catalytic dehalogenation with tritium gas (Ref. 1 and preceding papers in the series), we consistently observed small amounts (0.35-9.4%) of tritium incorporated into histidine in addition to that incorporated into the dehalogenated amino acid residue. Schwyzer and Karlaganis[2] observed secondary labelling of all amino acid residues but predominantly of histidine when preparing an analogue of corticotrophin containing norvaline at position 4 of a (1-24)-corticotrophin by catalytic hydrogenation of the corresponding allylglycine precursor. We decided to examine in detail this secondary labelling by exchange in the hope that we might develop a simple method for the incorporation of tritium into stable sites in synthetic peptides without the need to synthesise halogenated analogues. The existing evidence[2,3] suggested that histidine residues might be labelled to the extent of 1 Ci $mmol^{-1}$. However, for the method to be useful, it was essential to establish whether labelling was accompanied by racemisation and whether other amino acid residues were similarly labelled.

No report exists of the exchange labelling of protected peptides by stirring material in solution in an aprotic solvent in the presence of precious metal catalysts in an atmosphere of tritium gas, although water-soluble aromatic amino acids and the dipeptide L-phenylalanylglycine have been labelled[4] at the benzylic position without racemisation by catalytic exchange in aqueous solution at neutral or alkaline pH. Procedures used for peptides and similar materials which are related are the Wilzbach technique[5], in which solid is stored in contact with tritium gas for several days, and catalytic exchange with tritiated (protic) solvents. Wilzbach labelling of peptides is known to be accompanied by generalised decomposition of which racemisation of amino acid residues is but one aspect[6]. With such complex materials as polypeptides, determination of purity is difficult and it is probably fair to say that, to date, no Wilzbach-labelled polypeptide has been convincingly demonstrated to be pure by a sufficient range of analytical techniques.

Several publications exist of the exchange-labelling of aromatic materials (including some containing amino acids) by catalysis using tritiated solvent or tritium gas. It would be extremely difficult to try to draw any general conclusions from the literature because of the large number of variables which have to be considered. These include the nature of the catalyst, its

surface characteristics (which are generally unknown), the presence or absence of absorbents, the nature and purity of solvents and their pH, the temperature and time of reaction, the pressure of overlying gas (when appropriate) and the nature and reactivity of the substrate. However, some publications bear on our present investigation.

It is possible that the implied distinction made between exchange labelling in the presence of tritium gas or tritiated solvent is invalid. A study by Cerny and Hanus[7] showed that non-protic solvents (such as dioxan and ethyl acetate) accumulated isotope due to the presence of protic (hydroxylic) impurities such as ethanol and water. Marton and Kovacs[8] extended this observation and postulated a direct hydrogen transfer reaction at the catalyst surface between the tritiated solvent and the substrate being labelled. These studies may not have any bearing on our investigation because it was shown[7] that the presence of the substrate to be hydrogenated promoted the exchange of isotope between the solvent and the gas and in that instance the substrate was unsaturated. Our substrate is saturated.

A publication describing the labelling of folic acid by two different techniques[9] shows results not consistent with exchange from gas through water in the system. The first method labelled folic acid to a level of 1 Ci $mmol^{-1}$ using platinum and tritiated water containing potassium bicarbonate. The fact that 500 Ci of water was needed to achieve a high specific activity product demonstrates clearly both the inefficiency of the exchange and that this technique could not be recommended for general use. Apart from the restraints of licence which apply to laboratories outside the radioisotope industry, it should be noted that it has been stated that the suspected human LD_{50} of tritiated water is approx. 70 Ci[10]. The second method described the preparation of folic acid labelled to a level of 30 Ci $mmol^{-1}$ by catalytic exchange with tritium gas using palladium on calcium carbonate in potassium hydroxide solution. Only 25 Ci tritium gas, which is relatively non-toxic, was used and this procedure is obviously more attractive for consideration as the basis of a general method. It is obvious that the substrate is labelled to a much higher level than is consistent with gas equilibration with water. However, no comparative information can be deduced as, in the two experiments, different catalysts were used and the pH of the reaction solutions were different.

The results of a recent study[11] in which a range of (mostly) aromatic compounds in methanol or water solution were labelled using tritium gas in the presence of palladium oxide or platinum oxide can be explained by exchange via tritiated water formed during the reaction and need not, as assumed, be due to direct exchange with tritium gas. The authors consistently found that palladium was the more efficient catalyst which is at variance with a previous report[12] that platinum was the most useful catalyst for heterogeneous exchange using tritiated or deuteriated water.

It is our view that such apparent inconsistencies in the literature mostly reflect the great number of experimental variables over some of which the experimenters have little control. For our study, we considered it most useful to employ conditions which are reasonably practical. For instance, the DMF used was purified by azeotropic distillation and was stored over a molecular sieve, but no attempt was made to remove traces of moisture from the reaction vessel, the catalyst or the substrate.

At ambient temperatures and pressures, aromatic amino acids would be expected to label by exchange much more readily than aliphatic amino acids. The mechanism has been suggested[13] to be transfer from tritium gas via traces of water to the aromatic substrate by π-complex substitution involving a low-energy chemisorbed transition state of the aromatic system on the catalyst surface, which obviously is inoperable for aliphatic systems. We observed that all the aromatic residues in the compound under study (i.e. His, Phe, Trp and Tyr) were labelled by exchange and that methionine was the only non-aromatic amino acid residue labelled (Table 1). A trace of highly-labelled aminobutyric acid was present. This may have been formed from methionine by β-elimination of methane thiol and reduction of the generated double bond. The observed labelling of methionine suggests involvement of the sulphur atom in the exchange process. Determination of the distribution of label might indicate whether the two processes are linked, assuming they are initiated by proton abstraction.

The results given in Table 1 were obtained by counting the radioactivity eluted from the column of the amino acid analyser. We found it preferable to collect samples without addition of the colour reagents to simplify quantitation. After addition of reagent and development of the colour, the colour quenched the counting efficiency in an inverse but not proportional relationship. The results show that radioactivity is incorporated into sites of histidine, phenylalanine and tryptophan that are stable to hot

concentrated acid hydrolysis. A significant proportion of the combined radioactivity in methionine and tyrosine is exchangeable by hot acid. In the case of tyrosine this presumably represents the hydrogen atoms ortho to the hydroxyl group[14]. We have previously shown that label in the 2- and 5-positions in the histidine imidazole nucleus is stable at pH values up to 7.4 (at which enzymic digestion prior to analysis is carried out). This appears not to agree with the report[15] that, at pD 6.9, imidazole has a half-time of exchange of 109 minutes at 65° for the hydrogen atom at C-2 and the report[16] that this hydrogen atom in histidine and acetyl-histidine is exchangeable above pH 5. It may not be valid, however, to compare these data. A more detailed investigation of the exchange behaviour of the imidazole C-2 hydrogen atom in histidine with relationship to pH would seem to be warranted.

To determine the stereochemical purity of the labelled residues, it was merely necessary to separate the labelled residues that, on reaction with either leucine- or glutamic acid-N-carboxy-anhydride[17] (NCA) gave stereoisomeric mixtures which overlapped with one another on the amino acid analyser. In this instance, only aminobutyric acid and methionine needed to be absolutely separated from each other. Preparative paper chromatography in solvent system A gave sufficiently radiochemically pure samples of histidine, tyrosine and phenylalanine and a mixed fraction (d) of aminobutyric acid and methionine (Table 2). The fraction d afforded radiochemically pure preparations of methionine and aminobutyric acid on preparative paper chromatography in solvent system B (Table 3). Fraction c was not used for this purpose due to its contamination with tyrosine which chromatographs as a streak in solvent system B. Analysis of the stereochemical purities was by counting after derivatisation of the amino acids with an N-carboxy-anhydride and separation of the labelled diastereomers. This was achieved either by use of the amino acid analyser[17] but without addition of colour reagent as detailed earlier or by high pressure liquid chromatography (h.p.l.c.). A sample of authentic, unlabelled β-corticotrophin-(1-24)-tetracosapeptide was acid hydrolysed and the relevant amino acids were purified as described for the labelled acids. Estimation of their stereochemical purities gave the degree of racemisation which was due to acidic hydrolysis alone. The values (Table 4) show that it is likely that within the limits of experimental error none of the labelled residues is racemised. The presence of a small amount (1%) of aminobutyric acid (Abu) derived from methionine is more serious than this amount suggests

because the impurity is so highly labelled, having possibly involved the saturation of a double bond, that it accounts for 5% of the radioactive peptide. Provided that this Abu-containing impurity could be removed, the labelled peptide would appear to be chemically and stereochemically pure.

In this instance, exchange labelling gave a histidine residue of a specific activity of approximately 350 mCi $mmol^{-1}$ and the other aromatic residues, phenylalanine, tryptophan and tyrosine together with methionine were labelled to around 10-25 mCi $mmole^{-1}$. No other residues were labelled. We would have been able, under our experimental conditions, to detect labelling of a residue at a level of approximately 100 µCi $mmole^{-1}$. This is below the level at which Schwyzer and Karlaganis[2] claimed to have found labelling of every amino acid residue present in a corticotrophin tetracosapeptide. It is uncertain that their observation could be due to the extended time of reduction (24 h).

Our investigation suggests that the procedure described should be capable of development into a labelling technique for moderately complex synthetic polypeptides, provided that strict analytical criteria are adhered to.

EXPERIMENTAL

Catalytic exchange labelling - Protected β-corticotrophin-(1-24)-tetracosapeptide[18] (12.6 mg) in DMF (0.67 ml) was stirred with 10% palladium on charcoal (10.8 mg) and 5% rhodium on calcium carbonate (9.0 mg) in the presence of tritium gas (98%, 3.2 ml, 8 Ci) for 35 minutes at room temperature. Catalysts were removed by filtration through a pad of cellulose (Macherey-Nagel MN-300) and the eluate was evaporated to dryness. The residue was kept with 90% trifluoroacetic acid (5 ml) for 30 minutes at room temperature and the solution was evaporated to dryness. The residue was dissolved in water (1 ml) and passed through a column (1 ml) of Dowex 1 (acetate form) resin. The eluate was evaporated to dryness and the residue dissolved in water (1 ml). The solution was applied to a column (4 x 0.7 cm) of carboxymethylcellulose (trimethylammonium form) which was then eluted with a linear gradient (40 ml, 0-1M) of trimethylammonium acetate solution pH 5.5. Fractions (1 ml) were collected automatically and analysed for radioactivity. Fractions 22-29 were combined and repeatedly evaporated to dryness below 30° with addition of water until all the buffer had been removed. The residue (1.86 µmoles, 54%, 480 mCi $mmole^{-1}$) was dissolved in water (1.0 ml) and stored in liquid nitrogen. The material gave the

following amino acid analysis after acidic hydrolysis: Abu, 0.01; Arg, 3.03; Glu, 1.00; Gly, 2.03; His, 0.99; Lys, 3.99; Met, 0.96; Phe, 1.00; Pro, 2.90; Ser, 1.76; Trp, 0.56; Tyr, 2.00; Val, 3.01 and after hydrolysis with a mixture of carrier-bound enzymes[19]: Abu, 0.01; Arg, 2.92; Glu, 0.99; Gly, 1.95; His, 0.96; Lys, 3.89; Met, 0.91; Met(O), 0.05; Phe, 0.99; Pro, 2.53; Ser, 1.94; Trp, 0.96; Tyr, 1.89; Val, 2.61.

Analysis of distribution of tritium - Samples ($\sim$5 nmoles) of the acidic (1.24 µmoles) and enzymic (0.48 µmoles) hydrolysates of the product described above were separately chromatographed in the normal manner on the Beckman 120C amino acid analyser and the eluates were collected automatically (0.5 minute fractions) from the end of the column without addition of ninhydrin and passage through the colorimeter. After suitable dilution, the fractions were assayed for radioactivity. The quantities of tritium associated with amino acids are given in Table 1.

Table 1

The specific activity (mCi $mmol^{-1}$) of the amino acid residues measured after acidic or enzymic hydrolysis.

Amino Acid	Acid	Enzyme
Abu	3100	3400
His	364	359
Met	10	26
Phe	27	26
Trp	14	14
Tyr	5	7.5

Analysis of the optical purity of the tritiated amino acids - The acidic hydrolysate was dried, dissolved in water (0.15 ml) and applied as a band (15 cm) to Whatman No. 1 chromatography paper and developed (descending elution) in the solvent system (A) butan-1-ol:acetone:diethylamine:water (10:10:2:5, by vol.) for 12 hours. Marker strips were visualised using ninhydrin and radioactive fractions were recovered from paper strips by elution with water. The fractions were examined for the tritiated amino acids using the

Beckman analyser. The results are given in Table 2.

Table 2

Composition of the amino acid fractions recovered after preparative paper chromatography in solvent A.

	Content (in µmoles)					
Fraction	His	Tyr	Abu	Met	Trp	Phe
a	0.55	0.002	-	-	-	-
b	-	0.205	-	-	-	-
c	-	0.48	0.005	0.20	-	-
d	-	-	0.001	0.44	0.40	0.06
e	-	-	-	-	0.02	0.72

The fraction d was similarly separated into its components by preparative paper chromatography using the solvent system (B) t-amyl alcohol:pyridine:water (5:1:1, by vol.). The paper was eluted for 136 hours (descending). The results are given in Table 3.

Table 3

Composition of the amino acid fractions recovered after preparative paper chromatography in solvent B.

	Content (in µmoles)		
Fraction	Abu	Met	Trp
f	.001	-	0.18
g	-	0.24	0.005

Samples of each recovered fraction (1-2 μCi) were reacted with either leucine- or glutamic acid NCA[17] and the mixtures of diastereomers were separated using the amino acid analyser. Fractions were collected as described previously without addition of ninhydrin and were assayed for radioactivity. Alternatively, the diastereomers were separated by h.p.l.c. Detection was at 210 nm. Samples were chromatographed on a column (25 x 0.46 cm) of Nucleosil $10C_{18}$ using aqueous acetonitrile, 0.1N in triethylammonium phosphate as eluant. The solvent compositions used were: for Leu-Tyr, 12.5%; Glu-Phe, 10%; Leu-Met, 10% acetonitrile. The stereochemical purity of each tritiated amino acid is given in Table 4.

Table 4

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

Residue	Racemisation (%)	
	^3H	^1H
His	6.1 ± 0.3	4.0 ± 0.5
Tyr	4.7 ± 3.7	5.5 ± 2.0
Phe	3.2 ± 0.8	3.2 ± 0.1
Met	5.0 ± 1.7	7.6 ± 0.6
Trp	0 ± 2.4	-
Abu*	3.1 ± 0.4	-

* not present initially

Racemisation due to acidic hydrolysis - A sample (2.4 mg) of β-corticotrophin-(1-24)-tetracosapeptide was hydrolysed (16 hours, 110°, 6.7N-HCl) and the same amino acids isolated by preparative paper chromatography as previously described. The recovered amino acids were reacted with leucine- or glutamic acid-NCA and the compositions of the diastereomeric mixtures were determined either using the amino acid analyser or by h.p.l.c. The contents of D-amino acids are given in Table 4.

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