TNO

CENTRAAL LABORATORIUM
COMMUNICATION NO. 345

WILZBACH TRITIATION OF AMINO ACIDS AND ACTH

by

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Thesis, Leyden, 1968

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STELLINGEN

1. De conclusie van von Holt, Voelker en von Holt, dat het gerechtvaardigd schijnt volgens Wilzbach gemerkte insuline te gebruiken in biologische proeven, wordt niet gesteund door de resultaten van de hydrolyse van het gemerkte insuline.

   C. von Holt, I. Voelker, L. von Holt
   Biochim. Biophys. Acta 38 (1960) 88

2. Huang Wei-yuan, Hsu Jin-wen en Sun Shie-cherng hebben niet bewezen, dat bij hun poging tot synthese van 24-iso-β-sitosterol (γ-sitosterol) voornamelijk β-sitosterol ontstaan zou zijn.

   Huang Wei-yuan, Hsu Jin-wen, Sun Shie-cherng
   Acta Chimica Sinica 32 (1966) 57

3. Als in de formule van Debije voor de diëlectrische constante van vloeistofmengsels de uitdrukking $\frac{\varepsilon - 1}{\varepsilon + 2}$ wordt vervangen door $\varepsilon^{1/3} - 1$, wordt een formule verkregen die een betere overeenstemming geeft met de experimentele resultaten en eenvoudiger is toe te passen.

   H. Looyenga, Molecular Physics 9 (1965) 501
   E. Bock, E.F. Dojack, Can. J. Chem. 45 (1967) 1097
   F.I. Mopsick, Journal of Research of the N.B.S. 71 A (1967) 287

4. Pearson beweert ten onrechte, dat de veranderde vloeistofsamenstelling vlak bij het oppervlak van een gesuspendeerd deeltje in een dichtheidsgradiëntkolom het oppervlakte-energie-effect van Kawai en Keller te niet zou doen.

   J.R.A. Pearson, Polymer 9 (1968) 283
   T. Kawai, A. Keller, Phil. Magazine 8 (1963) 1973

5. De methoden, die gebruikt zijn om het pH-optimum van lipoxidase te bepalen, leiden tot aanvechtbare resultaten.

   G.R. Anes, T.A. King
   J. Sci. Fd. Agric. 17 (1966) 301

6. De methode van Belfield en Goldberg voor de bepaling van 5'-nucleotidase in serum is een verbetering ten opzichte van de gebruikelijke bepalingen en kan een belangrijk hulpmiddel zijn bij het aantonen van primaire of metastatische levertumoren.

   A. Belfield, D.M. Goldberg, Nature 219 (1968) 74
   K. Smith, H.H. Varon, G.J. Race, D.L. Paulson, H.C. Urschel, J.T. Mallams, Cancer 19 (1966) 1281

7. De wettelijke normen voor het lozen van radioisotopen in afvalwater missen in ten minste enkele gevallen een redelijke basis.

8. De duinen van Voorne-Putten zijn, nationaal zowel als internationaal gezien, zo belangrijk als natuurmonument, dat aantasting ervan door verdere uitbreiding van de Maasvlakte onaanvaardbaar is.

WILZBACH TRITIATION OF AMINO ACIDS AND ACTH

PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WISKUNDE EN NATUURWETENSCHAPPEN AAN DE RIJKS-UNIVERSITEIT TE LEIDEN, OP GEZAG VAN DE RECTOR MAGNIFICUS DR L. KUKENHEIM EZN, HOOGLERAAR IN DE FACULTEIT DER LETTEREN, TEN OVERSTAAN VAN EEN COMMISSIE UIT DE SENAAT TE VERDEDIGEN OP WOENSDAG 11 DECEMBER 1968 KLOKKE 16.15 UUR.

DOOR

JOHANNES HENDRIKUS PARMENTIER

geboren te Amersfoort in 1932

Promotor: Prof. Dr. E. Havinga.

Aan mijn Vrouw
Aan Elsbeth, Mieke
Jan-Pieter en Bart

C O N T E N T S

CHAPTER 1

INTRODUCTION

1.1. Wilzbach's method

In 1956, Wilzbach introduced a radiation-induced exchange method for the labelling of organic substances with tritium[1)2)]. An organic compound (gas, liquid or solid) is allowed to remain in contact with Curie quantities of pure tritium gas during a period of several days to several weeks. Under the influence of its beta-radiation, part of the hydrogen atoms are substituted by tritium atoms:

$$RH + T_2 \xrightarrow[\text{of } T_2]{\beta\text{-radiation}} RT + \text{other products}$$

Usually less than 1 % of the tritium gas present is incorporated per day. The specific activities obtained are in the range of 0.1 - 10 mCi/mmole.

The major problem in applying this method is that of purifying the labelled product. Due to such reactions as isomerization, hydrogenation and racemization, radioactive by-products may evolve in amounts exceeding 10-100 times the radioactivity found in the product desired. These by-products are then often found in chemically unimportant quantities and their specific activities may be extremely high. Other by-products are due to fragmentation and polymerization, but these can usually be removed easily. Several excellent reviews[3-6)] summarize results obtained upon application of the Wilzbach method.

1.2. Mechanism of the Wilzbach labelling process

Many investigations have been reported that focus attention on the mechanism of the Wilzbach labelling of gases (methane[7)], ethane[8)], ethylene[9)], cyclopropane[10)], C-6 hydrocarbons[11)], toluene[12)]. In this respect, the following general remarks have been made:

(a) For many of these gas reactions, it has been found that the number of T-atoms incorporated in organic molecules is equal to the number of excited ions generated by this nuclear reaction:

$$T_2 \rightarrow HeT^+ + e^-$$

The process based on it, is called decay-induced labelling. That decay-induced labelling is involved can be concluded from the G_T-value, which gives the number of T-atoms, incorporated into organic molecules per 100 eV of energy absorbed by these molecules. Per desintegrating T_2-molecule, a mean energy of 5500 eV is absorbed by the system. If the T-atom of each HeT^+-ion is incorporated into an organic molecule, a G_T-value of 100/5500 =

0.018 will be found. For methane, for instance, the following sequence of decay-induced reactions is proposed[7]:

$$1.\ HeT^+ + CH_4 \longrightarrow CH_4T^{+*} + He \quad (-89 < \Delta H_r < -67)$$
$$CH_4T^{+*} \longrightarrow CH_2T^+ + H_2 \quad (29 < \Delta H_r < 44)$$

$$2.\ CH_2T^+ + CH_4 \longrightarrow C_2H_4T^+ + H_2 \quad (\Delta H_r = -20)$$
$$C_2H_4T^+ + e^- \longrightarrow CH_2T^{\cdot *} + CH_2^* \quad (\Delta H_r = -134)$$
$$CH_2T^{\cdot *} + CH_4 \longrightarrow CH_3T + CH_3 \quad (\Delta H_r \sim 0)$$

or
$$3.\ CH_2T^+ + CH_4 \longrightarrow CH_3^+ + CH_3T \quad (\Delta H_r \sim 0)$$

Similar reactions are proposed for toluene.

The decay-induced labelling is independent of temperature and unimportant (low G_T-value) as a mechanism for tritiation of liquids and solids at room temperature[13].

At low temperatures (77 °K), decay-induced labelling seems to be the most important mechanism[14].

(b) Reactions between ionized or excited T_2-molecules or atoms with the organic compound are also possible:

$$T_2 \xrightarrow{\geq 4.6\ eV} 2\ T^{\cdot} \quad (\text{or } 2\ T^{\cdot *})$$
$$T^{\cdot} \xrightarrow{\geq 13.5\ eV} T^+ \quad (\text{or } T^{+*}) + e^-$$
$$T^+ + e^- \longrightarrow T^{\cdot *}$$

($T^{\cdot *}$ and T^{+*} are respectively a tritium radical and a tritium ion with an excess translation energy.)

These tritium atoms or ions may cause reactions like:

$$T^{\cdot *} + RH \longrightarrow RT + H^{\cdot *}$$

(c) Exchange with T_2 of excited or ionized molecules, formed by interaction of these molecules with beta-particles, seems to be an important mechanism. Schematically:

$$RH \rightsquigarrow RH^+ + e^-$$
$$RH \rightsquigarrow RH^*$$
$$RH^+ + T_2 \longrightarrow RT^+ + HT$$
$$RH^* + T_2 \longrightarrow RT + HT$$

Riesz and Wilzbach[11] consider the reactions mentioned under (c) as mainly responsible for the nature and the distribution of the carbon skeletons of labelled and unlabelled products. The correspondence between the products of gamma-ray radiolysis of benzene and n-hexane and the products obtained in the Wilzbach tritiation of these substances is an argument for this assumption.

The multitude of parameters involved, even in the case of methane, makes mechanistic studies very difficult. For larger molecules little is known about the ions and the excited states that can be present and about their reactivity. For the heterogeneous Wilzbach labelling, additional complications may be caused by surface phenomena, influence of the crystal lattice and traces of impurities. Labelling of a great number of solid substances has led to the following observations:

(1) Chemical structure and distribution of tritium in a molecule

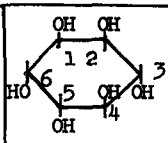
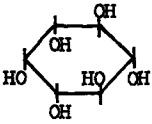
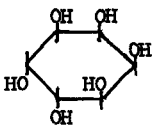
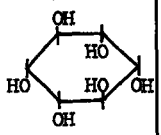
No relation has been reported between the distribution of tritium in a molecule and between its chemical structure[15)16)], except for the following features:

- Certain chemical bonds, especially C-J bonds, are very sensitive to radiation. Compounds with such bonds are preferentially labelled through replacement of iodine by tritium[17)].
- Saturation of double bonds takes place preferentially. Depending on the type of olefinic system, varying degrees of addition are found[18)]. Usually this is an important process, for instance with fatty acid esters[19)] and some steroids[20)], where most or all of the incorporated activity was found in the hydrogenated product.
- In most cases, substitution in aromatic systems takes place more readily than in aliphatic systems[13)16)].

(2) Stereochemistry of the Wilzbach substitution process

Knowledge about the stereochemistry of the tritium substitution in the Wilzbach process is of practical importance, because racemic or diastereoisomeric labelled products are often difficult to separate from the desired substance. Several studies were undertaken by different investigators to determine the extent of racemization taking place in the tritiation of organic compounds. The results of the tritiation of (crystalline) (-)inositol and (liquid) hexa-O-methyl(-)inositol, carried out by Angyal et al., are given in Table 1 [21)22)].

Table 1 Results of tritiation of (-)inositol and of hexa-O-methyl(-)inositol

substance				
	(-)inositol	myo-inositol	allo-inositol	muco-inositol
relative activity	1.0	0.09	0.06	0.03
substance	hexa-O-methyl (-)inositol	hexa-O-methyl myo-inositol	hexa-O-methyl allo-inositol	hexa-O-methyl muco-inositol
relative activity	1.0	0.65	0.47	0.33

They proved by degradation that (-)inositol and hexa-O-methyl(-)inositol were nearly equally labelled at each of the six carbon atoms of the ring[23], whereas myo-inositol contained tritium only at C-1. Assuming that allo-inositol and muco-inositol also contained tritium only at the inverted carbon atom, they calculated the ratios of inversion to retention in (-)inositol approximately as 1 : 4, 1 : 12 and 1 : 6 at C(1), C(2) and C(3). For hexamethyl-(-)inositol, these ratios are approximately 2 : 1, 1 : 1 and 1.5 : 1, respectively.

Inversion in both compounds is greatest at C(1), where an axial hydroxyl is converted into an equatorial one. A similar study was carried out by Crawford and Garnett with (crystalline) (+) and (-) oct-2-ylhydrogen phthalate and with (liquid) (+) octan-2-ol[24]. They observed predominant retention of configuration with (+) and (-) oct-2-ylhydrogen phthalate, whilst octan-2-ol racemized to the extent of 80 %. For L-mandelic acid (crystalline), tritiated by the Wilzbach method, Riesz found about 30 % inversion when the α-hydrogen was substituted by tritium[25]. Brown et al.[26] had not found inversion in the Wilzbach-labelling of 1,2,3,5-tetra-O-acetyl-D-ribofuranose, but it is quite possible that tritiated isomers were removed through their purification procedure. Evans[27] found (partial?) inversion of L-methionine, L-proline and L-tryptophan during Wilzbach labelling. Simon[56] observed high degrees of inversion when the OH-group in tartaric acid was replaced by tritium (78 - 98 %); in the calcium and copper salts retention prevailed (about 25 % inversion). In meso-tartaric acid, substitution of the carbon bound hydrogen caused 12 % inversion.

However, the data now available are still too scarce to draw general conclusions. At present, inversion in the liquid phase seems to be more important than in the solid phase. It is likely that intermolecular hydrogen bonding in crystal lattices restricts configurational changes during the labelling process.

(3) Crystal structure and distribution of tritium in a compound

The relation between the distribution of tritium and the crystal structure of a compound is very obscure. From a recent publication about the Wilzbach tritiation of sugars, one might infer that tritium is the more evenly distributed in a molecule as the crystal structure of the compound is the more irregular[28].

(4) Effects of exposure time, T_2-pressure, surface area and temperature

Different authors found that the amount of tritium incorporated in a compound increased linearly with the exposure time[28)29)30)]. An exponential increase has been found for cholic acid[31].

According to Riesz[11], G_T-values for hydrocarbons are roughly proportional to the pressure of tritium. Wilzbach found, however, that the labelling of benzoic acid, was independent of tritium pressure, but the formation of labelled by-products almost proportional to this pressure[32]. Garnett and Law[13] found a linear relationship between the specific activity of phenanthrene and the square of tritium pressure.

Rosenblum and Meriwether[33] found no obvious relation between crystal size and the specific activity of labelled compounds. They suggest that surface structure is more important than surface extent. Ebert[28], however, mentioned for saccharose an increase in specific activity of the glucose part with a factor of 2 when the crystal size increased from 0.08 to 1.5 mm. Wenzel et al.[34] found that increasing the surface of salicylic acid with factors of up to 10^6 by absorption of the compound on charcoal, 5-100 times higher specific activities were obtained. It is possible, however, that absorption on a carrier substance changes the reaction mechanism. Garnett and Law[13] found an exponential dependence of the specific activity of phenanthrene and the surface area of the sample.

Little information is available about the influence of temperature. Wilzbach[32] mentioned, for benzoic acid, that an increase in temperature from - 15 °C to + 60 °C doubled the amount of tritium incorporated per day, while the tritium incorporation in by-products remained the same. Ebert and Richter[28] found for the activity in the glucose part of saccharose at - 195, - 80, + 20 and + 60 °C respectively 1.3, 10.2, 13.7, and 31.5 x 10^{-2} μCi/mg. The first three values lie on a straight line in an Arrhenius plot,

the last value shows a deviation to higher activities.

1.3. Purpose of our investigation

The Wilzbach method for the labelling of organic compounds may well be seen as a last resort; it may sometimes prove useful when it is impossible or very difficult to label a compound by other methods (direct synthesis, catalyzed exchange in solution). Examples of possibly useful applications lie in the field of peptides and proteins.

So far, various investigators, including the present author, have labelled quite a number of amino acids, peptides and proteins by the Wilzbach method. A selection from their reported work is given in Table 2. In our opinion, the value of the Wilzbach method for the labelling of amino acids is limited. For most compounds, chemical synthesis or catalyzed exchange in solution are the methods of choice. For some amino acids, where exchange in solution gives bad results, the Wilzbach method is a useful substitute.
In the field of peptides and proteins, the Wilzbach method is more important because chemical synthesis or catalytic exchange are often very difficult or impossible.

A major disadvantage of the gas-exposure method, however, is the difficulty to establish the radiochemical purity of a tritiated product. The main criterion, used to establish this radiochemical purity, is the coincidence of the radioactive and the biologically active peak in chromatographic and/or electrophoretic studies. This coincidence of both peaks is a necessary, but not a sufficient, condition for radiochemical purity. Radiochemical impurities which are due to isomerization, racemization or hydrogenation may be difficult to detect and to remove. For instance, little is known about the effectiveness of the usual purification methods for removal of traces of highly radioactive, diastereo-isomeric forms of peptides and proteins.
The objective of our work under report is to obtain more information about the importance of racemization and hydrogenation caused by T-substitution and addition in the solid phase. As model compounds were chosen several amino acids and adrenocorticotropic hormone.

Chapter 2 of this thesis gives a general outline of procedures used and of results obtained with Wilzbach tritiated L-amino acids. We determined, for several amino acids, the amount of radioactive D-amino acid formed during the tritiation of a pure L-amino acid. In order to calculate also the degree of inversion that accompanies substitution of ^{1}H by ^{3}H, the distribution of tritium in two amino acids (valine and alanine) was determined. The importance of radiochemical impurities caused by hydrogenation was studied qualitatively by thinlayer radiochromatography. Part of this work has been published else-

where[52)54)].

Chapter 3 describes the tritiation of adrenocorticotropic hormone. A discussion of the results given in Chapters 2 and 3 is presented in Chapter 4. All experimental details are reported in Chapter 5.

Table 2 Wilzbach labelled peptides, proteins and amino acids

substance	author		reference
lysozyme ribonuclease	Steinberg Leonis	1957 1963	35 55
lysine-vasopressin	Fong	1959	36
human albumin γ-globulin	Pany	1959	37
γ_2-globulin	Rajam	1959	38
insulin	von Holt	1960	39
blood serum albumin	Ghanem	1962	40
oxytocin	Du Vigneaud	1962	41
tetanus toxin	Speirs	1962	42
L-valyl-L-leucine	Burnett	1963	43
thymus polypeptide	Frimmer	1964	44
bovine plasma albumin	Hill	1964	45
adrenocorticotropic hormone	Nishizawa	1965	46
DL-m-tyrosine DL-phenylalanine	Winstead	1960	47
L-lysine L-leucine L-isoleucine L-phenylalanine L-tryptophane DL-valine DL-methionine DL-threonine	Sato	1961	30
β-hydroxy-γ-aminobutyric acid	Verly	1963	48
L-cysteine	Klubes	1963	49
DL-leucine	Wenzel	1961, 1964	50, 51
L-valine DL-valine	Parmentier	1965	52
L-methionine L-tryptophane L-proline	Evans	1966	53
L- and D-phenylalanine L- and D-proline L-tyrosine L-glutamic acid L-alanine	Parmentier	1966	54

CHAPTER 2

RACEMIZATION AND HYDROGENATION OF AMINO ACIDS UNDER WILZBACH CONDITIONS

This chapter presents a general outline of the procedures used and of the results obtained. For details on experiments, see Chapter 5.

2.1. Determination of radioactive D-amino acid, formed during Wilzbach tritiation of the corresponding L-amino acid, by a reverse isotope dilution method.

The inactive amino acids were checked on optical purity. After labelling these amino acids by exposing them in the solid state to pure T_2-gas, labile tritium atoms were removed. The products obtained were carefully purified by repeated crystallization and by thin-layer chromatography until the purified product consisted only of D- and L-amino acid. Before purification, an equal amount of inactive D-amino acid had been added to the tritiated L-acid, in order not to remove traces of active D-amino acid by the crystallization procedure. The purified acid was dissolved and the distribution of the radioactivity between the L- and the D-acid was determined by a reverse isotope dilution method. The principle of this method is as follows.

We call the amount of D-acid m_D, with a total activity A_D, and the amount of L-acid m_L, with an activity A_L. This mixture of L- and D-acid is divided into three equal parts. To portion I is added an amount M_D of inactive D-acid, to portion II an amount M_L of inactive L-acid and to portion III an amount of M_{DL} of inactive DL-acid.

$$M_D = M_L = M_{DL} \qquad M_D \gg m_D + m_L$$

By repeated crystallizations, we remove all L-acid in portion I, and all D-acid in portion II. The specific activities of the three portions are now:

$$a_D = \frac{\frac{1}{3} A_D}{M_D + \frac{1}{3} m_D}, \quad a_L = \frac{\frac{1}{3} A_L}{M_L + \frac{1}{3} m_L} \quad \text{and} \quad a_{DL} = \frac{\frac{1}{3}(A_L + A_D)}{M_{DL} + \frac{1}{3}(m_D + m_L)}$$

The percentages of activity present in D- and L-amino acid, thus obtained, are given in good approximation by:

$$\frac{a_D}{a_{DL}} \times 100 \text{ and by } \frac{a_L}{a_{DL}} \times 100, \text{ respectively.}$$

Essentially, this method was first applied for amino acids by Rittenberg[1] and later by several other authors[2)3)4)5)6)7)]. The method must be used carefully, for co-precipitation of traces of D-acid with the L-acid, and vice versa, may introduce considerable errors[7)]. However, by carrying out the reverse isotope dilution as described here, co-precipitation can easily be detected; then $a_D + a_L$ will exceed a_{DL}.

In general, the results of this method can be influenced by:

(1) the D-amino acid content of the L-amino acid that is to be tritiated;
(2) radiochemical impurities in the labelled amino acids;
(3) chemical and optical impurities in the inactive D-, L- and DL-amino acids used in the isotope dilution;
(4) incomplete separation of the enantiomers by the crystallization procedure;
(5) the accuracy of the dilution and counting procedure.

The combined influence of factors (2), (3), (4) and (5) is reflected in the deviation of the expression: $100(a_D + a_L)/a_{DL}$ from 100. Errors caused by factors (2), (3) and (4) tend to make this sum higher than 100. By far, the largest uncertainties are caused by factor (1).

A number of pure L-amino acids were tritiated by the Wilzbach method, next purified and, finally, the amount of activity in the D-enantiomer was determined. The results are given in Table 3.

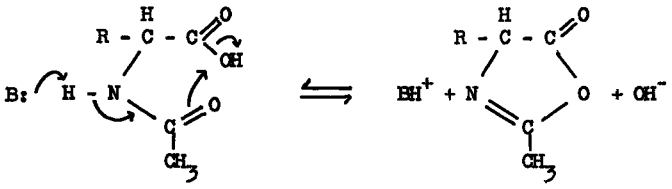
Table 3 Results of the reverse dilution analysis, with estimated maximum deviations

amino acid tritiated	tritiation time (days)	carrier added	percentage of activity	
L-phenylalanine	8	DL-phenylalanine	[100]	
		L-phenylalanine	96.6 ± 3	99.3
		D-phenylalanine	2.7 ± 0.5	
D-phenylalanine	8	DL-phenylalanine	[100]	
		L-phenylalanine	2.2 ± 1	99.7
		D-phenylalanine	97.5 ± 3	
L-tyrosine (first exp.)	8	DL-tyrosine	[100]	
		L-tyrosine	96.3 ± 3	97.4
		D-tyrosine	1.1 ± 0.4	
L-tyrosine (second exp.)	8	DL-tyrosine	[100]	
		L-tyrosine	95.9 ± 3	97.7
		D-tyrosine	1.8 ± 0.4	
L-proline	8	DL-proline	[100]	
		L-proline	92.6 ± 3	97.2
		D-proline	4.6 ± 1	
D-proline	8	DL-proline	[100]	
		L-proline	4.0 ± 1	101.1
		D-proline	97.1 ± 3	
L-glutamic acid	8	DL-glutamic acid	[100]	
		L-glutamic acid	88.9 ± 3	100.8
		D-glutamic acid	11.9 ± 1	
L-alanine (first exp.)	8	DL-alanine	[100]	
		L-alanine	78.5 ± 3	97.8
		D-alanine	19.3 ± 2	
L-alanine (second exp.)	8	DL-alanine	[100]	
		L-alanine	76.5 ± 3	99.5
		D-alanine	23.0 ± 2	
L-alanine (third exp.)	1	DL-alanine	[100]	
		L-alanine	75.8 ± 3	99.5
		D-alanine	23.7 ± 2	
L-valine	8	DL-valine	[100]	
		L-valine	95.4 ± 2	99.6
		D-valine	4.2 ± 0.5	

2.2. Distribution of tritium in alanine and valine

To determine the degree of inversion that accompanies the substitution of a hydrogen atom by a tritium atom, it is necessary to determine the radioactivity present on the α-site both of the D- and the L-amino acid. This was done for alanine-^{3}H and valine-^{3}H. After determination of the radioactivity present in the L- and D-amino acid by a reverse isotope dilution method, these pure L- and D-acids were racemized by heating them with a mixture of acetic acid and acetic anhydride, following the procedure given by Greenstein and Winitz[8].

The proton exchange at the α-site proceeds via the intermediate 5-oxazolone, after acetylation of the amino acid:



Under the influence of a weak base, 5-oxazolone splits off its proton at the asymmetric carbon atom, due to the stabilization of the formed anion.

After this exchange procedure, and removal of the solvent, the acylamino acid was purified by crystallization and the specific radioactivity measured.

For valine, it was proved by Crawhall and Smyth[9] that this procedure removed all the radioactivity at the α-position; moreover, hydrogen atoms in group R do not exchange under these conditions.

For alanine, we proved the same by refluxing inactive L-alanine with acetic acid and acetic anhydride. The acetic acid was labelled with tritium in the -COOH group. The acetylalanine formed was hydrolyzed with HCl, and the alanine purified and measured. The specific activity found was 0.37 μCi/millimol. If only the α-hydrogen had exchanged with tritium, the specific activity should have been 0.39 μCi/millimol; the difference between both figures lies well within the experimental error.

The results of our determination of the tritium distribution in labelled D- and L-valine and D- and L-alanine are presented in Table 4. All figures are given as a percentage of the total activity present in D- and L-amino acid. From this table it can be seen that tritium labelling of L-alanine and L-valine caused 20 and 10 % inversion, respectively.

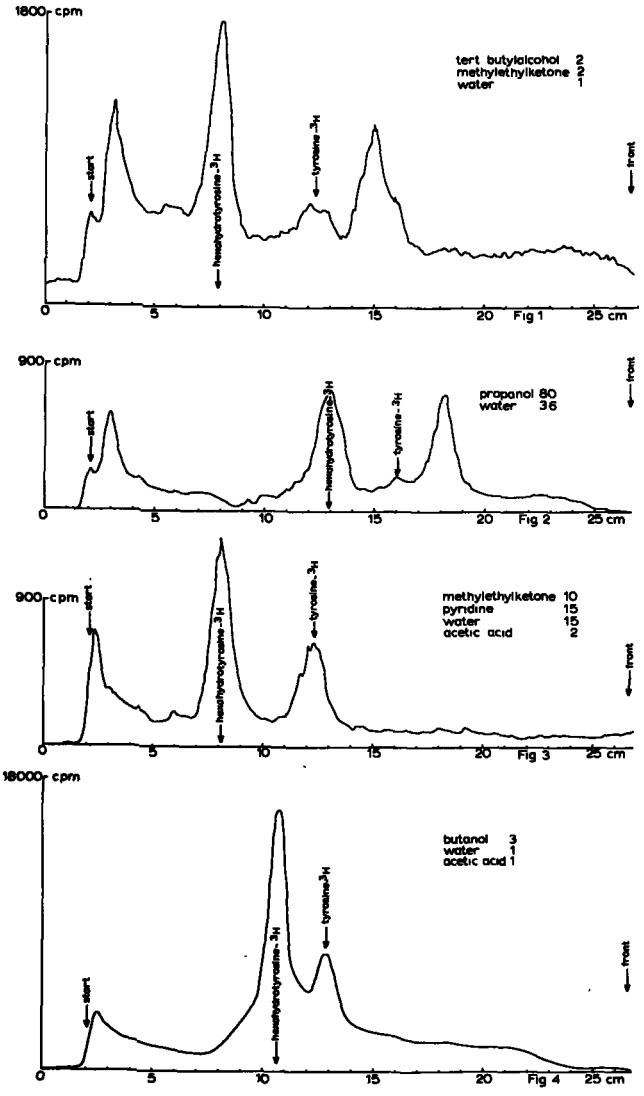
Table 4 Distribution of tritium in valine and alanine

amino acid tritiated	carrier added	percentage of ^{3}H-activity		percentage of ^{3}H-activity remaining after removal of α-H		percentage of ^{3}H-activity in α-H	
L-valine CH_3>$CHCHNH_2COOH$ CH_3>	DL-valine	[100]		79.8		20.2	
	L-valine	95.4		77.5		17.9	
	D-valine	4.2	} 99.6	2.4	} 79.9	1.8	} 19.7
L-alanine CH_3CHNH_2COOH	DL-alanine	[100]		44.8		55.2	
	L-alanine	78.5		36.4		42.1	
	D-alanine	19.3	} 97.8	8.9	} 45.3	10.4	} 52.5

2.3. Formation of hydrogenated amino acids

In the aromatic amino acids that were tritiated, possible radiochemical impurities might consist of hydrogenated products. To check this assumption for phenylalanine and tyrosine, hexahydro-β-phenylalanine and hexahydrotyrosine were prepared as reference substances. By means of these reference substances, and through thin-layer chromatography of the unpurified tritiated amino acids in several solvents, for phenylalanine as well as for tyrosine, presence of a large amount of the corresponding radioactive hydrogenated amino acid could be proved. Figures 1-4 give four radiochromatograms from tritiated, unpurified tyrosine. For each of the four solvents used, a radioactive peak coincided with the peak of hexahydrotyrosine. The absence could be proved of radioactive β-cyclohexylalanine; this compound might have been formed by simultaneous hydrogenation of the aromatic nucleus and substitution of the hydroxyl group.

For phenylalanine, presence of hexahydro-β-phenylalanine was proved by two-dimensional thin-layer chromatography. A radiochemical impurity, assumed to be hexahydro-β-phenylalanine, was isolated from a preparative chromatogram of unpurified phenylalanine by elution with water. β-Cyclohexylalanine was added as a carrier and a two-dimensional thin-layer chromatogram was made. The plate was sprayed with ninhydrin reagent. Only one coloured spot was visible; it coincided with the radioactive spot.



Figs 1 - 4. Radiochromatograms of tyrosine, tritiated by the Wilzbach method

CHAPTER 3

WILZBACH TRITIATION OF CORTICOTROPIN (α_p-ACTH)

To get more insight into the practical problem of obtaining a radiochemically pure "Wilzbach labelled" peptide, we thought it would be useful to carry out a Wilzbach labelling of a naturally occurring peptide hormone. After removal of radioactive fragmentation and polymerization products, we have thus tried to prove the presence of important radiochemical impurities of about the same molecular weight ($\sim$4500) as corticotropin has itself. Corticotropin was chosen as a model compound, because it was available to us in a rather pure form. Moreover, if a reasonable product could be obtained, several applications would then be feasible.

3.1. Outline of procedure

3.1.1. Tritiation and purification

A sample of natural pig corticotropin, mainly consisting of the A_1, A_2 and A_3 fractions obtained by De Jager et al.[1], remained in contact with 3 Ci of tritium gas during 8 days. After this time, labile bound tritium atoms were removed by dissolving the sample in a large excess of water. The water was removed by vacuum evaporation at room temperature.

The normal procedure, after a Wilzbach tritiation, is a purification by chromatography. The product is then assumed to be radiochemically pure if the peak of the desired material contains all radioactivity. This is a necessary, but not a sufficient, condition for radiochemical purity. Radiochemical impurities can be present due to racemization, isomerization and hydrogenation and little is known about the effectiveness of chromatographic methods in the separation of these very closely related molecules with high molecular weights. Clearly, the possible presence of a considerable amount of radioactivity in, for instance, diastereoisomeric or hydrogenated forms of the labelled peptide might reduce the value of a biological tracer substance very seriously. We chose a purification by means of a Sephadex column. We realized that such a purification could result in removal of fragmentation and polymerization products. We supposed that radiochemical impurities caused by racemization, hydrogenation and isomerization - reactions giving molecules with about the same molecular weight as corticotropin - were not likely to be removed. It was hoped that the presence or absence of these molecules could be seen more clearly after a partial hydrolysis as is described below, in 3.1.2. The radiochemical purity of the product after purification over Sephadex was evaluated by electrophoresis on cellulose acetate, followed by autoradiography.

3.1.2. Partial hydrolysis of tritiated corticotropin

It seemed reasonable to expect that at least some of the smaller peptides, resulting from possible radiochemical impurities in our tritiated corticotropin, would differ enough from the peptides obtained from corticotropin itself to enable a chromatographic and/or electrophoretic separation. By making a peptide map of the hydrolysate, and an autoradiogram of this map, spots on the autoradiogram not corresponding to a spot on the peptide map after chemical detection, very probably would have to be attributed to radiochemical impurities.

Partial hydrolysis of corticotropin was accomplished by use of HCl, and by using trypsin . The main component of the material that had been tritiated by us was found to consist of α_p-ACTH (corticotropin A, β-corticotropin, ACTH-A_1). The amino acid sequence of this component is as follows:

H-ser-tyr-ser-met-glu-his-phe-arg-|try-gly-lys-pro-val-gly-lys-|lys-¦-arg-¦-arg-
1 2 3 4 5 6 7 8 | 9 10 11 12 13 14 15 | 16 ¦ 17 ¦ 18

pro-val-lys-|val-tyr-pro-asp-gly-ala-glu-asp-glu-leu-ala-glu-ala-phe-pro-leu-
19 20 21 | 22 23 24 25 26 27 28 29 30 31 32 33 34 35 36 37

glu-phe-OH
38 39

NH_2

Indicated are the cleavages by trypsin; the dotted lines designate minor cleavages[2].

3.2. Results

Our results were obtained with two different batches of purified pig corticotropin, indicated here by numbers 1 and 2 (donated by N.V. Organon, Netherlands). Batch 2 was found to be more extensively purified than Batch 1.

Both batches had been tritiated, and were next purified, in the same way. After purification, Batch 1 was partially hydrolyzed by HCl, Batch 2 by trypsin. The results are given in Figures 5 - 10.

Figure 5 shows:

(a) an electropherogram of unlabelled corticotropin (Batch 1);
(b) an electropherogram of tritiated corticotropin (Batch 1) before purification;
(c) an autoradiogram of electropherogram (b);
(d) an electropherogram of tritiated corticotropin (Batch 1) after purification over Sephadex G-50 (material of the main peak, indicated in Fig. 7 a);
(e) an autoradiogram of electropherogram (d).

Figure 6 shows:

(a) an electropherogram of unlabelled corticotropin (Batch 2);
(b) an electropherogram of tritiated corticotropin (Batch 2) after purification over Sephadex G-50 (material of the main peak, indicated in Figure 7 b);
(c) an autoradiogram of electropherogram (b).

After purification of unlabelled corticotropin (Batch 2) over a Sephadex G-50 column, an electropherogram was obtained that was exactly identical (by chemical detection) with that of labelled corticotropin after purification (Figure 6 b).

Figure 7 shows the purification of corticotropin (Batches 1 and 2) over a Sephadex G-50 column. Electropherograms and autoradiograms were made from the material of the main peaks (5 d, 5 e, 6 b, 6 c).

Figure 8 shows an autoradiogram of the peptide map from the acid hydrolysate of corticotropin (Batch 2). Indicated are the spots that could be detected by spraying with ninhydrin reagent, or by means of ultraviolet light. Figure 10 a is a photograph of this peptide map.

Fig. 9 is an autoradiogram of the peptide map from the trypsin hydrolysate of corticotropin (Batch 2). Again, the spots that could be detected by ninhydrin, or by U.V., are indicated. Figure 10 b is a photograph of the corresponding peptide map. The peptide map of the trypsin hydrolysate of unlabelled corticotropin, purified over Sephadex, was found to be identical with the peptide map given in Figure 10 b.

From Figure 7 it will be clear already that our labelled and unlabelled material in the main peak behaved at least partially different; Figures 8 and 9 show that a substantial amount of the radioactivity, present on the peptide maps, does not coincide with chemically detectable spots. A minimum estimate for the amount of radioactivity not attributable to a chemically detectable spot for the acid hydrolysate, is about 40 % of the total activity present in these spots.

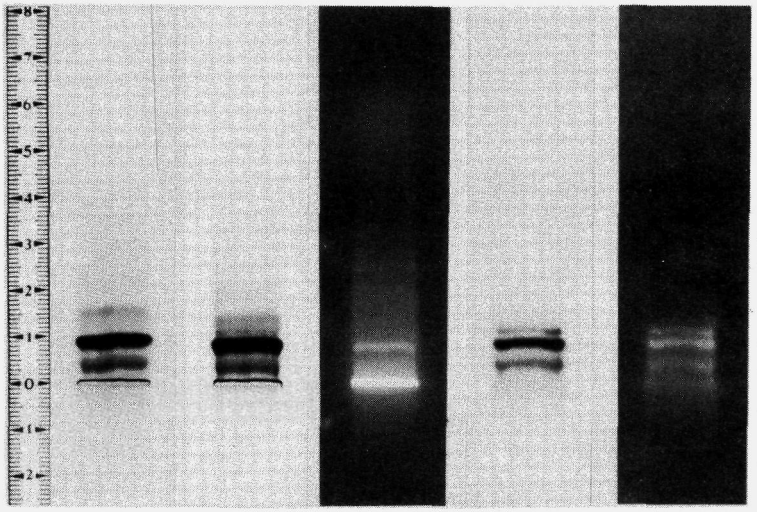


Fig. 5 a b c d e

a: electropherogram of unlabelled corticotropin (Batch 1)
b: electropherogram of tritiated corticotropin
c: autoradiogram of electropherogram b
d: electropherogram of tritiated, purified corticotropin
e: autoradiogram of electropherogram d

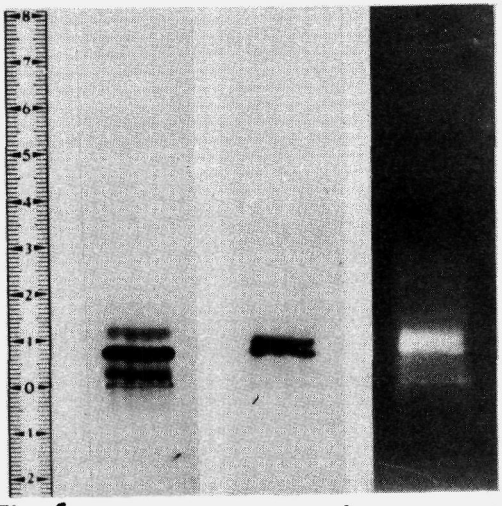
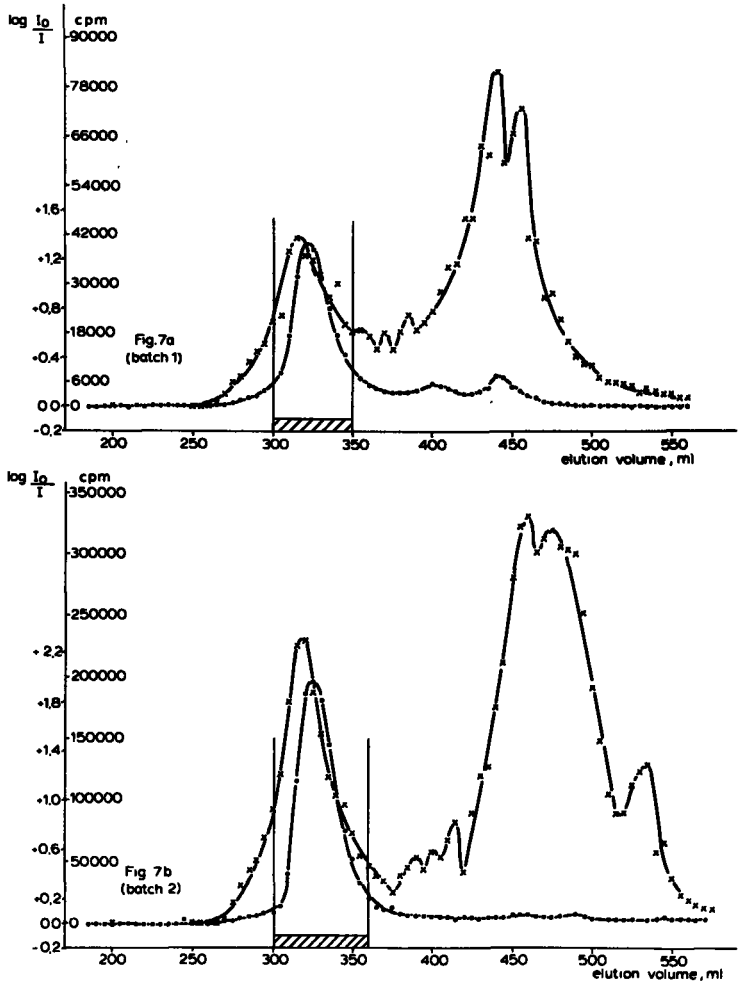


Fig. 6 a b c

a: electropherogram of unlabelled corticotropin (Batch 2)
b: electropherogram of tritiated, purified corticotropin
c: autoradiogram of electropherogram b



Figs 7 a and b Purification of corticotropin (Batches 1 and 2) over Sephadex G-50

x - x - x Elution of radioactivity
. - . - . Elution of corticotropin

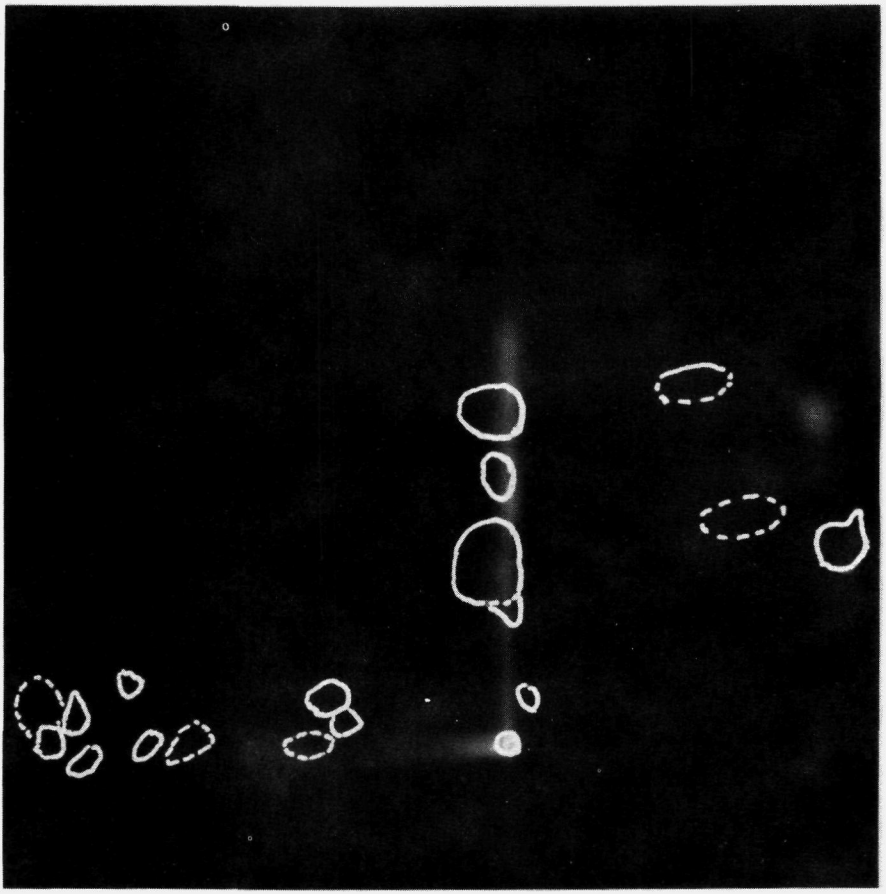


Fig. 8 Autoradiogram of the "peptide map", obtained after partial, acid hydrolysis of labelled, purified corticotropin. Indicated are the spots that could be detected with ninhydrin reagent, or by U.V.
(contact print of the original autoradiogram)

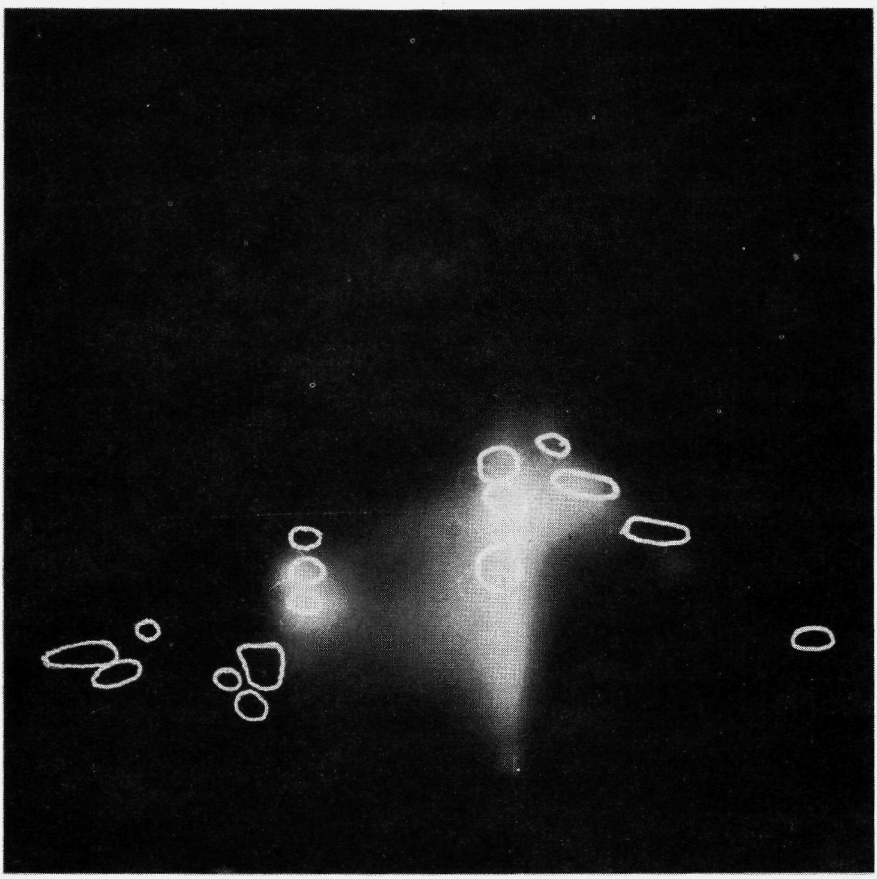
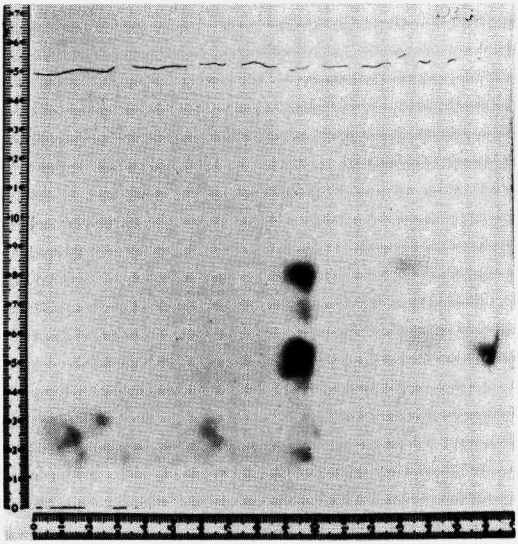


Fig. 9 Autoradiogram of the peptide map, obtained after partial hydrolysis (by trypsin) of labelled, purified corticotropin. Indicated are the spots that could be detected with ninhydrin reagent, or by U.V.
(contact print of the original autoradiogram)

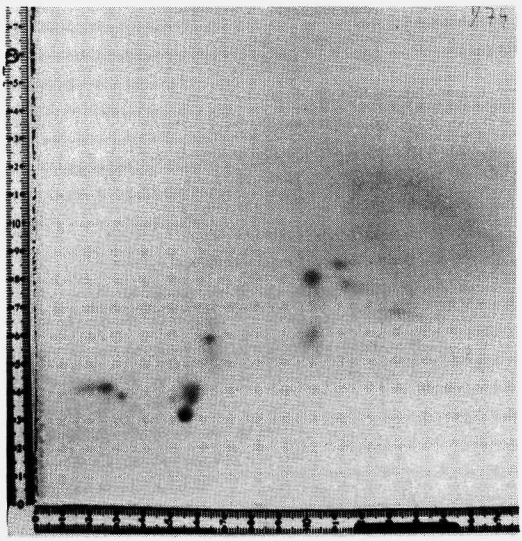


chromatography ↑

← electrophoresis

Fig. 10 a

Peptide map of the acid hydrolysate of corticotropin



chromatography ↑

← electrophoresis

Fig. 10 b

Peptide map of the trypsin hydrolysate of corticotropin

CHAPTER 4

DISCUSSION OF RESULTS

4.1. Results obtained with amino acids

4.1.1. Mechanism of racemization and hydrogenation processes

For amino acids we have demonstrated that the Wilzbach method causes formation of labelled racemized and hydrogenated products. Especially hydrogenation is an important process. As the purpose of this investigation was purely practical, we did not study the mechanism of these processes. From Chapter 1 it will be clear that mechanistic studies are very difficult. Even the reactions of solid glycine caused by γ-radiation are very complicated[1], and it can be expected that the reactions of other amino acids, under the influence of radiation and in the presence of T_2, will be far more complicated. However, the question arises if a plausible explanation of the racemization and hydrogenation can be given. As to the racemization process, each mechanism that we propose must serve to explain the following facts:

(1) The optical rotation of L-alanine before and after the irradiation is practically the same.

(2) The percentage of activity found in D-alanine after Wilzbach tritiation of L-alanine is independent of the reaction time.

(3) When, after exposure of pure L-alanine and L-valine to T_2, the activity distribution in the resulting labelled L- and D-alanine and L- and D-valine was determined, in D-alanine-^{3}H the tritium was found to be distributed roughly in a 1 : 1 ratio over the alpha place and the CH_3-group; in D-valine-^{3}H a similar ratio was found for the distribution of tritium over the alpha place and the $\begin{matrix} CH_3 \\ CH_3 \end{matrix}\!\!>\!CH$-group.

(4) The inversion in L-valine is less than in L-alanine.

The first two facts exclude the possibility that D-alanine-^{3}H is formed by a quick racemization of L-alanine under the influence of intense beta-radiation accompanied by a much slower substitution of hydrogen by tritium. So it seems probable that the racemization is caused by the labelling process. Further evidence for this mechanism comes from observation (3): the possibility that a molecule D-alanine-^{3}H, labelled on the α-place, is labelled in the CH_3-group in a second, independent labelling process, is negligible, as with the obtained specific activities only about one out of every 50,000 alanine molecules consists of D-alanine-^{3}H. So it is likely, that in the racemization process two tritium atoms are introduced (nearly) simultaneously into the molecule.

The numerous electron spin resonance studies of irradiated amino acids, performed in the last ten years, give only information about stable radicals in the crystal. We mention the studies of Miyagawa and Gordy[2] and Miyagawa and Itoh[3] about irradiated alanine and the publication of Shields about irradiated valines[4]. In alanine, the stable radical $CH_3\dot{C}HCOO^-$, and, in valine, the radical $(CH_3)_2\dot{C}CH(NH_3)^+COO^-$ were identified. These radicals, formed at room temperature, are the final products of a chain of events, involving several intermediate species. Even ESR-studies of amino acids irradiated at low temperatures only give information about relatively stable radicals. For L-alanine irradiated at 80 °K, a radical was found with the unpaired electron localized mainly on the carboxylgroup[5], while for DL-valine at this temperature the initial radical seems to be $(CH_3)_2\dot{C}HCHCOO^-$ [6]. Electron spin resonance studies of ^{3}H-labelled solid amino acids[7], and of amino acids exposed to thermal hydrogen atoms[8], show that, generally, under these conditions, the same stable radicals are formed as by X-ray or γ-ray irradiation. It is obvious that reactions of these stable radicals cannot account for the labelling pattern of alanine and valine.

We suppose that under the influence of the tritium beta-radiation the most important primary chemical process will be a rather indiscriminate process of C-H-bond rupture[9]. The total homolytic dissociation energy for this bond rupture will consist of two parts: the bond dissociation energy and the separation energy of the two radicals R˙ and H˙. The contribution of the separation energy to the total dissociation energy will be small; the motion of the hydrogen atom is not severely restricted in a crystal lattice. So a number of reactive radicals will be formed by C-H-bond rupture:

$$RH^* \rightarrow {}^{\cdot}R^* + {}^{\cdot}H^*$$

The ˙R* radical has an excess vibrational energy, the H-atom an excess translational energy. Quite a number of reactions are possible for the ˙R* radical: recombination, disproportionation, intramolecular hydrogen transfer to attain the energetically most favourable structure, or intermolecular hydrogen transfer. Combination of R˙ with H˙ or T˙ will be a far more important process than combination of two large R˙ radicals. The reorientation necessary for this last process will be energetically more unfavourable than disproportionation, where only a hydrogen shift is necessary, or than combination with a hydrogen atom. It is likely that, in the spurs of the beta-rays, the radical density is high enough for disproportionation reactions. (The total number of alanine molecules in an experiment is about 7×10^{20}. About 1.5×10^{16} molecules D-alanine-^{3}H are formed, when each molecule D-alanine is labelled with two tritium atoms. We estimate the total energy dissipation in alanine

as 10^{20} eV. With an estimated G-value of 1 for a $^{\cdot}R(NH_3^+)COO^-$ radical this means that about 10^{18} of these radicals are formed during the total irradiation time of 8 days. We suppose (see below) that D-alanine-^3H is formed via a disproportionation reaction of $^{\cdot}R(NH_3^+)COO^-$ radicals. So it is necessary to assume that several per cent. of the $^{\cdot}R(NH_3^+)COO^-$ radicals react via disproportionation reactions. This is possible only when the primary radicals are formed in clusters, as is generally assumed.)

Concluding, we assume that the following reactions of L-alanine under Wilzbach-conditions lead to labelled alanine:

(1) alanine $\rightsquigarrow$ $^{\cdot}CH_2CH(NH_3)^+COO^-$ $\xrightarrow{T^{\cdot}}$ $CH_2TCH(NH_3)^+COO^-$

(2) alanine $\rightsquigarrow$ $CH_3\dot{C}(NH_3)^+COO^-$ $\xrightarrow{T^{\cdot}}$ $CH_3CT(NH_3)^+COO^-$

(3) alanine $\rightsquigarrow$ $CH_3\dot{C}(NH_3)^+COO^-$ $\longrightarrow$ $CH_2{=}C(NH_3^+)COO^-$

$CH_2{=}C(NH_3)^+COO^-$ $\overset{T_2}{\rightsquigarrow}$ $CH_2TCT(NH_3)^+COO^-$

The radicals $^{\cdot}CH_2CH(NH_3)^+COO^-$ and $CH_3\dot{C}(NH_3)^+COO^-$ may be formed directly or by intramolecular and intermolecular hydrogen displacements.

We suppose that in the crystal lattice racemization by reaction (2) is not very important. In reaction (3), disproportionation leading to the unsaturated intermediate is not only possible between two $CH_3\dot{C}(NH_3)^+COO^-$ radicals, but also between two $^{\cdot}CH_2CH(NH_3)^+COO^-$ radicals or between a $^{\cdot}CH_2CH(NH_3)^+COO^-$ and a $CH_3\dot{C}(NH_3)^+COO^-$ radical. In reaction (3), $CH_2{=}C(NH_3)^+COO^-$, once formed, readily takes up tritium. We suppose this, because it is known that tritium adds quickly to double bonds. This racemization mechanism explains the even distribution of tritium in D-alanine and D-valine over the α-place and the rest of the molecule. The mechanism of the addition of T_2 to a double bond under the influence of its own radiation is still rather obscure. Peng[10] found, for the addition of tritium to oleate, 60 % participation of tritium ion molecules and 40 % participation by tritium radicals. Nystrom[11] found evidence for a free radical process in several unsaturated systems. We have postulated in reaction (3) a mechanism whereby T_2 adds to an activated double bond.

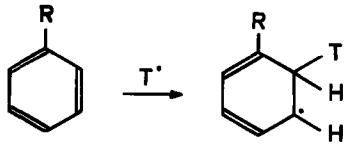
The fact that the percentage of D-alanine-^3H does not depend on the irradiation time suggests that, in the clusters of primary radicals, a constant fraction of these radicals reacts by disproportionation.

For valine, a similar scheme can be drawn. The reactions under (3) are then relatively less important, because in reaction (1) the radicals $(CH_3)_2\dot{C}CH(NH_3)^+COO^-$ as well as $^{\cdot}CH_2(CH_3)CH{-}CH(NH_3)^+COO^-$ play a role.

We emphasize that the mechanism suggested here for the racemization process is only a plausible one; other mechanisms are feasible. If it could be proved that in D-alanine-^{3}H two tritium atoms are located in each labelled molecule, this would provide strong support to the proposed mechanism. As to this latter question, one can oxidize alanine with ninhydrin without loss of tritium from the α-place[12]. The resulting acetaldehyde can be analyzed by mass spectrometry. By choosing suitable experimental conditions, D-alanine may be isolated with sufficiently high specific activity to detect the presence of $CH_2TC{=}O{\diagdown}T$ in the mass spectrum of acetaldehyde. [0.10 % of the P+4 mass peak of labelled acetaldehyde may be determined[13]; we think it is possible to isolate labelled acetaldehyde with about 1 % of its molecules doubly labelled.]

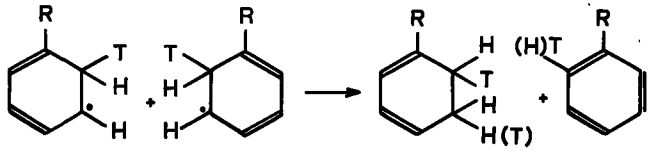
With regard to the mechanism of the hydrogenation of phenylalanine and tyrosine under Wilzbach conditions, we have found a large amount of tritium in products that behaved chromatographically like hexahydrophenylalanine and hexahydrotyrosine. As we may expect that the chromatographic behaviour of partially hydrogenated products does not differ very much from that of the fully hydrogenated compounds[14], it is possible that both fully and/or partially hydrogenated, labelled products were present.[After Wilzbach tritiation of polynuclear aromatic hydrocarbons, Lijinski and Garcia[17] found several partially hydrogenated products (di- and tetrahydro reduction products) of high specific activity.]

The initiating reaction:

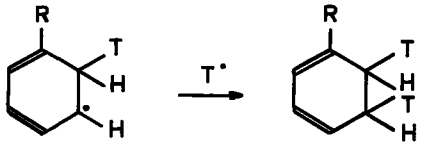


seems very probable[16)18)]. Disproportionation and combination reactions of this radical can lead to partially hydrogenated products:

Disproportionation :



Combination :



Once formed, the partially hydrogenated products may be further hydrogenated by similar processes. Direct addition of T_2 to activated aromatic nuclei may also play a role.

4.1.2. Practical implications of results obtained with amino acids

From our results, and those of Evans[15], we conclude that partial racemization is a common phenomenon during the Wilzbach tritiation of amino acids. Moreover, all other studies dealing with racemization of other types of compounds indicate that partial inversion during the labelling procedure is a general process. Obviously, this is a disadvantage of the Wilzbach method. For simple substances this disadvantage is mostly not insurmountable. A more serious disadvantage is the large amount of activity incorporated in hydrogenated products. This easy (partial) saturation of phenylgroups was rather unexpected. In our opinion, the value of the Wilzbach method is restricted to a number of relatively simple compounds for which labelling by other methods is laborious. A good example of such a compound is L-proline, which cannot be labelled satisfactorily with tritium by the common process of catalyzed exchange in solution. High specific activities can be obtained with the Wilzbach method; moreover, the labelled proline contains surprisingly little radiochemical impurities and is easy to purify.

4.2. Results obtained with corticotropin

The essence of our experiments carried out with ACTH was to detect whether any difference between the chemical and radiochemical behaviour of peptide molecules, tritiated by the Wilzbach method, could be found. After removal of radioactive fragmentation and polymerization products by Sephadex filtration (Figs 7a and 7b, Chapter 3), products were obtained which appeared radiochemically pure by an electrophoretic criterion. These products were partially hydrolyzed, and peptide maps were made of the hydrolysates. Autoradiograms of these peptide maps revealed that a substantial amount of the radioactivity did not coincide with chemically detectable spots (Figs 8 and 9, Chapter 3). Obviously, radiochemical impurities were present in the purified product with about the same molecular weight as corticotropin itself.

The experimental procedure used does not give any details about the type of impurities that were present. To assume that at least part of these impurities are caused by racemization and hydrogenation processes seems reasonable. Compared with amino acids, the picture may be complicated by molecular rearrangements. Our conclusion is that the Wilzbach method is not suited to obtain labelled peptides and proteins of known radiochemical purity. Results, obtained in the past with Wilzbach labelled peptides and proteins, may be

considerably in error due to the presence of radiochemical impurities.

CHAPTER 5

EXPERIMENTAL PART

5.1. Experiments relating to Chapter 2

5.1.1. Materials used

The amino acids used (L-, D- and DL-forms) were obtained from Fluka (puriss. quality) except D-, L- and DL-proline and D-, L- and DL-alanine; these were obtained from Calbiochem (puriss. quality). The optical purity of the amino acids was checked by determination of the optical rotation.

L-phenylalanine	: $[\alpha]_D^{24}$	$= -33.2^\circ$, c = 1 - 2	in water
D-phenylalanine	: $[\alpha]_D^{24}$	$= +33.7^\circ$, c = 0.8 - 2.2	in water
L-tyrosine	: $[\alpha]_D^{20}$	$= -8.8^\circ$, c = 4.44	in 6.3 N HCl
D-tyrosine	: $[\alpha]_D^{20}$	$= +7.8^\circ$, c = 3.59	in 6.3 N HCl
L-proline	: $[\alpha]_D^{20}$	$= -83.6^\circ$, c = 1	in water
D-proline	: $[\alpha]_D^{20}$	$= +82.9^\circ$, c = 1	in water
L-glutamic acid	: $[\alpha]_D^{24}$	$= +31.7^\circ$, c = 2.7	in 1.73 N HCl
D-glutamic acid	: $[\alpha]_D^{24}$	$= -31.6^\circ$, c = 2.7	in 1.73 N HCl
L-alanine	: $[\alpha]_D^{23.5}$	$= +14.4^\circ$, c = 5	in 1 N HCl
D-alanine	: $[\alpha]_D^{23.5}$	$= -14.3^\circ$, c = 5	in 1 N HCl
L-valine	: $[\alpha]_D^{24}$	$= +28.5^\circ$, c = 3	in 6 N HCl
D-valine	: $[\alpha]_D^{21}$	$= -29.3^\circ$, c = 3	in 6 N HCl

(c = grams of solute per 100 ml of solvent)

The optical purity of L-phenylalanine, L-tyrosine, L-glutamic acid and L-valine was also checked by enzymatic analysis with L-amino-oxidase. This analysis indicated a D-amino acid content of L-valine and L-phenylalanine of less than 0.5 %. L-tyrosine was completely digested; 4.1 % L-glutamic acid was found after the digestion. In a separate, identical check experiment with an optically very pure standard sample of L-glutamic acid, 4.1 % of this standard sample was also not digested in the reaction time chosen. The amino acids were used without further purification; they were all chromatographically homogeneous.

5.1.2. Exposure to tritium gas

Exposure to tritium gas was performed in an apparatus whose essence had been described by Wenzel[1]; it is shown in Figure 11. We used a modification, in which the uranium tube was replaced by an ampoule filled with molecular sieve, type 4 A. The tritium gas can easily be stored in this molecular sieve for short periods, and, if necessary, removed by sealing the ampoule[2]. The tritium gas had been obtained from the Radiochemical Centre, Amersham, in 3 C portions. The isotopic purity of the gas was about 98 %, its volume 1.2 ml at N.T.P. Each 3 C portion of the gas was used only two or, at most, three times. About 100 mg of the finely powdered, and thoroughly outgassed, amino acid was exposed for 7 - 8 days to 3 C tritium gas, at a pressure of about 20 cm Hg, at room temperature.

5.1.3. Purification of labelled amino acids

To remove labile bound tritium atoms, the labelled substances were dissolved in a large excess of water (for tyrosine, first experiment, 6 N HCl was used). The solvent was removed under vacuum. To each of the labelled L-amino acids, an exactly equal portion of the corresponding inactive D-amino acid was added (for tyrosine, 1st experiment: D-tyrosine-HCl) and to each of the labelled D-amino acids an exactly equal amount of inactive L-amino acid. The products so obtained were crystallized as follows:

Phenylalanine	:	4 x from ethanol-water
Tyrosine	: 1st exp.	: 1 x from 6 N HCl, 3 x from water
	2nd exp.	: 2 x from water
Proline	:	1 x from ethanol-ether
Glutamic acid	:	6 x from water
Alanine	:	2 x from ethanol-water
Valine	:	4 x from ethanol-water

After crystallization, the labelled amino acids were purified further by preparative thin-layer chromatography. Thin-layer plates (20 x 30 cm) with silica gel H were prepared (thickness 1 mm) and dried overnight, then 1 h at 110 °C. Per plate, 5 mg of an amino acid was dosed in a band. The amino acid was dissolved in 0.5 ml of water, or, for tyrosine, in 0.5 ml 1 N HCl. Chromatograms were developed with butanol/acetic acid/water 3 : 1 : 1, and then dried at a temperature of at least 50 °C. The distribution of radioactivity over the chromatogram was determined with a Berthold scanner. The amino acids were removed by extracting the silica gel with hot water; the water was removed under diminished pressure. For tyrosine, the extraction was done with 40 ml of hot water, acidified with 5 ml 0.1 N HCl. As and when necessary, the isolated

products were chromatographed a second or a third time. Depending on the yield, and on the activities of the amino acids, we started the first chromatographic purification with 5 mg (dosed on one plate), or with 10 mg (dosed on two plates). In this latter procedure, the extracts were added together after the first chromatogram had been made. L-glutamic acid was purified only by crystallization from water, as we had the impression that purification over SiO_2 caused additional impurities. Radiochemical purities of the final products were better than 99 %; except for D-phenylalanine (99 %) and for L-glutamic acid (97 %). These figures were estimated from the second or the third preparative chromatogram, or from an analytical thin-layer or paper chromatogram. These paper and thin-layer chromatograms were also developed with butanol/acetic acid/water 3 : 1 : 1 (ascending technique; Whatman paper no. 1, or a silica gel H thin-layer of 0.20 mm thickness). Radioactivity distributions on the analytical paper chromatograms were determined by cutting the paper in pieces of $\frac{1}{2}$ cm, and counting in a liquid scintillation counter (5.1.7.). From the thin-layer plate, the silica gel was removed in bands of $\frac{1}{2}$ cm, and the activity of each band was counted (5.1.7.).

We did not measure the precise specific activities of the undiluted, labelled amino acids. The tritiated amino acids were considered pure only after chromatographic purification and were then immediately diluted with carrier. The yield of this purification was not determined, and, as a consequence, the dilution factor was not known exactly. The specific activities of the impure substances, however, were known from determination by liquid scintillation counting, see 5.1.7., and based on the purities determined from the radiochromatograms, the specific activities of the pure amino acids were estimated. The results are given in Table 5.

Table 5 Specific activities of some tritiated amino acids, labelled by the Wilzbach method. Exposure time 8 days, with 3 Ci T_2

amino acid	spec. activity (μCi/mg)
L-alanine (exposure 1 day)	7
L-alanine	60
L-valine	51
L-proline	148
L-phenylalanine	91
L-glutamic acid	27

5.1.4. Reverse dilution analysis

The amino acids, obtained after the purification, were dissolved in about 6 ml of water. For tyrosine, 0.1 N HCl was used. If necessary, this solution was filtered over a G-3 glass filter and then divided in three portions of about 2 ml. These three portions were weighed, and a weighed amount of respectively L-, D- and DL-amino acid was added as a carrier (about 200 mg; for proline about 300 mg). The fractions were crystallized till the sum of the specific activities (corrected for slightly different dilutions) of the D- and the L-fractions was equal to the specific activity of the DL-fraction; then the fraction with the lowest specific activity was crystallized further to prove its constant specific activity. Specific activities were determined by liquid scintillation counting (5.1.7.).

Crystallization procedure:

Phenylalanine	: 3 - 5 x from ethanol-water
Tyrosine	: 5 - 6 x from water
Proline	: 12 x from absolute ethanol
Glutamic acid	: 6 x from water
Alanine	: 2 - 3 x from ethanol-water
Valine	: 4 x from ethanol-water

Given is the number of crystallizations, necessary to make the sum of the specific activities of the D- and the L-fraction approximately equal to the specific activity of the DL-fraction.

The results of the reverse isotopic dilution are given in Table 3, Chapter 2.

5.1.5. Determination of tritium distribution in alanine and valine

Portions of 100 mg of radioactive D-, L- and DL-alanine, resulting from the reverse isotopic dilution analysis, were boiled for 10 minutes with 2.5 ml acetic acid and 0.24 ml acetic anhydride. After cooling, the acetic acid was removed under vacuum at a temperature of 40 °C. When the acetylalanine was dry, water was added and removed under vacuum. This was repeated four times. Benzene was added and distilled off under vacuum to remove the last traces of water. The residue was purified by crystallization from acetone. Yields: 70 - 77 %. The samples were counted in a liquid scintillation counter. The results are given in Table 4, Chapter 2. To establish whether treatment with acetic acid and acetic anhydride did not remove radioactivity from the CH_3-group of alanine, a reversed exchange was carried out. Radioactive acetic acid (CH_3COOT) was made by treatment of acetic anhydride with HTO. Acetic anhydride (May and Baker, p.a) was doubly fractionated over sodium acetate[3]. The acetic acid

content, as judged by NMR, was less than 0.5 %. The anhydride (102.4 mmol) was stirred with HTO (102.4 mmol) for four hours at 40 °C; it was used after standing another 12 hours at room temperature. Its absolute specific activity was determined in triplo and found to be 0.433 μCi/mmol. 82.4 mmol of this radioactive acetic acid, 2.29 mmol L-alanine and 5.1 mmol acetic anhydride were used for an inverse exchange reaction, carried out in the same manner as described for the exchange of alanine-^{3}H. The total amount of exchangeable hydrogen present was 4 x 2.29 + 82.44 = 91.6 milli-atom, and the total activity 82.44 x 0.433 = 35.7 μCi. So the specific activity of the exchangeable hydrogen was 0.39 μCi/milli-atom. After isolation and purification, the acetylalanine contained an activity of 0.609 μCi/millimol. The acetylalanine was hydrolyzed[4] by refluxing 100 mg for 2 hours with 4.5 ml 2 N HCl. This was removed under vacuum. Water was added to the residue and evaporated under vacuum (3 x). The residue was treated with 2 N HCl and the resulting alanine-HCl was counted, after drying, in a liquid scintillation counter. A specific activity of 0.37 μCi/mmol was found, corresponding with the exchange of only one hydrogen atom.

Radioactive D-, L- and DL-valine, resulting from the reverse isotopic dilution analysis, were diluted with a two-fold amount of the corresponding inactive amino acids. About 1 mmol valine was then treated, in the same manner as described for alanine, with acetic acid (19 mmol) and acetic anhydride (16 mmol). The acetylvaline was crystallized from water and the activity determined by liquid scintillation counting. The results are given in Table 4, Chapter 2.

5.1.6. Detection of hydrogenated products in tritiated phenylalanine and tyrosine

Hexahydrophenylalanine and hexahydrotyrosine were prepared to serve as carriers, or as reference substances.

Hexahydrophenylalanine. 206 mg DL-phenylalanine was dissolved in 30 ml 4 N NCl, and 237 mg PtO_2 added. Reduction was carried out in a hydrogenation apparatus, under stirring. After uptake of the calculated amount of hydrogen, the solution was filtered off and the solvent evaporated under vacuum. The product was crystallized from 6 N HCl, yield 180 mg. Analysis: C 51.8 %, H 9.1 % (theoretically: C 52 %, H 8.9 %). Only one spot was visible after thin-layer chromatography. (Solvent butanol-acetic acid-water 3 : 1 : 1, ninhydrin detection.)

Hexahydrotyrosine was prepared by reduction of L-tyrosine[5]. 1.68 g PtO_2 was suspended in 65 ml H_2O and reduced by H_2. 2.18 g L-tyrosine was added, with 4 ml 0.1 N NaOH. The reaction was carried out in a hydrogenation apparatus.

The total hydrogen uptake exceeded that calculated for the formation of hexahydrotyrosine. A chromatogram of the reaction mixture showed the presence of several impurities. The main impurity consisted of hexahydrophenylalanine. 12 ml 1 N HCl was added to the reaction mixture. After evaporation, 22 ml absolute ethanol was added and the solution saturated with dry HCl. Next, 19 ml absolute ethanol was added and the solution refluxed for two hours. After evaporation of the alcohol, the residue was dried over KOH. This esterification procedure was repeated two times. The residue was dissolved in 5 ml H_2O (cooled) and 3.3 g K_2CO_3 was added at a temperature of - 3 to - 0 °C. The solution was extracted with four portions (10 ml) of cold ether, the ether was removed and the residue extracted five times with 5 ml pentane. After evaporation of the pentane, the residue was crystallized from ether and then from ethylacetate. The product had a melting point of 76 - 76.5 °C (uncorrected).

C: 61.5 %; H: 9.7 %; N: 6.4 % (calculated: C: 61.4 %; H: 9.8 %; N: 6.5 %).

The ester was hydrolyzed by heating 48 mg for four hours with 4 ml water at 95 °C. After addition of 0.5 ml HCl conc., the solvent was evaporated and the residue dried over KOH under vacuum. The product was homogeneous as judged by thin-layer chromatography (butanol-acetic acid-water 3 : 1 : 1, ninhydrin detection).

C: 46.9; H: 8.1; N: 6.2 (calculated: C: 48.3; H: 8.05; N: 6.26)

Scanning of the preparative chromatograms of tritiated phenylalanine revealed an important radioactive peak, not detectable by ninhydrin. This impurity was isolated from the preparative chromatogram (5.1.3.) by elution with water. After evaporation of the water, the residue, mainly SiO_2, was extracted with ethanol. Hexahydroalanine was added, as a carrier, and a two-dimensional thin-layer chromatogram was made. About 50 µg substance was dosed on a thin-layer plate of 20 x 20 cm, with a thickness of 0.25 mm. Development in the first direction was done with pyridine/water/tert.amylalcohol 35 : 30 : 35, in about five hours. After drying, development in the second direction took place with butanol-acetic acid-water 3 : 1 : 1 in 4½ hours. After drying, the activity distribution over the chromatogram was determined by scanning (5.1.7.), and finally, the plate was sprayed with ninhydrin reagent. Only one coloured spot was visible, which coincided exactly with the radioactive spot.

For tyrosine, four one-dimensional chromatograms were made with four different solvents. Tyrosine-^3H (not purified, only labile ^3H-atoms removed) was chromatographed on thin-layer plates (silica gel H, 20 x 30 cm, thickness 0.25 mm) with tyrosine and hexahydrotyrosine as reference substances. In most experiments spots of 2 µl were applied, containing 20 µg of the substance.

The solvents used were:

(1) t-butanol-methylethylketon-water 2 : 2 : 1;
(2) propanol-water 20 : 9;
(3) methylethylketon-pyridine-water-acetic acid 10 : 15 : 15 : 2;
(4) butanol-water-acetic acid 3 : 1 : 1.

Development times: about 20 hours; chemical detection by spraying with ninhydrin reagent; radioactive detection by scanning (see 5.1.7.). The results are given in Chapter 2, Figures 1, 2, 3 and 4.

5.1.7. Radioactivity measurements

The amino acids were measured by dissolving 0.5 - 2 mg of the acid in 1 ml of a hyamine hydroxide 1.0 M solution in methanol (purchased from Nuclear Enterprises Ltd., Edinburgh, Scotland); when the amino acid was completely dissolved 6 ml scintillation liquid was added. (Composition: 200 ml ethanol p.a., 800 ml toluene p.a., 5 g 2,5-diphenyloxazol, (PPO) and 0.5 g 2,2'-p-phenylen-bis-(5-phenyloxazol), (POPOP)). The measurements were carried out in an automatic liquid scintillation counter (Tritomat 6020, Isotope Development Ltd.; later in a Tri-Carb 3375, Packard).

Activity distributions on paper chromatograms were determined by cutting the chromatograms in strips of $\frac{1}{2}$ cm. These strips were put in the measuring bottles, as far as possible in the same positions, and covered with 5 ml of a scintillation mixture (composition: 60 g naphtalene p.a., 4 g PPO, 0.2 g POPOP, 100 ml ethanol, 20 ml ethyleneglycol; added up to a volume of 1000 ml with p-dioxane p.a.). Activities on most analytical thin-layer chromatograms were measured by removing the silica gel H in bands of $\frac{1}{2}$ cm, covering the gel with the dioxane-scintillation mixture, and counting in the scintillation counter.

Activity distributions on preparative thin-layer chromatograms, and on analytical thin-layer chromatograms with sufficient activities, were determined with a Berthold "Dünnschicht-scanner LB 2720". Activities, as measured on chromatograms by one of the methods described above, give only approximate estimations of radiochemical purities.

5.2. Experiments relating to Chapter 3

5.2.1. Material used

Two batches of the adrenocorticotropic hormone used had been donated by N.V. Organon, Oss, Netherlands. Both batches consisted mainly of the A_1, A_2 and A_3 fractions described by De Jager[6]; they had been prepared from pig anterior pituitary powder by batchwise purification over carboxymethylcellulose using pyridine-acetic acid as a buffer system. Batch 2 (indicated by Organon as

lot no. 27517-P2) was found to be more extensively purified than Batch 1 (indicated as lot no. 1027).

5.2.2. Exposure to tritium gas

Of Batch no. 1, 60 mg was exposed to 3 Ci of tritium gas for 8 days at room temperature. The product was dissolved twice in 15 ml distilled water, to remove labile bound tritium ($\sim$250 mCi).

Of Batch no. 2, 96 mg was exposed to 3 Ci of tritium gas for 7 days at room temperature. The product was dissolved in 50 ml distilled water. (Labile bound tritium: $\sim$ 150 mCi).

5.2.3. Purification over Sephadex

We used a Sephadex G-50 (fine) column with a length of 145 cm and a diameter of 2 cm (water regain, W_r, 5.0 $\pm$ 0.3 g/g, bed volume per g dry gel 10 ml). The void volume, V_o, (elution volume for a substance that is completely excluded from the gel), was determined with Blue Dextran and found to be 185 ml. The total volume of the gel bed was 455 ml. The inner volume, V_i, can be calculated as[7]:

$$V_i = \frac{W_r \cdot d}{W_r + 1} (V_t - V_o) = 241 \text{ ml}$$

(d = density of the gel in the swollen condition, = 1.07)

Purification of Batch 1: about 50 mg of the irradiated product was dissolved in 1.5 ml water; this sample was applied on top of the column and eluted with 0.02 n acetate buffer (p_H 5.2)[8].

Purification of Batch 2: about 80 mg was dissolved in 5 ml water and eluted with 0.02 n acetate buffer.

In both experiments, the irradiated product did not dissolve completely in the buffer; a sticky brown solid remained undissolved. Fractions of 5 ml were collected and measured in a Zeiss spectrophotometer at 280 Å. The radioactivity of each fraction was determined by measuring 5 µl in a liquid scintillation counter (5.1.7.). The elution curves are given in Chapter 3, Figures 7 a and 7 b. Elution volume for Batch 1: 305 ml; for Batch 2: 315 ml.
($K_d = \frac{V_e - V_o}{V_i}$, K_d Batch 1: 0.50; Batch 2: 0.54; K_d Batch 2, unlabelled: 0.52)

The fractions of Batches 1 and 2 that were used for further experiments are indicated in Figures 7 a and 7 b. The volume of these fractions was for Batch 1 reduced to about 2 ml, and for Batch 2 to about 10 ml. After removal of insoluble material, both solutions were desalted over a Sephadex G-15 column (length

85 cm, diameter 1.5 cm, water regain, W_r, 1.5, bed volume per g dry gel 3 ml; void volume, V_o, determined with Blue Dextran, 62.5 ml; inner volume V_1 = 62 ml; total bed volume 150 ml). Fractions of 5 ml were collected and measured in a Zeiss spectrophotometer at 280 Å; elution volumes of 80 ml for Batch 1 and 90 ml for Batch 2 were found. The hormone fractions were evaporated to dryness and used in this form for electrophoresis and partial hydrolysis. Complete desalting was not obtained, and the resulting product contained some sodium acetate.

The yield of corticotropin after purification over Sephadex G-50 was about 40 %; after desalting over Sephadex G-15 the overall yield was about 10-20 %; so it was found that adsorption is quite considerable[9)10)].

After a passage of labelled material, both columns were carefully washed out, until the radioactivity in the effluent was negligible.

5.2.4. Electrophoresis and autoradiography of corticotropin

Electrophoresis of corticotropin was carried out on strips of cellulose-acetate (2.5 x 17 cm, Gelman Sepraphore III). We used a Shandon universal electrophoresis apparatus (after Kohn) Mk II. Per strip we applied 1-2 λ solution (2 mg corticotropin, dissolved in 20 λ of a veronal-formamide buffer. Composition of buffer solution: 105 ml 0.2 m veronal, 45 ml 0.2 N HCl, 100 ml formamide, 350 ml distilled water; p_H = 8.2.)

Before application of the corticotropin, the strips were swollen for 15 minutes in the buffer solution in a refrigerator. Electrophoresis was carried out immediately after equilibration (30 minutes, ~ 200 V, ~ 1.5 mA per strip). The wet strips were coloured in a bath of 0.5 % amido black 10 B in methanol-water-acetic acid 5 : 5 : 1 for five minutes. After washing in methanol-water-acetic acid 5 : 5 : 1, the strips were photographed while they were still wet and then dried on a siliconated glass plate under filter paper. Autoradiography of the strips was carried out with Kodak No Screen X-ray films, the exposure times of most strips were about one week.

5.2.5. Partial hydrolysis of labelled corticotropin

In an acid hydrolysis of Batch 1, 3 mg labelled corticotropin, purified over Sephadex as described in 5.2.3., was hydrolyzed for five hours in 2 ml freshly distilled, constant boiling HCl (5.7 N) at 75 °C. The hydrolysis was carried out in sealed evacuated tubes. These tubes were carefully cleaned in boiling HCl. After hydrolysis, hydrochloric acid was evaporated under vacuum. The residue was dissolved in 0.5 ml water, and the water evaporated; this treatment was repeated four times.

In a hydrolysis of Batch 2 with trypsin, about 8 mg of purified, labelled corticotropin was dissolved in 8 ml water. The p_H of the solution was adjusted to 8 by means of 0.1 N NaOH. The solution was stirred for 25 min at 37 °C, and trypsin was added (0.08 mg in 10 µl, Worthington, 2 x crystallized, charge TRSF 789). For 2½ hours the temperature was maintained at 37 °C and the p_H at 8 by adding, as the hydrolysis proceeded, 0.01 N NaOH. Next, the solution was acidified with 0.1 N HCl to p_H 3. The reaction mixture was freeze-dried.

5.2.6. Peptide maps of corticotropin hydrolysates.

Silica gel-G plates (20 x 20 cm) were prepared and activated for one hour at 100 °C. The plates were sprayed with buffer (500 ml pyridine, 20 ml acetic acid, 4500 ml water, p_H = 6.4) and, next, saturated with the same buffer solution by means of thick paper strips (Whatman 3 MM) attached to two sides of the plates and hanging in the buffer solution which was contained in two electrode vessels. 2 λ of the sample (a saturated solution of the hydrolysate in the pyridine-acetic acid buffer) was applied to the plate. Electrophoresis was carried out for 40 minutes (≈ 1200 V, ≈ 40 mA) and, thereafter, the plate was dried at 60 °C. After drying, the plate was activated for 20 minutes at 100 °C. Chromatography in the second direction was carried out with butanol-pyridine-acetic acid-water 15 : 10 : 3 : 12 as a solvent, for three hours. Autoradiograms were made on Kodak No Screen X-ray film, with exposures of about one month.

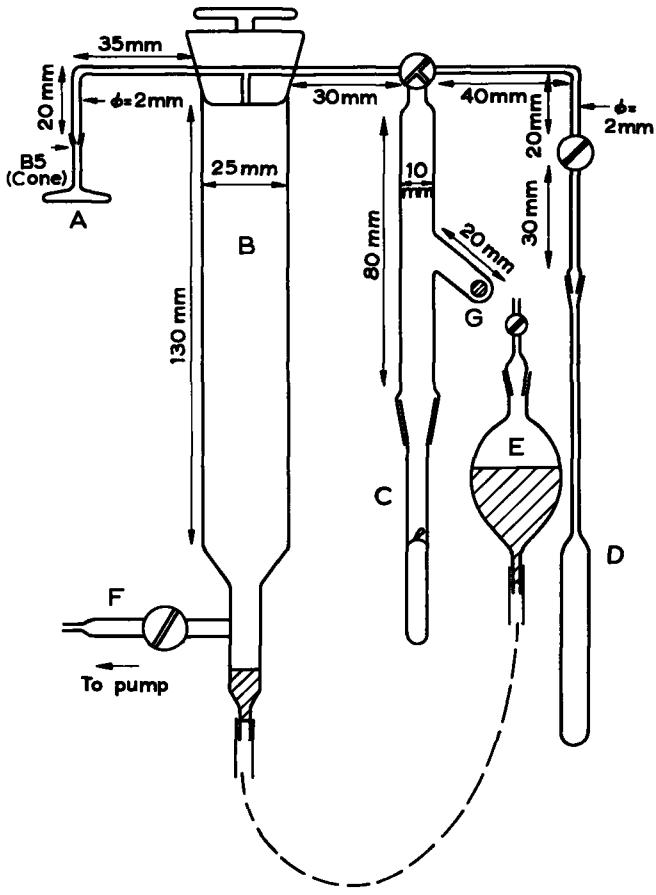


Fig. 11. Tritiation apparatus

A : reaction vessel

B-E : Toepler pump

C : break-seal ampoule with T_2

D : tube with molecular sieve

S U M M A R Y

In 1956, Wilzbach introduced a radiation-induced exchange method for the labelling of organic substances with tritium. The organic compound (gas, solid or liquid) is allowed to remain in contact with Curie quantities of pure tritium gas during a period of several days to several weeks. The major problem in applying the method is the purification of the labelled product. Due to such reactions as isomerization, hydrogenation and racemization, radioactive by-products may be present in chemically unimportant quantities, but with extremely high specific activities.

The purpose of the present investigation was to obtain information about the importance of racemization and hydrogenation of amino acids and peptides in the solid phase caused by tritium substitution and addition. As a peptide, adrenocorticotropic hormone was chosen. In Chapter 1, a survey is given about the relevant literature.

Chapter 2 describes how pure L-amino acids were labelled by the Wilzbach method. After a rigorous purification, removing all radiochemical impurities except the corresponding, labelled D-amino acid, the amount of radioactivity present in the D-amino acid was determined by a reverse isotope dilution method. 1 - 20 per cent. of the radioactivity in the pure amino acid was present in the D-enantiomer, depending on the structure of the compound. In tritiated alanine and valine, the distribution of the radioactivity over the α-place and the rest of the molecule was determined in the L- and in the D-enantiomer. With the values then obtained, the degree of inversion caused by substitution of the alpha-hydrogen by tritium could be calculated. In L-alanine, tritium labelling caused 20 % inversion, and in L-valine, 10 % inversion.

After Wilzbach tritiation of phenylalanine and tyrosine, a major part of the incorporated radioactivity was found in (partial) hydrogenation products of these aromatic amino acids.

In Chapter 4, a mechanism is proposed for the racemization process in amino acids as well as a mechanism for the incorporation of tritium in (partial) hydrogenated aromatic amino acids.

Chapter 3 describes the tritiation of adrenocorticotropic hormone by the Wilzbach method. This tritiation was carried out to get more insight into the practical problem of obtaining a radiochemically pure Wilzbach labelled peptide. After removal of radioactive fragmentation and polymerization products by Sephadex filtration, a product was obtained that appeared radiochemically pure by an electrophoretic criterion. This product was partially hydrolyzed, and peptide maps were made of the hydrolysates. Autoradiograms of these peptide maps revealed that a substantial amount of the radioactivity did not

coincide with chemically detectable spots. Obviously, radiochemical impurities were present in the purified product with about the same molecular weight as corticotropin itself, presumably caused by racemization, hydrogenation and isomerization. We conclude that in general it will be very difficult, or even impossible, to prove rigorously the radiochemical purity of any polypeptide or protein, labelled by the Wilzbach method. Results that were obtained in the past with Wilzbach labelled peptides and proteins may be considerably in error due to the presence of radiochemical impurities.

S A M E N V A T T I N G

In 1956 werd door Wilzbach een algemene methode aangegeven om organische verbindingen met tritium te merken. Hiertoe wordt de organische verbinding (als gas, vloeistof of vaste stof) gedurende een periode, uiteenlopend van enkele dagen tot enkele weken, in contact gebracht met Curie-hoeveelheden van zuiver tritiumgas. Onder invloed van de beta-tritium-straling vindt uitwisseling plaats van waterstof voor tritium. Het voornaamste probleem bij de toepassing van deze methode is de zuivering van het gemerkte produkt. Ten gevolge van bijvoorbeeld isomerisatie, hydrogenering en racemisatie kunnen radioactieve bijprodukten aanwezig zijn met extreem hoge specifieke activiteiten, terwijl chemisch gezien, de aanwezigheid er van niet of nauwelijks waarneembaar is.

Het doel van dit onderzoek was om gegevens te verkrijgen over de mate, waarin racemisatie- en hydrogeneringsreacties bij aminozuren en peptiden, die volgens Wilzbach worden gemerkt met tritium, een rol spelen. In hoofdstuk 1 wordt een overzicht gegeven van de literatuur, die betrekking heeft op dit onderwerp.

In hoofdstuk 2 wordt beschreven hoe zuivere L-aminozuren volgens de Wilzbach-methode werden gemerkt. Na een zuivering, waarbij alle radiochemische verontreinigingen werden verwijderd, behalve gemerkt D-aminozuur, werd het percentage gemerkt D-aminozuur in het verkregen produkt bepaald door middel van een isotopenverdunningsmethode. 1 - 20 procent van de totale radioactiviteit in het zuivere aminozuur was aanwezig in de D-enantiomeer, afhankelijk van de structuur van de verbinding. In volgens Wilzbach gemerkt alanine en valine werd bovendien de verdeling van de radioactiviteit over de alpha-plaats en de rest van het molecule bepaald, zowel in de L- als in de D-enantiomeer. Uit deze gegevens valt af te leiden, dat in L-alanine en in L-valine substitutie van waterstof op de alpha-plaats door tritium respectievelijk 20 en 10% inversie veroorzaakt. Na Wilzbach-tritiëring van fenylalanine en tyrosine werd een groot deel van de radioactiviteit, opgenomen door de vaste stof, teruggevonden in (partiële) hydrogeneringsprodukten.

In hoofdstuk 4 wordt een mechanisme voor de racemisatie van aminozuren onder invloed van T_2 voorgesteld. Tevens wordt aangegeven, op welke wijze men zich de inbouw van tritium in de fenylkernen van tyrosine en fenylalanine, onder gelijktijdige (partiële) hydrogenering van deze kernen, kan voorstellen.

In hoofdstuk 3 wordt de tritiëring van corticotropine beschreven. Deze tritiëring werd uitgevoerd om inzicht te krijgen in het probleem, of het mogelijk is een peptide, gemerkt volgens Wilzbach, radiochemisch zuiver in handen

te krijgen. Na verwijdering van radioactieve afbraak- en polymerisatieprodukten door middel van filtratie over Sephadex, werd een produkt verkregen dat, te oordelen naar het elektroferogram, radiochemisch zuiver was. Dit produkt werd partieel gehydrolyseerd. De peptiden in de hydrolysaten werden gescheiden op dunne lagen met behulp van electroforese in één richting en chromatografie in de tweede richting. Autoradiogrammen van deze "fingerprints" toonden aan, dat een belangrijk deel van de radioactiviteit niet samenviel met chemisch aantoonbare vlekken. Blijkbaar waren in het gezuiverde corticotropine radiochemische onzuiverheden aanwezig met ongeveer hetzelfde molecuulgewicht en vermoedelijk ontstaan door racemisatie, hydrogenering en isomerisering. Onze conclusie is, dat het in het algemeen uiterst moeilijk of zelfs onmogelijk zal zijn om de radiochemische zuiverheid van een polypeptide of eiwit, gemerkt volgens de Wilzbach-methode, te bepalen. Eerdere resultaten, verkregen met volgens Wilzbach gemerkte eiwitten en peptiden, kunnen tot geheel verkeerde conclusies hebben geleid, ten gevolge van de aanwezigheid van radiochemische verontreinigingen.

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A C K N O W L E D G E M E N T S

I wish to thank Professor Dr. A.J. Staverman, director of the Centraal Laboratorium TNO, Delft, for the permission, and opportunity, to publish the investigations described in this thesis.

I am grateful to Dr. P. Platzek, head of the Isotope Department of the said research institute, for his interest and encouragement. Many thanks are due to Dr. J.D.H. Homan, Dr. H.G. Wijmenga, Drs. E. de Jager and Ir. B.C. Goverde of N.V. Organon, for their help and for many helpful discussions. The enzymatic digestions with D- amino acid oxidase were carried out under the supervision of Ir. B.C. Goverde.

I have had stimulating and valuable discussions with my colleagues Dr. P.Ph.H.L. Otto and Dr. H. Looyenga and with Dr. W.A. Cramer of the Reactor Instituut, Delft.

Of the collaborators of the Centraal Laboratorium TNO who assisted in the performance of the experiments, I wish to thank especially Miss M.J.Th. Toby, Miss T. v.d. Marel and Mr. Th. Ambaum.

A part of this investigation, already described in a previous publication, was sponsored by Euratom under contract No.: 078-64-RISN.

Op verzoek van de faculteit der Wiskunde en Natuurwetenschappen volgt hier een overzicht van mijn academische studie.

Na het behalen van het einddiploma H.B.S. aan het St. Janscollege te 's-Gravenhage in juni 1952 en het vervullen van de militaire dienstplicht, werd in september 1954 de studie in de scheikunde aan de Rijksuniversiteit te Leiden aangevangen. Het candidaatsexamen F heb ik afgelegd in december 1959. Onder leiding van de hoogleraren Dr. E. Havinga, Dr. A.A.H. Kassenaar en Dr. A.J.Staverman werd het doctoraalexamen voorbereid, dat in december 1961 werd afgelegd. In maart 1958 trad ik in dienst van het Centraal Laboratorium TNO, waar ik tot op heden werkzaam ben bij de afdeling radio-isotopen.