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RADIOACTIVE LABELLING OF PEPTIDIC HORMONES

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ABSTRACT

The labelling of peptidic hormones requires stability, specificity and sensitivity of the label. Introduction of a radioactive atome is one way to satisfy these criteria. Several processes have been described to prepare radioactive TRF : synthesis of the peptide with labelled aminoacids or introduction of the label into the hormone. In that approach, tritium can be substituted in the imidazole ring, via precursors activating the proper carbon. Monoiodo TRF leads essentially to tritium labelling of the 5 position whereas monoazo TRF allows the preparation of ^{3}H TRF labelled in the 2 position. Di-substituted TRF leads to labelling into the 2 and 5 carbons. Labelled analogs of TRF can be prepared with labelled iodine ; further developments of peptide labelling, will be presented. In particular, the homolytic scission of the C-iodine bond by photochemical activation. The nascent carbon radical can be stabilized by a tritiated scavenger. This approach eliminates the use of heavy metal catalysts.

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"To follow a molecule step by step, to tell its story and its journey in the body of a dog, from its entrance until its elimination" was the wish of Claude Bernard as expressed in a letter to his friend Taine.

Progresses have been made in this direction by introducing a radioactive atom into the molecule investigated. In the field of polypeptidic hormones one faces the necessity of high specific radioactivity in order to cope with the minute amount present at the level of the target cell. More precisely, a specific radioactivity of 50 Ci tritium/mMole allows the detection of roughly 10^{-14} mole in the measured sample, a figure which seems often still above the required level. A factor of ca 20 can be gained by replacing tritium by iodine ^{125}I or ^{131}I leading to the detection of about 5.10^{-16} mole.

These remarks raise the question of the various routes permitting the introduction of a labelled atom and of the significance of the labelled hormone, for further applications.

Table 1 report the specific radioactivities of common radioactive isotopes.

Table 1

^{14}C	62 mCi/mC	5 600 y
^{3}H	29 Ci/mH	12.2 y
^{32}P	9100 Ci/mP	14 d
^{35}S	1500 Ci/mS	87 d
^{125}I	1700 Ci/mI	60 d
^{131}I	3800 Ci/mI	8 d
^{11}C	10^{7} Ci/mC	20 min

There are three different approaches to label a peptide : total synthesis, preparation of a derivative with a labelled reagent, replacement of an atom by its labelled counter part.

I - Labelling by total synthesis -

Synthesis of a peptide whereby labelled aminoacids are incorporated represents the most difficult solution of the question. Peptide synthesis requires large amounts of chemically protected aminoacids, of the

order of a millimole and exceptionnaly 10 times less. This means the handling of several curies throughout the whole synthesis, is very expensive and requires elaborated safety devices. This approach, however, has been used to prepare ^{3}H-angiotensin II /1/, ^{3}H-LRF /2/ and ^{3}H-TRF /3/ by incorporation of ^{3}H-tyrosine and ^{3}H-proline respectively. The final products had specific radioactivities of the order of 40 Ci/mMole, an achievement in the field.

It is of importance to remark that synthetic ^{3}H-TRF carried nearly two tritium atoms on the proline ring. By another procedure which shall be discussed later, ^{3}H-TRF was obtained by introduction of two tritium atoms on the histidine ring. Since proline and histidine represent critical features for the biological properties of TRF and since TRF is a very small molecule, the two species of ^{3}H-TRF represented a good example to study the isotopic effect resulting from the presence of two tritium atoms. None was found suggesting that the labelled compound was a true tracer of the present molecule, a very important conclusion indeed.

The synthesis of larger peptides, like ACTH, has been achieved by condensing a labelled fragment with the complementary part /4/. It is very likely that progresses are expected in this field of peptide synthesis with emphasis given to the handling of smaller quantities.

An interesting approach proposed by Schwyzer /5/ consists in the introduction by total synthesis of an ethylenic L-aminoacid into the peptide. Reduction of the double bond is a convenient mean to label the peptide, once its synthesis is completed.

II - Preparation of a labelled peptide derivative -

This second approach offers a family of possibilities which are characterized by their intrinsic simplicities : the label is carried by the group reacted with the preexisting peptide. Free aminogroups, terminal NH_2- in linear peptides and the ε- NH_2 - groups from lysine, free carboxylgroups, the hydroxyl functions, and the aromatic site chains are targets which can be considered for condensation with a labelled reagent. However, It should be emphasized that these derivatives are analogs of the parent hormone. They cannot be considered as true tracers of the relevant peptide.

This remark in mind, labelled hormone derivatives can be very useful especially in radioimmunological assays and in a variety of other investigation.

- Iodination -

A derivative commonly used is the iodinated peptide, labelled with ^{125}I or ^{131}I. Since the starting labelled material is the nearly carrier free iodide I^-, an oxidizing agent has to be used to transform the iodide into a reacting specie, either I°/6/ or I^+/7/. The latter

has been extensively used and is prepared by mixing a source of chlorine, chloramine T with iodide /8/. Chloramine T, in aqueous solution hydrolyses and produces hypochlorous acid, which beside its reaction with I^-, acts also on reducing groups of the peptide disulfide bridges, methionine, serine and threonine. If these residues are critical for biological activity, the latter is impaired or even abolished.

A clear cut example is afforded by the iodination of gastrin by I^-/chloramine T, leading to complete inactivation as a result of methionine oxidation as shown by Stagg et al /9/. This side reaction can be prevented by dimethylsulfoxyde 0,5 M, which act as an analog of methionine thioether and prevent by competition oxidation of the latter.

A very interesting approach to prepare an activated iodine from iodide is to associate glucose, glucose oxidase and a peroxidase, as proposed by Nunez et al /10/. The nature of the activated species is unknown as is the exact role of the peroxidase. This enzyme directs iodine under very mild conditions towards tyrosine residues of the peptide and exhibits a specificity, probably related to conformational characteristics of both partners. A careful analysis of iodination of insulin, comparing the results obtained with two peroxidases and with chemical reagents has been reported recently by Lambert and Jacquemin /11/.

If side reactions can be deleterious towards the biological activities of peptides, the main reaction, iodination of the aromatic rings is in many cases associated with an alteration of the fundamental characteristics of the molecule, for several reasons :

1 - Iodine is a bulky atom, of a size similar to a benzene ring,
2 - Iodine promotes a rearrangement of the πelectrons of the aromatic rings, which drastically lowers the pK of the associated ionizable groups : whereas the tyrosine hydroxylgroup has a pK of 10,2, monoiodotyrosine has a pK of 8,5 and di-iodotyrosine a pK of 6,5. Similary the imidazole side chain of TRF has a pK of 6,2 whereas the monoiodo derivative has a pK below pH5.
3 - The presence of iodine onto an aromatic side chain increases the hydrophobicity of the molecule and promotes adsorption. This effect becomes noticeable when partially iodinated peptides are filtrated through Sephadex or Biogel columns. The iodinated species are more retained than the parent molecules, and even to the extent that they required special treatments to be recovered.

Thus (monoiodotyrosyl) angiotensin II is eluted after angiotensin II on Biogel P_2 column /12/. (monoiodotyrosyl) ocytocin is more retained on Sephadex G15 than ocytocin /13/. The same is observed with iodo TRF /14/ on AG 11 A8 column. Furthermore, (monoiodohistidyl)TRF and (di-iodohistidyl)TRF migrate more (R_F : 0,53 and 0,85 respectively) than TRF (R_F : 0,37) on thin layer chromatography developed with n-butanol : acetic acid : water (75 : 10 : 25)

Even large peptides like insulin, carrying 1,6 I atoms per 6 000MW can be separated from the non iodinated form, on QAE Sephadex or similar cation exchangers /15/.

The iodination α neurotoxin form Naja Nigricollis represents an extreme case since it is retained on Biorex 70 column so firmly that even 0.2 M phosphate buffer does not elute this derivative/16/.

These examples are by no means exceptional . They represent the general behavior of iodinated peptides. These data show that the partition of peptides between two phases, which by no means exhibit conformational specificity, is altered following iodination. One has to expect therefore, that binding processes occuring at biological membranes might also be modified, and particularly the ratio between specific and non specific binding.

A very clear cut example is the binding of iodoocytocin to purified neurophysin II. Monoiodination of the tyrosyl residue of the hormone reduces the affinity and di-iodination abolishes it /17/. Furthermore, if the aromatic side chain is implicated in the triggering of the biological response, the presence of iodine on the cycle might interfer with the process.

Table II reports some data comparing the biological activity of purified mono and di-iodinated peptides with the intact material, as well as with the peptide recovered after replacement of the halogen by tritium. One shall notice that iodination is not necessarily deleterious. Iodinated glucagon is more active than glucagon /19/.

Table II

Peptide	Biological activity	Specific radioactivity Ci/mMole after replacement of the iodine by tritium
Angiotensin II(AGII)	100 (12)	
MIT - AGII	25-30	
DIT - AGII	0 (10% colon)	
^{3}H - AGII	100	56

Peptide	Biological activity	Specific radioactivity Ci/m'lole after replacement of the iodine by tritium
Castrine I (CI)	100 (18)	
MIT - CI	100	
DIT - CI	30-40	
^{3}H - CI	100	60
Ocytocin (OCY)	100 (13)	
MIT - Ocy	50	
DIT - Ocy	5-10	
^{3}H - Ocy	100	35
TRF	100 (a)	
NIH - TRF	100	
DIH - TRF	0	
^{3}H - TRF	100	60
LRF	100 (b)	
monoiodo LRF	90-100	
di-iodo LRF	10	
^{3}H - LRF	100	60-70
Glucagon (C)	100 (19)	
monoiodo G	480	
di-iodo G	570	
tri-iodo G	1 000	

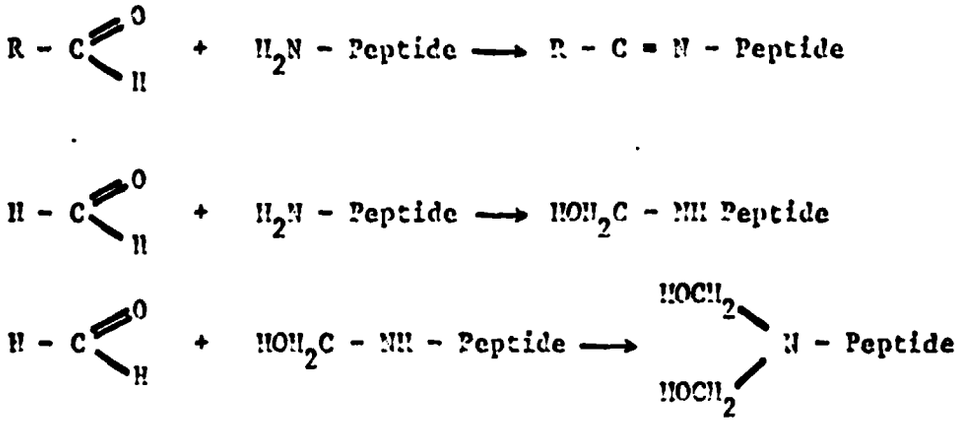
MIT, DIT : monoiodo and di-iodotyrosyl - MIH, DIH : monoiodo and di-iodohystidyl - a : data of Dr. C. Oliver (Marseille) - b : data of Dr. Kerdelhué (Gif sur Yvette).

When carrier free radioactive iodide, i.e. a very small amount, is used for iodination, in order to prepare a material of high specific radioactivity, it is frequent that only a fraction of the present peptide molecules is labelled. Consequently, testing the biological activity of the preparation without prior separation of the iodinated material from the non reacted peptide can be misleading, since the accuracy of the biological test may not detect the change of activity of the labelled molecules.

- Label carrying reagents -

If iodination is a convenient procedure to label peptides it requires the presence of at least one exposed aromatic side chain, tyrosine or histidine, and no free thiol group.

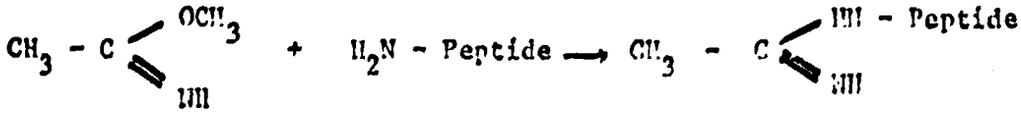
These limitations prompted a search for other approaches also leading to peptide derivatives, like reaction of aldehyde with free aminogroups



The reaction is reversible leading to Schiff bases or to methylol derivatives of the aminogroup. Reduction with tetrahydroborate stabilizes the substitution and results in a secondary amine. The label is introduced either on the aldehyde reagent, or during the reduction process which adds a hydrogen atom or a tritium on the aldehydic carbon.

Cooper and Reich /20/ labelled a snake neurotoxin with pyridoxal phosphate according to this line. De la Llosa et al (21) prepared methylated tritium labelled luteinizing hormone by reaction with formaldehyde and reduction with B^*H_4. In both examples, substitution of free aminogroups did not impair the biological characteristics of the peptides. Such favorable cases, however, cannot be the rule, since the charges carried by aminogroups and their hydrogen bonding properties are often of significance in the binding and the triggering of the biological response.

These remarks emphasizes the interest of methylacetimidate, a reagent proposed by Repke and Zull /22/. It condenses readily with free aminogroups.



Positioning in space an imide group in the vicinity of the previously existing aminogroup and carrying a similar charge the modification afforded is therefore minimal and methylacetimidate, labelled with tritium on the methyl group should find extensive uses.

Synthesis of peptide derivatives allows not only the introduction of radioactive atoms, but also that of other types of labels, fluorescent or possessing an electronic spin. These fields shall not be discussed here.

III-Labelling of peptide by replacement of a stable hydrogen by tritium -

Since incorporation of a labelled aminoacid into the sequence of a peptide is a difficult task, many efforts where devoted and still are to replace atoms of a peptide by their radioactive isotopes. These efforts are directed mostly towards hydrogen atoms, which fall into two classes separated by a somewhat blurred frontier : the labile hydrogens which exchange with the protons of the medium and the stable hydrogens which do not under physiological conditions.

Consequently, in order to replace a stable C-^{1}H bond by a C-^{3}H bond one has to overcome an energy barrier. This can be done presently, at the level of the aromatic hydrogens only, by two general processes.

The first corresponds to the activation of the C-H bond by chemisorption of the aromatic ring on heavy metal catalysts, like platinum or palladium black. When these catalysts are saturated with tritium, tritium is also activated and an exchange between the hydrogen isotopes takes place /23/.

A very efficient refinement of this general method has been recently proposed by Buchman et al /24/. It consist in the preparation of the platinum catalyst just before use, by reducing platinum oxide with tritium gas. The catalyst is then certainly deprived of previously adsorbed hydrogen and perhaps also more active, features leading to an improved labelling.

It should be noted that the molecules having experienced an exchange are diluted by those which have not. The contact with the catalyst should be therefore maintained long enough. The basic difficulty, however, is that the proper adsorption of the aromatic side chains on the catalyst is frequently hindered by the peptidic backbone.

The positions of the tritium atoms incorporated on the aromatic cycles are uncertain and do not always correspond to the predictions, nor to randomness, as shown recently by ^{3}H NMR /25/, a powerful extension of ^{1}H-NMR.

The second process corresponds to the preparation of a derivative R-C-^{1}H → R-C-X followed by the replacement of X by tritium, leading to R-C-^{3}H.

The potential interest of this approach is that the activated intermediate R-C-X can be isolated and characterised. Dilution of the final product R-C-^{3}H by the parent molecule R-C-^{1}H can therefore be avoided. The aromatic side chain to be considered are those of tyrosine, histidine a: tryptophane, since we know of no reagent X attacking under mild conditions the phenylalanine side chain.

The reagents X studied so far are iodine, diazonium derivatives, reacting both with tyrosine and histidine side chains, and O-nitrophenyl sulfenylchloride, directed towards the indol ring of tryptophane. They can all be removed and replaced by a tritium atom.

- Iodination -

The purpose is really to prepare a iododerivative of the peptide to be labelled, with no limitation as far as the iodinating species are concerned (limitations are experienced when iodine is the required labelled atom). To avoid side reactions, and specifically attack of disulfide, of thioether, and of tryptophane, the rate of reaction with tyrosine and histidine should be kept as rapid as possible. This is attained by the use of ICl, which corresponds to the I^{+} iodinating species. A control test is conveniently made by varying the iodination conditions of tyrosine or histidine containing peptides in the presence of the fragile groups. This allows to sort out in each particular cases the best conditions. A general feature is to add ICl very slowly, by numerous small aliquots.

It is proper to note that when ICl is brought in aqueous solution, it progressively hydrolysed giving raise to iodine, detectable by its brown color (at pH 4, and 20°C, iodine becomes detectable after 90 seconds). In presence of tyrosine, this does not occur until tyrosine is di-iodinated.

By contrast, histidine catalyses the hydrolysis of ICl into iodine, which is formed immediately and then reacts with the imidazole ring/26/. This catalytic formation of iodine from ICl by histidine containing peptides can lead to side reactions which where thought to be prevented by the choice of ICl.

It is very interesting to note that TRF, despite containing a histidyl residue, does not catalyse the hydrolysis of ICl in aqueous solutions.

This observation indicates that the histidine side chain in TRF is

not freely available to form a charge transfer complex with ICl, as does histidine or his-pro-phe, for instance. The structure of TRF, in aqueous solution, is therefore submitted to constrains which shall be defined by other approaches. It remains that the rate of hydrolysis of ICl in presence of histidine containing peptides is an index of the degrees of freedom surrounding the imidazole ring /26/. It is convenient to have the ICl weakly labelled with ^{125}I, both to follow the iodinated peptide, to quantify its formation and also the removal of iodine.

As mentionned it has been possible in all cases studied yet to separate the iodinated from the non reacted peptide.

Tyrosine is substituted symetrically in ortho of the hydroxyl group, and its maximum absorption is shifted to longer wave length, from 276 nm for tyrosine (pH 5,8) to 295 nm (pH 5,9) for the mono-iodo and to 301 nm, with a shoulder at 312 nm (pH 6,4) for the di-iodo derivative.

Histidine side chain can be substituted by iodine in two sites, carbon 2 and carbon 5, which are not equivalent. Kinetic studies with deuteroimidazoles showed to Grimison et al /27/ that the initial iodination occured at the 5 position, see also Bensusan et al /28/ and Holloway et al /29/. The same is true for histidine inserted in a peptide; mono-iodo TRF was whown by ^1H-NMR to be deprived of the proton carried by carbon 5 of the imidazole ring /30/.

One could imagine that according the molar ratio of iodinating reagent versus the aromatic aminoacid, a mono or disubstitution by iodine would occur. As a matter of fact, the situation is more complicated. The work of Choh Hao Li /31/ established that I_2 and IOH, hypoiodous acid react instantaneously with mono-iodo tyrosine, and that the rate limiting step is the monohalogenation of tyrosine. Therefore, irrespective of the stoechiometry, only di-iodotyrosine is found. By contrast, we observed that the rates of reaction of ICl with tyrosine and mono-iodotyrosine are of the same order of magnitude, allowing therefore the preparation of mono-iodotyrosyl derivatives.

In the presence of I_2 or IOH, mono-iodohistidine is also iodinated faster than is histidine /32/. Consequently di-iodohistidyl derivatives are the main products. Does IClin that case lead to different kinetics?

Since the imidazole side chain catalyses the hydrolysis of ICl into iodine and hypoiodous acid no answer can be given by direct measurement. We have only one example, that of TRF, where no enhancement of ICl hydrolysis takes place. That means that free iodine is formed with the same apparent initial kinetics, suggesting that the rate of reaction of ICl with TRF is slow and of the same order of magnitude than rate of reaction of ICl with water.

Analysis of the TRF derivatives made from ICl before the appearance of free iodine is reported in Table III./30/

Table III

Formation of iodo TRF as a function of ICl molar ratio. TRF 1 µMole. Sodium phosphate buffer 0.1 M, pH = 7, 4° C. Time of reaction, 60 seconds.

ICl (µMoles)	mono-iodo TRF (µMoles)	di-iodo TRF (µMoles)
0,5	0.22	0.05
1	0.39	0.15
1.5	0.5	0.27
2	0.35	0.58
4	0.05	0.95

Table III shows that the mode of reaction of ICl with the histidyl residue of TRF is different from what is known for iodine and histidine /32/. However, we are not in position to decide whether the data of Table III reflect the fact that ICl is able to react (since no free iodine was present) and expresses its one characteristics or whether the conformational restrictions preventing the catalytic effect of the imidazole ring in TRF also oriente the reaction of ICl.

Finally, one should recall that iodination of tyrosine is faster than that of histidine, when both residues are fully accessible. According Choh Hao Li /32/ at pH 6, the rate constant K for histidine is about one hundredth of that for tyrosine, I_2 being the halogenating reagent; at pH 7 and 7,2, the respective rate constants are in the ratio 1 : 20.

As mentionned before, ICl reacts still faster than I_2 on tyrosine and to the extent that in presence of histidine, ICl will iodinate the phenol ring in preference to the imidazole catalyzed hydrolysis. This explains why halogenation of a peptide containing both tyrosine and histidine, like angiotensin II, leads first to (mono-iodotyrosyl)angiotensin II then to (di-iodotyrosyl)angiotensin II. It explains also the possibility to iodinate with ICl the tyrosine residue of LRF, which contains histidine without degrading the tryptophane /33/, so sensitive to free iodine.

In short, this discussion indicates that iodination of peptides is seldom a simple process and that cautious analysis is required if defined compounds are wanted.

- p.benzene sulfonate diazonium chloride -

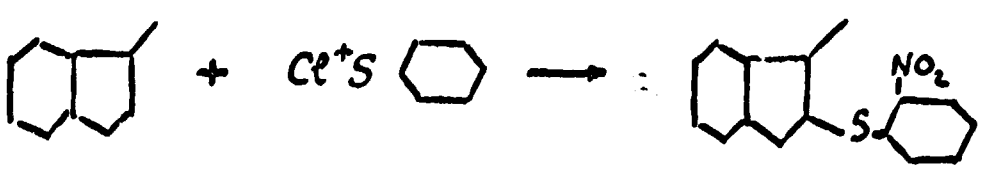
This reagent described by Rung and Behrend in 1892 /34/ was shown to react with phenol and imidazole derivatives by Pauly in 1904 /35/. Prepared by diazotisation of sulfanilic acid, the diazonium chloride has many features in common with the positive I^+ species, with respect to its reaction with tyrosine and histidine. The resulting azoderivatives are colored and like the iododerivatives can be separated from the non reacted peptidic material.

The interest in preparing azoderivatives for further tritiation arise from their specificity towards histidine. Whereas iodine, as mentionned, reacts first with carbon 5 and very rapidly then with carbon 2, leading to di-iodo histidyl derivatives, the diazonium salt reacts more slowly, giving first an azoderivative on carbon 2 /27//29/. This is very interesting since, after replacement by tritium, this labelled isotope will be located on that position. The proton carried by carbon 2 of imidazole rings is exchangeable provided the vicinal N_1 (or π) is not protonated (or hydrogen bonded) nor included in a hydrophobic area /36/. Tritium labelling in that position is therefore a tool in conformational and environmental studies of peptide containing histidine residues.

In addition diazonium salts seems to be less agressive towards fragile groups present in peptide and might allows tritiation in other wise forbidden cases. These features are under investigation.

- O.nitro phenyl sulfenyl chloride (NPS) -

There are biologically active peptides which contain tryptophane and no other aromatic side chain: Pentagastrin : gly-tryp-met-asp-phe(NH_2) is an example. Moreover, even if other aromatic groups are present, it might be interesting to introduce a label in different positions of the molecule. These reasons prompted the search for a reagent able to react specifically with tryptophan and able to be replaced by a tritium atom. Scoffone et al /37/ showed that O.nitrophenyl sulfenyl chloride reacts with indole derivatives and substitutes carbon 2, leading to the formation of a thioether.



The reaction is carried out in pure acetic acid at room temperature. A yellow derivative is formed and the extent of the reaction is controlled by measuring the absorbance at 280 nm. For instance, in pure acetic acid, L-tryptophan at 279 nm has a molar extinction coefficient ε_M : 5800, and as nitrophenyl thioether derivative : ε_M : 16 700.

The presence of the nitrophenyl thioether group promotes an increased solubility into organic solvents, allowing convenient separation of the derivatives from the unreacted peptidic material /30/.

- Tritiation -

After having prepared and characterized the peptidic derivatives, the activating reagent X is removed and a tritium atom introduced in replacement. This process is usually done by a catalytic process using platinum or palladium dispersed on a carrier like Al_2O_3, $BaCO_3$ or charcoal. The carrier has a role in neutralizing or adsorbing at least partially the removed activating group and preventing poisoning of the catalyst. The catalyst is added to the solution of the peptide derivative in the proper solvent, under pure tritium gas. Since gas handling requires a vacuum line, the peptide solution is frozen until the reaction is ready to start. The catalyst as available in the market is saturated with hydrogen. This is important to remember since the preparation of tritiated peptides of high specific radioactivities requires to keep the tritium dilution by the catalyst to a minimum. It is therefore necessary to extract and displace the adsorbed hydrogen by tritium gas in preliminary manipulations of the catalyst in the dry state.

The second point worth mentionning is that the catalyst promotes not only the removal of the activating group X, but also the exchange of the protons present in the solvent where the peptide derivative is dissolved. This exchange results in dilution of the tritium adsorbed on the catalyst. All efforts should therefore be taken to remove catalyst poisons which would slow down the removal of the activating group.

The procedure utilized, in its details depends of the compound considered, and these details can be found in the relevant litterature.

We shall note here only some general aspects.

1 - Removal of the activating group iodine, azoderivatives of O.nitrophenyl thioether occurs with elimination of the whole group. If this is obvious for iodinated derivatives, it is worth mentionning for the two other cases. In general, one tritium atom undiluted is introduced instead.

The new characteristics given to the peptide when the activating

group is introduced, are removed with the letter. For instance, when di-iodoangiotensin II is tritiated, full biological activity is recovered. The complete return to the initial properties of the peptide is a demonstration that neither the preparation of the intermediate, nor the tritiation step have altered the primary structure.

2 - The localisation of the tritium atom is in principle that of the removed activating group. However, since we are dealing with aromatic cycles which can be labelled by direct chemisorption on the catalyst /23//24/, some exchange takes place as secondary reactions.

This remark is more academical than practical, because of the different order of magnitude of the corresponding rate constants. The main reaction is over in a matter of minutes, the other in a range of days.

3 - Deleterious side reaction promoted by the catalyst itself are not numerous with respect to peptide chemistry. Reduction of disulfide bridges can be readily avoided by maintaining the tritium pressure below 500 mbars the pH below 8, and the temperature below 20°C.

Opening disulfide bridges however can occurs resulting from an oxidative scission. Cysteine sulfinic and cysteic acids are formed, as a consequence of the removal of iodine. This estonishing oxidative reaction takes place under reductive conditions. We believe that the catalyst adsorbs the iodotyrosyl residue and nearby the disulfide bridge. When iodine is removed it is transfered to the sulfur, leading to the formation of iodosulfonium derivatives. Their hydrolysis conducts then to the oxidized forms of the sulfur and to an opening of the bridge.

It has been proven that soluble iodine scavengers do not prevent the phenomenum, which is therefore meant to take place on the catalyst surface.

A source of complications is found with aged samples of tritium gas. The formation of helium belongs to the process of tritium desintegration. When helium accumulates it is present not only as an inert gas, but also in the form of activated species which react with the peptide in a variety of ways, and lead to inactivation and to polymerisation. The only way to avoid these troubles is to use freshly purified tritium.

4 - After tritiation, the material recovered is submitted to purification and the non tritiated derivatives are removed by applying the principle having allowed in the first step purification of the peptide derivatives from the non reacted material.

In general, the yields are low, of the order of 20%-30%, since

peptide are well retained on the catalyst and its carrier. When disulfide bridges are present, the yields are still lower.

IV - Storage -

Labelled material is stored in dilute solution and especially when histidin residues are carrying the label at pH close to 4, to avoid a slow exchange. It is often recommended to keep the material frozen in liquid nitrogen. This represents the best condition of storage, particularly for aqueous solution, for the following reason. When an aqueous solution crystallizes, the crystals are made of pure water and the solute concentrates dramatically. This increase in concentration favors the kinetics of radiochemical reactions, whereas the lowering of temperature has the opposite effect. What has to be considered is the balance between both factors and, as a rule, -30°C is a temperature where the concentration effect overcomes largely the temperature effect. It is only below -100° that the storage is the frozen state becomes meannigfull.

V - Conclusions -

The needs for labelled peptidic hormones are growing and the state of the art requires further developments. First of all, when a peptide does not contain aromatic aminoacids, direct labelling is presently not possible. One has to rely upon synthesis of at least part of the sequence, or upon preparation of an analog, the added group carrying the label. This situation should be improved.

An other aspect is the trend towards higher specific radioactivities. Tritium has its own limitations, in this respect. Iodine whose isotopes are convenient, raise other questions which have been discussed. One might think of ^{11}C, offering a tremendously high theoretical specific radioactivity, but of a half life of 20 min. Work with this isotope requires rapid chemical processes and some efforts have already been made in this direction for the labelling of luteinizing hormone /39/. One can predict that the present interest for drastic reduction of the time scale of chemical processes will find numerous applications in the field of molecular pharmacology.

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