PREPARATION OF HIGH SPECIFIC ACTIVITY DL-TRYPTOPHAN-2, 3-T AND ITS RESOLUTION TO L-TRYPTOPHAN-2, 3-T.

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SUMMARY

DL-Tryptophan-2, 3-T of high specific activity was obtained by reducing a suitable precursor with T_2 gas. It was resolved also into L-Tryptophan-2, 3-T by using the enzyme carboxypeptidase. The optical purity of L-Tryptophan-2, 3-T was greater than 99% and the specific activity was 15 Ci/mM.

RESUME

On a obtenu du DL-Tryptophan-2, 3-T de haute activité spécifique par la réduction d'un précurseur convenable par le gaz tritium. La résolution du DL-Tryptophan-2, 3-T a été réalisée par l'enzyme carboxypeptidase. La pureté optique a été supérieure à 99% et l'activité spécifique de 15Ci/mM.

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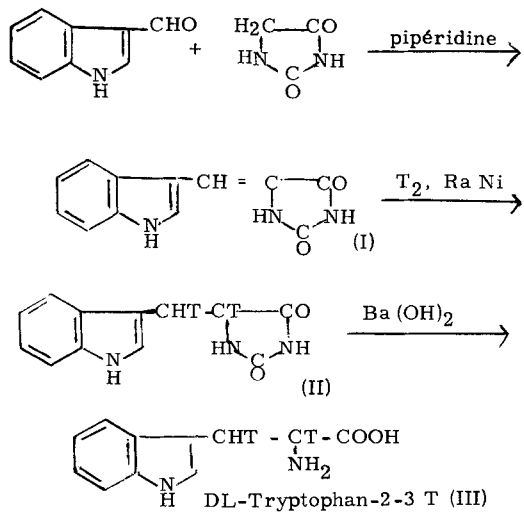
MATERIALS AND METHODS

Tryptophan labelled with Tritium has been used in the assay of Tryptophan hydroxylase (1) (8). DL-Tryptophan-5-T prepared by catalytic dehalogenation of 5 Bromo Tryptophan (2) has been the labelled compound in such investigations. Since the reaction site and position of the label were the same, a large amount of labile activity was introduced into the reaction medium when Tryptophan 5-T was used.

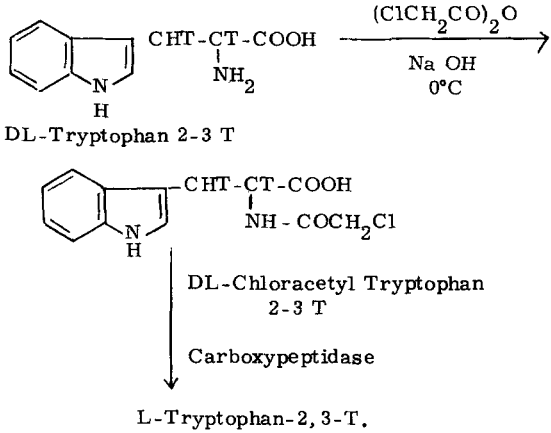
Therefore, we considered the possibility of tritium labelling of Tryptophan in positions, other than 5, preferably in the aliphatic side chain. Recently, the preparation L-Tryptophan-2-T was reported by a combination of racemisation technique and enzyme resolution, starting from L-acetyl Tryptophan (3). But the specific activity was very low (≃ 15mCi/mM). We have found one of the chemical methods of synthesis (4,5), could be conveniently adaptated to prepare DL-Tryptophan-2, 3-T of higher specific activity (15 Ci/mM).

The following reaction scheme was used :



Further, since the L form of the amino-acid was the substrate in enzyme assays, DL-Tryptophan-2, 3-T was resolved to L form without lowering of the specific activity.

The resolution of III was achieved by preparing its chloracetyl derivative and enzymatically hydrolysing the latter to L-Tryptophan with carboxypeptidase :



We did not find any experimental evidence to think that either of the tritium label was labile during the derivatisation of Tryptophan and its subsequent resolution.

EXPERIMENTAL

Indolydenehydantoin (I)

The precursor for tritiation (I) was prepared as per method (4). Indole-3 aldehyde and hydantoin were mixed in 1 : 1.2 molar amounts, 10 ml of piperidine was added and the mixture was refluxed for 30 minutes. After 15 minutes, the highly coloured solution began to deposit a yellow crystalline solide indolydene hydantoin piperidine salt. It was poured into a large bulk of water and acidified (pH~ 5) with acetic acid. The ppt. was collected, washed with methanol to remove any unchanged aldehyde and some colouring matter and finally recrystallised from glacial acetic acid. The chemical purity of the compound was checked by TCL of an aliquot of a solution in dimethyl formamide. Only a single spot was obtained having an Rf = 0.32, the solvent system for development being chloroform : acetic acid (95 : 5 ; v/v).

Tritiation of (I)

In a 3-5 ml round bottom flask, 15 mg of indolydene hydantoin, 0.7 ml of 1N NaOH and 0.7 to 1.0 ml, Raney Ni catalyst suspension (6) were placed and the flask was connected to tritium gas. The yellow suspension gradually turned colourless and the solid dissolved completely. After an hour when the absorption of tri-

tium ceased, the reaction vessel was disconnected and the catalyst was removed by filtration. The filtrate was rotary evaporated repeatedly, untill the labile activity was removed. The white solid left behind (II) was dissolved in small volume of water. The product hand an Rf of 0.22 when it was chromatographed by TLC in the solvent chloroform acetic acid (95 : 5 v/v). The radiochemical purity of the product was around 80%. The ultra-violet spectra of the product ressembled qualitatively with that of Tryptophan (λ_{max_1} : 278, 287 = λ_{max_2}).

Isolation of DL-Tryptophan-2,3-T

The reduced indolydene hydantoin (II) was hydrolysed by refluxing it with 90 mg of crystalline Ba $(OH)_2$ for about 20 hours. A TLC followed by radio scan of an aliquot of the hydrolysate revealed more than 70% radioactivity in the region corresponding to inactive Tryptophan. The hydrolysate was then applied to the top of a column of Dowex 1 x 8 in the acetate form (column height 30 cm, outer diameter of column : 1.2 cm and the flow rate 20ml/hr). After washing the column with about 100 ml water, dilute acetic acid (0.2 M) solution was used to displace the Tryptophan from the column. The fractions having high radioactive content were pooled together and subsequently aliquotes were withdrawn to measure, the radiochemical purity and specific activity. DL-Tryptophan-2, 3-T, had a specific activity of 15 Ci/mM while its radiochemical purity was more than 97% by paper chromatography in the solvent systems (1) Butanol-acetic acid-water (50 : 25 : 25) and (2) isopropanol-ammonia-water (85 : 5 : 15). About 400 mCi of DL-Tryptophan-2, 3-T was obtained in each tritiation experiment.

DL-Chloracetyl Tryptophan-2, 3-T

The solution of DL-Tryptophan-2, 3-T was concentrated to small volume (~ 0.2 ml) and to this was added 0.5 ml 2N NaOH. After chilling the reaction mixture in ice, with vigorous stirring, solid chloracetic anhydride (~ 100 mg) was added and the stirring continued. The reaction mixture was checked by TLC to know wheter Tryptophan had completely reacted to give the chloracetyl derivative (Rf Trypt. 0.51 ; Rf Chloracetyl Tryp. 0.85, TLC, the solvent-butanol acetic acid-water (30 : 10 :10)

In case Tryptophan had reacted only partially, more alkali and chloracetic anhydride were added and the mixture again analysed. When most of the Tryptophan had reacted, the reaction mixture was acidified (pH 5) with acetic acid, transfered to a liquid liquid extractor and continously extracted with ethyl acetate for 4 hours. The ethyl acetate phase contained pure chloracetyl Tryptophan with very little contamination by Tryptophan. Paper chromatography of an aliquot indicated less than 3% activity associated with Tryptophan. The radiochemical yield of chloracetylation was 66%.

Liquid Liquid Extraction :

Initial activity of DL-Tryptophan-2, 3-T	Activity in Ethyl Acet. phase	Activity in water phase
320 mCi	220 mCi	100 mCi

Enzymatic resolution by DL-Chloracetyl Tryptophan-2, 3-T

The ethyl acetate phase (220 mCi) was rotary evaporated to dryness and 10 ml water were added to dissolve the compound. The pH of the solution was adjusted to 7.2, using 2NLiOH solution. 0.2 ml (~ 4 mg) of a suspension of carboxy peptidase (carboxypeptidase A (No C-8750), Sigma Chemical Company, USA) was added to the solution and the mixture incubated at 38° C for two days. The enzymatic hydrolysis of DL-Chloracetyl Tryptophan to L-Tryptophan was measured periodically by withdrawing aliquots and running chromatogramme on TLC plates. It was observed that complete hydrolysis was rarely possible irrespective of incubation time and the quantity of enzyme used. The maximum conversion that we could ever have was 40%.

Recovery of L-Tryptophan-2, 3-T

The enzymatic reaction mixture was filtered and the filtrate after concentration to a small volume, was applied to a column of Dowex 1 x 8 in acetate form (Column length 30 cm, od. : 1,2 cm and flow rate 4 ml/hr). After washing the column with 50 ml water, dilute acetic acid (0,2 M) was used to elute out the Tryptophan, whereas the Chloracetyl Tryptophan was firmly held by the resin. The fractions corresponding to Tryptophan were pooled up. The total activity of L-Tryptophan was 60 mCi (50% theoretical yield for resolution). Aliquots were then used to measure the specific activity and radiochemical purity. The results were :

L-Tryptophan-2, 3-T

(A)

Paper Chromatography Solvent System			Radiochemical purity
1. Butanol	Acetic Acid	Water	
50	25	25	> 97 °/.
2. Isopropanol	Ammonia	Water	
85	5	15	> 97 °/.
3. Isoamyl alcohol	Pyridine	Acetic Acid (10)	
40	80	Water(40)	> 97 °/.

(B)

Ion Exchange Chromatography (by Amino Acid Analyser JEOL)	> 95 °/.
Specific Activity	15 Ci/mM

Optical Purity of L-Tryptophan-2, 3-T

The optical purity of L-Tryptophan-2, 3-T was estimated by reacting amino acid with the enzyme L amino acid oxidase as per the method (3, 7). To about 2mCi of the amino acid dissolved in 0.5 ml Tris buffer (pH 7.3), 0.1 to 0.2 ml L-amino acid oxidase suspension (crotalus adamenteus venom, Nutritional Biochemical Corpon, USA) was added, 5 mg catalase (NBC) was added and the mixture incubated at 38°C.

After 24 hours, an aliquot of reaction mixture was chromatographied by TLC. The radio scan of the plate indicated complete disappearance of activity from Tryptophan region and reappearance of all activity in a region corresponding to the oxidised product of Tryptophan. From the area of the two regions, the optical purity of L-Tryptophan-2, 3-T was computed as more than 99%.

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