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Preparation of Tritium-Labelled D,L-2,6-Diaminopimelic Acid

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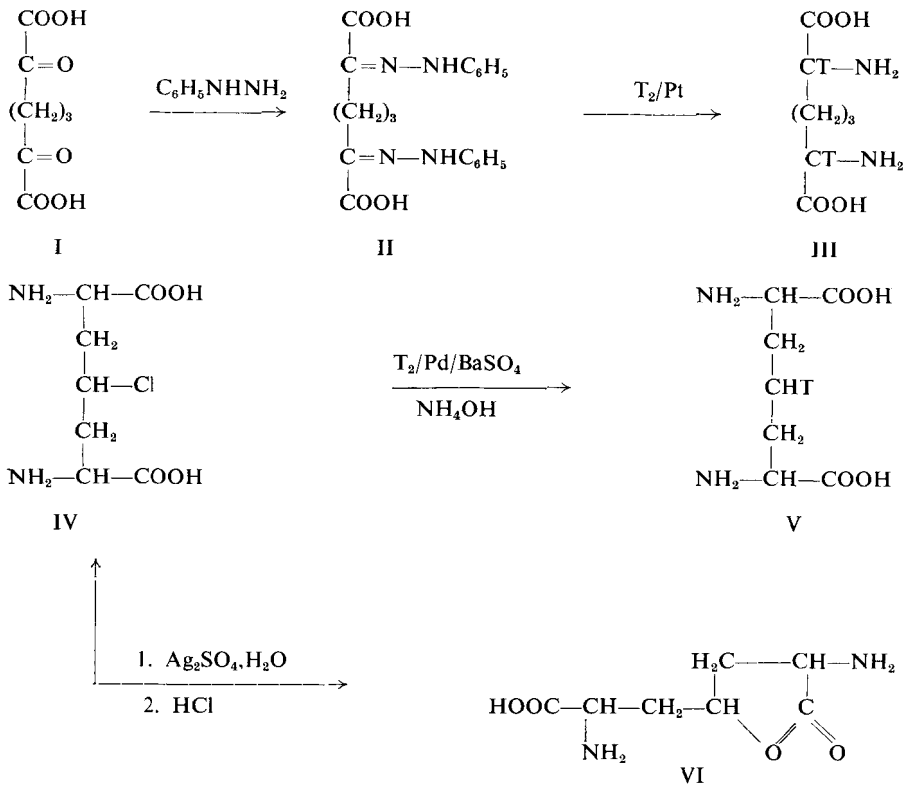
SUMMARY

On hydrogenation of the 2,6-bis(phenylhydrazone) of 2,6-diketopimelic acid with carrier-free tritium a very low specific activity was achieved, due to the labile C-T-bonds in the α-positions. 2,6-Diaminopimelic-4-^{3}H acid of spec. activity 790 mCi/mmole was obtained by catalytic dehydrohalogenation of 4-chloro-2,6-diaminopimelic acid in 1N ammonia. A new, simple apparatus for catalytic tritiation is also described.

2,6-Diaminopimelic acid (DAP) is the construction unit of peptides of the cell membranes of microorganisms. In our previous communication [1] we described the preparation of 2,6-diaminopimelic-2-^{14}C acid. For autoradiographic investigation of the localization of DAP in cell tissues, tritium-labelled DAP is more suitable; the preparation of DAP of high specific activity was not described so far. We tried first to introduce tritium by hydrogenation of the double bonds in the 2,6-bis(phenylhydrazone) of 2,6-diketopimelic acid.

From 2,6-diketopimelic acid I (Cope and Fournier) [2] we prepared its bis(phenylhydrazone) II and we hydrogenated it with carrier-free tritium. Hydrogenation took place most rapidly on PtO_2 (Adams catalyst) in ethyl acetate. After the consumption of approx. 3 equivalents of tritium (per one eq. of phenylhydrazone II) the hydrogenation was interrupted and the reaction (hydrogenation) was brought to completion with hydrogen. After elimination of the labile activity and purification of the product by paper chromatography, however, the specific activity of DAP was 8 mCi/mmole only. It is evident from the activity balance of the experiment that practically the total tritium in the positions 2 and 6 was exchanged under the conditions of hydrogenation (with hydrogen) to completion. During the elimination of the labile activity and subsequent chromatography practically all the activity was washed out from the product.



Diaminopimelic acid of satisfactory specific activity was obtained by catalytic dehydrohalogenation of 4-chloro-2,6-diaminopimelic acid IV, prepared earlier (Hanuš and Vereš) [3]. It is known that the exchange between gaseous tritium and water takes place substantially more slowly in alkaline medium. Therefore we used as solvent 1N ammonia which binds simultaneously the liberated hydrogen chloride. $Pd/BaSO_4$ (10 %) was used as catalyst. Great attention was devoted to the purification of the final product from the traces of 4-chloro-2,6-diaminopimelic acid. We made use of the aqueous hydrolysis of 4-chloro-2,6-diaminopimelic acid to 4-hydroxy-2,6-diaminopimelic acid described by us [3], and of the possibility to convert this 4-hydroxy acid into lactone (VI). This lactone can be separated from 2,6-diaminopimelic acid-4-T (V) with advantage by preparative electrophoresis and thus possible traces of the unreacted 4-chloro-2,6-diaminopimelic acid can be eliminated.

The hydrogenations were in both cases carried out in an apparatus enabling a more convenient handling of small volumes of gases and also the performance of the hydrogenation at constant pressure. The description of the apparatus is given in the experimental part.

EXPERIMENTAL.

2,6-Bis(phenylhydrazone) of 2,6-diketopimelic acid II.

1.13 g (6 mmole) of 2,6-diketopimelic acid (Cope and Fournier) [2] was dissolved in 15 ml of glacial acetic acid at 60° C. The undissolved small fraction was filtered off while warm and 1.4 ml of phenylhydrazine were added immediately to the filtrate. On standing in a refrigerator yellow crystals separated out. They were filtered off under suction, washed with glacial acetic acid and eventually with a mixture of ethanol and water (5 : 1). Recrystallization was carried out from ethanol-water. Yield 1 g (45 %) of 2,6-bis(phenylhydrazone) II, m.p. 174-176° C.

Analysis for $C_{19}H_{20}N_4O_4$ calculated : C, 62.0; H, 5.5 %; found : C, 62.0; H, 5.7 %.

Hydrogenation of 2,6-Bis(phenylhydrazone) of 2,6-diketopimelic acid with tritium.

Adams catalyst (29 mg of PtO_2) was prereduced with hydrogen in 0.3 ml of ethyl acetate. To the resulting suspension, 20 mg of 2,6-Bis(phenylhydrazone) II were added and the mixture was tritiated with carrier-free tritium at constant pressure of 650 torr. When 4 ml of tritium were absorbed, corresponding to 75 % of the theoretical consumption, the tritiation was interrupted; 0.3 ml of water, 0.2 ml of ethanol, and 0.02 ml of conc. HCl were added, and the hydrogenation was continued with an excess of hydrogen at 610 torr until the absorption ceased. The catalyst was filtered off and the solvent evaporated to dryness. The residue was evaporated twice with excess ethanol. The residue was chromatographed preparatively on Whatman No. 3 paper in n-butanol-acetic acid-water (4 : 1 : 5).

Detection was carried out by radioautography and the zone corresponding to 2,6-diaminopimelic acid was cut out and eluted with 3 % ammonia. Lyophilisation of the eluate to dryness gave 10 mg (96 %) of chromatographically pure 2,6-diaminopimelic acid. The product had specific activity 8 mCi/mM.

Activity balance of the experiment :

Tritium consumed for tritiation (4 ml) : approx. 10,000 mCi

Activity in the solvent and washed out activity : 914 mCi

Activity applied on the chromatogram : 130 mCi

Activity of the pure 2,6-diaminopimelic acid (10 mg) eluted from the paper : 0.42 mCi.

During the completion of the hydrogenation (with hydrogen) at least 9,000 mCi were therefore exchanged from the product.

Hydrogenation of 4-chloro-2,6-diaminopimelic acid IV.

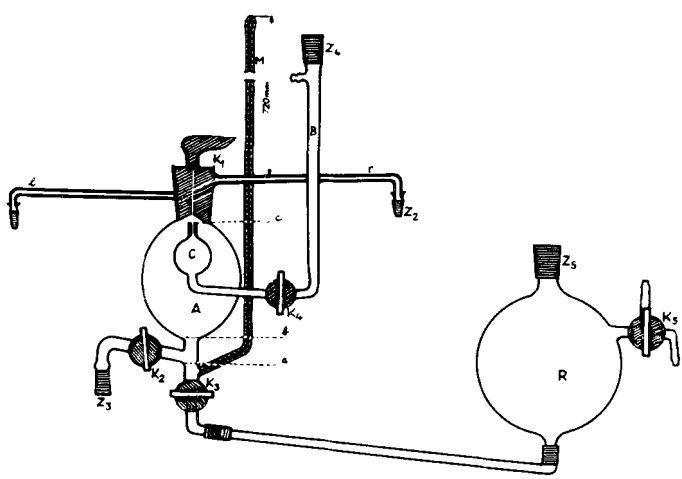
4-Chloro-2,6-diaminopimelic acid hydrochloride (Hanuš and Vereš) [3] (20.3 mg, 0.081 mmole) was dissolved in 0.5 ml of 1N ammonia and hydroge-

nated on 60 mg of $Pd/BaSO_4$ (10 %) with carrier-free tritium at constant pressure of 626 torr. After 90 minutes the absorption corresponded to the theoretical value (1.75 ml). The catalyst was filtered off and the solvent freeze-dried. The labile activity was washed out by double freeze-drying with an excess of water. The residue after the last freeze-drying was dissolved in 2 ml of water, additioned with 27 mg of silver sulfate, and stirred for 24 hours. The suspension was acidified with hydrochloric acid, the resulted silver chloride was filtered off and the filtrate evaporated to dryness. The dry crude 2,6-diaminopimelic acid was dissolved in a small amount of water and applied on a sheet of Whatman No. 3 paper (10 cm width). Preparative electrophoresis was carried out in water-pyridine-acetic acid (per 1 l of water, 2.5 ml of glacial acetic acid and 10 ml of pyridine). After 90 minutes at 600 V, the zone corresponding to 2,6-diaminopimelic acid was cut out and eluted with 5 % ammonia. The eluate was decolorized with active charcoal and the solvent was eliminated by freeze-drying. The 2,6-diaminopimelic acid-4-^{3}H obtained (14 mg; 95 %) had specific activity 790 mCi/mmole.

The radiochemical purity of the product was checked by scanning of a chromatogram of the product.

Activity balance of the experiment :

The activity of tritium consumed for tritiation (1.8 ml) : approx. 4,500 mCi
Activity in the solvent : 4,800 mCi
The labile, washed out activity : 130 mCi
Applied on the preparative electrophorogram : 60 mCi
2,6-diaminopimelic acid-4-^{3}H (14 mg) eluted : 64 mCi.



Hydrogenation apparatus.

All hydrogenations were carried out on an apparatus represented in Figure which was developed in our laboratory (Hanuš) [4].

The two main parts of the apparatus are the reaction chamber A and the mercury reservoir R. These two parts are connected by a tube through which mercury is transferred from one component to the other. The reaction chamber A is provided at the top with a stopcock K_1 which has a double oblique bore and whose plug is hollowed at the bottom in conical shape. Two arms, l and r, are fused at different heights on to the stopcock. These arms, ended with ground glass joints, serve for connecting the source of tritium and the hydrogenation vessel, respectively. At the bottom the reaction chamber A is provided with a side tube and stopcock K_2 by which the apparatus can be evacuated. Somewhat below this side tube, a capillary tube M, serving as a manometer, is fused on. The inlet of mercury from the reservoir R can be shut off by the capillary stopcock K_3. The vessel C inside the chamber A is connected with the burette B by a tube passing through the wall of A and by the stopcock K_4. The volumes of C and B are identical. The ground glass joint Z_4 serves for filling the burette B with mercury.

At the top the reservoir R is provided with a ground glass joint Z_5 (for filling with mercury) and with a three-way stopcock K_5 by means of which R can be either evacuated or connected with the atmosphere.

Procedure for the hydrogenation with carrier-free tritium :

The reservoir containing UT_3 is connected with arm l and the hydrogenation vessel is connected with r. After evacuating the apparatus the stopcock K_1 is turned to the left and by heating the reservoir with UT_3, tritium is forced above the mercury in A which is at the level b. By raising the level of mercury in A and turning K_1 to the opposite side, the gas is forced into the reaction vessel; the level of mercury is raised up maximally to the mark c. It is apparent that the maximum pressure attainable in the apparatus by connecting R with the atmosphere equals the atmospheric pressure less the difference in levels of mercury in A and R. In our construction this was 660 mm Hg. As long as the level of mercury in A is below the mark c it is possible by a small positive pressure in R to raise this level and to attain during hydrogenation pressures of tritium up to 720 mm Hg. In the course of hydrogenation, the tritium which is consumed is replaced by adding mercury from the burette B into vessel C so as to maintain constant the pressure indicated by the manometer M. The amount of tritium incorporated in the course of one hydrogenation into the substance treated is limited by the volume of C and therefore also of the burette B.

This tritiation apparatus is universal and serves very well also for the following operations : for transferring of tritium from ampoules and containers for active uranium, for the activation of uranium, for the precise quantitative dilution of carrier-free tritium with hydrogen, for inactive hydrogenations in

model experiments, for other processes in which gaseous tritium is employed (for example the Wilzbach reaction).

All activity measurements were carried out on a scintillation apparatus Mark I (Nuclear Chicago).

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