

RADIOLYSIS OF ^{14}C - AND ^3H - LABELLED THYMIDINE

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SUMMARY

10^{-3}M oxygenated aqueous solutions of thymidine preparations, labelled with ^{14}C and ^3H in various positions were gamma-irradiated. Radiation products formed were followed in dependence of irradiation doses. Their yields were confronted with products formed during the autoradiolysis of long time stored tritiated thymidine preparations, and results obtained were discussed.

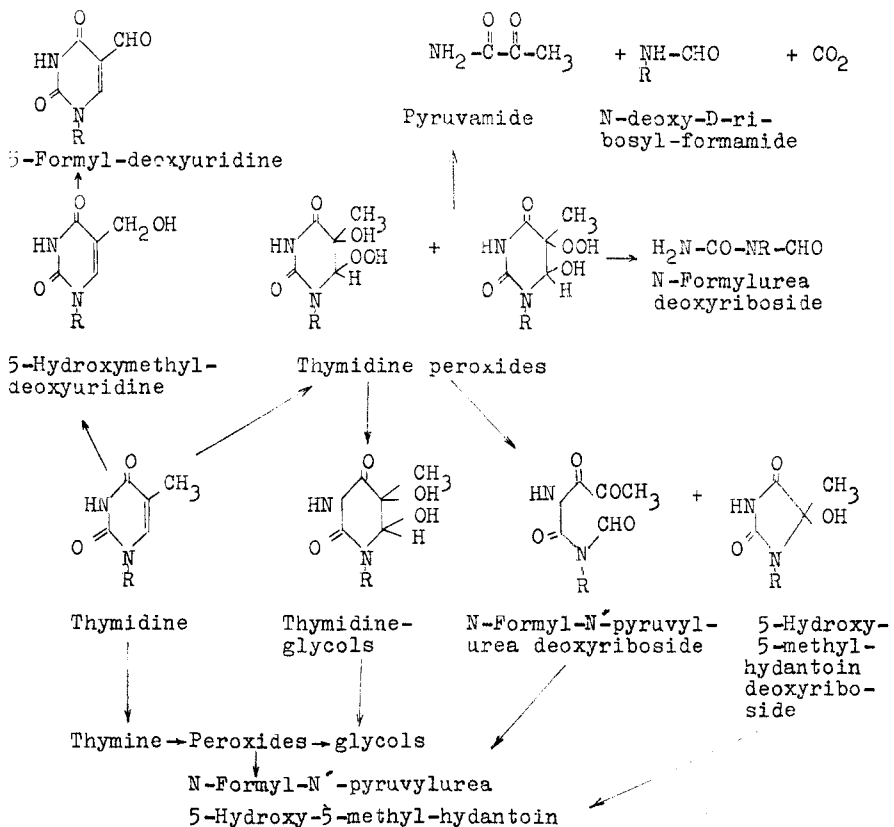
Introduction

Thymidine labelled in various positions with radioactive isotopes of carbon and hydrogen is widely used as a tracer substance in biochemical and biological research. Therefore the knowledge on radiation stability of thymidine as well as on radiation transformation of this substance has both a theoretical and a practical importance.

A number of papers(1,2) report on the studies of thymidine radiolysis, carried out by irradiation of its aqueous solutions. The autoradiolysis of ^3H -thymidine, stored at various specific activities and under various conditions(3,4,5), was also investigated. In all these studies distinct changes were demonstrated which took place in the thymidine molecules in consequence of the effect of reactive water particles, especially of OH radicals formed in the course of irradiation. From the above mentioned papers it followed that in oxygenated solutions thymidine hydropero-

xides and glycols are formed, and the cleavage of the pyrimidine nucleus takes place. As the main products, pyruvamide and N-deoxy-D-riboseyl-formamide have been described; N-formyl-deoxy-D-riboseyl-carbamide and 5-hydroxy-5-methyl-hydantoin deoxyriboside were found in a lower yield. 5-Hydroxymethyl-deoxyuridine and 5-Formyl-deoxyuridine in small amounts were also formed. In consequence of radiation a cleavage of the N-glycosidic bond takes place in the molecule of thymidine, setting free thymine, which undergoes its own radiolytic degradation(6,7,8).

Hence, the radiation decomposition of thymidine in oxygenated solution may be expressed in a simplified manner by the following schema:



The aim of our work was to carry out a qualitative and quantitative analysis of the radiation products of thymidine labelled with radiocarbon or tritium in various positions and to follow their formation in dependence on the irradiation dose. The comparison of the radioactivity of single radiation products, formed in irradiated solutions of 2- ^{14}C -thymidine, $^{14}\text{CH}_3$ -thymidine, 6- ^3H -thymidine and methyl- ^3H -thymidine, enabled us to obtain basic informations of the character of the radiation products.

The products, whose cyclic structure remains in the course of irradiation intact, are formed in all preparations of thymidine investigated with the same proportions.

The products which are formed in consequence of the cleavage of the pyrimidine nucleus have a different radioactivity which depends on the position of the radioactive atom in the original thymidine molecule.

The results obtained during the study of gamma-irradiated solutions of labelled thymidine preparations were confronted with the actual beta-autoradiolysis of the tritiated thymidine, and we would like to draw the attention of investigators on certain complications which may occur during the storage of labelled thymidine preparations in consequence of autoradiolysis.

Materials and methods:

2- ^{14}C -thymidine, $^{14}\text{CH}_3$ -thymidine, 6- ^3H -thymidine and methyl- ^3H -thymidine were prepared in the Institute for Research, Development, and Production of Radioisotopes, Czechoslovakia. The solutions of the preparations labelled with radiocarbon were diluted to radioactive concentration of 22 and 8.5 $\mu\text{Ci/ml}$, while the tritiated preparations were diluted to 640 $\mu\text{Ci/ml}$. On addition of unradioactive thymidine (Koch-Light) their chemical concentration was adjusted to the value 10^{-3}M .

For irradiation a ^{60}Co source of an intensity $4.7 \cdot 10^{18} \text{eV/ml/}$

hour was used. The radiation doses ranged from $4.7 \cdot 10^{16}$ to $30.55 \cdot 10^{18}$ eV/ml.

The irradiated solutions were analysed about 24 hours after irradiation by two-dimensional thin-layer chromatography on silica gel (Silufol, Kavalier, Czechoslovakia) in the systems: I. chloroform-formic acid-water (4-2-1) and II. ethyl acetate-isopropyl alcohol-water (75-16-9). The identification of single radiation products was carried out both using the corresponding standards, and on the basis of the data on their R_F values (9). In order to make the identification and quantitative determination of the radiation products easier, mixed solutions of ^{14}C -thymidine and ^3H -thymidine were irradiated in some instances. Autoradiography, which in this case was carried out by the two-phase method, i.e. before and after the impregnation of the chromatograms with the scintillation liquid, enabled an orientational difference to be made between the products labelled with ^{14}C from those labelled with ^3H .

Quantitative evaluation of these substances was carried out after their isolation from chromatograms by liquid scintillation method on a Mark II instrument.

For studying the autoradiolytic process an average sample from a mixture of tritiated preparations 6- ^3H -thymidine was used, which were supplied by the producer with a specific activity about 25 Ci/mM and had been stored in aqueous solution of radioactive concentration of 1 mCi/ml at $+2^\circ\text{C}$ for 30 months.

A parallel unradioactive solution of thymidine was also irradiated in order to obtain a larger amount of radiation products. In this case Fink's reagent (10), diphenylamine reagent for sugars, hydroxamate test for the determination of formamide, and a solution of 2,4-dinitrophenylhydrazine were used for their detection on thin-layer plates.

Results and discussion

The yields of single radiation products were determined, which

were formed on irradiation of oxygenated solutions of 2- ^{14}C -thymidine, $^{14}\text{CH}_3$ -thymidine, and 6- ^3H -thymidine with gradual doses of gamma-irradiation. The results are expressed in percentages of the original radioactivity of non-irradiated solutions of single preparations of thymidine.

The radiation products, in which degradation of original ring structure does not take place, i.e. hydroxyperoxides and glycols of thymidine, 5-hydroxymethyl-deoxyuridine, further thymine and its radiation products, give the same yields in all mentioned preparations of thymidine. Their dependence on the dose is represented in Fig.1, with the exception of hydroxyperoxides which are unstable in aqueous solution and were not evaluated quantitatively.

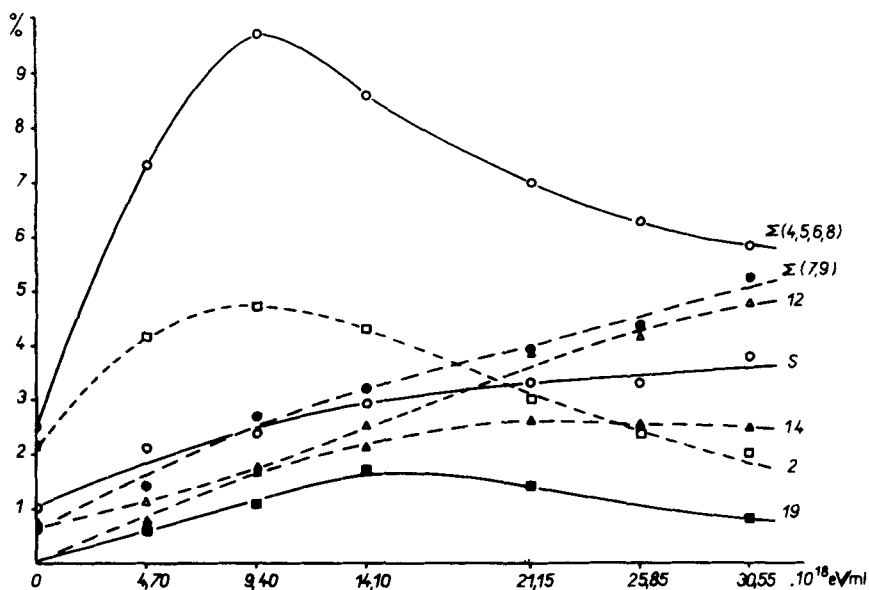


Fig. 1 Formation of radiation products in oxygenated gamma-irradiated solution of ^{14}C and ^3H labelled thymidine.
 x-axis: radiation dose in 10^{18} eV/ml
 y-axis: % of radioactivity of original unirradiated sample. Numbers of the curves correspond to the products listed in Table 1.

As is evident from the course of curve marked 4,5,6,8 in Fig.1, the formation of totally expressed glycols of thymidine increases first with the radiation dose. However having reached its maximum at a dose of $9.4 \cdot 10^{18}$ eV/ml a drop in their amount takes place - the glycols of thymidine are evidently decomposed in the course of further irradiation. A similar course, though in a much lower yield, is observed for the formation of 5-hydroxy-thymidine and 5-formyl-deoxyuridine (curve 19 in Fig.1). Thymine split off by radiation (curve 2 in Fig.1) undergoes its own radiation degradation. Its content decreases during irradiation, while the yields of total glycols (curve marked 7 and 9 in Fig.1) and further degradation products of thymine also given in Fig.1 increase proportionally.

This is primary N-formyl-N-pyruvylurea (curve 14 in Fig.1) whose initial increase gradually slows down, clearly in consequence of the cyclisation of this substance to 5-hydroxy-5-methyl-hydantoin the amount of which increases with the radiation dose (curve 12 in Fig.1).

In addition to the mentioned radiation products further substances are also formed on irradiation of thymidine. These substances remain on the start when submitted to two-dimensional chromatograms in the developing solvents mentioned. The yield of these products expressed as per cent of radioactivity of the original unirradiated solution is practically identical in all preparations of labelled thymidine used in these experiments, and it increases with the radiation dose (curve S in Fig.1). The identification of these radiation products has not yet been carried out.

Fig.2 shows the dependence of the radiation degradation of thymidine (curve 1, Fig.2) and the formation of radiation products formed on degradation of the pyrimidine nucleus on the dose. In the irradiated solution of $^{14}\text{CH}_3$ -thymidine radioactive pyruvamide is formed (curve 10, Fig.2). From the graph it follows that the yield of this substance increases proportionally with the radiation dose

and it belongs among the main products of radiolysis of thymidine. The formation of a further radioactive product in this solution, i.e. N-formyl-N-pyruvylurea deoxyriboside or 5-hydroxy-5-methylhydantoin deoxyriboside takes place in very low yield.

When the solution of 6- ^3H -thymidine is irradiated N-deoxy-D-ribose-formamide is formed as the main radioactive product(curve 3, Fig.2). Its increase stops after a maximum is attained, and it decreases in the course of further irradiation, while two other tritiated radiation products are formed; these products are the as yet unidentified fragments of thymidine(curves 11 and 15, Fig.2).

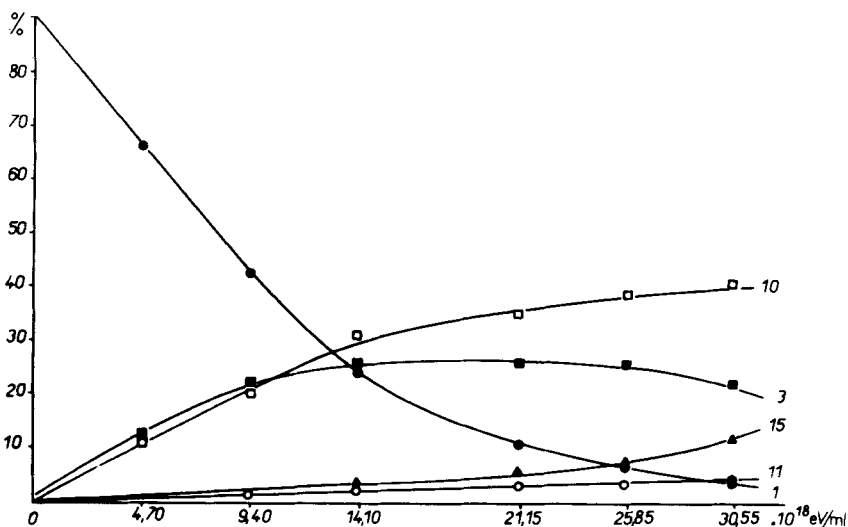


Fig.2 Decrease of thymidine and formation of radiation products in oxygenated gamma-irradiated solution.

x-axis: radiation dose in 10^{18} eV/ml

y-axis: % of radioactivity of original unirradiated solution. Curve numbering correspond to the products listed in Table 1.

N-Formylurea deoxyriboside also belongs to the radioactive products which were demonstrated in the irradiated solution of tritiated

Table 1

| Decrease of thymidine and formation of its radiation products (in % of radioactivity of unirradiated sample) | a.) External gamma-radiolysis | | | | | b.) Autoradiolysis | | |
|--|--|--------|---------|--------|--------|--------------------|------------|-------------|
| | Radiation doses ($\cdot 10^{18}$ eV/ml) | | | | | Time of storage | | |
| | Thd CH_3 - ^{14}C | | Thd-6-T | | | Thd- CH_2 T | Thd-6-T | |
| | 4.7 | 9.4 | 21 | 4.7 | 9.4 | 21 | (9 months) | (30 months) |
| 1. thymidine | 67.5 | 46.4 | 11.0 | 68.0 | 46.5 | 11.1 | 61.4 | 38.7 |
| 2. thymine | 4.9 | 4.9 | 3.3 | 3.8 | 4.4 | 2.8 | 3.5 | 4.1 |
| 3. N-deoxy-D-riboseyl formamide | 0 | 2.0 | 1.4 | 1.5 | 2.0 | 1.4 | 1.6 | 7.5 |
| 4. thymidine-glycol_cis (+) | 2.3 | 3.3 | 2.3 | 2.1 | 3.4 | 2.5 | 1.9 | 1.3 |
| 5. thymidine-glycol_cis (-) | 1.9 | 2.5 | 1.7 | 2.1 | 3.1 | 1.8 | 1.5 | 1.7 |
| 6. thymidine-glycol_trans (-) | 1.7 | 2.2 | 1.5 | 1.8 | 2.3 | 1.7 | 1.1 | 1.0 |
| 8. thymidine-glycol_trans (+) | 0.6 | 1.1 | 2.0 | 0.6 | 1.2 | 2.1 | 0.5 | 1.1 |
| 7. thymine-glycol_cis | 0.8 | 1.5 | 2.0 | 0.8 | 1.6 | 2.2 | 0.4 | 3.0 |
| 9. thymine-glycol_trans | 11.5 | 20.5 | 34.5 | 0 | 0 | 0 | 7.1 | 1.9 |
| 10. pyruvamide | 0 | 0 | 0 | 0.5 | 1.3 | 3.4 | 0 | 0 |
| 11. unidentified tritiated product | 1.0 | 1.6 | 4.0 | 0 | 0 | 0 | 2.1 | 2.1 |
| 12. 5-hydroxy-5-methyl-hydantoin | 0.7 | 1.7 | 2.6 | traces | traces | traces | traces | 0 |
| 14. N-formyl-N ² -pyruvylurea | 0 | 0 | 0 | 0.6 | 1.5 | 6.4 | 0 | traces |
| 15. unidentified tritiated product | 1.3 | 1.5 | 0.8 | 1.2 | 1.6 | 1.4 | 0.6 | 0.9 |
| 16. 5-hydroxymethyl-deoxyuridine | | | | | | | | 0.6 |
| 17, 18- 5-hydroxy-5-methyl-hydantoin (+) and (-) deoxyriboside | traces | traces | traces | 0 | 0 | 0 | 1.3 | 0 |
| 19. 5-formyldeoxyuridine | 0.7 | 1.0 | 1.4 | 1.1 | 1.5 | 2.2 | 0.7 | 1.1 |
| 20. formamide | 0 | 0 | 0 | traces | traces | traces | 0 | 0.6 |
| S unidentified products on the start | 2.4 | 2.6 | 2.8 | 1.9 | 2.2 | 3.2 | 0.8 | 5.4 |
| volatile tritiated products | 0 | 0 | 0 | 2.5 | 5.0 | 15.0 | traces | 12.0 |

thymidine. Its yield was very low (maximum 0.5%); therefore it was not recorded graphically.

The yields of radiation products formed in oxygenated solutions of ^{14}C - and ^3H -thymidine preparations irradiated with the doses 4.7, 9.4 and 21.10^{18} eV/ml are given in the Table 1a.

From the yields obtained a total balance of radiation decomposition of thymidine taking place during irradiation with a dose $9.4 \cdot 10^{18}$ eV/ml was carried out.

From the total loss of thymidine about 20 % are accounted for by the products formed on cleavage of the N-glycosidic bond which corresponds to the earlier papers(11). The radiation cleavage of N-glycosidic bond takes place not only in the molecules of thymidine but also in the molecule of its irradiation products especially thymidine glycols as it follows from the course of corresponding curves in Fig.1. The remaining part is due to the products of hydroxylation and oxydation reactions, i.e. hydroxy-, hydroperoxy- and formyl-derivatives of thymidine, and products formed by total degradation of the pyrimidine cycle. The mutual ratio of these products depends on the irradiation dose, on the time of storage in solution after irradiation and on the oxygen content in irradiated solutions.

The results were compared **with** those obtained on irradiation of oxygenated solutions of $^{14}\text{CH}_3$ -thymidine and 6- ^3H -thymidine and the same solutions previously saturated with nitrogen. From the comparison of the yields of pyruvamide and N-deoxy-D-ribosyl formamide (Table 2) a direct dependence of their formation on the content of oxygen in the irradiated solutions was shown obviously via the formation of hydroxyperoxides of thymidine.

The effect of oxygen on the radiation decomposition of the pyrimidine nucleus is also demonstrated by the yield of volatile radiation products formed in irradiated solutions of labelled thymidine preparations; in oxygenated solutions their proportion

Table 2

| | | | | | |
|--|----------------|------|------|-------|-------|
| Irradiation dose ($\cdot 10^{18}$ eV/ml) | | 4.7 | 9.4 | 21.15 | 25.85 |
| Pyruvamide | O ₂ | 11.5 | 20.5 | 34.5 | 38.3 |
| | N ₂ | 2.0 | 3.1 | 4.1 | 2.8 |
| (in % of radioactivity of unirradiated solution of $^{14}\text{CH}_3$ -thymidine) | | | | | |
| N-deoxy-D-ribo- syl formamide | O ₂ | 12.7 | 22.5 | 26.3 | 25.6 |
| | N ₂ | 3.6 | 5.8 | 5.4 | 4.2 |
| (in % of radioactivity of unirradiated solution of $6\text{-}^3\text{H}$ -thymidine). | | | | | |

is higher than in oxygen-free solutions. The formation of volatile radioactive products is manifest especially during the irradiation of aqueous solutions of $2\text{-}^{14}\text{C}$ -thymidine. After irradiation with a dose $9.4 \cdot 10^{18}$ eV/ml the yield of volatile radioactive products is up to 25%; the main part is due to $^{14}\text{CO}_2$. The formation of radioactive volatile products was also observed during the irradiation of $6\text{-}^3\text{H}$ -thymidine. At the dose $9.4 \cdot 10^{18}$ eV/ml their yield corresponds about to 5%. A detailed analysis of volatile radiation products was not carried out. For the sake of orientation it was found that during the irradiation of $6\text{-}^3\text{H}$ -thymidine tritiated formaldehyde, formic acid and tritiated water were the components concerned.

The splitting off of tritium atom takes place in consequence of the transformation of thymidine in irradiated solutions, for example during the formation of 5-hydroxy-methyl-hydantoin deoxyriboside which is formed by recyclisation of N-formyl-N-pyruvyl-urea deoxyriboside and its corresponding derivatives formed secondarily from the liberated thymine, or during other still unexplained processes.

To the increase in the amount of tritiated water in irradiated

solution of 6- ^3H -thymidine may also contribute the exchange of tritium in some secondary radiation products for example such as formic acid.

We tried to confront the results which were obtained during external gamma-irradiated aqueous solutions of thymidine with those of beta-autoradiolysis. We used an averaged sample of a mixture of 6- ^3H -thymidine stored for 30 months at 2°C (radioactive concentration 1mCi/ml). The content of thymidine decreased in consequence of autoradiolysis to 39% during this time.

Simultaneously, methyl- ^3H -thymidine was also checked. This preparation had been chromatographically purified and stored 9 months at 2°C (radioactive concentration 2mCi/ml). Its content decreased to 61% during this time.

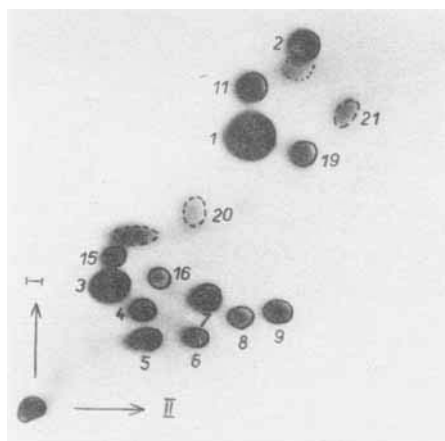


Fig. 3 Autoradiogram of the two-dimensional thin-layer chromatogram of 6- ^3H -thymidine preparation stored 30 months. I.system: chloroform-formic acid-water(4:2:1) II.system:ethyl acetate-isopropyl alcohol-water(75:16:9) Numbers of the spots correspond to the products listed in Table I with exception of product 21, whose qualitative and quantitative evaluation has not been yet carried out.

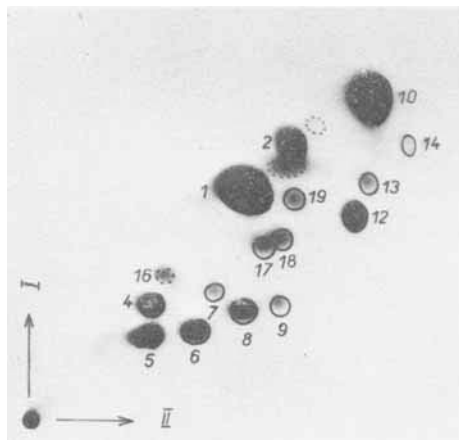


Fig.4 Autoradiogram of two-dimensional thin-layer chromatogram of methyl- ^3H -thymidine preparation stored for 9 months after purification. The same chromatographic systems and spot numbering as in Fig.3 were used. Product 13 remained qualitatively and quantitatively unidentified.

As follows from the Figures 3 and 4 the products of autoradiolysis, which were demonstrated in the solution of tritiated thymidine stored for a long period are qualitatively the same as the products found in gamma-irradiated solutions of these preparations. The radiation products formed were separated chromatographically, estimated, and tabulated. (Table 1,b).

The differences consist in quantitative relations of single radiation products which result from different concentration and quality of solutions used for the study of gamma- and beta-radiolysis of thymidine.

In the preparations of $6\text{-}^3\text{H}$ -thymidine stored for a long time the relatively low content of N-deoxy-D-ribosyl formamide and the increased yield of volatile radiation products is striking. These results may be explained by the fact that on prolonged storage of thymidine in aqueous solution hydrolysis of some radiation products

takes place, especially of N-deoxy-D-ribosyl formamide, which confirms the presence of free formamide in stored solutions. Formamide, which also undergoes hydrolysis, affords formaldehyde and formic acid whose tritium atom may be exchanged in the course of storage by the protium of water.

In consequence of these processes a gradual increase in the content of volatile radiation products, including tritiated water, takes place during the storage of tritiated preparations of thymidine.

During the control of their purity the possible presence of volatile radioactive products of beta radiolysis should be also checked.

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