THE STABILITY OF THYMIDINE, URIDINE AND THEIR RELATED NUCLEOTIDES, LABELLED WITH TRITIUM

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SUMMARY

Accurate knowledge of the stability of (3H)-uridine, (3H)-thymidine, and their triphosphates is important for their use as tracers in biological systems. Data for the stability of these compounds at different storage temperatures and in the presence of a stabiliser, ethanol, are presented. The effects observed are discussed in terms of current theories on radiation self-decomposition.

High specific activity tritium-labelled thymidine and uridine, and nucleotides containing these base moeties, are extensively used in life science research to label DNA (thymidine) and RNA (uridine). Thymidine and uridine are frequently used in the in vivo labelling of cells [1,2], especially in conjunction with autoradiographic techniques. [^{3}H]-Thymidine-5'-triphosphate (TTP) and [^{3}H]-uridine-5'-triphosphate (UTP) are used for in vitro labelling including the assays of DNA polymerase [3] and RNA polymerase [4].

For such applications a knowledge of the initial radiochemical purity of the tracer is of vital importance, and radiation self-decomposition is the most likely cause of the presence of impurities in such compounds. Misleading calculations of rates of uptake etc., may be caused by an invalid assumption of the initial purity of the radiotracer; in one case this has lead to the incorrect suggestion of the presence of an isotope effect [5,6]. More seriously, impurities may be preferentially

incorporated into cells and behave in a similar way to macromolecules, although they are incorporated into a unusual part of the cell structure[7,8].

The problems due to the self-decomposition of these radiotracers have been enhanced recently by the introduction and use of very high specific acitivity products (corresponding to more than one tritium atom per molecule of compound). This paper describes some recent observations on the self-decomposition of tritium-labelled thymidine, uridine, TTP and UTP at different specific activities and under various conditions of storage, for example, solvent and temperature. The results are discussed in the light of current theories of radiation self-decomposition.

Results and Discussion

1. The nature of decomposition products

The major decomposition products produced from [^{3}H]-thymidine were slow-moving impurities in the paper chromatography system V corresponding to glycols and other hydroxylation products [9]. A small amount of faster-moving impurities was also produced on prolonged storage after extensive decomposition. The same decomposition products were observed from all forms of thymidine, irrespective of the position of labelling, at both high and very high specific activity.

The decomposition products from [^{3}H]-uridine were both slower- and faster-running impurities. The latter were resolved into two separate impurities in the thin-layer system IV which gave the best estimate of the extent of decomposition. Similar patterns of decomposition were obtained for both singly- and doubly labelled [^{3}H]-uridine.

The decomposition products from [^{3}H]-UTP and [^{3}H]-TTP were hydrolysis products, principally nucleoside-5'-diphosphate and a small amount of 5'-monophosphate.

2. The effect of the method of sterilization on the stability of [^{3}H]-thymidine.

The data in Table I show the marked adverse effect which autoclaving (i.e. heating at 120°C for 20 minutes) has on the stability of [^{3}H]-thymidine. The rate of decomposition is increased by up to three times that of material which has been sterilised by filtration only. However, during the autoclaving process the radiochemical purity is not affected immediately and the impaired

Compound	Specific Activity (Ci/mmol)	Method of Sterilisation (a)	Time of Storage (Weeks)	Percentage Decomposition
[6-^{3}H]-Thymidine	2	A	6	4
	2	SF	19	5
[Methyl-^{3}H]-Thymidine	28	A	15	10
	28	SF	15	2

TABLE I

The effect of the method of sterilization on the stability of [^{3}H]-thymidine. Samples stored at + 2° in aqueous solution (1mCi/ml); mean of two determinations of purity in each case.

(a) A = Autoclaving
SF = Sterile Filtration

stability presumably results from the presence of chemical impurities which are introduced during the autoclaving process by leaking from the glass vial or from its rubber closure and which catalyse the radiolysis of thymidine.

3. The effect of temperature on the stability of [^{3}H]-thymidine at very high specific activity

The data in Table II show the effect of temperature on the stability of [methyl

Solvent	Storage Temperature (°C)	Time of Storage (Weeks)	Percentage Decomposition (a)
Water	- 140°	5	4
"	- 20°	5	17
"	+ 2°	5	4
2% Ethanol	+ 2°	8	ND
"	+ 2°	23	6
10% Ethanol	+ 2°	8	ND
"	+ 2°	23	3

TABLE II

The effect of temperature and solvent on the stability of [methyl-^{3}H]-thymidine; specific activity 44Ci/mmol, radioactive concentration 1mCi/ml - mean of two determinations of purity in each case.

(a) ND Not Detectable i.e. less than 1% decomposition.

^{3}H]-thymidine at a specific activity of 44Ci/mmol in aqueous solution. Optimum stability occurs at + 2° or - 140°; however at - 20° the rate of self-decomposition is greatly increased.

This is in agreement with the general principles of the effect of temperature on the stability of tritium-labelled compounds as described by Sheppard [10] and Bayly and Evans [11,12] and confirms the results obtained with low-specific activity [^{3}H]-thymidine in earlier work [13,14]. "Pockets" of high concentration of the labelled compound form during the gradual freezing process when the aqueous solution is frozen by being placed at -20°; due to the short path-length of the tritium beta emission most of the energy of the radiation is absorbed in the immediate vicinity of the labelled compound and hence the dispersal effect of the aqueous medium is diminished. Decomposition may take place both by primary (external) decomposition and by secondary decomposition due to the high concentration of free radicals, in particular the hydroxyl radical [15] in these "pockets".

It is a common practice to store radiochemicals in the deep-freeze at -20°, as for other biochemicals, and from the above data it can be seen that this can lead to decreased stability.

4. The effect of ethanol on the stability of [methyl-^{3}H]-thymidine at very high specific activity

Table II also shows the greatly beneficial effect that ethanol has on the stability of [^{3}H]-thymidine. At a low concentration (2%) ethanol is most effective as a stabiliser, decreasing the rate of decomposition approximately by a factor of 4; 10% ethanol is only slightly more effective.

Ethanol acts as a scavenger for the hydroxyl radical ($k \sim 1 \times 10^{9}$ $M^{-1}.s^{-1}$.) [16]; the reduction in self-decomposition observed in this case is in agreement with that predicted from the known rate of hydroxyl radical attack on thymidine, using the mathematical model proposed by Sheppard [10]. This confirms the suggestion that the hydroxyl radical is the major attacking agent in the self-decomposition of [^{3}H]-thymidine.

5. The effect of temperature on the stability of [5,6-^{3}H]-uridine

As can be seen from Table III (a), the effect of temperature on the stability of [^{3}H]-uridine at very high specific activity is similar to that for the

a) Effect of Temperature

Specific Activity (Ci/mmol)	Solvent	Temperature °C	Time of Storage (Weeks)	Percentage Decomposition (c)
55	Water	- 140°	10	ND
55	"	- 140°	15	6
55	"	- 140°	22	24
55	"	- 20°	10	25
55	"	+ 2°	10	6
55	"	+ 2°	15	9
55	"	+ 2°	22	24
51	"	+ 2°	6	5
43	"	+ 2°	6	5
53	"	+ 2°	12	10
51	"	+ 2°	5	ND
b) Effect of Additives				
55	2% Ethanol	+ 2°	15	3
55	"	- 140°	15	ND
55	10% Ethanol	+ 2°	22	ND
55	"	- 140°	22	ND
55	50% Ethanol	- 20°	10	ND
55	"	- 140°	10	ND
55	0.1% 2-mercapto-ethanol	+ 2°	10	ND
55	"	- 140°	10	ND

TABLE III

The effect of temperature and solvent on the stability of [5,6-^{3}H]-uridine. Radioactive concentration 1mCi/ml; mean of two determinations of purity in each case.

c) ND - Not detectable.

other pyrimidine nucleoside, thymidine. Thus the rate of self-decomposition is approximately 4 times greater at -20° than at +2°; storage at -140° may give rise to slightly less decomposition but the effect is not significant.

Data are also quoted in Table III (a) for the rates of self-decomposition for different batches of [5,6-^{3}H]-uridine at similar specific activities stored at +2°. They show a variation in rates of self-decomposition between batches, which indicate that studies of the effects of temperature of solvents on stability need to be carried out on samples from one batch of labelled compound to eliminate inter-batch variation.

6. The effect of additives on the stability of [5,6-^{3}H]-uridine

The data in Table (b) show the stability of [5,6-^{3}H]-uridine in the presence of different concentrations of stabilisers. At all concentrations, ethanol proved to be an excellent stabiliser, almost completely suppressing self-decomposition over a period of 3-4 months. Even a low concentration (0.1%) of 2-mercaptoethanol, a well-known reducing agent, proved to be an effective stabiliser.

The stabilising effect of ethanol on [^{3}H]-uridine is similar to that on [^{3}H]-thymidine. It has been suggested that the self-decomposition products of uridine are glycols and hydrates[17]; these are formed by the attack of the hydroxyl radical for which ethanol is a good scavenger.

Unfortunately the presence of ethanol in preparations of [^{3}H]-uridine interferes with RNA polymerase activity [18] and hence its use as a scavenger is restricted.

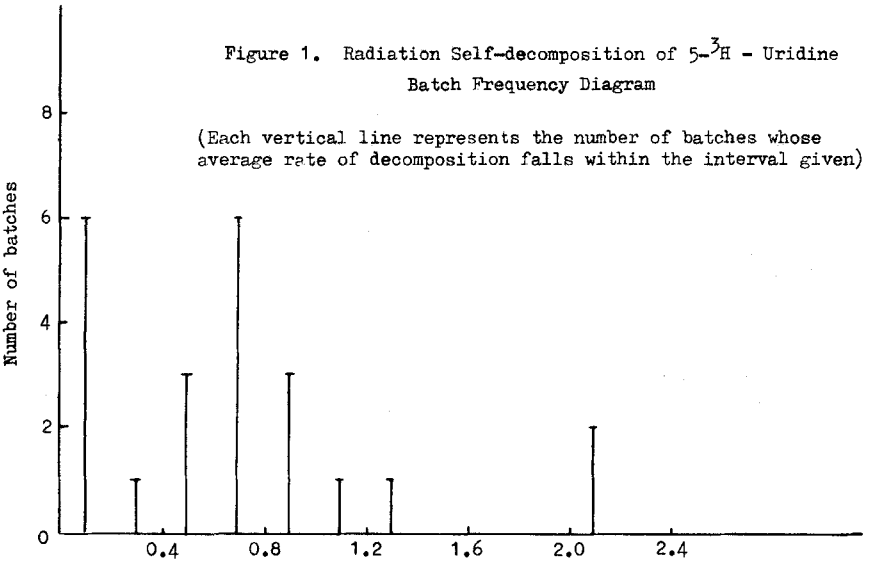
7. Batch-to-batch variation in the stability of [5-^{3}H]-uridine sterilised by autoclaving

Figure 1 shows the batch frequency diagram for the rates of self-decomposition for 23 batches of [5-^{3}H]-uridine.
Each vertical bar represents the number of batches whose average rate of decomposition lies within the interval given on the abscissa. There is a distribution of rates about a peak value (0.65 per cent per week) with some batches keeping exceptionally well, having virtually no decomposition, and others of exceptional instability.

This variation between batches is presumably caused by differences in the chemical impurities present in individual batches. The sterilisation by autoclaving of the labelled compound is known to induce instability (see Section 2) due to the formation of such impurities. The effect of such

Figure 1. Radiation Self-decomposition of 5-^{3}H - Uridine
Batch Frequency Diagram

(Each vertical line represents the number of batches whose average rate of decomposition falls within the interval given)



impurities in inhibiting or accelerating the rate of self-decomposition of [^{3}H]-thymidine has been observed previously [10].

It has also been shown that in many cases a labelled compound is apparently stable for a period followed by marked instability [10]. Differences in the length of the "plateau" period of stability explain the apparent wide variation in average rates of self-decomposition, calculated from radiochemical purity values measured at time intervals of 4 and 5 weeks.

The probable rate of self-decomposition of any one batch of [5-^{3}H]-uridine may be predicted from a statistical analysis of data presented in Figure 1; thus there is a 90% probability that the rate of self-decomposition is less than 0.9% per week. The variances included in such an analysis consist of those due to measurement variation, vial-to-vial variation (within one batch), and batch-to-batch variation.

8. The self-decompostion of [^{3}H]-UTP and [^{3}H]-TTP

The data in Table IV show the rates of self-decomposition for [^{3}H]-UTP and

Compound	Specific Activity (Ci/mmol)	Storage Temperature	Time of Storage (Weeks)	Percentage Decomposition (a)
[5,6-^{3}H]-Uridine-5'-triphosphate	42	- 20°	26	2
	42	- 140°	12	ND
	42	+ 2°	12	ND
	47	- 20°	35	3
[5-^{3}H]-Uridine-5'-triphosphate	18	- 20°	18	3
	14	- 20°	8	2
	11	- 20°	15	ND
	23	- 20°	22	2
[Methyl-^{3}H]-Thymidine-5'-triphosphate	35	- 140°	26	3
	54	- 140°	19	8
	45	- 140°	10	3
	50	- 140°	12	10
	26	- 140°	7	ND
	15	- 140°	24	6
	28	- 140°	19	6

TABLE IV

Rates of self-decomposition of [5,6-^{3}H]-uridine-5'-triphosphate, [5-^{3}H]-uridine-5'-triphosphate and [methyl-^{3}H]-thymidine-5'-triphosphate. Solvent ethanol:water (1:1); radioactive concentration 1mCi/ml. Mean of two determinations of purity in each case.

(a) ND - Not detectable, i.e. less than 1%

[^{3}H]-TTP at different specific activities. The solvent for these studies was ethanol:water (1:1) which remains liquid at -20°, so that the temperature of storage can be reduced to -20° to inhibit chemical decomposition (hydrolysis) without freezing the solution, which would cause an increase in self-decomposition due to radiolysis. Moreover, the presence of ethanol in such concentrations acts as a most effective radical scavenger.

From the data it can be seen that under these storage conditions the stability of [^{3}H]-UTP is exceptionally good (about three per cent decomposition in three to six months storage), despite the known lability of the triphosphate bond towards hydrolysis. It also appears that there is little dependence of the

rate of decomposition on specific activity. This has been noted before for decomposition which is chemical in nature [19] and is in agreement with kinetic theory [10].

The self-decomposition of [^{3}H]-TTP even when stored at -140°, is greater than that observed for [^{3}H]-UTP. The rate of decomposition (up to ten per cent in three months) is sufficiently small that the material has a quite acceptable shelf-life; however, the reason for the enhanced instability of TTP compared with UTP is not well understood yet. It appears to be due to differences in the ease of hydrolysis of the triphosphate bond, since the major decomposition product in both cases is the diphosphate; whether it is due to the change in base moiety or the presence of a deoxyribose sugar residue has not been established.

It should be noted again that there is no significant correlation between the rate of self-decomposition and specific activity.

Experimental

(a) Compounds

All the compounds studied were standard products from The Radiochemical Centre.

[5-^{3}H]-Uridine had code number TRK 178

[6-^{3}H]-Thymidine had code number TRA 61

[5,6-^{3}H]-Uridine had code number TRK 410

[Methyl-^{3}H]-Thymidine with a specific activity greater than 30Ci/mmole (i.e. doubly labelled) had code number TRK 418

[5-^{3}H]-Uridine-5'-triphosphate ([5-^{3}H]-UTP) had code number TRK 289

[5,6-^{3}H]-Uridine-5'-triphosphate ([5,6-^{3}H]-UTP) had code number TRK 412

[methyl-^{3}H]-Thymidine-5'-triphosphate ([methyl-^{3}H]-TTP) had code number TRK 424.

They were dispensed at radioactive concentrations of 1mCi.ml^{-1} into glass vials using solutions made up with different media according to the data given in the Tables of Results. The solutions were sterilised by autoclaving at 120° for 20 minutes, by sterile filtration using a bacterial filter during dispensing, or were self-sterile (containing 50% ethanol); the vials were stored at different temperatures, +2°, -20°, 140°C (the temperature of the vapour above liquid nitrogen) in an upright position.

(b) Methods

Each vial was opened for analysis and used once only. Approximately 4mg of

carrier material was added to each vial immediately before chromatography to prevent absorption on the chromatogram. The contents were then chromatographed in the systems given in Table V at 20° in a temperature-controlled room; after drying in even stream of air they were scanned by gas-flow proportional counter and the radiochemical purity value calculated from disc integration or by triangulation. Where purity values in chromatographic systems differed, the lowest radiochemical purity value was taken to by the one indicating the maximum extent of decomposition; the mean value from duplicate measurements was taken. A blank vial was analysed immediately after sterilization to give the zero-time value for calculating the extent of decomposition.

Compound	Type of chromatography and support	Solvent	Time of elution
Uridine	Descending; No.1 paper	I, II,	16 hr.
	Thin-layer on Silica GelG	III, IV	4 hr.
Thymidine	Descending; No.1 paper	V	8 hr.
UTP, TTP	Descending; No.1 paper	VI, VII	16 hr.
	Thin-layer on PEI cellulose	VIII	16 hr.

TABLE V

The methods for analysis of uridine, thymidine, UTP and TTP

Solvent
I n-butanol:ethanol:water. 52:33:15.
II t-butanol:butan-2-one:water:ammonia. 4:3:2:1.
III ethanol:borax solution (8%) : 0.5M EDTA; 5M ammonium acetate, 440:160:1:40
IV n-butanol saturated with water
V ethyl acetate saturated with pH 6 phosphate buffer.
VI iso-butyric acid:water:ammonia:0.1M EDTA. 100:56:4.2:1.6.
VII ethanol: M ammonium acetate. 5:2.
VIII 0.2M ammonium bicarbonate

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