Journal of Labelled Compounds — January-March 1965 — Vol. I, No. 1.

*Self-Radiolysis of Clathrates containing Tritium-labelled Molecules**

G. CIRANNI, A. GUARINO, R. PIZZELLA and E. POSSAGNO
Centro Nazionale di Chimica delle Radiazioni del C.N.R.
Istituto di Chimica Farmaceutica, University of Rome, Italy.

SUMMARY

Clathrates containing high specific activity methyl stearate-9,10-H^3 were stored for approximately 70 days.

The following « host » molecules were employed : desoxycholic acid, cycloveratryl, tri-o-thymotide and β-cyclodextrin. The gaseous self-radiolysis products decrease significantly up to ≃90 % (HT from chloeic clathrate). The total percentual decreases of the decomposition are : 16.95 % (choleic clathrate); 27.10 (tri-o-thymotide clathrate); 19.35 (cycloveratryl clathrate); 12.10 (β-cyclodextrin clathrate).

INTRODUCTION

Inclusion compounds, either of the channel type or of the cage structure can be usefully employed as protective media, owing to the fact that the radioactive molecules can be introduced, as « guests », into a cage of inactive « host » molecules. As consequence, each radioactive molecule is surrounded by a large number of inactive molecules and the primary external effect, due to the irradiation from the decay of other labelled compounds, can be minimized [1].

A second usefull feature is the trapping of reactive radiolytic fragments within a cage that prevents their diffusion and subsequent reactions with the labelled substance.

A third point to be considered is that some kind of energy transfer from the guest to the host compound could decrease the decomposition of excited molecules.

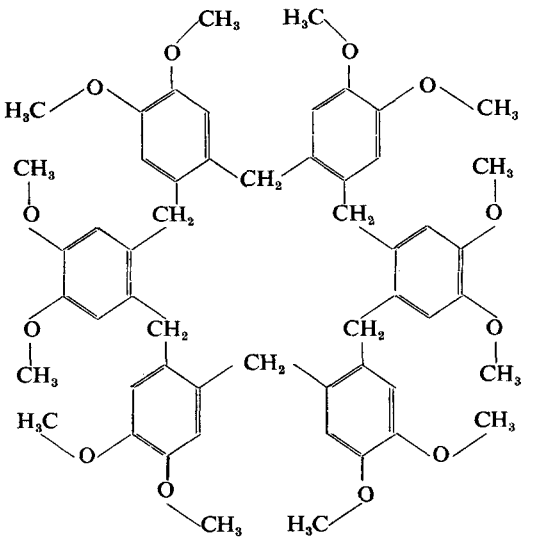
In order to check the possibility to use inclusion compounds for the storage of labelled molecules, the following adducts were employed :

1. — Desoxycholic acid.
2. — Cycloveratryl.
3. — Tri-o-thymotide.
4. — β-cyclodextrin.

* Received on December 21, 1964.

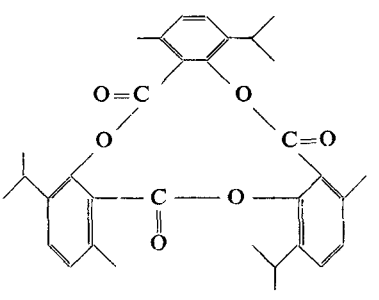
Carboxylic acids are known to form choleic adducts [2] and the number of carbon atoms in the acid molecule controls the composition of the clathrate : for example, eight molecules of desoxycholic acid enclathrate one molecule of a C_{15} fatty acid. The solvent used in the preparation of the adduct can be included too, and must be taken into account when calculating the G values of the gaseous radiolytic products.

Cycloveratryl has the formula :



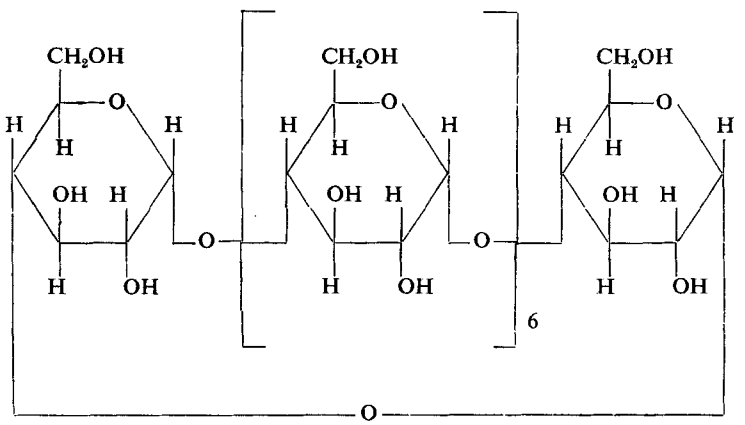
This compound seems to have a stable non planar configuration, in which the benzene rings are tilted with respect to the plane passing through the molecule. This peculiar shape leaves open spaces into which guest molecules can fit [3].

Tri-o-thymotide clathrates have been studied extensively [4], and they are known to form two species of adducts : cavity and channel clathrates, depending upon the shape of the included molecule.



In the case of a n-$C_{12}H_{25}COOCH_3$ molecule — its length, in extended form, is 20.2 Å — the structure of the tri-o-thymotide clathrate is the channel; probably, the methyl stearate adduct is also of the channel type.

The cyclodextrins prepared by the action of *Bacillus macerans* on amylose are cyclic oligosaccharides having chains of 6, 7 and 8 glucose units, joined head to tail. The difference between these cyclodextrins is that the diameter of their holes are 6, 8 and 10 Å, respectively [5].



In the case of methyl stearate the inclusion compound have been prepared with the β-cyclodextrins. The special interest of these adducts is the possibility they offer to introduce labelled compounds, stored as cyclodextrin clathrates, into biological systems. The enzymatic hydrolysis of the cyclodextrin can free the labelled compound directly inside the living system.

EXPERIMENTAL PART

Methyl stearate-9,10-^3H.

The synthesis of the ester has been described elsewhere [6]. The specific activity was 314.0 mC/mmole.

Clathrate preparations.

A. Choleic clathrate. — 700 mg of desoxycholic acid were added to a hot alcoholic solution of 60 mg of tritiated methyl stearate, and the solvent distilled off. The choleic adduct formed contained approximately 85 % of the ester, the remaining being simply adsorbed on the desoxycholic acid.

The choleic adduct was destroyed after storage by boiling with o-xylene, according to the observation of GIACOMELLO [7] that the hydrocarbon replaces the ester into the desoxycholic acid lattice; by filtration and distillation approximately 98 % of the ester was recovered.

B. Tri-o-thymotide clathrate. — The preparation of the tri-o-thymotide was carried out according to SPALLINO and PROVENZAL [8]. The tri-o-thymotide and methyl stearate-9,10-H^3 were dissolved into 2,2,4-trimethyl-pentane, under heating. After cooling, the tri-o-thymotide clathrate precipitated. The distillation of the solvent caused the ester which was not included in the clathrate to remain adsorbed on the adduct.

After storage, the tri-o-thymotide adduct was dissolved in 2,2,4-trimethyl-pentane, under heating. A known amount of inactive ester was added and the solution cooled.

This solution contained the first dilution methyl stearate-9,10-^3H; after distilling off the solvent, one aliquot of the ester was distilled under vacuum, diluted again with a known amount of inactive ester, purified by preparative gas-chromatography and the radioactive ester determined by the reverse isotope dilution analysis.

C. Cycloveratryl clathrate. — The cycloveratryl was prepared according to CASINOVI and OLIVIERO [9].

The clathrate was obtained by cooling a hot solution of cycloveratryl and methyl stearate-9,10-^3H in benzene. In this case again some ester remained adsorbed on the adduct, after the benzene distillation. The recovery of the methyl stearate after the storage was performed as in the case of the tri-o-thymotide, except that benzene was the solvent employed.

D. β-Cyclodextrin clathrate. — Cyclodextrins were prepared by the action of *Bacillus macerans* on the amylose, according to D. FRENCH *et al.* [10].

The clathrate was formed by shaking a 50 % water-ethanol solution of β-cyclodextrins and methyl stearate-9,10-^3H. The precipitated clathrate was filtered, washed with water and dried. After the storage it was dissolved in the same solvent mixture and boiled in the presence of a known amount of inactive ester.

The four clathrates were stored in « Pyrex » ampoules (10 ml) under vacuum in the conditions summarized in Table I.

The gases formed in the self-radiolysis were collected by a Toepler pump and analyzed with a radio-gas-chromatograph. Experimental details are reported elsewhere [6].

Weighed amounts of inactive methyl ester were then added to the adducts, which were then decomposed according to the various techniques previously described. Known aliquots of these first dilution samples were diluted and purified by preparative gas-chromatography, according to CACACE *et al.* [11]. The percentage of decomposition of the stored esters was calculated from the decrease of the specific activity by means of the reverse isotope dilution method.

In order to obtain further information on the influence of the host molecules, the G-values for the total acids were estimed for the four clathrates stored.

TABLE I. Self-radiolysis decomposition

System	Quantity (mg)	Time of Storage (days)	Absorbed dose (eV/g)	Decomposition (%)	Percentual decrease [a] of the decomposition
Methyl stearate-9,10-^{3}H (under vacuum)	314.15	71	$1.37\ 10^{21}$	24.90	—
Choleic clathrate	268.56 [b] 3542.20	72	$1.37\ 10^{21}$	19.75	16.95
Tri-o-thymotide clathrate	301.36 [b] 971.20	72	$1.37\ 10^{21}$	18.04	27.10
Cycloveratryl clathrate	314.80 [b] 3000.00	71	$1.37\ 10^{21}$	20.10	19.35
β-Cyclodextrin clathrate	322.37 [b] 1973.80	101	$1.91\ 10^{21}$	21.82	12.10

[a] In comparison with the storage under vacuum.
[b] Radioactive compound.

RESULTS AND DISCUSSIONS

The results for the gaseous products shown in Table II indicate that the activity contained in the H_2, CH_4 and C_2H_6 decreases significantly in the samples stored as clathrates, in comparison to the samples stored under vacuum.

TABLE II. Self-radiolysis gaseous products

Self-radiolysis compounds identified	mμC/mg starting Me-stearate-9,10-^{3}H				
	under vacuum	desoxycholic acid	cyclo-veratryl	tri-o-thymotide	β-cyclo-dextrin
H_2	494.00	27.20	232.00	224.00	225.00
CH_4	18.70	0.91	6.38	7.88	7.73
C_2H_6	3.60	0.20	1.20	1.77	1.32
Total acids	74,800	29,500	28,530	29,200	—

In Table III are shown the percentual decreases for the G-values for the four clathrates.

TABLE III

Activity decrease Δ % [a]	Clathrate			
	choleic	cycloveratryl	tri-o-thymotide	β-cyclodextrin
$\Delta(H_2)$	94.5	53.0	54.8	54.5
$\Delta(CH_4)$	95.2	65.9	58.2	58.6
$\Delta(C_2H_6)$	94.7	65.8	50.0	63.3
Δ(total acids)	60.6	61.9	61.0	—

[a] Referred to the storage under vacuum, according to the expression :

$$\Delta\ \% = \frac{\text{activity found under vacuum} - \text{activity found in clathrate } 100}{\text{activity found under vacuum}}$$

Experiments were also carried out in order to compare the gaseous products formed in the self-radiolysis and gamma radiolysis of methyl stearate.

All the samples were irradiated into a gamma facility at approximately 100 Mrad.

The following systems were irradiated :

(i) Methyl stearate

(ii) The stoichiometric mixture of methyl stearate and the clathrate-forming compounds

(iii) The clathrates of methyl stearate.

The results obtained, which are averages of many irradiations, are summarized in Table IV. Taking into account the electron fraction of the two components (methyl stearate, and one of the four clathrate-forming compound) the G-values for the stoichiometric mixture are, as an average, larger then the G-values for the clathrate. These G-values are of the same order of magnitude of the results obtained in the self-radiolysis, taking into account the contribute of the clathrate-forming compound to the total gaseous yield, the total dose difference for these two irradiations, (100 Mrad and 22 Mrad respectively), and the solvent effect, i.e. it was trapped during the preparation of the clathrates, but it was absent into the stoichiometric mixture.

Work is now in progress in our laboratory to get a better understanding about the radiation chemistry of clathrates prepared without solvents.

The percentage of initial methyl stearate-9,10-^{3}H destroyed, stored as clathrate, increases in the order : tri-o-thymotide $<$ cycloveratryl $<$ desoxycholic acid $<$ cyclodextrin.

TABLE IV. Radiolysis and self-radiolysis of Me-stearate

System	G_{H_2}		G_{CH_4}		$G_{C_2H_6}$	
	Self-radiolysis (22 Mrad)	Radiolysis (100 Mrad)	Self-radiolysis (22 Mrad)	Radiolysis (100 Mrad)	Self-radiolysis (22 Mrad)	Radiolysis (100 Mrad)
Methyl stearate-9,10-^{3}H (under vacuum)	1.36	1.55	0.82	0.72	0.03	0.040
Desoxycholic acid	—	1.03	—	0.04	—	0.007
Choleic mixture	—	1.19	—	0.06	—	0.005
Choleic clathrate	1.06	1.28	1.81	0.12	0.03	0.020
Tri-o-thymotide	—	0.06	—	0.03	—	0.001
Tri-o-thymotide mixture	—	0.38	—	0.10	—	0.010
Tri-o-thymotide clathrate	0.86	0.09	0.56	0.04	0.02	0.007
Cycloveratryl	—	0.05	—	0.05	—	0.003
Cycloveratryl mixture	—	0.23	—	0.10	—	0.015
Cycloveratryl clathrate	0.82	0.15	0.38	0.08	0.01	0.006
β-Cyclodextrin	—	1.07	—	0.03	—	0.003
β-Cyclodextrin mixture	—	1.32	—	0.16	—	0.010
β-Cyclodextrin clathrate	1.73	1.29	0.82	0.32	0.03	0.021

It is well known that, in the case of the tri-o-thymotide and cycloveratryl, the cage-forming molecules have an arrangement influenced by their interaction with the enclosed component. The G-values for H_2, CH_4 and C_2H_6, and the decrease of self-radiolysis, for these two clathrates, present similar behaviour under radiation. Probably the benzene rings of these two molecules protect the enclathrated active compound.

Cyclodextrin and choleic adducts show higher G-values for H_2, CH_4 and C_2H_6, and a lower total protection. The choleic adduct presents a peculiar ten fold decrease in activity of gaseous products.

G. O. PHILLIPS has found [12] that β-cyclodextrin clathrate with p-nitrophenol, under irradiation with Co^{60} gammas (dose 1.93 10^{20} eV/g) gives a different E.S.R. spectrum with respect to the same stoichiometric uncomplexed

mixture of these two compounds. He concluded that some energy transfer could take place from the α-D-glucose moiety to the p-nitrophenol.

The extent of the self-radiolysis of a tritiated compound decreases when it is stored as a clathrate. There are several possible reasons for this effect :

(i) In the first place the dilution of the active molecules with a large excess of inactive material must be considered. This, of course, decreases the primary radiolysis of the molecules and can be obtained in other ways, for instance dissolving the active compound in a large amount of a suitable solvent.

(ii) The secondary radiolysis is caused by the interaction with the labelled molecules of reactive intermediates produced by the β particles from the tritium decay.

In the case of a clathrate, these reactive intermediates are prevented from reaching a labelled molecule by the « cage » surrounding it. Therefore, the diffusion of radicals, ions etc. which could attack and destroy labelled molecules is substancially reduced in comparison, for instance, with a liquid solution.

(iii) An additional advantage of the clathrates is the possibility that the fragment from a given labelled molecule, destroyed by self-radiolysis, can cross-link with the surrounding cage. This prevents the labelled fragments from leaving the cage, once the clathrate is destroyed, to recover the stored substance, and therefore reduces the amount of radioactive impurities that contaminate the active substance.

The cross-linking of clathrates under irradiation has been described by Mc CLAIN and DIETHORN [13].

(iv) Finally, energy transfer from the guest to the host molecule could protect the labelled species.

Unfortunately, the procedures employed to prepare the clathrate compounds — i.e. crystallization from a solvent containing the host and the guest component, followed by evaporation of the solvent — leave some 20 % of the labelled compounds simply adsorbed on the adduct.

In order to get a better understanding of the self-radiolysis of the enclathrated labelled molecules, it would be interesting to remove any adsorbed labelled compound from the adduct. Work in this direction is in progress in our laboratory.

In conclusion, the clathrate formation may prove of practical interest for storing labelled molecules of high specific activity, provided that good techniques are available to prepare the adducts and simple methods can be used for a quantitative recovery after storage. In the case of cyclodextrins there is lesser necessity to free the labelled molecules, when employed for biological studies, owing to the possibility of an enzymic hydrolysis of the adduct. The phenomena connected with the cage structure, energy transfer and radiation stability of the clathrates do not seem to have been since now

extensively investigated and the use of labelled tracers seems to be an usefull approach to the study of these problems.

AKNOWLEDGEMENT

The Authors wish to express their gratitude to Prof. G. GIACOMELLO for helpful discussions. They are also indebted to Prof. F. CACACE for a critical review of the manuscript and to Dr. I. CACCIARI for the preparation of ß-cyclodextrins.

REFERENCES

1. GUARINO, A. — Proceedings of the Conference on Methods of preparing and storing merked molecules. EURATOM, p. 1059, Bruxelles (1963).
2. CAGLIOTI, V. and GIACOMELLO, G. — *Gazz. Chim. Ital.*, **79** : 245 (1939).
3. CAGLIOTI, V. *et al.* — *J. Inorg. and Nuclear Chem.*, **8** : 572 (1958).
4. NEWMANN, A. and POWELL, H. — *J. Chem. Soc.*, 3747 (1952).
5. CRAMER, F. and HENGLEIN, F. M. — *Chem. Ber.*, **90** : 2561 (1957).
6. GUARINO, A., PIZZELLA, R. and POSSAGNO, E. — *Journ. labelled molecules* (in press).
7. GIACOMELLO, G. and BIANCHI, E. — *Gazz. Chim. Ital.*, **73** : 3 (1943).
8. SPALLINO, R. and PROVENZAL, G. — *Gazz. Chim. Ital.*, **39** : 325 (1909).
9. OLIVIERO, A. and CASINOVI, C. — *Ann. Chim.*, **42** : 168 (1952).
10. FRENCH, D., LEVINE, M., PAZUR, J. and NORBERG, E. — *J. Am. Chem. Soc.*, **71** : 353 (1949).
11. ALIPRANDI, B., CACACE, F. and CIRANNI, G. — *Anal. Chem.*, **36** : 2445 (1964).
12. PHILLIPS, G. O. — *Discussions of the Faraday Soc.*, **36** : 281 (1963) (general discussions).
13. MC CLAIN, J. and DIETHORN, W. — *Int. J. Appl. Rad. and Isotopes*, **15** : 151 (1964).