REACTION OF A SPIN-LABELLED CARBODIIMIDE WITH NUCLEOSIDES, POLY U AND tRNA

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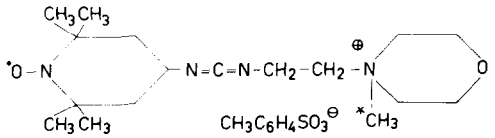
Received 22 February 1971

1. Introduction

A spin-labelled water-soluble carbodiimide (*S*-carbodiimide):



has been synthesized recently in this laboratory [1]. The compound was demonstrated to react with nucleosides, and the reaction products were characterized in a preliminary manner.

The present communication is concerned with the kinetics of the reaction of *S*-carbodiimide with the nucleosides uridine, guanosine, inosine, with poly U and with tRNA. Stability of the reaction products to mild alkaline hydrolysis has been also investigated.

2. Materials and methods

S-Carbodiimide and the products of its reaction with nucleosides (*S*-nucleosides) have been obtained as described earlier [1]. The preparation of *S*-carbodiimide contained no diamagnetic admixtures as revealed by PMR-spectrometry. *S*-Carbodiimide labelled with ^{14}C at the methyl grouping (see asterisk in formula) was obtained by the same method but starting with ^{14}C-methyl *p*-toluenesulphonate (about 1 Ci/mole). Sodium polyuridylate ("Reanal", Hungary) was purified by phenol extraction from 1 M NaCl, precipitation with ethanol from aqueous layer, dissolution in 0.1 M NaCl, precipitation with cetyltrimethylammonium bromide, dissolution in ethanol, precipitation with 1 M NaCl and three reprecipitations from 1 M NaCl with ethanol. Unfractionated yeast tRNA (acceptor activity 100 pmoles of valine per A unit) was obtained in the Technological Laboratory of this Institute. Nucleosides were recrystallyzed preparations homogenous in paper chromatography.

All the reactions were run at 25°. The reaction kinetics with nucleosides were studied by measuring the amounts of unreacted nucleosides. For the purpose, *S*-carbodiimide and *S*-nucleosides were removed from aliquots of reaction mixtures by passing through Sephadex C-50 Na^+ columns after appropriate dilution. The reactions were performed in 0.1 N *N*-methylmorpholinium chloride buffer pH 7.5 with 2×10^{-2} M *S*-carbodiimide and 2×10^{-3} M nucleosides.

Kinetics of hydrolysis of *S*-nucleosides were measured by the same method estimating the amounts of nucleosides liberated. The hydrolysis was run in 0.1 N sodium carbonate-bicarbonate pH 9.0 with 2×10^{-3} M *S*-nucleosides.

The reactions with polynucleotides were studied using ^{14}C-*S*-carbodiimide. The concentration of poly U was 2×10^{-3} M, of *S*-carbodiimide 2×10^{-2} M. The composition of reaction mixtures with tRNA is shown in fig. 1; the concentration of tRNA was 2 mg/ml. Both the reactions were performed in 0.02 M *N*-methylmorpholinium chloride pH 7.5. The extents of modification were calculated on basis of known polynucleotides concentration [2] and of the radioactivity of aliquots of reaction mixtures passed through Sephadex C-50 Na^+ (elution with

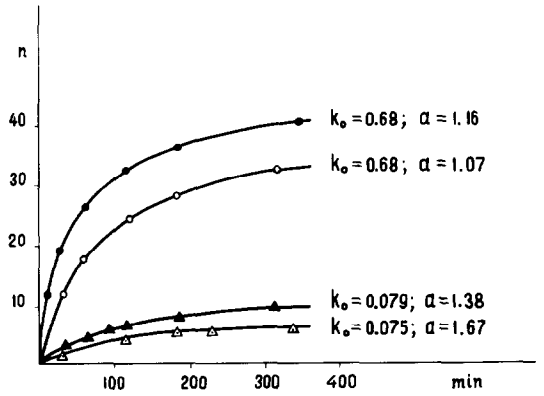


Fig. 1. Kinetics of the reaction of *S*-carbodiimide with tRNA. Solid lines are best fit functions obtained by choice of parameters k_0 and a in integral form of equations (1) and (2) combination [6]. Points are experimental data.
• 0.06 M *S*-carbodiimide; ⊙ 0.03 M *S*-carbodiimide;
▲ 0.06 M *S*-carbodiimide + 0.02 M $MgCl_2$ + 0.1 M NaCl;
△ 0.03 M *S*-carbodiimide + 0.02 M $MgCl_2$ + 0.1 M NaCl.
The values of k_0 and a are shown against the corresponding curves; units of k_0 are M^{-1} min^{-1}.

water). Radioactivities were determined with a Nuclear-Chicago Mark I scintillation counter in dioxan scintillation fluid.

Hydrolysis (demodification) of modified tRNA was performed in 0.1 N sodium carbonate–bicarbonate pH 10.6.

The concentrations of tRNA in stock solutions were determined spectrophotometrically after alkaline hydrolysis (0.3 N KOH, 25°, 24 hr). The molar extinction coefficient of hydrolysate in 0.1 N HCl was assumed to be 7.9×10^5 M^{-1} cm^{-1} at 260 nm.

The ESR-spectra were measured with E-3 Varian instrument at ambient temperature.

3. Results and discussion

The rate constants of the reactions of *S*-carbodiimide with nucleosides compared with those of the reactions of *N*-cyclohexyl, *N'*-β-(4-methylmorpholinium)ethylcarbodiimide (CME-carbodiimide) are presented in table 1; *S*-carbodiimide is about two orders of magnitude more reactive towards nucleosides compared with CME-carbodiimide.

The rate constants of the hydrolysis of *S*-nucleosides to original nucleosides in weakly alkaline medium are presented in table 2; *S*-nucleosides are about 4–5 times less stable compared with CME-nucleosides.

Table 1.
True rate constants of the reaction of *S*-carbodiimide and CME-carbodiimide with nucleosides.

Nucleoside	k_{II} at 25° (M^{-1} min^{-1})	
	with CME-carbodiimide	with *S*-carbodiimide
Uridine	3.8 [3]	260
Guanosine	1.4 [4]	170
Inosine	4.1 [4]	220

The values of k_{II} (true second-order rate constants) were determined as described in [3, 4] from the reaction kinetics and from the pK values of nucleosides at 25°.

Table 2
True second-order rate constants of the hydrolysis of *S*-nucleosides to starting nucleosides compared with those of CME-nucleosides.

Compound	k_{II}^h at 25° (M^{-1} min^{-1})
S-Uridine	290
CME-uridine	57 [5]
S-Guanosine	1500
CME-guanosine	380 [4]

The k_{II}^h values were calculated from the reaction kinetics, from the pH of reaction mixtures and from the ionic ratio of water at 25° as described in [4, 5].

The reaction of *S*-carbodiimide with poly U proceeded to completion (one mole of ^{14}C-label per mole of uridine residues). The apparent second-order rate constant of this reaction at pH 7.5 was 2 M^{-1} min^{-1}. Hydrolysis of modified poly U at pH 10 resulted in complete demodification.

According to our model [6], the kinetics of the reaction of tRNA with CME-carbodiimide were described by the following equations:

$$-\frac{dz}{dt} = kcz \quad (1) \qquad k = k_0 a^{-n} \quad (2)$$

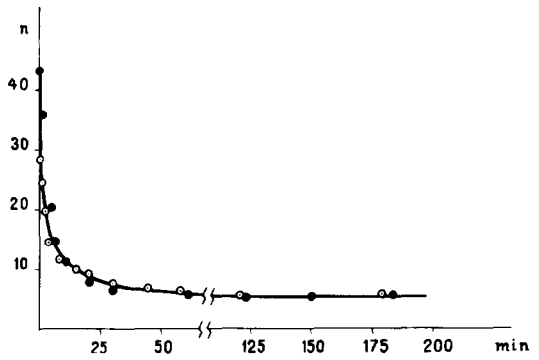


Fig. 2. Kinetics of alkaline demodification of modified tRNA. tRNA modified with *S*-carbodiimide to $n = 43$ (•) and to $n = 29$ (⊙).

Here z is the concentration of non-reacted potentially reactive nucleosides of tRNA (U, G, I, T, Ψ); c, concentration of the carbodiimide; t, time of reaction; a, inhibition coefficient; n, extent of tRNA modification (moles of CME-nucleoside residues per mole of tRNA); k_0, the value of k at zero time. Factor k is the apparent average rate 'constant':

$$k = \sum_{\{\Omega\}} k'_{\Omega} \cdot f_{\Omega} \qquad (3)$$

where k'_{Ω} are the apparent second-order rate constants of nucleoside residues in the state Ω; state Ω is determined by the nature of nucleoside, by primary structure of the corresponding tRNA (this term involves the extent and sites of previous modification) and by the conformation of this tRNA.

It was found (fig. 1) that the reaction kinetics of tRNA with *S*-carbodiimide are in a good accord with the assumption of exponential drop of apparent average second-order rate constant k in the course of modification (eq. (2)). This is an important observation because the good fit of experimental data by the empirical equation proposed earlier for the reaction with CME-carbodiimide proves that it is the decreasing apparent reactivity of tRNA nucleoside rather than time of conformational rearrangement that limits the rate of modification. Remembering that the logarithm of the apparent second-order rate constant of the reaction of CME-carbodiimide with nucleosides is directly proportional to pH [3, 4],

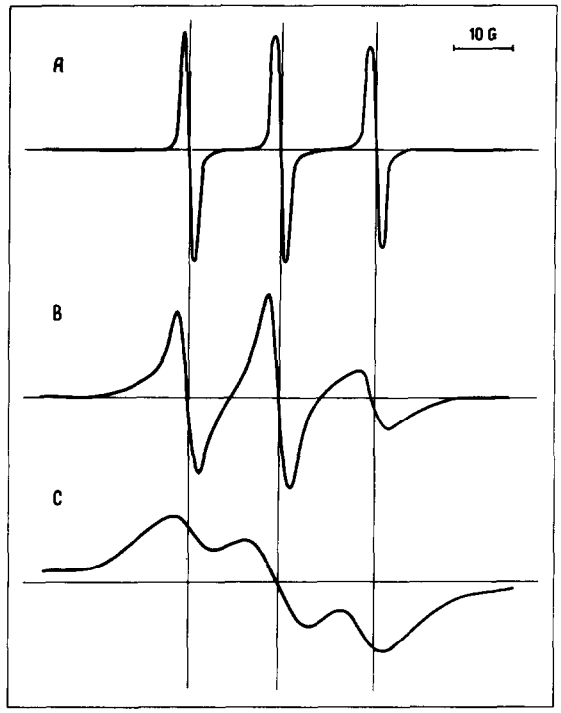


Fig. 3. ESR-spectra. (A) *S*-Carbodiimide in water, 10^{-3} M. (B) tRNA modified with *S*-carbodiimide to $n = 15$, in 0.1 M CH_3COONa pH 5.0, 14 absorbance units (260 nm) per ml. (C) Poly U completely modified with *S*-carbodiimide, in the same buffer, 2×10^{-3} M.

the values of k_0 obtained at pH 7.5 with *S*-carbodiimide indicate that the reagent is two orders of magnitude more reactive than CME-carbodiimide with respect to tRNA [cf. 6].

Mild alkaline treatment (fig. 2). of tRNA modified with *S*-carbodiimide results in removal of radioactive label. The remaining radioactivity ($n = 5$) must be partly due to the stability of *S*-pseudouridine residues [cf. 7].

The ESR spectra of *S*-carbodiimide, of completely modified poly U, and of modified tRNA are shown in fig. 3. Detailed interpretation of the ESR spectra will be subject of physical studies.

Acknowledgements

The authors are thankful to Drs. Yu. Molin and J. Backer for critical discussion.

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