161. Self-Replication of Oligonucleotides in Reverse Micelles

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The coupling between the tri(deoxynucleotides) d[(MeO)C-G-Ap] (**1**) and d[$(NH_2)T_{d5'}$-C-G] (**2**) to yield the phosphoramidate-linked (hexadeoxy-nucleotide) d[(MeO)C-G-Anh$^{5'}T_{d5'}$-C-G] (**3**) was investigated both in aqueous solution and in reverse micelles constituted of CTAB (cetyl(trimethyl)ammonium bromide) in hexane/pentan-1-ol 9:1. No significant difference was found concerning the yield and the kinetics of the reaction in the two systems. The coupling between **1** and **2** was also carried out in the presence of the template d[(MeO)C-G-A-T-C-G] (**4**), an analogue of **3**, so as to reproduce the conditions of template-directed self replication. It was shown that the trinucleotide coupling in the presence of a template obeys the so-called square-root law both in H_2O and in reverse micelles. No significant difference of the time course of the reaction in H_2O and in reverse micelles was observed. This shows that self-replication of oligonucleotides occurs within geometrically bounded structures, which represents a step forward in the mimicking of minimal life processes.

1. Introduction. – In the last few years, there has been a growing interest in the chemistry of self-replication. Here the term 'self' indicates that the population growth is caused by the structure itself. There are two lines of research in this field. One is the template-directed synthesis based on the complementarity of DNA or analogs thereof [1–6]. In this case, a longer oligonucleotide acts as a template for two shorter oligonucleotides, usually the two halves, which are bound *via* complementary base pears: binding and alignment induced by the template facilitates the chemical coupling of the two shorter units to yield a copy of the template itself. In the other approach, bound structures such as micelles or vesicles are used. In this case, there is a reaction inside the surfactant aggregate which produces the surfactant which makes up the aggregate itself [7–10]. The first approach is germane to DNA self-replication, whereas the second one is rather a model of the reproduction of the cellular shell. Having in mind the biological cell as the model, one can refer to these two mechanisms as 'core' replication and 'shell' replication, respectively.

Clearly a system more germane to synthetic models of the living cell would be one in which the template replication of oligonucleotides takes place in micelles. The aim of this paper is to describe how this can be done. To this purpose, we used the micellar system described in a previous paper [11], in which mononucleotide coupling was successfully described. The system consists of the surfactant CTAB (cetyl(trimethyl)ammonium bromide), the cosurfactant and cosolvent pentan-1-ol (9.9%; *v/v*) in hexane, in the presence of *Hepes* (0.1M, pH 7.5) as buffer at $w_o = 22$ ($w_o = [H_2O]/[CTAB]$). We have also shown that the physically relevant concentration for the nucleotide coupling is the H_2O-pool concentration (*i.e.* referred to the microphase of the H_2O pool).

The nucleotide self-replication in micelles was studied by coupling the two trinucleotides **1** and **2** as shown in the *Scheme*, in the presence of the hexanucleotide **4**, which has a structure only partly different from the reaction product **3**. This slight difference facilitates the analysis, as **3** and **4** elute in HPLC as two distinct peaks. The reaction shown in the *Scheme* is a minor modification of the reaction first described by *von Kiedrowski* and coworkers [2], which was actually the first report of sigmoidal self-replication of this sort. The difference lies in the fact that the German authors used the nucleotide sequence d[(MeO)C-C-G-C-G-G], whereas we used the sequence d[(MeO)C-G-A-T-C-G].

Since our reagents are slightly different from those of *von Kiedrowski*, it was necessary to repeat in our work the coupling in H_2O without reverse micelles, to compare H_2O and micellar results. Results were analyzed on the basis of the so-called square-root law of template catalysis proposed by *von Kiedrowski* and coworkers [2].

2. Results and Discussion. – 2.1. *Synthesis of the Oligonucleotides.* The preliminary steps in the self-replication reaction in reverse micelles were the synthesis of the trinucleotides **1** and **2**, as well as of the hexamers **3** and **4**. Hexamer **3** corresponds to the product of the condensation of **1** and **2** and was used to establish the existence of the expected reaction product by HPLC coinjection. The oligonucleotides **2**–**4** were prepared by solid-phase synthesis using phosphoramidite chemistry [12]. Compounds **2** and **3** were synthesized using the 3′-phosphoramidite of 5′-deoxy-5′-[(monomethoxytrityl)amino]-thymidine. The coupling of the 5′-methoxy-protected building block at the end of sequence **1**, **3**, and **4** was described previously [11].

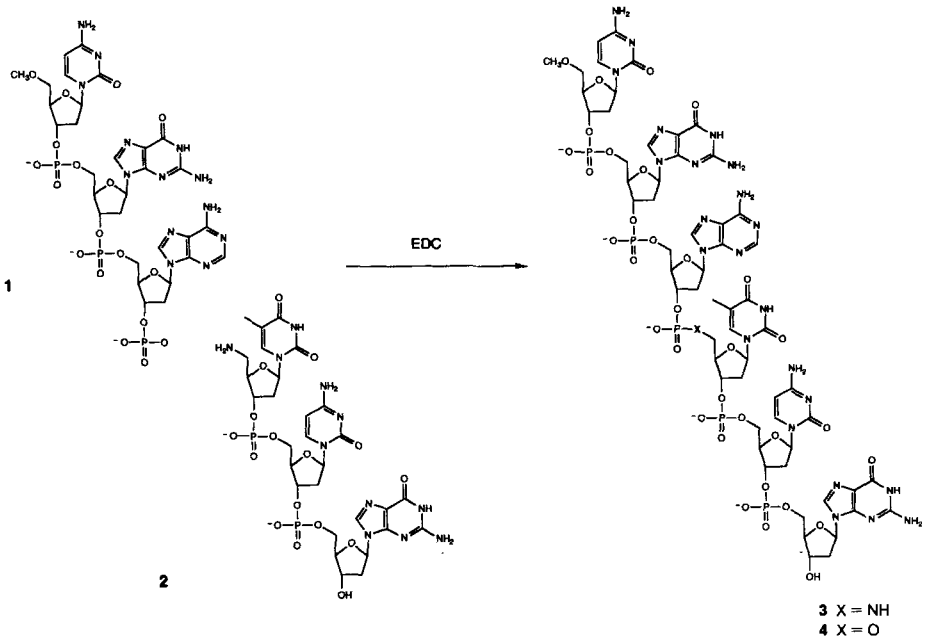
Trinucleotide **1** was synthesized on a specially functionalized CPG (controlled-pore glass) solid-support material to obtain a free phosphate group at the 3′-end of the trinucleotide, similarly as described recently [13]. The 5′-end of sequences **1**, **3**, and **4** was protected by a Me group to prevent side reactions, whereas the 3′-OH ends of sequences **2**–**4** were left unprotected (due to steric reasons, and due to the greater nucleophilicity of the NH_2 group, no side products had to be expected in the coupling reaction between a 5′-NH_2 group and a 3′-activated phosphate group in the presence of free 3′-OH groups).

2.2. *Nucleotide Coupling in Aqueous Solution.* The coupling reaction between the 5′-*O*-methylated, 3′-phosphorylated trinucleotide **1** and 5′-amino-5′-deoxytrinucleotide **2**, activated by the H_2O-soluble *N*-[3-(dimethylamino)propyl]-*N*′-ethylcarbodiimide hydrochloride (EDC) to yield hexamer **3** (*Scheme*) was first investigated in aqueous *Hepes* buffer (0.1M, pH 7.5). The reaction was carried out in an analogous way as *von Kiedrowski* and coworkers showed in a recent paper [2], although we used a different sequence (see *Scheme*). One C-G base pair in the hexanucleotide duplex is replaced by an A-T base pair, though the sequence is still palindromic. As a result, the duplex is expected to be more labile due to the loss of one H-bond.

The temperature optimum to follow the reaction was 5°. The reaction system was evaluated with different initial template concentrations in *Hepes* buffer and analyzed by the simulation and fitting program Simfit [2] developed by *von Kiedrowski*. The system obeyed the square-root law of template catalysis. The 'square-root law' means the equation $dc_T/dt = ac_T^{1/2} + b$, which describes the initial velocity of template synthesis as the sum of an autocatalytic ($ac_T^{1/2}$) and a nonautocatalytic (b) reaction path. The program Simfit uses the integrated form of this equation:

$$dc/dt = (c_A - c)(c_B - c)\,[k_a(c + c_T)^{1/2} + k_b] \exp(-k_c t)$$

Scheme



which derives from the square-root law under consideration of the mass conservation: c_A, c_B are the reactant, c_T the template, and c the product concentration, k_a and k_b are the rate constants for the autocatalytic and the non-autocatalytic reaction pathways, respectively, k_c is the rate constant for the carbodiimide hydrolysis [2].

The data for the aqueous replicating system are not shown, but the relation k_a/k_b which gives an information about the performance of the replicating system is 410. The value for the '*Kiedrowski* system' is 420 [2].

2.3. *Aqueous* vs. *Reverse-Micellar System.* The behaviour of the reaction system in CTAB reverse micelles at $w_o = 22$ is shown in *Fig. 1.* An aqueous reaction solution

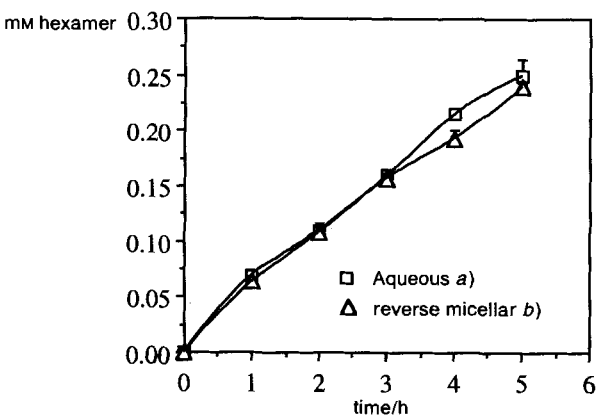


Fig. 1. *Trinucleotide coupling between* **1** *and* **2** *to yield* **3** (see *Scheme*) *as monitored by HPLC:* a) *in aqueous* Hepes *buffer* (0.1M, pH 7.5) *and* b) *in CTAB reverse micelles containing the trinucleotides dissolved in the same buffer at* $w_o = 22$. Aqueous concentrations in both cases 1mM **1** and **2**, 200 mM EDC; temperature 5°.

according to the *Scheme* was compared with a reverse-micellar reaction solution with the same H_2O-pool concentration (1 mM **1** and **2**; 200 mM EDC). The reaction was first carried out without any template.

The reverse-micellar reaction system was prepared by dissolving all reactants together (*i.e.* **1**, **2**, and EDC) first in aqueous *Hepes* buffer. Immediately afterwards, this stock solution was injected into the CTAB reverse-micellar system, assuming that no reaction occurred before solubilization. In fact, control experiments in *Hepes* buffer showed that no product formation could be detected within the first 3 min.

It is clear from *Fig. 1* that the kinetical behavior of the reaction is the same within experimental errors in H_2O and in reverse micelles. In conclusion, then, the micellar medium does not impair the coupling of trinucleotides.

2.4. *Self-Replicating Hexanucleotides in Reverse Micelles.* The interesting question was now, whether it would be possible to achieve self-replication of these oligonucleotides in reverse micelles. To check this point, the condensation of the trimers **1** and **2** was first carried out in the presence of template **4**, by using two different experimental configurations. In the first, we just followed the procedure described in the previous section, namely mixing together **1** and **2** in the presence of **4** in a stock aqueous solution and adding this to the reverse micelles. The experimental data for this configuration are not shown.

In the second experimental configuration, the reaction in micelles was accomplished by mixing two separated reverse-micellar systems. One contained the trinucleotide 3′-phosphate **1** and the activator EDC, the second contained the 5′-aminotrinucleotide **2** and the template **4**. The H_2O-pool concentration of the oligonucleotides and the activator

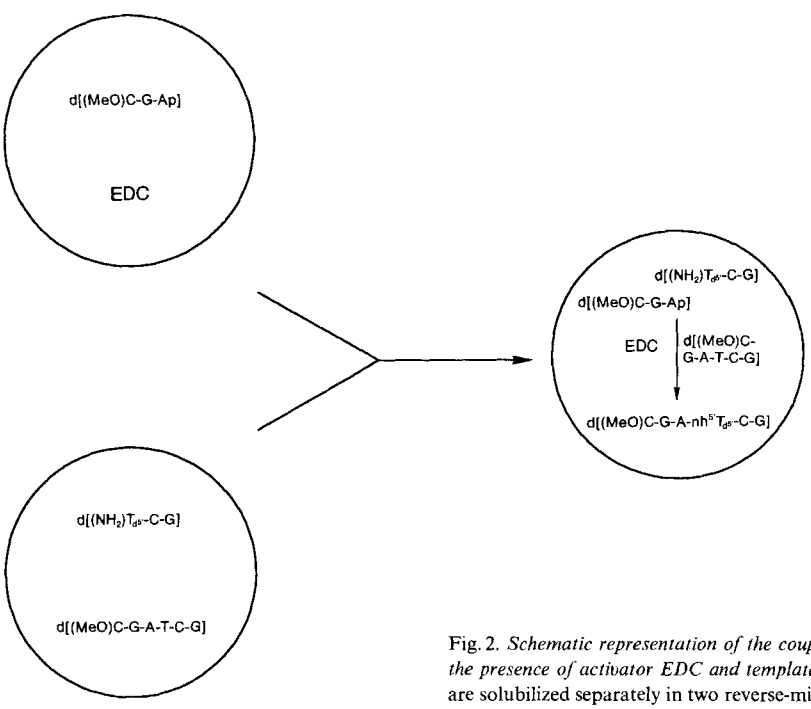


Fig. 2. *Schematic representation of the coupling of* **1** *and* **2** *in the presence of activator EDC and template* **4**. The reactants are solubilized separately in two reverse-micellar solutions.

in the two 'reactant-reverse-micellar' solutions had to be twice as high as the desired final total H_2O-pool concentration. In *Fig. 2*, an illustration of this concept can be seen. In this case, the micelles have to exchange their contents in order to make a chemical condensation between **1** and **2** possible. If the exchange rate of micellar material is much faster than the reaction rate, the two experimental setups should give the same results. This was in fact the expectation on the basis of literature. *E.g.*, *Jada et al.* determined the second-order rate constants for the exchange of material between the H_2O-pools of reverse micelles stabilized by cationic surfactants similar to CTAB to be in the range of $10^8\,M^{-1}\,s^{-1}$ [14]. The second-order rate constant for the coupling of **1** and **2** in the CTAB system was $7.08 \cdot 10^{-3}\,M^{-1}\,s^{-1}$ (see below), and in fact the kinetic results of the two reaction configurations yielded the same result.

In *Fig. 3*, the coupling reaction between **1** and **2** in CTAB reverse micelles as a function of different initial concentrations of template **4** is shown. One can see that the raction rate increased with increasing amount of pre-existing template **4** (*Curves a–d*). The system showed self-replication according to the scheme published by *von Kiedrowski* [2]. The so-

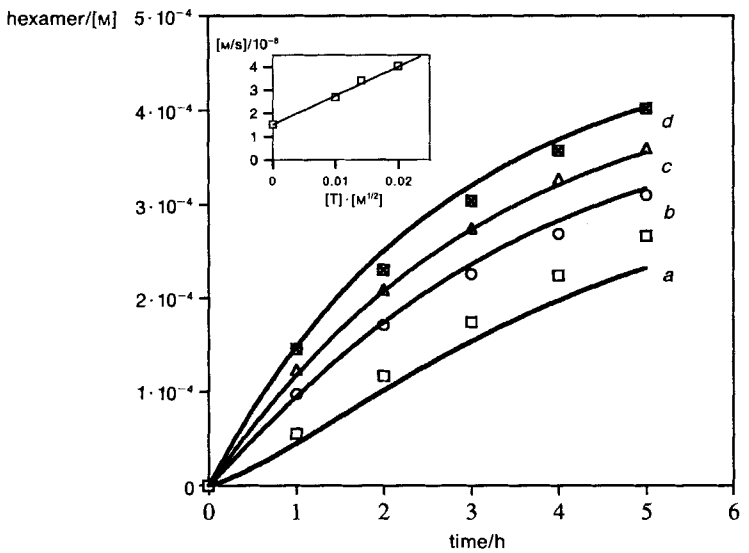


Fig. 3. *Time course of the formation of the (3'–5')-phosphoamidate* **3** *in presence of the template* **4** *in CTAB reverse micelles of* $w_0 = 22$ *determined by HPLC.* The *Curves a–d* represent the solution of the differential equation derived from a kinetical mechanism by *von Kiedrowski* [2]. The points are experimental mean values of the formation of **3**. The yield of **3** without template after 5 h was 25%. Initial concentrations and reaction conditions: [**1**] = [**2**] = 1 mM, [EDC] = 200 mM, [*Hepes*] = 0.01M, pH 7.5, *T* 5°, [**4**] = 0 (*a*), 0.1 (*b*), 0.2 (*c*), 0.4 mM (*d*). Of this solution, 10 µl were solubilized in 50 mM CTAB 50 mM; in 9.9% (*v*/*v*) pentan-1-ol/hexane.

called square-root law of template catalysis was valid also in reverse micelles under the experimental conditions. This. can clearly be seen from the inset in *Fig. 3*. A linear relationship between the square root of the template concentration and the initial velocity of the formation of compound **3** could be determined.

The rate constants were determined after analyzing the concentration data of all reactants and the product **3** by the program Simfit [2]. The rate constants for the reverse micellar system were: $k_a = 2 \pm 0.064$ $M^{-3/2}$ s^{-1}, $k_b = (7.08 \pm 0.58) \cdot 10^{-3}$ $M^{-1}s^{-1}$, and $k_c = (3.89 \pm 0.3) \cdot 10^{-5}$ s^{-1}. The relation k_a/k_b was 285. The efficiency of autocatalysis was smaller compared to the aqueous system (410), but was still in the same range.

Concerning the yield of the self-replication, it should be noted that for an initial concentration of 0.1 mM template, 0.31 mM **3** was additionally synthesized after 5 h, which corresponds to a 'self-replication factor' of 3. For 0.2 mM template, the yield of **3** was 0.36 mM, for 0.4 mM template, 0.402 mM, which corresponded to a factor of 1.8 and 1, respectively.

3. Conclusion. – We could show that self-replication of nucleotides can be mediated by a micellar reaction. The yield and kinetics of self-replication in micelles is very close to that in H_2O, and the fact that the time course of the reaction obeys the square-root law means that the self-replication mechanism postulated by *von Kiedrowski* [2] is not altered drastically under these conditions.

The fact that oligonucleotide self-replication can occur within a small, geometrically closed structure represents a step forward in the mimicking of the minimal life processes. The next, more challenging step would be the one in which the self-replication of the nucleotides proceeds simultaneously with the replication of the containing shell. In this case, we would have a 'shell replication' and a 'core replication' at once, which provides a much more elegant model for the cellular event. As it is well known, a few self-replicating micellar systems were described so far [7] [8]. Unfortunately, one cannot yet realize the self-replication chemistry of nucleotides within these self-replicating micelles. In fact, the self-replication of micelles takes place either under very high alkaline conditions or in the presence of permanganate, both prohibitive for the chemistry of nucleotide coupling. Also self-replication of CTAB micelles, utilized in this work as host for the nucleotide coupling, does not work in view of the unfavorable solubility relation of Me_3N and cetyl bromide [15]. It is our endeavor to continue our search for conditions which will allow to overcome this difficulty and thus permit a coupled 'shell and core' replication.

We would like to thank *Erich Küng* and *Patrick Iaiza* for their skilled technical assistance. Furthermore, we thank Prof. *Günter v. Kiedrowski* for his introduction to the program Simfit, written by himself, and *Matthias Matzen* for his help in evaluating our kinetic data with this program.

Experimental Part

1. *General.* All solvents were of highest quality available from *Fluka* and *Merck*. MeCN was refluxed over CaH_2 and disitilled just prior to use, 1*H*-tetrazole (*Fluka*) purified by sublimation. Anion-exchange chromatography: *Sephadex A25* (*Pharmacia*), $(Et_3NH)HCO_3$ buffer (pH 7.5) as eluent. HPLC: *Perkin-Elmer 4* machine *LCI-100* chromator-integrator for data analysis; linear-gradient elution 4.5–15% MeCN in NH_4OAc (0.1M, pH 7) in 16 min. After each HPLC run, the column was purified by applying a gradient up to 50% MeOH (particularly important for reverse-micellar reaction solutions as the CTAB molecule tends to stick to the reversed-phase material due to hydrophobic interactions). For each template concentration, three independent series were carried out at always the same times, and the mean values of the concentration of **3** were taken for kinetical analysis. The reproducibility of series of the same experimental conditions was very high ($\leq$ 5% standard deviation) due to the HPLC method which was used. The concentration of **3** was determined by calibration (concentration *vs.* peak area). The extinction coefficient at 260 nm was determined to be 57200 M^{-1} cm^{-1} [16].

2. *Synthesis of the Oligonucleotides.* 2.1. *d[(MeO)C-G-A_p]* (**1**). The fragment was synthesized on controlled-pore glass (CPG) modified in a similar way as described recently [13]. Ammonia treatment in the presence of 1,4-dithioerythritol (DTE) yielded directly the desired 3′-phosphate. Of this support material, 250 mg (14.5 μmol) were treated with 3% dichloroacetic acid/ethylene chloride yielding a free OH group. Standard phosphoramidites were coupled to this support by the phosphite method up to the desired sequence. The synthesis of the 5′-methoxyprotected 2′-deoxycytidine phosphoramidite d[(MeO)Cp′] (p′ = phosphoramidite) was described earlier [11]. After completion of the synthesis on the solid support, it was treated with 5 ml of 0.1M DTE in conc. NH_3 soln. at 70° for 4 h. After removal of the support material, the NH_3 soln. was evaporated on a speed-vac concentrator. The pellet was taken up in 400 μl (per tube) of 80% AcOH. Insoluble material was removed by filtration and the oligonucleotide precipitated by addition of 1 ml of Et_2O. After centrifuging (15 min, 0°), the supernatant was removed, and the pellets were dried in a speed-vac concentrator.

2.2. *d[(NH_2)$T_{d5'}$-C-G]* (**2**), *d[(MeO)C-G-A-nh$^{5'}T_{d5'}$-C-G]* (**3**), *and d[(MeO)C-G-A-T-C-G]* (**4**). The syntheses of **2**–**4** were performed on controlled-pore glass (CPG) modified with dG as solid support and starting with 250 mg (11.2 μmol) using phosphoramidite chemistry [17]. For the synthesis of **2**, we applied at the end the 3′-phosphoramidite of 5′-deoxy-5′-[(monomethoxytrityl)amino]thymidine. This building block was also used for the synthesis of **3** to create the internal phosphoramidate linkage, as described earlier [12]. Deprotection and workup was performed as described for **1**. The amino-linked fragment **3** had to be worked up differently because of acidic lability [12]. After evaporation of the NH_3 soln., the residues were taken up in 300 μl of H_2O/dioxane 1:2 per tube. Fragment **3** was precipitated after addition of 600 μl of THF. After centrifuging (15 min, 0°), the supernatant was removed, and the pellets were dried in a speed-vac concentrator.

2.3. *Purification.* After dissolving the crude compounds in doubly distilled H_2O, they were applied on a *Sephadex-A-25* column (35 cm) with the help of a peristaltic pump. Compounds **1** and **2** were gradient eluted with 0.1–1.4M $(Et_3NH)HCO_3$, compounds **3** and **4** with 0.1–1.0M $(Et_3NH)HCO_3$, resp. Pure fractions (checked by reversed-phase HPLC) were evaporated serveral times with H_2O/EtOH.

3. *Coupling of Oligonucleotides in Aqueous and in Reverse-Micellar Phase.* 3.1. *Aqueous Reaction Solutions.* They were prepared by the glass-capillary technique as recently published [11] [18]. The concentrations were 1 mM for **1** and **2** and 200 mM for the activator *N*-[(3-dimethylamino)propyl]-*N′*-ethylcarbomidiimide hydrochloride (EDC; *Fluka*). Before injection into the HPLC, the solns. were diluted by rinsing the capillaries into 249 μl of HPLC starting buffer (4.5% MeCN in $(Et_3NH)OAc$) containing 1M NaCl.

3.2. *Reverse Micellar Reaction Solutions.* Stock solns. of **1** and **2** (1.3 mM) and of template **4** (0.13 mM) were prepared separately in H_2O (stock *1*, *2*, and *4*). Aliquots (16 μl) of stock *1* were put into *Eppendorf* tubes and evaporated (stock *A*). Then, mixed solns. of stock *2* (always 16 μl) and different amounts of stock *4* (0, 16, 32, 64 μl) were prepared and evaporated (stock *B1*, *B2*, *B3*, and *B4*). To these stock solns. (*A*, *B1* to *B4*), 8 μl of an aq. *Hepes* buffer (10 mM; pH 7.5) were added. The buffer which was added to stock *A* contained 520 mM of EDC. Volumes of 7.75 μl of such prepared stock solns. were injected into 0.5 ml of the recently described CTAB-containing reverse-micellar system [11]. The resulting reverse-micellar solns. contained **1** at a final H_2O-pool concentration of 2 mM (400 mM EDC; soln. *I*), the corresponding reverse-micellar solns. contained **2** (2 mM), mixed with different concentrations of **4** (0, 0.2, 0.4, and 0.8 mM; soln. *II*). To start the reaction, soln. *II* was added to soln. *I* by a syringe. Note that by this procedure, all concentrations are being decreasing by a factor of 2. HPLC samples were prepared by diluting 20 μl of the reaction soln. with 80 μl of HPLC starting buffer containing 1M NaCl.

REFERENCES

[1] G. von Kiedrowski, *Angew. Chem.* **1986,** *10,* 932.
[2] G. von Kiedrowski, B. Wlotzka, J. Helbing, M. Matzen, S. Jordan, *Angew. Chem.* **1991,** *103,*4, 456.
[3] G. von Kiedrowski, A. Terfort, *Angew. Chem.* **1992,** *104,* 626.
[4] W.S. Zielinski, L.E. Orgel, *Nature (London)* **1987,** *327,* 346.
[5] T. Tjivikua, P. Ballester, J. Rebek, Jr., *J. Am. Chem. Soc.* **1990,** *112,* 1249.
[6] J.S. Nowick, Q. Feng, T. Tjivikua, P. Ballester, J. Rebek, Jr., *J. Am. Chem. Soc.* **1991,** *113,* 8831.
[7] P.A. Bachmann, P. Walde, P.L. Luisi, J. Lang, *J. Am. Chem. Soc.* **1990,** *112,* 8200.
[8] P.A. Bachmann, P. Walde, P.L. Luisi, J. Lang, *J. Am. Chem. Soc.* **1991,** *113,* 8204.
[9] P.A. Bachmann, P.L. Luisi, J. Lang, *Nature (London)* **1992,** *357,* 57.
[10] P. Schmidli, P. Schurtenberger, P.L. Luisi, *J. Am. Chem. Soc.* **1991,** *113,* 8127.
[11] Ch. Böhler, W. Bannwarth, M. Giustini, P.L. Luisi, *Helv. Chim. Acta* **1993,** *76,* 1341.

[12] W. Bannwarth, *Helv. Chim. Acta* **1988,** *71,* 1517.
[13] P. Kumar, N.K. Bose, K.C. Gupta, *Tetrahedron Lett.* **1991,** *32,* 967.
[14] A. Jada, J. Lang, R. Zana, R. Makhloufi, E. Hirsch, S.J. Candau, *J. Phys. Chem.* **1990,** *94,* 387.
[15] G. Mascolo, M. Giustini, P.L. Luisi, J. Lang, *J. Colloid Interface Sci.* **1990,** *140,* 401.
[16] C.R. Cantor, M.M. Warshaw, *Biopolymers* **1970,** *9,* 1059.
[17] H. Kiefer, W. Bannwarth, 'Immunological Methods', Eds. J. Lefkovits and B. Pernis, Academic Press, New York, 1985, Vol. 3, p. 69–83.
[18] B. Wlotzka, Dissertation, Universität Göttingen, 1992.