Dependence of the degree of association of mono- and disubstituted biologically active derivatives of fullerene C_{60} in aqueous solutions on the concentration and nature of substituents

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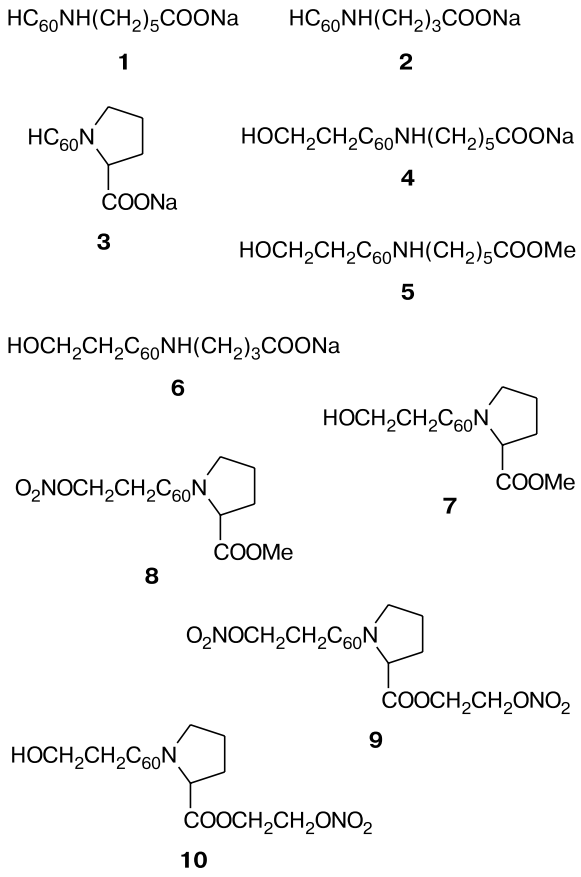
The degree of association of biologically active mono- and disubstituted fullerene derivatives in aqueous solutions was studied using the diffusion method. The degree of association, which exerts a strong effect on the biological activity of the fullerene derivatives, depends mainly on the nature of the substituents and, to a less extent, on the concentration of the substance in solution.

Key words: fullerene, amino acid, solubility in water, diffusion, associates.

Various aspects of biological activity of water-soluble amino acid derivatives of fullerene C_{60} (ADFs) and disubstituted fullerene derivatives derived from the monosubstituted derivatives were revealed in recent studies.[1,2] A wide range of biological activity of mono- and disubstituted ADFs is caused by the unique structure of the carbon spheroid and its ability to transform oxygen to the singlet state[3] and manifest the membranotropic and antiradical properties.[4] As shown by biological tests, these compounds exhibit the antiviral[5—7] and antotumor activities,[8,9] exert a positive effect on the cardiovascular system,[10] and are convenient adjuvants.[11] However, the state and behavior of these compounds in water should be studied to use aqueous solutions of the fullerene derivatives in biology.

The following compounds were chosen in this work as objects of the study: sodium salt of *N*-monohydrofullerenyl-ω-aminocaproic acid (**1**), sodium *N*-monohydrofullerenyl-γ-aminobutyrate (**2**), sodium L-*N*-monohydrofullerenylprolinate (**3**), sodium *N*-{[2-(hydroxy)ethyl]-fullerenyl}-ω-aminocaproate (**4**), methyl *N*-{[2-(hydroxy)-ethyl]fullerenyl}-ω-aminocaproate (**5**), sodium *N*-{[2-(hydroxy)ethyl]fullerenyl}-γ-aminobutyrate (**6**), methyl L-*N*-{[2-(hydroxy)ethyl]fullerenyl}prolinate (**7**), methyl L-*N*-{[2-(nitroxy)ethyl]fullerenyl}prolinate (**8**), 2-nitroxyethyl L-*N*-{[2-(nitroxy)ethyl]fullerenyl}prolinate (**9**), and 2-nitroxyethyl L-*N*-{[2-((hydroxy)ethyl]fullerenyl}-prolinate (**10**).



The most popular methods for the determination of molecular characteristics from sedimentation data are the absolute methods of sedimentation equilibrium[12] and approaching to sedimentation equilibrium (Archibald method[13]). In this case, these methods turned out to be inappropriate, because for high rotation rates of a rotor (≥40 000 min^{-1}) necessary for equilibration the substance precipitated partially or completely on the bottom of the cell under the centrifugal field of a centrifuge, which is

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characteristic of large nanoparticles in the microgel. In addition, the sedimentation patterns very rapidly diffused during the experiment, also indicating the presence of large particles and the non-true character of solutions of the compounds under study. Therefore, to study the molecular characteristics, we used the procedure of measuring the diffusion coefficient D.[14] Knowing of the diffusion coefficient makes it possible to monitor the stability of the molecular structure in solution and its possible changes and to calculate the translational friction coefficient of the molecules and their mean hydrodynamic radius, *i.e.*, the so-called Stokes radius. If molecules of the compounds under study exist in solution as spheres and no solvation of the molecules is observed, it is easy to calculate the volume and molecular weight of the particles based on the Stokes radius.

Experimental

Experiments were carried out on a MOM-3180 analytical ultracentrifuge (Hungary) using the Philpot—Svensson optics at a temperature of the rotor of 25±0.1 °C and water as the solvent. The diffusion coefficient (D) was measured in a boundary-forming cell by the deposition of the solvent on the solution with the final concentration in a wide concentration range. The boundary between the pure solvent and solution was photographed at specific time intervals thus detecting its expansion. The rotation rate of the rotor (4000—6000 min^{-1}) was chosen in such a way that the particles would not settle during the time of experiment and the boundary would take place only due to diffusion. The partial specific volume of particles in the solution ($\bar{V}$) was determined picnometrically. The density of water ρ_0 = 0.997 g cm^{-3} and the viscosity of water at 25 °C η_0 = 0.8937 cP are tabulated values. The gradient curves had rather close to the Gaussian shape and, hence, the imaginary diffusion coefficient D_c (diffusion coefficient at the final concentration) was calculated from the ratio of the surface area under the curve (Q) to the maximum ordinate of the curve (H) at the moment t (see Ref. 15)

$$D_c = (Q/H)^2/(4\pi t). \quad (1)$$

The D coefficient is determined by the translational friction coefficient f:

$$f_{sph} = kT/D_{sph}, \quad (2)$$

where k is the Boltzmann constant, and T is the absolute temperature (K).

According to the Stokes equation for spherical molecules, the translational friction coefficient f_{sph} is related to the diameter d

$$f_{sph} = 3\pi\eta_0 d, \quad (3)$$

where η_0 is the viscosity of the solvent.

If the diameter of the sphere is expressed through its volume $d = (6V/\pi)^{1/3}$, Eq. (3) can be written as follows:

$$f_{sph} = 3\pi\eta_0(6V/\pi)^{1/3}. \quad (3a)$$

When the solvation of particles is absent, *i.e.*, $V = M\bar{V}/N_A$ (M is the molecular weight, N_A is Avogadro´s number, and $\bar{V}$ is the partial specific volume),[14] we obtain for the sphere

$$f_{sph} = 3\pi\eta_0(6M\bar{V}/\pi N_A)^{1/3}. \quad (4)$$

Solving this equation with respect to M, we derive the formula for the calculation of the molecular weight

$$M = (f_{sph}/3\pi\eta_0)^3/(6\bar{V}/\pi N_A). \quad (5)$$

Results and Discussion

Using the measured values of the diffusion coefficient D_c, the partial specific volume $\bar{V}$, and above presented Eqs (2)—(5), we calculated the translational friction coefficients f, the molecular weight M of particles (named associates), the number of individual molecules with the molecular weight M_0 in the associate $n = M/M_0$, the volumes of the associates and individual molecules $V_{ass} = V_{sph}$ and $V_0 = V_{ass}/n$, and their diameter $d_{ass} = d_{sph}$ and $d_0 = (6V_0/\pi)^{1/3}$ for aqueous solutions of the compounds under study.

All compounds under study can be grouped according to the structure. The first, simplest in structure group contains sodium salts of ADFs **1**—**3**. These fullereneamino acids are not dissolved in water; however, their salts are highly soluble (solubility ~50 mg mL^{-1}) and possess high antiviral activity.[5,6] Therefore, their behavior in an aqueous solution should be studied.

As can be seen from the data in Table 1, the degree of association and the behavior of these compounds measured in the same concentration interval depend strongly on the nature of the amino acid residue. At high concentrations compound **1** with the longest hydrophobic fragment was most associated. At the same concentrations the degree of association for compound **2** is somewhat lower: the hydrophobic fragment in **2** is slightly shorter than that in compound **1**. They behave quite similarly during dilution: the associates decompose rapidly. Their partial specific volumes $\bar{V}$ are equal (0.541 $cm^3\ g^{-1}$) and the biological activities are resembling: the both compounds are antiviral drugs, but compound **1** is better against AIDS infection and compound **2** possesses the very high activity against the cytomegaloviral infection. Compound **3** stands apart: it is not associated so strongly at high concentrations and its degree of association with dilution decreases only by several times rather than by several orders of magnitude as for compounds **1** and **2**. The value $\bar{V}$ = 0.728 $cm^3\ g^{-1}$ indicates that the molecule is twisted not so strongly as the previous molecules.

To extend the spectrum of biological activity of the ADFs and to impart new properties to the known biologically active compounds, it is necessary to introduce into ADF molecules such fragments that knowingly possess biologically activity. This will possibly result in the ap-

Table 1. Effect of concentration on the degree of association of sodium salts of ADFs **1**—**3**

C/mg mL^{-1}	$D_c \cdot 10^7$ /cm^2 s^{-1}	f_{sph}	R_{sph}	$V_{sph} \cdot 10^{22}$ /cm^3	$M_{D_{sph}} \cdot 10^6$	n	$V_{ful} \cdot 10^{22}$ /cm^3	R_{ful}	d_{ful}
		10^{-8} cm						10^{-8} cm	
				Salt ADF **1**					
1.25	2.8	14.687	87.18	27755	2.97	3410	8.1410	5.80	11.6
0.75	3.45	11.920	70.74	14830	1.586	1820	8.1410	5.80	11.6
0.50	23.25	1.769	10.50	48.49	0.00519	5.96	8.1010	5.78	11.58
0.125	31.5	1.305	7.745	19.46	0.00208	2.4	8.1110	5.78	11.6
				Salt ADF **2**					
1.50	3.29	12.5	74.2	17100	1.03	1221	14	6.94	13.88
1.25	5.20	7.9	46.9	4330	0.261	309	14	6.94	13.88
0.75	7.64	5.38	31.9	1360	0.0822	97.5	13.9	6.93	13.86
0.50	13.36	3.08	18.3	255	0.0154	18.2	14	6.94	13.88
				Salt ADF **3**					
1.60	13.3	3.03	18.03	245	2.03	23.7	10.34	6.27	12.50
1.25	15.9	2.55	15.08	143.6	1.19	13.88	10.34	6.27	12.50
1.00	17.3	2.33	13.86	111.5	0.9225	10.76	10.36	6.28	12.58
0.75	18.5	2.18	12.96	91.2	0.754	8.8	10.36	6.28	12.55

pearance of drugs with new properties and higher efficiency. The first step is the modification of compounds **1**—**3** by the hydroxyethyl group, which is the initial moiety for the further chemical modification. Therefore, compounds **4**—**7**, *viz.*, disubstituted derivatives with the hydroxyethyl fragment in the molecule, should be ascribed to the next group (Table 2). The introduction of the hydroxyethyl fragment into a molecule of compounds **1** and **2** afforded compounds **4** and **6**, whose degree of association increased multiply even in more dilute solutions. However, the behavior of the solutions upon dilution remained the same: the associates decompose rapidly. The substitution of the Na^+ ion for the Me group produces compounds **5** and **7**, whose degree of association is higher than that of compounds **1**—**3** but lower than that of **4** and **6**. As can be seen from the data in Table 2, the degree of association of compounds **4** and **5**, which are ω-aminocaproic acid derivatives, differs drastically, indicating

Table 2. Effect of concentration of degree on association of hydroxyethyl-substituted ADFs **4**—**7**

C/mg mL^{-1}	$D_c \cdot 10^7$ /cm^2 s^{-1}	f_{sph}	R_{sph}	$V_{sph} \cdot 10^{22}$ /cm^3	$M_{D_{sph}} \cdot 10^6$	n	$V_{ful} \cdot 10^{22}$ /cm^3	R_{ful}	d_{ful}
		10^{-8} cm						10^{-8} cm	
				Compound **4**					
0.8	2.17	18.90	112	59000	4.20	$4586 \cdot 10^6$	12.9	6.74	13.5
0.6	2.68	15.30	90.7	31200	2.20	$2423 \cdot 10^6$	12.8	6.74	13.5
0.4	3.15	13.05	61.2	9560	0.68	743	12.9	6.74	13.5
0.2	5.80	7.08	42	3110	0.22	242	12.8	6.76	13.5
				Compound **5**					
0.8	2.06	19.950	118.32	69380	4.9000	5390	12.87	6.748	13.5
0.6	3.45	11.910	70.8	14730	1.0400	1144	12.87	6.750	13.5
0.4	4.77	8.616	51.10	5589	0.3950	434.5	12.86	6.748	13.5
0.2	8.60	4.779	28.34	953.4	0.0674	74.1	12.87	6.748	13.5
				Compound **6**					
0.750	1.15	35.76	212.22	400350	42.1000	47303	8.46	5.87	11.74
0.500	5.33	7.715	45.786	4020	0.4220	474.7	8.47	5.87	11.74
0.250	6.87	5.986	35.525	1878	0.1974	221.8	8.47	5.87	11.74
0.125	17.23	1.226	7.2976	16.28	0.0017	1.9	8.57	5.89	11.78
				Compound **7**					
2.150	5.7	6.847	40.6	2811	19.40	217.40	12.93	6.76	13.52
1.500	8.0	5.050	30.1	1128	7.79	87.25	12.93	6.76	13.52
1.075	8.9	4.540	27.0	819	5.65	63.30	12.94	6.76	13.52
0.750	9.9	4.080	24.2	595	4.11	46.00	12.94	6.76	13.52
0.500	11.2	3.611	21.4	411	2.84	31.80	12.94	6.76	13.52

the different mechanism of dissolution of these compounds in water and, hence, different mechanism of associate formation. Compound **4** is a salt dissociating in water and, therefore, charged particles with a large hydrophobic fragment are dissolved only due to the Coulomb interactions forming large associates. Electroneutral compound **5** is dissolved due to the hydration of the molecule in the presence of the hydroxyethyl fragment, because methyl *N*-(monohydrofullerenyl)-ω-aminocaproate is not dissolved in water. The values $\bar{V} = 0.846$ and 0.852 $cm^3\ g^{-1}$ for compounds **4** and **5**, respectively, show that the introduction of the hydroxyethyl fragment strongly changes the conformation of the molecules, *viz.*, makes them less twisted and more accessible for chemical modifications. The replacement of the ionic form of the molecule by the nonionic form affects the solubility of the compounds: the solubility in water for methyl derivatives **5** and **7** is ~10 mg mL^{-1}, which is much lower than that of Na salts **4** and **6** (~50 mg mL^{-1}). The degree of association of compound **7** also increases compared to that of derivative **3** but not so strongly as for compounds **4**—**6**. This parameter is very important, and we used it for choosing as the fullerene derivatives such amino acids that are most appropriate for the further modifications of the molecules by biologically active fragments, because the high degree of association exerts a negative effect on the biological activity. In addition, the value $\bar{V} = 0.872\ cm^3\ g^{-1}$ of compound **7** is highest, indicating a greater accessibility of the functional groups in such a molecule as compared to more compact molecules with lower $\bar{V}$. Therefore, we chose the prolin derivatives for further study.

Recent studies[16] revealed various aspects of the antitumor effect of the exogenic NO-donors. They enhance the efficiency of the known cytostatics, retard the development of metastases in experimental tumors, and modulate the sensitivity of the drug-resistant tumors to cytostatic therapy. Therefore, we synthesized[9] the nitrosubstituted derivatives of different structure, which, as it turned out, during biotransformation generate nitrogen monoxide and exhibit activity as antitumor drugs. The concentration dependence of the degree of association of compounds **8**—**10**, which are the mono- and dinitrosubstituted derivatives, is presented in Table 3. The introduction into the molecule of nitro groups in different amounts and into different positions increased the degree of association in all cases. The maximum increase was observed for compound **9** containing two nitro groups in the molecule, probably, due to the stronger intermolecular interaction created by two functional groups. This effect was somewhat weaker for compound **8** containing only one functional nitro group in the molecule, which results in a decrease in the intermolecular interaction compared to compound **9**. The behavior of solutions of compounds **8** and **9** upon dilution is the same: the associates decompose rapidly due to a decrease in the intermolecular interaction. The $\bar{V}$ values for compounds **8** and **9** do not differ strongly (0.849 and 0.830 $cm^3\ g^{-1}$, respectively). This indicates a similar conformation of the molecules. Compound **10** with two different functional groups behaves in quite a different manner. The value $\bar{V} = 0.646\ cm^3\ g^{-1}$ indicates strong intermolecular interactions and low accessibility of the nitro group for external effects. The associates become smaller only insignificantly upon dilution, which also impedes the functioning of the nitro group. The same is confirmed by the biological tests: the activity of compound **10** as an antitumor drug is minimum.[9]

Table 3. Effect of concenfion on the degree of association of nitro derivaties **8**—**10**

C/mg mL^{-1}	$D_c \cdot 10^7$ /$cm^2\ s^{-1}$	f_{sph}	R_{sph}	$V_{sph} \cdot 10^{22}$ /cm^3	$M_{D_{sph}} \cdot 10^6$	n	$V_{ful} \cdot 10^{22}$ /cm^3	R_{ful}	d_{ful}
		10^{-8} cm						10^{-8} cm^1	
				Compound **8**					
3.2	1.38	29.30	174	220000	1550	16640	13.2	6.81	13.6
2.5	3.23	12.50	74.2	27100	121.5	1296	13.2	6.81	13.6
2.0	6.54	6.18	36.7	2070	14.6	156.3	13.2	6.81	13.6
1.6	9.48	4.26	25.3	677.6	4.8	51.25	13.2	6.81	13.6
1.0	12.53	3.23	19.2	295	2.1	22.2	13.2	6.81	13.6
				Compound **9**					
2.00	2.4	16.830	99.90	41760	305	2991.5	13.95	6.93	13.86
1.60	4.5	8.978	53.28	6336	46	453.9	13.96	6.93	13.86
1.00	5.9	6.847	40.64	2811	20.4	201.4	13.95	6.93	13.86
0.75	8.4	4.809	28.54	232.5	1.69	16.6	14.00	6.94	13.86
				Compound **10**					
1.75	5.8	6.960	41.34	2960	27.6	285.0	10.4	6.28	12.56
1.36	6.5	6.220	36.92	2108	19.6	203.0	10.4	6.28	12.56
1.00	7.1	5.695	33.80	1617	15.0	155.3	10.4	6.28	12.56
0.75	7.5	5.390	32.00	1370	12.8	131.8	10.4	6.28	12.56
0.55	7.9	5.100	30.35	1174	10.9	112.8	10.4	6.28	12.56

Thus, the study of the behavior of the fullerene derivatives in aqueous solutions showed that the chemical structure exerts the maximum effect on the degree of association and molecular conformation. The concentration of the solution also affects but not so strongly. The data on a change in the degree of association at different concentrations are useful for the preparation and storage of aqueous solutions for biological research.

References

1. A. Bianco, T. Da Ros, M. Prato, and C. Toniolo, *J. Peptide Sci.*, 2001, **7**, 208.
2. S. Bosi, T. Da Ros, G. Spalluto, and M. Prato, *Eur. J. Med. Chem.*, 2003, **38**, 913.
3. H. Tokuyama, S. Nakamura, and E. Nakamura, *J. Am. Chem. Soc.*, 1993, **115**, 7918.
4. R. A. Kotelnikova, A. I. Kotelnikov, G. N. Bogdanov, V. S. Romanova, E. F. Kuleshova, Z. N. Parnes, and M. E. Vol´pin, *FEBS Lett.*, 1996, **389**, 111.
5. N. E. Fedorova, S. M. Adueva, A. A. Medzhidova, V. S. Romanova, Z. N. Parnes, G. A. Galegov, and A. A. Kushch, *Voprosy virusologii* [*Problems of Virology*], 2002, No. 1, 30 (in Russian).
6. G. G. Miller, V. S. Romanova, L. N. Pokidysheva, I. V. Titova, E. N. Kaliberda, L. D. Rumsh, O. I. Andreeva, and N. P. Rybalkin, *Antibiotiki i khimioterapiya* [*Antibiotics and Chemotherapy*], 2004, **49**, No. 12, 3 (in Russian).
7. S. M. Andreev, A. O. Petrukhina, A. A. Babakhin, A. V. Garmanova, V. S. Romanova, and L. M. DyuByuske, *Immunologiya* [*Immunology*], 2006, **27**, No. 6, 343 (in Russian).
8. Application RF N2005140680 of 25.12.2005, *Byul. Izobret.* [*Invention Bulletin*], 2007, 20.07, No. 0720 (in Russian).
9. European Application N PST/RU 2007/000337 of 19.06.2007; A. I. Kotel´nikov, R. A. Kotel´nikova, N. P. Konovalova, G. N. Bogdanov, I. I. Skhodkina, V. S. Romanova, and S. M. Adreev, *Ros. Bioterapevt. Zh.* [*Russian Biotherapeutical Journal*], 2007, **6**, No. 1, 76 (in Russian).
10. L. I. Serebryakova, O. I. Pisarenko, O. V. Tskitishvili, I. I. Faingol´d, R. A. Kotel´nikova, V. S. Romanova, N. A. Sanina, A. I. Kotel´nikov, S. M. Aldoshin, and E. I. Chazov, *Tez. dokl. Ros. nats. kongressa kardiologov "Ot dispanserizatsii k vysokim tekhnologiyam," Prilozhenie 1 k zhurn. "Kardiovaskulyarnaya terapiya i profilaktika"* [*Abstrs of the Russian National Congress of Cardiologists "From Clinical Examination to High Technologies," "Appendix 1 to the Journal "Cardiovascular Therapy and Prophylaxis"*], 2006, **5**, No. 6, 339 (in Russian).
11. Pat. RF 2129436; *Byul. Izobret.* [*Invention Bulletin*], 1999, 12 (in Russian).
12. W. D. Lansing and E. O. Kraemer, *J. Am. Chem. Soc.*, 1935, **57**, 1389.
13. W. J. Archibald, *J. Appl. Phys.*, 1947, **18**, 362.
14. V. N. Tsvetkov, V. E. Eskin, and S. Ya. Frenkel´, *Struktura makromolekul v rastvorakh* [*Structure of Macromolecules in Solutions*], Nauka, Moscow, 1964, pp. 354; 397 (in Russian).
15. H. G. Elias, *Ultrazentrifugen-Methoden*, Beckmann-Instruments GmbH, München, 1961, 96.
16. N. P. Konovalova, *Tekhnologii zhivykh sistem* [*Technology of Living Systems*], 2004, **1**, No. 3, 42 (in Russian).

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