Properties of Swollen Polymer Networks. Solvation and Swelling of Peptide-Containing Resins in Solid-Phase Peptide Synthesis

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Abstract: Peptide-resins containing linear peptides up to 6000 molecular weight were prepared by stepwise solid-phase synthesis of the repeating model sequence Leu-Ala-Gly-Val-oxymethylphenylacetic acid on copoly(styrene–1% divinylbenzene) resin beads. Initial substitutions of 0.22 and 0.95 mmol/g of polystyrene were used and the resulting peptide-resins contained 11–81% peptide. Representative samples were taken after the addition of each model sequence unit and the diameters of the dry beads were measured by direct microscopic examination. The volume of 1 g of the unsubstituted dry beads was 1.0 mL and was found to increase by more than fivefold as the peptide content approached 80%. Similar measurements of the samples in CH_2Cl_2 or DMF showed that the swollen volumes of 1 g of the unsubstituted beads were 6.2 and 3.3 mL, respectively. The volumes of the swollen peptide-resins showed a dramatic increase in the course of the synthesis, and at 80% peptide the volumes/g of polystyrene were 12 mL in CH_2Cl_2 and 28 mL in DMF. There was no indication that the upper limits of swelling of the peptide-containing resin had been reached. The solvation properties of the cross-linked polymer network and the pendant peptide chains mutually affect one another and at high loadings of peptide in a solvent such as DMF the peptide component has a dominating influence on the swelling of the peptide-resin beads. The swelling of the unsubstituted resin is due to a decrease in free energy from solvation of the polystyrene and, at equilibrium, is balanced by the elastic restraining force resulting from deformation of the loosely cross-linked polymer network. The increased swelling of the peptide-resin can then be attributed to the additional net decrease in free energy from solvation of the linear peptide chains, which is counteracted by an increase in the elastic restraining force arising from further deformation of the loosely cross-linked network structure of the polymer support. No such additional counterforce is expected to arise from deformation of the linear peptide chain imposed by the expansion of the polymeric support. In either CH_2Cl_2 or DMF, the space available for peptide chain growth within the swollen resin beads is *not* a limiting factor in solid-phase peptide synthesis. After the synthesis of the 60-residue model there was actually more space within the bead for chain growth than at the beginning of the synthesis. The results of this study allow a rational choice of the level of loading of peptide on the resin and of an appropriate protocol for the synthesis of a particular peptide.

Solid-phase synthesis[1] has been successfully used for the preparation of a wide range of peptides and for facilitating a number of synthetic organic transformations.[2,3] In order to effectively utilize this technique to its full potential it is important to understand the nature of the polymer support and its role in the course of a synthesis. The most fundamental and important property of the support in that respect is its macroscopic insolubility. This permits purification by filtration and provides the speed, simplicity, and high recoveries associated with the method. However, a very important further consideration for a successful solid-phase synthesis is the extent of solvation and swelling of the peptide-resin in the reaction medium.[4] It is well known that the resin beads must be freely permeated by solvent and reagent molecules and that the coupling of amino acids to the growing peptide chain will proceed to completion only if carried out in solvents which swell the resin.[1] In addition, it has been recommended that the polymeric support be designed to have solvation properties similar to those of the peptide product.[5] Inadequate views of the solvation and swelling behavior of the peptide-resin during synthesis have previously been invoked to explain instances of poor synthetic results as being inherently due to the polymer-supported nature of the reactions.[6-10] We now believe that these poor results have chemical explanations[11] and that a revised model for swelling is required.

Here we have attempted to describe and explain the swelling behavior of peptide-containing copoly(styrene–divinylbenzene) resin beads during the course of the synthesis, and in particular to answer the questions: (1) How are the swelling properties of the peptide-resin influenced by each of the components of the system (the peptide, the resin, and the solvent)? (2) Is there a fixed maximum volume for a swollen resin bead, which is eventually filled by the growing peptide?

The questions were examined by preparing a series of resins containing increasing amounts of peptide, up to very high levels, and measuring the volumes of the beads in the dry and swollen states. It was found that the peptide-resin beads swell in some solvents to a much greater extent than previously thought, and the breakdown in synthetic capacity predicted by previous models[12,13] was not observed even under extreme conditions. The implications of these findings with respect to peptide synthesis on cross-linked polymer supports in general and to specific reaction protocols in particular are discussed, and a new model of peptide-resin swelling is advanced.

Results

Assembly of the Test Peptides. Resin-bound test peptides composed of 1–12 repeating units of the model peptide Leu-

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Scheme I

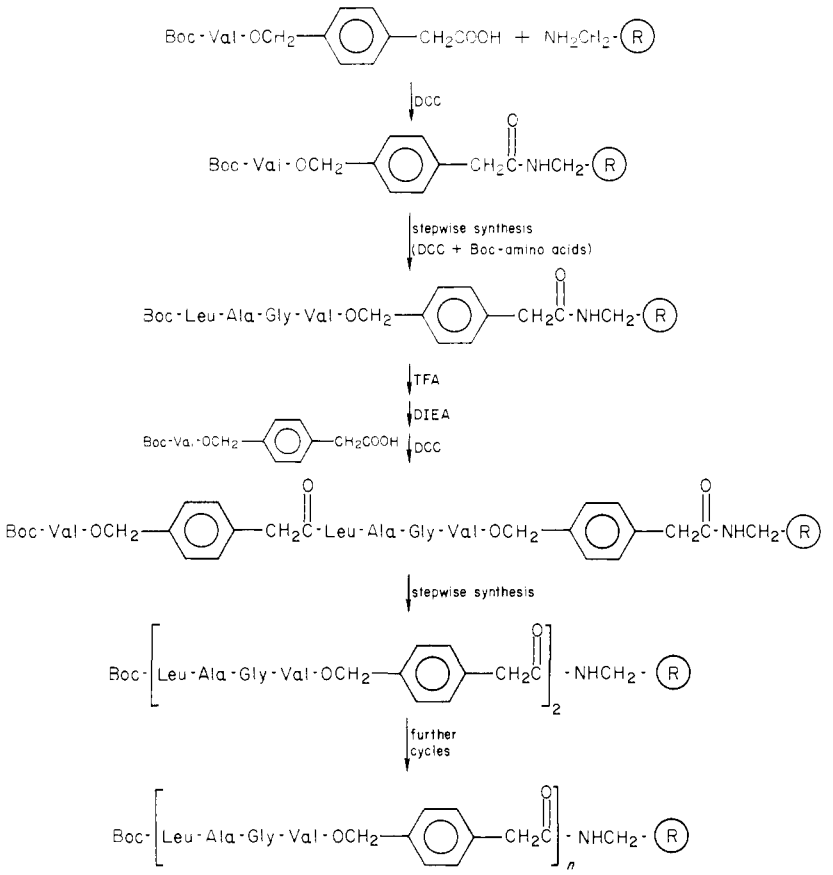

Table I. Synthetic Boc-(LAGV-OMPA)$_n$-NHCH$_2$-Resins

sample	n	initial substitution, mmol/g	amino acid analysis,[a] mmol/g pep-res					peptide content		
			Leu	Ala	Gly	Val	av	calcd[b] %	found[c] %	found[d] g pep/g styrene
1	0	0.22								
2	1	0.22	0.20	0.19	0.19	0.19	0.19	11.4	11.2	0.13
3	2	0.22	0.35	0.34	0.34	0.33	0.34	19.1	18.3	0.22
4	3	0.22	0.47	0.49	0.47	0.45	0.47	25.6	24.5	0.32
5	4	0.22	0.58	0.59	0.59	0.56	0.58	31.0	29.7	0.42
6	0	0.95								
7	1	0.95	0.57	0.55	0.56	0.58	0.57	35.5	33.6	0.51
8	2	0.95	0.86	0.88	0.88	0.85	0.87	50.1	46.8	0.88
9	3	0.95	1.10	1.11	1.12	1.07	1.10	59.3	57.4	1.35
10	4	0.95	1.25	1.25	1.26	1.23	1.25	65.7	64.2	1.79
11	6	0.95	1.44	1.42	1.43	1.43	1.43	73.9	72.2	2.60
12	7	0.95	1.48	1.45	1.48	1.59	1.50	76.6	75.4	3.06
13	8	0.95	1.52	1.50	1.52	1.62	1.54	78.9	77.1	3.37
14	9	0.95	1.54	1.54	1.55	1.66	1.57	80.7	78.4	3.63
15	10	0.95	1.56	1.56	1.57	1.68	1.59	82.4	79.2	3.81
16	11	0.95	1.60	1.60	1.58	1.67	1.61	83.6	80.0	4.00
17	12	0.95	1.65	1.60	1.65	1.65	1.64	84.7	81.4	4.38

[a] Hydrolyzed in 12 N HCl–propionic acid (1:1 v/v), 130 °C, 6 h, in duplicate. [b] Weight percent peptide in peptide-resin = [(mol wt × initial initial substitution)/(1 + mol wt × initial substitution)] × 100. [c] Average amino acid analysis × (mol wt/n) × 100. [d] g peptide/g polystyrene = % peptide/(100 − % peptide).

Ala-Gly-Val-oxymethylphenylacetic acid (LAGV-OMPA) were synthesized. This particular peptide unit was selected because of its special advantages in conjunction with our ongoing studies of synthetic efficiency as a function of distance from the polymer backbone. The peptides were synthesized on an aminomethylcopoly(styrene–1% divinylbenzene) resin support[14] according to Scheme I. The first residue of the peptide chain was attached through the oxymethylphenylacetic acid residue to give Boc-Val-OCH$_2$-Pam-resin.[15,16] Since this linkage is stable to prolonged trifluoroacetic acid treatment, no significant loss of chains was expected during the assembly of the peptide chain and, in addition, the major terminating side reaction, trifluoroacetylation, could be avoided.[11] This enabled the preparation of resins containing the high levels of peptide required for this study. The first mo-

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Table II. Dimensions of Dry Beads

sample no.	peptide content, %	measured diameter[a] no. beads	geometric mean, d_g, μm	geometric[b] standard deviation, σ_g	rel vol calcd from geometric diameter[c]	rel vol calcd from peptide content[d]
1	0	100	46.0	1.24	1	1
2	11.2	160	49.6	1.25	1.25	1.10
3	18.3	150	48.4	1.22	1.18	1.18
4	24.5	160	51.6	1.21	1.41	1.28
5	29.7	200	53.6	1.21	1.58	1.38
6	0	240	49.0	1.38	1	1
7	33.6	320	58.0	1.27	1.66	1.46
8	46.8	168	61.0	1.34	1.93	1.82
9	57.4	211	63.6	1.31	2.19	2.27
10	64.2	71	66.6	1.34	2.51	2.69
11	72.2	124	72.0	1.28	3.17	3.47
12	75.4	120	74.8	1.31	3.56	3.91
13	77.1	300	79.0	1.28	4.19	4.21
14	78.4	90	81.0	1.39	4.52	4.46
15	79.2	120	82.6	1.33	4.79	4.63
16	80.0	300	85.6	1.30	5.33	4.82
17	81.4	360	84.0	1.27	5.04	5.18

[a] These are averages of measurements on two separate samples of each peptide-resin preparation. The average agreement between measured mean diameters was ±5%. [b] For a single population, 68.26% of a very large number of repetitions of a measurement will fall within the range $d_g \pm \sigma_g$. [c] (Diameter of peptide-resin)^3/(diameter of unsubstituted resin)^3. [d] Calculated from the amino acid analysis, using the following densities: aminomethyl-resin, 1.04 g/mL; copoly(styrene–1% divinylbenzene), 0.99 g/mL; peptide, 1.00 g/mL.

nomer LAGV-OMPA unit ($n = 1$) was then assembled by normal stepwise synthesis, using dicyclohexylcarbodiimide activation. Additional units were assembled, up to $n = 12$, by further stepwise couplings. Initial substitutions of 0.22 and 0.95 mmol of aminomethyl/g of resin were selected for this study in order to obtain a wide range of ratios of peptide to resin. For each peptide-resin, the ratio was determined by hydrolysis and amino acid analysis. The data, which are assembled in Table I, show that the mole ratios of the component amino acids were very close to unity at every stage of the synthesis up to $n = 12$. The average deviation was approximately ±4%, which is within the limits of the automated ninhydrin method of analysis.[17] The agreement between the duplicate analyses of separate hydrolysates was also within this limit (data not shown). Therefore, the average amino acid analysis is a good representation of the amount of peptide present at each stage of the polypeptide synthesis. The average amino acid analyses shown in the table are expressed as mmol/g of peptide-resin and must be corrected for the weight gain due to the peptide before they will correspond directly with the values for initial substitution. Because this is a large correction at the higher sample numbers, the values for weight ratio of peptide/styrene are very sensitive to the amino acid analysis. The discrepancy between the percent peptide expected from the initial substitution together with the number of LAGV-OMPA units added and that found by amino acid analysis was approximately 4%. This may, in part, be due to a systematically low extent of hydrolysis by the HCl–propionic acid method,[18] but is primarily due to low levels of chain termination at each of the 48 synthetic cycles. The data show an average of about 1% termination per cycle. This level of termination was confirmed for the $n = 6$ sample by picrate titration,[19] and by coupling Boc-Phe to an aliquot of the peptide-resin and determining the amount of Phe/g of polystyrene. The series of peptide resins prepared for this study covered a loading range of 11.2–81.4% peptide. These correspond to weight ratios of peptide to polystyrene of 0.13:1 to 4.38:1. The lower levels are similar to the usual loadings for peptide synthesis, while the upper levels represent very high loadings that are not normally reached even in the synthesis of small proteins. They were expected to provide a rather extreme test of the questions posed in this study.

Density Measurements. The densities of dry, unswollen resin samples were determined by bulk measurements. A known mass of beads was covered with a nonswelling solvent such as petroleum ether which would fill the interstitial spaces between the beads without penetrating them. From the total weight and volume and the known density of the solvent, the volume occupied by the resin and hence its density could be calculated. The density of the copoly(styrene–1% divinylbenzene) resin was found to be 0.99 ± 0.08 g/mL (lit.[20] for polystyrene 1.04), the density of the aminomethyl-resin (0.95 mmol/g) was 1.04 ± 0.05 g/mL, and that of the Boc-(LAGV-OMPA)$_6$-NHCH$_2$-resin (0.95 mmol/g) was 1.23 g/mL. Since the latter sample was 72.2% peptide by amino acid analysis, the dry volume/g of polystyrene was determined to be 2.93 mL.

Dimensions of Dry Beads. Diameters of the unswollen, dry resin and peptidyl-resin beads were measured directly under the light microscope at low power. Representative samples were obtained by the following procedure. After the synthesis of each of the peptide-resins described in Table I the total sample was suspended in CH_2Cl_2 and shaken for 5 min to produce a uniform suspension. Immediately, an approximately 200-mg sample was withdrawn with a glass tube, filtered, washed with HOAc–CH_2Cl_2 (1:1), HOAc, 2-propanol, and CH_2Cl_2, and dried at room temperature overnight in vacuo. A sample of the dry beads was spread over a microscope slide and diameters of each of the 150–300 beads were measured by starting at one end of the slide and proceeding to the other. For better accuracy the microscope was focused on the outer edge of each bead. For each peptide-resin preparation the measurements were repeated on another sample of the dried aliquot. The average agreement between the geometric mean of the diameters of the beads in the two samples was ±3%. The data are given in Table II. The mean diameters ranged from 49 μm for the aminomethyl-resin to 84 μm for the most highly substituted peptide-resin with $n = 12$. Thus, the diameter of a dry bead approximately doubled when the content of peptide approached 80%. It should be emphasized that resin beads are not homogeneous in size. Therefore, for these data to be valid it was very important that it be shown that the sample of beads selected for the diameter measurement was representative of the entire batch of resin used for the synthesis. It was found that the sizes in a sample of beads were not normally, but log-normally distributed. It is well known[21] that in such a situation the arithmetic mean

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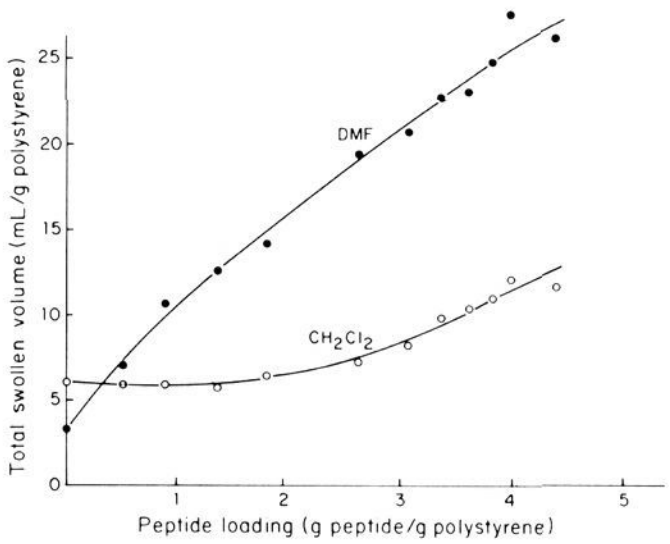

Figure 1. Total swollen volumes of peptide-resins calculated from measured bead diameters.

is not the true central value, and the particle distribution is more accurately described by the geometric mean particle size, d_g, and the geometric standard deviation, σ_g. The geometric standard deviation was essentially constant for all samples within each of the two lots of resin used and therefore the samples were not biased.

From the measured diameters of the beads the volumes could be calculated, and are expressed in Table II as the volume relative to the aminomethyl-resin taken as 1. It can be seen that the dry volume of the most highly loaded bead increased fivefold. The relative volume could also be calculated from the peptide content (obtained from the amino acid analyses), together with the measured density of the unloaded resin and the density of the peptide. For this purpose the best fit density of the peptide, 1.00 g/mL, was used. This is significantly lower than the theoretical close-packing density of 1.36 g/mL,[22] suggesting intimate mixing of the peptide and polystyrene within the peptide-resin. The scatter of the volumes calculated in this way compared with the measured volumes was ±5%.

For the $n = 6$ peptide-resin (sample 11, Table II) the volume calculated from bulk density measurements was 2.93 ± 0.15 mL/g, in agreement with the value of 3.14 ± 0.14 mL/g calculated from the bead diameters. This is further evidence that the sampling of beads for microscopic examination was not biased.

Dimensions of Swollen Beads. Swelling measurements were performed on individual beads by direct microscopic measurements. After the diameter of a dry bead was observed, a few drops of solvent were added to the same bead and its swollen diameter was observed. The ratio of the diameters of the swollen and dry bead was recorded. The averages of ten measurements for each sample are shown in Table III. The maximum swelling volume was attained within seconds for the nonviscous solvents, CH_2Cl_2 and DMF, used in this study. This procedure is more accurate than the simpler one of measuring the swollen diameter followed by remeasurement after allowing the volatile solvent to evaporate. We have observed that a bead swollen in methylene chloride does not shrink to its original volume when the solvent is allowed to evaporate in air, although the initial diameter was reached after drying in vacuo. Only the first procedure was used. Care was taken to examine beads representing all sizes within the sample, even though it was found that in every case the swelling ratio was the same for beads of all sizes within the same sample. The data on the swelling behavior of the peptide-resins in methylene chloride or dimethylformamide after addition of each LAGV-OMPA unit are shown in Table III. The average experimental values for the ratios of measured diameters, swollen to dry (swelling ratio, columns 4 and 9), are given. In CH_2Cl_2 the swelling ratio was 1.84 for unloaded aminomethyl-resin (0.95 mmol/g) and gradually decreased as the peptide content increased; by the time the peptide content reached 2.5 g/g of polystyrene, the ratio approached a constant value of 1.33. In DMF this swelling ratio gradually increased from 1.49 for unloaded beads to 1.84 at 2.5 g peptide/g

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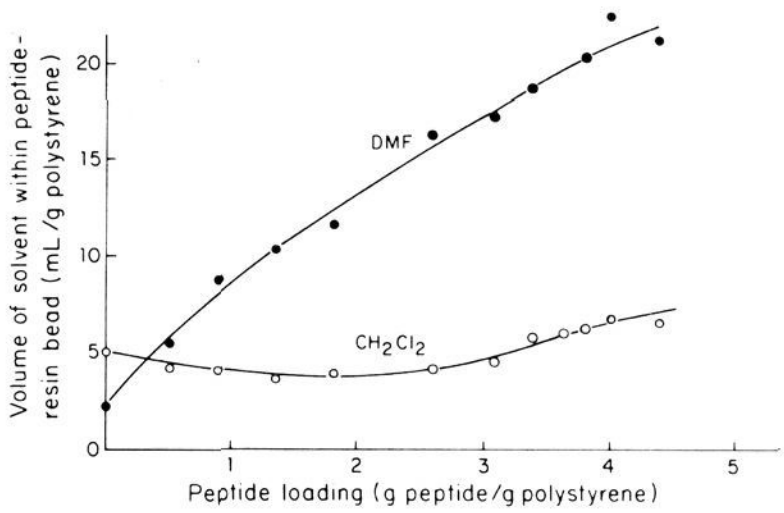

Figure 2. Volumes of solvent within the swollen peptide-resins.

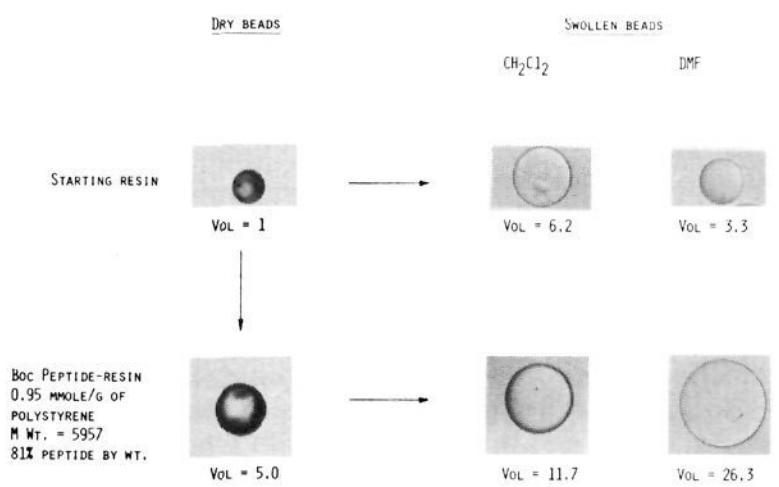

Figure 3. Starting aminomethyl-resin and peptide-resin beads showing relative sizes, dry and swollen. Beads representative of the geometric mean diameters were chosen.

of polystyrene and then remained nearly constant.

The mean diameters of swollen beads (columns 5 and 10) were obtained by multiplying the measured swelling ratio by the previously determined geometric mean dry diameter of each sample. These ranged from 90 to 113 μm in CH_2Cl_2, and 73 to 149 μm in DMF. The ratios of swollen to dry volumes (columns 6 and 11) were obtained by cubing the swelling ratios. The total swollen volumes/g of polystyrene (Figure 1) were obtained by multiplying the ratios of swollen to dry volumes by the previously determined volumes of dry beads/g of polystyrene. Finally, the volume of solvent retained inside the beads/g of polystyrene (Figure 2) was calculated by subtracting the dry volume from the corresponding total volume. At the highest loading of peptide (81%) the mean volume of the peptide-resin bead expanded to 11.7 mL/g of polystyrene in CH_2Cl_2 and to 26.3 mL in DMF. Thus, the volumes occupied by solvent were 6.7 and 21.3 mL, respectively. These numbers are a measure of the space within the swollen bead potentially accessible for further chain growth.

Figure 3 illustrates the relative sizes of the unsubstituted and highly loaded resin beads in the dry state and after swelling in CH_2Cl_2 or DMF. The photographs were taken under the microscope by phase contrast at low power. For each of the photographs a bead representing the average diameter of the sample was selected. The beads shown are typical. They were smoothly spherical in both the dry and swollen states for all the peptide-resin samples examined, up to those containing more than 80% peptide. There was no evidence of damaged or ruptured beads.

Autoradiographs of cross sections of peptide-resin beads[23] containing tritium-labeled valine were also obtained (Figure 4). The sample was Boc-[^{3}H]Val-(LAGV-OMPA)$_6$-resin containing 70% peptide by weight and 8 mCi/mmol. Within a resolution limit of 0.5 μm, the peptide chains were found to be distributed uniformly throughout the polymeric support, indicating that synthesis occurred throughout the swollen peptide-resin. Furthermore, since the amino terminus of the fully extended peptide

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Table III. Dimensions of Swollen Beads

sample no.	geometric mean diameter dry beads d_g, μm	vol dry beads/g styrene, mL	dichloromethane: ratio of diameters,[a] swollen/dry	dichloromethane: mean diameter swollen beads,[b] μm	dichloromethane: ratio of vols,[c] swollen/dry	dichloromethane: total swollen vol/g styrene,[d] mL	dichloromethane: vol of solvent/g styrene,[e] mL	dimethylformamide: ratio of diameters,[a] swollen/dry	dimethylformamide: mean diameter swollen beads,[b] μm	dimethylformamide: ratio of vols,[c] swollen/dry	dimethylformamide: total swollen vol/g styrene,[d] mL	dimethylformamide: vol of solvent/g styrene,[e] mL
1	46.0	0.99	1.63	75.0	4.49	4.45	3.46	1.37	63.0	2.57	2.55	1.56
2	49.6	1.24	1.68	83.3	4.74	5.88	4.64	1.52	75.4	3.51	4.35	3.11
3	48.4	1.15	1.57	76.0	3.87	4.45	3.30	1.54	74.5	3.65	4.20	3.05
4	51.6	1.40	1.56	80.5	3.80	5.32	3.92	1.53	78.9	3.58	5.01	3.61
5	53.6	1.56	1.41	75.6	2.80	4.37	2.81	1.55	83.1	3.72	5.80	4.24
6	49.0	0.99	1.84	90.2	6.23	6.17	5.18	1.49	73.0	3.30	3.27	2.28
7	58.0	1.64	1.54	89.3	3.65	5.99	4.35	1.63	94.5	4.33	7.10	5.46
8	61.0	1.91	1.47	89.7	3.18	6.07	4.16	1.78	108.7	5.64	10.77	8.86
9	63.6	2.17	1.39	88.4	2.69	5.84	3.67	1.80	114.5	5.83	12.65	10.48
10	66.6	2.48	1.38	91.9	2.63	6.52	4.04	1.79	119.2	5.74	14.23	11.75
11	72.0	3.14	1.33	95.8	2.35	7.38	4.24	1.84	132.5	6.22	19.53	16.39
12	74.8	3.52	1.33	99.5	2.35	8.27	4.75	1.81	135.4	5.93	20.87	17.35
13	79.0	4.15	1.34	105.9	2.40	9.96	5.81	1.77	139.8	5.54	22.99	18.84
14	81.0	4.47	1.33	107.7	2.35	10.50	6.03	1.73	140.1	5.18	23.15	18.68
15	82.6	4.74	1.33	109.9	2.35	11.13	6.39	1.74	143.7	5.27	24.98	20.24
16	85.6	5.28	1.32	113.0	2.30	12.14	6.86	1.74	148.7	5.27	27.82	22.54
17	84.0	4.99	1.33	111.7	2.35	11.73	6.74	1.74	146.2	5.27	26.30	21.31

[a] Measured swelling ratio. [b] Swelling ratio × geometric mean diameter of dry beads. [c] (Swelling ratio)³. [d] Volume of dry beads/g styrene × ratio of volumes, swollen/dry. [e] Total swollen volume/g styrene − volume dry beads/g polystyrene.

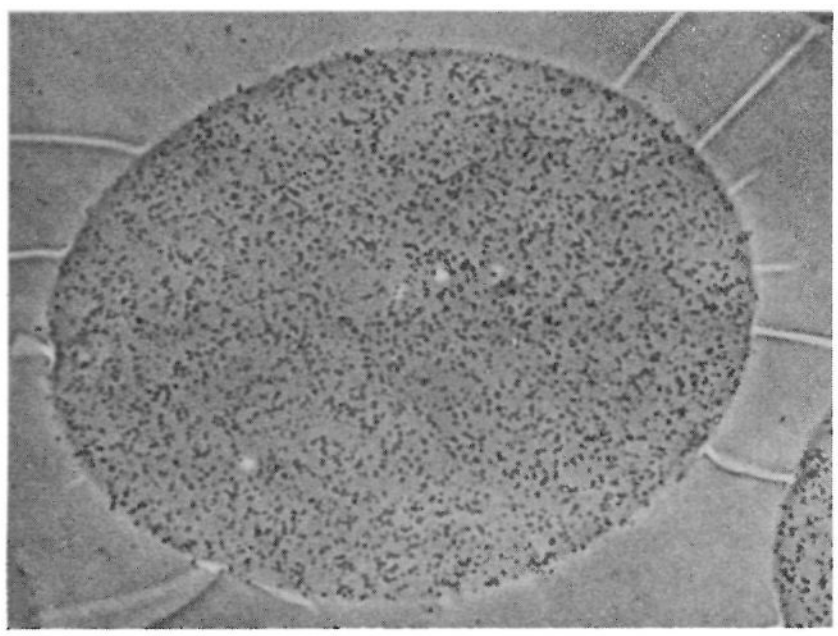

Figure 4. Autoradiograph of a cross section of a peptide-resin bead showing distribution of growing peptide chains. The bead contains 70% peptide with Boc-[^{3}H]valine coupled to the amino terminus. Approximate diameter 70 μm. The circular cross section of the bead has been distorted in the fixing process, and the radial lines are artifacts of the dried photographic emulsion.

chain must be within 0.012 μm of the polystyrene to which it is attached, the autoradiograph also represents the distribution of the polymer backbone and demonstrates that the dimensions of the peptide-resin beads correspond to the size of the cross-linked polystyrene network.

Discussion

In the past it has been assumed that the ultimate swollen volume of a synthetic peptide-resin would be approximately the same as the volume of the fully swollen unsubstituted resin bead in a given solvent.[23] If this were true, it would be expected that the space available for peptide growth would eventually become filled as the size of a synthetic peptide increased.[2] However, until now, there have been no adequate data to support or refute this assumption. Peptides large enough in molecular weight and at sufficiently high substitution to exceed the limits based on this assumption have never been studied. In addition, there have never been properly designed experiments to test the extent of swelling of peptide-resin preparations.

A Revised Model for Swelling of Peptide-Resins. It can be seen from the present results (Figure 1) that to a first approximation this simple view served as a useful guide for the planning of practical syntheses in dichloromethane. However, it is now clear that it is far from an accurate representation of the true situation and that a proper description of the system will require a new model that takes into account a swelling effect of the peptide in addition to that of the polystyrene support.

As shown in Table II the mean diameter (and therefore the volume) of the *dry* peptide-resin beads increased progressively with increasing peptide content. This fact alone is not inconsistent with the old view of the beads because the unloaded resin swells in CH_2Cl_2 (or in DMF up to 72% peptide) to a volume greater than these dry volumes and therefore could be expected to accommodate the increased mass of peptide simply by a displacement of solvent, with a resulting steady decrease in swelling ratio. However, the further results of the study emphatically contradict this idea. From Table III it can be seen that the swelling ratios of the peptide-containing beads were appreciable at all levels of loading examined. The result is that the total swollen volume in both solvents increased dramatically with increasing peptide content and was far greater than the initial swollen volume of unloaded resin. Furthermore, there was an increasing amount of solvent imbibed per bead as the amount of peptide increased. These results are shown in graphic form in Figures 1 and 2 and are illustrated photographically in Figure 3. It is obvious from these data that the amount of peptide that can be synthesized/g of resin is far greater than previously thought. Our former conclusion[12] that there would be just enough free space in the beads to accommodate the fully protected synthetic ribonuclease molecule at a substitution of 0.2 mmol/g was too conservative because we did not allow for solvation of the protected protein component and its effect on further swelling of the polystyrene beads. As seen from the data of Table III there actually can be even more space available after assembly of a large polypeptide than at the start.

Maximum Swollen Volume. What is the upper limit to the swelling of the loosely cross-linked polymer beads? There is no adequate theoretical treatment of this problem because of the various ill-defined factors that can affect the structure and swollen volume of a three-dimensional polymer network. If the simplifying assumption is made that copoly(styrene–divinylbenzene) resin forms a regular tetrahedral lattice and is therefore isomorphous with diamond, then the maximum volume of the fully extended resin can be estimated.[24] From the density (3.51 g/mL) and carbon-to-carbon distance (1.54 Å) of diamond, the number of tetrahedral centers/mL can be calculated. Substitution of the

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Table IV. Calculated Maximum Swelling of Cross-Linked Polystyrene[a]

degree of cross-linkage,[b] %	distance between cross-links,[c] Å	max vol/g,[d] mL
[diamond]	[1.54]	[0.285]
2	65	49
1	130	196
0.5	260	784
0.25	520	3136

[a] Assumes a fully extended regular tetrahedral structure, with no interpenetration of polystyrene chains. [b] Taken to be equivalent to the nominal weight percent of divinylbenzene in the styrene mixture. [c] Carbon–carbon bond distance in diamond; linear polystyrene chain length between cross-links for the resin, = $(n/2) \times 2.6$ Å. [d] Calculated on the assumption that the cross-linked polystyrene is isomorphous with diamond according to the expression max vol/g = $V_d(E_d/E_s n)[r_s n/2/r_d]^3$, where V_d = 1/density of diamond = 1/3.51 mL/g, E_d = equiv wt of diamond = 12.0 g/equiv, E_s = equiv wt of polystyrene = 104 g/equiv, r_d = C-C distance in diamond = 1.54 Å, r_s = repeat distance in polystyrene backbone = 2.6 Å, and n = mol styrene/mol divinylbenzene = 100/% DVB.

C–C distance by the distance between cross-links in the extended polystyrene chain then allows the density of the expanded resin to be calculated for a given degree of cross-linking from the equation in Table IV. It can be seen that the calculated maximum swollen volume/g (density^{-1}) is inversely proportional to the square of the percentage cross-linking. Table IV shows such data for diamond and for polystyrene resins of selected cross-linking. The data indicate that a very large expansion of the beads would be possible if no other limitations were imposed on the swelling, reaching 200 times the dry volume for 1% cross-linked resin.

The resin used in the studies reported here are nominally 1% cross-linked. This denotes the weight percent of crude divinylbenzene added to the polymerization mixture. Because of impurities and incomplete (monofunctional) reaction, the actual cross-link density, as defined in Table IV, may be about one-half to one-quarter of the nominal value.[25] In addition, we have neglected other factors, including interpenetration (entanglement) of the polymer network, the effect of which is in the same direction as cross-linking, but of unknown magnitude. The observed value of about 28 mL/g of resin for the highly loaded peptide-resin swollen in DMF would require about 2.5% total effective cross-linking due to divinylbenzene content, interpenetration, and all other factors. This therefore suggests that the ultimate limit of the swelling of the peptide-resin has not been approached in this study, even with 4 g of peptide/g of polystyrene (80% peptide). This conclusion is further indicated by the curves in Figure 1, where there is no evidence that the swollen volume as a function of peptide content is approaching a maximum in either solvent.

Explanation of the Swelling Behavior of the Peptide-Resin. The swelling of a cross-linked polymer network is analogous to the process of dissolving a linear polymer.[26] The driving force for either swelling or dissolving is made up of contributions from the normal entropy and enthalpy changes associated with mixing of solvent and solute molecules plus changes in configurational entropy due to dilution of flexible chain molecules.[27] The large configurational entropy change is unique to linear macromolecules and is always favorable.[28] If the sum of these contributions is favorable ($\Delta G < 0$), then for a non-cross-linked polymer the dissolving process continues until the entire available solvent volume is randomly occupied by polymer. However, for the corresponding polymer in a network structure this tendency to disperse is counteracted by an elastic restraining force due to a decrease in configurational entropy of polymer chains held between network junctions (cross-links). This arises because the chains forming the network are forced to assume more elongated, less probable configurations as the network expands. At equilibrium the incremental decrease in free energy due to dispersal is matched by the incremental decrease in configurational entropy due to the extended network structure of the polymer and there is no further swelling. Any contribution that perturbs this balance will change the equilibrium swollen volume of the resin.

For example, a change in the extent of cross-linking of the polymer will affect the degree of loss of configurational entropy in the extended network; the higher the cross-linking, the lower the final swollen volume of the resin. Similarly, a change in the free energy of interaction of the solvent and polymer will affect the swollen volume. A solvent structurally unrelated to the polymer, particularly one with dissimilar polarity, will swell the resin less than a related solvent.[29] If, instead, favorable interactions between the polymer and solvent exist compared with self-interactions, the result will be enhanced swelling. Thus, dichloromethane, which hydrogen bonds with the π electrons of the aromatic nuclei of polystyrene,[30] is an excellent swelling solvent for the resin.

For the case of the resin containing covalently attached linear peptide, the equilibrium swollen volume will be different from that of the resin alone because of the net free-energy change associated with solvation of the peptide chains within the swollen resin matrix. This change in free energy is added to the free energy of solvation of the polymer, providing an increased net driving force for swelling. The linear peptide chains are not cross-linked and are anchored at one end only, so that expansion of the cross-linked polymer network does not force them to assume more elongated, less probable configurations. Thus, the solvated peptide chains do not themselves become an additional elastic constraint on the system. At equilibrium, then, the increased driving force for swelling must be balanced by an increase in the elastic restraining force due to the cross-linked polystyrene network alone. The necessary increase in restraining force is generated by the further swelling of the resin.

In principle, the equilibrium swollen volume of the peptide-resin could be greater or less than that of resin alone, depending on whether the net free energy of solvation of the peptide is negative or positive, respectively. This net free energy of solvation of the peptide chains is determined by the same factors described above, namely, the normal enthalpy and entropy changes associated with mixing of solvent and peptide compared with pure solvent and peptide in the unsolvated (amorphous) state. Because the peptide is prevented, by its attachment to the cross-linked network, from diluting itself throughout the solvent volume, the maximum possible overall free-energy change is reduced. Nonetheless, the net free-energy change for solvation of the peptide within the swollen resin matrix will be more favorable (more negative) than for the unattached peptide diluted to the same concentration because, as discussed later, the amorphous state within the dry peptide-resin is less favorable than the amorphous state of the free peptide.

Because of the factors discussed above, which favor a negative free energy of solvation of the peptide in the peptide-resin, even a relatively poor solvent for protected peptide can lead to an enhanced swollen volume for the peptide-resin. Furthermore, solvents with particular affinity for the peptide chain, due to specific strong hydrogen bonding[31] or strong dipolar interactions,[32] will give rise to greater swelling of the peptide-resin, as can be seen from the results presented in this study where DMF swells the peptide-resin to a volume many times greater than that of the unsubstituted resin. In addition to the contributions to the free energy of solvation of the peptide discussed above, for longer

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peptides there can also be a large gain in configurational entropy on dilution with low molecular weight solvent.

Implications

Optimum Loadings. The present results show that the volume available for chain growth within the swollen resin is not the limiting factor in solid-phase peptide synthesis, in any practical sense, and is not the basis on which to decide the level of substitution to be used for a synthesis.[2] To decide on the desired loading of the resin for a synthesis, we must consider the effect of solvation of the peptide-resin on the reaction protocol to be used. For relatively small amounts of peptide/g of polystyrene the properties of the peptide-resin do not change dramatically from those of the unsubstituted resin. In particular, the total swollen volume remains approximately constant (Table III). Therefore, synthetic protocols with constant wash volumes and a single solvent (preferably dichloromethane) can be used throughout a synthesis. This has usually been done in practice for the synthesis of peptides, and even for proteins such as ribonuclease where the final protected peptide only reached 0.7 g/g of polystyrene support.

In contrast, the use of much higher substitutions that lead to the formation of several grams of peptide/g of resin will result in peptide-resins whose properties change substantially in the course of a synthesis. This effect must be taken into account in the synthetic protocol. If, because of increased swelling, the holdup of solvent increases, it will become necessary to increase the volume or number of washes in order to maintain efficient washing of the resin-bound peptide. This will be especially pronounced for DMF. Similarly, the increased holdup volume will require more solvent to cover the resin and will lower the concentrations of amino acids and other reagents if they are added in constant amount during a synthesis. To maintain a uniform high concentration needed for optimum reaction rates, the molar amounts would have to be increased. Although it has not been demonstrated experimentally in the present work, it is possible that for very high levels of peptide it would be necessary to change the solvent to maintain the highly swollen state of the peptide-resin that is needed for rapid and complete chemical reactions. This need could be evaluated by monitoring the swelling behavior of the peptide-resin in the course of the synthesis, either on the whole resin mixture or on samples which could be returned to the reaction vessel. In the current work, mixed solvents such as DMF plus CH_2Cl_2 were observed to have swelling properties intermediate between the two pure solvents. No enhanced (cooperative) effects[28] were seen.

For most purposes, then, it is simpler to use loadings such that the final peptide-resin contains less than about 1 g of peptide/g of resin. This is in accord with recommendations based on the simple model of swelling previously used, and is the current practice in most laboratories. In commercial syntheses, where substantially higher loadings are sometimes used,[33,34] the above considerations may become important.

Solubilizing Power of the Resin. It is a common observation that protected peptides that were highly solvated and available for reaction while covalently attached to the resin are insoluble in the same solvent after cleavage from the resin.[35,36] Normally, highly ordered intermolecular structures lead to extensive self-association, aggregation, and precipitation of protected peptides from solution in organic solvents.[37] A peptide such as the one assembled in this study would be expected to be insoluble and poorly solvated in a solvent such as dichloromethane. The observation that the peptide-resins are highly swollen even at high peptide contents indicates that the resin-bound peptide is highly solvated, as discussed above. For this to occur, the solvation of the peptide chains need only be favorable relative to the peptide in the amorphous, unsolvated state *within the peptide-resin matrix*. Because of the network structure of the cross-linked polymer to which the chains are covalently attached, self-aggregation of the peptide and polystyrene components is minimized. This leads to a structure for the amorphous peptide-resin in which the two dissimilar components are intimately mixed at a molecular level, and which is less favorable thermodynamically[38] than is the case for self-aggregated free components. Both the peptide and the polymer will tend to interact more favorably with added solvent, and will exert a mutual solubilizing effect on one another (see ref 39 for discussion of a related effect). Thus, solvents which normally would be unable to dissolve a protected peptide can be effective solvating agents for the same resin-bound peptide. Similar solubilizing properties of high molecular weight linear polymers for covalently attached moieties are well known,[28,40] but the effect will be greater for a cross-linked polymer network. Another inherent advantage in a swollen gel such as a loosely cross-linked polystyrene is that swelling stretches out individual polymer chains, thereby making the reactive sites more accessible to reagent molecules.[41]

This "solubilizing" effect is an essential consequence of the loosely cross-linked, highly swollen resin support and is an extremely important feature of solid-phase peptide synthesis, since it enables the synthesis of larger protected peptides than is generally possible by solution methods, where the principal obstacle has been the poor solubility of protected intermediates.[42,43]

Conclusions

From the experiments described here, it is possible to answer the two questions posed in the introduction. First, it is clear that the swelling properties of the peptide-resin are influenced by each of the components of the system, although in ways that have not been well documented before. During the course of peptide synthesis on a cross-linked polystyrene support the solvation is initially a property of the polymer that makes up the resin, while at the end of a long synthesis it is strongly influenced by the protected peptide, which will be more highly solvated than if it were not covalently attached to a loosely cross-linked polymer network. The polystyrene and protected peptide exert a complementary solubilizing effect on each other, and together they determine the swelling behavior of a peptide-resin in a given solvent. Dichloromethane is a better swelling solvent than DMF for polystyrene, whereas DMF is the better solvating medium for protected peptides, although both were effective swelling solvents for peptide-resins throughout the range of loading studied here.

It can now be definitely stated that the initial volume of the swollen unsubstituted resin bead is not the final volume of the swollen highly loaded peptide-resin bead, which can be much larger and can contain substantially more solvent. Over the range examined, up to 80% peptide, there was no evidence that a maximum swollen volume for the peptide-resin had been reached in either solvent and the space potentially available for peptide chain growth within the swollen beads actually increased rather than being gradually filled by peptide. These results are contrary to the previously held views of the solid-phase system and provide a new insight into the high synthetic efficiencies which are observed for peptide synthesis on polystyrene supports.

Experimental Section

Materials. Copoly(styrene–1% divinylbenzene) beads 200–400 mesh prepared by suspension polymerization, without diluent, were purchased from Bio-Rad Laboratories. Boc-amino acids were obtained from

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Chemical Dynamics. Tritiated Boc-valine was prepared by reacting valine-G-^3H with di-*tert*-butyl dicarbonate (Fluka).[44] *p*-Tolylacetic acid, *N*-(hydroxymethyl)phthalimide, and bromoacetophenone were obtained from Aldrich. Methylene chloride was distilled over sodium carbonate. DMF was MCB spectroquality and was stored over 4 Å molecular sieves. The materials and methods for solid-phase synthesis were similar to those described elsewhere[16] but modified as indicated. Hydrolysis of peptide-resins to amino acids was done by using 12 N HCl–propionic acid (1:1 v/v)[18] at 130 °C for 6 h with norleucine as the internal standard. Ion exchange chromatography was performed on a Beckman amino acid analyzer (Model 121). Buffers were prepared from Beckman concentrates.

Aminomethyl-resin of the desired substitution was prepared by the procedure of Mitchell et al.[16] Two batches of resin were prepared containing 0.31 (0.22 mmol/g) and 1.31% N (0.95 mmol/g), respectively, by elemental analysis. *tert*-Butoxycarbonylvalyl-4-(oxymethyl)phenylacetic acid was prepared as previously described,[45] isolated as the free acid, and recrystallized from ethyl acetate–petroleum ether to give a pure white solid, 76% yield, mp 72-74 °C.

Anal. Calcd: C, 62.46; H, 7.39. Found: C, 62.56; H, 7.38.

This product was used to prepare *tert*-butoxycarbonylvalyl-4-(oxymethyl)phenylacetamidomethyl-resin (Boc-Val-Pam-Res), as described previously.[16]

Synthesis of Boc-Leu-Ala-Gly-Val-OCH$_2$-Pam-Resin. The following protocol was used for the synthesis of the model tetrapeptide. Boc-L-Val-OCH$_2$-Pam-resin (1 g) was placed in a reaction vessel and for the introduction of each amino acid was treated with shaking with the following reagents for the times shown, followed by filtration: (1) 20 mL of CH_2Cl_2 (3 × 1 min); (2) 20 mL of trifluoroacetic acid–CH_2Cl_2 (1:1 v/v) (1 min); (3) 20 mL of trifluoroacetic acid–CH_2Cl_2 (1:1 v/v) (30 min); (4) 20 mL of CH_2Cl_2 (6 × 1 min); (5) 20 mL of 5% diisopropylethylamine in CH_2Cl_2 (5 min); (6) 20 mL of CH_2Cl_2 (3 × 1 min); (7) 20 mL of 5% diisopropylethylamine in CH_2Cl_2 (5 min); (8) 20 mL of CH_2Cl_2 (3 × 1 min); (9) Boc-Gly-OH (4 equiv) in 15 mL of CH_2Cl_2 (5 min), without filtration, followed by (10) DCC (4 equiv) in 5 mL of CH_2Cl_2 for 30 min; (11) 20 mL of CH_2Cl_2 (6 × 1 min). This synthetic cycle was repeated with Boc-L-Ala and then with Boc-L-Leu. In a double-coupling synthesis, steps 7-11 were repeated in each cycle. Successive LAGV-OMPA units were assembled by the above procedures.

Density Measurements of Dry Resin Samples. Unsubstituted copoly(styrene–1% divinylbenzene) resin (Biobeads S-X1, 200-400 mesh) (0.541 g) was weighed in a graduated conical centrifuge tube. Petroleum ether (30-60 °C) was added to just cover the beads and allowed to equilibrate for 1 h. The weight of added solvent was determined to be 0.326 g and the total volume of the mixture was 1.05 mL. From the density of petroleum ether (0.65 g/mL) the volume of solvent was 0.50 mL and therefore the volume of the resin was 0.55 mL, giving a density of 0.98 g/mL. By microscopic examination it was shown that no swelling of the beads occurred in this solvent. The densities of the peptide-resins were determined in the same way.

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Dipole-Supported States. A Very Low Lying Excited State of Acetaldehyde Enolate Anion

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Abstract: The potential surfaces of the ground-state anion, ground-state radical, and lowest excited anion states of acetaldehye enolate have been investigated by ab initio SCF calculations and configuration interaction. A (9s5p/4s2p) Dunning–Huzinaga basis set, augmented with 3s and diffuse p atomic orbitals, and in some cases with polarization functions, has been used. A low-energy Rydberg-like excited anion has been found, lying 1.82 eV above the anion ground state, after zero-point energy correction. This excited state is very similar to the ground state of the neutral radical, and is probably responsible for the sharp resonances observed in electron photodetachment experiments.

Introduction

Threshold resonances in the photodetachment cross sections of substituted acetophenones and acetaldehyde enolate anions have recently been reported.[2,3] Substituent effects, as well as semiempirical calculations,[3] do not support the hypothesis that these resonances are conventional n–π^* or π–π^* excited states. For this reason, an explanation based on the possibility of low-lying, dipole-supported excited states of the anions was suggested.[3] Photodetachment would then be enhanced by excitation of the anion to vibrational levels of this first excited state. If weakly coupled to the continuum, this state would account for the observed sharp resonances.

Dipole-supported states have received considerable theoretical[4-18] attention. It has been shown that a fixed dipole may bind an electron, provided that the dipole moment is greater than the critical value 1.625 D. Crawford[7] and Garrett[8] have analyzed critical values more adapted to real molecules. Taking into account

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