PHOTOLYSIS OF CROSSLINKED PEPTIDES FROM ELASTIN OF PORCINE AORTA

Monique DAVRIL, Kia-Ki HAN, Mireille Guay+ and François LAMY+

Laboratoire des Protéines Fibreuses, Unité de Recherches no 16 de l'INSERM sur la Biochimie des Protéines Place de Verdun, 59045, Lille Cédex, France and + *Département de Biochimie, Faculté de Médecine, Université de Sherbrooke Sherbrooke, Québec, J1H 5N4, Canada*

Received 31 August 1978

1. Introduction

Elastin, a fibrous protein found in connective tissue is a hydrophobic, highly crosslinked insoluble protein. The study of its primary structure has been greatly hindered because no specific enzymatic or chemical method is known which would release a finite number of homogeneous peptides. Since the major crosslinks are tetrasubstituted pyridinium compounds, the (iso)desmosines [1] (1, fig.1) we are exploring the possibility of breaking specifically the aromatic ring by photolysis. The photochemical decomposition of free (iso)desmosines has been studied [2] and it was found that the primary photochemical event is the formation of a tetrasubstituted azabicyclo compound 2, which is subsequently hydrolysed to give an open chain amino-aldehyde 3. This compound can either recyclise to give a novel isomer of (iso)desmosines, photo-desmosine 5 (in preparation) or be further hydrolysed to give free lysine and a trisubstituted analogue of glutaconic aldehyde 4. In this paper we wish to report on the photochemical behavior of (iso)desmosines present in highly reticulated peptides obtained by non-specific cleavage of aortic elastin [3].

2. Materials and methods

Elastin from pork aorta was purified by the method in [4]. The fibrous protein was digested with elastase and thermolysin exactly as in [3]. After each digestion the peptide mixture was filtered on a Sephadex G-25 column and the excluded fractions collected. The fraction obtained after elastase treatment was called fraction A and that obtained after thermolysin treatment, fraction B. Fraction B was submitted to partial acid hydrolysis in concentrated HCl at 37°C for 72 h (10–20 mg enzymatic digest/ml acid). The hydrolysate was placed on a SP-Sephadex C-25 column (2 × 70 cm) and eluted (30 ml/h) first with 750 ml buffer at pH 2.9 (pyridine 8 ml, formic acid 20 ml, water to 1 l) followed by a pH 2.9–5.0 gradient obtained by mixing in a 9-chamber Varigrad gradient apparatus suitable amounts of this buffer and of a buffer at pH 5.0 (pyridine 80.5 ml, formic acid 23 ml and water to 1 l).

A broad peak which eluted between pH 3.9 and pH 4.5, contained a material which had a high A_{278}. This fraction (fraction C) was lyophilysed and used in the experiments described below.

2.1. *Photolysis*

For photolysis at 254 nm an immersion lamp (model PCQ, Ultraviolet Products, San Gabriel, CA) was used. For irradiation with monochromatic wavelengths, a high pressure xenon–mercury lamp (model LH151N) Schoeffel inst. corp. was used with interference filters (280 nm and 288 nm).

2.2. *Absorbance spectra*

The ultraviolet absorbances of peptide solutions were measured on a Cary 118 C Spectrophotometer. The decomposition of (iso)desmosines was characterized by a decrease of the A_{268} and A_{278} peaks, which are the characteristic maxima of desmosine and isodesmosine, respectively. Photolysis of the pyridi-

Address correspondence to F. L.

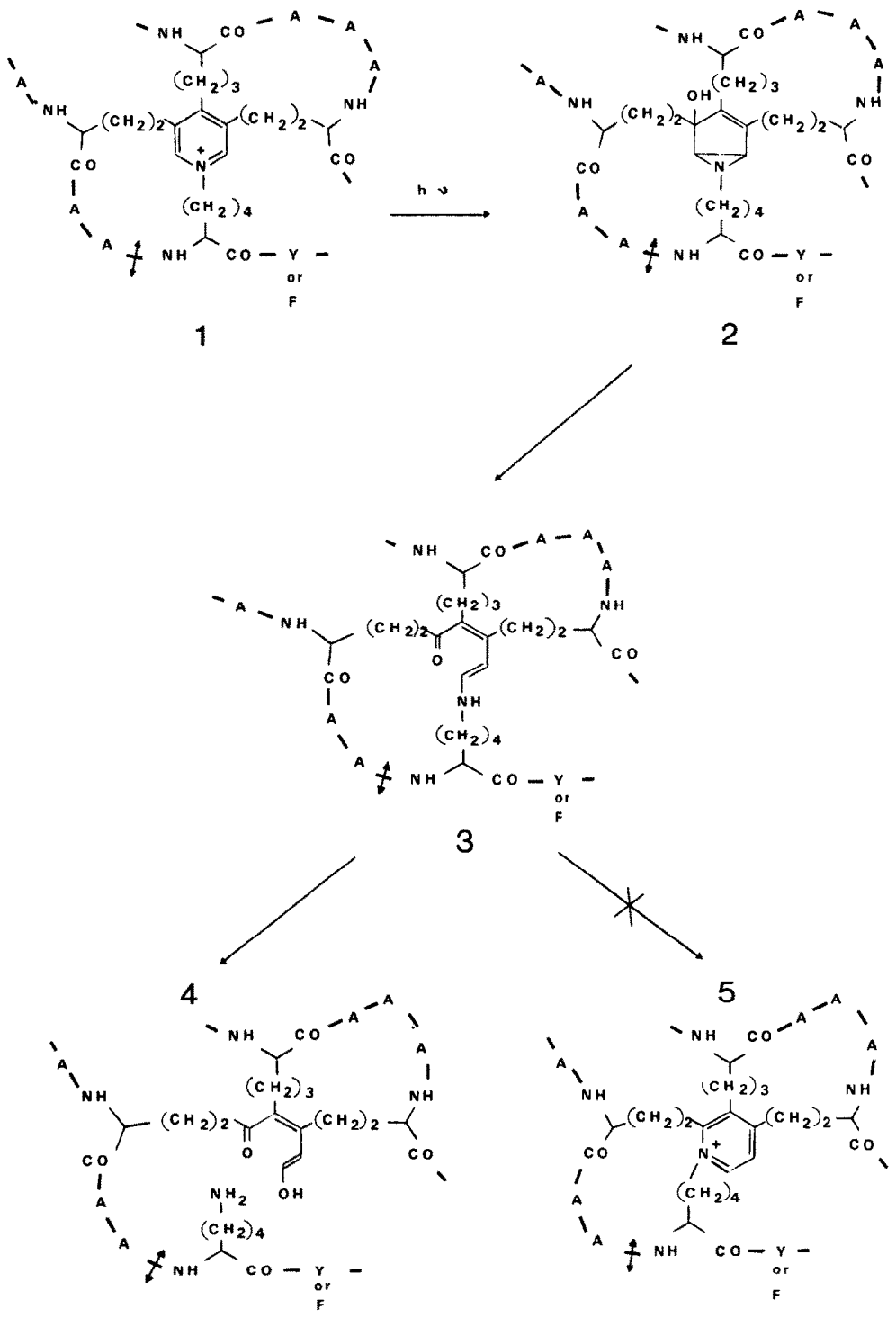

Fig.1. Scheme of the photolytic decomposition of a segment of a peptide crosslinked by desmosine.

nium rings was also followed by amino acid analysis after acid hydrolysis of the irradiated peptide solution [2].

2.3. *Analytical methods*

Peptide fractions were checked for homogeneity by paper electrochromatography on Whatman 3 MM (46 X 57 cm). Electrophoresis was performed in pyridine/acetic acid/water buffer (10:100:2800, by vol.) pH 3.6 at 2.2 kV for 80 min. Chromatography was carried out in the 2nd dimension, with solvent *n*-butanol/pyridine/acetic acid/water (150:100:30:120, by vol.). The spots were localized with a ninhydrin–cadmium reagent [5].

For amino acid analyses, samples were hydrolyzed at 100°C in vacuo for 25 h in 6 N HCl. In order to protect tyrosine from oxidation 1 drop of phenol solution (1% phenol in water) was added to each ml of HCl solution. The analyses were performed with a Jeolco 5-AH auto analyzer adapted for a single column separation of all amino acids including the elastin crosslinks.

Amino-terminal residues were determined by dansylation by the method in [6].

3. Results

Table 1 shows the amino acid composition of the various peptides fractions from aortic elastin. First one can see that the content in (iso)desmosines increases with each hydrolytic treatment. Peptides C show an 18-fold enrichment of each crosslink when compared with intact elastin. Two different purifications of peptides C were performed (columns C_1 and C_2). While these two identically purified fractions had essentially the same content of crosslinking amino acids, the concentration of some other residues (notably tyrosine and phenylalanine) varied by much as 100%. When compared with intact elastin, Fractions C_1 and C_2 were enriched in alanine but contained less glycine. It is evident that the chromatographic procedures used to obtain peptides C selects a class of peptides highly reticulated but otherwise not homogeneous. Table 2 shows the results of several photolytic experiments at different wavelengths and for different times of irradiation, carried out on peptide fractions purified from aortic elastin. After the

Table 1
Amino acid compositions of crosslinked peptide fractions at different stages of enrichment, expressed in residues/1000

	Elastin	Fractions			
		A	B	C_1	C_2
Asp	6.3	12.5	8.0	4.7	8.0
Thr	15.9	14.6	17.4	9.1	5.5
Ser	12.6	17.2	16.4	6.5	9.2
Glu	20.5	20.1	24.8	10.6	12.6
Pro	121.1	122.0	136.2	148.6	159.2
Gly	294.0	334.6	256.7	93.9	102.6
Ala	222.5	217.5	5.5	337.3	345.1
Val	128.3	130.3	59.5	26.5	38.0
Ileu	18.8	11.7	13.4	11.4	11.8
Leu	56.7	40.6	45.2	26.3	32.8
Tyr	17.3	15.1	23.4	52.8	26.7
Phe	33.7	10.6	17.6	20.9	10.3
Ides	4.2	12.7	15.2	83.0	86.8
Des	5.9	15.6	20.5	115.3	107.0
Lnl	0.9	3.2	5.5	12.8	16.0
Lys	9.3	6.8	8.5	14.8	22.3
Arg	7.4	2.5	7.2	25.7	6.1

specified times of irradiation the fractions were hydrolysed and their amino acids composition determined. The amino acids listed are those whose concentrations changed following photolysis. The first conclusion that one can draw from this table is that (iso)desmosines, in covalent linkages, are destroyed by ultraviolet light as they are when free in solution. Comparison of the results obtained with fractions C_{1a} and C_{1b} shows that this decomposition is reproducible when the irradiation is carried out at the same wavelength and for the same time. The most striking observation is that, during photolysis under all the experimental conditions tested, the amount of lysine produced is practically equal to the amount of (iso)desmosines cleaved (compare the two last lines of the table). This, coupled with the fact that we did not observe a peak of photodesmosine on the amino acid chromatograms, indicates that the pyridinium rings of (iso)desmosines in covalent linkages are cleaved to give lysine and an analogue or glutaconic aldehyde instead of being re-aromatized as was the case during the course of the photolysis of free crosslinks.

Two facts must be mentioned. Tyrosine and phenylalanine are potentially photosensitive. At 280 nm or

Table 2
Changes in concentrations of selected amino acids after photolysis[a] of various elastin peptide fractions[b]

	Fraction A		Fraction B		Fraction C_{1a}		Fraction C_{1b}		Fraction C2	
	C[c]	Δ[d]	C	Δ	C	Δ	C	Δ	C	Δ
Glu	24.9	+ 4.8	32.9	+ 8.1	16.1	+ 5.5	17.4	+ 6.8	21.6	+ 9.0
Tyr	9.5	− 5.6	14.2	− 9.2	48.2	− 4.6	49.4	− 3.4	26.6	− 0.1
Ides	1.7	−11.0	1.6	13.6	21.0	−62.0	21.4	−61.6	41.2	−45.6
Des	1.7	−13.9	0.5	−20.0	49.4	−65.9	41.9	−73.4	77.6	−29.4
Lys	13.3	+ 6.5	17.6	+ 9.1	43.0	+28.2	45.9	+31.1	44.4	+22.1
$\Sigma \frac{\text{Ides + Des}}{4}$		− 6.2		− 8.4		−32.0		−33.8		−18.8

[a] The photolysis of each fraction, dissolved in a mixture 1:1 of methanol–HCl (0.3) N, was carried out under the following conditions: Fraction A, 28 mg in 150 ml solvent, at 288 nm for 3.5 h; Fraction B, 44 mg in 150 ml solvent, at 280 nm for 7.5 h; Fraction C_{1a} and C_{1b}, 36 mg of each in 50 ml solvent at 280 nm for 2.25 h. These two fractions were aliquots of fraction C_1 and represent duplicates of the same photolytic experiment

[b] The preparation of each fraction is in section 2

[c] Concentrations after photolysis of a given amino acid in residues/1000 residues

[d] Changes in concentrations of amino acids after photolysis

288 nm, phenylalanine proved to be stable while tyrosine was only slightly attacked (~10% disappeared after 2 h irradiation). More puzzling was the appearance of new residues of glutamic acid. It is possible that it is a photoproduct derived from glutaconic aldehyde. However we have not studied this reaction in detail.

We have also examined by absorption spectrophotometry the kinetics of the photolysis at 254 nm or 288 nm of peptide fraction C_1. Figure 2 shows the ultraviolet spectra at different times of irradiation. In panel A (irradiation at 254 nm) the wide peak corresponding to the overlapping absorption maxima of (iso)desmosines, decreases as a function of time, while a transient intermediate appears at 364 nm. This compound reaches its maximum after 20 min irradiation and completely disappears after 105 min. This represents the apparition of the open chain amino aldehyde 3 which was shown to be produced during the course of the photolysis of the free crosslinks [2]. In panel B (irradiation at 288 nm) the same decrease in absorption due to the photolysis of the pyridinium ring is observed. However the transient intermediary at 264 nm is undetectable. We suppose that at the monochromatic wavelength of 288 nm, the ring of the primary azabicyclo photo-product II is open only by hydrolysis and not by photocleavage which could occur at the more energetic heterochromatic wavelength centered around 254 nm. Under these conditions the rate of hydrolysis of the open chain amino aldehyde would be greater than its rate of production and it would have no chance to accumulate and to be detected.

We have mapped peptide C_1 as shown at the top of fig.2. Before photolysis one major spot was detected with electrophoretic and chromatographic R_F slightly higher than those of a desmosine standard. After photolysis, under the conditions described in the legend of table 2, the major spot was still present but three new spots appeared in the vicinity of the position of free lysine. We have determined the amino terminal residue present in peptides C_1 before and after photolysis (fig.2, bottom). Before photolysis, proline and alanine were the main N-terminal residues. After photolysis a new residue appeared, namely bis-dansyllysine.

In order to explain these results we may recall that the photolysis of (iso)desmosines leads to the production of free lysine and glutaconic aldehyde. Since it has been shown that crosslink attaches only two polypeptide chains [7] we would expect that photolysis of reticulated peptides would result in the apparition of new lysine, as we report here but not in the splitting of the peptide itself. All the chains should remain attached to the backbone of the glutaconic aldehyde (see 4, fig.1). Indeed the peptide

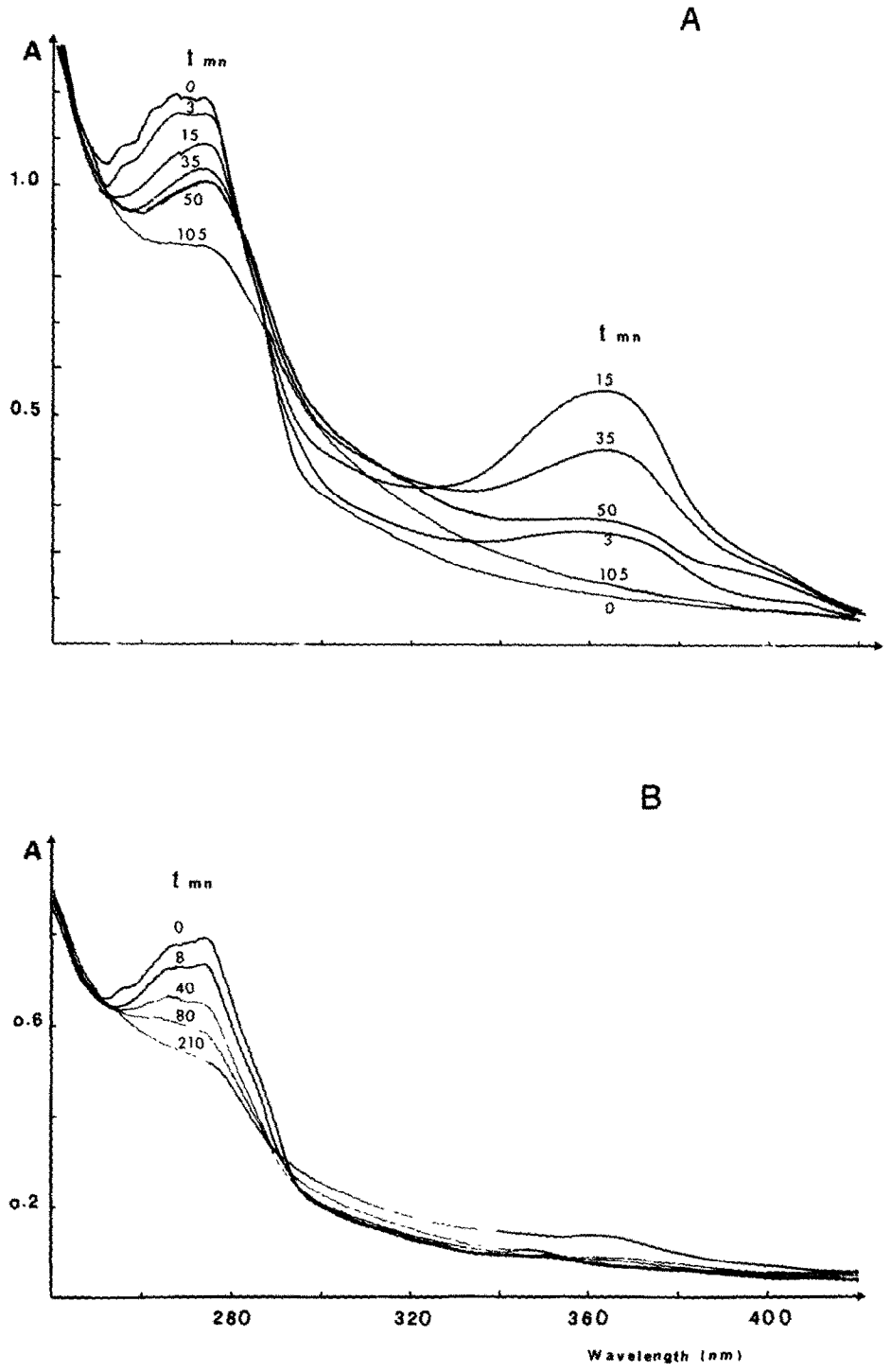

Fig.2. Photolysis of reticulated elastin peptides at 254 nm (A) and 280 (B). The numbers over the absorption peaks represent the lengths of time of irradiation (in minutes).

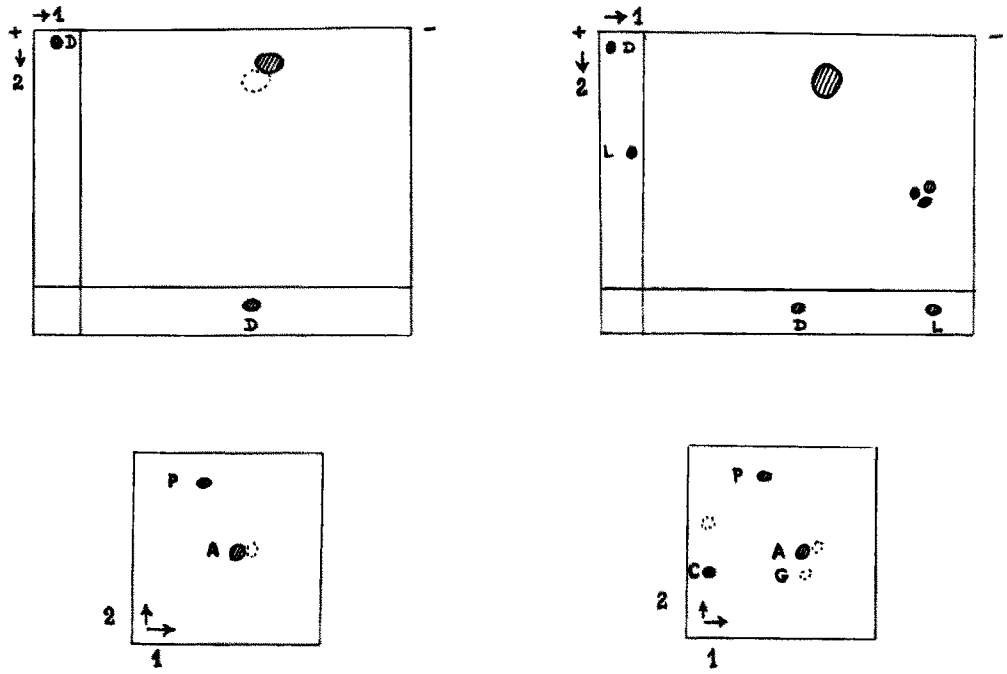

Fig.3. Mapping of reticulated peptides (upper part of the figure) and identification of their NH_2-terminal residues (lower part). On the left are the results before and on the right after photolysis. *Abbreviations:* D, desmosine; L, lysine; P, proline; A, alanine; G, glycine; C, bisdansyllysine.

map shows that the major portion of peptides C_1 remains intact after photolysis. In order to explain the appearance of additional peptides one must postulate that at least one peptide bond has been broken. This could readily occur in our experiments since one step in the purification of the reticulated peptides involved an incubation with elastase. This enzyme has a great affinity for the peptide bond alanyl-lysine in a sequence $(Ala)_4$–Lys–Tyr [8]. In our case such a bond exists and is indicated on fig.1 by an arrow. After photolysis a peptide having an N-terminal lysine should be produced. This sequence of events is supported by the fact that we do detect bisdansyllysine as an N-terminal residue after photolysis of peptides C_1.

In conclusion we have shown that (iso)desmosines can be photolysed even when they are part of the structure of elastin peptides. The mechanism of the reaction is similar to that observed during the photodecomposition of the free amino acids. However, when in covalent linkages, the decomposition proceeds entirely toward the cleavage of the pyridinium rings. No re-aromatization is observed. In order to render this specific method useful for obtaining large peptides, suitable for sequence studies, it will be necessary to develop another specific method which could complete the cleavage of the photochemically-produced glutaconic aldehyde.

Acknowledgements

We wish to thank Mmes M. Lohez and O. Henin for their excellent technical assistance. This work is subsidized by a financial aid of DGRST (grant no. 77-7-1.400 to K. K. H.) In Canada this work was supported by a grant from the Medical Research Council and in France by a Financial aid from INSERM A travel grant was awarded to M. G. by le Conseil de la Recherche en Santé du Québec.

References

[1] Partridge, S. M., Elsden, D. F. and Thomas, J. (1963) Nature 197, 1297–1298.
[2] Baurain, R., Larochelle, J. F. and Lamy, F. (1976) Eur. J. Biochem. 67, 155–164.
[3] Davril, M. and Han, K.-K. (1974) FEBS Lett. 43, 331–336.
[4] Moschetto, Y. and Havez, R. (1969) Bull. Soc. Chim. Biol. 51, 1171–1186.
[5] Dreyer, J. and Bynum, E. (1967) Methods Enzymol. 11, 32.
[6] Bruton, C. J. and Hartley, B. S. (1970) J. Mol. Biol. 52, 165–175.
[7] Foster, J. A., Rubin, L., Kagan, H. M., Franzblau, C., Bruenger, E. and Sandberg, L. B. (1974) J. Biol. Chem. 249, 6191–6196.
[8] Atlas, D. (1975) J. Mol. Biol. 93, 39–53.