SALT RESPONSE OF RIBOSOMES OF A MODERATELY HALOPHILIC BACTERIUM

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1. Introduction

Extremely halophilic bacteria are well adapted to grow in the high salt concentrations they require [1,2]. Their enzymes, membranes, ribosomes, and presumably other cell structures are able to function well in such environments. Thus, the ribosomes of *Halobacterium cutirubrum* need ionic conditions similar to those of their internal environment (3 M K^+ and other salts) for stability and activity [3–5].

The adaptation of moderately halophilic bacteria, which can grow over a wide *range* of salt concentrations (0.5–3.5 M NaCl, or more) poses special problems. The ability to grow over such ranges is not due to selection of sub-populations but individual cells can grow at all salt concentrations [6].

The internal ionic composition of *Vibrio costicola* varies when the cells are grown at different NaCl concentration. The internal ionic concentration is at least as high as the external concentration (D. Shindler, R. Wydro and D. J. Kushner in preparation.) The few studies carried out so far suggest that enzymes formed by such cells when growing at different salt concentrations have the same salt response. This holds for both the NADH oxidase (L. Hochstein, personal communication) and the threonine deaminase (D. B. Shindler and D. J. Kushner, unpublished results) of *Vibrio costicola* and for the extracellular amylase of a moderately halophilic *Micrococcus sp* [7]. Would cells growing at one salt concentration produce ribosomes able to function only at that concentration, or would such cells produce ribosomes able to function in a wide range of salt concentrations? As a first step we studied the sedimentation behaviour in different ionic environments of the ribosomes of *Vibrio costicola* grown in different NaCl concentrations.

2. Materials and methods

Vibrio costicola (NRC 37001) was obtained from the National Research Council of Canada, Ottawa, and maintained on nutrient agar slants containing 1.0 M NaCl. Cultures were grown on the saltsglucose medium of [6] with the following changes. The glucose concentration was raised to 1% (w/v) and 0.5% (w/v) Difco casaminoacids (vitamin-free) was added. For later experiments, shown in figs.2 and 3 table 1, the concentration of phosphate buffer (sodium salt) in the salts-glucose medium was also increased four-fold. Different amounts of NaCl, as indicated, were added to the media, and the final pH adjusted to 7.5. Cells were harvested in mid or late exponential growth at absorbance 660mm, (1 cm cuvette) of 0.7–1.1 corresponding to about 0.4–0.5 mg dry wt per ml, washed in fresh media, recentrifuged, and suspended in 10 mM Tris buffer, pH 7.5, with salts added as specified. Cells were disrupted in a French pressure cell (American Instrument Co.) at 20 000 psi. After the addition of 10 μl DNase (Sigma DNCL, 1 mg/ml) to the extract, unbroken cells and large membrane fractions were removed by centrifuging for 20 min at 27 000 *g*. Aliquots of the resulting supernatant were layered on top of 15–30% or 5–20% linear sucrose gradients containing 10 mM Tris, pH 7.5 and the appropriate salts and centrifuged in the SW41 rotor of a Spinco Ultracentrifuge (Beckman Instrument Co.) as indicated. Gradients were frac-

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tionated with an ISCO Model 640 Fractionator equipped with a Model UA-5 Analyzer, or by hand; absorbance was monitored at 254 or 260 nm.

In some experiments ribosomes were labelled by growing cells in the presence of [^{14}C]uracil, and the gradients were scanned by measuring radioactivity as well as ultraviolet absorbance. Both methods gave the same results.

The larger subunits (48S) of *V. costicola* ribosomes were used as markers in these gradients. These were prepared from a large batch of cells (25 litre of culture); 48S subunits were separated in a zonal centrifuge, using a 5 to 30% sucrose gradient and a 45% sucrose 'cushion' in media containing 1.0 M NaCl. The preparation was slightly contaminated with monosomes (64S) and smaller subunits (28 S), and also served to mark these positions.

For determination of sedimentation coefficients, the ribosomes were further purified by the method of [3]. Sedimentation analyses were carried out in a Spinco Model E analytical ultracentrifuge at 20°C. Ultraviolet optics were used on samples having an OD^{1cm}_{260} of 0.9. Ultraviolet scans were made at 265 nm every four min for a period of 50 min (at 36 000 rev/min). Viscosity was measured in an Ostwald–Fenske viscometer and density in a Gay-Lussac pycnometer. Corrections for density and viscosity were made as described by Chervenka [8] or by the extrapolation method described in the Results.

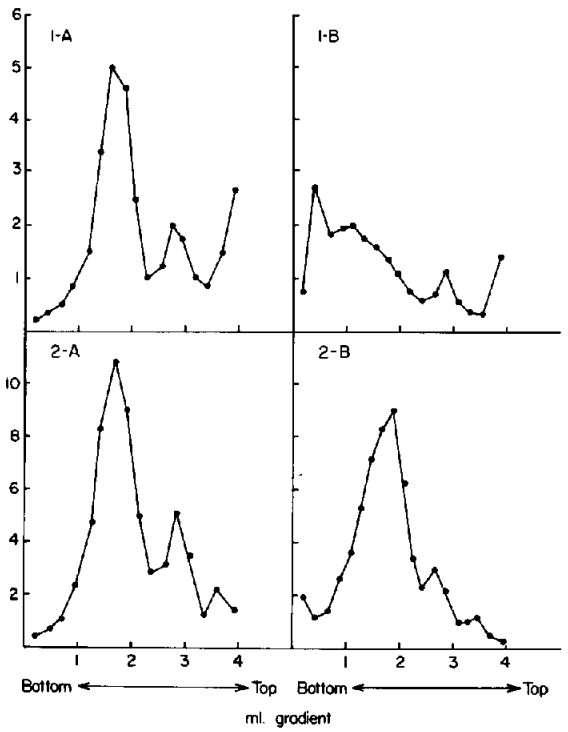

Fig.1. Sucrose density gradients of ribosomes from *Vibrio costicola*. Cells in the presence of: (1) 0.5 M NaCl; (2) 1.5 M NaCl. (A) Ribosomes prepared and centrifuged in gradients containing TMK (10 mM Tris, pH 7.4, 10 mM $MgCl_2$ and 60 mM KCl) + 0.5 M NaCl, for 3.5 h at 208 000 *g*, at 4°C. (B) Ribosomes prepared and centrifuged in gradients containing TMK + 1.5 M NaCl for 4 h at 208 000 *g*, at 4°C. Gradients (15–30% sucrose) were run in 4.5 ml tubes.

3. Results

Vibrio costicola was previously shown to grow in media containing NaCl of from 0.5–3.5 M, the possible range also depending on the composition of the growth medium [6]. Rates of growth were not extensively investigated. For the present work cells were harvested in the exponential phase. In the medium used, logarithmic growth could be maintained for 4 h or more at constant rates which varied with the NaCl concentrations: approx. 0.39 doublings/h at concentrations between 0.5 and 1.0 M, and approximately 0.31 doublings/h at NaCl concentrations of 1.5 and 2.0 M.

Initially ribosomes were prepared from cells grown in salts-glucose casaminoacids medium containing 0.5, 1.0 or 1.5 M NaCl, and sucrose gradients were run for each set of ribosomes in TMK buffer with different concentrations of NaCl (fig.1). If the NaCl concentrations for ribosome preparation and sucrose gradients were the same as, or much lower than, that in the growth medium two peaks were observed running in the same positions as 48 and 28 S markers; corresponding, that is, to the larger and smaller ribosomal subunits (see below.) When the NaCl concentration of the gradient was substantially higher than that of the growth medium (1.5 M for cells grown in 0.5 M NaCl (fig.1) or 2.0 M for cells grown in 1.0 M NaCl (not shown) more complex patterns with wider peaks appeared.

Subsequent experiments (D. B. Shindler, R. M. Wydro, and D. J. Kushner, in preparation) showed

that cells of *V. costicola* grown over a wide range of NaCl concentrations contain about 50 mM Mg^{2+} and substantial amounts of K^+ (from 50 to 100% of the Na^+ concentration, depending on the treatment of cells before analysis.) Sedimentation behaviour of ribosomes was therefore investigated in the presence of substantial quantitites of all three cations.

Sedimentation patterns of ribosomes from cells grown in a range of NaCl concentrations (0.5 M to 2.0 M) were measured in sucrose gradients containing different amounts of Na^+, K^+ and Mg^{2+} ions (fig.2). Regardless of the salt concentration of growth or isolation and separation, all preparations had substantial amounts of 48 and 28 S subunits. Except for ribosomes from cells grown and centrifuged in the highest salt concentrations (fig.2, frames 3D and 4D),

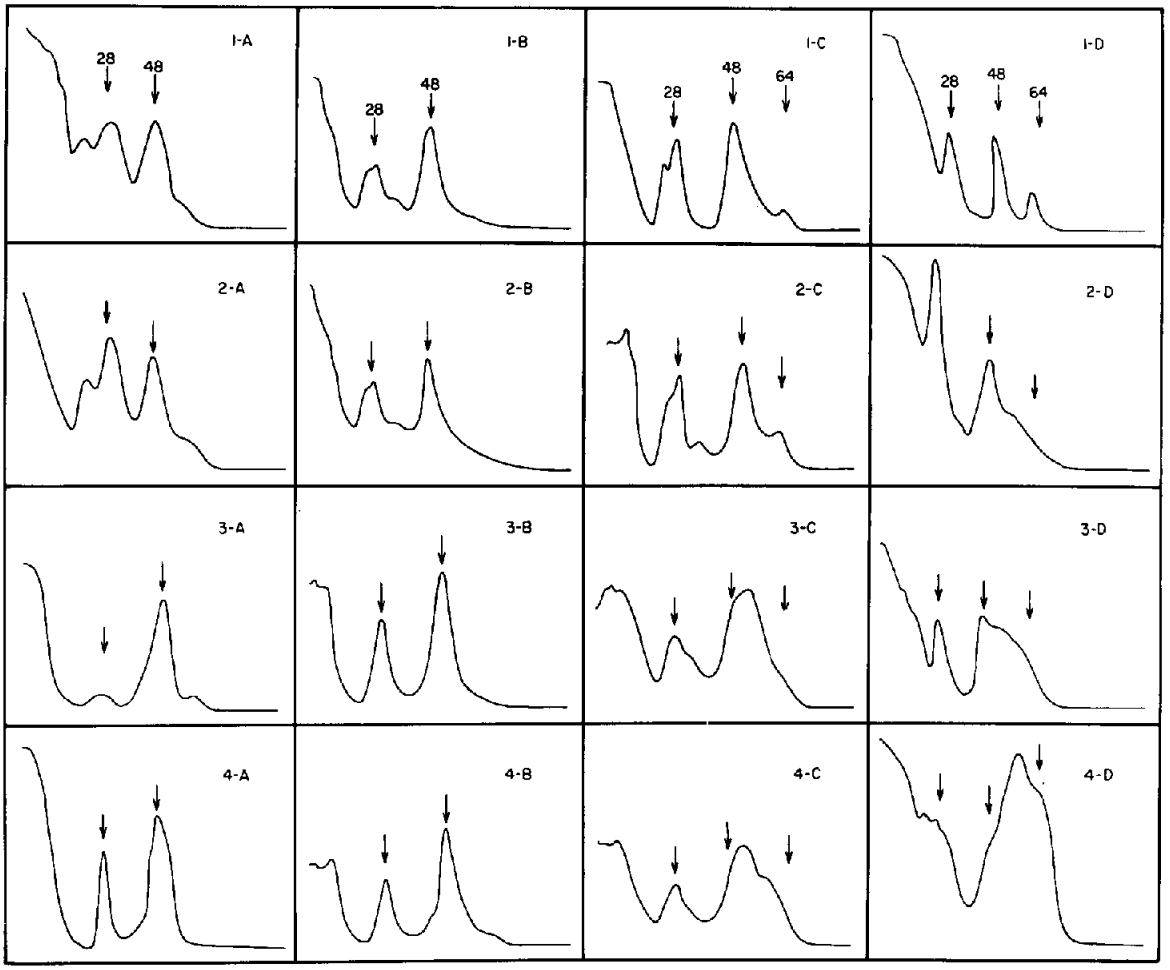

Fig.2. Sucrose density gradients of ribosomes from *Vibrio costicola*. Cells grown in the presence of: (1) 0.5 M NaCl; (2) 1.0 M NaCl; (3) 1.5 M NaCl; (4) 2.0 M NaCl. Ribosomes prepared and centrifuged in gradients containing 10 mM Tris, pH 7.5,+: (A) 3 mM Na-succinate, 10 mM $MgCl_2$; (B) 0.5 M NaCl, 0.25 M KCl, 25 mM $MgCl_2$; (C) 1.0 M NaCl, 0.5 M KCl, 50 mM $MgCl_2$; (D) 2.0 M NaCl, 1.0 M KCl, 100 mM $MgCl_2$. Gradients (5–20% sucrose) were run in 12.4 ml tubes at 208 000 *g*; 3 h for (A) and (B); 4 h for C and D. In each run a marker tube with 48 S *V. costicola* subunits (and small amounts of 28 and 64 S material) was included; positions of the markers are indicated. Material containing about 4 *A* units at 260 nm (1 cm cuvette) was placed on each gradient. The results are normalized so that all 48 S peaks have the same height. (*A* usually 0.4). The tops of gradients appear on the left of each graph.

the 48 and 28 S subunits were the predominant species.

Some differences in sedimentation pattern at different salt concentrations were noted following growth at different NaCl concentrations: if cells were grown in low NaCl concentrations and ribosomes prepared in low salt concentrations (frames 1A, 1B, 2A and 2B) some intermediate material between 28 and 48 S was observed, which disappeared at higher salt concentrations (frames 1C and 1D). At the higher salt concentrations (frames 1C and 1D, 2C and 2D) 64S monosomes begin to appear. Ribosomal preparations from cells grown at higher salt concentrations did not show intermediates between 28 and 48 S peaks when centrifuged in low salt concentrations (frames 3A, 3B, 4A and 4B) but showed greater proportions of 64S monosomes in high salt concentrations (frames 3C, 3D, 4C and 4D.)

A more quantitative determination of sedimentation values of the major peaks was carried out in an analytical ultracentrifuge equipped with u.v. optics. In order to estimate the $S_{w,20}$ values for each peak, the products of the values observed (S_{obs}) and the viscosity of each salt solution were plotted against the density of each solution [9,10], as done for *H. cutirubrum* ribosomes by Bayley and Kushner [3], (fig.3). In this plot the points in salt solutions more concentrated than 0.5 M extrapolated to, or near, the values observed in the most dilute buffer, whose viscosity and density were indistinguishable from those of water. Using all the points, including the very dilute buffer, the intercepts of the abscissa and ordinates were determined by linear regression, using the method of least squares. Intercepts on the ordinate give the $S_{w,20}$ values 64, 48 and 28, corresponding to 'standard' values for procaryotic ribosomes, except that the $S_{w,20}$ for monosomes is slightly but significantly lower than 70.

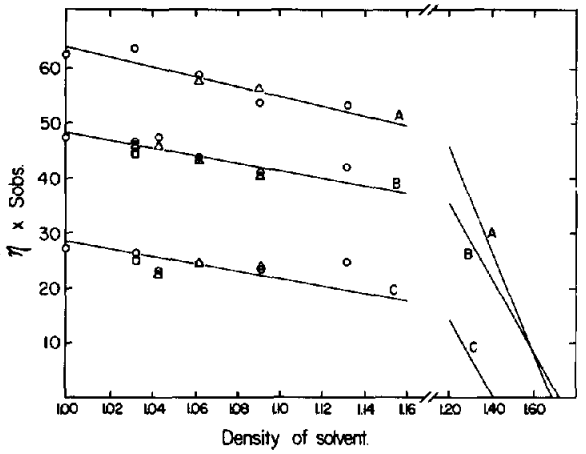

Fig.3. Plot of relative viscosity of solutions (η) times the sedimentation coefficients observed (S_{obs}) of ribosomal particles against the solution density. S_{obs} values were measured in an analytical ultracentrifuge with u.v. optics. Solutions A, B, C, and D of fig.2 (without sucrose) gave densities of 0.998, 1.032, 1.062, and 1.132 respectively. Solutions containing 0.5 M NaCl, 0.5 M KCl and 50 mM $MgCl_2$ gave a density of 1.043; solutions containing 1.5 M NaCl, 0.75 M KCl, and 75 mM $MgCl_2$ gave a density of 1.091. Different symbols indicate different experiments. Cells were grown in media containing 1.0 M NaCl.

Table 1
Effects of NaCl concentration in growth media, and composition of suspending media on sedimentation coefficients of *V. costicola* ribosomes

NaCl in growth media	Composition of suspending media	$S_{w,20}$ values[a]		
		Monosomes	Large subunit	Small subunit
0.5 M	0.5 M NaCl; 0.25 M KCl; 0.025 M $MgCl_2$		48.2 ± 0.9(2)	28.3 ± 0.5(2)
	0.5 M NaCl; 0.5 M KCl; 0.05 M $MgCl_2$		48.5 ± 0.7(2)	24.6 ± 1.2(2)
1.0 M	3 mM Succinate; 10 mM $MgCl_2$	62.5(1)	46.9 ± 1.7(2)	27.4(1)
	0.5 M NaCl; 0.25 M KCl; 0.025 M $MgCl_2$		49.4 ± 1.0(2)	29.0 ± 1.0(2)
	1.0 M NaCl; 0.5 M KCl; 0.05 M $MgCl_2$	62.8 ± 0.7(4)	46.8 ± 00.5(7)	28.4 ± 1.1(6)
	2.0 M NaCl; 1.0 M KCl; 0.1 M $MgCl_2$	65.0(1)	50.3(1)	35.6(1)
1.5 M	1.5 M NaCl; 0.75 M KCl; 0.075 M $MgCl_2$	62.7 ± 1.6(2)	45.7 ± 0.4(2)	30.2 ± 0.7(2)

[a] These values are means of a number (in brackets) of separate determinations ± average deviation from mean. Most were determined with less than 5% error. $S_{w,20}$ values are calculated from S observed and the measured density and viscosity of suspending buffer, assuming partial specific volumes for monosomes, 0.595; large subunit, 0.581; and small subunit, 0.713.

The intercept on the abscissa gives the density at which movement would stop, that is the density of the material itself. Results for the 64S and 48S material are quite close; those for 28S material somewhat lower. Partial specific volumes of 0.595, 0.581, and 0.713 were calculated as the inverse of the densities for the 64S, 48S and 28S material respectively. *E. coli* monosomes and subunits also have partial specific volume of about 0.60 [11]. The S values of the smaller subunits were more difficult to measure accurately because of their slower motion and lower absorbance. We think it unlikely that their apparent higher partial specific volume is real. When $S_{w,20}$ values for monosomes and subunits were calculated for each salt concentration used it was found (table 1) that the calculated $S_{w,20}$ values did not vary significantly with the ionic environment and they were not affected by the NaCl concentrations at which the cells had been grown. A determination of the $S_{w,20}$ value of *E. coli* ribosomes in very dilute buffer gave results indistinguishable from 70.

4. Discussion

Ribosomes of the moderately halophilic bacterium *Vibrio costicola* appear unusual in being able to maintain subunits and monosomes of constant sedimentation values over a wide range of salt concentration and composition. This is in contrast to the behaviour of ribosomes from the extreme halophile, *Halobacterium cutirubrum*, which maintain 'normal' monosomes and subunits in very high KCl concentrations but alter drastically in lower salt concentrations [3]. It is also in contrast to the behaviour of *Escherichia coli* ribosomes whose sedimentation behaviour and protein content are changed by salt concentrations of 0.5 M or higher [12–14] and which we have found to aggregate in gradients containing 1.0 M NaCl. Although *E. coli* grew almost as well in a salts glucose casamino acids medium containing 0.5 M NaCl as in the absence of this salt, analytical ultracentrifuge experiments suggested that, in TMK containing 0.5 M NaCl ribosomes from these cells fell apart into a mixture of ill-defined particles. These cells presumably had very different internal and external ionic environments. There is some evidence from our experiments that *V. costicola* ribosomes may aggregate at high concentrations of NaCl alone but not when both NaCl and Kcl are present in substantial amounts, as they are within the living cell. Ribosomes of *H. cutirubrum* also form ill-defined aggregates in high concentrations of NaCl alone [3].

Work with halophilic bacteria posesinteresting technical problems. Such were encountered in assigning S values to peaks in gradients, containing high salt concentrations which reduced sedimentation rates by increasing density and viscosity. 'Standard' markers, such as *E. coli* ribosomes could be used in gradients containing 0.01 or 0.1 M NaCl, but not in 0.5 M or higher. Preliminary results suggested that purified R_{17} phage of *E. coli* (78S) interacted with *V. costicola* ribosomes at high salt concentrations. It was only after analytical ultracentrifuge measurements had shown that *V. costicola* subunits retained the same S values over a wide range of salt concentration that these could be used as markers.

The S values of *V. costicola* monosomes are slightly lower than those of *E. coli*. This is not necessarily a reflection of the halophilic nature of *V. costicola*, since ribosomes of the extreme halophile *H. cutirubrum* had $S_{20,w}$ values of 70, and its subunits had values of 52 and 31 S [3].

The association of *V. costicola* subunits to monosomes is again unusual in taking place only in high salt concentrations. This association is also influenced by the NaCl concentration at which the cells are grown. A good deal of material intermediate between the two subunits was observed in cells grown in low salt concentrations. The nature of this material is unknown. Some preliminary experiments have suggested that the stage of growth can greatly influence the associability of isolated ribosomes from these bacteria; it might be expected that factors such as salt concentration which affect the rate of growth might also affect ribosomal properties. Sedimentation behavior is only one property of ribosomal subunits, and does not necessarily reflect the in composition or function. A. Matheson (personal communication) found that the 30S subunits of *H. cutirubrum* could lose a number of proteins without changing their sedimentation rate. Studies of the protein composition and of the activity in *in vitro* protein synthesizing systems, which should allow a more detailed assessment of ribosomal integrity, are now under way.

5. Conclusions

This paper describes the sedimentation behaviour and properties of ribosomes from the moderate halophile, *Vibrio costicola*. It was found that the salt concentration in the growth medium, and the composition of the buffer in which ribosomes were made and suspended had no effect on the sedimentation coefficients as measured in an analytical ultracentrifuge. However, the sedimentation values for monosomes and possibly the subunits were lower than the corresponding values for *E. coli*. Sedimentation patterns in sucrose gradients showed some differences both with the salt concentration in the growth medium and the ionic composition of the suspending medium. Thus, monosomes were best seen when growth of cells as well as centrifugation and isolation of ribosomes took place in media with the highest salt concentrations. At the lowest salt concentrations 'abnormal material' sedimenting between the larger and smaller subunits were seen.

We suggest that subunit interactions and hence the properties of the monosomes may be involved in the halophilic response of this organism.

Acknowledgements

Preliminary experiments on ribosomes of *V. costicola* began in 1968 when one of us (DJK) spent two months in the laboratory of Dr A. Tissiéres, Institut de Biologie Moleculaire, Geneva, Switzerland, a visit supported by the National Research Council of Canada. We are indebted to Dr Tissiéres and to Dr R. R. Traut for hospitality and advice at that time.

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References

[1] Larsen, H. (1967) in: 'Advances in Microbial Physiology' (A. H. Rose and J. F. Wilkinson, eds.), Vol 1, pp 97–132, Academic Press, New York.
[2] Kushner, D. J. (1968) Adv. Appl. Microbiol. 10, 73.
[3] Bayley, S. T. and Kushner, D. J. (1964) J. Mol. Biol. 9, 654.
[4] Bayley, S. T. (1966) J. Mol. Biol. 15, 420.
[5] Bayley, S. T. (1971) in: 'Protein Biosynthesis in Bacterial Systems' (J. A. Last and A. I. Laskin, eds.), pp 89–110, Marcel Dekker, New York.
[6] Forsyth, M. P. and Kushner, D. J. (1970) Can. J. Microbiol. 16, 253.
[7] Onishi, H. (1972) J. Bact. 109, 570.
[8] Chervenka, C. H. (1973) A manual of methods for the ultracentrifuge. Spinco Div., Beckman Instrument Co., Palo Alto, California.
[9] Schachman, H. K. and Lauffer, M. A. (1949) J. Am. Chem. Soc. 71, 538.
[10] Hill, J. and Cox, D. J. (1965) J. Phys. Chem. 69, 3032.
[11] Hill, W. E., Rosetti, G. P. and Van Holde, K. E. (1969) J. Mol. Biol. 44, 263.
[12] Spitnik-Elson, P. and Atsmon, A. (1969) J. Mol. Biol. 45, 113.
[13] Atsmon, A., Spitnik-Elson, P. and Elson, D. (1969) J. Mol. Biol. 45, 125.
[14] Dijoseph, C. G., Pon, C., Rapaport, N. D. and Kaje, A. (1971) Biochim. Biophys. Acta 240, 407.