The Effect of the Selective Reduction of Yeast Transfer Ribonucleic Acid with Sodium Borohydride on the Acceptance of Valine and Serine.

Peter Cerutti

Program in Biochemical Sciences,

Frick Chemical Laboratory, Princeton University,

Princeton, New Jersey.

Received February 2, 1968

We have recently focused our interest on studies of the biological function of the minor nucleoside 5,6-dihydrouridine, which has been detected in tRNA from yeast, E. coli, B. subtilis and rat liver (Madison and Holley, 1965; Sanger et al, 1965; Cerutti et al, 1968). Dihydrouridine is so far the only component of nucleic acids from natural sources, which has a partially saturated, non-planar heterocyclic portion. It has been demonstrated with model polymers that dihydrouridine does not participate in base-pairing (Cerutti et al, 1966). In agreement with this result it has in general been assumed that the dihydrouridine cluster in the 5'-half of the sequenced tRNA's for serine, tyrosine, phenylalanine and valine from yeast is contained in a non-base-paired, looped-out region (Holley et al, 1965; Zachau et al, 1966; Madison et al, 1966; RajBhandary et al, 1966; Bayev et al, 1966). One or two residues of dihydrouridine are additionally found in a similar topographical location in the 3'-half of the tRNA's for alanine, tyrosine and valine but not in $tRNA_{ser}$ or $tRNA_{phe}$. It is at present not known if any of the dihydrouridines are essential for the biological activity of yeast tRNA.

To study this problem we have recently developed a method for the reduction of dihydrouridine in yeast tRNA with sodium borohydride (Cerutti and Miller, 1967). A characteristic difference in the primary structure of $tRNA_{val}$ and $tRNA_{ser}$ is, as mentioned above the presence or absence of dihydrouridine in the 3'-half of the molecule. A comparison of the valine and serine acceptance of reduced yeast tRNA seemed therefore of special interest to us and is reported in this paper. A striking difference for both systems was in fact observed in that the chargeability with serine was unaffected by the modification whereas the chargeability with valine decreased to about one-half of a control.

RESULTS

Selective reduction of tRNA with sodium borohydride: The following tRNA preparations were used for the reductive modification with sodium borohydride in the dark: (1) commercial tRNA obtained from stationary yeast which consisted of a mixture of 65% tRNA-CCA and 35% tRNA-CC (determined according to Bell et al, 1964; tRNA-CCA, tRNA with a complete 3'-terminus; tRNA-CC, tRNA lacking its 3'-terminal adenosine) (2) tRNA-CCA which was obtained by readdition of AMP to commercial tRNA with tRNA-adenylyl-transferase, according to Lindahl et al (1967). (tRNA-adenylyl-transferase from yeast prepared according to Preiss et al (1961) was a gift of Dr. Mary Möller. The purification was carried as far as the precipitation with ammonium sulfate). For the reduction 45 mg tRNA in 12.5 ml 0.1 M boric acid-sodium hydroxide-potassium chloride buffer pH 9.9 were treated with 30 mg $NaBH_4$ at room temperature in the dark. After 3 and 5 hr incubation an additional 10 mg of $NaBH_4$ were added to make up for the loss in the reducing agent by decomposition. Aliquots were withdrawn after ½, 1½, 2, 3, 5 and 10 hr (for tRNA partially lacking its 3'-terminal adenosine) or after 1, 2, 3, 5 and 10 hr (for tRNA-CCA). After the reduction the tRNA was twice precipitated with

cold 95% ethanol and then further purified by a passage through Sephadex G-25 (column size 1 x 15 cm, solvent 10^{-3} M tris pH 7.0). The lyophilized preparations were then taken up in 1 ml buffer (0.15 M potassium chloride, 0.01 M potassium cacodylate, 0.005 M magnesium chloride, 0.0005 M Na_2-EDTA, pH 7.0) and applied on top of a Sephadex G-100 column (size 1.5 x 90 cm, flow rate 15 ml/hr) which had been equilibrated and was used with the same buffer at room temperature (cf. Lindahl et al, 1967). No significant differences in the elution profiles of reduced and unreduced tRNA were found for samples which had been treated up to 5 hr with $NaBH_4$. After prolonged treatment with the reducing agent (10 hr or longer) broadening of the profile was observed indicating partial degradation of the tRNA. All the preparations obtained from the reduction of tRNA-CCA, which was radioactively labeled on the 3'-terminal adenosine had nearly the same specific activity, indicating that no terminal adenosine was lost during the reduction or working-up procedure.

A set of control samples was treated under analogous conditions and carried through the same purification procedure, except that $NaBH_4$ was omitted in the incubation mixture.

The Effect of the Selective Reduction of Yeast tRNA on the Enzymatic Addition of Terminal Adenosine: The ability of tRNA-adenylyl-transferase from yeast to add AMP to tRNA-CC (in a mixture containing 65% tRNA-CCA and 35% tRNA-CC) decreased as a function of the length of the exposure of tRNA to sodium borohydride (Figure 1). The effect of the modification levelled off after about 3 hr treatment with the reducing agent and reached a value of 67% residual activity. No decrease in activity was observed for the control tRNA samples which had been exposed to buffer at pH 9.9 for an equivalent length of time and carried through the same purification steps. The initial rate of incorporation of AMP into tRNA was also slightly lower for reduced tRNA than for the control samples.

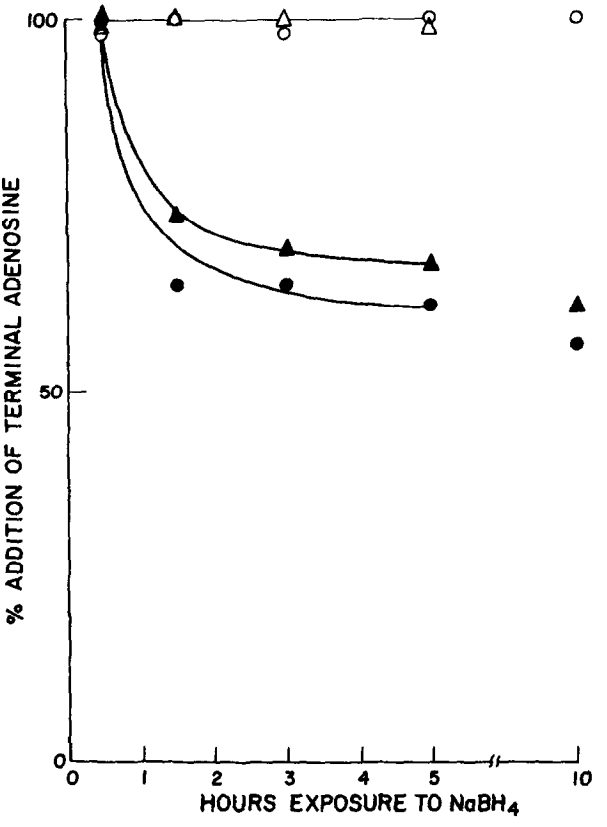

Figure 1 Effect of the reduction of yeast tRNA, lacking 35% of its 3'-terminal adenosine, on the addition of adenosine by yeast tRNA-adenylyl-transferase.
tRNA preparations which had been exposed to $NaBH_4$ for ½ to 10 hr: ▲—▲, incubation with the enzyme at pH 8.6; ●—●, incubation with the enzyme at pH 7.0. Control tRNA preparations which had been exposed to buffer pH 9.9 for ½ to 10 hr: Δ—Δ, incubation with the enzyme at pH 8.6; O—O, incubation with the enzyme at pH 7.0. Incorporation of terminal adenosine into tRNA was assayed by a modification of the method of Moller and Fresco (1968). The 0.05 ml reaction mixture contained 5 μmoles of Tris, pH 8.6, 1 μmole of magnesium chloride, 0.5 μmoles of β-mercaptoethanol, 1 μmole phosphoenolpyruvate, 10 μg pyruvate kinase, 0.2 μmoles

Na_2-EDTA, 0.2 μmoles C^{14}-ATP (0.5 mc/mmole), 30 μg tRNA-adenylyl-transferase from yeast and a limiting amount of tRNA. The reaction was stopped after 60 min incubation at 37° by the addition of 20 vol of cold 5% TCA. The precipitate was collected on Millipore filters and washed with a 150 vol of cold 5% TCA.

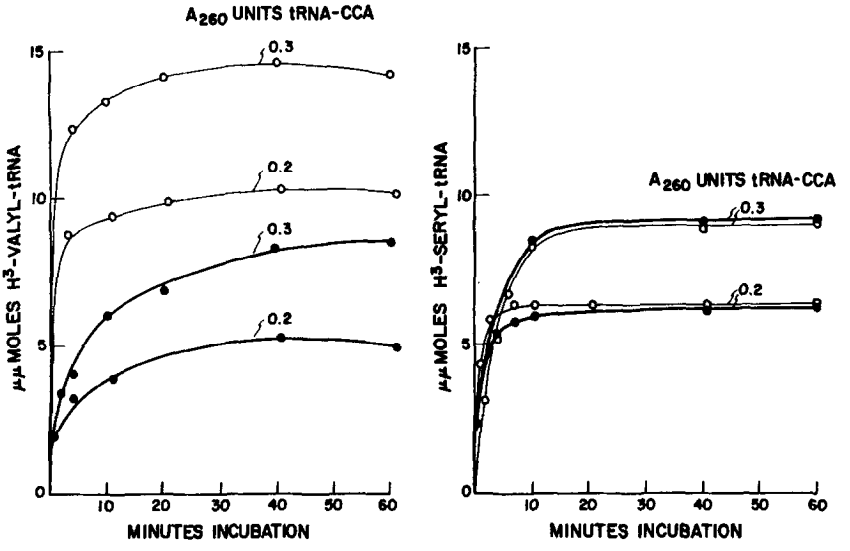

Figure 2 Effect of the reduction of yeast tRNA-CCA on the kinetics of acylation with serine and valine.

●—● , tRNA-CCA preparation which had been exposed to $NaBH_4$ for 5 hr. O—O , control tRNA-CCA preparations which had been exposed to buffer pH 9.9 for 5 hr.

Amino acid acceptor activity was assayed in 0.05 ml of a solution containing 0.22 μmoles of ATP, 0.022 μmoles of CTP, 0.0018 μmoles of C^{14}-valine or C^{14}-serine (50 mc/mmole) or 0.0023 μmoles of H^3-serine (870 mc/mmole) or 0.0034 μmoles of H^3-valine (290 mc/mmole), 0.0022 μmoles of 19 other C^{12}-amino acids, 0.55 μmoles magnesium chloride, 0.55 μmoles potassium chloride, 0.05 μmoles Na_2-EDTA, 11 μmoles of Tris, pH 7.0. To this mixture were added 0.2 to 0.4 A_{260} units of tRNA

(A_{260} units, optical density units per ml water as measured in a 1 cm light path cuvette at 260 mμ) and 0.002 ml enzyme solution. Aminoacyl-tRNA-synthetases from yeast were prepared according to Groves and Kempner (1967) and further purified by column chromatography on DEAE according to Yamane and Sueoka (1963). All assays were run in triplicates. The incubations were at 37° in a shaker-incubator for 40 min if not otherwise stated. The reactions were stopped and the samples assayed as described in the legend to Figure 1.

The Effect of the Selective Reduction of Yeast tRNA on the Aminoacylation with Serine and Valine: All the tRNA preparations used in these experiments had been purified on a Sephadex G-100 column. This purification step was included to eliminate, if present, any degradation products which could act as inhibitors of the aminoacyl-tRNA-synthetases (cf. Hayashi and Miura, 1964; Stulberg and Isham, 1967). Under the conditions used for the charging experiments, the rate of aminoacylation was proportional to the level of enzyme but the yield of aminoacyl-tRNA was only dependent on the amount of tRNA added.

Figure 2 shows the kinetics of acylation with serine and valine of tRNA-CCA which had been exposed to sodium borohydride for 5 hr. Whereas the modification had essentially no effect on the aminoacylation with serine, a marked decrease of the rate of formation of valyl-tRNA was observed and the yield of valyl-tRNA was lowered to approximately 51% of the control sample. Incubation of tRNA-CCA at pH 9.9 for the same length of time lead to a decrease in the chargeability with valine of only 7%.

The kinetics of the acylation with serine and valine of reduced tRNA in which 35% of the 3'-terminal adenosine are removed are shown

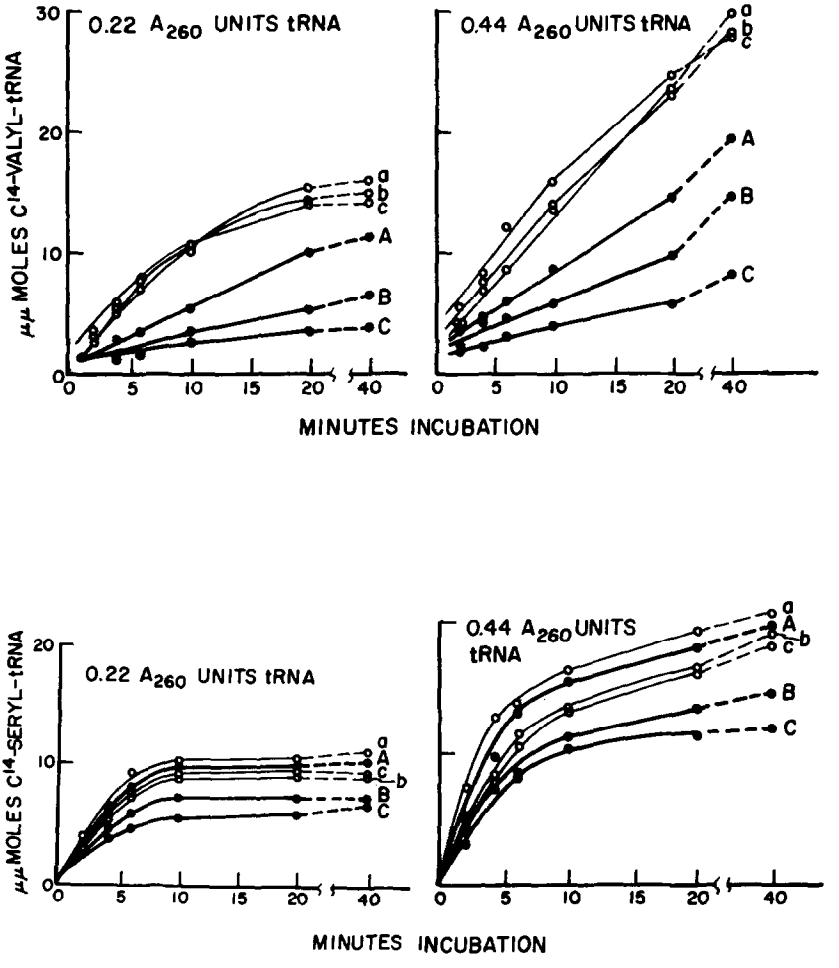

Figure 3 Effect of the reduction of yeast tRNA, lacking 35% of its 3'-terminal adenosine on the kinetics of acylation with serine and valine.

●—●, tRNA preparations which had been exposed to $NaBH_4$ for 30 min (curves A), 90 min (curves B) and 180 min (curves C).

○—○, control tRNA preparations which had been exposed to buffer ph 9.9 for 30 min (curves a), 90 min (curves b), 180 min (curves c).

Amino acid acceptor activity was assayed as described in the legend to Figure 2.

in Figure 3. In contrast to the undiminished chargeability of reduced tRNA-CCA with serine, a 26% loss of activity and a significant decrease in the rate of the reaction was now observed after 3 hr treatment with sodium borohydride. A 69% loss in chargeability with valine was found for the same tRNA preparation. This value should be compared to the 45% loss in valine acceptance for the corresponding tRNA-CCA preparation. Again a marked decrease in the rate of the aminoacylation upon reduction was found.

Figure 4 shows a plot of the decrease in the valine and serine acceptance as a function of the length of exposure to sodium borohydride for tRNA-CCA and tRNA lacking the terminal adenosine to 35%.

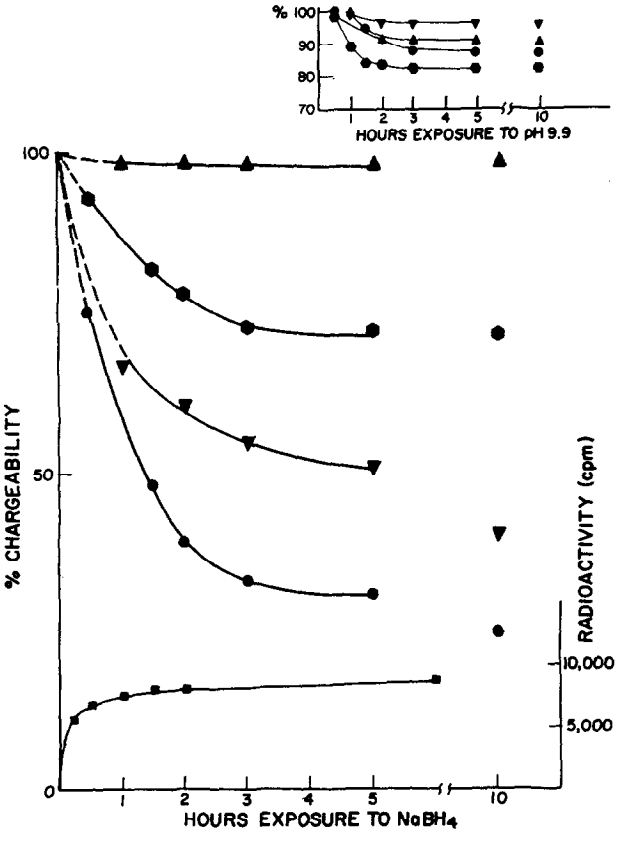

Figure 4 Effect of the reduction of tRNA-CCA and of tRNA, lacking 35% of its 3'-terminal adenosine, on the acceptance of

serine and valine as a function of the length of exposure to $NaBH_4$.

% residual chargeability corrected for the loss of activity of the corresponding control preparations, which had been exposed to buffer pH 9.9 for an equivalent length of time. ▲—▲ , serine acceptance of reduced tRNA-CCA; ●—●, serine acceptance of reduced tRNA, lacking 35% of its 3'-terminal adenosine. ▼—▼ , valine acceptance of reduced tRNA-CCA; ●—● , valine acceptance of reduced tRNA, which lacked 35% of its 3'-terminal adenosine. ■—■, kinetics of the incorporation of tritium into dihydrouridine in yeast tRNA upon reduction with sodium borotritiide (Cerutti and Miller, 1967).

Inset: Loss of serine and valine acceptance of control tRNA preparations which had been exposed to buffer pH 9.9 for equivalent lengths of time. Corresponding symbols are used.

Amino acid acceptor activity was measured as described in the legend to Figure 2.

The following summarizes these results. (1) Only the aminoacylation with valine is affected by the reduction of tRNA-CCA. A decrease in the chargeability with both amino acids, but far more pronounced with valine, is found for reduced tRNA partially lacking its 3'-terminal adenosine. (2) More than 90% of the final effect of the modification are observed after 3 hr treatment with the reducing agent.

DISCUSSION

Probably the most important finding in this work is the striking difference in the effect of the modification on the chargeability

with serine and valine. Since no effect of the reduction of yeast tRNA-CCA on the acylation with serine was observed it has to be concluded that an intact dihydrouridine cluster in the 5'-half of $tRNA_{ser}$ I & II (Zachau et al, 1966) is not essential for this reaction. An alternative interpretation, which cannot be excluded, is that the dihydrouridine residues in $tRNA_{ser}$ I & II are more resistant towards the reduction than the dihydrouridine residues in $tRNA_{val}$. Similarly the residue of N_4-acetyl-cytidine found at position 12 of $tRNA_{ser}$ I & II (counting starting at the 5'-end) which also should react rapidly with sodium borohydride (Cerutti and Miller, 1967; Miller and Cerutti, 1967) does not seem to be essential for the aminoacylation step. It is tempting to correlate the resistance of the serine acceptance and the susceptibility of the valine acceptance with the absence or presence of dihydrouridine in the 3'-half of $tRNA_{ser}$ I & II and $tRNA_{val}$. This may indicate that the dihydrouridine in the 3'-half in $tRNA_{val}$ is important for the recognition process with the synthetase. However, work with single species of tRNA and with highly purified enzyme preparations will be needed to substantiate this interpretation.

Since no loss in serine acceptance of reduced tRNA-CCA was detected, the decrease in activity of modified tRNA which partially lacked its terminal adenosine may be explained by the effect of the modification on the addition of terminal adenosine. Similarly an additional loss in the acceptance of valine was observed for the same preparation as compared to tRNA-CCA. An inhibitory effect of reduced tRNA-CC on the synthetase cannot be excluded but seems unlikely from the kinetics of the acylation (see Figure 2).

The effect of the modification on the acylation with valine levels off after approximately 3 hr treatment with the reducing agent. The kinetics of the tritium incorporation into the dihydrouridine residues of yeast tRNA upon treatment with sodium borotritiide

on the other hand shows that more than 90% of the reaction has occurred after 1 hr (Figure 4; cf. Cerutti and Miller, 1967). It has to be kept in mind that the tritium incorporation curve represents an average for unfractionated tRNA and that significant differences in the rates of reduction for each individual tRNA species may exist. This may explain the apparent discrepancy between the kinetics of the modification reaction and the deactivation of $tRNA_{val}$.

The reason that the deactivation curve for the valine acceptance of tRNA-CCA levels off and does not go to zero even after 5 hr exposure to sodium borohydride may be that not all of the two (Smith and Herbert, 1963) or three (Thiebe and Zachau, 1964) species of $tRNA_{val}$ of yeast are inactivated by the modification. Experiments with highly purified $tRNA_{val}$ should answer this question.

No effect of the reduction of yeast tRNA with sodium borohydride on the acceptance of valine was observed by Roy and Tener (1967). Much milder reduction conditions have been used by these authors as compared to this work, conditions which are expected to lead only to negligible modification of dihydrouridine according to our experience. No direct comparison is possible between the effect of the dark-reduction (as used in this paper) and the photoreduction (Adman and Doty, 1967) with sodium borohydride on the amino acid acceptance of yeast tRNA.

Acknowledgement

I would like to thank Dr. B. M. Alberts for critically reading the manuscript and Miss Patricia Mulligan for technical assistance. These studies were supported by grants GB 4894 from the National Science Foundation, GM 14090 from the National Institutes of Health and by a grant from Hoffmann-La Roche Inc., Nutley, New Jersey.

References

Adman, R. and Doty, P., Biochem. Biophys. Res. Commun. 27, 579 (1967).

Bayev, A., Venkstern, T., Mirzabekov, A., Krutilina, A., Axelrod, V., Li, L. and Engelhardt, V., Proc. 3rd. Fed. Europ. Biochem. Soc. Heidelberg: Springer Verlag (1966).

Bell, D., Tomlinson, R. V. and Tener, G. M., Biochemistry 3, 317 (1964).

Cerutti, P., Miles, H. T. and Frazier, J., Biochem. Biophys. Res. Commun. 22, 466 (1966).

Cerutti, P., Miller, N. and Holt, W., in manuscript (1968).

Cerutti, P. and Miller, N., J. Mol. Biol. 26, 55 (1967).

Groves, W. E. and Kempner, E. S., Science 156, 387 (1967).

Hayashi, H. and Miura, K-I., J. Mol. Biol. 10, 345 (1964).

Holley, R. W., Biochem. Biophys. Res. Commun. 10, 186 (1963).

Holley, R. W., Apgar, J., Everett, A. G., Madison, J. T., Marquisee, M., Merril, S. H., Penswick, J. R. and Zamir, A., Science 147, 1462 (1965).

Lindahl, T., Adams, A. and Fresco, J. R., J. Biol. Chem. 242, 3129 (1967).

Madison, J. T. and Holley, R. W., Biochem. Biophys. Res. Commun. 78, 153 (1965).

Madison, J. T., Everett, G. A. and Kung, H., Science 153, 531 (1966).

Miller, N. and Cerutti, P., J. Am. Chem. Soc. 89, 2767 (1967).

Möller, M. and Fresco, J. R., manuscript in preparation (1968).

Preiss, J., Dieckmann, M. and Berg, P., J. Biol. Chem. 236, 1748 (1961).

RajBhandary, U. L., Stuart, A., Faulkner, R. D., Chang, S. H. and Khorana, H. G., Cold Spr. Harb. Symp. Quant. Biol. 31, 425 (1966).

Roy, K. L. and Tener, G. M., Biochemistry 6, 2847 (1967).

Sanger, F., Brownlee, G. G. and Barrell, B. G., J. Mol. Biol. 13, 373 (1965).

Smith, C. J. and Herbert, E., Fed. Proc. 22, 230 (1963).

Stulberg, M. P. and Isham, R. R., Proc. Nat. Acad. Sci., Wash. 57, 1310 (1967).

Thiebe, R. and Zachau, H. G., Biochim. Biophys. Acta 103, 568 (1964).

Zachau, H., Dütting, D., Feldmann, H., Melchers, F. and Karau, W., Cold. Spr. Harb. Symp. Quant. Biol. 31, 417 (1966).