Biochem. J. (1978) **170**, 57–61
Printed in Great Britain

The Mode of Action of Alpha Sarcin and a Novel Assay of the Puromycin Reaction

By ADRIAN N. HOBDEN and ERIC CUNDLIFFE
Department of Biochemistry, University of Leicester, University Road, Leicester LE1 7RH, U.K.

(*Received* 2 *June* 1977)

A new technique was developed for measuring the amount of peptidyl-tRNA in a protein-synthesizing system *in vitro*. By this technique the course of the puromycin reaction may be followed and the modes of action of various inhibitors of protein synthesis readily determined. We conclude that the polypeptide alpha sarcin inhibits the binding of aminoacyl-tRNA into the ribosomal 'A' site, that sparsomycin inhibits the peptidyl-transferase reaction and that cycloheximide may block translocation.

Elongation of the nascent peptide chain during protein synthesis is a cyclic process involving, in eukaryotic systems, two protein factors, EF-1 and EF-2. Ribosomes carrying peptidyl-tRNA in the ribosomal 'P' site bind aminoacyl-tRNA into the adjacent 'A' site in response to appropriate mRNA codons (Cundliffe & McQuillen, 1967). Binding requires EF-1 and GTP, and subsequently EF-1 and GDP are released in a complex from which GDP is eventually displaced by GTP. Peptidyl-tRNA bound in the 'P' site then reacts with aminoacyl-tRNA bound in the 'A' site in a step that requires no extra-ribosomal factor or external source of energy. This is a peptidyltransferase reaction and results in the elongation of the nascent peptide chain by one amino acid residue. The peptidyl-tRNA is now located in the 'A' site and must move across to the 'P' site before the next aminoacyl-tRNA can bind. This occurs as part of the 'translocation' process, which also involves displacement of deacylated tRNA from the 'P' site and movement of the ribosome along the mRNA so as to position the next codon in the 'A' site. Translocation requires EF-2 and GTP, the latter being hydrolysed to GDP plus phosphate.

An agent which inhibits polypeptide-chain elongation may do so by affecting any one of the three component reactions outlined above, namely the binding of aminoacyl-tRNA to the 'A' site, the peptidyltransferase reaction or translocation. Whether or not such an agent inhibits the puromycin reaction depends on which of these three processes is inhibited.

Puromycin is an analogue of the 3′-end of aminoacyl-tRNA and accepts the transferred peptide in the peptidyltransferase reaction. This so-called 'puromycin reaction' does not require factors or GTP and results in the formation of peptidyl-puromycin, which detaches from the ribosome (Traut & Munro, 1964).

Various drugs which block the ribosomal 'A' site do not prevent the puromycin reaction (Cundliffe & McQuillen, 1967), evidently because puromycin has access to the 'A' site under conditions where aminoacyl-tRNA does not. In contrast, inhibitors of either translocation or peptidyltransferase prevent the puromycin reaction in systems synthesizing protein, although it is possible to distinguish between them on other grounds (for a discussion see Cundliffe & McQuillen, 1967).

Here we describe a new technique for following the puromycin reaction. This involves the use of the detergent cetyltrimethylammonium bromide, which precipitates peptidyl-tRNA, but not peptidyl-puromycin, and allows the progress of the puromycin reaction to be detected by a decrease in peptidyl-tRNA concentrations. This assay is rapid, convenient and allows the course of the reaction to be followed in detail.

Alpha sarcin is a polypeptide of mol.wt. 16000 isolated from the mould *Aspergillus giganteus* (Olson, 1963; Schuurmans *et al.*, 1964). It inhibits several induced tumours, including sarcoma 180 and carcinoma 755, but shows no anti-bacterial or anti-fungal activity (Olson & Goerner, 1965; Olson *et al.*, 1965). We show below that alpha sarcin does not inhibit the puromycin reaction in rabbit reticulocyte lysates, despite being a potent inhibitor of the elongation phase of protein synthesis.

The modes of action of two other inhibitors of protein synthesis, sparsomycin and cycloheximide, have also been studied. Whereas both inhibit the puromycin reaction when added singly to reticulocyte

Abbreviations used: EF-1 and -2, elongation factors 1 and 2.

lysates, only sparsomycin does so in lysates previously treated with alpha sarcin. The implications of these observations with regard to the modes of action of the inhibitors used are discussed.

Experimental

Materials

L-[4,5-^{3}H]Leucine (51Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; phosphocreatine and creatine kinase were from Boehringer und Soehne, Mannheim, Germany; yeast RNA and L-amino acids were from Sigma Chemical Co., Kingston-upon-Thames, Surrey, U.K.; other chemicals were of A.R. grade.

Preparation of rabbit reticulocyte lysates

The rabbit reticulocyte lysates used in these experiments were kindly given by Dr. R. J. Jackson and Dr. T. Hunt (Department of Biochemistry, University of Cambridge, Cambridge, U.K.). They were prepared as follows. Rabbits weighing 3kg were given four successive daily injections of 30mg of acetylphenylhydrazine dissolved in 2ml of warm saline (0.13M-NaCl/7.5mM-$MgCl_2$/5mM-KCl) and cardiac puncture was performed after 9 days. After centrifugation lysates were prepared from the washed, packed cells by adding 1.5vol. of cold water and centrifuging the debris down at 30000*g* for 15min at 4°C (Hunt *et al.*, 1972). Lysates were stored as 1ml batches under liquid N_2.

Incubation conditions

Frozen portions of lysates were thawed at 0–5°C and were made 0.025mM in haemin by using a stock solution of 1mM-haemin in 50% (v/v) ethylene glycol/20mM-Tris/HCl (pH8.2)/50mM-KCl. Creatine kinase at 0.05mg/ml (final concentration) was also added.

Standard incubation mixtures contained the following components: 0.75ml of lysate containing haemin, 0.05ml of salts solution (2M-KCl/10mM-$MgCl_2$), 0.05ml of 0.2M-phosphocreatine, 0.1ml of 0.3mM-L-[4,5-^{3}H]leucine (3Ci/mmol) and 0.05ml of a mixture of amino acids with the following composition: 3mM-alanine; 0.5mM-arginine; 0.5mM-asparagine; 2.0mM-aspartic acid; 0.5mM-cysteine; 0.5mM-glutamine; 2.0mM-glutamic acid; 2.0mM-glycine; 2.0mM-histidine; 0.5mM-isoleucine; 2.0mM-lysine; 0.5mM-methionine; 1.5mM-phenylalanine; 1.0mM-proline; 2.0mM-serine; 1.5mM-threonine; 0.5mM-tryptophan; 0.5mM-tyrosine; 3.0mM-valine. This amino acid mixture was adjusted to pH7.5 with KOH, and dithiothreitol (10mM final concn.) was added.

Inhibitors of protein synthesis were used at the following final concentrations: alpha sarcin, 100μg/ml (6μM); puromycin, 40μg/ml (0.09mM); sparsomycin, 40μg/ml (0.1mM); cycloheximide, 200μg/ml (0.7mM); edeine, 10μg/ml (2μM).

Preparation of samples for analysis

Protein synthesis in reticulocyte lysates was stopped by taking 5μl samples of incubation mixture into 0.5ml of water. Before assay of total protein synthesis by liquid-scintillation counting it was necessary both to bleach samples to prevent 'quenching' and also to remove aminoacyl-tRNA. This was done by addition of 0.5ml of 1M-NaOH containing approx. 1mg of unlabelled leucine/ml and 0.1ml of 20vol. H_2O_2. The mixture was then incubated at 30°C for several minutes until the solution was completely bleached and the aminoacyl-tRNA hydrolysed, whereupon 0.5ml of 50% (w/v) trichloroacetic acid was added. Precipitates were collected on Whatman GF/C filters, which were then washed three times with 5ml of 5% (w/v) trichloroacetic acid and then dried. Radioactivity present on filters was assayed by liquid-scintillation counting in a scintillant containing 4g of 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/litre of toluene. To distinguish, in other samples of the incubation mixture, peptidyl-tRNA from free peptide and

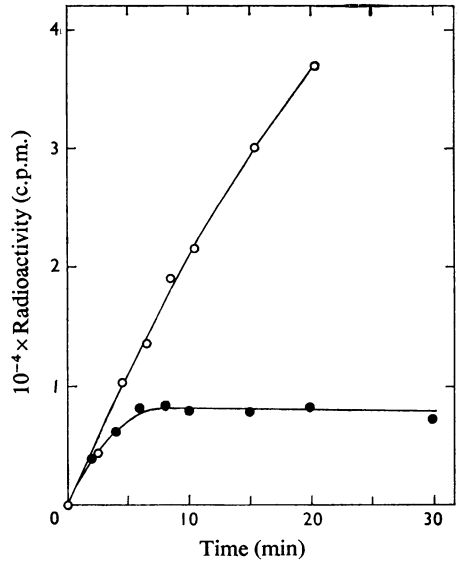

Fig. 1. *Incorporation of* L-[^{3}*H*]*leucine into peptide*
A mixture containing L-[^{3}H]leucine (51 Ci/mmol) in 0.3mM-L-leucine was incubated at 30°C, and 5μl samples were removed into water at various times. These samples were precipitated with either trichloroacetic acid (○) or cetyltrimethylammonium bromide (●) and radioactivity in the precipitates was measured by liquid-scintillation counting (see the Experimental section).

aminoacyl-tRNA, 1ml of 2% (w/v) cetyltrimethylammonium bromide solution in water was added instead of NaOH and H_2O_2, and this was followed by 1ml of 0.5M-sodium acetate/acetic acid buffer, pH5.4, containing 0.5mg of yeast RNA/ml as carrier. The resultant precipitate, which contains peptidyl-tRNA and aminoacyl-tRNA (Darnbrough *et al.*, 1973), was left to aggregate before being collected on Whatman GF/C filters, which were then washed thoroughly with water. Aminoacyl-tRNA was solubilized by heating the filters in 10% (w/v) trichloroacetic acid for 20min at 90°C, and finally the filters were washed three times with 5ml of 5% (w/v) trichloroacetic acid, dried and subjected to liquid-scintillation counting as above.

Results

Incorporation of label into peptides

Reticulocyte lysates incorporated L-[^{3}H]leucine into protein (as judged by trichloroacetic acid precipitation) linearly with time for over 20min (Fig. 1). Conversely, incorporation of L-[^{3}H]leucine into peptidyl-tRNA (as judged by cetyltrimethylammonium bromide precipitation) was linear for the first 4min and during that time was equivalent to the total protein synthesized. Shortly thereafter (Fig. 1), incorporation into peptidyl-tRNA slowed and soon reached a plateau. If edeine, a known inhibitor of initiation of protein synthesis in rabbit reticulocyte lysates (Obrig *et al.*, 1971), was then added to the incubation mixture during this plateau stage the amount of L-[^{3}H]leucine present in peptidyl-RNA fell, within 3min, to less than 10% of its previous value (results not shown). This implies that a steady state is reached after 6–10min in which initiation of the synthesis of new peptide chains on ribosomes is balanced by release of completed ones from other ribosomes.

The reticulocyte lysates' abilities to incorporate L-[^{3}H]leucine into protein or peptidyl-tRNA were found to vary considerably between preparations.

Inhibition of protein synthesis by alpha sarcin

Alpha sarcin inhibited by 95% the rate of protein synthesis in reticulocyte lysates when used at a final concentration of 10μg/ml (Fig. 2). In all other

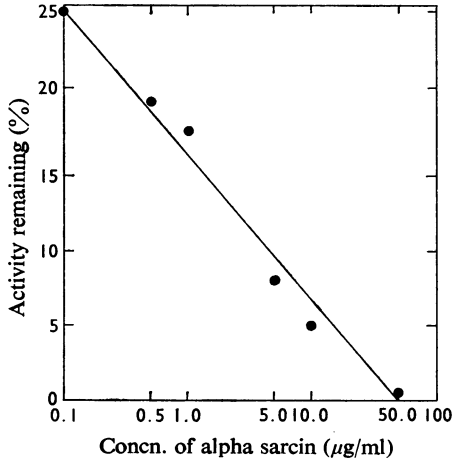

Fig. 2. *Inhibition of protein synthesis by alpha sarcin*
An incubation mixture was set up as in Fig. 1 except that no non-radioactive L-leucine was added. Alpha sarcin at various final concentrations was added before the incubation. Samples (5μl) were precipitated with trichloroacetic acid and radioactivity in the precipitates was measured by liquid-scintillation counting (see the Experimental section).

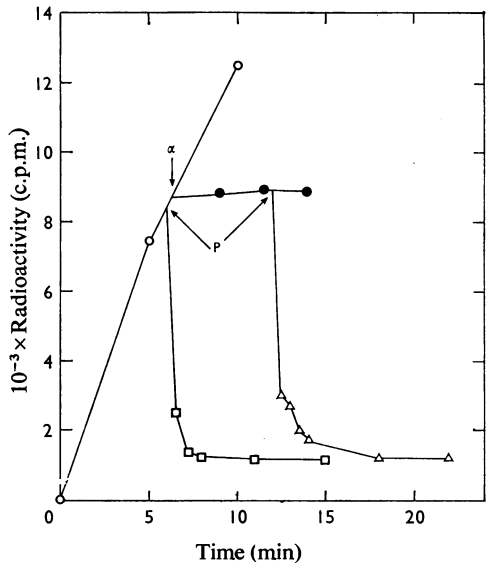

Fig. 3. *Assay of the puromycin reaction in control lysates or lysates inhibited by alpha sarcin*
An incubation mixture was set up as in Fig. 1 except that no non-radioactive L-leucine was added. After 6min portions of the control lysate were removed either into puromycin (P; 40μg/ml final concn.) or into alpha sarcin (α; 100μg/ml final concn.). At 13min a further portion of the lysate inhibited by alpha sarcin was removed into puromycin (40μg/ml final concn.). Samples (5μl) were precipitated with cetyltrimethylammonium bromide (see the Experimental section), and radioactivity in the precipitates was determined by liquid-scintillation counting. ○, Uninhibited control; ●, plus alpha sarcin; □, plus puromycin; △, plus alpha sarcin and puromycin.

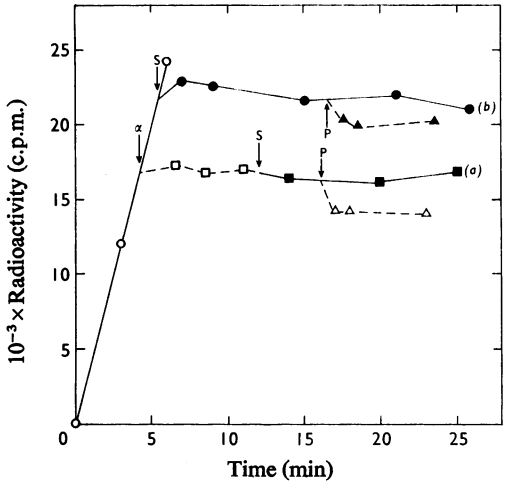

Fig. 4. *Inhibition of protein synthesis and the puromycin reaction by sparsomycin*

An incubation mixture was set up as in Fig. 1 except that no non-radioactive L-leucine was added. As indicated, portions were removed either into (*a*) alpha sarcin (α; 100 μg/ml final concn.) or (*b*) sparsomycin (S; 40 μg/ml final concn.). Sparsomycin (S; 40 μg/ml final concn.) was added to (*a*) at 12 min, and, finally, portions of preparations (*a*) and (*b*) were removed into puromycin (P; 40 μg/ml final concn.) at 16 min. Samples (5 μl) were precipitated with cetyltrimethylammonium bromide (see the Experimental section) and radioactivity in the precipitates was determined by liquid-scintillation counting. ○, Uninhibited control; ●, plus sparsomycin; ▲, plus sparsomycin and puromycin; □, plus alpha sarcin; ■, plus alpha sarcin and sparsomycin; △, plus alpha sarcin, sparsomycin and puromycin.

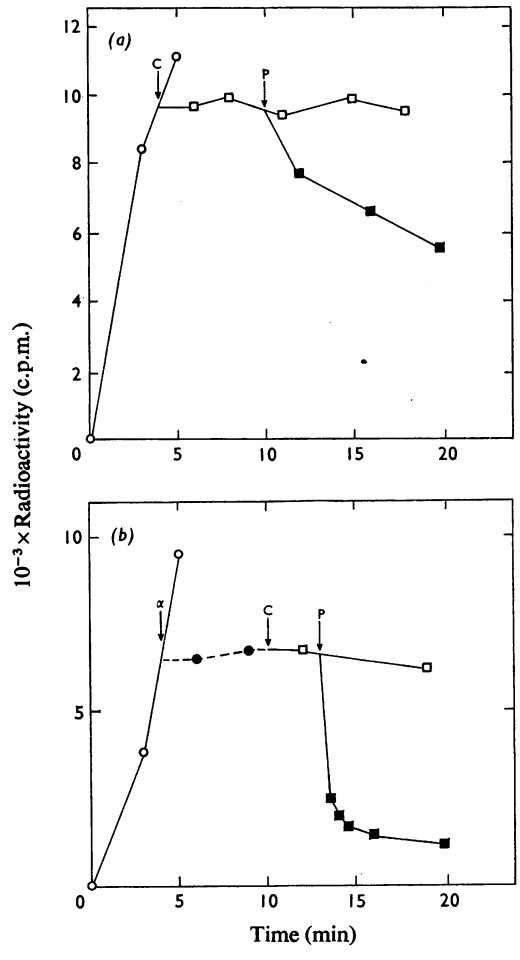

Fig. 5. *Effect of cycloheximide on the puromycin reaction in the presence or absence of alpha sarcin*

An incubation mixture was set up as in Fig. 1 except that no non-radioactive L-leucine was added. As indicated, portions were removed into cycloheximide (C; 200 μg/ml final concn., *a*) or alpha sarcin (α; 100 μg/ml final concn., *b*). At 10 min cycloheximide (C; 200 μg/ml final concn.) was added to the lysate inhibited by alpha sarcin (*b*). Finally, portions of the two inhibited lysates were removed into puromycin (P; 40 μg/ml final concn.). Samples (5 μl) were precipitated with cetyltrimethylammonium bromide (see the Experimental section) and radioactivity in the precipitates was determined by liquid-scintillation counting. ○, Control; (*a*) □, plus cycloheximide; ■, plus cycloheximide and puromycin; (*b*) ●, plus alpha sarcin; □, plus alpha sarcin and cycloheximide; ■, plus alpha sarcin, cycloheximide and puromycin.

experiments referred to here, alpha sarcin was used at 100 μg/ml (final concentration), under which conditions it inhibited protein synthesis totally and immediately (Figs. 2 and 3). Under these latter conditions, however, alpha sarcin did not prevent the rapid and almost quantitative release of nascent peptide chains from ribosomes in the presence of puromycin (Fig. 3). Fig. 3 also shows the effect of adding puromycin at the same concentration (40 μg/ml) to an otherwise uninhibited lysate. The effects of sparsomycin and cycloheximide on peptidyl-tRNA synthesis in reticulocyte lysates are shown in Figs. 4 and 5(*a*) respectively. Here these antibiotics were again used at high concentrations so that protein synthesis would be totally inhibited. Their effects on the puromycin reaction are also shown in Figs. 4 and 5(*a*). Sparsomycin almost completely prevented the decrease in the concentration of peptidyl-tRNA by puromycin, whereas cycloheximide (although strongly inhibitory) caused a slow decrease.

In a further series of experiments, sparsomycin or

cycloheximide was added to reticulocyte lysates in which protein synthesis had already been stopped by alpha sarcin, puromycin finally added and peptidyl-tRNA concentration monitored throughout. The results are shown in Figs. 4 and 5(*b*). The effect of sparsomycin, namely almost total inhibition, was unchanged by the prior addition of alpha sarcin (Fig. 4), whereas cycloheximide now failed to inhibit release (Fig. 5*b*).

Discussion

Precipitation of peptidyl-tRNA by cetyltrimethylammonium bromide affords an extremely convenient and accurate means of following the reaction of nascent polypeptides with puromycin. Using this assay, we have investigated the modes of action of three antibiotics which inhibit polypeptide chain elongation. Puromycin takes part in the ribosomal peptidyltransferase reaction whereby peptidyl-tRNA in the 'P' site is deacylated with the formation of peptidyl-puromycin. Obviously, therefore, any drug that inhibits the peptidyltransferase will prevent the puromycin reaction. In addition, inhibitors of translocation also inhibit the puromycin reaction in systems synthesizing protein by halting the elongation cycle so as to leave peptidyl-tRNA in the ribosomal 'A' site, where it is unable to react with puromycin. It has been argued previously (Cundliffe & McQuillen, 1967; Cundliffe, 1971) that the only way a drug can stop polypeptide-chain elongation without affecting the puromycin reaction is by blocking the 'A' site, so as to prevent the binding of aminoacyl-tRNA to that site. Evidently puromycin may still have access to the peptidyltransferase centre under such conditions. We conclude therefore that alpha sarcin (Fig. 3) inhibits the binding of aminoacyl-tRNA to the ribosomal 'A' site. In contrast, and in agreement with the findings of others, cycloheximide (Baliga *et al.*, 1970) and sparsomycin (Baglioni, 1966; Goldberg & Mitsugi, 1967) both inhibited the puromycin reaction (Figs. 4 and 5*a*). We therefore conclude that sparsomycin and cycloheximide were inhibiting either the peptidyltransferase or translocation in our lysates.

According to our hypothesis, after the addition of alpha sarcin to reticulocyte lysates, peptidyl-tRNA was confined to the ribosomal 'P' sites, from which it was readily removable by puromycin (Fig. 3) via the peptidyltransferase reaction. Therefore, one would expect a drug that inhibits the peptidyltransferase to prevent such puromycin reactions in the presence of alpha sarcin. Sparsomycin did so (Fig. 4), as it did in analogous experiments in a bacterial system (Cundliffe & McQuillen, 1967), in accordance with its established action as a peptidyltransferase inhibitor [for reviews, see Cundliffe (1972) and Vazquez (1974)]. In other experiments (Fig. 5*b*) cycloheximide did not inhibit the puromycin reaction after the prior addition of alpha sarcin to reticulocyte lysates. This result is consistent with an action of cycloheximide on translocation reactions, which, of course, cannot occur when peptidyl-tRNA is confined to the ribosomal 'P' site. The first report that cycloheximide inhibits translocation (McKeehan & Hardesty, 1969) has been followed by various conflicting suggestions, that the drug inhibits either this reaction or the peptidyltransferase [for reviews see Cundliffe (1972) and Vazquez (1974)]. Our results are consistent with the more recent general consensus that cycloheximide inhibits translocation. While the present work was in progress we learned that alpha sarcin prevents the binding of EF-1 but not EF-2 to eukaryotic ribosomes (R. D. Nolan, D. Schindler & J. E. Davies, unpublished work), in agreement with the findings reported here.

We thank the Medical Research Council for a Research Studentship (to A. N. H.) and for a Research Project Grant (to E. C.), and also the Wellcome Trust for a grant for research expenses (to E. C.). We are particularly grateful to Dr. Birger H. Olson for a gift of alpha sarcin.

References

Baglioni, C. (1966) *Biochim. Biophys. Acta* **129**, 642–645
Baliga, B. S., Cohen, S. A. & Munro, H. N. (1970) *FEBS Lett.* **8**, 249–252
Cundliffe, E. (1971) *Biochem. Biophys. Res. Commun.* **44**, 912–917
Cundliffe, E. (1972) in *The Molecular Basis of Antibiotic Action* (Gale, E. F., Cundliffe, E., Reynolds, P. E., Richmond, M. H. & Waring, M. J., eds.), pp. 278–379, John Wiley and Sons, London
Cundliffe, E. & McQuillen, K. (1967) *J. Mol. Biol.* **30**, 137–146
Darnbrough, C., Legon, S., Hunt, T. & Jackson, R. J. (1973) *J. Mol. Biol.* **76**, 379–403
Goldberg, I. H. & Mitsugi, K. (1967) *Biochemistry* **6**, 383–391
Hunt, T., Vanderhoff, G. A. & London, I. M. (1972) *J. Mol. Biol.* **66**, 471–481
McKeehan, W. & Hardesty, B. (1969) *Biochem. Biophys. Res. Commun.* **36**, 625–630
Obrig, T., Irvin, J., Culp, W. & Hardesty, B. (1971) *Eur. J. Biochem.* **21**, 31–41
Olson, B. H. (1963) *U.S. Patent* 3 104 204
Olson, B. H. & Goerner, G. L. (1965) *Appl. Microbiol.* **13**, 314–321
Olson, B. H., Jennings, J. C., Roga, V., Junek, A. J. & Schuurmans, D. M. (1965) *Appl. Microbiol.* **13**, 322–326
Schuurmans, D. M., Duncan, D. T. & Olson, B. H. (1964) *Cancer Res.* **24**, 83–89
Traut, R. R. & Munro, R. E. (1964) *J. Mol. Biol.* **10**, 63–72
Vazquez, D. (1974) *FEBS Lett.* **40**, *Suppl.* 563–584