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THE EFFECT OF ETHANOL ON POLYPEPTIDE CHAIN INITIATION IN RETICULOCYTE LYSATES

INHIBITION OF RECYCLING OF INITIATION FACTOR eIF-2

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Abstract—Using the reticulocyte cell-free system, we have investigated the mechanism by which ethanol inhibits the initiation of protein synthesis. Ethanol inhibited the formation of 40S-initiation complexes, and this effect correlated well with the inhibition by ethanol of overall peptide-chain initiation. Ethanol was a more potent inhibitor of translation at 37° than at 30°. The inhibition of peptide-chain initiation and 40S-initiation complex formation in reticulocyte lysates under other conditions is associated with increased phosphorylation of the α-subunit of protein synthesis initiation factor-2 (eIF-2α)† and the inhibition of recycling of this factor. Recycling of eIF-2 is mediated by another protein factor GEF (= guanine nucleotide-exchange factor). The addition of ethanol to reticulocyte lysates led to increased phosphorylation of eIF-2α and to a decrease in the rate of exchange of guanine nucleotides bound to eIF-2. This second finding indicated that recycling of eIF-2 was impaired probably due to decreased availability of GEF. Using purified components it was found that ethanol inhibited the ability of GEF to stimulate eIF-2 and that this inhibition showed a similar temperature dependence to the effect of ethanol on overall protein synthesis. Taken together, these results suggest that ethanol leads to inhibition of peptide-chain initiation both through increased phosphorylation of eIF-2α and by directly inhibiting the productive interaction of eIF-2 and GEF.

Ethanol inhibits protein synthesis in a variety of types of cells. In rodents ethanol ingestion inhibits brain protein synthesis [1]. Ethanol also inhibits protein synthesis in perfused liver, liver slices and hepatocytes [2–4]. Physiologically-achievable concentrations of ethanol inhibit protein synthesis in human bone-marrow and reticulocytes [5].

Several workers have studied the effect of ethanol on protein synthesis at the molecular level. In reticulocytes [6] and in isolated hepatocytes [7] ethanol caused disaggregation of polysomes indicating selective impairment of peptide-chain initiation. Wu [8] demonstrated that the ethanol-induced inhibition of translation in the reticulocyte cell-free system could be overcome by the addition of purified initiation factor-2 (eIF-2).† Changes in the activity of eIF-2 are involved in the regulation of protein synthesis under a variety of conditions in reticulocytes (reviewed in Refs 9–11).

The function of eIF-2 is to mediate the binding of the initiator tRNA, Met-tRNA$_i$, to the 40S-ribosomal subunit as a ternary complex which also contains GTP (i.e. [eIF-2·GTP·Met-tRNA$_i$]). The overall activity of eIF-2 can be regulated by changes in the phosphorylation state of its smallest subunit (eIF-2α [9–11]). For example, absence of haem in this system leads to the activation of a protein kinase specific for eIF-2α and hence to an increase in its level of phosphorylation. Phosphorylated eIF-2 binds tightly to the recycling factor GEF (= guanine nucleotide-exchange factor) and renders it unavailable for the recycling process. Since GEF is required to regenerate active [eIF-2·GTP] complexes after each round of initiation, the overall consequence of increased phosphorylation of eIF-2 is inhibition of peptide-chain initiation, and in particular of the recycling of eIF-2 and the binding of Met-tRNA$_i$ to 40S-subunits [12–15]. The effects of ethanol on protein synthesis in reticulocyte lysates have been re-investigated in the light of these recent advances in our understanding of the regulation of protein synthesis.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine (1000 Ci mmol^{-1}), [L-[4,5]-^{3}H]leucine (150 Ci mmol^{-1}) [8-^{3}H]GDP (15 Ci mmol^{-1}) and [γ-^{32}P]ATP (5000 Ci mmol^{-1}) were from Amersham International (U.K.). Calf liver tRNA was from Boehringer (Lewes, U.K.). All other chemicals and biochemicals were obtained as described in [16]. [^{35}S]Met-tRNA$_i$ and reticulocyte lysates were prepared as described by Clemens *et al.* [17]. Rat liver eIF-2 (>90% pure as judged by SDS–polyacrylamide gel electrophoresis) was isolated essentially as in [18]. The isolation procedure for rat liver GEF is described in [19]. The haem-controlled repressor (HCR, an eIF-2α kinase) was generously provided by Richard Jackson (Department of Biochemistry, University of Cambridge, U.K.).

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† Abbreviations used: eIF-2, cukaryotic initiation factor-2; eIF-2α, its α-subunit; GEF, guanine nucleotide exchange factor; HCR, haem-controlled repressor, a protein kinase specific for eIF-2α; TEA, triethanolamine.

Methods. Protein synthesis in the reticulocyte lysate cell-free system was assayed as described previously [20] but using 100 μl incubation volumes and taking 15 μl samples at the time-points indicated. The polyribosome profiles of reticulocyte lysate incubations were analysed on 15–30% (w/v, linear) sucrose gradients (in 20 mM TEA–HCl, pH 7.8; 5 mM $MgCl_2$; 0.25 M KCl) which were centrifuged at 150,000 *g* for 75 min in a Beckman SW50.1 rotor. Formation of [^{35}S]Met-tRNA$_i$-containing 40S-initiation complexes was assayed as follows. The incubations were identical to those for protein synthesis [20] but labelled leucine was omitted. After 15 min 10 μl (10^5 dpm) of [^{35}S]Met-tRNA$_i$ was added. Thirty seconds later the reactions were stopped by dilution with three volumes of ice-cold 20 mM TEA–HCl, pH 7.5 containing 2% (v/v) formaldehyde. Samples were applied to 20–50% (w/v) linear sucrose density gradients (in 0.1 mM sodium cacodylate pH 6.6; 5 mM $MgCl_2$; 0.1 M KCl [21]) and centrifuged in a Beckman SW50.1 rotor at 150,000 *g* for 4 hr. Fractions were collected and counted as described [20]. Post-ribosomal supernatants were prepared from reticulocyte lysates as described in [20].

The formation of ternary ({eIF-2·GTP·[^{35}S]Met-tRNA$_i$}) complexes was measured by their retention on nitrocellulose filters [20]. Exchange of [^{3}H]GDP for unlabelled GDP was followed by the method of Matts and London [22]. The phosphorylation of eIF-2 in reticulocyte lysates *in situ* was examined as described in [23] with the modifications given in the figure legend.

RESULTS AND DISCUSSION

In all the reticulocyte lysate preparations tested, 0.84 M ethanol completely inhibited the incorporation of amino acids under standard conditions (i.e. at 31°), after an initial lag period of 5–10 min during which protein synthesis proceeded at the control rate. These findings are in agreement with the data of Wu [8]. Half-maximal inhibition (at 30 min of incubation) was obtained with 0.42 M ethanol at this temperature, although at 37° ethanol inhibited more potently and 50% inhibition was obtained with 0.21 M ethanol. This concentration of ethanol inhibited only weakly at 31° (not shown).

The kinetics of the inhibition induced by ethanol (i.e. the initial lag period) suggested that peptide-chain initiation rather than elongation or termination was being inhibited. This interpretation was supported by the finding that in lysates incubated with ethanol polysomes were disaggregated relative to control samples (not shown). Disaggregation of polysomes was prevented by the addition of an inhibitor of elongation (cycloheximide) showing that it was not occurring as a result of activating a nuclease. Disaggregation of polysomes is also observed after ethanol treatment of intact reticulocytes [6], liver [2] or isolated hepatocytes [7].

The absence of haem is another condition which leads to impairment of initiation in reticulocyte lysates, and this system has been studied in detail (see Refs 9–11). The formation of 40S-initiation complexes containing Met-tRNA$_i$, which is mediated by initiation factor eIF-2, is an early stage in the initiation process and is inhibited in the absence of haem. The effect of ethanol on the formation of these complexes was therefore examined. Ethanol at 0.42 M inhibited the formation of 40S-initiation complexes by 60% and overall protein synthesis by 50% (Fig. 1). An ethanol concentration which inhibited protein synthesis maximally also gave almost complete inhibition of 40S-initiation complex formation. The inhibition by ethanol of 40S-initiation complex formation therefore correlated well with its overall effects on translation. Ethanol has also been found to inhibit the formation of 40S-initiation complexes in isolated hepatocytes [7].

The effect of haem-deprivation on peptide-chain initiation is exerted through increased phosphorylation of an α-subunit of eIF-2 due to activation of the haem-controlled eIF-2α kinase, HCR. Phosphorylation of eIF-2 results in an impairment of its recycling, i.e. of the exchange of bound GDP for GTP which regenerates active eIF-2 and for which GEF is required at physiological Mg^{2+}-concentrations [11–15, 24–27]. The degree of inhibition of eIF-2 activity is greater at higher Mg^{2+}-concentrations [13, 24]. As previously reported [20], haem-deficiency causes a fall in the rate and final extent of formation of ternary [eIF-2·GTP·Met-tRNA$_i$] complexes measured in dialysed post-ribosomal supernatants from pre-treated lysates. This effect is more marked when assays are performed at higher Mg^{2+}-concentrations (2.5 vs 0.25 mM, Fig. 2). To test whether ethanol had a similar effect on eIF-2 activity, formation of ternary complexes was assayed at two Mg^{2+}-concentrations in dialysed post-ribosomal supernatants from control or ethanol-treated lysates. The results obtained were similar to those

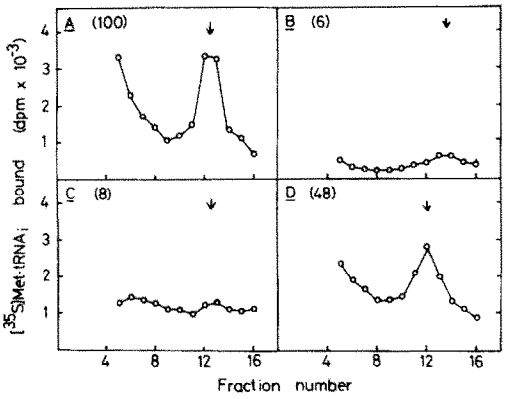

Fig. 1. Formation of 40S-initiation complexes containing [^{35}S]Met-tRNA$_i$ in reticulocyte lysates. Incubations were for 15 min and were pulsed with [^{35}S]Met-tRNA$_i$ (1×10^6 dpm) for 30 sec immediately prior to fixing with formaldehyde and density gradient analysis. Incubations contained 20 μM haemin and the following additions—panel A: no further addition; panel B: 2 U of HCR; panel C: 0.84 M ethanol; panel D: 0.42 M ethanol. Figures in parentheses show the relative rate of protein synthesis in parallel incubations containing [^{3}H]leucine (control—+ haemin = 100). The arrow indicates the position of the 40S-subunit peak in each gradient. (One U of HCR is defined as inhibiting a standard reticulocyte lysate incubation of 100 μl by 75% after 30 min.)

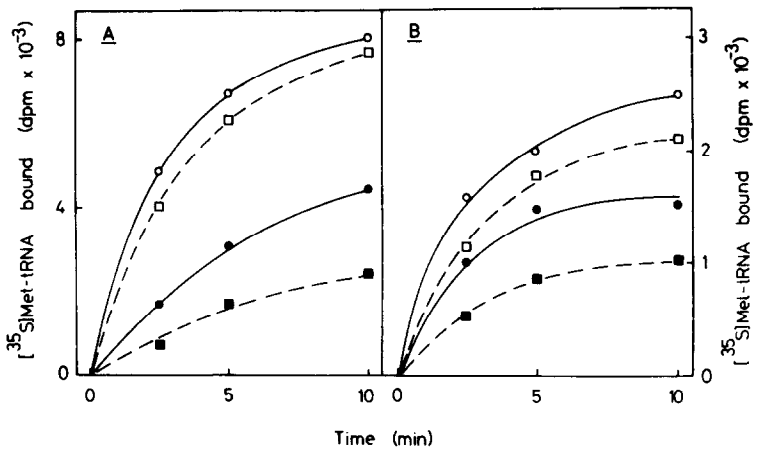

Fig. 2. Formation of ternary complexes containing [^{35}S]Met-tRNA$_i$ in post-ribosomal supernatants derived from reticulocyte lysates subjected to differing treatments. All lysates were incubated at 31° for 15 min. Panel A: samples from control (+ 20 μM haemin) (○, ●) or haem-deprived (□, ■) lysates were assayed for their ability to form ternary complexes in incubation mixtures containing 0.25 mM (open symbols) or 2.5 mM $MgCl_2$ (closed symbols). Panel B: as panel A but samples were derived from control (○, ●) or ethanol-treated (0.73 M) (□, ■) lysate incubations.

observed for haem-deficiency, i.e. decreases in both the rate and final plateau level of formation of ternary complexes with the differences being more accentuated at the higher Mg^{2+}-concentration (Fig. 2). Since the samples had been dialysed, this is presumably not a direct effect of ethanol but rather reflects a stable change in eIF-2. The effect of ethanol on the level of phosphorylation of eIF-2α was therefore examined more directly.

Reticulocyte lysate samples were incubated with

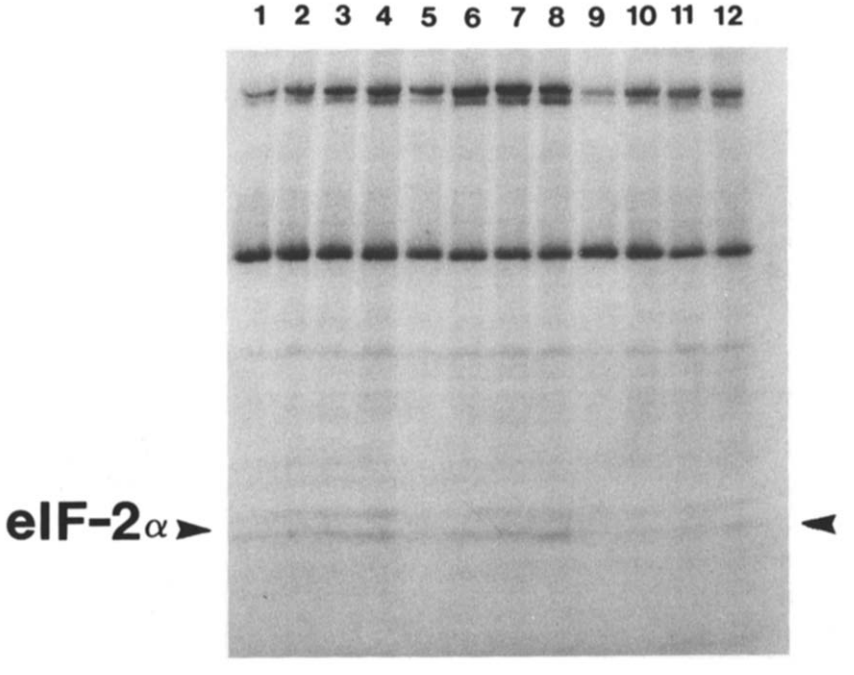

Fig. 3. Phosphorylation of eIF-2 in reticulocyte lysates. Standard lysate incubations (volume 60 μl, but containing only 20 nM added ATP) were carried out in the presence of 20 μM haemin plus 2 U HCR (tracks 1–4), 20 μM haemin plus 0.73 M ethanol (tracks 5–8), or 20 μM haemin alone (tracks 9–12). After 15 min at 31°, [γ-^{32}P]ATP (10 μCi) was added and the incubations were continued. Samples of 18 μl were removed at 1.5 (tracks 1, 5, 9), 3 (tracks 2, 6, 10), 4.5 (tracks 3, 7, 11) and 6 (tracks 4, 8, 12) minutes after addition of the labelled ATP, and immediately denatured in SDS-sample buffer for 5 min at 95°. Samples were analysed by SDS–polyacrylamide gel electrophoresis. The figure shows an autoradiograph of the dried gel.

or without ethanol, or with the addition of purified HCR, in the presence of [γ-^{32}P]ATP and the phosphorylated proteins were analysed by SDS–polyacrylamide gel electrophoresis and autoradiography. Both in the HCR- and the ethanol-treated lysates the level of radioactivity associated with eIF-2α was increased relative to the control samples (Fig. 3). The apparent rate of phosphorylation was faster in the HCR-treated samples than in the presence of ethanol, but the final level of labelling (at 6 min) was similar. This finding implies that ethanol inhibits initiation, at least in part, by increasing the level of phosphorylation of eIF-2α. This would be expected to have the effects on ternary complex formation and binding of Met-tRNA$_i$ to 40S-subunits which were described above. This conclusion also explains the observation of Wu [9], that the effect of ethanol on peptide-chain initiation is overcome by addition of purified eIF-2—a similar result is obtained for haem-deficient lysates [28–32].

Increased phosphorylation of eIF-2α is also expected to inhibit the rate of exchange of guanine nucleotides bound to eIF-2 since phosphorylation of eIF-2α decreases the availability of GEF. To test this the method of Matts and London [22] was used. Here, preformed {eIF-2·[^{3}H]GDP} complexes are added to reticulocyte lysate incubations and the time-dependent displacement of bound [^{3}H]GDP is followed. In the presence of 0.84 M ethanol, this process was completely blocked (Fig. 4) and a similar degree of inhibition of exchange was observed in lysates pre-treated with amounts of purified HCR giving maximal inhibition of translation (Fig. 4 and Ref. 22).

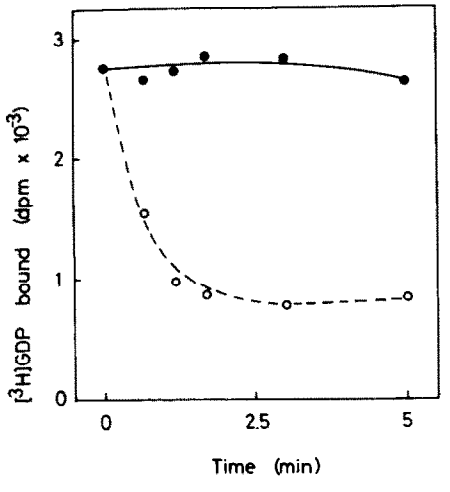

Fig. 4. Exchange of bound [^{3}H]GDP in reticulocyte lysates. ([^{3}H]GDP·eIF-2) was preformed by incubation for 10 min at 30° of a reaction mixture containing 20 mM Tris–HCl, pH 7.6, 100 mM KCl, 100 μg ml^{-1} creatine kinase, 2 μM [^{3}H]GDP (7000 dpm pmol^{-1}) and eIF-2 (about 5 pmol). The mixture was then made 1 mM in Mg(OAc)$_2$ and stored on ice until required [22]. Lysate incubations were carried out under standard conditions. After 15 min they were diluted two-fold with buffer (40 mM Tris–HCl, pH 7.6, 100 mM KCl, 50 mM NaF, 2 mM Mg(OAc)$_2$, 10% (v/v) glycerol, 40 μM cold GDP) and 10 μl of ([^{3}H]GDP·eIF-2]) (approx. 2 pmol) was then added immediately. Samples (30 μl) were taken at the times indicated and filtered immediately through cellulose nitrate filters for assessment of bound [^{3}H]GDP. The figure shows the results obtained with lysates incubated under control conditions (i.e. plus 20 μM haemin, ○) and with ethanol (0.84 M plus 20 μM haemin, ●).

The degree of inhibition of lysate protein synthesis by ethanol was not affected by the addition of 4-methyl pyrazole at concentrations which completely inhibit alcohol dehydrogenase (i.e. 0.5 mM), suggesting that ethanol metabolism is not required for its effects, and that it presumably acts directly on components of the translational machinery.

Using purified components, the direct effect of ethanol on eIF-2 and GEF was examined. Even at concentrations which completely inhibited protein synthesis, ethanol had little effect on eIF-2 activity as measured by formation of ternary complexes. In contrast, similar concentrations of ethanol strongly inhibited the stimulation of eIF-2 by added, purified GEF (Fig. 5). Ethanol therefore has a direct effect on these factors, and in particular on the eIF-2/GEF interaction which is important for recycling of eIF-2, in addition to its effect in increasing the level of phosphorylation of eIF-2α. Separate experiments (not shown) demonstrated that ethanol does not irreversibly inactivate GEF. The inhibition of ternary complex formation reported by Wu [9] using partially purified eIF-2 may reflect the presence of GEF in his eIF-2 preparation; these two proteins tend to co-purify [12, 13, 16, 23]. Ethanol inhibited the GEF-stimulation of eIF-2 more potently at 37° than at 31°, in line with its effects on overall protein synthesis (Fig. 6). Wu [8] has reported that ethanol activates an HCR-like translational inhibitor in reticulocyte lysates. In this work, ethanol did activate a translational inhibitor present in post-ribosomal supernatants treated with phosphocellulose to remove eIF-2(αP) which would sequester GEF. However, incubation at 37° in the presence of ethanol did not induce further activation of HCR.

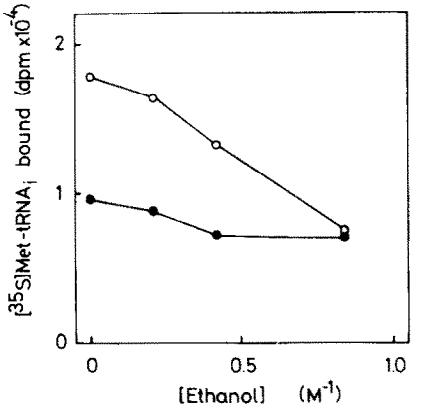

Fig. 5. Formation of ternary complexes by purified eIF-2. Homogeneous rat liver eIF-2 was assayed for its ability to form ternary complexes (binding of [^{35}S]Met-tRNA$_i$) at a range of ethanol concentrations in the presence (○) or absence (●) of added, purified GEF (1.5 μg per 50 μl incubation). Incubations were for 10 min.

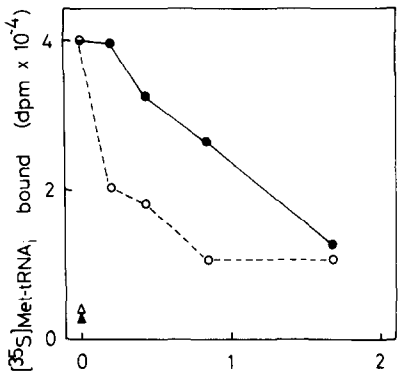

Fig. 6. Effect of ethanol on GEF-stimulated ternary complex formation. Formation of ternary complexes by purified eIF-2 (0.5 μg) in the presence of partially purified GEF (2.5 μg) was assayed over a range of ethanol concentrations at 31° (●) or 37° (○). Triangles show the activity of eIF-2 in the absence of added GEF, at 31° (▲) or at 37° (△). Assays were for 10 min in a final volume of 50 μl.

CONCLUSIONS

The inhibition of ethanol of peptide-chain initiation in reticulocyte lysates appears to result from a combination of two effects:

(i) increased phosphorylation of eIF-2α, similar to that observed in the absence of haem;

(ii) a direct effect on the interaction of eIF-2 and GEF, and hence on recycling of eIF-2.

Each of these effects could contribute to the inhibition by ethanol of guanine-nucleotide exchange and of the formation of ternary and 40S-initiation complexes. Indeed the two effects may be related since Crouch *et al.* [33] have shown that the interaction of eIF-2 with GEF renders eIF-2α less susceptible to phosphorylation by HCR. In particular, the increased sensitivity of the reticulocyte lysate system to ethanol at 37° compared to 31° would appear to be due to its effect on eIF-2/GEF rather than to activation of HCR. Gross and Redman [34] have recently shown that a specific antiserum against HCR only partially reverses the inhibition of protein synthesis induced by ethanol in reticulocyte lysates, although it completely blocks the inhibition due to haem-deficiency. This is in accord with the idea that only part of the effect of ethanol is mediated directly through activation of HCR.

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REFERENCES

1. S. Tewan and E. P. Noble, in *Alcohol and Abnormal Protein Synthesis* (Eds. M. A. Rothchild, M. Oratz and S. S. Schreiber), pp. 421–448. Pergamon Press, New York (1975).
2. M. A. Rothchild, M. Oratz, T. Morland, S. S. Schreiber, A. Burks and B. Martin, *Pharmacol. Biochem. Behav.* **13**, Suppl. 1, 31.
3. T. Morland and A. Bessessen, *Biochim. biophys. Acta* **474**, 312 (1977).
4. A. Perin, G. Scalobrino, A. Sessa and A. Amaboldi, *Biochim. biophys. Acta* **366**, 101 (1974).
5. M. A. M. Ali and M. C. Brain, *Br. J. Haematol.* **28**, 311 (1974).
6. M. L. Freedman, M. S. Cohen, V. Rasman and F. Y. Forte, *Br. J. Haematol.* **30**, 354 (1975).
7. J. Harbitz, B. Wallis, J. G. Hauge and J. Morland, *Biochem. Pharmac.* **33**, 3465 (1984).
8. J. M. Wu, *J. biol. Chem.* **356**, 4164 (1981).
9. S. Ochoa, *Archs Biochem. Biophys.* **223**, 345 (1983).
10. C. G. Proud, *Trends Biochem. Sci.* **11**, 73 (1986).
11. V. M. Pain, *Biochem. J.* **235**, 625 (1986).
12. A. Konieczny and B. Safer, *J. biol. Chem.* **258**, 3402 (1983).
13. R. L. Matts, D. H. Levin and I. M. London, *Proc. natn. Acad. Sci. U.S.A.* **80**, 2559 (1983).
14. J. Siekierka, V. Manne and S. Ochoa, *Proc. natn. Acad. Sci. U.S.A.* **81**, 352 (1984).
15. M. Salimans, H. Goumans, H. Amesz, R. Benne and H. O. Voorma, *Eur. J. Biochem.* **145**, 91 (1984).
16. C. G. Proud and V. M. Pain, *FEBS Lett.* **143**, 55 (1982).
17. M. J. Clemens, E. C. Henshaw, H. Rahamimoff and I. M. London, *Proc. natn. Acad. Sci. U.S.A.* **71**, 2946 (1974).
18. D. R. Colthurst and C. G. Proud, *Biochim. biophys. Acta* **868**, 77 (1986).
19. C. G. Proud, *Biochim. biophys. Acta* **914**, 64 (1987).
20. C. G. Proud, M. J. Clemens and V. M. Pain, *FEBS Lett.* **148**, 214 (1982).
21. R. G. Crystal, N. A. Elson and W. F. Anderson, *Meth. Enzymol.* **30**, 101 (1974).
22. R. L. Matts and I. M. London, *J. biol. Chem.* **259**, 6708 (1984).
23. V. Ernst, D. H. Levin and I. M. London, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2118 (1979).
24. R. Panniers and E. C. Henshaw, *J. biol. Chem.* **258**, 7928 (1983).
25. R. S. Ranu and I. M. London, *Proc. natn. Acad. Sci. U.S.A.* **76**, 1079 (1979).
26. J. Siekierka, V. Manne, L. Mauser and S. Ochoa, *Proc. natn. Acad. Sci. U.S.A.* **80**, 1232 (1983).
27. B. Safer, R. Jagus, A. Konieczny and D. Crouch, in *Interaction of Translational and Transcriptional Controls in the Regulation of Gene Expression* (Eds. M. Grunberg-Manago and B. Safer), pp. 311–325. Elsevier-Biomedical, New York (1983).
28. R. Kaempfer, *Biochem. Biophys. Res. Commun.* **61**, 591 (1974).
29. M. J. Clemens, *Eur. J. Biochem.* **66**, 413 (1976).
30. P. J. Farrell, T. Hunt and R. J. Jackson, *Eur. J. Biochem.* **89**, 517 (1978).
31. B. Safer, D. Peterson and W. C. Merrick, in *Translation of Synthetic and Natural Polynucleotides* (Ed. A. B. Legocki), pp. 24–31. University of Poznan (1977).
32. J. Siekierka, K-I. Mitsui and S. Ochoa, *Proc. natn. Acad. Sci. U.S.A.* **78**, 220 (1981).
33. D. Crouch and B. Safer, *J. biol. Chem.* **259**, 10363 (1984).
34. M. Gross and R. Redman, *Biochim. biophys. Acta* **908**, 123 (1987).