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# Phosphorylation and the Control of Protein Synthesis

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# Phosphorylation and the control of protein synthesis

By T. Hunt

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This paper reviews the evidence that protein synthesis in rabbit reticulocytes is regulated by the reversible phosphorylation of the initiation factor eIF-2 by protein kinases under the control of the cytoplasmic haemin concentration on the one hand, and double-stranded RNA on the other. A molecular mechanism is proposed to account for the observation that inhibition of protein synthesis occurs when considerably less than half the eIF-2 present has been phosphorylated. The question of whether phosphorylation regulates protein synthesis in other types of cell is discussed.

## The control of protein synthesis in reticulocytes

### *Haemin*

The reticulocyte is a highly specialized cell in a state of transition. It is in the last stages of becoming an erythrocyte, a cell that lacks not only a nucleus but virtually every other normal intracellular feature apart from an extremely high protein content. About 90% of the protein synthesis in the reticulocyte is devoted to globin synthesis, and this synthesis is highly coordinated with that of the prosthetic group haemin. If the supply of haemin fails, the synthesis of globin stops. When the supply resumes, so does the synthesis of globin. The analysis of the mechanism responsible for this coordination is made possible by the fact that the cell-free system prepared from rabbit reticulocytes maintains high activity, entirely comparable with the activity in intact cells, and shows the same regulatory phenomena as the intact cells, including the dependence of globin synthesis on the concentration of free haemin.

Studies of the cell-free system have revealed that besides haemin there are a number of other regulatory influences on protein synthesis, some of which have apparent physiological significance, though others have less obvious value to the cell. Three of these are listed below.

### *Double-stranded RNA*

Low levels (10–100 ng $ml^{-1}$) of accurately base-paired double-stranded RNA (dsRNA) inhibit protein synthesis; paradoxically, levels above 1 μg $ml^{-1}$ not only do not inhibit, but can reverse an established inhibition by low levels of dsRNA. The reversal of inhibition by high levels of dsRNA is specific for inhibition caused by low levels of dsRNA; however, dsRNA cannot stimulate protein synthesis that has been inhibited by any other cause (Hunter *et al.* 1975).

### *Intracellular oxidation*

Addition of oxidized glutathione (Kosower *et al.* 1971) or a number of similar oxidized thiol reagents, or removal of glucose, or indeed any condition that directly or indirectly depletes the level of sugar phosphates in the lysate causes inhibition of protein synthesis (Ernst *et al.* 1978). Connected with this mode of inhibition, depletion of glucose-6-phosphate dehydrogenase, or thioredoxin reductase, or thioredoxin, or NADPH, in short, any condition leading to failure of

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the thioredoxin-dependent reducing system, leads to inhibition of protein synthesis (Jackson *et al.* 1983).

### *Non-specific stress*

A variety of mild physical stresses, of which high hydrostatic pressure (Henderson & Hardesty 1978) and somewhat elevated temperature (Bonanou-Tzedaki 1978) are the canonical examples, cause an inhibition of protein synthesis that shares characteristics with the other conditions listed above.

### *Characteristics of the inhibition*

The characteristics shared by all these inhibitory conditions are as follows (reviewed by Hunt (1979), Jackson (1982), Jagus *et al.* (1982), Kramer & Hardesty (1980), London *et al.* (1981) and Ochoa & de Haro (1979)):

(1) the inhibition of protein synthesis is global: it is not confined to the α and β chains of globin;

(2) when protein synthesis is inhibited, it slows *abruptly* from its initial rate to a new rate that is usually 5–10% of the starting value;

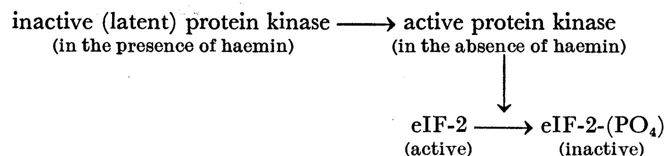
(3) inhibition specifically and only affects initiation of protein synthesis, at the level of formation of 40*S*–Met-$tRNA_f$ complexes;

(4) inhibition can be overcome by the addition of high levels of purine or purine derivatives, by addition of high levels of Mg-GTP, and by addition of various protein 'factors', which will be discussed further below;

(5) inhibition is accompanied by an increase in the level of phosphorylation of the α subunit of eIF-2, the initiation factor that catalyses the binding of initiator tRNA to the 40*S* ribosomal subunits;

(6) in the inhibition due to lack of haemin, the presence of dsRNA, high pressure or high temperature, but *not* in sugar phosphate deficiency or thioredoxin system failure, it is possible to detect and to purify an inhibitor of protein synthesis that has protein kinase activity directed against the α subunit of eIF-2.

These basic observations are widely interpreted in terms of scheme 1.




SCHEME 1

This simple scheme, although consistent with the observations, and expressive of the basic control mechanism, does not reveal the true complexity of the situation. For one thing, all of the inhibitory conditions are reversible, and mechanisms exist for dephosphorylating eIF-2 and for deactivating the inhibitory protein kinases. The second problem is to account in precise terms for the inactivity of phosphorylated eIF-2. The idea that phosphorylated eIF-2 is inactive in catalysing the binding of Met-$tRNA_f$ to 40*S* subunits is too simple, in fact incorrect. This is the crux of the matter, and will be discussed next.

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## Is phosphorylation of eIF-2 responsible for the inhibition of protein synthesis?

Almost as soon as it had been discovered that phosphorylation of eIF-2 occurred during inhibition by a lack of haemin or the presence of dsRNA, and that the inhibitor of protein synthesis that accumulated in lysates when they were incubated in the absence of haemin was a protein kinase specific for eIF-2, it also became apparent that by all available tests the phosphorylated eIF-2 was still active (Farrell *et al.* 1977). The factor not only bound GTP or GDP, Met-$tRNA_f$ and 40*S* ribosomal subunits, but it could also catalyse protein synthesis in an eIF-2-deficient protein synthesis system whether or not it had been phosphorylated (Safer *et al.* 1977; Trachsel & Staehelin 1978; Benne *et al.* 1979). The rates and affinities of these various binding reactions appeared to be unaltered by phosphorylation. It therefore seemed that if phosphorylation did have an effect on the activity of eIF-2 it must be a subtle one, which the various tests did not reveal. Doubts were even voiced as to whether phosphorylation of eIF-2 α subunit truly accounted for the inhibition of initiation (Jagus & Safer 1981 *a*, *b*).

The nature of the defect in phosphorylated eIF-2 is still not completely clear, but recent results from several laboratories seem to confirm earlier suggestions that phosphorylation of eIF-2 allows it to catalyse one round of initiation, but that the modified factor then seizes up with GDP bound to it (Siekierka *et* al. 1982; Safer *et al.* 1982; Clemens *et al.* 1982; Voorma & Amesz 1982; Gupta *et al.* 1982). It has been known for many years that the affinity of eIF-2 for GDP is about 100 times its affinity for GTP (Walton & Gill 1975), and that the nucleotide-binding subunit is the one that is phosphorylated by eIF-2 kinase (Barrieux & Rosenfeld 1977). The analogies between the behaviour of eIF-2 with respect to GTP and GDP and the bacterial elongation factor T suggested that a 'recycling factor' might exist whose function was to allow the displacement of the GDP retained on eIF-2 after one cycle with GTP in preparation for the next cycle. Phosphorylation would block the action of this hypothetical factor.

## The recycling factor, guanine nucleotides, and the phosphorylation of eIF-2

The factor known variously as ESP and, latterly, SP by Ochoa's group (Siekierka *et al.* 1982) and RF by others (Amesz *et al.* 1979; Gupta 1982) has the properties expected of such a recycling factor. It was first isolated as a protein that could rescue protein synthesis in reticulocytes from the inhibitory effects of a lack of haemin or dsRNA (though it should be cautioned that there may be more than one such factor; at least one rescue factor is probably an anti-kinase (Gross *et al.* 1981)). It has been convincingly shown that RF does not interfere with the phosphorylation status of eIF-2, and that it does nothing to alleviate a serious inhibition when a very high dose of kinase is present. Figure 1 shows that RF simply makes eIF-2 kinase approximately one quarter as effective as an inhibitor of protein synthesis, without affecting its kinase activity. This paradoxical result helps to resolve another puzzle, which is that protein synthesis can be completely inhibited when only a small fraction of the total eIF-2 present in the lysate is phosphorylated. For if the simple idea that phosphorylation of eIF-2 inactivated it, there ought to be a linear relation between the degree of phosphorylation and the degree of inhibition of protein synthesis. There is not. How can protein synthesis stop when 80% of the eIF-2 appears to be perfectly normal and functions perfectly normally in partial *in vitro* reactions? The answer

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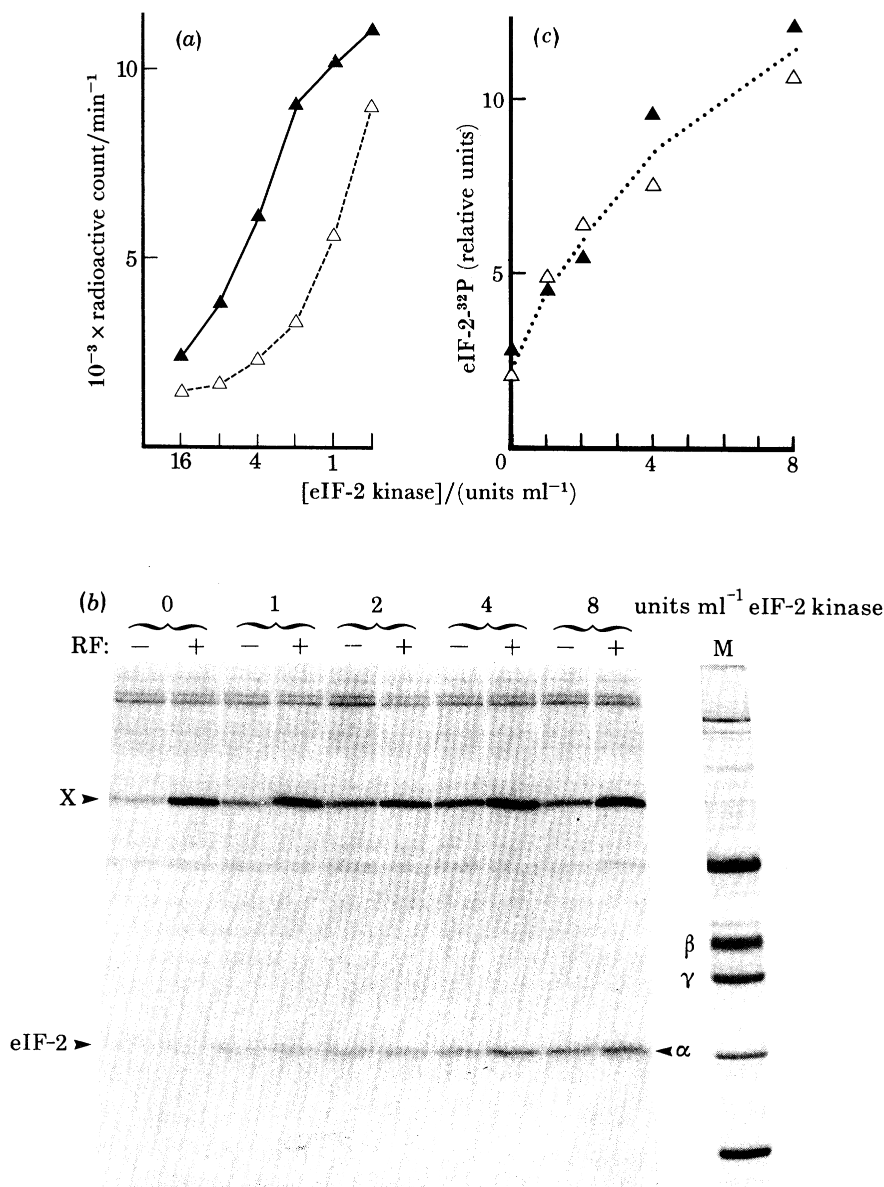


Figure 1. RF does not affect the phosphorylation of eIF-2 although it rescues protein synthesis from the effects of added eIF-2 kinase.

(*a*) A standard reticulocyte lysate incubation according to Jackson & Hunt (1983) was incubated in the presence of increasing concentrations of purified eIF-2 kinase (HCR), and the incorporation of L-[$^{14}$C]valine measured after 40 min at 30 °C (△). ▲, The incorporation found when purified RF (10 μg ml$^{-1}$) was also present.

(*b*) In a parallel set of tubes to (*a*), portions of the same incubations were incubated for 2 min in the presence of [γ$^{32}$P]ATP (2 mCi ml$^{-1}$) before stopping the reaction in SDS–polyacrylamide gel sample buffer. The samples were analysed on a 15% SDS–polyacrylamide gel which was dried and radioautographed as shown here. The right-most track (M, marker track) shows the position of the polypeptides of eIF-2. Band X is a protein whose phosphorylation appears to be stimulated by 2-mercaptoethanol. Its significance is unclear. The radioautograph was scanned in a densitometer to yield the data plotted in (*c*), which confirms that addition of RF (▲) does not alter the level of phosphorylation of eIF-2, even though it affords almost complete protection against the inhibitory effects of eIF-2 kinase (HCR) at 2 units ml$^{-1}$. △, RF absent from incubation. Purified RF was the generous gift of John Siekierka.

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is almost certainly that phosphorylated eIF-2 forms some kind of non-productive complex with the recycling factor, RF, such that the rest of the eIF-2, although apparently normal, becomes clogged up with GDP. Addition of extra RF alleviates the situation by allowing the non-phosphorylated eIF-2 to function and, only when a much higher dose of kinase is added does this stimulant of protein synthesis fail. A strong prediction of this model is that whereas normally one would find eIF-2 complexed with GTP, in inhibited lysates it would be complexed with GDP. Further, on isolating the eIF-2/RF complexes from a partly inhibited lysate, one ought to find an excess of phosphorylated eIF-2 compared with the free pool. These predictions remain to be tested.

The existing data concerning RF are all compatible with this view, but to my mind fall short of confirming it beyond doubt. The important points are as follows:

(1) RF is isolated as a complex with eIF-2 (Amesz *et al.* 1979; Safer *et al.* 1982; Siekierka *et al.* 1982);

(2) RF promotes the exchange of guanine nucleotides with eIF-2 (Clemens *et al.* 1982);

(3) guanine nucleotides promote the exchange of eIF-2 with RF (Safer *et al.* 1982);

(4) phosphorylation of eIF-2 inhibits the exchange of guanine nucleotides promoted by RF (Clemens *et al.* 1982; Siekierka *et al.* 1982);

(5) there is about 4–5 times as much eIF-2 as RF on a molar basis in reticulocytes lysates (Amesz *et al.* 1979; Safer *et al.* 1982).

This model explains almost all of the previously puzzling difficulties. It has some interesting features that have not as far as I know been found in other systems regulated by reversible phosphorylation. This system shows no regulation of eIF-2 phosphatase(s) whatsoever, and the activity responsible for dephosphorylating eIF-2 is, moreover, extremely vigorous in the unfractionated lysate (Safer & Jagus 1979; Jackson *et al.* 1983). Thus it would be impossible for the eIF-2 pool ever to become fully phosphorylated, and the actual control range of the system will exhibit extremely nonlinear properties, set in part by the activity of the kinase and in part by the relative levels of RF and eIF-2. This probably accounts for the suddenness of the shut-off in protein synthesis in one of the inhibitory states mentioned at the beginning of this paper Interestingly, the shut-off in intact cells appears less abrupt, but this could be due to cellular heterogeneity that is homogenized by lysis and mingling of the cytoplasms.

## Regulation of eIF-2 kinase

It is a pity that at least two undergraduate texts (Stryer 1981; Mainwaring *et al.* 1982) give a misleading account of the mode of activation of the haemin-regulated eIF-2 kinase, based on a discarded hypothesis from Ochoa's laboratory that proposed involvement of cyclic-AMP-dependent protein kinase in a cascade type of activation (Ochoa & de Haro 1979). The evidence for this proposal rested heavily on the result that cyclic-AMP-dependent protein kinase inhibited protein synthesis in the reticulocyte lysate. It subsequently emerged that the inhibition was due to a heat-stable contaminant of the particular preparation of this enzyme (de Haro *et al.* 1982). It should be stressed that the rabbit muscle cyclic-AMP-dependent protein kinase does not inhibit protein synthesis under any circumstances, though it phosphorylates several proteins in the lysate. Furthermore, although this enzyme is inhibited by haemin *in vitro* (Datta *et al.* 1977), the presence of haemin does not affect its activity in the physiological milieu of the lysate (Hunt 1979; Grankowski *et al.* 1979; Levin *et al.* 1979).



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This only goes to show what does not regulate the activity of the kinase, though, and one of the most intriguing aspects of this whole regulatory system remains the question of how the haemin-regulated kinase is turned on and off. Undoubtedly haemin stabilizes the latent, non-inhibitory form (Fagard & London 1981), and GTP also promotes the stability of this form as well as promoting the conversion of active kinase back to the latent form (Gross *et al.* 1981). Equally, activation of the kinase *in vitro* is accompanied by phosphorylation of the kinase with the kinetics of an intramolecular reaction (Hunt 1979; Gross & Mendelewski 1978). Neither we nor others have ever observed dephosphorylation of this protein, however, and I am far from persuaded that the enzyme is phosphorylated when it is switched on by physiological signals; there is simply no evidence one way or the other, despite arguments to the contrary by London *et al.* (1981).

It is interesting that the form of the kinase that is most easily switched off is the form that can be activated in the presence of haemin by mild oxidation (Jackson *et al.* 1983). Such oxidation occurs when a reticulocyte lysate is depleted of its ability to reduce –S–S– bonds by removal of the enzyme glucose-6-phosphate dehydrogenase by affinity chromatography, thereby preventing the reduction of NADP to NADPH (Hunt *et al.* 1983). Other experiments show that in the absence of a functional thioredoxin–thioredoxin-reductase system, which is present and active as a protein disulphide reducing system in reticulocyte lysates, eIF-2 becomes phosphorylated (Jackson *et al.* 1983). Because there is no detectable alteration in the activity of the phosphatase, and no oxidation of eIF-2 itself (though other proteins in the lysate do form –S–S– bonds under these conditions), we conclude that the kinase must be activated by this treatment, although it has proved impossible to demonstrate this directly owing to the tendency of the kinase either to lose activity (presumably by reverting to the latent form) or to become irreversibly activated. Because we have not succeeded in isolating the latent form of the kinase to a sufficient degree of purity, the details of the interconversion of these forms remain obscure, and we cannot answer the question of whether oxidation affects the kinase directly, or whether a system of interconverting enzymes exists. Gross *et al.* (1981) have evidence to suggest the existence of another kind of rescue factor that appears to be a GTP-dependent physiological inactivator of the kinase, and we have suspicions, but no good evidence, that accessory factors may be necessary to turn the kinase on. But very little work has been done in this area recently.

## Is protein synthesis in other cells regulated by phosphorylation?

There is scant evidence that the reversible phosphorylation of eIF-2 occurs anywhere else than reticulocytes, though scattered reports of eIF-2 kinases from liver (Delaunay *et al.* 1977), and especially from interferon-treated cells, have appeared (Nilson *et al.* 1982). Extensive studies made by my colleagues in Cambridge on cell-free systems derived from cultured cells and from mouse liver have not only failed to uncover active kinases, but also tend to suggest the existence of controls that affect steps in protein biosynthesis other than the binding of Met-tRNA$_f$ to 40*S* subunits. Defects in mRNA binding to ribosomes seem to occur instead, and there is evidence for a kind of mRNA 'masking' activity.

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## Phosphorylation of ribosomal protein S6

On the other hand, at least one other component of the protein-synthesis machinery does get reversibly phosphorylated: protein S6 of the 40*S* ribosomal subunit (Leader 1980; Gordon *et al.* 1982). The phosphorylation of this protein often accompanies or precedes the activation of cellular growth by hormones (in fact it is often one of the earliest detectable changes in stimulated cells (Rosen *et al.* 1981)). Unfortunately, however, there is no good direct evidence that phosphorylated 40*S* subunits differ in their capacity for protein synthesis from non-phosphorylated 40*S* subunits (Leader *et al.* 1981; Mastopaolo & Henshaw 1981). This need not necessarily be significant, for appropriate homologous systems to test this are lacking. The most convincing results that compare the activity of the two forms were obtained in reticulocyte lysates, which may lack the capacity to discriminate between them.

## Other steps in protein synthesis

A recent report from Cohen's laboratory supports earlier observations (Berg 1977) that the charging of tRNA with amino acids may be regulated by reversible phosphorylation (Damuni *et al.* 1982). However, it has yet to be shown directly that transfer of phosphate from ATP to protein occurs and is responsible for the observed inhibition of tRNA synthetases. Nor have the putative protein kinase activity or the factors that regulate its activity been identified. This area deserves further investigation.

## Conclusion

In conclusion, it is now beyond doubt that reversible phosphorylation can in some cells under some circumstances switch protein synthesis on and off. Whether the phosphorylation of ribosomes is of physiological significance is still doubtful. Even less certain is the answer to the question of whether the many other examples where eukaryotic protein synthesis is regulated (either in quantity or in kind) owe anything to the operation of regulated protein kinase–phosphatase couples.

It is a pleasure to thank Richard Jackson, Pam Herbert and Liz Campbell for all their help in various ways connected with these studies.

I should like to dedicate this paper to the memory of Stan Adamson, Vivian Ernst and Grace Vanderhoff.

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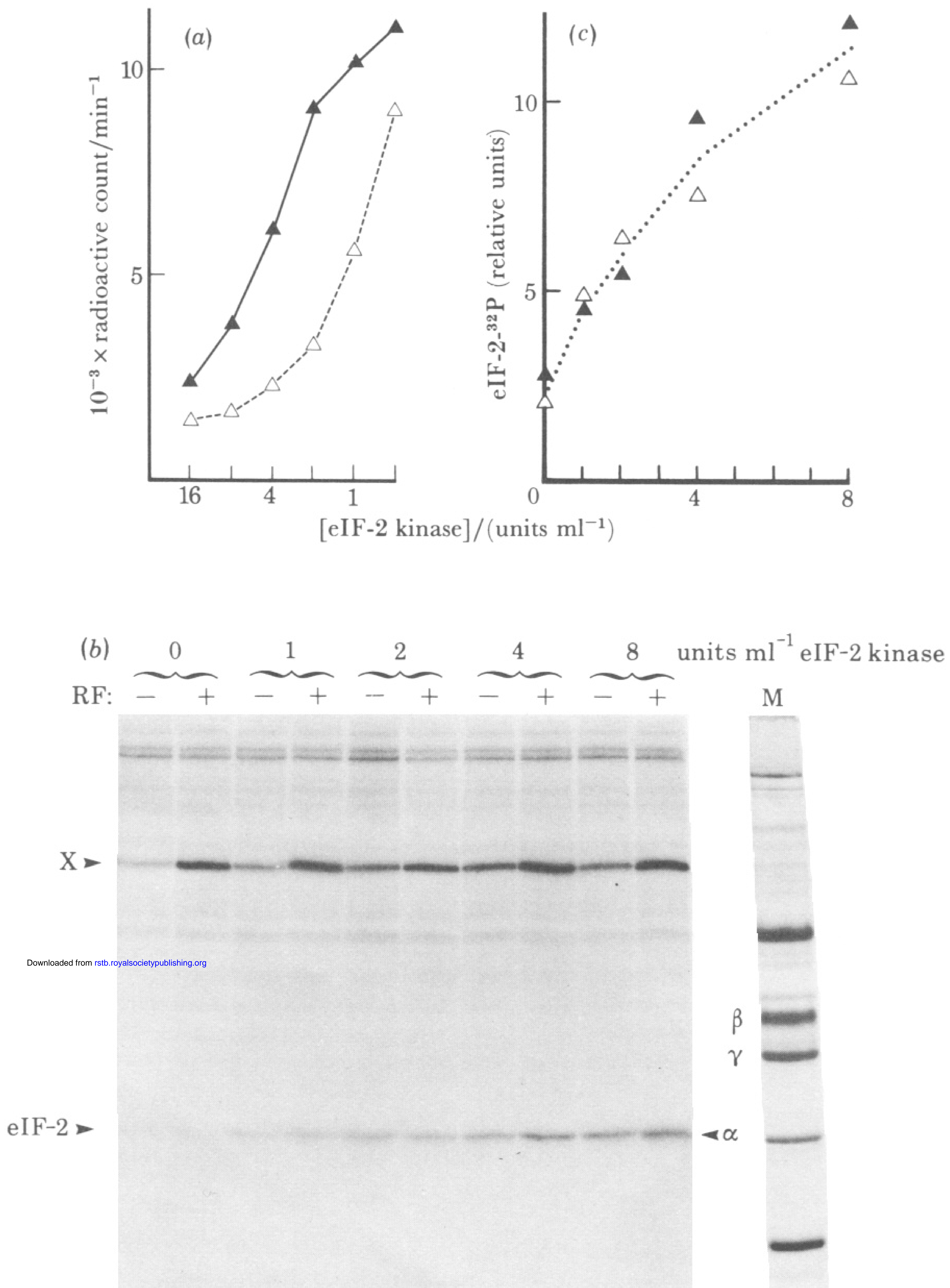


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FIGURE 1. RF does not affect the phosphorylation of eIF-2 although it rescues protein synthesis from the effects of added eIF-2 kinase.

(*a*) A standard reticulocyte lysate incubation according to Jackson & Hunt (1983) was incubated in the presence of increasing concentrations of purified eIF-2 kinase (HCR), and the incorporation of L-[$^{14}$C]valine measured after 40 min at 30 °C (△). ▲, The incorporation found when purified RF (10 μg ml$^{-1}$) was also present.

(*b*) In a parallel set of tubes to (*a*), portions of the same incubations were incubated for 2 min in the presence of [γ$^{32}$P]ATP (2 mCi ml$^{-1}$) before stopping the reaction in SDS–polyacrylamide gel sample buffer. The samples were analysed on a 15 % SDS–polyacrylamide gel which was dried and radioautographed as shown here. The right-most track (M, marker track) shows the position of the polypeptides of eIF-2. Band X is a protein whose phosphorylation appears to be stimulated by 2-mercaptoethanol. Its significance is unclear. The radioautograph was scanned in a densitometer to yield the data plotted in (*c*), which confirms that addition of RF (▲) does not alter the level of phosphorylation of eIF-2, even though it affords almost complete protection against the inhibitory effects of eIF-2 kinase (HCR) at 2 units ml$^{-1}$. △, RF absent from incubation. Purified RF was the generous gift of John Siekierka.