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PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES*: A RADIOIMMUNOASSAY METHOD FOR DIRECT DETERMINATION OF EIF-2 CONCENTRATIONS IN RETICULOCYTE LYSATES.

Bahram. GH. YAGHMAI and Naba.K.Gupta

Summary

A radioimmunoassay method for direct determination of eIF-2 concentration in reticulocyte lysate has been developed. This assay method measures eIF-2 concentrations in nanogram range. A value of 40 pmol eIF-2 per ml of reticulocyte lysate was obtained.

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School of Medicine Kerman-University

INTRODUCTION

It is now generally agreed that the eukaryotic peptide chain initiation factor eIF-2¹ plays a key role in regulation of protein synthesis initiation in eukaryotic cells, for a review, see Ref. 2. eIF-2 forms a ternary complex, Met-tRNA_f.eIF-2 GTP as the first step in peptide chain initiation (3,4). The eIF-2 concentrations in cell-free extracts and also in purified preparations are mostly determined by using a Millipore filtration assay method; the initiation complexes formed with Met-tRNA_f, eIF-2 and GTP are quantitatively retained on Millipore filters (3).

Recent report from our laboratory and elsewhere indicate that the ternary complex forming activity of eIF-2 is regulated by several factors which include Co-eIF-2A (5-9), Co-eIF-2B (3,10), Co-eIF-2C (11-13), eIF-2 kinases (14-16), sRF (17), Met-tRNA_f deacylase (1), and also Mg⁺⁺ (11,13,17). Consequently, the Millipore filtration assay method for eIF-2 activity in crude cell-free preparation represents the sum total activity of eIF-2 in the presence of ancilliary factors. To gain a better understanding of the precise roles of eIF-2 and its ancilliary factors in regulation of eukaryotic protein synthesis-

The abbreviations used are: eIF-2, eukaryotic peptide chain initiation factor 2, forms Met-tRNA_f.eIF-2.GTP complex (3,4); Co-eIF-2A, a factor stimulating Met-tRNA_f binding to eIF-2 (5,7). Co-eIF-2B (TDF), a factor promoting dissociation of ternary complex at high Mg⁺⁺ concentrations(3,10). Co-eIF-2C, relieves Mg⁺⁺ inhibition of ternary Complex formation(11). SRF, post ribosomal supernatant factor that reverses protein synthesis inhibition in heme-deficient lysates (17); anti-eIF-2, immune serum to eIF-2.

is, it will be necessary to develop an assay method which measures directly the concentrations of these factor activities as they are present in the cells and the changes in these factor activities under different physiological conditions.

Recently, we reported preparation of eIF-2 antibodies by injecting homogeneous reticulocyte eIF-2 preparations to chickens. We have now used these eIF-2 antibodies and have developed a sensitive radioimmunoassay method for direct measurement of eIF-2 activity in reticulocyte lysate. This assay method measures eIF-2 concentrations in the nanogram range. A value of 40 pmoles of eIF-2 per ml of reticulocyte lysate was obtained.

MATERIALS AND METHODS

Materials:

Bolton Hunter reagent was obtained from New England Nuclear. Rabbit Anti-Sera to chicken IgG was purchased from Miles Laboratories.

Reticuloctye Lysates and Initiation Factor:

The preparation of rabbit reticulocyte lysates and peptide chain initiation factor, eIF-2 has been described previously (3). The homogeneous eIF-2 preparation showed three protein bands upon sodium dodecyl sulfate polyacrylamide gel electrophorsis corresponding to approximate molecular weights of 54,000, 52,000, and 38,000 (3).

Immune Serum:

Anti-eIF-2 serum was prepared following the procedure described previously (7). A six-month-old chicken was immunized using homogeneous eIF-2. The first injec-

tion (Day 1), 0.5 ml containing 0.25 mg of homogeneous eIF-2 preparation was given with 0.6 ml of complete Freund's adjuvant (GIBCO). On day 12, the immunization was repeated using 0.5 ml of protein (0.25 mg) and 0.6 ml of complete Freund's adjuvant. The final injection was performed on Day 29 using 0.3 ml of eIF-2 and 0.6 ml of complete Freund's adjuvant. Blood was drawn from the wing vein before immunization (to prepare control serum) and on Day 31. Serum was stored in small aliquots in liquid nitrogen.

Iodination of eIF-2:

Homogeneous eIF-2 preparation was iodinated using the procedure of Bolton and Hunter (18). The reaction mixture contained (in a total volume of 50 μ l) 20 mM potassium phosphate, pH 7.6; 10 μ g homogeneous eIF-2 and dried iodinated ester, and was incubated at 0°C for 30 minutes with occasional stirring. The reaction mixture was then applied on to a Sephadex G-25 column (0.5 cm x 10cm) equilibrated with a buffer containing 20mM Tris-HCl, pH 7.6, 0.1 M potassium chloride and 1 mM dithiothreitol. Iodinated eIF-2 was eluted in the void volume and was separated from unbound radioactivity. The specific activity of eIF-2 was 1.9×10^7 cpm/ μ g.

Radioimmunoassay:

Chicken immune serum (5 μ l) (1:40 dilution) containing eIF-2 antibody was taken in a glass tube (10 mm x 75 mm) and was mixed with varying concentrations of (125 I)-labelled eIF-2 in a total volume of 50 μ l containing 10 mM Tris-HCl, pH 7.6, 0.1 MKCl, and 50 μ g bovine serum albumin. The reaction mixture was then incubated at

37° for 1 hr followed by a further incubation at 4° C for 14 hr. After the incubation, 5 μ l carrier non-immune chicken serum was added to each tube followed by another addition of 20 μ l of anti-chicken IgG rabbit serum. The reaction mixture was then incubated at 30° C for $\frac{1}{2}$ hr followed by a further incubation at 4° C for 8 hrs. The contents of each tube were then layered on 0.15 ml sucrose solution (1 M) contained in polyethylene centrifuge tubes (0.4 ml capacity) and were centrifuged for 1 minute in a Beckman microfuge. The bottom tips of the centrifuge tubes were carefully sheared off with a razor blade and counted in a Beckman gamma counter.

For measurement of eIF-2 concentrations in reticulocyte lysates, different amounts of reticulocyte lysates were added to 5 μ l containing 10 mM Tris-HCl, pH 7.6, 0.1 M KCl and 50 μ g bovine serum albumin. The reaction mixtures were preincubated for 1 hr at 30° C and 4 hrs at 4° C prior to the addition of 1.5 ng of (125 I)-eIF-2. The reaction mixtures containing (125 I)-labelled eIF-2 were again incubated at 37° C for 1 hr and at 4° C for 14 hrs. The antibody bound eIF-2 was separated from unbound eIF-2 by the double antibody technique as described above.

RESULTS

Recently, we have prepared eIF-2 antibodies by injecting homogeneous preparations of reticulocyte eIF-2 to chicken. The chicken immune serum thus prepared strongly inhibited protein synthesis in reticulocyte lysates and such inhibition was overcome by preincubating the immune serum specifically with eIF-2 and not with any other protein factor. To further test the specificity of eIF-2

antibody, we iodinated a partially purified preparation of eIF-2 (Fraction IV (3)) and incubated the iodinated eIF-2 with chicken immune serum. The immunoprecipitate, supernatant solution and also an original sample of iodinated eIF-2 were then analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography.

Fraction IV eIF-2 preparations showed, in addition to the characteristic eIF-2 protein bands, several other faint contaminating protein bands (track 3). However, the immunoprecipitate showed only the characteristic eIF-2 protein bands and no contaminating protein bands of the original eIF-2 preparation. In this experiment, the 52,000 and 54,000 protein bands of eIF-2 were not resolved and appeared as a single intense band. The supernatant solution after immunoprecipitation did not contain any eIF-2 protein band.

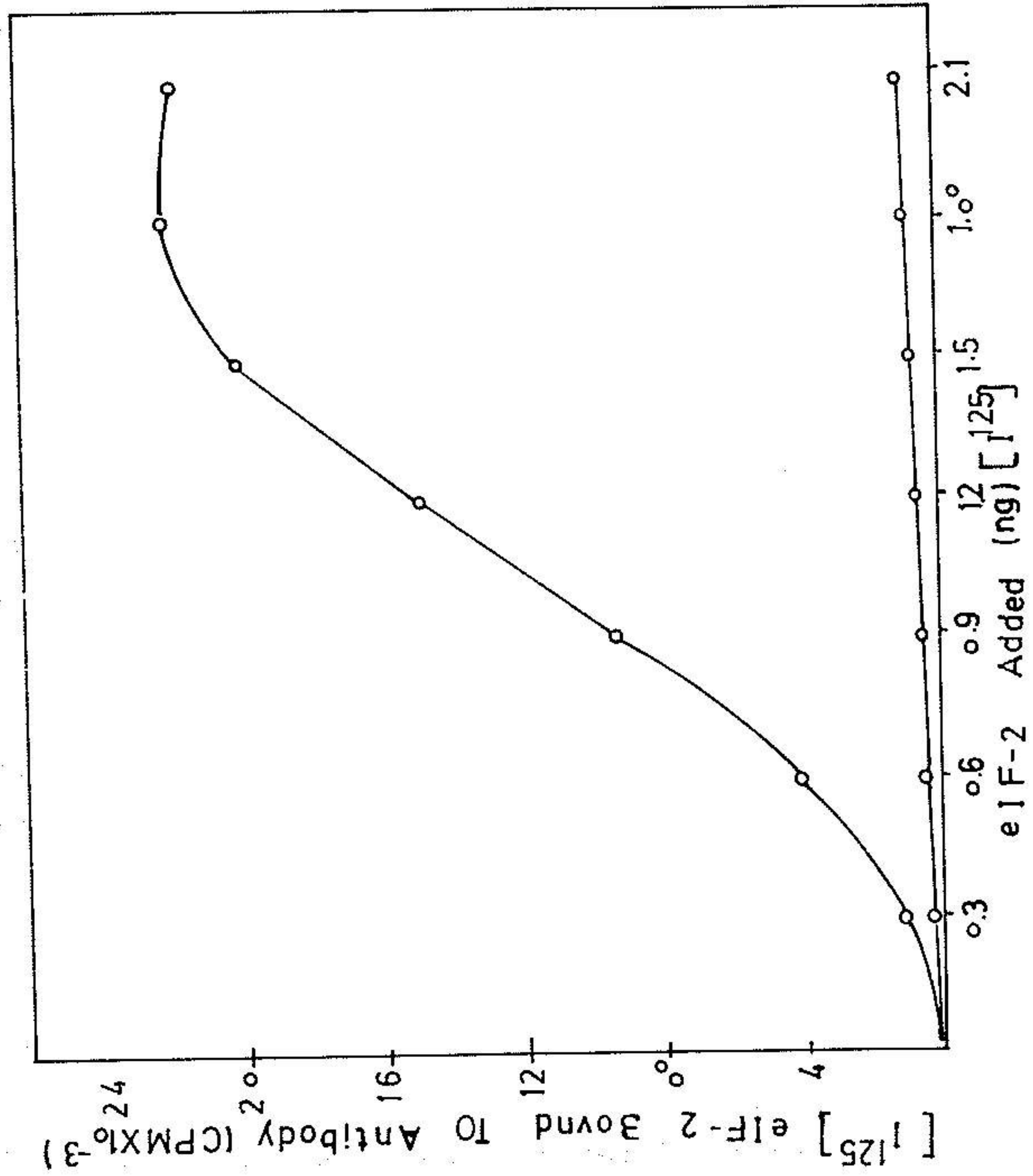
Fig.1 shows the extent of $[^{125}\text{I}]$ -labelled eIF-2 binding to a fixed amount of anti-eIF-2 serum (5 μl , 1:40 dilution) at different concentrations of added $[^{125}\text{I}]$ -labelled eIF-2. $[^{125}\text{I}]$ -eIF-2 binding to antibody showed a slight lag at low eIF-2 concentration (0.3 - 0.6 ng level) and then increased almost linearly with added eIF-2. eIF-2 binding to antibodies reached a plateau at an eIF-2 concentration above 1.5 ng presumably because of the limitation of antibodies. Under the experimental conditions and in the presence of 1.5 ng eIF-2, 70 percent of input $[^{125}\text{I}]$ -eIF-2 was bound to the antibodies. The binding of $[^{125}\text{I}]$ -eIF-2 is clearly specific to eIF-2 antibodies; no binding of $[^{125}\text{I}]$ -eIF-2 was observed when control serum was used in this experiment.

As expected, the binding of $[^{125}\text{I}]$ -labelled eIF-2 to anti-eIF-2 could be inhibited by preincubation of the an-

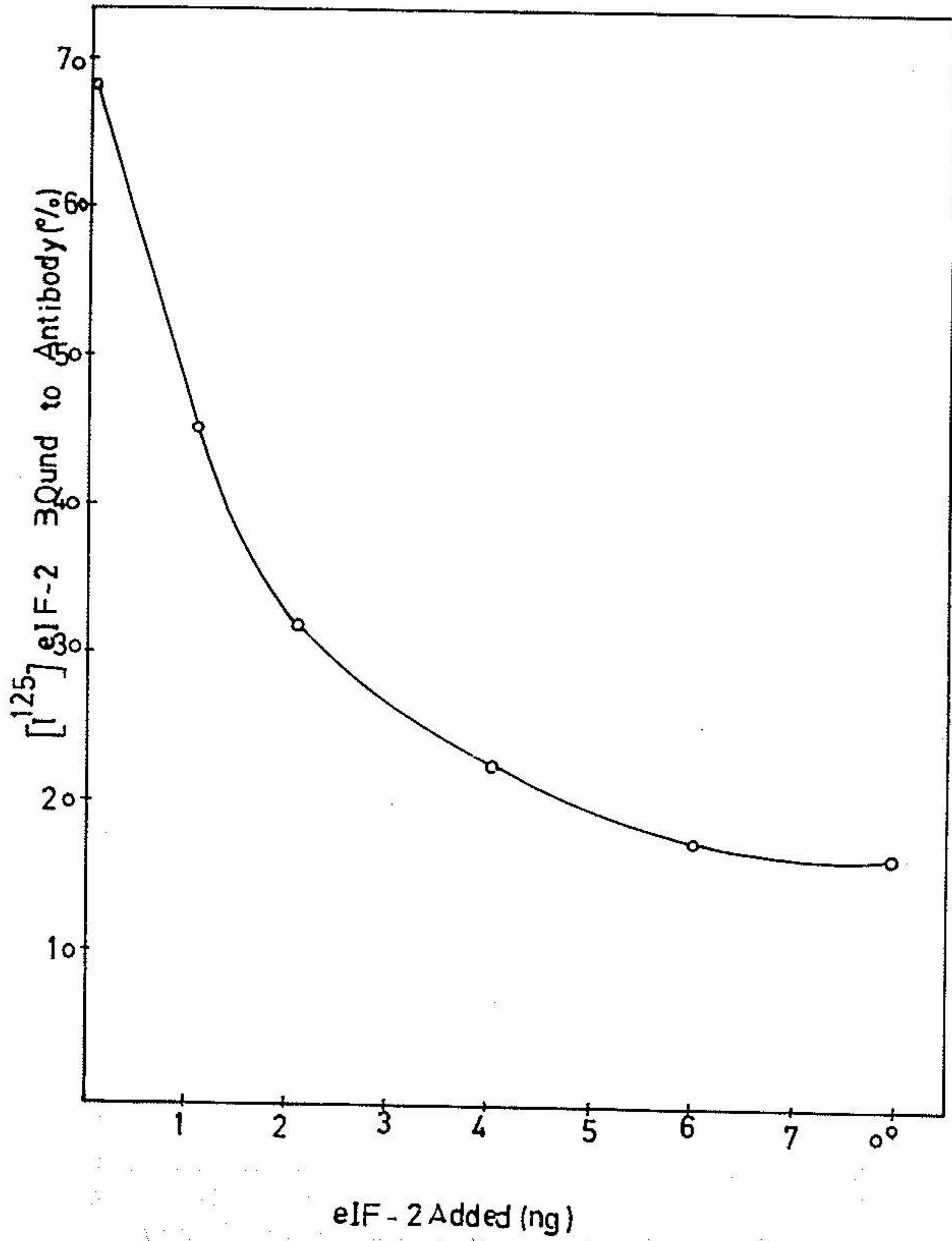
tiserum with unlabelled eIF-2. A typical inhibition curve for $\left[^{125}\text{I}\right]$ -eIF-2 binding to antibodies by prior incubation of unlabelled eIF-2 with antiserum is shown in Fig.2. As mentioned above, under the conditions of the experiment and in the presence of 1.5 ng $\left[^{125}\text{I}\right]$ eIF-2, 70 percent of input eIF-2 was bound to anti-eIF-2-serum. Prior incubation of anti-eIF-2 serum with unlabelled eIF-2 with increasing concentrations lowered progressively subsequent binding of $\left[^{125}\text{I}\right]$ eIF-2 to anti-eIF-2 serum. Under the experimental conditions, prior incubation of anti-eIF-2 serum with 0.8 ng of unlabelled eIF-2 reduced subsequent binding of input $\left[^{125}\text{I}\right]$ -eIF-2 to antiserum to 50 percent.

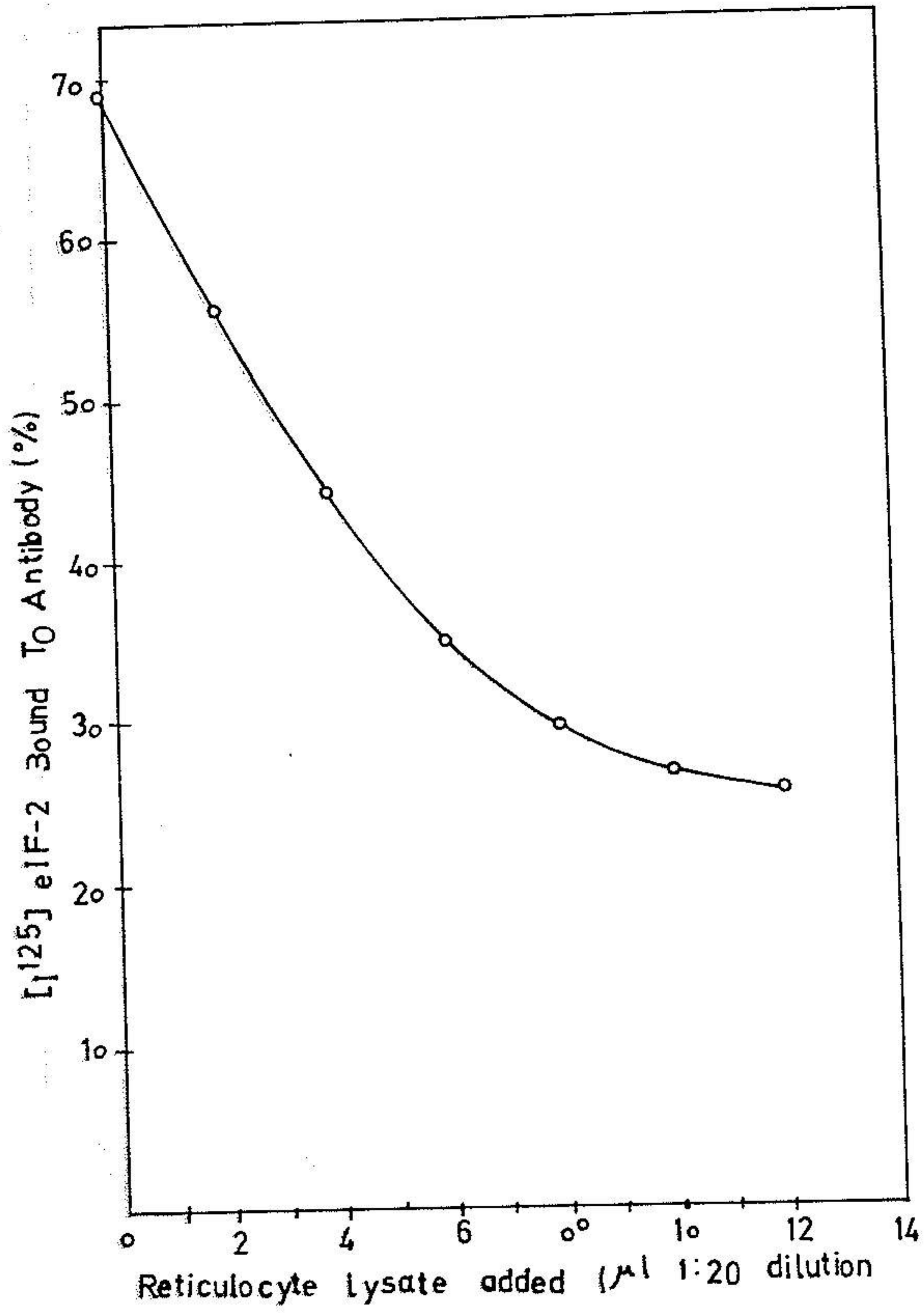
A similar inhibition of $\left[^{125}\text{I}\right]$ eIF-2 binding was also observed by prior incubation of anti-eIF-2 serum with reticulocyte lysates at different concentrations. A typical inhibition curve for $\left[^{125}\text{I}\right]$ -eIF-2 binding to anti-eIF-2 serum by added reticulocyte lysate (1:20 dilution) at different concentrations is shown in Fig.3. From this curve, it can be seen that addition of 3 μl reticulocyte lysate (1:20 dilution) lowered the binding of input $\left[^{125}\text{I}\right]$ eIF-2 to anti-eIF-2 serum to 50 percent. From the results described in Fig.2, this amount of inhibition was caused by 0.8 ng homogeneous eIF-2. From these results, we conclude that 3 μl reticulocyte lysate (1:20 dilution) contains 0.8 ng eIF-2. These results correspond to a value of 40 pmol eIF-2 per ml of reticulocyte lysate assuming a molecular weight of reticulocyte eIF-2 to be 140,000.

(Fig)



(Fig 2)



(Fig³)

Discussion

The radioimmunoassay method described in this paper measures nanogram concentrations of eIF-2 present in cell free extract. More importantly, this assay method can be used to determine directly the concentration of eIF-2 in the presence of other ancillary factors. As mentioned previously, several ancillary factors present in the cell regulate the extent of ternary complex formation by eIF-2 and the standard Millipore filtration assay method is not an accurate measure of eIF-2 concentration in the cell-free extract.

Using this radioimmunoassay method we obtained a value of 40 pmol of eIF-2 per ml of reticulocyte lysate. This value agrees closely to a value of eIF-2 concentration in reticulocyte lysates recently reported by Safer et al. (19). These authors measured eIF-2 concentrations in reticulocyte lysates using an isotope dilution technique (19).

However, the radioimmunoassay method described in this paper is more sensitive than the isotope dilution assay method and also can be used directly in the free extract independent of the presence of 40S ribosomes and other protein factors. Moreover, the isotope dilution assay method assumes that the activity of the entire endogenous eIF-2 population is the same as the added radioactively labelled eIF-2. Reports from our laboratory and elsewhere have clearly established that the activity of eIF-2 varies widely depending on the presence of other protein factors and also Mg^{++} concentrations.

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