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Regulation of protein synthesis in reticulocyte lysates: Immune serum inhibits heme-regulated protein kinase activity and differentiates heme-regulated protein kinase from double-stranded RNA-induced protein kinase

(phosphorylation of Met-tRNA$_f$ binding factor/95,000-dalton polypeptide)

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ABSTRACT **A specific immune serum to the heme-regulated inhibitor (HRI) has been prepared by immunizing chickens with highly purified reversible HRI prepared from rabbit reticulocyte lysates. Studies with this immune serum demonstrate that the behavior of purified reversible HRI is similar to that of the inhibitor activated in rabbit reticulocyte lysates: the immune serum (*i*) inhibits the phosphorylation of the small subunit (38,000 daltons) of the eukaryotic initiation factor eIF-2 by both crude and purified inhibitor preparations; (*ii*) prevents the concomitant inhibition of protein synthesis by both crude and purified inhibitor preparations; and (*iii*) prevents the autophosphorylation of the 95,000-dalton polypeptide in purified and crude HRI preparations. The protein kinase and inhibitory activities of crude and partially purified preparations of the double-stranded RNA-induced inhibitor of protein synthesis are not affected by the immune serum prepared to reversible HRI. These results indicate that the inhibitor induced by double-stranded RNA is antigenically distinct from the reversible HRI.**

In heme deficiency, protein synthesis in reticulocytes and reticulocyte lysates is inhibited (1–3). This inhibition occurs at the level of initiation of translation and is due to the activation of a cyclic 3′:5′-AMP-independent protein kinase which specifically phosphorylates eIF-2α [the small (38,000 daltons) subunit of eukaryotic initiation factor 2 (eIF-2)] (4–8). This inhibitory kinase is formed in the absence of heme and has been designated heme-regulated inhibitor (HRI) (8) or heme-controlled repressor (HCR) (9). HRI has been purified to various degrees in two distinct forms depending on how the inhibitor has been activated (4, 6, 8, 10, 11). The heme-irreversible HRI is formed by prolonged incubation (9) or treatment with sulfhydryl reagents (12) and is not inactivated by incubation with hemin; the heme-reversible HRI is activated in the absence of hemin and is inactivated by incubation with hemin (11).

Initiation of protein synthesis in rabbit reticulocyte lysates is inhibited by low levels of double-stranded RNA (dsRNA) (13, 14). The inhibition caused by the addition of dsRNA is similar to that of HRI. Both inhibitions are characterized by biphasic kinetics—i.e., in lysates, there is an initial period of synthesis at the control rate followed by an abrupt decline in the rate of synthesis (3, 14)—as well as polysome disaggregation (3, 14, 15), depletion of the 40S ribosomal subunit–Met-tRNA$_f^{Met}$ complex (16, 17), potentiation by ATP (4, 18), activation of a cyclic 3′:5′-AMP-independent protein kinase activity that phosphorylates eIF-2α (4–8), and reversal and prevention by the addition of exogenous eIF-2 (19–22). In contrast to HRI, dsRNA induces an inhibitor (dsI) that is found associated with ribosomes (4, 23) and can be extracted from ribosomes with solutions of high salt concentration (23). dsRNA-induced inhibition of protein synthesis and protein phosphorylation has also been observed in cell-free extracts derived from cells treated with the antiviral agent interferon (24–27).

Farrell *et al.* (4) have proposed that HRI and dsI are distinct molecular entities. Although dsRNA-induced protein kinase activities recently have been isolated from interferon-treated Ehrlich ascites tumor and mouse L cells (27, 28), as yet dsI has not been identified.

Here we report the preparation of a serum that contains antibodies to highly purified reversible HRI. This immune serum was used to study the mechanism of action of HRI, when it is activated in the lysate and when it is highly purified. The immune serum was also tested against dsI activity. Lysates in which dsI had been activated and partially purified dsI were unaffected by the immune serum as judged by (*i*) the phosphorylation of eIF-2α; and (*ii*) inhibition of protein synthesis. These results indicate that activated dsI is antigenically distinct from reversible HRI.

MATERIALS AND METHODS

Preparation of Immune Serum. One 6-month-old White Leghorn chicken was immunized three times by intramuscular injection (into the breast) with step 7 reversible HRI (11) as follows. The first injection (day 1) containing 0.75 ml of protein (0.25 mg) was given with 0.75 ml of complete Freund's adjuvant (GIBCO). On day 12 the immunization was repeated using 0.5 ml of protein (0.175 mg) and 0.75 ml of complete Freund's adjuvant. The final injection was performed on day 22 as described for the second injection. Blood was drawn from the wing vein before immunization (to prepare control serum) and on day 32. Serum were stored in small aliquots in liquid nitrogen.

Protein Kinase Assay and Sodium Dodecyl Sulfate/Polyacrylamide Gel Electrophoresis. Protein kinase assays (20 μl) contained 20 mM Tris·HCl (pH 7.6 at 25°C), 60 mM KCl, 2 mM Mg(OAc)$_2$, and 0.1 mM [γ-^{32}P]ATP [1–2 Ci/mmol (1 Ci = 3.7 × 10^{10} becquerels)]. For other additions and incubation

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Abbreviations: HRI, heme-regulated inhibitor; dsRNA, double-stranded RNA; dsI, dsRNA-induced inhibitor; PRS, post-ribosomal supernatant; MalNEt, *N*-ethylmaleimide; eIF-2, eukaryotic initiation factor 2 (the initiation factor that forms a ternary complex with Met-tRNA$_f$ and GTP, as adopted at the 1976 Fogarty International Symposium on Protein Synthesis National Institutes of Health, Bethesda, MD) (36).

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conditions, see the figure legends. After incubation the samples were diluted by the addition of 20 μl of sodium dodecyl sulfate sample buffer (29) and heated at 100°C for 1 min. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis (15% acrylamide, 0.09% bisacrylamide) and autoradiography were performed as described (5).

Protein Synthesis Assay. Reticulocytes were obtained from rabbits treated with 1-acetyl-2-phenylhydrazine (Sigma). Reticulocyte lysates and incubation mixtures for protein synthesis were prepared as described (30). Protein synthesis was determined by the incorporation of [^{14}C]leucine (specific activity, 150 mCi/mol) into protein in 5-μl aliquots (31).

Labeling of Reversible HRI with [γ-^{32}P]ATP. Phosphate-labeling of HRI was carried out in 25-μl reaction mixtures containing 20 mM Tris·HCl (pH 7.6 at 25°C), 60 mM KCl, 2 mM $Mg(OAc)_2$, 0.1 mM [γ-^{32}P]ATP (1 Ci/mmol), and 4 μl (≈2 μg of protein) of step 7 reversible HRI (11). The mixtures were incubated for 5 min at 37°C and used immediately as described in Fig. 2.

Isolation of Antigen–Antibody Complexes with IgG Sorb. A 10% solution of IgG Sorb (The Enzyme Center, Inc., Boston, MA) in H_2O was added to incubation mixtures containing antigen and control or immune serum. The mixture was incubated on ice for 15 min. The IgG Sorb-bound protein was then recovered by brief centrifugation in a Brinkmann model 3200 centrifuge. The sediment was washed three or four times with 0.3 ml of 20 mM Tris·HCl (pH 7.6 at 25°C)/100 mM KCl and then treated with 40 μl of sodium dodecyl sulfate sample buffer for 1 min at 90°C. The IgG Sorb was removed by centrifugation and the supernatant was analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography as described above.

Preparation of HRI and dsI Fractions. The reversible form of HRI was prepared from the postribosomal supernatant (PRS) of rabbit reticulocytes by the methods described by Trachsel *et al.* (11). Irreversible HRI was prepared by incubating reticulocyte lysates in the absence of added hemin for 6 hr at 37°C or by incubating the lysates in the presence of 5 mM *N*-ethylmaleimide (MalNEt) for 10 min at 30°C; the MalNEt-treated lysates were then treated with 10 mM 2-mercaptoethanol and cooled.

Induction of dsI activity in reticulocyte lysates and isolation on crude ribosomes were performed as described by Levin and London (23), except that poly(I)·poly(C) (100 ng/ml) was used as an inducer. The preparation of partially purified dsI activity from reticulocyte lysates will be described elsewhere.

The HRI- and dsI-containing fractions were assayed for their abilities to inhibit protein synthesis, and approximately equal amounts of inhibitory activity were used in the experiments described here.

RESULTS

Effect of Control and Immune Sera on Reversible HRI Kinase Activity. Antibodies to the irreversible form of HRI have been prepared in the guinea pig (32) and goat (6). Here we report the preparation of chicken antiserum to highly purified reversible HRI isolated from rabbit reticulocyte lysates.

To determine whether the serum obtained from the immunized chicken contained antibodies to HRI, the effects of control and immune sera on HRI activity were compared. Fig. 1 shows the effect of various dilutions of control and immune sera on HRI kinase activity. Control serum had no effect on HRI's ability to phosphorylate eIF-2α. In contrast, the immune serum greatly reduced the ability of HRI to phosphorylate eIF-2α. Furthermore, this inhibition of HRI kinase activity was dependent on the amount of immune serum added. Interestingly, in the presence of increasing concentrations of immune but not of control serum, there was a concomitant decrease in the phosphorylation of the 95,000-dalton polypeptide that is apparently autophosphorylated in association with activation of purified reversible HRI preparations (11).

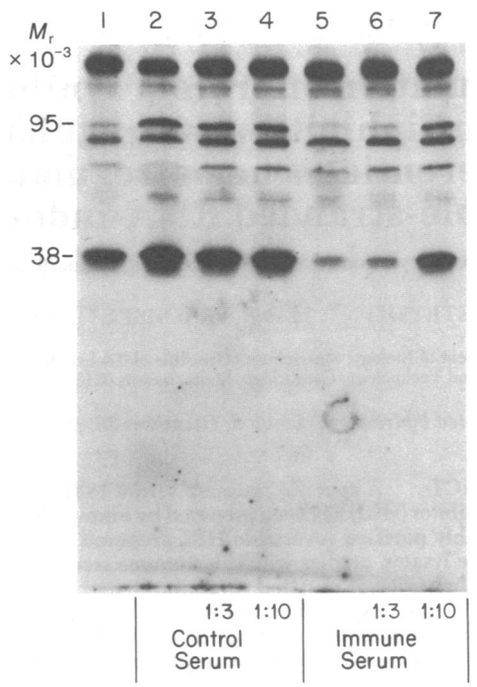

FIG. 1. Effect of control and immune sera on kinase activity of reversible HRI. Protein kinase reaction mixtures contained 0.5 μg of step 7 reversible HRI, 1.8 μg of eIF-2, and 1 μl of different concentrations of control or immune serum or buffer. Prior to the addition of eIF-2 and [γ-^{32}P]ATP, the reaction mixtures were preincubated on ice for 30 min. After the addition of eIF-2 and [γ-^{32}P]ATP, the incubation was continued at 30°C for 5 min and terminated by the addition of sample buffer. The samples were analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and autoradiography. Lanes: 1, preincubation with buffer [25 mM Tris·HCl (pH 7.7 at 25°C), 90 mM KCl, 0.1 mM EDTA, and 5% glycerol]; 2–4, preincubation in the presence of 1 μl of control serum, undiluted (lane 2), diluted 1:3 (lane 3), or diluted 1:10 (lane 4); 5–7, preincubations in the presence of 1 μl of immune serum as for lanes 2–4. The autoradiogram is shown.

There was a modest, but reproducible, increase in phosphorylation of eIF-2α by HRI in the presence of control serum (Fig. 1). The reason for this increased phosphorylation is not clear. The serum appeared to protect or maintain the activity of HRI; neither control nor immune serum alone had any significant kinase activity toward eIF-2 (see Fig. 3).

The eIF-2 preparation contained contaminating proteins that were phosphorylated (Fig. 1). However, the effect of the immune serum was specific for eIF-2α and the 95,000-dalton polypeptide of HRI; phosphorylation of the contaminating proteins was not affected by the immune serum.

Effect of Control and Immune Sera on Reversible HRI Activity in Protein Synthesis. Table 1 represents two separate experiments demonstrating the effect of preincubating reversible HRI (step 3, 15 μg) (11) with control and immune sera. Preincubation with immune, but not control, serum resulted in diminished inhibition of protein synthesis by HRI.

There was some inhibition of protein synthesis due to the addition of control or immune serum alone (with the control being less inhibitory than the immune serum). This effect is

Table 1. Effect of control and immune sera on reversible HRI activity in protein synthesis

Additions	[^{14}C]Leucine incorporated, cpm	
	Exp. 1	Exp. 2
None	17,320	18,812
Control serum (1:10)	13,720	13,053
Immune serum (1:10)	12,502	12,663
HRI	3,932	4,500
Control serum (1:10) + HRI	4,567	5,312
Control serum (1:20) + HRI	—	5,736
Immune serum (1:10) + HRI	10,582	12,376
Immune serum (1:20) + HRI	—	9,564

Preincubations (20 μl) contained 20 mM Tris·HCl (pH 7.6 at 25°C), 60 mM KCl, 2 mM $Mg(OAc)_2$, 1 μl of step 3 reversible HRI (15 μg of protein), and control or immune serum in the indicated final dilution. Preincubations were carried out for 30 min on ice. Then, 2 μl of the preincubation mixtures were added to a 25-μl protein synthesis lysate assay and protein synthesis was measured.

probably due to substances present in the crude serum preparations. However, in assays in which HRI was preincubated with immune serum, protein synthesis was restored to about the same level as in those assays in which immune serum was added alone. Because the inhibition of protein synthesis by serum alone was slight compared to that caused by HRI alone, the crude sera were used in subsequent experiments without further purification.

The activity antagonistic to HRI in the immune serum copurified with the IgG fraction prepared from immune serum (data not shown).

Immunoprecipitation of Reversible HRI. To determine the specificity of the immune serum for reversible HRI, partially purified ^{32}P-labeled HRI and HRI that had been phosphorylated directly in the PRS were precipitated by adsorption to insoluble protein A (IgG Sorb). The precipitates were analyzed by electrophoresis and autoradiography. ^{32}P-Labeled HRI that had been mixed with PRS displayed one major labeled polypeptide of 95,000 daltons (Fig. 2). Preincubation of the PRS, which contained added ^{32}P-labeled HRI, with immune serum followed by immunoprecipitation resulted in the removal of this 95,000-dalton polypeptide from the supernatant fraction (lane 3) and its quantitative recovery in the resulting precipitate (lane 5). Control serum under the same conditions had only a slight effect on this 95,000-dalton polypeptide (lanes 2 and 4).

In other experiments in which reversible HRI was phosphorylated directly in lysates and then treated with immune serum and precipitated, the 95,000-dalton polypeptide component of HRI was also found in the resulting precipitate (lane 6). When hemin (25 μM) was added, little radioactive 95,000-dalton phosphopeptide could be detected in the precipitate (lane 7).

Effects of Control and Immune Sera on Reversible HRI and dsI Kinase Activities. The effects of treatment with control or immune serum on the kinase activities of various preparations containing HRI and dsI were examined by measuring the phosphorylation of eIF-2α (Fig. 3). Control or immune serum alone showed little or no ability to phosphorylate eIF-2α. Lysates in which HRI had been activated by heat treatment or MalNEt treatment and partially purified reversible HRI produced a marked phosphorylation of eIF-2α after preincubation with control serum. However, preincubation of these same preparations with immune serum resulted in a complete, or nearly complete, inhibition of phosphorylation of eIF-2α. In contrast, preincubation, with control or immune serum, of

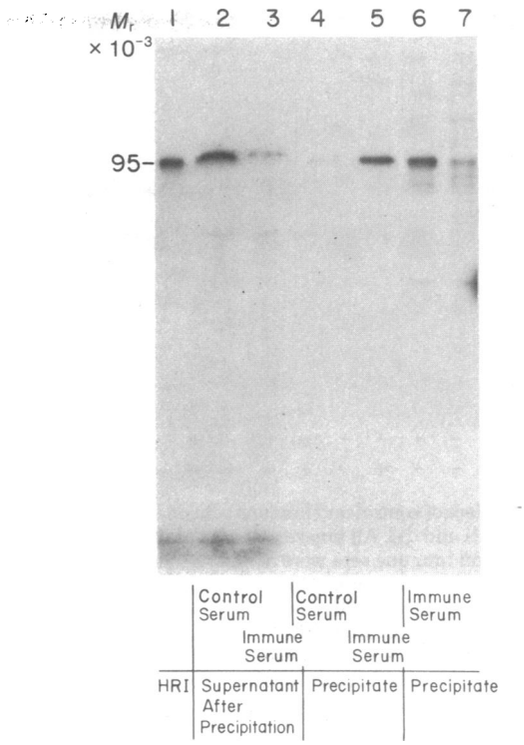

FIG. 2. Immunoprecipitation of reversible HRI. Step 7 reversible HRI was labeled with ^{32}P and 2 μl (≈1 μg of protein) of this material was mixed with 20 μl of reticulocyte PRS and 2 μl of control or immune serum. Incubations were carried out on ice for 30 min; then 20 μl of IgG Sorb was added. The IgG Sorb-bound material was separated. Lanes: 1, 2 μl of reversible [^{32}P]HRI before mixing with supernatant; 2, control serum, unbound material; 3, immune serum, unbound material; 4, control serum, IgG Sorb-bound material; 5, immune serum, IgG Sorb-bound material. For lanes 6 and 7, 40 μl of reticulocyte PRS was supplemented with 0.3 mM fructose 1,6-diphosphate, 0.03 mM NAD^+, 2 μl of $^{32}P_i$ (carrier-free), and 0.1 mM [γ-^{32}P]ATP (1 Ci/mmol) (33). The supernatants were incubated in the presence of 5 mM MalNEt (lane 6) or 25 μM hemin (lane 7) for 5 min at 30°C; then, 8 μl of immune serum was added to each incubation and the mixtures were further incubated on ice for 30 min. IgG Sorb (40 μl) was then added and the IgG Sorb-bound proteins were isolated. The autoradiogram is shown.

lysates activated by dsRNA, crude ribosome-bound dsI, or partially purified soluble dsI resulted in no significant decrease in the phosphorylation of eIF-2α.

The eIF-2 preparation used in the experiments described in Fig. 3 contained a minor endogenously phosphorylated polypeptide slightly larger than the 38,000-dalton subunit of eIF-2. The endogenous phosphorylation of this polypeptide was not affected by control or immune serum.

Effects of Control and Immune Sera on the Inhibition of Protein Synthesis by Reversible HRI and dsI. The addition of preparations containing HRI or dsI to reticulocyte lysates resulted in an inhibition of protein synthesis with biphasic kinetics (Fig. 4), in agreement with previous results (8, 14, 23). The cessation of protein synthesis occurred after about a 3-min lag with incubation at 30°C. The inhibition of protein synthesis by heat-activated HRI (Fig. 4*A*) or partially purified step 3 HRI [15 μg of protein (11)] (Fig. 4*B*) was significantly prevented by preincubating the preparations with immune serum, whereas preincubation with control serum had no effect on this inhibition. In contrast, the inhibition of protein synthesis caused by the addition of dsRNA-activated lysates or by the addition of partially purified dsI was not affected by preincubation with either control or immune serum (Fig. 4 *C* and *D*).

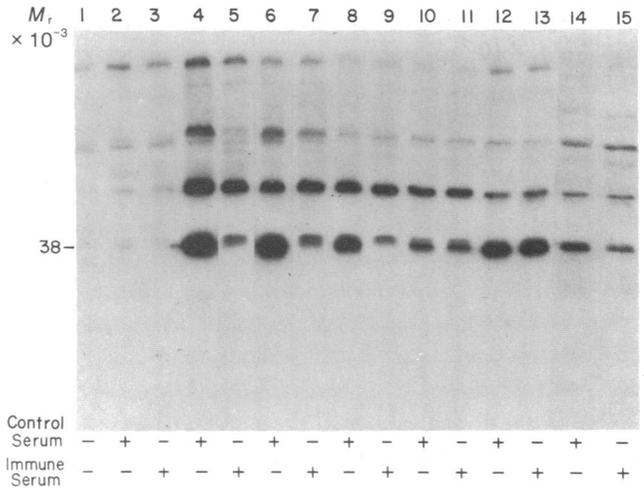

FIG. 3. Effect of control and immune sera on the phosphorylation of eIF-2 by HRI and dsI. All kinase assays contained 1 μg of eIF-2; 1 μl of control and immune sera were added as indicated on the autoradiogram. Fractions containing HRI and dsI were added as described below. Incubations were for 10 min at 37°C. Lanes: 1–3, no additions; 4 and 5, 0.5 μl of heat-activated HRI; 6 and 7, 1 μl of MalNEt-activated HRI; 8 and 9, 0.25 μg of step 3 reversible HRI; 10 and 11, 0.5 μl of dsRNA-activated lysate; 12 and 13, 0.025 A_{260} unit of dsI-associated ribosomes; 14 and 15, 18.7 μg of partially purified dsI.

DISCUSSION

Evidence for the existence of specific antibodies to reversible HRI consists of the following experimental observations. (*i*) The addition of immune but not control serum can prevent the phosphorylation of eIF-2α (Fig. 1). The phosphorylation of eIF-2α by the protein kinase activity of HRI appears to be the primary event in the inhibition of protein synthesis in reticulocyte lysates (4–8). (*ii*) The inhibition of protein synthesis by the addition of HRI to reticulocyte lysates can be prevented by prior incubation of the HRI fraction with immune serum (Table 1; Fig. 4*B*). (*iii*) The immune serum but not the control serum reacts with PRS containing HRI and serves to remove the 95,000-dalton polypeptide, which is quantitatively recovered in the resulting immunoprecipitate (Fig. 2). (*iv*) In addition to preventing the phosphorylation of eIF-2α by HRI, the immune serum concomitantly prevents the phosphorylation of the 95,000-dalton polypeptide of purified reversible HRI preparations (Fig. 1). In these preparations, the 95,000-dalton polypeptide of HRI apparently undergoes autophosphorylation, and this phosphorylation may be a requirement for the activation of purified heme-reversible HRI (11). It is not clear as yet whether HRI is similarly activated in lysates; however, other studies in this laboratory (V. Ernst, D. H. Levin, and I. M. London, unpublished data) and by other investigators (34) have demonstrated that in heme-deficient lysates there is a rapid *in situ* phosphorylation of HRI and of eIF-2α.

The data presented here support the concept that the 95,000-dalton polypeptide is an integral component of both purified HRI and HRI activated in the lysate; the immunoprecipitation technique demonstrates that this polypeptide is involved in the specific antibody–antigen reaction (Fig. 2, lanes 1–6). Furthermore, when hemin is added to lysates, the inhibitory effect of HRI on protein synthesis and the phosphorylation of the 95,000-dalton polypeptide (Fig. 2, lane 7) are prevented. These findings are in agreement with earlier studies carried out in this laboratory (11) and other recent studies (35).

As judged by its inhibition of the phosphorylation of eIF-2α (Fig. 3, lanes 4–9) and its prevention of the inhibition of protein

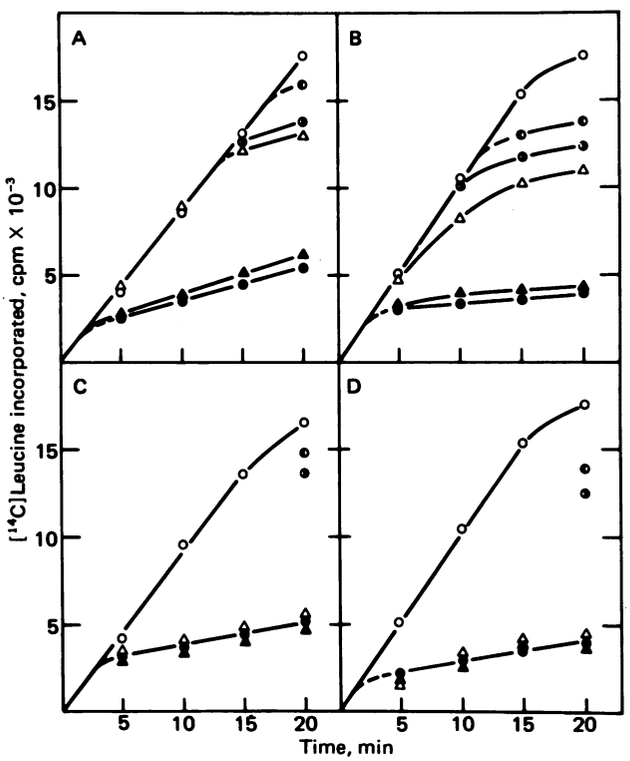

FIG. 4. Effect of control and immune sera on inhibition of protein synthesis by HRI and dsI fractions. Protein synthesis assays (30 μl) were carried out at 30°C. Aliquots (5 μl) were assayed for protein synthesis at the intervals indicated. The amount of inhibitory activity of the crude and partially purified HRI and dsI fractions added was approximately equal in all experiments. Control experiments included assays that had no additions and those in which control and immune sera (diluted and preincubated in the same fashion as the sera used to treat HRI and dsI fractions) were added. (*A*) Heat-activated HRI. Prior to addition to the protein synthesis assay, 4 μl of heat-activated HRI or buffer was preincubated with 1 μl of control or immune serum for 60 min at 0°C. Aliquots (0.6 μl) were added to the protein synthesis assay. ○, Control (no additions); ◐, control plus control serum; ◑, control plus immune serum; ●, heat-activated HRI; ▲, heat-activated HRI plus control serum; △, heat-activated HRI plus immune serum. (*B*) Partially purified HRI. Conditions for protein synthesis assay were as described in Table 1 except that the preincubation at 0°C with sera was as in *A*. ●, HRI; ▲, HRI plus control serum; △, HRI plus immune serum. (*C*) dsI lysate. Prior to addition to the protein synthesis assay, 4.5 μl of dsI lysate or buffer was diluted by the addition of 0.5 μl of control or immune serum and preincubated as described in *A*. Aliquots (1.5 μl) were added to the protein synthesis assay. Protein synthesis assays were as in *A* except that high concentrations of poly(I)·poly(C) (20 μg/ml) were added in this case. ●, dsI lysate; ▲, dsI lysate plus control serum; △, dsI lysate plus immune serum. (*D*) Partially purified dsI. First, 4.5 μl of partially purified dsI (84 μg) or buffer was diluted by the addition of 0.5 μl of control or immune serum and preincubated as described above. (The activity of the dsI preparation used in this experiment was lower than that usually found in other similar preparations.) Aliquots (3 μl) were added to the protein synthesis assay. ●, dsI; ▲, dsI plus control serum; △, dsI plus immune serum.

synthesis (Fig. 4 *A* and *B*), the immune serum is as effective against HRI that has been activated in lysates as it is against purified HRI. These data suggest that the purified reversible form of HRI is similar in nature to unprocessed HRI activated in reticulocyte lysates and that no major modifications occur during the purification process.

Although the protein kinase activated by dsRNA (dsI) has not yet been purified, it was of interest to determine whether it could be differentiated from HRI immunologically. The findings indicate that dsI, in both crude and more purified

preparations, is antigenically distinct from reversible HRI. The ability of dsI to phosphorylate eIF-2α and its ability to inhibit protein synthesis are unaffected by anti-HRI serum that inhibits these activities of HRI (Figs. 3 and 4).

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