Interferon-Induced (2′–5′)Oligoadenylate Synthetase: Adsorption to and Assay on Adenosine 2′,5′-Diphosphate–Sepharose†

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ABSTRACT: We have developed a new assay method for 2′,5′-oligoadenylate synthetase based on adsorption of crude cell extracts to adenosine 2′,5′-diphosphate (ADP)–Sepharose at 4 °C. After binding the enzyme from cell lysates, the resin was washed and incubated at 30 °C in the presence of adenosine 5′-triphosphate (ATP) and double-stranded ribonucleic acid (dsRNA). The 2′,5′-ppp(Ap)$_n$A [(2′–5′)oligo(A)] product was assayed by its ability to inhibit protein synthesis in a micrococcal nuclease pretreated mouse L cell extract programmed by encephalomyocarditis (EMC) viral RNA. By use of extracts from interferon-treated mouse L cells, the levels of (2′–5′)oligo(A) produced by this method were comparable to those obtained under identical conditions by using poly(inosinic acid)·poly(cytidylic acid) [(I)$_n$·(C)$_n$]–Sepharose and were higher than the amounts obtained in a direct solution assay. Preformed (2′–5′)oligo(A) was more stable under the conditions of the 2′,5′-ADP–Sepharose binding assay than under the solution assay conditions. In addition, the synthetase was relatively stable when attached to the resin; substantial activity remained after 24 h at 30 °C. Synthesis of (2′–5′)-oligo(A) was linear for ~5 h and was proportional to the amount of extract bound and to the concentration of ATP in the reaction incubation. The reaction was stimulated by Mg^{2+} concentrations up to ~10 mM and was independent of the K^+ concentration at levels below 0.1 M. The amount of (2′–5′)oligo(A) produced depended on the amount of and the nature of the dsRNA present. Activating nucleic acids that varied in potencies over a 10 000-fold range could be distinguished. The convenience and range of this assay make it suitable for the assay of 2′,5′-oligoadenylate synthetase in crude cell extracts when a direct solution assay does not provide high yields of (2′–5′)oligo(A) or when variation of the nature and/or concentration in the nucleic acid activator is desired.

Treatment of cells with interferon elicits the synthesis of new messenger RNAs, new proteins, and new enzymatic activities [Farrell et al., 1979; Knight & Korant, 1979; reviewed by Baglioni (1979)]. Two of these enzymes require activation by double-stranded RNA (dsRNA)[1] and have been implicated in the mechanism by which dsRNA inhibits protein synthesis in extracts from interferon-treated cells. These activities may also play a role in the establishment of the interferon-induced antiviral state in vivo. The addition of dsRNA to protein kinase containing extracts from interferon-treated cells or from rabbit reticulocytes results in the phosphorylation of a 67K protein and the small subunit of eIF2 (Roberts et al., 1976; Lebleu et al., 1976; Cooper & Farrell, 1977; Kimchi et al., 1979a,b; Samuel, 1979). A phosphatase that dephosphorylates the 67K protein has also been reported (Kimchi et al., 1979b; Epstein et al., 1980). Another dsRNA-dependent enzyme, (2′–5′)oligoadenylate synthetase, polymerizes ATP into the novel (2′–5′)-ppp(Ap)$_n$A [(2′–5′)oligo(A)][1] (Kerr & Brown, 1978). Extracts from a number of interferon-treated cell types, as well as extracts from rabbit reticulocytes and estrogen-treated and withdrawn chick oviduct, have shown high levels of this enzyme (Clemens & Williams, 1978; Williams et al., 1979a; Schmidt et al., 1978; Minks et al., 1979a; Ball & White, 1978; Stark et al., 1979). Since they show activity in the range of 0.3–5 nM, the 2′,5′-linked oligoadenylate molecules are potent inhibitors of cell free protein synthesis (Ball & White, 1978; Schmidt et al., 1978; Kerr & Brown, 1978; Minks et al., 1979a). The dimers are inactive, whereas the trimer, tetramer, and pentamer exhibit equal activities in some cell-free systems (Kerr & Brown, 1978; Ball & White, 1978).

Several reports have demonstrated that (2′–5′)oligo(A) reversibly activates an endonuclease in extracts from control or from interferon-treated cells; this results in the breakdown of mRNA and of polysomes (Schmidt et al., 1978; Clemens & Williams, 1978; Baglioni et al., 1978; Ball & White, 1979). Recently, Williams et al. (1979c) and Hovanessian et al. (1979) have demonstrated the ability of exogenously applied (2′–5′)oligo(A) to activate a nuclease and to inhibit reversibly protein synthesis in intact cells. In addition, (2′–5′)oligo(A) synthesized in vivo has been isolated from EMC virus infected mouse L cells that had been pretreated with interferon (Williams et al., 1979b). These observations suggested a role for the 2′,5′-oligoadenylate synthetase in the mechanism of the interferon-induced antiviral state.

To date two different methods have been used to assay (2′–5′)oligo(A) synthetase activity. The first involves adsorption of the synthetase to (I)$_n$·(C)$_n$–Sepharose (Hovanessian et al., 1977) or (I)$_n$·(C)$_n$–diazobenzyloxymethyl paper (Stark et al., 1979); the second assay of the synthetase is directly in solution (Minks et al., 1979a; Zilberstein et al., 1978). We report here a new method based on adsorption of the (2′–5′)oligoadenylate synthetase to 2′,5′-ADP–Sepharose.

Experimental Procedures

Nucleic Acids. (I)$_n$·(C)$_n$ was prepared by annealing (I)$_n$ ($s_{20,w}$ = 9.4) and (C)$_n$ ($s_{20,w}$ = 10.0) (P-L Biochemicals) as

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[1] Abbreviations used: (2′–5′)oligo(A) is used to designate 2′,5′-ppp(Ap)$_n$A or 5′-*O*-triphosphoryladenylyl(2′–5′)adenylyl(2′–5′)adenosine, where *n* has been reported to be between 1 and ~10; (I)$_n$, poly(inosinic acid); (C)$_n$, poly(cytidylic acid); *P. chrysogenum*, *Penicillium chrysogenum*; dsRNA, double-stranded ribonucleic acid; EMC, encephalomyocarditis. 2′,5′-ADP–Sepharose refers to adenosine 2′,5′-diphosphate linked covalently to Sepharose via the adenine 6-amino group. (I)$_n$·(C)$_n$–Sepharose refers to (I)$_n$·(C)$_n$ which has been linked covalently to cyanogen bromide activated Sepharose. The nature of the covalent linkage has not been established; mRNA, messenger RNA; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; DEAE, diethylaminoethyl; PEI, polyethylenimine; NADP, nicotinamide adenine dinucleotide phosphate.

described by Torrence & Friedman (1979). *Penicillium chrysogenum* dsRNA was from Beecham Pharmaceuticals (England), and Qβ RNA was purchased from Miles Laboratories (Elkhart, IN). EMC virus was grown in Krebs II cells, and the RNA was extracted and purified as described by Kerr & Martin (1972).

Affinity Resins. Resins based on Sepharose were obtained from Pharmacia Fine Chemicals (Upsala, Sweden). 2′,5′-ADP–agarose was purchased from Sigma Chemical Co. (St. Louis, MO). Matrix Gel Red A and Matrix Gel Blue A were obtained from Amicon (Lexington, MA).

Binding and Reaction Conditions. S100 extracts (21–30 OD_{280} mL^{-1}) were prepared from mouse L cells that had been pretreated overnight with 30 NIH reference units/mL (~2 × 10^7 NIH reference units/mg of protein) of mouse interferon as described previously (Torrence & Friedman, 1979). Binding was similar to that described by Hovanessian et al. (1977) for $(I)_n$·$(C)_n$–Sepharose. In a typical experiment, 100 μL of S100 was mixed at 0–4 °C for 2–18 h with an equal volume of packed 2′,5′-ADP–Sepharose or $(I)_n$·$(C)_n$–Sepharose equilibrated in a buffer of 20 mM Hepes, pH 7.5, 50 mM KCl, 1.5 mM $Mg(OAc)_2$, 7 mM β-mercaptoethanol, and 10–20% (v/v) glycerol. In most experiments binding was allowed to proceed overnight with an additional 20 μL of glycerol. After binding, the resin was washed 3 times with ~30 volumes of the above buffer and then incubated at 30 °C for 2–6 h with 100 μL of a fresh reaction buffer containing 20 mM Hepes, pH 7.5, 90 mM KCl, 10 mM $Mg(OAc)_2$, 7 mM β-mercaptoethanol, 10–20% (v/v) glycerol, 5 mM ATP, and 10^{-3}–10^{-7} M nucleic acid. Unless otherwise noted, 10^{-4}M $(I)_n$·$(C)_n$ was the activating nucleic acid. The supernatant was removed, heated at 95 °C for 5–15 min, and centrifuged to provide a (2′–5′)oligo(A)-containing supernatant that was then assayed for its ability to inhibit protein synthesis in an EMC viral RNA directed mouse L cell-free system.

The synthetase was assayed in solution essentially as described by Minks et al. (1979a). Reaction mixtures of 25–50 μL consisted of 10% S100 lysate, 0.12 M KOAc, 5–25 mM $Mg(OAc)_2$, 20 mM Hepes, pH 7.5, 1 mM dithiothreitol, 10^{-3}–10^{-7} M $(I)_n$·$(C)_n$, (usually 10^{-4} M), and 5 mM ATP. These mixtures were incubated at 30 °C and then heated and processed as described above.

Protein Synthesis. Untreated and preincubated–dialyzed extracts of mouse L_e or L_k cells were prepared as described previously (Torrence & Friedman, 1979). In the preparation of micrococcal nuclease pretreated extracts, S10 supernatants were digested with 2–10 μg/mL micrococcal nuclease (Worthington Biochemical Corp., Millipore) at 20 °C for 5–10 min in the presence of 1 mM $CaCl_2$ with occasional shaking. The reaction was quickly terminated by bringing the solution to 2 mM ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid (EGTA) (Pelham & Jackson, 1976). All extracts were stored in small aliquots in liquid nitrogen and were thawed once.

Serial three- or tenfold dilutions of (2′–5′)oligo(A)-containing solutions were assayed for their ability to inhibit protein synthesis by the addition of 2.5 μL of each dilution or of sterile distilled water into 25 μL of a cell-free synthesis mixture. Conditions for protein synthesis using EMC viral RNA as messenger were as described previously except that the $Mg(OAc)_2$ concentration was 1–3.5 mM (Torrence & Friedman, 1979). Protein synthesis, as measured by the incorporation of [^{3}H]leucine (New England Nuclear) into hot trichloroacetic acid insoluble counts, was plotted as a function of the dilution of inhibitor added. IC_{50} was defined as the dilution required to inhibit protein synthesis by 50%. The reciprocal of the IC_{50} was defined as the titer.

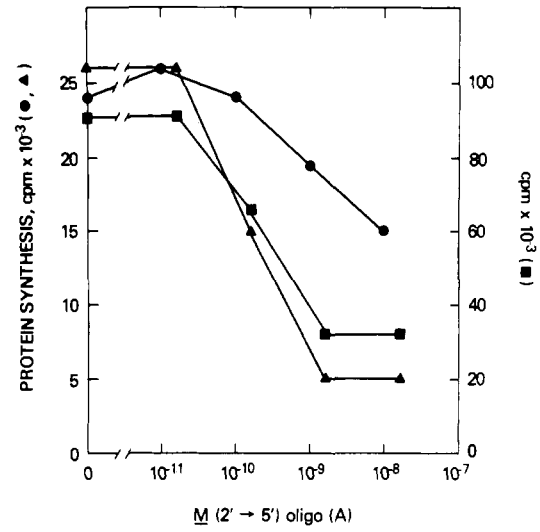

FIGURE 1: Sensitivity of differently prepared mouse L cell-free systems to the inhibitory effect of (2′–5′)oligo(A). Reaction mixtures were made 10% with dilutions of a solution of known concentration of (2′–5′)oligo(A) and incubated for 2 h with 1 μg of EMC viral RNA as described under Methods. Protein synthesis is expressed as counts per minute per 15 microliters of reaction mixture. The abscissa indicates the final concentrations of (2′–5′)oligo(A) in the reaction mixture. (●) Preincubated and dialyzed S10; (▲) micrococcal nuclease pretreated S10; (■) untreated S10. The endogenous levels of protein synthesis for these extracts were 1040, 400, and 29 000 cpm, respectively.

Analysis of Inhibitor Synthesized by the 2′,5′-ADP–Sepharose-Bound Enzyme. Tritiated inhibitor was synthesized by 2′,5′-ADP–Sepharose-bound enzyme at 30 °C for 8 h as described above with the addition of [^{3}H]ATP (29.9 Ci/mmol, New England Nuclear) at 100 Ci/mol. Inhibitor was purified by chromatography on DEAE-cellulose as described by Baglioni et al. (1979). Chromatographic analysis of the purified inhibitor was accomplished on a 0.9 × 29 cm DEAE-Sephacel column with a gradient of 50–300 mM KCl in 20 mM Hepes, pH 7.5, and 7 M urea (Kerr & Brown, 1978). Bacterial alkaline phosphatase (Worthington Biochemical Corp., Freehold, NJ) (15 units/mL, 37 °C, 4 h) was used to remove the 5′-triphosphate moiety of (2′–5′)oligo(A).

Results

Sensitivity of Various Extracts to (2′–5′)Oligo(A). A previous report by Williams et al. (1978) showed that the sensitivity of various cell-free systems to (2′–5′)oligo(A) varied with the cell source and method of preparation. Extracts prepared by different methods for use in the protein synthesis assay system were tested for their sensitivity to (2′–5′)oligo(A). As shown in Figure 1, EMC viral RNA directed protein synthesis in untreated and nuclease-pretreated extracts from control mouse L_e cells was inhibited 50% by 0.5–1.0 nM (2′–5′)oligo(A). In contrast, the extracts that had been preincubated and dialyzed were 10–100-fold less sensitive to inhibition by (2′–5′)oligo(A). The endogeneous protein synthesis in untreated extracts was not sensitive to inhibition by (2′–5′)oligo(A). Because of their low endogeneous levels of synthesis, nuclease-pretreated extracts were employed to assay the levels of (2′–5′)oligo(A) in all subsequent experiments.

Identification of Inhibitor. Radiolabeled inhibitor was synthesized by using [^{3}H]ATP. Analysis of the size distribution of DEAE-purified ^{3}H-labeled inhibitor was accomplished by chromatography on DEAE-Sephacel in the presence

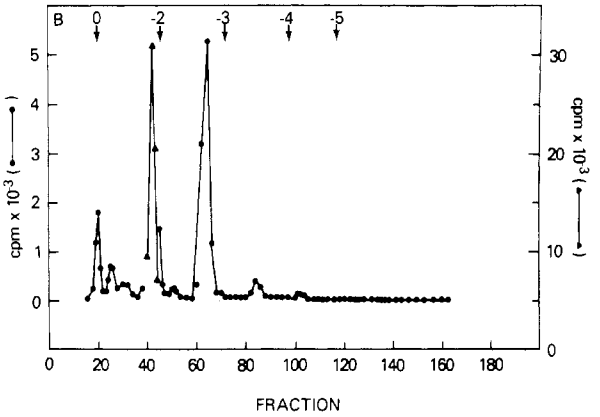

FIGURE 2: DEAE-cellulose chromatography of radiolabeled (2′–5′)oligo(A) in the presence of 7 M urea. Inhibitor was synthesized by the 2′,5′-ADP–Sepharose-bound enzyme in the presence of [^{3}H]ATP, purified, digested with bacterial alkaline phosphatase, and then chromatographed as described under Methods. Markers are adenosine, 3′,5′-$(Ap)_2$A, 3′,5′-$(Ap)_3$A, 3′,5′-$(Ap)_4$A, and 3′,5′-$(Ap)_5$A.

Table I: Comparison of (2′–5′)Oligoadenylate Synthetase Assay Procedures

expt	method	incubation time (h)	titer[a]
1	2′,5′-ADP–Sepharose	18	5000
	$(I)_n\cdot(C)_n$–Sepharose	18	5000
2	solution assay	0	<<100
		2	1250
		5	450
		7.5	<100
3	2′,5′-ADP–Sepharose	6	11000
	solution assay	2	1250

[a] Inhibitor synthesis was allowed to proceed for the specified time. The inhibitor-containing solution was then assayed by protein synthesis inhibition, and the titers were extrapolated from graphs of protein synthesis vs. dilution as described under Methods.

of 7 M urea. The inhibitor preparation eluted after the −4 charged marker and displayed a heterogeneous charge distribution. After digestion with bacterial alkaline phosphatase (Figure 2), two major sharp peaks, containing 74% and 19% of the label, eluted slightly ahead of the −2 and −3 markers, respectively. These results, suggesting that the major product was a trimer, were confirmed by chromatography of the digested material on PEI–cellulose plates (Kerr & Brown, 1978) (data not shown).

Synthesis of Inhibitor by Enzyme Bound to 2′,5′-ADP–Sepharose. S100 extracts from interferon-treated mouse L cells were bound to either 2′,5′-ADP–Sepharose or $(I)_n\cdot(C)_n$–Sepharose and assayed for (2′,5′)oligo(A) synthetase activity. The extract was also assayed for synthetase activity in a solution assay. As shown in Table I, the 2′,5′-ADP-bound enzyme produced as much inhibitor as did the $(I)_n\cdot(C)_n$-bound enzyme. Both titers were ~5000. (2′,5′-ADP–agarose gave identical results.) The titers obtained by the solution assay were about four- to tenfold lower and decreased after ~2 h. Similar results were obtained when an ATP-generating system was present in the solution assay (data not shown).

The stability of (2′–5′)oligo(A) under the conditions of the bound and solution assays was determined. Inhibitor purified by the method of Minks et al. (1979a) was incubated with either 2′,5′-ADP-bound enzyme or S100 extracts (in solution) under conditions identical with those used for the synthesis reactions, except that no ATP was present. After 2.5 h at 30 °C, the mixtures were heat-treated and centrifuged. The supernatants were rediluted and tested for their ability to

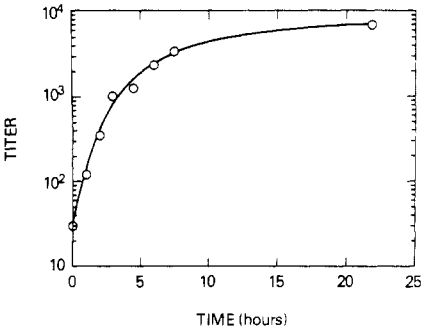

FIGURE 3: Time course of (2′–5′)oligo(A) synthesis by the 2′,5′-ADP–Sepharose-bound synthetase from extracts of interferon-treated mouse L cells. The amount of (2′–5′)oligo(A) synthesized at various times was determined by the protein synthesis inhibition assay and is expressed on the ordinate as the titer, which is defined under Methods.

inhibit protein synthesis. The degradation of (2′–5′)oligo(A) in the solution assay was virtually complete. Inhibitory activity decreased by greater than 200-fold during the 2.5-h incubation. In contrast, under the conditions of synthesis employing 2′,5′-ADP-bound enzyme, there was less than a 10-fold decrease in inhibitory capacity (data not shown).

Time Course and Enzyme Stability Experiments. The time course of synthesis of (2′–5′)oligo(A) by the mouse synthetase bound to 2′,5′-ADP–Sepharose was examined. The protein synthesis inhibitory activities of reaction supernatants obtained at various times were expressed as titers and are shown in Figure 3. Synthesis proceeded efficiently for ~5 h, after which time the titer of active (2′–5′)oligo(A) plateaued. Thin-layer chromatography of the reaction mixtures demonstrated that substantial amounts of ATP persisted after 5 h (data not shown). Also, reincubation of a washed enzyme–resin sample (previously incubated at 30 °C for 5 h) with fresh buffer, ATP, and $(I)_n\cdot(C)_n$ for a second 5 h resulted in the continued synthesis of (2′–5′)oligo(A) (data not shown). The observed plateau therefore was not due to the breakdown of substrate or to a rapid loss of synthetase from the resin.

The stability of the 2′,5′-ADP-bound synthetase was examined as follows. A reaction under standard conditions was allowed to proceed at 30 °C for 6 h. Half of the supernatant was removed and assayed for (2′–5′)oligo(A) by inhibition of protein synthesis. The remainder of the supernatant and the resin were incubated overnight at 30 °C. The resin was then washed 3 times with buffer and incubated with fresh buffer, ATP, and $(I)_n\cdot(C)_n$ for another 6 h, after which time a second sample was removed for inhibitor assay. This overnight incubation and reassay were continued for 2 more days. The results showed that substantial activity remained after incubation at 30 °C for 24 h and that some activity remained even after 72 h at 30 °C (Figure 4).

Dependence of Synthetase Activity on Concentrations of Extract, ATP, Mg^{2+}, K^+, and Nucleic Acid. The concentration of each constituent of the reaction mixture employing 2′,5′-ADP–Sepharose-bound synthetase was varied to determine the dependence of the synthetase activity on each.

S100 extracts from interferon-treated mouse L cells were mixed in varying proportions with extract from control mouse L cells and then assayed for synthetase activity by the 2′,5′-ADP-binding assay using 100 μL of combined extract. Figure 5 shows the inhibitory titers obtained as a function of the volume of extract from interferon-treated cells in the reaction mixture. For convenience, these results are plotted on a semilog scale. Synthesis of (2′–5′)oligo(A) was proportional to the amount of S100 from interferon-treated cells.

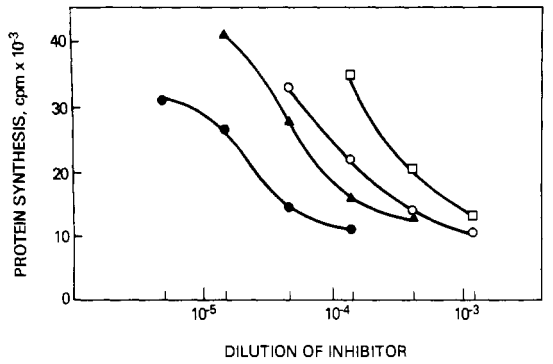

FIGURE 4: Ability of 2′,5′-ADP–Sepharose-bound extracts to synthesize (2′–5′)oligo(A) after incubation at 30 °C for various times. S100 supernatants from interferon-treated mouse L cells were bound at 0 °C for 2 h to 2′,5′–ADP–Sepharose as described under Methods. Incubation with 0.8×10^{-4} M $(I)_n \cdot (C)_n$ and 5 mM ATP was allowed to proceed for 6 h. Subsequent incubations were performed after overnight incubations at 30 °C as described in the text. The level of (2′–5′)oligo(A) in each mixture was measured by the protein synthesis inhibition assay. Control protein synthesis was 36 000 cpm. (●) First day; (▲) second day; (○) third day; (□) fourth day.

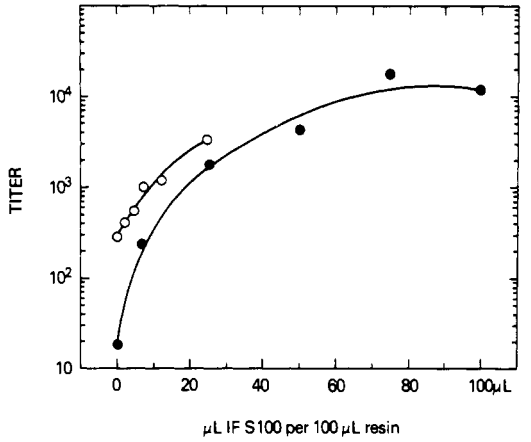

FIGURE 5: Synthesis of (2′–5′)oligo(A) by extracts from interferon-treated mouse L cells. Extract from interferon-treated mouse L cells was diluted with extract from control mouse L cells, and synthesis of (2′–5′)oligo(A) was accomplished by using 100 μL total of S100 per 100 μL of 2′,5′-ADP–Sepharose (●) or 100 μL of S100 per 200 μL of 2′,5′-ADP–Sepharose (○). The abscissa indicates the microliters of interferon S100 per 100 microliters of resin employed in the 5-h incubations, and the ordinate indicates the amount of (2′–5′)oligo(A) synthesized as measured by protein synthesis inhibition, indicated here as the titer.

The reaction also required ATP, and synthesis was linear up to concentrations of ~5 mM (Figure 6a); at ATP concentrations >10 mM, the titer of (2′–5′)oligo(A) declined.

As shown in Figure 6b, the concentration of Mg^{2+} also affected the reaction. Maximal amounts of protein synthesis inhibitor were obtained at concentrations of $Mg(OAc)_2$ >10 mM. At concentrations <10 mM, net yields of (2′–5′)oligo(A) were lower.

Synthesis of inhibitor by the 2′,5′-ADP-bound enzyme was completely independent of K^+ concentration up to ~0.1 M (Figure 6c). Only a twofold decrease in activity was measured at 0.35 M KOAc. Higher concentrations of salt elute protein from the resin (data not shown).

Synthetase activity was also dependent on the concentration and the nature of the activating dsRNA. Figure 7 presents illustrative data comparing $(I)_n \cdot (C)_n$ with *P. chrysogenum* RNA and with Qβ RNA, which are stronger and weaker activators, respectively, than $(I)_n \cdot (C)_n$. These RNAs varied over a 10 000-fold range in their abilities to activate the (2′–5′)oligoadenylate synthetase.

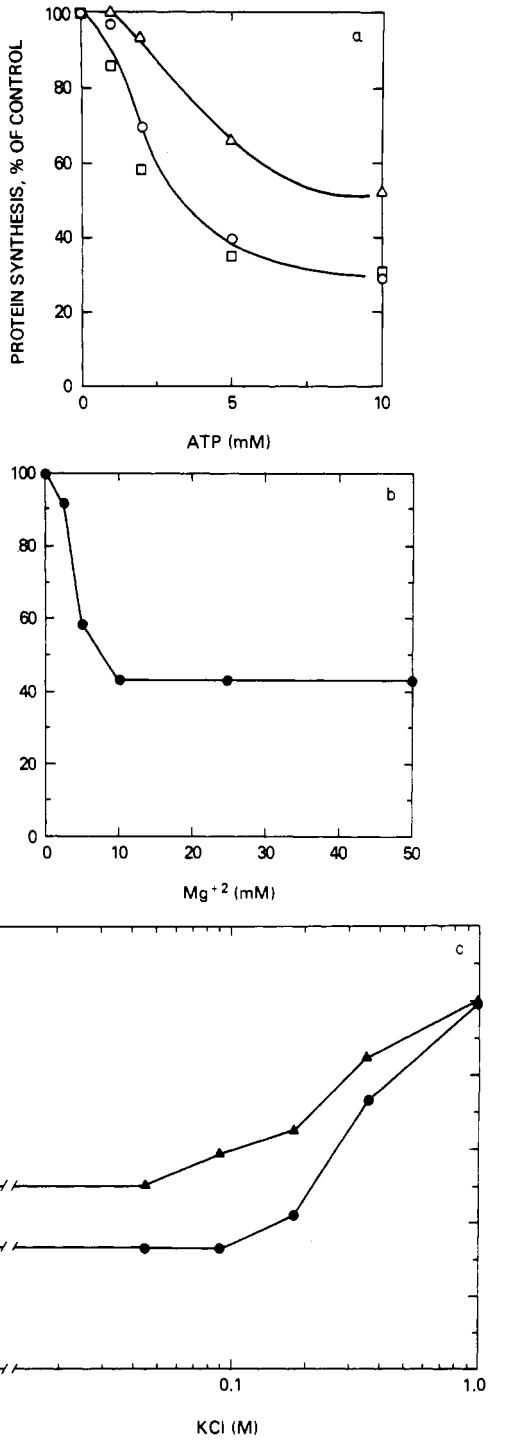

FIGURE 6: Dependence of (2′–5′)oligo(A) synthesis on ATP, $Mg(OAc)_2$, and KCl. The concentration of ATP, $Mg(OAc)_2$, or KCl was varied in the reaction buffer added to the 2′,5′-ADP–Sepharose-bound 2′,5′-oligoadenylate synthetase (see Methods). Incubations were for 5 h. Supernatants were then assayed at the indicated dilutions for inhibition of protein synthesis (see Methods). The extent of protein synthesis inhibition is represented as the percent of control [minus (2′–5′)oligo(A)] and is plotted against the concentration of the varied constituent in the original synthetase reaction. (a) ATP varied with the Mg^{2+} to ATP ratio held constant at 2: (□, ○) 3.7×10^{-3} dilution of reaction mixture; (△) 1.2×10^{-3} dilution. (b) Mg^{2+} varied: (●) 2×10^{-4} dilution. (c) KCl varied: (●) 4×10^{-4} dilution; (▲) 1×10^{-4} dilution.

Discussion

We have reported here a new assay method for 2′,5′-oligoadenylate synthetase, based on adsorption of the enzyme to 2′,5′-ADP–Sepharose at 4 °C. Activation of the enzyme

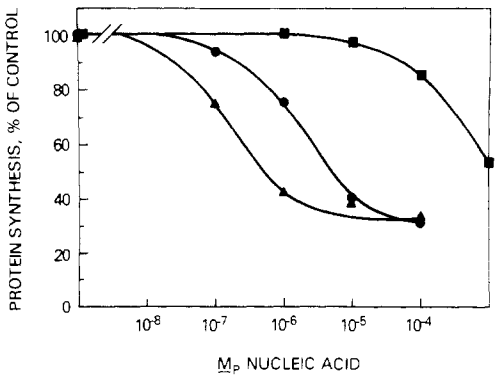

FIGURE 7: Dependence of (2′–5′)oligo(A) synthesis on the concentration and nature of the nucleic activator. (▲) *P. Chrysogenum* dsRNA; (●) $(I)_n \cdot (C)_n$; (■) Qβ RNA. Synthetase reaction mixtures were prepared by using the 2′,5′-ADP–Sepharose-bound enzyme as described under Methods except that the final concentration of the nucleic acid activator was varied. After a 5-h incubation at 30 °C, the reaction mixtures were processed as described and assayed at a final 10000-fold dilution for inhibition of protein synthesis. The extent of protein synthesis inhibition is represented as the percent of control [minus (2′–5′)oligo(A)] and is plotted against the concentration of nucleic acid in the original synthetase reaction.

by double-stranded RNA produced (2′–5′)oligoadenylate molecules which were assayed by their ability to inhibit protein synthesis. As reported previously by Williams et al. (1978), we have found that the sensitivity of cell-free systems varied with the methods used in their preparation. An EMC viral RNA directed, micrococcal nuclease pretreated cell-free system from mouse L cells was selected for use in the experiments described here. This system was sensitivie to subnanomolar amounts of (2′–5′)oligo(A), comparable to similar systems used by others (Ball & White, 1978; Kerr & Brown, 1978).

Extracts from interferon-treated or from control mouse L cells (as well as other cell types) contain enzymes that inactivate (2′–5′)oligo(A) (Schmidt et al., 1978; Williams et al., 1978; Minks et al., 1979b). The amount of (2′–5′)oligo(A) in any reaction mixture involving crude cell extracts therefore reflects the balance between synthesis and degradation. This complicates the quantitation and comparison of synthetase levels among different cell types in which the ratio of synthetic to degradative activities may vary. Also, variations in assay conditions may alter the ratio of these enzymatic activities (Minks et al., 1979b; P. F. Torrence and M. I. Johnston, unpublished observations).

In this study we observed very low levels of (2′–5′)oligo(A) synthetase activity in extracts from interferon-treated mouse L cells by using a solution assay similar to that described by Minks et al. (1979). Synthesis of the inhibitor by either $(I)_n \cdot (C)_n$-bound or 2′,5′-ADP-bound synthetase proceeded much better. The absence of substantial (2′–5′)oligo(A) synthesis in the solution assay was not due to degradation of the synthetase since the enzyme could be bound to 2′–5′-ADP–Sepharose and activated after incubation under the solution assay conditions (data not shown). Also, we observed that the inhibitory capability of purified (2′–5′)oligo(A) was significantly more stable under conditions of the 2′,5′-ADP–Sepharose assay than under the conditions used in the solution assay. These results suggest that inactivating enzymes were removed or inactivated to some extent in the binding procedure.

The 2′,5′-oligoadenylate synthetase activity was stable when bound to the 2′,5′-ADP–Sepharose, and synthesis proceeded at a linear rate for ~5 h. After 5 h potentially active synthetase and ATP persisted (≥3 mM, data not shown). The time course data (Figure 4) was plotted on a semilog scale to emphasize the increase in titers at early times. When the results are plotted on a linear scale, the plateau in titer is much less dramatic. As the reaction progresses, there may be a greater tendency for the synthetase to add AMP residues to preexisting molecules, rather than to synthesize new molecules. Since oligomers of three units or longer are reported to have equal protein synthesis inhibitory activity capabilities, the lengthening process would not be expected to result in an increase in inhibitory activity (Kerr & Brown, 1978; Ball & White, 1978). Indeed, time course samples which at later times plateau in protein synthesis titer do display a continuing linear increase in ATP incorporation (M. I. Johnston, K. Zoon, R. F. Friedman, E. De Clercq, and P. F. Torrence, unpublished experiments).

The amount of (2′–5′)oligo(A) produced in this assay was proportional to the amount of S100 applied; linearity was observed with about 0–20 μL of S100 per 200 μL of packed 2′,5′-ADP–Sepharose. Similar results have been reported for the $(I)_n \cdot (C)_n$–resin method. Using 250 μL of $(I)_n \cdot (C)_n$–Sepharose, Baglioni et al. (1979) reported a linear relationship between the amount of S100 applied (0–20 μL) and the amount of (2′–5′)oligo(A) synthesized. Experiments not shown here suggest that little or no synthetase remained in the supernatant after binding to 2′,5′-ADP–Sepharose; inactivation of the enzyme in solution during the binding reaction could not be discounted. It is also possible that some of the bound enzyme molecules could not express activity because of steric hindrance between protein molecules or between the long $(I)_n \cdot (C)_n$ activator chains. However, either 2′,5′-ADP–Sepharose or $(I)_n \cdot (C)_n$–Sepharose would be useful in the quantitation of activatable synthetase levels in crude extracts provided that appropriate resin levels are employed.

The 2′,5′-ADP–Sepharose-bound synthetase was dependent on the presence of Mg^{2+} and ATP, but not on K^+ levels below 0.1 M. Above 0.1 M the decrease in synthetase activity observed may have been due either to elution of the synthetase from the resin or to direct inactivation of bound enzyme molecules. The Mg^{2+} optimum of 10 mM was lower than that reported by Minks et al. (1979a) for the synthetase from interferon-treated HeLa cells assayed in solution (25 mM). Synthesis of (2′–5′)oligo(A) was proportional to the concentration of ATP up to 10 mM. The reaction also displayed a linear dose–response relationship with respect to the concentration of activator RNA present. This assay can distinguish nucleic acids that vary over a 10000-fold or greater range in activity potency. The single-stranded Qβ RNA probably was able to activate the synthetase by virtue of contaminating segments of dsRNA (Robertson et al., 1968).

The convenience, reproducibility, and range of this assay method makes it suitable for the study of (2′–5′)oligoadenylate synthetase. The assay has the advantages that (1) crude extracts may be employed, (2) as compared to the solution assay described by Minks et al. (1979), high titers of (2′–5′)oligo(A) can be obtained from extracts that contain high levels of inactivating enzymes, and (3) the nature and concentration of the nucleic acid activator can be varied readily. Although various nucleic acids can be coupled to Sepharose or to paper (Stark et al., 1979), a simple measurement of the nucleic acid concentration is not possible.

The observation that (2′–5′)oligo(A) synthetase can bind 2′,5′-ADP–Sepharose and retain activity was an unexpected one. 2′,5′-ADP would not be expected to be a substrate for the synthetase by virtue of the 2′-phosphate substituent (Ball, 1980). Instead, the resin may act as a product affinity ligand. During the reaction a small percentage of the enzyme mole-

cules may be removed from the column, and these molecules may be responsible for the synthesis of (2′–5′)oligo(A). [Such a leakage would account for the decreased activity of the bound enzyme over long (72-h) incubation periods. Alternatively, the bound molecules may become inactive.]

We have tested the ability of various molecules to decrease the synthetase activity bound to the column. Both KCl and 2′,5′-ADP were effective; after washes of 0.7 M and 5 mM, respectively, followed by equilibration with reaction buffer, the levels of (2′–5′)oligo(A) synthesized by both resins were <5% of the control buffer washed resin. Less effective was 50 mM NADP, which resulted in a decrease of (2′–5′)oligo(A) titer of 60%. The enzymatic activity in the washes was usually <10% of the activity of the bound enzyme. In addition, other supports were tested for their ability to replace 2′,5′-ADP–Sepharose in this assay. Blue Sepharose, Matrix Gel Red A, Matrix Gel Blue A, and dATP–Sepharose were inactive in the assay. In contrast, Sepharose containing 3′,5′-ADP, 5′-ATP, 5′-AMP, or adenosine ligands were active to varying degrees under the conditions described here, although all were less active than 2′,5′-ADP–Sepharose (P. F. Torrence and M. I. Johnston, unpublished observations). In addition to its use in the synthetase assay system described here, the 2′,5′-ADP–Sepharose resin and other singular resins may prove to be useful in the purification of (2′–5′)oligoadenylate synthetase.

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