noglobulin-induced conformational changes in proteins are certainly well established (Celada & Strom, 1972). Work designed to obtain information on these points is currently in progress.

Acknowledgments

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References

Abe, T., & Tamiya, N. (1979) *Toxicon 17*, 571–582.
Berzofsky, J. A., Hicks, G., Ferdoko, J., & Minna, J. (1980) *J. Biol. Chem. 255*, 11188–11191.
Boquet, P. (1979) *Handb. Exp. Pharmacol. 52*, 751–824.
Boulain, J. C. (1979) Thêse 3ême cycle, Paris VI.
Bystrov, V. F., Arseniev, A. S., & Gavrilov, Yu, D. (1978) *J. Magn. Reson. 30*, 151–184.
Celada, F., & Strom, R. (1972) *Q. Rev. Biophys. 5*, 395–425.
Chang, C. C. (1979) *Handb. Exp. Pharmacol. 52*, 309–376.
Faure, G. (1980) Diplôme d'Etudes Approfondies, Paris VI.
Faure, G., Mênez, A., Boulain, J. C., Sato, A., Tamiya, N., & Fromageot, P. (1981) *Toxicon* (in press).
Fryklund, L., & Eaker, D. (1975) *Biochemistry 14*, 2865–2871.
Grishin, E. V., Sukhikh, A. P., Lukyanchuk, N. N., Slobobyan, L. N., Lipkin, Yu. V. N., Ovchinnikov, A., & Sorokin, V. M. (1973) *FEBS Lett. 36*, 77–78.
Ishikawa, Y., Mênez, A., Hori, H., Yoshida, H., & Tamiya, N. (1977) *Toxicon 15*, 477–488.
Kimball, M. R., Sato, A., Richardson, J. S., Rosen, L. S., & Low, B. W. (1979) *Biochem. Biophys. Res. Commun. 88*, 950–959.
Köhler, G., & Milstein, C. (1975) *Nature (London) 256*, 495–497.
Low, B. W. (1979) *Handb. Exp. Pharmacol. 52*, 213–257.
Mênez, A., Morgat, J. L., Fromageot, P., Ronsseray, A. M., Boquet, P., & Changeux, J. P. (1971) *FEBS Lett. 17*, 333–335.
Mênez, A., Boulain, J. C., & Fromageot, P. (1979) *Toxicon 17 (Suppl. 1)*, 123.
Mênez, A., Boulain, J. C., Faure, G., Couderc, J., Liacopoulos, P., Tamiya, N., & Fromageot, P. (1982) *Toxicon 20*, 95–103.
Sobel, A., Weber, M., & Changeux, J. P. (1977) *Eur. J. Biochem. 80*, 215–224.
Tamiya, N., Takasaki, C., Sato, A., Menez, A., Inagaki, F., & Miyazawa, T. (1980) *Biochem. Soc. Trans. 8*, 753–755.
Weber, M., & Changeux, J. P. (1974) *Mol. Pharmacol. 10*, 1–14.
Yang, C. C. (1979) *Toxicon 17 (Suppl. 1)*, 205.

Dual Effects of Pyridoxal 5′-Phosphate on Glucocorticoid–Receptor Complexes†

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ABSTRACT: The ability of pyridoxal 5′-phosphate to inhibit DNA–cellulose binding of activated glucocorticoid–receptor complexes is pH and protein concentration dependent. At the tested pHs, all of the inhibitory activity of pyridoxal 5′-phosphate appears to be due to its ability to form a Schiff base. 2-Amino-2-(hydroxymethyl)-1,3-propanediol (100 mM) is unable to prevent or reverse the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding, while the same concentration of lysine is partially effective. Pyridoxal 5′-phosphate does not alter the elution profile of glucocorticoid–receptor complexes as ascertained by diethylaminoethyl (DEAE)-cellulose or DEAE-Sephadex chromatography. This observation permitted the use of these resins in detecting the previously unreported stimulation of glucocorticoid–receptor complex activation by pyridoxal 5′-phosphate. This stimulation is specific for pyridoxal 5′-phosphate and appears to be mediated via a Schiff base formation. Additionally, glucocorticoid–receptor complexes activated by pyridoxal 5′-phosphate treatment at low temperatures do not differ in size from thermally activated complexes. Thus, in vitro, pyridoxal 5′-phosphate can exert both a stimulatory effect on activation as well as an inhibitory effect on the binding of activated complexes to DNA–cellulose.

Glucocorticoid–receptor complexes undergo a two-step process in order to bind to nuclei, chromatin, or purified DNA (Baxter et al., 1972; Milgrom et al., 1973; Simons et al., 1976). The first step, termed "activation", is presumed to involve a conformational change resulting in a more positively charged molecular species with an increased affinity for DNA and other polyanions (Milgrom et al., 1973; Kalimi et al., 1975). This conformational change is reflected in the observed shift in the elution of glucocorticoid–receptor complexes from DEAE-Sephadex and DEAE-cellulose columns at lower salt concentrations (Parchman & Litwack, 1977; Sakaue & Thompson, 1977). The second step, termed "translocation", involves the movement and subsequent binding of the activated complex to a nuclear acceptor site. Thus, the receptor-mediated biological response will be observed only when activation is followed by translocation.

Endogenous inhibitors of either the activation or the

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translocation of glucocorticoid–receptor complexes have been reported [see Sekula et al. (1981) for a review]. When pyridoxal 5′-phosphate is added exogenously, it has been shown to be a structurally specific inhibitor of the binding of activated receptor complexes to DNA–cellulose or isolated nuclei (Cake et al., 1978; Cake & Litwack, 1978). In addition to its effect on glucocorticoid receptors, pyridoxal 5′-phosphate also inhibits other steroid hormone receptors [see Nishigori et al. (1978) and DiSorbo & Litwack (1982) for a review]. A comparison of reports on pyridoxal 5′-phosphate effects is sometimes complicated by the use of different experimental conditions and assays. For this reason, we have investigated the effect of pH and protein concentration on the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding. We have also observed the inability of pyridoxal 5′-phosphate to alter the elution profile of glucocorticoid–receptor complexes during DEAE-Sephadex[1] or DEAE-cellulose chromatography, suggesting a minimal number of coenzyme molecules bound per receptor. This observation was especially important since it afforded us the only possible way (i.e., through ion-exchange chromatography) of examining the direct effect of this translocation inhibitor on the activation of glucocorticoid–receptor complexes. Surprisingly, pyridoxal 5′-phosphate stimulated in vitro activation. Pyridoxal 5′-phosphate accomplished this previously unreported effect without altering receptor size and in a manner identical with its inhibition of DNA–cellulose binding (i.e., through Schiff base formation). Thus, it appears that pyridoxal 5′-phosphate can exert two opposing effects on glucocorticoid–receptor complexes. The implications of these novel findings are discussed.

Materials and Methods

Materials

Chemical Reagents. Pyridoxal, pyridoxal 5′-phosphate, pyridoxine, pyridoxamine, pyridoxamine 5′-phosphate, and sodium borohydride were obtained from the Sigma Chemical Co. One hundred millimolar stock solutions of pyridoxal 5′-phosphate and its analogues were prepared in distilled water and adjusted to pH 7.2. Sodium borohydride was prepared as a 1 M stock in distilled water just prior to its use. Tris[1] and lysine were prepared as 1 and 0.5 M stock solutions, respectively, and adjusted to pH 8.0.

Methods

Preparation of Cytosol. Adrenalectomized rats (CD Sprague-Dawley strain) were used 4–8 days following surgery. Animals were killed by decapitation and the livers perfused in situ with cold 0.15 M NaCl through the portal vein. The livers were removed and homogenized in an equal volume of either TSM buffer (0.05 M Tris-HCl, 0.25 M sucrose, and 5 mM $MgCl_2$, pH 8.0 at 0 °C) or BSM buffer (0.2 M boric acid, 0.25 M sucrose, and 5 mM $MgCl_2$, pH 8.0, at 0 °C), except for the pH dependency studies where the livers were homogenized in TSM buffer (pH 6.8) or BSM buffer (pH 6.2). The homogenate was centrifuged at 105000*g* for 1 h at 0–4 °C. After the upper lipid layer was discarded, the cytosol was removed and stored under liquid nitrogen until further use.

Specific Cytosolic Binding of Triamcinolone Acetonide. The cytosol was incubated for 2 h at 0–4 °C with 50 nM [^{3}H]triamcinolone acetonide (New England Nuclear, 37.0 Ci/mmol) in the presence or absence of a 1000-fold excess of nonradioactive steroid. Specific macromolecular bound steroid was determined by using the dextran-coated charcoal technique (Beato & Feigelson, 1972). Protein concentration was determined by the method of Lowry et al. (1951).

Assay of Receptor Binding to DNA–Cellulose. The binding of glucocorticoid–receptor complexes to DNA–cellulose was determined by the procedure of Kalimi et al. (1975). A 100-μL aliquot, preincubated with [^{3}H]triamcinolone acetonide (TA), was added to a test tube containing 50 μL of packed DNA–cellulose (P-L Biochemical Co., 1 mg of native DNA/mL of cellulose) and incubated for 45 min at 0 °C with occasional mixing. At the end of the incubation, 2 mL of ice-cold TE buffer (10 mM Tris-HCl and 1 mM EDTA, pH 8.0) was added, mixed, and centrifuged for 5 min at 600*g*. The final DNA–cellulose pellet obtained after two additional washes with the same buffer was resuspended in 0.8 mL of TE buffer, and an aliquot was added to 10 mL of Liquiscint (National Diagnostics) for determination of radioactivity. The average counting efficiency for tritium was 30%.

Chromatographic Procedures. All chromatographic procedures were performed in a 4 °C cold room. Sephadex G-25 chromatography was performed on prepacked PD-10 minicolumns (Pharmacia; 9.1-mL bed volume), which were equilibrated and eluted with the homogenization buffer. DEAE-Sephadex A-50 columns (8-mL bed volume) were prepared in 10-cm^3 glass syringes. Dextran-coated charcoal (0.5 mL) was mixed with the initial 2 mL of resin to adsorb free steroid during chromatography (Parchman & Litwack, 1977). The columns were equilibrated with 0.02 M potassium phosphate buffer (pH 6.8 at 0–4 °C). A 0.5-mL sample of [^{3}H]triamcinolone acetonide labeled cytosol was applied to the column which was then washed with 16 mL of the equilibration buffer. The wash was collected as eight 2-mL fractions. Adsorbed hormone–receptor complexes were eluted with a 0–1 M linear KCl gradient prepared in the equilibration buffer. Fractions (0.5 mL) were collected and analyzed for bound radioactivity and KCl concentration. The KCl concentration was determined by measuring the conductivity of appropriate samples with a Markson Model 10 conductivity meter and comparison with a standard KCl curve.

DEAE-cellulose (Whatman DE 52) columns (3-mL bed volume) were prepared in 5-cm^3 plastic, disposable syringes. The columns were equilibrated with KPD buffer (5 mM potassium phosphate and 0.5 mM dithiothreitol, pH 7.6, at 0–4 °C). Prelabeled cytosol was treated with dextran-coated charcoal, and the sample was applied to the column. The column was washed with 10 mL of the equilibration buffer, and the wash was discarded. Bound radioactivity was eluted with a linear 5–400 mM potassium phosphate gradient. Thirty 1-mL fractions were collected and analyzed for radioactivity and salt concentration as described above.

A Sephadex G-200 column (43 × 25 cm) was prepared and equilibrated with 0.05 M Tris buffer (pH 7.5 at 0 °C) containing 0.1 M NaCl at a pressure of 15 cm of H_2O. The column had a void volume (V_0) of 90.4 mL and a total volume (V_t) of 211.0 mL. The column was calibrated by using the purified proteins ferritin (61.5 Å), catalase (51.3 Å), albumin (35.5 Å), ovalbumin (28.6 Å), chymotrypsinogen A (20.9 Å), and RNase A (16.4 Å). [^{3}H]TA-labeled cytosol was treated with dextran-coated charcoal prior to Sephadex G-200 chromatography. Fractions (2 mL) were collected at a flow rate of 10 mL/h and then analyzed for radioactivity. The Stokes radii of the radioactive peaks were determined graphically from

[1] Abbreviations: Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid.

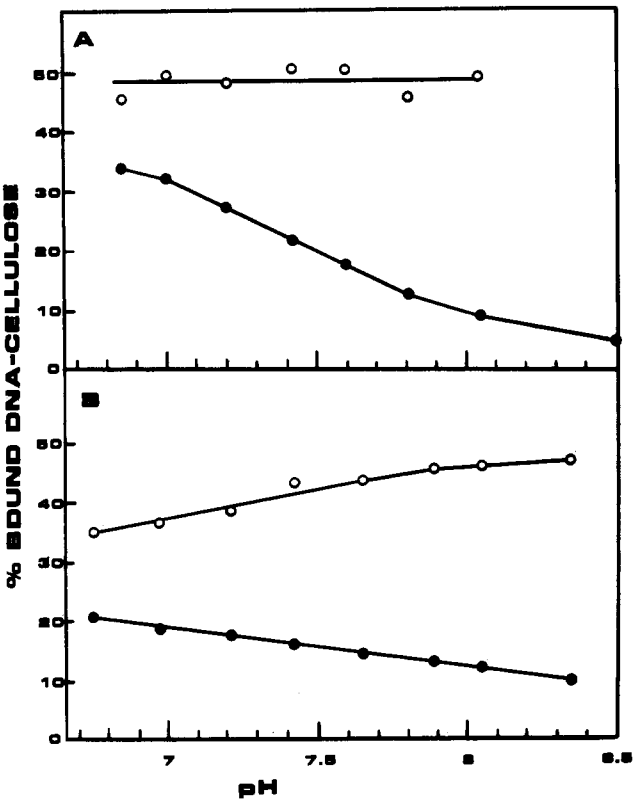

FIGURE 1: Effect of pH on the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding by activated [^{3}H]TA–receptor complexes. Experimental details are described under Results. Cytosols were prepared in BSM buffer (A) or TSM buffer (B) and incubated in the presence (●) or absence (○) of 5 mM pyridoxal 5′-phosphate prior to determining their DNA–cellulose binding ability.

a plot of K average [$K_{av} = (V_e - V_0)/(V_t - V_0)$] vs. Stokes radius in angstroms.

Results

Effect of pH on the Ability of Pyridoxal 5′-Phosphate To Inhibit DNA–Cellulose Binding of Activated Glucocorticoid–Receptor Complexes. The pH of rat liver cytosol was adjusted with 1 N NaOH and 1-mL aliquots were removed to determine if the ability of pyridoxal 5′-phosphate to inhibit DNA–cellulose binding of activated glucocorticoid–receptor complexes is pH dependent. Cytosols were labeled with [^{3}H]TA, heat activated (25 °C for 45 min), and then incubated (0 °C for 30 min) in the presence or absence of 5 mM pyridoxal 5′-phosphate. Bound radioactivity and DNA–cellulose binding were then determined (Figure 1). Under our experimental conditions, it appeared that pH affects the degree of activation or the extent of DNA–cellulose binding (or both) achieved in the untreated cytosol prepared in TSM buffer (B), whereas cytosol prepared in BSM buffer was independent of this pH effect (A). Regardless of this effect, the ability of pyridoxal 5′-phosphate to inhibit DNA–cellulose binding was pH dependent, and this dependency was observed in both BSM buffer (A) and TSM buffer (B). The greatest inhibition of DNA–cellulose binding was observed at the most alkaline pH tested. Pyridoxal 5′-phosphate's inhibitory activity was clearly related to its ability to block DNA–cellulose binding, since pyridoxal 5′-phosphate did not affect steroid binding in the pH range of 6.7–8.2 (data not shown). The addition of 5 mM pyridoxal 5′-phosphate prior to heat activation also resulted in a pH-dependent inhibition of DNA–cellulose binding (data not shown).

Reversibility of the Observed Pyridoxal 5′-Phosphate Mediated Inhibition of DNA–Cellulose Binding. Cake et al. (1978) have shown that the observed inhibition of DNA–cellulose binding by pyridoxal 5′-phosphate apparently operates through a Schiff base, since the inhibition cannot be reversed by gel filtration following reduction by sodium borohydride. Since these experiments were performed at pH 8.0, it was necessary to quantitate at other pHs the amount of the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding that is related to its Schiff base forming ability. Aliquots of rat liver cytosol prepared in BSM buffer were removed after pH adjustment, labeled with [^{3}H]TA, and heat activated (25 °C for 1 h). At each pH, the cytosol was incubated in the presence or absence of 5 mM pyridoxal 5′-phosphate (0 °C for 30 min), followed by reduction with 10 mM sodium borohydride (0 °C for 20 min). In this pH range, borohydride alone slightly reduced steroid binding. An aliquot from each treatment was chromatographed on a prepacked Sephadex G-25 column and the macromolecular fraction collected. Bound radioactivity and DNA–cellulose binding were then determined. Comparison of the DNA–cellulose binding ability of post G-25 samples with those receiving no G-25 treatment should reflect the amount of pyridoxal 5′-phosphate inhibition which is Schiff base related. The results are shown in Table I. The data suggest that at the tested pHs virtually all of the pyridoxal 5′-phosphate inhibitory activity is associated with its ability to form a Schiff base. Thus, only one mechanism of inhibition of DNA–cellulose binding by pyridoxal 5′-phosphate appears to be operating in this pH range.

Table I: Irreversibility of the pH-Dependent; Pyridoxal 5′-Phosphate Mediated Inhibition of DNA–Cellulose Binding by Activated [^{3}H]TA-Receptor Complexes following Reduction by Sodium Borohydride[a]

additions	pH	% bound DNA–cellulose[b] prior to gel filtration	% bound DNA–cellulose[b] after gel filtration
10 mM $NaBH_4$	7.0	48.4	48.0
5 mM pyridoxal-P + 10 mM $NaBH_4$	7.0	23.9 (49.4)[c]	26.7 (55.6)
10 mM $NaBH_4$	7.5	57.4	46.1
5 mM pyridoxal-P + 10 mM $NaBH_4$	7.5	19.2 (33.5)	16.3 (35.4)
10 mM $NaBH_4$	8.0	55.2	39.4
5 mM pyridoxal-P + 10 mM $NaBH_4$	8.0	12.4 (22.5)	11.0 (27.9)

[a] Following pH adjustment with 1 N NaOH, rat liver cytosol was labeled with [^{3}H]TA, heat activated (25 °C for 1 h), and then incubated in the presence or absence of 5 mM pyridoxal-P (0 °C for 30 min). All samples were reduced with 10 mM $NaBH_4$ (0 °C for 20 min). Aliquots were assayed for DNA–cellulose binding and bound radioactivity prior to and after gel filtration on Sephadex G-25 M. [b] (DNA–cellulose binding)/(total cytoplasmic [^{3}H]TA binding) × 100. [c] Values in parentheses represent the DNA–cellulose binding as a percentage of the appropriate control.

Since the observed effect of pyridoxal 5′-phosphate appears to be due to the formation of a Schiff base, the primary amines Tris and lysine were then examined for their ability to prevent the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding (Table II). The results show that the addition of 100 mM Tris either prior to or following the addition of 5 mM pyridoxal 5′-phosphate failed to block or reverse the observed inhibition of DNA–cellulose binding. Lysine (100 mM), on the other hand, partially blocked and reversed the pyridoxal 5′-phosphate inhibition to the same extent. The difference in the abilities of lysine and Tris to alter pyridoxal 5′-phosphate's inhibition of DNA–cellulose binding is perhaps best understood in light of the study on the stability constants

Table II: Effect of Tris and Lysine on the Pyridoxal 5′-Phosphate Mediated Inhibition of DNA-Cellulose Binding of Activated [^{3}H]TA-Receptor Complexes[a]

expt	sequence of additions	% bound DNA-cellulose[b]
I	control	55.7
	100 mM Tris	53.8 (97)[c]
	5 mM pyridoxal-P	9.7 (17)
	5 mM pyridoxal-P → 100 mM Tris	13.9 (25)
II	100 mM Tris[d]	31.4
	100 mM Tris → 5 mM pyridoxal-P	7.5 (24)
III	control	20.0
	100 mM lysine	22.4 (112)
	5 mM pyridoxal-P	5.4 (27)
	100 mM lysine → 5 mM pyridoxal-P	14.2 (71)
	5 mM pyridoxal-P → 100 mM lysine	15.8 (79)
IV	control	32.1
	5 mM pyridoxal-P	8.9 (28)
	100 mM lysine → 5 mM pyridoxal-P	17.2 (54)
	5 mM pyridoxal-P → 100 mM lysine	17.8 (55)

[a] Heat-activated [^{3}H]TA-receptor complexes were incubated at 0 °C for 30 min with the indicated compounds. DNA-cellulose binding and bound radioactivity were then determined. [b] Defined in Table I. [c] Values in parentheses represent the DNA-cellulose binding as a percentage of the appropriate control. [d] 100 mM Tris also served as the control since no significant difference was observed between control sample and one containing 100 mM Tris.

of Schiff bases formed between pyridoxal 5′-phosphate and amino compounds (Matsuo, 1957). Schiff bases formed by using Tris as the amino source were found to be less stable than those formed by using amino acids. Thus, lysine might be expected to compete to a greater extent than Tris for pyridoxal 5′-phosphate.

Effect of Protein Concentration on the Pyridoxal 5′-Phosphate Inhibition of DNA–Cellulose Binding. The ability of pyridoxal 5′-phosphate to block the 4.6S to 6S transition of rat uterine estrogen receptors has been reported to be dependent on the protein concentration of the cytosol (Traish et al., 1980). To determine whether protein concentration can affect the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding, we diluted previously activated (25 °C for 1 h) [^{3}H]TA-labeled cytosol (52 mg of protein/mL) prepared in BSM buffer with various amounts of the homogenization buffer. Various concentrations of pyridoxal 5′-phosphate were added (0 °C for 30 min), and DNA–cellulose binding was measured. For each dilution, the concentration of pyridoxal 5′-phosphate necessary for inhibition of 50% of the control DNA–cellulose binding was determined. As seen in Figure 2, the inhibitory activity of pyridoxal 5′-phosphate is protein concentration dependent.

Our inability to determine values for the 50% inhibition of DNA–cellulose binding at protein concentrations lower than 7 mg/mL resulted from the low [^{3}H]TA binding observed at this concentration. Determination of the minimum concentration of pyridoxal 5′-phosphate necessary for DNA–cellulose binding inhibition will have to await the purification of relatively stable, activated glucocorticoid–receptor complexes which are still capable of binding to DNA–cellulose.

Effect of Pyridoxal 5′-Phosphate on the Elution of Glucocorticoid–Receptor Complexes from Ion-Exchange Columns. The ability of pyridoxal 5′-phosphate to interact with activated complexes directed us to examine the effect of pyridoxal 5′-phosphate on the elution profile of these complexes from DEAE-cellulose columns. [^{3}H]TA-labeled, heat-activated (25 °C for 1 h) cytosol was incubated in the presence

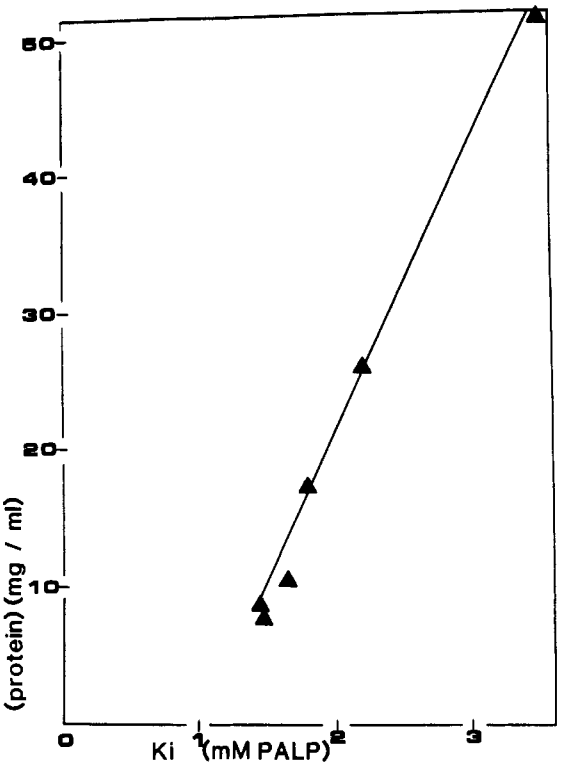

FIGURE 2: Effect of cytosolic protein concentration on the concentration of pyridoxal 5′-phosphate necessary to inhibit 50% of the binding of activated [^{3}H]TA–receptor complexes to DNA–cellulose. Experimental details are described under Results.

or absence of 5 mM pyridoxal 5′-phosphate. The cytosols were then treated with 10 mM sodium borohydride to prevent the reversible dissociation of pyridoxal 5′-phosphate from the glucocorticoid–receptor complex during gel filtration. Cytosols were subjected to gel filtration, the macromolecular fractions collected, and aliquots subjected to DEAE-cellulose chromatography (Figure 3). Activated receptor complexes normally elute at a potassium phosphate concentration of 0.06 M. Neither the addition of sodium borohydride alone (A) nor the addition of pyridoxal 5′-phosphate and sodium borohydride (B) alters the elution of the activated receptor complexes. Similarly, no changes in the elution profile were observed when cytosols, incubated in the presence or absence of 5 mM pyridoxal 5′-phosphate, were subjected to DEAE-Sephadex chromatography (data not shown). The elution of unactivated glucocorticoid–receptor complexes from ion-exchange columns was also unaffected by the presence of pyridoxal 5′-phosphate (data not shown).

Effect of Pyridoxal 5′-Phosphate on the Activation of Glucocorticoid–Receptor Complexes. Since pyridoxal 5′-phosphate inhibits the binding of activated receptor complexes to DNA–cellulose and does not alter the elution profile of glucocorticoid–receptor complexes during ion-exchange chromatography, DEAE-cellulose columns were used to investigate pyridoxal 5′-phosphate's effect on activation. [^{3}H]TA-labeled cytosol was incubated (15 °C for 30 min) in the presence or absence of 5 mM pyridoxal 5′-phosphate and an aliquot subjected to DEAE-cellulose chromatography (Figure 4). In agreement with earlier published results (Barnett et al., 1980), very little activation (A) is achieved under these experimental conditions in the control sample (13% of the bound radioactivity eluted as the activated form of the receptor). In the pyridoxal 5′-phosphate treated sample, however, 70% of the bound radioactivity was eluted as activated complexes (B). Since activation has been shown to occur

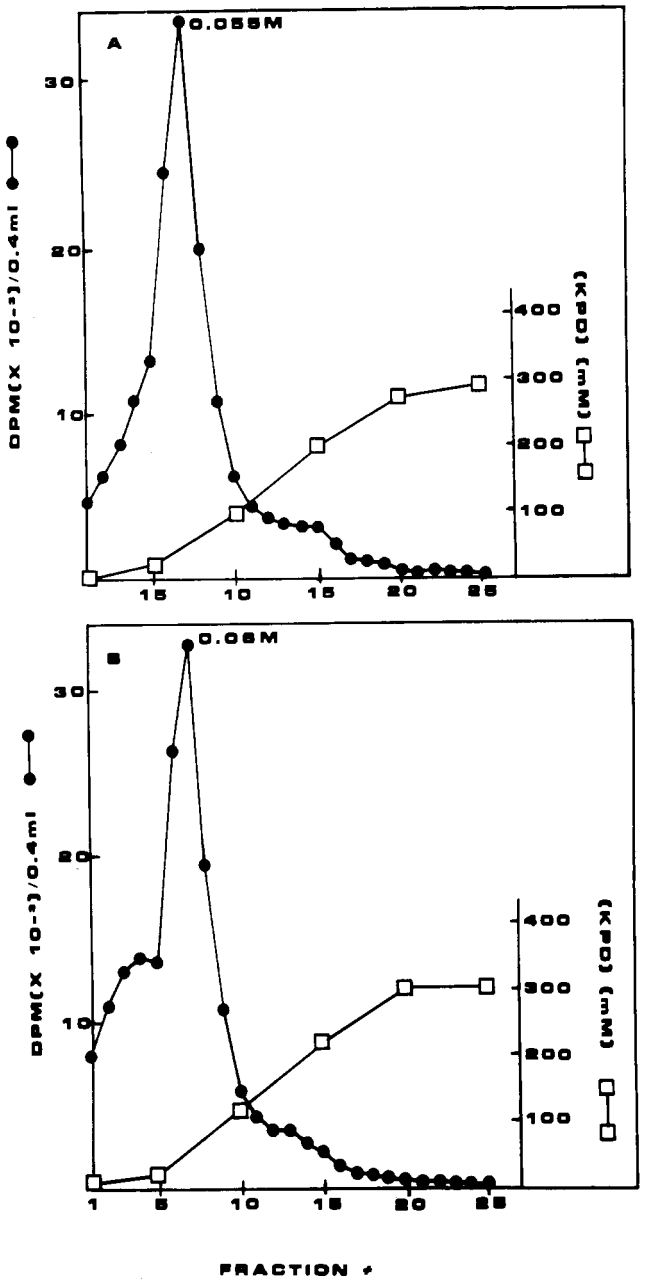

FIGURE 3: Effect of pyridoxal 5′-phosphate on the elution of activated [^{3}H]TA–receptor complexes from DEAE-cellulose column. Experimental details are described under Results. Cytosols were incubated in the absence (A) or presence (B) of 5 mM pyridoxal 5′-phosphate prior to treatment with 10 mM sodium borohydride, gel filtration, and DEAE-cellulose chromatography.

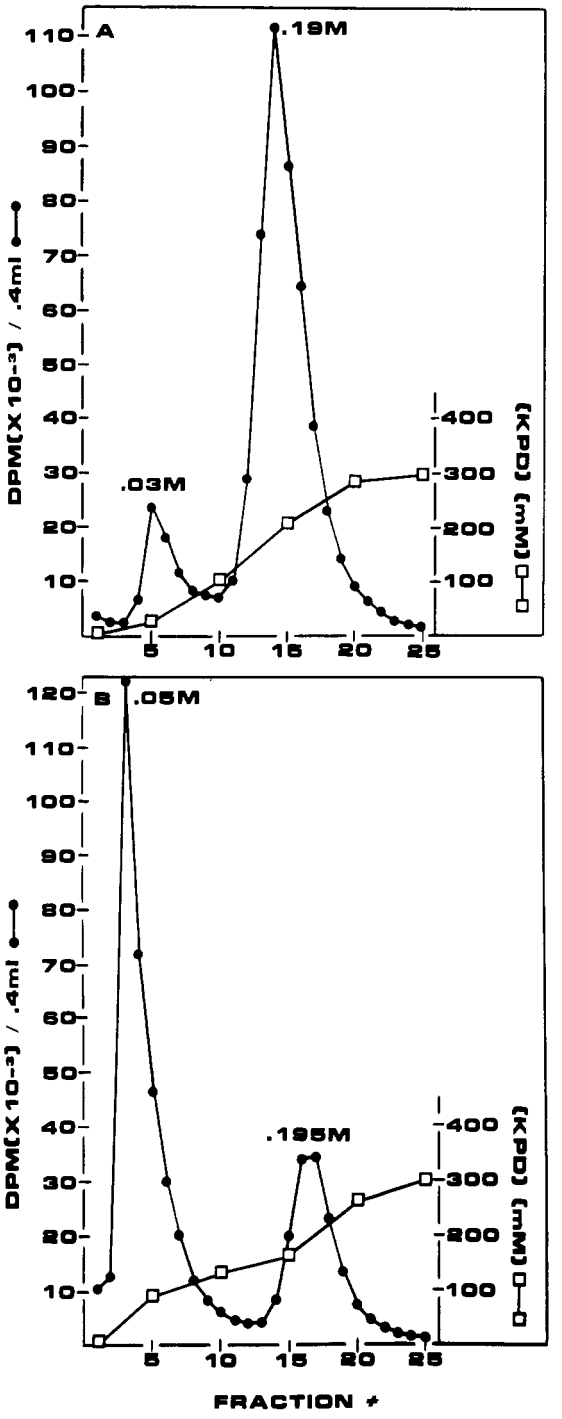

FIGURE 4: Effect of pyridoxal 5′-phosphate on the formation of activated glucocorticoid–receptor complexes at 15 °C. Experimental details are described under Results. [^{3}H]TA-labeled cytosols were incubated at 15 °C for 30 min in the absence (A) or presence (B) of 5 mM pyridoxal 5′-phosphate prior to DEAE-cellulose chromatography.

slowly at low temperatures (Milgrom et al., 1973; Kalimi et al., 1975; Goidl et al., 1977), [^{3}H]TA-labeled cytosol prepared in TSM buffer was incubated at 4 °C for 16 h in the presence or absence of 5 mM pyridoxal 5′-phosphate, and the degree of activation was determined by DEAE-cellulose chromatography (data not shown). Pyridoxal 5′-phosphate again significantly increased the proportion of activated glucocorticoid–receptor complexes formed (low salt eluting peak).

Previously published reports have suggested that pyridoxal 5′-phosphate treatment can alter the molecular properties of the glucocorticoid receptor (Cidlowski & Thanassi, 1979; Cidlowski, 1980; Kalimi & Love, 1980). For assessment of whether the size of the glucocorticoid–receptor complex is altered during pyridoxal 5′-phosphate mediated activation, [^{3}H]TA-labeled cytosol was incubated for 16 h at 4 °C (or for 30 min at 15 °C, data not shown) in the presence of 5 mM pyridoxal 5′-phosphate, treated with dextran-coated charcoal, and subjected to Sephadex G-200 chromatography (Figure 5). The major peak of radioactivity was eluted from the column with a calculated Stokes radius of 54 Å and an approximate molecular weight (assuming 5 S) of 120 000. Additionally, only one minor peak was eluted near the void volume (appears as a shoulder on the leading edge of the major peak). A similar chromatogram was observed by using heat-activated glucocorticoid–receptor complexes with the only detectable difference being a slight increase in the relative amount of the minor peak. Since the minor peak probably represents receptor aggregates, it appeared that, under our

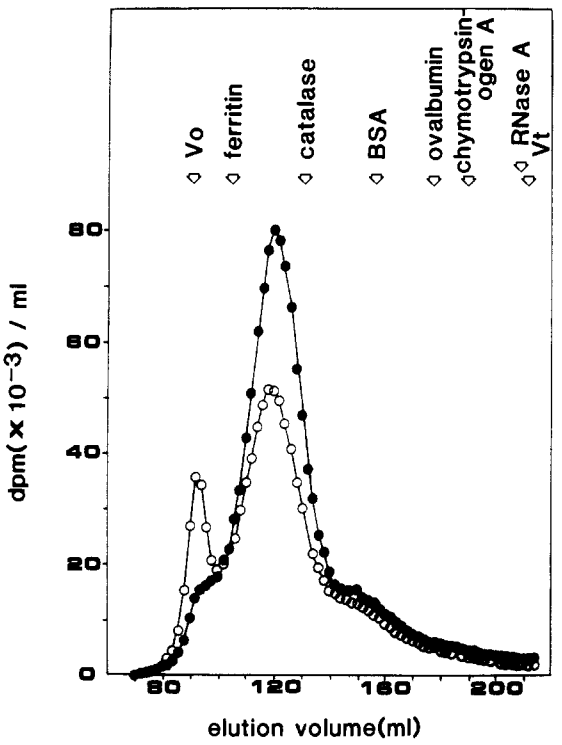

FIGURE 5: Effect of activating conditions on the size of the glucocorticoid–receptor complex. Sephadex G-200 chromatographic analyses of complexes which were activated either by 5 mM pyridoxal 5′-phosphate at 4 °C for 16 h (●) or by heating at 25 °C for 45 min (○).

experimental conditions, pyridoxal 5′-phosphate does not induce receptor fragmentation, although it may facilitate the previously observed disaggregation of glucocorticoid–receptor complexes (Dolan et al., 1980).

Structural Specificity for the Observed Stimulation of Activation. Pyridoxal 5′-phosphate's ability to stimulate the activation of glucocorticoid–receptor complexes necessitated the determination of the structural specificity of this effect. Although the precise mechanism is not clearly understood, it has been shown that some phosphorylated compounds and their dephosphorylated analogues stimulate activation (Barnett et al., 1980). If pyridoxal 5′-phosphate fits into the above class, then certainly pyridoxal and possibly pyridoxamine and pyridoxamine 5′-phosphate should also stimulate activation of glucocorticoid–receptor complexes. To test this possibility, we examined various B_6 analogues at 5 mM concentrations for their ability to stimulate activation (15 °C for 30 min) by using DEAE-cellulose chromatography and the DNA–cellulose binding assay. Under the experimental conditions employed, the inability of all B_6 analogues (with the exception of pyridoxal 5′-phosphate) to inhibit DNA–cellulose binding of activated receptor complexes (data not shown) permitted the use of this assay to measure the effect of these analogues on activation. As shown in Table III, with the exception of pyridoxal 5′-phosphate, none of the B_6 analogues stimulate activation significantly. At higher concentrations (20–25 mM), pyridoxal also stimulated activation of glucocorticoid–receptor complexes (data not shown), suggesting that this effect is mediated by the formation of a Schiff base. Thus, it appears that both the stimulation of activation and the inhibition of DNA–cellulose binding by pyridoxal 5′-phosphate are accomplished through the same mechanism.

Table III: Effect of Various B_6 Analogues on the Activation of [^{3}H]TA-Receptor Complexes[a]

addition	% bound DNA–cellulose[b]	activated peak[c]	unactivated peak[c]
control	6.9	366 000 (32)[d]	773 300 (68)
5 mM pyridoxine	8.6		
5 mM pyridoxamine	8.5		
5 mM pyridoxamine-P	7.4	289 700 (35)	555 700 (65)
5 mM pyridoxal	9.6	409 500 (39)	643 700 (61)
5 mM pyridoxal-P	5.5	698 500 (70)	299 000 (30)

[a] [^{3}H]TA-receptor complexes were incubated at 15 °C for 30 min in the presence or absence of the indicated B_6 analogues. DNA–cellulose binding and bound radioactivity were determined, and charcoal-treated aliquots were subjected to DEAE-cellulose chromatography as described under Materials and Methods. [b] Defined in Table I. [c] Bound radioactivity eluted at KPD concentrations identical with those used to elute activated (low salt) and unactivated (high salt) [^{3}H]TA-receptor complexes. [d] Values in parentheses represent the percent of the total bound radioactivity eluting in the activated or unactivated peak.

Discussion

In these studies, we have demonstrated that both pH (Figure 1) and cytosolic protein concentration (Figure 2) can alter quantitatively the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding by activated glucocorticoid–receptor complexes. While a cytosolic protein concentration dependency for the pyridoxal 5′-phosphate mediated blockage of the 4.6S to 6S transition of rat uterine estrogen receptor (Traish et al., 1980) has been observed, no pH dependency has ever been demonstrated for a pyridoxal 5′-phosphate effect on any steroid hormone receptor system. A pH-dependent response, however, has been reported for the inactivation of *Escherichia coli* RNA polymerase by pyridoxal 5′-phosphate (Bull et al., 1975).

Our investigation also showed that, following reduction by sodium borohydride, none of the pyridoxal 5′-phosphate mediated DNA–cellulose binding inhibition observed between pH 7 and 8 was reversible by gel filtration (Table I). This result indicated that pH only affects the extent to which pyridoxal 5′-phosphate may form a Schiff base with amino acid residues (presumably the ϵ-amino group of lysine) in activated receptor complexes. This interpretation is consistent with the observation that the pH curve for cytosol prepared in BSM buffer (Figure 1, panel A) resembles the titration of an amino acid. The apparent pK_a would appear to be approximately 7.4. While this pK_a is lower than the known value for the ϵ-amino group of lysine (pK_a = 10.5), it did not differ significantly from the value obtained from the pH-dependent curve of the pyridoxal 5′-phosphate mediated inactivation of purified *E. coli* RNA polymerase (pK_a = 7.9) (Bull et al., 1975). The results suggest that the lysine residue is either in an apolar environment or near another cationic group. The recent reports that histidine and arginine residues (DiSorbo et al., 1980a) and probably a metal ion (Schmidt et al., 1981) are located at or near the DNA binding site certainly support the latter interpretation.

Conflicting data can be found in the literature concerning the ability of primary amines to alter observed pyridoxal 5′-phosphate mediated effects on steroid hormone receptors or nucleic acid polymerases. This investigation demonstrated that 100 mM Tris is unable to prevent or reverse the pyridoxal 5′-phosphate mediated inhibition of DNA–cellulose binding, while the same concentration of lysine is partially effective (Table II). These results are in agreement with those found with the estrogen receptor (Müller et al., 1980) and DNA polymerase (Modak, 1976) but contrast with results found with the progesterone receptor (Nishigori & Toft, 1979) and *E. coli* RNA polymerase (Venegas et al., 1973). The observed dif-

ferences in the effects of primary amines may reflect cytosolic differences or slight differences in the microenvironment surrounding the reactive lysine residues between the various nucleotidyl binding proteins.

The observation that pyridoxal 5′-phosphate can cause progesterone receptor subunits to elute as more acidic forms from DEAE-cellulose (Nishigori & Toft, 1979) suggested that pyridoxal 5′-phosphate might also result in the elution of activated glucocorticoid receptors at a higher salt concentration. We found, however, that pyridoxal 5′-phosphate did not alter the elution profile of either activated or unactivated receptor complexes during DEAE-Sephadex or DEAE-cellulose chromatography (Figure 3). The data contrast with those obtained with 1,10-phenanthroline, a metal chelator, which like pyridoxal 5′-phosphate prevents the DNA–cellulose binding of activated glucocorticoid–receptor complexes, but shifts the elution profile of these complexes to a higher salt concentration similar to that required to elute unactivated complexes (Schmidt et al., 1981). The inability of pyridoxal 5′-phosphate to alter the elution profile of glucocorticoid–receptor complexes during anion-exchange chromatography was important, since it afforded us the only method of examining the direct effect of pyridoxal 5′-phosphate on activation.

The pyridoxal 5′-phosphate mediated stimulation of activation (Figure 4), which is reported here for the first time, resulted in receptors whose size, as determined by gel filtration chromatography, was identical with that of thermally activated receptors (Figure 5). Thus, the ability of pyridoxal 5′-phosphate to stimulate activation and to subsequently inhibit DNA–cellulose binding (Table III) did not result from its previously reported ability to induce receptor fragmentation (Cidlowski, 1980; Kalimi & Love, 1980). Our inability to demonstrate receptor fragmentation with an identical concentration of pyridoxal 5′-phosphate may reflect differences in tissue sources, cytosolic protein concentrations, or other experimental conditions. Additionally, pyridoxal 5′-phosphate's ability to stimulate activation appeared to be structurally specific (Table III) and mediated via a Schiff base formation. It is interesting to note that a structurally specific, pyridoxal 5′-phosphate induced shift in the sedimentation coefficient of glucocorticoid–receptor complexes to a value obtained with activated complexes has been reported (Cidlowski & Thanassi, 1979). Our studies demonstrated that since activation can occur in the presence of pyridoxal 5′-phosphate (data not shown) under the same conditions (16 h at 4 °C) utilized during sucrose density centrifugation, it seems possible that the observed shift in *s* value might have been due to a stimulation of activation rather than the suggested disaggregation of unactivated receptor complexes. However, we cannot exclude the possibility that disaggregation occurs as a consequence of activation.

Thus, it appears that in vitro pyridoxal 5′-phosphate can exert two seemingly opposite effects on glucocorticoid–receptor complexes—the stimulation of their activation and the inhibition of their binding to DNA–cellulose. Both effects seem to be structurally specific and dependent upon Schiff base formation. The ability of pyridoxal 5′-phosphate to exert these effects via the same mechanism is probably most simply explained in terms of mass action. By binding to activated glucocorticoid–receptor complexes, pyridoxal 5′-phosphate effectively removes these complexes from the cytosol, and the rate of activation is enhanced. One alternative explanation, however, is that pyridoxal 5′-phosphate exerts these effects by binding to two different lysine residues. Binding to one of these residues stimulates the conformational change associated with activation, while binding to the other results in the loss of DNA–cellulose binding by activated receptor complexes.

At this time, it is unknown as to what significance the pyridoxal 5′-phosphate mediated stimulation of activation has in vivo. It seems possible that at physiological temperatures (37 °C) activation occurs maximally, thus masking any stimulation by pyridoxal 5′-phosphate. On the other hand, activation has been shown to be a physiologically significant, time-dependent event (Munck & Foley, 1979; Litwack et al., 1980) and, therefore, may be under some type of regulation. Studies on pyridoxine-deficient animals (DiSorbo et al., 1980b) and on cell cultures with varying intracellular pyridoxal 5′-phosphate levels (DiSorbo & Litwack, 1981) have suggested that pyridoxal 5′-phosphate has a physiological role in the regulation of activated glucocorticoid–receptor complexes. While proof will await further experimentation, it is conceivable that pyridoxal 5′-phosphate's physiological role may extend to the regulation of the activation process.

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References

Barnett, C. A., Schmidt, T. J., & Litwack, G. (1980) *Biochemistry 19*, 5446–5455.

Baxter, J. D., Rousseau, G. G., Benson, M. C., Garcea, R. L., Ito, J., & Tomkins, G. M. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 1892–1896.

Beato, M., & Feigelson, P. (1972) *J. Biol. Chem. 247*, 7890–7896.

Bull, P., Zaldivar, J., Venegas, A., Martial, J., & Valenzuela, P. (1975) *Biochem. Biophys. Res. Commun. 64*, 1152–1159.

Cake, M. H., & Litwack, G. (1978) *Eur. J. Biochem. 82*, 97–103.

Cake, M. H., DiSorbo, D. M., & Litwack, G. (1978) *J. Biol. Chem. 253*, 4886–4891.

Cidlowski, J. A. (1980) *Biochemistry 19*, 6162–6170.

Cidlowski, J. A., & Thanassi, J. W. (1979) *Biochemistry 18*, 2378–2384.

DiSorbo, D. M., & Litwack, G. (1981) *Biochem. Biophys. Res. Commun. 99*, 1203–1208.

DiSorbo, D. M., & Litwack, G. (1982) in *Biochemical Actions of Hormones* (Litwack, G., Ed.) Vol. 9, pp 205–219, Academic Press, New York.

DiSorbo, D. M., Phelps, D. S., & Litwack, G. (1980a) *Endocrinology (Baltimore) 106*, 922–929.

DiSorbo, D. M., Phelps, D. S., Ohl, V. S., & Litwack, G. (1980b) *J. Biol. Chem. 255*, 3866–3870.

Dolan, K. P., Diaz-Gil, J. J., & Litwack, G. (1980) *Arch. Biochem. Biophys. 201*, 476–485.

Goidl, J. A., Cake, M. H., Dolan, K. P., Parchman, L. G., & Litwack, G. (1977) *Biochemistry 16*, 2125–2130.

Kalimi, M., & Love, K. (1980) *J. Biol. Chem. 255*, 4687–4690.

Kalimi, M., Colman, P., & Feigelson, P. (1975) *J. Biol. Chem. 250*, 1080–1086.

Litwack, G., Schmidt, T. J., Markovic, R. D., Eisen, H. J., Barnett, C. A., DiSorbo, D. M., & Phelps, D. S. (1980) in *Perspectives in Steroid Receptor Research* (Bresciani, F., Ed.) pp 113–131, Raven Press, New York.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Matsuo, Y. (1957) *J. Am. Chem. Soc. 79*, 2011–2015.

Milgrom, E., Atger, M., & Baulieu, E.-E. (1973) *Biochemistry 12*, 5198–5205.
Modak, M. J. (1976) *Biochemistry 15*, 3620–3626.
Müller, R. E., Traish, A., & Wotiz, H. H. (1980) *J. Biol. Chem. 255*, 4062–4067.
Munck, A., & Foley, R. (1979) *Nature (London) 278*, 752–754.
Nishigori, H., & Toft, D. (1979) *J. Biol. Chem. 254*, 9155–9161.
Nishigori, H., Moudgil, V. K., & Toft, D. (1978) *Biochem. Biophys. Res. Commun. 80*, 112–118.
Parchman, L. G., & Litwack, G. (1977) *Arch. Biochem. Biophys. 183*, 374–382.
Sakaue, Y., & Thompson, E. B. (1977) *Biochem. Biophys. Res. Commun. 77*, 533–541.
Schmidt, T. J., Sekula, B. C., & Litwack, G. (1981) *Endocrinology (Baltimore) 109*, 803–812.
Sekula, B. C., Schmidt, T. J., & Litwack, G. (1981) *J. Steroid Biochem. 14*, 161–166.
Simons, S. S., Jr., Martinez, H. M., Garcea, R. L., Baxter, J. D., & Tomkins, G. M. (1976) *J. Biol. Chem. 251*, 334–343.
Traish, A., Müller, R. E., & Wotiz, H. H. (1980) *J. Biol. Chem. 255*, 4068–4072.
Venegas, A., Martial, J., & Valenzuela, P. (1973) *Biochem. Biophys. Res. Commun. 55*, 1053–1059.

Antibody·Nucleic Acid Complexes. Immunospecific Retention of Globin Messenger Ribonucleic Acid with Antibodies Specific for 7-Methylguanosine†

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ABSTRACT: Antibodies specific for 7-methylguanosine (m^7G) were immobilized to Sepharose, and this adsorbent (anti-m^7G/Sepharose) was tested for its ability to select for globin mRNA on the basis of its 5′-terminal, m^7G-containing cap, i.e., $m^7G(5')ppp(5')N$. Preliminary studies with [^{3}H]m^7G indicated that the binding of ^{3}H-labeled hapten to immobilized antibody was (i) essentially complete within 30 min, (ii) not affected significantly by variations in pH (5.0–9.0), temperature (0–56 °C), or salt concentrations (0.01–1.0 M NaCl), and (iii) specific for m^7G-containing (oligo)nucleotides with no detectable cross-reactivity toward comparable unmethylated (oligo)nucleotides. Estimates based upon the amount of active antibody coupled to Sepharose (0.15 $\mu g/\mu L$) and its affinity constant ($K_a = 5 \times 10^7$ M^{-1}) revealed that 50 μL of anti-m^7G/Sepharose was sufficient to quantitatively bind the equivalent of 5 μg of globin mRNA, a value in excellent agreement with that experimentally obtained. In assessment of the immunospecific selection of mRNA, rabbit globin mRNA was specifically labeled at each terminus via periodate oxidation and NaB^3H_4 reduction. Upon recovery of oligo(dT)–cellulose, the ^{3}H-labeled mononucleotides derived from enzymatic digestion (P_1 nuclease and tobacco acid pyrophosphatase) of ^{3}H-labeled globin mRNA yielded a [^{3}H]-pm^7G:[^{3}H]pA ratio of 0.41. This result indicated the presence of 3′-terminal fragments of mRNA generated during the labeling reaction and enriched during recovery of oligo(dT)–cellulose chromatography. However, when incubated with anti-m^7G/Sepharose, 63% of the poly(A)-containing ^{3}H-labeled globin mRNA was immunospecifically retained and possessed a [^{3}H]pm^7G:[^{3}H]pA ratio of 0.95 in contrast to a 0.15 ratio for the nonretained preparation. Last, potential degradation of immunospecifically retained, unlabeled globin mRNA was not evident as evaluated by analysis of the resulting in vitro translational product, i.e., rabbit globin (ca. 15 000 molecular weight). Moreover, kinetic data from the latter experiments demonstrated that the translation of m^7G-selected mRNA was 30–50% more efficient than that of unfractionated mRNA preparations. As determined from sucrose gradient centrifugation data, increased translational efficiency was attributed to the removal of an 18S rRNA contaminant.

The unique poly(A) sequence at the 3′ terminus of most eukaryotic mRNAs provides an almost universal approach for isolating these mRNAs by oligo(dT)–cellulose (Edmonds et al., 1971; Aviv & Leder, 1972) or poly(U)–Sepharose (Lindberg & Persson, 1972) chromatography. Under conditions that promote base-pair formation [moderate to high salt concentrations, e.g., 0.1–0.5 M NaCl in 0.01 M tris(hydroxymethyl)aminomethane (Tris),[1] pH 7.4], these adsorbents select poly(A)-containing mRNAs yet exclude the bulk of tRNA and rRNA populations. While such chromatographic techniques have been used with considerable success, they are not amenable to the isolation of mRNAs lacking poly(A) sequences (Milcarek et al., 1974; Nemer et al., 1976). Further, it is probable that a fraction of the mRNAs selected on the basis of poly(A) content have undergone degradation either

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[1] Abbreviations: m^7G', pm^7G', and pA′, trialcohol (ribose ring-opened) derivatives of 7-methylguanosine, its 5′-phosphate, and adenosine 5′-phosphate, respectively; m^7G and pm^7G, 7-methylguanosine and its 5′-phosphate, respectively; m^7GpppN^m, m^7G linked by a 5′–5′-triphosphate to a 2′-O-methylated nucleoside; m_2^2G, N^2,N^2-dimethylguanosine; anti-m_2^2G/Sepharose and anti-m^7G/Sepharose, affinity-purified antibodies coupled to Sepharose; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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