References

Biesecker, G. (1973), *Biochemistry 12,* 4403.
Bohlen, P., Stein, S., Dairman, W., and Udenfriend, S. (1973), *Arch. Biochem. Biophys. 155,* 213.
Brockes, J. P., and Hall, Z. W. (1975a), *Biochemistry 14,* 2092.
Brockes, J. P., and Hall, Z. W. (1975b), *Biochemistry 14,* 2100.
Chang, H. W. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 2113.
Cuatrecasas, P. (1970), *J. Biol. Chem. 245,* 3059.
Devreotes, P. N., and Fambrough, D. M. (1975), *J. Cell. Biol. 65,* 335.
Devreotes, P. N., and Fambrough, D. M. (1976), *Proc. Natl. Acad. Sci. U.S.A. 73,* 161.
Devreotes, P. N., Gardner, J. M., and Fambrough, D. M. (1977), *Cell 10,* 365.
Dolly, J. O., and Barnard, E. A. (1975), *FEBS Lett. 57,* 267.
Fairbanks, G., Steck, T. L., and Wallace, D. F. H. (1971), *Biochemistry 10,* 2606.
Karlin, A. (1974), *Life Sci. 14,* 1385.
Karlin, A., and Cowburn, D. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3636.
Klett, R. P., Fulpius, B. W., Cooper, D., Smith, M., Reich, E., and Possani, L. D. (1973), *J. Biol. Chem. 248,* 6841.
Lindstrom, J., and Patrick, J. (1974), in Synaptic Transmission and Neuronal Interaction, New York, N.Y., Raven Press, p 191.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.
Merlie, J. P., Sobel, A., Changeux, J-P., and Gros, F. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 4028.
Meunier, J-C., Sealock, R., Olsen, R., and Changeux, J-P. (1974), *Eur. J. Biochem. 45,* 371.
Miledi, R., Molinoff, P., and Potter, L. T. (1971), *Nature (London) 229,* 554.
Ong, D. E., and Brady, R. N. (1974), *Biochemistry 13,* 2822.
Patrick, J., Heinemann, S. F., Lindstrom, J., Schubert, D., and Steinbach, J. H. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 2762.
Patrick, J., and Lindstrom, J. (1973), *Science 180,* 871.
Patrick, J., Lindstrom, J., Culp, W., and McMillan, J. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3334.
Patrick, J., McMillan, J., Wolfson, H., and O'Brien, J. C. (1977), *J. Biol. Chem. 252,* 2143.
Raftery, M. A., Vandlen, R. L., Reed, K. L., and Lee, T. (1976), *Cold Spring Harbor Symp. Quant. Biol. 41,* 193.
Rang, H. P. (1975), *Q. Rev. Biophys. 7,* 283.
Schmidt, J., and Raftery, M. (1973), *Biochemistry 12,* 852.
Schubert, D., Harris, A. J., Devine, C. E., and Heinemann, S. (1973), *J. Cell. Biol. 61,* 398.
Vogel, A., Sytkowski, A. J., and Nirenberg, M. W. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 3180.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Weill, C. L., McNamee, M. G., and Karlin, A. (1974), *Biochem. Biophys. Res. Commun. 61,* 997.

Effects of Neuraminidase on Lectin Binding by Wild-Type and Ricin-Resistant Strains of Hamster Fibroblasts†

Saul W. Rosen*,‡ and R. Colin Hughes

ABSTRACT: The nature of cell surface receptors for ricin on wild-type and ricin-resistant variants of baby hamster kidney fibroblasts has been studied. Neuraminidase stimulated ricin binding threefold by wild-type cells, and increased their susceptibility to ricin toxicity as measured by inhibition of [^{3}H]leucine uptake (LD_{30} fell from 5.0 to 0.5 μg/mL). Basal ricin binding by ricin-resistant variants (10–300% that of wild type) was also stimulated (2- to 17-fold) by neuraminidase in all seven clonal strains examined; susceptibility to ricin was greatly increased by neuraminidase in these variants. Neuraminidase did not affect the binding of concanavalin A by wild type or a ricin-resistant variant, but decreased the binding of wheat-germ agglutinin by 90% in both cell types. The trivial binding of peanut agglutinin by wild type and a ricin-resistant variant was markedly enhanced (14- to 22-fold) by neuraminidase. Neither collagenase (50 U/mL) nor Pronase (0.0001%) affected ricin binding by wild type or a ricin-resistant variant. These data suggest the existence of "exposed" and "cryptic" oligosaccharide receptors for ricin on the cell membrane glycoproteins of baby hamster kidney fibroblasts. The cryptic ricin receptors probably include at least the sequence D-galactosyl-β-(1→3)-*N*-acetylhexosamine substituted by sialic acid residues. Exposed and cryptic ricin receptors appear to be different and under separate genetic control.

The isolation from baby hamster kidney cells of stable variants resistant to ricin, the toxin lectin of castor beans (*Ricinus communis*), has been reported from this laboratory (Meager et al., 1975, 1976). Evidence has been presented for two major phenotypic classes: variants binding normal amounts of ricin and variants that bind this lectin poorly. Binding of the lectin to cell surface receptors is necessary for the toxic effects on intact cells (Olsnes et al., 1974a,b; Nicolson et al., 1975b; Olsnes and Pihl, 1976) and a deficiency in surface receptors could alone account for resistance. Although the steps subsequent to initial binding are not fully understood, it is clear that the toxin must enter the cell, perhaps by an endocytosis-like

† From the Division of Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, United Kingdom. *Received April 28, 1977.*

‡ Present address: Clinical Endocrinology Branch, National Institute of Arthritis, Metabolism and Digestive Diseases, National Institutes of Health, Bethesda, Md. 20014.

mechanism (Nicolson, 1974; Refsnes et al., 1974; Sandvig et al., 1976; Olsnes et al., 1976) to inactivate the 60S ribosomal subunit and inhibit protein synthesis (Lin et al., 1971; Olsnes and Pihl, 1973; Nicolson et al., 1975a). Resistance to lectin toxicity could also be mediated at the site of any one of these later steps.

Decreased binding of ricin might result from alterations in the cell membranes whereby (a) changes in carbohydrate chain assembly affect cell surface reception of ricin, a lectin known to require D-galactose or *N*-acetyl-D-galactosamine residues for binding (Nicolson and Blaustein, 1972; Hughes et al., 1973; Olsnes et al., 1974b); (b) unaltered ricin receptors on the cell surface are masked by sialic acid, hyaluronic acid, collagen, or some other protein coat, present in increased amount on the surface of resistant cells. Several lecitin-resistant cell lines have shown specific enzyme defects that could lead to defective assembly of receptors (Gottlieb et al., 1975; Gottlieb and Kornfeld, 1976). So far, however, no evidence has been obtained for "masking" of receptors in the ricin-resistant baby hamster kidney cell lines.

In this paper, we have treated wild-type baby hamster kidney cells and ricin-resistant variants with neuraminidase and have examined the effects on the binding of various lectins, and on the sensitivity of the cells to ricin. In addition, we have studied the effects of collagenase and Pronase on ricin binding. The data obtained are consistent with at least two types of receptor on baby hamster kidney membranes, one of which is masked by sialic acid. The roles of these receptor classes in mediating ricin toxicity in wild-type cells and their independent modulation during selection for resistance to ricin are discussed.

Materials and Methods

Cell Cultures. Baby hamster kidney fibroblasts (BHK21/C13) were obtained from Flow Laboratories, Irvine, Ayrshire, Scotland, U.K., and grown at 39 °C in Glasgow-modified minimal essential medium (Flow Laboratories) supplemented as previously described (Meager et al., 1976). Ricin-resistant (Ric^R)[1] variants, isolated from mutagenized cells (Meager et al., 1976), were grown and subcultured under identical conditions as wild-type cells. Variants were routinely examined for reversion to ricin sensitivity, as described below; no such reversion was ever detected.

Lectins, Enzymes, and Sugars. Highly purified preparations of ricin were either obtained from Miles-Yeda Ltd., Kiryat Weizmann, Rehovoth, Israel, or were generously given to us by Mr. P.Gillett and Dr. M. J. Crumpton, and by Ms. M. Holman and Dr. J. H. Humphrey of the National Institute for Medical Research, Mill Hill, U.K. The latter preparations, isolated by the procedure of Nicolson and Blaustein (1972), were more than 90% pure when examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and by gel chromatography on polyacrylamide (Bio-Gel P150; Bio-Rad Laboratories). All three preparations gave indistinguishable results in cell-binding studies (see below); their LD_{50} values in the mouse were essentially identical and in good agreement with published data (Olsnes and Pihl, 1973). Peanut agglutinin, purified from *Arachis hypogaea* (Lotan et al., 1975), was a gift of Dr. N. Sharon. Purified wheat-germ agglutinin (Allen et al., 1973) was a gift of Dr. A. Allen. Concanavalin A was obtained as a three-times crystallized freeze-dried powder from Miles-Yeda.

Vibrio cholerae neuraminidase (EC 3.2.1.18), obtained from Behringwerke-Hoechst, London, U.K., as a solution (500 U/mL) in 0.05 M sodium acetate buffer (pH 5.5) containing 9 mg/mL sodium chloride and 1 mg/mL calcium chloride, was diluted in this buffer to a concentration of 50 U/mL just before use. A unit of neuraminidase is defined (Schramm and Mohr, 1959) as the amount of enzyme required to release 1 μg of *N*-acetylneuraminic acid from human α_1-acid glycoprotein in 15 min at 37 °C. Collagenase (EC 3.4.99.5), purified from *Clostridium histolyticum,* was obtained from Advanced Biofactures Corp., Lynbrook, N.Y., as a frozen solution (2500 U/mL) in 0.33 M calcium acetate–0.025 M Tris buffer (pH 7.4) and was diluted in this buffer to a concentration of 50 U/mL just before use. A unit of collagenase is defined as the amount of enzyme required to release 1 μmol of leucine/min from undenatured collagen (achilles tendon) in the above buffer at 37 °C. *Streptomyces griseus* protease (EC 3.4.24.4) was obtained from Sigma as Pronase. The purified powder (Type VI) contained 4 U/mg; one unit catalyzes the hydrolysis of casein to produce Folin color equivalent to 1 μmol of tyrosine per min at 37 °C and pH 7.5.

Lactose was obtained from BDH Chemicals Ltd., Poole, Dorset, U.K.; methyl α-D-mannoside and *N*-acetyl-D-glucosamine were obtained from Sigma.

Iodination of Lectins. Ricin, concanavalin A, and wheat-germ agglutinin were iodinated, as previously described (Meager et al., 1976), to specific activities of 1–3 μCi/μg with carrier-free ^{125}I by lactoperoxidase and hydrogen peroxide (generated with glucose–glucose oxidase), in a solution made 0.1 M with an appropriate sugar hapten of the lectin. Lactose was used with ricin, methyl α-D-mannoside with concanavalin A, and *N*-acetyl-D-glucosamine with wheat-germ agglutinin. [^{125}I]Ricin and [^{125}I]concanavalin A were purified by affinity chromatography on agarose (Sepharose 6B, Pharmacia Fine Chemicals, Uppsala, Sweden) and dextran (Sephadex G-50, Pharmacia), respectively, and by elution with 0.1 M lactose and 0.1 M methyl α-D-mannoside, respectively. ^{125}I-Labeled wheat-germ agglutinin was purified by chromatography on polyacrylamide (Bio-Gel P150); four peaks of radioactivity were observed. The major peak, eluting with $K_{av} = 0.68$ and MW_{app} of <10 000, consistent with Allen et al. (1973), was used in the binding studies.

Peanut agglutinin, a homotetramer with subunit molecular weight of approximately 27 500 (Lotan et al., 1975), chromatographed on Bio-Gel P150 as a predominant (>60%) species of apparent molecular weight 63 000. The unfractionated lectin was lightly iodinated with carrier-free ^{125}I to a specific activity of 0.2 μCi/μg by a chloramine T method (Greenwood et al., 1963). The ^{125}I-labeled peanut agglutinin showed four peaks of radioactivity on Bio-Gel P150, with apparent molecular weights of >140 000, 70 000, 55 000, and 38 000. The three most excluded peaks (probably iodinated oligomers of the subunit) were pooled for the binding experiments.

Binding of ^{125}I-Labeled Lectins to Wild-Type and Variant Cells. Assays were performed on confluent or nearly confluent cell monolayers by a slight modification of the methods previously described (Meager et al., 1976). Lectin binding was carried out in duplicate or triplicate at 4 °C, in the presence and absence of an appropriate sugar inhibitor, i.e., lactose for ricin, methyl α-D-mannoside for concanavalin A, and *N*-acetyl-D-glucosamine for wheat-germ agglutinin; lactose was also included in the peanut agglutinin experiments, although its affinity for this lectin is low (Lotan et al., 1975). The monolayers were washed twice with a phosphate-buffered saline (pH 7.0) whose exact composition is given in Meager et al. (1976),

[1] Abbreviations used are: Ric^R, ricin-resistant clonal line derived from wild-type baby hamster kidney fibroblasts; PNA, peanut agglutinin; WGA, wheat-germ agglutinin; R_E, exposed receptors for ricin; R_C, cryptic receptors for ricin; Tris, tris(hydroxymethyl)aminomethane; MW_{app}, apparent molecular weight.

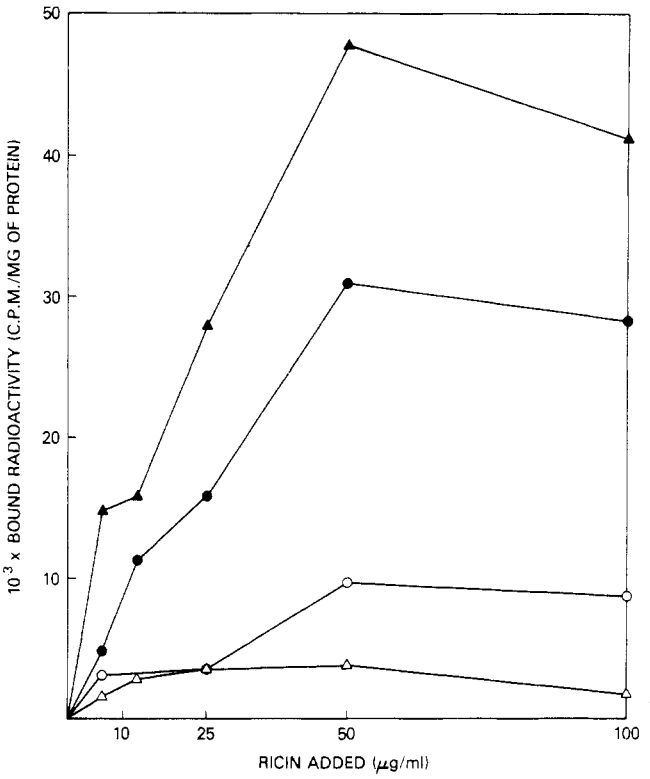

FIGURE 1: Effect of neuraminidase on ricin binding by wild-type and ricin-resistant baby hamster kidney cells. Cells (ca. 2×10^6) growing in monolayer culture on 35 mm diameter plastic dishes were incubated at 39 °C for 30 min with neuraminidase (50 U/mL) or control buffer. After washing, the monolayers were exposed for 30 min at 4 °C to ricin at the concentrations indicated. Bound ricin was estimated as described in the Materials and Methods section: wild-type cells (●, with neuraminidase; ○, control); ricin-resistant cell line Ric^R21 (▲, with neuraminidase; △, control).

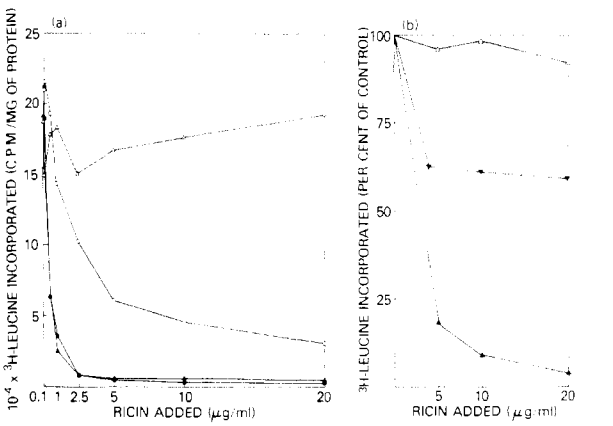

FIGURE 2: Effect of neuraminidase on ricin inhibition of protein synthesis by normal and ricin-resistant baby hamster kidney cells. Cells (ca. 2×10^6) approaching confluency in monolayer culture on 35 mm diameter plastic dishes were incubated (a) serially at 39 °C with neuraminidase (50 U/mL) or buffer for 30 min, ricin in the concentrations indicated for 60 min, and [^{3}H]leucine (1 μCi/mL) for 60 min. In b, the cells were washed twice with phosphate-buffered saline after neuraminidase and reincubated for 24 h with complete medium, washed, and treated with ricin and [^{3}H]leucine as in a. Control dishes contained no ricin. Incorporation into protein was assessed by measurement of acid-precipitable, ethanol-insoluble radioactivity: wild-type cells (●, with enzyme; ○, control); ricin-resistant cell line Ric^R21 (▲, with enzyme; △, control; ▼, with enzyme after reincubation).

exposed to 1 mL of a solution containing lectin ± 0.1 M sugar for 30 min, and washed twice more with phosphate-buffered saline. In experiments where the monolayers were exposed to an enzyme, the initial washes were carried out at room temperature and followed by an incubation at 39 °C with 1 mL of solution containing the enzyme or control buffer; the monolayers were then taken into the cold room, washed, exposed to lectin ± sugar, and washed again. Air-dried monolayers were dissolved in hot (80 °C) 1.0 N NaOH and aliquots counted for radioactivity in a well-type γ spectrometer (Packard Instrument Co.). Protein was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. Results were expressed as "specific" lectin binding (total bound counts per minute minus sugar-irreversible bound counts per minute) per 1.0 mg of cell protein.

Protein Synthesis by Wild-Type and Variant Cells. Protein synthesis was assessed by the incorporation of radioactive leucine into acid-precipitable material (Hughes and Gardas, 1976). Cells at touching confluence were washed twice with warm phosphate-buffered saline and incubated for 60 min at 36 °C with 1.2 μCi of [^{3}H]leucine (L-4,5-^{3}H, Radiochemical Centre, 55 Ci/mmol) in leucine-free Glasgow-modified minimal essential medium, supplemented as described (Meager et al., 1976). The monolayers were then washed three times with phosphate-buffered saline, twice with an ice-cold solution of 10% (v/v) perchloric acid–2% (w/v) phosphotungstic acid, and twice with ice-cold ethanol. The air-dried monolayers were dissolved in hot (80 °C) 1.0 N NaOH, and aliquots were taken for protein determination and for measurement of ^{3}H radioactivity. For the latter, 500 μL of alkaline extract was added to 10 mL of a colloidal silica gel scintillation fluid (Sargent and Campbell, 1965) and the solution counted in a liquid scintillation spectrometer (Packard Instrument Co.).

The effect of neuraminidase on ricin-inhibited protein synthesis was studied as follows: cells at touching confluence were washed twice with phosphate-buffered saline warmed to 36 °C and exposed to neuraminidase or control buffer for 30 min at 36 °C. The monolayers were then incubated with varying concentrations of ricin for 2 h at 36 °C, to permit penetration of the lectin into the cells (Olsnes et al., 1974a; Nicolson et al., 1975b; Olsnes and Pihl, 1976). Following two washes with warm phosphate-buffered saline, the monolayers were incubated with [^{3}H]leucine and incorporation was measured as described above.

Results

Effect of Neuraminidase on Ricin Binding and Ricin-Inhibited Protein Synthesis in Wild-Type and Ric^R21 Cells. Effects of neuraminidase on the binding of ricin by monolayers of wild-type baby hamster kidney fibroblasts and the ricin-resistant variant Ric^R21 are shown in Figure 1. At saturation, basal ricin binding by the variant was only 20% that of the parent cells, in good agreement with previously published data from our laboratory (Meager et al., 1976). Treatment with 50 U/mL of *Vibrio cholerae* neuraminidase resulted in a threefold rise in lectin bound by the wild-type cells (2.7 to 7.7 μg of ricin/mg of cell protein at 100 μg of ricin/mL) and in an even greater rise in ricin bound by the Ric^R21 variant (0.5 to 11.2 μg). There was no significant inhibition of protein synthesis in the ricin-resistant variant Ric^R21 at concentrations of ricin as high as 20 μg/mL (Figure 2a). Wild-type fibroblasts, on the other hand, were sensitive to the lectin and concentrations of ricin as low as 1 μg/mL significantly lowered [^{3}H]leucine incorporation into protein. When the wild-type cells were treated with neuraminidase, they became even more sensitive to the toxic action of ricin. Seventy percent inhibition of protein synthesis was achieved by only 0.5 μg/mL ricin in neuraminidase-treated cells, whereas ten times that concentration of

TABLE I: Effect of Neuraminidase on Ricin Binding and Ricin Toxicity in Wild-Type and Ricin-Resistant Variants.

Cell line	Ricin binding[a]		Ricin toxicity LD_{30}[b] (μg/mL)		
	Relative to wild type	Neuraminidase/ control	Control	Neuraminidase	Neuraminidase/ control
Wild type		3.0	5.0	0.5	0.1
Ric^R7	3.1	3.0	>20[c]	8.5	<0.4
Ric^R12	1.3	6.1	>20[c]	4.7	<0.2
Ric^R14	0.3	8.3	>20[c]	1.0	<0.05
Ric^R16	0.9	3.3	12.5[d]	0.5	0.04[d]
Ric^R17	1.15	5.2	>20	2.5	<0.1
Ric^R21	0.1	17.5	>20	0.5	<0.1
Ric^R22	0.85	1.8	7.4[d]	0.6[d]	0.08[d]

[a] Mean of values determined at ricin concentrations of 50 and 100 μg/mL. [b] Concentration of ricin required for 70% inhibition of [^{3}H]leucine uptake. [c] No inhibition of [^{3}H]leucine incorporation at any concentration of ricin examined (≤20 μg/mL). [d] LD_{30}; 70% inhibition not achieved at any concentration of ricin examined (≤20 μg/mL).

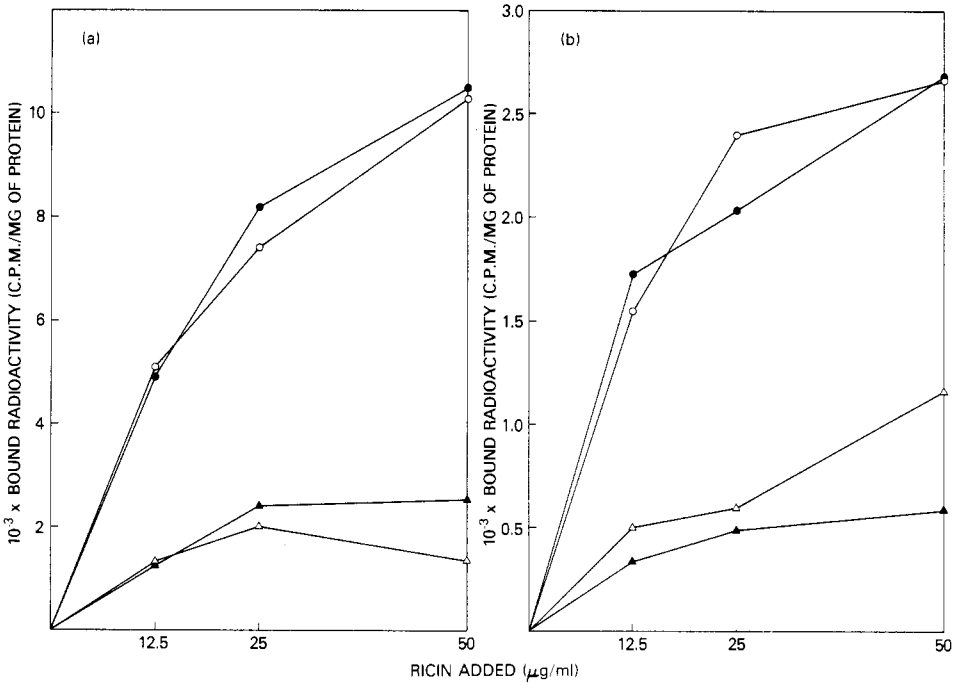

FIGURE 3: Effect of collagenase and Pronase on ricin binding by wild-type and ricin-resistant baby hamster kidney cells. Cells (ca. 2 × 10^6) growing in monolayer culture on 35 mm diameter plastic dishes were incubated at 39 °C for 30 min with solutions of (a) collagenase (50 U/mL) in 0.33 M calcium acetate–0.025 M Tris (pH 7.4); (b) Pronase, 0.0001% (w/v) in phosphate-buffered saline (pH 7.0) or control buffer. After washing, the monolayers were exposed for 30 min at 4 °C to ricin in the concentrations indicated. Bound ricin was estimated as described in the Materials and Methods section: wild-type cells (●, with enzyme; ○, control); ricin-resistant cell line Ric^R21 (▲, with enzyme; △, control).

ricin was required to achieve the same inhibition of wild-type cells not exposed to the enzyme (Figure 2a). The variant Ric^R21 became as sensitive to ricin as the wild-type cells, when monolayers of both cell types were incubated with neuraminidase before exposure to ricin (Figure 2a). When the neuraminidase-treated Ric^R21 monolayers were washed and reincubated for 24 h in nutrient medium in the absence of enzyme, resistance to ricin was partially restored (Figure 2b).

Effect of Neuraminidase on Ricin Binding and Ricin-Inhibited Protein Synthesis in Other Variant Cells. Several variant lines were treated with neuraminidase and their resistance to ricin tested subsequently. Each line was highly resistant to ricin but bound different amounts of the lectin, ranging from 30 to 300% compared with wild-type cells (Table I). In all cases there was at least a doubling of ricin binding and greatly increased inhibition of protein synthesis after neuraminidase treatment (Table I). In Ric^R14, -16, and -22 cells the inhibition was quantitatively similar to results obtained with Ric^R21 cells (Figure 2) in that <1 μg of ricin reduced leucine incorporation by 70% of control. In the case of Ric^R7 and Ric^R12, and to a lesser degree Ric^R17, although measurably increased sensitivity to ricin was observed, the treated cells maintained significantly greater resistance than either wild-type cells or the other neuraminidase-treated variant cells. Behavior resembling that of Ric^R7, Ric^R12, and Ric^R17 cells was reported for mouse L cell variants CL3 and CL6, whose resistance to ricin decreased tenfold after neuraminidase treatment but remained much higher than wild-type L cells (Gottlieb and Kornfeld, 1976).

Effect of Collagenase and Pronase on Ricin Binding by Wild-Type and Variant Cells. Collagenase treatment of wild-type and Ric^R21 fibroblasts had little or no effect on ricin binding, as shown in Figure 3a. Pronase, in concentrations of 0.0001% (w/v), also had no significant effect on ricin binding by wild-type or variant cells (Figure 3b). At higher concentrations, up to 0.01%, Pronase caused rounding and detach-

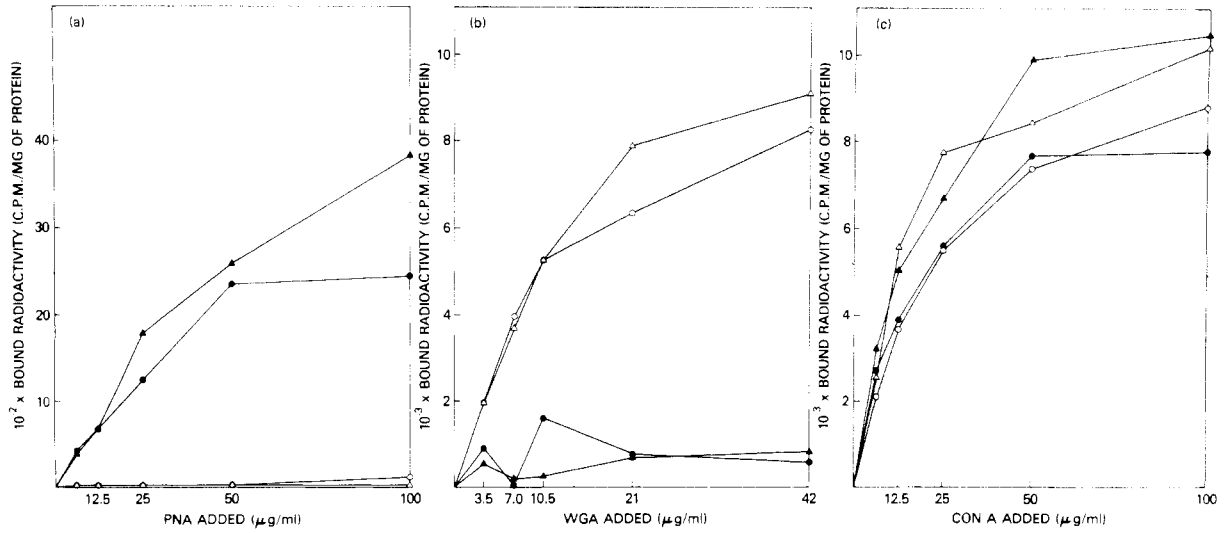

FIGURE 4: Effect of neuraminidase on the binding of peanut agglutinin, wheat-germ agglutinin, and concanavalin A by wild-type and ricin-resistant baby hamster kidney cells. Cells (ca. 2×10^6) growing in monolayer culture on 35 mm diameter plastic dishes were incubated at 39 °C for 30 min with neuraminidase or control buffer. After washing, the monolayers were exposed for 30 min at 4 °C to (a) peanut agglutinin (PNA); (b) wheat-germ agglutinin (WGA); (c) concanavalin A (Con A) in the concentrations indicated. Bound lectins were estimated as described in the Materials and Methods section: wild-type cells (●, with neuraminidase; ○, control); ricin-resistant cell line Ric^R21 (▲, with neuraminidase; △, control).

ment of cells from the monolayers. However, even the lower concentration of Pronase used in the ricin-binding experiment is known (R. Nairn and R. C. Hughes, manuscript in preparation) to remove cell surface material including the large external transformation-sensitive ("LETS" ("250K") glycoprotein (Hynes, 1974), although monolayer morphology was not grossly altered. The general lack of effect shown by each of these enzymes contrasts with the striking enhancement of ricin binding following exposure of both wild-type and Ric^R21 variants to neuraminidase (Figure 1, Table I).

Effect of Neuraminidase on the Binding of Other Lectins (Peanut Agglutinin, Wheat-Germ Agglutinin, Concanavalin A) by Wild-Type and Variant Cells. Binding of peanut agglutinin (PNA) by wild-type and Ric^R21 cells was trivial (<0.5 μg of lectin bound/mg of cell protein) at all concentrations of lectin examined (Figure 4a). Treatment of the monolayers with neuraminidase produced a striking increase in lectin binding, reaching levels of 7 μg of PNA bound/mg of protein for the wild-type cells and 11 μg of PNA/mg of protein for Ric^R21, at lectin concentrations of 100 μg/mL (Figure 4a).

With wheat-germ agglutinin (WGA), on the other hand, the high binding (5.0 μg of WGA/mg of cell protein) of the lectin by monolayers of wild-type and variant cells was greatly reduced (0.5 μg of WGA/mg of cell protein) after neuraminidase (Figure 4b). This is not unexpected in light of the known avidity of wheat-germ agglutinin for glycosides of sialic acid (Greenway and LeVine, 1973).

Exposure of the monolayers of both cell types to neuraminidase had little effect on the binding of concanavalin A (Figure 4c), a lectin with affinity for mannose-containing glycosides (Agrawal and Goldstein, 1967). Untreated Ric^R21 cells bound somewhat more of the lectin than did the parent cells, in agreement with previous results from this laboratory (Meager et al., 1976).

Discussion

Neuraminidase treatment of both wild-type and ricin-resistant variant cells produced an increase in binding of ricin (Figure 1, Table I), and a concomitant increase of ricin entry into the cells, as evidenced by the toxic effect of this lectin on protein synthesis (Figure 1, Table I). Indeed, after neuraminidase treatment, the dose–response curves for ricin-sensitive wild-type and ricin-resistant variant cells became closely similar (Figure 2a, Table I), except for the case of Ric^R7 cells, and to a lesser extent Ric^R12 and Ric^R17 cells. Enhancement of ricin binding by neuraminidase has also been noted by others studying different cell lines (Nicolson, 1973; Adair and Kornfeld, 1974; Gottlieb and Kornfeld, 1976). It does not appear to be due to contamination of the enzyme by proteases or endoglycosidases. Although such contamination has been observed in neuraminidase purified from *Clostridium perfringens* (Chien et al., 1975), the Behringwerke–Hoechst enzyme, purified from *Vibrio cholerae,* was found to be free of these activities (Y.-T. Li, personal communication).

The enhanced binding of ricin after neuraminidase treatment, like the "basal" binding of ricin, was blocked by lactose, a β-D-galactopyranoside. Since the binding of this lectin is known to be restricted to β-glycosides of D-galactose and of *N*-acetyl-D-galactosamine, neuraminidase treatment must have uncovered such residues. The "unmasking" by neuraminidase of ricin receptors is in keeping with what is known about the structure of cell membrane glycoproteins (Hughes, 1976) and glycolipids (Fishman and Brady, 1976). In many of these complex carbohydrates sialic acid is the terminal sugar residue; galactose is penultimate and is "unmasked" after removal of the sialic acid with neuraminidase.

Our data argue for the existence of at least two classes of ricin receptors on the surface of baby hamster kidney cells (Figure 5). One class is "exposed" and available for ricin binding without pretreatment with neuraminidase (R_E in Figure 5). Within this class there appear to be at least two subtypes. Binding to one subtype is "productive" of cytotoxic effects in the wild-type strain; this subtype is deficient in ricin-resistant variants. The other subtype binds ricin but the binding is "not productive" of toxic effects on the cell, a distinction pointed out by Nicolson et al. (1976) in their study of a ricin-resistant murine lymphoma. Nonproductive receptors are present on the surface of wild-type cells and, in reduced amounts, on the surface of Ric^R21. These receptors are expressed even more prominently in certain other ricin-resistant variants that, like Ric^R21, are resistant to ricin but bind

wild-type amounts of the lectin (Table I; Meager et al., 1976; Hughes, 1977).

The second class of ricin receptors is "cryptic" and exposed only after incubation with neuraminidase (R_C in Figure 5). The reason for crypticity is unknown but could be due to a carbohydrate chain bearing several sialic acid residues as shown in Figure 5. Reincubation of ricin-sensitive neuroaminidase-treated cells, after removal of neuraminidase by washing, partially restores the masking (Figure 2b) and the cells revert to a resistant state. This is presumably due to de novo regeneration of sialic acid containing complex carbohydrates at the cell surface (Hughes et al., 1972). Like the exposed and productive receptors, the cryptic receptors, or at least a fraction of them, are productive of cytotoxicity in neuraminidase-treated wild-type cells and ricin-resistant variants.

We have not excluded the possibility that the exposed or cryptic ricin receptors, or both, could be glycolipids. Most glycolipids contain galactose or *N*-acetyl-D-galactosamine, and the gangliosides (Fishman and Brady, 1976) have galactose penultimate to terminal sialic acid. Several glycolipids bind ricin (Surolia et al., 1975; Triche et al., 1975; Gardas, 1976b), and Hughes and Gardas (1976) found enhanced ricin binding and a lower threshold for cytotoxicity in both wild-type and Ric^R21 baby hamster kidney cells incubated with a ricin-binding glycolipid fraction extracted (Gardas, 1976a) from human erythrocytes. Although Yogeeswaran et al. (1974) found no change in patterns of major glycosphingolipids between wild-type and lectin-resistant Chinese hamster ovary cells, a definite increased sialylation of lactosyl ceramide to give a higher ganglioside content has been reported in a ricin-resistant mouse L cell variant, and this was correlated with loss of ricin-binding sites in these cells (Gottlieb and Kornfeld, 1976).

It is more likely from our results, however, that the ricin receptors of baby hamster kidney cells are glycoproteins, and the reduced binding of ricin by several of the ricin-resistant variants is due to alterations in glycoprotein structure. In cell-surface labeling experiments with lactoperoxidase, nine distinct glycosylated polypeptides bearing exposed ricin receptors have been detected by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of the fraction of cell extracts bound by a ricin–agarose affinity column (R. Nairn and R. C. Hughes, submitted for publication). Similarly, glycopeptides deriving from cell-surface glycoproteins, prepared from Pronase digestion of wild-type cells grown in labeled glucosamine, bind tightly to a ricin affinity column (S. W. Rosen, L. Wilson, and R. C. Hughes, manuscript in preparation). Complex oligosaccharides containing the peripheral sequence *N*-acetylneuraminic acid–galactose–*N*-acetyl-D-glucosamine linked to a "core" region made up of mannose and *N*-acetyl-D-glucosamine have been isolated from baby hamster kidney cell membranes (Ogata et al., 1976). Such chains may be present in the glycopeptide fraction obtained from wild-type cells and represent the putative exposed and productive ricin-binding sites (R_E in Figure 5) mediating internalization and toxicity of the lectin in sensitive cells, since it has been shown that ricin binds avidly to such sequences (Nicolson and Blaustein, 1972; Hughes et al., 1973). The sugar composition of glycopeptides from wild-type cells which bind to a ricin affinity column is consistent with these sequences (S. W. Rosen, L. Wilson, and R. C. Hughes, manuscript in preparation).

Certain blocks or alterations in assembly of these oligosaccharide chains would lead to reduced binding of ricin and decreased sensitivity to its toxic effects. In at least one variant clone, Ric^R14, the loss of ricin binding sites correlates with loss of activity of an *N*-acetylglucosaminyltransferase (Meager et al., 1975), the first enzyme necessary for assembly of the peripheral trisaccharide sequence mentioned above. Such a loss has been reported for other lectin-resistant cell variants (Gottlieb et al., 1975; Stanley et al., 1975) and presumably leads to a block in assembly of exposed receptors. It is striking, however, that such an enzymic defect does not affect assembly of the carbohydrate chains carrying the cryptic receptors. This suggests strongly that the structure of oligosaccharides bearing the exposed ricin receptors and those bearing the cryptic receptors are different and under separate genetic control. None our our data exclude the possibility that both exposed and cryptic ricin receptors may be part of a single cell membrane glycoprotein rather than on two separate glycoproteins.

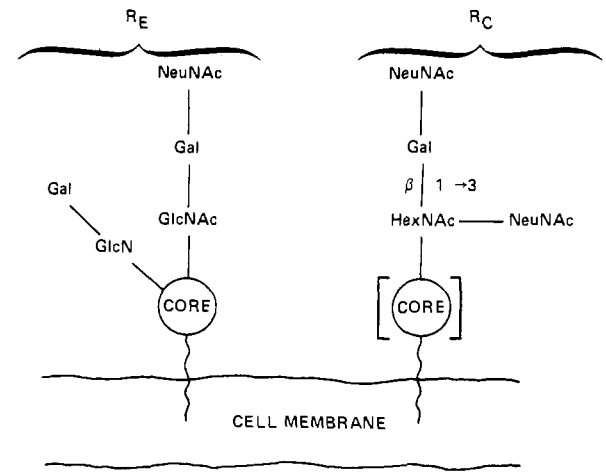

FIGURE 5: Model of ricin receptors on baby hamster kidney cells. Two types of oligosaccharide chains are shown; these could be in the same or different glycoprotein molecules in the membrane. The "exposed" ricin-binding receptors, R_E, contain a core region of mannose and *N*-acetyl-D-glucosamine linked to an asparagine residue, and terminated by sequences containing a ricin-binding galactosyl residue. These sequences resemble those recently described by Ogata et al. (1976). The "cryptic" ricin-binding receptors, R_C, which may or may not contain a core region, are perhaps more heavily sialylated than the R_E receptors and are unmasked by neuraminidase treatment; these desialylated residues also then bind peanut agglutinin. It cannot be ruled out that the R_E receptors may also bind peanut agglutinin after neuraminidase treatment. In ricin-resistant variants, however, assembly (or possibly function) of the R_E receptors is disturbed, while R_C receptors appear to be unaffected. Abbreviations: *N*-acetylneuraminic acid (NeuNAc), D-galactose (Gal), *N*-acetyl-D-glucosamine (GlcNAc), glucosamine (GlcN), *N*-acetyl-D-hexosamine (HexNAc).

The striking enhancement of peanut agglutinin binding to wild-type or ricin-resistant baby hamster kidney cells after neuraminidase treatment is similar to that reported for lymphocytes by Novogrodsky et al. (1975). The specificity of this lectin is well studied (Lotan et al., 1975; Pereira et al., 1976) and suggests that the cryptic receptors include at least the disaccharide sequences D-galactosyl-β-(1→3)-*N*-acetylgalactosamine or D-galactosyl-β-(1→3)-*N*-acetyl-D-glucosamine. Presumably, in the intact oligosaccharide chains, these sequences are heavily substituted by sialic acid (*N*-acetylneuraminic acid) residues (R_C in Figure 5), and only after neuraminidase treatment do they become terminal and available to peanut agglutinin, as seen by the negligible binding of this lectin by untreated cells (Figure 4a). Interestingly, when fully sialylated, these galactosyl sequences also do not bind to ricin, although other sequences, such as the peripheral trisaccharide sialic acid–galactose-β(1→4)-*N*-acetyl-D-glucosamine of α_1-acid glycoprotein (Hughes et al., 1973) or thyroglobulin (Fukuda and Egami, 1971; Toyoshima et al., 1972) present in the putative exposed receptors (R_E in Figure

5) bind ricin even without removal of the sialic acid residues. The sequence galactose β-(1→3)-*N*-acetylhexosamine, to which peanut agglutinin is most complementary (Pereira et al., 1976), is found in a glycoprotein ("glycophorin") of the human erythrocyte membrane (Marchesi and Furthmayr, 1976). The oligosaccharide containing this sequence is known to bind ricin only after neuraminidase treatment (Adair and Kornfeld, 1974) and therefore approximates the properties of the putative cryptic receptor of baby hamster kidney cells (R_C in Figure 5).

The ricin-binding properties of baby hamster kidney cells are unaffected by treatment with collagenase or Pronase. The cryptic ricin-binding sites, therefore, are not masked by a superficial layer of extraneous collagen or other protein. These results are consistent with the model suggested above. Binding of wheat-germ agglutinin, a lectin with affinity for glycosides of sialic acid (Greenway and LeVine, 1973), was decreased following neuraminidase incubation. Thus, the wheat-germ agglutinin receptors may, at least in part, be the very sialic acids masking the cryptic ricin receptors. Since there appears to be little, if any, difference in wheat-term agglutinin binding between wild-type and Ric^R21 cells, oversialylation in the variant cell line of ricin receptors normally exposed in wild-type cells (Gottlieb and Kornfeld, 1976) is unlikely as a mechanism of ricin resistance in these cells. Finally, that neuraminidase treatment had little or no effect on the binding of concanavalin A, a lectin showing particular complementarity for oligosaccharides containing three consecutive α-(1→2)-D-mannopyranosyl residues (So and Goldstein, 1968), follows from the internal or "core" position of such repeating mannose units in all known cell membrane glycoproteins (Hughes, 1976).

The main point to be made from our data is that alterations in cell-surface carbohydrate structure arising from selection with ricin does not, in any of the ricin-resistant variants examined, affect the expression of cryptic sites that bind ricin after exposure to neuraminidase. An explanation for ricin resistance in these cells, particularly the cell lines binding normal amounts of ricin, is a block of internalization of the lectin or its toxic subunit (Olsnes and Pihl, 1973). In this case the toxin molecules responsible for the biological activity on cytoplasmic protein synthesis are inactive. However, a total block in endocytotic uptake of ricin can probably be excluded as a mechanism of resistance in the variant lines studied in this paper, since these same cells, when supplied with new ricin-binding sites by neuraminidase treatment, clearly internalize the toxic lectin. We can conclude, therefore, that the genetic lesion in each of these lines is affecting directly the recognition by ricin of its productive surface receptors, although the mechanisms by which this is achieved are obviously extremely varied.

Acknowledgments

We thank Lionel Wilson for technical assistance, Terry Butters for analysis of the ricin preparations on polyacrylamide gels, and Peggy Jacobs and Elizabeth Watson for secretarial help.

References

Adair, W. L., and Kornfeld, S. (1974), *J. Biol. Chem. 249,* 4696.

Agrawal, B. B. L., and Goldstein, I. J. (1967), *Biochim. Biophys. Acta 147,* 262.

Allen, A. K., Neuberger, A., and Sharon, N. (1973), *Biochem. J. 131,* 155.

Chien, S.-F., Yevich, S. J., Li, S.-C., and Li, Y.-T. (1975), *Biochem. Biophys. Res Commun. 65,* 683.

Critchley, D. R., and Macpherson, I. (1973), *Biochim. Biophys. Acta 296,* 145.

Fishman, P. H., and Brady, R. O. (1976), *Science 194,* 906.

Fukuda, M., and Egami, F. (1971), *Biochem. J. 123,* 415.

Gardas, A. (1976a), *Eur. J. Biochem. 68,* 177.

Gardas, A. (1976b), *Eur. J. Biochem. 68,* 185.

Gottlieb, C., Baenziger, J., and Kornfeld, S. (1975), *J. Biol. Chem. 250,* 3303.

Gottlieb, C., and Kornfeld, S. (1976), *J. Biol. Chem. 251,* 7761.

Greenway, P. J., and LeVine, D. (1973), *Nature (London), New Biol. 241,* 191.

Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963), *Biochem. J. 89,* 114.

Hughes, R. C. (1976), Membrane Glycoproteins: a Review of Structure and Function, London, Butterworths.

Hughes, R. C. (1977), *J. Supramol. Struct.* (in press).

Hughes, R. C., and Gardas, A. (1976), *Nature (London) 264,* 63.

Hughes, R. C., Palmer, P. D., and Sanford, B. H. (1973), *J. Immunol. 111,* 1071.

Hughes, R. C., Sanford, B., and Jeanloz, R. W. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 942.

Hynes, R. O. (1974), *Cell 1,* 147.

Lin, J. Y., Liu, K., Chen, C. C., and Tung, T. C. (1971), *Cancer Res. 31,* 921.

Lotan, R., Skutelsky, E., Danon, D., and Sharon, N. (1975), *J. Biol. Chem. 250,* 8518.

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

Merchesi, V. T., and Furthmayr, H. (1976), *Annu. Rev. Biochem. 45,* 667.

Meager, A., Ungkitchanukit, A., and Hughes, R. C. (1976), *Biochem. J. 154,* 113.

Meager, A., Ungkitchanukit, A., Nairn, R., and Hughes, R. C. (1975), *Nature (London) 257,* 137.

Nicolson, G. L. (1973), *J. Natl. Cancer Inst. 50,* 1443.

Nicolson, G. L. (1974), *Nature (London) 251,* 628.

Nicolson, G. L., and Blaustein, J. (1972), *Biochim. Biophys. Acta 266,* 543.

Nicolson, G. L., Lacorbiere, M., and Eckhart, W. (1975a), *Biochemistry 14,* 172.

Nicolson, G. L., Lacorbiere, M., and Hunter, T. R. (1975b), *Cancer Res. 35,* 144.

Nicolson, G. L., Robbins, J. C., and Hyman, R. (1976), *J. Supramol. Struct. 4,* 15.

Novogrodsky, A., Lotan, R., Ravid, A., and Sharon, N. (1975), *J. Immunol. 115,* 1243.

Ogata, S.-I., Muramatsu, T., and Kobata, A. (1976), *Nature (London) 259,* 580.

Olsnes, S., and Pihl, A. (1973), *Biochemistry 12,* 3121.

Olsnes, S., and Pihl, A. (1976), in The Specificity and Action of Animal, Bacterial and Plant Toxins, Series B, Vol. 1. Cuatrecasas, P., Ed., London, Chapman and Hall.

Olsnes, S., Refsnes, K., and Pihl, A. (1974a), *Nature (London) 249,* 627.

Olsnes, S., Saltvedt, E., and Pihl, A. (1974b), *J. Biol. Chem. 249,* 803.

Olsnes, S., Sandvig, K., Refsnes, K., and Pihl, A. (1976), *J. Biol. Chem. 257,* 3985.

Pereira, M. E. A., Kabat, E. A., Lotan, R., and Sharon, N. (1976), *Carbohydr. Res. 51,* 107.

Refsnes, K., Olsnes, S., and Pihl, A. (1974), *J. Biol. Chem. 249,* 3557.

Sandvig, K., Olsnes, S., and Pihl, A. (1976), *J. Biol. Chem. 257,* 3977.

Sargent, J. R., and Campbell, P. N. (1965), *Biochem. J. 96,* 134.
Schramm, G., and Mohr, E. (1959), *Nature (London) 183,* 1677.
So, L. J., and Goldstein, I. J. (1968), *J. Biol. Chem. 243,* 2003.
Stanley, P., Narasimhan, S., Siminovitch, L., and Schachter, H. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 3323.
Surolia, A., Bachhawat, B. K., and Podder, S. K. (1975), *Nature (London) 257,* 802.
Toyoshima, S., Fukuda, M., and Osawa, T. (1972), *Biochemistry 11,* 4000.
Triche, T. J., Tillack, T. W., and Kornfeld, S. (1975), *Biochim. Biophys. Acta 394,* 540.
Yogeeswaran, G., Murray, R. K., and Wright, J. A. (1974), *Biochem. Biophys. Res. Commun. 56,* 1010.

Effect of Estradiol-17β on Preprolactin Messenger Ribonucleic Acid Activity in the Rat Pituitary Gland†

Roger T. Stone,‡ Richard A. Maurer,§ and Jack Gorski*

ABSTRACT: Rat pituitary RNA was translated in the wheat germ system. Preprolactin messenger RNA activity was estimated by adsorption of cell-free products to solid phase antiprolactin. When male rats were injected for 4 days with estradiol-17β, pituitary preprolactin mRNA activity was increased 2.5- to 3.0-fold over controls. This increase was evident when either total RNA, poly(adenylic acid) RNA, or polysomal RNA was translated in the cell-free system. In male rats receiving daily injections of estradiol-17β, preprolactin mRNA activity was increased to an apparent maximum of 300% of controls after 7 days of treatment. Our data also indicate that estradiol increases preprolactin mRNA activity per μg of RNA as well as the pituitary content of RNA. After estradiol treatment was discontinued, preprolactin mRNA activity declined to 50% of the maximum stimulation after approximately 2 days. In ovariectomized retired breeder female rats, a 5-fold increase in preprolactin activity over ovariectomized controls was obtained. In other studies, a 2-fold increase in preprolactin mRNA activity was obtained in male rats 24 h after a single injection of pimozide, a dopamine blocking drug.

Prolactin secretion and synthesis by the pituitary gland is influenced by hypothalamic factors and by estrogens. Estrogen treatment of either males or ovariectomized females results in an increase in the circulating levels, pituitary content, and rate of PRL[1] synthesis (Catt and Moffat, 1967; Neill, 1972; Yamamoto et al., 1976). It is clear that in the female estrogens function in part via the hypothalamus to mediate cyclic changes in PRL release (Neill, 1972). However, several lines of evidence also suggest that estrogens have direct effects on the pituitary. Gersten and Baker (1970) noted that estrogen pellets implanted into one side of the pituitary of ovariectomized rats resulted in hyperplasia and hypertrophy of lactotrophs only on the side ipsilateral to the implant. In experiments in which the pituitary was removed from hypothalamic control by transplantation to the kidney capsule of hypophysectomized rats estrogen treatment resulted in increased serum PRL levels (Lu et al., 1971). Estrogen binding studies using radioautographic techniques (Keffer et al., 1976) or tissue extracts (Leavitt et al., 1969; Notides, 1970) confirm the pituitary as a target tissue for estrogens.

We have recently demonstrated the synthesis of preprolactin in the wheat germ cell-free translation system directed by RNA from rat pituitaries or MtTW10 pituitary tumor tissue (Maurer et al., 1975, 1976). Preprolactin synthesized in the cell-free system shares major tryptic peptide fragments with authentic rat PRL and has a leucine-rich N-terminal addition of 29 amino acids (Maurer et al., 1977). At least two other laboratories have demonstrated a similar translation product with RNA from rate pituitary cell culture lines and in both instances it was shown that thyrotropin releasing hormone increased messenger RNA (mRNA) activity in RNA preparations from these cells (Evans and Rosenfeld, 1976; Dannies and Tashjian, 1976). In this report we present evidence that preprolactin mRNA activity in RNA isolated from the pituitary glands of male or ovariectomized female rats is increased by treatment with either estradiol or pimozide, a dopamine blocking drug.

Materials and Methods

Materials

[^{3}H]Leucine (55–57 Ci/mmol) and NCS tissue solubilizer were obtained from Amersham/Searle (Arlington Heights, Ill.). Optical grade CsCl and RNase-free sucrose were obtained from Schwarz/Mann (Orangeburg, N.Y.). Bio-Gel A_{15} agarose beads were obtained from Bio-Rad Laboratories (Richmond, Calif.) and wheat germ was a gift from General Mills (Vallejo, Calif.).

† From the Departments of Biochemistry and Animal Science, University of Wisconsin, Madison, Wisconsin 53706. *Received February 23, 1977.* Supported in part by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, Ford Foundation Grant 630-0505A, National Institutes of Health Grant CA 18110 (to J. G.), and a Population Council Fellowship and National Institutes of Health Fellowship F22 HD 03404 (to R. A. M.). A preliminary report of this work was presented at the 1976 ASBC Meetings in San Francisco.

‡ Present address: Department of Biochemistry, University of Texas Medical School, Houston, Texas 77025.

§ Present address: Department of Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, Iowa 52242.

[1] Abbreviations used are: EDTA, ethylenediaminetetraacetic acid; PRL, prolactin; Tris, tris(hydroxymethyl)aminomethane; GH, growth hormone; DEAE, diethylaminoethyl; PBS, phosphate-buffered saline.