Simplification of the ecdysteroid radioimmunoassay by the use of protein A from *Staphylococcus aureus*[1]

J.T. Warren, W. Smith and L.I. Gilbert

Department of Biology, Wilson Hall 046A, University of North Carolina, Chapel Hill (North Carolina 27514, USA), 30 June 1983

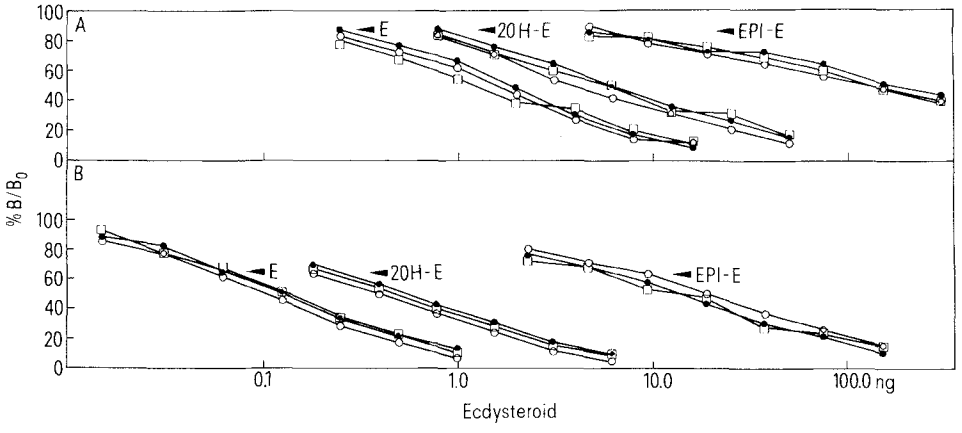
Summary. Three methods of separating antibody-bound ligand from free ligand were compared for an ecdysteroid radioimmunoassay. Ecdysteroid antibody concentration and ligand specific radioactivity were adjusted to measure 2 ranges of ecdysone concentrations (0.01–2.0 ng and 0.25–32.0 ng). In comparison with the traditional separating agent, ammonium sulfate, neither protein A nor polyethylene glycol altered sensitivity or specificity of the radioimmunoassays. The protein A immunoglobulin precipitation method is quick and simple, making it a preferred alternative protocol for terminating ecdysteroid radioimmunoassays.

One of the principal methodological advances in the field of insect endocrinology was the development of the ecdysteroid radioimmunoassay (RIA) by Borst and O'Connor[2]. This RIA has resulted in a plethora of papers in which insect molting hormone has been quantified during specific developmental stages, e.g. in *Manduca sexta*[3], *Calpodes ethlius*[4]. In addition, it has proven useful in identifying ecdysteroids collected from high performance liquid chromatography (HPLC)[5] and has resulted in the development of a specific and reproducible in vitro assay for the insect prothoracicotropic hormone[6].

Separation of antibody-bound from free ligand for the ecdysteroid RIA has been achieved in various ways including equilibrium dialysis[7], double-antibody precipitation[8], gel filtration[9], dextran-coated charcoal[10], and ammonium sulfate[2]. Due to the high cost and/or labor requirements of the first 3 of these methods, and the adverse effects of charcoal absorption on antibody ligand equilibria[11], ammonium sulfate precipitation is most commonly employed as a separating agent for ecdysteroid RIA. This method, however, is time-consuming for large scale processing of samples, such as has recently become necessary in our laboratory following HPLC of biological material. In an attempt to further simplify the ecdysteroid RIA, we have tested 2 additional methods of separating bound from free ligand: polyethylene glycol (PEG) 6000, recently reported to be of use for separating bound from free ecdysteroids[12], and immobilized protein A, currently in common use for vertebrate peptide radioimmunoassay[13–15].

Materials and methods. Ecdysone and 20-hydroxyecdysone were purchased from Rohto and Sigma while [^{3}H]-ecdysone (~ 60 Ci/mmol) was obtained from New England Nuclear Corp. The latter was repurified by HPLC when necessary. 3-Epi-ecdysone was a generous gift from Drs J. Svoboda and M. Thompson (U.S.D.A., Beltsville, Maryland). Antibody was produced in rabbits against an ecdysone-22-succinyl thyroglobulin synthesized by Dr D.H.S. Horn (C.S.I.R.O., Canberra, Australia)[16]. Protein A was prepared as a suspension of formaldehyde-treated *Staphylococcus aureus* cells of the Cowan I strain. Such cells are coated with protein A which binds specifically to the F_c portion of antibodies.

S. aureus cells were prepared according to the instructions of Kessler[17] using Tryptic soy broth (Bacto) as culture medium. Packed cells were suspended by weight (5%) in buffer (150 mM NaCl, 40 mM phosphate, 0.02% sodium azide, pH 7.2). [Similar material is available commercially from Calbiochem-Behring (San Diego, California)]. The ecdysteroid micro-assay (range of ecdysone sensitivity 0.01–2.0 ng/assay) contained 18,000 dpm [^{3}H]-ecdysone (62.7 pg, 60 Ci/mmol), 0.012% antibody serum, 0.05% bovine serum albumin (protein A procedure) or 5% normal rabbit serum (ammonium sulfate, PEG procedures) in 100 mM borate buffer (0.2 ml, pH 8.5). For the ecdysteroid macro-assay (range of ecdysone sensitivity 0.25–32.0 ng/assay)[18], the specific activity of the [^{3}H]-ecdysone tracer was reduced to 4 Ci/mmol (18,000 dpm, 941 pg), while the final antibody concentration was increased to 0.15%. Other aspects of the macro-assay were identical to the micro assay. In both cases, ecdysteroid standards were dissolved in Grace's medium (GIBCO, Grand Island, NY) and assayed in 10-µl aliquots; incubations were conducted in 6.5 × 55 mm glass test tubes at 4 °C overnight. Precipitation with ammonium sulfate was carried out as previously described[2,3]. This procedure involved addition of 200 µl of a saturated ammonium sulfate solution to assay tubes while vortexing vigorously in the cold (4 °C), followed by incubation at 4 °C for 15 30 min. Tubes were then centrifuged for 15 min at 1500 × g, the supernatant aspirated, and pellets rinsed (resuspended, incubated 15 min, centrifuged, supernatant aspirated) in 50% saturated ammo-



A comparison of standard curves using 3 methods to separate bound ecdysone from free (●—●, Staphylococcal protein A; ○—○, ammonium sulfate; □—□, PEG-6000). Panel A shows assay designed to measure 0.25–32 ng ecdysone (macroassay). Panel B shows assay designed to measure 0.01–2 ng ecdysone (microassay). Standard curves for both assays were performed for the following ecdysteroids: ecdysone (E); 20-hydroxyecdysone (20H-E); and 3-epiecdysone (EPI-E). Eachpoint represents the mean of 8 (E) or 4 (20 H-E, EPI-E) determinations. Co-efficients of variation averaged less than 10%.

nium sulfate. Rinsed pellets were suspended in 25 µl water. Protein A (20 µl of a 5% wet wt/vol solution) or PEG-6000 (200 µl of a 35% solution) were added to assay tubes followed by vortexing at room temperature. Particular care was required while vortexing the viscous PEG in order to obtain a homogeneous solution. For the protein A and PEG procedures, tubes were incubated at room temperature for 15 min, centrifuged at 1500 × g for 15 min, supernatants aspirated, and pellets suspended in 50 µl water. For all 3 procedures, samples were brought to a final volume of 500 µl with Scintiverse (Fisher) and counted by scintillation spectroscopy (LKB). Standard curves were analyzed by a logit-log transformation program.

Results and conclusions. The assay competition curves for ecdysone, 20-hydroxyecdysone and 3-epi-ecdysone obtained by the 3 procedures (PEG, ammonium sulfate, protein A) using both the micro- and macro-RIA protocols are shown in the figure. As evident from this figure, the 3 precipitating agents had little effect on assay sensitivity or specificity. The amounts of ecdysteroids required to inhibit 50% [^{3}H]-ecdysone binding (I_{50}) for the 3 procedures varied as follows: micro-assay – 0.11–0.13 ng ecdysone/assay, 0.39–0.49 ng 20-hydroxyecdysone/assay, 13.0–19.0 ng 3-epi-ecdysone/assay; macro-assay – 1.3–1.8 ng ecdysone/assay, 4.0–6.2 ng 20-hydroxyecdysone/assay, 120–150 ng 3-epi-ecdysone/assay.

The 3 procedures have been used in our laboratory to determine total ecdysteroid levels in tissues and hemolymph during the metamorphosis of *Manduca sexta*, as well as for the quantification of HPLC fractions of ecdysteroids from larval hemolymph and embryos. Although results were equivalent for the 3 methods, the protein A procedure was easier and more rapid. For example, precipitation did not have to be done in the cold as required with ammonium sulfate, and rapidity of vortexing was not critical. Indeed, one of the pitfalls in obtaining reliable results with ammonium sulfate is the tendency of inexperienced researchers to vary the time and/or intensity of the vortexing step after initial addition of the precipitating agent. Further, with protein A, 1 centrifugation step was sufficient to lower non-specific binding to less than 10% of total counts bound. This reduced the time required for assay termination by 30–50%. The protein A technique is, therefore, recommended highly as an alternative to the standard ammonium sulfate protocol for terminating ecdysteroid RIAs. In addition, it should be an efficient tool for use in vertebrate steroid hormone RIAs as well. To our knowledge, this is the first description of the use of protein A in a steroid hormone RIA but it may have even wider applicability since it also appears to be useful in the juvenile hormone (sesquiterpene) RIA[19].

1 Supported by grants AM-30118 from the National Institutes of Health and PCM-8116931 from the National Science Foundation.
2 Borst, D., and O'Connor, J.D., Science *178* (1972) 418.
3 Bollenbacher, W.E., Smith, S.L., Goodman, W., and Gilbert, L.I., Gen. comp. Endocr. *44* (1981) 302.
4 Dean, R.L., Bollenbacher, W.E., Locke, M., Smith, S.L., and Gilbert, L.I., J. Insect Physiol. *26* (1980) 267.
5 DeLaage, M.A., Hirn, M.H., and DeReggi, M.L, in: Methods in Enzymology, vol.84, p.350. Eds J.J. Langone and H. Van Vunakis. Academic Press, New York 1982.
6 Bollenbacher, W.E., Agui, N., Granger, N.A., and Gilbert, L.I., Proc. natl Acad. Sci. USA *76* (1979) 5148.
7 DeReggi, M.L., Hirn, M.H., and DeLaage, M.A., Biochem. biophys. Res. Commun. *66* (1975) 1307.
8 Lazarovici, P., Shapira, D., and Shaaya, E., Comp. Biochem. Physiol. *74A* (1983) 525.
9 Garen, A., Kauvar, L., and Lepesant, J.A., Proc. natl Acad. Sci. USA *74* (1977) 5039.
10 Porcheron, O., Foucrier, J., Gros, C., Pradelles, P., Cassier, P., and Dray, F., FEBS Lett. *61* (1976) 159.
11 Malvano, R., and Rolleri, E., in: Radioimmunoassay of Steroid Hormones, 2nd edn, p.27. Ed. D. Gupta. Weinheim, Deerfield Park, Florida, 1980.
12 Briers, T., and DeLoof, A., J. invert. Reprod. *3* (1981) 145.
13 Frohman, M.A., Frohman, L.A., Goldman, M.B., and Goldman, J.M., J. Lab. clin. Med. *93* (1979) 614.
14 Buckley, D.I., Hagman, J., and Ramachandran, J., Endocrinology *109* (1981) 10.
15 Conn, P.M., Rogers, D.C., and Sheffield, T., Endocrinology *109* (1981) 1122.
16 Gilbert, L.I., Goodman, W., and Bollenbacher, W.E., in: Biochemistry of Lipids II, p.1. Ed. T. Goodwin. University Park Press, Baltimore 1977.
17 Kessler, S.W., J. Immun. *115* (1975) 1617.
18 Bollenbacher, W.E., Katahira, E., O'Brien, M., and Gilbert, L.I., Molec. cell. Endocr. *32* (1983) 27.
19 Granger, N.A., personal communication.

0014-4754/84/040393-02$1.50 + 0.20/0

Effect of insulin on the synthesis and release of lipid peroxide by cultured hepatocytes isolated from normal and diabetic rats

K. Kosugi[1], Y. Harano, A. Kashiwagi, M. Suzuki and Y. Shigeta

The Third Department of Medicine, Shiga University of Medical Science, Ohtsu, Shiga 520-21 (Japan), 17 May 1983

Summary. Lipid peroxide content in hepatocytes isolated from ketotic diabetic rats was higher than normal, and the release of peroxide into the media was also elevated for the initial 18 h. Insulin suppressed both peroxide release and synthesis by cultured hepatocytes isolated from normal and from diabetic rats.

Lipid peroxide (LPO) in serum has been reported to be elevated in diabetic subjects, especially with macro- or micro-angiopathy[2]. Since LPO has been suggested to play an important role in tissue injury of vascular origin[3,4], elevated LPO in diabetes may therefore play a role in the pathogenesis of diabetic angiopathy[5]. Further, liver has been reported to be a major organ for both the synthesis and removal of LPO[6,7]. Therefore, insulin deficiency may possibly stimulate the formation and release of hepatic LPO in the diabetic. We report here our study of the effect of insulin on LPO production in liver, done in an effort to elucidate the mechanism causing high serum levels of LPO in diabetes. We have assessed LPO synthesis and release by isolated and cultured hepatocytes from normal and diabetic rats. In vitro effects of insulin on LPO synthesis and release by cultured hepatocytes have also been investigated.

Materials and methods. Diabetes was produced in male Sprague-Dawley rats, weighing 250–300 g, by the intravenous injection of streptozotocin, 10 mg/100 g b.wt. Those rats exhib-