in satiated animals the CCK peptides do not increase exploration but even possess central depressant effects[10]. Thus the increased exploration in the presence of decreased consuming activity seems to be an additional measure of hunger suppression.

1 J. Gibbs, R.C. Young and G.P. Smith, J. comp. physiol. Psychol. *84*, 488 (1973).
2 J. Antin, J. Gibbs, J. Holt, R.C. Young and G.P. Smith, J. comp. physiol. Psychol. *89*, 784 (1975).
3 D.N. Lorenz, G. Kreielsheimer and G.P. Smith, Physiol. Behav. *23*, 1065 (1979).
4 J.F. Rehfeld, N. Golterman, L.-J. Larsson, P.M. Emson and C.M. Lee, Fedn Proc. *38*, 2325 (1979).
5 E. Straus and R. Yalow, Fedn Proc. *38*, 2320 (1979).
6 E. Straus and R. Yalow, Science *203*, 68 (1979).
7 L.-J. Larsson and J.F. Rehfeld, Brain Res. *165*, 201 (1979).
8 J.F. Rehfeld, L.-J. Larsson, N.R. Goltermann, T.W. Schwartz, J.J. Holst, S.L. Jensen and J.S. Morley, Nature *284*, 33 (1980).
9 L. Halmy, C. Nyakas and J. Walter, submitted.
10 G. Zetler, Neuropharmacology *20*, 277 (1981).

PRO EXPERIMENTIS

Development of an enzyme linked immunosorbent assay (ELISA) for measurement of vitellogenin concentrations in cockroaches

M. Dumas, J. Buschor and B. Lanzrein[1]

Division of Zoophysiology, Institute of Zoology, University of Berne, Engehaldenstrasse 6, CH-3012 Berne (Switzerland), 7 January 1982

Summary. A competitive enzyme linked immunosorbent assay was developed for the quantification of a large lipoprotein, namely the yolk protein vitellogenin, in the haemolymph of cockroaches *(Nauphoeta cinerea).* This assay was found to be specific, reproducible and it has a high sensitivity (approximately 10 ng).

Vitellogenins are female specific proteins which in the cockroach *Nauphoeta cinerea* are synthesized in the fat body and incorporated into the oocytes under the control of juvenile hormone[2-4]. For our studies on the production and incorporation of vitellogenin in different physiological stages and under various experimental conditions we needed a sensitive and specific assay for the measurement of vitellogenin concentrations. An enzyme linked immunosorbent assay (ELISA) seemed to meet our demands[5,6]. Enzyme immunoassays depend on the assumption that either an antigen or an antibody can be linked to an enzyme whilst retaining both immunological and enzymic activity in the resultant conjugate. The technique described here involves the following steps: adsorbtion of antivitellogenin to polystyrene tubes, incubation of known quantities of standard vitellogenin or unknown samples together with enzyme-labelled vitellogenin (competition step), incubation with enzyme substrate and measurement of resulting colour change.

Material and methods. Nauphoeta cinerea is an ovoviviparous cockroach. Under the rearing conditions used (26 °C, 60% relative humidity) its oocyte maturation cycle lasts 12 days and the pregnancy 35–40 days.

For developing an ELISA, purified vitellogenin and a vitellogenin-specific antibody had to be prepared. For purification of vitellogenin, which is a large lipoprotein, haemolymph was collected from reproducing females, added on a DEAE column and eluted with a gradient of NaCl 0.15–0.6 M at pH 6.5 (for details see Buschor and Lanzrein[7]). Antibodies were raised in rabbits against egg case homogenates[2] and the antiserum was run over a sepharose affinity column containing male haemolymph in order to remove cross reacting antibodies. IgG fractions were obtained after precipitation with 45% $(NH_4)_2SO_4$ by chromatography on a protein A Sepharose column. The competitive ELISA established here was set up according to methods described[5,6]. Vitellogenin was conjugated to alkaline phosphatase; first the enzyme was activated using the cross linking agent glutaraldehyde[8,9], and then the activated enzyme was reacted with DEAE purified vitellogenin.

Assay procedure. 1 ml coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6) containing 180 ng IgG from an antivitellogenin serum was incubated in polystyrene tubes (3 ml volume) at 4 °C during 16 h whilst shaking. After 3 washes with phosphate buffered saline (PBS-Tween) the tubes were filled with 3 ml of PBS-Tween containing 0.5% bovine serum albumine and incubated in a shaking water bath for 8 h at 4 °C in order to saturate the polystyrene tube walls. Thereafter, the tubes were washed with PBS-Tween followed by the competition step. The vitellogenin standard solution and the diluted haemolymph samples respectively were incubated together with the vitellogenin-alkalinephosphatase conjugate (1:7000) in 1 ml PBS Tween during 15 h at 4 °C whilst shaking. This was followed by 3 washes with PBS-Tween. For quantification, 1 mg enzyme substrate (p-nitrophenylphosphate) in 1 ml diethanolamine buffer (pH 9.8) was incubated at 20 °C during 40 min. The reaction was stopped by the addition of 100 µl of 3 M NaOH. Photometric measurement was made using a PYE UNICAM spectrophotometer by reading the absorption at 405 nm.

Results and discussion. A typical standard curve obtained by using DEAE purified vitellogenin without and with addition of male haemolymph as control is shown in figure 1 and demonstrates a linear slope between concentrations of

Calculation of haemolymph vitellogenin (VG) concentrations from figure 2

	Dilution factor	VG (mg/ml)	$\bar{X} \pm SE$
Day-11 females	1.6×10^5	35.2	
	3.2×10^5	35.2	
	6.4×10^5	36.8	
	1.3×10^6	35.8	
			35.9 ± 0.33
Day-3 females	2.6×10^4	5.5	
	5.1×10^4	4.8	
	1.0×10^5	5.9	
	2.0×10^5	6.5	
			5.7 ± 0.36
Day-37 females	3.2×10^3	0.74	
	6.4×10^3	0.87	
	1.3×10^4	0.83	
	2.6×10^4	0.74	
			0.80 ± 0.03
Day-7 males	2×10^2	< 0.01	

4 ng/ml and 500 ng/ml. The assay is thus very specific and sensitive and it is also highly repruducible if one adheres carefully to the prescribed assay conditions. By investigating the effect of the vitellogenin-alkalinephosphatase conjugate concentration we found that lower concentrations (1:10,000 and 1:13,000) resulted in flattened curve slopes and lower maximum absorption values. Lowering of the antibody concentration influences the curve slope in like manner. Shaking after addition of antivitellogenin IgG turned out to increase IgG binding to the polystyrene tubes by approximately 90%, probably due to the increase in surface coming into contact with antibody. Incubation at low temperatures (4 °C) increases the reproducibility of the assay.

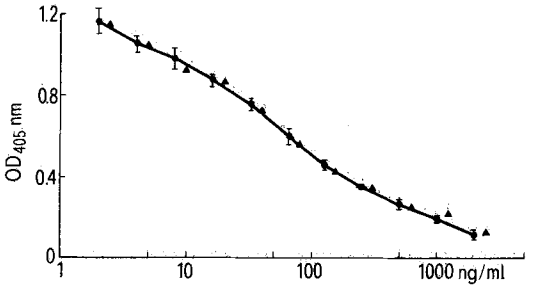

Figure 1. ELISA standard curve (mean values of 3–4 values ± SD) for purified vitellogenin (●) and for purified vitellogenin together with 10^2 times diluted male haemolymph (▲). For practical purposes the triangles are marked on the right side of the points although vitellogenin concentrations were for both the same. Abscissa: vitellogenin concentration; ordinate: absorption at 405 nm.

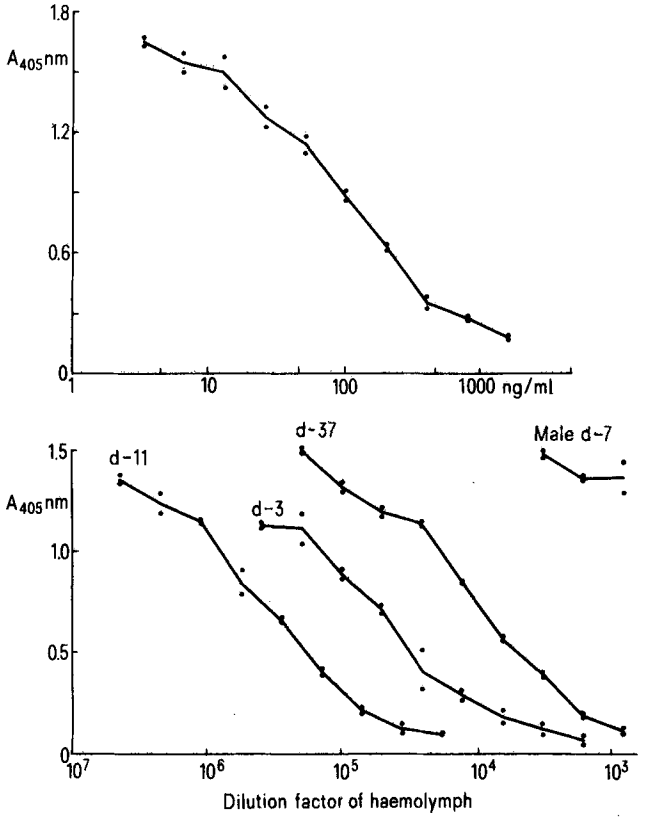

Figure 2. ELISA competition curves of haemolymph from females of different stages and from males (bottom) together with corresponding standard curve (top). Abscissa: vitellogenin concentration (top) or dilution factor of haemolymph (bottom); ordinate: absorption at 405 nm.

Typical titration curves for haemolymph samples from different physiological stages and from a vitellogenin-negative sample (male haemolymph) together with the corresponding standard curve of DEAE purified vitellogenin are given in figure 2. The negative results obtained with male haemolymph even at high concentrations indicate again a high specificity of the assay. The titration curves of 3 physiological stages, namely day 3 (early stage of vitellogenesis), day 11 (late stage of vitellogenesis) and day 37 (pregnancy, no vitellogenesis) display significant differences. The similar slopes to that of the standard curve indicate that the assay is applicable for samples containing both high or low vitellogenin concentrations, and the double values demonstrate a good reproducibility. Calculation from 4 values of the linear range of each curve into haemolymph vitellogenin concentrations according to the standard curve is given in the table and shows the applicability of this assay.

In order to further examine the quality and reliability of the ELISA developed here, we made a direct comparison between rocket-immunoelectrophoresis (another method frequently used in our laboratory[7,10] and ELISA by measuring same standards and biological samples in both assays. We observed that the ELISA results gave always somewhat higher values than the measurements with rocket-immunoelectrophoresis. We have no explanation for this phenomenon. However, the fact that rocket-immunoelectrophoresis, in contrast to ELISA, measures precipitable antibody only might play a role, and some observations suggest that the purity of the vitellogenin-alkalinephosphatase-conjugate might be of importance. Nevertheless, both assays appear to be suitable for measuring and comparing relative vitellogenin concentrations in biological samples; only calculation into absolute values reveals some differences between the 2 assay systems. ELISA has the following advantages over rocket-immunoelectrophoresis: large quantities of samples can be measured simultaneously, less antibody is necessary, the linear concentration range is much larger and the sensitivity is higher. ELISA, as described here, is rather time consuming and thus its application is particularly recommended only if many samples or samples of low vitellogenin concentration have to be tested. However, the procedure could be markedly improved and shortened by using specific ELISA equipment (plates, photometer) and would then be an excellent tool for routine analysis of vitellogenin.

1 Acknowledgments. We would like to express our thanks to Prof. H. Fey and Ms H. Pfister, Veterinary Bacteriological Institute, University of Berne, for introducing us into the ELISA methodology, to P. Beyeler for technical assistance and to M. Kaltenrieder for drawing the graphs. We also thank the Sandoz Foundation for a fellowship to M. Dumas and the Swiss National Science Foundation (grant No. 3.188-0.77) for financial support.

2 G. Bühlmann, Revue suisse Zool. *81*, 642 (1974).
3 B. Lanzrein, Gen. comp. Endocr. *39*, 69 (1979).
4 R. Wilhelm and M. Lüscher, J. Insect Physiol. *20*, 1887 (1974).
5 A. Voller, D.E. Bidwell and A. Bartlett, The enzyme linked immunosorbent assay (ELISA). Dynatech Europe, Borough House, Guernsey, G.B. 1979.
6 H. Fey and G. Stiffler-Rosenberg, Schweiz. Arch. Tierheilk. *119*, 437 (1977).
7 J. Buschor and B. Lanzrein, in preparation.
8 S. Avrameas, Immunochemistry *6*, 43 (1969).
9 S. Avrameas, T. Ternynck and J.-L. Guesdon, Scand J. Immun. *8*, suppl. 7, 7 (1978).
10 B. Lanzrein, R. Wilhelm and J. Buschor, in: Juvenile hormone biochemistry, p. 147. Eds G.E. Pratt and G.T. Brooks. Elsevier, North Holland Biomedical Press, Amsterdam 1981.