Biochem. J. (1978) **175**, 685–690
Printed in Great Britain

A Radioimmunoassay for Chicken Avidin

COMPARISON WITH A [^{14}C]BIOTIN-BINDING METHOD

By MARKKU S. KULOMAA, HEIKKI A. ELO and PENTTI J. TUOHIMAA
Department of Biomedical Sciences, University of Tampere, Box 607, SF-33101 Tampere 10, Finland

(*Received* 23 *March* 1978)

A double-antibody solid-phase radioimmunoassay for chicken avidin is reported. Avidin was labelled with ^{125}I by the chloramine-T method. The bound and free avidin were separated with a second antibody bound to a solid matrix. In the logit–log scale the standard curve was linear from 1–2 to 100–200ng of avidin/ml. Cross-reaction of ovalbumin was less than 0.015%. Saturation of biotin-binding sites of avidin with an excess of biotin decreased radioimmunoassay values by about 15%. Recovery studies indicated that avidin can be assayed from all chicken tissues studied with radioimmunoassay, whereas the [^{14}C]biotin/bentonite method gave poor recoveries for avidin in the liver and kidney. Radioimmunoassay and the [^{14}C]biotin/bentonite method gave similar concentrations for oviduct avidin.

Avidin is a biotin-binding protein found previously only in the eggs and oviducts of many species of birds and in the egg jelly of frogs at a maximum concentration of about 0.05% of the total protein (Green, 1975). It is induced by progesterone in the oviduct of oestrogen-primed chicks (Hertz *et al.*, 1943; O'Malley *et al.*, 1969). In addition, actinomycin D and tissue trauma cause avidin induction in the chick oviduct (Elo *et al.*, 1975).

Most of the methods used to assay avidin are based on its biotin-binding capacity. Microbial tests (Eakin *et al.*, 1941) are sensitive, but rather time consuming. In addition, their specificity is low, since tissue extracts may contain other antimicrobial agents besides avidin. A disadvantage of spectrophotometric methods (Green, 1963, 1965) is their low sensitivity. The radioactive-ligand-binding methods, which are based on the use of [^{14}C]biotin, are currently the most widely used assay systems. Unbound [^{14}C]biotin and the avidin–[^{14}C]biotin complex have been separated, e.g. by CM-cellulose (Green, 1963) or Sephadex gel (Wei & Wright, 1964). Korenman & O'Malley (1967) adsorbed the avidin–[^{14}C]biotin complex on to bentonite. We have modified this method to one that is easy and rapid (Elo *et al.*, 1975), or sensitive (Elo & Tuohimaa, 1974). O'Malley & Korenman (1967) have also developed an immunoprecipitation method for avidin, where the avidin–[^{14}C]biotin complex is precipitated with the antibody.

Because the avidin–biotin complex is very stable, all the methods based on biotin binding are disturbed by endogenous biotin, which prevents [^{14}C]biotin from being bound. Most of the [^{14}C]biotin methods may also be affected by any biotin-binding factor in the tissue. In recent studies we have found that tissue injury and acute inflammation induce avidin in the chick not only in the oviduct, but also in non-oviductal tissues (Elo *et al.*, 1978). Because the recoveries of the [^{14}C]biotin/bentonite method for avidin are low in the liver and kidney, it was necessary to develop a new and sensitive radioimmunoassay for chicken avidin. In the present paper we describe a method that is not based on the use of [^{14}C]biotin. Furthermore, we compare the value and accuracy of the radioimmunoassay method and the [^{14}C]biotin/bentonite method for the determination of avidin in different chicken tissues.

Experimental

Materials

Chemicals were obtained from the sources indicated: actinomycin D, avidin (highly purified; 11.2units/mg of protein), bovine serum albumin (96–99% pure), lysozyme (three-times-crystallized) and ovalbumin (99% pure) from Sigma Chemical Co., St. Louis, MO, U.S.A.; biotin, chloramine-T, diethylstilboestrol, PPO (2,5-diphenyloxazole) and progesterone from E. Merck, Darmstadt, Germany; bentonite and merthiolate (thiomersal) from BDH Chemicals, Poole, Dorset, U.K.; radioactive chemicals, D-[*carbonyl*-^{14}C]biotin (sp. radioactivity 57mCi/mmol) and carrier-free Na^{125}I (100mCi/ml) from The Radiochemical Centre, Amersham, Bucks., U.K.; Freund's complete adjuvant from Difco, Detroit, MI, U.S.A.; Sephadex G-50 (medium) from Pharmacia Fine Chemicals, Uppsala, Sweden; Triton X-100 from Kock–Light Laboratories, Colnbrook, Bucks., U.K.; and sheep anti-(rabbit

γ-globulin) immunoadsorbent from Organon, Oss, Holland.

Animals and tissue samples

Leghorn chicks (2–3 days old; strain Ti 13; Munkkila Hatchery, Paimio, Finland) were subcutaneously injected daily with 0.5mg of diethylstilboestrol in 50μl of propylene glycol for 7–10 days. At 1 day after the last diethylstilboestrol injection the animals were injected subcutaneously with 20mg of progesterone in 1ml of propylene glycol/kg body wt. or intraperitoneally with 0.2mg of actinomycin D/kg, or oviducts were ligated as described previously (Elo *et al.*, 1975). Local tissue injury to the intestine and liver was carried out by burning an area of about 1cm² in the liver and a section of 2–2.5cm in length of the intestine with a red-hot electrode (Kaustik-Gerät, Karlheinz Dosch, Heidelberg, West Germany). The animals were killed 24–25h after treatment. Tissues were removed and stored at −20°C until assayed.

[^{14}C]Biotin-binding method

The [^{14}C]biotin-binding method in the present study was a modification (Elo *et al.*, 1975) of the [^{14}C]biotin/bentonite method developed by Korenman & O'Malley (1967) for chick oviduct avidin. Tissue was homogenized on ice in 6–40vol. of a buffer (0.07M-KCl/0.004M-$MgCl_2$/0.07M-NaCl/0.02M-sodium phosphate buffer, pH7.1) with a motor-driven Teflon-pestle homogenizer. The homogenate was then centrifuged for 25min at 4°C and 2500*g*. Avidin was assayed in duplicate from the supernatant at room temperature (22–24°C). The samples corresponded to 25 and 50mg of the tissue in 2ml of homogenization buffer. [^{14}C]Biotin [0.3nmol in 0.5ml of 0.2M-$(NH_4)_2CO_3$] was mixed with the supernatant sample. After 15min incubation, 40mg of bentonite in 2ml of 0.2M-$(NH_4)_2CO_3$ was added to the tube, which was then mixed several times over a 15min period. The tube was centrifuged for 5min at 20°C and 300*g*. The bentonite sediment was washed four times with 3ml of 0.2M-$(NH_4)_2CO_3$ and transferred into a scintillation vial three times with 3.5ml of scintillation solution (3g of PPO, 37ml of ethylene glycol, 106ml of ethanol, 257ml of Triton X-100 and 600ml of xylene). Radioactivity was counted in a liquid-scintillation counter (LKB/Wallac 81000).

Radioimmunoassay

Preparation of antiserum. Avidin antiserum was prepared by immunizing rabbits with 1mg of avidin in 0.5ml of water and Freund's complete adjuvant. Subcutaneous injections were repeated at 2-week intervals for 4–6 months. Blood was taken by cardiac puncture and serum was separated by centrifugation for 15min at 4°C and 3000*g*. Antiserum was stored in small fractions at −80°C.

Labelling of avidin. The purity of highly purified avidin was checked on polyacrylamide-gel electrophoresis containing sodium dodecyl sulphate (Weber & Osborn, 1969). Another faint band, corresponding to a mol.wt. of about 37000, could be seen when a concentration of more than 100μg of avidin was used. Avidin was labelled with carrier-free $Na^{125}I$ by the chloramine-T method of Greenwood *et al.* (1963). $Na^{125}I$ (1mCi; 10μl), 0.5M-sodium phosphate buffer, pH7.5 (10μl), 20μg of avidin (10μl) and 50μg of chloramine-T (10μl) in 0.05M-sodium phosphate buffer (pH7.5) were mixed in a plastic tube. After 40s the reaction was stopped with 100μg of sodium metabisulphite (100μl) in 0.05M-sodium phosphate buffer, pH7.5.

^{125}I-labelled avidin was separated from the free radioactive iodide and damaged proteins on a Sephadex G-50 column (0.8cm×25cm) in 0.05M-sodium phosphate buffer/0.15M-NaCl/0.01M-EDTA/0.01% (w/v) merthiolate, pH7.5 (PB buffer) containing 0.1% (w/v) bovine serum albumin. The column was equilibrated with 50ml of the elution buffer, which contained bovine serum albumin to prevent adsorption of the labelled protein. The ^{125}I-labelled avidin fractions were pooled, diluted 1:5 with PB buffer containing 0.5% (w/v) bovine serum albumin and stored at −20°C until used in the assay. The purity of labelled avidin was checked on a second Sephadex G-50 column and on polyacrylamide-gel electrophoresis with sodium dodecyl sulphate (Weber & Osborn, 1969). The radioactivity of the gel pieces (3mm) was measured with a gamma radioactivity counter (LKB/Wallac, Ultrogamma II). No other labelled product was found besides avidin. The labelled avidin could be used for at least 6 weeks.

Assay procedure. All dilutions were made with PB buffer containing 0.5% bovine serum albumin. Avidin tissue samples were the same supernatants (2500*g*) as in the [^{14}C]biotin/bentonite method. The assay was made in a small plastic tube (12mm×75mm). Sample or standard (400μl), avidin antiserum (100μl) and ^{125}I-labelled avidin (50μl, 10–20ng) were mixed and incubated overnight (16–20h) at room temperature. The maximal binding control (B_0) contained 400μl of the buffer instead of the sample and the background control contained 500μl of the buffer instead of sample and antiserum. The standard curve ranged from 1ng to 500ng of avidin/ml. The standard avidin (250μg) was dissolved in 1ml of the oviduct supernatant, diluted 1:10 and stored at −80°C. The final dilution was made immediately before the assay.

After the first reaction, 1ml of sheep anti-(rabbit

γ-globulin) immunoadsorbent was added to each tube, which was then rotated slowly end-over-end for 2h at room temperature. The visible precipitate was separated by centrifugation with a swing-out rotor for 2–3min at 1000*g* and washed twice with 2ml of PB buffer containing 0.5% bovine serum albumin. The radioactivity of the pellet was measured with a gamma radioactivity counter (LKB/Wallac, Ultrogamma II).

Results

Properties of radioimmunoassay

About 10–20% of the total radioactivity was bound to avidin in the iodination process. The specific radioactivity of the product was 20–60 μCi/μg. Only one peak of radioactivity was found on a second Sephadex G-50 column. However, after 3–4 weeks of storage some degradation was found. This was also found on polyacrylamide-gel electrophoresis. The affinity of the product for anti-avidin antiserum did not change in 6 weeks.

Fig. 1 shows the titration of ^{125}I-labelled avidin with anti-avidin antiserum. The binding of ^{125}I-labelled avidin was assayed in the absence of unlabelled avidin (B_0). At the highest antibody concentration about 60%, and in the absence of antibody 1–2%, of the total radioactivity precipitated (B_0/T%). A dilution of antiserum ($1–2\times10^{-5}$) was used in the assay such that 20–30% of the ^{125}I-labelled avidin was bound.

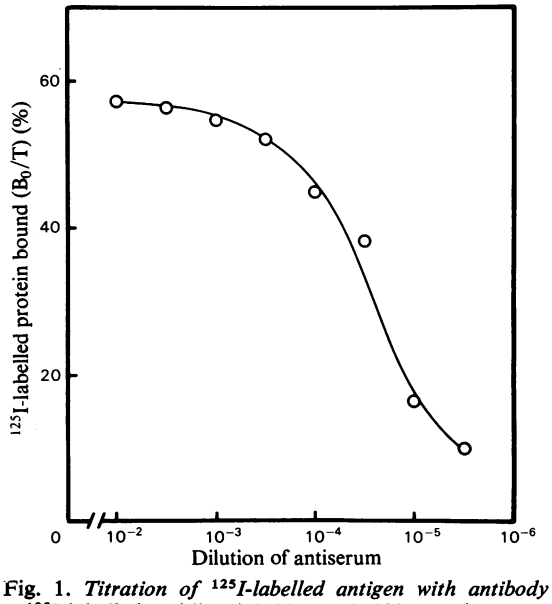

Fig. 1. *Titration of ^{125}I-labelled antigen with antibody* ^{125}I-labelled avidin (10–20ng; 27000c.p.m.) was mixed with antiserum diluted with PB buffer containing 0.5% bovine serum albumin. Tubes were incubated overnight at room temperature. Thereafter 1ml of the second antibody (diluted 1:20) was added and incubation was continued for 2 or 20h depending on the dilution of antiserum. Bound and free radioactivities were separated and the precipitate was counted for radioactivity in a gamma counter.

Although the equilibrium of antigen–antibody reaction was reached in 4h (Fig. 2), the incubation time was usually 16–20h (overnight). The time of the second antibody reaction was dependent on the titre of antiserum. It was normally 2h, but 20h at antiserum dilutions smaller than 1:5000. A typical standard curve, with antiserum diluted 1×10^{-5}, is shown in Fig. 3. Results were calculated on the basis of the linear part of the logit–log scale.

Specificity

The effect of the saturation of biotin-binding sites of avidin on radioimmunoassay was studied with an excess of biotin in the standard solutions. Biotin (0.1ng/standard) decreased the standard values by about 15%. Lysozyme showed no cross-reaction at concentrations up to 1μg/ml (Fig. 4). Ovalbumin displaced labelled avidin at a concentration of more than 50μg of ovalbumin/ml. The cross-reaction of ovalbumin was thus less than 0.015%. The avidin-containing supernatant of the oviduct homogenate gave a shape of curve similar to that of avidin standards.

Comparison of radioimmunoassay and the [^{14}C]biotin/bentonite method

Table 1 shows avidin concentration in the oviducts of oestrogen-injected chicks after different treat-

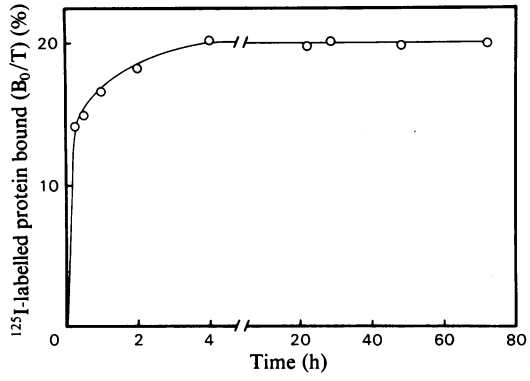

Fig. 2. *Kinetics of the antigen–antibody reaction* Reaction mixtures contained buffer, antiserum diluted 1×10^{-5} and 10–20ng of ^{125}I-labelled avidin (30000c.p.m.). Tubes were incubated at room temperature. The time of the second antibody reaction was 2h.

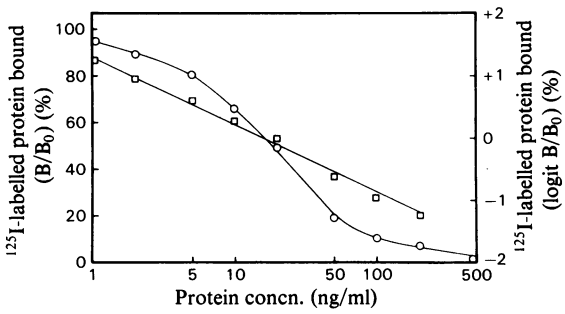

Fig. 3. *Avidin standard curve*
The procedure was as described in the text. Values were plotted on a semi-logarithmic paper. The ratio of the bound (B) over the maximal (B_0) radioactivity (○), and its logit value (□), were placed on the ordinate and the avidin concentration on the abscissa. The linear logit–log presentation was used to determine avidin concentration of samples.

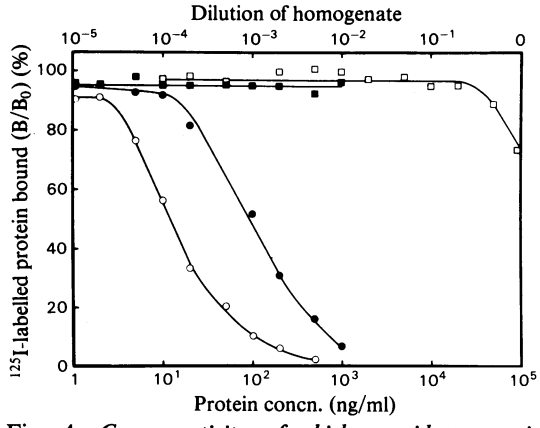

Fig. 4. *Cross-reactivity of chicken oviduct proteins*
Avidin standards (○) and oviduct supernatant (●) were made as described in the text. Ovalbumin (□) and lysozyme (■) were dissolved and diluted with PB buffer containing 0.5% bovine serum albumin. The oviduct supernatant of progesterone-treated oestrogen-injected chicks was diluted with the same buffer.

ments. Radioimmunoassay gave higher values for oviduct samples having very low avidin concentration, but usually results were essentially similar with both methods.

To study the recoveries in different chick tissues, a known amount of avidin was added to the tissue supernatants before measurement by both methods. Results in Table 2 show that the [^{14}C]biotin/bentonite method is not valid for the determination of avidin in liver and kidney because of the poor recoveries. But supernatants of these tissues can be assayed by radioimmunoassay. On the other hand radioimmunoassay gave an overestimation of the avidin concentration in the plasma and oviduct.

Table 1. *Avidin concentrations in the chick oviduct as measured with radioimmunoassay and the [^{14}C]biotin/bentonite method*
Oestrogen-injected chicks (for 7 days) were treated with 20mg of progesterone or 0.2mg of actinomycin D/kg body wt., or oviducts were ligated or the intestine and liver burned (for details see the Experimental section). Controls received diethylstilboestrol only. Avidin was assayed by radioimmunoassay and the [^{14}C]biotin/bentonite method. The means±S.E.M. for seven to 15 animals (the exact number is indicated in parentheses) are given.

	Avidin concn. (μg of avidin/g of wet tissue)	
Treatment	[^{14}C]Biotin/bentonite method	Radioimmunoassay
Control	0.5±0.5 (7)	0.7±0.4 (8)
Progesterone	35.9±4.5 (7)	28.6±5.2 (7)
Actinomycin D	19.2±4.0 (13)	21.4±4.1 (13)
Ligature	10.5±2.5 (8)	12.2±2.4 (7)
Burning injury	22.1±4.2 (15)	20.1±3.2 (15)

Table 2. *Recoveries, as measured by radioimmunoassay and the [^{14}C]biotin/bentonite method, of avidin in chick tissue supernatants*
The tissues of oestrogen-injected and uninjected chicks were used. In the radioimmunoassay recovery studies, 12.5 and 25ng of avidin/ml was added to the supernatant sample, which was incubated for 20min. Thereafter avidin was assayed by radioimmunoassay. The recovery is the mean for these two determinations. The recovery by the [^{14}C]biotin/bentonite method was studied by adding 0.5 or 1.0μg of avidin to the supernatant samples, corresponding to 25 or 50mg of tissue. After a 20min incubation, avidin was assayed by the [^{14}C]biotin/bentonite method. The means ±S.E.M. for eight to 17 chicks (exact number shown in parentheses) are given.

	Avidin recovery (%)	
Tissue	[^{14}C]Biotin/bentonite method	Radioimmunoassay
Plasma	106±3 (13)	124±8 (11)
Oviduct	92±1 (17)	131±4 (16)
Intestine	85±3 (13)	64±4 (18)
Liver	11±2 (13)	72±6 (12)
Kidney	35±6 (13)	75±6 (8)
Lung	83±3 (13)	80±7 (8)
Pectoral muscle	94±3 (13)	77±7 (8)
Brain	100±2 (13)	79±4 (9)

Discussion

The new radioimmunoassay for chicken avidin developed in the present study is more specific and 30–50-fold more sensitive than the [^{14}C]biotin/bentonite method. Since it is not based on the biotin-binding capacity of avidin, it is not essentially affected by the variable amounts of endogenous biotin in the tissues. Furthermore, unknown biotin-binding compounds in the tissue do not disturb avidin assay with radioimmunoassay. This is important, since some biotin-binding factor(s) has (have) been found in the egg yolk (White *et al.*, 1976) and in the kidney of the chicken (Elo *et. al.*, 1978). At least the latter differs immunologically from egg-white avidin.

In radioimmunoassay the range of the linear part in the standard curve (Fig. 3) was wide enough so that one or two dilutions of tissue samples were sufficient for the assay. Furthermore, the avidin radioimmunoassay is a fairly rapid method. If samples, standards and controls are assayed in duplicate, one technician can daily assay 30–60 supernatant samples and an avidin standard. With radioimmunoassay it is possible to assay avidin concentrations as low as 1–2ng/ml. Approximately the same sensitivity is also obtained with a micro-method of avidin assay in which [^{3}H]biotin is used as a radioactive ligand. The radioactivity of the bentonite sediment is measured in a gas counter with high efficiency and low background (Elo & Tuohimaa, 1974).

Contaminating radioactivity was not found in ^{125}I-labelled avidin after purification of labelled product by gel filtration. The second peak, which appeared after a few weeks' storage, may be due to damaged protein or a subunit of tetrameric avidin or both. However, the labelled product still bound to antibody. The immunological properties of labelled avidin seemed to depend greatly on the conditions of the iodination process. Deionized water was further purified by distillation in quartz-glass apparatus. In addition, the exact pH of the buffer was important. Avidin, as a tetrameric protein, seemed to change immunologically more easily during the iodination process than did monomeric ovalbumin (M. S. Kulomaa & P. J. Tuohimaa, unpublished work).

Ovalbumin comprises about 54% of the total protein in the chicken egg-white (Gilbert, 1971). Siva Sankar *et al.* (1958) reported that ovalbumin shows a certain amount of cross-reaction with anti-avidin antiserum. At high concentrations, ovalbumin showed cross-reaction also in our study (Fig. 4). Oestrogens cause differentiation of chick oviduct and ovalbumin induction in the mucosal glands (O'Malley *et al.*, 1969). It is therefore probable that cross-reaction observed in the oviduct samples of oestrogen-injected chicks is caused mainly by ovalbumin, but also some other oviductal protein(s) (e.g. the impurity of commercial avidin) may interfere with the assay. Cross-reaction in the oviduct samples can be avoided by diluting supernatants sufficiently (if possible) or by using short periods of oestrogen-injection (6–7 days) and low maximal binding capacity ($B_0/T\%$). Cross-reaction of lysozyme was studied, since lysozyme has a similar amino acid composition to avidin and it likewise binds biotin weakly (Green, 1968). However, lysozyme did not show any cross-reaction at the concentrations studied.

The [^{14}C]biotin/bentonite method used here has been modified by Elo *et al.* (1975). It is more rapid than the original [^{14}C]biotin/bentonite method developed by Korenman & O'Malley (1967). Ultracentrifugation of tissue homogenate has been replaced by a rapid centrifugation at a low *g* value, and the avidin–[^{14}C]biotin–bentonite complex is washed in a test tube instead of on a Millipore filter. The modified [^{14}C]biotin/bentonite method is very suitable for large numbers of tissue samples.

Radioimmunoassay gave essentially the same avidin concentrations in the chick oviduct after different treatments as the [^{14}C]biotin/bentonite method (Table 1). This further supports the finding that actinomycin D and tissue trauma induce avidin in the oviduct of oestrogen-injected chicks (Elo *et al.*, 1975). It has hitherto been thought that avidin is induced in the chick oviduct specifically by progesterone (O'Malley *et al.*, 1969). Oviduct samples having very low avidin contents gave slightly higher values with radioimmunoassay than with the [^{14}C]biotin/bentonite method. This can be explained by the better sensitivity of radioimmunoassay, but also by the low concentration of endogenous biotin or a little cross-reaction of unknown compound(s).

Recovery studies indicated that the [^{14}C]biotin/bentonite method is not a valid method to determine avidin in the liver and kidney (Table 2). The poor recoveries in these tissues could be due to a high endogenous biotin content. Avidin induction has been found in many chicken tissues (Elo *et. al.*, 1978), which suggests that avidin may have some physiological function in the chicken body. It is therefore important that avidin can be assayed with a radioimmunoassay from all tissues, including the liver and kidney. The overestimation in the plasma and oviduct suggests that these tissues contain some compound that inhibits the binding of ^{125}I-labelled avidin, or to which the tracer can bind besides the rabbit anti-avidin antibody.

In conclusion, a new radioimmunoassay for chicken avidin was developed. The radioimmunoassay and the [^{14}C]biotin/bentonite method were compared for avidin determination in different chick tissues. Radioimmunoassay can be used in all tissues studied, including the liver and kidney, where

the [^{14}C]biotin/bentonite method gives incorrect results.

We are grateful to Miss Tiina-Maija Mattila, Mrs. Outi Kurronen, Mr. Christoff Hartmann and Mr. Jari Myllyniemi for their excellent technical assistance. This work was supported by grant no. 560-0526 of the Ford Foundation.

References

Eakin, R. E., Snell, E. E. & Williams, R. J. (1941) *J. Biol. Chem.* **140**, 535–543

Elo, H. & Tuohimaa, P. (1974) *Biochem. J.* **140**, 115–116

Elo, H., Tuohimaa, P. & Jänne, O. (1975) *Mol. Cell. Endocrinol.* **2**, 203–211

Elo, H. A., Kulomaa, M. S. & Tuohimaa, P. J. (1978) *Comp. Biochem. Physiol.* in the press.

Gilbert, A. B. (1971) in *Physiology and Biochemistry of the Domestic Fowl* (Bell, D. J. & Freeman, B. M., eds.), vol. 3, pp. 1291–1329, Academic Press, London and New York

Green, N. M. (1963) *Biochem. J.* **89**, 585–591

Green, N. M. (1965) *Biochem. J.* **94**, 23c–24c

Green, N. M. (1968) *Nature (London)* **217**, 254–256

Green, N. M. (1975) *Adv. Protein Chem.* **29**, 85–133

Greenwood, F. C., Hunter, W. M. & Glover, J. S. (1963) *Biochem. J.* **89**, 114–123

Hertz, R., Fraps, R. M. & Sebrell, W. E. (1943) *Proc. Soc. Exp. Biol. Med.* **52**, 142–144

Korenman, S. G. & O'Malley, B. W. (1967) *Biochim. Biophys. Acta* **140**, 174–176

O'Malley, B. W. & Korenman, S. G. (1967) *Life Sci.* **6**, 1953–1969

O'Malley, B. W., McGuire, W. L., Kohler, P. O. & Korenman, S. G. (1969) *Recent Prog. Horm. Res.* **25**, 105–153

Siva Sankar, D. V., Cossano, B. J., Theis, H. W. & Marks, C. R. (1958) *Nature (London)* **181**, 619–620

Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412

Wei, R. D. & Wright, L. D. (1964) *Proc. Soc. Exp. Biol. Med.* **117**, 17–20

White, H. B., Dennison, B. A., Fera, M. A. D., Whitney, C. J., McGuire, J. C., Meslar, H. W. & Sammelwitz, P. H. (1976) *Biochem. J.* **157**, 395-400