Biochem. J. (1973) **132**, 459–463
Printed in Great Britain

Studies on the Assembly and Secretion of a Multicomponent Protein

THE BIOSYNTHESIS OF VITELLOGENIN, AN OESTROGEN-INDUCED GLYCOLIPOPHOSPHOPROTEIN

By A. H. MERRY, P. J. DOLPHIN, K. A. MUNDAY and M. AKHTAR
Department of Physiology and Biochemistry, University of Southampton, Southampton SO9 5NH, U.K.

(*Received* 20 *September* 1972)

1. Incorporation of [^{32}P]P_i and [^{3}H]leucine into vitellogenin secreted *in vitro* by liver slices from oestrogen-treated *Xenopus laevis* is accompanied by a 2h lag; no lag is apparent for the incorporation into total tissue protein. 2. The addition of cycloheximide was found immediately to inhibit further incorporation of radioactive leucine into total tissue protein. The incorporation into secreted vitellogenin, however, continued for 2h after the addition of cycloheximide. 3. Pulse-labelling of liver slices with [^{3}H]leucine for 30min, followed by a chase with a large excess of unlabelled leucine, resulted in the appearance of radioactivity in secreted vitellogenin from 90min after the end of the pulse period. 4. Evidence is presented which suggests that of the radioactivity from [^{3}H]leucine incorporated into proteins by the liver of oestrogen-treated *Xenopus* some 70% is present in the single protein vitellogenin. 5. The incorporation of [^{32}P]P_i into vitellogenin followed a pattern identical with that found for [^{3}H]leucine in the pulse-labelling experiments and this indicates that synthesis of the polypeptide chain and incorporation of P_i are closely linked processes. 6. The cumulative evidence suggests that the 2h lag phase represents the time required for the assembly and secretion of this multicomponent protein.

Recent studies from this laboratory have shown that upon treatment with oestrogen the South African clawed toad *Xenopus laevis* synthesizes, in large quantities, cholesterol, lipids (Dolphin *et al.*, 1972) and a serum component, vitellogenin, which has been characterized as a calcium-binding glycolipophosphoprotein (Munday *et al.*, 1968; Ansari *et al.*, 1971). Related studies have been reported by other workers (Wallace, 1970; Redshaw & Follett, 1971, and references cited therein).

To study the biosynthesis of this protein a technique *in vitro* involving the use of liver slices from oestrogen-treated animals has been previously developed (Dolphin *et al.*, 1971). With this method impressive amounts of radioactivity from several precursors were incorporated into vitellogenin, which was secreted into the incubation medium (Dolphin *et al.*, 1971). Similar studies *in vitro* have been reported by other workers (Wallace & Jared, 1969; Clemens *et al.*, 1972; Wittliff & Kenney, 1972). In our own studies it was noted that the appearance of radioactivity in secreted vitellogenin was attended by a lag phase of 90–120min (Dolphin *et al.*, 1971). In the present paper we have extended our previous studies on the specificity of incorporation of labelled precursors into vitellogenin and report experiments to evaluate various hypotheses for explaining the lag phase.

Experimental

Materials

The treatment of animals and their injection, radiochemicals, special chemicals, polyacrylamide-gel electrophoresis, Sepharose 4B column chromatography and measurement of radioactivity were all as previously described (Ansari *et al.*, 1971; Dolphin *et al.*, 1971).

Buffer. All incubations *in vitro* were performed with a phosphate–saline buffer, pH 7.3 (Dulbecco & Vogt, 1954), containing 4.09g of NaCl, 0.20g of KCl, 1.15g of Na_2HPO_4 (anhydrous), 0.20g of KH_2PO_4 (anhydrous), 0.10g of $CaCl_2,6H_2O$ and 0.10g of $MgCl_2,6H_2O$ in 1 litre of water. The buffer was supplemented with 20mM-sodium pyruvate as an energy source. In the pulse–chase labelling experiments 150mM-L-leucine or 10mM-P_i was added to the buffer after the pulse period.

Methods

Incubation of liver tissue. Animals were pithed and cut open from the ventral surface. The livers were washed twice in chilled buffer and cut into approx. 3mm cubes. For continuous labelling experiments portions (1g) of the liver were weighed into 25ml Erlenmeyer flasks, containing 12ml of buffer and radioactive precursor. Incubation was carried out at

22°C with constant shaking for various time-periods (indicated in the Results and Discussion sections). For total tissue protein-incorporation experiments portions (250mg) of liver cubes were weighed into 25ml flasks and 5ml of buffer was added. Sets of flasks prepared in this way were incubated at 22°C for various time-periods, after which duplicate 100mg samples were removed for estimation of incorporation into total tissue protein. The incubation medium was retained for determination of the incorporation of radioactivity into vitellogenin.

Pulse-labelling experiments. After the pulse-labelling period further incorporation into protein of radioactive precursors was inhibited by addition of either cycloheximide or of large amounts of unlabelled precursor. In the first case (Fig. 3) cycloheximide was added to a concentration of 100 μg/ml and the incubation continued in the same buffer. This concentration of cycloheximide has been shown previously to inhibit vitellogenin biosynthesis (Dolphin *et al.*, 1971). In the second case (Fig. 4) the liver was removed from the flasks and washed three times with fresh buffer. The liver cubes were then lightly blotted and transferred to a flask containing buffer and unlabelled precursor (150mM-L-leucine or 10mM-P_i) for the remainder of the incubation period.

Determination of the incorporation of radioactive precursors into vitellogenin. The method used was the dimethylformamide precipitation method B as previously described (Dolphin *et al.*, 1971), except that on removal of the sample from the incubation medium unlabelled precursor was added to a concentration of 100mM before the first protein precipitation. This addition was found to decrease the amount of non-covalently bound radioactive precursor.

Determination of the incorporation of labelled precursors into liver proteins. Incorporation of radioactivity into duplicate 100mg (wet wt.) tissue samples was determined as previously described (Dolphin *et al.*, 1971).

TEAE-cellulose chromatography. This was performed according to the method of Wallace (1965) on a column 30cm × 1.5cm. The absorbance of each fraction at 280nm was determined and the radioactivity associated with each 5ml fraction measured by the addition of an equal volume of Instagel (Packard Instrument Co. Inc., Downers Grove, Ill., U.S.A.).

Peptic hydrolysis of vitellogenin. A portion (25mg) of Sepharose 4B-purified [^{3}H]leucine-labelled vitellogenin was dissolved in 5ml of 10% (v/v) acetic acid and 1mg of recrystallized pepsin [Sigma (London) Chemical Co. Ltd., London S.W.6, U.K.] was added. The mixture was incubated at 30°C for 36h with constant shaking. The peptide fragments released were separated on a Sephadex G-25 column 2.6cm × 96cm. The eluent was 10% (v/v) acetic acid containing 1% (v/v) thiodiglycol [bis(hydroxyethyl) sulphide] and the flow rate was maintained at 27ml/h with a constant-pressure-head device. The radioactivity associated with each fraction was determined by addition of 1ml samples to 10ml of NE 250 scintillator solution (Nuclear Enterprises Ltd., Edinburgh, U.K.). The peptide content of each 5ml fraction was determined after hydrolysis in NaOH by using ninhydrin reagent.

Results

Specificity of incorporation of labelled precursors into vitellogenin

The work of Wallace & Dumont (1968) and Dolphin *et al.* (1971) strongly indicated that the incubation of labelled precursors with liver slices from oestrogen-treated *Xenopus* leads to the incorporation of radioactivity predominantly into a single protein component. To follow our projected approach for the study of vitellogenin biosynthesis, however, we required more definitive information on the specificity of incorporation of labelled precursors into vitellogenin. Some further experiments were therefore undertaken. It was shown that more than 95% of the protein-bound radioactivity extruded in the incubation medium of biosynthetic experiments was precipitated with dimethylformamide. When this precipitated protein was analysed either by TEAE-cellulose chromatography (Fig. 1) or by polyacrylamide-gel electrophoresis, under various acrylamide concentrations or pH values, all the protein-bound radioactivity was associated with a single protein peak (a typical scan is shown in Fig. 2). That this radioactivity was in fact associated with vitellogenin may be deduced from the fact that in all experiments the position of the radioactive peak corresponded to that of authentic vitellogenin.

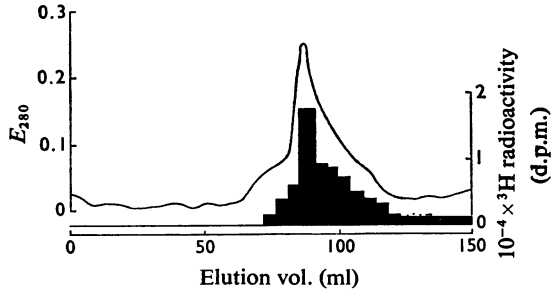

Fig. 1. *Analysis of dimethylformamide-precipitated protein on TEAE-cellulose*

Fractions (5ml) were collected and E_{280} (——) and ^{3}H radioactivity (histogram) determined.

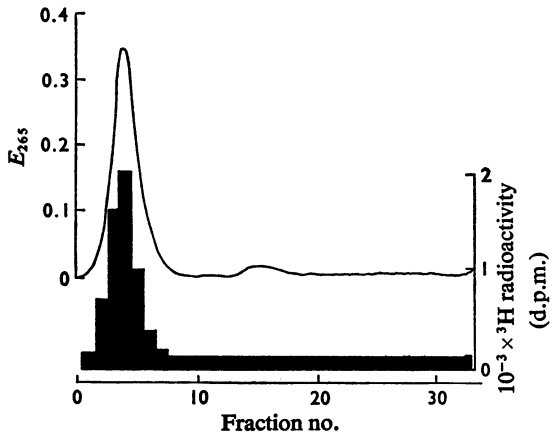

Fig. 2. *Analysis of dimethylformamide-precipitated protein on polyacrylamide-gel electrophoresis at pH 8.5*

Slices (2mm) were cut from the gels (acrylamide concentration 4%) after staining in Amido Black and the ^3H radioactivity (histogram) was determined. ——, E_{265}.

Possible hypotheses for explaining the lag phase

Our earlier work (Dolphin *et al.*, 1971) has shown that the incorporation of labelled precursors into secreted vitellogenin is attended by a 2h lag phase, and two broad hypotheses may be considered to rationalize this phenomenon. The first involved the assumption that the lag phase represents the time required for the intracellular pools of amino acid (or of a suitable precursor) to achieve the optimum specific radioactivity. The second envisaged that the lag phase represents the time taken for the assembly and secretion of the completed protein. The absence of a lag phase for the incorporation of labelled amino acids into total tissue protein showed that the contribution of intracellular pools of amino acids to the lag phase was small (Dolphin *et al.*, 1971) but gave no information about the contribution of intracellular pools of vitellogenin or some intermediates in its biosynthesis. These alternatives were evaluated in the following experiments.

Incorporation of L-[4,5-^3H]*leucine into secreted vitellogenin and total tissue protein after cycloheximide treatment at 2h*

Liver slices were incubated in the system described, in the presence of [^{3}H]leucine, and after 2h cycloheximide (100μg/ml) was added to inhibit further protein synthesis. Fig. 3(*a*) shows the complete inhibition of incorporation of label into total tissue protein immediately after the addition of cycloheximide.

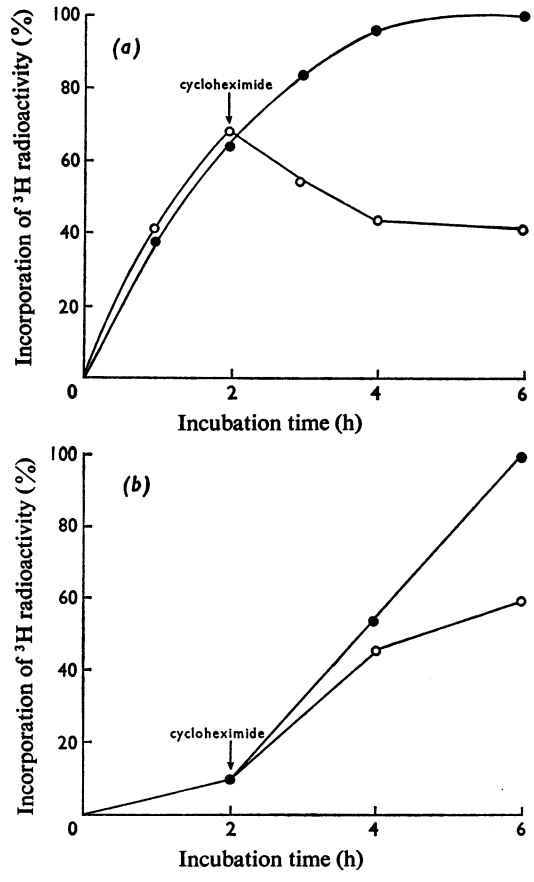

Fig. 3. *Incorporation of* L-[4,5-^3H]*leucine into (a) total tissue protein and (b) vitellogenin after cycloheximide treatment at 2h*

Liver slices were incubated with [^{3}H]leucine in parallel incubations. In one case the incubation medium was supplemented with cycloheximide at 2h. In the other no such addition was made. In (*a*) incorporation into total tissue protein was measured and in (*b*) incorporation into secreted vitellogenin in a 30μl sample was determined on polyacrylamide-gel electrophoresis. In both (*a*) and (*b*) the incorporation in the absence of cycloheximide at 6h was taken to be 100% and was 3.5×10^4 d.p.m./25mg wet wt. of tissue in (*a*) and 1.2×10^4 d.p.m./25mg wet wt. of tissue in (*b*). o, Cycloheximide added at 2h; •, no cycloheximide added.

The radioactivity in tissue proteins fell by 40% in the 2h after cycloheximide treatment.

In a parallel experiment the radioactivity of the secreted vitellogenin increased in the 2h period after treatment with cycloheximide, in a manner similar to

that noted for the experiments in the absence of cycloheximide (Fig. 3*b*). It is only after this period that the inhibitory effects of cycloheximide on incorporation of [^{3}H]leucine into vitellogenin become apparent. Attention is drawn to the fact that the 40% decrease in the total tissue protein radioactivity shown in Fig. 3(*a*) parallels the increase in the incorporation into vitellogenin (Fig. 3*b*).

Incorporation of radioactivity from [^{32}P]P_i and L-[4,5-^3H]leucine into vitellogenin after pulse–chase labelling

Samples of liver were incubated in the presence of [^{3}H]leucine. Further incorporation was stopped after 30min by changing the medium to one containing large amounts of unlabelled amino acid. Thus the actual protein synthesis was not inhibited as in the previous experiment. Flasks were removed after various incubation times and the incorporation of radioactivity into total tissue protein and secreted vitellogenin was determined.

Fig. 4 shows that the incorporation into total tissue protein remained at the same value for a further 1.5h after the pulse period but was subsequently decreased by 60% in the next hour. This loss of radioactivity associated with the tissue protein could be accounted for by the radioactive vitellogenin that was secreted over this period. After this time there was no further change in the extent of incorporation into either tissue protein or secreted vitellogenin.

Pulse-chase-labelling experiments of the above type were also carried out with [^{32}P]P_i, with [^{3}H]-leucine added as a reference label to facilitate comparison with the results obtained in the previous experiments. Fig. 5 shows that the pattern of incorporation obtained for both radioisotopes is the same. The appearance of radioactivity from both radioisotopes in secreted vitellogenin began 1h after the pulse period and ceased after a further 2h.

The effect of cycloheximide on phosphate incorporation was also studied and it was found that when the antibiotic was present throughout the incubation there was complete inhibition of incorporation of label into vitellogenin. When cycloheximide was added after 1h to tissue that had been incubated in the presence of labelled precursors the pattern of incorporation for [^{3}H]leucine and [^{32}P]P_i was identical (Fig. 6).

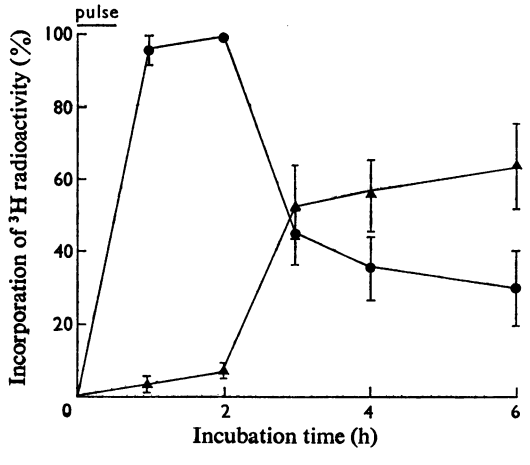

Fig. 4. *Incorporation of* L-[4,5-^3H]*leucine into total tissue protein and vitellogenin after pulse-labelling for* 30*min*

Incubation mixtures were set up as described and after 30min the slices were washed and transferred to a flask containing 150mM-L-leucine. Incorporation into secreted vitellogenin and total tissue protein was determined; all values are expressed as a percentage of that for total tissue protein at 2h, which was 5.0×10^3 d.p.m./100mg wet wt. of tissue. The mean ± S.D. for six experiments is shown. ●, Incorporation into total tissue protein; ▲, incorporation into secreted vitellogenin.

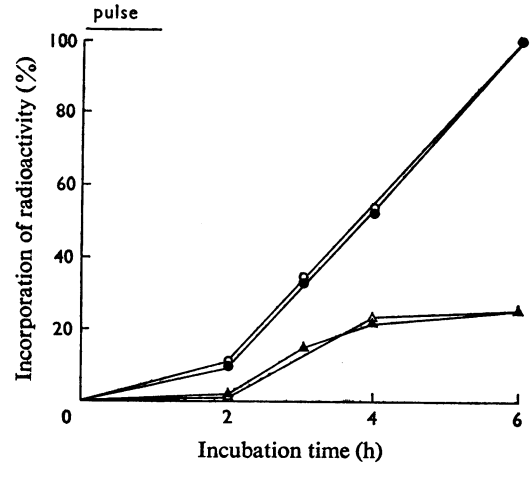

Fig. 5. *Incorporation of* L-[4,5-^3H]*leucine and* [^{32}P]P_i *into vitellogenin after pulse-labelling for* 1*h*

In this experiment the chase buffer was supplemented with 150mM-L-leucine and 10mM-P_i. The incorporation at 6h in the continued presence of labelled precursor is taken to be 100% and was 2.7×10^4 d.p.m./ml of incubation medium for ^{3}H and 1.1×10^4 d.p.m./ml of incubation medium for ^{32}P. ○, Incorporation of [^{32}P]P_i non-pulse label; ●, incorporation of [^{3}H]-leucine non-pulse label; △, incorporation of [^{32}P]P_i pulse label; ▲, incorporation of [^{3}H]leucine pulse label.

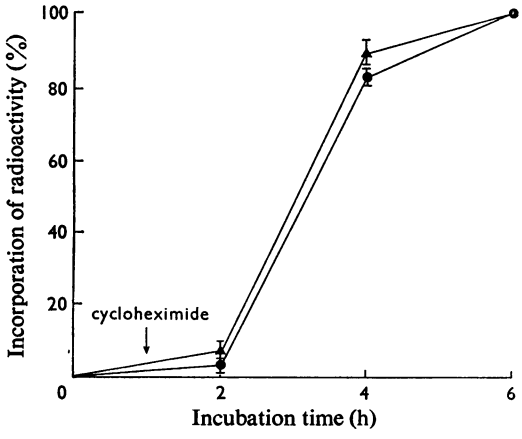

Fig. 6. *Incorporation of* L-[4,5-^{3}H]*leucine and* [^{32}*P*]P_i *into vitellogenin after cycloheximide treatment at* 1 *h*

Liver slices were incubated in the presence of labelled precursors for 1h and the incubation medium was then supplemented with cycloheximide. Incorporation into vitellogenin at 6h was taken to be 100% and was 6×10^3 d.p.m./ml of incubation medium for ^{3}H and 4.6×10^3 d.p.m./ml of incubation medium for ^{32}P. The mean ± S.D. for three experiments is shown. ▲, Incorporation of [^{32}P]P_i; ●, incorporation of [^{3}H]leucine.

Discussion

In this paper we have evaluated two hypotheses, which may explain the 2h lag phase found in the incorporation of radioactivity from [^{32}P]P_i and [^{3}H]leucine into secreted vitellogenin, namely that this lag phase represents either the time taken for the intracellular pool of amino acids (or of a suitable precursor) to achieve the optimum specific radioactivity or the time required for the assembly and secretion of the completed protein.

The experiment in Fig. 4 shows that transfer of the tissue from a medium containing radioactive leucine to one with a large excess of non-radioactive precursor makes very little difference to the radioactivity of either the secreted or the tissue proteins during the subsequent 90min period. Only after this time is there a rise in the radioactivity released in the secreted protein. Such a pattern would not be obtained if the lag phase of Fig. 4 reflected the time taken for the intracellular amino acid or precursor pools to obtain constant radioactivity. The cycloheximide experiments provide further support for this hypothesis and also indicate that the biosynthetic events occurring during the 90min lag phase do not require extensive formation of new peptide bonds.

The phosphate incorporation follows the same pattern as that found for leucine incorporation, both in the continuous labelling and in the pulse–chase-labelling experiments (Figs. 5 and 6). This suggests that the synthesis of the polypeptide chain and the incorporation of phosphate are very closely linked events and that if a pool of non-phosphorylated precursor exists it must be extremely small, or turning over very rapidly.

The cumulative evidence in this paper suggests that the best explanation for the 2h lag phase involves the assumption that vitellogenin or its precursors or both are bound to membranes in an orderly fashion during its biosynthesis and that translocation of the complete molecules through the cell in order of 'seniority' probably occurs.

A product–precursor relationship is apparent between the tissue proteins and the secreted vitellogenin, as shown in Fig. 4, as the decrease in total tissue protein radioactivity correlated with the rise in radioactivity present in secreted vitellogenin. An outstanding feature of this experiment is the demonstration that as much as 70% of the total protein synthesis by the oestrogen-treated *Xenopus* liver is directed towards the synthesis of vitellogenin. Furthermore the protein-bound radioactivity secreted into the incubation medium is almost exclusively associated with vitellogenin.

The help of Dr. C. B. Lazier in this work is gratefully acknowledged. P. J. D. thanks the Medical Research Council for a Postgraduate Studentship.

References

Ansari, A. Q., Dolphin, P. J., Lazier, C. B., Munday, K. A. & Akhtar, M. (1971) *Biochem. J.* **122**, 107–113

Clemens, M. J., Lofthouse, R. & Tata, J. R. (1972) *Biochem. J.* **128**, 97P–98P

Dolphin, P. J., Ansari, A. Q., Lazier, C. B., Munday, K. A. & Akhtar, M. (1971) *Biochem. J.* **124**, 751–758

Dolphin, P. J., Merry, A. H., Munday, K. A. & Akhtar, M. (1972) *J. Endocrinol.* **53**, li–lii

Dulbecco, R. & Vogt, M. (1954) *J. Exp. Med.* **99**, 167–182

Munday, K. A., Ansari, A. Q., Oldroyd, D. & Akhtar, M. (1968) *Biochim. Biophys. Acta* **166**, 748–751

Redshaw, M. R. & Follett, B. K. (1971) *Biochem. J.* **124**, 759–766

Wallace, R. A. (1965) *Anal. Biochem.* **11**, 297–311

Wallace, R. A. (1970) *Biochim. Biophys. Acta* **215**, 176–183

Wallace, R. A. & Dumont, J. N. (1968) *J. Cell. Physiol.* **72**, 73–90

Wallace, R. A. & Jared, D. W. (1969) *Develop. Biol.* **19**, 498–526

Wittliff, J. L. & Kenney, F. T. (1972) *Biochim. Biophys. Acta* **269**, 485–495