The Regulation of Haemoglobin Synthesis in Cultured Chick Blastoderms by Steroids Related to 5 β -Androstane

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(Received 12 June 1975)

1. After 24 h of incubation, the blastoderm may be dissected from the early developing chick embryo and successfully maintained under conditions of organ culture *in vitro*.
2. Low concentrations of steroids related to 5 β -androstane stimulate the synthesis of foetal haemoglobins, types E and P, in a highly steroid- and tissue-specific manner.
3. Our evidence suggests that the synthesis of haemoglobin is a relatively late developmental event, occurring after pronounced increases in the activity of δ -aminolaevulinic synthase and in DNA and RNA synthesis.
4. A poly(A)-rich species of RNA, sedimentation coefficient approx. 9S, was isolated which codes specifically for the synthesis of the polypeptide chains of early chick haemoglobins.
5. On the basis of several criteria, it appears that globin mRNA is present in the early chick blastoderm but is not translated.
6. All biochemical processes studied in the blastoderm were enhanced by low concentrations of 5 β -reduced steroids and the results are discussed with reference to the mechanism of action of biologically important steroids.

Over recent years, impressive advances have been made in the elucidation of the mechanism of action of steroid hormones [see the comprehensive review by King & Mainwaring (1974)]. There is now abundant evidence that the principal androgen, testosterone, is extensively metabolized in androgen-sensitive cells and 5 α -dihydrotestosterone (17 β -hydroxy-5 α -androstane-3-one) appears to be largely responsible for the manifestation of the androgenic response. Exceptions to this general premise are few, but notably include the effects mediated by testosterone on skeletal muscle and erythropoietic cells (King & Mainwaring, 1974). Muscle has a limited ability for forming 5 α -reduced metabolites of testosterone (Mainwaring & Mangan, 1973) and recent evidence supports the view that the anabolic effects of androgens on muscle are elicited directly by testosterone itself (Jung & Baulieu, 1972; Kreig *et al.*, 1974). The effect of testosterone on cells engaged in erythropoiesis is more complex and this is explained by the fact that haem and globin synthesis may be conducted in a diversity of cell types and is subject to extreme species differences and modulation during development.

Many systems have been adopted in the study of haem and globin synthesis, including chick blastoderm (O'Brien, 1961; Levere & Granick, 1965; Wainwright & Wainwright, 1966; Levere *et al.*, 1967), foetal chick liver (Granick & Kappas, 1967*a,b*), bone marrow of several species (Reisner, 1966; Necheles & Rai, 1969; Sierralta *et al.*, 1974) and

especially rabbit reticulocytes (Borsook *et al.*, 1955; Rabinovitz & Olsen, 1956). The involvement of testosterone and its metabolites in erythropoiesis has been attributed to two distinct mechanisms. On the one hand, testosterone was said to stimulate erythrocyte formation by elevating the circulating concentration of the erythropoiesis-stimulating factor, erythropoietin (Mirand *et al.*, 1965; Fried & Gurney, 1965; Rishpon-Meyerstein *et al.*, 1968), whereas other investigators established that erythropoiesis was specifically enhanced by steroids with the parent structure of 5 β -androstane (Levere *et al.*, 1967; Granick & Kappas, 1967*a*; Necheles & Rai, 1969) and was independent of erythropoietin. Gordon *et al.* (1970) studied erythrocyte formation in polycythaemic mice to distinguish between these hypotheses; by using an anti-serum raised against erythropoietin they negated the effect of testosterone on erythropoiesis but not that of 5 β -reduced steroids, such as 11-oxo/pregnanolone (3 α -hydroxy-5 β -pregnane-11,20-dione). Their study provided unequivocal evidence that certain steroids can influence erythropoiesis directly.

We decided to use the chick blastoderm system for many reasons. First, the onset of erythropoiesis may be studied without use of artificial means, e.g. as in the reticulocytic anaemia induced by phenylhydrazine (Lingrel & Borsook, 1963) or the polycythaemia promoted by lowered oxygen tension (Gordon *et al.*, 1970). Secondly, 5 β -androstane has an exceedingly angular structure because the A and B rings are in the *cis* orientation and we wished to establish the

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importance of the high-affinity binding mechanism or 'receptor' system for such steroids in the regulation of erythropoiesis. Spooner & Mainwaring (1973) have previously reported on the existence of a receptor system for 5β -reduced steroids in chick blastoderm. Thirdly, Parsons (1970) has demonstrated that chick blastoderm can extensively metabolize testosterone to 5β -reduced steroids at a time commensurate with the onset of erythropoiesis. Finally, chick blastoderm synthesizes a well-characterized protein, foetal haemoglobin, that may be modulated by steroids under conditions of organ culture *in vitro*.

Our principal objective was to evaluate further the steroidal regulation of erythropoiesis in chick blastoderm by using methods developed originally for studies on haemoglobin synthesis in rabbit reticulocytes (Labrie, 1969; Aviv & Leder, 1972; Williamson *et al.*, 1974).

Materials and Methods

Chemicals

[5- ^3H]Uridine (29 Ci/mmol), [Me- ^3H]thymidine (20 Ci/mmol), L-[U- ^3H]methionine (9 Ci/mmol), L-[^{35}S]methionine (380 Ci/mmol), L-[4,5- ^3H]leucine (53 Ci/mmol), L-[1- ^{14}C]leucine (50 mCi/mmol) and [1,4- ^{14}C]succinic acid were supplied by The Radiochemical Centre, Amersham, Bucks., U.K. The nutrient mixture, F-12, and the antibiotic mixture, fungizone, were supplied by the Grand Island Biological Co., Grand Island, N.Y., U.S.A. Crystamycin was obtained from Glaxo, Greenford, Middx., U.K. Sucrose, free of ribonuclease, was supplied by Schwarz/Mann, Orangeburg, N.Y., U.S.A. Poly-(A,U,G), poly(A) and poly(U) were obtained from Miles-Seravac, Maidenhead, Berks., U.K. Purified agarose (type A-45) was supplied by L'Industrie Biologique Française, Gennevilliers, France. Serva highly purified sodium dodecyl sulphate (low extinction at 260 nm) was obtained through Camlabs, Cambridge, U.K. Ribonuclease-free deoxyribonuclease was supplied by Worthington Chemical Co., Freehold, N.J., U.S.A. Starting cultures of *Rhodospseudomonas spheroides* were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, U.K. All other chemicals were of the highest available purity, and glass-distilled water was used in making up all solutions.

Fertilized eggs

All experiments were conducted on fertilized Brown Leghorn eggs produced in this Institute.

Cultures of chick blastoderm

The culture medium contained the following components (Chan & Ingram, 1973); 43.2 g of

powdered F-12 nutrient with glutamine, 1.34 g of NaHCO_3 , 14.3 g of 2-(N-2-hydroxyethylpiperazin-N-yl)ethanesulphonic acid and 13.4 mg of FeCl_3 dissolved in 4 litres of water. After sterilization by two cycles of filtration through Millipore membranes, the medium had a pH of 8.2 at 20°C. Before use, each 200 ml of medium was supplemented with 100 μl of fungizone (250 $\mu\text{g}/\text{ml}$) and 100 μl of crystamycin (1×10^6 i.u. of benzylpenicillin and 100 μg of streptomycin sulphate/ml). Eggs were incubated, air sac uppermost, at 38°C for 24 h at 60% humidity, without turning. The vitelline membrane was cut through on the periphery of the area opaca, and the blastoderm was removed from the egg yolk and placed in Petri dishes containing medium F-12. Gentle agitation of the dishes washed adherent yolk from the blastoderms and the embryos were staged by the system of Settle (1954). Only blastoderms from embryos corresponding to the primitive streak to the headfold stage were used. The vitelline membranes were removed from the blastoderms by gentle teasing and the embryos were then dissected out (Hell, 1964). The de-embryonated blastoderms were floated on to individual 1 cm squares of lens paper and mounted on stainless-steel grids in septate culture dishes, each compartment containing one blastoderm and 1 ml of medium F-12. The dishes were maintained at 38°C in an incubator circulated with sterilized air. Steroids were added, as indicated in the text, in 95% (v/v) ethanol such that the final alcohol concentration was less than 0.1%. An identical volume of alcohol was added to the control (steroid-free) cultures. In experiments involving the pulse-labelling of nucleic acids, [^3H]uridine or [^3H]thymidine was added to the culture medium to a final concentration of 10 $\mu\text{Ci}/\text{ml}$.

Isolation of erythrocytes and haemoglobin

Blastoderms were suspended in 0.15 M-NaCl/15 mM-sodium citrate and disrupted by gentle but repeated aspiration into a fine-tipped Pasteur pipette. After filtration through a 100-gauge stainless-steel grid, erythrocytes were collected by centrifuging the filtrate at 200g for 5 min and washed three times by further sedimentation in NaCl/sodium citrate solution. Lysis and the preparation of a clear haemoglobin extract were accomplished by the method of Bruns (1971). In the preparation of ^3H -labelled haemoglobin, blastoderms were incubated for 1 h in culture medium supplemented with 10 μCi of [^3H]methionine/ml.

Assay of δ -aminolaevulinic synthase (EC 2.3.1.37)

The assay requires a succinyl-CoA-generating system of bacterial origin. An extract of *R. spheroides* was prepared as directed by Lascelles (1956) and the succinyl-CoA-generating system was stable for

several weeks at 4°C without detectable loss of activity.

The assay depends on the formation of δ -amino-[^{14}C]laevulinic acid with [^{14}C]succinic acid as precursor. Our incubation system was based on that advocated by Irving & Elliott (1969). Each incubation mixture (total volume 0.5 ml) contained the following: 50 μmol of Tris/HCl buffer, pH 7.4; 1 μmol of EDTA; 100 μmol of glycine; 5 μmol of sodium arsenite; 20 μmol of MgCl_2 ; 1 μmol of pyridoxal 5'-phosphate; 1.25 μg of antimycin A; 5 μmol of sodium malate; 2 μmol of GSH; 0.4 μmol of CoA; 10 μmol of sodium succinate containing 0.1 μCi of [^{14}C]succinic acid and 100 μl (1 mg of protein) of *R. spheroides* enzyme extract. The reactions were initiated by the addition of 0.2 ml of blastoderm extract and incubation was continued for 1 h at 37°C in a metabolic shaking bath set at 120 cycles/min. As the source of δ -aminolaevulinic synthase, pooled blastoderms (12 or 18 per experimental group) were sonicated for 4 $\times$ 30 s at 20 kHz in 2.0 ml of 10 mM-Tris/HCl buffer, pH 7.4. The δ -amino[^{14}C]laevulinic acid formed in the reaction was ultimately measured as its chromatographically purified pyrrole derivative. This conversion was accomplished in two steps. First, pyrroles were formed by condensation with acetylacetone (Urata & Granick, 1963) and second, contaminating aminoacetone-pyrroles were removed by repeated extraction with diethyl ether (Granick, 1966). Samples containing δ -aminolaevulinic acid pyrrole were analysed by t.l.c. on Kieselgel G7731 plates (Merck, Darmstadt, W. Germany) as described by Irving & Elliott (1969). The δ -aminolaevulinic acid pyrrole was located with Ehrlich reagent (*p*-dimethylaminobenzaldehyde) and the appropriate area of silica gel was scraped from the plate and counted for radioactivity. The overall recovery of δ -aminolaevulinic acid through the entire procedure was 85% and the results were also corrected for the small amount of this compound synthesized in the *R. spheroides* extracts alone in the absence of added blastoderm extracts. The conversion of 10 pmol of [^{14}C]succinic acid into δ -amino[^{14}C]laevulinic acid was equivalent to 100 c.p.m. of chromatographically purified δ -aminolaevulinic acid pyrrole.

Extraction and analysis of blastoderm RNA

Pooled blastoderms were suspended in 5 ml of 25 mM-sodium acetate/acetic acid buffer, pH 5.2, containing 10 mM-EDTA and extracted repeatedly at 55°C with an equal volume of 85% (w/v) phenol, as described by Girard (1969). Contaminating DNA was digested with purified deoxyribonuclease as described by Mainwaring *et al.* (1974a). Fractionation and analysis of blastoderm RNA was conducted by the procedures oligo(dT)-cellulose chromatography, density-gradient ultracentrifugation and electrophoresis

in dilute polyacrylamide gels of Mainwaring *et al.* (1974a). In sucrose gradients, RNA samples (0.5 ml) were made 1% (w/v) with respect to sodium dodecyl sulphate, incubated at 37°C for 30 s, layered over 4.0 ml linear 8–40% (w/v) sucrose gradients prepared in 25 mM-sodium acetate/acetic acid buffer, pH 5.2, containing 0.1 M-NaCl, and centrifuged at 190 000 g_{av} for 3 h at 4°C. Electrophoretic analyses of RNA (50 μl samples) were conducted in gels (7.5 cm $\times$ 1.0 cm diam.), containing 2.6% (w/v) polyacrylamide and 2.0% (w/v) agarose at 3 mA/gel for 2 h at 4°C. RNA was located either by staining with Methylene Blue (Uriel & Avrameas, 1961) or by scanning at 260 nm in a Unicam SP 500 spectrophotometer with a Gilford scanning attachment. In both analytical procedures, liver polyribosomal RNA (essentially 18S and 28S rRNA) and 4S ascites-tumour tRNA served as markers [for their preparation, see Mainwaring *et al.* (1974a)].

Analysis of blastoderm proteins

(a) *Analyses for globin polypeptide chains.* Electrophoresis in 10% (w/v) polyacrylamide gels in the presence of sodium dodecyl sulphate was performed by the method of Weber & Osborn (1969). The molecular-weight markers, bovine serum albumin monomer (64 000), ovalbumin (47 000) and cytochrome *c* (12 800) were run in parallel and located by staining with Coomassie Brilliant Blue. Globin chains, essential for accurately monitoring the migration of these polypeptides, were produced from chick erythrocytes after 5 days of embryonic development by the acetone/HCl procedure of Rossi-Fanelli *et al.* (1958).

(b) *Specific electrophoretic analysis for chick haemoglobins.* This was performed on oxyhaemoglobins by isoelectric focusing in 5% (w/v) polyacrylamide gels (Wrigley, 1968) containing 2.5% (w/v) ampholytes (LKB Ltd., Selsdon, Surrey, U.K.), pH range 6.0–9.5. Blastoderm extracts, prepared as described above, were made 10% (v/v) with respect to glycerol and 2% (w/v) with respect to ampholytes, pH range 3–10, layered over the gels and a constant potential difference of 50 V was applied for 22 h at 2°C. The gels were fixed in 5% (w/v) trichloroacetic acid at 2°C for 10 min and scanned at the principal Soret band region of 415 nm. To ensure the authenticity of the localization of haem, the gels were immersed in 7% (v/v) acetic acid for 10 min, stained with 1:1 mixture of 10-vol. H_2O_2 and 1.4% (w/v) *o*-toluidine in acetic acid for 5 min and scanned at 600 nm (New, 1955).

Chemical analyses

Protein was determined by the Folin-Ciocalteu method as described by Lowry *et al.* (1951) with

bovine serum albumin as standard reference. Haemoglobin was quantitatively determined by the procedure devised by Hell (1964). DNA was assayed by the *p*-phenylhydrazine procedure of Webb & Levy (1955) with calf thymus DNA as standard. RNA was measured by the spectrophotometric procedure of Munro & Fleck (1966) with yeast tRNA as standard. Where indicated in the text, the procedure of Schmidt & Thannhauser (1945) was used in the separation of RNA and DNA.

Synthesis of protein in cell-free systems

Protein-synthesizing systems from the 30000g supernatant (S-30) fraction of wheat germ were prepared and assayed strictly in accordance with the directions of Roberts & Paterson (1973); the final assay volume was standardized at 25 μ l, including 10 μ l of added mRNA solution. Incubation was for 2 h at 22°C in air; [³⁵S]- or [³H]-methionine served as radioactive tracer. The products were analysed in three ways.

(a) *Direct precipitation.* After brief incubation in 3 M-KOH at 37°C to degrade aminoacyl-tRNA, the proteins were precipitated in 5% (w/v) trichloroacetic acid and collected in Whatman GF/A filters (Mainwaring & Wilce, 1973).

(b) *Polyacrylamide-gel electrophoresis.* Samples (50 μ l) were heated in a boiling-water bath for 5 min with 50 μ l of a mixture containing 0.1 M-2-mercaptoethanol, 20% (v/v) glycerol, 1% (w/v) sodium dodecyl sulphate in 50 mM-Tris/HCl buffer, pH 8.2, and then subjected to electrophoresis in polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulphate, as described above.

(c) *Analysis of tryptic digests.* After incubation with [³⁵S]methionine as tracer, samples were supplemented with 1.0 mg of chick haemoglobins from 5-day erythrocytes, labelled with [³H]methionine, and precipitated in 1.0 ml of 6 M-HCl in acetone for 3 h at -20°C. After sedimentation at 10000g for 5 min, the precipitate was dissolved in 2 ml of 5 mM-Na₂HPO₄/NaH₂PO₄ buffer, pH 6.8, and applied to a column (3 cm $\times$ 1 cm diam.) of Whatman CM-52 equilibrated with this buffer. The column was then eluted in a stepwise manner with Na₂HPO₄/NaH₂PO₄ buffer, pH 6.8, of gradually increasing molarity, as follows; 20 ml of 10 mM buffer, 20 ml of 25 mM buffer and finally 200 mM buffer, with collection of 0.5 ml fractions at this molarity only. Peak protein fractions, as judged by extinction analysis at 280 nm, were precipitated in 5% (w/v) trichloroacetic acid at 4°C. After resuspension in 2.0 ml of water, the precipitate was exhaustively extracted with ether and finally digested with trypsin as described by Mainwaring (1964). The trypsin-resistant 'core' was removed by sedimentation at 10000g for 30 min (Ingram, 1958) and the soluble

tryptic peptides (equivalent to 200 μ g of haemoglobin) were separated in the two-dimensional manner suggested by Sargent & Vadlamudi (1968) on t.l.c. plates (Kodak Chemical Co., Liverpool, U.K.; type 6064). Peptides were located with ninhydrin and counted individually for radioactivity.

Measurement of radioactivity

This was conducted in a Nuclear-Chicago Mark II liquid-scintillation spectrometer. Material recovered on t.l.c. plates or glass-fibre membranes was counted in a phosphor containing 6 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole/l of toluene. Aqueous samples, maximum volume 0.5 ml, were counted in 10 ml of a phosphor composed of 625 ml of Triton X-100, 1770 ml of toluene, 110 ml of water and 15 g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole. The channels-ratio method was used to determine the degree of quenching. Discrimination between ³⁵S and ³H in double-isotope mixtures was facilitated by use of a program for an Olivetti 602 computer (see Mainwaring *et al.*, 1974a).

Results

The standard reference for biochemical measurements on cultured blastoderms should be mentioned. Cell number cannot be used as the organs are maintained intact and wet weight is singularly inappropriate as the delicate structures are difficult to handle. The DNA content of blastoderm homogenates was adopted, therefore, as the standard reference.

Specificity in the stimulation of haemoglobin synthesis in chick blastoderm

In preliminary experiments, we incubated eggs for various times and observed the development of the blastoderm. After 24 h of incubation, the blastoderm was composed of colourless cells, totally devoid of haemoglobin and a further 24 h was required before haemoglobin was visible in a horseshoe pattern of blood islands posterior and lateral to the developing embryo. This embryonic stage was midway between the primitive streak and headfold development. Therefore haemoglobin-free blastoderms were removed after precisely 24 h of incubation and maintained *in vitro* for subsequent biochemical analysis, a practice consistent with that of other investigators (O'Brien, 1961; Levere & Granick, 1967; Levere *et al.*, 1967).

Levere *et al.* (1967) have provided convincing evidence that steroids related in structure to 5 β -androstane are particularly active in stimulating haemoglobin synthesis and we established a dose-response curve for this effect, by using aetiocholanolone (3 α -hydroxy-5 β -androstan-3-one) as the model

Fig. 1. Dose-response curve for the stimulation of haemoglobin synthesis in cultured chick blastoderm by aetiocholanolone

At 24h after incubation of fertilized eggs, the blastoderm was dissected from the embryo and maintained *in vitro*. See the Materials and Methods section for details. Groups of six blastoderms were cultured in the presence of various concentrations of aetiocholanolone for a further 48h. The haemoglobin content was quantitatively determined in homogenates of six pooled blastoderms.

compound. The chick blastoderm is extremely sensitive to low concentrations of aetiocholanolone and maximum stimulation was found at approx. 5 nM (Fig. 1).

We studied the enhancement of haemoglobin synthesis in terms of tissue specificity and steroid specificity in greater detail (Table 1). The findings on tissue specificity were clear cut. All of the embryonic tissues investigated, including the embryo itself, proliferated satisfactorily *in vitro*. Unlike blastoderm, however, the embryo and vitelline membrane are not sites of erythropoiesis and haemoglobin could not be induced even in the presence of aetiocholanolone. The results on steroid specificity of haemoglobin induction corroborated reports on the regulation of haem synthesis in chick blastoderm (Levere *et al.*, 1967) and foetal chick liver (Granick & Kappas, 1967a). A significant stimulation of haemoglobin synthesis was elicited by two steroids related to 5 β -androsterone; by contrast, cortisol and progesterone were much less effective and oestradiol-17 β inhibited haemoglobin synthesis. The stimulatory effect of progesterone may be attributable to its metabolism to 5 β -reduced steroids (Granick & Kappas, 1967a). Perhaps the most notable feature of this survey was the dissimilar biological activity of the stereoisomers of dihydro-

Table 1. Specificity in the stimulation of haemoglobin synthesis in chick blastoderm

Two experiments are summarized here. Expt. (a) Tissue specificity. After 24h of incubation, organs were dissected from fertilized eggs and maintained under controlled conditions *in vitro*, with six organs per experimental group. Aetiocholanolone was added to the culture medium, as indicated, and the cultures were continued for a further 48h. Pooled blastoderms were homogenized and the haemoglobin content was determined. Expt. (b) Steroid specificity. Blastoderms, dissected free from embryos, were maintained in culture for 48h in the presence of various steroids. The haemoglobin was determined in homogenates representing six blastoderms per experimental group. N.D., Haemoglobin could not be detected.

Tissue	Steroid (10nM)	Haemoglobin (μg/20 μg of DNA)
Expt. (a) Tissue specificity		
Vitelline membrane	None	N.D.
	Aetiocholanolone	N.D.
Embryo	None	N.D.
	Aetiocholanolone	N.D.
Blastoderm plus embryo	None	18.0
	Aetiocholanolone	28.1
Blastoderm minus embryo	None	20.3
	Aetiocholanolone	34.7
Expt. (b) Steroid specificity		
De-embryonated blastoderm throughout	None	10.5
	Oestradiol-17 β	7.1
	5 α -Dihydrotestosterone	11.5
	5 β -Dihydrotestosterone	37.3
	Aetiocholanolone	38.5
	Cortisol	15.4
	Progesterone	23.0

Fig. 2. Changes in important biochemical processes during the development of the chick blastoderm *in vitro*

Beginning 24h after incubation, blastoderms were dissected from fertilized eggs and maintained in culture *in vitro* in the presence or absence of 30nM-aetiocholanolone. (a) Changes in enzyme activity and haemoglobin content. δ -Aminolaevulinate synthase activity was determined with 18 blastoderms per experimental group (○, ●) and haemoglobin assayed with six blastoderms per experimental group (△, ▲). (b) Changes in the incorporation of radioactive precursors into RNA and DNA. Precisely 1h before analysis, groups of six blastoderms were incubated with [3 H]thymidine (○, ●) or [3 H]uridine (△, ▲) and the labelled tissue was fractionated in a manner distinguishing between DNA and RNA (Schmidt & Thannhauser, 1945). ○, △, Cultures in the absence of added steroid; ●, ▲, in the presence of 30nM-aetiocholanolone.

testosterone. The planar stereoisomer, 5 α -dihydrotestosterone, had been reported to be a weak inducer of porphyrins in foetal liver (Granick & Kappas, 1967a) and the data suggest that the extremely angular *cis* orientation of the A and B rings in 5 β -reduced steroids is a mandatory requirement for promoting haem synthesis.

A certain degree of experimental variation was observed in the chick blastoderm system (Table 1). We consistently found a fall in the number of successfully developing embryos, together with fluctuations in haemoglobin synthesis in culture, particularly in eggs from older hens. This problem was particularly evident in the extent of intrinsic haemoglobin synthesis in the absence of added steroid in different batches of eggs.

Time-course of biochemical events in the developing chick blastoderm in vitro

Changes in important biochemical parameters during the course of the differentiation and development of the blastoderm in culture are presented in Figs. 2(a) and 2(b). Determination of the activity of the enzyme δ -aminolaevulinate synthase was prompted by the suggestion of Levere & Granick (1965, 1967) that it catalyses the rate-limiting step of the biosynthetic pathway for porphyrin synthesis. There was a dramatic increase in the activity of δ -amino-

laevulinate synthase (Fig. 2a) and in the incorporation of radioactive precursors into DNA and RNA (Fig. 2b) far in advance of the onset of haemoglobin synthesis. Two aspects of this work warrant further comment. First, we were indubitably measuring haemoglobin synthesis *de novo* rather than simply the assembly of pre-formed globin chains with haem (see Levere & Granick, 1967; Kappas & Granick, 1968). Secondly, the use of DNA as the reference for the incorporation of precursors into both DNA and RNA is eminently justified under our conditions of analysis. The method of Schmidt & Thannhauser (1945) separates RNA from DNA; further, by using [3 H]thymidine, labelling of RNA will be negligible, and conversely, DNA cannot be labelled with [3 H]uridine as precursor (Hayhoe & Quaglino, 1965).

The 18h period immediately after the establishment of the blastoderm cultures was typified by intense metabolic activity save for the synthesis of haemoglobin (Figs. 2a and 2b). Low concentrations of aetiocholanolone dramatically enhanced all biochemical events, including the induction of haemoglobin, but did not modulate their temporal sequence. This suggests that the presence of 5 β -reduced steroids *in vitro* is amplifying events already set in train at a lower rate in the blastoderm after 24h development *in vivo*.

Erythropoiesis in the chick is an exceedingly complex process, with fundamental changes occur-

ring in the type of haemoglobin synthesized at particular developmental stages [see review by Wilt (1967)]. Electrophoretic evidence from several laboratories (Manwell *et al.*, 1963; Deucher & Dryland, 1964; Hashimoto & Wilt, 1966), indicates that two major forms of haemoglobin are present after 5 days of embryonic development and that these are structurally distinct from adult chick haemoglobins. These above studies were performed on circulating erythrocytes rather than the blastoderm itself and to facilitate the interpretation of experiments presented below, it was imperative to confirm that the haemoglobin present in the developing chick blastoderm *in vitro* was of the two early or primitive types. Only two forms of haemoglobin (pI 7.2 and 8.4) were identified in cultured blastoderm by isoelectric focusing (Figs. 3a–3d). In keeping with the nomenclature advocated

by Bruns (1971) and Brown & Ingram (1974), these will be termed haemoglobins E and P. The presence of 30 nM-aetiocholanolone in the cultures influenced the amount but not the type of haemoglobin synthesized (cf. Figs. 3a and 3c with Figs. 3b and 3d). Although not included in Fig. 3, we did confirm that haemoglobins in advanced embryos (17 days development) possessed isoelectric points distinct from haemoglobins E and P.

A more detailed enquiry into the stimulation of blastoderm RNA synthesis by aetiocholanolone

Analyses of blastoderm RNA in sucrose gradients, after labelling with [³H]uridine, are presented in Figs. 4(a)–4(c). Since the amounts of extracted blastoderm RNA were small, the samples were supplemented

Fig. 3. Analysis of foetal oxyhaemoglobins by isoelectric focusing in polyacrylamide gels

Blastoderms were dissected from 24 h chick embryos and maintained in culture *in vitro* for various times either in the presence or absence of 30 nM-aetiocholanolone. Blastoderms were lysed to release haemoglobin and soluble extracts were analysed by isoelectric focusing in the pH range 3–10 in polyacrylamide gels (see the Materials and Methods section for details). Extracts were derived from six pooled blastoderms and the direction of anodic migration is to the right. Haemoglobin was located either by scanning the gels directly at the principal Soret wavelength of 415 nm or haem was located by incubating gels with *o*-toluidine and H₂O₂ with subsequent scanning at 600 nm. In (a) and (b), gels were scanned at 415 nm and in (c) and (d) at 600 nm. Blastoderms after 12 h (·····), 24 h (– · – ·) or 36 h (—) in culture were used, (a) and (c) in the presence of 30 nM-aetiocholanolone, (b) and (d) in the absence of added steroid,

Fig. 4. Analysis of ^3H -labelled chick blastoderm RNA in sucrose-density gradients

After 24h incubation *in vivo*, chick blastoderms were maintained *in vitro* in either the presence or the absence of 30nM-aetiocholanolone, with six blastoderms per experimental group. Just 1h before analysis, the culture medium was supplemented with ^3H uridine and labelled RNA was extracted as described in the Materials and Methods section. The samples of ^3H -labelled blastoderm RNA were mixed with 5 E_{254} units of rat liver polyribosomal RNA as sedimentation marker and analysed in 5 ml linear 8–40% (w/v) sucrose gradients. The direction of centrifugation is from left to right. Gradients were monitored by extinction analysis at 254 nm and individual gradient fractions were counted for radioactivity. Blastoderms after culturing for (a) 12h, (b) 24h and (c) 36h. —, E_{254} ; ●, radioactivity from cultures incubated in the presence of 30nM-aetiocholanolone; ○, radioactivity from cultures incubated in the absence of steroid.

Fig. 5. Analysis of blastoderms for a poly(A)-rich RNA fraction

As described in the legend to Fig. 4(a), groups of nine blastoderms were maintained *in vitro* for 12h in the presence or the absence of 30nM-aetiocholanolone, labelled with ^3H uridine for 1h and the ^3H -labelled RNA was extracted. (a) Analysis by oligo(dT)-cellulose chromatography. Samples were analysed in a medium containing 0.5M-KCl and the poly(A)-rich RNA was selectively eluted by lowering of the ionic strength of the eluting medium to Tris buffer, pH7.4, only, at the point indicated by the arrow. RNA from cultures maintained in the presence of aetiocholanolone (●); RNA from cultures without added steroid (○). (b) Analysis by electrophoresis in polyacrylamide gels. Samples from the oligo(dT)-cellulose-fractionation procedure were supplemented with 20 μg of rat liver polyribosomal RNA (18S and 28S rRNA markers) and 5 μg of ascites-tumour tRNA (4S) and separated with the direction of migration to the right (anode). Gels were monitored at 254 nm to locate the RNA markers, sliced and counted for radioactivity. Poly(A)-rich RNA from cultures containing aetiocholanolone (●); poly(A)-rich RNA from steroid-free cultures (○); poly(A)-depleted RNA from cultures containing aetiocholanolone (■).

with rat liver polyribosomal RNA to provide accurate sedimentation markers. RNA synthesis was particularly conspicuous after 12h in culture and all species of blastoderm were actively labelled with ^3H uridine (Fig. 4a), which is consistent with the results presented

earlier in Fig. 2(b). The 18S and 28S rRNA components were labelled extensively but the most prominent feature of the gradient profiles was a highly labelled but diffuse peak of RNA remaining near the top of the gradients. It is important to

emphasize that the labelling of all blastoderm RNA species was enhanced by aetiocholanolone. After longer culture times, even in the presence of aetiocholanolone, all species of RNA were labelled to a much lower extent (Figs. 4*b* and 4*c*). Aetiocholanolone thus stimulates RNA synthesis by enhancing the rate of processes already in progress in the blastoderm after 24 h of development *in vivo* but does not evoke the synthesis of a novel steroid-sensitive species of RNA.

Since the globin mRNA in reticulocytes of rabbit (Labrie, 1969) and duck (Spohr *et al.*, 1974) has a sedimentation of 10S, we thought that the slowly sedimenting blastoderm RNA fraction described above in Fig. 4(*a*) may contain the mRNA species coding for the polypeptide chains of haemoglobins E and P. We therefore exploited the probable existence of poly(A)-rich sequences at the 3'-terminus of blastoderm globin mRNA as is the case with rabbit globin mRNA (Aviv & Leder, 1972). When ³H-labelled blastoderm RNA was fractionated on oligo(dT)-cellulose, two peaks of radioactivity were resolved (Fig. 5*a*). The principal peak of radioactive RNA was not retained under conditions of high ionic strength and a small peak of poly(A)-rich RNA was selectively eluted at low ionic strength. These RNA fractions were then analysed further by electrophoresis in dilute polyacrylamide gels (Fig. 5*b*). The poly(A)-depleted RNA was resolved into several components, including a peak of high sedimentation coefficient that barely penetrated into the gels, 18S and 28S rRNA and a broad 4S component (possibly 4S tRNA and 5S rRNA). In contrast, the poly(A)-enriched RNA was essentially recovered midway between the 4S and 18S markers, indicating an average sedimentation coefficient of approx. 10S. The labelling of the 10S, poly(A)-rich RNA fraction was markedly stimulated by the addition of 30 nM-aetiocholanolone to the blastoderm cultures.

Translation of blastoderm poly(A)-rich RNA in cell-free systems

The 10S poly(A)-rich blastoderm RNA stimulated protein synthesis in a mRNA-depleted system derived from wheat germ (Table 2). This property was not shared by other RNA species except poly(A,U,G), the synthetic mRNA coding for methionine. In additional experiments (Figs. 6*a*–6*c*) we determined the optimum conditions for the translation of poly(A)-rich RNA. As Roberts & Paterson (1973) reported in their development of the wheat germ S-30 system, remarkably sharp optima were established with respect to the MgCl₂ concentration (Fig. 6*a*) and KCl concentration (Fig. 6*b*) in the assay system at 1.75 mM-MgCl₂ and 90 mM-KCl respectively. Under these conditions, an exceedingly sensitive assay for blasto-

Table 2. *Specificity in the stimulation of protein synthesis by blastoderm poly(A)-rich RNA*

Samples of blastoderm RNA were fractionated by oligo(dT)-cellulose chromatography into poly(A)-depleted and poly(A)-enriched components. RNA fractions (0.5 µg) or synthetic mRNA species (5 µg) were incubated in triplicate with an S-30 system from wheat germ, with 3 µCi of [³H]methionine as tracer. The incorporation of amino acid into protein was determined and the results are given as means ± S.E.M.

RNA added	Incorporation (c.p.m.)
None (controls)	3100 ± 170
Poly(U)	2900 ± 200
Poly(A,U,G)	6000 ± 190
Liver polyribosomal RNA	3200 ± 60
Ascites-tumour tRNA	2700 ± 100
Blastoderm poly(A)-depleted RNA	3200 ± 200
Blastoderm poly(A)-enriched RNA	8700 ± 450

derm poly(A)-rich RNA was possible (Fig. 6*c*). Overall, the 10S blastoderm RNA satisfies the exacting criteria demanded of a putative mRNA.

The wheat germ S-30 system possesses two advantages over the ascites-tumour system used by Mainwaring *et al.* (1974*a,b*). First, endogenous protein synthesis in the absence of exogenous mRNA is exceedingly low; secondly, it provides a more efficient system for the translation of mRNA.

We next decided to delineate the stage in the development of the blastoderm at which globin mRNA was first detectable. Poly(A)-rich RNA was isolated from blastoderms cultures *in vitro* for various times, either in the presence or absence of 30 nM-aetiocholanolone. The mRNA from steroid-free cultures was translated in the presence of [³H]methionine, whereas that from steroid-stimulated cultures was translated with [³⁵S]methionine as tracer. The ³H- and ³⁵S-labelled proteins representing a given developmental stage were mixed and analysed concurrently (Figs. 7*a*–7*c*). The relative constancy of the ³⁵S/³H ratio suggests that aetiocholanolone does not enhance the relative amount of globin mRNA present in the blastoderm nor provoke its precocious appearance. The input of blastoderm mRNA in the experiments presented in Fig. 7 was held constant at 2.0 µg; identical results in qualitative terms were obtained at a lower input of 0.5 µg of mRNA. In additional experiments, we established that the amount of poly(A)-rich RNA remained within the range 12.9–14.1 µg/100 µg of blastoderm DNA at all developmental stages, even in the presence of aetiocholanolone. This strengthens the argument presented in Fig. 7 that despite changes in the synthesis or degradation of blastoderm mRNA during development, the amount of globin mRNA remains essentially constant.

Fig. 6. Optimum conditions for the translation of blastoderm poly(A)-rich RNA in the wheat germ S-30 systems

Blastoderm RNA was fractionated by oligo(dT)-cellulose chromatography and samples of poly(A)-rich RNA ($0.5 \mu\text{g}$, unless specified to the contrary) were added to the wheat germ S-30 system, total assay volume $25 \mu\text{l}$, with $3 \mu\text{Ci}$ of $[^3\text{H}]$ methionine as tracer. (a) Various MgCl_2 concentrations; KCl concentration constant at 75 mM . (b) Various KCl concentrations; MgCl_2 concentration constant at 1.7 mM . (c) Incorporation into protein relative to input of poly(A)-rich RNA; with 1.7 mM - MgCl_2 and 90 mM -KCl. In the latter experiment, the incorporation was corrected for a background of 2600 c.p.m. determined in the absence of added mRNA.

Fig. 7. Translation of blastoderm mRNA in a cell free system

After 24 h incubation of fertilized eggs, pooled groups of 18 blastoderms were maintained *in vitro* either in the absence or the presence of 30 nM -aetiocholanolone. Total RNA was extracted and poly(A)-rich mRNA isolated by oligo (dT)-cellulose chromatography. Samples ($2.0 \mu\text{g}$) of mRNA were incubated with a wheat germ S-30 system, as described in detail in the Materials and Methods section; mRNA from steroid-free cultures was incubated with $[^3\text{H}]$ methionine and that from steroid-stimulated cultures with $[^{35}\text{S}]$ methionine. $[^{35}\text{S}]$ Methionine (380 Ci/mmol) was diluted with non-radioactive L-methionine to a specific radioactivity identical with that of $[^3\text{H}]$ methionine (9 Ci/mmol). At the completion of incubation, ^{35}S - and ^3H -containing samples representing a given stage of development were mixed, treated with sodium dodecyl sulphate and analysed in 10% (w/v) polyacrylamide gels containing 0.1% (w/v) sodium dodecyl sulphate. The direction of anodic migration is to the right and electrophoresis was continued until the tracking dye, Bromophenol Blue, had just left the gels. The gels were sliced, solubilized in 0.5% (w/v) sodium dodecyl sulphate and counted for radioactivity under conditions discriminating between ^{35}S and ^3H . Molecular-weight markers were run in parallel: B, bovine serum albumin (64000), O, ovalbumin (45000) and G, mixed globin chains of chick haemoglobins E and P (approx. 17000 ; the α - and β -chains are not resolved under these conditions). Blastoderms from various times of culture; (a) 12 h, (b) 24 h, and (c) 36 h. $\bullet$, ^3H (steroid-free cultures) $\circ$, ^{35}S (cultures containing 30 nM -aetiocholanolone).

Fig. 8. A more rigorous identification of the polypeptide chains of haemoglobins E and P among the proteins formed in a wheat germ S-30 system in the presence of blastoderm mRNA

Syntheses were conducted in a total volume of 25 μ l in the presence of 1 μ g of mRNA and [35 S]methionine. Duplicate assays were pooled and supplemented with 1.0 mg of a mixture of haemoglobins E and P (20000 c.p.m.), labelled in intact erythrocytes of 5-day blastoderms with [3 H]methionine. Proteins labelled with [35 S]- and [3 H]-methionine, including dissociated globin chains, were precipitated in acid/acetone (1:95, v/v) at -20°C and analysed further. (a) Ion-exchange chromatography. Samples were applied to columns (2 cm $\times$ 1 cm diam.) of CM-52 equilibrated with 10 mM- $\text{Na}_2\text{HPO}_4/\text{NaH}_2\text{PO}_4$ buffer, pH 6.8, and washing was continued until no more ^{35}S was eluted; stepwise elution was performed with 25 and 200 mM-sodium phosphate buffer, as indicated by the arrows. The hatched area represents a peak of ^{35}S -labelled products that was not influenced by the presence or absence of mRNA. Proteins recovered within the bar in 200 mM-phosphate buffer were analysed further. $\bullet$, Blastoderm mRNA; $\circ$, poly(A,U,G) A; Δ , no addition of mRNA to S-30 system; —, ^3H . (b) Two-dimensional separation of tryptic digests. The ^3H - and ^{35}S -labelled proteins were incubated overnight with crystalline trypsin and after enzyme-resistant material was removed by centrifugation, the soluble digestion products (equivalent to approx. 200 μ g of protein) were subjected to two-dimensional analysis, first with electrophoresis and then with chromatography. Peptides were located with ninhydrin and counted for radioactivity. Only the numbered peptides, shown with hatching, contained ^3H and were also the only areas to give a positive reaction for methionine with the specific H_2PtCl_6 reagent (Easley, 1969). Complete details of all procedures may be found in the Materials and Methods section; the radioactivity (as ^3H and ^{35}S) recovered in individual peptides (1–5) is presented in Table 3.

Table 3. Radioactivity recovered in individual peptides of tryptic digests of authentic ^3H -labelled haemoglobins E and P and ^{35}S -labelled products from S-30 experiments *in vitro*

Details of this experiment are presented in the legend to Fig. 8. Of the 36 peptides present in tryptic digests (see Fig. 8b), only 5 contained methionine on the basis of a specific colorimetric reaction. The radioactivity represents ^3H from haemoglobins E and P labelled in intact erythrocytes with [^3H]methionine and products of S-30 reactions with [^{35}S]methionine as tracer.

Peptide	^3H (d.p.m.)	^{35}S (d.p.m.)		
		No added mRNA	Poly(A,U,G)	Blastoderm mRNA
1	1000	} <100	} <100	580
2	4770			2450
3	2090			3250
4	1010			1660
5	1090			1540
Others	<100			<100

As many proteins were being fabricated in chick blastoderm (Fig. 7) more convincing evidence was needed to confirm the authentic synthesis of the E and

P globin chains and therefore ion-exchange chromatography to isolate putative globin chains (Fig. 8a) followed by two-dimensional separation of tryptic

digests was used (Fig. 8b). The cell-free syntheses were conducted with [^{35}S]methionine as tracer and haemoglobins E and P, labelled with [^3H]methionine, were added as internal markers. As judged by two criteria, a specific stain for methionine (Easley, 1969) and the location of ^3H , only 5 of a total of 36 peptides contained methionine. ^{35}S could only be introduced into these peptides when the S-30 system was supplemented with blastoderm mRNA (Table 3); this property was not shared by the synthetic mRNA, poly(A,U,G).

Discussion

The acute steroid specificity demonstrated in the stimulation of erythropoiesis in cultured chick blastoderm agrees with published results (Levere & Granick, 1965; Levere *et al.*, 1967). Stimulation of erythropoiesis mandatorily requires the characteristically angular 5β -reduced steroids, whose unique structural feature is the *cis* orientation of the A and B rings. This stringent requirement emphasizes the importance of the metabolic studies conducted by Parsons (1970) and the significance of the high-affinity binding mechanism or receptor system, specific for 5β -reduced steroids, in chick blastoderm (Spooner & Mainwaring, 1973).

Most mechanisms through which steroids regulate important biochemical events can be described as either switch or amplification mechanisms (King & Mainwaring, 1974). The enhancement of haem synthesis by aetiocholanolone is an excellent example of amplification. It may be argued that 5β -reduced steroids recruit more cells into erythropoiesis or stimulate a constant population of erythropoietic cells to synthesize more haemoglobin. Distinction between these possibilities demands very precise histochemical or morphological techniques based on the specific location of haem or perhaps the use of radioactive DNA species complementary to the globin mRNA (Chan *et al.*, 1974).

It is noteworthy that many of the biochemical parameters studied were enhanced when blastoderm cells were entering a dramatic burst of mitosis or, at least, a period of intense incorporation of [^3H]thymidine into DNA, a phenomenon widely associated with developmental processes. Flexner *et al.* (1953) reported that the activities of certain enzymes in developing brain were greatly stimulated in critical periods of biochemical activity associated with functional and morphological changes. Similar observations have been made in embryonic development in amphibia (Gurdon & Woodland, 1968) and during the terminal stages of erythrocyte differentiation (Denton *et al.*, 1975). It is possible that certain structural genes can only be expressed when the genetic apparatus is in an open or activated form as, for example, before cell division. Such considerations

may be germane to the sudden appearance of δ -aminolaevulinic synthase activity in the developing blastoderm.

In appraising the influence of aetiocholanolone on processes related to erythropoiesis, Incefy & Kappas (1971, 1974) concentrated their attention on RNA polymerase enzymes of haem-producing cells. However, we have tried to identify and characterize the mRNA coding for the globin chains of the primitive haemoglobins E and P. Chan *et al.* (1974) identified the mRNA coding for these globin chains in chick erythrocytes after 5 days of embryonic development. Our present findings confirm and considerably extend the work of Chan *et al.* (1974) in two very important respects; first, globin mRNA is present at the very earliest stages of development and, secondly, haemoglobin synthesis may be stimulated specifically by 5β -reduced steroids. However, it is now evident that synthesis of globin *de novo* is not the rate-limiting step of haemoglobin production. Alternative mechanisms for the control of haemoglobin synthesis have been proposed for rabbit reticulocytes (Adamson *et al.*, 1969; Gross & Rabinovitz, 1972) and these may be applicable to the chick blastoderm also.

We are deeply indebted to Mrs. Margaret Barker for her assistance in the preparation of the manuscript and to our colleagues, Dr. Allan Smith and Dr. Tricia Wheeler, for the supply of the wheat germ S-30 system.

References

- Adamson, S. D., Herbert, E. & Kemp, S. F. (1969) *J. Mol. Biol.* **42**, 247–258
- Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1408–1412
- Borsook, H., Abrams, A. & Lowy, P. H. (1955) *J. Biol. Chem.* **215**, 111–124
- Brown, J. L. & Ingram, V. M. (1974) *J. Biol. Chem.* **249**, 3960–3972
- Bruns, L. (1971) Ph.D. Thesis, Massachusetts Institute of Technology
- Chan, L.-E. L. & Ingram, V. M. (1973) *J. Cell Biol.* **56**, 861–865
- Chan, L.-E. L., Weidmann, M. & Ingram, V. M. (1974) *Dev. Biol.* **40**, 174–185
- Denton, M. J., Spencer, N. & Arnstein, H. R. V. (1975) *Biochem. J.* **146**, 205–211
- Deucher, E. & Dryland, A. M. L. (1964) *Nature (London)* **201**, 832–833
- Easley, C. W. (1969) *Biochim. Biophys. Acta* **175**, 211–213
- Flexner, L. B., Belknap, E. L. & Flexner, J. B. (1953) *J. Cell. Comp. Physiol.* **42**, 151–161
- Fried, W. & Gurney, C. W. (1965) *Nature (London)* **206**, 1160–1161
- Girard, M. (1969) *Methods Enzymol.* **12A**, 581–588
- Gordon, A. S., Zanjani, E. D., Levere, R. D. & Kappas, A. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **65**, 919–924
- Granick, S. (1966) *J. Biol. Chem.* **241**, 1359–1367
- Granick, S. & Kappas, A. (1967a) *J. Biol. Chem.* **242**, 4587–4593

- Granick, S. & Kappas, A. (1967b) *Proc. Natl. Acad. Sci. U.S.A.* **57**, 1463-1467
- Gross, M. & Rabinovitz, M. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1565-1568
- Gurdon, J. B. & Woodland, M. R. (1968) *Biol. Rev.* **43**, 233-267
- Hashimoto, K. & Wilt, F. H. (1966) *Proc. Natl. Acad. Sci. U.S.A.* **56**, 1477-1483
- Hayhoe, F. G. J. & Quaglini, D. (1965) *Nature (London)* **206**, 151-154
- Hell, A. (1964) *J. Embryol. Exp. Morphol.* **12**, 609-619
- Incefy, G. S. & Kappas, A. (1971) *FEBS Lett.* **15**, 153-158
- Incefy, G. S. & Kappas, A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2290-2294
- Ingram, V. M. (1958) *Biochim. Biophys. Acta* **28**, 539-545
- Irving, E. A. & Elliott, W. H. (1969) *J. Biol. Chem.* **244**, 60-67
- Jung, I. & Baulieu, E.-E. (1972) *Nature (London) New Biol.* **237**, 24-26
- Kappas, A. & Granick, S. (1968) *J. Biol. Chem.* **243**, 346-351
- King, R. J. B. & Mainwaring, W. I. P. (1974) *Steroid-Cell Interactions*, Butterworth and Co., London
- Kreig, M., Szalay, R. & Voigt, K.-D. (1974) *J. Steroid Biochem.* **5**, 453-459
- Labrie, F. (1969) *Nature (London)* **221**, 1217-1222
- Lascelles, J. (1956) *Biochem. J.* **62**, 78-93
- Levere, R. D. & Granick, S. (1965) *Proc. Natl. Acad. Sci. U.S.A.* **54**, 134-137
- Levere, R. D. & Granick, S. (1967) *J. Biol. Chem.* **242**, 1903-1911
- Levere, R. D., Kappas, A. & Granick, S. (1967) *Proc. Natl. Acad. Sci. U.S.A.* **58**, 985-990
- Lingrel, J. B. & Borsook, H. (1963) *Biochemistry* **2**, 309-314
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265-275
- Mainwaring, W. I. P. (1964) Ph.D. Thesis, University of Sheffield
- Mainwaring, W. I. P. & Mangan, F. R. (1973) *J. Endocrinol.* **59**, 121-139
- Mainwaring, W. I. P. & Wilce, P. A. (1973) *Biochem. J.* **134**, 795-805
- Mainwaring, W. I. P., Wilce, P. A. & Smith, A. E. (1974a) *Biochem. J.* **137**, 513-524
- Mainwaring, W. I. P., Mangan, F. R., Irving, R. A. & Jones, D. A. (1974b) *Biochem. J.* **144**, 413-426
- Manwell, C., Baker, C. M. A., Roslansky, J. D. & Foght, M. (1963) *Proc. Natl. Acad. Sci. U.S.A.* **49**, 496-503
- Mirand, E. A., Gordon, A. S. & Wenig, J. (1965) *Nature (London)* **206**, 270-272
- Munro, H. N. & Fleck, A. (1966) *Methods Biochem. Anal.* **14**, 113-176
- Necheles, T. F. & Rai, U. S. (1969) *Blood* **34**, 380-384
- New, D. A. T. (1955) *J. Embryol. Exp. Morphol.* **3**, 326-330
- O'Brien, B. R. A. (1961) *J. Embryol. Exp. Morphol.* **8**, 202-221
- Parsons, I. C. (1970) *Steroids* **16**, 59-65
- Rabinovitz, M. & Olsen, M. E. (1956) *Exp. Cell Res.* **10**, 747-749
- Reisner, E. H. (1966) *Blood* **27**, 460-469
- Rishpon-Meyerstein, N., Kilbridge, T., Simone, J. & Fried, W. (1968) *Blood* **31**, 453-460
- Roberts, B. E. & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A.* **70**, 2330-2334
- Rossi-Fanelli, A., Antonini, E. & Capato, A. (1958) *Biochim. Biophys. Acta* **30**, 608-615
- Sargent, J. R. & Vadlamudi, B. P. (1968) *Anal. Biochem.* **25**, 583-587
- Schmidt, G. & Thannhauser, S. J. (1945) *J. Biol. Chem.* **161**, 83-89
- Settle, A. W. (1954) *Contrib. Embryol.* **241**, 223-237
- Sierralta, W., Gonzalez, M. C. & Minguell, J. (1974) *J. Steroid Biochem.* **5**, 645-648
- Spohr, G., Imazuimi, T. & Scherrer, K. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 5009-5013
- Spooner, P. M. & Mainwaring, W. I. P. (1973) *Acta Endocrinol. (Copenhagen)* **177**, 181
- Urata, G. & Granick, S. (1963) *J. Biol. Chem.* **238**, 811-820
- Uriel, J. & Avrameas, E. (1961) *C. R. Hebd. Séances Acad. Sci. Ser. D* **252**, 1524-1527
- Wainwright, S. D. & Wainwright, L. K. (1966) *Can. J. Biochem.* **44**, 1543-1560
- Webb, J. M. & Levy, H. B. (1955) *J. Biol. Chem.* **213**, 107-117
- Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406-4412
- Williamson, R., Crossley, J. & Humphries, S. (1974) *Biochemistry* **13**, 703-707
- Wilt, F. H. (1967) *Adv. Morphog.* **6**, 89-125
- Wrigley, C. V. (1968) *J. Chromatogr.* **36**, 362-365