A Chromatographic Preparation of Ox Growth Hormone

BY M. WALLIS* AND H. B. F. DIXON

Department of Biochemistry, University of Cambridge

(Received 20 December 1965)

1. A method is described for the chromatographic preparation of ox growth hormone. It involves chromatography of an extract of anterior pituitary lobes on DEAE-cellulose, followed by rechromatography on a dextran gel of low cross-linkage (Sephadex G-100). 2. The product is highly active in growth-hormone assays, and is obtained in good yield. It was homogeneous by several criteria, but showed some heterogeneity on starch-gel electrophoresis. 3. The molecular weight of the hormone was estimated from its behaviour on gel-filtration columns under various conditions. Evidence that the hormone may dissociate into sub-units under some conditions is presented.

The first isolation of an apparently homogeneous preparation of ox growth hormone was described by Li, Evans & Simpson (1945). Ammonium sulphate precipitation of alkaline pituitary extracts was followed by repeated salt fractionation and isoelectric precipitations. The material produced was a protein, and was homogeneous by several criteria. It was highly potent in growth-hormone assays, but the yield was low. A different method was reported shortly afterwards by Wilhelmi, Fishman & Russell (1948), who used an ethanol precipitation procedure. This gave considerably better yields.

Although several modifications of these procedures have been described (e.g. Campbell & Davidson, 1953; Li, 1954; Wilhelmi, 1955; Ellis, 1961) they all involve repeated precipitations, which are both tedious and liable to lead to considerable losses. In addition, several authors (e.g. Lewis, 1962; Ferguson & Wallace, 1963; Dellacha & Sonenberg, 1964) have reported that many ox growth-hormone preparations made by these methods show considerable heterogeneity.

It seemed probable that the application of chromatographic techniques might prove of value in the purification of ox growth hormone. Chromatography of material prepared by the Wilhelmi ethanol precipitation procedure has resolved the hormone into several fractions that are different in both physicochemical and biological properties (Ferguson & Wallace, 1963; Manchester & Wallis, 1963). Wallace & Ferguson (1963) showed that chromatography of pituitary extracts could be of considerable value in preparing sheep growth

hormone. The present paper describes the development of a chromatographic method for preparing ox growth hormone.

MATERIALS AND METHODS

Materials for chromatography. DEAE-cellulose was obtained from Whatman (DE-50). It was sieved before use, and only material passing 100 mesh was used. The ion-exchanger was washed with 0.5 N-HCl, with 0.5 N-NaOH and then extensively with water. Fines were removed during the water washing by repeated decantation. Sephadex G-100 was obtained from Pharmacia AB, Uppsala, Sweden. It was swelled for 2 days in 0.1 M-NaCl.

Procedure for column chromatography. Glass chromatography columns with sinters of porosity 2 or 3 were used. Columns were prepared by pouring in a fairly thick suspension of the chromatographic medium in the starting buffer and allowing it to settle. The top of a Sephadex column was protected by a disk of filter paper.

Before use columns were equilibrated by running the required buffer through until the pH of the effluent was equal to that of the influent (all pH values were measured at room temperature). Equilibration and chromatography took place in a cold room (1–4°). Buffers contained 0.03% of toluene or 0.1% of chloroform as a bacteriostat.

Fractions were collected in weighed test tubes and the volume of each fraction was calculated from its weight. The E_{280} (or sometimes E_{220} or E_{230} to increase sensitivity) was measured as an index of the amount of protein present.

DEAE-cellulose columns were used only once. Sephadex G-100 columns were used repeatedly.

Starch-gel electrophoresis. Gels were prepared (Smithies, 1955) in Perspex trays (20 cm. × 10 cm. × 0.6 cm.). Electrophoresis was performed with the gel in a horizontal position in the cold room. The gel and filter-paper bridges were covered with thin polythene sheet to reduce evaporation from the surface. A potential difference of 200–400 v was applied across the gel for 4–6 hr. Samples were applied to the gel on filter paper: a piece of Whatman 3MM paper was soaked in a solution of the sample (about 10 mg./ml. in the

* Present address: Department of Biophysics, The Johns Hopkins University School of Medicine, Baltimore, Md. 21205, U.S.A.

appropriate electrophoresis buffer) and inserted into a slit in the gel.

Buffers used for starch-gel electrophoresis were: (1) pH 3.8, continuous (LiOH-acetic acid, 0.6N with respect to acetic acid); (2) pH 8.8, discontinuous (gel buffer, 5.0mM-citric acid-76mM - tris-2.0mM - LiOH-7.6mM - H_3BO_3 , pH 8.8; electrode buffer, 0.1N-LiOH-0.38M- H_3BO_3 , pH 8.6). Buffer system (2) is similar to that used by Ferguson & Wallace (1961), but the gel buffer has a slightly higher pH.

After electrophoresis, gels were sliced with a fine wire and stained with 0.0125% (w/v) Nigrosine and 0.0125% (w/v) Naphthalene Black dissolved in methanol-acetic acid-water (5:1:4, by vol.).

Moving-boundary electrophoresis. A Perkin-Elmer model 38 instrument was used at 0-1°.

Ultracentrifuge analysis. All runs were performed on a Spinco model E analytical ultracentrifuge at room temperature at 59780 rev./min.

N-Terminal group determination. N-Terminal groups of growth-hormone preparations were determined by the fluorodinitrobenzene procedure of Sanger (1945). The DNP-amino acid derivatives obtained after hydrolysis of DNP-protein were separated into ether- and water-soluble fractions. The ether-soluble fraction was analysed by thin-layer chromatography (Brenner, Niederwieser & Pataki, 1961) and the water-soluble derivatives were analysed by high-voltage electrophoresis in tanks of the type described by Michl (1951). In some cases excess of dinitrophenol was removed from the ether-soluble fraction by sublimation on to a 'cold finger'. However, evidence was obtained that this procedure may lead to loss of some DNP-amino acids. Fortunately, dinitrophenol, always present after hydrolysis of a DNP-protein, runs well away from most DNP-amino acids in the chromatographic system used, so that the sublimation procedure could be omitted.

Assay of growth hormone. Weight gain in hypophysectomized female rats was used as the response in measuring growth-hormone activity (Marx, Simpson & Evans, 1942).

Female albino (Wistar) rats (150-200g.; from the laboratory colony or from Tucks Ltd.) were hypophysectomized and then left for at least 2 weeks before use. After operation the weights fell sharply and then levelled off; any rats that did not lose weight were discarded as probably having incomplete hypophysectomies.

Solutions of growth hormone for use in the assays were prepared by dissolving a weighed amount of material in 0.9% NaCl made alkaline with a little NaOH. Each solution was then diluted with 0.9% NaCl to 20-30 ml., adjusted to about pH 8.5 with dilute HCl and further diluted with 0.9% NaCl to give the required final concentration. Assay solutions also contained merthiolate (0.01%) as preservative and were kept in the cold room during the course of an assay.

Before beginning injections a preinjection mean weight was determined, for each rat, by weighing on 3 successive days. Rats were then randomly assigned to groups of about five animals each, one group being used per test solution. A 0.5 ml. portion of the assay solution was injected subcutaneously on each of 10 successive days into each rat of a group. The responses in the assay were measured by the increase in weight of each rat during the 10-day period (i.e. weight on the tenth day after the first injection minus the preinjection mean weight).

The assay data were evaluated by the statistical method described by Borth (1960) to cover assays with one standard

and several unknown preparations at one to four dose levels. The statistical validity tests described by this author justified the calculation of approximate fiducial limits.

The laboratory standard (55GH1, a growth-hormone preparation made by a slight modification of the ethanol precipitation method of Wilhelmi *et al.* 1948) was used as a reference preparation in the assays. This 55GH1 standard has been assayed against the International Standard ox growth-hormone preparation (containing 1.0 i.u./mg.), and was found to have a potency of 1.47 i.u./mg. (95% fiducial limits 1.04-2.01; index of precision for assay 0.19).

Crude extracts of ox pituitary glands. Glands were obtained frozen, and 75 glands were conveniently worked up at one time. These were allowed to thaw partially, and the anterior lobes were dissected (giving about 100g. of anterior lobes) and refrozen with solid CO_2 . All subsequent operations were performed in a cold room and solutions were cooled in ice. The anterior lobes were minced, ground with sand and extracted with 200 ml. of 0.25M-NaCl in borate-HCl buffer, pH 8.7 (6.5mM- $Na_2B_4O_7$ -5.5mM-HCl containing 0.03% of toluene). After being stirred for 3 hr. in an ice bath, the extract was spun (at about 7000g for 30 min.) and the supernatant was decanted and kept. The residue was re-extracted with a further 200 ml. of the NaCl solution for 1 hr. After a further centrifuging, the supernatants were combined, frozen and stored overnight. The extract was allowed to thaw and was then spun again (at about 7000g for 30 min.). The fairly-clear red supernatant was used as a crude growth-hormone extract in the subsequent studies.

RESULTS

Procedure for preparation of ox growth hormone, and yields and activities. The crude growth-hormone extract from 75 anterior pituitary lobes was dialysed

Fig. 1. Chromatography of an extract of ox anterior pituitaries on DEAE-cellulose. A column of DEAE-cellulose (55.5 cm. $\times$ 12.9 cm.²) was equilibrated with borate-HCl buffer, pH 8.7 (6.5mM- $Na_2B_4O_7$ -5.5mM-HCl containing 0.03% of toluene). The extract from 108g. of anterior pituitaries (about 400 ml.) was dialysed against the same borate-HCl buffer and applied to the column. Elution was continued with this buffer, and fractions (about 9.0 ml.) were collected. The flow rate was about 100 ml./hr. Subsequently fractions were pooled as indicated (A, B, C and D).

Table 1. *Yields and activities of fractions from the column shown in Fig. 1*

Fractions were injected into hypophysectomized rats daily for 10 days at the dose levels indicated. Estimated potencies are given relative to the laboratory standard (55 GH1). The index of precision for the assay is 0.34.

Fraction tested	Yield (mg.)	Dose/day (μ g.)	No. of rats	Mean wt. gain/loss by tenth day ($\pm$ s.e.m.) (g.)	Differences in mean wt. gain/loss from controls ($\pm$ s.e.m.) (g.)	Estimated potency	95% fiducial limits of potency
Controls	—	0.5 ml. of saline	5	-1.6 ($\pm$ 1.7)			
55 GH1 (laboratory standard)	—	4.7	4	+2.8 ($\pm$ 1.5)	+4.4 ($\pm$ 2.3)		
		15.0	5	+8.4 ($\pm$ 1.7)	+10.0 ($\pm$ 2.4)		
		47.4	4	+13.7 ($\pm$ 1.6)	+15.3 ($\pm$ 2.3)		
		150.0	5	+26.3 ($\pm$ 4.4)	+27.9 ($\pm$ 4.7)		
Fraction A (unretarded)	135.3	15.0	6	+1.8 ($\pm$ 2.8)	+3.4 ($\pm$ 3.3)	Low	
Fraction B	145.3	Not assayed	5	+3.4 ($\pm$ 1.2)	+5.0 ($\pm$ 2.1)		
Fraction C	282.5	15.0	5	+10.7 ($\pm$ 2.4)	+12.3 ($\pm$ 3.0)	1.07	0.57-2.00
		47.4	5	+15.4 ($\pm$ 1.0)	+17.4 ($\pm$ 2.0)		
Fraction D	112.0	15.0	5	+10.3 ($\pm$ 0.85)	+11.9 ($\pm$ 1.9)	1.17	

against five changes of borate-hydrochloric acid buffer (6.5 mm-sodium tetraborate-5.5 mm-hydrochloric acid containing 0.03% of toluene) in the cold room. The dialysed extract (300-400 ml.) was applied to a column of DEAE-cellulose (bed volume 500-700 ml.), equilibrated with the same buffer, and elution was continued with this buffer. The E_{280} of the fractions collected was read. Fig. 1 shows a typical elution pattern. Fractions were pooled as indicated (A, B, C and D), dialysed against water and freeze-dried.

Bioassay (Table 1) showed that the retarded peak (fractions B, C and D in Fig. 1) contained most of the growth-promoting activity, and had a potency similar to that of the laboratory standard. Starch-gel electrophoresis of fractions from the DEAE-cellulose column showed that the main band of Wilhelmi growth hormone (band I in Fig. 3) was heavily present in the retarded (active) peak but not in the unretarded peak. Material from the retarded peak was not electrophoretically homogeneous, but was not markedly more heterogeneous than Wilhelmi growth hormone.

The crude growth hormone (retarded peak from DEAE-cellulose) (500-600 mg.) was dissolved in sodium bicarbonate-carbonate buffer, pH 9.4 (67 mm-sodium hydrogen carbonate-10 mm-sodium carbonate containing 0.03% of toluene) at about 20 mg./ml., and the solution was clarified by centrifuging. It was then applied to a column of Sephadex G-100 (14.4 cm. $\times$ 70-80 cm.) equilibrated with the same buffer. Again, fractions were collected and E_{280} was followed. A typical elution pattern is shown in Fig. 2. Fractions were pooled (A, B and C), dialysed against water and freeze-dried.

Fig. 2. Gel filtration of fractions B, C and D from the column in Fig. 1. A column of Sephadex G-100 (75.7 cm. $\times$ 14.4 cm.²) was equilibrated with NaHCO₃-Na₂CO₃ buffer, pH 9.4 (67 mm-NaHCO₃-10 mm-Na₂CO₃ containing 0.03% of toluene). Fractions B, C and D from the column shown in Fig. 1 were pooled (515 mg.) and dissolved in 30 ml. of this buffer. The solution was applied to the column and eluted with the same buffer. The flow rate was about 55 ml./hr. Subsequently fractions were pooled as indicated (A, B, C and D).

Bioassay (Table 2) showed that the main peak (fraction B in Fig. 2) clearly contained the growth-promoting activity, whereas peaks A and D showed virtually no activity at the dose levels tested. The position of the strongly retarded peak D indicated that it was probably material small enough to pass through dialysis tubing. A part of this peak was

therefore assayed directly (7.5 ml. from the middle of peak *D* was diluted to 30 ml. with 1.2% sodium chloride, the whole was adjusted to pH 8.5 and 0.5 ml. portions were injected daily). Starch-gel electrophoresis of fractions from such Sephadex columns showed that again the band corresponding to the main band of Wilhelmi growth hormone is associated with the peak showing growth-promoting activity (*B* in Fig. 2). The material from peak *B* (eluted at about 0.5 column vol. of effluent) was purified growth hormone.

An alternative final purification step was used in some of the earlier preparations, based on that used by Wallace & Ferguson (1963) in the preparation of sheep growth hormone. The active material produced by chromatography on DEAE-cellulose was dissolved in water at a concentration of about 50 mg./ml., with the help of 2 drops of 0.1 N-sodium hydroxide, and dialysed against water. Much of the protein was precipitated during dialysis, and bioassay showed that this was a highly potent growth-hormone preparation.

Table 3 shows the yields and activities of several ox growth-hormone preparations made by these procedures. Preparations for which the final

purification step was gel filtration are indicated by the letters GF, whereas those for which a precipitation step was used are indicated by P.

Criteria of purity of chromatographically prepared growth hormone. Starch-gel electrophoresis (at pH 8.8) of several growth-hormone preparations made by this chromatographic method is illustrated in Fig. 3. All are more homogeneous than the preparation made by the ethanol precipitation method, though there are still some components that travel faster than the main band (I). The material purified after DEAE-cellulose chromatography by the precipitation method (P3) has more of the faster components (II and III) than that prepared by gel filtration. In none of the preparations made by the chromatographic method has there been any sign of the fast-moving bands (IV and V) that have been shown (Wallis & Kovačić, 1965) to correspond to ox prolactin. Starch-gel electrophoresis in the pH 3.8 system showed a single main band in all the preparations examined, but there was more trailing material in the preparations made by the Wilhelmi method and by the precipitation method.

Boundary electrophoresis of growth hormone

Table 2. *Yields and activities of fractions from the column shown in Fig. 2*

Fractions were injected into hypophysectomized rats daily for 10 days at the dose levels indicated. Estimated potencies are given relative to the laboratory standard (55GH1). The index of precision for the assay is 0.34.

Fraction tested	Yield (mg.)	Dose/day (μg.)	No. of rats	Difference in		Estimated potency	95% fiducial limits of potency
				Mean wt. gain/ loss by tenth day (± S.E.M.) (g.)	mean wt. gain/ loss from controls (± S.E.M.) (g.)		
Controls and laboratory standard	—	As in Table 1 (the results given in Tables 1 and 2 were derived in the same group of assays)					
Fraction A	11	15.0	5	−2.5 (± 1.2)	−0.9 (± 2.0)	Low	1.03–3.15
		47.4	4	+3.0 (± 1.7)	+4.6 (± 2.4)		
Fraction B	351	4.7	4	+6.6 (± 1.7)	+8.2 (± 2.4)	1.82	
		15.0	5	+11.9 (± 2.6)	+13.5 (± 3.1)		
		47.4	6	+19.8 (± 3.6)	+21.4 (± 3.9)		
Fraction C	22	Not assayed					
Fraction D	—	*	4	−2.7 (± 1.7)	−1.1 (± 2.4)	Low	

* The dose of fraction *D* was equivalent to 0.125 ml. from a tube at the peak (see the text).

Table 3. *Yields and activities of chromatographically purified ox growth hormone*

Potencies are given relative to the laboratory standard (55GH1).

Preparation	Yield/100 mg. of anterior pituitaries (mg.)	Potency in growth-hormone assay	95% fiducial limits of potency
Ox GHP3	307	1.54	1.04–2.21
Ox GHP4	444	1.15	0.85–1.57
Ox GHGF2	325	1.82	1.03–3.15
Ox GHGF4	310	1.32	0.95–1.84

Fig. 3. Starch-gel electrophoresis of several ox growth-hormone preparations in the discontinuous buffer system, pH 8.8, for 6 hr. at 250 v. (a) Ox GHGF2; (b) ox GHGF3; (c) ox GHP3; (d) a preparation of ox growth hormone made by Wilhelm's ethanol precipitation method.

(glycine-sodium hydroxide buffer, 0.38M with respect to glycine) showed a single slightly-skewed peak.

Ultracentrifugation showed a single peak in all the samples examined. This was the case in several different buffer solutions: pH 9.4 (glycine-sodium hydroxide buffer, 0.38M with respect to glycine; $S_{20,w}$ 3.1s), pH 4.0 (sodium acetate-acetic acid buffer, 36mM with respect to Na^+ ; $S_{20,w}$ 2.9s) and pH 7.4 (50mM-tris-0.1M-potassium chloride adjusted to pH 7.4 with hydrochloric acid; $S_{20,w}$ 2.9s).

Determination of the *N*-terminal amino acids of several preparations of growth hormone indicated in each case that the chief *N*-terminal amino acids were phenylalanine and alanine (as reported by Li & Ash, 1953) in approximately equal amounts (for one preparation: 0.74 μmole of phenylalanine and 0.76 μmole of alanine/ μmole of growth hormone, assuming a molecular weight of 45000 for the hormone, and correcting for losses on hydrolysis). In every case traces of other amino acids were found, but in only one of the four preparations examined (ox GHGF2) did these amount to more than 5% of the total DNP-amino acids recovered. Where removal of dinitrophenol on a 'cold finger' preceded chromatography of the DNP-amino acids, it was

found that the proportion of DNP-alanine was decreased, probably because some of this DNP-derivative sublimes under the conditions used. The values given above apply to a case where chromatography was performed without prior removal of dinitrophenol.

Rechromatography of a preparation of purified ox growth hormone on DEAE-cellulose with the same buffer as used in the preparative columns (Fig. 1) gave a single, rather asymmetric, retarded peak eluted in the starting buffer. A small peak (10–15%) was eluted by a salt gradient raising the concentration of sodium chloride (dissolved in starting buffer) to 0.1M. A further gradient (to 1.0M-sodium chloride) removed no more protein.

Rechromatography on Sephadex G-100 of material prepared by gel filtration showed a single almost symmetrical peak. This was the case in several different buffers (pH 2.5, 6.7, 9.4 and 11.0; see Table 4). Gel filtration of material purified by the precipitation method also showed a single peak, with possibly a trace of the faster peak seen in the preparative columns (e.g. in Fig. 2).

Molecular weight of ox growth hormone. Andrews (1964) and Whitaker (1963) have shown that it is possible to correlate the degree of retardation of a protein on Sephadex G-75 and G-100 with log (molecular weight). By using a method based on this correlation Andrews & Folley (1963) found that the molecular weights of growth hormones from ox, sheep and pig (measured by gel filtration on Sephadex G-75), were only about half the values previously reported. It was decided to determine molecular weights from the gel-filtration experiments described above for comparison with these results.

Columns of Sephadex G-100, similar to those used for the preparative work (Fig. 2), were equilibrated separately with four different buffers (Table 4). Each was calibrated in the way described by Andrews (1964) with the proteins ribonuclease, chymotrypsin (treated with 100-fold molar excess of di-isopropyl phosphorofluoridate for the columns run at alkaline pH, to inhibit the proteolytic action of the enzyme), horseradish peroxidase and bovine serum albumin. For each buffer the calibration curve [log (molecular weight) plotted against the volume of the eluate at the peak] proved to be a straight line, as expected from the results of previous workers. The slope varied slightly from buffer to buffer.

Table 4 shows the molecular weights calculated from the observed elution patterns of purified ox growth-hormone preparations by using the calibration curves. A value for the molecular weight of the hormone was also calculated from the elution patterns obtained (on calibrated columns) during two preparative runs on Sephadex G-100. The

Table 4. *Molecular weights of ox growth hormone derived from gel-filtration experiments*

Except for the cases marked * samples were applied to the Sephadex G-100 column in 4-5 ml. of buffer; * indicates preparative runs in which crude growth hormone from DEAE-cellulose was applied in 20-30 ml. of buffer.

Buffer solution	Wt. of sample applied (mg.)	Calculated mol. wt.
pH 2.5: 0.1 M-NaH ₂ PO ₄ (final concn.) adjusted to pH 2.5 with H ₃ PO ₄	6	37800
	6	39200
pH 6.7: 0.1 M-Na ₂ HPO ₄ -0.1 M-NaH ₂ PO ₄ containing 0.1% of CHCl ₃	6	33300
pH 9.4: 67 mM-NaHCO ₃ -10 mM-Na ₂ CO ₃ containing 0.03% of toluene or 0.1% of CHCl ₃	5	31200
	15	31000
	500*	34700
	515*	34300
pH 11.0: 0.1 M-Na ₃ PO ₄ (final concn.) adjusted to pH 11.0 with H ₃ PO ₄	7	25700

values obtained here were slightly higher than those obtained from analytical columns in the same buffer (Table 4). The molecular-weight value obtained varied considerably with pH, and appears to decrease as the pH increases. In no buffer, however, was the value obtained as high as that usually accepted for ox growth hormone (evidence for the value of 45 000 is summarized by Li, 1956) or as low as that found by Andrews & Folley (1963).

DISCUSSION

The biological activity of the chromatographically prepared growth hormone has always been higher than that of the laboratory standard 55GH1, although the difference was not always statistically significant (Table 3). Since this standard itself had a potency of about 1.47 i.u./mg. that of the chromatographically purified material was 1.5-2.7 i.u./mg. Dellacha & Sonenberg (1964) have published a modification of the Wilhelmi method for preparation of ox growth hormone that (after a gel-filtration step) gave material of activity 2-3 i.u./mg. These authors showed that their material was homogeneous by ultracentrifugation and boundary electrophoresis, the greatest resolution being achieved at pH 4.0. In the studies reported above, growth hormone prepared by gel filtration (after chromatography on DEAE-cellulose) appeared homogeneous on starch gel at pH 3.8 and in the ultracentrifuge at pH 4.0. The preparation made by the chromatographic method thus appears to be about as active and as homogeneous as that of Dellacha & Sonenberg (1964), although prepared by a simpler method, and obtained in much greater (eight- to ten-fold) yield.

An important feature of the chromatographic preparation is that growth hormone is retarded on the DEAE-cellulose column (Fig. 1), and thus is separated from that protein which passes un-

retarded through the column as well as that (the bulk of the protein in the original extract) which binds firmly to the column. Wallace & Ferguson (1963), chromatographing sheep pituitary extracts on DEAE-cellulose, found that the growth hormone was eluted in a virtually unretarded position and that there were two large retarded inactive protein peaks after that containing the hormone. There has been no indication in the present work of substantial quantities of protein eluted by the starting buffer after the peak of growth hormone.

Retardation of growth hormone on DEAE-cellulose under similar conditions has been noticed during chromatography of hormone made by the ethanol precipitation method (Ferguson & Wallace, 1963; Manchester & Wallis, 1963). These authors also showed that some growth-hormone activity was retained on the columns and could only be removed by increasing salt concentration. It is probable that this is true also of the preparative columns used in the present work, but no attempt has been made to recover growth-promoting material not eluted in the starting buffer. An important factor limiting the resolution that could be achieved on the preparative columns was the very large volume of extract (almost equal to the volume of the columns) that was applied.

The studies on the molecular weight of ox growth hormone by gel filtration support the conclusion of Andrews & Folley (1963) and Andrews (1966) that under some conditions the molecular weight of the hormone is considerably less than the value of 45 000 reported previously. The values obtained in the present study (Table 4) were all higher than that of 20 000 reported by Andrews & Folley (1963) but are fairly close to those reported by Andrews (1966).

There is some evidence from the experiments of Li & Pedersen (1953) that the hormone dissociates in solutions of high pH. A molecular weight of

29 000 was obtained at pH 11.5. At pH 10, although the molecular weight found was 45 000, the dependence of the sedimentation coefficient on the protein concentration indicated that a dissociation-association equilibrium was involved. A similar dependence of the molecular weight on pH has been found in the present experiments. The molecular weight indicated by gel filtration at pH 2.5 was 38 000–39 000, whereas that indicated at pH 11.0 was only about 26 000.

It seems possible therefore that solutions of ox growth hormone show a reversible equilibrium between monomers and dimers (and perhaps higher polymers) of the protein. The conditions used in the gel-filtration procedure may encourage dissociation. Andrews & Folley suggested that this was due to the pH of the buffer used and to the low concentrations of hormone, but the experiments of the present study indicate that at every pH value employed the molecular weight determined by gel filtration is lower than 45 000 (Table 4). Studies reported above showed that the sedimentation coefficient of ox growth hormone determined with low concentrations of the hormone in the buffer used by Andrews & Folley (1963) (the pH 7.4 buffer; $S_{20,w}$ 2.9 s) was very close to the values determined with higher concentrations of the hormone at pH 9.4 and 4.0 ($S_{20,w}$ 3.1 s and 2.9 s respectively), suggesting that the molecular weight of growth hormone in the pH 7.4 buffer was little different from that in the other buffers under these conditions. Thus the gel-filtration process itself may influence the association-dissociation equilibrium in favour of dissociation. A similar situation appears to apply with haemoglobin and lactoglobulin (Whitaker, 1963; Andrews, 1964). Andrews (1964) found that these proteins show markedly higher molecular weights when chromatographed at higher concentrations. The same effect has been observed with ox growth hormone (Table 4), although to a smaller extent.

The lowest molecular-weight value obtained in the present studies was 26 000 (at pH 11.0), which agrees well with the 'monomer molecular weight' derived by Andrews (1966), though rather higher than the value of about 20 000 obtained by Andrews & Folley (1963). Dellacha, Enero & Faiferman (1966) have reported a value of 20 800 from sedimentation and diffusion studies and 19 800 from gel-filtration studies (in both cases a buffer at pH 3.6 was used). If a value in this range of molecular weights (20 000–26 000) represents the smallest subunit of ox growth hormone, then a particle of molecular weight 45 000 would be composed of two such units. The data of Li & Pedersen (1953) suggest that this dimer may be the prevalent form in solution, at least at some pH values and at moderately high concentrations.

Since there is only one residue of each *N*-terminal

amino acid (phenylalanine and alanine) for each particle of molecular weight 45 000, the two subunits in the dimer may represent dissimilar chains. An alternative explanation, proposed by E. Marler, G. J. Ellis & A. E. Wilhelmi (unpublished work cited by Mills, 1965) is that the alanyl and phenylalanyl *N*-terminal residues of ox growth hormone derive from two forms of the hormone, possibly differing only near the *N*-terminus by the loss of a small peptide. In this case the dimer might be composed randomly of similar and dissimilar chains. Studies on the *C*-terminus of ox growth hormone (Santomé, Wolfenstein & Paladini, 1965*a,b*; Wallis, 1966) have shown that both the alanyl and phenylalanyl chains end in phenylalanine, and that the *C*-terminal sequences of both chains may be similar, supporting the idea that the chains themselves are similar.

We are grateful to Miss M. B. Thomas for performing the hypophysectomies and for help with the growth-hormone assays, and to Professor F. G. Young and Dr Anne Stockell Hartree for advice and encouragement. We thank the Medical Research Council for a scholarship to M. W.

REFERENCES

- Andrews, P. (1964). *Biochem. J.* **91**, 222.
- Andrews, P. (1966). *Nature, Lond.*, **209**, 155.
- Andrews, P. & Folley, S. J. (1963). *Biochem. J.* **87**, 3 p.
- Borth, R. (1960). *Acta endocr., Copenhagen*, **35**, 454.
- Brenner, M., Niederwieser, A. & Pataki, G. (1961). *Experientia*, **17**, 145.
- Campbell, J. & Davidson, I. W. F. (1953). *Abstr. Commun. 19th int. Physiological Congress*, p. 257.
- Dellacha, J. M., Enero, M. A. & Faiferman, I. (1966). *Experientia*, **22**, 16.
- Dellacha, J. M. & Sonenberg, M. (1964). *J. biol. Chem.* **239**, 1515.
- Ellis, S. (1961). *Endocrinology*, **69**, 554.
- Ferguson, K. A. & Wallace, A. L. C. (1961). *Nature, Lond.*, **190**, 629.
- Ferguson, K. A. & Wallace, A. L. C. (1963). *Recent Progr. Hormone Res.* **19**, 1.
- Lewis, U. J. (1962). *J. biol. Chem.* **237**, 3141.
- Li, C. H. (1954). *J. biol. Chem.* **211**, 555.
- Li, C. H. (1956). *Advanc. Protein Chem.* **11**, 101.
- Li, C. H. & Ash, L. (1953). *J. biol. Chem.* **203**, 419.
- Li, C. H., Evans, H. M. & Simpson, M. E. (1945). *J. biol. Chem.* **159**, 353.
- Li, C. H. & Pedersen, K. O. (1953). *J. biol. Chem.* **201**, 595.
- Manchester, K. L. & Wallis, M. (1963). *Nature, Lond.*, **200**, 888.
- Marx, W., Simpson, M. E. & Evans, H. M. (1942). *Endocrinology*, **30**, 1.
- Michl, H. (1951). *Mh. Chem.* **82**, 489.
- Mills, J. B. (1965). Ph.D. Dissertation: Emory University.
- Sanger, F. (1945). *Biochem. J.* **39**, 507.
- Santomé, J. A., Wolfenstein, C. & Paladini, A. C. (1965*a*). *Biochem. biophys. Res. Commun.* **20**, 482.

- Santomé, J. A., Wolfenstein, C. & Paladini, A. C. (1965*b*). *Biochim. biophys. Acta*, **111**, 342.
- Smithies, O. (1955). *Biochem. J.* **61**, 629.
- Wallace, A. L. C. & Ferguson, K. A. (1963). *J. Endocrin.* **23**, 285.
- Wallis, M. (1966). *Biochim. biophys. Acta*, **115**, 423.
- Wallis, M. & Kovačić, N. (1965). *J. Endocrin.* **33**, 323.
- Whitaker, J. R. (1963). *Analyt. Chem.* **35**, 1950.
- Wilhelmi, A. E. (1955). In *The Hypophyseal Growth Hormone, Nature and Actions*, p. 59. Ed. by Smith, R. W., Gaebler, O. H. & Long, C. N. H. New York: McGraw-Hill Book Co.
- Wilhelmi, A. E., Fishman, J. B. & Russell, J. A. (1948). *J. biol. Chem.* **176**, 735.