Biochem. J. (1986) 233, 207–213 (Printed in Great Britain)

Purification and partial sequence analysis of insulin-like growth factor-1 from bovine colostrum

Geoffrey L. FRANCIS,* Leanna C. READ,* F. John BALLARD,*‡ Christopher J. BAGLEY,† Faye M. UPTON,† Paula M. GRAVESTOCK† and John C. WALLACE†

*CSIRO (Australia), Division of Human Nutrition, and †Department of Biochemistry, University of Adelaide, Adelaide, South Australia 5000, Australia

1. Growth-promoting activity in bovine colostrum has been detected as the capacity to stimulate protein synthesis in L6 myoblasts. 2. By using this assay as a measure of bioactivity, a growth factor has been purified to near homogeneity from centrifuged colostrum by a series of steps including acid extraction, chromatography on sulphopropyl-Sephadex, followed by adsorption to, and elution from, C_{18} columns using acetonitrile and propan-1-ol gradients. 3. The purified growth factor has a low solubility at neutral and alkaline pH and has an M_r of 7800 by gel-permeation chromatography. 4. Sequence analysis of the first 30 amino acids from the *N*-terminus indicated complete identity in this region with human insulin-like growth factor-1. Accordingly we conclude that the purified growth factor is bovine insulin-like growth factor-1.

INTRODUCTION

Milk or colostrum can support the serum-free proliferation of mammalian cells (Klagsbrun, 1978, 1980; Carpenter, 1980; Steimer & Klagsbrun, 1981; Sereni & Baserga, 1981; Brown & Blakely, 1983; Read *et al.*, 1984). The active components are low-M_r acid-stable proteins that occur at especially high concentrations in colostrum (Klagsbrun, 1978, 1980; Brown & Blakely, 1983). Further characterization has identified: (*a*) epidermal growth factor as the major growth-promoting agent in human milk, at least for fibroblasts (Carpenter, 1980; Shing & Klagsbrun, 1984); (*b*) insulin in bovine colostrum as the key growth factor for H35 and MH_1C_1 hepatoma cells (Ballard *et al.*, 1982); and (*c*) platelet-derived growth factor or a closely related protein as an important mitogen in goat colostrum (Brown & Blakely, 1984). Insulin-like growth factor-1 (IGF-1) is also present at significant concentrations in human milk (Baxter *et al.*, 1984).

In a wide range of normal and transformed cells, bovine colostrum potently stimulates protein synthesis and inhibits protein breakdown, dual anabolic responses associated with the stimulation of cell growth (Ballard, 1982; Ballard *et al.*, 1982). The effects cannot be attributed to insulin or epidermal growth factor, because most of the cell lines tested do not respond to either growth factor at the low concentrations present in bovine colostrum (Ballard *et al.*, 1982; Gunn *et al.*, 1983; Read *et al.*, 1984). Since the identity of the major growth-promoting activity was not known, we elected to use one of these non-selective assays, the stimulation of protein synthesis (Read *et al.*, 1984), for screening purposes during the purification. The studies reported herein have resulted in the purification and characterization of IGF-1 from bovine colostrum.

MATERIALS AND METHODS

Materials

Colostrum was collected before suckling on the day of parturition and was generously provided by Ms. C. Twigger of the Northfield Research Centre, South Australian Department of Agriculture, Mr. C. Altmann, Balhannah, South Australia, Mr. J. Drummond, Woodside, South Australia, and Mr. C. Rothe, Echunga, South Australia. The colostrum was stored at −20 °C. [4,5-^{3}H]Leucine (specific radioactivity 40–60 Ci/mmol) was obtained from Amersham, Sydney, Australia. Foetal-bovine serum was from Commonwealth Serum Laboratories, Parkville, Vic., Australia. Sources for media, antibiotics and the L6 myoblasts have been given previously (Ballard, 1982). Bovine serum albumin was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Multiplication-stimulating activity (rat IGF-2) was kindly provided by Dr. J. Florini, Syracuse University, NY, U.S.A. Human placental membranes, isolated as described by Williams & Turtle (1979), were provided by Dr. R. C. Baxter, Royal Prince Alfred Hospital, Camperdown, N.S.W., Australia. The low-M_r-cut-off dialysis tubing (Spectrapor no. 3) was obtained from Spectrum Medical Industries, Los Angeles, CA, U.S.A.

SP-Sephadex C25 was purchased from Pharmacia Pty. Ltd., North Ryde, N.S.W., Australia,; Sep-Pak C_{18} cartridges and acetonitrile (u.v. cut-off 190 nm) from Waters Associates, Milford, MA, U.S.A.; heptafluorobutyric acid (sequencing grade) from Beckman Instruments, Palo Alto, CA, U.S.A.; trifluoroacetic acid (sequencing grade) from Pierce Chemical Co., Rockford, IL, U.S.A., and propan-1-ol (u.v. cut-off 203 nm) from Burdick and Jackson Laboratories, Muskegon, MI, U.S.A. H.p.l.c. solvents were passed through 0.5 μm-

Abbreviation used: IGF, insulin-like growth factor; SP-, sulphopropyl-.

‡ To whom correspondence and reprint requests should be addressed.

pore-size filters (type FH; Millipore Corp., Bedford, MA, U.S.A.) and degassed before use.

Bioassay for growth-factor activity

Confluent cultures of L6 myoblasts in 24-place multiwell dishes were incubated at 37 °C for 2 h under an atmosphere of CO_2/humidified air (1:19) in Dulbecco-modified Eagle's Minimal Essential Medium with the leucine concentration adjusted to 0.5 mM. The medium in each well was replaced with 950 μl of a similar solution, but containing 1 μCi of [^{3}H]leucine together with 50 μl of test sample diluted in phosphate-buffered saline/albumin [a solution, at pH 7.4, of 10 mM-potassium phosphate, 150 mM-NaCl and 0.1% bovine serum albumin, which was Cohn fraction V previously treated as described by Chen (1967)]. After incubation as described above for a further 17 h, the monolayers were washed at 0 °C, twice with Hanks' salts, twice with 5% trichloroacetic acid over a 10 min period, and once with water before dissolution in 0.5 M-NaOH containing 0.1% Triton X-100. Radioactivity in each extract was expressed as the percentage of that incorporated in the absence of a test sample. Where a quantitative assessment of the ability to stimulate protein synthesis (bioactivity) was required, a series of dilutions of the test sample was made and [^{3}H]leucine incorporation measured in triplicate wells at each dilution. The mean incorporation rate was plotted (see Fig. 1 below) and the volume of test sample required to increase protein labelling by 175% above the control value determined by interpolation. This volume was defined as 1 unit of bioactivity.

Three procedures were used for sample preparation before bioassay or radioreceptor assay. Iso-osmotic samples such as serum, centrifuged colostrum or pure growth factors dissolved in phosphate-buffered saline/albumin were used without modification. All fractions during the purification up to and including the eluate from SP-Sephadex chromatography were dialysed exhaustively against phosphate-buffered saline using Spectrapore no. 3 tubing before bioassay. Fractions from Sep-Pak and h.p.l.c. steps in the purification were freeze-dried to remove the volatile solvents and dissolved first in 10 mM-HCl and diluted as required with phosphate-buffered saline/albumin.

Radioreceptor assays

Details of competitive binding with ^{125}I-labelled rat IGF-2 to L6 myoblasts are given in the second of the two following papers (Ballard *et al.*, 1986), and for the competitive binding with ^{125}I-labelled bovine IGF-1 to human placental membranes in the first of the two following papers (Read *et al.*, 1986).

Protein measurements

Protein in cell samples up to and including the third h.p.l.c. step in the purification protocol was measured as described by Lowry *et al.* (1951), with crystalline bovine serum albumin as standard. Since scarcity of material prevented the use of this method at the final h.p.l.c. step, protein was measured by its A_{280}, porcine insulin being used as standard.

Protein sequencing

Proteins were sequenced by using the Applied Biosystems model 470A gas-phase sequencer and the procedures given by the manufacturers and described by Hunkapiller *et al.* (1983). For some analyses the proteins were *S*-carboxymethylated before sequencing. This was accomplished by dissolving freeze-dried protein in 50 μl of 10 mM-HCl and adding 100 μl of 6 M-guanidinium chloride in 0.1 M-Tris at pH 7.3, followed by 10 μl of 0.1 M-dithiothreitol. The solution was then incubated at 85 °C for 10 min, cooled to 20 °C, and 30 μl of 0.25 M-sodium iodoacetate in the above guanidinium chloride/Tris buffer added. After the solution had been left in the dark for 2 h, 10 μl of trifluoroacetic acid was added and the solution applied to a 2.1 mm × 30 mm C_{18} column equilibrated with 0.1% trifluoroacetic acid and eluted with a 0–60% (v/v) acetonitrile gradient in 0.1% trifluoroacetic acid. The reduced and carboxymethylated protein was detected by A_{215} (Hunkapiller *et al.*, 1983).

M_r measurements

Each protein sample dissolved in 40% (v/v) acetonitrile in 0.1% trifluoroacetic acid was applied in a volume of 150 μl to a TSK G3000 SW column (LKB) of dimensions 7.5 mm × 600 mm, equipped with a 7.5 mm × 75 mm guard column and equilibrated with the same solution. The flow rate was 0.4 ml/min, with absorbance measured at 210 nm. If required, 0.4 ml fractions were collected for measurement of growth-factor activity.

RESULTS

The purification described below is that carried out with a single batch of colostrum collected from cow A1.

Acid extraction

Approx. 2.5 litres of colostrum were centrifuged at 20000 *g* for 30 min at 5 °C. The infranatant (2.3 litres) was adjusted to pH 2.8 with acetic acid over a period of 1 h while it was stirred vigorously at 2 °C. Stirring was continued overnight at 2 °C, during which the viscous mixture became considerably more fluid. Centrifugation at 20000 *g* for 30 min at 5 °C yielded an opalescent extract (1.5 litres). Extraction of the large amount of pelleted protein with 1 M-acetic acid, although yielding additional growth-promoting material, was not routinely adopted because the specific bioactivity in the second extract was substantially lower than in the primary extract. In most purifications the primary acid extract resulted in the recovery of 60–80% of the bioactivity accompanied by removal of 90–95% of the protein. However, in the example given in Table 1, the bioactivity recovered was apparently very low and is not reported in Table 1. Presumably this situation was an artefact of loss of material in the sample taken for dialysis and assay, because later steps in the purification indicated amounts of bioactivity that were typical of other purifications.

Chromatography on SP-Sephadex

A 100 g portion of SP-Sephadex C-25 was hydrated with water at 95 °C for 2 h and converted into the hydrogen form by treatment with acetic acid at pH 2.5. The pH 2.8 colostrum extract was added to the gel layer and the mixture stirred overnight at 2 °C. The slurry was poured into a Pharmacia K50 column (5 cm diameter) at 2 °C, allowed to settle for 20 min and packed at a flow rate of 8 ml/min. The gel bed was washed with 0.5 litre of 1 M-acetic acid and subsequently with 50 mM-ammonium acetate, pH 5.5, at a flow rate of 4 ml/min until the A_{280}

Table 1. Purification of IGF-1 from bovine colostrum

The values given are from a single purification protocol from colostrum A1.

Step	Volume (ml)	Protein (mg)	10^{-3} × Bio-activity units	Specific activity (units/mg)	Recovery (%)
Centrifuged colostrum	2300	299×10^3	1050	3.5	100
pH 2.8 extract	1500	12.1×10^3	N.M.*	N.M.	N.M.
SP-Sephadex eluate	3950	2.45×10^3	(265)	108	25
Sep-Pak eluate	20	950	345	365	33
H.p.l.c					
I	6.0	105	41.6	395	4.6
II	3.0	16.1	22.3	1375	3.2
III	3.0	2.23	14.3	6410	2.3
IV Pool 1	3.0	0.300	0.29	970	< 0.1
Pool 2	2.0	0.123	2.11	17150	0.4
Pool 3	4.0	0.176	3.22	18300	0.6

* N.M., activity not measured; see the text.

(5 mm light path) was below 0.05. For the purification in Table 1 this required 6.6 litres of pH 5.5 buffer.

Elution of bioactivity was achieved with a 500 ml linear gradient from the pH 5.5 buffer to 0.25 M-NH_3 containing 0.5 M-NaCl, followed by a further 3.75 litres of the latter solution. The volume of eluate obtained with significant absorbance at 280 nm was 3.95 litres. Although this step typically results in an increase in specific activity of 5–7-fold and a yield of 50–70%, the illustrated example of colostrum A1 purification (Table 1) was somewhat less effective. Bioassays of the SP-Sephadex pool are shown in Fig. 1.

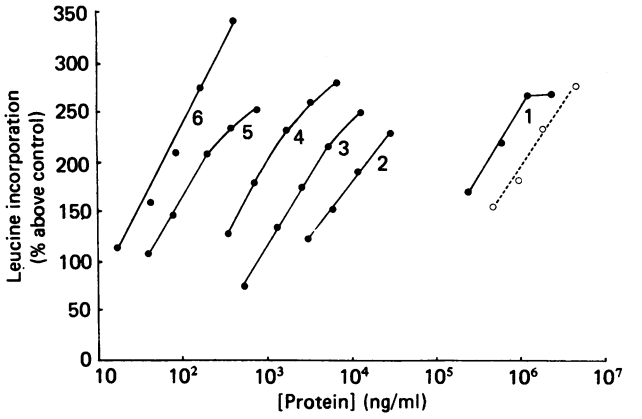

Fig. 1. Bioassays at different stages in the purification of growth factor from bovine colostrum (see Table 1)

Values plotted are the percentage stimulation of protein synthesis above that occurring in control wells. The curves indicated by closed symbols are, respectively: 1, centrifuged colostrum A1; 2, pooled fractions from SP-Sephadex chromatography; 3, the Sep-Pak eluate; 4, pooled fractions from the second h.p.l.c. step; 5, pooled fractions from the third h.p.l.c. step; 6, pool 3 from the fourth h.p.l.c. step. The bioactivity of foetal-bovine serum is indicated by open symbols.

Concentration on Sep-Pak C_{18} cartridges

This and all subsequent steps were carried out at 22 °C. The eluate from SP-Sephadex chromatography was adjusted to 0.1% with trifluoroacetic acid and the pH to 2.1 with concentrated HCl. This mixture was stirred for 15 h and the fine precipitate removed by centrifugation at 20000 *g* for 30 min. The supernatant was pumped through a series of ten Sep-Pak cartridges previously equilibrated with 0.1% trifluoroacetic acid by using a flow rate of 1 ml/min. The cartridge series was washed with 0.1% trifluoroacetic acid and eluted with 50% (v/v) of acetonitrile in 0.1% trifluoroacetic acid. The eluate (20 ml) retained 33% of the bioactivity in the starting colostrum (Fig. 1), together with 950 mg of protein, and represented a cumulative purification of 100-fold (Table 1). An additional 10–15% of the total colostrum bioactivity could be recovered from the solution passed through the Sep-Pak cartridges by passage through, and elution from, a further series of cartridges. This rechromatography was not used in the protocol shown in Table 1.

First h.p.l.c. step

The Sep-Pak eluate was diluted with 14.5 ml of 0.13% heptafluorobutyric acid to decrease the acetonitrile concentration to 29% (v/v), and 31.5 ml was injected on to Waters μ-Bondapak C_{18} column (7.8 mm × 300 mm), previously equilibrated with the same solution. Protein was eluted at a flow rate of 1 ml/min, with a 20 min linear gradient to 38% (v/v) acetonitrile followed by a 120 min linear gradient to 50% (v/v) acetonitrile, a 40 min linear gradient to 62% (v/v) acetonitrile and finally with a 40 min linear gradient to 65% (v/v) acetonitrile. The heptafluorobutyric acid concentration was 0.13% throughout. Protein elution was monitored at 280 nm and 2 min fractions were collected. The profiles of eluted absorbance and bioactivity are shown in Fig. 2(*a*). The recovery in fractions 14–16 was 105 mg of protein and 41.6×10^3 units of bioactivity, representing 12% and 13% respectively, and resulted in only a small increase in specific bioactivity.

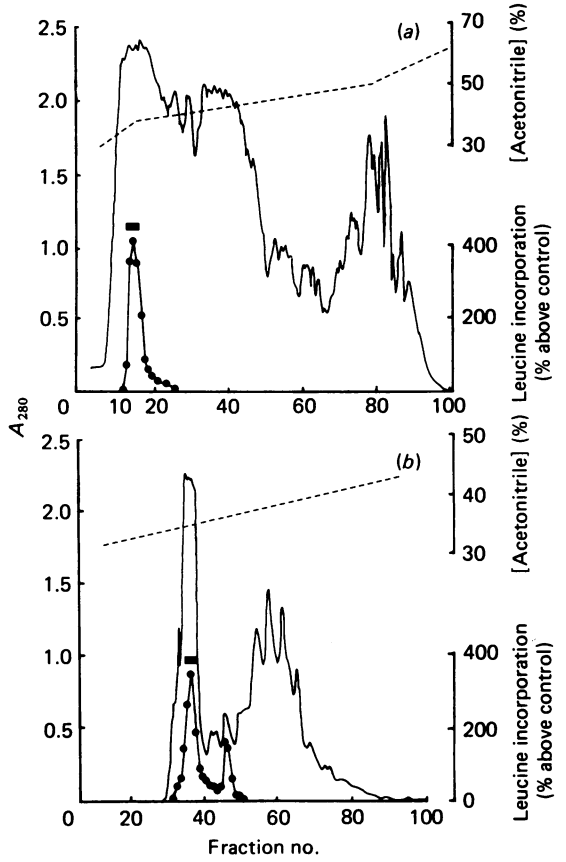

Fig. 2. Elution profiles at (*a*) the first and (*b*) the second h.p.l.c. steps

●, Bioactivity in 1 μl portions of eluate (% above control); ——, A_{280}; – – – –, acetonitrile gradients. Fractions pooled for subsequent purification steps are indicated by the solid bars.

The extremely poor recovery of bioactivity at the first h.p.l.c. step is reproducible. We considered that this situation could occur either because of chemical modification of the growth factor or because two or more components that act synergistically to stimulate protein synthesis were separated on the column. The second of these possibilities seemed more likely, because losses were very low at the Sep-Pak concentration step, even though this involved rather similar conditions. Moreover, if colostrum did contain several factors that produced a synergistic effect on bioactivity, the h.p.l.c. step is the first one in the purification that could be expected to result in the separation from each other of low-M_r acid-stable proteins with isoelectric points above 6. Three approaches were adopted to test the likelihood of this situation. First, the activity in the material loaded onto the column was compared with a pool obtained by recombining 25 μl portions of each eluted fraction. The amounts taken were calculated on the basis that the true bioactivity would be spread evenly throughout all fractions. The two solutions were freeze-dried and the proteins dissolved in 50 μl of 10 mM-HCl and diluted to 500 μl with phosphate-buffered saline/albumin. Measurement of protein synthesis-stimulating activities over 40-fold dilutions of the load and the recombination pool indicated approx. 50% recovery.

A second attempt to account for the low recovery in fractions 14–16 involved the addition of portions of this pool to equal volumes of other fractions. Bioactivity measurements gave results commensurate with the sum of each activity, except for fractions 69–87. When material from these fractions was added to an amount of the fraction 14–16 pool that itself produced only a small increase in protein synthesis, the resultant bioactivity was substantially higher than expected. Presumably this result reflects a synergistic stimulation of protein synthesis by one or more components in fractions 69–87 acting with the active substance in fractions 14–16.

The third test of recovery involved a radioreceptor assay using ^{125}I-labelled IGF-1 prepared from the final purification step (see below). Portions of the sample loaded on to the first h.p.l.c. column and the combined fractions 14–16 were freeze-dried, dissolved as above and added to human placental membranes, together with labelled IGF-1. Binding of radioactivity was measured over a wide concentration range, as described in the following paper (Read *et al.*, 1986). Parallel competition curves showed that the 31.5 ml loaded on to the h.p.l.c. column contained 2.54 mg of bovine IGF, whereas 1.31 mg or 52% was recovered in fractions 14–16. This and the previous two experiments show that the low recovery of bioactivity in the peak fractions is not associated with comparable losses of IGF, but rather is consistent with the separation of bioactivity into forms that act synergistically.

Second h.p.l.c. step

The pool of fractions 14–16 (5.5 ml) was diluted with 2 ml of 0.1% trifluoroacetic acid, and 5.7 ml was injected on to the same C_{18} column that was used for the first h.p.l.c. step, except that it had previously been equilibrated with 30.8% (v/v) acetonitrile in 0.1% trifluoroacetic acid. The flow rate was 1 ml/min during loading and 0.5 ml/min during elution. A linear gradient to 42.8% (v/v) acetonitrile in 0.1% trifluoroacetic acid was applied over 160 min, and the absorbance measured at 280 nm (Fig. 2*b*). Most of the bioactivity eluted at an acetonitrile concentration of 34.5%, with a further small peak at 36%. The bioactivity in the second peak, although also seen during the purification of growth factors from other batches of colostrum, has not been further investigated, nor has it been taken into account in the calculations of recovered activity. The second h.p.l.c. produced a 3–4-fold increase in specific activity and a recovery of 70% (Fig. 1, Table 1).

Third h.p.l.c. step

The pooled fractions (35–37, 3.0 ml) were diluted with 1.5 ml of 0.1% trifluoroacetic acid, and 4 ml was applied to an LKB LiChrosorb C_{18} column (5 μm particle size; 4 mm × 250 mm) previously equilibrated with 10% (v/v) propan-1-ol in 0.1% trifluoroacetic acid. The elution program at a flow rate of 0.5 ml/min involved a 7 min linear gradient to 17% (v/v) propan-1-ol followed by a 28 min linear gradient to 18.4% (v/v) propan-1-ol, an 85 min linear gradient to 20.5% (v/v) propan-1-ol and, finally, a 45 min gradient to 45% (v/v) propan-1-ol. The trifluoroacetic acid concentration was 0.1% throughout. The eluate was monitored at 280 nm (Fig. 3*a*) and portions of each fraction taken for freeze-drying and assay. The activity eluted at approx. 18% propan-1-ol in a single peak associated with the beginning of the protein

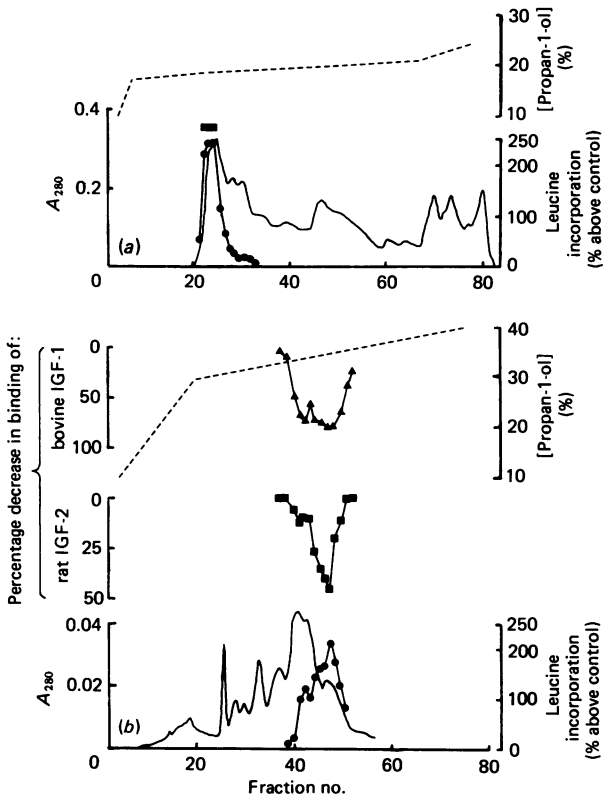

Fig. 3. Elution profiles at (*a*) the third and (*b*) the fourth h.p.l.c. steps

Details are as described in the legend to Fig. 2, except that the broken lines represent propan-1-ol gradients, the percentage decrease in ^{125}I-labelled-rat IGF-2 binding to L6 myoblasts in 4.5 μl portions of each gradient is indicated by squares and the percentage decrease in ^{125}I-labelled-bovine-IGF-1 binding to human placental membranes in 1.8 μl portions of each fraction is indicated by triangles.

profile. Fractions 22–24 (3 ml) contained 70% of the applied bioactivity and represented a 4.7-fold purification (see Fig. 1, Table 1).

Fourth h.p.l.c. step

The pooled fractions were diluted to 4.3 ml with 1.3 ml of 0.13% heptafluorobutyric acid and 4.1 ml was injected on to the same column used for the third h.p.l.c. step, but equilibrated with 10% (v/v) propan-1-ol in 0.13% heptafluorobutyric acid. The flow rate during elution was 0.5 ml/min and the program included a 29 min linear gradient to 29.6% (v/v) propan-1-ol followed by a 119 min linear gradient to 41.5% (v/v) propan-1-ol with the counter-ion maintained at 0.13%. The absorbance was monitored at 280 nm, and portions of each fraction freeze-dried for bioassay and radioreceptor assays (Fig. 3*b*). The protein-synthesis bioassay showed a broad region of activity from fractions 41 to 50, with peaks apparently at fractions 43 and 47. It should be stressed that the assay performed in this manner when only a single volume from each fraction tested is only semi-quantitative.

A radioreceptor assay with ^{125}I-labelled bovine IGF-1 using human placental membranes demonstrated a peak of activity in fraction 47, but again the activity was distributed over ten fractions (Fig. 3*b*). The pattern was very similar when fractions were screened by using a radioreceptor assay that measured competition with ^{125}I-labelled rIGF-2 binding to L6 myoblasts (Fig. 3*b*).

On the basis of the three measurements, the active fractions were combined into pool 1 (fractions 41–43), pool 2 (fractions 44 and 45) and pool 3 (fractions 46–49). The bioactivity in each of these pools was measured over a wide concentration range and is given in Table 1. The resultant curve for pool 3 is shown in Fig. 1 and indicates a 2.9-fold purification over the material loaded on to the fourth h.p.l.c. column. The corresponding purification factor for pool 2 is 2.7-fold.

Amino acid sequence of bovine growth factor

The sequence of the purified growth factor after *S*-carboxymethylation has been determined between the *N*-terminus and residue 30:

Gly-Pro-Glu-Thr-Leu-Cys-Gly-Ala-Glu-Leu-Val-Asp-
Ala-Leu-Gln-Phe-Val-Cys-Gly-Asp-Arg-Gly-Phe-Tyr-
Phe-Asn-Lys-Pro-Thr-Gly-

Comparison of these results with the reported sequences for human IGF-1 (Rinderknecht & Humbel, 1978) and rat IGF-1 (Rubin *et al.*, 1982) indicates no differences between the three growth factors. Clearly, the purified growth factor is bovine IGF-1.

M_r determination

The M_r of the growth factor was estimated by gel-permeation chromatography of a sample from a different purification (A2) from that reported in Table 1 (Fig. 4). A peak of material absorbing at 210 nm was detected at a mean elution volume of 17.9 ml, giving M_r of 7800 by reference to the elution volumes of ribonuclease A, lima-bean (*Phaseolus limensis*) trypsin inhibitor, mouse epidermal growth factor, insulin and insulin B chain (Fig. 4).

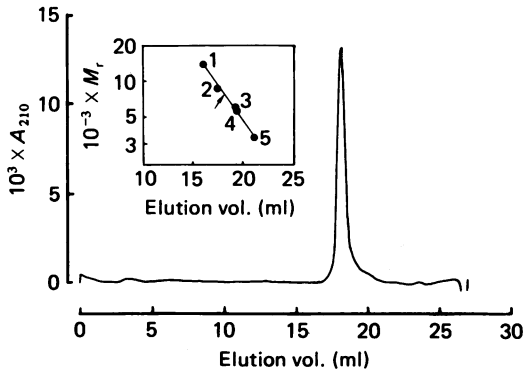

Fig. 4. Gel-permation chromatography of bovine IGF-1

The elution profile (A_{210}) of a 20 μl portion of pool-3 bovine IGF-1 (h.p.l.c. step 4, purification from colostrum A2) applied in 150 μl of 40% (v/v) acetonitrile containing 0.1% trifluoroacetic acid to a TSK G3000 SW column equilibrated and eluted with the same solution. The elution volumes of (1) pancreatic ribonuclease A, (2) lima-bean trypsin inhibitor, (3) mouse epidermal growth factor, (4) insulin and (5) insulin B-chain with M_r values respectively of 13700, 8400, 6000, 5700 and 3400 are plotted as an inset.

The absorbing material was essentially pure, as evidenced by peak shape. Moreover, bioactivity measurements confirmed that the growth factor was eluted at the same time as the peak absorbance. A comparable analysis of pool 3 from the purification described in Table 1 gave a similar M_r but with approx. 20% of the 210 nm-absorbing material appearing as shoulders before and after the major peak.

DISCUSSION

The major growth factor in bovine colostrum, assessed by its ability to stimulate protein synthesis in rat L6 myoblasts, has been purified to near homogeneity and identified by partial sequence analysis as IGF-1. Our initial attempts at purification utilized Sephadex G-100 chromatography under acid conditions as the first step (Francis *et al.*, 1982; Read *et al.*, 1983). This procedure had the advantage that the growth-promoting activity was separated from a high-M_r component that bound the bioactivity at neutral pH, but the disadvantage of being slow and unsuitable for large-scale application. Nevertheless, M_r fractionation in acid was required to remove binding protein, a situation comparable with that found for IGF in serum (Rinderknecht & Humbel, 1976; Rubin *et al.*, 1982). The acid-extraction method finally adopted as the first step in the purification of the bovine growth factor gave satisfactory enrichment of bioactivity and was suitable for the processing of several litres of colostrum.

After absorption of the extracted bioactivity to SP-Sephadex, elution was accomplished by a combination of high salt and very high pH, but, even under such conditions, the bioactivity was eluted in an extremely broad peak. Although gradual desorption may reflect the presence of several growth factors, each with a different alkaline pI, an additional explanation is that the growth factor is poorly soluble in aqueous solutions at neutral and alkaline pH. Indeed, bioactivity can be precipitated at later stages in the purification procedure by neutralizing the acid solutions used as h.p.l.c. solvents. This insolubility of the bioactivity contrasts with reports of the purification of human IGF-1 (Svoboda *et al.*, 1980), as well as rat IGF-1 and IGF-2 (Moses *et al.*, 1980; Marquardt *et al.*, 1981; Rubin *et al.*, 1982). In each of the above protocols, satisfactory separation was achieved on isoelectric-focusing gels. However, we found that the bovine bioactivity did not migrate on isoelectric-focusing gels above pH 4, owing to precipitation, unless high concentrations of urea were present. This property also applied to the purified bovine IGF-1. Since we found very poor recovery of bioactivity in steps subsequent to isoelectric focusing in urea, the technique could not be used for the purification of bovine IGF. As yet we have no explanation for the low relative solubility of the bovine form of IGF-1 at neutral or alkaline pH.

We have noted some variability in the elution pattern of bioactivity at the fourth h.p.l.c. step. Whereas for the purification illustrated in Fig. 3(*b*) the highest specific activity occurred in pool 3 and the lowest in pool 1, other purifications produced much higher specific activities for protein eluted at the same propan-1-ol concentration as pool 1. It seems unlikely that the broad region of bioactivity including pools 1, 2 and 3 reflects a variable proportion of IGF-2 at the fourth h.p.l.c. step, because the bioactivity pattern matches well with both the radioreceptor assay using human placental membranes and labelled IGF-1 as the radioligand and the radioreceptor assay with L6 myoblasts and IGF-2 as radioligand (Fig. 3*b*). These measurements are selective respectively for IGF-1 and IGF-2 [see the accompanying papers (Read *et al.*, 1986; Ballard *et al.*, 1986)], so that a significant amount of IGF-2 in one pool of the preparation would be readily detected. Moreover, isoelectric focusing of iodinated h.p.l.c.-step 4 material in the presence of urea gave no evidence of radioactive bands at the neutral pH expected for IGF-2 (results not shown).

A more likely explanation of the distribution of bioactivity between peaks 1, 2 and 3 is that two or more variants of IGF-1 are present. Partial sequence analysis has been carried out on material that is eluted at the same position as pool 1, but with a specific bioactivity equivalent to pool 3 in the purification summarized in Table 1. As indicated in the Results section, an *N*-terminal sequence identical with that of human IGF-1 was obtained (Rinderknecht & Humbel, 1978; Klapper *et al.*, 1983). The actual pool-3 sample from the protocol in Table 1 had the same *N*-terminal sequence, except for the omission of the *N*-terminal tripeptide Gly-Pro-Glu. Indeed, in another purification, where protein eluted in the regions of pool 1 and pool 3 had similar bioactivities, the pool-1 protein sequenced as complete IGF-1, whereas the pool-3 protein again lacked the *N*-terminal tripeptide. We conclude, therefore, that the broad region of bioactivity from pool 1 and pool 3 contained complete IGF-1 together with proteolytically modified material. Model-building studies by Blundell *et al.* (1983) predict that the *N*-terminal peptide is exposed, as would be expected if it can be removed by proteinases. Further evidence for proteolysis in the colostrum comes from the existence of α_{S2}-casein fragments in the purified IGF-1. These were detected as minor contaminants in three samples used for sequence analysis and differed from each other owing to the possession of *N*-termini at positions 166, 168 and 174 (Brignon *et al.*, 1977) as identified by reference to the NIH/NCI protein database.

We have compared the pool-3 material (Table 1) with purified human IGF-1, human IGF-2 and rat IGF-2 in a number of radioreceptor assays, radioimmunoassays and biological-responsiveness tests and report these results in the accompanying papers (Read *et al.*, 1986; Ballard *et al.*, 1986). In each test the bovine IGF-1 is much more similar in reactivity to human IGF-1 than to either of the IGF-2 preparations.

If allowance is made for the purity of pool-3 bovine IGF-1 being 60–70%, an estimate based on the minor contaminants detected by gel-permeation chromatography (Fig. 4) and the α_{S2}-casein fragment identified during sequence analysis, we find that bovine IGF-1 and human IGF-1: (*a*) have similar reactivity towards the anti-(human IGF-1) monoclonal antibody; (*b*) have similar reactivity towards type-1 IGF receptors, as noted in the human-placental-membrane assay; (*c*) differ in the polyclonal immunoassay by the human IGF-1 being 3–5-fold more potent; (*d*) differ in the type-2 IGF receptor assays (sheep placental membranes, foetal-human liver, L6 myoblasts, plasma membranes with IGF-2 as radioligand) by the bovine IGF-1 being twice as potent, and (*e*) differ in ability to evoke biological responses by the bovine IGF-1 being somewhat more potent.

The difference in reactivity towards the type-2 IGF

receptor could be explained if the bovine IGF contained a small contaminant of IGF-2, but evidence against this has been outlined above. It seems more probable that true differences occur between the two IGF-1 molecules, as also is indicated by the unusually low solubility of the bovine form at neutral or alkaline pH. The extent to which the differences relate to amino acid substitutions at positions beyond those sequenced here or to the deletion of the *N*-terminal tripeptide must await further study.

The placental-membrane-receptor assay indicated that 2.5 mg of IGF-1 was loaded on to the first h.p.l.c. column. Even if this represented a quantitative recovery of IGF-1 from colostrum, it reflects a concentration above 1 μg/ml in the starting material. In contrast, Baxter *et al.* (1984) have reported only 20 ng of IGF-1/ml in day-1 human colostrum. The opposite situation applies to epidermal growth factor, which is present in human colostrum at approx. 300 ng/ml (Read *et al.*, 1984; Beardmore *et al.*, 1983), but at no more than 5% of this concentration in bovine colostrum (Shing & Klagsbrun, 1984; Read *et al.*, 1984). Whether the altered pattern of growth-factor content in colostrum reflects species-specific differences in their site of synthesis has not been determined. Further analysis of the spectrum of growth factors in colostrum and milk, together with measurements of mammary-gland concentration or synthesis of growth factors, can be expected to help resolve the biological importance to the newborn animal of milk-derived IGF, epidermal growth factor or other growth factors.

We thank Mr. Mark Snoswell and Ms Joan Rowe for carrying out the sequence analyses. Support for this work was provided by the National Health and Medical Research Council of Australia, the Reserve Bank Rural Credits Development Fund, the Clive and Vera Ramaciotti Foundations and the J. S. Davies Bequest to the University of Adelaide.

REFERENCES

Ballard, F. J. (1982) Biochem. J. **208**, 275–287
Ballard, F. J., Nield, M. K., Francis, G. L., Dahlenburg, G. W. & Wallace, J. C. (1982) J. Cell Physiol. **110**, 249–254.
Ballard, F. J., Read, L. C., Francis, G. L., Bagley, C. J. & Wallace, J. C. (1986) Biochem. J. **233**, 223–230
Baxter, R. C., Zaltsman, Z. & Turtle, J. R. (1984) J. Clin. Endocrinol. Metab. **58**, 955–959
Beardmore, J. M., Lewis-Jones, D. I. & Richards, R. C. (1983) Pediatr. Res. **17**, 825–828
Blundell, T. L., Bedarkar, S. & Humbel, R. E. (1983) Fed. Proc. Fed. Am. Soc. Exp. Biol. **42**, 2592–2597
Brignon, C., Ribadeau-Dumas, B., Mercier, J.-C. & Pelissier, J.-P. (1977) FEBS Lett. **76**, 274–279.
Brown, K. D. & Blakely, D. M. (1983) Br. Vet. J. **139**, 68–78
Brown, K. D. & Blakely, D. M. (1984) Biochem. J. **219**, 609–617
Carpenter, G. (1980) Science **210**, 198–199
Chen, R. F. (1967) J. Biol. Chem. **242**, 173–181.
Francis, G. L., Read, L. C. & Ballard, F. J. (1982) Proc. Int. Congr. Biochem. 12th, 104
Gunn J. M., Bodner, J. B., Knowles, S. E. & Ballard, F. J. (1983) Biochem. J. **210**, 251–258
Hunkapiller, M. W., Hewick, R. M., Dreyer, W. J. & Hood, L. E. (1983) Methods Enzymol. **91**, 399–413
Klagsbrun, M. (1978) Proc. Natl. Acad. Sci. U.S.A. **75**, 5057–5081
Klagsbrun, M. (1980) J. Cell Biol. **84**, 808–814
Klapper, D. G., Svoboda, M. E. & Van Wyk, J. J. (1983) Endocrinology (Baltimore) **112**, 2215–2217
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) J. Biol. Chem. **193**, 265–275
Marquardt, H., Todaro, G. J., Henderson, L. E. & Oroszlan, S. (1981) J. Biol. Chem. **256**, 6859–6865
Moses, A. C., Nissley, S. P., Short, P. A., Rechler, M. M. & Podskalny, J. M. (1980) Eur. J. Biochem. **103**, 307–400
Read, L. C., Francis, G. L., Wallace, J. C. & Ballard, F. J. (1983) Proc. Aust. Biochem. Soc. **15**, 38
Read, L. C., Upton, F. M., Francis, G. L., Wallace, J. C., Dahlenburg, G. W. & Ballard, F. J. (1984) Pediatr. Res. **18**, 133–139
Read, L. C., Ballard, F. J., Francis, G. L., Baxter, R. C., Bagley, C. J. & Wallace, J. C. (1986) Biochem J. **233**, 215–221
Rinderknecht, E. & Humbel, R. E. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2365–2369
Rinderknecht, E. & Humbel, R. E. (1978) J. Biol. Chem. **253**, 2769–2770
Rubin, J. S., Mariz, I., Jacobs, J. W., Daughaday, W. H. & Bradshaw, R. A. (1982) Endocrinology **110**, 734–740
Sereni, A. & Baserga, R. (1981) Cell. Biol. Int. Rep. **5**, 339–345
Shing, J. W. & Klagsbrun, M. (1984) Endocrinology, **115**, 273–282
Steimer, K. S. & Klagsbrun, M. (1981) J. Cell. Biol. **88**, 294–300
Svoboda, M. E., Van Wyk, J. J., Klapper, D. G., Fellows, R. E., Grissom, F. E. & Schlueter, R. J. (1980) Biochemistry **19**, 790–797
Williams, P. F. & Turtle, J. R. (1979) Biochim. Biophys. Acta **579**, 367–374

Received 19 April 1985/9 July 1985; accepted 10 September 1985