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

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INTRODUCTION

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 sulphotropyl-Sepharose, followed by adsorption to, and elution from, C18 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 Mr 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.

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. [4,5-3H]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-Mr-cut-off dialysis tubing (Spectrapor no. 3) was obtained from Spectrum Medical Industries, Los Angeles, CA, U.S.A.

SP-Sepharose C25 was purchased from Pharmacia Pty. Ltd., North Ryde, N.S.W., Australia; Sep-Pak C18 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-, sulphotropl-
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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 CO₂/humidified air (1:19) in Dulbeccomodified
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 [³H]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. Radio-
activity 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 [³H]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 ex-
haustively 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 ¹²⁵I-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 ¹²⁵I-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 A₂₈₀, 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
TSK SW G3000 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 A₂₈₀
(Hunkapiller et al., 1983).

M₄ 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)
is 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 hydro-
gen 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 A₂₈₀

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Table 1. Purification of IGF-1 from bovine colostrum

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

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>10⁻³ × Bio-activity units</th>
<th>Specific activity (units/mg)</th>
<th>Recovery (%)</th>
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<tr>
<td>Centrifuged</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>colostrum pH 2.8 extract</td>
<td>2300</td>
<td>299 × 10⁶</td>
<td>1050</td>
<td>200</td>
<td>3.5</td>
</tr>
<tr>
<td>SP-Sephadex eluate</td>
<td>1500</td>
<td>12.1 × 10⁶</td>
<td>N.M.*</td>
<td>N.M.</td>
<td>N.M.</td>
</tr>
<tr>
<td>Sep-Pak eluate</td>
<td>3950</td>
<td>2.45 × 10⁶</td>
<td>(265)</td>
<td>108</td>
<td>25</td>
</tr>
<tr>
<td>H.p.l.c</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>6.0</td>
<td>105</td>
<td>41.6</td>
<td>395</td>
<td>4.6</td>
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<tr>
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<td>3.0</td>
<td>16.1</td>
<td>22.3</td>
<td>1375</td>
<td>3.2</td>
</tr>
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<td>6410</td>
<td>2.3</td>
</tr>
<tr>
<td>IV Pool 1</td>
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<td>0.300</td>
<td>0.29</td>
<td>970</td>
<td>&lt; 0.1</td>
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<tr>
<td>Pool 2</td>
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<td>2.11</td>
<td>17150</td>
<td>0.4</td>
</tr>
<tr>
<td>Pool 3</td>
<td>4.0</td>
<td>0.176</td>
<td>3.22</td>
<td>18300</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* 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-NH₄ 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.

Concentration on Sep-Pak C₁₈ 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% heptanfluorobutyric acid to decrease the acetonitrile concentration to 29% (v/v), and 31.5 ml was injected on to Waters µ-Bondapak C₁₈ 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 heptanfluorobutyric 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 × 10⁶ units of bioactivity, representing 12% and 13% respectively, and resulted in only a small increase in specific bioactivity.

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.

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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. 2b). 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 x 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 elute was monitored at 280 nm (Fig. 3a) 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 recovery.

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.

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**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.
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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. 3b). 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 ¹²⁵I-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. 3b). The pattern was very similar when fractions were screened by using a radioreceptor assay that measured competition with ¹²⁵I-labelled rIGF-2 binding to L6 myoblasts (Fig. 3b).

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-Pro-Thr-Gly-

Comparison of these results with the reported sequences for human IGF-1 (Rinderknecht & Hummel, 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.

Mr determination

The Mr 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 Mr 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-permeation chromatography of bovine IGF-1

The elution profile (A₂₁₀) 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 Mr 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 pH, 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. 3b). 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 $\alpha_{3\alpha}$-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 $\alpha_{3\alpha}$-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.

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