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Biochemistry

Platelet-derived growth factor: Purification and partial characterization

(sodium dodecyl sulfate gel electrophoresis/hydrophobic chromatography/radioiodination/cultured cells)

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ABSTRACT A cationic protein that stimulates DNA synthesis in human cultured cells was isolated from human platelets by ion exchange chromatography, hydrophobic chromatography, gel chromatography, and gel electrophoresis in sodium dodecyl sulfate. The electrophoretic behavior of biologically active or radioiodinated and reduced growth factor indicated that the native protein (≈30,000 daltons) was composed of two different polypeptides (≈13,000–14,000 and 16,000–17,000 daltons, respectively) linked via reduction-susceptible bonds. The stimulatory activity on human glial cells of the purified product at a concentration of ≈4 ng/ml (0.13 nM) was equal to that of 1% human serum.

The major multiplication-stimulating activity of serum for cultured cells originates from the blood platelets (1–3). Although partial purification of the platelet-derived growth factor (PDGF) has been described (4–7) it has not yet been purified to homogeneity, and the characterization of the factor is still incomplete. PDGF is a protein or proteins resistant to heat and to treatment with various dissociating agents such as 4 M guanidine·HCl, 6 M urea, or 2% sodium dodecyl sulfate ($NaDodSO_4$) but susceptible to reducing agents. The physiological function of PDGF is not known. PDGF is of interest both in relation to platelet function *in vivo* and as a representative of the group of potent growth-promoting substances for cultured cells (for a review, see ref. 8).

The present communication deals with the chemical properties of PDGF and the utilization of these properties in a purification protocol for PDGF leading to an electrophoretically pure product. Previous studies indicated that the growth-promoting activity of human platelets resides in at least two components, one anionic and the other cationic, that may be separated by ion exchange chromatography on CM-Sephadex (4, 5). The cationic PDGF served as starting point for the present experiments. A preliminary report has been presented elsewhere (9).

MATERIALS AND METHODS

Assay for multiplication-stimulating activity

Multiplication-stimulating activity was estimated by using the incorporation of [^{3}H]thymidine into trichloroacetic acid-precipitable material of serum-deprived, sparse cultures of a normal human glial cell line, U-787 CG (10), as described (5). Multiplication-stimulating activity was expressed relative to the activity given by medium containing 1% of a reference serum derived from a pool of healthy human subjects. The reference assay resulted in 10–18 × 10^3 cpm per dish, corresponding to 55–80% labeled nuclei. Controls, receiving no multiplication-stimulating activity, incorporated 1–3 × 10^3 cpm per dish (3–10% labeled nuclei).

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Purification of PDGF

General. Protein was determined as described (11). All operations were performed at 4°C unless otherwise specified. Plastic containers or siliconized glassware was used to minimize losses of multiplication-stimulating activity on vessel walls.

Ion Exchange Chromatography. Platelet lysate was prepared from outdated human platelets essentially as described (5). The 100,000 × *g* supernatants from 200 units of washed platelets were combined (800 ml, 4500 mg of protein) and dialyzed against 0.08 M NaCl/0.01 M phosphate, pH 7.4. After a small precipitate had been removed by centrifugation at 20,000 × *g* for 20 min, 20 g of dry CM-Sephadex (Pharmacia) was added and the slurry was mixed end-over-end overnight. The mixture was then poured into a column and washed with 0.01 M phosphate buffer at pH 7.4. The column was eluted with a linear gradient (1600 ml) of NaCl from 0–0.5 M in 0.01 M phosphate buffer at pH 7.4. Multiplication-stimulating activity was found both in the unadsorbed fraction and in the adsorbed fraction that eluted as a broad peak with the gradient (4, 5). Cationic (adsorbed) PDGF, eluted between 0.17 and 0.35 M NaCl, was pooled and used for further purification.

Blue Sepharose Chromatography. A 2 × 3.2 cm column (10 ml) of immobilized Cibacron blue F3GA (blue Sepharose; Pharmacia) was prepared and equilibrated with 0.3 M NaCl/0.01 M phosphate, pH 7.4. Cationic PDGF from the CM-Sephadex chromatography (600 ml, 35 mg of protein) was applied to the column, without prior dialysis, at a rate of 10–15 ml/hr. After the column was washed with ≈100 ml of 1 M NaCl/0.01 M phosphate, pH 7.4, it was eluted with 30 ml of 50% (vol/vol) ethylene glycol/1 M NaCl/0.01 M phosphate, pH 7.4.

Gel Chromatography. The effluent from the blue Sepharose chromatography was dialyzed against 1 M acetic acid and lyophilized. The material (2.7 mg of protein) was dissolved in 1 ml of 1 M acetic acid and applied to a 1 × 145 cm column of Bio-Gel P-150 (100–200 mesh; Bio-Rad) equilibrated with 1 M acetic acid. Elution was at room temperature at a flow rate of 2 ml/hour. Aliquots of the effluent were neutralized with 1 M NaOH before assay for multiplication-stimulating activity and protein. The column was calibrated with the following native protein standards: IgG (M_r 150,000), human serum albumin (M_r 68,000), ovalbumin (M_r 43,000), chymotrypsinogen A (M_r 25,700), lysozyme (M_r 14,300), RN (M_r 13,700) and cytochrome *c* (M_r 12,400).

Preparative $NaDodSO_4$/Polyacrylamide Gel Electrophoresis. This was performed according to the method of Laemmli (12). Gels consisting of 10% acrylamide (Merck) and 0.37% methylene bisacrylamide (Eastman) were prepared in

Abbreviations: PDGF, platelet-derived growth factor; $NaDodSO_4$, sodium dodecyl sulfate.

6 × 120 mm glass tubes. The active fractions of the Bio-Gel P-150 chromatography were pooled and lyophilized. The sample (about 150 μg of protein) was dissolved in 300 μl of 0.0625 M Tris, pH 6.8/2% $NaDodSO_4$/10% (vol/vol) glycerol. This solution was divided into two equal portions and applied to two identical gels along with a trace of bromophenol blue but no reducing agent. The sample was not boiled before application. After the run, one gel was fixed overnight in 20% sulfosalicylic acid and stained for protein with Coomassie brilliant blue R-250. The second gel was sliced (manually operated gel slicer) into 2-mm-wide pieces. Each slice was extracted with 0.5 ml of 0.15 M NaCl/0.01 M phosphate, pH 7.4/0.02% $NaDodSO_4$ for 20 hr or more at 4°C. The extracts were analyzed for multiplication-stimulating activity at a concentration of less than 0.5%. The resulting concentration of $NaDodSO_4$ was not toxic to the glial cells and did not interfere with the biological assay. The following reduced proteins were used as molecular weight markers: human serum albumin, heavy and light IgG chains, ovalbumin, chymotrypsinogen A, lysozyme, and cytochrome *c*. For comparison, the corresponding unreduced proteins (except IgG) were run in the same system. All of these protein standards migrated faster in nonreduced than in reduced form; the former had their molecular weights underestimated by about 10% (6–19%) when compared to the standard curve of the latter. Mobilities were calculated relative to that of bromophenol blue.

Iodination of PDGF

PDGF purified by $NaDodSO_4$/polyacrylamide gel electrophoresis was iodinated by using a slight modification of the method of Hunter and Greenwood (13). Radioiodinated PDGF was separated from excess ^{125}I on a 1.5 × 5 cm column of Sephadex G-25 equilibrated and eluted with 1 M acetic acid containing 1 mg of human serum albumin per ml. The radioactivity appearing in the void volume of the column was pooled, concentrated by lyophilization, dissolved in an appropriate buffer, and used for further experiments.

Binding of ^{125}I-labeled PDGF to glial cell cultures

Binding experiments were performed on 2.5-cm petri dishes with confluent human glial cell cultures (≈400,000 cells per dish). ^{125}I-Labeled PDGF (^{125}I-PDGF) preparation (175,000 cpm) in 1 ml of F-10 medium (14) containing 1 mg of human serum albumin per ml was added to the cultures and incubated for 60 min at 37 or 0°C. The cultures were then washed six times in ice-cold phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/8 mM Na_2HPO_4/1.5 mM KH_2PO_4/0.9 mM $CaCl_2$/0.8 mM $MgSO_4$, pH 7.3) containing fetal calf serum and solubilized in 0.25 ml of 0.025 M Tris, pH 6.8/2% $NaDodSO_4$/10% glycerol. After incubation at 37°C, 6300 cpm of ^{125}I-PDGF was found in the extract. In the presence of an approximate 10-fold molar excess of unlabeled PDGF, only about 1600 cpm of bound ^{125}I was obtained. Similar amounts of bound ^{125}I-PDGF were observed after incubation at 0°C.

Analytical $NaDodSO_4$/polyacrylamide gel electrophoresis

This was performed according to Laemmli (12), essentially as described above, with the same reduced standard proteins but a slightly higher gel concentration (13%). Samples of ^{125}I-PDGF were boiled for 2 min, with or without 5% 2-mercaptoethanol, prior to electrophoresis. After the run the gels were sectioned into 2-mm-wide pieces and the radioactivity in each slice was determined in a gamma spectrometer.

RESULTS

Purification

Hydrophobic Chromatography. When chromatographed in 0.3 M or 1 M NaCl/0.01 M phosphate, pH 7.4, on agarose gels substituted with neutral alkyl groups, such as octyl-Sepharose (Pharmacia), cationic PDGF bound to the gels; passage through a column of octyl-Sepharose removed essentially all of the biological activity from the solution whereas a major part of the protein contaminants passed unadsorbed (not shown). Most of the growth-promoting activity (≈75%) was recovered from the column by elution with 50% ethylene glycol. At a certain degree of substitution, pentyl-Sepharose (i.e., a less-hydrophobic gel matrix) would similarly bind most of the PDGF, provided that the ionic strength was kept sufficiently high; in this case, binding was demonstrable at 1 M NaCl but not at 0.5 M NaCl. This is in accord with the concept of a hydrophobic interaction between PDGF and the respective Sepharose derivatives and argues against electrostatic interaction (15, 16).

Under similar conditions, PDGF also bound to the immobilized dye Cibacron blue F3GA (blue Sepharose) (Fig. 1). The binding of proteins to this substance may occur by various mechanisms including hydrophobic interaction, nonspecific electrostatic interaction, and "dinucleotide-fold specific" interaction (17). Because the last two types of phenomena are not stable in high salt concentrations (1 M NaCl) (18) we conclude that PDGF was adsorbed to blue Sepharose via hydrophobic forces. This situation is thus analogous to that described for serum albumin (19) and fibroblast interferon (17).

The hydrophobic character of PDGF was utilized on a preparative scale. Chromatography on blue Sepharose (Table 1) and octyl-Sepharose (not shown) resulted in similar degrees of purification (usually ≈8-fold); blue Sepharose was used routinely for preparative purification because it yielded the most reproducible results. The recovery was about 65% in this step.

Gel Chromatography. Chromatography of PDGF in 1 M acetic acid on Bio-Gel P-150 separated the multiplication-stimulating activity from a major peak of high molecular weight (excluded) components (Fig. 2), leading to a further 7-fold increase in specific activity [recovery, ≈40% (Table 1)]. As indi-

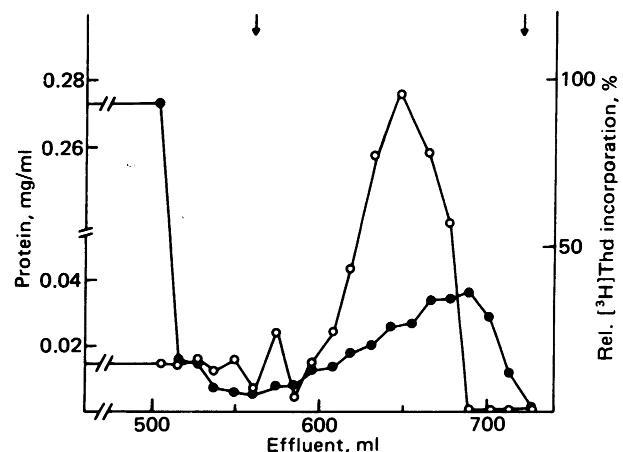


FIG. 1. Chromatography of PDGF on blue Sepharose. Cationic PDGF, obtained by CM-Sephadex chromatography of platelet lysate from 200 platelet units (135 mg of protein), was applied to a 10-ml column of blue Sepharose. After a wash with 1 M NaCl/0.01 M phosphate, pH 7.4, the adsorbed material was eluted with a gradient of ethylene glycol (0–50%) and assayed for protein (●) and multiplication-stimulating activity (○) (expressed as percentage of the activity given by 1% of human serum). The arrows indicate the beginning and end of the ethylene glycol gradient. Note that, in the routine preparation of PDGF, stepwise rather than gradient elution was used.

Table 1. Purification of PDGF from 200 platelet units

Stage	Total protein, mg	Recovery of activity, %	Protein conc. at reference conditions, μg/ml*
Human serum			700
Platelet lysate	4500	100	31
CM-Sephadex	35	30	1.1
Blue Sepharose	2.7	20	0.13
Bio-Gel P-150	0.15	8	0.02
Electrophoresis†	0.025	5	0.004

* At reference conditions, the multiplication-stimulating activity was equal to that obtained with medium supplemented with 1% human serum.

† $NaDodSO_4$/polyacrylamide gel electrophoresis. At this stage, protein was determined by amino acid analysis (after hydrolysis in 6 M HCl at 110°C for 24 hr). An internal standard of ^{125}I-PDGF was used to correct for losses during handling before hydrolysis.

cated by its elution from the column, the M_r of PDGF (28,000–40,000, by comparison with nonreduced standard proteins) was similar to that obtained previously with 1 M NaCl as an eluant (5); apparently, PDGF was not dissociated into low molecular weight components by exposure to low pH. Acetic acid, at a concentration of 1 M, like NaCl maintained the biological activity of PDGF in soluble form and, in addition, enabled simple concentration of the active material by lyophilization.

Electrophoresis. Further purification was obtained by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 3). Biologically active material was eluted from a region of the gel that exactly matched the position of a stainable component of an identical gel run in parallel. The estimated increase in specific activity was 5-fold and the recovery 50% or more (Table 1). The apparent M_r was 26,000–33,000 (Fig. 3). It should be pointed out that standard proteins were used in reduced form, whereas the preparation of PDGF was not reduced; reduction would irreversibly destroy the biological activity (4). This may lead to an underestimation of the M_r because intact disulfide bridges may constrain the size of the protein, thus enhancing its mobility in $NaDodSO_4$/polyacrylamide gel electrophoresis.

Stimulation of glial cells with various doses of PDGF (purified by electrophoresis) yielded the dose–response curve shown in Fig. 4. At a concentration of about 4 ng/ml (about 0.13 nM) the stimulatory effect of PDGF was equal to that of 1% human serum. Epidermal growth factor yields a similar response at

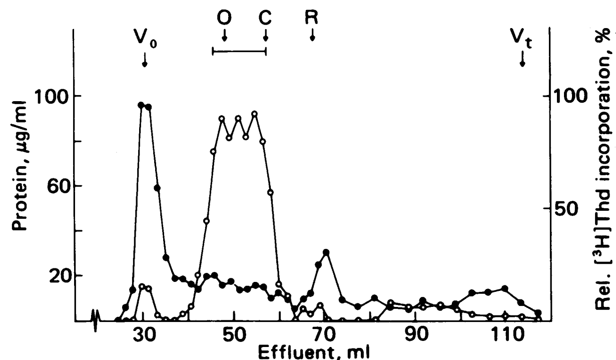
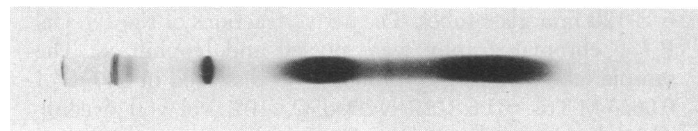


FIG. 2. Chromatography of PDGF, purified on blue Sepharose (2.7 mg of protein) on Bio-Gel P-150 in 1 M acetic acid. Effluent fractions were assayed for protein (●) and multiplication-stimulating activity (○) (expressed as described in the legend to Fig. 1). Material was pooled as indicated. Some of the molecular weight markers used (O, ovalbumin; C, chymotrypsinogen A; R, RNase) are indicated by arrows.



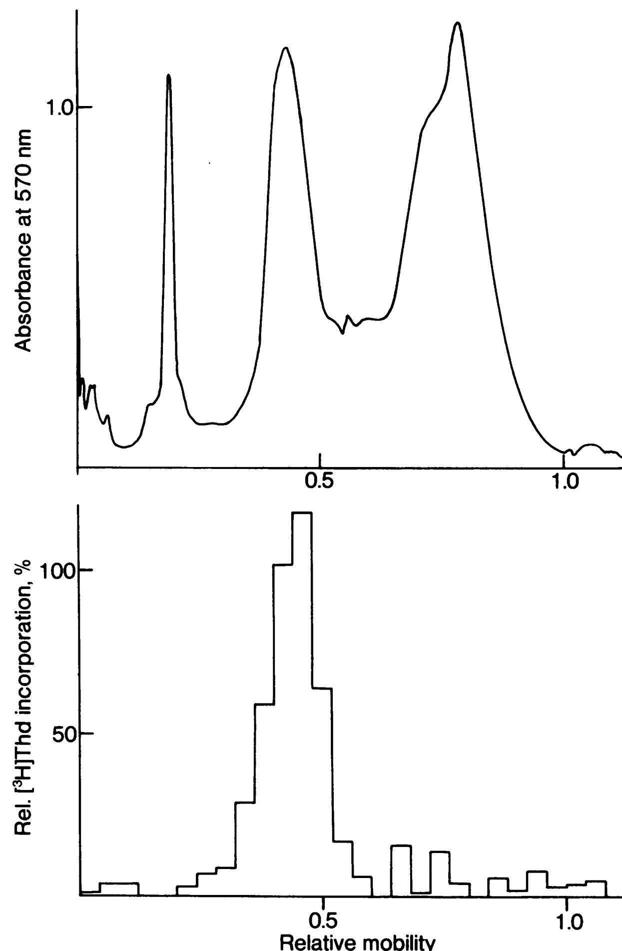


FIG. 3. Preparative $NaDodSO_4$/polyacrylamide gel electrophoresis of PDGF, derived from 200 platelet units, purified on Bio-Gel P-150 as described in Fig. 2 (75 μg of protein per gel). Parallel gels were stained for protein with Coomassie brilliant blue R-250 (a photograph of the stained gel and a scanning at 570 nm of this gel are shown in *Upper*) or sliced and assayed for multiplication-stimulating activity (*Lower*) (expressed as described in the legend to Fig. 1).

about 1 ng/ml (0.17 nM) (20). Thus, on a molar basis the specific activity of purified PDGF was in the same order of magnitude as that of epidermal growth factor.

^{125}I-labeled PDGF

$NaDodSO_4$/polyacrylamide gel electrophoresis of ^{125}I-PDGF ($\approx 14 \times 10^6$ cpm/μg of protein) resulted in the pattern shown in Fig. 5 *A* and *B*. A major radioactive component showed the same mobility as biologically active material with an apparent M_r of ≈30,000. In addition, a more slowly migrating component appeared; this material was also observed after "blank" iodination of gel eluates obtained from nonstainable regions of the gel. Because this material was precipitable with antibodies against bovine serum albumin, these findings were ascribed to artifactual incorporation of ^{125}I into carrier albumin (added after termination of the labeling reaction) rather than labeling of an endogeneous contaminant of the PDGF preparation. Reduction converted a large proportion (about 90%) of the 30,000-dalton component to faster migrating species. In the experiment shown in Fig. 5*B*, two or more components were obtained with apparent M_rs between 13,000 and 17,000. In

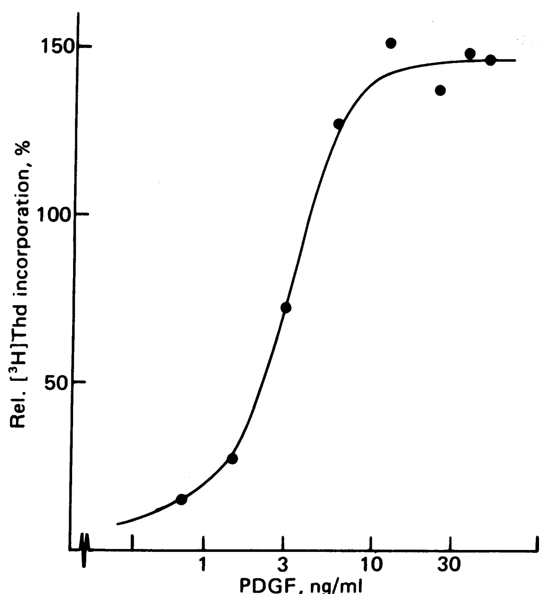


FIG. 4. Dose–response curve of PDGF purified by $NaDodSO_4$/polyacrylamide gel electrophoresis. The multiplication-stimulating activity of PDGF (expressed as described in the legend to Fig. 1) was recorded as a function of the concentration of PDGF. Determination of protein was as described in Table 1.

some experiments two distinct peaks were obtained, at M_r 13,000–14,000 and 16,000–17,000, respectively. The relative amounts of radioactivity in the two peaks varied from one experiment to another, probably due to differences in the extent of iodination of different tyrosine residues. The most likely explanation of these findings is that ^{125}I-PDGF was composed of two polypeptide chains, linked via disulfide bonds. The fact that the molecular weight of the reduction products add up to 30,000 supports this view; however it should be noted that only those polypeptide fragments carrying ^{125}I-labeled tyrosine are detectable by the present technique, and the involvement of more than two chains cannot be ruled out. A structure comprising one polypeptide chain only is unlikely but might be possible assuming an anomalous $NaDodSO_4$ binding of nonreduced PDGF.

Binding of ^{125}I-PDGF to Cell Cultures. Incubation of ^{125}I-PDGF with glial cell cultures at 37 or 0°C for 60 min resulted in significant binding of the growth factor to the cells. In the presence of an excess non-labeled factor, the binding was decreased by about 75%. $NaDodSO_4$/polyacrylamide gel electrophoresis of the solubilized cell cultures showed that only the 30,000-dalton component was involved in binding (Fig. 5*C*). As expected, it was split into material of lower apparent M_r (13,000–17,000) by treatment with a reducing agent (Fig. 5*D*). Negligible amounts of radioactivity retained the mobility of nonreduced PDGF.

DISCUSSION

The present protocol for purification of PDGF from a platelet lysate, utilizing charge fractionation, hydrophobic chromatography, and two different types of size separation, afforded an increase in specific activity of about 8000 times, with a total recovery of about 5% of biological activity. In the last step, $NaDodSO_4$/polyacrylamide gel electrophoresis, the biological activity of the product matched exactly with a major stainable component. The final specific activity of the present preparation of PDGF was of the same order of magnitude as that of epidermal growth factor, a pure multiplication-stimulating factor acting on cultured glial cells. In conjunction with the observations on ^{125}I-PDGF, these findings indicate that PDGF was close to purity (≈90% pure).

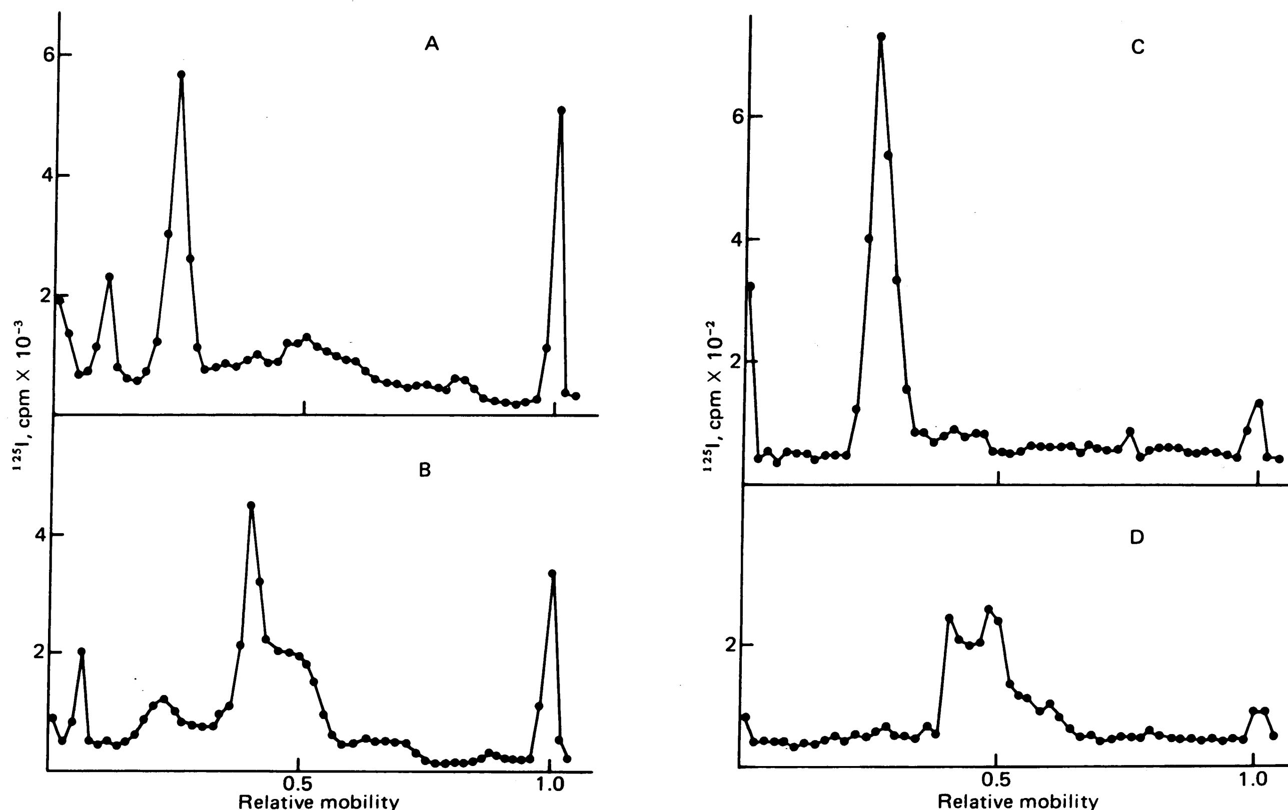


FIG. 5. $NaDodSO_4$/polyacrylamide gel electrophoresis of ^{125}I-PDGF before (*A* and *B*) or after (*C* and *D*) binding to glial cell cultures. Samples were analyzed before (*A* and *C*) or after (*B* and *D*) reduction with 5% 2-mercaptoethanol on 13% gels.

The picture of PDGF emerging from this and previous work is that of a basic [pI near 10 (5)] and somewhat hydrophobic protein with an apparent M_r of about 30,000. The broad distribution of PDGF in isoelectric focusing (5), gel chromatography (ref. 5; Fig. 2), and NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 3) suggest structural heterogeneity. Rechromatography of early and late portions of the gel chromatogram shown in Fig. 2 confirmed the original elution positions; therefore, the chromatographic behavior was not artifactual but was indicative of molecular size differences within the PDGF. Similar observations were made with PDGF obtained from different parts of the gel in electrophoresis.

After reduction, no stained component was observed in electrophoresis in the region corresponding to the biological activity of nonreduced PDGF; as expected, reduction also destroyed the biological activity. The nature of the reduction products therefore was examined by use of ^{125}I-PDGF. Again, reduction led to disappearance of most of the 30,000-dalton component. Instead, low molecular components appeared, indicating that ^{125}I-PDGF consisted of two or more polypeptide chains joined by disulfide bonds susceptible to the combined action of the reducing agent and NaDodSO$_4$. This conclusion was also supported by gel chromatographic experiments on Sephadex G-200 in 0.15 M NaCl/0.01 M phosphate, pH 7.4/0.1% NaDodSO$_4$ (not shown). In this system, ^{125}I-PDGF eluted at a position corresponding to a M_r slightly greater than 30,000, as compared with standard proteins, whereas reduced and alkylated ^{125}I-PDGF eluted as a protein of $M_r \approx 15{,}000$. Interestingly, gel chromatography in 4 M guanidine·HCl or 1 M acetic acid failed to reveal any major differences between the apparent size of reduced and nonreduced ^{125}I-PDGF. This may be due to molecular expansion of the fragments obtained from the reduced protein when they assume a more random coil-like structure—e.g., in 4 M guanidine·HCl (21). The proposed molecular make-up of PDGF is reminiscent of that of certain hormones (e.g., insulin) and may indicate the existence of a pro-form, from which PDGF derives by proteolytic modification.

Cell-associated ^{125}I-PDGF was found after incubation at 37°C and at 0°C, indicating that the labeled material was bound rather than taken up by pinocytosis. The binding pattern in the presence of a high concentration of unlabeled PDGF suggested saturability. The receptor nature of the PDGF binding structure on glial cells will be described elsewhere.

Antoniades and coworkers (22) have isolated a cationic growth factor from human serum that had a M_r of 13,000 as judged from the migration distance of a stainable component in NaDodSO$_4$/polyacrylamide gel electrophoresis. By using a radioimmunoassay for this component, immunological crossreactivity was observed between the latter and boiled platelet extract. It was concluded that the purified serum factor was likely to be a platelet protein (6). Certain properties, such as heat stability and basic pI, seem to be common to the serum-derived growth factor of Antoniades *et al.* and our preparation of PDGF, whereas the M_rs are apparently different. However, if it is assumed that the M_r determination of the serum-derived growth factor was performed under reducing conditions [which is not clear from the paper of Antoniades *et al.* (22)], good agreement with the 13,000- to 14,000-dalton component of PDGF is noted. It would be of interest to compare the electrophoretic behavior of the serum-derived growth factor with that of PDGF in terms of biological activity rather than stainable protein. Vogel *et al.* (7) recently reported on the partial purification of PDGF. Apparently their preparation had chromatographic properties on CM-Sephadex similar to those of the present PDGF (5). The wide M_r range given (10,000–40,000) would be compatible with both our M_r estimates and those of Antoniades *et al.* (22).

The combination of basic and hydrophobic properties of PDGF may explain the difficulties experienced in previous attempts at the purification of it. The present procedure permits the isolation of small amounts of PDGF with reproducible quantitative and qualitative yields of product. As more PDGF becomes available in pure form, it may find use in the development of defined media for normal cultured cells. Preliminary experiments along this line have been performed (9).

Note Added in Proof. After the submission of this paper, Antoniades *et al.* (23) reported the purification of a platelet-derived growth factor. This factor is probably identical to the present one, although the analytical data show some discrepancies.

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