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Evidence for a Low-Molecular-Weight Plasma Peptide Which Stimulates Chick Chondrocyte Metabolism

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Plasma contains a number of insulin-like activities (ILA) of molecular weights 7,000 to 90,000 (somatomedins and insulin-like proteins) which stimulate cellular metabolism and may function as growth factors. We have found evidence for the presence of an 800 Dalton peptide in human plasma which markedly stimulates the metabolism of chick chondrocytes.

This peptide was extracted from human Cohn fraction IV-1 by procedures similar to those used for somatomedin isolations. At the Sephadex G-50 column separation step, the fraction with molecular weights of 300–1,000 was found to markedly stimulate chick chondrocyte metabolism. Rechromatography on Sephadex G-25 concentrated activity in peptides of molecular weight of about 800. An HPLC separation on a silica C-18 reverse phase column gave elution of the active peptide at 18% acetonitrile in water. This bioactivity appears to be a peptide which is free of lipids, carbohydrates, nucleic acids, metal ions, and immunoreactive insulin. This factor markedly increased the metabolism of cultured chick chondrocytes, but had only marginal activity on rat chondrocytes. When added at 1 μg/ml to chick chondrocytes cultured in F-12 medium plus 1.5% fetal calf serum, the HPLC-purified activity increased DNA synthesis 7.3-fold, lipid synthesis 10.2-fold, and lactate production 2.9-fold after 48 h incubation.

However, unlike somatomedins A and C, this factor did not displace insulin from placental membranes. These results suggest that low-molecular-weight peptides, which are smaller than the somatomedins, may contribute to the total ILA of human plasma.

Key words: insulin-like, somatomedin, chick chondrocytes, peptides, HPLC

Human plasma contains a number of bioactivities which express themselves in cell culture systems as "insulin-like" (eg, increased utilization of energy-producing substrates, stimulation of lipid synthesis) and stimulatory to growth [1–5]. The insulin-like activities (ILA) of plasma have been attributed to a variety of polypeptide fractions with molecular weights ranging from 500 to 90,000 [1–5]. These non-insulin factors account for 90%–95% of the ILA of plasma, the remainder being due to immunoreactive insulin [2]. The

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best characterized of these peptides, somatomedins A and C [2, 3] and IGF I and II [1], are of molecular weights of about 7,000. In addition, a non-suppressible insulin-like 88,000 Dalton protein (NSILP) has recently been isolated [5]. However, the contribution of these various factors to overall plasma ILA is uncertain since the losses of activity (>95%) incurred in the course of isolation of such factors [1–3, 5] leave open the possibility that significant ILA may reside in unidentified factors.

In the course of isolating somatomedin-C activities from human plasma, we found evidence for a low-molecular-weight (~800) peptide which markedly stimulates lipid and DNA synthesis, and lactate production, of cultured chick chondrocytes. Our results support the observation of Bala and co-workers [4] that human plasma contains a molecule, smaller than the somatomedins, which enhances chondrocyte metabolism.

MATERIALS AND METHODS

Puck's saline G (calcium and magnesium free) [6], trypsin, fetal calf serum, Ham's F-10 and F-12 medium were purchased from Grand Island Biologicals; collagenase, type CLS, #4194 from Worthington Biochemicals; chicken serum, #407, from International Scientific Industries; Lactate Determination Kit #826, Sephadex G-25 and G-50, blue dextran from Sigma Chemical Company; ^{3}H-thymidine (17.6 Ci/M) and 1-[^{14}C]-acetate (2 Ci/M) from New England Nuclear; ^{125}I-Insulin (114 μCi/μg) from Cambridge Nuclear Co.; Human Cohn plasma fractions III-0, IV-1, and V plus the final supernatant (Cutter SE-1) remaining after the removal of proteins by the Cohn process from 200 L of plasma, from Cutter Laboratories; thin-layer high-performance silica gel plates #5631, spray reagents for detection of compounds from E. M. Laboratories; HPLC grade solvents, from J. T. Baker; high-performance liquid chromatography (HPLC) column (RSil HL, C-18 reverse-phase, 10 micron, 25 X 0.4 cm), from Alltech.

Isolation of Chondrocyte-Stimulating Activity From Plasma

Cohn fraction IV-1 from 20 L plasma was extracted with an HCl-ethanol solvent by the method of Van Wyk et al [8] for the initial purpose of isolating somatomedin-C. Solubilized material was precipitated with acetone, the dried precipitate extracted 3 times over a 2 h period with 20% formic acid, and polypeptides in the resultant extract fractionated by gel filtration on Sephadex. For these studies, we followed the procedure as published by Van Wyk et al [8], omitting the G-75 column step, in order to separate low-molecular-weight chondrocyte-stimulating activities on a single column for purposes of comparison (Fig. 1). The effect of extending the formic acid extraction step to 24 h on recovery of bioactivity was also determined. Molecular weights of eluted fractions were estimated by using blue dextran, ^{125}I-insulin, bacitracin, H-gly-his-lys-OH, and NaCl as markers. It was assumed that the exclusion peak on G-50 represented proteins with molecular weights of greater than 20,000.

The formic acid extract from the acetone powder was fractionated on G-50 (100 X 2.5-cm column) in 1% formic acid at 3°C. Fraction H from the G-50 column was rechromatographed in 1% formic acid at 3°C on G-25 (100 X 0.9-cm column). Sephadex columns were used for this purpose at least 10 times before estimation of molecular weights to minimize errors due to polypeptide adsorption. Active fractions from G-25 were further purified by HPLC using a linear gradient of acetonitrile. All fractions were lyophilized, re-

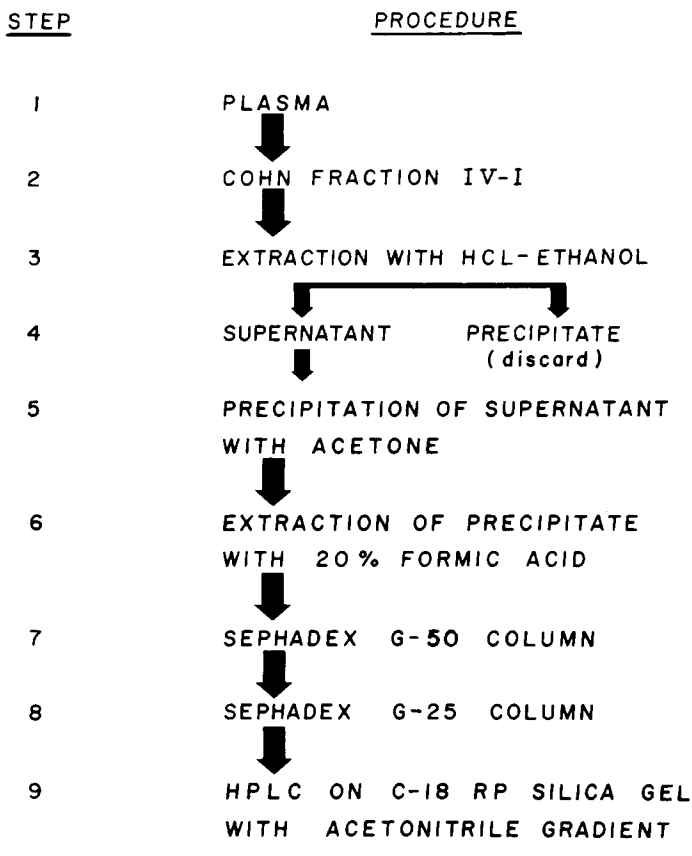

Fig. 1. Purification process.

dissolved on 0.01 N HCl, and relyophilized. Samples were stored at −86°C prior to bioassay, dissolved in Dulbecco's phosphate-buffered saline (pH 7.4), their protein content measured by the Lowry reaction, and diluted to desired concentrations for use in experiments.

In ancillary experiments, Cohn fractions III-0 and V in amounts equivalent to 20 L starting plasma, and the final supernatant remaining in an amount equivalent to 200 L starting plasma, after the removal of proteins by the Cohn process (Cutter SE-1), were also extracted similarly to fraction IV-1 to assess their potential as sources of chondrocyte-stimulating activity.

The direct isolation of this activity from plasma by methods similar to those employed by Bala et al [4] was also attempted. Citrated human plasma (20 ml) was fractionated on a G-50 column (2.5 X 100 cm) either in 0.05 M phosphate buffer, pH 7.4, or in 1% formic acid, pH 3.3, in a manner similar to the extract from Cohn fraction IV-1. Fractions with elution volumes similar to fractions G and H (Fig. 2) were dialyzed with distilled H_2O on an ultrafiltration membrane (Amicon UM-05), then lyophilized and stored at −20°C prior to testing.

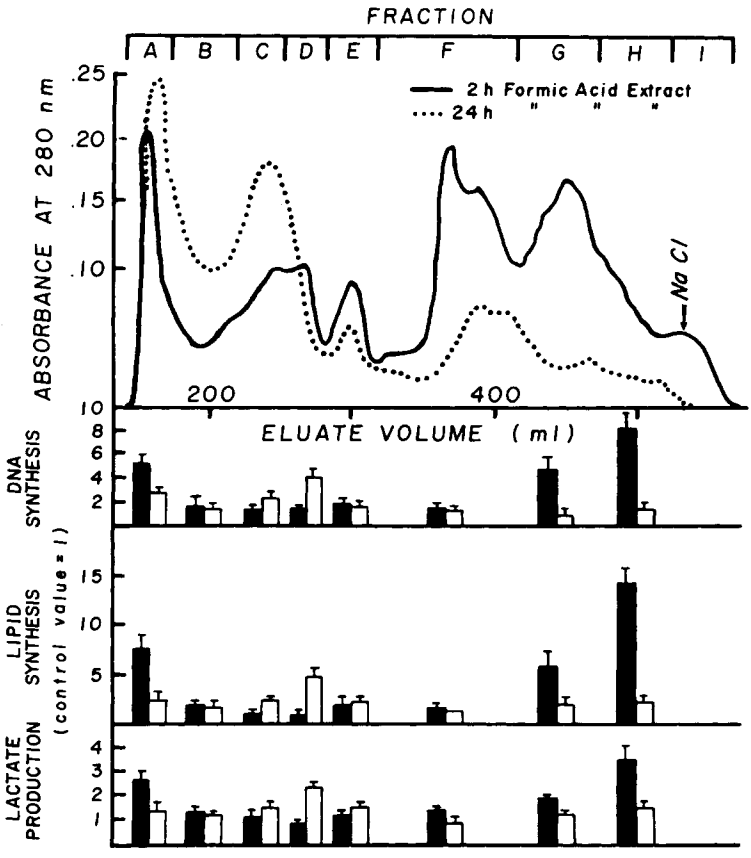

Fig. 2. Effect of fractions on G-50 column on DNA and lipid synthesis and lactate production by chick and rat chondrocytes. The elution pattern of the 2 h formic acid extract from plasma Cohn fraction IV-1 on a Sephadex G-50 column (2.5 X 100 cm) is shown by the solid line. Bar graphs represent stimulation of DNA and lipid synthesis and lactate production induced in chick chondrocytes (solid bars) and rat chondrocytes (open bars) induced by 5 μg/ml of proteinaceous material from the Sephadex G-50 fractions above each group of 3 bars. The elution pattern on G-50 obtained after extraction with formic acid for 24 h is given by the broken line. Fraction I could not be readily desalted and was not assayed.

Characterization of Bioactive Fraction From HPLC

Elemental analysis of the most active from HPLC was determined by x-ray photoelectron spectroscopy [9]. Specific tests were performed after drying this fraction on filter paper and staining with the following reagents: ninhydrin, triphenyl tetrazolium, diphenylamine, alkaline silver nitrate, iodine vapor, aniline phthalate, bromocresol green, 2′,7′-dichlorofluorescein, and dimethylaminobenzaldehyde. The ultraviolet spectrum of the fraction was determined.

Bioactive fractions from HPLC were chromatographed on thin-layer silica plates (solvent: chloroform/methanol/17% NH_4OH: 2/2/1 by volume), then stained with the detection reagents listed above.

Determination of Insulin-Like Activity and Immunoreactive Insulin in G-50 Fractions

Total insulin-like activity in fractions from the G-50 column was determined by radioactive receptor assay utilizing placental membranes and porcine ^{125}I-insulin, as described by Van Wyk et al [8]. Unlabeled porcine insulin (28.5 μU per nanogram) was used as a standard for insulin-like activity in the receptor titrations. Insulin concentrations were determined by standard radioimmunoassay [10].

Determination of DNA Synthesis, Lipid Synthesis, and Lactate Production

DNA synthesis was measured by incorporation of ^{3}H-thymidine into acid-precipitable protein by the procedure of Garland et al [11] for chondrocytes. Lipid synthesis was measured as the incorporation of ^{14}C-acetate into cellular lipids [12]. Lactate production was calculated from the lactic acid concentrations in the medium before and after incubation. Lactic acid was quantitated by a NAD-linked lactate dehydrogenase reaction [13].

Culture of Chick and Rat Chondrocytes

Chick chondrocytes were isolated by a modification of the method of Cahn et al [14]. Sterna from 12–15-day-old embryos were cleared of muscle tissue, excised, and submerged in ice-cold saline G containing 10% chicken serum. Fibroblasts and muscle cells were cleared from sterna by digestion in flasks (4 sterna per flask) containing 5 ml of saline G, pH 7.6, 0.2% collagenase, and 1.0% trypsin. After digestion for 15 min at 37°C on a rotary shaker (80 cycles/min), saline G (10 ml) was added and the solution decanted. Sterna were then washed and resuspended in saline G for examination under a dissecting microscope to select the cleanest fragments for isolation of chondrocytes by a repeat of the above digestion. The supernatant containing the isolated chondrocytes was decanted into 8 ml Ham's F-12 medium supplemented with 1.5 ml FCS. The digestion was repeated a third time and the final supernatant added to the previously isolated chondrocytes. Released cells and debris were sedimented by centrifugation at 1,000g for 10 min and resuspended in F-12 medium. Cellular debris was removed by centrifugation at 60g for 5 min, then the chondrocytes sedimented at 1,000g for 10 minutes. Chondrocytes were washed in F-12, then 2×10^5 cells incubated at 37°C in 2 ml Ham's F-12 containing 1.5% FCS in glass tubes (12 × 75 mm) on a roller drum (12 revolutions per hr) for 4 days. At the end of incubation, medium was removed and replaced with fresh F-12 medium containing 1.5% FCS, a peptide fraction to be tested, and 1 μCi of ^{3}H-thymidine or 1 μCi of ^{14}C-acetate, and incubation continued for an additional 48 h for determination of DNA and lipid synthesis and lactate production.

Rat chondrocytes were prepared from fragments of costal cartilage removed from 24-day-old male Sprague-Dawley rats. Adherent tissue was removed under a dissecting microscope and chondrocytes were isolated by the procedure used for chick chondrocytes. Experimental culture conditions and assays were similar to those employed with chick chondrocytes, except medium used was Ham's F-10 medium plus 1.5% FCS.

RESULTS

Chondrocyte-Stimulating Activity in Sephadex G-50 Fractions

The elution pattern of peptides in formic acid extracts chromatographed on Sephadex G-50 is shown in Figure 2. The approximate molecular weights of fractions containing major peaks were: A (exclusion peak), >20,000; C, 8,000–10,000; D, 5,000–7,000; E, 4,000–5,000; F, 2,000–4,000; G, 1,000–2,000; H, 300–1,000; I < 300.

TABLE I. Insulin-Like Activities of G-50 Fractions From Cohn IV-1 as Determined by Radioreceptor Assay and Radioimmunoassay

G-50 fractions	Insulin content (μU insulin/mg protein)	Insulin-like activity not due to insulin (μU ILA/mg protein)
A	60	450
B	30	1,470
C	120	6,080
D	530	12,080
E	470	5,230
F	ND[a]	650
G	ND	ND
H	ND	ND
I	ND	ND

Net ILA not due to insulin equals ILA determined by radioreceptor assay minus insulin content as measured by insulin radioimmunoassay. Details of procedure are given in text.
[a]ND, not detectable.

Optimal concentrations of bioactive protein fractions on cell cultures were determined in preliminary experiments. The effect of fractionated protein from Sephadex eluates on lipid synthesis in chick chondrocyte cultures was assayed at concentrations of 0.1 to 20 μg/ml incubation medium. It was found that most fractions gave nearly maximal stimulation at 5 μg/ml. When tested at this concentration, peptide fractions in the entire range of molecular sizes fractionated on Sephadex G-50 (500 to >20,000) stimulated DNA synthesis, lipid synthesis, and lactate production in chick and rat chondrocytes (Fig. 2). Chick chondrocytes were most responsive to fractions A, G, and H. In these cells, fraction A raised DNA synthesis 5.2-, lipid synthesis 7.7-, and lactate production 2.8-fold, while fraction H increased these processes 8.2-, 14.5-, and 3.5-fold, respectively. The effects of fraction G were intermediate between fractions A and H. In contrast with chick chondrocytes, rat chondrocytes responded maximally to fractions D, C, and A, in order of activity (Fig. 2). Fractions D and A increased DNA synthesis in rat chondrocytes 3.7- and 2.6-fold, lipid synthesis 4.8- and 2.5-fold, and lactate production 2.4- and 1.4-fold, respectively. Activities associated with fraction C were comparable to fraction A.

Insulin displacement activity was concentrated in fractions C, D, and E. Fraction D had the highest insulin-like activity (12,610 μU ILA/mg), as measured by ability to displace ^{125}I-labeled insulin from placental receptors (Table I). Displacement due to immunoreactive insulin was detectable in fractions A through D, ranging from approximately 2% of total displacement activity in fractions B and C, to 13% in fraction A. Fraction D contained the highest absolute amounts of immunoreactive insulin at concentrations of 530 μU ILA per mg protein or 19 ng insulin per mg protein, accounting for 4% of the total displacement activity in this fraction. However, the addition of insulin at concentrations ranging from 0.005 to 1.0 ng/ml had no detectable effect on DNA and lipid synthesis or lactate production in chick and rat chondrocytes.

Increasing the duration of the formic acid extraction to 24 h increased the recovery of peptides with molecular weights greater than 4,000 but had no effect on the production of smaller molecular species (Fig. 2). Thus, it is unlikely that the low-molecular-weight peptide fraction was generated by formic-acid-catalyzed proteolysis. Furthermore, the bioactivity of fractions G and H from G-50 was only marginally increased by the extended formic acid extraction.

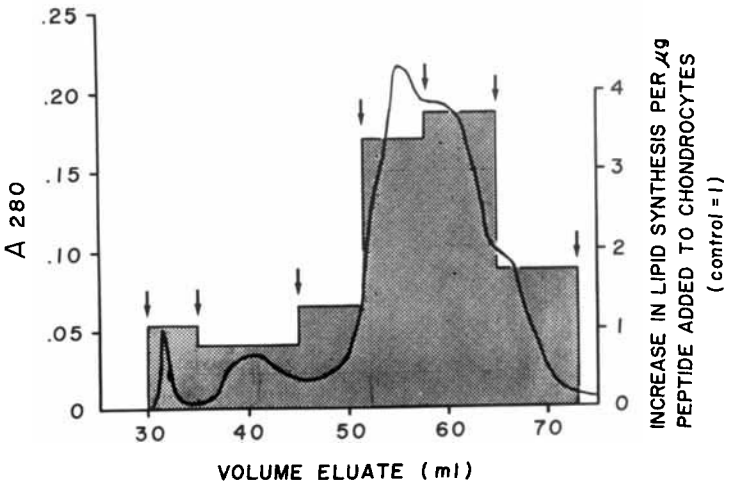

Fig. 3. Elution of G-50 fraction H on G-25. Bioactivity of fractions (stippled bars) measured as effect of peptide, when added at 1 µg/ml, on lipid synthesis in chick chondrocytes.

Cohn fractions III-0 and V, when processed in the manner of fraction IV-1, were found to possess similar bioactivities in G-50 fractions G and H, which stimulated chick chondrocyte metabolism. However, Cohn fraction III-0 yielded only 17% and fraction V only 46% of the comparable activity isolated from Cohn fraction IV-1. Extracts of Cutter fraction SE-1 were inactive.

G-50 fractions G and H from human plasma chromatographed at pH 3.3 possessed an activity which stimulated chick chondrocyte lipid and DNA synthesis, while similar fractions from plasma chromatographed at pH 7.4 was inactive. However, extraction of bioactive material by direct chromatography of plasma was impractical because of the low yield.

Chromatography of Sephadex G-50 Fraction H on Sephadex G-25

The most active low-molecular weight bioactive material isolated from Cohn IV-1 on G-50 (fraction H) was rechromatographed on a G-25 column and fractions lyophilized. The majority of activity associated with the main peak (Fig. 3). The addition of 1 µg/ml of the most active material to chick chondrocytes increased lipid synthesis approximately 3.5-fold. Comparison with standards gave a molecular weight of approximately 800.

Chromatography of Bioactive Fraction From Sephadex G-25 on HPLC

Material from the major peak of activity from G-25 was dissolved in 10% acetonitrile in H_2O, then chromatographed on a HPLC C-18 reverse-phase silica gel column into an acetonitrile gradient. Chick-chondrocyte-stimulating activity associated with a peak which eluted from the column in 18% acetonitrile (Fig. 4). The most active material, when added to chick chondrocytes at 1 µg/ml, increased lipid and DNA synthesis 10.2- and 7.3-fold, respectively, and lactate production 2.9-fold (Table II).

Elemental analysis, chemical tests, and ultraviolet absorbance spectra of the most active HPLC fraction indicated that it contained peptidic material which was relatively low in aromatic residues. Analysis by x-ray photoelectron spectroscopy found that hydro-

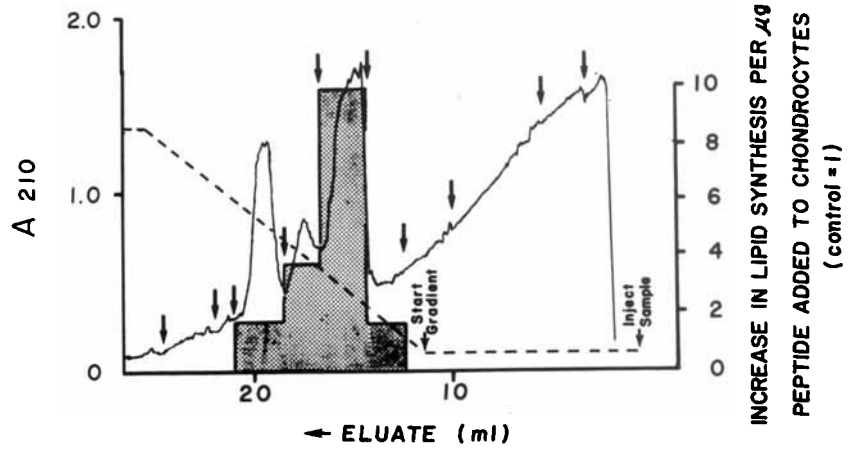

Fig. 4. HPLC of chick-chondrocyte-stimulating fraction from G-25. Bioactivity of fractions (stippled bars) measured as effect of peptide, when added at 1 μg/ml, on lipid synthesis in chick chondrocytes. Fractions without bars had no significant bioactivity. The dotted line represents the gradient which started at 10% acetonitrile and was increased to 70% acetonitrile.

TABLE II. Bioactivity of HPLC-Purified Isolates on Chick Chondrocytes

Peptide added, μg/ml	DNA synthesis	Lipid synthesis	Lactate production
0.0 (control)	1.0	1.0	1.0
0.1	1.9 ± 0.3	2.2 ± 0.2	1.3 ± 0.3
0.5	4.5 ± 0.7	6.4 ± 0.9	1.6 ± 0.2
1.0	7.3 ± 1.2	10.2 ± 2.3	2.9 ± 0.5

Values for control cultures normalized to unity for comparative purposes. Each determination is the average of 6 experiments.

gen, carbon, nitrogen, and oxygen in proportions characteristic of polypeptides comprised at least 99.5% of the sample. This fraction gave positive reactions to ninhydrin and iodine vapor; however, other staining reagents indicated that the fraction was free of carbohydrates, nucleic acids, reducing agents, steroids, and tryptophane. The ultraviolet spectra of the fraction had a typical peptide spectrum from 220–260 nanometers, with only a small increase in absorption at 280 nanometers, suggesting a low-content of aromatic residues (Fig. 5).

Chromatography of this fraction on TLC silica gel plates revealed three peptides as components. The fraction contained no amino acids. The major peptide migrated with an $R_f = 0.30$, gave a purple color with ninhydrin, and stained as a base with bromocresol green. The minor peptides, respectively, moved with R_f's of 0.09 and 0.37, stained orange and purple with ninhydrin, and gave acidic and neutral responses to bromocresol green. This isolate did not contain H-gly-his-lys-OH, a growth-modulating peptide previously isolated from human plasma [9]. Hydrolysis of this fraction in 6 N HCl for 24 h abolished the bioactivity and the three constituent peptides were replaced with a mixture of ninhydrin-staining spots characteristic of amino acids.

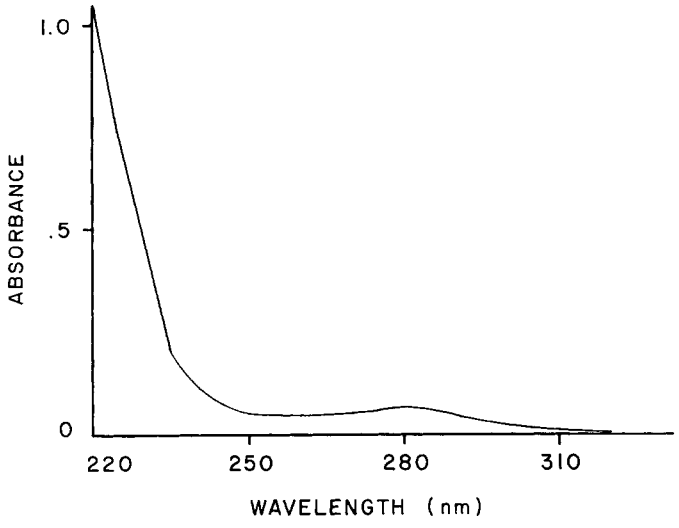

Fig. 5. Ultraviolet spectrum of chick-chondrocyte-stimulating activity from HPLC. The absorbance of bioactive material at a concentration of 0.2 mg/ml was determined in a 1-cm cell.

DISCUSSION

These studies demonstrate that diverse classes of peptides from human plasma possess insulin-like activities, as manifested by their stimulatory effects on chick and rat chondrocytes. When using plasma Cohn fraction IV-1 as a source, activities which stimulated chondrocyte metabolism were found to be associated with fractions of molecular weights greater than 20,000 (fraction A), approximately 6,000 (fraction D), and 300 to 2,000 (fractions G and H) (Fig. 2). These results are similar to the observations of Bala et al [4] who reported that sulfation of rat cartilage is stimulated by at least 6 polypeptide and protein fractions extracted from plasma which range in molecular size from 500 to 90,000. Insulin-like activities of molecular weights of approximately 7,000 isolated from acid-ethanol extracts of plasma have previously been attributed to a family of closely related peptides (somatomedins), while those of the high-molecular weight fractions to either a complex of binding proteins and somatomedins [1–3] or to a 90,000 Dalton protein with insulin-like activity [5]. It is clear that acid-ethanol extracts of plasma contain a variety of molecules with insulin-like properties.

The two cell types examined exhibited considerable variation in their ability to respond to eluted fractions. Maximal stimulation was obtained in chick chondrocytes with fractions A, G, and H and in rat chondrocytes with fractions A and D (Fig. 1). Estimations of insulin-like activities of peptide fractions extracted from plasma differed markedly when based on results of bioassays and receptor assays. Thus, among fractions exhibiting major stimulatory effects on lipid synthesis (fractions A, D, H), only fraction D produced significant displacement of ^{125}I-insulin from receptor-rich placental membranes (Table I). Conversely, fractions C and E possessed 40%–50% of the displacement activity of fraction D, while their bioactivities were relatively unimpressive compared with the effects of the more active fractions, especially in chick chondrocytes (Fig. 2). These findings suggest that attachment of plasma peptides to receptors with an affinity for insulin may not stimulate the expected "insulin-like" responses in certain cell types and tissues.

The low-molecular-weight (~800) chick-chondrocyte-stimulating activity isolated from Cohn fraction IV-1 appears to be a peptide and also may be present in other Cohn fractions (III-0 and V). Chromatography of human plasma on molecular sieving gels gives rise to a low-molecular-weight chick-chondrocyte-stimulating activity when processed at low pH (3.3), but not a physiological pH (7.4). This observation parallels the previous finding of Bala et al [4] that low-molecular-weight (500–2,000) chick-chondrocyte-stimulating activities are released from plasma under acidic conditions and suggests that such conditions may dissociate this activity from carrier proteins. It is possible that this factor is a bioactive breakdown product of the somatomedins; however, preliminary experiments have indicated that extension of the time of the acid-ethanol or the formic acid extraction steps does not increase the yield of this activity. This suggests that this factor may be a naturally occurring endogenous peptide which contributes to the ILA of human plasma.

In summary, these experiments give evidence for the existence in human plasma of a low-molecular-weight factor, peptidic in nature, which markedly stimulates the metabolism of cultured chick chondrocytes.

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