CHARACTERIZATION OF THE FACTOR DEFICIENT IN THE HUNTER SYNDROME BY POLYACRYLAMIDE GEL ELECTROPHORESIS

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SUMMARY: Fibroblasts cultured from skin of patients with the Hunter syndrome lack a genotype-specific macromolecular factor required for mucopolysaccharide degradation. From its electrophoretic mobility at several gel concentrations, this factor was estimated to have a molecular weight of 65,000 and a valence of -11 at pH 8. We conclude from these data that the factor is a protein.

The Hunter syndrome is an inheritable disorder of mucopolysaccharide metabolism, genetically distinct from the better known Hurler syndrome though phenotypically similar to it (1). The clinical manifestations of both diseases result from the massive intracellular deposits of dermatan sulfate and/or heparan monosulfate.

Fibroblasts cultured from the patients' skin likewise accumulate excessive amounts of dermatan sulfate (2,3), because of inadequate degradation of this polymer (4). However, the degradation can be accelerated and the accumulation reduced if Hunter or Hurler fibroblasts are supplied with secretions from fibroblasts of genotype other than their own (5). The corrective factors in these secretions are heat-labile and macromolecular. The data suggested that the factors were neither nucleic acids nor sulfated mucopolysaccharides, but evidence to identify them as proteins was not obtained (5).

We have now used analytical polyacrylamide gel electrophoresis (PAGE) to characterize one of these factors. Since the electrophoretic mobility

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[1] The system was computed using the theory of multiphasic buffer systems and ionic mobility values of T. M. Jovin and A. Chrambach (in preparation).

of charged molecules through gels of different pore size is a function of their size and charge, PAGE permits the estimation of molecular geometry and valence (6,7). When a specific assay is available for the substance under study, such estimates can be made without prior purification. The factor in which Hunter cells are deficient but which is present in normal cells - designated as the "Hunter factor" - was selected for this investigation because it is more stable and more readily available than the factor in which Hurler cells are deficient.

EXPERIMENTAL: Preparation of Hunter factor - Normal human skin fibroblasts were grown in 1410 cm^2 Bellco roller bottles in the presence of Eagle's Minimal Essential medium supplemented with fetal calf serum, non-essential amino acids and antibiotics (5). When the cells were confluent, serum-free medium (150 ml per bottle) was substituted (8). Twice a week, the medium was harvested, concentrated at 4° on a Diaflo XM-50 membrane in an Amicon Model 2000 Ultrafiltration Cell, washed with 0.9% NaCl and reconcentrated to one fiftieth the original volume. This preparation, containing 0.5 to 1 mg protein per ml, could be stored at -20° for several months. Before electrophoresis, it was clarified by centrifugation, diluted and reconcentrated in a smaller cell, to a final concentration of 2 to 3 mg protein per ml in 0.01 M NaCl.

Assay of Hunter factor activity - Fibroblasts from skin biopsies of Hunter patients maintained in culture as previously described (4,5), were used as test cells for the following assay. Cells at a density of 1 mg cell protein per 100 mm Petri plate were incubated in the presence of 5 ml medium containing 40 μC $^{35}SO_4$, plus one ml of 0.9% NaCl or of a suitably diluted Hunter factor preparation. After 48 hrs of incubation, radioactivity of the accumulated intracellular mucopolysaccharide was

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[2] The geometric mean radius is calculated from the known molecular weight, using the relationship $\bar{R} = \sqrt[3]{\frac{3\ (MW)\ \bar{v}}{4\ \pi\ N}}$. This assumes that the molecules are unhydrated spheres of partial specific volume, $\bar{v}$, 0.74 (6).

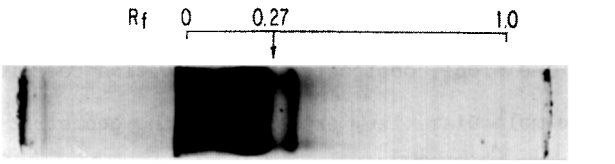
determined (4); reduction from the level attained by the saline control is the "correction" effected by the Hunter factor. A unit is defined as that activity which gives a half-maximal correction. This can be readily calculated from double reciprocal plots (1/correction vs. 1/concentration) which are linear.

Polyacrylamide gel electrophoresis - The methodology of PAGE was used as described (7), with the following exceptions. For the Hunter factor preparation, total gel concentration T = 3.5%, crosslinking C = 2% was used in the concentration gel. For the separation gels T was varied from 4 to 30% at constant C = 2%. Polymerization was initiated by 0.55 mM potassium persulfate, 0.014 mM riboflavin, and N,N,N',N'-tetramethylethylenediamine (TEMED) at the following concentrations: for concentration gels, 6.25 mM; for gels 4 to 17% T, 3.13 mM; for T $\geq$ 20%, 1.25 mM.

The instability of Hunter factor activity at pH's above 8 and the decreased mobility of proteins with decreasing pH led us to use the following multiphasic buffer system, operative at 0^{o} C^{1}: Upper Buffer: N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES), 0.04 M; 4-picoline, 0.044 M. Lower Buffer: 4-picoline, 0.063 M; HCl, 0.05 M. Concentration Gel Buffer: phosphoric acid, 0.049 M; 4-picoline, 0.054 M. Separation Gel Buffer: HCl, 0.0594 M; 4-picoline, 0.858 M. The operative pH of the separation phase was 7.95; ionic strength, 0.015; mobility of the front, $u_f = 4.83 \times 10^{-5}$ (cm/sec)/(volt/cm). 4-Picoline was redistilled under vacuum. Of the Hunter factor preparations, 240 μg of protein in 25% sucrose was applied to each gel in 200 μl.

Automated data processing was used to compute regressions and confidence limits by classical statistical least squares methods, as well as for the calculation of the free electrophoretic mobility and valence (7).

RESULTS: Molecular size of the Hunter factor - The relative mobility of the Hunter factor was determined at gel concentrations T = 9%, 11%, 12% and 17% (Fig. 1). From a plot (Fig. 2) of log R_f versus gel



Segment	I	II	III	IV	V
Activity (Units/ml)	< 0.10	1.80	< 0.10	0.15	0.10

Slice No.	4	5	6	7	8	9	10
Activity (Units/ml)	< 0.10	< 0.10	0.10	0.16	0.75	< 0.10	< 0.10

Figure 1. Determination of the R_f of the Hunter factor activity at a gel concentration of T = 12%. In a preliminary experiment, the approximate position of the activity was located by cutting the gel into 5 segments, each of which was homogenized in 2 ml of 0.9% NaCl, dialyzed, centrifuged; the 10,000 g supernatant was analyzed for activity. The activity was found in segment II (upper row). A second gel was cut into 1.3 mm slices with a wire cutter (9); assay for activity (without dialysis) in the slices corresponding to segment II showed it to be in slice 8. As the dye was in slice 30, this corresponds to an R_f of 0.27 (see arrow). Procedures for determining R_f at other gel concentrations were similar, except that the preliminary assay of large segments was omitted; T = 9%, R_f = 0.44; T = 11%, R_f = 0.31; T = 12% (repeat), R_f = 0.29; T = 17%, R_f = 0.09.

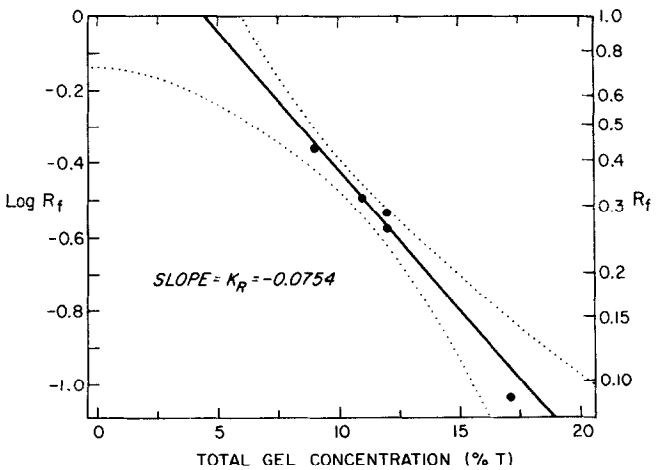


Figure 2. Ferguson plot for the Hunter factor activity. The logarithm of the R_f was plotted against gel concentration to obtain the retardation coefficient (slope of the regression line), K_R =-0.07544, and the intercept of the line with the ordinate, Y_o = 2.142. ——— weighted regression of log R_f on T; ..... 95% confidence limits for the line.

concentration ["Ferguson plot" (7,10)], the slope of the regression line was obtained. The slope, designated as retardation coefficient, K_R, is proportional to molecular size, expressed as the geometric mean radius, $\bar{R}$ (6).

A standard curve was constructed by plotting $\sqrt{K_R}$, obtained experimentally for 13 compounds (12 proteins and 1 dye) versus their geometric mean radii[2] (Fig. 3). From the standard curve the $\sqrt{K_R}$ of the Hunter factor (= 0.2747) is found to correspond to $\bar{R}$ = 2.67 nm, lower and upper 95% confidence limits being 2.01 and 3.23 nm, respectively. Conversion of molecular radius to molecular weight yields a value of 65,000, with lower and upper 95% confidence limits of 32,000 and 115,000.[3]

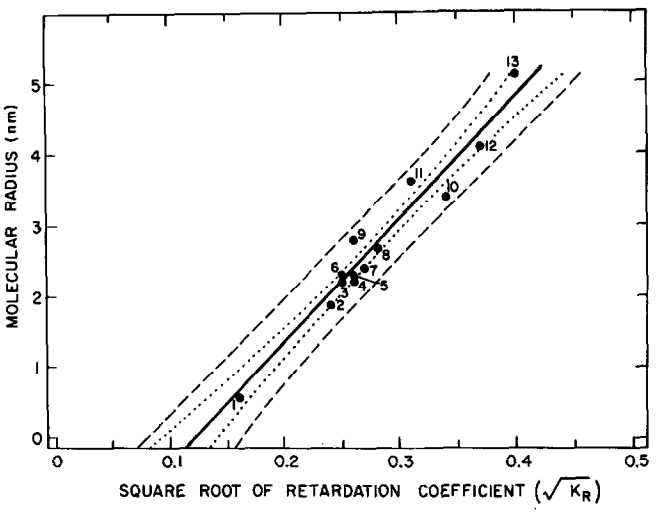


Figure 3. The square root of the experimentally determined retardation coefficients of 13 substances of known molecular weight is plotted against their geometric mean radii, $\bar{R}$. The compounds used and their molecular weights and radii (nm) are as follows: 1 = bromphenol blue, 670, 0.58; 2 = soybean trypsin inhibitor, 22,700, 1.88; 3 = pepsin, 35,500, 2.18; 4 = pepsinogen, 40,400, 2.28; 5 = ovalbumin, 43,500, 2.34; 6 = acid glycoprotein, 44,100, 2.34; 7 = fetuin, 48,400, 2.42; 8 = bovine serum albumin (BSA) monomer, 67,000, 2.70; 9 = transferrin, 74,000, 2.79; 10 = BSA dimer, 134,000, 3.40; 11 = ceruloplasmin, 160,000, 3.61; 12 = catalase, 232,000, 4.08; 13 = ferritin, 450,000, 5.09. ——— unweighted regression of radius $\bar{R}$ on $\sqrt{K_R}$; ..... 95% confidence limits for the regression line; ------ 95% confidence limits for a single observation around the line, which were used to compute the confidence limits for the radius of the Hunter factor.

[3]The wide limits for the molecular weight estimate results from a more conservative statistical treatment than is commonly used (7).

Free electrophoretic mobility and valence - Extrapolation of the Ferguson plot to zero gel concentration (Fig. 2, Y_o) provides the basis for calculating the free electrophoretic mobility (6,7) $M_o = 1.034 \times 10^{-4}$ (cm/sec)/(volt/cm). Together with the geometric mean radius, $\bar{R}$, this is used to compute, under a set of assumptions stated elsewhere (6,7), the valence of the Hunter factor molecule. The valence was found to be -11 at the operative pH of 7.95.

DISCUSSION: The physical properties of the Hunter factor derived from polyacrylamide gel electrophoresis - a molecular weight of 65,000 and a valence of -11 at pH 8 - are compatible with the structure of a protein but of no other macromolecular substance found in mammalian tissues. The negative charge per unit weight would be an order of magnitude higher for nucleic acids and for mucopolysaccharides. It is possible that the factor activity resides in a small molecule tightly bound to a protein of the stated size. The distinction, however, appears largely semantic, since the complex could be considered as the factor.

The protein nature of the factor suggests that it might be the "missing enzyme" in the Hunter syndrome. Since the band of factor activity is closely surrounded by other proteins, chemical proof of its proteinaceous nature, as well as determination of its function, must await isolation. It should be noted that in reversal of the usual order, a protein has first been recognized as such by its physical properties.

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