Huntington's-chorea fibroblasts

CELLULAR PROTEIN GLYCOSYLATION

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(Received 2 January 1980)

Five cell cultures of Huntington's-chorea fibroblasts exhibit greater than normal protein and lipid glycosylation when labelled with [U-¹⁴C]glucosamine. Oligosaccharide–polypeptide chains from all molecular-weight ranges are labelled differentially on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. This difference in protein glycosylation is not accompanied by any apparent difference in general cellular protein synthesis or by a differential rate of glucosamine uptake or decreased degradation of [¹⁴C]glycosylated macromolecules. Additionally [U-¹⁴C]glucosamine exclusively labels hexosamines and sialic acid of cellular macromolecules.

Huntington's chorea is a hereditary degenerative disease of the central nervous system transmitted as an autosomal dominant phenotype. Although Huntington's chorea is clinically expressed as a brain disease with pathological and biochemical predilection for the cortex and basal ganglia (Perry *et al.*, 1973; Bird & Iversen, 1974; Enna *et al.*, 1977), a number of studies on non-central-nervous tissues suggest that the mutation of Huntington's chorea also expresses itself in peripheral tissues (Barkley *et al.*, 1977; Butterfield *et al.*, 1977; Markesberry & Butterfield, 1977; Tourian & Hung, 1977a,b).

Nutritional studies from our laboratory have previously characterized Huntington's-chorea fibroblasts by a number of interesting differences from normal fibroblasts (Tourian & Hung, 1977a,b). Huntington's-chorea fibroblasts, for example, express a defect in attachment to the plastic substratum when grown in a nutritionally defined medium in the presence of the macromolecular fraction of serum. This defect of attachment can be corrected when Huntington's-chorea fibroblasts are grown in whole non-filtered serum. In a preliminary report we noted that this defined growth condition can be correlated with decreased protein glycosylation as measured by periodate/Schiff stain reaction and by [U-¹⁴C]glucosamine incorporation into protein (Tourian & Hung, 1978).

The nutritional studies suggest that [U-¹⁴C]glucosamine metabolism can be used to char-

acterize the differences between normal and Huntington's-chorea fibroblasts. In the present paper, we report a marked difference in the incorporation of [U-¹⁴C]glucosamine between five lines of Huntington's-chorea fibroblasts and controls matched as to age, sex, growth rate and passage number. In addition, we present preliminary characterization of their glycoproteins.

Materials and methods

Materials

Skin fibroblasts were obtained from patients ranging in age from 33 to 59 years old. The Huntington's-chorea fibroblasts were types GM-305, GM-1061, GM-1085, GM-1169 and from a 33-year-old female from our clinic and the normal fibroblasts were types GM-124, GM-967, GM-275, GM-2169 and GM-185. Additionally fibroblasts from an autosomal dominant Alzheimer's disease type GM-364 patient (53 years old), an autosomal dominant myotonic muscular-dystrophy patient (33 years old) and an autosomal recessive Charcot-Marie-Tooth disease patient (18 years old) were used for controls. The GM-designated fibroblasts were obtained from the Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ, U.S.A. Periodic checks by culture method showed the fibroblasts to be free of pleuro-pneumonia-like organisms. Culture media and dialysed foetal calf serum were obtained from GIBCO.

[U-¹⁴C]Glucosamine (sp. radioactivity 300 Ci/

Abbreviation used: SDS, sodium dodecyl sulphate.

mol), [U-¹⁴C]leucine (sp. radioactivity 310Ci/mol), [4,5,6,7,8,9-¹⁴C]CMP-sialic acid (sp. radioactivity 229Ci/mol) and Omnifluor were purchased from New England Nuclear Corporation. Ion-exchange resins (AG1; X4 and AG 50W; X4), SDS, acrylamide, methylenebisacrylamide, ammonium persulphate and Coomassie Brilliant Blue were purchased from Bio-Rad. Dithiothreitol and phenylmethanesulphonyl fluoride were obtained from Sigma. Calibration proteins (mol.wts. 20000–200000) for SDS/polyacrylamide-gel electrophoresis were purchased from Boehringer-Mannheim Biochemicals.

Growth of fibroblasts

Age, sex, passage number and growth rate of Huntington's-chorea fibroblasts were matched with normal controls. Fibroblasts were grown in Eagle's Minimal Essential medium and 20% dialysed foetal calf serum as previously described (Tourian & Hung, 1977a,b). The cell number and protein concentration of matched pairs were not statistically different (Tourian & Hung, 1977a,b). The passage number of the fibroblasts includes fibroblasts between tenth and fifteenth passage.

[U-¹⁴C]Glucosamine labelling of fibroblasts

Protein glycosylation of cells in culture varies during the cell cycle (Tenner *et al.*, 1977). Therefore it was critical to label cells in the same phase of growth. Exponentially growing fibroblasts, which were matched for growth rate, age, sex and passage number, were plated in duplicate at 3×10^5 cells per 25 cm² flask in 5 ml of Minimal Essential medium and 20% dialysed foetal calf serum. Fresh medium was added 48 h later. On the fifth day the flasks were labelled with 2 ml of [U-¹⁴C]glucosamine at 1.0 μCi/ml in fresh media, except when indicated otherwise. The following parameters were evaluated. (a) The time course of [U-¹⁴C]glucosamine uptake into cells. This fraction represents both labelled acid-soluble and acid-insoluble material. (b) Rate of accumulation into acid-soluble compounds. This fraction, which represents hexosamines and nucleotide sugars, was also evaluated by paper chromatography. (c) Rate of accumulation into acid-insoluble compounds. This fraction represents incorporation into macromolecules, which was further characterized by extraction with chloroform/methanol into glycoprotein and lipid. (d) The rate of decay of cellular acid-insoluble macromolecules was determined. (e) Rate of accumulation into acid-insoluble macromolecules in the culture medium.

SDS/polyacrylamide-gel electrophoresis

Cell monolayers from two tissue-culture flasks

(Falcon; 25 cm²) were washed twice in cold phosphate-buffered saline, pH 7.2, and were either directly solubilized in 0.5 ml of 10 mM-sodium phosphate buffer containing 2% SDS and 1 mM-phenylmethanesulphonyl fluoride or were suspended by scraping into 1.0 ml of the same buffer without SDS and precipitated by adding an equal volume of cold 10% trichloroacetic acid. The precipitates after washing twice with both 2 ml of cold 5% trichloroacetic acid and 2 ml of water were stored in a freezer as acid-insoluble material. For SDS/polyacrylamide-gel electrophoresis the acid-insoluble material was re-suspended in 0.5 ml of 10 mM-phosphate buffer containing 2% SDS and 1 mM-phenylmethanesulphonyl fluoride. Both whole-cell extract and acid-insoluble material were placed in boiling water for 3 min followed by the addition of dithiothreitol, glycerol and Bromophenol Blue to final concentrations of 0.1 M, 10% and 0.01% respectively, and were heated for a further minute. Proteins of the extracts after boiling for 3 min were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Protein (50 μg) was applied to 5% SDS/polyacrylamide cylindrical gels and electrophoresed at 8 mA/gel for 7 h in the buffer containing 0.1% SDS and 0.1 M-sodium phosphate (pH 7.2). Calibration of proteins for SDS/polyacrylamide-gel electrophoresis was routinely done. Gels were fixed and stained with 0.25% Coomassie Brilliant Blue. After destaining with methanol/acetic acid/water (5:1:5, by vol.) the gels were then scanned at 620 nm using an ISCO gel scanner. For the radioisotope-incorporation studies unfixed gels were sliced into 2 mm segments. Each segment was placed in a scintillation vial containing 100 μl of 30% H₂O₂. The vials were capped and heated at 100°C for 1.5 h in an oven. The samples were cooled and the radioactivity was counted in an Intertechnique SL-4000 liquid-scintillation counter, with 0.4% Omnifluor in a mixture of toluene/Triton X-100 (2:1, v/v).

Chloroform/methanol extract of trichloroacetic acid-insoluble material

The acid-insoluble material labelled with [U-¹⁴C]glucosamine was extracted twice with chloroform/methanol (2:1, v/v), dried under a stream of N₂ and directly counted for radioactivity in a liquid scintillation counter. The chloroform/methanol extract was designated as glycolipid.

Chemical analysis of acid-insoluble material

The [U-¹⁴C]glucosamine-labelled trichloroacetic acid-insoluble fraction was subjected to mild acid hydrolysis in 0.1 M-H₂SO₄ for 1 h at 80°C. A portion of the hydrolysate containing an internal standard of sialic acid was then put on a Dowex AG1 (X4;

formate form) column, and after washing with water, was eluted with 0.4 M-formic acid (Svennerholm, 1963). Portions of the collected fractions were assayed by the thiobarbituric acid test (Aminoff, 1961) and were counted for radioactivity in a liquid-scintillation counter.

The remaining portion of the neutralized hydrolysate was characterized after further acid hydrolysis in a vacuum-sealed test tube in 6 M-HCl for 24 h

Fig. 1. Time course of $[U-^{14}C]$ glucosamine uptake into the total cellular acid-soluble and acid-insoluble fraction of normal and Huntington's-chorea fibroblasts (a) and time course of $[^{14}C]$ glucosamine incorporation into cellular acid insoluble macromolecules (glycoprotein and glycolipid) (b) and secretion into the culture medium (c) (a) Monolayers were grown and labelled as described in the Materials and methods section. At each time point the culture medium was decanted, and the cell monolayers were washed twice with 5 ml of cold phosphate-buffered saline. The cells were then scraped off the flask in 1 ml of cold phosphate-buffered saline and total cellular radioactivity was measured in a liquid-scintillation counter. Duplicate samples were done for each time point. A portion of cells from each time point was used for protein determination. Symbols: $\times$, Huntington's-chorea fibroblasts; $\bullet$, normal fibroblasts. (b) The acid-insoluble fraction was obtained by adding cold trichloroacetic acid (10%, w/v) to a portion of cells and centrifugation of the pellet. The acid-insoluble material was further fractionated with chloroform/methanol (2:1, v/v) into glycoprotein and lipid. Duplicate samples were done for each time point. A portion of cells from each time point was used for protein determination. Symbols: $\times$, Huntington's-chorea fibroblasts; $\bullet$, normal fibroblasts. The linear regression analysis of the slopes of total ^{14}C -labelled macromolecules and ^{14}C -labelled glycoproteins and ^{14}C -labelled glycolipid accumulation in fibroblasts was calculated from $y = a_0 + a_1x$, where a_0 and a_1 are regression coefficients and s_0 and s_1 are their respective standard errors. The coefficient of determination r^2 can be interpreted as the proportion of total variation about the mean y explained by the regression.

Total cellular		Normal fibroblasts		Huntington's-chorea fibroblasts	
Expt. 1c (0-24h)					
^{14}C -labelled macromolecules	$y = 0.15x - 0.01$	0.09	0.01	$y = 0.27x - 0.08$	0.99
Expt. 2 (3-48h)					
^{14}C -labelled glycoproteins	$y = 0.27x + 1.49$	0.99	0.07	$y = 0.39x + 1.50$	1.00
^{14}C -labelled lipid	$y = 0.76x + 5.32$	0.97	0.07	$y = 1.54x + 1.45$	0.98
Total culture media					
Expt. 1 (3-48h)					
^{14}C -labelled macromolecules	$y = 12.22x + 10.3$	0.99	0.59	$y = 17.78x + 5.05$	1.00

In (c), the culture medium was treated in a similar manner as in 1(b), except 0.1 ml of medium was used instead of the fibroblasts. Symbols: $\times$, Huntington's-chorea fibroblasts; $\bullet$, normal fibroblasts.

at 100°C. The hydrolysate was put on a Dowex AG50W (X4; H⁺ form) column and eluted with 0.4M-HCl, after washing with water, by a modification of Gardell's procedure (Gardell, 1953). The eluate was dried by flash evaporation, re-dissolved in a known volume and identified by high-voltage paper electrophoresis on CAMAG 68 paper (39.4 cm × 15.2 cm) at 2500 V for 2.5 h in a pyridine/acetic acid solvent system (200 ml of pyridine and 8 ml of acetic acid made up to 3 litres with water, pH 6.4).

Chromatographic identification of hexosamines and nucleotide sugars of the cellular acid-soluble fraction

The acid-soluble fraction from the trichloroacetic acid-precipitated cell suspensions were neutralized with 1M-NaOH. Equal counts of radioactivity from both normal and Huntington's-chorea fibroblast extracts were chromatographed on Whatman 3 MM paper by ascending chromatography by the method of Plageman & Erbe (1973). The developed chromatograms were cut into segments (1.5 cm × 3 cm) and were counted for radioactivity directly by liquid-scintillation spectrometry with a counting efficiency of 62%. Non-labelled and labelled standard sugars and nucleotide sugars were used for reference.

Results

The time course of [U-¹⁴C]glucosamine labelling of fibroblasts and ¹⁴C-labelled macromolecules secreted into the culture medium

The time course of [U-¹⁴C]glucosamine uptake (acid-soluble and acid-insoluble) into exponentially growing fibroblasts was biphasic (Fig. 1a). This biphasic time course has also been previously observed by Bosman *et al.* (1969) in human cell cultures. A time-dependent difference of [U-¹⁴C]glucosamine incorporation into acid-insoluble macromolecules between Huntington's-chorea and normal fibroblasts was observed (Fig. 1b). More [U-¹⁴C]glucosamine was incorporated into glycoprotein and lipid by Huntington's-chorea fibroblasts (see legends to Fig. 1b). Three pairs of Huntington's-chorea and normal fibroblasts were tested for the time-course experiments with similar results.

The culture media were also evaluated for the accumulation of acid-insoluble ¹⁴C-labelled macromolecules. There was a time-dependent difference of ¹⁴C-labelled macromolecules secreted into the medium by Huntington's-chorea fibroblasts, which can be observed at 24 and 48 h (Fig. 1c).

It should be noted that the acid-insoluble macromolecular material secreted into the culture medium represents a major fraction of [¹⁴C]glucosamine-

labelled macromolecules synthesized by the cells (1C).

The decay of ¹⁴C-labelled macromolecules

Matched pairs of exponentially growing fibroblasts were labelled with 1 μCi of [U-¹⁴C]glucosamine/ml for 48 h. The labelled medium was removed and the cells were washed twice with fresh medium followed by addition of fresh medium and the decay of ¹⁴C-labelled acid-insoluble material in cells was evaluated at 0, 6, 12, 24, 48 and 68 h. The ¹⁴C-labelled acid-soluble material decays to negligible values after 12 h of the removal of [U-¹⁴C]glucosamine from the culture medium, indicating that re-utilization of label will not result in an underestimate of the true degradation rate between 12 and 68 h. The total ¹⁴C-labelled cellular and macromolecular material of Huntington's-chorea fibroblasts showed no differential rate of degra-

Fig. 2. The rate of decay of ¹⁴C-labelled macromolecules by fibroblasts

Exponentially growing fibroblasts were labelled for 48 h with 1 μCi of [U-¹⁴C]glucosamine/ml in an identical manner to that described in the legend to Fig. 1. The medium was changed to one not containing the isotope and the decay of ¹⁴C acid-insoluble macromolecular radioactivity evaluated at 0, 6, 12, 24, 48 and 68 h. Symbols: ×, Huntington's-chorea fibroblasts; ●, normal fibroblasts.

dation to account for the greater [^{14}C]glycosylation of Huntington's-chorea glycoproteins and lipids (Fig. 2).

SDS/polyacrylamide-gel electrophoresis of [^{14}C]glucosamine-labelled fibroblasts

Whole-cell extracts or acid-insoluble material were re-solubilized as described in the Materials and methods section, and were electrophoresed on SDS/polyacrylamide gels. A representative example

of SDS/polyacrylamide-gel electrophoresis of whole-cell extracts and acid-insoluble macromolecular fraction re-solubilized in SDS are shown (Figs. 3a and 3b). Repeated testing of such labelled fibroblasts showed a consistent 100–200% greater radiolabelling of oligosaccharide–polypeptide chains by five cell cultures of Huntington's-chorea fibroblasts than by normal fibroblasts. This greater labelling of Huntington's-chorea fibroblasts was not observed in fibroblasts similarly labelled from whole-cell extracts

Fig. 3. Representative SDS/polyacrylamide gels of (a) [^{14}C]glucosamine-labelled fibroblasts from normal, Huntington's-chorea and other neurologically diseased subjects and (b) [^{14}C]glucosamine-labelled acid-insoluble macromolecules from normal and Huntington's-chorea fibroblasts

(a) Cells were plated and grown as described in the Materials and methods section and on the fifth day the flasks were labelled with 2 ml of [^{14}C]glucosamine (sp. radioactivity 300 Ci/mol; $8.37 \mu\text{M}$) at $2.5 \mu\text{Ci/ml}$ for 48 h. The cell monolayers were treated as described in the Materials and methods section. —, Huntington's chorea; ----, normal; - - - - -, Dominant Alzheimer's Disease; $\times \times \times \times$, Charcot-Marie-Tooth disease. (b) —, Huntington's-chorea fibroblasts; ----, normal fibroblasts. Abbreviation used: TD, tracking dye.

from fibroblasts from patients with autosomal dominant Alzheimer's disease, autosomal dominant myotonic muscular dystrophy or recessive homozygote Charcot-Marie-Tooth disease (Fig. 3a).

Fibroblasts from patients with dominant Alzheimer's disease and Charcot-Marie-Tooth Disease do show differences in protein glycosylation of glycopeptides, especially those with molecular weights greater than 150 000. Such differences were never observed in normal fibroblasts. The resolution of the 2-mm sliced gels did not allow the detailed analysis of minimal mobility differences of individual oligosaccharide-polypeptide chains for normal and Huntington's-chorea fibroblasts, even when [^3H]- and [^{14}C]-glucosamine-labelled cell extracts were mixed in SDS/polyacrylamide-gel-electrophoresis profile studies.

It can be observed that there is some variation between whole-cell extracts and re-solubilized acid-insoluble material on SDS/polyacrylamide-gel-electrophoresis profiles (Figs. 3a and 3b). This consistent variation could result from incomplete acid-precipitation or re-solubilization of macromolecules.

Recovery of macromolecular ^{14}C radioactivity on SDS/polyacrylamide-gel electrophoresis was in the range of 60–80% as judged by evaluating re-solubilized acid-insoluble macromolecules. One-third of the cellular acid-insoluble macromolecular label is in the chloroform/methanol-extracted material. Therefore, the recovery of oligosaccharide-poly-

peptide on SDS/polyacrylamide-gel electrophoresis is very good. The recovery of ^{14}C radioactivity on SDS/polyacrylamide-gel electrophoresis cannot explain the consistent difference of greater radio-labelling of oligosaccharide-polypeptide in Huntington's-chorea fibroblasts compared with matched normal fibroblasts.

Non-labelled whole-cell extract (50 μg) was electrophoresed with a mixture of [^{14}C]glucosamine (25 000 c.p.m.), [^{14}C]glucosamine 6-phosphate (25 000 c.p.m.), [^{14}C]N-acetylglucosamine 6-phosphate (15 000 c.p.m.) and [^{14}C]CMP-sialic acid (15 000 c.p.m.). All of the soluble label from the exogenously added [^{14}C]hexosamine migrated ahead of the tracking dye. One-third of the [^{14}C]CMP-sialic acid migrated just behind the tracking dye. However, this low-molecular-weight region (gel slices 35–41) of oligosaccharide-polypeptide on SDS/polyacrylamide-gel electrophoresis was not the region where the large difference of [^{14}C]glucosamine labelling by fibroblasts was observed.

Quantification of the fate of the ^{14}C -labelled material resulted in no unaccountable label on top of the gel, such as high-molecular-weight material not entering the gel, from either Huntington's-chorea or normal fibroblasts. The acid-insoluble material was extracted with chloroform/methanol, followed by four times re-extraction with saline and separation into upper and lower phases (Folch *et al.*, 1957). The extracts were dried in a flask evaporator and

Fig. 4. SDS/polyacrylamide gels of [$U\text{-}^{14}\text{C}$]leucine-labelled normal, Huntington's-chorea and other neurologically diseased fibroblasts

The fibroblasts were plated and grown identically as described in Fig. 1 except that on the fifth day the cells were fed with 2 ml of the same medium containing [$U\text{-}^{14}\text{C}$]leucine at $2.5 \mu\text{Ci/ml}$. At the end of 48 h incubation cells were harvested and prepared as described in Fig. 1. —, Huntington's chorea; ----, normal; - · - · -, Dominant Alzheimer's disease; x x x x, Charcot-Marie-Tooth disease. Abbreviation used: TD, tracking dye.

Table 1. *Sialic acid and hexosamine analysis of cellular acid-insoluble material*

The chemical analysis of cellular acid-insoluble material was done as described in the Materials and methods section.

Fibroblasts	[¹⁴ C]Hexosamine (c.p.m.)	Acid-insoluble radioactivity (% of total)	[¹⁴ C]Sialic acid (c.p.m.)	Acid-insoluble radioactivity (% of total)
Normal	160 654	93	5986	3
Huntington's chorea	199 796	96	7740	3

Fig. 5. Densitometric scan of stained SDS/polyacrylamide gels

Fibroblasts were plated, grown and labelled identically as described in Fig. 3 under three sets of conditions: (1) no addition of labelled [¹⁴C]leucine or [¹⁴C]glucosamine, but addition of fresh media; (2) [¹⁴C]leucine (2.5 µCi/ml); (3) [¹⁴C]glucosamine (2.5 µCi/ml). The densitometric scans of Coomassie Blue-stained gels were made at A₆₂₀ with an ISCO gel scanner. (a) represents normal and (b) Huntington's-chorea fibroblasts.

were combined and electrophoresed. All of the ¹⁴C-labelled lipid migrated ahead of the tracking dye, indicating that none of the labelled peaks on the gel represented glycolipid.

The trichloroacetic acid-insoluble macromolecules (labelled with [¹⁴C]glucosamine for 48h) were further analysed by acid hydrolysis in 0.1 M-H₂SO₄ for 1 h at 80°C, followed by acid hydrolysis for 24 h in 6 M-HCl, followed by high-voltage paper electrophoresis. The ¹⁴C radioactivity incorporated in the trichloroacetic acid-insoluble residue could be

accounted for by sialic acid and hexosamines (Table 1). No ¹⁴C-labelled amino acids were detected in the acid hydrolysates, showing that there was no detectable conversion of labelled hexosamines into labelled amino acids of the polypeptide chains.

[U-¹⁴C]Leucine incorporation by Huntington's-chorea and normal fibroblasts

Exponentially growing fibroblasts were labelled with [U-¹⁴C]leucine at 2.5 µCi/ml for 48h under identical conditions described above for [U-¹⁴C]-glucosamine experiments. The cells were processed in an identical fashion as for [U-¹⁴C]glucosamine experiments. There was no significant difference in the amount of total cellular protein from normal and Huntington's-chorea fibroblast cultures. The profiles of [U-¹⁴C]leucine-labelled polypeptide on SDS/polyacrylamide-gel electrophoresis were identical for Huntington's-chorea and normal and other neurological-disease fibroblasts (Fig. 4). Densitometric scans of Coomassie Blue-stained gels of Huntington's-chorea and normal fibroblast extracts were similar under three sets of conditions: (1) no addition; (2) [U-¹⁴C]leucine, 2.5 µCi/ml; and (3) [U-¹⁴C]glucosamine, 2.5 µCi/ml. Since the densitometric scans under the three conditions were the same only one scan is shown for control and one for Huntington's-chorea (Fig. 5). Thus, no apparent difference in general cellular protein synthesis could be detected that would account for increased [U-¹⁴C]glucosamine incorporation into glycoprotein.

Discussion

Huntington's-chorea fibroblasts are characterized by greater protein and lipid glycosylation compared with normal and other neurological diseases when labelled with [U-¹⁴C]glucosamine. Additionally, ¹⁴C-labelled acid-insoluble macromolecules secreted into the culture medium are also labelled to a greater extent in Huntington's-chorea fibroblasts. We have shown that [U-¹⁴C]glucosamine exclusively labels hexosamines and sialic acid of cellular macromolecules. Increased glycosylation of proteins in Huntington's-chorea fibroblasts is not accompanied by any differential rate of glucosamine uptake into cells or degradation of ¹⁴C-labelled macromolecules.

The profile of [U-¹⁴C]leucine-incorporated oligopeptides on SDS/polyacrylamide-gel electrophoresis is identical for Huntington's-chorea, normal and other neurological disease fibroblasts, which indicates that there is no apparent difference in general cellular protein synthesis.

There are a number of hypotheses that can be advanced to explain increased [U-¹⁴C]glucosamine incorporation into glycopeptides and glycolipids in Huntington's-chorea fibroblasts, some of which are discussed below.

(a) The most obvious explanation for the results as they stand is that the specific radioactivity of cellular [U-¹⁴C]glucosamine is higher in Huntington's-chorea fibroblasts, since all glycoprotein components are affected, whereas protein synthesis remains normal.

There does not appear to be any apparent difference in incorporation of [U-¹⁴C]glucosamine into cell-associated trichloroacetic acid-insoluble macromolecules in the first 3 h of labelling or for the first 12 h for the secreted macromolecules in culture medium. There are many sequential steps before the incorporation of [U-¹⁴C]glucosamine into glycopeptides. The labelled [U-¹⁴C]glucosamine has to proceed from a precursor to product pool of molecules for each of these sequential steps. Equilibration of these pools with [U-¹⁴C]glucosamine $\rightarrow$ nucleotide sugars $\rightarrow$ dolichyl sugars $\rightarrow$ glycopeptides may require time to reflect a pool-size difference between a normal and a heterozygote cell line. The gene-dosage principle would predict that the locus for the hemizygous HD gene would express 50% of a given enzyme or structural protein due to mutation since one of its alleles for the mutated site is normal. Thus any observed difference from normal cannot be absolute but a relative one. It has been observed that abnormal accumulation of cellular ³⁵S-labelled mucopolysaccharides cannot be recorded for the first 4 h of time-course measurements in the autosomal recessive homozygous Hurler fibroblasts in culture. Since the level of cellular macromolecules at any given time is the net result of synthesis, degradation, secretion and re-uptake of the macromolecules of interest (Fratantoni *et al.*, 1968), it is not surprising that even in an autosomal recessive mutation, it requires time to observe a fundamental aberration of cellular macromolecular metabolism.

It follows that if the pool size of hexosamines is smaller in Huntington's-chorea fibroblasts, the culture-added [U-¹⁴C]glucosamine will have a higher specific radioactivity and result in an apparent increase in [¹⁴C]hexosamine incorporation. Our calculations show that the total uptake of [U-¹⁴C]glucosamine at the end of 48 h was 0.74 nmol/mg of protein (Fig. 1a). The [U-¹⁴C]glucosamine concentration in the medium in our experiments is

one to two orders of magnitude lower than that of recorded values of concentrations of glucosamine in the medium that can expand the UDP-*N*-acetylhexosamine pool size of mammalian cells (Kornfeld & Ginsberg, 1966; Bekesi & Winzler, 1969). This hypothesis remains to be verified.

(b) If the [¹⁴C]glycosylated macromolecules are degraded at a lower rate in Huntington's-chorea fibroblasts, ¹⁴C-labelled glycopeptides and lipid will be accumulated in Huntington's-chorea fibroblasts, resulting in an apparent greater incorporation of [¹⁴C]hexosamines. We have shown this mechanism not to be the case in the ¹⁴C-labelled-macromolecule decay experiments.

(c) Protein glycosyltransferases are generally specific and do not utilize lipid substrates (Kaufman *et al.*, 1967; Den *et al.*, 1970, 1975). Since in Huntington's-chorea fibroblasts both protein and lipid glycosylation rates were higher, it would be unlikely that increased glycosylation of peptides and lipids is due to an aberrant increase in the activity of a specific glycosyltransferase, even though we have not eliminated such a possibility.

(d) [¹⁴C]Glucosamine, [¹⁴C]hexosamines or [¹⁴C]sialic acid may be rapidly depleted in normal fibroblasts because of utilization through pathways not available to Huntington's disease fibroblasts, which will then result in an apparent increase in [¹⁴C]hexosamine incorporation by Huntington's-chorea fibroblasts. The analysis of total ¹⁴C-accumulated radioactivity in acid-insoluble and macromolecular fractions, the subfractions examined and the chromatographic analysis of hexosamines, and nucleotide sugars in the soluble compartment of the cell and the culture media do not support this hypothesis.

(e) Finally, there is always the possibility that protein and lipid glycosylation rates are different in Huntington's-chorea for unknown reasons, but will become obvious as work progresses in this field.

We thank Dr. Bernard Kaufman of the Biochemistry Department for his encouragement and advice during the progress of this project. This work was supported in part by The Committee to Combat Huntington's Chorea, by the National Foundation March of Dimes C.R.B.S. 283, U.S.P.H.S. grant no. 2 R01 AM 14834, and by the North Carolina United Way. W.-Y.H. is a Post-Doctoral Fellow of the NIH on Aging (grant no. 5T32 AG00007).

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