Alterations in Copper and Collagen Metabolism in the Menkes Syndrome and a New Subtype of the Ehlers–Danlos Syndrome†

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ABSTRACT: Cultured fibroblasts of 13 patients with the Menkes syndrome and two with a new subtype (type IX) of the Ehlers–Danlos syndrome (E-D IX patients) showed many very similar abnormalities in their copper and collagen metabolism. Both cell types had markedly increased copper concentrations and ^{64}Cu incorporation, and this cation accumulated in metallothionein or a metallothionein-like protein, as previously established for Menkes cells. Histochemical staining indicated that copper was distributed diffusely throughout the cytoplasm in both cell types, this location being consistent with the accumulation in metallothionein. Both fibroblast types also had markedly low lysyl oxidase activity and distinctly increased extractability of newly synthesized collagen, whereas no abnormalities were present in cell viability, duplication rate, prolyl 4-hydroxylase activity, or collagen synthesis rate. A high negative correlation ($P < 0.001$) was found in the pooled group of Menkes and E-D IX cells between cellular copper concentration ($r = 0.804$) or ^{64}Cu incorporation ($r = 0.863$) and the logarithm of lysyl oxidase activity. There was also a high positive correlation ($P < 0.001$) between cellular copper concentration and incorporation ($r = 0.869$). One of the two E-D IX patients was also shown to have similar changes in lysyl oxidase activity and collagen extractability in the skin biopsy specimen, suggesting that the abnormalities observed in cultured cells are similar to those present in vivo. The only distinct abnormality found in the cells of the parents of the E-D IX patients was an increased ^{64}Cu incorporation in those of the mother, this finding being consistent with X-linked inheritance of the disorder.

The Menkes steely hair syndrome is characterized by abnormalities of the hair, arteries, and bones and by progressive cerebral degeneration, with death usually by 3 years of age (Danks, 1983). A similar array of abnormalities is found in the mottled series of allelic mutant mice (Danks, 1983). In both X-linked disorders, intestinal copper absorption is deficient, serum copper and ceruloplasmin concentrations are low, and cultured fibroblasts and many other cells have markedly elevated amounts of this cation (Goka et al., 1976; Horn, 1976, 1981, 1983a; Danks, 1977, 1983; Camakaris et al., 1980; Kivirikko & Peltonen, 1982). The mutant cells also have increased amounts of metallothionein, but it is not known whether this is a primary alteration or an event secondary to abnormal copper metabolism (Bonewitz & Howell, 1981; Labadie et al., 1981a,b; Horn, 1983a). The connective tissue abnormalities have been attributed to a reduction in the activity of lysyl oxidase, a copper enzyme that initiates the cross-linking of collagen and elastin (Siegel, 1979). Lysyl oxidase activity is low in the mottled mice mutants both in tissue extracts (Rowe et al., 1977; Starcher et al., 1977) and in the medium of cultured fibroblasts (Starcher et al., 1977, 1978). Assays in the medium of cultured Menkes fibroblasts have given conflicting results, however, as the activity has been found to be unaltered (Siegel, 1979), only slightly decreased (Starcher et al., 1978), or markedly low (Royce et al., 1980).

Abnormalities in copper metabolism closely resembling those in the Menkes syndrome have recently been reported by us in two brothers with a heritable connective tissue disorder tentatively classified as one subtype of the Ehlers–Danlos syndrome (Kuivaniemi et al., 1982), termed here type IX of the syndrome. The clinical manifestations included bladder diverticula with spontaneous ruptures, inguinal hernias, slight skin laxity and hyperelasticity, and several skeletal abnormalities, a peculiar feature being occipital horn-like exostoses (Kaitila et al., 1982). Copper and ceruloplasmin concentrations in the serum and the copper concentration in the hair were all low, whereas the amount of this cation in cultured skin fibroblasts was markedly increased (Kuivaniemi et al., 1982). Lysyl oxidase activity was low in the medium of cultured skin fibroblasts, and this abnormality was accompanied by a reduced conversion of the newly synthesized collagen to the insoluble form (Kuivaniemi et al., 1982).

The present work examines further the changes in copper and collagen metabolism in the Menkes syndrome and the new subtype IX of the Ehlers–Danlos syndrome (E-D IX patients).[1] Special emphasis is laid on the question of whether there are any distinct differences in the biochemical abnormalities between the two disorders. Most of the work was carried out on cultured cells, but some measurements of lysyl oxidase activity and collagen solubility were also performed on skin biopsy specimens from one E-D patient to find out whether the changes observed in cultured cells are similar to those present in vivo.

Materials and Methods

Cell Lines and Skin Specimens. Menkes fibroblast cultures were established from skin biopsies of 11 definite and typical patients (Danks, 1983) aged 1–29 months (mean 9 months), one atypical patient aged 11 years, and one fetus of 21 weeks of gestation. The atypical patient had low serum copper and ceruloplasmin concentrations, pili torti, slowly processing mental and neurological manifestations, and consistently abnormal ^{64}Cu incorporation (Horn, 1976, 1981) into cultured skin fibroblasts. The fetus had a positive family history and abnormal ^{64}Cu incorporation (Horn, 1976, 1981). The Menkes

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Received June 21, 1983. This work was supported in part by grants from the Medical Research Council of the Academy of Finland and the Paulo Foundation.

[1] The terms E-D IX patients, E-D IX cells, and E-D IX family are used to indicate the two patients with the new subtype IX of the Ehlers–Danlos syndrome and their cells and family.

0006-2960/83/0422-6156$01.50/0

cell lines were numbered M1–M13 according to the severity of the abnormality in ^{64}Cu incorporation, M1 having the most severe degree of abnormality and M13 the mildest. The cells of the atypical case are M12 and the fetal cells M11.

The E-D family fibroblast cultures were established from skin biopsies of two patients aged 23 (E-D IX 1) and 10 years (E-D IX 2) having abnormal copper metabolism and various connective tissue manifestations (Kuivaniemi et al., 1982; Kaitila et al., 1982), and their clinically healthy parents aged 45 (mother) and 52 years (father). Control fibroblast cultures were established from skin specimens from 12 apparently healthy control subjects and three fetuses. Seven of the controls were aged 1–28 months (mean 10 months), and 5 were aged 7–46 years (mean 24 years). All the primary Menkes cultures and six of the control cultures were established in Copenhagen and subcultured in Oulu.

Skin specimens for the study of lysyl oxidase activity and collagen extractability were obtained from one E-D IX patient (E-D IX 1) and 11 control subjects with no detectable abnormalities in copper metabolism or connective tissues. The E-D biopsy and one control sample were used for studying both lysyl oxidase activity and collagen extractability, whereas the other samples were used only for one of the tests each. The ages of the controls are given under Results. The skin specimens were stored at −20 °C until used for the assays. All skin specimens used to establish cell cultures or to perform direct assays had been obtained either for diagnostic purposes or with the permission of the persons concerned or in connection with surgical operations and followed the principles of the Declaration of Helsinki.

Cell Cultures. The cells were grown in 65-cm^2 plastic tissue culture dishes in 10 mL of Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 290 μg/mL L-glutamine. For the measurement of lysyl oxidase activity, fibroblasts that had just reached confluent density were washed twice with a solution of 0.01 M sodium phosphate and 0.14 M NaCl, pH 7.4 (phosphate-buffered saline), and then cultured for 24 h in 5 mL of the above medium without serum but supplemented with 5 mg/mL bovine serum albumin (corresponding to an albumin concentration of 10% serum).

Metallothionein-like Protein. A total of 2 × 10^6 fibroblasts was inoculated onto the 65-cm^2 plastic dishes in the medium described above and allowed to attach for 4 h. The medium was then replaced with fresh medium containing 5 μCi of [^{35}S]cystine (300 Ci/mol, Amersham), and the incubation was continued for 96 h (Bonewitz & Howell, 1981). In some experiments, $ZnCl_2$ was present during this incubation in a concentration corresponding to 5 μg/mL Zn. The cells were then scraped into 6 mL of 10 mM tris(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl) buffer, pH 8.6, at 4 °C, homogenized with a Teflon/glass homogenizer (20 strokes), sonicated 4 times for 10 s in an ice bath, and centrifuged at 10000*g* for 30 min. The supernatant was bubbled with N_2, and 4 mL was applied to a column (2.5 × 80 cm) of Sephadex G-75 equilibrated and eluted with 10 mM Tris-HCl buffer, pH 8.6, at 4 °C. Fractions of 5 mL were collected, and aliquots of 0.5 mL were assayed for radioactivity in 5 mL of Lumagel (Wallac). Fractions of the metallothionein-like protein peak were pooled, lyophilized, dissolved in 2 mL of 0.5% sodium deoxycholate, and assayed for copper concentration.

Localization of Copper in Cultured Cells. The cells were grown to confluency on coverslips, fixed for 5 min in a solution of 4% glutaraldehyde and 0.2 M sodium cacodylate, pH 7.2, stained by a sulfide–silver method (Scheuer et al., 1967), and examined by light microscopy.

Extraction of Newly Synthesized Collagen from Cell Layers of Cultured Fibroblasts. Fibroblast cultures just reaching confluency were labeled with 8 μCi of [^{14}C]proline (320 Ci/mol, Amersham) for 48 h. The cell layers were then washed twice with phosphate-buffered saline, scraped into 1 mL of 1 M NaCl, homogenized with a Teflon/glass homogenizer (50 strokes), and extracted at 4 °C for 24 h. The pellet remaining after centrifugation at 15000*g* for 30 min at 4 °C was then extracted sequentially at 4 °C for 24 h with 1 mL of 0.5 M acetic acid and 1 mL of 8 M urea. The extracts and the insoluble residue were hydrolyzed in 6 M HCl at 120 °C for 24 h and assayed for hydroxy[^{14}C]proline (Juva & Prockop, 1966).

Extraction of Collagen from Skin Biopsy Specimens. The frozen skin specimens were homogenized with an Ultra-Turrax homogenizer 4 times for 5 s at 0 °C in a solution of 1 M NaCl and 50 mM Tris-HCl, pH 7.4 (1 mL/20 mg of skin). The specimens were then extracted sequentially for 24 h with this solution, 0.5 M acetic acid and 8 M urea, as in the case of the cell layers. The extracts and residue were hydrolyzed in 6 M HCl at 120 °C for 24 h and assayed for hydroxyproline (Kivirikko et al., 1967).

Assays of Enzyme Activities. Lysyl oxidase activity was measured in a final volume of 0.6 ml with 0.8 × 10^6 dpm of [6-^3H]lysine-labeled purified chick embryo calvaria collagen substrate (Siegel, 1974), the incubation time with the enzyme being 10 h. The substrate was prepared as described elsewhere (Siegel, 1974), except that 500 μCi of the isotope was used per 200 16-day chick embryo calvaria, this having a specific activity of about 6 × 10^{15} dpm/mol of collagen. In the case of the cell cultures, the media, which had been stored frozen at −20 °C for up to 1 month, were dialyzed for 3 h at 4 °C against a solution of 0.15 M NaCl and 0.1 M sodium phosphate, pH 7.8, and aliquots of 0.25 and 0.50 mL were then used for the assays without any prior concentration. In the case of the skin biopsy specimens, frozen samples of about 25 mg were cut into small pieces with scissors, homogenized with an Ultra-Turrax homogenizer at 0 °C 6 times for 5 s in 3 mL of a solution of 0.15 M NaCl and 0.1 M sodium phosphate, pH 7.8, and centrifuged at 10000*g* for 30 min at 4 °C. The pellets were resuspended into 1.0 mL of 6 M urea in 0.05 M Tris-HCl buffer, pH 7.8, at 4 °C and stirred at 4 °C for 16 h. The supernatants obtained by centrifugation at 10000*g* for 30 min were dialyzed at 4 °C against a solution of 0.15 M NaCl and 0.1 M sodium phosphate, pH 7.8, for 8 h and assayed for lysyl oxidase activity. All lysyl oxidase assays were carried out under conditions in which the relationship between the amount of enzyme added and the product formed was linear. Prolyl 4-hydroxylase activity in the cultured cells was determined with [^{14}C]proline-labeled protocollagen as the substrate (Kivirikko & Myllylä, 1982).

Other Assays. Copper-64 incorporation into cultured fibroblasts over 20 h (Horn, 1981) and cellular copper concentrations (Royce et al., 1980) were determined as described previously. The numbers of the cells were measured both by the counting of viable cells in a hemocytometer and by the assay of DNA content (Brunk et al., 1979). The cellular protein concentration was also determined in some experiments (Lowry et al., 1951; Horn, 1981).

Results

Cellular Copper Concentrations and Incorporation. Cultured skin fibroblasts from patients with the Menkes syndrome (Goka et al., 1976; Horn, 1976, 1981, 1983a; Danks, 1977,

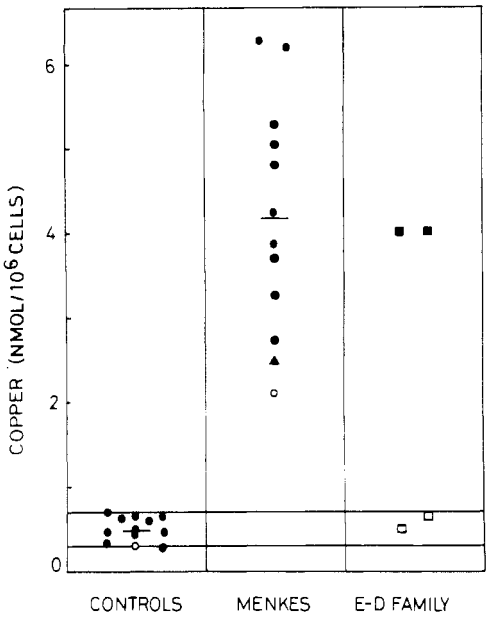

FIGURE 1: Copper concentrations in cultured fibroblasts. Each value is the mean of two to five duplicate assays. One Menkes cell line (M10) was lost before assay of the copper concentration. The long horizontal lines indicate the ranges of the control values and the short lines the means for the various groups. (○) Fetal fibroblasts; (▲) atypical Menkes fibroblasts (M12); (●) other control and Menkes fibroblasts; (■) E-D IX fibroblasts; (□) fibroblasts of the parents of the E-D IX patients (father on the left).

1983; Camakaris et al., 1980; Kivirikko & Peltonen, 1982) and the two E-D IX patients (Kuivaniemi et al., 1982) have increased cellular copper concentrations. Exact comparison of the published values is impossible, however, as the copper concentrations vary greatly with relatively minor differences in the culture conditions. A comparison was therefore made of copper concentrations in cultured Menkes and E-D IX fibroblasts grown and assayed in the same laboratory under identical conditions. No difference was found between these two groups, the mean for both of them being about 8 times that of the controls (Figure 1). The copper concentrations in the cells of the clinically healthy E-D IX parents were not abnormal, although the cells of the mother had a concentration close to the upper range of the control values (Figure 1).

Incorporation of radioactive copper into cellular proteins during an incubation for 20 h is known to be increased in cultured Menkes fibroblasts (Horn, 1976, 1981), whereas no data are available for E-D IX cells. The present experiments to study this aspect indicated a distinct increase in the ^{64}Cu incorporation of E-D IX cells (Figure 2) when compared with the 95% confidence limits determined in the same laboratory for 37 control cell lines (Horn, 1983b). The values in the 13 Menkes cell lines were again very similar to those in the E-D IX fibroblasts. The cells of the father of the E-D IX patients showed an incorporation close to the mean of the normal values, whereas the cells of the mother had an incorporation distinctly above the 95% confidence limit (Figure 2) and even above the 99% confidence limit (Horn, 1983b).

A highly significant correlation ($r = 0.869$, $P < 0.001$) was found between copper concentration and incorporation for the pooled group of Menkes and E-D IX cells (Figure 3), the regression line calculated with the control cells omitted passing close to the intersection point of the two control means. The cells of the two E-D IX patients did not show any distinct deviation from the regression lines calculated either for only the Menkes fibroblasts or for the pooled group of both cell types (Figure 3).

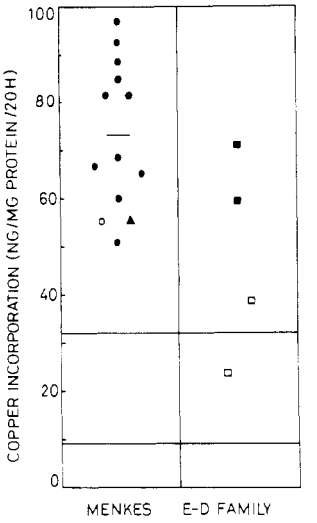

FIGURE 2: Copper incorporation into cellular proteins of cultured fibroblasts during an incubation of 20 h. Each value shown is the mean of two to ten duplicate assays. The long horizontal lines indicate the 95% confidence limits determined in the same laboratory for 37 control cell lines, and the short line indicates the mean for the Menkes fibroblasts. (○) Fetal Menkes fibroblasts; (▲) atypical Menkes fibroblasts (M12); (●) other Menkes fibroblasts; (■) E-D IX fibroblasts; (□) fibroblasts of the parents of the E-D IX patients (father on the left).

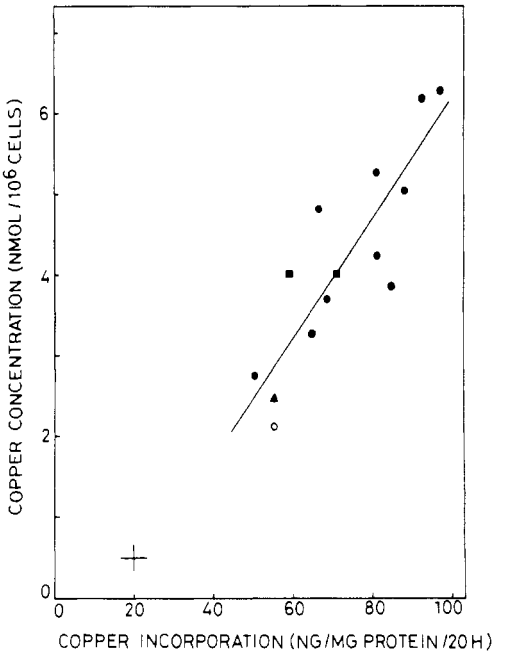

FIGURE 3: Relationship between cellular copper concentration and incorporation in cultured Menkes and E-D IX fibroblasts. The regression line was calculated for the pooled group of Menkes and E-D IX cells ($r = 0.869$, $P < 0.001$). (○) Fetal Menkes fibroblasts; (▲) atypical Menkes fibroblasts (M12); (●) other Menkes fibroblasts; (■) E-D IX fibroblasts. The short horizontal and vertical lines indicate the two control means.

Synthesis of a Metallothionein-like Protein. When cultured Menkes fibroblasts or Zn- or Cu-supplemented normal cells are incubated with [^{35}S]cysteine or [^{35}S]cystine, this amino acid becomes incorporated into a cytosolic protein that can be separated as a radioactivity peak in gel filtration (Bonewitz & Howell, 1981; Labadie et al., 1981a). This protein is synthesized in much smaller amounts by normal fibroblasts cultured without metal supplementation, and the protein has been identified as metallothionein by a number of criteria (Bonewitz & Howell, 1981; Labadie et al., 1981a,b; Horn,

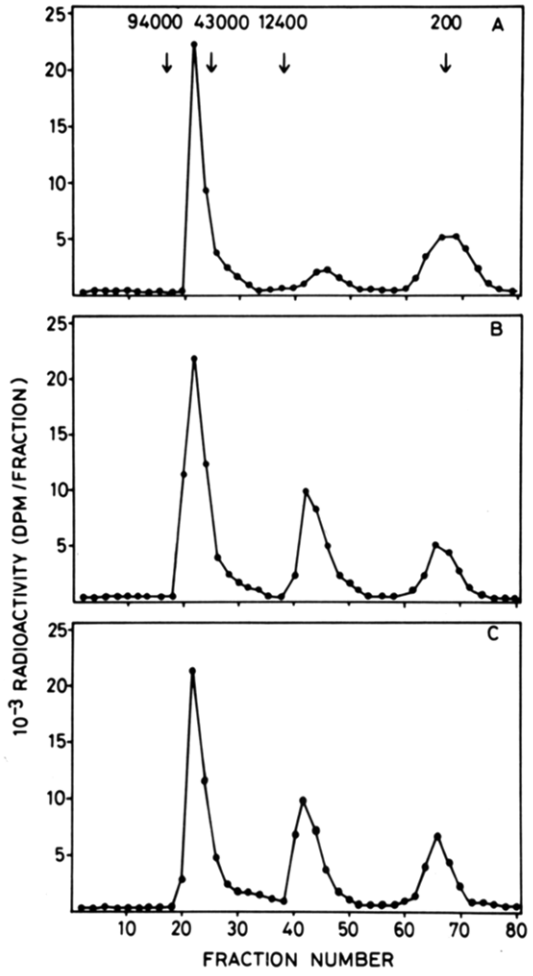

FIGURE 4: Gel filtration on Sephadex G-75 of [^{35}S]cystine-labeled cytosolic proteins of cultured control (A), Menkes (B), and E-D IX (C) fibroblasts. The cells were labeled with [^{35}S]cystine for 96 h, and the cytosolic proteins were chromatographed as described under Materials and Methods. The elution positions of the molecular weight standards are indicated with arrows.

1983a). A peak of radioactivity with identical elution behavior (second peak in Figure 4) with that studied by others (Bonewitz & Howell, 1981; Labadie et al., 1981a,b) was found when cytosolic proteins from cultured Menkes fibroblasts (Figure 4B) or Zn-supplemented control cells (not shown) were labeled by incubation of the cells with [^{35}S]cystine for 96 h and then studied by gel filtration. Only a small radioactivity peak was found in the control fibroblasts incubated without Zn supplementation (Figure 4A), whereas the peak was very distinct in the E-D IX cells incubated without any metal supplementation (Figure 4C).

Pooled fractions of this peak were concentrated by ultrafiltration and analyzed for copper by atomic absorption spectrophotometry. The peak from the normal cells contained 0.19 ± 0.07 nmol of this cation per 10^6 cells (mean ± SD of four chromatographies), while those from the E-D IX and Menkes cells contained 3.30 ± 0.61 and 3.10 ± 0.55 nmol, respectively.

Histochemical Localization of Accumulated Copper. Since the intracellular location of the excess copper in cultured Menkes and E-D IX fibroblasts is unknown, this aspect was studied by using silver staining techniques similar to those employed in work on copper localization in liver specimens (Scheuer et al., 1967). Little staining was found in the control cells (Figure 5A), whereas the Menkes (Figure 5B) and E-D IX (Figure 5C) fibroblasts consistently showed strong positive staining diffusely throughout the cytoplasm.

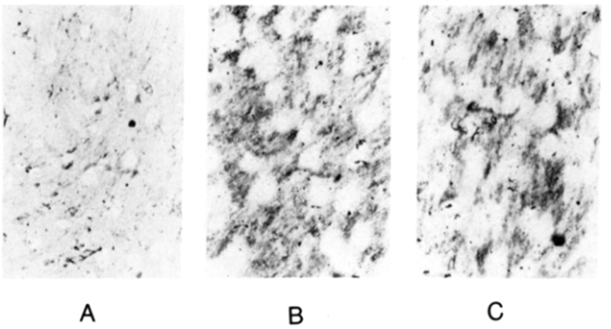

FIGURE 5: Histochemical localization of copper in cultured control (A), Menkes (B), and E-D IX (C) fibroblasts. The copper was demonstrated by using the sulfide–silver method (Scheuer et al., 1967) after fixation of the cell layer with 4% glutaraldehyde (magnification ×72).

Lysyl Oxidase Activity in the Medium of the Cultured Fibroblasts. Since some serum proteins inhibit lysyl oxidase activity, cells have frequently been cultured for 24 or 48 h in a serum-free medium, and this medium has been collected and used for assays of the enzyme activity (Byers et al., 1980; Royce et al., 1980; Kuivaniemi et al., 1982). Our experiments on various culture conditions indicated, however, that the enzyme activity is doubled in both control and Menkes and E-D IX fibroblasts when 5 mg/mL bovine serum albumin is added to the serum-free medium for the 24-h incubation. Concentration of the medium proteins by ammonium sulfate precipitation (Byers et al., 1980; Kuivaniemi et al., 1982) resulted in a loss of about one-third of the enzyme activity, as also found by Royce et al. (1980), and consequently, the medium samples were dialyzed without any concentration step and then used for the assays. Under the conditions used here, about 90% of the total lysyl oxidase activity in all the fibroblast types was in the medium. The remaining 10% could be extracted from the cell layers with 4 M urea in all fibroblast types, but no further studies were carried out on this activity.

In contrast to the conflicting data reported concerning lysyl oxidase activity in small groups of Menkes cell lines (Starcher et al., 1978; Siegel, 1979; Royce et al., 1980), the present results indicated a consistent decrease in lysyl oxidase activity in all 13 Menkes fibroblast lines (Figure 6). The activity values for the two E-D IX cell lines were within the range of the Menkes group, only a minor difference being found between the two means. The values for the E-D IX parents were below the control mean but were not abnormal. No dependence on donor age was found in the lysyl oxidase activity values of the controls, except in that the fetal cells had slightly higher activity. These latter values are therefore indicated by different symbols in Figure 6, and the mean and ranges for the control group are shown, excluding the fetal cells.

A high negative correlation was found between cellular copper concentration (Figure 7A, $r = 0.804$, $P < 0.001$) or copper incorporation (Figure 7B, $r = 0.863$, $P < 0.001$) and the logarithm of lysyl oxidase activity when calculated for the pooled group of Menkes and E-D IX cells or for the Menkes cells alone (these latter details are not given). The values for the two E-D IX cells did not show any distinct deviation from the regression lines.

Prolyl 4-Hydroxylase Activity of the Cultured Cells. This intracellular enzyme activity catalyzes the formation of 4-hydroxyproline in collagen by the hydroxylation of certain proline residues in peptide linkages (Kivirikko & Myllylä, 1980). No abnormality in this enzyme activity was found in any of the Menkes and E-D IX fibroblast lines (details not

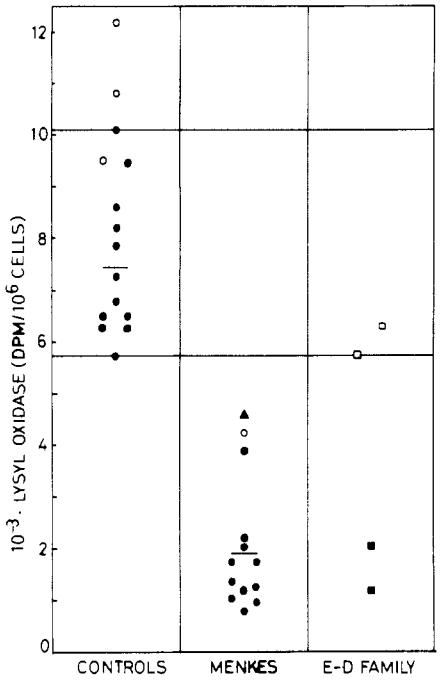

FIGURE 6: Lysyl oxidase activity in medium of cultured fibroblasts. Each value shown is the mean of three to five duplicate assays. The long horizontal lines indicate the ranges of the control values, excluding the fetal cells, and the short lines the means for the various groups, again excluding the fetal cells. (○) Fetal fibroblasts; (▲) atypical Menkes fibroblasts (M12); (●) other control and Menkes fibroblasts; (■) E-D IX fibroblasts; (□) fibroblasts of the parents of the E-D IX patients (father on the left).

Table I: Extractability of Newly Synthesized Collagen from Cell Layers of Cultured Fibroblasts

	hydroxy[^{14}C]proline (% of total hydroxy[^{14}C]proline)[a]		
fraction	E-D IX 1	Menkes[b]	control
expt I			
1 M NaCl	52.3	51.5	30.3
0.5 M acetic acid	5.7	4.1	2.6
8 M urea	22.9	35.6	21.7
total extractable	90.9	91.2	54.6
residue	9.1	8.8	45.4
expt II			
1 M NaCl	43.1	32.4	20.1
0.5 M acetic acid	1.9	2.3	2.6
8 M urea	40.3	35.4	20.4
total extractable	85.3	70.1	43.1
residue	14.7	29.9	56.9

[a] Fibroblast cultures just reaching confluency were labeled with [^{14}C]proline for 48 h, and the cell layers were extracted sequentially with 1 M NaCl, 0.5 M acetic acid, and 8 M urea, as described under Materials and Methods. All extracts and the residue were assayed for hydroxy[^{14}C]proline. The values are shown as percentages of total hydroxy[^{14}C]proline. [b] Menkes cell line M3 in experiment I and M8 in experiment II.

shown), suggesting that the differences in the lysyl oxidase activity between the various cell lines do not simply reflect a nonspecific toxic effect of the copper accumulation.

Extractability of Newly Synthesized Collagen from Cell Layers of Cultured Fibroblasts. A decrease has been found in the percentage of the newly synthesized collagen that becomes insoluble during incubation of cultured E-D IX fibroblasts with [^{14}C]proline for 24 h (Kuivaniemi et al., 1982). As no corresponding data are available for the Menkes fibroblasts and as a relatively small percentage of the collagen became insoluble during the 24-h incubation even in the control cells (Kuivaniemi et al., 1982), experiments were carried out to study the conversion of newly synthesized collagen to the insoluble form during an incubation with [^{14}C]proline for 48 h. There was some variation in the rate of this conversion between various experiments in both the control and the E-D IX cells, and thus, the values are comparable only within the same experiment. A distinct difference in this conversion was found between the control cells and the E-D IX or Menkes cells in the two experiments shown in Table I and in two additional experiments (not shown). The Menkes cell line M8

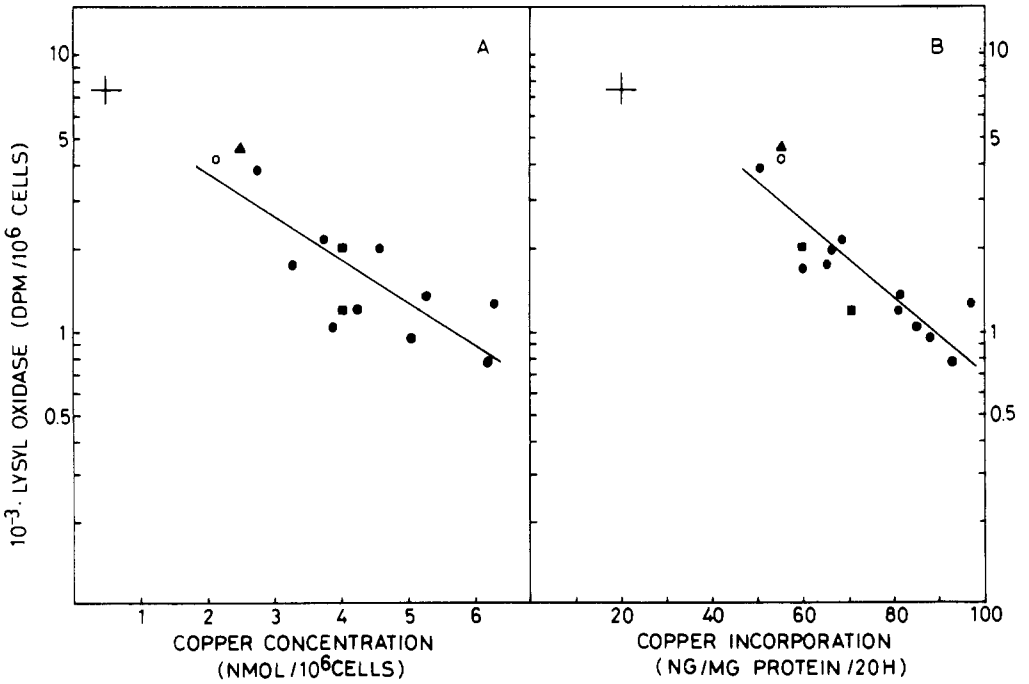

FIGURE 7: Relationship between lysyl oxidase activity (logarithmic scale) and cellular copper concentration (A) or incorporation (B) in the cultures of the Menkes and E-D IX fibroblasts. The regression lines were calculated for pooled groups of Menkes and E-D IX cells [(A) $r = 0.804$, $P < 0.001$; (B) $r = 0.863$, $P < 0.001$]. (○) Fetal Menkes fibroblasts; (▲) atypical Menkes fibroblasts (M12); (●) other Menkes fibroblasts; (■) E-D IX fibroblasts. The short horizontal and vertical lines indicate the control means.

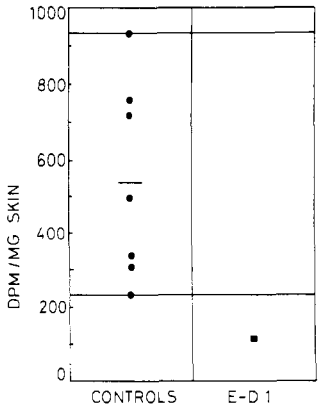

FIGURE 8: Lysyl oxidase activity in urea extracts of skin biopsy specimens (dpm/mg wet wt of skin). The long horizontal lines indicate the ranges of the control values, and the short line indicates the control mean. (●) Controls; (■) E-D IX patient 1.

used in experiment II had a milder deficiency in lysyl oxidase activity than the M3 line used in experiment I, the activity values being about 27 and 13% of the control mean, respectively. No consistent difference was found in the total hydroxyproline radioactivity (the sum of all fractions) per 10^6 cells between the various cell types, and therefore, the values are shown only as percentages of total hydroxyproline.

Lysyl Oxidase Activity and Collagen Extractability in Skin Biopsy Specimens. In order to study whether the abnormalities described above in cultured cells bear any relation to those present in vivo, a new skin biopsy was taken from E-D IX patient 1 at the age of 24 years and used for studies on lysyl oxidase activity and collagen extractability. Part of this biopsy and seven control biopsies were homogenized in a cold solution of 0.15 M NaCl and 0.1 M sodium phosphate, pH 7.8, the homogenates were centrifuged at 10000*g* for 30 min, and the residues were extracted with buffered 6 M urea, as described under Materials and Methods. No lysyl oxidase activity could be detected in the first extracts, and therefore, the values are given only for the urea-extracted activity (Figure 8). A considerable variation was found in the lysyl oxidase activity of the control skin biopsies. In accordance with previous reports (Hayakawa et al., 1976), no correlation was found between this enzyme activity and the age or sex of the subjects, and therefore, persons with an age range of 6–71 years (mean 30 years) were included in the control group. The activity value in the biopsy of E-D IX patient 1 was 22% of the control mean (Figure 8), this value being in a reasonably good agreement with the value of 16% determined in cultured skin fibroblasts from the same patient (Figure 6).

Since numerous previous studies have indicated that the extractability of skin collagen is higher in children than in adults (Kivirikko, 1970), the control group used in the extractability experiments included only adults (21–34 years, mean 28 years). No difference was found in the concentration of total hydroxyproline per gram wet weight of skin and therefore, the values are given only per milligram of total hydroxyproline. The relative amount of extractable collagen in the skin biopsy of E-D IX patient 1 was about 3 times the mean for the five controls, the values for all the solubilized fractions being distinctly above the upper control range (Table II).

Table II: Extractability of Skin Collagen

fraction	hydroxyproline (μg/mg of total hydroxyproline)[a] E-D IX 1	mean for controls (n = 5) (range)
1 M NaCl	12.53	2.95 (0.70–5.34)
0.5 M acetic acid	16.75	6.41 (1.95–12.4)
8 M urea	61.10	20.42 (14.4–30.4)
total extractable	90.38	29.78 (25.0–48.1)
residue	910	970 (952–975)

[a] The skin specimens were homogenized and extracted sequentially with 1 M NaCl, 0.5 M acetic acid, and 8 M urea as described under Materials and Methods. The extracts and residue were assayed for hydroxyproline. The values are shown as micrograms of hydroxyproline per milligram of total hydroxyproline.

Discussion

The data reported here indicate that Menkes and Ehlers–Danlos type IX cells show a number of very similar abnormalities in their copper and collagen metabolism. Both cell types have markedly increased copper concentrations and incorporation, this cation accumulating in metallothionein or a metallothionein-like protein. Both cell types also have low lysyl oxidase activity and increased extractability of the newly synthesized collagen. These abnormalities are not only found in cultured cells, as E-D IX patient 1 also had similar changes in lysyl oxidase activity and collagen extractability in vivo. The findings probably cannot be explained by nonspecific toxicity of the accumulated copper, as there were no disturbances in cell viability, duplication rate, prolyl 4-hydroxylase activity, or the rate of collagen synthesis per cell.

Gel-filtration studies on [^{35}S]cystine-labeled cell lysates indicated that copper accumulated in the E-D IX cells in metallothionein or a metallothionein-like protein, as previously established for Menkes cells (Bonewitz & Howell, 1981; Labadie et al., 1981a,b). The only previous report on the histochemical location of copper in Menkes cells indicates that this cation is heavily concentrated in the brush border of the intestinal epithelium and in the plasma membrane of cultured fibroblasts (Horn & Jensen, 1980). The present data suggest a different location in cultured fibroblasts, however, since at the light microscope level the copper was distributed diffusely throughout the cytoplasm. It may be noted that the present histochemical findings are consistent with accumulation of the cation in metallothionein.

It is not known at present whether the high concentration of metallothionein in Menkes cells is a primary defect or whether it is induced by copper accumulation caused by an unknown primary defect. The metallothionein of Menkes fibroblasts is not abnormal in its copper binding properties and is not even maximally induced, those data being in favor of the possibility that the induction of this protein may be a secondary event (Bonewitz & Howell, 1981; Labadie et al., 1981a,b; Horn, 1983a). The finding that the structural gene for metallothionein in the mouse is located on an autosome (Cox & Palmiter, 1982) also argues against a primary mutation in this protein. The accumulation of metallothionein in E-D IX cells may similarly be secondary to some unidentified abnormality in the cellular copper transport or utilization.

The changes in collagen solubility in cultured cells and in vivo are likely to be due to a deficiency in lysyl oxidase activity. The mechanism of the latter is so far unknown. A high negative correlation was found here between the logarithm of the activity of this copper enzyme and the concentration and incorporation of copper, suggesting a distinct relation between these alterations. Royce et al. (1980) propose that the decreased lysyl oxidase activity in the Menkes syndrome may be secondary to a deficiency in functional intracellular copper, even though the cellular concentrations of this cation are high.

Data on lysyl oxidase deficiency in mottled mouse mutants (Rowe et al., 1977), on the other hand, have raised the possibility that the genes coding for lysyl oxidase, some other copper-dependent enzymes, and an intracellular copper transport protein are linked by some mechanism. Thus, a single mutation would affect the function of all of them to varying degrees. Recent experiments performed in our laboratory indicate that Menkes and E-D IX cells contain and secrete reduced amounts of the lysyl oxidase protein (H. Kuivaniemi, L. Peltonen, A. Palotie, and K. I. Kivirikko, unpublished results), a finding that would be consistent with the latter alternative. These data do not exclude the possibility, however, that the newly synthesized enzyme protein may be rapidly degraded after synthesis.

A recently reported X-linked subtype of the Ehlers–Danlos syndrome (MacFarlane et al., 1980) appears to be very similar, if not identical, to the disorder in our E-D IX patients. Patients with the X-linked Ehlers–Danlos syndrome (MacFarlane et al., 1980) have bladder diverticula, skin changes, skeletal dysplasia, and occipital horn-like exostoses. Copper and ceruloplasmin concentrations in the serum are low, lysyl oxidase activity in skin extracts is reduced, and the desmosine cross-links of skin elastin are fewer in number (D. W. Hollister, R. C. Siegel, and J. Clark, personal communication). Similar clinical manifestations have also been found in two male cousins with an X-linked form of cutis laxa (Byers et al., 1980). Copper and ceruloplasmin concentrations in the serum are again reported to be reduced, lysyl oxidase activity is low in skin extracts and in the medium of cultured skin fibroblasts, and the amounts of lysine-derived aldehydes and cross-links are reduced in cultured skin fibroblasts (Byers et al., 1980). In view of the great similarities between these three diseases, it has been suggested that it would seem appropriate to classify them as one subtype of the Ehlers–Danlos syndrome (subtype IX) with abnormal copper metabolism (Kivirikko & Peltonen, 1982; Kuivaniemi et al., 1982). As no data are yet available on copper and metallothionein concentrations in cultured cells from patients with the diseases originally termed the X-linked subtype of the Ehlers–Danlos syndrome (MacFarlane et al., 1980) and X-linked cutis laxa (Byers et al., 1980), it is not known whether all these three disorders have identical abnormalities.

The clinical findings in cases of the subtype IX of the Ehlers–Danlos syndrome are in many respects different from those in the Menkes syndrome. In particular, there is no evidence of arterial or neurological changes, nor is the disease lethal in childhood. Nevertheless, there are also several common features such as mild neonatal hypothermia, infantile hypotonia, urinary tract infections, bladder diverticula (Harcke et al., 1977; Daly & Rabinovitch, 1981), mildly increased skin laxity and extensibility, and skeletal abnormalities, including wormian bones of the skull (Kaitila et al., 1982; Danks, 1983). The mottled series of mutant mice likewise show a wide array of abnormalities, some strains having mainly a neurological disorder without evidence of connective tissue abnormalities, while some have mainly the latter manifestations (Rowe et al., 1977; Danks, 1983). Serum copper and ceruloplasmin concentrations are usually distinctly lower in the Menkes syndrome (Danks, 1983) than in the subtype IX of the Ehlers–Danlos syndrome (Byers et al., 1980; MacFarlane, 1980; Kuivaniemi et al., 1982), which might suggest that the different clinical manifestations reflect differences in the severity of the abnormality in copper metabolism. This suggestion is nevertheless inconsistent with the present data on cellular copper and metallothionein concentrations and cellular copper incorporation in these two disorders. Neither does it concur with a recent report on a mild form of the Menkes syndrome (Procopis et al., 1981) in which the clinical manifestations are distinctly different from those found in our E-D IX patients, although serum copper and ceruloplasmin concentrations are only slightly reduced. Various mottled mouse mutants likewise do not show any clear relation between serum copper concentration and the presence or absence of signs of impairment of copper-dependent enzymes other than lysyl oxidase (Rowe et al., 1977). Thus the Menkes syndrome and the type IX Ehlers–Danlos syndrome do not seem to represent different degrees of severity of the same biochemical defect.

The only distinct abnormality found in the cells of the E-D IX parents was a clearly abnormal incorporation of radioactive copper in those of the mother. All the other diseases discussed above, including the other families with the subtype IX of the Ehlers–Danlos syndrome, have X-linked inheritance. Therefore, the single definitely abnormal value found in the mother of our family is in agreement with X-linked inheritance.

Acknowledgments

We gratefully acknowledge the expert technical assistance of Eeva Makkonen and Aila Jokinen.

Registry No. Cu, 7440-50-8; lysyl oxidase, 9059-25-0.

References

Bonewitz, R. F., & Howell, R. R. (1981) *J. Cell. Physiol. 106*, 339–348.

Brunk, C. F., Jones, K. C., & James, T. W. (1979) *Anal. Biochem. 92*, 497–500.

Byers, P. H., Siegel, R. C., Holbrook, K. A., Narayanan, A. S., Bornstein, P., & Hall, J. G. (1980) *N. Engl. J. Med. 303*, 61–65.

Camakaris, J., Danks, D. M., Ackland, L., Cartwright, E., Borger, P., & Cotton, R. G. H. (1980) *Biochem. Genet. 18*, 117–131.

Cox, D. R., & Palmiter, R. D. (1982) *Clin. Res. 30*, A117.

Daly, W. J., & Rabinovitch, H. H. (1981) *J. Urol. 126*, 262–264.

Danks, D. M. (1977) *Inorg. Perspect. Biol. Med. 1*, 73–100.

Danks, D. M. (1983) in *The Metabolic Basis of Inherited Disease* (Stanbury, J. B., Wyngaarden, J. B., Fredrickson, D. S., Goldstein, J. L., & Brown, M. S., Eds.) pp 1251–1268, McGraw-Hill, New York.

Goka, T. J., Stevenson, R. E., Hefferan, P. M., & Howell, R. R. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 604–606.

Harcke, H. T., Capitanio, M. A., Grover, W. D., & Valdes-Dapena, M. (1977) *Pediatr. Radiol. 124*, 459–461.

Hayakawa, T., Hino, M., Fuyamada, H., Nagatsu, T., Aoyama, H., & Izava, Y. (1976) *Clin. Chim. Acta 71*, 245–250.

Horn, N. (1976) *Lancet 1*, 1156–1158.

Horn, N. (1981) *Prenatal Diagn. 1*, 107–120.

Horn, N. (1983a) in *Metabolism of Trace Metals in Man: Developmental Biology and Genetic Implications* (Rennert, O. M., & Chan, W.-Y., Eds.) CRC Press, Cleveland, OH (in press).

Horn, N. (1983b) *J. Inherited Metab. Dis.* (in press).

Horn, N., & Jensen, O. A. (1980) *Ultrastruct. Pathol. 1*, 237–242.

Juva, K., & Prockop, D. J. (1966) *Anal. Biochem. 15*, 77–83.

Kaitila, I. I., Peltonen, L., Kuivaniemi, H., Palotie, A., Elo, J., & Kivirikko, K. I. (1982) in *Skeletal Dysplasia* (Papadotos, C. J., & Bartsocas, C. S., Eds.) pp 307–315, Liss, New York.

Kivirikko, K. I. (1970) *Int. Rev. Connect. Tissue Res. 5*, 93–163.
Kivirikko, K. I., & Myllylä, R. (1980) in *The Enzymology of Posttranslational Modifications of Proteins* (Freedman, R. B., & Hawkins, H. C., Eds.) pp 53–104, Academic Press, London.
Kivirikko, K. I., & Myllylä, R. (1982) *Methods Enzymol. 82*, 245–304.
Kivirikko, K. I., & Peltonen, L. (1982) *Med. Biol. 60*, 45–48.
Kivirikko, K. I., Laitinen, O., & Prockop, D. J. (1967) *Anal. Biochem. 19*, 249–255.
Kuivaniemi, H., Peltonen, L., Palotie, A., Kaitila, I., & Kivirikko, K. I. (1982) *J. Clin. Invest. 69*, 730–733.
Labadie, G. U., Beratis, N. G., Price, P. M., & Hirschhorn, K. (1981a) *J. Cell. Physiol. 106*, 173–178.
Labadie, G. U., Hirschhorn, K., Katz, S., & Beratis, N. G. (1981b) *Pediatr. Res. 15*, 257–261.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 256–275.
MacFarlane, J. D., Hollister, D. W., Weaver, D. D., Brand, K. D., Luzzatti, L., & Biegel, A. A. (1980) *Am. J. Hum. Genet. 32*, 118A.
Procopis, P., Camakaris, J., & Danks, D. M. (1981) *J. Pediatr. (St. Louis) 98*, 97–99.
Rowe, D. W., McGoodwin, E. B., Martin, G. R., & Grahn, D. (1977) *J. Biol. Chem. 252*, 939–942.
Royce, P. M., Camakaris, J., & Danks, D. M. (1980) *Biochem. J. 192*, 579–586.
Scheuer, P. J., Thorpe, M. E. C., & Marriott, P. (1967) *J. Histochem. Cytochem. 15*, 300–301.
Siegel, R. C. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4826–4830.
Siegel, R. C. (1979) *Int. Rev. Connect. Tissue Res. 8*, 73–118.
Starcher, B. C., Madaras, J. A., & Tepper, A. S. (1977) *Biochem. Biophys. Res. Commun. 78*, 706–712.
Starcher, B., Madaras, J. A., Fisk, D., Perry, E. F., & Hill, C. H. (1978) *J. Nutr. 108*, 1229–1233.

Bacterial Phosphoenolpyruvate-Dependent Phosphotransferase System. Mechanism of the Transmembrane Sugar Translocation and Phosphorylation†

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ABSTRACT: The phosphoryl-group transfer from PHPr to glucose or α-methylglucose and from glucose 6-phosphate to these same sugars catalyzed by membrane-bound $E_{II}B^{Glc}$ of the bacterial phosphoenolpyruvate-dependent phosphotransferase system has been studied in vitro. Kinetic measurements revealed that both the phosphorylation reaction and the exchange reaction proceed according to a ping-pong mechanism in which a phosphorylated membrane-bound enzyme II acts as an obligatory intermediate. The occurrence of a phospho-IIB^{Glc}/III^{Glc} has been physically demonstrated by the production of a glucose 6-phosphate burst from membranes phosphorylated by phosphoenolpyruvate, HPr, and E_I. The observation of similar second-order rate constants for the production of sugar phosphate starting with different phosphoryl-group donors confirms the catalytic relevance of the phosphoenzyme IIB^{Glc} intermediate. The in vitro results, together with data published by other investigators, have led to a model describing sugar phosphorylation and transport in vivo.

The phosphoenolpyruvate (PEP)[1]-dependent phosphotransferase system catalyzes the concomitant transport and phosphorylation of PTS sugars across the cytoplasmic membrane of a great variety of bacteria [for recent reviews, see Hays (1978) and Robillard (1982)]. The transport process can be described by a minimum of two enzyme catalyzed reactions:[2]

$$\text{PEP} + \text{HPr} \underset{Mg^{2+}}{\overset{\text{enzyme I}}{\rightleftharpoons}} \text{PHPr} + \text{pyruvate} \quad (1)$$

$$\text{sugar}_{out} + \text{PHPr} \underset{Mg^{2+}}{\overset{\text{enzyme II complex}}{\rightleftharpoons}} \text{sugar–P}_{in} + \text{HPr} \quad (2)$$

Reaction 1 describes the phosphorylation of the phosphoryl-group carrier protein HPr, catalyzed by enzyme I. In this reaction, enzyme I, like HPr, undergoes a cycle of (de)-phosphorylation. In previous reports, we have studied the mechanisms of the phosphorylation of HPr and enzyme I (Dooijewaard et al., 1979b; Misset et al., 1980; Misset & Robillard, 1982; Hoving et al., 1981, 1982).

Sugar phosphorylation and translocation are mediated by several sugar-specific membrane-bound enzyme II complexes which all use PHPr as the phosphoryl group donating substrate

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[1] Abbreviations: PEP, phosphoenolpyruvate; PTS, PEP-dependent phosphotransferase system; DTT, dithiothreitol; Glc, glucose; Glc-6-P, glucose 6-phosphate; α-MeGlc, methyl α-glucopyranoside; BSA, bovine serum albumin; Gal-6-P, galactose 6-phosphate; TMG, methyl β-thiogalactoside.

[2] Throughout this paper, we will use the following nomenclature for the different reactions: phosphoryl-group transfer involves the overall, enzyme II catalyzed reaction PHPr + S ↔ S–P + HPr whereas phosphoryl-group exchange refers to the partial reaction in Scheme I (Ic). For reasons of simplicity, both the homologous and the heterologous systems are termed phosphoryl-group exchange. In the literature, other nomenclature is used as well: isotope exchange (homologous system), transphosphorylation, and exchange group translocation [both systems, cf. Saier et al. (1977a,b)].

0006-2960/83/0422-6163$01.50/0