Reduced lysyl oxidase activity in skin fibroblasts from patients with Menkes' syndrome

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Lysyl oxidase activity against both collagen and elastin substrates has been examined in the culture medium of skin fibroblasts derived from unrelated patients with Menkes' syndrome and from control subjects. The medium of three Menkes' fibroblast lines showed 3–30% of the activity present in the medium of control fibroblasts, against a purified collagen substrate. Lysyl oxidase activity in the culture medium of two of the Menkes' fibroblast lines was also examined by using a crude aortic-elastin substrate and was similarly decreased in comparison with that in the medium of control fibroblasts. Lysyl oxidase activity in the medium of a fourth fibroblast line, derived from a foetus with Menkes' syndrome, was 42% of that in the medium of control fibroblasts derived from a 1-day-old baby against a collagen substrate, and 26% of that in control fibroblast medium against an elastin substrate. The copper content of the cell layers of the Menkes' fibroblast cultures was elevated in comparison with normal fibroblast cultures, as has previously been reported to be characteristic of such cells. It is suggested that the decrease in lysyl oxidase activity would help to explain the connective tissue defects observed in Menkes' syndrome, and that this reduction, in conjunction with the elevated concentrations of cellular copper, would support the hypothesis that a functional intracellular copper deficiency exists in Menkes' syndrome.

Menkes' syndrome is a lethal X-linked recessively inherited disorder, characterized by severe progressive cerebral degeneration, pili torti, hypothermia, scurvy-like bone changes, elongation and tortuosity of the cerebral arteries with a tendency to subdural haematoma, and generalized arterial disease (Danks, 1977; Sass-Kortsak & Bearn, 1978). These features resemble those occurring in copper deficiency and are explicable on the basis of copper deficiency (Danks *et al.*, 1972a,b; Danks, 1977). Low concentrations of serum copper and caeruloplasmin were first demonstrated in patients with Menkes' syndrome by Danks *et al.* (1972a,b) and were apparently consequent upon defective intestinal absorption of copper (Danks *et al.*, 1972a, 1973). The arterial disease is manifested as gross ab-

normality of the elastic laminae of the arterial walls, and has been suggested as being due to decreased lysyl oxidase activity resulting from overall copper deficiency (Danks *et al.*, 1972a,b; Oakes *et al.*, 1976). Bone changes similar to those occurring in Menkes' syndrome have also been observed in copper-deficient infants (Al-Rashid & Spangler, 1971; Yuen *et al.*, 1979), which, on the basis of their resemblance to bone changes seen in scurvy, have been attributed to a deficiency in the activity of copper-dependent ascorbic acid oxidase (Al-Rashid & Spangler, 1971). Although it has been suggested that the bone changes occurring in Menkes' syndrome might be similarly explained (Danks *et al.*, 1972b; Danks, 1977) it seems reasonable to suppose that they might rather result from decreased lysyl oxidase activity, as has been suggested to be the case in copper-deficient animals (Underwood, 1977).

Lysyl oxidase§ initiates the cross-linking of collagen and elastin, a pre-requisite for the structural integrity of the connective tissues, through the oxidative deamination of the ϵ -NH₂ group of

§ No EC number has yet been assigned to lysyl oxidase.

Abbreviations used: Dulbecco NaCl/P_i, Ca- and Mg-free phosphate-buffered saline; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; NaCl/P_i, phosphate-buffered saline; Tris, 2-amino-2-hydroxy-methylpropane-1,3-diol.

lysyl or hydroxylysyl residues within the non-helical telopeptides of the collagen α -chains, or of specific lysyl residues in elastin, to form the aldehydic precursors of the cross-links (Bailey *et al.*, 1974; Sandberg, 1976; Tanzer, 1976; Narayanan *et al.*, 1978; Siegel, 1979). The copper dependency of lysyl oxidase has been established (Siegel *et al.*, 1970; Harris *et al.*, 1974) and its activity has been shown to be decreased in cartilage (Siegel *et al.*, 1970) and aorta (Harris *et al.*, 1974) from chicks with dietary copper deficiency. Although it has not been definitely established that the same enzyme functions with both collagen and elastin as a substrate, highly purified preparations of lysyl oxidase have been shown to do so (Siegel, 1979).

The production of lysyl oxidase activity by cultured human-skin fibroblasts has been demonstrated and, consistent with its extracellular function, has been reported to be secreted almost entirely into the culture medium, negligible activity being associated with the cell layer (Layman *et al.*, 1972). In this paper are presented results of a study of lysyl oxidase activity in the culture medium of skin fibroblasts from unrelated patients and from a foetus affected with Menkes' syndrome. Lysyl oxidase activity in the culture medium of Menkes' fibroblasts was found to be considerably decreased against both collagen and elastin substrates in comparison with the activity in the medium of the control fibroblasts.

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Experimental

Materials

L-[4,5- ^3H]Lysine monohydrochloride (sp. radioactivity 75–100 Ci/mmol) was purchased from The Radiochemical Centre (Australia), Sydney, Australia. 'Aquasol' scintillation cocktail was purchased from New England Nuclear, Boston, MA, U.S.A. Eagle's basal medium (Eagle, 1955), Dulbecco's modification of Eagle's medium (Morton, 1970) and Ca- and Mg-free phosphate-buffered saline (NaCl/P_i) (Schmidt, 1969), were obtained from Commonwealth Serum Laboratories, Melbourne, Australia. Lysine-free Eagle's basal medium and Dulbecco's modification of Eagle's medium were obtained from the same source. Foetal calf serum was obtained from Flow Laboratories Australasia, Stanmore, N.S.W., Australia. Phosphate-buffered saline (NaCl/P_i , 0.1 M- $\text{NaH}_2\text{PO}_4/0.15\text{ M-NaCl}$ adjusted to pH 7.7 with NaOH, was passed through Chelex resin (Chelex 100, Bio-Rad Laboratories, Richmond, CA, U.S.A.) before use. The copper content, as determined

by atomic absorption spectrophotometry, was decreased from $0.02\mu\text{g/ml}$ to $<0.002\mu\text{g/ml}$ by this treatment. All glassware and dialysis tubing were soaked in 1% (w/v) EDTA and thoroughly washed with de-ionized water before use.

Methods

Cell culture

Fibroblast cultures were established from skin biopsies obtained from unrelated patients with Menkes' syndrome and from a foetus which was diagnosed as affected and aborted at 23 weeks, after a study of the copper content and copper metabolism of foetally-derived cells obtained by amniocentesis (Camakaris, J. & Danks, D. M., unpublished work). Skin biopsies for control fibroblasts were obtained at *post mortem* from unrelated control subjects who had died of causes unrelated to connective tissue defects, and from a 30-year old normal adult volunteer, with standard procedures. Fibroblasts were grown in Eagle's basal medium buffered with 20 mM-Hepes and supplemented with 10% foetal calf serum. Cell lines were demonstrated to be free of mycoplasma contamination by using the method of Tomkins *et al.* (1975). Before assay of lysyl oxidase stocks of each line were grown in Dulbecco's modification of Eagle's medium buffered with 20 mM-Hepes and supplemented with 10% foetal calf serum. All subsequent experiments were performed with Dulbecco's modification of Eagle's medium. For each cell line 0.4×10^6 fibroblasts were seeded into each of five flasks (75 cm^2 , Lux Scientific, Newbury Park, CA, U.S.A.), and grown in 10 ml of medium as above, at 37°C . After allowing 4 h for adhesion, the serum-containing medium was removed from one flask of each line and, after the cell layer had been rinsed twice with Dulbecco NaCl/P_i , was replaced with 5 ml of serum-free medium, buffered with 20 mM-Hepes and supplemented with freshly-prepared ascorbic acid solution to give a final concentration of $25\mu\text{g/ml}$. Incubation was continued for a further 48 h after which time the medium was collected and stored frozen until assayed for lysyl oxidase activity. Freezing and thawing the medium had no effect on the lysyl oxidase activity. After 1, 2, 4 and 7 days in culture the serum-containing medium in one further flask of each line was replaced with serum-free medium, which was collected after 48 h as shown in Fig. 1. One flask from each line was thus treated at each time point and the cells were always allowed to reach confluency before the medium in the last flask of each cell line was replaced with serum-free medium. Within any one experiment all cell lines were grown at the same time and the same batches of medium and foetal calf serum were used. After collection of the serum-free medium the cell layer

was rinsed with Dulbecco NaCl/P_i and the cells were harvested by treatment with 2 ml of 0.025% (w/v) trypsin in Versene (37°C for approx. 6 min). The cell pellet was washed twice with Dulbecco NaCl/P_i and either resuspended in Dulbecco NaCl/P_i for cell counting in a haemocytometer, which was difficult because of clumping of cells, or in 2 ml of sodium deoxycholate (0.5% w/v) for protein determination. Dissolution of the cell layer in deoxycholate was facilitated by heating in a boiling-water bath for 20 min. In two experiments the cell layer was scraped directly into deoxycholate with a 'rubber policeman', without prior trypsinization. The protein content of the cell layer was determined by using a modification (Hess *et al.*, 1978) of the method of Lowry *et al.* (1951), with the slight difference that instead of the use of two 300 μ l portions of the Folin-Ciocalteu reagent, two 150 μ l portions were used, and the volume was made up with water. Bovine serum albumin (stock solution 100 mg/ml, Sigma Chemical Co., St. Louis, MO, U.S.A.) was used as a standard.

In certain experiments in which larger amounts of culture medium were required, to provide sufficient samples for characterization of the assay system, fibroblasts were grown in roller bottles (660 cm²). Cells were seeded at 3–4 $\times 10^6$ cells per bottle and were grown in medium containing 10% foetal calf serum to approx. 70% confluency, at which stage the medium was replaced after washing the cell layer with Dulbecco NaCl/P_i, with 20 ml of serum-free medium containing freshly prepared ascorbic acid (25 μ g/ml). The serum-free medium was collected after 48 h and stored frozen until assayed.

Preparation of collagen and elastin substrates for lysyl oxidase assay

Crude aortic elastin and purified collagen substrates were prepared from the aortae and calvaria respectively, of 14–16-day chick embryos, essentially as described by Siegel (1974) and Stassen (1976). Aortae (24 per flask) were incubated at 37°C in an atmosphere of O₂/CO₂ (95:5, v/v) in lysine-free Eagle's basal medium (10 ml/flask) buffered with Hepes (25 mM), containing β -aminopropionitrile fumarate (50 μ g/ml), ascorbic acid (50 μ g/ml), penicillin (2000 units/ml) and streptomycin sulphate (0.2 mg/ml) and supplemented with glutamine (2 mM), glycine (50 μ g/ml), proline (40 μ g/ml) and Fe(NO₃)₃ · 9H₂O (10 μ g/ml). Alternatively lysine-free Dulbecco's modification of Eagle's medium was used, buffered with Hepes, containing β -aminopropionitrile fumarate, ascorbic acid, penicillin and streptomycin, as above, and supplemented with glycine and proline to the final concentrations given above. After 30 min pre-incubation, to decrease free lysine pools, 250 μ Ci of [³H]lysine was added to each flask. Incubation was

continued for a further 24 h and terminated by freezing. Subsequently the labelled aortae were washed thoroughly with cold water, blotted dry and powdered in a Spex freezer-mill (Spex Industries, Metuchen, NJ, U.S.A.). The powder was suspended in NaCl/P_i by sonication with an MSE instrument equipped with a needle probe 3 mm in diameter (3 $\times$ 1 min, 10 μ m peak to peak). To inactivate endogenous lysyl oxidase activity (Kagan *et al.*, 1974) the suspension was stood in a boiling-water bath for 15 min. After cooling, the suspension was centrifuged (25 000 g for 15 min) and the pellet was resuspended in 0.5 M-HCl and stood at 4°C for 16 h. Subsequently the pellet was washed twice with NaCl/P_i and stored frozen after resuspending in NaCl/P_i.

Calvaria (36 bones/10 ml of medium) were labelled with [³H]lysine as above, thoroughly washed with cold water and powdered in the Spex freezer-mill. The powder was extracted for 36 h at 4°C with 1.0 M-NaCl/0.05 M-Tris, pH 7.5, which had been passed through Chelex. The extract was centrifuged (30 000 g for 30 min) and collagen was precipitated from the supernatant by the addition of solid NaCl to a final concentration of 20% (w/v). The precipitate was collected by centrifugation (30 000 g for 30 min), resuspended in NaCl/P_i and dialysed exhaustively against NaCl/P_i, initial changes containing unlabelled lysine (1 mg/ml). The non-diffusible material was clarified by centrifugation (30 000 g for 30 min) and was stored frozen.

Lysyl oxidase assay

Lysyl oxidase activity in serum-free fibroblast culture medium was assayed by using the tritium release procedure of Pinnell & Martin (1968). Samples were dialysed against NaCl/P_i and 1.0 ml portions were mixed with either [³H]elastin substrate (0.5 ml containing 1–1.5 $\times 10^6$ c.p.m.) or [³H]-collagen substrate (0.5 ml containing approx. 0.5 $\times 10^6$ c.p.m.) in screw cap vials. Vials were flushed with oxygen, for which lysyl oxidase has a requirement (Narayanan *et al.*, 1974), and incubated at 37°C for 6 h in a shaking water-bath. Before addition of samples, collagen substrates were pre-incubated at 37°C for 1 h to promote fibril formation (Siegel, 1974). Enzyme control samples contained β -aminopropionitrile fumarate (50 μ g/ml) to inhibit lysyl oxidase activity (Pinnell & Martin, 1968). Reactions were terminated by plunging of the vials into ice, by addition of β -aminopropionitrile fumarate to the experimental samples and by freezing. The ³H₂O formed during the reaction was collected by a modification of the method of Melet *et al.* (1977). Reaction mixtures were transferred to tubes, bovine serum albumin solution (0.15 ml, 2% w/v) was added to each and protein was pre-

precipitated by the addition of trichloroacetic acid (0.15 ml, 60% w/v). After being mixed, the tubes were stood on ice for approx. 1 h. The precipitate was removed by centrifugation (200 g for 5 min) and 1.0 ml portions of the supernatants were passed through Dowex columns (0.5 cm $\times$ 2.0 cm, Dowex AG-50 W-X8, H⁺ form, 200–400 mesh, Sigma Chemical Co., St. Louis, MO, U.S.A.) in Pasteur pipettes plugged with glass wool, and previously equilibrated with trichloroacetic acid (1.0 ml, 5% w/v). The columns were washed with trichloroacetic acid (0.75 ml, 5% w/v) and 1.0 ml of the total eluate was mixed with 10 ml of Aquasol. Radioactivity was determined in a Packard Tri-Carb liquid-scintillation spectrometer, at a counting efficiency of 29%, as determined with ³H₂O (Packard Instrument Co., Downers Grove, IL, U.S.A.) as an internal standard. Lysyl oxidase activity was calculated as the amount of tritium, determined as c.p.m. of ³H₂O, released from the substrate by the sample, corrected for the non-specific background release of tritium determined in the enzyme control samples containing β -aminopropionitrile fumarate. Activity is thus expressed as c.p.m./ml of medium, or total activity in the medium is expressed as either c.p.m./100 μ g of cell layer protein, or c.p.m./10⁶ cells. All incubations were performed in duplicate, and results are mean values. In each assay substrate controls were included in which the substrate was incubated with 1.0 ml of NaCl/P_i in the presence and absence of β -aminopropionitrile fumarate to check for the absence of endogenous lysyl oxidase activity. Within any particular experiment all assays were performed at the same time, with the same batch of substrate. Assays were performed with substrates prepared as freshly as possible, and in no case were these stored for longer than 3 weeks, since background release of radioactivity from the substrates, in the presence of β -aminopropionitrile fumarate, increased linearly with the length of time for which they were stored frozen. Background release of radioactivity in the presence of β -aminopropionitrile fumarate was about 225 per 0.5 $\times$ 10⁶ c.p.m. for freshly prepared collagen substrate and about 550 per 10⁶ c.p.m. for freshly prepared elastin substrate.

Preliminary experiments performed in selecting assay conditions

In preliminary experiments normal fibroblasts were grown in 75 cm² flasks in medium containing 10% foetal calf serum. However, when lysyl oxidase activity was assayed directly in this medium poor duplicate values were obtained, and release of radioactivity as a result of lysyl oxidase activity was low in relation to background release from substrates in the presence of β -aminopropionitrile fumarate. Fibroblasts were then grown in roller

bottles in medium containing 10% foetal calf serum, and lysyl oxidase activity in the medium was assayed against a collagen substrate, directly and after concentration by (NH₄)₂SO₄ precipitation (Layman *et al.*, 1972), and by freeze-drying (DiFerrante *et al.*, 1975) and resuspension in NaCl/P_i. Loss of activity was apparent after either method of concentration, and the background release of radioactivity in the presence of β -aminopropionitrile fumarate was increased from 375 c.p.m., in the case of direct sampling of the medium, to 1820 c.p.m. after freeze-drying and to 3752 c.p.m. after (NH₄)₂SO₄ precipitation. Assay of foetal calf serum showed no β -aminopropionitrile fumarate-inhibitable activity, but the background release of radioactivity in the presence of β -aminopropionitrile fumarate was doubled in relation to that in NaCl/P_i controls. Subsequently fibroblasts were grown to 75% confluency in medium containing 10% foetal calf serum which was then replaced with serum-free medium, which was collected after 48 h. Direct assay of lysyl oxidase activity in this serum-free medium gave better duplicate values. However, about 30% of the activity was lost upon concentration by (NH₄)₂SO₄ precipitation and about 60% upon freeze-drying, although the background release of radioactivity in the presence of β -aminopropionitrile fumarate remained at about 300 c.p.m. Consequently, serum-free medium was used in further experiments, as described above, and the need for concentration of lysyl oxidase activity was avoided by use of a minimum volume of medium.

Assay characteristics

When lysyl oxidase activity was assayed in pooled medium from four roller bottles of normal fibroblasts, tritium release from both collagen and elastin substrates was linear for at least 7 h. Multiple simultaneous determinations of lysyl oxidase activity in pooled medium from two roller bottles of normal fibroblasts, with a collagen substrate, resulted in a value of 313.8 (s.d. $\pm$ 37.4, n = 9) c.p.m./ml of medium; coefficient of variation = 11.9%. In a further experiment, each of nine flasks was seeded simultaneously with 0.4 $\times$ 10⁶ fibroblasts from a stock of one line. Cells were grown in medium containing 10% foetal calf serum for 2 days, until they had reached 75% confluency, at which time the medium was replaced with serum-free medium for 48 h. Subsequent assay of the medium from each flask for lysyl oxidase activity with a collagen substrate resulted in a value of 720.6 (s.d. $\pm$ 76.3 n = 9) c.p.m./100 μ g of cell layer protein; coefficient of variation = 10.6%.

Determination of copper content of fibroblasts

The copper content of cell layers solubilized in deoxycholate was determined with a Varian-

Techtron CRA-90 atomic absorption spectrophotometer equipped with a BC-6 automatic background-subtract module.

Results

Lysyl oxidase activity in medium from normal and Menkes' fibroblasts

The relationship of lysyl oxidase activity in the culture medium of normal and Menkes' fibroblasts to duration of culture, assayed with a collagen substrate, is shown in Fig. 1. Such a profile of activity was consistently observed against both collagen and elastin substrates in five separate experiments, in which a total of four Menkes' lines and four normal lines were examined. It is apparent that lysyl oxidase activity in the medium of fibroblasts from two patients with Menkes' syndrome (Me-1 and Me-2), was considerably decreased in comparison with that in the medium of control fibroblast cultures from both a 1-day-old infant (C-1) and a 30-year-old adult (C-2). All four

cell lines were confluent after 4 days in culture, including those cells which were placed in serum-free medium after 2 days.

A quantitative assessment of the lysyl oxidase activity in the medium of the fibroblast lines illustrated in Fig. 1, produced by summation of the activity in each of the five flasks for each cell line, is given in Table 1 (Expt. 1), together with results from other determinations of lysyl oxidase activity in the medium of normal and Menkes' fibroblasts. The decreased lysyl oxidase activity in the medium of control fibroblast line C-2, originating from a 30-year-old adult, in relation to that in the medium of line C-1 originating from a 1-day-old infant, shown in Fig. 1, is apparently a reflection of the age of the donor rather than of the later passage of the cells, since line C-1 showed activity at passage 20 which was 80% of that at passage five, in a separate experiment. The Menkes' fibroblast lines Me-1, Me-2 and Me-3 showed considerably decreased lysyl oxidase activity in the medium against a collagen

Fig. 1. *Production of lysyl oxidase activity by normal and Menkes' fibroblasts*

Lysyl oxidase activity in culture medium of normal and Menkes' fibroblasts, as determined by assay against a collagen substrate, was calculated as the amount of tritium, determined as c.p.m. of $^3\text{H}_2\text{O}$, released from the substrate by the sample, with appropriate corrections. Activity is expressed as c.p.m./100 μg of cell layer protein. Control line C-1 (10) (●—●) originated from a 1-day-old infant and control line C-2 (22) (○—○) from a 30-year-old adult. Menkes' lines Me-1 (12) (□---□) and Me-2 (8) (■---■) were from affected infants aged <12 months and 8½ months respectively. The passage number of the cultures is indicated in parentheses.

substrate in relation to control lines derived both from infants (lines C-1, C-3 and C-4) and from an adult (line C-2), although, as also shown in Fig. 1, the extent of the decrease is less when comparison is made with the fibroblasts derived from an adult. In Menkes' lines Me-1, Me-2 and Me-3 lysyl oxidase activity in the medium was between 3 and 30% of that in the relevant control lines derived from 1- and 5-day-old infants used in each experiment. The extent of the decrease of lysyl oxidase activity in the medium of Menkes' fibroblasts apparently increases with passage number. Line Me-3 showed activity at passage 22, which was 46% of that shown at passage 8 (Table 1, Expt. 2), whereas, as mentioned, normal cells (line C-1) showed activity at passage 20, which was 80% of that at passage 5. Fibroblasts derived from a foetus with Menkes' syndrome (Me-4f) also displayed decreased lysyl oxidase activity in the medium against a collagen substrate in comparison with a control line (Table 1, Expt. 3).

Lysyl oxidase activity in the medium of Menkes' fibroblast lines Me-1, Me-2 and Me-4f was also assayed with an elastin substrate. In each case the activity was considerably decreased in comparison with that in the medium of the control fibroblasts, derived from a 1-day-old infant (Table 1, Expts. 4, 5), being between 8 and 41% of the control line activity.

The copper content of the cell layers was determined after their dissolution in deoxycholate for Menkes' cell lines Me-1, Me-2 and Me-4f and for control lines C-1 and C-2 in each experiment for which they were used. At confluency the copper content of the control lines was between 0.0027 and 0.015 μg of copper/100 μg of cell layer protein, whilst for the Menkes' lines it was between 0.016 and 0.045 μg of copper/100 μg of cell layer protein, indicating accumulation of copper by the Menkes' fibroblasts.

Discussion

The results presented demonstrate a considerable decrease of lysyl oxidase activity in the culture medium of Menkes' fibroblasts in comparison with that in the medium of control fibroblasts from age-matched subjects, against both purified collagen and crude elastin substrates. The Menkes' lines showed activity which was between 3 and 42% of that in control lines. The production of lysyl oxidase activity was monitored over the whole period of the growth cycle of the fibroblasts and in agreement with the results of Layman *et al.* (1972) and of Starcher *et al.* (1977) the greatest activity was found in the medium of the cells immediately preconfluence. The use of serum-free medium, and the destructive harvesting of the cell layers, necessary to relate lysyl oxidase activity in the medium to an index of the

Table 1. *Lysyl oxidase activity in the medium of normal and Menkes' fibroblasts*

Total lysyl oxidase activity in culture medium of five flasks from each cell line. Each of the five flasks for each cell line was seeded with 0.4×10^6 cells. At stages throughout the growth cycle the medium from one flask of each line was replaced with serum-free medium which was collected after 48 h and assayed for lysyl oxidase activity as described in the Experimental section. Lysyl oxidase activity was calculated as the amount of tritium, determined as c.p.m. of $^3\text{H}_2\text{O}$, released from the substrate by the sample, with appropriate corrections. In Expt. 2 activity is expressed as c.p.m./ 10^6 cells. In all other experiments activity is expressed as c.p.m./100 μg of cell layer protein. The passage number of the cultures in each experiment is indicated in parentheses. In the final column, the total activity in the medium of the Menkes' fibroblasts is given as a percentage of the total activity in the medium of the control lines within each experiment. Control lines originated as follows: C-1 from a 1-day-old infant; C-2 from a 30-year-old adult, C-3 and C-4 from 5-day-old infants. Menkes' lines Me-1, Me-2 and Me-3 originated from affected infants aged <12 months, 8½ months and 1 week respectively, and Me-4f originated from a 23-week-old foetus.

Collagen substrate (Expt. no.)	Control lines		Menkes' lines		Activity in Menkes' lines as % of controls	
	Line	Activity	Line	Activity		
1 (See also Fig. 1)	C-1 (10)	2862	Me-1 (12)	95	3.3	16.8
	C-2 (22)	566	Me-2 (8)	235	8.2	41.5
	C-3 (8)	8507	Me-3 (8)	1648	19.4	29.9
	C-4 (12)	5503	Me-3 (22)	754	8.9	13.7
3	C-1 (5)	1972	Me-4f (5)	837	42.4	—
Elastin substrate						
(Expt. no.)						
4	C-1 (20)	983	Me-1 (15)	80	8.1	—
5	C-1 (9)	1834	Me-2 (13)	744	40.6	—
			Me-4f (11)	485	26.4	—

number of cells in the culture at each stage of the growth cycle, made it necessary to use a separate culture at each time. It was felt reasonable to quantitate the production of lysyl oxidase activity throughout the growth cycle by summing the activity in each of the cultures of each cell line.

The decreased lysyl oxidase activity in the Menkes' fibroblasts would, perhaps, explain the gross abnormalities in the elastic laminae of the arteries apparent in Menkes' syndrome (Danks *et al.*, 1972a,b; Oakes *et al.*, 1976) and resembling those observed in copper deficiency, which are considered to result from decreased lysyl oxidase activity (Carnes, 1971; Underwood, 1977), and the abnormal elastin fibres present in the skin (Oakes *et al.*, 1976). The panacinar emphysema which has been reported in Menkes' syndrome (Daish *et al.*, 1978) might be similarly explained. Decreased lysyl oxidase activity in the lungs of 'blotchy' mice which have defective elastin and emphysema has been reported (Starcher *et al.*, 1977). No definite collagen defect has been reported in Menkes' syndrome although the scurvy-like bone changes might result from decreased lysyl oxidase activity and consequent collagen abnormality. It might be that decreased activity of lysyl oxidase has less effect on the maintenance of the structural integrity of collagen fibres than on that of elastin fibres, since the major intermolecular cross-links of collagen require the participation of only one lysyl- or hydroxyllysyl-aldehyde precursor (Tanzer, 1976) whereas one elastin cross-link requires the participation of three aldehydic lysyl precursors (Sandberg, 1976). In support of this suggestion is the observation in an ultrastructural study of the aorta of one patient with Menkes' syndrome, who had been receiving copper therapy, of increased amounts of collagen (Oakes *et al.*, 1976). It is not known if this collagen was abnormally soluble, but it presumably indicates the increased synthesis of collagen as a generalized repair response to the abnormal formation of elastin fibres.

The finding of decreased lysyl oxidase activity in the culture medium of Menkes' fibroblasts is in contrast to the report of Starcher *et al.* (1978) that lysyl oxidase activity was only slightly decreased in fibroblast cultures from two patients with Menkes' syndrome, being 84% of that in the medium of fibroblasts from normal subjects. The age of the donors of the normal human fibroblasts was not indicated in the latter study, nor did lysyl oxidase activity in the medium appear to be related to the number of cells in the cultures, and either of these factors may account for this discrepancy.

The Menkes' fibroblasts showed increased copper concentrations in comparison with normal cells as previously described (Goka *et al.*, 1976; Camakaris *et al.*, 1980). Despite this, the copper, which is

presumably intracellular (Danks, 1977; Camakaris *et al.*, 1980), is apparently unavailable for lysyl oxidase activity. Although the possibility of a mutation primarily affecting lysyl oxidase cannot be ruled out, the finding of decreased lysyl oxidase activity in the medium of Menkes' fibroblasts in the presence of increased intracellular copper supports the idea of a basic defect in copper transport. Danks (1977) has proposed that the fundamental defect in Menkes' syndrome might involve the increased affinity for copper of an intracellular copper-binding protein, which results in the intracellular copper being unavailable for copper-requiring enzymes. The same effect could be brought about by other disturbances of intracellular transport processes (Camakaris *et al.*, 1980), but the net effect would seem to be that of a functional copper deficiency in Menkes' syndrome. It might reasonably be expected that other copper-dependent enzymes would also show decreased activity in Menkes' syndrome, as suggested by Danks (Danks *et al.*, 1972a,b; Danks, 1977).

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