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Abnormal collagen metabolism in cultured skin fibroblasts from patients with Duchenne muscular dystrophy

(collagen synthesis/collagen degradation/hydroxyproline-to-proline ratio/prolyl hydroxylation)

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ABSTRACT **Total collagen synthesis is decreased by about 29% ($P < 0.01$) in skin fibroblasts established *in vitro* from male patients with Duchenne muscular dystrophy (DMD) as compared with that in normal male skin fibroblasts *in vitro*. The reduction in collagen synthesis is associated with an approximately 2-fold increase in collagen degradation in DMD fibroblasts. Correlated to these alterations in the metabolism of collagen, DMD fibroblasts express a significantly higher hydroxyproline/proline ratio (DMD: 1.36–1.45; $P < 0.01$) than do normal fibroblasts (controls: 0.86–0.89). The increased hydroxylation of proline residues of collagen (composed of type I and type III) could be the cause for the enhanced degradation of collagen in DMD fibroblasts.**

The muscular dystrophies are genetically determined diseases with variable expressions in different organ systems, mainly characterized by progressive degeneration in the principal target organ, the muscle (1, 2). The most severe and best defined form of these disorders is the X-chromosome-linked recessive Duchenne muscular dystrophy (DMD) (1, 2). Muscle weakness and dystrophy of skeletal and cardiac muscles in DMD are regarded to be the result of genetically induced secondary abnormalities in the structure and function of cellular membranes (1) and/or of an abnormal protein metabolism (3–8). Cell biological and biochemical abnormalities have been found also in erythrocytes (9–13) and lymphocytes (14–16). Until now, the primary genetic and biochemical defect has not been defined in this disorder (1, 2). Quantitative changes are recorded for a great number of biochemical parameters; however, no qualitative changes have been detected in any of the constituents in DMD tissues (1).

Biochemical analysis of DMD has been hampered by the shortage of affected tissues and the extensive range of responses of muscle to injury (1). Such changes make the selection of an appropriate reference base and of an adequate control extremely difficult (1). Because fibroblasts *in vitro* established from explants of human skin biopsies have proven to be very useful tools for investigations of various genetic errors of metabolism, cultured skin fibroblasts from patients with DMD have been used for studies into the syndrome-specific primary and/or secondary cell biological and biochemical defects (17–25).

Since collagen is the best defined component of the major structural proteins synthesized by fibroblasts and since alterations in the collagen synthesis have been described for DMD fibroblasts (25), experiments were undertaken to elucidate the mechanisms that lead to the changes in collagen metabolism of DMD fibroblasts *in vitro*.

MATERIALS AND METHODS

Cultures. For the present studies, fibroblast populations established from skin biopsies, excised from the inner aspect of the left upper arm of eight male patients (age 6–15 yr) with late to very late manifestations of the DMD syndrome and of eight normal male probands (in the same age range), were analyzed. The biopsy material was obtained from R. Beckmann (Abteilung für Muskelkrankheiten, Universitätskinderklinik, Universität Freiburg, Freiburg, F.R.G.). Skin biopsy material was cut into fragments of 1 mm^3; transferred to 25-cm^2 tissue culture flasks (Falcon); covered with Dulbecco's modified Eagle's medium (DME medium, Microbiological Associates) supplemented with 20% fetal calf serum (Seromed, München, F.R.G.; lots 107 and 139), penicillin (557 units/ml), and streptomycin (740 units/ml); and incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2. The culture medium was renewed once weekly. After 3–4 weeks, primary skin fibroblasts were treated with 0.25% trypsin. The cell number was determined, and skin fibroblasts were subcultured. For serial subcultivations, normal and DMD skin fibroblasts were transferred once weekly and plated at a constant density of 1×10^6 cells per 55-cm^2 tissue culture dish (Falcon) in 10 ml of DME medium supplemented with 10% fetal calf serum, penicillin (557 units/ml), and streptomycin (740 units/ml). For determination of growth rates and cumulative population doublings (CPD), cells were counted at each transfer in a Fuchs–Rosenthal hemocytometer.

Collagen Synthesis. Subconfluent cultures were incubated up to 48 hr with 2.5 ml of DME medium without serum but containing L-[^{3}H]proline (10 μCi; 30 Ci/mmol, New England Nuclear; 1 Ci = 3.7×10^{10}Bq), 2-aminopropionitrile (100 μg/ml), and Na ascorbate (100 μg/ml) (26). After the incubation periods, media were collected and dialyzed against 0.5% acetic acid. Cell layers were taken up in 2.5 ml of 1 M NaCl/Tris buffer, pH 7.4, sonicated, and dialyzed extensively against 0.5% acetic acid. Combined nondialyzable materials of cell layers and media were digested with pepsin for 4 hr at 13°C as described by Kontermann and Bayreuther (27). After an exhaustive dialysis against 0.5% acetic acid, pepsin-resistant radioactive material was determined and correlated to the cell number. In order to examine whether protein-bound radioactivity, as determined by pepsin digestion, represents collagen, a collagenase digestion was performed as described by Ionasescu *et al.* in some additional experiments (25).

Collagen Degradation. Collagen degradation was measured by following the remaining collagen-bound radioactivity in a "pulse–chase" experiment. Fibroblast cultures were incubated for 2 hr with 20 μCi of L-[^{3}H]proline as described above. After labeling, the radioactive incubation medium was removed, cell layers were washed five times with warm (37°C) phosphate-buffered saline, and 2.5 ml of chase medium (identical to labeling medium, except that 1 mM unlabeled L-proline was substituted for L-[^{3}H]proline) was added. At time t = 0, 0.5, 2, 6, and 24 hr after the addition of the

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Abbreviations: DMD, Duchenne muscular dystrophy; CPD, cumulative population doublings.

chase medium, the incubation was terminated and the medium was removed. Cell layers were resuspended in cold (4°C) 1 M NaCl/Tris buffer, pH 7.4, and the cells were removed from the culture dish with a rubber policeman and sonicated. Combined media and cell layer suspensions were digested with pepsin as described above. After determination of pepsin-resistant radioactive material, the percentage of collagen degradation was calculated from the following formula: % collagen degraded = [(cpm of radioactivity in the sample at $t = 0$) − (cpm of radioactivity in the sample at $t = x$)] × 100/(cpm of radioactivity in the sample at $t = 0$).

Collagen degradation was measured by determining the amount of dialyzable [^{14}C]hydroxyproline. Fibroblast cultures were incubated for 2 hr with 10 μCi of L-[^{14}C]proline (225 mCi/mmol, New England Nuclear) in medium as described above; 2 hr later, after repeated washing of the cell layers, 2.5 ml of chase medium was added. At various time points (t = 0.5, 2, 8, and 24 hr), the incubation was stopped by subsequent sonication and heating at 100°C for 10 min to inactivate protease activity. Collagen degradation was determined by calculating the percentage of dialyzable hydroxyproline after amino acid analysis of nondialyzed and dialyzed samples (28). Amino acid analysis was performed on a Durum DC-4 column with an automated Biotronic BT 6110 amino acid analyzer. The percentage of dialyzable [^{14}C]hydroxyproline was calculated from the following formula: % dialyzable [^{14}C]hydroxyproline = [(cpm of total [^{14}C]hydroxyproline in the sample) − (cpm of [^{14}C]hydroxyproline in the sample after dialysis)] × 100/(cpm of total [^{14}C]hydroxyproline in the sample).

Extraction of Cell Culture Collagen and Separation of Collagen Chains by CM-Cellulose Chromatography. Skin fibroblast cultures were labeled for 24 hr with 20 μCi of L-[^{3}H]proline as described above. Purification of collagen was performed as described by Kontermann and Bayreuther (27). In brief, lathyrythic rat skin collagen (1 mg/ml; type I and III) was added to the combined cell layers and media. Collagens were precipitated by two subsequent fractionated salt precipitations (30% $(NH_4)_2SO_4$; 2.5 M NaCl). Precipitated proteins were digested with pepsin as described above and prepared for CM-cellulose chromatography (Whatman CM 52). Samples were further analyzed by measuring the ionic strength of the different α chains and determining the total radioactivity. Hydroxyproline/proline ratios of pooled α-chain peaks of type I and type III collagen were determined by amino acid analysis.

Determination of Type I/Type III Collagen Ratios. Pepsin-resistant material was submitted to $NaDodSO_4$/polyacrylamide gel electrophoresis as described by Goldberg *et al.* (29). After electrophoresis, radioactive gels were cut into 1-mm discs with a Mickle gel slicer, swollen, and eluted in a mixture of Lipoluma/Lumasolve, 9:1 (vol/vol) (Baker), and the radioactivity was assayed. The percentage of type III collagen was measured by the determination of the amount of 2-mercaptoethanol-reducible γ components.

RESULTS

Overall Collagen Synthesis of DMD and Normal Skin Fibroblasts. To evaluate the relevance of the data for collagen biosynthesis obtained by the determination of pepsin-resistant material in total protein, collagenase digestion of total protein and $NaDodSO_4$/polyacrylamide gel electrophoresis of pepsin-resistant material was performed. Measurements of pepsin-resistant and collagenase-sensitive radioactive material both indicated the same amount of collagen in the samples (Table 1). $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1) revealed that pepsin-resistant material was essentially pure collagen. Thus, pepsin-resistant material was used as a measure for the collagen content of the samples studied.

Table 1. Amount of collagen synthesized by normal and DMD fibroblast cultures

Cell line	Total radioactivity, cpm	Pepsin-resistant radioactivity, cpm	Collagenase-sensitive radioactivity, cpm
HH-4	20,387 ± 1276	3846 ± 297	3265 ± 465
DMD-1	16,093 ± 989	2735 ± 213	2381 ± 224

Normal (HH-4) and DMD (DMD-1) fibroblast cultures were labeled for 24 hr with L-[^{3}H]proline at CPD number 18, as described. Aliquots of the combined media and cell-layer fractions were taken for determination of pepsin-resistant and collagenase-sensitive radioactivities; cpm were correlated to the cell number (10^6 cells).

In order to analyze the overall collagen synthesis of DMD and normal fibroblasts, the incorporation of L-[^{3}H]proline into extracellular and intracellular collagen (pepsin-resistant material) was studied after a 24-hr labeling period in six DMD and six normal fibroblast cell lines of approximately the same number of CPDs *in vitro*. Incorporation of labeled proline into extracellular and intracellular collagen was significantly reduced by about 29% and 35%, respectively, in fibroblast cultures derived from DMD patients as compared to those from controls (Table 2). In these experiments, cell cultures used were in the range of 20 (DMD) and 22–27 (HH, controls) CPDs.

Kinetics of Collagen Synthesis of DMD and Normal Skin Fibroblasts. Since the described data on overall collagen synthesis were obtained in a 24-hr pulse-labeling experiment, in an additional experiment the kinetics of collagen synthesis in both DMD and normal fibroblasts were investigated. Fig. 2 shows the results obtained from two DMD fibroblast cell lines (DMD-1 and DMD-2) and from two normal fibroblast cell lines (HH-4 and HH-6). During the first 10 hr, the slope of the kinetic curves for DMD fibroblasts was significantly lower than that of control fibroblasts. Moreover, overall col-

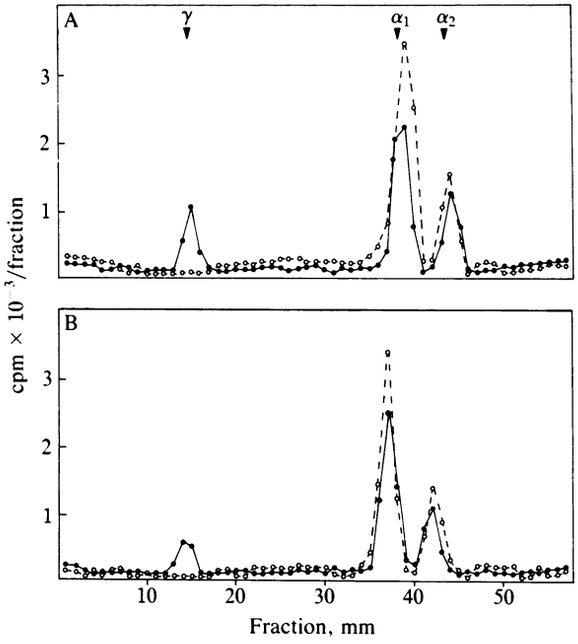

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoresis of pepsin-resistant proteins from normal (HH-6) (*A*) and DMD (DMD-1) (*B*) fibroblasts. Before reduction with 2-mercaptoethanol (●), approximately 15–18% of the labeled collagen is found in the large molecular weight peak, representing the γ component (unreduced type III collagen). After reduction (○), this material comigrates with the α_1-chain peak.

Table 2. Overall collagen synthesis in normal and DMD fibroblast cultures

Cell line	CPD	Extracellular collagen, cpm	Intracellular collagen, cpm
HH-4	23	1772	1356
HH-5	27	1485	1202
HH-6	25	1515	1698
HH-7	22	1447	1589
HH-12	26	1694	—
HH-13	24	1781	—
Mean ± SEM		1616 ± 61	1461 ± 112
DMD-1	20	1222	887
DMD-2	19	1310	1189
DMD-3	19	1033	909
DMD-4	21	1232	801
DMD-5	20	1020	—
DMD-6	21	1083	—
Mean ± SEM		1149 ± 50 (−29%)*	946 ± 84
% change		−29%*	−35%*

Incorporation of L-[^{3}H]proline into collagen (pepsin-resistant material) of normal (HH) and DMD fibroblasts after a 24-hr pulse; cpm were correlated to the cell number (10^6 cells).
*$P < 0.01$.

lagen synthesis in DMD fibroblasts reached the plateau after 20 hr, whereas control fibroblasts only reached the plateau after 30 hr. Similar results were obtained for other DMD (DMD-3 and DMD-5) and normal (HH-5 and HH-7) fibroblast cell lines (data not presented).

Collagen Degradation in DMD and Normal Skin Fibroblasts. In order to test whether the observed reduction in overall collagen synthesis in DMD fibroblasts could be the result of an elevated collagen degradation, degradation was determined by "pulse–chase" experiments with DMD cell lines of three different patients (DMD-1, DMD-3, and DMD-4) and three control fibroblast cell lines (HH-4, HH-6 and HH-10) at comparable CPD numbers. After a 2-hr pulse, the remaining collagen-bound radioactivity (pepsin-resistant material) was determined during various chase periods. According to the data (Table 3, experiment I), DMD fibroblasts expressed a higher rate of collagen degradation as compared to control fibroblasts. Approximately 60% of the newly synthesized collagen was degraded within 2 hr in DMD fibroblasts, whereas control fibroblasts only degraded 28% of the collagen synthesized; 24 hr after the addition of the chase medium, DMD fibroblasts showed a degradation rate of

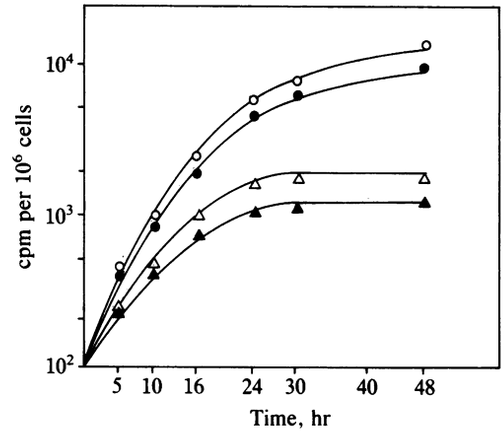

FIG. 2. Kinetics of overall collagen synthesis. Normal fibroblast cell lines HH-4 (○) and HH-6 (●) at CPD number 23 and DMD fibroblast cell lines DMD-1 (△) and DMD-2 (▲) at CPD number 19 were incubated in the presence of L-[^{3}H]proline for various times. After labeling, incorporation of L-[^{3}H]proline into collagen (pepsin-resistant material) was determined as described.

about 74%, as compared to 61% for control fibroblasts. Similar results for collagen degradation were obtained by following the release of dialyzable [^{14}C]hydroxyproline (Table 3, experiment II). Within 2 hr after the addition of the chase medium, approximately 63% of the total hydroxyproline of DMD fibroblasts was dialyzable, whereas in the same period of time in control fibroblasts, only 41% of the total hydroxyproline was dialyzable; 24 hr after the addition of the chase medium, approximately 70% of the total hydroxyproline was dialyzable in DMD fibroblasts as well as in control fibroblasts. Thus, these data suggest that a significant amount of newly synthesized collagen is degraded within hours of its synthesis in DMD and control fibroblasts. The almost 2-fold increase in the degradation rate of collagen in DMD fibroblasts as compared to control fibroblasts during the first 4 hr of the chase period suggests that there is an increase in the fraction of total collagen of DMD fibroblasts that rapidly turns over.

Collagen Type Analysis in DMD and Normal Skin Fibroblasts. In order to test whether DMD fibroblasts show differences in the amount and quality of type I and type III collagen as compared to normal fibroblasts, NaDodSO$_4$/polyacrylamide gel electrophoresis and CM-cellulose chromatography were performed. As determined by both methods, DMD fibroblasts (DMD-1, DMD-2) expressed no differences in the relative content of type I (α chains) and type III

Table 3. Collagen degradation in normal and DMD fibroblast cultures

Exp.	Assay	Time after chase, hr	% change in cell line					
			HH-4	HH-6	HH-10	DMD-1	DMD-3	DMD-4
I	Collagen degraded	0.5	12.7	16.5	14.2	24.1	23.8	27.8
		2	20.2	29.3	33.9	61.0	60.3	69.5
		6	28.4	39.2	47.2	74.0	71.2	71.1
		24	56.5	64.8	61.5	76.1	74.4	72.9
II	Dialyzable [^{14}C]Hyp	0.5	28.2	20.9	—	52.3	53.5	—
		2	40.3	42.7	—	66.8	59.1	—
		8	64.4	69.1	—	63.2	69.6	—
		24	74.7	67.4	—	71.2	70.0	—

Experiment I: rates of collagen degradation were determined by measuring the remaining collagen-bound radioactivity ([^{3}H]proline) in pepsin-resistant material in a pulse–chase experiment (2-hr pulse; 0.5- to 24-hr chase). Normal fibroblasts (HH) were used at CPD number 19. DMD fibroblasts were studied at CPD number 20. Experiment II: proportions of newly synthesized collagen were determined by following the amount of [^{14}C]hydroxyproline ([^{14}C]Hyp) in a pulse–chase experiment (2-hr pulse; 0.5- to 24-hr chase). Normal fibroblasts (HH) were studied at CPD number 21. DMD fibroblasts were analyzed at CPD number 22.

Table 4. Type I/type III collagen ratio in normal and DMD fibroblast cultures

Cell line	Method	CPD	Collagen, % Type I	Type III	Total
HH-6	CMC	18	87	13	100
DMD-1	CMC	23	83	17	100
HH-6	$NaDodSO_4$/PAGE	20	83	15.5	98.5
DMD-1	$NaDodSO_4$/PAGE	19	82	18.5	100.5
DMD-2	$NaDodSO_4$/PAGE	24	81	18.0	99.0

Numbers were obtained by two different methods using purified total collagen; CMC, carboxymethyl-cellulose chromatography.

(2-mercaptoethanol-reducible γ components) collagen when compared to controls (HH-6) (Table 4, Fig. 1).

However, during the purification of type I and type III collagen by CM-cellulose chromatography, the α_1(I) and α_2(I) chains of type I collagen from DMD fibroblasts were eluted reproducibly at lower LiCl concentrations [α_1(I): 0.065 ± 0.005 M LiCl; α_2(I): 0.099 ± 0.012 M LiCl] than the α_1(I) and α_2(I) chains of type I collagen obtained from normal skin fibroblasts [α_1(I): 0.074 ± 0.008 M LiCl; α_2(I): 0.111 ± 0.010 M LiCl]. This indicates a change in the net charge of single collagen chains.

Hydroxyproline/Proline Ratio in DMD and Normal Skin Fibroblasts. In order to investigate whether the elevated degradation of collagen in DMD fibroblasts is the consequence of structural changes in the collagen molecules, the degree of hydroxylation of proline residues in collagen (composed of type I and type III) of DMD and normal skin fibroblasts was analyzed. For this purpose, hydrolysates of purified collagen were examined by means of amino acid analysis. In these experiments the ratios of hydroxyproline/proline were determined in two separate studies of hydrolysates from parallel cultures of eight DMD and eight normal skin fibroblast cell lines. Collagen of DMD fibroblasts expressed a significantly higher ratio of hydroxyproline/proline (1.36–1.45; $P < 0.01$) as compared with the collagen of control fibroblasts (0.86–0.89) (Table 5).

Table 5. Hydroxyproline/proline (Hyp/Pro) ratio of total collagen (types I and III) of normal and DMD fibroblast cultures

Cell line	CPD	Hyp/Pro ratio Series 1	Series 2
HH-4	17	0.87	0.91
HH-5	23	0.93	0.79
HH-6	19	0.91	0.86
HH-7	16	0.90	0.90
HH-10	19	0.85	0.80
HH-11	22	—	0.83
HH-13	25	0.92	0.94
HH-14	24	0.86	0.86
Mean ± SEM		0.89 ± 0.01	0.86 ± 0.02
DMD-1	15	1.68	1.77
DMD-2	13	1.41	1.45
DMD-3	15	1.15	1.02
DMD-4	15	1.01	1.02
DMD-5	16	1.51	1.74
DMD-6	16	1.40	1.56
DMD-7	17	1.37	1.49
DMD-8	17	—	1.50
Mean ± SEM		1.36 ± 0.08*	1.45 ± 0.10*

Hyp/Pro ratios were determined by two independent series of measurements of the hydrolysates of total collagen from parallel cultures by means of amino acid analysis. The first series of amino acid analysis was undertaken at the Institut für Biochemie, Universität Stuttgart (G. Pfleiderer and K. Jany). A second series of amino acid analysis experiments was performed at the Max-Planck-Institut für Biochemie (K. Kühn and J. Mollenhauer).
*$P < 0.01$.

DISCUSSION

Abnormalities in the collagen metabolism in *in vivo* (30) and in *in vitro* (25) systems of the DMD syndrome have been reported previously. An excessive deposition of type III collagen was observed in the fibrosis in perimysium and endomysium in dystrophic muscle of DMD origin (30). In DMD fibroblasts *in vitro*, Ionasescu *et al.* (25) described a decrease in the synthesis of noncollagen proteins, a reduction in the incorporation of L-[^{3}H]proline into intracellular collagen, and an increase in the incorporation of radioactively labeled L-proline into extracellular collagen. Preliminary data on net protein synthesis of four DMD fibroblast cell lines indicate a significant decrease in the synthesis of total protein as measured by the incorporation of L-[^{3}H]leucine (unpublished data). In the present studies, a decrease in the synthesis of collagen of six DMD fibroblast cell lines was observed as determined by the amount of pepsin-resistant proteins. The reason for the discrepancy between these findings and the results of Ionasescu *et al.* (25) is unclear. They could be the outcome of differences in the *in vivo* and *in vitro* biological material studied.

In the present investigations, however, the measurements of overall collagen synthesis did not discriminate between synthesis and degradation. To elucidate the correlation between synthesis and degradation, collagen degradation was analyzed by measuring the amount of remaining collagen-bound radioactivity as well as the release of dialyzable hydroxyproline in pulse–chase experiments. These studies revealed a 50–100% increase in the combined extracellular and intracellular degradation of collagen in DMD fibroblasts compared to normal fibroblasts. Although it can not be excluded by the methods used in these studies that a decrease in the primary synthesis of collagen takes place, the reduction of overall collagen synthesis in DMD fibroblasts could very well be a corollary of the enhanced degradation of this protein.

In vivo (31) and *in vitro* (32) studies on protein degradation in DMD muscle tissues indicate an enhanced degradation of muscle proteins. One important function of protein degradation in mammalian cells is the selective removal of proteins with abnormal conformations (33, 34). Therefore, the molecular composition of the collagen types synthesized by DMD fibroblasts was analyzed. A significant change in the type I/type III collagen ratio and in α_1/α_2 chain ratios of type I collagen (data not shown) was not observed. A change in the net charge of the α chains of type I collagen could be demonstrated by CM-cellulose chromatography.

The major discrepancy, however, between collagen of DMD and normal fibroblasts was the unusual degree of hydroxylation (hydroxyproline/proline ratio, 1.36–1.45) in the collagen of DMD fibroblasts. As discussed above, a change in the hydroxylation of collagen derived from DMD fibroblasts based on different amounts of type III collagen can be excluded. Therefore, an increase in the hydroxylation of proline residues of single α chains can be discussed. Normally, hydroxyproline is found in vertebrate tissues almost exclusively in collagen molecules. Most of the amino acid hydroxyproline is present in the collagen molecules in the form of the *trans*-4-isomer, but all collagens also contain some *trans*-3-hydroxyproline (35, 36). The hydroxylation of proline residues is catalyzed by two distinct enzymes, prolyl-4-hydroxylase and prolyl-3-hydroxylase (37, 38). However, hydroxylation in a given sequence is incomplete (39). In normal type I collagen, only proline in the Y position of the Gly-X-Y sequence is 4'-hydroxylated (35). This leads to a maximal theoretical hydroxyproline/proline ratio of 1:1. The

maximal theoretical value of the 4'-hydroxyproline/proline ratio in type III collagen is 1:1.2 (40). In two independent amino acid analyses, no significant amounts of 3'-hydroxyproline that normally is located in the X position (35) and could lead to a further increase in the hydroxyproline/proline ratio was detected. Thus, one has to assume an unusual 4'-hydroxylation of proline residues in the X position.

The implications of the increased hydroxylation of proline residues, demonstrated in collagen of DMD fibroblasts *in vitro*, for the expression of the pathological manifestations in different organ systems *in vivo* can not be assessed at the present time. However, since collagen and other collagen-like polypeptides such as elastin and acetylcholinesterase (41, 42) are major muscle proteins, a single biochemical abnormality of this kind in several different proteins can be discussed to constitute a molecular basis for the pathological manifestation of DMD *in vivo*.

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