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# Alkali-Treated Collagen Retained the Triple Helical Conformation and the Ligand Activity for the Cell Adhesion *via* $\alpha 2\beta 1$ Integrin

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**Alkaline treatment is a good method for extracting collagen with high recovery even from an aged animal specimen. However, the properties of collagen treated under alkaline conditions have not been well established yet. By the treatment with a solution of 3% sodium hydroxide and 1.9% monomethylamine, the isoelectric point of type I collagen was lowered from 9.3 to 4.8 because of the conversions of Asn and Gln to Asp and Glu. With the acidification of the pI, the denaturation temperature of the collagen was decreased from 42 to 35°C after 20 d treatment, but the collagen-specific triple helical conformation was maintained. Human keratinocytes and fibroblasts adhered to the alkali-treated collagen *via* the collagen receptor integrin $\alpha 2\beta 1$. This indicates that the alkali-treated collagen maintained its property as a biological adherent molecule. Unlike acid-soluble collagen, alkali-treated collagen lost the ability to form fibrils at neutral pH under physiological conditions. This ability was lost even after 4 h of alkaline treatment, when the denaturation temperature of the collagen did not change. On the other hand, the alkali-treated collagen formed a fibrous precipitate with a uniform diameter of 50–70 nm under acidic conditions at 30°C.**

**Key words: alkaline treatment, BSE, collagen, fibril formation, integrin.**

The method of extracting collagen from connective tissue with a solution of sodium hydroxide and monomethylamine was developed about 30 years ago (*1*). This method has several benefits compared with acid or enzymatic extraction: (i) almost all collagen is solubilized even from aged animal skin, from which collagen is hardly extracted by pepsin treatment; (ii) the procedure is simple and inexpensive because most proteins other than collagen are destroyed under the conditions; and (iii) the collagen is sterilized during alkaline treatment. Alkaline extraction of collagen is also valuable from the standpoint of preparation of safer biomaterials for human use, because it is an effective way of inactivating the transmitting agent (prion) causing bovine spongiform encephalopathy (BSE) (*2, 3*).

Collagen provides mechanical strength to tissues (*4*) and forms a favorable matrix for cell attachment *in vivo*. For application, type I collagen is used for medical and cosmetic purposes, for example, as the base of artificial organs (*5, 6*), as a covering layer for burned skin, and in creams for cosmetic use (*7*). Extraction with acid or pepsin treatment is commonly used for collagen preparation from animal skin, of which bovine and porcine skin are the most common sources. Extraction with alkaline solution is also used, but the effects on collagen of treatment with a solution of sodium hydroxide and monomethylamine have not been fully characterized. It was known only: (i) the isoelectric point (pI) was shifted to an acidic value probably because of the conversion of the Gln and Asn residues to Glu and Asp residues (*1, 8*); (ii) some Arg residues were converted to ornithine residues by deamination (*1*); and (iii) degradation of collagen telopeptide was suspected from the observation of a decreased amount of Tyr residues by amino acid analysis, but triple helical conformation was not destroyed (*1, 8*).

When we use alkali-solubilized collagen for medical purposes, information on the effect of alkaline reagents on collagen is indispensable. In this report, we examined the changes in the chemical and biological properties of collagen by alkaline treatment. We found that the alkaline treatment affected the thermal stability and the fibril formation of collagen at neutral pH, but the restricted cutting site of collagenase (matrix metalloproteinases 1, MMP1) and the ligand activity for integrin were retained.

## MATERIALS AND METHODS

*Preparation of Collagen*—Collagens were prepared from bovine hide. Acid-soluble collagen was prepared by extraction with 0.5 or 0.05 M acetic acid at 4°C for 1 d. Pepsin-solubilized collagen was prepared by pepsin (0.02%) digestion in 0.5 M acetic acid at 4°C for 1 d. Type I collagen was

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Abbreviations: BSE, bovine spongiform encephalopathy; CD, circular dichroism; MMP, matrix metalloproteinases; PBS, phosphate buffered saline.

purified by salt fractionation (*9*). Alkali-solubilized collagen was prepared by treatment with 3.0% NaOH (w/v)-1.9% monomethylamine (v/v) solution at 20°C for 2 wk. Collagen was precipitated by dialysis against 50 mM Tris-HCl buffer (pH 7.4) in the absence of other salts. The precipitate was dissolved in 5 mM acetic acid.

The amount of extracted collagen was estimated by comparing the hydroxyproline content in the whole skin with that in the extracted collagen. The method of hydroxyproline assay from the hydrolyzed sample was as described (*10, 11*).

*Alkaline Treatment of Acid-Soluble Collagen*—Acid-soluble type I collagen was treated with 3.0% NaOH (w/v)-1.9% monomethylamine (v/v) solution at 20°C. The conditions of alkaline treatment were the same as those described for producing alkali-solubilized collagen (*1*). The alkaline solution was dialyzed against 0.5 M acetic acid at 4°C to stop the reaction, lyophilized, and dialyzed against distilled water.

*Determination and Calculation of the Isoelectric Point of Collagen*—The isoelectric point of collagen was measured by the method of Janus *et al.* (*12*) with slight modification. In brief, distilled and deionized water was added to lyophilized collagen, which was dissolved by boiling for 2 min to form a 1% denatured collagen solution. To 500 $\mu$l of denatured collagen solution, an equal volume of a mixture of 2 parts of activated anion exchange resin Amberlite IRA400 (Organo, Tokyo) and 1 part of cation exchange resin Amberlite IR120B (Organo) was added, and the whole mixture was incubated at 40°C with gentle shaking. After incubation for 2 h, the pH of the solution was measured. The acid-base equilibrium for the collagen-water system in the absence of salt is such that the pH of the solution is indistinguishable from the isoionic point of denatured collagen. Under these conditions, the isoionic and isoelectric points of the denatured collagen are identical (*12*).

The pI (isoelectric point) was calculated from the amino acid composition data using Compute pI/Mw software, which was provided by the ExPASY Proteomics tools site through the Internet (http://expasy.hcuge.ch/www/tools.html). This software, which predicts the pI of protein, is based on the method of Bjellquvist *et al.* (*13, 14*). Amino acid composition of collagen was calculated as the sum of two parts of the amino acid residues of the $\alpha$1 (*15*) and one part of those of the $\alpha$2 (*16*) chain of bovine type I collagen.

*Collagen Digestion with Collagenase*—Acid-soluble collagen or alkali-treated collagen was digested with human MMP1 (Yagai) in the collagenase assay buffer (*17*) at 20°C. The reaction was stopped by dialyzing against 5 mM acetic acid solution. The rate of digestion was estimated using densitometry by comparing the amount of digested fragments (TCa) with the undigested $\alpha$ chain after SDS-PAGE.

*Amino Acid Sequencing*—Collagen $\alpha$ chains or collagen-derived fragments by the enzymatic digestion were separated by SDS-PAGE. After electrotransference to an Immobilon-P$^{SQ}$ membrane (Millipore, MA, USA), amino acid sequencing was performed with a 491 Protein Sequencer (Applied Biosystems).

*Adhesion of Cells to Collagen Surface and Immunofluorescence Staining*—Human keratinocytes and fibroblasts from foreskin were grown on a heavy Teflon-coated slide with 12 wells (Cell-line Associates, NJ) in serum-free medium. Before the cell culture, the glass surface was coated with collagen (10 $\mu$g/ml PBS), then blocked with 0.25% heat-denatured BSA as described (*18*). After the incubation, the cells were fixed in 2% formaldehyde in PBS for 20 min. After treatment with 1% Triton X-100 in PBS for 1 min, the cells were blocked with 1% heat-denatured BSA in PBS. The slides were then incubated with monoclonal anti-human $\alpha 2\beta 1$ integrin antibody (P4B4) (*18*) and species-specific FITC-labeled secondary antibody. Cells were examined using a confocal laser scan microscope LSM 410 system (Zeiss, Jana, Germany).

*Detection of Collagen Conformational Change Using CD Spectroscopy*—Acid-soluble collagen or alkali-treated collagen (0.2 mg/ml) in PBS (pH 7.4) containing 0.8 M glucose to inhibit the fibril formation (*19*) was placed in a spectropolarimeter Jasco-600 (Japan Spectroscopic). The CD (circular dichroism) ellipticity of the collagen solution at 221 nm was monitored. The denaturation temperature ($T_m$) was measured by monitoring the ellipticity of the collagen solution (0.2 mg/ml PBS) in a cell in which the temperature was increased at a constant rate (1°C/4 min) from 20 to 50°C. $T_m$ was defined as the temperature of the mid-point of ellipticities at 20 and 50°C.

*Collagen Fibril Formation*—To survey the fibril-forming ability of collagen over a wide range of pH or salt concentration, McIlvaine buffer (0.1 M $Na_2HPO_4$, 0.05 M citrate) was used. The collagen was dissolved in the McIlvaine buffer (pH 2.5-8.0) in the presence of various concentrations of NaCl (final concentration 0-1.2 M) and placed in a 96-well immuno plate (Nunc, Denmark) (100 $\mu$l/well) on an ice-bath. The final concentration of collagen was adjusted to 1 mg/ml. After warming in a water bath at 30°C, the turbidity of the collagen solution was monitored by measuring the absorbance at 415 nm with a microplate reader MTP-32 (Corona Electric).

*Electron Microscopy*—A collagen fibril was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 30°C to avoid dissolving the fibril at low temperature, then stained with 0.5% uranyl acetate (pH 4.4) and with 2% phosphotungstic acid (pH 1.8). The samples were examined with an electron microscope as previously described (*20*).

## RESULTS

*Extractability of Collagen by Alkaline Treatment from the Skin*—We compared several methods for solubilizing collagen from bovine skin of different ages (Table I). From the newborn bovine skin, about 50% of the collagen was extracted by acetic acid treatment, while the pepsin treatment and alkaline treatment were able to solubilize almost of the collagen. From adult bovine skin (18 months), the solubility of the collagen by the acid treatment decreased to less than 10%. From aged bovine skin (8 years), only 10% of the collagen was extracted even by the pepsin treatment; but most of the collagen (82%) was extracted by the alkaline treatment using 3.0% NaOH (w/v)-1.9% monomethylamine (v/v) solution. This indicates that the alkaline solubilization method is a powerful way to produce a large amount of collagen solution. This collagen specimen consisted of type I collagen containing a small amount of type III collagen (about 10%). The mobilities on SDS-PAGE of alkali-solubilized type I and III collagen were slower than those of pepsin- or acid-soluble collagen because of the reduced hydrophobicity as a result of the

deamination of the acid amide group during the alkaline treatment (*21, 22*). The collagen extracted from the skin by alkaline solubilization was indistinguishable from the acid-soluble collagen treated in 3.0% NaOH (w/v)-1.9% monomethylamine (v/v) solution (Fig. 1, lane 1, acid-soluble collagen; lane 8, 20-d alkali-treated acid-soluble collagen; lane 9, alkali-solubilized collagen from the adult bovine skin). Thus, in the following experiments, we used the alkali-treated acid-soluble type I collagen to examine the effect of alkaline treatment on collagen molecules.

*Change in Primary Structure of Collagen during Alkaline Treatment*—The change in collagen with the duration of alkaline solution was examined. To estimate the degradation of the collagen by the alkaline treatment, a fixed volume of alkali-treated collagen solution was dialyzed against 5 mM acetic acid and the lyophilized collagen was weighed. No loss of collagen was detected during the alkaline treatment for up to 20 d (data not shown). The loss of the telopeptide could not be detected by this method. The mobilities of the collagen $\alpha$ chain on SDS-PAGE gradually decreased during alkaline treatment up to 8 d (Fig. 1).

TABLE I. **Extractability of collagen from bovine skin of different ages.**

| Extraction method | Extracted collagen from skin (%)[a] | | |
|---|---|---|---|
| | Newborn | 18 mo | 8 yr |
| Acetic acid[b] | 44.8 | 5.2 | 4.5 |
| Pepsin[c] | 90.8 | 93.2 | 10.0 |
| Alkali[d] | 91.0 | 99.2 | 81.9 |

[a]Total collagen was estimated by measurement of hydroxyproline content after hydrolysis of the skin. [b]0.05 M acetic acid at 4°C for 1 d. [c]Pepsin (0.02%) in 0.5 M acetic acid solution at 4°C for 1 d. [d]3.0% NaOH (w/v)-1.9% monomethylamine (v/v) solution at 20°C for 14 d.

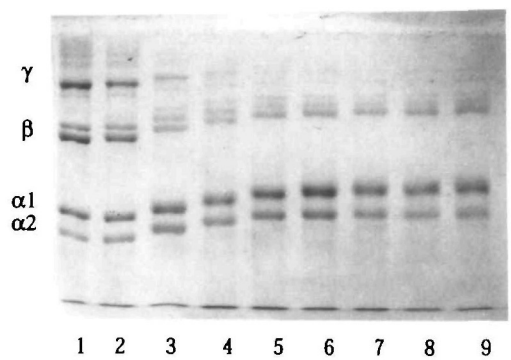


Fig. 1. **SDS-PAGE of alkali-treated collagen.** Acid-soluble collagen was treated in 3.0% NaOH (w/v)-1.9% monomethylamine (v/v) solution for the indicated period at 20°C and analyzed by SDS-PAGE (5% gel). Lane 1, acid-soluble collagen; lane 2, 4 h; lane 3, 1 d; lane 4, 2 d; lane 5, 5 d; lane 6, 8 d; lane 7, 12 d; lane 8, 20 d; lane 9, alkali-solubilized collagen from skin.

TABLE II. **Content of Gly residues in each cycle of amino acid sequencing of 20-d alkali-treated collagen.**

| | | Cycle | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Content of Gly (%) | $\alpha$1 | 24 | 23 | 29 | 30 | 31 | 31 | 32 |
| | $\alpha$2 | 24 | 27 | 29 | 31 | 33 | 32 | 33 |

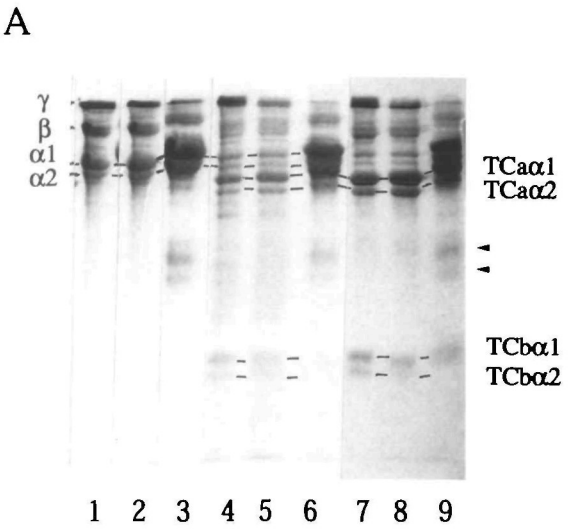


Fig. 2. **Alkali-treated collagen digested with MMP1 and the N-terminal amino acid sequences of the TCb fragments.** A: Acid-soluble collagen (lanes 1, 4, and 7), 4-h alkali-treated collagen (lanes 2, 5, and 8), and 20-d alkali-treated collagen (lanes 3, 6, and 9) were digested with human MMP 1 at 20°C for 0 d (lanes 1-3), 1 d (lanes 4-6), and 6 d (lanes 7-9). After the digestion, collagen was analyzed by SDS-PAGE (10% gel). The $\alpha$ chain of collagen is indicated on the left margin and the larger fragments (TCa) and the smaller fragments (TCb) are indicated on the right margin. The minor doublet bands indicated by the arrowheads may be degradative products of collagen by alkaline treatment. B: TCb fragments were subjected to amino acid sequencing. The number of amino acid residues in the figure was counted from the beginning of the triple helical region. The letter showed in bold was the different amino acid residues between acid-soluble collagen and alkali-treated collagen. X indicates unidentified amino acid residues.

TABLE III. **Change of collagen during alkaline treatment.**

| | Period of alkaline treatment at 20°C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 4 h | 1 d | 2 d | 5 d | 8 d | 12 d | 16 d | 20 d |
| Isoelectric point[a] | 9.3 | 7.8 | 5.6 | 5.1 | 4.9 | 4.8 | 4.8 | 4.8 | 4.8 |
| Change of Asn, Gln to Asp, Glu[b] | – | 26% | 45% | 72% | 92% | 100% | 100% | 100% | 100% |
| Mobility of $\alpha$1 chain on SDS PAGE[c] | 0.67 | 0.66 | 0.64 | 0.60 | 0.58 | 0.57 | 0.57 | 0.56 | 0.55 |

[a]Isoelectric point of denatured collagen estimated experimentally. [b]Isoelectric point of collagen was predicted by the method of Bjellquvist *et al.* (*13, 14*) by substituting Asn and Gln with Asp and Glu. Conversion ratio was determined by the matching of the predicted pI and experimental pI. [c]Relative mobility of the $\alpha$1 chain of collagen is from Fig. 1.

TABLE IV. **Thermal stability of alkali-treated collagen.**

| | Period of alkaline treatment at 20°C | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | 0 | 4 h | 1 d | 2 d | 5 d | 8 d | 12 d | 16 d | 20 d |
| Ellipticity at 20°C (milli deg)[a] | 113.4 | 106.6 | 106.4 | 99.7 | 95.4 | 98.8 | 95.4 | 90.8 | 100.9 |
| Denaturation temperature (°C)[b] | 40.4 | 40.3 | 38.0 | 36.7 | 35.5 | 35.1 | 34.8 | 34.6 | 34.4 |

[a]Collagen concentration was adjusted to 0.2 mg/ml in PBS. [b]Denaturation temperature ($T_m$) was defined as the temperature of the mid-point of ellipticities at 20 and 50°C.

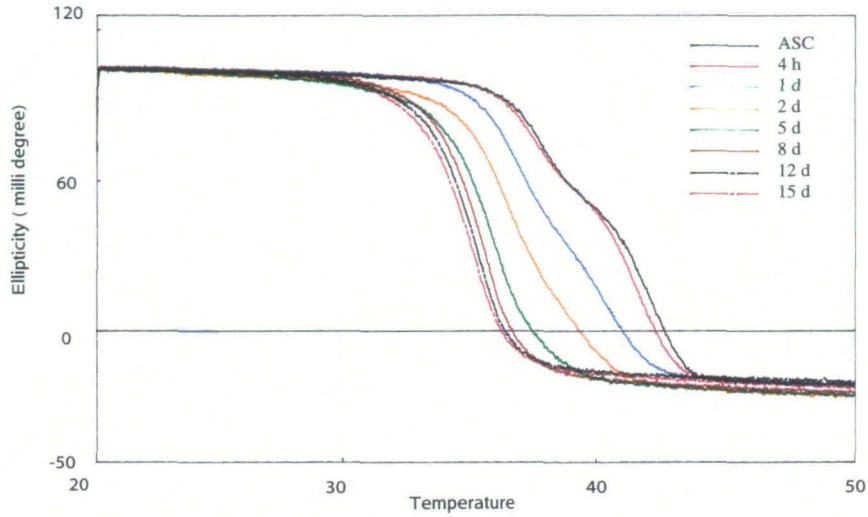


Fig. 3. **Denaturation curve of alkali-treated collagen by CD measurement.** The CD ellipticity at 221 nm of the acid-soluble collagen or alkali-treated collagen (treatment period is shown in the figure) was monitored by increasing the temperature from 20 to 50°C at a constant rate (1°C/4 min). ASC in figure means acid-soluble collagen.

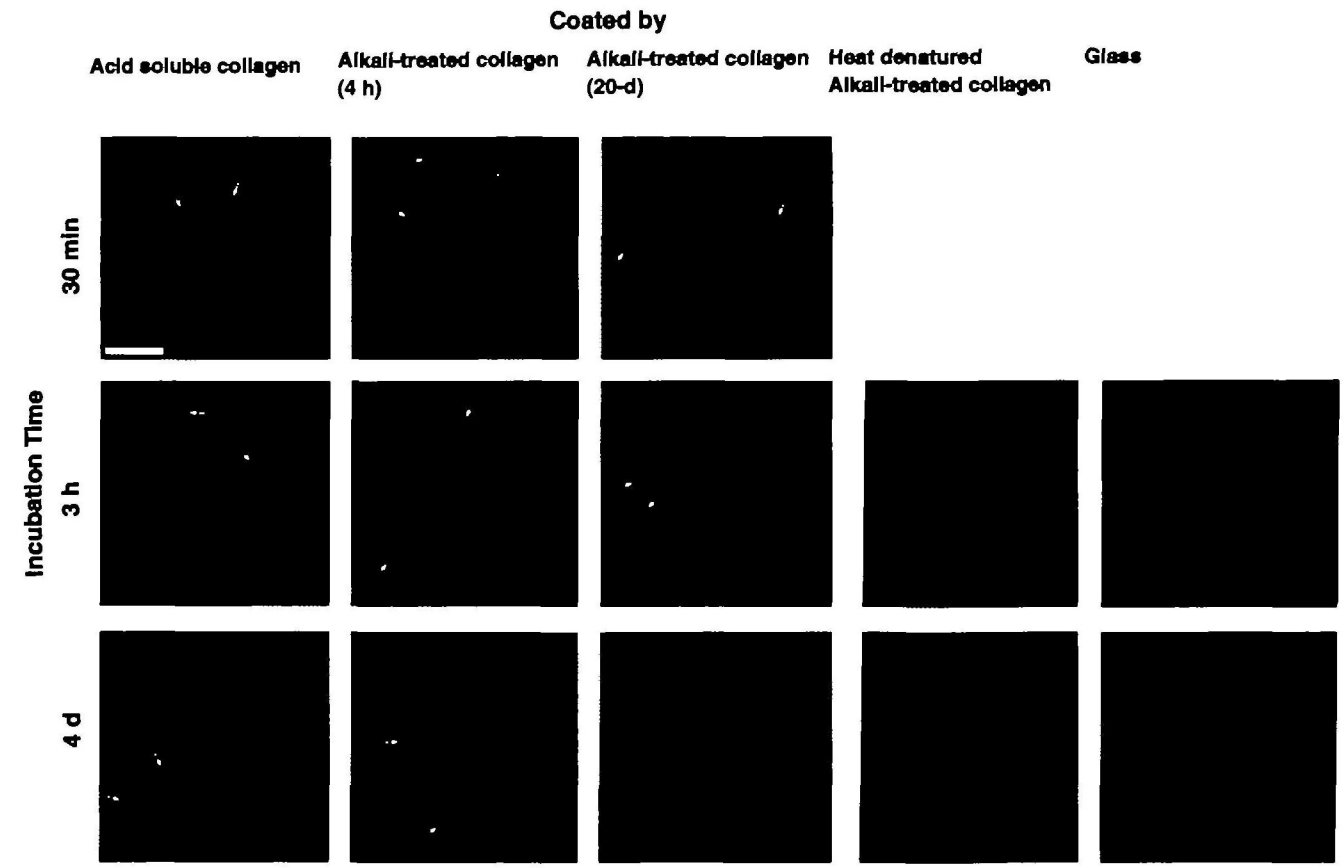


Fig. 4. **Immunofluorescence staining of keratinocytes on collagen with anti-$\alpha 2\beta 1$ integrin antibody.** Human keratinocytes on the collagen surface were fixed after 30 min, 3 h, and 4 d of incubation. Cells were immunofluorescence-stained with anti-$\alpha 2\beta 1$ integrin (P4B4) antibody. There were no adhered cells on the glass and denatured collagen after 30 min of incubation. Arrows show the focal contacts stained by the anti-$\alpha 2\beta 1$ integrin antibody. Bar in figure shows 20 $\mu$m.

After more than 8 d of treatment, the mobilities became constant. The retardation rates of the $\alpha 1$ and $\alpha 2$ chains were almost the same (Fig. 1). The decreasing amount of the $\gamma$ chain during alkaline treatment indicated the degradation of the telopeptide that is involved in crosslinking and/or degradation of the crosslinks in the triple helical region (Fig. 1). To determine the newly emerged N-terminal of the collagen molecule by the alkaline treatment, we tried amino acid sequencing of the alkali-treated collagen. Amino acid sequencing of acid-soluble collagen was unsuccessful, possibly because of blocking of the N-terminal amino acid. The emergence of various amino acids in the first cycle (mainly Gly, Pro, and hydroxyproline) of amino acid sequencing of the alkali-treated collagen indicated the absence of a specific cutting site in the sequence. However, when the amino acid compositions in each cycle of the Edman reaction were estimated, the content of Gly after the third cycle became 30-33% (Table II). This indicates that alkaline treatment cut the collagen molecule near the beginning of the triple helical region (Gly-X-Y) with some randomness.

The isoelectric point (pI) of collagen measured using the mixture of ion exchange resins was lowered by the alkaline treatment (Table III). This acidification of the pI was believed to be the result of deamination of the acid amide of the Asn and Gln residues (*1, 8, 21*). The calculated conversion rates of Asn and Gln to Asp and Glu by the method of Bjellquvist *et al.* (*13, 14*) for 8- to 20-d alkali-treated collagens were 100% (Table III). In the calculation, we assumed that the conversion rates of Asn and Gln were the same. Indeed, the peptide sequencing of the TCb fragment produced by MMP1 digestion showed that all Gln residues on the $\alpha 1$ chain at positions 779 and 787 were converted to Glu residues (Fig. 2B). We also observed the disappearance of Arg residues by the alkaline treatment, but we could not confirm the existence of ornithine, which was previously suggested to be a modified product of Arg (*1*).

*Susceptibility to Collagenase Digestion*—Susceptibility of the alkali-treated collagen to enzymatic digestion was examined. Fifty micrograms of collagen was incubated with 0.3 unit of MMP1 for several days at 20°C and analyzed by SDS-PAGE. Acid-soluble collagen and 4-h alkali-treated collagen showed the same susceptibility (Fig. 2A, lanes 4, 5, 7, and 8). On the other hand, collagen treated in alkaline solution for 20 d was less than 6 times as susceptible (Fig. 2A, lanes 6 and 9). The cutting site with MMP1 was determined by the amino acid sequencing. The amino terminal sequences of smaller peptides (TCb fragments) from the acid-soluble collagen and 20-d alkali-treated collagen were the same (Fig. 2B), although some amino acid residues were converted by chemical modification during alkaline treatment. The faint doublet bands between the TCa and the TCb fragments in the 20-d alkali-treated collagen (Fig. 2A, lanes 3, 6, and 9 indicated by the arrowheads) could not be identified by amino acid sequencing; however, the amino acid composition (rich in Gly, Pro, and hydroxyproline) in each cycle suggested that those two peaks also derived from collagen molecules. The amount of these two bands was less than 5% of total collagen. Except for these two bands, no degradation products of collagen by alkaline treatment were detected on SDS-PAGE analysis. This indicates that the collagen triple helical conformation is stable toward the alkaline hydrolysis, as previously assumed (*1, 8*).

*Conformational Change and Thermal Stability of Alkali-Treated Collagen*—The value of ellipticity of collagen at 20°C did not change during the alkaline treatment, but the thermal stability decreased (Table IV). Collagen preparations left in the alkaline solution for more than 1 d were denatured at the physiological temperature (37°C). Denaturation patterns with increasing temperature are shown in Fig. 3. Acid-soluble collagen and 4-h alkali-treated collagen showed a biphasic denaturation pattern, while the

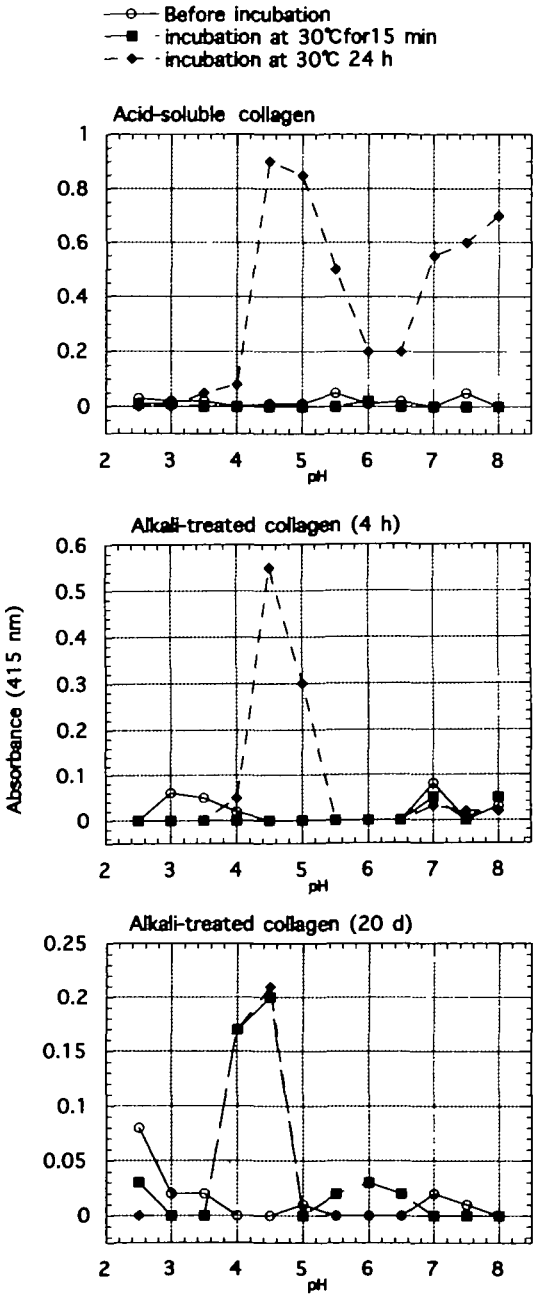


Fig. 5. **Effect of pH on collagen fibril formation.** Collagen in the solution of indicated value of pH in the presence of 0.15 M NaCl on the microtiter plate was warmed at 30°C and the absorbance at 415 nm was measured. Upper, acid-soluble collagen; middle, 4-h alkali-treated collagen; lower, 20-d alkali-treated collagen.

denaturation curves of collagen treated for more than 1 d became monophasic. There were small changes in the $T_m$ and the pattern of the denaturation curve of the collagens alkali-treated for longer than 5 d.

*Cell Adhesion to Alkali-Treated Collagen*—Collagen is the one of the cell adhesion molecule *via* the integrin receptor on the cell surface. The most common receptor for collagen on the keratinocytes is integrin $\alpha 2\beta 1$, which recognizes the specific arrangement of the amino acid residues defined by collagen triple helical conformation (*23-25*). Because alkali-treated collagen retained the collagen-specific conformation in spite of the modification of some amino acid residues, the ligand activity of alkali-treated collagen for the integrin on the keratinocyte was examined. Keratinocytes adhered well within 30 min to the acid-soluble collagen and alkali-treated collagen, but adhesion to the heat-denatured alkali-treated collagen surface was rather worse than to the glass surface. Focal contact

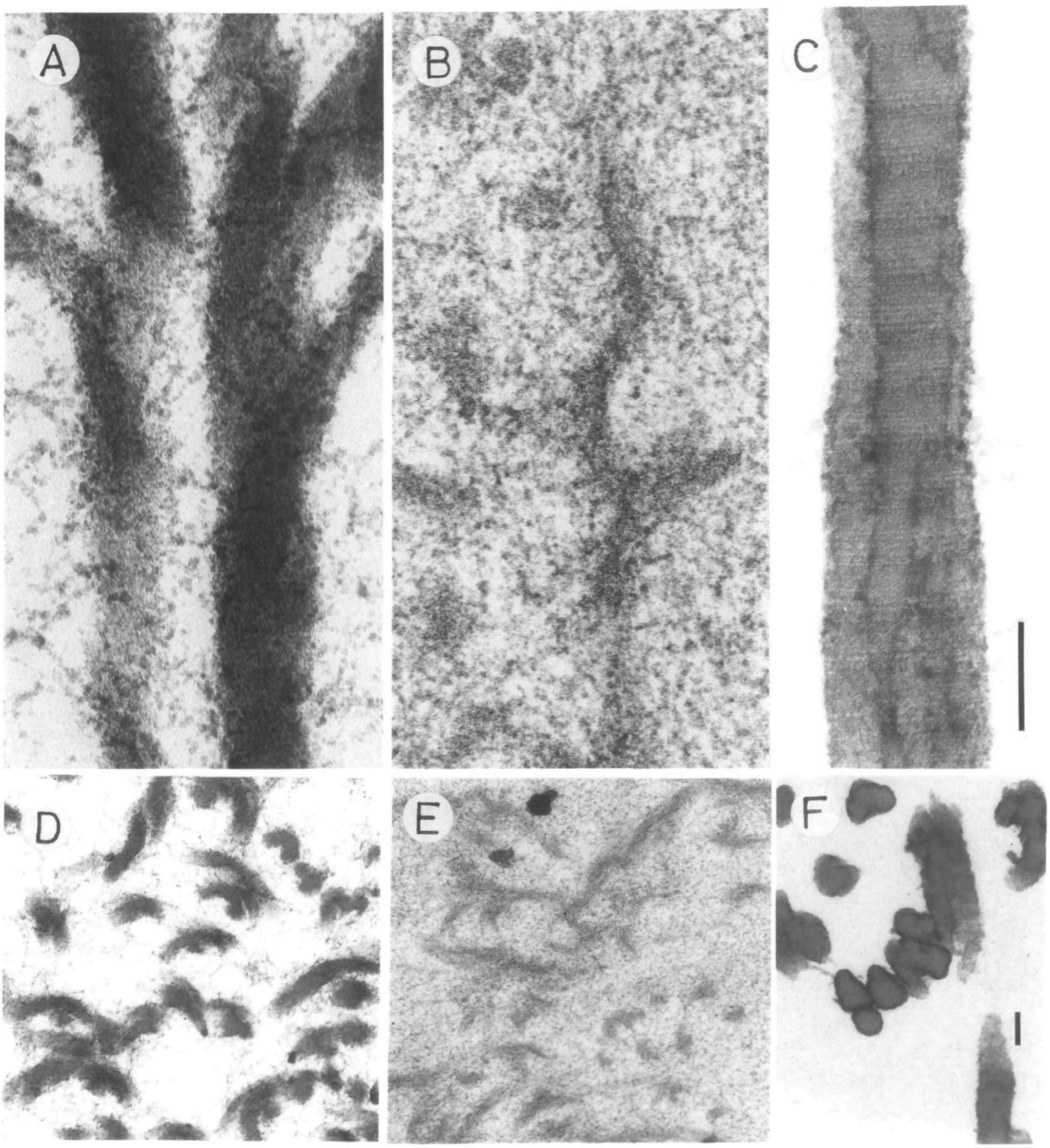


Fig. 6. **Electron microscopy of the fibrils of alkali-treated collagens.** Alkali-treated collagen in McIlvaine buffer or acid-soluble collagen in PBS was incubated at 30°C for 2 h. The precipitate was fixed with warmed glutaraldehyde (30°C) and observed under the electron microscope. A and D show the 20-d alkali-treated collagen precipitate in McIlvaine buffer at pH 4.5 in the presence of 0.3 M NaCl. B and E show the 20-d alkali-treated collagen precipitate in McIlvaine buffer at pH 4.5 in the presence of 0.8 M NaCl. C and F show the acid-soluble collagen fiber formed in PBS. A to C show higher magnification on the same scale. D to F show lower magnification on the same scale. The bars in C and F indicate 100 nm.

regions of keratinocytes on the alkali-treated collagen (4-h and 20-d treatments) and acid-soluble collagen were stained by anti-integrin $\alpha 2\beta 1$ for immunofluorescent microscopy (Fig. 4, 30-min and 3-h incubation). Adhesion of keratinocytes to 4-h alkali-treated collagen was comparable to that to acid-soluble collagen. This indicates that alkaline treatment does not destroy the ligand activity of collagen molecules. After 4 d of incubation, integrin shifted to the cell-cell contact region, as described by Carter *et al.* (*18, 26*) (Fig. 4, 4 d). On the glass and denatured-collagen surface, cells adhered to self-deposited laminin 5 *via* $\alpha 3\beta 1$ (*26*). When fibroblasts were used, the adhesion and the staining pattern of focal contact on the alkali-treated collagen after 1-h incubation were similar to those of keratinocytes.

*Fibril Formation of Alkali-Treated Collagen*—Acid-soluble type I collagen forms fibrils at neutral pH with physiological salt concentration *in vitro* between 20 and 40°C, but alkali-treated collagen lost the ability to form fibrils at neutral pH. Considering the acidification of the isoelectric point by the alkaline treatment, we examined the ability of alkali-treated collagen to form fibrils over a wider range of pH (Fig. 5) in the presence of 0.15 M NaCl. Because collagen that had been treated in the alkaline solution for more than 2 d was denatured above 37°C, the warming temperature was set at 30°C. As the difference in salt species is known to effect the fibril formation (*27*), McIlvaine buffer was chosen to cover the wider range of pH without changing the species of the salts. No alkali-treated collagen preparations were observed to undergo fibril formation at neutral pH. When we changed the concentration of NaCl to 0.3 M and 0.4 M, fibril formation by the alkali-treated collagen was not still observed at neutral pH. On the other hand, the turbidities of the solutions of both acid-soluble collagen and alkali-treated collagen increased at 30°C under acidic conditions (Fig. 5). The final turbidity of the alkali-treated collagen and acid-soluble collagen differed. When the concentration of NaCl in the solution was increased, fibril formation of all collagen preparations at acidic pH was accelerated. Increasing turbidity of the alkali-treated collagen was also observed in sodium acetate buffer at pH 4.5 with 0.3 M NaCl. The increase in turbidity under acidic conditions was much faster than that under neutral conditions. Increasing turbidity of 20-d alkali-treated collagen was observed within 15 min of incubation, while the fibril formation of acid-soluble collagen was observed after 2 h of incubation at 30°C.

To determine whether the insoluble material generated from the alkali-treated collagen solution was an amorphous precipitate or an organized fibril, the precipitate was examined by electron microscopy (Fig. 6). The precipitate formed by 20-d alkali-treated collagen at pH 4.5 showed a twisted fibrous structure with fibrils of 40–60 nm in diameter (Fig. 6, A and D). In Fig. 6A, two or three fibrils twisted together, so that the diameter of the fibril seems larger than 60 nm. When the salt concentration was increased to 0.8 M, precipitation was observed even before the incubation, but the turbidity was increased more by warming, which may be caused by additional fibril formation. Under the electron microscope, the precipitate at 30°C in the presence of 0.8 M NaCl was a mixture of fibrous and amorphous structures (Fig. 6, B and E). Compared with the fibril from acid-soluble collagen at neutral pH (Fig. 6, C and F), the diameter of the 20-d alkali-treated collagen fibril (40–60 nm) was smaller than that of the acid-soluble collagen (100–120 nm), and the collagen-specific banding pattern with a D period (Fig. 6C) was not observed in the fibril from the alkali-treated collagen (Fig. 6, A and B).

These results indicate that the alkali-treated collagen retained fibril-forming activity under acidic conditions as did acid-soluble collagen. On the other hand, collagen lost the ability of assembly at neutral pH after a few hours of alkaline treatment.

## DISCUSSION

The extractability of collagen by the alkaline treatment was much higher than that by the enzymatic or acidic extraction, especially from an aged sample. This is one of the merits of alkaline solubilization of collagen. In addition to the SDS-PAGE (Fig. 1), the alkali-solubilized collagen was indistinguishable from alkali-treated type I collagen in following properties (data not shown): (i) thermal stability of the triple helical conformation, (ii) acidification of pI, (iii) susceptibility to collagenase, (iv) keratinocyte adhesion *via* integrin, and (v) the features of fibril formation at neutral and acidic pH. This indicates that the properties of alkali-solubilized collagen is emerged by the result of alkaline treatment of type I collagen, so we regarded the those two collagen preparations were comparable materials.

Amino acid sequencing data of collagen $\alpha$ chain showed the emergence of the free amino terminal of collagen by the alkaline treatment. However, the precise cutting site could not be identified because of the heterogeneity of the amino terminal. Removing the telopeptide that is involved in crosslinking of the collagen molecule may be the primary effect of alkaline treatment to solubilize the collagen, as in the case of pepsin treatment; however, the higher extractability by alkaline treatment than by the enzymatic treatment may suggest the additional breakdown of crosslinks in the triple helical region that are resistant to the pepsin treatment (Table I). By the alkaline treatment, almost all protein other than triple helical collagen was hydrolyzed. This simplifies the collagen purification procedure. Also, it is known that this alkaline treatment destroys the infectious ability of the transmitting agent causing BSE (*2, 3*).

To evaluate the effect of alkaline treatment on the collagen, we examined the properties of alkali-treated acid-soluble type I collagen. The isoelectric point (pI) was gradually lowered during alkaline treatment (Table III). This may occur because of deamination of the acid amides of Asn and Gln as previously suggested (*1, 8*). We detected the change of Gln to Glu directly by the amino acid sequencing of TCb fragments generated by MMP1 digestion (Fig. 2B). The change in mobility of the $\alpha$ chain on SDS-PAGE (Fig. 1 and Table III) and the decreasing denaturation temperature (Table IV) paralleled the change of the pI. The decreasing mobility of the $\alpha$ chain on SDS-PAGE may occur because of the decreasing hydrophobicity of collagen due to the deamination (*21, 28*). The modification of amino acid residues also reduced the thermal stability of collagen, but the ellipticity at 20°C remained constant. This indicated that the triple helical conformation and the helicity of the collagen molecule were main-

tained throughout the period of the alkaline treatment.

The susceptibility of collagen to the collagenase digestion was decreased after the 20-d alkaline treatment. This indicated that the enzymatic recognition or catalytic activity of MMP1 was affected by the change in the amino acid residues, but the cutting site of MMP1 was not changed. The 4-h alkali-treated collagen showed an equivalent susceptibility to MMP1 to the acid-soluble collagen (Fig. 2). In practical applications, soluble collagen is known to be a better substrate than insoluble collagen for collagenase assay; for example, glucose, which inhibits collagen fibril formation, is added to the collagenase assay buffer to increase the sensitivity of the assay (*19*). The 4-h alkali-treated collagen can also be used as a good substrate for collagenase assay, because this collagen preparation loses its fibril-formation activity at neutral pH, as shown in Fig. 5.

As a biologically active substrate for the cell, alkali-treated collagen retained the ligand activity for the collagen receptor integrin $\alpha 2\beta 1$ on the cell surface. Human foreskin keratinocytes adhered to the alkali-treated (both 4-h and 20-d treatments) collagens and formed the focal contact with integrin $\alpha 2\beta 1$ as well as they did on acid-soluble collagen, but they did not adhere to the heat-denatured alkali-treated collagen that had lost the triple helical conformation (Fig. 4). Two possibilities have been suggested for the recognition site on collagen by $\alpha 2\beta 1$ integrin: one is that the conformational $\alpha 2\beta 1$ recognition site is a combination of Asp ($\alpha 1$ chain), Arg ($\alpha 2$ chain), and Asp ($\alpha 1$ chain) of type IV collagen at position 461 (*24, 25*); the other that the tetrapeptide in the $\alpha 1$-CB3 fragment of type I collagen is the recognition site for the $\alpha 2\beta 1$ integrin (*29*). Our results indicate that the collagen-specific triple helical conformation was essential for $\alpha 2\beta 1$ integrin recognition of type I collagen, as in the case of the recognition of type IV collagen by $\alpha 2\beta 1$ integrin (*24, 25*). However, our results also showed a discrepancy with Kühn's paper (*24*). Our amino acid sequencing data showed the Arg residues converted to other amino acid residues (possively ornithine) (Fig. 2B). There are several possible explanations for this: for example, the Arg residue may not be essential for the cell adhesion, or the modified Arg residue (ornithine is also a positively charged amino acid) may be able to substitute for Arg in this function. The definitive explanation must await the identification of the adhesion site of type I collagen molecules with integrin. Another curious feature of cell adhesion is that the 20-d alkali-treated collagen, whose $T_m$ (34.4°C) was lower than the incubation temperature of the cell (37°C), retained the ability to bind integrin. This may indicate that the triple helical conformation of collagen became more stable against the thermal disturbance by attaching to the solid surface.

We stated above that the thermal stability, susceptibility to MMP1 digestion, and cell adhesion of 4-h alkali-treated collagen were indistinguishable from those of acid-soluble collagen. However, alkaline treatment even for a few hours destroyed the ability of collagen to form fibrils at neutral pH. From the pI change, we estimated that about 25% of Asn and Gln residues were deaminated in 4-h alkali-treated collagen. This amount of deamination has less effect on the thermal stability of the triple helical conformation, but fibril formation was much more sensitive to the pI change of the collagen molecule. On the other hand, both alkali-treated collagen and acid-soluble collagen formed a precipitate under acidic condition at 30°C (Fig. 5). Ionic repulsion between the negatively charged residues which are increased by the alkaline treatment may inhibit the association of collagen molecules to form fibrils at neutral pH; but collagen molecules assembled with each other when the ionic repulsion was minimized under acidic conditions. By electron microscopic observation, this precipitate exhibited a fibrous nature with fibrils of uniform diameter, but it did not have the collagen-specific banding pattern as seen in the acid-soluble collagen fibrils formed in PBS (Fig. 6, C and F). The reason for the absence of the banding pattern, whether the difference in conditions for fibril formation or the difference in collagen preparation, is not yet clear.

Alkali-solubilization of collagen is an efficient way to prepare collagen as a safer biomaterial. By this treatment, collagen underwent some modification in its primary structure, but we can control the changes of collagen to maintain its biological activity by changing the period of alkaline treatment.

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