# Nutrient Metabolism

# Oral Administration of [14]C Labeled Gelatin Hydrolysate Leads to an Accumulation of Radioactivity in Cartilage of Mice (C57/BL)

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ABSTRACT   Several investigations showed a positive influence of orally administered gelatin on degenerative diseases of the musculo-skeletal system. Both the therapeutic mechanism and the absorption dynamics, however, remain unclear. Therefore, this study investigated the time course of gelatin hydrolysate absorption and its subsequent distribution in various tissues in mice (C57/BL). Absorption of ^{14}C labeled gelatin hydrolysate was compared to control mice administered ^{14}C labeled proline following intragastric application. Plasma and tissue radioactivity was measured over 192 h. Additional "gut sac" experiments were conducted to quantify the MW distribution of the absorbed gelatin using SDS-electrophoresis and HPLC. Ninety-five percent of enterally applied gelatin hydrolysate was absorbed within the first 12 h. The distribution of the labeled gelatin in the various tissues was similar to that of labeled proline with the exception of cartilage, where a pronounced and long-lasting accumulation of gelatin hydrolysate was observed. In cartilage, measured radioactivity was more than twice as high following gelatin administration compared to the control group. The absorption of gelatin hydrolysate in its high molecular form, with peptides of 2.5–15kD, was detected following intestinal passage. These results demonstrate intestinal absorption and cartilage tissue accumulation of gelatin hydrolysate and suggest a potential mechanism for previously observed clinical benefits of orally administered gelatin.   J. Nutr. 129: 1891–1895, 1999.

KEY WORDS:  • *gelatin hydrolysate* • *peptide absorption* • *organ distribution* • *cartilage* • *mice (C57/BL)*

Gelatin is a heterogeneous mixture of polypeptides which is produced by the hydrolysis of collagen. A further enzymatic degradation of this hydrolyzed collagen results in a product which is called gelatin hydrolysate, and which contains peptides with a mean MW of 3–6 kD.

From a nutritional point of view, gelatin is considered to be a protein of lower quality since nearly all essential amino acids are either underrepresented or, in the case of cysteine, completely absent (Eastoe and Leach 1977).

Medically, gelatin has been in use for therapeutic purposes for a long time. In several studies, a positive effect with regard to the growth of hair and nails was demonstrated subsequent to oral administration of gelatin hydrolysate (Brodie 1984, Gehring 1992, Morganti and Randazzo 1984). In recent years, improvement of degenerative joint disease after oral administration of gelatin hydrolysate received increasing attention. In addition to animal experiments, clinical investigations also demonstrated a positive therapeutic effect after orally administered gelatin hydrolysate, e.g., in the treatment of osteoarthritis (Adam 1991, Seeligmüller and Happel 1993). The question as to gelatin absorption from the gut and its therapeutic mechanism remain essentially unsolved. Apart from a metabolic stimulation leading to an increase of collagen synthesis (Lippiello et al. 1977, Nagler-Anderson et al. 1986), a modulation of immunological processes was also discussed

(Adam 1991, Trentham et al. 1993). Before speculating about the mechanism of the therapeutic effectiveness of gelatin hydrolysate, the question must be clarified as to whether gelatin hydrolysate can be absorbed from the intestine and furthermore in what form and quantity. In this investigation the time course of gelatin hydrolysate absorption was studied as well as its subsequent distribution in various tissues and organs. This study also included an investigation into the differential enrichment by certain tissues of gelatin hydrolysate or separately administered proline.

## MATERIALS AND METHODS

*Test substances.*   The ^{14}C labeled gelatin hydrolysate was produced by intraperitoneal application of ^{14}C-proline to Wistar rats (Animal Service, Charles University, Prague, Czech Republic). The skins of these animals were collected and hydrolyzed (50 min at 50°C) by enzymes (DGF Stoess, Eberbach, Germany) to obtain ^{14}C labeled gelatin hydrolysate with a mean MW of 6.1–6.8 kD. The specific radioactivity was 57.4 kBq/g. Uniformly labeled ^{14}C-proline was obtained from Hartmann Analytic (Braunschweig, Germany), with a specific activity of 8.3 GBq/mmol. Before administration, ^{14}C-proline was diluted with 9 g/L NaCl to achieve preparations with the desired radioactivity. Gelatin hydrolysate (batch no.: 830814) was obtained from DGF Stoess (Eberbach, Germany).

*Animal model.*   The experiments were performed on male mice (C57/BL; Harlan Winkelmann, Paderborn, Germany) with a body weight of 21–25 g. The animals were housed under standardized conditions with free access to nonpurified diet (Altromin standard

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diet 1310, containing 22% crude protein, 5% crude fat, 4% crude fiber average content; Altomin GmbH, Lage, Germany) and water. At the beginning of the experiment, the animals were randomly assigned to either the control or the test group. The test substances were administered via a gastric feeding tube. Mice of the gelatin group received 10 mg of ^{14}C labeled gelatin hydrolysate/g body weight (580 Bq/g body wt). In the control group ^{14}C labeled proline (580 Bq/g body wt) along with unlabeled gelatin hydrolysate (10 mg/g body wt) was administered. Thus all animals received standard doses of radioactivity of 580 Bq/g and 10 mg of gelatin hydrolysate/g body wt, respectively. Both before and after administration of the above substances, the animals had free access to nonpurified diet and water. The mice from each group were sacrificed 3, 6, 12, 24, 48, 96 and 192 h after oral administration, and samples of blood and various types of tissues and organs were collected. A volume of 0.6 mL of blood (35 IU heparin/mL) was taken from the portal vein. From each animal plasma, cartilage, skin, liver, kidney, skeletal muscle and spleen were removed, and radioactivity of the samples was measured in aliquots. Furthermore, the remaining ^{14}C-activity in the whole gastrointestinal tract was analyzed after the end of absorption time to determine the intestinal uptake of the test substances.

This study was approved by the Animal Research Committee at Kiel University. Care and handling of the animals were in accordance with the National Institutes of Health guidelines.

*Sample preparation and measurement of radioactivity.* The radioactivity of plasma samples was determined following the addition of liquid scintillator Aquasafe 300 Plus (Zinsser Analytic, Frankfurt/Main, Germany), using polystyrene scintillation vials (Zinsser). Tissues were minced with scissors, and weighed aliquots of each tissue were digested in Biolute S (Zinsser Analytic) for 12 h at 50°C. Samples were bleached with hydrogen peroxide (30%) and radioactivity determined following the addition of Aquasafe 300 Plus.

For the measurement of radioactivity, a liquid scintillation counter (Packard Tricarb 2000 CA; Camberra-Packard, Dreieich, Germany) with external standardization was used. All analyses were conducted in triplicate taking the respective background values into account. Counting was performed for 15 min or up to a statistical confidence level of 2 sigma error equal or less than 5%. The counting efficiency was 88–93% for all samples.

*Gut-sac technique.* Qualitative investigations of the absorption of the gelatin hydrolysate were carried out using the "gut-sac" method (Schilling and Mitra 1990, Wilson and Wisemann 1954). C57/BL mice were anesthetized by an i.p. injection of 90 mg/kg body wt ketamine hydrochloride (Ketanest; Parke-Davis Pharmaceutical Research, Morris Plains, NJ). After making a midline incision in the abdomen, 4–6-cm pieces of small intestine were removed beginning 5-cm distal to the stomach. The pieces of intestine were rinsed, closed on one end and filled with 0.8 mL of gelatin hydrolysate solution (500 g/L). The end of the segment was ligated with silk, and then the gut sac was immediately immersed in the test tube containing 5 mL of 37°C 9 g/L NaCl solution (absorption medium) and continuously bubbled with 95% $O_2$/5% $CO_2$. After 30 min, an aliquot of the absorption medium was removed for subsequent analysis of the protein profile by SDS gel electrophoresis and HPLC. In control experiments the intestinal segments were filled with 9 g/L of NaCl solution.

*Electrophoresis.* A discontinuous SDS-PAGE was performed as described by Schägger and Jagow (1987) with 16% acrylamide in the separating gel using a Mini-Protean II Cell (Bio-Rad Laboratories, München, Germany). Gels were stained by the method of Heukeshoven and Dernick (1985). The MW markers of 2.5–17 kD were obtained from Sigma-Aldrich (Deisenhofen, Germany).

*HPLC.* GPC–HPLC measurements were performed using a Pharmacia LKB 2150 system equipped with a UV-detector operating at 214 nm and a column TSK 2000 SW (XL) (Haas, Heidelberg, Germany) with 200 mmol/L of sodium phosphate buffer, pH 5.3, as solvent.

*Statistical analysis.* All results are expressed as mean values ± SEM. The significance of differences in values was assessed by the Mann-Whitney U-test. Differences with $P < 0.05$ were considered significant.
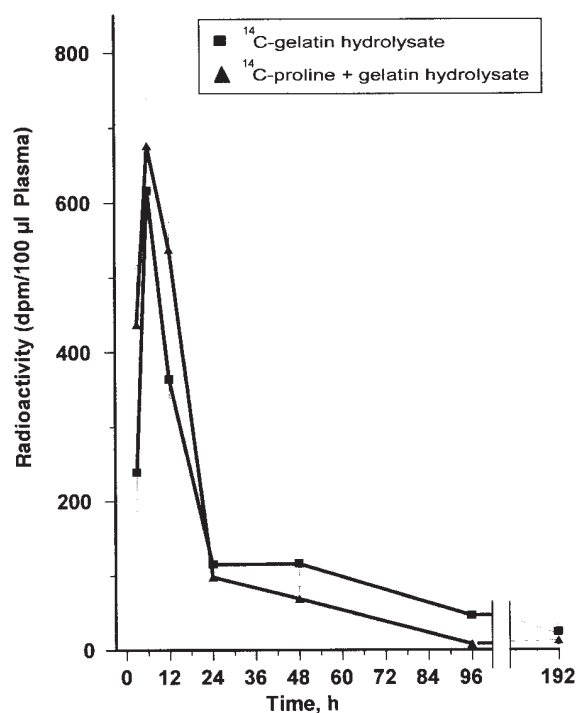


**FIGURE 1** Time course of radioactivity in plasma of mice subsequent to absorption of orally administered ^{14}C labeled gelatin hydrolysate and ^{14}C labeled proline in the control group. The mice received a standard dose of radioactivity of 580 Bq/g body weight and 10 mg gelatin hydrolysate/g body weight. The results are presented as mean ± SEM, $n = 6$.

## RESULTS

*Absorption and organ distribution of ^{14}C labeled gelatin hydrolysate.* Subsequent to oral administration of ^{14}C labeled gelatin hydrolysate, a rapid increase of radioactivity was observed in plasma, reaching a maximal concentration 6 h after the beginning of the observation period (**Fig. 1**). This peak was followed by a marked decrease of radioactivity. After beginning of the experiment (24 h) more than 85% of radioactivity in plasma disappeared. Later (3 d) the radioactivity declined to values near the limits of detectability in the gelatin group.

Radioactivity in skin attained its peak values 12 h after the administration of ^{14}C labeled gelatin hydrolysate (**Fig. 2**) and, in contrast to plasma, ^{14}C-activity remained relatively high (467 ± 107 dpm/100 mg) up to 96 h. At the end of the observation period (192 h), measured radioactivity declined to ~58% of the peak value. In plasma as well as in tissues and organs under investigation (**Table 1**), radioactivity revealed no significant differences between the values obtained following administration of ^{14}C labeled gelatin hydrolysate and the control group animals, which had received ^{14}C-proline together with unlabeled gelatin hydrolysate.

In cartilage, however, considerable differences could be determined between the gelatin group and the control group (**Fig. 3**). As early as 12 h after the beginning of the observation period, radioactivity in cartilage was significantly ($P < 0.05$) higher in mice which had received ^{14}C labeled gelatin hydrolysate than in control animals. In the experimental group, a pronounced increase in radioactivity could be observed reaching a peak value (246 ± 34 dpm/100 mg) at 48 h. At 96 h, ~2.6-fold more radioactivity was detected in cartilage
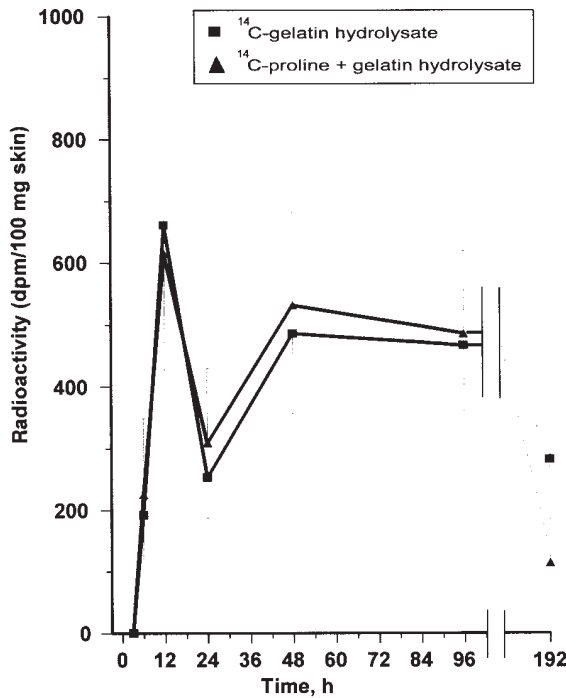
**FIGURE 2** Time course of radioactivity in skin of mice subsequent to absorption of orally administered $^{14}$C labeled gelatin hydrolysate and $^{14}$C labeled proline in the control group. The mice received a standard dose of radioactivity of 580 Bq/g body weight and 10 mg gelatin hydrolysate/g body weight. The results are presented as mean ± SEM, $n = 6$.



**FIGURE 3** Time course of radioactivity in cartilage of mice subsequent to absorption of orally administered $^{14}$C labeled gelatin hydrolysate and $^{14}$C labeled proline in the control group. The mice received a standard dose of radioactivity of 580 Bq/g body weight and 10 mg gelatin hydrolysate/g body we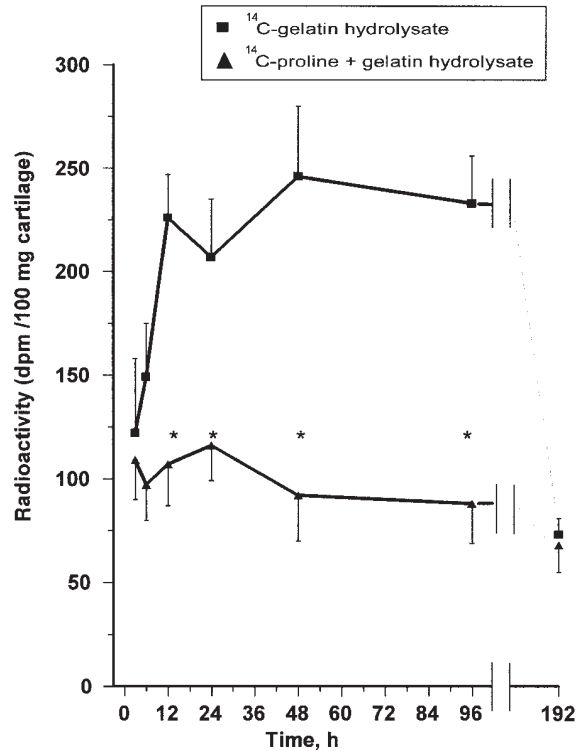ight. The results are presented as mean ± SEM, $n = 6$. Asterisks indicate $P < 0.05$ significantly different from the control group (Mann-Whitney U-test).

from the gelatin group compared with the control group. At the end of the observation period, only low radioactivity could be determined in cartilage of both groups. Investigations of the radioactivity remaining in gastrointestinal tract revealed a rapid and pronounced depletion of $^{14}$C-activity in the intestine, which correlated well with the initial increase of radioactivity in plasma as well as in all tissues. After 6 h of absorption time, a residual activity of <10% of the initial activity was determined in the gastrointestinal tract in both

groups. Subsequent to administration (12 h) of the test substances, more than 95% of the administered radioactivity had been absorbed. The amount of the remaining radioactivity did not differ significantly in both groups.

*Molecular distribution of the absorbed gelatin.* To gain more information about the qualitative absorption of gelatin

## TABLE 1

*Time course of the accumulation of radioactivity in liver, kidney, spleen and skeletal muscle after oral administration of $^{14}$C labeled gelatin hydrolysate (GH) and $^{14}$C labeled proline (P) in mice[1,2]*

| Tissue | | Time after administration, $h$ | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 12 | 24 | 48 | 96 | 192 |
| | | | | | dpm/100 mg wet tissue | | | |
| Liver | GH | 1520 ± 411 | 1993 ± 352 | 1617 ± 417 | 767 ± 314 | 1022 ± 97 | 529 ± 255 | 36 ± 12 |
| Liver | P | 2188 ± 626 | 2519 ± 547 | 1513 ± 191 | 679 ± 407 | 1283 ± 325 | 638 ± 136 | 58 ± 29 |
| Kidney | GH | 464 ± 230 | 570 ± 114 | 870 ± 501 | 1092 ± 231 | 704 ± 232 | 616 ± 242 | 0 |
| Kidney | P | 600 ± 181 | 828 ± 64 | 1195 ± 176 | 998 ± 340 | 685 ± 77 | 872 ± 149 | 0 |
| Spleen | GH | 210 ± 144 | 236 ± 65 | 524 ± 186 | 608 ± 189 | 332 ± 148 | 775 ± 127 | 250 ± 60 |
| Spleen | P | 301 ± 119 | 153 ± 71 | 1223 ± 178 | 517 ± 112 | 286 ± 91 | 488 ± 98 | 200 ± 111 |
| Skeletal Muscle | GH | 94 ± 69 | 119 ± 70 | 581 ± 351 | 282 ± 84 | 204 ± 65 | 314 ± 58 | 0 |
| Skeletal Muscle | P | 101 ± 22 | 432 ± 212 | 219 ± 104 | 218 ± 132 | 149 ± 37 | 256 ± 48 | 115 ± 12 |

[1] Values are means ± SEM, $n = 6$.
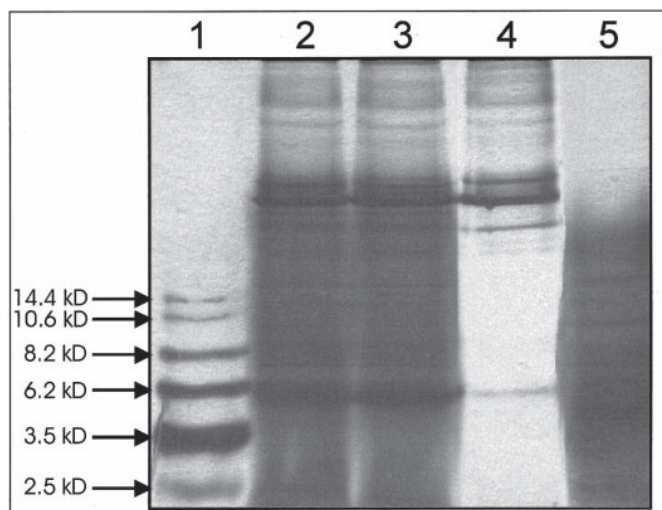[2] All mice received standard doses of radioactivity of 580 Bq/g body weight.

**FIGURE 4** SDS-PAGE separation of the absorption medium from "mice gut sac" experiments 30 min after the administration of gelatin hydrolysate and 9 g/L NaCl solution in the control experiment. Lane 1: Calibrating proteins; lanes 2+3: aliquot subsequent to absorption of gelatin hydrolysate; lane 4: aliquot subsequent to absorption of 9 g/L NaCl solution; lane 5: gelatin hydrolysate as reference. Illustration is the results of one characteristic absorption experiment ($n = 4$).

hydrolysate in the intestinal tract, "gut-sac" experiments were performed.

Subsequent to absorption of gelatin hydrolysate, proteins in a range from 2.5 kD to <15 kD MW were detected in the absorption medium ($n = 4$) by means of SDS electrophoresis (**Fig. 4**). The determined protein profile corresponded to the protein profile of gelatin hydrolysate (<1–25 kD) with a mean M.W. of 3.5 kD. A marked difference could be observed compared to the control experiments (absorption of 9 g/L of NaCl solution) despite the insufficient separation of the proteins. Subsequent to absorption of NaCl solution, almost no proteins of M.W. <25 kD were detected in the absorption medium.

To obtain a more exact qualitative analysis of transmural transit from mucosa to serosa side of gelatin hydrolysate, GPC–HPLC was performed to determine the protein profile in the absorption medium ($n = 4$). In agreement with the results of the determination of the MW distribution of peptides in the absorption medium by SDS-PAGE, peptides in the approximate range of 0.5–15 kD were detected by GPC–HPLC (**Fig. 5**). In the control group without gelatin absorption, no proteins of MW <10 kD could be determined.

## DISCUSSION

As shown in the present study, more than 90% of the administered radioactivity was removed from the gastrointestinal tract within the first 6 h subsequent to oral administration of gelatin hydrolysate. This rapid transmucosal transit of gelatin correlates well with experiments done by Asghor and Sela (1982) and Laser-Reutersward et al. (1985) concerning the digestibility of collagen. In addition to the fact that gelatin hydrolysate is absorbed from the intestine, it is of considerable importance to analyze the quality of gelatin uptake. It is commonly assumed that peptides are hydrolyzed in the gastrointestinal tract prior to absorption, so that free amino acids enter the circulation predominantly (Boullin et al. 1973, Mathews and Laster 1965). In contrast, however, there is

considerable evidence that peptides or even macromolecules can also be absorbed intact (Seifert and Sass 1990, Urao et al. 1997, Warshaw et al. 1974). Earlier investigations suggested that both peptides (Gardener 1982, 1983, Matthews 1975, Webb 1986) and macromolecular proteins (Hemmings and Williams 1978, Seifert et al. 1979) are absorbed from the gut and retain some biological function (Atisook and Madara 1991, Castell et al. 1997, Gardener et al. 1991, Hara et al. 1984). More recently, investigations of the mechanism of peptide (Daniel 1997) and macromolecule (Terpend et al. 1998) absorption and the influence on immune system response (Castro and Arntzen 1993, Kagnoff 1993, Pusztai 1993) suggested a therapeutic effect for orally applied peptides and proteins (Trentham et al. 1993, Weiner 1997).

In the "gut-sac" experiments, we identified peptides within a MW range from 1 kD to ~10 kD on the serosal side of the intestine after the application of gelatin hydrolysate, indicating that gelatin is also absorbed in the high molecular form to some extent. Peptides measured in the absorption medium with a MW of more than 10 kD seem to be of intestinal origin since they could be detected in all gut-sac experiments and controls, respectively. These results could be confirmed by SDS-electrophoresis as well as by HPLC. Although the interpretation of the gut-sac experiments is limited, due to the method, it is evident that in addition to degradation of gelatin and subsequent amino acid uptake, a significant amount of gelatin hydrolysate is absorbed in the high molecular form.

With regard to the distribution of radioactivity in the various tissues and organs, our current results demonstrate that almost no differences could be observed after the administration of ^{14}C labeled gelatin hydrolysate and ^{14}C labeled proline, respectively. In cartilage, however, a significantly ($P < 0.05$) higher degree of radioactivity could be determined for at least 96 h subsequent to administration of labeled gelatin than was the case after the application of ^{14}C labeled proline. The distribution of proline in cartilage after oral administration is consistent with observations of Tonna et al. (1971). Whereas for proline only a slight and transient uptake of ^{14}C-activity could be observed in cartilage, accumulation of radioactivity was more pronounced after the administration of gelatin hydrolysate persisting over a longer period than after the application of proline. The possible reason for this phenomenon might be due to the fact that, subsequent to intestinal absorption of gelatin hydrolysate, a heterogeneous mixture of peptides and, to some extent, polypeptides enters the circulation,
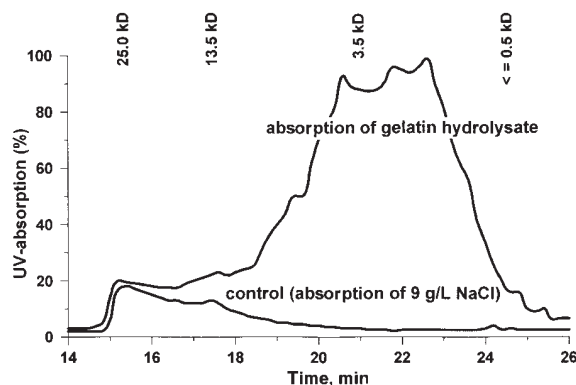


**FIGURE 5** GPC–HPLC chromatograms of the absorption medium from "mice gut sac" experiments 30 min subsequent to absorption of gelatin hydrolysate and 9 g/L NaCl solution in the control experiment. Illustration is the results of one characteristic absorption experiment ($n = 4$).

and that these peptides are preferably accumulated in cartilage. Another explanation of the accumulated values in cartilage could be a reduced metabolic rate of peptides as well as polypeptides compared to amino acids per se. If this is the case, similar differences in radioactivity should be expected in liver, the main organ of metabolism. Since the determined $^{14}$C-activity in the liver of animals treated with gelatin hydrolysate is not different from the radioactivity in animals treated with proline, it must be concluded that differences in metabolism could not be responsible for the accumulation in cartilage.

Although the beneficial effect of orally administered gelatin hydrolysate in the improvement of degenerative joint diseases could be demonstrated in several investigations, the therapeutic mechanism remains unsolved. One possible mechanism might involve the modulation of immunological processes. For the treatment of rheumatoid arthritis, it was shown in clinical trials that the oral administration of cartilage-derived type II collagen can improve the symptoms (Barnett et al. 1998, Trentham et al. 1993). This positive effect was explained by an orally induced tolerance in which mainly T-helper cells are involved (Kalden and Sieper 1998). In order to generate this tolerance in immunocompetent cells, high-M.W. peptides would have to be absorbed from the intestine. In addition to the immunological modulation, a metabolic stimulation was also discussed in connection with the therapeutic efficacy of orally administered gelatin hydrolysate. The pronounced accumulation of radioactivity in cartilage subsequent to administration of gelatin observed in the present study might possibly be caused by a selective modification of cell metabolism. In summary, this study indicates that gelatin hydrolysate is absorbed from the intestine and preferentially accumulated in cartilage. These results suggest that the unique amino acid and peptide profile of gelatin may be responsible for the previous observations of therapeutic efficacy of orally administered gelatin in degenerative conditions of the musculo-skeletal system (Adam 1991, Seeligmuller and Happel 1993). Additional research is needed to identify the mechanisms responsible for the preferential accumulation of gelatin hydrolysate in cartilage tissue.

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