

# Collagen Cross-Link Excretion during Space Flight and Bed Rest\*

SCOTT M. SMITH, JEANNIE L. NILLEN, ADRIAN LEBLANC, ALLAN LIPTON,  
LAURENCE M. DEMERS, HELEN W. LANE, AND CAROLYN S. LEACH

*Life Sciences Research Laboratories (S.M.S., H.W.L., C.S.L.), National Aeronautics and Space Administration, and Enterprise Advisory Services, Inc. (J.L.N.), Johnson Space Center, Houston, Texas 77058; Baylor College of Medicine (Ad.L.), Houston, Texas 77030; and The Milton S. Hershey Medical Center (Al.L., L.M.D.), Hershey, Pennsylvania 17033*

## ABSTRACT

Extended exposure to weightlessness results in bone loss. However, little information exists as to the precise nature or time course of this bone loss. Bone resorption results in the release of collagen breakdown products, including N-telopeptide and the pyridinium (PYD) cross-links, pyridinoline and deoxypyridinoline. Urinary pyridinoline and deoxypyridinoline are known to increase during bed rest. We assessed excretion of PYD cross-links and N-telopeptide before, during, and after long (28-day, 59-day, and 84-day) Skylab missions, as well as during short (14-day) and long (119-day) bed-rest

studies. During space flight, the urinary cross-link excretion level was twice those observed before flight. Urinary excretion levels of the collagen breakdown products were also 40–50% higher, during short and long bed rest, than before. These results clearly show that the changes in bone metabolism associated with space flight involve increased resorption. The rate of response (*i.e.* within days to weeks) suggests that alterations in bone metabolism are an early effect of weightlessness. These studies are important for a better understanding of bone metabolism in space crews and in those who are bedridden. (*J Clin Endocrinol Metab* 83: 3584–3591, 1998)

**B**ONE MASS is lost during space flight (1–5), as reflected by densitometry (1–3, 5) and by high concentrations of calcium and other minerals in urine (1–3, 6, 7). Both space flight and bed rest result in negative calcium balance (4, 8–12). The bone-resorption process releases minerals and collagen fragments; the amounts of collagen-breakdown products present in serum and urine constitute a marker of bone resorption (13, 14). Mature collagen contains residues of the pyridinium (PYD) cross-links, pyridinoline and deoxypyridinoline (DPD). Pyridinoline is found in several tissues, including cartilage and ligaments, but DPD is found almost exclusively in bone (15). N-telopeptide consists of the PYD cross-link moiety and peptide fractions of the collagen fibril and is considered a highly specific marker for bone resorption (16, 17). As collagen is degraded, PYD cross-links and N-telopeptide are released into the circulation, cleared by the kidneys, and excreted in the urine. These collagen metabolites are neither metabolized nor absorbed from the diet (18); and thus, their excretion directly reflects the rate of bone resorption (13, 16, 17). Further evidence of this is that treatment with antiresorptive bisphosphonates has been shown to reduce the amounts of these metabolites in urine (19, 20).

Previous studies have shown that bed rest is associated with increases in urinary pyridinoline and DPD (10, 21–24). In one 17-week study in which significant bone was lost (8),

urinary pyridinolines and DPD were almost 40% higher than before the bed rest (10). In that study, the PYD cross-links were measured with high-performance liquid chromatography, as opposed to the enzyme-linked immunosorbent assay (ELISA) techniques reported here. The temporal data for the 17-week study have not been presented previously. N-Telopeptide excretion has not been reported from bed rest or space flight studies. Collagen cross-links are extremely stable in urine, and it has been suggested that they retain that stability even in samples frozen for more than 25 yr (25).

The objective of the present study was to assess bone resorption during weightlessness, by measuring PYD cross-links and N-telopeptide in urine samples collected before, during, and after space flight or bed rest. The flight studies consisted of the 28-, 59-, and 84-day Skylab missions; the bed-rest studies lasted either 2 or 17 weeks.

## Materials and Methods

### Experimental Subjects

Subjects for the bed-rest studies, all volunteers, were included after passing an Air Force Class III physical examination and signing informed-consent forms. All experimental procedures were reviewed and approved by the Johnson Space Center Human Research Policy and Procedures Committee and by institutional review boards at either the University of Texas Medical Branch (the 2-week bed-rest study) or Baylor College of Medicine (the 17-week bed-rest study).

### Methods

*Exp 1. Skylab flights.* The three crewed Skylab missions (28, 59, and 84 days in length) were flown in 1973–1974. Results from the extensive bone and metabolic studies completed on these missions have been published elsewhere (1, 2). The nine subjects, three per mission, were all men, ages  $41 \pm 2$  yr (mean  $\pm$  SD), weighing  $71.5 \pm 8.8$  kg. The metabolic studies were such that inflight dietary intake was carefully maintained as close

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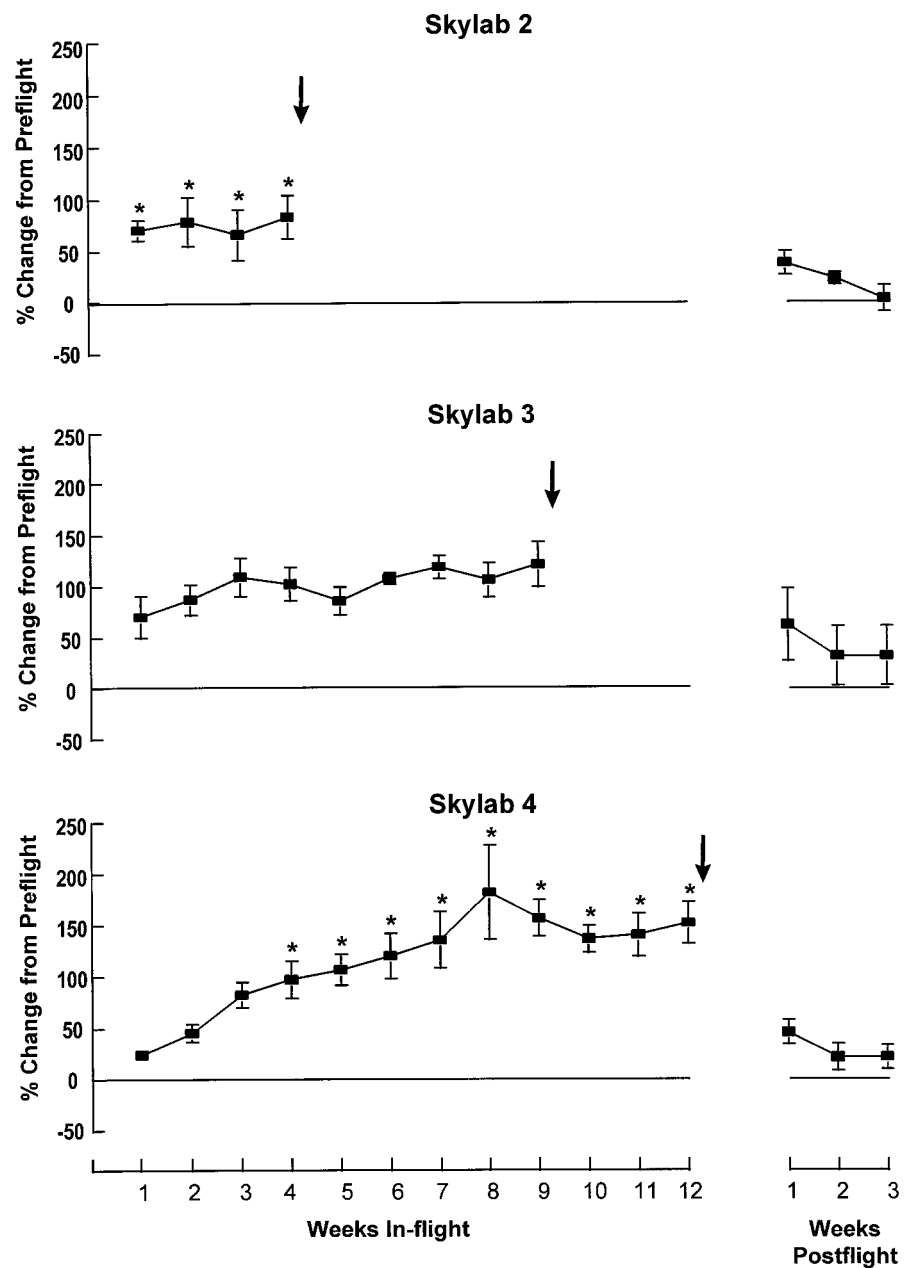
Address all correspondence and requests for reprints to: Scott M. Smith, Life Sciences Research Laboratories/SD3, National Aeronautics and Space Administration, Johnson Space Center, Houston, Texas 77058. E-mail: smsmith@ems.jsc.nasa.gov.

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**TABLE 1.** Skylab individual baseline (preflight) data

Subject	Mission	No. of 24-h Urine collections	N-Telopeptide (nmol/day <sup>-1</sup> )	Deoxypyridinoline (nmol/day <sup>-1</sup> )	PYD (nmol/day <sup>-1</sup> )
1	Skylab 2	31	1346 ± 334 (25%)	72 ± 18 (25%)	328 ± 67 (20%)
2		31	1361 ± 506 (37%)	67 ± 21 (31%)	311 ± 106 (34%)
3		31	761 ± 284 (37%)	42 ± 13 (31%)	261 ± 105 (40%)
4	Skylab 3	21	438 ± 78 (18%)	43 ± 7 (16%)	129 ± 23 (18%)
5		21	524 ± 104 (20%)	56 ± 10 (18%)	169 ± 26 (15%)
6		21	1248 ± 259 (21%)	62 ± 10 (16%)	240 ± 50 (21%)
7	Skylab 4	27	357 ± 68 (19%)	38 ± 9 (24%)	164 ± 39 (24%)
8		27	596 ± 192 (32%)	64 ± 15 (23%)	242 ± 56 (23%)
9		27	621 ± 153 (25%)	54 ± 17 (31%)	235 ± 69 (29%)

Data are mean ± SD (CV, %).



**FIG. 1.** Urinary N-telopeptide for the nine Skylab astronauts. Each line represents the mean of three subjects. The vertical arrow indicates landing. Data are expressed as mean ( $\pm$ SEM) of the percent change from preflight values for each subject. Asterisks indicate significant ( $P < 0.05$ ) difference, when compared with preflight. Statistical analyses were run on the actual data (*i.e.* not the percent change). See *Methods* for details.

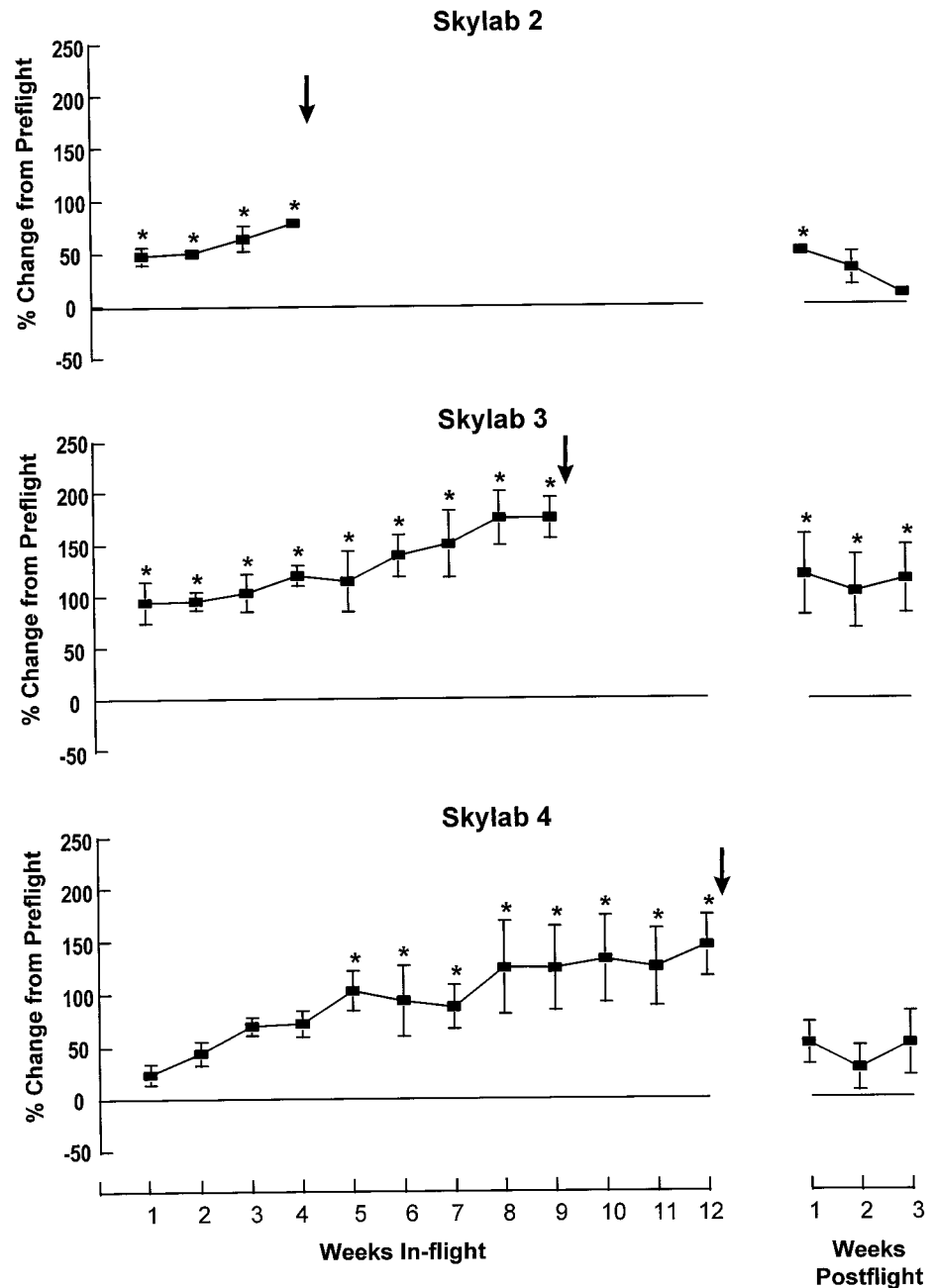


FIG. 2. Urinary PYD cross-links for the nine Skylab astronauts. Each line represents the mean of three subjects. The vertical arrow indicates landing. Data are expressed as mean ( $\pm$ SEM) of the percent change from preflight values for each subject. Asterisks indicate significant ( $P < 0.05$ ) difference, when compared with preflight. Statistical analyses were run on the actual data (*i.e.* not the percent change). See *Methods* for details.

to preflight levels as possible (2). Each crew member reported his food intake to the ground controllers on a daily basis. Urine was collected into 24-h pools for 3–5 weeks before launch, throughout the flight, and for 3–6 weeks after return. The samples were stored at  $-20^{\circ}\text{C}$  during the flight and at  $-70^{\circ}\text{C}$  thereafter. Samples were analyzed in 1996–1997 for cross-links. For statistical and graphical purposes, data from individuals were averaged over weekly periods.

**Exp 2. Two-week bed rest.** Urine samples in this experiment were collected in association with a study of protein metabolism during bed rest (26). Six adult men ( $30 \pm 6$  yr old, weighing  $64.8 \pm 6.8$  kg) were admitted to the General Clinical Research Center at the University of Texas Medical Branch at Galveston. After a 7-day ambulatory period, subjects were confined to bed in a head-down tilt position of  $-6$  degrees. All urine voids were refrigerated immediately after collection; voids were pooled over 24-h periods and frozen at  $-70^{\circ}\text{C}$  for batch analysis of cross-link excretion after the study was completed. Throughout the study, subjects

consumed a 3-day-cycle metabolic diet that provided recommended dietary allowances of all nutrients and was designed to maintain body weight.

**Exp 3. Seventeen-week bed-rest study.** Details of this study have been published elsewhere (8, 10, 27). Briefly, after 4, 5, or 10 weeks of ambulatory control periods, eight male subjects ( $32 \pm 12$  yr old, weighing  $72.0 \pm 7.6$  kg) were confined to horizontal bed rest for 17 weeks. Post-bed-rest results were available for six of the eight subjects. Urine voids were pooled over weekly periods and frozen at  $-70^{\circ}\text{C}$  until analysis after the study was completed. Subjects consumed a metabolic diet that met all nutrient recommended dietary allowances during all phases of the experiment. A multivitamin supplement was given to all subjects daily.

Group and phase means for the pyridinoline and DPD results from these subjects have been published elsewhere (10); the results presented here show individual values and the changes in those values over time, as well as values for N-telopeptide.

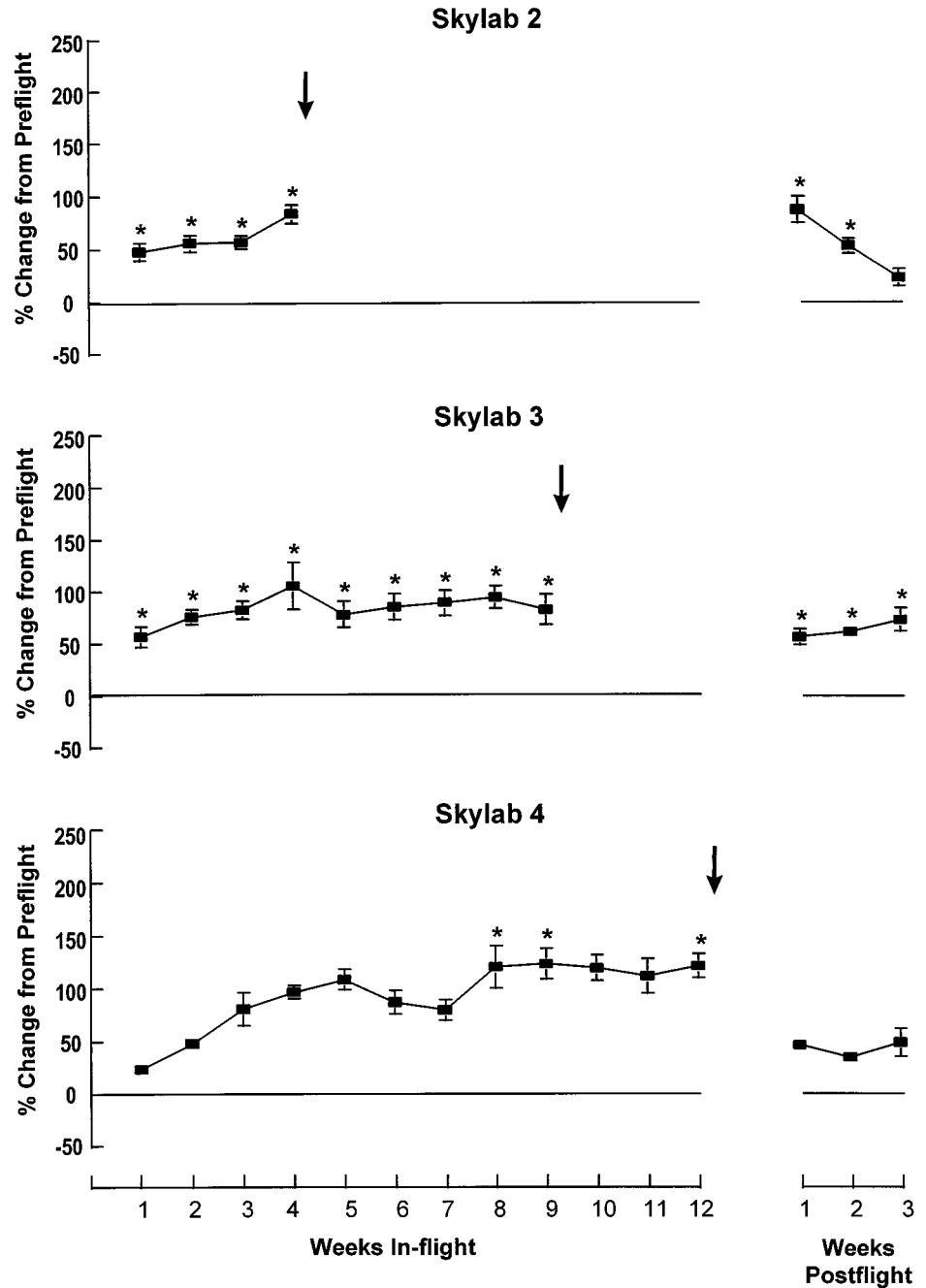


FIG. 3. Urinary DPD for the nine Skylab astronauts. Each line represents three subjects. The vertical arrow indicates landing. Data are expressed as mean ( $\pm$ SEM) of the percent change from preflight values for each subject. Asterisks indicate significant ( $P < 0.05$ ) difference, when compared with preflight. Statistical analyses were run on the actual data (*i.e.* not the percent change). See *Methods* for details.

**Cross-link analyses.** Samples were analyzed for PYD cross-links with the Pylinks kit (Metra Biosystems, Palo Alto, CA). This ELISA assay detects PYD cross-links (*i.e.* both pyridinoline and DPD) in urine. DPD was analyzed with the Pylinks-D kit (also from Metra Biosystems). N-Telopeptide (nTX) in urine was determined with the Osteomark ELISA kit (Ostex International, Inc., Seattle, WA), which specifically detects the N-telopeptide region of bone collagen in human urine. The accuracy and reproducibility of the assays were monitored by analyzing control sera (supplied by the manufacturers) with every assay. Coefficients of variation (CVs) for the low-level control for PYD, DPD, and nTX were 11.4%, 8.6%, and 10.2%, respectively; the high-level control yielded CVs of 11.2%, 9.9%, and 9.9%, respectively.

Results are presented as nanomoles of cross-link excreted per day, because samples were pooled over 24-h periods. Because lean body mass typically is lost during long space flight (5) or bed rest (27), we did not normalize the results in terms of creatinine.

**Statistical analysis.** Each subject served as his own control, and results were compared between before-flight (or bed rest) and after-flight (or bed rest). Statistical analyses of the Skylab data were done with Super-Anova (Abacus Concepts, Berkeley, CA). A multivariate approach to repeated-measures ANOVA was used, with the within-subject independent variable being: week. Differences among flight weeks were investigated using *a priori* contrasts. Probability values were adjusted for statistical significance by using the Dunn-Bonferroni-Sidak Procedure. Observed probabilities from ANOVAs were adjusted according to the Huynh-Feldt technique.

For the bed-rest results, one-way repeated-measures ANOVA was used to identify any differences from before-bed-rest results (*i.e.* during and after bed rest). When differences were significant ( $P < 0.05$ ), *post hoc* comparisons were performed with the Bonferroni *t* test. Statistical analyses were done with SAS (SAS Institute, Inc., Cary, NC) and SigmaStat (Jandel Scientific Software, San Rafael, CA). All values are ex-

pressed as mean  $\pm$  SD. Variables from the 2-week bed-rest subjects had considerably greater CVs between subjects (PYD = 24.2%, DPD = 26.1%, nTX = 52.4%) than within subjects (PYD = 14.9%, DPD = 17.0%, nTX = 16.0%). This was also true for nTX from the 17-week bed-rest subjects (36.7% between-subject variability, 13.9% within-subject). The between-subject variation was calculated as the SD divided by the mean value from individual subjects; within-subject variability was calculated from the daily values for each subject. Because of the relatively high between-subject variability, either the individual data are presented or data are expressed as the percent change from baseline.

## Results

### Exp 1. Skylab flights

The preflight cross-link values (Table 1) were within the manufacturer's normal ranges, and they showed day-to-day and subject-to-subject variations similar to published values. Thus, there does not seem to have been degradation of the cross-links, despite storage for an extended period.

N-telopeptide (Fig. 1), DPD (Fig. 2), and PYD (Fig. 3) were generally all higher during flight than before, and they returned to baseline soon after flight. Whether the increase reached statistical significance varied somewhat between markers and between flights (see figures for details). The excretion of collagen cross-links essentially doubled during space flight, leveling off at approximately 4 weeks of flight.

After flight, nTX excretion returned to preflight levels the first week, whereas PYD and DPD did not return to preflight levels until the second and third weeks after flight, respectively.

### Exp 2. Two-week bed rest

The cross-link values before bed rest (Table 2) were within the manufacturer's normal ranges and showed day-to-day and subject-to-subject variations similar to published values. Urinary PYD and DPD were higher ( $P < 0.01$ ) during bed rest than before (Table 2). N-telopeptide was higher for five of the six subjects during bed rest, compared with before (Table 2), but this difference was not statistically significant ( $P > 0.05$ ). In general, collagen cross-link excretion during this bed-rest study was 20–30% higher than pre-bed-rest levels, at the end of both the first and the second week.

### Exp 3. Seventeen-week bed rest

The pre-bed-rest nTX (Table 3), PYD and DPD (see Fig. 5) were within the manufacturer's normal ranges and showed

day-to-day and subject-to-subject variations similar to published values.

Urinary nTX concentrations for the eight subjects rose approximately 20% above the pre-bed-rest mean, by the end of the second week of bed rest, and were consistently 40% above the pre-bed-rest mean by the fourth week of bed rest (Fig. 4). This is quite similar to the results from the 2-week study, and it is about half of the response seen during space flight.

Urinary PYD and DPD were higher during bed rest than before (Fig. 5). Amounts of both markers peaked between weeks 3 and 8 (as did that of nTX), at approximately 40–50% above control values. This is approximately half of the magnitude of the response seen in space flight.

## Discussion

Urinary markers of bone resorption revealed that this process was elevated as early as the first week of space flight or bed rest. The Skylab results presented here are the first to demonstrate that bone resorption is elevated during space flight. Preliminary data from joint US-Russian flights aboard the Russian space station Mir support this finding (Smith *et al.* unpublished data), but urine was collected only sporadically on these later flights. Indeed, the comprehensive urine collections from the Skylab program likely represent the last time that this duration of collection will ever be conducted during space flight.

Although changes in bone mass cannot be detected after relatively short Space Shuttle flights, the collagen cross-link data presented here clearly demonstrate that bone resorption is elevated as early as the first few weeks of simulated or actual weightlessness. Typically, studies of bone loss have been conducted only on long flights; nonetheless, the biochemical changes that precede bone loss seemingly occur quite rapidly.

Urinary cross-link excretion provides a whole-body picture of bone resorption, whereas the loss of bone during space flight is regional (*i.e.* limited primarily to the weight-bearing bones). This, however, provides more convincing evidence for the role of bone resorption, because the response must be even greater if it is from a smaller portion of the skeleton.

Cross-links (PYD and DPD), but not nTX, increased during the 2-week bed-rest study; however, nTX excretion in the 17-week bed-rest study was significantly increased, com-

**TABLE 2.** Excretion of collagen cross-links before (prebed rest) and during a 2-week head-down bed-rest period

	Pyridinium cross-links (nmol/day <sup>-1</sup> )			Deoxyypyridinoline (nmol/day <sup>-1</sup> )			N-Telopeptide (nmol/day <sup>-1</sup> )		
	Before bed rest	Week 1 bed rest	Week 2 bed rest	Before bed rest	Week 1 bed rest	Week 2 bed rest	Before bed rest	Week 1 bed rest	Week 2 bed rest
Subject 1	217 $\pm$ 30 <sup>a</sup>	282 $\pm$ 33	280 $\pm$ 67	40.7 $\pm$ 7.9	56.8 $\pm$ 5.5	57.0 $\pm$ 13.0	339 $\pm$ 36	446 $\pm$ 64	397 $\pm$ 123
Subject 2	196 $\pm$ 26	240 $\pm$ 29	281 $\pm$ 34	39.5 $\pm$ 5.9	50.6 $\pm$ 4.1	63.5 $\pm$ 9.2	250 $\pm$ 21	321 $\pm$ 26	331 $\pm$ 45
Subject 3	341 $\pm$ 53	378 $\pm$ 46	332 $\pm$ 19	66.6 $\pm$ 9.4	70.4 $\pm$ 14.0	65.2 $\pm$ 4.5	846 $\pm$ 114	788 $\pm$ 118	678 $\pm$ 102
Subject 4	349 $\pm$ 47	418 $\pm$ 54	410 $\pm$ 78	74.7 $\pm$ 10.8	82.4 $\pm$ 9.6	88.1 $\pm$ 14.3	745 $\pm$ 130	886 $\pm$ 174	742 $\pm$ 125
Subject 5	241 $\pm$ 50	294 $\pm$ 43	250 $\pm$ 67	49.0 $\pm$ 7.8	54.8 $\pm$ 4.5	50.8 $\pm$ 10.7	363 $\pm$ 52	450 $\pm$ 119	417 $\pm$ 105
Subject 6	250 $\pm$ 33	306 $\pm$ 23	378 $\pm$ 96	54.5 $\pm$ 12.6	63.6 $\pm$ 6.7	84.0 $\pm$ 23.6	326 $\pm$ 105	531 $\pm$ 78	595 $\pm$ 187
Group Mean	266 $\pm$ 64	319 $\pm$ 66 <sup>b</sup>	322 $\pm$ 62 <sup>b</sup>	54.2 $\pm$ 14.2	63.1 $\pm$ 11.8	68.1 $\pm$ 14.9 <sup>b</sup>	478 $\pm$ 251	570 $\pm$ 219	526 $\pm$ 167

<sup>a</sup> Data are mean  $\pm$  SD of the daily excretion for each 7-day period.

<sup>b</sup> Significantly different from before bed rest ( $P < 0.05$ ).

pared with pre-bed-rest. The simplest explanation for this difference may be higher variability and smaller sample size in the 2-week study. Alternatively, elevated PYD and DPD, in the absence of elevated nTX, may represent the breakdown of collagen from sites other than bone (*e.g.* muscle). Urinary 4-pyridoxic acid, associated with muscle glycogen-phosphorylase breakdown, also was greater during the first 2 weeks of the long study but not the shorter study (28). Subjects in the 2-week study were smaller and leaner than those in the 17-week study, but that difference should not affect the response of bone resorption to bed rest.

The differences among the three assays (nTX, PYD, and DPD) cannot be fully explained. nTX did not increase during Skylab-3, for example, but it did during Skylab-2 and -4 and during the long bed-rest study. The lack of significance may be attributed to the conservative nature of the statistical analysis. Moreover, there were differences in the rate at which the three markers returned to normal after the test condition. This may reflect the influence of nonbone collagen

breakdown on the recovery process. Additional data are needed, with detailed postflight rehabilitation profiles, before detailed conclusions can be drawn.

Measuring collagen cross-links in urine provides the opportunity to monitor bone resorption without invasive and costly procedures, such as bone biopsies or the use of isotopic tracers. Cross-link excretion also provides information on bone metabolism far in advance of changes measurable by absorptiometry techniques. The advantages of measuring collagen cross-links over other markers, such as hydroxyproline, are that PYD cross-links are formed only in mature collagen; and thus, they reflect the breakdown of the extracellular matrix. Moreover, they are not confounded by dietary intake of collagen products (18). Thus, and perhaps most important, these markers provide tools for assessing the efficacy of treatments intended to reduce bone loss (19, 20).

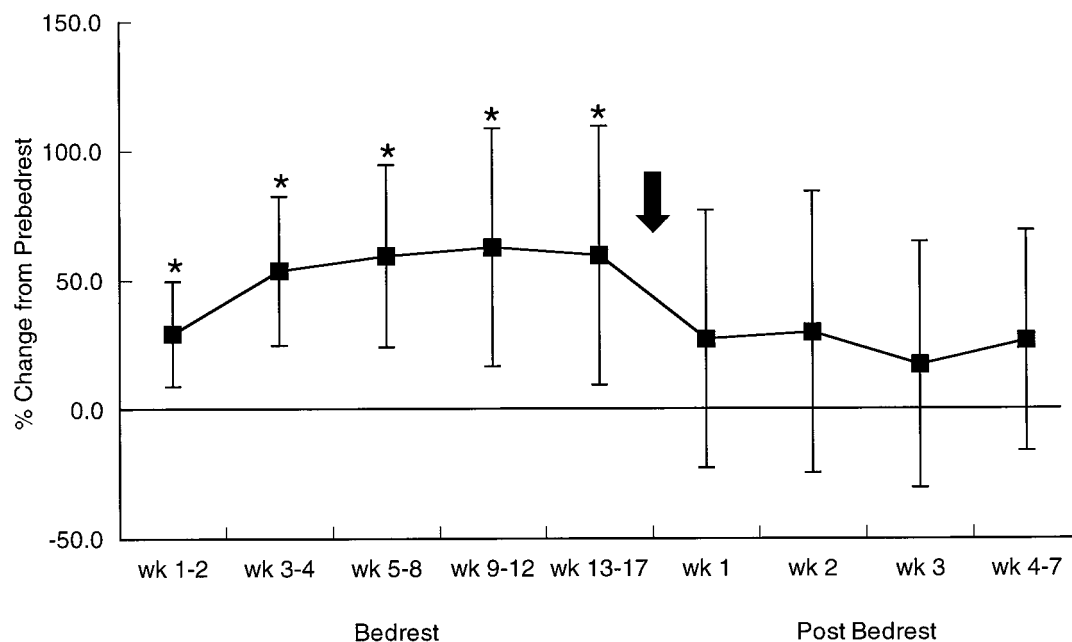
Exercise, diet, and pharmacologic interventions are the traditional means of counteracting the bone loss induced by weightlessness in both the US and Russian Space programs. However, neither the extensive exercise program (29) nor the carefully controlled diet (2) on Skylab prevented bone loss. Thus, bone was lost despite maintenance of energy, protein, and calcium intakes. Preliminary studies are under way to determine whether the newer generation of bisphosphonates (*e.g.* alendronate) can prevent bone loss in space travelers. It is likely that some combination of countermeasure techniques will be necessary to truly minimize in-flight bone loss.

The results presented here support the thesis that the cross-link compounds are stable for periods in excess of 25 yr of frozen storage (25). The range and variability of the Skylab samples were not unlike those of samples that were assayed within weeks of collection. This finding may help to uncover findings from previous epidemiological studies where urine samples may still be available.

**TABLE 3.** Individual baseline (prebed rest) N-telopeptide excretion from the subjects in the 17-week study

Subject	N-telopeptide (nmol/day <sup>-1</sup> )
1	842 ± 71 (8%)
2	428 ± 74 (17%)
3	838 ± 84 (10%)
4	1065 ± 118 (11%)
5	646 ± 64 (10%)
6	811 ± 96 (12%)
7	375 ± 66 (18%)
8	602 ± 42 (7%)

Data are mean ± SD (CV %). Values for subjects 1–6 are based on averages from 5 weeks of prebed samples, and those for subjects 7 and 8 are from 10 weeks of prebed rest samples.



**FIG. 4.** Urinary N-telopeptide during 17 weeks of bed rest. The vertical arrow indicates the end of the bed-rest period. Data are expressed as mean (±SD) of the percent change from pre-bed-rest values for each subject. Asterisks indicate significant ( $P < 0.05$ ) difference, when compared with pre-bed-rest. Statistical analyses were run on the actual data (*i.e.* not the percent change). See *Methods* for details.

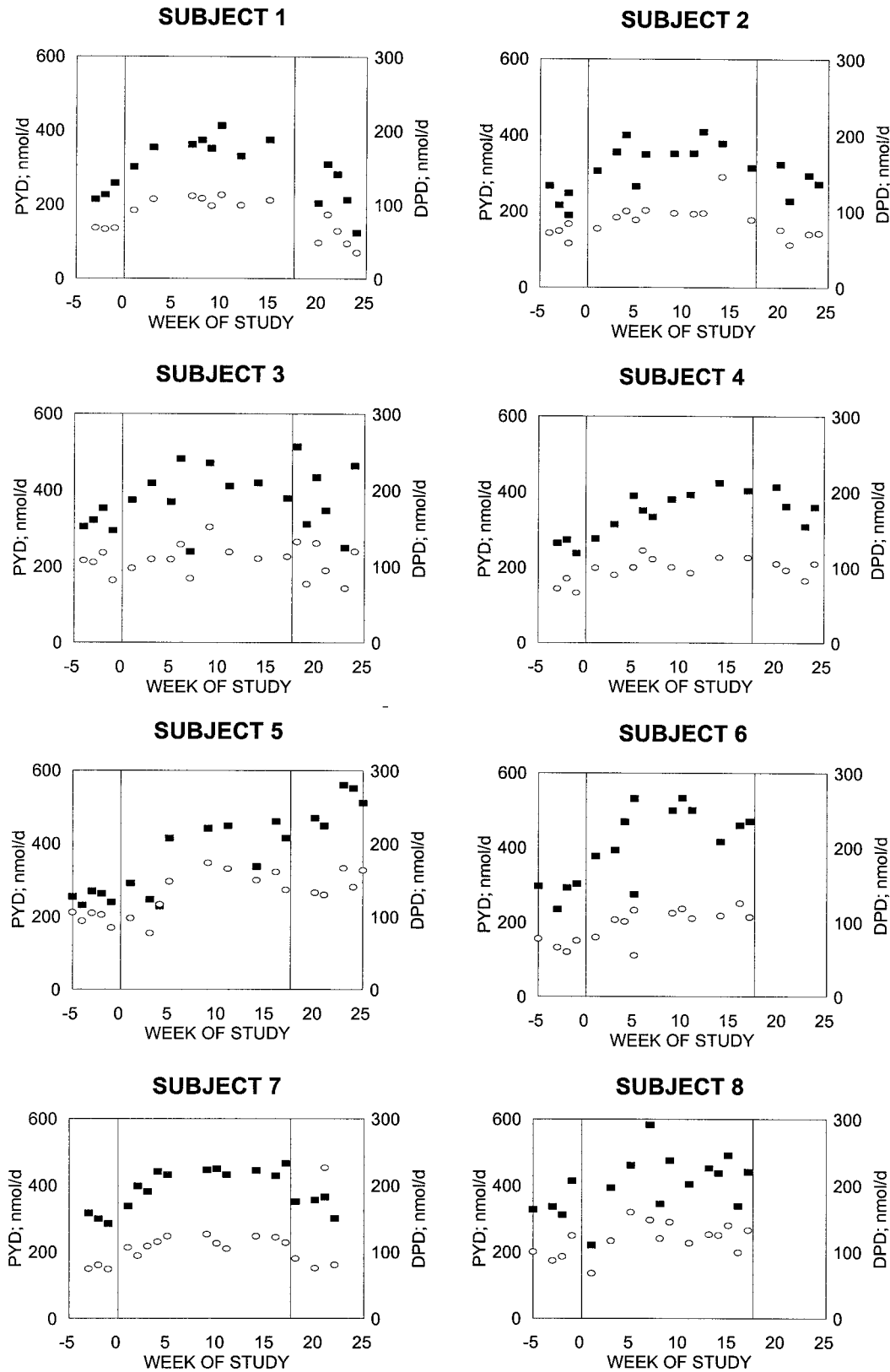


FIG. 5. Urinary pyridinoline (PYD, ■) and DPD (○) during 17 weeks of bed rest. The vertical lines represent the beginning and end of the bed-rest period. As discussed in *Methods*, these data have been previously published in condensed form (10). These data represent analysis by high-pressure liquid chromatography.

In summary, our results demonstrate that the loss of bone during weightlessness is associated with increased bone resorption. Whether diet, exercise, or pharmacologic means can alleviate this effect remains to be seen. Nevertheless, the ability to assess bone resorption, by following urinary collagen metabolite excretion in urine, represents a significant improvement in the ability to assess treatment efficacy. Effective treatments will not only assist in maintaining the health of space crews while they explore the galaxy, but they will also assist in treating bone disorders on Earth.

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