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Age Estimation by Measuring the Racemization of Aspartic Acid from Total Amino Acid Content of Several Types of Bone and Rib Cartilage: A Preliminary Account

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ABSTRACT: Age estimation has been often performed based on the rate of aspartic acid racemization using bones. In this study, we investigated various kinds of bone and a cartilage for suitability to estimate age by racemization. Ten male cadavers aged 22 to 77 years at death were selected, and bone specimens and cartilage were taken from seven sites in each individual. The rate of racemization of aspartic acid among total amino acid contained in each specimen was analyzed by gas chromatography. The correlation coefficient between the rate of racemization and chronological age was relatively high in the sternum, skull, and femur. The rates of aspartic acid racemization were high in the costal cartilage, femur, and skull. In addition, we found that the rate of racemization was only slightly lower after the second irrigation than after the first irrigation in femur and skull bone specimens, but those of others were significantly lower. These findings showed that among the six different bones and rib cartilage, the skull and femur might be used most effectively for age estimation using total amino acid fraction.

KEYWORDS: forensic science, forensic anthropology, estimation of age, bone, racemization, D-aspartic acid, gas chromatography

To date, there have been various methods of age estimation using bones, among which investigation of the morphological changes such as estimation of the degree of cranial suture closure (1,2), morphology of the pubic symphysis (3,4), and observation of trabecular structure of spongy bones are most popular (5). However, the estimated age using these methods is probably closer to the biological age than the chronological age.

Amino acids in tissues are generally present in L-forms (6), but in tissues presenting slow metabolism, such as teeth (7–15), crystalline lens (16,17), myelin protein of brain tissue (18), and compact bones (19–25), amino acids change from L-forms into D-forms, and from D-forms into L-forms with increasing age, ultimately resulting in an increase in the proportion of D-forms.

The reaction rate of racemization of aspartic acid is the highest among the amino acids and decreases in the order: aspartic acid > alanine = glutamic acid > isoleucine ≧ leucine. This information is used for dating fossils (26,27). In 1994, Ritz et al. (20,23) showed a high correlation (correlation coefficient $r = 0.99$) between the extent of aspartic acid racemization in osteocalcin in human skulls and chronological age, indicating that this could be applied to age estimation in humans in general. Using rib cartilage and cortical bone, Pfeiffer et al. (21,22) examined the ratio of D/L aspartic acid by dividing bones into two different fractions, namely an acid-soluble peptide fraction, and an acid-insoluble collagen-rich fraction, and showed that the correlation coefficient between the extent of racemization, and age ranged from 0.72 to 0.97.

In a previous study (24), the authors investigated the correlation between the rate of aspartic acid racemization in the femur and age, and found that this was higher in males, revealing gender differences. Therefore, we selected only males for this study, and examined the rate of aspartic acid racemization from total amino acid content of several bones and rib cartilage to determine which material was most valuable for age estimation. In addition, D-forms are found frequently among free amino acids, and it is possible that a substantial amount of free amino acids was removed during the process of irrigation and polishing of fragile bones, resulting in lower measurement of racemization. To investigate such influences, we irrigated specimens twice to compare the rate of racemization after the first irrigation with that after the second.

Materials and Methods

Collection and Treatment of Specimens

The examined materials were ten male cadavers aged 22, 37, 44, 52, 54, 56, 64, 65, 72, and 77 years old at death, donated specially for medical research and whole body fixed in 10% formalin, with a postmortem period of less than 1 year. We previously reported that this fixative had almost no effect on D/L ratios (28). Specimens were taken from six kinds of bone and cartilage, namely the skull (squamosal parts of the os temporale), sternum (manubrium sternum), lumbar spine (processus spinous of the first lumbar), coxal bone (a lateral portion of the fossa iliaca), sacral spine (crista

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sacralis median), femur (an upper portion of the corpus femoris), and rib cartilage (cartilage costalis of the seventh rib). The bones and cartilage were first cut with an anatomical saw, then further divided with a low-speed saw (Isomet, 11-1180, Buhler, Chicago, IL) into pieces measuring about 1 cm^2. The surface of each fragment was then polished with a whetstone removing soft tissues and spongy bone to obtain compact bone. Soft tissues adhering to the cartilage were also removed. Next, fragments were cleaned in distilled water, ethanol, and ether using an ultrasonic washer for 5 min each. After the bone and cartilage fragments were dried, they were reduced to powder (105 to 150 μm in diameter) using a grinder (Fritsch, Idav-oberstein, Germany) and 10 mg of powdered specimen from each material was used to determine the rate of aspartic acid racemization.

Irrigation Effect on D/L Ratios

One cadaver (56-years-old) was used for this comparison. Seven kinds of material fragments after removal of soft tissues and spongy bone in the case of bone were washed for 5 min in distilled water with an ultrasonicator as described above. The fragments were washed one more time for another 5 min with the ultrasonicator to obtain "twice-washed" samples.

Measurement of Racemization

The detailed procedure of amino acid analysis is given in Fig. 1. Amino acids were analyzed with a gas chromatograph (GC-17A, Shimadzu, Kyoto Japan) (11). The column used was a glass capillary column coated with chirasil-val (25 m in length, 0.3 mm in diameter). The rate of racemization (*D*/*L* ratio) was determined by the ratio of peak areas of *D*- to *L*-forms, which was transformed to the following formula:

$$\ln[(1 + D/L)/(1 - D/L)]$$

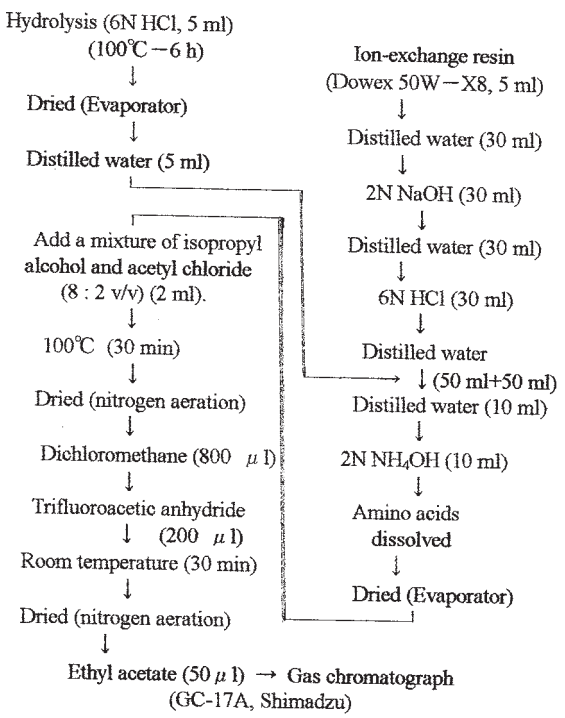

FIG. 1—*The procedure for analyzing amino acids from hydrolysis of specimens to injection of specimens into a gas chromatograph.*

Measurement was performed at least three times (three to five times) for each material and mean values were used as individual *D*/*L* ratios.

Derivation of the Rate Equation for Racemization (Age Estimation)

Racemization of amino acids is a reversible first-order reaction. Thus, racemization of aspartic acid is:

$$L\text{-Asp} \underset{k'}{\overset{k}{\rightleftarrows}} D\text{-Asp}$$

where k and k' are the reaction rate constants of the racemization reaction rate.

When the concentrations of *L*- and *D*- forms are expressed as [*L*] and [*D*], the rate equation of racemization is:

$$-d[L]/dt = k[L] - k'[D] \quad (1)$$

On integration, the general formula is derived:

$$\ln[(1 + D/L)/(1 - D/L)] = 2kt + \text{constant} \quad (2)$$

where t is the age and the formula is the rate equation of racemization. The correlation coefficient (r) is calculated by the following formula.

$$r = \{\sum_{i=I}^{n}(tiyi) - [\sum_{i=I}^{n}(ti)\ \sum_{i=I}^{n}(yi)/n]\}/\{\sum_{i=I}^{n}(ti - \bar{t})^2\ \sum_{i=I}^{n}(yi - \bar{y})^2\}$$

were t is the age, y is the *D*/*L* ratio, and $\bar{t}$ and $\bar{y}$ are each means. The t-test was calculated by the following formula {Here θ was used instead of t to avoid confusion to the t meaning time (age)}.

$$\theta = |r| \sqrt{n-2}/\sqrt{1-r^2}$$

Results

Gas chromatograms of amino acids from the femur showed the most abundant amounts of glycine, alanine, proline, and hydroxyproline. The *L*- and *D*-forms of aspartic acid were clearly isolated as we reported elsewhere (11,24,29). The racemization on rate equation and rate constant (k) were calculated using Formula 2.

Skull,	$\ln[(1 + D/L)/(1 - D/L)] = 0.000623t + 0.0363;$
Sternum,	$\ln[(1 + D/L)/(1 - D/L)] = 0.000820t - 0.0017;$
Rib cartilage	$\ln[(1 + D/L)/(1 - D/L)] = 0.000260t + 0.0380;$
Lumbar spine,	$\ln[(1 + D/L)/(1 - D/L)] = 0.000743t + 0.0035;$
Coxal bone,	$\ln[(1 + D/L)/(1 - D/L)] = 0.000534t + 0.0150;$
Sacral spine,	$\ln[(1 + D/L)/(1 - D/L)] = 0.000150t + 0.0388;$
Femur,	$\ln[(1 + D/L)/(1 - D/L)] = 0.000558t + 0.0361;$

The correlation coefficient between the chronological age and *D*/*L* ratio was relatively high in the sternum (0.974), skull (0.977), and femur (0.985) (Fig. 2*a*–*g*). On the other hand, it was lower in the sacral spine (0.739), rib cartilage (0.763), and coxal bone (0.881).

Figure 3 shows a comparison of the D/L ratios after the first and second irrigations of bones and cartilage (56-years-old). In the fe-

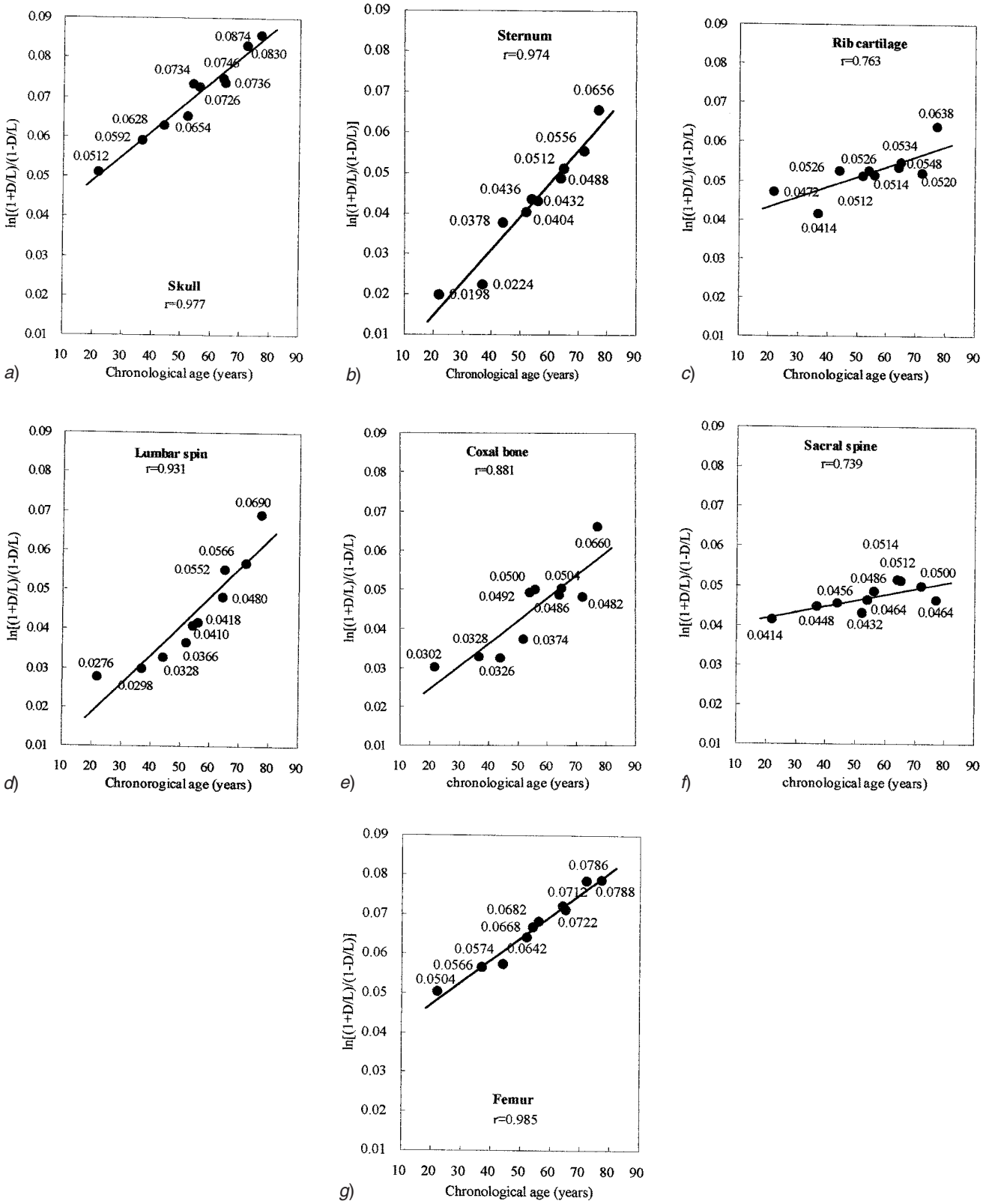

FIG. 2—*G Correlation between the D/L ratio of aspartic acid in each specimen and chronological age.* a) *skull;* r = *0.977;* $\theta_{(9)}$ = *13.08;* P < *0.001.* b) *sternum;* r = *0.974;* $\theta_{(9)}$ = *12.16;* P < *0.001.* c) *rib cartilage;* r = *0.763;* $\theta_{(9)}$ = *3.34;* P: *0.02 − 0.01.* d) *lumbar spine; r = 0.931;* $\theta_{(9)}$ = *7.21;* P < *0.001.* e) *coxal bone;* r = *0.881;* $\theta_{(9)}$ = *5.27;* P < *0.001.* f) *sacral spine;* r = *0.739;* $\theta_{(9)}$ = *3.10;* P: *0.02 − 0.01.* g) *femur;* r = *0.985;* $\theta_{(9)}$ = *16.15;* P < *0.001. The* r *is the coefficient of correlation.*

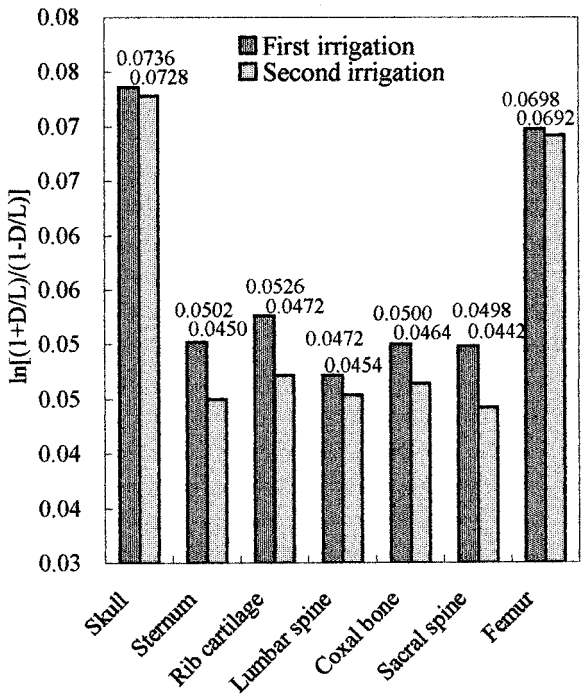

FIG. 3—*Differences in D/L ratio resulting from first and second irrigations. For the* t-*test,* θ = d / Sd (d, *means of differences between first and second irrigations;* Sd, *standard error);* d = *−0.003286,* Sd = *0.000834,* $\theta_{(6)}$ = *3.94,* P: *0.01 − 0.001.*

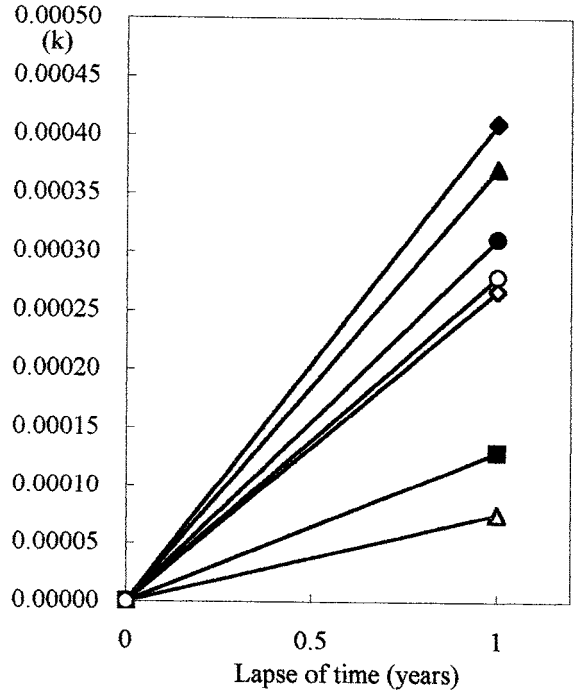

FIG. 4—*Comparison of the reaction rate constant* ($k \cdot y^{-1}$) *of aspartic acid racemization among different bone specimens and rib cartilage.* ◆, *sternum,* k = 4.10×10^{-4}; ▲, *lumbar spine,* k = 3.72×10^{-4}; ●, *skull,* k = 3.12×10^{-4}; ○, *femur,* k = 2.79×10^{-4}; ◇, *coxal bone,* k = 2.67×10^{-4}; ■, *rib cartilage,* k = 1.30×10^{-4}; △, *sacral spine,* k = 7.50×10^{-5}.

mur and skull specimens, the *D/L* ratio was only slightly lower after the second irrigation than after the first while in other bone specimens and rib cartilage it was significantly lower (P = 0.01 to 0.001) after the second irrigation than after the first irrigation, disclosing differences among bones.

The reaction rate of racemization of aspartic acid was found to increase in the order: sacral spine < rib cartilage < coxal bone ≦ femur < skull < lumbar spine < sternum (Fig. 4).

Discussion

One of the most important aspects of studies on racemization is the adjustment of the conditions so that the *D*- and *L*-forms of amino acids are clearly isolated and sharp peaks are obtained on chromatograms. Despite the fact that gas chromatography was performed, chromatograms were not presented in the reports by Ritz et al. (13,20,23), and amino acids were not clearly isolated in reports by Maroudas et al. (19) and Pfeiffer et al. (21,22). When the isolation is not clear, data might not be very accurate or reproducible. On the other hand, the chromatograms in this study showed clear isolation of *D*- and *L*-aspartic acid and a high amount of hydroxyproline, which suggests a major contribution of collagen to the total amino acid fraction.

A high correlation between the *D/L* ratio and age as well as rapid racemization are important factors for accurate age estimation. Since the reaction rate of racemization of aspartic acid is the highest among the amino acids, resulting in sufficient amounts of *D*-form, it is the best suited for age estimation.

Since the rate of aspartic acid racemization in total amino acid in dentin is very closely correlated with age, it has been applied to age estimation (29). Ritz et al. (20,23) found a very close relationship between the rate of aspartic acid racemization in osteocalcin in human skulls and age and almost no gender difference. These results suggest that this can generally be used as a measure of age in humans. Although this method of age estimation seems effective, not only large quantities of samples need to be collected, but also special facilities are required, making such a test less practicable. Furthermore, all proteins are exposed to autolysis and digestion by others after death. It is doubtful that osteocalcin is steady for a long time even though it is relatively steady in hard tissues. When age estimation is required, the time of death of cadavers is sometimes unknown. If the unknown body died a long time ago, all proteins are likely to be modulated. Thus, simple and applicable methods for cadavers left for an unspecified time need to be developed.

In our previous study (24) using femurs, we divided total amino acids into acid-soluble peptide and acid-insoluble collagen fractions. It was found that there was a difference in the correlation between the *D/L* ratio and age, and that the correlation was higher in males than in females for both fractions. We therefore examined the rate of aspartic acid racemization in total amino acids in male bones to determine which bones were the most suitable for age estimation. It seems that total amino acid can also be used simply as a measure of age estimation. The correlation coefficient between the chronological age and rate of racemization was relatively high in the sternum, skull, and femur. While the skull and femur were thick and strong, the other bones examined were comparatively thin and fragile. As several *D*-forms are contained in free amino acid (30) and a considerable amount of the free amino acids can be removed during polishing and irrigation of fragile bones, measurements obtained for *D*-forms can be lower than the real values. However, the *D/L* ratio was only slightly lower after the second irrigation than after the first irrigation in the femur and skull specimens, but others were not. These

results show that the process of irrigation can considerably affect the *D*/*L* ratio in some bones.

These findings were obtained from total amino acid of bones taken from male cadavers. We speculated that the *D*/*L* ratio is lower in specimens taken from females, because the incidence of osteoporosis and bone diseases is higher in females. Racemization is considered to occur more rapidly in tissues with slow metabolism (6). It has been noted that after menopause, the bone metabolic cycle increases even in healthy females, in addition to patients with osteoporosis, where the bone metabolic cycle is further increased (31,32). As we noted above, gender differences are present, but these were not discussed in the papers by Pfeiffer et al. (21,22), in which *D*/*L* ratios were determined using rib cartilage.

Our results indicated that when racemization in total amino acid from bones is the method adopted, among the six kinds of bone and a cartilage used in this study, the skull and femur are relatively suitable bones for age estimation in view of correlation coefficients and minimum effect of irrigation.

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