Biochemical Markers in Renal Bone Disease

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The identification of active renal osteodystrophy is imprecise without bone biopsy: X-rays may not detect it nor reflect its progression. Whilst plasma biochemical values may show elevated alkaline phosphatase the measurement of calcium and phosphorus is often unhelpful, particularly in dialysed patients (Katz et al, 1969). Bone biopsy is a troublesome procedure and requires a specialist experienced in its preparation and interpretation. We therefore need further biochemical measurements on plasma to assist in the initial assessment and to show subsequent response to treatment of bone disease.

The amino-acid hydroxyproline is virtually confined to collagen and more than half the body's collagen is in bone matrix, where it is more metabolically active than that found elsewhere. When collagen breakdown products are released into extracellular fluid the hydroxyproline cannot be re-utilised for collagen synthesis and most is oxidised. However, a proportion is excreted, mainly in the peptide-bound form, so that when gelatine is excluded from the diet, urine total hydroxyproline excretion is a good index of bone collagen turnover (Prockop & Kivirikko, 1967). Gross impairment of renal function may alter this relationship and so limit the value of urine hydroxyproline in the diagnosis of bone disease. In such circumstances a better index may be provided by measurement of blood hydroxyproline. This is present in the plasma in three forms: protein-bound, peptide-bound and as the free amino-acid (Le Roy et al, 1964). The protein-bound fraction is unaltered in bone disease (Le Roy & Sjoerdsm, 1965) and can be removed by alcohol precipitation. The fraction remaining after alcohol treatment of the plasma comprises most of the peptide-bound and free hydroxyproline, and these together are referred to here as 'plasma hydroxyproline.'

In this study plasma hydroxyproline and plasma alkaline phosphatase are related to features selected from iliac crest bone biopsies taken from two groups of patients (dialysed and undialysed).
PATIENTS AND METHODS

Twenty-eight patients with creatinine clearance less than 20ml/minute and thirty-four patients established on maintenance haemodialysis treatment were studied. Major sources of gelatine were excluded from their diets. Blood was taken near to the time of the biopsy and in the haemodialysed patients this was a pre-dialysis specimen. Three patients had had total parathyroidectomy for severe progressive osteitis fibrosa. Blood from these patients was taken before operation, 12 hours later (but before the first post-operative haemodialysis) and one week following surgery.

Plasma hydroxyproline was measured after precipitation of the plasma proteins by a modification of the method of Prockop and Udenfriend (1960). The free fraction was estimated in five non-dialysed patients with chronic renal failure and four dialysed patients after omitting the stage of acid hydrolysis of hydroxyproline peptide. Plasma alkaline phosphatase and creatinine were measured by conventional methods.

Iliac crest biopsies were taken under local anaesthetic with a bone trephine of internal diameter 5mm (Williams & Nicholson, 1963). Trabecular bone was prepared by a double embedding technique and examined before and after decalcification (Woods et al, 1968). For this study sections were reviewed for signs of excessive osteoclastic activity and osteoid area was measured in relation to the total area of bone using a point counting technique.

Patients were arranged into three groups according to simple criteria of histological abnormality designed to separate those with resorptive disease from the remainder (Table 1). Biopsies showing excess resorption with or

<table>
<thead>
<tr>
<th>Table 1. Histological types of bone abnormality in the three groups of patients</th>
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<tr>
<td>1. Active Resorption + Excess Osteoid</td>
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<tr>
<td>2. Osteoid Area &gt; 5% Total Bone Area: No Resorption</td>
</tr>
<tr>
<td>3. No Active Resorption, Osteoid Area &lt; 5% Bone Area</td>
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without increased osteoid were said to have had Type 1 histological changes. Those without excess resorption but with osteoid area more than 5% of the total bone were designated Type 2. Biopsies classified as Type 3 showed neither active resorption nor osteoid excess, and for purposes of this study were termed 'normal.' Evidence of previous resorptive disease such as irregular cement lines was found in some biopsies in each group but was not used as a criterion for classification.
RESULTS

Controls
Normal values were defined from twenty-four laboratory staff approximately matched for age and sex with the patients studied. Plasma hydroxyproline was \(1.4 \pm 1.3\) mg/l (mean \(\pm 2SD\)) and plasma alkaline phosphatase was \(5.9 \pm 4.0\) KA units/100 ml (mean \(\pm 2SD\)). The highest control values were 2.9 mg/l and 11 KA units/100 ml, respectively.

Non-dialysed Patients (Figure 1)

![Graph showing plasma hydroxyproline and alkaline phosphatase in 28 non-dialysed patients. Upper and lower limits of normal are shown. Plasma hydroxyproline 0.4 - 2.9 mg/l. Plasma alkaline phosphatase 3 - 11 KA units/100 ml.]

Figure 1. Plasma hydroxyproline and alkaline phosphatase in 28 non-dialysed patients. Upper and lower limits of normal are shown. Plasma hydroxyproline 0.4 - 2.9 mg/l. Plasma alkaline phosphatase 3 - 11 KA units/100 ml.

Fifteen of the twenty-eight patients had Type 1 bone changes and the remainder were normal (Type 3). Plasma hydroxyproline was higher in all patients with Type 1 bone histology than in those with Type 3 (mean \(\pm\) SEM 6.9 \(\pm\) 1.34 and 3.27 \(\pm\) 0.37 mg/l respectively; \(p = <0.01\)). Alkaline phosphatase was elevated in only eight of the fifteen patients with bone disease, but the difference in mean concentration between patients with Type 1 and Type 3
Figure 2. Relation between plasma hydroxyproline and creatinine in 28 non-dialysed patients.

Figure 3. Plasma hydroxyproline and alkaline phosphatase in 34 patients with chronic renal failure on haemodialysis. Normal values as in Figure 1.
histology was significant ($13.67 \pm 2.55$ and $7.0 \pm 0.73$ KA units/100 ml respectively, $p = < 0.01$).

Plasma creatinine did not correlate with either plasma hydroxyproline or the presence or absence of bone disease (Figure 2).

**Dialysed Patients (Figure 3)**

This group of thirty-four subjects contained seventeen with Type 1, ten with Type 2 and seven with type 3 bone histology.

Plasma hydroxyproline was raised in all with resorptive (Type 1) disease (mean $\pm$ SEM 6.2 $\pm$ 0.89 mg/l). In contrast the mean plasma hydroxyproline was normal in patients with excess osteoid (Type 2) and with 'normal' bones (Type 3) (mean $\pm$ SEM 2.54 $\pm$ 0.28 and 2.59 $\pm$ 0.29 mg/l) respectively. The difference between groups with Type 1 and Type 2 bone disease was significant ($p = < 0.001$) but there was no significant difference between mean plasma hydroxyproline in patients with Types 2 and 3 changes. In both these groups some of the individual values were elevated.

Mean alkaline phosphatase was elevated in the presence of both Types 1 and 2 bone histology (mean $\pm$ SEM Type 1: 12.24 $\pm$ 2.1; Type 2: 12.5 $\pm$ 0.21). The difference was not significant. However many individual values were within the range of normal in both groups.

**Total Parathyroidectomy (Figure 4)**

![Graph](image)

*Figure 4. Plasma hydroxyproline and alkaline phosphatase in 3 patients before and after total parathyroidectomy*
These patients initially showed the highest values for hydroxyproline and alkaline phosphatase. In all, hydroxyproline fell abruptly after surgery reaching normal values by the seventh day in two cases. Alkaline phosphatase increased post-operatively, returning to normal only after several months.

**Plasma Hydroxyproline Fractions (Table II)**

In this small group of patients in chronic renal failure, peptide-bound hydroxyproline was the major fraction of plasma hydroxyproline and free hydroxyproline was of the same order in non-dialysed and dialysed patients.

**Table II.** Peptide and free hydroxyproline in plasma of 5 non-dialysed and 4 dialysed patients with chronic renal failure

<table>
<thead>
<tr>
<th>Patients</th>
<th>Plasma Hydroxyproline mg/l</th>
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<tbody>
<tr>
<td>Non-dialysed</td>
<td>Peptide</td>
</tr>
<tr>
<td>D.R.</td>
<td>4.4</td>
</tr>
<tr>
<td>D.W.</td>
<td>&gt; 8.6</td>
</tr>
<tr>
<td>M.T.</td>
<td>&gt; 4.3</td>
</tr>
<tr>
<td>Y.D.</td>
<td>3.0</td>
</tr>
<tr>
<td>W.H.</td>
<td>2.9</td>
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<thead>
<tr>
<th>Dialysed</th>
<th>Plasma Hydroxyproline mg/l</th>
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<tbody>
<tr>
<td>R.L.</td>
<td>14.4</td>
</tr>
<tr>
<td>A.W.</td>
<td>&gt; 3.1</td>
</tr>
<tr>
<td>J.T.</td>
<td>10.3</td>
</tr>
<tr>
<td>E.T.</td>
<td>3.1</td>
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**DISCUSSION**

The results suggest that elevation of plasma hydroxyproline and alkaline phosphatase are associated with certain forms of bony abnormality in patients with chronic renal failure. In deciding how useful this relationship might be in the clinical context we need to know what is the effect of renal insufficiency upon these levels. It is assumed that renal function plays no significant part in determining the plasma alkaline phosphatase. On the other hand plasma hydroxyproline is normally filtered at the glomerulus and the free fraction almost entirely reabsorbed by the renal tubules. It might be expected therefore that in chronic renal failure the peptide fraction at least might accumulate in parallel with creatinine. Dubovsky et al. (1968) found elevated levels of both free and peptide-bound hydroxyproline in a proportion of patients with chronic renal failure, but some of their patients were known to have bone disease. In this study there was no correlation between plasma
creatine and hydroxyproline (whether or not patients had been dialysed) (Figure 2). Increases in plasma hydroxyproline, largely of the peptide fraction (Table II), are therefore likely to reflect changes in bone metabolism rather than renal glomerular function.

In both dialysed and non-dialysed patients excessive resorptive activity on bone biopsy was consistently associated with raised levels of plasma hydroxyproline. In contrast, in those patients with osteoid changes alone (Type 2) plasma hydroxyproline was not different from the concentrations found in patients without bone disease (Type 3). However some patients with chronic renal failure, whether dialysed or not, had slightly elevated plasma hydroxyproline concentrations in the absence of obvious bone disease on biopsy. We have no definite explanation for this, but it is likely that bony abnormality is a continuous variable and, by imposing artificial limits of normality on resorption and osteoid area measurements, a number of biopsies may have been mis-diagnosed as normal. Previous workers have shown that quantitative measurements may reveal abnormality in a qualitatively normal bone biopsy (Byers & Smith, 1971).

Another explanation is that iliac crest bone is not truly representative of changes occurring throughout the skeleton at all times. The idea that plasma hydroxyproline is related mainly to active resorption is further supported by the data from patients with renal failure who underwent total parathyroidectomy (Figure 4). The removal of hyperplastic parathyroid glands is presumably followed by cessation of bone resorption when plasma hydroxyproline falls very rapidly. Alkaline phosphatase rises post-operatively perhaps as a result of increased osteoblastic activity during the process of bone healing.

Alkaline phosphatase has hitherto been regarded as the most useful chemical index of renal osteodystrophy (Kyle, 1969), reflecting its osteoblastic origin with excessive matrix formation by these cells. Unfortunately alkaline phosphatase may be normal in the presence of gross bone disease with abnormal quantities of unmineralised osteoid. This is consistent with the idea that chronic azotaemia causes osteoblastic dysfunction with formation of abnormal bone matrix (Jowsey, 1969). Alkaline phosphatase was generally a poor index of the presence and type of bone disease in our patients. It did not discriminate between erosive and osteoid disease and was normal in eight non-dialysed and in sixteen dialysed patients with obvious bone disease on biopsy. Our findings differ from those of Sagar et al (1971) who found neither heat-labile nor total alkaline phosphatase to be elevated in patients with osteoid disease unaccompanied by excessive erosion. In our hands raised plasma hydroxyproline with normal alkaline phosphatase is likely to reflect resorptive disease, while elevated alkaline phosphatase without change in hydroxyproline reflects rather predominant osteoid change (Table III).

Whilst we cannot suggest that these biochemical measurements can displace
bone histology in the diagnosis of osteodystrophy they may be useful in following its course once it has been identified on biopsy.

Table III. Clinical significance of plasma hydroxyproline and alkaline phosphatase in renal bone disease

<table>
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<tr>
<th></th>
<th>Hydroxyproline</th>
<th>Alkaline Phosphatase</th>
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<tbody>
<tr>
<td>Excess Resorption</td>
<td>Always increased</td>
<td>May be normal</td>
</tr>
<tr>
<td>Excess Osteoid</td>
<td>Slight increase</td>
<td>May be increased</td>
</tr>
<tr>
<td>'Normal'</td>
<td>Slight increase</td>
<td>Normal</td>
</tr>
</tbody>
</table>

ACKNOWLEDGMENTS

We thank the staff of the Metabolic Unit for help with the biochemical estimations and are grateful to Dr Colin Woods and the technicians in the Pathology laboratory for assistance in the preparation and interpretation of the histological specimens.

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OPEN DISCUSSION

V PARSONS (London): I would like to congratulate Dr Bishop on these findings. I can say that we have been doing these measurements as well and find a mean of around 9 mg/l in patients on chronic dialysis which I think agrees quite closely to your figures. What I would like to question, however, is whether, when one finds something raised in the plasma in renal failure, it is all the result of increased bone turnover. How do you know that this
rise is not due to the failure of degradation of hydroxyproline peptides in the liver (where they are normally degraded)? Have you given any of your patients a single injection of hydroxyproline to see what its half life is in the normal patient, or in the anephric patient who is just entering dialysis therapy, compared with the patient who has been on it for some time? This would answer the question about degradation of hydroxyproline.

BISHOP: In answer to the second part of your question, we have not done this and I realise that there is patchy evidence from some centres that the metabolism of hydroxyproline itself is altered in renal failure, but in fact the evidence has been conflicting from different centres. In answer to the first part of the question, I think that one can only quote the second group, the group with osteoid excess alone, that did not have elevated plasma hydroxyproline — or at least, there was no extra elevation. Certainly this slight elevation above normal in both the osteoid and normal groups may be due to some change in the metabolism of hydroxyproline, but I think that bone resorption adds to this extra rise in the resorptive group.

PARSONS: Can I ask a final question about vitamin D? Did these patients at any time receive this steroid-like hormone, or were they completely off it?

BISHOP: None of the patients in the groups that were plotted in the first three slides had had vitamin D, but of course, the parathyroidectomised patients had been covered post-operatively with large doses of vitamin D.

M H MAXWELL (Los Angeles): Dr Bishop, I just want to ask one ingenuous question. You mention that the hydroxyproline is bound — at least a fraction is bound to peptides. Now what you call normal is in your uraemic patients without evidence of bone disease — what is the normal hydroxyproline value for normal individuals without uraemia? Does it compare with the values in these patients?

BISHOP: In fact, I quoted those in the second slide; in the normal subjects the highest value found was 2.9 mg/l.

A C KENNEDY (Glasgow, Chairman): Are there any technical problems in the estimation of plasma hydroxyproline in this setting of uraemic plasma?

BISHOP: There is no technical problem that we have found. It is not an easy determination, and it is quite a long determination, and admittedly there are stages in the technique in which the recovery might be reduced, but in fact there is no extra diminution of recovery in renal failure plasma. The recovery experiments we have done have shown 80% in both normal and renal failure groups.