# Quantitative method for determining serum alkaline phosphatase isoenzyme activity I. Guanidine hydrochloride: new reagent for selectively inhibiting major serum isoenzymes of alkaline phosphatase

M D S SHEPHARD, M J PEAKE

*From the Department of Biochemistry and Chemical Pathology, Flinders Medical Centre, Bedford Park, South Australia*

SUMMARY The potential use of the protein denaturant guanidine hydrochloride to inhibit selectively the enzyme activity of serum alkaline phosphatase isoenzymes from liver, bone, intestine, and placenta was investigated. Inhibition of each isoenzyme was shown to be dependent on time and concentration of inhibitor. In the presence of 0·3 mol/l (28·7 g/l) guanidine hydrochloride for 170 seconds 14%, 47%, and 90% of the total alkaline phosphatase activity remained in samples of bone, liver, and intestinal origins, respectively. In contrast, the activity of the placental isoenzyme increased by 24%. The degree of inhibition was shown to be independent of total alkaline phosphatase activity. Investigations were performed at 37°C using the Cobas Bio centrifugal analyser. We conclude that this reagent has several practical advantages over urea as a selective inhibitor of alkaline phosphatase isoenzymes, including a faster and more reproducible inhibition at a much lower reagent concentration.

An increase of total serum alkaline phosphatase activity (EC 3.1.3.1) is a common finding in the clinical chemistry laboratory and is usually indicative of either osteoblastic activity, hepatobiliary disease, the presence of a tumour, or pregnancy. Four major serum isoenzymes of alkaline phosphatase have been described, their tissue origins being liver, bone, intestine, and placenta. In recent years much work has focused on the development of methods to differentiate between these isoenzymes, particularly those of liver and bone, to locate the tissue source of increased alkaline phosphatase activities.

Selective inhibition by urea has been used by several workers[1-5] to differentiate isoenzymes of alkaline phosphatase. A wide range of concentrations has been used to study the effect of urea, ranging from 0·5 mol/l (30 g/l)[2] to 4·8 mol/l (288 g/l).[5] The degree of inhibition reported for each isoenzyme, however, has varied greatly; even using the same concentration of urea, there is no consistency in published results.[4,5] Most methods entailing urea require the use of highly concentrated urea solutions and

extended inhibition times, and these factors can impair analytical performance.

We therefore investigated the suitability of guanidine hydrochloride for the selective inactivation of alkaline phosphatase isoenzymes on the basis that this protein denaturant may provide a faster and more reliable differentiation of the four main serum isoenzymes of alkaline phosphatase at lower reagent concentrations. Guanidine hydrochloride has not been previously used for this purpose. Moss[6] recently stated that, "selective inactivation methods provide what is probably the best current approach to the quantitative determination of liver and bone phosphatases," and "quantitative analysis based on selective inactivation has received less attention than its potential diagnostic advantages would suggest might be appropiate." We report our investigation of a new reagent for use in such studies.

## Material and methods

### MEASUREMENT OF TOTAL ALKALINE PHOSPHATASE
A single vial Monotest alkaline phosphatase (AMP buffer) kit (Boehringer Mannheim, Mannheim, West

Table 1    *Reaction conditions on Cobas Bio for measuring liver and bone alkaline phosphatase isoenzyme activity*

| Instrument settings | A<br>Total<br>activity | B<br>Inhibition<br>studies | C<br>Urea<br>inhibition | D<br>Guanidine<br>hydrochloride<br>inhibition |
|---|---|---|---|---|
| 1  Units | U/l | U/l | U/l | U/l |
| 2  Calculation factor | 2688 | 2688 | 2688 | 2688 |
| 3  Standard 1 concentration | 0 | 0 | 0 | 0 |
| 4  Standard 2 concentration | 0 | 0 | 0 | 0 |
| 5  Standard 3 concentration | 0 | 0 | 0 | 0 |
| 6  Limit | 1·5 | 1·5 | 1·5 | 1·5 |
| 7  Temperature (°C) | 37 | 37 | 37 | 37 |
| 8  Type of analysis | 2 | 2 | 2 | 2 |
| 9  Wavelength (nm) | 405 | 405 | 405 | 405 |
| 10  Sample volume (μl) | 05 | 05 | 05 | 05 |
| 11  Diluent volume (μl) | 30 | 30 | 30 | 30 |
| 12  Reagent volume (μl) | 150 | 150 | 150 | 150 |
| 13  Incubation time (seconds) | 120 | 300 | 300 | 300 |
| 14  Start reagent volume (μl) | 0 | 0 | 0 | 0 |
| 15  Time of first reading (seconds) | 30 | 0·5 | 250 | 170 |
| 16  Time interval (seconds) | 10 | 20 | 10 | 10 |
| 17  No of readings | 20 | 30 | 07 | 07 |
| 18  Blanking mode | 1 | 1 | 1 | 1 |
| 19  Printout mode | 1 | 3 | 2 | 2 |

Germany; catalogue number 396494) was used to determine the total alkaline phosphatase activity in our test samples.

To each reagent vial containing 11·5 ml of AMP buffer (0·9 mol/l (80·2 g/l), pH 10·5) one tablet each of disodium p-nitrophenylphosphate substrate and magnesium chloride (final concentrations 16 mmol/l (3·51 g/l) and 1·0 mmol/l (95 mg/l) respectively) was added. After prewarming this vial for five minutes at 37°C total activity was determined on the Cobas Bio

centrifugal analyser (Roche Analytical Instruments, Nutley, New Jersey, USA) using the instrument settings shown in table 1 (column A).

### INHIBITION STUDIES WITH GUANIDINE HYDROCHLORIDE

Guanidine hydrochloride (grade 1, water soluble, Sigma, St Louis, Missouri, USA) was accurately weighed out and dissolved in AMP buffer from the above kit to the desired final concentration. Substrate
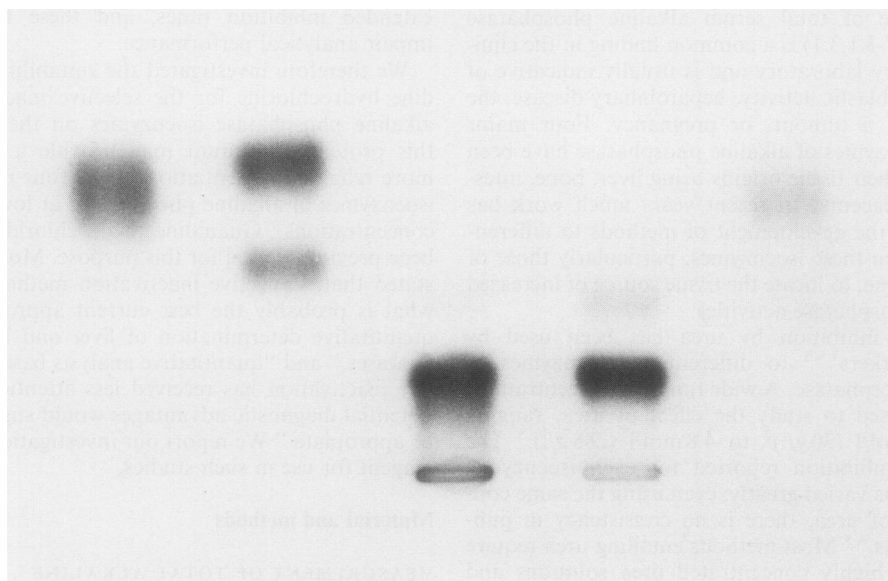


Fig 1    *Electrophoretic patterns (from left to right) of representative bone, liver, duodenal, jejunal, and placental source material used in experimental work. Total activities of these samples were 798, 857, 995, 976, and 350 U/l, respectively.*

and magnesium chloride tablets were then added to 11·5 ml of this reagent. This combined reagent was mixed on a stirring wheel until completely dissolved and prewarmed for a minimum of 10 minutes at 37°C immediately before use. The inhibitor did not change the pH of the reagent at the recommended concentration. Desiccated urea (Ajax Chemicals, Sydney, Australia) was similarly used for comparative purposes.

### SOURCES OF ALKALINE PHOSPHATASE ISOENZYMES
*Bone*
Serum from five patients, clinically and biochemically diagnosed as having Paget's disease, were used as source material for bone (B) alkaline phosphatase throughout this study. Total alkaline phosphatase activities in these sera ranged from 1500 to 3200 U/l. Increased activities were confirmed by conventional agarose gel electrophoresis (fig 1) as being of bone origin. This was further supported using wheat germ studies.[7] All sera behaved identically under the experimental conditions tested.

*Liver*
Serum from five patients, clinically and biochemically diagnosed as having primary biliary cirrhosis (n = 3), primary hepatic metastases (n = 1), and liver damage due to hypervitaminosis (n = 1) were used as source material for liver alkaline phosphatase in the study. Total activities in these sera ranged from 1250 to 1500 U/l. Increased activities were confirmed by conventional electrophoretic techniques as being of liver origin (fig 1). All sera behaved identically under the experimental conditions described later. Liver extracts were not used for inhibition studies because of previously reported analytical problems[8] and variable results.[9]

*Intestinal*
Sections of duodenum, jejunum, and ileum were obtained at necropsy from a patient who had died from myocardial infarction: necropsy showed no evidence of liver or bone disease.

To extract the intestinal alkaline phosphatase 20 ml of 0·9% sodium chloride was added to each section. After gentle mixing for 45 minutes the saline, into which the intestinal alkaline phosphatase had eluted, was decanted, spun at 4000 rpm for 10 minutes, and assayed for total alkaline phosphatase on the Cobas Bio centrifugal analyser. Activities obtained for the duodenal, jejunal, and ileal fractions were 3150, 2650, and 50 U/l, respectively. The duodenal and jejunal fractions were therefore chosen for subsequent experimental work. Both sources of intestinal alkaline phosphatase behaved identically under all experi-

mental conditions tested; the results for the jejunal fraction only are reported here.

*Placental*
Human placental alkaline phosphatase (P), in liquid form, was obtained from the Commonwealth Serum Laboratories (CSL; Parkville, Victoria, Australia). This material was stored at −70°C until analysis and had a total alkaline phosphatase activity of about 60 000 U/l.

Agarose gel electrophoresis confirmed the absence of other isoenzyme components in the intestinal and placental samples (fig 1).

When stored at 4°C, the total activity and isoenzyme composition of all samples described remained stable for more than three months. Dilutions in saline were prepared to give working activities for each sample of about 500 U/l and 1000 U/l for use in subsequent experimentation.

### ELECTROPHORESIS METHOD
Agarose gels (1%) (agarose; BDH Chemicals Ltd, Poole, England) containing 0·5% Triton X100 (Ajax Chemicals, Sydney, Australia) in 0·06 mol/l barbital buffer, pH 8·6 (20·6 g sodium barbitone and 3·7 g barbitone; Sigma Chemicals) were prepared for electrophoresis. Sample (2 μl) was applied to the gel and electrophoresed at 250 volts for 50 minutes at room temperature. After electrophoresis the gel was overlayed with a membrane that had been soaked with the chromogenic substrate 5-bromo-4-chloro-3 indoyl phosphate, p-toluidine salt (Sigma) at a final concentration of 4·6 mmol/l (2 g/l) in 2-amino-2-methyl-1, 3-propanediol buffer (0·4 mol/l (35·7 g/l), pH 10·4) containing magnesium chloride (0·01 mmol/l (0·95 mg/l)). The gel was incubated with this membrane for 45 minutes at 37°C to allow the bands to resolve. The membrane was then removed and the gel dried in an oven at 75°C for one hour.

For lectin studies samples were mixed with an equal volume of a 5 g/l aqueous solution of wheat germ lectin, incubated at 37°C for 30 minutes, and centrifuged at 4000 rpm for 10 minutes. The pellet was resuspended in saline and applied to the gel.

## Results

### SELECTIVE INACTIVATION OF BONE AND LIVER ISOENZYMES USING GUANIDINE HYDROCHLORIDE
Most requests from clinicians for alkaline phosphatase isoenzyme determinations require differentiation between liver and bone as the origin of an increased serum enzyme activity. Therefore a major component of this study was to investigate the ability of guanidine hydrochloride to discriminate between
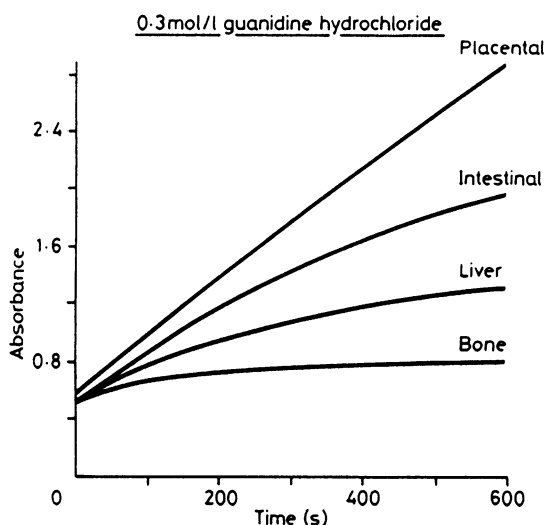
Fig 2 *Inhibition of bone, liver, intestinal, and placental isoenzymes using 0·3 mol/l guanidine hydrochloride at 37°C. Total activities of these fractions were 490, 505, 514, and 521 U/l, respectively.*

these two isoenzymes.

Initially, the kinetics of the inhibition of the bone and liver alkaline phosphatase activity by guanidine hydrochloride were investigated on Cobas Bio using the instrument settings shown in table 1 (column B).

The inhibitory effects of a wide range of guanidine hydrochloride concentrations were examined; final concentrations in the reaction cuvette ranged from 0·025 mol/l to 1·2 mol/l. At the extremes of these concentration ranges inhibition was found to be either too small or too great and, as a result, guanidine hydrochloride concentrations from 0·15 mol/l (11·7 g/l) to 0·35 mol/l (14·3 g/l) were used for detailed investigations. For comparative purposes, urea

concentrations from 1·3 mol/l (78 g/l) to 1·7 mol/l (102 g/l) were similarly used.

Because exact temperature control is essential for reproducible results, the substrate containing inhibitor was preincubated at 37°C for five minutes in the centrifugal analyser before the sample was added. The kinetics of the inhibition of the bone and liver samples at the working concentrations indicated were found to be non-linear for both guanidine hydrochloride (fig 2) and urea.

Using the absorbance data obtained, the alkaline phosphatase activity remaining for both the liver and bone specimens at different time intervals and final concentrations of guanidine hydrochloride were calculated (table 2). The concentrations of inhibitory agent specified represent final concentrations in the reaction cuvettes.

The non-linear inhibition pattern, in combination with the pronounced changes in the percentage residual activity of liver and bone isoenzymes caused by minor differences in either time or concentration, may explain why previous workers have been unable to produce consistent results using urea as a selective inhibitor.

SELECTION OF CONDITIONS FOR OPTIMAL
DISCRIMINATION BETWEEN LIVER AND BONE
ISOENZYMES USING GUANIDINE
HYDROCHLORIDE

From these preliminary data, analytical conditions for further investigation were selected, based on the following criteria:

1   Residual activity of the bone isoenzyme should be sufficient to permit precise measurement and should, therefore, exceed 10% of the original total alkaline phosphatase activity.

2   Maximum absorbance must not exceed two absorbance units over the time interval during which enzyme activity is monitored.

Table 2   *Activity remaining and percentage (in parentheses) of liver (L) and bone (B) alkaline phosphatase of original activity remaining following inhibition by guanidine hydrochloride.*

| Final concentration of guanidine hydrochloride (mol/l) | | Time interval (seconds) | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 10–70 | 90–150 | 170–230 | 250–310 | 330–390 | 410–470 | 490–550 |
| 0·15 | L | 882* (100) | 819 (96) | 767 (89) | 719 (84) | 677 (79) | 624 (73) | 586 (68) |
| | B | 804* (100) | 674 (84) | 575 (72) | 490 (61) | 424 (53) | 362 (45) | 312 (39) |
| 0·20 | L | 877 (100) | 776 (91) | 695 (81) | 627 (73) | 571 (66) | 513 (60) | 466 (54) |
| | B | 735 (92) | 555 (70) | 431 (54) | 338 (42) | 269 (34) | 215 (27) | 174 (22) |
| 0·25 | L | 825 (96) | 671 (78) | 562 (66) | 478 (56) | 409 (47) | 349 (41) | 301 (35) |
| | B | 632 (79) | 380 (48) | 244 (31) | 163 (20) | 114 (14) | 84 (11) | 61 (8) |
| 0·30 | L | 716 (84) | 533 (62) | 412 (47) | 324 (38) | 259 (30) | 209 (24) | 170 (20) |
| | B | 482 (60) | 204 (26) | 109 (14) | 64 (8) | 45 (6) | 31 (4) | 24 (3) |
| 0·35 | L | 658 (77) | 403 (47) | 277 (32) | 194 (23) | 142 (17) | 105 (12) | 79 (9) |
| | B | 348 (44) | 107 (13) | 53 (7) | 30 (4) | 23 (3) | 17 (2) | 13 (2) |

The total alkaline phosphatase activity of the liver and bone specimens used in this experiment was 857 U/l and 798 U/l, respectively.

3 The residual percentage activity of the liver iso-
enzyme must exceed that of the bone isoenzyme by a
factor of 3 during the designated reading interval.

The concentrations of guanidine hydrochloride and
the time intervals that satisfied the above criteria
were: 0·3 mol/l from 170–230 seconds; 0·25 mol/l from
330–390 seconds; and 0·35 mol/l from 90–150 sec-
onds. This last option was not chosen because of the
rapid change in bone alkaline phosphatase activity
over this period (fig 2). To further investigate the first
two options intrarun precision studies (n = 7) were
carried out using the liver and bone samples together
with a commercial lyophilised enzyme quality control
material used in our routine laboratory (Omega
Assayed Chemistry Control Serum II; Hyland Diag-
nostics, Malvern, Pennsylvania, USA). Table 3 sum-
marises these results.

Guanidine hydrochloride (0·3 mol/l) was selected
as the concentration of choice over a reading interval
of 170–230 seconds to minimise both the possibility of
abnormally high absorbance readings and to reduce
analysis time.

The instrument settings finally selected for the
quantitative discrimination of liver and bone alkaline
phosphatase isoenzymes using guanidine hydro-
chloride are shown in table 1 (column D). To sum-
marise, the reaction is carried out at 37°C, the time of
the first reading is 170 seconds using a final inhibitor
concentration of 0·3 mol/l, the reaction is monitored
for one minute at 10 second intervals, and the activity
is automatically calculated by linear regression. For
the bone isoenzyme, 14% of the total activity
remained in the presence of inhibitor, while for the
liver isoenzyme, 47% of the total activity remained.
Optimal reaction conditions for urea were similarly
determined, using the same criteria and intrarun pre-
cision studies. Final instrument settings selected for
use with this reagent are shown in table 1 (column C).

INTESTINAL AND PLACENTAL ISOENZYME
STUDIES WITH GUANIDINE HYDROCHLORIDE
Having optimised reaction conditions and obtained
maximal discrimination between the liver and bone
isoenzymes using 0·3 mol/l guanidine hydrochloride,
intestinal and placental fractions were then tested
under these final experimental conditions.

Table 4 *Percentage activity remaining for each isoenzyme
in the presence of 0·3 mol/l guanidine hydrochloride**

| Isoenzyme | Percentage activity remaining | |
| | Guanidine hydrochloride (0·3 mol/l) Time interval (170–230 seconds) | Urea (1·3 mol/l) Time interval (250–310 seconds) |
|---|---|---|
| Bone | 14 | 15 |
| Liver | 47 | 50 |
| Intestinal | 90 | 67 |
| Placental | 124 | 83 |

*For comparative purposes similar data for 1·3 mol/l urea are also
shown.

For the intestinal fraction, 90% of the total activity
remained in the presence of guanidine hydrochloride,
and inhibition was non-linear. In contrast, the activ-
ity of the placental isoenzyme fraction increased by
24%, and activation of placental alkaline phos-
phatase was linear with time (fig 2). Quality assurance
sera (Australian Quality Assurance General Serum
Chemistry Programme, supplied by the Common-
wealth Serum Laboratories), which contained over
90% placental alkaline phosphatase, displayed a simi-
lar activation of total activity in the presence of
0·3 mol/l guanidine hydrochloride.

SUMMARY OF THE ACTION OF GUANIDINE
HYDROCHLORIDE OR ALKALINE PHOSPHATASE
ISOENZYMES
Table 4 summarises the inhibition or activation of
each isoenzyme of alkaline phosphatase with
0·3 mol/l guanidine hydrochloride. Table 4 shows the
inhibition obtained under optimised conditions using
1·3 mol/l urea included for comparative purposes.
The pronounced differences observed between frac-
tions with guanidine hydrochloride not only for the
bone and liver isoenzymes but also for the intestinal
and placental fractions corroborate the usefulness of
this agent in the selective inhibition of isoenzymes of
alkaline phosphatase

REPRODUCIBILITY OF ISOENZYME INHIBITION
Day to day precision studies were then carried out
using all four isoenzyme fractions. The results of 12

Table 3 *Intrarun precision studies*

| Conditions | | Intrarun precision (coefficient variation %) | | |
| Guanidine hydrochloride (mol/l) | Time interval (seconds) | Liver | Bone | Commercial QC |
|---|---|---|---|---|
| 0·3 | 170–230 | 2·37 | 4·00 | 1·01 |
| 0·25 | 330–390 | 2·37 | 3·13 | 1·41 |

Table 5  *Percentage activity remaining for four isoenzymes of alkaline phosphatase in presence of guanidine hydrochloride: results of day to day precision studies*

| Condition | Percentage activity remaining | | | | | |
|---|---|---|---|---|---|---|
| | 0·3 mol/l guanidine hydrochloride Time interval (170–230 seconds) | | | | 1·3 mol/l urea Time interval (250–310 seconds) | |
| Sample | Bone | Liver | Intestinal | Placental | Bone | Liver |
| Mean | 13·6 | 46·6 | 90·2 | 124·4 | 15·3 | 50·3 |
| SD | 0·73 | 1·40 | 2·53 | 5·35 | 1·16 | 1·83 |
| CV (%) | 5·4 | 2·9 | 2·8 | 4·3 | 7·4 | 3·6 |
| No | 12 | 12 | 12 | 12 | 12 | 12 |

assays (table 5) show that the method using guanidine hydrochloride is reproducible and precise. Similar data for urea using the liver and bone isoenzymes are also shown.

## Conclusion

Investigation of a new selective inhibitor, guanidine hydrochloride, and its action on the four major serum isoenzymes of alkaline phosphatase, have been described. Investigations were performed at 37°C using the Cobas Bio centrifugal analyser. Optimal reaction conditions were determined and a precise reproducible assay suitable for use in combination with other selective inhibitors in routine diagnostic situations was developed. Simplicity was improved by the direct inclusion of guanidine hydrochloride in the alkaline phosphatase substrate.

Advantages of guanidine hydrochloride over urea as a selective inhibitor of alkaline phosphatase isoenzymes include:

### 1  More potent inhibitor
Similar inhibition is produced by guanidine hydrochloride at less than one quarter the molar concentration required for urea, thus avoiding the use of viscous and highly saturated solutions.

### 2  Reduced inhibition times
Faster inhibition allows activity measurements soon after the addition of guanidine hydrochloride and minimises the possibility of abnormally high absorbance measurements, especially when activity is measured at 37°C. Long incubation times have previously been required with urea.

### 3  Stability
The reagent containing guanidine hydrochloride is stable for three days as opposed to one day for urea.

### 4  Improved precision
The assay using guanidine hydrochloride is more precise.

### 5  Better discrimination
This inhibitor provides for better discrimination between intestinal and placental isoenzymes.

In the following paper we will show how this new inhibitor has been coupled with other selective inhibition techniques to quantitate all four major serum alkaline phosphatase isoenzymes in patient specimens, develop age and sex related reference ranges, and show its usefulness in a variety of clinical situations.

**References**

1 Birkett DJ, Conyers RAJ, Neale FC, Posen S, Brudenell-Woods J. Action of urea on human alkaline phosphatases with a description of some automated techniques for the study of enzyme kinetics. *Arch Biochem Biophys* 1967;**121**:470–9.
2 Bahr M, Wilkinson JH. Urea as a selective inhibitor of human tissue alkaline phosphatases. *Clin Chim Acta* 1967;**17**:367–70.
3 Statland BE, Nishi H, Young DS. Serum alkaline phosphatase. Total activity and isoenzyme determinations made by use of the centrifugal fast analyser. *Clin Chem* 1972;**18**:1468–74.
4 O'Carroll D, Statland BE, Steele BW, Burke MD. Chemical inhibition method for alkaline phosphatase isoenzymes in human serum. *Am J Clin Pathol* 1975;**63**:564–72.
5 Buttery JE, Milner CR, Nenadovic P, Pannal PR. Detection of alkaline phosphatase/immunoglobulin complexes. *Clin Chem* 1980;**26**:1620–1.
6 Moss DW. Alkaline phosphatase isoenzymes. *Clin Chem* 1982;**28**:2007–16.
7 Rosalki SB, Foo AY. Two new methods for separating and quantifying bone and liver alkaline phosphatase isoenzymes in plasma. *Clin Chem* 1984;**30**:1182–6.
8 Walker AW, Pollard AC. Observations of serum alkaline phosphatase electrophoretic patterns on polyacrylamide gel with particular reference to the effects of butanol extraction. *Clin Chim Acta* 1971;**34**:19–29.
9 Horne M, Cornish CJ, Posen S. The use of urea denaturation in the identification of human alkaline phosphatases. *J Lab Clin Med* 1968;**72**:905–15.

Requests for reprints to: Mr MDS Shephard, Department of Biochemistry and Chemical Pathology, Flinders Medical Centre, Bedford Park, South Australia 5042, Australia.