Application of a Continuous Spectrophotometric Assay for 5'Nucleotidase Activity in Normal Subjects and Patients with Liver and Bone Disease

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Serum 5'nucleotidase activity has been measured by a coupled kinetic assay in which adenosine formed by hydrolysis of adenosine 5'-monophosphate in the presence of 150-fold excess of β-glycerophosphate is converted to inosine by adenosine deaminase, with a consequent decrease in absorbance at 265 nm. The method gives activity in proportion to enzyme concentration so long as the rate of decrease of absorbance at 265 nm does not exceed 0.025/min, and not more than 30% of the substrate is consumed. The normal range established in 517 healthy adults was 0–15 mU/ml. A significant correlation between enzyme activity and age was found in females but not in males. Raised levels of 5'nucleotidase activity were found in 92% of patients with obstructive jaundice, 70% of patients with parenchymal liver disease, 81% of patients with hepatic metastases, and 11% of patients with bone disease. The estimation is useful in aiding the elucidation of raised serum alkaline phosphatase activity, and is of value as a liver function test, but is not as frequently increased as alkaline phosphatase in all classes of hepatobiliary disease.

Human serum is capable of hydrolyzing adenosine 5'-monophosphate (5'AMP) to adenosine and inorganic phosphate at alkaline pH as a result of the action of two distinct enzymes. The first, alkaline phosphatase (EC 3.1.3.1, orthophosphoric monoester phosphohydrolase, APase) splits a wide range of monophosphates and is elevated in many diseases affecting the bones and the hepatobiliary system. The other, 5'nucleotidase (EC 3.1.2.5, 5'-ribonucleotide phosphorylase, 5Nase) is specific for nucleoside monophosphates (1) and is raised in diseases of the hepatobiliary system, but seldom in diseases of bone. It has, therefore, been recommended for elucidating the origin of raised serum

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APase activity (2, 3) and is preferred to the latter as a routine test of liver function by some investigators (4, 5). The 5Nase is especially useful in children and adolescents whose values, unlike those of APase, do not differ from normal adults (6).

To estimate 5Nase activity, the hydrolysis of 5'AMP by APase must be suppressed, or deducted from the total hydrolysis of 5'AMP by serum. Attempts to realize these aims have met with variable success (2, 7–10) and have the disadvantage of employing measurement of phosphorus in test and blank at the end of an arbitrary incubation period.

In a preliminary study, we showed that the liberation of adenosine could be measured kinetically using adenosine deaminase as a marker enzyme, and that addition of β-glycerophosphate suppressed the hydrolysis of 5'AMP by APase of tissues and serum (11). We now report the application of these technics to the assay of 5Nase activity in the serum of normal subjects and of patients with diseases of bone, and of the hepatobiliary system.

**Clinical Material**

Serums were obtained from adults with jaundice or with raised APase activity. A total of 236 patients were examined, and fell into the following categories as defined by previously described criteria (10):

1. *Extrahepatic obstruction* (48 patients). These were subdivided into 18 with malignant obstruction without hepatic metastases; 13 with obstruction due to stones diagnosed clinically and radiologically, plus 7 diagnosed surgically; and 10 with acute cholecystitis with jaundice.

2. *Parenchymatous liver disease* (76 patients). These were subdivided into 31 with acute hepatitis; 6 with portal cirrhosis diagnosed clinically and radiologically; 20 diagnosed histologically; 10 with primary biliary cirrhosis; and 9 other miscellaneous conditions.

3. *Bone disease* (54 patients). These were subdivided into 25 with Paget's disease; 16 with metastatic bone cancer; and 13 with miscellaneous bone diseases, including osteomalacia and hyperparathyroidism.


5. *Reticulosis* (19 patients). These were subdivided into 5 patients with jaundice; 8 with hepatomegaly but no jaundice; and 6 with no evidence of liver disease.

Estimations were carried out on 7 subjects in whom a final diagnosis could not be made, and were, therefore, not considered further. A
normal series was provided by 517 healthy individuals undergoing routine examination including measurement of the following biochemical parameters in serum: urea, uric acid, APase, inorganic phosphate, cholesterol, total protein, calcium, magnesium, aspartate and alanine aminotransferase, creatinine phosphokinase, and hydroxybutyrate dehydrogenase.

All serums were examined within 4 days of storage at 4° or within 18 weeks of storage at −20°. No loss of 5Nase activity occurred in 30 serums maintained under the former conditions; under the latter, activity tended to decrease slightly but was never less than 92.5% of the original in 20 serums tested. Serum APase activity was determined by the method of Kind and King (12) as adapted for the AutoAnalyzer (Technicon Corp.) with the normal adult range in our laboratory being 3–15 King Units.

**Estimation of 5Nase Activity**

**Principle**

Adenosine liberated by hydrolysis of 5'AMP is converted to inosine by adenosine deaminase present in 300-fold excess relative to the highest nucleotidase activity expected. This causes a decrease of absorbance at 265 nm which is linear with time and with the adenosine produced, or the enzyme activity of the added serum keeping within the rate of 0–0.025 A units/min and 0–30% consumption of substrate.

\[
5'AMP + H_2O \xrightarrow{5'Nucleotidase} \text{Adenosine} + \text{Pi} \\
\text{Adenosine} + H_2O \xrightarrow{\text{Adenosine deaminase}} \text{Inosine} + \text{NH}_3
\]

In the presence of β-glycerophosphate, hydrolysis of 5'AMP by APase is progressively reduced and ceases when the concentration of the former is 150-fold that of 5'AMP (11).

1. 0.1 M Magnesium chloride prepared by dissolving 403 mg ‘Analar’ magnesium oxide in the minimum volume 1 N HCl and adjusting to 100 ml.

**Reagents**

2. 0.1 M Tris-HCl buffer, pH 7.9.
3. 0.041 M sodium β-glycerophosphate.
4. Calf intestinal adenosine deaminase. 2 mg protein/ml (Boehringer) freshly diluted 1 in 15 with buffer.
5. 0.01 M 5'AMP in water.
6. 0.01 M Inosine in water.
Method

To a silica glass cuvet with a 1 cm light path were added 1.5 ml buffer, 1.1 ml β-glycerophosphate, 0.3 ml MgCl₂, 30 µl 5′AMP, and 50 µl adenosine deaminase. The reference cuvet substituted inosine for 5′AMP. The contents were allowed to reached 37° in an SP 800 spectrophotometer with SP 20 external recorder and range expander (Unicam Instruments, Cambridge, England). The reaction was initiated by adding 20 µl serum to reference and test. The initial reaction rate was measured as the fall in absorbance at 265 nm over a period of 20 min. This was converted to nmole AMP hydrolyzed/min/ml serum using the observed difference in extinction coefficients of AMP and inosine of 8.0 × 10⁶ cm² mole⁻¹ under the assay conditions; ie

\[ ΔA_{265}/\text{min} \times 18,760 = \text{mU/ml}. \]

It is permissible to omit the reference cuvet with serums that are neither turbid nor jaundiced, and to read against air or water using the backoff control to bring the recorder pen to a suitable starting position. Where this facility is lacking, a solution of inosine or dichromate may be used in the reference side. Under these conditions, serum is present from the outset, and when the A₂₆₅ has stabilized, 5′AMP is added to start the reaction. With serums of low activity, or with large batches, the incubation mixture may be prewarmed to 37° and A₂₆₅ read immediately after addition of serum; the contents can then be returned to a waterbath and A₂₆₅ read once more after 50 min. A manual UV spectrophotometer is suitable for this technic, and best results are obtained if readings are taken at 37° instead of the IUB recommendation of 30°. A linear reaction rate can be assumed, provided the limits previously described are not exceeded. It is important to ensure scrupulous cleanliness of glassware during transfer, and to guard against volume changes by evaporation. The reproducibility of the method as gauged by 18 replicate analyses on the same sample gave a mean and Standard Error of 31.0 ± 0.6.

Results

Normal Subjects

The mean 5Nase activity for 250 males was 5.4 mU/ml with a SD of ±4.4 and an observed range of 0–24. The mean activity for 267 females was 5.0 mU/ml with a SD of ±4.8 and an observed range of 0–25. Only 18 (3.5%) of the 517 subjects exceeded 15 mU/ml which may be regarded as the upper-normal limit.

A significant correlation of serum 5Nase activity with age was found in females (R = 0.218, p < 0.01) as shown in Table 1. In females, significant correlations were also found between 5Nase activity and
serum cholesterol (R = 0.141, p < 0.05), APase (R = 0.173, p < 0.05), alanine aminotransferase (R = 0.140, p < 0.05), and calcium (R = 0.185, p < 0.01). Poor correlation existed with all other biochemical parameters tested, as well as blood pressure, weight, and percentage overweight. In the males, 5Nase was significantly related to blood pressure (R = 0.175, p < 0.05) and to serum APase (R = 0.178, p < 0.05), but to no other parameter.

Abnormal Subjects

The detailed results are presented in Table 2. Few patients had serum APase levels within the normal range; the highest incidence of

<table>
<thead>
<tr>
<th>Male</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>Number</td>
</tr>
<tr>
<td>10-19</td>
<td>6</td>
</tr>
<tr>
<td>20-29</td>
<td>50</td>
</tr>
<tr>
<td>30-39</td>
<td>40</td>
</tr>
<tr>
<td>40-49</td>
<td>59</td>
</tr>
<tr>
<td>50-59</td>
<td>50</td>
</tr>
<tr>
<td>60-69</td>
<td>36</td>
</tr>
<tr>
<td>70-79</td>
<td>9</td>
</tr>
</tbody>
</table>

Table 2. Serum APase and 5Nase Activities in Disease States

<table>
<thead>
<tr>
<th>Clinical State</th>
<th>APase (Kmg Units/100 ml)</th>
<th>5Nase (mU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td>Obstruction (48)*</td>
<td>73 ± 31</td>
<td>16-138</td>
</tr>
<tr>
<td>Malignant (18)</td>
<td>72 ± 28</td>
<td>25-114</td>
</tr>
<tr>
<td>Clinical lithiasis (13)</td>
<td>59 ± 38</td>
<td>16-138</td>
</tr>
<tr>
<td>Operative lithiasis (7)</td>
<td>73 ± 22</td>
<td>60-120</td>
</tr>
<tr>
<td>Cholycystitis (10)</td>
<td>83 ± 34</td>
<td>30-136</td>
</tr>
<tr>
<td>Liver disease (76)</td>
<td>47 ± 39</td>
<td>8-216</td>
</tr>
<tr>
<td>Hepatitis (31)</td>
<td>32 ± 23</td>
<td>8-100</td>
</tr>
<tr>
<td>Clinical portal cirrhosis (6)</td>
<td>65 ± 77</td>
<td>20-216</td>
</tr>
<tr>
<td>Histologic portal cirrhosis (20)</td>
<td>31 ± 21</td>
<td>11-100</td>
</tr>
<tr>
<td>Primary biliary cirrhosis (10)</td>
<td>79 ± 28</td>
<td>36-135</td>
</tr>
<tr>
<td>Miscellaneous (9)</td>
<td>60 ± 49</td>
<td>21-139</td>
</tr>
<tr>
<td>Bone Disease (54)</td>
<td>82 ± 82</td>
<td>9-390</td>
</tr>
<tr>
<td>Metastases (16)</td>
<td>106 ± 105</td>
<td>26-390</td>
</tr>
<tr>
<td>Paget's disease (25)</td>
<td>91 ± 79</td>
<td>13-333</td>
</tr>
<tr>
<td>Miscellaneous (13)</td>
<td>37 ± 10</td>
<td>9-50</td>
</tr>
<tr>
<td>Hepatic metastases (32)</td>
<td>81 ± 53</td>
<td>9-270</td>
</tr>
<tr>
<td>Reticulosis (19)</td>
<td>61 ± 30</td>
<td>22-130</td>
</tr>
<tr>
<td>Nonhepatic (6)</td>
<td>37 ± 17</td>
<td>22-69</td>
</tr>
<tr>
<td>Hepatic (8)</td>
<td>71 ± 27</td>
<td>40-100</td>
</tr>
<tr>
<td>Jaundiced (5)</td>
<td>72 ± 33</td>
<td>50-130</td>
</tr>
</tbody>
</table>

* No. of subjects is in parentheses.
normal serum APase was in patients with parenchymal liver disease, with the normal values restricted to patients with hepatitis or histologically proven portal cirrhosis.

Serum 5Nase activity was increased in 90% of patients with extrahepatic obstruction; and most normal values occurred in patients with clinically diagnosed cholelithiasis. By contrast, raised levels were seldom seen in bone disease, with metastatic bone disease showing the highest incidence. More than half the patients with reticulosis had raised 5Nase activity although this did not correlate with the presence or degree of hepatic involvement. In all, two-thirds of patients with parenchymal liver disease had raised values; normal levels were found frequently in patients with hepatitis or cirrhosis. More than 80% of patients with hepatic metastases had elevated serum 5Nase activity. In one patient, the repeatedly checked value was 800 mU/ml. When this case was omitted, the mean, SD, and range for the group became 43, 29, and 0–122 respectively.

Discussion

The method presented is rapid and requires half the time necessary for assays based on phosphorus determination. Accuracy is enhanced by reading test against blank. The pH of assay and Mg++ concentration are optimal for serum 5Nase (10). Adaptability is assured, since the reaction can be followed during a period appropriate to the activity being measured, and it is possible to handle a number of samples as a "batch."

Previous normal ranges for serum 5Nase activity have been determined on very small samples (2–5, 13, 14). The present series advances our knowledge of the behavior of this enzyme in the normal subject. A significant relationship between 5Nase and age was found in the female but not in the male. A similar correlation with respect to APase has previously been described (15) and was confirmed in our data where the values for R were 0.409 (p < 0.001) in females and 0.074 in males. In both sexes there was a relationship between 5Nase and APase, significant at the 5% level, and independent of age. The origin of normal serum APase is controversial, and is believed to be liver (16) or intestine (17). The origin of serum 5Nase is not known. Bone has 4× as much 5Nase as liver (18). It would not be surprising if both tissues contributed to the normal serum level; that this may be so in females is suggested by the correlation between 5Nase and calcium (R = 0.185, p < 0.01) as well as with alanine aminotransferase (R = 0.140, p < 0.05). The correlation between 5Nase and cholesterol in females was shown by statistical analysis to be due to the influence
of age on both, but we have no explanation for the correlation between 5Nase and systolic blood pressure in males.

Previous authors with one exception (19) have found the estimation of 5Nase activity to be of clinical value, although there is disagreement on the appropriate status for the investigation within a laboratory service. Some consider it superior to APase as a test of liver function (4, 5) while others would restrict its application to children and adolescents (6). It has been reported to be a more sensitive index of biliary obstruction (20, 21) and of hepatic metastases (22) than APase, though our data do not support these conclusions. Its superiority over APase in the diagnosis of pericholangitis complicating ulcerative colitis has also been claimed (23); we have seen only one patient with this condition, and in this instance, both enzymes were consistently above 100 units. Young (7) was impressed by the fact that 5Nase was much less frequently increased than APase in his patients with parenchymatous liver disease and considered the former estimation more valuable in discriminating between medical and surgical jaundice. Subsequent reports are not in accord with this view (5, 14, 20) and describe raised levels of 5Nase in greater than 90% of patients with hepatitis or cirrhosis. Our results are closer to those of Young (7), but we do not find serum 5Nase levels as reliable as serum adenosine deaminase and transaminase ratios (24, 25) in distinguishing the two main categories of jaundice.

The levels of both 5Nase and APase were higher in the subjects with portal cirrhosis without liver biopsy than in those in whom a biopsy specimen was obtained (Table 2). Both enzymes were lower in patients with cholelithiasis who were not operated upon than in those who were. This latter difference may reflect the greater severity and duration of obstruction in cases subsequently relieved at operation, but it is possible that the differences between the two portal cirrhosis groups and the two cholelithiasis groups reflect an element of inaccuracy in the purely clinical diagnosis—hence our decision to present the subgroups independently.

The incidence of raised 5Nase and APase levels in cases of reticulosis without evidence of hepatic involvement is intriguing. We have previously observed a closer correlation between the two enzymes in such patients than in reticuloses complicated by hepatic involvement or jaundice (10). This may reflect a high incidence of subclinical hepatic involvement in the reticuloses, though bone involvement would also explain the raised APase. The demonstration of raised leucocyte APase in Hodgkin's disease (26, 27) raises yet another possibility and would justify study of the relationship between the two enzymes in these cells.
The present series shows an overall incidence of 11% in bone disease with raised 5Nase activity (Table 2). Metastatic bone disease accounts for a disproportionate number of these, and as the three patients in question have not come to autopsy, the possibility of subclinical hepatic metastases cannot be excluded. The infrequency of raised serum 5Nase activity in bone disease has impressed previous authors, and its value in excluding liver disease in patients with raised APase is widely agreed (2–7, 10, 20). Alternative methods of gaining this information include isoenzyme electrophoresis (28) which is time-consuming, imprecise, and often disappointing as a routine procedure (10, 29). Study of heat stability (30) and l-phenylalanine sensitivity (17) are of considerable value in solving this problem. Our results indicate that with the method herein described, the distinction between liver disease and bone disease as the cause of raised serum APase activity can often be made, with the advantage that where raised, further insight may be gained into the type of liver disease concerned (6).

References


