Routine determination of 5′ nucleotidase activity of human serum using the LKB 8600 reaction rate analyser

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We have described a method (Ellis, Belfield, and Goldberg, 1970) for assaying the activity of 5′ nucleotidase (EC 3.1.3.5; 5′-ribonucleotide phosphohydrolase; 5Nase) in human serum based upon the following linked reactions:

\[
\begin{align*}
5′\text{AMP} & \xrightarrow{\text{5Nase}} \text{Adenosine} + \pi \\
\text{Adenosine} & \xrightarrow{\text{Deaminase}} \text{Inosine} + \text{NH}_4^+ \\
\text{NH}_4^+ + 2\text{-oxoglutarate} & \xrightarrow{\text{Dehydrogenase}} \text{L-glutamate} \\
\text{NADH} & \xrightarrow{\text{Dehydrogenase}} \text{NAD}^+ 
\end{align*}
\]

The overall reaction is monitored by the fall in \text{E}_{240} consequent upon oxidation of NADH, and \text{β}-glycerophosphate is present in large excess to prevent hydrolysis of 5′AMP by non-specific phosphatases. We also indicated the feasibility of employing automatic rate analysis to measure this reaction (Goldberg, Ellis, and Wilcock, 1971). The chemistry and technology of the method have now been improved (Ellis and Goldberg, 1972a). This paper describes the system operating in our laboratory to provide a routine automated 5Nase assay.

We use the LKB reaction rate analyser (LKB Instruments, Croydon, Surrey) at 37°C, modified to include an on/off switch for pump and stirring motor (Ellis and Goldberg, 1972b). The following are added to a final volume of 1 litre adjusted to pH 7.2 at 37°C: 22.9 g triethanolamine HCl (Sigma, cat. no. T-1502), 29 g NaCl, 361 mg 2-oxoglutarate (Boehringer, cat. no. 15673 SKAA), 19.5 g sodium \text{β}-glycerophosphate (Sigma, Gd. I, cat. no. G6251). The mixture is stored at −20°C in aliquots. To each 2.415 ml of the above are added daily 30 μl 100 mM MnCl₂, 50 μl glutamate dehydrogenase in 50% glycerol (GDH, Boehringer, cat. no. 15324 EGAH), 50 μl NADH disodium salt (Boehringer, cat. no. 15142 CNAB) 10 mg per ml water prepared fresh daily, and 5 μl adenosine deaminase in 50% glycerol (Boehringer, cat. no. 15069 EAAT). Of the final mixture 0.85 ml and 100 μl serum are dispensed into plastic cuvettes which are loaded into the cuvette block and incubated in a specially designed close-fitting water-tight stainless steel container suspended in a water bath at 37°C. Thirty to forty minutes are allowed for consumption of endogenous ammonia and the cuvettes are automatically fed through the instrument with the pump and stirring motors off and a one-minute programme to measure the blank, the slider previously having been set to a value of 0.7. The cuvette block is replaced in the heating tunnel and after allowing three minutes for the temperature to equilibrate the cuvettes are re-cycled by rapid manual feeding with the pump and stirring motors on. During this process 50 μl 20 mM 5′AMP is added to each cuvette. The reaction is not monitored at this stage because the initial lag period is wasteful of machine time, but the blanking peaks are inspected to ensure that NADH consumption by endogenous ammonia has not been excessive. The cuvettes are re-cycled for a final run with pump and stirring motors off and an automatic one-minute reading programme to measure the reaction rate. This is linear provided the readings are taken three to 13 minutes after adding 5′AMP, and 5Nase activity is not excessively high.

A plastic template is used to convert the slope of the test and blank run to International Units per litre (IU/l) and true enzyme activity obtained by difference. The limiting rate is an absorbance fall of 0.025 per minute corresponding to 40 IU/l. Samples with high activity may oxidize so much NADH before activity is recorded that the absorbance lies outside the limits of 0.7 to 1.8 imposed by the slider setting. This will be reflected in the position of the blanking peak and no effective trace will be obtained. It is therefore advisable to place samples suspected to have high 5Nase activity in the first positions of the cuvette block. We also recommend the running of a reagent blank in which water replaces serum at the beginning of each batch, as a precaution against contamination of the substrate by adenosine.

Figure 1 demonstrates typical traces of tests and blanks during a routine run. Data indicating within-batch precision are presented in Table I. Carryover was assessed in the recommended manner (Laboratory Equipment and Methods Advisory Group 1969) and the results showed that carryover was not significantly different from the within-batch variation.

Finally, the normal range was determined by analysis of sera from 65 healthy laboratory staff aged 16 to 60 years. There were equal numbers of males and females in this series. Sera from 30 patients attending an outpatient thyroid clinic, who were...
Fig. 1 Recorder traces obtained during routine 5Nase assays with LKB 8600 reaction rate analyser. Chart speed 50 mm per minute and full scale deflection 0.05 absorbance. Each serum sample is labelled to left of its blanking peak during blank determination (above) and test determination (below). Blanking peaks are higher during test determination, reflecting consumption of NADH during addition of substrate, temperature equilibration, and re-cycling. The blanking peak for sample F during both blank and test runs was off limits (E<sub>440</sub> < 0.700) due to excessive amount of endogenous ammonia in sample and no reaction trace was obtained; this would normally be corrected by adding more NADH and rechecking blank before re-cycling. The activities (IU/l) of the other samples (test-blank) were as follows: A = 39; B = 5; C = 5; D = 21; E = 6.
Present day practice

Sample | Mean (IU/l) | SD (IU/l) | Coefficient of Variation (%)
--- | --- | --- | ---
**First**<br>Test | 50·1 | 2·7 | 5·4
Blank | 4·9 | 0·9 | 19·0
Test-blank | 45·3 | 2·9 | 6·3
**Second**<br>Test | 27·4 | 0·9 | 3·4
Blank | 2·7 | 1·0 | 39·6
Test-blank | 25·1 | 1·3 | 5·0
**Third**<br>Test | 12·8 | 1·3 | 10·2
Blank | 0·7 | 0·6 | 86·0
Test-blank | 12·1 | 1·3 | 10·7

Table I Within-batch precision of 5'-nucleotidase assay based on 10 determinations of each sample

Clinically euthyroid and had normal values for PBI and 12 determinations on the SMA 12/60 (Technicon Instruments Ltd) were also analysed, and the results are presented in Table II. In practice the upper limit of normal is taken as 11 IU/l.

Table II Normal range for 5'-nucleotidase activity (IU/l at 37°C)

<table>
<thead>
<tr>
<th>Population</th>
<th>Number</th>
<th>Mean</th>
<th>SD</th>
<th>Mean + 2 SD</th>
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<td>Laboratory staff</td>
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<td>7·7</td>
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<tr>
<td>Euthyroid patients</td>
<td>30</td>
<td>6·0</td>
<td>2·25</td>
<td>10·5</td>
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References


