Inhibition by Concanavalin A as the Basis for a Specific Assay of Serum 5'-Nucleotidase Activity

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Concanavalin A inhibits serum 5'-nucleotidase activity, without causing significant inhibition of alkaline phosphatase activity. This observation serves as the basis for a new method for assaying the 5'-nucleotidase activity in serum, which depends upon the difference between the enzymic hydrolysis of adenosine-5'-monophosphate in the presence and absence of concanavalin A. Adenosine released by the 5'-nucleotidase reaction is deaminated by a coupled reaction with adenosine deaminase to liberate inosine and ammonia, and ammonia is measured colorimetrically by the Berthelot reaction. In sera from 40 healthy adult persons, 5'-nucleotidase activity averaged 6.4 U/liter (SD, ±2.0; range, 3–12). In sera from 100 patients, measurements of 5'-nucleotidase activity by the new assay averaged 8% lower than by a generally accepted method in which phenyl phosphate is used to suppress hydrolysis of adenosine-5'-monophosphate by alkaline phosphatase activity. The clinical validity of the new assay was tested by measuring serum 5'-nucleotidase activities in rats with bile duct ligation and in rats treated with thiocetamide to induce hepatocellular injury.

Additional Keyphrases: alkaline phosphatase • differentiation of hepatobiliary and osseous disease • optimal conditions • normal values • suggested reference method

5'-Nucleotidase (5'-N; EC 3.1.3.5; 5'-ribonucleotide phosphohydrolase) activity was first described by Reis (1) in 1934, based upon the presence in muscle homogenates of an enzyme that specifically hydrolyzes adenosine-5'-monophosphate (5'-AMP)⁴ to release inorganic phosphate at pH 7, but does not hydrolyze adenosine-3'-monophosphate under the same reaction conditions. The presence of 5'-N activity in human serum was first reported by Dixon and Purdom (2) in 1954. These workers observed that serum 5'-N assays are clinically useful for differential diagnosis of hepatobiliary and osseous diseases. They found that 5'-N activity is increased only in hepatobiliary diseases, whereas serum alkaline phosphatase (AP; EC 3.1.3.1; orthophosphoric monoester phosphohydrolase) activity is increased in diseases of both the hepatobiliary and osseous systems, as well as during childhood, adolescence, and pregnancy. These observations have been confirmed by numerous investigators (3–11). The clinical interpretation and diagnostic significance of serum 5'-N assays have been summarized in several authoritative reviews (12–17).

Techniques for assay of serum 5'-N activity may be classified in six primary categories.

Category A comprises radioisotopic procedures in which the 5'-AMP substrate is labeled with ¹⁴C, ³H, or ³²P, and the labeled reaction products are separated from the labeled substrate by ion-exchange chromatography, coprecipitation on barium sulfate, or adsorption on Norit (18–21).

Category B comprises procedures in which 5'-N hydrolyzes 5'-AMP to produce adenosine, which is deaminated by adenosine deaminase (ADA; EC 3.5.4.4) to yield inosine and ammonia. These coupled enzymic reactions are measured kinetically by recording the decrease in absorbance of adenosine at 265 nm (5, 22–25).

Category C comprises procedures in which the 5'-N and ADA reactions are coupled with the NADH-dependent glutamate dehydrogenase (EC 1.4.1.2) reaction, so that ammonia produced by the ADA reaction com-

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⁴ Nonstandard abbreviations used: ADA, adenosine deaminase; 5'-AMP, adenosine-5'-monophosphate; AP, alkaline phosphatase; con A, concanavalin A; MADP, α,β-methyleneadenosine-5'-diphosphate; and 5'-N, 5'-nucleotidase
bines with 2-oxoglutarate to yield L-glutamate. These three coupled enzymic reactions are measured kinetically by recording the decrease in absorbance of NADH at 340 nm (26–29).

Category D comprises procedures in which inorganic phosphorus that is released from 5'-AMP by 5'-N is measured colorimetrically by the Fiske–SubbaRow reaction (13, 22, 30–34). Category E comprises procedures in which 5'-AMP is hydrolyzed and deaminated by the coupled 5'-N and ADA reactions to yield ammonia, which is measured colorimetrically by the Berthelot reaction (35–40).

Category F is basically similar to category E, except that cytosine-5'-monophosphate is substituted for 5'-AMP as the substrate, and cytosine deaminase (EC 3.5.4.1) is substituted for ADA as the second enzyme in the coupled reactions (41).

In applications of all these techniques for assays of 5'-N activity in serum, there is the troublesome problem of correcting, inhibiting, or otherwise compensating for nonspecific hydrolysis of the substrate by serum AP activity. There have been four major approaches to solving this problem: First, nonspecific hydrolysis of 5'-AMP by AP can be largely suppressed by adding an excess of a preferential AP substrate such as β-glycerophosphate, phenyl phosphate, or p-nitrophenyl phosphate (18, 26, 37, 42–45). Second, nonspecific hydrolysis of 5'-AMP by AP can be partially compensated for by simultaneous assay of AP, with use of an empirical correction factor (24), or by use of a correction factor derived by simultaneous assay of a control cuvet that contains as a substrate a mixture of adenosine-2'-monophosphate and adenosine-3'-monophosphate (22). Third, amino acids (e.g., L-histidine or L-cysteine) can be added to the reaction mixture in an endeavor to inhibit hydrolysis of 5'-AMP by AP (12, 31). Fourth, a nonspecific inhibitor of 5'-N activity (for example, Ni²⁺) can be used to differentiate the hydrolysis of 5'-AMP by AP and 5'-N (13, 32, 34, 46, 47). The controversy concerning the relative merits of these four approaches and doubts about the specificity of the various analytical techniques (3, 10, 12, 17, 34, 44, 48–50) have discouraged many clinical chemists from performing measurements of serum 5'-N activity.

α,β-Methyleneadenosine-5'-diphosphate (MADP) and concanavalin A (con A) reportedly inhibit 5'-N activity in subcellular components of tissue homogenates (19, 45, 51–56). These compounds have not heretofore been tested as inhibitors of 5'-N activity in serum. As will be described in this paper, we have evaluated the use of MADP and con A in order to differentiate specific and nonspecific hydrolysis of 5'-AMP. We have found that MADP is not suitable for this purpose, since it inhibits hydrolysis of 5'-AMP by serum AP as well as by serum 5'-N. On the other hand, we have found that con A is entirely satisfactory as a reagent for specific differentiation of hydrolysis of 5'-AMP by serum AP and serum 5'-N. Moreover, we have observed that a concentration of con A that completely inhibits serum 5'-N activity inhibits erythrocyte 5'-N activity by only a third. Therefore, use of con A as a differential inhibitor in assays of serum 5'-N reduces the artifacts interference that is caused by hemolysis. In the present paper, we describe the use of con A as an inhibitor for determination of serum 5'-N activity in an assay system that is based upon the method of Persijn et al. (38, 39, 43). We anticipate that con A may also be suitable as an inhibitor in many of the other published procedures for analysis of serum 5'-N activity.

**Methods and Materials**

**Assay of Serum 5'-Nucleotidase**

**Principle**

Serum 5'-N catalyzes hydrolysis of adenosine-5'-monophosphate in the presence of Mg²⁺ to yield adenosine and inorganic phosphorus:

\[
5'-N - H_2O \rightarrow \text{adenosine} + P_i
\]

Adenosine produced by reaction 1 is deaminated by coupled reaction with ADA to yield inosine and ammonia:

\[
\text{Adenosine} + H_2O \rightarrow \text{inosine} + \text{NH}_4\text{OH}
\]

Reactions 1 and 2 are performed in barbital buffer at pH 7.2. Hydrolysis of 5'-AMP by serum AP is differentiated from serum 5'-N activity by simultaneous assays in the presence and absence of con A. The concentrations of the components of the enzymic reaction mixture are specified in Table 1.

Ammonia that is produced by reaction 2 is reacted with Berthelot's reagents (phenol/nitroprusside solution and alkaline hypochlorite solution) to yield indophenol. Interference of Mg²⁺ in the Berthelot reaction is prevented by addition of ethylenediaminetetraacetic acid. The blue color of indophenol is measured by spectrophotometry at 630 nm. Serum 5'-N activity is proportional to the difference between the absorbances that develop after Berthelot's reagents are added to two enzyme reaction mixtures that have been incubated respectively in the presence and absence of con A. Serum 5'-N activity (in U/liter) is computed by reference to the absorbance that develops with a standard solution of adenosine.

**Reagents**

**Adenosine deaminase stock solution.** A 1 MU/liter solution containing ADA from calf intestine, dissolved in equal volumes of glycerol and water, is purchased from Biodynamics/bmc, Indianapolis, Ind. 46250. The manufacturer specifies that contaminations with AP, 5'-N, and nucleotide phosphohydrolase (EC 3.1.3.31) activities are each less than 0.01%.

**Concanavalin A stock solution.** A solution containing con A from *Canavalia ensiformis* is purchased from Miles Laboratories, Inc., Elkhart, Ind. 46514. The solution contains 40 g of con A per liter, dissolved in saturated NaCl solution. The manufacturer specifies that the con
A has been twice recrystallized, and has been standardized on the basis of its absorptivity \( A_{280nm}^{\%} = 13.0 \).

Adenosine standard solution (3.74 mmol/liter). Crystalline adenosine, 100 mg, and 150 mg of benzoic acid are weighed into a 100-ml volumetric flask, dissolved in water, and diluted to 100 ml with water. This solution can be used even after storage for several months at 4 °C.

Barbital buffer/Mg\(^{2+}\) solution. Sodium diethylbarbital, 4.2 g, and 6.3 g of magnesium sulfate (MgSO\(_4\) 7H\(_2\)O) are dissolved in distilled water in a 1-liter volumetric flask. The solution is adjusted to pH 7.2 with HCl solution (1 mol/liter), and diluted to 1 liter with water. This solution can be stored at 4 °C for several months, but the pH should be checked before use and adjusted if necessary to pH 7.2.

ADA/Mg\(^{2+}\)-buffer solution. ADA stock solution, 0.1 ml, is added to 150 ml of barbital buffer/Mg\(^{2+}\) solution. This solution is stable for two weeks at 4 °C.

5'-AMP/ADA/Mg\(^{2+}\)-buffer solution. Exactly 62.5 mg of sodium adenosine-5'-monophosphate (C\(_{10}\)H\(_{12}\)N\(_5\)O\(_{7}\)PNa\(_2\).6 H\(_2\)O) is weighed into a 25-ml volumetric flask and dissolved in ADA/Mg\(^{2+}\)-buffer solution. This solution is prepared immediately before use. The solution is placed in a water bath at 37 °C. After equilibration, the solution is checked with a pH meter and is adjusted to pH 7.2 with NaOH or HCl solutions (1 mol/liter).

5'-AMP/ADA/Mg\(^{2+}\)-buffer/con A solution. Con A stock solution (2.5 ml) is placed in a 25-ml volumetric flask that contains exactly 62.5 mg of sodium adenosine-5'-monophosphate. The contents are diluted to 25 ml with ADA/Mg\(^{2+}\)-buffer solution. This solution is prepared immediately before use. The solution is placed in a water bath at 37 °C. After temperature equilibration, the solution is checked with a pH meter and is adjusted to pH 7.2 with NaOH or HCl solutions (1 mol/liter).

Phenol/nitroprusside stock solution. Crystalline phenol, 50 g, and 250 mg of sodium nitroprusside are dissolved in water and diluted to 1 liter. This solution is placed in an amber-colored bottle and can be stored for two months at 4 °C.

Ethylendiaminetetraacetic acid stock solution. Dipotassium ethylendiaminetetraacetate (C\(_{10}\)H\(_{14}\)N\(_3\)O\(_8\)K\(_2\)O\(_2\).2 H\(_2\)O), 5.6 g, is dissolved in water and diluted to 50 ml. This solution can be stored for several months at 4 °C.

**Phenol/nitroprusside/ethylenediaminetetraacetic acid working solution.** Into a 100-ml volumetric flask are placed 20 ml of phenol/nitroprusside stock solution and 2 ml of ethylendiaminetetraacetic acid stock solution. The contents of the flask are diluted to 100 ml with water. This solution is prepared immediately before use.

**Alkaline hypochlorite stock solution.** Twenty five grams of sodium hydroxide pellets and 72 ml of sodium hypochlorite reagent solution (52.5 g/liter; Fisher Scientific Co., Pittsburgh, Pa. 15238) are dissolved in water and diluted to 1 liter. The alkaline hypochlorite solution is placed in an amber-colored bottle and can be stored for two months at 4 °C.

**Alkaline hypochlorite working solution.** Twenty milliliters of alkaline hypochlorite stock solution is diluted to 100 ml with water. This solution is prepared immediately before use.

**\( a,\beta\)-Methyladenosine-5'diphosphate (MADP) solution.** For evaluation of MADP as a differential inhibitor of serum 5'-N and AP activities, 0.2 g of \( a,\beta\)-methyladenosine-5'-diphosphate (Miles Laboratories, Inc.) was dissolved in 25 ml of 5'-AMP/ADA/Mg\(^{2+}\)-buffer solution immediately before use.

**Procedures**

**Procedure for 5'-N assay.** To a series of glass-stoppered Pyrex test tubes, placed in a water bath at 37 °C, is added 0.5 ml of either (a) 5'-AMP/ADA/Mg\(^{2+}\)-buffer solution, or (b) 5'-AMP/ADA/Mg\(^{2+}\)-buffer/con A solution, as listed in Table 2. To the specified tubes is added 50 \( \mu \)l of serum, water (blank), or adenosine solution (standard). The contents of the tubes are mixed and the tubes are stopped. After the tubes have been incubated in the water bath at 37 °C for exactly 60 min, 2.5 ml of phenol/nitroprusside/ethylenediaminetetraacetic acid working solution is added to each tube. Immediately after mixing, 2.5 ml of alkaline hypochlorite working solution is added to each tube. The time between these additions should be as short as possible. The tubes are stopped and their contents then mixed. The tubes are incubated in the water bath at 37 °C for 20 min. The samples are transferred to spectrophotometer cuvettes (light path = 1 cm) and absorbances are measured at 630 nm with a spectrophotometer such as the Beckman Model 25 (Beckman Instruments Inc., Fullerton, Calif. 92634), which provides stable read-out of absorbance within ±0.001 \( A \) throughout the range from 0 to 2 \( A \).

**Calculations.** Serum 5'-N activity, expressed as IUB units (U) per liter at 37 °C (micromoles of substrate transformed/min per liter of serum), is calculated as follows:

\[
5\text{-}N \text{ activity, U/liter} = \frac{(A_1 - A_3) - (A_2 - A_4)}{(A_5 - A_3)} \times \text{factor (f)}
\]
Table 2. Protocol for Assay of Serum 5'-Nucleotidase Activity

<table>
<thead>
<tr>
<th>Tube no.</th>
<th>Serum without con A</th>
<th>Serum with con A</th>
<th>Blank without con A</th>
<th>Blank with con A</th>
<th>Standard without con A</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.5</td>
<td>—</td>
<td>0.5</td>
<td>—</td>
<td>0.5</td>
</tr>
<tr>
<td>2</td>
<td>—</td>
<td>0.5</td>
<td>—</td>
<td>0.5</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>0.05</td>
<td>0.05</td>
<td>—</td>
<td>0.05</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
<td>0.05</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.05</td>
<td>—</td>
</tr>
</tbody>
</table>

Solutions
- 5'-AMP, ADA, Mg²⁺, buffer
- 5'-AMP, ADA, Mg²⁺, Buffer, con A
- Serum
- Water
- Adenosine std.

Incubate for 60 min at 37 °C
Phenol/EDTA/nitroprusside
Alkaline HClO₄
Incubate for 20 min at 37 °C
Measure absorbance at 630 nm

where $A_1$ = absorbance of the serum sample without con A; $A_2$ = absorbance of the serum sample with con A; $A_3$ = absorbance of the reagent blank sample without con A; $A_4$ = absorbance of the reagent blank sample with con A; $A_5$ = absorbance of the adenosine standard sample without con A; and

$$f \text{ (factor)} = \frac{\text{concn of std (µmol/ml)} \times \text{vol of std (ml)}}{\text{vol of serum (liter)} \times \text{time (min)}}$$

$$= \frac{3.74 \times 0.05}{0.00005 \times 60} = 62.3 \text{µmol-min}^{-1}\text{-liter}^{-1}$$

Note: Contamination of the glassware and laboratory environment with NH₄⁺ should be strictly avoided. It is advisable to use the two reagent blanks (with and without con A) because the con A reagent contains traces of ammonia. Serum or heparinized plasma should be used, because anticoagulants (e.g., citrate or ethylenediaminetetraacetic acid) that form complexes with Mg²⁺ interfere in 5'-nucleotidase assays.

Other assays of serum constituents. Certain sera that were tested for 5'-N activity by the present method were also analyzed for (a) total bilirubin concentration by the method of Meites and Hogg (57); (b) AP activity by the method of Bowers and McComb (58), (c) γ-glutamyltransferase (EC 2.3.2.2) activity by the method of Szasz (59), (d) alanine aminotransferase (EC 2.6.1.2) activity by the method of Wroblewski and LaDue (60), and (e) 5'-nucleotidase (5'-N) activity by Sunderman and Hork's (50) adaptation of the technique of Persijn et al. (38, 39), with use of phenyl phosphate to suppress hydrolysis of 5'-AMP by serum AP (43). Bilirubin and AP were assayed with a Model ABA-100 bichromatic analyzer (Abbott Scientific Products, South Pasadena, Calif. 91030); the other assays were done with a Model 2400S spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio 44074).

Preparation of hemolysates for study of erythrocyte 5'-N activity. Erythrocytes were separated from leukocytes and platelets in heparinized samples of whole blood by passage through a chromatographic column containing a mixture of microcrystalline cellulose and α-cellulose, as described by Beutler et al. (61). The isolated erythrocytes were washed three times with NaCl solution (154 mmol/liter), and then were suspended in an equal volume of the NaCl solution. The erythrocyte suspension was hemolyzed by rapid freezing and thawing, which was repeated three times. The erythrocyte stroma was sedimented by centrifugation for 15 min at 1500 × g. The supernatant hemolysates were removed for immediate measurement of 5'-N activities by the present method and by the method of Persijn et al. (38, 39, 43). Concentrations of hemoglobin in the hemolysates were measured by the method of MacFate (62).

Induction of biliary obstruction in rats. Acute biliary obstruction was induced in male Sprague–Dawley rats (mean body weight, 250 g; range, 200–300 g) by bile duct ligation, as described by Issa et al. (63) and Kryszewski et al. (64). Control rats were anesthetized with pentobarbital and their abdominal cavities opened by midline incision. The bile duct was sham-ligated by passing a ligature loosely around the common bile duct in each control rat, the ligature being removed before the abdominal incision was closed. The test rats and control rats were exsanguinated from the vena cava at two, three, or four days after the operation.

Induction of hepatotoxicity in rats. Acute toxic injury of hepatic parenchymal cells was induced in male Sprague–Dawley rats (mean body weight, ~250 g; range, 200–300 g) by oral (gavage) administration of thioacetamide, 25 or 50 mg/kg body weight, dissolved in 2.5 ml of water, as described by Korsrud et al. (65). Control rats received 2.5 ml of water by gavage. The rats were then fasted, but permitted free access to water. The test rats and control rats were exsanguinated from the vena cava 24 h after the gavage.

Results

Differential Inhibition of 5'-N and AP Activities

Con A and MADP were evaluated as differential inhibitors of specific and nonspecific enzymic hydrolysis of 5'-AMP by 5'-N and AP. Three sera with increased AP activities were selected for this study (Table 3). Serum A was from a patient with Paget’s disease, who had no clinical or laboratory evidence of hepatobiliary disease; Serum B was from a healthy woman during the
last trimester of pregnancy; and Serum C was from a patient with choledocholithiasis without any clinical or radiographic evidence of osseous disease. Reaction conditions for this study were as listed in Tables 1 and 2, except that we varied the concentrations of con A and MADP used as the differential inhibitor: con A was added to the enzyme reaction mixtures in concentrations of 0, 1, 2, 3, 4, and 5 g/liter; MADP in concentrations of 0, 1.25, 2.5, 5.0, and 7.5 g/liter. As shown in Figure 1, plateaus of inhibition of enzymic hydrolysis of 5'-AMP were obtained in each of the three sera at con A concentrations ranging from 3 to 5 g/liter and at MADP concentrations ranging from 2.5 to 7.5 g/liter. As shown in Table 3, the 5'-N activities that were obtained with Sera A, B, and C by use of con A (3 to 5 g/liter) as a differential inhibitor agreed well with results obtained by the procedure of Persijn et al. (38, 39, 43), in which phenyl phosphate is used to suppress AP hydrolysis of 5'-AMP. Moreover, as anticipated from clinical evaluations of the three subjects, the 5'-N activities of Sera A and B were within the reference values for healthy persons (as will be described later), while the 5'-N activity in Serum C was greatly increased. From these findings, it appeared that con A might serve as a differential inhibitor of m'-AMP hydrolysis by 5'-N and AP. On the other hand, the 5'-N activities that were obtained with Sera A and B by use of MADP (2.5 to 7.5 g/liter) as a differential inhibitor were in gross disagreement with the results obtained by the procedure of Persijn et al. (38, 39, 43) and were inconsistent with the clinical evaluations of these two subjects. Little residual enzymic hydrolysis of 5'-AMP was found in Sera A and B when MADP was used. Evidently MADP is not suitable as a differential inhibitor for 5'-N assays, because it inhibits hydrolysis of 5'-AMP by 5'-N and also by bone and placental AP.

Con A and MADP were evaluated as inhibitors of serum AP activity, as measured by the Bowers-McComb technique (58) with p-nitrophenyl phosphate as the substrate. Three sera were selected for this study (Table 4). Serum A was the same serum from the patient with Paget's disease that was tested in the previous study, Serum D was from a healthy male laboratory worker with no clinical evidence of hepatobiliary disease, and Serum E was from a patient with choledocholithiasis. In addition, a solution of purified human placental AP was tested (Sample F). The AP assay was performed in the presence of con A (4.0 g/liter) and MADP (7.5 g/liter). As indicated in Table 4, addition of con A to the enzyme reaction mixture did not significantly inhibit AP activity in any of the specimens. In contrast, addition of MADP caused 10 to 33% inhibition of AP activity in the samples. On the basis of this study, MADP was rejected for use as a differential inhibitor, and only con A was used in subsequent experiments.

Optimal Concentration of Con A

Sera from seven patients with various hepatic and (or) biliary diseases (Sera G to M) were used to evaluate the optimal concentration of con A for inhibition of 5'-N activity (Table 5). The reaction conditions were as listed

**Table 3. Con A and MADP Compared as Differential Inhibitors of Enzymic Hydrolysis of 5'-AMP by Serum 5'-Nucleotidase (5'-N) and Alkaline Phosphatase (AP)**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Subject</th>
<th>AP activity by ref. 58</th>
<th>Total activity without inhibitor</th>
<th>Residual activity with con A inhibition</th>
<th>Residual activity with MADP inhibition</th>
<th>Activity, U/liter</th>
<th>5'-N activity based on con A inhibition</th>
<th>5'-N activity based on MADP inhibition</th>
<th>By method of ref. 38, 39, 43</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MJ, δ, 49 (Paget's disease)</td>
<td>1140</td>
<td>19.6</td>
<td>10.7</td>
<td>1.1</td>
<td>8.9</td>
<td>18.5</td>
<td>9.4</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>NB, δ, 27 (normal pregnancy)</td>
<td>169</td>
<td>19.1</td>
<td>9.4</td>
<td>1.4</td>
<td>9.7</td>
<td>17.7</td>
<td>9.7</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>GA, δ, 65 (choledocholithiasis)</td>
<td>240</td>
<td>95</td>
<td>14.6</td>
<td>18.3</td>
<td>78</td>
<td>77</td>
<td>80</td>
<td></td>
</tr>
</tbody>
</table>

* Reference interval for healthy adult persons, 20 to 90 U/liter (58).

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**Fig. 1. Inhibitory effects of con A (●) and MADP (○) on enzymic hydrolysis of 5'-AMP by sera from (A) a patient with Paget's disease of bone; (B) a healthy pregnant woman; and (C) a patient with choledocholithiasis**
in Tables 1 and 2, except that con A was added to the enzyme reaction mixture in concentrations ranging from 0.5 to 5 g/liter. As shown in Figure 2, the residual hydrolysis of 5'-AMP reached plateaus at concentrations of con A ranging from 3 to 5 g/liter. Preincubation of these sera for 10 min at 37°C in reaction mixtures containing con A, before substrate (5'-AMP) was added, did not significantly change the con A inhibition curves.

Determination of pH Optimum

We determined the pH optimum for measurements of serum 5'-N activity under the reaction conditions specified in Tables 1 and 2, using sera from three patients with hepatobiliary diseases. Before the sera were added, the enzyme reaction mixtures were equilibrated at 37°C and were adjusted to pH values from 6.2 to 7.6. Plots of the enzymic hydrolysis of 5'-AMP vs. the pH values of the reaction mixtures are shown in Figure 3. The pH optimum of 5'-N activity in sera from patients

Table 4. Inhibitory Effects of Con A and MADP on Enzymic Hydrolysis of Phenyl Phosphate by Alkaline Phosphatase (AP)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Subject or preparation</th>
<th>Total activity without any inhibitor</th>
<th>Residual activity with con A inhibition</th>
<th>Residual activity with MADP inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MJ, δ, 49 (Paget’s disease)</td>
<td>1140 762</td>
<td>(95%) (67%)</td>
<td>429 205</td>
</tr>
<tr>
<td>D</td>
<td>AA, δ, 43 (Healthy person)</td>
<td>119 107</td>
<td>(102%) (90%)</td>
<td>293 149</td>
</tr>
<tr>
<td>E</td>
<td>BD, δ, 57 (choledocholithiasis)</td>
<td>953 649</td>
<td>(97%) (68%)</td>
<td>429 205</td>
</tr>
<tr>
<td>F</td>
<td>Purified placental AP</td>
<td>1474 1297</td>
<td>(100%) (88%)</td>
<td>762 429</td>
</tr>
</tbody>
</table>

* Measured by the method of Bowers and McComb (56). Values in parentheses: percentages of the total AP activity remaining in the presence of con A (4 g/liter) or MADP (7.5 g/liter).

* A solution of purified alkaline phosphatase from human placenta was kindly provided by Dr. Allyn Rule, Tufts University School of Medicine, Boston, Mass.

with hepatobiliary diseases was found to be 7.2, consistent with the results of a previous investigation by Hill and Sammons (66). Residual hydrolysis of 5'-AMP that occurred in the presence of con A continued to increase as the pH was raised from 6.2 to 7.6. This finding supports the inference that the residual activity represents nonspecific hydrolysis of 5'-AMP owing to serum AP activity.

Optimal Co-Factor, Substrate, and ADA Concentrations

Optimum Mg2+ concentration. Enzyme reaction mixtures were prepared to contain Mg2+ in concentrations of 0, 7.5, 15, 22.5, 30, and 37.5 mmol/liter, and, except for the concentration of Mg2+, assayed for serum 5'-N under the reaction conditions specified in Tables 1 and 2. Data obtained with sera from two patients with hepatobiliary diseases are plotted in Figure 4. 5'-N activities were maximum with Mg2+ concentrations of 22.5 to 37.5 mmol/liter.

Optimum 5'-AMP concentration. A series of ADA/Mg2+-buffer solutions was prepared as specified

Table 5. Measurements of Total Bilirubin, Alkaline Phosphatase (AP), Alanine Aminotransferase (AT) and 5'-Nucleotidase Activities in Sera from Seven Patients with Hepatobiliary Diseases

<table>
<thead>
<tr>
<th>Serum</th>
<th>Subjects</th>
<th>Total bilirubin</th>
<th>AP</th>
<th>AT</th>
<th>Method of Peraldo et al.</th>
<th>Present method</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>RM, δ, 42 (primary biliary cirrhosis)</td>
<td>316</td>
<td>436</td>
<td>92</td>
<td>124</td>
<td>101</td>
</tr>
<tr>
<td>H</td>
<td>GM, δ, 85 (chloropropazine hepatitis)</td>
<td>12</td>
<td>189</td>
<td>48</td>
<td>81</td>
<td>79</td>
</tr>
<tr>
<td>I</td>
<td>WA, δ, 42 (choledocholithiasis)</td>
<td>299</td>
<td>171</td>
<td>51</td>
<td>70</td>
<td>66</td>
</tr>
<tr>
<td>J</td>
<td>FG, δ, 28 (liposarcoma with hepatic metastases)</td>
<td>5</td>
<td>352</td>
<td>115</td>
<td>51</td>
<td>42</td>
</tr>
<tr>
<td>K</td>
<td>MW, δ, 72 (ovarian carcinoma with hepatic metastases)</td>
<td>6</td>
<td>71</td>
<td>14</td>
<td>39</td>
<td>35</td>
</tr>
<tr>
<td>L</td>
<td>LW, δ, 48 (Laennec's cirrhosis)</td>
<td>31</td>
<td>336</td>
<td>16</td>
<td>38</td>
<td>32</td>
</tr>
<tr>
<td>M</td>
<td>WG, δ, 54 (cardiac failure with hepatic congestion)</td>
<td>12</td>
<td>285</td>
<td>—</td>
<td>36</td>
<td>30</td>
</tr>
</tbody>
</table>

The reference intervals for serum total bilirubin, alkaline phosphatase, and aspartate aminotransferase in sera from healthy adult persons are 1 to 12 mg/liter, 20 to 90 U/liter, and 8 to 29 U/liter, respectively, based upon reference 58 and unpublished studies in our laboratory.

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above, and 5'-AMP was added in order that the final reaction mixtures would contain 0.046, 0.46, 2.3, 4.6, and 9.2 mmol of 5'-AMP per liter. Figure 5 shows data obtained with two sera from patients with hepatobiliary diseases. 5'-N activities were maximum with 4.6 or 9.2 mmol of 5'-AMP per liter of reaction mixture.

Optimum ADA concentration. A series of enzyme reaction mixtures was prepared as specified in the procedure, except that the concentrations of ADA were 0, 0.015, 0.15, 0.3, 0.6, and 1.2 kU/liter, respectively. Standard solutions containing adenosine (3.74 and 7.48 mmol/liter) were analyzed. An ADA concentration of 0.6 kU/liter was sufficient to achieve maximum hydrolysis of adenosine. Additions of con A (3.6 g/liter) did not significantly affect the adenosine deaminase reaction, based upon measurements of enzymic deamination of adenosine in reaction mixtures that contained ADA in concentrations from 0.015 to 1.2 kU/liter.

Comparisons of Con A from Various Suppliers

Crystalline con A was purchased from three suppliers to ascertain whether or not there were any significant differences in con A inhibition of serum 5'-N activity. The preparations that were tested were: "Concanavalin A-Grade IV" (Sigma Chemical Co., St. Louis, Mo. 63178); "Concanavalin A, 3X crystallized" (Miles Laboratories, Inc.), and "Concanavalin A, 3X crystallized" (Pharmacia Fine Chemicals Co., Piscataway, N.J. 08854). Each of these preparations of con A was dissolved in saturated NaCl solution and added to enzyme reaction mixtures in a concentration of 2.5 g/liter. No significant differences were observed in 5'-N activities in five sera from patients with hepatobiliary diseases that each were analyzed with the three different preparations of con A. However, the crystalline preparations of concanavalin A all yielded slightly cloudy solutions when they were added to saturated NaCl solution, a turbidity not observed when the commercially prepared solution of 2X-recrystallized concanavalin A (Miles Laboratories, Inc.) was used. Accordingly, we used the commercially prepared solution of con A routinely, although the presence of traces of ammonia in it necessitated routine testing of a reagent blank containing con A. The presence of NaCl in the commercially prepared solution of con A did not significantly affect the 5'-N assay.

Color Development and Spectrophotometry

The rates of color development after additions of the phenol/EDTA/nitroprusside and alkaline HClO4 reagents to enzyme reaction mixtures containing sera or adenosine standards was monitored at 37 °C with a Beckman Model 25 recording spectrophotometer with temperature regulated cuvettes. The color developed by the Berthelot reaction was consistently most intense within 20 min of incubation at 37 °C, regardless of the presence or absence of con A and remained stable for at least 3 h at 25 °C. Between 550 to 700 nm the absorbance maximum of the final colored solution was 630 nm, regardless of the presence or absence of con A. Under

![Fig. 3. Enzymic hydrolysis of 5'-AMP by sera from three patients with hepatobiliary diseases at pH values ranging from 6.2 to 7.6. The total hydrolysis of 5'-AMP is designated by Δ; the residual hydrolysis of 5'-AMP in the presence of con A (3.6 mg/ml) is designated by ◇; 5'-N activity (calculated as the difference between the total and residual hydrolysis of 5'-AMP) is designated by ■.](image)

![Fig. 4. Effect of the concentration of Mg2+ in the enzyme reaction mixture upon 5'-N activities obtained with sera from two patients with hepatobiliary diseases.](image)

![Fig. 5. Effect of the concentration of the substrate, 5'-AMP, in the enzyme reaction mixture on 5'-N activities obtained with sera from two patients with hepatobiliary diseases.](image)
the specified conditions (Tables 1 and 2), the calibration curve obtained with adenosine standard solutions adhered strictly to the Beer–Lambert relationship throughout the range of adenosine concentrations from 0 to 1.0 mmol/liter of enzyme reaction mixture, corresponding to absorbance values from 0 to ~1.9 A, and serum 5'-N activities from 0 to ~167 U/liter.

Computations of 5'-N Activities

Two techniques for computing serum 5'-N activities were tested. The first technique was specified in the Methods and Materials section under Calculations. The second technique required two additional serum "blank" tubes (with and without con A) which were not incubated at 37 °C before the Berthelot color reagents were added. In the second technique of computation we used the following equation:

5'-N activity, U/liter

\[
= \frac{(A_{1A} - A_{1B}) - (A_{2A} - A_{2B})}{(A_5 - A_3)} \times 62.3
\]

where

- \( A_{1A} \) = absorbance of serum sample without con A, incubated for 60 min at 37 °C before color development.
- \( A_{1B} \) = absorbance of serum sample without con A, not incubated before color development.
- \( A_{2A} \) = absorbance of serum sample with con A, incubated for 60 min at 37 °C before color development.
- \( A_{2B} \) = absorbance of serum sample with con A, not incubated before color development.
- \( A_3 \) = absorbance of reagent blank without con A, incubated for 60 min at 37 °C before color development.
- \( A_5 \) = absorbance of adenosine standard without con A, incubated for 60 min at 37 °C before color development.

5'-N activities in 125 sera from hospital patients were calculated by both of the computational techniques, and the results were evaluated statistically by means of the paired-sample t-test and by linear regression analysis by the least-squares method (67). 5'-N activities in these sera ranged from 4.1 to 98 U/liter. There was no significant difference between the paired results obtained by the two techniques of computation. The correlation coefficient was 0.999. The regression equation was \( y = 1.007x - 0.126 \), where \( x \) = results by the second technique with use of nonincubated serum "blanks," and \( y \) = results by the computational technique that is specified in the procedure. None of the paired results of 5'-N activities in the 125 sera differed by more than 1 U/liter in the range from 4.1 to 48 U/liter or by more than 2 U/liter in the range from 49 to 95 U/liter.

Linearity of 5'-N Activity with Respect to Serum Volume

Two sera with 5'-N activities of 12 and 55 U/liter, respectively, were analyzed under the conditions specified in Tables 1 and 2, except that the enzymatic reaction was terminated after intervals of 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, and 2 h. Observed absorbance at 630 nm and duration of incubation at 37 °C were linearly related throughout the 2-h period. An incubation period of 1 h was chosen for routine use, because it provides a sensitivity of ~0.011 A per unit of enzymic activity (U/liter). When sera with 5'-N activities that exceed 100 U/liter are assayed, the duration of the incubation period can satisfactorily be decreased to 0.5 or even 0.25 h with multiplication of the analytical results by two or four, respectively.

Comparisons with Analyses by a Generally Accepted Method

One hundred consecutive sera from hospital patients were analyzed for 5'-N activity by the present method and by the method of Persijn et al. (38, 39, 43). The method of Persijn et al. was performed at pH 7.2 with procedural details and precautions that have been described by Sunderman and Horak (50). The results are plotted in Figure 6. Measurements of serum 5'-N activity by the present procedure averaged 8.4% lower than by the method of Persijn et al. (38, 49, 43) (\( P < 0.001 \), based upon a paired-sample t-test) (67). The correlation coefficient was 0.989; the equation of the regression line was \( y = 0.906x + 0.169 \) (where \( x = 5'\-N \)

\[ y = 0.91x + 0.17 \]

Fig. 6. Correlation obtained with sera from 100 hospital patients that were analyzed for 5'-N activity (A) by the present method with use of concanavalin A as an inhibitor of 5'-N activity; and (B) by the method of Persijn et al. (38, 39, 43) with use of phenyl phosphate to suppress nonspecific hydrolysis of 5'-AMP by serum AP activity
activities by the present method and \( y = 5'-N \) activities by the method of Persijn et al.), and the standard error of the difference was ±2.58 U/liter. The mean 5'-N activity in the 100 sera was 18.4 U/liter by the present method, 20.1 U/liter by the method of Persijn et al. (38, 39, 43).

**Analytical Precision**

*Within-run precision* of the present method of serum 5'-N assays was estimated on the basis of differences between duplicate analyses of 67 sera from hospital patients. The mean 5'-N activity was 13.2 U/liter; the standard deviation of the differences between duplicates was ±0.74 U/liter, computed by the method of Henry (68), and the within-the-run coefficient of variation was 5.6%.

*Day-to-day precision* of the present method for serum 5'-N assays was measured on the basis of analyses of a single pooled serum on 25 consecutive working days. One-milliliter aliquots of the pooled serum were frozen in 25 tubes, which were stored at -20 °C. One tube was thawed for each day's analysis of 5'-N activity. The mean 5'-N activity in the pooled serum was 8.3 U/liter. The standard deviation of the 25 replicate analyses was ±0.50 U/liter, and the day-to-day coefficient of variation was 6.0%.

**Tests for Interference**

**Bilirubin, lipemia, and ammonia.** Fifteen sera that were grossly icteric, lipemic and (or) ammonia-contaminated were analyzed by the present method, with the additional use of serum "blank" tubes (with and without con A) that were not incubated at 37 °C before the Berthelot color reagents were added. 5'-N activities computed by the technique specified in the procedure and by the second technique that is described earlier in the *Results* section were not significantly different. Therefore, under the reaction conditions that are specified in Tables 1 and 2, the absorbance reading of tube no. 2 (which is derived from an enzyme reaction mixture that contains serum and buffer/substrate/cofactor solution with con A) completely compensates for any nonspecific absorbance in tube no. 1 (which contains the same ingredients except for con A). As a further test for interference, we did mixing experiments in which a pooled serum was mixed in proportions of 5:0, 4:1, 3:2, 1:4, and 0:5 by volume with each of 15 sera that were grossly icteric, lipemic and (or) ammonia-contaminated. We measured 5'-N activities in the 75 serum mixtures under the conditions specified in Tables 1 and 2. The observed 5'-N activities yielded consistently linear relationships to the predicted 5'-N activities that were calculated on the basis of the relative proportions of the serum mixtures. On the basis of both sets of tests, we concluded that icterus, lipemia, and ammonia contamination do not interfere with assays of serum 5'-N activity by the present method.

**Hemolysis.** Van der Slik et al. (17) reported that hemolysis is a source of interference in measurements of serum 5'-N activity by the method of Persijn et al. (38, 39, 43). We performed the following test to determine whether or not hemolysis also causes interference in 5'-N assays by the present procedure. Venepunctures were performed on two healthy volunteers by use of syringes with 20-gauge needles. The needles were removed, and half of each blood sample was allowed to flow gently into test tubes. The remaining blood in each syringe was purposely hemolyzed by forcefully expelling the blood via a 25-gauge needle into test tubes. The blood samples were allowed to clot at room temperature, and specimens of nonhemolyzed and grossly hemolyzed sera were removed. The nonhemolyzed and hemolyzed samples of serum from the same person were then mixed in proportions of 5:0, 4:1, 3:2, 2:3, 1:4, and 0:5 by volume. 5'-N activities in the serum mixtures were measured by the present method and by the method of Persijn et al. (38, 39, 43) at pH 7.2. Figure 7 shows illustrative data obtained for the serum mixtures from one of the subjects. The results were similar for the second subject. In analyses of the two sera by the present method, gross hemolysis (serum hemoglobin concentrations of 2.4 and 2.5 g/liter, respectively) caused increases of serum 5'-N activity of 5.5 and 6.6 U/liter. In comparison, in analyses of the two sera by the method of Persijn et al. (38, 39, 43), gross hemolysis caused increases of serum 5'-N activity of 21.6 and 21.8 U/liter, respectively. Therefore, in the present method the interference by hemolysis in assays of 5'-N activity was only 25 to 30% of that observed by use of the method of Persijn et al. (38, 39, 43). In 5'-N assays by the present method and by the method of Persijn et al. (38, 39, 43), spectrophotometric interference by hemoglobin was ruled out, based upon measurements of non-incubated enzyme reaction mixtures that contained the hemolyzed sera. Therefore, we inferred that the interference caused by hemolysis was due to enzymatic hydrolysis of 5'-AMP by 5'-N in the erythrocytes.
Blood samples from two healthy volunteers were treated with heparin to prevent coagulation. Erythrocytes were separated from leukocytes and platelets by chromatography on columns containing cellulose, and erythrocyte hemolysates were prepared as described under Methods and Materials. The two hemolysates were diluted to hemoglobin concentrations of ~20 g/liter. We assayed for 5'-N activity under the reaction conditions that are specified in Tables 1 and 2, except that con A was added to the enzyme reaction mixtures in concentrations ranging from 0 to 5 g/liter. As shown in Figure 8, enzymic hydrolysis of 5'-AMP by erythrocyte hemolysates was progressively inhibited by increasing concentrations of con A. However, no plateau of residual hydrolysis of 5'-AMP was reached within the range of con A concentrations that was tested in this study. The con A inhibition curves that we obtained with the erythrocyte hemolysates (Figure 8) were less steep and differed in configuration from the curves that we previously had obtained with serum samples (Figure 2). At the con A concentration selected for routine assay of serum 5'-N activity (3.6 g/liter), the inhibition by con A of enzymic hydrolysis of 5'-AMP by the two erythrocyte homogenates was 31 and 35%, respectively. Thus con A is not very effective as an inhibitor of erythrocyte 5'-N activity. As a result, hemolysis causes less interference in assays of serum 5'-N activity by the present method than by the method of Persijn et al. (38, 39, 43).

**Reference Values**

Serum samples were obtained from apparently healthy adult residents of the vicinity of Hartford, Connecticut, 20 men and 20 women employed as clinical scientists or technologists in a hospital clinical laboratory. The mean age of the women was 32 years (range, 22 to 53 years), that of the men 34 years (range, 25 to 53 years). Subjects were excluded from the study (a) if they had any illness or had been pregnant within the previous year; (b) if they had any history of hepatic or biliary diseases, or (c) if they were receiving any medications, including oral contraceptives. 5'-N activity averaged 6.8 (SD ± 2.4) U/liter (range, 3.1 to 11.6) in sera from the 20 men, and 6.1 (SD ± 1.5) U/liter (range, 4.0 to 10.0) in sera from the 20 women, not a significant sex-related difference according to statistical comparisons by the t-test (67). 5'-N activity for the entire group of 40 healthy persons averaged 6.4 (SD ± 2.0) U/liter (range, 3.1 to 11.6). The median value was 5.9 U/liter, and the reference interval (5th to 95th percentiles) for serum 5'-N activity was 3.6 to 11.1 U/liter, calculated by the nonparametric method of Herrera (69).

**Clinical Validity**

Experiments were performed in rats in order to test the validity of the present serum 5'-N assay for the differentiation of biliary obstruction from acute hepatocellular injury. Acute biliary obstruction was induced in 17 rats by ligating the common bile duct, and blood was sampled on the second, third, or fourth days thereafter. Fourteen control rats were subjected to a similar surgical procedure with sham ligation of the bile duct, and blood samples were collected after corresponding intervals. The results of measurements of serum bilirubin, γ-glutamyltransferase, AP, and 5'-N are summarized in Table 6. The mean bilirubin concentration in the sera from the rats with acute biliary obstruction averaged 45-fold that in the sera from the

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**Table 6. Effects of Bile Duct Ligation on Concentrations of Total Bilirubin and Activities of γ-Glutamyltransferase (γ-GT), Alkaline Phosphatase (AP), and 5'-Nucleotidase (5'-N) in Rat Sera**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Days to collection</th>
<th>No. rats</th>
<th>Serum bilirubin (g/liter)</th>
<th>γ-GT</th>
<th>Serum enzyme activities AP (U/liter)</th>
<th>5'-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Sham</td>
<td>2</td>
<td>4</td>
<td>2 ± 2</td>
<td>0.2 ± 0.2</td>
<td>83 ± 9</td>
<td>17 ± 4</td>
</tr>
<tr>
<td>B</td>
<td>Sham</td>
<td>3</td>
<td>5</td>
<td>1 ± 2</td>
<td>0.4 ± 0.5</td>
<td>84 ± 12</td>
<td>12 ± 5</td>
</tr>
<tr>
<td>C</td>
<td>Sham</td>
<td>4</td>
<td>5</td>
<td>2 ± 2</td>
<td>0.3 ± 0.5</td>
<td>98 ± 17</td>
<td>17 ± 3</td>
</tr>
<tr>
<td>[A,B,C]</td>
<td>Sham</td>
<td>2,3,4</td>
<td>14</td>
<td>1 ± 2</td>
<td>0.3 ± 0.4</td>
<td>88 ± 14</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>D</td>
<td>Ligation</td>
<td>2</td>
<td>6</td>
<td>100 ± 30^b</td>
<td>5 ± 2^b</td>
<td>305 ± 74^b</td>
<td>70 ± 22^b</td>
</tr>
<tr>
<td>E</td>
<td>Ligation</td>
<td>3</td>
<td>5</td>
<td>90 ± 50^a</td>
<td>8 ± 4^a</td>
<td>198 ± 59^a</td>
<td>42 ± 12^a</td>
</tr>
<tr>
<td>F</td>
<td>Ligation</td>
<td>4</td>
<td>6</td>
<td>70 ± 30^a</td>
<td>6 ± 4^a</td>
<td>196 ± 37^a</td>
<td>33 ± 7^a</td>
</tr>
<tr>
<td>D,E,F</td>
<td>Ligation</td>
<td>2,3,4</td>
<td>17</td>
<td>90 ± 40^b</td>
<td>6 ± 3^b</td>
<td>236 ± 77^b</td>
<td>49 ± 22^b</td>
</tr>
</tbody>
</table>

^a P < 0.01 and ^b P < 0.001 vs. Groups [A,B,C] by t-test (67).
Table 7. Effects of Acute Hepatotoxicity Induced by Thioacetamide on Activities of Alanine Aminotransferase (AT), Alkaline Phosphatase (AP), and 5'-Nucleotidase (5'-N) in Rat Sera

<table>
<thead>
<tr>
<th>Group</th>
<th>Thioacetamide mg/kg body wt</th>
<th>No. Rats</th>
<th>Serum enzyme activities (U/liter)</th>
<th>5'-N activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>20</td>
<td>23 ± 5</td>
<td>105 ± 6</td>
</tr>
<tr>
<td>B</td>
<td>25</td>
<td>10</td>
<td>65 ± 25&lt;sup&gt;a&lt;/sup&gt; 117 ± 14&lt;sup&gt;b&lt;/sup&gt; 19 ± 5&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>9</td>
<td>116 ± 98&lt;sup&gt;a&lt;/sup&gt; 132 ± 32&lt;sup&gt;c&lt;/sup&gt; 18 ± 6&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Thioacetamide, p.o. by gavage 24 h before blood collection. <sup>b</sup> P < 0.05; <sup>c</sup> P < 0.001 vs. Group A by t test (67).

Discussion

Concanavalin A

Concanavalin A (con A) is a plant lectin from the jack bean (Canavalia ensiformis) that binds to α-D-mannose and related saccharide residues in surface glycoproteins of certain viruses, bacteria, protozoa, and cells from higher organisms (70). The binding of con A to cell membranes leads to mitogenesis of lymphocytes and to agglutination of erythrocytes and various tumor cells in tissue culture. Con A is a dimeric or tetrameric protein at pH >7, composed of identical protomers with 237 amino acid residues (mol wt, 25 500), (5, 16). Each protomer of con A has binding sites for Mn<sup>2+</sup>, Ca<sup>2+</sup>, and α-D-mannose (70, 71).

In 1974, Riordan and Slavik (56) reported that con A inhibits 5'-N activity of rat hepatocytes. Stefanovic et al. (45) observed that con A powerfully and reversibly inhibits 5'-nucleotidase of intact glialoma cells in tissue culture, and they suggested that con A inhibition might provide a means to explore the role of 5'-N in cellular metabolism. This suggestion was confirmed by Little and Widnell (72), who used con A to study in vivo translocation of 5'-N across cisternal membranes in rat hepatocytes. Riemer and Widnell (73) showed that con A inhibited 5'-N that had been solubilized from membrane-enriched fractions of several rat tissues, including liver, spleen, kidney, heart, lung, brain, and muscle. Carraway et al. (52–54) investigated the mechanism of con A inhibition of 5'-N activity in plasma membranes from mammary glands of lactating

Table 8. Reference Intervals for Serum 5'-Nucleotidase Activity in Healthy Adult Persons

<table>
<thead>
<tr>
<th>Reference</th>
<th>Year</th>
<th>Method</th>
<th>pH</th>
<th>Correction for AP act. a</th>
<th>No. subjects</th>
<th>5'-Nucleotidase Mean ± SD (U/liter at 37 °C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1965</td>
<td>Phosphorimetric</td>
<td>7.5</td>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>15</td>
<td>7.1 ± 2.4</td>
</tr>
<tr>
<td>32</td>
<td>1966</td>
<td>Phosphorimetric</td>
<td>7.4</td>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>21</td>
<td>—</td>
</tr>
<tr>
<td>76</td>
<td>1968</td>
<td>Phosphorimetric</td>
<td>7.5</td>
<td>Ni&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>110</td>
<td>3.4 ± 2.0</td>
</tr>
<tr>
<td>24</td>
<td>1969</td>
<td>ADA (kinetic)</td>
<td>7.5</td>
<td>Corr. factor</td>
<td>40</td>
<td>10.3 ± 3.1</td>
</tr>
<tr>
<td>5</td>
<td>1969</td>
<td>ADA (kinetic)</td>
<td>7.9</td>
<td>β-Glycerophosphate</td>
<td>516</td>
<td>5.2 ± 4.8</td>
</tr>
<tr>
<td>35</td>
<td>1970</td>
<td>ADA (Berthelot)</td>
<td>7.9</td>
<td>β-Glycerophosphate</td>
<td>56</td>
<td>7.9 ± 4.9</td>
</tr>
<tr>
<td>28</td>
<td>1970</td>
<td>ADA (kinetic)</td>
<td>7.2</td>
<td>β-Glycerophosphate</td>
<td>65</td>
<td>5.0 ± 1.4</td>
</tr>
<tr>
<td>40</td>
<td>1971</td>
<td>ADA (Berthelot)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.5</td>
<td>Phenylphosphate</td>
<td>100</td>
<td>7.9 ± 3.1</td>
</tr>
<tr>
<td>27</td>
<td>1972</td>
<td>ADA (kinetic)</td>
<td>7.5</td>
<td>Phenylphosphate</td>
<td>31</td>
<td>5.5 ± 1.9</td>
</tr>
<tr>
<td>49</td>
<td>1974</td>
<td>ADA (Berthelot)</td>
<td>7.5</td>
<td>Phenylphosphate</td>
<td>58</td>
<td>—</td>
</tr>
<tr>
<td>37</td>
<td>1975</td>
<td>ADA (Berthelot)</td>
<td>7.5</td>
<td>L-Cysteine</td>
<td>76</td>
<td>9.3 ± 2.2</td>
</tr>
<tr>
<td>31</td>
<td>1975</td>
<td>Phosphorimetric</td>
<td>9.5</td>
<td>L-Cysteine</td>
<td>204</td>
<td>9.0 ± 4.0</td>
</tr>
<tr>
<td>26</td>
<td>1976</td>
<td>Phosphorimetric</td>
<td>7.6</td>
<td>β-Glycerophosphate</td>
<td>35</td>
<td>—</td>
</tr>
</tbody>
</table>
| This paper | 1977 | ADA (Berthelot) | 7.2 | Concana
cavalin A | 40 | 6.4 ± 2.0 | 3–12 |

<sup>a</sup> Methods for correction of nonspecific hydrolysis of the substrate (5'-AMP) by alkaline phosphatase included con A or Ni<sup>2+</sup> inhibition of 5'-nucleotidase; β-glycerophosphate, phenylphosphate, or L-cysteine suppression of alkaline phosphatase; or use of a correction factor derived by simultaneous assay of serum alkaline phosphatase activity. <sup>b</sup> Analyses by the method of Persijn et al. (38, 39, 43).

control rats. The mean activities of the three enzymes in sera from the rats with acute biliary obstruction averaged respectively 20-, 2.7-, and 3.3-fold the corresponding mean values in sera from the control rats.

Acute hepatotoxicity was induced in two groups of 10 and 9 rats by oral administration (by gavage) of thioacetamide in doses of 25 and 50 mg/kg (body weight), and blood was sampled 24 h thereafter. Twenty control rats were administered the vehicle by gavage, and blood samples were collected after the same interval. The results of the measurements of serum alanine aminotransferase, AP and 5'-N activities are summarized in Table 7. In sera obtained from rats 24 h after oral administration of 25 mg of thioacetamide per kilogram, the mean activities the three enzymes averaged, respectively, 2.8-, 1.1-, and 1.5-fold the corresponding mean activities in sera from control rats. In sera obtained from rats 24 h after oral administration of 50 mg/kg of thioacetamide, the mean activities averaged, respectively, 5.0-, 1.3-, and 1.4-fold the corresponding mean activities in sera from the control rats. The measurements of serum 5'-N listed in Tables 6 and 7 demonstrate that serum 5'-N activity, as measured by the present method, undergoes a greater increase in rats with acute biliary obstruction than in rats with acute toxic injury of hepatic parenchymal cells. These findings are consistent with the reports of previous workers who have used other methods to assay serum 5'-N activity (63–65).
rats. Carraway and Carraway (52) showed that con A inhibition of 5'-N activity is prevented by α-methyl-mannose, and they inferred that con A inhibition of 5'-N activity results from a direct interaction of con A with the enzyme. The inhibition appears to be mediated by specific binding of the lectin to an oligosaccharide constituent of the enzyme, which is known to be a glycoprotein (55, 74). Moreover, Carraway and Carraway (52) showed that con A did not inhibit nonspecific hydrolysis of 5'-AMP by alkaline phosphatase activity in plasma membranes of lactating mammary glands. The present paper demonstrates that con A inhibits 5'-N activity in human and rat sera, and indicates that con A may be a useful reagent in clinical chemistry laboratories for differentiating enzymic hydrolysis of 5'-AMP by 5'-N and AP activities in serum. Studies that are in progress in our laboratory have revealed that con A also inhibits enzymic oxidation of p-phenylenediamine by serum ceruloplasmin under reaction conditions described by Sunderman and Nomoto (75). Thus, con A is not a specific inhibitor of serum 5'-N activity, because it also inhibits at least one other glycoprotein enzyme in serum.

Reference Intervals

The reference intervals for measurements of 5'-N activity in sera from healthy subjects that we obtained by the present method agree reasonably with reference values reported by others (Table 8). Seitanidis and Moss (77) found that serum 5'-N activity does not increase during normal pregnancy, in contrast to the increase in AP activity in serum during pregnancy. Belfield and Goldberg (78) reported that serum 5'-N activities are lower in children than in adults, the reverse of the situation for serum AP. Belfield and Goldberg (78) recommend 0 to 8 U/liter as the reference interval for serum 5'-N activity in healthy infants and in children who are younger than 16 years.

Advantages and Disadvantages of the Present Method

The method for assay of serum 5'-N activity described here appears to be more specific than any of the previously reported techniques. We envision that this method may eventually be recognized as a reference procedure. Advantages of the present method include: (a) excellent sensitivity and precision; (b) freedom from interference by bilirubin, lipemia, ammonia, and hemoglobin; and (c) low susceptibility to interference by erythrocyte 5'-N activity. The present method has two disadvantages: (a) it is relatively time-consuming, and (b) it is not readily adaptable to automation.

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References


