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A New Spectrophotometric Method for the Determination of 5′-Nucleotidase[1])

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Summary: A spectrophotometric method is described for the determination of 5′-nucleotidase.

In combination with the enzymes nucleoside phosphorylase and xanthine oxidase, inosine, formed by hydrolysis of 5′-IMP by 5′-nucleotidase, is cleaved phosphorolytically to hypoxanthine, which is oxidized to uric acid. In the presence of ethanol, the hydrogen peroxide formed is reduced by catalase and equivalent amounts of acetaldehyde are produced. The aldehyde is dehydrogenated (NADP-dependent) by aldehyde dehydrogenase and the production rate of NADPH is recorded at 334 nm. The inhibition of the unspecific cleavage of 5′-IMP by phosphatases is examined critically.

Eine neue spektrophotometrische Methode zur Bestimmung von 5′-Nucleotidase

Zusammenfassung: Eine spektrophotometrische Methode für die Bestimmung der 5′-Nucleotidaseaktivität wird beschrieben.

In Kombination mit den Enzymen Nucleosidphosphorylase und Xanthinoxidase wird Inosin, das bei der Hydrolyse von 5′-IMP durch die 5′-Nucleotidase entsteht, phosphorolytisch zu Hypoxanthin gespalten, das weiter zu Harnsäure oxidiert wird. Das entstandene Wasserstoffperoxid wird mit Katalase in Gegenwart von Ethanol reduziert. Dabei entstehen äquimolare Mengen an Acetaldehyd. Der Aldehyd wird NADP-abhängig durch die Aldehyddehydrogenase dehydriert und das gebildete NADPH bei 334 nm automatisch registriert. Die Inhibierung der unspezifischen 5′-IMP-Hydrolyse wird kritisch untersucht.

Introduction

5′-nucleotidases catalyze the hydrolysis of nucleoside-5′-monophosphates to nucleosides and inorganic phosphate.

5′-nucleotidase activity has been detected in a great number of human and animal tissues (1, 2).

The activity present in sera is released mainly from the membrane of liver cells by bile salts and can be used as a marker for liver disease (1). Elevated enzyme levels are detectable in sera particularly if the hepatobiliary tract is involved; generally, the rise in 5′-nucleotidase activity is accompanied by that of alkaline phosphatase, glutamyltransferase and other marker enzymes for liver disease. However, alkaline phosphatase is also elevated in some bone diseases and during normal pregnancy, whereas 5′-nucleotidase remains unaltered under these conditions (1).

The diagnostic value of 5′-nucleotidase is still under discussion, but the enzyme has been shown to be superior to other liver enzymes, especially in cases of liver metastasis (1, 3, 4, 5).

[1]) This work contains part of the dissertation of *Renate Pilz*, Medizinische Hochschule Hannover.
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For the determination of 5′-nucleotidase activity, the following methods have been described:

For the detection of especially low activities, radioactive methods with ^{14}C, ^{3}H and ^{32}P labelled nucleoside-5′-monophosphates, particularly 5′-AMP, are used, but special equipment is needed (6, 7, 8).

One of the oldest methods uses the determination of inorganic phosphate liberated from the substrate according to *Fiske-Subbarow* (9, 10). This method has been adapted to automation (11) and modified by several authors (12, 13, 14, 15).

With 5′-AMP as substrate, the reaction product adenosine can be deaminated to inosine by coupling with the adenosine deaminase reaction, and the disappearance of adenosine is measured at 265 nm (8, 16, 17, 18). The ammonia formation can be determined by the *Berthelot* reaction (19, 20, 21, 22, 23) or kinetically by coupling with the glutamate dehydrogenase reaction (24, 25, 26, 27, 28).

Considerable problems occur with the high absorbances of the substrate and the serum samples at 265 nm, and the high blanks caused by ammonia normally present in sera.

With 5′-IMP as the substrate, the reaction product inosine can be converted to urate by the coupled nucleoside phosphorylase and xanthine oxidase reactions which may be monitored at 293 nm (29), but at this wavelength, the serum samples exhibit high absorbances.

We describe a simple kinetic assay with 5′-IMP as the substrate, using the following reaction sequence:

(1) inosine-5′-*P* + H_2O $\xrightarrow{\text{5′-nucleotidase}}$ inosine + P_i

(2) inosine + P_i $\xrightarrow[\text{phosphorylase}]{\text{nucleoside}}$ hypoxanthine + ribose-1-*P*

(3) hypoxanthine + $2H_2O$ + $2O_2$ $\xrightarrow{\text{xanthine oxidase}}$ uric acid + $2H_2O_2$

(3) $2H_2O_2$ + 2 ethanol $\xrightarrow{\text{catalase}}$ 2 acetaldehyde + $4H_2O$

(5) 2 acetaldehyde + $2NADP^+$ + $2H_2O$ $\xrightarrow[\text{dehydrogenase}]{\text{aldehyde}}$ 2 acetic acid + 2NADPH + $2H^+$

Sum:

inosine-5′-*P* + $2NADP^+$ + 2 ethanol + $2O_2$ → uric acid + ribose-1-*P* + 2 acetic acid + 2NADPH + $2H^+$

Reaction (4) and (5) were described originally for the determination of uric acid in human serum (30, 31) and later for the measurement of several other substances (32) and enzymes of purine metabolism (33, 34).

A special problem of 5′-nucleotidase assay derives from the interference of alkaline phosphatase. Different possibilities for the elimination of this interference are analyzed critically.

Materials and Methods

Sera

Sera were used from patients with liver diseases (containing high 5′-nucleotidase and high alkaline phosphatase activities), from patients with bone disease, and from healthy persons. All sera were stored overnight at 4 °C.

Reagents

Stock solutions

NADP (Boehringer Mannheim 128031; 12 mmol/l): 10 mg were dissolved in 1 ml bidist. water.

NAD (Boehringer Mannheim 127302; 15 mmol/l): 10 mg were dissolved in 1 ml bidist. water.

Magnesium chloride (Merck 5833): 1 mol/l.

Manganese-II-chloride (Merck 5927): 1 mol/l.

Glycerol-2-phosphate (disodium salt, Sigma G-6521): 1 mol/l, adjusted to pH 7.5.

R 8231[2]) (Janssen, Düsseldorf): 33.9 mg were dissolved in 1 ml 0.1 mol/l triethanolamine buffer (Boehringer Mannheim 127426) pH 7.5 containing 0.1 mol/l KCl (Merck 4936).

Dithioerythritol (Merck 24511; 1 mmol/l): 1.54 mg dithioerythritol were dissolved in 10 ml H_2O bidist.

Inosine-5′-monophosphate (Boehringer Mannheim 106704; 40 mmol/l): 5 mg were dissolved in 0.25 ml 0.1 mol/l triethanolamine buffer pH 7.5 containing 0.1 mol/l KCl.

Adenosine-5′-monophosphate (Boehringer Mannheim 102199; 40 mmol/l): 5 mg were dissolved in 0.25 ml 0.1 mol/l triethanolamine buffer pH 7.5 containing 0.1 mol/l KCl.

Buffer-mixture

To a mixture of 9 ml 0.1 mol/l triethanolamine buffer pH 7.5 containing 0.1 mol/l KCl and 1 ml ethanol (Merck 972) were added 0.01 ml catalase suspension (EC 1.11.1.6; Boehringer Mannheim 106810; 10 mg/ml; ca. 13000 U) 5 mg NADP, 135 mg glycerol-2-phosphate (disodium salt), 0.02 ml of 1 mol/l potassium dihydrogenphosphate (Merck 4873) and 0.1 ml of 1 mol/l magnesium chloride (Merck 5833). pH was readjusted to 7.5.

Enzymes

Aldehyde dehydrogenase (EC 1.2.1.5; Sigma A-6758): 10 mg of the lyophilized powder (ca. 5 mg protein) were dissolved in 1 ml H_2O (ca. 50 U/ml). If necessary to remove phosphate, the enzyme was dissolved in 0.1 mol/l triethanolamine buffer pH 7.5 containing 0.1 mol/l KCl and glycerol 3 mol/l and dialyzed overnight at 4 °C against the same solution. The dialyzed enzyme was stable for one week at 4 °C.

Xanthine oxidase (EC 1.2.3.2; Boehringer Mannheim 110442; 10 mg/ml; ca. 0.4 U/mg): For elimination of $(NH_4)_2SO_4$, which produces magnesium ammonium phosphate precipitation, the enzyme suspension was dialyzed overnight at 4 °C against 0.1 mol/l KCl. The dialyzed enzyme showed an activity of about 3 U/ml and was stable for one week at 4 °C.

[2]) *D,L*-6(*m*-bromophenyl)-5,6-dihydroimidazo(2,1-b)thiazole oxalate.

Nucleoside phosphorylase (EC 2.4.2.1; Boehringer Mannheim 107964; 5 mg/ml; ca. 20 U/mg): If necessary, the enzyme suspension was dialyzed phosphate-free overnight at 4 °C against 0.1 mol/l triethanolamine buffer containing 0.1 mol/l KCl and glycerol 3 mol/l (stable for ten days at 4 °C).

Adenosine deaminase (EC 3.5.4.2; Boehringer Mannheim 102121; 10 mg/ml; 200 U/mg): The suspension was used without further dilution.

Antibodies

NaCl-polyethyleneglycol-solution: 0.15 mol/l NaCl containing 6% polyethyleneglycol 6000 (Serva 33137).

Anti-alkaline phosphatase from liver/bone (Merck 13772): 10 mg lyophilized powder were dissolved in 1 ml NaCl-polyethyleneglycol solution.

Instrumentation

In the manual versions, the NAD(P)H reduction rates were measured in an Eppendorf photometer at Hg 334 nm at 30 °C. The change in absorbance was registered continuously. The mechanized version was performed with a Gemsaec analyzer (ElectroNucleonics, Stuttgart).

The determination of alkaline phosphatase activity was performed with an Eppendorf photometer at Hg 405 nm. The absorbance at 690 nm, used for the determination of phosphate in the test system according to *Campbell* (11) and *Rieder* (10), was measured with a spectrophotometer Zeiss PMQ-II.

Methods

5'-Nucleotidase

For optimisation experiments, the reagents listed in table 1 were pipetted into a semimicro-cuvette with 10 mm optical path-length, thoroughly mixed, and incubated for 15 minutes at 30 °C. During this time, the reaction temperature was reached and traces of acetaldehyde present in the ethanol were dehydrogenated. Then a small linear rise of absorbance was registered at 334 nm, which represents the blank and has to be subtracted. The reaction was started by the addition of 5'-IMP. After a lag period of one to three minutes, depending on the 5'-nucleotidase activity, a linear rise of absorbance for at least 10 minutes was observed.

If one of the components was varied, a calculated volume of a stock solution was added to the mixture lacking this particular component. Thus, manganese replaced the magnesium ion, R 8231 the glycerol-2-phosphate, and NAD replaced NADP or was added additionally.

For routine application in clinical chemistry in the manual and mechanical version, dithioerythritol, which stabilizes the aldehyde dehydrogenase, was omitted and EDTA was added. In this case, the magnesium concentration had to be elevated. For simplification, the enzymes were added to the buffer mixture. The test composition, shown in table 2, is stable for 6 h in an ice bath.

Alkaline phosphatase

Alkaline phosphatase was measured using the test kit "alk. Phosph. opt." from Boehringer Mannheim (123862), but the reaction temperature was kept at 30 °C.

Precipitation of alkaline phosphatase by antibodies

0.5 ml serum were incubated with 0.025 ml dissolved antibodies (sufficient for at least 1 U alkaline phosphatase) and 0.475 ml NaCl-polyethyleneglycol-solution for 1 h at 37 °C and then stored for 16–18 h at 4 °C. After centrifugation, the supernatant was tested for alkaline phosphatase activity. For the control, 0.5 ml of the same serum were mixed with 0.5 ml NaCl-polyethyleneglycol solution and treated identically.

Absorption coefficients

For NADPH, the molar absorption coefficient ($\epsilon_{334\ nm}$) of 6.18×10^2 ($l \cdot mol^{-1} \cdot mm^{-1}$) and for *p*-nitrophenol ($\epsilon_{405\ nm}$) of 18.5×10^2 ($l \cdot mol^{-1} \cdot mm^{-1}$) according to *Walter* et al (35) were used.

Tab. 1. Reaction mixture for optimisation-experiments.

Reagents	µl	Concentration in the assay	Activities under present conditions
Buffer-reagent-mixture:			
9 ml 0.1 mol/l triethanolamine, pH 7.5, containing 0.1 mol/l KCl		0.09 mol/l	
		0.09 mol/l	
1 ml ethanol		1.7 mol/l	
0.01 ml catalase (10 mg/ml)			13000 U
5 mg NADP	400	0.64 mmol/l	
135 mg glycerol-2-phosphate, disodium salt		0.043 mol/l	
0.02 ml potassium phosphate (1 mol/l)		0.002 mol/l	
0.1 ml magnesium chloride (1 mol/l)		0.01 mol/l	
Dithioerythritol (1 mmol/l)	5	0.01 mmol/l	
Aldehyde dehydrogenase (10 mg/ml)	25		0.3 U
Xanthine oxidase (10 mg/ml, dialyzed)	40		0.1 U
Nucleoside phosphorylase (5 mg/ml)	5		0.25 U
Sample	50		
Incubate for 15 min, after registration of the blank, start with			
Inosine-5'-monophosphate (0.04 mol/l)	5	0.4 mmol/l	

Enzyme units

The enzyme activities are expressed in international units (U). 1 U 5'-nucleotidase represents the disappearance of 1 µmole of 5'-IMP, and 1 U of alkaline phosphatase the splitting of 1 µmole of *p*-nitrophenylphosphate per minute at 30 °C. For comparison with our method, a commercially available test kit (5'-nucleotidase, Biomérieux 6-1251 and 6-1261) was used. The determination and calculation were carried out according to the manufacturer.

Results and Discussion

Choice of substrates

In general, 5'-AMP is used as a substrate for 5'-nucleotidase. We prefer 5'-IMP at a final concentration of 0.4 mmol/l, because the same K_m-values of 0.03 mmol/l were found for 5'-IMP and 5'-AMP, and the maximal velocities were also identical. However, in the case of 5'-AMP, an extra 2 U of adenosine deaminase had to be added to the test system.

Tab. 2. Reaction mixture for the manual and mechanized version in routine application.

Reagents	µl	Concentration in the assay	Activities under present conditions
Buffer-reagent-mixture:			
9 ml 0.1 mol/l triethanolamine, pH 7.5,		0.09 mol/l	
containing 0.1 mol/l KCl		0.09 mol/l	
and 1 mmol/l EDTA		0.9 mmol/l	
1 ml ethanol		1.7 mol/l	
0.01 ml catalase (10 mg/ml)			13000 U
5 mg NADP		0.64 mol/l	
135 mg glycerol-2-phosphate, disodium salt		0.043 mol/l	
0.02 ml potassium phosphate (1 mol/l)	450	0.002 mol/l	
0.15 ml magnesium chloride (1 mol/l)		0.015 mol/l	
7 mg aldehyde dehydrogenase			0.3 U
0.14 ml nucleoside phosphorylase (5 mg/ml)			0.1 U
1 ml xanthine oxidase (10 mg/ml), dialyzed overnight against 0.1 mol/l KCl			0.25 U
Sample	150		
Start with			
Inosine-5'-monophosphate (0.04 mol/l)	5	0.4 mmol/l	
Respectively blank with			
Water	5		

pH-optimum

The optimal pH, reported by most authors between 7.2 and 7.9 (11, 13, 23, 25, 27) was found to be 7.5 in our assay with a plateau of activity between 7.25 and 7.75.

Influence of phosphate

For optimisation, it was necessary to establish the influence of phosphate; purine nucleoside phosphorylase needs phosphate as a substrate, whereas phosphate inhibits 5'-nucleotidase.

The results shown in figure 1 demonstrate that 5'-nucleotidase present in a constant volume of serum is not inhibited by up to 5 mmol/l phosphate. At a concentration of 3 mmol/l phosphate, the nucleoside phosphorylase possesses 50% of the normal activity. For this reason, a total of 3 mmol/l phosphate was chosen, so that even in the case of elevated phosphate levels in sera or tissue extracts, 5'-nucleotidase would not be inhibited.

Choice of the coenzyme

The potassium-dependent aldehyde dehydrogenase from yeast accepts NAD and NADP as coenzymes.

According to *Ellis* et al (26), the NADH-dependent test system with glutamate dehydrogenase gives only 65% of the activity obtained by measurement at 265 nm (18). We therefore examined the influence of NAD, NADP, NADH and NADPH.

Figure 2 shows the dependence of the 5'-nucleotidase activity on NAD and NADP, when the coenzymes were added separately. NADP-concentrations from 0.63 mmol/l to 1.89 mmol/l do not influence the 5'-nucleotidase activity, whereas increasing amounts of NAD from 0.11 mmol/l to 2.25 mmol/l cause increasing inhibition. If NAD, NADH or NADPH were added to the NADP-containing test system, only NAD and NADH were found to be inhibitors.

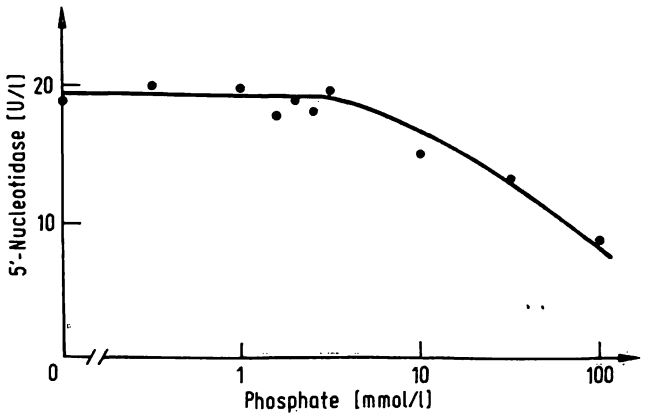

Fig. 1. Influence of phosphate on the activity of 5'-nucleotidase. Known amounts of phosphate were added to the test system of table 1. The activity of nucleoside phosphorylase, even in the samples with the lowest phosphate content, was not rate limiting.

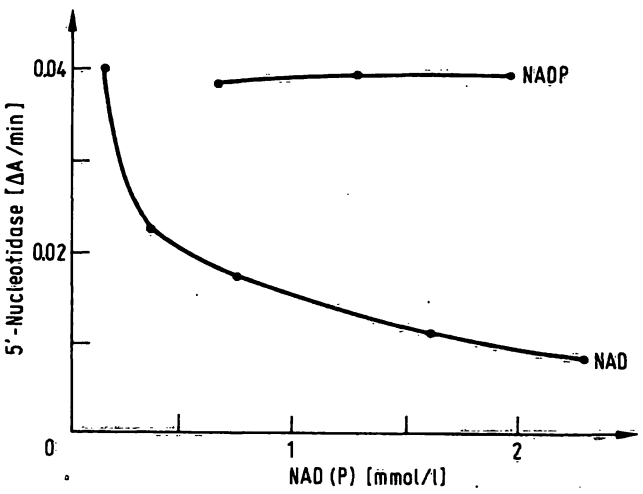

Fig. 2. Influence of NAD and NADP on the activity of 5'-nucleotidase.
Different amounts of NAD and NADP were added to the test system of table 1.

As in the assay for nucleoside phosphorylase, based on the same test principle (34), no differences between NAD and NADP is observed, the decrease of activity in the 5'-nucleotidase assay is not due to an inhibition of the indicator enzymes.

Influence of divalent cations

The activity of 5'-nucleotidase is dependent on divalent cations. Manganese and magnesium are reported to be the best activators (16, 27, 36).

With magnesium ions a plateau of maximal activity is reached at concentrations higher than 10 mmol/l.

Manganese is even more effective and would increase the sensitivity of our method, but difficulties occur with the low solubility of manganese phosphate. To introduce manganese in concentrations up to the optimum of 1 mmol/l, it is necessary to lower the phosphate concentration to 0.1 mmol/l. In this case, all indicator enzymes have to be dialyzed phosphate-free and the amount of nucleoside phosphorylase has to be increased six-fold for the same test capacity.

The manganese method cannot be recommended for the determination of 5'-nucleotidase in sera, as the sensitivity with magnesium is high enough even in sera of healthy persons. However, it is necessary for the determination of 5'-nucleotidase in lymphocyte extracts (37).

Prevention of 5'-IMP hydrolysis by unspecific phosphatases

One of the critical points in the determination of 5'-nucleotidase activity is the unspecific splitting of nucleoside-5'-monophosphates, which is reported to be caused by alkaline phosphatase present in sera and tissue extracts. Several methods have been described to bypass this difficulty.

5'-nucleotidase may be inhibited by nickel ions (10, 11, 15), concanavalin A (22, 23) or α,β-methyleneadenosine-5'-diphosphate (6), and the activity may be calculated by the difference in the presence and absence of the inhibitor. The use of nickel ions as a "specific" inhibitor for 5'-nucleotidase has already been criticized by several authors (5, 14, 16), because the activity of alkaline phosphatase from serum (14) and human bone (38) was found to be considerably effected by nickel ions. The same is said for α,β-methyleneadenosine-5'-diphosphate (23). In our hands, the addition of concanavalin A to our test system containing the serum sample caused a precipitate probably due to glycoproteins and was not suitable for a spectrophotometric assay.

Alternatively, the activity of alkaline phosphatase may be inhibited by *L*-amino acids such as *L*-histidine (36, 38, 39), *L*-cysteine (14, 39) or *L*-glycine (7, 40). The influecne of amino acids is obscure, as detailed studies have only been described for bull seminal plasma (36, 38).

Another possibility for the elimination of nucleoside-5'-monophosphate hydrolysis is the simultaneous determination of alkaline phosphatase at pH 7.5 with 2'-AMP and 3'-AMP as substrates, or at pH 9.8 with *p*-nitrophenylphosphate, from which the interference of alkaline phosphatase with the 5'-nucleotidase assay can be calculated using an empirical correction factor (16).

Furthermore, the technique of "enzyme diversion" according to *Belfield & Goldberg* (41) may be applied where high concentrations of glycerol-2-phosphate are offered to the alkaline phosphatase resulting in a competitive displacement of the nucleoside-5'-monophosphate from the active center; phenylphosphate may substitute for glycerol-2-phosphate (11), but there is no advantage (41). Among these possibilities, we chose first the technique of "enzyme diversion". Figure 3 shows some typical results with a serum of a patient suffering from liver disease. Identical figures were obtained with sera of patients suffering from bone diseases with high osteoblastic activity.

Our results agree well with the findings of *Belfield* et al. (19, 41, 42) and *Ellis* et al. (26). For further experiments, we chose a concentration of 43 mmol/l glycerol-2-phosphate, corresponding to a 100-fold excess over 5'-IMP.

The phosphate production from glycerol-2-phosphate by unspecific phosphatases during the incubation period and during the test time, which might have an inhibitory effect on 5'-nucleotidase (see figure 1), is negligible. According to *Beckman* et al. (16) we can estimate the phosphate production by a 100 µl sample of pathologic serum containing 1000 U/l alkaline phosphatase at 50 nmol/l phosphate after 15 minutes.

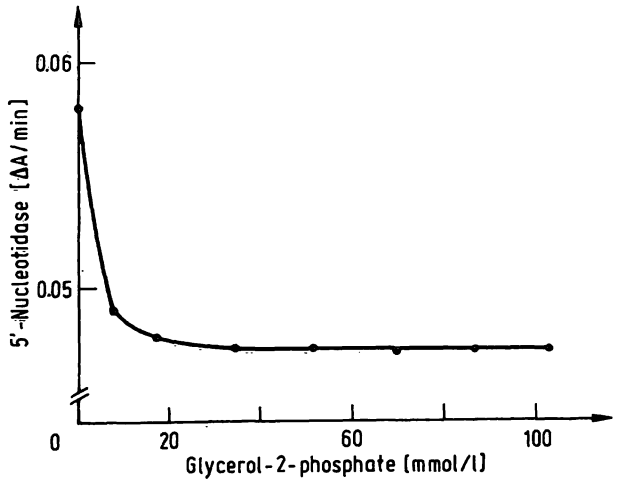

Fig. 3. Inhibition of unspecific phosphatase activity by glycerol-2-phosphate.
Known amounts of glycerol-2-phosphate were added to the test system of table 1, which contains a serum sample with high activity of alkaline phosphatase.

The commercial availability of sheep antibodies against human alkaline phosphatase from liver and bone offers the possibility of a specific elimination of the interfering alkaline phosphatase. By treatment of sera containing high activities of alkaline phosphatase with antibodies, the activity was reduced to 1–20%. Table 3 shows the results for a selection of sera. Of course, the treatment with antibodies reduces the unspecific hydrolysis of 5'-IMP at pH 7.5. However, the addition of glycerol-2-phosphate to antibody-treated sera reduces the hydrolysis even further, an effect, which could no longer be explained by the small residual activity of alkaline phosphatase. In the presence of glycerol-2-phosphate, 5'-IMP hydrolysis was nearly identical in antibody-treated and untreated sera.

A new possibility for the very specific and effective inhibition of alkaline phosphatase is the application of the anthelmintic levamisole analogue R 8231 (43, 44). With R 8231 in a concentration of 3 mmol/l, we could block the alkaline phosphatase activity at pH 9.8 in sera almost completely (99%). In our test system, we found a reduction of total 5'-IMP hydrolysis at pH 7.5 with R 8231 corresponding well to the diminution of 5'-IMP hydrolysis after antibody elimination of alkaline phosphatase.

From the data cited above, we assume that the unspecific 5'-IMP hydrolysis at pH 7.5 is not only caused by alkaline phosphatase, but may be due to other phosphatases, e.g. acid prostatic phosphatase, present in sera, the activity of which was eliminated by glycerol-2-phosphate. For this reason, we prefer the technique of "enzyme diversion" with glycerol-2-phosphate.

Tab. 3. Influence of glycerol-2-phosphate before and after treatment of sera with anti-alkaline phosphatase from liver/bone.

	Activities (U/l)			
Alkaline phosphatase before antibodies	1300	280	3500	1075
Alkaline phosphatase after antibodies	10	48	126	5
Difference	1290	232	3374	1070
5'-Nucleotidase before antibodies				
Without glycerol-2-*P*	6.4	10.2	40.3	2.0
Glycerol-2-*P* added	4.8	9.5	36.8	0.5
Difference ± glycerol-2-*P*	1.6	0.7	3.5	1.5
5'-Nucleotidase after antibodies				
Without glycerol-2-*P*	5.2	9.8	37.6	0.5
Glycerol-2-*P* added	4.8	9.3	37.2	0.4
Difference ± glycerol-2-*P*	0.4	0.5	0.4	0.1

Tab. 4. The within-run imprecision of the determination of the catalytic 5'-nucleotidase activity (n = 10).

	I (sample blank)	II[1] (I + substrate)
Manual procedure		
Mean value (U/l)	0.92	12.8
Standard deviation (U/l)	0.45	0.85
CV[2] (%)	48.6	6.6
Mean value (U/l)	0.14	3.05
Standard deviation (U/l)	0.31	0.30
CV (%)	22.1	9.7
Mechanized procedure (Gemsaec)		
Mean value (U/l)	1.48	12.75
Standard deviation (U/l)	0.11	0.38
CV (%)	7.6	3.0
Mean value (U/l)	0.39	3.25
Standard deviation (U/l)	0.11	0.11
CV (%)	28.2	3.3

[1]) Individual sample blank subtracted
[2]) CV, coefficient of variation

Precision

Several series of ten determinations were performed with human serum samples manually and with a Gemsaec analyzer. The coefficient of variation varied between 3.0 and 9.7% (tab. 4).

Detection limit

The minimal enzyme activity detectable with our method is 0.05 U in a cuvette volume of 0.6 ml corresponding to 0.24 U/l sample if 0.2 ml of sample was added.

Linearity

Figure 5 shows a linear correlation between the amount of sample added and the change of absorbance up to 0.04 ΔA/min.

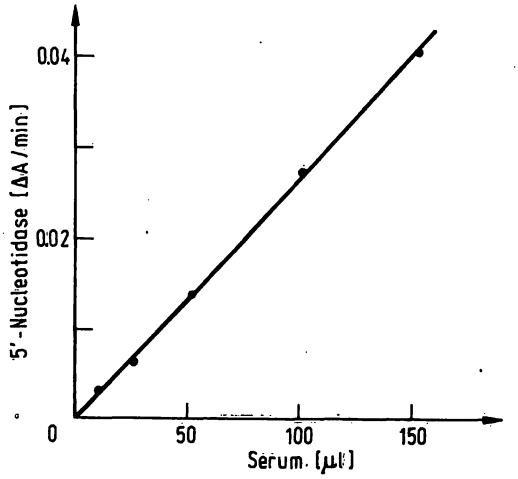

Fig. 4. Dependence of the absorbance change per minute at 334 nm on the amount of 5'-nucleotidase added.

Comparison of methods

For comparison, we chose a commercially available test kit from Biomérieux, Nürtingen, which uses the liberation of phosphate from 5'-AMP as an indicator for 5'-nucleotidase activity. The 5'-nucleotidase activity is calculated from the amount of phosphate liberated in the presence and absence of nickel ions (tab. 5).

The correlation between our method and the commercially available method was examined by means of linear regression analysis; the resulting coefficient value of $r = 0.88$ ($n = 6$) indicated a poor correlation.

Tab. 5. Comparison of our method with a commercially available test kit, using the liberation of phosphate from 5'-AMP in the presence and absence of nickel ions according to *Campbell* (11) and *Rieder* (10).

Serum	5'-Nucleotidase activity (U/l)	
	Determined with our method	Determined with the commercially available method
1	1.89	8.56
2	2.27	11.73
3	2.89	12.29
4	2.60	10.42
5	1.47	5.40
6	1.89	5.77

The high values obtained for 5'-nucleotidase with the assay according to *Campbell* (11) and *Rieder* (10) may have several causes. One reason may be the higher temperature in the commercially available method (37 °C) compared with ours (30 °C).

Furthermore, not only 5'-nucleotidase, but alkaline phosphatase (14, 16) as well as other unspecific phosphatases, which are not yet well characterized, may be inhibited by nickel ions. In this way, the activity of alkaline phosphatase and unspecific phosphatases is diminished. An additional decrease of colour development is found in the presence of nickel ions, because of precipitation or complex-binding of phosphate by Ni^{2+}; this is not controlled by an appropriate blank containing nickel but no sample.

Clinical application and reference values will be reported separately.

Acknowledgement

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