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Simultaneous Determination of 5'-Nucleotidase and Alkaline Phosphatase Activities in Serum

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Summary: A simple method is described for the simultaneous determination of alkaline phosphatase (EC 3.1.3.1) and 5'-nucleotidase (EC 3.1.3.5) in serum. The method is based on the determination of inorganic phosphorus released by the action of the two enzymes on adenosine-5'-monophosphate at pH 9.5 (200 mmol/l tris-buffer) in the presence and absence of *L*-cysteine. This amino acid at a concentration of 2–10 mmol/l was found to be a specific inhibitor for alkaline phosphatase but with no effect on 5'-nucleotidase activity.

Simultanbestimmung der Aktivitäten von 5'-Nucleotidase und alkalischer Phosphatase im Serum

Zusammenfassung: Eine einfache Methode zur gleichzeitigen Bestimmung von alkalischer Phosphatase (EC 3.1.3.1) und 5'-Nucleotidase (EC 3.1.3.5) im Serum wird beschrieben. Die Methode beruht auf der Bestimmung des durch beide Enzyme aus 5'-AMP bei pH 9,5 (200 mmol/l Trispuffer) freigesetzten Phosphats in Gegenwart und Abwesenheit von *L*-Cystein. 2–10 mmol/l Cystein erwiesen sich als spezifischer Hemmstoff für alkalische Phosphatase ohne Effekt auf die Aktivität von 5'-Nucleotidase.

Introduction

Phosphatase was the first enzyme of diagnostic importance to be recognized (1). Alkaline phosphatase and 5'-nucleotidase determination in serum have proved to be satisfactory diagnostic enzymes. Serum alkaline phosphatase is raised in a variety of conditions particularly in hepatobiliary or osteoblastic disorders (2–5). Serum activity can be determined by measuring the rate of hydrolysis of a suitable primary phosphate ester at an alkaline pH (6–14).

5'-Nucleotidase is an alkaline phosphomonoesterase which was first discovered by *Reis* (15), who showed that it is activated by magnesium and inhibited by nickel ions. Its activity is confined to the hydrolysis of nucleotide pentose-5'-monophosphate group. Elevated levels of serum 5'-nucleotidase activity are almost exclusively confined to hepatobiliary disease, especially obstructive jaundice (2–5). In contrast to serum alkaline phosphatase, normal activities of 5'-nucleotidase occur in *Paget*'s and other bone diseases (4). These observations have been confirmed by *Young* (3). The main difficulty in determing 5'-nucleotidase activity is the interference of alkaline phosphatase. Trials have been carried out by many investigators to eliminate this interference, but without satisfactory results (2, 3, 4).

The aim of the present study is to establish a method for measuring simultaneously the activities of 5'-nucleotidase and alkaline phosphatase in serum. The method is based on the determination of the inorganic phosphorus released by the action of the two enzymes on adenosine-5'-monophosphate at pH 9.5 in tris-buffer (200 mmol/l) in the presence and absence of *L*-cysteine. This amino acid was found to be a specific inhibitor for serum alkaline phosphatase but with no effect on 5'-nucleotidase activity.

Materials and Methods

Reagents required

for the established method mentioned in table 1, are:

1. Buffer/activator (0.4 mol/l tris-HCl buffer pH 9.5/0.02 mol/l Mg^{++})

   Dissolve 24.2 g. Tris(hydroxymethyl)methylamine, and 2.0 g of $MgCl_2 \cdot 6H_2O$ (A.R grade) in about 480 ml distilled water. Adjust the pH to 9.5 with HCl and dilute to 500 ml with water. The solution is stable at 4 °C.

2. Alkaline phosphatase inhibitor (0.1 mol/l *L*-cysteine)

   Dissolve 0.31 g of *L*-cysteine hydrochloride in about 15 ml distilled water. Adjust the pH to 9.5 with 100 g/l NaOH, dilute to 20 ml with water. The solution is stable for only four days, in closed container at 4 °C.

Tab. 1.

Procedure:

To a series of test tubes the amounts of the following reagents are added

	A	B	Test blank	standard	Reagent blank
	ml	ml	ml	ml	ml
Tris-buffer/Mg^{++}	0.5	0.5	0.5	–	–
Distilled water	0.1	–	0.1	–	–
L-cysteine	–	0.1	–	–	–
Serum	0.2	0.2	0.2	–	–
	Mix, incubate at 37 °C for 5 min then add,				
Substrate	0.2	0.2	–	–	–
	Incubate at 37 °C for 60 min, stop the reaction by adding				
Cold trichloroacetic acid	3.0	3.0	3.0	–	–
Substrate	–	–	0.2	–	–
	Mix, stand for 5 min, centrifuge take aliquote of,				
	2.0	2.0	2.0	–	–
Standard	–	–	–	0.5	–
Trichloroacetic acid	1.0	1.0	1.0	2.5	3.0
Ammonium molybdate	0.5	0.5	0.5	0.5	0.5
Ascorbic acid	0.5	0.5	0.5	0.5	0.5
	Mix, stand for 20 min, read at 640–720 nm against the test blank for the test and against reagent blank for the standard				

A = Total phosphatase activities
B = 5′-Nucleotidase activity
A–B = Alkaline phosphatase activity

Calculation:

$$\frac{\text{E 640 test}}{\text{E 640 standard}} \times 0.64\ (\mu\text{mol } P_i) \times \frac{1000\ (\text{per litre})}{60\ (\text{time}) \times 0.1\ (\text{ml of serum used})}\ [\text{U/l}]$$

3. Buffered substrate (0.025 mol/l adenosine-5′-monophosphate)

   Dissolve 0.4 g of adensoine-5′-monophosphate di-sodium salt in 40 ml of 0.05 mol/l tris-HCl pH 9.5. This solution is stable at – 20 °C.

4. Trichloroacetic acid

   Dissolve 100 g in one litre of distilled water and keep at 4 °C.

5. Ammonium molybdate.

   Dissolve 10 g in one litre of distilled water. The solution is stable at room temperature.

6. Reducing agent solution

   Use freshly prepared ascorbic acid at a concentration of 2 g/l.

7. Standard phosphorus solution.

   Dissolve 43.92 mg of potassium dihydrogen phosphate in 250 ml distilled water and add a few drops of chloroform. Use 0.5 ml of this solution which contains 0.64 µmol phosphorus. Keep at 4 °C.

Enzyme assay

5′-Nucleotidase and alkaline phosphatase activities were assayed according to the method outlined in Table 1. Through the course of this studies, unless stated otherwise, F^- was 10 mmol/l; CN^- was 5 mmol/l and *L*-cysteine was 10 mmol/l. Adenosine-5′-monophosphate (5 mmol/l) and β-glycerophosphate (5 mmol/l) were used in parallel in this investigation. Tris-buffer (200 mmol/l) was used to cover the range pH 7.5 to 9.5 while glycine buffer (50 mmol/l) was used for the range pH 9.5 to 10.5. The pH was checked after the addition of all reagents and no change was observed. The activity assayed in the presence of 5′-AMP as substrate corresponds to both 5′-nucleotidase and alkaline phosphatase while that assayed in the presence of β-glycerophosphate corresponds to alkaline phosphatase.

Results

The pH activity curves of human liver homogenate with 5′-AMP and β-glycerophosphate as substrate are shown in figure 1 and 2 respectively. While those for kidney homogenate are shown in figure 3 and 4. As shown in figure 1, 5′-nucleotidase was completely inhibited by F^- while alkaline phosphatase of liver was inhibited by 35 % at pH 9.5 (fig. 2). Also it was found that 5′-nucleotidase of both liver and kidney were not affected by CN^- or *L*-cysteine while alkaline phosphatase was almost completely inhibited by these agents. As shown in figure 1 and 3, 5′-nucleotidase showed a broad pH-activity curve (pH 7.5–9.5) while alkaline phosphatase showed a narrower range (pH 9.5 to 10.5) as illustrated in figure 2 and 4. It is worth noting that the activity of alkaline phosphatase in glycine buffer at pH 9.5 is lower than that in tris-buffer at the same pH; an exception was found with liver and 5′-AMP where the higher values were obtained with glycine buffer (fig. 1).

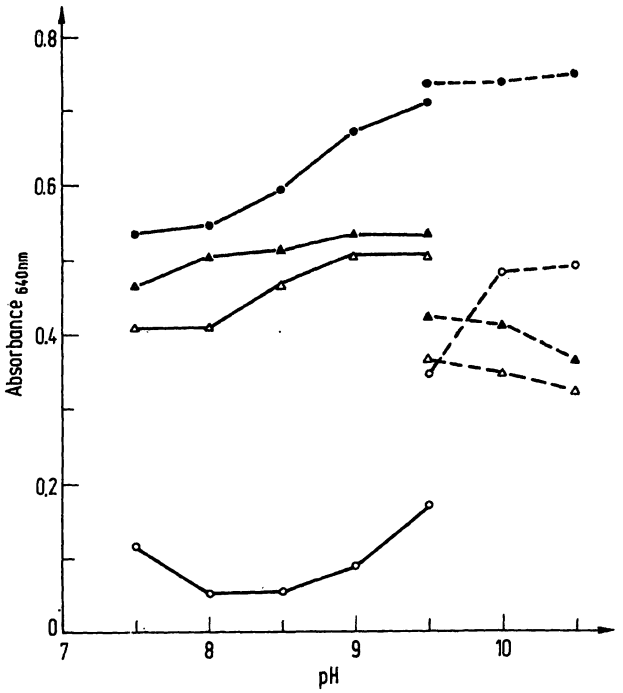

Fig. 1. pH-Activity curve of human liver homogenate, 5'-AMP (5 mmol/l) as substrate. (——) Tris buffer 200 mmol/l and (– – –) glycine buffer 50 mmol/l. (•) None, (o) 10 mmol/l F^-, (▲) 5 mmol/l CN^- and (△) 10 mmol/l *L*-cysteine.

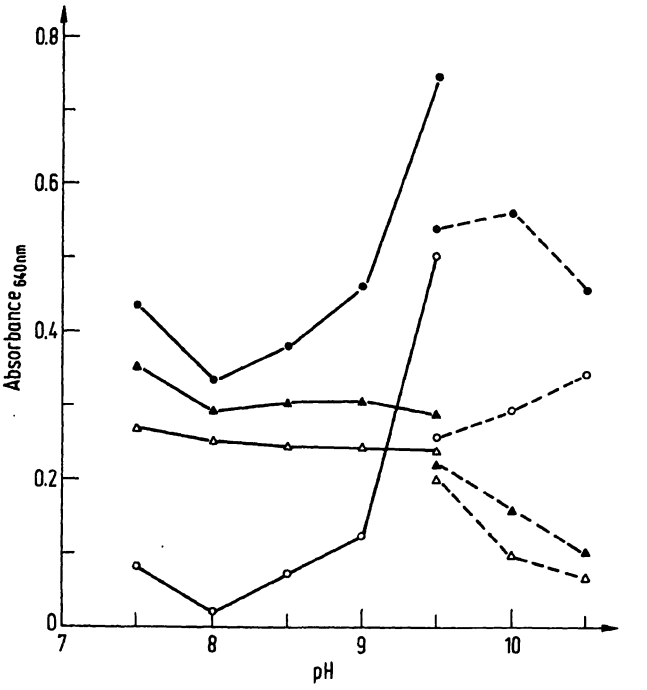

Fig. 3. pH-Activity curve of human kidney homogenate, 5'-AMP (5 mmol/l) as substrate. (——) Tris-buffer 200 mmol/l and (– – –) glycine-buffer 50 mmol/l. (•) None, (o) 10 mmol/l F^-, (▲) 5 mmol/l CN^-, and (△) 10 mmol/l *L*-cysteine.

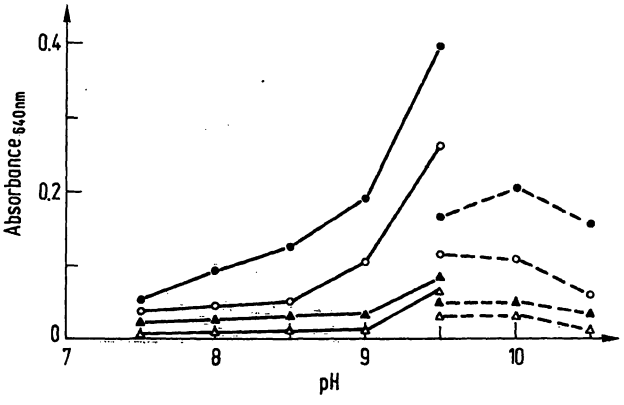

Fig. 2. pH-Activity curve of human liver homogenate, β-glycerophosphate (5 mmol/l) as substrate. (——) Tris buffer 200 mmol/l) (– – –) glycine buffer 50 mmol/l. (•) None, (o) 10 mmol/l F^-, (▲) 5 mmol/l CN^-, and (△) 10 mmol/l *L*-cysteine.

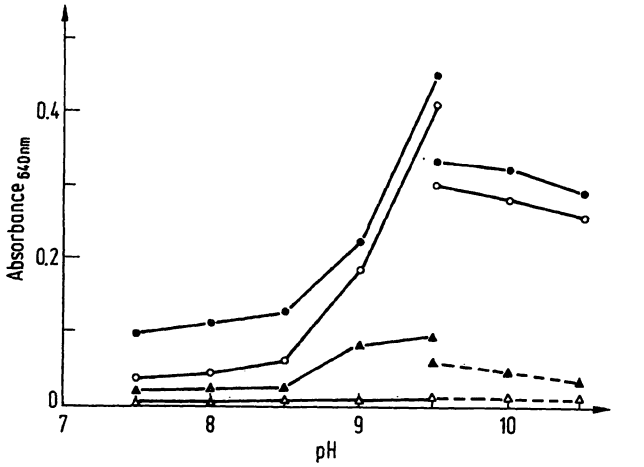

Fig. 4. pH-Activity curve of human kidney homogenate, β-glycerophosphata (5 mmol/l) as substrate. (——) Tris-buffer 200 mmol/l and (– – –) glycine-buffer 50 mmol/l. (•) None, (o) 10 mmol/l F^-, (▲) mmol/l CN^-, and (△) 10 mmol/l *L*-cysteine.

A comparative study was carried out to determine whether alkaline phosphatase isoenzymes of animal tissues are different in their behaviour from those of human, and whether the different alkaline phosphatase isoenzymes of human tissue homogenates or purified human alkaline phosphatase preparation showed different sensitivities towards F^-, CN^- and cysteine. The result is represented in table 2. Activities of 5'-nucleotidase and alkaline phosphatase were measured at pH 9.5 which was found to be in the optimal range for both enzymes. Generally, F^- inhibit 5'-nucleotidase with no or little effect on alkaline phosphatase while *L*-cysteine or CN^- inhibit alkaline phosphatase but not 5'-nucleotidase. However, only in mouse liver homogenate was alkaline phosphatase inhibited by 60 % in the presence of F^- and not affected by CN^- or *L*-cysteine. Alkaline phosphatase of washed heavy particles (which contain nuclei and plasma membrane, the latter being the site of alkaline phosphatase and 5'-nucleotidase) prepared from mouse liver homogenate was not affected by F^- and it was completely inhibited either by CN^- or *L*-cysteine.

In the homogenate, *L*-cysteine or CN^- inhibit the alkaline phosphatase of human liver by 75 %, and the alkaline

Tab. 2. The effect of fluoride, cyanide and *L*-cysteine on 5′-nucleotidase and alkaline phosphatase activities in animal and human tissues, at pH 9.5 (tris-buffer)

Agent	Type of tissue		Liver			Kidney			Intestine		
			5′-AMP	β-GP	*p*-NPP	5′-AMP	β-GP	*p*-NPP	5′-AMP	β-GP	*p*-NPP
None	Mouse	Homogenate[a]	3.0	0.71	0.68	17.0	19.0	20.0	28.0	34.0	21.0
		Heavy particles[b]	0.7	0.15	–	–	–	–	–	–	–
	Human	Homogenate[a]	2.45	1.25	1.26	3.05	1.50	2.30	23.0	25.5	22.5
		Purified[c]	3.2	4.0	3.8	1.12	1.37	1.07	3.80	5.8	4.0
10 mmol/l F^-	Mouse	Homogenate[a]	32	38	37	59	93	100	93	95	100
		Heavy particles[b]	11	85	–	–	–	–	–	–	–
	Human	Homogenate[a]	50	84	92	33	80	70	98	100	98
		Purified[c]	100	100	100	100	100	100	100	100	100
5 mmol/l CN^-	Mouse	Homogenate[a]	108	95	95	58	14	31	15	3	18
		Heavy particles[b]	91	10	–	–	–	–	–	–	–
	Human	Homogenate[a]	80	25	27	84	10	43	16	11	48
		Purified[c]	10	10	14	8	7	0	3	3	6
10 mmol/l *L*-Cysteine	Mouse	Homogenate[a]	153	95	90	50	8	31	15	1	23
		Heavy particles[b]	130	10	–	–	–	–	–	–	–
	Human	Homogenate[a]	82	26	23	84	10	39	16	7	48
		Purified[c]	0	0	0	3	4	0	0	0	3

Results tabulated as:

Activity values expressed as μmol P_i/min · g wet tissue and as μmol P_i/min · mg protein purified enzyme resp. as per cent, taking activity with Mg^{++} alone as 100 per cent

a) Tissues were homogenized in distilled water and aliquots equivalent to 10 mg of liver and 2 mg of kidney or intestine were used for the enzyme assay with an appropriate period of incubation.

b) Heavy particles was prepared by centrifuging mouse liver homogenate at 1000 *g* for 10 min; then the pellet was washed three times with distilled water.

c) Purified preparation of alkaline phosphatase from human liver, kidney and intestine according to *Schlamowitiz* (16).

β-GP = β-glycerophosphate; *p*-NPP = *p*-nitrophenylphosphate

phosphatase of kidney and intestine by 90 %. A preparation of purified human alkaline phosphatase isoenzymes was completely inhibited when 5′-AMP, β-glycerophosphate or *p*-nitrophenylphosphate were used as substrates. These results indicate that mouse liver alkaline phosphatase in tissue homogenates differs from that of human with respect to the effect of F^-, CN^- and *L*-cysteine, whereas in human tissue homogenate the liver alkaline phosphatase isoenzyme was less inhibited by *L*-cysteine than the kidney or intestine isoenzymes.

The effect of different concentrations of Ni^{++}, F^-, CN^- and *L*-cysteine on the activities of serum 5′-nucleotidase and alkaline phosphatase are shown in figure 5 and 6. When 5′-AMP was used as substrate (fig. 5), F^- (5–10 mmol/l) inhibited 5′-nucleotidase but not alkaline phosphatase. In the presence of *L*-cysteine (2–10 mmol/l) or CN^- (2–5 mmol/l) alkaline phosphatase, but not 5′-nucleotidase was completely inhibited. Nickel ions at a concentration of 1–10 mmol/l were found to inhibit both enzyms, (under these condition Ni^{++} was not precipitated).

Using 200 mmol/l tris-buffer and 5′-AMP as substrate, in the presence of *L*-cysteine or CN^-, a broad range for the maximal activity of serum 5′-nucleotidase was obtained between pH 7.5–9.5 and it was completely inhibited by F^- as shown in figure 7. On the other hand alkaline phosphatase showed a narrower range (pH 9.5–10.5) in the presence of F^- and was completely inhibited by CN^- and *L*-cysteine as illustrated in figure 8.

The time course of 5′-nucleotidase and alkaline phosphatase activities in normal serum, using 5′-AMP, was linear over three hours incubation as demonstrated in figure 9.

The rates of hydrolysis of 5′-AMP, β-glycerophosphate and *p*-nitrophenylphosphate by serum phosphatases and by purified alkaline phosphatase of human liver, kidney and intestine, in the presence and absence of F^- or *L*-cysteine are shown in table 3.

Serum 5′-nucleotidase was found to be activated by Mg^{++} more than alkaline phosphatase. The optimal Mg^{++} concentration for maximal 5′-nucleotidase and alkaline phosphatase activities was found to be in the range of 5–10 mmol/l. 5′-AMP, at a concentration of 20 mmol/l slightly inhibited alkaline phosphatase, while a concentration of 5–10 mmol/l was found to be sufficient for the assay of both 5′-nucleotidase and alkaline phosphatase.

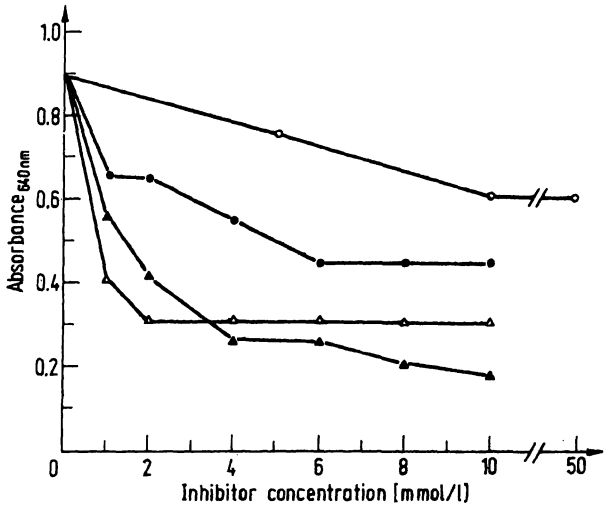

Fig. 5. Effect of different inhibitor concentrations on the activities of serum 5'-nucleotidase and alkaline phosphatase at pH 9.5 tris-buffer (200 mmol/l). 5'-AMP (5 mmol/l as substrate. (o) F^-, (•) Ni^{++}, (▲) CN^-, and (△) *L*-cysteine.

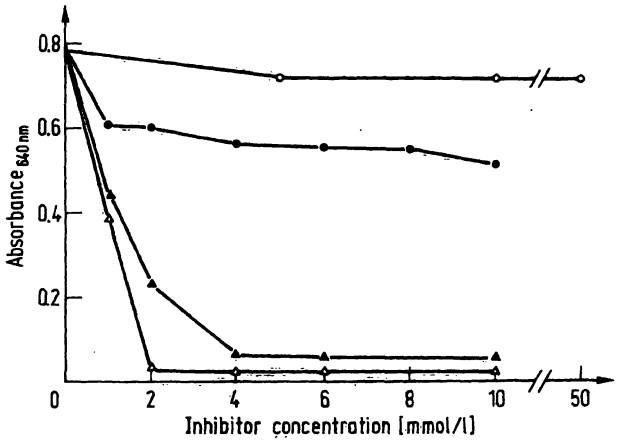

Fig. 6. Effect of different inhibitor concentrations on the activity of serum alkaline phosphatase at pH 9.5 tris-buffer (200 mmol/l), β-glycerophosphate (5 mmol/l) as substrate. (o) F^-, (•) Ni^{++}, (▲) CN^- and (△) *L*-cysteine.

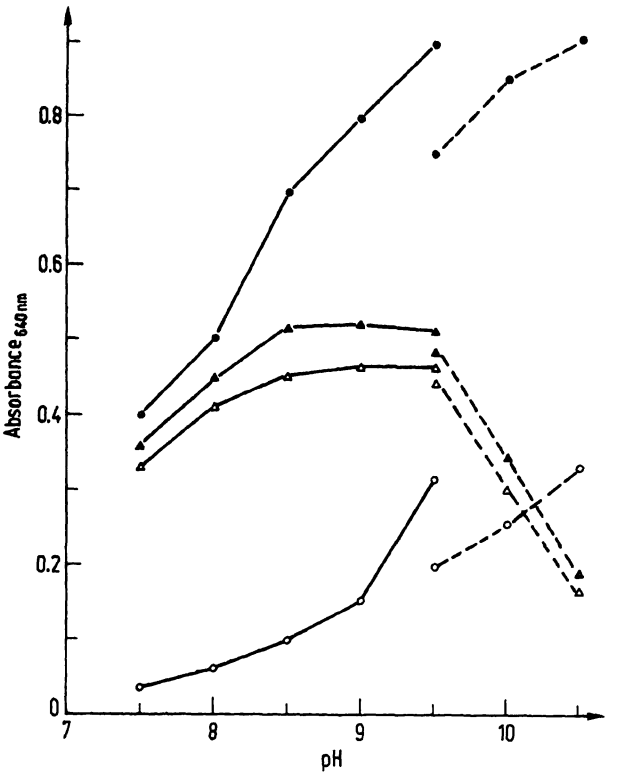

Fig. 7. pH-Activity curve of human serum of patient with liver metastases, 5'-AMP (5 mmol/l) as substrate. (——) Tris buffer 200 mmol/l and (– – –) glycine buffer 50 mmol/l. (•) None, (o) 10 mmol/l F^-, (▲) 5 mmol/l CN^- and (△) 10 mmol/l *L*-cysteine.

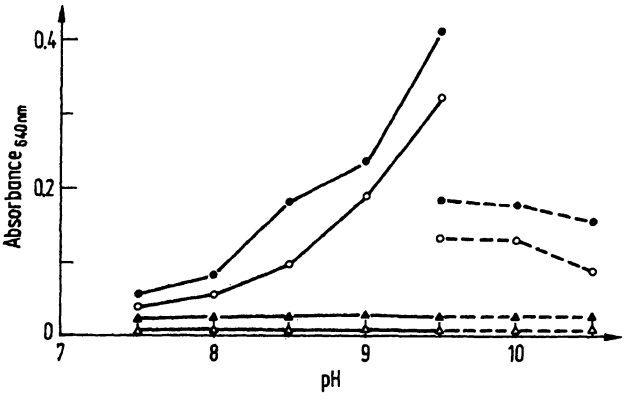

Fig. 8. pH-Activity curve of human serum of patient with liver metastases., β-glycerophosphate (5 mmol/l) as substrate. (——) Tris-buffer, 200 mmol/l, (– – –) glycine-buffer 50 mmol/l. (•) None, (o) 10 mmol/l F^-, (▲) 5 mmol/l CN^- and (△) 10 mmol/l *L*-cysteine.

Tab. 3. Rate of hydrolysis of 5'-AMP, β-glycerophosphate (β-GP) and *p*-nitrophenylphosphate (*p*-NPP) with human serum (liver metastases) and purified alkaline phosphatase of human liver, kidney, and intestine.

Substrate 5 mmol/l	Serum			Liver			Kidney			Intestine		
	None	F^-	*L*-cysteine	None	F^-	*L*-cysteine	None	F^-	*L*-cysteine	None	F^-	*L*-cysteine
5'-AMP	(232)	(132)	(104)	(3.2)	(3.2)	(0.0)	(1.0)	(1.1)	(0.0)	(3.8)	(3.8)	(0.0)
	100	100	100	100	100	–	100	100	–	100	100	–
β-GP	70	140	2	127	130	–	155	150	–	150	140	–
p-NPP	56	107	2	82	80	–	100	88	–	100	100	–

Activity with 5'-AMP substrate was taken as 100 %

( ) Activity expressed as μmol P_i/min · mg protein for purified enzyms, and as U/l for serum.

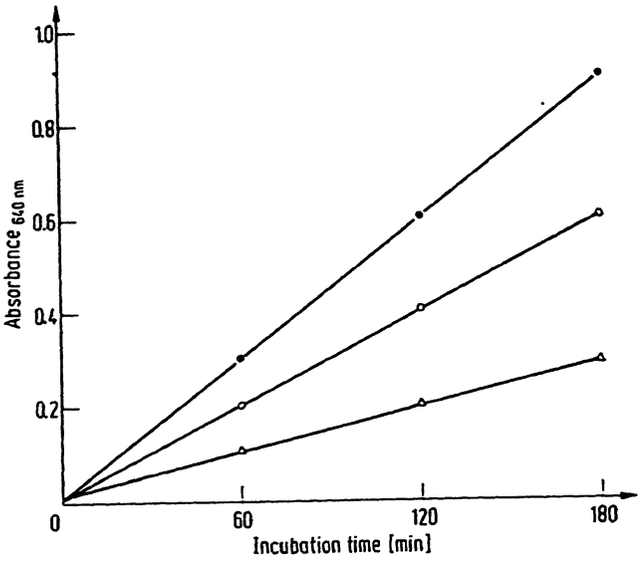

Fig. 9. Time-activity course of human serum 5'-nucleotidase and alkaline phosphatase in absence (•) and presence of 10 mmol/l F^- (o) and 10 mmol/l *L*-cysteine (△), 5'-AMP (5 mmol/l) as substrate. 200 mmol/l tris-buffer pH 9.5.

The effect of different concentration of tris-buffer (pH 9.5) on the activities of 5'-nucleotidase and alkaline phosphatase was tested. The activity of 5'-nucleotidase in the presence of 25–200 mmol/l tris was not affected, while 25–50 mmol/l was not sufficient for optimal activity. Accordingly 100–200 mmol/l was found to be convenient for the assay of both enzymes.

The level of serum 5'-nucleotidase and alkaline phosphatase activities in normal and different pathological conditions are represented in table 4. Cases with elevated 5'-nucleotidase and alkaline phosphatase (hepatoma, and liver metastases) as well as cases with normal 5'-nucleotidase and elevated alkaline phosphatase (lymphoma with bone metastases) are included to evaluate the applicability of the method.

Tab. 4. The level of serum 5'-nucleotidase and alkaline phosphatase activities in normal and in different pathological conditions of Egyptian subjects.

Clinical diagnosis	No. of cases	U/l 5'-Nucleotidase	U/l Alkaline Phosphatase
Normal adult	204	9.0 ± 4	21 ± 6
Carcinoma of head of pancreas	5	144.0 ± 63	130 ± 54
Hepatoma	10	63.0 ± 46	49 ± 26
Breast cancer with liver metastases	10	63.0 ± 33	54 ± 35
Lymphoma with bone metastases	8	13.5 ± 4.5	55 ± 22
Lymphoma with liver metastases	10	79.0 ± 38	87 ± 24

Activity measured using 5'-AMP as substrate in presence and absence of 10 mmol/l *L*-cysteine.

Discussion

Determination of 5'-nucleotidase activity in serum is based on either measuring the amount of inorganic phosphorus released by the action of the enzyme according to *Fiske & Subbarow* (17, 18), the decrease in the optical density of adenosine, released by deamination of the adenosine-5'-monophosphate in the presence of adenosine deaminase (19), or by measuring the amount of ammonia, (which is proportional to the 5'-nucleotidase activity) released after deamination of adenosine, by the indophenol reaction (20, 21) first reported by *Berthelot* (22).

The phosphorus method used for 5'-nucleotidase determination in serum is essentially based on measuring the enzyme activity in the presence and absence of 1 mmol/l Ni^{++}. The difference in activities is assumed to represent the specific 5'-nucleotidase activity. Criticism had been made against using Ni^{++} (21) since it was found to inhibit human bone and liver alkaline phosphatase (23, 24). *Young* (3) corrected for non-specific phosphatase by preincubating serum with 1.5 mmol/l EDTA which should inactivate more alkaline phosphatase than 5'-nucleotidase. *Kowlessar* and coworkers (2) estimate 5'-nucleotidase in the presence of high concentration of Mg^{++} which would inhibit alkaline phosphatase completely. Any proof for these conditions of inactivation is lacking in their reports. *Dixon & Purdon* (4) estimated the alkaline phosphatase contribution to the hydrolysis of 5'-AMP by measuring the hydrolysis of phenyl phosphate at pH 7.5. By subtracting the latter activity from the activity with 5'-AMP the specific 5'-nucleotidase activity would be determined. The disadvantages of this correction lies in the fact that the rate of hydrolysis of both substrates by the different isoenzymes of alkaline phosphatase is not equal (25).

All methods mentioned above for determination of 5'-nucleotidase were inadequate in preventing the interference of alkaline phosphatase. In the present study we were able to completely and specifically inhibit alkaline phosphatase in serum by *L*-cysteine at a concentration of 2–10 mmol/l, without any effect on 5'-nucleotidase activity (Table 3, fig. 7 and fig. 8).

Agus et al (26) reported that cysteine and its analogues inhibit alkaline phosphatase of human kidney and that from different tissues, cell cultures and *E. coli*. They found that the inhibition can be reversed by H_2O_2, Zn^{++} or passage through Sephadex G-25. They suggested that *L*-cysteine inhibits alkaline phosphatase by chelation of the zinc atom at the active site of the enzyme. *Cox & Macleod* (27) reported also that alkaline phosphatase of established human cell cultures was repressed by cysteine or cystine. They claimed that this repression is caused by the interference with enzyme synthesis or activation rather than by SH inhibition of alkaline phosphatase.

It was reported by *Fishman* et al. (28) that *L*-cysteine and analogues inhibit intestinal alkaline phosphatase but spare that of liver. Our results on tissue homogenates of mouse agreed with their findings, but we found that *L*-cysteine inhibits alkaline phosphatase of human liver homogenate by only 75 %.

Purified human liver alkaline phosphatase (Table 2), serum alkaline phosphatase of liver origin (fig. 8) and alkaline phosphatase of washed heavy particles isolated from mouse liver homogenate were completely inhibited by *L*-cysteine (Table 2). These data indicate the possibility of an endogenous component in liver homogenate which stops or abolishes the action of *L*-cysteine and CN^- on liver alkaline phosphatase.

Therefore, in so far as the different alkaline phosphatase isoenzyme is present in the soluble form (serum) or free from other cell components (washed heavy particle from liver homogenate or purified alkaline phosphatase), *L*-cysteine can be used as a specific alkaline phosphatase inhibitor, especially if we take into consideration that *L*-cysteine (2–10 mmol/l) has no inhibitory effect on serum 5′-nucleotidase.

It is of interest to notice that *L*-cysteine activates 5′-nucleotidase in liver homogenate by 30–50 %, but not in kidney, intestine or serum (Table 2). Although the mechanism of activation is not yet clear, it might be due to the removal of endogenous inhibitor.

Using tris-buffer (200 mmol/l), serum 5′-nucleotidase showed a broad pH optimum (7.5–9.5) which allows the enzymatic assay of both alkaline phosphatase and 5′-nucleotidase to be carried at pH 9.5. Our results with glycine buffer agreed with those reported by *Hausamen* et al (29) who found that glycine had an inhibitory effect on alkaline phosphatase. The rate of hydrolysis of 5′-AMP by alkaline phosphatase under these condition at pH 9.5 was equal to that of *p*-nitrophenylphosphate and less than β-glycerophosphate by about 40 %. In normal adult Egyptian subjects (204 cases), using this method, the 5′-nucleotidase level in serum was found to be 9.0 ± 4 U/l (ranging from 2–14) which is in good agreement with the reported values (18, 20). However the serum alkaline phosphatase level was found to be 21 ± 6 U/l. In the serum of different pathological conditions the results were in good agreement with the clinical diagnosis as shown in cases with high 5′-nucleotidase and alkaline phosphatase (hepatoma or liver metastases), or with normal 5′-nucleotidase but high alkaline phosphatase (bone metastases) as shown in Table 4.

This method has many advantages over the methods in current use. Firstly, one can assay the activity of 5′-nucleotidase and alkaline phosphatase simultaneously without the interference of one enzyme with the other. It saves time especially when there are many samples to be assayed. Secondly, the method is sensitive providing it is applied as mentioned above. Under these conditions we were able to estimate phosphorus in concentrations as low as 0.09 μmol. Thirdly, few reagents are needed to run the assay for the two enzymes, which makes it cheap for routine analysis.

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