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A New Method for the Determination of Serum Nucleotidase

III. Inhibition of alkaline phosphatase

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The inhibition of the Nucleotidase effect of bone alkaline phosphatase by phenyl disodium orthophosphate is described. The conditions for inhibition and the effect on the normal 5N range and the elevated 5N values in liver diseases and in cases of high osteoblastic activity are reported.

Die Hemmung des Nucleotidase-Effekts von alkalischer Phosphatase aus Knochen durch Dinatrium-phenylorthophosphat wird beschrieben. Die Bedingungen für die Hemmung sowie der Effekt auf 5'-Nucleotidase im Normbereich als auch auf erhöhten Aktivitäten bei Lebererkrankungen und Fällen hoher Osteoblasten-Aktivität werden dargestellt.

Since REIS (1) reported that human liver homogenate hydrolyses AMP at pH 7.5 more rapidly than other phosphoric esters, serum activity towards AMP at pH about 7.5 is usually considered to be the result of two enzyme systems, i. e. a 5-nucleotidase system having only affinity for 5-nucleotides and a non-specific phosphatase which is able to split AMP as well. The measured total activity towards AMP is commonly simply referred to as 5-nucleotidase (5N)¹⁾.

Various attempts have been made to eliminate the interference of non-specific phosphatase in the 5N assay, a critical review of these has been given in this journal by BECKMANN and coworkers (2) and PERSIJN and coworkers (3). Summing up the conclusions of this discussion it may be stated that there is a lot of criticism regarding the conditions postulated as inhibiting the concomitant AP¹⁾ in the 5N assay.

Clinically the 5N assay is of interest since 5N activity increases greatly in liver diseases (4—11). In cases of bone disease 5N activity has been reported normal in cases where AP levels are elevated (8, 9). It has been pointed out, however, by BECKMANN and coworkers (2) that high levels of bone phosphatase can be associated with moderately elevated 5N levels.

It is obvious that bone phosphatase has what could be called a "5-nucleotidase effect".

Clinically this does not alter the diagnostic significance of the 5N assay as much as would seem at first sight, since in liver diseases, contrary to bone diseases, high levels of AP are commonly associated with high levels of 5N, presumably as a result of liver metastases.

The differentiation of bone and liver diseases on the basis of moderately elevated 5N levels, is difficult, especially if the AP levels are not known.

¹⁾ The following abbreviations are used: ADA = adenosine deaminase (EC 3.5.4.4), AP = alkaline phosphatase (EC 3.1.3.1), 5N = activity towards AMP (EC 3.1.3.5), PhP = phenylphosphate, SGPT = serum alanine aminotransferase (EC 2.6.4.2), GP = β -glycerophosphate.

This is the reason why 5N assays are often combined with AP determinations. It is obvious that the 5N assay as such would be clinically a more independent parameter if the 5N effect of bone phosphatase could be inhibited. The correction for the 5N effect of liver phosphatase on the other hand seems to be more interesting from the biochemical than from the diagnostical point of view.

Examples of selective inhibition of the 5N effect of bone phosphatase are to be found in studies on cancer. In these cases bone phosphatase appears in the blood and its 5N effect is superimposed on the elevated 5N activities originating from liver metastases.

Such cases are found

- a) if osteoblastic metastases are present, and
- b) if recalcification in osteolytic metastases takes place as a response to chemotherapy.

The decrease of the 5N level resulting from tumour regression in the liver is masked to some degree by the 5N effect of the increasing amount of bone phosphatase. A 5N assay in which the residual activity of bone phosphatase on AMP is suppressed would therefore give more exact information about the condition of the liver during the course of therapy etc.

The study of these problem led us to apply a completely new approach to the inhibition of the nucleotidase effect of non-specific phosphatases, especially bone phosphatase.

This was achieved by adding to the incubation medium a second substrate possessing a higher affinity for AP than AMP (e. g. GP or PhP¹⁾). In this way the AMP was repressed from AP by the competitive second substrate and the net effect was an apparent inhibition of AP in the assay of 5N activity. Thus in fact the AP is left in full activity but this is directed towards a substrate of which the hydrolysis products are not measured in the 5N assay itself. We have proposed the name of "competitive substrate retention" for this approach. It is obvious that under these conditions the assay of

5N activity has to be based on measurements not of the inorganic phosphate but of the adenosine released from AMP. In 1968 we introduced such a new method for the assay of 5N in serum, which was 3–4 times more sensitive than methods based on the determination of inorganic P. In the new method the 5N is measured by a colorimetric reaction with NH_3 liberated from adenosine by the enzyme ADA¹) (3). As can be deduced from data of IPATA (12) and LEYBOLD and coworkers (13) our method is about eleven times more sensitive than those methods in which adenosine is determined from its rate of deamination by ADA at 265 nm. Moreover the effect of PhP on 5N activity cannot be studied with such a method since PhP has a very strong absorbance at 265 nm. A sensitive method for 5N is a prerequisite for the investigation of small but important 5N effects of bone phosphatase. The new colorimetric method for 5N as introduced by us, has been shown to be sufficiently sensitive.

The present paper describes the results of experiments to establish the reaction conditions for the competitive substrate, its concentration and pH for inhibiting the nucleotidase effect of bone phosphatase and its effect on the normal 5N range and on the elevated 5N values in cases of liver disease.

Methods and Materials

Measurement of the 5N activity

The 5N activity was measured using either dialyzed ADA in ammonium sulphate (3) or ADA in 50% glycerol (14). It should be pointed out that in ref. 14 two corrigenda to ref. 3 are given.

The extinction values of the colour products were read at 625 nm in a 10 mm cuvet.

The ADA and AMP (adenosine disodium monophosphate) were purchased from BOEHRINGER Mannheim, the PhP (phenyl disodium orthophosphate, M. W. = 254) and most of the chemicals used, from British Drug House in England.

The pH for the study of activity-pH relationship (fig. 5) was measured in the test medium with serum at 37°.

For the pH range 8.5 to 9.7, veronal- Na_2CO_3 buffers were used. The serum was diluted with four or five times its own quantity of water and the concentration of ADA doubled because of diminishing ADA activity at the higher pH values. By separate experiments it was confirmed that ADA had sufficient activity at the higher pH values for the rapid conversion of adenosine liberated from AMP.

Measurement of alkaline phosphatase activity

The AP activities in this paper are quoted in mU/ml according to the procedure of MORGENSTERN and coworkers (15) using the Auto-Analyzer. Incubation takes place in 2-amino-2-methylpropanol buffer (pH 10.25) with p-nitrophenyl phosphate as a substrate (normal values 25–116 mU/ml).

Results

Figures 1 and 2 show the 5N activities, expressed as the amounts of adenosine released from the same concentration of AMP in the presence of various concentrations of a second substrate (PhP). PhP did not influence the colorimetric reaction for adenosine as applied in our method for 5N determination (tested up to 300 μMol

Fig. 1
Effect of various concentrations of PhP (μMol per ml incubation medium) on the 5N activity at pH 7.5

Fig. 2
Effect of various concentrations of PhP (μMol per ml incubation medium) on the 5N activity of sera from patients with high osteoblastic activity

A = patient with hyperparathyroidism (AP = 1440 mU/ml)

B = patient with M. Paget (AP = 1000 mU/ml)

The arrows indicate the normal range

per ml). The inhibition by the competitive substrate was found to be almost entirely dependent on the type and concentration of this substrate and the pH of the incubation mixture.

Figure 1 shows the effect of PhP on the 5N activity at pH 7.5 of a pool of serums of patients with liver diseases. The addition of PhP to the incubation mixture results in the slowly increasing inhibition of 5N activity. At a concentration of 300 μMol the inhibitive effect of PhP was only approx. 25%.

Results of inhibition experiments in established cases of liver disease using PhP in concentrations of 8 μMol per ml incubation fluid appear in Table 1.

The 5N level of 576 mU/ml (patient No. 3) was the highest that had been found in our laboratory for years.

Tab. 1
Inhibition of 5N activities by 8 $\mu\text{Mol/ml}$ phenyl phosphate in the incubation medium at pH 7.5 in cases of liver diseases.

patient No. diagnosis	activity AP in mU/ml	5N in mU/ml	percentage inhibition by 8 μMol PhP/ml
1. cholangitis	462	84.1	1.8
2. liver cirrhosis	439	191	1.0
3. liver cirrhosis	747	576	2.3
4. biliary cirrhosis	670	110.7	5
5. biliary cirrhosis	447	175.5	1.4
6. main bile duct obstruction	423	71.1	3.7
7. cirrhosis	146	17.6	4.0
8. infectious hepatitis	780	118.8	8.0

Tab. 2

Effect of 8 $\mu\text{Mol/ml}$ PhP in the incubation medium on 5N activity of sera with low AP levels. Extinction values at 625 nm read against appropriate blanks. Conversion factor to 5N activity in mU: 0.92

	AP activity (mU/ml)	no PhP		5N assay		8 $\mu\text{Mol/ml}$ PhP mean 5N activity (mU/ml)	% inhibition
		E_{625} nm	mean 5N activity (mU/ml)	E_{625} nm			
Patient B	75	0.335	0.336	30.8	0.327 0.324	30.0	2.6
Patient F	129	0.374	0.383	34.9	0.375 0.368	34.2	2.0

In Table 2 are data on the effect of PhP on elevated 5N levels of two serums which had normal or only slightly elevated AP levels.

To illustrate the sensitivity of our method the extinction values of the duplicate experiments have also been included in the table. It will be seen that PhP has a very slight inhibiting effect on 5N activity.

Patient B was followed up after operation for breast carcinoma. There were no clinical signs of bone or liver metastases. In May 1968 the levels of 5N, AP and SGPT were normal. In October the 5N and SGPT levels were elevated and were still so in Jan. 1969, whereas AP had remained normal.

The bromsulphophthalein test was normal on 20-3-1969.

On the basis of the 5N levels there is strong suspicion of liver metastases.

The occurrence of elevated 5N and normal AP levels in the case of metastatic liver diseases has also been described by SMITH and coworkers (11). It has been observed that a rise of 5N can be the precursor of a rise of AP level (16).

Patient F is suffering from M. Hodgkin, which was treated radiologically in June 1968; in the summer of 1968 hypersplenism developed.

On 17-10-1968 extirpation of the spleen took place, after which event 5N and AP values increased sharply to 70 and 300 mU/ml respectively. Leucocyte and thrombocyte counts increased rapidly to normal values. After 12 weeks AP (and SGPT) levels returned to normal, but the 5N levels decreased more slowly.

During splenectomy the liver was found normal in appearance and on palpation; the changes in AP and 5N values were tentatively interpreted as the expression of the adaptation of the liver to the new demand for RES function after the splenectomy.

The effect of GP on liver phosphatase activity has been published by us in an earlier communication (17). GP

represses 5N activity to a very slight extent exceeding the s. d. of the test up to about 10 $\mu\text{Mol/ml}$. At higher concentrations however GP—unlike to PhP—reduces the 5N activity almost to zero. The degree of inhibition has been shown to be directly proportional to concentration (17).

In Table 3 the effect of GP on the 5N activity of bone and liver phosphatase at pH 7.5 and 8.2 is shown.

Tab. 3

Effect of GP on the 5N activity of bone phosphatase (AP = 1440 mU/ml) and liver phosphatase (AP = 590 mU/ml) at pH 7.5 and 8.2

$\mu\text{Mol/ml}$ GP in the incubation medium	5N activity (mU/ml)			
	Bone phosphatase pH 7.5	Bone phosphatase pH 8.2	Liver phosphatase pH 7.5	Liver phosphatase pH 8.2
0	24.8	61.1	64.8	78.3
GP 2	21.6	—	62.8	74.4
GP 4	18.9	—	61.5	70.5
GP 6	18.5	36.3	61.5	—

The data concern the serum of a patient with hyperparathyroidism and a pool of serums of liver diseases.

A shift of pH to 8.2 has a strong elevating effect (approx. 140%) on the 5N activity (without GP) of bone phosphatase, whereas the 5N activity of liver phosphatase is only increased by about 20%. The data show that GP is able to suppress the 5N activity of bone phosphatase to the normal range only to a limited extent, especially at pH 8.2. The effect of PhP on the 5N activity at pH 7.5 of bone phosphatase is given in Figure 2. It will be seen from Figure 2 that the presence of PhP unlike that of GP, reduces the elevated 5N effect of bone phosphatase to the normal range.

Table 4 shows that PhP selectively inhibits the 5N effect of bone phosphatase in a mixture of serums containing elevated levels of liver and bone phosphatase.

In Table 5 the results are given of further inhibition experiments on serums from patients with osteoblastic

Tab. 4

Inhibition of serum bone phosphatase added to serum liver phosphatase

	no PhP found	5 N activity (mU/ml)		
		8 $\mu\text{Mol/ml}$ PhP in the incubation medium found	calculated	recovery %
I A 0.1 ml serum (liver disease)	80.5	78.6	—	—
B 0.1 ml serum (bone disease)	18.2	11.0	—	—
0.05 ml serum A + 0.05 ml serum B	51.6	45.5	44.8	101.5
II A 0.1 ml serum (liver disease)	179.9	175.4	—	—
B 0.1 ml serum (bone disease)	50.7	12.8	—	—
0.07 ml serum A + 0.03 ml serum B	147.5	128.2	126.7	101.2
0.02 ml serum A + 0.08 ml serum B	84.5	47.1	45.5	103.4

Tab. 5

Effect of 8 $\mu\text{Mol/ml}$ PhP in the incubation medium on 5N level of sera from patients with elevated phosphatase from osteoblastic activities

patient	AP (mU/ml)	5N (mU/ml)	
		no PhP	with 8 $\mu\text{Mol/ml}$ PhP
R	820	16.3	10.6
D	570	16.0	10.2
C	3570	50.7	12.8
B	715	15.7	9.2

Tab. 6

Inhibition of 5N activities by phenyl phosphate (8 $\mu\text{Mol/ml}$) in cases of patients with tumours

patient diagnosis	AP (mU/ml)	5N (mU/ml)	
		without PhP	with PhP
G breast carcinoma	900	172.0	165
K breast carcinoma	1337	108.0	89.8
M —	154	16.2	9.2
Me breast carcinoma	150	28.1	26.6
Z cervix carcinoma	133	23.0	21.1
W breast carcinoma	150	27.5	26.6

AP activities (morbus Paget). Patient C and B were both aged 75.

In Table 6 data are presented from a group of cancer patients. For patient M, suspected to have a tumour in the duodenum, the diagnosis was doubtful.

The reason for including this case in the table is that in the serum of patient M an *intestinal* AP could be demonstrated. The effects of PhP suggest that it could also be used to inhibit intestinal phosphatase.

In the X-ray films of patient K the presence of osteoblastic metastases could clearly be demonstrated, whereas patient G was found to have many osteolytic metastases. All patients—except M—were suspected of having liver metastases.

In Table 7 the results of 5N assays with and without PhP are given. These were performed on serums from healthy women who had used contraceptive pills for some years. It has been observed by VAN DER SLIK and PERSIJN (16) in a preliminary investigation that long

Tab. 7

Effect of PhP on 5N activities of sera from women who use contraceptive pills.

Duration of intake (years)	5N activity (mU/ml)	
	no PhP	8 $\mu\text{Mol/ml}$ PhP
5	11.6	11.3
3	21.1	19.2
3½	17.1	16.7

Tab. 8

5N activities (mU/ml) of normal subjects (n = 10)

subject no	no PhP	8 $\mu\text{Mol/ml}$ PhP
1	9.2	8.5
2	8.4	7.2
3	8.4	8.2
4	9.0	8.8
5	8.5	8.0
6	8.5	7.7
7	9.2	8.4
8	8.4	8.0
9	6.2	5.4
10	9.1	8.6

term use of this pill may result in a significant rise in the 5N level.

This matter is now being studied intensively.

To have any diagnostic value the addition of 8 μMol PhP to 1.0 ml incubation medium of the 5N assay should lower the normal range of 5N far less than in cases of bone disease. The results of 5N assays, with and without PhP, in sera of healthy persons whose ages varied from 29 to 43 appear in Table 8. These results indicate that the serum 5N activities of healthy persons are suppressed to a very slight extent.

The suppression by PhP amounts to an average of 7%.

5N assays in the presence of 8 μMol PhP were made on 60 apparently healthy persons of both sexes, aged 20–60 who declared that they had not recently had any complaint of the liver or the digestive tract, and that they (women) did not use contraceptive pills. The range of individual values was 2.9–10.5 mU/ml² (mean $\pm 2 \times \text{s. d.}$).

The extinction at 625 nm as a measure of serum activity of 5N is proportional to the amount of serum assayed in the presence of 8 μMol PhP per ml incubation medium (Fig. 3).

Fig. 3

Relation of optical density to enzyme activity in the presence of PhP (8 $\mu\text{Mol/ml}$).

Discussion

It was stated in the introduction that to investigate the 5N effect of bone phosphatase and its subsequent elimination for diagnostic purposes, the method applied must satisfy two criteria.

These are:

- a high sensitivity
- non-interference of the inhibitor, e. g. PhP, with the reactions of adenosine on which the 5N assay is based.

² The normal range (4–14 mU/ml) reported by us in ref. 3 was based on results obtained from phosphate determinations. If with our new method corrections were applied for impurities such as are found in some batches of ADA (see footnote ref. 3) the normal range without PhP was found to be 5.5–11.5 mU/ml.

The results show that our method completely fulfills these requirements.

While our work was in progress a report appeared in *Nature* by BELFIELD and GOLDBERG (18), describing a similar approach to suppressing the 5N activity of bone phosphatase. In this approach GP in a concentration of 22 μMol was added to an incubation medium of pH 7.9. In a communication dealing with the determination of 5N in the presence of GP we have made several critical remarks on this approach. Briefly these remarks refer to the insufficient sensitivity of the method employed for the 5N assay by these authors, and the fact that GP inhibits true 5N at concentrations higher than 10 μMol (17). In addition the results presented here (Table 3) suggest that a slight shift in the pH of the incubation medium will enlarge the discrepancy between 5N level in the presence of GP and the normal range. This makes the approach of BELFIELD and GOLDBERG still more indefensible.

It can be assumed that the amount of reduced activity by a competitive substrate represents the 5N activity of non-specific phosphatase.

Thus, if we consider Figure 2 A the depth of the plateau, corresponding to 17.6 mU/ml is the 5N effect of alkaline bone phosphatase. The remaining activity (7.2 mU/ml) most likely represents a different phosphatase activity, which is present in the serum of normal subjects, i. e. 5-nucleotidase.

From Figure 1 it can be seen that, at pH 7.5 liver enzymes—unlike those from osteoblasts—have far more affinity for AMP than for PhP, which is convenient if it is desired to repress selectively bone phosphatase activity towards AMP by addition of PhP. Starting from pH values of about 7.9 and higher, a distinct suppression of non-specific liver phosphatase becomes demonstrable. For pH 8.2 this suppression is illustrated in Figure 4. There are no clinical reasons known at the moment for a 5N assay in which non-specific liver phosphatases are to be suppressed. We are therefore in agreement with BECKMANN and coworkers (2), that a pH value of 7.5 should be taken for the 5N assay and that this pH is also to be preferred if PhP is used.

For the sake of completeness the pH-activity relationship of liver phosphatase and bone phosphatase is presented in Figure 5. This Figure explains the fact that at lower

Fig. 4

Effect of PhP on the 5N activity at pH 8.2 in cases of liver diseases

Fig. 5

Effect of pH on 5N activity

- pooled serum from patients with liver diseases.
(AP = 587 mU/ml; 5N = 95 mU/ml)
- serum with high activity of bone phosphatase
(AP = 1440 mU/ml; 5N = 24.8 mU/ml)
Activity at pH 8.9 was taken as 100%.

pH (e. g. 7.5) diseases with high osteoblastic activity show only moderately elevated 5N levels unlike liver diseases with high AP levels. This discrepancy becomes much less at higher pH values. Since the results (Fig. 5) show that the direct relationship between optical density and the amount of enzyme is not influenced by 8 μMol PhP we would propose changing in ref. 3 the preparation of buffered substrate/ADA solution to read: Dissolve 0.250 g adenosine-5'-monophosphate ($\text{C}_{10}\text{H}_{12}\text{O}_7\text{N}_5\text{PNa}_2\cdot 6\text{H}_2\text{O}$) and 0.215 g phenyl disodium orthophosphate ($\text{C}_6\text{H}_5\text{Na}_2\text{PO}_4\cdot 2\text{H}_2\text{O}$) in 100 ml buffer/ADA. The solution is prepared fresh before use. By this modification a standard procedure is obtained for 5N assay in which interference by bone phosphatase is reduced.

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