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Comparative Studies on Subcellular Organelles and their Marker Enzymes in Selected Marine Animal Species and in the Rat

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Report on work carried out under the Indo-German collaboration on peaceful uses of atomic energy, project CHEM-11:

"Subcellular Distribution and Mobilization of Lanthanides and Actinides"

This report describes the research activities of Dr. R. Winter and Priv.Doz. Dr. A. Seidel during their stay in Bhabha Atomic Research Centre, Bombay in 1980/81. The work was performed in collaboration with Dr. S. Patel, M.C. Balani and Dr. B. Patel.

Abstract

The concentration of several marker enzymes for subcellular organelles has been determined in some marine animal species, which specifically accumulate heavy metals or radionuclides. The dependence of the enzyme activity on the incubation conditions was tested as well as the integrity of the lysosomes after cell homogenization. In several cases, significant differences in the enzyme concentrations between the animal species were observed. The data indicate subcellular studies of the heavy metal accumulation in marine invertebrates require more sophisticated methods for the preservation of the lysosomes, which seem to be much more fragile than in mammalien systems. In addition, several of the marker enzymes used for mammalian organelles appear to be unsuitable for marine invertebrates.

Vergleichende Untersuchungen an subzellulären Organellen und deren Leitenzymen in ausgewählten Meerestieren und der Ratte

Zusammenfassung

In marinen Tierspezies, die jeweils spezifisch wichtige Schwermetalle und Radionuklide akkumulieren, wurde die Konzentration bestimmter Leitenzyme für Zellorganelle untersucht. Die Abhängigkeit der Enzymaktivität von den Inkubationsbedingungen wurde geprüft, ebenso die Intaktheit der Lysosomen. In einigen Fällen gibt es große Unterschiede in der Enzymkonzentration zwischen den Tierarten. Es zeigte sich, daß für biochemische subzelluläre Studien der Schwermetallakkumulation in marinen Wirbellosen vor allem die vergleichsweise extrem hohe Zerbrechlichkeit der Lysosomen noch ein Hindernis darstellt. Außerdem erwiesen sich einige Leitenzyme bei diesen Tiergruppen nicht als geeignet.

Contents

1. Introduction

Stable and radioactive ions of heavy metals are released into the human environment, both terrestrial and aquatic, by conventional and nuclear industries. It is well known, that these elements are concentrated by several orders of magnitude by aquatic species, which form a part of the food chain to man. Some of these organisms living in the marine environment, can be used as "indicator/sentinel species" to detect the presence of stable/radioactive toxic elements in the marine biosphere (Pa 73, Pa 75, Pa 78, Pa 80). Studies on the mechanisms of the bioaccumulation of many trace and heavy metals and their radionuclides have assumed a great significance, therefore, during the last few years.

One of the tools used to obtain a clearer understanding of the behaviour and incorporations of these ions is given by the biochemical analysis of the involved tissues, especially of the binding sites in the cell. This concept has been applied successfully to the study of the fate and effect of transuranium elements in mammalian liver (Bo 70, Gr 81, Se 80). In principle all these methods require as a prerequisite a knowledge of the occurrence, specific concentration and optimal assay conditions for specific marker enzymes, used to monitor the presence of subcellular organelles in the respective cell fractions. Furthermore, the cellular organelles should of course remain intact from a morphological as well as functional point of view; this is especially important for the fragile lysosomes.

Biochemical methods for detecting subcellular organelles for studies of the binding of radionuclides to them have already been

developed for mammalian liver (Bo 70, Gr 81). However, there are relatively few such studies for marine species. One of the major aims of the present study was to investigate the degree to which the methods used for mammalian tissues can be applied or optimized for the study of the subcellular behaviour of certain heavy metals/radionuclides in marine benthic species. The enzymes which were tested represent the usual markers for lysosomes (acid phosphatase and aryl sulfatase), mitochondria (glutamate dehydrogenase), pericellular membranes (5'-nucleotidase) and endoplasmic reticulum membranes (glucose-6-phosphatase). The marine species, which were selected, have earlier been recognized as "indicator species" (Pa 73, Pa 75, Pa 78). A major part of the report deals with the blood clam, Anadara granosa Linn., hepato-pancreas (digestive glands), which has been used as a model tissue.

This species concentrates several trace and heavy metals and radionuclides including plutonium-239 in its tissues (Pa 78, Pa 79). Also several physiological and radiobiological parameters of this species have already been studied (Pa 71, Pa 75, Pa 78, Pa 79), and it is available all the year round in Indian coastal waters. The data presented in this study are also of importance in other fields such as aquatic toxicology and the radio/physioecology of marine species and food sciences (e.g. Dh 80, Do 79, Mo 78).

2. Sample collection, experimental techniques and abbreviations

The common sponge (Spirestrella cuspidifera and Prostylyssa foetida) and sea hare (Aplysia benedicti) were collected from the intertidal zone of Tarapur about 100 km north of Bombay (Pa 73). The blood

clam (Anadara granosa), rock crab (Scylla serrata) and gobiid mudskipper fish (Boleophthalmus boddaerti) were collected from the intertidal zone of Trombay in the Bombay harbour (Pa 75). Wistar-rats were obtained from the stock maintained at BARC.

The experiments with the clam tissues were performed throughout the year, those with the other species were carried out during January-March, 1981. The experimental animals were collected freshly and sacrificed just prior to homogenization and analysis. Sponge tissues were homogenized in a Potter-Elhvehjem homogenizer using ∿10 up and down strokes at ∿1000 rpm. Unless otherwise stated all other homogenizations were performed using ∿5 strokes at 500 rpm. The hepatopancreas (digestive gland) from clam, crab and sea hare was separated from the intestinal mass, and liver from mudskipper and rats was dissected out. In one experiment with the blood clam the subcellular fractions from digestive gland were obtained by differential centrifugation. All the methods are based on those described by Gruner et al. (Gr 81). The basic conditions followed are listed in Table 1. Special details are given in the respective sections. Calibration curves were prepared for the assay of inorganic phosphate, p-nitrocatechol and protein. For acid phosphatase, 5'-nucleotidase and glucose-6-phosphatase the following incubation mixtures were used:

0.1 ml sample (homogenate at different dilutions)
0.1 ml substrate solution (with variable concentrations as indicated in the Tables and Figures)

1.0 ml Buffer (with variable pH)

0.2 ml $HClO_4$ to stop the reaction

For aryl sulfatase the conditions were as follows:

0.1 ml sample (homogenates at different dilutions)

0.1 ml NCS solution (with variable concentration as indicated in the Tables and Figures)

2.0 ml Buffer

1.0 ml PTA to stop the reaction

Enzyme activity is expressed as "Units": One unit catalyses the reaction of 1 µmol of substrate per min at 25°C.

Special details are indicated in the respective Tables or Figures.

Abbreviations:

AMP	-	Adenosine-5'- monophosphate
NCS	-	p-nitrocatecholsulfate
Pi	-	inorganic phosphate
PTA	-	Phosphotungstic acid

3. Studies with blood clam - Anadara granosa

3.1. Digestive gland

3.1.1. Acid phosphatase

The influence of the β-glycerophosphate concentration in the enzyme activity is shown in Fig.1. Clearly substrate inhibition occurs at molarities above 0.2. In experiments of this type, self-decomposition of the substrate itself must be considered, especially if it was stored at high temperatures. This is illustrated by Fig.2 which gives the results of blank determinations of the free inorganic phosphate in a charge of β-glycerophosphate (different from that used for Fig.1.)

Table 2 shows the dependence of acid phosphatase activity on pH. A maximum occurs at pH ∿ 3.5, but some activity is found at pH ∿ 8 which probably comes from β-glycerophosphate hydrolysis by an alkaline phosphatase. The amount of phosphate liberated at pH 8 is less than one third of that at pH 3.5. The influence of the incubation temperature on phosphate liberation is illustrated in Fig. 3. A distinct maximum occurs at ∿ 35 C, whereas there is not much difference between 5 and 25 C.

The dependence of phosphate liberation on incubation time is shown in Fig. 4 and 5 for different conditions. In Fig.4, the conditions correspond to those adopted for rats and different sample volumes were used. The reaction becomes nonlinear for sample volumes above 300 µl. In Fig.5 the line A was obtained under the conditions used

for rats and line B by using different substrate molarities and pH. Under both sets of conditions, the reaction was linear with the usual sample volumes (0.1 ml, homogenate 1:10).

The latency of the acid phosphatase activity was tested in most of the experiments by addition of Triton X100. However, latency could not be detected unequivocally in any case by this standard procedure. Acid phosphatase could be inhibited almost completely by 0.1 M NaF, as has been observed for the same enzyme from rat liver. A very high percentage of acid phosphatase appears in the cytosol after subcellular fractionation (Table 17).

3.1.2. Aryl sulfatase

Fig.6 shows the influence of p-nitrocatecholsulfate concentration on the enzyme activity. The rate of liberation appears to be linear up to 0.02 mol/l. The pH dependence of enzyme activity is illustrated in Fig.7. There is a clear maximum at pH 6 and virtually no activity is found at the maximum of acid phosphatase (pH 3.5, Table 2).

The effect of the incubation temperature is qualitatively similar to that observed for acid phosphatase with a sharp maximum at 40 C(Fig.8). The linearity of the enzyme reaction with time was established for the usual conditions, the result is shown in Fig.9. In the same figure we present the results of different inhibition tests. Aryl sulfatase from clam digestive gland can be inhibited to about 50 % by $Na_2\ SO_4$ and completely by $KH_2\ PO_4$ or KCN (Cha 74). The results of the latency tests were not consistent. Sometimes,

no latency at all was observed, in other experiments a latency of 20 - 40% was seen. This remarkably low latency does not depend on the homogenization procedure: In one experiment, homogenization was performed by 2 up and down strokes with the pestle revolving only at 150 rpm (instead of 5 strokes at $\sim$ 500 rpm). Nevertheless, no latency of arylsulfatase was seen (Table 3). In another experiment, using 3 up and down strokes at 150 rpm, different sucrose molarities of the medium were tested (Table 3) but no marked increase of aryl sulfatase latency was observed. As with acid phosphatase, the relative content of the enzyme is very high in the cytosol (Table 17).

3.1.3 5'-nucleotidase (Adenosine monophosphatase, AMP'ase)

It has been shown with rat liver homogenates that the total liberation of inorganic phosphate from adenosine monophosphate is due in about equal extent to a specific 5'-nucleotidase and to non-specific acid phosphatases with a small contribution from alkaline phosphatase. We have shown that also with clam digestive gland homogenates the amount of inorganic phosphate liberated is reduced to $\lesssim$ 25% of the initial value in the presence of 1m M NaF as inhibitor of acid phosphatase.

The dependence of AMP dephosphorylation on pH is shown in Fig.10. Two maxima occur. The maximum at the acid side is at a distinctly different pH-value as that for acid phosphatase (pH 3.5 Table 2), which also indicates that different enzymes really are present.

The temperature dependence (Fig.11) for 5'-nucleotidase is quite different from that for acid phosphatase and aryl sulfatase (Figs.3

and 8). There is a steady increase up to 60 C. This result could not have been due to nonspecific decomposition of the substrate AMP by heat, as was shown by a separate experiment in which the substrate was incubated at the same temperatures in the absence of the enzyme. The influence of substrate molarity was studied in a limited experiment (Table 4), performed without addition of NaF, which indicated that little difference exists between concentrations of 0.05 and 0.5 mol/l AMP. However, at 0.01 mol/l, significantly less phosphate was liberated. In a further experiment also without added NaF, the linearity with time was tested at pH 7.4. Even with a possible contribution from acid phosphatase, the reaction is linear under these conditions (Fig.12).

3.1.4 Glucose-6-phosphatase

Table 5 shows the influence of temperature on glucose-6-phosphatase activity in the clam digestive gland. The data indicate a broad maximum of activity lying between 35 and 45 C. The effect of substrate concentration can bee seen in Table 6 and the dependence of the rate of phosphate liberation on time in Fig.13. However, in all the experiments with glucose-6-phosphatase, the background values were very high. This strongly suggests a high concentration of free inorganic phosphate in the native substrate, probably due to self-decomposition. The data for glucose-6-phosphatase must, therefore, be regarded with caution. However, enzyme activity is present, though in very low concentrations.

3.2 Other organs

Table 7 gives the enzyme concentrations in several organs of the clam. Clearly, digestive gland and intestine show the highest activity of the two lysosomal markers. The results for 5'-nucletidase may be influenced somewhat nonspecifically by acid phosphatase since no NaF was added.

4. Sponges

Analysis for acid phosphatase and arylsulfatase as lysosomal markers and for 5'-nucleotidase as marker for pericellular membranes were performed in two indicator sponges occuring at Tarapur. One of them (Spirestrella cuspidifera) is a sponge boring through coral; it selectively concentrates ^{60}Co. Histochemically the presence of acid phosphatase has been demonstrated in etching cells of this sponge. The other species (Prostylyssa foetida) concentrates iodine but does not bore; it lives in symbiosis with algae.

As can be seen from Table 8, neither aryl sulfatase nor acid phosphatase could be detected in Spirestrella, whereas sufficient acid phosphatase activity was found in Prostylyssa. Thus, Spirestrella may either not have been in an active "boring" stage or acid phosphatase activity is confined only to the etching cell which may have remained on the surface of the coral. The percentage of non-bound acid phosphatase was very high also in Prostylyssa (Table 13 and Fig.14). In Fig.14 the influence of β-glycerophosphate concentration on the acid phosphatase activity is shown. The

activity rises towards a plateau at a molarity between 1.0 and 1.5. Some latency of acid phosphatase, though relatively low, has been found consistently.

5. Sea hare - Aplysia benedicti

Aplysia benedicti is a gastropod mollusc occuring during certain winter months in Tarapur coastal waters. Its hepatopancreas selectively concentrates ^{60}Co and its shell interior concentrates ^{131}I.
Acid phosphatase as well as aryl sulfatase are present to a large extent in the hepatopancreas of this species (see Table 13). None of these enzymes was detected in bound form in the homogenate to a significant extent (s.also Tables 9 and 10). Table 9 illustrates the influence of p-nitrocatecholsulfate molarity and in Table 10 that of β-glycerophosphate on the activity of the respective enzymes. Glycerophosphate concentration was optimal at 0.4-0.5 M/l, no definite optimum or plateau was reached in case of aryl sulfatase (Fig.15, Table 9). With respect to the other marker enzymes, neither 5'-nucleotidase nor glutamate dehydrogenase could be detected (Table 14).

6. Crab - Scylla serrata

In the hepatopancreas of a crab species from Bombay waters, both lysosomal marker enzymes are present in sufficient amounts to allow quantitative determinations (Table 13, Fig.16). However, latency tests did not give consistent results in the case of acid

phosphatase and in most cases, no bound lysosomal marker enzyme activity was found.

Glucose-6-phosphatase and glutamate dehydrogenase activity was not detectable (Table 14). However, in the case of glucose-6-phosphatase, the influence of nonspecific factors, disturbing the enzyme assay cannot be excluded. The background values for the determination of inorganic phosphate are very high in crab hepatopancreas homogenates. The marker enzyme for pericellular membranes (5'-nucleotidase) is present in crab hepatopancreas (Table 14) in measurable amounts.

7. Gobiid mudskipper fish - Boleophthalmus boddaerti

Tables 11 and 14 and Fig.17 give an account of the results obtained from studies of mudskipper liver. This fish species can easily be obtained in Bombay harbour and may be a potential species for further studies of radionuclide bioaccumulation mechanisms.

In Table 11 and Fig.17, the results of two different investigations of lysosomal markers are presented in detail. The assay conditions for acid phosphatase used for rats yield enzyme concentrations higher than those found for clam digestive gland (Table 11). After 30 minutes incubation under these conditions the acid phosphatase assay may no longer be linear (compare 30 min.- and 60 min.-values for "rat conditions" in Table 11). This is in contrast to the results with aryl sulfatase (Fig.17). The presence of NaF inhibited the acid phosphatase activity to a considerable extent, but not

completely. In case of aryl sulfatase, similar values for enzyme concentration were obtained independent of the conditions chosen. The percentage of non-bound aryl sulfatase seemed to be somewhat lower in mudskipper homogenates as compared to acid phosphatase (Table 13).

In two different experiments with several different assay conditions neither 5'- nucleotidase, nor glutamate dehydrogenase activities could be found in mudskipper liver (Table 14).

8. Rat

The results for the assays of several marker enzymes in liver of the Wistar-rat are shown in Table 12. In case of the two lysosomal markers, the reaction was linear with time up to 60 min. Arylsulfatase could be inhibited completely by KH_2PO_4 and partially by Na_2SO_4. These experiments were performed mainly for comparison with data already obtained in other rat species in IGT, KFK.

9. Interspecies comparison

The activities of the lysosomal marker enzymes and of the other markers are compared for the different animal species in Tables 13 and 14.. Lysosomal enzymes are present in measurable concentrations in all species except sponges, but the lysosomes are not well preserved in many cases. There may even be some differences in the fragility of lysosomes which either contain acid phosphatase or arylsulfatase.

With respect to the other markers, it becomes obvious, that those usually adopted for rat liver will not be suitable for all other animals. It is surprising, that no 5'-nucleotidase was found in the mudskipper, and this result requires further investigation.

The conditions which were used to obtain the results given in Table 13 are listed in Table 15. At present some of them may seem to be the optimal ones for assay of these enzymes in hepatic tissues of these animal species.

Data for the protein concentrations are given in Table 16 together with the specific enzyme activites (in units per mg of protein). These specific activity of acid phosphatase is of the same order of magnitude with the lowest value being found in the crab (Scylla serrata). However the specific activity of aryl sulfatase in Anadara granosa hepatopancreas is distinctly higher than in the other tissues studied. The subcellular distribution of protein in Anadara granosa hepatopancreas (Table 17) is similar to that in rat liver (Se 79).

10. Summary and conclusions

During the stay of Dr. R. Winter and Priv. Doz. Dr. A. Seidel in the Health Physics Division BARC, the following investigations were performed in collaboration with Dr. B. Patel, Dr.(Mrs.) S. Patel and Mr. M.C. Balani.

a) Calibration curves for the determination of protein organic phosphate and p-nitrocatechol for enzyme determinations were set up and detailed analyses of the occurrence and the optimal assay conditions for the usual marker enzymes for subcellular organelles in the digestive gland of a blood clam Anadara granosa were made.

b) In whole animals and hepatic tissues detailed analyses were made of the specific concentrations (per unit fresh weight and per/mg protein) and latency of two lysosomal marker enzymes in two spezies of sponge (Spirestrella cuspidifera and Prostylyssa foetida), a blood clam (Anadara granosa), sea hare (Aplysia benedicti) crab (Scylla serrata), a gobiid fish (Boleophthalmus boddaerti) and rat (Wistar). These species have previously been identified as "indicators" to detect heavy metal and radionuclide contamination of the marine environment. 5'-Nucleotidase, glucose-6-phosphatase, glutamate dehydrogenase, protein concentrations and the subcellular distribution of enzyme in these animal species were also studied.

Results:

1. The calibration curves and enzyme analyses in rat liver showed that work at BARC performed under similar conditions as at KFK yielded similar results.

2. Some of the usual marker enzymes for rat liver cannot be used for several of the animal species of interest.

3. The optimal assay conditions for enzymes in invertebrates are different from those in vertebrates.

4. In sharp contrast to the results with rat liver (and to some extend also fish liver), no intact lysosomes can be obtained by the usual homogenization procedures from hepatic tissues of the invertebrates tested.

The results obtained so far are, on one hand, interesting from the more theoretical point of view of the comparative biology of lysosomes or of general marine biology. In connection with the increasing interest in the role of lysosomes during deterioration of marine foodstuffs our results have also a more practical application, especially if the higher fragility of lysosomes from marine organisms as compared to terrestrial mammals is confirmed. However, in view of the original scope of the study, we shall focus our interest on the lysosomes as the most probable candidates for the binding of several important heavy metals, as for example ^{239}Pu. In that respect, our data show, that the usual

marker enzymes for mammalian lysosomes can be assayed with minor modifications of the procedures developed for mammals. Presently, the most important obstacle to meaningful biochemical analysis of the subcellular distribution of heavy metal accumulation in marine (invertebrate) species is the fact that lysosomes become broken during the first cell fractionation steps. This can lead to serious artifacts due to secondary redistribution of the elements primarily deposited in lysosomes. For further progress, the properties of the lysosomes in the respective marine animal tissues as well as the reasons for their extreme fragility need to be better understood.

In view of these results as well as of other developments at BARC and KFK, the following proposals for future work are made:

1. Further efforts should be made to obtain well preserved lysosomes from marine invertebrates.
2. Suitable marker enzymes should be sought for the different cell components in the different marine species and tissues.
3. Considering the difficulties of preparing well defined subcellular fractions from tissues of several important indicator species, procedures for the electron microscopic localisation of non-radioactive and radioactive heavy metals should be adopted. As a first step towards this techniques for electron microscopic histochemistry should be developed.
4. Facilities should be developed to keep sea hare Aplysia benedicti under laboratory conditions. This would provide the

possibility of studying the distribution retention and (ultimately) subcellular distribution of Plutonium and other radionuclides in a species in which the blood transportation does not depend on transferrin.

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11. References

Bo 70 Boocock,G., Danpure,C.J., Popplewell,D.S., Taylor,D.M.: The subcellular distribution of plutonium in rat liver. Radiat.Res. 42 (1970) 381-396.

De 80 Dhevendaran,K., Kannupandi,T., Natarajan,R.: Arylsulfatase Activity in Marine Gastropods. Nahasagar - Bull.Natl.Inst. Oceanogr. 13 (1980) 173-178.

Do 79 Doke,S.N., Warrier, S.B.K., Ninjoor,V., Nadkarni,G.B.: Role of Hydrolytic Enzymes in the Spoilage of Fish. J.Food Sci.Technol. 16 (1979) 223-226.

Gr 78 Gruner,R.: Die Verwendung von Triton-WR-1339 (TWR) zur quantitativen Bestimmung der lysosomalen Bindung von Transuranen in der Rattenleber. Thesis, University of Karlsruhe, 1978.

Gr 81 Gruner, R., Seidel,A. and Winter,R.: The Initial Early Distribution of Monomeric ^{239}Pu and ^{241}Am in Rat Liver as Determined by Triton WR 1339 Injection - Radiat.Res. 85 (1981) 367-379.

Mo 78 Moore,M.N., Loewe ,D.M., Fieth,P.E.M.: Response of Lysosomes in Digestive Cells of the Common Mussel, Mytilus edulis, to Lex Steroids and Cortisol. Cell Tiss.Res.188 (1978) 1-9.

Pa 71 Patel,S., and Patel,B.: Effect of ionizing radiation on haemoglobin of marine lamellibranchs. Mar.Biol., 10, (1971) 272-279.

Pa 73 Patel,B., Valanju, P.G., Mulay, C.D., Balani,M.C. and Patel,S.: Radioecology of certain molluscs in Indian coastal waters. In "Radioactive contamination of the marine environment", IAEA, Vienna, (1973) STI/PUB/313, 307-330.

Pa 75 Patel,B., Mulay,C.D. and Ganguly,A.K.: Radioecology of Bombay harbour- a tidal estuary. Estuarine and Coastal Marine Science, 3 (1975) 13-42.

Pa 78 Patel,B., Patel,S., Balani,M.C. and Pawar,S.S.: Flux of certain radionuclides in the blood clam Anadara granosa Linn. under environmental conditions. J.exp.mar.bio.ecol. 35, (1978) 177-195.

Pa 79 Patel,B., Patel,S. and Balani,M.C.: Biological responses of a tropical coastal ecosystem to releases from electro-nuclear installations. In "Biological implications of radionuclides released from nuclear industries". IAEA,

Vienna (1979) SM-237/12, 335-349.

Pa 80 Patel,B., Pawar,S.S., Balani,M.C. and Patel,S.: Macroalgae as sentinel of trace and heavy metals in the management of coastal environment. In "Management of Environment" (B.Patel, edit.), Wiley Eastern, New Delhi, 1980, 371-388.

Se 79 Seidel,A., Winter,R., Jentzsch,C., Gruner,R., Heumann,H.-G., Hanke,S.: The Biochemical Mechanisms Responsible for Differences in the Half-Life of Monomeric ^{239}Pu in Rat and Syrian Hamster Liver. In: "Biological Implications of Radionuclides Released from Nuclear Industries." IAEA, Vienna (1979), 401-415.

Se 80 Seidel,A.and Taylor,D.M.: Inorganic Biochemistry in Environmental Research: Methods, Results, Perspectives. In: Management of the Environment (B.Patel edit.), Wiley Eastern, Neu Delhi (1980), 203-216.

12. Tables and Figures

Table I

Markerenzymes and assay procedures[a]

Enzyme	EC-Code	Organelle	Substrate	Buffer	Special Conditions	Ref.
Acid phosphatase	3.1.3.2.	Lysosome	0.1 M β-glycero-phosphate	0.05 M acetate pH 5.0	0.005 M EDTA	15
Aryl-sulfatase	3.1.6.1.	Lysosome	0.019 M 2-hydroxy-5-nitro-phenylsulfate	0.5 M acetate pH 6.0	-	16
5'-nucleo-tidase	3.1.3.5.	Pericellular Membranes	0.1 M adenosine-5'-monophosphate	0.1 M Tris[b] pH 7.4	0.008 M[c] $MgCl_2$	17
Glucose-6-phosphatase	3.1.3.9.	Endoplasmic Reticulum	0.3 M glucose-6-phosphate	0.05 M K-maleinate pH 6.0	0.001 M EDTA 0.001 M NaF	(15) 18
Glutamate-dehydrogenase	1.4.1.2.	Mitochondria	0.1 M ketoglutarate	0.035 M triethanolamine-HCl pH 8.0	0.01 M EDTA 0.05 M ammon. acetate ~0.002 M ADP ~0.3 mM NADH	19,20

[a] Triton X100 was added before the assays (final concentration 0.1 %) (14).

[b] Tris (hydroxymethyl)-aminomethan

[c] No tartrate or other enzyme inhibitors were added

Table 2

Influence of pH on acid phosphatase activity in digestive gland homogenate of the blood clam Anadara granosa

pH	nmol phosphate liberated per min	percent of maximum
2.3	0	0
2.9	39	67
3.5	58	100
4.1	53	91
4.8	4.6	8
6.9	0	0
8.3	4.3	7
10.3	0	0

Homogenate 1:10, 0.07 M β-glycerophosphate, Incubation time 120 min, values representative for four experiments.

Table 3

Bound and non-bound (values in brackets) arylsulfatase activity in clam (Anadara granosa) digestive gland under mild homogenization conditions and increased medium molarity

Homogenization conditions	Sucrose molarity	Triton X 100	Relative Units	n
Homogenate 1:100, 2 up and down strokes at 150 RPM	0.25 M	+	3.4	3
		-	3.3 (∿100)	3
Homogenate 1:50, 3 up and down strokes at 150 RPM	0.25 M	+	5.7	2
		-	4.4 (∿ 80)	2
	0.5 M	+	4.3	2
		-	4.7 (100)	2
	0.75 M	+	5.5	2
		-	4.2 (76)	2

0.04 M NCS, incubation time 20 min.

Table 4

Influence of substrate (AMP, adenosin -5'-monophosphate) molarity on inorganic phosphate liberation by 5'-nucleotidase (no NaF added) from clam (Anadara granosa) digestive gland

AMP molarity	nmol phosphate liberated per sample
0.5	777
0.25	815
0.10	699
0.075	704
0.05	632
0.01	329

Homogenate 1:10, pH 7.4, incubation time 20 min, (two determinations per value).

Table 5

Influence of temperature on inorganic phosphate liberation by glucose -6-phosphatase in clam (Anadara granosa) digestive gland

Temperature (^{o}C)	nmol phosphate liberated per sample
4	256
25	198
30	183
35	479
40	509
45	460

Homogenate 1:10, 1.3 M glucose-6-phosphate solution , pH 6.0, Incubation time 120 min.
Two determinations per point.

Table 6

Influence of glucose -6-phosphate concentration on liberation of inorganic phosphate by Glucose -6-phosphatase in clam (Anadara granosa) digestive gland

Glucose-6-phosphate molarity	nmol phosphate liberated per sample
1.50	726
1.25	954
1.00	280
0.75	134
0.50	140
0.25	271

Homogenate 1:10, buffer pH 6.0; Incubation time 120 min. Two determinations per point.

Table 7

Enzyme concentration in different tissues of Anadara granosa (Units/g fresh tissue)

Tissue	Acid phosphatase	n	Aryl-sulfatase	5'-nucleo-tidase	n
Mantle	0.09	4	0	0.41	2
Foot	0.09	4	0	0.11	3
Labial palps	0.45	2	0.79	0.47	3
Gills	0.24	4	0.79	1.14	2
Intestine	1.40	4	2.80	0.46	2
Digestive gland	1.61	4	14.1 [a]	1.35	2
Adductor muscles	0.09	2	0	0.09	3

Arithmetic means of n experiments, in case of aryl sulfatase only one experiment

[a] n = 11

Table 8

Assay of marker enzymes in two species of siliceous sponges

a) Spirestrella cuspidifera and b) Prostylyssa foetida

Experiment	Sponge	Acid phosphatase U/g fresh weight	Acid phosphatase % Non-latent	5'-nucleotidase	Arylsulfatase
1	(a)	0	-		
	(b)	1.14(+TX, n=2)	-	not detected	not detected
2	(a)	0			
	(b)	0.54(+TX, n=2)			
		0.39(-TX, n=1)	72		
		0 (+NaF, n=2)			
3	(a)	0			
	(b)	1.48(+TX, n=1)			
		1.29(-TX, n=1)	87		

+ or - TX = Triton X 100 added or not added respectively, n = number of determination
β-glycerophosphate 0.1 - 0.15 M; Incubation time 2 - 3 hours.

Table 9

Influence of p-nitrocatecholsulfate concentration on rate of liberation of catechol in Aplysia hepatopancreas

p-nitrocatechol sulfate molarity	Triton X100	U/g [b]
0.075	+	1.9
	-	1.9
		(100)
0.050	+	1.7
	-	1.6
		(95)
0.025	+	1.3
	-	1.2
		(89)
0.01	+	0.6
	-	0.6
		(100)
0.0075	+	0.4
	-	0.4
		(100)
0.005	+	0.3
	-	0.3
		(100)
0.0025	+	0.1
	-	0.1
		(100)
0.001	+	0.1
	-	0.1
		(100)

[a] Two determinations per value

[b] Units (U) refer to g fresh weight. Values in brackets give percentage of non-latent enzyme. Homogenate 1:20, buffer pH 6.0, incubation time 60 min.

Table 10

Effect of concentration of β-glycerophosphate on acid phosphatase activity in homogenate of Aplysia hepatopancreas.

β-glycerophosphate molarity	Special Conditions[b]	U/g[c]	non-latent percentage
1.5	-TX	2.2	86
	+TX	2.5	
	+NaF	0	
0.75	-TX	3.4	98
	+TX	3.4	
	+NaF	0	
0.50	-TX	4.6	100
	+TX	4.6	
	+NaF	0	
0.38	-TX	4.6	86
	+TX	5.4	
	+NaF	0.7	
0.15	-TX	4.0	100
	+TX	3.9	
	+NaF	0.2	
0.075	-TX	2.4	89
	+TX	3.0	
	+NaF	0.2	
0.05	-TX	2.4	100
	+TX	2.0	
	+NaF	0.5	
0.038	-TX	1.8	64
	+TX	2.8	
	+NaF	0.1	
0.015	-TX	0.4	37
	+TX	1.1	
	+NaF	0.1	
0.0075	-TX	1.7	
	+TX	0.4	
	+NaF	0	

[b] Two determinations per value.
+ and - TX = with and without Triton X100, respectively
+ NaF = 1 M NaF in buffer

[c] Units (U) refer to g fresh weight.

Homogenate 1:20, buffer pH 3.6, incubation time 120 min.

Table 11

Lysosomal enzymes in fish liver homogenate (1:20) (Boleophthalmus boddaerti)

Enzyme	Exp.No.	General-conditions	Incubation-time (min)	Spezial-conditions	U/g	n
Acid Phos-phatase	1	R[a]	30	+TX	3.98	2
				-TX	3.20	
					(80)	2
				+NaF	0.63	3
			60	+TX	2.16	2
				-TX	1.34	3
					(62)	
				+NaF	0.29	3
		C[a]	30	+TX	1.18	2
				-TX	1.71	2
					(100)	
				+NaF	0.32	2
			60	+TX	1.70	
	2	R[a]	120	+TX	1.54	4
				-TX	0.72	4
					(47)	
Aryl-Sulfatase	1	R[b]	60	+TX	0.14	3
				-TX	0.07	3
					(74)	
			30	+TX	0.15	3
				-TX	0.07	1
					(50)	
		C[b]	30	+TX	0.13	2
				-TX	0.07	2
					(100)	
			60	+TX	0.14	2
				-TX	0.07	2
					(46)	
	2	R[b]	120	+TX	0.13	3
				-TX	0.03	2
					(24)	

U/g = Units per g fresh weight. Values in brackets give percentage of non-latent enzyme

TX = Triton X100

R[a] = Conditions for rats: 0.15 M β-glycerophosphate solution, buffer pH 5.0.

R[b] = Conditions for rats: 0.02 M nitrocatecholsulfate solution, buffer pH 6.0

C[a] = Conditions for clams: as above, substrate 0.07 M, buffer pH 3.6

C[b] = Conditions for clams: as above, substrate 0.04 M.

Table 12

Marker enzymes in rat liver homogenate

Enzyme	Incubation time (min.)	Special conditions	U/g fresh weight	Assay conditions
Acid phosphatase	20	+TX	4.16	s.Table 15
		-TX	0.36	
			(9)	
	60	+TX	5.40	
			0.61	
			(11)	
Aryl sulfatase	20	+TX	0.39	s.Table 15
		-TX	0.20	
			(51)	
	60	+TX	0.34	
		-TX	0.19	
			(56)	
		$+KH_2PO_4$	0	
		$+Na_2SO_4$	0.10	
Glucose -6-phosphatase	20		22.3	substrate 0.2 M buffer pH: 6.0
5'-nucleotidase	20		3.75	
	60		4.35	substrate 0.1 M buffer pH 7.4

Three determinations per value

Table 13

Lysosomal marker enzymes in various animal species (values in brackets indicate percentage of non-sedimentable enzyme activity). (Units per g fresh weight)

Species	Tissue	Acid phosphatase	n	Aryl-sulfatase	n
Spirestrella cuspidifera	whole animal	n.d.		n.d.	
Prostylyssa foetida	whole animal	0.5 - 1.5 (80)	5	n.d.	
Anadara granosa	digestive gland	3.5 ± 0.7 (100)	7	14.1 (100)	11
Aplysia benedicti	hepato-pancreas	4.8 (100)	4	1.7 (100)	2
Scylla serrata	hepato-pancreas	0.45 (41/100)	3	3.0 (∿100)	2
Boleophthalmus boddaerti	liver	4.0 (80)	2	1.4 (24-74)	13
Rat (Wistar)	liver	4.8 (10)	6	0.4 (54)	6

Arithmetic means, n=number of determinations.
n.d. = enzyme activity not detectable. Values in brackets also correspond to percentage of broken lysosomes. In case of 100% broken lysosomes, data for homogenates with and without added Triton X100 were combined, the other enzyme concentrations correspond to experiments with Triton X100.

Note: The conditions for the respective determinations are listed in a seperate table 14.

Table 14

Concentration of several non-lysosomal marker enzymes in various animal species (same organs as Table 12).
(Units per g fresh weight)

Species	5'-nucleotidase	n	Glucose-6-phosphatase	n	Glutamate dehydrogenase [c]	n
Spirestrella cuspidifera	n.d.	3	n.a.		n.a.	
Prostylyssa foetida	n.d.	3	n.a.		n.a.	
Anadara granosa	6.2[a]	6	0.2[b] 0.5	3 4	n.d.	6
Aplysia benedicti	n.d.	3	n.a.		n.d.	2
Scylla serrata	1.8[c]	2	n.d.	6	n.d.	2
Boleophthalmus boddaerti	n.d.	4.	0.8	2	n.d.	2
Rat (Wistar)	4.1	6	22.3	2	166	1

Arithmetic means, n=number of determinations
n.d. = not detectable
n.a. = not analysed

[a] Substrate 0.3 M, incubation time 2 hours.
[b] Substrate 1.3 M, incubation time 3-4 hours.
[c] Conditions as for rats (s.Table 1)

Table 15

Proposed assay conditions for lysosomal markers in various species of marine invertebrates, fish and rat.

Species	(a) Acid phosphatase		(b) Aryl sulfatase		
	Substrate molarity [c] (mol/l)	Buffer pH	Substrate molarity[c]	Buffer pH	Homogenate dilution
Sponges	0.1 - 0.15	3.6	-	-	-
Anadara granosa	0.07	3.6	0.04	6.0	1 : 100
Aplysia benedicti	0.4 - 0.5	3.6	0.05	6.0	1 : 20
Scylla serrata	0.15	5.0	0.02	6.0	1 : 20
Boleophthalmus boddaerti	0.15	5.0	0.02 - 0.04	6.0	1 : 20
Rat (Wistar)	0.15	5.0	0.02	6.0	1 : 20

a 100 µl sample, 100 µl substrate solution, 1 ml buffer, 200 µl $HClO_4$, Incubation 30 - 60 min (-120 min). Homogenate 1:10.

b 100 µl sample, 100 µl substrate solution, 2 ml buffer, 1 ml PTA, Incubation time 20 - 60 min.

c refers to substrate solution

Table 16

Protein- and enzyme concentration in different animal species

Spezies (organ)	Protein concentration (mg/g fresh weight)		Enzyme concentration[a] (U/mg Protein) Acid phosphatase	Arylsulfatase
S.Cuspidifera (whole sponge)	13.0	(1)		
P. foetida (whole sponge)	14.0	(1)	0.07	
A. granosa (hepatopancreas)	81± 6	(12)	0.04	0.17
A. benedicti (hepatopancreas)	54	(1)	0.09	0.03
S. serrata (hepatopancreas)	67 ± 13	(5)	0.01	0.04
B. boddaerti (liver)	88 ± 9	(3)	0.05	0.02
Rat (liver)	167 ± 4	(44)[b]	0.03	0.002

[a] Estimated by using values from Table 13 and this Table

[b] from Gruner (Gr 78), Table 9

Table 17

Relative distribution of protein and enzymes in subcellular fractions of Anadara granosa digestive gland (Values are expressed as percentage of the total protein or enzyme content in the total homogenate (% N+E) or in the postnuclear supernatant (% E)).

Fraction [a]	Protein		Acid phosphatase	Aryl sulfatase
	% N+E	% E	% N+E	% N+E
N	25 ± 1	-	8	21
E	74 ± 2	-	94	81
MLP	36 ± 2	50 ± 5	34	15
S	39 ± 5	48 ± 4	81	69

Arithmetic means ± S.E., 4-5 experiments per value

[a] N= nuclei and debris; E= postnuclear supernatant; MLP= fraction normally containing most of midochondria, Lysosomes and membranes; S= cytosol

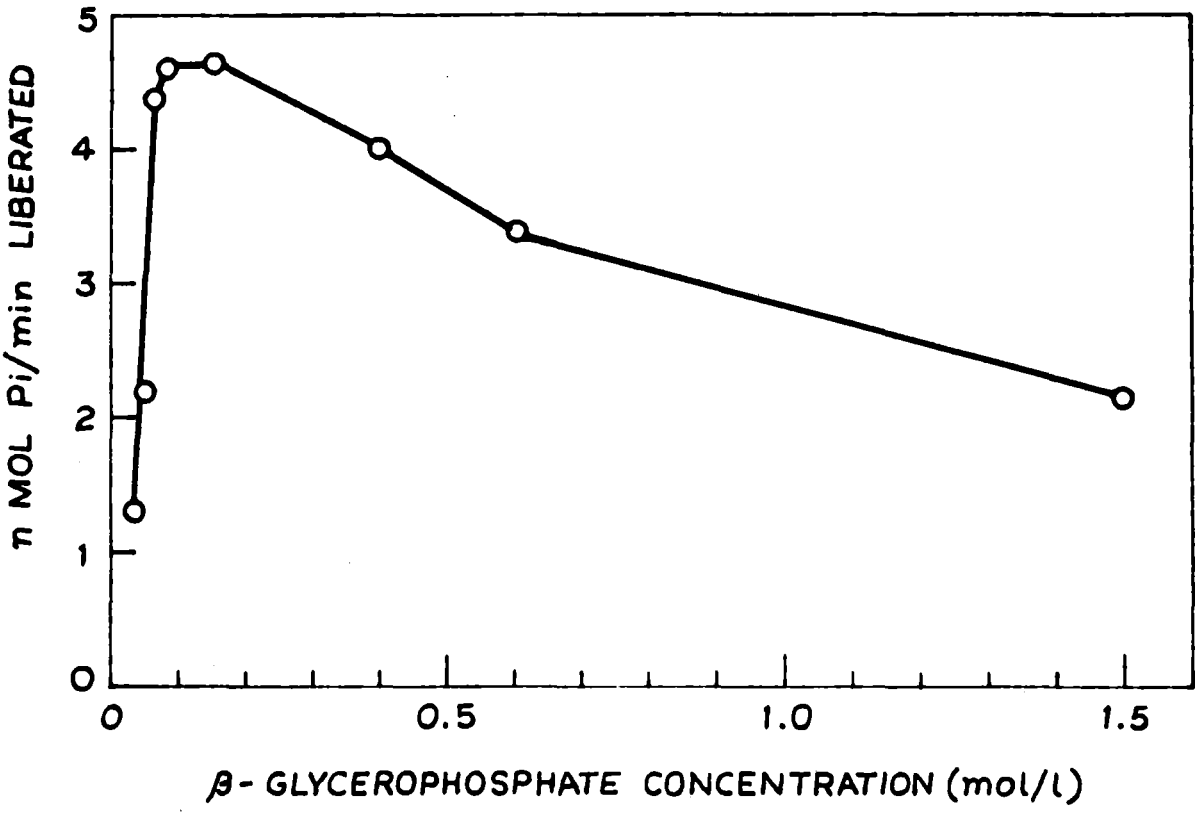

Fig. 1 Influence of substrate concentration on acid phosphatase activity from a clam digestive gland homogenate (representative for 3 qualitatively similar experiments). 1:10 homogenate, incubation 60 min, pH 5.0.

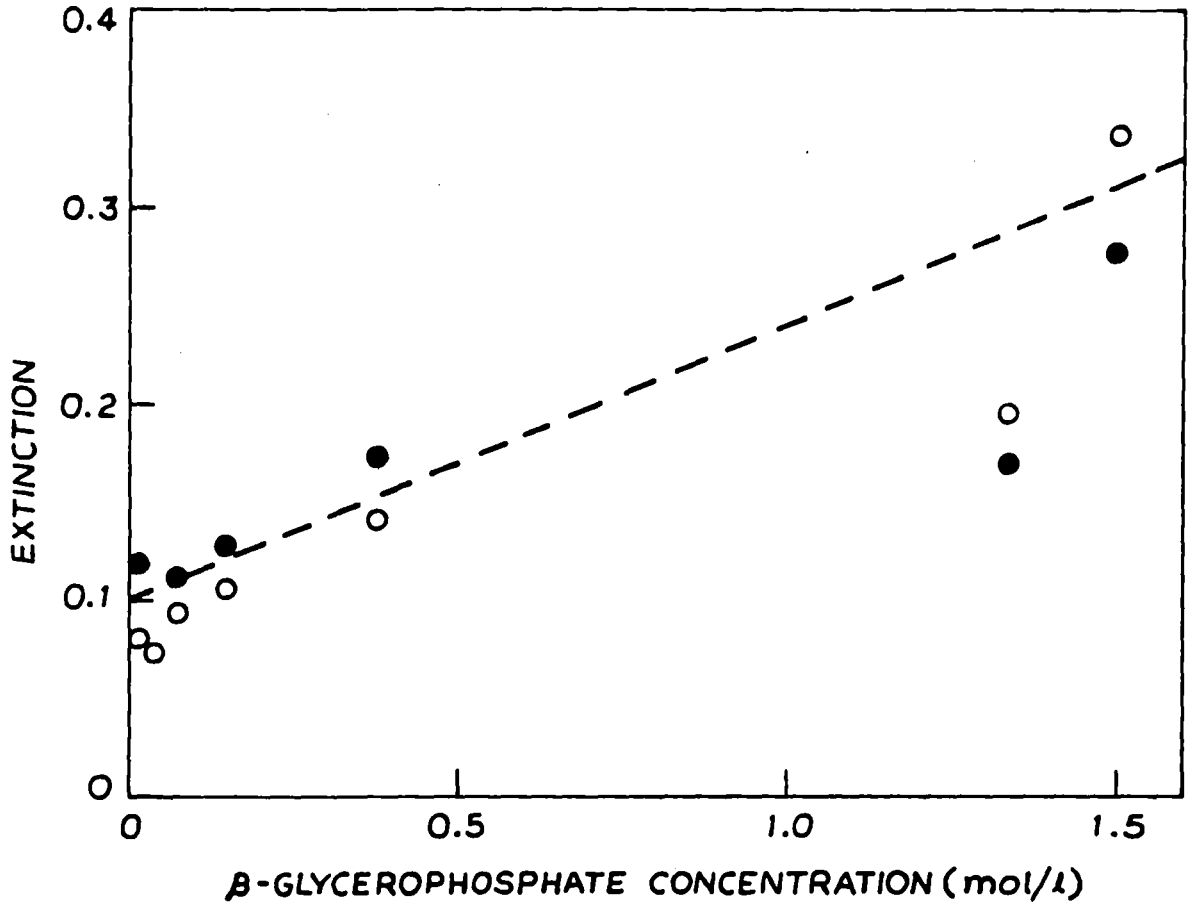

Fig. 2 Influence of β-glycerophosphate concentration on extinction of blank samples after determination of free inorganic phosphate.

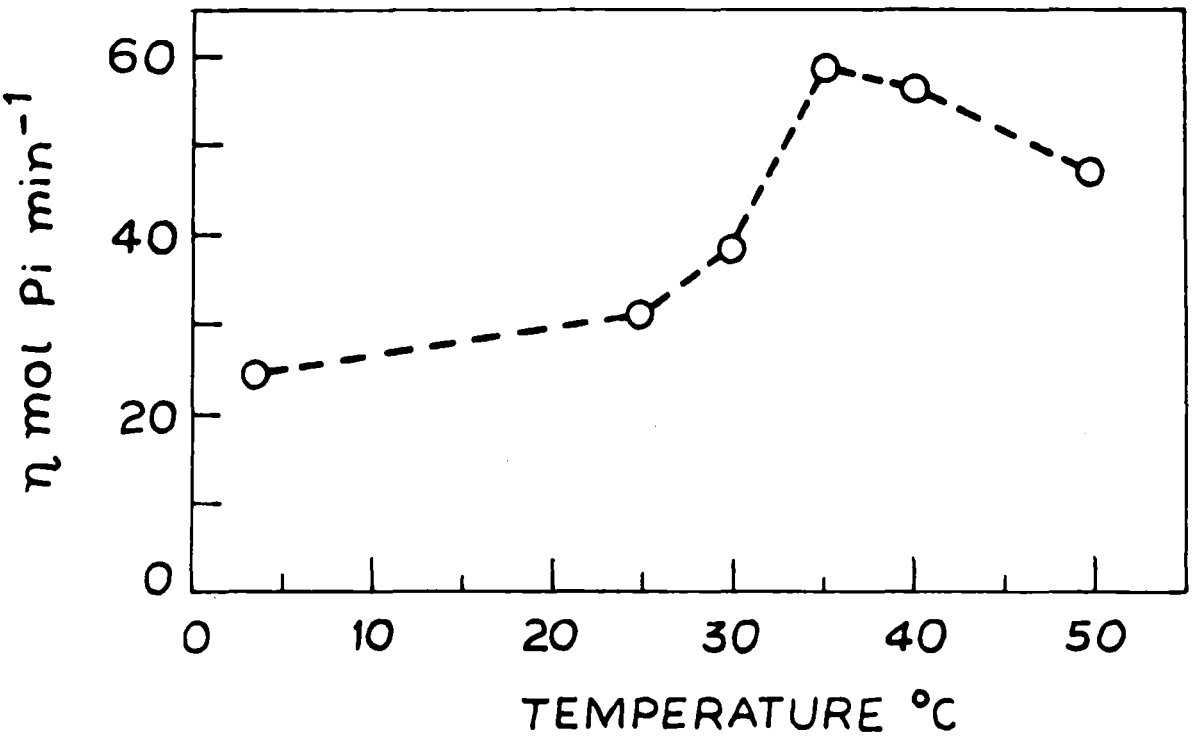

Fig. 3 Influence of incubation temperature on the activity of acid phosphatase from clam digestive gland homogenate (1:20, 20 min incubation, β-glycerophosphate 0.07 M, pH 3.6).

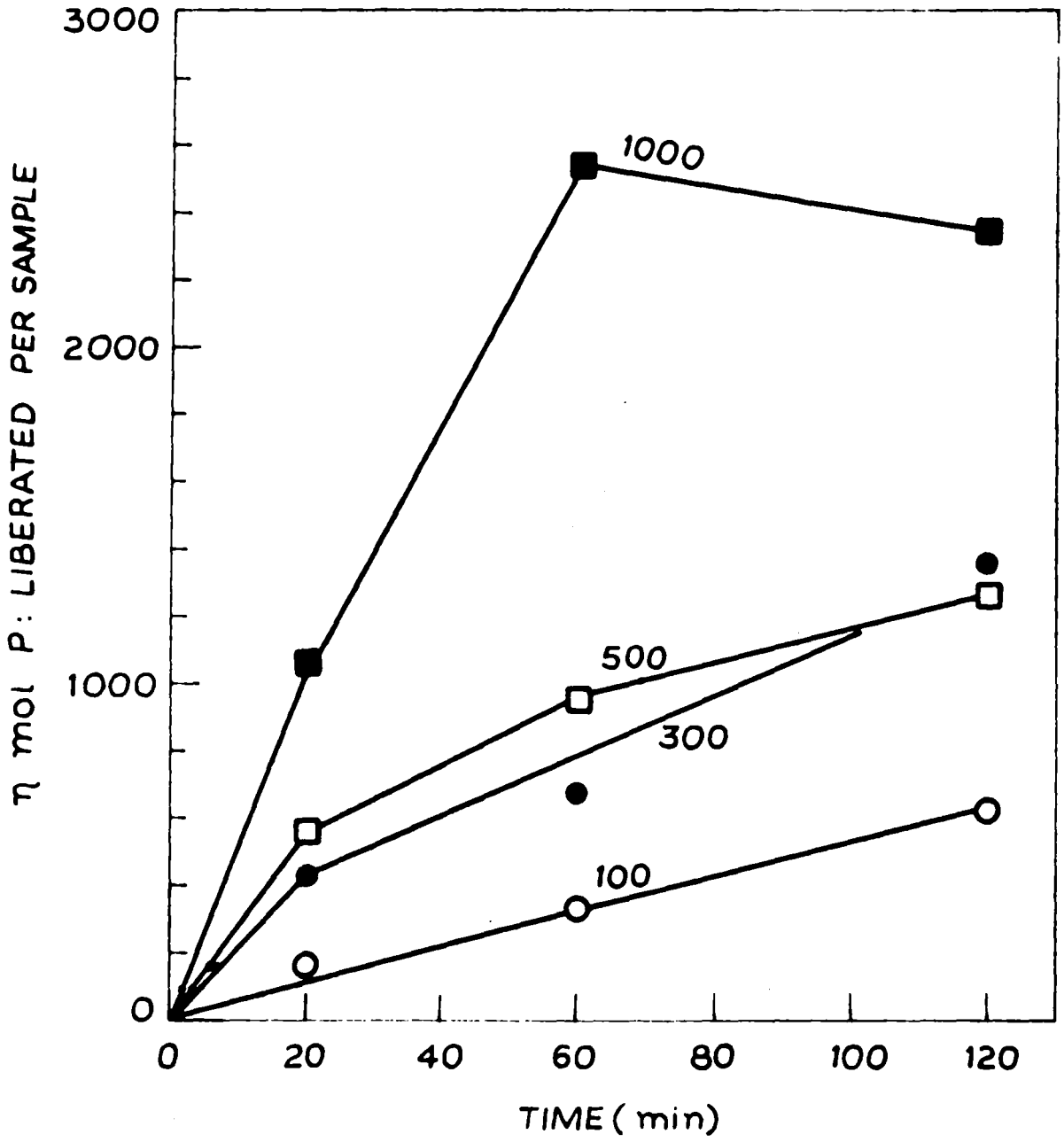

Fig. 4 Dependence of liberation of inorganic phosphate on incubation time and sample volume (numbers in the graph give µl of sample; clam digestive gland homogenate 1:10, β-glycerophosphate 0.15 M, pH 5.0).

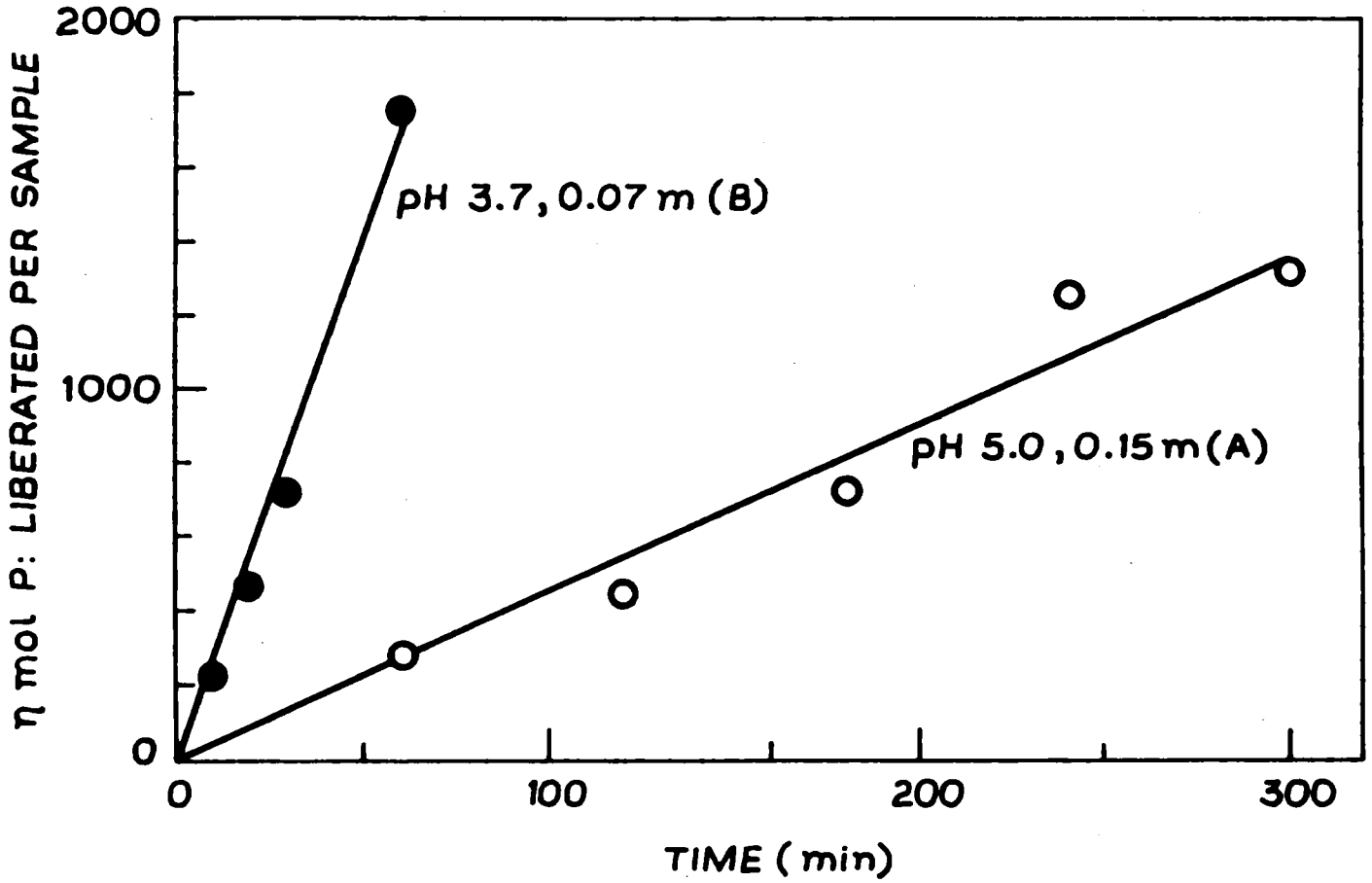

Fig. 5 Dependence of liberation of inorganic phosphate on incubation time, pH and β-glycerophosphate concentration (clam digestive gland homogenate 1:10).

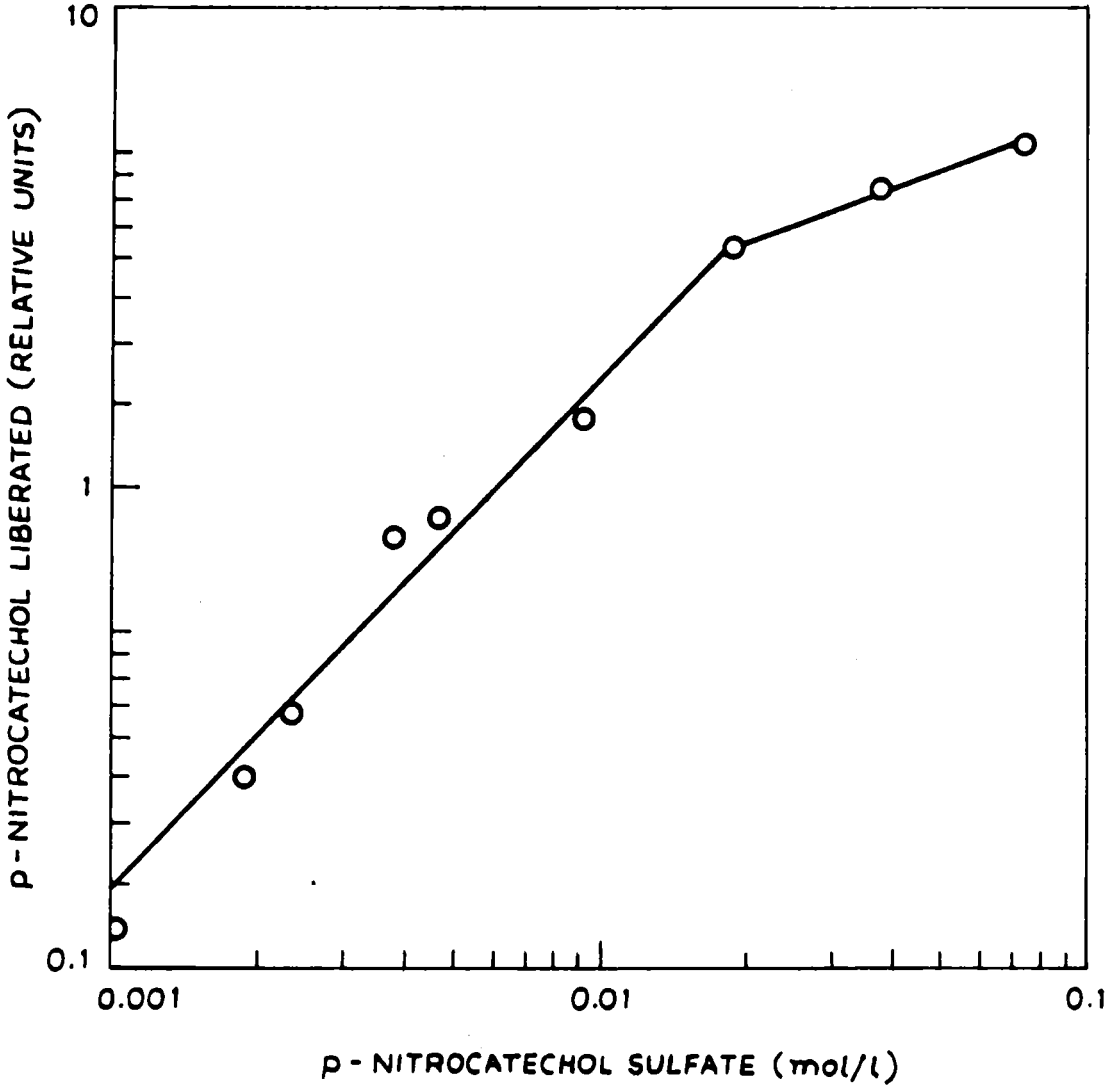

Fig. 6 Influence of p-nitrocatecholsulfate concentration on amount of p-nitrocatechol released by arylsulfatase from clam digestive gland homogenate (1:20; pH 20 min incubation). Representative for 3 experiments.

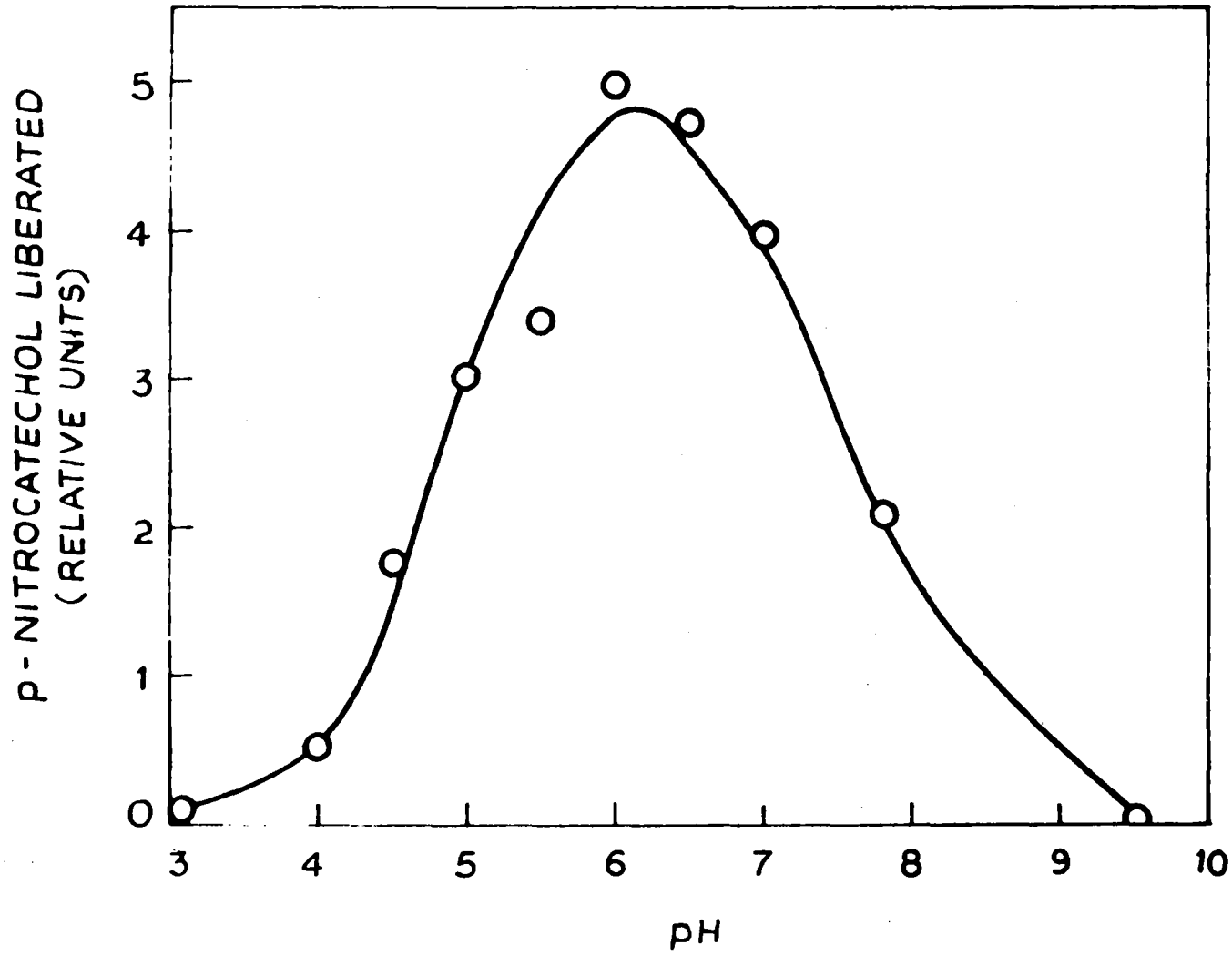

Fig. 7 Influence of pH on arylsulfatase activity in clam digestive gland homogenate (1:50; 0.05 M, incubation time 20 min). Representative for 3 experiments.

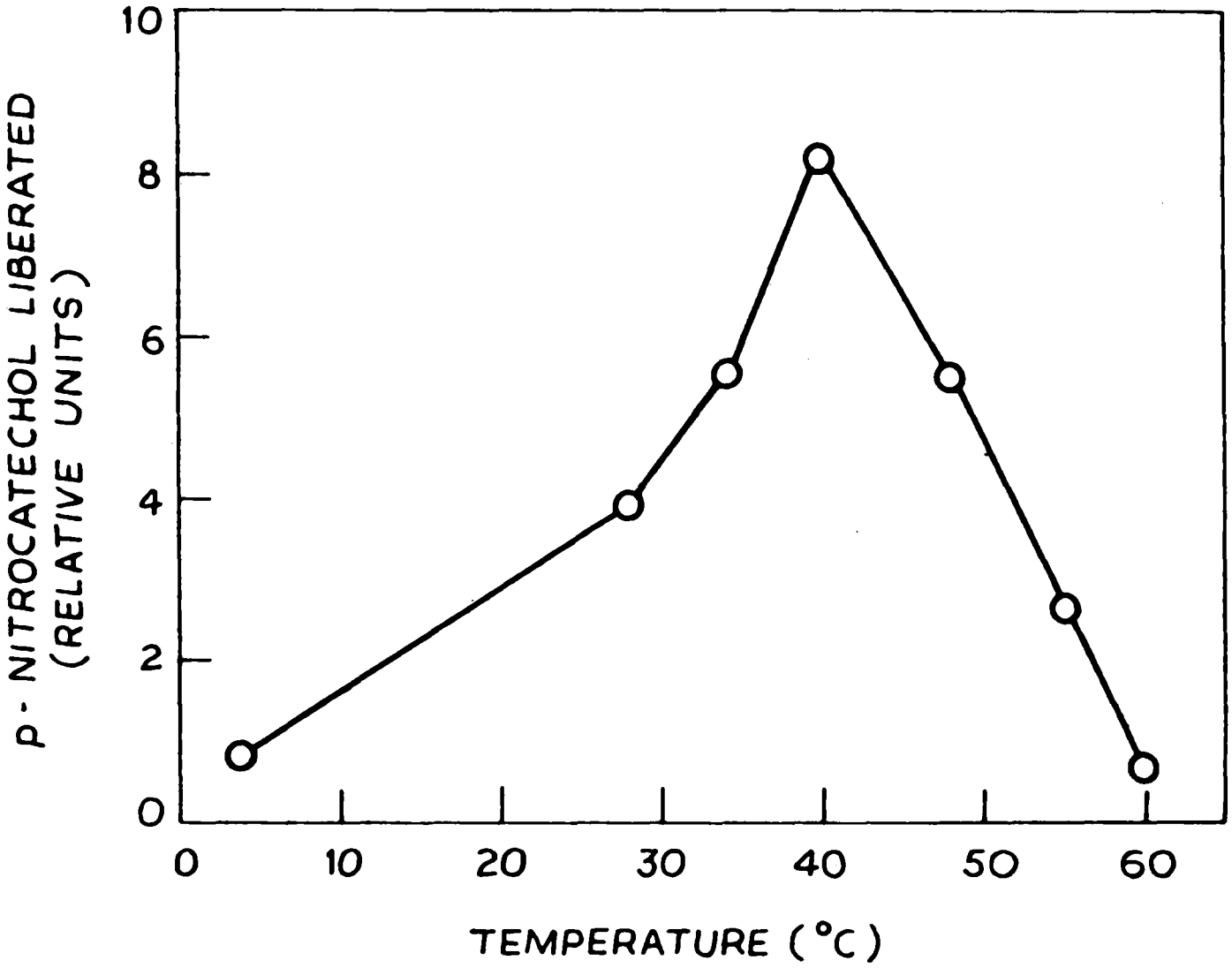

Fig. 8 Influence of incubation temperature on the activity or arylsulfatase from clam digestive gland homogenate (1:50, NSC 0.03 M, pH 6.0, incubation time 20 min). Two values per point, representative for two different experiments.

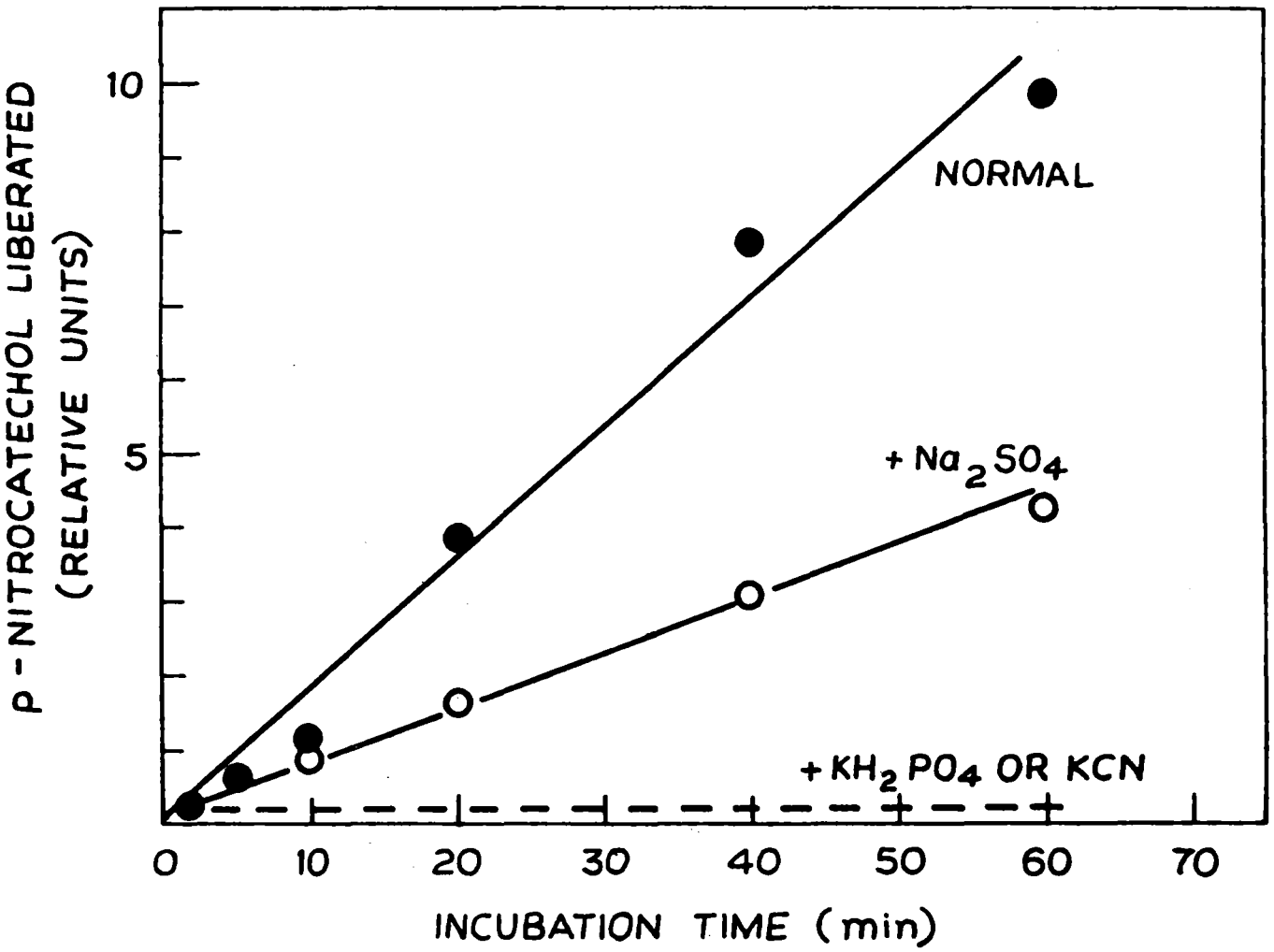

Fig. 9 Influence of incubation time on p-nitrocatechol liberation by arylsulfatase from clam digestive gland homogenate (1:100, NCS 0.03 M). Representative for two determinations, results of inhibition tests are included.

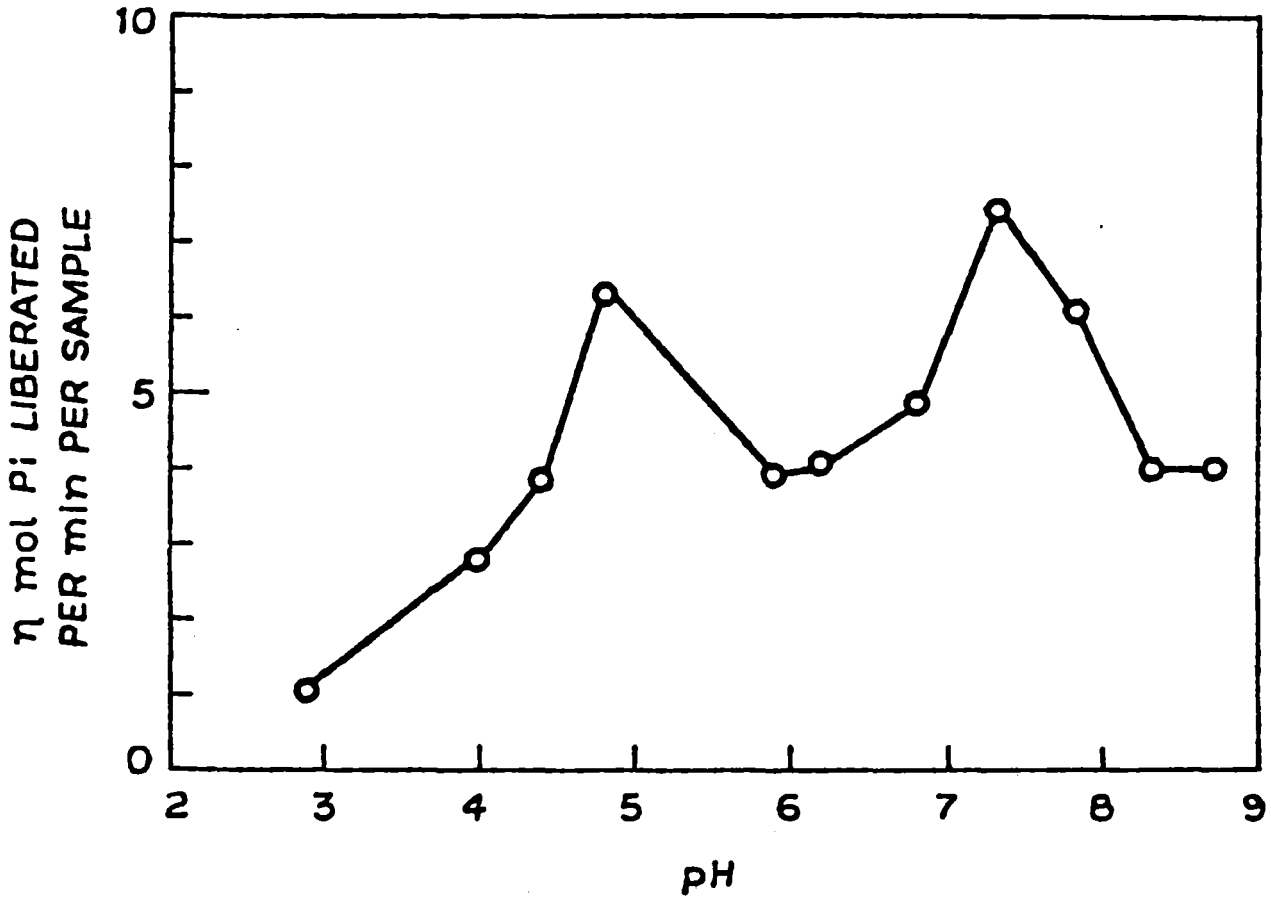

Fig. 10 Influence of pH on AMP dephosphorylation by clam digestive gland homogenate (1:10, AMP 0.3 M, buffer including 10 mM NaF, incubation time 120 min). Two determinations per point.

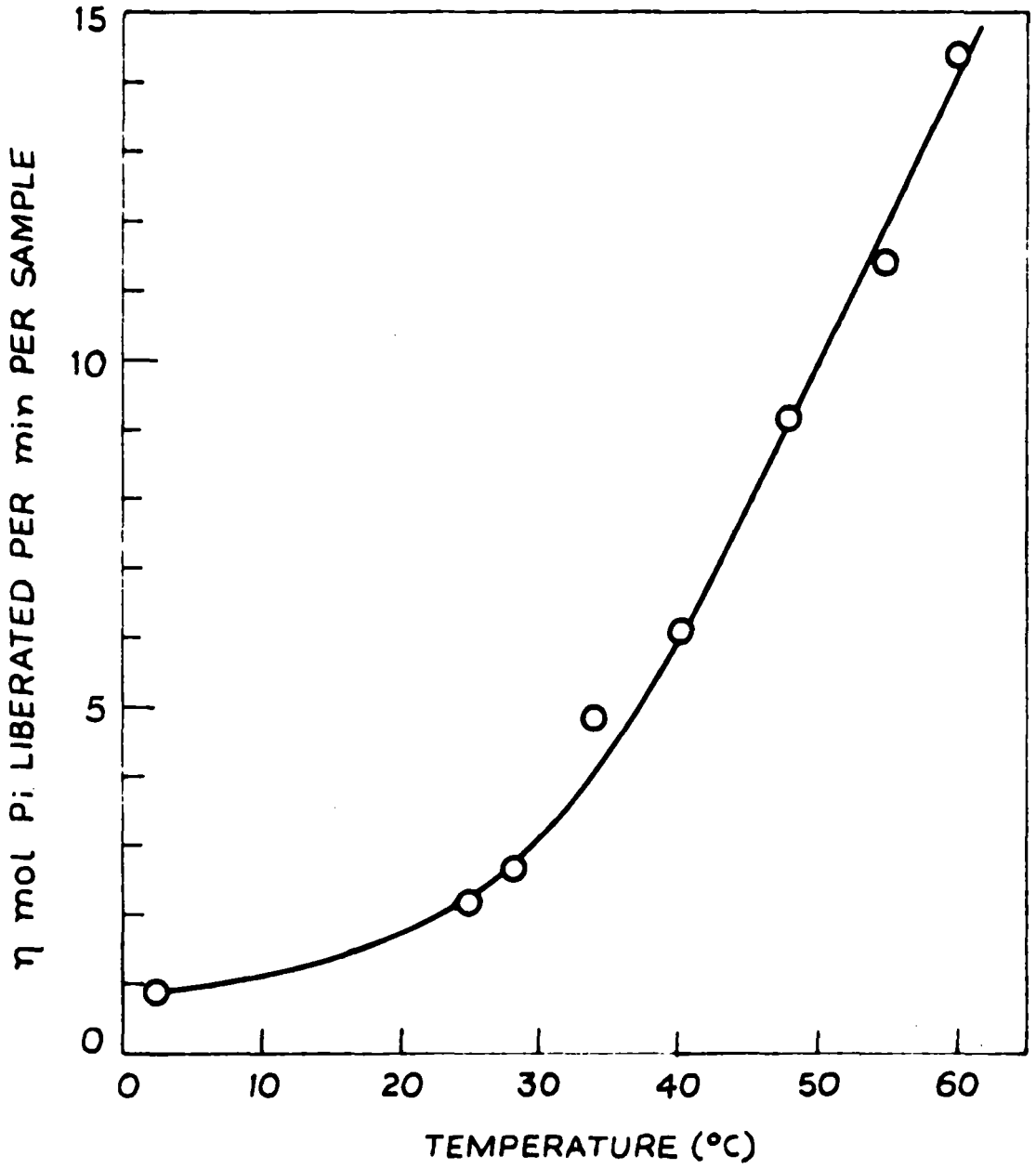

Fig.11 Influence of incubation temperature on AMP dephosphorylation by 5'-nucleotidase from clam digestive gland homogenate (1:10, AMP 0.3 M, pH 7.4 with 10 mM NaF, incubation time 120 min).

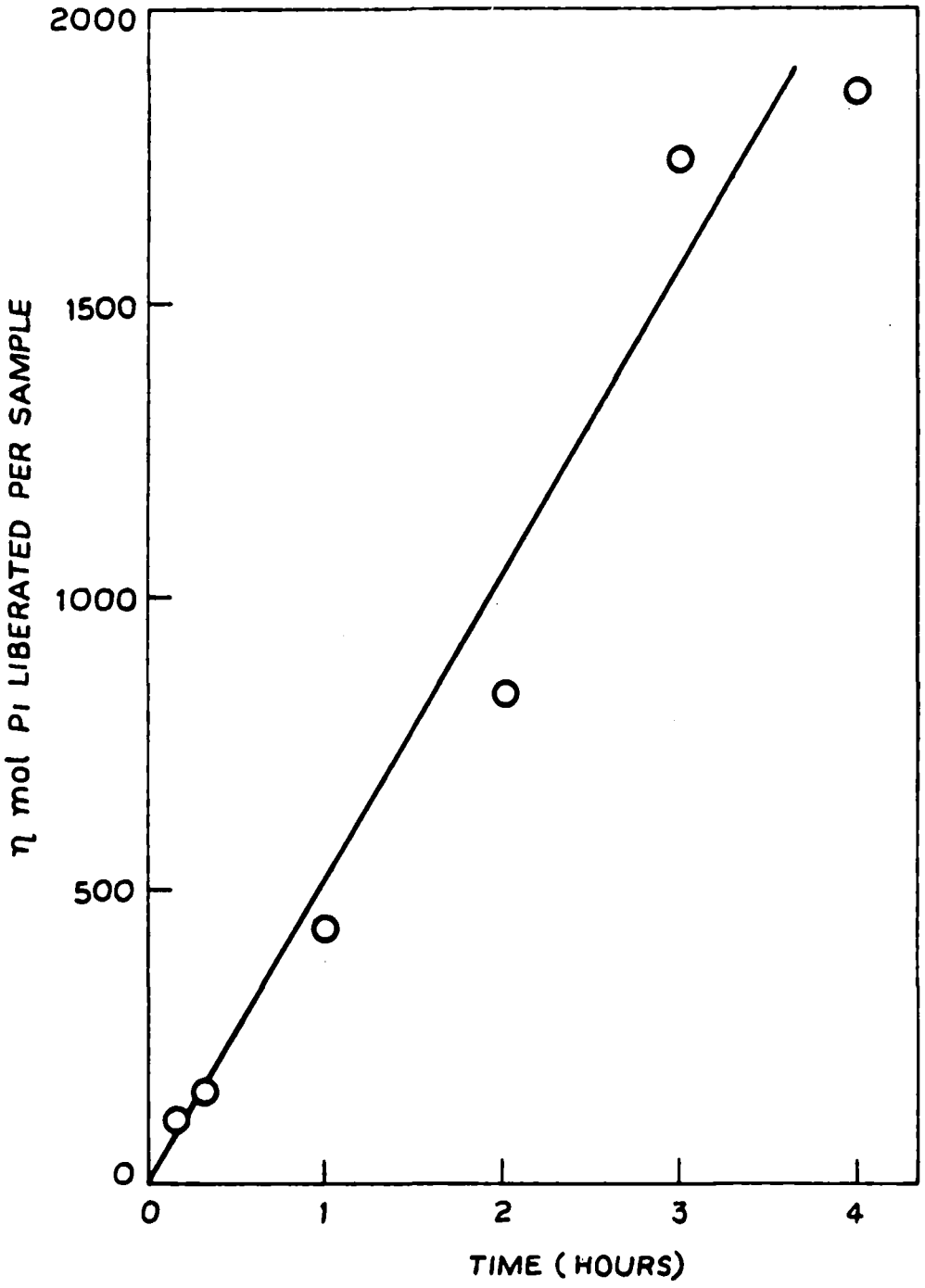

Fig.12 Influence of incubation time on 5'-nucleotidase activity (AMP dephosphorylation) from clam digestive gland homogenate (1:10, data for 0.1 and 0.3 M AMP combined, no NaF added). Two determinations per point,

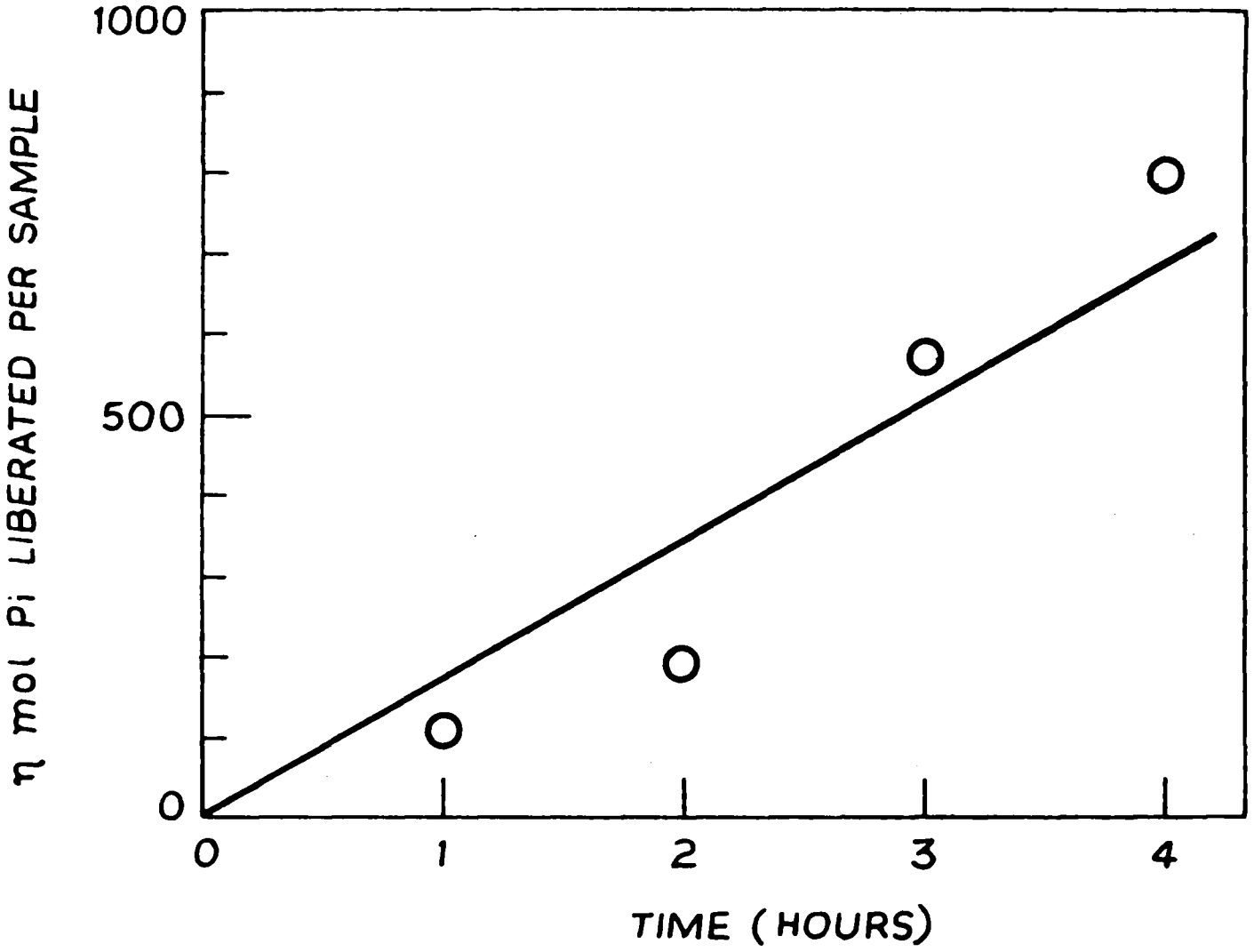

Fig.13 Influence of incubation time on glucose-6-phosphatase activity in clam digestive gland homogenate (1:10, substrate 1.3 M, pH 6.0). Two determinations per point.

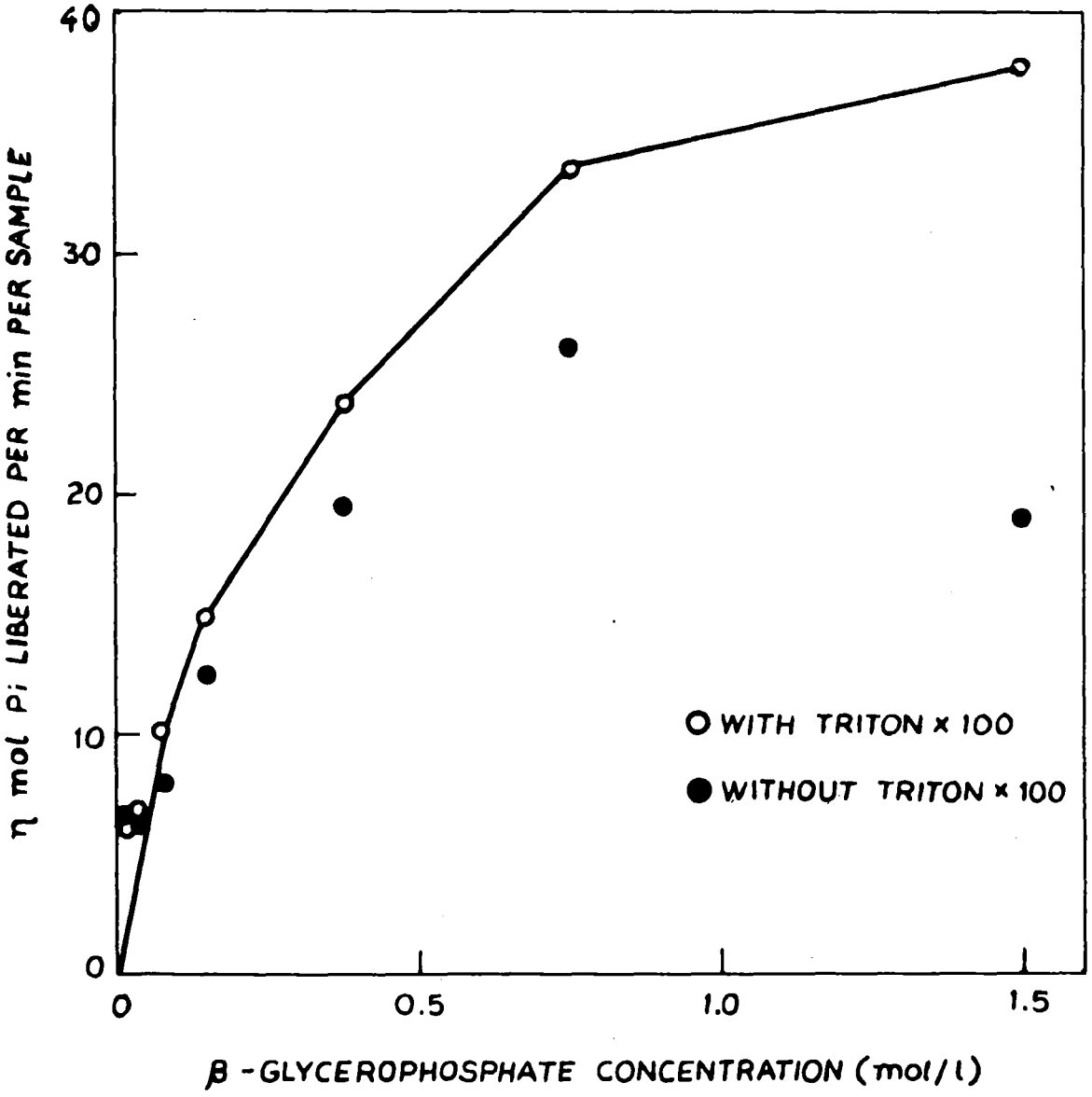

Fig.14 Influence of β-glycerophosphate concentration on liberation of inorganic phosphate by acid phosphatase of the sponge Prostylyssa foetida (1:10 homogenate of whole animal, pH 3.5). One determination per point.

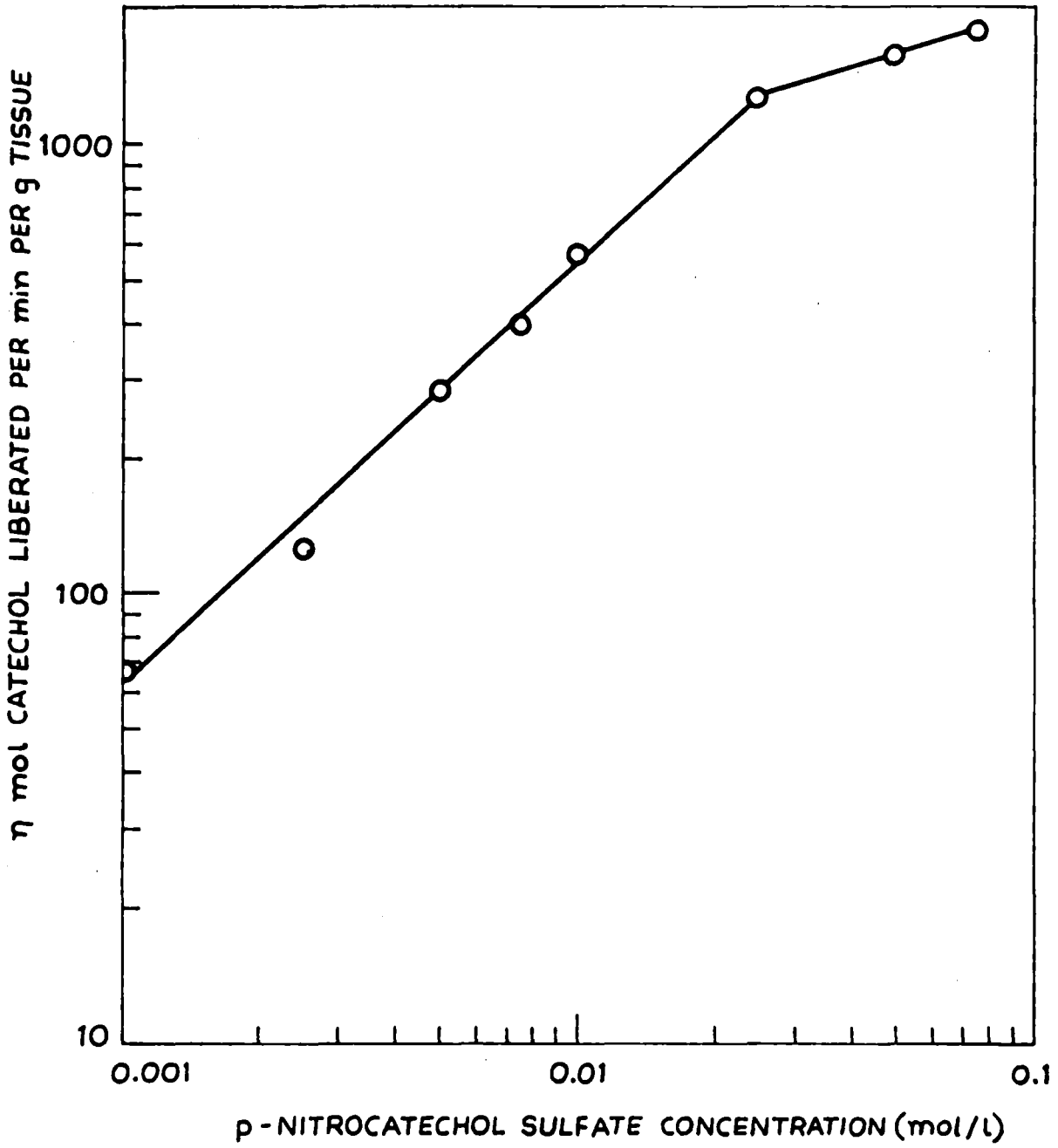

Fig.15 Influence of p-nitrocatecholsulfate concentration on amount of p-nitrocatechol released by arylsulfatase from Aplysia (sea hare) hepatopancreas homogenate (1:20 pH 6.0, incubation time 60 min). Four determinations per point.

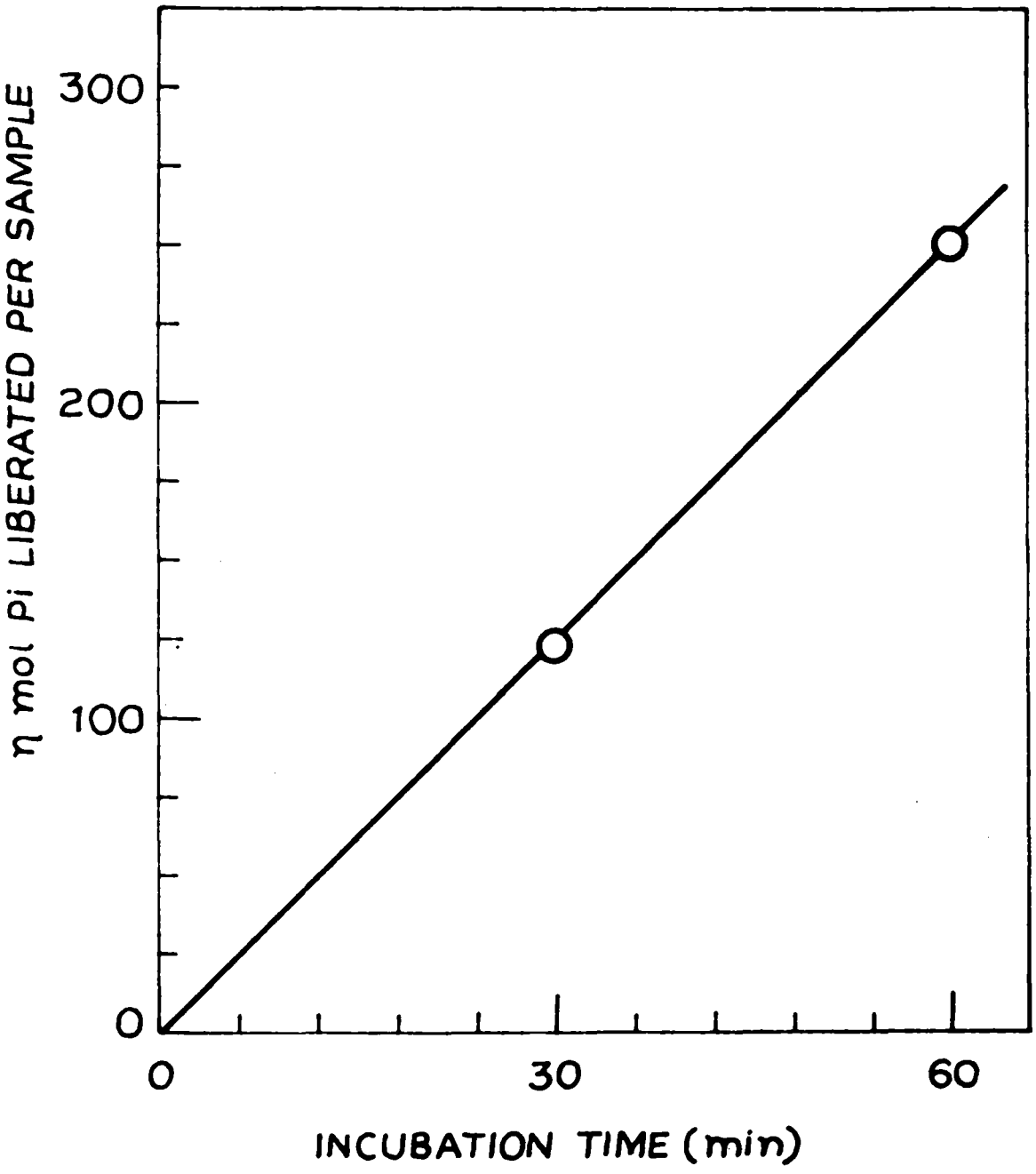

Fig.16 Influence of incubation time on liberation of inorganic phosphate by acid phosphatase from crab hepatopancreas homogenate (1:10, substrate 0.15 mM, pH 5.0). Two determinations per point.

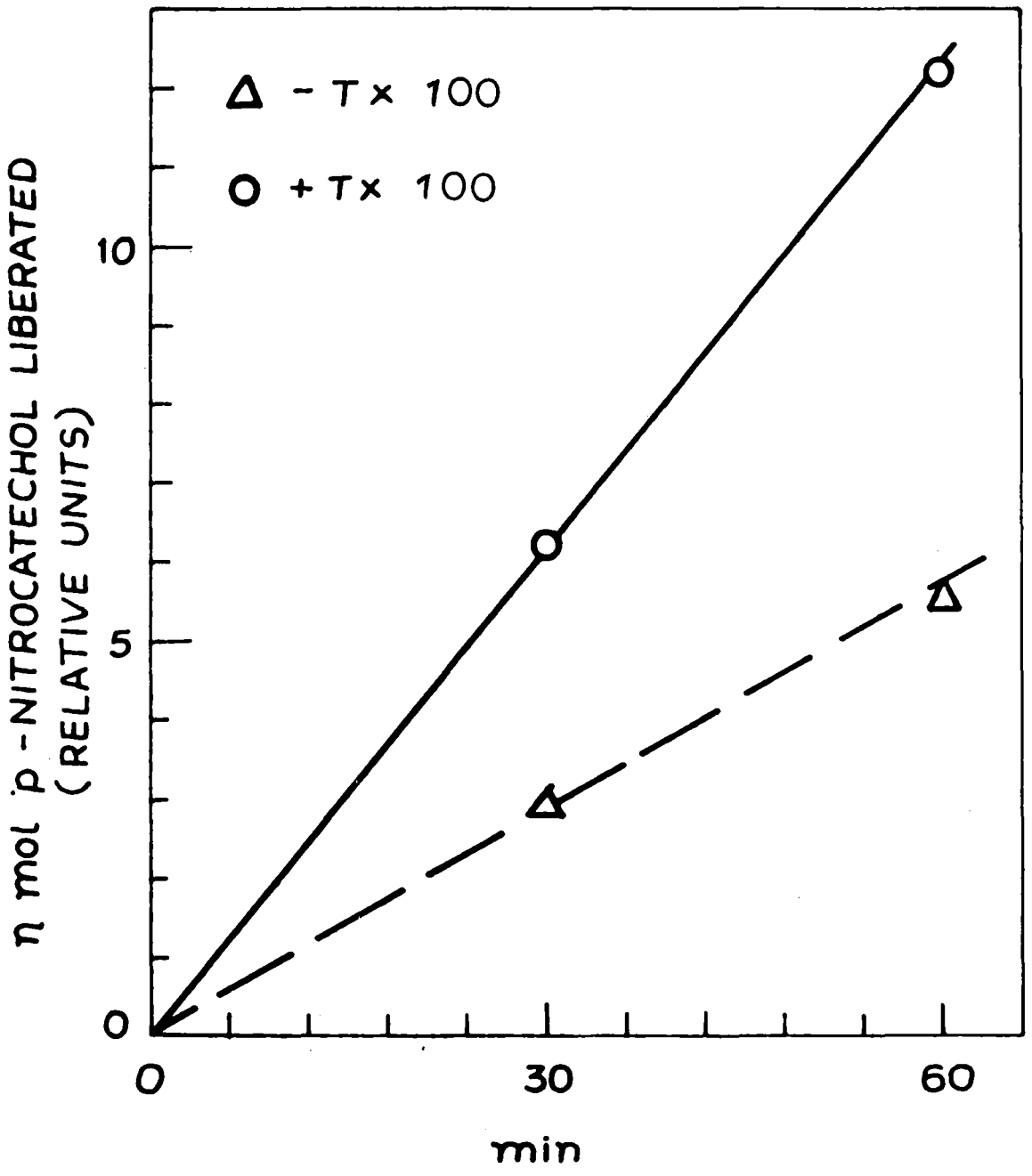

Fig.17 Influence of incubation time on arylsulfatase activity in mudskipper liver homogenate (1:20; pH 6.0, 0.019 M NCS). Two-three determinations per point.