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BREAKDOWN OF ADENOSINE AND INOSINE NUCLEOTIDES IN BONE AT PHYSIOLOGICAL pH

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SUMMARY

The breakdown of adenosine and inosine nucleotides and nucleosides have been studied in articular and epiphyseal cartilage, epiphyseal and metaphyseal cancellous bone, diaphyseal compact bone and periosteum. Dephosphorylating, deaminating, aminating and adenylate-kinase activities have been demonstrated.

INTRODUCTION

Much attention has recently been given to the role of ATP in bone formation. The enzyme mechanism responsible for this phenomenon, however, is not clear. Suggested

Abbreviations: ATP, adenosinetriphosphate; ADP, adenosinediphosphate; AMP, muscle adenylic acid; AS, adenosine; Ad, adenine; ITP, inosinetriphosphate; IDP, inosinediphosphate; IMP, inosinemonophosphate; IS, inosine.

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reaction patterns involve either a transphosphorylation between ATP and an unidentified acceptor[1,2] or the splitting of the pyrophosphate group from ATP, the deposition of pyrophosphate and its subsequent transformation into orthophosphate[3]. Neither of these hypotheses has so far been substantiated by direct evidence.

In a previous paper the pyrophosphatases of bone have been studied[4]. In the present paper the enzymic activities leading to the breakdown of ATP and ITP in the different parts of the bone at physiological pH are described.

EXPERIMENTAL

Homogenate preparation

Six distinct, histologically uniform fractions were prepared from lamb's metatarsum as described in detail elsewhere[4]. 1 g of each fraction was homogenized in a mortar in the presence of 5 ml of water and traces of quartz sand. The volume of the homogenate was made up to 24 ml with water. Since wet cartilage and bone have an approximately equal non-protein content (75 %[5] and 79 %[6] respectively), comparison of the homogenates on the wet weight basis was considered sufficiently accurate. In some experiments thin slices of known weight were prepared from each fraction and used without being homogenized; the results were in all cases identical to those obtained with homogenates.

Materials and methods

The incubation medium was as follows: 0.5 ml 0.1 *M* tris(hydroxymethyl)amino methane buffer pH 7.3, 0.4 ml homogenate, 0.1 ml 0.005 *M* $MgCl_2$, 1.5 mg substrate in 0.15 ml H_2O. Controls were made without the addition of the enzyme or the substrate.

Each fraction was incubated with the following substrates: chromatographically pure sodium salt of adenosinetriphosphoric acid ($ATPNa_2$) and diphosphoric acid ($ADPNa_2$) (Sigma Chem. Corp.); AMP (Schwarz); AS (Light and Co.); ITP, IDP and IMP (California Foundation for Biochem. Research); IS (Fluka).

Incubation was carried out at 38° for 8 min with constant agitation and the reaction was stopped by heating at 100° for 4 min. The pH was controlled before and after incubation. The reaction mixtures were centrifuged and 0.03 ml of the supernatant fluid was analyzed according to CERLETTI *et al.*[7]. Where a sharper separation of AMP from IS was required, bidimensional chromatography was employed; the first solvent was: *n*-propanol–water–100 % trichloroacetic acid–NH_3 (75:20:5:0.5); the second solvent was: *n*-propanol–butanol–water–conc. NH_3 (36:30:24:10).

In order to follow the course of the reaction, samples of a reaction mixture prepared with tissue slices were taken and analyzed as previously indicated; determinations were made after 10 min, 3, 6, 13 and 20 h of incubation.

No breakdown of the substrate, even after 20 h incubation was detectable in controls, to which no homogenate had been added.

RESULTS

The chromatographic and electrophoretic procedures employed provide the means of ascertaining the fate of any adenosine or inosine compound by following their interconversion or disappearance. The presence of the enzymic activities listed below

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was therefore studied in all the 6 homogeneous fractions of bone tissue: nucleotide dephosphorylation, conversion of nucleosides into the corresponding purine bases, transfer of phosphoric radicals between nucleotides or to nucleosides, interconversion of adenosine derivatives to inosine derivatives and *vice versa* by deamination and amination, respectively.

A typical chromatogram is reproduced in Fig. 1. Results are summarized in Figs. 2–7. Each of them has been confirmed in several experiments.

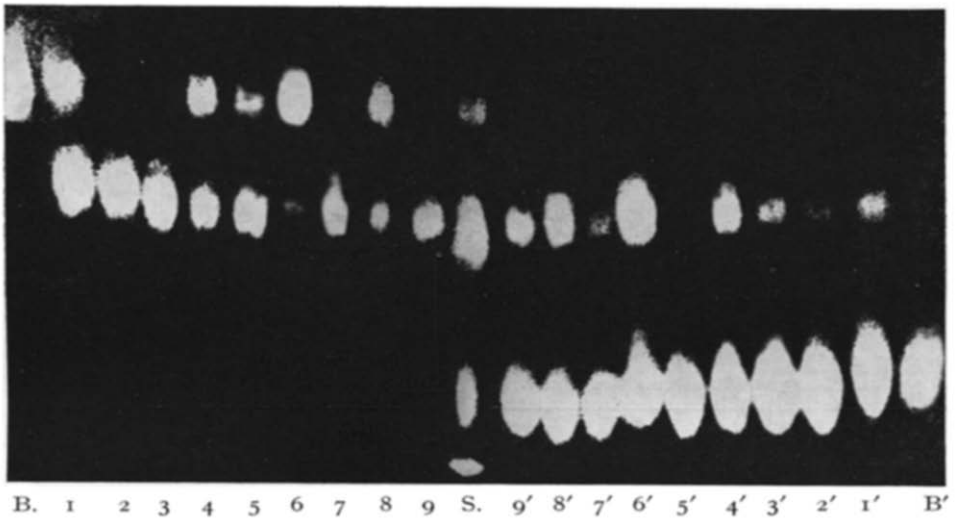

Fig. 1. Descending chromatography of IMP and AS incubated with homogenates of different bone fractions. Experimental conditions as in Fig. 2–7. Substrate from 1 to 9 IMP, from 1′ to 9′ AS. 1,1′ Articular cartilage, 2,2′ epiphyseal cartilage plate, 3,3′ metaphyseal spongiosa, 4,4′ epiphyseal spongiosa, 5,5′ compact bone, 6,6′ diaphyseal bone marrow, 7,7′ marrow of metaphyseal spongiosa, 8,8′ marrow of epiphyseal spongiosa, 9,9′ periosteum. Blanks (no enzyme added) B: IMP, B′: AS. S = Standard mixture. Top to bottom IMP, IS, AS, Ad.

(1) *Articular cartilage*

As shown in Fig. 2, only inosinephosphates are weakly dephosphorylated. The formation from ADP of both ATP and AMP in equal amounts shows the presence of an adenylate kinase. IS is aminated and AS weakly deaminated.

(2) *Epiphyseal spongiosa*

Fig. 3 shows dephosphorylation of ATP and ADP and (more intense) of inosinephosphates. AS is deaminated. IS is transformed into hypoxanthine and also weakly aminated.

(3) *Epiphyseal cartilage plate*

Inosine phosphates and ATP are actively dephosphorylated, while IMP and AMP undergo little or no breakdown (Fig. 4). An adenylate kinase is evidenced by the formation of both ATP and AMP from ADP. From the relative amounts formed (AMP > ATP) ADPase activity might also be inferred in addition to ATPase activity.

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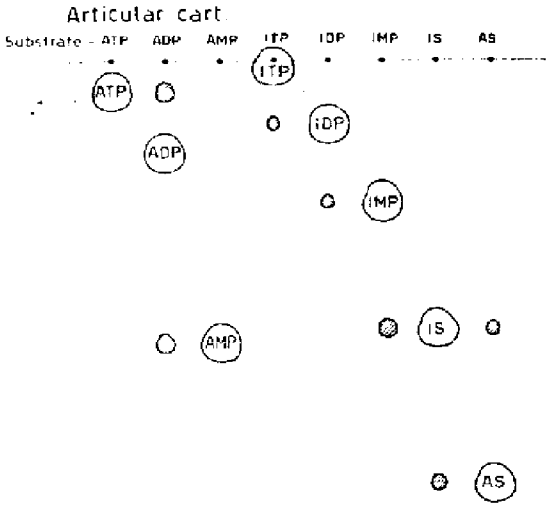

Fig. 2.

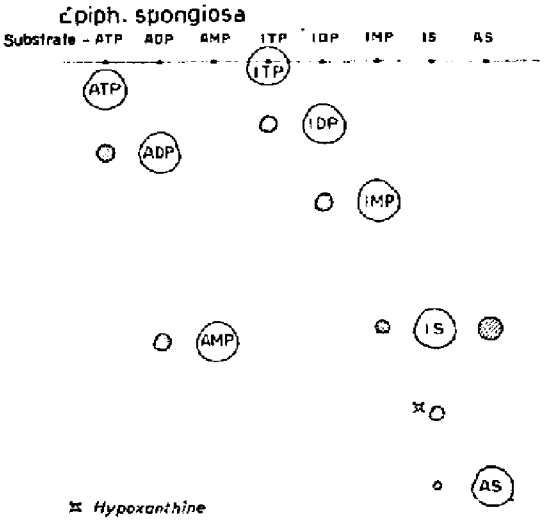

Fig. 3.

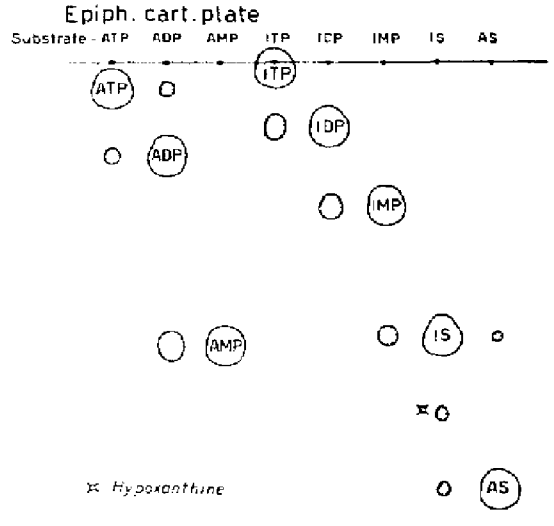

Fig. 4.

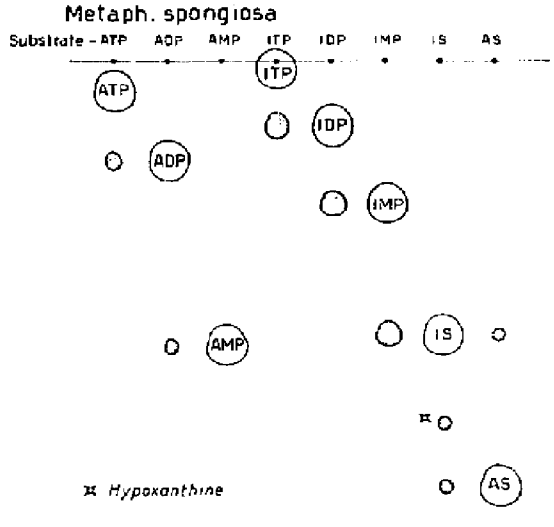

Fig. 5.

With short incubation periods only a little inosine is formed from adenosine. After 6 h of incubation nucleotides are also deaminated. More inosinenucleotide is formed by deamination of the corresponding adenosinenucleotide than by dephosphorylation of the higher homologous phosphate.

Moderate amounts of hypoxanthine and of adenosine are formed from inosine.

(4) *Spongiosa of metaphysis*

As shown in Fig. 5, the dephosphorylation of inosinephosphates is active, that of ATP and ADP weak. The rate of the IMP → IS reaction is particularly high, so that when ITP is incubated for a long period of time only IDP and IS are present, and

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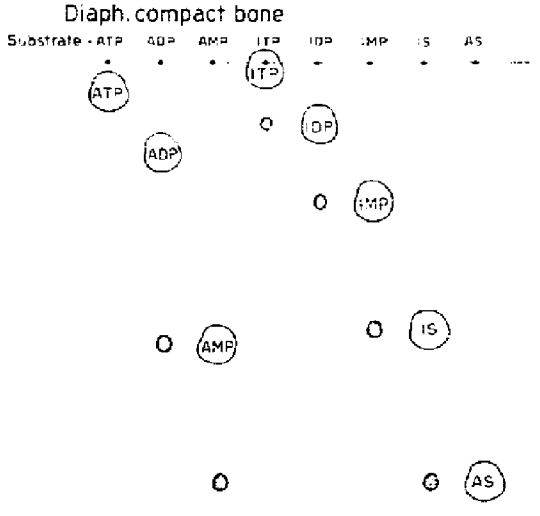

Fig. 6.

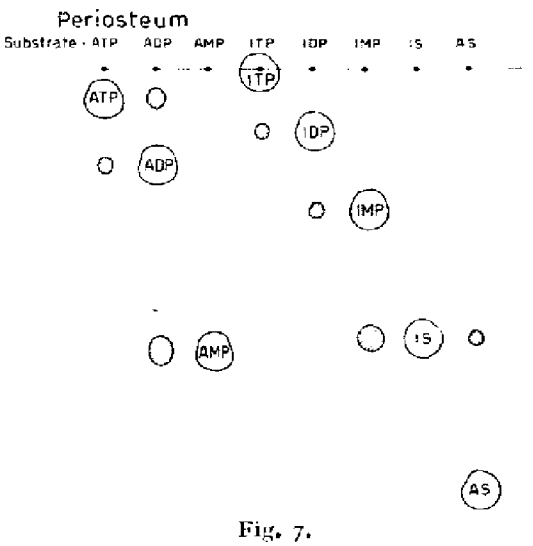

Fig. 7.

Figs. 2–7. Action of bone constituents on adenine and inosine nucleotides and nucleosides. Dots represent the substrate not metabolized, dashes the products of reactions; their areas correspond to the amount formed. Each sample contained: 1.5 mg substrate in 0.15 ml H_2O, 0.5 ml tris buffer pH 7.3, 0.1 ml 0.005 *M* $MgCl_2$ 0.4 ml homogenate. Incubation 8′ at 38°.

IMP does not appear among the reaction products, probably because it is dephosphorylated as soon as it is formed.

AS and hypoxanthine are formed from inosine. Traces of IS are formed from AS.

(5) *Diaphyseal compact bone*

Only inosinephosphates, ADP and AMP are dephosphorylated. IS is rather intensely aminated (Fig. 6).

(6) *Periosteum*

Fig. 7 shows intense dephosphorylation of ATP and inosinephosphates and deamination of AMP and AS. Hypoxanthine is formed from IS. Adenylate kinase activity transforms ADP to ATP and AMP. Since the amounts of AMP formed are only slightly larger than those of ATP, ATPase activity is sufficient to account for the difference. At any rate, ADPase activity should be rather weak.

In experiments carried out at pH 9.2 with AMP or IMP as substrates, a strong dephosphorylation of AMP was evidenced in all fractions. IMP is intensely dephosphorylated in epiphyseal spongiosa, compact bone and periosteum, while in articular and epiphyseal cartilage and in metaphyseal spongiosa the dephosphorylation is not so active.

DISCUSSION

A more extensive study of the enzymes connected with the breakdown of ATP and ITP can be carried out after establishing their optimum activity over all the pH range.

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This work is now in progress. We thought, however, that a first substantial approach could be made by studying the breakdown of ATP and ITP at physiological pH.

The results reported above provide evidence for the existence of several enzymic activities differing both for the reaction products and for their localization within the bone.

Evidence has been obtained for the presence of nucleotidases, nucleosidases, deaminases and aminases. The occurrence in each tissue preparation of reactions interrelated so that the product of one is the substrate of another, makes it difficult to establish the number and type of active enzymes; some of the properties of these enzymes, however, have been ascertained by the present work.

Nucleotidases are variously distributed and are always specific either for adenosine or for inosine derivatives; the action on the di- and triphospho-derivatives of each series is similar and, generally, quite different from their action on the corresponding monophosphates. In most fractions in fact, adenosinenucleotidases are active either on ATP and ADP or on AMP; when ADP, ATP and AMP are dephosphorylated, the reaction rate for AMP is strikingly different from that for ADP and ATP. Inosinenucleotidases, on the other hand, usually attack all the three phosphorylated derivatives; also in this case, however, the reaction rates for the di- and triphosphates differ from that for IMP.

This different reaction rate for monophosphates can be accounted for by the phosphomonoesterase, which has been found in ossifying cartilage and in newly ossified bone by MARTLAND AND ROBINSON[8] and by ROCHE AND BULLINGER[9]. An enzyme of this type has also been purified from bone by several authors[10,11]: it does not split internucleotide phosphoryl groups[12]. Unfortunately, none of these authors clearly indicates its localization within the different bone constituents. A diesterase activity is outlined in the work of GULLAND AND JACKSON[11], and LIEBKNECHT[13] describes phosphatase activity of bone extracts on ATP, ADP and AMP, while OTTA[14] obtained hystochemical evidence of a 5-nucleotidase specific for AMP in articular cartilage. All these enzymes have pH optima in the alkaline range (about pH 9) and their wide distribution in different bone constituents is shown by the enhanced dephosphorylation we observed in all fractions when samples were incubated at pH 9.2.

The presence among the reaction products of compounds with two phosphoric radicals less than the substrate has never been observed until after the appearance of the homologous compound with one phosphoric radical less than the substrate. Direct evidence exists, therefore, for the presence of enzymes which split one phosphoric radical at a time. It cannot be excluded, however, that the dephosphorylation of a part of the substrate could occur by a loss of a pyrophosphoric radical; direct data in favour of this hypothesis are, for the time being, lacking. This is in agreement with LIEBKNECHT's results[13] on bone extracts, at pH 9.5.

Kinase activity of miokinase type ($2ADP \longrightarrow ATP + AMP$) has been found only in articular and epiphyseal cartilage and in periosteum and not in ossified bone fractions. In articular cartilage adenylate kinase is not accompanied by ADPase activity. In fractions 3 and 6 nucleotidase activity is suggested by the relative amounts of ATP and AMP formed ($AMP > ATP$).

Deaminases are rather specific: generally only one compound is actively deaminated by a histologically homogeneous fraction.

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The nucleoside is the most common substrate of deamination, and the reaction seems generally to be reversible, except in fractions 5 and 6. AMP is deaminated only by fraction 6, ATP and ADP only by fractions 3. Irreversible amination of inosine occurs in fraction 5. The amination of IDP and ITP, moreover, could never be demonstrated.

LUTWAK-MANN[15] reports for articular cartilage dephosphorylation of muscle and yeast adenylic acids and of ATP, accompanied by deamination of these compounds and of adenosine. These data are in good agreement with ours, considering that the long incubation time used by this author (3 h) allows deamination of adenosine compounds followed by dephosphorylation of the inosine derivatives formed. On the other hand we were unable to confirm the results of PERKINS[16]. The dephosphorylation activity found by this author in bone metaphysis is probably ascribable to the marrow present among the trabeculae rather than to the bone tissue itself.

It should finally be noted that epiphyseal cartilage plate and both spongiosas are able to metabolize ATP to hypoxanthine, and that although several fractions contain a nucleosidase that transforms inosine to hypoxanthine, no fraction is capable of producing adenine from adenosine.

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