ADENINE CYCLE IN HEPATOPANCREOCYTES OF HELIX POMATIA (GASTROPODA)*

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Intact hepatopancreocytes were obtained from hibernating or active purinotelic snails, H. pomatia (Gastropoda). When incubated with \[^{14}C\]\textit{ glycine or \[^{14}C\]}\textit{formate, they synthesized de novo purine compounds, including also adenylates, adenosine and adenine. Hepatopancreocytes resynthesized also adenylates and other purine compounds from \[^{3}H\]adenine or from \[^{3}H\]adenosine split by the H. pomatia cell enzyme to adenine; the resynthesis of ADP+ATP was proportional to adenine concentration. Thus all reactions of the postulated adenine cycle: AMP→adenosine→adenine→AMP occur in the intact hepatopancreocytes; this cycle could probably be responsible for maintenance of the high level of adenylates during winter sleep.

The pattern of purine nucleotide metabolism in Helix pomatia hepatopancreas presented in Scheme 1 has been postulated on the basis of investigations \textit{in vitro} (Jeżewska & Barankiewicz, 1977). The occurrence of a specific adenosine phosphorylase, adenosine phosphoribosyltransferase and 5'-nucleotidase in this tissue suggested the operation of the adenine cycle: AMP→adenosine→adenine→AMP. It seems that in purinotelic species the adenine cycle in liver may provide some self-sufficiency of the adenine nucleotide pool, and its independence of the presumable main pathway of protein catabolism: small precursors→IMP→inosine→hypoxanthine→xanthine→uric acid. In the present study, intact hepatopancreocytes were obtained for the first time from the H. pomatia hepatopancreas during winter sleep (hibernation) and active life, and were used to investigate the postulated metabolic pathways.

MATERIALS AND METHODS

Reagents. Chemicals were purchased from the following sources: Fisher’s medium for leukemic cells of mice, Cat. No. H-11 (Grand Island Biol. Comp., Grand Island, N.Y., U.S.A.); purine bases, nucleoside and nucleotides (Calbiochem. Corp.,

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[11]
Los Angeles, Calif., U.S.A.); 1,4-bis-2-(5-phenyloxazolyl)benzene and bovine serum albumin (Fluka AG, Buchs S.G., Switzerland); 2,5-diphenyloxazole (Serva, Heidelberg, G.F.R.); 5-amino-4-imidazolecarboxamide (Sigma Chemical Co., St. Louis, Mo., U.S.A.); polyethyleneimine cellulose sheets (Macherey-Nagel Co., Düren, G.F.R.); Eastman Kodak Chromagram sheets (Eastman Kodak Co., Rochester, U.S.A.); collagenase (Wytównia Surowiec i Szczepionek, Warszawa, Poland). All other reagents were purchased from Polskie Odczynniki Chemiczne (Gliwice, Poland).


Animals. Adult Helix pomatia (Gastropoda) were used in March (winter sleep) and in May (active period).

Preparation of hepatopancreocytes. The snail’s nerve ring (ganglia cerebralia) was cut and the shell was discarded. The hepatopancreas (digestive gland) was taken and the intestine coiled between its lobes was carefully removed. The hepatopancreatic tissue (1 - 1.5 g) was immersed in Fisher’s medium containing 0.15% collagenase and 4% albumin, and finely minced with scissors. The suspension was transferred to a 25 ml beaker and shaken at 30°C. At intervals of about 5 min the suspension was aspirated into Pasteur’s pipettes with progressively smaller bore-size (Topper et al., 1975). After 30 min the cell suspension was homogeneous when microscopically examined. Cells were centrifuged at 3000 g in a cold-room. The pellet was washed 3 times with 5 ml of Fisher’s medium containing 4% albumin. Finally the same medium was added to make a 5% (v/v) cell suspension, which was used in further experiments.

Viability of the cells was tested by staining with Trypan Blue (Belleman et al., 1977). The cells were able to synthesize nucleic acids from radioactive precursors. The energy charge (Atkinson, 1968) as well as the ATP/ADP ratio, calculated taking into account quantities of radioactive AMP, ADP and ATP synthesized during 2 h of incubation (not their total concentrations in the cells), were within the range of values found in hepatopancreas of Mytilus edulis (Wijmsan, 1976).

The hepatopancreocytes obtained from hibernating and active snails are further referred to as hepatopancreocytes H and A, respectively.

Incubation. The incubation mixture contained in a final volume of 0.2 ml: 80 μl of 5% suspension of hepatopancreocytes in Fisher’s medium and: 0.01 μmole of [8-3H]adenine (10 μCi), or 0.15 μmole of [8-3H]adenosine (10 μCi), or 0.1 μmole of [14C]glycine (1.0 μCi), or 0.01 μmole of [14C]formate (0.56 μCi) and 0.1 μmole of AIC. All mixtures used in one experiment contained the same suspension of hepatopancreocytes H or A, and were incubated simultaneously in a water bath for 2 h at 30°C, with shaking. All precursors were used at concentrations exceeding several times their endogenous concentrations.

1 Abbreviations: AIC, 5-amino-4-imidazolecarboxamide; PPO, 2,5-diphenyloxazole; POPP, 1,4-bis-2-(5-phenyloxazolyl)benzene; hepatopancreocytes H, obtained from snails during winter sleep; hepatopancreocytes A, obtained from active snails.
Determination of radioactive products and precursors. Extraction of radioactive compounds, separation of purine ribonucleotides, ribonucleosides and bases, as well as the radioactivity measurements were performed essentially as described by Burridge et al. (1977). In the separation of purine compounds from each other and from glycine or formate, the third washing of the polyethyleneimine cellulose sheet in formic acid/methanol/water (35:90:15, by vol.) was performed according to an unpublished method of Dr. J. F. Henderson (personal communication), used at the Cancer Research Unit, University of Alberta, Canada. The sum of radioactivities of all purine compounds synthesized, including nucleic acids, and of the radioactivity of the remaining precursor, was found to be almost equal to the initial precursor radioactivity.

Content of radioactive purine compounds were calculated from the radioactivity measurements and expressed as nmoles synthesized by 1 g of packed cells during 2 h. These data are presented in Schemes 2, 3, 4 and 5.

RESULTS AND DISCUSSION

Total adenine compound pool and the enhancement of IMP biosynthesis de novo. In the present experiments the hepatopancreocytes synthesized IMP either entirely de novo from [14C]glycine (and from other small precursors provided by Fisher’s medium) or from [14C]formate and AIC. The effect of enhanced IMP biosynthesis on the metabolism of other purine compounds was investigated in two ways:

1. Incorporation of glycine and formate into purine compounds by hepatopancreocytes H and A were compared. Enhancement of the IMP biosynthesis was achieved by using a 10 times higher concentration of glycine than of formate (25 and 2.5 μmoles per 1 g of packed cells, respectively).

Table 1

Radioactive purine compounds in hepatopancreocytes from hibernating (H) and active (A) *H. pomatia* snails after incubation with radioactive precursors.

<table>
<thead>
<tr>
<th>Substrate (μmoles/g of packed cells)</th>
<th>Total purine compounds synthesized*</th>
<th>Pools of adenine compounds</th>
<th>IMP+Ino+Hyp</th>
<th>xanthine+guanine compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H</td>
<td>A</td>
<td>H</td>
<td>A</td>
</tr>
<tr>
<td>-------------------------------------</td>
<td>----</td>
<td>----</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Formate (2.5)</td>
<td>94</td>
<td>165</td>
<td>39</td>
<td>41</td>
</tr>
<tr>
<td>Glycine (25.0)</td>
<td>560</td>
<td>1132</td>
<td>233</td>
<td>249</td>
</tr>
<tr>
<td>Adenine (2.5)</td>
<td>746</td>
<td>232</td>
<td>375</td>
<td>67</td>
</tr>
<tr>
<td>Adenosine (37.5)</td>
<td>18 040</td>
<td>14 857</td>
<td>6571</td>
<td>6957</td>
</tr>
</tbody>
</table>

* Uric acid included.
Scheme 1. Purine-nucleotide metabolism in the hepatopancreas of *H. pomatia* (Gastropoda), after Jeżewska & Barankiewicz (1977), modified

Enzymes found in vitro:
1. 5'-Nucleotidase (EC 3.1.3.5) and unspecific phosphatase
2. Adenosine phosphorylase**
3. Adenosine kinase (EC 2.7.1.20)
4. AMP aminohydrolase (EC 3.5.4.6)
5. Adenosine aminohydrolase (EC 3.5.4.4)
6. Adenine aminohydrolase (EC 3.5.4.2)
7. Adenine phosphoribosyltransferase (EC 2.4.2.7)
8. Inosine-guanosine phosphorylase**
9. Hypoxanthine-guanine phosphoribosyltransferase (EC 2.4.2.8)
10. Dehydrogenase hydroxylating hypoxanthine and xanthine**
11. Guanine aminohydrolase (EC 3.5.4.3).

Abbreviations used: Ade, adenine; Ado, adenosine; Guo, guanine; Guo, guanosine; Hyp, hypoxanthine; Ino, inosine; Uri, uric acid; Xan, xanthine, Xiao, xanthosine. * Denotes end-products of protein catabolism; ** the enzymes characteristic for *H. pomatia*, which at present have no EC number.

Scheme 2. Biosynthesis of purine compounds from formate by hepatopancreocytes from *H. pomatia*

<table>
<thead>
<tr>
<th>Formate</th>
<th>6.7 ATP</th>
<th>7.4</th>
<th>5.2 GTP</th>
<th>8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.3 ADP</td>
<td>11.8</td>
<td>8.5 GDP</td>
<td>7.8</td>
<td></td>
</tr>
<tr>
<td>5.2 AMP</td>
<td>6.4</td>
<td>6.2 IMP</td>
<td>8.1</td>
<td>2.9 XMP</td>
</tr>
<tr>
<td>4.5 Ado</td>
<td>8.9</td>
<td>3.5 Ino</td>
<td>7.2</td>
<td>2.8 Xiao</td>
</tr>
<tr>
<td>8.6 Ade</td>
<td>6.8</td>
<td>5.2 Hyp</td>
<td>12.9</td>
<td>2.3 Xan</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.6 Uri</td>
<td>8.1</td>
<td></td>
</tr>
</tbody>
</table>

The values represent the number of moles derived from the precursor added (in this case from 2.5 μmoles of formate) in 1 g of packed cells after 2 h of incubation. The values to the left of the name of the compound refer to hepatopancreocytes from hibernating snails (H), those to the right, to the hepatopancreocytes from active snails (A). Conditions of the experiment and methods of the purine compound determination were described in Materials and Methods. This and following schemes are superimposable on Scheme 1, and the same sequence of enzymic reactions is implied. For abbreviations see Scheme 1.
Scheme 3. Biosynthesis of purine compounds from glycine by hepatopancreocytes from *H. pomatia*

<table>
<thead>
<tr>
<th>Glycine</th>
<th>ATP 26.5</th>
<th>ADP 38.8</th>
<th>AMP 112.2</th>
<th>Ado 18.4</th>
<th>Ade 36.7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28.2</td>
<td>54.4</td>
<td>100.0</td>
<td>35.4</td>
<td>31.3</td>
</tr>
<tr>
<td>32.3 GTP</td>
<td>107.4</td>
<td>22.4 GDP</td>
<td>27.2</td>
<td>49.0 GMP</td>
<td>56.8</td>
</tr>
<tr>
<td>38.4 XMP</td>
<td>55.1</td>
<td>24.8 Xao</td>
<td>31.3</td>
<td>18.4 Guo</td>
<td>41.1</td>
</tr>
<tr>
<td>17.7 Hyp</td>
<td>120.4</td>
<td>15.6 Xan</td>
<td>88.7</td>
<td>14.3 Gua</td>
<td>158.1</td>
</tr>
<tr>
<td>23.1 Uri</td>
<td>22.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Precursor added — 25 μmoles of glycine/1 g of packed cells. For explanations see Scheme 2.

Scheme 4. Biosynthesis of purine compounds from adenosine by hepatopancreocytes of *H. pomatia*

<table>
<thead>
<tr>
<th>Adenosine</th>
<th>ATP 508</th>
<th>ADP 363</th>
<th>AMP 306</th>
<th>Ade 5394</th>
</tr>
</thead>
<tbody>
<tr>
<td>7431 Ino 5216</td>
<td>128 IMP 63</td>
<td>38 XMP 47</td>
<td>3046 Hyp 1867</td>
<td></td>
</tr>
<tr>
<td>92 Gua 143</td>
<td>61 GTP 66</td>
<td>59 GDP 34</td>
<td>182 Gua 89</td>
<td>35 Uri 40</td>
</tr>
</tbody>
</table>

Precursor added — 37.5 μmoles of adenosine/1 g of packed cells. For explanations see Scheme 2.

Scheme 5. Biosynthesis of purine compounds from adenine by hepatopancreocytes from *H. pomatia*

<table>
<thead>
<tr>
<th>Adenine</th>
<th>ATP 132.3</th>
<th>ADP 193.4</th>
<th>AMP 28.6</th>
<th>Ado 20.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>224.1 Hyp 88.6</td>
<td>11.9 IMP 4.0</td>
<td>37.0 Ino 13.6</td>
<td>22.1</td>
<td>11.9 IMP 4.0</td>
</tr>
<tr>
<td>21.1 Gua 12.3</td>
<td>6.8 GTP 4.6</td>
<td>17.4 GDP 8.1</td>
<td>16.0 GMP 4.2</td>
<td>4.2 Uri 3.3</td>
</tr>
</tbody>
</table>

Precursor added — 2.5 μmoles of adenine/1 g of packed cells. For explanations see Scheme 2.

2. The incorporation of glycine or formate by hepatopancreocytes H was compared with the incorporation by hepatopancreocytes A. Hepatopancreocytes A can be expected to synthesize IMP more actively, because during the active life the excretion of purines by purinotetic Gastropoda is increased (Jeżewska, 1969), as a result of enhanced protein metabolism (Jeżewska, 1971).

It was found that the synthesis of purine compounds from glycine both by hepatopancreocytes H and A was 6 - 7 times more intense, as compared with the synthesis from formate (Table 1). Thus the ability of cells to synthesize IMP *de novo* remained high during winter sleep. The pool of adenine compounds as well as the other pools (Table 1) were also 6 - 7 times greater than upon the incubation with formate. This indicates that the increase of the IMP biosynthesis from small precursors did not change the distribution of IMP between these pools. However, the distribution of IMP in hepatopancreocytes H differed from that in hepatopancreocytes A.
Hepatopancreocytes A incorporated both glycine and formate twice as intensely as hepatopancreocytes H did (Table 1). This difference concerned first of all the intensity of following processes (Schemes 1, 2 and 3): 1, IMP degradation to inosine and hypoxanthine by 5'-nucleotidase acting preferentially on IMP (Barankiewicz & Jeżewska, 1978), and by inosine-guanosine phosphorylase activated by inosine itself (Barankiewicz & Jeżewska, 1973); 2, IMP conversion to GMP and subsequent degradation to guanine, one of the end products of the nitrogen catabolism in terrestrial snails (Jeżewska et al., 1963); 3, formation of xanthine which can arise from hypoxanthine (Barankiewicz & Jeżewska, 1972) and from guanine (unpublished).

The intensity of these three processes was several times higher in hepatopancreocytes A; this is consistent with the increased purine excretion in vitro during active life. On the other hand, changes in the intensity of the IMP biosynthesis de novo, resulting from the passage from hibernation to active life, did not affect the total adenine compound pool; hepatopancreocytes H and A incorporated labelled formate or glycine into adenine compounds with the same intensity (Table 1). 14C from both precursors was found in adenosine mono-, di- and triphosphates as well as in adenosine and adenine.

Free adenine as a product of AMP catabolism. It is generally accepted that in animal cells adenosine is deaminated to inosine rather than split into free adenine and ribose-1-phosphate. Intense formation of radioactive adenine in H. pomatia hepatopancreocytes (Schemes 2 and 3) reflects the high activity of adenosine phosphorylase present in the hepatopancreas (Barankiewicz & Jeżewska, 1973, 1976). On the other hand, adenosine aminohydrolase also occurs in the H. pomatia hepatopancreas (Jeżewska & Barankiewicz, 1976). Therefore, an attempt was made to compare the efficiency of adenosine degradation to adenine and inosine by these two enzymes.

In the H. pomatia hepatopancreas both inosine and adenine can be transformed further to hypoxanthine by inosine-guanosine phosphorylase (Barankiewicz & Jeżewska, 1976) and adenine aminohydrolase (Barankiewicz, 1973), respectively. The activity of the former enzyme is high, and that of the latter, low; therefore, it is assumed in further considerations that hypoxanthine was derived mainly from inosine.

In hepatopancreocytes H nearly equal amounts of adenine and inosine+hypoxanthine were formed (Schemes 2 and 3). Thus, on the assumption that inosine and hypoxanthine were exclusively derived from adenosine, the activities of adenosine aminohydrolase and adenine phosphorylase could be equal. In fact, dephosphorylation of IMP occurred as well (Scheme 1). Therefore, it seems that in hepatopancreocytes H adenosine is split to adenine rather than to inosine.

In hepatopancreocytes A, in which the level of adenosine was twice as high as in hepatopancreocytes H, the content of adenosine remained the same and that of inosine and hypoxanthine increased (Schemes 2 and 3). This increase could be due either to enhanced adenosine deamination or to dephosphorylation of IMP synthesized with greater intensity (Scheme 1). Therefore, the activities of adenosine aminohydrolase and adenosine phosphorylase were compared in conditions of low
IMP dephosphorylation and saturating adenosine concentration (Scheme 4). The activity of the former enzyme was calculated as the sum of inosine + hypoxanthine, the activity of the latter enzyme—as the sum of adenine + all other purine compounds (except inosine and hypoxanthine) formed by reutilization of adenine (cf Scheme 1 and the next paragraph). The two enzymes exhibited approximately the same activity both in hepatopancreocytes H and A.

The above data suggest that, at low concentration of adenosine, its degradation to adenine predominates; this may ensure the stability of the adenine compound pool during the winter sleep. During active life, 5'-nucleotidase and adenosine aminohydrolase may remove the excess of AMP formed under conditions of highly enhanced IMP biosynthesis, and thus can control the levels of adenine and adenylylates. The high AMP accumulation and low IMP concentration in hepatopancreocytes A during the incubation with adenosine (Scheme 4) suggest that AMP aminohydrolase is not involved in the regulation of the AMP level (the possible role of this enzyme has been discussed by Barankiewicz & Jeżewska, 1978).

Fate of free adenine in hepatopancreas. Adenine has not been found in the nephridial excreta of H. pomatia (Jeżewska et al., 1963); thus, it is either degraded further or reutilized. The H. pomatia hepatopancreas was shown to contain the activity of adenine aminohydrolase (as mentioned above), as well as the activities of adenine phosphoribosyltransferase (Barankiewicz & Jeżewska, 1975) and adenosine phosphorylase (synthesizing activity, Barankiewicz & Jeżewska, 1976)+adenosine kinase (unpublished). In vitro the activity of adenine phosphoribosyltransferase was highest of all these activities.

The activity of adenine aminohydrolase in the hepatopancreocytes is difficult to evaluate. When adenine was incubated with the cells (Scheme 5), rather high radioactivity was found in hypoxanthine, but the incorporation of 14C into purine nucleotides and nucleosides was twice as high. Thus, the label in hypoxanthine could be derived from direct adenine deamination as well as from the catabolism of newly synthesized AMP and IMP. Probably at physiological concentrations of adenine, its deamination by adenine aminohydrolase is of little importance.

High level of radioactivity in other compounds (except hypoxanthine) indicated that adenine was efficiently reutilized by the hepatopancreocytes. Adenine reutilization was 3 times as intense in the case of hepatopancreocytes H as with hepatopancreocytes A. This difference concerned first of all the level of adenine nucleotides accumulating in hepatopancreocytes H (Scheme 5). A similar increase in AMP, ADP and ATP levels occurred also when hepatopancreocytes H were incubated with adenosine (Scheme 4). Synthesis of ADP and ATP was roughly proportional to the level of adenine, irrespective whether adenine or adenosine was used as precursor. With either precursor the ADP+ATP pool accounted for 13 - 16% of the adenine pool in hepatopancreocytes H and for 1.3 - 1.7% in hepatopancreocytes A. This implies the involvement in adenine reutilization of phosphoribosyltransferase rather than of the two-step action of adenosine phosphorylase and adenosine kinase.

Since adenine and adenosine were found to occur in the tissues, including the hepatopancreas, of some molluscs (RaghupathiRamireddy & Swami, 1967; Seki...
et al., 1968), it seems that the adenylate pool may be supplemented by the action of adenine phosphoribosyltransferase, at least in hibernating snails. During the active life the adenylate pool may rather be supplemented by the AMP synthesis from IMP and, in fact, the activity of adenine phosphoribosyltransferase decreased.

Possible role of the adenine cycle. All reactions of the postulated adenine cycle were found to occur in the intact H. pomatia hepatopancreocytes, and it seems that this cycle may operate in vitro, at least during winter sleep. In experiments with adenine and adenosine, hepatopancreocytes H accumulated ADP and ATP. This is in agreement with the accumulation of adenylates in the tissues of various hibernating or aestivating animals (Raghupathiramireddy & Swami, 1967). Several factors as anaerobiosis, starvation, temperature shock and so on, occurring during winter sleep, are known to cause a decrease of adenylate pool. Experiments with glycine and formate showed that the level of adenylates in hepatopancreocytes depended on the intensity of the IMP synthesis de novo, but this process was considerably reduced during winter sleep. Therefore, it seems probable that the high adenylate level may be maintained owing to the adenine cycle operation. This cycle can provide for the total restoration of AMP degraded to adenine at a minimal energy cost of 1 mole of ATP per 1 mole of AMP.

It seems that the operation of the adenine cycle may not be limited to the tissues of H. pomatia. In mammals adenosine is also split to adenine and then reutilized for the biosynthesis of adenylate (Snyder & Henderson, 1973). The occurrence of 2,6-dihydroxyadenine lithiasis in man, resulting from total lack of adenine phosphoribosyltransferase and from hydroxylation of accumulating adenine by xanthine oxidase (Simmonds et al., 1977), suggests the importance of the adenine cycle also for mammals.

We thank Prof. Dr. F. J. Henderson from the Cancer Research Unit, University of Alberta, Canada, for the helpful discussion.

REFERENCES


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**CYKL ADENINOWY W HEPATOPANKREOCYTACH HELIX POMATIA (GASTROPODA)**

**Streszczenie**

Z purynotlenicznych ślizaków *H. pomatia* (Gastropoda) w okresie snu zimowego i aktywnego życia otrzymywano nienaruszone hepatopankreocytę. Komórki inkubowane z [14C]glicyną lub [14C]mrowczanem syntetyzowały *de novo* związki purynowe, m.in. adenylną, adenozynę i adenenę. Hepatopankreocytę także syntetyzowały adenylną i inne związki purynowe z [1H]adeniną i [1H]adenozyną, która była rozkładana do adeniiny przez enzym występujący w komórkach *H. pomatia*; syntezta ADP + ATP była proporcjonalna do stężenia adenyiny. Wszystkie reakcje postulowane w cyklu adenosinowym: AMP → adenozyna → adenina → AMP, zachodziły zatem w nienaruszonych hepatopankreocytach; być może cykl ten umożliwia utrzymanie wysokiego poziomu adenylną w czasie snu zimowego.

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