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The Properties and Extracellular Location of 5'-Nucleotidase of the Rat Fat-Cell Plasma Membrane

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1. A phosphohydrolase specific for 5'-nucleotides was characterized by using a particulate fraction from isolated fat-cells. 2. The activity of intact cells towards 5'-AMP was studied. 3. The activity in either situation had the same K_m for AMP (45 μM) and was inhibited by low concentrations of ATP (<50 μM), but less potently by the ATP analogues AMP-*P*(CH_2)*P*[adenylyl ($\beta\gamma$-methylene)diphosphonate] and AMP-*P*(NH)*P* (adenylylimidodiphosphate). 4. Homogenization of intact fat-cells caused no increase in activity and at least 85% of the activity was recovered in the particulate preparation. 5. The preparation of fat-cells used in this work was not freely permeable to AMP. 6. The ability of intact fat-cells to hydrolyse AMP implies that 5'-nucleotidase is an ectoenzyme in fat-cells. 7. Concentrations of ATP 100 times lower than intracellular concentrations inhibit the enzyme when added extracellularly to intact fat-cells, implying that this effect is also mediated at the extracellular face of the membrane. 8. Antibodies raised to whole liver cells and whole fat-cells inhibit 5'-nucleotidase in intact cells. 9. Incubation of intact fat-cells with adrenaline (1 μg/ml) or insulin (50 μi.u./ml) failed to alter the K_m or $V_{max.}$ of the enzyme.

5'-Nucleotidase (EC 3.1.3.5) is frequently found to behave as a plasma-membrane enzyme in subcellular fractionations of mammalian tissues (de Pierre & Karnovsky, 1973), although in rat liver a small proportion may be associated with the intralysosomal matrix (Pletsch & Coffey, 1972). Studies on partially purified preparations from bull seminal plasma (Levin & Bodansky, 1966), rat liver (Song & Bodansky, 1967), rat heart (Edwards & Maguire, 1970) and porcine smooth muscle (Burger & Lowenstein, 1970) have been reported. A purified enzyme from detergent-solubilized mouse liver membranes has been described (Evans & Gurd, 1973). The enzyme has been demonstrated in the plasma-membrane fraction of rat fat-cells (Avruch & Wallach, 1971; Combret & Laudat, 1972), although it may not be exclusively confined to this fraction (Avruch & Wallach, 1971).

A physiological role has not been firmly established although a function in adenosine release has been proposed (Levin & Bodansky, 1966). Adenosine is vasodilatory and the possible role of 5'-nucleotidase in the regulation of blood flow has been discussed (Baer *et al.*, 1966; Baer & Drummond, 1968; Nakatsu & Drummond, 1972). Adenosine has also been implicated as a neurohumoral agent in brain (Pull & McIlwain, 1972).

Work by Fain *et al.* (1972) and Schwabe *et al.* (1973) with isolated fat-cells has focused on the possible involvement of adenosine in the process of hormonal stimulation of lipolysis and posed questions as to the mechanism of adenosine release and its control. The present studies were undertaken to define more precisely the properties of the enzyme in rat fat-cells with a view to exploring its role in the metabolism and regulation of rat adipose tissue and as part of a larger study to characterize the enzymic properties of the rat fat-cell plasma membrane.

Materials and Methods

[2-^{3}H]AMP (5000mCi/mmol), [^{32}P]AMP (500–3000mCi/mmol), [^{32}P]P_i, ^3H_2O (5Ci/ml) and inulin [^{14}C]carboxylic acid were from The Radiochemical Centre, Amersham, Bucks., U.K. AMP-*P*(CH_2)*P** and AMP-*P*(NH)*P* were from Boehringer (London) Ltd., Ealing, London W5 2TZ, U.K. Non-immune rabbit serum (pooled inactivated) was obtained from Wellcome Reagents Ltd., Beckenham BR3 3BS, Kent, U.K. Siliclad is a product of Clay Adams Ltd., Parsippany, N.J., U.S.A. A standard solution of 0.15M-$Ba(OH)_2$ is available from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.

* Abbreviations: AMP-*P*(CH_2)*P*, adenylyl($\beta\gamma$-methylene)diphosphonate; AMP-*P*(NH)*P*, adenylylimidophosphate; Hepes, 2-(*N*-2-hydroxyethylpiperazin-*N*'-yl)-ethanesulphonic acid.

Isolated fat-cells

Isolated fat-cells were prepared from the epididymal fat-pads of male Wistar rats (120–180g) by the method of Rodbell (1964). Rats were either laboratory-bred or obtained from Ralph Tuck, Rayleigh, Essex, U.K. at least 2 days before use. All rats were fed on stock laboratory diet *ad lib* until decapitation. All glassware with which fat-cells came into contact had been previously coated with Siliclad by immersion in a 2% solution.

When used for the assay of 5'-nucleotidase fat-cells were washed three times in 15ml of buffer containing 120mM-NaCl, 6mM-KCl, 1.3mM-$CaCl_2$, 1.2mM-$MgCl_2$, 20mM-Hepes buffer and 4% (w/v) bovine serum albumin (Armour Pharmaceutical Co., Eastbourne, Sussex, U.K.; fraction V) adjusted to pH7.4 with NaOH, and were finally resuspended in this medium to a concentration of approx. 30mg dry wt./ml. This buffer was chosen because it is iso-osmotic with fat-cells, has a high buffer capacity at pH7.4 (pK_a of Hepes is 7.31 at 37°C) and has a low concentration of polyanions which interfere with the precipitation of AMP by $BaSO_4$ and $Zn(OH)_2$. In experiments to determine the effect of homogenization on whole-cell 5'-nucleotidase one-half of a cell suspension was homogenized by aspiration through a steel grid as described by Avruch & Wallach (1971).

Particulate fraction from fat-cells

Fat-cells from four to eight rats were washed three times in 10ml of buffer containing 45mM-Tris–HCl and 45mM-sodium β-glycerophosphate (pH7.4), and then resuspended in 10ml of the same buffer and homogenized at room temperature by 20 strokes of a Dounce all-glass homogenizer. The homogenate was centrifuged at 1000*g* for 30s to separate the congealed fat, and the infranatant was collected. After this was centrifuged at 15000*g* for 20min at 4°C, the pellet was resuspended in homogenizing buffer to a protein concentration of 2–4mg/ml, measured by the method of Lowry *et al.* (1951).

Enzyme assays of particulate preparations

(1) *Release of [^{3}H]adenosine from [^{3}H]AMP.* This assay was a modification of the radioassay of Avruch & Wallach (1971). It was performed at 37°C in a 500μl volume containing 200μM-AMP, 50mM-Tris–HCl, pH8.0, and tracer [^{3}H]AMP (20000–30000c.p.m.). When the medium contained EDTA it was necessary to add an excess of $MgSO_4$ to obtain maximum activity. The reaction was initiated by adding 10μl of particulate preparation and terminated with 100μl of 0.15M-$ZnSO_4$. Subsequent addition of 100μl of 0.15M-$Ba(OH)_2$ precipitated 98±1% of added AMP but only 11±1% of adenosine. Supernatant (500μl) was added to 5ml of scintillant and radioactivity determined in a liquid-scintillation counter. The scintillant was prepared by adding 8g of PPO (2,5-diphenyloxazole), 200mg of POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene] and 1litre of Triton X-100 to 2litres of toluene.

(2) *Release of P_i.* The breakdown of unlabelled substrates was assayed by measuring the associated release of P_i. The reaction was carried out at 37°C in a 500μl volume containing 200μM-AMP or other phosphorylated compound and 50μM-Tris–HCl, pH8.0, and was initiated by adding enzyme in 10μl. The reaction was terminated by addition of 500μl of 0.4M-$HClO_4$. Supernatant (500μl) was added to 2.5ml of Itaya & Ui's (1966) reagent and the E_{660} measured after addition of 100μl of 1.5% Tween 20. A linear standard curve was obtained by adding 1–50nmol of sodium orthophosphate to the reaction mixture and processing as above.

Enzyme assays in intact fat-cells and whole homogenates

(3) *Release of [^{3}H]adenosine from [^{3}H]AMP.* The reaction was started by adding 100nmol of AMP + [^{3}H]AMP (20000–30000c.p.m.) in 10μl to 500μl of fat-cell suspension or homogenate and was carried out at 37°C in capped polythene test tubes in a shaking incubator. Addition of 200μl of 0.15M-$ZnSO_4$ terminated the reaction and was followed by 200μl of 0.15M-$Ba(OH)_2$ and a further 100μl of 0.15M-$ZnSO_4$. This difference in procedure from assay (1) was necessary to precipitate AMP in the presence of large quantities of protein. A 500μl portion of supernatant was assayed for radioactivity as in assay (1).

(4) *Release of [^{32}P]P_i from [^{32}P]AMP.* The incubation was carried out as in assay (3), but was terminated by addition of 500μl of 1M-$HClO_4$. Supernatant (500μl) was mixed with 100μl of 25mM-ammonium molybdate and the mixture extracted with 700μl of isobutanol–benzene (1:1, v/v) (Burger & Lowenstein, 1970). Then 500μl of the organic phase was mixed with 2ml of the same solvent containing 4g of PPO and 100mg of POPOP /litre, and radioactivity determined in a liquid-scintillation counter. Recoveries of 55±1% of added [^{32}P]P_i (500–600c.p.m./nmol) were achieved in the range 1–20nmol per incubation.

Where adenosine was added the reaction was initiated by addition of 50μl containing 4.4mg of adenosine/ml and substrate at 11 times the final concentration. Adrenaline was added in 10μl from a solution prepared the same day in 0.01M-HCl. Standard solutions of bovine insulin were made by diluting solutions containing 50i.u./ml (kept as frozen samples at −20°C) with incubation buffer containing 0.1% bovine serum albumin and were added in 10μl. In experiments to test the effect of nucleotides on 5'-nucleotidase they were added to assays (1) and (2) in 10μl containing 10mM-$MgSO_4$

to ensure that they were presented in the chelated form (Dixon & Webb, 1966).

Assay of ATP

Samples for ATP assay were prepared by terminating the incubation of assays (1)–(4) with 500 μl of 0.4 M-$HClO_4$. Supernatant (500 μl) was neutralized with 5 M-K_2CO_3 and the precipitate formed at 0°C. Supernatant (10 μl) was assayed by a modification of the luciferase luminescence assay of Strehler & Totter (1952) as described by Siddle *et al.* (1973).

Measurement of distribution spaces in fat-cells

Suspensions of fat-cells prepared as described above were incubated with either ^3H_2O (5 μCi/ml) or with [^{3}H]AMP (5 μCi/ml) in the presence of unlabelled 0.2 mM-AMP and 0.5 mg of unlabelled adenosine/ml. In each case 2.5 μCi of inulin [^{14}C]carboxylic acid/ml was also included. Samples (200 μl) of cell suspensions were layered over 50–100 μl of dinonylphthalate and centrifuged, and the radioactivity in the cell cake was measured as described by Gliemann *et al.* (1972). By the use of a Packard Tri-Carb liquid-scintillation spectrometer it was possible to measure both ^{3}H and ^{14}C in the same samples.

Raising of and use of antisera

Antisera were raised to isolated rat fat-cells, to isolated rat liver cells, produced by the tetraphenylboron method of Gershenson & Casanello (1968), and to rat erythrocytes. The antisera were raised in mixed-strain rabbits fed *ad libitum* and kept at the Department of Biochemistry, Cambridge, U.K. Cells prepared as described were washed in albumin-free Krebs–Ringer bicarbonate buffer [1.3 mM-Ca^{2+}, pH 7.4 (Cohen, 1957)] before injection. Rabbits were injected at monthly intervals with the isolated fat-cells from four epididymal fat-pads, the isolated liver cells from one rat liver or 1 ml of packed erythrocytes. For the first three monthly injections approx. 0.5 mg of heat-killed Mycobacteria were injected with the rat cells to act as adjuvant. Then 10 days after the third and subsequent injections rabbits were bled from an ear vein, approx. 20 ml of blood being collected. This was allowed to clot for 30 min at room temperature and then stored for 24 h at 4°C. The serum was then removed and heated to 56°C for 45 min to inactivate complement (Lachmann, 1968).

γ-Globulin fractions were prepared by the method of Deutch (1967) and resuspended in the same volume of 0.154 M-NaCl as the original serum.

Antiserum (50 μl) or γ-globulin solution was added to 500 μl of fat-cells and incubated for 60 min before assay of 5'-nucleotidase.

Results

Kinetics of 5'-nucleotidase in particulate preparations. The enzyme activity determined by assays (1) and (2) was linear over the first hour and with protein concentration in the range 10–100 μg/ml. The activities obtained by the two techniques were never significantly different. Lineweaver–Burk plots constructed in the concentration ranges 8–40 μM-AMP and 40–200 μM-AMP were linear and indicated a K_m of 43±4 μM (mean±S.E.M. of six separate experiments). The activity was not stimulated by up to 10 mM-Mg^{2+} at pH 8.0 and varied little over the range pH 7.4–9.0 in the presence or absence of this ion.

The activity of the preparation towards nucleotide monophosphates, glucose 6-phosphate and β-glycerophosphate was determined by the release of P_i in assay (2). The results are shown in Table (1) which also shows the effect of these compounds on the hydrolysis of AMP measured by assay (1). All the nucleotide 5'-monophosphates used were appreciably degraded but the 2'- and 3'-monophosphates, glucose 6-phosphate and β-glycerophosphate were not. In addition, these compounds inhibited AMP breakdown in proportion to their own reactivity. This suggested the presence of a single enzyme of broad specificity for the nucleoside moiety but high specificity for a 5'-phosphate.

The enzyme was powerfully inhibited by a broad range of nucleoside di- and tri-phosphates (Table 2). In general triphosphates were more potent than diphosphates at the same concentration. In the presence of Mg^{2+}, ATP in the range 10–40 μM inhibited the enzyme competitively (Fig. 1) with a K_i of 9±2 μM (mean±S.E.M. of three separate experiments). These experiments were complicated by the rapid breakdown of ATP catalysed by the preparation. However, the protein concentration in the assay could be lowered to a value where 80% of the initial ATP, assayed by the luciferase method, was still present at the end of an incubation. The ATP analogues AMP-*P*(CH_2)*P* and AMP-*P*(NH)*P* inhibited 5'-nucleotidase only weakly at 50 μM although larger effects were observed at higher concentrations (Table 3). Since it is not clear whether these compounds act in a way similar to ATP at these high concentrations the detailed kinetics of the inhibition were not determined. The possibility that these effects were due to contamination by ATP was not supported by measurement of the ATP content of samples of these compounds by the luciferase technique.

Apparent inhibition of the production of [^{3}H]-adenosine in assay (1) by ATP would have been observed if there was rephosphorylation of the product. This possibility was tested by observing the effect of a large excess of unlabelled adenosine on the

Table 1. *Substrate specificity of 5'-nucleotidase*

The activity of the particulate preparation to various substrates present singly at 200 μM was measured by the release of P_i, or by their ability to inhibit the release of [^{3}H]adenosine from [^{3}H]AMP. The competing substrate and AMP were presented at concentrations of 200 μM. In these experiments Tris-β-glycerophosphate buffer was replaced by 0.1 M-Tris-HCl (pH 8.0) in the preparation of the particulate fraction. Values are means ± S.E.M. of four observations. Abbreviation: AMPase, adenosine monophosphatase.

Substrate	P_i released (nmol/h per mg of protein)	% activity towards AMP	Competing substrate (200 μM) present with 200 μM-[^{3}H]AMP	AMPase activity (nmol of [^{3}H]adenosine released/h per mg of protein)	% inhibition
5'-AMP	580±10	—	—	640±10	—
5'-GMP	460±10	79±4	5'-GMP	380±10	41±2
5'-CMP	240±10	41±2	5'-CMP	580±10	9±3
5'-UMP	370±10	64±3	5'-UMP	430±10	32±2
2'-CMP	0	0	2'-CMP	630±10	0
3'-CMP	0	0	3'-CMP	620±20	0
3'-AMP	0	0	3'-AMP	600±10	7±3
Glucose 6-phosphate	0	0	Glucose 6-phosphate	630±10	0
Sodium β-glycerophosphate	0	0	Sodium β-glycerophosphate	640±10	0

Table 2. *Effect of di- and tri-nucleotides on 5'-nucleotidase in particulate preparations*

5'-Nucleotidase was assayed by assay (1) in the presence of 200 μM-AMP ± 50 μM-nucleotides. Values are means ± S.E.M. of four observations.

Nucleotide at 50 μM	[^{3}H]Adenosine produced (nmol/h per mg of protein from 200 μM-AMP)	% of control
None	547±7	
ATP	190±10	35±2
ADP	227±7	42±2
UTP	237±7	43±2
UDP	314±7	58±2
GTP	330±10	61±2
GDP	426±7	78±2
CTP	311±3	57±2
CDP	430±20	79±5

assay. Both 0.4 mg of adenosine/ml and 50 μM-ATP inhibited 5'-nucleotidase and the inhibition caused by both agents together was the sum of their individual effects (Table 4). It was therefore concluded that ATP inhibited 5'-nucleotidase directly. The inhibition by adenosine could be due to product inhibition (Cleland, 1970).

Intact fat-cells

The intactness of isolated fat-cells used in these experiments was assessed by their ATP content, their ability to sustain normal rates of adrenaline-stimulated lipolysis and their failure to release the

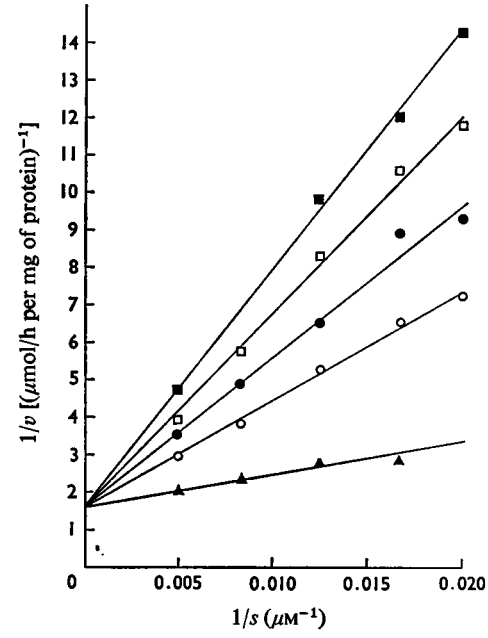

Fig. 1. *Inhibition of 5'-nucleotidase in particulate preparations by ATP*

5'-Nucleotidase was assayed in particulate preparations by incubating portions with 40–200 μM-AMP by assay (1). The reactions were carried out in the presence of 10 μM-ATP (○), 20 μM-ATP (●), 30 μM-ATP (□), 40 μM-ATP (■), and in the absence of ATP (▲), and Lineweaver–Burk plots were constructed from the data.

Table 3. *Inhibition of 5'-nucleotidase in intact cells and particulate preparations*

The 5'-nucleotidase activity of intact fat-cells and a particulate preparation produced from the same batch of cells was measured by production of [^{3}H]adenosine from [^{3}H]AMP; 0.5mg of adenosine/ml was present in either assay (1) or assay (3) as appropriate. Values are means± S.E.M. of four observations.

Inhibitor	Activity of whole cells (%)	Activity of particulate preparations (%)
None	100	100
200μM-GMP	71±6	58±5
200μM-CMP	85±4	79±4
200μM-UMP	70±6	71±5
50μM-ATP	52±3	54±3
50μM-ADP	53±4	56±3
50μM-AMP-*P*(NH)*P*	85±5	100±6
200μM-AMP-*P*(NH)*P*	72±6	85±5
50μM-AMP-*P*(CH_2)*P*	88±5	92±6
200μM-AMP-*P*(CH_2)*P*	75±5	83±4

intracellular cytosol enzyme lactate dehydrogenase (EC 1.1.1.27) into the incubation medium (Rodbell, 1966). Less than 20% of the activity released on homogenizing the fat-cells in the presence of 1% Triton X-100 was found in the medium at the start of an incubation. A further 5–10% was present in the medium after an hour's incubation. Similar proportions of 5'-nucleotidase activity were also found in the medium supporting the conclusion that these activities represent cell breakage. This also indicates that 5'-nucleotidase is not excreted into the medium on incubation of fat-cells.

5'-Nucleotidase activity in intact fat-cells

Assay (3) exhibited a markedly non-linear time-course. The initial rate could be restored by washing the cells, resuspending them and adding fresh substrate, but not by addition of further substrate to the original incubation. The enzyme was therefore not irreversibly inactivated nor was the decreased reaction rate due to exhaustion of substrate. Addition of a tracer amount of labelled AMP (20000–30000c.p.m.) after 30min incubation with unlabelled 200μM-AMP showed that the initial rate of conversion of the label was the same as that observed when labelled and unlabelled AMP were added simultaneously. The non-linearity was therefore not caused by the production of a reversible inhibitor during the reaction. It was also possible that the non-linear time-course was the result of conversion of the product [^{3}H]adenosine, either by rephosphorylation or further degradation, into a product not precipitable by $BaSO_4$ and $Zn(OH)_2$. To test this possibility an excess of unlabelled adenosine was added to decrease loss of the labelled product. In the presence of 0.4mg of unlabelled adenosine/ml the time-course became linear for 45min. This assay was further validated by measuring the appearance of the other product P_i in assay (4). Assay (4) had a linear time-course which agreed quantitatively with assay (3) in the presence of 0.4mg of adenosine/ml. By using this modification of assay (3) a Lineweaver–Burk plot was constructed in the range 40–200μM-AMP and was linear, yielding a K_m of 60±5μM. Assay (4) also yielded a linear plot (Fig. 2) and a K_m of 44±4μM. (Values are mean±S.E.M. of four separate experiments in each case). The difference between these values was probably due to the inhibitory effect of adenosine on the enzyme in assay (3) (Table 4). In experiments to test the effect of homogenization of intact fat-cells on their 5'-nucleotidase activity samples from a fat-cell suspension and a homogenate of the same suspension were assayed either by assay (4) or by assay (3) in the presence of 0.4mg of adenosine/ml. In neither assay was the activity of the homogenate and the intact cell preparation significantly different. If a whole homogenate was centrifuged at 15000*g* for 20min at 4°C and the pellet resuspended as for the particulate preparation at least 85% of the activity of the homogenate was recovered in the particulate preparation (five experiments).

Table 4. *Mechanism of ATP inhibition of 5'-nucleotidase*

Samples of particulate preparations were incubated with 200μM-AMP+[^{3}H]AMP with or without the additions shown below. The amount of [^{3}H]adenosine produced in 1h was measured. Values are means±S.E.M. of four observations.

Addition	5'-Nucleotidase specific activity (nmol/h per mg of protein)	% control activity
None	638±8	100
0.5mg of adenosine/ml	564±5	88±2
50μM-ATP	460±10	72±2
50μM-ATP+0.5mg of adenosine/ml	400±10	63±3

The 5'-nucleotidase of intact fat-cells was inhibited by the compounds shown in Table 3. The production of [^{3}H]adenosine from [^{3}H]AMP was inhibited by unlabelled GMP, UMP and CMP at 200μM. The activity was also inhibited by 50μM-ATP and -ADP, ATP in general being slightly more inhibitory although this was only seen under conditions where ATP breakdown was minimized. Comparable inhibition by AMP-*P*(NH)*P* and AMP-*P*(CH_2)*P* was only achieved at higher concentrations. Table 3 also compares the inhibition observed in intact fat-cells with that observed in a particulate preparation

from the same batch of cells. The pattern of inhibition was qualitatively and quantitatively similar.

Attempts to measure the kinetics of the ATP inhibition in intact cells were confounded by the breakdown of added ATP which was never less than 75% even at cell concentrations of 20mg dry wt./ml. In view of the observation that fat-cells became more fragile at lower cell concentrations these were avoided. AMP-*P*(NH)*P* or AMP-*P*(CH_2)*P* at a concentration of 200μM did not significantly prevent ATP breakdown and were themselves inhibitory. ATP-regenerating systems could not be used owing to the presence of myokinase in the cell preparations which together with the regenerating system, caused conversion of the substrate AMP into ATP. It was therefore impossible to establish the type of inhibition by ATP although the magnitude of the effect was similar to that observed in particulate preparations.

Distribution space for AMP in fat-cells

Since intact fat-cells appeared to hydrolyse AMP at the same rate as homogenates it was important to determine whether fat-cells are permeable to AMP. The measurement of the distribution space for AMP is complicated in view of the rapid hydrolysis of the nucleotide to adenosine which does permeate fat-cells (Humes *et al.*, 1969). Table 5 shows the spaces for ^3H_2O or [^{3}H]AMP and inulin [^{14}C]carboxylic acid determined simultaneously in the same samples. The intracellular water space was 4.0±0.3μl/100mg of fat-cells which is in the range of values reported by other workers (see Gliemann *et al.*, 1972). The AMP space is clearly smaller than the water space but is increasing over the 10min incubation. This increase is consistent with the rate of hydrolysis of AMP expected in the preparation. At short time-intervals it is clear that the space for AMP closely approaches the inulin space indicating that the fat-cells are not freely permeable to AMP. It is difficult with this technique to rule out the possiblity that a small proportion of the cells (<20%) are leaky to AMP. These data are consistent with the hypothesis that AMP hydrolysis takes place at the outer face of the membrane.

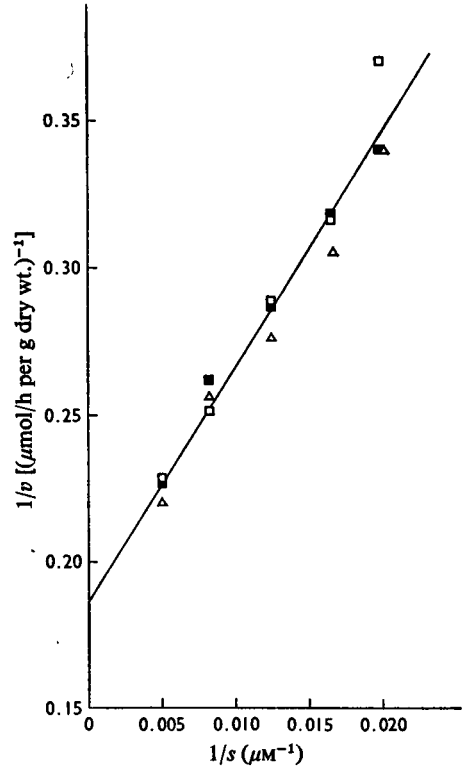

Fig. 2. *Effect of hormones on 5'-nucleotide in whole fat-cells*

Samples from the same fat-cell suspension were incubated with 200μM-AMP+[^{32}P]AMP for 30min. The [^{32}P]P_i produced was assayed. Assays were carried out in the presence of 1μg of adrenaline/ml (■), 50μi.u of insulin/ml (□) and in the absence of hormones (△).

Table 5. *Distribution spaces of isolated fat-cells*

Distribution spaces for pairs of substances ^3H_2O or [^{3}H]AMP with inulin[^{14}C]carboxylic acid were determined simultaneously (by the method of Gliemann *et al.*, 1972) on 200μl samples of fat-cell suspensions. Values are means±S.E.M. of four observations.

Incubation time (min)	Distribution spaces (μl/100mg of fat-cells)					
	^3H_2O	Inulin[^{14}C]-carboxylic acid	^3H_2O−inulin [^{14}C]carboxylic acid	[^{3}H]AMP	Inulin[^{14}C]-carboxylic acid	[^{3}H]AMP−inulin [^{14}C]carboxylic acid
2	5.6±0.4	1.8±0.1	3.8±0.5	2.32±0.08	1.7±0.1	0.6±0.2
	6.6±0.2	2.12±0.8	4.5±0.3	2.40±0.06	1.7±0.1	0.7±0.2
10	6.0±0.2	2.06±0.06	3.9±0.5	4.2±0.2	2.8±0.1	1.4±0.4
	6.6±0.2	2.68±0.06	3.9±0.5	3.4±0.2	2.28±0.08	1.1±0.3

Studies with antibodies

Of six anti-(liver cell) sera screened, four significantly inhibited 5'-nucleotidase when preincubated with fat-cells for 1 h at a dilution of 1 in 12 (Table 6). Two of nine anti-(fat-cell) sera also inhibited the enzyme under these conditions. Ten anti-erythrocyte sera and six non-immune sera were all ineffective.

In view of the low titre of the inhibiting antisera it was necessary to eliminate the possibility that the inhibition was caused by adventitious nucleotides or other small molecules contaminating the sera. To this end the γ-globulin fractions of two inhibiting sera and one non-immune serum were prepared. The anti-liver and anti-fat γ-globulins were effective inhibitors of 5'-nucleotidase. These two inhibiting sera were also tested for their ability to lyse fat-cells. Neither caused significant lysis, supporting the conclusion that they exert their effects at the outer membrane surface.

Fat-cells that had been coated with anti-liver serum at a dilution of 1 in 12 and then washed and resuspended still contained an inhibited 5'-nucleotidase. Homogenization of these cells produced no significant increase in activity compared with control cells (Table 7). This observation supports the hypothesis that all the 5'-nucleotidase is an ectoenzyme. The inhibition produced by the antiserum under these conditions is only modest and therefore the conclusion is not unequivocal. Further confirmation will only be possible when antibodies of high titre are available.

Effect of hormones

Owing to the known antilipolytic effects of adenosine on fat-cells (Fain *et al.*, 1972) assay (4) was used for this study. Adrenaline (1 μg/ml) or insulin (50 i.u./ml) were added simultaneously with substrate to suspensions of fat-cells. Lineweaver–Burk plots in the concentration range 50–200 μM-AMP were constructed (Fig. 2) and indicated no effect of either hormone on the K_m or $V_{max.}$ of the enzyme.

Discussion

The substrate specificity of the rat fat-cell 5'-nucleotidase activity and the observation that it may be inhibited by nucleotides is in agreement with the work on this enzyme in other systems (Levin & Bodansky, 1966; Song & Bodansky, 1967; Baer & Drummond, 1968; Burger & Lowenstein, 1970; Edwards & Maguire, 1970). Competitive inhibition of rat heart 5'-nucleotidase by ATP has been reported by Baer & Drummond (1968), in the presence of Mg^{2+}. Edwards & Maguire (1970), however, found mixed inhibition in the absence of Mg^{2+}. In contrast with the present work these observations were with solubilized preparations.

The most interesting finding to emerge from this study was clear evidence that the active site of the fat-cell enzyme faced mainly, if not exclusively, the external medium rather than the cytoplasm of the cell, i.e. that the enzyme was an ectoenzyme. Rigorous proof of such a location for an enzyme after the demonstration that it is located in the plasma membrane requires the satisfaction of a number of criteria: (i) action on extracellularly supplied substrate; (ii) extracellular release of product; (iii) demonstration of cell integrity; (iv) lack of penetration of cells by substrate; (v) no release of enzyme into the extracellular medium; (vi) failure of homogenization to increase activity (if the enzyme is exclusively an ectoenzyme); (vii) selective inhibition by the extracellular addition of protein reagents that do not penetrate the cell.

Table 6. *Effect of antisera and γ-globulins on 5'-nucleotidase in intact fat-cells*

Antisera or γ-globulins at a dilution of 1 in 12 were incubated for 60 min with 500 μl of fat-cell suspensions and then 5'-nucleotidase assayed by assay (3).

Antiserum or γ-globulin at a dilution of 1 in 12	5'-Nucleotidase (μmol/h per g dry wt.)	% control
None	2.32±0.05	
Non-immune	2.32±0.02	100±3
Anti-liver, 21	0.81±0.05	35±2
Anti-liver, 23	0.77±0.01	33±1
Anti-fat, 27	1.07±0.02	46±1
None	2.16±0.05	
Non-immune γ-globulin	2.04±0.02	94±3
Anti-liver, 23, γ-globulin	0.93±0.05	43±3
Anti-fat, 27, γ-globulin	1.10±0.05	51±3

Table 7. *Effect of homogenization on control fat-cells and fat-cells incubated with antisera*

Fat-cell suspensions were incubated plus and minus antisera for 30 min before the cells were washed and resuspended. 5'-Nucleotidase was then assayed by assay (3) in the presence of 0.5 mg of adenosine/ml.

Antiserum at a dilution of 1 in 12	5'-Nucleotidase in intact cells (μmol/h per g dry wt.)	5'-Nucleotidase in an homogenate (μmol/h per g dry wt.)	Activity in homogenates / Activity in cells
None	5.1±0.2	4.4±0.1	0.86±0.05
Non-immune	4.5±0.1	3.9±0.1	0.87±0.05
Anti-liver, 23	2.7±0.1	2.3±0.1	0.85±0.05

The suggestion that 5'-nucleotidase may be extracellular in the liver was made several years ago on histochemical grounds (Essner *et al.*, 1958) and this finding has been confirmed by Farquhar *et al.* (1974). However, the latter workers point out that the localization of a histochemical deposit does not necessarily reflect the true localization of an enzyme. Indeed histochemical localization of adenylate cyclase, an enzyme thought to function on the cytosol surface of the plasma membrane, has also shown an exclusive extracellular deposition of precipitate (Reik *et al.*, 1970). While this work was being prepared for publication other papers appeared claiming to demonstrate that 5'-nucleotidase may be an ectoenzyme (Trams & Lauter, 1974; Gurd & Evans, 1974). Trams & Lauter (1974) studied cultured cell lines and showed hydrolysis of extracellular AMP without apparent penetration of substrate. Gurd & Evans (1974) raised an antiserum against 5'-nucleotidase and obtained inhibition of the enzyme in a membrane preparation that was not rigorously characterized with regard to membrane orientation and permeability. They were not therefore able to unequivocally localize the enzyme to one side of the plasma membrane of intact cells.

In the present work it has been demonstrated that isolated rat fat-cells hydrolyse extracellular AMP with the stoicheiometric production of adenosine and phosphate. The cells were not freely permeable to AMP and were judged to be largely intact on the basis of ATP content, release of intracellular lactate dehydrogenase and retention of hormonal sensitivity. The small proportion of 5'-nucleotidase activity detected in the medium paralleled the medium activity of lactate dehydrogenase. No increase in activity was detected after homogenization. The enzyme activity of intact cells could be inhibited by γ-globulin fractions of antisera raised against rat liver and fat-cells. In addition the enzyme activity of intact cells was potently inhibited by concentrations less than 50μM-ATP added to the extracellular medium under conditions in which the intracellular ATP content (200–400nmol/g dry wt.) would yield a cytosol ATP concentration of 5–10mM if it were uniformly distributed throughout the intracellular water space of 40μl/g dry wt.

The question remains whether all the 5'-nucleotidase of fat-cells is an ectoenzyme. The activity in the intact cells and homogenate appeared to be due to the same enzyme as judged by the K_m values for AMP, effects of ATP, AMP-*P*(CH_2)*P* and AMP-*P*(NH)*P* and activity towards a variety of substrates. The inability to reveal further activity on homogenization could be explained by inactivation or sequestration of part of the activity during homogenization. Inactivation seems an unlikely explanation since both in our hands and in those of other workers the enzyme is particularly stable even to the effects of detergents. Our experiments with antibodies failed to reveal any intracellular component of the activity, although owing to the low titre of the antibodies it is not possible to rule out a minor intracellular component. Indeed the existence of a plasma-membrane precursor would make the presence of such activity likely (Farquhar *et al.*, 1974). The kinetics of ATP inhibition which have been established in the present work for the enzyme in particulate form, would indicate that any enzymic activity on the inner face of the plasma membrane if such exists is likely to be totally inhibited at normal cytosol ATP concentrations. Unless the kinetics of ATP inhibition of the enzyme inside the cell are considerably modified by other factors a very large decrease in ATP concentration would be required to allow significant breakdown of AMP to occur by this route within the cell. The present work suggests therefore that in considering the physiological role of the enzyme the possibility that its activity is normally expressed on the outer surface of the plasma membrane must be entertained. The source of substrate for this activity is at present unknown.

Lack of hormonal sensitivity of the enzyme activity in intact fat-cells although not surprising is of interest. It has been suggested in view of the wide range of substances which possess insulin-like activity on fat-cells that insulin effects may be mediated by a general change in membrane structure (Rodbell *et al.*, 1968). Further, insulin effects have been reported on several fat-cell plasma-membrane systems including adenylate cyclase (Illiano & Cuatrecasas, 1972), the 'sodium pump' (Hales & Perry, 1970) and the glucose-transport system (Avruch *et al.*, 1972). The present results with 5'-nucleotidase clearly show that not all aspects of fat-cell membrane function are subject to regulation by this hormone.

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