INTRODUCTION

5′-Nucleotidase (EC 3.1.3.5) is an ectoenzyme in many tissues concerned with the conversion of AMP into adenosine. This activity is probably part of a metabolic pathway for removing extracellular adenine nucleotides released during processes such as neurotransmission (Burnstock, 1981), strenuous exercise (Forrester & Lind, 1969), platelet thrombus formation (Gaarder et al., 1961) and shock (Trams et al., 1980). In turn, besides being further metabolized, the product adenosine has effects at A₁- or A₂-type adenosine receptors on various tissues, including vasculature (a possible role of 5′-nucleotidase in the regulation of blood flow has been proposed for several years; see, e.g., Baer et al. (1966); Baer & Drummond (1968); Nakatsu & Drummond (1972)). 5′-Nucleotidase is interesting in other respects. As a cell-surface glycoprotein it undergoes various stages of post-translational processing (Wada et al., 1986; van den Bosch et al., 1986), appears to circulate between the cell surface and an intracellular pool (Stanley et al., 1980; Wilcox et al., 1982; Widnell et al., 1982), possibly interacts with elements of the cytoskeleton (Mannherz & Rohr, 1978; Carraway et al., 1979), and is attached to the plasma membrane either as a short-stalked integral membrane protein (Baron et al., 1986) or through a glycosyl-phosphatidylinositol lipid anchor (Low et al., 1986; Low, 1987). In addition, in white adipose tissue 5′-nucleotidase activity shows sex differences and adaptivity in several pathophysiological states (Green et al., 1981; Vernon et al., 1983; Newsholme et al., 1985; Jamal & Saggerson, 1987).

Over the past two decades 5′-nucleotidase has been assayed in various ways. These include measurement of orthophosphate release (colorimetric or radiochemical; Widnell, 1974), spectrophotometric measurement by coupling to adenosine deaminase (Widnell & Unkeless, 1968; Belfield & Goldberg, 1968; Burger & Lowenstein, 1970), or measurement of the conversion of radiolabelled AMP into adenosine. In the last method, product and substrate have been separated by ion-exchange chromatography (Glastris & Pfeiffer, 1974), by paper chromatography (Widnell & Unkeless, 1968) or by precipitation of AMP with ZnSO₄ + Ba(OH)₂ (Avruch & Wallach, 1971; Newby et al., 1975).

Here we demonstrate a novel simple assay of 5′-nucleotidase that exploits the fluorescent properties of etheno-AMP and ethenoadenosine, and we also make some comments on the metabolism and pharmacological action of ethenoadenosine.

MATERIALS AND METHODS

Chemicals

These were obtained and treated as described by Woodward & Saggerson (1986) and Jamal & Saggerson (1987). In addition, 1,N⁶-etheno-AMP and concanavalin A were from Sigma Chemical Co., Poole, Dorset, U.K.

Isolation of adipocytes

White adipocytes were isolated from the epididymal adipose tissues of male Sprague-Dawley rats (160–180 g) essentially as described by Rodbell (1964). Brown adipocytes were isolated from the interscapular depot of the same animals (Woodward & Saggerson, 1986) by the procedure originally described by Fain et al. (1967) and elaborated by Nedergaard & Lindberg (1982).

Preparation of white-adipocyte extracts

Preparations of white adipocytes from two rats were washed twice in 10 ml of buffer (45 mM-Tris/HCl/45 mM-β-glycerophosphate, pH 7.4). The cells were then resuspended in 10 ml of the same ice-cold buffer and homogenized at 500 rev./min by five up-and-down strokes in a Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle (radial clearance 0.2 mm). The homogenate was first centrifuged at 300 gav for 1 min at 4 °C to remove fat. Portions of this defatted homogenate were stored frozen at −40 °C, and the remainder was re-centrifuged for 30 min at 3000 gav to yield a 'particulate protein' pellet, which was re-suspended in 1 ml of the Tris/β-glycerophosphate buffer and also stored at −40 °C. Protein contents of extracts were measured by the method of Lowry et al. (1951), with bovine serum albumin as standard.

Assays for 5′-nucleotidase

These were based on the method of Newby et al. (1975) and were performed at 37 °C in a shaking water bath (approx. 100 cycles/min). The final volume of 0.5 ml contained 50 mM-Tris/HCl buffer (pH 8.0) and appropriate concentrations of [2-⁳H]AMP (0.6 μCi/
Assay of adenosine deaminase

Calf intestine adenosine deaminase (EC 3.5.4.4) supplied in 3.2 mM(NH₄)₂SO₄ was centrifuged for 2 min at 6500 gₑ in an Eppendorf 5412 centrifuge and dissolved in 10 ml of 0.15 M-Ba(OH)₂. The enzyme was assayed spectrophotometrically at 265 nm and 25 °C in 0.1 M-glycylglycine buffer (pH 7.4) as described by Kalckar (1947), with additions of adenosine or ethenoadenosine as appropriate.

Experiments with isolated adipocytes

Measurements of white-adipocyte lipolysis and brown-adipocyte O₂ uptake were made as described by Saggerson (1986) and Woodward & Saggerson (1986) respectively.

T.I.C.

Ethenoadenosine was separated from etheno-AMP on Kieselgel 60 F₂₅⁴ plates in a solvent system consisting of propan-2-ol/ethyl acetate/N₂H₄ (9:4:3, by vol.) (Arch & Newsholme, 1978).

Spectra

Absorption spectra and fluorescence spectra were obtained with a Pye–Unicam SP8-100 spectrophotometer and a Perkin–Elmer 3000 fluorimeter respectively.

RESULTS AND DISCUSSION

Assay of 5'-nucleotidase using etheno-AMP as substrate

It was found that the fluorescent analogue etheno-AMP could be conveniently substituted for [³H]AMP in the assay initially devised by Newby et al. (1975). In preliminary experiments (results not shown), with either defatted homogenates or 30000 g particulate fractions from white adipocytes, it was established that production of fluorescent material not precipitated by ZnSO₄ + Ba(OH)₂ was linear both with protein concentration and with time for at least 15 min. T.I.C. of the supernatant from such assays showed the formation of a single detectable product (Rₚ = 0.46) which appeared to be identical with chromatographed standard amounts of ethenoadenosine. The background in the assay was extremely small, since 99.7% of 200 μM-etheno-AMP was precipitated by ZnSO₄ + Ba(OH)₂. On the other hand, when samples containing 1-10 μM-ethenoadenosine were treated with ZnSO₄ + Ba(OH)₂, negligible precipitation of fluorescent material was observed in the presence or the absence of adipocyte protein. Using 30000 g particulate material as enzyme source, the rate
Assay of 5'-nucleotidase

Fig. 2. Fluorescence spectra of ethenoadenosine after incubation with adenosine deaminase

Ethenoadenosine (150 μM) was incubated for 30 min at 25 °C in 3.0 ml of 0.1 M-glycylglycine buffer (pH 7.4) with 0.2 unit of calf intestine adenosine deaminase. (a) Excitation spectrum, emission measured at 410 nm. (b) Emission spectrum, excitation at 235 nm. Both spectra are identical with those observed before the addition of adenosine deaminase.

Fig. 3. Activity of adenosine deaminase in the presence and absence of ethenoadenosine

Adenosine deaminase activity was assayed at 25 °C as described in the Materials and methods section by using 0.2 μg of calf intestine enzyme and the indicated concentrations of adenosine: ○, ●, without and with 40 μM-ethenoadenosine respectively.

of conversion of 200 μM-etheno-AMP into ethenoadenosine was inhibited by 10 μM-concanavalin A by 89 %, which is characteristic of membrane-associated 5'-nucleotidase (Riordan & Slavik, 1974; Reimer & Widnell, 1975). In our hands, with rat adipocyte material as enzyme source, the fluorescence assay was sensitive enough to detect the formation of 500 pmol of ethenoadenosine. Table 1 shows that the apparent Km of the adipocyte particulate 5'-nucleotidase for etheno-AMP was appreciably lower than for the physiological substrate AMP. However, the intrinsic activity, with a near-saturating concentration (200 μM) of the fluorescent substrate, was only 26 % of that with AMP.

Although commerically etheno-AMP is more expensive than the equivalent amount of AMP (+[3H]AMP), the fluorescence assay has the advantage of speed and simplicity. A very large number of samples can be read with a fluorimeter in a short time. No radioactive sample preparation or liquid-scintillation-counting time is needed. A second possible advantage of the fluorescence assay is that further metabolism of the product, ethenoadenosine, is restricted and its pharmacological effects may be less than those of adenosine.

Ethenoadenosine and adenosine deaminase

Adenosine deaminase is present at quite high activity in most tissues, including blood, and represents a potential...
problem when 5'-nucleotidase is assayed in crude systems, since adenosine, inosine, hypoxanthine, xanthine and even uric acid may appear as products of the assay. With etheno-AMP as substrate it is perfectly feasible to assay 5'-nucleotidase activity in whole homogenates (see above), and it should be possible to assay the ectoenzyme reliably in cell suspensions or even in tissue perfusions. This conclusion is based on the following findings.

After incubation of 200 μM-ethenoadenosine with calf intestine adenosine deaminase, the only spot detected on t.l.c. was ethenoadenosine (results not shown). In addition, whereas deamination of adenosine by adenosine deaminase produced characteristic changes in absorption spectrum (Fig. 1), there was no change in the absorption spectrum of ethenoadenosine between 190 and 300 nm when adenosine deaminase was added (Fig. 1). Similarly, neither the fluorescence excitation nor the emission spectrum changed on treatment of ethenoadenosine with adenosine deaminase (Fig. 2). Fig. 3 shows that ethenoadenosine had no appreciable effect on adenosine deaminase activity at any tested concentration of the physiological substrate for this enzyme. It was therefore concluded that ethenoadenosine is neither a substrate nor an inhibitor of adenosine deaminase.

**Effects of ethenoadenosine at adipocyte adenosine receptors**

If etheno-AMP were to be used to assay 5'-nucleotidase in whole-tissue preparations, it is of use to know whether the resulting ethenoadenosine has effects at tissue adenosine receptors which could cause extensive changes in tissue metabolism. Adenosine is antilipolytic in white and brown adipose tissues, and decreases O₂ uptake in brown adipocytes (Saggerson, 1986; Woodward & Saggerson, 1986), and these effects appear to be mediated through A₁-type receptors. Fig. 4 shows that the A₁-receptor agonist phenylisopropyladenosine inhibited up to 85% of the lipolysis elicited by 0.1 μM-noradrenaline, with an IC₅₀ (conc. giving 50% inhibition) of approx. 0.1 nM. By contrast, ethenoadenosine had only small effects, and the decrease in lipolysis was never more than 40%. Fig. 5 shows that phenylisopropyladenosine decreased by 70% the brown-adipocyte respiration elicited by 10 nM-noradrenaline, with an IC₅₀ of approx. 5 nM. By contrast, ethenoadenosine had no effect at any tested concentration. In addition (results not shown), 1 μM-ethenoadenosine did not affect the IC₅₀ for phenylisopropyladenosine, suggesting that ethenoadenosine does not act as an antagonist at the A₁ receptors in brown adipocytes.

**Conclusions**

We propose a novel and simple assay for 5'-nucleotidase which has the advantages that the product, ethenoadenosine, is not further metabolized by adenosine deaminase and, in adipose tissue at least, has little effect at adenosine receptors.

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**REFERENCES**

Assay of 5'-nucleotidase


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