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THE RELEASE OF ADENOSINE AND INOSINE FROM CANINE SUBCUTANEOUS ADIPOSE TISSUE BY NERVE STIMULATION AND NORADRENALINE

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SUMMARY

1. Plasma and adipose tissue purine nucleosides were assayed by reversed phase high-performance liquid chromatography after purification of the samples on phenylboronate affinity gel.

2. The adenosine content of unstimulated subcutaneous adipose tissue was close to 1 n-mole/g. The concentrations of adenosine and inosine in canine arterial plasma were $0{\cdot}26 \pm 0{\cdot}03$ and $0{\cdot}16 \pm 0{\cdot}03$ μM, respectively. In venous plasma from the canine subcutaneous adipose tissue the corresponding values were $0{\cdot}32 \pm 0{\cdot}04$ and $0{\cdot}28 \pm 0{\cdot}06$ μM under basal conditions. The arterio-venous concentration difference of adenosine was linearly dependent upon the arterial adenosine concentration. At arterial concentrations below 0·3 μM there was a net production of adenosine; above 0·3 μM there was a net extraction of approximately 77 % of the adenosine.

Adenosine was extensively eliminated in blood. The major part of this elimination could be accounted for by metabolism to inosine, hypoxanthine and uric acid.

3. Following sympathetic nerve stimulation (4 Hz for 20 min) the rate of adenosine outflow from adipose tissue increased from $0{\cdot}33 \pm 0{\cdot}22$ to a peak value of $1{\cdot}2 \pm 0{\cdot}26$ n-mole/min. This corresponds to a net release of $8{\cdot}7 \pm 3{\cdot}0$ n-mole/100 g tissue. Inosine outflow rose from $0{\cdot}64 \pm 0{\cdot}37$ to $5{\cdot}3 \pm 1{\cdot}4$ n-mole/min, corresponding to a net release of $24{\cdot}6 \pm 8{\cdot}7$ n-mole/100 g.

Nerve stimulation also increased the release of [^{3}H]purines from [^{3}H]adenine pre-labelled adipose tissue. The fractional release increased 15-fold after stimulation. The radioactivity was mainly in the form of hypoxanthine, inosine and uric acid while adenosine was a minor component. When metabolism in blood was inhibited by dipyridamole and an adenosine deaminase inhibitor nerve-stimulation-induced release of [^{3}H]purines was mainly in the form of adenosine.

4. Noradrenaline injection also induced a release of radioactive purines and of inosine. On the other hand, the outflow of endogenous adenosine was very small.

5. The present results demonstrate that under basal conditions adenosine is present in arterial and venous canine plasma. The free extracellular tissue level may be similar to the basal arterial adenosine concentration. Sympathetic nerve stimulation and noradrenaline induces a marked release of adenosine which is rapidly metabolized in the tissue and blood stream to inosine, hypoxanthine and uric acid. In adipose tissue the levels of adenosine reached after adrenergic stimulation appear high enough to be of physiological relevance.

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INTRODUCTION

There is evidence that adenosine is an endogenous modulator of lipolysis and cyclic AMP accumulation in isolated fat cells *in vitro* (see Schwabe, Ebert & Erbler, 1975). The possibility exists that adenosine could also be a physiologically important regulator of adipose tissue function (see Fredholm, 1978*a*). Thus, lipolysis induced by sympathetic nerve stimulation in canine subcutaneous adipose tissue *in situ* is inhibited by exogenous adenosine (Fredholm & Sollevi, 1977). This appears to be partly due to inhibited transmitter release and partly to inhibited effect of the released transmitter (Hedqvist & Fredholm, 1976). Two inhibitors of adenosine uptake, dipyridamole (Bretschneider, Frank, Bernard, Kochsiek & Scheler, 1959) and dilazep (Pohl & Brock, 1974), antagonize lipolysis induced by sympathetic nerve stimulation (Fredholm & Sollevi, 1977, 1978) even though these drugs do not inhibit lipolysis *per se* (Fredholm, 1978*b*). Possibly these drugs act as antilipolytic agents *in vivo* because they increase the levels of endogenous adenosine which inhibits lipolysis.

Stimulation of the sympathetic nerves to canine subcutaneous adipose tissue increases the outflow of radioactive compounds from the [^{3}H]adenine labelled tissue (Fredholm, 1976). [^{3}H]adenine is incorporated into the adenine nucleotide pool in adipose tissue. The release of radioactive purines by nerve stimulation was accompanied by a small but significant fall in ATP and [^{3}H]ATP (Fredholm, Belfrage & Blaschke, 1977). The overflow of radioactive purines was blocked by α-adrenoceptor blockade, suggesting release from the target cells rather than from nerve endings (Fredholm, 1976). While these studies with radioactive techniques demonstrate that purines may be released by nerve stimulation they do not give quantitative data.

There are several methods described for the determination of adenosine and inosine in biological materials. The most widely used in the past is a modification (Olsson, 1970) of the spectrophotometric assay of Kalckar (1947). This method is, however, laborious and rather insensitive, thus requiring large plasma samples. To overcome these shortcomings other methods have been developed, such as radioenzymatic (Namm & Leader, 1974), competitive binding to erythrocyte binding protein (Olsson, Davis, Gentry & Vomaka, 1978), radioimmunoassay (Schrader, Nees & Gerlach, 1978), enzymatic fluorometric (Gardiner 1979), and finally high performance liquid chromatography (Anderson & Murphy, 1976; Hartwick & Brown, 1976; Davis, Suits, Kuo, Gehrke, Waalkes & Borek, 1977; Gehrke, Kuo, Davis & Suits, 1978; Kuttesch, Schmalstieg & Nelson, 1978; Nordström, Rehncrona, Siesjö & Westerberg, 1977). Since the latter technique offers the possibility of simultaneous determination of both adenosine and inosine in small plasma samples it was chosen for the present studies.

In the present communication we report the determination of inosine and adenosine in canine plasma and adipose tissue following purification of the samples on a phenylboronate affinity gel (Uziel, Smith & Taylor, 1976), and isocratic separation of nucleosides on reversed phase high performance liquid chromatography. Simultaneously the release of radioactive purines have been followed in order to evaluate the uptake and release of adenosine and inosine by the canine subcutaneous adipose tissue under basal *in vivo* conditions and following adrenergic stimulation. A preliminary account of some of these data has been given (Sollevi & Fredholm, 1979).

METHODS

Experimental procedure

The experiments were performed on twenty female mongrel dogs, weighing 13–20·5 kg. They were anesthetized with sodium pentobarbitone (30 mg/kg I.V.) with supplement as required. After tracheotomy the dogs were mechanically ventilated with an Braun Melsungen model 74052 respirator. Subcutaneous adipose tissue in the inguinal region was isolated from skin and other surrounding tissues as described by Rosell (1966). The adipose tissue preparation was connected to the animal by one artery, one vein and one nerve containing adrenergic fibres (*cf.* Fredholm, 1970). The weight of the preparation was between 26 and 71 g (average 45 g). The preparation was protected from drying by a thin plastic film. Arterial blood was diverted from the femoral artery to the adipose tissue via a drop counter and venous outflow was returned to the femoral vein via polyethylene catheter containing a three-way stop cock, which was used for venous blood sampling. Heparin (2500 i.u./kg) was administered to prevent coagulation in the drop-chamber and tubings I.V. at least 2 hr before the first experimental run. Fluid losses due to sampling and trauma were replaced with isotonic saline. Systemic blood pressure was measured with Statham P23AC transducer and recorded together with adipose tissue blood flow on a Grass model 7B polygraph. Vascular conductance was calculated by dividing adipose tissue blood flow expressed per 100 g tissue by the blood pressure, and is thus given in the units $ml \cdot min^{-1} \cdot mm\ Hg^{-1} \cdot 100\ g^{-1}$.

The nerve supplying the adipose tissue was sectioned close to the hiatus of the inguinal canal in all experiments. The distal part of the sectioned nerve was placed on a bipolar silver electrode and protected from drying with plastibase (Squibb). The nerve was stimulated at 4 Hz during 20 min with impulses of supramaximal duration (1 msec) and intensity (12V), which were delivered by a Grass model S4 stimulator. In twelve experiments adenosine (3–40 n-mole/min) was administered to the adipose tissue by close intra-arterial infusions via a sidearm. In nine experiments 120 μc ^{3}H-adenine (corresponding to 7 n-mole) was administered by close I.A. infusion during 15 min, leading to labelling of the tissue adenine nucleotides as described earlier (Fredholm, 1976). This infusion was given more than 1 h before the first experimental run, at which time there was an essentially constant fractional outflow of radioactivity from the tissue.

Adipose tissue biopsies

Skin and mammary tissue was removed from the inguinal fat pad. Adipose tissue samples were cut out and immediately frozen in liquid nitrogen, after which they were stored at −80 °C. Biopsies weighing 46–356 mg (average 164 mg) were crushed under liquid nitrogen and homogenized in ice-cold 0·4 M-perchloric acid. Internal standard N^2N^2-dimethylguanosine (500 p-mole) was added to the homogenate. After centrifugation at 2000 *g* (0 °C, 10 min) the supernatant was removed, neutralized and processed as described below.

Blood sampling

Blood samples (1·5–2 ml.) were collected in ice-cold centrifuge tubes, kept on ice and centrifuged at 0 °C (3000 rev/min for 5 min) within 15 min. Plasma was removed for the determination of glycerol (Laurell & Tibbling, 1966) and in an aliquot radioactivity was measured (see below). Plasma for the determination of nucleosides was deproteinized by 0·4 M-perchloric acid (PCA). After centrifugation the supernatant was removed, frozen and stored at −20 °C.

The venous sampling followed a standardized protocol as illustrated in Figs. 2 and 3. Arterial samples were withdrawn at regular intervals for the determination of the hematocrit and the above-mentioned parameters. The net rate of release or uptake of the various metabolites was calculated from the arterio-venous concentration differences and plasma flow values and expressed per 100 g tissue.

Determination of nucleosides

(a) *Purification of samples.* Plasma and tissue samples were purified essentially as described for urinary samples by Gehrke *et al.* (1978). The purification is performed by using an affinity-gel, containing immobilized phenylboronic acid (Uziel *et al.* 1976), which selectively binds *cis*-diols, such as ribose.

The deproteinized plasma samples were adjusted to pH 8·8 by 2·5 M-NH_4Ac pH 9·5, followed by the addition of 500 p-mole of an internal standard (I.S.), N^2N^2-dimethylguanosine, for the calculation of recovery throughout the purification procedure. Each sample was run over columns

(150 × 6 mm) containing 0·8 ml. phenylboronate gel, which was prepared as described in detail by Gehrke *et al.* (1978). Ribonucleosides were attached to the gel in alkaline solution (0·25 M-NH_4Ac, pH 8·8) and eluted by addition of a weak acid solution (0·1 M-HCOOH) (cf. Gehrke *et al.* 1978). The acid elutes containing nucleosides were evaporated to dryness and reconstituted in 250 μl. double distilled water and stored at −20 °C until analysis. Purine bases, cyclic nucleotides as well as several other interfering substances are lost during this purification procedure.

As described by Davis *et al.* (1977) the recovery of added nucleosides on the columns is close to 100 % when 10 n-mole is added. We found a recovery of 80–100 % over the range 10 p-mole–10 n-mole added adenosine. As described by Gehrke *et al.* (1978) the affinity columns are quite stable for several months of repeated use.

(b) *Chromatography.* The reconstituted purified plasma samples were injected in a total volume of 25–100 μl. via a Waters U6K injector. The chromatograph was operated in a reverse phase mode using a Waters μ-Bondapak C_{18} column (30 cm, prepacked) and 0·01 M-$NH_4H_2PO_4$ pH 6·0 containing 14 % methanol as the mobile phase. The buffer solution was made up fresh each day from stock solutions, degassed and filtered through Millipore filters (HAWP 0·45 μM). In most experiments a short (4 cm) precolumn packed with the same material was used. A Waters model 6000A solvent delivery system gave a flow of usually 1·5 ml./min at an operating pressure of 1200–2000 psi. Absorbance at 254 nm was continuously measured by a Waters model 440 absorbance detector and recorded on a Kipp & Zonen strip chart recorder.

TABLE 1. Typical retention times for some purine nucleotides on a Waters μ-Bondapak C_{18} column using 0·01 M-NH_4PO_4 pH 6·0 containing 14 % methanol as the mobile phase. Flow rate was 1·5 ml./min. Results of several independent runs, in which the retentiontimes for adenosine varied between 8·0 and 9·25 min

	Retention time (min)	Peak height (mm) (100 p-mole)
GMP	2·25	220
Uric acid	2·40	122
AMP	3·0	108
7-methyl-inosine	3·10	117
Adenosine-*N'*-oxide	3·15	77
1-methyl-adenosine	3·40	112
Hypoxanthine	3·55	167
3',5'-cyclic GMP	4·0	154
Inosine	4·05	178
Guanosine	4·25	172
2-deoxy-inosine	4·45	127
1-methyl-inosine	5·20	86
3',5'-cyclic AMP	8·15	76·5
Adenosine	8·85	106
N_2^2-dimethyl-guanosine	10·3	72
3-deoxy-adenosine	10·45	54
2-deoxy-adenosine	11·0	70
2'-*O*-methyl-adenosine	13·85	48
N^6-methyl-adenosine	18·65	29

The retention-times as well as the peak-heights for a number of adenosine analogues are given in Table 1. The peak-hights were linearly related to amount of purine over a wide range (5–1000 p-mole) and we have therefore used peak-hights to quantify the purines. As seen in Table 1 it is possible to assay inosine and adenosine and I.S. in mixtures within 12 min, thereby enabling us to assay thirty to forty samples in a day on a single chromatograph.

Adenosine and inosine in samples were primarily identified by the retention times. In addition three different procedures were used to ascertain the identification in selected samples. First by adding adenosine and inosine to samples earlier analysed in the chromatograph, increasing the peaks corresponding to these two substances in the chromatogram. Secondly, by adding [^{14}C]adenosine or [^{3}H]inosine to samples, before purification procedure, analysing the samples by continuous

collection of fractions from the mobile phase for radioactivity content. All radioactivity was recovered at the chromatogram-peak for adenosine or inosine, supporting a degradation-free purification. Finally, samples (75 μl.) were incubated with and without adenosine deaminase (ADA, 0·1 μg/μl., deaminating adenosine to inosine) at 30 °C for 30 min. The enzymatic reaction was terminated by heating (80 °C for 2 min). ADA-treatment eliminated the adenosine peak and simultaneously increased the inosine peak in the chromatogram (Fig. 1).

A plasma volume of 0·5 ml. was generally purified for the determination of nucleosides. 25–100 μl. of the reconstituted sample was injected into the chromatograph. The detection limit of the nucleosides is approximately 5 p-mole. Consequently the sensitivity of the method using 0·5 ml. plasma is approximately 0·025 μM.

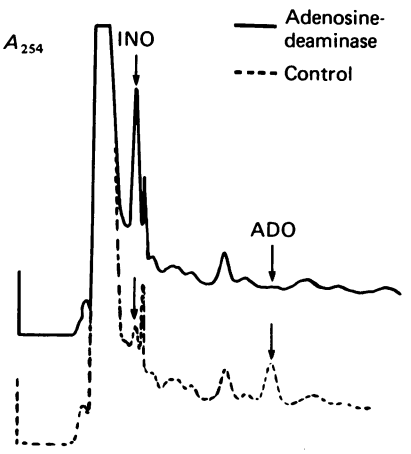

Fig. 1. Identification of adenosine (ADO) and inosine (INO) in plasma. One aliquot of the purified plasma samples was injected directly (dashed line) another aliquot was pretreated with adenosine deaminase (0·1 mg/ml.) as described in the text (continuous line). Note the disappearance of the adenosine peak and the increase of the inosine peak after adenosine deaminase.

Stability of purine compounds

Purine nucleotides and nucleosides are unstable compounds, especially in the presence of biological material. It was therefore necessary to ascertain that the levels found represent the biologically relevant concentrations, not influenced by alterations occurring during sampling, purification or storage.

It is known that the phosphate ester bond of the nucleotides may hydrolyse (see Henderson & Le Page, 1958). The nucleotides AMP and IMP may therefore be hydrolysed to adenosine and inosine, respectively. Nucleotide levels in canine plasma are low (Dobson, Rubio & Berne, 1971). We have estimated the levels of AMP and IMP in PCA-extracts of canine plasma by HPLC using 0·025 M-KH_2PO_4 pH 6·0 as mobile phase. Only after haemolysis did we detect measurable amounts (above 2 μM) of AMP. And not even in these samples were there any marked increase in adenosine levels. Therefore, artifactual production of adenosine and inosine after sampling is unlikely. Breakage of the glycosidic linkage, which occurs most readily in acid, could lead to breakdown of adenosine to form adenine, which is not recovered by our purification procedure. However, it was found that incubation of [^{3}H]adenosine for 24 hr in acid solution at 20 °C did not lead to formation of [^{3}H]adenine. It was found, however, that the radioactive compound as supplied by the manufacturer contained some 16% adenine. Moreover, there was evidence of ^3H_2O formation (see Evans, Sheppard & Turner, 1970). The variability of samples assayed 3 months apart was random amounting to 5·4 $\pm$ 0·3%, and did not show systematically lower values for the second assay, as would be expected had instability during storage been a real problem.

Recovery of added adenosine

In agreement with earlier results (e.g. Namm & Leader, 1974) we found that the recovery of adenosine added to PCA treated plasma was essentially complete. The data presented in Table 2 show that the recovery of adenosine added to untreated plasma was also close to 100% in the range 0·2–1·1 μM, i.e. the relevant concentration range. A small amount of the added adenosine was recovered as inosine, however. When adenosine was added to whole blood, on the other hand, only about 70% was recovered as adenosine. The remaining 30% was recovered as inosine. Rapid disappearance of adenosine from whole blood has previously been reported by Rubio *et al.* (1969), even though these authors reported a larger disappearance (about 50%).

TABLE 2. Recovery of adenosine added to plasma or to arterial whole blood. A sample of heparinized carotid arterial blood was drawn. An aliquot was centrifuged at 2000 *g* at 0 °C and the plasma collected. The remaining blood was kept at 0 °C. Adenosine (0·2–2·9 n-mole) was added either to 1 ml. plasma or to 1 ml. whole blood. The latter was centrifuged either immediately or after 20 min at 0 °C. The centrifugation and removal of plasma took approximately 10 min.

The plasma samples obtained were thereafter deproteinized and analysed for adenosine and inosine as described under methods. The recovery was calculated by subtracting endogenous adenosine and inosine levels. The figures are means of duplicate incubations

		Recovery as adenosine		Recovery as adenosine + inosine	
	n-mole adenosine	(n-mole)	(%)	(n-mole)	(%)
Addition to plasma	0·20	0·25	125	0·25	125
	0·79	0·73	92	0·81	102
	1·10	1·07	96	1·09	98
	2·40	2·11	88	2·30	96
	2·90	2·44	84	2·46	85
Addition to whole blood.	0·20	0·15	76	0·24	119
Centrifuged immediately	0·79	0·51	65	0·73	92
	2·40	1·82	77	2·40	100
Addition to whole blood.	0·20	0·15	75	0·24	119
Left standing 20 min at	0·79	0·51	65	0·78	99
0 °C before centrifugation	2·40	1·60	66	2·26	94

The results shown in Table 2 demonstrate that the recovery of adenosine added to blood at 0° is not altered even if the sample is left standing for 20 min. The adenosine deamination in dog blood is very temperature sensitive (Van Belle, 1969). It is therefore possible that the initial deamination found is due to the fact that it takes some time for the temperature of the blood cells to fall to 0 °C. This could also explain why we get a better recovery than those who have used larger volumes of blood. For example, Rubio *et al.* (1969) used samples of 100 ml. blood and found a recovery of adenosine of about 50%. Thus it is a distinct advantage to use a method of sufficient sensitivity to permit assay in small samples such as the present one. In summary, we conclude that the present method offers an essentially adequate measure of the adenosine and inosine level in plasma at the site of sampling.

Measurements of radioactivity

Radioactivity was measured by liquid scintillation spectrometry by mixing 50 μl. plasma sample with 10 ml. scintillator (4 g omnifluor^{R}, Packard Instrument) in toluene–Triton X-100 (2:1).

Statistics

The results are usually expressed as mean ± S.E. of mean, calculated by conventional methods. Statistical hypotheses were tested by Student's *t* test for paired or unpaired variates. Linear regression was calculated by the least-squares method.

Materials

L-noradrenaline hydrochloride, adenosine, adenosine deaminase, (EC 3.4.5.4.), 5′-adenosine-monophosphate (AMP), 5′-inosine-monophosphate (IMP), 7-methyl-inosine, 1-methyl-adenosine, adenosine-N^1-oxide, 2-deoxy-inosine, 1-methyl-inosine, 2′-*O*-methyl-adenosine, N^6-methyl-adenosine, 1-ethyl-3-(3-dimethyl-amino-propyl)carbodiimide hydrochloride (EDAC) from Sigma, Saint Louis, U.S.A. 3-deoxy-adenosine, 2-deoxy-adenosine, inosine, Boehringer, Mannheim, Germany. Dipyridamole, Boehringer, Ingelheim, Germany. Hydrazide Bio-Gel P2 (200–400 mesh lot no. 16007), Bio Rad Lab., Richmond, Calif. U.S.A. Succinic anhydride, *m*-aminophenylboronic acid hemisulphate, Aldrich-Europe, Beerse, Belgium. N^2N^2-dimethylguanosine, PL-Biochemicals, International Enz. Limited, Widsor, England. Erythro-9-(2-hydroxy-3-nonyl) adenine-hydrochloride (EHNA) a gift from Wellcome, U.S.A. Hypoxanthine, Merck, Germany, heparin (5000 i.u./ml.), Vitrum, Sweden, Sodium pentobarbitone ACO, Solna, Sweden. [2,8-^{3}H]adenosine (30·4 c/m-mole), [2-^{3}H]adenine (16·6 c/m-mole, [8-^{14}C]adenosine (54·7 mc/m-mole), New England Nuclear, Boston, U.S.A. Other chemicals were reagent grade from various sources.

RESULTS

Circulation and lipolysis

The resting blood flow in the acutely denervated canine s.c. adipose tissue was $9{\cdot}5 \pm 1{\cdot}8$ ml . min^{-1} . 100 g^{-1} (range 3·8–23·5).

Prolonged sympathetic nerve stimulation (4 Hz, 20 min) elicits a characteristic vascular response. There is a pronounced initial vasoconstriction which fades during continued stimulation indicating an autoregulatory escape. After the stimulation there is a post-stimulatory hyperaemia (see Fig. 2*a*). When noradrenaline was given by close intraarterial injection (20 n-mole) there was a marked vasoconstriction (Fig. 3*A*), which was of long standing and the blood flow only gradually reverted to and above control levels.

Since fatty acids are rapidly reutilized in canine s.c. tissue (cf. Fredholm, 1970) lipolysis has been assessed by measuring the rate of glycerol outflow from the tissue. Under basal conditions this rate was $0{\cdot}30 \pm 0{\cdot}08$ μmole . min^{-1} . 100 g^{-1}. During sympathetic nerve stimulation there was a progressive increase in the rate of glycerol outflow (Fig. 2*B*). After the stimulation the major release of glycerol occurred. The total amount of glycerol released by nerve stimulation (20 min, 4 Hz) was $55{\cdot}0 \pm 1$ μmole . 100 g^{-1}, which is similar to the values reported earlier (Fredholm, 1970). Noradrenaline (20 n-mole I.A.) similarly increased lipolysis (Fig. 3*B*). The total amount of glycerol released was $118{\cdot}0 \pm 21{\cdot}1$ μmole . 100 g^{-1} ($n = 3$).

Purine nucleosides under basal conditions

The concentrations of adenosine and inosine in arterial plasma averaged $0{\cdot}26 \pm 0{\cdot}03$ (15) and $0{\cdot}16 \pm 0{\cdot}03$ (12) μM, respectively. These levels are similar to those found earlier (Bockman, Berne & Rubio, 1976). In the same dogs the concentrations in venous plasma from the canine s.c. adipose tissue were $0{\cdot}32 \pm 0{\cdot}04$ and $0{\cdot}28 \pm 0{\cdot}06$ μM. Thus, there was no significant net release or formation of adenosine under resting conditions. On the other hand, there was a net rate of inosine release corresponding to $0{\cdot}45 \pm 0{\cdot}18$ n-mole . min^{-1} . 100 g^{-1} ($P < 0{\cdot}05$). In eleven biopsies from four different

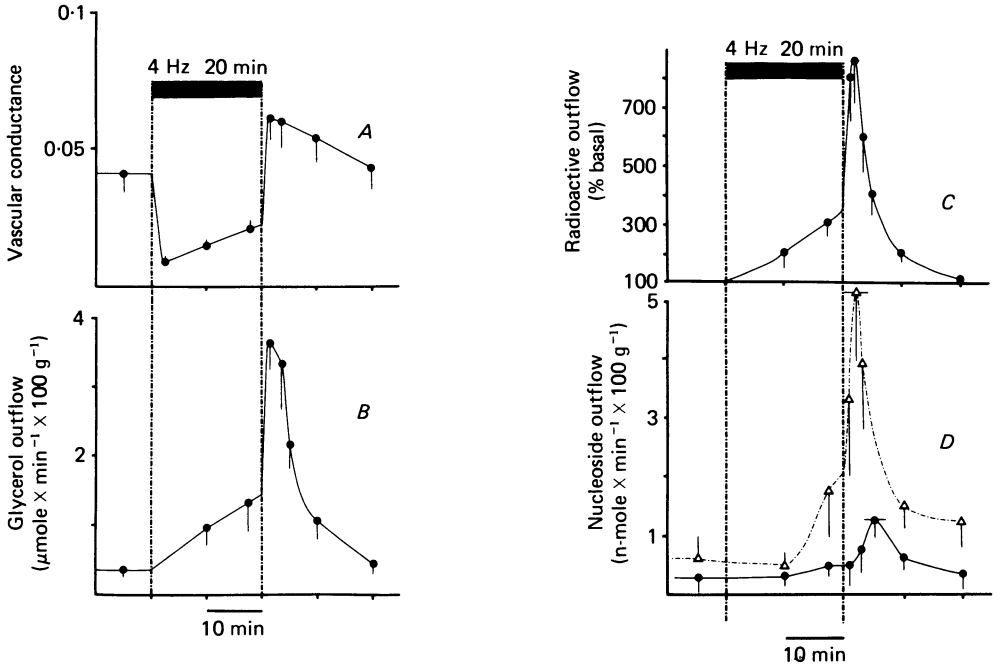

Fig. 2. The effects of sympathetic nerve stimulation (20 min, 4 Hz) on adipose tissue vascular conductance (*A*) and outflow of glycerol (*B*), ^{3}H radioactivity (*C*) and endogenous nucleosides (●, adenosine and △, inosine) (*D*). Mean S.E. from ten experiments.

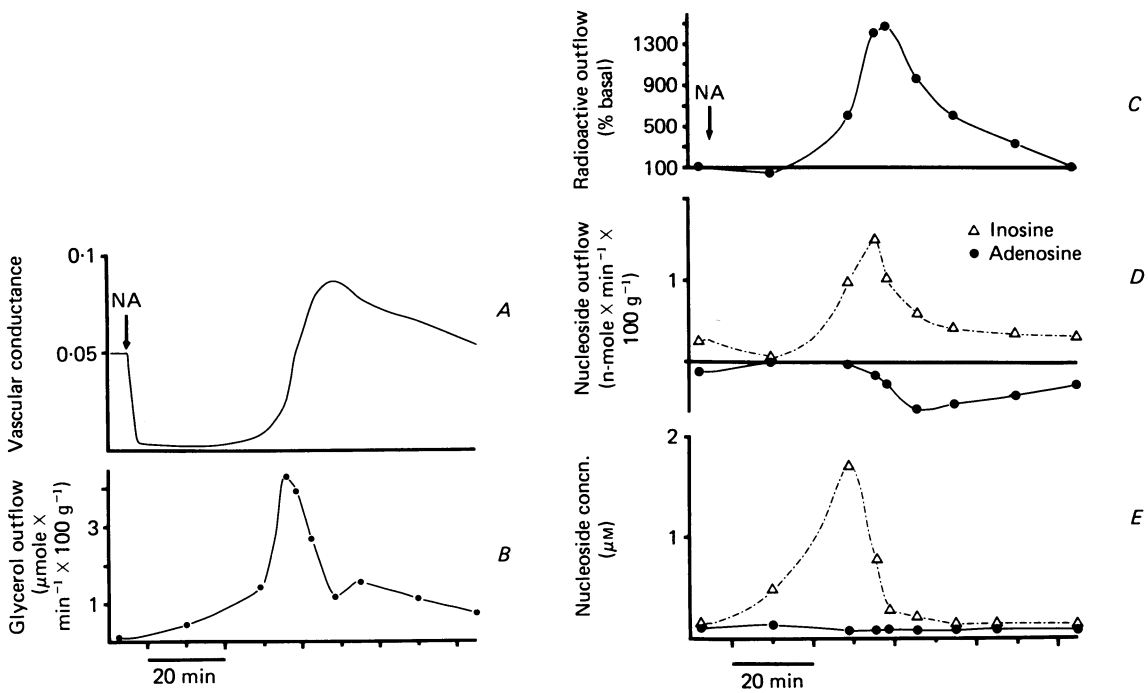

Fig. 3. The effect of close I.A. noradrenaline (NA) injection (20 n-mole) on the vascular conductance (*A*), rate of glycerol release (*B*), rate of outflow of radioactive purines (*C*), rate of outflow of inosine and adenosine (*D*) and the venous concentration of inosine and adenosine (*E*). Results from one typical experiment. The other experiments showed essentially similar results, but the differences in the duration precluded a meaningful compilation of average results.

animals the levels of adenosine and inosine in adipose tissue were $1{\cdot}1 \pm 0{\cdot}1$ and $0{\cdot}7 \pm 0{\cdot}1$ n-mole/g, respectively.

After labelling of the tissue adenine nucleotide stores by [^{3}H]adenine there was a basal fractional release rate of ^{3}H-radioactivity corresponding to $0{\cdot}48 \pm 0{\cdot}10 \times 10^{-3}$ of the tissue content per minute. The total content of adenine nucleotides in canine subcutaneous tissue is approximately 10 μmol $\times$ 100 g^{-1} (Fredholm *et al.* 1977). If we therefore assume uniform labelling one may calculate that 5 n-mole purines are released per min and 100 g tissue, a value considerably higher than the observed rate of inosine release.

TABLE 3. Elimination of adenosine in blood. Heparinized canine blood was pumped through polyethylene tubing at a rate of 4·4 ml./min. Adenosine was infused at a rate of 39·5 n-mole/min. After a distance corresponding to 40 sec samples of blood were drawn. The levels of adenosine and inosine determined as described under Methods. Results are means of duplicate determinations. Adenosine was infused alone or in the presence of increasing concentrations of dipyridamole together with a fixed concentration of the adenosine deaminase inhibitor EHNA

	Concentration (μM)		Recovery (%)†	
	Adenosine	Inosine	As adenosine	As ADO + INO
No infusion	0·13	0·29	—	—
Adenosine	1·6	2·85	5·3	19·9
Adenosine + EHNA (10 μM) + dipyridamole (0·2 μM)	12·86	0·16	72·5	71·7
Adenosine + EHNA (10 μM) + dipyridamole (0·8 μM)	14·06	0·08	80·0	78·2
Adenosine + EHNA (10 μM) + dipyridamole (1·9 μM)	16·83	< 0·10	95·0	94·0
Adenosine + EHNA (10 μM) + dipyridamole (20·0 μM)	16·94	< 0·10	95·8	94·8

† The recovery was calculated by subtracting the measured value in plasma from the value found without adenosine infusion, multiplied by the rate of plasma flow ($4{\cdot}4 \times 0{\cdot}51 = 2{\cdot}25$ ml./min) and divided by the infusion rate of adenosine.

Elimination of adenosine

It is well known that there is a rapid uptake and metabolism of adenosine by tissues and blood cells (see Fox & Kelley, 1978). We have tried to evaluate the importance of such elimination in several ways. In the first experiment, the results of which are shown in Table 3, adenosine was infused into blood pumped through a loop of plastic tubing. After approximately 40 sec contact with blood only 5 % of the adenosine was recovered intact and about 15 % was recovered as inosine. The addition of EHNA at 10 μM together with a low concentration of dipyridamole (0·2 μM) markedly enhanced the recovery of adenosine, but reduced the recovery of inosine. When 2 μM dipyridamole was present the recovery was 95 %. When instead only 10 sec contact with blood was allowed the recovery of intact adenosine was 90 % and following 20 sec contact it was about 40 % (results not shown). It has been shown that the mean transit time of blood in adipose tissue is 10–20 sec (Linde, Chisholm & Rosell, 1974). It may

therefore be concluded that when adenosine is infused close intra-arterially the blood cells do not eliminate more than 50% of the adenosine.

In another set of experiments, the arteriovenous difference in adenosine level was studied as a function of the adenosine concentration in arterial plasma. These results are summarized in Fig. 4. It may be seen that there is an excellent linear relationship between the two parameters. The slope of the curve was 0·77, indicating that more than 75% of the adenosine was eliminated in a single passage. Another interesting feature of the plot is that it intercepts with the x-axis at an arterial concentration of 0·3 μM. This indicates that the tissue and/or blood cells are in equilibrium with a free plasma concentration of adenosine of 0·3 μM.

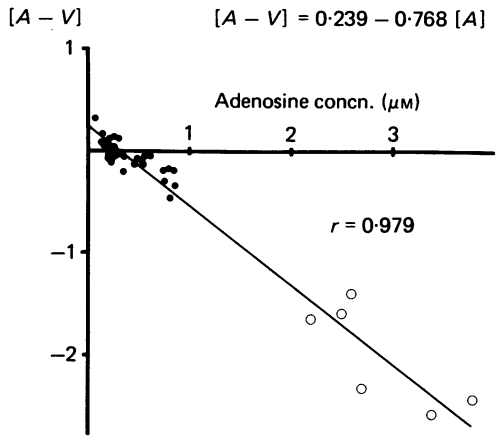

Fig. 4. The relation between adenosine concentrations in arterial plasma and the arteriovenous (A–V) difference during control conditions from ten adipose tissue preparations. Adenosine was also infused in six of these experiments to increase arterial plasma concentration (open circles). The equation of the line is expressed in the Figure. The correlation coefficient was 0·979 and the x-intercept at the arterial adenosine concentration 0·31 μM.

In a third set of experiments adenosine was infused close intra-arterially at rates between 3 and 40 n-mole/min. On the venous side the plasma levels of adenosine and inosine were assayed. In twelve experiments the average recovery of adenosine was 10·8 ± 2·3%. In addition 10·5 ± 3·0% of the infused adenosine was recovered as inosine. The elimination of adenosine infused intra-arterially to adipose tissue is clearly higher than that which can be accounted for by the blood cells (10–60%, see above). Therefore, both the adipose tissue itself and the blood cells seem to contribute to the over-all elimination of adenosine administered to adipose tissue.

When the adenosine deaminase inhibitor EHNA (2–5 μM) was administered the recovery of adenosine was 29·8 ± 9·5% (n = 5). Under these circumstances little or no adenosine was recovered as inosine. In seven experiments dipyridamole was in addition given to the animal at a dose of 0·5–1·0 mg/kg. In these experiments 51·8 ± 9·7 of the adenosine given was recovered on the venous side. Finally, in three experiments dipyridamole was infused intra-arterially at a rate giving a concentration of 1–2 μM in blood together with EHNA at 5 μM. In these experiments the recovery of infused adenosine was 68·5 ± 3·3%. This figure is significantly lower than the figure for recovery of adenosine in blood at these drug levels (Table 3).

Effect of nerve stimulation on purine release

The outflow of radioactivity following [^{3}H]adenine prelabelling increased already during nerve stimulation (4 Hz, 20 min). After about 15 min of stimulation the overflow was 3 times that seen before stimulation (Fig. 2*C*). Immediately following stimulation there was a peak rate of radioactive release corresponding to $0{\cdot}76 \pm 0{\cdot}2$ ($n = 9$) per cent of the total content per minute. The total release of radioactivity induced by a nerve stimulation was $5{\cdot}0 \pm 2{\cdot}3$ ($n = 9$) per cent of the tissue content. Assuming uniform labelling this corresponds to a total net release of 0·5 μmole of purine material per 100 g tissue during and following nerve stimulation at 4 Hz for 20 min. The composition of the purine material was determined in several experiments. In no instance was any significant part of the radioactivity accountable for by nucleotides. After the phenylboronate affinity step 75–95% of the radioactive materials were removed (not shown). Since these columns retain nucleosides and

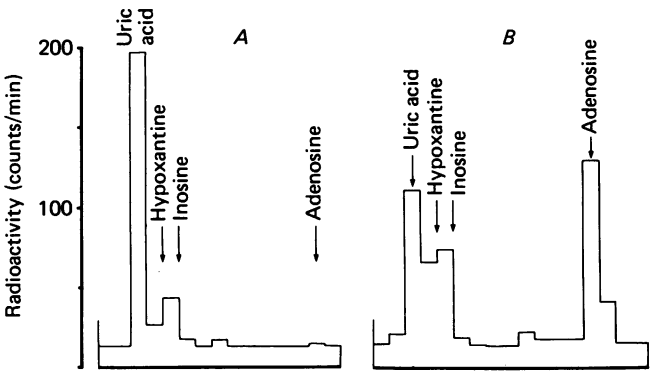

Fig. 5*A*, the distribution of radioactivity in a venous plasma sample collected from a canine s.c. adipose tissue preparation in which the nucleotide stores were prelabelled by [^{3}H]adenine. The plasma sample was collected during a tissue stimulation with a close I.A. noradrenaline injection (20 n-mole). The sample was deproteinized by perchloric acid and an aliquot was injected directly on the HPLC-system. Radioactive content in the collected fractions was plotted. Adenosine concentration in the same sample, analyzed in the HPLC-system after purification, was 0·2 μM. *B*, the same experiment and stimulation as in (*A*). However, the tissue was continuously treated with the adenosine uptake blocker dipyridamole (0·5–1 μM) in combination with the adenosine-deaminase-blocker (EHNA, 3 μM). Adenosine concentration analysed after purification, was 2·9 μM.

nucleotides (except cyclic nucleotides) this means that a very large part of the radioactivity was comprised on non-nucleoside, non-nucleotides material. On silica gel thin layer chromatography this material chromatographed with hypoxanthine, xanthine and uric acid. In general these products accounted for more than 90% of the radioactive purines. Of the nucleosides far more radioactivity was accounted for by inosine than by adenosine (Fig. 5*A*). However, after treatment with dipyridamole and EHNA the radioactivity was shifted so that nucleosides constituted more than half of the radioactivity (Fig. 5*B*). Under these circumstances authentic adenosine was more than ten times higher than under control conditions.

The outflow of endogenous inosine coincided in time with the outflow of radioactivity (Fig. 2). The peak release rate of inosine was $5{\cdot}3 \pm 1{\cdot}4$ n-mole . min^{-1} . 100 g^{-1} and

occurred at about 2 min past stimulation. The concentration of inosine in venous plasma increased from 0·43 μM (range 0·15–1·18) to 0·95 μM (range 0·38–2·69) (Fig. 6). The total amount of inosine released amounted to 24 ± 8 n-mole/100 g. Nerve stimulation caused a smaller increase in adenosine overflow. The concentration in venous plasma increased from 0·26 μM (range 0·17–0·51) to 0·44 μM (range 0·10–0·77) (Fig. 5). The peak rate was $1{\cdot}2 \pm 0{\cdot}3$ n-mole . min^{-1} 100 g^{-1} and the total net release was $8{\cdot}7 \pm 3$ n-mole/100 g tissue. It should also be noted that the peak adenosine outflow from the tissue (5 min after stimulation) occurred later than inosine.

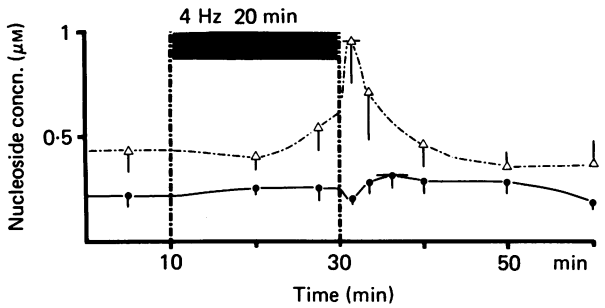

Fig. 6. The effect of sympathetic nerve stimulation (20 min, 4 Hz) on adenosine and inosine concentrations in venous plasma. Mean S.E. from ten experiments. The outflow pattern is given in Fig. 2.

Effect of noradrenaline on purine release

In three experiments exogenous noradrenaline was injected close intra-arterially in a dose of 20 n-mole. In these experiments there was a marked (15-fold) increase in the outflow of radioactive purines (Fig. 3). A total release of $8{\cdot}7 \pm 4{\cdot}9$ % of the tissue radioactivity was released. There was also a marked increase in the rate of inosine outflow and a peak inosine release rate of $2{\cdot}8 \pm 0{\cdot}8$ n-mole . min^{-1} . 100 g^{-1} was found. There was a net release of $14{\cdot}6 \pm 4{\cdot}6$ n-mole inosine/100 g tissue, but no increase in adenosine overflow following noradrenaline (Fig. 3). However, a slight increase in the tissue content of adenosine from 0·86 n-mole/g ($n = 2$) to 1·42 n-mole/g ($n = 2$) was found.

DISCUSSION

The aim of the present study was to quantitate the amounts of purines, especially adenosine, that is formed in subcutaneous adipose tissue under basal conditions and following adrenergic stimulation. To this end we have combined a previously described radioactive technique (Fredholm, 1976) with determinations of endogenous purines by HPLC. The latter technique is shown to provide adequate data on the levels of endogenous adenosine and inosine in canine blood plasma.

We found that the concentration of adenosine was similar in arterial and venous plasma under basal conditions, and there was no net outflow or extraction of adenosine by canine subcutaneous tissue. However, several lines of evidence suggest that even so adenosine is continuously produced by the cells in subcutaneous tissue. First, there is a negative arterio-venous concentration difference for the first break-down product, inosine, corresponding to a release rate of about 0·5 n-

mole . min^{-1} . 100 g^{-1}. Secondly, there was a continuous overflow of radioactivity from tissue where the adenine nucleotide stores had been labelled. Assuming homogeneous labelling of these stores a total release rate of 5 n-mole . min^{-1} . 100 g^{-1} can be calculated. The radioactivity corresponded to about 10% of nucleoside material, of which inosine dominated. Thus, the actually determined inosine release rate and that calculated, with many assumptions, from the overflow of radioactivity are in general agreement. It therefore seems reasonable to assume that already under basal conditions there is a release of adenosine from the adipose tissue in the range of 10–100 p-mole . min^{-1} . g^{-1}. The adenosine formed is rapidly taken up by blood cells, deaminated or phosphorylated, (Schrader, Berne & Rubio, 1972).

We found a tissue level close to 1 n-mole/g. Since the canine subcutaneous adipose tissue contains 70–75% inert material in the form of fat droplets and only 15–20% is water (Fredholm, 1970) this corresponds to a concentration of adenosine in the water space of 5–6 μM. In view of the very high potency of adenosine as a vasodilator and as an antilipolytic compound this figure is clearly not a reasonable estimate of the biologically active concentration of adenosine. It may be argued that most of the adenosine in the adipose tissue is intracellular in a free form, but data using radio-active tracers (Fredholm & Hjemdahl, 1979; Fain & Malbon, 1979) do not support his idea. On the other hand, Ueland & Saebø (1979) have shown that several tissues contain a considerable binding capacity for adenosine amounting to between 0·1 and 10 n-mole/g tissue. Such a bound fraction of adenosine could probably account for most of the adenosine content in the tissue biopsies. Thus, measurement of biopsy adenosine content may not give a true estimate of a free biologically active level of the nucleoside. In view of the rapid inactivation of adenosine (and inosine) in the blood stream it is also not possible to assess tissue concentrations of the nucleosides from their concentration in venous plasma.

When the relationship between the arterial concentration of adenosine and arteriovenous concentration difference was plotted an excellent linear relationship was found (Fig. 4). The straight line intercepted with the arterial concentration at 0·3 μM. At concentrations below this level there was a net production of adenosine by the adipose tissue at higher concentrations there was a net uptake. One possible interpretation of this finding is that 0·3 μM-adenosine corresponds to the concentration of adenosine in subcutaneous tissue in equilibrium with the blood plasma. The finding that there was a net production of inosine by adipose tissue clearly suggests that the concentration of inosine and/or its precursors is above the arterial concentration. Hence a steady-state concentration of adenosine in adipose tissue above some 0·25 μM seems likely from the present data.

Sympathetic nerve stimulation caused a marked increase in the release of labelled purines, in agreement with our previous results (Fredholm, 1976; Fredholm *et al.* 1977). The purines released were almost exclusively in the form of adenosine metabolites. After the administration of an adenosine deaminase inhibitor, however, adenosine was a major constituent. Therefore it is likely that the purines released all ultimately derived from adenosine which was rapidly deaminated and further metabolized in the absence of the adenosine deaminase inhibitor. From the maximal release rate of radioactive purines a peak release rate of close to 50 n-moles . - min^{-1} . 100 g^{-1} may be calculated, corresponding to a peak plasma concentration

of purines approaching 10 μM. The peak rate of endogenous inosine overflow average 5 n-mole . min^{-1} . 100 g^{-1}, and the concentration of inosine was close to 1 μM. As mentioned in the results inosine was not the major purine metabolite and further breakdown products on the average constituted some 90% of the total. Thus, estimates from both radioactive purine release and from release of endogenous purines are in general agreement. A reasonable conclusion is that the peak concentration of the adenosine metabolites in plasma following nerve stimulation is somewhere between 1 and 10 μM. At least in some part of the tissue, the concentration of purines must therefore have been at least as high.

Inosine (and the further metabolites hypoxanthine, xanthine and uric acid) could be formed from adenosine by deamination or from 5′-IMP by the action of 5′-nucleotidase. Since an adenosine deaminase inhibitor, EHNA, eliminates much, if not all, of the inosine and the other metabolites (Fig. 5) it can be concluded that most of the inosine, hypoxanthine and uric acid ultimately derives from adenosine. Thus, it does seem possible to conclude that the adenosine concentration must have reached 1–10 μM somewhere in the tissue. Arch & Newsholme (1978) have in detail discussed the kinetics of inosine formation and concluded that the rate of inosine formation is a direct measure of the concentration of adenosine. The present results demonstrate that the rate of inosine outflow is increased about 10-fold after nerve stimulation or after injection of noradrenaline. It is therefore probable that the concentration of adenosine in the tissue has also increased about 10-fold. As discussed above the biologically aetive concentration of adenosine may be close to 0·25 μM in adipose tissue under basal conditions. A 10-fold increase would bring the concentration up above 1 μM.

We have previously suggested that most of the radioactive purines released by nerve stimulation in adipose tissue derive from non-neuronal elements, since the release was essentially abolished by α-adrenoceptor blockade (Fredholm, 1976). In the present experiments release of purines could be induced not only by nerve stimulation but also by noradrenaline, further supporting the non-neuronal origin of the purines. In the kidney (Fredholm & Hedqvist, 1978), heart (Fredholm, Hedqvist, Vernet & Wennmalm, 1979), nictitating membrane (Luchelli-Fortis, Fredholm & Langer, 1979) and vas deferens (Fredholm, Fried & Hedqvist, 1980) the bulk of purines released following nerve stimulation seem to derive from non-neuronal elements. It is, however, not possible to generalize since there are several reports suggesting that release of purines may also occur from nerves. For example, Su (1975) found that release of purines from the rabbit aorta induced by nerve stimulation could not be abolished by receptor blockade. Burnstock, Cocks, Kasakov & Wong (1978) found that exogenous noradrenaline did not cause purine release in the guinea-pig taenia coli.

Adenosine has a dose-dependent antilipolytic effect in isolated fat cells (see Schwabe *et al.* 1975). At the concentrations of noradrenaline that cause lipolysis *in vivo* the antilipolytic effect is marginal at 10^{-7} M and maximal at 10^{-5} M-adenosine (Fredholm, 1978). Adenosine is also an inhibitor of the release of noradrenaline from sympathetic nerve endings in adipose tissue (Hedqvist & Fredholm, 1976). At least in some systems the presynaptic effect of adenosine is half maximal at 1 μM (Hedqvist & Fredholm, 1976, Hedqvist *et al.* 1979). However, the sensitivity of the nerve

terminals in canine subcutaneous adipose tissue may be lower (Sollevi, Hjemdahl & Fredholm, 1980). Adenosine is a vasodilator in adipose tissue just as it has been shown to be in the coronary circulation (cf. Olsson & Pattersen, 1976). In the anaesthetized dog the ED_{50} for coronary vasodilatation is 1·0 μM (Olsson, Khouri, Bedynek & McLean, 1979). The vasodilatory potency in adipose tissue is similar (Sollevi & Fredholm, 1981). Thus, changes in the concentration of adenosine from 0·1 to 10 μM causes a full swing from no to maximal effect. The present results suggest that the adenosine levels in adipose tissue vary within this important concentration interval.

In conclusion, our results show that endogenous purine nucleosides are continuously formed by the isolated perfused canine subcutaneous tissue in parallel with the release of labelled purine compounds. The basal level of free adenosine in the tissue appears to be in the 10^{-7} M range. In addition there probably is a large biologically inert (bound?) fraction of adenosine so that the total tissue level is more than ten times higher. Following stimulation of the sympathetic supply or injection of noradrenaline the rates of purine release increase 10-fold. The data suggest a corresponding 10-fold increase in tissue adenosine level which therefore seems to vary over a range that correspond to virtually no effect to about 50% effect on lipolysis and blood flow. The results therefore provide further evidence that endogenous adenosine plays a physiological role in the regulation of adipose tissue metabolism and circulation.

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