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# METABOLIC EFFECTS OF $N^6$-SUBSTITUTED ADENOSINES IN RATS*

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**Abstract**—*In vitro* studies using isolated fat cells of fed rats demonstrated that certain $N^6$-substituted adenosine derivatives are highly effective antilipolytic agents. The compounds are thought to interfere with the binding of ATP to adenylate cyclase. Enzymatic reactions which provide or depend on ATP were not influenced. Substances which exhibit antilipolytic activity *in vitro* lowered serum free fatty acids (FFA) in normal rats after oral administration. No symptoms of tachyphylaxis were observed after oral treatment for four weeks. Blood glucose, cholesterol, triglycerides, corticosterone and adrenal wt were unaltered. Liver glycogen was decreased, presumably as a consequence of FFA depression. Isolated fat cells from treated animals exhibited a higher response towards added dibutyryl-cAMP than controls. The mobilization of glucose from glycogen by glucagon in the anesthetized rat was not altered after treatment with $N^6$-substituted adenosines. Despite good tolerance after oral treatment, rats died after i.v. injections of low doses of adenosine derivatives, presumably of heart failure.

CLINICAL investigations have shown that the disturbed metabolic state of maturity-onset diabetics is considerably improved when inhibitors of triglyceride hydrolysis are given together with sulfonylureas. Geyer[1] and Neumann *et al.*[2] used derivatives of isoxazole- and pyrazole-5-carboxylic acid as blocking agents for lipolysis. However, this treatment was restricted due to development of an increasing loss of effectiveness of the compounds. These symptoms have been described as tachyphylaxis and prevented a broader clinical application. In 1969, a new class of lipolytic inhibitors was described[3–6] which did not exhibit tachyphylactic properties. The compounds were derivatives of adenosine and were thought to interfere with the binding of ATP at the adenylate cyclase system, thus decreasing the levels of cyclic AMP and the rate of triglyceride break-down. Since alterations in the levels of cAMP may lead to serious consequences in the organism a careful study of the metabolic effects of such compounds is needed.

The effects of new $N^6$-substituted adenosine derivatives on intermediary metabolism in the rat and on the lowering of serum free fatty acids after chronic administration are reported.

## MATERIALS AND METHODS

NADH, NADP, ADP, ATP, phosphoenolpyruvate, dibutyryl-cyclic-AMP (DB-cAMP) and the enzymes hexokinase, glucose-6-phosphate dehydrogenase, pyruvate kinase, adenosine deaminase, and lactate dehydrogenase were obtained from Boeh-

* Dedicated to Prof. H. Langecker on the occasion of her 80th birthday.

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ringer GmbH, Mannheim, L-epinephrine from E. Merck AG, Darmstadt, crude collagenase from Worthington, Freehold, New Jersey, bovine serum albumin from Behring-Werke, Marburg, and crystalline glucagon from E. Lilly, Giessen.

Adenosine derivatives were synthesized[17] and kindly provided by Dr. H. Vorbrüggen, 3-methylisoxazole-5-carboxylic acid by Dr. Biere from Schering AG, Berlin. Their antilipolytic effect was determined in intact female Wistar rats (100–120 g body wt). Blood was obtained by decapitation from 24-hr fasted animals before and 90 min after oral administration of the test compounds. The substances were dissolved in saline or given as aq. microsuspensions by gavage. Controls were treated with the vehicle. Serum FFA were determined according to Duncombe.[7] Details for the determination of blood constituents as well as glycogen in liver are given elsewhere.[9] Corticosterone was measured according to Moncloa *et al.*[10] The effect of glucagon on the liver of fasted rats was investigated in anesthetized animals. Blood was collected by interruption of a shunt between the arteria and vena femoralis. Blood glucose was determined enzymatically.[12] The *in vivo* results are expressed as means and statistically evaluated by the parameter-free *u*-test.[8]

The isolation of fat cells followed the method of Rodbell[11] with minor modifications.[9] The results are given as mean ± S.E. and Student's *t*-test was used for statistical examination.

## RESULTS

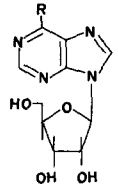
From a series of $N^6$-substituted adenosine derivatives a few characteristic compounds have been selected for a study of their metabolic effects in the rat (Table 1). In more detailed experiments compound VIII has been arbitrarily chosen to demonstrate its influence on intermediary metabolism.

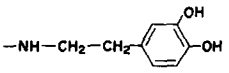
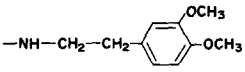
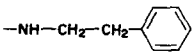
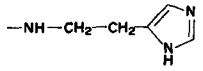
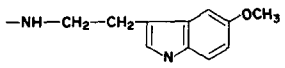
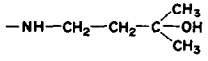
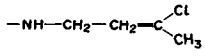
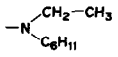
Basal lipolysis in isolated fat cells of the rat was slightly elevated by VIII, whereas marked inhibition was observed when lipolysis was stimulated with L-epinephrine. The most pronounced inhibition was achieved at a concentration of as little as $10^{-5}$ M (Table 2). Based on kinetic studies, Westermann[6] proposed that the antilipolytic properties of adenosine derivatives resulted from the inhibition of cAMP formation.

This hypothesis could be further substantiated by the fact that no inhibition of lipolysis was seen in the presence of DB-cAMP. Moreover, an increase in the release of FFA was observed (Table 2) suggesting that high concentrations of adenosine derivatives have intrinsic cAMP-like activity.[19] The results of Ebert and Schwabe[13] and Fain *et al.*[20] with adenosine support this hypothesis. The suggestion of Westermann and others[5,6] that adenosine analogues may displace ATP from adenylate cyclase prompted us to investigate the influence of compound VIII and others on enzymatic reactions which provide or depend on ATP. The conversion of glucose to glucose-6-phosphate in the hexokinase reaction was not altered in presence of VIII in concentrations of $10^{-3}$ to $10^{-5}$ M. Similarly, the formation of ATP from ADP and phosphoenolpyruvate catalyzed by pyruvate kinase was not affected.

In addition, a series of experiments was conducted to demonstrate that none of the substituted adenosine derivatives so far tested were substrates for adenosine deaminase, an enzyme which converts adenosine to inosine. The stability of $N^6$-substituted adenosines[18] to enzymatic conversion might explain the high antilipolytic efficacy in animals.

TABLE 1. STRUCTURE AND FFA-LOWERING EFFECT OF A SERIES OF $N^6$-SUBSTITUTED ADENOSINE DERIVATIVES



| Compound | R | Dose (mg/kg) | FFA, mval/l after 90 min | |
|---|---|---|---|---|
| | | | Control | Treated |
| Adenosine | $-NH_2$ | — | — | — |
| I |  | 10<br>50 | 0·71 | 0·38*<br>0·29* |
| II |  | 10<br>50 | 0·65 | 0·42<br>0·20* |
| III |  | 10<br>50 | 0·65 | 0·38*<br>0·19* |
| IV |  | 10<br>50 | 0·72 | 0·40*<br>0·28* |
| V |  | 10<br>50 | 0·53 | 0·34*<br>0·21* |
| VI |  | 10<br>50 | 0·62 | 0·27*<br>0·13* |
| VII |  | 50 | 0·88 | 0·27* |
| VIII |  | 10 | 0·84 | 0·41* |
| IX |  | 10<br>50 | 0·92 | 0·80<br>0·57* |
| X |  | 10<br>50 | 0·69 | 0·26*<br>0·24* |
| XI |  | 50 | 0·88 | 0·53* |

Fasted female rats were treated orally with 10 and 50 mg/kg body wt. Values are expressed as means, $n = 10$.

* $P < 0{\cdot}05$.

TABLE 2. LIPOLYSIS IN FAT CELLS OF FED FEMALE RATS IN THE PRESENCE OF VIII WITHOUT AND AFTER STIMULATION WITH DB-cAMP AND EPINEPHRINE

| | μmole FFA/100 mg cell dry wt in 2 hr |
|---|---|
| No addition | 0·08 ± 0·04 |
| VIII, $10^{-3}$ M | 0·18 ± 0·02 |
| $10^{-4}$ M | 0·15 ± 0·01 |
| L-Epinephrine (0·6 μg/ml) | 3·46 ± 0·16 |
| + VIII, $10^{-4}$ M | 1·40 ± 0·14* |
| $10^{-5}$ M | 0·67 ± 0·03* |
| $10^{-6}$ M | 1·14 ± 0·04* |
| DB-cAMP ($5 \times 10^{-4}$ M) | 4·50 ± 0·05 |
| + VIII, $10^{-3}$ M | 10·78 ± 1·05* |
| $10^{-4}$ M | 8·68 ± 0·75* |

Values are expressed as means ± S.E., $n = 3$.
* Significantly different ($P \leq 0{\cdot}05$) from value without addition of VIII.

When the antilipolytic properties of adenosine analogues had been established *in vitro*, the FFA lowering effect in the animal was investigated. Table 1 summarizes the results in intact rats after oral administration. Most of the tested compounds were effective at a dose of 10 mg/kg body wt.

Since glycogen mobilization via the phosphorylase system involves the participation of cAMP, the peripheral increase in glucose concentration was measured after i.v. administration of glucagon. As seen from Fig. 1 the single treatment of rats with compound VIII in a concentration which consistently leads to a FFA depression did not significantly alter glucose output from the liver. It is concluded that the liver appears to be less sensitive to adenosine analogues than adipose tissue.

The answer to the most pertinent question as to the loss of effectiveness of the compounds after chronic oral treatment is demonstrated in Table 3. The FFA-lowering effect of VIII is fully maintained after 30 days of treatment. Under the same experimental conditions, already after 8 days, rats developed complete resistance towards

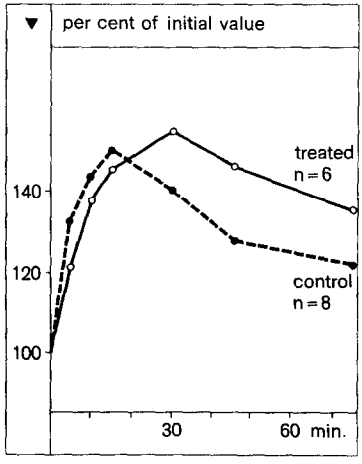



FIG. 1. Changes in blood glucose of fasted female rats after i.v. administration of 10 μg/kg glucagon 2 h after oral treatment with 10 mg/kg VIII. The basal blood glucose concn was 72·8 mg/100 ml for the controls and 78·9 mg/100 ml for the treated animals and not statistically different. Values are expressed as means.

TABLE 3. FREE FATTY ACIDS (in mval/l) IN SERUM OF FASTED INTACT RATS AFTER DAILY ORAL TREATMENT WITH 10 mg/kg VIII OR 5 mg/kg 3-METHYL-ISOXAZOLE-5-CARBOXYLIC ACID

| | Days after treatment | | | |
|---|---|---|---|---|
| | 1 | 8 | 15 | 30 |
| Control | 0·84 | 0·84 | — | 0·91 |
| Compound VIII (10 mg/kg) | 0·41* | 0·42* | 0·49 | 0·42* |
| 3-Methyl-isoxazole-5-carboxylic acid (5 mg/kg) | 0·55* | 0·79 | — | — |

FFA were determined 2 hr after administration of the test compounds. Values are expressed as means, $n = 10$.
* $P \leq 0{\cdot}05$.

3-methylisoxazol-5-carboxylic acid. This compound caused tachyphylaxis in rats already reported for 3-methylpyrazole-5-carboxylic acid.[14,15] The morphology of treated rats, body wt and weight of the adrenals, blood glucose, cholesterol, triglycerides and corticosterone were not different from controls. Glycogen in liver was decreased (Table 4). The reason for this reduction is currently under investigation.

Isolated fat cells of animals treated for 30 days responded normally to epinephrine but showed increased sensitivity towards DB-cAMP (Table 5). It is conceivable that the continuous depression of cAMP levels in fat cells caused a reduction in the activity and/or amount of phosphodiesterase. Even though adenosine derivatives appeared to be well tolerated after chronic treatment rats were highly sensitive to i.v. infusion of the compounds. 0·1 mg/kg i.v. was well accepted and associated with a reduction in serum FFA levels. After 0·5 mg/kg the animals died, presumably of heart failure.

TABLE 4. BLOOD GLUCOSE, SERUM LIPIDS, CORTICOSTERONE, LIVER GLYCOGEN AND ADRENAL WT IN FASTED (16 hr) FEMALE RATS AFTER ORAL TREATMENT FOR 30 DAYS WITH 10 mg/kg VIII

| | Controls | Treated |
|---|---|---|
| Blood glucose (mg/100 ml) | 66·8 | 60·2 |
| Serum triglycerides (mg/100 ml) | 66·7 | 52·4 |
| Serum cholesterol (mg/100 ml) | 67·1 | 66·8 |
| Serum corticosterone ($\mu$g/100 ml) | 34·8 | 42·2 |
| Glycogen in liver (mg/100 g body wt) | 108·1 | 11·9* |
| Adrenal wt (mg/100 body wt) | 39·3 | 38·6 |

The last dose of VIII was given 24 hr before the experiment. Values are expressed as means, $n = 10$.
* $P \leq 0{\cdot}01$.

TABLE 5. LIPOLYSIS IN ISOLATED FAT CELLS OF FED FEMALE RATS AFTER ORAL TREATMENT WITH 10 mg/kg OF VIII FOR 30 DAYS

| | Controls | Treated |
|---|---|---|
| | μmole FFA/100 mg cell dry wt in 2 hr | |
| No addition | 0·13 ± 0·02 | 0·16 ± 0·01 |
| L-Epinephrine (0·2 μg/ml) | 12·98 ± 0·40 | 11·55 ± 0·64 |
| DB-cAMP ($5 \times 10^{-4}$ M) | 6·73 ± 0·30 | 10·47 ± 0·28* |

Values are expressed as mean ± S.E., $n = 6$.
* $P \leq 0{\cdot}01$.

## DISCUSSION

A number of $N^6$-substituted adenosine derivatives have been shown to be potent inhibitors of epinephrine-stimulated lipolysis in rat adipose tissue cells. In intact animals FFA were consistently reduced. Westermann *et al.*[3] and Stork *et al.*[21] achieved a depression of FFA with similar compounds with considerably lower concentrations. The different results may be due to the use of a different strain of rats. The mechanism of the antilipolytic action appeared to reside in an inhibition of cAMP formation, presumably by competitive interference with ATP-binding at the adenylate cyclase system.[6]

Ebert and Schwabe[13] pointed out that in addition to its antilipolytic effect adenosine exerted lipolytic properties at high concentrations *in vitro*.The slight stimulation of basal lipolysis with compound VIII, the absence of a dose-response relationship in the presence of L-epinephrine at high concentrations and the stimulation of FFA release into the medium in the presence of DB-cAMP support the concept of an intrinsic cAMP-like activity.

In contrast to previously described antilipolytic substances like isoxazole and pyrazole derivatives, adenosine analogues did not exhibit reduced efficacy after chronic administration. In a certain dose range, one representative of this class of compounds was tolerated well in the rat during 4 weeks of treatment. Since it is well established that cAMP exerts ubiquitously important regulatory functions in the organism it is necessary that the test substances should be effective in adipose tissue in a dose which does not influence other organs or enzymatic reactions. A preliminary attempt was made to show that the action of glucagon in liver was not appreciably affected at a dose which consistently caused a reduction in serum FFA. Similarly, ATP dependent enzymes were not disturbed in the presence of a representative of the adenosine derivatives.

Preliminary results after i.v. administration, however, indicated that the therapeutic range might not be sufficient with the compounds so far investigated. Further attempts should be directed towards the synthesis of compounds which exhibit a wider dissociation between biological efficacy and unwanted side effects. More detailed studies on the effects of adenosine derivatives on heart function in relation to their FFA-lowering effect will be presented elsewhere.[16]

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