ADENOSINE RECEPTORS:

PHARMACOLOGY AND ADAPTIVE CHANGE

BY

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TO MY FAMILY

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Contents

List of Abbreviation

ATP	:	Adenosine triphosphate
cAMP	:	Cyclic adenosine 3',5'-monophosphate
ACTH	:	Adrenocorticotropic hormone
BSA	:	Bovine serum albumin
CHA	:	Cyclohexyladenosine
DHA	:	Dihydroalprenolol
DPX	:	1,3-Diethyl-8-phenylxanthine
FFA	:	Free fatty acid
cGMP	:	Cyclic guanosine 3',5'-monophosphate
HSL	:	Hormone sensitive lipase
IBMX	:	1-Methyl-3-isobutylxanthine
KRB	:	Krebs-Ringer bicarbonate buffer
NE	:	Norepinephrine
NECA	:	5'-N-Ethylcarboxamide-adenosine
PIA	:	Phenylisopropyladenosine
POPOP	:	1,2-Bis(2,5-phenyloxazolyl)benxene
PPO	:	2,5-Diphenyloxazole
TSH	:	Thyroid stimulating hormone

ABSTRACT

After chronic caffeine treatment (6 weeks, 0.2% caffeine solution), the average body weights of rats decreased by 6%, from 439 g in control to 412 g in caffeine-treated group. There was a larger degree of reduction (22%) in epididymal fat pad weight from 4.7 g in control to 3.65 g in caffeine-treated group. In addition, chronic caffeine treatment also induced a 10% decrease in the mean cell diameter of adipocytes (86um vs 77um). Nevertheless, there was no significant change in the mean cell weight as well as the triglyceride content of adipoyctes from caffeine-treated rats.

Adenosine appeared to be an important inhibitor of lipolysis. Indeed, the addition of adenosine deaminase can induce lipolysis in isolated adipocytes presumably by lowering the endogenous adenosine level. Half-maximal adenosine deaminase-induced lipolysis occurred at a concentration of 1 U/ml and maximal response was obtained at a concentration of 30 U/ml. There was no significant difference in the adenosine deaminase (30 U/ml) stimulated lipolysis between the control and caffeine-treated rats. However, significant increase in the anti-lipolytic potency of 2-chloroadenosine, a metabolically stable adenosine analogue, was observed after chronic caffeine treatment. The IC_{50} of 2-

chloroadenosine on adenosine deaminase (1 U/ml) stimulated lipolysis decreased from 2.68 nM in control rats to 1.09 nM in caffeine-treated rats.

Scatchard analysis of ^{3}H-phenylisopropyladenosine (PIA) binding in adipocyte membranes revealed an increase in the maximal number (Bmax) of adenosine receptors from 1.45 to 2.08 pmole/mg protein, while the dissociation constant (K_D) remained unchange after chronic caffeine treatment. A consistent increase (10-20%) in the brain cerebral cortex ^{3}H-PIA binding sites was also observed in caffeine-treated rats but they were not significantly different from the controls.

Besides these adaptive changes in adenosine receptors, there was also a significant change in the lipolytic response stimulated by isoproterenol, a beta-adrenergic receptor agonist, after chronic caffeine treatment. The EC_{50} of isoproterenol increased from 77 nM in control rats to 110 nM in caffeine-treated rats, accompanied by a 35% decrease in the maximal lipolytic rate stimulated with 1 uM isoproterenol (6.5 vs 4.2 umole glycerol/g cells/30 min).

In agreement with these changes in the lipolytic response to isoproterenol, chronic caffeine treatment also diminished cAMP accumulation in intact adipocytes stimulated by 100 uM isoproterenol, both in the presence and absence of adenosine deaminase. On the

other hand, there was no significant difference in the inhibitory potency of 2-chloroadenosine on the isoproterenol (100 uM) stimulated cAMP accumulation in adipocytes from control and caffeine-treated rats.

Radioligand binding analysis showed that the binding of ^{3}H-dihyroalprenolol (DHA) to beta-adrenergic receptors of adipocyte membranes demonstrated a cooperativity. However, after 6 weeks 0.2% caffeine treatment, no cooperativity was observed for ^{3}H-DHA binding to adipocyte membranes. Radioligand binding analysis revealed a decrease in the number of beta-adrenergic receptors on adipocyte membranes after chronic caffeine treatment. However, there was no significant difference in the ^{3}H-DHA binding in brain cerebral cortex membranes prepared from control and caffeine-treated rats.

In summary, these data suggest that chronic caffeine treatment sensitizes the adenosine A1 receptors, but desensitizes the beta-adrenergic system in adipocytes. Since activation of A1 adenosine and beta-adrenergic receptors lead to an inhibition and activation of lipolysis respectively, thus these adaptive changes represent a homeostatic mechanism acting in concert to counteract the lipolytic action of caffeine treatment.

CHAPTER 1

INTRODUCTION

1.12 The background

Besides alcohol and nicotine, caffeine is probably one of the most widely used psychoactive substance in our society. It is present in large amounts in coffee, tea, and cocoa as a natural product and as an additive in many soft drinks. Chemically, caffeine is a xanthine derivative. Pharmacologically, caffeine produces bronchodilatory, cardiotonic and diuretic effects. Biochemically, caffeine is a weak phosphodiesterase inhibitor and a relatively more potent adenosine receptor antagonist. Indeed, recent evidence suggest that many biological effects of caffeine can be attributed to its antagonism of adenosine receptors (Fredholm, 1980; Snyder and Sklar, 1984).

Chronic exposure of receptors to its antagonist usually leads to an increase in the receptor number. This adaptive change may be responsible for the pathophysiological syndrome sometimes observed upon withdrawal of the antagonist as well as the development of drug tolerance. For instance, after long-term treatment of ischaemic heart disease with propranolol, an increase in beta-adrenergic receptor was found (Aaron *et al* , 1980). Clinically, upon abrupt cessation of propranolol treatment, an increase in anginal symptoms, occurrence of myocardial infarction and ventricular arrhythmias has been reported in some

patients (Grahame-Smith, 1985). Powers (1925) reported a syndrome associated with chronic heavy consumption of caffeine, including vertigo, headache, nervousness, visual disturbance and tachycardia. However, the effect of chronic caffeine consumption on peripheral organs has not yet been characterized. As adenosine is a well-known anti-lipolytic agent in adipose tissues, the present project was designed to study the effect of chronic caffeine consumption on adenosine receptors in adipocytes as well as the biological consequence of such treatment.

1.2) The physiological roles of adenosine

Chemically, adenosine is a nucleoside, consisting of a ribose and an adenine moiety linked together by a N-glycosidic bond between the C-1 carbon atom of the pentose and the N-9 nitrogen atom of the purine base (Fig. 1.1). Biochemically, adenosine is a precursor of ATP and GTP, and thus is an important substrate for both energy metabolism and RNA/DNA synthesis. Recent evidence suggest adenosine may also be a neuro-transmitter in the central and peripheral nervous system. Its possible involvement in the regulation of many physiological functions is listed in Table 1.1.

In the nervous system, adenosine inhibits the spontaneous neuronal firing of brain cerebral cortex

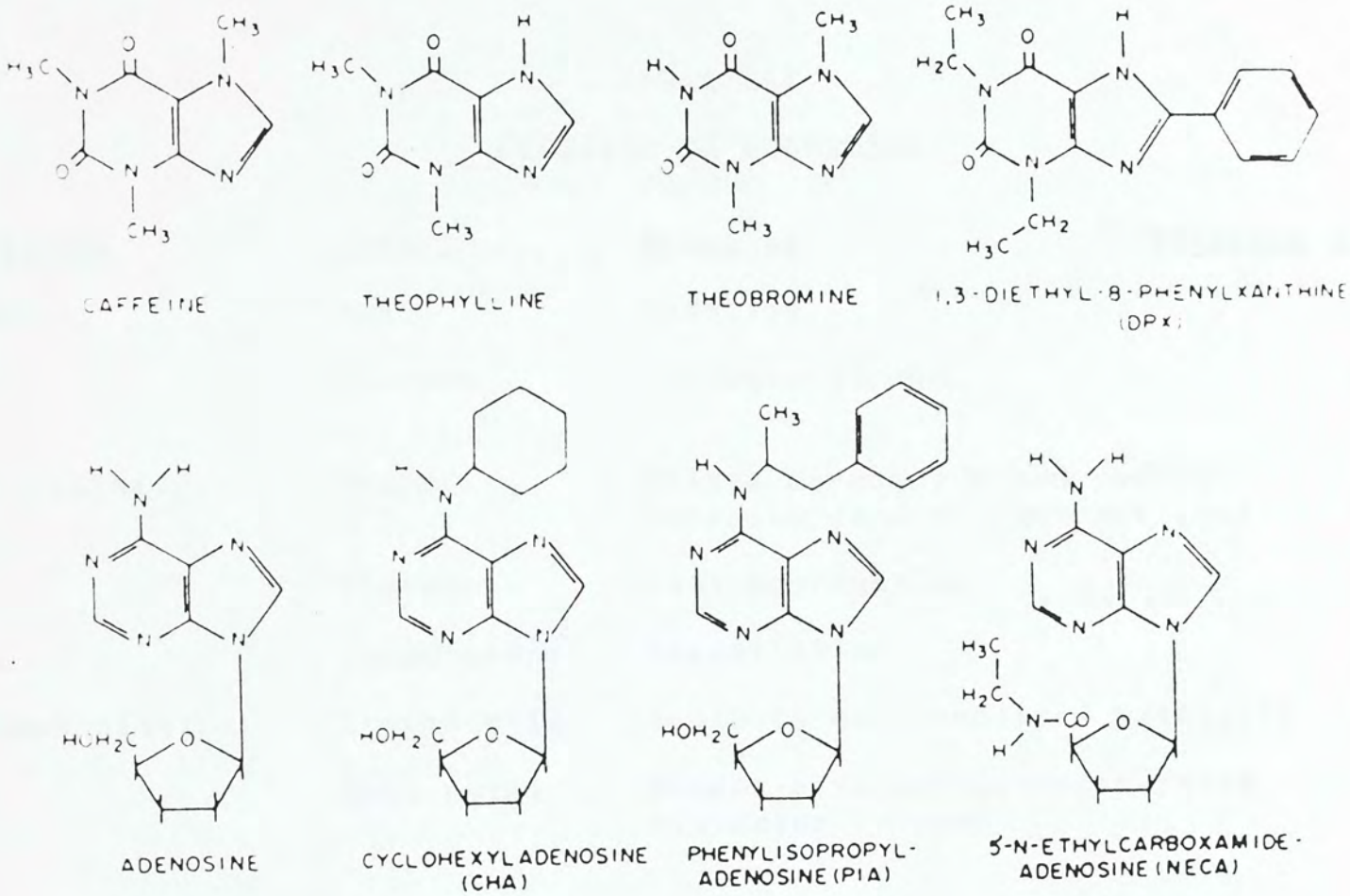


Fig. 1.1 Structure of some xanthine and adenosine derivative. (Adopted from Snyder and Sklar, 1984)

Table 1.1

Function of adenosine

System	Target	Response	Receptor type
CNS	Brain	Sedation	A2
	Neurons	Inhibits firing	A2
Circulatory	Heart	Dilate coronary blood vessel	A2
		Decrease cardiac contractility	A1
	Platelets	Anti-aggregation	A2
	Vasculature	Vasodilation	A2
Immunological	Lymphocytes	Inhibits cell mediated cytolysis	A2
	Mast cells	Potentiates antigen-stimulated histamine release.	
Respiratory	Trachea	Constriction	A2
Others	Adrenals	Stimulates steroidogenesis	A2
	Adipocytes	Anti-lipolysis	A1
	Kidney	Vasoconstriction	
	Leydig cells	Stimulates steroidgenesis	A2
	Vas deferens	Inhibits electrically stimulated constriction	A1

(Phillis and Wu, 1983), and the release of norepinephrine and acetylcholine (Paton, 1979; Hayashi *et al*, 1983). Furthermore, the sedative and anti-anxiety effects of neuroleptics, benzodiazepines and anti-depressants seemed to correlate with their ability to potentiate central adenosine action by inhibiting the adenosine uptake system (Phillis and Wu, 1983)

In the cardio-vascular system, adenosine is an active vasodilator in the heart, brain, skeletal muscle, intestine and lung, but it is a vasconstrictor in the kidney and liver (Mentzer *et al*, 1975; Arch and Newsholme, 1978; Berne *et al*, 1979). Adenosine also produces negative chronotropic and negative inotropic effects in the heart, and tracheal contraction in the lung (Fredholm *et al*, 1979; Prasad *et al*, 1980). Indeed, abnormality in adenosine regulation has been suggested to be involved in the pathogenesis of hypertension and asthma (Kamikawa *et al*, 1983). There is also evidence in support of its role as a mediator of hypoxic and reactive hyperemic coronary vasodilation (Raberger, 1979).

At the cellular level, adenosine inhibits aggregation of blood platelets (Haslam *et al*, 1979), facilitates antigen-stimulate histamine release from mast cells (Marquardt *et al*, 1978), inhibits lymphocyte-mediated cytolysis (Wolberg *et al*, 1978),

and stimulates steroidogenesis in adrenal and Leydig cells (Londos *et al*, 1980). In adipocytes, adenosine inhibits both lipolysis (Fredholm, 1978) and glucose oxidation (Wong *et al*, 1985).

1.3) Metabolism of adenosine

1.3.1) Sources of adenosine

1.3.1.1) ATP pathway:

In the nervous system, ATP can be released either from the purinergic nerves as a neurotransmitter, or from the adrenergic nerves as a co-transmitter (Burnstock, 1979; Su, 1983). The released ATP can be degraded into ADP and AMP by adenosine triphosphatase (ATPase). The AMP formed can be further metabolized to adenosine by the extracellular 5'-nucleotidase (Fig. 1.2), which is primarily an ecto-enzyme. Intracellularly, when the cellular AMP level is high as a consequence of large energy demand, they can be degraded into adenosine by the intracellular 5'-nucleotidase. The adenosine thus formed can passed through the cell membrane by facilitated diffusion into the extracellular space and acts on the adenosine receptors. Indeed, in view of the close relationship between adenosine and the energy state of the cell, Newby (1984) suggested that adenosine may function as a 'retaliatory metabolite': a metabolite which act as a

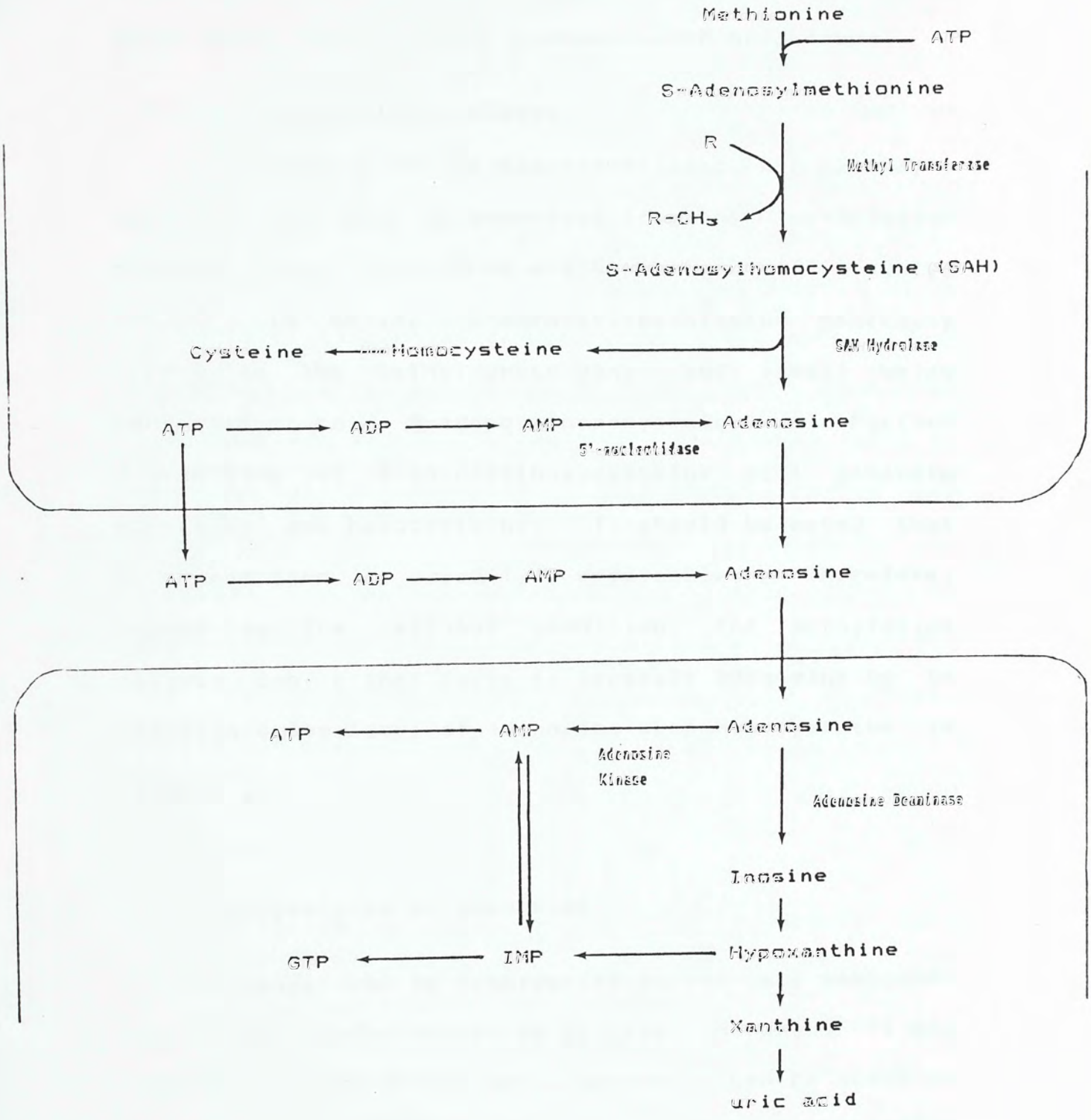


Fig. 1.2 Metabolic pathway of Adenosine

local hormone to retaliate against an external stimulus which would tend to cause excessive ATP breakdown.

1.3.1.2) Methylation pathway:

In addition to the afore-mentioned 'ATP pathway', adenosine can also be generated from the 'methylation pathway' (Fig. 1.2) (Arch and Newsholme, 1978; Daly, 1982). In cells, S-adenosyl-methionine generally serves as the methyl group donor and itself being converted to S-adenosylhomocysteine. Further degradation of S-adenosylhomocysteine will generate adenosine and homocysteine. It should be noted that this reaction is readily reversible. Therefore, depend on the cellular condition, the methylation pathway can either serve to generate adenosine or to maintain a low level of adenosine when homocysteine is high.

1.3.2) Disposition of adenosine

Adenosine can be transported across cell membranes via a facilitated diffusion process (Marangos *et al*, 1982). Once inside the cell, adenosine can be acted on by two enzymes: adenosine deaminase and adenosine kinase (Fig. 1.2). Adenosine deaminase converts adenosine into inosine, which can be further metabolized to hypoxanthine. If cellular nucleotides are present in abundance, hypoxanthine will be

converted to xanthine and then uric acid. Otherwise, hypoxanthine will be recruited into the metabolic pool through IMP by the salvage enzyme hypoxanthine-guanine phosphoribosyl transferase.

Adenosine kinase converts adenosine to AMP, which then enter the metabolic pool (Fig. 1.2). The Km value of adenosine kinase is about 50 fold lower than that of adenosine deaminase. Moreover, the adenosine concentration in tissues is larger than the Km value of adenosine kinase in general. Therefore, most of the adenosine will be rephosphorylated. However, in situations when the adenosine level increases drastically, adenosine deaminase will play an important role in its removal. This is physiologically important, because it prevents large amount of ATP being consumed for the removal of adenosine by adenosine kinase (Arch and Newsholme, 1978).

Currently, stable analogues of adenosine were obtained by modifying the parent adenosine skeleton (Fig. 1.1). Since both A1 and A2 receptors can tolerate only a very minor modification in the ribose moiety of adenosine analogue for activity (see section 1.4), most stable analogues of adenosine was obtianed by modifying the purine ring of adenosine. For instance, the stable analogue 2-chloroadenosine is obtained by displacement of the hydrogen atom with a

chloride atom at the C-2 carbon of the purine ring.

1.3.3) Cytotoxicity of adenosine

High cellular levels of adenosine can be cytotoxic (Fox, 1982). When adenosine is accumulated in the cell, owing to adenosine deaminase deficiency or other reasons, it will be converted into deoxy-ATP. This derivative can inhibit the ribonucleotide reductase, an enzyme which converts ADP, GDP, UDP and CDP to the corresponding deoxyribonucleotides for DNA and/or RNA synthesis (Fig. 1.3). For an actively growing cell, a decrease in DNA and/or RNA synthesis is expected to hinder the growth and proliferation of the cell.

An accumulation of adenosine will also increase the cellular level of S-adenosylhomocysteine, which in turn can inhibit the cellular methylation reaction via a feedback regulation (Fig. 1.4) (Fox, 1981). Like phosphorylation and dephosphorylation reactions, the methylation and demethylation reactions are biologically important processes in the regulation of enzyme activities in the cell. Methylation is also important for the synthesis of many biomolecules including phosphatidylcholine, norepinephrine, creatine, and vitamin B12 (Alberts *et al*, 1983; Lehninger, 1970). Therefore, an inhibition of this process may contribute towards the cytotoxic effects of

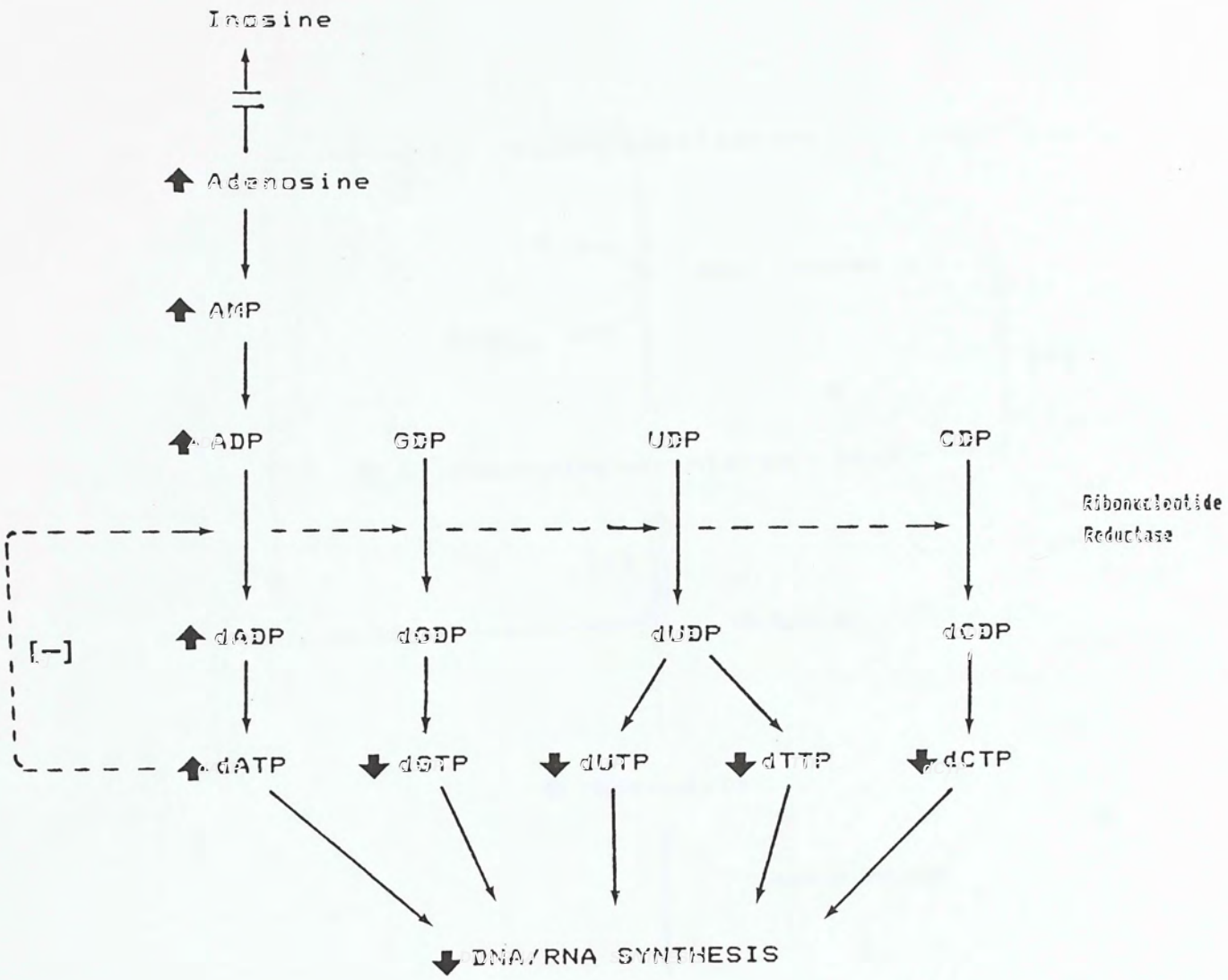


Fig. 1.3 Possible mechanism of feedback inhibition of ribonucleotide reductase during intracellular accumulation of adenosine

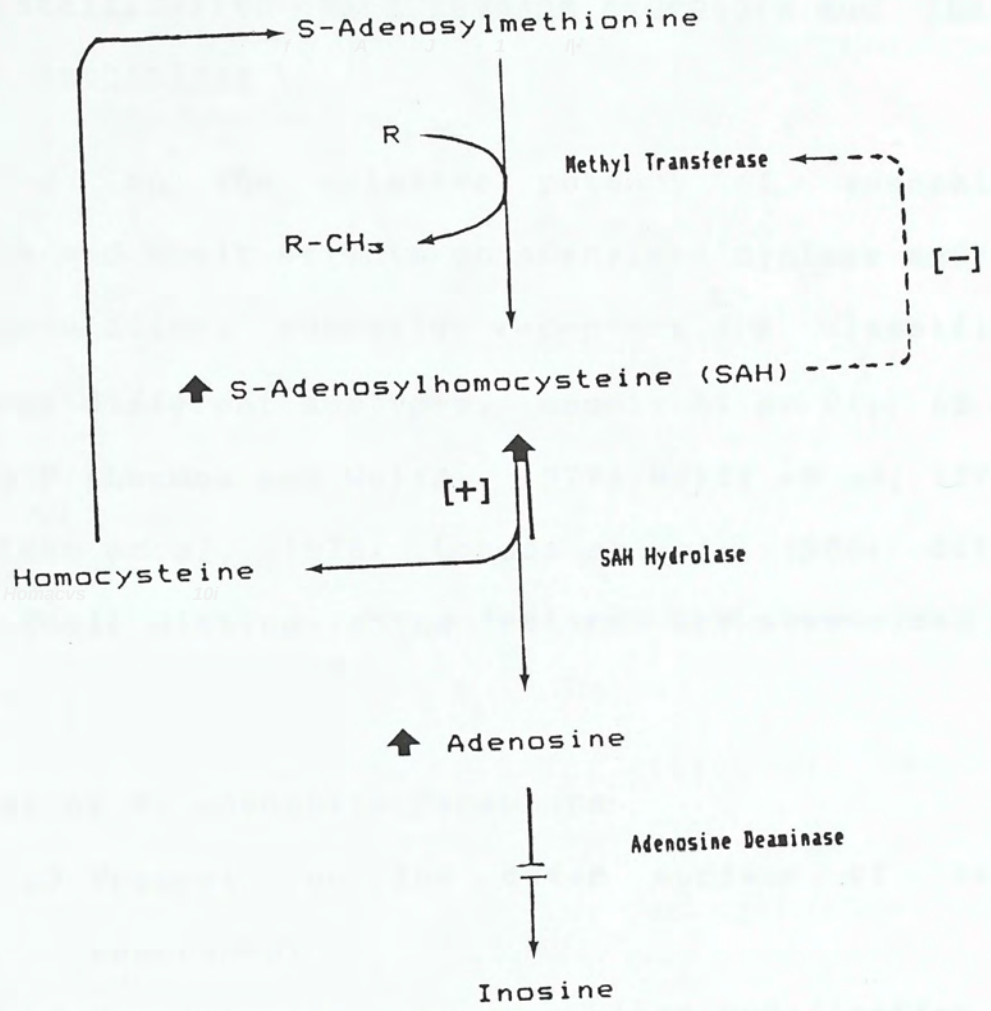


Fig. 1.4 Possible mechanism of feedback inhibition of methyltransferation during intracellular accumulation of adenosine.

high cellular adenosine.

1.4) Classification of adenosine receptors and their cellular mechanisms

Based on the relative potency of adenosine analogues and their effects on adenylate cyclase and/or cAMP accumulation, adenosine receptors are classified into three different subtypes, namely A1 or Ri, A2 or Ra, and P (Londos and Wolff, 1977; Wolff *et al*, 1978; Van Calker *et al*, 1979; Londos *et al*, 1980; Daly, 1983). Their distinguishing features are summarized as follows:

(A) A1 or Ri adenosine receptors

a) Present on the outer surface of cell membranes.

b) Can tolerate only very minor modification in the ribose moiety of adenosine analogue for activity.

c) Mediate an inhibitory effect on adenylate cyclase and/or cAMP accumulation.

d) Possess a high affinity for adenosine (submicromolar)

e) Have higher stereoselectivity (prefer L-PIA to D-PIA by 30 to 100 fold)

f) Drug potency:

L-PIA=CHA>2-chloroadenosine=

adenosine>NECA=D-PIA.

g) Antagonized by xanthines.

(B) A2 or Ra adenosine receptors

a) Present on the outer surface of cell membranes.

b) Can tolerate only very minor modification in the ribose moiety of adenosine analogue for activity.

c) Mediates a stimulatory effect on adenylate cyclase and/or cAMP accumulation.

d) Possess a low affinity for adenosine (high micromolar)

e) Have lower stereoselectivity (L-PIA only < 3 fold more active than D-PIA).

f) Drug potency:

NECA>2-chloroadenosine=

adenosine>L-PIA=CHA>D-PIA.

g) Antagonized by xanthines.

(C) P adenosine receptors

a) Present inside the cell, with a possible association with the catalytic unit of adenylate cyclase.

b) Can tolerate only minor modification in the purine moiety for adenosine analogues for activity.

c) Mediate an inhibitory effect on adenylate cyclase and/or cAMP accumulation.

d) Possess a low affinity for adenosine (high micromolar)

e) Drug potency:

2',5'-dideoxyadenosine>

5'-deoxyadenosine>2'-deoxyadenosine>

adenosine>2-chloroadenosine

f) Not antagonized by xanthines.

There is a differential distribution of A1 and A2 adenosine receptors in different tissue/organs. In general, the A2 subtype seems to have a wider distribution than the A1 subtypes (Table 1.1). Besides biological assays, radioligand binding studies using ^{3}H-PIA, ^{3}H-CHA and ^{3}H-DPX have been used successfully to identify and characterise the A1 adenosine receptors in neurons and adipocytes (Bruns *et al*, 1980; Trost and Schwabe, 1981; Murphy and Snyder, 1982). On the other hand, ^{3}H-NECA can be used to label the A2 adenosine receptors (Yeung and Green, 1984; Huttemann, 1984).

In addition to the well-known linkage of adenosine receptors to adenylate cyclase, there are also evidence indicating that adenosine receptor activation can

result in the regulation of Ca^{++} flux across cell membranes (Stone, 1981; Silinsky, 1981). Whether the effect on Ca^{++} mobilization is secondary to its action on adenylate cyclase or is mediated by a separate mechanism remains to be investigated.

1.5) Triglyceride metaboloism

1.5.1) General aspect

Following feeding excess carbohydrates and fatty acids will be transferred to adipose tissues, where they will be stored in the form of triglycerides. During fasting and exercise they can be released for energy supply.

There are two types of adipose tissues, namely the brown and white adipose tissues. The brown adipose tissue, characterized by its high mitochondrial content, is capable of oxidizing fatty acids at high rate and generates heat. Mobilization of fatty acids in the brown adipose tissue is controlled by the sympathetic nervous system. Norepinephrine is released from the nerve endings, which in turn activates the lipase in adipocytes. Physiologically, brown adipose tissues are important in thermogenesis in hibernating animals and in neonates of many species including man (Joel, 1965).

Unlike the brown adipose tissue, the white adipose tissue contains fewer mitochondria. Mobilization of fatty acid is controlled by both the nervous system and circulatory hormones. Physiologically, white adipose tissue serves as an energy store. Upon complete oxidation, fat gives more than twice the energy available from protein or glycogen on equal weight basis. Thus, when compared with glycogen and protein, energy stored in white adipose tissue is much more economical. Furthermore, white adipose tissue contains much less water than other tissues, such as the liver and muscle, thus making it a compact and rich storehouse for energy.

1.5.2) Biosynthesis of triglyceride

Usually, adipose tissue of the land animal contains 5-30% water, 2-3% protein, and 60-85% lipid. Among the lipids, 90-99% are triglycerides, which consist of three long-chain fatty acids linked through ester bonds to the hydroxyl group of the glycerol. Generally, one triglyceride molecule consists of two to three different types of fatty acids of different chain length and different degree of unsaturation. In rats, similar to men, the ratio of saturated to unsaturated fatty acid is about 3 : 7. Sixteen and eighteen carbons fatty acids are the major forms of saturated and

unsaturated fatty acids respectively (Jeanrenaud, 1965).

Adipose tissue has little or no glycerol kinase, thus could not reutilize the glycerol moiety released from the triglyceride. Instead, alpha-glycerophosphate, the active form of glycerol during triglyceride synthesis, is derived from dihydroxyacetone phosphate which is an intermediate in the glucose oxidation pathway (Fig. 1.5). In contrast, the free fatty acids (FFA) released from triglycerides by hormone sensitive lipase (HSL) can be activated with ATP and CoA by pyrophosphatase, and reused for triglyceride synthesis (Fig. 1.5). Dietary fat is another source of FFA. After digestion, FFA can be absorbed in the intestine, and be incorporated into triglycerides in the liver where the triglyceride can combine with proteins to form chylomicrons. These lipid-rich droplets are then circulated in the body together with other blood components. When they reach the adipose tissues, FFA is released from chylomicrons by the action of a lipoprotein lipase. The FFA released is then taken into adipocytes for triglyceride synthesis. In addition, fatty acids can also be synthesized *de novo* in adipocytes using carbohydrates and acetyl CoA (Fig. 1.5) In human, *de novo* fatty acid synthesis occurs mainly in the liver, where they are then transported in the form of lipoprotein to adipose

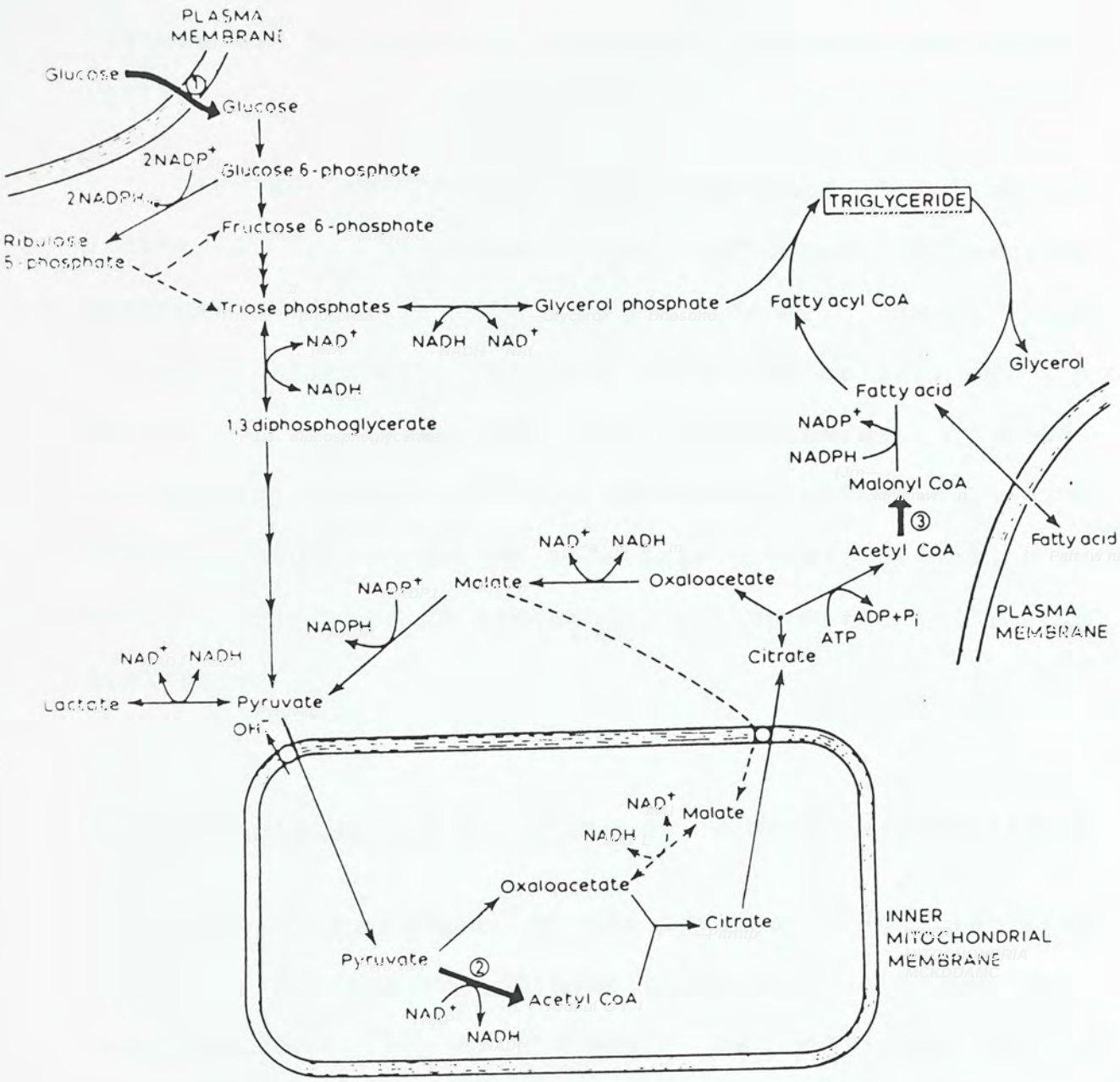


Fig. 1.5 Metabolism of triglyceride in rat epididymal adipose tissue (Adopted from Denton and Pogson, 1976).

tissues for triglyceride synthesis (Meisner and Carter, 1977).

Glucose oxidation, fatty acid and triglyceride synthesis are all under the influence of various hormones (Rodbell, 1965a). For instance, insulin and oxytocin stimulate glucose oxidation as well as the formation of fatty acid and triglyceride. Thyroid-stimulating hormone (TSH), adrenocorticotropic hormone (ACTH), and epinephrine stimulate glucose oxidation as well as triglyceride synthesis, but decrease fatty acid synthesis.

1.5.3) Mobilization of triglyceride from adipose tissue

Lipolysis refers to the process which mobilize triglyceride from the adipose tissues. In adipocytes, the breakdown of triglyceride is catalyzed by a hormone-sensitive lipase (HSL). Currently, it is widely believed that lipolytic hormones exert their effects by increasing cAMP generation in adipocytes. An elevated cellular cAMP level converts an inactive cAMP-dependent protein kinase into an active form, which in turn activates the HSL by a phosphorylation reaction (Fig. 1.6) (Meisner and Carter, 1977; Steinberg, 1976). Evidence in support of the 'cAMP hypothesis' include the following:

a) Lipolytic hormones, including ACTH, TSH, and NE,

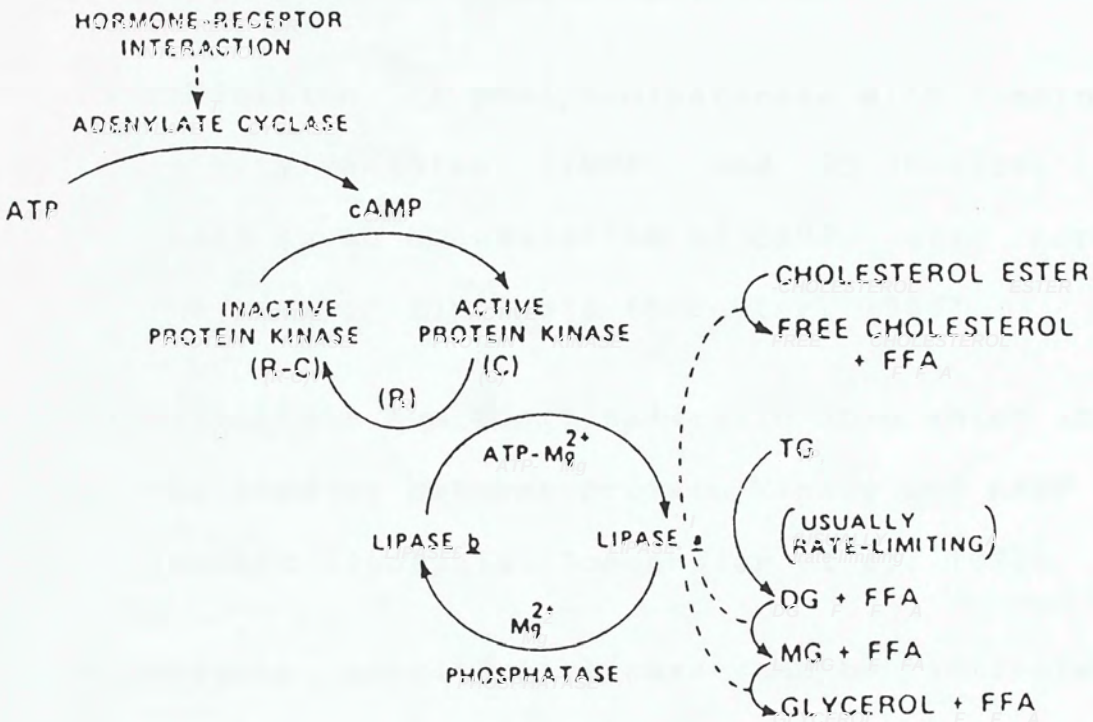


Fig. 1.6 Possible mechanism of activation of hormone sensitive lipase (Adopted from Steinberg, 1976)

are able to increase cAMP levels in adipocytes (Butcher *et al*, 1968)

b) Forskolin which activates the catalytic unit of adenylate cyclase can induce lipolysis in adipocytes (Litosch *et al*, 1982)

c) Inhibition of phosphodiesterase with 1-methyl-3-isobutylxanthine (IBMX) and RO-20-1724, which leads to an accumulation of cAMP, also increases the rate of lipolysis (Shechter, 1984).

d) Salicylate and acetylsalicylic acid which inhibit the binding between protein kinase and cAMP also inhibit lipolysis (Schonhofer *et al*, 1973)

e) Hormone sensitive lipase can be activated by exogenous protein kinase prepared from bovine skeletal muscle. However, HSL prepared from adipose tissues that has been pre-incubated with NE gave a lower degree of activation by the protein kinase. The results suggested that most HSL has been activated by the NE-stimulated cAMP accumulation, thus a lower degree of activation of HSL by exogenous protein kinase was obtained (Khoo and Steinberg, 1974).

In addition to the hormone sensitive lipase, diglyceride and monoglyceride hydrolase activities are also present in adipose tissues (Steinberg, 1976).

Although the degree of activation by cAMP of these glyceride hydrolase is much lower than that of HSL, their enzyme activity against di-, and monoglycerides is much higher than that of HSL. Consequently, it is likely that the hydrolysis of the first ester bond of triglyceride by HSL may be the rate-limiting step in FFA mobilization.

1.6) Regulation of lipolysis

1.6.1) Intrinsic factors

1.6.1.1) Age

The rate of lipolysis stimulated by epinephrine and ACTH is age-dependent, being higher in younger rats. On the other hand, there is no age difference in the theophylline-stimulated lipolysis (Miller and Allen, 1973). Dax and co-worker (1981) found that there was no difference in the beta-adrenergic receptor number and the response of cAMP accumulation to epinephrine , they therefore concluded that the age - dependent decrease in lipolysis to epinephrine and ACTH may be due to an alternation of the lipolytic pathway beyond the level of receptors occupancy and its coupling to adenylate cyclase.

1.6.1.2) Cell size

When the rate of lipolysis is expressed in term of umole FFA/10^5 cells it was found that the rate of lipolysis correlated linearly and positively with the cell diameter (Zinder and Shapiro, 1971; Reardon *et al*, 1973; Vernon and Finley, 1985). However, when the rate of lipolysis is expressed in umole FFA/umole triglyceride, there is a negative linear correlation between the cell diameter and lipolytic rates (Reardon *et al*, 1973).

1.6.2) Extrinsic factors

1.6.2.1) Free fatty acid

FFA produced in lipolysis can be removed either by re-esterification with alpha-glycerophosphate, or by diffusion out of the adipocyte. Since FFA is hydrophobic, it usually binds to a carrier such as albumin for transport. Rodbell (1965b) showed that, in the absence of glucose, there was a decrease in the efflux of FFA and glycerol when the molar ratio of FFA-to-albumin exceeds 3. The efflux of FFA was completely inhibited when the ratio exceeds 6. If glucose was added to the medium to increase the re-esterification of FFA, an enhancement of lipolysis was observed (Bally *et al*, 1965). These experiments suggest FFA may act as a feedback inhibitor to regulate lipolysis.

1.6.2.2) Hydrogen ion (H^+)

Hydrogen ion and FFA are released stoichiometrically during lipolysis, therefore, an accumulation of FFA in the medium is accompanied by an acidification of the extracellular space. Meisner (1977) demonstrated that there was about 50% decrease in FFA efflux when adipocytes were suspended in a buffer at a pH of 6.8, instead of 7.4. Poyart and co-worker (1967) showed that an acidic environment would favor the accumulation of FFA in adipocytes which will in turn inhibit lipolysis (see section 1.6.1.1)

1.6.2.3) Lipolytic hormone

Norepinephrine, epinephrine, TSH, ACTH and glucagon are all able to mobilize fatty acids from adipocytes in *in vitro* experiments. All these lipolytic hormones stimulate cAMP accumulation in adipocytes and only ACTH requires Ca^{++} ion for its lipolytic activity (Allen *et al*, 1977). Although the level of circulating catecholamine is low, a physiological role of neuronal catecholamine in lipolysis was demonstrated by stimulating the sympathetic nerve *in vitro* (Correll, 1963).

Growth hormone, thyroid hormone, and corticosteriods do not mobilize fatty acid directly,

but their presence are important for hormone stimulated lipolysis. For instance, hypophysectomized and adrenalectomized animals have a lower basal rate of lipolysis as well as diminished response to lipolytic stimuli (Shafrir *et al*, 1960). Adipose tissues from hypothyroid animals also show a markedly lower response to lipolytic stimuli (Deykin and Vaughan, 1963).

1.6.2.4) Anti-lipolytic agents

Insulin, adenosine, and prostaglandins are all able to inhibit the hormone stimulated lipolysis. All three hormones inhibit the hormone stimulated cAMP accumulation in adipocytes (Meisner and Carter, 1977; Fredholm, 1978). However, the effects of prostaglandin synthesis inhibitors and antagonists on lipolysis and cAMP accumulation are controversial. A role of prostaglandins as a feedback regulator of lipolysis is still an open question (Fredholm, 1978). Adenosine can potentiate the effect of insulin on both lipolysis and glucose oxidation, even though different mechanisms are involved in these two processes (Schwabe, 1974).

CHAPTER 2

METHODOLOGY

2.11 Rat

Both male and female Sprague-Dawley rats were bred and grew in the animal house of Chinese University of Hong Kong. They were kept in rooms with a light-dark cycle of 12 h time interval. Drinking solution and pellet diet (Formerly Laboratory Chow 5001C, Dean's Animal Feeds) were given *ad libitum*. The body weight of rats increase rapidly between the sixth and the twelfth weeks of age (Fig. 2.1).

2.12 Chronic Caffeine Treatment

For each treatment, 8 male rats (200-250 g) from two age-matched (about 7 weeks) litters were equally divided into two groups. The control group received tap water while the caffeine group was given a solution of caffeine (Merck) for 6 weeks or for as long as indicated. The amount of water and caffeine solutions consumed were recorded on alternate days. When comparing with the control group, it was found that the daily fluid consumption of the experimental group given 0.2% caffeine solution was lowered by about 20%. In contrast, there was a 35% increase in daily fluid intake in the group given 0.05% caffeine solution. No significant difference, however, was observed between the controls and those received 0.1% caffeine solution (Table 2.1). There was a trend of decrease in fluid

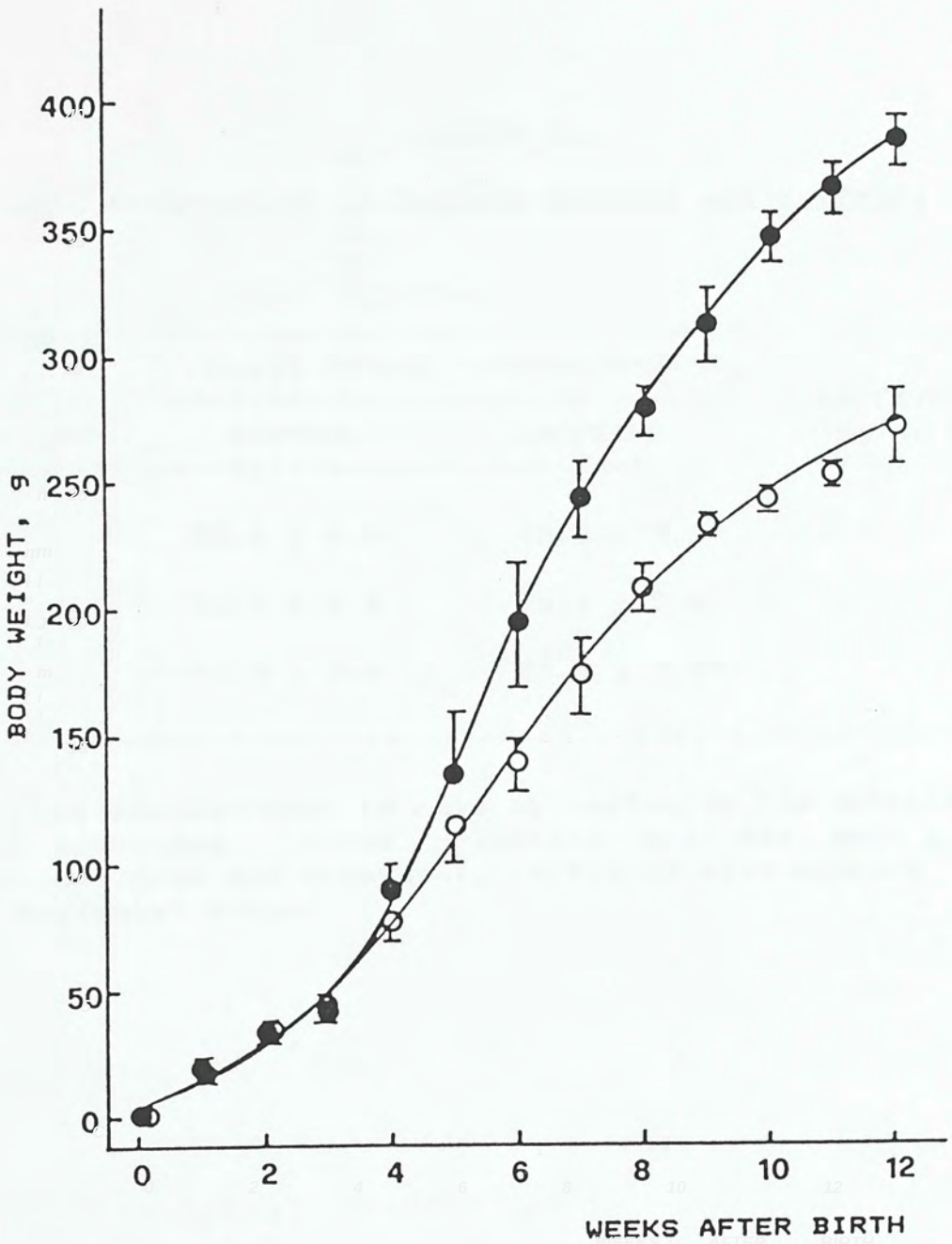


Fig. 2.1: Growth curves (in terms of body weight) of male (●) and female (○) rats after birth. Data presented are obtained from the animal house of Chinese University of Hong Kong.

Table 2.1

Fluid consumption in various control and caffeine groups

CAFFEINE CONCENTRATION	FLUID INTAKE (ml/rat/day)		CAFFEINE CONSUMED (mg/kg body weight)
	CONTROL	CAFFEINE	
0.05 %	33.4 ± 5.5	45.3 ± 7.3*	64
0.10 %	33.4 ± 5.5	36.3 ± 3.9	107
0.20 %	46.0 ± 2.6	35.9 ± 2.5*	287

Caffeine was adminstrated to rats by replacing the drinking water with caffeine solutions. Value presented are the mean ± S.D. of 21 determination from one treatment. * $P<0.05$ when compare with control by using Students' t-test.

consumption (about 10%) towards the end of the 6-weeks treatment period in both control and caffeine treated groups (Fig. 2.2).

2.3) Isoproterenol Treatment

D,L-Isoproterenol (0.2 mg/ml, Sigma) was administered to male rats (300-350 g) by subcutaneous injection at a dosage of 0.2 mg/kg body weight every 12 h for 6 days. Control male rats were injected with the same volume of saline vehicle.

2.4) Adipocyte Counting

An improved Neubauer haemocytometer was used for fat cell counting. When compared with blood cells, adipocytes have larger cell diameters. In order to obtain higher cell counts, the whole ruled area, instead of just the central ruled area was used for adipocyte counting. Since the whole ruled area is 3 mm x 3 mm with a depth of 0.1 mm, the total volume of the ruled area is 9×10^{-4} cm^3. Thus the cell concentration :

$$\frac{\text{Cell number in the ruled area}}{9 \times 10^{-4}} \text{ cells/ml}$$

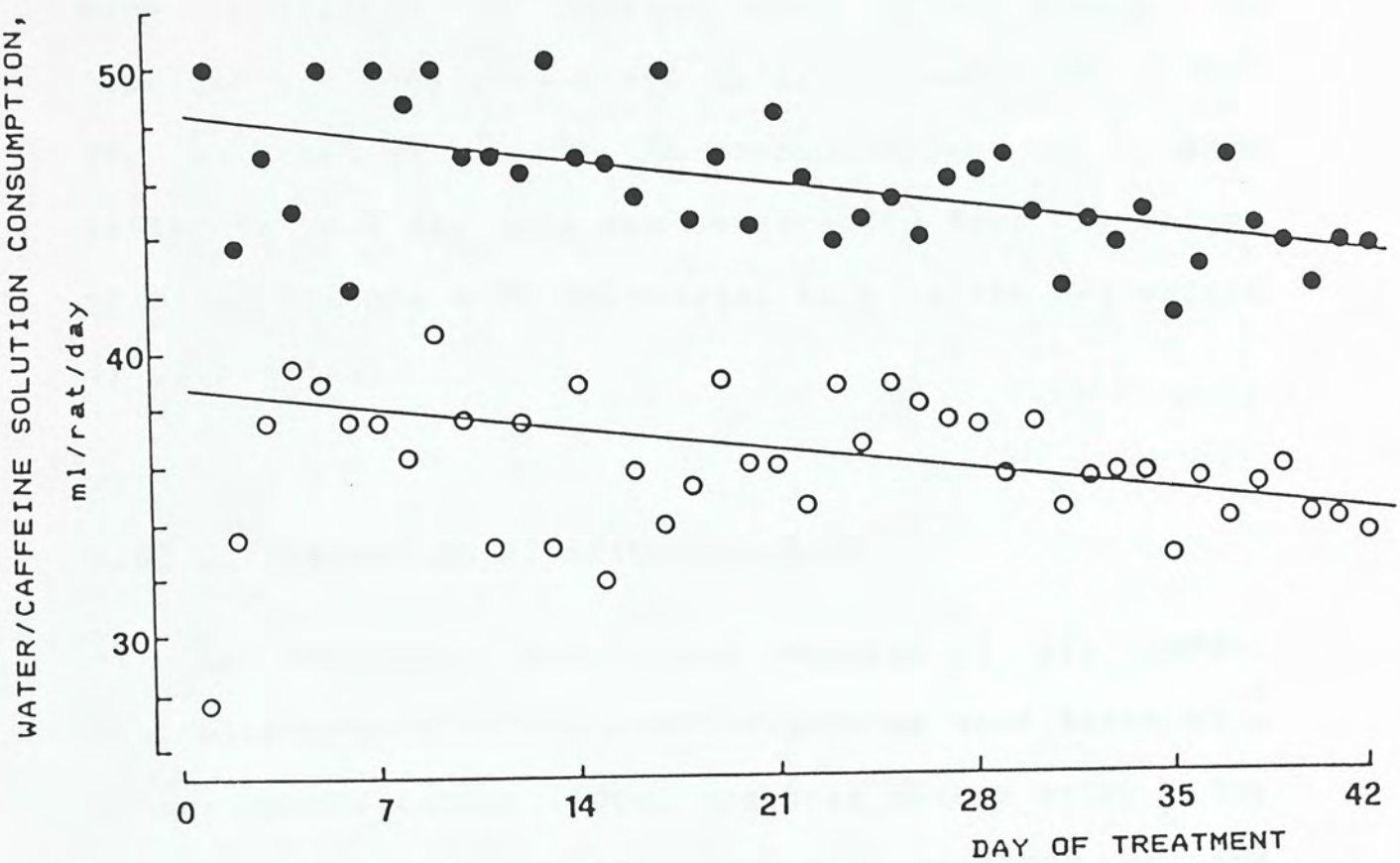


Fig. 2.2: Consumption of tap water (●) and 0.2% caffeine solution (○) in the control and caffeine-treated group respectively, over the 6 weeks experimental period. Data showed are the mean of 2-4 separated treatments, with deviation less than 10% of the mean.

2.5) Measurement of Adipocyte Weight

Under vacuum filtration, aliquots of fat cells were collected on Whatman GF/B glass fibers in duplicate. They were dried in an incubator set at 80° for at least 24 h. As the average weight of a GF/B filter is 70.1 mg, this was substracted from the weight of dried filters with adipocytes to give the dry weight of adipocytes.

2.6) Determination of Adipocyte Size

As described previously (Ashwell *et al*, 1976), photomicrographs of isolated adipocytes were taken at a proper magnification (100x) together with a ruler. The diameter of isolated adipocytes were measured on the photographs. To avoid bias in sampling, three photomicrographs were prepared for each adipocytes sample, and the number of adipocytes distributed around the central horizontal line in photographs was counted. Assuming the adipocytes are spherical and their cell volumes can be calculated according to equation 2.1 as described by Goldrick (1967):

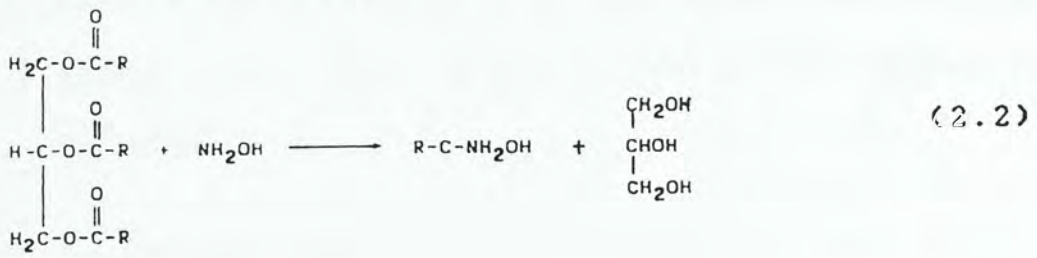
$$\text{Cell volume} = \left(\frac{\pi}{6}\right)\left(3\sigma^2 + \overline{X}^2\right)\overline{X} \qquad (2.1)$$

where $\overline{X}$ = mean cell diameter.

σ^2 = variance of the cell diameters.

2.7) Triglyceride Determination

A hydroxamic acid reaction was initially developed by Goddu *et al* (1955) for the spectrophotometric determination of esters and anhydrides. This reaction was adopted by Rapport and Alonzo (1955) for triglyceride determination. In principle, triglyceride is hydroxylaminolyzed and the fatty acids formed can react with hydroxylamine to form hydroxamic acid (equation 2.2). The resulting hydroxamic acid can combine with ferric acid to give a characteristic red to purple chelated complex (equation 2.3).



(2.2)

(2.3)

Maximal colour yield was obtained when the final ferric ion concentration was greater than 2 mM and that of hydrogen ion concentration was less than 0.6 M (Goddu *et al*, 1955). It should be noted that this assay is not specific for triglycerides. A positive result can be obtained with any fatty acid ester. Furthermore, high concentrations of carbonyl compounds,

transition elements, ions or any compound which can complex with ferric ion may affect the intensity of the colour. The rate of formation of hydroxamic acid is affected by both temperature and the type of ester used, so colour yield is also temperature sensitive.

2.7.1) Stock reagents

a) Ethanol hydroxylamine hydrochloride:

Hydroxylamine hydrochloride (0.6 g) was dissolved in 1 ml water and then mixed with 19 ml absolute ethanol.

b) Ethanolic sodium hydroxide:

Sodium hydroxide (0.6 g) was dissolved in 1 ml water and then mixed with 19 ml absolute ethanol.

c) Ferric perchlorate:

Iron wire (0.8 g) in 10 ml of 70% perchloric acid was dissolved by heating on a hot plate. Upon cooling, the solid residue was redissolved in 10 ml water and diluted with absolute ethanol to 100 ml.

2.7.2) Working reagents

a) Alkaline hydroxylamine:

Equal volumes of ethanolic hydroxylamine hydrochloride and ethanolic sodium hydroxide

were mixed. After a few minutes, the sodium chloride precipitate was removed by filtration. The reagent was stable for about 5 h.

b) Ethanolic acid ferric prechlorate:

Ferric perchlorate (4 ml) was mixed with 1.2 ml perchloride acid and then diluted with absolute ethanol to 100 ml.

2.7.3) Preparation of triglyceride standards

a) Glycerol triacetate was used as standard. It was dissolved in chloroform to give a stock concentration of 5 mM.

b) The stock solution was diluted with chloroform to give the following concentrations: 1, 2, 3, 4, and 5 mM.

2.7.4) Extraction of triglyceride

a) Equal volumes of adipocyte suspension and chloroform were mixed vigorously. After centrifugation, the top aqueous layer was aspirated off.

b) The chloroform extracts were diluted 20 to 40 fold and their tirglyceride contents were determined.

2.7.5) Assay protocol

a) Ether (3 ml) was added to tubes containing 100 ul samples or standard.

b) Alkaline hydroxylamine (100 ul) was added and mixed.

c) The tubes were incubated in a water bath (65-70) in a fume-hood to facilitate the formation of hydroxamic acid and to evaporated the ether.

d) Residual ethanol was removed by suction under reduced pressure.

e) Ethanolic acid ferric perchlorate (2 ml) was added and the mixture kept to stand for 30 min.

f) Absorbance was read at 530 nm.

A typical standard curve for triglyceride determination is shown in Fig. 2.3.

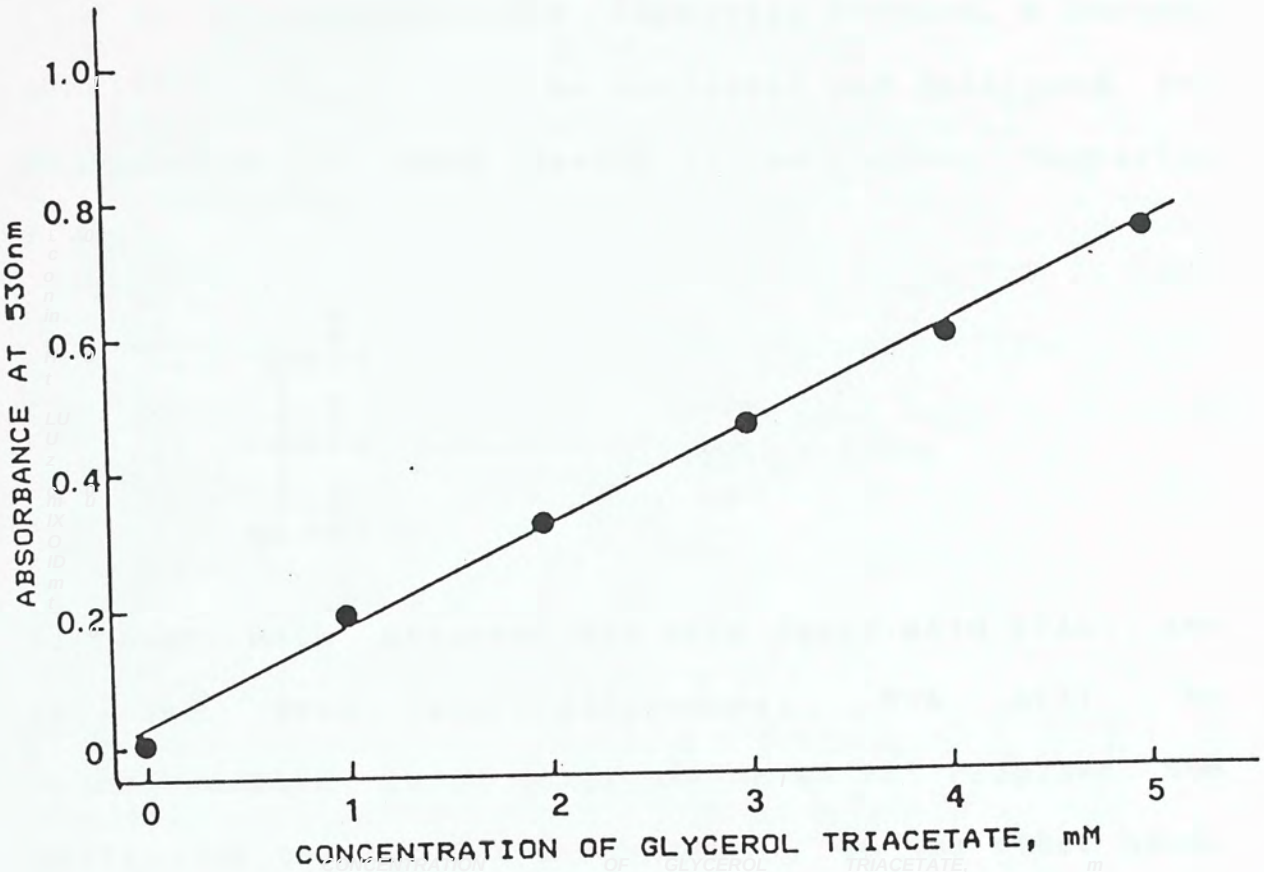
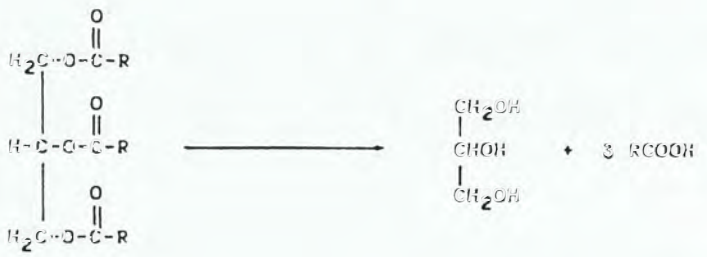


Fig. 2.3: A typical calibration curve for triglyceride determination. Values presented are the mean of duplicate determinations from one typical experiment. The straight line was determined by linear regression analysis. Each experiment has an individual calibration curve performed in parallel.

2.8) Lipolysis Assay

In the presence of a lipolytic hormone, a hormone sensitive lipase will be activated and catalyzed the degradation of triglyceride in adipocytes (equation 2.4):



(2.4)

Although both glycerol and free fatty acid (FAA) are released from the adipocytes, FFA will be reincorporated into triglyceride after reuptake and activation to acyl CoA derivative. On the other hand, because of the extremely low level of glycerol kinase, the glycerol cannot be incorporated significantly after its release (Steinberge and Khoo, 1977). Therefore, in the present study, lipolysis was determined by measuring the amount of glycerol released.

2.8.1) Buffer

2.8.1.1) Krebs-Ringer Bicarbonate Buffer (KRB)

KRB was prepared freshly on the day of experiment. The buffer had the following composition (mM): $CaCl_2$ (2.5), $MgSO_4$ (2.4), KH_2PO_4 (1.2), KCl (4.8), NaCl (119), $NaHCO_3$ (32.5). It was gassed with 95% O_2 - 5% CO_2 for 30 min to give a pH of 7.4 and then kept in

a 37° water bath.

2.8.1.2) Bovine serum albumin supplemented Krebs-Ringer bicarbonate buffer (BSA – KRB)

KRB after prepared according to section 2.8.1.1 was supplemented with 1-4% bovine serum albumin (Fraction 5, 96-98% purity, Sigma). After the BSA was completely dissolved, pH of BSA-KRB was adjusted with 1 N NaOH or 1 N HCl to 7.4, and then kept in a 37° water bath.

2.8.2) Preparation of adipocytes

Adipocytes were prepared according to the method described by Rodbell (1964) with some modifications:

a) Rats were killed by cervical dislocation. Epididymal fat pads were removed immediatedly and rinsed with 0.9% NaCl solution.

b) After dissecting free of blood vessels, the fat pads were weighed and minced.

c) The minced tissues were suspended in 4% BSA-KRB (1 ml/g tissue) containing collagenase (1 mg/ml, from Clostridium histolyticum, Sigma) in 50 ml or 20 ml plastic tubes. They were incubated at 37° in a water bath with continuous shaking (80-100 cycles/min) for 60-90 min.

d) Collagenase treatment was terminated by adding 2 volumes of KRB at 37^0. Adipocytes were collected by filtering the mixture through a layer of cheesecloth.

e) The filtrate was centrifuged with a clinical table-top centrifuge (ICE) at low speed (about 250xg) for about 30s at room temperature.

f) After centrifugation, adipocytes floated to the surface which facilitated the removal of buffer using a Pasteur pipette. Adipocytes were resuspended in 10 ml of 1% BSA-KRB and swirlled gently to disperse the cells.

g) Step (e) and (f) were repeated 2-3 times.

h) For lipolysis assay, adipocytes were resuspended in 4% BSA-KRB (3 ml/ml isolated adipocytes).

i) To give a final volume of 500ul, 350ul-450ul of the adipocyte suspension were dispensed into 1.5 ml plastic microcentrifuge tubes (Eppendorf) with various test drug(s) at 37^0 in a water bath with continuous shaking (about 35 cycles/min) and gassing (95% O_2-5% CO_2). Assays were performed in duplicate.

j) After an appropriate interval (30-90 min), incubation was terminated by adding 0.5 ml of 10% trichloroacetic acid. The reaction mixture was

centrifuged at about 10000xg for 5 min at room temperature.

k) Three layers were obtained after centrifugation. The top layer was fat and the bottom layer was denatured protein. Two hundred microlitres of the middle aqueous layer were used for glycerol determination within 24 h.

2.8.3) Glycerol determination

The method for glycerol determination was adopted from Lambert and Neish (1950) with some modifications. In principle, 1 mole of glycerol is oxidized by periodate to form 1 mole of formic acid and 2 moles of formaldehyde. After neutralization of the excess periodate with metabisulphite, the formaldehyde formed can be determined colorimetrically by reacting with chromotropic acid (MacFadyner, 1945).

Compounds that have two hydroxy group, or a hydroxyl and an amino group, or two carbonyl group, or a hydroxyl and a carbonyl group in the adjacent carbon atom can be oxidized by periodate to form formaldehyde. Oxidation has also been reported to occur with cystine, methionine and tryptophan which contain nitrogen but possess no alpha-amino-alcohol structure (Jackson, 1944). Therefore, samples for glycerol determination

should be free of sugar, protein and other compounds with the aforementioned chemical structures.

2.8.3.1) Reagents

a) 12.5 N H_2SO_4

b) 1 N H_2SO_4

c) 50 mM sodium periodate

d) 10% sodium metabisulphite

2.8.3.2) Preparation of chromotropic reagent

Chromotropic reagent (500 ml) was prepared freshly by dissolving one gram of 4,5-dihydroxynaphthalene-2,7-disulfonic acid (Sigma) in 100 ml water, and then 400 ml 12.5 N sulfuric acid were added and mixed thoroughly.

2.8.3.3) Preparation of glycerol standard

Stock glycerol solution (2 umole/100 ul) was diluted in water to give a working solution of 40 nmole/100 ul. The working solution was serially diluted to give the following concentrations: 40, 20, 10, 5, and 2.5 nmole/100 ul.

2.8.3.4) Assay protocol

a) Fifty microlitres of 1 N sulfuric acid were added to tubes containing 200 ul samples or standards

b) Fifty microlitres of 50 mM sodium periodate were added, mixed and left to stand for 5 min.

c) Fifty microlitres of 10% sodium metabisulphite were added, mixed and left to stand for 5 min.

d) Two millitres of chromotropic reagent were added.

e) Tubes were covered with tin foil and boiled for 30 min.

f) Water (0.5 ml) was added and the reaction mixture allowed to cool down to room temperature.

g) Absorbance was read at 570 nm.

A typical standard curve for glycerol determination is shown in Fig. 2.4.

2.8.4) Data analysis by the Hill plot

For a receptor mediated biological response, it can be described as:

$$R + nL \xrightarrow{K_D} RL_n \longrightarrow (E-E0)$$

where R = (receptor)

L = (drug)

n = theoretical number of drug-binding site per receptor molecule.

E_o = basal response

$(E-E_o)$ = stimulated response

K_D = dissociation constant

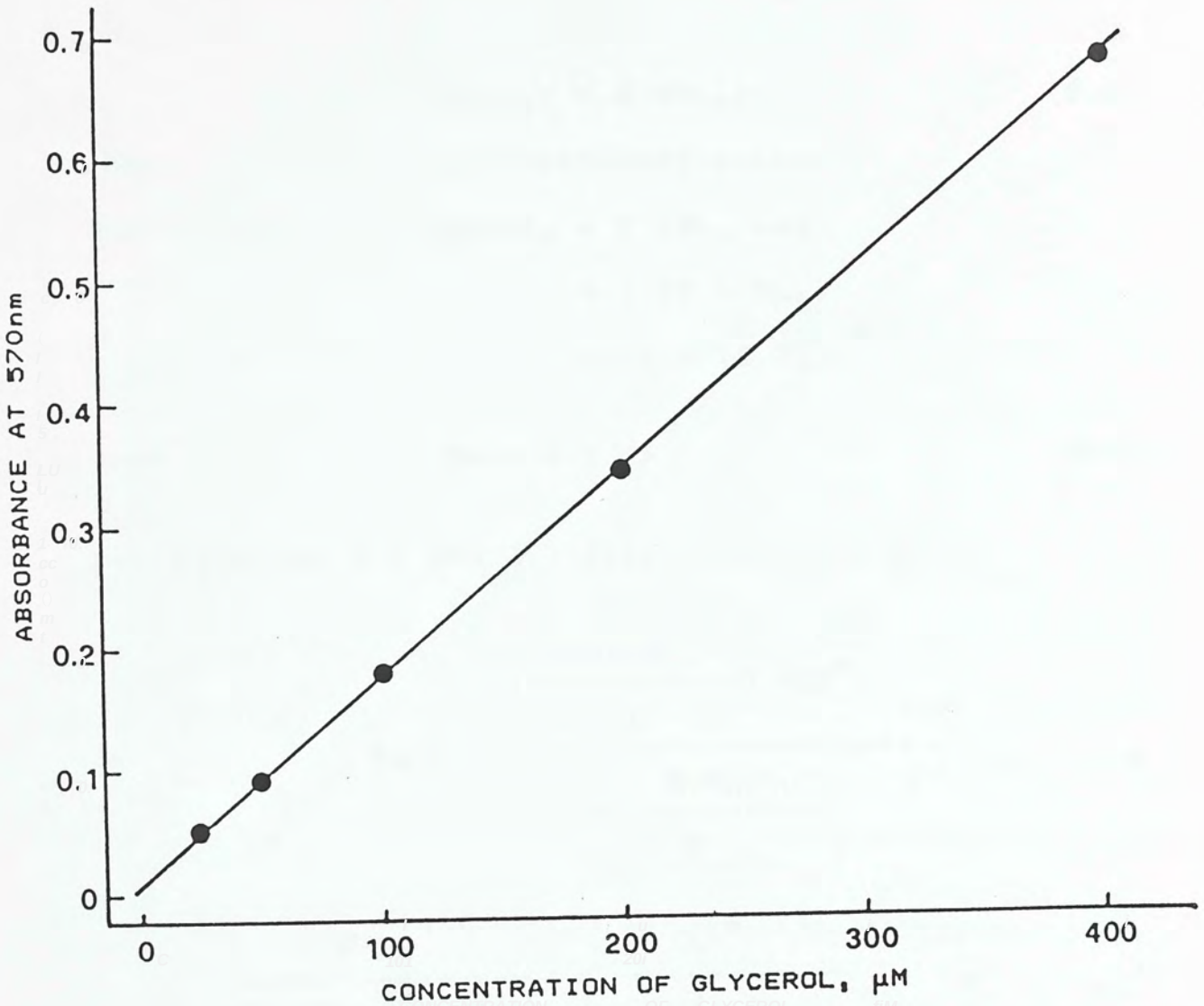


Fig. 2.4: A typical calibration curve for glycerol determination. Values presented are the mean of duplicate determinations from one typical experiment. The straight line was determined by linear regression analysis. Each experiment was accompanied by an individual calibration curve.

as

$$K_D = \frac{(R)\ (L)^n}{(RL_n)} \qquad (2.5)$$

$$(E-E_o) = f\ (RL_n) \qquad (2.6)$$

where f = efficacy constant

therefore

$$E_{max}-E_o = f\ (RL_n)_{max}$$

$$= f\ (R + RL_n)$$

$$= fR + (E-E_o)$$

thus

$$E_{max}-E = fR \qquad (2.7)$$

put equation 2.6 and 2.7 into equation 2.5:

$$K_D = \frac{\left(\frac{E_{max}-E}{f}\right)(L)^n}{\left(\frac{E-E_o}{f}\right)}$$

$$\frac{E-E_o}{E_{max}-E} = \frac{(L)^n}{K_D}$$

$$\log\left(\frac{E-E_o}{E_{max}-E}\right) = n \log (L) - \log K_D \qquad (2.8)$$

Therefore, when a dose response curve is transformed to a Hill plot according to equation 2.8 by plotting $\log((E-E_o)/(E_{max}-E))$ vs $\log (L)$, a straight line can be obtained. At half-maximal response $(E-E_o)/(E_{max}-E) = 1$, and $\log 1 = 0$. Therefore, the concentration of drug that gives half-maximal response (IC_{50}, or EC_{50}) can

be obtained from the Hill plot by finding corresponding value of log (L) at log $((E-E_0)/(Emax-E)) = 0$.

2.9) cAMP Accumulation Assay

2.9.1) Stimulation of cAMP accumulation

a) Isolated adipocytes were prepared as described in section 2.8.2 and resuspended in equal volumes (v/v) of 4% BSA-KRB.

b) To give a final volume of 1 ml, 700-900 ul of adipocyte suspension were dispensed into 1.5 ml plastic microcentrifuge tube and incubated with various test drug(s) in a water bath at 37^0. Reaction was started by adding isoproterenol.

c) After 5 min or as indicated, incubation was terminated by heating in a boiling water bath for 5 min.

d) Upon cooling, the reaction mixture was centrifuged at about 10000xg for 20 min at room temperature.

e) Three layers were obtained after centrifugation and 50 ul of the middle aqueous layer were taken out for cAMP determination performed within 3 h.

2.9.2) cAMP determination

In principle, the assay is based on competition for binding to an adrenal cAMP binding protein between unlabelled cAMP and a fixed amount of ^{3}H-cAMP . With increasing levels of cAMP in the sample, the amount of ^{3}H-cAMP bound to the cAMP-binding protein decreases proportionally. After removal of unbound ^{3}H-cAMP by adsorption onto charcoal, the protein-bound radioactivity cab be determined. The amount of cAMP in sample can be calculated by comparing with a standard curve performed in parallel.

Although cGMP and ATP are the two naturally occurring nucleotides most likely to interfere with the binding of cAMP to its binding protein (Albano *et al*, 1974; Brown *et al*, 1972), it was found that ATP did not interfere at concentrations up to 5 mM (Tovey *et al*, 1974). Moreover, 200 times as much cGMP as cAMP is required to give an equivalent reducton in the binding of tritated cAMP (Tovey *et al*, 1974). In mammalian tissues, the concentration of cAMP is generally about 100 times higher than that of cGMP (Steiner *et al*, 1972), therefore the normal tissue cGMP is unlikely to affect the present cAMP assay. Divalent ions such as Mg^{++} and Ca^{++} have been reported to reduce the binding of ^{3}H-cAMP to the cAMP-binding protein (Tovey *et al*, 1974). The intereference of these

divalent ions was prevented by adding 4 mM EDTA to the assay buffer in the present study.

2.9.2.1) Preparation of cAMP binding protein

Binding protein was prepared according to the method of Brown *et al* (1972). Briefly,

a) Bovine adrenal glands (20 pairs) were freshly collected from a slaughter house and kept on ice.

b) After removal of fats, the adrenal cortex was separated from the medulla.

c) The medulla was homogenized with 1.5 vol (v/w) of Medium A (0.25 M sucrose, 25 mM KCl, 5 mM $MgCl_2$ and 50 mM Tris-HCl, pH=7.4 at room temperature).

d) The homogenate was centrifuged at 10000xg for 5 min, the pellet was discarded.

e) The supernatant was centrifuged again at 10000xg for 15 min, the pellet was discarded.

f) The supernatant was divided into 500 ul portions and stored at -20^0.

g) For cAMP measurement, the binding protein was thawed and diluted 20 fold with the assay buffer to give a total binding of around 6000 cpm at a final ^3H-cAMP concentration of 4.5 nM.

2.9.2.2) Preparation of charcoal solution

Activated charcoal (neutral, Sigma) was stirred in the assay buffer (50 mM Tris-HCl, 4 mM Na_2EDTA, pH 7.4 at room temperature) containing 2% BSA to give a charcoal concentration of 75 mg/ml.

2.9.2.3) Preparation of working 3H-cAMP solution

^3H-cAMP (specific activity: 23.6 uCi/mmole, Amersham) was diluted with assay buffer to give an 18 nM solution. This solution was freshly prepared before each experiment.

2.9.2.4) Preparation of cAMP standard

Stock cAMP solution (16 pmole/50 ul, Amersham) was diluted serially with assay buffer to give the following concentrations: 1, 2, 4, and 8 pmole/50 ul.

2.9.2.5) Assay protocol

a) The following aliquots (ul) in duplicate was added to 1.5 ml plastic microcentrifuge tubes:

TUBE	ASSAY BUFFER	STANDARD cAMP	SAMPLE
BLANK	150 ul	-	-
STANDARD ZERO	50 ul	-	-
STANDARD	-	50 ul	-
SAMPLES	-	-	50 ul

b) Fifty microlitres of working ^{3}H-cAMP solution was added to each tube.

c) One hundred microlitres of the cAMP binding protein solution was added to each tube except the blank.

d) After mixing, the tubes were kept at 40 for 2 h.

e) One hundred microlitres of a well-stirred charcoal suspension was added to each tube, mixed, and then centrifuged within 2 min, at 10000xg at 40 for 10 min.

f) Without disturbing the sediment, 200 ul of the supernatant were taken out for radioactivity measurement by liquid scintillatin counting in a home-made scintillation fluid (0.4 g POPOP, 4 g PPO, in 1 litre mixture of 666 ml toluene and 333 ml Triton X-100).

2.9.2.6) Data calculation

a) Specific binding of ^{3}H-cAMP was calculated by subtracting the value of blank.

b) The specific binding in the absence of unlabelled cAMP (standard zero) was designated as Co, while the specific binding in the presence of unlabelled cAMP from standard or sample was designated as Cx.

c) The ratio of Co/Cx was calculated for each tube.

d) A linear calibration curve was obtained by plotting Co/Cx against pmole of standard cAMP.

e) The amount of cAMP in the sample was read off from the standard curve using their Co/Cx values.

A typical calcibration curve for cAMP determination is shown in Fig. 2.5.

2.10) Radioligand Binding Analysis

A biological response is elicited by the occupancy of receptors with its specific agonist. In 'test tube' experiments, the association of receptors and its specific agonists can be demonstrated by using radioactive isotope labelled agonist as the tracer.

The binding of a radiolabelled ligand to its receptor is saturable and reversible. Kinetically, the binding reaction obeys the Law of Mass Action and will attend an equilibrium state after an appropriate period of incubation. After reaching equilibrium, the unbound radiolabelled ligand can be separated from the bound radiolabelled ligand by rapidly passing the reaction mixture through a glass microfibre filter which retain the membranes as well as the bound radiolabelled ligand (Bennett, 1978).

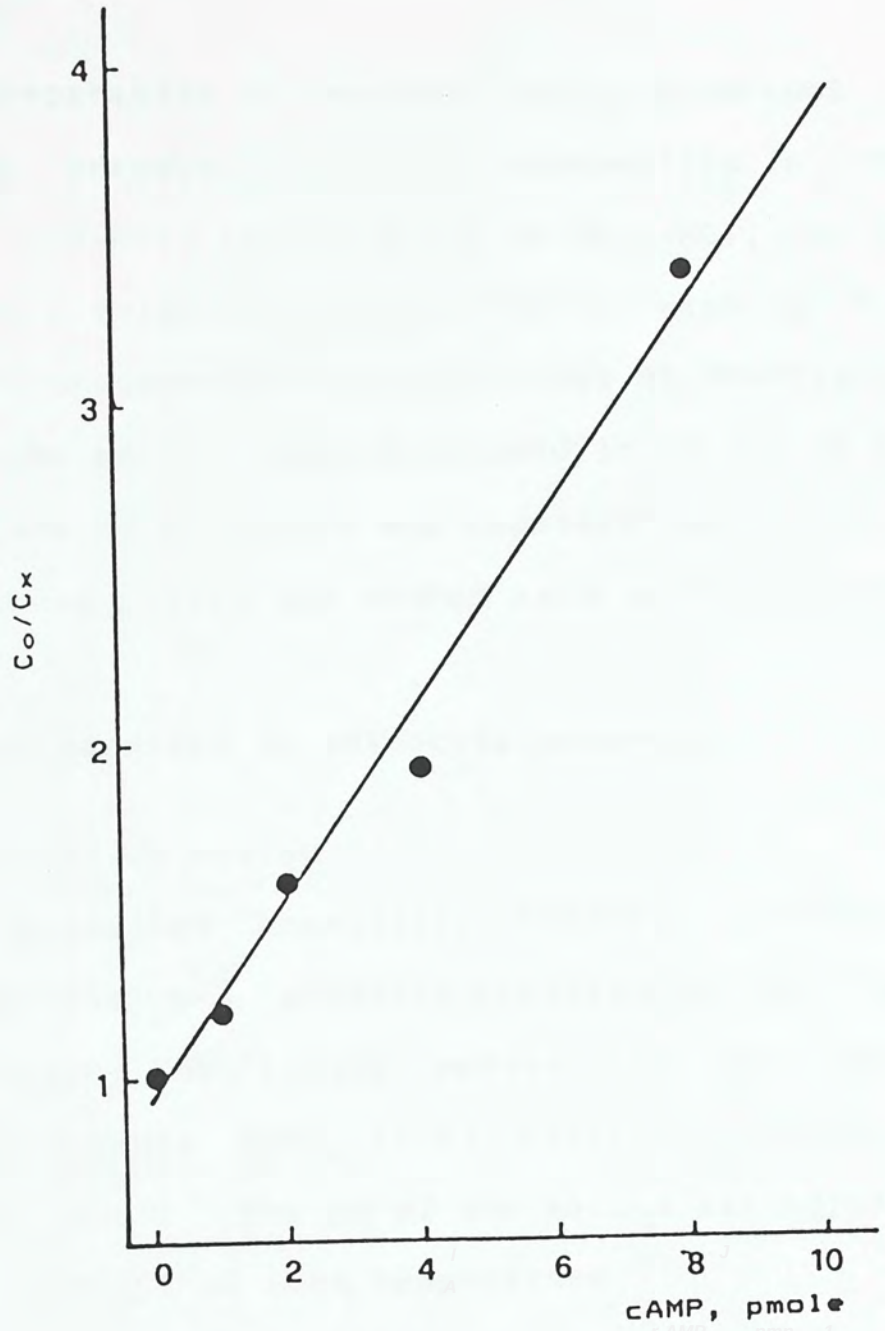


Fig. 2.5: A typical calibration curve for cAMP determination. Values presented are the mean of duplicated determinations from one typical experiment. The straight line was determined by linear regression analysis. Each experiment was accompanied by an individual calibration curve. Co and Cx are the binding of ^{3}H-cAMP in the absence and presence of unlabelled cAMP respectively.

2.10.1) Preparation of cerebral cortex membranes

Brain cerebral cortex was homogenized in 10 vol (v/w) of ice-cold buffer B (50 mM Tris-HCl, pH 7.4 at 25°) using a Brinkmann polytron PT-10 (setting 6, 10-15s). The homogenate was centrifuged at 45000xg for 10 min and the pellet was resuspended in 20 vol of buffer B. This washing procedure was repeated twice. The crude membrane preparation was stored as a pellet at -20°.

2.10.2) Preparation of adipocyte membranes

2.10.2.1) Lysing medium

As described previously (Rodbell, 1972), the lysing medium was prepared freshly on the day of experiment. The lysing medium had the following composition (mM): $MgCl_2$ (2.5), $CaCl_2$ (0.1) $KHCO_3$ (1.0) and Tris (2.0). The pH of the medium was adjusted to 7.4 with 1 M HCl at room temperature.

2.10.2.2) Protocol

a) Adipocytes were isolated as described in secton 2.8.2 and washed twice with 1% BSA-KRB.

b) Ice-cold lysing medium (10 ml) was added to the tube containing washed adipocytes, and the cells were dispersed by gentle swirlling.

c) The suspension was centrifuged at about 250xg in a table-top centrifuge for 30s and the infranatant fluid was aspirated off.

d) Another 10 ml of ice-cold lysing medium were added. Adipocytes were broken by inverting the tubes 20 times, using vigorous shaking motion at each downward stroke.

e) The tubes were centrifuge at about 1600xg for about 60s.

f) The cloudy infranatant was transfered to a 50 ml centrifuge tube with a Pasteur pipette.

g) Steps (d), (e) and (f) were repeated, and the infranatants were combined.

h) The combined infranatant was centrifuged at 45000xg for 15 min at 4°.

i) The supernatant was discard. The pellet was homogenized in 10 ml ice-cold buffer B with a Brinkmann polytron PT-10 (setting 6, 10-15s)

j) The homogenate was centrifuged at 45000xg for 15 min and the pellet was washed two more times in 10 ml ice-cold buffer B.

k) For radioligand receptor binding analysis, the pellet was resuspended in ice-cold buffer B to give a protein concentration of about 0.8 mg/ml.

2.10.3) Adenosine receptor binding

Similar to published methods (Lohse *et al*, 1984; Trost and Schwabe, 1981), binding experiment was initiated by adding 140 ul of adipocyte or cerebral cortex membranes in duplicate to 0.5-20 nM of ^{3}H-phenylisopropyladenosine (^{3}H-PIA, Amersham) at 37^{o} for 1 h in final volume of 200 ul containing 50 mM Tris-HCl (pH 7.4) and 0.5 U/ml adenosine deaminase. Incubation was terminated by filtration on glass fibre filter (Whatman GF/B) under suction. The filter was washed four times with 3 ml aliquots of ice-cold buffer B. Radioactivity retained on the filter was determined by liquid scintillation spectrometry. Non-specific binding was defined as binding in the presence 100 uM 2-chloroadenosine. Specific binding was calculated by subtracting non-specific binding from total binding.

2.10.4) Beta-adrenergic receptor binding

Binding assay was performed as described previously (Bylund and Snyder, 1976; Williams *et al*, 1976) with some modifications. Assay was initiated by adding 140 ul adipocyte or cerebral cortex membrane in duplicate to 0.3-80nM ^{3}H-dihydroalprenolol (^{3}H-DHA, Amersham) at 37^{o} for 30 min in a final volume 200 ul containing 50 mM Tris-HCl (pH 7.4). Incubation was terminated as described in section 2.10.3. Non-specific binding was defined as binding in the presence of

100 uM propranolol (adipocyte membrane) or 1 mM isoproterenol (cerebral cortex membrane). Specific binding was calculated by subtracting non-specific binding from total binding.

2.10.5) Binding data analysis - Scatchard plot

Radioligand binding to a single population of non-interacting site usually follows kinetics similar to those of classic enzyme-substrate interaction and obeys the Law of Mass Action. For a reversible ligand-receptor interaction:

(R) = concentration of unoccupied receptor sites

(L) = concentration of free ligand

(RL) = concentration of receptor-ligand complex

K_D = dissociation constant

$$(R) + (L) \rightleftharpoons (RL)$$

at equilibrium:

$$K_D = \frac{(R)\,(L)}{(RL)} \qquad (2.9)$$

Since only a finite number of specific receptor sites exist per unit tissue, the ligand-receptor interaction is saturable. The maximum number of specific receptor sites is usually designated as Bmax, where

$$(R) + (RL) = Bmax \qquad (2.10)$$

$$\text{therefore} \qquad K_D = \frac{(B_{max}-RL)\,(L)}{(RL)}$$

$$(RL) = \frac{B_{max}\,(L)}{(L) + K_D}$$

let (RL) = bound ligand = B

(L) = free ligand = F

$$\text{then} \qquad B = \frac{B_{max}\,F}{K_D + F}$$

$$\frac{B}{F} = \frac{B_{max} - B}{K_D} \qquad (2.11)$$

According to the Scatchard equation (2.11), if one plots B/F vs B, a striaght line can be obtained which allows the determination of both the dissociation constant (K_D = -1/slope) and the number of binding sites (Bmax = X-axis intercept).

2.11) Protein Determination

Protein was determined as described by Lowry *et al* (1951) with bovine serum albumin (Fraction 5, 98-99% purity, Sigma) as the protein standard. A typical standard curve for protein determination is shown in Fig. 2.6.

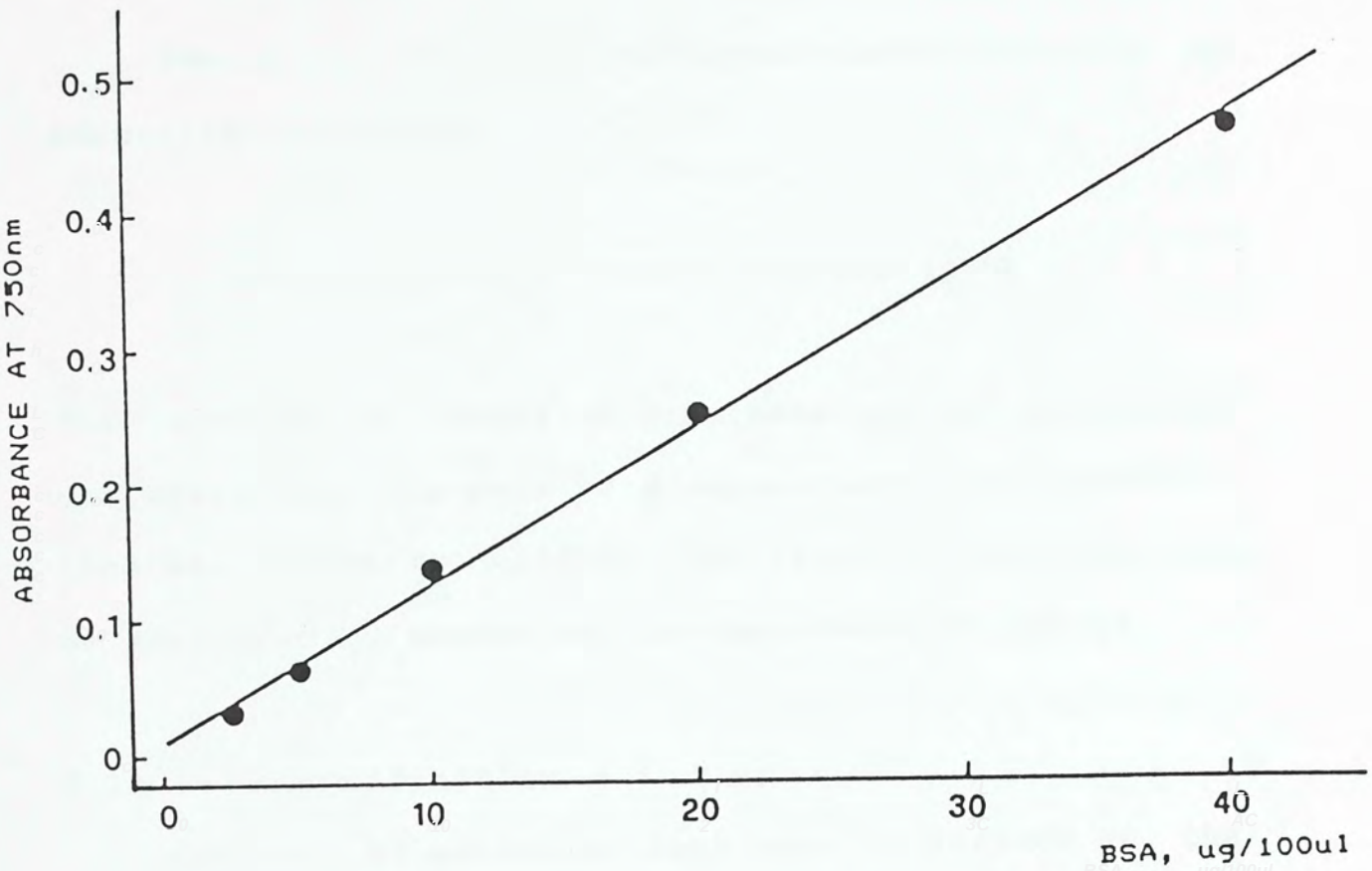


Fig. 2.6: A typical calibration curve for protein determination using bovine serum albumin (BSA) as standard. Values presented are the mean of duplicated determinations from one typical experiment. The straight line was determined by linear regression analysis. Each experiment has an individual calibration curve performed in parallel.

2.12) Measurement of Adenosine Deaminase Activity

Adenosine deaminase catalyzed the deamination of adenosine to inosine:

$$\text{Adenosine} + H_2O \longrightarrow \text{inosine} + NH_3$$

The activity of adenosine deaminase can be determined by measuring the rate of disappearance of adenosine (Agarwal and Parks, 1978). The level of adenosine can be monitored by measuring its absorbance at 265 nm.

2.12.1) Unit definition

One unit of adenosine deaminase is defined as the amount of enzyme that catalyzed the deamination of 1 umole of adenosine at pH 7.5 per min at 250.

2.12.2) Preparation of glycerol free adenosine deaminase

Glycerol free adenosine deaminase (from calf intestinal, in 50% glycerol-10 mM potassium phosphate, Sigma) was prepared by dialysing against KRB for 24 h with three changes of 1 litre of buffer before used in lipolytic assay.

2.12.3) Assay protocol

a) Adenosine solution was prepared freshly in KRB (section 2.8.1) at the following concentrations: 100, 50, 10, and 5 uM.

b) An aliquot (5 ul) of a properly diluted dialysed adenosine deaminase was added to a sample cuvette containing 1 ml of 5 uM adenosine solution. The reaction mixture was mixed by inverting the cuvette with its top on for 3-4 times.

c) By using 5 uM adenosine solution as the reference blank, The rate of adenosine disappearance was recorded by monitoring the decrease in absorbance at 265 nm (slit width 1 nm) with a pen recorder.

d) Step (b) and (c) were repeated for 10, 50, and 100 uM of adenosine.

2.12.4) Calculation

Since $$A = e\ C\ l$$

$$\Delta A = e\ l\ \Delta C$$

$$\frac{\Delta A}{\Delta t} = e\ l\ \frac{\Delta C}{\Delta t}$$

Therefore

$$V = \frac{\Delta C}{\Delta t} = \frac{\frac{\Delta A}{\Delta t}}{e\ l} = \frac{\frac{\Delta A}{\Delta t}}{8.1} \qquad (2.12)$$

where:

V = enzyme activity (umole adenosine/min)

l = light path = 1 cm

e = molar extinction coefficient of adenosine = 8.1

c = concentration of adenosine

A = absorbance at 265 nm

t = time interval (min)

The initial velocities of the enzyme reaction at different adenosine concentrations were calculated according to equation 2.12. A double reciprocal plot was obtained by plotting 1/V vs 1/C, and the maximal velocity of adenosine deaminase (Vmax) was determined as the Y-axis intercept from the double reciprocal plot. A typical double-reciprocal plot from one experiment is shown in Fig. 2.7.

2.13) Statistical methods

Comparisons of data were performed using the following standard statistical methods: correlation analysis, linear regression analysis, and Students' t-test (Phillips, 1978).

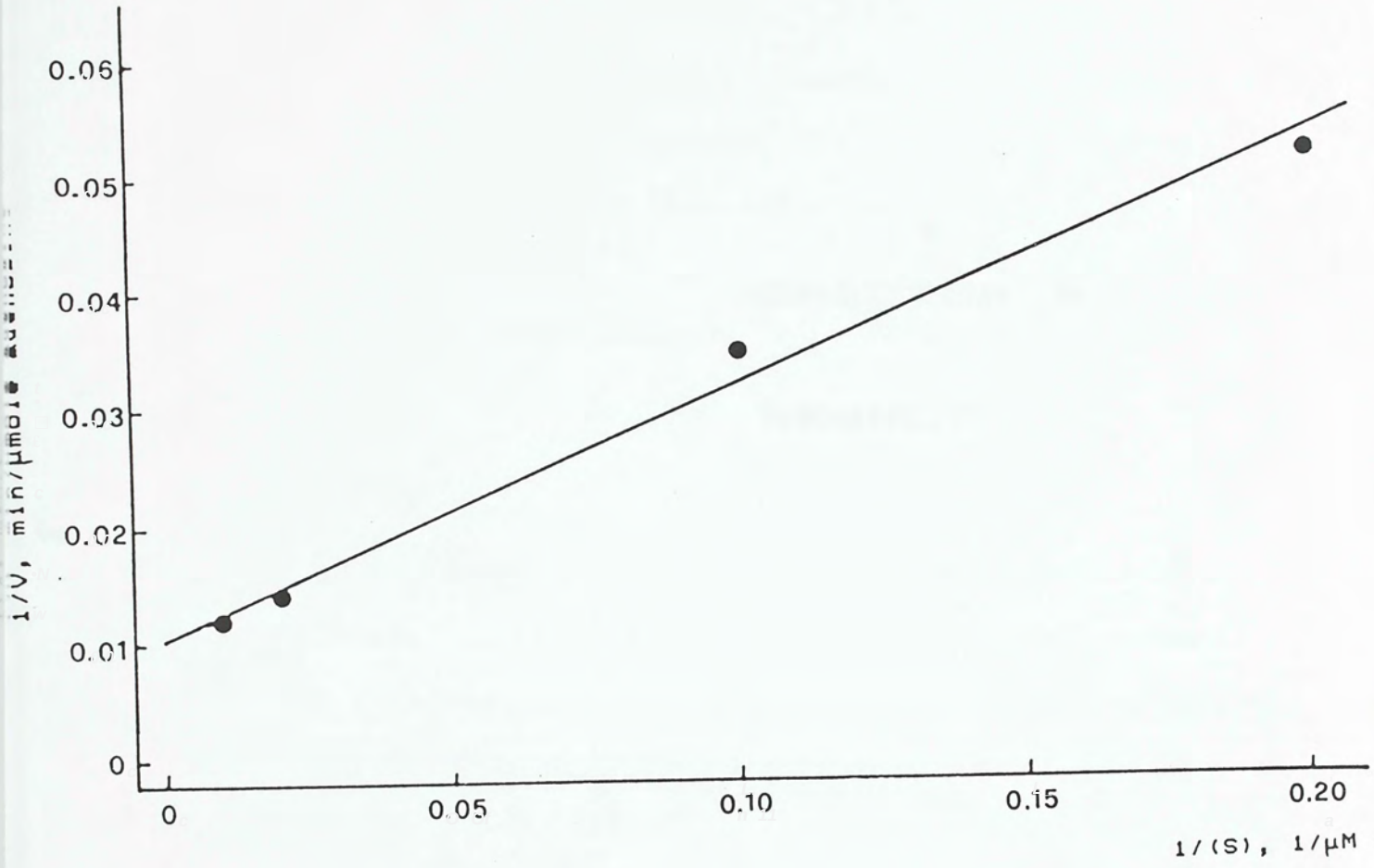


Fig. 2.7: A double reciprocal plot for adenosine deaminase. Data presented were that of one typical experiment. The straight line was determined by linear regression analysis.

CHAPTER 3

RESULT

In preliminary dose-response (0.05%, 0.1% and 0.2%) and time course (3 weeks, 6 weeks, and 9 weeks) experiments, significant increase in the anti-lipolytic potency of 2-chloroadenosine was observed after 6 weeks 0.2% caffeine treatment (Section 3.3). Therefore, this paradigm was adopted for in-depth evaluation.

3.1) Morphological Aspects of Chronic Caffeine Treatment

3.1.1) Body weight

After 6 weeks of caffeine (0.2%) treatment, the body weights of rats were decreased by about 6% when compared with the controls (Table 3.1). Furthermore, the caffeine-treated rats displayed a narrower body weight distribution than the controls (Fig. 3.1).

3.1.2) Epididymal fat pad weight

There was a linear correlation between the body weight and the epididymal fat pad weight (Fig. 3.1 A,B). However, when compared with the body weight, a larger decrease in the epididymal fat pad weight (30% against control) was observed among the caffeine-treated rats (Table 3.1). As a consequence, an 18% decrease in the weight proportion of epididymal fat pad to body weight was obtained in the caffeine-treated rats (Table 3.1). Furthermore, similar to the body

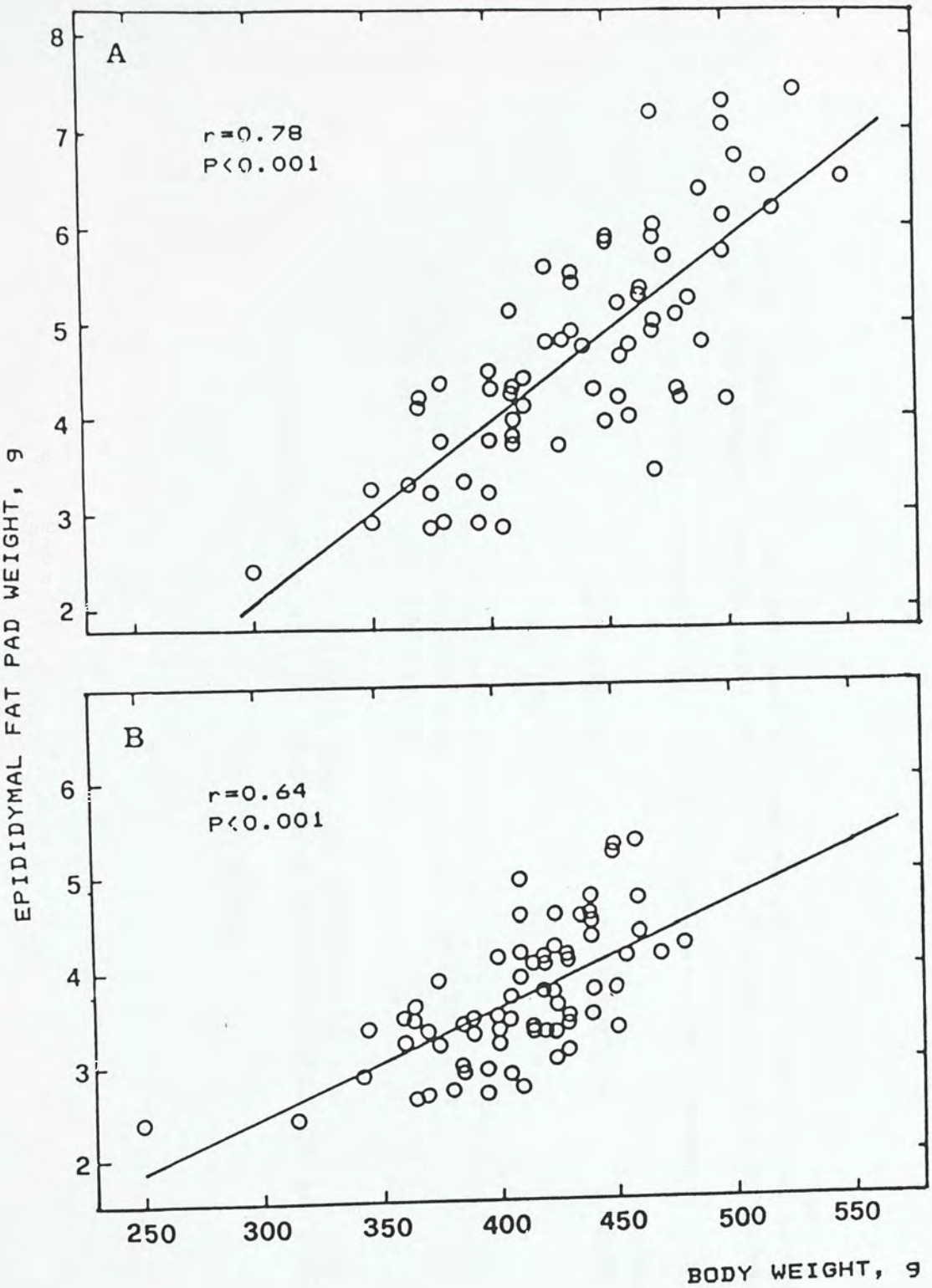


Fig. 3.1: Scatterplot of body weight vs epididymal fat pad weight of (A) control and (B) 0.2% caffeine-treated (6 weeks) rats. The correlation coefficients (r) were determined by linear regression analysis.

TABLE 3.1

Effect of chronic caffeine treatment on rat body weight and epididymal fat pad weight

RAT	BODY WEIGHT (g)	EPIDIDYMAL FAT PAD WEIGHT (g)	WEIGHT PROPORTION
CONTROL	438.93±49.32	4.7±1.20	0.011
CAFFEINE-TREATED (0.2%, 6 weeks)	411.82±36.67*	3.65±0.67*	0.009

Values presented are the mean ± S.D. of 68 rats Weight proportion are the ratio of epididymal fat pad weight to body weight * P<0.0005 with Students' t-test.

weight, the caffeine-treated rats also displayed a narrower epididymal fat pad weight distribution than the controls (Fig. 3.1 A,B)

3.1.3) Mean cell size of adipocytes

After chronic caffeine treatment, there was no apparent difference in the morphology of adipocytes (Fig 3.2 A,B), but the cell diameter and cell volume of adipocytes from 0.2% caffeine-treated rats were about 10% and 30% respectively smaller than that of the controls (Table 3.2).

3.1.4) Mean cell weight of adipocytes

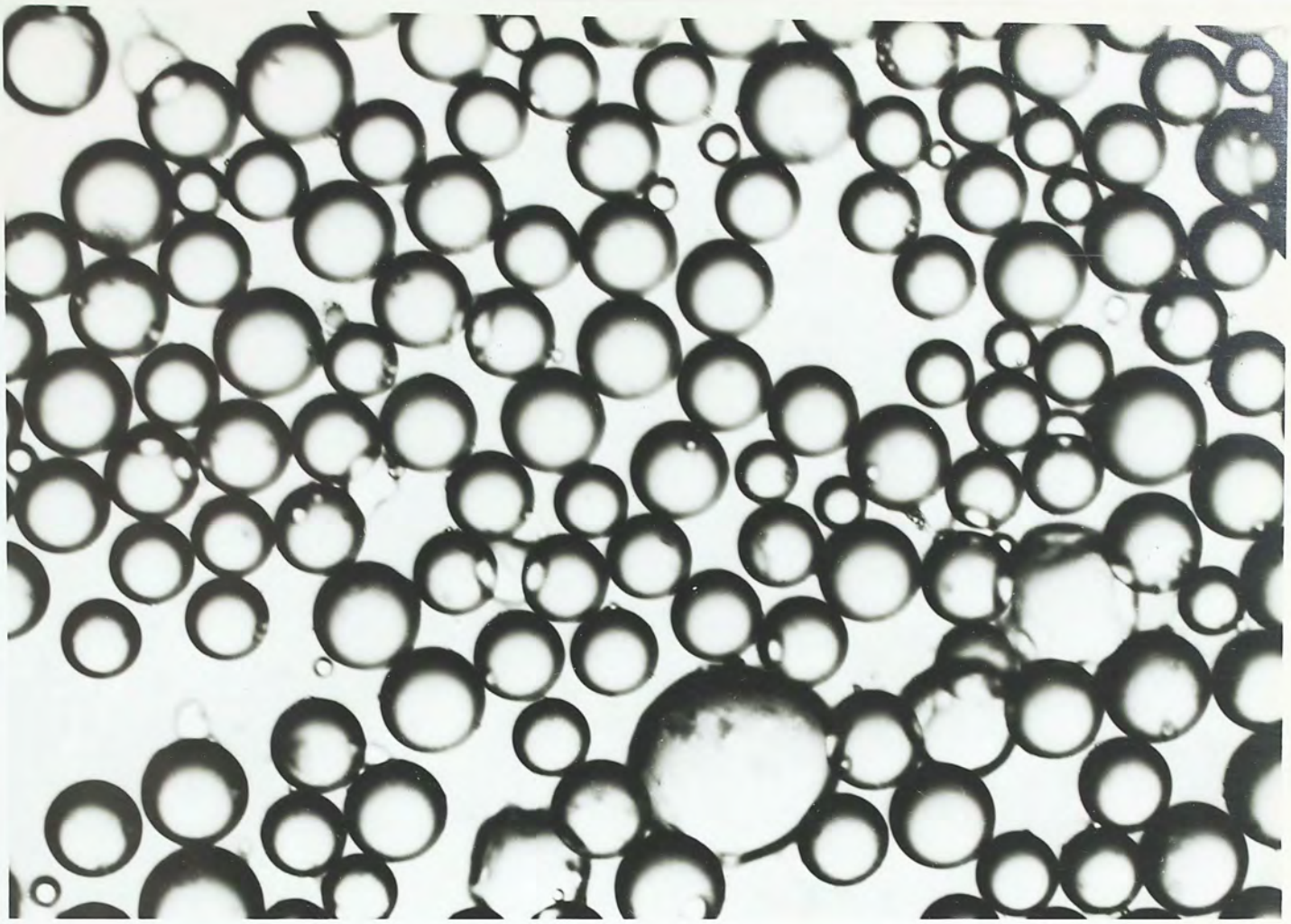
There was no statistically significant difference in the mean cell weight of adipocytes between the control and the caffeine-treated rats (Table 3.2).

3.1.5) Triglyceride content of adipocytes

No statistically significant difference in the triglyceride content was found between the control and the caffeine-treated (0.2%) rats expressed either in term of per mg cell weight or per 10^5 cell number (Table 3.3)

Fig. 3.2: Photomicrographs of free fat cells of (A) control and (B) 0.2% caffeine-treated (6 weeks) rats. The adipocytes were obtained from collagenase-treated rat epididymal adipose tissue.

A



B

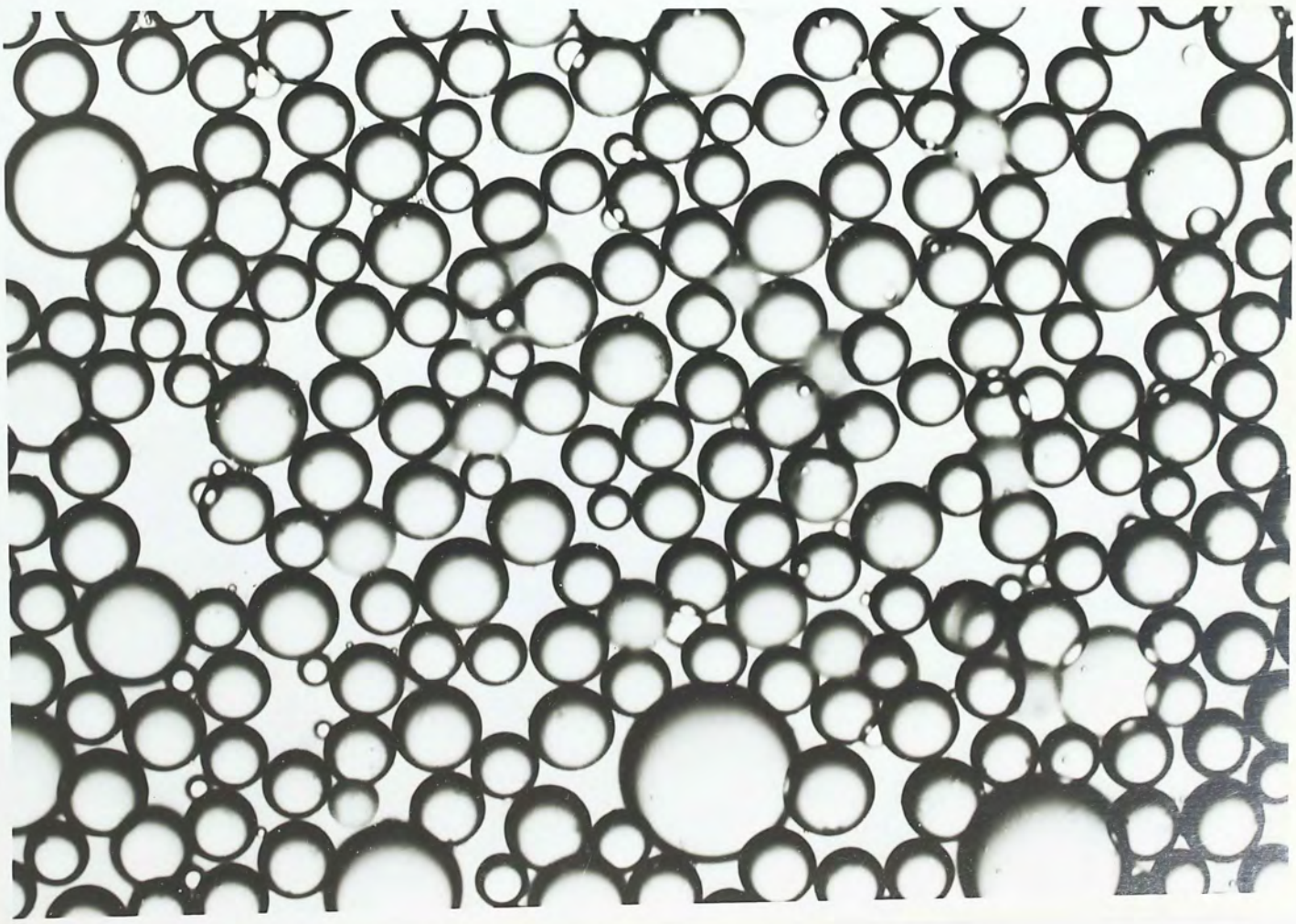


TABLE 3.2

Effect of chronic caffeine treatment on cell weight and cell size of adipocytes

RAT	CELL WEIGHT (mg/10^5 cells)	CELL DIAMETER (um)	CELL VOLUMN (um^3)
CONTROL	22.67	8.63±4.96	339,870
CAFFEINE-TREATED (0.2%, 6 weeks)	24.25*	7.26±5.41**	245,022

Values presented are the mean ± S.D. of 4 rats. * No statistical significant difference ** $P<0.05$ when compare with controls using Students' t-test.

TABLE 3.3

Effect of chronic caffeine treatment on triglyceride content of adipocytes

RAT	TRIGLYCERIDE CONTENT	
	umole/10^5 cells	umole/mg cells
CONTROL	19.61±6.54	1.23±0.23
CAFFEINE-TREATED (0.2%, 6 weeks)	17.91±1.98	1.28±0.15

Values presented are the mean ± S.D. of four rats. There is no significant difference between control and caffeine treated rats when tested by Students' t-test.

3.2) Some Characteristics of Adipocytes Related to Lipolysis

3.2.1) Dependence of glycerol release on cell concentration

In the presence of either adenosine deaminase (1 U/ml) or isoproterenol (300 nM), there was a positive linear correlation between the amount of glycerol released and cell concentration. This was true whether the cell concentration was expressed in terms of cell number or cell weight (Fig. 3.3 and Fig. 3.4) Since the amount of glycerol released correlated better with cell weight and the determination of cell weight was more objective, lipolytic rates were expressed as glycerol release per g cell weight per unit time.

3.2.2) Characteristics of the adenosine receptors on adipocytes

In initial experiments, it was observed that the methylxanthines including caffeine and DPX can stimulate the release of glycerol from adipocytes (Figure 3.5). This suggested that adenosine may normally exert an inhibitory effect on lipolysis. Indeed, lowering endogenous adenosine by adenosine deaminase (1 U/ml) enhanced the lipolytic potency of both caffeine and DPX (Fig. 3.5). Furthermore,

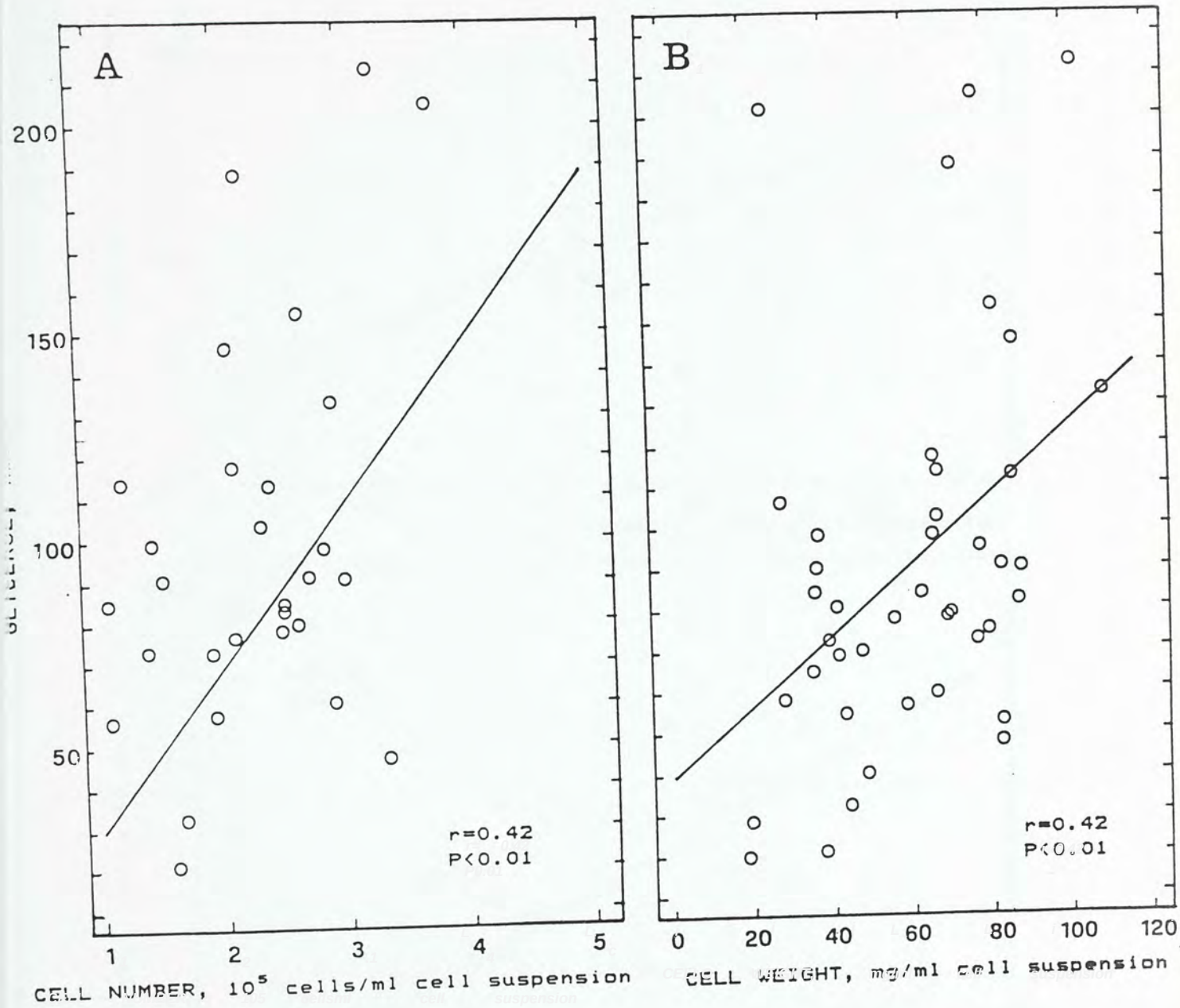


Fig. 3.3: Scatterplot of (A) cell number, or (B) cell weight vs glycerol release stimulated by lowering endogenous adenosine with 1 U/ml adenosine deaminase. The correlatrion coefficients (r) were determined by linear regression analysis.

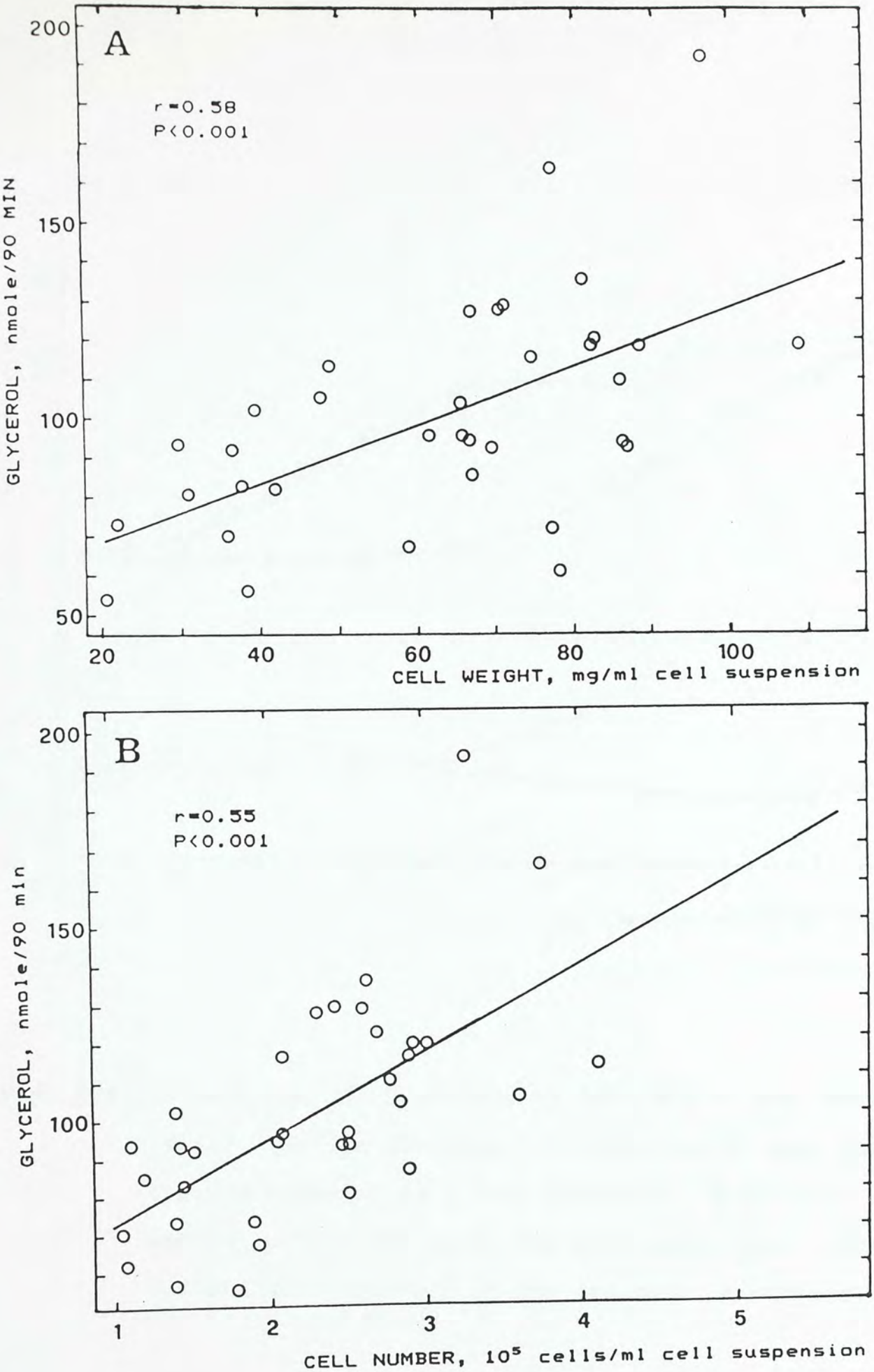


Fig. 3.4: Scatterplot of (A) cell number, or (B) cell weight vs glycerol release stimulated by 300 nM isoproterenol. The correlation coefficient (r) were determined by linear regression analysis.

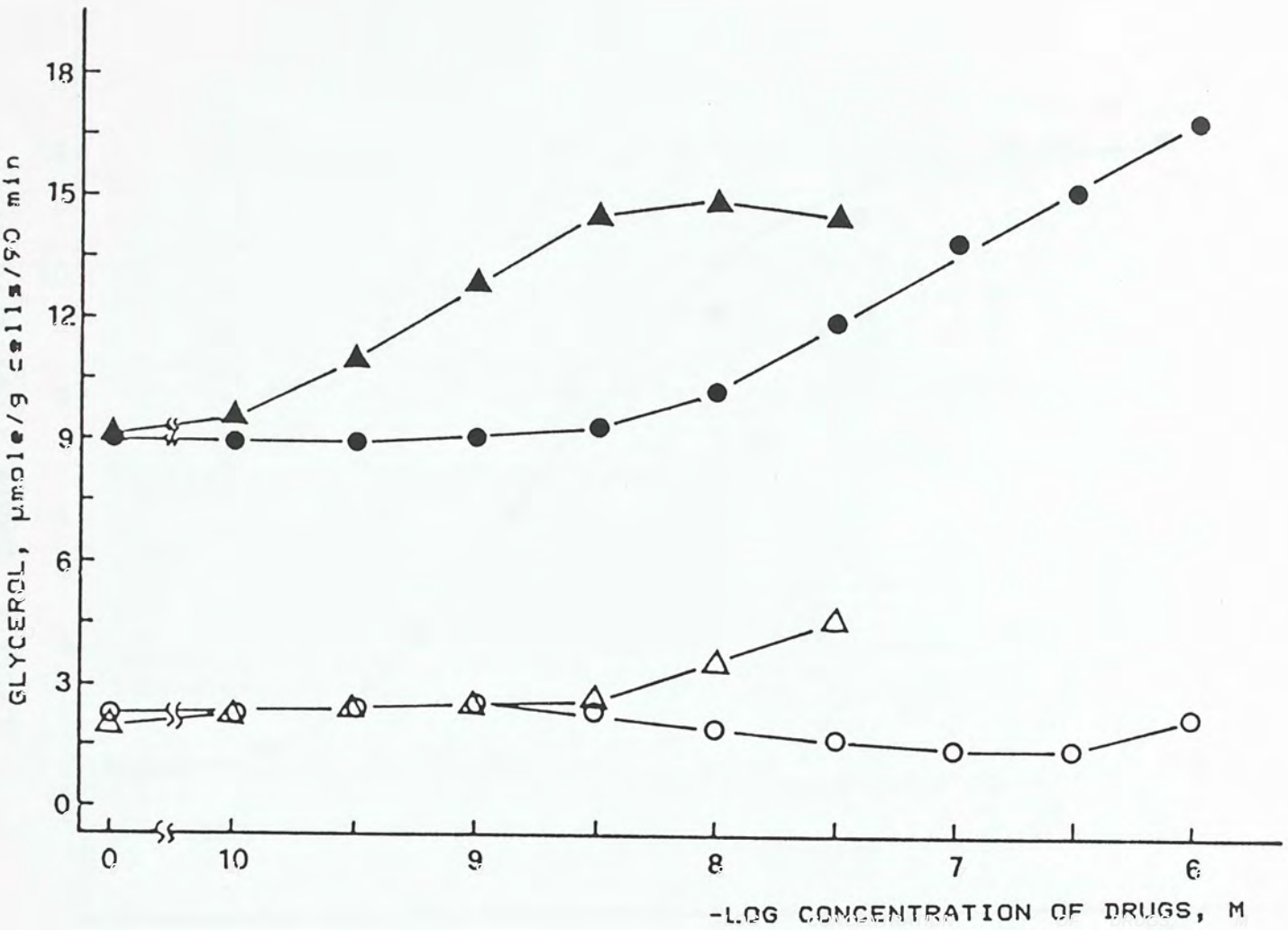


Fig. 3.5: Stimulation of lipolysis by DPX (▲△) and caffeine (●○) in the presence (closed symbols) and absence (opened symbols) of 1 U/ml adenosine deaminase. Data showed are that of a typical experiment. Similar results were obtained in two separate experiments.

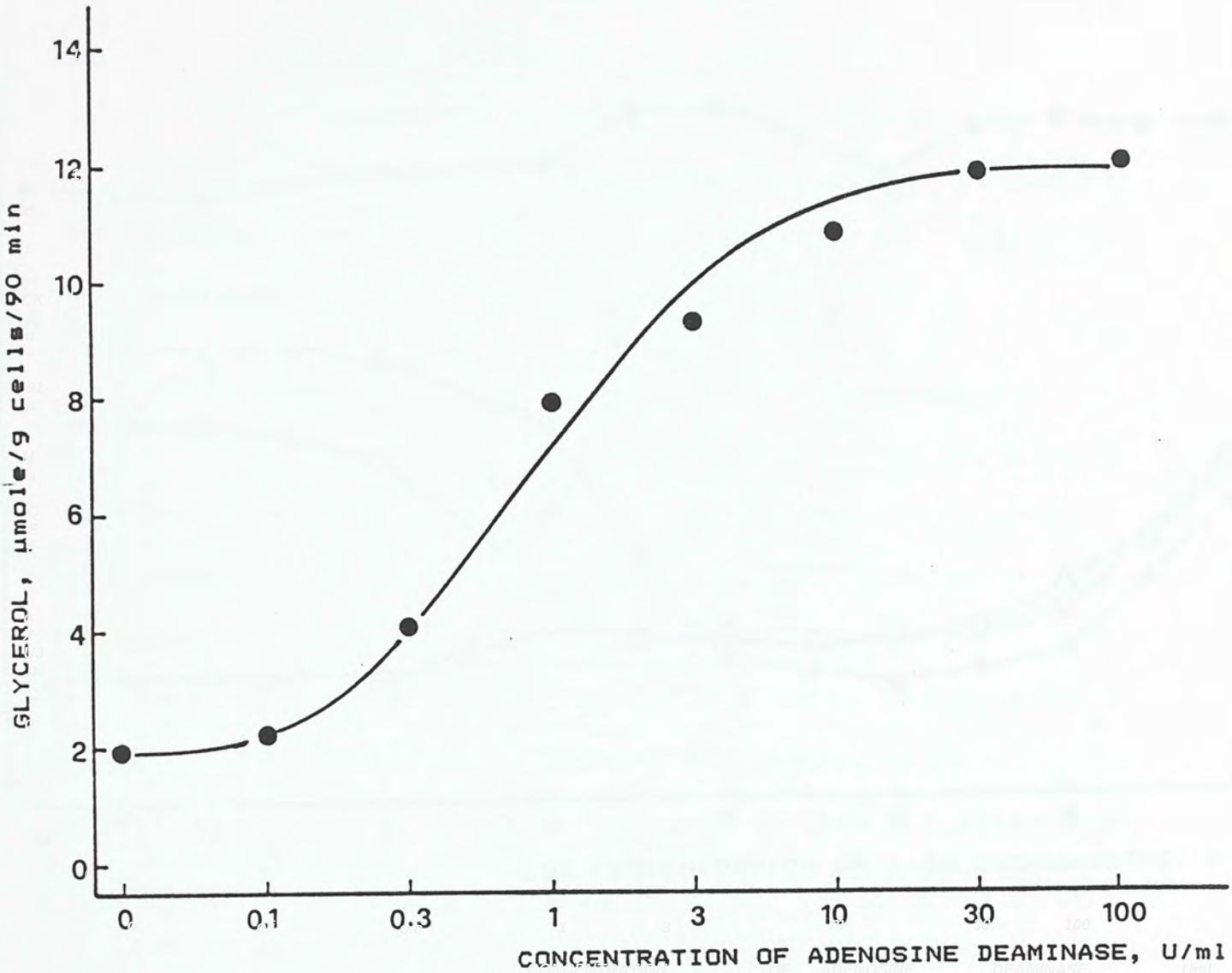


Fig. 3.6: Dose dependence of adenosine deaminase in the stimulation of lipolysis. Data showed are that of a typical experiment. Similar results were obtained in two separated experiments.

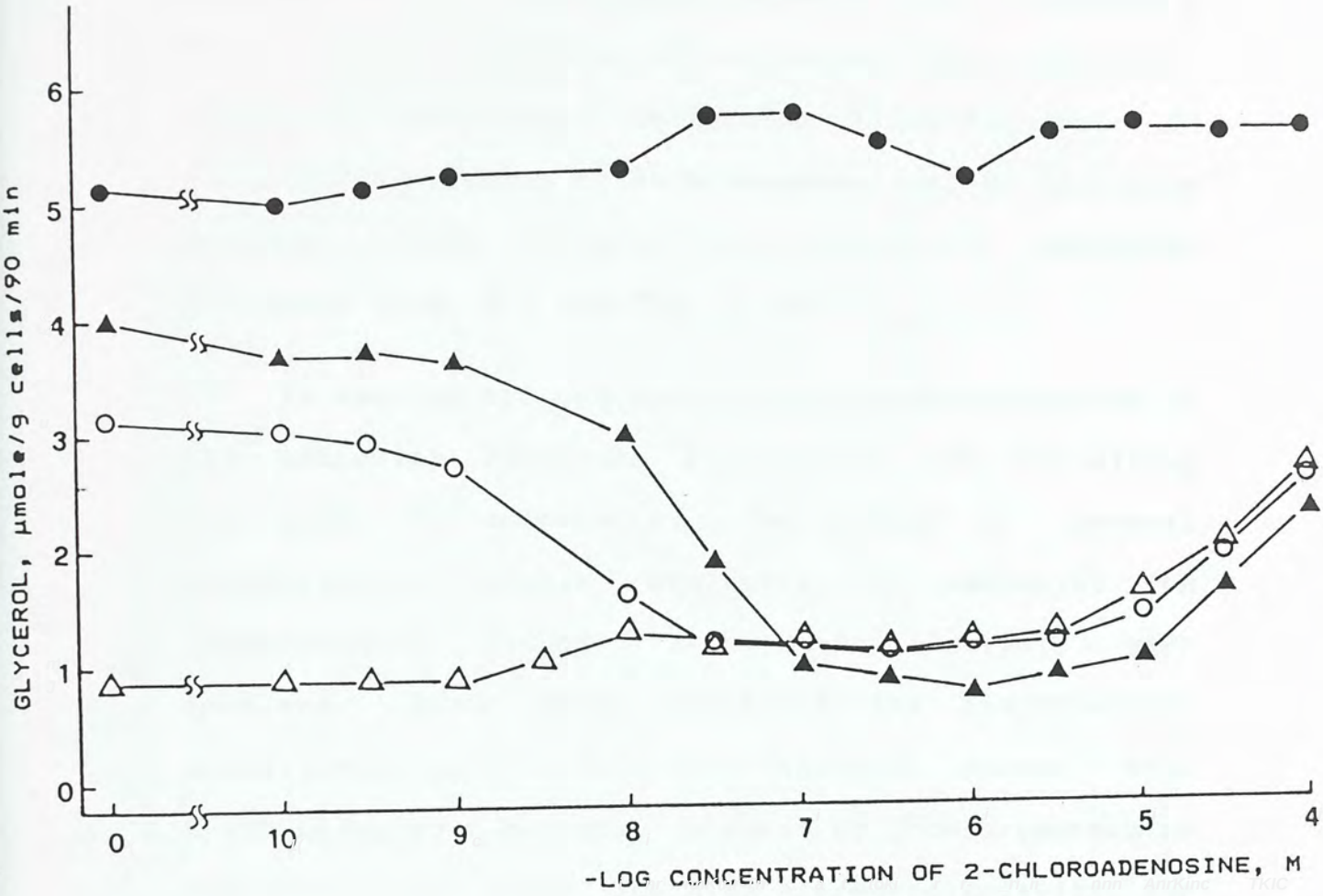


Fig. 3.7: Effect of 2-chloroadenosine on glycerol release of basal (△), or stimulated by 1 U/ml adenosine deaminase (○); 10 nM isoproterenol + 1 U/ml adenosine deaminase (▲); 1 uM isoproterenol + 1 U/ml adenosine deaminase (●). Data showed are that of a typical experiment. Similar results were obtained in two separate experiments.

lipolysis in adipocytes can be induced by adenosine deaminase in a dose-dependent manner, and it reached a plateau at about 30 U/ml of the enzyme (Fig. 3.6). This adenosine deaminase stimulated lipolysis can be inhibited completely by 2-chloroadenosine, an adenosine analogue which resisted the action of adenosine deaminase (Fig. 3.7 and Fig. 3.13).

To examine the pharmacololgical characteristics of the adenosine receptors responsible for inhibiting lipolysis in adipocytes, the effect of several metabolically stable analogues of adenosine on isoproterenol (10 nM) stimulated lipolysis were examined. These drugs inhibited the isoproterenol stimulated lipolysis in a dose-dependent manner, with L-PIA being most potent, followed by 2-chloroadenosine and D-PIA (Fig. 3.8). The high stereo-selectivity for L-PIA vs D-PIA (approximate 100 fold), conformed with the pattern suggested for an A1 adenosine receptors mediated response.

3.2.3) Characteristics of the adrenergic receptors on adipocytes

Norepinephrine (NE) is well-known as a lipolytic agent. It was, however, ten fold less potent in promoting the release of glycerol from adipocytes than isoproterenol (Fig. 3.9). Isoproterenol is a selective beta-adrenergic agonist, and a higher lipolytic potency

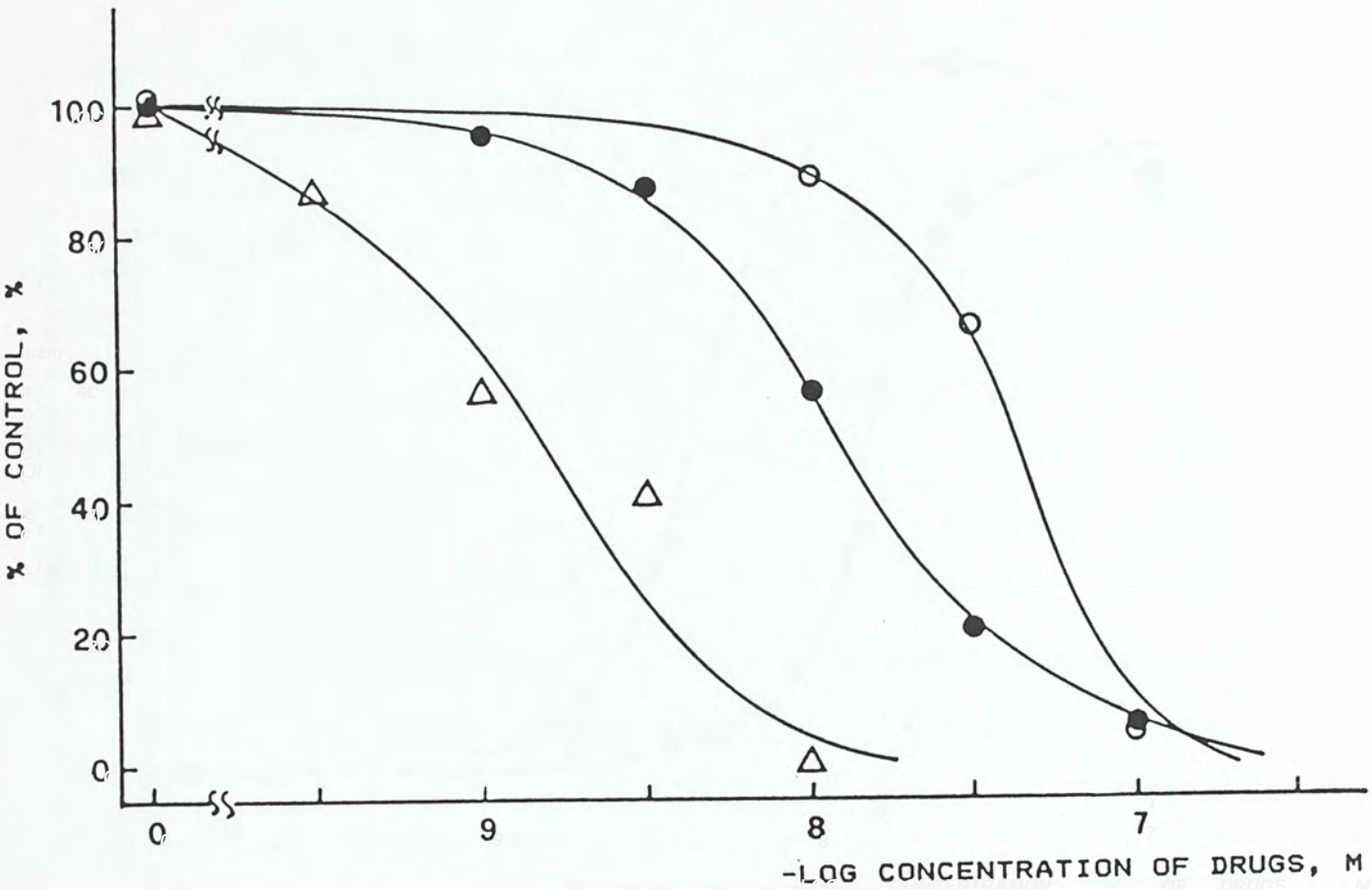


Fig. 3.8: Inhibition of 10 nM isoproterenol stimulated lipolysis in the presence 1 U/ml adenosine deaminase by L-PIA (△), 2-chloroadenosine (●) and D-PIA (○).

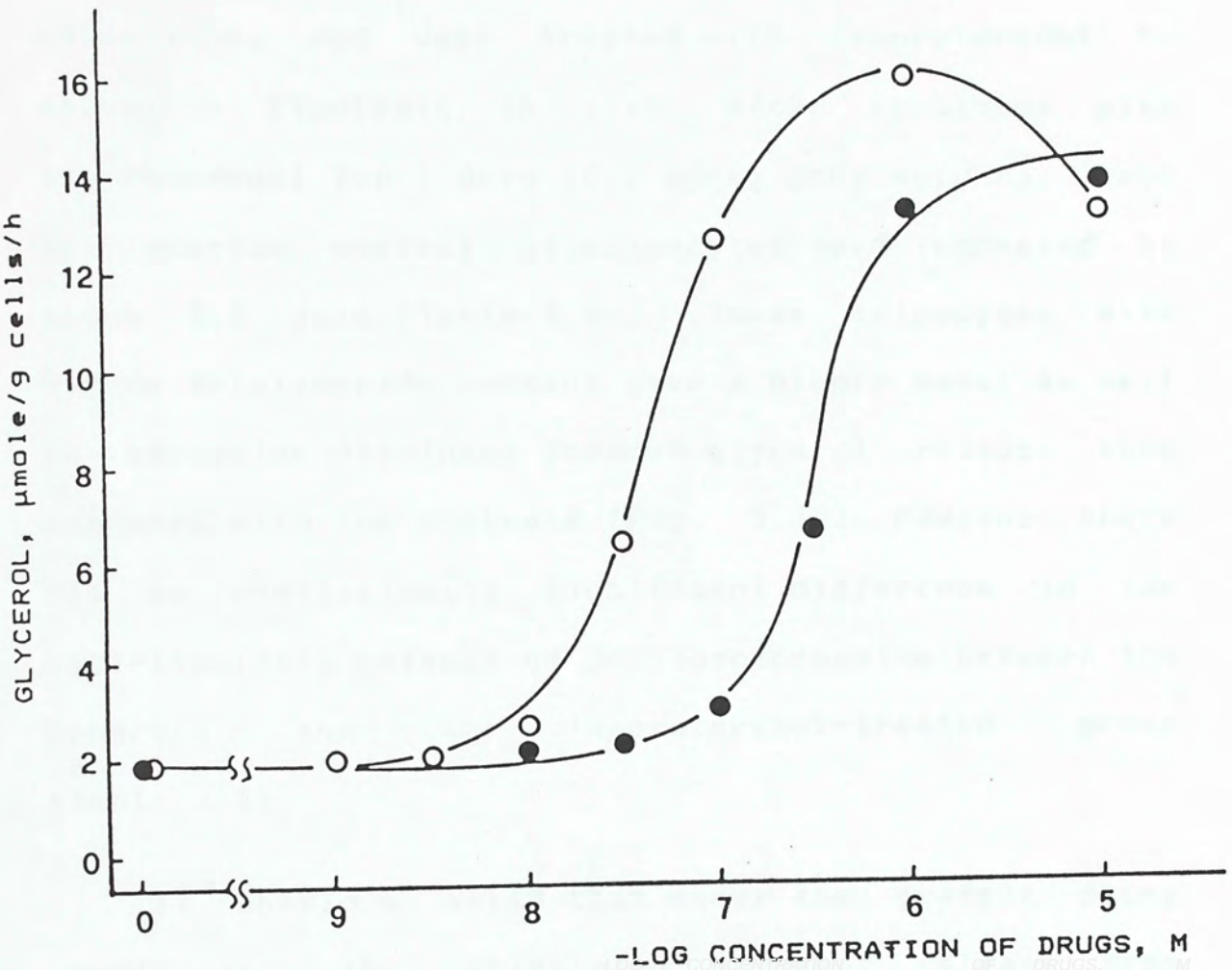


Fig. 3.9: Dose dependence of isoproterenol (○) and norepinephrine (●) in the stimulation of lipolysis

3.2.4) Effect of triglyceride content on lipolysis

To investigate whether the amount of glycerol released is affected by the triglyceride content of adipocytes, rat were treated with isoproterenol to stimulate lipolysis *in vivo*. After treatment with isoproterenol for 6 days (0.2 mg/kg body weight), cell triglyceride content of adipocytes were increased by about 2.5 fold (Table 3.4). These adipocytes with higher triglyceride content gave a higher basal as well as adenosine deaminase induced glycerol release when compared with the controls (Fig. 3.12). However, there was no statistically significant difference in the anti-lipolytic potency of 2-chloroadenosine between the controls and the isoproterenol-treated group (Table 3.5).

It should be noted that under the present assay conditions, the stimulated glycerol release from adipocytes was linear up to 90 min when induced with 3 U/ml adenosine deaminase (Fig. 3.13). It is also linear up to 75 min when stimulated with 1uM isoproterenol in the presence of 1U/ml adenosine deaminase (Fig. 3.14). Thus, to avoid any interference as a result of depletion of triglyceride content in adipocytes, the rate of lipolysis was measured over a period of 30 min to 90 min only.

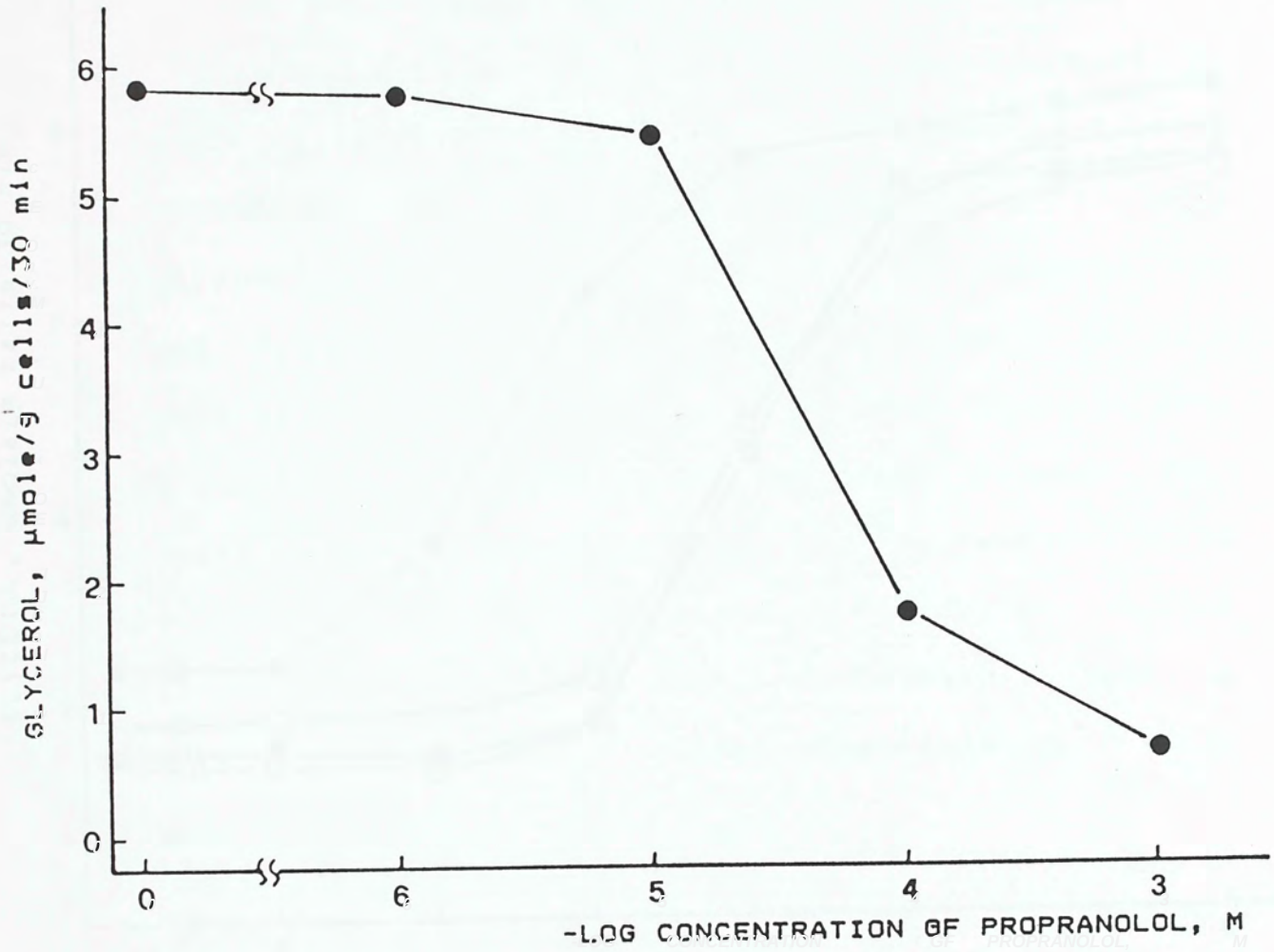


Fig. 3.10: Dose dependence of propranolol in the inhibition of lipolysis stimulated by 10 uM isoproterenol.

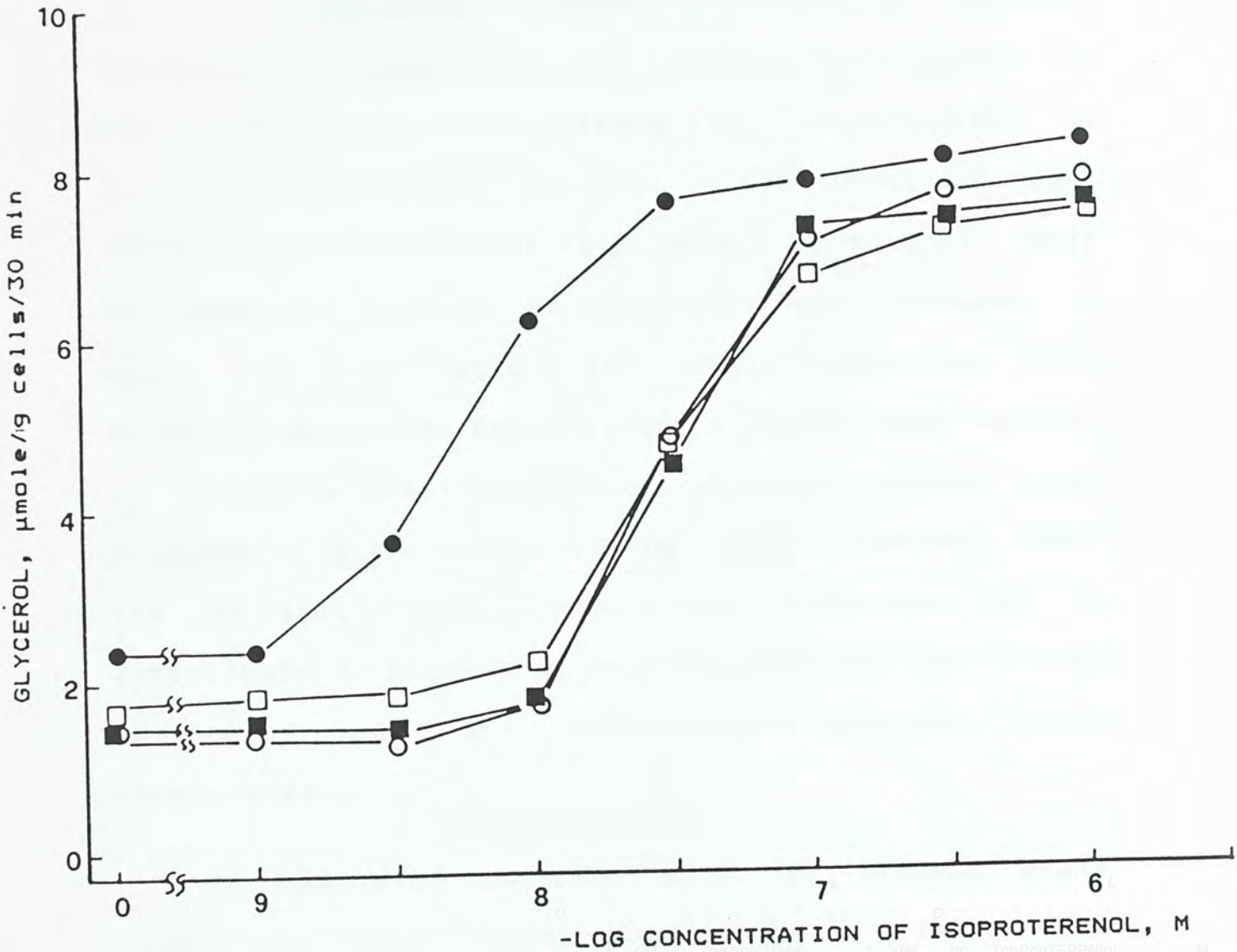


Fig. 3.11: Dose dependence of isoproterenol in the stimulation of lipolysis in control (■); 1 U/ml 2-chloroadenosine (□); 1 U/ml adenosine deaminase (●); 1 U/ml adenosine deaminase + 1 uM 2-chloroadenosine (○). Data showed are that of a typical experiment. Similar results were obtained in three separate experiment.

3.2.4) Effect of triglyceride content on lipolysis

To investigate whether the amount of glycerol released is affected by the triglyceride content of adipocytes, rat were treated with isoproterenol to stimulate lipolysis *in vivo*. After treatment with isoproterenol for 6 days (0.2 mg/kg body weight), cell triglyceride content of adipocytes were increased by about 2.5 fold (Table 3.4). These adipocytes with higher triglyceride content gave a higher basal as well as adenosine deaminase induced glycerol release when compared with the controls (Fig. 3.12). However, there was no statistically significant difference in the anti-lipolytic potency of 2-chloroadenosine between the controls and the isoproterenol-treated group (Table 3.5).

It should be noted that under the present assay conditions, the stimulated glycerol release from adipocytes was linear up to 90 min when induced with 3 U/ml adenosine deaminase (Fig. 3.13). It is also linear up to 75 min when stimulated with 1uM isoproterenol in the presence of 1U/ml adenosine deaminase (Fig. 3.14). Thus, to avoid andy interference as a result of depletion of triglyceride content in adipocytes, the rate of lipolysis was measured over a period of 30 min to 90 min only.

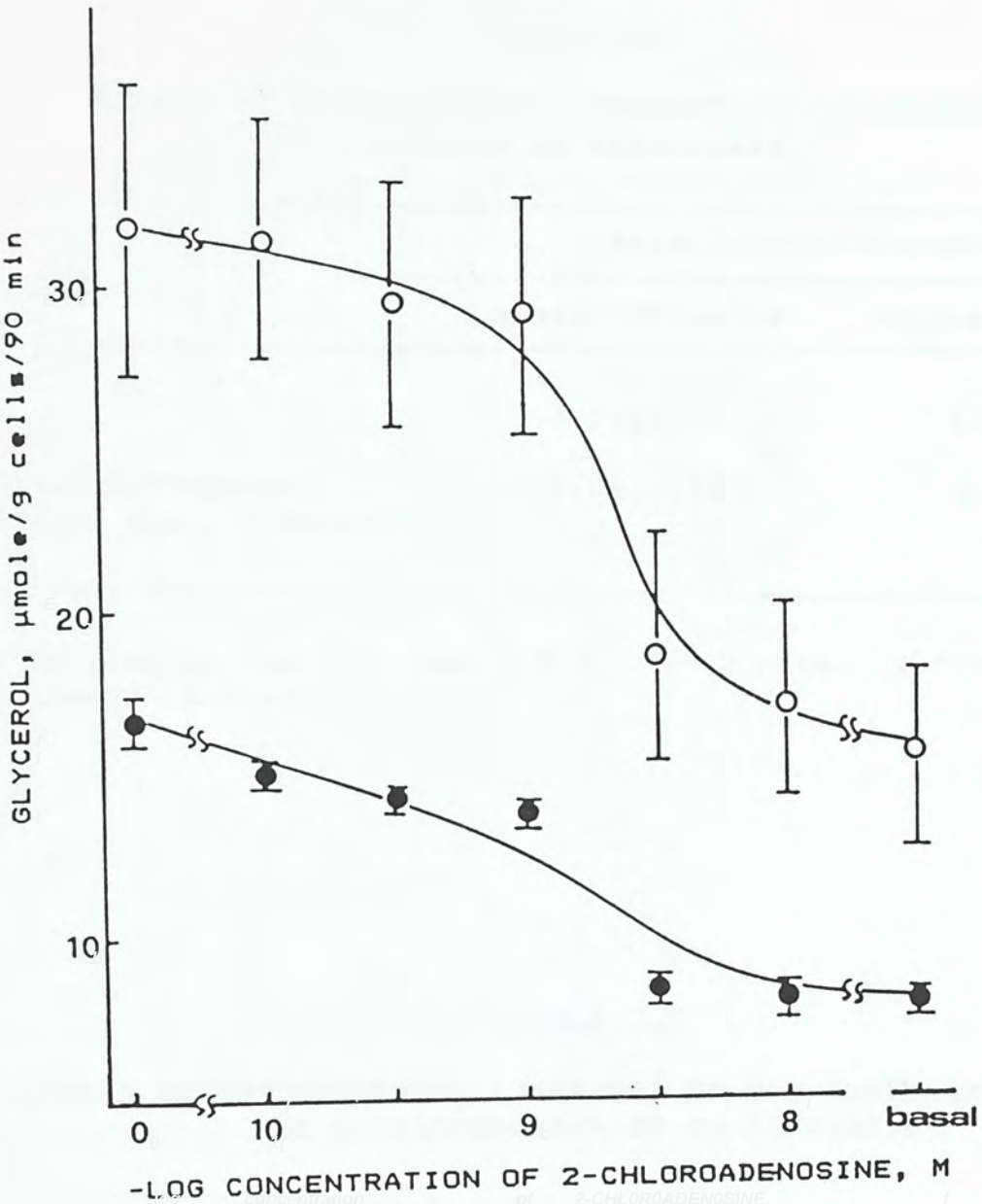


Fig. 3.12: Dose dependence of 2-chloroadenosine in the inhibition of lipolysis stimulated by 1 U/ml adenosine deaminase in control (●) and isoproterenol-treated (0.2 mg/kg body weight/12 h for 6 days) (○) rats. Data showed are mean ± S.E.M. of 3 rats.

TABLE 3.4

Effect of isoproterenol treatment on triglyceride content of adipocytes

RAT	TRIGLYCERIDE CONTENT	
	umole/10^5 cells	umole/mg cells
CONTROL	4.76±0.62	0.50±0.10
ISOPROTERENOL-TREATED (0.2 mg/kg, s.c., 6 days)	11.06±3.15 *	1.29±0.10**

Values presented are the mean ± S.D. of 3 rats. * P<0.05, ** P<0.005 with Students' t-test.

TABLE 3.5

Effect of isoproterenol treatment on the inhibitory potency of 2-chloroadenosine on lipolysis

RAT	IC_{50} (nM)
CONTROL	0.71±0.41
ISOPROTERENOL-TREATED (0.2 mg/kg, s.c., 6 days)	1.63±0.49

IC_{50} is the concentration of 2-chloroadenosine required to inhibite 50% lipolysis stimulated by 1 U/ml adenosine deaminase. Values presented are the mean ± S.D. of 3 rats. There is no significant difference between control and isoproterenol-treated rats when tested by Students' t-test.

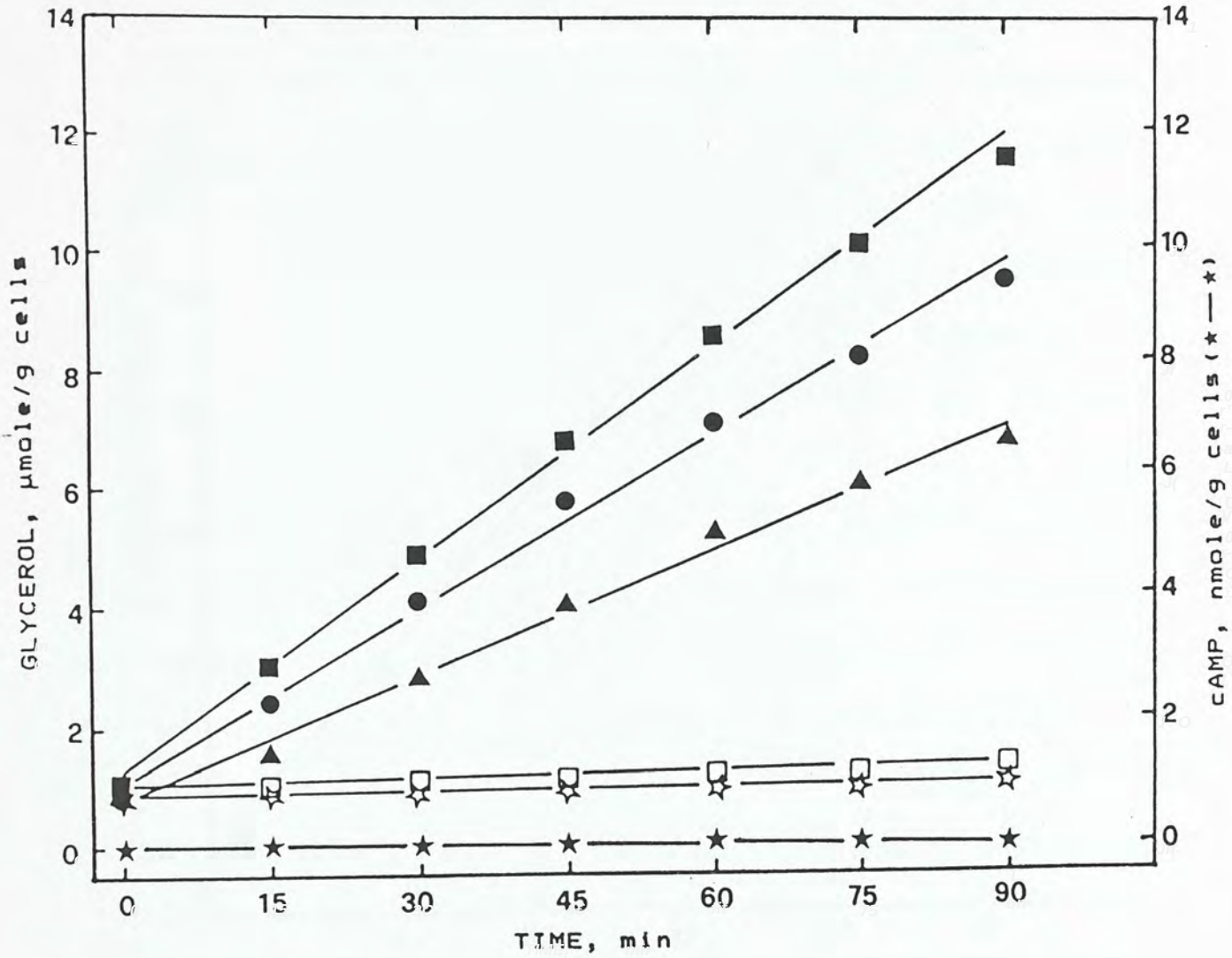


Fig. 3.13: Time dependence of glycerol release from and cAMP accumulation in adipocytes. Glycerol release was measured in control (☆); 0.5 U/ml (▲), 1 U/ml (●), 3 U/ml (■) adenosine deaminase; and 3 U/ml adenosine deaminase + 1 uM 2-chloroadenosine (□). cAMP accumulation (★) was determined in the presence of 1 U/ml adenosine deaminase.

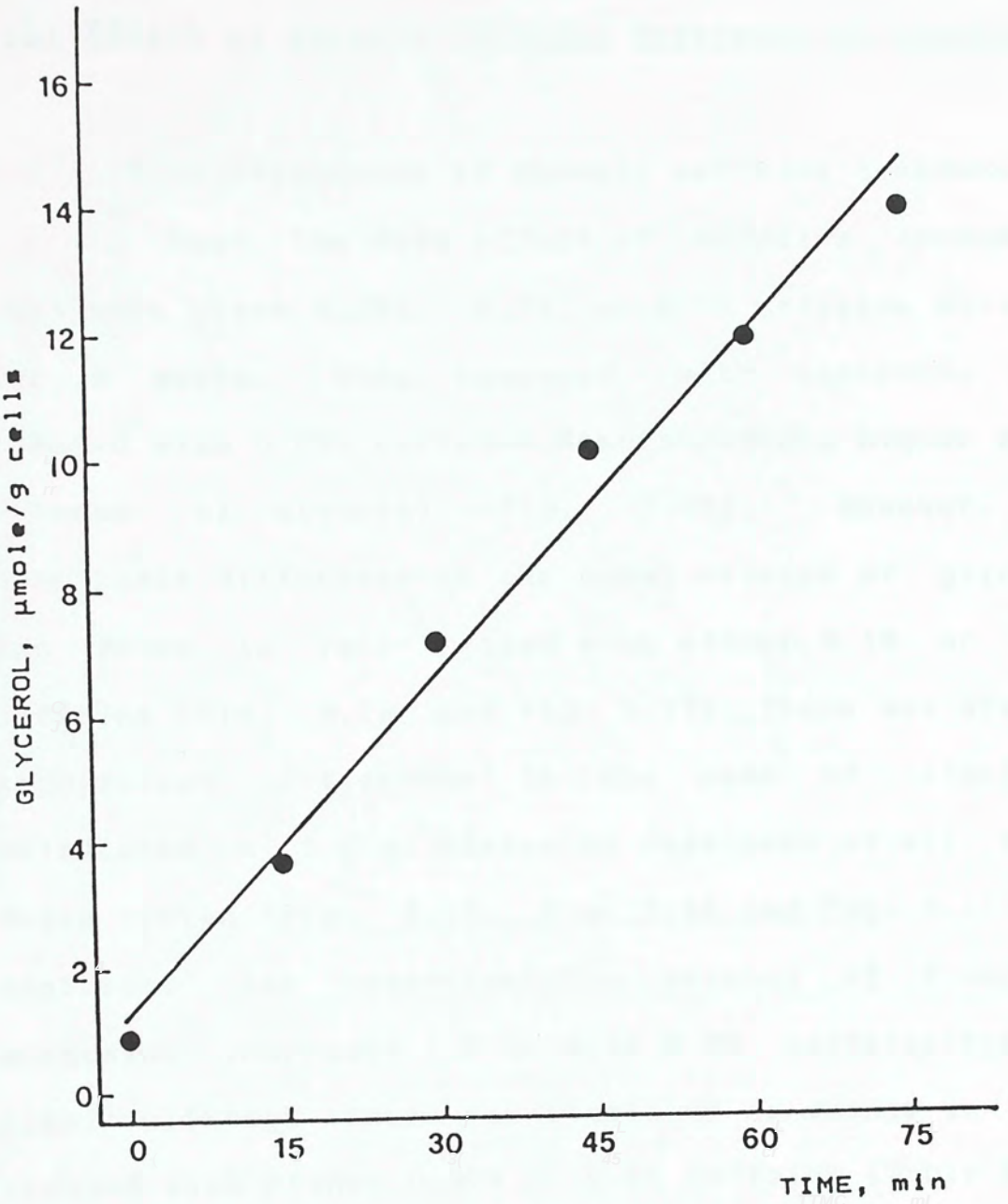


Fig. 3.14: Time dependence of glycerol release stimulated by 1 uM isoproterenol in the presence of 1 U/ml adenosine deaminase. Data showed are that of a typical experiment. Similar results were obtained in two separate experiments.

3.3) Effect of Chronic Caffeine Treatment on Lipolysis

3.3.1) Dose dependence of chronic caffeine treatment

To test the dose effect of caffeine treatment, rats were given 0.05%, 0.1%, or 0.2% caffeine solution for 6 weeks. When compared with controls, rats treated with 0.05% caffeine demonstrated a higher basal release of glycerol (Fig. 3.15). However, no observable difference in the basal release of glycerol was found in rats treated with either 0.1% or 0.2% caffeine (Fig. 3.16 and Fig. 3.17). There was also no significant difference in the rate of lipolysis stimulated by 1 U/ml adenosine deaminase at all three doses tested (Fig. 3.15, Fig. 3.16 and Fig. 3.17). In contrast, the anti-lipolytic potency of 2-chloro-adenosine increased 2.5 fold in 0.2% caffeine-treated rats, although there was little or no change in rats treated with either 0.05% or 0.1% caffeine (Table 3.6).

3.3.2) Time dependence of 0.2% caffeine treatment

To evaluate the effect of time course of caffeine treatment, rats were treated with 0.2% caffeine solution for 3, 6 and 9 weeks. There was no significant difference in the basal release of glycerol throughout these periods. However, when the rate of lipolysis stimulated by 1 U/ml adenosine deaminase was compared, rats treated with 0.2% caffeine for 9 weeks displayed a

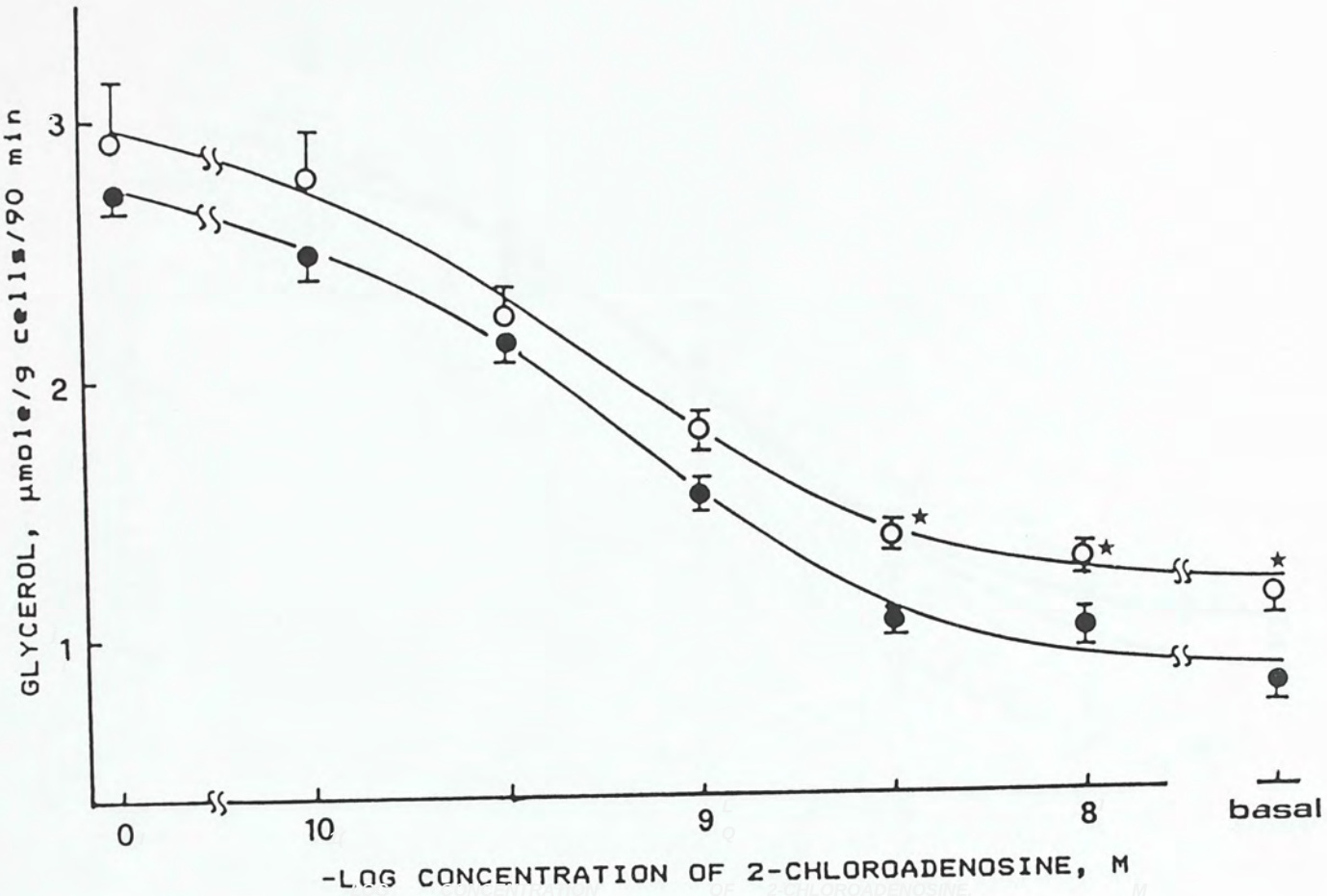


Fig. 3.15: 2-chloroadenosine inhibition of lipolysis stimulated by 1 U/ml adenosine deaminase in control (●) and 6 weeks 0.05% caffeine-treated (○) rats. Data showed are the mean ± S.E.M. of 4 rats. * P<0.05 with Students' t-test.

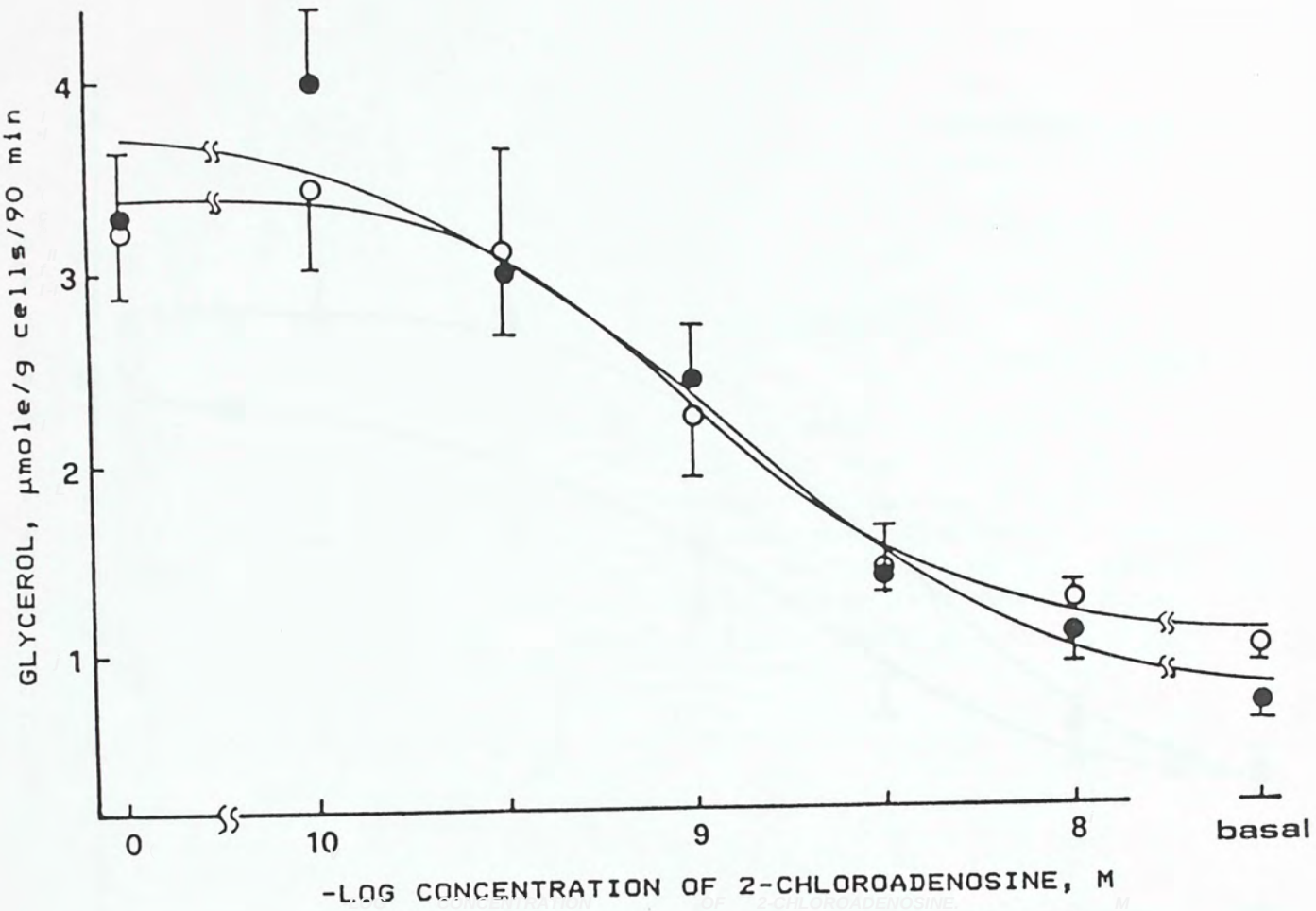


Fig. 3.16: 2-chloroadenosine inhibition of lipolysis stimulated by 1 U/ml adenosine deaminase in control (●) and 6 weeks 0.1% caffeine-treated (○) rats. Data showed are the mean ± S.E.M. of 4 rats.

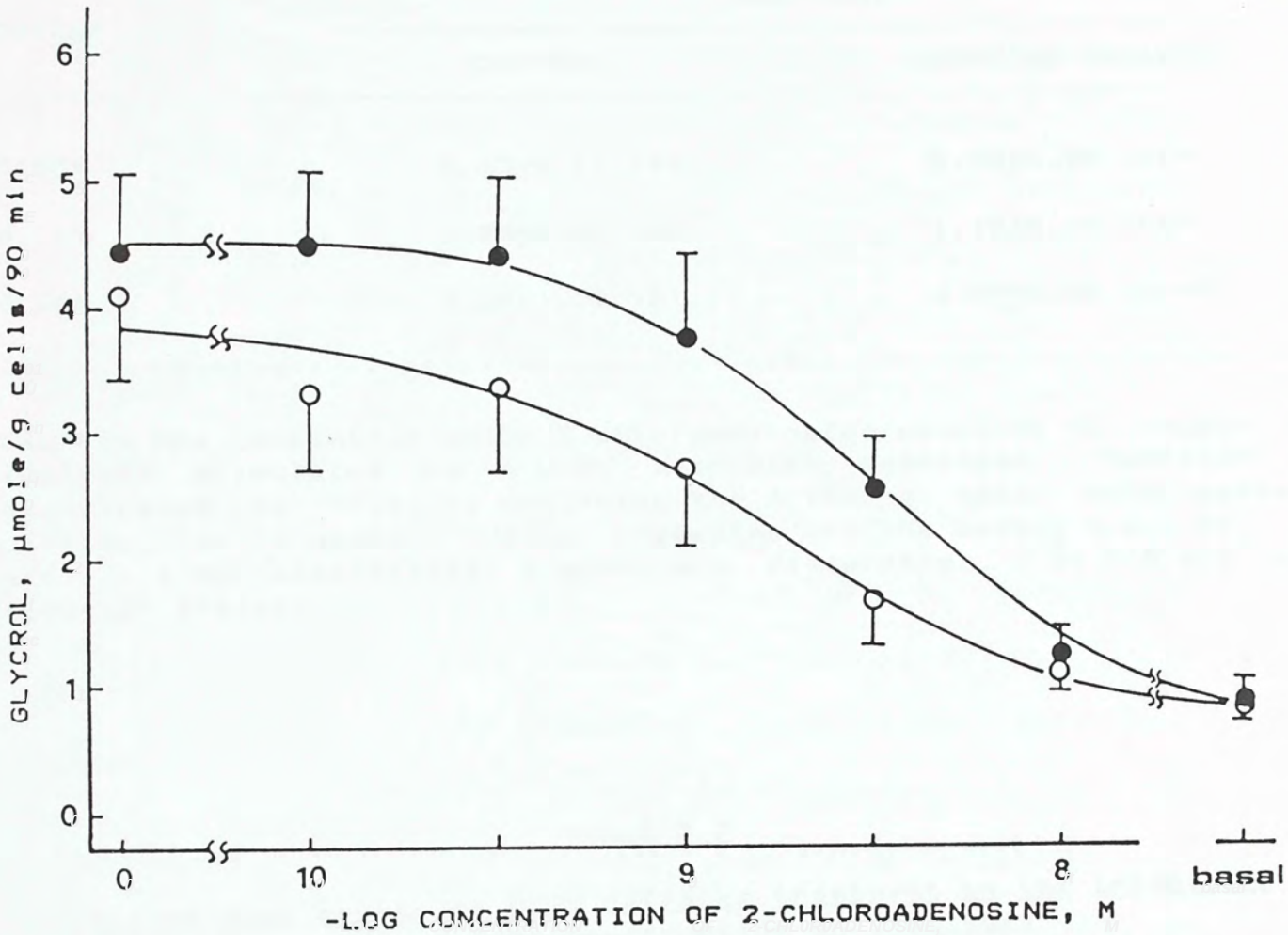


Fig. 3.17: 2-chloroadenosine inhibition of lipolysis stimulated by 1 U/ml adenosine deaminase in control (●) and 6 weeks 0.2% caffeine-treated (○) rats. Data showed are the mean ± S.E.M. of 8 rats.

TABLE 3.6

Effect of dosage of caffeine treatment (6 weeks) on the inhibitory potency of 2-chloroadenosine on lipolysis

DOSAGE	IC_{50} (nM)	
	CONTROL	CEFFEINE-TREATED
0.05%	0.63±0.13 (4)	0.55±0.90 (4)*
0.10%	1.53±0.41 (4)	1.13±0.14 (4)*
0.20%	2.68±1.02 (8)	1.09±0.52 (8)**

IC_{50} is the concentration of 2-chloroadenosine required to inhibit 50% lipolysis stimulated by 1 U/ml adenosine deaminase. Caffeine was adminstrated to rats by replacing the drinking water with caffeine solution for 6 weeks. Values presented are the mean ± S.D. of (n) rats. * No statistical significant difference. ** P<0.002 with Students' t-test.

TABLE 3.7

Effect of time course of 0.2% caffeine treatment on the inhibitory potency of 2-chloroadenosine on lipolysis

TIME COURSE OF TREATMENT	IC_{50} (nM)	
	CONTROL	CAFFEINE-TREATED
3 WEEKS	1.48±0.97 (4)	1.24±0.42 (4)*
6 WEEKS	2.68±1.02 (8)	1.09±0.52 (8)***
9 WEEKS	2.41±0.61 (4)	1.14±0.42 (4)**

IC_{50} is the concentration of 2-chloroadenosine required to inhibit 50% lipolysis stimulated by 1 U/ml adenosine deaminase. Caffeine was administrated to rats by replacing the drinking water with 0.2% caffeine solution for the time period indicated. Values presented are the mean ± S.D. of (n) rats. * No statistical significant difference. ** P<0.02, *** P<0.002 with Students' t-test.

lower rate of lipolysis than that of rats treated for 3 and 6 weeks (Fig.3.17, Fig. 3.18 and Fig. 3.19). Furthermore, significant difference in the anti-lipolytic potency of 2-chloroadenosine was observed after 6 and 9 weeks of 0.2% caffeine treatment (Table 3.7). Therefore, 6 weeks of 0.2% caffeine treatment was chosen as a model for further study.

3.3.3) Effect of chronic caffeine treatment (0.2%, 6 weeks) on isoproterenol stimulated lipolysis

Although there was no significant difference in the rate of lipolysis stimulated by 30 U/ml adenosine deaminase (Table 3.8), there was about a 40% decrease in the rate of lipolysis stimulated by 1uM isoproterenol in caffeine-treated rats (Fig. 3.20). A significant decrease in the lipolytic potency of isoproterenol was also noticeable after chronic caffeine treatment (Table 3.9).

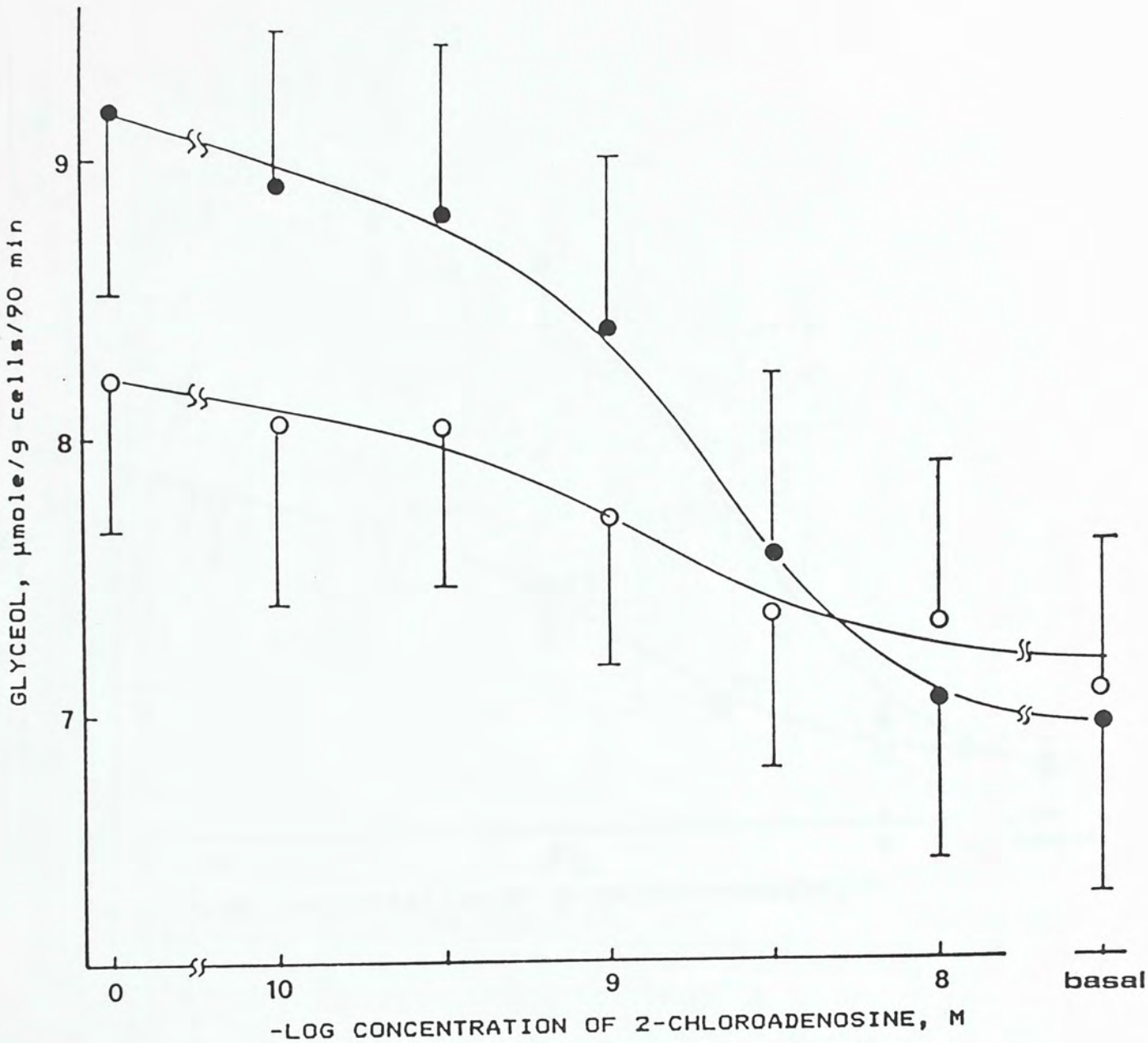


Fig. 3.18: 2-chloroadenosine inhibition of lipolysis stimulated by 1 U/ml adenosine deaminase in control (●) and 3 weeks 0.2% caffeine-treated (○) rats. Data showed are the mean ± S.E.M. of 4 rats.

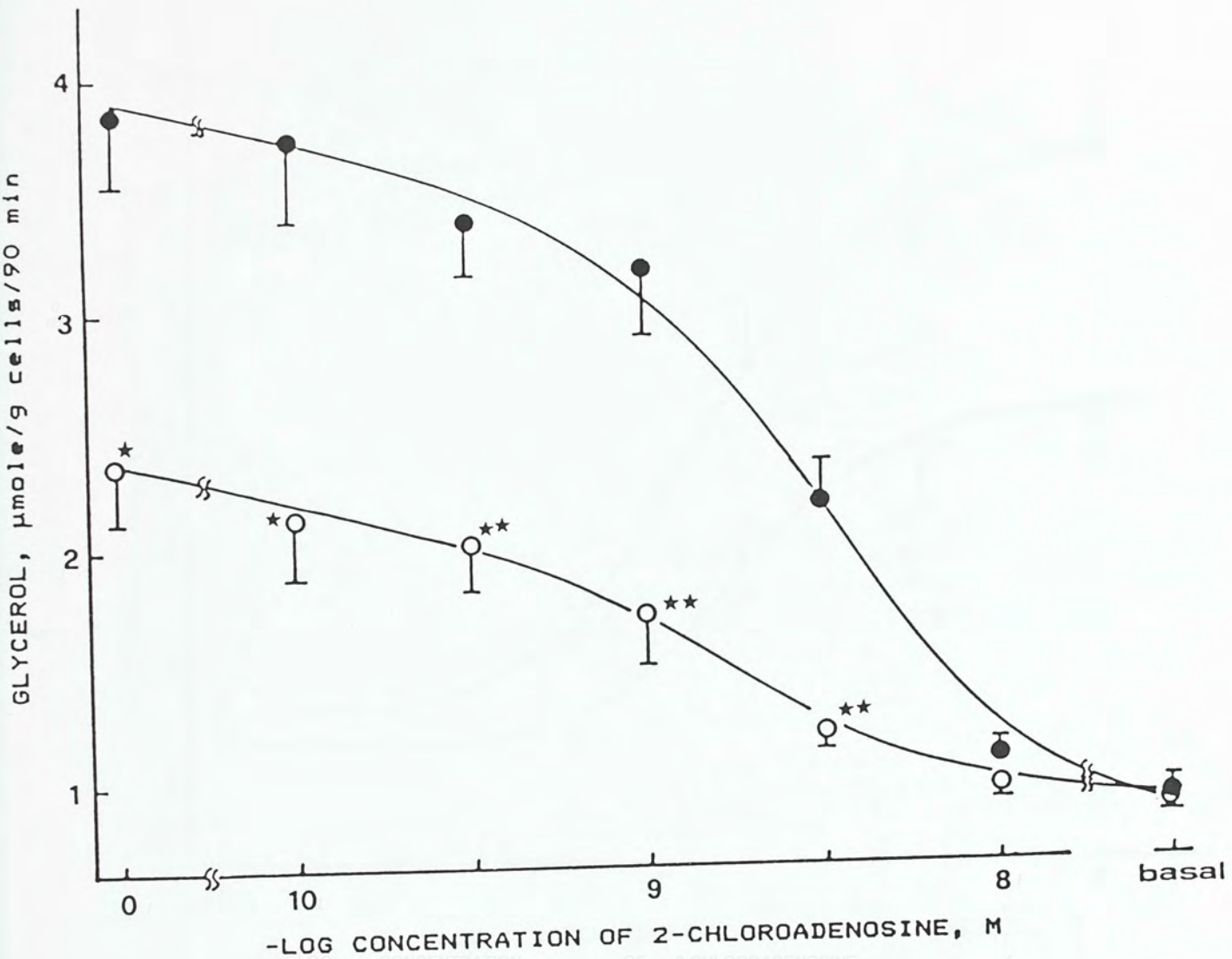


Fig. 3.19: 2-chloroadenosine inhibition of lipolysis stimulated by 1 U/ml adenosine deaminase in control (●) and 9 weeks 0.2% caffeine-treated (○) rats. Data showed are the mean ± S.E.M. of 4 rats. ★ $P<0.05$, ★★ $P<0.01$ with Students' t-test.

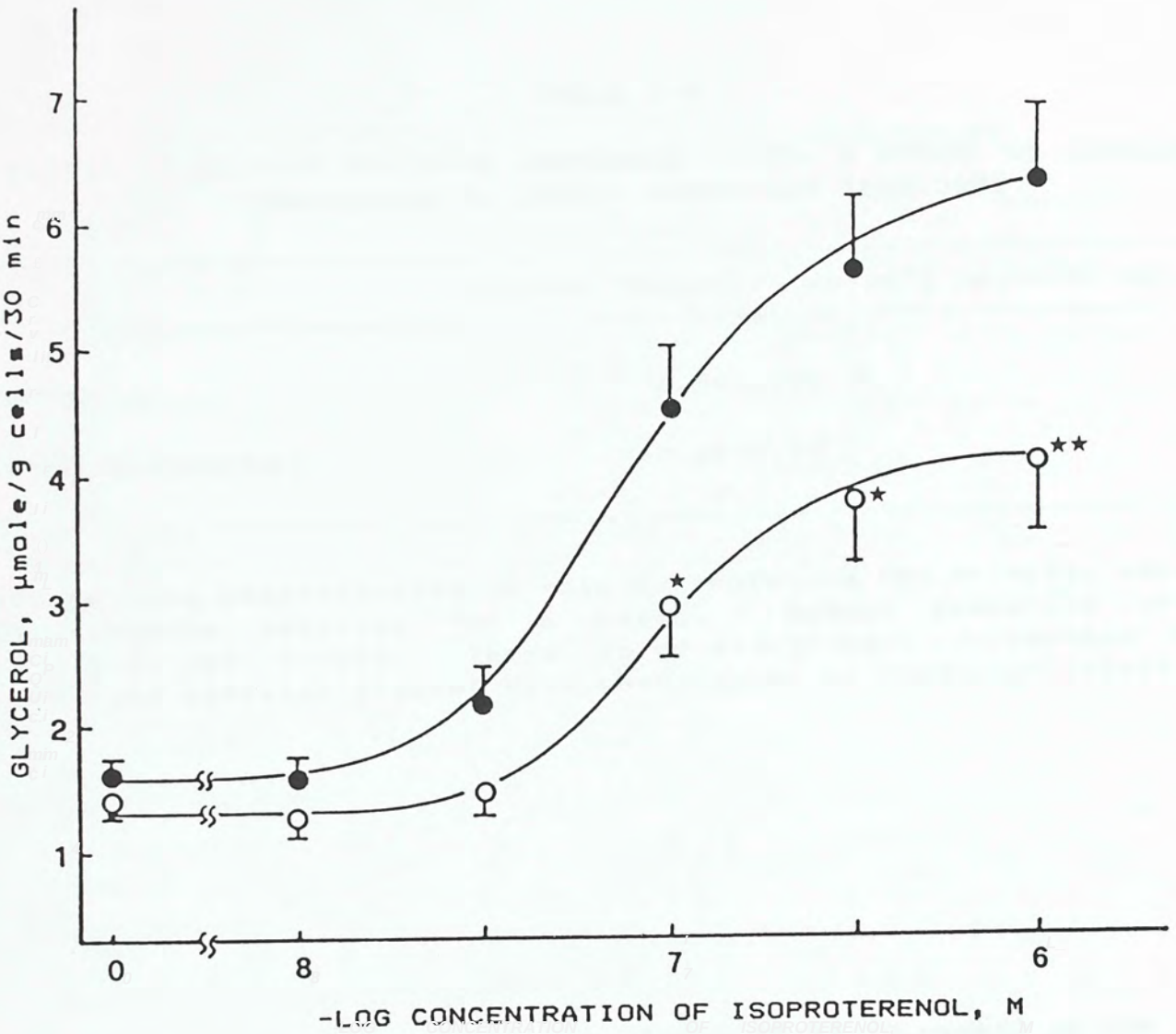


Fig. 3.20: Dose dependent stimulation of lipolysis by isoproterenol in control (●) and 6 weeks 0.2% caffeine-treated (○) rats. Data showed are the mean ± S.E.M. of 6 (control) and 7 (caffeine-treated) rats. * P<0.05 **P<0.06 with Students' t-test.

TABLE 3.8

Effect of chronic caffeine treatment (0.2%, 6 weeks) on lipolysis stimulated by 30U/ml adenosine deaminase

RAT	GLYCEROL RELEASED (umole/g cells/90 min)
CONTROL	6.04±2.32
CAFFEINE-TREATED	5.65±2.90

Caffeine was administrated to rats by replacing the drinking water with 0.2% caffeine solution for 6 weeks. Values presented are the mean ± S.D. of 4 rats. There is no significant difference between control and caffeine-treated rats when tested by Students' t-test.

TABLE 3.9

Effect of chronic caffeine treatment (0.2%, 6 weeks) on the lipolytic potency of isoproterenol

RAT	EC_{∞} (nM)
CONTROL	76.56±22.51 (6)
CAFFEINE-TREATED	109.61±31.62 (7)*

EC_{∞} is the concentration of isoproterenol required to obtain half-maximum stimulated lipolysis. Caffeine was administrated to rats by replacing the drinking water with 0.2% caffeine solution for 6 weeks. Values presented are the mean ± S.D. of (n) rats. * P<0.06 with Students' t-test.

3.4) Lipolysis and cAMP Accumulation

3.4.1) Accumulation of cAMP in intact adipocytes

In agreement with previously reports (Schimmel *et al*, 1981; Bojanic and Nahorski, 1984), isoproterenol stimulated cAMP accumulation in adipocytes in a dose dependent manner (Fig. 3.21). The accumulation of cAMP in adipocytes stimulated by 100 uM isoproterenol in the presence of 1 U/ml adenosine deaminase, can be inhibited by 2-chloroadenosine in a dose dependent manner (Fig. 3.24).

3.4.2) Relationship between cAMP level and lipolysis

Although there is evidence suggesting that the intracellular cAMP level may modulate lipase activity, which in turn regulates the rate of lipolysis, the relationship between cAMP level and lipolysis is not a straight forward one. For instance, while isoproterenol stimulated cAMP accumulation in dose-dependent manner up to 100 uM, this was not so for lipolysis which decreased when the concentration of isoproterenol was greater than 1 uM (Fig. 3.21). Moreover, in the presence of 100 uM isoproterenol, cAMP level reached maximum level after 6-8 min (Fig. 3.22) and then gradually returned to control level by 24 min but lipolysis was maintained at a steady rate for at least 30 min (Fig. 3.23).

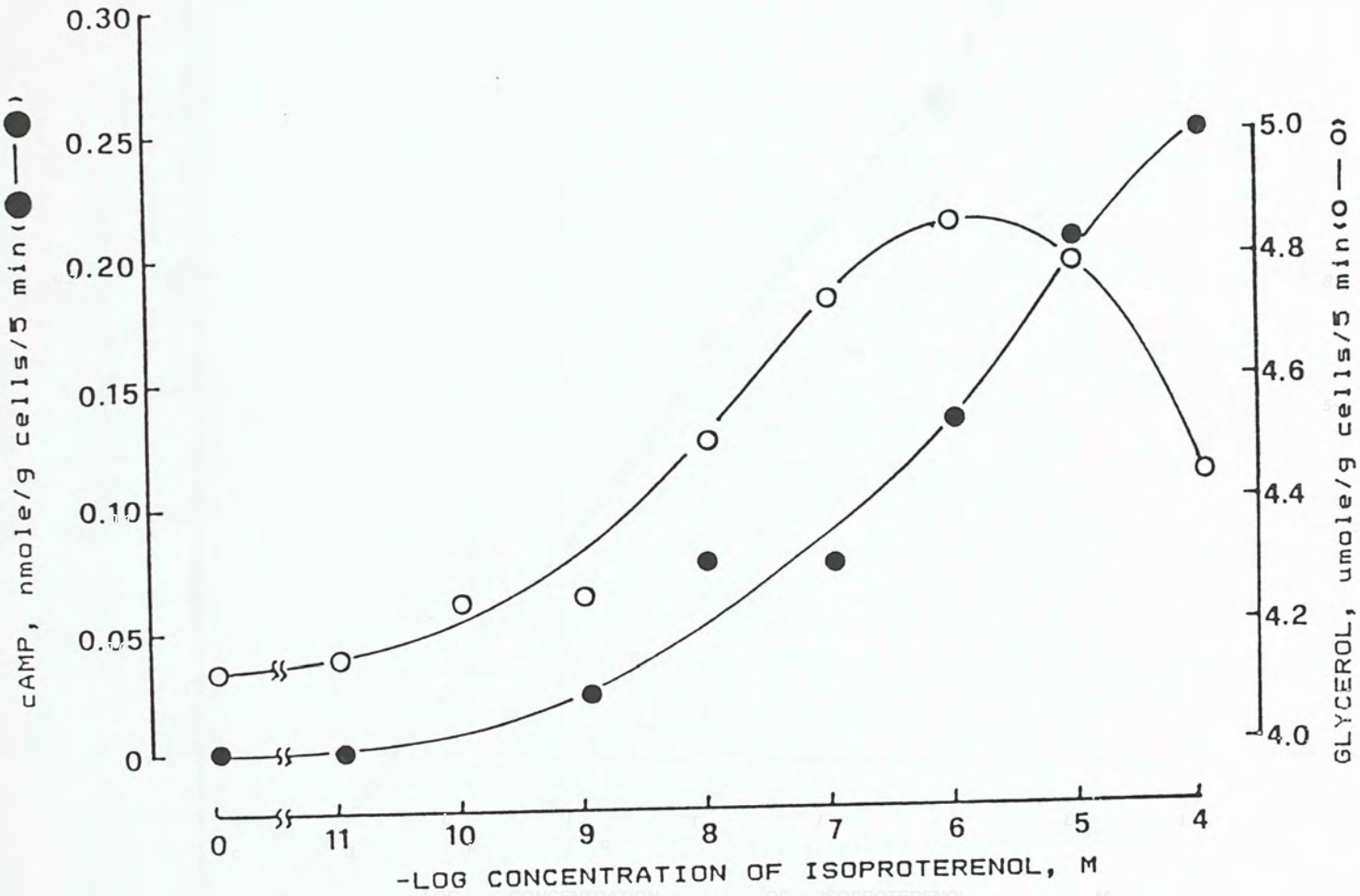


Fig. 3.21: Dose dependent stimulation of cAMP accumulation (●) and glycerol release (O) by isoproterenol.

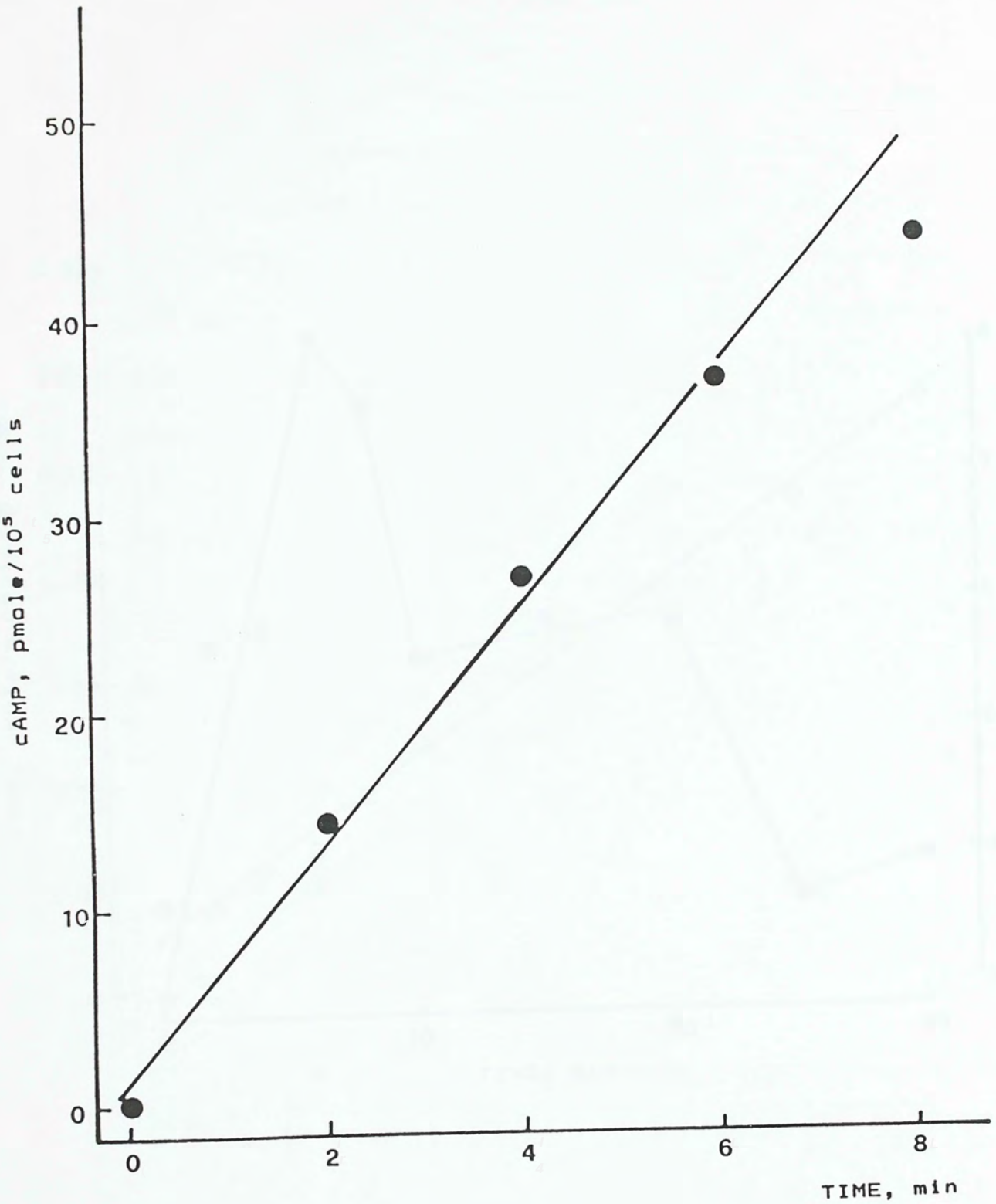


Fig. 3.22: Time dependent accumulation of cAMP in intact fat cell stimulated by 100 uM isoproterenol.

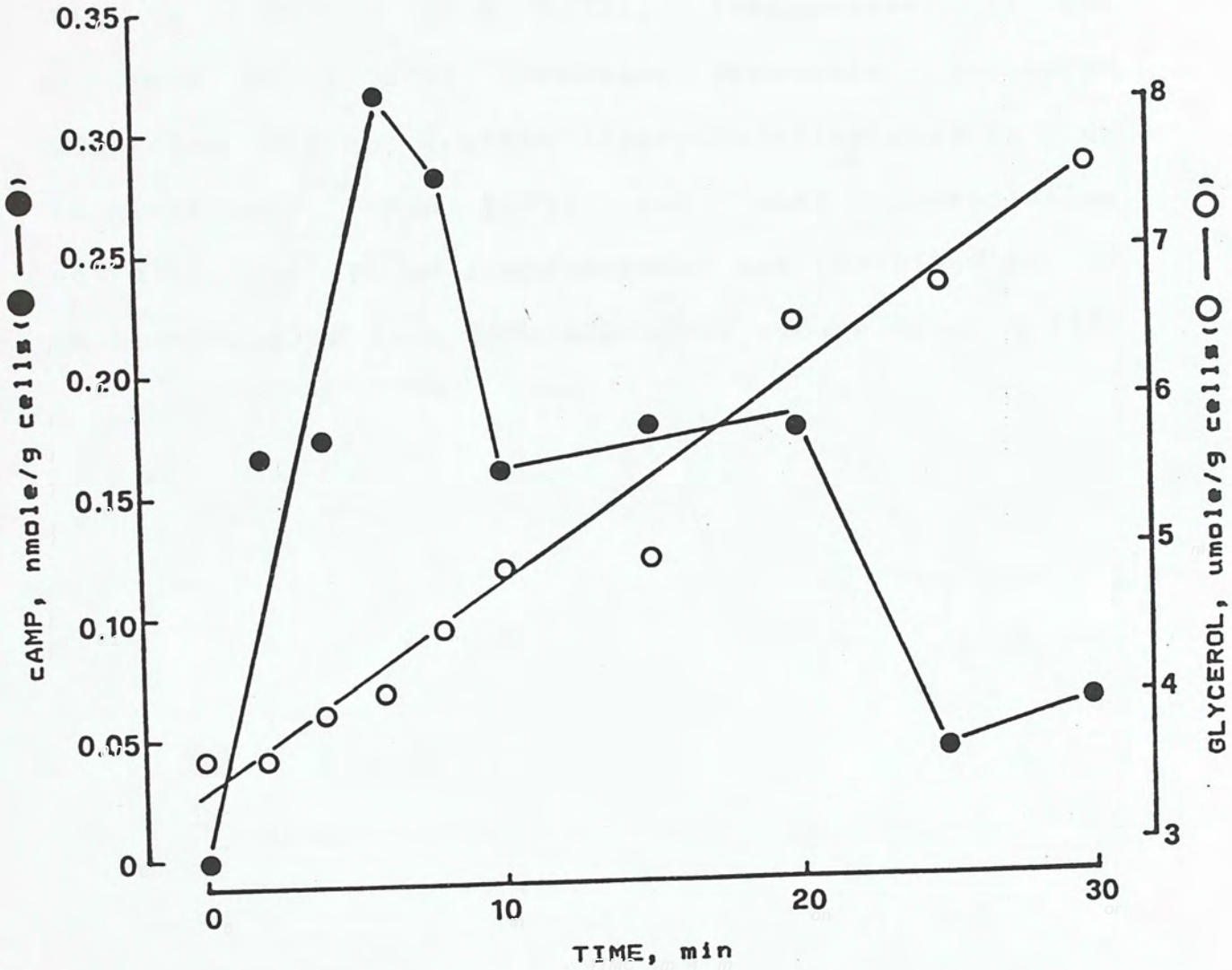


Fig. 3.23: Time dependent accumulation of cAMP (●) and glycerol release (○) stimulated by 100 uM isoproterenol in intact adipocytes .

The presence of 1 U/ml adenosine deaminase can stimulate lipolysis (Fig. 3.7), however, no accumulation of cAMP was detected under this condition for up to 90 min (Fig. 3.13). Furthermore, in the presence of 1 U/ml adenosine deaminase, 2-chloro-adenosine did not inhibit lipolysis stimulated by 1 uM isoproterenol (Fig. 3.7), but cAMP accumulation stimulated by 100 uM isoproterenol was inhibited by 2-chloroadenosine in a dose-dependent manner (Fig. 3.24).

3.5) Effect of Chronic Caffeine Treatment (0.2%, 6 weeks) on cAMP Accumulation in Intact Adipocytes

3.5.1) Effect of 2-chloroadenosine on isoproterenol stimulated cAMP accumulation

Although there was an increase in the anti-lipolytic potency of 2-chloroadenosine after chronic caffeine treatment (Table 3.7), no significant difference in the inhibitory potency of 2-chloro-adenosine was observed for its inhibitory effect on 100 uM isoproterenol-stimulated cAMP accumulation in the presence of 1 U/ml adenosine deaminase (Fig. 3.24 and Table 3.10). There was also no difference in the basal level of cAMP in adipocytes prepared from the control and the caffeine-treated rats.

3.5.2) Effect on cAMP accumulation stimulated by isoproterenol

In agreement with the decrease of lipolytic response to isoproterenol stimulation, chronic caffeine treatment also reduced the isoproterenol-stimulated cAMP accumulation in intact adipocytes (Fig. 3.25). In addition, in the presence of 1 U/ml adenosine deaminase, cAMP accumulation stimulated by 100 uM isoproterenol decreased about 50% when compared with that of the controls (Fig. 3.24).

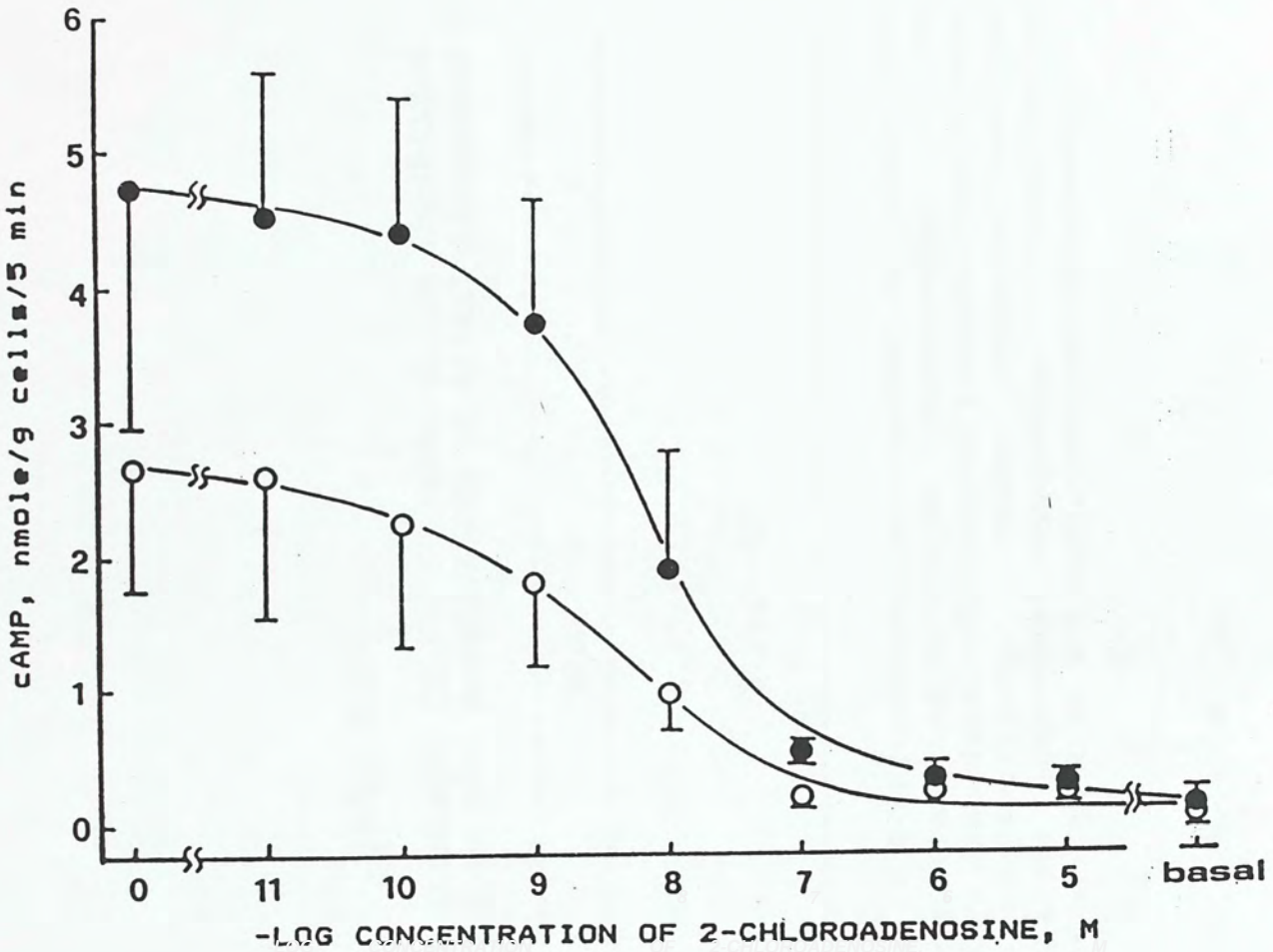


Fig. 3.24: Inhibition of cAMP accumulation stimulated by 100 uM isoproterenol in the presence 1 U/ml adenosine deaminase in control (●) and 6 weeks 0.2% caffeine-treated (○) rats. Adipocytes obtained from 4 rats were pooled for the assays. Data showed are the mean ± S.E.M. of two separate experiments.

TABLE 3.10

Effect of chronic caffeine treatment (0.2%, 6 weeks) on the inhibitory potency of 2-chloroadenosine on cAMP accumulation of intact adipocytes

RAT	IC_{50} (nM)
CONTROL	3.70±1.13
CAFFEINE-TREATED	3.71±1.17

IC_{50} is the concentration of 2-chloroadenosine required to inhibit 50% of the cAMP accumulation stimulated by 100 uM isoproterenol in the presence of 1 U/ml adenosine deaminase. Adipocytes isolated from 4 rats were pooled for assay in duplicate. Values presented are the mean ± S.D. of two separete caffeine treatments. There is no significant difference between control and caffeine-treated groups.

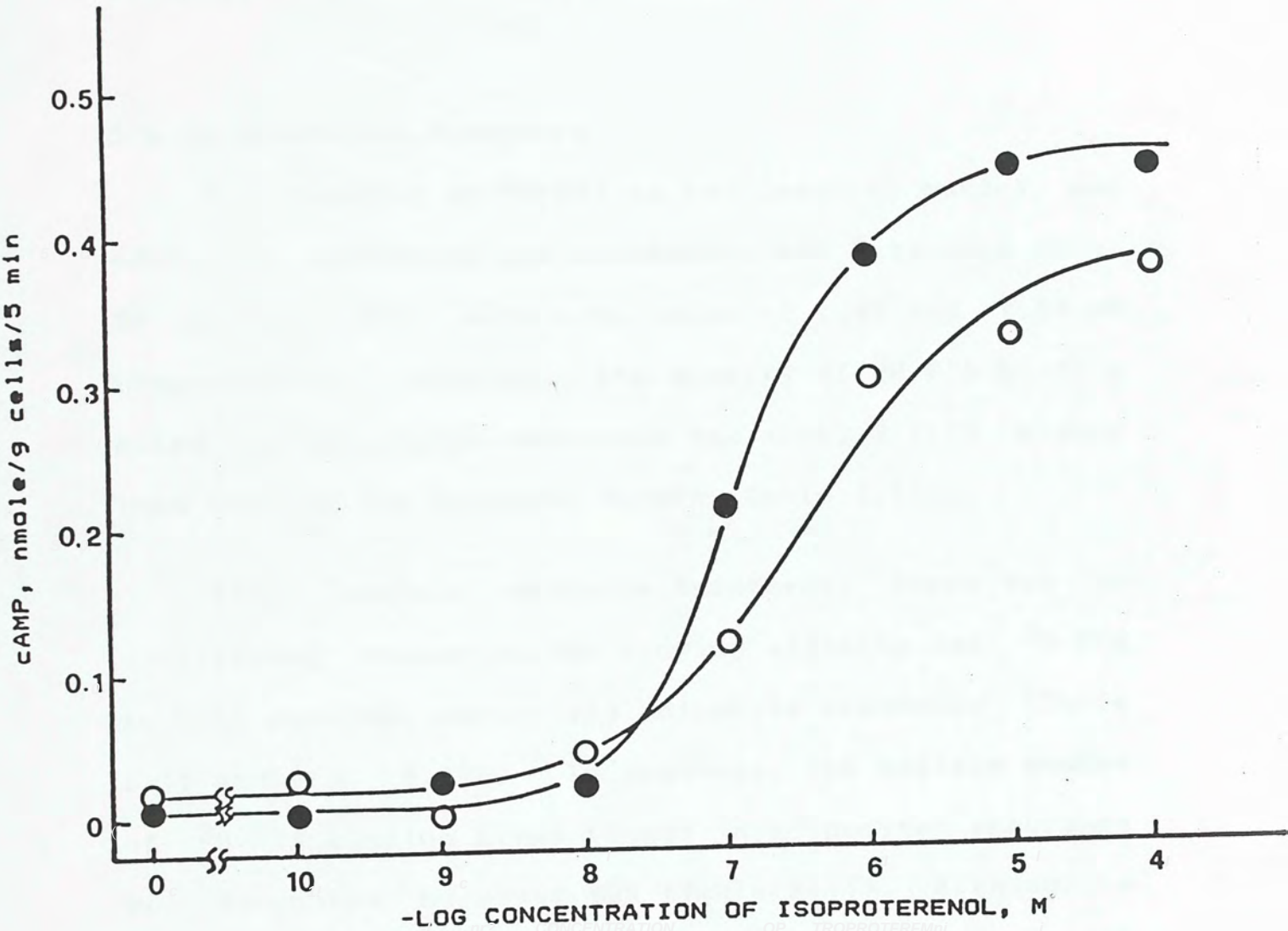


Fig. 3.25: Dose dependent stimulation of cAMP accumulation by isoproterenol in control (●) and 6 weeks 0.2% caffeine-treated (○) rats. Adipocytes obtained from 4 rats were pooled for the assays. Data showed are that of a typical experiment. Similar results were obtained in two separate experiments.

3.6) Adaptative Changes of Receptors After Chronic Caffeine Treatment (0.2%, 6 weeks)

3.6.1) Adeonsine receptors

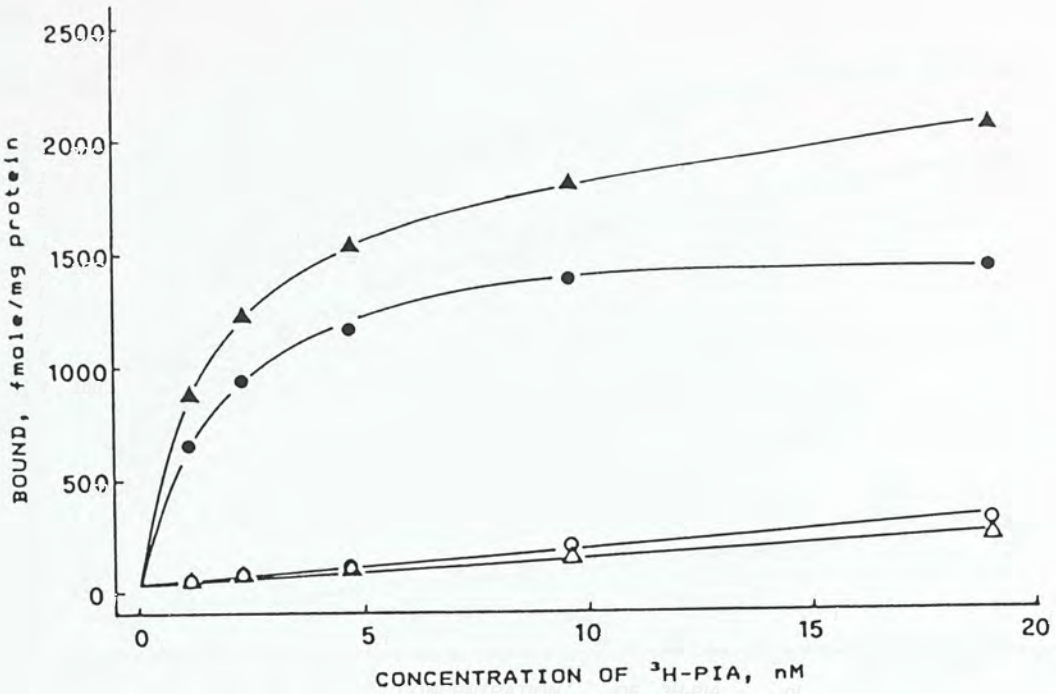
The binding of ^{3}H-PIA to rat cerebral cortex and adipocyte membranes was monophasic and saturable (Fig. 26 and Fig. 27), with a K_D value of 1.45 and 1.54 nM respectively. However, the density of ^{3}H-PIA binding sites in adipocytes membranes was about 2 fold higher than that of the cerebral cortex (Table 3.11).

After chronic caffeine treatment, there was no significant change in the binding affinity for ^{3}H-PIA in both cerebral cortex and adipocyte membranes (Table 3.11 and Fig. 3.27). In contrast, the maximum number of ^{3}H-PIA binding sites (Bmax) in adipocyte membranes was increased by about 45% (Table 3.1f). Although a consistent 10-20% increase in the ^{3}H-PIA binding sites was also observed in cerebral cortical membranes, it did not reach statistical significance(Fig. 3.26).

3.6.2) Beta-adrenergic receptors

The binding of ^{3}H-DHA to cerebral cortex membrane was monophasic and saturable (Fig. 3.28), with a K_D value of 2 nM and a Bmax value of 227 fmole/mg protein (Table 3.12). In contrast, the binding of ^{3}H-DHA to

A



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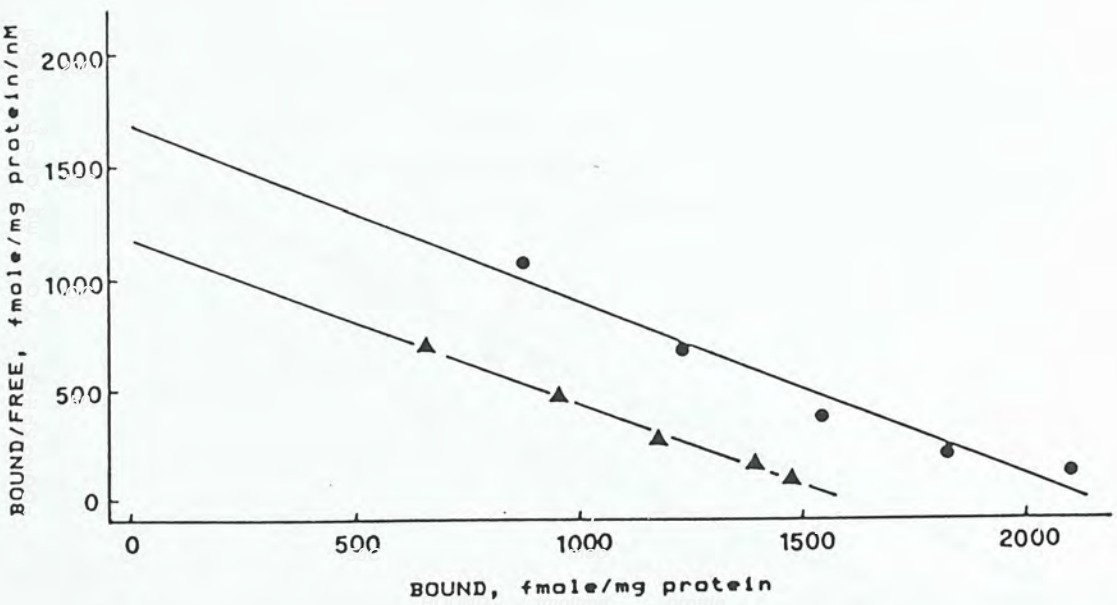
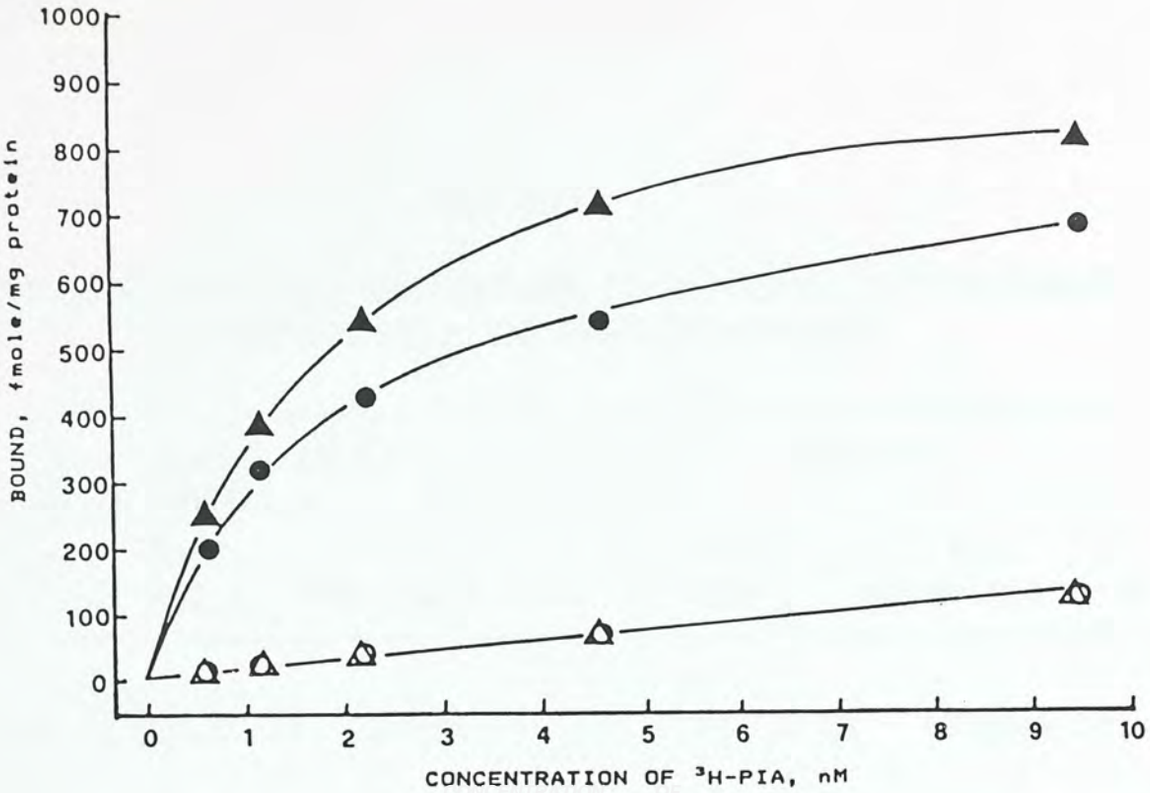


Fig. 3.26: (A) Saturation curve of ^{3}H-PIA binding to rat fat cell membranes of control (● ○) and 6 weeks 0.2% caffeine-treated (▲ △) rats. Adipocytes obtained from 4 rats of each group were pooled for the assays. Non-specific binding (○ △) was defined as the binding in the presence of 100 uM 2-chloroadenosine. Specific binding (● ▲) was obtained by substraction of non-specific binding from total binding. (B) Scatchard plot of the same data is showed. Data showed are that of a typical experiment. Similar results were obtained in three separate experiments.

A



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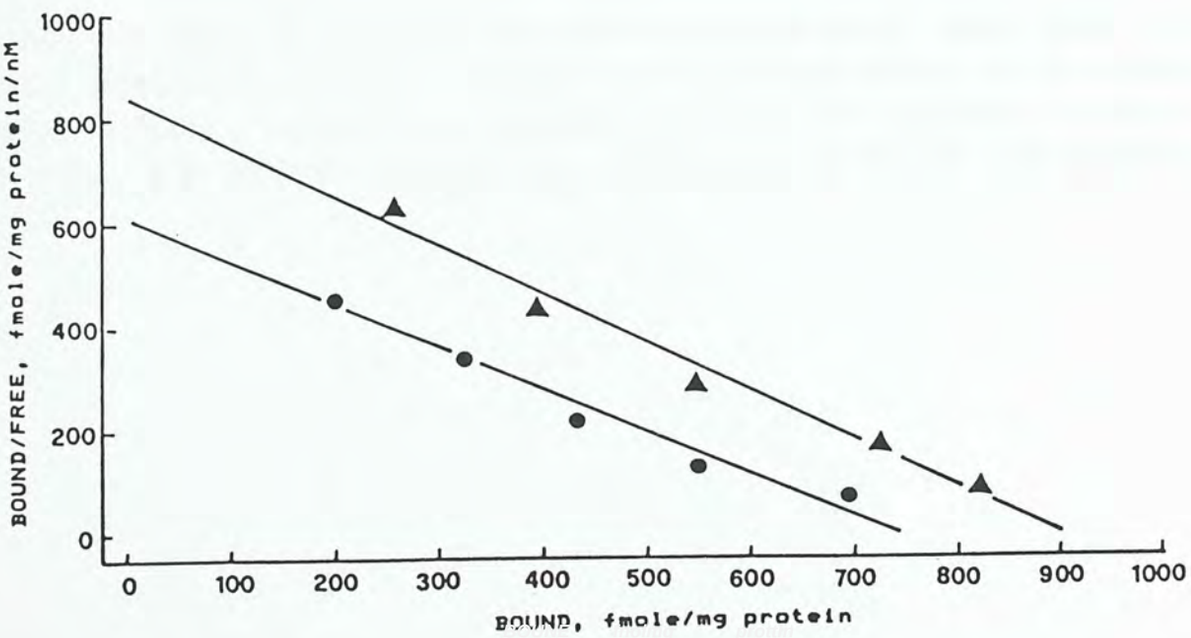

Fig. 3.27: (A) Saturation curve of ^{3}H-PIA binding to rat cerebral cortex membranes of control (● ○) and 6 weeks 0.2% caffeine-treated (▲ △) rats. Tissue from 4 rats of each group were pooled for the assays. Non-specific binding (○ △) was defined as the binding in the presence of 100 uM 2-chloroadenosine. Specific binding (● ▲) was obtained by substraction of non-specific binding from total binding. (B) Scatchard plot of the same data is showed. Data showed are that of a typical experiment. Similar results were obtained in 4 separate experiments.

TABLE 3.11

Effect of chronic caffeine treatment (0.2%, 6 weeks) on ^{3}H-PIA binding to rat cerebral cortex and adipocytes membranes

RAT	CEREBRAL CORTEX		ADIPOCYTES	
	K_D (nM)	B_{max} (fmole/mg protein)	K_D (nM)	B_{max} (fmole/mg protein)
CONTROL	1.45±0.38 (4)	630±97 (4)	1.54±0.36 (3)	1450±107 (3)
CAFFEINE-TREATED	1.49±0.45 (4)*	750±116 (4)*	1.53±0.24 (3)*	2076±65 (3)**

Caffeine was administrated to rats by replacing drinking water with 0.2% caffeine solution for 6 weeks. Tissues from 4 rats were pooled for the binding assays. Values presented are the mean ± S.D. of (n) separate caffeine-treatments. * No statistical significant difference. ** P<0.001 with Students' t-test.

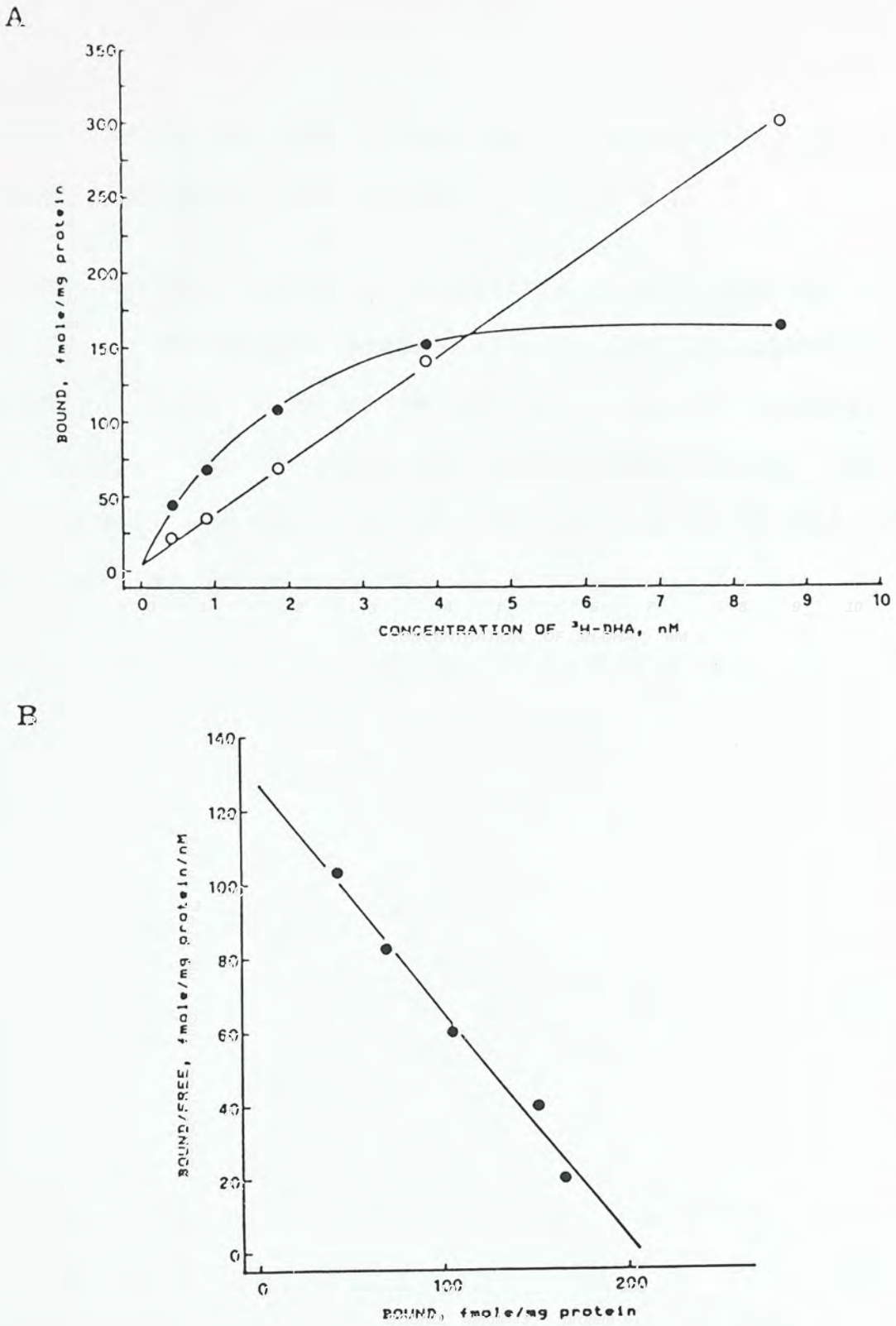

Fig. 3.28: (A) Saturation curve of ^{3}H-DHA binding to rat cerebral cortex membranes. Tissue from 4 rats were pooled for the assay. Non-specific binding (○) was defined as the binding in the presence of 1 mM isoproterenol. Specific binding (●) was obtained by substraction of non-specific binding from total binding. (B) Scatchard plot of the same data is showed. Data showed are that of a typical experiment. Similar results were obtained in 5 separate experiments.

adipocyte membrane was sigmoidal, suggestive of a cooperative process (Fig. 3.29).

After chronic caffeine treatment, the binding of ^{3}H-DHA to adipocyte membranes became monophasic, accompanied with a decrease in the receptor density (Fig. 3.29). No significant difference in K_D and Bmax, however, was observed in the binding of ^{3}H-DHA to cerebral cortex membranes (Table 3.12).

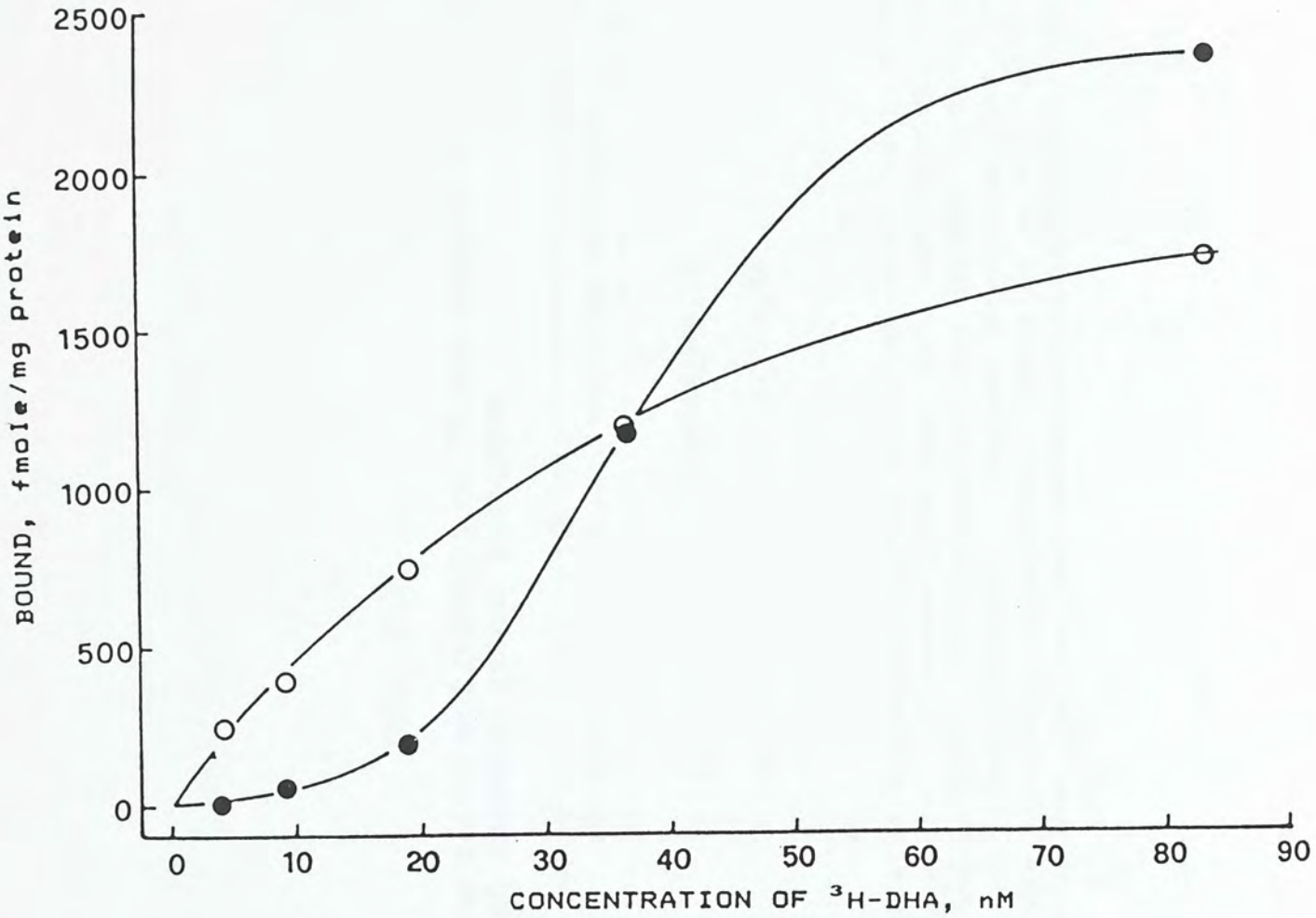

Fig. 3.29: Saturation curve of ^{3}H-DHA binding to rat fat cell membranes prepared from control (●) and 6 weeks 0.2% caffeine-treated (○) rats. Adipocytes from 4 rats of each group were pooled for the assays. Non-specific binding was defined as the binding in the presence of 100 uM propranolol. Data showed are that of a typical experiment. Similar results were obtained in two separate experiments.

TABLE 3.12

Effect of chronic caffeine treatment on ^{3}H-DHA binding to rat cerebral cortex membranes

RAT	K_D (nM)	B_{max} (fmole/mg protein)
CONTROL	2.11±0.65	226.85±99.12
CAFFEINE-TREATED	2.08±0.72	208.63±30.86

Caffeine was administrated by replacing the drinking water with 0.2% caffeine solution for 6 weeks. Tissues from four rats were pooled for the binding assays. Non-specific binding was defined as the binding in the presence of 1 mM isoproterenol. Values presented are the mean ± S.D. of 5 separate caffeine treatments. There is no statistical significant difference between control and caffeine-treated groups when tested with Students' t-test.

CHAPTER 4

DISCUSSION

4.1) The morphological aspect of chronic caffeine consumption.

Similar to a previous report (Johansson, 1978), rats were found to have a lower body weight when compared with age matched controls after chronic caffeine consumption (Table 3.1). In addition, the present study revealed a smaller epididymal fat pad weight as well as a lower adipocytes size (Table 3.1 and Table 3.2). These results tempt to suggest there may be a retardation of growth after chronic caffeine treatment. Since caffeine can block the cellular re-uptake of adenosine at high concentration (Marangos *et al*, 1982), therefore, chronic caffeine treatment may decrease the re-uptake and hence the re-utilization of adenosine. Thus more energy is required for the *de novo* synthesis of adenosine during caffeine treatment and this may slow down the normal growth rate.

Usually, adipose tissue contains 60-85% of lipid, of which 90-95% are triglycerides (Jeanrenaud, 1955). A decrease in the epididymal fat pad weight may be the result of a decrease in the adipocyte triglyceride content or a decrease in the adipocyte number. In spite of a smaller adipocytes size, there was no change in the mean cell weight and cell triglyceride content in adipocytes after the chronic caffeine treatment (Table 3.2 and Table 3.3), Thus, the lower epididymal

fat pad weight was most likely due to a decrease in adipocyte number.

4.2) Up regulation of adenosine receptors after chronic caffeine treatment

Similar to a previous report (Fredholm *et al*, 1984), the present study demonstrated that adenosine exerted its anti-lipolytic action via the A1 adenosine receptors in rat adipocytes. However, a small lipolytic activity of 2-chloroadenosine and adenosine can be obtained at high concentration of these compounds. This may suggest the existence of other adenosine receptors subtypes in adipocytes. More studies are, therefore, required to characterise the lipolytic activity of adenosine and its analogues.

In the present study, it was found that the anti-lipolytic potency of 2-chloroadenosine increased 2.5 fold after chronic 0.2% caffeine treatment (Table 3.6 and Table 3.7). This is in agreement with the observation that the hypotensive action of adenosine is increased after chronic caffeine consumption (Borstek *et al*, 1983). Furthermore, the present study also confirmed Fredholm's observation (1982) that no change in the inhibition of lipolysis by 2-chloroadenosine was obtained after 0.1% caffeine treatment for 1 weeks. Adaptive changes in adenosine receptors can, however,

be obtained by increasing the caffeine dosage and prolonging the duration of treatment (Table 3.6 and Table 3.7).

Although a consistent 10-20% increase in brain adenosine receptors was observed in the 0.2% caffeine-treated rats, it was not significantly different from the control (Table 3.10). In contrast, significant up-regulation of brain adenosine receptors has been reported previously (Fredholm, 1982; Murray, 1982; Boulenger *et al*, 1983; Wu and Coffin, 1984; Green and Stiles, 1986). This discrepancy may be the result of storage of the cerebral cortex at -20^{o} for about 48 h before assayed for receptors in the present study, while receptor assays were performed on fresh tissue in the previous reports.

In contrast to the cerebral cortex, an increase in the number of adenosine receptors with no change in affinity for ^{3}H-PIA was observed in adipocytes membranes after chronic 0.2% caffeine treatment (Table 3.10). According to the 'Law of Mass Action', at a fixed drug concentration, the number of receptors being occupied is determined by the total number of receptors present as well as by its affinity for the drug. Since a biological response is proportional to the number of receptors being occupied (Stephenson, 1956), an increase in the anti-lipolytic potency of 2-chloro-

adenosine upon chronic caffeine treatment may be attributed to an increase in receptor occupancy as a result of increase in adenosine receptor number in adipocytes.

Adenosine deaminase can stimulate lipolysis presumably by removing endogenous adenosine which is released from adipocytes (Fig. 3.7). In the present study, when lipolysis was stimulated with 30 U/ml adenosine deaminase, there was no difference in the lipolytic rate between the control and caffeine-treated rats (Table 3.8), suggesting that there was no change in the lipolytic pathway. This finding further supports the notion that the increase in the anti-lipolytic potency of 2-chloroadenosine may be due to an increase of adenosine receptor number in the adipocytes. Although an increase in the coupling efficiency of adenosine receptor with its effector machinery cannot be ruled out in the present study.

In spite of an increase in the potency of 2-chloroadenosine to inhibit adenosine deaminase stimulated lipolysis after chronic caffeine treatment, there was no change in the inhibitory potency of 2-chloroadenosine on cAMP accumulation stimulated by isoproterenol (Fig. 3.24 and Table 3.10). This discrepancy may suggest that the anti-lipolytic action of 2-chloroadenosine and the inhibition of cAMP accumulation by 2-chloroadenosine are two separate

events with no causal relationship. This notion was further supported by the absence of cAMP accumulation in the intact adipocytes when lipolysis was stimulated by the addition of adenosine deaminase (Fig. 3.13).

4.3) Down regulation of beta-adrenergic receptors after chronic caffeine treatment

In the present study, the adrenergic receptors in adipocytes was characterized to be a beta-adrenergic type. Previous reports suggested the beta-adrenergic receptors of adipocytes should be the $beta_1$-adrenergic receptors (Fain, 1973; Moisner and Carter, 1977; García-sáinz and Fain, 1980). However, recent evidence suggested the beta-adrenoceptor of rat adipocytes was neither a $beta_1$ nor a $beta_2$ type, but rather a hybrid of $beta_1$ and $beta_2$-adrenoceptors (Bojanic and Nahorski, 1984; Vente *et al*, 1980).

The increase of anti-lipolytic potency of 2-chloroadenosine after chronic caffeine treatment, there was accompanied by a decrease in the lipolytic response to isoproterenol (Fig. 3.25). A corresponding decrease in ^{3}H-DHA binding sites on adipocytes membranes (Fig. 3.29) was also observed together with a parallel decrease in the cAMP accumulation stimulated by isoproterenol either in the presence or absence of adenosine deaminase (Fig. 3.24 and Fig. 3.25). Thus,

upon chronic caffeine treatment, the adipocytes are less responsive to the beta-adrenergic stimulation for lipid mobilization.

Previously, it has been demonstrated that chronic activation of beta-adrenergic receptors may lead to a down regulation of these receptors as reflected by a decrease in receptor numbers (Chuang and Costa, 1979; Reisine, 1981). The down-regulation of ^{3}H-DHA binding sites on adipocytes membrane observed in the present study suggested that the adrenergic system of adipose tissue was overactivated during chronic caffeine treatment.

Both brown and white adipose tissue are innervated by adrenergic nerve fibers (Wirsén, 1965; Meisner and Carter, 1977). As reviewed by Paton (1979), adrenergic neurotransmission can be inhibited presynaptically by adenosine (Fig. 4.1). Since caffeine is an adenosine receptor antagonist, it is conceivable that the presynaptic inhibition of adrenergic transmission by adenosine can be decreased by chronic caffeine treatment, resulting in an increased release of norepinephrine when stimulated. Thus the adrenergic system of adipose tissue may be indirectly over-activated during chronic caffeine treatment.

It was found in the present study that, in the presence of 1 uM 2-chloroadenosine, there was no

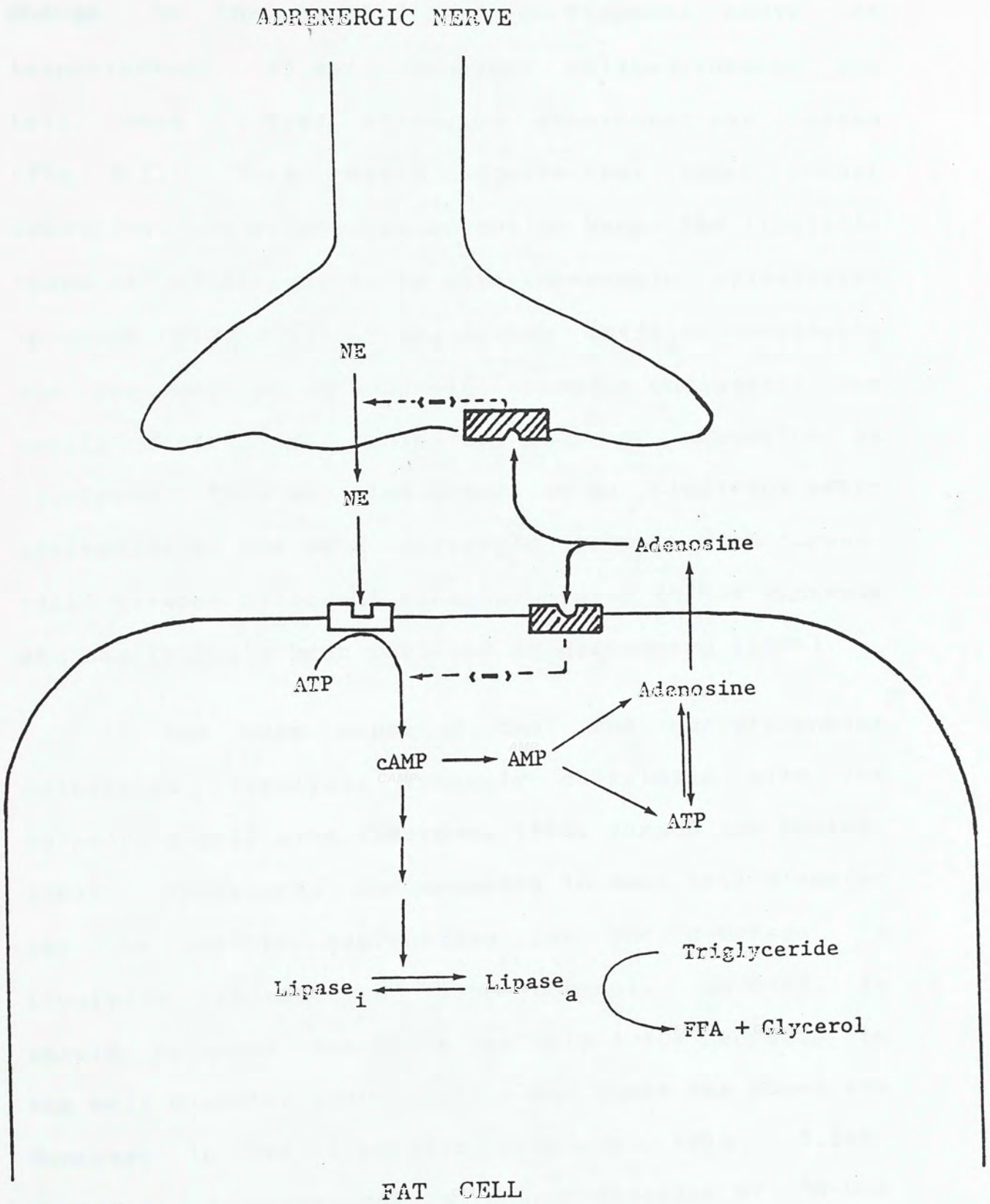

Fig. 4.1: Roles of adenosine in the regulation of norepinephrine release from adrenergic nerve endings and lipolysis.

change in the lipolytic dose response curve of isoproterenol. It was, however, shifted towards the left when 1 U/ml adenosine deaminase was added (Fig. 3.11). This result suggests that under normal condition, the endogenous adenosine keeps the lipolytic response of adipocytes to beta-adrenergic stimulation in check (Fig. 4.1). Thus, during caffeine treatment, the presence of an adenosine receptor antagonist can partly offset the inhibitory tone of adenosine on lipolysis. This may also result in an indirect over-activation of the beta-adrenergic system. Such 'cross-talk' between different receptor systme is not uncommon and has recently been reviewed by Hollenberg (1985).

It has been reported that the norepinephrine stimulated lipolysis linearly correlated with the adipocytes cell size (Reardon, 1973; Vernon and Finley, 1985). Therefore, the decrease in mean cell diameter may be another explanation for the decrease in lipolytic response to isoproterenol. However, it should be noted that there was only a 10% decrease in the cell diameter (Table 3.2), but there was about 40% decrease in the lipolytic response (Fig. 3.20). Moreover, a corresponding down-regulation of ^{3}H-DHA binding site was observed (Fig. 3.29). Therefore, the decrease in mean cell diameter after chronic caffeine treatment may account for only part of the decrease in the lipolytic response to isoproterenol.

It has been reported previously that there was a down regulation of brain beta-adrenergic receptors after long-term administration of caffeine or theophylline (Goldberg *et al*, 1982; Fredholm *et al*, 1984; Green and Stile, 1986). However, no significant change in the cerebral cortex beta-adrenergic receptors was observed in the present study (Table 3.11). As the radioligand used for labelling beta-adrenergic receptors and the brain region examined in the present study were different from those reported previously, the discrepancy may be related due to the difference of the tissues as well as the radioligand used.

4.4) General discussion on the chronic caffeine treatment

A cup of coffee contains 75 - 150 mg caffeine (Fredholm, 1980). For a person with a body weight of 75 kg who consumes 3 cups of coffee each day, his caffeine consumption amounts to 3 - 6 mg/kg. In the present study, the effect of chronic ingestion of 60 - 280 mg/kg/day of caffeine was examined in rats. As a matter of fact, rats have a higher metabolic rate. Indeed, pharmacokinetics studies illustrated that a 2.5 fold higher caffeine dose is required to elicit a similar pharmacological response in rats when compared with men (Cardinali, 1980). Furthermore, a relatively

shorter time period of caffeine treatment is requried for experimental purpose. Thus the present caffeine dosage were adopted as an animal model for habitual caffeine consumption in men.

Long-term heavy caffeine consumption has been report to induce syndromes including vertigo, headache, and nervousness (Powers, 1925). Recent evidence suggest the biological effect of caffeine may be due to its antagonism of adenosine receptors (Fredholm, 1980; Snyder and Sklar, 1984). Since adenosine is an anti-lipolytic agent in adipocytes, an increase in lipolysis in response to lipolytic stimuli is expected during caffeine treatment. The present finding of a 'heightened' adenosine inhibition together with a 'dampered' adrenergic stimulation in adipocytes after chronic caffeine treatment suggests that an adaptive response has been in operation to compensate for the lipolytic action of caffeine.

It has been reported that long-term caffeine consumption also induced an up-regulation of brain benzodiazepine receptors (Boulenger *et al*, 1983; Wu and Coffin, 1984). Therefore, the biological effect of long-term caffeine consumption in the central nervous system may involve a more complicate adaptive changes of receptors in addition to the adenosine and beta-adrenergic receptors.

In addition to chronic caffeine treatment, the responsiveness of adenosine receptors on adipocytes was reported to be modulated in some patho-physiological conditions:

(1) Anti-lipolytic potency of N^6-phenylisopropyl-adenosine (PIA), a metabolic stable adenosine analogue of adenosine, increases during lactation in rat (Vernon *et al*, 1983) and sheep (Vernon and Finley, 1985).

(2) Anti-lipolytic action of PIA decreases after starvation (Chohan *et al*, 1984)

(3) Anti-lipolytic action of PIA increases in hypothyroidism (Chohan *et al*, 1984)

The results of these studies suggest that adenosine is an important physiological regulator of lipolysis. However, the relative importance of adenosine and insulin as a physiological anti-lipolytic agent remains to be investigated.

Although the present study suggests that caffeine may be effective for slimming purpose, its stimulatory effect on the central nervous system and cardiovascular system should not be overlooked (Colton, 1967; Snyder and Sklar, 1984). In fact, long-term treatment with caffeine has been reported to cause myocardial fibrosis and markedly reduced life-span in rats (Johansson,

1981). Thus, caffeine is not recommended for the purpose of slimming.

4.5) Lipolysis and cAMP accumulation

Currently, it is widely accepted that lipolytic hormones stimulate the accumulation of cAMP in adipocytes. An elevated intracellular cAMP level will dissociate the regulatory unit from the catalytic unit of a cAMP dependent kinase, which in turn will activate the hormone-sensitive lipase by transferring a phosphate group from ATP to the lipase. However, the present study and several previous reports indicate that the relationship between cAMP level and lipolysis is not a straight forward one. For instance:

(1) Isoproterenol stimulated cAMP accumulation peaked at 6-8 min and then gradually return to the pre-stimulated level, whereas lipolysis continued at a steady rate for at least 30 min (Fig. 3.23; Schimmel, 1974; Butcher *et al*, 1968).

(2) Lipolysis stimulated by supramaximal concentration of isoproterenol was not inhibited by adenosine or its stable analogues. However, isoproterenol stimulated cAMP accumulation was

susceptible to inhibition by 2-chloroadenosine (Fig. 3.7 and Fig. 3.25; Trost and Stock, 1979).

(3) Adenosine deaminase and methylxanthine can stimulate lipolysis, however, no noticeable accumulation of cAMP was detected under these conditions (Fig. 3.13; Butcher *et al*, 1968).

(4) After pre-treatment of adipose tissue with an lipolytic agent, one can obtain further stimulation of lipolysis upon a second application of the lipolytic agent. This second stimulated response, however, was not accompanied by cAMP accumulation (Schimmel, 1973).

(5) After two hour incubation of adipocytes with tritium labeled cAMP or dibutyryl-cAMP, same amount of radiolabeled cAMP and dibutyryl-cAMP was found in the adipocyte extract. This result suggested that both cAMP and dibutyryl-cAMP can penetrate into the adipocytes and to the same extent, although a higher degradation rate of cAMP was expected. However, only dibutyryl-cAMP induced lipolysis *in vitro* (Okuda *et al*, 1975)

(6) Local anaesthetics inhibited lipolysis but stimulated cAMP accumulation in adipocytes (Siddle and Hales, 1974)

In addition to the "cAMP theory", calcium ion has recently been implicated in the regulation of lipolysis (Schimmel, 1973; Siddle and Hales *et al*, 1974; Hales *et al*, 1974; Goko *et al*, 1984). However, there was no observable increase in the turnover rate of phosphatidylinositol in adipocytes when lipolysis was stimulated with ACTH and dibutyryl-cAMP (García-Sáinz and Fain, 1980).

It has also been suggested by Okuda and co-workers (1974) that lipolysis was stimulated by alteration in the interaction between a lipase and the triglyceride globule in adipocytes rather than as a result of an activation of lipase by a cAMP-dependent protein kinase. Nevertheless, a thorough understanding of the mechanism underlying hormone induced lipolysis will require the successful purification and characterization of the hormone-sensitive lipase as well as other lipolytic enzymes of adipocytes.

CONCLUSION

After chronic caffeine treatment (0.2%, 6 weeks), there was a decrease in the body weight, epididymal fat pad weight, and mean cell diameter of adipocytes when compared with that of the controls. However, there was no difference in the triglyceride content and the mean cell weight of adipocytes between the control and the caffeine-treated rats.

Lipolysis induced by adenosine deaminase was not different between the control and the caffeine-treated rats. However, an increase in the anti-lipolytic potency of 2-chloroadenosine, a stable adenosine analogue, was observed after chronic caffeine treatment. This was accompanied by an increase in the number of adenosine receptors in the adipocytes.

On the other hand, a diminished lipolytic response to isoproterenol, an beta-adrenergic agonist, was obtained in the caffeine-treated rats. Furthermore, cAMP accumulation stimulated by isoproterenol in intact adipocytes was decreased both in the presence and absence of adenosine deaminase after the chronic caffeine treatment. In agreement with the decrease in lipolytic response and cAMP accumulation to isoproterenol, ecreas[illegible] the numbe[illegible]

of beta-adrenergic receptor was also observed in these adipocytes.

Since caffeine is an adenosine receptor antagonist, an increase in lipolysis is expected during caffeine treatment. The present finding of a 'heightened' adenosine inhibition together with a 'dampened' adrenergic stimulation in adipocytes after chronic caffeine treatment, suggests an adaptive response has been in cooperation to compensate for the lipolytic action of caffeine.

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