Biochem. J. (1970) **120**, 187–193
Printed in Great Britain

An Effect of Insulin on Adipose-Tissue Adenosine 3′:5′-Cyclic Monophosphate Phosphodiesterase

By E. G. LOTEN and J. G. T. SNEYD
Department of Clinical Biochemistry, University of Otago Medical School, Dunedin, New Zealand

(*Received* 8 *June* 1970)

1. 3′:5′-Cyclic nucleotide phosphodiesterase activity was measured in homogenates prepared from epididymal fat-pads and isolated fat-cells incubated in the absence and presence of insulin. 2. Homogenates of insulin-treated tissues showed an increase in phosphodiesterase activity compared with controls. No effect of insulin was observed when the hormone was added directly to homogenates. 3. There was kinetic evidence for the presence of two 3′:5′-cyclic nucleotide phosphodiesterases in adipose tissue. Insulin raised the maximal velocity of the low-K_m enzyme and lowered the K_m of the higher-K_m enzyme. 4. It is suggested that the effect of insulin on adipose tissue phosphodiesterase accounts for the ability of this hormone to lower cyclic-AMP concentration in the tissue.

Insulin has been shown to lower the cyclic AMP concentration in adipose tissue incubated with lipolytic hormones (Butcher, Sneyd, Park & Sutherland, 1966; Butcher, Baird & Sutherland, 1968). It is likely that this lowering of cyclic AMP concentration accounts for the anti-lipolytic action of insulin and for its effects on phosphorylase and glycogen synthetase in adipose tissue (Jungas, 1966). It is not clear how insulin lowers cyclic AMP concentration, but it seems that it must either decrease the rate of formation or increase the rate of destruction of the cyclic nucleotide. This work was undertaken to determine whether insulin's ability to lower cyclic AMP concentration might be caused by stimulation of the specific phosphodiesterase (EC 3.1.4.c) that breaks down the cyclic nucleotide.

MATERIALS AND METHODS

Male albino rats (Wistar strain, 150–200g) were obtained from the Otago Medical School Animal Department. Crystalline porcine insulin was a gift from Dr J. McGuire of Eli Lilly and Co. (Indianapolis, Ind., U.S.A., lot PH 5589). The cyclic [^{3}H]AMP (1.4Ci/mmol) was purchased from Schwarz BioResearch (Orangeburg, N.Y., U.S.A.) This preparation was satisfactory on arrival but after storage at 0°C for several months required repurification. This was done by sequential passage through cation- and anion-exchange resins (Bio-Rad AG 50W, X8, 200–400 mesh, H^+ form, and Bio-Rad AG 1, X2, 200–400 mesh, Cl^- form). Cyclic AMP, adenosine and *Crotalus atrox* snake venom were purchased from Sigma Chemical Company (St Louis, Mo., U.S.A.). The ion-exchange resins were purchased from Bio-Rad Laboratories (Richmond, Calif., U.S.A.) and used without further purification. Bovine serum albumin (fraction V) was supplied by Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). All other chemicals used were of analytical grade.

Preparation of isolated fat-cells. Isolated fat-cell suspensions were prepared by the method of Rodbell (1964) and dispensed in 1ml portions into plastic scintillation vials for incubation. Dry-cell weights were determined by pipetting 1ml portions of the cell suspension on to preweighed Millipore filter pads (0.8μm pore size; Millipore Corp., Bedford, Mass., U.S.A.) and drying, initially by suction and then in a desiccator before reweighing.

In all experiments with isolated cells, the cell preparation was checked by incubating samples with 0.3mM-[U-^{14}C]glucose in modified Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing half the recommended amount of calcium and 3.5% (w/v) bovine serum albumin, in the presence and absence of insulin (100μU/ml). Insulin caused a threefold or greater stimulation of $^{14}CO_2$ production in all experiments.

It is well known that there is considerable variation in metabolic activity of adipose tissue from one animal to another (Steinberg & Vaughan, 1965; Cahill & Renold 1965). To allow for this variation all experiments with fat-pads employed paired tissue, i.e. the control and insulin-treated tissues were obtained from the same rat. In experiments with isolated fat-cells all experimental treatments were carried out on the same batch of fat-cells.

Incubation of adipose tissue. Rat epididymal fat-pads were excised and cut longitudinally into two pieces. They were incubated in plastic scintillation vials in pairs, in such a way that each vial contained two pieces of tissue, one from each side of the same animal. Incubation was carried out in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing one half the recommended amount of calcium, under an atmosphere of O_2+CO_2 (95:5). After a preliminary incubation of 45min at 37°C

the pads were removed, transferred to fresh medium and incubated for 10min at 37°C in the presence or absence of insulin. The pads were then homogenized in 4ml of water at 30°C and this homogenate was used immediately in the assay system.

Isolated fat-cells were incubated in Krebs–Ringer bicarbonate buffer containing one half the recommended amount of calcium and 3.5% (w/v) bovine serum albumin. After incubation for 10min at 37°C in the presence or absence of insulin the vials were removed from the incubator and a volume of 60μM-cyclic AMP in water equal to the volume of isolated fat-cell suspension was added to each vial. The tissue was then rapidly homogenized while still in the plastic scintillation vials with a Silverson Homogenizer (Silverson Machines Ltd. London S.E.1, U.K.). This crude homogenate was used immediately in the assay system.

Preparations obtained by homogenizing isolated fat-cells in water showed a variable and often rapid decline in phosphodiesterase activity with time. This was prevented by the addition of cyclic AMP to the homogenizing medium.

The time-delay between addition of non-radioactive cyclic AMP and commencement of the assay was less than 1min and was kept constant for all samples. The amount of substrate broken down in this time was less than 0.5% of that initially present which would not appreciably affect the final values obtained for enzyme activity.

In kinetic experiments where it was necessary to manipulate substrate concentrations in the assay system, the isolated fat-cells were homogenized in a final concentration of 0.25M-mannitol instead of the cyclic AMP solutions. Mannitol prevented the decline in phosphodiesterase activity observed in homogenates made in water.

Assay of phosphodiesterase activity. The phosphodiesterase assay mixture consisted of 6μmol of tris–HCl buffer, pH7.5, 0.3μmol of $MgCl_2$ and cyclic [^{3}H]AMP (about 10000c.p.m.) in a volume of 100μl. The reaction was started by the addition of 50μl of the tissue homogenate containing non-radioactive cyclic AMP (1.5nmol). In experiments with fat-pads, or kinetic experiments with isolated fat-cells when the tissue homogenate contained no non-radioactive cyclic AMP, this was included in the 100μl sample. The mixture was incubated at 30°C for a total of 30min. After the first 20min, 20μl of a solution of *C. atrox* snake venom (0.5mg/ml in 40mM-tris–HCl buffer, pH7.5) was added. The snake venom contains a powerful nucleotidase which is added in excess to convert 5′-AMP formed by the phosphodiesterase into adenosine. Phosphodiesterase action was terminated by the addition of 50μl of 5mM-adenosine in 50mM-EDTA, pH4.6, and the samples were kept at 0°C until chromatographed. Storage at this temperature for up to 4h did not alter the amount of adenosine present. Times greater than 4h were not tested, since samples were chromatographed within 2h. In experiments with insulin-treated cells the hormone was present during all steps at the same concentration as in the incubation medium.

Adenosine formed during the assay by the actions of phosphodiesterase and the nucleotidase of snake venom was separated from unchanged cyclic AMP by a simple anion-exchange chromatography procedure. The sample (0.22ml) was applied to a column (2.5cm×0.5cm) of anion-exchange resin (Bio-Rad AG 1; X2, 200–400 mesh, Cl^- form) and washed through with 3.5ml of water. Unchanged cyclic AMP remained on the column, and adenosine was eluted. Recovery of adenosine was calculated from the extinction at 260nm and was better than 96% as a routine.

The validity of this separation method was checked in two ways. First, a sample from the assay was subjected to high-voltage paper electrophoresis (5kV for 30min) in 0.02M-sodium phosphate buffer, pH8.0. The areas corresponding to cyclic AMP and adenosine were identified under a u.v. lamp, cut out and their radioactivity was counted. By this method the amount of adenosine formed was found to correspond exactly to the amount of cyclic AMP that had disappeared. The second check consisted of eluting the anion-exchange column with 0.02M-HCl after the water elution. This removed cyclic AMP, the radioactivity of which could then be counted. By this method also, the formation of adenosine was found to correspond to the disappearance of cyclic AMP.

Radioactivity in the aqueous effluent from the columns was determined by counting a sample (usually 2ml) in 10ml of scintillation fluid consisting of naphthalene (125g), 2,5-diphenyloxazole (7.5g) and *p*-bis-(*O*-methylstyryl)benzene (0.37g) in dioxan (1 litre). The radioactivity of samples was counted in a Packard model 3375 scintillation spectrometer and corrected for quenching by use of external standards.

The formation of adenosine in the assay was linear for 90min at substrate concentrations of 10μM or higher and linear for 60min at the lowest substrate concentration used, provided less than 20% of the substrate was utilized.

Denaturation of insulin. Porcine insulin (40mU/ml) was incubated overnight at room temperature with 10mM-2-mercaptoethanol at pH8.7 and then dialysed for 24h at 4°C against three changes of water. This preparation failed to stimulate $^{14}CO_2$ production from [U-^{14}C]glucose by isolated fat-cells.

RESULTS

Effect of insulin on phosphodiesterase activity. In preliminary experiments with epididymal fat-pads incubated *in vitro*, the addition of insulin (800μU/ml) to the incubation medium produced an increase in the activity of phosphodiesterase in homogenates of the pads. In 12 such experiments with paired pads, ten showed a clear increase in phosphodiesterase activity in pads incubated with insulin and two showed no significant change (Table 1). The increase in phosphodiesterase activity in the insulin-treated tissue was significant at the $P=0.005$ level (Student's t test for paired variates).

The effect of insulin could be clearly demonstrated on isolated fat-cells, showing that the effect was on the adipocyte itself rather than on other cell types in the fat-pads. This preparation gave more consistent results than fat-pads, probably because phosphodiesterase from other cell types was not present to cause interference. In 51 consecutive experiments with control and insulin-treated cells

Table 1. *Effect of insulin on phosphodiesterase activity of adipose tissue*

Rat epididymal fat-pads (1–2g) were incubated for 45min in modified Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932), transferred to fresh medium with or without insulin and incubated for a further 10min. The pads were then homogenized in 4ml of water at 30°C and phosphodiesterase activity was assayed immediately at a substrate concentration of 10μM as described in the text. Isolated fat-cells were prepared, dispensed in 1ml portions into plastic vials and incubated for 10min in modified Krebs–Ringer bicarbonate buffer containing 3.5% (w/v) bovine serum albumin in the presence or absence of insulin. At the end of the incubation 1ml of 60μM-cyclic AMP was added to each vial and the cells were homogenized and immediately assayed for phosphodiesterase activity as described in the text. Values are means±S.E.M., for the number of observations in parenthesis.

Preparation	Additions	Phosphodiesterase activity (nmol of adenosine formed/min per g dry wt.)	
Fat-pads	None	3·49 ± 0·23	(12)
	Insulin (800μU/ml)	4·33 ± 0·28	
Isolated cells	None	4·42 ± 0·23	(38)
	Insulin (800μU/ml)	5·34 ± 0·33	
Isolated cells	None	4·62 ± 0·39	(13)
	Insulin (100μU/ml)	5·50 ± 0·48	

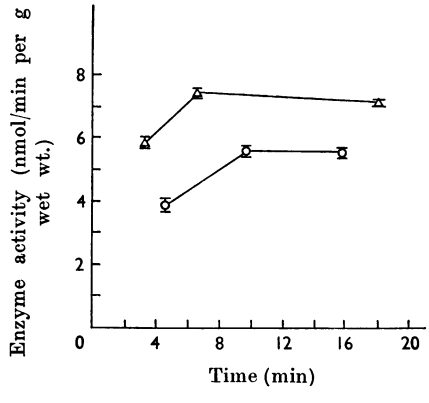

Fig. 1. Effect of incubation time of isolated cells on phosphodiesterase activity in homogenates from the cells. Isolated fat-cells were prepared and incubated, as described in Table 2, for various times in the presence (△) and absence (○) of insulin. Homogenates were prepared and assayed as described in the text. Values are means ±S.E.M. of triplicate observations.

all but two showed a clear increase in enzyme activity with insulin, whereas one showed a decrease and one no change. The insulin concentration was either

Table 2. *Effect of insulin on phosphodiesterase prepared from cells incubated in the absence of insulin*

Isolated fat-cells were dispensed in 1ml portions into plastic vials and incubated for 15min in the absence of insulin. After incubation, 1ml of 60μM-cyclic AMP was added to each vial and the cells were homogenized. This homogenate was assayed for phosphodiesterase activity as described in the text both with and without insulin (800μU/ml) in the reaction mixture. Values are means± S.E.M. of triplicate determinations.

Expt. no.	Additions to assay mixture	Phosphodiesterase activity (nmol of adenosine formed/min per g dry wt.)
1	None	4·66 ± 0·26
	Insulin (800μU/ml)	4·40 ± 0·10
2	None	4·74 ± 0·44
	Insulin (800μU/ml)	4·79 ± 0·10

Table 3. *Effect of zinc sulphate and denatured insulin on phosphodiesterase activity from isolated fat-cells*

Isolated fat-cells (2ml) were incubated with the addition of various compounds for 15min before being homogenized as described in Table 4. The cells were homogenized in medium containing the same concentration of the compounds that they were incubated with. This concentration was maintained also in the assay mixture. Values are means±S.E.M. of triplicate determinations.

Additions	Phosphodiesterase activity (nmol of adenosine formed/min per g dry wt.)
None	3·53 ± 0·15
$ZnSO_4$(1μM)	1·94 ± 0·17
None	4·53 ± 0·14
Insulin (800μU/ml)	5·85 ± 0·51
$ZnSO_4$ (0.1μM)	4·22 ± 0·39
None	3·59 ± 0·18
Insulin (800μU/ml)	5·86 ± 0·01
Denatured insulin (800μU/ml)	3·22 ± 0·29

800μU/ml or 100μU/ml, both concentrations being equally effective (Table 1). The increase in phosphodiesterase activity was highly significant ($t=6·547$, $P \ll 0·001$; by using Student's t test for paired variates). The effect was manifest rapidly (Fig. 1); times shorter than 3min were not tested.

Fig. 1 shows that phosphodiesterase activity from control cells increased over the first few minutes of incubation. At the earliest times measured the fractional increase in activity is therefore greater than at a time when phosphodiesterase activity from control cells is steady. The possibility that this early rise in control activity was due to insulin

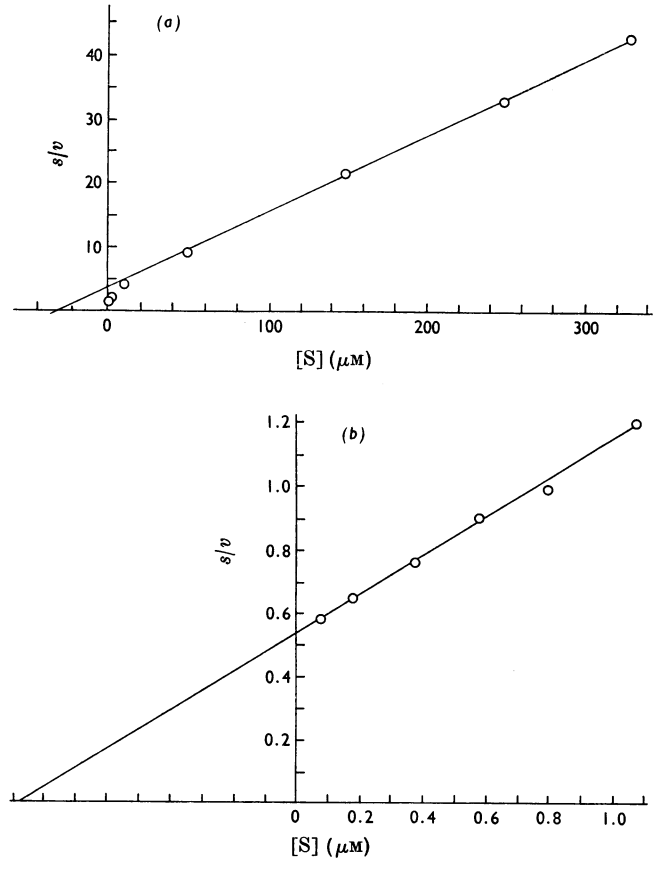

Fig. 2. Kinetic data for adipose-tissue phosphodiesterase. Enzyme activity was measured in homogenates of isolated fat-cells as described in the text, except that the substrate concentration varied from 1 to 330 μM (*a*), and from 76 nM to 1·08 μM (*b*). Values for points on the graph were calculated from observed velocities at various substrate concentrations.

contamination of the bovine serum albumin was considered unlikely since the cells had been isolated and washed in the medium in which they were subsequently incubated.

Since insulin was present not only when cells were incubated but also during the homogenization and assay procedure it was not clear at which stage it was exerting its effect. Insulin added to the homogenate prepared from cells incubated in the absence of insulin was without effect (Table 2). It thus appears that the effect of insulin requires the presence of intact fat-cells. The effect appeared to be specific for insulin. Zn^{2+} (0·1 μM) had no stimulatory effect and a higher concentration (1 μM) was inhibitory. Insulin that had been inactivated by incubation with 2-mercaptoethanol was also without effect (Table 3).

Kinetics of adipose-tissue phosphodiesterase. All the initial experiments were performed at a substrate concentration of 10 μM, since, if insulin were to increase the affinity of the enzyme for cyclic AMP, phosphodiesterase activation might be manifest only at substrate concentrations below the K_m. K_m for phosphodiesterase in a homogenate of isolated fat-cells was determined from a plot of s/v against s and found to be 29 μM (Fig. 2*a*), which is in fair agreement with the findings of Blecher, Merlino & Ro'Ane (1968) who obtained a value of 23.5 μM. The plot appeared to deviate from linearity at low substrate concentrations. When the kinetics were studied at substrate concentrations lower than 10 μM it became evident that in the Lineweaver–Burke plot or the plot of s/v against s there were two separate linear portions of the graph with different slopes (Figs. 2*a* and 2*b*). The simplest explanation for this effect is the presence of two proteins with

Table 4. *Kinetic data for adipose-tissue phosphodiesterase*

Enzyme activity was measured in homogenates of isolated fat-cells incubated for 10 min in the presence or absence of insulin (100 μU/ml). The values for the low-K_m enzyme were derived from measurements made at substrate concentrations below 1 μM, and those for the high-K_m enzyme from measurements made at substrate concentrations between 50 and 330 μM.

	Low-K_m enzyme		High-K_m enzyme	
Addition	K_m (μM)	$V_{max.}$ (pmol of adenosine formed/min per g dry wt.)	K_m (μM)	$V_{max.}$ (pmol of adenosine formed/min per g dry wt.)
None	0.88	1700	41	6860
Insulin	0.87	2640	27	6830

phosphodiesterase activity, although one enzyme displaying negative co-operativity cannot be excluded (Levitski & Koshland, 1969). On the assumption that two enzymes were present, two sets of values for K_m and $V_{max.}$ were estimated (Table 4). The $V_{max.}$ and K_m of the low-K_m enzyme were determined from a plot of s/v against s at substrate concentrations below 1 μM. The reaction velocity of the high-K_m enzyme was determined by subtracting the $V_{max.}$ of the low-K_m enzyme from the observed velocities at substrate concentrations above 50 μM, and by using these values in a s/v against s plot. This procedure assumes (*a*) the reaction velocities of the two enzymes are additive; (*b*) at substrate concentrations above 50 μM an increase in the substrate concentration does not appreciably increase the velocity of the low-K_m enzyme; (*c*) at substrate concentrations below 1 μM

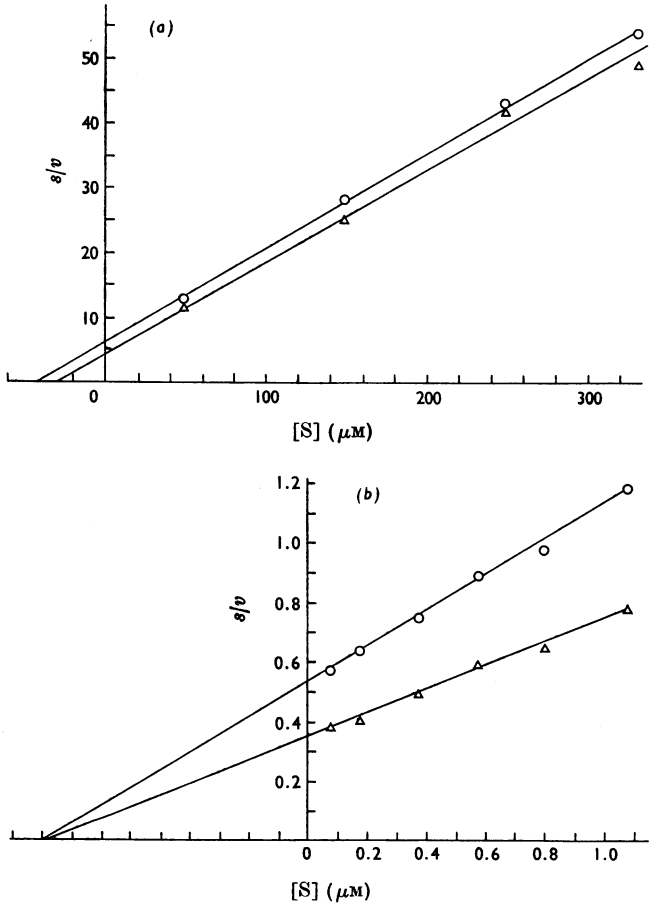

Fig. 3. Kinetic data for phosphodiesterase from control (○) and insulin-treated (△) tissue. Enzyme activity was measured in homogenates of isolated fat-cells that had been incubated for 10 min in the presence or absence of insulin (100 μU/ml). Values for points on the graphs were calculated from velocities derived as described in the text, at substrate concentrations from 50 to 330 μM (*a*), and 76 nM to 1.08 μM (*b*).

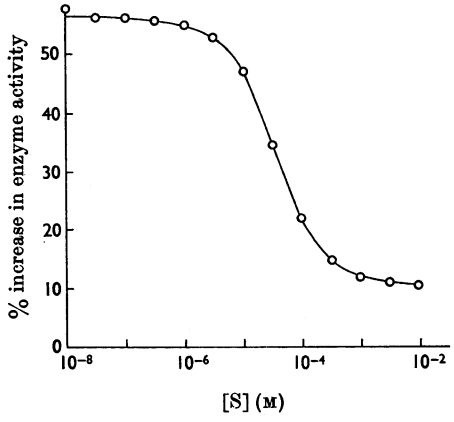

Fig. 4. Dependence on substrate concentration of the insulin effect on adipose-tissue phosphodiesterase. The values were derived by calculating the total activity of two enzymes with the kinetic parameters given in Table 4 acting on the same substrate at a range of substrate concentrations.

the high-K_m enzyme does not contribute appreciably to the observed velocity.

The effect of insulin on the kinetics of phosphodiesterase was then studied by using homogenates prepared from control and insulin-treated isolated fat-cells. Insulin raised the $V_{max.}$ of the low-K_m enzyme without affecting the K_m. Insulin also lowered the K_m of the high-K_m enzyme without changing the $V_{max.}$ (Figs. 3*a* and 3*b*, Table 4).

DISCUSSION

The finding of two apparent K_m values for adipose-tissue phosphodiesterase follows reports from Brooker, Thomas & Appleman (1968) of a similar phenomenon in rat brain, and from Beavo, Hardman & Sutherland (1970) who found two K_m values for cyclic AMP phosphodiesterase in ox heart. The latter group published values of 1 μM and 50 μM, which are close to the values in the present work. It appears that either there are two enzymes present in the crude homogenate, or that only one enzyme is present but that it displays unusual kinetic behaviour. This type of behaviour has been reported by Levitski & Koshland (1969) for enzymes displaying negative co-operativity. Distinction between these possibilities must await purification of the enzyme and demonstration of either one or two catalytic proteins. In the discussion that follows it will be assumed that two separate enzymes exist, but the argument would remain substantially the same for a single enzyme displaying negative co-operativity.

The K_m values obtained for the enzymes must be regarded as approximate only, since interference between the two enzyme activities tends to alter each value towards a mean. The values obtained here were from substrate concentrations below 1 μM for the low K_m value and concentrations between 50 and 330 μM for the higher K_m value. The main effect of insulin appeared to be to increase the $V_{max.}$ of the lower K_m enzyme, without changing the K_m. The decrease in the K_m of the high-K_m enzyme is small and of doubtful significance, but our results do not allow us to exclude an effect of insulin here.

Other workers (Blecher *et al.* 1968; Hepp, Menahan, Wieland & Williams, 1969) have been unable to detect any alteration of phosphodiesterase activity after insulin treatment of adipose tissue. Both these groups used substrate concentrations considerably higher than those used in the present work. Blecher *et al.* (1968) used a saturating substrate concentration in their assay, and Hepp *et al.* (1969) used two different substrate concentrations, 1 μM and 0·1 mM. From the results of our work it can be shown that since insulin is acting to increase $V_{max.}$ of the low-K_m enzyme, the magnitude of the observed effect is dependent on the substrate concentration. If the percentage increase in enzyme activity with insulin treatment is plotted against the substrate concentration, a curve as in Fig. 4 is obtained. This curve shows that the insulin effect is maximal at concentrations below the lower K_m and declines to a minimum slightly above the higher K_m. At the lowest substrate concentration used by other workers only a relatively small increase in activity is observable; this could easily be obscured by small errors in the assay results, and possibly not recognized as significant.

Hepp *et al.* (1969) were unable to show an action of insulin on lipolysis induced by exogenous cyclic AMP, a finding that would at first sight argue against an action of insulin on phosphodiesterase. However, Goodman (1969) demonstrated a clear action of insulin on lipolysis stimulated by exogenous cyclic AMP. It is difficult to reconcile these two results although different incubation media were used. Hepp *et al.* (1969) tested insulin only against 'maximal' doses of exogenous cyclic AMP, conditions which could mask an anti-lipolytic effect of insulin. Since in these experiments the intracellular concentration of cyclic AMP was unknown, and could have been very high, it is possible that insulin lowered the intracellular cyclic AMP concentration without affecting the degree of activation of the lipolytic system. It has for instance been reported that insulin will lower cyclic AMP concentrations that have been raised with lipolytic hormones, without bringing the cyclic AMP down to a value where it is rate-limiting for lipolysis (Butcher *et al.* 1966, 1968). Further, Hepp *et al.* (1969) showed a clear-cut competitive

antagonism between caffeine and insulin on lipolysis, a finding that points to a common site of action for these agents.

The present results do not indicate how insulin might be exerting its effect on phosphodiesterase. The effect is manifest within 3min, which would argue against an enzyme induction. Preliminary experiments with cycloheximide have shown no effect of this agent on insulin-mediated stimulation of phosphodiesterase, making it unlikely that we are observing the same effect as that described by Senft, Schultz, Munske & Hoffman (1968). These workers observed a decreased activity of phosphodiesterase from adipose tissue of diabetic rats and demonstrated that insulin administration returned the activity towards normal. This effect of insulin was blocked by actinomycin D.

It appears that intact fat-cells are necessary since the addition of insulin to homogenates was without effect. The increase in phosphodiesterase activity cannot be secondary to increased glucose transport since there was no glucose present in the incubation medium of either the fat-pads or isolated cells.

Although Jungas (1966) showed that labelled cyclic AMP accumulation from labelled ATP was decreased in homogenates from epididymal fat-pads that had been incubated with insulin, other workers using isolated fat-cells (Williams, Walsh, Hepp & Ensinck, 1968) fat-cell 'ghosts' (Rodbell, 1967; Cryer, Jarett & Kipnis, 1969) and homogenates of adipose tissue (Vaughan & Murad, 1969) have not shown an influence of insulin on adenyl cyclase activity.

Although the effect on phosphodiesterase is small it is probably sufficient to account for the decrease in cyclic AMP concentration in response to insulin. The formation of cyclic AMP by adenyl cyclase in cells in the basal state is slow (Birnbaumer, Pohl, & Rodbell, 1969) whereas the activity of phosphodiesterase is comparatively high. Consequently only a small increase in the activity of the latter enzyme could significantly alter the amount of cyclic AMP present.

We thank the New Zealand Medical Research Council and the Golden Kiwi Medical Research Committee for financial assistance. E.G.L. is the holder of a New Zealand Medical Research Council Training Fellowship.

REFERENCES

Beavo, J. A., Hardman, J. G. & Sutherland, E. W. (1970). *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 2077.

Birnbaumer, L., Pohl, S. L. & Rodbell, M. (1969). *J. biol. Chem.* **244**, 3468.

Blecher, M., Merlino, N. S. & Ro'Ane, J. T. (1968). *J. biol. Chem.* **243**, 3973.

Brooker, G., Thomas, L. J. & Appleman, M. M. (1968). *Biochemistry, Easton*, **7**, 4177.

Butcher, R. W., Baird, C. E. & Sutherland, E. W. (1968). *J. biol. Chem.* **243**, 1705.

Butcher, R. W., Sneyd, J. G. T., Park, C. R. & Sutherland, E. W. (1966). *J. biol. Chem.* **241**, 1652.

Cahill, G. F. & Renold, A. E. (1965). In *American Handbook of Physiology*, Section 5, p. 683. Ed. by Renold, A. E. & Cahill, G. F. Washington D.C.: American Physiological Society.

Cryer, P. E., Jarett, L. & Kipnis, D. H. (1969). *Biochim. biophys. Acta*, **177**, 586.

Goodman, H. M. (1969). *Proc. Soc. exp. Biol. Med.* **130**, 97.

Hepp, K. D., Menahan, L. A., Wieland, O. & Williams, R. H. (1969). *Biochim. biophys. Acta*, **184**, 554.

Jungas, R. L. (1966). *Proc. natn. Acad. Sci. U.S.A.* **56**, 757.

Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyler's Z. physiol. Chem.* **210**, 33.

Levitski, A. & Koshland, D. E. (1969). *Proc. natn. Acad. Sci. U.S.A.* **62**, 1121.

Rodbell, M. J. (1964). *J. biol. Chem.* **239**, 375.

Rodbell, M. J. (1967). *Biochem. J.* **105**, 2P.

Senft, G., Schultz, G., Munske, K. & Hoffman, M. (1968). *Diabetologia*, **4**, 322.

Steinberg, D. & Vaughan, M. (1965). In *American Handbook of Physiology*, Section 5, p. 343. Ed. by Renold, A. E. & Cahill, G. F. Washington, D. C.: American Physiological Society.

Vaughan, M. & Murad, F. (1969). *Biochemistry, Easton*, **8**, 3092.

Williams, R. H., Walsh, S. A., Hepp, D. K. & Ensinek, J. W. (1968). *Metabolism*, **17**, 653.