Biochem. J. (1983) **212**, 189–195
Printed in Great Britain

Glucagon inhibition of insulin-stimulated 2-deoxyglucose uptake by rat adipocytes in the presence of adenosine deaminase

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(*Received 29 November 1982/Accepted 3 December 1982*)

Effects of adenosine deaminase and glucagon on insulin-stimulated 2-deoxyglucose uptake by rat adipocytes are reported. (1) Adenosine deaminase (10μg/ml) caused a rightward shift in the dose–response curve for the stimulation by insulin of 2-deoxyglucose uptake, but the enzyme did not alter either the basal or the maximally insulin-stimulated uptake rate. (2) In adipocytes obtained from 24h-starved rats, glucagon inhibited the effect of insulin on 2-deoxyglucose uptake in the presence (but not in the absence) of adenosine deaminase. Basal uptake rates were unaffected. (3) Glucagon inhibited insulin-stimulated 2-deoxyglucose uptake to a greater extent in cells isolated from starved rats than in cells from fed rats. (4) Adipocytes isolated from fed and from starved rats did not differ in their capacity for degradation of ^{125}I-labelled glucagon. The results suggest that adenosine and glucagon are regulators of insulin action in adipose tissue.

Glucagon is known to antagonize the effects of insulin on glucose metabolism in the liver (for reviews see Alford & Chisholm, 1979; Unger & Orci, 1981). However, glucagon has not been demonstrated to antagonize insulin action in other tissues. In fact, the only known effect of glucagon on peripheral tissues is its ability to stimulate lipolysis in adipose tissue and isolated adipocytes (see Lefebvre, 1975). The rather high concentration of glucagon required to elicit this lipolytic effect has raised questions as to its physiological significance (Blecher *et al.*, 1969; Fernandez & Saggerson, 1978). However, Honnor & Saggerson (1980) have demonstrated that the sensitivity of isolated adipocytes to the lipolytic effect of glucagon is greatly enhanced in the presence of adenosine deaminase. This effect of adenosine deaminase was presumed to result from removal of adenosine from the incubation medium, since it is known that adenosine is released by isolated adipocytes (Schwabe *et al.*, 1973).

In addition to the effects described above, it is known that adenosine alters the response of isolated adipocytes to insulin. Thus it has been demonstrated that adenosine deaminase decreases the sensitivity of adipocytes to the effects of insulin on glucose oxidation (Schwabe *et al.*, 1975), total glucose utilization (Green & Newsholme, 1979) and lipolysis (Schwabe *et al.*, 1975; Green & Newsholme, 1979).

Adenosine modifies the response of isolated adipocytes to both insulin and glucagon, and glucagon antagonizes the effects of insulin on the liver. Therefore it was decided to determine whether glucagon might antagonize the effect of insulin on adipocyte glucose transport if adenosine is removed from the incubation medium.

In these studies, uptake of 2-deoxyglucose was measured as an index of the rate of glucose transport (Olefsky, 1978). The results demonstrate that glucagon inhibits the effect of insulin on 2-deoxyglucose uptake in the presence of adenosine deaminase. Adipocytes isolated from 24h- or 48h-starved rats are more sensitive to glucagon than are those isolated from fed rats. The results suggest that glucagon and adenosine are physiologically important regulators of insulin action in adipose tissue.

Materials and methods

Chemicals

Pig insulin and bovine glucagon were generously given by Dr. Ronald Chance of Eli Lilly and Co. (Indianapolis, IN, U.S.A.). Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co. (Phoenix, AZ, U.S.A.). Collagenase

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was from Worthington Biochemical Corp. (Freehold, NJ, U.S.A.). Silicone oil (density 0.99 g/ml) was purchased from A. H. Thomas (Philadelphia, PA, U.S.A.). $Na^{125}I$, L-[1-^{3}H]glucose and 2-deoxy-D-[1-^{3}H]glucose were from New England Nuclear Corp. (Boston, MA, U.S.A.). 2-Deoxy-D-glucose, Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], Tricine {*N*-[2-hydroxy-1,1-bis-(hydroxymethyl)ethyl]glycine} and adenosine deaminase (EC 3.5.4.4) were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Adenosine deaminase was supplied as a suspension in 3.2 M-$(NH_4)_2SO_4$. Before use the enzyme suspension was dialysed against 2 × 500 vol. of 154 mM-NaCl/10 mM-Hepes/NaOH buffer, pH 7.4, to remove $(NH_4)_2SO_4$.

Animals

Male Sprague–Dawley rats weighing 190–230 g were purchased from Charles River (Cambridge, MA, U.S.A.).

Preparation of isolated adipocytes

Animals were killed by cervical dislocation, and epididymal fat-pads were removed. Isolated fat-cells were prepared by shaking at 37°C for 1 h in Krebs–Ringer bicarbonate buffer (Krebs & Henseleit, 1932) containing 5 mM-glucose, collagenase (3 mg/ml) and bovine serum albumin (40 mg/ml), in accordance with the method of Rodbell (1964). Cells were filtered through 250 μm nylon mesh, centrifuged (25 *g* for 90 s) and washed three times in the incubation buffer (see below).

Incubation of adipocytes

Adipocytes (approx. 250000 cells in 1 ml) were incubated with shaking for 1 h at 37°C in a buffer containing 137 mM-NaCl, 5 mM-KCl, 4.2 mM-$NaHCO_3$, 1.3 mM-$CaCl_2$, 0.5 mM-KH_2PO_4, 0.5 mM-$MgCl_2$, 0.4 mM-$MgSO_4$, 10 mM-Hepes, 10 mM-Tricine and bovine serum albumin (10 mg/ml), pH 7.4, and additions as described in the text and Figure legends.

Measurement of 2-deoxyglucose uptake

At the end of the incubation period (see below), 2-deoxy-D-[1-^{3}H]glucose (sp. radioactivity 1.6 Ci/mol) was added (final concn. 0.1 mM). The assays were terminated 3 min later by transferring 200 μl samples of the cell suspension to plastic micro-tubes containing silicone oil (100 μl). The tubes were centrifuged for 30 s in a Beckman Microfuge, and the assay was considered terminated when centrifugation began. The tubes were cut through the oil layer with a razor blade, and the radioactivity in the cell pellet was measured in a liquid-scintillation counter. In each experiment similar incubations were performed with L-[1-^{3}H]glucose in place of 2-deoxyglucose to determine the amount of sugar trapped in the extracellular water space of the cell layer. This amount was always less than 0.05 nmol/2.5 × 10^5 cells, after a 3 min incubation. All data of 2-deoxyglucose uptake have been corrected for this factor.

Iodination of glucagon

Bovine glucagon was iodinated by the chloramine-T method (Hunter & Greenwood, 1962) and purified by chromatography on QAE (quaternary aminoethyl)-Sephadex A-25. The purified ^{125}I-labelled glucagon was >96% precipitable with trichloroacetic acid.

Measurement of ^{125}I-labelled glucagon degradation

Adipocytes were incubated, as described above, with ^{125}I-labelled glucagon (100 ng/ml). At the times indicated in Fig. 5, the amount of degraded glucagon was determined as follows. A sample (200 μl) of the cell suspension was centrifuged with silicone oil (as described above in the method for measurement of 2-deoxyglucose uptake) to separate cells from buffer. A sample (100 μl) of the infranatant was immediately added to ice-cold incubation medium (400 μl). This was followed by addition of iced 15% (w/v) trichloroacetic acid (500 μl). The resulting precipitate was removed by centrifugation (1800 *g* for 10 min), and the radioactivity in the supernatant (representing degraded material) and in the pellet was measured in a γ-radiation counter.

Measurement of cell concentration

Adipocytes were counted by a modification of Method III of Hirsch & Gallian (1968). The cells were fixed in 2% osmium tetroxide in 50 mM-collidine buffer (made iso-osmotic with NaCl) for 24 h at 37°C, and then counted with a Model ZB Coulter Counter.

Expression of results

All results have been normalized to a cell concentration of 2.5 × 10^5 cells/ml. Each value in the Figures represents the mean ± S.D. for three determinations. *P* values were calculated either by analysis of variance or by Student's non-paired *t* test as appropriate.

Results

Effect of adenosine deaminase on the response of 2-deoxyglucose uptake to insulin

Fig. 1 shows the effect of insulin on the rate of 2-deoxyglucose uptake by adipocytes in the presence or in the absence of adenosine deaminase. Insulin produced an approx. 15-fold increase in the rate of uptake. Neither basal nor maximally stimulated rates of uptake were affected by the presence of adenosine deaminase. However, at submaximally effective concentrations of insulin, the effect of the

hormone was lower in the presence of adenosine deaminase. Thus adenosine deaminase decreased the insulin sensitivity of 2-deoxyglucose uptake; the half-maximally effective concentration of insulin was approx. 0.2 ng/ml in the absence of adenosine deaminase and 0.5 ng/ml in the presence of the enzyme. The effect of adenosine deaminase on insulin sensitivity was not observed when N^6-phenylisopropyladenosine (an adenosine analogue that is not deaminated by adenosine deaminase) was included together with the enzyme.

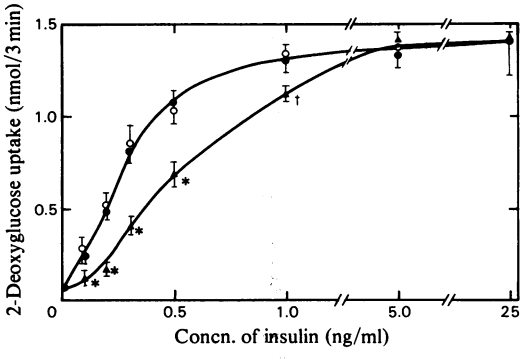

Fig. 1. *Effect of insulin, in the presence or in the absence of adenosine deaminase, on 2-deoxyglucose uptake by adipocytes*

Adipocytes isolated from fed rats were incubated for 1 h with the indicated concentrations of insulin, plus: ●, no further additions; ▲, adenosine deaminase (10 μg/ml); ○, adenosine deaminase (10 μg/ml) and N^6-phenylisopropyladenosine (0.1 μM). 2-Deoxyglucose uptake was measured as described in the Materials and methods sections. Values that are significantly different from controls are indicated by: † $P < 0.01$; * $P < 0.001$. A concentration of insulin of 1 ng/ml equals approx. 0.167 nM.

Effects of glucagon on 2-deoxyglucose uptake

In the absence of adenosine deaminase, glucagon, at concentrations up to 1000 ng/ml, had no effect on the basal rate of 2-deoxyglucose uptake by adipocytes (Fig. 2*a*). Similarly, there was no effect of glucagon on 2-deoxyglucose uptake stimulated by a submaximally effective concentration of insulin (0.5 ng/ml) or a maximally effective concentration (25 ng/ml). In the presence of adenosine deaminase, however (Fig. 2*b*), glucagon completely inhibited the effect of both the submaximal and maximal insulin concentrations. Again, there was no effect on basal uptake. Thus glucagon inhibits the effect of insulin on 2-deoxyglucose uptake, but only in the presence of adenosine deaminase.

The effect of glucagon on insulin-stimulated 2-deoxyglucose uptake was not observed when N^6-phenylisopropyladenosine (0.1 μM) was included together with adenosine deaminase (Fig. 2*b*). This suggests that in the presence of adenosine deaminase it is the low adenosine concentration, rather than the presence of the enzyme itself, that allows glucagon to inhibit the insulin effect.

Effect of starvation on glucagon inhibition of insulin-stimulated 2-deoxyglucose uptake

The experiments described in the preceding section, demonstrating glucagon's antagonism of insulin action, were performed with adipocytes prepared from rats that had been starved for 24 h

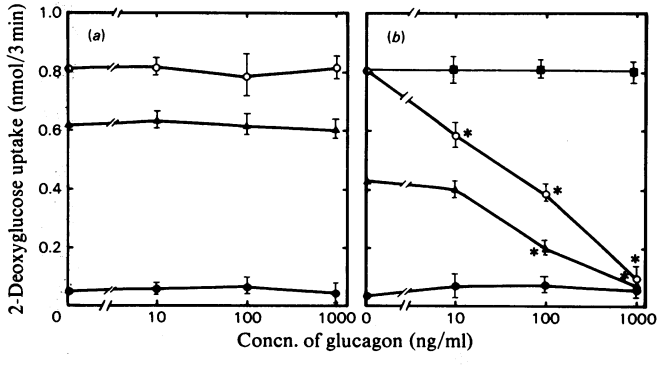

Fig. 2. *Effect of glucagon on basal and insulin-stimulated rates of 2-deoxyglucose uptake by adipocytes (a) in the absence or (b) in the presence of adenosine deaminase*

Adipocytes isolated from 24 h-starved rats were incubated for 1 h with the indicated concentrations of glucagon plus: ●, no further additions; ▲, insulin (0.5 ng/ml); ○, insulin (25 ng/ml); ■, insulin (25 ng/ml) and N^6-phenylisopropyladenosine (0.1 μM). 2-Deoxyglucose uptake was measured as described in the Materials and methods section. Statistically significant effects of glucagon are indicated by: * $P < 0.001$. A concentration of glucagon of 10 ng/ml equals approx. 2.78 nM.

(see the legend to Fig. 2), since preliminary experiments with cells isolated from fed rats had been less successful. Consequently the effects of starvation were more rigorously examined by using cells isolated from fed, 24h-starved and 48h-starved rats in parallel (Fig. 3). All cells were incubated with adenosine deaminase in the presence or in the absence of insulin (25ng/ml). In the absence of glucagon, there was a progressive fall in the rates of both basal and insulin-stimulated 2-deoxyglucose uptake during starvation. This effect of starvation is well documented (Olefsky, 1976). Glucagon had no effect on the basal rates of 2-deoxyglucose uptake by cells from fed or starved rats. In the cells from fed rats (Fig. 3*a*) glucagon had a small inhibitory effect on insulin-stimulated 2-deoxyglucose uptake. However, in the cells from starved rats (Figs. 3*b* and 3*c*) glucagon completely inhibited the effect of insulin.

In order to examine the effect of starvation on the glucagon response independently of the direct effects of starvation on 2-deoxyglucose uptake, the data from Fig. 3 were plotted as percentages of the rates observed in the absence of glucagon (Fig. 4). From this analysis it is clear that there is a progressive increase in the sensitivity of the cells to glucagon during starvation. Thus in the cells from fed rats there was only a 20% inhibition of insulin-stimulated 2-deoxyglucose uptake at the highest glucagon concentration tested, namely 1000ng/ml. Half-maximal inhibition was seen at glucagon concentrations of approx. 56ng/ml in cells from 24h-starved and 8ng/ml in cells from 48h-starved rats.

Glucagon degradation by adipocytes from fed and starved rats

One possible explanation for the difference in glucagon sensitivity between fed and starved rats is that the cells differ in their capacity for glucagon degradation. To evaluate this possibility adipocytes isolated from fed, 24h-starved and 48h-starved rats were incubated, under conditions identical with those used in the preceding experiments, in the presence of ^{125}I-labelled glucagon (100ng/ml). Degraded ^{125}I-labelled glucagon was determined by its solubility in trichloroacetic acid (Fig. 5). Cells from fed and from starved rats degrade glucagon at approximately the same rates.

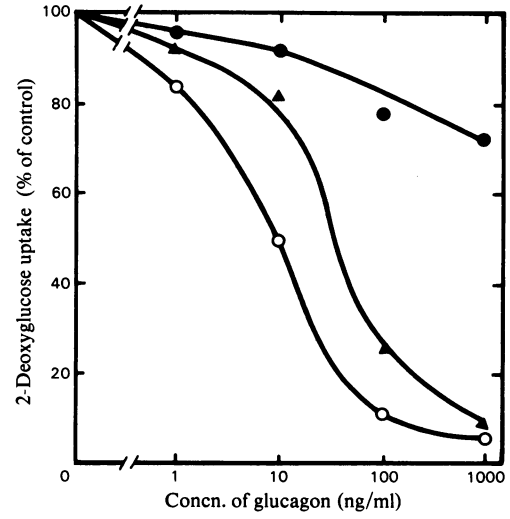

Fig. 4. *Effect of starvation on the response of insulin-stimulated 2-deoxyglucose uptake to glucagon in the presence of adenosine deaminase*

The data on insulin-stimulated 2-deoxyglucose uptake from Fig. 3 were normalized such that the rate in the absence of glucagon is set at 100%. Cells from: ●, fed rats; ▲, 24h-starved rats; ○, 48h-starved rats.

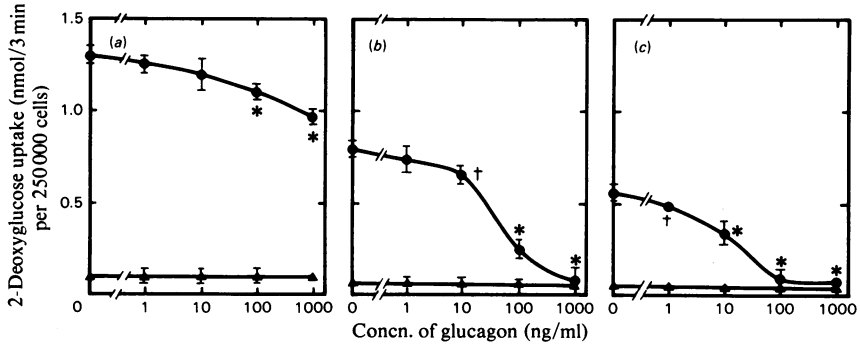

Fig. 3. *Effect of glucagon on 2-deoxyglucose uptake by adipocytes from (a) fed, (b) 24h-starved and (c) 48h-starved rats in the presence of adenosine deaminase*

Adipocytes were isolated, in parallel, from fed, 24h-starved and 48h-starved rats. The cells were incubated for 1h with adenosine deaminase plus the indicated concentrations of glucagon, in the absence (▲) or in the presence (●) of insulin (25ng/ml). 2-Deoxyglucose uptake was measured as described in the Materials and methods section. Statistically significant effects of glucagon are indicated by: †$P < 0.01$; *$P < 0.001$.

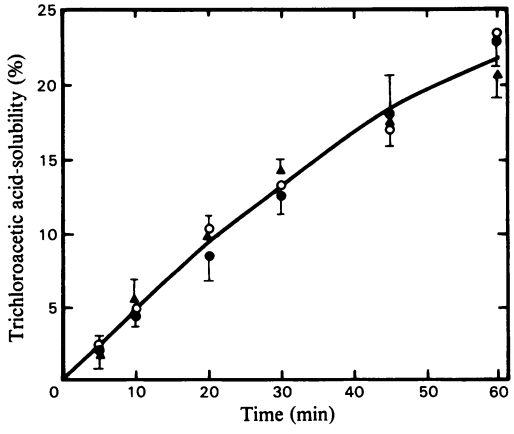

Fig. 5. *Glucagon degradation by adipocytes isolated from fed or starved rats*

Adipocytes were isolated, in parallel, from fed (●) 24h-starved (▲) or 48h-starved (○) rats. The cells were incubated with adenosine deaminase (10μg/ml), insulin (25ng/ml) and ^{125}I-labelled glucagon (10ng/ml). At the indicated times the trichloroacetic acid-solubility of the ^{125}I-labelled glucagon was determined as described in the Materials and methods section. None of the differences approached statistical significance.

Discussion

2-Deoxyglucose is taken up by cells by the D-glucose-transport system and provides a good index of the rate of glucose transport (Olefsky, 1978). The results presented in Fig. 1 demonstrate that the sensitivity of adipocyte 2-deoxyglucose uptake to insulin is decreased by the presence of adenosine deaminase. This suggests that the enzyme decreases the insulin-sensitivity of adipocyte glucose transport. The finding that the non-metabolizable adenosine analogue N^6-phenylisopropyladenosine reverses this effect of adenosine deaminase suggests that the decreased insulin sensitivity in the presence of the enzyme is due to the low adenosine concentration rather than the presence of the enzyme itself. Previous workers have demonstrated that the sensitivity of both glucose oxidation (Schwabe *et al.*, 1975) and total glucose utilization (Green and Newsholme, 1979) to insulin are increased by adenosine. The current findings suggest that this is at least partly due to changes in the insulin sensitivity of the adipocyte glucose-transport system.

The major finding of the present investigation is that glucagon inhibits insulin-stimulated 2-deoxyglucose uptake by adipocytes in the presence of adenosine deaminase. Furthermore, this glucagon effect is not observed in the presence of the enzyme plus N^6-phenylisopropyladenosine (Fig 2*b*). Taken together, these findings strongly suggest that glucagon can antagonize the effect of insulin on glucose transport, and that adenosine can block this effect of glucagon. Thus it appears that glucagon and adenosine can interact to regulate the response of adipocyte glucose transport to insulin.

Honnor & Saggerson (1980) have demonstrated that the lipolytic effect of glucagon in adipocytes is markedly potentiated in the presence of adenosine deaminase. Thus it appears that adenosine can decrease the effect of glucagon on the rate of lipolysis as well as insulin-stimulated glucose transport. This suggests that adenosine has a general effect to decrease the response of adipocytes to glucagon in addition to its known effects to increase the response to insulin (see above). Thus adenosine may be an important regulator of the response of adipocytes to both these hormones.

Another interesting finding made during the present study is that the glucagon-sensitivity of adipocytes from starved rats is greater than in cells from fed rats (Figs. 3 and 4). Honnor & Saggerson (1980) reported that adipocytes from 24h-starved rats showed a higher lipolytic response to glucagon than did those from fed rats. In addition, Caro & Amatruda (1980) have demonstrated that hepatocytes from 72h-starved rats have a greater response to glucagon than have those from fed rats (with stimulation of amino acid uptake being used as a measure of glucagon action). A possible explanation for the apparent increased glucagon-sensitivity in cells from starved rats is that they degrade less glucagon than do those from fed animals. This seemed a particularly attractive possibility, since it has been suggested that changes in rates of glucagon degradation play a significant role in the increase in glucagon concentration that occurs in man during fasting (Fisher *et al.*, 1976). However, the results (Fig. 5) demonstrate that adipocytes from fed, 24h-starved and 48h-starved rats degrade ^{125}I-labelled glucagon at approximately equal rates. It should be pointed out that the method used for measurement of glucagon degradation (solubility in trichloroacetic acid) only measures total degradation to low-molecular-weight products. It is possible that differences in rates of degradation to a biologically inactive, but trichloroacetic acid-insoluble, intermediate are involved in producing the apparent differences in glucagon sensitivity in cells from starved rats. Indeed, it is known that such inactive, but trichloroacetic acid-insoluble, intermediates are produced during degradation of glucagon by adipocytes. Thus Sonne & Gliemann (1977) demonstrated that the rate of degradation of ^{125}I-labelled glucagon by adipocytes is higher when measured by its ability to bind to fresh adipocytes than when measured by trichloroacetic acid solubility.

A second possible explanation for the increased glucagon sensitivity of cells from starved rats is that they might bind more glucagon than do cells from fed rats. However, since glucagon concentrations probably rise during starvation, it would be expected that cells from starved rats would, if anything, bind less glucagon than those from fed rats, owing to down-regulation of receptors. Indeed, Caro & Amatruda (1980) have demonstrated that hepatocytes from 72h-starved rats bind significantly less glucagon than do those from fed rats. This decrease in binding appeared to be due to a decrease in the number of glucagon-binding sites on the cells. Thus it seems likely that deprivation of food 'primes' the cells for glucagon action at a site distal to the glucagon receptor.

Before considering the possible physiological significance of these effects of glucagon and adenosine, it is important to consider the concentrations used in the present studies. Adipose-tissue adenosine concentrations *in vivo* are unknown, as are the changes in adenosine concentrations resulting from addition of adenosine deaminase in the current studies. However, the fact that the present findings result from removal of adenosine produced spontaneously by isolated adipocytes suggests that the cells are capable of producing sufficient adenosine to modify their response to insulin and glucagon. Furthermore, indirect evidence has been presented (Green *et al.*, 1981) suggesting that adipose-tissue adenosine concentrations change in a number of physiological conditions, including starvation. Thus it seems likely that changes in adenosine concentration are involved in regulating the response of adipose tissue to hormones *in vivo*.

It is also important to consider the concentrations of glucagon used in the current studies. In the presence of adenosine deaminase, inhibition of insulin-stimulated 2-deoxyglucose uptake was detectable at glucagon concentrations of approx. 1–10ng/ml in adipocytes from starved rats (Fig. 4). Serum concentrations of approx. 0.3–2ng/ml have been reported in rats (Helman *et al.*, 1980; Woodson & Potter, 1979). To my knowledge there have been no reports of glucagon concentrations in starved rats. However, assuming the situation is analogous to that in man (Marliss *et al.*, 1970), it is likely that the concentrations will rise significantly in serum from starved rats. Furthermore, the interstitial concentration of glucagon in adipose tissue is unknown. In many tissues the glucagon concentration has been reported to be significantly higher than that in serum (Perez-Castillo & Blazquez, 1980). Therefore the glucagon concentrations found to be effective in the current study, although higher than reported serum concentrations, may be within the physiological range. This becomes more apparent when the extent of glucagon degradation during the 1h incubation period (Fig. 5) is considered, since the amounts of biologically active glucagon present during the experiments may have been considerably lower than the added concentrations. However, any physiological interpretation of the findings should be made with caution.

The possible physiological importance of this effect of glucagon may be related to the well-known 'glucose-sparing' effect that occurs during starvation (see Newsholme & Start, 1973). Thus it is likely that during starvation the glucagon concentration rises, as does the sensitivity of adipocytes to this hormone. Assuming that the adenosine concentration in the tissue is fairly low, this situation would result in inhibition of the action of endogenous insulin on glucose transport. This effect, together with the well-established fall in insulin concentration, could act to lower the rate of glucose utilization and thus contribute to the overall decrease in glucose utilization that is known to occur during starvation *in vivo*. However, it should be pointed out that adipose tissue may contribute little to the total rate of glucose utilization *in vivo*. Thus similar studies to those presented here, with more important tissues for glucose utilization, especially muscle, might be of interest.

I am grateful to Dr. J. M. Olefsky, Dr. S. Marshall and Dr. O. G. Kolterman for valuable advice and review of the manuscript, and to Dr. O. G. Kolterman for providing me with ^{125}I-labelled glucagon. This work was supported by Grants AM 25241 and AM 25242 from the National Institute of Arthritis, Metabolism and Digestive Disease.

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