Regulation of Activated Lipolysis by Albumin, Glucose, and H^+.* (32225)

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It is now well established that the addition of epinephrine (E), norepinephrine (NE) and other lipolytic agents to rat epididymal adipose tissue incubated *in vitro* causes a marked increase in the rate of hydrolysis of triglycerides stored in the adipose cells(1,2). The increased rate of triglyceride breakdown may be measured by the determination of glycerol concentration in the medium or by the amount of free fatty acids (FFA) contained in the medium and the tissue after a certain period of time. One portion of the FFA liberated from triglycerides is released into the medium where it is bound to albumin. The other portion remains in the cells where it will undergo re-esterification in the presence of glycerophosphate. It has been established that the relative amount of intracellular or extracellular newly liberated FFA depends upon albumin and glucose. The role of albumin in binding FFA is well documented and it has been demonstrated that in conditions of activated lipolysis when albumin was absent from the medium, FFA accumulates in the cell and inhibits glycerol release and oxygen consumption(3,4). It was also shown that the lipolytic effect of drugs on FFA release from adipose tissue was dependent on the concentration of albumin in the medium(5,6). The role of glucose on the lipolytic effects of E has been studied by Jungas and Ball(3). These authors found that glycerol production by adipose tissue incubated *in vitro* in the presence of E was stimulated by the addition of glucose. On the other hand, FFA accumulation in the tissue during the same experimental conditions was decreased by glucose. It can be postulated that glucose increases the rate of FFA re-esterification by furnishing glycerophosphate which is not produced in adipose tissue(5,7). This hypothesis may also explain why glucose reversed partially the inhibition of O_2 consumption produced by E in adipose tissue incubated in albumin free media(4). Experiments from this laboratory performed *in vivo*(8,9) and *in vitro*(10) have indicated that a third factor influences catecholamine or theophylline induced lipolysis, namely acid pH: Acidosis significantly inhibits catecholamine-induced FFA mobilizaton and glycerol release from adipose tissue. The mechanism by which acidoses inhibits lipolysis is not, however, completely understood. In experiments reported earlier(10), it was suggested that acidoses might act on the enzymatic process which activates cyclic 3′,5′-AMP formation in the cells. Other experiments have shown that, when dogs were infused with 1.5 μg/kg/min of l-norepinephrine, pH fell from 7.42 to 7.34, while FFA concentration increased from a control value of 0.30 mEq/l to 2.0 mEq/l and no change in blood lactic acid occurred(8). Recently, Rudman and Shanks made the same observations in rabbits(11).

The present experiments were designed with a 2-fold purpose: (1) to determine if induced lipolysis and resulting fall in pH due to FFA release could in itself be a rate limiting reaction; and (2) to establish the relative importance of albumin, glucose and (H^+) interaction in the activation of lipolysis.

Methods. Male Sherman rats (average weight 150-200 g) maintained on a Purina Laboratory diet, were used in all experiments. They were permitted free access to food and water until decapitated. The distal portions of epididymal fat pads were removed, cut into pieces and fat cells were prepared as described by Rodbell(12). Adipose tissue was incubated in a shaking waterbath for 90 minutes at 37°C in a Krebs-Ringer phosphate buffered medium containing 5 g%

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TABLE I. Glycerol Production (μMol/ml Fat Cells) in Media Having Two Different Buffer Capacities and with Glucose Omitted.

Media		30 min	60 min	90 min
I (a)	Alb, 1 g/100 ml; NE, 10^{-5} M	1.94 ± .18*	2.51 ± .23	3.13 ± .31
		pH = 6.86	pH = 6.70	pH = 6.65
II (b)		2.64 ± .22	3.82 ± .23	4.56 ± .35
(n=12)†		pH = 7.15	pH = 7.06	pH = 7.00
	P	.02	<.001	<.005
I (c)	Alb, 1 g/100 ml; NE, 10^{-5} M; Th, 10^{-2} M	3.51 ± .17	4.80 ± .07	6.02 ± .06
		pH = 6.92	pH = 6.80	pH = 6.60
II (d)		3.49 ± .05	5.27 ± .10	6.75 ± .09
(n=20)		pH = 7.14	pH = 7.10	pH = 7.08
	P	>.1	<.05	<.001

* Mean ± 1 SEM. † n = No. of experiments.

TABLE II. Effects of Glucose and Albumin on Glycerol Production by Isolated Fat Cells (μMol/ml Fat Cells) Incubated in Media Having Two Different Buffer Capacities.

Media		30 min	60 min	90 min
I (e)	Alb, 1 g/100 ml; NE, 10^{-5} M; Th, 10^{-2} M; glucose, 100 mg /100 ml	2.78 ± .27	4.56 ± .45	5.80 ± .25
II (f)		3.00 ± .36	4.85 ± .45	6.41 ± .31
P (n=10)		NS	NS	NS
I (g)	Alb, 5 g/100 ml; NE, 10^{-5} M; Th, 10^{-2} M	6.50 ± .59	10.52 ± .60	13.06 ± 1.02
II (h)		6.95 ± .34	11.81 ± .70	13.74 ± 1.07
P (n=6)		NS	NS	NS

bovine albumin with 10 mg of bacterial collagenase added to each vial (final volume 3 ml). The mixture contained no glucose. At the end of incubation in the fat cells were washed twice with saline, pooled and 0.1 ml of packed cells were pipetted for incubation in various media.

Solution I, of low buffer capacity, had the following composition (per ml): 2.0×10^{-3} mmol Na_2HPO_4, 1.3×10^{-3} mmol KH_2PO_4, 0.14 mmol NaCl, 5.5×10^{-3} mmol KCl, 6×10^{-4} mmol $MgSO_4 \cdot 7\ H_2O$, 1.5×10^{-3} mmol $CaCl_2$. (The final ionic concentration in mmol/l was: Na 144.0, K 6.8, Mg 0.6, Ca 0.5, PO_4 3.3, Cl 148.5 and SO_4 0.6).

Solution II, of "normal buffer capacity", contained the usual amount of Na phosphate buffer and had the following composition (per ml): 1.8×10^{-2} mmol Na_2HPO_4, 1.3×10^{-3} mmol KH_2PO_4, 0.12 mmol NaCl, 4.7×10^{-3} mmol KCl, 6×10^{-4} mmol $MgSo_4 \cdot 7\ H_2O$, and 1.5×10^{-3} mmol $CaCl_2$. (The final ionic concentration in mmol/l was: Na 156.0, K 6.0, Mg 0.6, Ca 1.5, PO_4 19.3, Cl 127.7 and SO_4 0.6).

Four groups of experiments were performed with the two media of low and normal buffer capacity (Tables I and II): In the first group, fat cells were incubated in the two different media containing the optimal concentration of 10^{-5} M NE and 1 g/100 ml albumin. In the second group, 10^{-5} M NE and 10^{-2} M theophylline (Th) were added to the media containing 1 g/100 ml of albumin. In a third group, the media contained, in addition to albumin (1 g/100 ml) 100 mg/100 ml glucose. In the last group, the media contained 10^{-5} M NE, 10^{-2} M Th, and 5 g/100 ml albumin but no glucose. After the addition of albumin and drugs, pH was adjusted to 7.40 in both media. In some experiments pH was also determined at the end of the incubating period. The final volume in each vial was 2 ml and in all experiments the gas phase was air.

Suspensions of corresponding fat cells were

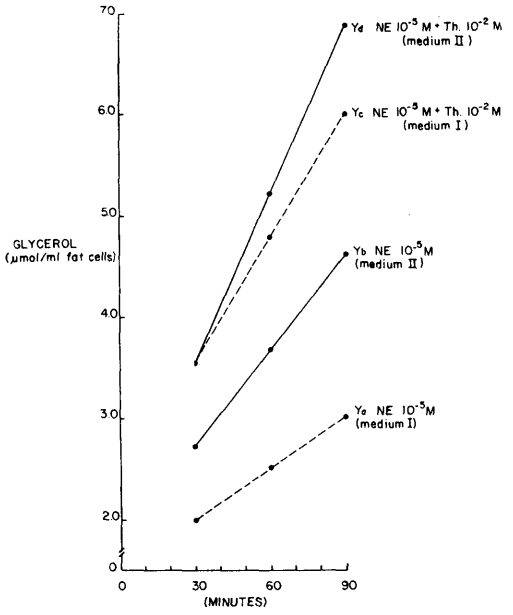

FIG. 1. Norepinephrine (NE) and Theophylline (Th) activated lipolysis as a function of the incubating time. Isolated fat cells were incubated in media of low (medium I) and normal (medium II) buffering capacities. The equations of the lines are: $Y_a = 0.018x + 1.45$ and $Y_b = 0.032x + 1.76$ (Y_a not equal to Y_b). In the second series, the equations of the upper lines are $Y_c = 0.042x + 2.28$ and $Y_d = 0.055x + 1.90$ (Y_c not equal to Y_d). Statistical comparison between slopes: Y_a/Y_b Variance ratio 1.16; $F < 0.05$; b_a not equal to b_b ($n = 68$); $2P > 0.05$; Y_c/Y_d Variance ratio 1.40; $F < 0.05$; b_c not equal to b_d ($n = 116$); $2P > 0.01$.

incubated separately for each interval of time. Glycerol concentration (expressed in micromoles per ml of fat cells) in the media was determined at the end of the incubating period according to the colorimetric method of Lambert and Neish(13) with Korn's modification(14). When glucose was added, glycerol was determined by the enzymatic method(15). Close agreement was found between the colorimetric and enzymatic methods in control experiments using glucose-free media. pH was measured with a micro glass electrode (Radiometer). Bovine albumin (fraction V, Armour Laboratories) was used without further purification (FFA content: 2.8 μEq/g albumin). Concentrations of NE (Winthrop Laboratories) and Th (from commercial purchase) are expressed in moles of free base. Statistical evaluation of the results was made by the student "t" test and P was evaluated within the 95% limit of confidence with $n_x + n_y - 2$ degree of freedom. Calculations of the regression lines were obtained by the method of least squares. Comparisons between the regression coefficience and the variances of the lines were made according to current methods(16).

Results. a. Effect of buffer capacity on glycerol production induced by NE (Table I, Fig. 1): In the first group of experiments fat cells were incubated in media containing 1% albumin and 10^{-5} M NE. In solution II which had the "normal" buffer capacity, glycerol production was in each group of samples significantly higher than in solution I with the low buffer capacity. In 8 out of 12 experiments, pH was measured at the end of incubation (Table I): pH fell from 7.40 to 7.0 in solution II, and to 6.65 in solution I. The two regression lines (Fig. 1) (Y_a corresponding to solution I and Y_b to solution II) fitted the test of linearity. The regression coefficient (b_b) in solution II is $b_b = 0.0318 \pm 0.0061$ ($2P < 0.005$) and significantly different from $b_a = 0.0184 \pm 0.0061$ ($2P < 0.001$) ($n = 34$). The equations of the lines Y_a and Y_b are also significantly different.

b. Effect of buffer capacity on glycerol production induced by theophyllines and NE: In the second group of experiments, 10^{-2} M theophylline, 10^{-5} M NE and 1% bovine albumin were added to each medium (Table I). In both conditions, the glycerol production was much higher than in the first series. At the 60th and 90th minute of incubation, glycerol production was still significantly higher in medium II than in medium I and the corresponding changes in pH at the end of incubation were of the same magnitude as in the first series. The regression lines (Y_c and Y_d, Fig. 1) are also linear. The regression coefficient for solution II is: $b_d = 0.0552 \pm 0.0021$ ($2P < 0.001$) and for solution I $b_c = 0.0419 \pm 0.0025$ ($2P < 0.001$). Coefficient b_c is significantly different from b_d (Fig. 1).

c. Effect of glucose added to the medium. When glucose was added to the media containing 10^{-5} M NE, 10^{-2} M Th and 1 g/100 ml albumin, glycerol production was iden-

tical for each period of time in both media (Table II). The equations of the lines were respectively for solution II with regular buffer capacity $Y_f = 0.0560x + 1.345$ and the regression coefficient $b_f = 0.0560 \pm 0.0083$; for solution I with low buffer capacity $Y_e = 0.0504x + 1.386$ and $b_e = 0.0504 \pm 0.0075$. Both lines were found to be statistically linear, parallel and identical ($Y_e = Y_f$). Variance ratio = 1.40 ($F<0.05$), $b_e = b_f$ ($2P = 0.6$) ($\bar{n} = 56$).

d. Effect of increased albumin concentration: In the last group of experiments, 5% bovine albumin was added to the two different media containing both 10^{-5} M NE and 10^{-2} M Th. In these conditions the glycerol production was significantly increased when compared to the previous series, but it was no longer dependent upon the buffer capacity of the medium. The equation of the lines were $Y_g = 0.0955x + 4.30$ with $b_g = 0.0955 \pm 0.0060$ (solution I) and $Y_h = 0.1030x + 4.05$ with $b_h = 0.1030 \pm 0.0070$ (solution II), respectively, where x is the time in minutes (see legend Fig. 1). Both equations fitted the test of linearity, parallelism and identity ($Y_g = Y_h$). Variance ratio = 1.22 ($F<0.05$) $b_g = b_h$ ($2P = 0.8$) ($\bar{n} = 32$).

Discussion. Because of the absence of α glycerol kinase in adipose tissue(17), glycerol may be considered as the end product of triglyceride breakdown. In these experiments, the glycerol production was taken as the index of the lipolytic activity of the fat cells. In each group of experiments, the rate of glycerol production was constant during the period of the study as shown by the strict linearity of the regression lines of the rate of glycerol production. In these conditions the equation of the line will express the total glycerol production while the slope of the line will give the rate of glycerol production per unit of time. Rudman and Shanks(11) have shown that in the presence of albumin in the incubation medium a marked acidosis occurs during hormonal stimulation of lipolysis. According to these authors, FFA molecules newly formed from triglycerides breakdown are continuously released in the medium where they dissociate to be bound by albumin molecules. The H^+ resulting from the dissociation of FFA may account for the decrease in pH of the medium. In the present experiments a marked decrease of pH in both media was also observed when lipolysis was activated by NE. When pH fell from 7.40 to 6.60 (medium I) both glycerol production and lipolytic activity were inhibited as compared to medium II. This inhibition is comparable to that reported elsewhere(10), but in the present case accumulation of H^+ was produced by lipolysis itself. It seems, therefore, that lipolysis may be a self limiting process with H^+ produced by the FFA dissociation playing a definitive role.

It has been demonstrated *in vitro*(10) that, when 10^{-2} M Th was added to 10^{-5} M NE, maximal activation of lipolysis may be achieved in a medium containing 5 g albumin/100 ml but no glucose. In these conditions the rates of lipolysis were similar at pH 7.4 or 6.6. This observation was interpreted as indicating that, when intracellular lipases were completely activated, the lipolytic activity of the system was not pH dependent, or that increased $[H^+]$ might exert in normal conditions its inhibitory effect by affecting lipase activation. This hypothesis was tested under the present experimental conditions by the addition of 10^{-2} M Th to the media. In the second series of experiments, glycerol production was potentiated as compared to the first series (Fig. 1) in spite of similar changes in pH. However, the two slopes of the lines Y_c and Y_d are still significantly different; this means that the inhibition of the lipolytic activity observed in the first series is partially but not completely reversed by the addition of theophylline. It is assumed that maximal activation of lipases was achieved through the combination of the drugs, then one may postulate that under these particular conditions, the activating process of lipolysis is no longer the rate limiting step and that some other factor is involved in the inhibition. Furthermore, lines Y_b and Y_c (Fig. 1, Table III) have similar slopes b_b and b_c, *i.e.*, the lipolytic activity due to NE alone in the normal buffered medium was the same as that due to NE and Th combined in the acidotic medium. Different factors may be responsible for the

inhibition of the lipolytic rate observed in the second group of experiments. Phosphate, besides being a substrate for phosphorylation reactions, may enhance the lipolytic activity (2,5). As the concentration of phosphate in medium I was much less than in medium II, this could account for the inhibition.

The greater $[H^+]$ observed in medium II may influence lipolysis in two ways: first, FFA are weak acids with pKs between 4.5 and 5.5(18). Therefore, when pH decreases, less FFA will dissociate and more should remain in the cell. One may, however, calculate that this would account for a decrease of only 5 to 6% in the percentage of ionization in a medium of pH 6.6 compared to a medium of pH 7.0. A weakening of the ionic bonds which link the anionic part of dissociated FFA to albumin may occur in acidotic media as discussed elsewhere(18,19). Both mechanisms would result in an increase of undissociated FFA either in the medium or in the tissue. Recently, Rudman *et al* have shown that under conditions of hormonal activation, undissociated FFA are not released in the medium but accumulate in the cell(11). Accumulation of FFA in the cell has been demonstrated in numerous experimental conditions and is known to be a rate-limiting factor of lipolysis(4,6). One may, therefore, postulate that the inhibition observed in the second series of experiments, in spite of the addition of Th, is due to intracellular accumulation of FFA.

Glucose oxidation by adipose tissue leading to the formation of α glycerophosphate is increased in the presence of catecholamines (3,7,17). This, in turn, enhances resynthesis of triglycerides and lowers the amount of FFA in the tissue(4,5). In the third series (Table II), when glucose was added, the same lipolytic activity was found in media of low and normal buffer capacities and the two regression lines (Y_e and Y_f) are identical. This finding indicates that glucose enhances glycerol release under conditions of acidosis (lines Y_c and Y_e). This effect did not occur at normal pH (lines $Y_d = Y_f$) which may indicate that maximal lipolytic rate was reached when NE and Th were combined. Jungas and Ball(3) have also reported that glucose increases glycerol release from rat adipose tissue stimulated *in vitro* with epinephrine. They suggested that an increase in tissue FFA re-esterification through dihydroxyacetonephosphate provided by glucose oxidation might account for this effect. This interpretation has been confirmed(5).

It has also been reported that the rate of release of FFA and glycerol from adipose tissue was controlled by the concentration of albumin in the blood *in vivo*(20) and in perfused adipose tissue(21). Because of its affinity for FFA high albumin concentration in the medium decreases the accumulation of FFA in the tissue and thereby enhances the rate of lipolysis. As reported by Rodbell, isolated fat cells are still more sensitive to the presence of albumin in the medium than adipose tissue, probably because of their limited capacity to bind FFA in excess of the binding capacity of albumin(6). Hagen (4) also reported that O_2 consumption of adipose tissue incubated with epinephrine without albumin was inhibited, and that this inhibition was reversed when albumin was added. Long chain FFA are known to be potent uncoupling agents(22) and these effects on electron transport may be antagonized by albumin(23,24). This same inhibitory action of FFA may also have occurred in the present experiments when low albumin concentrations were used. Indeed, in the fourth series (Table II), when albumin concentration was increased from 1 to 5 g/100 ml, the lipolytic rate was the same in the media of low and normal buffer capacity. Since glucose as well as albumin reverses the inhibition of lipolysis observed at low pH under conditions of maximal activation (line Y_c, Fig. 1), one may postulate that acidosis might also exert its inhibitory effect on lipolytic activity indirectly by promoting accumulation of undissociated FFA in the cells. Since, however, FFA were not directly determined either in the media or in the cells, such a feedback control of FFA production by H^+ released by FFA requires further confirmation.

Summary. Isolated fat cells were incubated in two Krebs-Ringer media containing normal and low phosphate buffer concentra-

tions with 1 g/100 ml albumin. When lipolysis was activated with 10^{-5} M NE, a marked acidosis (final pH = 6.6) occurred in the medium of low buffer capacity and the lipolytic activity was significantly inhibited as compared to lipolysis in the medium with normal buffer capacity. This inhibition was partially reversed when theophylline 10^{-2} M was added. When fat cells were incubated in these two media, but with glucose present or an albumin concentration of 5 g/100 ml, lipolysis proceeded at a similar rate independently of the buffer capacity of the medium.

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Significance of Increased Alkaline Phosphatase Activity in Viral-Induced Thymic Lymphoma.* (32226)

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Murine thymic lymphoma cells have been shown to have a high concentration of alkaline phosphatase enzymatic activity, whereas normal thymic lymphocytes have none(1,2). This finding is of interest because of the possibility that it might represent a specific metabolic alteration related to the neoplastic change. Further, the histochemical alkaline phosphatase staining of these cells might offer a way to identify positively these neoplastic cells in tissue sections for study of cell proliferation kinetics(2). Smith, studying the progression of leukemogenesis in irradiated C57BL mice, noted a positive correlation between the regions of phosphatase activity and the development of lymphoma in the thymuses, and suggested that the phosphatase activity was related to the lymphoma(1). Metcalf *et al* correlated alkaline phosphatase activity in AKR leukemogenesis, and found that the phosphatase levels

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