

# Effect of ketone bodies on lipolysis in adipose tissue in vitro

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**ABSTRACT** Norepinephrine-sensitive lipase activity was measured in rat epididymal fat pads by determining release either of free fatty acids or of glycerol. Stimulation of the lipase activity by norepinephrine in vitro could not be duplicated by injecting norepinephrine into the rats before sacrifice. A reliable method for assay of lipase deactivation rate was developed in which the tissue is incubated for 80 min, norepinephrine is added for a further incubation of 10 min, and the decay of lipase activity is measured during the next 10 min in the absence of hormone.

Of the ketone bodies tested,  $\beta$ -hydroxybutyrate and probably acetoacetate inhibited the activation of lipase by norepinephrine but had no effect on lipase deactivation rate, whereas acetone increased lipase activity stimulated by norepinephrine when tested at the concentration at which acetoacetate gave an inhibition.

Substances other than  $\beta$ -hydroxybutyrate that produce reduced nucleotides— $\alpha$ -glycerophosphate, malate, and ethanol—had no effect on lipase activity as tested in the present system.

**KEY WORDS** ketone bodies ·  $\beta$ -hydroxybutyrate · acetoacetate · lipolysis · adipose tissue · lipase · norepinephrine-stimulated activation · deactivation · assay · rat

**A**HORMONE-SENSITIVE lipase system present both in rat and human adipose tissue has been found and characterized by several investigators (1–6). Factors other than hormones responsible for the control of this lipolytic activity are only partially known. Cyclic 3',5'-AMP (7) seems to be involved, as in other hormone-induced activation of enzymes (8). Furthermore, glucose seems either to be connected with lipase activation (9, 10) or to be necessary for maintenance of lipase activity (1). Nicotinic acid (10) and lactic acid (11) intervene in lipase activation in the presence of glucose, and insulin (12) in its absence. The present work describes the effects on the

lipase system of other physiologically occurring substances, namely the ketone bodies.

Previous studies (13) have shown that  $\beta$ -hydroxybutyrate decreased glycerol outflow from rat epididymal fat pads during a presumed decay of lipase activity in these tissues after removal from the rat. One possible explanation for this was thought to be an effect of  $\beta$ -hydroxybutyrate on a lipase deactivation system, particularly since no effect on lipase activation could be detected. For this reason a system for assay of both lipase activation rate and lipase deactivation rate was elaborated from the lipase assay system of Vaughan, Berger, and Steinberg (4).

A preliminary report of the effect of  $\beta$ -hydroxybutyrate on norepinephrine-stimulated release of glycerol from the epididymal fat pad of the rat has been presented (14).

## EXPERIMENTAL PROCEDURE

Male rats of a Sprague-Dawley strain, weighing 200–300 g, were fed ad lib. and killed by a blow on the head and exsanguination. The epididymal fat pads were removed and placed in 0.9% NaCl at room temperature. The distal part was immediately cut into 2 or 3 approximately equal sections and incubated after having been weighed on a torsion balance.

Tissue was incubated in a Dubnoff-type incubator at 37°C in Krebs-Ringer bicarbonate buffer with 4% bovine serum albumin (Fraction V, Armour and Company, Ltd., Eastbourne, England, batch KC 2271). The gas phase was 5% CO<sub>2</sub>-95% O<sub>2</sub>, pH 7.4 and final volume 3.0 ml. Additions to the incubation medium, all from the beginning of incubations unless indicated otherwise, and times for incubations are given for each experiment. Glucose was not present except when indicated. Glycerol was determined on aliquots from the incubation medium by the method of Lambert and Neish (15).

Lipase assay was performed as described by Vaughan

et al. (4) by measuring fatty acid release during 20 min of the reaction (10).

Norepinephrine (bitartrate) was obtained from Astra (Södertälje, Sweden); DL- $\beta$ -hydroxybutyrate (sodium salt), DL- $\alpha$ -glycerophosphate (disodium salt, grade X), and DL-malic acid from Sigma Chemical Co. (St. Louis, Mo.); ethanol (analytical grade) from AB Svenska Vin- & Spritcentralen (Stockholm, Sweden); and acetone (analytical grade) from E. Merck A. G. (Darmstadt, Germany). Acetoacetate was obtained by redistilling ethyl acetoacetate (Merck, analytical grade) at 180°C and then hydrolyzing and neutralizing according to Ljunggren (16). The acetoacetate concentration of the final solution was determined manometrically (17), both immediately after preparation and at the time it was used in experiments. It contained equimolar concentrations of acetoacetate and ethanol, and also, increasing with time, acetone as a decomposition product. All reagents except norepinephrine were adjusted to pH 7.4 before use by the addition of KOH or HCl.

## RESULTS

### *Glycerol Release and Lipase Activity*

In Fig. 1 are shown the glycerol release and lipase activity at 20 min intervals after the rat was sacrificed. Glycerol release from the fat pad decreased as described earlier by Vaughan (18) and reached a plateau about an hour after incubation. The high initial release of glycerol might have been caused, at least partly, by a discharge of glycerol present in the tissue at excision. Lipase activity, although at another order of magnitude, seemed to follow the glycerol release. When norepinephrine was added, a sharp increase in lipase activity and glycerol release occurred, the ratio between them being maintained. The level of activity reached after the addition of norepinephrine in vitro was higher than that found immediately after removal of the pad from the rat.

### *Assay of Lipase Deactivation Rate*

Wide variations in activity were observed both for endogenous lipase activation and for the rate of decrease of activity after removal of the tissue from the rat: Fig. 1 shows the high values for SEM. Vaughan et al. (4) also noted this variability.

Since the initial lipase activity was lower than that obtained by stimulation with norepinephrine in vitro (Fig. 1), different means of increasing it were tried, both in vivo and in vitro.

Injection of large doses of norepinephrine subcutaneously, 15 min before the rat was sacrificed, yielded levels of lipase activity (measured immediately after removal of tissues) not significantly different from those obtained in control rats injected with saline. In another attempt, the

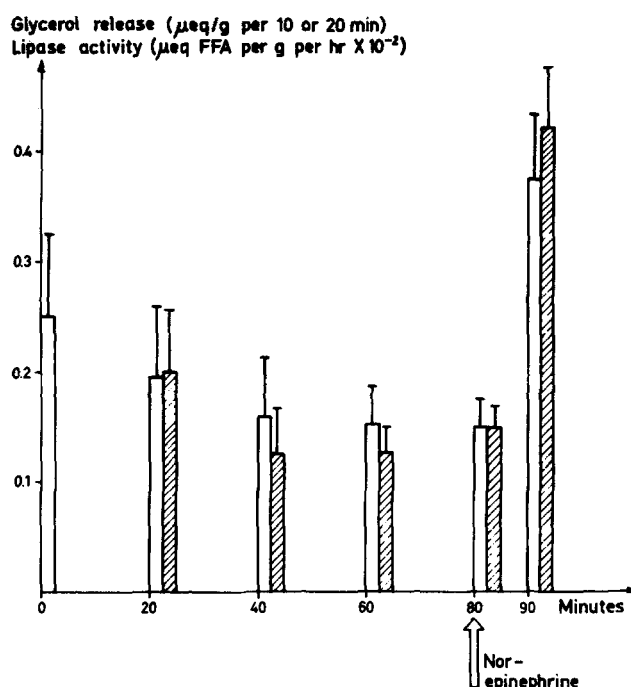


Fig. 1. Epididymal fat pad lipase activity and glycerol release at different times after sacrificing of rats. The rat was sacrificed and pads were immediately divided into 6 parts, incubated from 0 min, and assayed for lipase activity (open bars) at indicated times. Glycerol release (hatched bars) was measured during a 20 min period preceding the time point (10 min period before 90 min measurement). Norepinephrine (0.1  $\mu\text{g}/\text{ml}$ ) added at 80 min. Means  $\pm$  SEM of five experiments.

fat pad was removed and immediately incubated for 10 min in the presence of norepinephrine, after which it was transferred to another incubation flask without norepinephrine. Results were just as irregular as in the absence of in vitro stimulation.

Finally, the following procedure was found to give reproducible results. The fat pad was removed and incubated for 90 min, with norepinephrine present during the last 10 min. After this period, the tissues were transferred to media that were free from norepinephrine and the decay of lipase activity with time was measured (Fig. 2). Data from Fig. 1 have also been included so that events during the whole incubation procedure may be easily compared. The lipase activity leveled off during 80 min; addition of norepinephrine increased this activity markedly. When the tissue was transferred (at 90 min) to a medium free from norepinephrine, activity decayed quickly and almost linearly to the basal level that had been present before norepinephrine activation.

In what follows, lipase activation rate refers to the difference between lipase activity at 80 and at 90 min in the procedure described, while lipase deactivation rate refers to the difference in lipase activity at 90 and at 100 min.

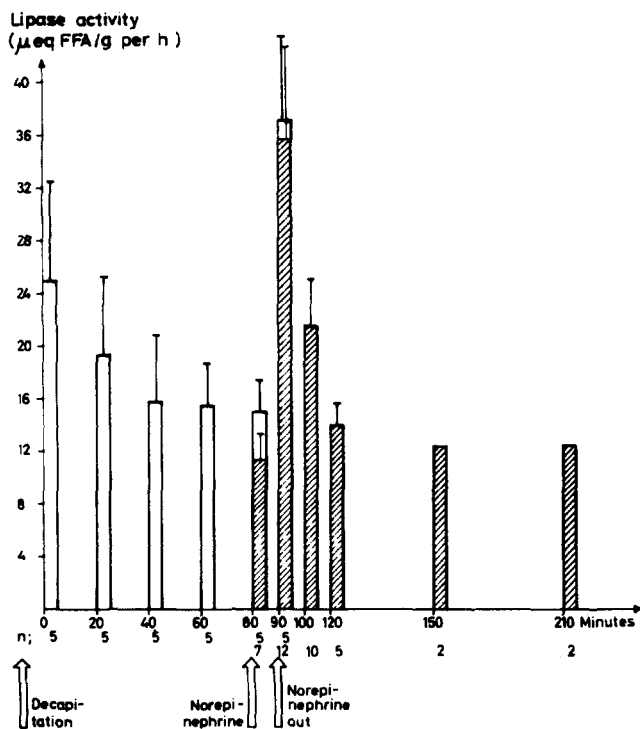


FIG. 2. Lipase activation and deactivation in rat epididymal fat pads. The rat was sacrificed when indicated, pads were divided into 6 parts and incubated from 0 min, without norepinephrine, for 80 min. At 80 min  $0.1 \mu\text{g/ml}$  norepinephrine was added. At 90 min pads were rinsed and transferred to new media without norepinephrine. Assay for lipase activity as indicated (hatched bars). Open bars transferred from Fig. 1. Means  $\pm$  SEM of indicated number of experiments.

#### Effect of $\beta$ -Hydroxybutyrate on Glycerol Release and Lipase Activation and Deactivation

Fig. 3 shows the effect of  $\beta$ -hydroxybutyrate, 96 mM, on release of glycerol from the fat pad in the basal state and after stimulation by norepinephrine, measured after different periods of time and in the presence and absence of glucose. It may be seen that  $\beta$ -hydroxybutyrate inhibited glycerol release both under basal conditions and after norepinephrine stimulation. The inhibition was more pronounced in the absence of glucose than in its presence and this difference seemed to increase with duration of incubation. That the norepinephrine-stimulated release of glycerol was higher in the absence of glucose than in its presence seems to be in contrast to earlier findings (10), but may be explained by the fact that rats could not be matched in different series of experiments.

In Fig. 4 the results of experiments with  $\beta$ -hydroxybutyrate at 10 mM in the absence of glucose are given. Here also an inhibition was found, which increased with duration of incubation.

The cause of this inhibition was investigated by determination of the lipase activation rate in the absence of glucose (Table 1). There was a significant decrease in

glycerol release and lipase activation by norepinephrine when  $\beta$ -hydroxybutyrate was present. Lipase deactivation rate, however, was not affected by  $\beta$ -hydroxybutyrate (Table 2).

#### Effect of Other Metabolites on Glycerol Release and Lipase Activation

As can be seen in Table 3, the preparation of acetoacetate used markedly inhibited lipase activity and glycerol release. Acetone, on the other hand, at 76 mM significantly increased glycerol release as well as lipase activity stimulated by norepinephrine.

In the absence of glucose, lipase activity was not inhibited by  $\alpha$ -glycerophosphate (Table 4). Malate or ethanol, in the concentrations tested, did not inhibit glycerol release or lipase activity either (Table 4).

## DISCUSSION

As suggested previously (4) and demonstrated both earlier (1, 4) and by the results reported here, there probably exists a balance between active and inactive lipase in adipose tissue. When the fat pad is removed from the rat that has been killed by concussion and exsanguination, various degrees of lipase activity are obtained. This is suggested by the glycerol measurements of Vaughan (18). The endogenous activity seems not only to decrease irregularly, but also to disturb the activation in vitro by norepinephrine. Although the contribution of lipoprotein lipase to this effect could not be ascertained because conditions for its assay were not optimal, activity changes of this enzyme might have been responsible. Only after a period in which the endogenous activity has decayed is it

TABLE 1 EFFECTS OF  $\beta$ -HYDROXYBUTYRATE ON NOREPINEPHRINE-STIMULATED LIPASE IN RAT EPIDIDYMAL FAT PADS IN VITRO

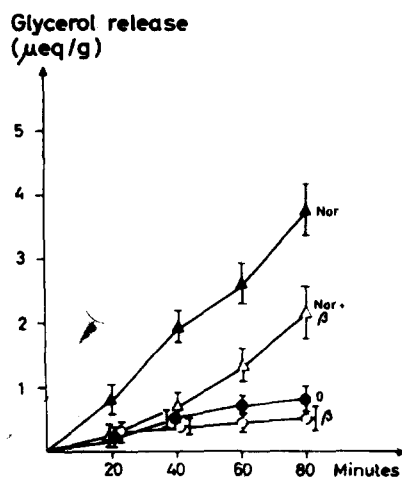
Added to Incubation System	Glycerol Release*	Lipase Activity at 90 Min	Increase in Lipase Activity, 80-90 Min (Lipase Activation Rate)
—	$0.69 \pm 0.15$ $P < 0.01$	$9.8 \pm 2.4$ $P < 0.01$	$-0.2 \pm 2.1$ $P < 0.01$
Norepinephrine†	$1.40 \pm 0.09$	$30.2 \pm 2.0$	$21.2 \pm 2.2$
Norepinephrine† + $\beta$ -hydroxybutyrate (96 mM)	$0.52 \pm 0.05$ $P < 0.01$	$22.7 \pm 2.2$ $P < 0.025$	$13.7 \pm 1.7$ $P < 0.02$

Means  $\pm$  SEM for 11 experiments in each instance. Norepinephrine, where added,  $0.1 \mu\text{g/ml}$ . Statistical differences as compared with norepinephrine-containing system.

\* Measured during 0-90 min, see Fig. 2.

† Added at 80 min, see Fig. 2.

## WITH GLUCOSE



## NO GLUCOSE

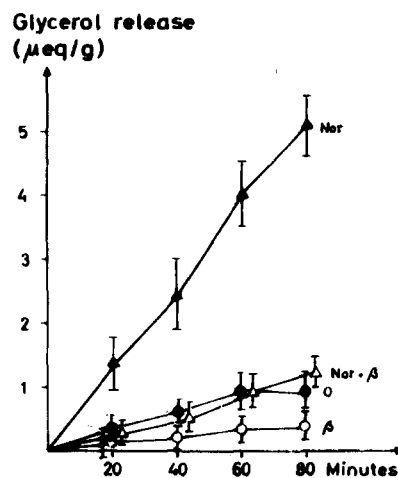


FIG. 3. Effect of  $\beta$ -hydroxybutyrate (96 mM) on glycerol release from rat epididymal fat pads in vitro. Rats were sacrificed and pads divided into 4 parts incubated from 0 min. Additions as indicated in following concentrations: norepinephrine (Nor) 0.1  $\mu$ g/ml;  $\beta$ -hydroxybutyrate ( $\beta$ ) 96 mM; glucose 10 mM. Means  $\pm$  SEM of six (with glucose) and seven (no glucose) experiments.

TABLE 2 EFFECT OF  $\beta$ -HYDROXYBUTYRATE ON LIPASE ACTIVITY AFTER NOREPINEPHRINE STIMULATION AND LIPASE DEACTIVATION RATE IN RAT EPIDIDYMAL FAT PADS IN VITRO

Added to Incubation System	Lipase Activity*	Lipase Deactivation Rate,
	at 100 Min	90-100 Min †
	$\mu$ eq FFA/g/hr	$\mu$ eq FFA/g/hr
Norepinephrine ‡	16.2 $\pm$ 0.9	18.1 $\pm$ 1.4
Norepinephrine ‡ + $\beta$ -hydroxybutyrate (96 mM)	16.1 $\pm$ 1.0	18.5 $\pm$ 1.0

Means  $\pm$  SEM for six experiments.

\* See Fig. 2.

† Decrease in activity from that at 90 min (34.5  $\pm$  0.8  $\mu$ eq of FFA per g per hr, n = 5) to that at 100 min (see Fig. 2).

‡ Added at 80 min, see Fig. 2.

possible to activate the lipase reproducibly (Fig. 1), as shown earlier by Vaughan et al. (4). The decrease of lipase activity after such activation is not only reproducible but also initially close to linear (Fig. 2).

Activation of the lipase system in vitro seemed to be more effective than activation in vivo caused either by the trauma at killing or by injection of a large dose of norepinephrine plus the trauma. It is possible that the in vitro concentrations of norepinephrine are far greater than the concentrations, either of this hormone or of other factors causing lipase activation, that occur in vivo. Furthermore, the in vitro system is probably free from antilipolytic factors present in vivo.

It seems likely that the increase in lipase activity after norepinephrine is added is the algebraic sum of the effects on lipase activation rate and on deactivation rate; in the presence of hormone, the former is probably much

TABLE 3 EFFECT OF ACETOACETATE AND ACETONE ON NOREPINEPHRINE-STIMULATED LIPASE IN RAT EPIDIDYMAL FAT PADS IN VITRO

Added to Incubation System	Glycerol Release, 0-90 Min*	Lipase Activity at 90 Min*
	$\mu$ moles/g/90 min	$\mu$ eq FFA/g/hr
—	0.87 $\pm$ 0.09	7.4 $\pm$ 0.8
	$P < 0.01$	$P < 0.01$
Norepinephrine †	1.94 $\pm$ 0.15	21.1 $\pm$ 1.3
Norepinephrine † + acetoacetate (76 mM)	0.85 $\pm$ 0.10	11.6 $\pm$ 2.1
	$P < 0.01$	$P < 0.01$
—	0.78 $\pm$ 0.07	11.0 $\pm$ 1.8
	$P < 0.01$	$P < 0.01$
Norepinephrine †	1.32 $\pm$ 0.08	27.6 $\pm$ 1.6
Norepinephrine † + acetone (76 mM)	1.78 $\pm$ 0.08	33.8 $\pm$ 2.0
	$P < 0.01$	$P < 0.05$

Means  $\pm$  SEM for six experiments in each instance. Norepinephrine, where added, 0.1  $\mu$ g/ml. Statistical differences as compared with norepinephrine-containing system.

\* See Fig. 2.

† Added at 80 min.

more pronounced. When tissue is transferred to hormone-free medium, the procedure described presumably measures mainly deactivation rate, since activation rate in the absence of hormone probably is very small.

It also seems conceivable that activation and deactivation of the lipase occur by way of two independent enzyme systems, since  $\beta$ -hydroxybutyrate affects only lipase activation (Table 1) but not deactivation (Table 2).

Glycerol production in adipose tissue seems, with certain assumptions, to give a correct estimation of triglyceride hydrolysis in this tissue (18). The increase in

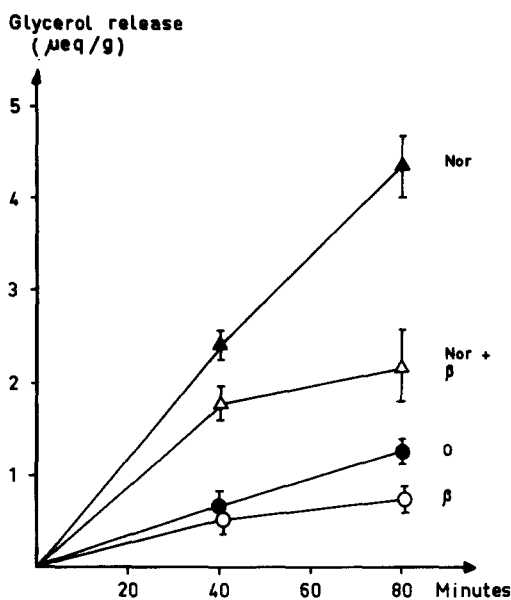


FIG. 4. Effect of  $\beta$ -hydroxybutyrate (10 mM) on glycerol release from rat epididymal fat pads in vitro. Conditions and additions as in legend to Fig. 3 except that no glucose was present and the concentration of  $\beta$ -hydroxybutyrate was 10 mM. Means  $\pm$  SEM of six experiments.

lipase activity parallels that in glycerol production induced by different hormones (4). Furthermore, the inhibition of lipase activation is also followed by similar changes in glycerol outflow from the rat epididymal fat pad. Such inhibition is caused by widely different substances via, presumably, different mechanisms. Inhibitory substances include a  $\beta$ -adrenergic blocking agent (19), nicotinic acid (10), and glycolysis inhibitors (12), as well as physiologically occurring substances such as insulin (13), lactic acid (11), and in the present work  $\beta$ -hydroxybutyrate and probably acetoacetate. The similarity in the fluctuations of the activity of the lipase system and glycerol outflow in the present work (Fig. 1) adds to earlier evidence that indicates that the lipase investigated is at least partially the trigger for the hormone effects on adipose tissue lipolysis.

When glucose is absent from the system, the inhibition of glycerol release by  $\beta$ -hydroxybutyrate is more pronounced than in its presence (Fig. 3). The inhibition-antagonizing effect of glucose in these experiments becomes increasingly evident as incubation is prolonged (Fig. 3). Furthermore, at a concentration of 10 mM  $\beta$ -hydroxybutyrate does not interfere with glycerol release or lipase activation in the presence of glucose (12), but in the absence of glucose glycerol outflow was inhibited at this concentration, also here seemingly more pronounced after prolonged incubation (Fig. 4). As suggested by earlier work (1, 9, 10, 12) these observations seem to give further support to the proposition that glucose plays a role in the balance of lipase activity. The

mechanism of this effect of glucose in the present work might be the removal of  $\beta$ -hydroxybutyrate, but might be partially explained by an alteration, caused by glucose, in  $\beta$ -hydroxybutyrate uptake.

Recently, inhibition of lipase activation by  $\beta$ -hydroxybutyrate could not be demonstrated in the presence of glucose (12). It seems probable that the mechanism for inhibition of glycerol outflow by  $\beta$ -hydroxybutyrate is the same in the presence or absence of glucose, but that it is, for technical or other reasons, possible to demonstrate an inhibition of lipase activation only when it is more pronounced, namely in the absence of glucose. A time factor may also be of importance for this inhibition.

The acetoacetate preparation used caused inhibition of glycerol release and of lipase activity (Table 3). It contained ethanol in equimolar concentration, but ethanol itself had no inhibitory properties at the concentration employed (Table 4). Incubation systems with acetoacetate also probably contained acetone to an unknown extent, produced when the acetoacetate solution was stored or by decarboxylation in the incubation flask; but since acetone increased both glycerol release and lipase activity stimulated by norepinephrine in the concentration tested (Table 3), it seems unlikely that its presence would invalidate the conclusion that aceto-

TABLE 4 EFFECT OF  $\alpha$ -GLYCEROPHOSPHATE, MALATE, AND ETHANOL ON NOREPINEPHRINE-STIMULATED LIPASE IN RAT EPIDIDYMAL FAT PADS IN VITRO

Added to Incubation System	Glycerol Release, 0-90 Min*	Lipase Activity at 90 Min*
	$\mu\text{moles/g/90 min}$	$\mu\text{g FFA/g/hr}$
—		$9.8 \pm 2.4$
		$P < 0.01$
Norepinephrine†	‡	$30.2 \pm 2.0$
Norepinephrine† + $\alpha$ -glycerophosphate (58 mM)	‡	$30.0 \pm 1.8$
		n = 11
—	$0.70 \pm 0.10$	$7.2 \pm 0.9$
	$P < 0.01$	$P < 0.01$
Norepinephrine†	$1.94 \pm 0.22$	$21.4 \pm 1.5$
Norepinephrine† + malate (12.5 mM)	$1.56 \pm 0.14$	$22.1 \pm 2.0$
	n = 6	n = 6
—	$1.11 \pm 0.10$	$7.8 \pm 2.1$
	$P < 0.01$	$P < 0.01$
Norepinephrine†	$1.93 \pm 0.11$	$19.7 \pm 1.6$
Norepinephrine† + ethanol (76 mM)	$1.92 \pm 0.18$	$19.3 \pm 1.8$
	n = 6	n = 6

Means  $\pm$  SEM of indicated number of experiments. Norepinephrine, where added, 0.1  $\mu\text{g/ml}$ . Statistical differences as compared with norepinephrine.

\* See Fig. 2.

† Added at 80 min.

‡ Data deleted because of high basal glycerol values due to  $\alpha$ -glycerophosphate.

acetate is an inhibitor of glycerol release and lipase activity stimulated by norepinephrine. Because of the complex composition of the acetoacetate preparation used, a more detailed analysis of its effect on lipase activation and inactivation rates was not performed.

In contrast to  $\beta$ -hydroxybutyrate,  $\alpha$ -glycerophosphate does not inhibit lipase activity in the absence of glucose (Table 4), just as it does not when glucose is present (12).

Insulin (13), nicotinic acid (10), lactic acid (11), and  $\beta$ -hydroxybutyrate inhibit lipase activation. They also presumably produce reduced nucleotides in adipose tissue. Other substances that also presumably cause an increase in reduced nucleotides, viz.  $\alpha$ -glycerophosphate, malate, and ethanol, were therefore tested; none of them interfered with lipase activation (Table 4). Although this is an indirect way of testing whether reduced nucleotides inhibit lipase activation, it seems to be the only one possible until the separate enzymic steps in the lipase activation system can be directly assayed. This question must therefore be left open for the time being.

Madison, Mebane, Unger, and Lochner (20) have shown that  $\beta$ -hydroxybutyrate and acetoacetate decrease plasma free fatty acids and blood glucose in the dog by inducing insulin secretion from the pancreas. The present results suggest that these ketone bodies also have a direct effect on adipose tissue lipolysis, which would cause a decrease in plasma free fatty acids. This question is now being investigated.

The excellent technical assistance of Miss M. Karlsson and Mrs. K. Jarlevid is gratefully acknowledged.

This work was supported partly by a grant from the Swedish Medical Research Council (Y 495) and partly from the Swedish National Association against Heart and Chest Diseases.

*Manuscript received 28 December 1965; accepted 12 May 1966.*

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