enzyme loaded 4fold, using columns of increasing capacity (45 × 2.4 cm, 45 × 4 cm), or adding 0.5% TX-100 and 1 M NaCl to the wash (to disassociate aggregates). These results indicate that the appearance of this fraction in the wash is not due to overloading or aggregation. We designated this fraction: F_0. The remaining activity was quantitatively eluted in 3 fractions: F_1, F_2 and F_3 (figure). This chromatogram was quantitatively and qualitatively reproducible, e.g., the average coefficient of peak height variation was 5%. Isolation and rechromatography of an individual peak fraction resulted in elution at the same point it eluted from originally. This result shows that any possible interconversion between the isolated forms does not take place detectably within a 36 h time frame. Freshly isolated 11S enzyme quantitatively eluted from the column as 1 peak at a point identical with F_2.

The table shows some of the properties of these multiple forms. The forms all have a sedimentation coefficient of 11.1 showing that they are not sizeozymes. The K_m values for acetylthiocholine iodide are slightly different. Fresh 11S has a K_m value of 1.5×10^{-4} M which is similar to the average K_m value of the forms. The electrophoretic mobility of the chromatographically isolated forms (normalized with respect to F_0) does not show a simple linearity of migration versus fraction number. Freshly prepared 11S AChE migrates identically to F_2. Examination of a gel electrophoretogram of the 5-year-old preparation done when the preparation was fresh showed only 1 enzyme activity band. Since electrophoresis conditions were slightly different, we do not know whether it migrated identically to F_2. The original material was shown to be a true acetylcholinesterase since it hydrolyzed butyryl choline at a rate orders of magnitude below the rate it hydrolyzed acetylcholine. Similar tests on F_0, F_1, F_2 and F_3 confirmed that they, too, were true acetylcholinesterases. The apparent identity of mol. wt, utilization of the same substrate and difference in electrophoretic mobility clearly allow F_0, F_1, F_2 and F_3 to be termed isozymes. The origin of this multiplicity and charge difference is not clear. In this connection, the following treatments were tried in an attempt to produce isozymes from fresh 11S enzyme: 30 min incubation at 45 °C; repeated (5–10 times) freeze thawing; 60 min incubation at 37 °C with either 50 μg papain, 25 μg trypsin or 25 μg pepsin (at a protease to AChE ratio of 1/50, 40–60% loss of activity was observed); addition of soybean trypsin inhibitor or phenylmethylfulfonyl fluoride (1.5 mg/100 ml of extract); extraction with 1% TX-100; chromatography over a 10fold range of protein concentration; and incubation with 1 M Guanidine-HCl at 4 °C followed by dialysis against 5000 volumes of 0.01 M Na-phosphate (pH 7.6) in 1 M NaCl (96% loss of activity resulted). Some of these treatments are well known to produce isozymic forms from tetrameric enzymes with different subunits. The others were designed to investigate the effects of exogenous proteases, detergent removal of lipids, and protein concentration on the formation of the isozymes. In no case was more than 1 electrophoretic or chromatographic band seen. These experiments rule out such simple origins for the isozyme bands and suggest that further research is necessary to elucidate the generative process. Though we do not know the actual time dependence for the appearance of each of the forms, it is clear that in studies of genetic and/or tissue specific AChE isozymes, a possible storage problem should be considered and tissues examined at as early a date as is possible.

Modification of theophylline-induced lipolysis in human fat cells after trypsination

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Summary. Trypsin-treatment of human fat cells results in the potentiation of the lipolytic response and the cAMP accumulation induced by theophylline ($5 \cdot 10^{-4}$ M) but not of those induced by theophylline ($5 \cdot 10^{-3}$ M). The amount of cAMP formed after exposure to theophylline ($5 \cdot 10^{-3}$ M) plus norepinephrine ($5 \cdot 10^{-6}$ M) remains, however, 2.6fold higher in trypsin-treated human fat cells than in the control ones.

Recently, we have shown that the exposure of human fat cells to trypsin, although not affecting the lipolytic response of these cells to dibutyryl cyclic AMP, resulted in a marked increase in both lipolysis and 3′–5′ cyclic AMP (cAMP) synthesis induced by catecholamines[2]. Contrasting with these findings, we found that trypsin-treatment of rat fat cells maintained a normal lipolytic response to catecholamines[2].

In the present studies, we have investigated the influence of trypsin-digestion on the lipolytic response of human adipocytes exposed to theophylline, a lipolytic agent inhibiting phosphodiesterases (PDE)[3] and acting therefore on the lipolytic process at a step localized between the sites of action of catecholamines and cAMP.

Materials and methods. Human omental adipose tissue was obtained from overnight fasted, non-obese patients of both sexes (30–50 years old), having no clinical and biochemical evidence of endocrine disease and undergoing abdominal surgery. Procedure for the premedication, anesthesia and adipose tissue collection were as described previously[4].

Human fat cells were isolated following a modification[2] of the method of Rodbell[5]. Procedures used for the preparation of trypsin-treated fat cells, their incubation, the determination of lipolysis, the extraction and the determination of cAMP, as well as the origin of the material used, have been described previously in detail[2]. As the

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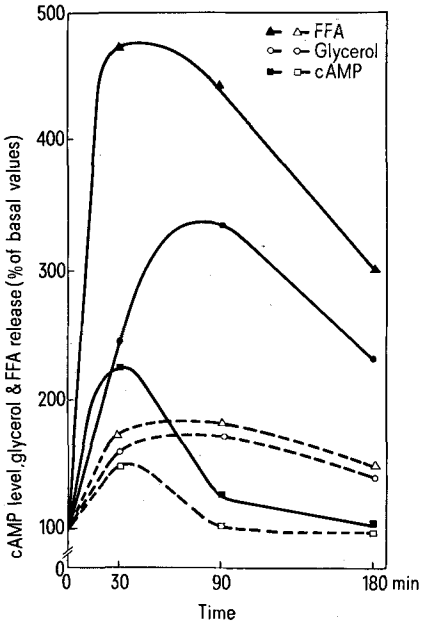
Influence of trypsin-treatment on the lipolytic response and on the intra- and extracellular cAMP accumulation induced by theophylline or theophylline + norepinephrine in human fat cells

Addition to the medium	Control cells				Trypsin-treated cells			
	Glycerol release	Intracellular cAMP	Extracellular cAMP	Total cAMP	Glycerol release	Intracellular cAMP	Extracellular cAMP	Total cAMP
Theophylline (5×10^{-4} M)	203 ± 35	150 ± 11	180 ± 17	163 ± 17	415 ± 32*	202 ± 12*	233 ± 15*	224 ± 15*
Theophylline (5×10^{-3} M)	374 ± 26	251 ± 28	211 ± 23	234 ± 26	395 ± 35**	276 ± 24**	172 ± 28**	222 ± 29**
Theophylline (5×10^{-3} M) + norepinephrine (5×10^{-6} M)	339 ± 18	555 ± 63	389 ± 51	477 ± 62	370 ± 29**	957 ± 78*	803 ± 191*	936 ± 124*

Trypsin-treated and control fat cells were prepared and incubated in the presence or absence of theophylline as described in the figure. Determinations of cAMP and glycerol were performed after 30- and 60-min- incubation,respectively. When indicated, norepinephrine (5×10^{-6} M) was added after a 30-min-incubation with theophylline; in these experiments, cAMP levels and glycerol release were determined 5 min and 30 min later, respectively. Experiments were performed in duplicate, using fat cells from 2 different patients. In each experiment, incubations were carried out in quadruplicate and the data obtained were expressed as percent of the corresponding basal values. Results are thus the mean ± SE of 8 percentages. *means that $p < 0.01$ vs control cells; **means that $p > 0.05$ vs control cells.

biological variability of preparations of human fat cells was large, most of the metabolic data were expressed as percent of the corresponding basal values. Results are given as mean ± SE and Student's t-test was used for comparison of mean values.

Results. The influence of trypsin-digestion on the time-course of both lipolysis and cAMP accumulation induced in human fat cells by theophylline (5×10^{-4} M) is shown in the figure. As can be seen, trypsin-treated fat cells had an increased lipolytic response to theophylline (2–4 times), an effect which persisted at least during the first 3 h following the end of the trypsin-digestion period.



Influence of trypsin-treatment on the time-course of lipolysis and cAMP accumulation induced by theophylline (5×10^{-4} M) in human fat cells. Trypsin-treated fat cells were obtained by digestion of adipose tissue with collagenase plus trypsin[2] and their metabolic responses were compared to those of fat cells isolated with collagenase only. Fat cells were incubated in Krebs-Ringer bicarbonate buffer containing albumin (4%, w/v), glucose (5 mM) and when indicated, theophylline (5×10^{-4} M). Each point represents the mean value of 4 determinations. The data concerning the effects of theophylline in trypsin-treated and control fat cells are expressed as percent of the corresponding basal values. Trypsin-treated and control fat cells are represented by solid and broken lines respectively.

Under these conditions, however, trypsin-digestion failed to modify the basal glycerol and free fatty acid (FFA) release. Although unchanged in the basal state after trypsination, the intracellular cAMP level found after 30- or even 90-min-exposure to theophylline was significantly higher in trypsin-treated fat cells than in the control ones; however, after 3-h-incubation with theophylline, the intracellular cAMP levels in both control and trypsin-treated fat cells were equivalent and not statistically different from the basal levels.

As shown in the table, the trypsin-induced increase in the lipolytic response and in the rate of cAMP accumulation in fat cells exposed to theophylline 5×10^{-4} M, was not due to an inhibition of the release of cAMP from the cell. Moreover, the ability of trypsin to increase the sensitivity of human fat cells to theophylline appeared to be dependent on the concentration of theophylline present in the incubation medium: in fact, the trypsin-induced increase in both lipolysis and cAMP accumulation in response to theophylline (5×10^{-4} M) was completely abolished when the theophylline concentration was 10fold higher (5×10^{-3} M). Finally, when norepinephrine (5×10^{-6} M) was added after a 30-min-preincubation with theophylline (5×10^{-3} M), the amounts of cAMP accumulated in and out the trypsin-treated cells were markedly enhanced compared with the control ones, although the lipolytic responses of both groups of cells were similar.

Since the conditions used (high concentration of theophylline) were shown to induce an almost complete inhibition of PDE[6], adenylate cyclase activity may be assessed from the above data by subtracting from the cAMP accumulated in the presence of both norepinephrine and theophylline, the amount of cAMP found 5 min before in the presence of theophylline alone. Following these calculations, the estimated adenylate cyclase activation induced by norepinephrine was 2.6fold higher (584 ± 188 pmoles cAMP/g cell lipid/5 min) in trypsin-treated fat cells than in the control ones (222 ± 53 pmoles cAMP/g lipid/5 min.

Discussion. In a recent study[2], we have shown that treatment of human fat cells by trypsin results in an increase of their lipolytic response to catecholamines but not to dibutyryl-cyclic AMP. As the integrity of these cells appeared unaltered under these conditions, we suggested that these trypsin-induced modifications could be localized on the cell surface and could be due to a 'permissive' effect of trypsin on the catecholamine-induced

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activation of human fat cell adenylate cyclase[2]. Although adenylate cyclase was not directly investigated, the present results argue strongly in favour of such a mechanism, which, as shown previously, does not involve the destruction of α-adrenergic receptors[2].

The present study also shows that such a 'permissive' effect of trypsin does not apply only to catecholamine-induced lipolysis. In fact, we found that the trypsin-treatment of human fat cells also increased the lipolytic response and the cAMP accumulation induced by theophylline concentration producing half-maximal stimulation of lipolysis. Nevertheless, the maximal lipolytic response of human fat cells to theophylline was found unaltered after trypsin-treatment.

The above-described modifications induced by trypsin in human fat cells are comparable with those exerted by growth hormone (GH) + glucocorticoid in the rat ones[7]. In fact, it was reported that these hormones increase norepinephrine- and theophylline-induced lipolysis, but fail to affect both the dibutyryl-cyclic AMP-induced one and the maximal lipolytic response of rat adipocytes to theophylline and catecholamines[7,8]. Furthermore, despite its non-effectiveness on the basal adenylate cyclase activity of rat fat cell ghosts, GH was shown to increase the sensitivity of this enzyme to catecholamines[9], an effect which was prevented by inhibitors of RNA-synthesis[9]. It was thus concluded that GH affects lipolysis through increased synthesis of a plasma membrane protein(s) which may enhance the ability of catecholamines to activate adenylate cyclase[9].

In the case of human fat cells, synthesis of such a protein could also explain the modifications of lipolysis found after trypsination. In a previous report[2], we suggested that trypsin inactivates a membranous protein(s) which may usually prevent the binding of catecholamines to human fat cells or inhibit the transmission of the appropriate signals from the β-receptors to adenylate cyclase. Recent experiments[10] have shown, however, that catecholamine-binding to human fat cells was unimpaired after trypsin-treatment.

Anyhow, such modifications in plasma membrane proteins would not explain the present results concerning the 'permissive' effect of trypsin on theophylline-induced lipolysis. In fact, because of the well-known inhibitory action of theophylline upon PDE[3] and of the cytosolic localization of these enzymes in human fat cells[11], it appears unlikely that the 'permissive' effect of trypsin on theophylline-induced lipolysis could result from a direct action of trypsin on PDE. Explanation of this 'permissive' effect requires the establishment that the inhibition of PDE may be the only way of action of theophylline on lipolysis in human fat cells.

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Feeding an insect through its respiration: Assimilation of alcohol vapors by *Drosophila melanogaster* adults

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Summary. Ethanol given to otherwise starved *Drosophila* adults can increase their survival from 2.5 to 8 days. Similar results were obtained when only alcohol vapor was accessible to the flies, demonstrating the possibility of significant feeding through their respiration. This physiological capacity could be useful in wild conditions.

Among all presently known *Drosophila* species, *D. melanogaster* is characterized by a very high tolerance to ethanol[2] (and unpublished data) which allows its development on the surface of fermenting wine jars, on substrates often containing 10% of alcohol. This tolerance is due to a very active alcohol dehydrogenase (ADH)[3]: after its detoxification, alcohol is used as an energy source as shown by the increase in lifespan of starved adults[4,5].

Since alcohol is volatile, long experimental tests are difficult to run and it is not easy to know the exact concentration which reaches the flies. In our previous studies, toxicity measurements were made by placing the adults in air tight plastic tubes containing an alcoholic solution adsorbed on cellulose wool[2,3]. For the study of alcohol utilization, flies were kept in ventilated cages[4] and the alcoholic solution was incorporated to an agar gel. In these experiments, the flies were able to ingest alcohol but it was also supposed that some alcohol could also penetrate directly into the body through respiration.

The present work was undertaken to test this hypothesis: we show that the feeding value of ethanol is almost the same when only vapors are provided as when adults have a direct access to the solution. Two experimental techniques were compared. In the first, flies were kept in a double, ventilated cage. In the second, they were put in the upper compartment of an air-tight tube. Schemes and further explanations are given in figure 1. With both techniques, control experiments were made in which flies had a direct access to the alcoholic solution.

The results, obtained with F_1 heterozygotes issued from a cross between 2 laboratory strains, are shown in figure 2. With the ventilated cage technique (figure 2A), life duration of controls increased with alcohol concentration up to an optimum of about 10% and then decreased almost linearly. When flies had no access to the alcoholic solution, a fairly similar curve was observed but the toxic effects

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