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LIPOGENESIS AND INSULIN SENSITIVITY OF SINGLE FAT CELLS*

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SUMMARY

1. A method for measuring the lipogenesis from [^{14}C]glucose by single fat cells is described: (i) after incubation with 'carrier-free' [U-^{14}C]glucose (0·55 μ-mole/ml.), collagenase-isolated fat cells were fixed with osmium tetroxide; (ii) similarly incubated pieces of epididymal fat pads were treated with osmium tetroxide for 90 sec, whereby only the superficial cells are fixed, and the tissue was then disintegrated by shaking with collagenase. The osmium-fixed free cells were washed, sucked into a micropipette, measured under a microscope and assayed individually for ^{14}C-activity.

2. There was a quantitative recovery of ^{14}C-lipid activity from osmium-fixed single cells.

3. Both collagenase-isolated cells and *in situ* fixed surface cells were normally distributed with respect to diameters (for both cell groups from *ad lib.* fed rats of *ca.* 110 g; mean diameter, about 55 μm; S.D. about 7 μm).

4. Frequency distribution curves (number of fat cells versus ^{14}C-lipogenesis per cell) were asymmetric and very broad (coefficient of variation about 50 %) for collagenase-isolated cells incubated with insulin (10^3 μ-u./ml.). Frequency distribution curves for surface cells obtained from similarly incubated pieces of epididymal fat pads showed a coefficient of variation of the same magnitude, whereas the mean lipogenesis of these cells was only about one third of that of the isolated cells.

5. Collagenase-isolated cells incubated in the presence of insulin (10^3 μ-u./ml.) showed a weak but highly significant positive correlation between fat cell diameter and ^{14}C-lipogenesis (eight rats, r about 0·5 and

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$P < 0{\cdot}001$ for each rat). Analysis of the relationship: lipogenesis = k × diameter to the exponent of β showed that the estimates of β varied significantly from rat to rat (range: 1·3–2·9). Similar correlations between cell size and lipogenesis were found both for cells incubated with insulin in various submaximal concentrations and for cells incubated without insulin.

6. Small and large cells from the same rat were equally sensitive to insulin.

7. Statistical analysis of frequency distribution curves (number of cells versus ^{14}C-lipogenesis per unit surface area) representing cells from the same rat incubated with insulin 0, 2·5, 5, 10, and 10^3 μ-u./ml., respectively, suggests that insulin exerts a graded influence on the lipogenesis of each fat cell.

INTRODUCTION

Previous studies on adipose tissue *in vitro* have been carried out either with pieces of adipose tissue or with suspensions of isolated fat cells prepared by treating the tissue with collagenase (recent reviews: Jeanrenaud (1968) and Gliemann (1969)). These preparations contain a large number of cells per sample, typically 10^5 or more, and studies of metabolic parameters on the single cell level have not been carried out so far.

The present paper describes a technique for the measurement of lipogenesis from [^{14}C]glucose by one single fat cell obtained either from a suspension of free fat cells or from the surface of a piece of fat tissue.

One purpose of the study has been to analyse the variation among fat cells with respect to synthesis of lipids from glucose in the absence and in the presence of insulin in various concentrations in an attempt to distinguish between the hypothesis of a continuous and the hypothesis of a quantal cellular response to insulin. Another purpose has been to study whether a relation exists between the cell size and the rate of lipogenesis for fat cells obtained from the same animal. This question has attracted considerable interest during the past few years since it has been observed that the number of fat cells of a rat remains constant after the 15th week of age (Knittle & Hirsch, 1968) and from this it follows that changes in the total fat volume of the adult rat takes place through changes in cell volume rather than in cell number.

METHODS

Chemicals. [U-^{14}C]glucose was obtained from the Radiochemical Centre, Amersham, and used without further purification. The specific activity was 288 m-c/m-mole, i.e. isotopic abundance was 78%. Bovine insulin (monocomponent, 25 u./mg) was a gift from NOVO Research Institute. Crude collagenase (Lot no. 127B-0240) was obtained from Sigma and human albumin from Swiss Red Cross.

Treatment of fat cells. Male Wistar rats, 105–120 g, fed *ad lib.* were used. The isolated fat cells were prepared according to previously described methods (Gliemann,

1967). The incubations were performed as follows: 2000–4000 fat cells (about 0·2 mg cell lipid) were incubated with shaking in 3 ml. plastic tubes with 100 μl. Krebs–Ringer bicarbonate buffer containing albumin (10 mg./ml.) and [U-^{14}C]glucose 0·55 μ-mole/ml. (equals 160 μ-c/ml.). Each of these samples was accompanied by four low specific activity (3·2 μ-c/ml.) but otherwise identical samples. In a few experiments, some samples were also incubated in a volume of 1 ml. The incubation with high specific activity glucose was stopped after 150 min by the addition of osmium tetroxide (approximately 1 mg/mg cell lipid) in a buffer volume of 2 ml., and at the same time the cells incubated with low specific activity were extracted by the method of Dole & Meinertz (1960). The fixed cells appeared dark (Pl. 1*A*) and their specific gravity exceeded that of the suspending medium. These cells were then washed repeatedly in water until no significant radioactivity could be detected in 20 μl. wash water.

The following processes were carried out under a stereomicroscope: about 20 μl. suspension containing approximately 150 cells were sucked into a micropipette (tip diameter 80–100 μm) connected to a 100 μl. syringe with the plunger driven by a micrometer screw (Pl. 1*B*). The individual cells were pipetted into droplets of water which were lined up in rows on a piece of Parafilm® supported by a microslide.

After pipetting about thirty cells the water was allowed to evaporate and the slide transferred to an ordinary microscope. The diameter of each cell, which appeared as a solid black ball, was measured to the nearest number of graduation marks of an ocular micrometer with intervals equivalent to 2·5 μm at a magnification of 400×. Finally the Parafilm® was cut into pieces about 3 mm × 4 mm, each with one cell, and placed in scintillation fluid. Inspection under the stereomicroscope revealed that the fixed cell was released from the Parafilm® and could be recognized as a black ball for some time. However, after 4–8 hr, the black material had dissolved in the fluid and only a ghost of the cell was left. Therefore, the fixed single cells were extracted for at least 24 hr before the radioactivity was measured.

Single cells from the surface of epididymal fat tissue were recovered as follows: *A ca.* 5 mg tip of the fat pad (most of the surface cells belonging to the natural surface) was incubated in the same way as the isolated fat cells. After incubation the tissue was washed, immersed for 90 sec in osmium tetroxide (1% of 0·9 % NaCl) and again washed repeatedly. Only the cells located superficially were fixed by this procedure (Pl. 1*C*). Finally, the tissue was disintegrated by shaking for 3 hr with collagenase (1 mg/ml.). The fixed cells were recovered from the bottom of the vessel, washed, resuspended in water and sucked into the micropipette. The fixed surface cells were somewhat irregular in shape, in contrast to the spherical collagenase-isolated cells (Pl. 1*D*). For this reason, their size could be assessed only approximately by measurement of the 'average diameter'.

Measurement of radioactivity. Counting of ^{14}C-activity was carried out in a Packard Tricarb liquid scintillation spectrometer, model 3375, using Bray's scintillation fluid (Bray, 1960) and 20 ml. polyethylene vials. Reduction in the volume of scintillation fluid from 20 to 0·5 ml. had no significant effect on the recovery of ^{14}C-lipid counts, whereas the blank value decreased from about 23 counts per minute (cpm) to about 10 cpm. A volume of 2 ml. was chosen for the extraction and counting of single cells. Addition of pieces of Parafilm® (1 cm × 1 cm) to the scintillation vials did not influence the recovery of ^{14}C-activity. The blank value was 12·6 cpm ± 0·2 cpm (S.E.), as determined by repeated counting of the same sample until 5000 counts had accumulated. All samples containing single cells were assayed for radioactivity until at least 2000 counts had accumulated. Every tenth sample contained no cell but 5 μl. water, in which the fixed cells were suspended. Twenty-two such blank values, representing a total of 12000 counts, showed a mean of 12·53 cpm and a S.E. of the

mean of 0·11 cpm. It was concluded, therefore, that the cells were sufficiently washed. In the absence of insulin, 25 % of the cells counted as little as 10 cpm or less. It should be noted that under these conditions, the coefficient of variation was 10 % for cells with a ^{14}C-activity of 4 cpm and 5 % for cells with 10 cpm. In the great majority of cases (75 % of cells without insulin and all cells with insulin) the coefficient of variation on single cell counts was between 1·5 and 5 %.

Calculations. The statistical calculations were carried out on a Univac 1108 computer (Recku, Copenhagen). The likelihood ratio test (see Appendix) was designed by Aa. Vølund.

Evaluation of the methods

The mean ^{14}C-lipogenesis of cells incubated in a volume of 100 μl. at either high or low specific activity was not different from that of cells incubated in a volume of 1 ml. (data not shown). The coefficient of variation for 100 μl. cell samples incubated with insulin 10^3 μ-u./ml. was 4·0 % (n = 8).

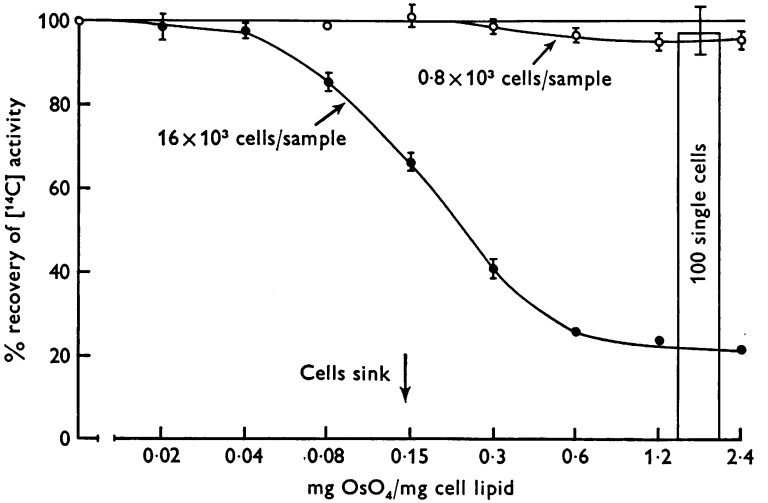
The error variance of the optically measured cell diameters was estimated by the variance between four repeated measurements of each of 100 cells. This quantity was adjusted by taking into consideration that the nearest graduation mark of the ocular micrometer was recorded as the cell diameter (a Sheppard type of correction). The estimate of the error variance thus obtained accounted for about 8 % of the total variance of the diameters of the cell sample and was equivalent to a measurement error of 1·8 μm.

The possible influence of the fixation with osmium tetroxide on the size of collagenase-isolated cells was examined in the following ways: to two concentrated suspensions, where cells accounted for about 30 % of the volume, was added a small volume of osmium tetroxide solution (5 %) and the same volume of buffer, respectively. Subsequent centrifuging of the suspensions in haematocrit tubes showed that the difference between the volumes occupied by treated and untreated cells was less than 5 %.

Furthermore, the osmium fixation did not cause any significant change in the mean or variance of the diameters of a sample of 250 fat cells (comparison of variances: F = 1·16; $P > 0·05$; comparison of means: standard normal deviate = 0·02, $P > 0·10$). These observations are not in agreement with the findings of Hirsch & Gallian (1968), who reported a 20–30 % swelling of the fat cells released from a piece of fat tissue incubated with osmium tetroxide in collidine buffer for 48 hr. To exclude the possibility of changes in the diameters of osmium-fixed cells caused by drying, forty cells were measured before and after drying using an inverted microscope. No significant difference was found between the two measurements (t = 1·17, $P > 0·10$, paired differences). The incubation for 2·5 hr caused no observable changes in the diameter distribution of osmium-fixed cells. The maximal increase in diameter due to accumulation of lipids synthesized from glucose was calculated to 0·1 μm (i.e. negligible).

The recovery of ^{14}C-activity was tested with cells which were pre-incubated with [^{14}C]glucose plus insulin and washed. With unfixed cells the recovery of ^{14}C-activity was practically identical after immersion directly in Bray's scintillation fluid and after extraction by the method of Dole & Meinertz (1960) followed by counting of the lipid phase. This was regarded as 100 % recovery. Text-fig. 1 shows that the ^{14}C-activity of samples containing 16×10^3 cells decreased progressively when more than 0·05 mg osmium tetroxide was present per mg of cell lipid. This reduction of counting efficiency was avoided by decreasing the number of fixed cells per counting vial to about 800. The mean ^{14}C-activity of 100 fixed cells, extracted and counted individually, was not different from that of unfixed cells immersed directly in the

scintillation fluid. Considering that at least half of the triglycerides synthesized from glucose by epididymal fat pad contain one or more double bonds (Hollenberg, 1966), it can be concluded that [^{14}C]triglyceride-osmium complexes are extracted and counted with a close to 100 % efficiency.



Text-fig. 1. The efficiency of extraction of [^{14}C]lipids from osmium-fixed cells. Isolated fat cells were incubated with [^{14}C]glucose and insulin for 2 hr and washed until no significant radioactivity could be detected in the medium. 100 μl. samples containing various amounts of osmium tetroxide were added to 50 μl. suspension containing 16×10^3 cells (4·4 mg cell lipid) and $0{\cdot}8 \times 10^3$ cells, respectively. The extraction was carried out in 2 ml. Bray's scintillation fluid and the recovery of activity from unfixed cells was regarded as 100 %. The arrow indicates the conditions where the cells sink in buffer. The points on the curves represent the mean of four values ± S.E. of mean when this exceeds the size of the symbol. The column represents the mean of 100 single cells ± S.E. of mean.

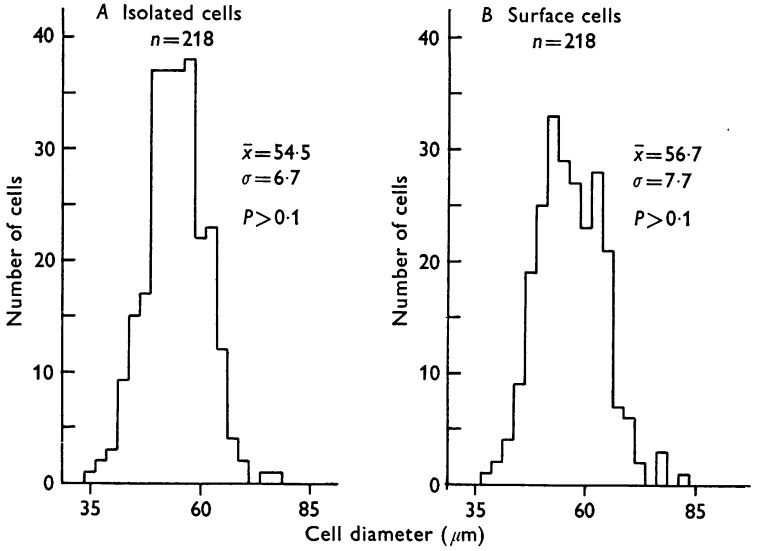
RESULTS

Size distribution of fat cells

Text-fig. 2 shows frequency distributions of the observed diameters for (*A*) collagenase-isolated cells prepared from one epididymal fat pad and (*B*) tissue surface cells obtained from the paired pad. Both distributions are normal and they are statistically significantly different. However, the difference is small, and furthermore, the fact that the observed mean 'diameter' and the S.D. are slightly larger for the somewhat irregular surface cells than for the spherical collagenase-isolated cells does not necessarily reflect a difference in cell volume between the two groups.

Distribution of ^{14}C-lipids

Text-fig. 3 shows frequency distribution curves of the synthesized ^{14}C-lipids for (*A*) collagenase-isolated cells incubated in the presence of insulin in a maximal stimulating concentration (10^3 μ-u./ml.) and (*B*) surface cells obtained from similarly incubated pieces of the paired pad. It appears that both distributions are positively skew. The relative dispersion in lipogenesis is about the same for the two cell populations and



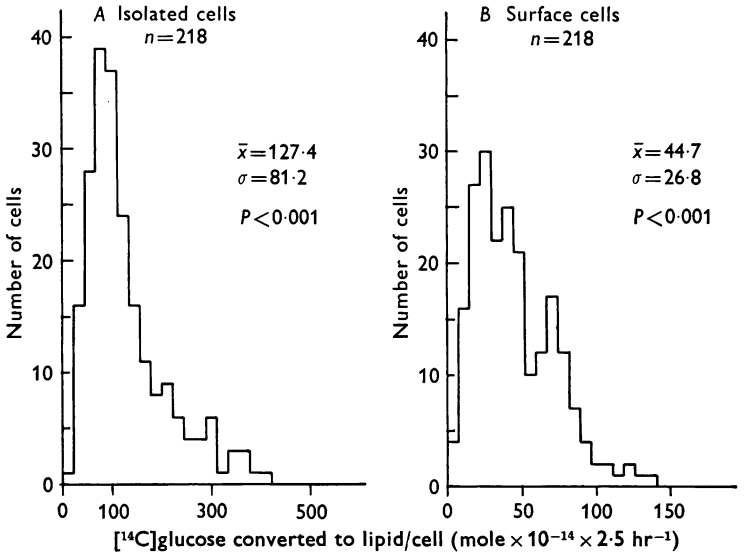
Text-fig. 2. Frequency distributions of fat cell diameters. The diameters were measured with an ocular micrometer with intervals equivalent to 2·5 μm. n indicates the number of cells measured. $\bar{x}$ and σ indicate the mean value and the S.D. respectively. P indicates the probability level for χ^2 tests of goodness of fit to normal distributions. Variance and mean of the surface cell distribution are significantly higher than those of the collagenase-isolated cell distribution ($F = 1{\cdot}32$, $P < 0{\cdot}05$) and ($t = 3{\cdot}3 \sim$ standard normal deviate, $P \sim 0{\cdot}001$).

this phenomenon seems therefore unrelated to the collagenase treatment. On the other hand, the mean ^{14}C-lipogenesis of the surface cells was only about one-third of that of the collagenase-isolated cells, a phenomenon which was reproduced in three other experiments (data not shown). The following studies were carried out with collagenase-isolated cells.

Relation between cell size and lipogenesis

Text-fig. 4*A* shows a scatter plot of lipogenesis per cell against cell diameter for a sample of 252 fat cells incubated with insulin in a maximally

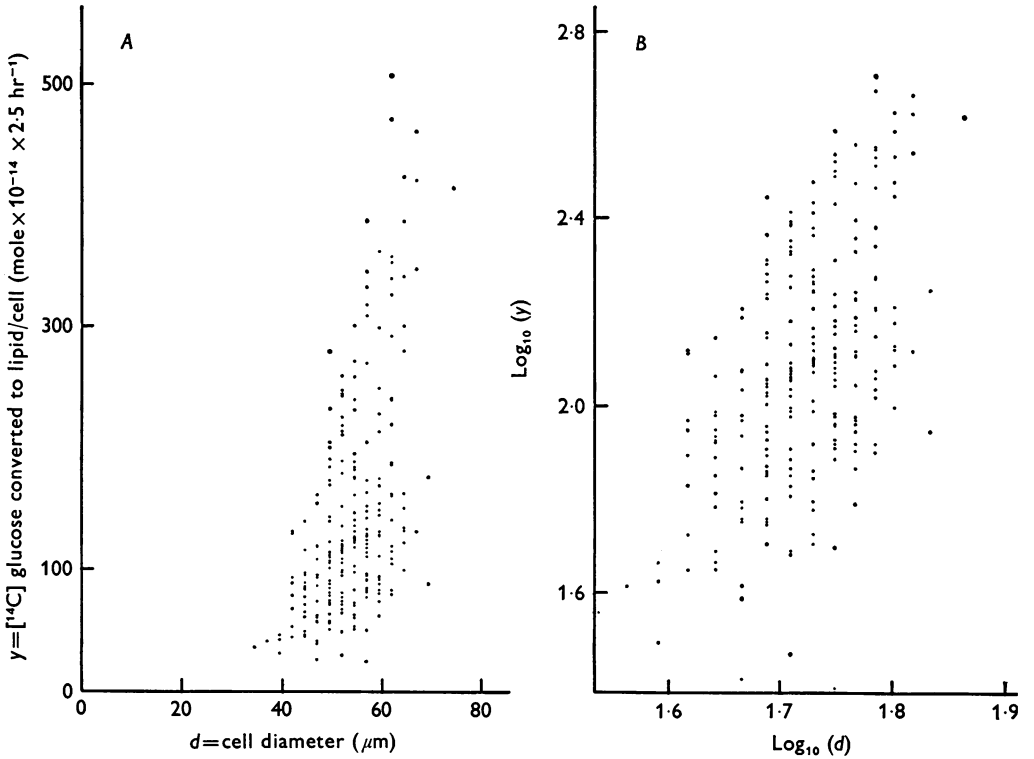
stimulating concentration. It is seen that the points exhibit a large scatter, apparently around some downward convex curve, and a hypothesis of linearity was rejected (Text-fig. 4, text). It is further seen (Table 1) that the regression lines of lipogenesis on cell diameter for each of the twelve samples from eight different rats had a negative intercept with the ordinate.



Text-fig. 3. Frequency distributions of [^{14}C]lipids in collagenase-isolated cells and fat pad surface cells. 100 μl. suspension of cells obtained from one fat pad was incubated with 'carrier-free' glucose (0·55 μ-mole/ml.) and insulin (10^3 μ-u./ml.). 7 mg of tissue from the paired pad was incubated under identical conditions except that the insulin concentration was 10^4 μ-u./ml. in order to ensure maximal stimulation of all tissue cells. The data are from the same experiment as that shown on Text-fig. 3. $\bar{x}$ and σ indicate the mean value and the S.D., respectively and P indicates the probability level for χ^2 tests of goodness of fit to normal distributions.

The model $y = kd^{\beta}$, where y is lipogenesis per cell and d is cell diameter, was fitted to the data by linear regression of log y on log d. A scatter plot of these transformed variables is shown in Text-fig. 4*B*. It is seen from Table 2 that the estimates of β obtained from different rats (samples 1–8a) range from 1·30 to 2·84. If these estimates had been in the neighbourhood of 2·00, this would have indicated that the lipogenesis was proportional to the cell surface area. However, the variation between the exponents for samples 1–8a is significantly greater than what can be attributed to the within sample error of estimate ($P < 0{\cdot}005$, cf. text to Table 2); thus, using this model, no exponent common to samples 1–8a can be found.

The coefficient of determination (r^2 in Table 2) ranges from 0·22 to 0·37. The fraction of the variance of log y which could not be attributed to variation in log d or measurement errors was calculated to range from 0·75 to 0·58 (Hald, p. 615, 1967), i.e. approximately 4/5 of the observed S.D. of the frequency distribution of lipogenesis per cell remained unexplained by the diameter variation and the measurement errors of d and y.



Text-fig. 4. Scatterplots of the observations of sample no. 1, Tables 1 and 2 on linear scales (A) and logarithmic scales (B). Since the cell diameters were grouped in classes of 2·5 μm width, an approximate F-test of the hypothesis of linearity could be carried out (Hald, p. 537 (1967)). According to such test, the untransformed data could not be fitted adequately by a straight line ($P \approx 0{\cdot}02$), whereas the transformed data could ($P \approx 0{\cdot}20$).

Cell size and sensitivity to insulin

It was shown in Table 1 (rat no. 8) that the addition of insulin caused an increase in the mean lipogenesis to about 24 times that of the 'basal'. Text-fig. 5 shows the lipogenesis per unit surface area in relation to the insulin concentration for a group of small cells and a group of large cells. There was no significant difference in the maximal response to insulin.

Furthermore, it can be seen from the curves that half of the maximal stimulation for both groups would be obtained with nearly the same concentration of insulin, namely about 7 μ-u./ml.

TABLE 1. Correlation between cell diameter and lipogeneses. The samples 1–8a were prepared from different rats and incubated with the same insulin concentration (10^3 μ-u./ml.), whereas the samples 8a–8e were prepared from the same rat, but with different insulin concentrations (10^3, 10, 5, 2·5, 0 μ-u./ml.). The lipogenesis was significantly correlated to the cell diameter ($P < 0{\cdot}001$ in all experiments)

Linear regressions of lipogenesis on cell diameter. Equation of regression line:

$$\text{lipogenesis} = \alpha \times \text{diameter} + q$$

Sample no.	Insulin concn. (μ-u./ml.)	No. of cells	Mean lipogenesis/cell (mole glucose $\times 10^{-14} \times 2{\cdot}5$ hr^{-1})	α(mole $\times 10^{-14}$/ 2·5 hr$^{-1} \times \mu$m)	q(mole $\times 10^{-14}$ $\times 2{\cdot}5$ hr^{-1})	Coeff. of correlation
1	10^3	252	126·5	6·2	−211·8	0·52
2	10^3	100	79·9	3·4	−73·9	0·59
3	10^3	101	109·6	4·1	−63·3	0·62
4	10^3	101	89·6	2·5	−19·6	0·47
5	10^3	68	87·3	3·3	−43·9	0·49
6	10^3	130	144·4	4·1	−93·1	0·46
7	10^3	97	130·0	4·0	−57·8	0·52
8a	10^3	118	119·2	3·2	−35·7	0·42
8b	10^1	102	75·1	2·6	−44·4	0·49
8c	5	105	44·6	1·6	−34·5	0·48
8d	2·5	107	19·7	0·9	−25·6	0·44
8e	0	100	4·7	0·2	−5·7	0·39

Nature of the insulin dose–response relation

The shape of the usual insulin dose–response curve obtained with samples containing a large number of fat cells can theoretically be explained in two principally different ways.

I. Each cell either responds or does not respond depending upon whether the insulin concentration is above or below a certain cellular threshold level. The distribution of the cellular threshold concentrations and the response distributions of the stimulated and non-stimulated cells determine the usual dose–response curve.

II. Each cell is characterized by a continuous dose–response curve. This is equivalent to saying that insulin exerts a graded effect on the single cell.

These two explanations may be regarded as simple models. It is possible to construct more complex models representing stepwise changes from I to II by assuming a certain number of intermediate stimulation states placed in between the non-stimulated and the highest stimulated state and a corresponding number of cellular threshold distributions and response distributions. Furthermore, it is possible to derive intermediate models between I and II by assuming a heterogeneous cell population, i.e. a

TABLE 2. Nature of the correlation between cell size and lipogenesis. The numbers refer to the same samples as those shown in Table 1. To test for heterogeneity (with respect to β') between the identically treated samples 1–8a, G was calculated as

$$\sum_{i=1}^{8} \frac{1}{e_i^2}(\beta_i - \bar{\beta})^2,$$

where β_i is the slope of the regression line for the ith rat, e_i is the S.E. of β_i and $\bar{\beta}$ is the weighted mean slope calculated as

$$\sum_{i=1}^{8} \frac{1}{e_i^2} \cdot \beta_i \Big/ \sum_{i=1}^{8} \frac{1}{e_i^2}.$$

Assuming all values of β_i to be estimates of the same exponent, the quantity G is approximately distributed as a χ^2 statistic with (8–1) degrees of freedom, the goodness of the approximation being ensured by the fact that each estimate of e_i is based on a large number of degrees of freedom (minimum 66, maximum 250), and that $1/e_i^2$ is therefore a good estimate of the ideal weighting factor. Since

$$G = 21{\cdot}50 > \chi^2_{0{\cdot}995}\,(7) \approx (P < 0{\cdot}005),$$

the hypothesis about a common slope was rejected. The 'corrected exponents' are the estimates of the regression coefficients of log (lipogenesis) on the true value of log (diameter), i.e. the slope of the regression corrected for random errors in the independent variable. The formula $\beta = \beta'\,(1 + [\sigma_V^2/\sigma_X^2])$ (Hald, pp. 615–616, 1967), where β is the true regression coefficient, β' the observed, σ_X^2 the variance of the property log (diameter) and σ_V^2 is the measurement error of log (diameter), was used. The estimate of σ_V^2 was obtained as described in 'Evaluation of the methods'.

Linear regressions of log (lipogenesis) on log (diameter).

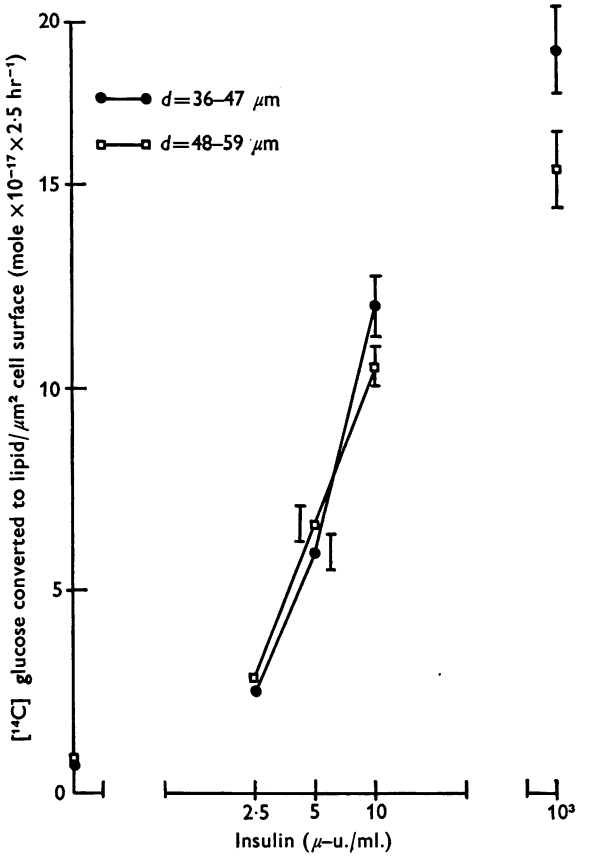
Equation of regression line: log (lipogenesis) $= \beta' \times$ log (diameter) $+ k$

Sample no.	No. of cells (n)	Exponent ($\beta' i$)	S.E. of $\beta' i$ (e_i)	Coeff. of correlation (r)	r^2	Corrected exponent (β)
1	252	2·61	0·25	0·55	0·30	2·84
2	100	1·98	0·26	0·61	0·37	2·16
3	101	1·54	0·22	0·58	0·34	1·68
4	101	1·19	0·23	0·47	0·22	1·30
5	68	1·80	0·40	0·48	0·23	1·96
6	130	1·90	0·29	0·50	0·25	2·07
7	97	1·60	0·24	0·57	0·33	1·74
8a	118	1·46	0·25	0·48	0·23	1·59
8b	102	1·79	0·31	0·50	0·25	1·95
8c	105	2·00	0·31	0·54	0·29	2·18
8d	107	2·70	0·47	0·49	0·24	2·94
8e	100	2·67	0·50	0·47	0·22	2·90

fraction of the cells might behave as under I and the remaining fraction as under II. Such heterogeneity may further be combined with intermediate response states producing models of high complexity.

If model I is true it follows that any observed distribution of cellular responses is a mixture of two distributions, i.e. a fraction of the cells represents a sample from the distribution of stimulated cells and the rest

a sample from the distribution of the non-stimulated cells. Since the response distributions of the stimulated and non-stimulated cells are unknown, at least three different observed distributions are required to test the statistical hypothesis (H_0) that these three distributions are mixtures of two response distributions (stimulated and non-stimulated cells) against the alternative hypothesis (H_1) that the three distributions are not



Text-fig. 5. Insulin dose–response relationship for cells of different size. The data are from the experiment also shown in Table 1, rat no. 8. The abscissa shows the insulin concentration on a log scale. The numbers on the curves indicate the range of diameters. The data are expressed per unit surface area with mean values ± 1 S.E. of the mean when this exceeds the size of the symbol. The ratio

$$\frac{\text{lipogenesis } (10^3\ \mu\text{-u./ml.}) \text{ minus lipogenesis } (0\ \mu\text{-u./m.})}{\text{lipogenesis } (0\ \mu\text{-u./ml.})}$$

is with 95% confidence limits in brackets: 25 (20–33) for the small cells and 20 (15–26) for the large cells, respectively.

TABLE 3. Observed and calculated numbers of observations in the classes of grouped distributions of cellular lipogenesis per unit surface area. Data from rat no. 8. Different class intervals are used by each set of distributions at three successive insulin concentrations. The calculated numbers shown inside the parentheses are the expected numbers determined under H_0 by the likelihood maximization. The lack of fit between the calculated and the corresponding observed numbers shown outside the parentheses is quantitatively expressed in the significantly high values of χ^2 leading to rejection of H_0

Class limits	Insulin concn. (μ-u./ml.) 0·0	2·5	5·0	Class limits	Insulin concn. (μ-u./ml.) 2·5	5·0	10·0	Class limits	Insulin concn. (μ-u./ml.) 5·0	10·0	10^3
0·00–0·79	92 (88)	30 (34)	1 (1)	0·00–0·99	43 (32)	4 (16)	1 (1)	0·00–1·79	15 (14)	4 (5)	1 (1)
0·80–0·99	5 (7)	13 (7)	3 (7)	1·00–1·79	29 (26)	11 (14)	3 (3)	1·80–2·99	36 (30)	4 (11)	3 (2)
1·00–1·39	3 (5)	20 (10)	5 (13)	1·80–2·99	30 (41)	36 (23)	4 (6)	3·00–4·99	44 (49)	29 (22)	8 (10)
1·40–1·79	1 (1)	9 (6)	6 (9)	3·00–4·99	8 (11)	44 (28)	29 (42)	5·00–6·99	9 (11)	39 (24)	22 (35)
1·80–∞	1 (1)	39 (53)	93 (79)	5·00–∞	1 (1)	13 (28)	66 (52)	7·00–∞	4 (4)	27 (41)	84 (70)
χ^2	30·3				54·7				33·4		
D.F.	1				1				1		
P-value	< 0·0005				< 0·0005				< 0·0005		

mixtures of two. The details of the statistical likelihood ratio test are given in the Appendix.

The distributions of lipogenesis per unit surface area of the cells from rat no. 8 were used for testing H_0 against H_1. The test was carried out using the three groups of data corresponding to the successive insulin concentrations (0, 2·5, and 5 μ-u./ml.), (2·5, 5 and 10 μ-ul/ml.), and (5, 10 and 10^3 μ-u./ml.). The results of the likelihood ratio rests are shown in Table 3. It appears that H_0 is rejected in each case ($P < 0{\cdot}0005$). The lack of agreement between the observed distributions and the best fitting mixtures of two distributions is also shown in Table 3.

The experiment was repeated twice with two other rats using the insulin concentrations 2·5, 5 and 10 μ-u./ml., and without correcting the lipogenesis for the surface area of the cell. This resulted in slightly lower values of the approximate χ^2-statistic, but H_0 was still rejected at the 0·0005 level.

It can thus be concluded that the observed distributions at different insulin concentrations along the dose–response curve cannot be explained according to model I. At least three discrete cellular stimulation states are required for some cells. It seems more reasonable to assume the simplest of the alternative models, i.e. that insulin exerts a graded effect on each single cell.

DISCUSSION

Comparison of collagenase–isolated cells and tissue surface cells

Fat cells from the surface of a piece of tissue exhibited about the same relative dispersion with respect to lipogenesis from glucose as did isolated fat cells. This observation indicates that the marked relative dispersion could not be caused by the collagenase treatment.

The mean lipogenesis from glucose of the tissue surface cells was about one-third of that of collagenase-isolated cells (Text-fig. 3). Since the natural surface of a fat pad is covered with mesothelium, the reason for the observed difference in mean lipogenesis might be that the diffusion of glucose through this layer is rate limiting even for the lipogenesis of the most superficially located fat cells. It also seems possible, however, that the collagenase treatment changes the characteristics of the fat cell membrane and facilitates the access of glucose to the carrier sites.

The relationship between cell size and lipogenesis

In the present work, a positive correlation between lipogenesis and cell diameter to an exponent of β was demonstrated for young *ad lib.* fed rats. The estimates of β on cell samples from different rats varied significantly; it seems reasonable to assume that this phenomenon is due to a biological

variation between rats, although other factors, such as the collagenease treatment, cannot be excluded.

The finding of a positive correlation between lipogenesis and cell diameter is in agreement with the results of Björntorp & Karlsson (1970), who utilized the different flotation rates of large and small fat cells to separate isolated fat cells from the same piece of tissue into populations with different mean diameters. With human subcutaneous fat cells these authors observed an increase in the mean lipogenesis per cell from [^{14}C]-glucose with the mean diameter. However, the effect of insulin was small and varying. Similar results were obtained when pieces of tissue were incubated with radioactive glucose and the cells then separated into populations with different mean sizes by treatment with collagenase followed by differential flotation.

The growth of fat cells will largely be proportional to the net lipogenesis, i.e. lipogenesis minus lipolysis. According to our results with short-term experiments *in vitro*, the lipogenesis will be proportional to the diameter to the exponent of β where β has a value between 1 and 3. Zinder & Shapiro (1971), using the technique of Björntorp & Karlsson (1970) for separation of isolated fat cells, found that the epinephrine-induced release of free fatty acids was proportional to the surface area of the cell. Extrapolation from such *in vitro* results to cell growth *in vivo* must obviously be interpreted with caution. It seems noteworthy, however, that the change in the distribution of fat cell diameters seen during the growth of young, *ad lib.* fed rats, as published by Di Girolamo, Mendlinger & Fertig (1971), are in agreement with the changes in diameter distribution which we have calculated under the assumption that the net lipogenesis is proportional to the diameter to exponents ranging from about 1·2 to 1·8 and clearly in disagreement with changes in distributions obtained with exponents below 0·8 and above 2·2.

The above assumptions also imply that the turnover rate of the triglycerides located within small fat cells will be greater than that of the triglycerides located within large fat cells. This may contribute to the functional compartmentation of adipose tissue which was first proposed by Hirsch, Farquhar, Ahrens, Peterson & Stoffel (1960).

Other workers have attempted to analyse the relationship between cell size and lipogenesis by using pieces of fat tissue or collagenase-isolated fat cells prepared from animals of different ages and therefore with different mean cell sizes: Zinder, Arad & Shapiro (1967), using isolated epididymal fat cells from rats weighing less than 220 g, incubated both with and without insulin, found that the rates of lipogenesis from [^{14}C]glucose and from [^{14}C]palmitate in the presence of glucose increased with the cell size. On the other hand, Knittle & Hirsch (1968), using rat epididymal fat pads, found

the mean lipogenesis per cell independent of the cell size in the absence of insulin. Salans, Knittle & Hirsch (1968), using human fat tissue and isolated rat fat cells, Greenwood, Johnson & Hirsch (1970), using isolated mouse fat cells, and Salans & Dougherty (1971), using isolated rat fat cells and fat cell 'ghosts', reproduced this result and found in addition that the lipogenesis in the presence of insulin in a maximally stimulating concentration decreased with increasing cell size. Smith (1970), using pieces of human subcutaneous fat tissue, found that the lipogenesis from glucose in the absence of insulin increased with the cell size, whereas the insulin-induced percentage change in lipogenesis over the basal value was negatively correlated to the cell size. The above studies are, however, not directly comparable with the present one, since populations of fat cells from different individuals (with different mean cell sizes) were compared.

Nielsen, Hansen & Gliemann (1972), using a filter technique to fractionate osmium-fixed collagenase-isolated fat cells according to size, studied the lipogenesis of fat cells prepared from *ad lib.* fed rats of different ages and weighing from 100 to 500 g. These authors were able to demonstrate a positive correlation between cell size and lipogenesis, both in the absence and in the presence of insulin for cells prepared from any one animal, irrespective of its weight. In the presence of insulin, however, the lipogenesis of cells prepared from large rats (weight above 300 g) was found to be markedly smaller than the lipogenesis of cells of the same size but prepared from smaller rats. Considering the results of the latter study, it would be hazardous to draw conclusions about the relationship between cell size and lipogenesis from experiments where changes in cell size cannot be dissociated from changes in the age or weight of the animals.

The present results show that the lipogenesis of cells prepared from the same piece of tissue varied markedly even after the best correction for differences in cell size. This variation is presumably largely due to differences in the rates of glucose transport per unit surface area, since carrier-facilitated transport appears to be rate-limiting for the glucose metabolism under the conditions employed in the present study (Gliemann, 1970). It remains to be seen whether this residual variation is only present in such relatively short term *in vitro* experiments.

The nature of the insulin action

The log insulin dose–response curve is an approximately symmetrical sigmoid curve, and such a relationship would be obtained if the fat cells responded to insulin in an 'all or none' fashion with their log threshold concentrations approximately normally distributed. However, the present results suggest that increasing concentrations of insulin cause a continuous increase in the glucose uptake of each single cell.

Fisher & Gilbert (1970), studying the kinetics of permeation of pentoses into the rat heart, found that the maximal transport capacity of the carrier system was altered by insulin in a graded fashion whereas the apparent affinity of the pentoses to the carrier system was decreased by insulin in a manner which was independent of the insulin concentration. These effects could not be accounted for by a graded action of insulin on the pentose transfer of each carrier whereas they were consistent with the hypothesis that a fraction of the available carriers were converted to another form in the presence of insulin in a submaximal concentration.

In principle, these converted carriers of the rat heart could be the carriers of a certain number of cells or they could be a fraction of carriers of each cell. The present demonstration of a graded effect of insulin on the single fat cell raises the question as to whether or not the insulin effect on the glucose carrier of fat cells is of a quantal nature. Experiments to elucidate this question are at present in progress in this laboratory.

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APPENDIX

By Aa. Vølund

A likelihood ratio test for mixtures of distributions

Any sampling distribution grouped into k classes can be represented by the multinomial distribution:

$$p(x_1, x_2, \ldots, x_k) = \left(\sum_{j=1}^{k} x_j\right)! \prod_{j=1}^{k} \pi_j^{x_j} / x_j!$$

π_j is the probability that an observation falls into the j-th class, and x_j is the number of observations in that class. The well-known problem of testing whether n grouped distributions are samples from the same population is readily solved by means of a likelihood ratio test or a χ^2-test. In the following a likelihood ratio test is suggested to test whether n grouped distributions are samples from mixtures of r populations ($n > r \geqslant 2$).

It is assumed that the parameters of the n multinomial distributions are given by

$$\pi_{ij} = \sum_{\nu=1}^{r} \gamma_{i\nu}\theta_{\nu j}; \quad \sum_{\nu=1}^{r} \gamma_{i\nu} = 1; \quad \sum_{j=1}^{k} \theta_{\nu j} = 1;$$

$$i \in (1, 2, \ldots, n); \quad j \in (1, 2, \ldots, k).$$

The problem of testing H_0: $r \leqslant r_0$ against H_1: $r > r_0$ is considered. The likelihood ratio (LR) is

$$LR = \frac{\sup_{H_0}\left\{\prod_{i=1}^{n}\prod_{j=1}^{k}\left(\sum_{\nu=1}^{r_0}\gamma_{i\nu}\theta_{\nu j}\right)^{x_{ij}}\right\}}{\sup_{H_1}\left\{\prod_{i=1}^{n}\prod_{j=1}^{k}\left(\sum_{\nu=1}^{r}\gamma_{i\nu}\theta_{\nu j}\right)^{x_{ij}}\right\}}. \tag{1}$$

The number of independent parameters under H_0 is: $r_0(k-1)+(r_0-1)\,n$ and under $H_1\,(n = r)$: $n(k-1)$. It is thus required that

$$n(k-1) > r_0(k-1)+n(r_0-1). \tag{2}$$

From the general theory of likelihood ratio tests (see, for example, Lehmann (1959)) it is known that the asymptotic distribution of $-2\log(LR)$ is a χ^2 distribution with $n(k-1)-r_0(k-1)-n(r_0-1)$ degrees of freedom.

The simplest case; $n = 3$, $r_0 = 2$ requires according to (2) that $k \geqslant 5$. The numbers of independent parameters under H_0 and H_1 are 11 and 12 respectively. The numerator of (1) can be calculated by a computer maximization procedure, whereas the denominator is readily calculated as the maximum likelihood of n multinomial samples ($n = r$).

Table 3 shows three sets of grouped distributions of lipogenesis at successive insulin concentrations. The five classes are chosen so as to obtain approximately the same total number of observations in each class. The maximization under H_0 was carried out by means of a simplex algorithm (O'Neill, 1971). Repeated runs with different starting points gave the same approximate χ^2 value, but the values of the parameters under H_0 were slightly different. The calculated expected number of observations in each class ($\hat{x}_{1j}$) under H_0 are shown in the brackets in Table 3:

$$\hat{x}_{1j} = \left(\sum_{j=1}^{k} x_{1j}\right)\sum_{\nu=1}^{r_0}\hat{\gamma}_{i\nu}\hat{\theta}_{\nu j}.$$

The high values of $-2\log(LR)$ which is approximately χ^2-distributed with 1 degree of freedom under H_0 lead to rejection of H_0; i.e. the observed distributions cannot be regarded as samples from mixtures of two populations.

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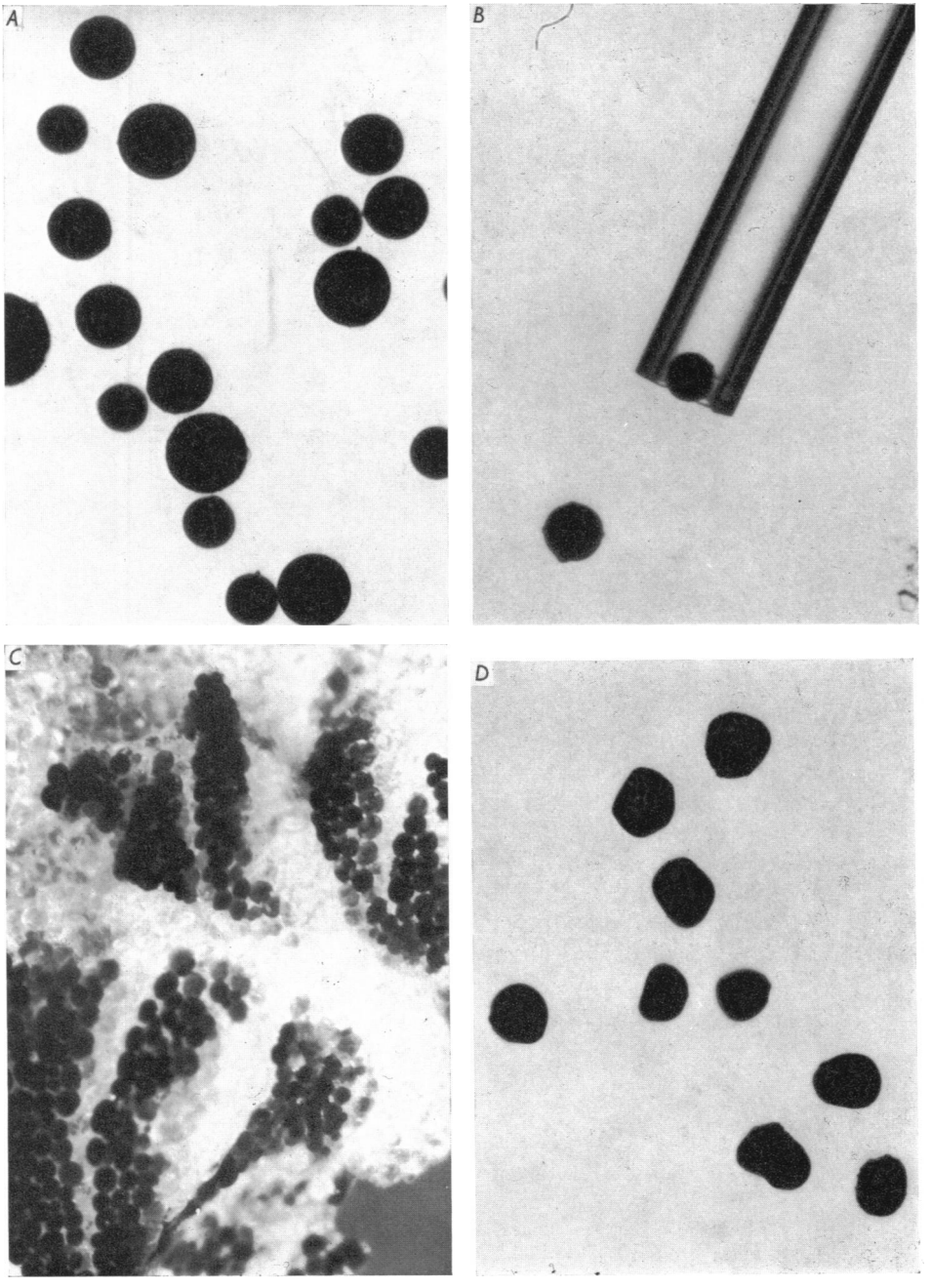
EXPLANATION OF PLATE

A: collagenase-isolated fat cells fixed with osmium tetroxide.

B: cell leaving the tip of the pipette.

C: piece of epididymal fat tissue with osmium-fixed surface cells. Note that the tissue has been partially torn apart in order to expose the underlying unfixed cells.

D: single osmium-fixed surface cells.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