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Dynamic Aspects of Insulin Action: Synchronization of Oscillatory Glycolysis in Isolated Perifused Rat Fat Cells by Insulin and Hydrogen Peroxide†

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ABSTRACT: Glucose oxidation to CO_2 was investigated in isolated perifused rat epididymal fat cells. Insulin stimulated rates of oxidation up to 30-fold. Multiple pulses of insulin or prolonged perifusion with the hormone led to a time-dependent desensitization of the cells. The action of insulin could be mimicked by H_2O_2. Reversal of H_2O_2 effects was associated with a damped oscillation of large initial amplitude. Initiation of perifusion with insulin induced rates of glucose oxidation that oscillated around a mean elevated rate with an amplitude of about ±4% of the mean, significantly larger than the measurement error. Basal rates did not show clear oscillations. The oscillations after insulin had a statistically significant period of around 14 min. The results were the same with C1- or C6-labeled glucose and occurred in the presence of both 0.275 and 5.5 mM glucose in the perifusion medium. The oscillations were interpreted as the result of insulin- or H_2O_2-induced synchronization of oscillatory glycolysis by individual fat cells. The similarity of the observed oscillatory period with the period of oscillatory insulin secretion by pancreatic β cells suggests that oscillatory glycolysis may constitute the internal pacemaker for the latter process.

Glycolysis is well-known to oscillate in a variety of systems. The best studied case is yeast (*Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae*) where oscillations could be observed in individual cells, in cell populations, and in cell extracts [for a review, see Hess (1979)]. Evidence for oscillations has also been found in mammalian systems, i.e., cell-free tissue extracts from beef heart (Frenkel, 1965, 1966, 1968a-c) or rat leg muscle (Tornheim & Lowenstein, 1973-1975). Evidence that such oscillations occur in intact mammalian cells is still very limited, however, and consists of observations in cultured mouse fibroblasts (L cells) (Werrlein & Glinos, 1974) and cell suspensions of Ehrlich ascites tumor cells (Ibsen & Schiller, 1967, 1971). Because individual cells may oscillate out of phase, oscillations in cell populations are usually only seen after synchronization. The present report demonstrates that glycolysis in isolated rat epididymal fat cells oscillates and that perifusion with insulin or hydrogen peroxide can synchronize the oscillations.

Materials and Methods

Epididymal fat cells were isolated from 120-150-g Sprague-Dawley rats (Taylor Laboratories, Bellevue, WA) according to Rodbell (1964). Materials were those specified in Muchmore et al. (1981), except that bovine serum albumin (BSA)[1] was a product of Armour Pharmaceutical Co. (CRG-7, lot TX-3). Fat cells were perifused in the apparatus described by Little & de Haën (1980), with the following modifications: the pump was a Technicon Pump 1 equipped with a main buffer line (0.9 mL/min) and a line (0.1 mL/min) for additions of insulin, H_2O_2, or control medium, which joined the main buffer line 1 cm before the entrance to the perifusion chamber. Another line (0.1 mL/min) for addition of NaOH (0.35 M) containing EDTA (26 mM) joined the perifusion

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[1] Abbreviations: BSA, bovine serum albumin; KRB, Krebs-Ringer bicarbonate buffer modified as described in the text; KRP, Krebs-Ringer phosphate buffer modified as described in the text; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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effluent 1 cm distal to the chamber exit. The addition of NaOH elevated the pH of the perifusate to pH 10.5 for trapping CO_2. EDTA prevented calcium from precipitating. So that loss of CO_2 and consequent pH changes in the perifusion medium during prolonged perifusions could be prevented, the medium was kept under a slight pressure of 5% CO_2/95% air with the help of a rubber balloon.

Except where otherwise noted, fractions of 2 mL were collected directly into plastic 15-mL scintillation vials by using a fraction collector (Isco, Model 328) and were capped immediately. No loss of counts occurred if the samples were left open for up to 30 min. $^{14}CO_2$ was collected after acidification of the effluent with 0.5 mL of 1 M citric acid containing 5% sodium azide by a modification of the methods of Gliemann (1965) and Muchmore et al. (1981). Chimneys with improved fit and lifetime were prepared as follows. Polyallomer centrifuge tubes ($^5/_8 \times 3$ in., Beckman) were cut with a razor blade at an angle of 116° from the long axis 3 mm below the mark between the straight and the round portion of the tube. The cut was initiated opposite to the thinner and thus softer side of the straight portion of the tube. The top of the tube was slightly rounded by pressing it into a 8 × 8 × 4 cm aluminum block with a mold in the form of a spherical cap of diameter 19 mm and of depth 8 mm, which had been preheated over a Bunsen burner. The chimneys were used to hold reagents and to join two scintillation vials in the manner described by Gliemann (1965), except that both ends of the chimney were inserted just enough for a tight fit. The increased volume of the system helped to reduce pressure buildup and consequent leakage of $^{14}CO_2$ during collection. Spuriously low counts were further minimized if perifusate samples were limited to 2 mL. It can be calculated that 1.12 mL of CO_2 is liberated under these conditions.

Unless specifically stated otherwise, the standard perifusion medium was a modified Krebs–Ringer bicarbonate medium and contained 128 mM NaCl, 5.16 mM KCl, 1.04 mM $CaCl_2$, 1.30 mM KH_2PO_4, 1.30 mM $MgSO_4$, and 10 mM Hepes. In addition, it contained 0.5% (w/v) BSA, ^{14}C-labeled glucose at 0.275 mM (sp act. 45.5 μCi/mmol) or at 5.5 mM (sp act. 2.27 μCi/mmol), 100 units/mL penicillin, and 100 μg/mL streptomycin. The pH was adjusted to 7.4 and the solution allowed to equilibrate with the perifusion apparatus by passing 64–78 mL of buffer through the apparatus prior to initiation of experiments. Each perifusion used the fat cells from two to three rats, which varied between 0.5 and 1 mL of packed cells.

For the experiment in bicarbonate-free medium, the above buffer was replaced by Krebs–Ringer phosphate with half the recommended concentration of calcium. The buffer was maintained under CO_2-free air with the aid of a rubber balloon.

Data Evaluation. Following the lead of Ookhtens et al. (1974) and Hansen et al. (1982), data were evaluated quantitatively by a variety of smoothing and detrending techniques as well as by techniques specifically suited for the analysis of time series (Jenkins & Watts, 1968). Specifics concerning the adaptation of these techniques to our data are presented together with the results. Fortran IV programs were written for the various techniques and implemented on Digital Equipment Corp. PDP12 and VAX 11/780 computers. All analyses and programs were tested on fully evaluated time series in the literature, synthetic time series of random numbers that were normally distributed around zero, and oscillatory metabolic functions of complex wave form continuously monitored in yeast extracts (Hess & Boiteux, 1968).

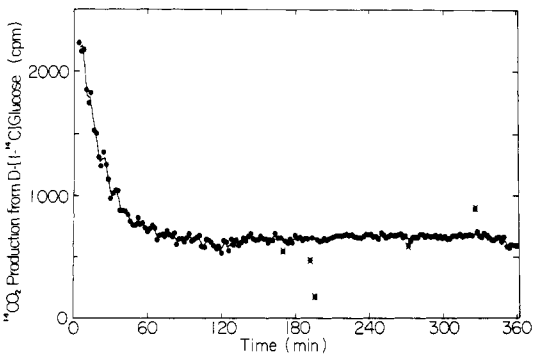

FIGURE 1: Basal glucose C1 oxidation rate of perifused fat cells. Fractions of 2 min were collected. Crossed out data points are considered outliers on the basis of criteria cited in the text. The solid line is the two point moving average.

One recent technique of data smoothing, the optimal segments technique, was developed by Finegood & Bergman (1983). This technique minimizes simultaneously the changes in slopes between adjacent point to point segments and the deviations between fitted and experimental values, with the constraint that no more of the variation in the data is lost than what is allowed by the measurement error. The changes are well distributed along the time axis. The authors kindly provided us with a description of the technique and a corresponding BASIC program. The program was translated into Fortran IV with some modifications. The method requires matrix inversion. Fifteen points were analyzed at a time in order to keep the size of the matrices reasonable when treating large numbers of data points. The reading frame was moved one point at a time until all the data were covered. The midpoints of the reading frame were accepted as the smoothed values. At the ends, the $(15 - 1)/2$ beginning and ending smoothed values, respectively, were also accepted.

Results

Basal Glucose Oxidation. Fat cells were perifused with 0.275 mM D-[1-^{14}C]glucose for 360 min, and every 2 min, the total amount of $^{14}CO_2$ produced during the period was determined (Figure 1). Initial rates of glucose oxidation were often elevated by a variable amount and, as observed in this experiment, decayed exponentially to a base-line level. In this experiment, the decay time was $t_{1/2} = 13.5$ min. These initial decay rates varied substantially from experiment to experiment, and $t_{1/2}$ could be as high as 25.3 min. In the example shown in Figure 1, the initial activity was high and essentially reached average basal levels after 90 min. The average basal rate remained constant for the remaining duration of the experiment, i.e., 270 min, indicating that there was no significant cell death during this phase. Individual measurements were associated with a total analytical error expressed as coefficient of variation, $(\sigma/\mu)_{tot}$, composed of an experimental error in fraction and CO_2 collection, $(\sigma/\mu)_v$, and a statistical error in counting, $(\sigma/\mu)_c$, where μ is the mean and σ is the standard deviation. If a constant rate of CO_2 production between 212 and 324 min (Figure 1) is assumed, the total error associated with each measurement was estimated as $(\sigma/\mu)_{tot} = 1.84\%$, whereas the error in counting was $(\sigma/\mu)_c = 1.23\%$. The error in fraction and CO_2 collection was thus estimated as $(\sigma/\mu)_v = [(\sigma/\mu)_{tot}^2 - (\sigma/\mu)_c^2]^{1/2} = 1.37\%$. Since in all other experiments the method of fraction and CO_2 collection was identical but the counting error varied, the same value of $(\sigma/\mu)_v$ and the above propagation of error formula were used to evaluate the total error in experiments where glucose oxidation oscillated. In view of the error given, it also appears

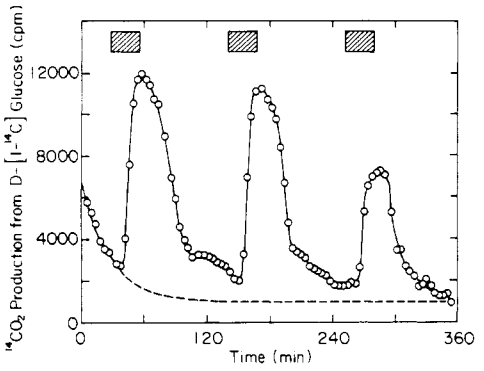

FIGURE 2: Repetitive stimulation of glucose Cl oxidation by insulin in perifused fat cells. Three pulses of insulin in submaximally stimulating concentrations (5×10^{-10} M) were given as indicated by the bars. Data are the two point moving average after elimination of a few outlying data points. Fractions of 4 min were collected. The dashed line is the continuation of the exponential decay of the unstimulated rate as obtained by the method of least squares using the first nine data points.

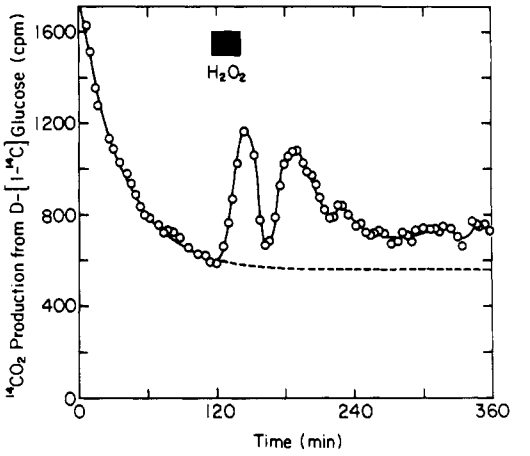

FIGURE 3: Stimulation of glucose Cl oxidation by H_2O_2. After a stable base line was approached a pulse of H_2O_2 (200 μM) was applied. Fractions of 4 min were collected. The dashed line is the continuation of the exponential decay of the unstimulated rate as obtained by the method of least squares using the first 23 data points.

that in the early parts of the experiment in Figure 1 there may have been fluctuations not accountable on the basis of error alone, but we do not want to make a point about that.

Figure 1 also shows a number of fractions with large deviations from their neighbors which we attribute to systematic error (CO_2 leakage, radioactive contamination). When outliers were more than three standard deviations away from the mean surrounding data points, they were excluded from consideration. The frequency of such erroneous measurements in Figure 1, i.e., 5 in 180, was typical. When points were required at these positions for data analysis, the mean of the two adjacent ordinates was substituted.

Repetitive Insulin Pulses. Three consecutive 24-min pulses of insulin (5×10^{-10} M) separated by 80-min washout intervals resulted in three response peaks of glucose oxidation (Figure 2). These early experiments had a higher variation in the data than that reported above. Because for current purposes only the gross shape of the responses is relevant, we chose to report two point moving averages. The first response was variable in magnitude and dependent on insulin concentrations. It was on occasion as large as 30 times the basal rate. Subsequent peaks decreased in magnitude with time. Stimulated glucose oxidation after cessation of perifusion with insulin decayed in a complex fashion with two apparent phases. The time between insulin pulses was of insufficient duration to attain the base line anticipated from the initial exponential decay. The decay of insulin responsiveness in the absence of cell death, as indicated by the constant base line documented in Figure 1, is consistent with a process of time-dependent desensitization. The rate of such desensitization varied greatly between experiments and could not be simply related to the insulin concentrations used. Thus, we could not decide whether desensitization was insulin induced, perhaps via receptor internalization, or whether other metabolic alterations in the course of the perifusion were responsible.

Stimulation by H_2O_2. H_2O_2 is well-known to mimic insulin action in many respects [e.g., see Little & de Haën (1980)]. To test whether H_2O_2 stimulation of glucose oxidation was reversible, we exposed perifused fat cells to a 24-min pulse of 200 μM H_2O_2 (Figure 3). Glucose Cl oxidation was rapidly stimulated. Upon cessation of the H_2O_2 perifusion, glucose oxidation rates decayed in a form similar to a damped oscillation of large initial amplitude to a value somewhat above basal rates. Multiple consecutive pulses of H_2O_2 showed this phenomenon to be reproducible (data not shown). Thus, the

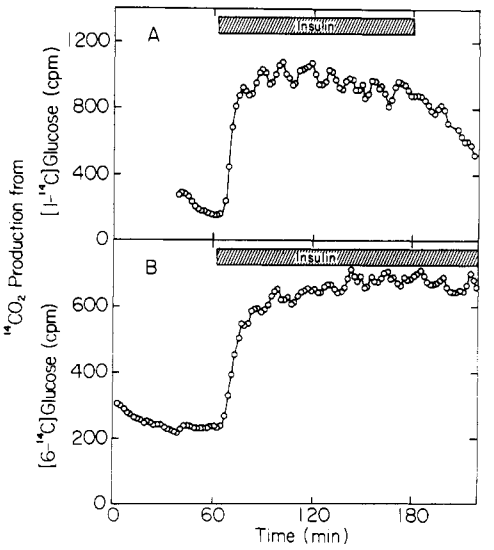

FIGURE 4: Prolonged stimulation of glucose oxidation by insulin in perifused fat cells. Insulin was perifused at a concentration of 5×10^{-9} M for the periods indicated by the bars. Data are two point moving averages of 2-min fractions. (A) Tracer was D-[1-^{14}C]glucose; (B) tracer was D-[6-^{14}C]glucose.

H_2O_2 effect on glucose oxidation is in part reversible, but not in a simple fashion.

Prolonged Perifusion with Insulin. After an initial perifusion period during which an approximately stable basal glucose oxidation rate was reached, insulin was suddenly included in the perifusion medium to a concentration of 5×10^{-9} M. Figure 4 shows two such experiments in which glucose labeled at position 1 or 6 was used. The data from these experiments are presented as moving averages of 2 to facilitate initial visual analysis. The mean rate tended to fall slightly with time, after approaching a maximally stimulated glucose Cl oxidation rate in an exponential fashion. Glucose oxidation rates decayed after stopping insulin perifusion. Glucose C6 oxidation showed a similar pattern of onset of stimulation but less decay as a function of time (Figure 4B). Stimulated rates appeared to oscillate around a mean with a period of about 14 min. When insulin perifusion was longer than in the experiments shown in Figure 4, the amplitude of the oscillations decreased (data not shown). Systematic error due to variations in temperature, pumping speed, or batchwise handling of samples could not be correlated with these oscillations in any

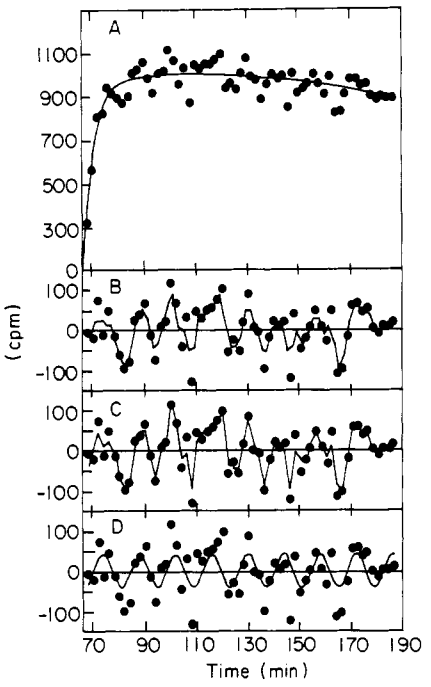

FIGURE 5: Illustration of data analysis for the period of insulin stimulation in the experiment shown in Figure 4A. (A) Data are original counts from 2-min fractions after subtraction of the basal rate estimated as 151 cpm. The solid line is the least-squares fit or eq 1 to the data. The constants were $A = 818.7 \pm 42.7$ cpm, $B = 1.40 \pm 1.40$ cpm min^{-1}, $C = 0.019 \pm 0.010$ cpm min^{-2}, $t_0 = 70.0 \pm 0.4$ min, and $k = 0.25 \pm 0.05$ min^{-1}. (B–D) Data are the values shown in panel A minus the value of the mean predicted by eq 1. (B) The solid line is the two point moving average. (C) The solid line is the result of the optimal segments smoothing technique described under Materials and Methods. (D) Least-squares fit of a sine wave to the data.

of the reported experiments, leading us to conclude that the oscillations were real. In order to evaluate the observed oscillations in more detail and to assess their statistical significance, we performed seven such glucose Cl oxidation experiments and evaluated them completely. For illustrative purposes, we have chosen to present the complete analysis of a particular experiment, but we discuss the others and the extent to which the chosen experiment was typical.

Modeling of the Gross Features of the Stimulated State. The original unsmoothed data were the basis of any further analysis. The gross features of the time dependence of glucose oxidation after a sudden inclusion of insulin in the perifusion buffer could be described by the equation:

$$\tilde{y}_t = (A + B\tau + C\tau^2)[1 - \exp(-k\tau)] + b_0 \qquad (1)$$

where $\tilde{y}_t$ is the least-squares estimate of the glucose oxidation rate at time t and $\tau = t - t_0$ (Figure 4A). The curve-fitting parameters were A, B, C, t_0, and k. Thus, t_0 is the fitted time at which glucose oxidation rates suddenly increased in response to insulin. The basal rate, b_0, was estimated separately. The factor $A + B\tau + C\tau^2$ reflects a quadratic decay of the mean amplitude of stimulation, similar to the phenomenon of desensitization described above for repetitive insulin pulses. The factor $1 - \exp(-k\tau)$ describes an exponential approach to the stimulated rate (constants given in the legend to Figure 5A). The fluctuations around the mean rates, shown in Figure 5B–D, were obtained by subtraction of the mean, $\tilde{y}_t$, estimated for a given time by the above equation, from the data in Figure 5A.

Analysis of Fluctuations. For the following analyses, we have chosen the term fluctuations to describe any deviations in excess of measurement error regardless of any periodicity and to reserve the term oscillations for periodic fluctuations. The data in Figure 5 had a coefficient of variation around $\tilde{y}_t$ of 5.79%. The combination of volume and counting measurement error estimated as described above could only account for a coefficient of variation of 1.72%. Thus, fluctuations corresponding to a coefficient of variation of 5.53% of $\tilde{y}_t$ remained to be explained in this experiment. In seven experiments, 4.22 ± 3.88% (sample standard deviation) could not be accounted for by error.

The one-tailed F-ratio test for equality of variances showed that six of seven experiments, including the one in Figure 5B, showed fluctuations in excess of measurement error with significance at the $p < 0.01$ level. It must be noted that such significance might also have resulted from an inadequate definition of the mean by the curve-fitting procedure described above. However, in the experiment that showed no significant fluctuations by this test, the observed variance was precisely that expected from the error analysis.

Moving Point Averaging. The solid line in Figure 5B shows the moving average of two points. This data smoothing technique, used also in Figure 4, brought to the fore an oscillatory pattern with some suggestive regularity. This procedure reduced the coefficient of variation as expected by a factor of $1/2^{1/2}$.

Optimal Segment Smoothing. Figure 5C shows the result of applying the adaptation of the optimal segment smoothing technique described under Materials and Methods. The smoothed pattern was remarkably similar to the one obtained by two point moving averaging (Figure 5B). This strongly suggested that the excess variation found was neither due to inaccuracy in our estimate of the measurement error nor due to an inadequate description of the gross features of the insulin stimulated state, but reflected fluctuations inherent in glucose oxidation of fat cells.

Sinusoidal Approximation. Figure 5D shows a least-squares fit of a sine wave to the data. Although the fit is poor, maxima and minima of the sine curve coincided with those obtained by the two smoothing techniques. This correlation suggests that there was an oscillation in glucose oxidation with a period of about 14 min.

Autocorrelation and Spectral Density Function. In order to further characterize oscillations, i.e., fluctuations with significant periodicities, we obtained autocorrelation and spectral density functions from either the raw deviations from $\tilde{y}_t$ or the derived data from the various smoothing techniques described above. The essence of the results was not affected by smoothing of the original data. Presented in detail is the analysis of data smoothed by two point moving averaging followed by quadratic detrending. The autocorrelation function was estimated according to eq 5.3.35 of Jenkins & Watts (1968) together with the 95% confidence intervals for individual autocorrelation coefficients based on Fisher's transformation of r_k to z_k [see p 185 of Snedecor & Cochran (1967)] (Figure 6A). Seven autocorrelations had 95% confidence intervals that did not include zero. The spectral density function was estimated by using Tukey and Parzen windows of various sizes (Jenkins & Watts, 1968). Figure 6B shows the results for our sample experiment and two Tukey window sizes.

The statistical significance of the observed peaks was evaluated by a limited randomization test (Edgington, 1969) performed on the raw deviations from $\tilde{y}_t$. However, because the original data for the experimental spectrum were two point moving averages and were quadratically detrended, it was

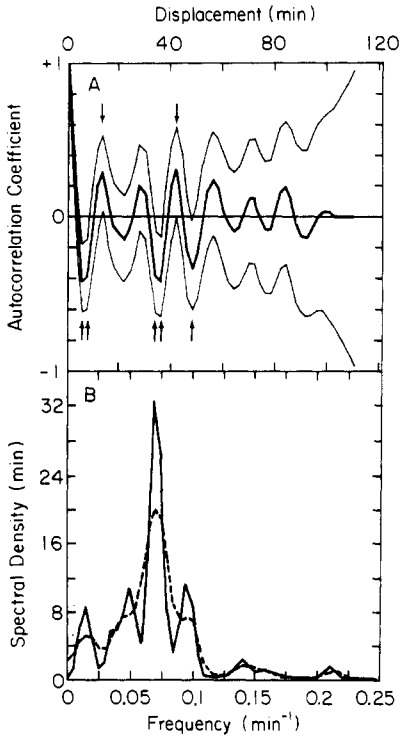

FIGURE 6: Autocorrelation and spectral analysis of the data in Figure 4B–D. (A) The autocorrelation function (thick line) and 95% confidence intervals of individual autocorrelations (thin lines) were estimated as described in the text. Arrows indicate autocorrelation coefficients significant at the $p \leq 0.05$ level. (B) The spectral density function was estimated from the autocorrelation function in panel A by methods referenced in the text, using the Tukey windows with lags of 112 (solid line) and 56 min (dashed line), corresponding to bandwidths of 0.012 and 0.024 min^{-1}, respectively.

necessary that after each randomization the data were similarly smoothed and detrended.[2] Peaks in spectra from randomized data were placed into five categories covering the following ranges of periods: 4–5.9, 5.9–7.4, 7.4–9.5, 9.5–20, and 20–∞ min. One hundred randomizations were performed. If N peaks were observed in the random data within one category, of which M were equal to or greater than the experimental peak of interest, the probability that a spectral peak equal to or greater than that found experimentally would be obtained by chance was given by $p = (1 + M)/(1 + N)$ (Birnbaum, 1973).

In our example, only the spectral density peak corresponding to a period of 14.0 min was statistically significant ($p < 0.004$, using a Tukey window with lag of 112 min) (Figure 6B). One experiment out of six with significant fluctuations was aperiodic in the sense that no single frequency was statistically significant. This appeared to result from a shortening of the period during the course of the experiment. The other five, including the one illustrated, had a single statistically significant ($p < 0.05$) periodicity in the 9.5–20- and 20–∞-min categories. The mean period of four of these experiments was 13.5 ± 2.1 min (range 11.0–17.2 min). Inexplicably, the fifth experiment had a period about twice that, i.e., 27.6 min.

It is noteworthy that none of the experiments in medium based on KRB buffer had significant oscillations with periods in the range from 4 to 9.5 min. If the fluctuations in our data would have reflected measurement error alone, observed periodicities would have been equally distributed along the scale of periodicities. This was verified by synthetic data and

[2] This was not done by Hansen et al. (1982).

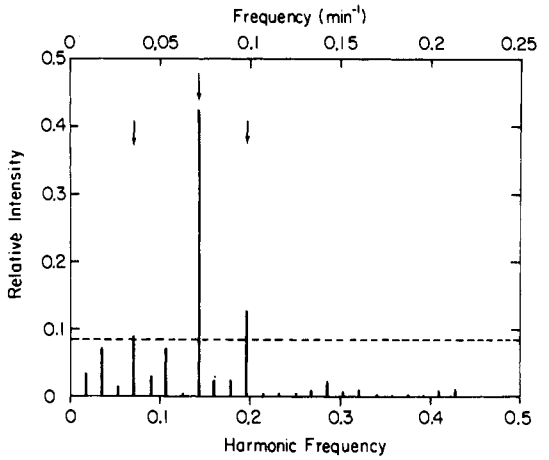

FIGURE 7: Periodogram of the data in Figure 4B–D after two point moving averaging and quadratic detrending. The relative intensity is the fractional contribution to the variance given the 95% confidence limit (dashed line) evaluated according to Siegel (1980) with the parameter $\lambda = 0.4$. Arrows indicate the frequencies significant at the $p \leq 0.05$ level.

provided additional evidence for fluctuations, and in fact oscillations, in excess of measurement error. Thus, our previous conclusion based on a propagation of error argument was confirmed by an analysis independent of an estimate of such measurement error.

Fourier Transform Analysis. Raw and smoothed deviations from $\tilde{y}_t$ were analyzed by direct Fourier transform, and peaks in the periodogram were analyzed for statistical significance according to Siegel (1980) (Figure 7). Three frequencies were significant at the $p < 0.05$ level. By far the largest, at a frequency of 0.0714 min^{-1} and thus corresponding to a period of 14 min, agreed nicely with the period indicated by the maximum in the spectral density function (Figure 6B). The smallest of the three significant peaks at a frequency of 0.0357 min^{-1}, or a period of 28 min, appeared to be a harmonic of the major frequency. The third peak had a frequency of 0.0982 min^{-1} or a period of 10.2 min. It is noteworthy, however, that the contributions of these three frequencies to the observed variance around $\tilde{y}_t$ accounted only for 65% of the observed variance. A possible explanation for this is that the nonsignificant peaks are important contributors to a wave form that deviates from a sinusoid. Alternatively, the underlying process is not described by the exact repeat of a specific wave form, but by a stochastic process. The periodograms of the other experiments showed considerable variation in peak patterns. For this reason, we have principally relied on analysis of spectral density functions.

Glucose Concentration Dependence. All studies thus far were performed with 0.275 mM glucose since studies at the physiologic concentration (5.5 mM) require large quantities of costly tracer. However, to verify that the observations made at the low glucose concentration had some relevance to more physiologic conditions, we performed one experiment involving a step function increase of insulin concentration at 5.5 mM glucose. Except for elevations in absolute basal and insulin-stimulated oxidation rates, the results were virtually the same as those obtained at a glucose concentration of 0.275 mM, including oscillations with a similar period (data not shown).

Bicarbonate-Free Medium. It has been reported that in bicarbonate-free medium insulin stimulation of lipogenesis and hexose monophosphate shunt activity is blunted when compared to bicarbonate-containing medium (Richardson & Czech, 1978). Therefore, fat cells were perifused with medium based on Krebs–Ringer phosphate equilibrated with CO_2-free

air. The results of insulin stimulation were very similar to those observed in bicarbonate-containing medium, but the dominant period of oscillations was 9.5 min.

Discussion

Development of a method for the accurate measurement of glucose oxidation to CO_2 in perifused rat fat cells has made quantitative examination of dynamic aspects of glucose metabolism in these cells possible. Glucose oxidation rates of freshly isolated fat cells were variably elevated and decayed to a stable base line (Figure 1). If insulin in maximally stimulating concentrations (10^{-7} M) was perifused after a stable base line was reached, glucose oxidation rates were stimulated up to 30-fold (data not shown). Such large ratios of stimulated to basal rates are rarely obtainable in fixed-volume incubations. The present data extend the observation in fixed-volume incubations that basal rates are much more variable than insulin-stimulated rates and suggest some technical solutions; i.e., fat cells could be perifused for 1 h before use.

Multiple pulses of submaximally stimulating concentrations of insulin (5×10^{-10} M) resulted in decreasing responses (Figure 2), that in view of the steady base line (Figure 1) could not be explained by cell death. A similar decay of insulin action with time, i.e., desensitization, was frequently also observed during prolonged perifusions with insulin, even when insulin concentrations (5×10^{-9} M) much in excess of those required for maximal stimulation were used (Figure 4A). Desensitization can be the result of either an insulin-induced down regulation of insulin receptors, which results in a shift of the insulin concentration effect curve for glucose oxidation to higher concentrations, or a post-receptor alteration in the glucose transport system (Marshall & Olefsky, 1980). At the submaximal insulin concentrations (5×10^{-10} M) used in the experiment of Figure 2, both effects would result in a lowering of the observed insulin effect. However, with the insulin concentrations (5×10^{-9} M) used in the experiments shown in Figure 4A, this decay could only result from the second mechanism.

Hydrogen peroxide is known to mimic insulin action on fat cells in many respects, including stimulation of glucose transport (Czech et al., 1974a; Livingston et al., 1978; Sørensen et al., 1980; Kono et al., 1982), preferential stimulation of glucose Cl oxidation over glucose C6 oxidation (Czech et al., 1974b), enhancement of glucose incorporation into glycogen (Lawrence & Larner, 1978) and lipids (Cascieri et al., 1979; May & de Haën, 1979) accompanied by activation of pyruvate dehydrogenase (May & de Haën, 1979; Paetzke-Brunner & Wieland, 1980), inhibition of hormone-stimulated lipolysis (Cascieri et al., 1979; Little & de Haën, 1980), and down regulation of insulin receptors (Caro & Amatruda, 1980). Because prolonged exposure of fat cells to H_2O_2 might be damaging to cells, we perifused them with H_2O_2 only for a short period (Figure 3). However, on cessation of perifusion with H_2O_2, glucose oxidation rates decayed in association with a damped oscillation of very large initial amplitude.

The most interesting finding of this study was that rates of glucose oxidation to CO_2 after a step-function increase in insulin concentration oscillated around a new mean level (Figure 4). These oscillations could be shown to be significantly larger than that accountable for by measurement error alone. Using the techniques of time series analysis, they were shown to have a statistically significant dominant frequency of around 14 min. As will be discussed below, we attribute these oscillations to oscillatory glycolysis. The fact that oscillations could only be observed after fat cell metabolism had been suddenly altered by the addition of insulin (Figure 4) or H_2O_2 (Figure 3) but were not clearly discernable in the basal state of freshly isolated cells (Figure 1) or in cells that were isolated and maintained in perifusion in the continuous presence of insulin (data not shown) indicated that synchronization of the fat cell population was a prerequisite for the observation of oscillations. A step-function increase in insulin was apparently a suitable means of synchronizing fat cell metabolism. The effects of insulin, which stimulates transport as well as metabolism of glucose in fat cells, may be considered equivalent to the effects of sudden addition of glucose to the medium of Ehrlich ascites tumor cells, which have previously been found to result in synchronization of metabolism (Ibsen & Schiller, 1967).

Thus, upon appropriate synchronization of a population of isolated rat epididymal fat cells, glucose oxidation to CO_2 shows oscillations with a period of around 14 min and very little damping for the duration of about 2 h, i.e., eight periods. In the absence of synchronization, no oscillations could be observed. The simplest interpretation is that glucose oxidation to CO_2 by individual fat cells oscillated in both basal and insulin-stimulated states, but phase differences in cell populations prevented their observation in the absence of synchronization. Alternatively, oscillations may occur only in the insulin-stimulated state and are absent in the basal state. We believe oscillatory CO_2 production reflects oscillatory glycolysis in a manner similar to the case of oscillatory CO_2 production by yeast extracts (Hess & Boiteux, 1968). The similarity in amplitude, frequency, and even phase of oscillations observed with Cl- and C6-labeled glucose (Figure 4) suggests that the source of the oscillations is not the hexose monophosphate shunt. This is the case despite the fact that insulin and H_2O_2, the synchronizing agents used, are known to activate the latter pathway more than glycolysis (Czech et al., 1974b). This localized the source of CO_2 to the pyruvate dehydrogenase reaction and meant that the oscillophore was in the glycolytic pathway. In yeast [for a review, see Hess (1979)] and muscle extracts (Frenkel, 1965, 1966, 1968a–c; Tornheim & Lowenstein, 1973–1975), phosphofructokinase, an enzyme regulated by multiple allosteric regulators, has been identified as the primary oscillophore, although resonances with pyruvate kinase may also partake (Termonia & Ross, 1982). Attempts at modeling the system have met with reasonable success [e.g., see Termonia & Ross (1981)] although the role of the only recently discovered major allosteric regulator of phosphofructokinase, fructose 2,6-diphosphate (Van Schaftingen et al., 1980; Uyeda et al., 1981), has yet to be considered. The regulatory properties of phosphofructokinase from different organisms and tissues are very similar, in keeping with the early evolution of this enzyme. It thus appears safe to conclude that also in rat fat cells glycolysis oscillates primarily due to the allosteric properties of that enzyme.

The amplitude of the oscillations is not easily interpretable. Its small size could reflect the genuine amplitude of individual cells, perhaps resulting from oscillatory CO_2 production by pyruvate dehydrogenase superimposed on a more constant rate of CO_2 production by the tricarboxylic acid cycle. Alternatively, the small amplitude is caused by incomplete synchronization of the cells. Most likely, the latter mechanism plays a major role and also explains the observed variability of amplitudes.

The observed oscillations could not be described completely by a pure sine wave plus measurement error (Figure 5D). The multiple peaks in the periodogram (Figure 7) also indicated that a wave form of higher complexity may be involved. This

is in keeping with the observations in yeast cell extracts (Hess & Boiteux, 1968), where the wave form was shown to be complex and to change as a function of substrate type and concentration. It is also possible that the shape of the oscillations was determined by the combination of a deterministic and a stochastic process. Attempts to model the data as such a system by using autoregression technology have thus far not yielded clear-cut results.

Periods observed (around 14 min) were longer than those of oscillatory glycolysis in other intact mammalian cells, i.e., Ehrlich ascites tumor cells with a period of around 2 min (Ibsen & Schiller, 1967) and cultured mouse fibroblasts (L cells) with a period of 1–3 min (Werrlein & Glinos, 1974). However, the glycolytic oscillator of broken cells has the capacity to vary the period from 2 s to 3 h depending on substrate conditions (Rapp, 1979). There is currently no known role for the oscillation of glycolysis in fat cells except for a suggestion that metabolic efficiency may be optimized under such conditions (Richter & Ross, 1981).

Of particular interest were the length of the dominant period of around 14 min and the similarity of this period with the period of oscillatory insulin secretion by pancreatic β cells, which is around 10–13 min [e.g., see Goodner et al. (1977) and Hansen et al. (1982)]. The insight gained from the fat cell system described here offered two new potential explanations pertaining to the insulin secretion system. First, considering the crucial role glycolysis plays in controlling insulin secretion (Ashcroft, 1980), it is quite possible that glycolysis constitutes the internal pacemaker of that oscillating system (Lipkin et al., 1982). A relationship between oscillatory glycolysis and periodic phenomena in the pancreatic β cell had been suggested before (Matthews & O'Connor, 1979); however, the electrophysiological signals of the β cell these authors considered have a frequency much higher than that of oscillatory glycolysis. Only recently has the electrophysiologic signature corresponding to the periodicity of insulin secretion been identified (Daniel L. Cook, unpublished results).

Second, the present study illustrates that sudden changes in the metabolic milieu can synchronize glycolysis in populations of cells independent of fixed physical contact or a shared vascular or neuronal network. This suggests that the synchronization of insulin secretion by the population of islets of Langerhans of the perfused dog pancreas (Stagner et al., 1980) may have been the result of the interruption of perfusion associated with surgical preparation of the pancreas followed by the sudden change in perfusion medium at the beginning of the experiment. Furthermore, stimulated fat cells retain synchrony for several hours, i.e., at least as long as the period over which oscillatory insulin secretion by the perfused dog pancreas is observed.

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Structure and Function of Myosin Subfragment 1 As Studied by Tryptic Digestion†

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ABSTRACT: Limited tryptic digestion of myosin subfragment 1 (S-1) was carried out under a high concentration of trypsin (weight ratio of trypsin to S-1 of 1:20) in the absence and presence of actin and/or ADP, and changes in the Mg^{2+}-ATPase activities of S-1 and acto-S-1, binding of S-1 to actin, and structure were followed during the course of proteolysis. Two of the three main products of lesser hydrolysis, the 50K and 27K fragments, converted into 45K and 22K fragments, respectively. The 20K fragment also degraded into smaller fragments but more slowly. When the acto-S-1 complex was digested, the 70K fragment degraded into 50K and 20K fragments (the 70K is a stable fragment under milder proteolysis). Addition of ADP accelerated every breakdown and was more effective for the breakdown of the 70K and 27K fragments. The 70K fragment was also converted into a 34K fragment when ADP was added to the acto-S-1 complex. In every case except the acto-S-1 complex without ADP, the Mg^{2+}-ATPase activities of S-1 and acto-S-1 and the binding of S-1 to actin decreased during the course of proteolysis. On the other hand, in the case of acto-S-1 without ADP, the Mg^{2+}-ATPase activity and the binding of S-1 to actin were protected from tryptic digestion, whereas the turbidity drastically fell to half of the initial value within 15 min of digestion and remained constant even after 60 min. The latter result suggests that large conformational changes were induced in the trypsinized S-1 by the binding of actin and may support the idea of two actin binding sites on S-1 [Mornet, D., Bertrand, R., Pantel, P., Audemard, E., & Kassab, R. (1981) *Nature (London) 292*, 301–306]. That is, we may speculate that on proteolysis of acto-S-1 one of the actin bonds was broken and one was retained. If so, the binding of actin to half of the sites of S-1 may be enough for actin activation.

It is generally believed that myosin subfragment 1 (S-1)[1] is the segment of the myosin molecule containing the active site for ATPase and also the site at which actin interacts. These sites are interacting with, but distinct from, each other (Barany & Barany, 1959; Highsmith, 1976). So, studies of S-1 conformational changes accompanying these interactions are highly significant. Here we pursue these studies using a method based on proteolysis. Balint et al. (1978) showed that limited tryptic proteolysis of the heavy chain of S-1 produced mainly three fragments (called 27K, 50K, and 20K). The 27K and 20K fragments contain the N and C termini of S-1 heavy chain, respectively (Lu et al., 1978). Mornet et al. (1979) and Yamamoto & Sekine (1979a,b) showed that the 50K/20K cut was abolished in the acto-S-1 complex and that the cut reduced the activation of S-1 Mg^{2+}-ATPase by actin (but not the Mg^{2+}-ATPase of S-1 alone). During the course of tryptic digestion of S-1, neither the binding property of S-1 to actin nor the Mg^{2+}-ATP-induced dissociation of the complex was significantly changed (Mornet et al., 1979). The three fragments have become a valuable frame work to which one can assign specific groups and functionalities. Szilagyi et al. (1979) showed that a photoaffinity analogue of ATP was specifically incorporated into the 27K fragment. It was found that both reactive thiols, SH_1 and SH_2, and the reactive lysyl residue resided on the 20K (Balint et al., 1978) and the 27K (Mornet et al., 1980; Miyanishi & Tonomura, 1981; Hozumi & Muhlrad, 1981) fragments, respectively. In previous papers, we reported the distributions of tryptophan (Hozumi, 1981) and cysteine (Hozumi, 1982) residues over the three fragments. Mornet et al. (1981b) showed that the 95K heavy chain of S-1 intimately contacts with two neighboring actin monomers; one monomer is bound to the 50K domain and the other to the 20K domain. Recently, we studied the proteolytic generation of the 27K fragment, i.e., the 27K/50K cut. It was found that the 27K fragment was generated by two routes proceeding in parallel; in one case, a 29.5K fragment was first produced and then degraded to 27K; in the other, a 27K fragment was directly produced without any precursor (Hozumi & Muhlrad, 1981). In addition, it was observed that

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[1] Abbreviations: S-1, myosin subfragment 1; TPCK-trypsin, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone treated trypsin; DACM, *N*-[7-(dimethylamino)-4-methyl-3-coumarinyl]maleimide; 1,5-IAEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; NaDodSO$_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance.

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