The Role of Carbon Dioxide and the Influence of pH on the Insulin Stimulated Glucose Metabolism of Isolated Cells from Rat Adipose Tissue

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Summary. The role of CO_2 and the influence of pH on the insulin-stimulated glucose metabolism of cell suspensions from rat adipose tissue were investigated in a Tris-buffered system. CO_2 was assimilated in presence of glucose, fructose or pyruvate in the medium. The fixed CO_2 is mainly localized in the C-1 position of lactate. Insulin enhances the turnover rates only when cells are incubated in glucose-containing medium. The influence of the extracellular CO_2 concentration on CO_2 fixation, on glucose oxidation, and on the transfer of glucose-6- ^{14}C to lipid compounds follows saturation kinetics. The same metabolic reactions show a defined maximum at an extracellular pH of 7.5. On both sides of the pH-peak the turnover rates decrease very rapidly.

Le rôle de l'anhydride carbonique et l'influence du pH sur le métabolisme du glucose stimulé par l'insuline, des cellules isolées du tissu adipeux de rat.

Résumé. Les auteurs ont étudié le rôle du CO_2 et l'influence du pH sur le métabolisme du glucose stimulé par l'insuline, de suspensions de cellules du tissu adipeux de rat, dans un système tampon Tris. Le CO_2 était assimilé en présence de glucose, de fructose ou de pyruvate dans le milieu. Le CO_2 fixé est localisé principalement dans la position C-1 du lactate. L'insuline augmente les vitesses de renouvellement seulement lorsque les cellules sont incubées dans un milieu contenant du glucose. L'influence de la concentration extracellulaire en CO_2 sur la fixation

du CO_2 , sur l'oxydation du glucose et sur la transformation du glucose-6- ^{14}C en composés lipidiques suit des cinétiques de saturation. Les mêmes réactions métaboliques montrent un maximum caractérisé à un pH extracellulaire de 7.5. Des deux côtés du pH maximum, les vitesses de renouvellement décroissent très rapidement.

Die Rolle von Kohlendioxid und der Einfluß des pH auf den durch Insulin stimulierten Glucosestoffwechsel isolierter Fettzellen von Ratten.

Zusammenfassung. In einem mit Tris gepufferten System wurden die Rolle des CO_2 und der Einfluß des pH auf den insulinstimulierten Glucosemetabolismus von Zellsuspensionen des Rattenfettgewebes untersucht. CO_2 wird bei Anwesenheit von Fructose, Glucose oder Pyruvat im Medium assimiliert. Das fixierte CO_2 ist hauptsächlich in der C-1-Position der Milchsäure lokalisiert. Insulin erhöht die Umsatzraten nur bei der Inkubation in einem glucosehaltigen Medium. Der Einfluß der extracellulären CO_2 -Konzentration auf die CO_2 -Fixierung, auf die Glucoseoxidation und auf den Übergang von 6- ^{14}C -Glucose in die Verbindungen der Lipidfraktion läßt sich durch Substratsättigungskurven beschreiben. Die gleichen Stoffwechselreaktionen zeigen ein ausgeprägtes pH-Maximum bei pH 7.5. Beiderseits der Gipfelpunkte fallen die Kurven steil ab.

Key-words: Isolated fat cells, CO_2 fixation, glucose utilization (kinetics), pH dependency insulin activity.

It is well established that insulin influences several metabolic pathways of various tissues *in vitro*. For the elucidation of the insulin effect, pieces or isolated cells from rat adipose tissue have an important role as test objects. During these investigations *in vitro* the turnover of various substrates is usually pursued in bicarbonate-containing buffer systems. Little is known about the influence of two environmental factors, i.e. CO_2 and pH. The present studies, therefore, deal with the fixation of CO_2 as well as with the influence of CO_2 concentration in the medium, and with the effect of changes in the extracellular pH on the insulin-stimulated glucose metabolism of isolated cells from rat adipose tissue.

Materials and Methods

Chemicals

Collagenase prepared from C. histologicum, and sodium pyruvate were obtained from Serva, Heidelberg; the scintillation materials from Merck, Darmstadt. Crystalline bovine insulin (25 IU/mg) and $\text{Na}_2^{14}\text{CO}_3$ (1.25 mc/mmole) are our own products. Glucose-1- ^{14}C and glucose-6- ^{14}C with specific activities of 20–30 mc/mmole were purchased from Amersham.

Haemaccel® is a plasma volume substitute of the Behring-Werke, Marburg. Phenylpyruvic acid was obtained from Fluka AG, Buchs; Altromin „R“ from Altrogge, Laage (Lippe); hydroxide of Hyamine® 10-X from Packard Instrument Company.

Preparation of adipose cells

Male Ivanovas rats (Invanovas, Kisslegg, Allgäu), weighing 80–150 g, had free access to Altromin „R“. Animals were killed by a blow on the head, followed by decapitation and removal of the fat pads. Fat cells were isolated as described by RODBELL (1964) except that the incubation fluid contained 5.1 g NaCl, 1.5 g K_2HPO_4 , 2.4 g Tris and 100 ml Haemaccel per litre. The pH was adjusted to 7.8. After the incubation with collagenase the cells were washed three times by suspending them in a 20 mM Tris buffer, pH 7.2, now containing 3.5 g NaCl, 1.5 g K_2HPO_4 and 100 ml Haemaccel per litre. Finally the cells were suspended in 20 ml of this fluid. The fat cell content was determined by titrating the fatty acids after saponification of an aliquot with 0.2 N methanolic KOH: 1 ml 0.1 N KOH is equivalent to 26.9 mg of glyceroltripalmitate (TP).

Incubation Technique

We used pieces of fat tissue only for examination of CO_2 fixation into tissue contents; all other experiments were undertaken with isolated fat cells. The cell suspension was drawn up into a 20 ml plastic syringe. Portions of 1 ml with a cell content equivalent to 20–50 mg TP were put into 25 ml polyethylene vials. After the addition of 1 ml supplement-containing Tris buffer pH 7.2, the vials were closed with sleeve-type rubber stoppers, and the bicarbonate plus labelled substrate ($3.5 \mu\text{C Na}_2^{14}\text{CO}_3$, $0.2 \mu\text{C}$ glucose-1- ^{14}C or $0.2 \mu\text{C}$ glucose-6- ^{14}C in 0.1 ml H_2O was injected. A NaHCO_3 addition of 48 μmoles per vial at 37°C causes a pH shift from 7.2 to 7.5. The various values of pH used in experiments investigating the influence of pH, and the constant pH of 7.5 used in the measurements of the effect of various NaHCO_3 concentrations, were obtained by injection of 0.1 N HCl or 0.1 N NaOH into the closed system. The volume was then corrected to 2.5 ml with Tris buffer of specified pH and the samples were agitated in a Dubnoff shaker at 37°C for three hours.

In separate control experiments we checked the accuracy of the adjustment of pH, and its constancy during incubation by a glass electrode drawn through the rubber stopper keeping the system gas-tight. To determine the pCO_2 of the gas phase and the "total CO_2 "¹ content of the medium at equilibrium state, 2 ml was removed from the gas phase and 0.2 ml from the fluid with a syringe containing 0.25 ml 0.5 N NaOH for trapping the radioactive $^{14}\text{CO}_2$. The influence of the medium pH on the distribution of CO_2 between fluid and the gas phase is shown in Fig. 1.

Fig. 1. Influence of the pH on the distribution of CO_2 between medium (I) and gas phase (II). Conditions were the same as marked in Fig. 4a

Analytical procedures

For the determination of the incorporated $^{14}\text{CO}_2$, 0.2 ml of 1 N H_2SO_4 was added to the vials, and CO_2 was then passed through the medium to ensure quantitative removal of free $^{14}\text{CO}_2$. 0.2 ml of the incubated cell suspensions was dissolved in dioxane containing 10% naphthalene, 0.5% PPO and 0.01%

POPOP and counted in a liquid scintillation spectrometer (Packard Instrument Company). Toluene with 17% methanol, 10% naphthalene, 0.5% PPO and 0.01% POPOP was utilized for counting pieces of fat tissue. The radioactivity present in the medium was separated by column chromatography (Dowex 1×8 , 200–400 mesh, HCOO^- -form) and the radioactive peak (Fig. 2) was identified by descending paper chromatography on SCHLEICHER & SCHÜLL 2043 bMgl (with the solvent n-butanol:benzyl alcohol: $\text{HCOOH}:\text{H}_2\text{O} = 7:1:1:2$ (SCHORMÜLLER and MEZGER 1966). The radioactivity localized in the C-1 of lactic acid was determined after oxidation with dichromate (ARONOFF, 1956).

Studies of glucose oxidation were performed in the incubation flasks described by RENOLD et al. (1960). After the incubation 0.2 ml 1 N Hyamine in methanol was injected into the plastic cup and 0.2 ml of 1 N H_2SO_4 was added to the medium. The flasks were kept at 38°C for one hour to complete the absorption of $^{14}\text{CO}_2$. For determination of the activity transferred from glucose-6- ^{14}C to lipids the cell suspensions were extracted twice with 3 ml of ether for five hours after the pH of the medium had been adjusted to 2.0. The radioactivity of the Hyamine- $^{14}\text{CO}_2$ -solutions and of the ether extracts was measured in the toluene scintillation fluid.

Results

CO_2 Fixation. Table 1 shows the levels of fixed $^{14}\text{CO}_2$ localized in the fat tissue contents and in the incubation fluid. 85% of the incorporated radioactivity appears in the incubation medium. Insulin addition causes a threefold increase of CO_2 fixation.

Reaction rates are based on the specific activity of the added NaHCO_3 . CO_2 produced from glucose during the incubation caused a maximal decrease of only 2% of the initial value.

Table 1. CO_2 fixation by rat adipose tissue

	$^{14}\text{CO}_2$ fixed ($\mu\text{moles}/\text{mmole TP}$) ¹	
	Medium	Tissue
Control	0.75 ± 0.25	0.15 ± 0.04
200 μU Insulin/ml	2.16 ± 0.35	0.38 ± 0.06

30–70 mg of fat tissue was incubated for three hours at 37°C in 2.5 ml of 20 mM Tris buffer pH 7.5 containing 25 μmoles glucose and 48 μmoles CO_2 per vial. $\text{pCO}_2 = 12$ mm Hg. Each value is the mean of four replicates $\pm$ standard error (SE).

¹ 1 mmole is equivalent to 808 mg wet tissue.

For the following studies we used cell suspensions. The influence of different additions on the CO_2 fixation is shown in Table 2.

In the Tris buffer medium without any substrate, only a small amount of $^{14}\text{CO}_2$ was incorporated. When glucose or fructose was added to the medium the

¹ CO_2 , H_2CO_3 , HCO_3^- , and CO_3^{2-} .

fixed levels were much higher, but equivalent for both substrates. In the presence of pyruvate, however, a remarkably enhanced turnover was measured. A stimulating effect of insulin on CO_2 fixation has been

Table 2. Effect of various substrates on CO_2 fixation of cell suspensions of rat adipose tissue

Additions	$^{14}\text{CO}_2$ fixed ($\mu\text{moles/ml} \times \text{mmole TP}$)	
	Control	200 μU Insulin/ml
—	0.044 ± 0.005	0.045 ± 0.009
Fructose 10 mM	0.50 ± 0.05	0.48 ± 0.02
Glucose 10 mM	0.48 ± 0.05	1.10 ± 0.04
Pyruvate 4 mM	1.38 ± 0.10	1.50 ± 0.12
Glucose 10 mM Phenylpyruvate 1 mM	0.06 ± 0.004	0.05 ± 0.007
Pyruvate 4 mM		
Phenylpyruvate 1 mM	0.06 ± 0.006	—

Cells equivalent to 50 μmoles TP were incubated for three hours at 37°C in 2.5 ml of 20 mM Tris buffer pH 7.5 with 48 μmoles CO_2 per vial. $p\text{CO}_2 = 12$ mm Hg. Each value is the mean of four replicates $\pm$ SE.

observed only in presence of glucose in the medium. Phenylpyruvate, a potent inhibitor of reactions catalyzed by malic enzyme and pyruvate carboxylase (SEUBERT and HUTH, 1965) suppresses CO_2 incorporation to almost the control value. When ^{14}C -containing substances were separated by column- (Fig. 2) and

Fig. 2. Separation of ^{14}C -substances present in medium by anion exchange chromatography. Reaction mixture contained the same components as marked in Table 1

paper-chromatography, the main part of the activity coincided with the position of lactic acid. No radioactivity could be detected in citrate or other acids of the citrate cycle.

Degradation studies revealed that the radioactivity of lactic acid is quantitatively localized in C-1 position. Peak "a" contains distinct substances that have not yet been identified.

Influence of "total CO_2 " concentration on the glucose metabolism. The effect of "total CO_2 " concentration of the medium on CO_2 fixation, on glucose oxidation and on the transfer of radioactivity from glucose-6- ^{14}C to

lipids is demonstrated in Fig. 3a—3c. At low concentrations, CO_2 additions cause a nearly proportional increase of the turnover rates. In the absence of insulin a CO_2 concentration of more than 5 mM causes no further increase of CO_2 fixation and lipid synthesis.

Fig. 3a—3c. Effect of CO_2 concentration in the medium on CO_2 fixation (3a), on glucose oxidation (3b) and on transfer from glucose-6- ^{14}C to lipids (3c). Cells equivalent to 50 μmoles TP were incubated for three hours in 2.5 ml of 20 mM Tris buffer pH 7.5 containing 25 μmoles glucose. Osmotic pressure was balanced by variation of NaCl concentration in the Tris buffer. Each value is the mean of four replicates, $\pm$ SE

The saturation level for glucose oxidation, however, is reached at 7.5 mM CO_2 . Insulin stimulates the examined metabolic pathways considerably; simultaneously, the maximal CO_2 levels necessary for maximal turnover rates are enhanced by about 60%. Glucose oxidation alone shows a slight increase between concentrations of 8–15 mM CO_2 per ml.

Fig. 4a–4d. Effect of the medium-pH on CO_2 fixation in presence of glucose (4a) or pyruvate (4d), on glucose oxidation (4b) and on transfer of glucose-6- ^{14}C to lipids (4c). Cells equivalent to 50 μmoles TP were incubated for three hours at 37°C in 2.5 ml of 20 mM Tris buffer with 25 μmoles glucose (4d, 4 μmoles pyruvate) and 48 μmoles CO_2 per vial. Each value is the mean of four replicates; $\bar{x} \pm \text{SE}$. Distribution of CO_2 between medium and gas phase see Fig. 1

Influence of extracellular pH on the glucose metabolism. pH studies were performed in the range from 6.0 to 9.0. The incorporation of CO₂, the glucose oxidation and the transfer of glucose-6-¹⁴C to lipids showed a definite pH-maximum at 7.5 in the presence, as well as in the absence, of insulin, with the exception of the control curve of glucose oxidation, which had a pH-maximum at 7.3. The turnover rates decrease very rapidly on both sides of the pH-peaks. In the presence of pyruvate in lieu of glucose as substrate (Fig. 4d), however, CO₂ fixation is insulin-independent but shows the same pH dependency.

Discussion

For metabolic investigations under conditions *in vitro*, tissue pieces or isolated cells are conventionally incubated in bicarbonate buffer with 5% CO₂ in the supernatant gas phase. If the preparation of these buffers is performed in the usual manner, it is not easy to introduce a definite quantity of CO₂, and, in the case of CO₂ fixation studies, a definite specific radioactivity of ¹⁴CO₂. It is also difficult to adjust distinct pH values over the range of pH 6.0 to pH 9.0. Based on the findings of BAKER and RUTTER (1964), we believe that the employment of a Tris buffer medium and the injection of definite quantities of CO₂ in form of a NaHCO₃ solution offers a convenient possibility to overcome these difficulties. It may be added that in presence of 3.5% albumin in the incubation medium the turnover rates of the examined reactions do not alter significantly (HEPTNER in preparation).

The strongly marked CO₂ fixation in the presence of pyruvate as the sole substrate (Table 2), and its inhibition by phenylpyruvate indicate that CO₂ is fixed by β -carboxylation, probably catalyzed by pyruvate carboxylase, which has been detected in rat adipose tissue (WISE and BALL, 1964). The fact that the main part of the fixed CO₂ is localized in the C-1 position of lactate leads to the suggestion that the C-1-labelled oxaloacetate is transferred into 1,4-labelled malate if this compound is in equilibrium with fumarate (WOOD and UTTER, 1965; SEUBERT and HUTH, 1965; WISE and BALL, 1964). Malate-1,4-¹⁴C gives rise to C-1-labelled pyruvate and finally to lactate-1-¹⁴C. The studies of YOUNG (1964) and WISE and BALL (1964) indicate that this reaction sequence may serve a role in lipogenesis by generating NADPH from NADH. The lack of an insulin effect on CO₂ fixation in the presence of pyruvate shows that there is no direct influence of insulin on CO₂ fixation. The accelerated CO₂ incorporation during the incubation in a glucose-containing medium may be interpreted as stimulation of glucose transfer into the cell by insulin, causing an increase of pyruvate production.

When the influence of various extracellular CO₂ concentrations on CO₂ fixation, on glucose oxidation

and on transfer of glucose-6-¹⁴C to lipids was measured, a saturation type of kinetics was observed in both the presence and the absence of insulin. At CO₂ concentrations up to about 5 mM in the absence of insulin, and up to about 10 mM in the presence of insulin, CO₂ fixation reactions are probably the rate determining factors. With regard to the turnover of C-1 and also of C-6-labelled glucose, the malonyl-CoA synthesis catalyzed by acetyl-carboxylase may be considered as the rate limiting step in the reaction sequence from glucose to fatty acids (NUMA et al., 1961).

Above these CO₂ concentrations some other step becomes rate limiting. It may be assumed that this concerns a process preceding CO₂ fixation, glucose oxidation, lipid synthesis and also the CO₂-independent formation of glycogen (LYNN et al., 1959), i.e. glucose transport into the cell stimulated by insulin. When the results were plotted according to LINEWEAVER and BURK (1934), remarkable deviations from linearity were obtained, indicating that some other process may interfere with the reactions of substrate with enzymes.

The reactions examined show an unexpectedly sharp pH-maximum and a very rapid decrease of reaction rate on either side of the peak. In preliminary studies performed without pH control in the closed systems, we obtained — in agreement with GLIEMANN (1965) — maximal turnover rates within the range of pH 7.0—pH 8.5. Later we found that these results were based on insufficient pH adjustment, indicating the necessity of an accurate pH control of the medium in the closed system after CO₂ is in equilibrium state between fluid and supernatant. One may speculate that the increase of CO₂ concentration by variation of the pH from 7.0 to 7.5 causes the increase of the turnover rates within this range. This is not true, since at pH 7.0 the "total CO₂" concentration is 8 mM (Fig. 1, curve I), and this concentration is sufficient for maximal turnover rates at pH 7.5 (Fig. 3a—3c). The results indicate that probably there exists a special effect on the maintenance of an effective cellular structure, especially on the permeability of the cell membrane. This does not concern only the insulin-stimulated glucose consumption, as is indicated by the effect of pH on the insulin-independent fixation of CO₂, using pyruvate as substrate. On the other hand, the results demonstrate that using insulin-dependent reactions of adipose tissue comparable values for insulin activities are obtained only under strictly constant pH conditions from the beginning to the end of the incubation period. All possible care must be taken to ensure an accurate pH adjustment of the samples with various buffer capacity.

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