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INHIBITION OF MITOCHONDRIAL METABOLISM BY THE DIABETOGENIC THIADIAZINE DIAZOXIDE—I

ACTION ON SUCCINATE DEHYDROGENASE AND TCA-CYCLE OXIDATIONS

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Abstract—Diazoxide exerts a non-competitive inhibition on succinate dehydrogenase in intact and disintegrated mitochondria. This inhibition causes a diminished rate of citric acid cycle turnover, as demonstrated by a decrease in pyruvate and fatty acid oxidation in mitochondria from heart and liver. The inhibition of total oxidation of long chain fatty acids produces a relative increase of acetoacetate formation. The inhibition of insulin release from the pancreas, considered as the predominant reason for the diabetogenic action of the drug, is discussed in view of the inhibitory effects on aerobic energy generation.

ONE OF the reasons for the induction of hyperglycaemia by diazoxide (7-chloro-3-methyl-1,2,4-benzo-thiadiazine) is its inhibiting insulin release from the islets of Langerhans,[1–5] an effect which has not yet been explained on a molecular basis. In a previous paper[6] we have shown that diazoxide is also an effective inhibitor of succinate oxidation in isolated mitochondria, and that due to inhibition of succinate dehydrogenase a diminished turnover of the citric acid cycle may be expected. This should result in a decrease in energy output from the main oxidative pathway, and in addition, significant changes of related pathways may be noted. In fact, we recently obtained evidence, that in subcellular systems in the presence of diazoxide the predicted metabolic shifts can be observed.[7] The present paper deals with the action of diazoxide on oxidation of pyruvate and fatty acids by isolated mitochondria, elucidating the fact that a general inhibition of oxidative energy metabolism is induced by the drug. The relation of the specific inhibition of insulin release and other responses of cellular metabolism to the described effects is also discussed.

MATERIALS AND METHODS

Liver mitochondria were prepared from the livers of male Wistar rats by the conventional methods of differential centrifugation[8] in a medium containing 0·25 M sucrose, 1 mM triethanolamine, 0·1 mM EDTA, pH 7·2. For incubations two types of buffer were used as described previously;[9] a sucrose medium when respiration was measured (RC-buffer: 0·25 M sucrose, 10 mM triethanolamine, 10 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4; pH 7·2), and a phosphate buffer for experiments with fatty acids as substrates (FA-buffer; 16 mM KH_2PO_4, 12 mM NaF, 26 mM NaCl, 58 mM KCl, 6 mM $MgCl_2$, pH 7·2). Rabbit heart mitochondria were isolated as described previously[10] in a KCl medium. For incubations of heart mitochondria a medium

consisting of 110 mM KCl, 4·3 mM $MgCl_2$, 3·3 mM KH_2PO_4; pH = 7·4 (referred to as HM buffer) was used.

[^{14}C]labeled compounds were obtained from The Radiochemical Centre, Amersham. $^{14}CO_2$-liberation from incubations of rabbit heart mitochondria was determined according to Glieman;[11] radioactivity was measured by liquid scintillation counting. Oxygen consumption was recorded polarographically with a platinum micro electrode.[12] Reduction of mitochondrial flavoproteins was measured fluorometrically in suspensions of intact mitochondria, using a modified Eppendorf fluorometer.[13] Protein content was determined by a biuret method. Succinate dehydrogenase was measured in mitochondria, disrupted by osmotic shock, using cytochrome *c* as terminal electron acceptor.[8] Acetoacetate was determined enzymatically as described in the literature.[14] L-Palmitoylcarnitine was synthesized according to Bremer.[15] Enzymes and substrates were obtained from Boehringer, Germany. Diazoxide was a generous gift from Byck, Essex.

RESULTS

(1) *Succinate oxidation in presence of diazoxide*

From our previous report[6] it is obvious that the ability of mitochondria for oxidation of exogenous succinate is decreased with increasing concentrations of diazoxide. The specificity of the effect to the dehydrogenase level was demonstrated by the fact that the oxidation of NADH by submitochondrial particles is not influenced by the inhibitor.

Figure 1 (right) shows a Lineweaver–Burk plot of succinate-linked respiration of liver mitochondria in presence and absence of diazoxide. The regression curves clearly

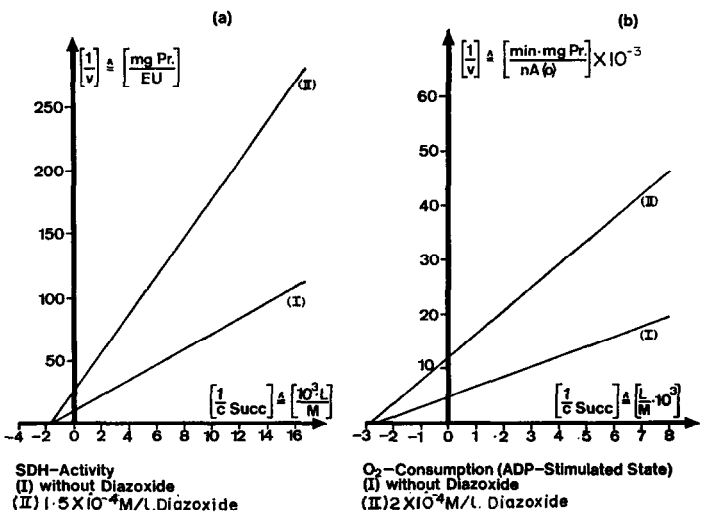

FIG. 1. Action of diazoxide on the kinetics of succinate oxidation. (a) Lineweaver–Burk plot of SDH-activity in disintegrated rat liver mitochondria (experimental condition as in Ref. 16, (b) Lineweaver–Burk plot of succinate dependent respiration in intact rat liver mitochondria. Incubation conditions: 2·4 ml RC-buffer, 25°, 3–4 mg mitochondrial protein, 1 μmole ADP. The regression lines are mathematically fitted by the least square method to the experimental data of the individual determinations; n = 6–9; r = 0·968–0·997.

reveal a non-competitive type of inhibition. The apparent values of the K_m are $3{\cdot}9 \times 10^{-4}$ (control) and $3{\cdot}6 \times 10^{-4}$ M (+diazoxide), the V_{max} are 213 nA(O)/min/mg for the control, but 83 nA(O) per min and mg protein with diazoxide. At concentrations of diazoxide exceeding 10^{-3} M the inhibition is converted to the uncompetitive type.

Measurements of succinate dehydrogenase (SDH) in disrupted mitochondria with cytochrome *c* as terminal electron acceptor gave corresponding results. The Lineweaver–Burk plots for SDH are given in Fig. 1 (left). The V_{max} effect is reproduced yielding the exact values of $V_{max} = 0{\cdot}104$ EU/mg (control) and $V_{max} = 0{\cdot}041$ EU/mg (+diazoxide) with apparent K_m of $6{\cdot}26$–$6{\cdot}37 \times 10^{-4}$ M in both cases.

(2) *Comparison of diazoxide and malonate effects*

Diazoxide, despite being a non-competitive inhibitor, closely resembles the effect of malonate on succinate oxidizing mitochondria. Figure 2 shows the reduction of mitochondrial flavoprotein by succinate, as measured fluorometrically with intact

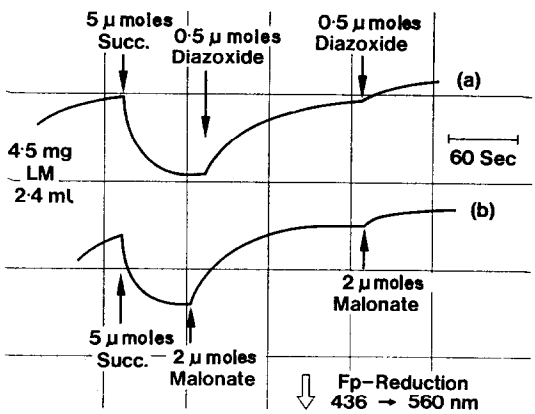

FIG. 2. Action of diazoxide and malonate on flavoprotein reduction in rat liver mitochondria. Incubation mixture 2·4 ml RC-buffer, 25°. Other conditions indicated in the figure.

liver mitochondria. On addition of malonate (lower trace) succinate dehydrogenase becomes inhibited and flavoproteins return to a more oxidized state. Diazoxide (upper trace) exerts the same effect, as expected from the above results. Whereas malonate had to be added in the same range of concentration as the substrate, a ten times lower concentration of diazoxide induced the same extent of flavoprotein oxidation. This is in accordance with the kinetic studies demonstrating a non-competitive site of action.

(3) *Diazoxide action on pyruvate oxidation*

Figure 3 shows a registration of mitochondrial oxygen uptake. Rabbit heart mitochondria, which can rapidly oxidize pyruvate, initially show almost no oxygen uptake with pyruvate. ADP induced a burst of respiration illustrating a high degree of respiratory control. After exhaustion of ADP the system returned to the resting state,[16] but oxygen uptake was reactivated by addition of an uncoupler, causing maximum rate of respiration. With addition of diazoxide the oxidation became

inhibited again. This is interpreted as a decrease of oxaloacetate formation, limiting pyruvate utilization via the Krebs-cycle. Evidence for this theory is obtained by the reactivation of respiration after addition of malate, serving as a source of oxaloacetate. Parallel determinations of pyruvate consumption are in complete agreement with the proposed mechanism.

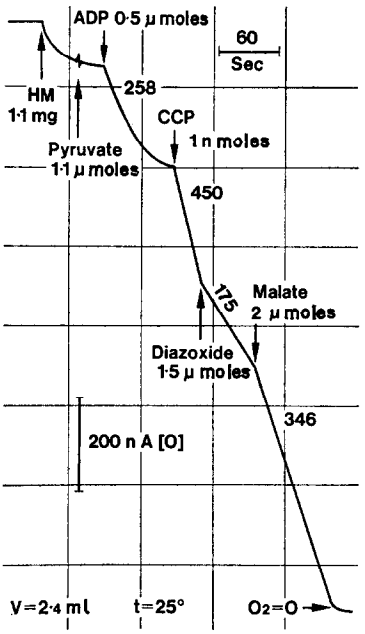

FIG. 3. Registration of respiration of rabbit heart mitochondria, oxidizing pyruvate. 2·4 ml RC-buffer, 25°. Other conditions as indicated. Figures along the trace indicate respiratory rate as nA (O) per min and mg protein.

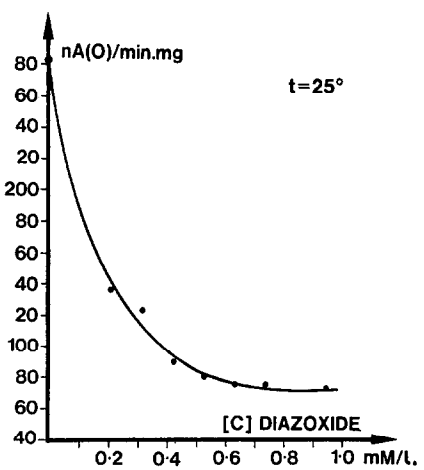

FIG. 4. Effect of various concentrations of diazoxide on pyruvate dependent respiration of rabbit heart mitochondria. Conditions: 2·4 ml HM-buffer, 1·76 mg mitochondrial protein, 1·5 µmoles pyruvate, 2·25 µmoles ADP, 25°. Each figure is the mean of double determinations of respiration, 1 min past diazoxide addition.

Figure 4 gives the correlation between the degree of inhibition of pyruvate oxidation and the concentration of diazoxide, indicating 1/2 maximal inhibition at 0·13 mM diazoxide.

4) *Oxidation of* [^{14}C]*pyruvate*

Both, 1-[^{14}C]pyruvate and 2- or 3-[^{14}C]pyruvate have been used in order to study the formation of CO_2 from pyruvate. With 1-[^{14}C]pyruvate the above results were easily reproduced. As shown in Fig. 5, the basic rate of pyruvate oxidation by rabbit

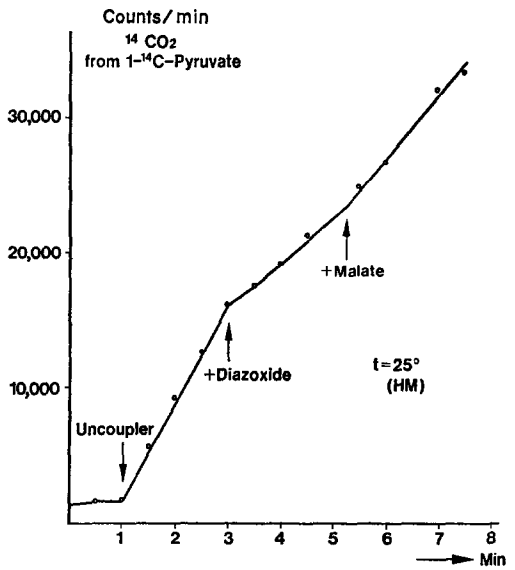

FIG. 5. $^{14}CO_2$-liberation from 1-[^{14}C]pyruvate during oxidation by heart mitochondria. Batch incubation of 19·6 mg rabbit heart mitochondria with 40 ml HM-buffer, 22·5 μmoles 1-[^{14}C]pyruvate, specific activity 83·7 mc/mole, 40 nmoles *m*-Cl-CCP as uncoupler. Diazoxide was added to give a final concentration of 0·385 mM, malate 0·75 mM. Samples were withdrawn at the times indicated.

heart mitochondria is almost negligible. When rapid respiration is induced by addition of an uncoupler a concomitant rise in $^{14}CO_2$ production occurs, which is diminished by diazoxide. Again the inhibition is released by malate.

The inhibition by diazoxide could also be demonstrated with 3-[^{14}C]pyruvate, as summarized in Table 1. Malate, however, failed to stimulate CO_2-labeling, when CO_2 originated from decarboxylations within the Krebs-cycle. This phenomenon is explained by the drastic isotope dilution of Krebs-cycle intermediates by addition of relatively large amounts of malate. As shown in Fig. 6, where no diazoxide is present, malate has no effect on $^{14}CO_2$-liberation from 1-[^{14}C]pyruvate, but considerably lowers the label in CO_2 from 3-[^{14}C]pyruvate. From the dependency of this effect on malate concentration, as presented in Fig. 7, it is evident that in the presence of 10^{-4} M malate only half of the original label is found in respiratory CO_2, suggesting a rough estimate for endogenous malate concentration of about $0{\cdot}5 \times 10^{-4}$ M; this is in good agreement with the literature.[17]

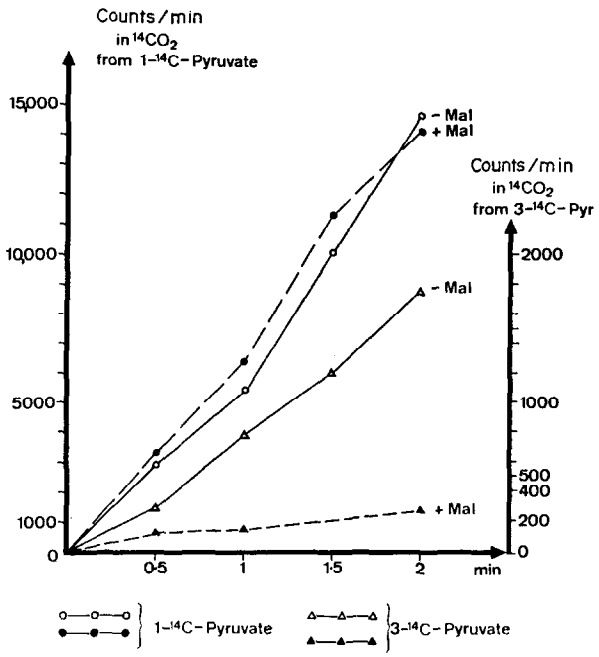

FIG. 6. Effect of malate on $^{14}CO_2$-liberation from 1-[^{14}C] pyruvate and 3-[^{14}C]pyruvate in rabbit heart mitochondria. Each incubation contained 2·0 ml HM-buffer, 0·95 mg mitochondrial protein, 1 nmole mCl-CCP as uncoupler, and 2 μmoles malate when present. 1·5 μmoles pyruvate with specific acitivity of 83·7 mc/mole (1-^{14}C-), and 71·4 mc/mole (3-^{14}C-).

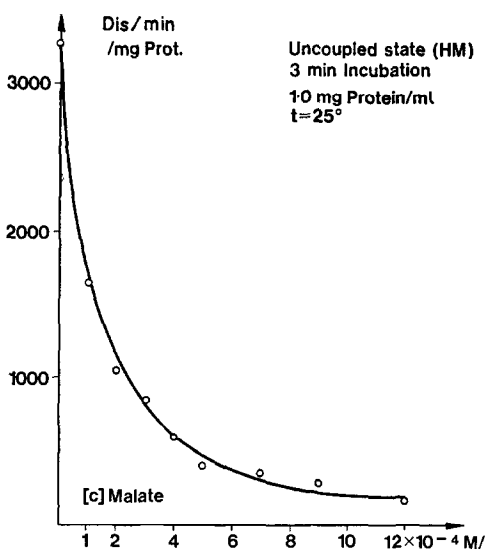

FIG. 7. Influence of malate concentration on $^{14}CO_2$-liberation from 3-[^{14}C]pyruvate by heart mitochondria. Each figure is the mean of double determinations, containing: 2·0 ml HM-buffer, 3·0 μmoles pyruvate-3-[^{14}C], 1 nmole uncoupler (*m*-Cl-CCP), 1·0 mg mitochondrial protein, and malate as indicated. Specific activity of pyruvate 73 mc/mole, 25°.

TABLE 1. GRADUAL INHIBITION OF 3[^{14}C]PYRUVATE OXIDATION IN HEART MITOCHONDRIA BY INCREASING AMOUNTS OF DIAZOXIDE

Diazoxide (mM/l.)	0	0·12	0·23	0·34	0·45	0·56
$^{14}CO_2$ from pyruvate (counts/min/mg protein	4439	3741	3228	2796	2975	1369

Experimental conditions as in Fig. 6; no exogenous malate added.

(5) *Action of diazoxide on fatty acid oxidation*

The oxidation of fatty acids has been investigated under various conditions in liver and heart mitochondria. Mitochondria from rabbit heart muscle catalyse complete oxidation of long chain fatty acids. In accordance with the above findings in these mitochondria the formation of $^{14}CO_2$ from uniformly labeled palmitate was largely inhibited by diazoxide.[7] Since no ketone bodies can be accumulated, these results suggest also that the turnover of the citric acid cycle was inhibited.

Liver mitochondria in the uncoupled state and in presence of ATP and carnitine have a high affinity for fatty acid oxidation.[18] However, the main product of this oxidation is acetoacetate when malate is absent. Diazoxide therefore should have no effect on the conversion of long chain fatty acids to acetoacetate under these conditions. The results of Fig. 8 demonstrate, that in fact an equal rate of acetoacetate

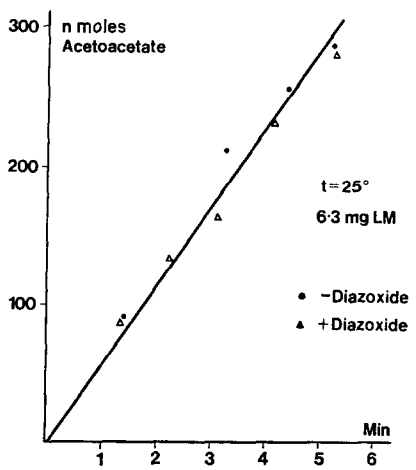

FIG. 8. Effect of diazoxide on acetoacetate formation from palmitate in liver mitochondria. Each determination is the mean of double experiments containing: 2·4 ml FA-buffer, 6·3 mg rat liver mitochondria, 1·5 μmoles palmitate, 2 μmoles D,L-carnitine, 30 nmoles DNP, 1·5 μmoles ATP, and diazoxide when present in a concentration of 0·9 mM.

formation has been measured in liver mitochondria with diazoxide present or absent.

Simultaneous determination of oxygen uptake, however, again revealed that the contribution of the citric acid cycle to fatty acid utilization is diminished by diazoxide. An inhibition of oxygen consumption occured which was released by excess malate,

as a source of oxaloacetate. This is summarized in Table 2, including the analogous effect of malonate.

TABLE 2. EFFECTS OF DIAZOXIDE AND MALONATE ON THE RATE OF FATTY ACID OXIDATION IN RAT LIVER MITOCHONDRIA

Expt No.	Substrate (mM/l.)	Additions (mM/l.)	Oxygen uptake (nA/mg/min)
1*	palmitate (0·21)	—	76·8 ± 1·9
	palmitate	diazoxide (0·94)	51·8 ± 5·1
	palmitate	diazoxide + malate (0·94) (1·26)	78·3 ± 3·2
2	palmitate (0·21)	—	60·6 ± 1·8
	palmitate	malonate (1·2)	44·3 ± 1·0
	palmitate	malonate + malate (1·2) (1·26)	75·7 ± 6·3
3	L-palmitoyl carnitine (0·22)	—	77·8 ± 2·9
	L-palmitoyl carnitine (0·22)	diazoxide (0·94)	52·1 ± 8·8
	L-palmitoyl carnitine (0·22)	diazoxide + malate (0·94) (1·26)	75·8 ± 6·8

* Experiment numbers signify individual preparations. FA-buffer was used for incubations; vol. 2·4 ml; additions: 30 nmoles 2,4-DNP; and 1·5 μmoles ATP and 2 μmoles D,L-carnitine, when palmitate was the substrate. Mitochondrial protein 7·3 mg; 25°. (n=4–6.)

The ratio of oxygen consumed to acetoacetate generated during a fixed time interval of fatty acid oxidation can be taken as a measure whether fatty acids are mainly converted to ketone bodies or fully oxidized via the Krebs-cycle. As demonstrated by

TABLE 3. RELATIVE FORMATION OF ACETOACETATE FROM PALMITATE IN LIVER MITOCHONDRIA

Expt No.	Additions			O_2-uptake (nA(O)/mg)	Acetoacetate (nM/mg)	Δ O/Δ AcAc ratio
	Palm.	Mal.	Diaz.			
1*	+	—	—	159·6	51·5	3·1
	+	+	—	279·7	18·8	14·9
	+	+	+	226·6	35·9	6·4
2	+	—	—	117·3	26·43	4·4
	+	+	—	177·6	12·12	14·6
	+	+	+	146·8	28·85	5·1
3	+	—	—	186·5	54·9	3·4
	+	+	—	344·7	19·0	18·1
	+	+	+	273·6	44·9	6·1

* The experiment numbers represent individual mitochondrial preparations. All figures are means of three to five determinations. Incubation conditions as in Table 2. Diazoxide, when present 0·9 mM; 25°; mitochondrial protein varied between 6 and 10 mg; vol. = 2·45 ml. The time interval of observation was defined from addition of ATP (to initiate fatty acid oxidation) to the moment when the mixture was sucked from the polarographic cell into perchloric acid. Malate, when added, 0·3 mM.

Garland[19,20] the ratio Δ O/Δ AcAc is 3·5 when palmitate is converted to acetoacetate only; it becomes greater than 3·5 when a mixed formation of acetoacetate, citrate and/or CO_2 occurs, approaching infinity when no acetoacetate is formed. The experiments of Table 3 show, that in absence of malate the citric acid cycle is not active during palmitate oxidation. When liver mitochondria were supplemented with traces of malate the ratio Δ O/Δ AcAc increased, indicating an activation of the TCA cycle. By diazoxide the Δ O/Δ AcAc ratio decreased again, due to inhibition of the turnover of the cycle. Thus, by inhibiting the recovery of oxaloacetate in the Krebs cycle, diazoxide causes a relative increase of acetoacetate formation from free fatty acids.

DISCUSSION

The above investigation makes it obvious that in mitochondria from heart and liver the oxidative utilization of acetyl-CoA is diminished by diazoxide.

The degree of inhibition of succinate dehydrogenase (SDH) correlates well with that of pyruvate oxidation. From the Lineweaver–Burk plots (Fig. 1) a 60 per cent inhibition of V_{max} of SDH is seen with $1{\cdot}5\text{–}2{\cdot}0 \times 10^{-4}$ M diazoxide. As calculated from Fig. 4 the same extent of inhibition is achieved on pyruvate oxidation (when corrected for the endogenous respiration, not suppressible by diazoxide).

The release of catecholamines from adrenal and extra adrenal sources[21,22] together with the observed inhibition of Krebs-cycle linked oxidations may explain some metabolic effects of diazoxide. An increase of free fatty acids in blood, a decrease of carbohydrate utilization[5] and even enhanced ketogenesis following administration of high dosages of the drug,[23–25] have been reported. The relative increase of ketogenesis, reported here, may also stimulate the rate of gluconeogenesis in the liver via an increased level of acetyl-CoA, thus activating pyruvate carboxylation.

The inhibition of insulin release from Langerhans' islets may also be considered under these aspects. Actually, liberation of insulin from the islets is closely related to oxidative metabolism. This follows from the ratios of specific enzyme activities in isolated islets,[26] and from the fact, that induction of insulin release from single islets causes a significant stimulation of oxygen consumption.[27] Experiments have been reported, suggesting that oxidations via the Krebs cycle provide both, the signal and the energy required for the process of insulin secretion.[28,29] It may be postulated therefore, that the effect of diazoxide is mainly mediated by an inhibition of oxidative generation of energy. Some support is given to this concept by our finding that other inhibitors of the terminal oxidative pathway are also capable of blocking the release of insulin from isolated islets.* However, it has not yet been investigated whether the islet cells exhibit a specific susceptibility for diazoxide, causing a relative specificity of the drug to block insulin liberation. The antagonism of diazoxide and certain sulfonylureas on insulin release cannot be explained on the same basis. None of the metabolic effects of diazoxide was released by tolbutamide or other sulfonylureas.

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