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The Effect of Streptozotocin-induced Diabetes on the Early Steps of Glucagon Action in Isolated Rat Liver Cells

H. Chamras[1], M. Fouchereau-Peron, and G. Rosselin

Unité de Recherche de Diabétologie et d'Etudes Radio-Immunologiques des Hormones Protéiques (INSERM, U. 55; CNRS ERA 494), Hôpital Saint-Antoine, Paris, France

Summary. This study was undertaken to investigate the effect of experimental diabetes on the early steps of glucagon action. The binding of glucagon and glucagon-stimulated cyclic AMP accumulation in the presence of a potent phosphodiesterase inhibitor (IBMX, 0.1 mmol/l) were studied in liver cells isolated from control and streptozotocin-induced (65 mg/kg) diabetic rats. Comparative studies of insulin binding indicated that hepatocytes of diabetic rats bound twice as much ^{125}I-insulin (10.8 ± 2.0%) as those of control rats (5.7 ± 1.3%). Scatchard analysis and the competition plots of the data suggested that this was due to an increased number of receptors rather a change in their affinity. No significant change was observed in ^{125}I-glucagon binding of diabetic liver cells (5.8 ± 0.5%) as compared to controls (6.8 ± 0.4%). The number of molecules of glucagon bound to high and low affinity binding sites of control liver cells was (51 ± 2) × 10^3 and (1300 ± 134) × 10^3 sites/cell, respectively. The corresponding numbers in streptozotocin-treated rats were (45 ± 5) × 10^3 and (1000 ± 167) × 10^3 sites/cell, respectively. Cyclic AMP response to concentrations of glucagon below 1 nmol/l was significantly lower in diabetics than in normals: for 0.3 nmol/l and 0.6 nmol/l of glucagon, cyclic AMP production was 48 ± 7 pmol/10^6 cells and 78 ± 8 pmol/10^6 cells in diabetics, as compared to 72 ± 9 and 110 ± 9 pmol/10^6 cells in normals. At concentrations of glucagon that are maximally efficient (≥ 7 nmol/l) cyclic AMP production was higher in diabetic (202 ± 20 pmol/10^6 cells) than in normal rats (156 ± 7 pmol/10^6 cells). Thus, diabetes seems to increase the quantity of adenylate cyclase and decrease its affinity for glucagon. Those changes are not related to a modification of the glucagon binding sites and are associated to an increase of insulin receptors.

Key words: Hepatocytes, streptozotocin-treated rats, normal rats, insulin receptors, glucagon receptors, cyclic AMP production.

Changes in specific binding of insulin and glucagon have been observed in experimental diabetic animals. The number of insulin receptors in liver membranes from streptozotocin diabetic rats is increased, and is associated with hypoinsulinaemia [1, 2]. Similar observations have been made in diabetic Chinese hamsters [3]. In contrast, studies of glucagon binding in diabetic rat liver membranes have yielded contradictory results [1, 2, 4]. Davidson et al. reported no change in glucagon binding [1] while other authors found a decreased [4] or an increased [2] glucagon binding to liver membranes of streptozotocin-treated rats. This has prompted us to investigate the early steps of glucagon action in experimental diabetes, using isolated liver cells. In each animal, the binding of insulin by liver cells was determined as a control of receptor modification due to diabetes. The characteristics of glucagon receptors regarding the kinetics and stoichometry of the binding process were simultaneously measured in comparative experiments.

Materials and Methods

Animals

Male Sprague-Dawley rats (Iffa Credo) weighing 120–130 g were fed ad libitum on a standard laboratory diet, and were given free access to water. Diabetes was induced in rats fasted overnight by a single IV injection of streptozotocin (65 mg/kg) in 0.1 mol/l citrate buffer, pH 4.0. Control rats received the diluent only. The streptozotocin-treated rats developed glycosuria and hyperglycaemia

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within 48 h after injection. The animals were sacrificed 7 days later, when blood glucose levels were higher than 400 mg/100 ml. All animals were studied in the fed state and sacrificed at about 10 h.

Materials

Highly purified porcine insulin (MCS 821506) and porcine glucagon (B 66) were used for iodination and as standards (generously supplied by Dr. J. Schlichtkrull, Novo Research Institute, Copenhagen). Carrier free $Na^{125}I$, IMS 300, was purchased from the Radiochemical Centre (Amersham, England), bovine serum albumin (BSA, fraction V, lot 294) from Miles Laboratories, crude collagenase (type I, 140 IU/mg, lot 86 C 0075) from Sigma, N-2 hydroxymethyl piperazide N'2 ethane-sulfonic acid (HEPES, lot 28c-5026) from Sigma, and streptozotocin (lot 9681-GGS 118 F IU 9889) from Upjohn Laboratories. The 2'0-succinyl cyclic AMP (cyclic-Suc-AMP), its tyrosine methyl ester (cyclic-Suc-AMP-tyr-0-Me), the radioiodinated derivative (^{125}I-labelled-cyclic-Suc-AMP-tyr-0-Me) and antibodies against the albumin-conjugated cyclic-Suc-AMP were prepared in our laboratory [5, 6]. Cyclic AMP, AMP, ADP, ATP, adenosine were purchased from Calbiochem, bacitracin (lot 124c, 01841) from Sigma, trasylol (R) (6600 IKIU/mg) from Bayer AG, and 3-isobutyl-l-methylxanthine, IBMX (lot 121247) from Aldrich Chemical Company Inc. The guinea pig anti-porcine insulin and rabbit anti-porcine glucagon sera were prepared in our laboratory. Caffeine and other chemicals were obtained from Merck.

Isolation of Liver Cells

The method of liver cell preparation was the "two-step procedure" described by Seglen involving a perfusion of liver with a calcium free buffer followed by perfusion with collagenase [7, 8]. The following modifications of that procedure were made: 1) collagenase buffer 100 ml, containing 50 mg collagenase, was supplemented with 5 mg of soybean trypsin inhibitor (Sigma) [9]; 2) after collagenase digestion, the liver was immediately flushed to remove collagenase with 100 to 150 ml of calcium and magnesium free Krebs Ringer Phosphate (KRP), pH 7.5, containing 0.2 g/100 ml bovine serum abumin (BSA) dialysed (against KRP): 3) once separated, the cells were centrifuged at 200 rpm for 1 min. The cell pellet was washed in KRP containing 3 g/100 ml BSA, pH 7.5. Following the third centrifugation the purified parenchymal cell pellet was resuspended in fresh incubation medium (KRP 3 g/100 ml BSA, pH 7.5) at a concentration of 800000 cells/ml. Cells were counted in a Malassez haemocytometer. This procedure yielded 400 to 500 $\times$ 10^6 cells/liver.

The viability of the cells was similar in diabetic and control animals. The proportion of cells excluding Trypan Blue was about 90–95% before incubation and not lower than 80% after 4 h at 20° C. Extracellular lactic dehydrogenase activity [10] was not significantly increased after 2 h incubation in either diabetic (4.5 $\pm$ 1.0% vs 5.5 $\pm$ 1.0%) or control (4 $\pm$ 1% vs 5 $\pm$ 2%) rat liver cells. The ATP content [11] of the diabetic and control hepatocytes was respectively 18 $\pm$ 3 and 18 $\pm$ 2 nmol/10^6 cells before incubation and 14 $\pm$ 5 and 14 $\pm$ 2 nmol/10^6 cells after 2 h incubation at 20° C.

Hormone Binding Assay

^{125}I-insulin and ^{125}I-glucagon were monoiodinated, using the chloramine-T method under the conditions previously described [12, 13]. The specific activity of labelled insulin and glucagon was 350 and 600 Ci/g, respectively. Studies of insulin and glucagon binding were performed at 20° C in calcium-free KRP buffer as previously described [14] with the following modifications. Each incubation tube contained in a final volume of 0.5 ml, ^{125}I-labelled hormone at about 0.3 nmol/l, unlabelled hormone at the concentrations indicated in the figures, BSA at 3 g/100 ml and 0.5 $\times$ 10^6 cells/ml. The medium of the cells incubated with glucagon also contained 2,000 IU/ml of kallikrein inhibitor (Trasylol) and 100 μg/ml of bacitracin [15] as inhibitors of glucagon degradation, 0.1 mmol/l of IBMX, a potent inhibitor of phosphodiesterase activity, and 10 mmol/l of alanine. The cell bound hormone was separated by centrifugation as described previously [16, 17]. The supernatant was removed and the radioactivity of the washed pellet was determined by gamma spectrometry.

Hormone Degradation Assay

Hormone degradation was measured by the method previously described [18]. ^{125}I-labelled hormone (0.3 nmol/l) was exposed to liver cells of control and diabetic rats. After 2 h incubation at 20° C, the ^{125}I-labelled hormone remaining intact in the medium was tested for its ability to rebind to specific receptors in liver membranes. Appropriate controls without cells represented 100% of the substrate available for degradation.

Measurement of Cyclic AMP

Endogenous cyclic AMP was measured by radioimmunoassay in the methanol extract of the whole mixture (cells + medium) as previously described [19]. For concentrations lower than 20 nmol/l the sensitivity of the radioimmunoassay was increased by the succinylation of the cyclic AMP following the method described by Cailla et al. [20].

Other Determinations

Plasma immunoreactive glucagon was measured using the Unger glucagon antibody 30 K [21]. Plasma insulin concentration was estimated by radioimmunoassay [22, 23], the results being expressed in terms of a porcine insulin standard (20 IU/mg). Plasma glucose was determined by the glucose oxidase method (Boehringer Mannheim Test, FRG). Protein concentration was measured by the method of Lowry et al. using BSA as standard [24].

Expression of Results

Specific binding was obtained by subtracting from the total binding the amount of labelled hormone which is not displaced by a large excess of unlabelled hormone (17 μmol/l for insulin and 14 μmol/l for glucagon). The number of specific binding sites of the hormone-receptor complexes was obtained from competition data at steady state by plotting the amount of hormone specifically bound against the concentration of free hormone. The number of binding sites and the corresponding apparent dissociation constant were also determined by Scatchard plots of the same data. Results were expressed by mean $\pm$ SEM. Statistical analysis was performed by using Student's unpaired t-test. In some instances Wilcoxon's test for coupled differences was used.

Results

Experimental Animals

The presence of diabetes, 7 days after injection of streptozotocin was attested in each animal by gly-

Table 1. Characteristics of the control and diabetic rats. Measurements were performed 7 days after injection of streptozotocin (65 mg/kg)

	Body weight g	Glycosuria g/l	Plasma glucose mg/100 ml	Plasma insulin mU/l	Plasma glucagon ng/l
Controls (n = 8)	169 ± 3	not detectable	161 ± 3	100 ± 11	335 ± 41
Streptozotocin treated rats (n = 8)	124 ± 3[a]	9.0 ± 0.6	522 ± 11[a]	50 ± 2[a]	856 ± 87[a]

[a] $p < 0.001$

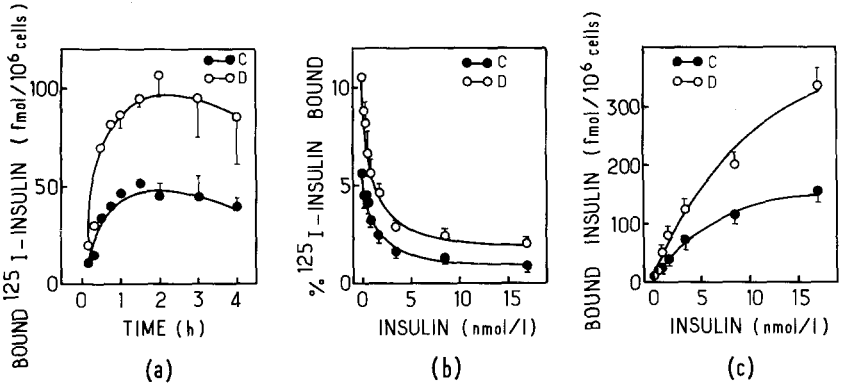

Fig. 1. Time-course **a** and competition curve **b** of insulin binding to isolated hepatocytes from control (C) and diabetic (D) rats. Incubation conditions were as described under Methods. Each point is the mean ± SEM of 4 separate cell preparations for control and diabetic rats. The panel **c** represents at each hormone concentration, the amount of insulin bound, calculated from the competition curve

cosuria, loss of body weight, high plasma glucose level, low plasma insulin and high plasma glucagon (Table 1).

Effect of Diabetes on Insulin Binding

The binding of ^{125}I-insulin increased with time in cells of control and diabetic rats (Fig. 1 a). In diabetic rats, the specific binding of ^{125}I-insulin was about two-fold greater than in control rats. As insulin binding was maximal and stable in both groups between 60 and 180 min of incubation, the binding of ^{125}I-insulin was studied as a function of increasing unlabelled hormone concentration after 120 min incubation (Fig. 1 b). The initial binding was 5.7 ± 1.3% (n = 4) and 10.8 ± 2.0% (n = 4) of the total ^{125}I-insulin with cells from control and diabetic rats, respectively. The 50% inhibition of ^{125}I-insulin binding was achieved with about 1.25 nmol/l insulin in both types of cells, suggesting an identical affinity.

When the data were expressed as quantities of insulin bound per 10^6 cells (Fig. 1 c) hepatocytes of diabetic rats bound about twice as much insulin as those of controls ($p<0.05$). At 0.6 nmol/l (4 ng/ml) the quantity of insulin bound was 42.2 ± 6.5 (n = 4) and 26.4 ± 6.0 (n = 4) fmol/10^6 cells in diabetic and control rats, respectively.

To determine whether the increased insulin binding in the cells of diabetic rats was due to an increased affinity or to a higher number of receptor sites, the binding data were analyzed by the method of Scatchard [25]. The curves obtained for diabetic and control rats were curvilinear and parallel (data not shown), suggesting that the binding affinity for insulin was similar. The number of occupied binding sites was significantly higher in diabetics than in controls at both concentrations tested. The number of molecules of insulin bound per liver cell of control rats, calculated from data shown in Figure 1, at 0.3 or 17.8 nmol/l insulin were 1800 ± 350 and 93000 ± 7600, respectively. The corresponding numbers in streptozotocin-treated rats were 3400 ± 500 and 205000 ± 5000, respectively.

To rule out the possibility that increased insulin binding in diabetic rats could have been due to decreased hormone degradation, the degradation of insulin by hepatocytes was examined. There was no significant difference in the amount of insulin degraded by hepatocytes of control and diabetic rats. After 2 h incubation at 20°C, the percent intact ^{125}I-insulin was 81 ± 2% and 75 ± 4% (n = 4) in control and diabetic rats, respectively.

Effect of Diabetes on Glucagon Binding and Glucagon-stimulated Cyclic AMP Accumulation

Glucagon binding was time dependent in control and diabetic rats (Fig. 2 a). In both these groups maximal binding was attained by 45 min and apparent equilibrium was maintained until 240 min. The quantity of

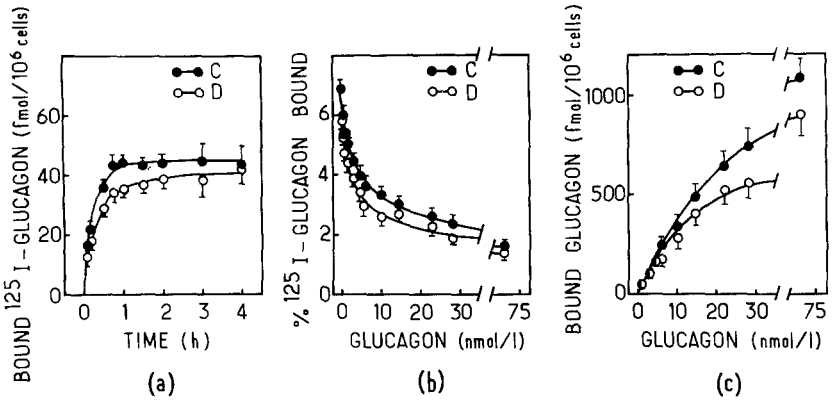

Fig. 2. Time-course **a** and competition curve **b** of glucagon binding to isolated hepatocytes from control (C) and diabetic (D) rats. Incubation conditions were described under Methods. Each point is the mean ± SEM of 10 separate cell preparations for control and diabetic rats. In panel **c** the amount of glucagon bound, calculated from the competition curve was plotted as a function of hormone concentrations

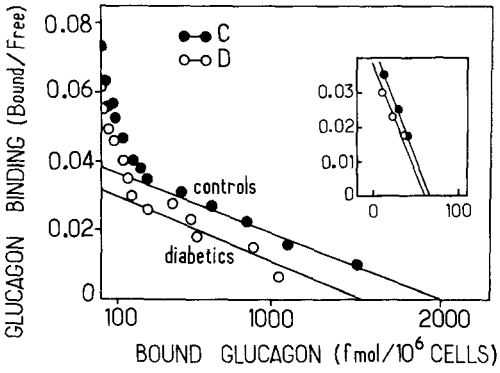

Fig. 3. Scatchard analysis of glucagon to hepatocytes from control (C) and diabetic (D) rats. The ratio of bound to free glucagon (B/F) was plotted as a function of bound glucagon (B) (Data from Fig. 2b). The inset represents the points corresponding to the high affinity binding sites, obtained by subtraction from the total sites the contribution of the low affinity binding sites

Table 2. Dissociation constants and number of glucagon binding sites per liver cell calculated from data shown in Figure 3

		Glucagon binding sites	
		High affinity	Low affinity
Dissociation constant (Kd) (mol/l × 10^{-9})	C	2.4 ± 0.2	52 ± 8
	D	2.5 ± 0.3	50 ± 7
Number of sites per cell (× 10^3)	C	51 ± 2	1,300 ± 134
	D	45 ± 5	1,000 ± 167

bound ^{125}I-glucagon in diabetic hepatocytes was not significantly different from controls. Binding of glucagon was studied in the presence of increasing concentrations of unlabelled hormone (1 to 75 nmol/l) (Fig. 2b). The amount of ^{125}I-glucagon bound after a 60 min incubation period was 6.8 ± 0.4% (n = 10) and 5.8 ± 0.5% (n = 10) in control and diabetic rat liver cells, respectively. The 50% inhibition of the ^{125}I-glucagon binding was achieved with about 7 nmol/l glucagon in both conditions. This suggests that the apparent average affinity is similar in both cases. The amount of glucagon bound at equilibrium was calculated from the competition curve and was not significantly different in diabetics and controls (Fig. 2c). At 0.1 nmol/l (0.3 ng/ml) glucagon, a concentration close to that present in portal vein of normal rat [26], the quantity of bound glucagon was 12.4 ± 3.0 (n = 10) and 10.2 ± 2.0 (n = 10) fmol/10^6 cells in control and diabetic rats, respectively.

Scatchard analysis of the same data (Fig. 3) showed that diabetic and control curves were curvilinear and parallel to each other, indicating that the binding affinities for glucagon were similar in diabetic and control hepatocytes (Table 2). Neither was the degradation of labelled glucagon significantly different. After 60 min of incubation at 20°C, the percent intact ^{125}I-glucagon was 86 ± 5.5% and 82 ± 6.4% in control and diabetic rats, respectively.

The glucagon-stimulated cyclic AMP accumulation was studied at 20°C in the presence of 0.1 mmol/l IBMX. These conditions are optimal for relating the accumulation of cyclic AMP to the activation of adenylate cyclase by glucagon. In these conditions, the rate of cyclic AMP production induced by glucagon was constant between 0 and 10 min in diabetics and in controls (Fig. 4, left panel). After 30 min a plateau of cyclic AMP level induced by glucagon was observed. In both groups, the addition of 3-isobutyl-1-methylxanthine at a concentration higher than 0.1 mmol/l did not result in any further stimulation of cyclic AMP by glucagon (Fig. 4, right panel), suggesting maximal inhibition of phosphodiesterase activity.

The mean dose-response curves to glucagon are shown in Figure 5. In the absence of glucagon the level of cyclic AMP did not differ significantly in control (5.7 ± 0.7 pmol/10^6 cells, n = 10) and diabetic (6.5 ± 1.0 pmol/10^6 cells, n = 10) liver cells. After

addition of glucagon the following was observed: 1) Cyclic AMP response to concentrations of glucagon below 1 nmol/l was lower in diabetics than in normals: for 0.3 nmol/l and 0.6 nmol/l of glucagon, cyclic AMP production was 48 ± 7 and 78 ± 8 pmol/10^6 cells in diabetics, as compared to 72 ± 9 ($p<0.05$) and 110 ± 9 pmol/10^6 cells in normals ($p<0.05$). 2) At concentrations of glucagon that are maximally efficient cyclic AMP production was higher in diabetic than in normal rats; for example at 7 nmol/l glucagon cyclic AMP production was 202 ± 20 pmol/10^6 cells and 156 ± 7 pmol/10^6 cells ($p<0.05$), respectively. 3) Half-maximum glucagon-induced cyclic AMP level was generated by a concentration of glucagon which was about 3 times higher than that observed in controls.

When the glucagon-stimulated cyclic AMP accumulation is compared to the percentage of glucagon bound at steady state (Fig. 6), it is apparent that most cyclic AMP accumulation occurred when only a fraction of the glucagon binding sites was occupied. Furthermore, for a proportion of glucagon bound ranging from 2 to 30%, the percentage of the cyclic AMP accumulation was lower in cells of diabetic rats than those of controls (Fig. 6).

Discussion

The data reported here indicate that in streptozotocin diabetes, glucagon-induced cyclic AMP production in liver cells is decreased without significant change in the number or in the affinity of the glucagon binding sites. This decrease is observed at concentrations of glucagon in the range of those found in the portal vein of the rat [26], dog [33] and man [34], concentrations that are estimated not to exceed 1.7× those found peripherally. The validity of our results is strengthened by the following: 1) the isolated cell preparation from both diabetic and control rat liver consisted of about 90% single hepatocytes with well preserved structure as judged by the phase microscopy, their ability to exclude Trypan Blue, and absence of leakage of intracellular lactic dehydrogenase; 2) the inactivation of ^{125}I-glucagon and the degradation of receptors were minimized by performing the incubation at 20°C; 3) in accordance with data from different laboratories [1, 2, 3], the insulin binding capacity was significantly higher in diabetic animals than in controls in our experimental conditions (Fig. 1).

Studies of glucagon binding in streptozotocin diabetic rats have presented contradictory results. A two-fold decrease [4] and a 95% increase [2] of the binding capacity for glucagon to liver plasma mem-

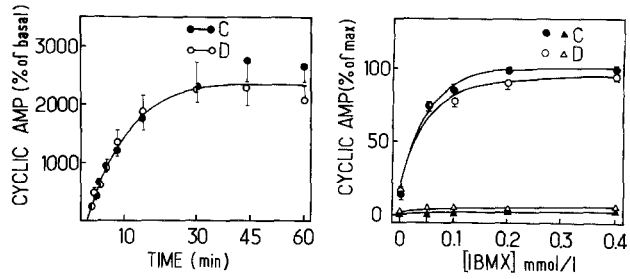

Fig. 4. *Left panel:* Time-course of glucagon-stimulated cyclic AMP accumulation in isolated liver cells from control (C) and diabetic (D) rats. The cyclic AMP level is expressed as percent of the basal value. The basal level is 100 ± 13 (n = 10) for control and 100 ± 26 (n = 10) for diabetic rats. *Right panel:* The cyclic AMP accumulation after 30 min of incubation is plotted as a function of the IBMX concentration. Each point is the mean ± SEM of 3 separate experiments. In both studies, cells (0.5 × 10^6/ml) were incubated at 20°C in the absence or in the presence of 1 nmol/l of glucagon. The incubation buffer was identical to that used for glucagon binding assay (see Methods)

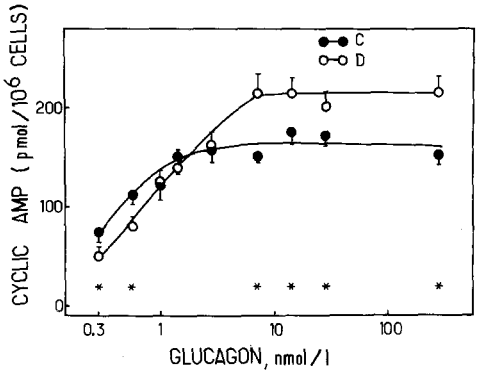

Fig. 5. Dose-response of glucagon-stimulated cyclic AMP accumulation in liver cells of control (C) and diabetic (D) rats. Cells (0.5 × 10^6/ml) were exposed to various concentrations of glucagon and incubated for 30 min at 20 °C. Each point is the mean ± SEM of 10 separate experiments. *, $p<0.05$ between diabetic and control rats

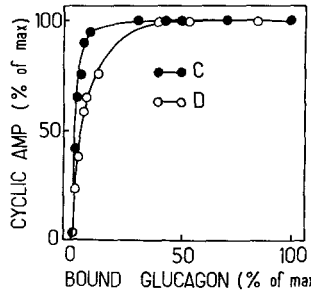

Fig. 6. Relationship between glucagon-stimulated cyclic AMP accumulation (Fig. 5) and glucagon specifically bound to isolated liver cells (Fig. 2b) from control (C) and diabetic (D) rats

branes of diabetic rats have been reported. These discrepancies may be due to differences that occur in the preparation of liver plasma membranes. Indeed, as reported [2], membranes from untreated diabetic rat differ from normal in terms of enzymatic activities, namely of 5' nucleotidase and glucose-6-phosphatase. Furthermore, it has been demonstrated that the receptor protein content of cell membranes does not necessarily reflect the hormone binding by intact hepatocytes [27].

To our knowledge, the only previous study on glucagon binding to diabetic hepatocytes was presented by Bhathena et al. [28]. These authors reported that glucagon binding was decreased about two-fold in diabetic rats compared to controls, whereas no significant change was found in our studies. Severity of diabetes was similar in the two studies. However, Bhathena et al. [28] studied glucagon binding after 30 min incubation at 30°C. Their data indicated that in these conditions no alteration in glucagon degradation occurred in diabetic rat hepatocytes compared to normal rats when tested by the rebinding of glucagon to charcoal. In our study under similar experimental conditions, the exact measurement of the binding capacity of glucagon at 30°C was difficult. No steady state of binding was obtained and the degradation of glucagon after 30 min incubation was as high as 50%, when measured by the rebinding of the glucagon to plasma membranes. Since in such conditions the comparative study of the hormone binding was meaningless, the experiments were performed at 20°C. In that respect, we have verified that the number of binding sites of normal hepatocytes was much higher at 20°C (data not shown) in accordance to the results obtained by Sonne et al. [29].

Our data on the number of insulin binding sites are in agreement with others [1, 2, 3, 28] who have observed an increase in diabetes with low plasma insulin levels. Such results are consistent with the observation that an inverse correlation exists between insulin binding and the circulating hormone concentration [30, 31]. However, our data on glucagon binding does not support the possibility of a down regulation of glucagon receptors related to the high plasma glucagon level present in the streptozotocin diabetic rats. Such a down regulation mechanism has been observed with fasting, where the high plasma glucagon level is associated with a significant decrease of glucagon binding [32]. However, the hyperglucagonaemia in fasting is of short duration as compared to that of diabetic state. It will be noted that even in the studies that reported a decrease in glucagon receptors in association with hyperglucagonaemia [4], no correlation was found between the plasma glucagon level and the reduction in receptor concentration.

The glucagon-stimulated cyclic AMP production in liver cells was studied in the conditions that are optimal for relating the cyclic AMP level to activation of adenylate cyclase. Most, if not all, the phosphodiesterase activity was inhibited by lowering the temperature to 20°C and with addition of IBMX. Our results clearly demonstrated that streptozotocin diabetes affects the glucagon-induced cyclic AMP production in liver cells. They also indicate that the concentration of glucagon used and its efficiency, the magnitude of glucagon-induced stimulation, are not affected in the same way. Briefly, the potency of glucagon is lower and the efficiency of glucagon is higher in liver cells of diabetics compared to controls. Such data may explain the apparent discrepancy in results that indicate an increase [2] or a decrease [28] of the glucagon-induced cyclic AMP accumulation, or the adenylate cyclase activity, in diabetic liver. The higher efficiency of glucagon in diabetics suggests that the quantity of adenylate cyclase is increased in the liver cells of diabetics. Our observation that low concentrations of glucagon are less potent in promoting cyclic AMP production in liver cells of diabetics than in those of controls strongly suggests a decrease in the affinity of adenylate cyclase for glucagon. The number of the binding sites being unaltered, the decrease in affinity could be related to a defect beyond the binding process.

The abnormality of the early steps of glucagon action observed in diabetes is different to that found in fasting animals. In fasting state, the decrease in the glucagon-induced cyclic AMP is related to a decrease in the number of glucagon binding sites [32], and the effect of hyperglucagonemia is counterbalanced by the persistance of insulin production. In diabetes, the increased number of insulin binding sites may partially balance the lack of insulin. Such regulation is not evident for glucagon. However, the diminished sensitivity of the diabetic liver cells to glucagon can provide a mechanism for reducing to some extent the excessive gluconeogenesis and hyperglycaemia associated with the diabetic state.

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Dr. H. Chamras
Unité de Recherche de Diabétologie
et d'Etudes Radio-Immunologiques des Hormones Protéiques
INSERM U. 55
Hôpital Saint-Antoine
184 Rue du Faubourg Saint-Antoine
F-75012 Paris
France