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Escherichia coli (Lilly) and *Saccharomyces cerevisiae* (Novo) rDNA Glucagon: An Assessment of Their Actions When Supplied Selectively to Periportal Cells in the Bivascularly Perfused Rat Liver

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Abstract: The actions of Eli Lilly-rDNA glucagon and Novo Nordisk-rDNA glucagon on glycogen catabolism and related parameters were investigated using the bivascularly perfused rat liver. The technique allows glucagon to be supplied to a selective portion of the hepatic periportal region (≈39%) when the former is infused into the hepatic artery in retrograde perfusion. Both glucagon preparations were equally effective in influencing metabolism (glucose output, glycolysis and O_2 uptake) when supplied to all cells along the liver sinusoids. When only a selective periportal region of the liver was supplied with the hormone, however, the action of Novo Nordisk-rDNA glucagon was proportional to the accessible cell space, whereas the action of Eli Lilly-rDNA glucagon greatly exceeded the action that was expected for the accessible space. Chromatographically, both rDNA preparations were not pure, but their impurities were not the same. The impurities in Eli Lilly-rDNA glucagon resembled those found in the similarly acting pancreatic Eli Lilly glucagon. It was concluded that the space-extrapolating action of Eli Lilly-rDNA glucagon is caused by a yet-to-be-identified impurity. The hypothesis was raised that an impurity in certain glucagon preparations can enhance cell-to-cell propagation of the glucagon signal, possibly via gap junctional communication.

Keywords: rDNA glucagon; liver; bivascular perfusion; signaling propagation; glycogenolysis; glycolysis; oxygen uptake

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1. Introduction

The microcirculation of the rat liver presents an interesting feature that has been used to supply, in a selective way, periportal cells with substrates or metabolic effectors. This feature is highlighted in the scheme of Figure 1 [1,2]. As a dual-entry organ, most blood enters the liver via the ramifications of the portal vein, with a smaller contribution coming from the oxygen-rich hepatic artery ramifications. The confluences of the ramifications of the hepatic artery with the ramifications of the portal vein present a relatively complex pattern. In general, however, there are two such confluences, as illustrated by Figure 1, a presinusoidal confluence and an intrasinusoidal one. This particular feature of the liver microcirculation allows to investigate selectively the response of a substantial region of the periportal parenchyma, provided that the liver is perfused in the retrograde mode (hepatic vein → portal vein) and the modifying agents are introduced into the hepatic artery [1,2]. Since the accessible cell spaces can be quantified using the multiple-indicator dilution technique [3], the modified metabolic fluxes can be easily normalized for comparative purposes. This strategy was used to corroborate, for example, the predominance of lactate, alanine and glutamine gluconeogenesis in the periportal region [4–7], and to demonstrate the predominance of vasopressin action in the perivenous region [2]. Furthermore, it was also possible to show that the stimulatory action of glucagon on gluconeogenesis is more

pronounced in the periportal region in absolute terms only because this metabolic pathway also predominates in the same region [8]. In the latter study, a commercial bovine/porcine preparation obtained from Eli Lilly and Company [9] was used. This pancreatic glucagon preparation was commonly used for a long period of time for treating acute hypoglycemic episodes. When this glucagon preparation was used for investigating the zonation of the effects of the hormone on glycogen catabolism in the bivascularly perfused rat liver [10], a surprising disproportion between the accessible cell spaces and the modifications in glycogenolysis and oxygen uptake was observed. The increases in glucose output caused by the hormone were practically the same, irrespective of the perfusion mode (antegrade and retrograde) and the infusion route (portal vein hepatic vein and hepatic artery), a set of observations that was quite difficult to interpret in terms of a periportal predominance of the action of glucagon on glycogenolysis. Retrograde cell-to-cell communication involving intracellular cAMP [11] was hypothesized to occur, but no definitive clarification of the phenomenon has yet been presented. It is worth mentioning, however, that a similar phenomenon was found when highly penetrating cAMP analogs (N^6,2′-*O*-dibutyryl cAMP and N^6-monobutyryl cAMP) were supplied via the microcirculation to periportal cells, although no such behaviour was seen with exogenous cAMP [12].

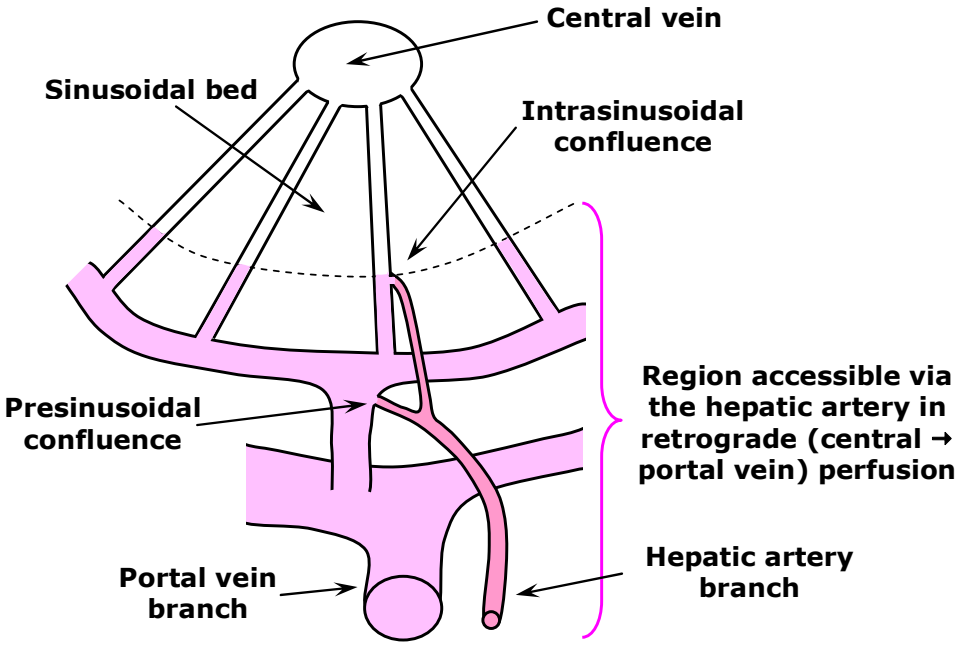

Figure 1. Diagram illustrating some of the main features of the hepatic microcirculation. The colored area represents the liver space that is accessible via the hepatic artery in retrograde perfusion, i.e., in the direction hepatic vein → portal vein [1,2].

Presently, glucagon preparations of pancreatic glucagon are no longer used in both clinics and experimental laboratories, as they have been replaced mostly by either synthetic or rDNA glucagon [13]. Readily available on the market are rDNA glucagon (human glucagon for injection) from Eli Lilly (Indianapolis) and rDNA glucagon (GlucaGen®) from Novo Nordisk (Bagsværd, Denmark). They are produced in genetically modified microorganisms, the first one in a non-pathogenic *Escherichia coli* strain [14] and the latter in a recombinant *Saccharomyces cerevisiae* strain [15]. Both have been successfully used for treating emergency hypoglycemia and are said to be safer than porcine or bovine glucagon because there is no risk of disease transmission. Nonetheless, a question that can still be raised is one concerned with the abovementioned disproportion between the accessible cell space in the liver and the effects of glucagon on glucose output that was observed earlier using glucagon of pancreatic origin [10]. Whether this phenomenon also occurs with rDNA glucagon is still an open question, and its reexamination with both

currently available rDNA glucagon forms is exactly the aim of the present work. For this purpose, rat livers from fed rats were bivascularly perfused using the technique described earlier, and the output of glucose and glycolysis products (lactate and pyruvate) was measured in addition to oxygen uptake. Analysis and interpretation of the perfusion experiments were accomplished in terms of the previously determined accessible cell spaces, and chromatographic analyses were done in order to examine the purity of the various glucagon preparations [3]. The results are expected to inform further about the equivalence of the various glucagon preparations and their impurities in terms of their interactions with the liver cells.

2. Materials and Methods

2.1. Materials

The liver perfusion apparatus was built in the workshops of the University of Maringá. Bovine glucagon and *Escherichia coli* recombinant DNA glucagon (human glucagon for injection) were obtained from Eli Lilly and Company (Indianapolis, IN 46285, USA). *Saccharomyces cerevisiae* recombinant glucagon (GlucaGen®) was obtained from Novo Nordisk A/S (2880 Bagsværd, Denmark). Stock solutions of glucagon were prepared to contain 1 mg of glucagon (plus the excipient lactose) per mL of alkaline Krebs/Henseleit-bicarbonate solution. The stock solutions were stored at 4 °C and diluted to the required concentrations in the Krebs/Henseleit-bicarbonate buffer (pH 7.4) prepared for the perfusion experiments or for the chromatographic analyses. NAD^+ and all enzymes and coenzymes used in the enzymatic assays were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All standard chemicals were of the best available grade (98–99.8% purity).

2.2. Liver Perfusion

Male albino rats (*Rattus novergicus*, Wistar strain), weighing 190–220 g, were maintained on a regulated light–dark cycle under constant temperature (22 ± 3 °C) and fed ad libitum with a standard laboratory diet (Nuvilab®). Anesthesia was brought about by the intraperitoneal administration of ketamine (70 mg/kg) + xylazine (7 mg/kg) before manipulating the liver for perfusion. The criterion for anesthesia was the lack of body or limb movement in response to a standardized tail-clamping stimulus. All experiments were done in accordance with internationally accepted recommendations for the care and use of animals. The protocols were approved by the Ethics Committee for Animal Experimentation of the University of Maringá.

Hemoglobin-free, non-recirculating bivascular liver perfusion was performed either in the antegrade mode (entry via the portal vein plus the hepatic artery and exit via the hepatic vein) or in the retrograde mode (entry via the hepatic vein plus the hepatic artery and exit via the portal vein). The following surgical procedure was employed [16]. After exposing the abdominal cavity and excising the connective tissue between the liver and the stomach, two ligatures were tightened around the esophagus. The latter was excised between these ligatures, and both the stomach and the intestines were displaced to the right. The splenic vein, the left renal artery, and the ramification of the coeliac trunk leading to the pancreas and spleen were closed with tight ligatures. The stomach and the intestines were again displaced to the left, and the right renal artery was closed. Open ligatures were disposed around the following vessels: (1) gastroduodenal artery; (2) mesenteric artery; (3) dorsal aorta, both below the left renal vein and above the coeliac trunk; (4) inferior vena cava and (5) portal vein. Perfusion was initiated by cannulating the portal vein under reduced flow until complete exsanguination. Perfusion of the hepatic artery was established by cannulating the dorsal aorta below the renal vein. Immediately, the gastroduodenal artery, the mesenteric artery and the dorsal aorta above the coeliac trunk were closed. This procedure deviates the perfusion fluid into the hepatic artery. The thorax was opened para-externally, and the diaphragm was excised just below the ribs. The superior branch of the vena cava was tightened and the inferior branch was cannulated. The perfusion was carried out in situ, with flow provided by two peristaltic pumps. The perfusion

fluid was Krebs/Henseleit-bicarbonate buffer (pH 7.4) containing 25 mg% bovine-serum albumin, saturated with a mixture of oxygen and carbon dioxide (95:5) by means of a membrane oxygenator with simultaneous temperature adjustment (37 °C). The portal flow was adjusted to a rate between 28 and 32 mL/min, and the arterial flow was between 2 and 3 mL/min. All perfusion experiments were initiated in the antegrade mode. Retrograde perfusion, when required, was established by changing the direction of flow for 15–20 min before initiating sampling of the effluent perfusate.

2.3. Analytical

Oxygen concentration in the effluent perfusate was monitored continuously, employing a teflon-shielded platinum electrode connected to a polarographic device [17]. Samples of the effluent perfusion fluid were collected according to the experimental protocol and analyzed for their metabolite content. The following compounds were measured by standard enzymic procedures: glucose [18], lactate [19] and pyruvate [20]. The total metabolic rates were expressed as μmol min^{-1} (g liver wet weight)$^{-1}$. Changes in the metabolic fluxes caused by glucagon were expressed as μmol $minute^{-1}$ (mL accessible cell space)$^{-1}$. The wet liver weight was taken as corresponding to 4.4% of the animal weight. The accessible cell spaces determined in previous work were used [3].

2.4. Chromatographic Analyses

Three different glucagon samples were analyzed by means of high-performance liquid chromatography: (a) rDNA glucagon (GlucaGen®) from Novo Nordisk; (b) rDNA glucagon from Eli Lilly and (c) bovine glucagon from Eli Lilly. A Shimadzu liquid chromatograph, equipped with an HRC-ODS-Shimadzu ion exchange column (15 cm), was utilized (Shimadzu, Kyoto, Japan). The flow rate was 1.2 mL/min, the temperature was 25 °C, and 20 μL of samples were injected. The mobile phase was 180 mM phosphate buffer, pH 4.75. The glucagon samples were diluted with ultra-pure water to the desired concentration (2 μg/μL). The absorbance of the eluates was monitored simultaneously at 280 and 214 nm.

2.5. Treatment of Data

Data are presented as means ± mean standard errors of the mean. Statistical analysis was done by means of the Statistica® software version for Windows 98 Edition (StatSoft, Tulsa, OK, USA). Parametric statistical analysis was done based on the normal distribution of the metabolic data obtained in our laboratory, as revealed by the Shapiro–Wilk test. One-way variance analysis with Student–Newman–Keuls post hoc testing or Student's *t* testing was done, according to the context. The Newman–Keuls test has been routinely used in previous work by our group because of its medium power without being excessively conservative.

3. Results

3.1. Time Courses of the Effects of rDNA Glucagon Preparations

Figure 2 shows the time courses of the changes caused by Eli Lilly (a) and Novo (b) rDNA glucagon when infused into the portal vein in antegrade perfusion at a rate of 40 pmol min^{-1} (g liver wet weight)$^{-1}$. This infusion rate is largely saturating for the glucagon receptor, so no limitations occur at this level. The livers were from fed rats, and the perfusion medium (Krebs/Henseleit-bicarbonate buffer) contained no substrates. Under these conditions, the liver respires mainly at the expense of endogenous fatty acids and releases glucose and glycolysis products (lactate and pyruvate) derived from its glycogenolytic and glycolytic activities [21,22]. On the top of both graphs in Figure 2, a scheme informs in pictorial form that all cells along the sinusoidal bed were supplied with glucagon, which was introduced into the portal vein starting at a 10 min perfusion time. The immediate responses to glucagon infusion are increases in glucose output and oxygen consumption and a decrease in lactate + pyruvate release. The latter is the consequence of glycolysis inhibition [23]. All changes tended to reach new steady states during the

last minutes of the glucagon infusion. It is apparent that the responses to both types of glucagon can hardly be distinguished from each other, neither in terms of their intensities nor in the kinetics of their development over time.

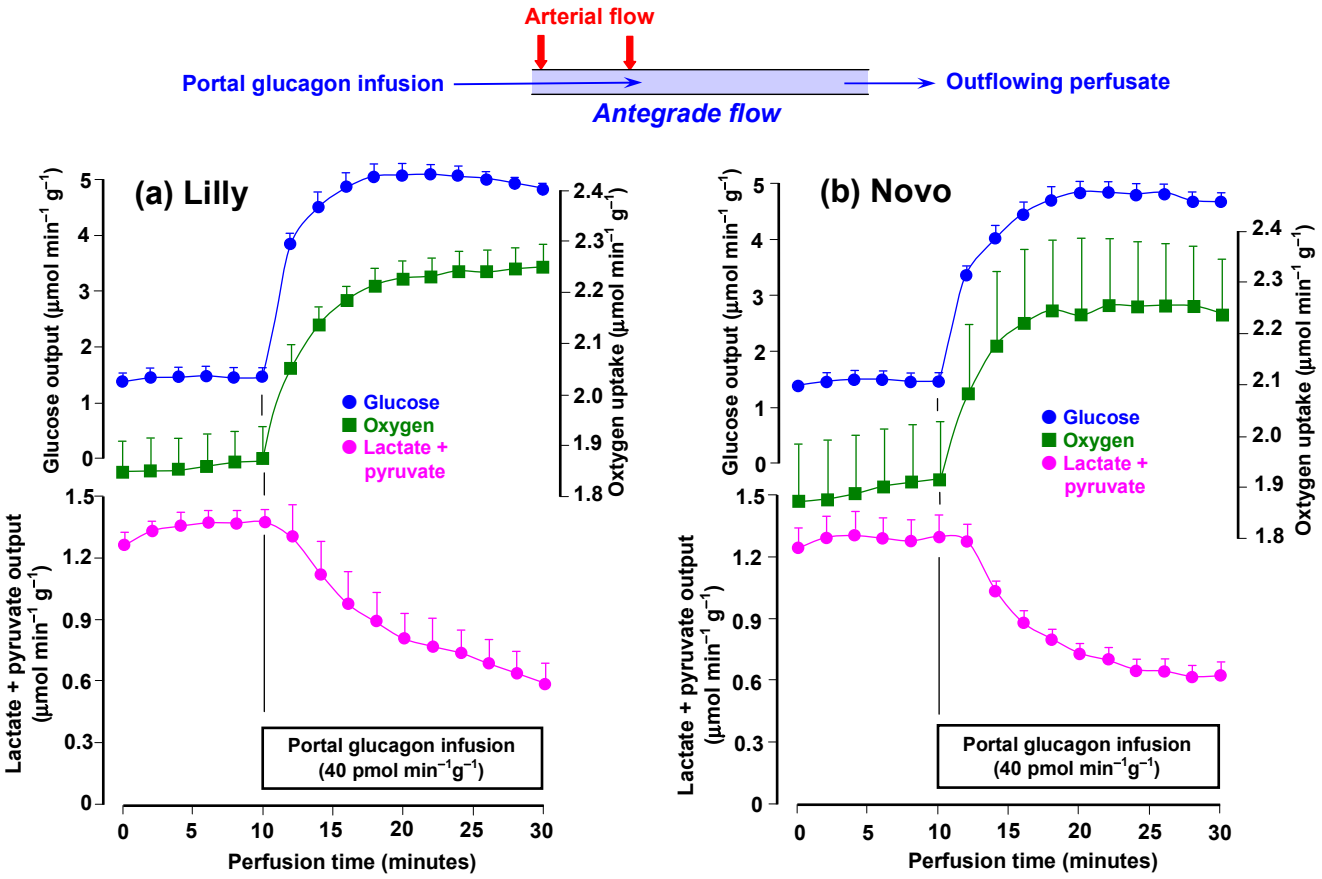

Figure 2. Time courses of the actions of Eli Lilly and Novo Nordisk rDNA glucagon on liver glycogen catabolism and oxygen consumption when infused into the portal vein in antegrade perfusion. Livers from fed rats were perfused bivascularly in the antegrade mode (portal vein + hepatic artery → hepatic vein). rDNA glucagon samples from Lilly (**a**) or Novo (**b**) were infused into the portal vein at the indicated times and infusion rates. Samples of the outflowing perfusate were taken for the measurement of glucose, lactate and pyruvate. Oxygen in the outflowing perfusate was measured polarographically. Data points are the means ± MSE of 5 liver perfusion experiments.

Figure 3 shows the time courses of the analogous experiments that were done with both types of glucagon when they were introduced into the hepatic artery under retrograde perfusion (i.e., in the direction of the hepatic vein → portal vein). The scheme at the top of the graphs informs us that only a fraction of the cells along the sinusoidal bed were supplied with glucagon; actually, only cells situated periportally. It is worth mentioning that changing the direction of perfusion did not produce significant modifications in the basal metabolic rates that were monitored (not shown). The introduction of both types of glucagon into the hepatic artery, however, did not elicit the same response. The introduction of Eli Lilly rDNA glucagon (a) produced changes that were in all cases comparable to those found in the experiments in which antegrade perfusion was done (compare to Figure 2a). This behavior of Eli Lilly rDNA glucagon is almost the same as that reported previously for Eli Lilly pancreatic glucagon [10]. The response to Novo rDNA glucagon of all parameters, however, was clearly reduced (compare to Figure 2b), irrespective of the parameter that was measured.

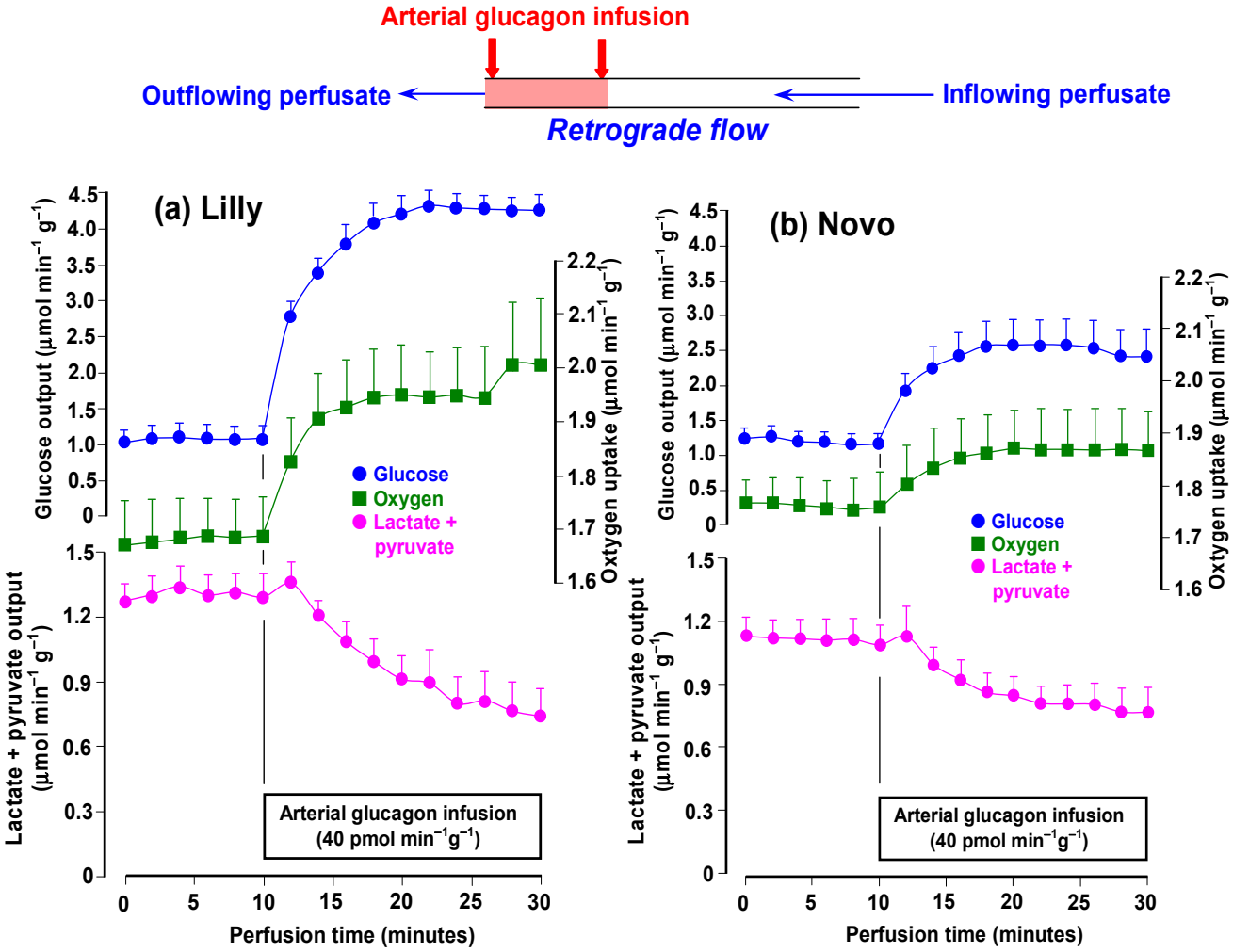

Figure 3. Time course of the actions of Eli Lilly and Novo Nordisk rDNA glucagon on liver glycogen catabolism and oxygen consumption when infused into the hepatic artery in retrograde perfusion. Livers from fed rats were perfused bivascularly in the retrograde mode (hepatic vein + hepatic artery → portal vein). rDNA glucagon samples from Lilly (**a**) or Novo (**b**) were infused into the hepatic artery at the indicated times and infusion rates. Samples of the outflowing perfusate were taken for the measurement of glucose, lactate and pyruvate. Oxygen in the outflowing perfusate was measured polarographically. Data points are the means ± MSE of 4 (**a**) or 5 (**b**) liver perfusion experiments.

3.2. Cell Space-Normalized Changes of the rDNA Glucagon Preparations

A useful analysis of the modifications caused by both types of glucagon would be proportioned by a normalization of the changes in terms of the cells that were effectively supplied with the hormone in the two experimental protocols that were employed. The basal rates represent the metabolism of all cells in the sinusoidal bed, irrespective of the perfusion direction and the subsequent route of glucagon infusion. However, the changes caused by glucagon should, at least in principle, be restricted to the cells that are effectively supplied. If this is so, the modifications caused by the different types of glucagon preparations in the experiments shown in Figures 2 and 3 should be proportional to the cell volumes that were supplied in each case. These cell volumes have already been determined using multiple-indicator dilution experiments in which [^{14}C]sucrose and [^{3}H]water were used as indicators of the extracellular and total aqueous spaces, the cellular space being given by the difference between the total and extracellular spaces [3]. In the perfused liver of fed rats, 0.68 mL of cells per gram of liver can be reached via the portal vein in antegrade perfusion and 0.27 mL/g via the hepatic artery in retrograde perfusion. Using these previously determined parameters and the changes caused by glucagon in the

experiments shown in Figures 2 and 3, normalized values were calculated and represented by bars in Figure 4. For comparison purposes, Figure 4 also shows the normalized changes caused by bovine glucagon that were obtained in our laboratory in a previous work where the corresponding figures were displayed in tabular form [10]. Figure 4a reveals that the increases in glucose output per mL of accessible cell space caused by all three preparations when they are introduced into the portal vein in antegrade perfusion (green bars) are practically the same. An almost identical value was also found for Novo rDNA glucagon infused into the hepatic artery (orange bar) in retrograde perfusion. This means that with Novo rDNA glucagon, the effects were proportional to the accessible cell spaces in both perfusion modes. A completely different picture emerges, however, when the same analysis is applied to both pancreatic and rDNA glucagon from Eli Lilly: an almost tripled response was observed in both cases, meaning a strong lack of proportionality to the cell space that was accessible via the microcirculation. Almost the same pattern was found for the increase in oxygen uptake (Figure 4b) caused by the various glucagon preparations: proportionality to the accessible space for Novo rDNA and no proportionality in the case of the other two glucagon preparations. The difference is a somewhat more pronounced response of oxygen uptake to pancreatic glucagon when the latter is infused into the portal vein. Glycolysis, given as lactate + pyruvate production (Figure 4c), showed a similar pattern, although the differences were generally less pronounced and the data showed a somewhat enhanced statistical dispersion.

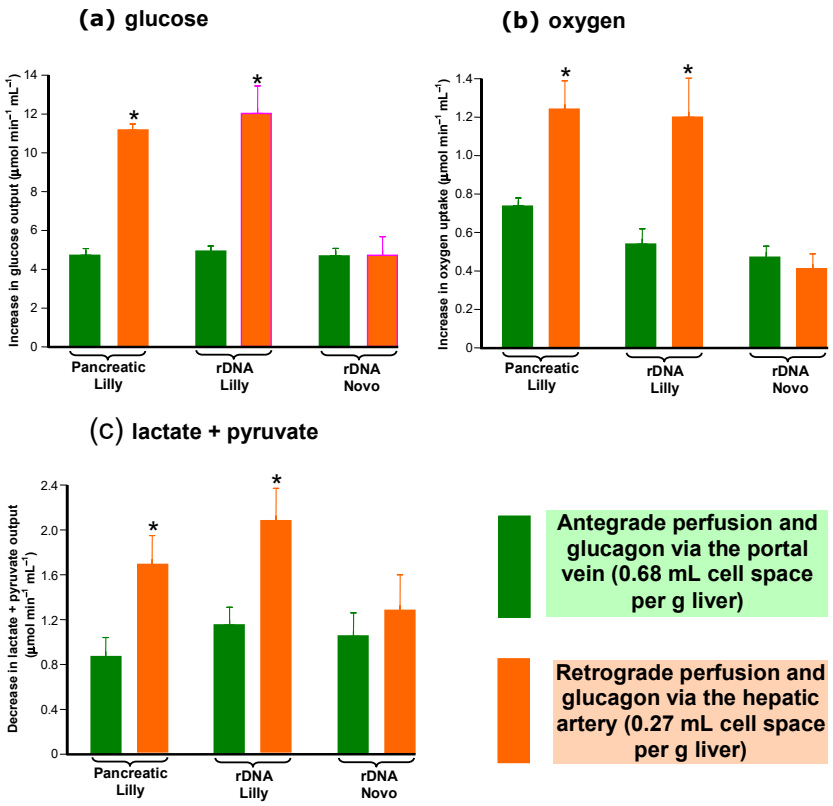

Figure 4. Normalized changes in glucose output (**a**), oxygen uptake (**b**) and lactate + pyruvate output (**c**), expressed as µmol min^{-1} mL^{-1}, in perfused livers caused by three different glucagon preparations. The perfusion mode and the route of glucagon infusion are indicated, as is the type of glucagon preparation that was infused. The data on Eli Lilly rDNA glucagon and Novo rDNA glucagon were computed from the experiments shown in Figures 2 and 3. Normalization was done by dividing the changes in the metabolic fluxes, expressed as µmol min^{-1} $(g\ liver)^{-1}$, by the corresponding accessible cell spaces in each perfusion mode given in the inset next to (**c**) [3]. The data on pancreatic glucagon were obtained from Constantin et al. [10]. Asterisks indicate statistical significance with respect to the data obtained in antegrade perfusion.

3.3. HPLC Chromatography of the Glucagon Preparations

The differences in response revealed by Figure 4 deserve, at least as a preliminary approach, a chromatographic analysis of the three glucagon preparations. The main purpose of the chromatographic analysis was to verify if the amounts of glucagon in each preparation were comparable and if each of them presented distinct or similar chromatographic patterns. The HPLC eluate was monitored spectrophotometrically at two different wavelengths, 214 and 280 nm. The latter was selected because glucagon, due to its aromatic amino acids, is known to absorb at this wavelength [9,24]. Monitoring at 280 nm should also allow for the detection of fragments that possess these amino acids in their structures. The peptide linkage can be detected at 214 nm as well as lactose (or lactose adjuncts), which was added as an excipient and stabilizer by the suppliers of the three glucagon preparations. The amounts of glucagon applied to the columns were always the same (40 µg), but the various preparations contained different amounts of lactose. Figure 5 shows typical chromatograms, and Table 1 details the overall analysis of the most prominent peaks detected at 280 nm and common to the three preparations. The scale in Figure 5 was adjusted to the peak value detected at 214 nm. Lactose always appears as the first 214 nm-absorbing peak. Additionally, there were several 214 nm absorbing peaks, several of them coinciding with the peaks detected at 280 nm. Eli Lilly rDNA glucagon was the preparation that presented the greatest number of impurities, absorbing at 214 nm. Figure 5 shows the tracings obtained up to 40 min after the sample injection. Prolongation of the elution for one hour did not reveal further components absorbing at 214 or 280 nm. Table 1 reveals that all three preparations had a major 280 nm peak with a retention time of around 32 min (peak 5), which is most certainly the peptide glucagon [9,24]. In relative terms, it was more abundant in pancreatic glucagon, whereas Eli Lilly and Novo rDNA presented similar relative abundances. The second most prominent 280 nm peak (peak 4) had a retention time slightly above 25 min. It was fairly abundant in pancreatic and Eli Lilly rDNA glucagon, but much less so in Novo rDNA. The contributions of peaks two and three were relatively modest for all preparations. Peak one, however, was the second most prominent 280 nm peak in Novo rDNA glucagon (Figure 5c). Its retention time coincided with that of a 214 nm peak whose height was more than half of the peak of free lactose.

Table 1. Chromatographic analysis of three glucagon preparations. The chromatographic procedures (HPLC) are described in the Section 2. All values are the means ± mean standard errors of three chromatographic separations. Detection was done at 280 nm. Values labeled with the same superscript letters in each line are statistically different according to Student–Newman–Keuls post hoc testing ($p < 0.05$).

Peaks at 280 nm	Pancreatic Glucagon Elli Lilly		rDNA Glucagon Elli Lilly		rDNA Glucagon Novo	
	R_f (min)	Relative Area (%)	R_f (min)	Relative Area (%)	R_f (min)	Relative Area (%)
1	4.02 ± 0.01	1.12 ± 0.08 [a]	-	-	3.98 ± 0.02	15.08 ± 0.12 [a]
2	4.52 ± 0.01	1.29 ± 0.01 [a]	4.50 ± 0.01	4.03 ± 0.90 [a]	4.49 ± 0.01	2.49 ± 0.38
3	10.0 ± 0.03	0.56 ± 0.08 [a]	10.10 ± 0.03	4.02 ± 0.43 [a]	-	-
4	25.34 ± 0.03	12.33 ± 0.11 [a,b]	25.54 ± 0.19	9.04 ± 0.29 [a,c]	25.26 ± 0.05	2.36 ± 0.25 [b,c]
5	32.09 ± 0.08	83.42 ± 0.11 [a,b]	32.15 ± 0.14	78.14 ± 2.44 [a]	32.02 ± 0.04	77.15 ± 0.05 [b]

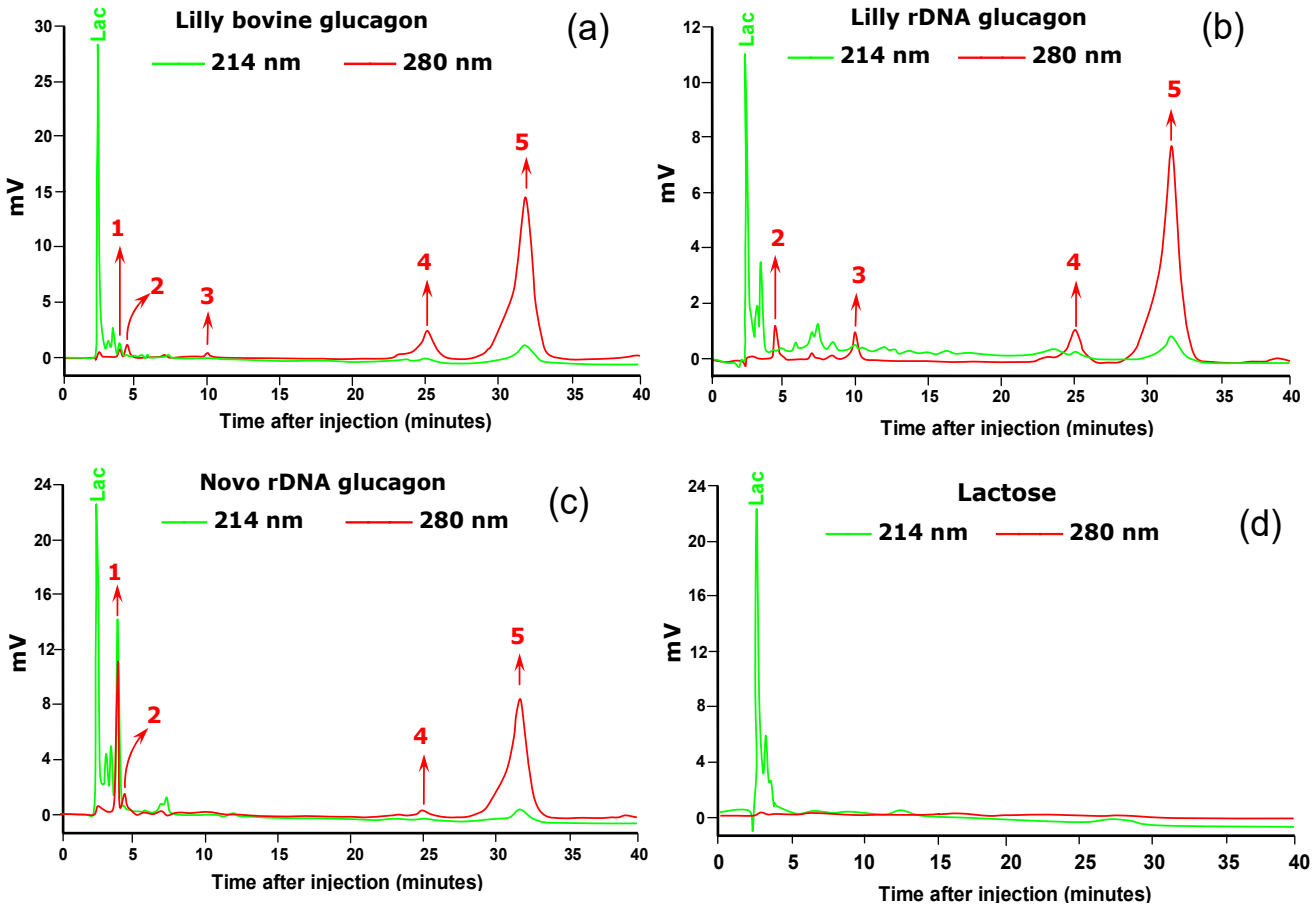

Figure 5. (**a**–**d**) Typical chromatograms of three commercial glucagon preparations. The samples that were injected (20 µL) contained 40 µg of glucagon, calculated according to the supplier's specifications (Eli Lilly and Company, Indianapolis, IN, USA; Novo Nordisk A/S, Bagsvaerd, Denmark). The spectrophotometric detector was put into dual-wavelength mode (214 and 280 nm). The peaks at 280 are numbered and their relative areas are given in Table 1 in addition to the mean retention times.

4. Discussion

The lack of proportion between the accessible cell spaces and the metabolic action of pancreatic glucagon observed in earlier experiments [10] was again reproduced, this time with rDNA glucagon synthesized in a genetically modified non-pathogenic *Escherichia coli* strain [14], manufactured by Eli Lilly Co. The rDNA glucagon synthesized in a modified strain of *Saccharomyces cerevisiae* and commercialized by Novo Nordisk, on the contrary, showed effects that were in all cases proportional to the cell spaces that are accessible via the microcirculation in two different perfusion modes and infusion routes. The results obtained earlier with pancreatic glucagon [10] and in the present work with *Escherichia coli* rDNA glucagon are certainly puzzling and difficult to interpret. They cannot be simply dismissed as an artifact, however, because (1) exactly the same perfusion system was used in all experiments, (2) the samples were all solubilized and stored in the same way, (3) three metabolic parameters were measured with corroborating results and (4) similar results were obtained in the same experimental system with the cAMP analogs *N*,2′-*O*-dibutyryl cAMP and *N*-monobutyryl cAMP [12]. On the other hand, since all preparations obviously contain the same active peptide known as glucagon in high proportion, it is extremely unlikely that the actions observed with the pancreatic and the *E. coli*-rDNA glucagon preparations, which seem to go beyond the accessible space, are caused by this peptide itself, as originally suggested [10]. An alternative hypothesis would be that this particular phenomenon, which possibly occurs in addition to the adenylate cyclase activation in the

cells directly supplied with glucagon, could be caused by another substance, present as an impurity in both the pancreatic and *E. coli*-rDNA glucagon, which is absent or not present at the required amount in the *S. cerevisiae* glucagon manufactured by Novo Nordisk.

It is long known that glucagon preparations, used in both research and clinics, contain impurities to various degrees. These impurities may be, for example, peptide fragments and lactose adducts that are formed during storage [24–27]. As shown by a recent study, these impurities may vary considerably depending on the storage conditions and the origin of the glucagon sample [28]. According to the latter study, synthetic glucagon had an average purity of 92.8–99.3%, whereas recombinant glucagon had an average purity of 70.3–91.7%. Our comparative chromatographic analyses of the samples used in the perfusion experiments suggested purities between 77 and 83% when monitoring was done at 280 nm. Perhaps, more importantly, is the fact that, chromatographically, both Eli Lilly products presented more similarities, differing in several aspects from Novo rDNA glucagon. The main difference is the very low level of peak 4 in the latter when compared to the other two. This contaminant has already been observed previously [24] in glucagon samples purified from biological material but not in synthetic glucagon. Its absorbance at 280 nm suggests that it could be a peptide containing aromatic amino acid side chains. Another significant difference, although it is unlikely to be related to the main phenomenon observed in this work, is the presence of peak 1 in the Novo rDNA glucagon sample. This peak also absorbs very strongly at 214 and 280 nm, suggesting that it might be a lactose adduct. The latter has been demonstrated to appear in various forms in several glucagon preparations stored under several conditions [26]. The chromatographic differences detected in the present work do not allow us to infer the molecular species that could be involved in the different responses. However, they could be a starting point for future investigations on the subject.

With the available data, it is very difficult to devise the mechanism underlying the phenomenon described herein. Zonation of the metabolic modifications induced by the putative contaminant in the periportal region can be excluded. In the case of this interpretation, the magnitude of the modifications that were observed in retrograde perfusion would imply that even the actions found when all cells were supplied (portal vein infusion) were totally restricted to the small cell space that is accessible via the hepatic artery in retrograde perfusion. This is unlikely and would be, in fact, extremely coincidental. Furthermore, experiments in which the cytochrome c oxidase of the cells in the accessible periportal region was blocked by cyanide were not impeditive for oxygen uptake stimulation when Eli Lilly pancreatic glucagon was supplied via circulation to the same cells [10]. This suggests that the metabolic effects did not occur solely in the cells that were directly supplied with the various components of the pancreatic [10] and *Escherichia coli* rDNA glucagon preparations but also in cells that are situated retrogradely, as illustrated by Figure 6. It should be noted that Figure 6 attributes the retrograde action of the *Escherichia coli* rDNA glucagon to the impurities in this preparation and not to the 29-amino acid peptide itself, for the reasons already mentioned above. A possible mechanism underlying the phenomenon could be a kind of cell-to-cell communication, as suggested earlier [10]. Although Figure 6 illustrates retrograde perfusion with glucagon infusion into the hepatic artery, which is the way by which the phenomenon can be experimentally demonstrated, there is a priori no reason for the mechanism not being operative during antegrade perfusion and glucagon entry via the portal vein. Crucial for the mechanism displayed in Figure 6 is the question of whether cell-to-cell communication in the liver parenchyma has already been described. In this respect, it has been shown that cell-to-cell communications can in fact occur in the liver parenchyma via gap junctions. These structures are composed of connexins and have been proposed to propagate signals from periportal to perivenous hepatocytes generated by electrical stimulation of liver nerves [29]. There are indeed at least two reports showing that commercial glucagon preparations and cAMP analogs can increase gap junctional intercellular communication [11,30]. Unfortunately, none of the authors of these studies were informed about the source of the hormone that was used in their experiments. How-

ever, the results were perfectly reproduced and even exceeded by 8-Br-cAMP [30] and N^6,2′-*O*-dibutyryl cAMP [11]. It is perhaps significant that the latter cAMP analogs were shown to elicit the same response as Eli Lilly rDNA glucagon when supplied to the liver according to the protocol illustrated by Figure 3 [12], an indication that these analogs may be involved in cellular activities that extrapolate their mere role as cAMP sources.

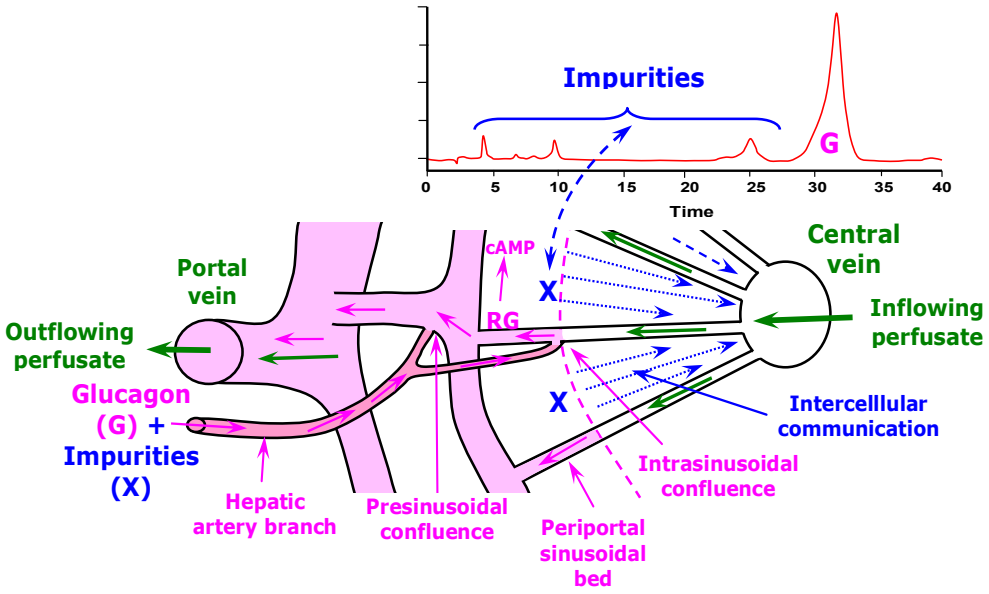

Figure 6. Mechanistic interpretation of the amplified actions of *E. coli*-rDNA glucagon from Eli Lilly and its impurities in the liver. G represents glucagon, R is the glucagon receptor and X the impurities.

As stated above, the differential effects of the two glucagon preparations investigated in the present work are probably due to the presence of impurities. When impurities are detected in medicinal and therapeutic preparations, there is an immediate questioning about their undesirable consequences. For the contaminating peptides in glucagon preparations detected previously, for example, concerns have been raised about the possibility of adverse immunological reactions [27,28]. Concerns of this kind seem not to apply to the observations of the present work for the simple reason that no new effects on the metabolism have been found. Glycogenolysis, oxygen uptake stimulation and glycolysis inhibition are normally observed with any glucagon preparation [21,23], and the fact that impurities may extend this effect to a greater number of cells can be regarded, in principle at least, as a positive event in clinical terms. Even so, the possibility of adverse consequences cannot be ruled out with absolute certainty, and studies in this respect are certainly welcome. On the other hand, the fact that the present study was performed using a preparation in which the microcirculation, the relationships between cells, the polarity of the cells, and other features are well preserved is certainly a positive aspect that gives credibility to the proposed cell-to-cell propagation of metabolic signals in the liver. Nonetheless, one should not ignore the fact that studies of this kind are only possible in the livers of animals, rats (*Rattus novergicus*) in the present case. Translation to humans of any observation of this kind is evidently always somewhat uncertain and requires a difficult experimental approach. In this respect, confirmation with other animal species is desirable insofar as this can increase the likelihood of its significance for the human liver.

Glucagon has been regarded for many years mainly as an insulin antagonic hormone in as much as several types of its preparations have been used to treat insulin-induced hypoglycemic episodes. More recent investigations, however, indicate that its action encompasses a much more ample set of physiological roles, which may have clinical significance [31–33]. With reference to the latter, there is presently intense research being done using various glucagon agonists, which are in most cases chemically modified peptides [34].

The strategy behind these studies is to obtain greater peptide solubility, stability and circulating half-life in addition to gaining insight into structure-function relationships [35]. The glucagon preparations used in the present work are likely to be contaminated by peptides of diverse natures, whose isolation and identification may prove to be a difficult and laborious task. An alternative starting point for new experiments would be to investigate the selective response of the periportal cells to glucagon agonists [34] in the search for an amplified response similar to that found for both the pancreatic and *E. coli*-rDNA glucagon preparations.

5. Conclusions

It is long known that glucagon preparations in general contain impurities of various kinds. However, to our knowledge, this is the first time that evidence has been brought to light that impurities can affect metabolic fluxes. The simplest explanation for the observations of the present work, in combination with the results of previous studies, is that a contaminating by-product in certain commercial glucagon preparations, or cAMP derivatives with high penetration power, can enhance cell-to-cell propagation of the glucagon signal, possibly via gap junctional communication. At the present stage, the interpretation given to the phenomenon should be regarded as a working hypothesis. However, the search for an explanation may be a rewarding enterprise if it deciphers hitherto unknown mechanisms of hepatic metabolism regulation. Finally, the results of past or future experiments with cellular or in vivo systems to which a given glucagon preparation was supplied should always be interpreted with care, considering the possibility that the effects were not necessarily caused solely by a single peptide.

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