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The Effect of Biguanides on Secretion and Biosynthesis of Insulin in Isolated Pancreatic Islets of Rats

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Summary. Based on the clinical observation that biguanide treatment of obese patients may alter insulin levels, the influence of metformin and phenformin on basal and glucose stimulated insulin secretion, as well as on insulin biosynthesis, was studied in isolated islets of rats. — Biguanide concentrations of 100 μg/ml, or higher, significantly reduced glucose stimulated insulin secretion. Both dose dependence and a difference in the intrinsic activities of metformin and phenformin were demonstrated. Incubating the same islets for a second period without biguanides, glucose stimulated insulin secretion was still decreased. Addition of glibenclamide during this second period increased insulin secretion, but did not overcome complete inhibition achieved after incubation at very high biguanide concentrations. Glucose stimulated biosynthesis of proinsulin and insulin was decreased in the presence of biguanides and completely suppressed at very high concentrations. Inhibition of cell respiration in the islet cells effected by high biguanide doses may be the reason for the inhibition of secretion and biosynthesis of insulin. — On the other hand, an insulin release was found at the highest phenformin concentration of 10 mg/ml and during perfusion of the isolated rat pancreas with higher biguanide doses. — Biguanide concentrations found to be effective in this study are very high compared with therapeutic levels. Moreover, biguanide actions are known to be highly dependent on species, concentration and metabolic situation. — Definite conclusions from these findings regarding clinical significance, therefore, seem unwarranted.

Effet des biguanides sur la sécrétion et la biosynthèse de l'insuline dans les îlots pancréatiques isolés de rats

Résumé. Partant de l'observation clinique selon laquelle le traitement biguanidique des obèses peut altérer les taux réactifs d'insuline, les auteurs ont étudié l'influence de la metformine et de la phenformine sur la sécrétion basale d'insuline et sur la sécrétion stimulée par le glucose, ainsi que sur la biosynthèse de l'insuline dans les îlots isolés de rats. — Des concentrations de biguanide de 100 μg/ml ou plus réduisaient ou inhibaient significativement la sécrétion d'insuline stimulée par le glucose. Un effet proportionnel à la dose et une différence dans l'activité intrinsèque de la metformine et de la phenformine ont été démontrés. Lors de l'incubation des mêmes îlots pendant une seconde période sans biguanide, la sécrétion d'insuline stimulée par le glucose s'est avérée diminuée également après préincubation avec les biguanides. L'addition de glibenclamide au cours de cette seconde période augmentait la sécrétion d'insuline, mais ne surmontait pas l'inhibition complète provoquée après incubation dans des concentrations très élevées de biguanides. La biosynthèse de la proinsuline et de l'insuline stimulée par le glucose était diminuée en présence de biguanides ou, pour de très fortes concentrations, complètement supprimée. L'inhibition de la respiration cellulaire dans les cellules des îlots, provoquée par de fortes doses de biguanides est considérée comme la cause de l'inhibition de la sécrétion et de la biosynthèse de l'insuline. — D'autre part, une libération d'insuline a été trouvée pour une concentration très élevée de phenformine de 10 mg/ml. Une libération d'insuline, décrite dans la littérature, qui se produit au cours d'une perfusion du pancréas isolé du rat avec des doses plus élevées de biguanides, a également été observée. — Les concentrations de biguanide qui se sont révélées efficaces dans cette étude sont très élevées par rapport aux taux thérapeutiques. En outre, on sait que l'action des biguanides dépend de l'espèce, de la concentration et de la situation métabolique. — D'après ces résultats, des conclusions définitives en ce qui concerne la signification clinique ne semblent donc pas justifiées.

Die Wirkung der Biguanide auf Sekretion und Biosynthese des Insulins in isolierten Pankreas-Inseln von Ratten

Zusammenfassung. Ausgehend von der klinischen Beobachtung, daß bei übergewichtigen Patienten die reaktiven Insulinspiegel durch Biguanidbehandlung veränderbar sind, wurde an isolierten Langerhansschen Inseln von Ratten der Einfluß von Metformin und Phenformin auf die basale und glucosestimulierte Insulinsekretion sowie auf die Insulinbiosynthese untersucht. — Biguanidkonzentrationen von 100 μg/ml aufwärts erniedrigten bzw. hemmten die glucosestimulierte Insulinsekretion isolierter Inseln, wobei sowohl eine Dosis-Wirkungsbeziehung als auch Unterschiede in der intrinsischen Aktivität von Metformin und Phenformin nachweisbar waren. Wurden dieselben Inseln in einer 2. Periode ohne Biguanidzusatz inkubiert, so war die glucosestimulierte Insulinsekretion auch nach der Vorinkubation mit Biguaniden erniedrigt. Zusatz von Glibenclamide in dieser 2. Inkubationsperiode steigerte die Insulinsekretion, konnte jedoch nicht die komplette Hemmung durchbrechen, die nach Vorinkubation der Inseln mit sehr hohen Biguaniddosen erzielt wurde. — Die glucosestimulierte Biosynthese von Proinsulin und Insulin wurde durch Biguanide in höherer Konzentration reduziert, bei sehr hoher Konzentration völlig unterdrückt. Eine Atmungshemmung der Inselzellen durch hohe Biguaniddosen könnte zur Erklärung der Hemmung von Sekretion und Biosynthese von Insulin herangezogen werden. — Bei der extrem hohen Phenforminkonzentration von 10 mg/ml wurde andererseits eine Insulinfreisetzung aus den isolierten Inseln beobachtet. Die in der Literatur beschriebene Insulinfreisetzung während der Perfusion des isolierten Rattenpankreas mit höheren Biguaniddosen wurde ebenfalls gefunden. Die Biguanidkonzentrationen, die sich in dieser Arbeit als wirksam erwiesen, liegen im Vergleich zu therapeutisch erreichbaren Spiegeln sehr hoch. Weiters ist aber auch die Abhängigkeit der Biguanidwirkung von Species, Konzen-

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tration und Stoffwechselausgangslage bekannt. Es sollten daher aus diesen Befunden keine definitiven Schlüsse hinsichtlich ihrer klinischen Bedeutung gezogen werden.

Key words: Biguanides, glibenclamide, insulin secretion, insulin biosynthesis, isolated pancreatic islets, isolated perfused pancreas.

The site and mode of action of biguanides are still a matter for discussion although these drugs have been widely used in the management of diabetes mellitus. Whereas a large number of reports deal with the influence of biguanides on liver (Altschuld and Kruger, 1968; Willms, Moshagen and Söling, 1968), the gastrointestinal tract (Czyzyk, Lawecki, Sadowski, Ponikowska and Szczepanik, 1968; Kruger, Altschuld and Hollobaugh, 1970), adipose tissue (Ditschuneit, Rott and Faulhaber, 1968; Söling, Zahlten, Böttcher and Willms, 1967) and muscle tissue (Butterfield, 1968), only little attention has been given to the possibility of direct action by biguanides on the endocrine pancreas as such. Using extracorporal perfusion of the dog pancreas, no influence of biguanides on insulin secretion was found by Mehnert, Schäfer, Kaliampetsos, Stuhlfauth and Engelhardt (1962). On the other hand, Grodsky, Karam, Pavlatos and Forsham (1963) were the first to report a decrease of elevated serum insulin levels in obese patients under biguanide treatment. Using the transumbilical venous catheter and an intraduodenal glucose infusion, Berger and Künzli (1969) demonstrated less increase of both insulin and glucose levels in portal vein and peripheral venous blood after biguanide application. According to these authors, the smaller increase of insulin might have been a consequence of lower blood sugar levels. Our previous studies in obese patients treated with biguanides resulted in most cases in a reduction of elevated insulin levels following intravenous glucose injection (Schatz, Doci and Höfer, 1970). However, from our data it might be assumed that this reduction of insulin levels under biguanide medication was not in all cases due to an increased peripheral glucose uptake, and thus a diminished secretory stimulus to the pancreas alone. Therefore, we investigated the influence of biguanides on secretion and biosynthesis of insulin in isolated pancreatic islets of rats. For the purpose of investigating the nature of inhibition of glucose stimulated insulin secretion found in the presence of higher biguanide doses, glibenclamide, as a recognized β-cytotrophic agent, was also used.

Material and Methods

Male rats of the strain FW 49 Lemgo-Kirchb.-Bib. weighing 200 g were used. The animals had free access to food (rat pellets Altromin®, Altrogge, Lippe, Germany) and water. Isolated pancreatic islets were obtained by digestion of the pancreas with collagenase (Serva, Heidelberg) according to Lacy and Kostianovsky (1967).

Insulin secretion: Batches of 5 isolated islets each were incubated in 1 ml Krebs-Ringer-bicarbonate buffer, pH 7.4, supplemented with bovine serum albumin (5 mg/ml) and a protease inhibitor (Transylol®, Bayer, 1000 KIU/ml). The samples were kept under a constant gas phase of O_2:CO_2 (95:5%, v/v), and a metabolic shaker was used at a temperature of 37°C. The islets were incubated for two periods of 60 min each:

During the first incubation period (*period I*) the medium contained 50 or 300 mg% glucose and metformin (N,N-dimethyl-biguanide. HCl, obtained from Laboratoires Aron, France) or phenformin (phenethylbiguanide, obtained from Farbwerke Hoechst AG, Germany). Biguanide concentrations ranged from 0.1 µg/ml to 10 mg/ml.

After washing, the islets were incubated for a second period (*period II*) at a glucose concentration of 300 mg% without biguanide. During this second period glibenclamide (HB 419, obtained from Farbwerke Hoechst AG, Germany) was added to one half of the batches. Glibenclamide concentration was 2.5 µg/ml (in some experiments 10 µg/ml).

After the first and second hour samples of the incubation medium were taken for determination of the immunologically measurable insulin (Melani, Ditschuneit, Bartelt, Friedrich and Pfeiffer, 1965).

Insulin biosynthesis: Batches of 25 islets were incubated in 1 ml of Krebs-Ringer-bicarbonate buffer, pH 7.4, supplemented with 17 naturally occurring amino acids (20 µg/ml of each amino acid, leucine excluded), bovine serum albumin (2 mg/ml), a protease inhibitor (1000 KIU/ml Trasylol®, Bayer) and 50 µCi ^{3}H-L-leucine (19 Ci/mmol, Amersham). Glucose was present at a concentration of 300 mg%. The concentrations of metformin in the incubation media were 1 and 10 mg/ml, those of phenformin 0.1 and 1 mg/ml. Incubations were carried out under a constant gas phase of O_2:CO_2 (95:5%, v/v) for 4 h, using a metabolic shaker at 37°C.

After deep freezing and thawing, followed by ultrasonic disintegration, the islet proteins were precipitated with ice-cold trichloroacetic acid at a final concentration of 10%. The precipitate was dissolved in 0.5 ml acetic acid (1 M) and separated on a calibrated Sephadex-G-50-fine column, 1.2 × 55 cm, equilibrated and eluted with 1 M acetic acid. Aliquots of the 1 ml fractions were assayed for radioactivity in a liquid scintillation counter. Immunologically measurable insulin was determined in each fraction. The radioactivity peaks were further identified by polyacrylamide gel electrophoresis and rechromatography after trypsination (Schatz, Abdel Rahman, Hinz, Fehm, Nierle and Pfeiffer, 1972). Following treatment of peak P (see Fig. 4) with trypsin, most of the radioactivity was eluted from the Sephadex column in the position of insulin (peak I).

Since the stereochemical structure of biguanides endows them with pronounced chelating properties which is also the case for calcium (Sterne, 1969), the following determinations[1] were carried out in the incubation media, containing albumin and all tested concentrations of metformin and phenformin: Total calcium by flame photometry, titrable calcium by titration with Na_2EDTA and competitive calcium binding using Chelex 100 (Bio-Rad, München) and radioactive ^{45}Ca., pH was determined in all incubation media.

Results

Insulin secretion: During the first incubation period (Fig. 1) metformin in concentrations of up to 1 mg/ml

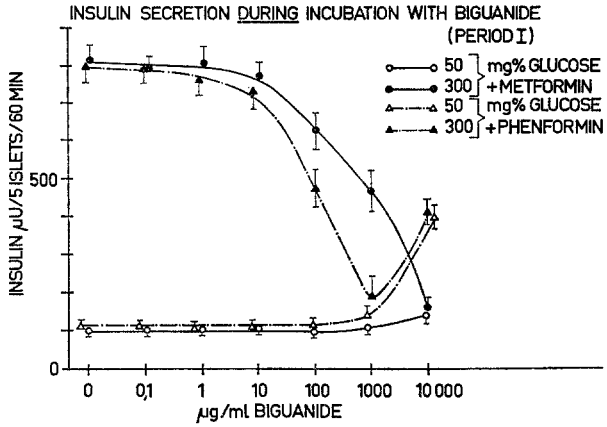

Fig. 1. Insulin secretion from isolated pancreatic islets of rats incubated for one hour (period I) in the presence of different concentrations of metformin and phenformin. $\bar{x} \pm SEM$ ($n = 14 - 22$) Inhibition of glucose-stimulated insulin secretion is significant at biguanide concentrations of 100 µg/ml and higher

ml and being almost completely suppressed at concentrations of 10 mg/ml. With 100 µg/ml phenformin, glucose stimulated insulin secretion was decreased to a significantly greater extent than with the same concentration of metformin. Maximum inhibition occurred with 1 mg/ml phenformin, whereas 10 mg/ml led to significantly elevated insulin levels in the incubation media as compared with the values obtained with 1 mg/ml.

During the second incubation period (Fig. 2 and 3) all islets were incubated in fresh medium containing 300 mg% glucose without biguanides. Decrease of glucose stimulated insulin secretion was in parallel to that found during the first period, regardless of the glucose concentration used in the first period. Addition of glibenclamide led to further stimulation of insulin secretion in those islets which showed a reduced insulin output following incubation with biguanides. However, it did not overcome the maximum inhibition of glucose stimulated insulin secretion after exposure

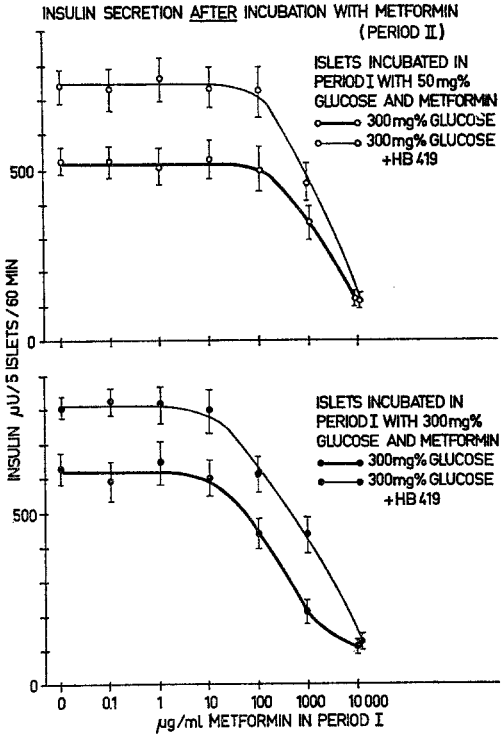

Fig. 2

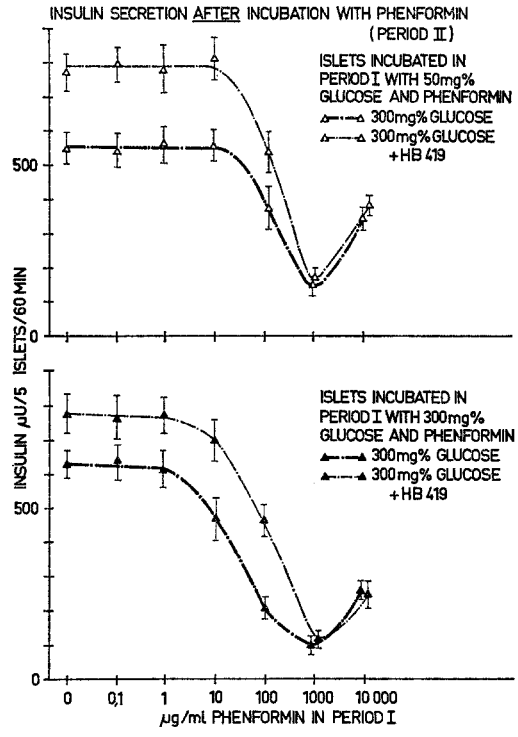

Fig. 3

Fig. 2 and 3. Insulin secretion from identical islets as in Fig. 1 during a second hour of incubation (period II): After washing, all batches of islets were incubated in fresh medium at a glucose concentration of 300 mg% without biguanide. To one half of the batches glibenclamide (Hb 419, 2.5 µg/ml) was added

did not influence basal insulin secretion occurring at 50 mg% glucose; with 10 mg/ml metformin, a slight elevation of insulin levels in the incubation medium was observed. With phenformin, this elevation was found already at a concentration of 1 mg/ml and was highly significant at 10 mg/ml. Insulin secretion stimulated with 300 mg% glucose was significantly reduced by metformin starting from concentrations of 100 µg/ of the islets to very high concentrations of biguanide. Insulin levels observed at phenformin concentrations of 10 mg/ml were not altered by glibenclamide.

Insulin biosynthesis: Fig. 4 shows the incorporation pattern of ^{3}H-leucine into the proinsulin and insulin fraction of islet cells. Metformin at a concentration of

1 We wish to thank Dr. Minne, Dr. Piazolo and Dr. Ziegler, Ulm, for carrying out the calcium investigations.

1 mg/ml reduced the incorporation of ^{3}H-leucine into all protein fractions. Relatively more radioactivity was found in the insulin than in the proinsulin fraction as compared with 300 mg% glucose alone. 10 mg/ml metformin completely abolished the incorporation of radioactive leucine into proinsulin and insulin. With phenformin almost identical patterns were observed with concentrations of 0.1 and 1 mg/ml, respectively.

Both titrable calcium levels and calcium bound to Chelex 100 as well as the pH remained unchanged after the addition of biguanides to the incubation media even at the highest concentrations of 10 mg/ml.

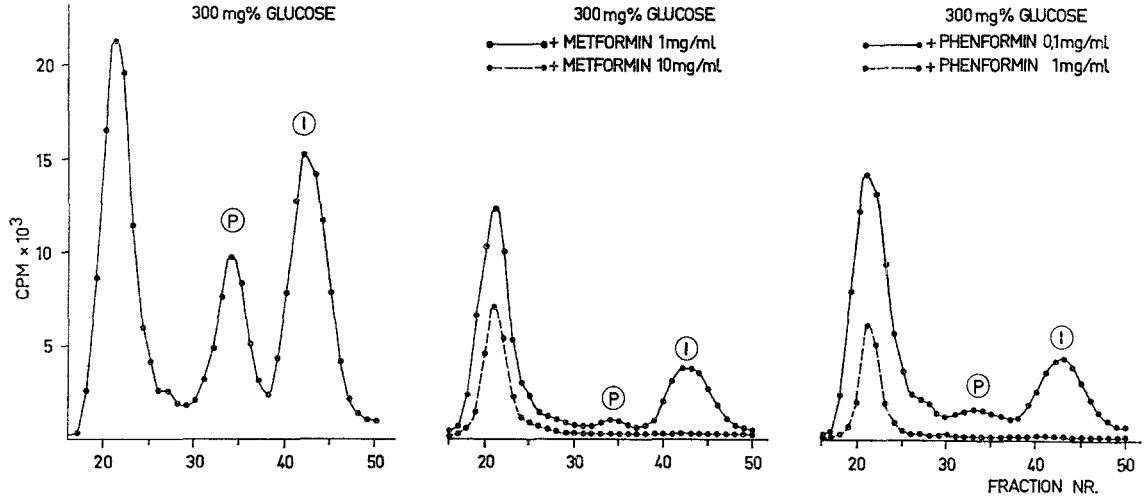

Fig. 4. Incorporation of ^{3}H-leucine into islet proteins during 4 h of incubation. Separation on a Sephadex-G-50 fine column, 1.2 × 55 cm, 1 ml fractions. The first peak corresponds to islet proteins excluded from Sephadex-G-50. P = proinsulin, I = insulin

Discussion

Our results demonstrate a dose related inhibition of glucose stimulated insulin secretion from isolated pancreatic rat islets effected by metformin and phenformin. This inhibition was observed in the presence of the drugs as well as after preincubation. However, at extremely high biguanide concentrations, especially with phenformin at 10 mg/ml, a rise of insulin levels in the incubation media was observed, essentially independent of the glucose concentration or the presence or absence of glibenclamide.

Large doses of phenformin have been shown to inhibit cell respiration (Steiner and Williams, 1958). On the other hand, incubation of pancreatic tissue in an atmosphere of nitrogen or under aerobic conditions in the presence of 2.4,-dinitrophenol or cyanide, abolishes the stimulatory effect of glucose on insulin secretion (Malaisse, Malaisse-Lagae and Wright, 1967). Thus, inhibition of glucose stimulated insulin secretion by large doses of phenformin might be a consequence of an inhibition of islet cell respiration. On the other hand, insulin release observed during or after incubation of the islets with excessive amounts of phenformin might be attributed to a leakage of insulin from the islet cells damaged by the high concentration of the drug. Concerning metformin, however, some evidence against this hypothesis might be seen in the findings reported by Meyer in 1960. Differences between effective doses of metformin and phenformin which we observed reflect the well known differences in intrinsic activities of these drugs (Beckmann, 1969).

The action of biguanides on the pentose shunt in the islet cells has to be considered as another possible explanation of biguanide induced inhibition of glucose stimulated insulin secretion. The results concerning direct glucose oxidation in the presence of biguanides, however, are contradictory (see: Söling *et al.*, 1967). Furthermore, the role of the pentose shunt for glucose stimulated insulin secretion has recently been questionned (Snyder, Kashket and O'Sullivan, 1970).

Glibenclamide was able to increase glucose stimulated insulin secretion in islets preincubated with biguanides. The secretion curve due to glibenclamide, however, exhibited a decrease similar to the curve without glibenclamide. These findings, the observation that maximum inhibition was not overcome by this sulphonylurea even at the concentration of 10 µg/ml and, finally, the lack of effect of glibenclamide on insulin release observed with 10 mg/ml phenformin, could be explained by the effects of large doses of biguanide on cell function as described above.

Loubatières, Mariani and Jallet (1971) observed a stimulation of insulin output from the pancreas with biguanides both *in vivo* (dogs) and *in vitro* (perfusing the isolated rat pancreas with biguanides in concentrations of 200 µg/ml metformin and 50 or 100 µg/ml phenformin for 30 min each). The authors suggested that stimulation of insulin secretion effected by biguanides might be one of the factors involved in the mode of action of these drugs. Studying the effect of metformin on insulin secretion (Schatz, Katsilambros,

Hinz and Pfeiffer, 1971) we tested metformin at a range of concentration from 1 μg/ml to 10 mg/ml in 6 perfusion experiments of the isolated rat pancreas. During 10 min of perfusion with 1, 10 and 100 μg/ml metformin the insulin secretion due to 150 mg% glucose remained unchanged. However, we found an increase of insulin output with 1 mg/ml metformin. With a concentration of 10 mg/ml this increase amounted to 400% of basal insulin values. Insulin levels decreased again after cessation of the metformin perfusion.

A significant release of insulin from islets was observed only with very high concentrations of phenformin. Differences between the results obtained in isolated islets and the isolated perfused pancreas may be ascribed to the nature of the two experimental models. The lack of inhibition of insulin secretion during perfusion of the isolated pancreas with smaller biguanide doses might also be due to differences in glucose concentrations used (cf. Fußgänger and Pfeiffer, 1971).

In the second part of this study a decrease of ^{3}H-leucine incorporation into the proinsulin and insulin fraction of rat islets was demonstrated in the presence of biguanides (Fig. 4). Obviously intrinsic activity accounts for the quantitative differences between metformin and phenformin. A decrease of insulin secretion by biguanides is thus closely in parallel with inhibition of insulin biosynthesis. The amount of radioactivity incorporated into the first elution peak, which represents islet proteins different from proinsulin and insulin, is also reduced in the presence of high concentrations of biguanides. Therefore, it may be concluded that protein synthesis, in general, is inhibited. The fact that with 1 mg/ml metformin and 0.1 mg/ml phenformin, respectively, relatively more radioactivity is incorporated into the insulin fraction than into proinsulin, as compared with glucose alone, might be ascribed to the duration of the incubation (4 h): after 2 to 3 h exposure of the islets to the large concentrations of biguanides, the islet cell metabolism might seriously have deteriorated so that proinsulin was not synthesized any longer whereas already labelled proinsulin was still converted to insulin.

A decrease of calcium ions in the incubation media due to a chelation of calcium by biguanides was not demonstrable. Omission of calcium ions abolishes insulin secretion from isolated islets whereas insulin biosynthesis is not affected (Hinz, Schatz, Meier, Nierle and Pfeiffer, 1971). If, therefore, a chelation of calcium had been the cause for biguanide induced inhibition of insulin secretion, insulin biosynthesis should not have been hindered in our experiments.

Although biguanide concentrations have been described as being several times higher in pancreatic tissue than in serum (Beckmann, 1969) the concentrations found to be effective in this study are still very high when compared with therapeutic levels. Biguanide action is highly dependent on species as well as on concentration (Sterne, 1969). Furthermore, the primary metabolic situation has proved to be of great influence (Schatz *et al.*, 1970). Therefore, it seems to us that it is not feasible to draw any clinical conclusion from these findings obtained in isolated islets from normoglycemic rats.

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