Stimulatory Effect of Chelating Agents and Mesoxalate on the In Vivo Release of Insulin in the Pancreas of the Dog

The Possible Role of Amino Acids

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SUMMARY

Plasma ILA has been assayed in the blood of the pancreatic vein in dogs by measuring the fall in blood glucose level in hypophysectomized-adrenodemedullated rats. The infusion of EDTA, L-histidine, glycine and L-leucine into the pancreatic artery increased the ILA in the pancreatic effluent, the increase being most pronounced fifteen to thirty minutes after the cessation of a ten-minute infusion. No increase in the ILA was observed in the pancreatic vein draining the nonperfused pancreatic area. Mesoxalate stimulated the release of insulin, but its effect was not characteristic of the chelating agents with respect to time course of action. Vagotomy abolished the effect of both chelating agents and mesoxalate. These findings indicate the in vivo stimulation of the release of biologically active insulin by chelating agents and mesoxalate. DIABETES *15*:44-50, January, 1966.

Based on the physicochemical properties of the insulin molecule, derived from in vitro studies, it has been suggested by Maske[1] that in vivo insulin is stored in the beta cells as a zinc complex, and that the dissociation of this complex is the first step in the mobilization of insulin. It was thus our purpose to present evidence that would clarify the basis of this assumption.

If the insulin-zinc complex is dissociated prior to the release of insulin, it might be anticipated that a perfusion of the pancreas with chelating agents would initiate the release of insulin. It was, therefore, thought to be of interest to study this problem directly by measuring changes of the insulin-like activity (ILA) in the pancreatic vein of dogs in response to various chelating agents administered into the pancreatic artery.

In the present studies, ethylenediaminetetraacetate (EDTA) and three amino acids have been selected according to their chelating capacities, and their effects on the insulin release have been compared with that of mesoxalate, a known hypoglycemic agent.[2] A possible role of the vagus nerve in insulin secretion[3] has also been investigated.

METHODS

Experimental procedures

Adult mongrel dogs of 7 to 10 kg. body weight were fed a high carbohydrate diet for several days prior to the experiments. After an overnight fast the dogs were anesthetized with sodium thiopental. One of the main vessels of the pancreas, either the superior or the inferior pancreatico-duodenal vessels, or the pancreatic branch of the splenic vessels, was carefully exposed.

When blood samples were taken from the superior pancreatico-duodenal vein, the inferior pancreatico-duodenal vein and the gastroepiploic vein were ligated to prevent the dilution of the venous blood in the particular portion of the pancreas.

In twenty-three dogs, chelating agents were infused into a pancreatic artery, and the ILA in the effluent was assayed. About one hour following laparotomy, 0.5 millimoles of the chelating agent was infused over a ten-minute period into the pancreatic artery through a fine needle attached to a flexible polyethylene tube (figure 1).

The chelating agents, disodium or disodium-monocalcium EDTA, L-histidine, glycine and L-leucine, were dissolved in de-ionized water immediately before infusion. EDTA was infused to ten dogs, L-histidine to six dogs, glycine to three dogs and L-leucine to four dogs.

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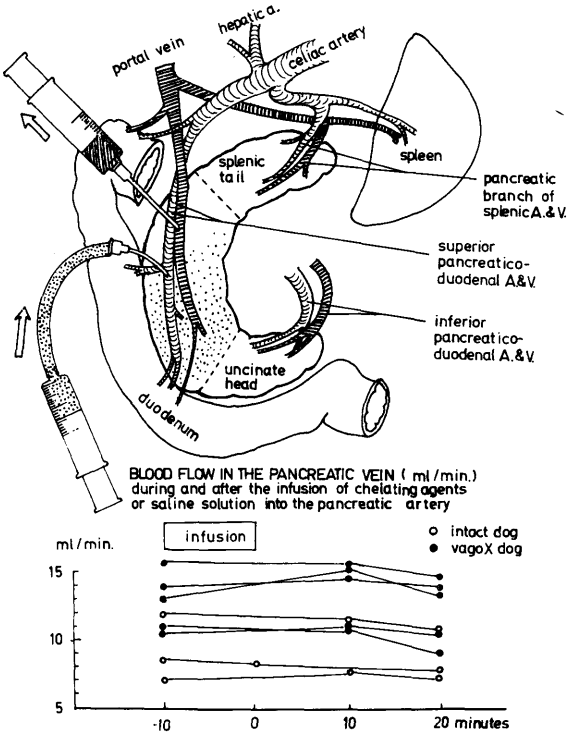

FIG. 1. Schematic diagram of the experimental procedure in the dog and a graph of blood flow before and after infusion. The tested biochemical substances were infused into either the superior or the inferior pancreatico-duodenal artery, or the pancreatic branch of the splenic artery. Blood was collected from a pancreatic vein into a heparinized syringe. The blood flow in the pancreatic vein was measured in eight dogs before and after the infusion of the substances.

At selected intervals, usually 0, 10, 20, 30 and 40 min. after the end of the infusion, 20 ml. of blood was collected from the pancreatic vein. The samples were centrifuged, and ILA in the plasma was determined by an assay using hypophysectomized-adrenodemedullated rats.[3]

In order to show the direct effect of the stimulatory agent only on the perfused area, the following procedure was used. In five dogs, ILA was measured in the pancreatic vein draining the nonperfused area and compared with ILA from the vein draining the perfused area. In three dogs, EDTA was infused into the splenic *vein* towards the liver and the blood samples were taken from the superior pancreatico-duodenal vein. In two dogs, a mixture of L-histidine and glycine was infused into either superior or inferior pancreatico-duodenal artery, and the blood samples were taken from both the superior and the inferior pancreatico-duodenal vein.

In order to study the combined effects of EDTA infusion and vagotomy, the vagus nerve of three dogs was cut just above the diaphragm about one hour before the infusion of EDTA. Similarly, the combined effects of L-histidine and vagotomy were studied in three dogs.

For comparison, a total of one millimole of sodium mesoxalate was similarly infused into the superior pancreaticoduodenal artery of five dogs and into the pancreatic branch of the splenic artery of one dog. ILA in the blood from a nonperfused portion of the pancreas was also assayed from three dogs in which mesoxalate was infused into either the superior pancreatico-duodenal artery, the pancreatic branch of the splenic artery or the splenic *vein.* In a similar manner to the above treatments with chelating agents, the ILA in the superior pancreatico-duodenal vein was assayed in six vagotomized dogs.

In control experiments comprising three dogs, a 0.9 per cent solution of sodium chloride was infused into the superior pancreatico-duodenal artery and changes in the ILA of the effluent were examined.

Assay of ILA in the plasma

Adult male albino rats weighing 120 to 140 gm. were hypophysectomized by parapharyngeal approach and were kept at 25 to 29° C. after surgery. Seven to ten days later, both adrenals were demedullated, and a 2 per cent glucose solution was administered as drinking water thereafter. These rats were used for the assay three to four days after the adrenodemedullation. After two hours of fasting, each rat was injected intraperitoneally with a one-milliliter sample of a dog plasma per 100 gm. rat body weight. The glucose level in the samples taken from the tail vein was determined before, and one hour after, the injection according to Hagedorn and Jensen. The ILA of the dog plasma was estimated using the standard regression line which relates the logarithm of the dose of standard insulin to the average fall of blood glucose level in rats. Four to seven rats were used for the assay of the ILA of a dog plasma sample. Rats which survived after the first assay were used for the second and occasionally for the third assay at two- to three-day intervals. The validity of this assay has been firmly established.[3]

Blood flow in the pancreatic vein

In three normal and five vagotomized dogs, blood flow was measured before and after the infusion of saline or chelating agents. An air bubble was injected into a transparent polyethylene tube which connected the superior pancreatico-duodenal vein with the femoral

vein, and the blood flow was calculated from the speed of the traveling bubble.

Statistical analysis

The variances in fall of blood glucose from the treatment, from the dogs and from the rats were determined by an analysis of variance, and the significance of the difference between the means of ILA, expressed as the average fall of the blood glucose levels in rats, was tested.[4]

RESULTS

A. *Fasting ILA in the pancreatic vein of normal and vagotomized dogs*

Table 1 shows the average fasting ILA in the superior pancreatico-duodenal vein and the pancreatic branch of the splenic vein of sixteen and three normal dogs, respectively. The ILA is shown as the average fall of blood glucose level (mg. per 100 ml.) in the rat. The fall in the superior pancreatico-duodenal venous plasma corresponds to the hypoglycemic effect of 0.27 to 0.50 mU/ml. of insulin, and is essentially the same as the concentration which had previously been reported.[3] The ILA in the pancreatic branch of the splenic vein was slightly higher than that in the superior pancreatico-duodenal vein, but this difference was not significant. Vagotomy did not affect the fasting level of the ILA.

TABLE 1

Fasting ILA in the pancreatic veins of normal and vagotomized dogs

Blood taken from:	Number of dogs	Insulin-like activity: Decrease in blood glucose concentration* (mg. per 100 ml.)	Insulin-like activity: Corresponding insulin concentration† (mU./100 ml.)
Superior pancreatico-duodenal vein (normal dogs)	16	8.1±0.49	0.27—0.50
Superior pancreatico-duodenal vein (vagotomized dogs)	7	9.7±0.84	0.31—0.53
Pancreatic branch of splenic vein (normal dogs)	3	10.8±1.54	0.41—0.48

*Hypoglycemic effect determined in hypophysectomized-adrenodemedullated rats (mean and standard error).

†Insulin concentration which corresponds to the hypoglycemic effect of the dog plasma samples. These values were estimated from the standard regression line of the logarithm of the dose of standard insulin and the average fall of blood glucose level in rats. The range of individual values is shown.

B. *Blood flow in the pancreatic vein*

The blood flow in the pancreatic vein was measured in three normal and five vagotomized dogs. As shown in figure 1, no appreciable changes occurred during the course of the experiment.

C. *ILA in pancreatic effluent blood*

a. *Normal dogs*

(1) Effluent from the perfused area:

0.9 per cent sodium chloride: As shown in table 2 and figure 2, the infusion of saline did not change the ILA of pancreatic effluent blood.

Chelating agents and mesoxalate: Table 2 also shows that the infusion of the chelating agents results in a significant increase in the ILA. The ILA in the effluents showed a slight but significant increase about twenty minutes after the cessation of the infusion of L-leucine. A larger increase in the ILA was found after the infusion of glycine, the response being most pronounced fifteen to thirty minutes after the infusion (figure 2). A similar increase in the ILA was observed after the infusion of L-histidine, which was a stronger chelating agent than either leucine or glycine, the rise in the concentration of ILA commencing more steeply than in the case of glycine. EDTA was found to increase ILA to the same degree (figure 3), whereas in the case of mesoxalate, the ILA increased sharply immediately after cessation of the infusion, as also shown in table 2 and figure 4.

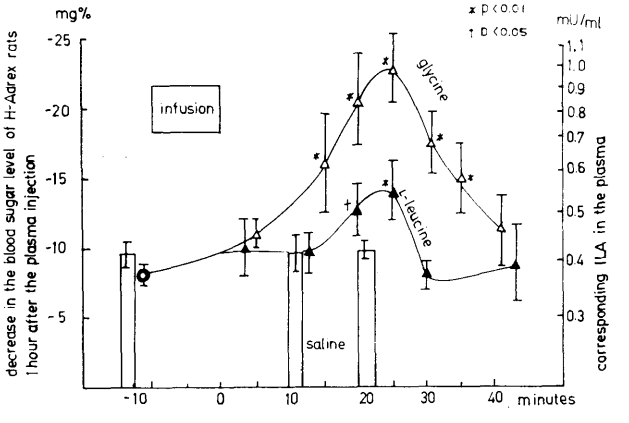

FIG. 2. The effect of glycine and L-leucine on the ILA in pancreatico-duodenal venous blood plasma. The average fall in rat blood sugar and its 95 per cent fiducial limit after the injection of dog plasma are shown, and the related insulin concentration is indicated. The circle, at time equals minus ten minutes, shows the fasting ILA in sixteen normal dogs. The ILA was unaffected by infusion of 0.9 per cent sodium chloride and increased after the infusion of glycine and of L-leucine. *($P<0.01$) and † ($P<0.05$) indicate statistical significance when compared to the fasting ILA.

TABLE 2

The ILA (as mg. per 100 ml. fall of rat blood glucose level) in the pancreatic effluents following the intra-arterial infusion of saline, chelating agents and mesoxalate (means and standard errors)

Substance infused Number of dogs	Saline 3	L-leucine 4	Glycine 3	L-histidine 6	EDTA 10	Mesoxalate 6
The fasting ILA (the average fall of blood glucose level in the rats by the pancreatic plasma of the fasting dog)					8.1±0.49	
Five minutes before the cessation of the infusion	—	—	—	—	—	9.0±1.8
Minutes after infusion						
0	—	—	—	7.0±1.7	12.7±4.4*	26.7±1.3†
5	—	10.0±2.1	11.2±1.2	—	19.1±0.9†	—
10	9.7±1.3	9.6±1.5	—	18.1±2.1†	16.4±1.4†	19.4±2.0†
15	—	—	16.1±3.6†	22.6±1.1†	21.5±1.2†	—
20	9.8±0.8	12.8±2.0*	20.7±3.3†	18.0±1.8†	21.7±1.0†	13.2±1.5†
25	—	14.0±2.0†	22.9±2.4†	14.1±1.6†	13.0±2.5	—
30	—	8.1±0.9	17.6±2.2†	11.9±3.6	5.1±0.9	7.5±1.3
40	—	9.0±2.7	11.2±2.7	8.5±1.9	6.0±1.2	5.0±1.3

*$p<0.05$ when compared to the fasting ILA.
†$p<0.01$

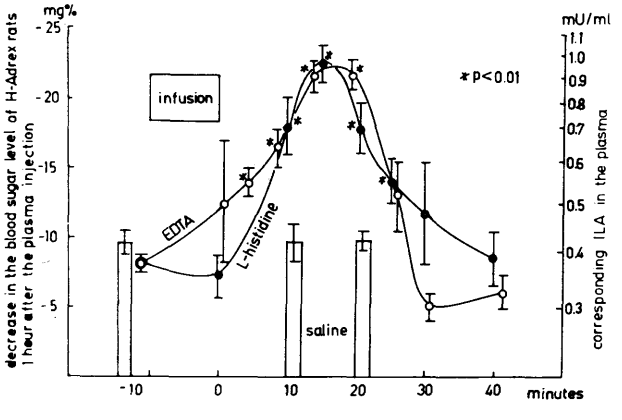

FIG. 3. The effect of EDTA and L-histidine on the ILA in pancreatico-duodenal venous blood plasma. Both EDTA and L-histidine were found to increase ILA. Means and 95 per cent fiducial limits are shown.

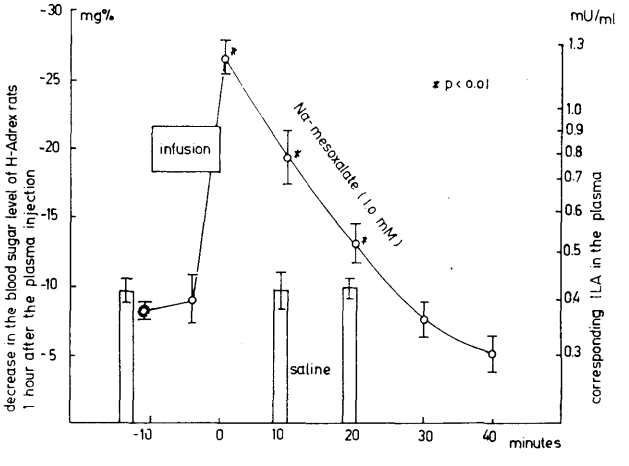

FIG. 4. The effect of mesoxalate on the ILA in pancreatico-duodenal venous blood plasma. The infusion of sodium mesoxalate was found to increase the ILA sharply immediately after the cessation of the infusion. Means and 95 per cent fiducial limits are shown.

(2) Effluent from the nonperfused area:

Table 3 and figure 5 show the ILA in the effluent from nonperfused pancreatic areas and area perfused with mesoxalate, EDTA and a mixture of L-histidine and glycine. It can be seen that the plasma taken from a vein draining a nonperfused area of the pancreas did not show any significant increase in the ILA, in contrast to the plasma taken from a perfused area.

b. Vagotomized dogs

No significant increase in the ILA in the effluents from the perfused pancreatic area of vagotomized dogs was found after the infusion of individual chelating agents (i.e., EDTA and L-histidine) and mesoxalate as shown in figures 6 and 7.

D. *Blood glucose level of dogs*

There was no significant change in the blood glucose level of the dogs used throughout all of these experiments.

E. *Correlation between chelating capacities and effects on the release of insulin*

Figure 8 shows the computer-fitted curves of the ILA in the pancreatic venous blood versus time fol-

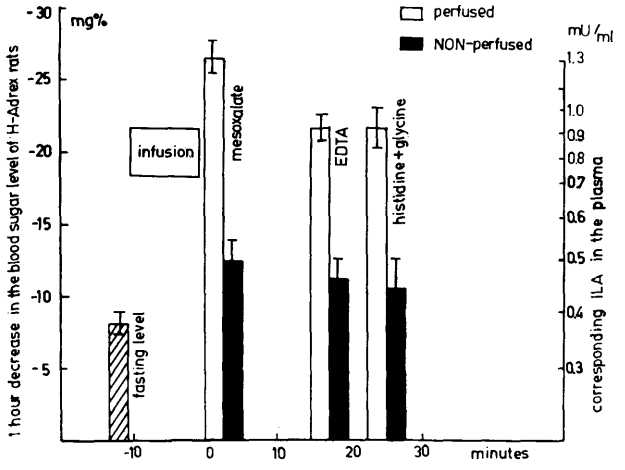

FIG. 5. The ILA in the venous effluent from nonperfused pancreatic areas and areas perfused with chelating agents and mesoxalate. The infusion of mesoxalate, EDTA or a mixture of L-histidine and glycine increased the ILA in the effluents from the perfused area. In contrast, the plasma taken from a vein draining a nonperfused area of the pancreas did not show any significant increase in the ILA. Means and 95 per cent fiducial limits are shown.

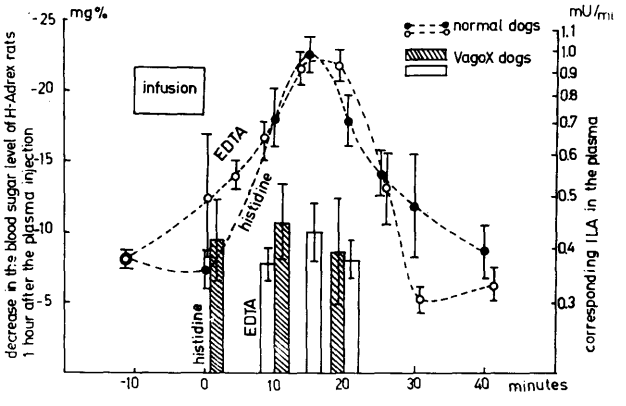

FIG. 6. The effect of EDTA and L-histidine on the ILA in pancreatico-duodenal venous blood plasma in vagotomized dogs. In contrast to the effect in normal dogs, vagotomy abolished the effect of EDTA and L-histidine on the ILA. Means and 95 per cent fiducial limits are shown.

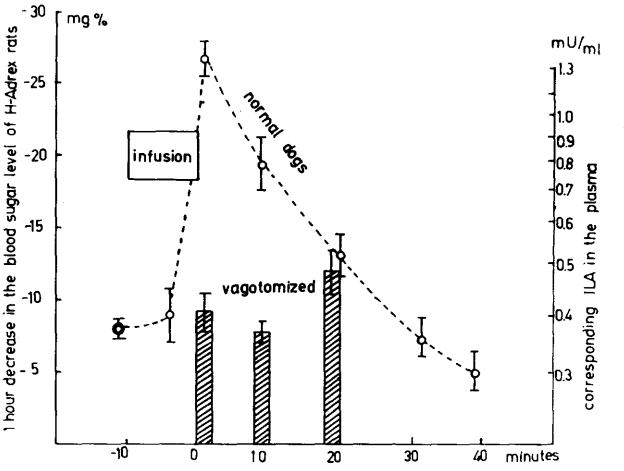

FIG. 7. The effect of mesoxalate on the ILA in pancreaticoduodenal venous blood plasma in vagotomized dogs. In contrast to the effect in normal dogs, vagotomy abolished the effect of sodium mesoxalate on the ILA. Means and 95 per cent fiducial limits are shown.

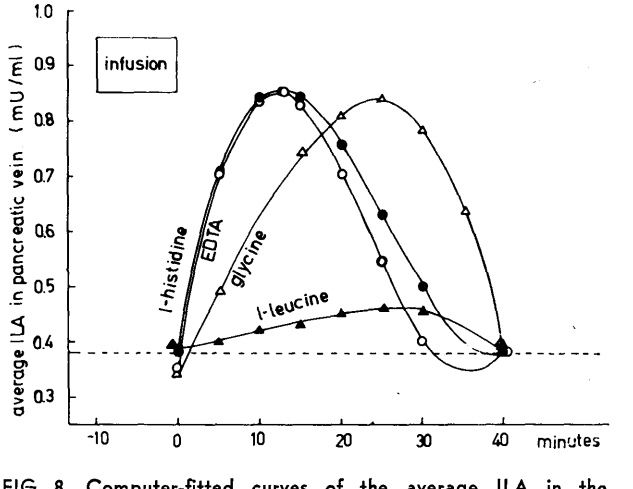

FIG. 8. Computer-fitted curves of the average ILA in the pancreatic vein (mU./ml.) following the infusion of chelating agents. The polynomial curves are fitted to the ILA in the pancreatic venous blood following the infusion of EDTA, L-histidine, glycine and L-leucine, respectively. The broken line shows the fasting ILA level.

lowing the infusion of EDTA, L-histidine, glycine and L-leucine, respectively. From these curves, the total amount of insulin released above normal values was computed as the area above the base line (blood flow assumed to be 10 ml. per minute), and the initial slope was used to compute the rate of release at the end of the infusion.

Figure 9 relates the initial rate of insulin release and the total amount of insulin released to the chelating capacity of the infused agent. The chelating capacity of the infused agents is expressed by the stability constant of the zinc chelate,[5] which increased with increasing initial rate of insulin release. However, the total amount of insulin released is the same for all substances except leucine.

DISCUSSION

The results of the present studies show that the infusion of EDTA, L-histidine, glycine and L-leucine into the pancreatic artery increases the ILA in pan-

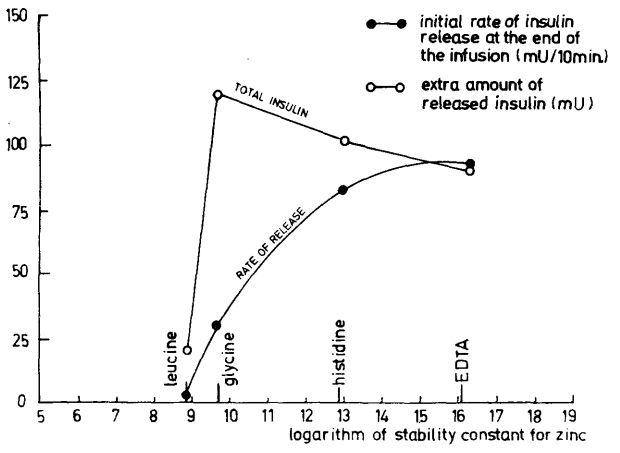

FIG. 9. Relationship between chelating capacities and the effects on the release of insulin. The total amount of insulin released above normal values and the initial rate of insulin release at the end of the infusion were computed from the polynomial curves shown in figure 8. Blood flow of the pancreatic vein was assumed to be 10 ml. per minute for the computation. The initial rate of insulin release and the total amount of insulin released are related to the chelating capacity of the infused agent. The chelating capacity is expressed as the logarithm of the stability constant of the zinc chelate. The abscissa indicates the units for the initial rate of insulin release at the end of the infusion and for the extra amount of released insulin.

creatic effluent blood. In all cases, the increase in the ILA was most pronounced fifteen to thirty minutes after the cessation of the infusion. The present studies also confirm the direct stimulatory effect of mesoxalate on the pancreatic beta cells.[2] In contrast to the chelating agents, mesoxalate increased sharply the ILA immediately after the cessation of the infusion. In vagotomized dogs, the tested substances were found to be ineffective. No change in blood glucose concentration occurred in any of these experiments.

From these findings it has been concluded that the increase in ILA observed in intact dogs was due neither to a change in blood glucose level, an interaction between any of the infused agents and insulin in the plasma nor to a direct effect of these agents on the blood glucose level of the rats used in the assay of insulin, but to an in vivo stimulation of the release of biologically active insulin by the tested chelating agents and mesoxalate.

In addition, when these agents were infused into the splenic vein or into a branch of the pancreatic artery and the blood samples were taken from a vein draining an area different from the perfused one, no increase in ILA was observed in the effluent blood. This suggested a direct effect of these agents on the beta cells.

In this connection, it is to be noted that the vagotomy abolished insulin release following the infusion of the tested agents, even though the effects observed seem to depend upon direct stimulation of the beta cells. Therefore, it seems likely that the vagus nerve may exert a permissive effect on the release of insulin. This effect, together with that of electrical stimulation of the vagus nerve on insulin release in dogs[3] and beta granulation and reactive zinc content in rabbits,[6] support the possible role of the vagus nerve in insulin release from the pancreas.

Since no hypoglycemic effect was observed following the infusion of any of the tested agents, it seems likely that the rate of insulin release during this period (maximum rate about 10 mU. per minute) is insufficient to decrease the blood glucose level in the anesthetized dog.

Although induction of hypoglycemia by chelating agents such as dimercaprol (BAL),[7] EDTA[8] and L-leucine[9] has been reported, the relation between this effect and the release of an extra amount of insulin is still not clear. The hypoglycemic effect of BAL has been shown to be independent of insulin.[10] The hypoglycemic effect of EDTA has been obtained only in insulin-treated diabetics, and is attributed to the rapid dissociation of administered insulin.[8] The hypoglycemic effect of L-leucine in humans and in dogs has been

TABLE 3

The ILA in the venous effluent from nonperfused pancreatic areas and areas perfused with chelating agents and mesoxalate

Substance infused	Minutes after the cessation of the infusion	Insulin-like activity* In the effluent from perfused area	In the effluent from nonperfused area	Significance of difference
EDTA	10	16.4±1.4 (5)	10.2±1.4 (3)	$p<0.05$
	20	21.7±1.0 (7)	11.2±1.5 (3)	$p<0.01$
L-histidine and glycine	20	21.7±1.4 (2)	10.7±2.0 (2)	$p<0.05$
Mesoxalate	0	26.7±1.3 (4)	12.5±1.4 (3)	$p<0.01$

*ILA shown as mg. per 100 ml. fall of rat blood glucose level with standard error.
( ) Number of dogs

observed after an oral dose of 750 mg./kg.,[11] but a dose of 150 to 300 mg. per kg., which is markedly effective on patients with insulinoma,[12] has little, if any, effect on the blood glucose level[11,13-15] and on the plasma ILA level[13,16] in normal subjects. Therefore, the hypoglycemic effect of L-leucine in patients with either insulinoma[12] or idiopathic hypoglycemia,[9] might be due to an increased ability of the beta cells to mobilize insulin,[16] or it might be totally unrelated to such a mechanism.[17,18] The marked hypoglycemic effect of mesoxalate, infused into the pancreatic artery of dogs, has been shown only after a prolonged infusion of two to six hours.[2]

The insulin releasing capacity of chelating agents such as EDTA, L-histidine, glycine and L-leucine and the more rapid response of the beta cells to the stronger chelating agents seem to be consistent with the hypothesis of Maske[1] on the competitive action of chelating agents with insulin. An alternative possibility for the explanation of the effect of chelating agents has been offered by the experiments of Fujii et al.,[19] suggesting that the release of minute quantities of zinc from the spermatozoa of the starfish, *asterias amurensis,* is responsible for the initiation of cellular activity. However, the manner in which any of insulin-releasing substances, including those tested in this study, act on the beta cells of the pancreas is still unknown and requires further study.

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