Production of Nonimmunoassayable Insulin-like Material by Perfused Rat Liver

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SUMMARY

The perfusion of isolated rat liver with media containing 74 μU. bovine crystalline insulin per ml. resulted in a rapid loss of immunoassayable insulin accompanied by the appearance of nonimmunoassayable insulin-like activity as measured in vivo by a rat intraperitoneal assay for insulin activity. The material with insulin-like activity could be absorbed and separated from insulin on Dowex-50W × 8 resin (Na^+ cycle). Fractionation on Sephadex G-100 indicated that it has a molecular weight above 40,000 with possible peaks of activity between 40,000 to 60,000 and above 90,000. Small amounts of nonimmunoassayable insulin-like activity were released by perfused livers even when insulin was not added to the perfusion media. When insulin was added to the media, there was a marked increase in the release of nonimmunoassayable insulin-like activity. DIABETES *19*:296-301, May, 1970.

Previous studies from this laboratory have suggested that insulin circulates in an active, low molecular weight "free" form similar to crystalline insulin and in an inactive, high molecular weight "bound" form.[1] It has been proposed that the regulation of insulin activity in human beings and animals might involve mechanisms at the tissue level, catalyzing the transformation of "free" insulin into its inactive "bound" form or the activation and utilization of "bound" insulin. In the present study evidence has been obtained that the perfusion of rat livers with media containing crystalline insulin results in the production of nonimmunoassayable material with insulin-like activity with many characteristics similar to those ascribed to "bound" insulin.

MATERIAL AND METHODS

Isolated livers from 300 to 400 gm. male Charles River CD rats were perfused as described by Hems et al.[2] The perfusion media consisted of 2.4 per cent human serum albumin (Cohn Fraction V further purified by a second alcohol precipitation), 0.1 per cent glucose and 10 per cent hemoglobin (from human red blood cells washed three times in 0.85 per cent saline) in Krebs Ringer bicarbonate buffer (KRB), pH 7.4. The albumin used in these studies was dialyzed before use for forty-eight hours at 2° C. against 100 volumes of KRB with two buffer changes. The total perfusion volume was 150 ml., after the first 20 ml. of perfusate which passed through the liver was discarded. Crystalline bovine insulin was then slowly added over thirty to sixty seconds directly to the portal cannula in 1 ml. of KRB. The perfusate in the reservoir was constantly mixed by a magnetic stirring rod, by aeration with 5 per cent CO_2, 95 per cent O_2 and by recirculation through the perfusion system. Approximately 20 ml. of perfusate passed through the livers each minute. Enough bovine insulin was added to provide 74 or 200 μU. of insulin to each ml. of perfusion media.

Preparation of partially purified concentrates with insulin-like activity from liver perfusates

Partially purified concentrates with insulin-like activity were obtained from liver perfusates by resin absorption and elution. Dowex-50W × 8 resin (Na^+ cycle) was placed in glass columns 28 cm. long and 5.0 cm. wide. One volume of perfusate (about 80 ml.) was passed slowly (1 ml./min.) through 2.5 resin volumes (200 ml.). Following passage of the perfusate the resin was washed twice, each time with one resin volume of 0.15 M NaCl. The material which was retained on the column was eluted from the resin with two resin volumes of cold, dilute ammonium hydroxide (0.02 N). During elution the flow rate was increased to 60 ml. per minute. The pH of the eluate was monitored with a continuous recording pH meter and it was kept between 5 and 8 with the help of 0.2 N sulfuric acid. The final pH of the eluate was adjusted to 7.4 ± 0.4. The eluate was then lyophilized and the dry powder was dissolved in cold distilled water and dialyzed at 2° C. for forty-eight hours against 100 volumes of Gey and Gey bicarbonate buffer with two changes. The concentrates thus prepared exhibited about 200 to 300-fold protein purification compared with the original perfusate.

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Chromatography of whole liver perfusates and partially purified concentrates with insulin-like activity from perfusates on Sephadex columns.

Sephadex G-100 was suspended in borate buffer, pH 8.0, and stirred for seventy-two hours at room temperature. The mixture was placed in a column 50 × 10 cm. and the buffer was slowly discharged with a hydrostatic head of 15 cm. The column was transferred to a cold room (2° to 5° C.) and was washed with borate buffer for forty-eight hours before use. The final bed volume of the Sephadex was about 800 ml. Eight to 12 ml. of whole liver perfusates or their partially purified concentrates were layered on top of the column and the effluent was collected in tubes. The various components were eluted from the Sephadex column with the borate buffer. The rate of flow was between 7 to 10 ml./hour.

The fractions obtained from the Sephadex column were lyophilized, dissolved in a minimum volume of cold distilled water and then dialyzed for forty-eight hours at 2° to 5° C. against 100 volumes of Gey and Gey bicarbonate buffer with four buffer changes.

The Sephadex column used in these studies was standardized with the use of molecular markers including human γ-globulin (M.W. 160,000), albumin (M.W. 69,000), α-chymotrypsin (M.W. 22,500) and cytochrome C (M.W. 12,400).

Immunoassay of insulin

Immunoassayable insulin in the perfusion media was determined by the Morgan and Lazarow technic[3] as modified by Soeldner and Slone.[4]

Intraperitoneal insulin assay of liver perfusates and of concentrates with insulin-like activity obtained from liver perfusates

Insulin-like activity in whole perfusion media and in extracts obtained from the perfusates was determined in vivo by the intraperitoneal assay of Rafaelsen et al.[5] as modified by Cahill et al.[6] The whole perfusates or perfusate extracts in 5 per cent human plasma albumin to be assayed were injected intraperitoneally into groups of four to six intact fed male Charles River CD rats (110 to 115 gm.) along with 4 mg. glucose and 2 μc. glucose-U-C-14 (specific activity about 3.3 millicuries/millimole). In each assay either 5 ml. of whole perfusion media or 5 ml. of 5 per cent human plasma albumin were also assayed. Before injection the rats were lightly anesthetized with 50 per cent CO_2:50 per cent O_2, the testes displaced into the abdominal cavity and the communication between peritoneal and scrotal spaces closed by a transcutaneous suture. Two hours after injection, the rats were anesthetized with 50 per cent CO_2:50 per cent O_2, decapitated, and both hemidiaphragms and epididymal pads removed rapidly. The epididymal fat pads were weighed immediately, placed in separate tubes each containing 20 ml. chloroform: methanol (2:1) and shaken for four hours. The hemidiaphragms were cooled rapidly on iced aluminum foil, weighed, placed in tubes containing 30 per cent KOH at 100° C., hydrolyzed, and the glycogen precipitated and transferred to planchettes for counting. The chloroform-methanol extract of the adipose tissue containing the total fat was also counted. Glycogen was then isolated from the extracted adipose tissue and its radioactivity determined.

RESULTS

Table 1 shows the effect of liver perfusion time on the amount of immunoassayable insulin in twelve perfusates to which 74 μU. of bovine crystalline insulin per ml. were added. One-ml. samples for immunoassay were obtained by sampling the circulating perfusion media before the addition of insulin and at five, thirty, sixty and ninety minutes after the addition of insulin to each perfusion system. It can be seen that the continuous passage of the perfusion media though the rat livers resulted in a rapid loss of immunoassayable insulin activity. Immunoassay of the perfusion media prior to the addition of insulin and to the start of the perfusion gave an immunoassayable insulin value of 8 μU./ml. It is believed that this value was an artifact related to the effects of the media on the assay not related to immunoassayable insulin in the media. When 8 μU. were subtracted from the mean insulin values obtained during the perfusion and the logarithm of the insulin was plotted against time, a straight line was obtained indicating that the half life of the immunoassayable insulin added to the perfusion media was approximately 26½ minutes under the perfusion conditions used.

Table 2 shows the effect of perfusion time on the total insulin-like activity of perfusion media to which 74 μU. of crystalline insulin per ml. were added. In this study 8 to 10 ml. samples of perfusion media were

TABLE 1

The effect of rat liver perfusion on immunoassayable insulin

Time, minutes	Initial perfusate —insulin	0	5	30	60	90
μU. insulin/ml.	8±2	82*	62±5	41±6	22±2	16±2

Values represent means ± S.E.M. for twelve perfusions.
*Obtained by adding value for initial perfusion media to the amount of crystalline insulin added to the perfusate (8 + 74).

TABLE 2

The effect of rat liver perfusion on insulin-like activity measured in vivo

Sample, perfusion time in minutes after addition of insulin	No. of rats	Diaphragm glycogen cpm/gm. tissue ± S.E.M.	Adipose tissue glycogen cpm/gm. tissue ± S.E.M.	Adipose tissue fat cpm/gm. tissue ± S.E.M.
Control media, no insulin	6	4,052±220	301±12	5,867±619
5	6	7,353±206*	543±81†	10,033±1,272†
30	8	8,934±956	452±48	10,800±1,390
60	8	8,293±1,648	500±96	9,033±1,127
90	6	8,759±780	713±48	8,800±327

The values represent the means of the CPM incorporated per gm. of the two epididymal fat pads or two hemidiaphragms in each rat. *$p < .001$, † $p < .02$ when compared to control values. Values obtained after five minutes were not statistically different than those at five minutes.

removed from the perfusion system five, thirty and sixty minutes after the crystalline insulin was added to the media. Perfusion media from four perfusions were pooled and assayed for insulin activity in vivo by the intraperitoneal assay. It can be seen from table 2 that as expected following the addition of crystalline insulin to the perfusion media there was a marked increase in the insulin-like activity of the perfusion media as measured by the in vivo assay system used. Unlike the immunoassayable insulin (table 1), the biologic insulin-like activity did not significantly decrease during the ninety-minute perfusion period. These data indicate that the passage of crystalline insulin through isolated perfused liver resulted in the loss of immunoassayable insulin activity but not in a similar loss of biological insulin activity, and suggest that the disappearance of immunoassayable insulin from the perfusion media was accompanied by a continuous addition of nonimmunoassayable insulin-like material into the media during the period of perfusion.

The data of table 3 and figure 1 show the insulin-like activity (determined by the in vivo technic) of fractions of the perfusates partitioned according to molecular size on Sephadex G-100. In these studies 200 μU. of crystalline bovine insulin were added to the perfusion system for each ml. of perfusion media. In one series of studies, ninety-minute perfusates from three perfusions were fractionated on Sephadex. Identical fractions from the Sephadex runs were pooled and bioassayed. In the other, ninety-minute perfusates of three perfusions were first treated with Dowex-50W and the extracted material from the perfusates was then fractionated on Sephadex G-100 as described above and pooled. It can be seen that the insulin-like activity

TABLE 3

Insulin activity in Sephadex G-100 fractionated rat liver perfusates treated and untreated with Dowex 50

	No. of rats	Sephadex fraction elution volume, ml.	Molecular size × 10^{-3}	Diaphragm glycogen cpm/gm. tissue ± S.E.M.	Adipose tissue glycogen cpm/gm. tissue ± S.E.M.	Adipose tissue fat cpm/gm. tissue ± S.E.M.
Untreated perfusate						
	4		Control*	4,775± 132	340± 26	6,810± 990
	5	230—310	90	10,840± 908	576±110	11,120±2,397
	5	311—400	60—90	6,360± 479	582± 56	12,250±1,040
	5	401—480	40—60	13,500±2,111	696± 74	13,800±1,686
	5	481—600	20—40	4,340± 490	418± 83	8,000± 912
	5	601—750	<20	4,075± 487	442± 23	9,000± 482
Perfusate treated with Dowex 50						
	4		Control*	2,800± 316	214± 11	3,300± 300
	5	230—310	90	7,825± 864	275± 16	7,000± 408
	5	311—400	60—90	5,750± 175	266± 31	5,450± 499
	5	401—480	40—60	8,700± 919	330± 24	8,200± 258
	5	481—600	20—40	3,390± 333	228± 17	4,000± 316
	5	601—750	<20	3,300± 500	250± 16	4,460± 328

*Control values were obtained by injecting each rat with 5 ml. of 5 per cent human serum albumin containing approximately 2 to 4 μC. of glucose-U-C-14. Differences in control values of the two series of experiments were due primarily to differences in the amount of labeled glucose used.

Each rat received the equivalent of 7 ml. of pooled perfusate from three perfusions.

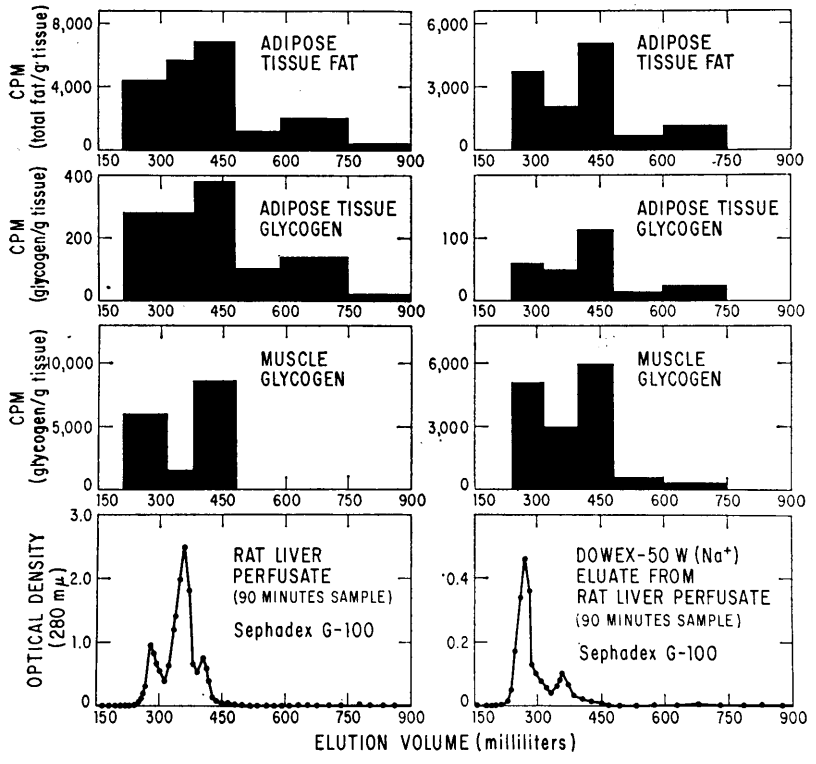

FIG. 1. Insulin activity and protein concentration in Sephadex G-100 fractionated rat liver perfusates treated and untreated with Dowex-50. Insulin activities have been plotted minus control values (table 3).

appeared in fractions with a molecular weight of more than 40,000. It appears from the data that there were possibly two peaks of activity: one with a molecular weight of approximately 40,000 to 60,000, the other above 90,000. Similar results were obtained whether the perfusates were first treated with Dowex-50W or not. However, as shown by figure 1, the absorption and elution of the material on the Dowex-50W column resulted in a marked concentration of activity due mainly to the removal of the excess human serum albumin in the perfusate.

In other studies shown in table 4, media with (74 μU./ml. of perfusion media) and without crystalline insulin were perfused in isolated rat livers from fed or fasted (forty-eight-hour) rats. Perfusion media collected at ninety minutes were passed through Dowex-50-W × 8 resin (Na^+ cycle) and the material with insulin-like activity was eluted from the resin and concentrated as described above. These bound insulin-like concentrates were assayed in vivo for insulin activity by the intraperitoneal assay. Each preparation was assayed in groups of four to five rats. Each rat was injected with 5 ml. of concentrate corresponding to 10 ml. of perfusate.

At the time the data in table 4 were collected, the in vivo assay was being used routinely in this laboratory in a variety of studies and had been standardized so that data could be more readily compared from day to day. Data from a large number of studies in which crystalline insulin standards (100, 1,000 and 10,000 μU.) were used in the in vivo assay were pooled and standard curves prepared. When the log of the counts per minute of diaphragm glycogen, adipose tissue glycogen and adipose tissue fat per gram of tissue were plotted against the logs of the insulin concentrations used, regression lines were obtained with the following formulae: for diaphragm glycogen $y = .436 \times +2.767$; for adipose tissue glycogen $y = .678 \times +1.131$; and for adipose tissue fat, $y = .367 \times +3.014$. The data in table 4 consist of the logs of the counts per minute ± S.E.M. obtained in the in vivo assay and an estimate of the insulin-like activity using the counts per minute observed and the regression lines obtained when crystalline insulin standards had been used. The estimates of insulin-like activity were derived by converting the logs of the counts per minute in each experiment to μU. of insulin using the regression line formulae and then calculating the mean insulin-like activities rather than calculating the insulin-like activities from the means of the logs since the two methods give slightly different results and the latter is mathematically incorrect. The insulin-like activities obtained were further corrected

TABLE 4

The production of nonimmunoassayable insulin-like material by perfused rat liver

Sample tested	No. of samples	Diaphragm glycogen	Adipose tissue glycogen	Adipose tissue fat
5 ml. of 5% human serum albumin	6	3.432±.022	2.304±.020	3.579±.019
Perfusates — insulin fed rats	5	3.632±.066* (84)	2.396±.028† (21)	3.661±.020* (24)
Perfusates + insulin fed rats	7	3.812±.029 (268)	2.560±.036 (80)	3.843±.022 (155)
Perfusates — insulin fasted rats	5	3.632±.025 (65)	2.444±.013 (33)	3.746±.033 (71)
Perfusates + insulin fasted rats	5	3.894±.050 (417)	2.617±.035 (107)	4.014±.016 (518)

The perfusate data represent the effect of injecting the equivalent of 10 ml. of Dowex treated perfusate in 5 ml. of 5 per cent human serum albumin. Values are means of logarithms ± S.E.M. of the counts per minute per gm. tissue. Figures in parentheses indicate estimated nonimmunoassayable insulin per 10 ml. of perfusate in μU. as described in the text.

*$p < .02$,† $p < .05$ when compared to 5 per cent HSA. Values obtained when insulin was added to the perfusion media were all significantly higher, $p < .01$, than values obtained when insulin was not added to the media.

by subtracting the small amount of insulin-like activity obtained in these studies when 5 ml. of 5 per cent human serum albumin were assayed by the in vivo technic.

It can be seen from table 4 that a small amount of insulin-like activity was obtained from perfused livers even when insulin was not added to the perfusion media. This varied from 2.1 to 8.4 μU./ml. of perfusate depending on which of the three criteria—diaphragm glycogen, adipose tissue glycogen or adipose tissue fat synthesis—were used. Whether the liver donor rats were fed or fasted had no statistically significant effect on the release of these small amounts of insulin-like activity into the perfusates. The amount of insulin-like activity was markedly increased when crystalline insulin had been added to the perfusion media. It should be emphasized that the insulin-like activity in these samples was not immunoassayable because the Dowex treatment they received was designed to remove the immunoassayable activity. This was confirmed by immunoassay of all perfusate concentrates used in this study.

It is obvious from the data that the calculated insulin-like activities varied with the end points in the assay being measured. This is a problem of the in vivo assay and has been observed by others.[6] It provides difficulties in quantifying the effect of adding insulin to the perfusion media on the appearance of nonimmunoassayable insulin-like activity in the perfusates. In general values calculated from the adipose tissue glycogen data were lower than from diaphragm glycogen or adipose tissue fat. When insulin was added to the perfusion media concentrates from liver perfusates of fasting rats were somewhat more active than those of fed rats in stimulating the utilization of labeled glucose. However, statistical significance was only achieved ($p < .001$) in the incorporation of glucose into adipose tissue fat and not into diaphragm or adipose tissue glycogen.

DISCUSSION

During recent years there has been considerable interest in the nature of circulating insulin and whether insulin may circulate in part in a nonactive "bound" form of high molecular weight convertible into active insulin at tissue sites when needed. Antoniades et al.[7] have suggested in a study of diabetic and nondiabetic subjects that the pancreas secretes "free" insulin and that extrapancreatic mechanisms possibly in the liver catalyze the binding of "free" insulin into a "bound" form. Other studies have supported this concept. Egdahl and Goldberg[8] suggested that the livers of dogs extract insulin-like activity (ILA) from portal blood and then release it under appropriate metabolic conditions such as hyperglycemia. Thus, they concluded that the liver seems to play an important role in the homeostasis of ILA. Samaan et al.[9] measured levels of typical and atypical ILA in ten normal human subjects and six patients with liver disease. Levels of atypical ILA were low in the patients although levels of typical ILA were normal or else after glucose administration were high. In a later experiment Samaan et al.[10] infused insulin into the portal veins of four pancreatectomized dogs. Using an isolated fat pad assay on hepatic vein serum, they reported a striking rise in total ILA and a rise in the atypical ILA of 200 to 300 per cent above the pre-infusion level. When livers of two similar dogs were excluded from the circulation and insulin was infused into the femoral vein, no rise in atypical insulin was observed in serum taken from the brachial vein. Bürgi

et al.[11] and Solomon et al.[12] studied the fate of insulin in a rat liver perfusion system by adipose-tissue assay. Although as in the present study they demonstrated a rapid inactivation of insulin, they were unable to demonstrate the formation of nonsuppressible ILA by the methods they used. On the other hand, Siess et al.[13] reported that perfusion of rat liver, whether or not insulin was added to the circulating medium, resulted in the release of a substance with properties similar to those of nonsuppressible ILA.

In the present study the addition of crystalline insulin to a rat liver perfusion system resulted in a rapid loss of immunoassayable ILA (half life of about 26½ minutes) accompanied by the appearance of nonimmunoassayable ILA. As in the studies of Seiss et al.[13] detectable ILA was released from the perfused rat livers whether or not crystalline insulin was added to the perfusion media. However, the addition of crystalline insulin to the perfusion media resulted in a marked increase in nonimmunoassayable ILA production. There appeared to be a tendency for more nonimmunoassayable ILA to be produced when crystalline insulin was added to the media perfused through livers from fasted than fed rats. These data, however, are not secure and further work will be necessary to determine whether fasting really increases the ability of a perfused rat liver to produce nonimmunoasayable ILA when insulin is added to the perfusion media. Previous observations have shown that fasting results in increased levels of circulating "bound" insulin[14] and that fasting rats are less able to utilize "bound" insulin than fed animals.[15,16]

The use of cation exchanger and Sephadex purification procedures in these studies provides evidence that the liver produces a substance with biological and physical properties reported for "bound" insulin.[1] The material obtained from rat liver perfusates is not immunoassayable using the conventional immunoassay methods for insulin. It is, however, biologically active, stimulating the incorporation of C-14 from labeled glucose into adipose tissue fat and adipose tissue and diaphragm glycogen when injected intraperitoneally. It has a molecular weight above 40,000 with possibly two peaks of activity, one in a fraction with a molecular weight of about 40,000 to 60,000, the other over 90,000. Unlike crystalline insulin, an anion at physiological pH, it can be absorbed at pH 7.4 on Dowex-50W (Na^+ cycle), a cation exchanger.

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