"Bound" Insulin: in vivo and in vitro Biologic Activity*

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Summary. Partially purified preparations of human serum "bound" insulin were injected intraperitoneally or intravenously into intact fed, fasted or fasted-refed rats, or incubated *in vitro* with their isolated epididymal adipose tissue. The biologic activity of "bound" insulin on muscle and adipose tissue, *in vivo* and *in vitro*, was compared with the activity of crystalline insulin standards. "Bound" and crystalline insulin injected intraperitoneally into rats stimulated the incorporation of glucose- $u\text{-}^{14}\text{C}$ into the glycogen of muscle and into the glycogen and fat of adipose tissue. The activities of both "bound" and crystalline insulin were affected by the nutritional state of the animals. Intravenous administration of partially purified "bound" insulin or crystalline insulin stimulated the incorporation of glucose- $u\text{-}^{14}\text{C}$ into the muscle glycogen of intact fed, fasted or fasted-refed rats and into the fat of the adipose tissue of fed or fasted-refed rats. "Bound" insulin, but not crystalline insulin stimulated fat synthesis in the adipose tissue of fasted rats. "Bound" or crystalline insulin, at the concentration used, failed to stimulate glycogen synthesis in adipose tissue. "Bound" insulin stimulated the oxidation of glucose into CO_2 in isolated rat adipose tissue and the incorporation of glucose- $u\text{-}^{14}\text{C}$ into the glycogen and fat of the adipose tissue. The effect of "bound" or crystalline insulin on isolated adipose tissue was also affected by the nutritional state of the animal.

Résumé. Cette étude porte sur l'injection intrapéritonéale ou intraveineuse de préparations partiellement purifiées d'insuline «liée» de sérum humain. Des rats nourris ad libitum, mis à jeun, ou renourris après une période de jeûne, ont servi pour ces injections, et leur tissu adipeux épiddidymaire a servi à l'étude de l'effet *in vitro* de ces préparations. L'activité biologique de cette insuline «liée» a été comparée avec celle d'une insuline cristalline standard. L'insuline «liée» tout comme l'insuline cristalline stimule l'incorporation de glucose- $u\text{-}^{14}\text{C}$ dans le glycogène du muscle et dans le glycogène et la graisse du tissu adipeux, à la suite de leur injection intrapéritonéale. L'état de nutrition des animaux injectés modifie les activités et de l'insuline «liée» et de l'insuline cristalline. L'injection intraveineuse d'insuline «liée» partiellement purifiée, ou d'insuline cristalline, stimule l'incorporation de glucose- $u\text{-}^{14}\text{C}$ dans le glycogène musculaire de rats nourris ad libitum, à jeun, ou renourris après un jeûne prolongé. Elle

stimule également l'incorporation du glucose dans la graisse du tissu adipeux de rats nourris ou renourris après jeûne. L'insuline «liée» seulement, non l'insuline cristalline, stimule la lipogénèse du tissu adipeux de rats à jeun. Tant l'insuline cristalline que l'insuline «liée» est restée sans effet, pour les dosages utilisés, sur la synthèse du glycogène dans le tissu adipeux. L'insuline «liée» stimule l'oxydation du glucose par le tissu adipeux isolé ainsi que son incorporation dans le glycogène et la graisse de ce tissu. L'action de l'insuline «liée» ou cristalline est également modifiée par l'état de nutrition de l'animal dont le tissu adipeux est utilisé *in vitro*.

Zusammenfassung. Teilweise gereinigte Präparate von „gebundenem“ Insulin aus menschlichem Serum wurden normalen gefütterten, hungernden oder nach Hungern wiederaufgefütterten Ratten intraperitoneal oder intravenös injiziert oder *in vitro* mit dem isolierten epididymalen Fettgewebe dieser Ratten inkubiert. Die biologische Aktivität des „gebundenen“ Insulins am Muskel und Fettgewebe, *in vivo* und *in vitro*, wurde mit der Aktivität von Standardlösungen kristallinen Insulins verglichen. Wurde „gebundenes“ und kristallines Insulin Ratten intraperitoneal injiziert, kam es zu einer vermehrten Einlagerung von Glukose- $U\text{-}^{14}\text{C}$ in Muskelglykogen und in das Glykogen und Fett des Fettgewebes. Sowohl die Aktivität des „gebundenen“ als auch des kristallinen Insulins wurden durch den Ernährungszustand der Tiere beeinflusst. Die intravenöse Verabreichung des teilweise gereinigten „gebundenen“ Insulins oder des Kristallinsulins führte zu einer verstärkten Einlagerung von Glukose- $U\text{-}^{14}\text{C}$ in das Muskelglykogen der gefütterten, hungernden oder der nach Hungern wiederaufgefütterten Ratten und auch in das Fett des Fettgewebes der gefütterten und nach Hungern wiederaufgefütterten Ratten. Nur das „gebundene“ Insulin, nicht das kristalline Insulin steigerte die Fettsynthese des Fettgewebes der hungernden Ratten. Weder das „gebundene“ noch das kristalline Insulin führten bei den verwendeten Konzentrationen zu einer Steigerung der Glykogensynthese des Fettgewebes. Das „gebundene“ Insulin stimulierte die Oxydation von Glukose zu CO_2 am isolierten Fettgewebe der Ratte und die Einlagerung von Glukose- $U\text{-}^{14}\text{C}$ in das Glykogen und Fett des Fettgewebes. Die Wirkung des „gebundenen“ und des kristallinen Insulins auf das isolierte Fettgewebe wurde ebenfalls durch den Ernährungszustand der Tiere beeinflusst.

In a previous report we have described some of the properties of circulating "free" and "bound" insulin¹.

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"Free" insulin appears to be similar in many respects to crystalline insulin extracted from pancreas. It exerts biologic activity on isolated adipose tissue and muscle, its biologic activity being inhibited by anti-insulin antisera. The molecular weight of "free" insulin is less than 40,000 and is probably similar to that of crystalline insulin-¹³¹I added *in vitro* to serum (sedimentation

coefficient 2.8 s)¹. The electrophoretic mobility of "free" insulin, like that of crystalline insulin-¹³¹I added *in vitro* to serum, is similar to α_1 -globulins and albumin¹.

It has been suggested that "bound" insulin represents a combination of "free" insulin with other substances or a combination of "free" insulin molecules, such a combination being catalyzed by tissues *in vivo*. "Bound" insulin is not generated *in vitro* by the addition of crystalline insulin to serum. The electrophoretic mobility of "bound" insulin is similar to that of β and γ globulins and its molecular weight is between 60,000 and 100,000, as estimated by chromatography on Sephadex G-75 and G-100¹.

The biologic activity of partially purified "bound" insulin preparations was originally tested *in vivo*, in hypophysectomized-alloxanized rats primed with glucose². A decrease in the blood glucose levels of these animals, following intraperitoneal injection of "bound" or of crystalline insulin standards, was used as an index of insulin activity⁸. Subsequently "bound" insulin was assayed *in vitro* on isolated rat epididymal adipose tissue and isolated rat diaphragm^{3, 4, 11}. "Bound" insulin was shown to exert activity on isolated adipose tissue but not on isolated diaphragm. However, addition of adipose tissue extracts (ATE) could "activate" "bound" insulin on isolated diaphragm^{3, 4, 18, 19}.

This report presents *in vivo* and *in vitro* evidence that the biologic effects of "bound" insulin are similar to those of crystalline insulin in fed, fasted and fasted-refed rats.

Materials and Methods

Preparation of "Bound" Insulin from Pooled Human Sera: Partially purified preparations of "bound" insulin were obtained from pooled normal human sera by chromatography on Dowex-50 W resin (Na⁺ cycle) as described elsewhere^{1, 5}. One milligram protein of the "bound" insulin preparation used in these studies was obtained from 4 milliliters of pooled sera and assayed about 500 microunits of insulin activity on isolated rat diaphragm in the presence of ATE⁴. One mg protein of the "bound" insulin contained less than 10 microunits of immunoassayable insulin, as measured by the MORGAN and LAZAROW technique¹⁴.

To achieve high concentrations of "bound" insulin, the "bound" insulin was first dialyzed against cold H₂O at 2°C, to remove the excess salt, and then concentrated by dialyzing against polyethylene glycol (Carbowax) at 2°C. The concentrated material was in turn dialyzed for 24 hours against 100 volumes of Gey and Gey bicarbonate buffer equilibrated with 95% O₂ : 5% CO₂.

Intraperitoneal Injections: Studies on the biologic activity of "bound" or crystalline insulin injected intraperitoneally into intact rats were carried out by the technique described by RAFAELSEN, LAURIS and REYNOLD¹⁵ as modified by CAHILL et al.⁹. Partially puri-

fied serum "bound" insulin or crystalline insulin in 5% human plasma albumin was injected intraperitoneally into intact rats along with glucose-u-¹⁴C (specific activity about 3.3 millicuries per millimole). The human plasma albumin was dissolved in Gey and Gey bicarbonate buffer and equilibrated with 95% O₂ : 5% CO₂. Five milliliters of the 5% albumin solution, with or without "bound" insulin or crystalline insulin, 4 mg glucose and 2 μ C glucose-u-¹⁴C were injected per rat (Charles River Laboratories, male CD rats). Before injection the rats were lightly anesthetized with 50% CO₂ : 50% O₂, 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% CO₂ : 50% O₂, decapitated, and both hemidiaphragms and epididymal pads removed rapidly. The epididymal pads were weighed immediately, placed in separate tubes each containing 20 ml. chloroform:methanol (2:1) and shaken for 4 hours. The hemidiaphragms were cooled rapidly on iced aluminum foil, weighed, placed in tubes containing 30% KOH at 100°C, hydrolyzed, and the glycogen precipitated¹⁵ and transferred to planchettes for counting. The chloroform-methanol extract of the adipose tissue containing the fat was counted in a liquid scintillation counter, the number of ester bonds measured⁶ and the incorporation of glucose carbon into fat expressed as counts per milliequivalent of ester bonds of fat. Glycogen was then isolated from the extracted adipose tissue and its radioactivity determined.

In these studies groups of 6–10 rats were injected with up to 3 levels of crystalline insulin and 3 levels of "bound" insulin. Control rats were injected with 5 ml of 5% human plasma albumin. The rats were either fed Purina chow *ad libitum*, fasted for 48 hours or fasted for 72 hours and then fed for 48 hours. The body weight of the fed rats ranged from 106 to 114 g, of the fasted (48 hrs) rats 84 to 93 g and of the fasted-refed rats 98 to 116 g.

Intravenous Injections: "Bound" insulin or crystalline insulin in Gey and Gey bicarbonate buffer or buffer alone was injected into groups of fed, fasted (48 hrs) or fasted (72 hrs) — refed (48 hrs) rats. The animals were lightly anesthetized with 50% CO₂ : 50% O₂ and the samples were injected into the jugular vein. Each rat received 1 ml of control buffer, partially purified "bound" insulin (7 or 10 mg protein) or crystalline insulin (1 mu or 10 mu) along with 2 μ C of glucose-u-¹⁴C (specific activity about 3.3 millicuries per millimole). Two hours after the injection the animals were anesthetized with 50% CO₂ : 50% O₂, decapitated and both hemidiaphragms and fat pads removed and processed for glycogen and fat extraction as described¹⁵.

Isolated Rat Epididymal Adipose Tissue Assay^{7, 13}: Pieces of isolated adipose tissue (120–150 mg) were placed in Warburg flasks and incubated at 37°C for 3 hours in Krebs-Ringer bicarbonate buffer, equilibrated with 95% O₂ : 5% CO₂, containing 0.05 g per cent

gelatin, 3 mg of glucose per ml, 0.6 μ C of glucose-u- 14 C, and either bovine crystalline insulin or partially purified serum "bound" insulin. Blank values were obtained by incubating pieces of each epididymal fat pad without crystalline or "bound" insulin. Net gas exchange was measured for 3 hours in all flasks and expressed as μ l CO₂ per 100 mg of wet weight adipose tissue. At the end of 3 hours, each piece of adipose tissue was washed 6 times in cold buffer and placed in tubes containing chloroform: methanol (2:1) and processed as described above.

In these studies, "bound" or crystalline insulin was incubated with adipose tissue obtained from fed rats, fasted rats (48 hrs) or fasted (48 hrs) — refed rats (48 hrs).

Results

Intraperitoneal Injections: "Bound" insulin or crystalline insulin injected intraperitoneally into fed, fasted or fasted refed rats stimulated the incorporation of

glucose-u- 14 C into the glycogen of muscle and adipose tissue and into the fat of adipose tissue (Table 1).

"Bound" insulin compared with crystalline insulin, appeared to be less active in stimulating glycogen than fat synthesis by the adipose tissue of fed rats.

In fasted (48 hrs) rats, "bound" insulin exerted less activity on muscle than crystalline insulin, but more on adipose tissue when compared with the results obtained in fed rats (Table 1).

In fasted-refed rats the activity of "bound" and crystalline insulin on muscle was similar to that of fed rats. However, the incorporation of glucose-u- 14 C into the fat of adipose tissue in the presence of "bound" or crystalline insulin was markedly increased. "Bound" insulin compared with crystalline insulin was less effective in stimulating glycogen synthesis than fat synthesis in the adipose tissue of these rats (Table 1).

Intravenous Injections: "Bound" or crystalline insulin stimulated the incorporation of glucose-u- 14 C into the muscle glycogen of fed, fasted and fasted-refed rats

Table 1. The effect of "bound" or crystalline insulin injected intraperitoneally into intact rats on the incorporation of radioactivity from glucose-u- 14 C into glycogen of diaphragm and glycogen and fat of adipose tissue

Amount Injected Into Each Rat	Diaphragm glycogen		Adipose tissue glycogen		Adipose tissue fat	
	cpm/g tissue ± SEM	P <	cpm/g tissue ± SEM	P <	cpm/mEq ester bond of fat ± SEM	P <
Control	2200 ± 161 (36)		<i>Fed Rats</i> 163 ± 12 (34)		3525 ± 623 (10)	
"Bound" Insulin:						
0.4 mg protein	3188 ± 274 (10)	NS	200 ± 32 (10)	NS	—	—
1.25 mg protein	8030 ± 673 (24)	0.001	370 ± 12 (21)	0.001	9383 ± 1499 (10)	0.001
2.5 mg protein	14100 ± 973 (24)	0.001	550 ± 33 (24)	0.001	13583 ± 1087 (10)	0.001
Crystalline Insulin:						
100 μ u	3800 ± 521 (10)	0.01	258 ± 24 (10)	0.01	5380 ± 618 (10)	0.01
1000 μ u	15550 ± 1298 (30)	0.001	2061 ± 238 (28)	0.001	14081 ± 2812 (10)	0.001
10000 μ u	28300 ± 1913 (24)	0.001	5857 ± 473 (24)	0.001	29200 ± 2221 (10)	0.001
Control	5440 ± 510 (24)		<i>Fasted (48 hrs) Rats</i> 102 ± 8 (23)		5176 ± 748 (10)	
"Bound" Insulin:						
0.4 mg protein	9051 ± 618 (10)	0.01	280 ± 14 (10)	0.001	—	—
1.25 mg protein	22000 ± 2029 (23)	0.001	300 ± 12 (24)	0.001	9125 ± 658 (10)	0.01
2.5 mg protein	31760 ± 4400 (24)	0.001	391 ± 26 (23)	0.001	12500 ± 1223 (10)	0.001
Crystalline Insulin:						
100 μ u	13526 ± 1134 (10)	0.001	103 ± 8 (10)	NS	5193 ± 658 (10)	NS
1000 μ u	63592 ± 3999 (24)	0.001	112 ± 5 (24)	NS	6860 ± 740 (10)	NS
10000 μ u	108560 ± 6169 (16)	0.001	122 ± 13 (16)	NS	9495 ± 629 (10)	0.05
Control	2360 ± 284 (10)		<i>Fasted (72 hrs) — Refed (48 hrs) Rats</i> 115 ± 15 (10)		2795 ± 327 (10)	
"Bound" Insulin:						
1.25 mg protein	8600 ± 754 (10)	0.001	296 ± 36 (10)	=0.001	32900 ± 3545 (10)	0.001
2.5 mg protein	12520 ± 1354 (10)	0.001	647 ± 68 (10)	0.001	41600 ± 4757 (10)	0.001
Crystalline Insulin:						
100 μ u	3940 ± 378 (10)	0.01	301 ± 37 (10)	0.001	15925 ± 1718 (10)	0.001
1000 μ u	12920 ± 879 (10)	0.001	2412 ± 151 (10)	0.001	52300 ± 3201 (10)	0.001
10000 μ u	30910 ± 1013 (10)	0.001	5620 ± 572 (10)	0.001	73500 ± 6014 (10)	0.001

Each rat was injected with 2 μ C of glucose-u- 14 C (3.3 mC/millimole) in 5 ml of 5% human serum albumin containing either no insulin (control) or various amounts of partially purified "bound" insulin or crystalline bovine insulin. The rats were sacrificed two hours after the injection. The values are means of the average counts per minute incorporated in the two epididymal pads or two hemidiaphragms per gm tissue in each rat.

Numbers in parenthesis represent the number of rats used. One mg of the partially purified "bound" insulin preparation used was obtained from 4 ml of pooled human sera and assayed about 500 μ u insulin activity on isolated rat diaphragm in the presence of adipose tissue extract (ATE)⁴.

Table 2. The effect of "bound" or crystalline insulin injected intravenously into intact rats on the incorporation of radioactivity from glucose- u - ^{14}C into glycogen of diaphragm and glycogen and fat of adipose tissue

Amount injected into each rat	Diaphragm glycogen		Adipose tissue glycogen		Adipose tissue fat	
	cpm/g tissue $\pm$ SEM	P <	cpm/g tissue $\pm$ SEM	P <	cpm/ μ Eq ester bond of fat $\pm$ SEM	P <
Control	3196 $\pm$ 603 (10)		<i>Fed Rats</i> 20 $\pm$ 4 (10)		2494 $\pm$ 408 (10)	
"Bound" Insulin: 10 mg protein	5169 $\pm$ 591 (9)	0.05	28 $\pm$ 6 (9)	NS	2575 $\pm$ 129 (9)	NS
Crystalline Insulin: 1000 μ u	3190 $\pm$ 485 (10)	NS	22 $\pm$ 5 (10)	NS	2665 $\pm$ 266 (10)	NS
10000 μ u	7558 $\pm$ 847 (10)	0.001	22 $\pm$ 3 (10)	NS	2820 $\pm$ 408 (10)	NS
Control	7133 $\pm$ 1238 (19)		<i>Fasted (48 hrs) Rats</i> 118 $\pm$ 8 (19)		7068 $\pm$ 709 (19)	
"Bound" Insulin: 10 mg protein	16054 $\pm$ 1834 (15)	0.001	141 $\pm$ 15 (15)		14965 $\pm$ 2375 (15)	0.01
Crystalline Insulin: 1000 μ u	10080 $\pm$ 1170 (10)	NS	116 $\pm$ 9 (10)	NS	6444 $\pm$ 555 (10)	NS
10000 μ u	29280 $\pm$ 2770 (10)	0.001	115 $\pm$ 10 (10)	NS	8410 $\pm$ 709 (10)	NS
Control	1993 $\pm$ 386 (10)		<i>Fasted (72 hrs) — Refed (48 hrs) Rats</i> 101 $\pm$ 17 (10)	NS	14200 $\pm$ 869 (10)	
"Bound" Insulin: 7 mg protein	3635 $\pm$ 447 (10)	0.02	70 $\pm$ 7 (10)	NS	19800 $\pm$ 663 (10)	0.01
Crystalline Insulin: 1000 μ u	2658 $\pm$ 422 (9)	NS	71 $\pm$ 18 (9)	NS	16330 $\pm$ 1213 (9)	NS
10000 μ u	6586 $\pm$ 1218 (10)	0.01	98 $\pm$ 26 (10)	NS	19800 $\pm$ 663 (10)	0.01

Each rat was injected with 2 μ C of glucose- u - ^{14}C (3.3 mC/millimole) in 1 ml of physiological saline containing either no insulin (control) or partially purified "bound" insulin or crystalline bovine insulin. The rats were sacrificed two hours after the injection. The values are means of the average counts per minute incorporated in the two epididymal pads or two hemidiaphragms per gm tissue in each rat.

Numbers in parenthesis represent the number of rats used. One mg of the partially purified "bound" insulin preparation used was obtained from 4 ml of pooled human sera and assayed about 500 μ u of insulin activity on isolated rat diaphragm in the presence of ATE⁴.

Table 3. The effect of "bound" or crystalline insulin incubated with isolated epididymal adipose tissue of intact rats on the oxidation of glucose into CO_2 and the incorporation of radioactivity from glucose- u - ^{14}C into glycogen and fat

Amount per ml incubating medium	Net Gas Exchange in μ l CO_2 /100 mg Tissue $\pm$ SEM	P <	Adipose tissue glycogen		Adipose tissue fat	
			cpm/gm Tissue $\pm$ SEM	P <	cpm/ μ Eq of ester bond of fat $\pm$ SEM	P <
Control	-9 $\pm$ 4		<i>Fed Rats</i> 1500 $\pm$ 293		163 $\pm$ 41	
"Bound" Insulin: 0.4 mg protein	61 $\pm$ 9	0.001	8460 $\pm$ 1890	0.001	312 $\pm$ 51	0.05
1.25 mg protein	99 $\pm$ 17	0.001	12700 $\pm$ 2710	0.01	498 $\pm$ 17	0.001
Crystalline Insulin: 100 μ u	96 $\pm$ 10	0.001	14220 $\pm$ 2620	0.001	325 $\pm$ 44	0.02
500 μ u	125 $\pm$ 11	0.001	40310 $\pm$ 7460	0.001	485 $\pm$ 57	0.001
Control	4 $\pm$ 3		<i>Fasted (48 hrs) Rats</i> 1024 $\pm$ 95		152 $\pm$ 15	
"Bound" Insulin: 0.4 mg protein	54 $\pm$ 7	0.001	1570 $\pm$ 253	NS	243 $\pm$ 12	0.001
1.25 mg protein	93 $\pm$ 4	0.001	2088 $\pm$ 103	0.001	312 $\pm$ 12	0.001
Crystalline Insulin: 100 μ u	45 $\pm$ 10	0.001	1576 $\pm$ 218	0.05	186 $\pm$ 28	NS
500 μ u	71 $\pm$ 9	0.001	2086 $\pm$ 152	0.001	269 $\pm$ 33	0.01
Control	-20 $\pm$ 6		<i>Fasted (48 hrs) — Refed (48 hrs) Rats</i> 2400 $\pm$ 82		112 $\pm$ 17	
"Bound" Insulin: 0.4 mg protein	108 $\pm$ 18	0.001	15590 $\pm$ 2480	0.001	626 $\pm$ 98	0.001
1.25 mg protein	153 $\pm$ 17	0.001	31850 $\pm$ 6400	0.001	176 $\pm$ 68	0.001
Crystalline Insulin: 100 μ u	134 $\pm$ 15	0.001	27280 $\pm$ 3726	0.001	636 $\pm$ 79	0.001
500 μ u	153 $\pm$ 23	0.001	56660 $\pm$ 440	0.001	834 $\pm$ 95	0.001

Values are means of six experiments. One mg of the partially purified "bound" insulin preparation used was obtained from 4 ml of pooled human sera and assayed about 500 μ u of insulin activity on isolated rat diaphragm in the presence of ATE⁴.

and into the fat of the adipose tissue of fasted-refed rats (Table 2). "Bound" insulin also stimulated the incorporation of glucose carbon into the fat of the adipose tissue of fasted-refed rats. "Bound" or crystalline insulin in the amounts used, did not stimulate the incorporation of glucose- u - ^{14}C into the glycogen of the adipose tissue of intact rats.

Isolated Rat Epididymal Adipose Tissue: Both "bound" and crystalline insulin stimulated the oxidation of glucose to CO_2 and the incorporation of glucose carbon into the fat and glycogen of isolated adipose tissue from intact rats (Table 3). The response of the adipose tissue of fasted-refed rats to "bound" or crystalline insulin was increased when compared with that of fed rats. On the other hand the response of the adipose tissue of fasted rats to "bound" or crystalline insulin was lower than that of fed or fasted-refed rats (Table 3).

Discussion

Partially purified preparations of "bound" insulin exerted *in vivo* and *in vitro* biologic activities qualitatively similar to those of crystalline insulin. Intraperitoneal injections of "bound" insulin into rats stimulated the incorporation of glucose- u - ^{14}C into the glycogen of muscle and adipose tissue and into the fat of adipose tissue. Intravenous injections of "bound" insulin stimulated glycogen synthesis by muscle in fed, fasted and fasted-refed rats. "Bound" or crystalline insulin injected intravenously failed to stimulate the incorporation of glucose- u - ^{14}C into the glycogen of adipose tissue of these rats or into the fat of the adipose tissue of fed rats. Rats appear to be relatively insensitive to intravenous administration of "bound" or crystalline insulin, an observation consistent with that reported earlier by RAFAELSEN^{16,17}.

The biologic activity of both "bound" and crystalline insulin was affected by the nutritional state of the animals. In both *in vivo* and *in vitro* studies the activity of "bound" and crystalline insulin on rat adipose tissue was greatly suppressed by fasting. On the other hand refeeding after fasting markedly increased their activity on adipose tissue. These findings confirm similar observation reported earlier by GOLDMAN and CAHILL¹⁰, RAFAELSEN et al.¹⁵ and HUBER et al.¹².

Quantitative differences in the biologic activity of "bound" and crystalline insulin were observed in these studies. When amounts of crystalline or "bound" insulin which had quantitatively similar effects on fed or fasted-refed rats were injected intraperitoneally into fasted rats the "bound" insulin was only half as effective as the crystalline insulin in promoting muscle glycogen synthesis, but more effective in stimulating fat synthesis (Table 1). The differences in activity between "bound" and crystalline insulin may reflect changes in their relative rates of "utilization" by various tissues under different metabolic conditions, differences in their molecular specificity on cell mechanisms

under various conditions, modification of their activities through molecular changes by tissues, or other phenomena.

The present studies show that *in vivo* "bound" insulin exerts biologic activity on the diaphragm of intact rats. Earlier studies have shown that *in vitro* "bound" insulin exerted biologic activity on isolated adipose tissue but not on isolated rat diaphragm except in the presence of adipose tissue extracts (ATE)⁴. The difference between the *in vivo* and *in vitro* effect of "bound" insulin on muscle tissue indicates the presence of mechanisms in the intact animal which *in vivo* catalyze the activation of "bound" insulin. The nature of such mechanisms is unknown. The fact that "bound" insulin exerts biologic activity on muscle when injected intraperitoneally may suggest the possibility that *in vivo* the epididymal or mesenteric fat or other tissues lining the intraperitoneal cavity of the intact rats contribute to the activation of the injected "bound" insulin. Whatever the nature of the mechanisms which activate "bound" insulin, its utilization *in vivo* appears to be affected by the nutritional conditions of the animals.

The present *in vivo* and *in vitro* studies provide further evidence that the biologic activity of "bound" insulin corresponds to that of crystalline insulin. "Bound" insulin in these studies was used in a partially purified form. The animals received intraperitoneal injections of between 400 and 2,500 μg protein per rat and intravenous injections of 10,000 μg protein per rat. These amounts seem too far below the protein concentration of 1 ml of whole serum (about 60,000 μg protein per ml) for the effects to represent non-specific insulin-like affects of serum proteins.

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