Effect of Insulin on FFA Mobilization and Ketosis in Fasting Pregnant Rats

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SUMMARY

The concentrations of FFA and ketone bodies in plasma increased more in rats pregnant for eighteen days than in nonpregnant rats when both groups were fasted for two days. The immunoreactive insulin content of plasma was the same in fasted nonpregnant and pregnant rats whether the latter were ketotic or not. Hence, decreased circulating insulin, per se, did not appear to be the primary cause of ketosis in fasting pregnant rats. FFA mobilization was not suppressed completely by insulin in ketotic pregnant rats.

The adipose tissue of pregnant rats showed impaired metabolism of glucose and increased FFA release in vitro. Lipolysis, in the presence or absence of insulin, was greater in adipose tissue of pregnant than in that of nonpregnant rats. Ketogenesis by liver slices of pregnant rats was greater than that of nonpregnant control rats and was not reduced thirty minutes after insulin injection despite a 50 per cent lowering of the concentration of plasma FFA and ketone bodies during this time.

It is suggested that the ketosis of fasting in pregnant rats is caused by increased lipolysis in their adipose tissues and that increased FFA release is due to the absence of sufficient glucose to support re-esterification in the adipose tissues. The small effect of insulin (0.2 and 1.0 U., subcutaneously) on the plasma FFA may be due to the very low plasma glucose in fasting pregnant rats. DIABETES *19*: 563-70, August, 1970.

It was suggested that insulin lack, secondary to hypoglycemia, may increase fat mobilization and cause severe ketosis in pregnant rats fasted during the last part of gestation.[1] Subsequently it was found that cortisone and growth hormone are also necessary for this syndrome to develop.[2]

The present experiments were designed to study the relationship between plasma insulin concentration and lipid mobilizations in pregnant rats fasted near term and to determine the response of these rats to exogenous insulin.

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METHODS

The preparation and care of pregnant rats have been described previously.[1] Serial blood samples (0.7 to 0.8 ml.) were taken from the tip of the tail into a spot plate containing heparin and transferred to cold plastic centrifuge tubes (Beckman Microfuge tubes, capacity 0.35 to 0.4 ml.). Usually two tubes were filled and centrifuged (Beckman Microfuge, 15,000 rpm for three minutes). The plasma was immediately aliquoted: 25 μl. were deproteinized for determination of glucose[3] and ketone bodies,[4] 50 μl. were taken for free fatty acids (FFA), and 25, 50 and 100 μl. for insulin immunoassay (IRI).[5] Remaining plasma and tubes containing the various aliquots were stored frozen (—20° C.) until they were needed for analysis.

In some experiments the rats were anesthetized with ether and aortic blood drawn into a heparinized syringe. The blood was cooled in an ice bath and centrifuged at 2° C. The plasma was analyzed for ketone bodies and glucose as before. An aliquot of plasma was extracted for determination of free fatty acids by titration.[6]

Direct determination of free fatty acids. The method of Novak[7] was modified to permit the direct measurement of plasma FFA without prior extraction. The cobalt reagent contained 10 ml. of 6 per cent Co $(NO_3)_2 \cdot 6H_2O$ in saturated K_2SO_4 solution, 7 ml. of saturated Na_2SO_4 solution and 1.35 ml. of triethanolamine. To 50 μl. of plasma in a 16 × 100 mm. screw-cap culture tube (Kimax #45066A) were added 50 μl. of saturated sodium acetate (adjusted to pH 7.0 with glacial acetic acid) and 0.3 ml. of cobalt reagent (prepared daily). The contents of the tube were mixed (Vortex mixer) and allowed to stand at room temperature for fifteen minutes. Three milliliters of chloroform-hexane (1:1) were added. The tubes were

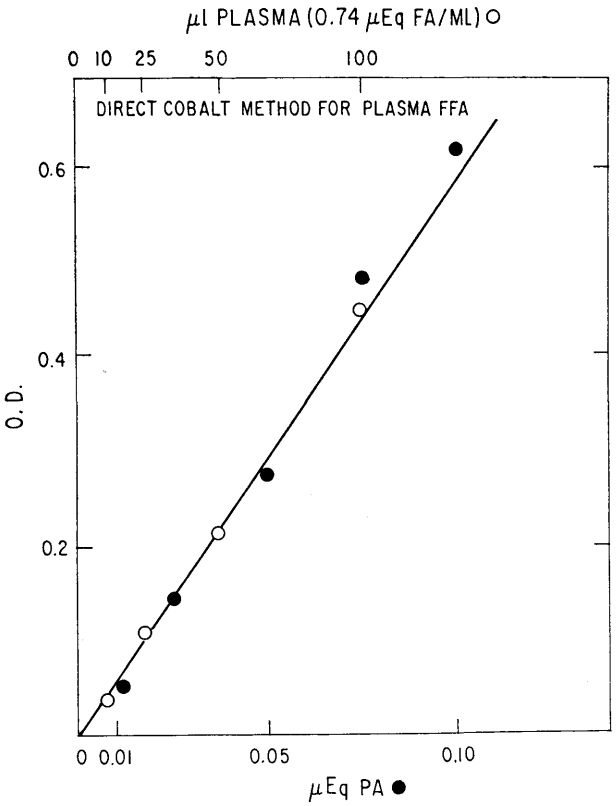

FIG. 1. Comparison of a standard curve for palmitic acid (PA) with aliquots of a plasma pool (FFA = 0.74 μEq./ml.) employing the method of direct determination of plasma FFA described in the text.

tightly closed with Teflon-lined caps and placed in a high speed shaker for three to five minutes. The tubes were centrifuged at 2,500 rpm for five minutes to separate the aqueous layer from the chloroform-hexane phase. Aliquots of the latter were mixed with 0.5 ml. of 0.03 per cent 1-nitroso-2-naphthol in ethanol. After fifteen minutes, the samples were read at 500 mu. in a spectrophotometer and compared with appropriate standards. The optical densities of various aliquots of plasma and of palmitic acid standards carried through this procedure are compared in figure 1.

The data suggest that the direct determination of FFA is not affected by variation in protein and other plasma constituents over the range studied. Plasma FFA concentrations measured by the direct procedure are compared in table 1 with those obtained by the method of Dole and Meinertz,[6] which involves extraction of the fatty acids into heptane and titration of the washed heptane phase, and with those obtained with the cobalt procedure on the heptane phase.[7] The similarities between the results of the three procedures demonstrate that the cobalt methods are not sensitive to phospholipids and other acidic contaminants present in the plasma. Hemolysis did not interfere with the determination of FFA by the direct cobalt method until one third or more of the red blood cells were broken (Novak, unpublished findings). A report employing the direct cobalt procedure has appeared.[8]

In vitro procedures. Parametrial adipose tissue (50 to 100 mg.) was incubated for two hours at 37° C. in a 2 ml. bicarbonate buffer solution containing 4 per cent albumin and 3 mM. glucose (0.5 μc. of glucose-1-C-14). The gas phase was 5 per cent CO_2—95 per cent O_2. The incubation and analytic procedures have been described previously.[9]

Release of fatty acids to the medium was measured by a modification of Dole's procedure.[6] After absorption of CO_2 the flask was opened, and the tissue was removed and placed in 10 ml. of 2:1 chloroform-methanol. To the 2 ml. of acidified medium remaining in the incubation flask, 5 ml. of 4:1 isopropanol-hexane were added and the contents mixed vigorously. Hexane (3 ml.) and water (1 ml.) were added and the contents transferred to a 13 ml. centrifuge tube. Appropriate blanks and palmitic acid standards were treated in the same manner. Aliquots of the hexane phase were evaporated in a conical centrifuge tube to a volume of less than 0.5 ml. One milliliter of Nile blue indicator (0.001 per cent in ethanol) was added and the fatty acids (0.05-1.0 μEq.) titrated with 0.01 N NaOH (Menisco-matic buret, American Instrument Co., Silver Spring, Md.).

Ketogenesis by liver slices was determined as described previously.[4,11] Representative slices were extracted in chloroform-methanol (2:1) for determination of triglyceride content.[10]

TABLE 1

Comparison of cobalt methods with a titration method for determination of FFA in plasma

No. of plasmas*	Heptane extract†		Direct cobalt method‡
	Titration	Cobalt	
	μEq. FFA per ml. plasma		
9	0.51±0.04	—	0.54±0.03
7	0.65±0.05	0.71±0.04	0.73±0.06

*Aorta blood plasma from normal rats fasted two days; blood drawn while rat was under ether anesthesia.

†1 ml. plasma extracted by the method of Dole and Meinertz.[6] About 2 ml. of the extract, washed with lower phase, was titrated and 0.2-0.5 ml. was assayed by the method of Novak.[7]

‡50 μl. of plasma assayed; see Methods.

TABLE 2

Effects of pregnancy and fasting on plasma concentration of glucose, ketone bodies, FFA and IRI in tail blood

Group	No. of rats	Days pregnant	No. of fetuses	Days fasted	Glucose mg./100 ml.	Ketone bodies mg./100 ml.	FFA μEq./ml.	IRI† μU./ml.
I	15	none	none	0	119±5*	4±2	0.5±0.1	157±15
				1	110±10	7±2	1.1±0.1	118±10
				2	100±3	12±1	1.5±0.1	84±10
II	7	18	2-5	0	106±7	3±2	0.4±0.1	172±19
		20		2	101±6	13±3	1.2±0.2	103±17
III	15	18	7-14	0	96±2	2±0.4	0.4±0.04	178±11
		19		1	64±3	24±2	1.2±0.1	83± 6
		20		2	55±3	56±3	1.4±0.1	88± 6

*Mean ± S.E.M.
†Immunoreactive insulin.

RESULTS

Effect of fasting. The effects of fasting on the plasma concentration of glucose, total ketone bodies, FFA and IRI in tail blood are recorded in table 2. The values in the fed state were similar for nonpregnant (Group I) and pregnant rats (Groups II and III) except for lower plasma glucose levels in the pregnant rats with more than seven fetuses (Group III). Fasting for one day caused hyperketonemia and lowered the concentrations of plasma glucose and IRI in Group III. Ketonemia increased further after the second day of fasting, whereas the concentrations of plasma glucose, FFA and IRI remained the same. Plasma values of rats with less than five fetuses (Group II) did not differ after two days of fasting from those of the nonpregnant groups. The plasma IRI of pregnant and nonpregnant rats were the same after two days of fasting but the pregnant rats in Group III were ketotic (table 2).

Some of the ketotic rats (Group III) were anesthetized with ether and the fetuses delivered by cesarean section. The results in table 3 show that the ketone body concentration of fetal plasma was similar to that of maternal plasma in agreement with earlier findings.[12] Glucose and FFA concentrations were lower and IRI levels were higher in fetal than in maternal circulating plasma. These results support the view that the ketone bodies of the fetus are derived from the maternal circulation.[12]

Effects of insulin. Since plasma IRI of ketotic pregnant rats was the same as in nonketotic animals (table 2), it seems unlikely that the ketosis of fasting in pregnant rats was due primarily to the concentration of circulating insulin. The effect of exogenous insulin was then tested in ketotic pregnant rats and in nonpregnant controls also fasted for two days. Within fifteen to thirty minutes after the subcutaneous injection of 0.2 or 1 U. of Regular Insulin (Iletin, Lilly), the plasma IRI rose to its peak value (figure 2). The patterns of plasma IRI of fasted pregnant and control rats were similar after injection of either dose.

Insulin injection lowered the plasma glucose of nonpregnant and pregnant rats to about 40 mg. per 100 ml. plasma in thirty minutes (figure 2). The higher dose of insulin prolonged the period of hypoglycemia for both groups.

Insulin lowered the ketone body concentration in fasted pregnant rats from 45 to 15 mg. per 100 ml. plasma in sixty minutes whether 0.2 or 1.0 U. of insulin was injected (figure 3). Three hours after the injection of insulin the plasma ketone body concentration had risen to 30 mg. per 100 ml. in the rats injected with 0.2 U. but was 12 mg. per 100 ml. in those receiving 1 U. of insulin. The plasma ketone body concentration

TABLE 3

Glucose, ketone bodies, FFA and IRI in maternal and fetal plasma

Group (n)	Concentration in plasma			
	Glucose mg./ 100 ml.	Ketone bodies mg./ 100 ml.	FFA μEq./ml.	IRI μU./ml.
Maternal (6)	70±6	65±3	0.84±0.12	65± 9
Fetal (6)	30±6	51±6	0.24±0.06	133±18

Rats pregnant eighteen to nineteen days were fasted two days and then anesthetized with ether. Blood was drawn from the maternal aorta into a heparinized syringe and the fetuses removed immediately. Blood from eight to ten fetuses of each of the six litters was collected after decapitation and pooled for analysis. Results are given as mean ± S.E.M.

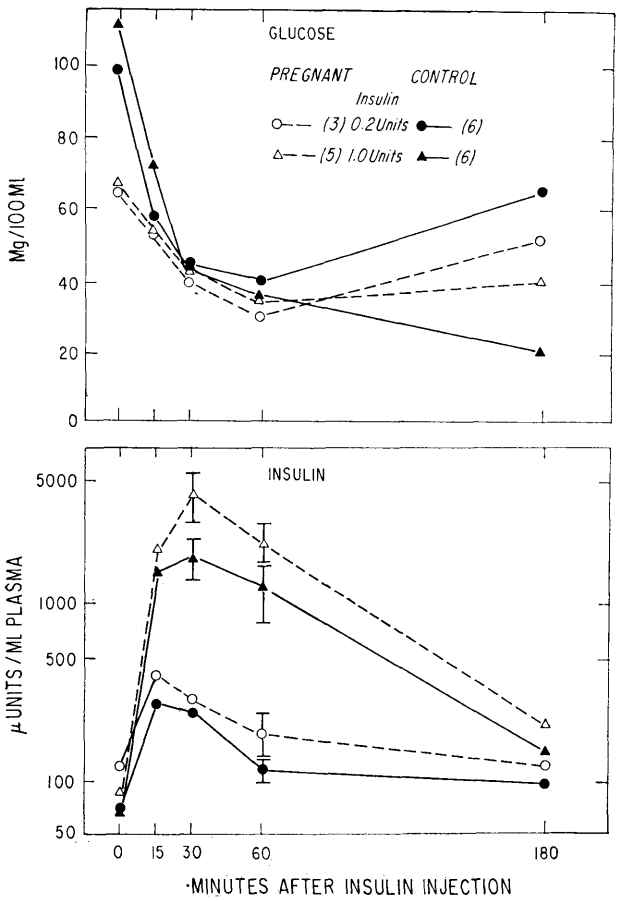

FIG. 2. Effect of insulin injection (subcutaneously) on the glucose and immunoreactive insulin concentration of the tail plasma of twenty-day pregnant and of nonpregnant rats. The pregnant rats had more than seven fetuses. All rats were fasted two days. The number of rats in each group is given in parentheses.

in nonpregnant rats was decreased from 15 to 8 and 4 mg. per 100 ml. in sixty minutes by 0.2 and 1 U. of insulin, respectively (figure 3). By three hours after insulin the plasma ketone body concentration had returned to near the preinsulin values.

The concentration of FFA in tail blood plasma was the same in pregnant and nonpregnant rats after two days of fasting (table 2 and figure 3). After insulin injection the concentration of plasma FFA was lower in the nonpregnant rats; after thirty and sixty minutes the differences in the concentrations of plasma FFA of pregnant and nonpregnant rats were highly significant ($p < 0.01$). These results suggested that fatty acid release might not be completely inhibited by insulin in pregnant rats.

Effect of glucose. Glucose (3 gm./kg.) was given by stomach tube to fasted pregnant and nonpregnant rats (figures 4 and 5). Glucose administration rapidly increased the plasma glucose and IRI content, perhaps more in pregnant than nonpregnant rats, and markedly decreased hyperketonemia as reported earlier.[1] The FFA content of tail plasma of pregnant rats was lower than that of nonpregnant rats from fifteen to sixty minutes after glucose administration ($p < 0.01$).

The response to injection of isotonic saline demonstrates that handling and bleeding from the tail had little or no effect on the four parameters studied.

Effect of insulin on FFA, ketone bodies and glucose in aorta blood plasma. Comparison of the data for two-day fasted pregnant rats in tables 2 and 3 showed that the plasma obtained from aortic blood, under ether anesthesia (table 3) contained less FFA than did plasma from tail blood.

A comparison of tail and aortic plasma (table 4) suggested that the difference in FFA concentration was less when the rats were anesthetized. Anesthesia pro-

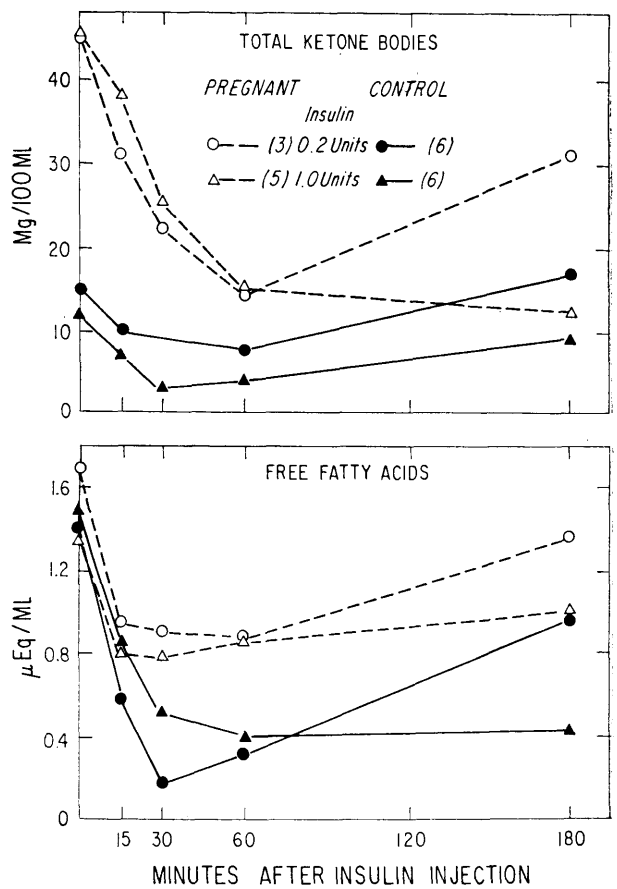

FIG. 3. Effect of insulin injection on the total ketone body and FFA concentration of the tail plasma. Same experiment as figure 2.

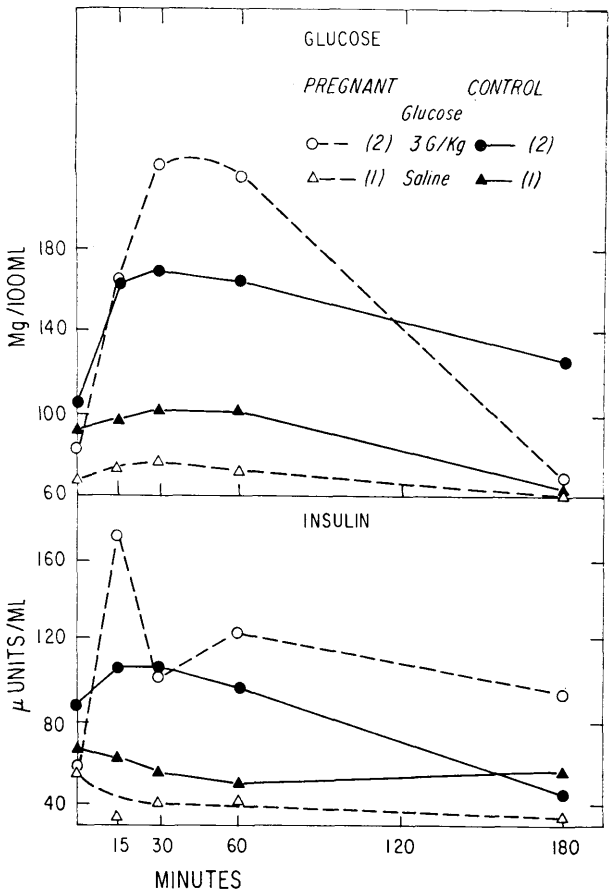

FIG. 4. Effect of glucose (3 gm./kg., per os) on the glucose and immunoreactive insulin concentration of the tail plasma of twenty-day pregnant and of nonpregnant rats. Pregnant rats had more than seven fetuses. All rats were fasted for two days. The saline-injected rats received 1 ml., subcutaneously, of a 0.9 per cent NaCl solution. The number of rats in each group is given in parentheses.

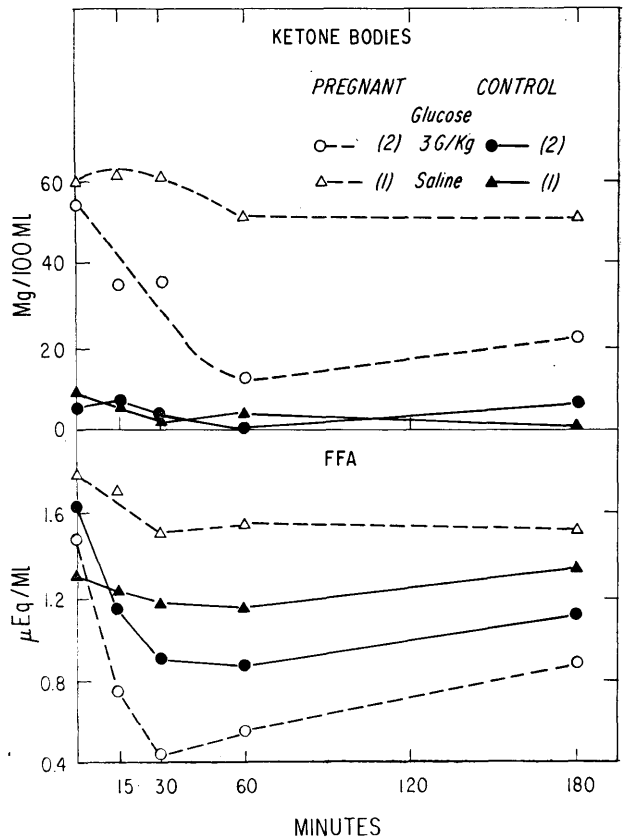

FIG. 5. Effect of glucose on the total ketone body and FFA concentration of tail plasma. Same experiment as figure 4.

motes vasodilation in the tail resulting in a faster blood flow. It is, therefore, suggested that the tail blood sample of unanesthetized rats is largely venous while that of anesthetized rats is largely arterial. The area drained by the tail vein may contain cells that release relatively large amounts of FFA. Therefore, the effects of insulin on the aortic plasma levels of FFA, ketone bodies and glucose of fasted pregnant and nonpregnant rats were tested. Since the maximum response of the FFA concentration in tail plasma to the administration of glucose and insulin occurred at about thirty minutes, this period after insulin was used in studies with aortic plasma. The selection and treatment of the rats were the same as before except that twenty-eight minutes after insulin the rats were anesthetized with ether and at thirty minutes after insulin aortic blood was drawn into a heparinized syringe.

The plasma glucose and ketone body concentrations in the aortic blood of two-day fasted rats (table 5) are in good agreement with those obtained with the tail plasma of similar rats (Groups I and III, table 2). The plasma FFA levels, however, were much lower in aortic than in tail blood, as noted above. Insulin (1 U., subcutaneously) lowered the concentrations of glucose and ketone bodies in aortic blood plasma to the same degree as had been observed earlier (table 5 and figures 2 and 3, thirty minutes after insulin). Although insulin lowered the plasma FFA concentration of aortic blood, the values for the pregnant rats were significantly higher than those of the nonpregnant group. These results (table 5) support the view that FFA release might not be completely suppressed by insulin in fasted pregnant rats. Therefore, the effect of insulin on the in vitro metabolism of parametrial adipose tissue of fasted pregnant and nonpregnant rats was investigated.

Response of adipose tissue to insulin, in vitro. The

TABLE 4

Comparison of methods of blood collection on the concentration of plasma glucose and FFA

Rat no.	Anesthesia	Blood collection†	Plasma glucose mg./100 ml.	FFA‡ μEq./ml.
1*	none	tail	102	1.33
	ether	tail	135	0.53
	ether	aorta	140	0.55
2*	none	tail	119	1.37
	ether	tail	152	0.67
	ether	aorta	140	0.55
3	none	tail	122	0.62
	pentobarb.	tail	147	0.43
4	none	tail	130	0.61
	pentobarb.	tail	147	0.25
5	none	tail	130	0.46
	ether	tail	156	0.18
6	none	tail	130	0.70
	ether	tail	156	0.36
7*	none	tail	110	1.25
	none	decap§	114	0.54
8*	none	tail	119	1.14
	none	decap§	119	0.50

*Rats fasted overnight; others were fed.
†Blood was collected with heparin and cooled immediately.
‡Direct cobalt procedure (see Methods).
§Decapitation and collection of blood in a chilled beaker containing heparin.

triglyceride content of the parametrial adipose tissue of fasted pregnant rats was the same as that of nonpregnant controls (0.7 to 0.8 millimole per gm. tissue). The adipose tissue of pregnant rats converted less of the added glucose to CO_2 and total lipid (largely glyceride-glycerol) than did the tissue of nonpregnant rats (figure 6). Insulin (60 and 120 μU./ml.) increased the metabolism of glucose by the adipose tissue of both groups. However, even in the presence of 120 μU. of insulin per ml. of medium the adipose tissue of pregnant rats oxidized less glucose to CO_2 and converted little or no glucose to fatty acid. The major effect of insulin on the adipose tissue of pregnant rats was to increase the formation of glyceride-glycerol from glucose.

TABLE 5

Effect of insulin on plasma concentration of glucose, ketone bodies and FFA in aortic blood of pregnant and nonpregnant rats fasted two days

Group	Insulin injected‡ Units	No. of rats	Aortic plasma concentration* Glucose mg./100 ml.	Ketone bodies mg./100 ml.	FFA μEq./ml.
Non-pregnant	0	9	115±4	11.6±1.2	0.69±0.5
	1	8	41±2	2.9±0.3	0.27±0.02
Pregnant†	0	7	69±3	50.4±5.8	1.02±0.05
	1	7	40±4	24.8±4.2	0.43±0.04

*Mean ± S.E.M.
†Pregnant eighteen days at start of fast; seven to twelve fetuses/rat.
‡Insulin injected subcutaneously, thirty minutes earlier.

The release of FFA to the medium was measured also in these experiments. The parametrial adipose tissue of fasted pregnant and nonpregnant rats released FFA to the medium (figure 7). The amount of FFA released by adipose tissue of the pregnant rats was significantly larger. The stimulatory effect of insulin on glyceride-glycerol formation paralleled its action in decreasing FFA release in vitro.

Ketogenesis by liver slices. Since the plasma concentrations of both FFA and ketone bodies of pregnant rats fall after insulin, the effect of insulin on ketogenesis by liver slices was studied.

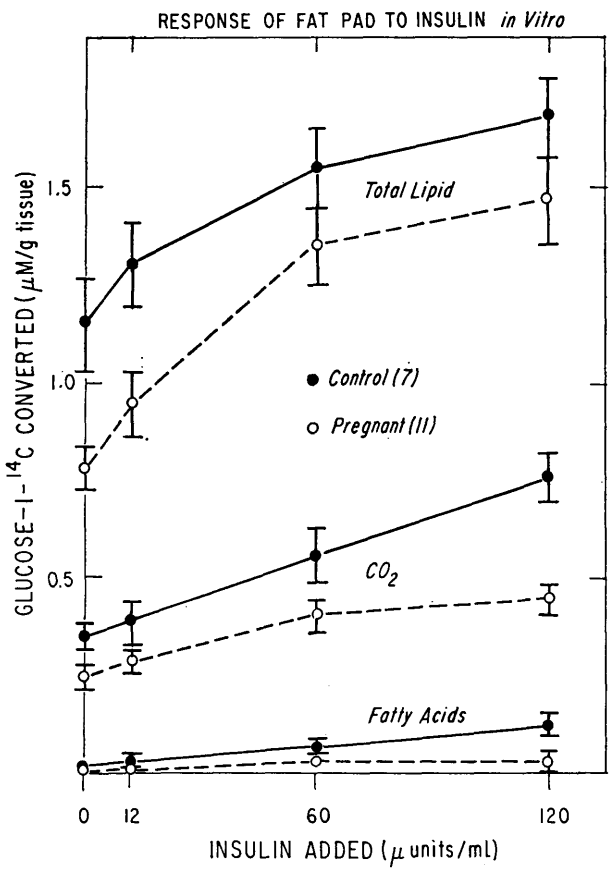

FIG. 6. In vitro response to insulin by adipose tissue from pregnant and nonpregnant rats. Pieces of parametrial adipose tissue were incubated at 37° C. in 2 ml. of 4 per cent albumin-bicarbonate buffer with 3 mM glucose for two hours. Values are given as mean ± S.E.M. The number of rats in each group is given in parentheses.

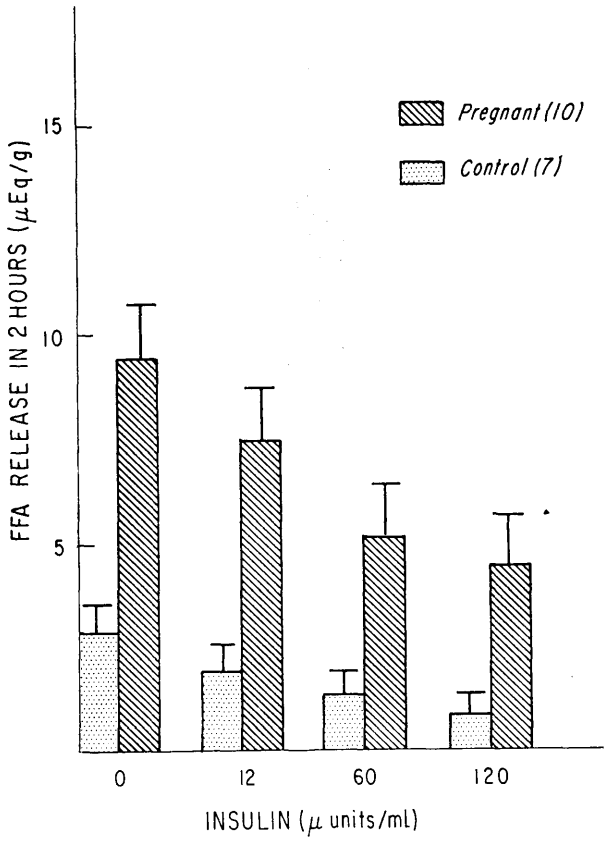

FIG. 7. Fatty acid release by parametrial adipose tissue from pregnant and nonpregnant rats. Same experiment as in figure 6.

Livers were removed from the rats used in the aorta plasma study one-half hour after subcutaneous injection of 1 U. of Regular Insulin. The data in table 6 confirm the finding that fasted pregnant rats have twice the liver triglyceride concentration of nonpregnant controls.[1] Slices from livers of fasted pregnant rats produced ketone bodies at a rate 1.5 times that of slices from livers of nonpregnant controls. The increased rate of ketogenesis may be related to the higher triglyceride content of the livers of fasted pregnant rats.[11] Insulin injection, which reduced the plasma concentration of ketone bodies and FFA by at least 50 per cent in thirty minutes, did not affect in vitro ketogenesis by slices from the livers of either pregnant or control rats. The rate of ketogenesis in this study (4 to 7 μmoles/gm. hr.) is in agreement with the previously published values for slices of normal rat liver (5 μmoles/gm. hr.) and much less than that of fatty livers of diabetic rats (70 μmoles triglyceride/gm.; 35 μmoles ketone bodies/gm. hr.).[11]

DISCUSSION

The plasma FFA concentration of tail blood of unanesthetized rats is considerably higher than that of rats anesthetized with ether or pentobarbital. However, the plasma FFA concentration of tail blood of ether anesthetized rats is similar to that of aortic blood of the same rats (table 4). The lower values are not due to ether anesthesia since similar values were found in blood of unanesthetized rats collected by decapitation. The discrepancy between the plasma FFA concentration of tail blood and aortic blood of unanesthetized rats may be due to the venous blood collected from the tail draining some tissue releasing FFA whereas the aortic blood represents the circulating levels of FFA in the animal. The blood collected from the tail of anesthetized rats resembled arterial blood in color, rate of flow and FFA content. The concentrations of plasma glucose, ketone bodies, and insulin were similar in tail and aortic blood.

Reduction in circulating insulin, on the basis of the data in table 2, does not appear to be the principal cause of lipid mobilization and ketosis in fasting pregnant rats. It may be a contributing cause, however, since the administration of insulin alone decreased the plasma concentrations of FFA and ketone bodies (figure 3, table 5). Previous studies on pregnant rats deprived of adrenal or pituitary suggested that growth hormone and glucocorticoid are required for the development of ketosis during fasting in late-term pregnant rats.[2] The participation of some hormones from the placenta, also, is possible, since neither hypophysectomy nor adrenalectomy blocked completely the rise in ketonemia in this condition. Recently, it has been shown that the plasma corticosterone levels in rats do not increase until the twentieth day of pregnancy.[13] However, hypoglycemia in fasting pregnant rats may increase the secretion of glucagon and growth hormone, both of which increase lipolytic activity in adipose tissue.[8,14,15] In addition, lipolytic hormones may be derived from the placenta.[16]

Increased FFA release from the adipose tissue of fasted pregnant rats is suggested by the higher FFA levels in their plasma (table 5), by in vitro FFA release by adipose tissue (figure 7) and by increased triglyceride in plasma and liver[1] (table 6). Lipolysis, that is the hydrolysis of triglyceride, in the adipose tissue of pregnant rats appeared to be greater, also. In the absence of data on the release of glycerol from the adipose tissue, the rate of lipolysis was estimated from the sum of the fatty acids re-esterified by the tissue

TABLE 6

Ketogenesis by slices of liver of pregnant and nonpregnant rats*

Group	Insulin injected	No. of rats	Liver TG	Ketogenesis†
	U.		μmoles/gm.	μmoles/gm. hr.
Non-pregnant	0	5	10 ±0.9	4.7±0.5
	1	5	9.0±1.5	4.4±0.6
Pregnant	0	7	23 ±2	6.9±0.8
	1	7	21 ±4	6.0±0.6

*See legend, table 5.

†100 mg. liver slices incubated in 2 ml. bicarbonate buffer pH 7.4, for one hour at 37° C.; gas phase 95 per cent 0_2 — 5 per cent CO_2. Duplicate flasks stopped at 0 time and one hour by addition of 1 ml. of 5 per cent $ZnSO_4$, followed by 1 ml. 0.3 N $Ba(OH)_2$.[9]

(glyceride-glycerol formation, figure 6) and the FFA release from the tissue (figure 7). By this method the rate of lipolysis in the adipose tissue of fasted pregnant rats is 1.5 to 2 times that of fasted nonpregnant rats (0.032 and 0.017 μmoles triglyceride hydrolyzed per gm. tissue per minute respectively). These values are in good agreement with those obtained by measuring the release of glycerol by the adipose tissue from fasted rats.[17] Insulin decreased the release of FFA by adipose tissue in vitro (figure 7), but did not alter appreciably the rate of lipolysis. Hence the main effect of insulin and glucose on FFA release by adipose tissue was to increase re-esterification to triglyceride. Insulin injection into fasted pregnant rats, with hypoglycemia, only partially corrected the augmented FFA release from adipose tissue. Glucose administration, which was accompanied by increased plasma IRI, may be more effective in reducing the plasma FFA, presumably, by promoting re-esterification of FFA within the adipose tissue and thus reducing FFA release.

It is concluded from the present study and from those that preceded it[1,2,12] that lipid mobilization and ketogenesis are augmented in fasting pregnant rats. Increased lipolysis in adipose tissues of pregnant rats is probably in response to the hormones of the adrenal, pituitary and placenta. The glucose uptake by adipose tissue is reduced when the concentrations of plasma glucose and insulin are low and this limits the re-esterification of fatty acids in the adipose tissue of the fasting pregnant rat. The net result of increased lipolysis and decreased re-esterification of fatty acids is a greatly increased release of FFA by adipose tissue. Although the ketosis of fasting in pregnancy is a consequence of increased FFA mobilization, a specific action of hormones on the fate of fatty acids in the liver has not been ruled out.

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