Some Aspects of Lipid Metabolism in Rats Treated with Anti-Insulin Serum

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Rats can be made diabetic by pancreatectomy (Scow, 1957) or by the administration of alloxan (Lukens, 1948) or of anti-insulin serum (Armin, Grant & Wright, 1960*b*). Some of the metabolic abnormalities in pancreatectomized (Scow & Chernick, 1960) and alloxan-diabetic (Lukens, 1948; Spiro & Hastings, 1958) rats have been studied, but little is known about the serum-treated animals. The observations reported here show that lipid accumulates rapidly in the liver and blood of rats rendered diabetic by anti-insulin serum.

MATERIALS AND METHODS

Experimental procedure

The rats (albino Wistar; 170–210 g.) were divided into four groups: (a) Diabetic rats, which were injected intraperitoneally with anti-insulin serum (5 ml., sufficient to neutralize 10–16 units of insulin) obtained from guinea pigs treated with recrystallized bovine insulin incorporated into an oily emulsion containing lanolin and paraffin oil (Robinson & Wright, 1961); in later experiments a second injection was given after 4 hr., as some (2 out of 4) rats given a single injection were no longer ketonuric after 24 hr. (b) Control rats, which received control serum (5 ml.) obtained from guinea pigs treated with the oily emulsion (see above) but containing no insulin. (c) Fed normal rats, which were untreated animals kept under the same conditions as the diabetic and control groups, with free access to water and a standard rat-cake diet. (d) Starved normal rats, which, since diabetic rats do not eat (Armin *et al.* 1960*b*), were deprived of food but had free access to water.

During the experimental period food intake was measured and urine was tested for glucose and ketones. Blood-sugar concentrations were determined in samples taken immediately before death. The methods were as described by Armin *et al.* (1960*b*). The rats were decapitated 1–48 hr. after injection of serum or withdrawal of food. Blood was collected from the severed necks and cooled in ice to prevent hydrolysis of esterified fatty acids during clotting and separation of serum. The serum and the livers (wrapped in aluminium foil) were stored at -15° .

Estimations

Serum lipids. Esterified fatty acids were estimated by the method of Stern & Shapiro (1953).

Non-esterified fatty acids were estimated by the method of Dole (1956), modified to produce a one-phase titration

system; the heptane extract (3 ml.) was titrated with 5 *M*-NaOH in 90% (v/v) methanol with cresol red as indicator [1 ml. of a 0.001% soln. in 98% (v/v) ethanol].

Liver lipids. The frozen livers were minced and a chloroform-methanol (2:1, v/v) extract was made and washed as described by Folch, Lees & Sloane-Stanley (1957). The solvent was removed in a rotary evaporator at 50° under reduced pressure. The residue, after being dissolved in chloroform and filtered, yielded solution A (50 ml.), containing the total liver lipid.

A sample (10 ml.) of solution A was evaporated and the dry weight of the residue determined after it had stood for 12 hr. over P_2O_5 and paraffin wax. A further part (20 ml.) of solution A was evaporated in a rotary evaporator at 50° under reduced pressure and the residue mixed with 10*N*-KOH (2 ml.) and ethanol (18 ml.). The mixture was kept overnight at room temperature under nitrogen; water (75 ml.) and 10*N*-hydrochloric acid (5 ml.) were then added, and this mixture was extracted four times with light petroleum (b.p. $40-60^{\circ}$). The combined extracts (50 ml.) formed solution B, containing the saponified liver lipid.

Samples of solutions A and B were evaporated and the residues submitted to the following estimations by the methods stated: (a) Phosphorus (Strickland, Thompson & Webster, 1956), after digestion with 60% perchloric acid. (b) Total cholesterol, as reported by Varley (1954), after solution in chloroform. (c) Free cholesterol (Davison, Dobbing, Morgan & Wright, 1958). (d) Esterified fatty acids (Stern & Shapiro, 1953), after solution in ethanol-ether (3:1, v/v). (e) Non-esterified fatty acids: the residue was kept *in vacuo* over solid KOH for at least 6 hr., and then extracted with acetone; the filtered extract was evaporated, and the residue was dissolved in acetone-ethanol (1:1, v/v), and immediately titrated with 0.02*N*-NaOH in 90% (v/v) methanol with cresol red as indicator.

To express the fatty acid contents of the lipid in absolute units, the mean equiv. wt. of the fatty acids is taken to be 280 (see Results section).

Fatty acid composition of liver lipid. Samples of solution B, stored at -15° under nitrogen, were evaporated, and the residues were kept for 12 hr. *in vacuo* over P_2O_5 and paraffin wax and then dissolved in ether and treated with diazomethane (Baker, 1960). The resultant methyl esters of the fatty acids were purified by sublimation at low pressure and analysed by gas chromatography as described by R. W. R. Baker (in preparation).

Fatty acid composition of adipose-tissue lipid. Pieces (0.2–0.3 g.) of mesenteric fat were removed from normal fed rats. The lipid was extracted and saponified, and the methyl esters of the fatty acids were prepared and analysed by gas chromatography by the methods used for liver lipids.

RESULTS

The rats responded to the injection of anti-insulin serum in the way described by Armin *et al.* (1960b). They ate very little and lost weight at the same rate as starved normal animals (Table 1). Glucose appeared in the urine within 2 hr. and persisted until death. The time of onset of ketonuria was variable, ranging from 5 to 16 hr. Free fluid was found in the peritoneal cavities 1 and 3 hr. after injection, but not thereafter unless a

second injection had been given. By 12–24 hr. their stomachs were grossly distended with food and fluid and their livers were pale and mottled. No gross signs of infection were found in the peritoneum or elsewhere. The control animals behaved like normal fed animals in all respects.

Blood-sugar and serum-lipid concentrations (Table 1). The blood-sugar and serum-non-esterified-fatty acid concentrations rose rapidly in the diabetic rats, the former reaching a maximum of about 300 mg./100 ml. in 3 hr. and the latter continuing to rise for

Table 1. *Food consumption, weight changes and blood constituents of diabetic, control, fed normal and starved normal rats*

Experimental details are described in the text. The results are given as means $\pm$ S.D., with the number of animals in parentheses. NEFA, non-esterified fatty acids; EFA, esterified fatty acids.

Group	Time killed (hr.)	Food eaten (g./kg. of initial body wt./hr.)	Weight lost (g./100 g. of initial body wt.)	Concn. of blood sugar at death (mg./100 ml.)	Concn. of serum NEFA at death (μ equiv./ml.)	Concn. of serum EFA at death (μ equiv./ml.)
Fed normal rats	—	*3.4 $\pm$ 0.66 (16)	*0.9 $\pm$ 2.0 (16)	89 $\pm$ 9.4 (20)	0.32 $\pm$ 0.13 (17)	7.1 $\pm$ 0.84 (12)
Starved normal rats	3	Nil	3.3 $\pm$ 1.7 (4)	90 (2)	0.34 (2)	7.7 (2)
	6		4.2 $\pm$ 3.0 (4)	93 $\pm$ 8.6 (4)	0.30 (2)	6.0 (2)
	12		6.9 $\pm$ 3.1 (4)	73 $\pm$ 8.9 (4)	0.64 (2)	6.5 (2)
	24		11.6 $\pm$ 2.5 (4)	65 $\pm$ 6.5 (4)	0.70 (2)	5.7 (2)
	48		15.4 $\pm$ 1.4 (4)	67 $\pm$ 4.4 (4)	0.70 $\pm$ 0.10 (4)	6.6 $\pm$ 0.94 (4)
Control rats (injected with normal serum)	1	—	—	87 $\pm$ 3.5 (6)	0.33 $\pm$ 0.13 (3)	—
	24	3.4 $\pm$ 1.4 (6)	—0.1 $\pm$ 1.6 (6)	85 $\pm$ 3.5 (6)	—	—
Diabetic rats (injected with anti-insulin serum)	1	—	—	†148 $\pm$ 26 (4)	0.97 $\pm$ 0.15 (7)	9.2 $\pm$ 2.1 (3)
	3	—	3.0 $\pm$ 1.3 (8)	310 $\pm$ 21 (8)	1.29 $\pm$ 0.34 (4)	11.2 $\pm$ 0.78 (4)
	6	0.5 $\pm$ 0.45 (24)	5.1 $\pm$ 2.0 (8)	302 $\pm$ 27 (8)	1.40 $\pm$ 0.16 (4)	9.8 $\pm$ 0.87 (4)
	12		6.5 $\pm$ 2.6 (8)	304 $\pm$ 32 (8)	2.26 $\pm$ 0.26 (4)	8.9 $\pm$ 0.81 (4)
	24		11.8 $\pm$ 3.8 (8)	276 $\pm$ 27 (8)	2.36 $\pm$ 0.16 (4)	14.9 $\pm$ 1.8 (4)

* Measured over a period of 24 hr.

† Measured 40 min. after injection.

Table 2. *Phospholipid, cholesterol and total lipid contents of the livers of diabetic, control, fed normal and starved normal rats*

Experimental details are described in the text. The results are given as means $\pm$ S.D., with the number of animals in parentheses.

Group	Time killed (hr.)	Liver weight (g./100 g. of initial body wt.)	Total liver lipid		Liver phospholipid* (mg./100 g. of initial body wt.)	Total liver cholesterol† (mg./100 g. of initial body wt.)
			(g./100 g. of liver wt.)	(mg./100 g. of initial body wt.)		
Fed normal rats	—	4.08 $\pm$ 0.60 (22)	4.45 $\pm$ 0.61 (22)	180 $\pm$ 27 (22)	104 $\pm$ 24 (8)	7.4 $\pm$ 1.7 (8)
Starved normal rats	3	4.25 $\pm$ 0.70 (4)	4.26 $\pm$ 0.74 (4)	185 $\pm$ 61 (4)	88 (2)	6.1 (2)
	6	3.51 $\pm$ 0.73 (4)	4.79 $\pm$ 0.50 (4)	165 $\pm$ 19 (4)	95 (2)	5.8 (2)
	12	3.16 $\pm$ 0.17 (4)	5.90 $\pm$ 0.80 (4)	183 $\pm$ 22 (4)	108 (2)	8.4 (2)
	24	3.28 $\pm$ 0.44 (4)	5.65 $\pm$ 0.58 (4)	185 $\pm$ 26 (4)	89 (2)	6.4 (2)
	48	2.72 $\pm$ 0.20 (4)	5.86 $\pm$ 0.86 (4)	160 $\pm$ 27 (4)	—	—
Control rats (injected with normal serum)	24	4.56 $\pm$ 0.21 (6)	4.06 $\pm$ 0.25 (6)	185 $\pm$ 18 (6)	—	—
Diabetic rats (injected with anti-insulin serum)	1	3.84 $\pm$ 0.37 (4)	4.59 $\pm$ 0.40 (4)	176 $\pm$ 14 (4)	—	—
	3	4.26 $\pm$ 0.65 (8)	5.37 $\pm$ 0.54 (8)	232 $\pm$ 50 (8)	86 $\pm$ 19 (4)	6.6 $\pm$ 0.77 (4)
	6	3.06 $\pm$ 0.55 (8)	6.96 $\pm$ 1.4 (8)	233 $\pm$ 24 (8)	83 $\pm$ 10 (4)	7.1 $\pm$ 0.73 (4)
	12	3.46 $\pm$ 0.17 (8)	11.1 $\pm$ 2.3 (8)	383 $\pm$ 84 (8)	103 $\pm$ 8.0 (4)	7.8 $\pm$ 0.64 (4)
	24	3.57 $\pm$ 0.27 (8)	12.0 $\pm$ 2.5 (8)	428 $\pm$ 111 (8)	93 $\pm$ 5.3 (4)	7.9 $\pm$ 2.0 (4)

* Assumed to contain 4% of phosphorus.

† The proportion of total cholesterol which was esterified did not vary; the mean value for all rats (24) was 92 $\pm$ 7.2%.

12 hr. to reach a concentration ($2.26 \mu\text{equiv./ml.}$) seven times normal. A significant increase ($P < 0.05$) in the serum-esterified-fatty acid concentration was detected in 1 hr.

During the first 12 hr. of starvation the blood-sugar concentration of normal rats fell slightly to about 70 mg./100 ml. and the serum-non-esterified-fatty acid content doubled, but the serum-esterified-fatty acid concentration remained steady. No changes occurred thereafter.

Liver lipid. The lipid contents of the livers of diabetic rats increased rapidly, but no changes occurred in the starved or control groups (Table 2). The increase was first detected at 3 hr. ($P < 0.001$) and at 12 hr. the lipid content was more than twice normal. The small increase in the liver-lipid concentration of starved normal rats is attributed to a decrease in the size of the livers.

This increase in liver lipid in diabetic rats is predominantly due to a large accumulation of esterified fatty acids (Table 3), but partly also to a small rise in non-esterified-fatty acid content. No changes ($P > 0.10$) were detected in the phospholipid and cholesterol contents (Table 2). Starved normal rats showed no consistent changes in the composition of their liver lipid.

The fatty acid contents of the saponified liver lipid were almost identical with the total fatty acid contents (esterified plus non-esterified) of the total lipid from which they were obtained (Table 3). The esterified-fatty acid content of the saponified lipid was less than 1 %.

The fatty acid composition of the liver lipids of diabetic rats (Table 4) was abnormal. Normal-rat livers contained roughly equal weights of palmitic acid, stearic acid, oleic acid, arachidonic acid and

Table 3. *Esterified, non-esterified and total fatty acids in liver lipid of diabetic, fed normal and starved normal rats*

Experimental details are given in the text. The results are stated as mg./100 g. of initial body wt., and are given as means $\pm$ S.D., with the number of animals in parentheses. NEFA, non-esterified fatty acids; EFA, esterified fatty acids.

Group	Time killed (hr.)	Total liver lipid	EFA in total lipid	NEFA in total lipid	Total fatty acids after saponification
Fed normal rats	—	182 ± 29 (12)	116 ± 17 (12)	6.7 ± 1.3 (12)	128 ± 18 (8)
Starved normal rats	3	238 (2)	154 (2)	5.7 (2)	161 (2)
	6	181 (2)	109 (2)	7.9 (2)	115 (2)
	12	164 (2)	106 (2)	10.4 (2)	107 (2)
	24	198 (2)	122 (2)	7.1 (2)	125 (2)
	48	160 ± 27 (4)	106 ± 24 (4)	6.3 ± 0.40 (4)	—
Diabetic rats	1	176 ± 14 (4)	113 ± 11 (4)	6.7 ± 0.79 (4)	—
	3	276 ± 15 (4)	177 ± 8.7 (4)	6.2 ± 0.52 (4)	194 ± 16 (4)
	6	225 ± 12 (4)	150 ± 12 (4)	9.8 ± 0.72 (4)	160 ± 20 (4)
	12	408 ± 83 (4)	342 ± 83 (4)	16.1 ± 1.0 (4)	344 ± 84 (4)
	24	470 ± 21 (4)	399 ± 25 (4)	13.4 ± 1.3 (4)	401 ± 26 (4)

Table 4. *Proportions of fatty acids in the liver of diabetic, fed normal and starved normal rats*

Experimental details are given in the text. The results are stated as mg./100 g. of initial body wt., and are given as means $\pm$ S.E.M. NEFA, non-esterified fatty acids; EFA, esterified fatty acids.

No. of rats	Fed normal rats	Starved normal rats	Diabetic rats
...	4	2	4
Total liver lipid	201 ± 17	198	477 ± 22
Total fatty acids in liver (EFA + NEFA in the total lipid)	134 ± 14	123	407 ± 23
Fatty acid (no. of C atoms and double bonds)			
Myristic acid (14-0)	1 ± 0.11	1	6 ± 1.6
Palmitic acid (16-0)	30 ± 6.6	26	111 ± 15
Palmitoleic acid (16-1)	3 ± 0.87	2	16 ± 3.2
Stearic acid (18-0)	26 ± 2.8	26	23 ± 2.1
Oleic acid (18-1)	20 ± 2.7	14	119 ± 8.4
Linoleic acid (18-2)	19 ± 1.6	18	75 ± 2.8
Arachidonic acid (20-4)	20 ± 2.0	27	23 ± 1.2
Clupanodonic acid (22-5)	7 ± 1.2	7	11 ± 0.71
Others*	8 ± 1.7	4	23 ± 4.9

* The total of up to 16 minor components.

linoleic acid, together with lesser amounts of clupanodonic acid, palmitoleic acid and myristic acid. The diabetic-rat livers contained much more oleic acid, palmitic acid and linoleic acid, but no more ($P > 0.10$) stearic acid or arachidonic acid; there were small but significant ($P < 0.05$) rises in palmitoleic acid, myristic acid, and clupanodonic acid. From the results in Table 4 the mean molecular weights of the fatty acids from the livers of normal and diabetic rats were calculated to be 281 and 275 g. respectively.

Adipose-tissue lipid. Table 5 shows that adipose-tissue lipid from normal rats contains proportionately much more oleic acid and much less stearic acid and arachidonic acid than the corresponding liver lipid.

DISCUSSION

These results show that the diabetic syndrome of polyuria, glycosuria, ketonuria and hyperglycaemia, occurring in rats treated with anti-insulin serum, is accompanied by rapid accumulations of lipid in the liver and blood. Similar accumulations of liver lipid have been described in pancreatectomized rats (Chernick & Scow, 1959) and in alloxan-diabetic rats after withdrawal of insulin (Spiro, Ashmore & Hastings, 1958).

Serum-treated rats become diabetic more rapidly than do either alloxan-diabetic or pancreatectomized rats. Immediately after the injection, their blood-sugar concentrations begin to rise at rates which are greater in well-nourished (190 mg./100 ml./hr.) than in starved (83 mg./100 ml./hr.) animals (Armin, Grant & Wright, 1960a). After pancreatectomy the blood-sugar concentrations of starved rats rise at only about 20 mg./100 ml./hr. (Chernick & Scow, 1959), and in alloxan-diabetic rats no rise in blood-sugar concentration occurs for

at least 4 hr. after the last subcutaneous injection of soluble insulin (Spiro *et al.* 1958). The total lipid and total fatty acid (esterified plus non-esterified) contents of the livers of serum-treated rats both increase at mean rates of about 12 mg./100 g. of body wt./hr. during the first 24 hr. Again this is more rapid than the rate (4.2 mg./100 g. of body wt./hr.) calculated from the results of Chernick & Scow (1959) for the first 18 hr. after pancreatectomy. The fatty acid contents of the livers of alloxan-diabetic rats do not change for at least 4 hr. after the last injection of insulin and then rise slowly (5.7 mg./100 g. of body wt./hr.) during the next 16 hr. (Spiro *et al.* 1958). These differences are probably due to variations in the rate of onset of insulin deficiency, which is fastest after serum administration and slowest in alloxan-diabetic rats. In the former case the injected anti-insulin serum rapidly induces insulin deficiency by neutralizing endogenously secreted insulin (Armin, Cunningham, Grant, Lloyd & Wright, 1961), but in the latter signs of diabetes do not appear until the effects of the injection of a depot of soluble insulin have worn off. Pancreatectomized animals probably fall between these two extremes, for the source of endogenous insulin has been removed and only a small amount of hormone remains to circulate in the blood and disappear in an unknown but probably short time.

The lipid accumulating in the livers of serum-treated rats is composed almost entirely of esterified fatty acids but contains no phosphorus or cholesterol, and therefore consists predominantly of neutral triglycerides; this has also been shown in pancreatectomized rats (Chernick & Scow, 1959). With respect to fatty acid composition this triglyceride is very similar to depot fat (Table 5) and resembles normal-rat-liver triglyceride (Creasey,

Table 5. *Fatty acid compositions of rat lipids*

The results are stated as percentages of the total fatty acids, and are given as means $\pm$ S.E.M. Those in columns (a) and (b) show the fatty acid compositions of the lipids in the liver and adipose tissue of fed normal rats. The proportions of fatty acids in the triglyceride accumulating in the livers of diabetic rats, shown in column (c), were calculated from the results in Table 4. The percentage of fatty acid in accumulating triglyceride is $100 (F_D - F_N) / (T_D - T_N)$, where F_D and F_N are the weights of the individual fatty acids, and T_D and T_N are the total weights of fatty acids, in the livers of diabetic and fed normal rats respectively.

Fatty acid	(a) Normal-liver lipid	(b) Normal-adipose-tissue lipid	(c) Additional lipid in diabetic livers
Myristic acid	0.5 $\pm$ 0.07	2.3 $\pm$ 0.06	2.1 $\pm$ 0.16
Palmitic acid	22.6 $\pm$ 0.99	28.5 $\pm$ 1.5	29.5 $\pm$ 1.0
Palmitoleic acid	2.3 $\pm$ 0.23	6.8 $\pm$ 0.52	4.7 $\pm$ 0.60
Stearic acid	19.8 $\pm$ 1.0	4.7 $\pm$ 0.32	-0.9 $\pm$ 1.1
Oleic acid	14.1 $\pm$ 0.69	35.3 $\pm$ 0.72	36.5 $\pm$ 0.90
Linoleic acid	14.2 $\pm$ 0.64	18.2 $\pm$ 1.6	20.3 $\pm$ 0.88
Arachidonic acid	15.1 $\pm$ 0.77	0.6 $\pm$ 0.18	1.1 $\pm$ 0.87
Clupanodonic acid	5.2 $\pm$ 0.56	0	1.5 $\pm$ 0.58
Others*	6.0 $\pm$ 0.68	3.6 $\pm$ 0.43	5.3 $\pm$ 1.3

* The total of up to 16 minor components.

Hankin & Handschumacher, 1961), but differs from the liver lipid of normal rats (Table 5).

In theory it could accumulate in three ways: (a) by increased synthesis of fatty acids in the liver; (b) by decreased breakdown of fatty acids in the liver; (c) by increased transport of fatty acids to the liver. The first two possibilities may be discounted as being inconsistent with the behaviour of liver slices from diabetic rats. These synthesize fatty acids very slowly or not at all (Spiro *et al.* 1958; J. Ashmore & R. F. Mahler, unpublished work) but produce large amounts of ketone bodies (Scow & Chernick, 1960; J. Ashmore & R. F. Mahler, unpublished work) and can oxidize palmitic acid to carbon dioxide at a normal rate (Milstein & Driscoll, 1959). The third possibility is supported by the raised concentrations of free fatty acids in the serum of diabetic rats (Table 1). The rapid depletion of body fat during diabetes (Armin *et al.* 1960*b*) and the absence of fatty livers in pancreatectomized rats previously depleted of body fat by starvation (Chernick & Scow, 1959) suggest adipose tissue as the source of the transported fatty acids. This conclusion is supported by the close similarity in fatty acid composition between the new triglyceride and depot fat (Table 5) and by the rapid release of non-esterified fatty acids *in vitro* by adipose tissue taken from diabetic rats (Wertheimer & Shafir, 1960; R. F. Mahler, W. L. Stafford & M. E. Tarrant, unpublished work).

SUMMARY

1. In normal rats made diabetic by the injection of guinea-pig anti-insulin serum, an increase in the liver-lipid content was detected within 3 hr.; by 12 hr. the content had doubled.

2. The concentration of free fatty acids in the serum of diabetic rats also rose rapidly, trebling in the first hour.

3. The increase in liver-lipid content was due to an accumulation of neutral triglyceride, with a fatty acid composition nearly the same as that of depot fat from normal rats.

4. This similarity supports other evidence that the accumulation of triglyceride in the livers of diabetic rats results from increased mobilization of lipid from adipose tissue.

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