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THE ACTION OF SALICYLATE IN REDUCING PLASMA FREE FATTY ACIDS AND ITS PHARMACOLOGICAL CONSEQUENCES

BY

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Accumulating evidence suggests an inverse relationship between utilization of glucose and release of free fatty acids from adipose tissue (Dole, 1956; Gordon & Cherkes, 1956; Randle, Garland, Hales & Newsholme, 1963). When glucose is available less free fatty acids are released into the blood stream, probably because they are re-esterified with the formed glycerolphosphate in the adipose tissue (Shafrir & Gorin, 1963). Therefore drugs able to increase the glucose oxidation could lower the level of plasma free fatty acids. The lowering of circulating free fatty acids may have a pharmacological interest because the level of these is one of the factors which may regulate the level of tissue triglycerides (Feigelson, Pfaff, Karmen & Steinberg, 1961; Carlson & Liljedahl, 1963) and the degree of hypercholesteraemia when cholesterol is added to a normal diet (Banerjee & Bandyopadhyay, 1963; Kritchevsky & Tepper, 1962). Furthermore an increase of plasma free fatty acids usually follows conditions of emergency in order to provide enough energy for the increased metabolic demands of the various tissues (Steinberg, 1963; Paoletti, Maickel, Smith & Brodie, 1961).

In looking for agents able to lower plasma free fatty acids our attention has been directed to salicylate. This drug is known to be effective in increasing oxidative processes (Cochran, 1952; Meade, 1954; Tenney & Miller, 1955; Johnson, Sellers & Schönbaum, 1963) and it has hypoglycaemic and hypocholesteraemic properties (Reid, 1961). However, contradictory results on the effect of salicylate on plasma free fatty acids are reported in the literature; Carlson & Östman (1961) found in human beings a significant decrease of plasma free fatty acids after administration of salicylate. Gilgore, Drew & Rupp (1963) could not confirm these results.

This paper will report the effect of salicylate on normal and elevated levels of plasma free fatty acids in rats. Furthermore some pharmacological and biochemical consequences of this effect will be described.

METHODS

Male Sprague-Dawley rats of average weight 150 g were kept in Makrolon plastic cages (40 × 26 × 16 cm), six per cage, at a room temperature of 22° C. The experiments were begun usually in the morning and during treatment the animals were kept in quiet surroundings. Food (pellets ALAL 56) and water were given *ad libitum* up to the beginning of the experiments unless otherwise specified. The standardization of

the experimental conditions is very important, as recently reported by Barrett (1964). Free fatty acids were determined by the method of Trout, Estes & Friedberg (1960) and also by thin-layer chromatography (Kieselgel G, Merck) according to Vogel, Doizaki & Zieve (1962). Dole's (1956) method was used only in preliminary experiments. Serum and liver triglycerides were determined according to Van Handel, Zilversmit & Bowman (1957). Blood salicylate was analysed according to Grinschgl & Eichhorn (1953). Salicylate was always used as the sodium salt. Corticotrophin as a long-lasting form (ACTHormongel) was a generous gift of Ormonoterapia Richter, Milano.

RESULTS

Salicylate and plasma free fatty acids in fed resting rats

In preliminary trials Dole's (1956) method was used to titrate free fatty acids. Salicylate simulated an increase of plasma free fatty acids because the drug is extracted in heptane and is therefore titrated. This was confirmed by experiments carried out *in vitro* by adding known concentrations of salicylate to rat serum. In the method of Trout *et al.* (1960) salicylate is washed out by two successive extractions with 0.05% sulphuric acid. This was shown by checking for the presence of salicylate in the heptane phase by thin-layer chromatography. The results obtained by the quantitative methods are presented in Table 1. Further confirmation that free fatty acids were really reduced was obtained also by a qualitative estimation with thin-layer chromatography.

TABLE 1

LEVEL OF PLASMA FREE FATTY ACIDS 1 HR AFTER SALICYLATE ADMINISTRATION IN FED AND RESTING RATS

Injections were intraperitoneal. Values are means and standard errors

Treatment	Plasma free fatty acids (μequiv./l.) estimated according to	
	Dole (1956)	Trout *et al.* (1960)
Saline	292±35	302±31
Salicylate (100 mg/kg)	604±28	224±26
Salicylate (200 mg/kg)	823±30	183±17

Table 2 summarizes the duration of the lowering effect on plasma free fatty acids after a large dose of salicylate. The peak of the effect occurs after about 1 hr whilst after 2 hr the value returns to normal. The increase observed 3 hr after salicylate administration is probably related to the fact that the animals were fasted at the beginning of the treatment.

TABLE 2

DURATION OF ACTION OF SALICYLATE ON PLASMA FREE FATTY ACIDS IN FED AND RESTING RATS

Each figure represents the average of at least five determinations. Values are means and standard errors. Injections were intraperitoneal; all results with salicylate refer to 300 mg/kg

Treatment	Time until killing (min)	Plasma free fatty acids (μequiv./l.)	Significance between controls and treated *P*
Saline	—	215±21	—
Salicylate	30	137±6	<0·01
Salicylate	60	110±11	<0·01
Salicylate	90	143±15	<0·01
Salicylate	120	220±20	>0·05
Salicylate	180	292±16	<0·01

Salicylate and plasma free fatty acids in animals of different age

Salicylate showed a significant effect in reducing free fatty acids not only in adult animals but also in suckling rats (17 days old) and in aged mice (19 months old). The results obtained are summarized in Table 3.

Effect of salicylate on plasma free fatty acids in rats with reduced adrenal or thyroid function

Since the activity of adrenal and thyroid glands is an important factor in determining the level of plasma free fatty acids, salicylate was given to rats adrenalectomized 3 days before the experiment and to suckling rats, the mothers of which received after delivery a diet containing 0.4% of propylthiouracil. This treatment reduces considerably the thyroid function in the litters.

Table 4 shows that salicylate also lowers plasma free fatty acids in rats with reduced thyroid or adrenal function.

TABLE 3

EFFECT OF SALICYLATE IN ANIMALS OF DIFFERENT AGE SUBMITTED TO 6 TO 12 HR FASTING

Each figure represents the average of at least six determinations, with standard errors. Animals were killed 30 min after the administration of salicylate. Injections were intraperitoneal

	Plasma free fatty acid (μequiv./l.) for		
Treatment	Suckling rats	Adult rats	Old mice
Saline	630±50	671±64	596±87
Salicylate (100 mg/kg)	380±18	342±19	—
Salicylate (300 mg/kg)	315±21	341±11	359±27

TABLE 4

EFFECT OF SALICYLATE ON PLASMA FREE FATTY ACIDS IN ADRENALECTOMIZED OR HYPOTHYROID RATS

Adrenalectomized rats received saline *ad libitum*. Each figure represents the average of at least five determinations, with the standard error. Injections were intraperitoneal

Condition	Treatment	Plasma free fatty acids (μequiv/l.)
Adrenalectomized	Saline	380±60
Adrenalectomized	Salicylate (300 mg/kg)	143±22
Hypothyroid	Saline	623±31
Hypothyroid	Salicylate (100 mg/kg)	382±47
Hypothyroid	Salicylate (300 mg/kg)	331±43

TABLE 5

EFFECT OF SALICYLATE ON RAISED LEVELS OF PLASMA FREE FATTY ACIDS IN RATS EXPOSED DURING 4.5 HR TO COLD (2° C) OR SUBMITTED TO 48 HR FASTING

Salicylate was given 30 min before killing. Each figure represents the average of at least five determinations, with the standard error. Injections were intraperitoneal

		Plasma concentrations for		
		Cooled rats		Fasting rats
Treatment	Dose (mg/kg)	Salicylate (mg/ml.)	Free fatty acids (μequiv/l.)	Free fatty acids (μequiv/l.)
Saline		0	738±60	617±20
Salicylate	25	0·79±0·6	574±34	—
Salicylate	50	1·26±0·3	419±26	709±27
Salicylate	100	1·99±0·4	362±28	489±26
Salicylate	200	2·92±0·8	268±26	379±21
Salicylate	300	—	164±28	295±10

Effect of salicylate on plasma elevated free fatty acids by cold or fasting

Exposure to cold or prolonged fasting are typical conditions leading to a considerable increase in the level of plasma free fatty acids. Salicylate decreases free fatty acids also in these experimental conditions proportionally to their level in blood (Table 5). The effect of salicylate on plasma free fatty acids is more marked and longer lasting in animals exposed to cold than in rats submitted to fasting.

Salicylate is effective also when given orally. For instance, 1 hr after administration to rats exposed to cold, there is an inhibition of 36% at the dose of 50 mg/kg, 53% at 100 mg/kg and 76% at 300 mg/kg. Furthermore, in rats submitted to 15 hr of fasting, salicylate (300 mg/kg, intraperitoneally) did not change the blood glucose level when the determinations were made 10, 20 or 40 min after administration.

Effect of salicylate on plasma free fatty acids increased by drug treatment

Table 6 summarizes a number of treatments able to increase plasma free fatty acids and the effect of salicylate on these treatments. Salicylate antagonizes or prevents the activity of amphetamine, noradrenaline, corticotrophin and chlorpromazine.

Experiments, not reported in detail here, show that salicylate antagonizes the effect of amphetamine on free fatty acids but not its effects on anorexia. The antagonism toward noradrenaline is not present when effects on blood pressure and heart rate are measured. Furthermore, salicylate prevents the effect of chlorpromazine on free fatty acids but not its effects on hyperglycaemia (unpublished results).

TABLE 6

EFFECT OF SALICYLATE ON PLASMA FREE FATTY ACIDS INCREASED BY DRUG TREATMENT

Noradrenaline was given in oil at the same time as salicylate, 1 hr before killing. Amphetamine or corticotrophin were given 90 min before salicylate and 2 hr before killing. Chlorpromazine was given 10 min after salicylate and 30 min before killing. Each figure represents the average of at least five determinations, with the standard error. S.c., subcutaneous; i.v., intravenous. Salicylate-treated animals received 300 mg/kg of salicylate, intraperitoneally

Treatment	Route and dose	Plasma free fatty acids (μequiv/l.) for Controls	Salicylate-treated
Saline	—	256±34	149±23
Noradrenaline	1 mg/kg, s.c.	723±54	252±36
Amphetamine	5 mg/kg, s.c.	492±74	237±23
Corticotrophin	100 U, s.c.	957±42	201±12
Chlorpromazine	1.5 mg/kg, i.v.	505±22	138±29

Effect of salicylate congeners or derivatives on plasma free fatty acids

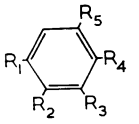
Salicylate appears to be the most effective drug of the series of compounds reported in Table 7. Salicylamide is without effect, showing that the effect of salicylate on free fatty acids is probably not related to its anti-inflammatory properties. Acetylsalicylate has a similar activity to salicylate. Substitutions in the ortho position with chlorine, nitro or amino groups lead to a reduction in activity. *p*-Aminosalicylic acid has only a small effect on plasma free fatty acids. The introduction of a second carboxyl group (phthalic acid) results, also, in a lack of activity.

Benzoic acid has considerable activity, although it is less effective than salicylate (Table 8).

TABLE 7

EFFECT OF SALICYLATE CONGENERS OR DERIVATIVES ON PLASMA FREE FATTY ACIDS IN RATS FASTED FOR 18 HR

All compounds were given intraperitoneally at a dose of 300 mg/kg as sodium salts. Each figure represents the average of at least five determinations, and is the percentage decreased with respect to controls



Compound	R_1	R_2	R_3	R_4	R_5	Plasma free fatty acids (% decrease)	P
Salicylic acid	COOH	OH	H	H	H	49	<0·01
Benzoic acid	COOH	H	H	H	H	43	<0·01
Acetylsalicylic acid	COOH	OCH_3	H	H	H	37	<0·01
o-Chlorobenzoic acid	COOH	Cl	H	H	H	36	<0·01
o-Nitrobenzoic acid	COOH	NO_2	H	H	H	23	<0·01
Anthranilic acid	COOH	NH_2	H	H	H	18	<0·05
Sulphosalicylic acid	COOH	OH	H	H	SO_3Na	14	>0·05
p-Aminosalicylic acid	COOH	OH	H	NH_2	H	11	>0·05
Gallic acid	COOH	H	OH	OH	OH	34	<0·01
Phthalic acid	COOH	COOH	H	H	H	4	>0·05
Salicylamide	$CONH_2$	OH	H	H	H	+35	<0·01

TABLE 8

EFFECT OF SALICYLATE AND BENZOATE ON PLASMA FREE FATTY ACIDS IN RATS EXPOSED TO COLD DURING 4.5 HR

Drugs were given 30 min before killing. Figures show the changed plasma free fatty acids as a percentage of controls values (=100). Injections were intraperitoneal

Dose of drug (mg/kg)	Plasma free fatty acids (% of control) after	
	Salicylate	Benzoate
0	100	100
25	62	79
50	55	78
100	26	28

Effect of salicylate on liver triglycerides

Salicylate decreases accumulation of liver triglycerides in various experimental conditions although it does not change liver triglycerides in normal rats. Tables 9 and 10 summarize some results obtained when liver triglycerides were increased either by exposure to cold or by administration of corticotrophin.

Salicylate reaches the peak of its activity on liver triglyceride accumulation induced by cold when administered 4 hr before death, but it is without effect when given 6 hr before death (results not reported in the table). There is also a decrease of plasma triglycerides which requires further investigation.

In Table 10 salicylate is shown to counteract the increase of liver triglycerides which is induced by large doses of corticotrophin. The lack of effect on free fatty acids in these experiments is explained by the fact that salicylate was given 4 hr before the determinations.

Effect of salicylate on body temperature

Since the animals exposed to cold derive the energy for maintenance of body temperature from mobilization of free fatty acids, it seemed reasonable to establish the effect of salicylate

TABLE 9

EFFECT OF SALICYLATE (300 MG/KG, INTRAPERITONEALLY) ON LIVER AND PLASMA TRIGLYCERIDES AND ON PLASMA FREE FATTY ACIDS IN RATS EXPOSED TO COLD (2° C) FOR 4.5 HR

Each figure represents the average of at least five determinations, and the standard error. Plasma free fatty acids have been analysed on pooled samples

Time before killing (min)	Free fatty acids in plasma (μequiv/l.)	Triglycerides in Plasma (mg/l.)	Triglycerides in Liver (mg/100 g)
Controls	657	37±4	1,063±78
30	142	30±3	1,022±65
90	257	25±2	821±41
120	422	36±2	738±81
240	440	40±4	659±48

TABLE 10

EFFECT OF SALICYLATE ON ACCUMULATION OF LIVER TRIGLYCERIDES AFTER CORTICOTROPHIN

Salicylate (300 mg/kg intraperitoneally) was given together with corticotrophin (subcutaneously) 4 hr before killing. Each figure represents the average of five determinations and the standard error. Free fatty acids were determined on pooled plasma

Treatment	Plasma free fatty acids (μequiv/l.) for Controls	Plasma free fatty acids (μequiv/l.) for Salicylate-treated	Liver triglycerides (mg/100 g) for Controls	Liver triglycerides (mg/100 g) for Salicylate-treated
Saline	400	472	559±39	557±49
Corticotrophin (100 U/kg)	875	750	1,399±105	689±51
Corticotrophin (200 U/kg)	820	750	1,529±96	872±92

TABLE 11

EFFECT OF SALICYLATE ON BODY TEMPERATURE OF RATS FASTED FOR 4 HR AND KEPT AT A TEMPERATURE OF 22° OR 2° C

Each figure represents the average of at least five determinations, and the standard error. Injections were intraperitoneal

Treatment	Dose (mg/kg)	Environmental temperature (°C)	Body temperature (°C) after 4 hr	4·5 hr	5 hr	6 hr
Saline		22	37·5±0·1	37·3±0·09	37·3±0·13	37·5±0·15
Salicylate	300	22	37·5±0·04	37·1±0·10	37·0±0·10	36·6±0·21
Saline		2	34·8±0·1	34·4±0·3	35·0±0·1	35·0±0·1
Salicylate	25	2	34·2±0·3	34·3±0·5	34·1±0·4	33·4±0·3
Salicylate	50	2	33·6±0·5	33·3±0·2	32·9±0·3	32·5±0·4
Salicylate	100	2	33·6±0·1	32·5±0·1	31·4±0·04	31·4±0·3
Salicylate	200	2	34·0±0·4	32·2±0·4	30·8±0·2	29·7±0·4

TABLE 12

EFFECT OF SALICYLATE (300 MG/KG SUBCUTANEOUSLY, TWICE A DAY) ON SURVIVAL TIME OF FASTED OR FED RATS

Each pair of figures shows the number of rats which died and the number tested

Treatment	Food provided	Cumulative mortality after (hr) 48	72	96	120	144	160
Saline	Yes	0/10	0/10	0/10	0/10	0/10	0/10
Salicylate	Yes	0/10	0/10	0/10	0/10	0/10	0/10
Saline	No	0/26	0/26	1/26	3/26	23/26	26/26
Salicylate	No	6/28	15/28	21/28	28/28	—	—

on body temperature of rats kept either at room temperature or in cold room. Table 11 reports the results obtained, which show that salicylate lowers body temperature of animals exposed to cold at doses almost inactive in animals kept at room temperature. Fig. 1 reports the relationship between the drop in body temperature and the lowering effect on free fatty acids induced by salicylate.

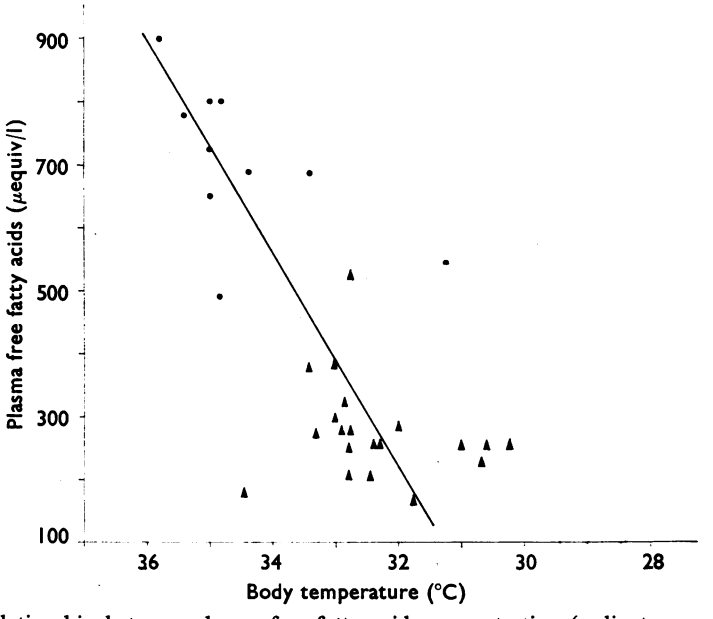


Fig. 1. The relationship between plasma free fatty acids concentration (ordinate, μequiv/l.) and body temperature (abscissa, °C) of rats exposed to cold (2° C) for 4 hr. Filled circles, control rats; filled triangles, rats injected intraperitoneally with salicylate (200 mg/kg).

Effect of salicylate on prolonged starvation

If animals submitted to starvation (with water available) derive their energy by mobilizing free fatty acids from adipose tissue, salicylate should be expected to reduce the survival time of these animals. Table 12 shows that the survival time of starved animals is considerably shortened by the administration of salicylate, even though the same dose of salicylate is not toxic over a longer time in fed animals.

DISCUSSION

The results show that salicylate is effective in lowering plasma free fatty acids in rats when suitable methods for the determination are used. It is likely that the contradictory results reported by Gilgore *et al.* (1963) and by Carlson & Östman (1961) arise from the fact that the former used Dole's (1956) method, in which salicylate interferes. It has been shown here that Dole's method is not suitable for the determination of free fatty acids in the presence of salicylate and many of its congeners and derivatives.

Salicylate decreases plasma free fatty acids in fed resting rats as well as in fasted animals and in rats exposed to cold. In fed animals the duration of action is less than 2 hr, but in animals exposed to cold is more than 4 hr. The doses of salicylate required to reduce the increase in plasma free fatty acids are higher in fasted than in cold-exposed animals. In

the latter condition salicylate is effective at 25 mg/kg, a dose very close to that used in human therapy.

The effect of salicylate on reducing plasma free fatty acids is present also in rats deprived of adrenal or thyroid function. High activity is exerted by salicylate also when plasma free fatty acids have been increased by noradrenaline, corticotrophin, amphetamine or chlorpromazine.

Salicylate does not exert a general sympatholytic effect because it does not affect the cardiovascular actions of noradrenaline. It is unlikely therefore that salicylate acts, in these conditions, by inhibiting the catechol amine-induced mobilization of free fatty acids.

After 48 hr of fasting, catechol amines do not play any role in the lipolysis of triglycerides in adipose tissue (Stern & Maickel, 1963). However, catechol amines may be involved in lipolysis after exposure to cold (Gilgen, Maickel, Nikodjevic & Brodie, 1962) as well as after treatment with chlorpromazine (Bonaccorsi, Garattini & Jori, 1964). In these conditions there is also an activation of the corticotrophin release as shown by the marked increase of plasma corticosterone (Smith, Paoletti & Brodie, 1962). It is interesting that, recently, corticotrophin has been demonstrated to act on the mobilization of free fatty acids without corticosterone or catechol amines acting as mediators of its effect (Schotz, Masson & Page, 1959; Ho & Meng, 1964).

It is remarkable that salicylate is acting here as an inhibitor of corticotrophin when other evidence suggests that salicylate releases it (Wexler, 1963). Several substances related to salicylic acid have been found effective, including benzoic acid, acetylsalicylic acid, *o*-chlorobenzoic acid, *o*-nitrobenzoic acid and gallic acid. Salicylamide is not effective, reminiscent of nicotinic acid and nicotinamide which respectively are active and inactive in lowering free fatty acids (Eaton, 1963). However, the simple acidic function is not responsible for the effect of salicylate because other monocarboxylic acids (*p*-aminobenzoic acid and *p*-aminosalicylic acid) and a dicarboxylic acid (phthalic acid) are inactive.

As a result of the reduction of plasma free fatty acids, salicylate reduces the accumulation of triglycerides in liver in conditions of increased mobilization, for example after cold exposure or administration of corticotrophin. It is possible that salicylate may serve as a tool to distinguish different types of liver triglyceride accumulation (" fatty liver "). For an exact evaluation of this point it will also be necessary to consider the effect of salicylate on plasma corticosteroids.

Decreased availability of free fatty acids may be responsible for the lowering effect of salicylate on body temperature of rats exposed to cold and for the decreased survival time of animals submitted to fasting. Further investigations are required, however, to establish the relationship between these effects and the reduction of plasma free fatty acids.

Work is now in progress to obtain information about the mechanism of action of salicylate. Obviously there are two possibilities: either an increased utilization or a reduced mobilization of free fatty acids. Against the first hypothesis is the fact that in the cold the body temperature of animals treated with salicylate falls. This is contrary to what would be expected if free fatty acids were utilized at higher rates. In favour of a reduced mobilization are preliminary results showing that salicylate prevents also the release of glycerol in plasma. Whether this is due to a direct effect of salicylate on the adipose tissue lipase or to an increased esterification of free fatty acids through greater availability of glycerophosphate remains to be demonstrated. The

fact that salicylate does not lower the blood glucose level is not necessarily against the hypothesis that salicylate increases the utilization of glucose. It may also be significant that salicylate is strongly bound to proteins (Gordon, Boyle, Brown, Cherkes & Anfinson, 1953) and that free fatty acids are transported into the plasma by an albumin fraction (Goodman & Gilman, 1955).

The reduced level of free fatty acids in plasma may have some bearing in explaining some of the therapeutic effects of salicylate, such as the hypoglycaemic effect in diabetics and the hypocholesterolaemic action in atherosclerotic patients (Reid, 1961). It is possible that also the antipyretic effect of salicylate may have some connection with a reduced availability of free fatty acids necessary to keep up with the increased metabolic needs during conditions of fever. It is more difficult to establish a relation between this effect on plasma free fatty acids and its anti-inflammatory action. It may be pertinent to mention here that the anti-inflammatory drugs salicylamide and phenylbutazone do not share the action of salicylate on plasma free fatty acids (unpublished).

Salicylate, nicotinic acid (Carlson, Havel, Ekelung & Holmgren, 1963; Vertua, Usardi, Bombelli, Farkas & Paoletti, 1964) and 3,5-dimethylpyrazole (Gerritsen & Dulin, 1963; Bizzi, Jori, Veneroni & Garattini, 1964) are representative of a new class of drugs which can lower plasma free fatty acids. They may be useful in assessing the biological significance of free fatty acids in normal conditions as well as in situations of emergency.

SUMMARY

1. The possibility of interference with free fatty acid mobilization by means of salicylates, which affect carbohydrate metabolism, has been investigated in male Sprague-Dawley rats. Plasma free fatty acids, serum salicylate and serum and liver triglycerides have been determined.

2. Salicylate can decrease plasma free fatty acids in fed resting rats as well as in conditions of increased mobilization such as fasting, cold and treatment with noradrenaline, corticotrophin, amphetamine or chlorpromazine.

3. Salicylate is also active in adrenalectomized animals or those with reduced thyroid function and at doses having no effect on blood glucose level.

4. Accumulation of liver triglycerides by exposure to cold or administration of corticotrophin is prevented by salicylate.

5. Salicylate decreases body temperature in rats exposed to cold and shortens survival time in starved animals, probably because of its effect on plasma levels of free fatty acids.

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