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The Effect of Oxytetracycline on Insulin Resistance in Obese Mice

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1. Chronic oxytetracycline treatment was found to improve the insulin resistance of the obese–hyperglycaemic mouse. 2. The improved response to insulin was accompanied by decreased concentrations of circulating insulin and glucose, by a decrease in the lipid content of the liver and by an increase in the insulin-receptor sites of the liver and adipose tissue. 3. The increase in insulin-receptor sites preceded the fall in blood glucose. 4. Comparable studies done on food-restricted animals indicated that although chronic food restriction corrected the hyperinsulinaemia it did not restore the insulin-receptor sites or the hyperglycaemia.

The obese–hyperglycaemic mouse is characterized by obesity, non-ketotic hyperglycaemia, hyperinsulinism, islet-cell hyperplasia and resistance to exogenous insulin. Insulin resistance in the obese–hyperglycaemic mouse has been attributed to several factors. (1) The number of insulin-binding sites on the membranes of the liver (Kahn *et al.*, 1972, 1973*a*) and adipose tissue (Freychet *et al.*, 1972*a*) is decreased. In the liver this decreased insulin binding was evident whether it was expressed per cell, per unit surface area or per receptor site for counter insulin hormones. This decrease in insulin receptors could be a primary genetic defect or secondary to obesity, hyperinsulinism or some other factor. (2) The islet cells themselves change; the experiments of Strautz (1968, 1970) and Gates *et al.* (1972) have shown that after transplantation of islet cells from normal animals into *ob/ob* animals and NZO mice respectively the body weight and the concentrations of glucose and insulin decreased and glucose tolerance was improved. That the hyperinsulinism itself may be a factor in the production of insulin resistance is indicated by the work of Solomon & Mayer (1962) and by that of Mahler & Szabo (1971), who showed that injections of alloxan into the *ob/ob* mouse resulted in lower blood glucose concentrations accompanied by a decreased resistance to exogenous insulin and regranulation of the islet cells. This work also indicated that the use of pharmacological agents to alter the metabolism of the obese animals would be useful in elucidating the nature of the abnormal metabolic processes that characterize this syndrome.

The antibiotic oxytetracycline, which is known to inhibit mitochondrial protein synthesis in proliferating tissues (De Vries & Kroon, 1970; Himms-Hagen, 1971) also profoundly affects glucose metabolism and the action of insulin. In both humans and animals which received either tolbutamide or exogenous insulin, oxytetracycline potentiated the action of insulin (Hiatt & Bonorris, 1970; Miller, 1966). We therefore postulated that oxytetracycline may decrease both the high concentrations of insulin and the insulin resistance seen in the obese–hyperglycaemic mice.

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Materials and Methods

Animals

Male C_{57}B1/6J *ob/ob* mice and their lean controls were obtained from Jackson Laboratories, Bar Harbor, Maine, U.S.A. The mice were 9–11 weeks of age at the beginning of the experiments. They were maintained on Purina Chow and water *ad libitum*, except as otherwise stated.

Oxytetracycline treatment

Unless otherwise indicated in the legends to Tables and Figures, groups of lean and obese mice were treated for 10 days with a daily intramuscular injection of oxytetracycline (Terramycin; Pfizer Co. Ltd., Montreal, Quebec, Canada) suspended in olive oil. All animals received the same daily dose of 100 mg/kg, calculated on the basis of the body weight of the untreated lean mice. The control animals received placebo injections. The injections were given between 16:00 and 16:30 h.

Glucose- and insulin-tolerance tests

The treated animals were given their last oxytetracycline injection between 16:00 and 16:30 h and were starved from 21:00 h. After starvation for 12 h, blood samples were withdrawn from the tail vein with a heparinized capillary pipette (zero-time sample). In some experiments, glucose (1 g/kg) was administered intraperitoneally and blood samples were withdrawn 15, 30 and 60 min after the glucose injection. The blood samples were kept cold until they

could be processed. For the glucose-tolerance tests, the animals were anaesthetized with pentobarbital (45mg/kg). Care was taken to keep the animals warm by placing them over an electric heating pad set at the lowest setting.

The insulin-tolerance tests were performed essentially as the glucose-tolerance tests, except that the animals were not anaesthetized and 1 or 2i.u. of regular insulin/kg body weight was administered by intraperitoneal injection, after removal of the zero-time sample.

Measurements of glucose and insulin concentrations

The concentrations of glucose in the blood were measured by an enzymic assay (Glucostat, Worthington Biochemical Corp., Freehold, N.J., U.S.A.) on a 1:20 dilution of whole blood to which $Ba(OH)_2$ and $ZnSO_4$ were added to precipitate the protein (Somogyi, 1945).

The plasma immunoreactive insulin concentrations and some of the blood glucose concentrations were measured in blood samples obtained by decapitation of the animals. In this case, the blood was collected in heparinized test tubes. A portion of whole blood (0.1–0.2ml) was removed and processed for the determination of glucose as above, and the remainder was centrifuged to separate the formed elements from the plasma. The plasma was then stored frozen until it was assayed for insulin by radioimmunoassay with a kit (Schwartz/Mann, Orangeburg, N.Y., U.S.A.) with human insulin as a standard. Human insulin and mouse insulin do not have the same immunoreactivity. For this reason, the values reported here are only relative and they differ from those of other workers who used mouse insulin as a standard. This problem has been discussed by Abraham *et al.* (1971). In the present case, this limitation does not affect the validity of the results.

Extraction of tissue lipids

The livers were removed from the animals and immediately frozen on solid CO_2. They were weighed and homogenized in chloroform–methanol (3:1, v/v). The total lipids were extracted by the method described by Rabin (1969), which is based on the method of Folch *et al.* (1957). The extracts were evaporated under N_2 at 60°C. Three successive portions (10ml) of benzene were added and evaporated, and finally the residues were dried and weighed.

Preparation of membrane fractions

Membrane fractions were prepared from the livers of both lean and obese animals. The mice were killed by decapitation and their livers were removed and placed in ice-cold sucrose medium (0.25M-sucrose–10mM-Tris–1mM-EDTA, pH7.4) in an ice bath. After being washed and the connective tissue removed, the livers were processed by the method described by Illiano & Cuatrecasas (1972). The isolated membranes were resuspended in 0.05M-Tris–HCl, pH7.4, and stored at −60°C until used. Membranes were also prepared from adipose tissue essentially as described by Cuatrecasas (1971*a*).

Insulin-binding assays

The insulin-binding assay system contained 0.2mg of membrane protein, 2% albumin in Krebs–Ringer phosphate buffer (118mM-NaCl–1.2mM-$MgSO_4$–5mM-KCl–100mM-sodium phosphate buffer, pH7.4). [^{125}I]Iodinated insulin (New England Nuclear Corp., Boston, Mass., U.S.A.) was added to start the reaction. The total volume was 0.5ml. The incubations were carried out for 25min at 25°C in polystyrene tubes. Each assay was done in triplicate. Non-specific binding was determined for each sample also in triplicate by adding 40μg of unlabelled crystalline porcine insulin to the reaction mixture. The porcine insulin was a generous gift of Dr. Mary Root, Eli Lilly Co. Ltd., Indianapolis, Ind., U.S.A. At the end of the incubation period, 4ml of ice-cold Krebs–Ringer phosphate buffer containing 0.1% albumin was added to each tube and the contents of the tubes were filtered on EAWP Millipore filters. The tubes and filters were rinsed with two 4ml portions of buffer. The filters were then placed in polystyrene tubes and the radioactivity was counted in a gamma counter (Packard Instruments, La Grange, Ill., U.S.A.). This method was based on those already described in the literature (Kahn *et al.*, 1973*a*; Cuatrecasas, 1971*a*).

Isoproterenol-binding assays

The binding of [^{3}H]isoproterenol was measured as described by Lefkowitz *et al.* (1972) in membranes from liver and adipose tissue. Membrane protein (0.1mg) was suspended in an incubation mixture consisting of 1ml of 5mM-Tris–HCl, pH7.4, containing 100nM-[^{3}H]isoproterenol bitartrate. The tubes were incubated for 90min at 37°C. The membrane-bound hormone was separated from the medium by filtration on Millipore filters and washed with three 4ml portions of 5mM-Tris–HCl buffer. The filters were dissolved in Bray's (1960) scintillation mixture and counted for radioactivity in a Beckman liquid-scintillation counter. In some of the experiments, the incubation medium, free of the membranes, was submitted to ascending paper chromatography in butanol–acetic acid–water (4:1:5, by vol.), with unincubated [^{3}H]isoproterenol as a standard. The chromatograms were cut into equal strips (2.5cm × 1.5cm), which were placed in individual scintillation vials. To each vial 3.5ml of water was added. The vials were allowed to stand for 60min, after which 11.5ml of Aquasol (New England Nuclear Corp.) was added and the vials were counted for radioactivity. More than 90% of the radioactivity was found to be asso-

ciated with a spot corresponding to genuine isoproterenol.

5'-Nucleotidase assays

These assays were done on the following fractions obtained during membrane isolation: crude homogenate (fraction I); 10000*g* supernatants (fraction 2); NaCl–Mg precipitates (fraction 3); and washed membrane preparations (fraction 4). The assay mixtures contained, in a final volume of 1ml, 50mM-Tris–HCl, pH7.5, 5mM-5'-AMP and 10mM-$MgCl_2$. Enzyme fractions equivalent to 100μg of protein were added to start the reaction. After incubation at 37°C for 30min, the reaction was terminated by adding 1ml of 10% trichloroacetic acid. Portions (1ml) of the deproteinized supernatant were analysed for P_i. The amount of P_i released was determined colorimetrically by the method of Fiske & Subbarow (cf. Chaykin, 1966). Blank values were obtained for each fraction from tubes where the reaction was stopped at zero time. These values were subtracted from the experimental values. By running tubes which contained only the respective enzyme fractions without 5'-AMP and tubes containing 5'-AMP alone, it was found that the blank value was almost entirely contributed by the enzyme source.

Protein determinations

These were done by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Results

Our initial observations after treating obese and lean mice with oxytetracycline for 14 days were the following (Table 1). The weight of the fat-pads was decreased in both lean and obese mice. There was a dramatic decrease in the weight and in the lipid content of the liver in the obese oxytetracycline-treated mice, whereas there was no significant decrease in the weight of the liver and an actual increase in the amount of lipid/g of liver in the lean animals.

These findings prompted us to measure the concentrations of glucose and insulin in the blood of these animals (Table 2). The results indicate that oxytetracycline treatment affected the blood glucose and insulin concentrations from fed and starved lean mice very little, although it had a marked effect on both these parameters in the obese mice. In fact, both the glucose and insulin concentrations were found to be lower in the fed treated obese animals than in the starved obese control animals. We therefore investigated the effects of this treatment on the glucose and insulin tolerance of these four groups of animals (Figs. 1 and 2). Whereas the control obese animals had a typically diabetic glucose-tolerance curve the obese oxytetracycline-treated animals had a curve which was comparable with those of the control and treated lean animals. There was no statistically significant difference between the results obtained for the treated obese and control lean animals although the values obtained for the former tended to be slightly higher. The removal of glucose from the blood was slower than is normally observed and we ascribe this to the fact that for these tests the animals were kept under pentobarbital anaesthesia (Davidson, 1971).

The insulin-tolerance curves (Fig. 2) supported the above results. In this instance as well, the treated obese mice responded nearly normally to the injection of 1i.u. of insulin/kg (Fig. 2*a*) whereas their response was indistinguishable from that of the lean animals when 2i.u. of insulin/kg was administered (Fig. 2*b*). In both instances, the response of the untreated obese animals was completely different from that of the

Table 1. *Weight of liver and epididymal fat-pads and lipid content of the liver in oxytetracycline-treated mice and their controls*

The mice were treated with oxytetracycline for 14 days. At the end of the experiment, they were decapitated and their tissues were removed, frozen on solid CO_2 and weighed. The livers were homogenized in chloroform–methanol and the total lipids were extracted by the method of Folch *et al.* (1957). The lipid extract was placed in weighed vials, the solvent was removed by evaporation under N_2 and the residue was dried and weighed. The results are given as means±S.D. $n = 24$ in all groups. N.S., not significant.

Group	Weight of fat-pad (g)	Liver		
		Weight (g)	Total lipids	
			mg/liver	mg/g of liver
Lean				
Control	0.49±0.18	1.18±0.20	38±1.2	34±0.6
Treated	0.20±0.10	0.96±0.18	45±1.5	47±1.0
	$P<0.001$	$P<0.01$	N.S.	$P<0.001$
Obese				
Control	3.54±0.30	3.53±0.47	950±22	280±6
Treated	2.89±0.44	1.52±0.31	140± 6	100±4
	$P<0.001$	$P<<0.001$	$P<<0.001$	$P<<0.001$

Table 2. *Concentrations of glucose and insulin in the blood of lean and obese mice and their modification by starvation and/or oxytetracycline treatment*

The mice were killed by decapitation between 08:00 and 10:00h and the blood was collected in heparinized tubes. A portion of the blood was used to prepare a barium–zinc filtrate for the determination of glucose concentration. The remainder of the blood was centrifuged and the plasma was collected and stored frozen until the insulin concentrations were determined by radioimmunoassay. The starved animals had been without food for 12h since 21:00h the previous night. The results are given as means±S.D. $n = 12$–20.

	Glucose (mg/100ml)	Insulin (μunits/ml)
Lean		
Control fed	120±26	45.0± 4.4
starved	79±22	39.5± 4.5
Treated fed	108±30	55.6± 7.7
starved	85±15	— —
Obese		
Control fed	261±72	242 ±13.9
starved	198±58	132 ±11.4
Treated fed	73±12	66.3± 5.4
starved	64±11	— —

oxytetracycline-treated obese mice as well as the lean mice.

It was important to evaluate whether this normalized response of the obese mice with respect to the above parameters might be associated with variations of the insulin-binding capacity of the liver. The results presented in Table 3 indicate that treatment with oxytetracycline increased the insulin-binding capacity of a membrane fraction isolated from liver, both in the lean mice, where the increase was of the order of 25%, and in the obese mice, where the insulin-binding capacity was approximately doubled as a result of treatment. It is noteworthy that, at the insulin concentration used, in the samples obtained from the treated obese mice the binding was restored to that observed in the samples obtained from the control lean mice. Different membrane fractions and different batches of insulin were used for Expts. 1 and 2.

We also tested the effect of oxytetracycline added *in vitro* on the capacity of the liver membranes to bind insulin. At the concentrations tested (ranging from 0.2 to 2.0mg/ml) oxytetracycline had an inhibitory effect on the binding of insulin in membranes isolated from both the obese and the lean mice. In both cases, the maximum inhibition was of the order of 75% and was obtained with 1mg of oxytetracycline/ml. The results indicate that the changes *in vivo* were not due to a direct effect of oxytetracycline on the membranes.

The possibility that oxytetracycline acted simply by lowering the food intake of the animals was next explored. For these studies, the obese animals were

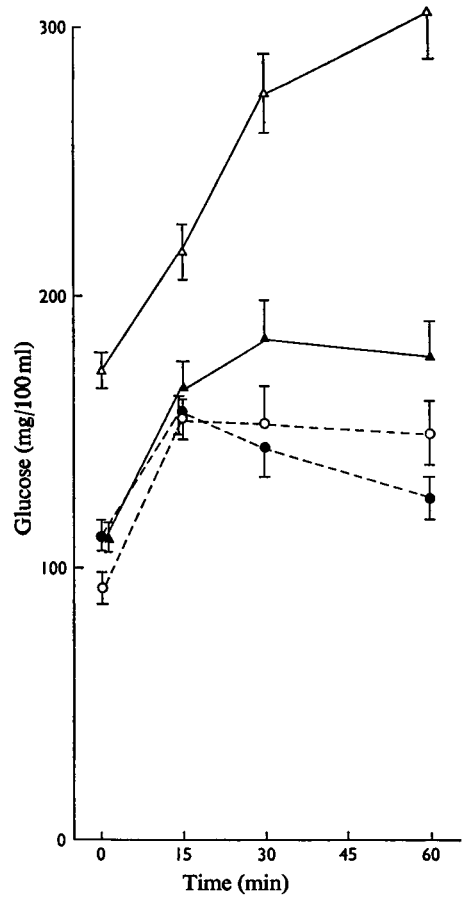

Fig. 1. *Response of lean and obese mice to a glucose load and the effect of treatment with oxytetracycline*

The mice were starved for 12h starting at 21:00h. The following morning a blood sample was removed from the tail into a heparinized capillary tube (zero-time sample). Samples were removed in the same manner at 15, 30 and 60min after the administration of glucose (1g/kg) via intraperitoneal injection. Barium–zinc filtrates of the blood were prepared and used for the determination of glucose. The results given are means±S.D. $n = 8$ in each group. ○, Untreated lean mice; ●, treated lean mice; △, untreated obese mice; ▲, treated obese mice.

divided into three groups: some animals served as controls, some were treated with oxytetracycline and some were restricted to 2.5g of food per day, which is somewhat less than the intake that we had measured for the lean animals eating *ad libitum*. The lean animals were divided into the usual groups, control and oxytetracycline-treated. The experiment lasted for a total of 10 days, but some of each group of animals were killed at days 1, 2, 3, 5, 7 and 10 after the onset of treatment. The food intake of the control and

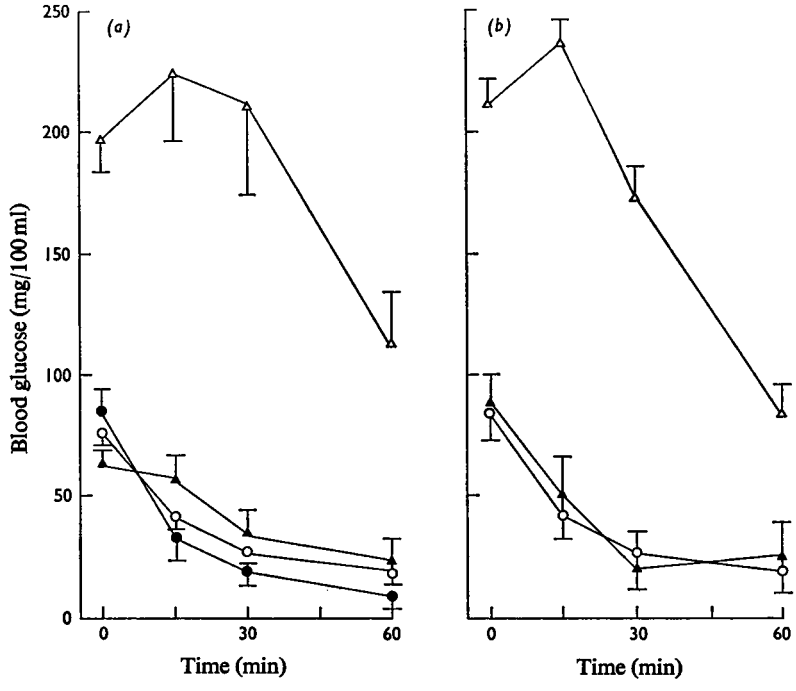

Fig. 2. *Response of lean and obese mice to insulin*

The mice were starved for 12h overnight and treated as described in the legend to Fig. 1 except that regular insulin was injected subcutaneously instead of glucose. The dose of insulin was (*a*) 1 unit/kg; (*b*) 2 units/kg. The results shown are means ±S.E.M. of at least four observations. ○, Untreated lean mice; ●, treated lean mice; △, untreated obese mice; ▲, treated obese mice.

Table 3. *Binding of insulin to isolated liver membranes*

The membrane fractions were prepared from the pooled livers of three animals, by the method of Illiano & Cuatrecasas (1972). The binding of insulin to the membranes was measured as described in the Materials and Methods section. The insulin concentration was 100nmol/l. The specific radioactivity of the radioactive insulin was 45 Ci/g for Expt. 1 and 47.2Ci/g for Expt. 2. The results given are means of three observations corrected for non-specific binding. Expts. 1 and 2 were done with different membrane preparations. The results are expressed as fmol of insulin bound/mg of membrane protein.

	Insulin bound	
	Expt. 1	Expt. 2
Lean		
Control	660	620
Treated	810	890
Obese		
Control	230	390
Treated	690	653

oxytetracycline-treated animals was measured over a period of 10 days. The results obtained show that oxytetracycline treatment somewhat decreased the food intake of both groups of mice. In the lean animals, the decrease in food intake was observed in the early days of treatment, but it was only temporary and the daily food intake was restored to that observed in the control animals (3.0g/day) by day 4. The oxytetracycline-treated obese mice consumed an average of 4.3g/day compared with 5.8g/day for the control obese animals. The food-restricted animals consumed their full ration of 2.5g/day. Over a 10-day period the food-restricted obese animals were those which had the greatest weight loss. They lost an average of 8g, if the normal weight gain of the control animals during that period is taken into consideration. On the same basis the obese oxytetracycline-treated animals lost an average of 4g and the lean oxytetracycline-treated animals also lost an average of 4g.

The blood sugar concentrations were measured during the same period and the results presented

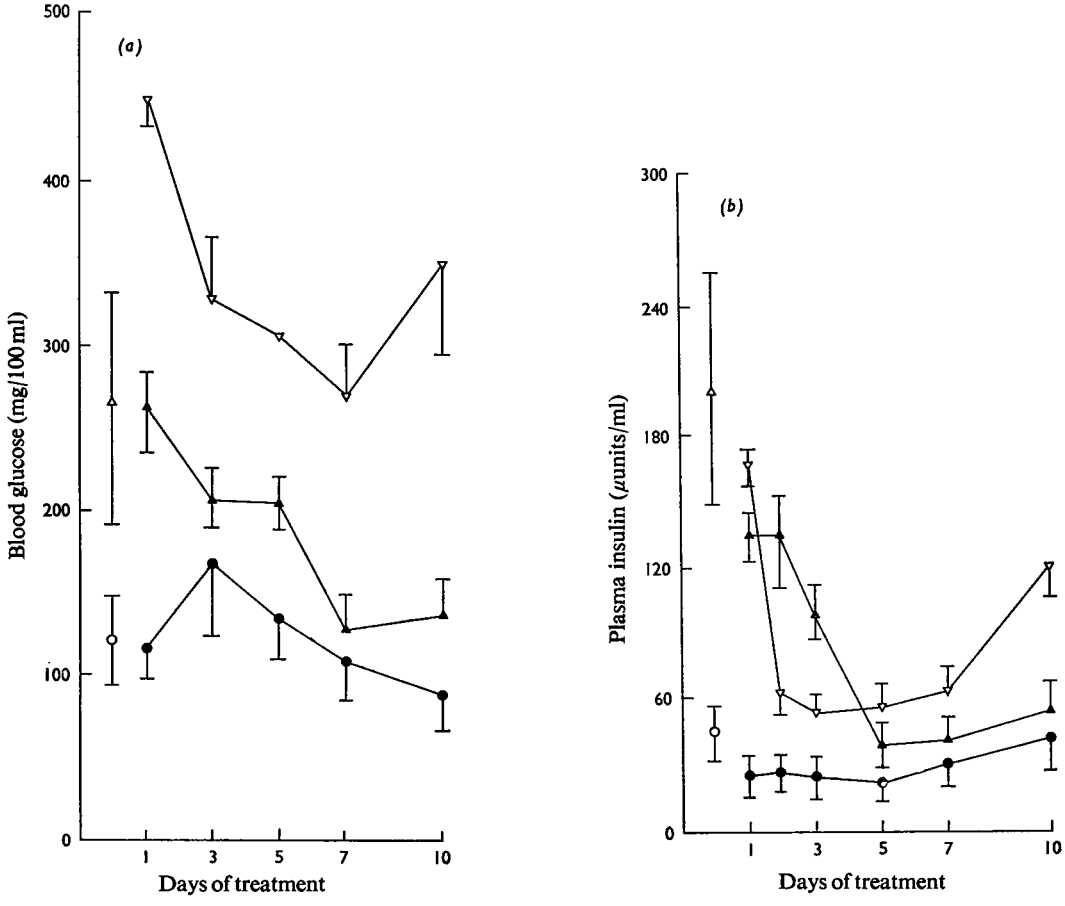

Fig. 3. *Effects of treatment on the concentrations of (a) glucose and (b) insulin in the blood*

After decapitation of the animals, the blood was collected and processed as described in the Materials and Methods section and in the legend to Table 2. The results are given as means±S.E.M. for three to four observations except for the control value which is given as mean±S.D. for 18 observations. ○, Lean control; ●, lean oxytetracycline-treated; △, obese control; ▲ obese oxytetracycline-treated; ▽, obese food-restricted.

(Fig. 3*a*) indicate that, of the five groups of animals studied, the obese oxytetracycline-treated animals were the only group which showed significant decreases in blood glucose concentrations. The significant fall in the concentrations of blood glucose occurred between day 5 and day 7 of treatment and was maintained through day 10. After day 5, the blood glucose concentrations of the oxytetracycline-treated obese animals were within the range observed for the lean animals. The plasma insulin concentrations were also measured over the same time-course and the results are given in Fig. 3(*b*). Dramatic decreases occurred in both the oxytetracycline-treated and the food-restricted obese animals. The effect of both treatments was very swift; the insulin concentrations decreased to near normal within the first days of the treatment with food restriction producing its effects earlier than oxytetracycline-treatment. There was a return to the higher concentrations of insulin by day 10 of food restriction. The differences between the values obtained for days 7 and 10 are statistically significant ($P<0.05$). In the oxytetracycline-treated obese mice, the decrease in insulin concentrations was more gradual up to the third day of treatment. There was then a sharp decrease between day 3 and day 5, which brought the plasma insulin concentrations of the oxytetracycline-treated obese animals within the range observed for the lean mice. These low values were maintained for the remainder of the treatment.

Fig. 4 represents the results obtained for the weight and the composition of the liver during a similar time-course of treatment. In the case of the total

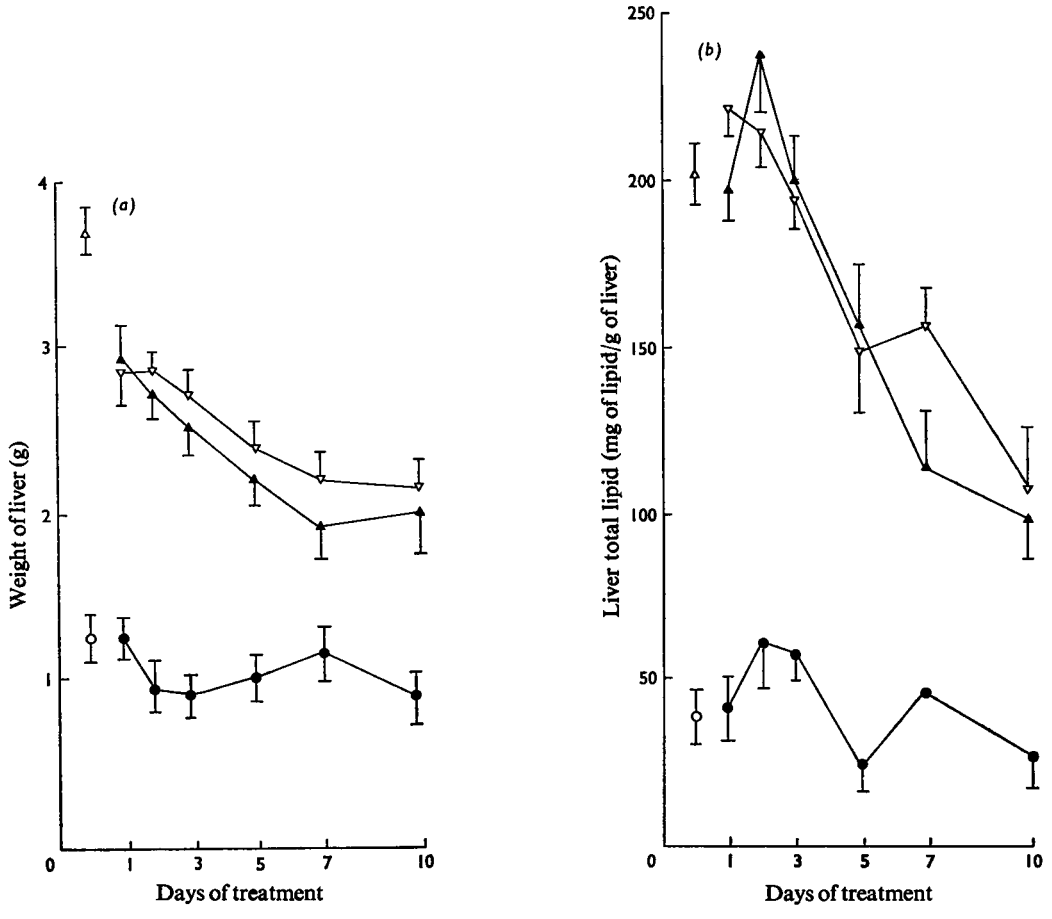

Fig. 4. *Effect of treatment on (a) the weight of the liver and (b) the total lipid content of the liver*

The lean mice were divided into two groups: control and oxytetracycline-treated. The obese mice were divided into three groups, control: oxytetracycline-treated and food-restricted. The animals were submitted to their respective treatments for various periods from 1 to 10 days as specified on the ordinate. After the animals were killed, the livers were removed and processed as described in the Materials and Methods section and in the legend to Table 1. Each point on the graph represents the mean of three observations±S.E.M. except for the controls where $n = 18$. ○, Lean control; ●, lean oxytetracycline-treated; △, obese control; ▲, obese oxytetracycline-treated; ▽, obese food-restricted.

weight of the liver (Fig. 4*a*) and of the lipid concentration in the liver (Fig. 4*b*) the results obtained were similar. In both the oxytetracycline-treated and the food-restricted animals, there was a rapid decrease in the weight of the liver during the first few days after the onset of treatment. There were significant differences ($P < 0.001$) between the obese controls and both groups of treated animals (oxytetracycline and food-restricted) within the first 18h after the beginning of the treatment. The concentration of lipids in the livers did not decrease immediately but there was a rapid decrease after the third day of treatment. It is evident that substances other than lipids contribute to the increase in the liver weight in obese animals. The pattern observed for the oxytetracycline-treated animals differed very little from that observed in the food-restricted animals. No notable changes occurred in the lean oxytetracycline-treated animals during the same period.

We also investigated the time-course of oxytetracycline treatment and of food restriction on the binding of insulin to membranes isolated from the livers. The results of these experiments are reported in Table 4. During the first 3 days of treatment, either by oxytetracycline or by food restriction, the treated animals did not differ from their respective controls.

Between day 3 and day 5, however, there was an increase in the insulin binding by the membranes obtained from the oxytetracycline-treated animals, either lean or obese. The increase in binding was maintained thereafter. In the lean oxytetracycline-treated animals, the concentrations were 40% above those observed for the lean control animals whereas in the obese oxytetracycline-treated animals, the concentrations represented approx. a 2.5-fold increase over the values observed for the obese control animals. No such trend was noted in the food-restricted obese group. On day 10, it was never possible to obtain a value for insulin binding to the membranes since the counts obtained in the presence of large amounts of unlabelled insulin were always greater than those obtained with labelled insulin alone. This may indicate that the membranes obtained after 10 days of food restriction degrade the insulin more (cf. Freychet *et al.*, 1972*b*).

Two orders of insulin-binding sites have been described in the liver and it was shown that in the *ob/ob* mice the high-affinity low-capacity site was the more altered (Kahn *et al.*, 1973*a*). We measured the binding of radioactive insulin in the presence of increasing concentration of unlabelled insulin. The results of these experiments are reported in Fig. 5. It is evident from the results obtained that oxytetracycline treatment restored the binding nearly to the values obtained in the lean mice. This is particularly true of the values obtained at the low concentrations of insulin. Also, at all the insulin concentrations tested, the binding of insulin was consistently greater in the treated lean animals compared with the control lean animals.

In view of the observed effects of oxytetracycline treatment on insulin binding and in order to ascertain further that the oxytetracycline effect was not due to secondary effects on food intake, or to differences in the membrane preparations, we measured the binding of isoproterenol by the various membrane fractions obtained from the liver. These results are reported in Table 5. There was little difference between the five groups of animals over the period surveyed, except for the lean treated animals where the isoproterenol binding increased in parallel with the insulin binding.

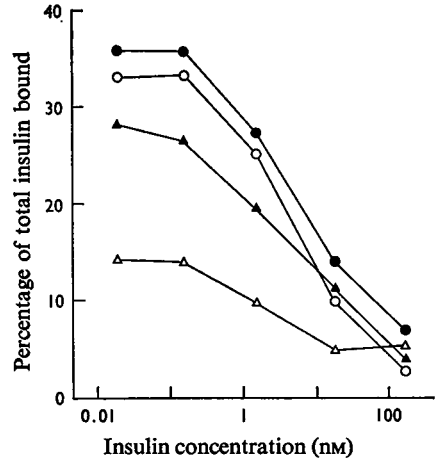

Fig. 5. *Effect of increasing concentrations of insulin on the binding capacity of liver membranes from oxytetracycline-treated lean and obese mice and their controls*

The binding assays were done as described in the Materials and Methods section. The concentration of [^{125}I]iodinated insulin was 40pM (sp. radioactivity 45Ci/g). The concentration of unlabelled insulin was increased in the range 0.1nM to 0.1μM. The amount of insulin bound/mg of protein per total insulin in 1ml of incubation medium was measured and is given on the ordinate as percentage of total insulin bound. Each point represents the mean of three to six observations. ○, Lean controls; ●, lean treated; △, obese control; ▲, obese treated.

Table 4. *Effect of treatment length on the binding of insulin by liver membrane preparations*

The animals were submitted to their treatments for the times indicated. Membranes were prepared from the pooled livers of three animals. The techniques were as described in the Materials and Methods section and in the legends to Table 3 and Fig. 5. The insulin concentration was 40pmol/l and the specific radioactivity was 54 Ci/g. The results are expressed as fmol of insulin bound/mg of membrane protein. Each number is the mean of three observations. The numbers in parentheses represent percentages of the values obtained for the lean control animals.

Group	Days of treatment 1	3	5	7	10
Lean					
Control	3.0 (100)	3.8 (100)	3.5 (100)	3.8 (100)	2.7 (100)
Oxytetracycline-treated	3.1 (103)	3.5 (92)	5.0 (142)	5.7 (150)	3.7 (137)
Obese					
Control	0.94 (31)	1.7 (45)	0.89 (25)	0.67 (18)	0.78 (27)
Oxytetracycline-treated	0.79 (26)	1.3 (37)	2.4 (69)	2.5 (66)	2.1 (78)
Food-restricted	1.1 (37)	1.5 (39)	1.3 (37)	1.1 (29)	—

Table 5. *Binding of isoproterenol to liver membranes and the ratio of isoproterenol to insulin binding*

The isoproterenol-binding assays were done as described in the Materials and Methods section. The isoproterenol concentration was 100nmol/l (sp. radioactivity 10Ci/g). The results are expressed as pmol of isoproterenol bound/mg of protein. The isoproterenol/insulin ratios were calculated taking the ratio obtained from the lean control animals as 1. The insulin-binding values are those reported in Table 4. Each number represents the mean of three observations.

Group	Days of treatment ...	[^{3}H]Isoproterenol bound			Isoproterenol/insulin		
		1	5	7	1	5	7
Lean							
Control		1.9	2.0	2.2	1	1	1
Oxytetracycline-treated		2.1	2.8	4.9	1.1	1.1	1.5
Obese							
Control		1.5	1.9	1.7	2.5	3.7	4.4
Oxytetracycline-treated		1.8	1.7	1.7	3.6	1.2	1.2
Food-restricted		1.6	2.1	1.8	2.3	2.8	2.8

Table 6. *5′-Nucleotidase activity as an estimate of membrane purification*

The enzyme assays are described in the Materials and Methods section. The values quoted represent the average of two experiments each done on duplicate samples.

	5′-Nucleotidase activity (μmol of P_i released/h per mg of protein)		
	Obese	Oxytetracycline-treated Obese	Lean
Fraction 1	0.52	0.57	0.42
Fraction 2	0.27	0.33	0.3
Fraction 3	0.33	0.42	0.42
Fraction 4	0.94	0.94	0.97

In consequence, the ratio isoproterenol binding/insulin binding is much higher in the obese control animals than in the lean control animals and this ratio decreases as a result of oxytetracycline treatment but not as a result of food deprivation. These results are in accord with the statement of Kahn *et al.* (1973*a*), who reported that the binding of isoproterenol was not altered in the obese–hyperglycaemic mice.

5′-Nucleotidase activities (Table 6) were similar in lean, obese and oxytetracycline-treated obese animals. The specific activity as well as the degree of purification of the enzyme were similar in all three cases.

The binding of insulin and isoproterenol were also measured in adipose tissue. The results obtained were similar to those reported above for the liver. Again, oxytetracycline treatment improved the binding of insulin by the membrane fractions obtained from the oxytetracycline-treated obese mice but food restriction did not improve the insulin binding. The isoproterenol binding was similar in all cases (Table 7).

Discussion

In addition to obesity the obese–hyperglycaemic mouse is characterized by high circulating concentrations of insulin and glucose and tissue resistance to insulin. Because of their exaggerated insulinism, these animals appeared to be a logical model in which to study the manner by which oxytetracycline potentiates the action of insulin and, in turn, to study the inter-relationships between the altered metabolism of these animals and their resistance to insulin action. It is obvious that oxytetracycline has profound effects on the metabolism of the obese mice and that at least two of its effects are different from those brought about by food restriction. Oxytetracycline treatment almost completely restored the binding of insulin to the membranes of the liver, but food restriction did not produce this effect. Qualitatively, the results which we have obtained for the insulin binding to liver membrane preparations of untreated lean and obese mice are essentially similar to those already reported (Kahn *et al.*, 1973*a*). Quantitatively, the main difference between the results quoted above and the results reported here reside in the fact that the membrane preparations used for the present study were less purified and therefore the specific activities obtained were lower.

The finding that food restriction did not improve the binding of insulin to liver membranes is, however, in apparent disagreement with the report of Kahn *et al.* (1973*b*), who stated that 'acute and chronic dietary restriction of the *ob*/*ob* mouse ameliorates the insulin resistance and is accompanied by an increase in insulin-receptor sites toward normal'. It is impossible to evaluate this statement in view of our results since the experimental data of Kahn *et al.* (1973*b*) are not available. The results presented for adipose tissue are in agreement with those reported by Freychet *et al.* (1972*a*).

Table 7. *Binding of insulin and isoproterenol to membranes from adipose tissue*

The membrane preparation and the hormone-binding assays were performed as described in the Materials and Methods section. The hormone concentration in the assay mixture was for insulin 80pmol/l (sp. radioactivity 56.4Ci/g) and for isoproterenol 100nmol/l (sp. radioactivity 10Ci/g). The insulin binding represents the results of two experiments each done in triplicate samples on two different membrane preparations. The isoproterenol binding represents the mean±S.E.M. of four experiments each done on triplicate samples involving four different membrane preparations. Treatment was for 7 days.

	[^{125}I]Iodinated insulin bound (fmol/mg of protein)	[^{3}H]Isoproterenol bound (pmol/mg of protein)
Lean		
Control	1.8, 1.9	1.3±0.11
Oxytetracycline-treated	1.4, 1.2	1.4±0.22
Obese		
Control	0.60, 0.55	1.7±0.21
Oxytetracycline-treated	1.6, 1.3	1.8±0.25
Food-restricted	0.50, 0.58	1.9±0.6

Our results indicate that the effects of oxytetracycline on insulin-binding are not dependent on the decrease in food consumption caused by oxytetracycline; the oxytetracycline-treated obese animals consumed almost twice as much food as the food-restricted animals.

The patterns obtained for the displacement of labelled insulin by unlabelled insulin for the lean and the obese mice (Fig. 5) is in agreement with that found by Kahn *et al.* (1973*a*). In addition, we have noted that the profile observed in the obese oxytetracycline-treated animals was similar to that found for the lean animals.

It is noteworthy that in the case of the oxytetracycline-treated mice, the return to normal glycaemia followed the improvement in insulin binding by the liver, whereas in the food-restricted animals, where the insulin binding was not restored, the glycaemia always remained in the same range as that of the obese animals. In both groups of treated obese mice (oxytetracycline or food restriction), however, the insulin concentrations were within the normal range soon after the onset of treatment. Some authors (Chlouverakis & White, 1969; Batt & Miahle, 1966) have reported that food restriction completely corrected the hyperglycaemia in the obese mouse, so that the blood sugar concentrations fell within the normal range. Our results and those of Genuth *et al.* (1971) do not agree with the results quoted above. In obese animals, food restriction leads to 'stuff–starve' cycles; thus the animals are in various metabolic states throughout the day and the scheduling of the experiments may influence the results. Our conditions resemble those of Abraham *et al.* (1971) and our results are also similar to theirs.

We can only speculate on the mechanisms by which oxytetracycline can increase insulin sensitivity in the obese animals. This antibiotic has been shown to interfere with phospholipid metabolism and decrease the choline content of the liver (Mukherjee & Mukherjee, 1969). It is possible that changes in the phospholipid metabolism may lead to an alteration of the membrane, leading in turn to an alteration of the insulin-binding properties. This may be an action analogous to that of phospholipase C on preparations *in vitro* (Cuatrecasas, 1971*b*). It was also reported that chlortetracycline, an analogue of oxytetracycline, binds to insulin in the islets of Langerhans (Prochazka *et al.*, 1965).

The observation that oxytetracycline treatment can alter the insulin binding of the liver in both obese and lean mice and that this increased binding is consistent with the known action of oxytetracycline as a potentiator of insulin action in dogs (Hiatt & Bonorris, 1970), humans (Miller, 1966) and rodents indicates that the development of useful pharmacological agents capable of modifying hormone binding is a distinct possibility.

The primary genetic defect in the *ob/ob* mouse is not known. Our experiments indicate that oxytetracycline tends to correct many of the metabolic defects of these animals, especially those of the liver. However, certain lines of evidence indicate the muscle or the islet cells as the sites of the primary defect. It is generally thought that the insulin resistance of the muscle is more marked in comparison with that of other tissues (Stauffacher & Renold, 1969; Genuth *et al.*, 1971). In turn, experiments with transplanted islet cells point to an abnormality in islet-cell function (Strautz, 1968, 1970; Gates *et al.*, 1972). The normalization of glucose tolerance in our oxytetracycline-treated animals is consistent with a decrease in insulin resistance of muscle. However, more direct experiments are necessary to demonstrate this. Our present experiments are directed towards examining specifi-

cally the effects of oxytetracycline treatment on muscle and islet-cell metabolism.

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